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Document Title

A Nature of the Residue Study with [14C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

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Reviewer(s) and ID(s)

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Key Terms including compound numbers (ex: XDE-123)

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SUMMARY

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

STUDY TITLE

A Nature of the Residue Study with [14C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

DATA REQUIREMENTS

OECD Guidance Document 501 for Metabolism in Crops (Issued 8 January 2007)

AUTHORS

M. Ma, Y. A. Adelfinskaya

STUDY COMPLETED ON

09 July 2010

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LABORATORY STUDY ID

090058

A Nature of the Residue Study with [14C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

SUMMARY

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Forage was collected on 25 August 2009 (74, 54, and 42 days after the first, second, and third applications, respectively). Forage contained 3.020 µg acid equivalents/g. Mature grain, cobs, and fodder were collected 18 September 2009 (98, 78, and 66 days after the first, second, and third applications, respectively). At maturity, the grain contained 0.032 µg a.e./g. The mature cobs and fodder contained 0.014 µg a.e./g and 4.179 µg a.e./g, respectively. A portion of each sample was sequentially extracted with neutral solvent then methanolic base and the non-extractable residues of forage and fodder were subjected to bound residue determinations including pectin, acid-detergent fiber, lignin, and cellulose isolation. Starch was isolated from a separate portion of grain.

The major radioactive component identified in forage and fodder was 2,4-D which comprised, 77.4% (2.338 mg a.e./kg) and 56.5% (2.362 mg a.e./kg) of the Total Radioactive Residue (TRR), respectively. Glucose-conjugated 2,4-Dichlorophenol (2,4-DCP) was identified in forage and fodder. Approximately 21% of the TRR in grain (0.007 mg a.e./kg) was associated with starch. Extensive metabolism of 2,4-D was observed as demonstrated by incorporation or encapsulation of radioactivity into natural plant constituents such as starch, pectin, and lignin.

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DATA REQUIREMENTS

OECD Guidance Document 501 for Metabolism in Crops (Issued 8 January 2007)

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090058

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: 2,4-D DMA

Title: A Nature of the Residue Study with [14C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA sec. 10(d)(1)(A), (B), or (C) and which pertains to a registered or previously registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA sec. 10(g).

Company: Dow AgroSciences LLC

Company Agent: D. Fonseca

Title: Regulatory Manager

Signature: 

Date: 17 May 2010

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: A Nature of the Residue Study with [14C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

Study Initiation Date: 04-MAY-2009

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

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

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

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160 with the exceptions noted in the in-life report: weather data and equipment used for plot maintenance was calibrated prior to use but not GLP verified.

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17 May 2010
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Submitter
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Study Completion Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Study ID: 090058

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Study Initiation Date: 04-May-2009 **Study Completion Date:** 09-Jul-2010

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
01-May-2009	01-May-2009	Protocol Review
18-Nov-2009	18-Nov-2009	Analysis of 2,4-D Content in Corn Stover (Fodder)
22, 23, 25, 28, 29, 30-Jun & 01, 06-Jul-2010	06-Jul-2010	Raw Data and Report review and Test Substance and sample verification

AUDITS CONDUCTED BY RESEARCH FOR HIRE AND ABC LABORATORIES QUALITY ASSURANCE ARE LISTED ON THE QUALITY ASSURANCE STATEMENTS OF THE SUB-REPORTS.

QUALITY ASSURANCE STATEMENT:

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

Val Gartner, RQAP-GLP
Dow AgroSciences, Quality Assurance

09-Jul-2010
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A Nature of the Residue Study with [14C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

1.0 SUMMARY ABSTRACT

[^{14}C]-2,4-D (2,4-dichlorophenoxy acetic acid dimethylammonium salt) was applied to AAD-1 maize at the maximum seasonal rate of 3.36 kg a.e./ha (three applications, each 1.12 kg a.e./ha at pre-emergence, V4, and V8). The maize was grown outdoors to maturity at Research For Hire. Plot maintenance simulated typical cultural practices.

Forage was collected on 25 August 2009 (74, 54, and 42 days after the first, second, and third applications, respectively). Forage contained 3.020 µg acid equivalents/g. Mature grain, cobs, and fodder were collected 18 September 2009 (98, 78, and 66 days after the first, second, and third applications, respectively). At maturity, the grain contained 0.032 µg a.e./g. The mature cobs and fodder contained 0.014 µg a.e./g and 4.179 µg a.e./g, respectively. A portion of each sample was sequentially extracted with neutral solvent then methanolic base and the non-extractable residue of forage and fodder was subjected to bound residue determinations such as pectin, acid-detergent fiber, lignin, and cellulose isolation. Starch was isolated from a separate portion of grain.

The major radioactive component identified in forage and fodder was 2,4-D which comprised, 77.4% (2.338 mg a.e./kg) and 56.5% (2.362 mg a.e./kg) of the TRR, respectively. Glucose-conjugated 2,4-Dichlorophenol was identified in forage and fodder. Approximately 21% of the TRR in grain (0.007 mg a.e./kg) was associated with starch. Extensive metabolism of 2,4-D was observed as demonstrated by incorporation or encapsulation of radioactivity into natural plant constituents such as starch, pectin, and lignin. Residue levels are summarized below.

Sample	2,4-D		2,4-DCP		2,4-DCP-conjugate	
Forage	% TRR	a.e. mg/kg	% TRR	a.e. mg/kg	% TRR	a.e. mg/kg
Neutral Extraction	66.5%	2.007	2.2%	0.067	18.0%	0.543
Methanolic Base Extraction	11.0%	0.331	0.1%	0.003	2.0%	0.060
Total Identified	77.4%	2.338	2.3%	0.069	20.0%	0.604

	2,4-D		2,4-DCP		2,4-DCP-conjugate	
Fodder	% TRR	a.e. mg/kg	% TRR	a.e. mg/kg	% TRR	a.e. mg/kg
Neutral Extraction	45.9%	1.917	1.7%	0.072	17.8%	0.743
Methanolic Base Extraction	10.6%	0.444	0.0%	0.000	2.4%	0.102
Total Identified	56.5%	2.362	1.7%	0.072	20.2%	0.845

2.0 INTRODUCTION

2.1. Objective of Study and Guidelines Followed

2.1.1. Purpose of Study

The purpose of this study was to characterize the radioactive residue in immature and mature AAD-1 corn (event 278) following the maximum seasonal application of ^{14}C-2,4-D DMA. One plot was treated with three applications, including one pre-emergence and two foliar spray applications. The ^{14}C-2,4-D was formulated as a soluble liquid (SL), the current proposed commercial formulation, and applied at a target rate of 3.36 kg a.e./ha (three applications, each 1.12 kg a.e./ha at pre-emergence, V4, and V8).

2.1.2. Relevant History and Background Information

2,4-D (2,4-dichlorophenoxy acetic acid dimethylammonium salt), is an herbicide developed primarily for control of broad-leaved weeds (dicotyledons) in cereal crops (monocotyledons), a variety of other crops and on non-crop land. 2,4-D is a selective, systemic herbicide. It is a synthetic auxin.

When applied as the salt or as an ester formulation, 2,4-D undergoes rapid dissociation or hydrolysis to the acid. Previous studies (*1*) have demonstrated that 2,4-D is readily degraded by soil microorganisms. The side chain is cleaved to the phenol which is further oxidized to a catechol derivative. The labeled carbon is postulated to degrade completely to $^{14}CO_2$ and enter the metabolic pool via the Krebs cycle.

Hydroxylation, decarboxylation, cleavage of the acid side chain, ring opening, and conjugation with amino acids or sugars are the major metabolic pathways of 2,4-D in plants (*1*).

A nature of residue study with [^{14}C]-2,4-D applied to AAD-1 corn (event 474) was conducted in 2008 (*2*). [^{14}C]-2,4-D DMA salt was applied to AAD-1 maize (event 474) at the maximum

seasonal rate of 3.36 kg a.e./ha (three applications, each 1.12 kg a.e./ha at pre-emergence, V4, and V8). Immature forage and the mature fodder, cobs and grain samples were collected and analyzed. The study concluded that 2,4-D is metabolized to 2,4-DCP and glucose conjugates of 2,4-DCP.

2.1.3. Guidelines

This study was conducted to fulfill requirements for nature of the residue in plant as outlined in the OECD Guidance Document 501 for Metabolism in Crops (Issued 8 January 2007).

2.1.4. Guideline Deviations

None

2.2. Justification of Study Application Rate

The maximum seasonal application rate for 2,4-D is 3363 g a.e./ha (equivalent to 4048 g a.i./ha in terms of 2,4-D DMA). This study was conducted at an approximate 1X rate.

3.0 MATERIALS AND METHODS

3.1. Test, Reference, and Control Substances

3.1.1. Test Substance

The radiolabeled test substance of the technical product was obtained from the Dow AgroSciences Specialty Synthesis Group. See Figure 1 for structure, radiolabel position, and nomenclature. Physico-chemical properties of 2,4-D can be found in Table 1.

3.1.2. Reference Substances

Non-radiolabeled 2,4-D and available metabolite standards were acquired from the Test Substance Coordinator at Dow AgroSciences for use as reference standards for chromatographic and mass spectral comparison. Non-radiolabeled test material was also used to dilute the specific activity of the dosing solution. All reference standards were received in solid form. Structures, purity, ID numbers, chemical names and abbreviations for reference substances are presented in Figure 2. Reference standards were prepared in acetonitrile, methanol or acetronitrile: water (50:50) at a concentration of approximately 1 mg/mL. Prepared bulk standard solutions were stored refrigerated when not in use.

The chromatogram in Figure 3 presents the UV retention times of the test and reference materials.

3.1.3. Control Substance

A soluble liquid formulation blank that is comparable to the formulation currently being developed for commercial use was added to test material spray solutions. Details of the formulation blank are provided in Figure 2.

3.2. Test Site

The in-life phase of this study was conducted at Research For Hire (RFH). The address of the RFH is: 1696 South Leggett Street, Porterville, California 93257, USA.

3.3. Test System

Genetically modified AAD-1 corn seed (event-construct pDAS1740-278) was obtained for this study. The modification makes the corn resistant to both 2,4-D and “fop” grass herbicides including quizalofop and haloxyfop. Corn is representative of the pulses & oilseeds group. The soil type, transplanting, and maintenance may be found in the in-life report (Appendix A).

The experimental design is detailed in Table 2.

3.4. Preparation and Application of Test Substance

3.4.1. Preparation of Application Solutions

The ^{14}C-2,4-D DMA, nominally 7.2 mCi, was received from Specialty Synthesis. The entire sample was diluted to 10-mL in methanol, using a volumetric flask. Aliquots (0.025 mL) were diluted in 10-mL volumetric flasks, using acetonitrile to dilute to volume. Aliquots of the dilutions were analyzed by LSC and to determine the actual amount of radioactive test substance received as well as the purity (Figure 4).

The specific activity of the ^{14}C-2,4-D DMA, was adjusted by combining 579 mg of non-radiolabeled 2,4-D DMA with 48 mg (7.1 mCi) of the ^{14}C-2,4-D DMA. Individual test substance samples were prepared for each of the three applications by dispensing the appropriate amount of this diluted specific activity solution. The individual test substance samples were evaporated to dryness under a stream of nitrogen, and shipped to Research For Hire (RFH).

Upon receipt at RFH, each of the test substance solution aliquots was dissolved in 0.2279 g (47% w/w) of formulation blank XRM-4436, E2868-54. Each test substance solution was shaken, swirled, and sonicated until all solids went into solution then stored in a refrigerator.

Each application solution was prepared separately, on the day of application. Details of each preparation can be found in the in-life report (Appendix A). In general, the appropriate vial of application solution was warmed up to room temperature, rinsed multiple times with water and brought to a known volume with water, then transferred to an application container. Aliquots were taken to determine the concentration and confirm homogeneity.

3.4.2. Application Procedures

Details of each application can be found in the in-life report (Appendix A).

The applications were made using an R&D wand sprayer pressurized with CO_2, where the container was covered with aluminum foil, evenly spraying in two passes per row of corn. The spray solution container was then rinsed with 30 mL of water, swirled, then sprayed evenly onto the plot in the same manner.

3.4.3. Significant Events

Table 3 lists the significant events for this study. More detailed information on the in-life phase of the study can be found in the in-life report (Appendix A).

3.5. Sample Collection

Details of each harvest can be found in the in-life report (Appendix A), while harvest dates are listed in Table 3.

Immature plant samples, R4 growth stage (milky inner fluid in kernels) were harvested on 25 August 2009. The plants were cut approximately 4 inches above the soil surface, then cut into approximately 6 inches segments to fit into bags, weighed, and stored frozen pending shipment to ABC Laboratories.

Mature crop was harvested on 18 September 2009. The cobs were removed from the stalks then the stalks cut approximately 6 inches above the soil surface. The grain and cobs were separated by hand. Each sample was individually placed in tared bags and weighed. The samples were stored frozen prior to frozen shipment to ABC Laboratories.

3.6. Sample Milling

Details of the milling procedures can be found in the ABC Laboratories report (Appendix B). In general, all samples were milled using the Straub grinding mill with dry ice. A Robot Coupe was used to break down the grain, cobs, and fodder samples before the milling.

3.7. Measurement of Total Radioactive Residue (TRR)

Details of the oxidative combustion procedures can be found in the ABC Laboratories report (Appendix B). Aliquots (5 x approximately 0.2 g) of the milled samples were analyzed by oxidative combustion as described in Appendix B to determine the radioactive residues in the samples. The results from the combustion assay of the treated samples can be found in Appendix B.

3.8. Sample Extraction, Analysis, Characterization, and Identification

In general, the milled samples were analyzed by the sequence of extractions described in Table 4 and Figure 5. Details of each step are provided below.

3.8.1. Neutral Organic Solvent Extraction (EX1)

Approximately 10 g of homogenized forage and fodder, or 20 g cobs, were extracted with approx. 50 mL of 90/10 methanol/water (75-100 mL for grain and cobs), as described in Figure 5 and Table 4. The mixture was blended using a Polytron homogenizer for approx. 5 minutes at ≥10,000 rpm. The mixture was then shaken on a horizontal shaker for approx. 30 minutes (low speed). After vacuum filtering, the solids were transferred back into the original jar. The extraction procedure was repeated two more times but without Polytron homogenization, pooling the extract. The weight of the extract was measured and triplicate aliquots (0.25-3.0 mL) were analyzed by liquid scintillation counting as described in Section 3.9.2. Additional aliquots of the forage and fodder extracts were cleaned-up using the Strata-X SPE procedure described in Section 3.9.3, then analyzed by HPLC as described in 3.9.4.

Approximately 20 g of grain were extracted in a similar manner, first using hexane (3 times, 175 mL total, Polytron homogenization 5 minutes during first extraction only) followed by 90/10 methanol/water (3 times, 175 mL total). The extracts were pooled by solvent per sample, the volume of each measured and triplicate aliquots (3.0 mL) were analyzed by liquid scintillation counting as described in Section 3.9.2.

3.8.2. Methanolic Base Extraction (EX2)

The tissue remaining after the neutral extraction (3.8.1) was further extracted with approx. 25-75 mL of 90/10 methanol/1.0 N NaOH, as described in Figure 5 and Table 4. The mixture was heated to approximately 50 ºC for one hour, then shaken at room temperature on a horizontal shaker for approximately 60 minutes (low speed). After vacuum filtering (or centrifuging and decanting), the solids were transferred back into the original jar. The weight of the combined extract was measured and triplicate aliquots (0.5-3.0 mL) were analyzed by liquid scintillation counting as described in 3.9.2. Additional aliquots of the forage and fodder extracts were cleaned-up using the Strata-X SPE described in Section 3.9.3, then analyzed by HPLC as described in 3.9.4.

3.8.3. Determination of the Non-Extractable Residue (NER)

The tissue remaining after the second sequential extraction (Section 3.8.2) was air-dried and weighed. Triplicate aliquots (0.05 or 0.10 g) were analyzed by oxidative combustion to determine the amount of non-extractable radioactive residue.

3.8.4. Bound Residue Determination of the Non-Extractable Residue (NER)

The general bound residue characterization scheme used was a modification of the IUPAC Technical Report (*3*).

3.8.4.1. Pectin Solubilization

The pectin substances in the NRR were solubilized using ethylenediaminetetraacetic acid (EDTA), 50 mM in pH 4.5 buffer (*4*) as described in Figure 6 and Table 4. An aliquot (1 g) of the non-extractable tissue (Section 3.8.3) was heated (approx. 80 ºC) with 100 mL of the buffered EDTA while stirring for approximately 5 hours. After cooling then vacuum filtering, the solids were transferred back into the original jar. The volume of the extract was measured and triplicate aliquots (1.0 mL) were analyzed by liquid scintillation counting as described in 3.9.2.

3.8.4.2. Lignin Extraction

The lignin was removed from the solids remaining after the pectin solubilization using a procedure adapted by Hatfield (*5*). First, the solids were transferred to flasks and covered with water (40 mL). Sodium chlorite (1.25 g $NaClO_2$) and glacial acetic acid (150 µL of CH_3COOH) were added to each solid sample, stirred, and heated in a hot water bath (approx. 70 ºC) for one hour. Additional $NaClO_2$ (0.4 g) and acetic acid (150 µL) were added to each sample, mixed thoroughly, and incubated for another hour. The solids were vacuum filtered and washed several times with water. The total amount of radioactivity in the liquid fraction, which included dissolved lignin, was determined by LSC analysis. After air-drying overnight, the remaining solids were weighed and used in the ADF Isolation procedure, below. This procedure is also described in described in Figure 6 and Table 4.

3.8.4.3. Acid-Detergent Fiber (ADF) Isolation

The ADF fraction was isolated from the solids remaining after the lignin extraction step, using a procedure adapted by Van Soest (*6*) and is also described in Figure 6 and the second page of Table 4. The pellet from the lignin extraction step (Section 3.8.4.2) was refluxed for approximately one hour in acid detergent solution (20 g hexadecyltrimethyl-ammonium bromide in 1 L 2.0 N H_2SO_4). Following the reflux period, the solids were removed by vacuum filtration through a tared glass fiber filter. The resulting filter cake was washed with water, then acetone. After drying in an oven at 100 ºC overnight, the remaining solids (this is the ADF fraction) were weighed and combusted as described in Section 3.9.1. The ADF fraction consists of cellulose and includes radioactivity encapsulated by cellulose. The total amount of radioactivity in the liquid fraction, which includes hemicellulose and dissolved plant proteins, was determined by LSC analysis.

3.8.4.4. Starch Isolation

The procedure for isolating starch was adapted from Wargo *et al* (7). Unextracted grain tissue (5 g) was weighed into a centrifuge jar and covered with 100 mL dimethyl sulfoxide (DMSO)/water (90/10, v/v) and blended at 10,000 rpm for 5-10 minutes using a Polytron homogenizer. The mixtures were shaken overnight on a horizontal shaker (low speed). The

samples were centrifuged 30 minutes at 600*g* and the supernatant decanted. Anhydrous ethanol was used to precipitate the starch from the supernatant. The starch was filtered and washed several times with anhydrous ethanol. The weight of the combined supernatant and washes was recorded and aliquots analyzed by LSC. The non-extractable residue was dried under warm air and submitted for oxidative combustion analysis. The isolated starch was dried in a 60-70 ºC oven overnight and aliquots submitted for combustion analysis.

3.8.5. Sample Storage Conditions

Samples, including milled tissue, extracts, and post-extracted samples, were stored in freezers when not in the process of analysis.

3.8.6. Metabolite Identification Procedures

Metabolites were isolated from the methanol/water extracts of the forage tissue. First, aliquots (50 mL) of the extract replicates were purified using a Strata X SPE, using the procedures described in Section 3.9.3, eluting with 20 mL methanol/water/1.0 N NaOH (80/20/1, v/v/v). The eluate was concentrated to dryness on the TurboVap (40 ºC and 10 psi nitrogen) and reconstituted in water/methanol/acetic acid (80/20/0.1, v/v/v) (3.0 mL). The reconstituted solution was analyzed by HPLC while collecting 30-second fractions. Fractions from nine such injections were collected and the fractions with the same retention time were combined. Fraction vials 27 (13.0-13.5 min), 33 (16.0-16.5 min) and 53 (26-26.5 min) were individually concentrated to dryness (TurboVap, 40 ºC) and reconstituted in 0.2-0.5 mL of water/methanol/acetic acid (80/20/0.1, v/v/v). These three concentrated fractions were submitted for MS analysis: F-A-EX1-EL-ID-F27, F-A-EX1-EL-ID-F33, and F-A-EX1-EL-ID-F53.

3.9. Instrumental Methods

3.9.1. Oxidative Combustion

Determination of TRR levels is detailed in Appendix B. The amount of total ^{14}C activity in other samples, particularly post-extracted tissue, was determined by combusting aliquots in an oxygen

atmosphere to give $^{14}CO_2$ which was trapped in an alkaline trapping reagent. The ^{14}C activity was then measured by LSC.

3.9.2. Liquid Scintillation Counting (LSC)

The liquid scintillation counters automatically converted the radioactivity counting rate in counts per minute (cpm) to disintegrations per minute (dpm) using an external standard to correct for sample quenching. The instrument was calibrated at least every six months with a set of ten quenched standards. Each day of use, the instrument was normalized and its performance was checked with respect to background cpm value, unquenched standard cpm value, and quenched standard dpm value for a range of quenched standards. The dpm value for an extraction sample was determined by LSC after diluting an appropriate aliquot of the sample with scintillation cocktail and counting for at least five minutes.

3.9.3. Strata-X Solid Phase Extraction (SPE)

Neutral organic extracts were made basic (pH >9) using 1 N NaOH. The samples were concentrated on a Turbovap (40 ºC water bath, 10 psi nitrogen) to remove the majority of the organic solvent. After acidifying the sample with 2 N HCl (pH <2.5) and diluting with water (4 mL), the sample was mixed. The Strata-X SPE cartridges (500 mg, 8B-S100-HDG, Phenomenex Inc., Torrance, California, USA) were conditioned with methanol (5 mL) followed by water (2 x 5 mL). The prepared sample was applied to the conditioned SPE, eluted at approx. 2 mL/min, collecting the eluate. The SPE was dried for 10 seconds after the SPE had eluted. The sample vial was rinsed with water/1.0 N HCl (99/1, v/v, 5 mL), transferred to the SPE cartridge, and eluted at approx. 2 mL/min, pooling with the load eluate. The SPE cartridge was dried under full vacuum for 20 seconds. The Strata-X SPE was eluted with methanol/water/1.0 N NaOH (80/20/1, v/v/v) in three aliquots (4 mL, 4 mL, 2 mL), pooling the elution aliquots.

The elution samples were concentrated to near dryness in a Turbovap (40 ºC water bath and 10 psi nitrogen). The elution samples were reconstituted in water/methanol/acetic acid

(80/20/1, 1.0 mL), and mixed well. Triplicate aliquots of each load and reconstituted elution sample were analyzed by LSC. The concentrated elution sample was also analyzed by HPLC.

3.9.4. High Performance Liquid Chromatography

The primary HPLC system (ARC-3) used for this study consisted of an Agilent 1200 Series autoinjector, degasser, binary pump, a 1200 Series variable wavelength detector, and a ν.ARC Radio-LC System on-line radioactivity detector (AIM Research Co., Hockessin, Delaware, USA). The ν.ARC sample cell was 0.8 mL, and the efficiency was approximately 75%. All components were controlled by ARC Data System software on a Dell Optiplex computer.

The primary reversed phase HPLC method used for sample analysis is presented in Table 5. The typical HPLC column used was a Synergi 4 µm Hydro-RP, 150 x 4.6 mm (Phenomenex). A direct spike of each sample analyzed by HPLC was compared to the sum of the radioactivity eluted from the column and used to determine chromatographic recovery.

Typical retention times for 2,4-D and metabolites are shown in Table 5. A typical UV chromatogram showing the retention times for 2,4-D and reference standards used in this study is provided in Figure 3.

3.9.5. Liquid Chromatography with Mass Spectral Detection (LC/MS)

The LC/MS instrumentation and conditions are described in the mass spectral report, Appendix D. In general, analysis used liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) with electrospray ionization (ESI). A Berthold radioactivity monitor (RAM) was used to assist in location of the metabolite peaks.

3.10. Method Verification and Data Handling

3.10.1. Detection Limits

The formulas used to estimate the reliability of the radioactive counting data were obtained from Currie (*8*).

$$\text{Limit of Detection (LOD)}_{(\text{dpm})} = \frac{2.71 + \left(4.65\sqrt{\text{bkg dpm} \times \text{count time}}\right)}{\text{count time}}$$

$$\text{LOD}_{(\text{ppm})} = \frac{\text{LOD}_{\text{dpm}}}{\text{Sample Weight}_{\text{g}} \times \text{Specific Activity}_{\text{dpm/µg}}}$$

$$\text{Limit of Quantitation (LOQ)}_{(\text{dpm})} = \frac{50\left(1 + \sqrt{1 + \frac{\text{bkg dpm} \times \text{count time}}{12.5}}\right)}{\text{count time}}$$

$$\text{LOQ}_{(\text{ppm})} = \frac{\text{LOQ}_{\text{dpm}}}{\text{Sample Weight}_{\text{g}} \times \text{Specific Activity}_{\text{dpm/µg}}}$$

Example: For the combustions, background was 77 dpm (forage) or 97 dpm (cobs, grain and fodder), typical aliquot weight was 0.2 g, and count time was 5 minutes.

$$\text{LOD, tissue}_{(\text{dpm})} = \frac{2.71 + \left(4.65\sqrt{77\ \text{dpm} \times 5\ \text{min}}\right)}{5\ \text{min}} = 19\ \text{dpm over background}$$

$$\text{LOD, tissue}_{(\text{ppm})} = \frac{19\ \text{dpm}}{0.2\ \text{g} \times 25{,}470\ \ \text{dpm}/\mu g} = 0.0037\ \mu\text{g/g}$$

$$\text{LOQ, tissue}_{(\text{dpm})} = \frac{50 \times \left(1 + \sqrt{1 + \left(\frac{77\ \text{dpm} \times 5\ \text{min}}{12.5}\right)}\right)}{5\ \text{min}} = 66\ \text{dpm over background}$$

$$\text{LOQ, tissue}_{(\text{ppm})} = \frac{66\ \text{dpm}}{0.2\ \text{g} \times 25{,}470\ \ \text{dpm}/\mu g} = 0.0130\ \mu\text{g/g}$$

For HPLC analyses, the background was the integration average of the last four minutes of each run.

3.10.2. Statistical Methods

Statistical analyses included calculations of means and standard deviations for the interpretation and summarization of results. Means and standard deviations were calculated using Microsoft Excel™. More decimal places than are shown in tables were used to calculate values presented in this report. Therefore, minor differences due to rounding may be found when calculating values from data in tables presented here.

3.10.3. Sample Calculations

Sample calculations may be found in Appendix C.

3.10.4. Material Balance

No material balance determinations were made. However, individual recovery results are reported for each sample analyzed by extraction.

3.10.5. Reference Values

HPLC reference values for 2,4-D and metabolites may be found in Table 5.

4.0 RESULTS AND DISCUSSION

4.1. In-Life Summary

The average radiochemical purity of the ^{14}C-2,4-D DMA test substance prior to application was determined to be 99.8% by HPLC (Figure 4). The specific activity was calculated to be 25,470 dpm/µg (3.05 mCi/mmol) for the applied ^{14}C-2,4-D DMA.

Each application solution contained 101-107% of the target amount of formulated ^{14}C-2,4-D DMA (Table 6). Overall, the plot received 589.2 mg 2,4-D DMA, equivalent to 3512 g a.e./ha or 104.4% of the target. The applications were made at an approximate 1X seasonal rate.

The average radiochemical purity (^{14}C-2,4-D) of the formulated application solutions post-application was 99.8% (99.6-100.0%). Pre-application retainer sample analyses were similar, with an average of 99.7% ^{14}C-2,4-D (98.8-100.0%), indicating stability of the test substance during storage and application. An example chromatogram, a post-retainer sample from the third application, is provided in Figure 7.

The report of the in-life phase of this study is presented in Appendix A, including weights of the harvested crop samples.

4.2. Distribution of Total Radioactive Residue

Table 7 presents the distribution of the total tissue residues within the crop harvests. Immature forage contained 3.020 mg a.e./kg. Mature grain and cobs contained 0.032 and 0.014 mg a.e./kg, respectively. Mature fodder contained 4.179 mg a.e./kg. Therefore, very little 2,4-D translocated into the grain and cobs.

4.3. Characterization and Identification of Residues

4.3.1. Neutral Organic Extraction (EX1)

The residue was initially extracted with neutral organic solvent (90:10 methanol:water) as described in Section 3.8.1 and the results are summarized in Table 8. Approximately 91% of the TRR (2.750 mg a.e./kg) was extracted from the immature forage plants using this procedure. Lower levels of radioactivity were extracted from the mature fodder (70.2% of the TRR, 2.934 mg a.e./kg). The neutral extraction removed approximately 12.3% (0.004 mg a.e./kg) and 36.5% of the TRR (0.005 mg a.e./kg) from mature grain and cobs, respectively.

An aliquot of forage and fodder neutral organic extract was prepared for HPLC as described in Section 3.8.1. The average SPE recoveries were 93.7-94.6% for the forage and fodder samples.

HPLC results are summarized in Table 9. The neutral organic extract of immature forage contained primarily parent 2,4-D, 66.5% of the TRR (2.007 mg a.e./kg), as shown in Figure 8. Low levels of the 2,4-dichlorophenol (2,4-DCP), 2.2% of the TRR, were present. The disaccharide and glucose conjugates of 2,4-DCP were also present at 12.4% and 5.6% of the TRR, respectively. No other single component accounted for more than 4% of the chromatogram (≤ 3.0% TRR).

The neutral organic extract of fodder showed a similar HPLC profile to the forage extract, as shown in Figure 9. The major components of the fodder extract were 2,4-D (45.9% of the TRR), the disaccharide and glucose conjugates of 2,4-DCP (7.7% and 10.0% of the TRR, respectively). 2,4-DCP was present at very low levels (1.7% of the TRR).

The neutral extraction removed approximately 12.3% (0.004 mg a.e./kg) and 36.5% of the TRR (0.005 mg a.e./kg) from mature grain and cobs, respectively. Two replicates of neutral extract of grain and cobs were combined, respectively, and run on a Strata X SPE. The results showed 51% and 83% of the radioactivity of grain and cobs were not retained on the SPE. The eluates were not analyzed by HPLC due to the low levels of radioactivity. The 2008 study (AAD-1 corn event 474) (*2*) can be used as a reference for the HPLC analysis results of grain and cob

neutral organic extracts. In the 2008 study, 2,4-D was detected in the grain at less than 10% of the TRR (<0.01 mg a.e./kg) and the rest of the radioactivity did not co-elute with any other reference compound.

HPLC results are summarized in Table 9. The neutral organic extracts of forage and fodder consisted of multiple components, including 2,4-D, glucose conjugates, and 2,4-DCP. The forage and fodder HPLC results of neutral organic extracts are similar to the results from the 2008 study (AAD-1 corn event 474) (*2*).

4.3.2. Methanolic Base Extraction (EX2)

The pellet remaining after the neutral extraction was next extracted with 90/10 methanol/1.0 N NaOH, as described in Sections 3.8.2 and summarized in Table 8. From the immature forage, 14.4% of the TRR was extracted with this procedure. From the mature fodder, an additional 15.6% of the radioactive residue was extracted. From mature grain and cobs, an additional 12.1% and 4.8% of the TRR was extracted with methanolic base, respectively.

Following Strata X SPE clean-up of the methanolic base extracts, the average recovery in the eluates were good (94-96%); SPE recovery values were not used in the calculation of the percentage and mg/kg levels of each metabolite in the extract.

HPLC analysis of the extract of the forage and fodder showed primarily 2,4-D and low level components of the glucose conjugates of 2,4-DCP as shown in Figure 10 (forage) and Figure 11 (fodder). Due to the low overall levels (≤ 0.005 mg a.e./kg) in grain and cobs, these extracts were not analyzed by HPLC. HPLC results for the forage and fodder are summarized in Table 9.

4.3.3. Extraction Summary

Table 8 and Table 9 summarize the amount of the TRR that was extractable and the HPLC results, respectively. More than 85% of the forage and fodder TRR was extracted and analyzed by HPLC. Less than 42% of the grain and cob TRR was extracted and the extracts were not analyzed by HPLC due to the low overall levels.

Following three applications of 2,4-D DMA to AAD-1 corn, the primary residue in the forage and fodder was 2,4-D. Forage and fodder contained approximately 20% of the TRR as the combined disaccharide of 2,4-DCP and the glucose conjugate of the 2,4-DCP, and very low levels of 2,4-DCP (<2.5% of the TRR). Overall, 99.7% of the forage TRR and 78.4% of the fodder TRR, respectively, was characterized, as shown in Table 9. These results were similar to the 2008 study (AAD-1 corn event 474) (*2*).

4.3.4. Bound Residues

As shown in Table 8 the amount of non-extractable, or bound radioactivity, exceeded 70% of the TRR in grain and cobs (0.029 and 0.011 mg a.e./kg). However, the bound residues were not evaluated as bound radioactivity was lower than 0.050 mg a.e./kg. Refer to the 2008 study (AAD-1 corn event 474) (*2*) for bound residue analysis of grain and cobs.

The bound residues of forage and fodder were evaluated as described in Section 3.8.4, and the results are shown in Table 10. In the forage and fodder, the non-extractable radioactivity was associated with and distributed amongst the natural components; pectin (3.4% and 5.9% of the TRR, respectively), lignin (1.7% and 2.2% of the TRR, respectively), ADF soluble fraction (1.2% and 1.3% of the TRR, respectively), and approximately 0.4-0.5% of the TRR in the ADF. The 2008 study (AAD-1 corn event 474) (*2*) demonstrated similar levels of incorporation or encapsulation of radioactivity into pectin and lignin of forage and fodder tissue. The 2008 study also demonstrated that the grain radioactivity was associated with primarily ADF soluble and lignin fractions.

Table 11 shows the results of the starch isolation. Overall recoveries were 176%, and were therefore normalized to 100%. Approximately 11.9% (6.1% when normalized to 100% recovery) of the radioactivity was extracted with DMSO, while 36.7% of the TRR precipitated as starch (20.6% when normalized to 100% recovery). The results indicate that a significant portion of the radioactivity, approximately 21% of the TRR in grain, is closely associated with starch. The 2008 study (AAD-1 corn event 474) (*2*) also demonstrated approximately 30% of the radioactivity in grain was associated with starch.

4.4. Degradate and Metabolite Isolation and Identification

Metabolites were isolated from the forage tissue, as described in Section 3.8.6. These three samples were submitted for LC/MS/MS analyses. In addition to parent 2,4-D, 2,4-DCP and two glucose conjugates of 2,4-DCP were detected, as described in the mass spectral report, Appendix D. 2,4-DCP was observed under negative ESI condition (MW 162). The glucose conjugate of 2,4-DCP was observed as the formate adduct (MW 370) and was observed in both AAD-1 corn and AAD-12 soybeans (*9*). The disaccharide conjugate of 2,4-DCP (also observed as a formate adduct, MW 532) contains two glycosides. The mass spectral data is provided in Appendix D.

4.5. Sample Storage Stability

All samples and extracts were stored frozen at approximately -20 ºC when not in use. Initial analyses of extracts occurred within about 7-12 weeks, as shown in Table 3. Repeat analyses of extracts stored frozen showed results similar to the initial analyses, demonstrating stability of the extracts under these storage conditions.

4.6. Metabolic Pathway

The proposed metabolic pathway is presented in Figure 12 and metabolite structures are provided in Table 12. As shown in the diagram, the metabolism of 2,4-D DMA in AAD-1 transformed maize proceeds rapidly through dissociation to the acid. After cleavage of the acid side chain, the resulting 2,4-dichlorophenol (2,4-DCP) is rapidly conjugated with glucose. Very low levels of free 2,4-DCP compared to conjugated 2,4-DCP indicate that conjugation is rapid and a preferential route of metabolism. Metabolism proceeds through natural incorporation of the radiolabeled carbon into natural plant constituents, such as pectin, lignin, and starch. The proposed metabolic pathway is the same as for the 2008 study (AAD-1 corn event 474) (*2*).

Plant metabolism studies conducted on similarly transformed AAD-12 soybeans (*9*) also concluded that metabolism proceeds to 2,4-DCP and glucose conjugates of 2,4-DCP.

Overall, the ^{14}C-residues in the AAD-1 transformed maize were similar to those observed on the transformed soybeans and conventional crops.

5.0 CONCLUSIONS

Three applications of 2,4-D DMA salt at approximately the maximum proposed seasonal application rate resulted in immature forage and mature grain, cobs, and fodder that contained 3.020, 0.032, 0.014, and 4.179 mg acid equivalents per kg, respectively.

The major component in forage and fodder was identified as parent, 77.4% and 56.5% of the TRR (2.338 mg a.e./kg and 2.362 mg a.e./kg), respectively. Glucose-conjugated 2,4-DCP was identified in forage and fodder. Approximately 21% of the TRR in grain (0.007 mg a.e./kg) was associated with starch. Extensive metabolism of 2,4-D was demonstrated by incorporation or encapsulation of radioactivity into natural plant constituents such as pectin, lignin, and starch.

In summary, the majority of the radioactive residue was characterized as 2,4-D and free or conjugated 2,4-DCP.

6.0 RETENTION OF RECORDS

Original raw data, as defined by 40 CFR 160, the signed protocol original, amendments, deviations, and the signed original of the final report are retained in the archives of Dow AgroSciences located at 9330 Zionsville Road, Indianapolis, Indiana 46268-1054.

7.0 REFERENCES

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Table 1. Physico-chemical properties of 2,4-D DMA Salt or 2,4-D

Parameter	Values	Comments
Water solubility	@ 25 ºC	
pH 5	321 g/L	Reference 1
pH 7	729 g/L	Reference 1
pH 9	664 g/L	Reference 1
unbuffered		
Vapor Pressure	$<1 \times 10^{-7}$ mm Hg @ 26 ºC	Reference 2
pK_a	acid: 2.87	Reference 3
Log K_{ow}		
Unbuffered	N/A	
pH 5	acid: 0.04	Reference 4
pH 7	acid: -0.91	Reference 4
pH 9	acid: -1.04	Reference 4
Organic Solvent Solubilities		
Acetone	N/A	
Acetonitrile	1.06 g/100 mL	Reference 5
Methanol	>50 g/100 g	Reference 5
Octanol	5.41 g/100 mL	Reference 5
Toluene	0.165 g/100 mL	Reference 5
Hexane	0.00357 g/100 mL	Reference 5

N/A = not available

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Table 2. Experimental Design

Parameter	Description – Foliar Plot
Test Site	Research For Hire (RFH) 1696 South Leggett Street, Porterville, California 93257, USA
Soil type	sandy clay loam
Crop type	AAD-1 corn, event-construct pDAS1740-278
Application formulation	soluble liquid
Application timing and target rate (crop stage)	1121 g a.e./ha – pre-emergent 1121 g a.e./ha – V4 1121 g a.e./ha – V8 or 48 inches, whichever comes first
Immature harvests	forage (R4)
Mature harvest	grain, cobs, and fodder
Other details	none

Table 3. Significant Events

Event	Date	Days after First Application	Days after Harvest
Pre-emergent Application	12 June 2009	0	Not Applicable
Foliar Application #1 (V4)	02 July 2009	20	Not Applicable
Foliar Application #2 (V8)	14 July 2009	32	Not Applicable
Immature Harvest – Forage (R4)	25 August 2009	74	Not Applicable
Milling Completed	01 September 2009	Not Applicable	7
Combustion Analysis	04 September 2009	Not Applicable	10
Initiate Extraction	16 October 2009	Not Applicable	52
HPLC characterization init.	26 October 2009	Not Applicable	62
HPLC charact. complete	26 October 2009	Not Applicable	62
Mass Spectral Analysis initial	12 March 2010	Not Applicable	199
Mass Spectral Analysis final	19 March 2010	Not Applicable	206
Mature Harvest - Grain	18 September 2009	98	Not Applicable
Milling Completed	13 October 2009	Not Applicable	25
Combustion Analysis	23 October 2009	Not Applicable	35
Initiate Extraction	25 November 2009	Not Applicable	68
Mature Harvest - Cobs	18 September 2009	98	Not Applicable
Milling Completed	13 October 2009	Not Applicable	25
Combustion Analysis	23 October 2009	Not Applicable	35
Initiate Extraction	19 November 2009	Not Applicable	62
Mature Harvest - Fodder	18 September 2009	98	Not Applicable
Milling Completed	15 October 2009	Not Applicable	27
Combustion Analysis	23 October 2009	Not Applicable	35
Initiate Extraction	18 November 2009	Not Applicable	61
HPLC characterization init.	11 December 2009	Not Applicable	84
HPLC charact. complete	05 March 2010	Not Applicable	168

Table 4. Typical Sample Collection and Analysis Procedure for All Tissue Samples Except Where Noted (See Also Figure 6)

Parameter		Description
Neutral Extraction (EX1) *	Solvent	Methanol/water (90/10, v/v)
	Procedure*	Weigh approximately 10 or 20 g milled tissue* Add extraction solvent (approx. 25-75 mL) Polytron homogenize (~5 min @ ≥10K rpm) Shake on horizontal shaker (low speed) ~30 min Vacuum filter Record weight of the extract Transfer tissue back into original jar
	Method of analyses	LSC of triplicate aliquots of the extract
Methanolic Base Extraction (EX2)	Solvent	Methanol/1.0 N NaOH (90/10, v/v)
	Procedure	Add extraction solvent (approx. 25 or 50 mL) Heat to approximately 50 ºC for 1 hour Shake on horizontal shaker (low speed) ~60 min Vacuum filter Record weight of the extract
	Method of analyses	LSC of triplicate aliquots of the extract
Extracted Tissue Combustion	Solvent	NA
	Procedure	Obtain weight of remaining tissue sample
	Method of analyses	Oxidative combustion of triplicate aliquots

*Extracted approximately 10 g of forage or fodder tissue, or 20 g of grain or cob tissue.

Table 4, Cont. Typical Sample Collection and Analysis Procedure for All Tissue Samples Except Where Noted (See Also Figure 6)

Bound Residue Analysis

Pectin Extraction	Solvent	Ethylenediaminetetraacetic acid (EDTA), 50 mM in 50 mM pH 4.5 buffer
	Procedure	Reference 4
		Sonicate 2 minutes Heat (80 ºC) & stir approx. 5 hours Cool Vacuum filter, record volume
	Method of analyses	LSC of triplicate aliquots of the filtrate Continue analysis of tissue with Lignin Extraction
Lignin Extraction	Solvent	Sodium chlorite
	Procedure	Reference 5
		Transfer solids from step above to a flask Cover with 40 mL water Add sodium chlorite (1.25 g) and glacial acetic acid (150 µL) to flask and mix well Heat to 70 ºC for 1 hour Add additional sodium chlorite (0.4 g) and glacial acetic acid (150 µL), and mix well Heat to 70 ºC for 1 hour Vacuum filter Wash solids several times with water
	Method of analyses	LSC of triplicate aliquots of the filtrate Continue analysis of tissue with ADF Isolation
ADF Isolation	Solvent	Hexadecyltrimethylammonium bromide (20 g) in 1 L 2.0 N H_2SO_4
	Procedure	Reference 6
		Reflux 1 hour Vacuum filter Rinse solids with water and acetone Record volume of filtrate
	Method of analyses	LSC of triplicate aliquots of the filtrate Combust ADF

Table 5. HPLC Conditions

Program:	ν.ARC System (LC-ARC-3)	
Column	Synergi 4µm Hydro-RP, 150 x 4.6 mm	
Flow Rate	1.0 mL/min	
Radioactivity Detection	Agent (Cocktail)	StopFlow AD
	Ratio	1.0
	Efficiency	approximately 75%
	Stop-Flow Mode	DynamicFlow
	DynamicFlow Start	0.00 min
	DynamicFlow Stop	50.00 min
UV Detection	280 nm	
Solvent A	0.1% acetic acid in water	
Solvent B	0.1% acetic acid in 80/20 acetonitrile/methanol	
Time (min)	Solvent Elution	
0.0	90/10 A/B initial conditions, begin linear gradient	
20.0	50/50 A/B, linear gradient	
30.0	5/95 A/B, begin 10 minute hold	
40.0	5/95 A/B, linear gradient to original conditions	
41.0	90/10 A/B, begin re-equilibration	
50.0	90/10 A/B, end run	

Compound	HPLC Retention Time (min)[a]
4OH 2,5-D	14.2
X11963417	14.4
4-CPAA	18.4
4-CP	20.2
2,4-D	22.3
phenol	24.6

[a] All reference values are approximate, and may vary slightly due to temperature, column age, matrix, sample size, etc.

Table 6. Application Rate of 2,4-D DMA Applied to AAD-1 Corn (event 278)

Application	Actual (dpm/0.10 mL)	Volume (mL)[a]	Amount Applied (mg a.i.)[b]	Application Rate (g a.e./ha)[c]	Target Application Rate (g a.e./ha)[d]	% of Target
1 - pre-emergent	3,436,400	148.7	200.6	1196	1121	106.7
2 - foliar	3,403,449	148.7	198.7	1184	1121	105.7
3 - foliar	3,252,408	148.7	189.9	1132	1121	101.0
Total Applied (Seasonal):			589.2	3512	3363	104.4

[a] Volume actually applied, after aliquots removed for storage stability and LSC, if applicable.

[b] Amount applied = $\frac{\text{amount (dpm/mL)} \times \text{volume (mL)}}{\text{specific activity (dpm/µg)} \times 1000\ \mu g / mg}$, where specific activity is 25,470 dpm/µg.

[c] Application rate = $\frac{\text{Amount Applied (mg)}}{1000\ \text{mg/g} \times \text{plot size (ha)}} \times 0.831$, where plot size was 1.39 x 10^{-4} ha and factor 0.831 converted from a.i. to a.e. (see sample calculations) (rounding differences noted).

[d] Target does not include any overages.

Table 7. Total Radioactive Residues in Plant Samples Collected for 2,4-D DMA Nature of Residue in AAD-1 Corn (event 278) Study

Sample	dpm/g	mg a.e./kg (ppm)
forage (immature plants)	92,596	3.020
mature grain	984	0.032
mature cobs	422	0.014
mature fodder	128,137	4.179

Table 8. Fractionation of the Residues in 2,4-D Treated AAD-1 Corn (event 278) RACs (Average of Duplicates)

	TRR[a]	Neutral Organic Extract		Basic Methanol Extract		Non-Extractable		Total Recovered	
Sample ID	mg a.e./kg	%TRR	mg a.e./kg	%TRR	mg a.e./kg	%TRR	mg a.e./kg	%TRR	mg a.e./kg
Forage	3.020	91.1%	2.750	14.4%	0.436	8.3%	0.252	114	3.438
Grain[b]	0.032	12.3%	0.004	12.1%	0.004	90.3%	0.029	118	0.038
Cobs	0.014	36.5%	0.005	4.8%	0.001	78.4%	0.011	120	0.016
Fodder	4.179	70.2%	2.934	15.6%	0.651	11.8%	0.491	98	4.075

[a] From Table 7.
[b] Grain replicates were each first extracted with hexane, followed by the neutral organic extract. The hexane removed an average of 2.7% of the TRR, 0.001 mg a.e./kg.

Table 9. 2,4-D and Metabolite Levels (Acid Equivalents) In Extracts of 2,4-D Treated AAD-1 Corn (event 278) RACs (Average of Duplicates)

Sample ID	2,4-D 22.2 min		disaccharide conjugate of 2,4-DCP 11.7 min		glucose conjugate of 2,4-DCP 14.7 min		2,4-dichlorophenol (2,4-DCP) 24.7 min		unidentified[a] extractable residue	
	% TRR	mg a.e./kg	% TRR	mg a.e./kg	% TRR	mg a.e./kg	% TRR	mg a.e./kg	% TRR	mg a.e./kg
Forage										
neutral extract	66.5%	2.007	12.4%	0.373	5.6%	0.170	2.2%	0.067		
base extract	11.0%	0.331	0.6%	0.018	1.4%	0.042	0.1%	0.003		
total	77.4%	2.338	13.0%	0.391	7.0%	0.212	2.3%	0.069	0.3%	0.009
Fodder										
neutral extract	45.9%	1.917	7.7%	0.324	10.0%	0.419	1.7%	0.072		
base extract	10.6%	0.444	0.6%	0.024	1.9%	0.078	0.0%	0.000		
total	56.5%	2.362	8.3%	0.347	11.9%	0.497	1.7%	0.072	21.6%	0.901

[a] Unidentified radioactivity did not elute with 2,4-D, the glucose conjugates of 2,4-DCP or 2,4-DCP.

Table 10. Fractionation of the Bound Residues in 2,4-D Treated AAD-1 Corn (event 278) (Average of Duplicates)

	Bound Residue[a]		Pectin		Lignin		ADF soluble		ADF		Recovery
Sample ID	%TRR	mg a.e./kg	%TRR	mg a.e./kg	%TRR	mg a.e./kg	%TRR	mg a.e./kg	%TRR	mg a.e./kg	%[b]
Forage	8.3	0.252	3.4	0.104	1.7	0.053	1.2	0.037	0.4	0.011	81.2
Fodder	11.8	0.491	5.9	0.246	2.2	0.091	1.3	0.052	0.5	0.021	83.7

[a] Values from Table 8
[b] Procedural recovery for the 4-step process.

Table 11. Starch Isolation from Mature Grain of AAD-1 Corn (event 278) Treated with ^{14}C-2,4-DMA (Average of Duplicates)

Sample ID	Non-Extractable		Extractable (non-starch)		Starch		Recovery
	% TRR	mg a.e./kg	% TRR	mg a.e./kg	% TRR	mg a.e./kg /g	
Grain[a]	73.7	0.024	6.1	0.002	20.6	0.007	100%

[a] Recovery was 176% prior to normalization.

Table 12. Metabolites of 2,4-D Applied to AAD-1 Maize (event 278)

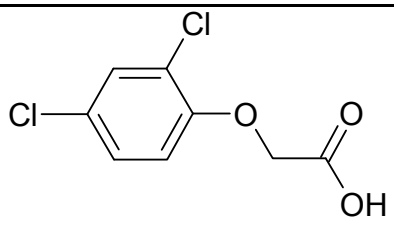
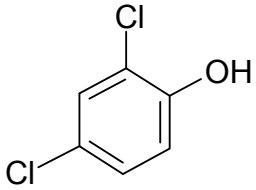
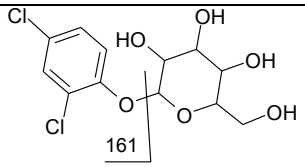
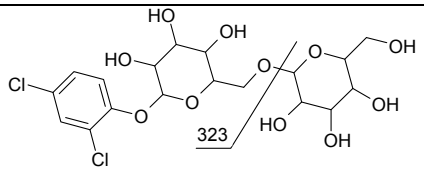
Common name/code or ID	Chemical Name	Chemical Structure
2,4-D	2,4-dichlorophenoxy acetic acid	
2,4-DCP	2,4-dichlorophenol	
X11963417, glucose conjugate of 2,4-DCP	2,4-dichlorophenyl β-D-glucopyranoside	
disaccharide of 2,4-DCP	2,4-dichlorophenyl 6-*O*-hexopyranosylhexopyranoside	 or isomer

Figure 1. Chemical Nomenclature and Structures of 14C-2,4-D

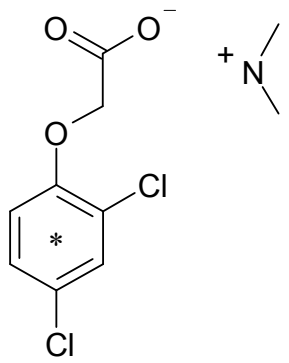
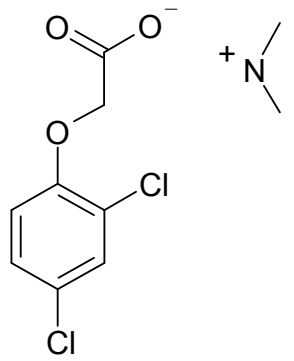
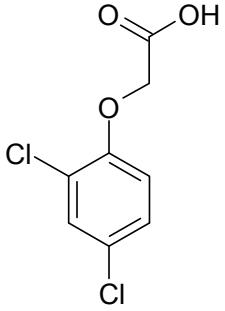
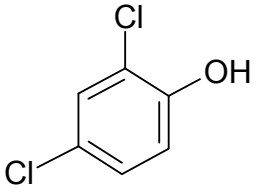
	Test Substance	Structure
Common Name	14C-2,4-D DMA salt	
Synonyms	^{14}C-2,4-D	
Chemical Name	2,4-dichlorophenoxy acetic acid-dimethylammonium salt, Phenyl ring-14C (UL)	
Inventory Number	INV027475-0002	
FA & PC Reference	FAPC-024455-0	
SPS Reference	DE3-100044-13	
Specific Activity	39.4 mCi/mmol	
Radiochemical purity	99.1%	
GLP analysis	Yes, 4/16/2009	* denotes ^{14}C

Figure 2. Chemical Nomenclature and Structures of 2,4-D Reference Standards and Formulation Blank

	Reference Substances	Structure
Common Name	**2,4-D DMA**	
Synonyms	TSN100485	
CAS Nomenclature	acetic acid, (2,4-dichlorophenoxy)-,compd. with N-methylmethanamine (1:1)	
IUPAC Nomenclature	2,4-dichlorophenoxy acetic acid dimethylamine salt	
CAS Number	2008-39-1	
Molecular Weight	266.12 g/mole	
Molecular Formula	$C_8H_5Cl_2O_3 \cdot C_2H_8N$	
Purity	>99%	

Common Name	**2,4-D**	
Synonyms	AGR275828	
CAS Nomenclature	(2,4-dichlorophenoxy)acetic acid	
IUPAC Nomenclature	2,4-dichlorophenoxy acetic acid	
CAS Number	94-75-7	
Molecular Weight	221.04 g/mole	
Molecular Formula	$C_8H_6Cl_2O_3$	
Purity	99.5%	

Common Name	**2,4-dichlorophenol**	
Synonyms	AGR182992, 2,4-DCP	
CAS & IUPAC Nomenclature	2,4-dichlorophenol	
CAS Number	000120-83-2	
Molecular Weight	163.00 g/mole	
Molecular Formula	$C_6H_4Cl_2O$	
Purity	99%	

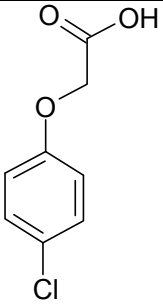
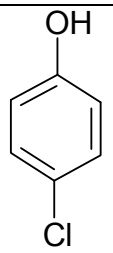
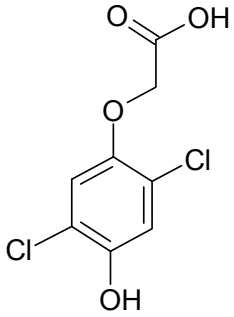
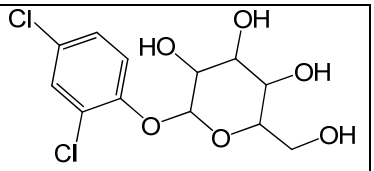
Common Name	**4-CPAA**	
Synonyms	TSN100163, 4-CPA	
CAS Nomenclature	acetic acid, (4-chlorophenoxy)-	
IUPAC Nomenclature	4-chloro-phenoxyacetic acid	
CAS Number	122-88-3	
Molecular Weight	186.60 g/mole	
Molecular Formula	$C_8H_7ClO_3$	
Purity	99%	

Figure 2, Cont. Chemical Nomenclature and Structures of 2,4-D Reference Standards and Formulation Blank

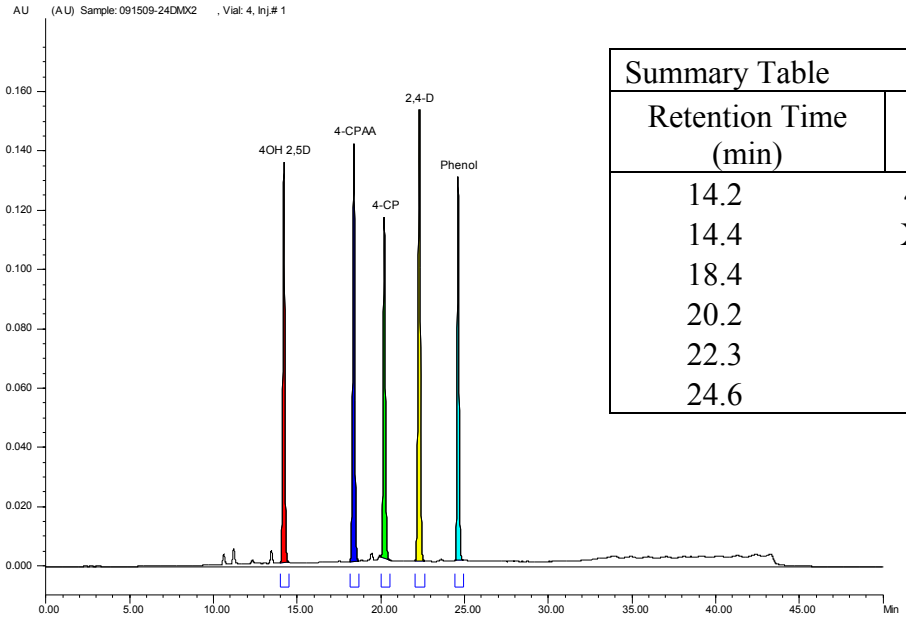
	Reference Substances	Structure
Common Name	**4-CP**	
Synonyms	TSN100174	
CAS & IUPAC Nomenclature	4-chlorophenol	
CAS Number	000106-48-9	
Molecular Weight	128.56 g/mole	
Molecular Formula	C_6H_5ClO	
Purity	99.7%	

Common Name	**4-OH 2,5-D**	
Synonyms	TSN100130	
CAS Nomenclature	not available	
IUPAC Nomenclature	2,5-dichloro-4-hydroxyphenoxyacetic acid	
CAS Number	not available	
Molecular Weight	275.13 g/mole	
Molecular Formula	$C_8H_5Cl_2O_4 \cdot K$	
Purity	91.5%	

Common Name	**Glucose conjugate of 2,4-dichlorophenol (**X11963417)	
Synonyms	TSN033048-0001	
CAS & IUPAC Nomenclature	not available	
CAS Number	not available	
Molecular Weight	325.15 g/mole	
Molecular Formula	$C_{12}H_{14}Cl_2O_6$	
Purity	100%	

	Formulation Blank		
Common Name	XRM-4436 Formulation Blank		
Formulation Type	soluble liquid (SL)		
Description	Component	Technical Wt%	Batch (g)
	dimethylamine (DMA)	5.00	11.80
	Polyglycol P-4000	0.10	0.24
	Versene Acid (EDTA)	3.00	7.08
	Water	45.34	107.00
	2,4-D (added later)	46.56	
	total	100.00	

Figure 3. Chromatograms of Test and Reference Substances with Retention Times Clearly Indicated for Each Substance (See Table 5 for HPLC Conditions)



Summary Table	
Retention Time (min)	Reference
14.2	4OH 2,5-D
14.4	X11963417
18.4	4-CPAA
20.2	4-CP
22.3	2,4-D
24.6	phenol

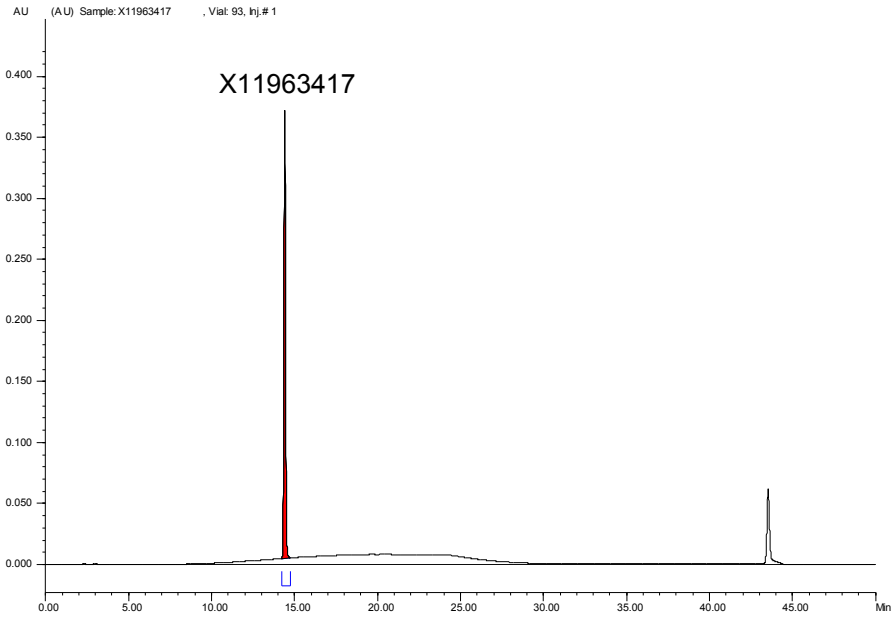
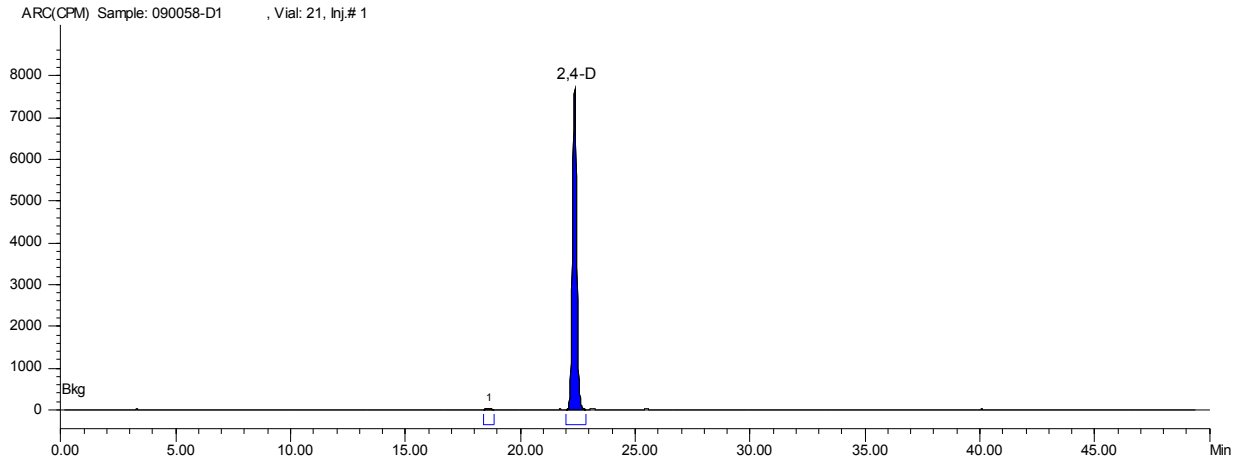


Figure 4. Radio-chromatogram of ^{14}C-2,4-D Test Substance (Dilution A)



Summary Table		
Retention Time (min)	% of HPLC	Reference
22.4	99.8	2,4-D (free acid)
HPLC recovery 106%		

Figure 5. Schematic Flowchart for the Analysis of Crop Fractions (See Also Table 4)

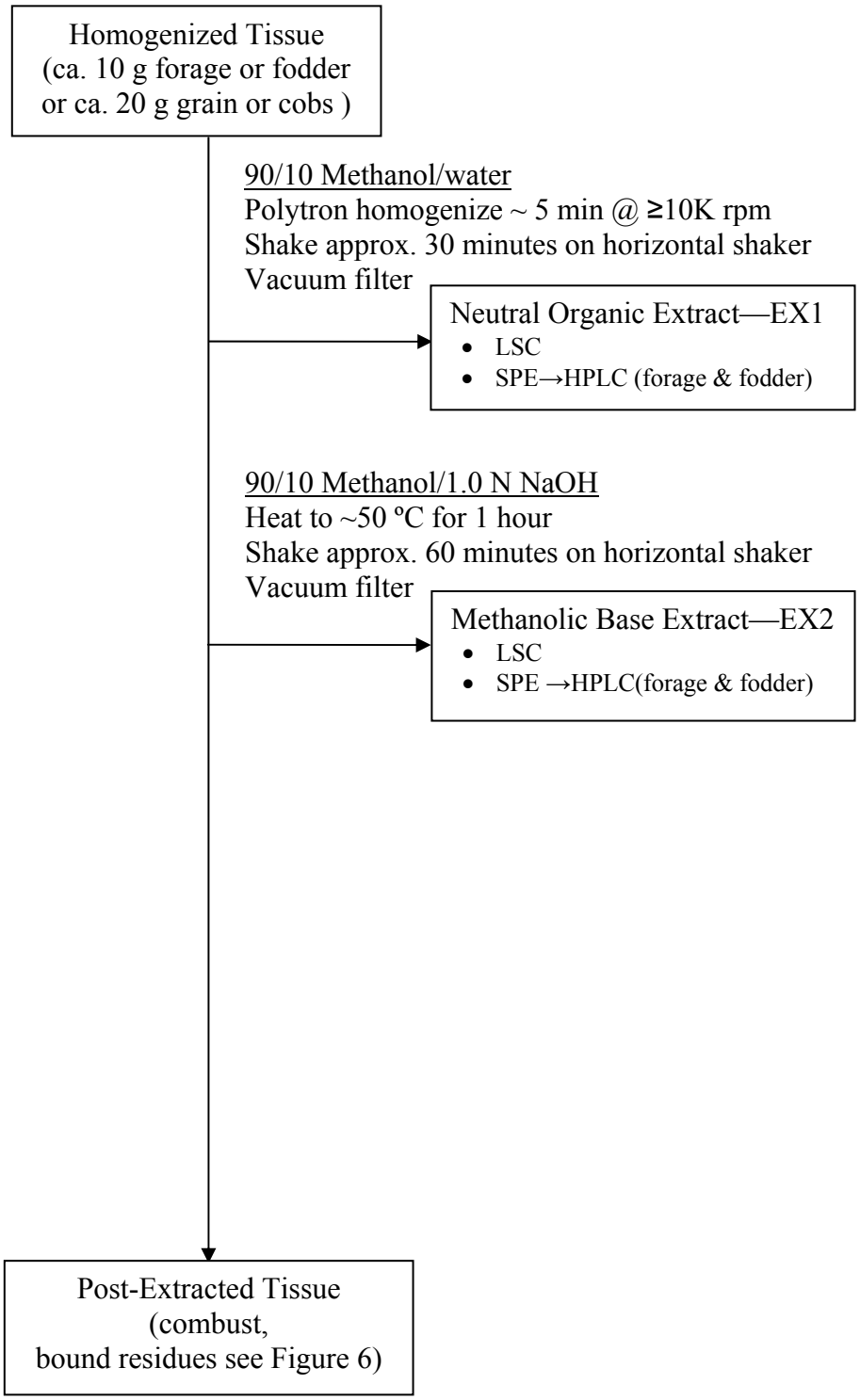


Figure 6. Schematic Flowchart for the Analysis of Bound Residues in Crop Fractions

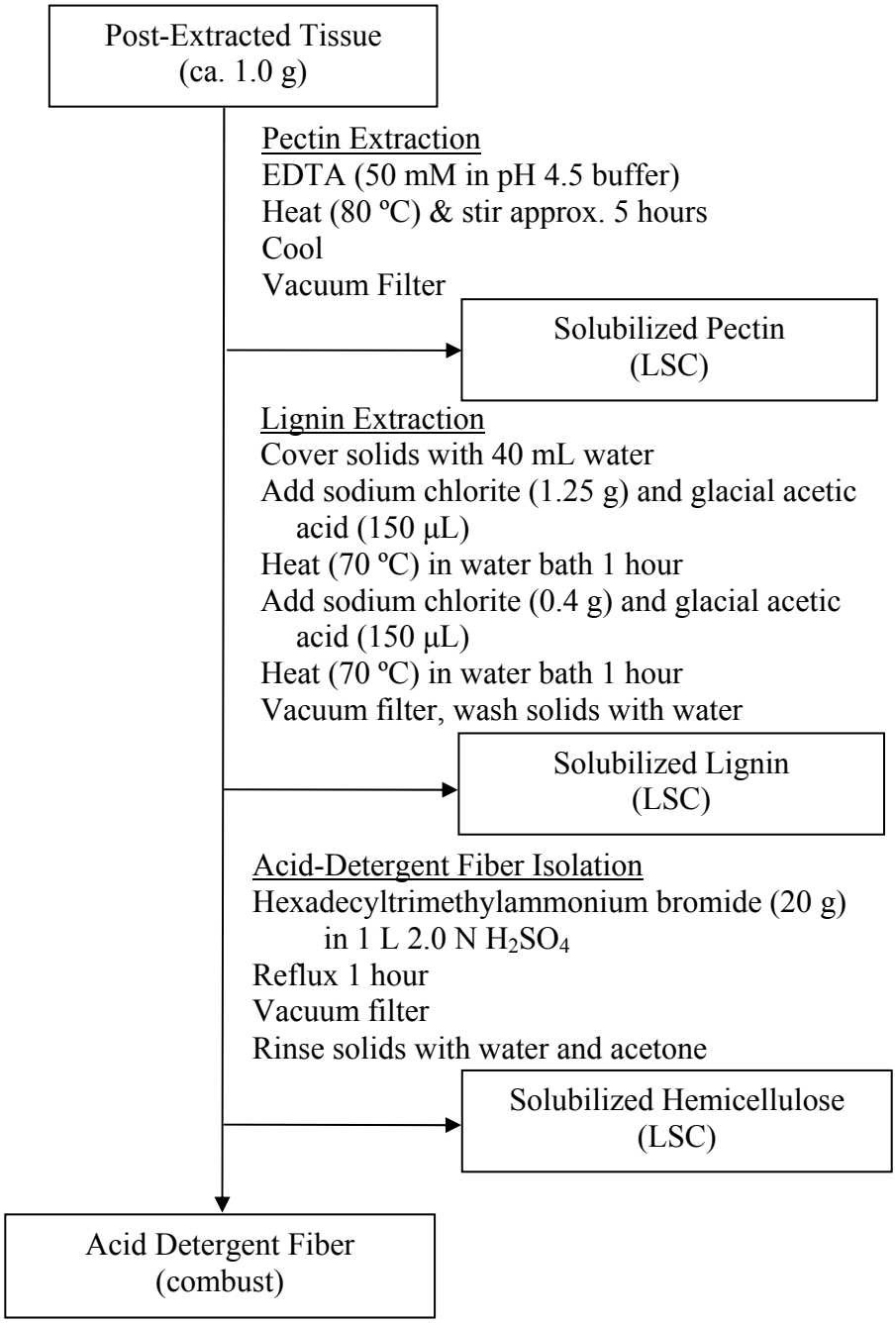
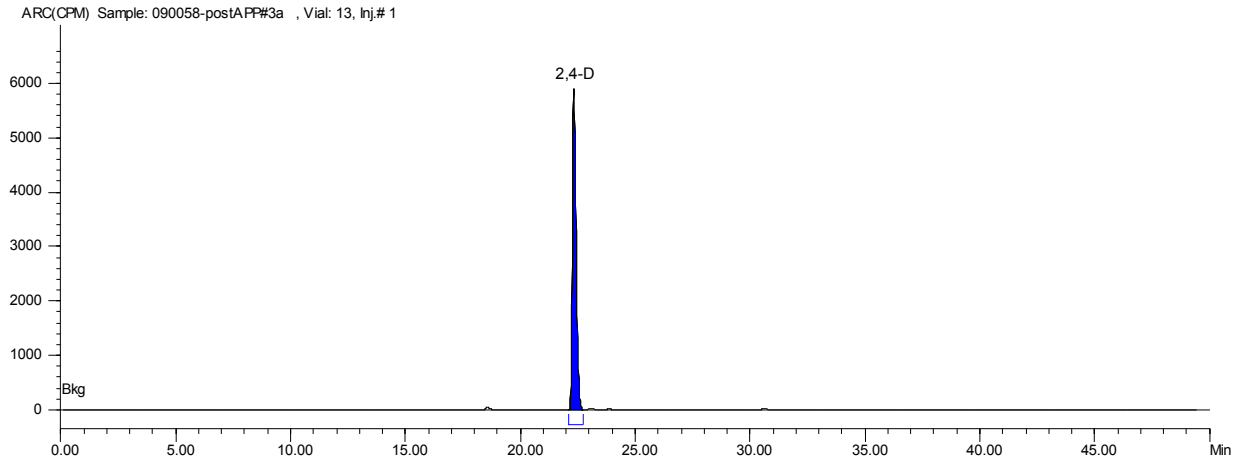
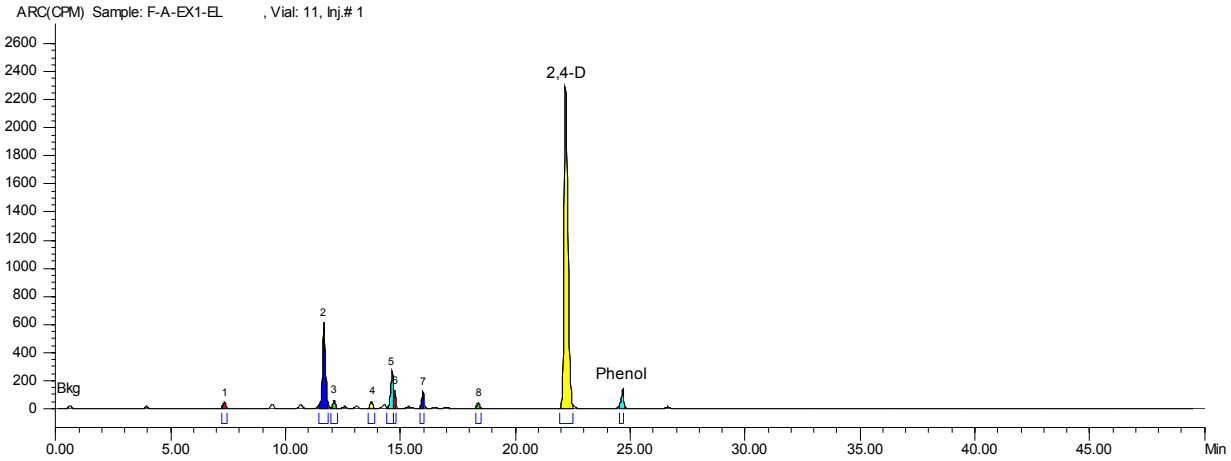


Figure 7. HPLC Chromatogram Indicating Radiochemical Purity of the ^{14}C-2,4-D DMA After the Third Application



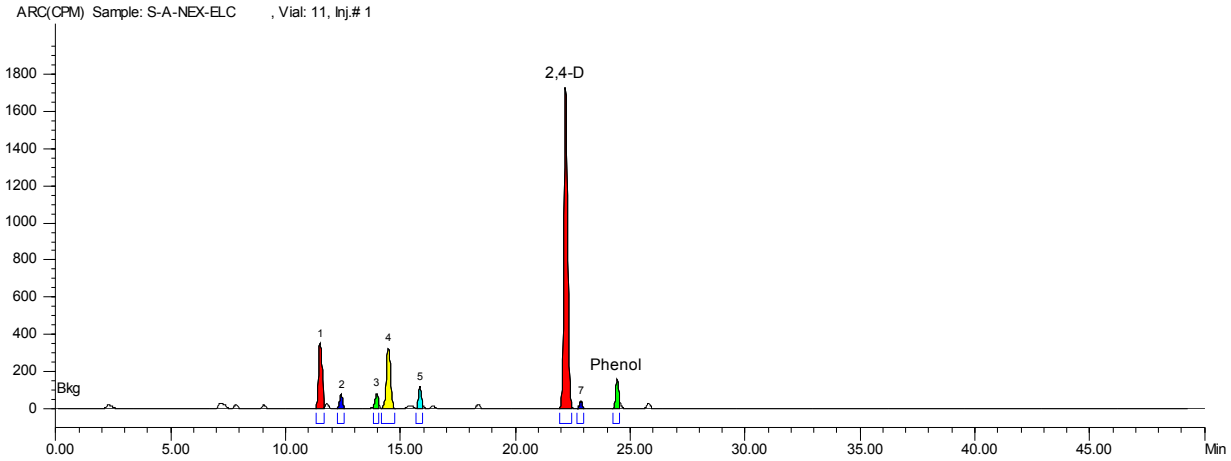
Summary Table		
Retention Time (min)	% of HPLC	Reference
22.3	99.6	2,4-D
HPLC recovery 97.6%		

Figure 8. HPLC Radio-Chromatogram of the Immature Forage – Neutral Organic Extract (Replicate A)



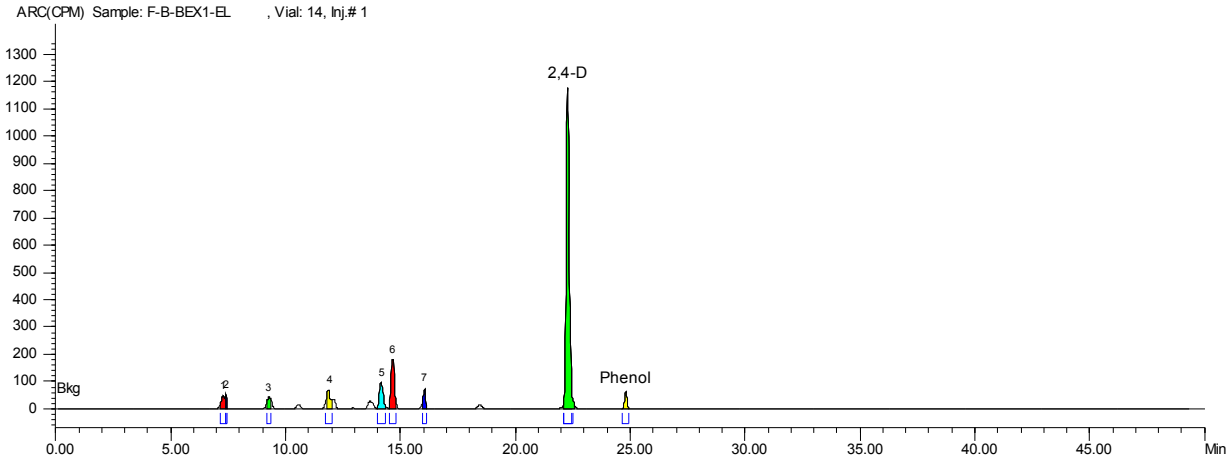
Summary Table			
Retention Time (min)	% of HPLC		Peak ID (#)
7.3	0.5		(1)
11.7	14.4		disaccharide conjugate of 2,4-DCP (2)
12.1	0.6		(3)
13.7	0.5		(4)
14.6	4.1	5.8	glucose conjugate of 2,4-DCP (5,6)
14.7	1.7		
16.0	1.4		(7)
18.4	0.4		(8)
22.2	73.7		2,4-D
24.7	2.7		phenol
HPLC recovery 81.7%			

Figure 9. HPLC Radio-Chromatogram of the Fodder – Neutral Organic Extract (Replicate A)



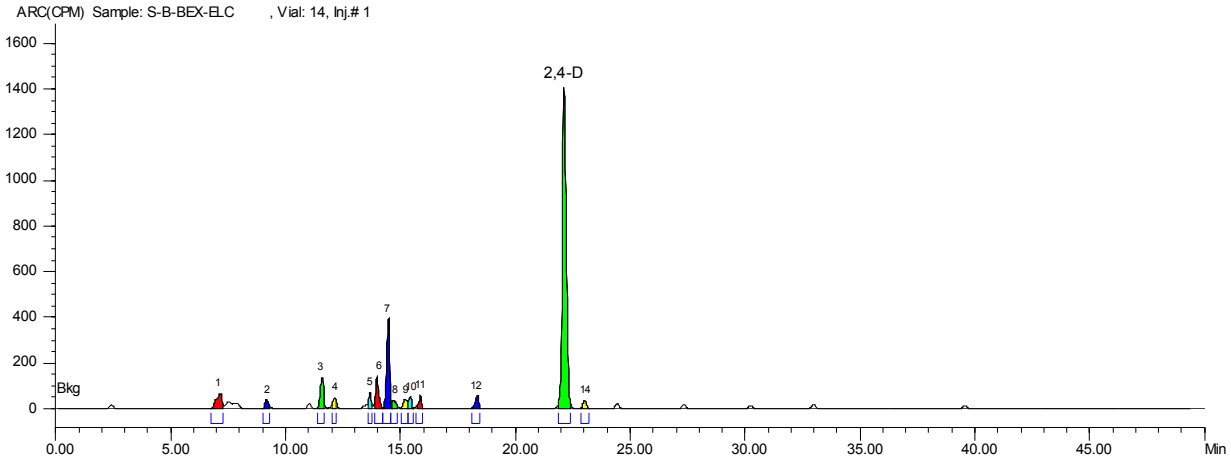
Summary Table		
Retention Time (min)	% of HPLC	Peak ID (#)
11.5	10.3	disaccharide conjugate of 2,4-DCP (1)
12.4	1.1	(2)
14.0	1.5	(3)
14.5	14.9	glucose conjugate of 2,4-DCP (4)
15.8	2.4	(5)
22.2	67.2	2,4-D (6)
22.9	0.5	(7)
24.4	2.2	phenol (8)
HPLC recovery 77%		

Figure 10. HPLC Radio-Chromatogram of the Immature Forage – Methanolic Base Extract (Replicate B)



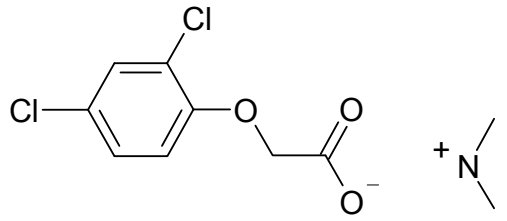
Summary Table		
Retention Time (min)	% of HPLC	Peak ID (#)
7.3	1.4	(1)
7.4	1.6	(2)
9.3	2.2	(3)
11.9	3.1	disaccharide conjugate of 2,4-DCP (4)
14.1	3.8	(5)
14.6	9.4	glucose conjugate of 2,4-DCP (6)
16.1	1.6	(7)
22.3	75.5	2,4-D
24.8	1.4	phenol
HPLC recovery 91%		

Figure 11. HPLC Radio-Chromatogram of the Fodder – Methanolic Base Extract (Replicate B)

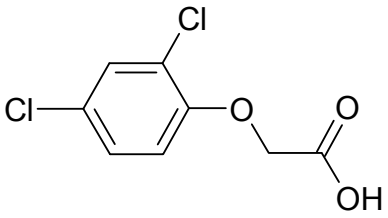


Summary Table			
Retention Time (min)	% of HPLC		Peak ID (#)
7.2	3.0		(1)
9.2	0.8		(2)
11.6	3.7		disaccharide conjugate of 2,4-DCP (3)
12.1	1.3		(4)
13.7	1.3		(5)
14.0	3.8		(6)
14.5	12.0		glucose conjugate of 2,4-DCP (7)
14.7	0.9		(8)
15.2	0.8		(9)
15.4	1.0		(10)
15.9	1.6		(11)
18.4	1.4		(12)
22.1	68.0		2,4-D (13)
23.0	0.6		(14)
HPLC recovery 87%			

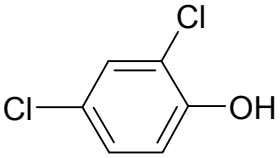
Figure 12. Proposed Metabolic Pathway for 2,4-D DMA salt in AAD-1 Maize



2,4-D DMA salt



2,4-D



2,4-dichloro-phenol (2,4-DCP)

glucose conjugates of 2,4-DCP
and natural incorporation/encapsulation

Appendix A—In-Life Report

(Research For Hire)

IN-LIFE PHASE FINAL REPORT

STUDY TITLE

A Nature of the Residue Study with [^{14}C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

SPONSOR

Dow AgroSciences, LLC
9330 Zionsville Road
Indianapolis, IN 46268

DATA REQUIREMENT

EPA (OPPTS 860.1300), OECD 501 Metabolism in Crops
(8 January 2007)

PRINCIPAL INVESTIGATOR AND IN-LIFE PHASE TEST SITE

Larissa Tersteege
Research For Hire
1696 South Leggett Street
Porterville, CA 93257

STUDY DIRECTOR AND TESTING FACILITY

Mingming Ma
Regulatory Sciences and Government Affairs-Indianapolis Lab
Dow AgroSciences, LLC
9330 Zionsville Road
Indianapolis, IN 46268

IN-LIFE PHASE COMPLETION DATE

September 18, 2008

STUDY IDENTIFICATION

Dow AgroSciences: Protocol No. 090058 Project No.10001686-5051-1
Research For Hire: Study Number R050907

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

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

These data are the property of Dow AgroSciences, LLC and as such are considered to be confidential for all purposes other than compliance with FIFRA Section 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality, which may exist under any other statute or in any other country.

Company: Research For Hire

Company Agent: John S. Corkins

Title: General Manager

John S. Corkins
General Manager

2/25/10
Date

REGULATORY COMPLIANCE STATEMENT

Study Title: A Nature of the Residue Study with [^{14}C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

I was directly involved with the conduct and supervision of the above-captioned study and do herby certify that, with the following exceptions, this study was conducted in accordance with the Environmental Protection Agency's Good Laboratory Practice Regulations (40 CFR 160) with the following exceptions:

1. Weather Data

2. Equipment used for plot maintenance is calibrated prior to use but not GLP verified.

Larissa Tersteege
Principal Field Investigator

2/25/10
Date

QUALITY ASSURANCE STATEMENT

The in-life phase of this study was monitored by the Quality Assurance Unit in accordance with the GLP Standards set forth in 40 CFR 160. The following list describes the inspections made and the dates that the findings were reported.

Summary of Inspections

Date of Inspection or Audit	Phase Inspected	Date Findings Reported to Study Director and Test Facility Management
06/12/09	Application #1	06/22/09
08/25/09	Forage Sampling	08/31/09
02/19/10	Raw Data Audit/In Life Report	02/25/10

Mary Jones
Quality Assurance

2/25/10
Date

CERTIFICATION OF AVAILABILITY OF RAW DATA

Original study specific raw data will be sent to Dow AgroSciences, LLC. Research For Hire will maintain certified copies.

Research For Hire
1696 South Leggett Street
Porterville, CA 93257

Mary Jones
Quality Assurance

Date

PROJECT PERSONNEL

RESEARCH FOR HIRE

Personnel	Position
John Corkins	General Manager, Contract Research
Larissa Tersteege	Principal Field Investigator
Denise M. McQuarrie	Research Assistant
Blaine Turner	Research Biologist
Thomas Sukut	Technician I
Sandy Medina	Office Assistant
Stephanie Phipps	Office Coordinator
Kayla Padilla	Research Assistant

DOW AGROSCIENCES

Personnel	Position
Mingming Ma	Study Director

REPORT APPROVAL

Mingming Ma
Study Director
Dow AgroSciences, LLC

06 Jul 2010
Date

John S. Corkins
General Manager
Research For Hire

2/25/10
Date

Larissa Tersteege
Principal Field Investigator
Research For Hire

2/25/10
Date

STUDY IDENTIFICATION PAGE

In-Life Phase Study Site:	Research For Hire 1696 South Leggett Street Porterville, CA 93257
Sponsor:	Dow AgroSciences, LLC 9330 Zionsville Road Indianapolis, IN 46268
Sponsor Representative:	D. Fonseca Dow AgroSciences, LLC 9330 Zionsville Road Indianapolis, IN 46268
Testing Facility:	Dow AgroSciences, LLC Indianapolis Lab 9330 Zionsville Road Indianapolis, IN 46268
Study Director:	Mingming Ma Dow AgroSciences, LLC 9330 Zionsville Road Indianapolis, IN 46268
Principal Field Investigator:	Larissa Tersteege Research For Hire 1696 S. Leggett Street Porterville, CA 93257
Study Initiation Date: RFH Experimental Start Date: RFH Experimental End Date:	May 4, 2009 June 12, 2009 September 18, 2009

Research for Hire shall send all original study specific raw data to Dow AgroSciences LLC at the Sponsor's request.

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INTRODUCTION

In this study, the radiolabeled test substance, [^{14}C]-2,4-D DMA salt was applied to corn plants, which were grown in a sandy clay loam soil.

Dow AgroSciences, LLC sponsored the study. The testing facility was Dow AgroSciences, LLC (DAS), and the DAS protocol number was 090058. The in-life phase was contracted to Research For Hire (RFH), and the RFH study number was R050907.

OBJECTIVE

To determine the nature, amount and distribution of residues in the forage and mature crop fractions of AAD-1 corn following three application of [^{14}C]-2,4-D DMA salt (one pre-plant and two foliar applications. All phases of this study were conducted to meet the standards of Good Laboratory Practices (GLP).

CONDUCT OF THE STUDY

The in-life phase of this study was conducted at Research For Hire according to Dow AgroSciences, LLC protocol, "A Nature of the Residue Study with [^{14}C]-2,4-D-DMA Applied to AAD-1 Corn (Event 278)" (protocol no. 090058) and amendments. The study was also conducted in accordance with U.S. EPA, OECD 501 guidelines of the testing of chemicals for Metabolism in Crops (Issued 8 January 2007). The study also adhered to the Good Laboratory Practices Standards (GLP) (40 CFR Part 160) with exceptions noted on the compliance statement.

MATERIALS AND METHODS - IN-LIFE STUDY

EXPERIMENTAL FACILITIES

Research For Hire, 1696 S. Leggett Street, Porterville, California 93257, conducted the in-life phase of the study from June 12, 2009 to September 18, 2009. The [^{14}C]-2,4-D DMA salt treated box was secured in an outdoor area that was enclosed with a wire mesh security fence with a locked gate and was marked with a weatherproof radioactive materials placard. The area was accessible to authorized personnel only.

APPARATUS

Appendix A lists the analytical and field instruments used in the study.

TEST MATERIAL

Test Material

- [^{14}C]-2,4-D DMA salt

Chemical name:	2,4-dichlorophenoxy acetic acid dimethylammonium salt, Phenyl ring-14C(UL)
Common name:	2,4-D-DMA salt-Phenyl-UL-14C (or 14C-2,4-D)
Lot or Identification Nos.:	DE3-100044-13
Stated specific activity:	3.07 mCi/mmol
Radiopurity:	99.1%
Expiration date:	December 2009

TEST MATERIAL RECEIPT AND DISTRIBUTION

All radioactive materials were handled in accordance with Nuclear Regulatory Commission regulations and with RFH Standard Operating Procedures (SOP's). For research involving the use of radioactive materials, RFH operates under NRC License No. 1433-54.

The test materials received at Research For Hire were logged in per RFH SOP's. The test materials for the applications were received in good condition. The packing material was monitored with a survey meter, and radiation levels were below background levels and documented into the raw data. Three vials of [^{14}C]-2,4-D DMA salt, containing a total of 6.873 mCi were received.

Upon receipt, 0.2279 g of blank formulation (XRM-4436, E2868-54) was added to each vial. The test substances were stored in the RFH lab refrigerator (EQP 28-4) with a set temperature between 1.67 °C and 8.89 °C (35°F and 48 °F). Refer to Table 1 - Test Material Receipt and Distribution.

TEST SITE

The test site was located at Research For Hire, 1696 S. Leggett Street, Porterville, Tulare County, California.

One planting box was used to conduct this study, with inside dimensions of 1.5 m long x 0.91 m wide x 0.46 m deep (5 feet x 3 feet x 1.5 feet), was double-lined with 6-mil plastic and filled with sandy clay loam soil to within approximately 5 cm (2 inches) from the top. The one box was treated with ^{14}C-2,4-D DMA salt.

The treated box was placed inside a secured fence, with a locked gate that was marked with a radioactive materials weatherproof placard. A plastic drape was installed around the treated box and was raised at the time of the test material application to prevent cross contamination. The test plot (box) was identified with a placard bearing the study number, project number, test material ID (treated), date of first application, transgenic ID and the name of the Research For Hire (RFH) principal field investigator.

The maps for the test site and plot diagrams are documented in the raw data.

SOIL HISTORY

This soil came from an area at Research For Hire facility where no radiolabeled substances of any kind had previously been applied. The absence of any radioactivity in the soil was confirmed by combustion analysis of a representative aliquot.

TEST CROPS AND PLOT MAINTENANCE

TEST CROP GROUP CLASSIFICATION/VARIETY

The treated box was planted with variety AAD-1 (event 278) on June 12, 2009. The corn seeds were received from Dow AgroSciences (DAS), LLC on June 3, 2009.

PLANTING OF CROP

On June 12, 2009, the corn was planted into the box into 2 rows, with a row spacing of 24 inches, and a plant spacing of approximately 4 inches.

CONFIRMATION OF AAD-1 GENE

On July 1, 2009, each corn plant was tested for the presence of the AAD-1 gene using a strip test provided by DAS. In general, one leaf was folded so that four pieces were punched into a micro-centrifuge tube. AAD1 leaf extraction buffer (0.5mL) was added to the tube and the leaf was crushed into the buffer. An AAD1 test strip was inserted for approximately 5 minutes. The presence of the gene was indicated with two lines. All plants contained the AAD-1 gene.

FERTILIZATION

The test box was fertilized on June 22, 2009 with Miracle Gro at a rate of 2 tablespoons per 5 gallons of water, 5 gallons per plot. On July 23, 2009, July 30, 2009, August 6, 2009 and August 20, 2009 with Miracle Gro at a rate of 5 tablespoons per 5 gallons of water, 5 gallons per plot. The test box was also fertilized on June 30, 2009 and July 8, 2009 with UN32 at a rate of 40 units per acre.

PLANT MAINTENANCE

On July 2, 2009, the plants were thinned to a spacing of 6 inches, as per typical agricultural practices.

IRRIGATION

Irrigation water was applied by hand using a spray wand. The water was carefully added to the soil in order to prevent washing the test substance off of the treated leaves and to minimally disturb the soil. RFH well water was applied as necessary to grow a healthy crop.

CLIMATIC DATA

Climatic data were collected from the California Irrigation Management Information System Weather Station number 169. The station was approximately 5 miles Southwest of the test site. On-site rainfall was monitored by a Tru-Check rain gauge during the study. Table 2 summarizes the climatic data.

TEST MATERIAL PREPARATION AND APPLICATION

GENERAL PREPARATION

The vials containing the test substance aliquots for applications were received at Research For Hire on June 9, 2009.

FIRST APPLICATION

The spray solution for the first application was prepared on June 12, 2009 using the test substance labeled "090058 application solution #1". The [^{14}C]-2,4-D DMA test substance was removed from the refrigerator and allowed to come to room temperature. A glass beaker with a minimum capacity of 200 mL was used to prepare the application solution. Distilled water, 150 mL, was measured and approximately half was transferred to the glass beaker reserving the other half for rinsing and the final dilution. Using a transfer pipette, the [^{14}C]-2,4-D DMA (in approximately 0.2279 g formulation blank XRM-4436, E2868-54) was quantitatively transferred from the shipping vial into the

beaker and swirled to dissolve/suspend. The vial was then rinsed with ten sequential 2 mL portions of the reserved water, shaken well, and transferred to the beaker. The remaining amount of water needed to bring the spray solution to the desired volume (150 mL) was then added. The solution was mixed on a magnetic stir plate, at medium speed, with no heat for 5 minutes.

Three (3) 0.1 mL aliquots were removed from the application solution to clean LSC vials. Exactly 9.90 mL of acetonitrile were added to each of the three aliquots, bringing their final volume to 10 mL. Each vial was capped and mixed thoroughly by inversion. Three (3) 0.10 mL aliquots were removed from each of the three dilutions to a pre-counted LSC vial containing 10 mL of Ready-Solv. The vials were counted in the Beckman LS6500 LSC for one minute and five minute counts.

Four (4) 0.25 mL aliquots (2 pre-aliquots and 2 post-aliquots) were taken. The pre-aliquots were placed immediately into the RFH walk-in freezer EQP 28-2 and the post-aliquots were transported to the field along with the spray solution. After application the post-application retention aliquots were stored in the RFH walk-in freezer EQP 28-2.

TIME AND RATE OF FIRST APPLICATION

The application was made on June 12, 2009 to the treated box. The spray solution was applied to the bare soil, at planting. The spray solution was applied with a plastic bottle covered in aluminum foil (to protect against photodegradation) that was equipped with an R&D sprayer, model GS (EQP 11-4), using a single aluminum spray wand and a flat fan 8001 nozzle. The system was pressurized with CO_2 at 25 PSI. The R&D sprayer was connected to the spray vessel with a flexible hose approximately three feet in length. The air supply hose was approximately 30-feet long so that the CO_2 tank and regulator could remain outside the treated area. The radiolabeled treatment solution contained 150 mL of final spray solution and 148.7 mL was actually applied to the plot. The spray solution was applied evenly in two passes per row of corn. Following the application the empty spray container was rinsed with 30 mL of distilled water which was sprayed on the plot in a manner similar to the application solution.

SECOND APPLICATION

The spray solution for the second application was prepared on July 2, 2009 using the test substance labeled "090058 application solution #2". The solution was prepared in the same manner as the first application solution.

TIME AND RATE OF SECOND APPLICATION

The application was made on July 2, 2009 to the treated box. The spray solution was applied to the corn plants at the V4 growth stage. The application was conducted in the same manner as the first application.

THIRD APPLICATION

The spray solution for the third application was prepared on July 14, 2009 using the test substance labeled "090058 application solution #3". The solution was prepared in the same manner as the first application solution.

TIME AND RATE OF THIRD APPLICATION

The application was made on July 14, 2009 to the treated box. The spray solution was applied to the corn plants at the V8 growth stage. The application was conducted in the same manner as the first application.

SAMPLE COLLECTION

Table 3 summarizes the sample collection dates, sample weights, and the sample shipment dates.

IMMATURE FORAGE CORN SAMPLES

On August 25, 2009 harvest of immature corn forage occurred. The corn plants were at the R4 (milky inner fluid in kernel) growth stage. Two entire plants were cut 4 inch above the soil surface. The plants were cut into sections approximately 6 inches long to fit into Ziploc bags. Each plant was placed into a pre-labeled, tared plastic Ziploc bag. After weighing on scale EQP 13-1, the samples were placed into labeled cloth residue bags and then into the RFH walk-in freezer (EQP 28-2) until shipment to ABC Laboratories. The clippers used for harvesting were cleaned before and after harvest with a 50% solution of isopropyl alcohol and water. Samplers wore disposable gloves and lab coats.

MATURE CORN HARVEST

On September 18, 2009, the mature corn samples were harvested. The corn plants were harvested at growth stage BBCH 89 (fully ripe: kernels hard and shiny, about 65% dry matter). The cobs were removed from stalks then the husks were removed; stalks were cut approximately 6 inch above the soil surface. The plants were then cut into sections to fit into Ziploc bags. The grain and cobs were separated by hand and placed into aluminum foil lined tubs. The grain and cobs were then placed into tared and labeled Ziploc bags, weighed, and then placed into labeled cloth residue bags. The weights were recorded in the trial notebook, and the samples were stored in the RFH walk in freezer (EQP 28-2) until shipment to ABC Laboratories. The clippers used for harvesting were cleaned before and after harvest with a 50% solution of isopropyl alcohol and water. Samplers wore disposable gloves and lab coats.

SAMPLE HANDLING

Table 3 summarizes the dates of sample shipment.

SAMPLE SHIPMENTS

All treated plant samples were shipped in coolers containing approximately twenty-five pounds of dry ice via Federal Express to the following address:

Clark Chickering and RSO
ABC Laboratories, Inc.
7200 East ABC Lane
Columbia, MO 65202

All pre- and post-application retention aliquots were shipped in coolers containing approximately twenty-five pounds of dry ice via Federal Express to the following address.

Attention: Mingming Ma and DAS RSO
Dow AgroSciences, LLC
9330 Zionsville Road
Indianapolis, IN 46268

RESULTS AND DISCUSSION

As shown in Table 4, LSC analysis of the aliquots taken from the spray solution for the application events showed the solution to be within the desired range of radioactivity and homogeneous. This served as confirmation that the targeted amount of the ^{14}C test material was applied to the test box.

All plants contained the AAD-1 gene. The treated crops showed no signs of phytotoxicity during the course of the study.

Photographs were taken on the days of applications and sampling events, and stored in the raw data. Treated corn forage, stover, and mature corn grain and cobs were harvested to enable determination of the nature of the residue of [^{14}C]-2,4-D applied to AAD-1 corn.

TABLE 1 - TEST MATERIAL RECEIPT AND DISTRIBUTION

Material	Date	Distribution	Purpose	Quantity
[^{14}C]-2,4-D DMA 1st Application	6-9-09	Receipt	Received	2.291 mCi
	6-12-09	Application	1st Application	2.291 mCi
[^{14}C]-2,4-D DMA 2nd Application	6-9-09	Receipt	Received	2.291 mCi
	7-2-09	Application	2nd Application	2.291 mCi
[^{14}C]-2,4-D DMA 3rd Application	6-9-09	Receipt	Received	2.291 mCi
	7-14-09	Application	3rd Application	2.291 mCi

TABLE 2 - CLIMATIC DATA

Date Range	Minimum Temperature (°F)[2]	Maximum Temperature (°F)[2]	Minimum Humidity (%)[2]	Maximum Humidity (%)[2]	Precipitation (inches)[1]
06/01/08-06/30/08	56.37	85.94	31.70	89.22	0.00
07/01/08-07/31/08	61.31	95.78	25.04	85.16	0.00
08/01/08-08/31/08	58.59	92.34	27.06	85.48	0.00
09/01/08-09/30/08	57.15	90.38	29.22	86.20	0.00

[1] Information obtained from RFH Station Tru-Check Raingauge.
[2] Information obtained from RFH Experimental Farm Weather Station ~5.25 miles Southwest.

TABLE 3 - SAMPLING, SHIPPING DATES AND WEIGHTS OF TREATED SAMPLES

Sample Number	Sample Description	Plot ID	Sample Weight (g)	Date Sampled	Date Shipped
R050907-1	Pre App. Retention Aliquot App. #1, (a)	2-4-D	NA	6/12/09	6/15/09
R050907-2	Pre App. Retention Aliquot App. #1, (b)	2-4-D	NA	6/12/09	6/15/09
R050907-3	Post App. Retention Aliquot App. #1, (a)	2-4-D	NA	6/12/09	6/15/09
R050907-4	Post App. Retention Aliquot App. #1, (b)	2-4-D	NA	6/12/09	6/15/09
R050907-5	Pre App. Retention Aliquot App. #2, (a)	2-4-D	NA	7/2/09	7/7/09
R050907-6	Pre App. Retention Aliquot App. #2, (b)	2-4-D	NA	7/2/09	7/7/09
R050907-7	Post App. Retention Aliquot App. #2, (a)	2-4-D	NA	7/2/09	7/7/09
R050907-8	Post App. Retention Aliquot App. #2, (b)	2-4-D	NA	7/2/09	7/7/09
R050907-9	Corn Forage	2-4-D	1357	8/25/09	8/26/09
R050907-10	Pre App. Retention Aliquot App. #3, (a)	2-4-D	NA	7/14/09	7/15/09
R050907-11	Pre App. Retention Aliquot App. #3, (b)	2-4-D	NA	7/14/09	7/15/09
R050907-12	Post App. Retention Aliquot App. #3, (a)	2-4-D	NA	7/14/09	7/15/09
R050907-13	Post App. Retention Aliquot App. #3, (b)	2-4-D	NA	7/14/09	7/15/09
R050907-14	Corn Stover	2-4-D	3888	9/18/09	9/21/09
R050907-15	Corn Cobs	2-4-D	599	9/18/09	9/21/09
R050907-16	Corn Grain	2-4-D	1036	9/18/09	9/21/09

TABLE 4 - TEST MATERIAL FORMULATED SPRAY SOLUTION VERIFICATION RESULTS (DPM'S)

Plot ID	App #	Sub-Sample No.	dpm/100 µL of App. Solution Dilution [a]	Mean dpm/100µL of App. Solution Dilution	% of Theoretical
^{14}C-2,4-D DMA salt	1	1	34300.62	34445.77	101.57
		2	34544.66		
		3	34568.00		
		4	34268.91		
		5	34511.76		
		6	34709.95		
		7	33539.56		
		8	34757.35		
		9	34811.16		
^{14}C-2,4-D DMA salt	2	1	33802.41	34034.49	100.36
		2	33659.34		
		3	33693.46		
		4	34069.05		
		5	34336.94		
		6	34193.97		
		7	34109.16		
		8	34185.33		
		9	34260.73		
^{14}C-2,4-D DMA salt	3	1	32859.07	32524.08	95.90
		2	32599.22		
		3	32581.21		
		4	30430.18		
		5	33183.64		
		6	33264.10		
		7	30363.12		
		8	33242.02		
		9	34194.14		

[a] Values shown in this column represent the five minute counts from the LSC analysis.

APPENDIX A: LIST OF EQUIPMENT USED FOR GENERATING IN-LIFE PHASE RAW DATA

Liquid Scintillation Counter - Model LS 6500 (EQP 30-2)
Mfg: Beckman Instruments, Inc., 3500 Harbor Blvd., Fullerton, California 92134-3100 (714) 871-4848

Psychro-Dyne (Psychrometer)- (EQP 27-15)
Mfg: Environmental Tectonics Corporation, County Line Industrial Park, Southampton, Pennsylvania 18911

Top Loading Balance - Model AB-87 (EQP 13- 1)
Mfg: Abbeon Cal, Inc., 123-21T Gray Avenue, Santa Barbara, California 93101-1895

Wind Speed Indicator/Turbo Meter – Model 271 (EQP 47-2)
Mfg: Davis Instruments, 3415 Diablo Avenue, Hayward, California 94545

Survey Monitor – Model AB-87 (EQP 14-1 and 3)
Mfg: Technical Associates, 7051 Eton Avenue, Canoga Park, CA 91303

Todd Windshield Thermometer – (EQP 35-34)
Mfg: Todd Windshield Thermometer, 1221 W. Ontario St., Corona, CA 91720

Lindberg Sola Basic Oxidizer - Model 55035 (EQP 15-1)
Mfg: Lindberg, 2450 W. Hubbard Street, Chicago, Illinois 10112

Appendix B—Milling and TRR Determination Report

(ABC Laboratories)

SAMPLE PROCESSING REPORT FOR

STUDY TITLE

A Nature of the Residue Study with [^{14}C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

DATA REQUIREMENT

OECD Guidance Document 501 for Metabolism in Crops
(Issued 08 January 2007)

AUTHOR

Brett Clark

SAMPLE PROCESSING INITIATION DATE

27 Aug 2009

SAMPLE PROCESSING REPORT COMPLETION DATE

21 Dec 2009

SPONSOR

Dow AgroSciences, LLC
9330 Zionsville Road
Indianapolis, Indiana 46268

PERFORMING LABORATORY

ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202

STUDY IDENTIFICATION

ABC Study No. 64993
DAS Protocol No. 090058

STATEMENT OF GLP COMPLIANCE

Compound: [^{14}C]-2,4-D DMA

Study Title: A Nature of the Residue Study with [^{14}C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)

The sample processing portion of this study, described in this report, was conducted in compliance with the following Good Laboratory Practice Standards:

United States Environmental Protection Agency, (EPA-FIFRA)
Title 40 of the US Code of Federal Regulations Part 160
(August 17, 1989)

The original raw data was provided to Dow AgroSciences LLC with the final sample processing report. Copies of all data in support of this report were retained at ABC Laboratories, Inc. along with facility records and a copy of the final report and the study plan.

Study Director:

29Dec09

Brett Clark Date
Associate Chemist, Chemical Services
ABC Laboratories, Inc.

Sponsor:

09 Jul 2010

Mingming Ma Date
Study Director
Dow AgroSciences LLC

Submitter:

1-July-2010

D. Fonseca Date
Regulatory Manager
Dow AgroSciences LLC

QUALITY ASSURANCE STATEMENT

ABC's Quality Assurance Unit reviewed the Sample Processing Report and Raw Data for Study No. 64993 entitled "A Nature of the Residue Study with [^{14}C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)" for Dow AgroSciences LLC. The following inspections/audits were conducted on this study.

Date of Study Based Inspection	Phase Inspected	Date Reported to Principal Investigator	Date Reported to ABC Management	Date Reported to Study Director/Study Director Management
01 Sep 2009	Procedure: Sample Preparation	02 Sep 2009	02 Sep 2009	03 Sep 2009
15 Oct 2009	Procedure: Sample Preparation	16 Oct 2009	19 Oct 2009	19 Oct 2009
20 Nov 2009	Raw Data & Draft Sample Processing Report	24 Nov 2009	04 Dec 2009	07 Dec 2009
17 Dec 2009	Final Sample Processing Report	17 Dec 2009	22 Dec 2009	22 Dec 2009

These audits indicate that the report is an accurate reflection of the study as it was conducted by ABC Laboratories, Inc.

Jeri Hofen FOR — 29Dec2009

Chris Hughes — Date
Manager, Quality Assurance, Chemical Services
ABC Laboratories, Inc.

SIGNATURE PAGE

Prepared by: ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202

Prepared by: Brett Clark _____ 29 Dec 09 Date

Brett Clark
Associate Chemist
ABC Laboratories, Inc.

Approved by: Jon E. Rhodes _____ 29 Dec 09 Date

Jon E. Rhodes, M.S.
Director, Chemical Services
ABC Laboratories, Inc.

SAMPLE PROCESSING SUMMARY REPORT

Study Sponsor:	Dow AgroSciences LLC
Study Title:	A Nature of the Residue Study with [^{14}C]-2,4-D DMA Applied to AAD-1 Corn (Event 278)
Study Director:	Mingming Ma
Location of Study:	ABC Laboratories, Inc. 7200 East ABC Lane Columbia, Missouri 65202

SAMPLE RECEIPT

Corn samples shipped frozen on dry ice by FedEx were received from Research for Hire (RFH), Porterville, California as stated in Table 1. Upon receipt, all samples received were verified against the RFH shipping transmittal document and placed into frozen storage pending sample milling.

SAMPLE PROCESSING

Homogenization

All samples were homogenized as per ABC SOP CD.EQ.1.40. In general, the samples were removed from frozen storage, pre-weighed, broken down as needed and milled (entire sample) with dry ice, to maintain a frozen state during milling, returned to frozen storage to allow for sublimation of the dry ice (typically 2 days), post weighed, and returned to frozen storage pending combustion and total radioactive residue (TRR) analysis. Weighing and milling procedures were documented using ABC Laboratories' "Sample Preparation Log" form.

All samples were milled using the Straub grinding mill. A robot coupe was used to break down Sample Nos R050907-14, R050907-15, and R050907-16 as the nature of the matrices was too coarse to be immediately milled with the Straub grinding mill. The Straub grinding mill (Straub Company, Warminster, Pennsylvania) was equipped with grinding plates that could be adjusted in a horizontal plane relative to each other. The closer the plates were set to each other, the finer the samples were ground. In order to obtain a good homogeneous sample, the grinding plates were adjusted as close together as possible. Dry ice was passed through the Straub mill to chill the machine prior to milling the samples. Frozen samples (two of which were broken down with the robot coupe) were passed through the mill a minimum of three times, along with enough dry ice to maintain a frozen state throughout the milling process. As the sample/dry ice mixture was being passed through the mill, it was captured in a stainless steel pan, with continuous stirring (using a plastic spatula) of the sample during the milling process. At the completion of the milling process, the homogenized sample/dry ice mixture was transferred to labeled containers, loosely capped, and then placed in a holding freezer to allow sublimation of the dry ice to occur. Depending upon the mass of the sample received, multiple bottles may have been required.

The homogenization equipment was cleaned after each sample was processed.

Sample homogeneity using the sample processing procedure above was assessed using the results obtained from the combustion and TRR analysis of five 0.2-g aliquots of each sample (used

to determine TRR, below). Overall, combustion results indicated that the samples were homogeneous with CVs of ≤18%.

MEASUREMENT OF RADIOACTIVITY

Total radioactive residue (TRR) and combustion results are found in Table 2.

Oxidation analyses (combustion) of plant tissues were performed on a Harvey OX 500 (R.J. Harvey Instruments Corporation, Tappan, New Jersey). Oxidized samples were counted in a Carbon-14 cocktail (R.J. Harvey Instruments Corporation, Tappan, New Jersey).

Five, 0.2-g aliquots of each homogenized sample were combusted to determine the total ^{14}C residues (4-minute burn time). Evolved $^{14}CO_2$ was collected, and the radioactivity determined by LSC. Total ^{14}C-residues in the samples were reported as dpm/g or µCi/g.

Prior to and after use of the oxidizer for all sample analyses, the oxidizer efficiency was determined by combusting known levels of ^{14}C-benzoic acid standard spiked on cellulose and determining the amount of ^{14}C-activity recovered versus the amount applied. The efficiency of the oxidizer was determined to be within 95 and 105% prior to and after use, indicating the oxidizer was functioning properly during sample analysis.

Radioactivity measurements were made with a Beckman Model 6000 Liquid Scintillation Counting System (Beckman Coulter, Inc., Fullerton, California). The quench curve was obtained by counting a set of Beckman quenched carbon-14 liquid scintillation quench standards. The amount of quench in a sample was determined by analyzing the position of its Compton spectrum. In this LSC system, the defining parameter was the H-number. The value of the H-number was equal to the difference between the inflection points of the Compton spectrum of the unquenched standard and the sample. As quench increases, so does the H-number. Each combustion sample was counted for 5.0 minutes. The single-label dpm program was designed to establish the quench curve and to resolve the sample count to dpm by the relationship:

$$\text{dpm} = \frac{(\text{cpm} - \text{background cpm})}{\text{counting efficiency}}$$

Sample dpm/g = sample dpm/aliquot size in g

Table 1 Sample Information in AAD1 Corn (Event 278) Samples from Plots Treated with [^{14}C]-2,4-D DMA

Sample ID	Matrix	Plot	Sampling Time Point	Date Received at ABC	Date Prepared	Date of TRR Determination	Date Shipped to DAS
R050907-09	Forage	2,4-D DMA	Immature	27-Aug-09	01-Sep-09	04-Sep-09	15-Sep-09
R050907-14	Stover	2,4-D DMA	Mature	22-Sep-09	15-Oct-09	23-Oct-09	27-Oct-09
R050907-15	Cobs	2,4-D DMA	Mature	22-Sep-09	13-Oct-09	23-Oct-09	27-Oct-09
R050907-16	Grain	2,4-D DMA	Mature	22-Sep-09	13-Oct-09	23-Oct-09	27-Oct-09

Table 2 Combustion Results in AAD-1 Corn (Event 278) Fraction Samples from Plots Treated with [^{14}C]-2,4-D DMA

Sample ID	Sample Type	Analysis Date	Rep #	Mean Oxidizer Efficiency (%)	Sample Weight Combusted (g)	dpm Found	Mean Efficiency Blank dpm Found	Net Sample dpm Found[a]	dpm/g[b]	Mean dpm/g
R050907-09	Treated Corn Forage	04-Sep-09	1	97.9	0.208	18,821.76	77.17	18,744.59	92,055.70	92,595.69
		04-Sep-09	2		0.203	17,773.35		17,696.18	89,047.47	
		04-Sep-09	3		0.210	19,295.54		19,218.37	93,483.58	
		04-Sep-09	4		0.209	18,104.85		18,027.68	88,111.31	
		04-Sep-09	5		0.200	19,711.13		19,633.96	100,280.39	
R050907-14	Treated Corn Stover	23-Oct-09	1	95.3	0.203	28,041.51	97.04	27,944.47	144,509.74	128,137.25
		23-Oct-09	2		0.204	24,356.79		24,259.75	124,839.91	
		23-Oct-09	3		0.202	23,367.11		23,270.07	120,932.66	
		23-Oct-09	4		0.204	23,144.13		23,047.09	118,599.60	
		23-Oct-09	5		0.203	25,584.61		25,487.57	131,804.33	
R050907-15	Treated Corn Cobs	23-Oct-09	1	95.3	0.205	176.50	97.04	79.46	406.92	421.97
		23-Oct-09	2		0.201	195.33		98.29	513.36	
		23-Oct-09	3		0.203	191.41		94.37	488.03	
		23-Oct-09	4		0.202	165.10		68.06	353.72	
		23-Oct-09	5		0.201	163.63		66.59	347.80	
R050907-16	Treated Corn Grain	23-Oct-09	1	95.3	0.205	281.35	97.04	184.31	943.84	984.17
		23-Oct-09	2		0.206	300.30		203.26	1,035.83	
		23-Oct-09	3		0.201	278.06		181.02	945.44	
		23-Oct-09	4		0.202	294.68		197.64	1,027.14	
		23-Oct-09	5		0.204	285.26		188.22	968.59	

[a] Net dpm/aliquot combusted = sample dpm found/aliquot combusted in g – (average Efficiency Blank dpm found)
[b] dpm/g in aliquot combusted = net dpm/aliquot combusted ÷ (% oxidizer efficiency) ÷ (aliquot weight, g)

Appendix C—Sample Calculations

Specific Activity Determinations

The specific activity is the amount of radioactivity per unit of mass of 2,4-D DMA in the test substance. First, the total amount of radioactive 2,4-D DAM was determined (dpm and µg). Then the specific activity was calculated as the sum of the radioactivity divided by the sum of the mass.

$$\text{Total Radioactivity}_{(\text{dpm})} = \left(\frac{\text{average dpm}}{\text{original aliquot}_{\text{mL}}}\right) \times \left(\frac{\text{dilution volume}_{\text{mL}}}{\text{dilution aliquot}_{\text{mL}}}\right) \times \text{original volume}_{\text{mL}}$$

$$\text{Total Radioactive 2,4-D DMA}_{(\mu\text{g})} = \frac{\text{Total Radioactivity}_{\text{dpm}}}{\text{Original Specific Activity}_{\text{dpm/µg}}}$$

$$\text{New Specific Activity}_{(\text{dpm/µg})} = \frac{\text{total radioactivity}_{\text{dpm}}}{\text{total mass 2,4 - D DMA (radioactive + non - radioactive)}_{\mu\text{g}}}$$

Example for ^{14}C-2,4-D DMA:
Where the radiolabeled test substance (nominally 7.2 mCi) was diluted to 10.0 mL and 0.025 mL aliquots were diluted to 10.0 mL, and 0.025 mL aliquots were taken for LSC. The average dpm/aliquot was 99,358 dpm/0.025 mL. The original specific activity was 328,679 dpm/µg. The majority of the test substance solution, 9.925 mL, was mixed with 578.5 mg non-radiolabeled 2,4-D (98.8% purity resulting in 571.6 mg or 571,558 µg).

$$\text{Total Radioactivity}_{(\text{dpm})} = \left(\frac{99358\ \text{dpm}}{0.025\ \text{mL}}\right) \times \left(\frac{10\ \text{mL}}{0.025\ \text{mL}}\right) \times 9.925\ \text{mL} = 1.58 \text{ x } 10^{10}\ \text{dpm}$$

$$\text{Total Radioactive 2,4-D DMA} = \frac{1.58 \times 10^{10}\ \text{dpm}}{328679\ \text{dpm/µg}} = 48{,}004\ \mu\text{g (99.8\% pure = 47,917 µg)}$$

$$\text{Specific Activity 2,4-D DMA}_{(\text{dpm/µg})} = \frac{1.58 \times 10^{10}\ \text{dpm}}{47{,}917\ \mu\text{g} + 571{,}558\ \mu\text{g}} = 25{,}470\ \text{dpm/µg}$$

Oxidative Combustion Calculations

All oxidative combustion results were corrected for oxidizer recovery (determined on the day of use) and background dpm values.

$$\text{Net dpm/g value} = \frac{\text{net combustion dpm value}}{\text{combustion recovery} \times \text{aliquot weight}_{g}}$$

Example combustion calculation for forage (replicate A) post-extracted pellet combustion:
Where oxidizer recovery = 93.1%, combustion value #1 = 2535 dpm, and aliquot weight = 0.0504 g.

$$\text{Net dpm value} = \frac{2535\ \text{dpm}}{0.931 \times 0.0504\ \text{g}} = 54{,}003\ \text{dpm/g}$$ (rounding difference noted)

Calculation of TRR Levels

a) *ABC Labs determined dpm/g, see* Appendix B

b) *Converting dpm/g to µg a.i./g (or mg a.i./kg)*

To determine the total radioactive residue level in each sample, the average dpm/g value for the sample was converted to µg active ingredient/g (equivalent to mg ai/kg) by dividing the dpm/g value by the specific activity value of the applied ^{14}C-2,4-D DMA (25,470 dpm/µg).

For example, average dpm/g in the forage was 92,596. This was converted to a µg a.i./g (or mg a.i./kg) value as follows:

$$\frac{92{,}596\ \text{dpm/g}}{25{,}470\ \text{dpm/µg}} = 3.635\ \text{µg a.i./g (or mg a.i./kg)}$$

c) *Converting mg a.i./kg to mg a.e./kg*

To determine the total radioactive residue level in each sample in terms of acid equivalents, the mg a.i./kg value was multiplied by a conversion factor. The conversion factor was calculated by dividing the molecular weight of the acid by the molecular weight of the active ingredient (2,4-D DMA), in this case, 221.04/266.12 = 0.831 (see

Figure 2).

For example, forage contained 3.635 mg a.i./kg and was converted to mg a.e./kg as follows:

$3.635\ \text{mg a.i./kg} \times 0.831 = 3.0202\ \mu\text{g a.e./g}$ (or mg a.e./kg) (rounding difference noted)

d) *TRR Distribution among Fractions Generated by the Extraction of the Samples*

For Table 8, the percentage distribution of the total radioactive residues in the samples among the fractions generated by the extraction procedure was calculated below.

$$\text{Amount Extracted (dpm)} = \frac{\text{dpm}}{\text{aliquot (mL)}} \times \text{extract volume (mL)}$$

$$\text{Extraction Recovery (\% TRR)} = \frac{\text{Amount Extracted (dpm)}}{\text{extracted tissue weight (g) x TRR (dpm/g)}}$$

Extracted mg/kg = % TRR x TRR (mg/kg)

An example for the Neutral Organic Extract of the forage (replicate A):

$$\text{Amount Extracted (dpm)} = \frac{1535\ dpm}{0.25\ \text{mL}} \times 141\ \text{mL} = 847{,}831\ \text{dpm}$$ (rounding difference noted)

$$\text{Extraction Recovery} = \frac{847{,}831\ \text{dpm}}{10.04\ \text{g x}\ 92{,}596\ \text{dpm/g}} = 91.2\%$$ (rounding difference noted)

Extracted mg a.e./kg = 0.912 x 3.020 mg a.e./kg = 2.754 mg/kg

$$\text{Normalized Extraction Recovery} = \frac{2.754\ mg/kg}{2.754\ \text{mg/kg} + 0.460\ \text{mg/kg} + 0.259\ \text{mg/kg}} = 79.3\%$$

(where 0.460 and 0.259 mg a.e./kg are the amounts in EX2 and the post-extracted pellet, respectively)

e) *TRR Distribution among 2,4-D and Its Metabolites Following HPLC Analysis*

The percentage distribution of the TRR among 2,4-D and its metabolites following HPLC analysis of the sample extracts was calculated as follows:

% of TRR = (% of TRR in the Extract Being Assayed) x (% Distribution of Radioactivity in the Extract among the Fractions of Interest as Determined by the HPLC Analysis)

For example in Table 9, the percent of the TRR accounted for as parent 2,4-D in the Neutral Organic extract of the forage (replicate A):

% of TRR = 91.2% x 0.737 (73.7% of the radioactivity in this extract that eluted with 2,4-D – see Figure 8)
= 67.2% of the TRR (rounding difference noted)

To convert the total percentage distribution value for each component of the residue profile to a mg/kg value, the TRR value the sample of interest (expressed as mg/kg of 2,4-D acid equivalents) was multiplied by the percentage value at which the component of interest was present.

For the 2,4-D in the above sample the calculation:
2,4-D = 67.2% of the TRR x 3.020 mg a.e./kg (TRR – see Table 7)
= 2.030 mg a.e./kg

Appendix D—Mass Spectral Report

Mass Spectral Analysis Summary

Three radiolabeled samples and two reference standards from *A Nature of the Residue Study with [^{14}C]-2,4-D Applied to AAD-1 Corn, Event 278* (Protocol ID: 090058) were submitted for mass spectral analysis. Three compounds of interest were detected in the analyzed samples and are discussed in this report.

Liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) with electrospray ionization (ESI) were used to analyze the samples and the standards. A Berthold radioactivity monitor (RAM) was used to assist in location of the metabolite peaks.

Calibration standard

Prior to analysis, calibration of the Thermo LTQ FT Ultra mass spectrometer was verified using a solution of caffeine, the tetrapeptide MRFA, and Ultramark 1621.

Mass Measurement Reporting

Mass measurements made using the ion trap (IT-MS) for the MS and MS/MS mass spectra were measured to +/- 0.1 Da accuracy (for example *m/z* 300.1 +/- 0.1). Mass measurements made using the FTICR (FT-MS) for the MS and MS/MS mass spectra were measured to +/- 0.001 Da accuracy (for example, *m/z* 300.001 +/- 0.001). The reporting of these values is based on the typical known accuracy limitations of each mass analyzer.

Chromatographic and Mass Spectral Parameters

The LC, MS, and MS/MS parameters used to analyze the samples and reference standards from *A Nature of the Residue Study with [^{14}C]-2,4-D Applied to AAD-1 Corn, Event 278* (Protocol ID: 090058) are shown in **Table 1**. The mass spectrometer instrumental parameters including gas flow and applied voltages were optimized prior to the analyses.

Table 1. Chromatographic and Mass Spectral Parameters

Instrumentation	Mass Spectrometer	Thermo LTQ FT Ultra, s/n: SN06120F	
	HPLC	Thermo Accela	
Liquid Chromatography	Column	Supelco Ascentis C_{18}, 150 x 4.6 mm, 2.7 micron, serial number: USHW002792	
	Solvent A	0.1% formic acid in water	
	Solvent B	0.1% formic acid in acetonitrile	
	Flow rate	1.0 mL/minute	
	HPLC split flow ratio	Approximately 20:80 (MS:RAM)	
LC Gradient	Time (minutes)	% Solvent A	% Solvent B
	0.00	95	5
	5.00	95	5
	20.00	50	50
	25.00	50	50
	40.00	5	95
	42.00	5	95
	43.00	95	5
	50.00	95	5
MS and MS/MS parameters	Mode	Negative electrospray (-ESI)	
	MS/MS Isolation width	7.0 Da	
	CE	35%	
	Activation Q	0.250	
	Activation Time	30.0 msec	

Mass Spectral Results and Discussion

A summary of the mass spectral results for the reference standards that were analyzed as part of this work is shown in **Table 2**. The mass spectral results for the sample analyses are shown in **Tables 3 - 5.**

The RAM trace depicting **peak A** in sample *F-A-EX1-EL-ID-F27* is shown in **Figure 1.** The RAM trace depicting **peak B** in sample *F-A-EX1-EL-ID-F33* is shown in **Figure 2.** The RAM trace depicting **peak C** in sample *F-A-EX1-EL-ID-F53* is shown in **Figure 3.**

Peak A in the sample *F-A-EX1-EL-ID-F27*, which eluted at approximately 12.78 minutes (RAM trace), produced a deprotonated formate adduct ion, $[M-H+formate]^-$, at *m/z*: 531.067 +/- 0.001 under negative ESI conditions. The chromatographic and mass spectral results for **peak A** are shown in **Figure 4**. This accurate mass measurement for the unknown (not adduct) was 486.069 Da, which was consistent with the molecular formula $C_{18}H_{24}Cl_2O_{11}$. This formula is consistent with a structure containing 6 rings and double-bonds. Based on this evidence, the proposed structure for **peak A** is a disaccharide conjugate of dichlorophenol (DCP) metabolite of 2,4-D, presented in **Table 3**. A reference standard of this compound was not available for chromatographic and mass spectral comparison.

Peak B in the sample *F-A-EX1-EL-ID-F33*, which eluted at approximately 14.71 minutes (RAM trace), was compared to the standard of X11963417 (TSN033048-0001). The chromatographic and mass spectral results for both **peak B** and X11963417 are shown in **Figure 5. Peak B** was identified as X11963417, the glucose conjugate of DCP, based upon relative retention time and mass spectral match with the known standard.

Peak C in the sample *F-A-EX1-EL-ID-F53*, which eluted at approximately 20.85 minutes (RAM trace), was compared to the standard of DCP (AGR182992). The chromatographic and mass spectral results for both **peak C** and DCP are shown in **Figure 6** and **Figure 7. Peak C** was identified as DCP based upon relative retention time and mass spectral match with the known standard.

Table 2. Summary of mass spectral results observed from the reference standards used in *A Nature of the Residue Study with [^{14}C]-2,4-D Applied to AAD-1 Corn, Event 278*

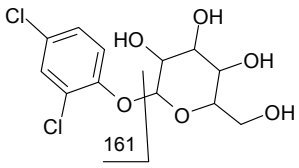
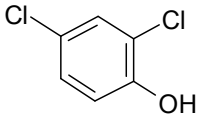
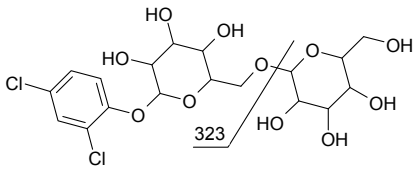
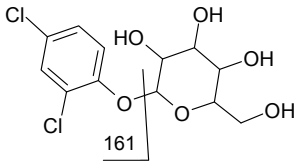
Standard	**-ESI/MS *m/z*** **-ESI/MS/MS *m/z***	**Structure** **MW (Da) and Formula**
TSN033048-0001 RT: 14.71 min (MS) *X11963417* *glucose conjugate of dichlorophenol, metabolite of 2,4-D*	369.014 $[M-H+formate]^-$ 161.0, 322.9	 324, $C_{12}H_{14}Cl_2O_6$
AGR182992 RT: 20.97 min (MS) *X195116* *dichlorophenol (DCP)*	160.956 $[M-H]^-$ 160.957	 162, $C_6H_4Cl_2O$

Table 3. Summary of mass spectral results observed from *A Nature of the Residue Study with [^{14}C]-2,4-D Applied to AAD-1 Corn, Event 278* sample *F-A-EX1-EL-ID-F27*

Peak ID **Retention Time (min)** **RAM / MS**	**-ESI/MS *m/z*** **-ESI/MS/MS *m/z***	**Proposed Structure** [1] **MW (Da) and Formula**
A 12.78 / 12.86	531.067 [M-H+formate]$^-$ 323.1, 485.0	 Or isomer 486, $C_{18}H_{24}Cl_2O_{11}$

[1] structure assignment based on mass assignment and MS/MS fragments.

Table 4. Summary of mass spectral results observed from *A Nature of the Residue Study with [^{14}C]-2,4-D Applied to AAD-1 Corn, Event 278* sample *F-A-EX1-EL-ID-F33*

Peak ID **Retention Time (min)** **RAM / MS**	**-ESI/MS *m/z*** **-ESI/MS/MS *m/z***	**Proposed Structure** [1] **MW (Da) and Formula**
B 14.71 / 14.70	369.014 [M-H+formate]$^-$ 160.9, 322.8	 324, $C_{12}H_{14}Cl_2O_6$

[1]Structure assignment based upon relative LC retention time and mass spectral match to a reference standard

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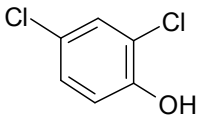
Table 5. Summary of mass spectral results observed from *A Nature of the Residue Study with [^{14}C]-2,4-D Applied to AAD-1 Corn, Event 278* sample *F-A-EX1-EL-ID-F53*

Peak ID **Retention Time (min)** **RAM / MS**	**-ESI/MS *m/z*** **-ESI/MS/MS *m/z***	**Proposed Structure** [1] **MW (Da) and Formula**
C 20.85 / 20.83	160.957 $[M-H]^-$ 160.957	 162, $C_6H_4Cl_2O$

[1]Structure assignment based upon relative LC retention time and mass spectral match to a reference standard.

Figure 1. Chromatographic results showing the RAM trace for the sample

F-A-EX1-EL-ID-F27

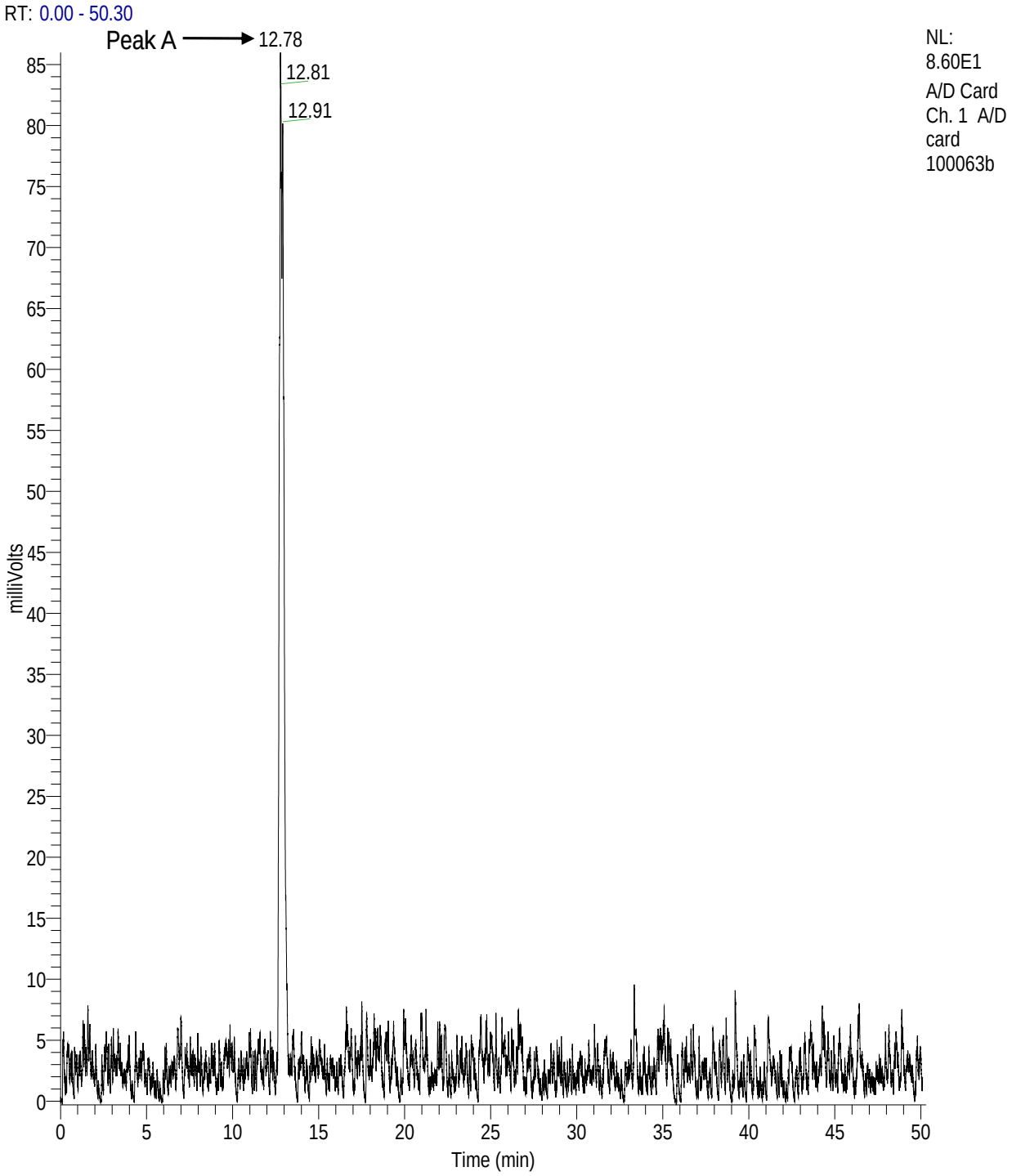


Figure 2. Chromatographic results showing the RAM trace for the sample *F-A-EX1-EL-ID-F33*

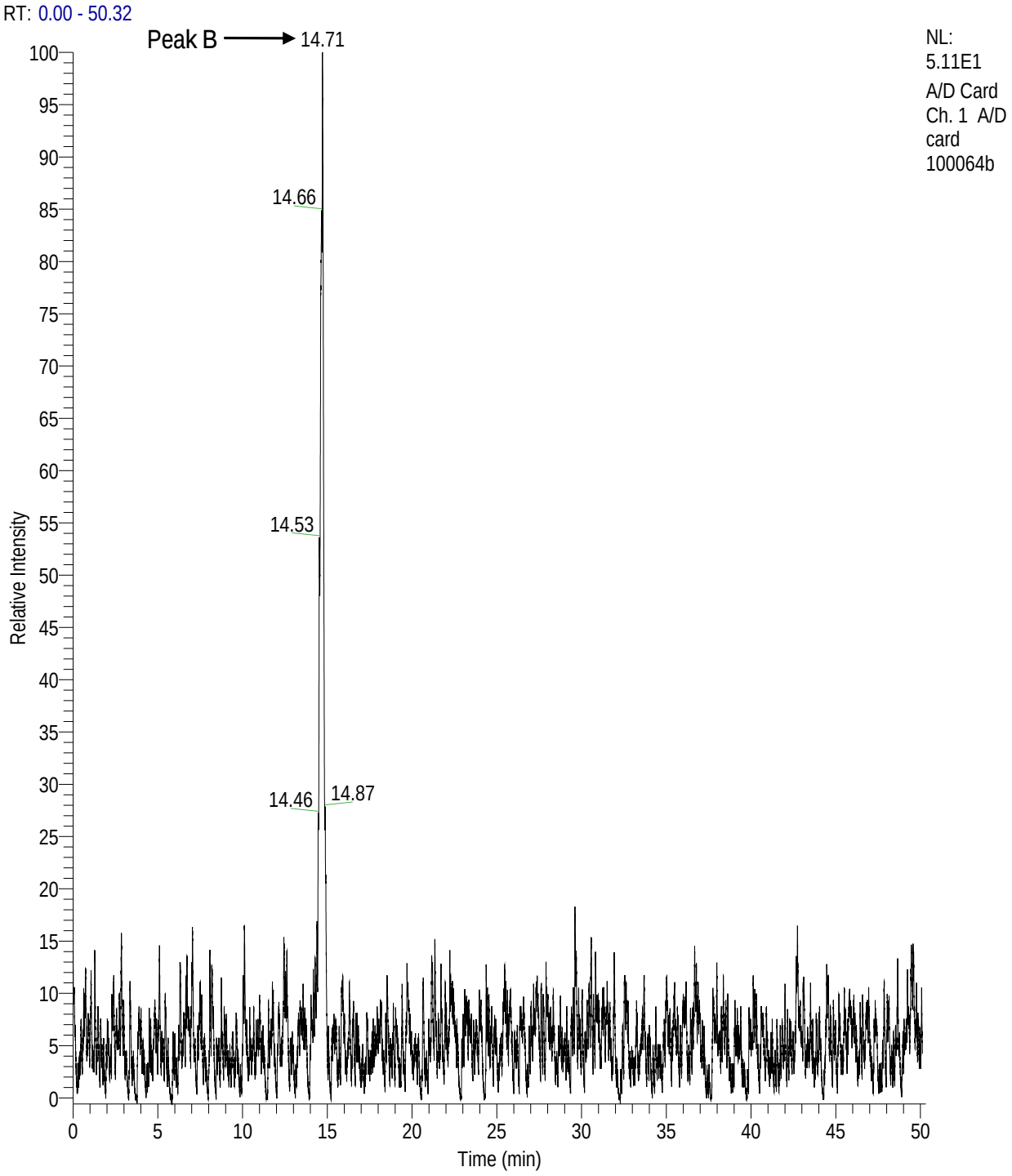


Figure 3. Chromatographic results showing the RAM trace for the sample *F-A-EX1-EL-ID-F53*

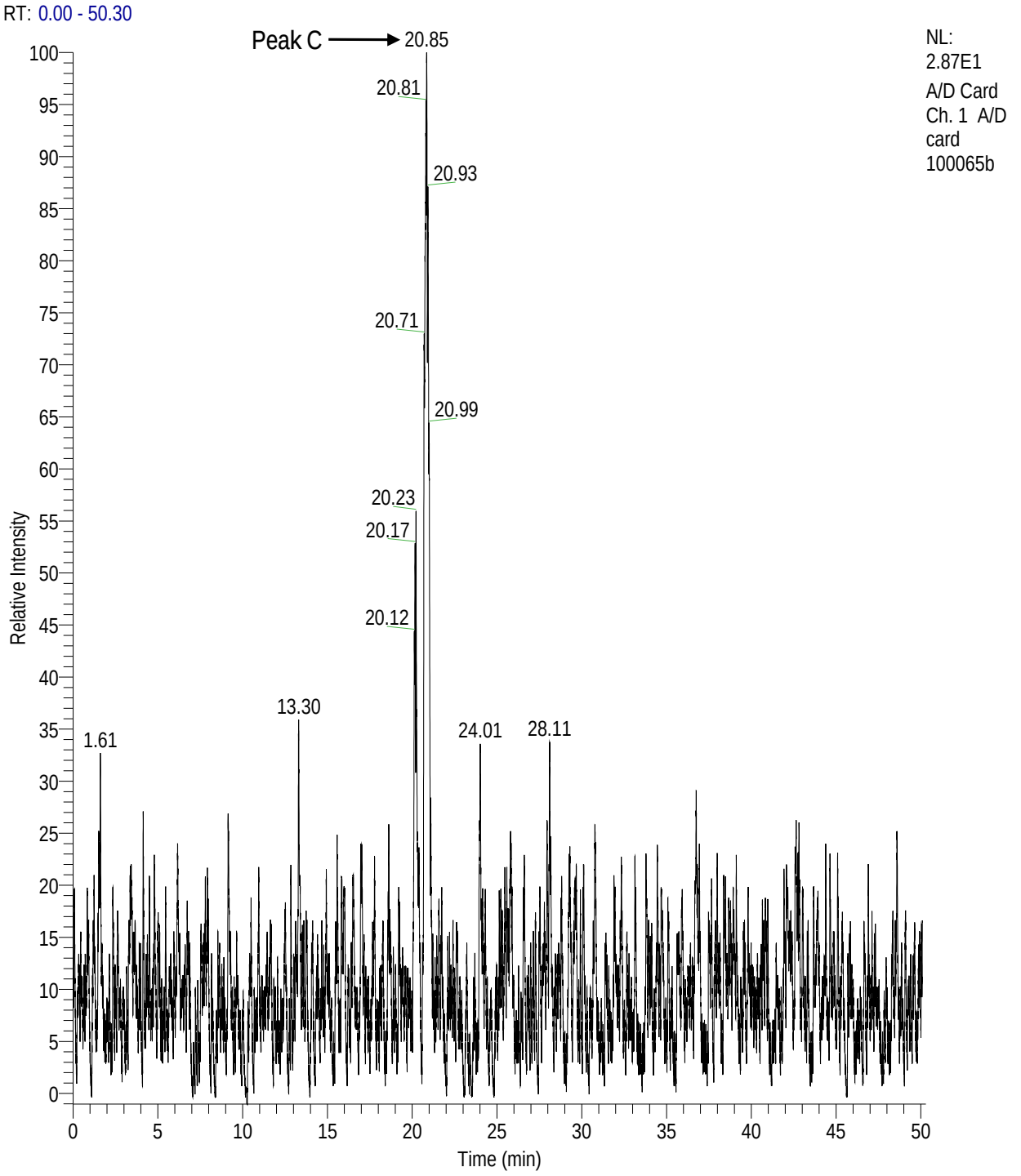
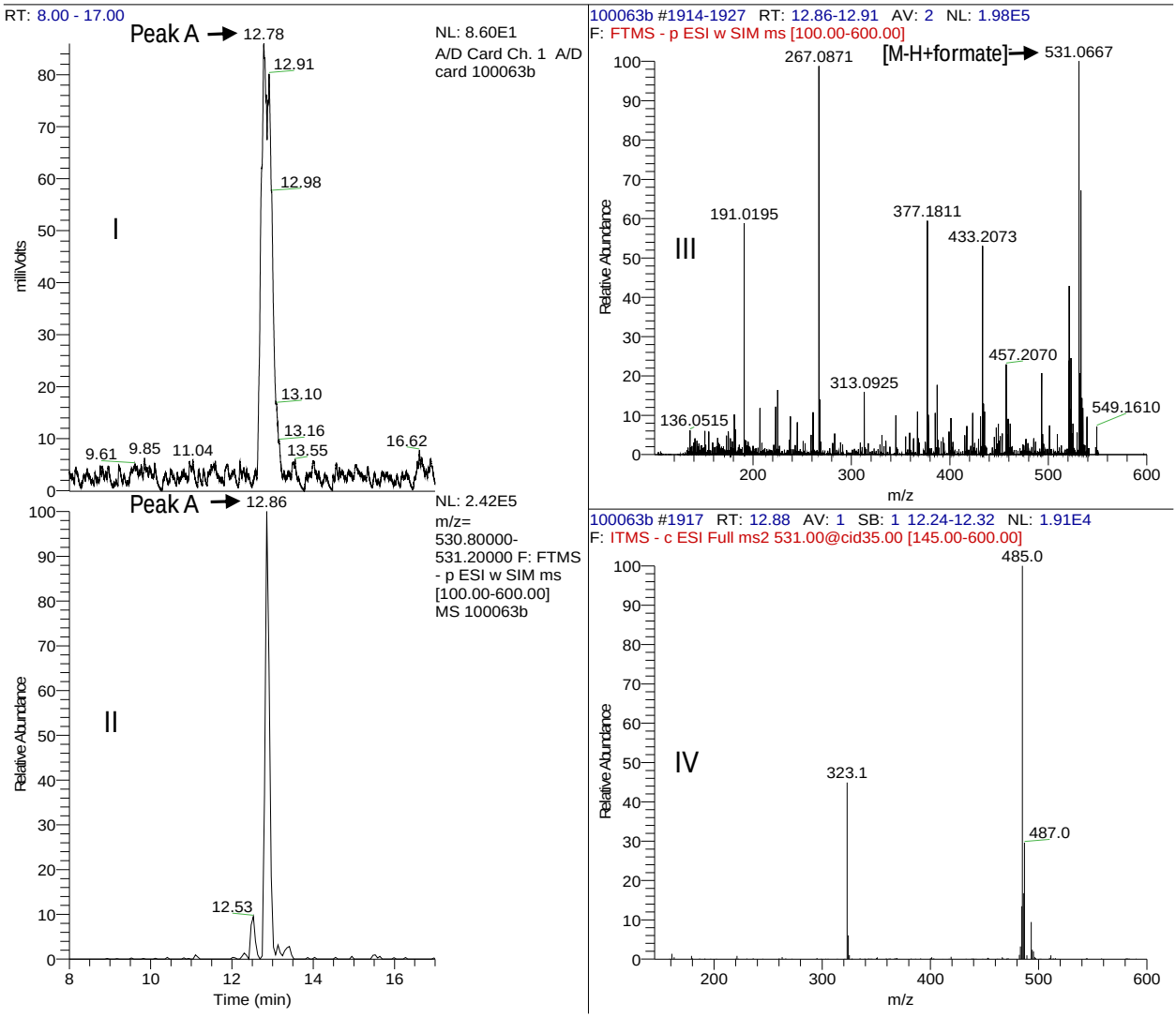


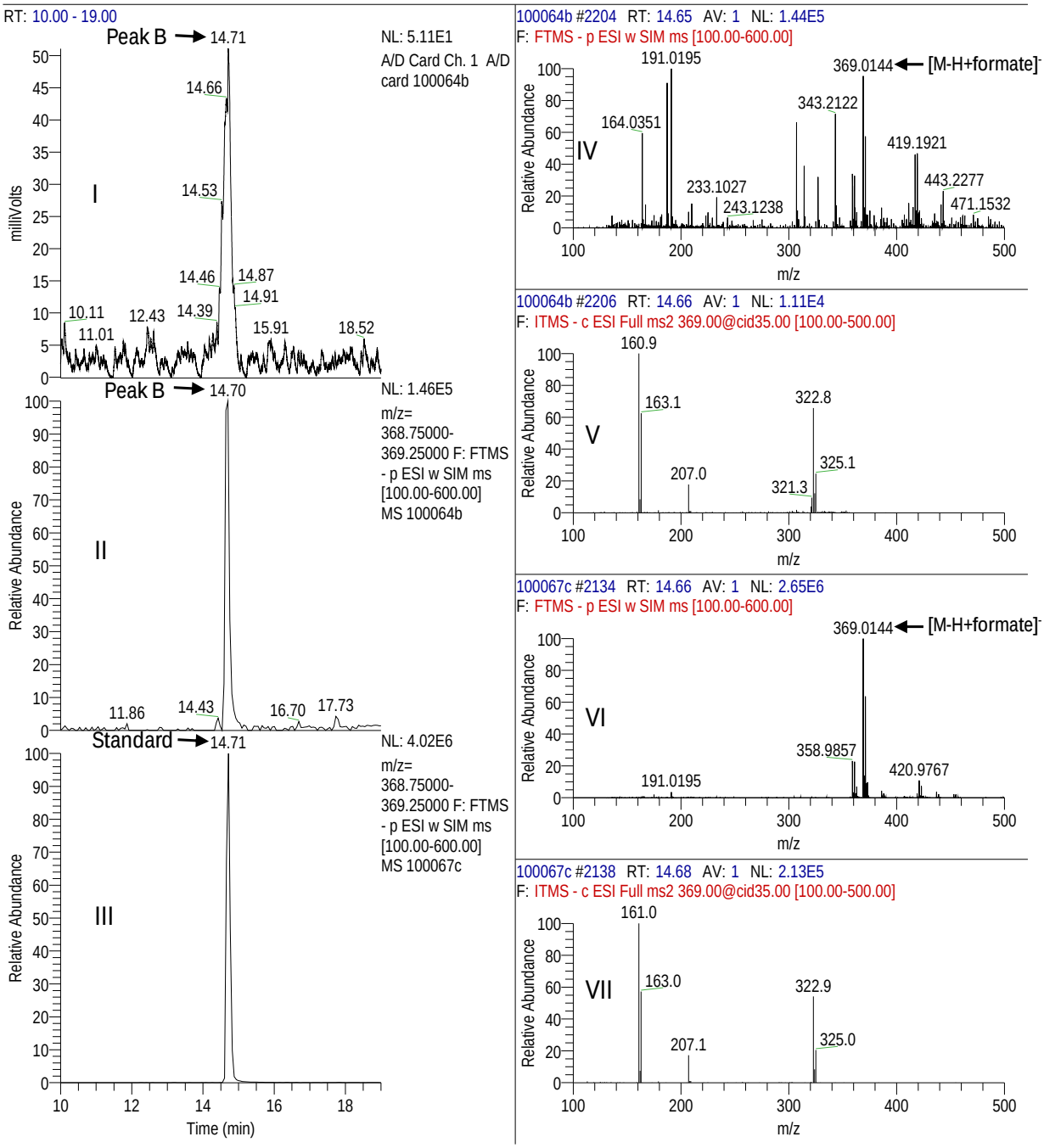
Figure 4. Chromatographic and mass spectral results for **peak A** in sample *F-A-EX1-EL-ID-F27*



I-II: sample – RAM, extracted ion chromatogram
III-IV: sample – mass spectrum, MS/MS spectrum of m/z 531.067

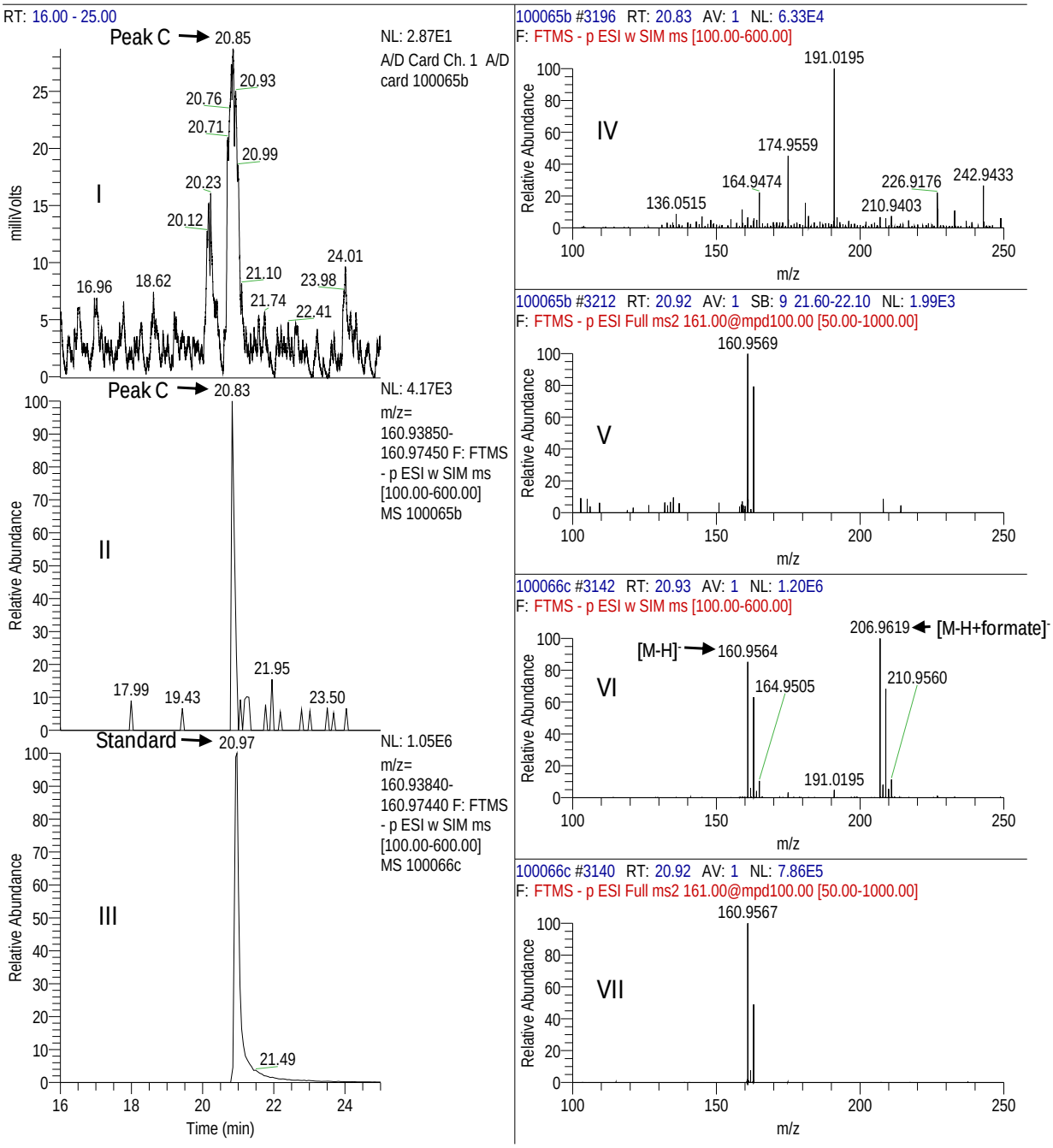
Observed $[M-H+formate]^-$	531.067
Observed M (-formate+H: 44.998)	486.069
Proposed Theoretical M	486.070
Measured Error from Proposed Theoretical M	0.001 Da or 2 ppm
Proposed Molecular Formula	$C_{18}H_{24}Cl_2O_{11}$
Rings and Double Bonds for Proposed Formula	6

Figure 5. Chromatographic and mass spectral results for **peak B** in sample *F-A-EX1-EL-ID-F33* and X11963417 standard (*TSN033048-0001)*



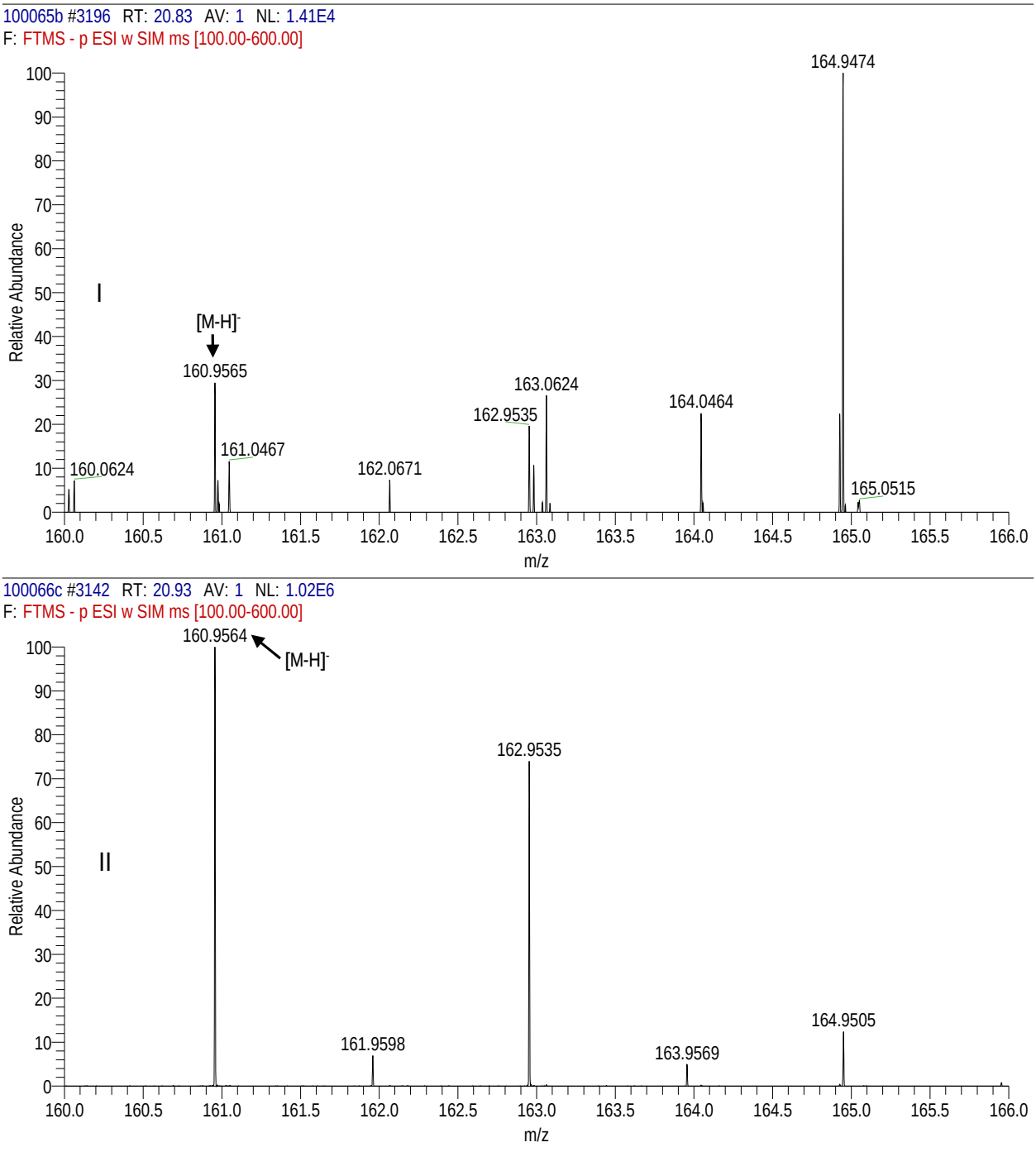
I-II: sample – RAM, extracted ion chromatogram
III: standard – extracted ion chromatogram
IV-V: sample – mass spectrum, MS/MS spectrum of m/z 369.014
VI-VII: standard – mass spectrum, MS/MS spectrum of m/z 369.014

Figure 6. Chromatographic and mass spectral results for **peak C** in sample *F-A-EX1-EL-ID-F53* and DCP standard *(AGR182992)*



I-II: sample – RAM, extracted ion chromatogram
III: standard – extracted ion chromatogram
IV-V: sample – mass spectrum, MS/MS spectrum of m/z 160.957
VI-VII: standard – mass spectrum, MS/MS spectrum of m/z 160.956

Figure 7. Expanded mass spectral results for **peak C** in sample *F-A-EX1-EL-ID-F53* and DCP standard *(AGR182992)*



I: sample – mass spectrum
II: standard – mass spectrum