

STUDY TITLE

Metabolism of Dicamba in Dicamba-Tolerant Soybeans

DATA REQUIREMENT

Nature of the Residue – Plants, Livestock, US EPA OPPTS 860.1300
Metabolism in Crops, OECD Guideline for the Testing of Chemicals No. 501

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Amendment 1

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This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160 with the following exceptions:

1. No claim of compliance is made for the off-site climatic data provided by the California Irrigation Management Information System (CIMIS) and Research For Hire Experimental Farm Data Logger CR 10.
2. Test site information (crop history, pesticide history, maintenance practices and irrigation data).

None of these exceptions had any effect on the integrity of the study.

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Quality Assurance Statement

Study Title: Metabolism of Dicamba in Dicamba-Tolerant Soybeans

Study Number: 06-98-M-1

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed, and accurately reflects the raw data for the portion of the study conducted by Monsanto Company. This confirmation excludes data generated by PTRL West, Inc. and Research For Hire.

Reviews conducted by the PTRL West, Inc. and Research For Hire Quality Assurance Units are enclosed within the respective sub-report and are specified on their individual QA Statements.

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

Dates of Inspection/Audit	Phase	Date Reported To:	
		Study Director	Management
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07/18/2006	Radiolabelled TS Dispensing	11/20/2006	11/20/2006
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03/29/2010	Amended Report Audit	03/29/2010	03/29/2010


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Certification

This report is an accurate and complete representation of the study activities. The study director may be contacted at the address or phone number below in regard to technical inquiries concerning this report.

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Sample Storage: Any study samples that are to be retained will be stored at Monsanto Company, St. Louis, MO USA.

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Abbreviations and Acronyms

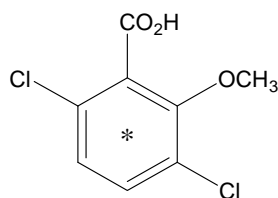
Ac: acetyl
ae: acid equivalents
ACN: acetonitrile
amu: atomic mass units
CAS: Chemical Abstracts Service
conj.: conjugate/conjugates
cpm: counts per minute
DAT: days after treatment
DCGA: 3,6-dichlorogentisic acid (2,5-dichloro-3,6-dihydroxybenzoic acid)
DCSA: 3,6-dichlorosalicylic acid (3,6-dichloro-2-hydroxybenzoic acid)
dpm: disintegrations per minute
EI: electron ionization
ESI: electrospray ionization
EPA: (US) Environmental Protection Agency
ESTC: (Monsanto) Environmental Sciences Technology Center
x g: relative centrifugal force (gravitational units)
GC: gas chromatography
gluc.: glucoside/glucosides
HMG: 3-hydroxy-3-methylglutaryl
HMGA: 3-hydroxy-3-methylglutaric acid
ISP: ionspray ionization
HPLC: high performance liquid chromatography
HPLC/LSC: HPLC with fraction collection and liquid scintillation counting
HPLC/RAD: HPLC with radioactivity flow detection
HPLC/UV: HPLC with UV detection
LC: liquid chromatography
LSC: liquid scintillation counting
MS: mass spectrometry
MW: molecular weight
NA: not applicable
NMR: nuclear magnetic resonance
OECD: Organization for Economic Cooperation and Development
PRE-T: preemergence treatment
POE-T: postemergence treatment
RAD: radioactivity flow detection
rpm: revolutions per minute
RT: room temperature
TMS: trimethylsilyl (i.e., TMSdiazomethane = trimethylsilyldiazomethane)
TOF: time-of-flight
TRACE II: **The RadioActivity Counting System, 2nd generation**
TRR: Total Radioactive Residues
Unk: unknown
UV: ultraviolet
v/v: volume to volume

1 Introduction and Summary

Dicamba, 3,6-dichloro-2-methoxybenzoic acid, is a foliar or soil-applied herbicide in the auxin family and is utilized for the control of many broadleaf weeds in a number of crops including corn, sorghum, small grains, grasses and asparagus. This report describes a metabolism study conducted with [^{14}C]dicamba in dicamba-tolerant soybean. The study was conducted to support the registration of dicamba for use in dicamba-tolerant soybean in the United States and other world areas in accordance with the requirements of EPA residue chemistry test guideline OPPTS 860.1300, "Nature of the Residue – Plants, Livestock" and OECD Guideline for the Testing of Chemicals No. 501, "Metabolism in Crops". All phases of the study were conducted under the U.S. EPA FIFRA Good Laboratory Practice Standards. The in-life portion of the study, consisting of the treatment, sample collection and processing, radiochemical analysis (TRR determination) and initial chromatographic analysis for stability purposes was conducted by PTRL West, Inc., Hercules, CA, USA. Soybean plants were grown and treated at the Research For Hire field site in Porterville, CA, USA, under the supervision of PTRL West, Inc. Soil analyses were conducted by AGVISE Laboratories, Northwood, ND, USA. The metabolite analysis phase of the study was conducted within the Environmental Sciences Technology Center of Monsanto Company in Creve Coeur, MO, USA.

The purpose of this study was the determination of the nature of residues found in/on agricultural commodities of dicamba-tolerant soybean following treatment with dicamba. Specific objectives of the study were: 1) determination of the total radioactive residues (TRR) in dicamba-tolerant soybean following treatment with radiolabeled dicamba; 2) determination of the efficiency of extraction of the residue components; 3) identification and quantification of the major components of the terminal residue, and delineation of the major routes of metabolism of dicamba in dicamba-tolerant soybean.

The test material, consisting of a mixture of unlabeled dicamba and dicamba uniformly labeled with ^{14}C in the ring carbons, was formulated as an aqueous solution of the diglycolamine salt.



[Phenyl-U- ^{14}C]dicamba

* uniformly ring-labeled with carbon-14

Dicamba-tolerant soybean (*Glycine max* L., event GM_A90617, lot # GLP-0604-17294-S) was utilized as test system in this study. Dicamba-tolerant soybean event GM_A90617 expresses a modified dicamba mono-oxygenase (DMO) gene derived from the soil bacterium *Stenotrophomonas maltophilia*. The GM_A90617 soybean event used in this study contained the same DMO gene expression cassette as dicamba-tolerant soybean event MON 87708. The soybean plants were grown in 12-inch pots in two greenhouses. The treated groups were designated PRE-T (preemergence treatment, 29 pots) and POE-T (postemergence treatment, 32 pots). The untreated groups each consisted of 8 pots and were designated UNT-C (untreated), PRE-C (untreated, interspersed amongst the PRE-T pots after the preemergence application) and POE-C (untreated, interspersed amongst the POE-T pots after the postemergence application). The PRE-T and POE-T plants, after treatment, were housed in the same greenhouse, but physically separated. Applications were conducted using a hand-held sprayer that fit directly on the spray bottles. Separate bottles containing the required amount of test substance formulation were used for each pot. The preemergence application was made directly to the soil of the PRE-T group pots on the day of planting after the soybean seeds were planted. The postemergence application was made to the foliage of the plants of the POE-T group 29 days after planting at the R1 growth stage (first flower). The target application rate was 2.5 lbs ae/acre (2.80 kg/ha) compared to a maximum intended seasonal use rate in the US of 2.0 lb ae/acre (2.24 kg/ha). The actual achieved application rates were 2.55 and 2.52 lb ae/acre (2.86 and 2.82 kg/ha), for the PRE-T and POE-T applications, respectively.

Immature foliage (pre-forage) samples were collected as thinnings from UNT-C and PRE-T plants 14 days after planting and the preemergence application. Forage samples were collected from all pots 7 days after the postemergence application and 36 days after the preemergence application. Two individual POE-T forage plants were collected separately and were washed with water for determination of surface residues. Hay was collected from UNT-C, PRE-T and POE-T groups 27 days after the postemergence treatment and 56 days after the preemergence treatment. Seed was collected from all groups 83 days after postemergence treatment (112 days after planting and preemergence treatment).

Samples were processed by grinding and were stored frozen. Total radioactive residues (TRR) in the samples were determined by combustion analysis. Foliage (pre-forage, forage and hay) samples were each extracted four times with 40:60 (v/v) acetonitrile:water. Seed samples were each extracted three times with hexane to remove oils, then once with acetonitrile followed by four extractions with 40:60 (v/v) acetonitrile:water. The levels of [¹⁴C]dicamba-derived residues found in the soybean matrices, and their extractabilities are summarized in the following table:

Soybean Matrix	TRR mg/kg (ppm)*	Percent Extracted (Normalized)
PRE-T Pre-forage	3.248	91.09
PRE-T Forage	1.433	91.21
PRE-T Hay	1.056	90.88
PRE-T Seed	0.291	59.35
POE-T Forage	134.147	93.79
POE-T Hay	39.149	95.30
POE-T Seed	0.389	63.67
PRE-C Forage	0.080	75.64
PRE-C Seed	0.170	43.89
POE-C Forage	0.280	93.50
POE-C Seed	0.138	44.16

*Dicamba equivalents

Extractabilities were greater than 90% for all foliage matrices except the PRE-C forage. For the PRE-T seed and POE-T seed, extraction efficiencies were 59.35% and 63.67%, respectively, of which 10.35% and 8.41% of the TRR was extracted in the oil (hexane) fraction, respectively. Residue levels in PRE-C and POE-C forage and seed collected from untreated control plants interspersed amongst the PRE-T or POE-T plants ranged from 0.080 to 0.280 mg/kg indicating uptake and/or deposition of dicamba or volatile soil metabolites, e.g., $^{14}\text{CO}_2$, from the air. Extraction efficiencies were generally lower for these matrices, and the proportion of residues in the oil fraction of the PRE-C and POE-C seed was generally higher, reflecting a higher degree of incorporation of radioactivity (i.e., $^{14}\text{CO}_2$ or other small molecules from extensive degradation of dicamba) into natural products.

Release of unextracted residues in soybean seed was investigated through extractions with acid and base, or through a series of chemical and enzymatic digestions. Dilute acid or base extraction released very little of the unextracted residues. The chemical and enzymatic digestions, however, released considerable amounts of the unextracted residues resulting in 7.09% of TRR (0.021 mg/kg) and 7.60% of TRR (0.030 mg/kg) remaining unextracted in the PRE-T and POE-T seed, respectively. The hemicellulose (12.90-13.92% of TRR) and protein (8.18-10.06% of TRR) fractions contained the highest amounts of released residues. Organic solvent partitioning or HPLC analysis of the released residues in the protein fraction indicated that the residues were polar water-soluble materials. The released residues in the hemicellulose fraction partially

partitioned into organic solvents but proved to be of an insoluble nature and therefore not related to dicamba. These results indicated that the unextracted residues in seed were likely derived by reincorporation of small molecules, e.g., $^{14}\text{CO}_2$, into natural plant constituents.

Extracts of PRE-T pre-forage and PRE-T/POE-T forage, hay and seed were concentrated and analyzed by HPLC with radioactivity flow detection or fraction collection and detection by liquid scintillation counting (LSC) to quantitate metabolites. All metabolites constituting 2% or more of the TRR in any matrix were isolated and purified by preparative HPLC and were identified. Metabolite identification was conducted through a combination of spectral techniques, primarily mass spectrometry, as well as chemical degradations and derivatizations, and comparisons to available or synthesized reference standards. In all, five discrete metabolites of dicamba and non-metabolized parent dicamba were identified. Two additional metabolites of dicamba in the range of 1-2% of TRR were isolated and characterized. The names and structures of identified metabolites are shown in Table 1. Unchanged dicamba was a significant component of the residue only in the POE-T forage and hay (24.21% of TRR and 12.33% of TRR, respectively). The results of washing experiments of two POE-T forage plants indicated that the unchanged dicamba residues in forage (and likely hay as well) were virtually entirely surface residues. For pre-forage, forage and hay, the identified and characterized metabolites constituted 87.29-92.17% of the TRR in these matrices. For seed, the identified and characterized radioactivity comprised 50.21-53.21% of the TRR.

The proposed pathways for the metabolism of dicamba in dicamba-tolerant soybean are shown in Figure 112. Metabolism of dicamba in dicamba-tolerant soybean proceeds by initial demethylation to form DCSA **22** primarily by the action of the dicamba *O*-demethylase enzyme which is the product of the dicamba mono-oxygenase gene introduced to confer dicamba tolerance. Little free DCSA is observed in soybean matrices; rather, the DCSA is converted to its 2-*O*- β -glucoside (DCSA glucoside **9**) some of which is further acylated with 3-hydroxy-3-methylglutaric acid (HMGA) on the 6-hydroxyl of the glucose moiety to form DCSA HMGglucoside **11**. As a minor pathway, DCSA **22** is converted by ring 5-hydroxylation to DCGA which is not observed in its free form but is present in soybean matrices as the 5-*O*- β -glucoside (DCGA glucoside **3**) and the corresponding malonylglucoside (DCGA malonylglucoside **8**).

DCSA glucoside **9** was the major metabolite in dicamba-tolerant soybean foliage (pre-forage, forage and hay) from the pre- or postemergence treatments constituting 60.32-74.48% of TRR. DCSA HMGglucoside **11** constituted 5.21-7.62% of TRR in PRE-T pre-forage, forage and hay, and 1.14 and 2.48% of TRR in the POE-T forage and hay, respectively. DCGA glucoside **3** constituted 0.75-4.32% of TRR in pre-forage, forage and hay, with larger amounts present in the hay compared to the forage. The DCGA

malonylglucoside **8** represented 5.46% of TRR in the PRE-T pre-forage, but only 0.73-1.61% of TRR in forage and hay. DCSA **22** represented only 1.54-1.93% of TRR in hay, but somewhat larger amounts were observed in forage (3.19-4.08% of TRR). Metabolite fractions **14** and **18**, characterized as mixtures of unknown DCSA and DCGA conjugates, each constituted less than 2.0% of the TRR in soybean foliage.

Similar to the treated plants, DCSA glucoside **9** was the major metabolite observed in the forage of the PRE-C and POE-C (untreated) plants. Parent dicamba was a significant percentage of the residues of both the PRE-C and POE-C forage, apparently due to evaporation of dicamba from the soil or plant surfaces of the treated plants in close proximity and deposition on the surfaces of the interspersed control plants. However, dicamba represented a low overall residue level (0.015 and 0.073 mg/kg, respectively) in/on these plants. Significant residues corresponding to the component characterized as sugars **1** were present in the PRE-C forage presumably due to uptake and incorporation of $^{14}\text{CO}_2$ from the soil.

While the DCSA glucoside **9** dominated the metabolite profiles of soybean foliage matrices, there were four prominent metabolite peaks observed in seed profiles of treated plants. DCSA glucoside **9** constituted 11.55 and 15.27% of TRR (0.034 and 0.059 mg/kg) in the PRE-T and POE-T seed, respectively. DCSA HMGglucoside **11** represented 8.73 and 9.61% of TRR (0.025 and 0.037 mg/kg), and DCGA malonylglucoside **8** constituted 4.73 and 4.64% of TRR (0.014 and 0.018 mg/kg) in the PRE-T and POE-T seed, respectively. The fourth prominent peak was a polar material characterized as sugars **1** representing 8.42 and 9.15% of TRR (0.025 and 0.036 mg/kg) in PRE-T and POE-T seed, respectively. DCGA glucoside **3** was also present in the seed constituting 1.60 and 2.07% of TRR (0.005 and 0.008 mg/kg) in the PRE-T and POE-T seed, respectively. DCSA **22** and dicamba **23** were very minor components of the seed residues (<1% of TRR each in PRE-T and POE-T seed). Compared to forage and hay, extractable seed residues, while lower in magnitude, contained relatively higher proportions of the DCSA HMGglucoside **11** and the two DCGA conjugates **3** and **8**. One other significant radioactive component of the seed residues, present in the hexane extracts (oil fraction), was triglycerides. These were characterized by acid-catalyzed transesterification of the oil triglycerides with methanol and HPLC analysis of the resulting fatty acid methyl ester mixture. The sugar and triglyceride residues in seed are presumed to result by uptake of $^{14}\text{CO}_2$ from metabolism of dicamba in the soil, and possibly by metabolism of dicamba to $^{14}\text{CO}_2$ and/or other small molecules in the plant, and incorporation into plant natural products through normal metabolic processes.

The major radioactive components of the PRE-C and POE-C seed were sugars **1** (12.61 and 12.50% of TRR, respectively) and triglycerides (16.89 and 11.07% of TRR, respectively) reflecting significant uptake of $^{14}\text{CO}_2$. DCSA glucoside **9** represented 2.18

and 5.75% of TRR, and DCSA HMGglucoside **11** represented 1.33 and 4.12% of TRR for PRE-C and POE-C seed, respectively.

O-Demethylation and ring-hydroxylation, the processes by which DCSA and DCGA are formed in dicamba-tolerant soybean, are common metabolic processes in plants, soil and animals. Glycosylation and subsequent malonylation are common routes of metabolism of xenobiotics as well as natural products in plants. Although the acylation of a xenobiotic or xenobiotic glycoside with HMGA has not, to our knowledge, been previously reported, HMGA is a common plant constituent and there are many plant natural product glycosides, e.g., flavonol glycosides, conjugated with HMGA. Metabolism of dicamba in dicamba-tolerant soybean is also similar to the metabolism of dicamba in soil and other plant species. DCSA is observed as the major aerobic soil metabolite of dicamba; DCGA is also observed in soil as a minor metabolite. Although the glucose conjugate of 5-hydroxydicamba is the major dicamba metabolite in crops such as wheat and grasses, which are naturally tolerant to dicamba, *O*-demethylation to form DCSA is also observed as a minor pathway in these crops.

The dicamba metabolites identified in this study are converted by acid hydrolysis to the chemophores DCSA and DCGA. Current residue enforcement methodology, which incorporates an acid hydrolysis step followed by methylation and analysis by GC, would be expected to be adequate for analysis of dicamba residues in dicamba-tolerant soybean. DCSA residues would be converted to the same dimethylated analyte as dicamba while DCGA residues would be converted to the same trimethylated analyte as 5-hydroxydicamba.

Assessment of the stability of dicamba residues in soybean matrices by reanalysis of samples at the end of the analysis phase of the study and comparison to the original analyses indicated, qualitatively, that dicamba-derived residues in dicamba-tolerant soybean are stable for a period of two years in frozen storage. Additional quantitative storage stability data on the endogenous radioactive residues were obtained in this study to support the residue study of dicamba in dicamba-tolerant soybean (Monsanto study REG-08-096). The results of these quantitative storage stability analyses provide assurance that the DCGA and DCSA residues in forage, hay and seed, and dicamba in forage and hay, were stable in the samples generated in study REG-08-096 over the storage intervals in that study.

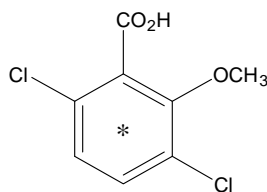
2 Materials and Methods

2.1 Test Substance

The test substance for this study was dicamba (3,6-dichloro-2-methoxybenzoic acid, CAS number 1918-00-9). Dicamba has a molecular formula of $C_8H_6Cl_2O_3$ and a molecular weight of 221.0 g/mol. Dicamba is a solid that is soluble in organic solvents, especially moderately polar solvents. It is moderately soluble in water (4.5 g/L at 25 °C) as the free acid; salts of the acid, such as sodium or amine salts, are very soluble in water (400 g/L at 25 °C for the Na salt).

Two separate lots of dicamba test substances were utilized for this study, one for the preemergence treatment and one for the postemergence treatment. The test substances were prepared by mixing unlabeled dicamba with sufficient [^{14}C]dicamba to give a target specific activity of approximately 5.4 mCi/mmol (0.90 MBq/mg). The [^{14}C]dicamba (specific activity of 45.0 mCi/mmol, 7.53 MBq/mg) used for mixing was uniformly labeled with carbon-14 in the ring carbons and was synthesized and purified by GE Healthcare (Amersham).

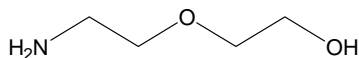
For the preemergence treatment, the test substance (lot number 6103-01A) had a specific activity of 5.39 mCi/mmol (54190 dpm/ μ g, 0.903 MBq/mg). A mass spectrum of the test substance is shown in Figure 1. The radiochemical purity of the test substance was determined to be 99.2% by reverse phase HPLC using Method A with radiochemical detection (HPLC/RAD, Figure 2). The chemical purity of the test substance was 99.4%. The test substance (lot number 6103-01C) for the postemergence treatment had a specific activity of 5.43 mCi/mmol (54560 dpm/ μ g, 0.909 MBq/mg). A mass spectrum of the test substance is shown in Figure 3. The radiochemical purity of the test substance was 99.5% (Figure 4). The chemical purity of the test substance was 99.1%. Due to the presence of two chlorines in the molecule, dicamba (and its metabolites) exhibited distinctive mass spectral doublets separated by two mass units in an approximate 3:2 ratio. This was utilized to distinguish mass spectral peaks of dicamba metabolites from mass spectral peaks of matrix contaminants.



[Phenyl-U- ^{14}C]dicamba

* uniformly ring-labeled with carbon-14

For each of the two treatments, the dicamba test substance was formulated in water as the diglycolamine salt by dissolving the solid test substance in water containing approximately 1.05 molar equivalents of diglycolamine [2-(2-aminoethoxy)ethanol]. The test substance formulation procedures are described in detail on pages 19 and 20 of the in-life report (Appendix B).



Diglycolamine (DGA)
2-(2-aminoethoxy)ethanol

The test substance formulations were analyzed by LSC to verify homogeneity and were analyzed by HPLC/LSC prior to transfer to the field site and following the respective preemergence and postemergence applications. The test substance formulations were determined to be homogeneous, and the test substances were stable during formulation, transport and application (see page 29 of the in-life report in Appendix B).

2.2 Reagents and Standards

2.2.1 Reagents and Solvents

Common reagents were generally analytical reagent grade or A.C.S. certified grade. Solvents used for HPLC were HPLC grade. Water was Burdick and Jackson HPLC Grade or EMD OmniSolv® High Purity Solvent. Ultima-Flo™ AP and Permafluor® E⁺ scintillation cocktails, CarboSorb® E combustion trapping solution and Spec-Chec-¹⁴C combustion standard were obtained from PerkinElmer Life and Analytical Services (Shelton, CT).

2.2.2 Reference Standards

Reference compounds were employed for retention time comparisons with radioactive metabolites, as mass spectral standards, and as HPLC retention time standards to monitor HPLC system performance. Their names and structures are given in Table 2 and their sources are listed below. Representative retention times for the primary reference standards (**I-IV**) under HPLC method B are shown in Table 3 and a representative HPLC chromatogram is shown in Figure 5. The following standards were certified reference standards obtained from the Monsanto ESTC Reference Standards Officer for use in this study:

Report Number	CAS Registry Number	Name	Lot Number	Purity
I	1918-00-9	Dicamba	GLP-0202-12068-A	99.16%
			GLP-0605-17340-A	99%
II	3401-80-7	3,6-Dichlorosalicylic Acid (DCSA)	GLP-0601-16849-A	95%
			GLP-0605-17341-A	100%
III	7600-50-2	5-Hydroxydicamba	GLP-0606-17478-A	100%
			GLP-0704-18561-A	99%
IV	18688-01-2	3,6-Dichlorogentisic Acid (DCGA)	GLP-0708-18951-A	96%
XIV	112-39-0	Palmitic Acid, Methyl Ester	GLP-0708-18967-A	99%
XV	112-61-8	Stearic Acid, Methyl Ester	GLP-0708-18953-A	99%
XVI	112-62-9	Oleic Acid, Methyl Ester	GLP-0605-17404-A	100%
XVII	112-63-0	Linoleic Acid, Methyl Ester	GLP-0605-17373-A	99%
XVIII	301-00-8	α -Linolenic Acid, Methyl Ester	GLP-0605-17374-A	100%

Q = Qualitative

The following reference standards were either purchased or synthesized in small quantities for use only in this study. They were characterized for structure and certified for use as retention time or mass spectral standards. Their purities were not formally determined at Monsanto.

Report Number	Supplier	CAS Registry Number	Name	Catalog/Lot Number	Purity
V	Monsanto	NA	5-Hydroxydicamba, Methyl Ester	822904D	Q
VI	Monsanto	NA	5-Acetyloxydicamba, Methyl Ester	822926A	Q
VII	Monsanto	62059-39-6	Trimethyl DCGA	815808A	Q
VIII	MP Biomedicals, Inc.	815-92-9	D-[U- ¹⁴ C]Glucose	11049/329167	>98%
IX	Sigma-Aldrich	141-82-2	Malonic Acid	M1750/114K3648	100%
X	Monsanto	1190-39-2	Dibutyl Malonate	815837B	Q
XI	Monsanto	1187-99-1	γ -Hydroxy- α -ketoglutaric Acid (HKGA)	815864A	Q
XII	Fluka	503-49-1	3-Hydroxy-3-methylglutaric Acid (HMGA)	55695/1330941	Q
XIII	Monsanto	55590-95-9	Tris(trimethylsilyl)HMGA	839328B	Q

Q = Qualitative

The following reference standards were purchased and used as mass spectral standards for high resolution exact mass determination. They were not specifically characterized, but their parent ion masses were verified at the time of their use.

Report Number	Supplier	CAS Registry Number	Name	Catalog/Lot Number	Purity
XIX	Aldrich	345909-26-4	Taurocholic Acid, Sodium Salt Hydrate	861960/02315HE	97%
XX	Sigma-Aldrich	153439-40-8	Fexofenadine HCl	F9427/047K47131	>98%
XXI	Sigma-Aldrich	67368-29-0	MRFA (Met-Arg-Phe-Ala) Acetate Salt	M-1170/084K5116	≥90%

The characterization of reference standards purchased specifically for use in this study is described below.

D-[U-¹⁴C]Glucose (VIII): Radiolabeled glucose was purchased from MP Biomedicals, Inc. and was characterized by positive and negative ion Loop/ESI/MS (mobile phase 1:1 0.5% aq. formic acid:acetonitrile, 0.2 mL/min). The positive ion mass spectrum exhibited a formate adduct ion at m/z 227 $[M+HCOOH+H]^+$. However, the spectrum was complex and contained several additional ions. In negative ion mode a cleaner and more definitive spectrum was obtained exhibiting a parent ion at m/z 179 $[M-H]^-$ and a formate adduct at m/z 225 $[M+HCOO]^-$ consistent with the molecular weight of glucose of 180.

Malonic Acid (IX): Malonic acid (purchased from Sigma-Aldrich) was characterized by conversion to the dibutyl ester (dibutyl malonate) with BF_3 /butanol and GC/EI mass spectral analysis (GC Method D except 60 °C initial temperature, 220 °C injector) of the derivative. The spectrum was consistent with the published spectrum (98.7% match, NIST/EPA/NIH v2.0a Mass Spectral Library) (see preparation and characterization of dibutyl malonate below).

HMGA (3-Hydroxy-3-methylglutaric Acid, XII): HMGA was purchased from Fluka and was characterized by negative ion LC/ESI/MS (HPLC Method G with Beckman Coulter Ultrasphere ODS C18 column 150 x 2 mm, 5 μ m) as well as ¹H and ¹³C NMR spectroscopy. The mass spectrum exhibited a parent ion peak at m/z 161 $[M-H]^-$ consistent with the molecular weight of HMGA of 162. Both the proton and carbon NMR spectra were consistent with the structure of HMGA: ¹H NMR (DMSO- d_6) δ 1.32 (s, 3H, CH₃), 2.60, 2.65 (ABq, J = 15 Hz, 4H, CH₂); ¹³C NMR (DMSO- d_6) δ 28.4, 45.9, 69.5, 173.1.

The synthesis and characterization of reference standards prepared on small scale specifically for use in this study is described below.

Trimethyl DCGA (Methyl 2,5-Dichloro-3,6-dimethoxybenzoate, VII) was synthesized by treating 5-hydroxydicamba in methanol with a 10% hexane solution of trimethylsilyldiazomethane for 40 min at RT. The reaction product was analyzed by HPLC Method B

(retention time 37.3 min). It was also characterized by GC/EI/MS (GC Method B) and displayed a parent ion doublet at m/z 264/266 $[M]^+$ as well as fragment ion doublets at m/z 249/251 $[M-CH_3]^+$ and m/z 233/235 $[M-OCH_3]^+$ (Figure 37). The spectrum matched the published spectrum of methyl 2,5-dichloro-3,6-dimethoxybenzoate (NIST/EPA/NIH v2.0a Mass Spectral Library, 98.8% match).

5-Hydroxydicamba, Methyl Ester (Methyl 2,5-Dichloro-3,6-dihydroxybenzoate, V) standard was synthesized by refluxing 5-hydroxydicamba reference standard (III) with concentrated sulfuric acid (1 mL) in methanol (10 mL) for approximately 3 days. The product was purified using HPLC Method B at a retention time of 30.9 min. The standard was characterized by negative ion LC/ESI/MS (Method G) indicating a parent ion two-chlorine doublet at m/z 249/251 $[M-H]^-$ and a fragment ion at m/z 234/236 $[M-CH_3]^-$ (Figure 52) consistent with the structure.

5-Acetyloxydicamba, Methyl Ester (Methyl 2,5-Dichloro-3-acetyloxy-6-methoxybenzoate, VI): This standard was prepared by treating 5-hydroxydicamba, methyl ester standard (V) with pyridine and acetic anhydride for 2 hours at RT. It was analyzed by HPLC Method B and had a retention time of 36.6 min. The standard was characterized by GC/EI/MS (GC Method A) exhibiting a parent ion two-chlorine doublet at m/z 292/294 $[M]^+$ and fragment ion doublets at m/z 250/252 $[M-CH_2CO]^+$ and 219/221 $[M-CH_2CO-OCH_3]^+$ consistent with the expected product (Figure 42).

Tris(trimethylsilyl)HMGA (3-Methyl-3-[(trimethylsilyl)oxy]pentanedioic acid, 1,5-Bis(trimethylsilyl) Ester, XIII): This standard was prepared in a similar manner to the derivatization of the hydrolysis product of Peak 11. HMGA (3-hydroxy-3-methylglutaric acid, XII) was treated with 0.1 N NaOH at RT for 1 h followed by acidification with 3.6 N H_2SO_4 and extraction of the resulting solids with acetone (to mimic the hydrolysis of Peak 11). The resulting solution was treated with *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and pyridine at 72 °C for 3.5 hours. The solution was analyzed by GC/EI/MS (Figure 91) (GC Method E) to characterize the standard. The GC/MS spectrum exhibited ions at m/z 363 $[M-CH_3]^+$, 273 $[M-CH_3-HOSi(CH_3)_3]^+$, 247 $[M-CH_2C(O)OSi(CH_3)_3]^+$, 147 $[(CH_3)_2SiOSi(CH_3)_3]^+$ and 73 $[Si(CH_3)_3]^+$ consistent with the structure of tris(trimethylsilyl)HMGA (MW 378). The spectrum matched the published spectrum (93.9%, NIST/EPA/NIH v2.0a Mass Spectral Library).

Dibutyl Malonate (Malonic Acid, Dibutyl Ester, X): The dibutyl ester of malonic acid was prepared by treating malonic acid IX (which had been exposed to 1 N NaOH for 1 hour at room temperature and neutralized with HCl to mimic the Peak 8 hydrolysis conditions) with BF_3 /butanol at 56 °C for 10 minutes. The reaction mixture was partitioned with hexane after adding sat. NaCl, and the hexane layer was washed with sat.

K₂CO₃. The hexane solution was dried with Na₂SO₄ and concentrated to a small volume for mass spectral analysis. The sample was characterized by GC/EI/MS (Figure 59) (GC Method D). The spectrum exhibited ions at m/z 187 [M-CH₂CH₃]⁺, 161 [M-CH₂CH₂CH=CH₂]⁺, 143 [M-OCH₂CH₂CH₂CH₃]⁺, 105 [m/z 161-butene]⁺, 87 [m/z 105-H₂O]⁺ and 57 [CH₃CH₂CH₂CH₂]⁺, consistent with the dibutyl malonate structure (MW 216) with a match (95.7%, NIST/EPA/NIH v2.0a Mass Spectral Library) to the published library spectrum.

HKGA (γ -Hydroxy- α -ketoglutaric Acid, XI): HKGA was synthesized from oxaloacetic acid and glyoxylic acid by the method of Ruffo, *et al.*² The material was characterized by mass spectrometry and proton NMR. The negative ion electrospray mass spectrum exhibited a parent ion at m/z 161 [M-H]⁻, as well as ions corresponding to gas-phase dimers and trimers, and fragment ions at m/z 143 [M-H-H₂O]⁻ and m/z 99 [M-H-H₂O-CO₂]⁻. ¹H NMR (D₂O) δ 2.95 (dd, 1H, J = 8.4, 17.6 Hz, CH), 3.07 (dd, 1H, J = 3.6, 17.6 Hz, CH₂), 4.31 (dd, 1H, J = 3.6, 8.4 Hz, CH₂).

2.3 Study Design

This study consisted of five groups of soybean plants: untreated control (UNT-C), preemergence treated pots/plants (PRE-T), postemergence treated plants (POE-T), and untreated control plants interspersed amongst the preemergence treated plants (PRE-C) or the postemergence treated plants (POE-C). Immature foliage (thinnings), forage, hay and seed were collected. The study design is described in detail in the protocol attached as Appendix A to this report and in the in-life report attached as Appendix B to this report. The schedule of events for the in-life portions of the study is given on page 121 and pages 140-141 of the in-life report (Appendix B).

2.4 Test System

Dicamba-tolerant soybean (*Glycine max* L., event GM_A90617, seed lot # GLP-0604-17294-S) was utilized as test system for this study. Dicamba-tolerant soybean event GM_A90617 expresses a modified dicamba mono-oxygenase (DMO) gene derived from the soil bacterium *Stenotrophomonas maltophilia*. The GM_A90617 soybean event used in this study contained the same DMO gene expression cassette as dicamba-tolerant soybean event MON 87708.

The crop was grown in 12-inch diameter pots containing a loamy sand soil in two greenhouses at the Research for Hire field site in Porterville, California, USA. Untreated control (UNT-C) plants and plants intended for the POE-C and POE-T groups prior to the postemergence treatment were grown in a separate greenhouse from that housing the treated plants. Four seeds were planted per pot. At 14 days after planting, the PRE-C and PRE-T pots were thinned to two plants per pot, and the POE-C and POE-T pots were

thinned to one plant per pot. Details regarding the field site location, crop planting and maintenance, and climate and soil data are given in the field report (pages 122-151 of the in-life report in Appendix B).

2.5 Test Substance Preparation and Application

Applications of the test substance (separate pre- and postemergence applications) were conducted using a hand-held sprayer that fit directly on the spray bottles. A separate bottle containing the required amount of test substance formulation (aqueous solution of the diglycolamine salt of [¹⁴C]dicamba) was used for each pot. The preemergence application was made directly to the soil of the PRE-T group pots on the day of planting after the soybean seeds were planted. The postemergence application was made to the foliage of the plants of the POE-T group 29 days later at the R1 (first flower) growth stage. Details of the test substance preparation and application may be found on pages 19-21, Table I (page 35), pages 75-82 and page 137 of the in-life report (Appendix B).

2.6 Sample Collection

Immature foliage (pre-forage) was collected as thinnings from UNT-C and PRE-T plants 14 days after planting and the preemergence application. Forage samples were collected from all pots of all treatments 7 days after the postemergence application and 36 days after the preemergence application. Two individual forage plants (plant #13 and plant #28) were collected separately from the POE-T group for surface residues determination. Hay was collected from UNT-C, PRE-T and POE-T groups 27 days after the postemergence treatment (56 days after the preemergence treatment). Seed was collected from all groups 83 days after the postemergence treatment (112 days after planting and the preemergence treatment). Details regarding the collection and storage of the soybean samples, and dates of collection, are given on page 17, pages 137-138, pages 140-141 and page 144 of the in-life report (Appendix B).

2.7 Sample Preparation

Immature foliage (pre-forage), forage, hay and seed specimens were ground in a food processor or blender in the presence of dry ice at PTRL West, Inc.

At the field site at the time of harvest, forage collected from individual POE-T plants #13 and #28 was rinsed (separately) in three consecutive vessels each containing 1 L of HPLC grade water. An aliquot of the first rinse was transported to PTRL West for HPLC analysis. Aliquots (3 x 1 mL) from each rinse were collected at the field site for LSC analysis. These aliquots were then transported to PTRL West for repeat LSC analysis. Details of the sample preparation for radioactivity analysis are given on pages 21-22 of

the in-life report (Appendix B). Processed sample weights are presented in Table VIII (page 42) of the in-life report.

2.8 Sample Handling

All samples were stored frozen at ≤ -10 °C prior to and after processing when not in use. Samples were shipped from Research For Hire to PTRL West, Inc., and from PTRL West, Inc. to Monsanto Company, overnight in coolers with dry ice. At Monsanto Company the samples were stored at approximately -20 °C and were thawed only for short periods of time necessary to obtain samples for analysis. Shipment dates are given on pages 42 and 144 of the in-life report (Appendix B).

2.9 Sample Analysis

Definitive combustion analyses of all processed (ground) soybean matrices for total radioactive residue (TRR) determination were conducted at PTRL West, Inc. Initial extractions and HPLC analyses of PRE-T and POE-T forage and seed were conducted at PTRL West, Inc. to establish an initial baseline for assessment of sample storage stability through the course of the study. Details and results of the analyses conducted at PTRL West, Inc. are given on pages 22-27, pages 29-31, Tables IV-VII (pages 38-41), and Figures 8-13 (pages 54-68) of the in-life report (Appendix B). Definitive extractions of all matrices including the associated combustion analyses, quantitative HPLC analyses for metabolite quantitation, and isolation, purification and identification of metabolites was conducted at Monsanto Company. The methods utilized for these analyses are described herein.

2.9.1 Analytical Instrumentation

2.9.1.1 Sample Oxidizer

Combustion analysis was performed using Packard/PerkinElmer Model 307 sample oxidizers with or without Oximate 80 robotics. Samples were weighed into combustion cones and oxidized in a continuous flow of oxygen to $^{14}\text{CO}_2$ which was trapped in a solution of CarboSorb[®] E and Permafluor[®] E⁺. Performance of the oxidizer was routinely monitored by combustion assays of ^{14}C -stearic acid (Spec-Chec- ^{14}C) spiked onto blank cones and pads.

2.9.1.2 Liquid Scintillation Counters

Liquid scintillation counting (LSC) was performed with Packard Tri-Carb Model 2750TR/LL or PerkinElmer Tri-Carb 2900TR liquid scintillation analyzers. For the initial portion of the study, Packard Tri-Carb Model 2750TR/LL counters interfaced to the TRACE system were utilized. TRACE, an acronym for **T**he **R**adio**A**ctivity **C**ounting

SystEm, is a hardware and computer software system developed by Monsanto. TRACE collected data from the counters, performed data reduction, and generated reports and histogram plots. On retirement of the TRACE data collection system, data were printed directly from the Model 2750 TR/LL liquid scintillation analyzer software as an interim solution. For the final portions of the study, the liquid scintillation counting system TRACE II (The **R**adio**A**ctivity Counting Syst**E**m, 2nd generation), a multi-component hardware/software system for LSC data collection, storage, processing, and reporting, was utilized. This system incorporates PerkinElmer Tri-Carb 2900TR liquid scintillation analyzers with QuantaSmart 2.02 software interfaced to a data collection server running Jane™ (Ver. 4.2.18.30), a liquid scintillation counting data acquisition, processing, and reporting software application developed by LabLogic Systems Limited and modified to Monsanto Company specifications.

Quench curves for conversion of cpm to dpm values were established for each instrument using sets of sealed vials containing a fixed quantity of ¹⁴C-radioactivity ([¹⁴C]toluene), increasing quantities of a quenching solvent (nitromethane), and an appropriate counting fluid. The sealed vials were purchased from PerkinElmer Life and Analytical Sciences. The instrument performance (counting efficiency and background) was assessed each day prior to usage by counting sealed background and standard (¹⁴C and ³H) vials.

For each set of vials, the background or noise value was determined by counting vials (typically one or three) containing only scintillation cocktail (and solvent, optionally) to obtain an average cpm background level. The computed value is used by the TRACE or TRACE II software to establish a background threshold that is employed to determine if the sample radioactivity is distinguishable from background. All LSC data were corrected for background.

Samples were counted using the automatic external standard pulse method. Vials were generally counted using a single five-minute pass. Samples were primarily aliquoted by weight and generally analyzed in triplicate, although fewer replicates were used in some cases, by admixture with Ultima-Flo™ AP scintillation cocktail. Samples derived from lignin extraction experiments were mixed with 10% aqueous sodium thiosulfate to reduce the chlorite prior to counting. Very acidic or basic samples were neutralized prior to counting.

2.9.1.3 High Performance Liquid Chromatography

An HP1100 HPLC system (Agilent Technologies, Palo Alto, CA) was used for the metabolite analysis phase of this study (an additional HPLC system utilized for LC/MS analyses is described below in Section 2.9.1.4). The HPLC system consisted of the following: G1322A solvent degasser; G1311A quaternary pump; G1315A diode array

(UV) detector; G1313A autosampler with a 900- μ L loop; G1316A column heater; and a Packard 150TR Flow Scintillation Analyzer radioactive flow detector (RAD). ChemStation software (Agilent Technologies, version A.10.02) was used to control injection volumes and gradient conditions. An ISCO (Lincoln, NE) Foxy fraction collector was used for collection of fractions for HPLC/LSC analysis and for metabolite isolation or purification. Refer to Section 2.9.2.1 for descriptions of HPLC methods.

Two methods of detection and quantitation of radioactivity in the HPLC effluent were employed in this study. In one method, designated herein as HPLC/RAD, the HPLC effluent issuing from the UV detector was fed into the mixing tee of a radioactive flow detector (RAD). Ultima-FloTM AP scintillation fluid was simultaneously pumped into the RAD mixing tee at a ratio of approx. 3:1 with the HPLC effluent (3.6:1 for HPLC Method C). The outflow of the mixing tee was directed into the detection cell of the RAD. The HPLC solvent flow rate was 3 mL/min or 2.5 mL/min (HPLC Method C). Data collection (radioactive flow detection and UV detection), analysis and report generation were performed by the Atlas 2003R1.1 chromatography data collection system (ThermoElectron Corporation, Altrincham, UK). The second method of detection, designated herein as HPLC/LSC, consisted of collection of the HPLC effluent employing a fraction collector with subsequent liquid scintillation counting of the fractions after admixing with Ultima-FloTM AP scintillation fluid. Fractions were generally collected in 0.3-minute intervals for HPLC/LSC analyses.

One additional HPLC system was utilized for HPLC Method E (analysis of fatty acid methyl esters). The HPLC system consisted of the following: two Waters 515 pumps, a Waters 2487 Dual UV detector at 206 nm and a Rheodyne 7125 manual injector with a 2-mL loop. A Foxy 200 fraction collector (ISCO, Lincoln, NE) was used for collection of fractions for HPLC/LSC analysis (0.5 min per vial). Atlas 2003R1.1 chromatography data collection system (ThermoElectron Corporation, Altrincham, UK) was used for collection, processing and reporting of UV data.

2.9.1.4 Mass Spectrometry

Liquid Chromatography / Electrospray Ionization (LC/ESI) Mass Spectrometry:

Electrospray mass spectra were recorded with a ThermoFinnigan LCQ ion trap mass spectrometer interfaced with an Agilent HP1100 HPLC consisting of a G1322A solvent degasser, G1312A binary pump, G1313A autosampler and G1315A diode array detector. Metabolite samples and reference standards were generally chromatographed using HPLC Methods F or G (see Section 2.9.2.1). On occasion, loop injections (designated Loop/ESI) were conducted in which the sample solution (5 μ L) was injected directly into HPLC mobile phase bypassing the HPLC column. The liquid chromatograph eluent was directed into the mass spectrometer *via* an electrospray ionization source consisting of a

stainless steel sprayer held at 4.5 kV that was connected to a source of high purity nitrogen at 100 psi (sheath gas flow rate setting of 60). The spray generated in the source was directed onto a stainless steel capillary that was held at a temperature of either 220 or 230 °C throughout the analysis. The mass spectrometer was typically scanned from 50 or 100 amu to 800 or 1000 amu at a rate determined by the data system to maintain an acceptable ion population in the ion trap. Negative or positive ions were analyzed. MS/MS analyses were conducted by collision-induced dissociation using helium gas at a normalized collision energy of 50%. Prior to each set of mass spectral analyses, a reference standard was analyzed to verify system performance.

Control of the mass spectrometer, as well as mass spectral data acquisition and processing, was accomplished with ThermoFinnigan Xcalibur software, version 1.0, on a Gateway G6-200 computer running Windows NT. A hand-held Agilent HP 1100 Series Control Module running firmware version A.02.02 was utilized for control of the HPLC instrument. During the course of the study, the system computer and software were upgraded to a Dell Optiplex GX-620 workstation running Windows XP Professional Version 2002 SP2 with ThermoFinnigan Xcalibur software version 2.0 SR2, LCQ MS software version 2.0 and LC Devices software version 1.4 for mass spectral and HPLC instrument control and data processing, and Agilent ChemStation software version A.10.02 for optional HPLC instrument control.

Liquid Chromatography / IonSpray Ionization (LC/ISP) Mass Spectrometry:

IonSpray MS/MS spectra of metabolite Peak 11 were acquired on an Applied Biosystems/MDS Sciex API 5000 triple quadrupole MS/MS instrument equipped with a Waters ACQUITY ultra-performance liquid chromatograph. Samples were chromatographed on an ACQUITY BEH C₁₈ column (50 mm x 2.1 mm, 1.8 µm) held at 60 °C at a flow rate of 0.6 mL/min. The mobile phase consisted of 1% (v/v) formic acid in water (A) and 1:1 acetonitrile:methanol (B). Gradient elution (90% to 5% solvent A, linear over 10 minutes) was utilized. The liquid chromatograph eluent was introduced into the mass spectrometer by means of an articulated ion spray source using nitrogen as the nebulizing gas. The source temperature was maintained at 600 °C. MS/MS spectra were generated by collision-induced dissociation at a relative collision energy setting of -30. Data were collected and processed using Analyst version 1.4.2 software.

High Resolution IonSpray Ionization Time-of Flight (TOF) Mass Spectrometry:

IonSpray ionization TOF mass spectrometric analysis was conducted in negative ion mode for exact mass determination of metabolite Peak 11. A PerkinElmer/Sciex QSTAR quadrupole time-of-flight mass spectrometer with a TurboIonSpray source and Analyst software was used. Samples were introduced by infusion utilizing a Harvard Apparatus Pump II syringe pump at a flow rate of approx. 20 µL per minute. Prior to exact mass determination, the instrument was tuned and calibrated using taurocholic acid.

For high resolution accurate mass determination of metabolite Peak 11, the standards taurocholic acid (**XIX**), fexofenadine (**XX**) and MRFA (**XXI**, Met-Arg-Phe-Ala tetrapeptide) were added to the Peak 11 sample solution and all four components were infused and analyzed simultaneously. For each exact mass determination, the sample solution was infused and data were collected and averaged over a period of 2-5 min. The exact mass experiment was repeated three times. Fexofenadine and MRFA, whose negative ion parent exact masses (500.28008 and 522.24986) closely bracket the metabolite Peak 11 parent ion mass (511), served as calibration standards. Taurocholic acid (negative ion parent exact mass of 514.28385) was included as a check of the accuracy of the instrument. The high resolution data were processed by setting the masses of the bracketing fexofenadine and MRFA parent ion peaks to their theoretical values and reading the exact masses of the Peak 11 unknown and the taurocholic acid standard. For the three experiments, the exact masses measured for the anion of the taurocholic acid standard, and the associated deviations from the theoretical mass were 514.2833 (1.1 ppm), 514.2833 (1.1 ppm) and 514.2828 (2.0 ppm) demonstrating very good mass accuracy for this method.

Gas Chromatography / Electron Ionization (GC/EI) Mass Spectrometry: Electron ionization (EI) mass spectrometric analyses were conducted in positive ion mode at 70 eV using either a Finnigan TRACE MS mass spectrometer equipped with a TRACE GC 2000 gas chromatograph and an AS 2000 autosampler or a Finnigan TRACE DSQ MS mass spectrometer equipped with a TRACE GC Ultra gas chromatograph and an AS 3000 autosampler. Instrument control and data processing were performed with Finnigan Xcalibur™ software, version 1.2 (TRACE MS) or version 1.4.1 (TRACE DSQ MS). High purity helium was employed as the carrier gas. Samples were injected in split/splitless mode. The mass spectrometer was scanned from 30 to 300 amu (TRACE MS) or 41 to 400 amu (TRACE DSQ MS). Various chromatography methods were used as listed below (Methods A-D utilized the TRACE MS, Method E utilized the TRACE DSQ MS):

- A) The gas chromatograph was equipped with a Restek Rxi-1 MS capillary column (20 m x 0.18 mm i.d., 0.18 µm film). The column oven temperature was held at initial temperature (50 °C) for 1 min and was raised to 260 °C at 15 °C/min with a final 3 min hold. Helium flow 0.5 mL/min. Injector 250 °C.
- B) The gas chromatograph was equipped with a Restek Rtx-1 MS capillary column (30 m x 0.25 mm i.d., 0.25 µm film). The column oven temperature was held at initial temperature (70 °C) for 1 min and was raised to 260 °C at 10 °C/min with a final 3 min hold. Helium flow 1.0 mL/min. Injector 220 °C.

- C) The gas chromatograph was equipped with a Restek Rtx-1 capillary column (20 m x 0.18 mm i.d., 0.2 μ m film). The column oven temperature was held at initial temperature (50 °C) for 1 min and was raised to 260 °C at 15 °C/min with a final 3 min hold. Helium flow 0.5 mL/min. Injector 250 °C.
- D) The gas chromatograph was equipped with a Restek Rtx-1 capillary column (20 m x 0.18 mm i.d., 0.2 μ m film). The column oven temperature was held at initial temperature (90 °C) for 1 min and was raised to 260 °C at 10 °C/min with a final 3 min hold. Helium flow 1.0 mL/min. Injector 250 °C.
- E) The gas chromatograph was equipped with a Restek Rtx-1 MS capillary column (30 m x 0.25 mm i.d., 0.25 μ m film). The column oven temperature was held at initial temperature (70 °C) for 1 min and was raised to 260 °C at 10 °C/min with a final 3 min hold. Helium flow 1.0 mL/min. Injector 220 °C.

Prior to each set of mass spectral analyses, a reference standard was analyzed to verify system performance.

Nuclear Magnetic Resonance Spectrometry: NMR spectra were obtained on a Varian Inova 400 MHz NMR spectrometer. The instrument was controlled and data were collected and processed by Varian VNMRJ v2.1b software. Standard Varian proton and carbon pulse sequences were utilized. For proton NMR spectra of metabolite Peak 11 in D₂O, 5 mm BMS3 Shegemi NMR tubes were utilized with the Varian PRESAT pulse sequence for water peak suppression.

2.9.2 Analytical Methods and Procedures

2.9.2.1 HPLC Methods

The following HPLC methods were utilized in this study. All gradient segments were linear. Mobile phase solvents and columns were maintained at ambient temperature for all methods. Detection of reference standards by UV using HPLC Methods B and C was at 280 nm. Detection of reference standards and metabolites by UV using LC/MS Methods F and G was at 280 nm (typical) or 254 nm.

Method B was the primary method used in this study. Typical retention times of the primary reference standards (**I-IV**) under reverse phase HPLC Method B are listed in Table 3, and a representative chromatogram of a mixture of these four reference standards is shown in Figure 5.

Method A	
Uses	Test Substance Purity and Stability Analyses
Column	Beckman Ultrasphere ODS, 5 µm, 4.6 x 250 mm
Pre-Column	None
Flow Rate	1.0 mL/min
Solvent A	0.1% Trifluoroacetic Acid in Water
Solvent B	Acetonitrile

TIME (min)	%A	%B
0	90	10
5	90	10
30	0	100
35	0	100

Method C	
Uses	Purification of Metabolites, Metabolite Co-elutions
Column	Beckman Ultrasphere ODS, 5 µm, 10 x 250 mm
Pre-Column	Brownlee NewGuard RP-18
Flow Rate	2.5 mL/min
Solvent A	0.5% (v/v) Formic Acid in Water
Solvent B	Methanol

TIME (min)	%A	%B
0	90	10
5	90	10
50	0	100
60	0	100

Method E	
Uses	Fatty Acid Methyl Ester Analysis
Column	Xpertek Xper-SIL SCX 21.2 x 25 cm, impregnated with AgNO ₃
Pre-Column	None
Flow Rate	18 mL/min
Solvent A	1% Acetonitrile in Hexanes
Solvent B	Hexanes

TIME (min)	%A	%B
0	44.4	55.6
45	44.4	55.6

Method B	
Uses	Soybean Forage, Hay and Seed Profiles, Metabolite Isolation
Column	Beckman Ultrasphere ODS, 5 µm, 10 x 250 mm
Pre-Column	Brownlee NewGuard RP-18
Flow Rate	3.0 mL/min
Solvent A	0.5% (v/v) Formic Acid in Water
Solvent B	Acetonitrile

TIME (min)	%A	%B
0	90	10
5	90	10
50	0	100
60	0	100

Method D	
Uses	Purification of Metabolite Peak 11
Column	Beckman Ultrasphere ODS, 5 µm, 10 x 250 mm
Pre-Column	Brownlee NewGuard RP-18
Flow Rate	3.0 mL/min
Solvent A	Water
Solvent B	Acetonitrile

TIME (min)	%A	%B
0	90	10
5	90	10
50	0	100
60	0	100

Method F	
Uses	LC/Electrospray Mass Spectral Analyses
Column	Keystone Scientific Betasil-C18, 2 x 100 mm, 5 µm, 100 Å pore
Pre-Column	None
Flow Rate	0.20 mL/min
Solvent A	0.5% Formic Acid in Water
Solvent B	Acetonitrile

TIME (min)	%A	%B
0	90	10
10	10	90
15 (or 20)	10	90

Method G	
Uses	LC/Electrospray Mass Spectral Analyses
Column	Keystone Scientific Betasil-C18, 2 x 100 mm, 5 µm, 100 Å pore
Pre-Column	None
Flow Rate	0.20 mL/min
Solvent A	0.5% Formic Acid in Water
Solvent B	Acetonitrile

TIME (min)	%A	%B
0	100	0
1	100	0
20	10	90
25	10	90

Method H	
Uses	Analysis of Metabolite Peak 1 (Sugar Analysis)
Column	Shodex Asahipak NH2P-50 4D
Pre-Column	None
Flow Rate	0.75 mL/min
Solvent A	0.1% NH ₄ OH in Water
Solvent B	Acetonitrile

TIME (min)	%A	%B
0	10	90
5	10	90
50	90	10

2.9.2.2 Analysis of Soybean Pre-forage, Forage, Hay and Seed for Contained Radioactivity Levels

Combustion analyses of soybean pre-forage, forage, hay and seed for TRR determination were conducted by PTRL West, Inc. and are described in the in-life report (Appendix B). Additional combustion analyses of these matrices were conducted during the analytical phase of the study, described herein, associated with the sample extractions in order to determine accountabilities for the extractions, and for the storage stability analyses. These combustion analyses employed quintuplicate aliquots (approx. 70-330 mg each) of sample, except for POE-T forage for which 10 replicates of 18-31 mg each were analyzed and PRE-C and POE-C seed (10 replicates each). The samples were weighed into combustion cones containing a cellulose pad. The combustion cones were then capped with a cellulose pad and stored in a plastic bag in a freezer until combusted. The samples were typically thawed and air-dried for approximately 1 h prior to combustion. The samples were combusted as described in Section 2.9.1.1. The vials that were generated by the oxidizer were counted by LSC. Radioactivity analysis results for pre-forage, forage, hay and seed are presented in Table 4.

2.9.3 Preparation of Soybean Samples for HPLC Analysis

2.9.3.1 Extraction of Residues

Residues were extracted from processed pre-forage, forage and hay, as well as washed forage plant #28, using 40:60 (v/v) acetonitrile:water. Initially, seed was also extracted with 40:60 (v/v) acetonitrile:water. However, due to difficulties in obtaining high quality HPLC profiles of the seed extracts, the definitive seed extractions were conducted by

extracting the seed first with hexane to extract oils followed by an acetonitrile wash and finally extraction with 40:60 (v/v) acetonitrile:water. The following procedures are representative.

Extraction with Acetonitrile:Water

A weighed aliquot (4-5 g) of the thawed pre-forage, forage, hay or seed sample was transferred to a 50-mL plastic centrifuge tube. Extraction solvent (20-35 mL of 40:60 (v/v) acetonitrile:water) was added, and the mixture was shaken on a Burrell wrist-action shaker (maximum setting) for 20 minutes. The tube was then centrifuged at 9,000 rpm (approx. 8,500 x g) in a Sorvall RC-5B refrigerated centrifuge for 10-20 minutes at 4 °C or in a Dynac bench-top unit at the maximum setting for 5 min at RT. The clear extract supernatant was transferred by Pasteur pipette to a 50-mL centrifuge tube and weighed. Three aliquots of each extract were weighed into liquid scintillation vials, mixed with Ultima-Flo™ AP scintillation cocktail, and counted by LSC to determine the extracted radioactivity. This extraction procedure was repeated three times (total of four extractions) for each sample. Vigorous hand-shaking of the mixture was conducted, as required, in order to re-suspend the extraction pellet prior to shaking on the wrist-action shaker. In some cases extracts were stored at ≤ -20 °C for short periods of time (typically overnight) prior to preparation for HPLC analysis.

Exceptions to this procedure were immature foliage (PRE-T pre-forage), for which a 1 g sample was extracted with 4 x 5 mL, PRE-T forage (1.6 g sample extracted with 4 x 20 mL) and PRE-C forage (8 g sample extracted with 4 x 30 mL).

Extraction of Seed with Hexane

For the definitive metabolite quantitation profiles, seed samples were extracted three times with hexane followed by a single acetonitrile extract to remove oils prior to extraction with 40:60 acetonitrile:water. A weighed aliquot (4-5 g) of the thawed seed sample was transferred to a 50-mL plastic centrifuge tube, 25 mL of hexane was added, and the mixture was shaken and centrifuged as described above. The clear extract supernatant was decanted, weighed, aliquoted and counted by LSC to determine extracted radioactivity. This hexane extraction procedure was repeated twice (total of 3 hexane extractions) for each sample. The extraction pellet was then extracted with 35 mL of acetonitrile after drying the pellet with a stream of nitrogen gas to remove the visible hexane. The acetonitrile extract was decanted, weighed, aliquoted and counted by LSC. Finally, four extractions with 40:60 (v/v) acetonitrile:water were conducted as described immediately above.

Combustion of Extraction Pellets (Post-Extraction Solids)

For determination of unextracted radioactivity, each final extraction pellet (following the acetonitrile:water extractions) was dried to remove visible liquid using a gentle flow of nitrogen and/or air-dried for approximately 1 to 5 hours in a fume hood. The dried extraction pellet was mixed well with a spatula, 4-10 combustion samples (100-370 mg each) were weighed into combustion cones containing a cellulose pad, and each cone was capped with an additional cellulose pad. The combustion samples were generally frozen in a plastic bag in a freezer prior to combustion then were thawed, briefly air-dried and combusted as described in Section 2.9.1.1 above.

Extraction results for all soybean matrices are summarized in Table 5 and Table 6.

2.9.3.2 Preparation of Extracts for HPLC Analysis

For each of the individual soybean matrices, each extract (excluding hexane extracts), or a percentage of each extract, was combined into a round bottom flask and weighed (for the PRE-T pre-forage, only the first 3 of the 4 extracts were combined). The extract solution was rotary evaporated under moderate vacuum to remove the acetonitrile and part of the water (bath temperature ≤ 30 °C, except < 38 °C for PRE-C and POE-C forage). Evaporation was stopped when the volume of water remaining was approx. 0.6 gram to 45 grams (depending on the amount of radioactivity present in the initial extract). Aliquots of the distillate were analyzed by LSC to determine if any volatile radioactive components were lost in the evaporation step (no significant amounts of radioactivity were found in any of the extract evaporation distillates). Water was added to the extract concentrate in the flask, as needed, to obtain the required concentration of radioactivity in solution. Acetonitrile (8-10% by weight) was added to the extract concentrate in the round bottom flask for the following matrices: PRE-T pre-forage, POE-T forage, PRE-T hay and POE-T hay. Addition of 10% acetonitrile and 0.5% formic acid by weight was made to the extract concentrate in the round bottom flask for the following: PRE-T forage, PRE-C forage and POE-C forage. The addition of acetonitrile or formic acid made very little difference in the chromatography of the samples. A small portion (approximately 0.5-1.4 mL) of the extract concentrate solution was transferred to a plastic microfuge tube. To the PRE-T, POE-T, PRE-C and POE-C seed extract concentrates, and the washed forage plant #28 extract concentrate, in the microfuge tube was added a small amount of formic acid and acetonitrile equivalent to approximately 0.5% and 10% by weight, respectively, of the weight of the extract concentrate in the microfuge tube. Reference standard was sometimes added to the microfuge tube and the samples were reweighed; however, the UV standards were generally not distinguishable from the matrix background in the HPLC profile of the extract material. Nothing was added to the extract concentrates for PRE-T forage final and extended stability and POE-

T seed final and extended stability samples. The solutions were centrifuged in a bench-top microcentrifuge for 1-1.5 min (the seed extract concentrates required 2 centrifugation steps) resulting in a clear supernatant for HPLC analysis (see Section 2.9.4 below). Only a very small pellet was produced in the centrifugation of the hay and forage extract concentrates while the seed extract concentrates produced a more substantial pellet. Small aliquots of the clear supernatant were removed and counted by LSC to determine preparative (evaporation and centrifugation) recovery and to determine the radioactivity concentration in the solution for determination of HPLC column recovery. Preparative recoveries for samples used to generate the quantitation profiles are found in Table 7.

For the initial profile (initial stability profile) of the POE-T seed extract, a solid-phase extraction (SPE) clean-up step was added to improve the chromatography of the sample. The combined extract solution was evaporated as above to about 35 g, acidified, applied to an SPE cartridge and eluted as described below in Section 2.9.6. Aliquots were counted by LSC of the application solution and SPE cartridge eluate to calculate recovery. The resulting sample from the SPE was then evaporated (with antifoam B) until nearly dry. Acetonitrile and formic acid (10% and 0.5% by weight, respectively) were added to the concentrate. Approximately 1 mL of the sample was transferred to a microfuge tube, 10 µL of a solution of dicamba reference standard (**I**) was added and the tube was centrifuged to give a clear supernatant for HPLC analysis.

2.9.4 Quantitative HPLC Analyses

Reverse phase HPLC/LSC and HPLC/RAD analyses of extract concentrates were conducted using HPLC Method B. HPLC/UV data were also collected at 280 nm; however the UV reference standard (if added) was not generally visible due to the matrix background. Semi-preparative HPLC columns were employed in the HPLC analyses to allow injections of sufficiently large samples. Peaks in the HPLC/LSC and HPLC/RAD quantitation chromatograms are labeled “Peak 1” through “Peak 26” based on their retention times as shown in the quantitation profiles (Figure 6 through Figure 17).

As a general procedure, the prepared sample (Section 2.9.3.2) was transferred to an autosampler vial, which was then weighed. The sample was injected on the HPLC and the vial was again weighed to determine the weight of sample injected. The volume injected was 250 to 900 µL (except for POE-T forage for which the volume injected was 15 µL) depending on the amount of radioactivity in the prepared sample. Weighed (applied) aliquots of the sample were counted by LSC to determine the amount of radioactivity injected onto the column.

For HPLC/LSC analyses (PRE-T, POE-T, PRE-C and POE-C seed, POE-T seed final and extended stability profiles, and PRE-T forage final and extended stability profiles),

fractions of 0.3 min each were collected in scintillation vials over the course of the HPLC analysis (except for the final and extended stability profiles for which 0.5 min fractions were collected). The fractions were mixed with Ultima-Flo™ AP scintillation cocktail (6 mL each), and were counted by LSC. The Jane software produced a histogram plot showing the radioactivity in each fraction and a tabular report showing the total radioactivity in the analyzed fractions, the percent of the total distribution in each fraction, and the HPLC column recovery based on the sum of radioactivity in all fractions and the radioactivity in the applied aliquots. Radioactivity was assigned to the various peaks in the HPLC/LSC quantitation profiles by integration using the Jane software. For the HPLC/LSC quantitation profiles, all peaks with an area of 10 or more dpm were integrated and quantitated, although in some cases smaller peaks were also integrated. In general, all peaks constituting approx. 0.5% of the profile or greater were quantitated. Peaks generally consisted of three to five vials and attempts were made to integrate based on peak symmetry. In cases where unresolved peaks were present, the Jane software allowed integration such that half of the radioactivity in the vial common to the two peaks is assigned to one peak and half to the other peak. The Jane software produced a table of integrated peaks listing the percent of the total distribution of radioactivity (percent of chromatogram) for each peak.

For HPLC/RAD analyses (PRE-T pre-forage; PRE-T, POE-T, PRE-C, POE-C and POE-T washed plant #28 forage; PRE-T, POE-T, PRE-T final and extended stability, POE-T final and extended stability hay; and POE-T initial seed stability), the peaks were integrated using the Atlas software (either automatic or manual integration, or a combination). For each profile, the chromatogram was expanded visually within the software and all peaks discernible above the noise were integrated. This typically resulted in all peaks greater than 0.2-0.5% of the profile being quantitated. The Atlas software produced a chromatogram and a tabular report showing the percent of the total distribution in each peak. For each profile, the HPLC/RAD column effluent was collected and aliquots were counted by LSC to determine the HPLC column recovery based on the radioactivity in the effluent and the radioactivity in the applied aliquots.

Radioactivity recoveries for the quantitation analyses are summarized in Table 7. Quantitation results are shown for each profile with the respective chromatogram (Figure 6 through Figure 17). Summaries of the quantitation of identified metabolites and unidentified metabolites are shown in Table 8 through Table 13.

2.9.5 Partitioning of Extract Concentrates with Ethyl Acetate

Partitioning of soybean matrix (seed or hay) extracts was utilized to obtain information on the organic/aqueous partitioning behavior of dicamba residues and to facilitate quantitation of the metabolites or to facilitate isolation and purification of metabolites.

The following procedure is representative. The combined acetonitrile:water extracts from extraction of 4 g of POE-T seed or 5 g of POE-T hay (extracted as described above in Section 2.9.3.1) were rotary evaporated to remove the acetonitrile. The remaining aqueous solution was acidified to pH 1-2 with conc. HCl and was partitioned with three 30-mL portions of ethyl acetate. The combined ethyl acetate partitions and the remaining aqueous layer were analyzed by LSC to determine the amount of radioactivity in each phase. The ethyl acetate phase was evaporated to dryness and the residue was reconstituted in 0.5% aq. formic acid and acetonitrile for HPLC analysis. The results of the partitioning experiments are shown in Figure 18 and Figure 19 for POE-T seed and Figure 97 for POE-T hay.

2.9.6 Solid Phase Extraction (SPE) of Seed Extract Concentrates

Solid phase extraction on reverse phase (C-18) cartridges was utilized in some cases to clean up and clarify the POE-T and PRE-T seed extracts prior to HPLC/RAD analysis (to allow injection of a sufficiently large sample) and to facilitate the isolation of the very polar Peak 1 from POE-T seed extracts. After extracting the seed with 40:60 acetonitrile:water and rotary evaporation of the combined extracts to remove acetonitrile, the aqueous solution was acidified to pH 2-3 with conc. HCl and applied to a pre-conditioned 60 cc Mega Bond Elut™ C18 SPE cartridge (pre-conditioned with methanol then 0.5% aq. formic acid). The cartridge was eluted step-wise with 0.5% aq. formic acid and acetonitrile mixtures containing progressively higher amounts of acetonitrile from 100% aq. 0.5% formic acid to 60:40 acetonitrile:0.5% formic acid followed finally by 100% acetonitrile. All of the eluent was collected, evaporated to dryness and used for HPLC analysis.

2.9.7 Isolation and Purification of Metabolites

For the purpose of identification, metabolite peaks were isolated primarily from the POE-T forage except where noted. All metabolite peaks constituting greater than 1.0% of the TRR in any matrix and also present at levels greater than 0.05 mg/kg were isolated and purified for characterization or identification. Extraction and partitioning methods utilized for isolation of metabolites were very similar to those described in Sections 2.9.3 and 2.9.5 except, in many cases, larger samples were extracted and larger HPLC injections were conducted in order to obtain sufficient quantities of metabolites for identification. Metabolites were isolated by preparative HPLC fractionation using the primary HPLC method (Method B). The isolated metabolite fractions were typically purified by preparative HPLC using Method C. Method C utilized methanol as the organic component of the mobile phase providing a different selectivity than the acetonitrile mobile phase organic component of Method B. Method C was effective in

further separating the isolated metabolites from much of the background soybean matrix contaminants present in the initial isolates.

All isolates and purified metabolites were stored frozen at approximately -20°C and were thawed only for short periods of time as needed.

Following are details of the isolation and purification of metabolites identified or characterized in this study. The peak names are based on their designations in the HPLC quantitation profiles.

Peak 1

Metabolite Peak 1 was isolated from the 40:60 (v/v) acetonitrile:water extracts of POE-T seed (with or without prior hexane extraction) by HPLC fractionation using HPLC Method B. Optionally, an SPE clean-up of the extract concentrate on a 60 cc Mega Bond Elut™ C18 column eluting successively with 30:70, 60:40 and 100:0 acetonitrile:0.5% aq. formic acid was conducted prior to HPLC fractionation.

Peak 3

Metabolite Peak 3 was isolated from the POE-T forage extract concentrate using HPLC Method B. The isolated metabolite fraction was further purified using HPLC Method C.

Peak 8

Metabolite Peak 8 was isolated from the POE-T forage extract concentrate using HPLC Method B. The isolated metabolite fraction was purified using HPLC Method C.

Peak 9

Metabolite Peak 9 was isolated from the PRE-T pre-forage extract concentrate and the POE-T forage extract concentrate by fractionation using HPLC Method B. The isolated metabolite fractions were further purified using HPLC Method C.

Peak 11

Metabolite Peak 11 was isolated from the POE-T forage extract concentrate using HPLC Method B. The isolated metabolite fraction was purified using HPLC Method C. For NMR analysis, a relatively large quantity (approx. 2.9 μg) of the metabolite was isolated from 6 g of POE-T forage using HPLC Method B. The isolate was extensively purified to remove matrix contaminants by chromatography using HPLC Method C followed by Method D and finally Method B.

Peak 14

Metabolite Peak 14 was isolated from the ethyl acetate partition of acidified POE-T hay extract concentrate using HPLC Method B.

Peak 17

Metabolite Peak 17 was isolated from the ethyl acetate partition of acidified POE-T hay extract concentrate using HPLC Method B followed by purification using HPLC Method C.

Peak 18

Metabolite Peak 18 was isolated from the POE-T forage extract concentrate using HPLC Method B followed by purification using HPLC Method C.

Peak 22

Metabolite Peak 22 was isolated from the POE-T forage extract concentrate using HPLC Method B. Peak 22 also was isolated from the ethyl acetate partition of acidified POE-T hay extract concentrate using HPLC Method B. In both cases, the isolate was further purified using HPLC Method C.

Peak 23

Peak 23 was isolated from the POE-T forage extract concentrate using HPLC Method B. The isolated fraction was purified using HPLC Method C.

2.9.8 Metabolite Identification and Characterization

Metabolites were identified and/or characterized by a variety of methods. Examination of the aqueous/organic partitioning properties of metabolites and comparison of their HPLC retention times to those of authentic reference standards aided in the characterization and identification of metabolites. The primary technique for identification was mass spectral analysis which provided molecular weights and some structural information. Extensive utilization of hydrolysis (acid, base or enzymatic) and chemical derivatization, especially methylation and acetylation, was employed to provide additional structural information on the metabolites. Comparison of the chromatographic and mass spectral properties of the metabolite derivatives or hydrolysates to those of synthetic reference standards was used to confirm the identities of the metabolites. In one case (Peak 11), high resolution mass spectrometry and NMR spectroscopy were required to firmly establish the identity of the metabolite.

General methods and procedures for metabolite identification are described in the sections below. The specific methods and experiments employed to identify each individual metabolite are detailed in Section 3.6.1 of the Results and Discussion section. The identities and structures of metabolites identified in this study are shown in Table 1.

2.9.8.1 Mass Spectrometry

LC/Electrospray mass spectrometry using HPLC Methods F or G was the primary characterization/identification method used for metabolite identification. Typically, negative ion mass spectrometry was utilized for the metabolites and their hydrolysis products since dicamba metabolites generally contained a free ionizable carboxylic acid or phenol group. Neutral derivatives (methyl esters, acetates) of the metabolites were generally analyzed by positive ion mass spectrometry. GC/EI mass spectrometry was utilized for volatile derivatives of the metabolites or their hydrolysis products. Tandem mass spectrometry (MS/MS) was utilized, in select cases where sufficient sample was available, to obtain fragmentation and additional structural information.

Mass spectra for metabolites and derivatives are shown in the figures (Section 6). For clarity, the LC/MS figures generally present a lower mass range (typically 100-600 amu) than the full range of data collected. In many cases, gas-phase dimers or sodium adducts of the dimers were observed as minor ions but may not be shown in the figures. These dimer ions, if observed, are included in the discussion of individual metabolite identifications (Section 3.6.1).

2.9.8.2 HPLC Coinjection of Isolated Metabolites or Derivatives with Analytical Reference Standards

Coinjection analysis with reference standards by HPLC was performed in a limited number of cases on isolated metabolites or the products of hydrolysis and/or derivatization of metabolites. For these analyses, the metabolite or derivative was detected by radioactive flow detection (RAD) while the reference standard was detected by UV. Thus, it is necessary to consider the lag time between the UV and RAD detectors to determine if the RAD and UV peaks coelute. This lag time, which is the amount of time necessary for the HPLC eluent to travel from the UV detector to the RAD detector, was generally in the range of 0.2-0.4 min. In these analyses, peaks were considered to coelute if the RAD peak was 0.2-0.4 min later in retention time than the UV peak.

2.9.8.3 Derivatization of Metabolites

Metabolites were methylated with trimethylsilyldiazomethane. Methylation combined with mass spectrometry of the derivatives gave information indicating the presence of carboxylic acid and/or phenolic groups. Acetylation with acetic anhydride/pyridine

combined with mass spectrometry of the derivatives gave information indicating the presence of hydroxyl or phenolic groups. The following procedures are representative.

Methylation

The sample in a reaction vial was evaporated to dryness under a stream of nitrogen and the residue was taken up in 300 μ L of methanol and 1 μ L of trifluoroacetic acid. Trimethylsilyldiazomethane (TCI America, 10% solution in hexane) was added (300-500 μ L) until the yellow color persisted and the solution was allowed to stand at RT for 35 min. The reaction mixture was then evaporated to dryness under nitrogen and the residue was taken up in an appropriate solvent for chromatographic and/or mass spectrometric analysis.

Acetylation

Acetylation was conducted using acetic anhydride and pyridine as follows. To the sample (evaporated to dryness under nitrogen) in a reaction vial was added 0.2 mL of pyridine and 0.4 mL of acetic anhydride. The solution was maintained at RT for 1-2 h or overnight and then was evaporated to dryness under a stream of nitrogen. The derivatized sample was taken up in an appropriate solvent for analysis by HPLC or mass spectrometry. For metabolites that were difficult to acetylate (e.g., Peak 11), the reaction mixture was heated at 60 $^{\circ}$ C for 3 h or, preferably, a small amount (3.8 mg) of 4-(dimethylamino)pyridine was added to the acetic anhydride/pyridine reaction mixture and the mixture was maintained at RT for at least 2 h.

Silylation

A solution of the metabolite hydrolysate (e.g., base hydrolysis product of Peak 11) or carboxylic acid standard (HMGA), containing Na_2SO_4 from NaOH hydrolysis and neutralization with H_2SO_4 , in a reaction vial was evaporated to dryness under a stream of nitrogen. The residue was taken up in 0.2 mL of acetone, and the solution and solids were sonicated for 5 min then heated at approx. 72 $^{\circ}$ C for approx. 10 min. The solids were broken up with a needle as necessary. The acetone solution was transferred to an autosampler vial along with a 0.1 mL acetone wash of the solids and was evaporated to dryness. Acetonitrile (25 μ L) and pyridine (25 μ L) were added to the vial and the mixture was vortexed. The vial was sealed with a septum cap and 175 μ L of a mixture of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (Supelco) was injected by syringe through the septum. The vial was heated at approx. 72 $^{\circ}$ C for approx. 3.5 h, cooled to RT and the solution was analyzed directly by GC/EI/MS.

Butylation

The metabolite hydrolysate solution (e.g., base hydrolysis product of Peak 8) or carboxylic acid standard (e.g., malonic acid), containing NaCl from NaOH hydrolysis and neutralization with HCl, was evaporated to dryness in a reaction vial and was treated with 1 mL of boron trifluoride/butanol reagent (Supelco) at 60 °C for 10 min. After cooling, 1 mL of hexane and 1 mL of saturated aqueous NaCl was added, the mixture was shaken, and the layers were allowed to separate. The top (hexane) layer was removed and the aqueous mixture was partitioned once more with hexane. The combined hexane solutions were washed with 2 mL of saturated aqueous K₂CO₃ and were dried over Na₂SO₄. The solution was evaporated to a volume of approx. 300 µL and was analyzed by GC/EI/MS.

2.9.8.4 Hydrolysis of Metabolites

Hydrolysis of metabolites, often coupled with derivatization, was utilized to obtain information on the identity of the metabolite exocon and the nature and position of attachment of conjugating moieties, the endocon (i.e., sugars such as glucose or carboxylic acids such as malonic acid). Very mild base hydrolysis generally effected the removal of conjugating carboxylic acids. More stringent basic conditions often effected removal of conjugating sugars. Acid hydrolysis was utilized to effect removal of sugar moieties from the metabolites. In limited experiments, the enzyme β-glucosidase was utilized to obtain information on the nature of conjugating sugar moieties. The following procedures are representative.

Hydrolysis with Acid

The sample in a reaction vial was evaporated to dryness. After addition of 100 µL of 2 N HCl, the mixture was heated at 97-100 °C for 2 h. The solution was neutralized (final pH 1-2) with 100 µL of 2 N NaOH and was analyzed by HPLC. A milder hydrolysis was effected by treatment of the metabolite with 1 N HCl at 60-62 °C for 1 h.

Hydrolysis with Base

The metabolite sample in a reaction vial was evaporated to a small volume (approx. 25 µL) under a stream of nitrogen and was treated with 200 µL of 1 N NaOH at 58-65 °C for 5 h. The mixture was neutralized/acidified with 220 µL of 1 N HCl and was analyzed by HPLC. A very mild base hydrolysis (for cleavage of conjugating carboxylic acids such as malonic acid from glucose conjugates) was effected by treatment of the metabolite (evaporated to dryness in a reaction vial) with 1 N NaOH at RT for 1 h. The solution was neutralized/acidified by addition of 2 N HCl or 3.6 N H₂SO₄, was evaporated to dryness

and the reaction product was analyzed by HPLC and/or derivatized (butylated or trimethylsilylated) for GC/MS analysis.

Hydrolysis with β -Glucosidase

The metabolite solution in a reaction vial was evaporated to dryness and taken up in 0.1 mL of 0.05M sodium acetate buffer (pH 5) solution plus 0.1 mL of a solution of 0.6 mg of β -glucosidase (Sigma, from almonds, 26 units/mg) in 1 mL of 0.05M sodium acetate buffer (pH 5). The mixture was incubated at 37 °C for 69 h then was analyzed by HPLC. The activity of the enzyme was verified by incubation of p-nitrophenyl- β -D-glucopyranoside (positive control) resulting in formation of an intense yellow color (p-nitrophenol). A blank solution of the enzyme in buffer alone did not produce the yellow color.

2.9.8.5 Confirmation of Metabolite Identification in Seed Extracts

Due to the relatively low residue levels in the seed and the difficulty in obtaining sufficient quantities of pure metabolites from the seed extracts, metabolites were not specifically isolated from the seed. It can be reasonably assumed that metabolite peaks in the seed extracts represent the same metabolites as those peaks in forage and hay with the same or similar retention times. However, the retention times of the metabolites in seed extracts were slightly different than those in the other matrices due to larger amounts of matrix in the seed extract HPLC injections and the resulting matrix effects on the chromatography. In order to confirm the identities of these peaks, seed extracts (seed was extracted with hexane, acetonitrile and acetonitrile:water as described in Section 2.9.3.1) were spiked individually with the metabolites which had been isolated and purified from POE-T forage and were analyzed by HPLC/LSC using either Method B or C. The spiked profile retention times were compared to the unspiked profile retention times to verify the peak identities. The results of these analyses are presented in Table 17 and Figure 20 through Figure 22.

2.9.8.6 Confirmation of the Identities of Diacid Conjugating Moieties in Metabolites

Metabolite Peaks 8 and 11 were identified as glucose conjugates that were further modified with dicarboxylic acid moieties esterified to the glucose. Since these conjugating moieties were derived endogenously they were not radioactive and could not be identified by normal methods. The following procedures were used to identify or confirm the identity of the dicarboxylic acid moieties in these metabolites.

The purified metabolite fraction isolated using HPLC Method B and purified using HPLC Method C (Peak 8 was further purified using HPLC Method B) was subjected to

mild base hydrolysis (Section 2.9.8.4) to effect hydrolytic removal of the conjugating diacid to be identified. The base was neutralized (HCl or H₂SO₄) and the solution was subjected to either the butylation (Peak 8 hydrolysate) or silylation (Peak 11 hydrolysate) procedure described in Section 2.9.8.3. The resulting derivative was analyzed by GC/EI/MS. For each analysis, four samples were analyzed: a solvent blank, a negative control, the metabolite derivative, and a positive control. The negative control was included to insure that the conjugating moiety was derived from the metabolite and not underlying matrix material. It was prepared in an identical manner to the metabolite derivative using an HPLC fraction collected near the metabolite fraction in the final purification, but not containing the metabolite. The negative control consisted of a solvent blank for the Peak 8 analysis. For the Peak 11 analysis, a blank vial was subjected to the base hydrolysis and silylation procedures to create the negative control. The positive control for each analysis consisted of a reference standard of the suspected diacid moiety that was subjected to the identical hydrolysis and derivatization procedures, at a similar level (10-400 ng) to that of the metabolite.

2.9.9 Analysis of Hexane Extracts of Soybean Seed

2.9.9.1 Exhaustive Hexane Extraction of Soybean Seed and Fatty Acid Analysis

Samples of PRE-T and POE-T soybean seed (approx. 4 gram each) were extracted with 25 mL of hexane by shaking for 20 minutes in a centrifuge tube and centrifuging at 9,000 rpm in a Sorvall RC-5B centrifuge for 15-20 minutes. The hexane extract was decanted and the extraction was repeated 2 more times. The resulting extraction pellet was transferred to a round bottom flask and was further extracted by refluxing with hexane for 2 hours with stirring. The mixture was then transferred to a centrifuge tube and centrifuged as above. The four hexane extracts were aliquoted and counted by LSC to determine the total radioactivity extracted by hexane. For POE-T seed, a small portion of the extracts were filtered, aliquoted and counted. The resulting activity was similar to the unfiltered extracts. The hexane fractions were frozen.

For POE-T seed, the combined hexane extracts were rotary evaporated to near dryness at room temperature. Methanol (100 mL) and concentrated sulfuric acid (2 mL) were added to the round bottom flask and the sample was refluxed for 2.5 hours with stirring. The solution was cooled, partitioned three times with 50 mL of hexane, and the combined hexane extracts were rotary evaporated to near dryness. The residue was taken up in 10 mL of hexane. At each step, the sample was weighed and weighed aliquots were counted by LSC. Approximately 1 mL of the final hexane solution was transferred into an autosampler vial and 400 µL of the solution was analyzed by HPLC/LSC (0.5 min fractions collected, 11 mL of cocktail added to each) using the fatty acid methyl ester method (Method E). A mixture of fatty acid methyl ester reference standards (**XIV**-

XVIII) corresponding to the fatty acids known to be present in soybean seed was injected prior to the radioactive sample using the same HPLC method. The results of the analysis of the seed hexane extract are presented in Table 15 and Figure 23.

2.9.9.2 Analysis of Hexane Extract Residues for Dicamba and DCSA

To characterize radioactivity in the hexane extracts of control (PRE-C and POE-C) soybean seed, the combined hexane extracts of each matrix were partitioned with 1:1 acetonitrile:aqueous sodium bicarbonate (pH 8). Prior tests showed that dicamba and DCSA partitioned out of hexane into 1:1 acetonitrile:bicarbonate virtually quantitatively (>97%). The hexane extracts of PRE-C and POE-C seed were each partitioned with acetonitrile:sodium bicarbonate. No significant radioactivity partitioned out of the hexane into the aqueous acetonitrile:bicarbonate solution, indicating that no dicamba or DCSA was present in the hexane extracts. Results of these experiments are presented in Table 16.

2.9.10 Analysis of Unextracted Residues

The extraction pellets remaining after 40:60 acetonitrile:water extractions of the PRE-T and POE-T soybean seed were subjected to additional extractions and enzymatic or chemical digestions to release unextracted residues. In the first set of experiments, samples of PRE-T or POE-T soybean seed were extracted with hexane, acetonitrile and 40:60 acetonitrile:water as described in Section 2.9.3.1. The extraction pellets were then subjected to sequential extraction with dilute acid (0.1 N HCl) and base (0.1 N NaOH). The results of the dilute acid and base extractions are presented in Table 18. In the second set of experiments, the extraction pellets from freshly extracted PRE-T or POE-T soybean seed were subjected to a series of enzymatic and chemical digestions to sequentially release various classes of biomolecules into the soluble phase. Initially, the enzymatic/chemical digestions were conducted on seed samples that had been extracted only with acetonitrile:water and included a lignin extraction step. The experiment was repeated with samples that had been extracted with hexane, acetonitrile and acetonitrile:water (as described in Section 2.9.3.1). The results of the enzymatic and chemical digestion experiments are reported in Table 19. Typical procedures for the dilute acid and base extractions, and the enzymatic and chemical digestions are described below. Values (percent extracted or unextracted) presented in the tables are normalized to 100% accountability.

2.9.10.1 Extraction with Dilute Acid and Base

The pellet (PRE-T or POE-T seed) remaining after hexane, acetonitrile and 40:60 acetonitrile:water extractions of a 4-gram sample was extracted in a 50-mL plastic

centrifuge tube with 30 mL of 0.1 N HCl by shaking on a wrist-action shaker for 20 min. The tube was centrifuged for 5 minutes in a bench-top unit, the supernatant was decanted and weighed, and aliquots were counted by LSC after neutralizing. Next, 30 mL of 0.1 N NaOH was added to the pellet. The tube was shaken on a wrist-action shaker for 20 min then centrifuged for 5 min in a bench-top unit. The supernatant was weighed and aliquots were counted by LSC after neutralizing. The pellet was then combusted to determine remaining radioactivity.

2.9.10.2 Enzymatic and Chemical Digestion

For the enzymatic and chemical digestions, PRE-T and POE-T soybean seed samples (approx. 4 g each) were first extracted either with 40:60 (v/v) acetonitrile:water alone (4 x 30-35 mL) or with hexane (3 x 25 mL), acetonitrile (1 x 35 mL) and 40:60 (v/v) acetonitrile:water (4 x 30-35 mL) as described in Section 2.9.3.1. The pellets resulting from these extractions were stored frozen for a short period of time (generally overnight) and then were subjected to the procedures described below. All extraction steps were conducted in a 50-mL plastic centrifuge tube.

Phosphate Rinse

The pellet remaining after multiple extractions with 40:60 (v/v) acetonitrile:water was sonicated for 20 min with 30 mL of 0.05 M phosphate buffer, pH 7.0, then was shaken on a wrist-action shaker for 20 min. The suspension was centrifuged at 9,000 rpm (approx. 8,500 x g) for 20 min and the supernatant was decanted. The supernatant was weighed and weighed aliquots were analyzed by LSC.

Hydrolysis with α -Amylase (Starch Fraction)

The pellet remaining from the phosphate rinse was suspended in 30 mL of 0.05 M phosphate buffer, pH 7.0, and was gently shaken at 30 °C for approx. 17-18 h in the presence of α -amylase (Sigma type 1-A diisopropylfluorophosphate (DFP) treated saline solution from porcine pancreas, 200 μ L, 1325 units/mg). The mixture was hand-shaken approx. every hour during the first 5-6 hours of incubation to disburse the pellet. The suspension was centrifuged at 9,000 rpm (approx. 8,500 x g) for 20 min and the supernatant was decanted. The extract was weighed and weighed aliquots were analyzed by LSC. The pellet was frozen until the next step.

The enzymatic activity of the α -amylase was validated by subjecting a negative control (approx 1 g of starch and 30 mL of 0.05 M phosphate buffer, pH 7.0) and a positive control (1 g of starch, 30 mL of 0.05 M phosphate buffer pH 7.0 and 50 μ L α -amylase solution) to the same digestion procedure as described above. The two controls were developed on TLC plates along with a maltose standard. The spots were visualized by

heating the TLC plate on a hot plate after spraying with sulfuric acid. Maltose was present in the positive control containing the enzyme and not in the negative control, thereby validating the enzyme activity.

Hydrolysis with Protease (Protein Fraction)

The pellet following the amylase digestion was suspended in 30 mL of 0.05 M tris[hydroxymethyl]aminomethane (Tris) buffer (Sigma), pH 7.0, and was gently shaken at 37 °C for approx. 24 h (hand-shaken every hour during the first 5-6 hours to disburse the pellet) in the presence of protease (Sigma type 1 from bovine pancreas, 300 mg, 6.9 units/mg). The suspension was centrifuged at 9,000 rpm (approx. 8,500 x g) for 20 min and the supernatant was decanted. The liquid was weighed and weighed aliquots were analyzed by LSC.

The enzymatic activity of protease was validated by subjecting a negative control (approx. 0.01 g of casein and 35 mL of 0.05 M Tris buffer, pH 7.0) and a positive control (0.01 g of casein, 35 mL of 0.05 M Tris buffer, pH 7.0, and 300 mg protease) to the same digestion procedure as described above. Aliquots (2 mL) of the digested controls were combined with 1 mL of Sigma 2% ninhydrin reagent and boiled in a water bath for 10 min. After cooling, 5 mL of ethanol was added. This ninhydrin procedure indicates the presence of amino acids by the color purple. Amino acids were present in the positive control containing the enzyme (purple) and not in the negative control (amber), thereby validating the enzyme activity.

EDTA Extraction (Pectin Fraction)

The pellet following the protease digestion was suspended in 30 mL of 1:1 0.05 M EDTA:0.05 M sodium acetate buffer, pH 4.9, and was gently shaken at 80 °C for approx. 5 h (the mixture was mixed by hand approx. every hour to disburse the pellet), then allowed to stand at RT overnight. The suspension was centrifuged at 9,000 rpm (approx. 8,500 x g) for 20 min and the supernatant was decanted. The liquid was weighed and weighed aliquots were analyzed by LSC. The pellet was stored in the freezer until the next step.

Oxidation with Chlorite (Lignin Fraction)

This step was conducted only for the PRE-T and POE-T seed samples that had not been pre-extracted with hexane. The pellet remaining after the EDTA extraction was suspended in 28 mL of water. Glacial acetic acid (100 µL) and sodium chlorite (0.3 g) were added and the sample was gently shaken at 70 °C for 1 h. The suspension was centrifuged for 10 min in a Dynac bench-top unit and the supernatant was decanted. This digestion was repeated three more times by adding water, acetic acid and sodium chlorite

to the pellet. After the last digestion, the pellet was mixed with 25 mL of water, centrifuged and decanted as before. The liquid from the 5 extracts was weighed and weighed aliquots were analyzed by LSC. The addition of 10% aqueous sodium thiosulfate to the aliquots was necessary to reduce the remaining chlorite for counting by LSC. The pellet was stored in the freezer until the next step.

Hydrolysis with Cellulase (Cellulose Fraction)

The pellet following the pectin/EDTA extraction (or chlorite oxidation) was suspended in 30 mL of 0.05 M sodium acetate buffer, pH 5.0, and was gently shaken at 37 °C overnight (shaken by hand every hour for the first 5 hours) in the presence of cellulase (Sigma, from *Trichoderma viride*, 400 mg, 7.5 units/mg). The suspension was centrifuged at 9,000 rpm (approx. 8,500 x g) for 20 min, the liquid was decanted, weighed, and weighed aliquots were analyzed by LSC. The pellet was stored in the freezer until the next step.

The enzymatic activity of cellulase was validated by subjecting a negative control (approx. 1 g of cellulose powder and 35 mL of 0.05 M sodium acetate buffer, pH 5.0) and a positive control (1 g of cellulose, 35 mL of 0.05 M sodium acetate buffer, pH 5.0, and 400 mg of cellulase) to the same digestion procedure as described above. The two controls were developed on TLC plates along with a glucose standard. The spots were visualized by heating the TLC plate on a hot plate after spraying with sulfuric acid. Glucose was present in the positive control containing the enzyme and not in the negative control, thereby validating the enzyme activity.

Hydrolysis with Strong Base (Hemicellulose Fraction)

The pellet following the cellulose digestion was suspended in 20 mL of 24% aqueous KOH and was stirred at RT overnight. The suspension was centrifuged at 9,000 rpm (approx. 8,500 x g) for 20 min (did not clarify), then filtered, and the filtrate was centrifuged as above for 5 min. The liquid filtrate was weighed, and weighed aliquots were analyzed by LSC after neutralizing the aliquots with concentrated HCl and diluting with water.

Combustion of Final Pellet

The air-dried filter paper and pellet remaining after hemicellulose digestion was divided into small pieces and weighed. Weighed aliquots were analyzed by combustion/LSC.

Organic Solvent Partitioning of Unextracted Residue Digests

The aqueous extract solutions resulting from digestion of the PRE-T and POE-T seed extraction pellets (following hexane/ACN/ACN:water extraction) with protease (protein fraction), 0.05 M EDTA/sodium acetate (pectin fraction), cellulase (cellulose fraction) and 24% KOH (hemicellulose fraction) were each acidified to approx. pH 1 by addition of conc. HCl. Each solution was then partitioned with two 25-mL portions of ethyl acetate (samples were centrifuged to separate the layers). For each digest, the combined ethyl acetate extracts and the remaining aqueous layer were aliquoted for LSC analysis. The results of the partitioning experiments are presented in Table 20. The hemicellulose fractions of PRE-T and POE-T seed extract pellets (without hexane extraction) were diluted with water and acidified with conc. HCl to pH 2 (cloudy suspensions developed on acidification). The mixtures were each partitioned with two 20-mL portions of diethyl ether. The ether was evaporated to dryness (solids formed). To the solids was added 3 mL of 10:90 (v/v) acetonitrile:0.5% aq. formic acid (Method B starting mobile phase), then the mixtures were centrifuged and the supernatants were counted by LSC. The results of the partitioning experiments are shown in Table 21.

Organic Solvent Partitioning and HPLC Analysis of the Hemicellulose Fraction of PRE-T Seed Unextracted Residues

The hemicellulose digest of the PRE-T seed extraction pellet (without hexane extraction) was further treated with 1 N KOH at 60 °C for 1 h to hydrolyze potential glucoside conjugates that were not hydrolyzed in the hemicellulose (24% KOH) digestion. The resulting solution was neutralized to pH 2 with conc. HCl and partitioned twice with 40 mL of ethyl acetate. The combined organic phases and the aqueous phase were counted by LSC. The combined organic phases were evaporated, HPLC mobile phase (1 mL of 10:90 ACN:0.5% aq. formic acid) was added (solids formed), the liquid was centrifuged, counted by LSC and analyzed by HPLC/LSC using Method B (see Table 21 and Figure 24).

HPLC Analysis of the Hemicellulose Fraction of POE-T Seed Unextracted Residues

The hemicellulose digest of the POE-T seed unextracted residue (following hexane/ACN/ACN:water extraction) was acidified with conc. HCl to about pH 1 and was rotary evaporated to a small volume (a large amount of white solid formed in the acidification and evaporation). The liquid was removed and centrifuged. The supernatant was analyzed by HPLC/LSC using Method B (Figure 25).

HPLC Analysis of the Protease Digest of PRE-T Seed Unextracted Residues

The protease digest of the PRE-T seed unextracted residue (following hexane/ACN/ACN: water extraction) was evaporated to a small volume (<2 mL) and

acetonitrile (10% by weight) was added. The sample was centrifuged and analyzed by HPLC/LSC using Method B (Figure 26).

2.9.11 Storage Stability Analyses

Shortly after collection and processing of the harvested soybean samples, combustions were conducted at PTRL West, Inc. to establish a baseline for stability determinations. Extractions and HPLC analyses of the extracts were conducted shortly after processing (grinding) either by PTRL West, Inc. (forage and seed) or by Monsanto (pre-forage and hay). Analyses of selected matrices were conducted by Monsanto Company at the completion of the analysis phase of the study to provide stability data. Stability of [¹⁴C]dicamba residues in soybean forage, hay and seed in frozen storage during the approximately 2-year interval between the initial and final analyses may be assessed by comparing the respective combustion and extraction data (Table 14), and the HPLC profiles (Figure 108 through Figure 111) obtained initially and after approx. 2 years of storage. Additional quantitative storage stability analyses of selected soybean matrices, including an extended analysis (approx. 3 years of storage) are included in Appendix C.

2.10 Calculations

$$\text{PRE-T Test Substance Specific Activity (dpm/}\mu\text{g)} = 5.39 \text{ mCi/mmol} / 221.0 \text{ mg/mmol} * 2.22 \times 10^9 \text{ dpm/mCi} * 0.001 \text{ mg/}\mu\text{g)} = 54,190 \text{ dpm/}\mu\text{g}$$

$$\text{POE-T Test Substance Specific Activity (dpm/}\mu\text{g)} = 5.43 \text{ mCi/mmol} / 221.0 \text{ mg/mmol} * 2.22 \times 10^9 \text{ dpm/mCi} * 0.001 \text{ mg/}\mu\text{g)} = 54,560 \text{ dpm/}\mu\text{g}$$

For soybean matrices:

Matrix dpm/g is determined by combustion analysis.

Matrix TRR (mg/kg, dicamba equivalents) = (Matrix dpm/g) / (Test Substance Specific Activity)

Extracted dpm = sum of dpm in extracts

Sample dpm = (weight of sample extracted) * (Matrix dpm/g)

Percent Extracted = (Extracted dpm) / (Sample dpm) * 100

Pellet dpm = (weight of extraction pellet) * (pellet dpm/g determined by combustion)

Percent Unextracted = (Pellet dpm) / (Sample dpm) * 100

$$\text{Accountability} = (\text{Percent Extracted}) + (\text{Percent Unextracted})$$

$$\text{Normalized Percent Extracted (Extractability)} = (\text{Percent Extracted}) / (\text{Accountability}) * 100$$

$$\text{Normalized Percent Unextracted} = (\text{Percent Unextracted}) / (\text{Accountability}) * 100$$

$$\text{Extracted mg/kg (ppm)} = (\text{Normalized Percent Extracted}) * (\text{Matrix TRR}) / 100$$

$$\text{Unextracted mg/kg (ppm)} = (\text{Normalized Percent Unextracted}) * (\text{Matrix TRR}) / 100$$

For metabolite peaks:

Pre-forage, forage and hay:

$$(\text{Peak}) \text{ Percent of Matrix TRR} = (\text{Percent of Chromatogram}) * (\text{Extractability})$$

Seed:

$$(\text{Peak}) \text{ Percent of Matrix TRR} = (\text{Percent of Chromatogram}) * (\text{Extractability}) * (\text{Evaporation and Centrifugation Recovery}) * (\text{HPLC Column Recovery})$$

$$\text{Peak mg/kg (ppm)} = (\text{Matrix TRR}) * ((\text{Peak}) \text{ Percent of Matrix TRR})$$

Triglyceride (Triacylglycerol) Calculations:

Percent in Partition is determined by LSC of hexane and methanol phases from the hexane partitioning of the methanolysis solution of the POE-T seed oil fraction.

For an individual partition (i.e., hexane or acidic methanol):

$$\text{Normalized Percent in Partition} = (\text{Percent in Partition}) / [(\text{Percent in Hexane Phase}) + (\text{Percent in Acidic Methanol Phase})]$$

$$\text{Fatty Acids in Oil (Percent)} = \text{Normalized Percent in Partition (hexane)} = 89.64\%$$

Glycerol in Oil Calculation:

$$\text{Carbon Contribution (of an individual fatty acid in soybean oil)} = (\text{Number of Carbons}) * (\text{Oil Percent}) / (\text{Total Oil Percent of all Fatty Acids})$$

e.g., for palmitic acid (C16, 10.70% of soybean oil, 95.70% total fatty acids in soybean oil): Carbon Contribution = $(16 * 10.70 / 95.70) = 1.79$

[soybean oil fatty acid composition – Reference 1:

lauric (C12:0): 0.10%; myristic (C14:0): 0.16%; palmitic (C16:0): 10.70%; palmitoleic (C16:1): 0.29%; stearic (C18:0): 3.87%; oleic (C18:1): 22.80%; linoleic (C18:2): 50.80%; linolenic (C18:3): 6.76%; arachidic (C20:0): 0.22%]

Average Number of Carbons in Soybean Oil Fatty Acids = Sum of Carbon Contribution of all individual fatty acids = 17.76

Average Number of Carbons in Soybean Oil Triglycerides (3 fatty acids per triglyceride) = $3 * 17.76 = 53.29$

Number of Glycerol Carbons in Soybean Oil Triglycerides = 3

Glycerol Carbons as Percent of Fatty Acid Carbons in Soybean Oil Triglycerides = (Number of Glycerol Carbons in Soybean Oil Triglycerides) / (Average Number of Carbons in Soybean Oil Triglycerides) * 100% = $3 / 53.29 * 100\% = 5.63\%$

Glycerol in Oil (Percent) = [Normalized Percent in Partition (acidic methanol)] * (Glycerol Carbons as Percent of Fatty Acid Carbons in Soybean Oil Triglycerides / 100 = $10.36\% * 5.63\% / 100 = 5.05\%$

Total Triglycerides in Oil (Percent) = [Fatty Acids in Oil (Percent)] + [Glycerol in Oil (Percent)] = $89.64\% + 5.05\% = 94.68\%$

Total Unidentified in Oil (Percent) = $100\% - \text{Total Triglycerides in Oil (Percent)} = 100\% - 94.68\% = 5.32\%$

For a matrix (PRE-T, POE-T, PRE-C or POE-C Seed):

Triglyceride Percent of TRR = (Normalized Percent of TRR in Hexane Extract of Seed) * (Percent of Oil as Triglycerides) / 100

e.g., for PRE-T seed: Triglyceride Percent of TRR = $14.65\% * 94.68\% / 100 = 13.87\%$

Oil Unidentified Percent of TRR = [Total Unidentified in Oil (Percent)] * (Normalized Percent of TRR in Hexane Extract of Seed) / 100

e.g., for PRE-T seed: Oil Unidentified Percent of TRR = $5.32\% * 14.65\% / 100 = 0.78\%$

Triglyceride mg/kg (ppm) = (Triglyceride Percent of TRR) * (Matrix TRR) / 100

Oil Unidentified mg/kg (ppm) = (Oil Unidentified Percent of TRR) * (Matrix TRR) / 100

Calculations were performed using Microsoft® Excel software. Numbers in the tables (Section 5) of this report are rounded values. Use of the rounded values in manual calculations may give results differing slightly from those calculated using Excel since the Excel calculations were performed using non-rounded numbers.

2.11 Statistical Methods

No statistical analyses were performed for the metabolite analysis phase of the study except for calculation of averages and standard deviations.

2.12 Archival of Raw Data, Specimens and Test Substances

The raw data for the portions of the study conducted at Monsanto Company, including research notebooks, hard copies of chromatography, LSC, NMR and mass spectrometry data, and the final report, were archived in the Monsanto Regulatory Archives. The raw data, or certified exact copies, for the portions of the study conducted at PTRL West, Inc., Research for Hire and AGVISE Laboratories, were transferred to Monsanto Company and were archived in the Monsanto Regulatory Archives. All study specimens will be retained until after Quality Assurance verification, in accordance with 40 CFR § 160.190 (a). Reserve samples of test and reference substances will be retained as long as they are suitable for evaluation.

3 Results and Discussion

3.1 Test Strategies

Soybean planting, test substance application, sample collection and processing, as well as initial combustion, extraction and HPLC analyses of selected matrices for stability purposes, were conducted by or under the supervision of PTRL West, Inc. The portions of the study conducted by PTRL West, Inc. are referred to as the “in-life” phase. Definitive determinations of soybean radioactivity levels, extractions, metabolite quantitation and metabolite characterization and identification were conducted in the Environmental Sciences Technology Center of Monsanto Company.

3.2 Protocol, Amendments and Deviations

The original protocol and two amendments are included in Appendix A of this report. Amendment #1 to the protocol was written to correct minor errors, to incorporate a change in principal field investigator at the Research For Hire facility, and to accommodate an additional sampling (immature seed) from extra POE-T plants that had been treated to utilize excess test material. The immature seed was analyzed for method development and business purposes. Data from the analyses of the immature seed samples are included in the study file, but are not included in this report. Amendment #2 was written to clarify the matrices to be used for storage stability assessment and to add additional storage stability analyses, including quantitation of the stability profiles and addition of a later stability time point to support the dicamba residue study in dicamba-tolerant soybean.

Several minor protocol deviations occurred during the course of the in-life portion of the study. These are described in the in-life and field reports (Appendix B). None of the protocol deviations had a negative impact on the study results.

3.3 Rejected and Unused Data

Rejected or unused data generated during this study were retained in the study file. Much of the rejected data resulted from unacceptable instrument performance, equipment malfunction or operator error. There are also data that are considered method development in which experiments were attempted but did not give a positive or interpretable result, data related to optimizations of techniques or reaction conditions, or data for experiments that did not give a quality result and were repeated.

3.4 Dicamba Residue Levels in Dicamba-Tolerant Soybean

Radioactive residues in soybean matrices resulting from treatment of dicamba-tolerant soybean with [^{14}C]dicamba are summarized in Table 4. Two sets of TRR values are reported in Table 4 – those determined initially by PTRL West, Inc. as reported in the in-life report (Appendix B) and those determined at Monsanto Company at the time that the definitive extractions and metabolite quantitation profiles were conducted. The latter are included for comparison purposes. The TRR values used for all mg/kg (ppm) calculations in this report are those determined by PTRL West, Inc. (except the quantitative stability analyses summarized in Appendix C). The differences between the TRR values determined at Monsanto during the analytical phase and the initial TRR values determined by PTRL were all less than 10% except for two values (PRE-T and PRE-C forage) for which the differences were less than 15%.

Levels of radioactive residues (expressed as parent dicamba equivalents) in soybean foliage for the preemergence (at planting) treatment were relatively low and declined with time. Residue levels were 3.248 mg/kg in PRE-T pre-forage (immature foliage) harvested at 14 DAT, 1.433 mg/kg in PRE-T forage (36 DAT) and 1.056 mg/kg in PRE-T hay (56 DAT). Levels of radioactive residues in soybean foliage for the postemergence treatment (29 days after planting) were relatively high decreasing from 134.147 mg/kg in POE-T forage (7 DAT) to 39.149 mg/kg in POE-T hay (27 DAT). A considerable amount (approx. 33%) of the residues in the POE-T forage was due to surface residues comprised of non-metabolized parent dicamba as determined by analysis of water washes of two selected POE-T forage plants. It is likely that the non-metabolized dicamba in the POE-T hay also was largely due to dicamba surface residues. Residues in the seed from both the pre- and postemergence treatments were low indicating limited translocation of dicamba foliage residues to the seed. Residue levels in PRE-T seed, collected at 112 DAT, were 0.291 mg/kg compared with residues of 0.389 mg/kg in POE-T seed collected at 83 DAT.

Radioactive residue levels in untreated controls were low, but significant, in the forage and seed harvested from PRE-C and POE-C untreated plants that were interspersed amongst the PRE-T and POE-T plants, respectively. This is likely due both to uptake of dicamba evaporating from the soil and/or plant surfaces and to uptake of $^{14}\text{CO}_2$ produced by metabolism (mineralization) of dicamba in the soil, and possibly the plants, of the treated pots. A very small amount of radioactivity was detected in the untreated (UNT-C) hay and seed (0.001 and 0.013 mg/kg, respectively). Although the untreated control plants were grown in a separate greenhouse from that housing the treated plants, the greenhouses were in very close proximity (adjoining). It is conceivable that a very small amount of $^{14}\text{CO}_2$ released by mineralization of dicamba in the treated pots and/or

[¹⁴C]dicamba due to volatility was drawn into the greenhouse containing the untreated plants.

3.5 Extraction of Dicamba Residues from Soybean Matrices

3.5.1 Optimization of Extraction Conditions

Solvent extraction conditions for extraction of dicamba-derived residues from soybean tissues were optimized by conducting extractions of immature foliage (PRE-T pre-forage) with mixtures of acetonitrile and water of varying composition. Extraction with 25:75, 40:60, 60:40, or 75:25 (v/v) acetonitrile:water extracted 79.8, 92.8, 90.5 and 88.1 percent of the radioactive residues from the PRE-T pre-forage. As 40:60 (v/v) acetonitrile:water gave the highest extractability, this solvent mixture was selected for extraction of all soybean matrices. For each matrix (immature foliage, forage, hay and seed), multiple (typically four) extractions with this solvent mixture were conducted. Initially, soybean seed also was extracted with this solvent mixture. However, significant difficulties were experienced in the preparation (evaporation/centrifugation) of the seed extract samples for HPLC analysis, as well as in the actual chromatography, due to the presence of lipids in the extract concentrates. For this reason, the seed samples for metabolite quantitation were first extracted three times with hexane to extract oils followed by an intermediate acetonitrile extraction to remove the excess hexane and then four 40:60 acetonitrile:water extractions.

3.5.2 Extraction of Soybean Samples

A summary of the extraction results is presented in Table 5 (percent) and Table 6 (mg/kg, ppm). Normalized extraction efficiencies (extractabilities) for foliage (pre-forage, forage and hay) were all above 90% except for the PRE-C forage which likely contained significant residues from uptake and incorporation of liberated ¹⁴CO₂ into natural products. Extractabilities for the POE-T forage and hay were somewhat higher (93.79% and 95.30%, respectively) than for the PRE-T forage and hay (91.21% and 90.88%, respectively), likely a reflection of additional easily-extracted surface residues in the POE-T forage and hay. Extractabilities (including hexane extraction) were approximately 60% for seed from the treated pots (59.35% PRE-T, 63.67% POE-T) and approximately 44% for seed from the untreated pots (43.89% PRE-C, 44.16% POE-C). Of the extracted seed radioactivity, the amount in the hexane extracts (oil fraction) ranged from 8.41% of TRR in POE-T seed to 17.84% of TRR in PRE-C seed. The larger proportion of residues in the oil fraction of the PRE-C seed relative to the PRE-T, POE-T and POE-C seed likely reflects increased uptake of ¹⁴CO₂ by the PRE-C plants due to mineralization of dicamba in the treated soil of the PRE-T pots in close proximity to the

PRE-C pots. Extractions of UNT-C hay and seed, which contained very low residues (0.001 and 0.013 mg/kg, respectively), were not conducted.

3.5.3 Extraction of Unextracted Residues

Extraction of additional (unextracted) residues from the seed extraction pellets (post-extraction solids) was investigated for the PRE-T and POE-T seed. Unextracted residues, following extraction with hexane, acetonitrile and acetonitrile:water comprised 40.65% (0.118 mg/kg) and 36.33% (0.141 mg/kg) of the TRR in the PRE-T and POE-T seed, respectively. Extraction of unextracted residues of the PRE-C and POE-C seed was not investigated since the PRE-C and POE-C control groups were included in the study only to provide information on the origin (i.e., uptake from the soil, plant surface or air) of dicamba residues in the treated plants.

Two methods were investigated for release of additional residues: 1) extraction with dilute acid and base, and 2) chemical or enzyme digestions designed to release various classes of biomolecules (e.g., starch, protein, cellulose) into the soluble phase. The results of the acid and base extractions are shown in Table 18; the results of the chemical and enzymatic digestions are shown in Table 19. Note that for each set of extractions (acid/base or chemical/enzymatic) separate fresh seed samples were extracted with hexane, acetonitrile and acetonitrile:water before initiating the unextracted residue extractions. In other words, the extraction pellets containing the unextracted residues were not the exact pellets derived from the extractions conducted for quantitative metabolite profiling. Thus, small differences may exist in the normalized extractabilities reported in Table 5, Table 18 and Table 19.

3.5.3.1 Acid and Base Extraction of Unextracted Residues

After frozen storage, the extraction pellets remaining from the acetonitrile:water extractions of PRE-T and POE-T seed were each extracted with 0.1 N HCl followed by 0.1 N NaOH. For the PRE-T seed, the acid and base extractions released an additional 1.55% (0.005 mg/kg) and 1.30% (0.004 mg/kg) of the TRR, respectively (35.04% of TRR, 0.102 mg/kg, remained unextracted). For the POE-T seed, the acid and base extractions released an additional 1.39% (0.005 mg/kg) and 1.42% (0.006 mg/kg) of the TRR, respectively (31.58% of TRR, 0.123 mg/kg, remained unextracted). Thus, dilute acid or base extractions did not release a significant amount of the unextracted seed residues.

3.5.3.2 Chemical and Enzymatic Digestion of Unextracted Residues

The chemical and enzymatic digestion procedure for extraction of unextracted residues consisted of an initial phosphate rinse (to remove acetonitrile), amylase and protease

digestions to release starch and protein, respectively, an EDTA extraction to release pectin, a cellulase digestion to release cellulose, and finally a 24% KOH extraction to release hemicellulose. Initially, the digestion procedure was conducted on seed samples that had been extracted with acetonitrile:water without hexane pre-extraction and included a chlorite lignin extraction step after the pectin extraction step. The results for this procedure are included in Table 19 for completeness. However, the discussion below pertains primarily to the digestion procedure conducted on extraction pellets derived from seed samples extracted first with hexane and acetonitrile then acetonitrile:water.

Chemical and enzymatic digestion of PRE-T and POE-T seed extraction pellets resulted in release of small amounts of residues (0.25-3.75% of TRR) in each of the phosphate rinse, starch, pectin and cellulose fractions. Relatively larger amounts of residues (10.06% and 8.18% of TRR for PRE-T and POE-T seed, respectively) were released in the protein fraction. The largest portion of unextracted residues was released in the hemicellulose fraction (13.92% and 12.90% of TRR for the PRE-T and POE-T seed, respectively). In all, the series of chemical and enzymatic digestions released an additional approx. 30% of TRR from the seed extraction pellets resulting in total extractabilities of 92.91% and 92.40% for the PRE-T and POE-T seed, respectively. For the PRE-T and POE-T seed, respectively, 7.09% of TRR (0.021 mg/kg) and 7.60% of TRR (0.030 mg/kg) remained unextracted.

Chemical and enzymatic digestion of extraction pellets from seed that had not been pre-extracted with hexane gave similar results to those described above except that larger amounts (19.11-20.53% of TRR) were released in the hemicellulose fraction. This difference presumably represented lipids that remained with the extraction pellet because hexane extractions were not conducted and were extracted/hydrolyzed with the strong base of the hemicellulose digestion. The extra lignin extraction step conducted on the extraction pellets from non-hexane extracted seed released only small additional amounts of residues (4.39-4.81% of TRR, 0.014-0.017 mg/kg).

3.5.3.3 Partitioning of Released Unextracted Residues

Fractions (protein, pectin, cellulose and hemicellulose) from the chemical and enzymatic digestion of seed unextracted residues that contained released residues at levels greater than 0.01 mg/kg were acidified and partitioned with ethyl acetate to characterize the nature of the released residues. The results of these partitioning experiments are shown in Table 20. Virtually none of the residues from the protein, pectin and cellulose fractions partitioned into the organic phase demonstrating that the residues were highly water-soluble, were unlikely to be dicamba-related, and were likely due to extensive metabolism of dicamba and incorporation of resulting small molecules (e.g., $^{14}\text{CO}_2$) into

plant constituents. A significant portion (12.77-18.96%) of the released residues in the hemicellulose fractions from PRE-T and POE-T seed partitioned into the respective organic phases. However, this represented a residue level of less than 0.01 mg/kg in both cases.

Organic solvent partitioning of the hemicellulose fractions from seed that had not been pre-extracted with hexane was also investigated as summarized in Table 21. Partitioning of the acidified fractions and partitioning with Et₂O resulted in 32.64-37.45% of the radioactivity in the organic phases. Significant amounts of solids also formed containing 11.58-14.60% of the residues in the hemicellulose fractions. Concentration of the organic phases and redissolution of the residues in starting HPLC mobile phase (90:10 0.5% aq. HCOOH:ACN) resulted in redissolution of only approx. 7% (7.00-7.58%) of the radioactivity in the organic phases demonstrating that the organic-extractable materials were primarily not dicamba-related.

Further treatment of the hemicellulose digest of PRE-T seed with 1 N KOH at 60 °C (to attempt to hydrolyze any esterified or glycosylated dicamba residues) and partitioning with EtOAc resulted in transfer of 44.85% of the hemicellulose residues into the organic phase. Concentration and attempted redissolution in HPLC mobile phase resulted in dissolution of only 6.84% of the radioactivity originally in the organic phase. Thus, the majority of the radioactivity in the organic phase of the EtOAc partition of the PRE-T seed hemicellulose fraction does not correspond to dicamba metabolites and is likely due to incorporation of small molecules into natural plant constituents.

3.6 Metabolism of Dicamba in Soybean

3.6.1 Identification and Characterization of Radioactive Components of Soybean Forage, Hay and Seed

Metabolites for identification or characterization were isolated and purified as described in Section 2.9.7. All metabolites constituting greater than 1.0% of the TRR in any matrix that were also present at a level exceeding 0.05 mg/kg in that matrix were isolated, purified and characterized, and they were identified when possible. Metabolites were isolated primarily from POE-T forage since it contained the highest residues and also contained most of the metabolites of interest. Methods utilized for the characterization and identification of metabolites are described in Section 2.9.8. The specific experiments conducted for the characterization and identification of the isolated metabolites, and the results of these analyses, are described in detail below.

Peak 1 – Sugars

Peak 1 eluted as a broad band of polar radioactivity near the column void volume (4-6 min) using HPLC Method B. This peak only constituted a significant percentage of the TRR in seed where it represented 8.42% of TRR (0.025 mg/kg) in the PRE-T seed and 9.15% of TRR (0.036 mg/kg) in the POE-T seed. Peak 1 remained in the aqueous phase when the POE-T seed extract concentrate was acidified and partitioned with ethyl acetate (Figure 19) indicating a polar, water-soluble material. The Peak 1 isolate following C18 SPE cleanup was subjected separately to acid hydrolysis (2 N HCl, 100 °C, 2 h) and base hydrolysis (1 N NaOH, 60 °C, 1 h). Analysis of the hydrolysates by HPLC/RAD (Method B) showed that no significant change in Peak 1 had occurred (Figure 27). No recognizable dicamba-related metabolites (e.g., DCSA or DCGA) were released by acid or base hydrolysis.

Because it was surmised that Peak 1 was a mixture of soluble carbohydrates (sugars), the Peak 1 isolate was analyzed by HPLC/LSC using a carbohydrate analysis method (Method H) to give a mixture of peaks eluting from approx. 16 to 32 min with significant peaks at approx. 23, 26 and 29 min (Figure 28, top). A radioactive glucose reference standard (**VIII**) eluted at 19 min under these conditions (Figure 28, bottom). The Peak 1 isolate was hydrolyzed with acid (1 N HCl, 60 °C, 20 min) using a published method³ designed to hydrolyze sugars (e.g., sucrose) to glucose without degrading glucose. The hydrolysate was analyzed by HPLC/LSC (Method H). The HPLC profile showed one major peak with minor trailing peaks (Figure 29, top). The retention time of the major peak was consistent with that of glucose as evidenced in the profile of the mixture spiked with radioactive glucose standard (**VIII**) (Figure 29, bottom). This provided evidence that components of Peak 1 were hydrolyzed by mild acid conditions to glucose (or other similar monosaccharides). The hydrolysate was analyzed by negative ion loop/ESI/MS (50:50 0.5% aq. formic acid:acetonitrile, 0.2 mL/min). The mass spectrum exhibited two chloride adduct doublet ions (approx. 3:1 ratio) at m/z 215/217 and 539/541 $[M+Cl]^-$ and possibly a third weak doublet at m/z 377/379 (Figure 30). These doublet ions may correspond to the chloride adducts of glucose (MW 180, formed by hydrolysis), and unhydrolyzed raffinose (MW 504) and sucrose (MW 342), sugars that are known to be present in soybean seed.⁴

A definitive identification of Peak 1 was not achieved. However, the chromatographic, partitioning and hydrolysis behavior of Peak 1, and the chromatographic and mass spectral analysis of its acid hydrolysate, indicate that Peak 1 is a mixture of very polar materials, likely sugars. These sugars are likely produced from extensive metabolism of dicamba to small molecules which are incorporated into natural plant constituents (such as carbohydrate, protein and oil) through normal plant metabolic processes. The sugars representing Peak 1 may be a mixture of the di-, tri- and tetrasaccharides sucrose,

raffinose and stachyose as these are known to be the predominant soluble carbohydrates present in soybean seed.⁴

Peak 3 – DCGA Glucoside

Peak 3 was observed in all treated matrices up to a maximum of 4.32% of TRR (1.690 mg/kg) in POE-T hay. It is a rather polar metabolite eluting at a retention time of approx. 6.5-7.0 minutes on HPLC Method B. The metabolite remained primarily in the aqueous phase on ethyl acetate partitioning of the POE-T hay or seed acidified extract concentrates. The purified Peak 3 isolate from POE-T forage was analyzed by negative ion LC/ESI/MS (LC Method F). The mass spectrum (Figure 31) exhibited a parent ion two-chlorine doublet at m/z 383/385 $[M-H]^-$ and a very small triplet gas-phase dimer ion at m/z 767/769/771 (not shown) corresponding to a nominal molecular mass of 384. Hydrolysis was utilized to determine if the metabolite was a hydrolysable conjugate and to determine the nature of the exocon. Acid hydrolysis (2 N HCl, 100°C, 2 h) of the metabolite produced a hydrolysate that was less polar than the metabolite and whose retention time (HPLC Method B) was consistent with that of the co-injected DCGA reference standard **IV** (Figure 32). The acid hydrolysate was analyzed by negative ion LC/ESI/MS (HPLC Method G). The spectrum exhibited a parent ion two-chlorine doublet at m/z 221/223 $[M-H]^-$ (Figure 33) and was consistent with the negative ion LC/ESI/MS spectrum (Figure 34) of the DCGA reference standard (**IV**) whose mass spectrum showed a parent ion two-chlorine doublet at m/z 221/223 $[M-H]^-$, fragment ion at m/z 177/179 $[M-H-CO_2]^-$ and a sodium adduct of the gas-phase dimer at m/z 465/467/469 $[2M-2H+Na]^-$ (not shown). The nominal mass (222) for the hydrolysate indicates loss of 162 mass units upon hydrolysis which is indicative of loss of a hexose moiety (likely glucose). Thus, mass spectral and hydrolysis data indicated that Peak 3 was likely a glucose conjugate of DCGA.

Further verification of the DCGA moiety in Peak 3 was achieved through acid hydrolysis followed by methylation (of the carboxylate and both hydroxyl groups) and comparison of the product to the trimethylated DCGA reference standard. The purified metabolite was hydrolyzed with acid (HCl) as above and the resulting hydrolysate was methylated with TMSdiazomethane. The methylated product was analyzed by HPLC/RAD using Method B giving a retention time of 37.50 min (RAD) for the major peak (Figure 35, top) which is consistent with the retention time (37.27 min, UV) of synthetic Trimethyl DCGA reference standard (**VII**, Figure 35, bottom). A positive ion GC/EI mass spectrum (GC method B) was obtained for the methylated product giving a parent ion two-chlorine doublet at m/z 264/266 $[M]^+$ and fragment ion doublets at m/z 249/251 $[M-CH_3]^+$, 233/235 $[M-OCH_3]^+$, 203/207 $[M-OCH_3-CH_2O]^+$ and 175/177 $[M-OCH_3-CH_2O-CO]^+$ (Figure 36). The spectrum matched that of the Trimethyl DCGA reference standard (**VII**, Figure 37) and gave a good match (94.8%) to the published library spectrum for

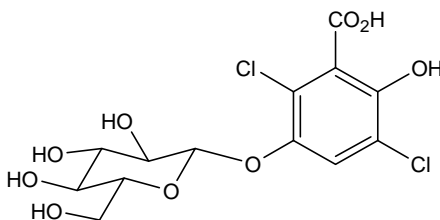
methyl 2,5-dichloro-3,6-dimethoxybenzoate (Trimethyl DCGA). The hydrolysis and methylation of Peak 3 confirmed DCGA as the exocon in this metabolite.

The glucose moiety in Peak 3 could potentially be attached to the DCGA moiety at the carboxyl group or either of the two hydroxyl groups. To determine the position of attachment of the glucose moiety the following experiments were conducted. The isolated Peak 3 was analyzed both by HPLC Method C, which utilizes 0.5% formic acid as the aqueous component of the mobile phase, and by the same method using water as the aqueous mobile phase instead of 0.5% formic acid. The retention time in the acidic mobile phase was 15.3 min while the retention time using water rather than formic acid was approx. 6.5 min earlier at 8.8 min indicating the presence of a free acidic group in the molecule. A similar experiment with 5-hydroxydicamba methyl ester (**V**), which contains a free phenolic group showed no change in retention time. These results suggest that the glucose moiety in Peak 3 is attached to one of the hydroxyls rather than the carboxylic acid. Methylation followed by hydrolysis to remove the glucose moiety was then investigated to provide additional evidence for the position of the glucosylation. Peak 3 was methylated with TMSdiazomethane to give a derivative with a retention time (HPLC/RAD, Method B) of 21.56 min and this derivative was subjected to moderate acid hydrolysis (2 N HCl as above, conditions under which methyl ester hydrolysis would not be expected to occur). The HPLC/RAD retention time (31.10 min) of the hydrolysate using Method B matched that (30.75 min, UV, Figure 38) of 5-hydroxydicamba methyl ester reference standard (**V**). This indicated that the 2-hydroxyl (as well as the carboxyl group) of the DCGA moiety of Peak 3 had been methylated (and thus non-conjugated in the metabolite) and that the glucose moiety is attached to the 5-hydroxyl of DCGA (the 3-position when naming DCGA as 2,5-dichloro-3,6-dihydroxybenzoic acid).

To further confirm the position of attachment of the glucose moiety of Peak 3, the methylated hydrolysis product of Peak 3 was further derivatized by acetylation and compared to a standard. The derivative of Peak 3 (5-hydroxydicamba methyl ester) resulting from methylation followed by acid hydrolysis was treated with acetic anhydride and pyridine (1 h at RT). The resulting acetylated product had an HPLC/RAD retention time of 36.91 min using Method B (Figure 39). The acetylated product was analyzed by positive ion GC/EI/MS (GC method A) along with the 5-acetyloxydicamba methyl ester reference standard (**VI**). The GC/MS retention time of the methylated hydrolyzed acetylated derivative of Peak 3 was identical to that of the 5-acetyloxydicamba reference standard (Figure 40). The mass spectrum of the derivative (Figure 41) displayed a weak parent ion two-chlorine doublet at m/z of 292/294 $[M]^+$ with a fragment ion at m/z 261/263 $[M-OCH_3]^+$ and intense fragment ions at m/z 250/252 $[M-CH_2=C=O]^+$ and 219/221 $[M-OCH_3-CH_2=C=O]^+$ and was virtually identical to the mass spectrum (Figure 42) of the synthetic reference standard of 5-acetyloxydicamba methyl ester (**VI**). This

firmly establishes that the glucose moiety of Peak 3 is attached to the 5-hydroxyl of DCGA.

The chromatographic, hydrolysis and derivatization, and mass spectral data are thoroughly consistent with the identity of Peak 3 as a 5-hexose conjugate of DCGA. Although the hexose moiety was not specifically identified, it is most likely glucose as this is most common in plants and by analogy to Peaks 9 and 11 (see below) in which the presence of a β -linked glucose moiety was established. Thus, Peak 3 is identified as the 5-*O*- β -glucoside of 3,6-dichlorogentisic acid (DCGA Glucoside, **3**) as shown below.



DCGA Glucoside, **3**

Peak 8 – DCGA Malonylglucoside

Peak 8 was present in all soybean matrices examined. As a percentage of TRR, the highest levels were observed in the PRE-T pre-forage (5.46% of TRR, 0.177 mg/kg) and PRE-T and POE-T seed (4.73 and 4.64 % of TRR, 0.014 and 0.018 mg/kg, respectively). The metabolite eluted at approx. 14 min using HPLC Method B and partitioned into the organic phase of the ethyl acetate partitioning of POE-T seed acidified extract concentrate. The purified Peak 8 isolate from POE-T forage was analyzed by negative ion LC/ESI/MS (HPLC Method F with 0.5% HOAc instead of HCOOH in mobile phase) giving a parent ion two-chlorine doublet at m/z 469/471 $[M-H]^-$ and gas-phase dimer at m/z 939/941/943 $[2M-H]^-$ corresponding to a nominal molecular mass of 470 (Figure 43). Mild base hydrolysis (1 N NaOH, RT, 1 h) of Peak 8 and HPLC/RAD analysis of the hydrolysate (Method B) gave a single peak at 7.21 min (Figure 44) consistent with the retention time of the DCGA glucoside metabolite (**3**). Similarly, analysis of the hydrolysate using HPLC Method C (Figure 45) gave a single peak at 14.97 min consistent with the retention time of the DCGA glucoside (**3**). Negative ion LC/ESI mass spectral analysis of the hydrolysate (HPLC Method G) gave a doublet ion at m/z 383/385 $[M-H]^-$ with a two-chlorine isotope pattern (Figure 46) and gas-phase dimer at m/z 789/791/793 $[2M-2H+Na]^-$ (not shown in figure). The retention time and mass spectrum of the mild base hydrolysate of Peak 8 are consistent with those for the DCGA glucoside metabolite (Peak 3) and indicate that Peak 8 is likely a derivative of Peak 3 in which Peak 3 has been esterified with a low molecular weight carboxylic acid. The difference in molecular mass (86 mass units) between Peak 8 and the DCGA glucoside suggests that Peak 8 is a malonyl conjugate of the DCGA glucoside.

The following experiments were conducted to confirm the DCGA core of the metabolite. Mild base (NaOH) hydrolysis of Peak 8 followed by acid hydrolysis (HCl) removed the malonic acid moiety and the glucose moiety, respectively, converting the metabolite to a less polar material. The retention time (HPLC Method B) of the hydrolysate was consistent with that of coinjected DCGA reference standard (**IV**) confirming that Peak 8 was a conjugate of DCGA (Figure 47). The acid/base hydrolysate was then methylated with TMSdiazomethane as described for Peak 3 above. The product was analyzed by HPLC/RAD using Method B (Figure 48) and had a retention time (37.51 min, RAD) consistent with that of the synthetic Trimethyl DCGA reference standard **VII** (37.27 min, UV). A positive ion GC/EI mass spectrum (GC method B) of the methylated sample exhibited a parent ion two-chlorine doublet at m/z 264/266 $[M]^+$ and fragment ion doublets at m/z 249/251 $[M-CH_3]^+$, 233/235 $[M-OCH_3]^+$, 203/207 $[M-OCH_3-CH_2O]^+$ and 175/177 $[M-OCH_3-CH_2O-CO]^+$ (Figure 49) and matched the spectrum (Figure 50) of the Trimethyl DCGA reference standard (**VII**). Both the methylated hydrolysate and the reference standard eluted at 14.65 min by GC/MS (data not shown). These results firmly establish that DCGA is the core (exocon) of metabolite Peak 8.

The position(s) of conjugation on the DCGA moiety of Peak 8 were determined in a similar manner to that utilized for Peak 3. The purified Peak 8 isolate was methylated with TMSdiazomethane to methylate the non-conjugated phenol or carboxylic acid moieties. The methylated derivative was then subjected to mild base (NaOH) hydrolysis followed by acid (HCl) hydrolysis to cleave the malonic acid moiety and the glucose moiety, respectively. Analysis by HPLC/RAD using Method B (not shown) gave a peak at 30.99 min which corresponded in retention time to the co-injected synthetic 5-hydroxydicamba methyl ester reference standard (**V**) at 30.79 min (UV). In addition to the peak at 30.99 min peak in the HPLC/RAD chromatogram of the metabolite derivative, a second peak of approximately equal intensity was observed at 20.89 min consistent with the retention time of 5-hydroxydicamba. It is likely that some 5-hydroxydicamba was formed by hydrolysis of the methyl ester formed in the first step of the sequence during the base hydrolysis step. A negative ion LC/ESI mass spectrum (Figure 51, HPLC Method G) of the methylated metabolite derivative exhibited a parent ion doublet at m/z 249/251 $[M-H]^-$ and fragment ion doublet at m/z 234/236 $[M-H-CH_3]^-$ consistent with the mass spectrum (Figure 52) of the 5-hydroxydicamba methyl ester standard (**V**). The identity of the second peak was confirmed to be 5-hydroxydicamba as it exhibited the characteristic parent ion doublet at m/z 235/237 $[M-H]^-$ and large fragment ion at m/z 191/193 $[M-H-CO_2]^-$ (not shown).

The formation of 5-hydroxydicamba methyl ester by methylation then base and acid hydrolysis of Peak 8 establishes that the 5-hydroxyl of DCGA is conjugated and that the 2-hydroxyl and carboxylic acid are free in Peak 8. Nevertheless, the experiment was

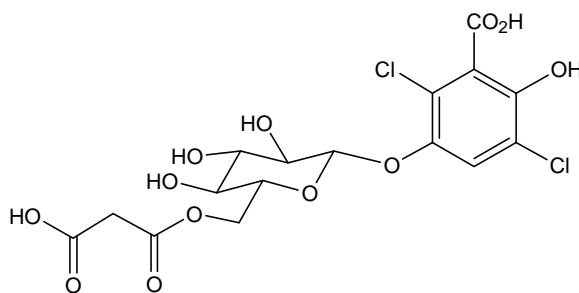
repeated, as follows, with reversal of the order of the methylation and base hydrolysis steps to avoid hydrolysis of the methyl ester of the derivative. A purified isolate of Peak 8 was subjected to mild base hydrolysis to remove the malonate group and the resulting product (7.00 min retention time, HPLC Method B – DCGA glucoside) was purified using HPLC Method B. The purified hydrolysate was methylated with TMSdiazomethane giving a major peak at 21.5 min (HPLC Method B) which was purified using HPLC Method B and then hydrolyzed with HCl to remove the glucose moiety. The resulting derivative eluted at 30.99 min (RAD) using HPLC Method B consistent with the retention time (30.75 min, UV) of coinjected 5-hydroxydicamba methyl ester standard **V** (Figure 53).

As further confirmation of the position of the conjugation in Peak 8, the 5-hydroxydicamba methyl ester derivative of Peak 8 was acetylated and the acetylated product was compared to 5-acetyloxydicamba methyl ester standard (**VI**). Acetylation of the product of mild base hydrolysis, methylation and acid hydrolysis of Peak 8 (prepared directly above) with acetic anhydride and pyridine (RT, 2 h) gave a single product with a retention time of 36.86 min by HPLC/RAD using Method B (Figure 54). The acetylated derivative had a virtually identical GC/MS retention time (Figure 55) to that of the 5-acetyloxydicamba reference standard (**VI**). The positive ion GC/EI mass spectrum (Figure 56) displayed a weak parent ion two-chlorine doublet at m/z of 292/294 $[M]^+$ with a fragment ion at m/z 261/263 $[M-OCH_3]^+$ and intense fragment ions at m/z 250/252 $[M-CH_2=C=O]^+$ and 219/221 $[M-OCH_3-CH_2=C=O]^+$ and was virtually identical to the mass spectrum (Figure 57) of the synthetic reference standard of 5-acetyloxydicamba methyl ester (**VI**). The hydrolysis and derivatization results firmly establish that the glucose moiety of Peak 8 is attached to the 5-hydroxyl of DCGA and that the 2-hydroxyl and the carboxyl group are free. Therefore, the malonate group in Peak 8 is bonded to the glucose moiety.

To verify that the low molecular weight carboxylic acid moiety esterified to the glucose of Peak 8 is malonic acid, this moiety was cleaved from the metabolite by base hydrolysis and converted to a volatile derivative for analysis by GC/EI/MS and comparison to a standard. Because this small acid moiety was derived endogenously and therefore did not contain significant radioactivity, a sensitive non-radioactive detection method with appropriate positive and negative control samples was required. Because it was suspected that the small acid moiety of Peak 8 was malonic acid, method development experiments were conducted investigating the esterification (butylation) of malonic acid at very low levels with BF_3 /butanol to confirm that malonic acid could be converted to dibutyl malonate and detected at the level expected to result from hydrolysis of a small amount of Peak 8. A purified isolate of Peak 8 was subjected to mild base hydrolysis conditions to remove the small acid moiety. Analysis of the reaction product

by HPLC/RAD (Method B) gave a peak at 7.2 min confirming successful removal of the acid moiety. The reaction mixture was then dried, treated with BF_3 /butanol, worked up and analyzed using GC Method D along with positive and negative control samples by GC/MS as described in Sections 2.9.8.3 (butylation) and 2.9.8.6. The hydrolyzed and butylated metabolite sample gave a sharp GC/MS peak at 6.00 min for the extracted ion chromatogram corresponding to the base peak (m/z 105) of dibutyl malonate (Figure 58). A corresponding peak in the GC/MS extracted ion chromatogram of the positive control sample was observed at a virtually identical retention time (6.01 min). The negative control sample gave a negligible peak at 6.00 min. The mass spectra corresponding to the 6-min peak of the sample and positive control (Figure 59) were virtually identical and gave a good match to the published library spectrum of dibutyl malonate. The mass spectrum corresponding to the very small 6-min peak in the negative control sample did not match that of dibutyl malonate. This result firmly establishes malonic acid as the small carboxylic acid moiety esterified to the glucose in Peak 8.

Based on the mass spectral, hydrolysis and derivatization results for Peak 8, it is concluded that Peak 8 is a malonylglucose conjugate of DCGA in which the malonylglucose moiety is attached to the 5-hydroxyl of DCGA. Although the position of malonylation of the glucose has not been determined, it is proposed to be the 6-hydroxyl (hydroxymethyl group) since this is most common in plant malonylglucosides of natural products and xenobiotics and by analogy to Peak 11 (discussed below) in which the position of esterification of the glucose moiety was established. Peak 8 is therefore identified as DCGA Malonylglucoside (**8**) shown below.



DCGA Malonylglucoside, **8**

Peak 9 – DCSA Glucoside

Peak 9 was present in all soybean matrices examined and was the major metabolite in all PRE-T and POE-T matrices, comprising approx. 60-74% of the TRR in pre-forage, forage and hay (up to 80.913 mg/kg in POE-T forage), and 12-15% of the TRR in seed (up to 0.059 mg/kg in POE-T seed). It eluted at approx. 15.5 min using HPLC Method B and distributed between the organic and aqueous phases in the ethyl acetate partitioning of the POE-T seed acidified extract concentrate indicating a moderately polar metabolite.

Initial experiments investigating the acid and base hydrolysis of the extract concentrate of PRE-T pre-forage gave a major peak with an HPLC/RAD retention time of 25.19 min (acid) or 25.22 min (base). HPLC coinjection analysis (Figure 60 and Figure 61) of the acid or base hydrolysate, respectively, with reference standards indicated that the major peak in the hydrolysates (25.19/25.22 min, RAD) coeluted with the DCSA reference standard **II** (24.82/24.89 min, UV) and thus Peak 9 was likely a conjugate of DCSA. A purified isolate of Peak 9 from POE-T forage was also subjected to acid hydrolysis resulting in a product with a retention time of 25.90 min (Figure 62) consistent with the retention time of DCSA (25.21 min, UV) and consistent with the hydrolysis results for the pre-forage extract. Note that for Figure 62 the acid hydrolysate and reference standards were analyzed in different injections, i.e., they were not coinjected, so there is a somewhat larger difference between the retention times of the major hydrolysate peak and the DCSA standard peak than the normal 0.2-0.4 min lag between the UV and RAD detectors. The analysis of the acid hydrolysis reaction also showed a minor peak at 32.21 min which may be the hydrolysate of non-DCSA containing metabolites or due to breakdown (e.g. decarboxylation) of the DCSA hydrolysis product during the hydrolysis reaction.

Mass spectral analysis of the purified Peak 9 isolates from PRE-T pre-forage and POE-T forage (Figure 63 and Figure 64) by negative ion LC/ESI/MS (LC Method F) gave spectra with a parent ion two-chlorine doublet at m/z 367/369 $[M-H]^-$, a minor fragment ion doublet at m/z 323/325 $[M-H-CO_2]^-$ and a significant fragment ion doublet at m/z 161/163 $[M-CO_2\text{-anhydroglucose}]^-$. Ions corresponding to gas-phase dimers were observed at m/z 735/737/739 $[2M-H]^-$ and m/z 757/759/761 $[2M-2H+Na]^-$. In addition, sodium formate adducts of the parent ion were observed in the spectrum of the POE-T forage isolate. These mass spectral data indicate a nominal molecular mass of 368 for Peak 9. Negative ion LC/ESI tandem (MS/MS) analysis of the m/z 367 parent ion of the POE-T forage isolate (Figure 65) generated daughter ions at m/z 323 $[M-H-CO_2]^-$ and 161 $[M-CO_2\text{-anhydroglucose}]^-$ confirming loss of CO_2 from the parent ion and then further loss of 161 mass units. Loss of CO_2 indicates that the carboxylic acid is free in the metabolite. Further loss of 162 mass units is indicative of a hexose moiety. The nominal molecular mass of Peak 9 and the fragmentation results suggest that Peak 9 is a hexose conjugate (likely glucose) of DCSA in which the hexose moiety is attached to the phenol of DCSA. Together with the hydrolysis results, above, these results are consistent with a phenolic glucoside of DCSA for the structure of metabolite Peak 9.

To confirm the structure of Peak 9 and to verify the position of attachment of the hexose moiety, the following experiments were conducted. The purified POE-T forage isolate was analyzed by HPLC/RAD using Method B and Method D (Figure 66). Method D is identical to Method B except water alone is utilized as the aqueous component of the

mobile phase instead of 0.5% formic acid. The metabolite eluted much earlier on Method D (6.14 min) compared to Method B (15.59 min) which indicates the presence of a free acidic (carboxylic acid) group. Methylation of the Peak 9 isolate with TMSdiazomethane gave a derivative that was less polar than the metabolite eluting at 21.09 min (Method B). Positive ion LC/ESI mass spectral analysis (HPLC Method F) did not provide a spectrum of the derivative; however, negative ion analysis (Figure 67) gave a somewhat weak parent ion two-chlorine doublet at m/z 381/383 $[M-H]^-$ with intense chloride and formate adduct ions at m/z 417/419 $[M+Cl]^-$ and 427/429 $[M+HCOO]^-$ and a fragment ion doublet at m/z 219/221 $[M-H-anhydroglucose]^-$ corresponding to loss of the hexose moiety. The nominal mass of 382 for the derivative is 14 mass units higher than that for the metabolite (Peak 9) and is consistent with the addition of a single methyl group to the metabolite (consistent with a single acid moiety in the metabolite). Negative ion MS/MS analysis of the m/z 427 formate adduct ion gave a very weak spectrum with a daughter ion peak at m/z 219 consistent with the fragment observed in the spectrum above.

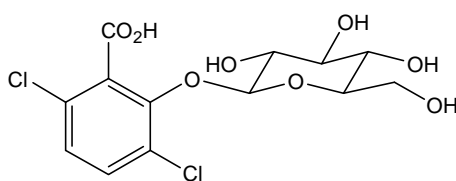
The methylated derivative of Peak 9 was subjected to acid hydrolysis (2 N HCl) to remove the glucose moiety which resulted in a derivative with a retention time of 33.78 min (HPLC Method B, Figure 68). The hydrolysate was analyzed by negative ion LC/ESI/MS (HPLC Method F) and gave a two-chlorine parent ion doublet at m/z 219/221 $[M-H]^-$ (Figure 69). This corresponds to a nominal molecular mass of 220 for the derivative which is consistent with a monomethylated derivative of DCSA. This methylated derivative of DCSA, which has the same nominal mass as dicamba, did not coelute with the dicamba reference standard **I** (Figure 68) and the mass spectrum did not show a loss of CO_2 , whereas loss of CO_2 is observed in the mass spectrum of dicamba (Figure 1). Thus, the methylated derivative of DCSA formed by methylation and hydrolysis of Peak 9 must be DCSA methyl ester. This firmly establishes the position of conjugation in Peak 9 as the phenol group, not the carboxylic acid group. To confirm that this methylated derivative is DCSA methyl ester, the derivative was treated with base (1 N NaOH, 50-60 °C, 3 h) to hydrolyze the ester group. The major component in the hydrolysate (81% hydrolyzed by HPLC) eluted at 25.15 min (RAD) using HPLC Method B, which is consistent with the retention time of DCSA. Negative ion LC/ESI/MS analysis (HPLC Method F) gave a parent ion two-chlorine doublet at m/z 205/207 $[M-H]^-$ and fragment ion doublet at m/z 161/163 $[M-H-CO_2]^-$ and a minor ion corresponding to the sodium adduct of the gas-phase dimer (not shown in figure) at m/z 433/435/437 $[2M-2H+Na]^-$ (Figure 70) matching very well the mass spectrum of DCSA reference standard **II** (Figure 102).

Confirmation of the glucose moiety in Peak 9 was obtained as follows. The methylated derivative of the Peak 9 POE-T forage isolate was acetylated using acetic anhydride and

pyridine giving a product with a retention time of 37.34 min (HPLC Method B). Positive ion LC/ESI/MS analysis (HPLC Method F) of the acetylated product (Figure 71) gave a parent ion two-chlorine doublet at m/z 573/575 $[M+Na]^+$, the sodium adduct of the parent ion, consistent with a nominal mass of 550 for the derivative. This mass is 168 mass units higher than the mass of the methylated derivative of Peak 9 and corresponds to addition of four acetyl units as would be expected for a glucoside containing four hydroxyl groups. The mass spectrum also exhibited fragment ion peaks that were singlets and did not contain chlorine at m/z 331 $[M+H-DCSA]^+$ and 353 $[M+Na-DCSA]^+$ corresponding to tetraacetylanhydroglucose and its sodium adduct, respectively.

Enzymatic hydrolysis confirmed that the hexose moiety of Peak 9 was glucose attached via a β -linkage. The purified Peak 9 isolate was treated with β -glucosidase from almonds for 69 hours at 37 °C in 0.05M sodium acetate buffer pH 5. The product was analyzed by HPLC Method B and indicated that hydrolysis to DCSA to the extent of approx. 87% had occurred (Figure 72). A control reaction without the enzyme gave no reaction demonstrating that hydrolysis in the reaction with enzyme was due to the enzyme and not chemical hydrolysis. Although the hydrolysis of the glucose linkage in Peak 9 by β -glucosidase is rather slow, hydrolysis does occur which demonstrates that the hexose moiety of Peak 9 is glucose and that the configuration at the anomeric center of the glucose is the β configuration. Further evidence for the β configuration at the anomeric center of the glucose moiety was obtained for the related metabolite Peak 11 (see discussion below).

The hydrolysis, derivatization and mass spectral data for Peak 9 provide convincing evidence for the following structure, the phenolic 2- O - β -glucoside conjugate of DCSA (DCSA Glucoside, **9**), as the identity of Peak 9.



DCSA Glucoside, **9**

Peak 11 – DCSA HMGglucoside

Peak 11 was present at moderate levels (as a percentage of the TRR) in all matrices. In the treated matrices Peak 11 constituted 1.14-9.61 % of the TRR with the highest levels (as %TRR) in seed and PRE-T forage or hay. The metabolite had a retention time of approx. 18 min using HPLC Method B and distributed between the organic and aqueous phases in the ethyl acetate partitioning of the POE-T seed acidified extract concentrate indicating a moderately polar metabolite. The purified Peak 11 isolate from POE-T

forage was analyzed by negative ion LC/ESI/MS (HPLC Method F) and exhibited a parent ion two-chlorine doublet at m/z 511/513 $[M-H]^-$ (Figure 73) and a gas-phase dimer ion triplet at m/z 1023/1025/1027 $[2M-H]^-$ consistent with a nominal molecular mass of 512. A minor fragment ion doublet at m/z 161/163 $[M-H-350 \text{ amu}]^-$ also was observed in the mass spectrum, consistent with the same fragment observed in the mass spectra of the DCSA reference standard **II** (Figure 102) and DCSA Glucoside **9** (Figure 63). Analysis of the metabolite by positive ion LC/ESI/MS did not provide a useful spectrum indicating that the metabolite was poorly ionized under positive ion conditions. The peak 11 isolate was also analyzed by negative ion LC/ESI tandem (MS/MS) mass spectrometry isolating both the m/z 511 and 513 parent ions in separate experiments (Figure 74). Comparison of the two daughter ion spectra allowed identification of those ions that were chlorine-containing and those that were not, which facilitated assignment of structures to the fragment ions. The m/z 511 and m/z 513 daughter ion spectra (m/z 513 daughter ions are listed in parentheses) exhibited an intense fragment ion at m/z 467(469) and an additional fragment at m/z 423(425) corresponding to loss of first one then a second molecule of CO_2 indicating the presence of two carboxylic acid moieties in the metabolite. A significant fragment ion was observed in both spectra at m/z 305 which did not contain chlorine and corresponds to a neutral loss of 206(208) mass units (the nominal mass of DCSA) from the parent ion. As well, a significant chlorine-containing fragment ion was observed at m/z 205(207). The presence of the m/z 305 and 205(207) fragments indicate that DCSA is the core of the metabolite structure. A fairly intense fragment ion was observed at m/z 161 (163) which corresponds to the dichlorophenol anion resulting from loss of CO_2 from the 205(207) DCSA fragment. Coincidentally, both daughter ion spectra exhibited the m/z 161 ion indicating that a second non-chlorinated species also contributed to this ion. This mass corresponds to the mass of the anion of anhydroglucose (or other anhydrohexose) indicating the likely presence of a hexose in the metabolite. A related minor chlorine-containing fragment was observed at m/z 323(325) which corresponds to a structure containing both the dichlorophenol and hexose moieties. Interestingly, although this was not appreciated at the time that these data were obtained, if one assumes that the structure of Peak 11 is a DCSA glucoside esterified on the glucose moiety to a dicarboxylic acid, the m/z 161 anion also may be derived from this dicarboxylic acid. Based on the mass spectral data for Peak 11, a DCSA phenolic glucoside structure in which the glucose is esterified by a small unknown dicarboxylic acid was proposed for Peak 11.

Hydrolysis experiments designed to verify that DCSA was the core (exocon) of the Peak 11 metabolite were conducted. Acid hydrolysis (2 N HCl, 100 °C, 2 h) resulted in a mixture of products, one of which appeared to be DCSA based on its negative ion LC/ESI mass spectrum. One of the byproducts showed an m/z 239/241/243 ion (apparent nominal molecular mass of 240) in its negative ion LC/ESI mass spectrum with an

isotopic pattern consistent with the presence of three chlorines in the molecule. This may be a trichlorinated hydroxybenzoic acid derived by chlorination of DCSA in the presence of HCl. Longer reaction times gave larger amounts of the main byproduct eluting at approx. 29 min (HPLC Method B) and less DCSA. Use of H₂SO₄ rather than HCl also resulted in a mixture of hydrolysis products. Finally, milder hydrolysis conditions (1 N HCl, 60 °C, 4 h) resulted in a cleaner conversion of Peak 11 to a product whose retention time (HPLC Method B) was consistent with that of DCSA (Figure 75). However, some byproducts were still formed in the reaction.

In contrast to the acid hydrolysis results, hydrolysis of the purified Peak 11 metabolite with base was more effective and gave a much cleaner reaction. Treatment with base under somewhat stringent conditions (1 N NaOH, 60 °C, 2-5 h) resulted in a hydrolysis product whose retention time matched that of DCSA by HPLC coinjection analysis (Figure 76). Negative ion LC/ESI mass spectral analysis (HPLC Method F) gave a spectrum (Figure 77) exhibiting an m/z 205/207 [M-H]⁻ parent ion and an m/z 161/163 [M-H-CO₂]⁻ fragment ion that matched the spectrum of DCSA reference standard **II** (Figure 102). Under these hydrolysis conditions, the conjugated hexose moiety is removed from the DCSA core of the metabolite. Although acid hydrolysis is generally more effective for hydrolysis of glycosides, in this case, considering that DCSA is highly electron withdrawing and thus a very good leaving group, hydrolysis with base by a substitution mechanism at the hexose anomeric center is rather facile.

Treatment of the Peak 11 isolate with base under mild conditions (1 N NaOH, RT, 1 h) resulted in a hydrolysis product whose retention time (15.63 min, HPLC Method B, Figure 78) was nearly identical to the retention time (15.64 min) of the DCSA Glucoside **9** (Peak 9). A small amount of DCSA (retention time 26.23 min) was also formed in the reaction even under these mild hydrolysis conditions. The mild base hydrolysis mixture was analyzed by negative ion LC/ESI/MS (HPLC Method G with acetic acid instead of formic acid). The mass spectrum (Figure 79) of the hydrolysate exhibited a parent ion two-chlorine doublet at m/z 367/369 [M-H]⁻ with sodium chloride adducts at m/z 425/427 [M-H+NaCl]⁻ and 483/485 [M-H+2NaCl]⁻ and gas-phase dimer ions at m/z 735/737/739 [2M-H]⁻ and m/z 757/759/761 [2M-2H+Na]⁻. A weak fragment ion doublet was observed at m/z 323/325 [M-H-CO₂]⁻ and a significant fragment was observed at m/z 161/163 [M-H-CO₂-anhydroglucose]⁻. The spectrum was a very good match to that of DCSA Glucoside **9** (Figure 64). Also observed in the mass spectral analysis was a peak in the total ion chromatogram whose spectrum was consistent with DCSA (not shown) as expected based on the HPLC analysis of the hydrolysis mixture that indicated the presence of a small amount of DCSA. The results of the acid and base hydrolyses indicated that Peak 11 is a derivative of the DCSA Glucoside **9** in which the glucose moiety is esterified (by inference due to removal of the moiety by mild base hydrolysis)

with a dicarboxylic acid. The molecular weight of the dicarboxylic acid must be 162 based on the molecular weight difference between Peaks 9 and 11 ($512 - 368 = 144$ mass units) plus one mole of water (18 mass units). One alternative possibility was that the glucose moiety was esterified with two moles of a dicarboxylic acid with a molecular weight of 90 (i.e., oxalic acid).

Derivatization experiments were conducted to obtain additional structural information on the metabolite, especially the dicarboxylic acid moiety (or moieties). Methylation of the metabolite was conducted to establish the number of acidic groups (carboxylic acid or phenol) in the metabolite. Methylation of the Peak 11 isolate with TMSdiazomethane resulted in a compound with an HPLC/RAD retention time of 24.28 min (Figure 80, Method B) which was analyzed by both positive and negative LC/ESI/MS (HPLC Method F). The positive ion spectrum (Figure 81) exhibited a rather weak two-chlorine doublet at m/z 563/565 $[M+Na]^+$ consistent with the sodium adduct of the parent ion of a derivative with a nominal mass of 540. The negative ion spectrum (Figure 82) gave an intense ion doublet at m/z 575/577 $[M+Cl]^-$ and a less intense doublet at m/z 585/587 $[M+HCOO]^-$ corresponding to the chloride and formate ion adducts of the derivative and consistent with a nominal mass of 540 for the derivative. A fragment ion doublet was observed at m/z 219/221 corresponding to the anion of DCSA methyl ester formed by loss of the conjugate group. A nominal mass of 540 for the methylated derivative of Peak 11 is 28 mass units higher than the nominal mass (512) of the metabolite corresponding to addition of two methyl groups in the methylation reaction and consistent with the presence of two carboxylic acid groups in the metabolite.

Similar to the results obtained for Peak 9, methylation of Peak 11 with TMS diazomethane followed by acid hydrolysis (2 N HCl, 100 °C, 2 h) gave a product with a retention time of 33.97 min (HPLC/RAD, Method B, not shown), which compares well with the retention time (33.78 min, Figure 68) of the hydrolysis product of Peak 9 identified as DCSA methyl ester.

Acetylation reactions of the Peak 11 metabolite were investigated to determine the number of free hydroxyl groups in the molecule. Acetylation of Peak 11 proved to be difficult and generally resulted in mixtures of several products as determined by HPLC/RAD and LC/ESI/MS. In retrospect, with full knowledge of the structure of Peak 11, acetylation of the tertiary hydroxyl group of the diacid sidechain would be expected to be difficult as is known for tertiary hydroxyls; also, since the tertiary hydroxyl group is in a beta position relative to both carbonyl groups, it would be expected to be prone to elimination. The acetylation results described below are fully consistent with these expectations.

Acetylation with acetic anhydride and pyridine (RT, 1 h) resulted in incomplete reaction (starting material remained). Attempts to force the reaction to completion using longer reaction times or heat (60 °C) resulted in mixtures of products. Negative ion LC/ESI/MS analysis showed peaks with doublet ions at m/z 679/681, 637/639, 619/621 and 601/603 corresponding to the Peak 11 tetraacetate, triacetate, triacetate minus water and triacetate minus two waters, respectively. Isolation of the major component and mass spectral analysis by negative ion LC/ESI/MS (Figure 83) indicated that the triacetate elimination product (m/z 619/621 parent ion and minor m/z 575/577 ion corresponding to loss of CO₂) was the major product of the reaction. In another attempt, acetylation at room temperature for 2 h followed by methylation (to methylate the carboxyl groups) gave a mixture which was again subjected to acetylation conditions overnight. HPLC/RAD analysis (Method B, not shown) gave a single peak at 39.9 min. Positive ion LC/ESI/MS analysis gave peaks with ion doublets at m/z 731/733 and 671/673 corresponding to the sodium adducts of the Peak 11 dimethyl ester tetraacetate and the elimination product (triacetate minus water), respectively.

Finally, a relatively clean acetylation of Peak 11 was effected by first methylating the carboxylic acid groups followed by acetylation under mild conditions in the presence of the catalyst 4-(dimethylamino)pyridine (DMAP), which is known to accelerate the acetylation of tertiary hydroxyl groups. Methylation of Peak 11 with TMSdiazomethane followed by acetylation (RT, 2h) with pyridine (0.2 mL), Ac₂O (0.4 mL) and DMAP (4 mg) gave a relatively clean reaction product with an HPLC/RAD retention time (Method B) of 39.82 min (Figure 84). The methylated/acetylated product was purified using HPLC Method D. Analysis by positive ion LC/ESI/MS (LC Method G) gave a mass spectrum (Figure 85) exhibiting a parent ion sodium adduct (two-chlorine doublet) at m/z 731/733 [M+Na]⁺ and singlet fragment ions at m/z 489 [M+H-DCSA]⁺, 447 [M+H-DCSA-CH₂=C=O]⁺, 429 [M+H-DCSA-HOAc]⁺ corresponding to loss of DCSA from the protonated parent ion followed by loss of ketene or elimination of acetic acid. A significant fragment ion singlet was observed at m/z 141 [CH₃O(CO)CH=C(CH₃)CH₂C≡O]⁺ apparently corresponding to the acetic acid elimination product of the methylated/acetylated diacid moiety. The spectrum corresponded to a nominal molecular mass for the methylated/acetylated derivative of 708 which is 196 mass units higher than the nominal mass of Peak 11 (512) consistent with addition of two methyl groups (2 x 14 = 28 mass units) and four acetyl groups (4 x 42 = 168 mass units). Significantly, the acetylation data indicate that the diacid moiety must contain one hydroxyl group since four acetyl groups were added and the esterified glucose contains only three hydroxyl groups.

The data obtained for Peak 11 to this point established that Peak 11 is a metabolite of DCSA Glucoside **9** that is esterified on the glucose moiety with an unknown dicarboxylic

acid moiety of molecular weight 162 that contains one hydroxyl group. Considerable effort was expended in experiments designed to determine the identity of this small diacid moiety. The following discussion describes the experiments and results that ultimately resulted in identification of this diacid. Negative ion IonSpray tandem (MS/MS) mass spectrometric analysis of Peak 11 was conducted using an ABI/Sciex API 5000 instrument. This instrument has the capability of analyzing low-mass fragments in MS/MS experiments. In separate experiments, the m/z 511 and 513 parent ion masses were isolated and fragmented. The MS/MS spectra and proposed mass assignments are shown in Figure 86 and Figure 87. The spectra were consistent with those obtained previously on the LCQ instrument (Figure 74) and, in addition, several low-mass fragment peaks were observed. These peaks are consistent with fragmentation of the glucose moiety of Peak 11 and/or fragmentation of the diacid moiety (none of the fragments contained chlorine). However, the fragmentation did not clearly indicate a structure for the diacid moiety because assignment of structures to all of the fragments proved to be difficult without first knowing the structure of the diacid moiety. Once the structure of the diacid moiety was known, structures could be assigned to the fragments that were consistent with the proposed structure for Peak 11.

Initially, the diacid moiety of Peak 11 was thought to be γ -hydroxy- α -ketoglutaric acid (HKGA) since this diacid is a known natural product and has a molecular weight of 162. Numerous experiments were conducted to attempt to verify this structure. An attempt to establish the presence of a ketone functionality in Peak 11 by FT-IR was not conclusive. HKGA was synthesized and several attempts were made to convert the very polar HKGA to a volatile derivative that could be analyzed by GC/MS or a less polar derivative that could be analyzed by LC/ESI/MS with a plan to hydrolyze the diacid moiety from Peak 11, derivatize the hydrolysate and compare to the HKGA derivative. These attempts included methylation or butylation (to form the dimethyl or dibutyl ester) and acetylation of the hydroxyl of HKGA, or formation of the 2,4-dinitrophenylhydrazone (2,4-DNP) derivative by reaction of the ketone of HKGA. Although some of the experiments with HKGA were successful on a relatively large scale, at the very low levels required for analysis of the diacid moiety derived from a small amount of Peak 11, the analyses generally failed. Attempts to directly convert Peak 11 to a 2,4-DNP or semicarbazone derivative did not give positive results (and in retrospect would not be expected to give positive results considering the absence of a ketone functionality in Peak 11. An attempt to form the phenethylamide of Peak 11 was also unsuccessful.

The key experiment that finally led to identification of the diacid moiety of Peak 11 was a high resolution exact mass determination for Peak 11. An initial attempt at obtaining an exact mass for Peak 11 by matrix assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry was unsuccessful because Peak 11 was very poorly

ionized by the MALDI method. Success was obtained using a PE Sciex QSTAR IonSpray TOF instrument in negative ion mode. The procedure used for the exact mass determination of Peak 11 on the QSTAR instrument is described in Section 2.9.1.4. The Peak 11 isolate was analyzed together with three reference standards: taurocholic acid (**XIX**) whose nominal mass (515 amu) is close to that of Peak 11 and served as an internal mass check; and fexofenadine (**XX**, nominal mass 501) and MRFA (**XXI**, Met-Arg-Phe-Ala tetrapeptide, nominal mass 523) which closely bracketed the mass of Peak 11 (and taurocholic acid) and served as internal calibration standards. A solution containing the three standards and purified Peak 11 was infused into the mass spectrometer, analyzing all four simultaneously, averaging a large number of scans over several minutes in order to obtain the highest precision and mass accuracy achievable by the instrument. In total, three exact mass determinations for Peak 11 were conducted with all three giving very similar results. The mass spectrum for one of the three determinations is shown in Figure 88.

For the three exact mass determinations, the exact masses measured for the anion of the taurocholic acid standard, and the associated deviations from the theoretical mass, were 514.2833 (1.1 ppm), 514.2833 (1.1 ppm) and 514.2828 (2.0 ppm) demonstrating very good mass accuracy for this method. The exact masses measured for the anion of Peak 11 (m/z 511, ^{35}Cl peak) in the three experiments were 511.0390, 511.0405 and 511.0404 (average = 511.0400). The measured masses for the corresponding ^{37}Cl isotope peak (m/z 513) were 513.0370, 513.0382 and 513.0389 (average = 513.0380). An elemental composition search using Xcalibur Qual Browser v2.0 software was conducted for mass 511.0400 constraining the search to the minimum number of each element known to be present in the molecule based on the DCSA glucoside core. The best match for the anion of Peak 11 was an elemental composition of $\text{C}_{19}\text{H}_{21}\text{O}_{12}\text{Cl}_2$ (for both the m/z 511 and 513 exact masses) with an exact mass of 511.0410, just 2.0 ppm different than the measured mass. There were no other reasonable elemental compositions within 20 ppm of the measured mass. This elemental composition corresponds to a molecular formula of $\text{C}_{19}\text{H}_{22}\text{O}_{12}\text{Cl}_2$ for the neutral metabolite Peak 11. Subtracting the elements of the DCSA glucoside from this formula and adding one OH, gives a molecular formula of $\text{C}_6\text{H}_{10}\text{O}_5$ for the diacid molecule that is esterified to DCSA glucoside in Peak 11.

A search of the CAS database for the molecular formula $\text{C}_6\text{H}_{10}\text{O}_5$, and refining the search to structures containing two carboxylic acids and one hydroxyl, resulted in 20 unique structures consisting primarily of isomeric hydroxyadipic, dimethyl- or ethylmalic, or hydroxymethylglutaric acids. Of these 20 structures, the most well-known structure based on the number of literature citations, and one which is a known modifier of flavonol glycosides,^{5,6} is 3-hydroxy-3-methylglutaric acid (HMGA). Thus, it seemed that HMGA was the most likely structure for the diacid moiety of Peak 11.

In order to verify that HMGA was indeed the diacid moiety of Peak 11, a relatively large amount (approx. 2.9 μg) of Peak 11 was isolated from POE-T forage and purified to a high degree by HPLC using Methods B, C and D successively and finally, again, Method B. A proton NMR spectrum of Peak 11 (Figure 89) was obtained in D_2O (44,000 transients). Resonances marked with 'x' in the figure are due to matrix and/or solvent contaminants and were determined to be unrelated to Peak 11 by comparison to a spectrum of a negative control sample (a sample collected from a chromatographic fraction near the Peak 11 chromatographic peak, but not containing Peak 11). Peak assignments, chemical shift and coupling information for Peak 11 are tabulated below (refer to Figure 89 for proton numbering scheme). The NMR spectrum is consistent with a 3-hydroxy-3-methylglutaryl (HMGA) glucoside of DCSA. Notable are the resonances at δ 1.14 and δ 2.44/2.53 corresponding to the HMGA protons (H11 and H9/H10) and consistent with the chemical shifts of the HMGA standard (δ 1.22 and δ 2.53/2.62, respectively) and with shifts observed in other HMGA glycoside esters (δ 2.04-2.65 and δ 0.99-1.36, respectively).^{5,6,11b} Also, the coupling constant of 7.2 Hz for the proton at the anomeric center (H3) indicates a β -configuration (the coupling constant for the α -configuration would be about 3.8 Hz);⁷ and the downfield shift of the two H8 protons indicates that the HMGA moiety is attached to the hydroxyl of the methylene carbon of glucose (the 6-position using glucose numbering).

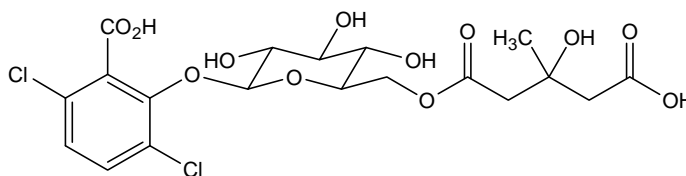
Proton	Chemical Shift (δ , ppm)	Multiplicity* (coupling, Hz)	Identification
H1 or H2	7.22	d ($J_{1,2} = 9.2$)	aromatic CH
H1 or H2	7.07	d ($J_{1,2} = 8.0$)	aromatic CH
H3	4.99	d ($J_{3,4} = 7.2$)	glucose CH (anomeric)
H8a or H8b	4.27	d ($J_{8a,8b} = 12.0$)	glucose CH_2
H8a or H8b	4.09	m ($J_{8a,8b} = 12.0$, $J_{6,8} = 6$)	glucose CH_2
H4, H5, H6, H7	3.346-3.464	m	glucose CH
H9 or H10	2.53	ABq ($J \approx 16$ Hz)	HMGA (CH_2)
H9 or H10	2.44	s (collapsed ABq)	HMGA (CH_2)
H11	1.14	s	HMGA (CH_3)

*s = singlet, d = doublet, m = multiplet, q = quartet

To further verify the HMGA moiety of Peak 11, the diacid was cleaved from the metabolite by mild base hydrolysis, derivatized and analyzed by GC/EI/MS in comparison to a reference standard. Similar to the malonic acid moiety of Peak 8, because the small HMGA moiety was derived endogenously and therefore did not contain significant radioactivity, a sensitive non-radioactive detection method with appropriate positive and negative control samples was required. Literature reports indicated that HMGA had been analyzed at low levels (e.g., in urine) by a number of methods, most notably by GC analysis after silylation. Method development experiments determined that silylation of HMGA to tris(trimethylsilyl)HMGA at the low levels expected by hydrolysis of Peak 11 was most effective in providing a volatile derivative that could be analyzed by GC/MS. The purified isolate of Peak 11 was subjected to mild

base hydrolysis conditions to remove the small acid moiety. Analysis of the reaction product by HPLC/RAD (Method B) gave a peak at 15.9 min confirming cleavage of the diacid moiety. The sample was then derivatized using the silylation procedure described in Section 2.9.8.3 and the product was analyzed by positive ion GC/EI/MS. As shown in Figure 90, peaks corresponding to the tris(trimethylsilyl) derivative of the sample and the positive control (HMGA reference standard) eluted at identical retention times (13.01 min) and had virtually identical mass spectra (Figure 91) which matched the published library spectrum for tris(trimethylsilyl)HMGA. No corresponding peak was observed in the negative control sample. These results firmly establish that HMGA is the diacid moiety esterified to glucose in Peak 11.

The substantial data obtained on Peak 11 are fully consistent with a 3-hydroxy-3-methylglutaryl DCSA glucoside structure (DCSA HMGglucoside, **11**, below) in which the glucose is bonded to DCSA through a β linkage and the HMGA moiety is bonded to the 6-position of the glucose moiety.



DCSA HMGglucoside, **11**

Peak 14 – Unknown DCSA and DCGA Conjugates

Peak 14 was present at low levels in all matrices constituting a maximum of 1.75 % of the TRR (0.686 mg/kg) in POE-T hay. It was a somewhat broad peak whose retention time (approx. 21.5 min on HPLC Method B) was near that of the 5-hydroxydicamba reference standard. The isolate from POE-T forage was analyzed by HPLC as a coinjection with the 5-hydroxydicamba reference standard using HPLC Method B (Figure 92). The somewhat broad radioactive peak eluted at 21.40 min while the reference standard was a sharp peak at 20.41 min. This one-minute difference in retention time between the radioactive peak and the standard is much larger than the lag time (0.25-0.4 min) between the UV and RAD detectors indicating that 5-hydroxydicamba is not a significant component of Peak 14. The Peak 14 sample was analyzed by HPLC Method C indicating a mixture of at least five radioactive components (Figure 93). These HPLC analyses indicate that Peak 14 from POE-T forage is not 5-hydroxy dicamba and is a mixture.

Peak 14 also was isolated from the organic phase of the ethyl acetate partition of the POE-T hay acidified extract concentrate using HPLC Method B. The isolate was analyzed by HPLC/RAD using Method C to give a chromatogram that indicated that it

was a mixture of at least three main components (Figure 94). Hydrolysis of the isolate with acid (2 N HCl, 100 °C, 2 h) and analysis of the hydrolysate using HPLC Method B (Figure 95) indicated two major peaks (along with four minor peaks) at 16.8 and 25.7 min consistent with DCGA and DCSA, respectively. No peak was observed at 20.5-21.0 min where 5-hydroxydicamba would be expected to elute (which should not have hydrolyzed under the acid hydrolysis conditions). The chromatographic and hydrolysis data for Peak 14 from POE-T forage and hay indicate that this peak is a mixture of unknown metabolites derived from DCSA and DCGA; 5-hydroxydicamba is not a significant component of Peak 14.

Peak 17 – Unknown

Peak 17 was observed at low levels in POE-T forage, hay and seed and constituted less than 1% of the TRR in each matrix. Negative ion electrospray mass spectral analysis of Peak 17 isolated from the ethyl acetate partition of POE-T hay gave a weak two-chlorine doublet at m/z 543/545 (Figure 96, top) corresponding to a probable molecular mass of 544. The m/z 543 extracted ion chromatogram (HPLC Method G) indicated that the material giving the m/z 543/545 ion eluted as a mixture of two components (Figure 96, bottom). The mass spectrum and extracted ion chromatogram are very similar to those obtained for Peak 18 (see discussion below) and probably correspond to Peak 18 rather than Peak 17. It is likely that a small amount of Peak 18 was isolated together with the Peak 17 isolate from the POE-T hay ethyl acetate partition because there appears to be a shoulder on Peak 17 corresponding to Peak 18 that is not sufficiently resolved to quantitate (see Figure 97). Although the Peak 17 isolate was purified using a second gradient (HPLC Method C), Peaks 17 and 18 appear to co-elute at 37.2 min under these conditions.

Peak 18 – Unknown DCSA and DCGA Glucoside Conjugates

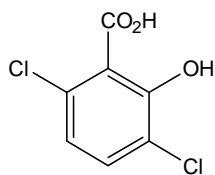
Peak 18 was present at low levels in PRE-T and POE-T forage, and PRE-T hay, constituting less than 1% of the TRR in each matrix. The metabolite peak isolated from forage using HPLC Method B was analyzed using HPLC Method C giving a major peak at 37.2 min along with several minor peaks. Acid hydrolysis of the isolate (2 N HCl, 100 °C, 2 h) and HPLC/RAD analysis using HPLC Method B (Figure 98, top) gave a major peak at 25.5 min consistent with DCSA and a minor peak at 16.7 min consistent with DCGA. The metabolite isolate was also hydrolyzed under mild base conditions (1 N NaOH, RT, 1 h). The reaction product was analyzed by HPLC/RAD (Method B) and yielded a major peak at 15.6 min consistent with DCSA glucoside and a minor peak at 7.0 min consistent with DCGA glucoside (Figure 98, bottom). Following further purification of the isolate using HPLC Method C, a negative ion LC/ESI mass spectrum was obtained which exhibited a two-chlorine doublet at m/z 543/545 (Figure 99, top)

corresponding to a probable molecular mass of 544. The m/z 543 extracted ion chromatogram (HPLC Method G) indicated that the material giving the m/z 543/545 ion eluted as a mixture of two components (Figure 99, bottom). The mass spectrum and extracted ion chromatogram are very similar to those obtained for the Peak 17 isolate, above. Peak 18 was not conclusively identified; however, the major component of Peak 18 appears to have a molecular mass of 544 and is a derivative of the DCSA glucoside (**9**). Based on the hydrolysis data, it is likely that this major component of Peak 18 is the DCSA glucoside in which the glucose moiety is esterified with a carboxylic acid other than malonic acid or HMGA. Likewise, the minor component of Peak 18 appears to be a derivative of the DCGA glucoside (**3**) in which the glucose moiety is esterified with a carboxylic acid. Peak 18 is therefore characterized as a mixture of unknown DCSA and DCGA glucoside conjugates.

Peak 22 – DCSA (3,6-Dichlorosalicylic Acid)

Peak 22 was a relatively minor metabolite in all soybean matrices with a maximum of 4.08% of TRR (5.473 mg/kg) in POE-T forage. It was a somewhat broad, slightly tailing peak with an HPLC retention time of approx. 25-27 min (Method B). The retention time and peak shape of this metabolite were somewhat variable depending on the injection matrix and condition of the HPLC column. Coinjection of the purified metabolite from POE-T forage with the DCSA reference standard using HPLC Method B indicated that the metabolite had a retention time consistent with that of DCSA (Figure 100). The metabolite partitioned out of the POE-T seed acidic aqueous extract concentrate into ethyl acetate. Negative ion LC/ESI/MS analysis (HPLC Method F) of the purified POE-T forage metabolite gave a spectrum (Figure 101) consistent with that of the DCSA reference standard (Figure 102) exhibiting a parent ion two-chlorine doublet at m/z 205/207 $[M-H]^-$ and an m/z 161/163 $[M-H-CO_2]^-$ fragment ion corresponding to a nominal molecular mass of 206.

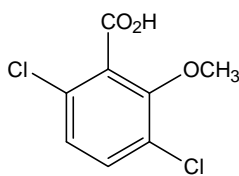
Peak 22 isolated from the ethyl acetate partition of the acidified POE-T hay extract concentrate was coinjected with the DCSA reference standard using a second HPLC Method (Method C). The metabolite gave a peak at 36.9 min (RAD) and the DCSA standard eluted at 36.6 min (UV) indicating coelution (Figure 103). LC/ESI/MS (HPLC Method G) analysis (Figure 104) of the metabolite from POE-T hay gave a mass spectrum with a parent ion doublet at m/z 205/207 $[M-H]^-$ and fragment ion at m/z 161/163 $[M-CO_2]^-$ that matched the mass spectra of the POE-T forage metabolite (Figure 101) and the DCSA reference standard (Figure 102). The retention time under two different reverse phase methods, partitioning behavior and mass spectral data for Peak 22 from POE-T forage and hay are consistent with 3,6-dichlorosalicylic acid (DCSA) as the identity of Peak 22.



DCSA, **22**

Peak 23 – Dicamba

Peak 23 was the latest-eluting (least polar) of the significant radioactive peaks in the HPLC profiles of soybean matrices eluting at 28 min using HPLC Method B. This peak was a minor component in most of the matrices except POE-T forage (24.21% of TRR, 32.473 mg/kg), POE-T hay (12.33 % of TRR, 4.828 mg/kg), PRE-C forage (19.27% of TRR, 0.015 mg/kg) and POE-C forage (26.18 % of TRR, 0.073 mg/kg). Peak 23 partitioned out of the POE-T seed acidic aqueous extract concentrate into ethyl acetate (Figure 19). The HPLC retention time of Peak 23 isolated from POE-T forage was consistent with that of parent dicamba (Figure 105). Washing experiments indicated that virtually all of Peak 23 could be washed off of POE-T forage plants suggesting that Peak 23 in the forage was entirely in surface residues and therefore likely to be parent dicamba. Mass spectral analysis of the purified material by negative ion LC/ESI/MS (HPLC Method F) gave a spectrum (Figure 106) exhibiting a two-chlorine parent ion doublet at m/z 219/221 $[M-H]^-$ and a fragment ion at m/z 175/177 $[M-H-CO_2]^-$ corresponding to loss of the carboxyl group. The mass spectrum indicated a nominal mass of 220 and was consistent with the mass spectrum of the dicamba reference standard (Figure 107). The chromatographic retention time, partitioning properties and mass spectral data are entirely consistent with dicamba for the identity of Peak 23.



Dicamba, **23**

Triglycerides

The hexane extracts (oil fraction) of PRE-T, POE-T, PRE-C and POE-C soybean seed contained 10.35, 8.41, 17.84 and 11.69% of the TRR, respectively. To investigate the nature of the residues in the hexane extracts, two experiments were conducted. First, the oil obtained by evaporation of the POE-T seed hexane extract was subjected to acidic methanolysis to convert triglycerides in the oil to fatty acid methyl esters (and glycerol)

by transesterification. The fatty acid methyl esters were then partitioned into hexane and analyzed by HPLC Method E, a method that utilizes a silver-impregnated column and is specific for analysis of fatty acid methyl esters. The results of the transesterification and partitioning are summarized in Table 15 and the HPLC analysis is shown in Figure 23. Although the oil fraction had a rather low specific activity (i.e., the amount of radioactivity relative to the weight of the oil was low), a sufficient amount of the transesterified oil could be injected on the HPLC to obtain an HPLC/LSC profile using 10-minute LSC counts of the collected fractions. Three radioactive peaks were observed in the HPLC profile of the esterified oil. The retention times of the peaks were consistent with those of the fatty acid methyl ester reference standards of palmitic and stearic acid, oleic acid and linoleic acid. These results indicate that the radioactive residues in the oil fraction of the seed are very likely triglycerides resulting from extensive metabolism of dicamba in the soil, and also possibly the plant, with subsequent incorporation, through normal plant metabolic processes, of small molecules such as $^{14}\text{CO}_2$ into the fatty acids comprising the oil triglycerides.

As shown in Table 15, 89.64% of the radioactivity in the oil fraction of POE-T seed partitioned into hexane following methanolysis and 10.36% remained in the acidic methanol solution. From this, it is calculated that 94.68% of the radioactivity in the POE-T seed hexane extract is comprised of triglycerides (includes 89.64% due to fatty acids in the hexane partition and 5.05% due to the glycerol moiety that remains in the acidic methanol phase – see Section 2.10 for calculations). Only 5.32% of the radioactive residues in the oil fraction are calculated to be due to components other than triglycerides. It was assumed that the value of 94.68% (the percent of radioactive residues in the hexane extract comprised of triglycerides) calculated for POE-T seed was the same for the other seed matrices (PRE-T, PRE-C and POE-C).

The second experiment that was conducted on the soybean hexane extracts involved partitioning of the extracts with aqueous bicarbonate. The results of this experiment are shown in Table 16. Method development experiments indicated that dicamba (and thus other more polar dicamba metabolites) could be partitioned out of hexane into aqueous bicarbonate. Partitioning of the PRE-C and POE-C hexane extracts with bicarbonate resulted in virtually none of the radioactivity partitioning into the aqueous phase from the hexane extracts. This is further evidence that the radioactivity in the seed hexane extracts (oil fraction) is not due to dicamba metabolites but is due to incorporation into the oil triglycerides.

3.6.2 Confirmation of the Identities of Metabolites in Soybean Seed

Metabolites for identification were generally isolated from PRE-T pre-forage or POE-T forage and hay. Except for Peak 1, which was present only in the seed at significant

levels, metabolites were not isolated for identification from seed due to the low levels of metabolites and the large amount of matrix material co-extracted with the seed residues. Due to the similarity of the seed profiles to the forage and hay profiles, it can be reasonably assumed that the seed metabolites are identical to the forage and hay metabolites. However, due to minor retention time differences in the seed profiles relative to the forage and hay profiles, the identities of the seed metabolite peaks were confirmed by HPLC coinjections of the POE-T seed extract concentrate with isolated POE-T forage metabolites using HPLC Method C, a method different than the method utilized for the metabolite quantitation profiles. The coelution analyses for Peaks 8, 9 and 11 are shown in Figure 20 through Figure 22, and the coelution results are summarized in Table 17. The coelutions confirmed the identities of the major peaks (Peaks 8, 9 and 11) in the seed. Confirmation of the identities of the minor peaks (Peaks 22 and 23) was somewhat ambiguous since these two peaks were not well separated on the particular column utilized since the column efficiency had degraded at the time of these analyses. Nevertheless, the retention times of these two minor peaks in the original seed quantitation profiles are fully consistent with their identities as DCSA and dicamba.

3.6.3 Quantitation of Metabolites

Quantitation of individual metabolite peaks was conducted for all soybean matrices (PRE-T pre-forage and PRE-T or POE-T forage, hay and seed) derived from the pre- or postemergence treatments as well as those derived from control plants interspersed amongst the treated plants (PRE-C and POE-C forage and seed). Names and structures of identified metabolites are tabulated in Table 1. The metabolite quantitation profiles are shown in Figure 6 through Figure 17. For HPLC/RAD quantitation profiles, all discernible peaks above the background noise were quantitated. This generally resulted in quantitation of all peaks greater than approx. 0.2-0.5% of the HPLC/RAD profile. For HPLC/LSC profiles, all peaks containing ≥ 10 dpm were quantitated (in some cases somewhat smaller peaks were quantitated) resulting in quantitation of all peaks constituting $>0.5\%$ of the HPLC/LSC profile. The quantitation of identified or characterized metabolites is summarized in Table 8 (PRE-T pre-forage, PRE-T/POE-T forage and hay), Table 9 (PRE-T/POE-T seed) and Table 10 (PRE-C/POE-C forage and seed). Likewise, quantitation of unidentified metabolites is summarized in Table 11, Table 12 and Table 13.

Analytical radioactivity recoveries for the quantitative HPLC analyses of soybean extracts are summarized in Table 7. The sample preparation (evaporation and centrifugation) recoveries and HPLC column recoveries for pre-forage, forage and hay were virtually quantitative at 97.11-104.20% and 96.38-110.10%, respectively. The recoveries for seed were somewhat lower (88.04-92.49% and 88.94-94.64% for sample preparation and HPLC column recovery, respectively). Because the sample preparation

and HPLC column recoveries for seed were not quantitative, these values were included in the metabolite percent of TRR calculations for the seed metabolites. The sample preparation and HPLC column recoveries were not included in the metabolite percent of TRR calculations for pre-forage, forage and hay metabolites.

3.6.3.1 Metabolites in Soybean Pre-forage and Forage

The major, and predominant, metabolite in soybean PRE-T pre-forage and PRE-T or POE-T forage was DCSA glucoside **9**, representing 68.96, 74.48 and 60.32% of the TRR (2.240, 1.067 and 80.913 mg/kg), respectively. In the PRE-T pre-forage and forage, the next most abundant metabolite was DCSA HMGglucoside **11** constituting 7.62% of TRR (0.247 mg/kg) and 5.21% of TRR (0.075 mg/kg), respectively. This metabolite represented only 1.14% of the TRR (1.535 mg/kg) in POE-T forage. For the POE-T forage, non-metabolized parent dicamba, which was shown to be on the plant surfaces by washing experiments, constituted 24.21 % of TRR (32.473 mg/kg). Only small amounts of parent dicamba (<2% of TRR) were observed in the PRE-T pre-forage or forage. Free (non-conjugated) DCSA **22** represented 3.19 and 4.08% of TRR (0.046 and 5.473 mg/kg) in PRE-T and POE-T forage, respectively, but only 1.46% of TRR (0.047 mg/kg) in PRE-T pre-forage. The PRE-T pre-forage contained DCGA malonylglucoside **8** (5.46% of TRR, 0.177 mg/kg) and DCGA glucoside **3** (2.77% of TRR, 0.090 mg/kg); however, these were minor metabolites in the PRE-T and POE-T forage each constituting <2% of TRR. Minor amounts of radioactive component **1**, characterized as sugars, were observed in the PRE-T pre-forage and forage, but not in the POE-T forage. All other metabolites in pre-forage or forage each accounted for less than 2.0% of the TRR.

The PRE-C and POE-C (untreated) forage metabolite profiles were quite similar to each other. The most abundant radioactive components of these matrices were DCSA glucoside **9** (40.66 and 55.49% of TRR, 0.033 and 0.155 mg/kg, respectively) and, somewhat surprisingly, dicamba **23** (19.27 and 26.18% of TRR, 0.015 and 0.073 mg/kg, respectively). Presumably, the dicamba in PRE-C and POE-C forage represents surface residues arising due to evaporation of dicamba from the soil and/or plant surfaces of the treated soil/plants in close proximity and deposition on the untreated PRE-C and POE-C plants. DCSA **22** represented 3.80 and 4.76% (0.003 and 0.013 mg/kg) of the TRR in the PRE-C and POE-C forage, respectively. The rather low extractability for the PRE-C forage (75.64%) as well as the presence of component **1** (sugars, 5.57% of the TRR) in the PRE-C forage indicates uptake and reincorporation of ¹⁴CO₂ from metabolism of dicamba in the soil of adjacent treated pots.

The HPLC profile for washed POE-T forage plant #28 was similar to the POE-T forage profile except that only a very small dicamba peak was observed in the former indicating

that virtually all of the dicamba had been removed in the surface washes. Somewhat more DCSA **22** was observed in the washed forage plant than in the POE-T forage.

In all, identified or characterized metabolites constituted 88.82-92.50% of the TRR in PRE-T pre-forage, and PRE-T, POE-T or POE-C forage. Identified or characterized metabolites constituted only 72.52% of the TRR in PRE-C forage due to the relatively large proportion of unextracted residues. Unidentified metabolites constituted 1.00-3.12% of the TRR in pre-forage and forage. Only one unidentified metabolite exceeded 1% of the TRR in any forage matrix (metabolite **16** in PRE-C forage, 2.44% of TRR, 0.002 mg/kg).

3.6.3.2 Metabolites in Soybean Hay

As in the forage, the major metabolite in soybean hay was DCSA glucoside **9** constituting 70.81 and 67.26% of the TRR (0.748 and 26.333 mg/kg) in the PRE-T and POE-T hay, respectively. In PRE-T and POE-T hay, respectively, DCSA HMGglucoside **11** represented 6.67 and 2.48% of TRR, and DCGA glucoside **3** represented 3.45 and 4.32% of TRR. Dicamba **23** constituted 12.33% of the TRR in the POE-T hay but was a very minor component (0.85% of TRR) in the PRE-T hay. Several other metabolites observed in hay, each of which constituted less than 2% of the TRR in either matrix, were DCSA **22**, DCGA malonylglucoside **8**, unknown DCSA/DCGA conjugates **14** and **18**, sugars **1** and unknown **16**. All other metabolites were less than 1.0% of the TRR.

Identified or characterized metabolites in PRE-T and POE-T hay represented 87.29 and 92.17% of the TRR, respectively, while unidentified metabolites represented 3.59 and 3.14% of the TRR, respectively.

3.6.3.3 Metabolites in Soybean Seed

The metabolite profiles of the PRE-T and POE-T seed extracts were very similar to each other. While the forage and hay metabolite profiles were dominated by the DCSA glucoside **9**, there were four prominent metabolite peaks in the seed profiles (DCSA glucoside **9**, DCSA HMGglucoside **11**, sugars **1** and DCGA malonylglucoside **8**). The major metabolite in PRE-T and POE-T soybean seed was DCSA glucoside **9** constituting 11.55% (0.034 mg/kg) and 15.27% (0.059 mg/kg) of TRR, respectively. Other PRE-T and POE-T seed metabolites were DCSA HMGglucoside **11** (8.73% TRR/0.025 mg/kg, 9.61% TRR/0.037 mg/kg, respectively), triglycerides (13.87% TRR/0.040 mg/kg, 10.76% TRR/0.042 mg/kg, respectively), sugars **1** (8.42% TRR/0.025 mg/kg, 9.15% TRR/0.036 mg/kg, respectively), DCGA malonylglucoside **8** (4.73% TRR/0.014 mg/kg, 4.64% TRR/0.018 mg/kg respectively) and DCGA glucoside **3** (1.60% TRR/0.005 mg/kg, 2.07% TRR/0.008 mg/kg respectively). Small amounts (<1.0% of TRR) of

DCSA **22** and dicamba **23** were observed in the seed profiles. No other metabolites, identified, or unidentified, exceeded 2.0 % of the TRR or 0.01 mg/kg in the PRE-T or POE-T seed. In the seed, a larger proportion of the DCSA residues were present as the DCSA HMGglucoside **11** compared to forage and hay. Also, a higher proportion of the extractable seed residues were DCSA metabolites compared to forage and hay. The ratios of DCSA metabolites to DCSA metabolites were approx. 30:1, 10-20:1 and 3-4:1 for forage, hay and seed, respectively.

The PRE-C and POE-C seed profiles were somewhat different than those of the treated samples. The profiles were dominated by the peak corresponding to sugars **1** which represented 12.61% of the TRR (0.021 mg/kg) in PRE-C seed and 12.50% of the TRR (0.017 mg/kg) in POE-C seed. Although several additional minor peaks were observed in the PRE-C and POE-C seed profiles, only DCSA glucoside **9** (2.18% of TRR/0.004 mg/kg and 5.75% TRR/0.008 mg/kg, respectively) and DCSA HMGglucoside **11** (1.33% TRR/0.002 mg/kg and 4.12% TRR/0.006 mg/kg, respectively) were prominent in the profiles. Triglycerides, in the hexane extracts of PRE-C and POE-C seed, constituted 16.89% (0.029 mg/kg) and 11.07% (0.015 mg/kg) of the TRR, respectively. All other PRE-C or POE-C seed metabolites constituted less than 2.0% of the TRR and ≤ 0.002 mg/kg. The relatively large proportion of the PRE-C and POE-C seed residues represented by sugars and triglycerides, as well as the low acetonitrile:water extractabilities (26.05% and 32.47%, respectively), indicates that residues in the PRE-C and POE-C seed are likely derived in large part from uptake and reincorporation of $^{14}\text{CO}_2$.

3.6.3.4 Metabolites in Soybean Seed Unextracted Residues

As described in Section 3.5.3.2, enzymatic and chemical digestions of the extraction pellets remaining after extraction of PRE-T and POE-T seed samples resulted in release of unextracted residues in several fractions. The largest percentages of unextracted residues were released in the hemicellulose and protein fractions. Partitioning of the hemicellulose fraction with ether or ethyl acetate resulted in transfer of a significant portion of the residues into the organic phase. Evaporation of the organic phase and redissolution resulted in very low recoveries of radioactivity indicating that the radioactivity was not soluble and therefore not due to dicamba metabolites. HPLC/LSC analysis of the organic partition of the PRE-T seed hemicellulose fraction (Figure 24) or the POE-T seed hemicellulose fraction without partitioning (Figure 25) indicated that the residues in the hemicellulose fraction were polar in nature. No peaks that could be ascribed to discrete dicamba metabolites were observed. Partitioning of the protein fraction with ethyl acetate resulted in virtually none of the residues transferred to the organic phase. HPLC/LSC analysis of the unextracted residues protein fraction (Figure 26) indicated that the radioactive residues released by protease treatment were quite polar

in nature. No significant peaks corresponding to identified metabolites of dicamba were observed in the profile. This is consistent with release of radioactive amino acids and/or peptide fragments from proteins during the protease digestion. Radioactivity in plant proteins would result from metabolism of dicamba to small organic molecules (e.g., $^{14}\text{CO}_2$) and incorporation of the label into plant proteins through normal metabolic processes.

3.6.4 Proposed Metabolic Pathways

Besides non-metabolized parent dicamba, which was observed primarily in foliage of plants from the postemergence treatment, five metabolites of dicamba were identified in dicamba-tolerant soybean. These were DCSA glucoside **9**, DCSA HMGglucoside **11**, DCSA **22**, DCGA glucoside **3** and DCGA malonylglucoside **8**. Names and structures of the identified metabolites are presented in Table 1. Two other minor metabolite peaks were characterized as mixtures of conjugates of DCSA and DCGA but were not conclusively identified: unknown DCSA/DCGA conjugates **14** and unknown DCSA/DCGA glucose conjugates **18**. Other significant characterized radioactive soybean residue components were the mixture of sugars **1** and triglycerides. These were derived from extensive metabolism of dicamba to $^{14}\text{CO}_2$ in the soil, and possibly to some extent in the soybean plants, followed by subsequent reincorporation of the $^{14}\text{CO}_2$ into plant natural products. The sugars were observed primarily in the acetonitrile:water extracts of soybean seed while the triglycerides were present in the hexane extracts (oil fraction) of soybean seed.

The proposed pathways for the metabolism of dicamba in dicamba-tolerant soybean are shown in Figure 112. The metabolism of dicamba in dicamba-tolerant soybean proceeds by initial demethylation of dicamba to form 3,6-dichlorosalicylic acid (DCSA, **22**) through the action of the dicamba *O*-demethylase enzyme resulting from introduction of the dicamba mono-oxygenase (DMO) gene into dicamba-tolerant soybean. Only small amounts of free DCSA are observed in soybean matrices; rather, the DCSA exists largely as its conjugate DCSA glucoside **9** some of which is further modified by esterification with 3-hydroxy-3-methylglutaric acid (HMGA) to form DCSA HMGglucoside **11**. As a minor pathway, DCSA is hydroxylated at the 5-position, presumably by a P-450 enzyme or other oxygenase, to form 2,5-dichloro-3,6-dihydroxybenzoic acid (DCGA). DCGA is not observed as a free metabolite in soybean matrices but is converted to the glucose conjugate DCGA glucoside **3** in which the glucose moiety is attached to the 5-hydroxyl of DCGA. The glucose conjugate **3** is further converted by malonylation of the glucose moiety to DCGA malonylglucoside **8**.

The primary metabolic reactions involved in metabolism of dicamba in dicamba-tolerant soybean (i.e., *O*-demethylation and ring-hydroxylation) are common metabolic processes

in plants, soil and animals. Glycosylation of xenobiotics, as well as phenolic natural products such as flavonols, followed by esterification of the glucose moiety with malonic acid (malonylation) is a common metabolic pathway in plants.⁸ To our knowledge, though, there have been no previous reports of xenobiotic glucose (or other hexose) conjugates modified by esterification with HMGA such as is observed for the metabolite DCSA HMGglucoside **11**. However, incorporation of HMGA into plant natural products is relatively well-known. A number of glycosides of plant natural products, including flavonols, betacyanins and steroidal saponins, acylated with HMGA have been identified. For example, a 3-hydroxy-3-methylglutaryl (HMG) glucoside of the flavonol kaempferol has been isolated from the Chinese herb *Polygala Japonica*,⁶ and an HMG galactoside of the flavonol quercetin has been identified in blackberries.⁵ A hydroxylated metabolite of abscisic acid that is esterified with HMGA has been identified in seeds of *Robinia pseudoacacia* (black locust).⁹ Red-violet betacyanin glycoside pigments in plants of the *Amaranthus* family are also known that are acylated with HMGA.¹⁰ In addition, the oligomeric lignans in flaxseed, which are considered to have health benefits, contain HMGA as a linking agent between the lignan monomer units.¹¹ Although there have apparently been no previous reports of xenobiotic metabolites containing glycosides acylated with HMGA, HMGA conjugates are quite common in plant natural products and it is likely that other HMGA-acylated glycoside conjugates of xenobiotics will be identified in the future.

Metabolism of dicamba in dicamba-tolerant soybean is similar to the metabolism of dicamba in soil and other plant species. DCSA is observed as the major aerobic soil metabolite of dicamba, and DCGA is also observed in soil as a minor metabolite.¹² The glucose conjugate of 5-hydroxydicamba is the major metabolite in crops such as wheat and grasses, which are naturally tolerant to dicamba; *O*-demethylation to form DCSA is also observed as a minor pathway in these crops.¹²

3.7 Residue Analysis for Dicamba Residues in Dicamba-Tolerant Soybean

As determined in this study, residues of dicamba in dicamba-tolerant soybean consist primarily of glucose conjugates of DCSA and DCGA (and their HMGA or malonate derivatives) as well as non-metabolized dicamba in postemergence treated foliage. It has been demonstrated that the major metabolites of dicamba in dicamba-tolerant soybean are converted to DCSA or DCGA upon acid hydrolysis (see Figure 32, Figure 47, Figure 60, Figure 62 and Figure 75. For the residue study of dicamba in dicamba-tolerant soybean (Monsanto study REG-08-096),¹³ a residue analytical method (AG-ME-1321-01)¹⁴ incorporating an acid hydrolysis step has been developed in which dicamba soybean residues are quantitated as the separate analytes DCSA, DCGA, 5-hydroxydicamba and dicamba by LC/MS/MS. Radiovalidation¹⁴ of the method was also conducted and

demonstrated very good conversions of radiolabeled dicamba-derived soybean residues to the DCSA, DCGA and dicamba analytes with no other significant radioactive products observed following hydrolysis.

The current enforcement analytical method for dicamba residues in crops¹⁵ consists of an acid hydrolysis/extraction step followed by methylation of the residues and GC/ECD analysis of the methylated analytes. In the method, dicamba and DCSA are quantitated together as methyl 3,6-dichloro-2-methoxybenzoate and 5-hydroxydicamba is quantitated as methyl 2,5-dichloro-3,6-dimethoxybenzoate. DCGA, or its conjugates, if present, would be converted to the latter analyte and analyzed together with 5-hydroxydicamba. Thus, the current enforcement method for soybean is adequate for analysis of dicamba residues in dicamba-tolerant soybean since virtually all residue components identified in this study would be converted to the two analytes of the method.

3.8 Storage Stability

The processed (ground) soybean pre-forage, forage, hay and seed samples received from PTRL West, Inc., were stored frozen at approx. -20 °C for approx. two years during the analysis phase of the study at Monsanto Company. The stability of dicamba soybean residues in frozen storage may be assessed by comparing the radioactivity analyses (TRR determinations), extraction efficiencies and HPLC profiles determined during the various phases of the study. As an initial stability baseline, extractions and HPLC profiles were conducted within 30 days of harvest for forage and seed by PTRL West, Inc. and for hay at Monsanto Company. On completion of the analytical phase of the study, four matrices (PRE-T forage, PRE-T and POE-T hay, and POE-T seed) were re-analyzed for assessment of stability over the two-year storage period. A comparison of the radioactivity analyses and extraction efficiencies determined at the beginning of the study with those obtained upon completion of the analytical phase of the study is presented in Table 14 along with associated dates of analyses. Comparisons of the initial and final HPLC metabolite profiles for PRE-T forage, PRE-T and POE-T hay, and POE-T seed are shown in Figure 108 through Figure 111. Additional quantitative storage stability analyses intended to support the residue study of dicamba in dicamba-tolerant soybean (Monsanto study REG-08-096)¹³ are described in Appendix C.

The combustion (TRR) values and extraction efficiencies for forage, hay and seed obtained after more than two years of frozen storage compare very well with the values obtained at the beginning of the study indicating no loss of residues or desiccation of the matrices, or gross changes in the properties of the residues. Note that the TRR value obtained initially at Monsanto for PRE-T forage is somewhat lower than the original value obtained by PTRL. The Monsanto number is somewhat low due to inefficient

combustion of this very wet matrix. This is evidenced by the high extraction accountability (115.86%, Table 5) observed for the PRE-T forage matrix.

The HPLC profiles for PRE-T forage, PRE-T hay, POE-T hay and POE-T seed obtained after two years of frozen storage are very similar to those obtained initially. The overall pattern of metabolism and the relative proportions of the major and minor metabolites are very similar comparing the initial profiles to the final profiles. However, there are two exceptions that are related to the chromatography and not due to instability. First, due to degradation of the HPLC column performance at the end of the study, the DCSA peak (Peak 22) in the final profile of PRE-T forage (Figure 108, bottom) was broad (27-31 min) and overlapped with the dicamba peak (Peak 23). No additional peaks or significant loss of any peak was observed, however. Secondly, in the original POE-T seed profile obtained at PTRL (Figure 111, top) Peak 8 (DCGA malonylglucoside) was not resolved from Peak 9 (DCSA glucoside). However, the peak was resolved in the initial Monsanto profile (Figure 111, middle) obtained approx. six months after harvest. Resolution of Peaks 8 and 9 in the POE-T seed proved difficult when conducting relatively large injections of extracts of seed that had not been pre-extracted with hexane due to large amounts of matrix material that degraded the chromatography. The Monsanto initial POE-T seed profile was obtained by using SPE to clean up the extract prior to injection while the final profile was the result of a smaller injection necessitating collection of fractions. Peak 8 is present and partially resolved in the PRE-T seed profile obtained initially at PTRL (Figure 12, pg 63, of the in-life report in Appendix B) indicating that this metabolite was present originally in the PRE-T seed (and certainly in the POE-T seed, as well, considering the similarity of the seed metabolite profiles) and providing additional evidence that the absence of the peak in the original PTRL POE-T seed profile is due to chromatographic difficulties.

Overall, the storage stability results indicate very good stability of dicamba-derived residues in soybean forage, hay and seed during two years of frozen storage. Additional storage stability analyses including quantitation of the metabolite profiles and an additional (extended) time point intended to support the residue study of dicamba in dicamba-tolerant soybean (Monsanto study REG-08-096)¹³ are described in Appendix C.

The stability of dicamba residues in extracts of soybean forage, hay and seed that were stored frozen was not assessed because extracts were not stored for long periods of time. Typically, extracts were analyzed by HPLC on the day of extraction or the following day, but occasionally were stored frozen for a maximum of one week prior to HPLC analysis or isolation of metabolites.

The stability of reference standards in solution was assessed informally by examination of periodic HPLC injections of solutions of the four primary reference standards

[dicamba (**I**), DCSA (**II**), 5-hydroxydicamba (**III**) and DCGA (**IV**)]. The reference standards were qualitatively stable. However, both DCSA and DCGA formed small amounts of later-eluting unidentified impurities of a dimeric nature in solution on storage. These impurities did not negatively affect the qualitative use of the standards in the study.

4 Conclusions

[¹⁴C]Dicamba was applied preemergence (PRE-T) to the soil on the day of planting or postemergence (POE-T) to foliage at the R1 (first flower) growth stage of dicamba-tolerant soybean in pots at a target rate of 2.5 lb ae/acre (2.80 kg/ha) compared to a maximum intended seasonal use rate in the US of 2.0 lb ae/acre (2.24 kg/ha). The actual achieved application rates were 2.55 and 2.52 lb ae/acre (2.86 and 2.82 kg/ha), respectively. Total radioactive residues (TRRs) for PRE-T immature foliage (pre-forage), forage, hay and seed (collected at 14, 36, 56 and 112 DAT, respectively) were 3.248, 1.433, 1.056 and 0.291 mg/kg (dicamba equivalents), respectively. For POE-T forage, hay and seed (collected at 7, 27 and 83 DAT, respectively) TRRs were 134.147, 39.149 and 0.389 mg/kg, respectively. The efficiency of extraction of the radioactive residues was high (90.88-95.30%) for pre-forage, forage and hay (utilizing 40:60 acetonitrile:water extractions). Extraction efficiencies for the PRE-T seed and POE-T seed (using hexane, acetonitrile and 40:60 acetonitrile:water extractions) were 59.35% and 63.67%, respectively, with 10.35% and 8.41% of the TRR in the oil (hexane) fraction, respectively. Residue levels in PRE-C and POE-C forage and seed collected from untreated control plants interspersed amongst the PRE-T or POE-T plants ranged from 0.080 to 0.280 mg/kg indicating uptake and/or deposition of dicamba or volatile soil metabolites, e.g., ¹⁴CO₂, from the air. Extraction efficiencies were generally lower for these matrices reflecting a higher degree of incorporation of radioactivity (presumably ¹⁴CO₂) into natural products.

Release of unextracted residues in soybean seed was investigated through extractions with acid and base, or through a series of chemical and enzymatic digestions. Dilute acid or base extraction released very little of the unextracted residues. The chemical and enzymatic digestions released considerable amounts of the unextracted residues resulting in 7.09% of TRR (0.021 mg/kg) and 7.60% of TRR (0.030 mg/kg) remaining unextracted in the PRE-T and POE-T seed, respectively. The hemicellulose (12.90-13.92% of TRR) and protein (8.18-10.06% of TRR) fractions contained the highest amounts of released residues. Organic solvent partitioning or HPLC analysis of the released residues in the protein fraction indicated that the residues were polar water-soluble materials. The released residues in the hemicellulose fraction partially partitioned into organic solvents but proved to be of an insoluble nature and therefore not related to dicamba. These results indicated that the unextracted residues in seed were likely derived by reincorporation of small molecules, e.g., ¹⁴CO₂, into natural plant constituents.

Extracts of PRE-T pre-forage and PRE-T/POE-T forage, hay and seed were concentrated and analyzed by HPLC to quantitate metabolites. All metabolites constituting 2% or more of the TRR in any matrix were isolated and purified by preparative HPLC, and

were identified by a combination of spectral techniques, primarily mass spectrometry, as well as chemical degradations and derivatizations, and comparisons to available or synthesized reference standards. In all, five discrete metabolites of dicamba and non-metabolized parent dicamba were identified. Two additional metabolites of dicamba in the range of 1-2% of TRR were isolated and characterized. Unchanged dicamba was a significant component of the residue only in the POE-T forage and hay (24.21% of TRR and 12.33% of TRR, respectively). The results of washing experiments on two POE-T forage plants indicated that the dicamba residues in forage (and likely hay as well) were virtually entirely surface residues. For pre-forage, forage and hay, the identified and characterized metabolites constituted 87.29-92.17% of the TRR in these matrices. For seed, the identified and characterized radioactivity comprised 50.21-53.21% of the TRR.

Metabolism of dicamba in dicamba-tolerant soybean proceeds by initial demethylation to form DCSA **22** by the action of the dicamba *O*-demethylase enzyme which is the product of the dicamba mono-oxygenase gene introduced to confer dicamba tolerance. Little free DCSA is observed in soybean matrices; rather, the DCSA is converted to its 2-*O*- β -glucoside (DCSA glucoside **9**) some of which is further acylated with 3-hydroxy-3-methylglutaric acid (HMGA) on the 6-hydroxyl of the glucose moiety to form DCSA HMGglucoside **11**. As a minor pathway, DCSA **22** is converted by 5-hydroxylation to DCGA which is not observed in its free form but is present in soybean matrices as the 5-*O*- β -glucoside (DCGA glucoside **3**) and the corresponding malonylglucoside (DCGA malonylglucoside **8**).

DCSA glucoside **9** was the major metabolite in dicamba-tolerant soybean foliage (pre-forage, forage and hay) constituting 60.32-74.48% of TRR. DCSA HMGglucoside **11** constituted 5.21-7.62% of TRR in PRE-T pre-forage, forage and hay, and 1.14 and 2.48% of TRR in the POE-T forage and hay, respectively. DCGA glucoside **3** constituted 0.75-4.32% of TRR in pre-forage, forage and hay, with larger amounts present in the hay compared to the forage. The DCGA malonylglucoside **8** represented 5.46% of TRR in the PRE-T pre-forage, but only 0.73-1.61% of TRR in forage and hay. DCSA **22** represented only 1.54-1.93% of TRR in hay, but somewhat larger amounts were observed in forage (3.19-4.08% of TRR).

While the DCSA glucoside **9** dominated the metabolite profiles of soybean foliage matrices, there were four prominent metabolite peaks observed in seed profiles. DCSA glucoside **9** constituted 11.55 and 15.27% of TRR (0.034 and 0.059 mg/kg) in the PRE-T and POE-T seed, respectively. DCSA HMGglucoside **11** represented 8.73 and 9.61% of TRR (0.025 and 0.037 mg/kg), and DCGA malonylglucoside **8** constituted 4.73 and 4.64% of TRR (0.014 and 0.018 mg/kg) in the PRE-T and POE-T seed, respectively. The fourth prominent peak was a polar material characterized as sugars **1** representing 8.42 and 9.15% of TRR (0.025 and 0.036 mg/kg) in PRE-T and POE-T seed,

respectively. DCGA glucoside **3** was also present in the seed constituting 1.60 and 2.07% of TRR (0.005 and 0.008 mg/kg) in the PRE-T and POE-T seed, respectively. DCSA **22** and dicamba **23** were very minor components of the seed residues (<1% of TRR each in PRE-T and POE-T seed). Compared to forage and hay, extractable seed residues, while lower in magnitude, contained relatively higher proportions of the DCSA HMGglucoside **11** and the two DCGA conjugates **3** and **8**. One other significant radioactive component of the seed residues, present in the hexane extracts (oil fraction), were triglycerides. These were characterized by acid-catalyzed transesterification of the oil triglycerides with methanol and HPLC analysis of the resulting fatty acid methyl ester mixture. The sugar and triglyceride residues in seed are presumed to result by uptake of $^{14}\text{CO}_2$ from metabolism of dicamba in the soil, and possibly by metabolism of dicamba to $^{14}\text{CO}_2$ and/or other small molecules in the plant, and incorporation into plant natural products through normal metabolic processes.

O-Demethylation and ring-hydroxylation, the processes by which DCSA and DCGA are formed in dicamba-tolerant soybean, are common metabolic processes in plants, soil and animals. Glycosylation and subsequent malonylation are common routes of metabolism of xenobiotics as well as natural products in plants. Although acylation of xenobiotics with HMGA has not, to our knowledge, been reported previously, there are many known plant natural product glycosides conjugated with HMGA.^{5-6,9-11} Metabolism of dicamba in dicamba-tolerant soybean is also similar to the metabolism of dicamba in soil and other plant species. DCSA is observed as the major aerobic soil metabolite of dicamba; DCGA is also observed in soil as a minor metabolite. The glucose conjugate of 5-hydroxydicamba is the major metabolite in crops such as wheat and grasses, which are naturally tolerant to dicamba; *O*-demethylation to form DCSA is also observed as a minor pathway in these crop plants.¹²

The dicamba metabolites identified in this study are converted by acid hydrolysis to the chemophores DCSA and DCGA. Thus, current residue enforcement methodology, which incorporates an acid hydrolysis step followed by methylation and analysis by GC, would be expected to be adequate for analysis of dicamba residues in dicamba-tolerant soybean.

Assessment of the stability of dicamba residues in soybean matrices by reanalysis of samples at the end of the analysis phase of the study and comparison to the original analyses indicated that residues of dicamba in dicamba-tolerant soybean are stable for a period of two years in frozen storage.

5 Tables

Table 1. Names and Structures of Identified or Characterized Dicamba Metabolites and Radioactive Residue Components in Dicamba-Tolerant Soybean

Report Number	Trivial Name and Chemical Name	Structure
1	Sugars (e.g., sucrose, raffinose, stachyose)	Various
3	DCGA Glucoside 2,5-dichloro-3-(β -D-glucopyranosyloxy)-6-hydroxybenzoic acid	
8	DCGA Malonylglucoside 3-[[6-O-(2-carboxyacetyl)- β -D-glucopyranosyloxy]-2,5-dichloro-6-hydroxybenzoic acid	
9	DCSA Glucoside 3,6-dichloro-2-(β -D-glucopyranosyloxy)benzoic acid	
11	DCSA HMGglucoside 2-[[6-O-(4-carboxy-3-hydroxy-3-methylbutyryl)- β -D-glucopyranosyl]oxy]-3,6-dichlorobenzoic acid	
14	Unknown DCSA/DCGA Conjugates	

Table 1 (cont). Names and Structures of Identified or Characterized Dicamba Metabolites and Radioactive Residue Components in Dicamba-Tolerant Soybean

Report Number	Trivial Name and Chemical Name	Structure
18	Unknown DCSA/DCGA Glucose Conjugates	
22	DCSA 3,6-dichloro-2-hydroxy benzoic acid	
23	Dicamba 3,6-dichloro-2-methoxybenzoic acid	
NA	Triglycerides (Triacylglycerols)	<p> $\left(\text{R} - \text{C}(=\text{O}) - \right) =$ palmitoyl, stearoyl, oleoyl, linoleoyl, linolenoyl, etc. </p>

Table 2. Names and Structures of Reference Standards

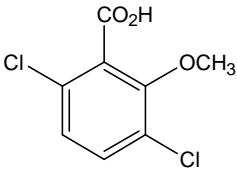
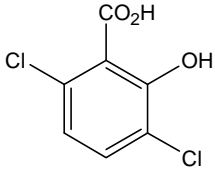
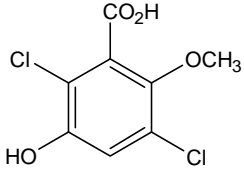
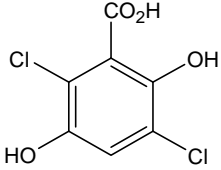
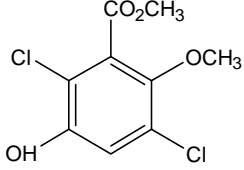
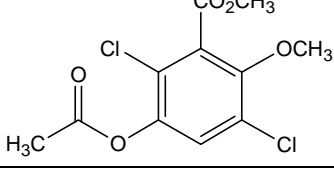
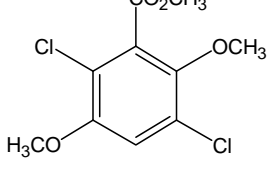
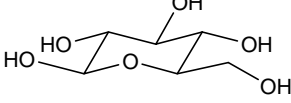
Report Number	Trivial Name and Chemical Name	Structure
I	Dicamba 3,6-dichloro-2-methoxy benzoic acid	
II	DCSA 3,6-dichloro-2-hydroxy benzoic acid	
III	5-Hydroxydicamba 2,5-dichloro-3-hydroxy-6-methoxybenzoic acid	
IV	DCGA 2,5-dichloro-3,6-dihydroxybenzoic acid	
V	5-Hydroxydicamba Methyl Ester 2,5-dichloro-3-hydroxy-6-methoxybenzoic acid, methyl ester	
VI	5-Acetyloxydicamba Methyl Ester 3-acetyloxy-2,5-dichloro-6-methoxybenzoic acid, methyl ester	
VII	Trimethyl DCGA 2,5-dichloro-3,6-dimethoxy benzoic acid, methyl ester	
VIII	¹⁴ C-D-Glucose	

Table 2 (cont). Names and Structures of Reference Standards

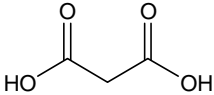
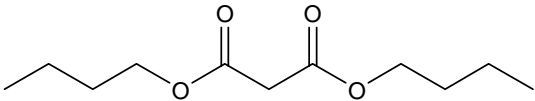
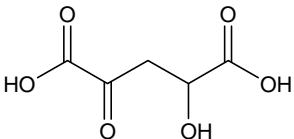
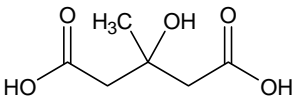
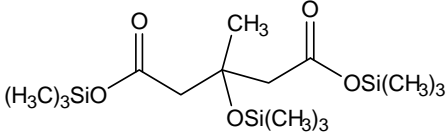
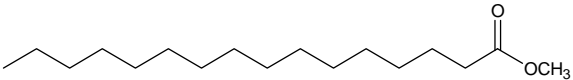
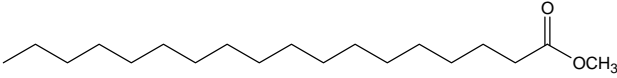
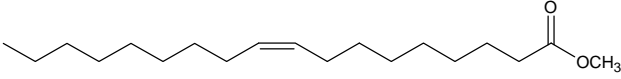
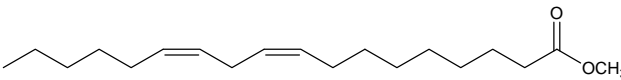
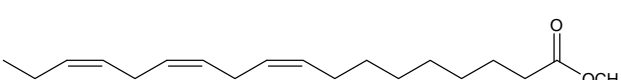
Report Number	Trivial Name and Chemical Name	Structure
IX	Malonic Acid propanedioic acid	
X	Dibutyl Malonate propanedioic acid, 1,3-dibutyl ester	
XI	HKGA γ -hydroxy- α -ketoglutaric acid	
XII	HMGA 3-hydroxy-3-methylglutaric acid 3-hydroxy-3-methyl pentanedioic acid	
XIII	Tris(trimethylsilyl)HMGA 3-methyl-3-[(trimethyl silyl)oxy]pentanedioic acid, 1,5-bis(trimethylsilyl) ester	
XIV	Palmitic Acid, Methyl Ester hexadecanoic acid, methyl ester	
XV	Stearic acid, Methyl Ester octadecanoic acid, methyl ester	
XVI	Oleic Acid, Methyl Ester 9-octadecenoic acid (9Z)-, methyl ester	
XVII	Linoleic Acid, Methyl Ester 9,12-octadecadienoic acid (9Z,12Z)-, methyl ester	
XVIII	α -Linolenic Acid, Methyl Ester 9,12,15-octadecatrienoic acid (9Z,12Z,15Z)-, methyl ester	

Table 2 (cont). Names and Structures of Reference Standards

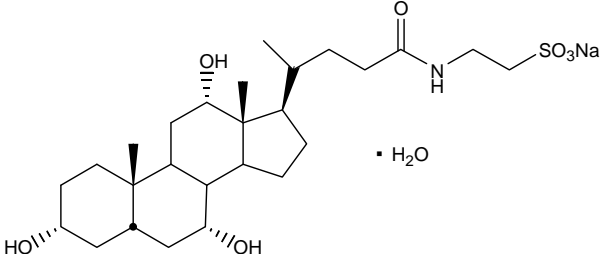
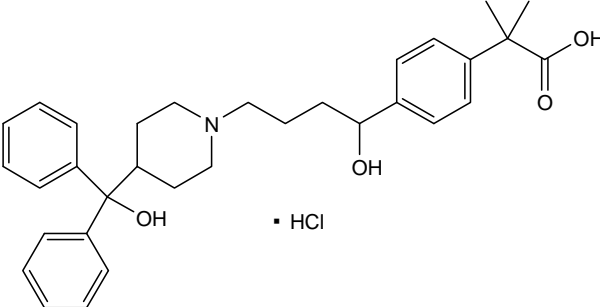
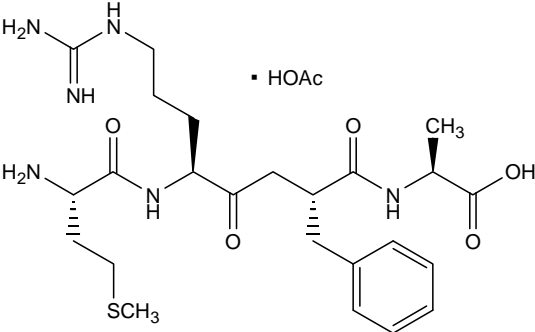
Report Number	Trivial Name and Chemical Name	Structure
XIX	<p>Taurocholic Acid, Na Salt Hydrate</p> <p>2-[[[(3α,5β,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]ethanesulfonic acid</p>	
XX	<p>Fexofenadine HCl</p> <p>4-[1-hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidiny]butyl]-α,α-dimethylbenzeneacetic acid</p>	
XXI	<p>MRFA (Met-Arg-Phe-Ala) Acetate Salt</p> <p>L-methionyl-L-arginyl-L-phenylalanyl-L-alanine</p>	

Table 3. Representative Reverse Phase HPLC Retention Times of Dicamba Reference Standards Using HPLC Method B

Number	Trivial Name	Retention Time (min)
I	Dicamba	28.03
II	DCSA	26.15
III	5-Hydroxydicamba	20.88
IV	DCGA	16.64

Table 4. Summary of Radioactive Residues in Soybean Pre-forage, Forage, Hay and Seed

Matrix	TRR (mg/kg, ppm)	
	PTRL*	Monsanto
UNT-C Pre-forage	0.000	NA
UNT-C Forage	0.000	NA
UNT-C Hay	0.001	NA
UNT-C Seed	0.013	NA
PRE-C Forage	0.080	0.071
PRE-C Seed	0.170	0.180
POE-C Forage	0.280	0.269
POE-C Seed	0.138	0.139
PRE-T Pre-forage	3.248	3.255
PRE-T Forage	1.433	1.237
PRE-T Hay	1.056	1.098
PRE-T Seed	0.291	0.288
POE-T Forage	134.147	125.512
POE-T Hay	39.149	39.030
POE-T Seed	0.389	0.410
Washed Forage Plant #28	81.786	82.600

* PTRL values were utilized for mg/kg (ppm) calculations

Table 5. Extraction Summary (Percent) for Soybean Pre-forage, Forage, Hay and Seed

Matrix	Sample Weight (g)	Sample dpm/g	Sample dpm	Extracted dpm	Pellet dpm	Percent Extracted	Percent Unextracted	Accountability	Normalized Percent Extracted	Normalized Percent Unextracted
PRE-T Pre-forage	1.0449	176375	184294	170962	16718	92.77	9.07	101.84	91.09	8.91
PRE-T Forage	1.5933	67015	106774	112828	10876	105.67	10.19	115.86	91.21	8.79
PRE-T Hay	5.0548	59502	300770	276587	27767	91.96	9.23	101.19	90.88	9.12
PRE-T Seed	4.1926	15621	65493	41088	28137	62.74	42.96	105.70	59.35	40.65
Hexane						10.94			10.35	
ACN, ACN/H ₂ O*						51.79			49.00	
POE-T Forage	5.0722	6847923	34734035	31864393	2111397	91.74	6.08	97.82	93.79	6.21
POE-T Hay	4.9834	2129499	10612146	9860947	486570	92.92	4.59	97.51	95.30	4.70
POE-T Seed	3.9993	22360	89424	56131	32022	62.77	35.81	98.58	63.67	36.33
Hexane						8.29			8.41	
ACN, ACN/H ₂ O*						54.48			55.27	
Washed Forage Plant #28	4.1130	4504172	18525661	16527336	NA**	89.21	10.79	NA**	89.21	10.79
PRE-C Forage	8.0906	3856	31199	30199	9723	96.79	31.16	127.96	75.64	24.36
POE-C Forage	4.1367	14691	60772	60123	4178	98.93	6.87	105.81	93.50	6.50
PRE-C Seed	4.1892	9749	40842	18064	23094	44.23	56.54	100.77	43.89	56.11
Hexane						17.98			17.84	
ACN, ACN/H ₂ O*						26.25			26.05	
POE-C Seed	3.9943	7576	30262	14112	17842	46.63	58.96	105.59	44.16	55.84
Hexane						12.35			11.69	
ACN, ACN/H ₂ O*						34.28			32.47	

* The ACN and ACN/H₂O extracts were combined, concentrated and analyzed by HPLC.

** The pellet was not combusted.

Table 6. Extraction Summary (mg/kg, ppm) for Soybean Pre-forage, Forage, Hay and Seed

Matrix	Matrix TRR mg/kg (ppm)*	Extracted mg/kg (ppm)	Unextracted mg/kg (ppm)
PRE-T Pre-forage	3.248	2.959	0.289
PRE-T Forage	1.433	1.307	0.126
PRE-T Hay	1.056	0.960	0.096
PRE-T Seed	0.291	0.173	0.118
POE-T Forage	134.147	125.811	8.336
POE-T Hay	39.149	37.308	1.841
POE-T Seed	0.389	0.248	0.141
Washed POE-T Forage Plant #28	81.786	72.964	8.822
PRE-C Forage	0.080	0.061	0.019
POE-C Forage	0.280	0.262	0.018
PRE-C Seed	0.170	0.075	0.095
POE-C Seed	0.138	0.061	0.077

* TRR values are from the PTRL West, Inc. in-life report (Appendix B).

Table 7. Summary of Analytical Recoveries for Quantitative HPLC Analyses of Soybean Pre-forage, Forage, Hay and Seed

Matrix	Evaporation and Centrifugation Recovery (Percent)	HPLC Column Recovery (Percent)
PRE-T Pre-forage	97.11	101.27
PRE-T Forage	98.82	100.50
PRE-T Hay	100.32	98.39
PRE-T Seed	92.04	90.72
POE-T Forage	100.09	99.80
POE-T Hay	99.61	98.34
POE-T Seed	91.21	94.64
Washed POE-T Forage Plant #28	99.75	96.38
PRE-C Forage	98.69	110.10
POE-C Forage	104.20	100.07
PRE-C Seed	88.04	88.94
POE-C Seed	92.49	91.60

Table 8. Metabolite Quantitation Summary – Identified or Characterized Metabolites in Soybean Pre-forage, Forage and Hay from Preemergence and Postemergence Treatments

Peak Number	Identification	Soybean Forage Matrix					
		PRE-T Pre-forage		PRE-T		POE-T	
		Percent of TRR	Metab. mg/kg (ppm)*	Percent of TRR	Metab. mg/kg (ppm)*	Percent of TRR	Metab. mg/kg (ppm)*
1	Sugars	1.47	0.048	0.96	0.014	NQ	NQ
3	DCGA Glucoside	2.77	0.090	1.14	0.016	0.75	1.007
8	DCGA Malonylglucoside	5.46	0.177	1.40	0.020	1.11	1.485
9	DCSA Glucoside	68.96	2.240	74.48	1.067	60.32	80.913
11	DCSA HMGglucoside	7.62	0.247	5.21	0.075	1.14	1.535
14	Unk DCSA/DCGA Conj.	0.29	0.009	1.26	0.018	0.38	0.503
18	Unk DCSA/DCGA Gluc.	NQ	NQ	0.55	0.008	0.12	0.164
22	DCSA	1.46	0.047	3.19	0.046	4.08	5.473
23	Dicamba	0.80	0.026	1.61	0.023	24.21	32.473
Totals		88.82	2.885	89.81	1.287	92.10	123.552

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

Peak Number	Identification	Soybean Hay Matrix			
		PRE-T		POE-T	
		Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
1	Sugars	1.08	0.011	0.49	0.190
3	DCGA Glucoside	3.45	0.036	4.32	1.690
8	DCGA Malonylglucoside	0.73	0.008	1.61	0.631
9	DCSA Glucoside	70.81	0.748	67.26	26.333
11	DCSA HMGglucoside	6.67	0.070	2.48	0.970
14	Unk DCSA/DCGA Conj.	1.64	0.017	1.75	0.686
18	Unk DCSA/DCGA Gluc.	0.51	0.005	NQ	NQ
22	DCSA	1.54	0.016	1.93	0.757
23	Dicamba	0.85	0.009	12.33	4.828
Totals		87.29	0.922	92.17	36.085

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

Table 9. Metabolite Quantitation Summary – Identified or Characterized Metabolites in Soybean Seed from Preemergence and Postemergence Treatments

Peak Number	Identification	Soybean Seed Matrix			
		PRE-T		POE-T	
		Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
NA	Triglycerides	13.87	0.040	10.76	0.042
1	Sugars	8.42	0.025	9.15	0.036
3	DCGA Glucoside	1.60	0.005	2.07	0.008
8	DCGA Malonylglucoside	4.73	0.014	4.64	0.018
9	DCSA Glucoside	11.55	0.034	15.27	0.059
11	DCSA HMGglucoside	8.73	0.025	9.61	0.037
14	Unk DCSA/DCGA Conj.	0.75	0.002	0.62	0.002
18	Unk DCSA/DCGA Gluc.	NQ	NQ	NQ	NQ
22	DCSA	0.37	0.001	0.46	0.002
23	Dicamba	0.20	0.001	0.64	0.003
Totals		50.21	0.146	53.21	0.207

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

Table 10. Metabolite Quantitation Summary – Identified or Characterized Metabolites in Control (PRE-C and POE-C) Soybean Forage and Seed

		Soybean Forage Matrix			
		PRE-C		POE-C	
Peak Number	Identification	Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
1	Sugars	5.57	0.004	1.76	0.005
3	DCGA Glucoside	1.82	0.001	1.30	0.004
8	DCGA Malonylglucoside	0.70	0.001	0.71	0.002
9	DCSA Glucoside	40.66	0.033	55.49	0.155
11	DCSA HMGglucoside	0.69	0.001	1.46	0.004
14	Unk DCSA/DCGA Conj.	NQ	NQ	0.84	0.002
22	DCSA	3.80	0.003	4.76	0.013
23	Dicamba	19.27	0.015	26.18	0.073
Totals		72.52	0.058	92.50	0.259

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

		Soybean Seed Matrix			
		PRE-C		POE-C	
Peak Number	Identification	Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
NA	Triglycerides	16.89	0.029	11.07	0.015
1	Sugars	12.61	0.021	12.50	0.017
3	DCGA Glucoside	0.11	0.0002	0.28	0.0004
8	DCGA Malonylglucoside	0.29	0.0005	1.32	0.002
9	DCSA Glucoside	2.18	0.004	5.75	0.008
11	DCSA HMGglucoside	1.33	0.002	4.12	0.006
14	Unk DCSA/DCGA Conj.	0.37	0.001	0.56	0.001
18	Unk DCSA/DCSA Gluc.	NQ	NQ	0.10	0.0001
22	DCSA	0.24	0.0004	0.25	0.0003
23	Dicamba	0.37	0.001	0.23	0.0003
Totals		34.38	0.058	36.18	0.050

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

Table 11. Metabolite Quantitation Summary – Unidentified Metabolites in Soybean Pre-forage, Forage and Hay from Preemergence and Postemergence Treatments

Peak Number	Soybean Forage Matrix					
	PRE-T Pre-forage		PRE-T		POE-T	
	Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
2	0.26	0.008	0.40	0.006	NQ	NQ
5	0.56	0.018	NQ	NQ	0.36	0.478
6	0.64	0.021	0.58	0.008	NQ	NQ
7	0.20	0.007	NQ	NQ	NQ	NQ
10	NQ	NQ	NQ	NQ	0.14	0.189
12	NQ	NQ	NQ	NQ	0.18	0.239
13	NQ	NQ	NQ	NQ	0.18	0.239
16	0.62	0.020	0.41	0.006	0.40	0.541
17	NQ	NQ	NQ	NQ	0.26	0.352
19	NQ	NQ	NQ	NQ	0.18	0.239
Totals	2.28	0.074	1.40	0.020	1.70	2.277

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

Peak Number	Soybean Hay Matrix			
	PRE-T		POE-T	
	Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
2	NQ	NQ	0.18	0.071
6	0.53	0.006	0.71	0.280
7	0.50	0.005	NQ	NQ
12	0.74	0.008	NQ	NQ
15	0.75	0.008	NQ	NQ
16	1.07	0.011	0.95	0.373
17	NQ	NQ	0.91	0.354
19	NQ	NQ	0.38	0.149
Totals	3.59	0.038	3.14	1.227

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

Table 12. Metabolite Quantitation Summary – Unidentified Metabolites in Soybean Seed from Preemergence and Postemergence Treatments

Peak Number	Soybean Seed Matrix			
	PRE-T		POE-T	
	Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
NA**	0.78	0.002	0.60	0.002
2	1.22	0.004	NQ	NQ
5	NQ	NQ	0.31	0.001
6	0.33	0.001	0.62	0.002
7	0.72	0.002	0.57	0.002
10	1.26	0.004	0.84	0.003
12	0.38	0.001	0.61	0.002
13	NQ	NQ	0.48	0.002
16	0.18	0.001	0.36	0.001
17	NQ	NQ	0.33	0.001
24	NQ	NQ	0.52	0.002
25	NQ	NQ	0.11	0.0004
Totals	4.85	0.014	5.37	0.021

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

** Unidentified Oil Components in Hexane Extracts

Table 13. Metabolite Quantitation Summary – Unidentified Metabolites in Control (PRE-C and POE-C) Soybean Forage and Seed

Peak Number	Soybean Forage Matrix			
	PRE-C		POE-C	
	Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
12	NQ	NQ	0.34	0.001
13	0.68	0.001	NQ	NQ
16	2.44	0.002	0.66	0.002
Totals	3.12	0.002	1.00	0.003

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

Peak Number	Soybean Seed Matrix			
	PRE-C		POE-C	
	Percent of TRR	Metabolite mg/kg (ppm)*	Percent of TRR	Metabolite mg/kg (ppm)*
NA**	0.95	0.002	0.62	0.001
2	NQ	NQ	0.32	0.0004
4	0.10	0.0002	NQ	NQ
5	0.08	0.0001	NQ	NQ
6	0.16	0.0003	NQ	NQ
10	0.53	0.001	0.73	0.001
12	0.30	0.001	NQ	NQ
13	0.14	0.0002	0.18	0.0003
15	0.25	0.0004	NQ	NQ
16	NQ	NQ	0.21	0.0003
17	0.11	0.0002	NQ	NQ
19	0.14	0.0002	NQ	NQ
20	0.08	0.0001	NQ	NQ
21	0.11	0.0002	NQ	NQ
25	0.34	0.001	0.15	0.0002
26	0.17	0.0003	NQ	NQ
Totals	3.45	0.006	2.21	0.003

NQ - Not Quantified (not present or too small to quantify)

* Dicamba Equivalents

** Unidentified Oil Components in Hexane Extracts

Table 14. Comparison of Initial Combustion Results (top) and Extraction Results (middle) Obtained at the Beginning of the Study and the End of the Analytical Phase, and Dates of Analyses (Bottom)

Matrix	TRR (mg/kg, ppm)		
	PTRL	Monsanto Initial	Monsanto Final
PRE-T Forage	1.433	1.237	1.355
PRE-T Hay	1.056	1.098	1.087
POE-T Hay	39.149	39.030	39.342
POE-T Seed	0.389	0.388	0.383

Matrix	Normalized Extracted (Percent)		
	PTRL	Monsanto Initial	Monsanto Final
PRE-T Forage	93.6	91.21	90.02
PRE-T Hay	NA	90.88	90.64
POE-T Hay	NA	95.30	95.68
POE-T Seed	57.5	57.00	55.52

Matrix	Collection Date	Processing Date	Extraction and Analysis Date		
			PTRL	Monsanto Initial	Monsanto Final
PRE-T Forage	7/7/06	7/13/06	7/27-28/06	12/8-12/06	11/11-14/08
PRE-T Hay	7/27/06	8/7/06	NA	8/18-23/06	11/11-13/08
POE-T Hay	7/27/06	8/7/06	NA	8/18-24/06	11/11-13/08
POE-T Seed	9/21/06	9/27/06	10/5,19/06	3/14-15/07	11/11-18/08

Table 15. Summary of Fatty Acid Analysis of Hexane Extracts of Soybean Seed

Hexane Partitioning of Methanolysis Solution from POE-T Seed Hexane Extract:

Partition	Percent in Partition	Normalized Percent in Partition	Fatty Acids in Oil (Percent)	Glycerol in Oil (Percent)	Total TAGs* in Oil (Percent)	Total Unidentified in Oil (Percent)
Hexane	94.36	89.64	89.64	0	94.68	5.32
Acidic Methanol	10.91	10.36	0	5.05		

* 'TAGs' = triacylglycerols (triglycerides). 'Glycerol Percent' is the percent of radioactivity as glycerol derived from hydrolysis of triglycerides (see Calculations, Section 2.10)

Matrix	Matrix TRR	Percent of TRR in Hexane Extract	Percent of Oil as TAGs*	TAG Percent of TRR	Oil Unident Percent of TRR	TAG mg/kg (ppm)**	Oil Unident mg/kg (ppm)
PRE-T Seed	0.291	14.65	94.68	13.87	0.78	0.040	0.002
POE-T Seed	0.389	11.36	94.68	10.76	0.60	0.042	0.002
PRE-C Seed	0.170	17.84	94.68	16.89	0.95	0.029	0.002
POE-C Seed	0.138	11.69	94.68	11.07	0.62	0.015	0.001

* 'TAGs' = triacylglycerols (triglycerides). 'Percent of oil as TAGs' determined to be 94.68% for POE-T seed (see table above) – all others assumed to be the same.

** Dicamba equivalents

Table 16. Summary of Bicarbonate Partitioning of Hexane Extract of Untreated (Control) Soybean Seed

Partition	Percent of Hexane-Extracted Residues	
	PRE-C Seed	POE-C Seed
Hexane	99.50	103.76
Bicarbonate	0	3.61

Table 17. Summary of Spikes of Seed Extracts with Isolated POE-T Forage Metabolites for Confirmation of Soybean Seed Metabolite Identification

HPLC Method	Metabolite	Retention Time (min) Unspiked	Retention Time (min) Spiked	Treatment
C	Peak 8	24.15	24.15	PRE-T
	Peak 9	27.45	27.15	
	Peak 11	31.35	31.35	
C	Peak 22	38.85	38.55	POE-T
	Peak 23	38.55	38.55	
B	Peak 22	27.15	27.75-29.85	POE-T
	Peak 23	28.65	28.65	

Table 18. Summary of Dilute Acid and Base Extractions of Soybean Seed Unextracted Residues

PRE-T Seed

Extract	Normalized Percent of TRR	Normalized mg/kg (ppm)*
Hexane	10.98	0.032
Acetonitrile	0.65	0.002
40:60 Acetonitrile:Water	50.47	0.147
0.1 N Aqueous HCl	1.55	0.005
0.1 N Aqueous NaOH	1.30	0.004
Total	64.96	0.189
Pellet (unextracted)	35.04	0.102

* Dicamba equivalents

POE-T Seed

Extract	Normalized Percent of TRR	Normalized mg/kg (ppm)*
Hexane	8.40	0.033
Acetonitrile	0.71	0.003
40:60 Acetonitrile:Water	56.49	0.220
0.1 N Aqueous HCl	1.39	0.005
0.1 N Aqueous NaOH	1.42	0.006
Total	68.42	0.266
Pellet (unextracted)	31.58	0.123

* Dicamba equivalents

Table 19. Summary of Enzymatic and Chemical Digestion of Soybean Seed Unextracted Residues

PRE-T Seed

Fraction	With Hexane		Without Hexane	
	Normalized Percent of TRR	Normalized mg/kg (ppm)*	Normalized Percent of TRR	Normalized mg/kg (ppm)*
Hexane	10.54	0.031	NA	NA
Acetonitrile	0.64	0.002	NA	NA
ACN/H ₂ O	49.20	0.143	51.88	0.151
Phosphate	0.25	0.001	1.96	0.006
Starch	1.29	0.004	1.09	0.003
Protein	10.06	0.029	8.34	0.024
Pectin	3.26	0.009	4.74	0.014
Lignin	NA	NA	4.81	0.014
Cellulose	3.75	0.011	2.21	0.006
Hemicellulose	13.92	0.040	20.53	0.060
Total Extracted	92.91	0.270	95.55	0.278
Pellet (unextracted)	7.09	0.021	4.45	0.013

* Dicamba equivalents

POE-T Seed

Fraction	With Hexane		Without Hexane	
	Normalized Percent of TRR	Normalized mg/kg (ppm)*	Normalized Percent of TRR	Normalized mg/kg (ppm)*
Hexane	8.75	0.034	NA	NA
Acetonitrile	0.76	0.003	NA	NA
ACN/H ₂ O	52.89	0.206	56.62	0.220
Phosphate	1.42	0.006	1.88	0.007
Starch	1.27	0.005	1.06	0.004
Protein	8.18	0.032	6.15	0.024
Pectin	2.78	0.011	3.84	0.015
Lignin	NA	NA	4.39	0.017
Cellulose	3.46	0.013	2.03	0.008
Hemicellulose	12.90	0.050	19.11	0.074
Total Extracted	92.40	0.359	95.08	0.370
Pellet (unextracted)	7.60	0.030	4.92	0.019

* Dicamba equivalents

Table 20. Summary of Organic Solvent Partitioning of Enzymatic and Chemical Digests of Unextracted Residues

Fraction	PRE-T Seed		POE-T Seed	
	Percent Partitioned	mg/kg (ppm)*	Percent Partitioned	mg/kg (ppm)*
Protein		0.029		0.032
Aqueous	90.42	0.026	83.47	0.027
Organic	0.20	0.0001	0.36	0.0001
Pectin		0.009		0.011
Aqueous	84.53	0.008	84.91	0.009
Organic	0.88	0.0001	3.62	0.0004
Cellulose		0.011		0.013
Aqueous	80.70	0.009	80.92	0.011
Organic	0	0	0	0
Hemicellulose		0.040		0.050
Aqueous	61.05	0.025	72.21	0.036
Organic	18.96	0.008	12.77	0.006

* Dicamba equivalents

Table 21. Summary of Organic Solvent Partitioning of Hemicellulose Digests of Unextracted Residues (No Hexane), Followed by Concentration of the Organic Phase and Redissolution

Fraction	PRE-T Seed			POE-T Seed		
	Percent Partitioned	%TRR	mg/kg (ppm) [†]	Percent Partitioned	%TRR	mg/kg (ppm) [†]
<u>Hemicellulose</u>		20.53	0.060		19.11	0.074
Ether	32.64	6.70	0.019	37.45	7.16	0.028
Concentrate*	7.00	0.47	0.0014	7.58	0.54	0.002
Aqueous	50.42	10.35	0.030	51.75	9.89	0.038
Solids	11.58	2.38	0.007	14.60	2.79	0.011
<u>Hemicellulose**</u>		20.53	0.060	NA		NA
Ethyl Acetate	44.85	9.21	0.027			
Concentrate*	6.84	0.63	0.0018			
Aqueous	50.61	10.39	0.030			

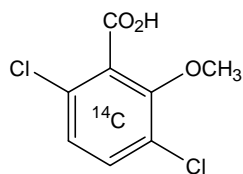
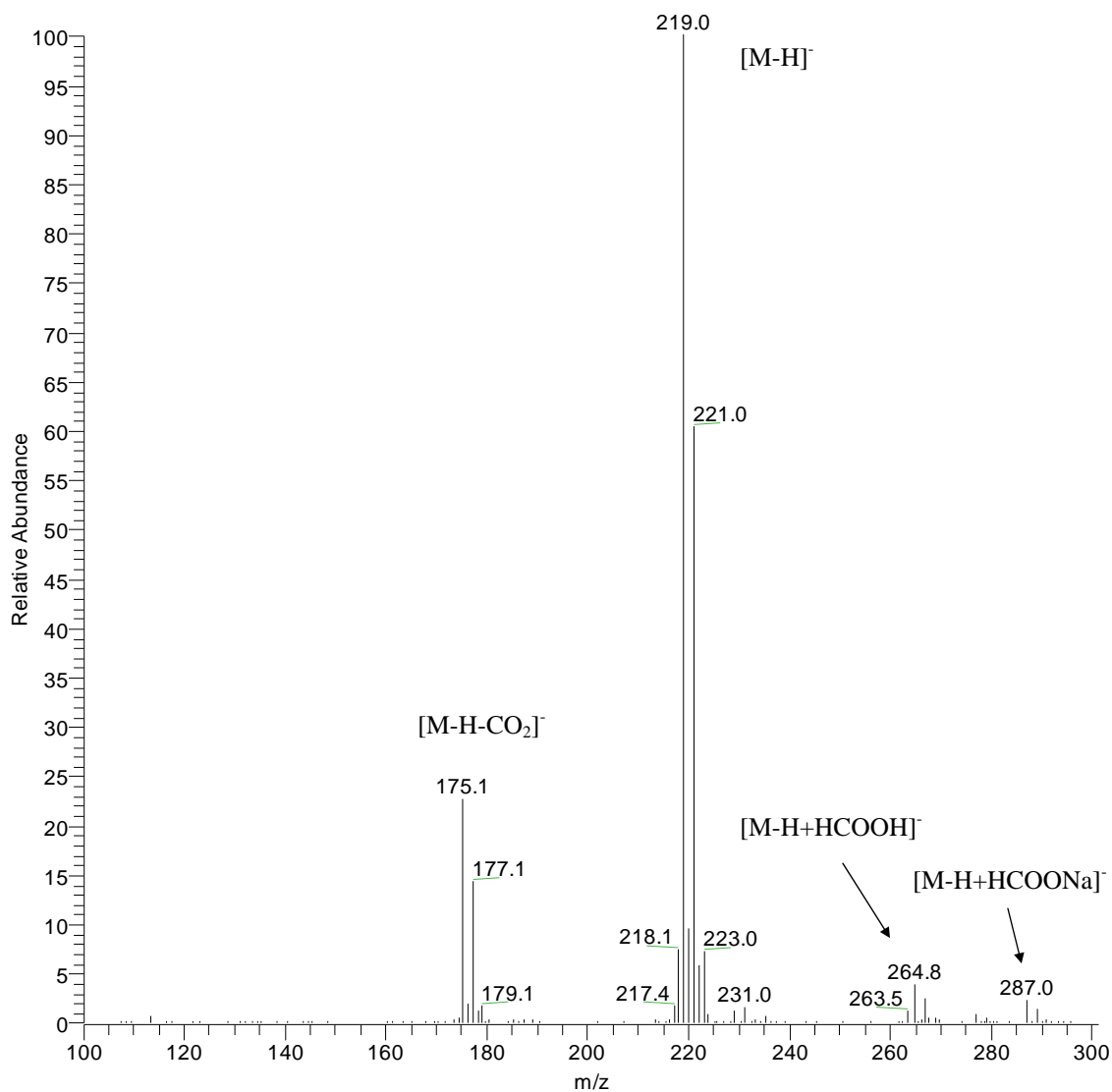
* The organic phase (ether or ethyl acetate) was evaporated to dryness and taken up in starting mobile phase

** The hemicellulose digest was treated with 1 N KOH at 60 °C prior to partitioning

[†] Dicamba equivalents

6 Figures

Figure 1. Negative Ion Electrospray Mass Spectrum of [^{12}C , ^{14}C]Dicamba Test Substance (Lot 6103-01A) Utilized for the Preemergence Application



[Phenyl-U- ^{14}C]Dicamba
Nominal Mass 220

Figure 2. HPLC/RAD Radiochemical Purity Analysis (Method A) of [^{12}C , ^{14}C]Dicamba Test Substance (Lot 6103-01A) Utilized for the Preemergence Application

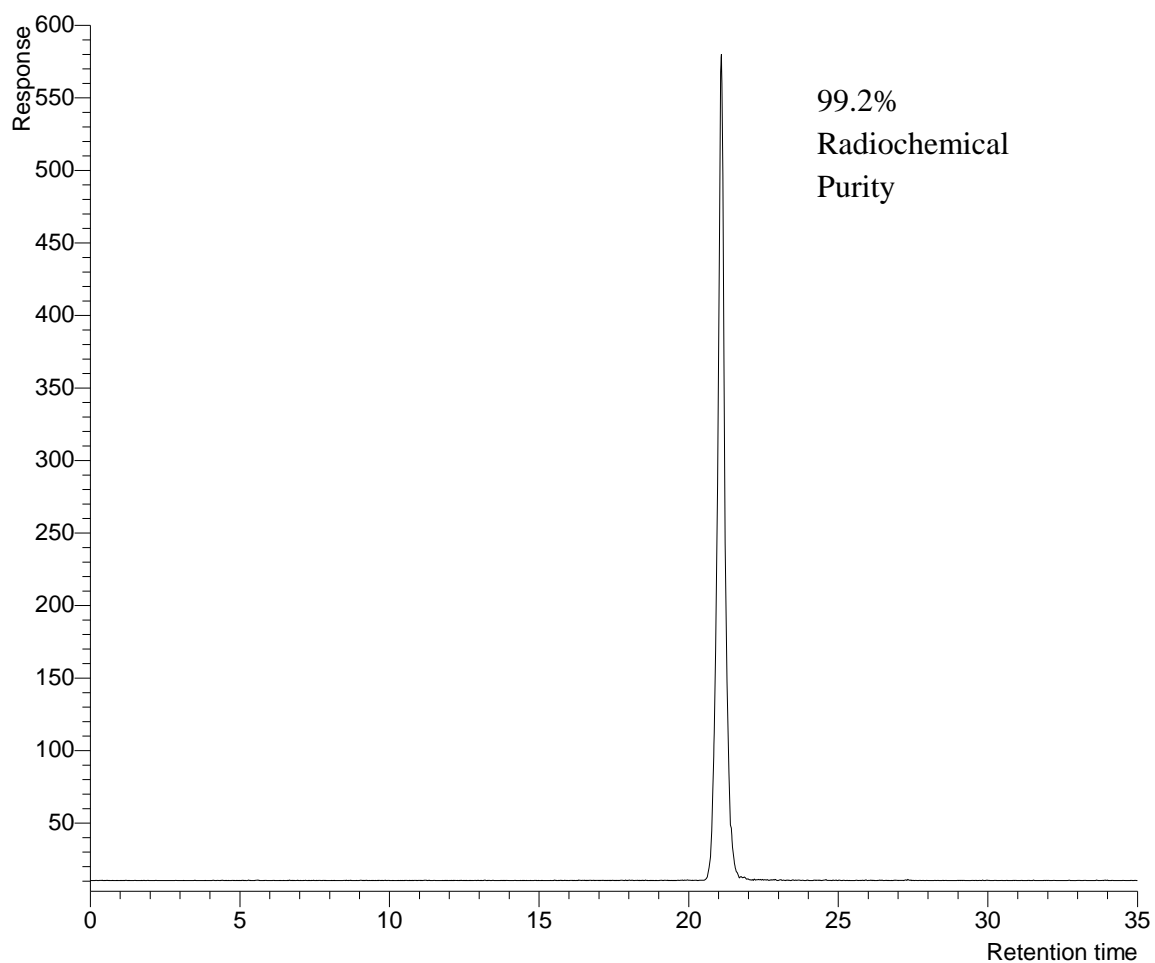
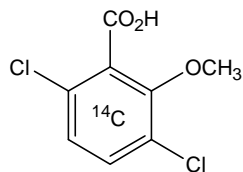
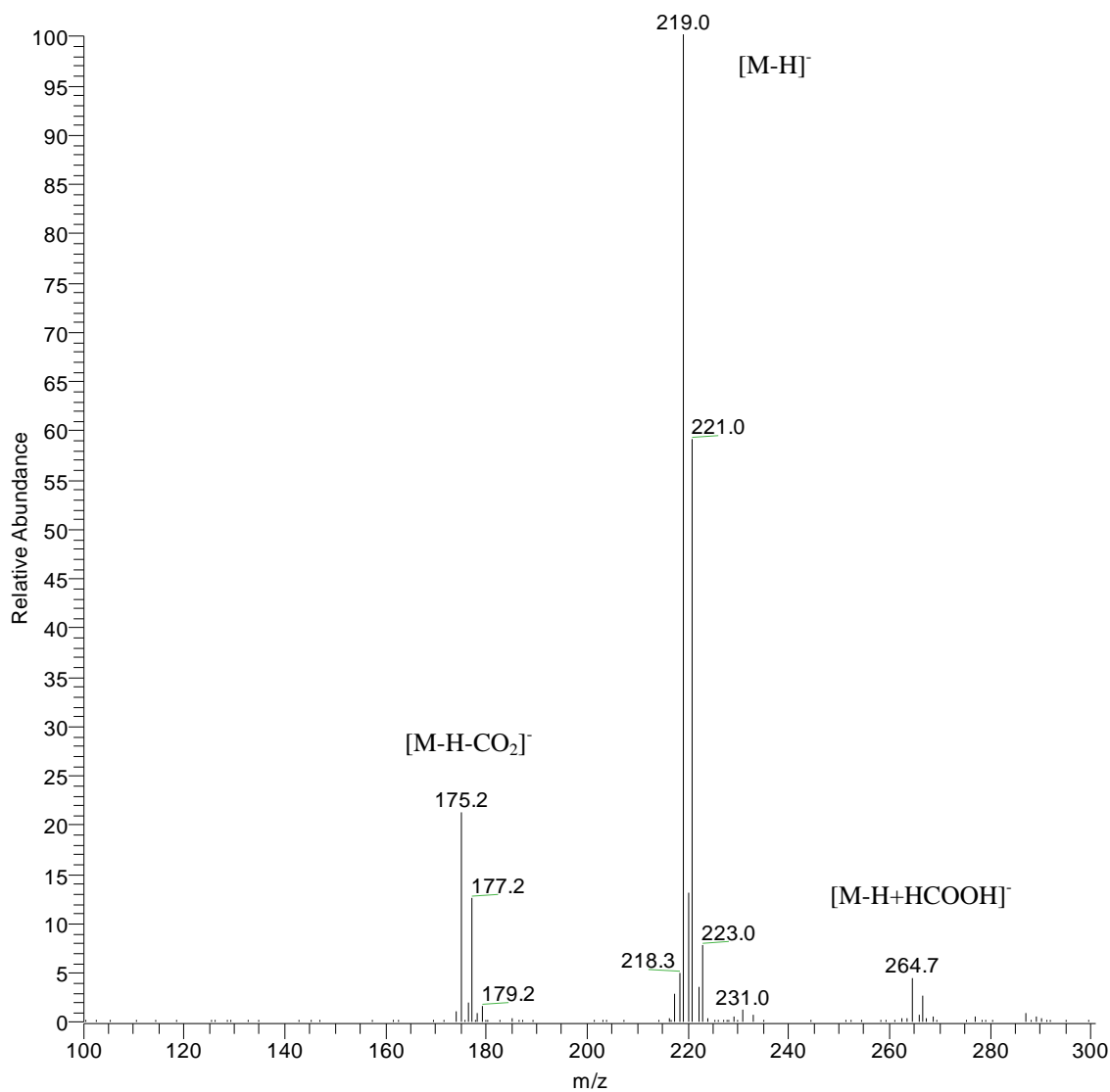


Figure 3. Negative Ion Electrospray Mass Spectrum of [^{12}C , ^{14}C]Dicamba Test Substance (Lot 6103-01C) Utilized for the Postemergence Application



[Phenyl- ^{14}C]Dicamba
Nominal Mass 220

Figure 4. HPLC/RAD Radiochemical Purity Analysis (Method A) of [^{12}C , ^{14}C]Dicamba Test Substance (Lot 6103-01C) Utilized for the Postemergence Application

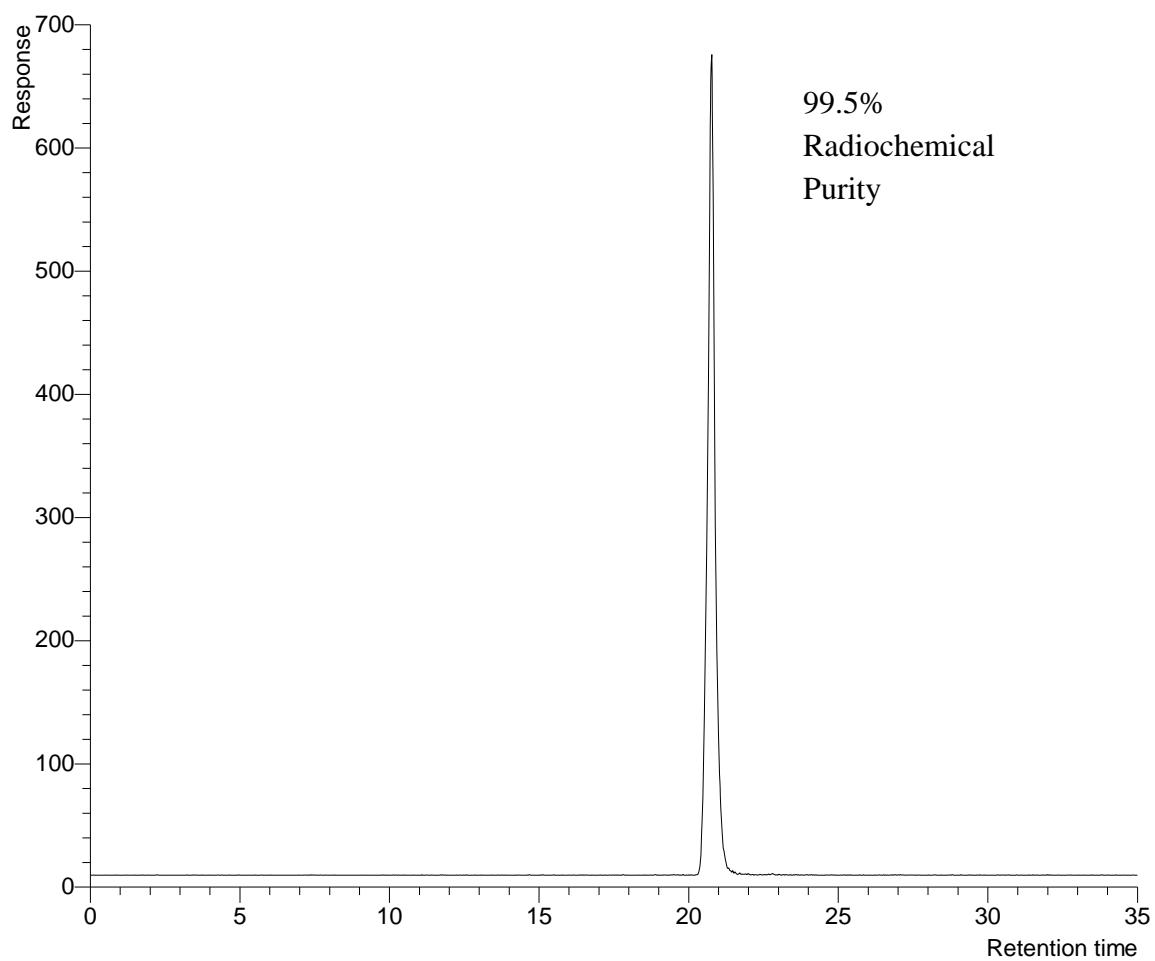
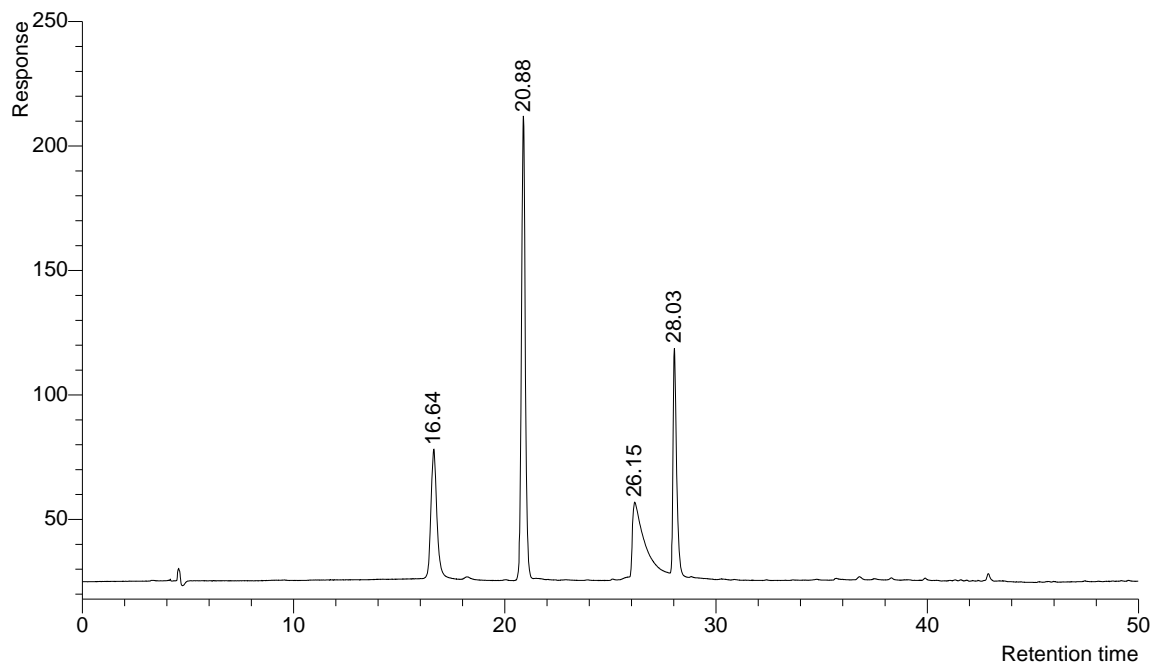
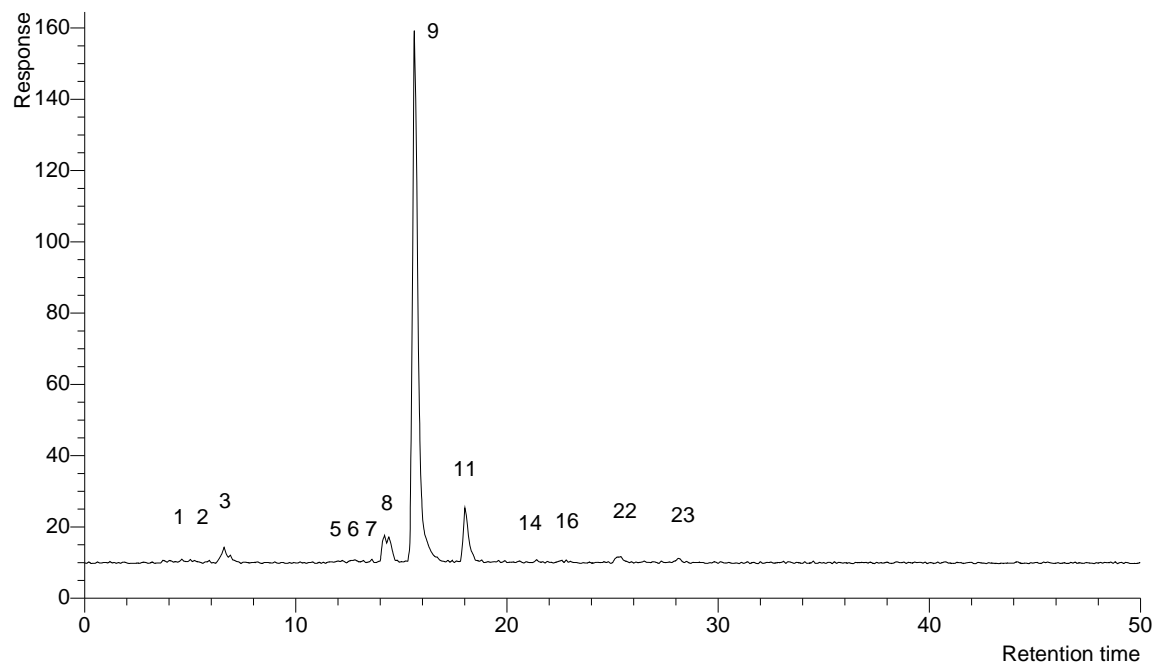


Figure 5. Representative HPLC/UV Chromatogram of Reference Standards Using Method B



Retention Time (min)	Name
16.64	DCGA (IV)
20.88	5-Hydroxydicamba (III)
26.15	DCSA (II)
28.03	Dicamba (I)

Figure 6. HPLC/RAD Quantitation Profile of PRE-T Immature Foliage (Pre-forage)

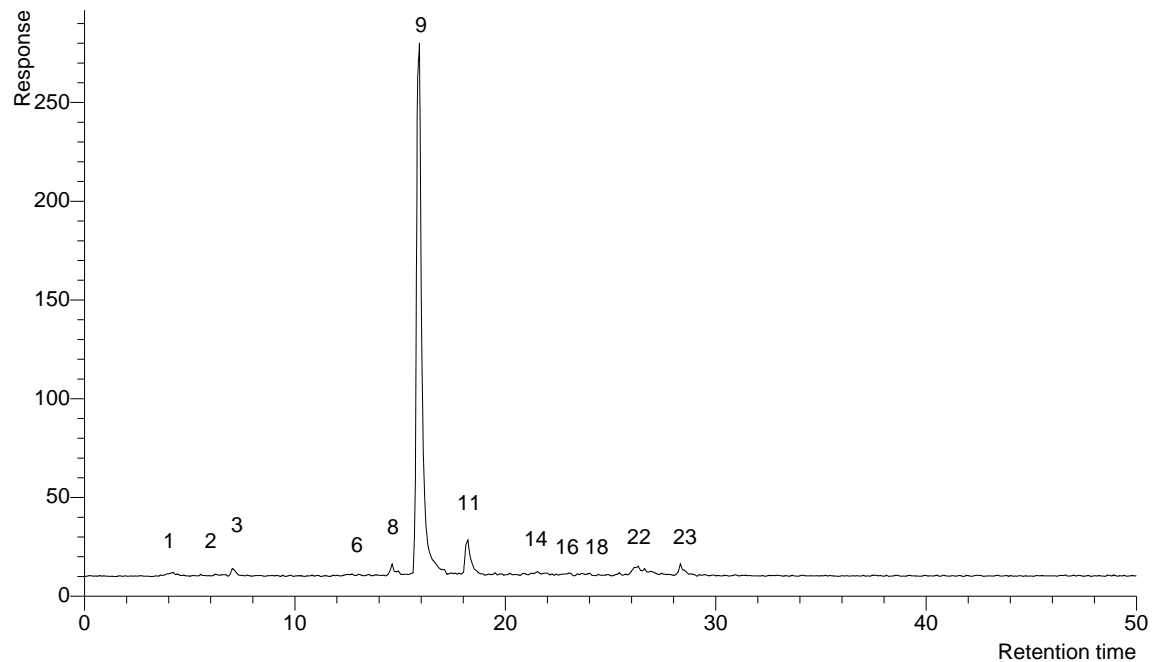


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	3.248	91.09%	97.11%	34840	101.27%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	4.6	Sugars	1.61	1.47	0.048
2	5.9	Unknown	0.28	0.26	0.008
3	6.6	DCGA Glucoside	3.04	2.77	0.090
5	12.2	Unknown	0.62	0.56	0.018
6	12.8	Unknown	0.70	0.64	0.021
7	13.6	Unknown	0.22	0.20	0.007
8	14.2	DCGA Malonylglucoside	5.99	5.46	0.177
9	15.6	DCSA Glucoside	75.71	68.96	2.240
11	18.0	DCSA HMGglucoside	8.36	7.62	0.247
14	21.4	Unk DCSA/DCGA Conj.	0.32	0.29	0.009
16	22.8	Unknown	0.68	0.62	0.020
22	25.4	DCSA	1.60	1.46	0.047
23	28.1	Dicamba	0.88	0.80	0.026
Totals			100.01	91.10	2.959

* Dicamba equivalents

Figure 7. HPLC/RAD Quantitation Profile of PRE-T Forage

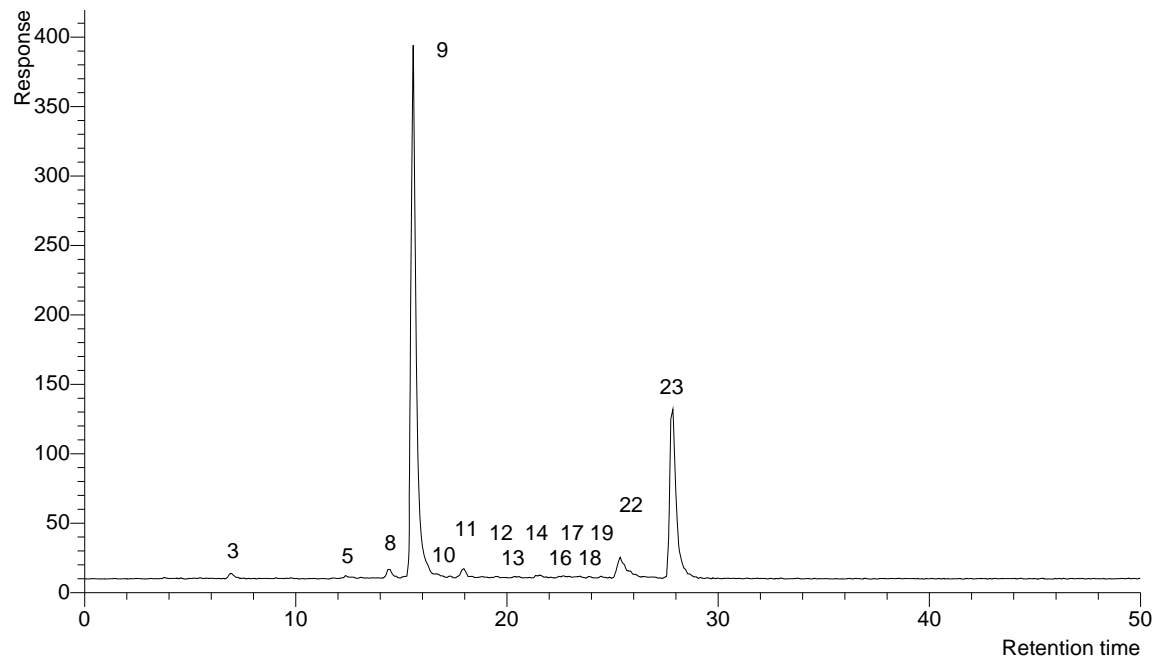


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	1.433	91.21%	98.82%	58920	100.50%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	4.2	Sugars	1.05	0.96	0.014
2	6.2	Unknown	0.44	0.40	0.006
3	7.0	DCGA Glucoside	1.25	1.14	0.016
6	12.7	Unknown	0.64	0.58	0.008
8	14.6	DCGA Malonylglucoside	1.54	1.40	0.020
9	15.9	DCSA Glucoside	81.66	74.48	1.067
11	18.2	DCSA HMGglucoside	5.71	5.21	0.075
14	21.5	Unk DCSA/DCGA Conj.	1.38	1.26	0.018
16	23.0	Unknown	0.45	0.41	0.006
18	24.0	Unk DCSA/DCGA Gluc.	0.60	0.55	0.008
22	26.3	DCSA	3.50	3.19	0.046
23	28.3	Dicamba	1.77	1.61	0.023
Totals			99.99	91.20	1.307

* Dicamba equivalents

Figure 8. HPLC/RAD Quantitation Profile of POE-T Forage

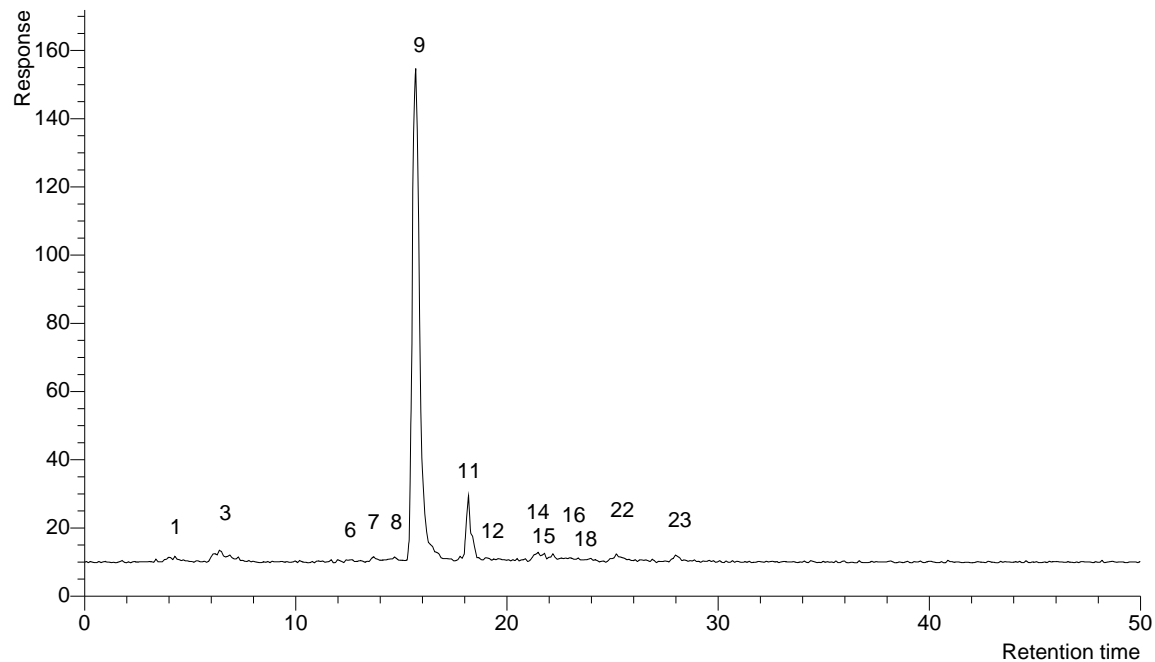


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	134.147	93.79%	100.09%	99369	99.80%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	7.0	DCGA Glucoside	0.80	0.75	1.007
5	12.4	Unknown	0.38	0.36	0.478
8	14.4	DCGA Malonylglucoside	1.18	1.11	1.485
9	15.6	DCSA Glucoside	64.31	60.32	80.913
10	17.3	Unknown	0.15	0.14	0.189
11	18.0	DCSA HMGglucoside	1.22	1.14	1.535
12	19.6	Unknown	0.19	0.18	0.239
13	20.4	Unknown	0.19	0.18	0.239
14	21.6	Unk DCSA/DCGA Conj.	0.40	0.38	0.503
16	22.7	Unknown	0.43	0.40	0.541
17	23.5	Unknown	0.28	0.26	0.352
18	23.9	Unk DCSA/DCGA Gluc.	0.13	0.12	0.164
19	24.5	Unknown	0.19	0.18	0.239
22	25.4	DCSA	4.35	4.08	5.473
23	27.9	Dicamba	25.81	24.21	32.473
Totals			100.01	93.80	125.829

* Dicamba equivalents

Figure 9. HPLC/RAD Quantitation Profile of PRE-T Hay

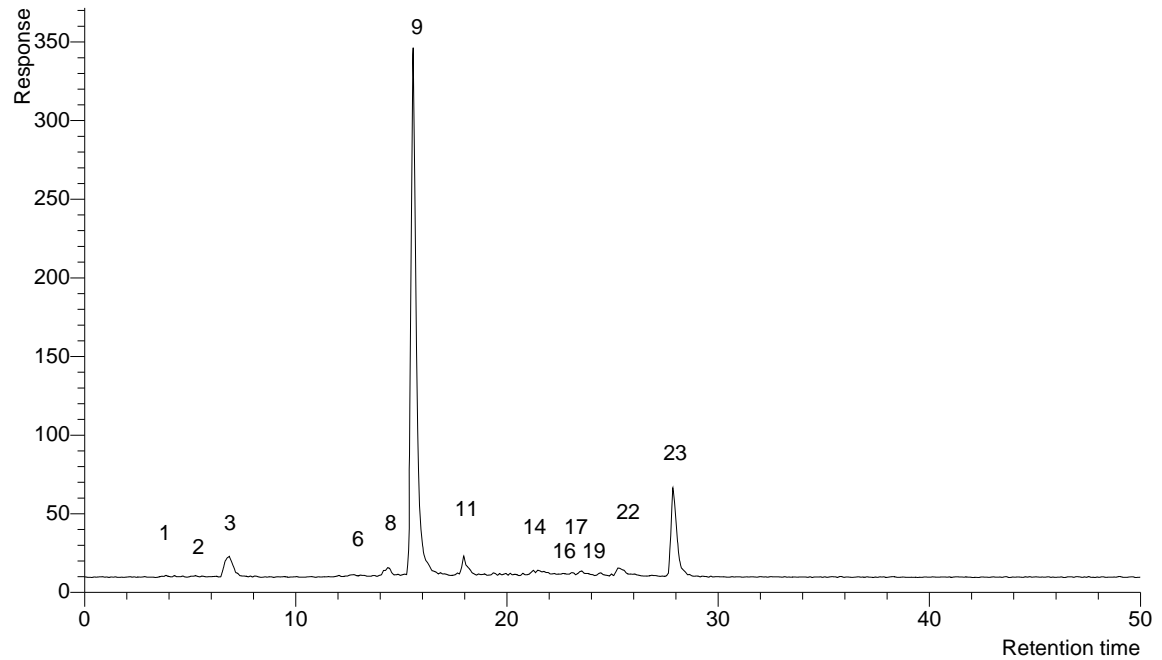


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	1.056	90.88%	100.32%	43913	98.39%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	4.3	Sugars	1.19	1.08	0.011
3	6.4	DCGA Glucoside	3.80	3.45	0.036
6	12.7	Unknown	0.58	0.53	0.006
7	13.7	Unknown	0.55	0.50	0.005
8	14.7	DCGA Malonylglucoside	0.80	0.73	0.008
9	15.7	DCSA Glucoside	77.92	70.81	0.748
11	18.2	DCSA HMGglucoside	7.34	6.67	0.070
12	19.0	Unknown	0.81	0.74	0.008
14	21.5	Unk DCSA/DCGA Conj.	1.80	1.64	0.017
15	22.2	Unknown	0.83	0.75	0.008
16	23.0	Unknown	1.18	1.07	0.011
18	24.0	Unk DCSA/DCGA Gluc.	0.56	0.51	0.005
22	25.2	DCSA	1.70	1.54	0.016
23	28.0	Dicamba	0.94	0.85	0.009
Totals			100.00	90.88	0.960

* Dicamba equivalents

Figure 10. HPLC/RAD Quantitation Profile of POE-T Hay

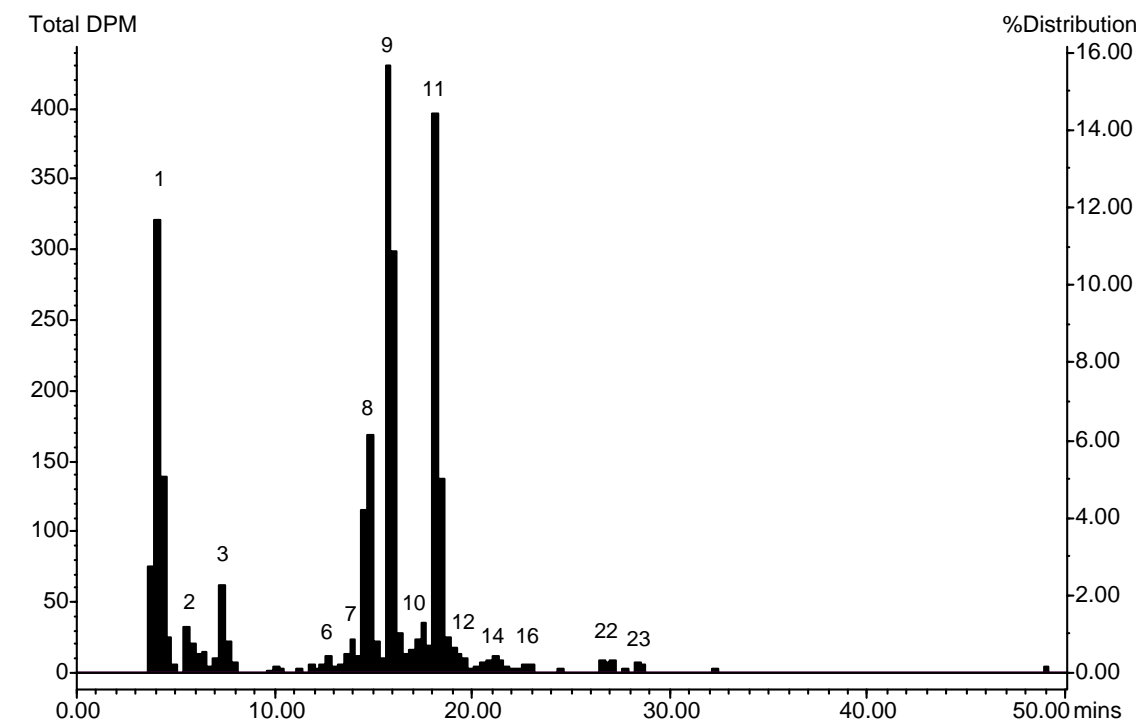


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	39.149	95.30%	99.61%	86113	98.34%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	3.9	Sugars	0.51	0.49	0.190
2	5.3	Unknown	0.19	0.18	0.071
3	6.9	DCGA Glucoside	4.53	4.32	1.690
6	12.8	Unknown	0.75	0.71	0.280
8	14.4	DCGA Malonylglucoside	1.69	1.61	0.631
9	15.6	DCSA Glucoside	70.58	67.26	26.333
11	18.0	DCSA HMGglucoside	2.60	2.48	0.970
14	21.5	Unk DCSA/DCGA Conj.	1.84	1.75	0.686
16	23.1	Unknown	1.00	0.95	0.373
17	23.6	Unknown	0.95	0.91	0.354
19	24.5	Unknown	0.40	0.38	0.149
22	25.3	DCSA	2.03	1.93	0.757
23	27.9	Dicamba	12.94	12.33	4.828
Totals			100.01	95.31	37.313

* Dicamba equivalents

Figure 11. HPLC/LSC Quantitation Profile of PRE-T Seed

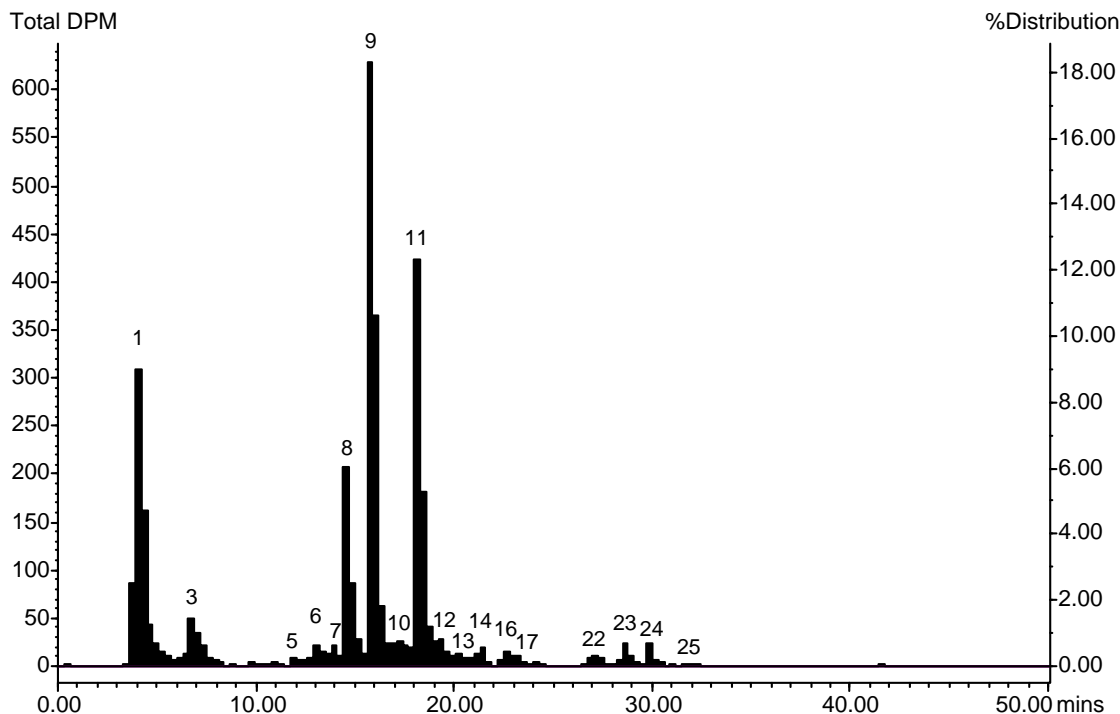


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	0.291	49.00%	92.04%	3030	90.72%

Peak Number	LSC Fractions Comprising Peak	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	13-17	4.1	Sugars	20.58	8.42	0.025
2	19-22	5.6	Unknown	2.98	1.22	0.004
3	23-27	7.4	DCGA Glucoside	3.90	1.60	0.005
6	42-44	12.8	Unknown	0.80	0.33	0.001
7	45-48	14.0	Unknown	1.76	0.72	0.002
8	48-52	14.9	DCGA Malonylglucoside	11.55	4.73	0.014
9	52-56	15.8	DCSA Glucoside	28.22	11.55	0.034
10	57-60	17.6	Unknown	3.07	1.26	0.004
11	60-64	18.2	DCSA HMGglucoside	21.34	8.73	0.025
12	65-67	19.4	Unknown	0.92	0.38	0.001
14	68-75	21.2	Unk DCSA/DCGA Conj.	1.84	0.75	0.002
16	76-77	22.7	Unknown	0.43	0.18	0.001
22	89-91	27.2	DCSA	0.91	0.37	0.001
23	95-96	28.4	Dicamba	0.48	0.20	0.001
Totals				98.78	40.42	0.118

* Dicamba equivalents; 'Extractability' includes the ACN and 40/60 ACN:H₂O extracts only

Figure 12. HPLC/LSC Quantitation Profile of POE-T Seed

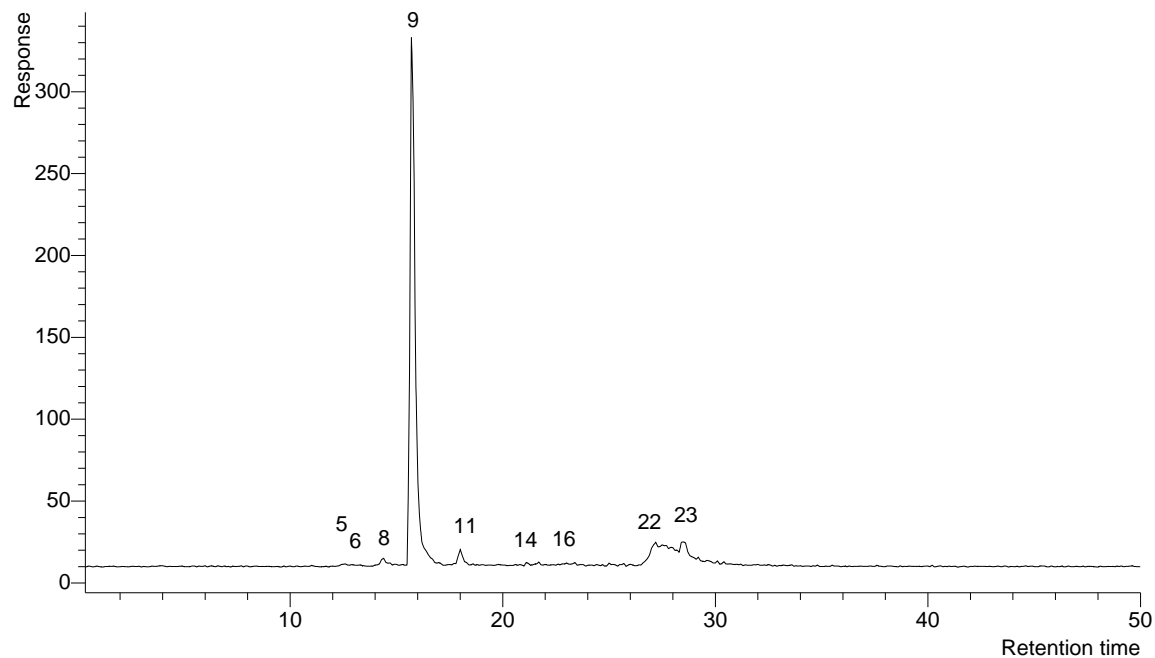


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	0.389	55.27%	91.21%	3631	94.64%

Peak Number	LSC Fractions Comprising Peak	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	12-20	4.1	Sugars	19.18	9.15	0.036
3	21-28	6.8	DCGA Glucoside	4.33	2.07	0.008
5	40-42	11.9	Unknown	0.66	0.31	0.001
6	43-45	13.1	Unknown	1.31	0.62	0.002
7	46-48	14.0	Unknown	1.19	0.57	0.002
8	48-52	14.6	DCGA Malonylglucoside	9.72	4.64	0.018
9	52-57	15.8	DCSA Glucoside	32.00	15.27	0.059
10	57-59	17.3	Unknown	1.76	0.84	0.003
11	60-64	18.2	DCSA HMGglucoside	20.14	9.61	0.037
12	65-66	19.4	Unknown	1.28	0.61	0.002
13	67-69	20.3	Unknown	1.00	0.48	0.002
14	70-73	21.5	Unk DCSA/DCGA Conj.	1.30	0.62	0.002
16	75-77	22.7	Unknown	0.76	0.36	0.001
17	77-80	23.3	Unknown	0.69	0.33	0.001
22	89-94	27.2	DCSA	0.96	0.46	0.002
23	94-98	28.7	Dicamba	1.35	0.64	0.003
24	99-102	29.9	Unknown	1.09	0.52	0.002
25	106-108	32.3	Unknown	0.24	0.11	0.0004
Totals				98.96	47.21	0.184

* Dicamba equivalents; 'Extractability' includes the ACN and 40/60 ACN/H₂O extracts only

Figure 13. HPLC/RAD Quantitation Profile of Washed POE-T Forage Plant #28

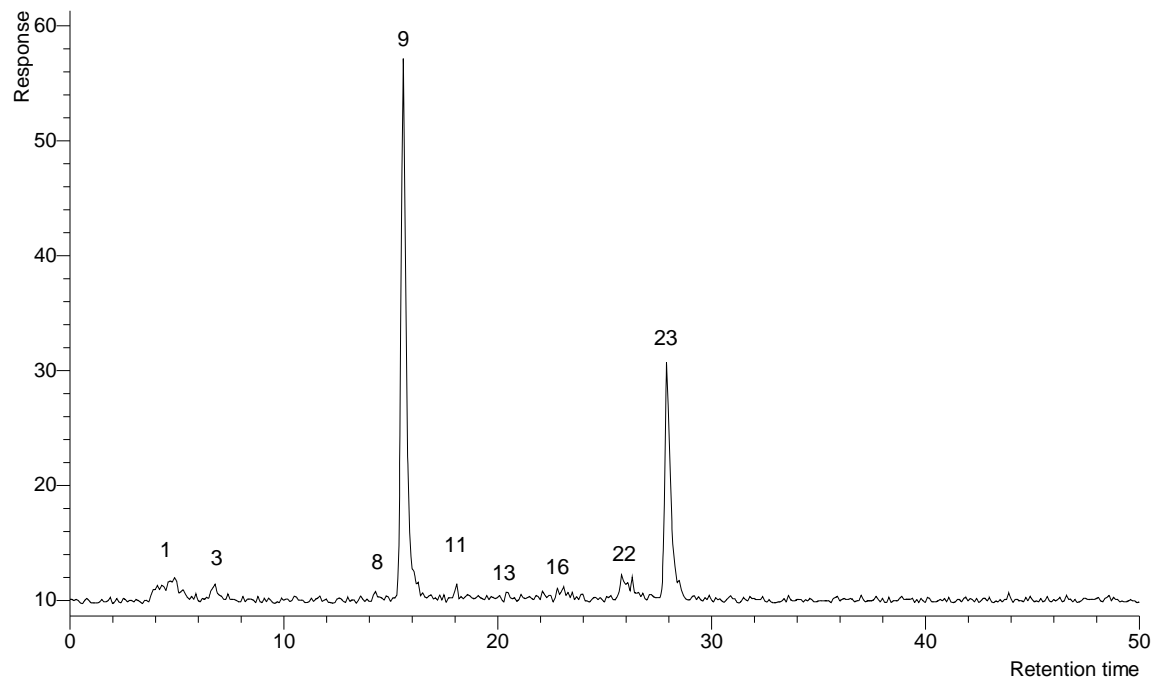


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	81.786	89.21%	99.75%	123805	96.38%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
5	12.4	Unknown	0.53	0.47	0.387
6	12.8	Unknown	0.42	0.37	0.306
8	14.2	DCGA Malonylglucoside	1.39	1.24	1.014
9	15.7	DCSA Glucoside	72.24	64.45	52.707
11	17.9	DCSA HMGglucoside	2.24	2.00	1.634
14	21.6	Unk DCSA/DCGA Conj.	0.74	0.66	0.540
16	22.9	Unknown	1.01	0.90	0.737
22	27.0	DCSA	19.54	17.43	14.257
23	28.5	Dicamba	1.90	1.69	1.386
Totals			100.01	89.22	72.969

* Dicamba equivalents; 'Matrix TRR' is the TRR in the washed forage plant (does not include wash)

Figure 14. HPLC/RAD Quantitation Profile of PRE-C Forage

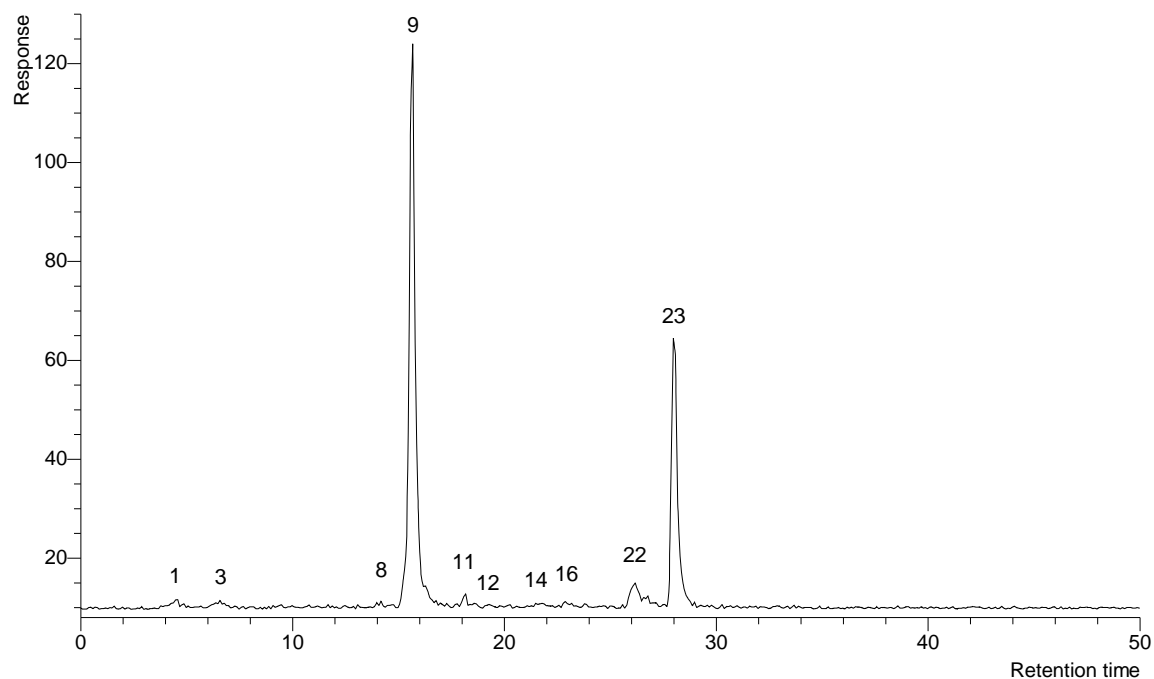


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	0.080	75.64%	98.69%	13138	110.10%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	4.9	Sugars	7.36	5.57	0.004
3	6.8	DCGA Glucoside	2.41	1.82	0.001
8	14.3	DCGA Malonylglucoside	0.93	0.70	0.001
9	15.6	DCSA Glucoside	53.75	40.66	0.033
11	18.1	DCSA HMGglucoside	0.91	0.69	0.001
13	20.4	Unknown	0.90	0.68	0.001
16	23.1	Unknown	3.22	2.44	0.002
22	25.8	DCSA	5.03	3.80	0.003
23	27.9	Dicamba	25.48	19.27	0.015
Totals			99.99	75.63	0.061

* Dicamba equivalents

Figure 15. HPLC/RAD Quantitation Profile of POE-C Forage

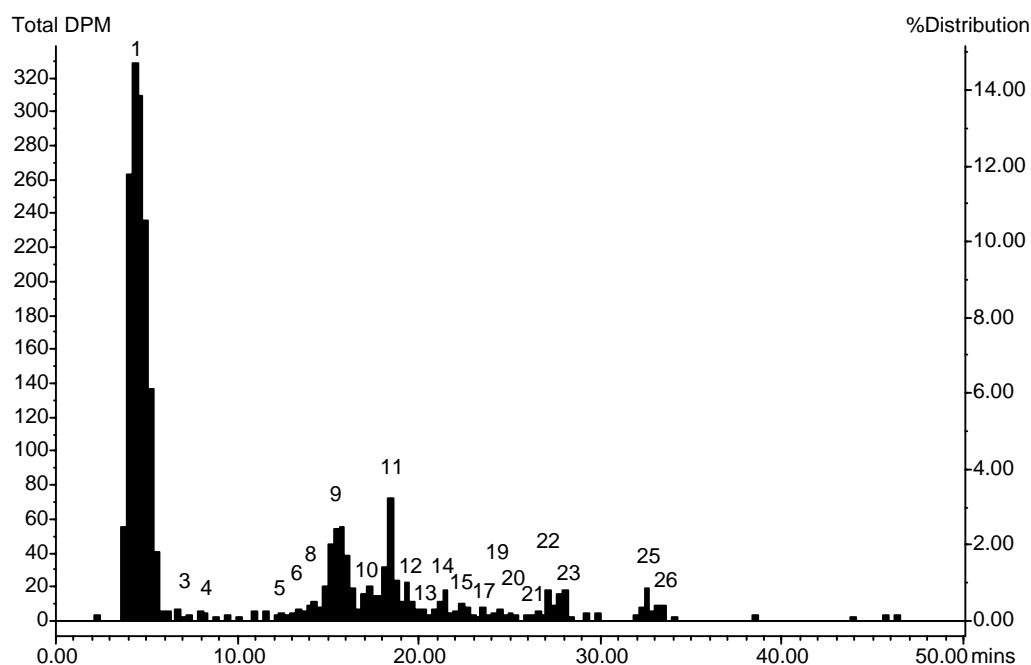


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	0.280	93.50%	104.20%	38850	100.07%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	4.6	Sugars	1.88	1.76	0.005
3	6.6	DCGA Glucoside	1.39	1.30	0.004
8	14.2	DCGA Malonylglucoside	0.76	0.71	0.002
9	15.7	DCSA Glucoside	59.35	55.49	0.155
11	18.2	DCSA HMGglucoside	1.56	1.46	0.004
12	19.3	Unknown	0.36	0.34	0.001
14	21.8	Unk DCSA/DCGA Conj.	0.90	0.84	0.002
16	22.9	Unknown	0.71	0.66	0.002
22	26.2	DCSA	5.09	4.76	0.013
23	28.0	Dicamba	28.00	26.18	0.073
Totals			100.00	93.50	0.262

* Dicamba equivalents

Figure 16. HPLC/LSC Quantitation Profile of PRE-C Seed

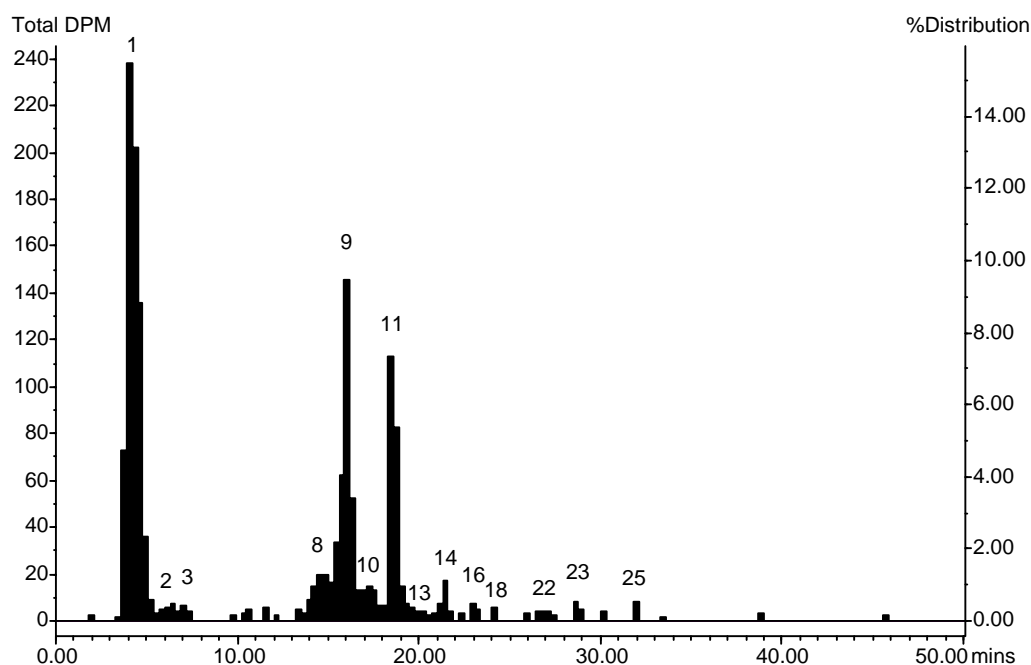


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	0.170	26.05%	88.04%	2513	88.94%

Peak Number	LSC Fractions Comprising Peak	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	13-21	4.4	Sugars	61.82	12.61	0.021
3	23-25	6.8	DCGA Glucoside	0.55	0.11	0.0002
4	27-28	8.0	Unknown	0.47	0.10	0.0002
5	41-42	12.5	Unknown	0.37	0.08	0.0001
6	43-46	13.4	Unknown	0.76	0.16	0.0003
8	46-49	14.3	DCSA Malonylglucoside	1.41	0.29	0.0005
9	50-56	15.8	DCSA Glucoside	10.67	2.18	0.004
10	57-60	17.3	Unknown	2.61	0.53	0.001
11	60-64	18.5	DCSA HMGglucoside	6.51	1.33	0.002
12	65-66	19.4	Unknown	1.49	0.30	0.001
13	67-69	20.3	Unknown	0.71	0.14	0.0002
14	70-73	21.5	Unk DCSA/DCGA Conj.	1.80	0.37	0.001
15	74-77	22.4	Unknown	1.22	0.25	0.0004
17	78-80	23.6	Unknown	0.55	0.11	0.0002
19	80-83	24.5	Unknown	0.68	0.14	0.0002
20	83-85	25.1	Unknown	0.39	0.08	0.0001
21	87-89	26.6	Unknown	0.55	0.11	0.0002
22	90-92	27.2	DCSA	1.16	0.24	0.0004
23	92-95	28.1	Dicamba	1.81	0.37	0.001
25	107-110	32.6	Unknown	1.65	0.34	0.001
26	111-112	33.5	Unknown	0.81	0.17	0.0003
Totals				97.99	19.99	0.034

* Dicamba equivalents; 'Extractability' includes the ACN and 40:60 ACN:H₂O extracts only

Figure 17. HPLC/LSC Quantitation Profile of POE-C Seed



HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	0.138	32.47%	92.49%	1681	91.60%

Peak Number	LSC Fractions Comprising Peak	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
1	12-19	4.1	Sugars	45.43	12.50	0.017
2	20-22	6.5	Unknown	1.16	0.32	0.0004
3	23-25	7.1	DCGA Glucoside	1.00	0.28	0.0004
8	46-51	14.9	DCGA Malonylglucoside	4.80	1.32	0.002
9	51-57	16.1	DCSA Glucoside	20.90	5.75	0.008
10	57-60	17.3	Unknown	2.64	0.73	0.001
11	61-66	18.5	DCSA HMGglucoside	14.96	4.12	0.006
13	67-69	20.0	Unknown	0.66	0.18	0.0003
14	70-73	21.5	Unk DCSA/DCGA Conj.	2.05	0.56	0.001
16	77-78	23.0	Unknown	0.78	0.21	0.0003
18	81	24.2	Unk DCSA Gluc Conj.	0.37	0.10	0.0001
22	89-92	27.2	DCSA	0.92	0.25	0.0003
23	96-97	28.7	Dicamba	0.84	0.23	0.0003
25	107	32.0	Unknown	0.54	0.15	0.0002
Totals				97.05	26.70	0.037

* Dicamba equivalents; 'Extractability' includes the ACN and 40:60 ACN:H₂O extracts only

Figure 18. HPLC/LSC Analysis of the Organic Phase of the Ethyl Acetate Partitioning of POE-T Seed Extract Concentrate Using HPLC Method B

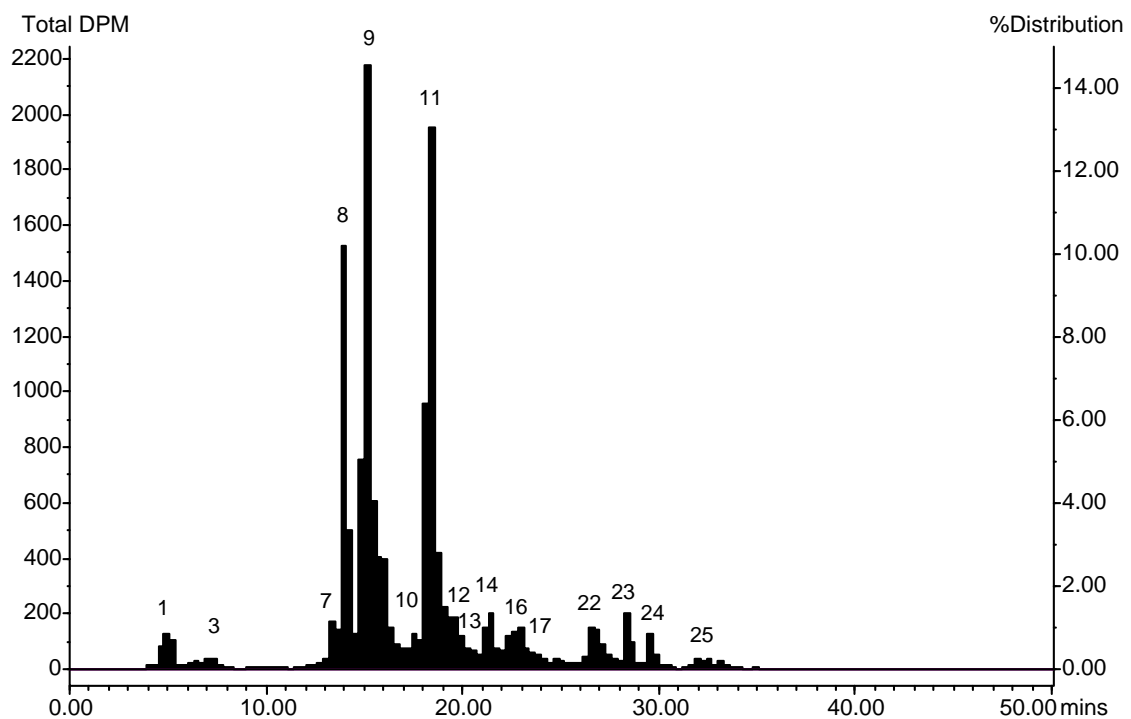


Figure 19. HPLC/RAD of the Organic (top) and Aqueous (bottom) Phases from the Ethyl Acetate Partitioning of the POE-T Seed Acidified Extract Concentrate Using HPLC Method B

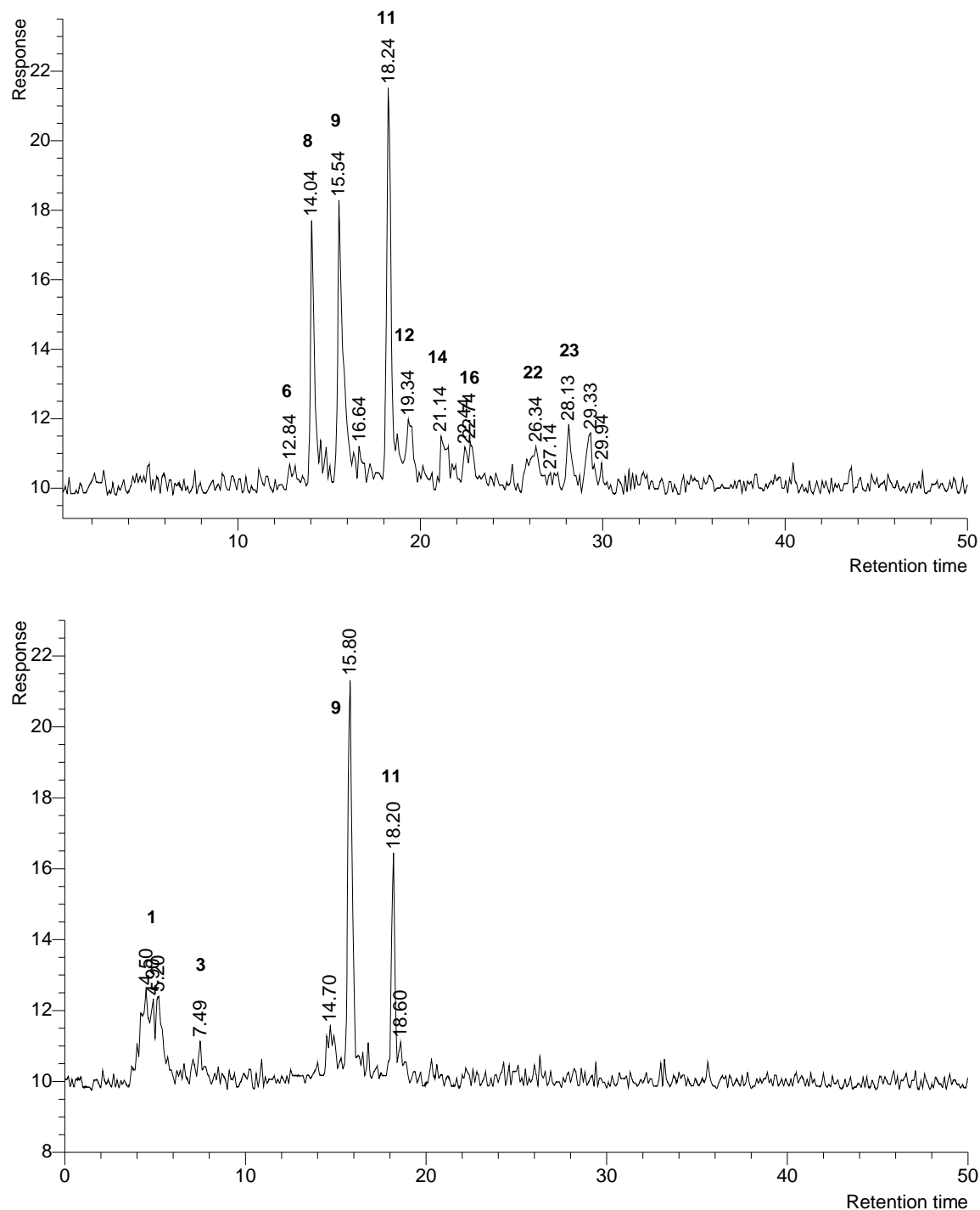


Figure 20. HPLC/LSC Coelution Analysis of PRE-T Seed Extract Concentrate and Peak 8 from POE-T Forage Using HPLC Method C

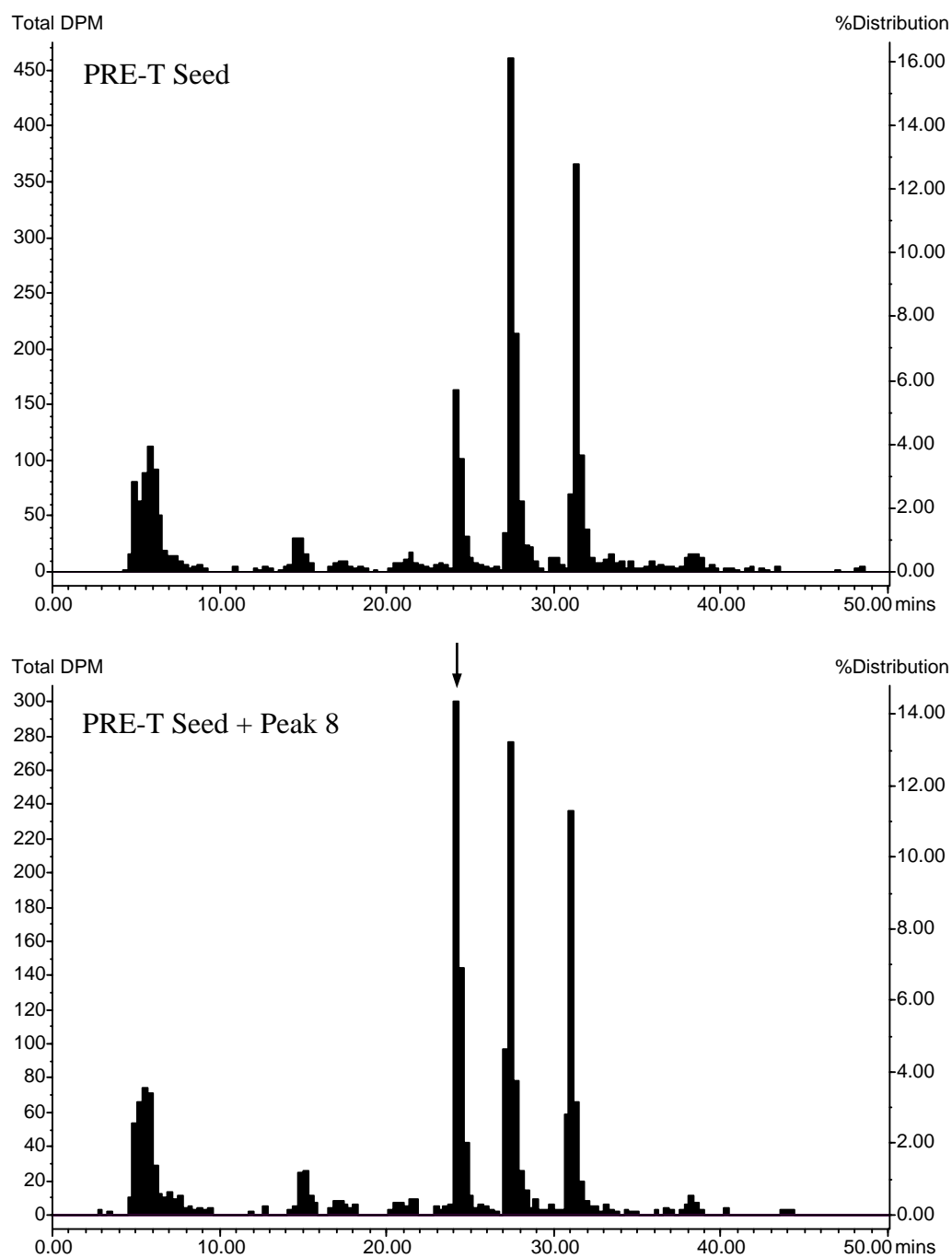


Figure 21. HPLC/LSC Coelution Analysis of PRE-T Seed Extract Concentrate and Peak 9 from POE-T Forage Using HPLC Method C

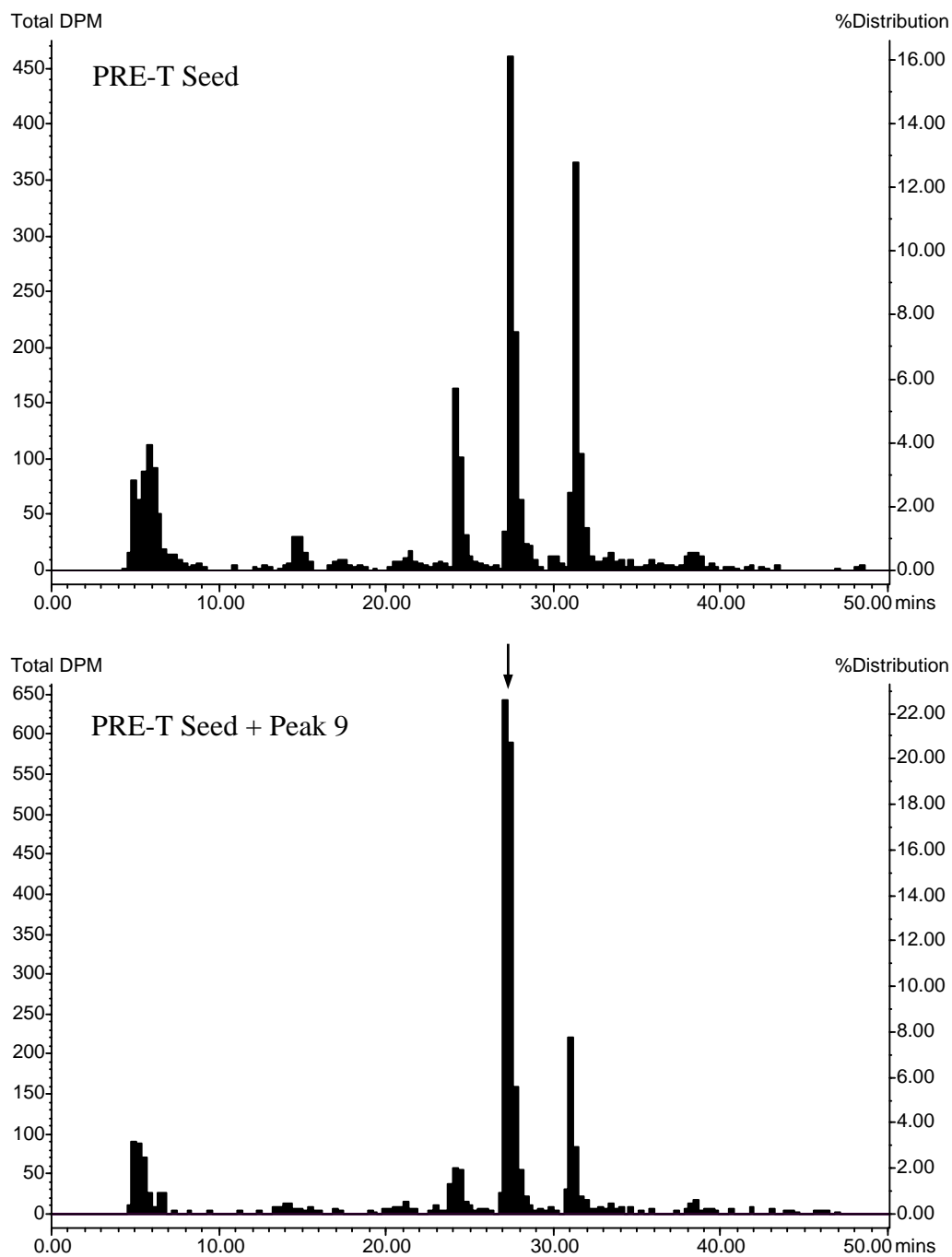


Figure 22. HPLC/LSC Coelution Analysis of PRE-T Seed Extract Concentrate and Peak 11 from POE-T Forage Using HPLC Method C

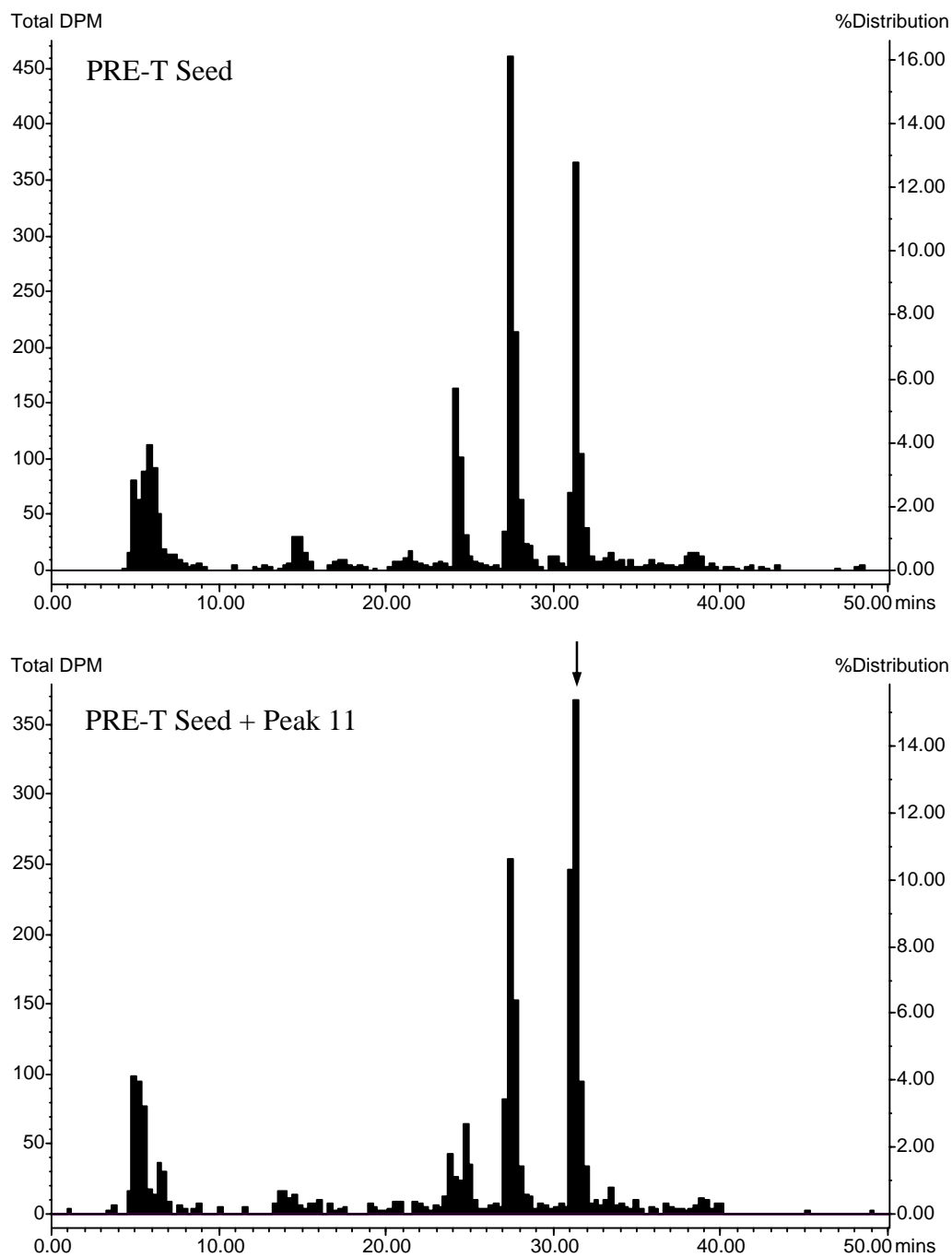


Figure 23. HPLC/LSC of Transesterified Oil from POE-T Seed Hexane Extract (top) and HPLC/UV of Fatty Acid Methyl Ester Reference Standards (bottom) Using HPLC Method E

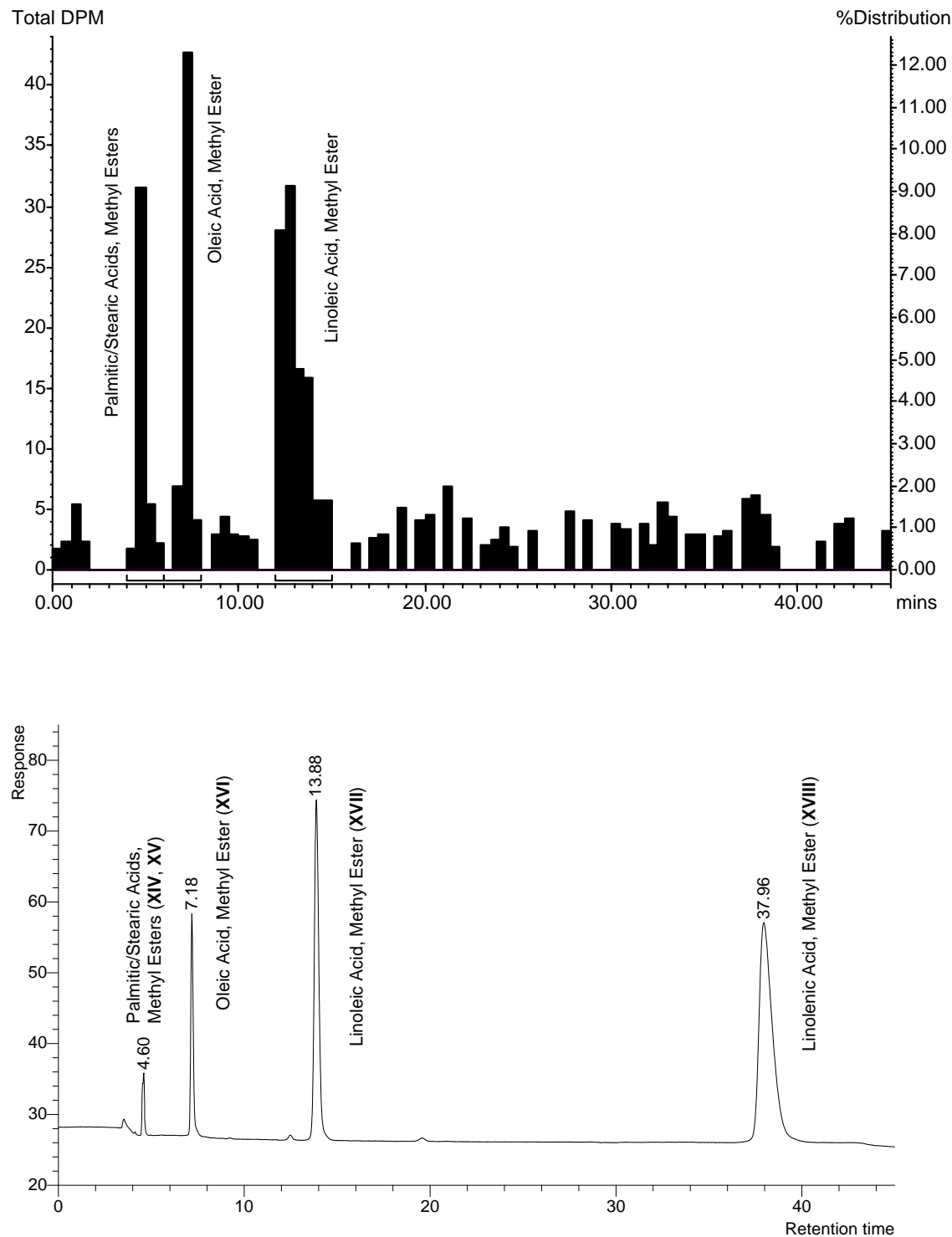


Figure 24. HPLC/LSC Analysis of the Organic Phase of the Ethyl Acetate Partitioning of the Hemicellulose Fraction of PRE-T Seed Using HPLC Method B

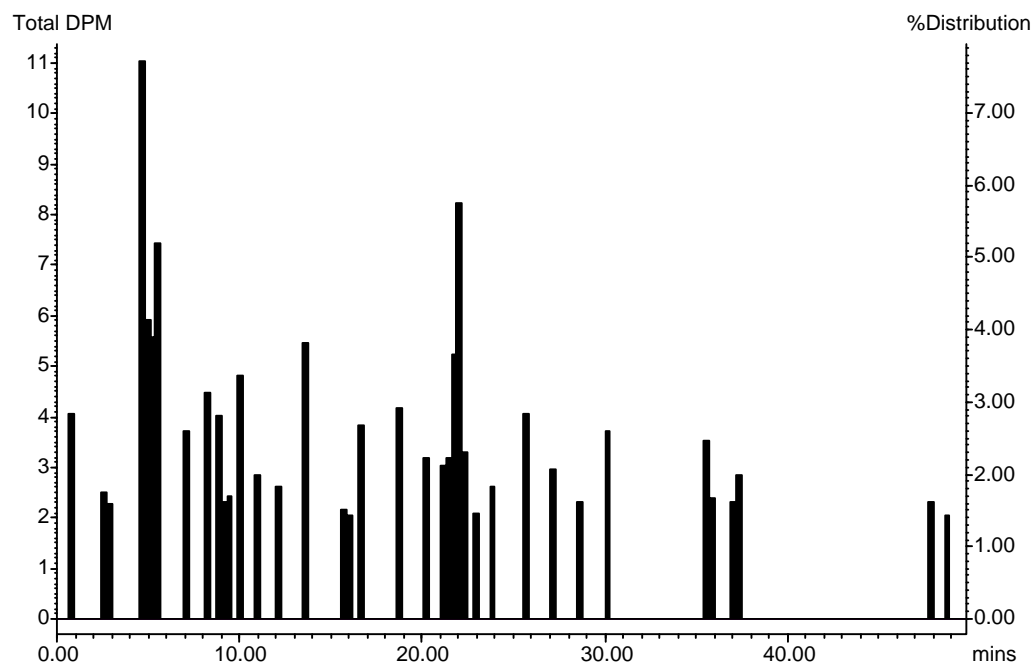


Figure 25. HPLC/LSC Analysis of the Hemicellulose Fraction of POE-T Seed Using HPLC Method B

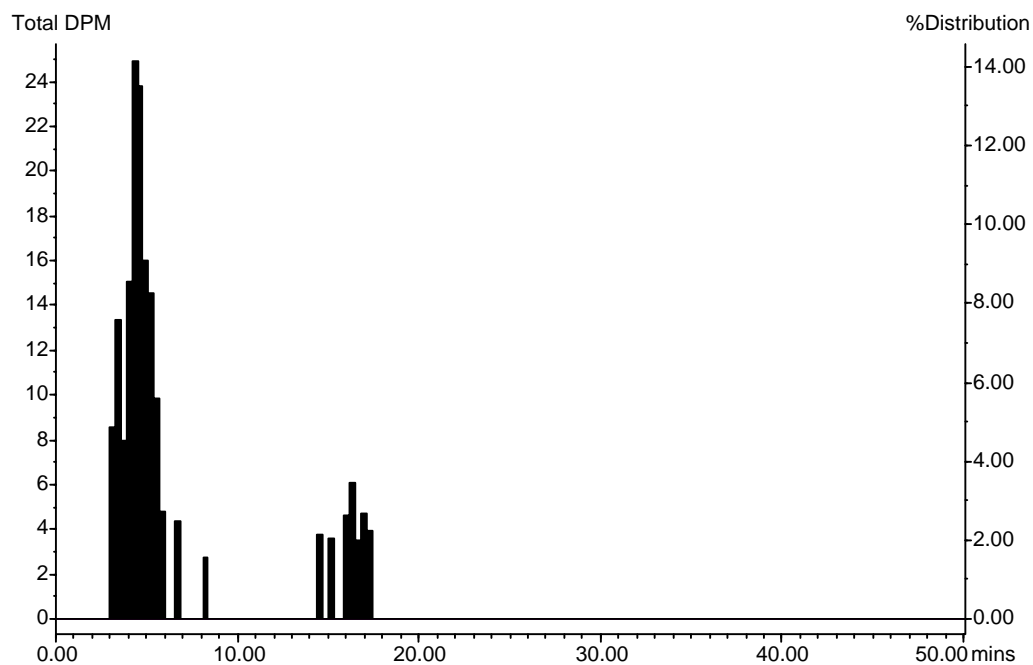


Figure 26. HPLC/LSC Analysis of the Protease Digest of PRE-T Seed Using HPLC Method B

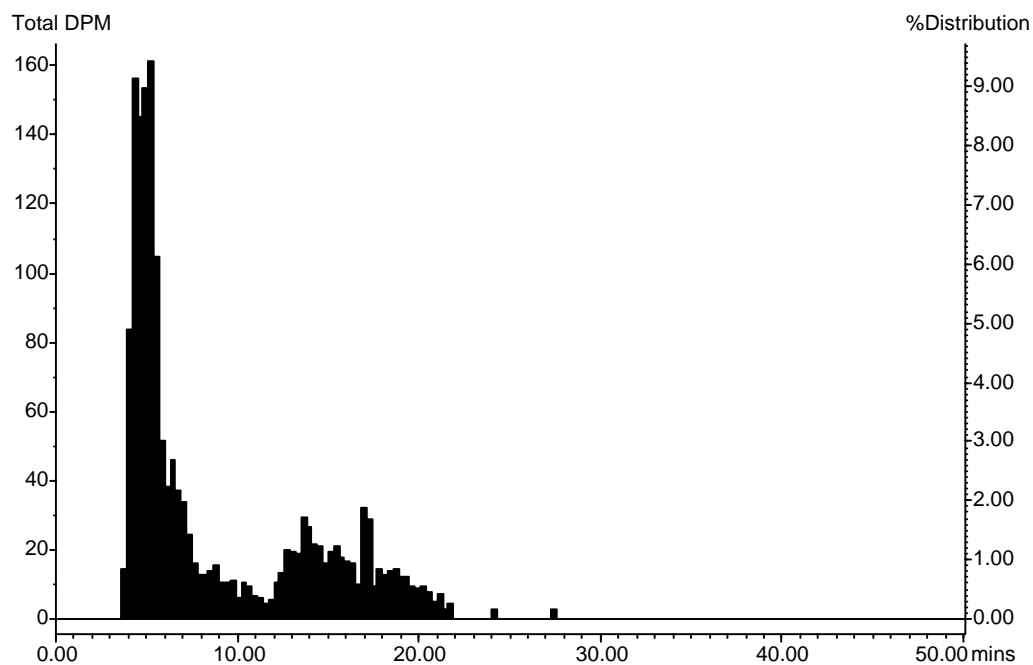


Figure 27. HPLC/RAD Analysis of the Acid Hydrolysate (top) and Base Hydrolysate (bottom) of Peak 1 from POE-T Seed Using HPLC Method B

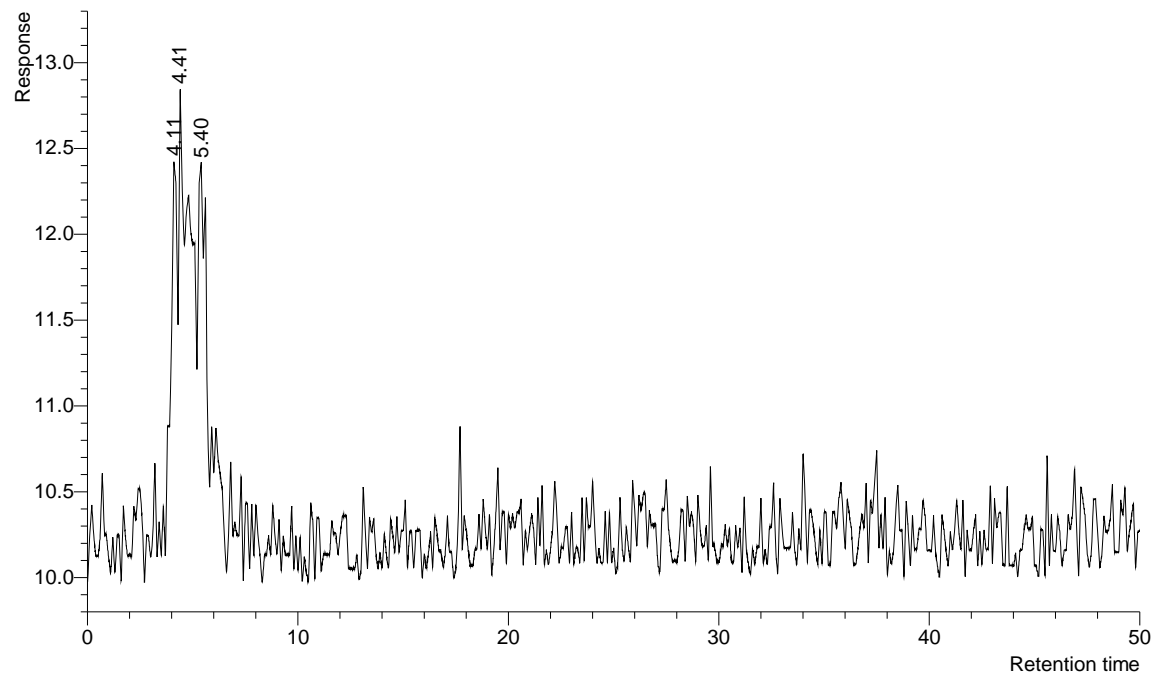
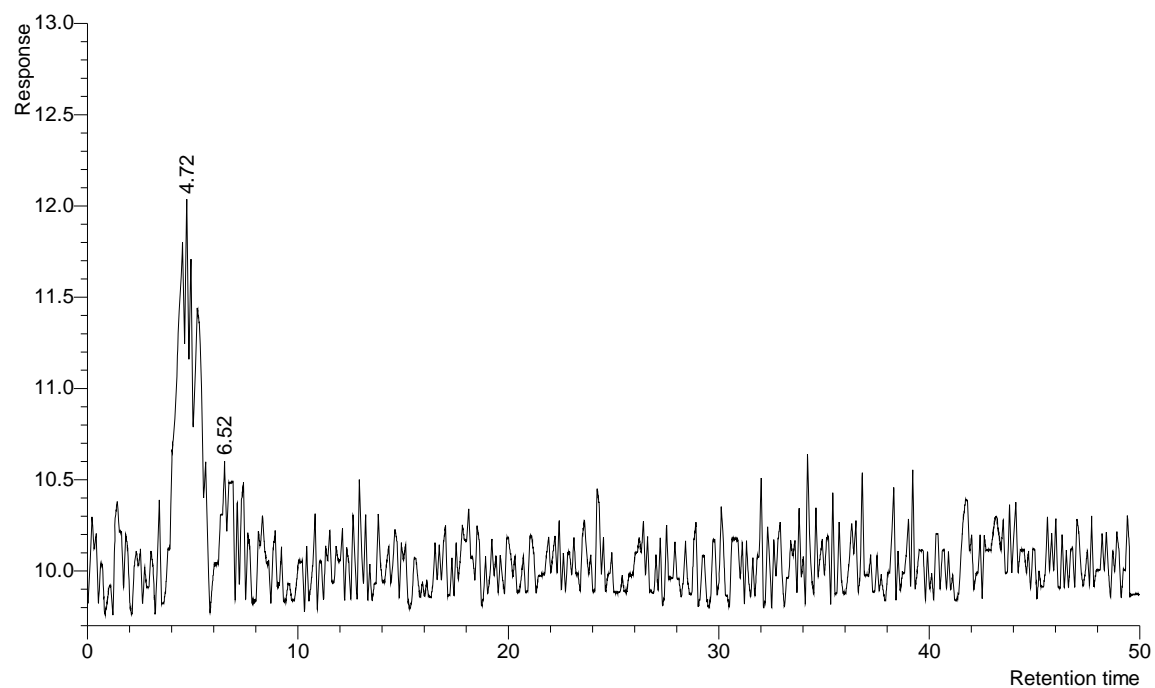


Figure 28. HPLC Analysis of Peak 1 from POE-T Seed (top) and [^{14}C]Glucose Reference Standard (bottom) Using HPLC Method H

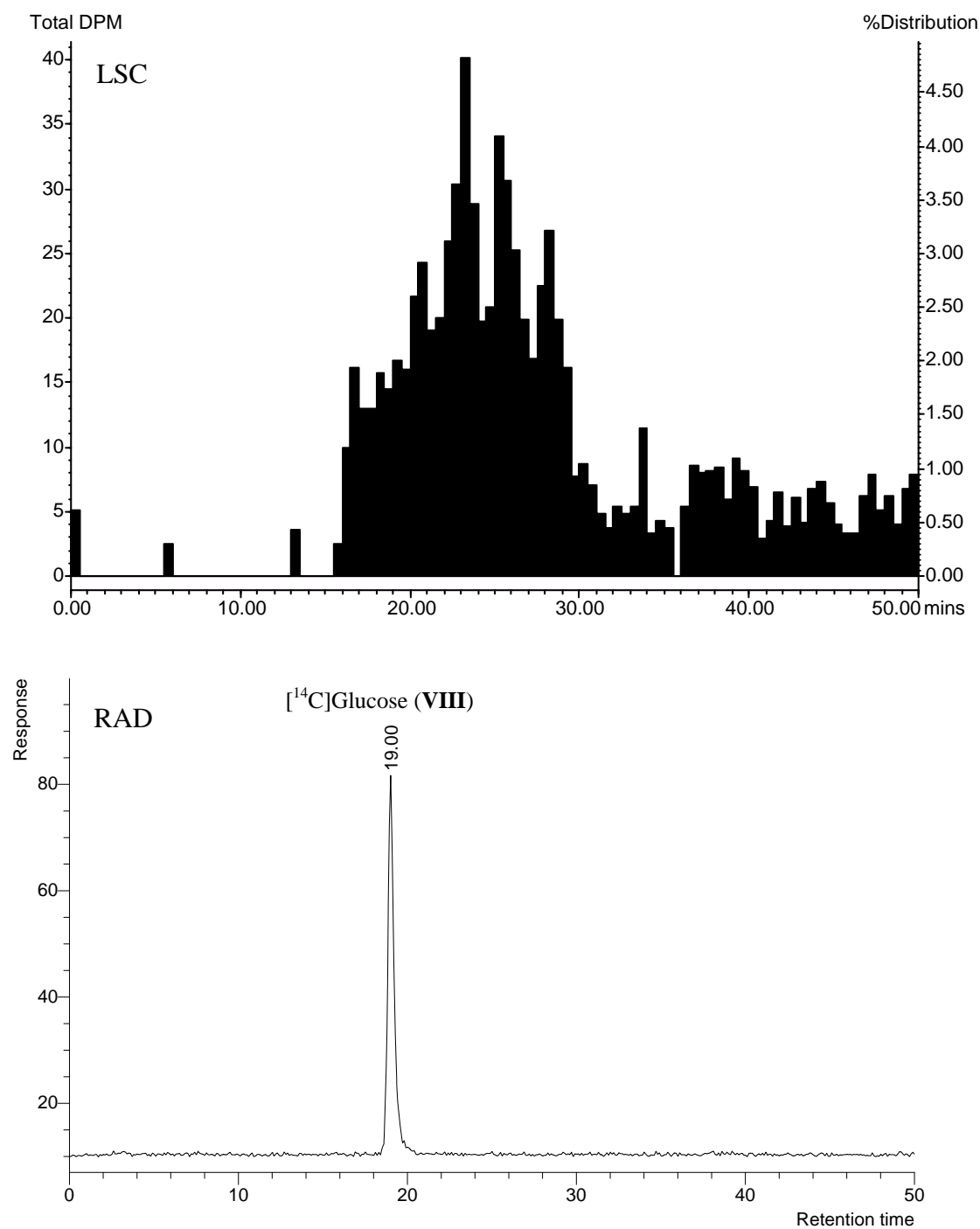


Figure 29. HPLC/LSC Analysis of the Acid Hydrolysate of Peak 1 from POE-T Seed Alone (top) and Spiked with [^{14}C]Glucose (bottom) Using HPLC Method H

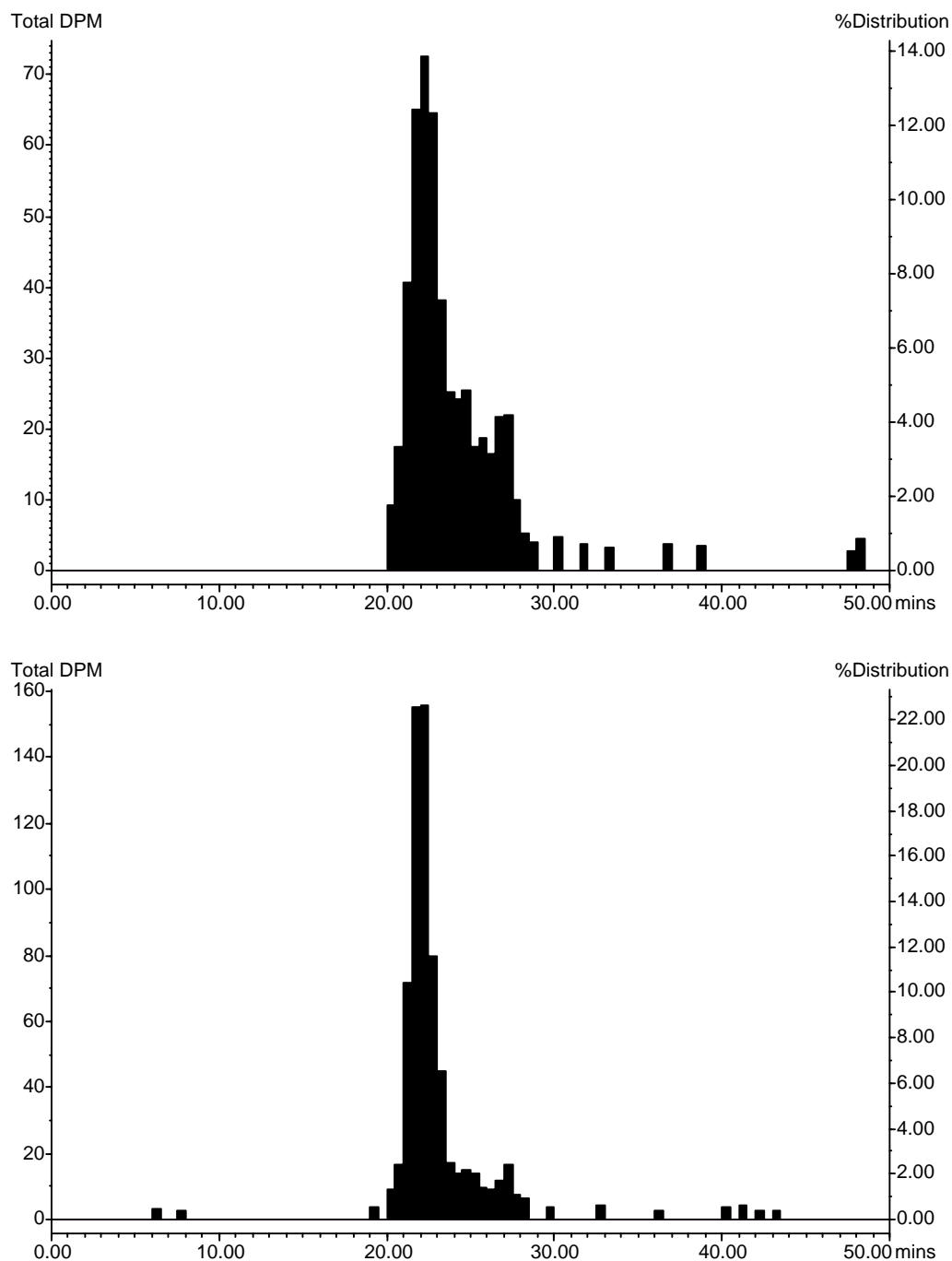


Figure 30. Negative Ion Electrospray Mass Spectrum of the Acid Hydrolysate of Peak 1 from POE-T Seed

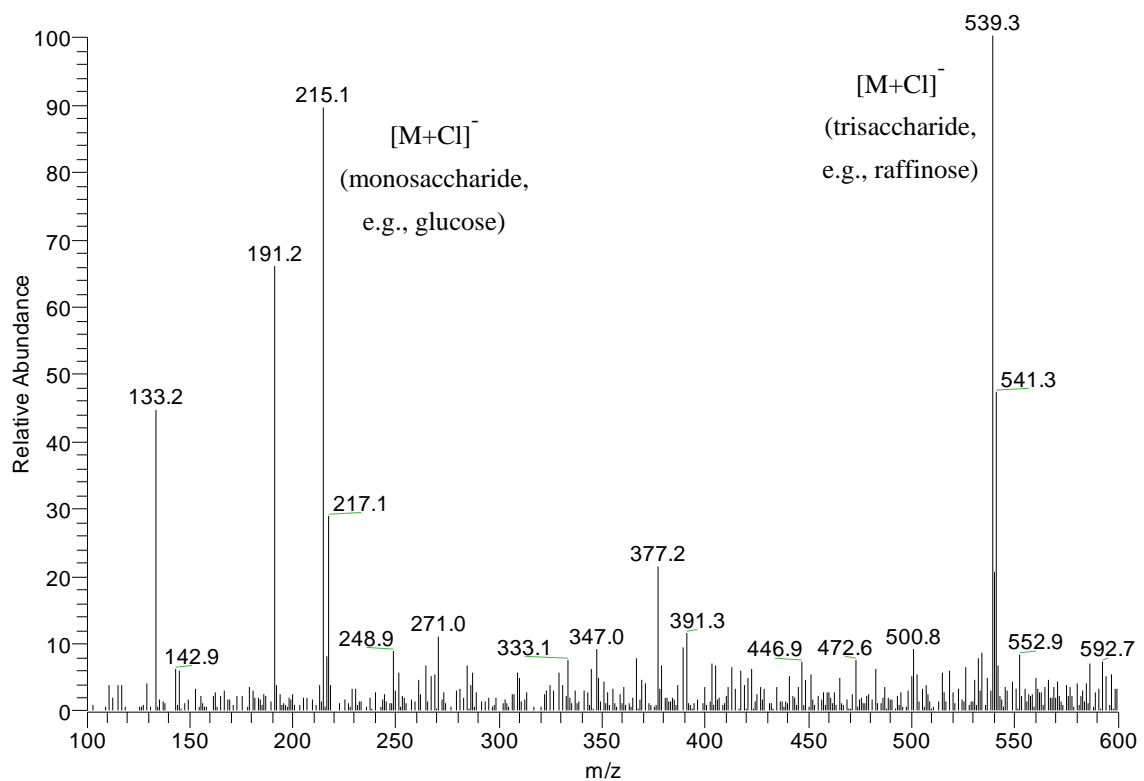
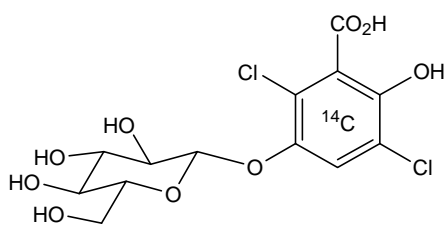
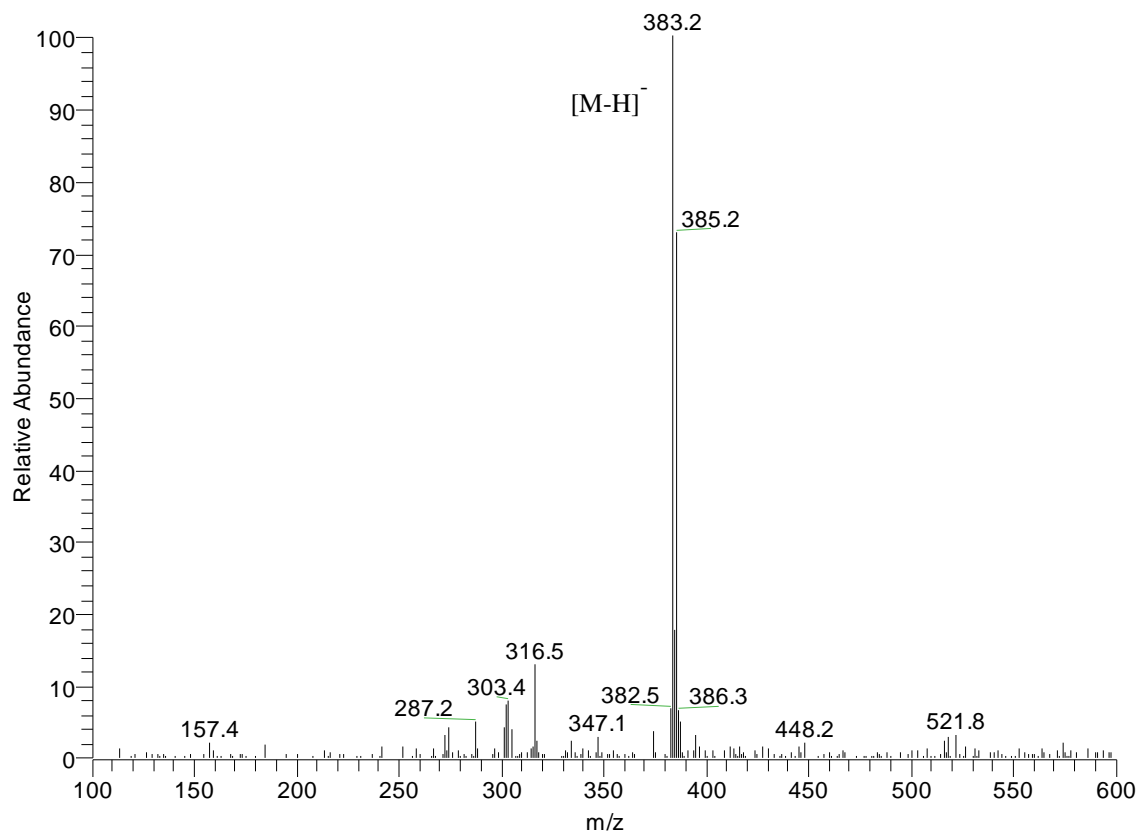


Figure 31. Negative Ion Electrospray Mass Spectrum of Peak 3 from POE-T Forage



DCGA Glucoside (**3**)
Nominal Mass 384

Figure 32. HPLC Coinjection Analysis of the Acid Hydrolysate of Peak 3 from POE-T Forage (top) and DCGA Reference Standard (bottom) Using HPLC Method B

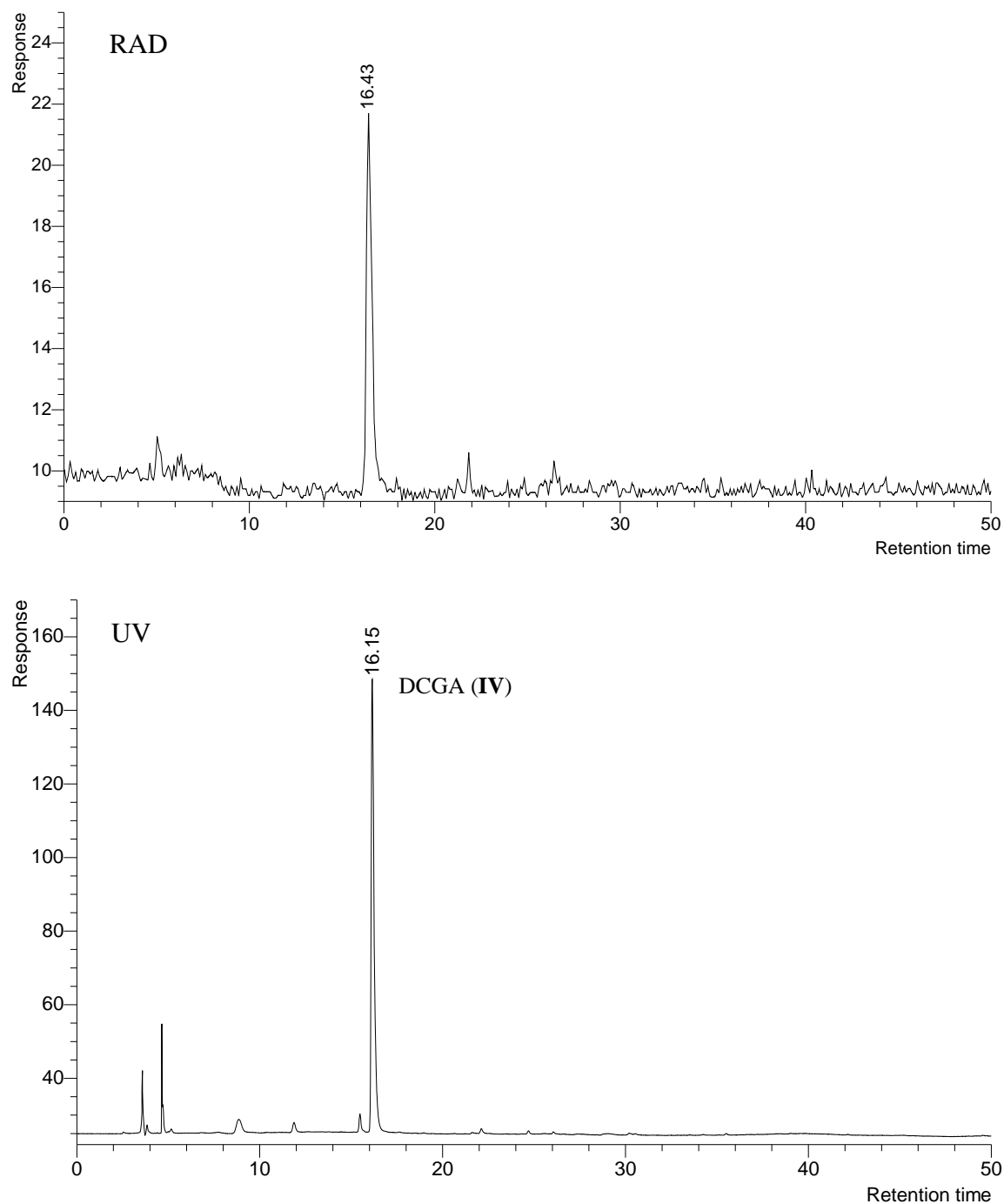
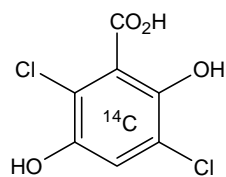
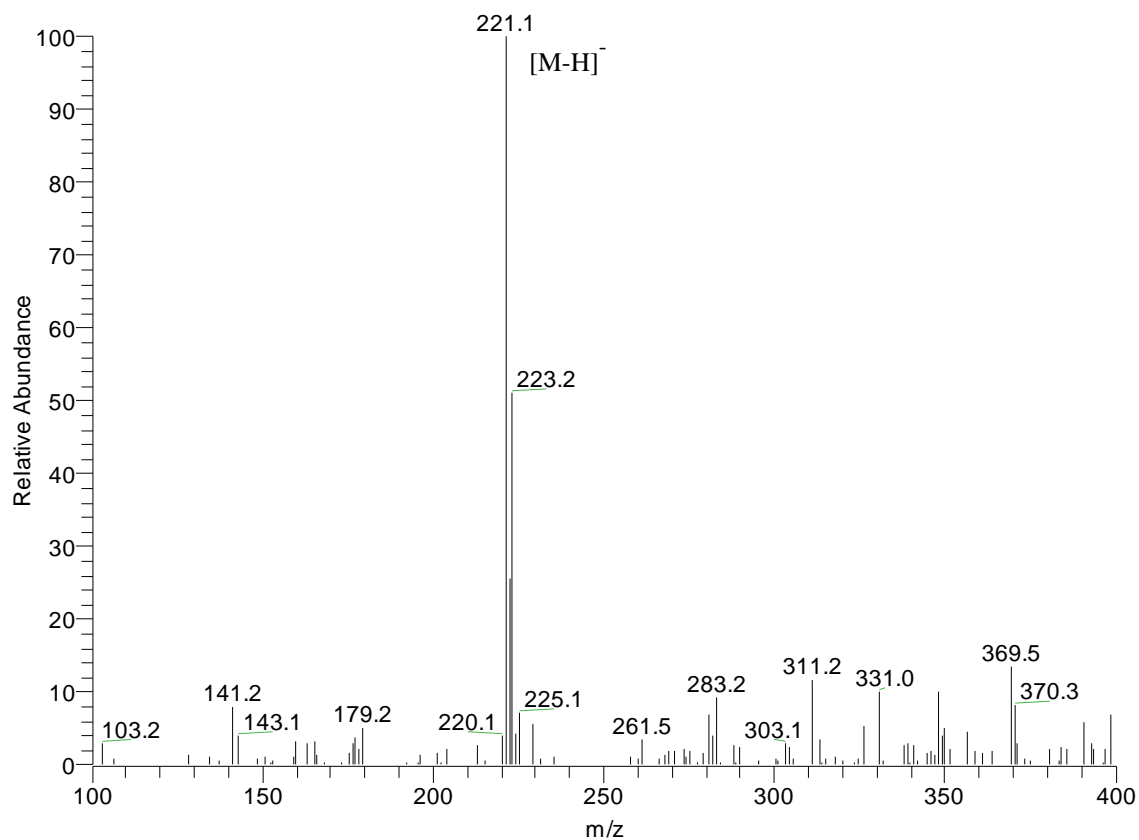
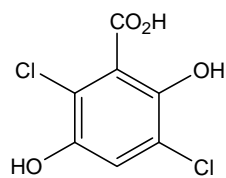
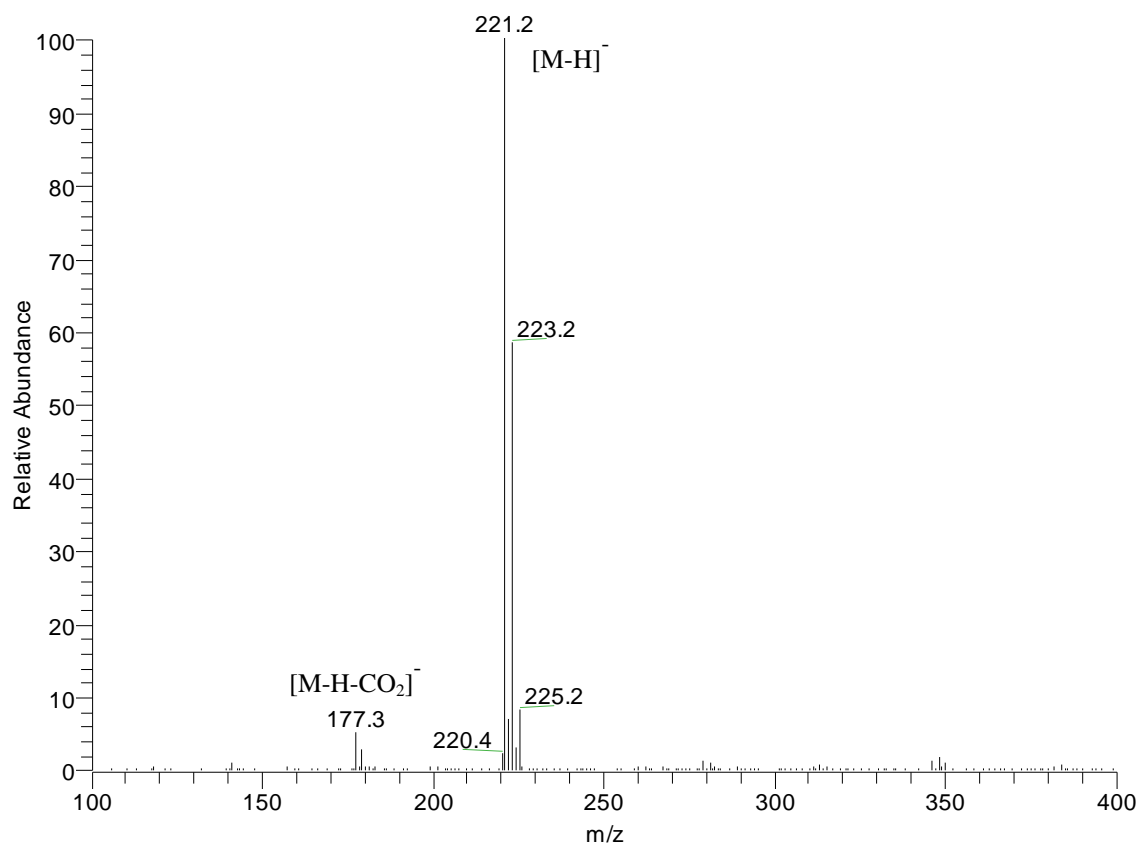


Figure 33. Negative Ion Electrospray Mass Spectrum of the Acid Hydrolysate of Peak 3 from POE-T Forage



DCGA
Nominal Mass 222

Figure 34. Negative Ion Electrospray Mass Spectrum of DCGA Reference Standard



DCGA (IV)
Nominal Mass 222

Figure 35. HPLC Analysis of the Methylated Acid Hydrolysate of Peak 3 from POE-T Forage (top) and Comparison to Trimethyl DCGA Reference Standard (bottom) Using HPLC Method B

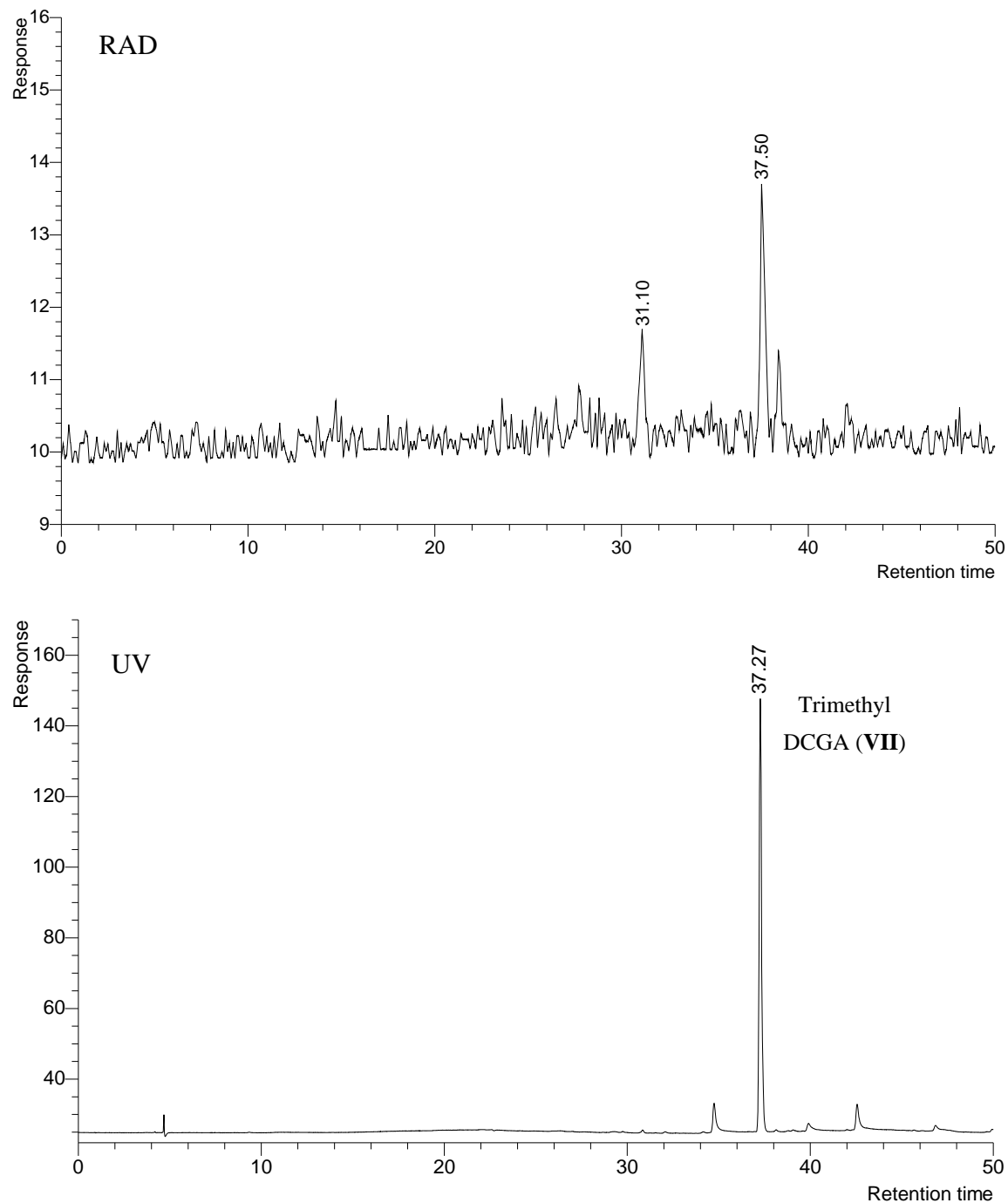
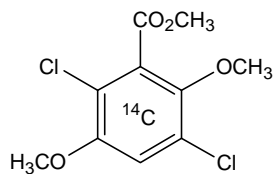
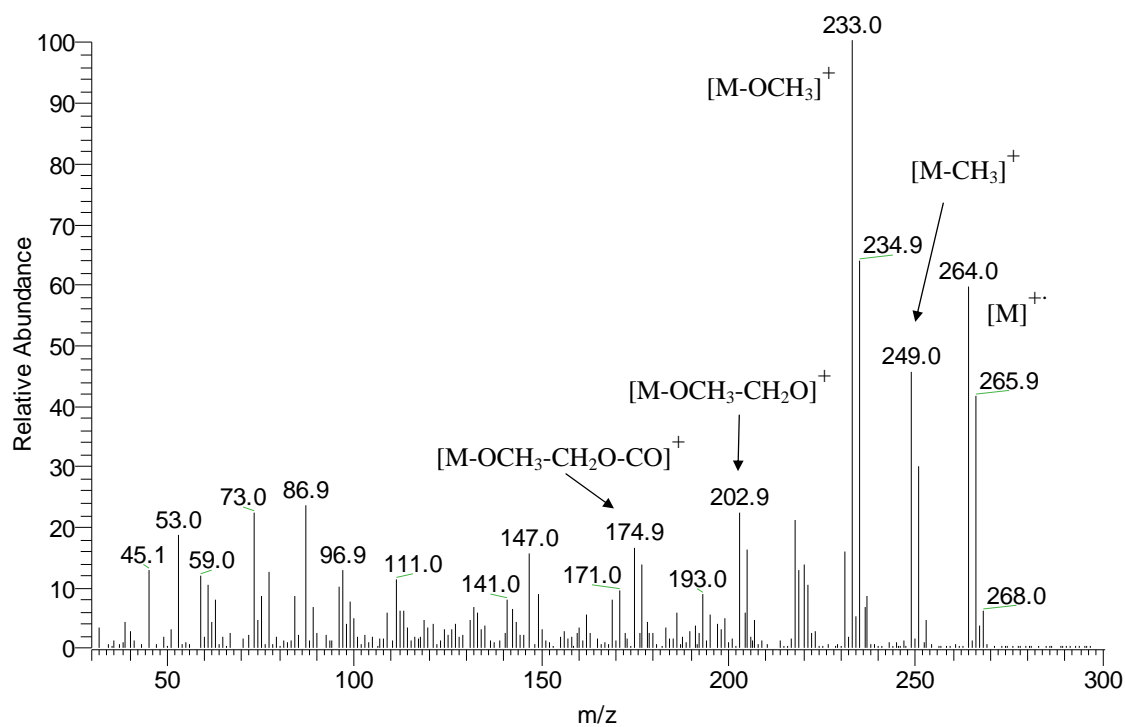
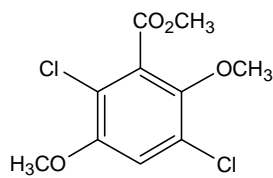
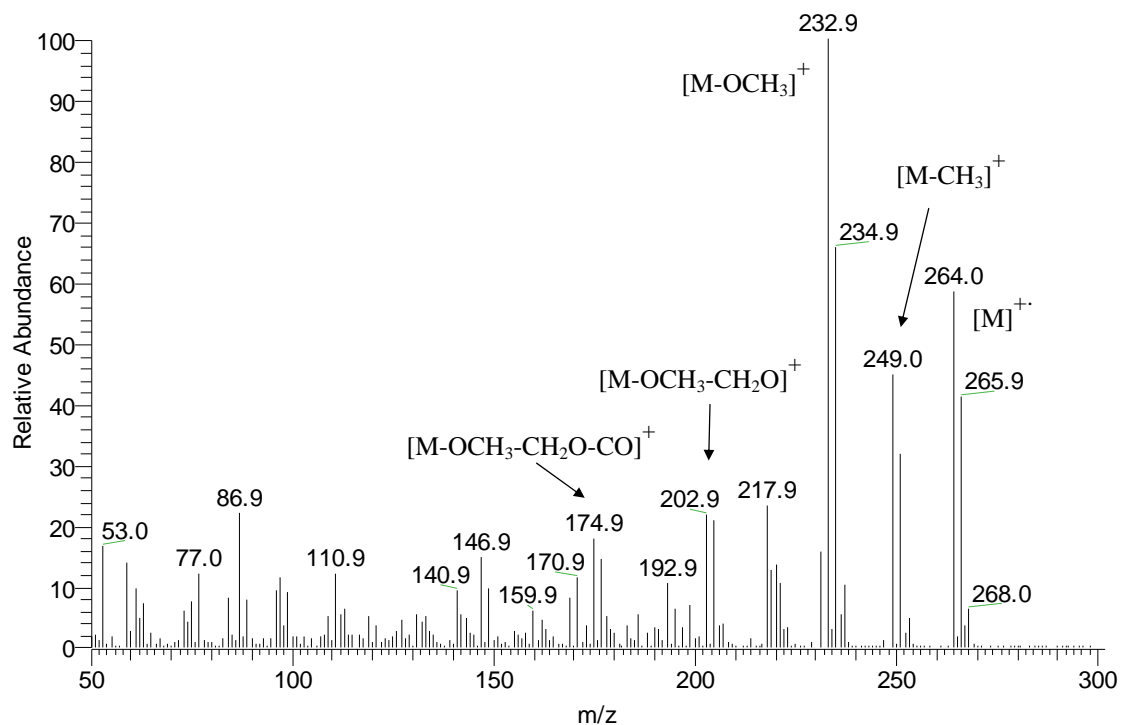


Figure 36. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of the Methylated Acid Hydrolysate of Peak 3 from POE-T Forage



Trimethyl DCGA
Nominal Mass 264

Figure 37. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of Trimethyl DCGA Reference Standard



Trimethyl DCGA (**VII**)
Nominal Mass 264

Figure 38. HPLC Analysis of the Methylated Acid Hydrolysate of Peak 3 from POE-T Forage and Comparison to 5-Hydroxydicamba Methyl Ester Reference Standard Mixture Using HPLC Method B

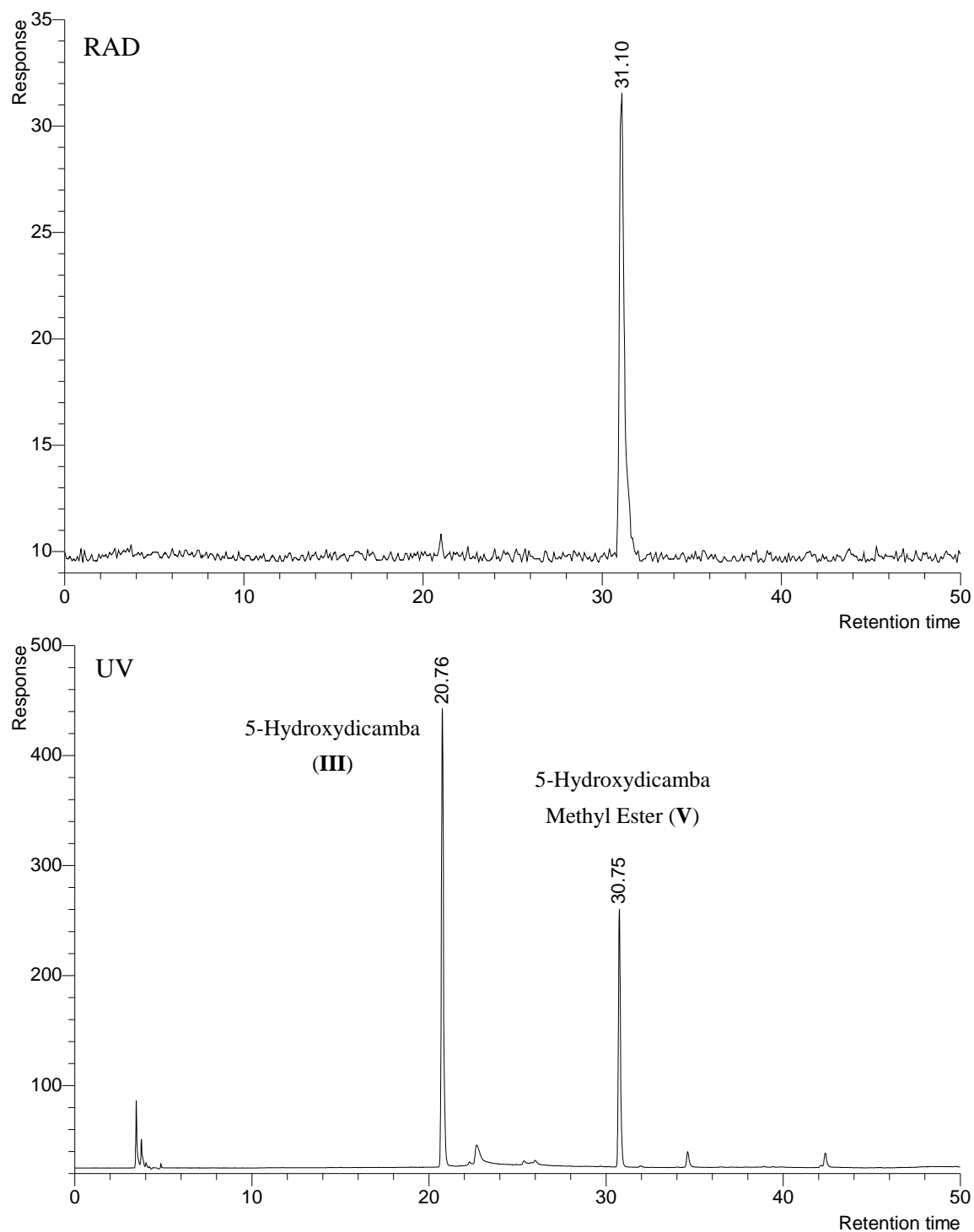


Figure 39. HPLC/RAD Analysis of the Methylated and Acetylated Hydrolysate of Peak 3 from POE-T Forage Formed by Methylation, Acid Hydrolysis and Acetylation Using HPLC Method B

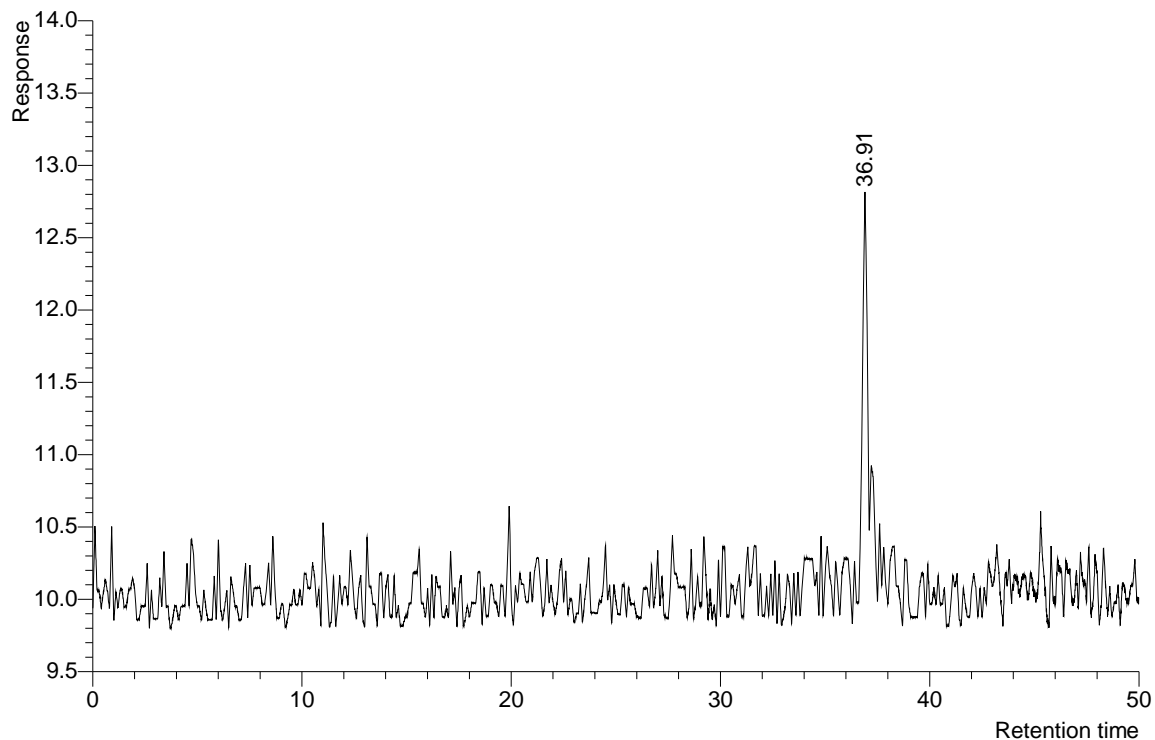


Figure 40. Positive Ion GC/EI/MS Selected Ion Chromatograms (m/z 250) for the Methylated and Acetylated Hydrolysate of Peak 3 from POE-T Forage Formed by Methylation, Acid Hydrolysis and Acetylation (top) and 5-Acetyloxydicamba Methyl Ester Reference Standard (bottom)

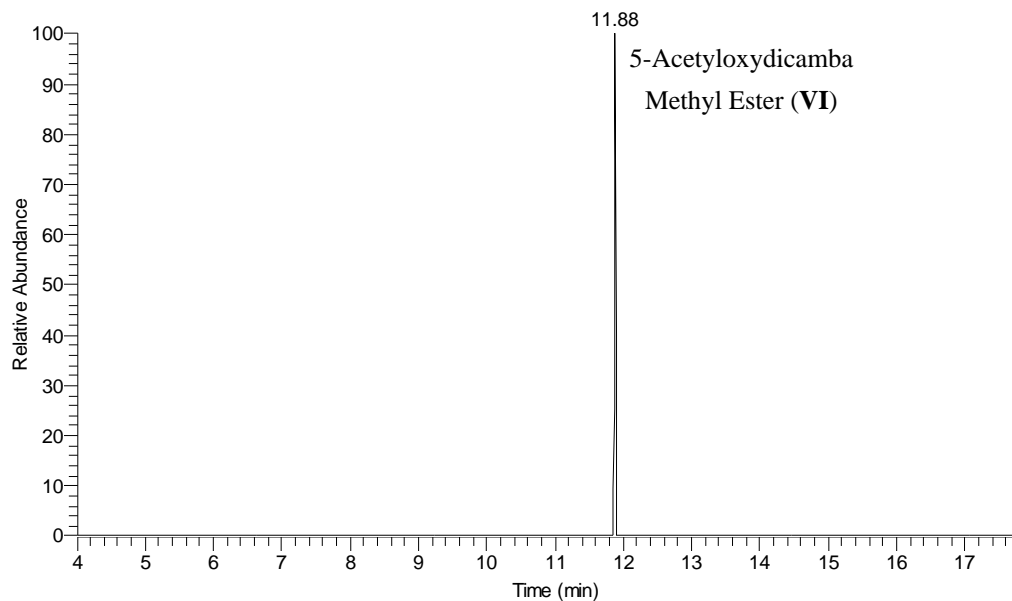
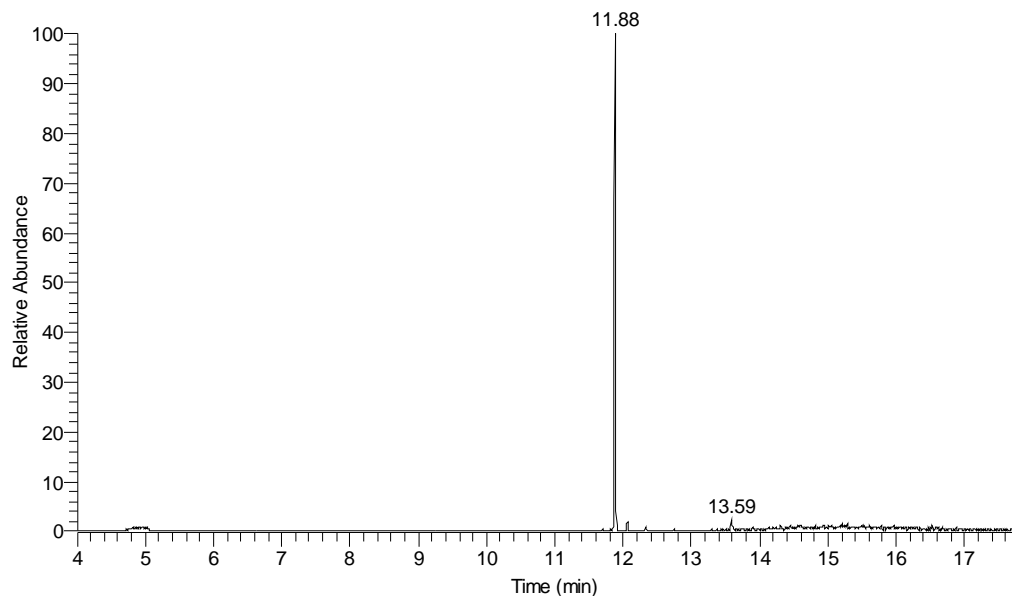
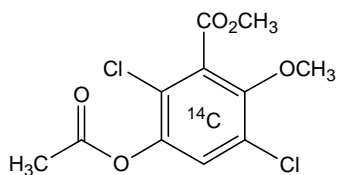
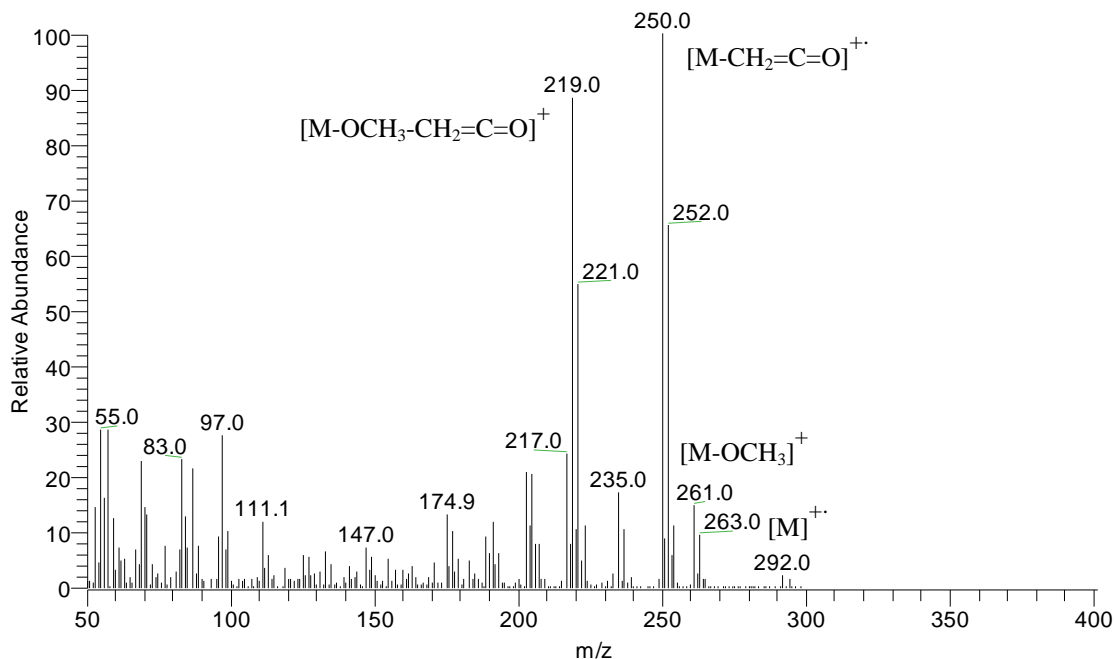
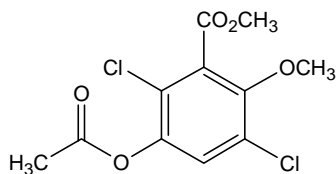
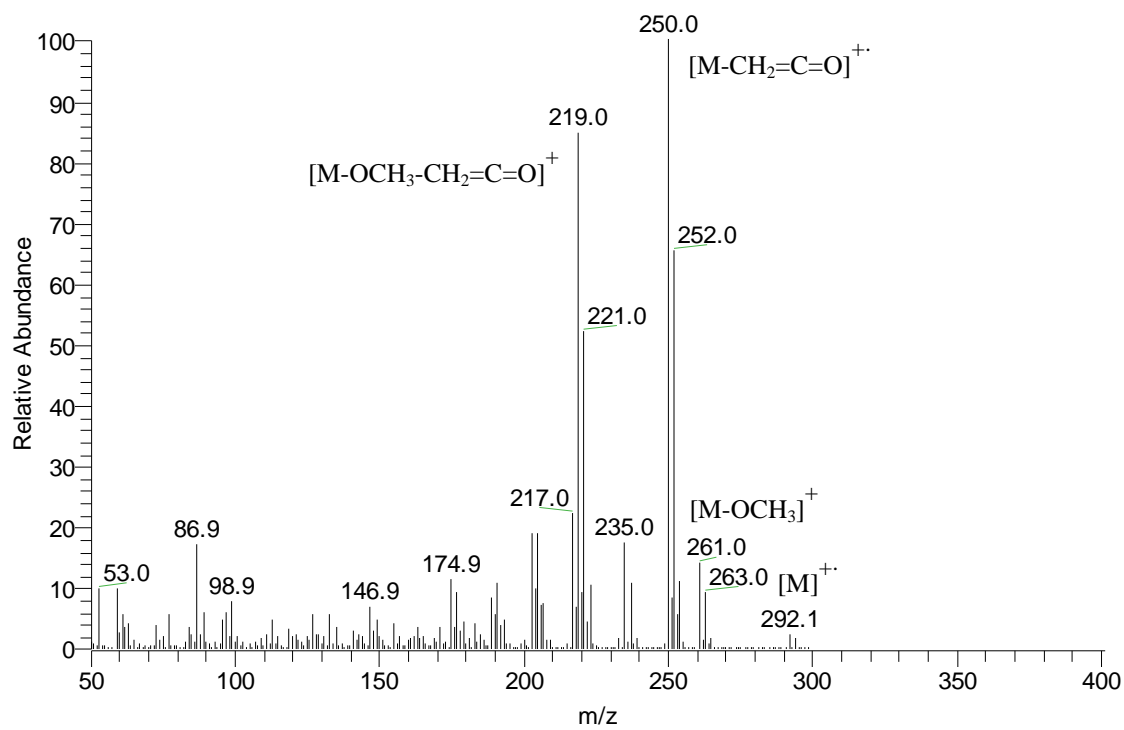


Figure 41. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of the Methylated and Acetylated Hydrolysate of Peak 3 from POE-T Forage Formed by Methylation, Acid Hydrolysis and Acetylation



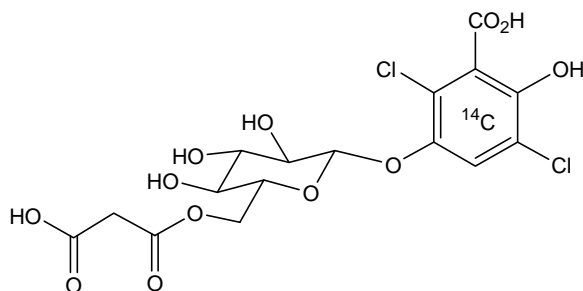
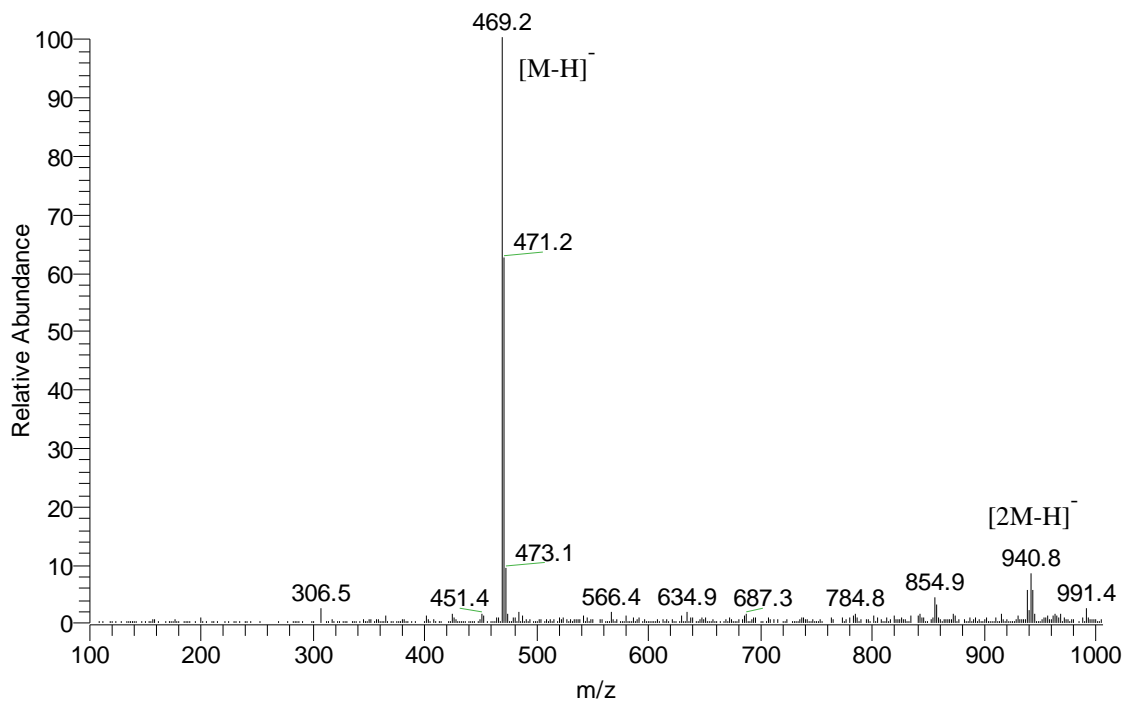
5-Acetyloxydicamba, Methyl Ester
Nominal Mass 292

Figure 42. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of 5-Acetyloxydicamba Methyl Ester Reference Standard



5-Acetyloxydicamba, Methyl Ester (VI)
Nominal Mass 292

Figure 43. Negative Ion Electrospray Mass Spectrum of Peak 8 from POE-T Forage



DCGA Malonylglucoside (8)
Nominal Mass 470

Figure 44. HPLC/RAD of the Mild Base Hydrolysate of Peak 8 from POE-T Forage (top) and Comparison with Peak 3 from POE-T Forage (bottom) Using HPLC Method B

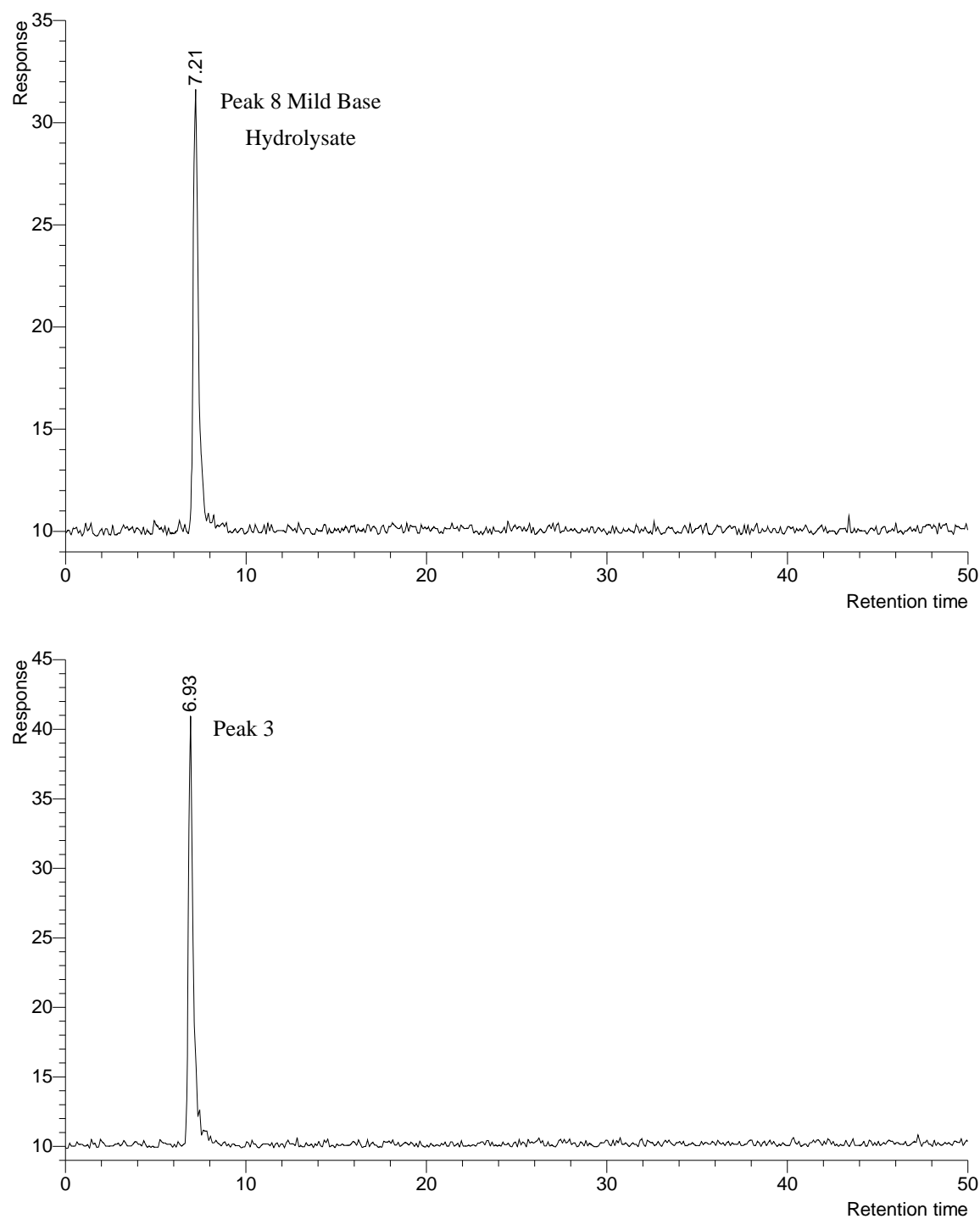


Figure 45. HPLC/RAD of the Mild Base Hydrolysate of Peak 8 from POE-T Forage (top) and Comparison with Peak 3 from POE-T Forage (bottom) Using HPLC Method C

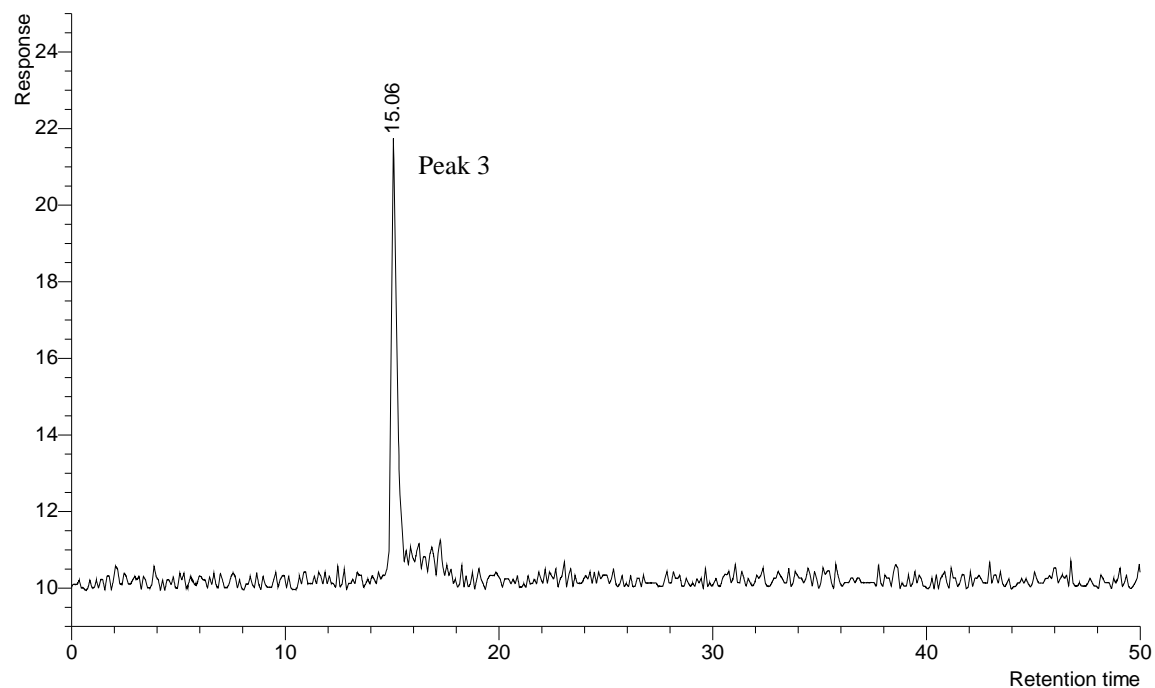
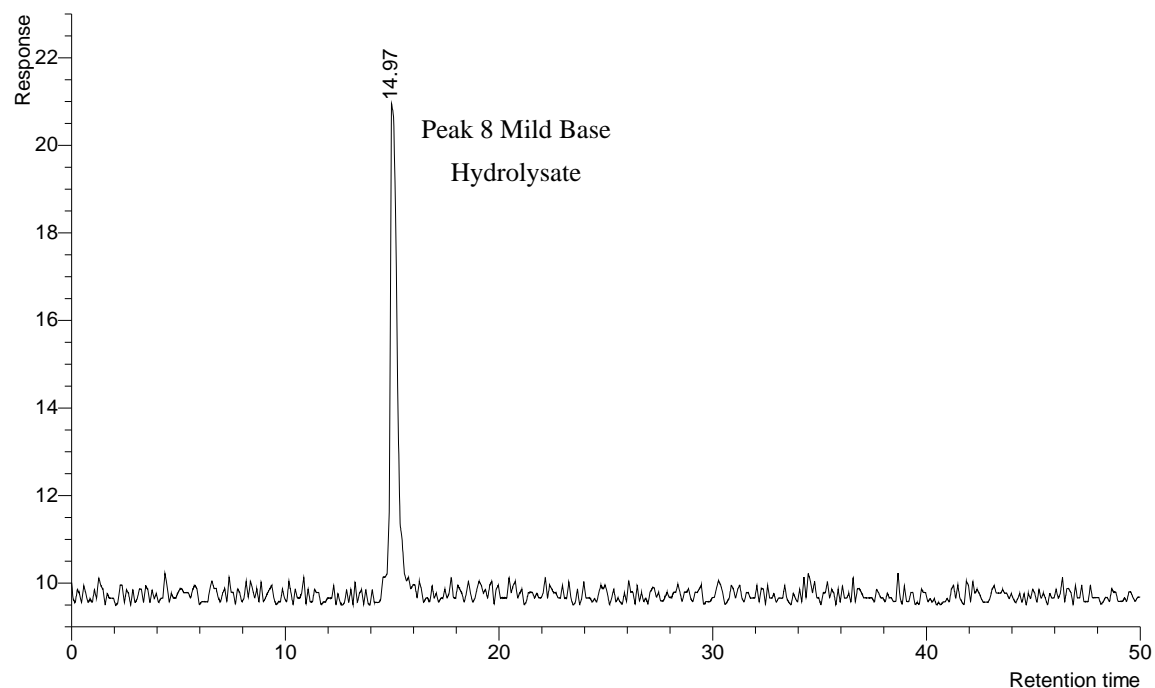
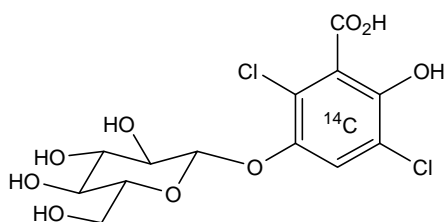
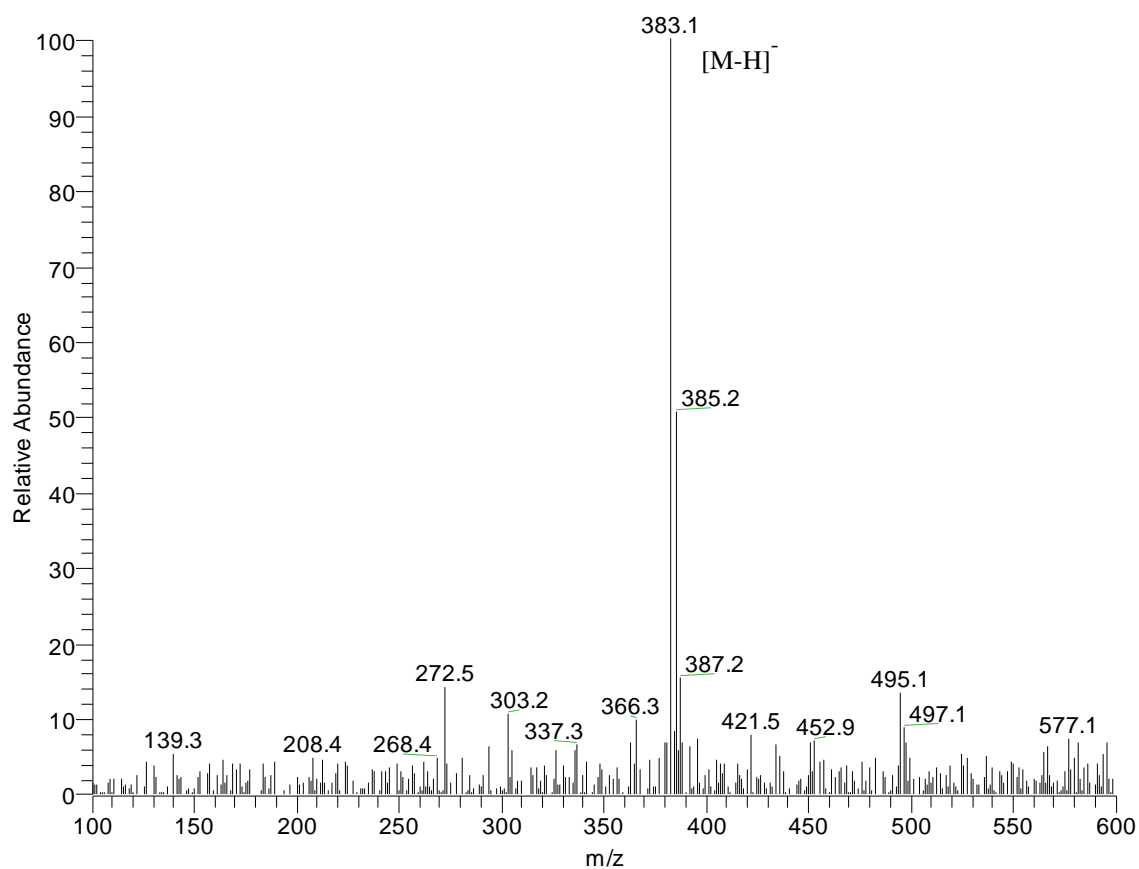


Figure 46. Negative Ion Electrospray Mass Spectrum of the Mild Base Hydrolysate of Peak 8 from POE-T Forage



DCGA Glucoside
Nominal Mass 384

Figure 47. HPLC Coinjection Analysis of the Hydrolysate of Peak 8 from POE-T Forage after Mild Base and Acid Hydrolysis (top) and DCGA Reference Standard (bottom) Using HPLC Method B

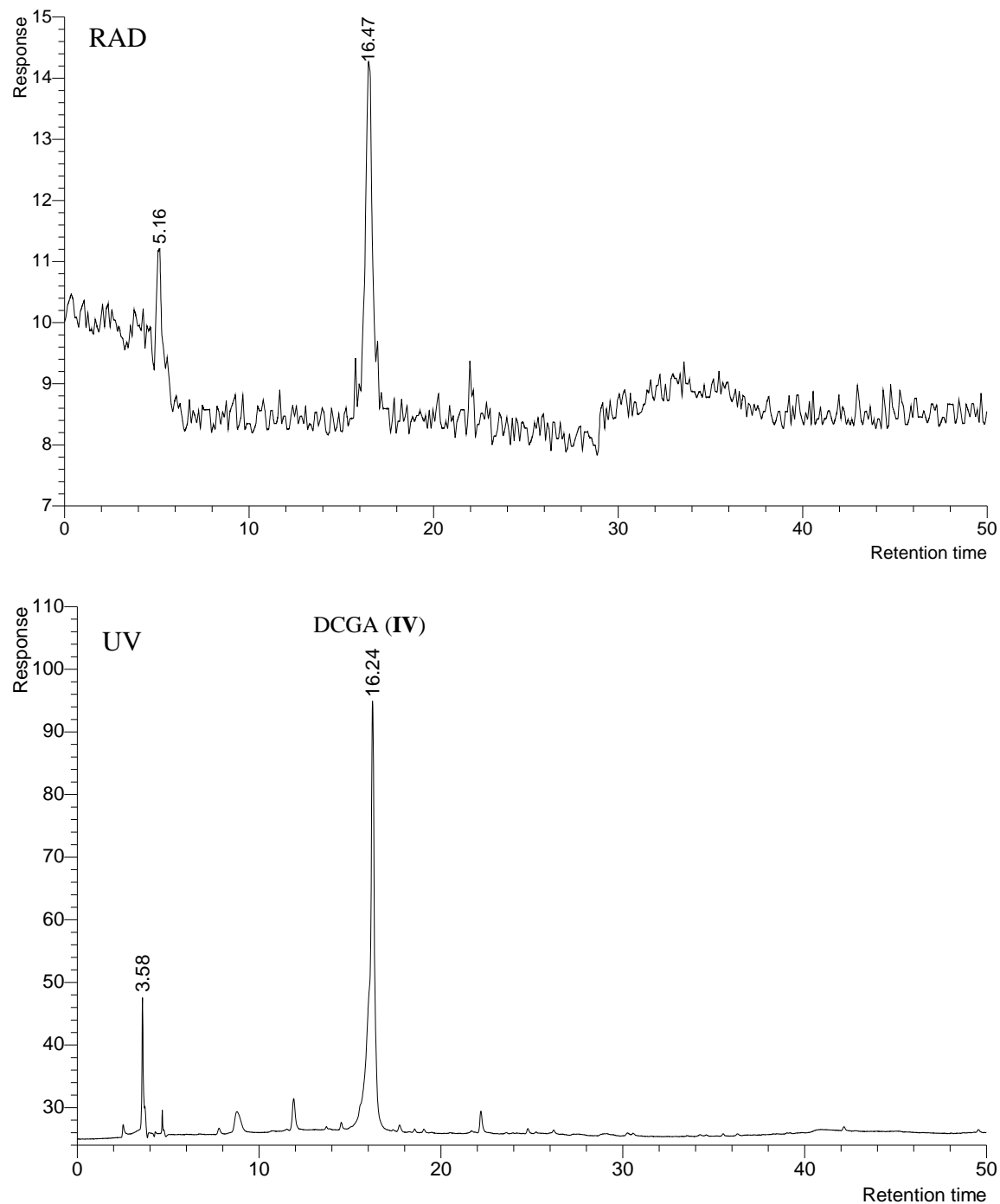


Figure 48. HPLC Analysis of the Methylated Mild Base and Acid Hydrolysate of Peak 8 from POE-T Forage (top) and Trimethyl DCGA Reference Standard (bottom) Using HPLC Method B

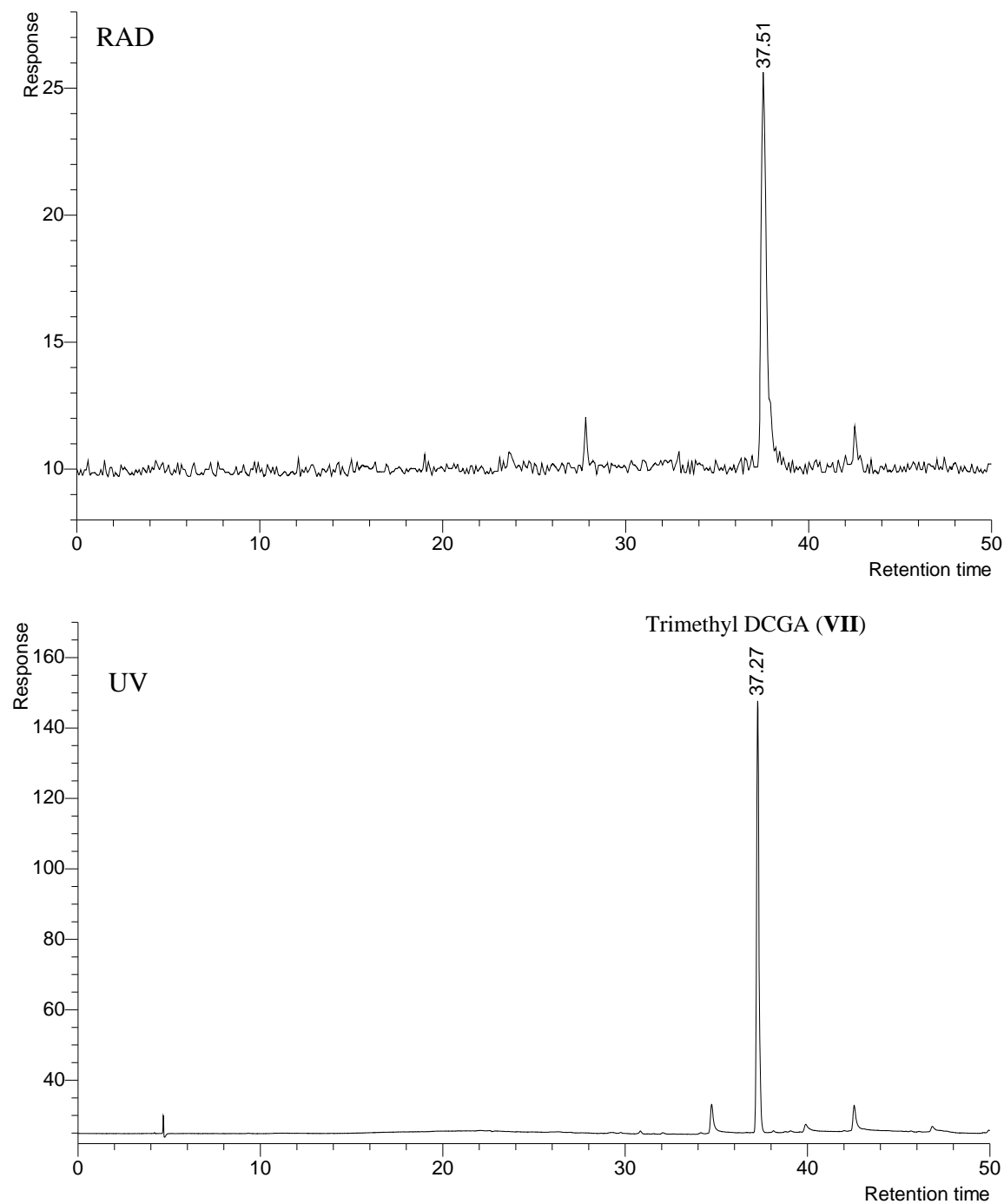
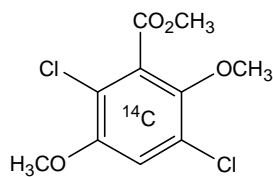
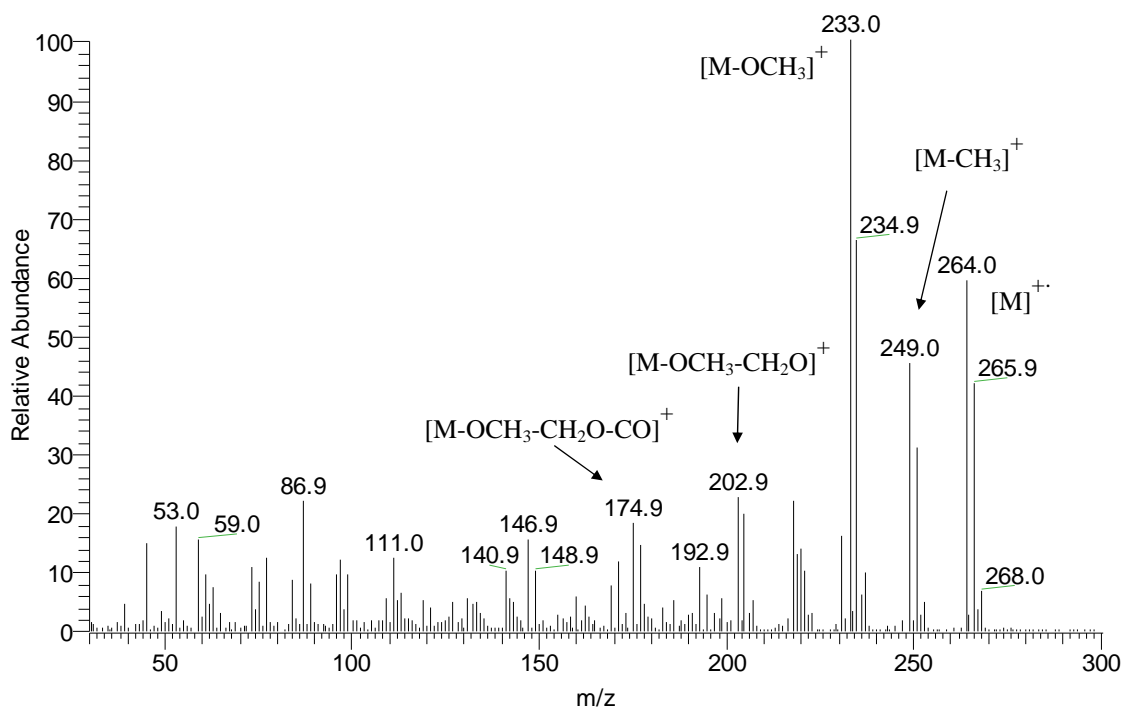
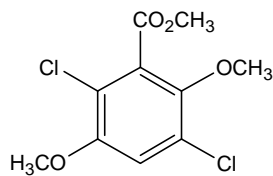
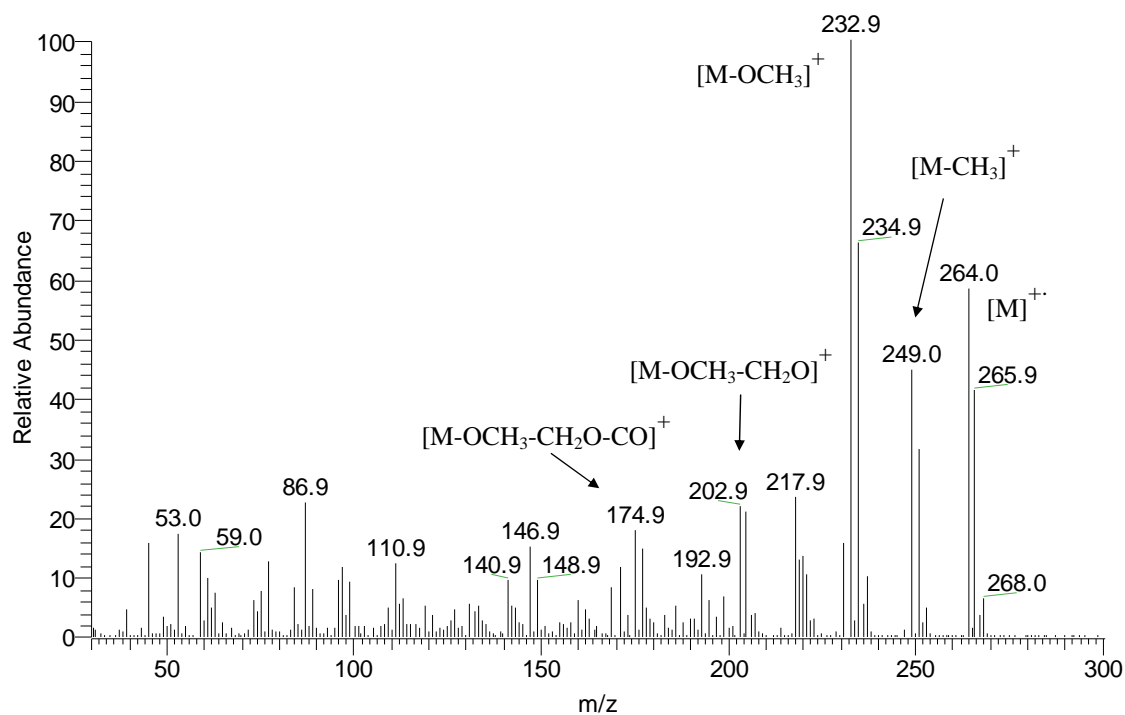


Figure 49. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of the Methylated Mild Base and Acid Hydrolysate of Peak 8 from POE-T Forage



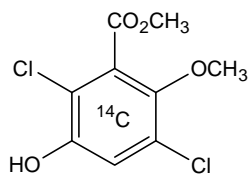
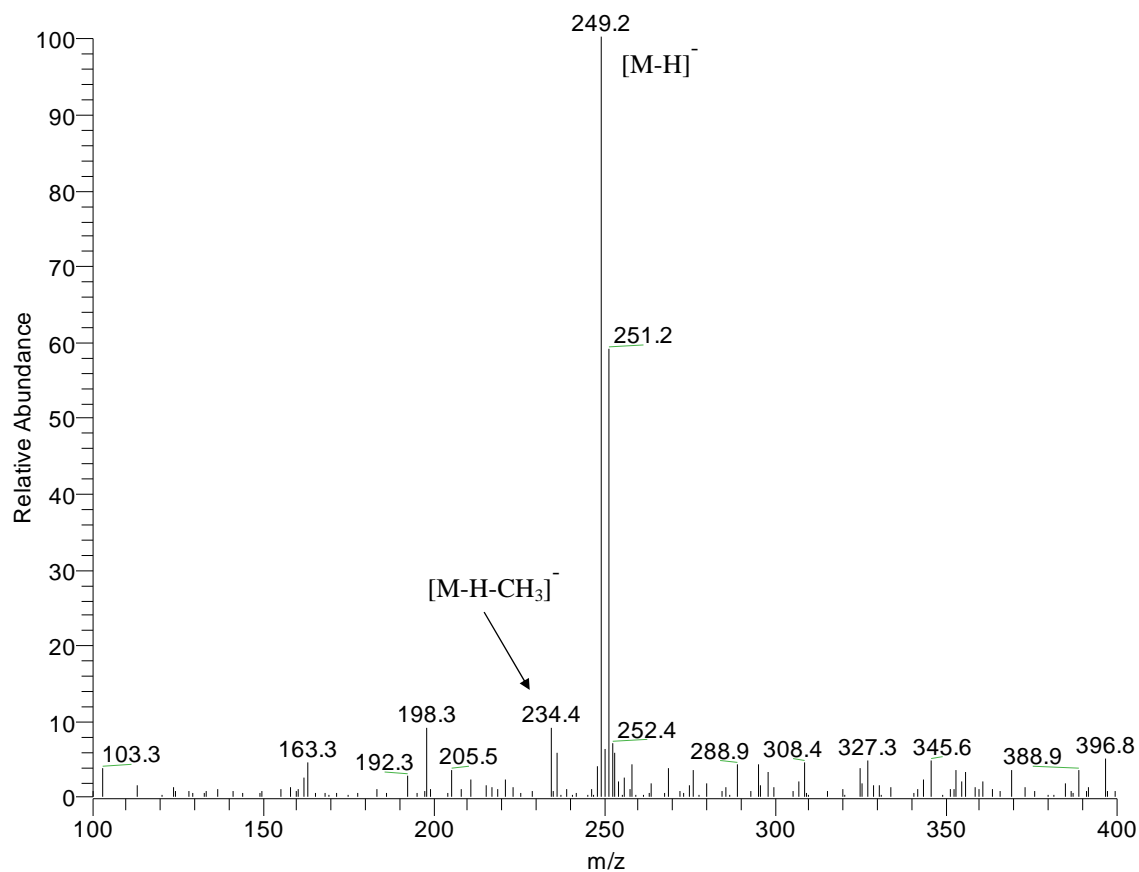
Trimethyl DCGA
Nominal Mass 264

Figure 50. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of Trimethyl DCGA Reference Standard



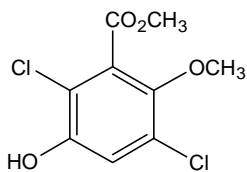
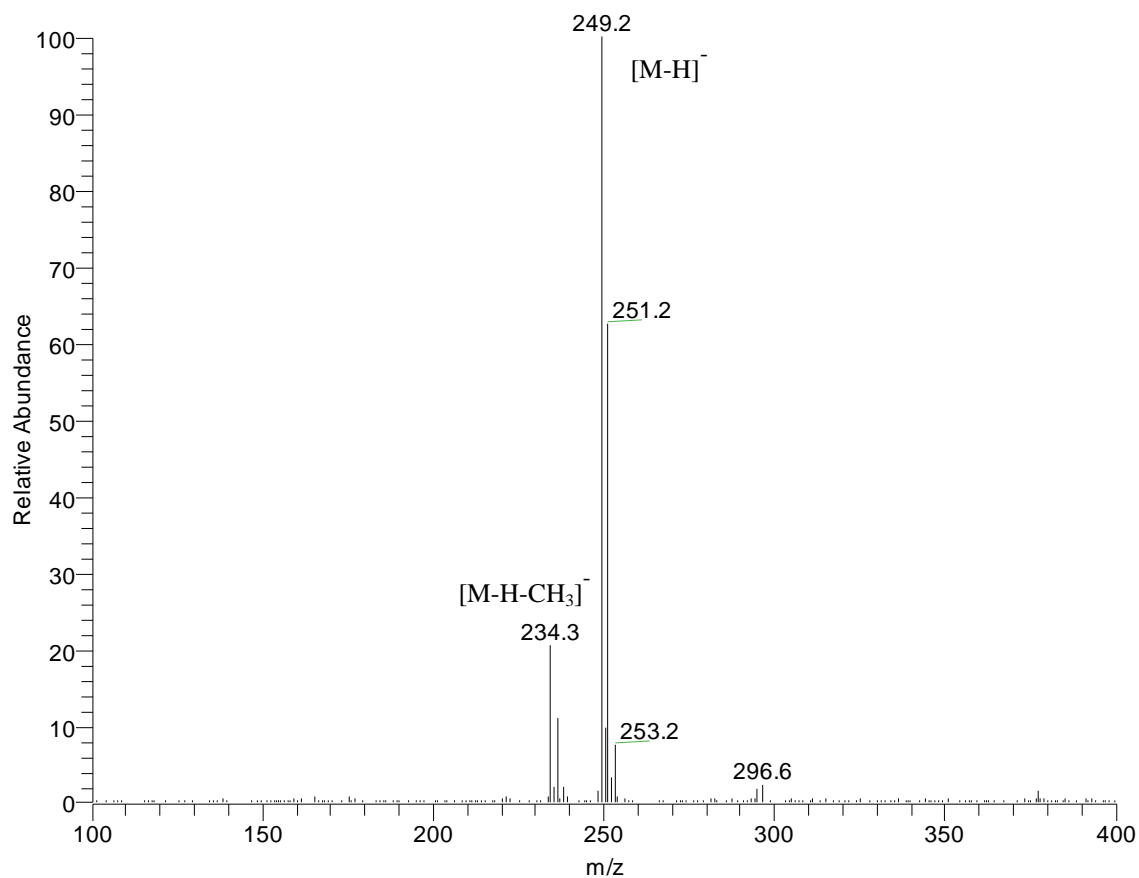
Trimethyl DCGA (VII)
Nominal Mass 264

Figure 51. Negative Ion Electrospray Mass Spectrum of the Mild Base and Acid Hydrolysate of Methylated Peak 8 from POE-T Forage



5-Hydroxydicamba, Methyl Ester
Nominal Mass 250

Figure 52. Negative Ion Electrospray Mass Spectrum of 5-Hydroxydicamba Methyl Ester Reference Standard



5-Hydroxydicamba, Methyl Ester (V)
Nominal Mass 250

Figure 53. HPLC Coinjection Analysis of the Mild Base and Acid Hydrolysate of Methylated Peak 8 from POE-T Forage and 5-Hydroxydicamba Methyl Ester Reference Standard (Coelution and Alone) Using HPLC Method B

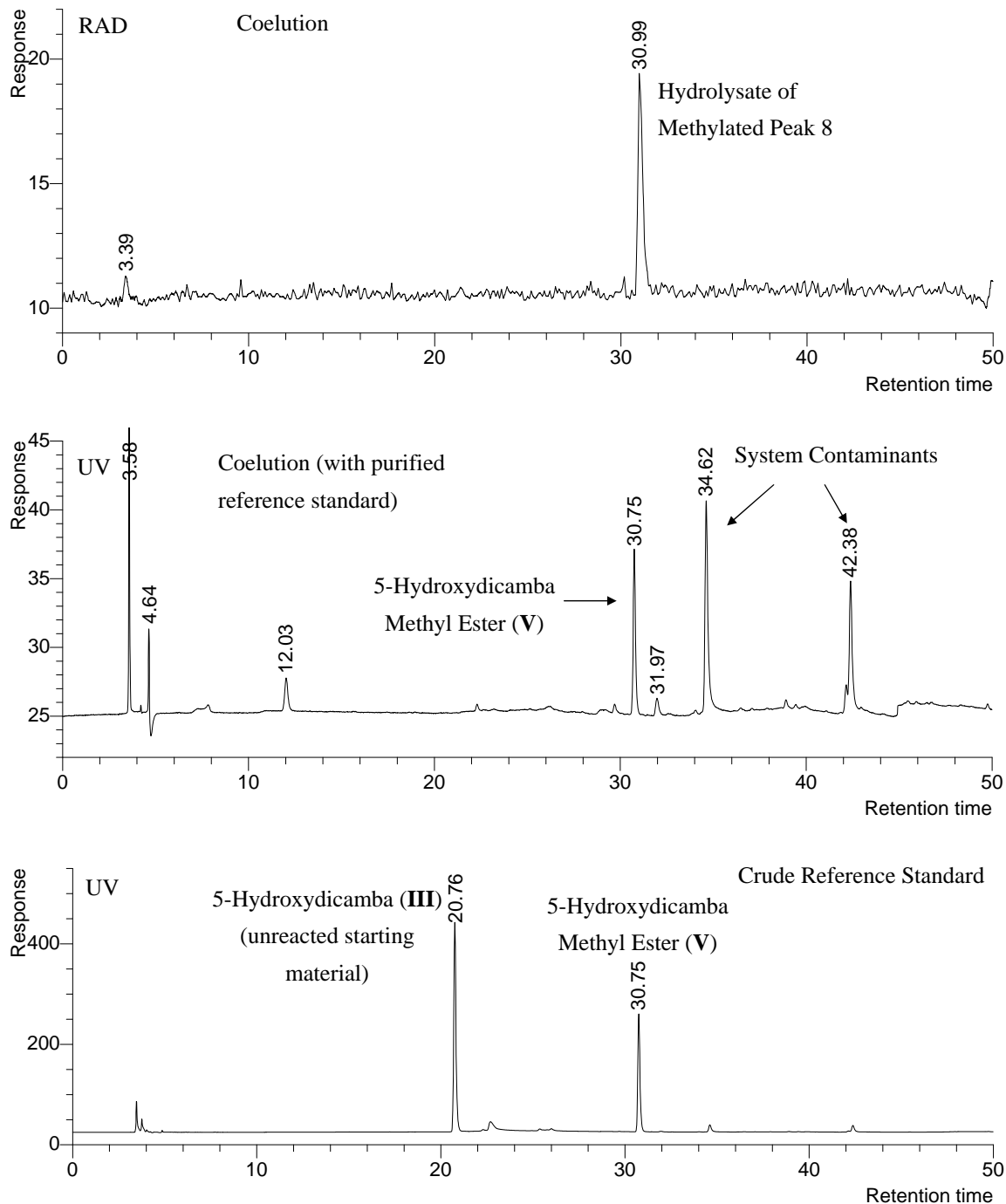


Figure 54. HPLC/RAD Analysis of the Methylated and Acetylated Hydrolysate of Peak 8 from POE-T Forage formed by Mild Base Hydrolysis, Methylation, Acid Hydrolysis and Acetylation Using HPLC Method B

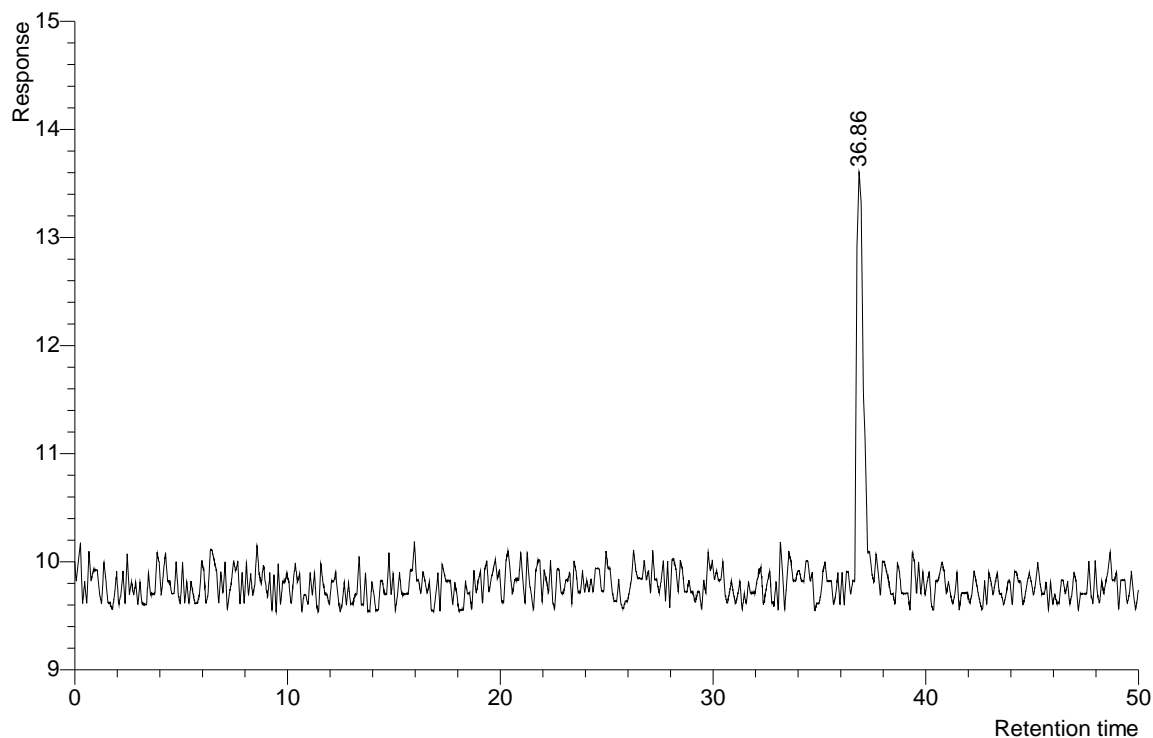


Figure 55. GC/EI/MS Selected Ion Chromatograms (m/z 250) for the Methylated and Acetylated Hydrolysate of Peak 8 from POE-T Forage Formed by Mild Base Hydrolysis, Methylation, Acid Hydrolysis and Acetylation (top) and 5-Acetyloxydicamba Methyl Ester Reference Standard (bottom)

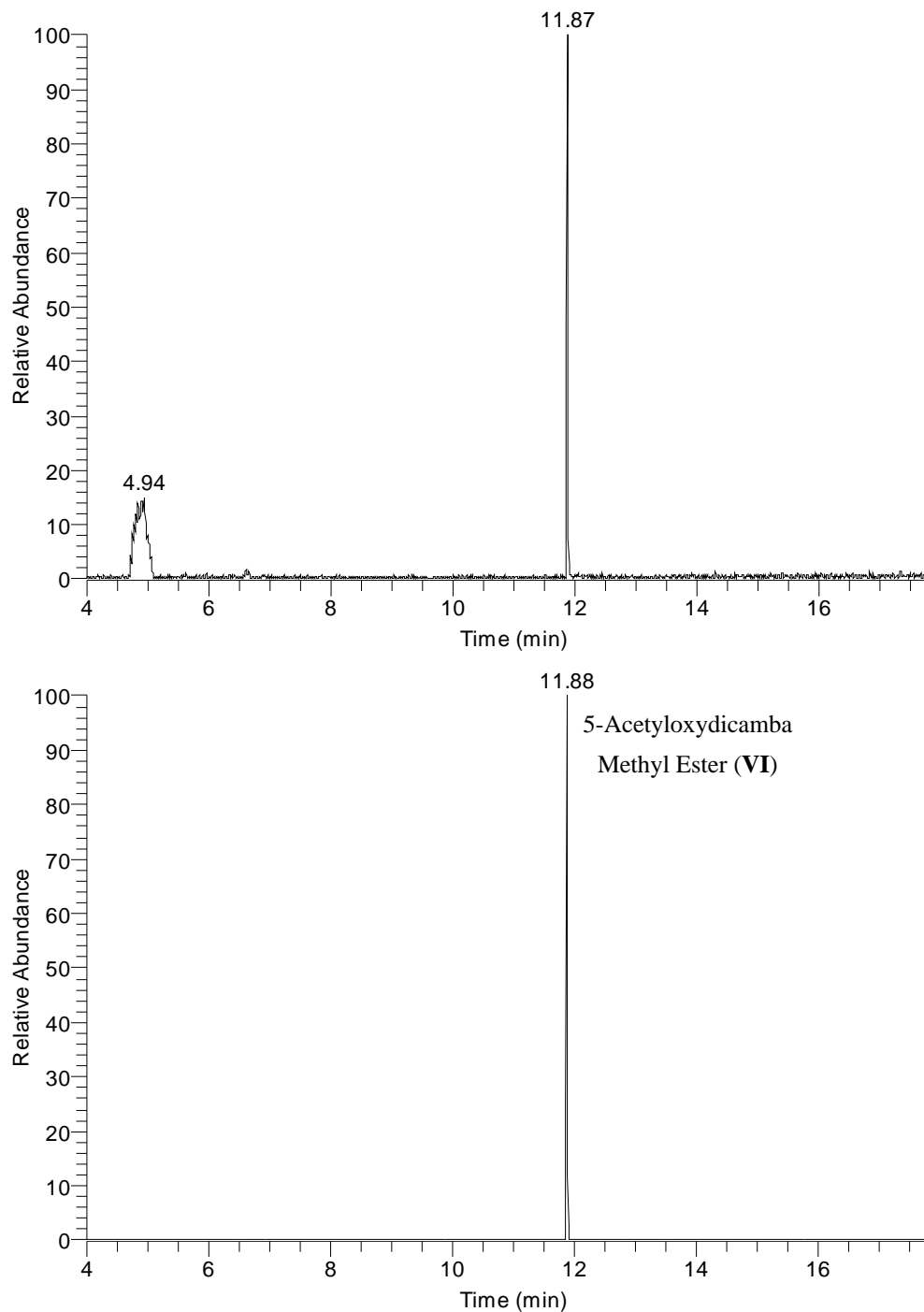
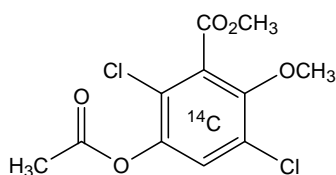
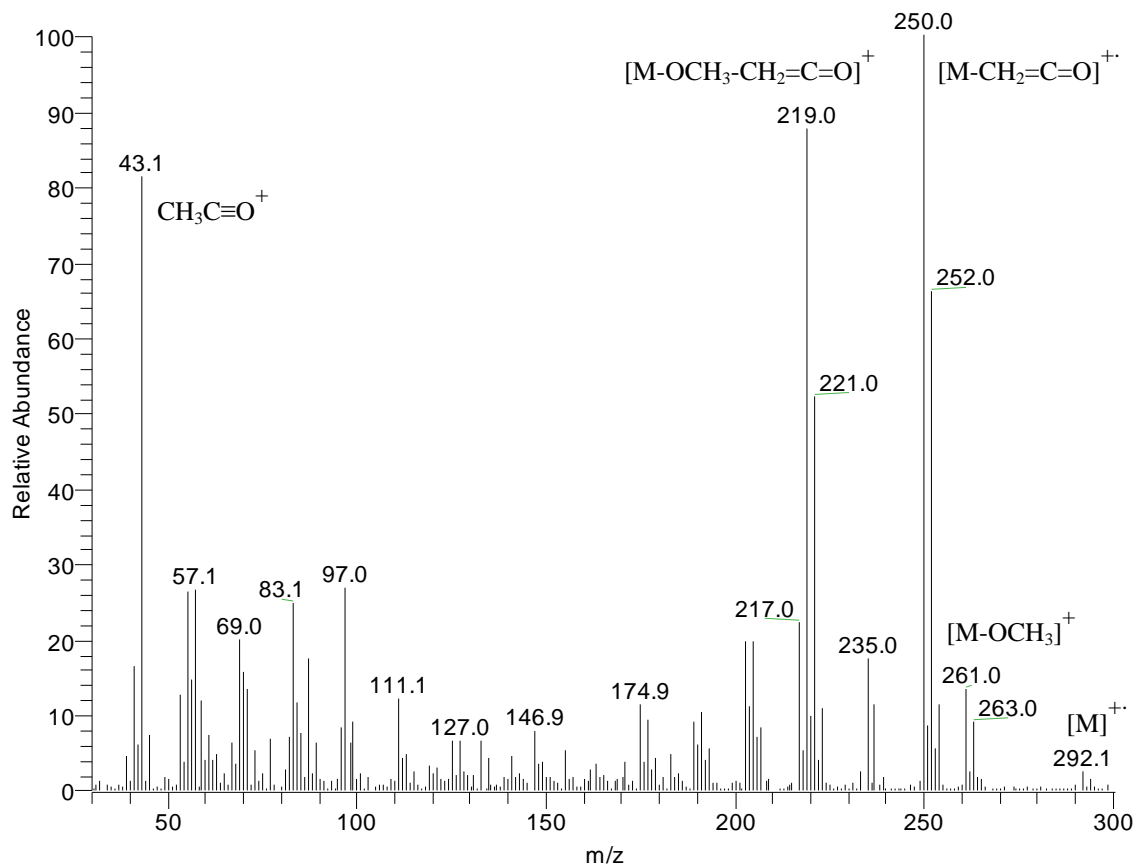
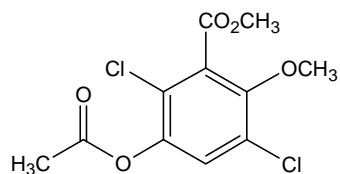
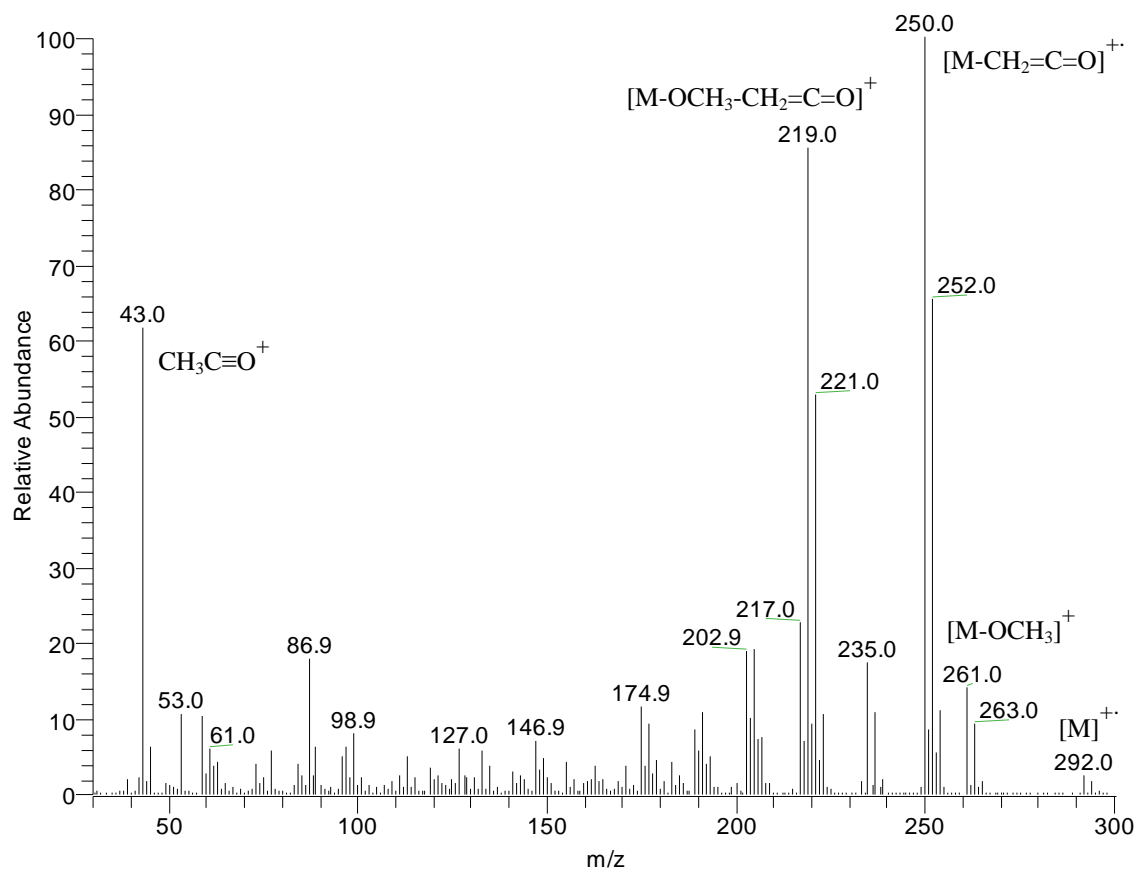


Figure 56. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of the Methylated and Acetylated Hydrolysate of Peak 8 from POE-T Forage Formed by Mild Base Hydrolysis, Methylation, Acid Hydrolysis and Acetylation



5-Acetyloxydicamba, Methyl Ester
Nominal Mass 292

Figure 57. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of 5-Acetyloxydicamba Methyl Ester Reference Standard



5-Acetyloxydicamba, Methyl Ester (VI)
Nominal Mass 292

Figure 58. Positive Ion Electron Ionization (GC/EI) Extracted Ion Chromatograms (m/z 105) of the Butylated Derivative of the Mild Base Hydrolysate of Peak 8 (top) with Dibutyl Malonate Positive Control (middle) and Negative Control (bottom)

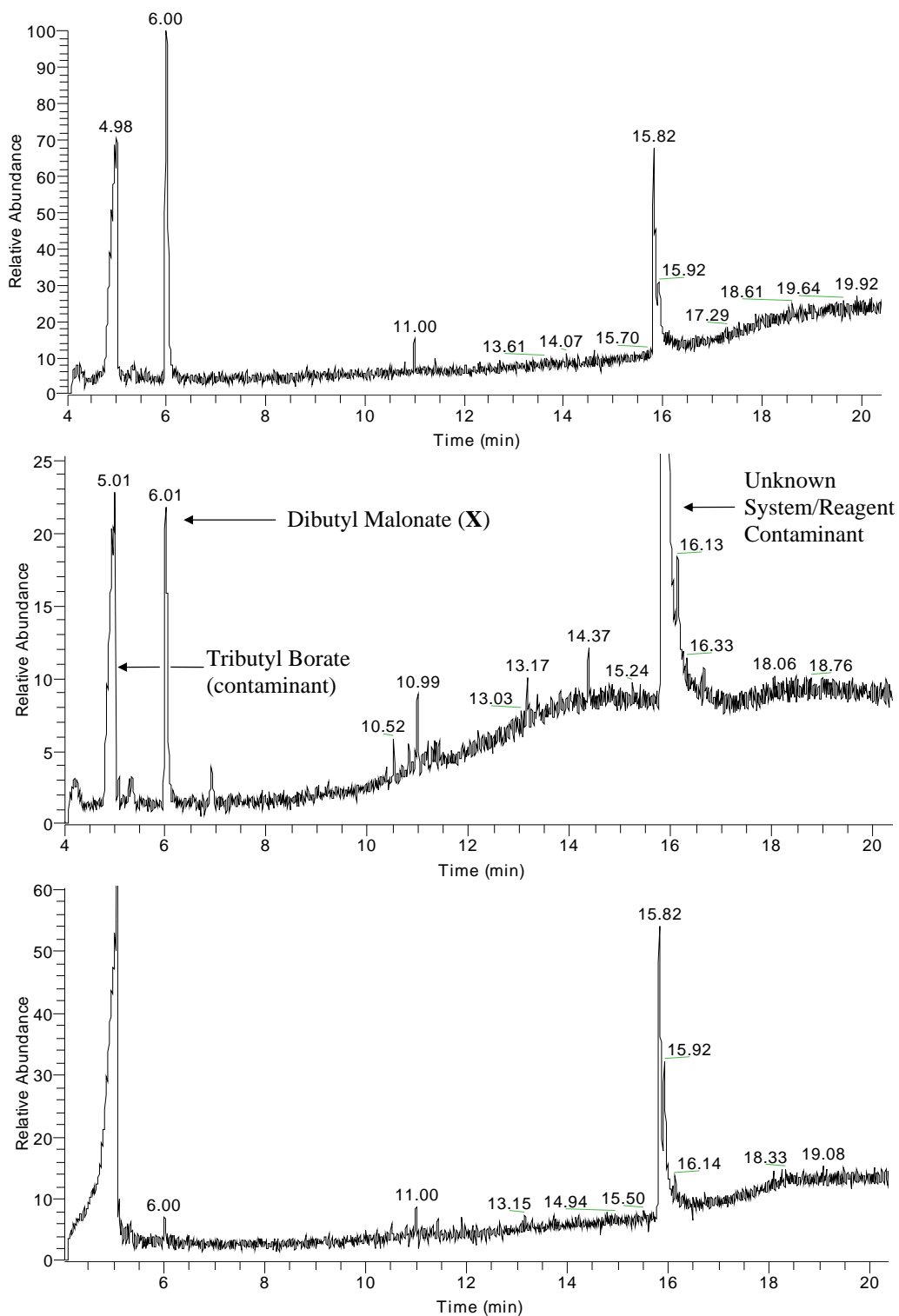


Figure 59. Positive Ion Electron Ionization (GC/EI) Mass Spectrum of the Butylated Derivative of the Mild Base Hydrolysate of Peak 8 from POE-T Forage (top) with Positive Control (Dibutyl Malonate Reference Standard, middle) and Negative Control (bottom)

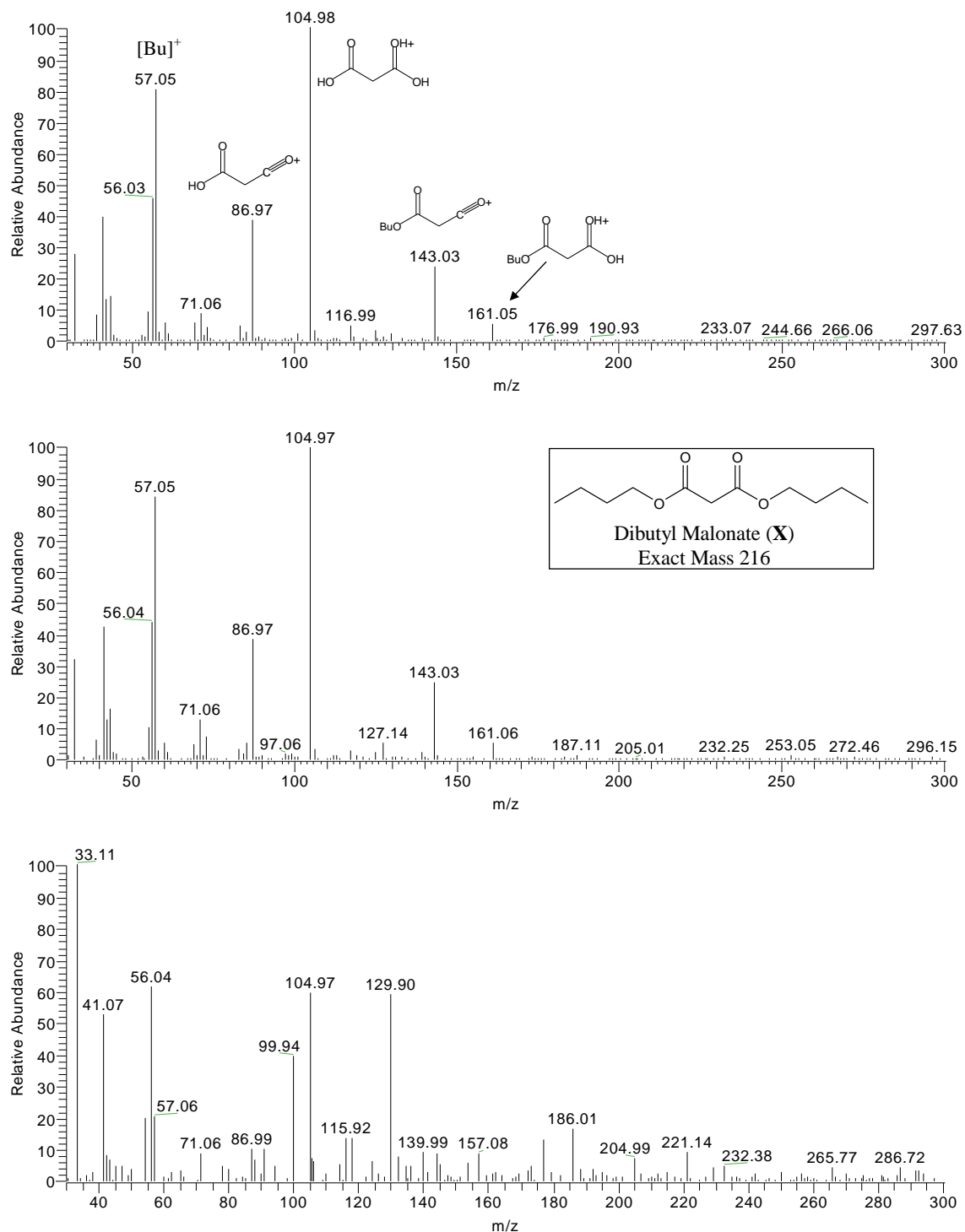


Figure 60. HPLC Coinjection Analysis of the Acid Hydrolysate of PRE-T Pre-forage Extract Concentrate with Reference Standards Using HPLC Method B

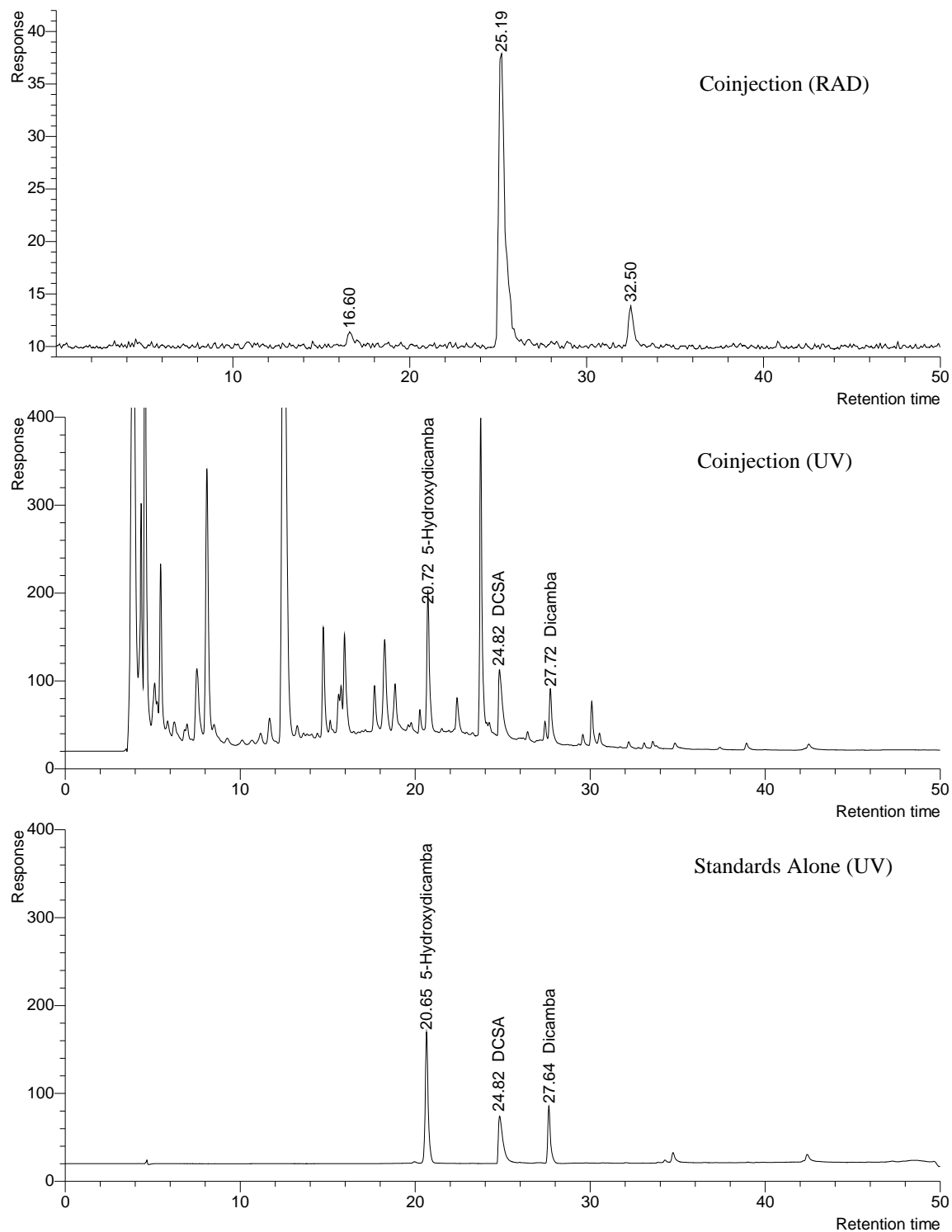


Figure 61. HPLC Coinjection Analysis of the Base Hydrolysate of PRE-T Pre-forage Extract Concentrate with Reference Standards Using HPLC Method B

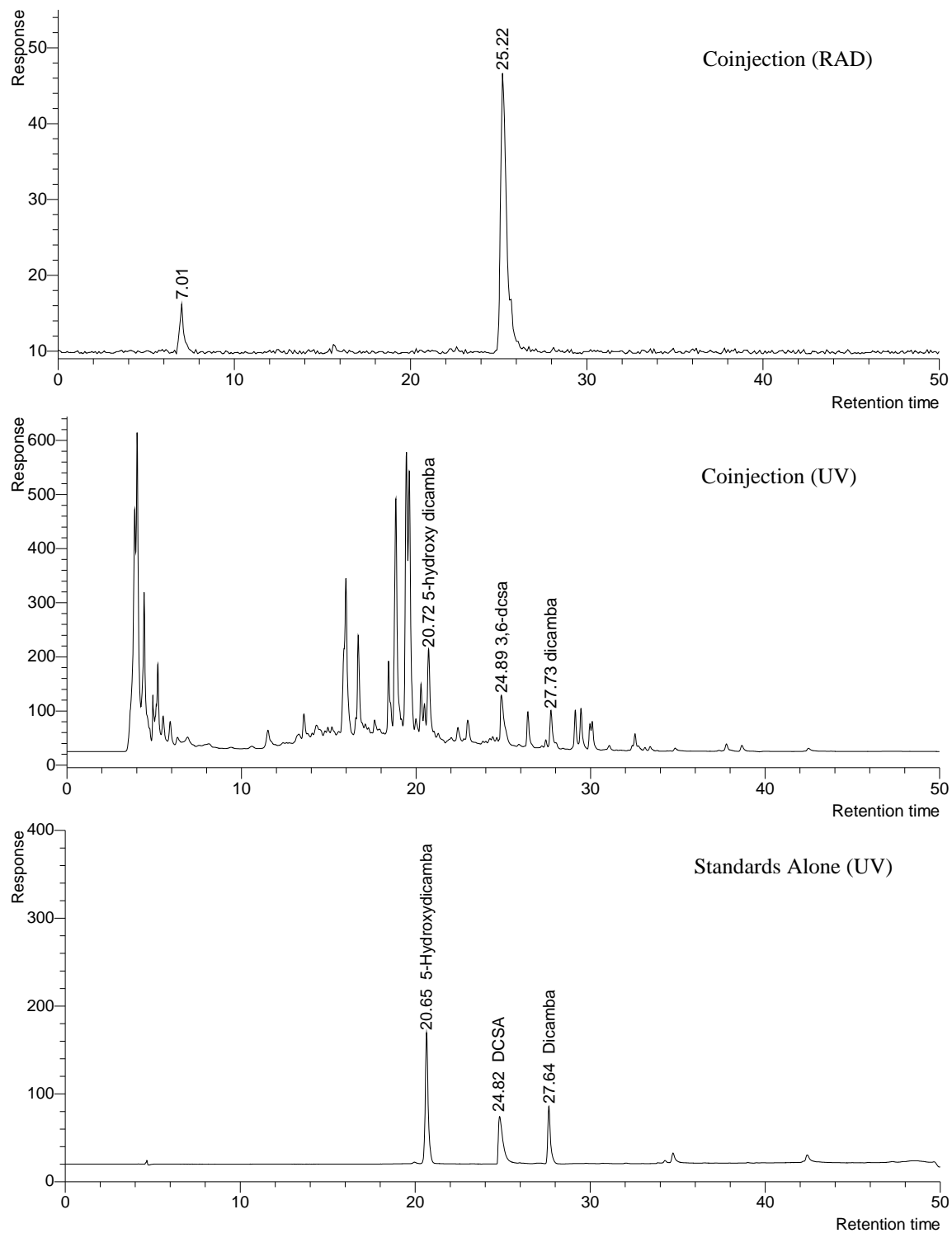


Figure 62. HPLC/RAD Analysis of the Acid Hydrolysate of Peak 9 from POE-T Forage (top) and Comparison to DCSA and Dicamba Reference Standards (bottom) Using HPLC Method B

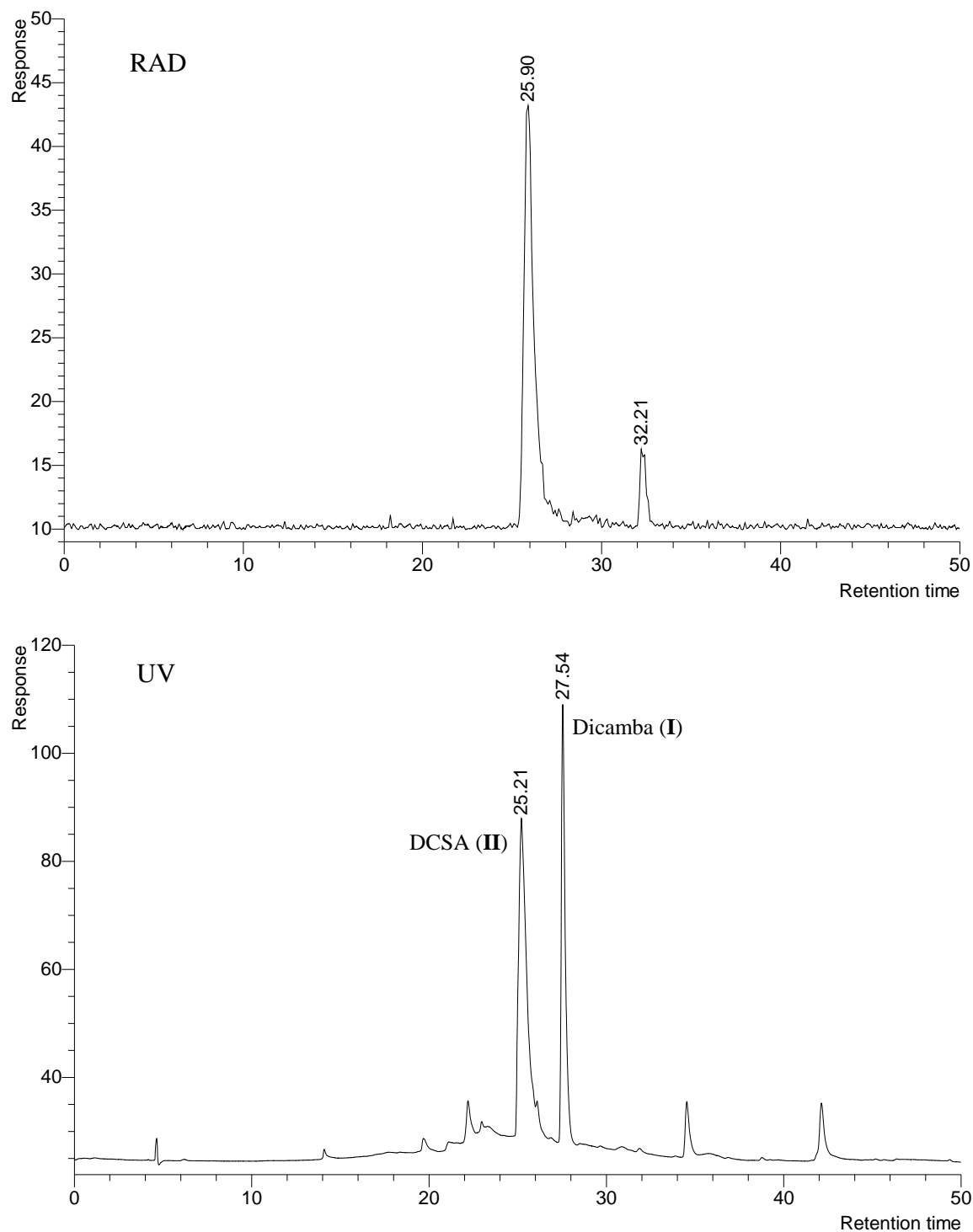
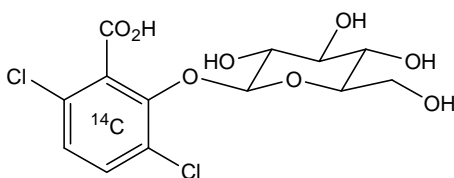
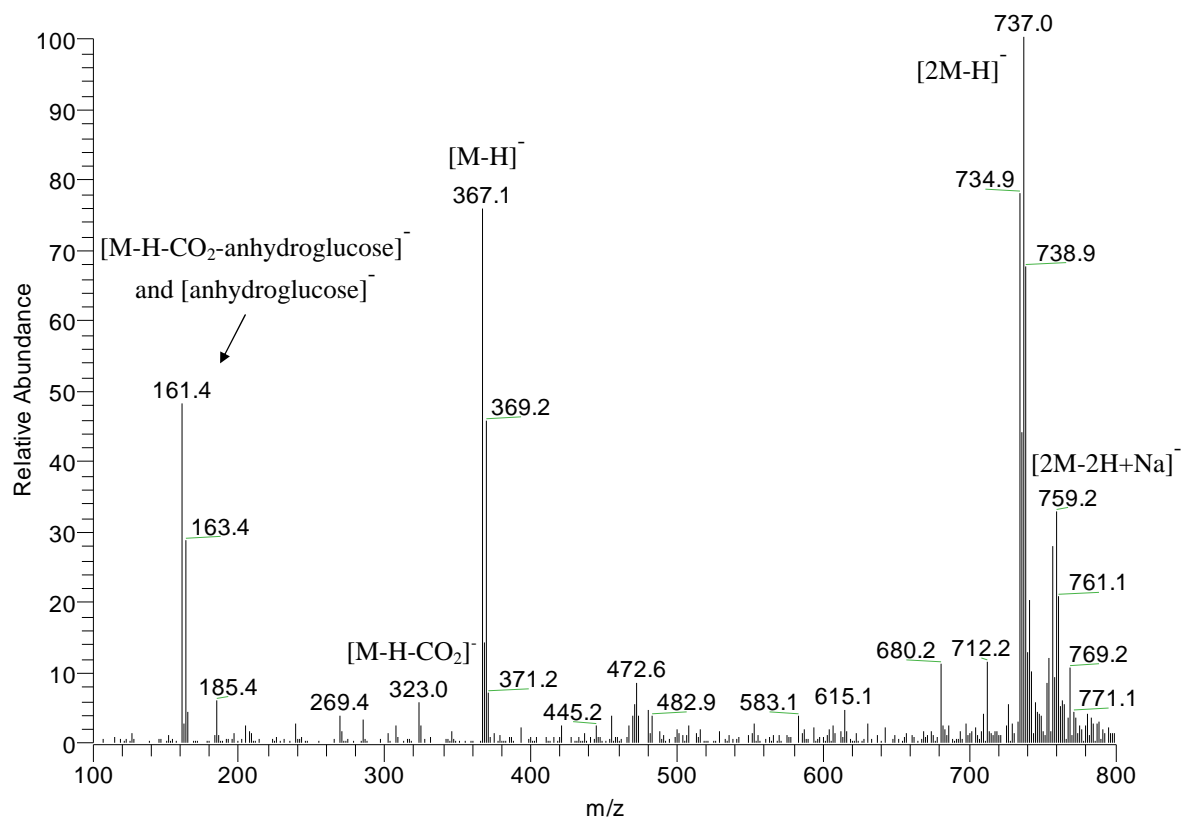
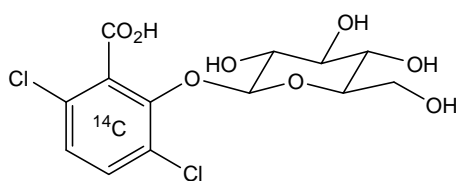
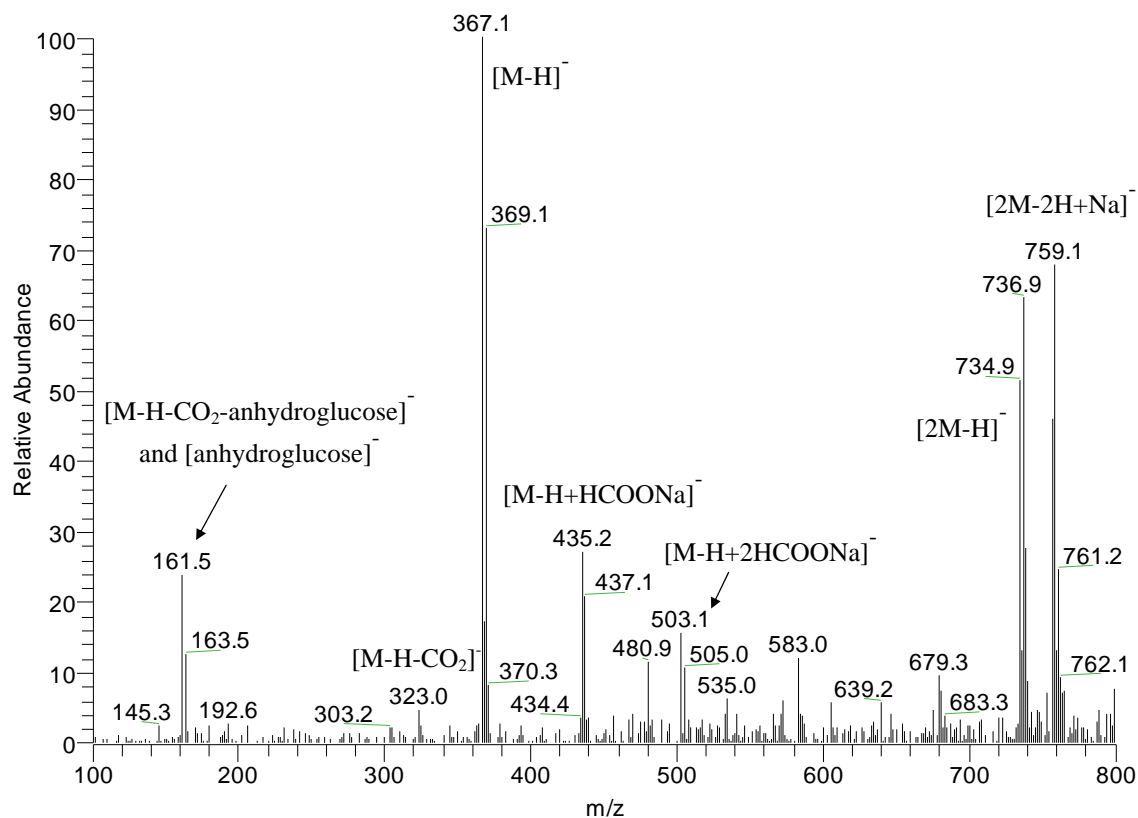


Figure 63. Negative Ion Electrospray Mass Spectrum of Peak 9 from PRE-T Pre-forage



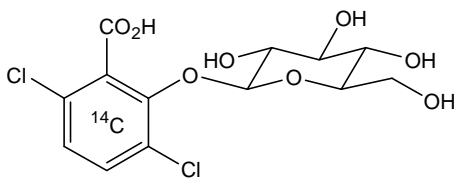
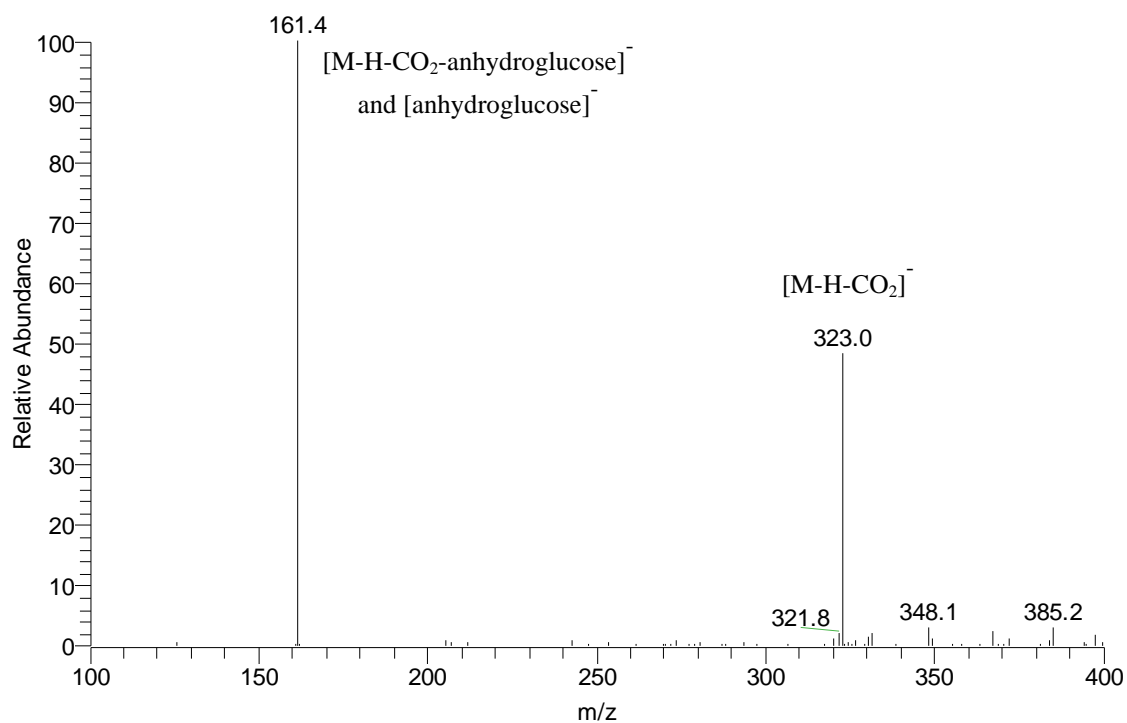
DCSA Glucoside (9)
Nominal Mass 368

Figure 64. Negative Ion Electrospray Mass Spectrum of Peak 9 from POE-T Forage



DCSA Glucoside (9)
Nominal Mass 368

Figure 65. Negative Ion Electrospray Tandem (MS/MS) Mass Spectrum of the m/z 367 Parent Ion of Peak 9 from POE-T Forage



DCSA Glucoside (9)
Nominal Mass 368

Figure 66. HPLC/RAD Analysis of Peak 9 from POE-T Forage Using HPLC Method B (top) and HPLC Method D (bottom)

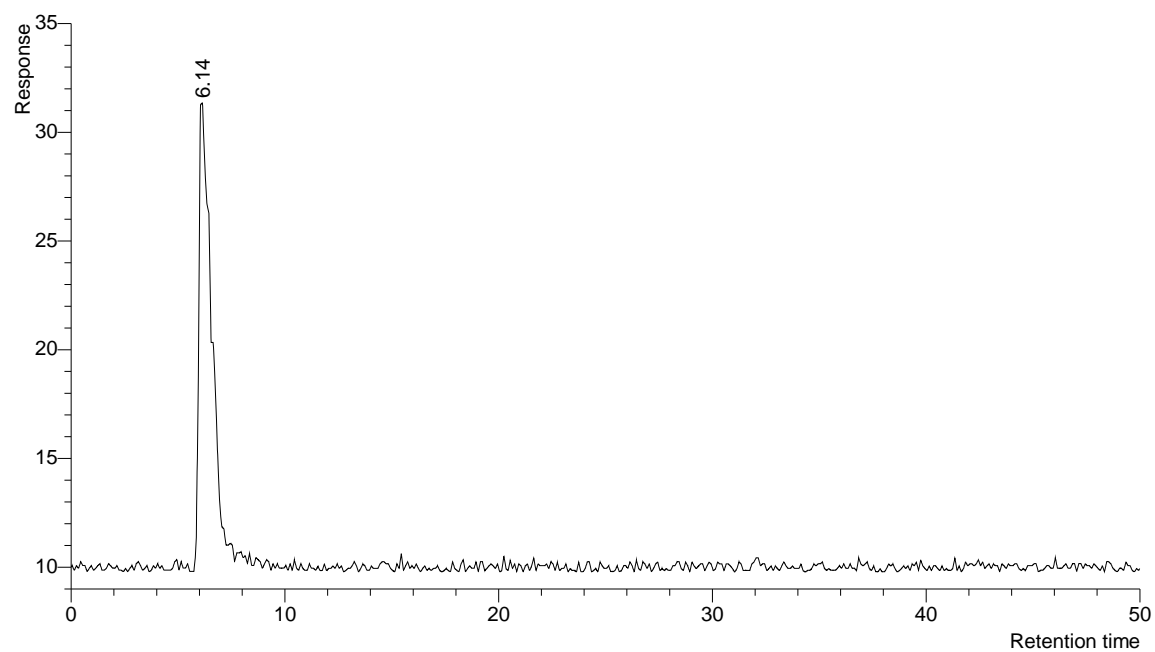
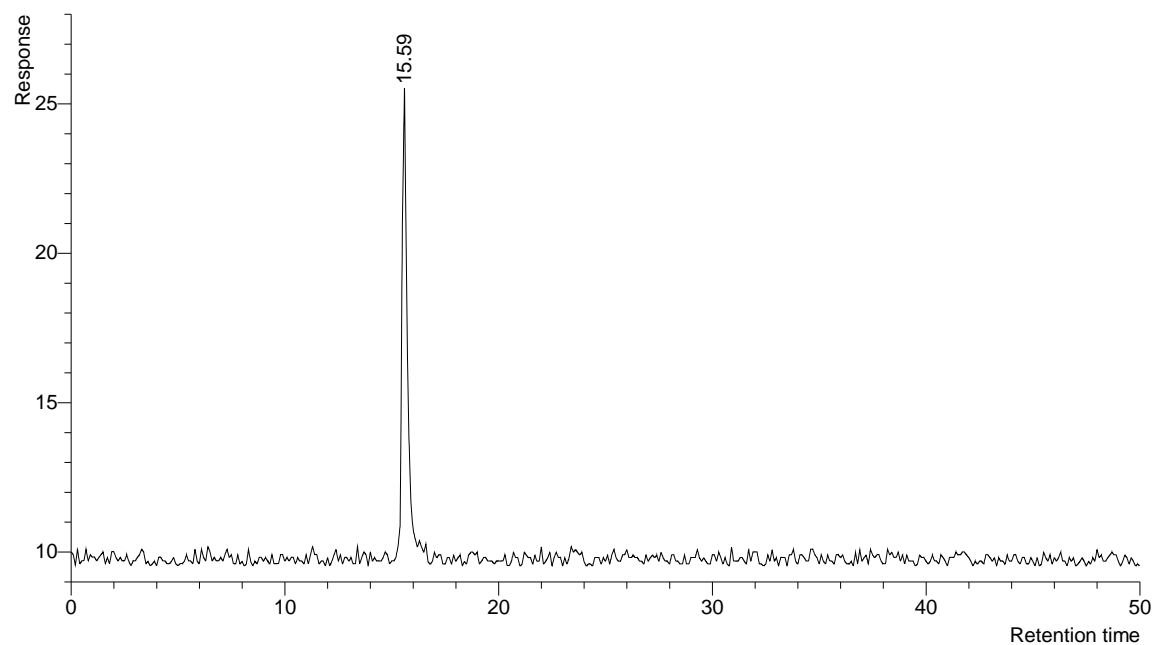
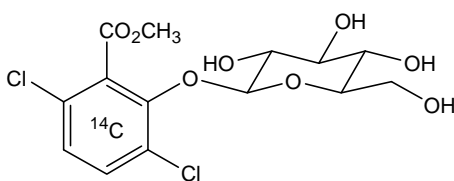
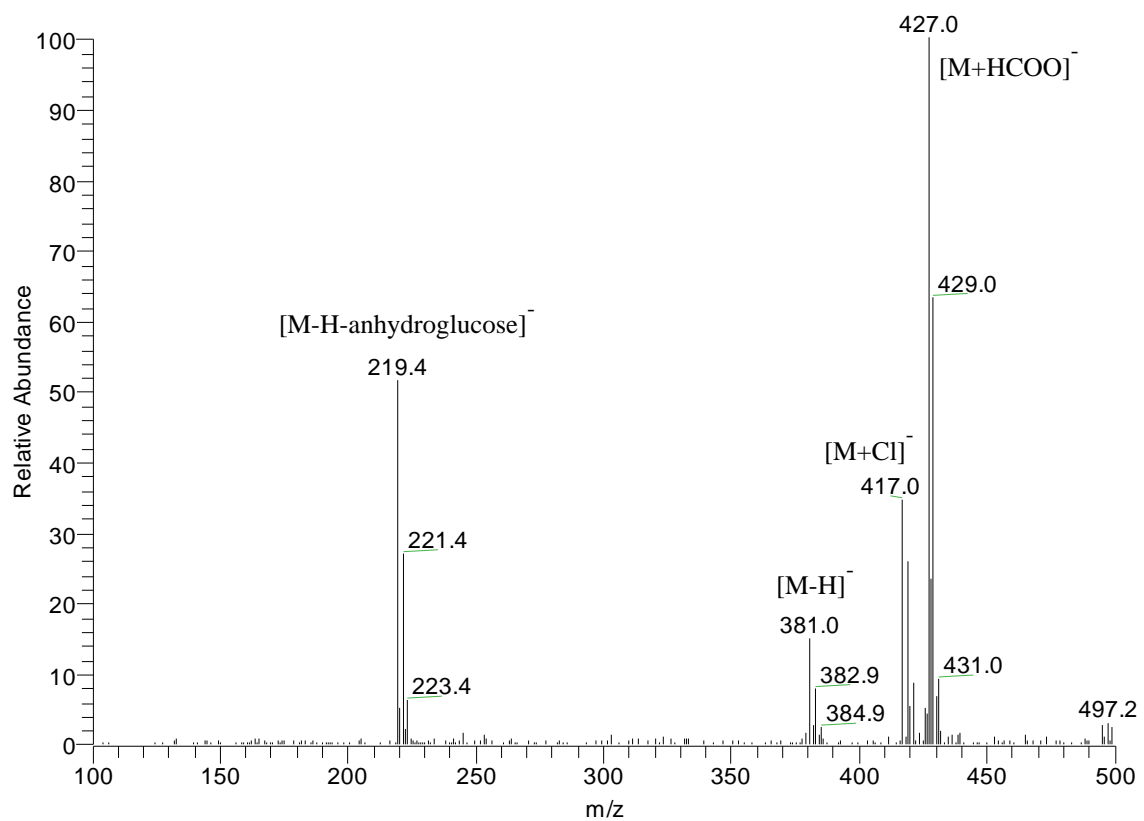


Figure 67. Negative Ion Electrospray Mass Spectrum of Methylated Peak 9 from POE-T Forage



DCSA Glucoside, Methyl Ester
Nominal Mass 382

Figure 68. HPLC/RAD Analysis of the Derivative from Methylation and Acid Hydrolysis of Peak 9 from POE-T Forage (top) and Comparison to Reference Standards (bottom) Using HPLC Method B

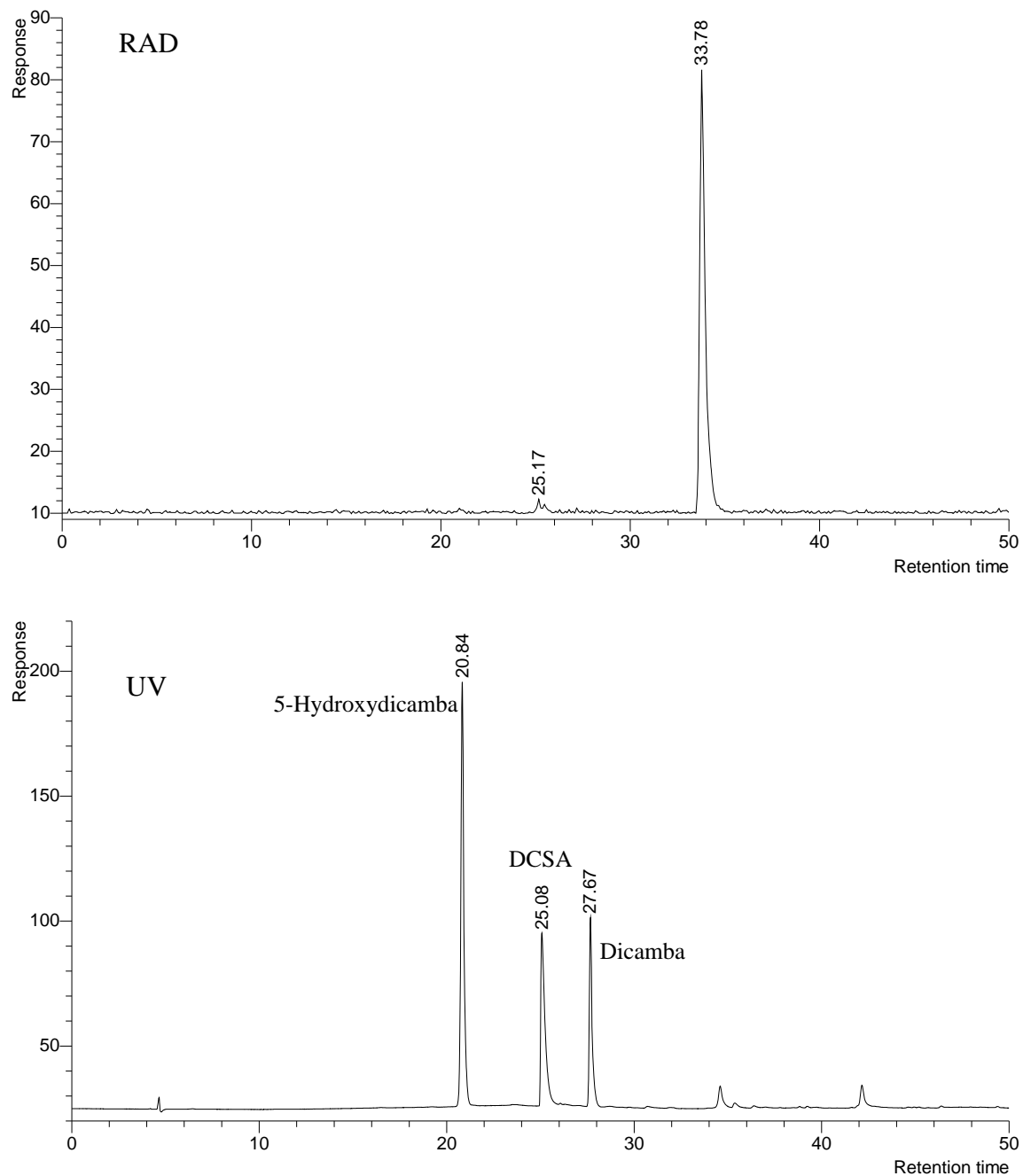
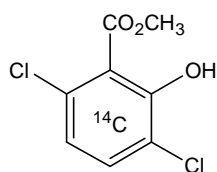
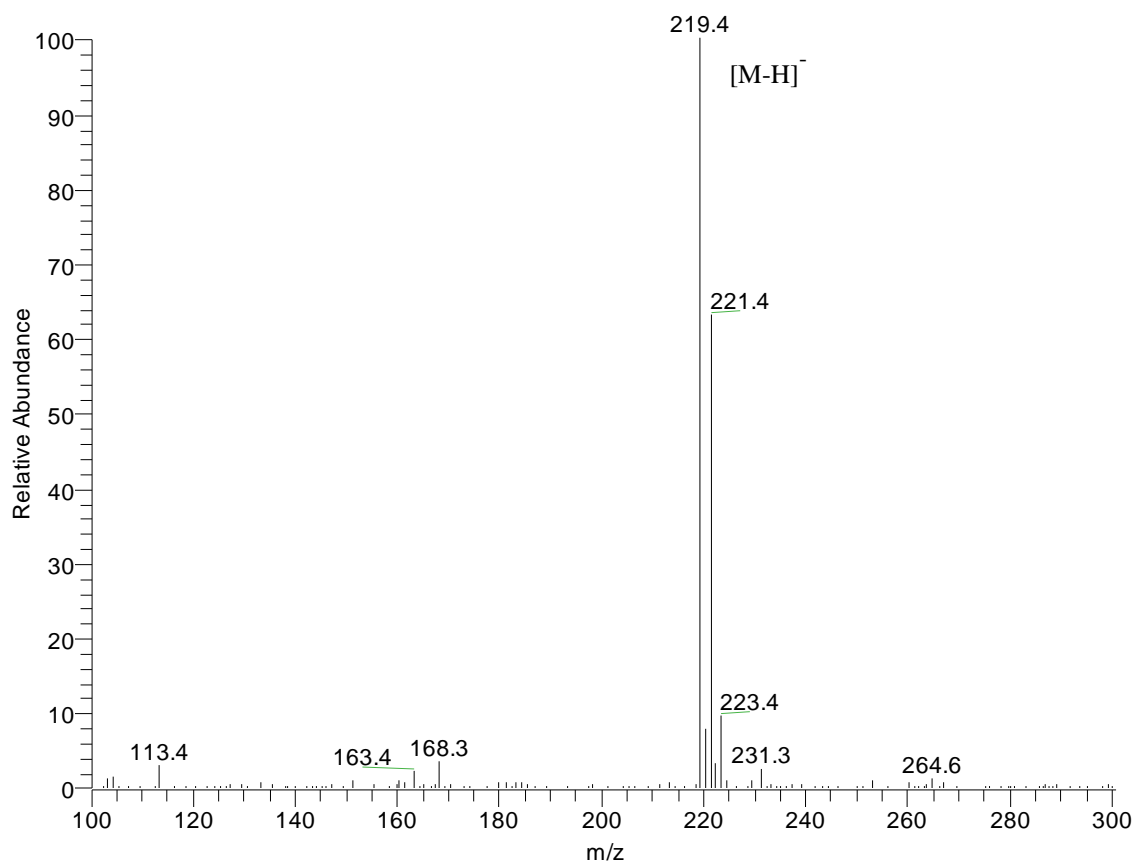
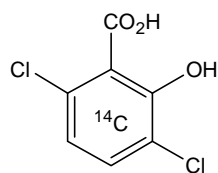
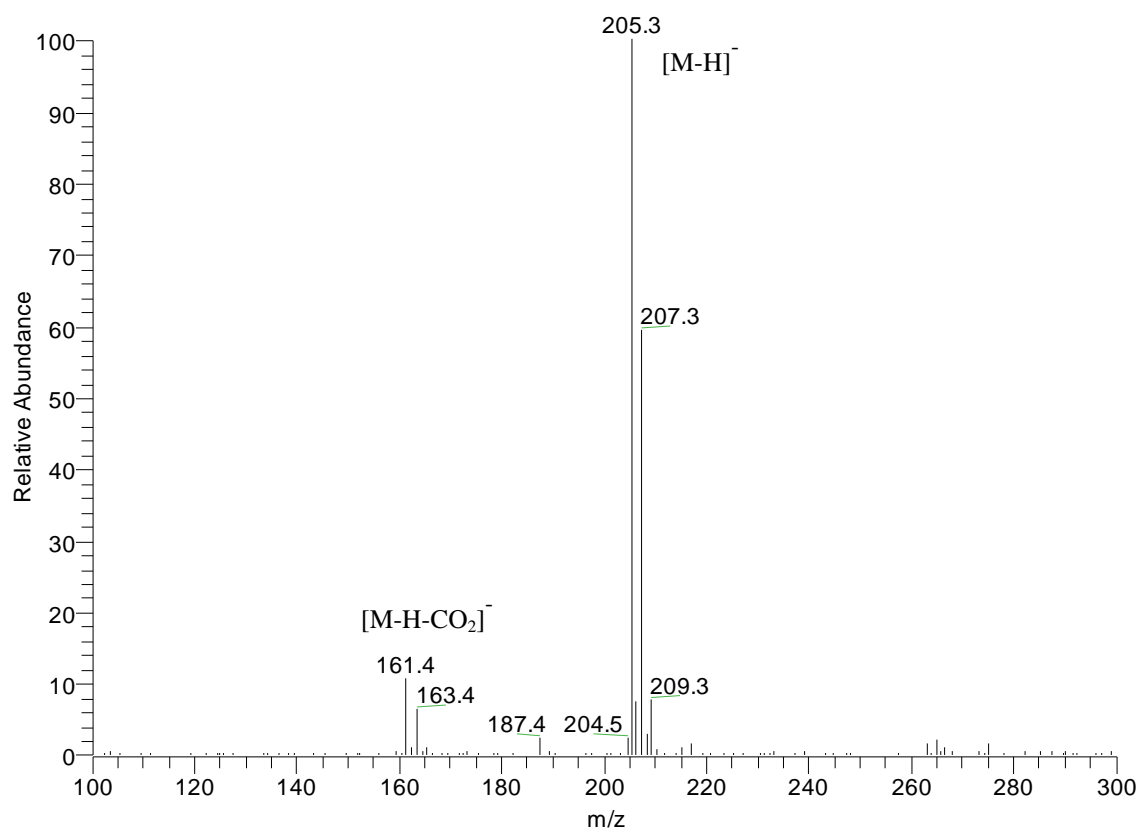


Figure 69. Negative Ion Electrospray Mass Spectrum of the Derivative from Methylation and Acid Hydrolysis of Peak 9 from POE-T Forage



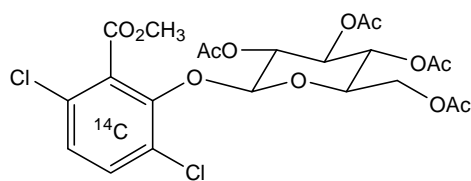
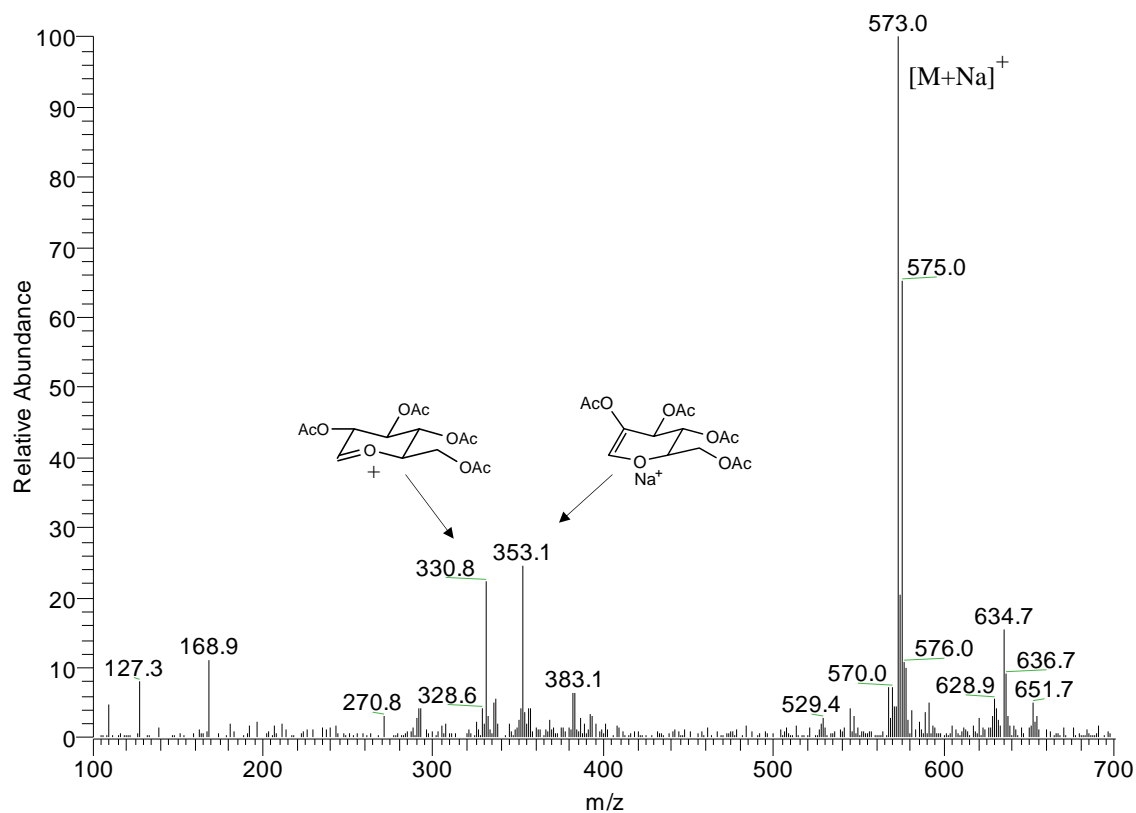
DCSA Methyl Ester
Nominal Mass 220

Figure 70. Negative Ion Electrospray Mass Spectrum of the Derivative of Peak 9 from POE-T Forage formed by Methylation, Acid Hydrolysis and Base Hydrolysis



DCSA
Nominal Mass 206

Figure 71. Positive Ion Electrospray Mass Spectrum of the Methylated and Acetylated Derivative of Peak 9 from POE-T Forage



DCSA Glucoside Tetraacetate, Methyl Ester
Nominal Mass 550

Figure 72. HPLC/RAD of the Reaction Mixture from β -Glucosidase Treatment of Peak 9 from POE-T Forage (top) and Control Reaction (No Enzyme, bottom) Using HPLC Method B

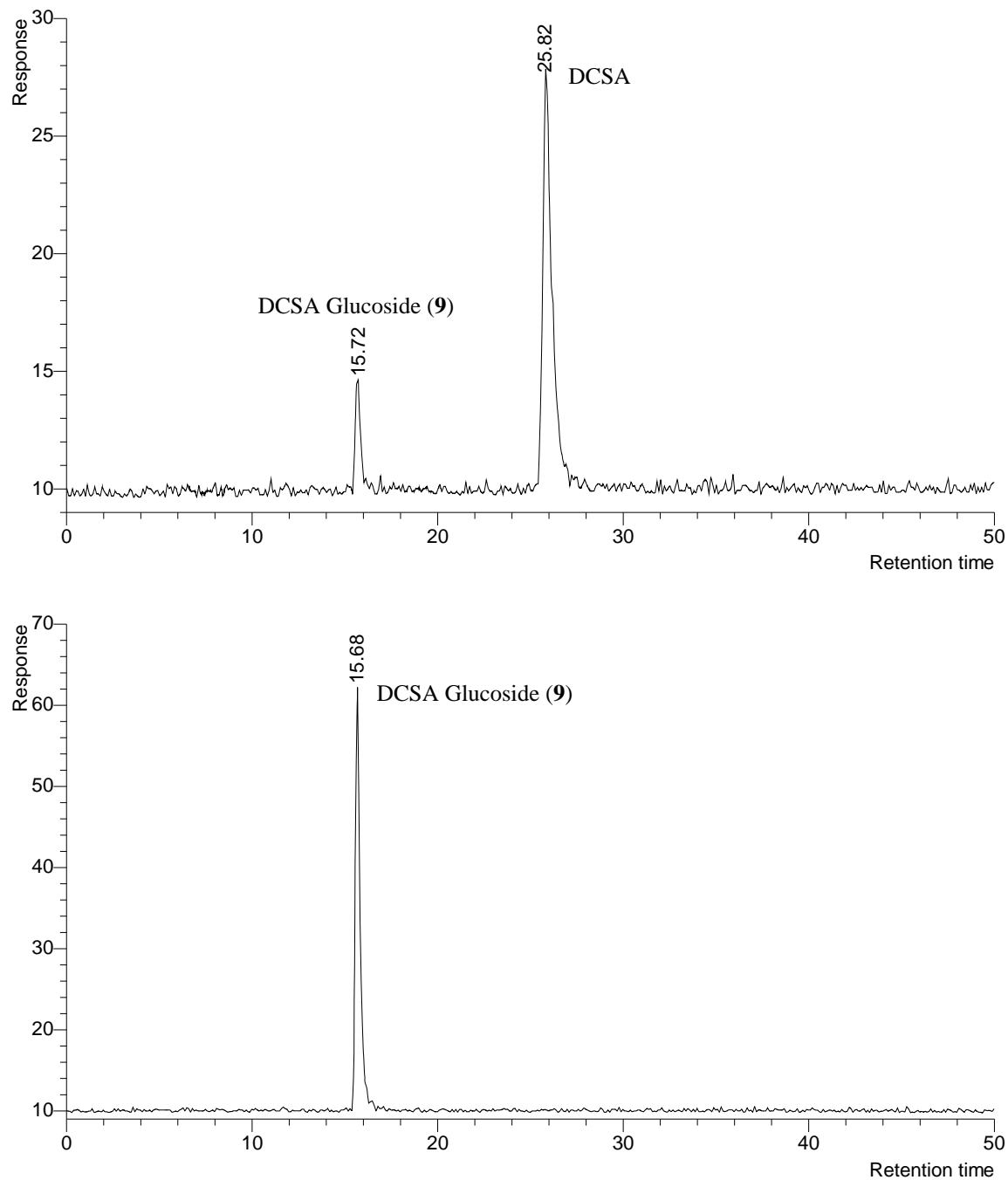
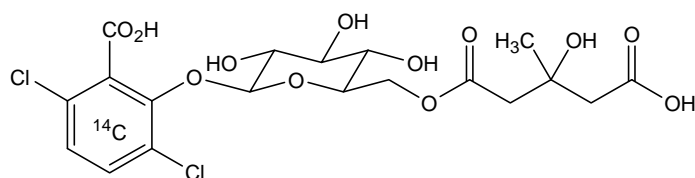
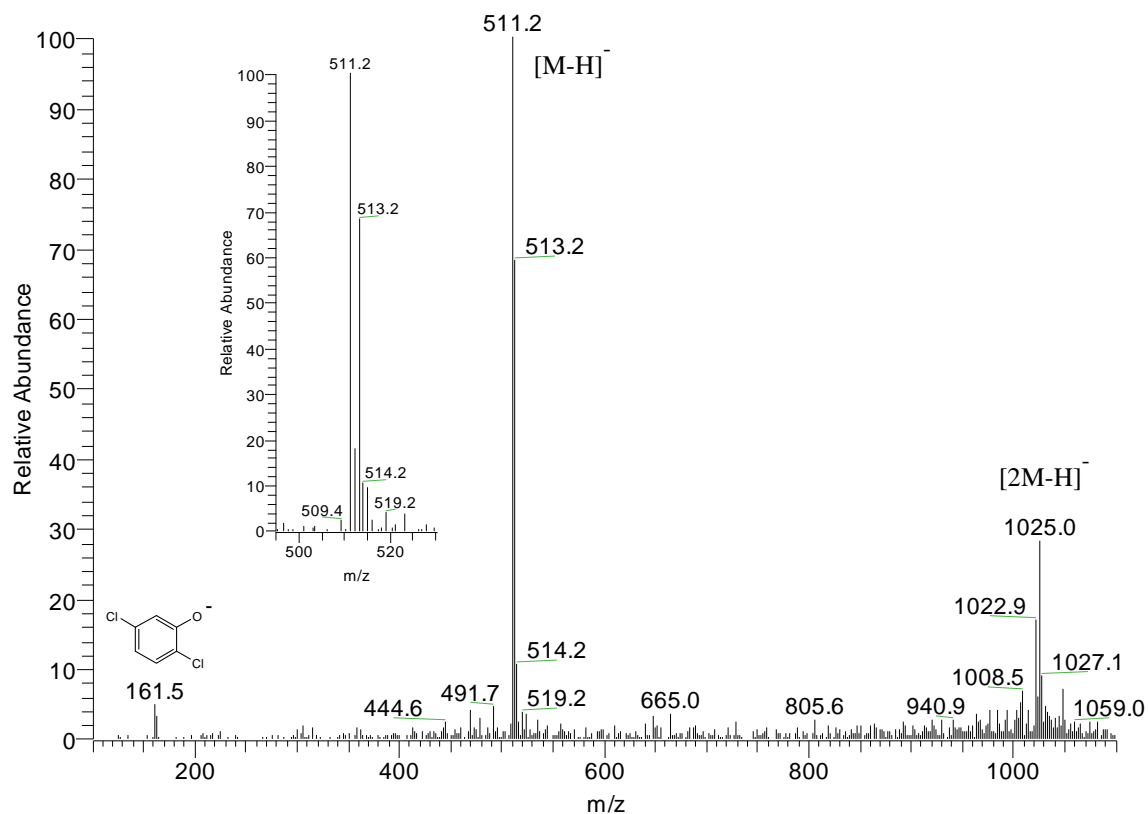


Figure 73. Negative Ion Electrospray Mass Spectrum of Peak 11 from POE-T Forage



DCSA HMGglucoside (**11**)
Nominal Mass 512

Figure 74. Negative Ion Electrospray Tandem (MS/MS) Mass Spectra of the m/z 511 Parent Ion (top) and m/z 513 Parent Ion (bottom) of Peak 11 from POE-T Forage

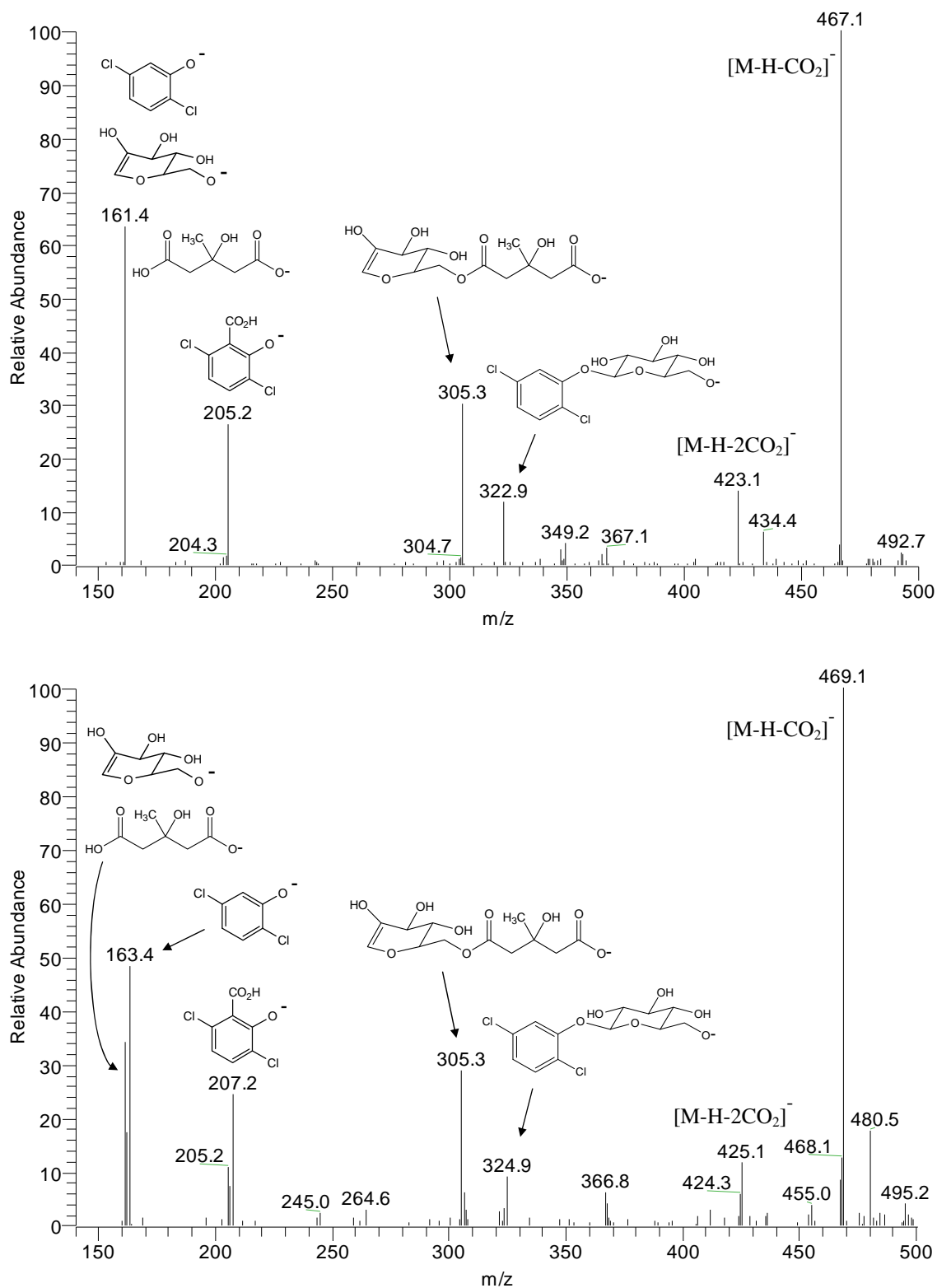


Figure 75. HPLC/RAD Analysis of the Acid Hydrolysate of Peak 11 from POE-T Forage and Comparison to DCSA and Dicamba Reference Standards Using HPLC Method B

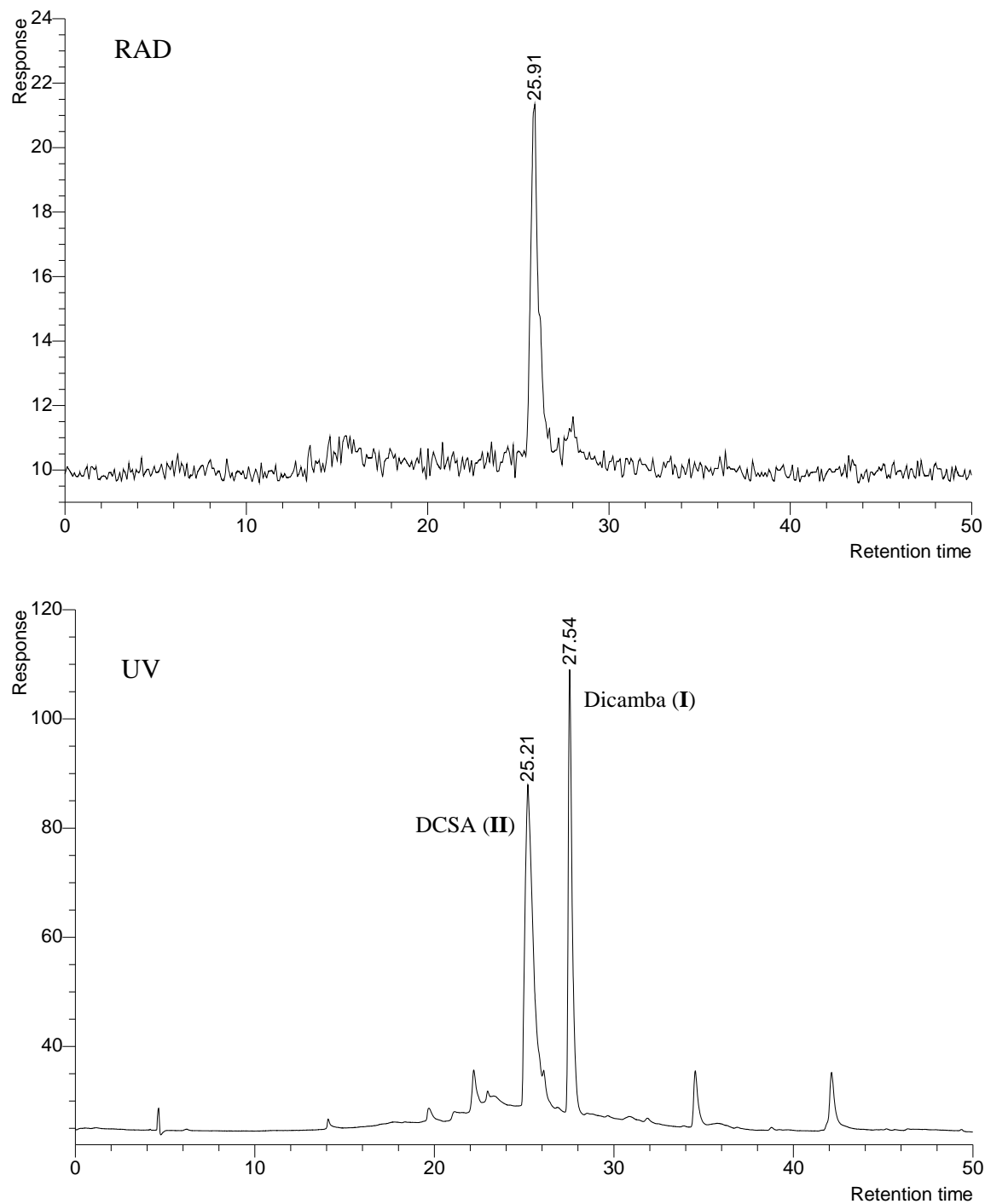


Figure 76. HPLC Coinjection Analysis of the Strong Base Hydrolysate of Peak 11 from POE-T Forage and DCSA Reference Standard Using HPLC Method B

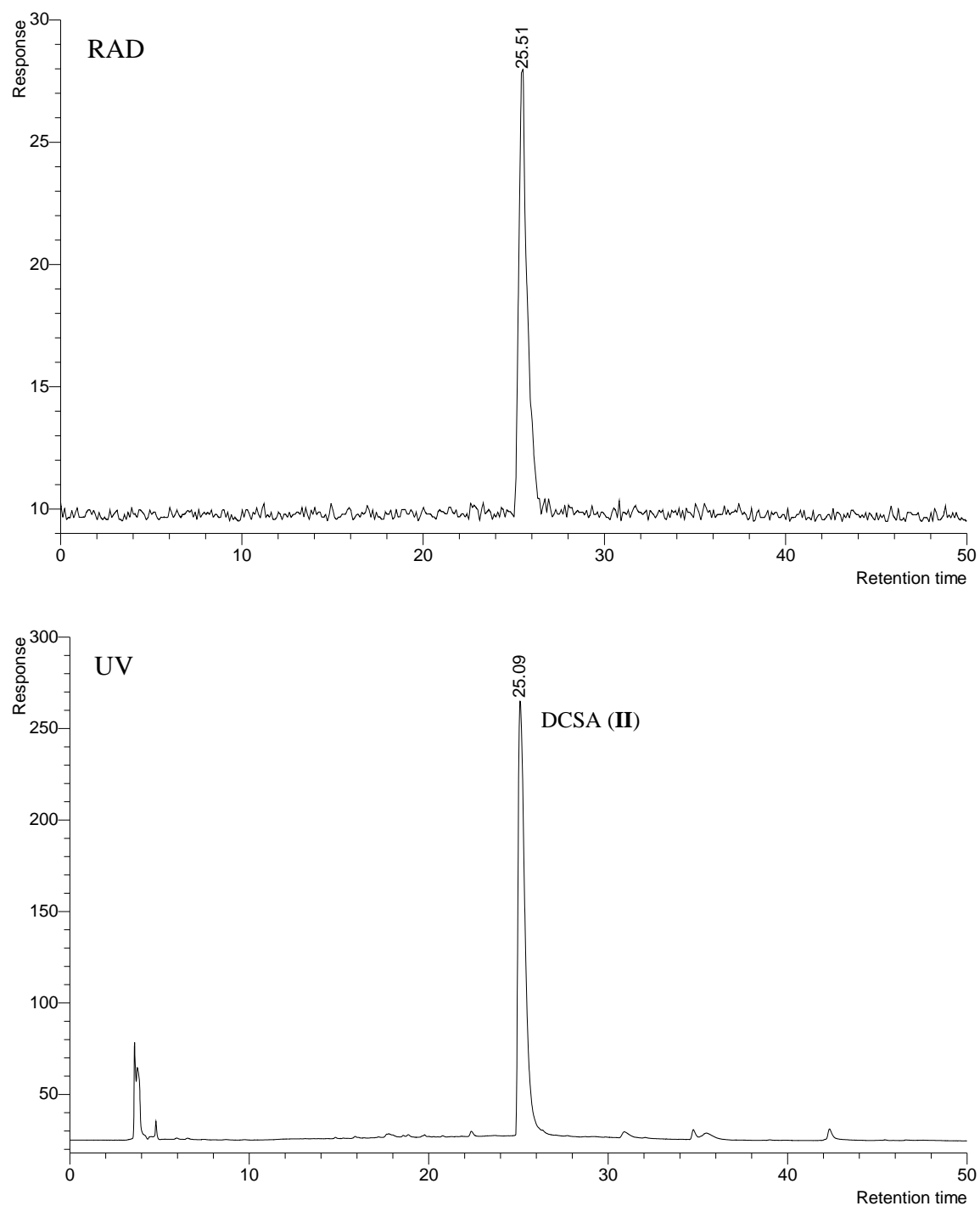
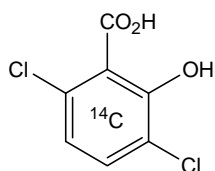
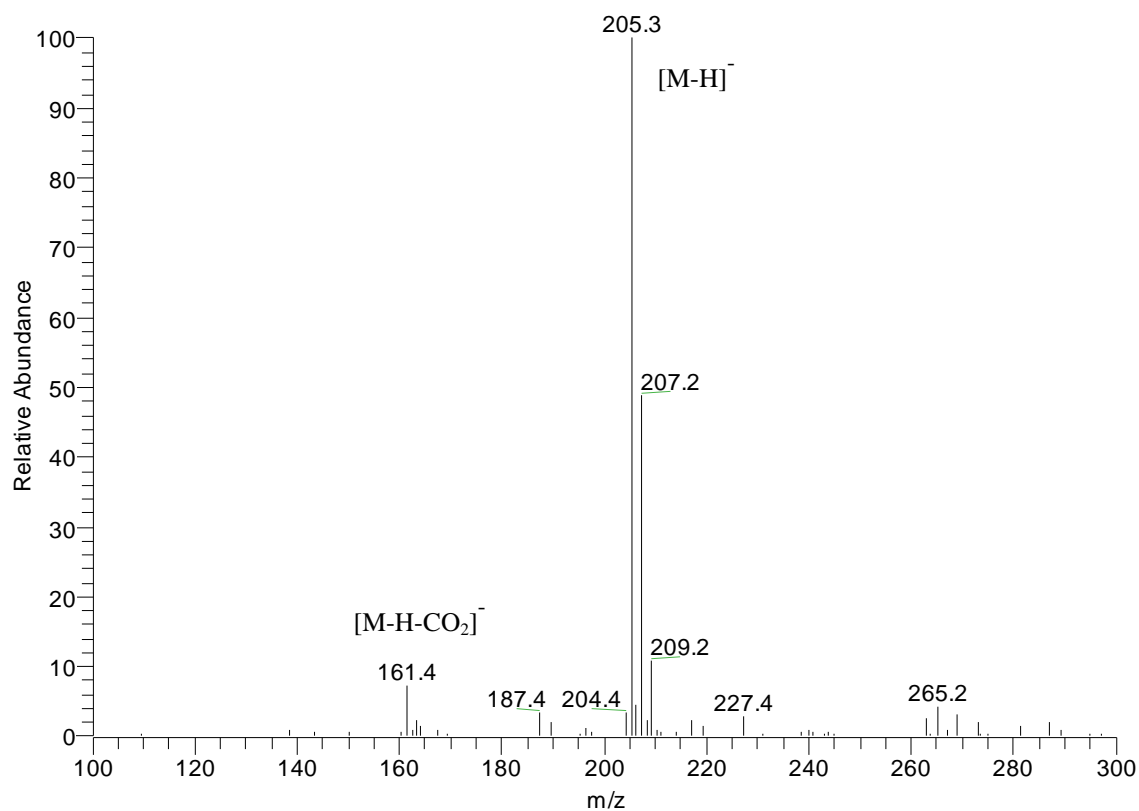


Figure 77. Negative Ion Electrospray Mass Spectrum of the Strong Base Hydrolysate of Peak 11 from POE-T Forage



DCSA
Nominal Mass 206

Figure 78. HPLC/RAD Analysis of the Mild Base Hydrolysate of Peak 11 from POE-T Forage and Comparison to DCSA Glucoside Metabolite (Peak 9) Using HPLC Method B

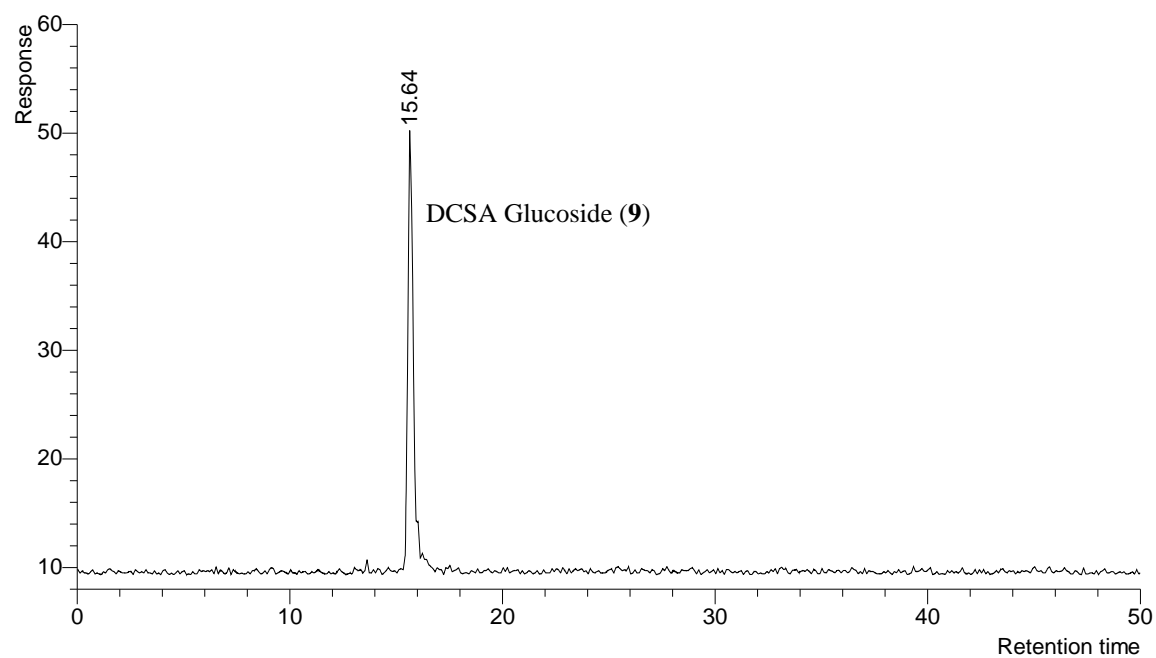
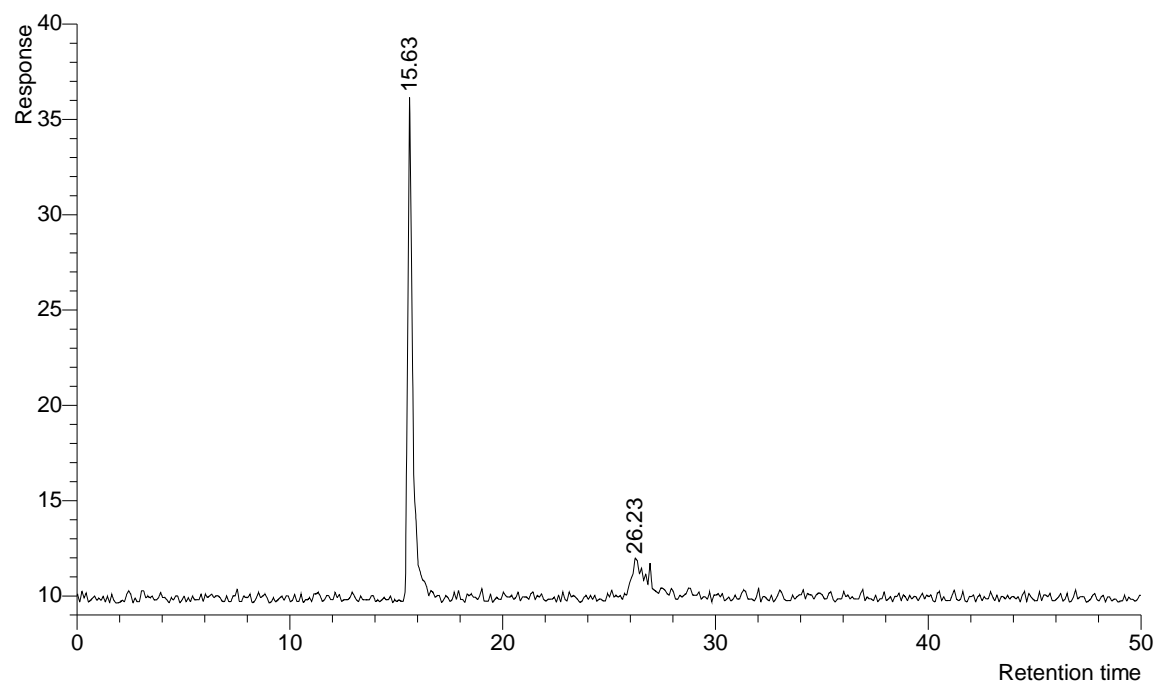
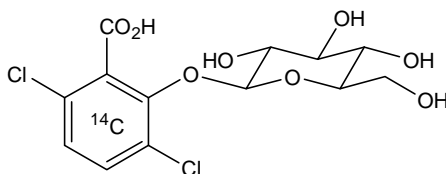
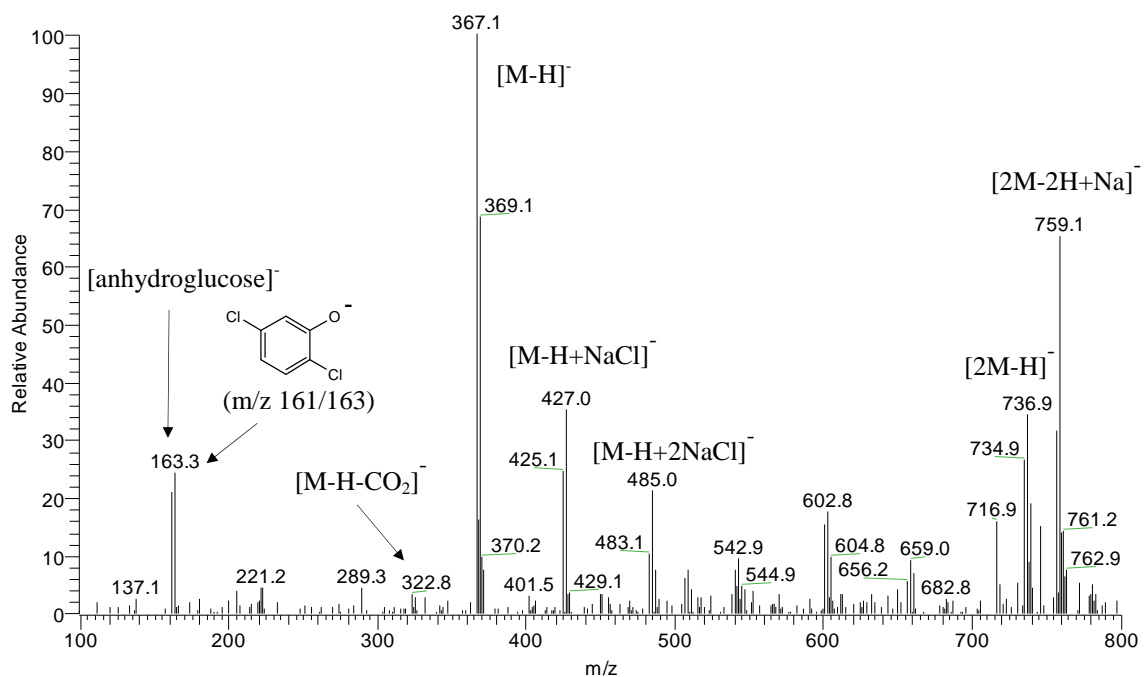


Figure 79. Negative Ion Electrospray Mass Spectrum of the Mild Base Hydrolysate of Peak 11 from POE-T Forage



DCSA Glucoside
Nominal Mass 368

Figure 80. HPLC/RAD Analysis of Methylated Peak 11 from POE-T Forage Using HPLC Method B

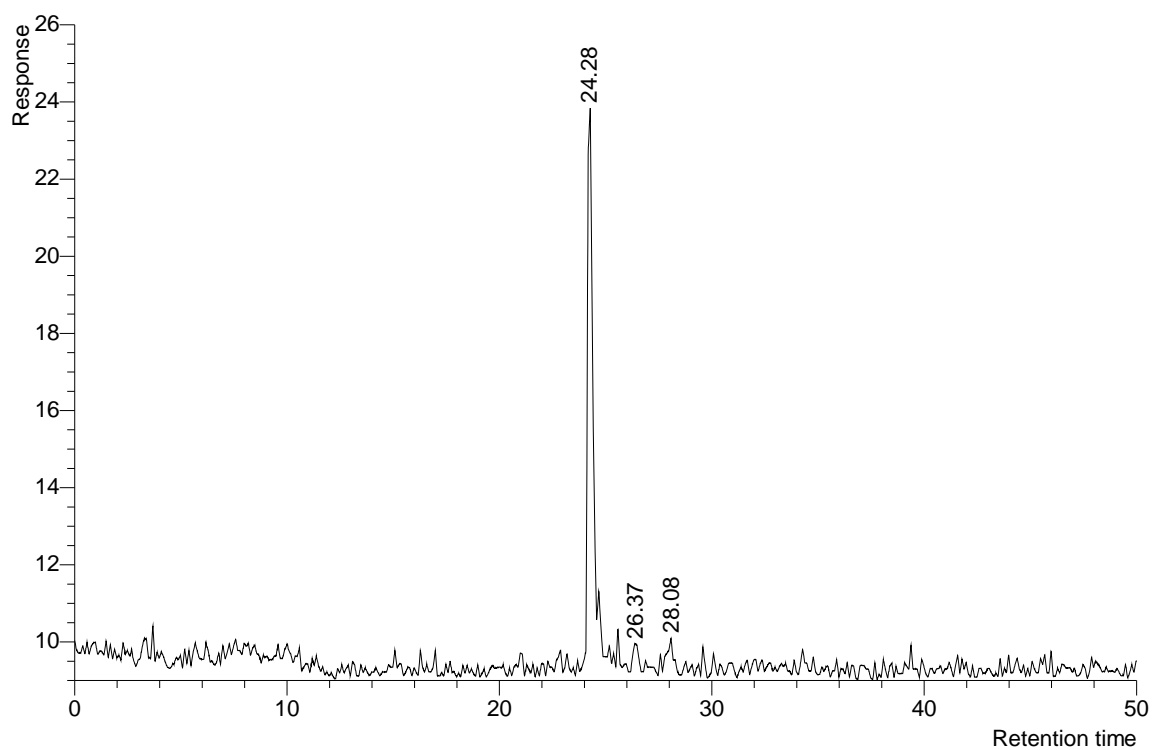
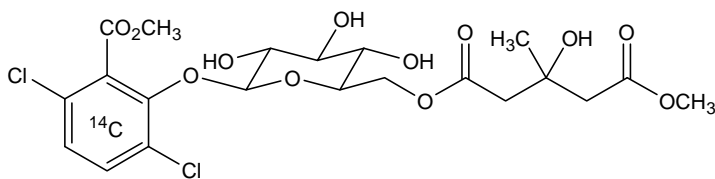
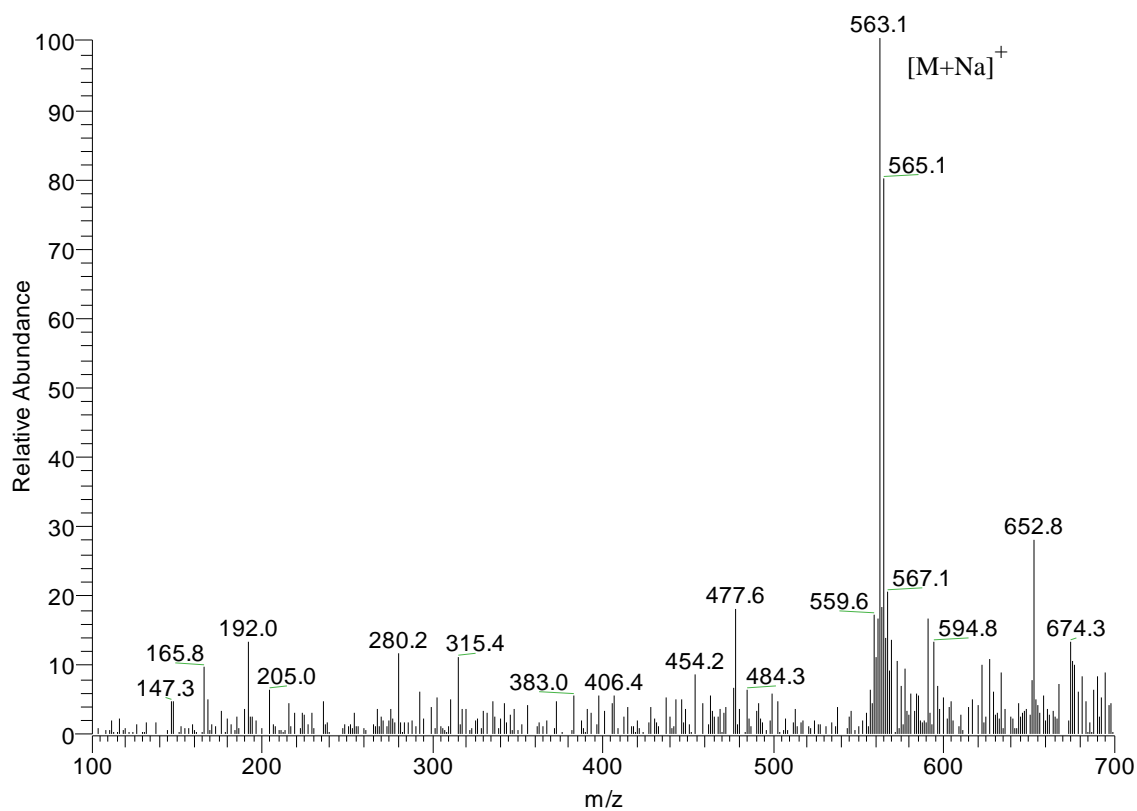
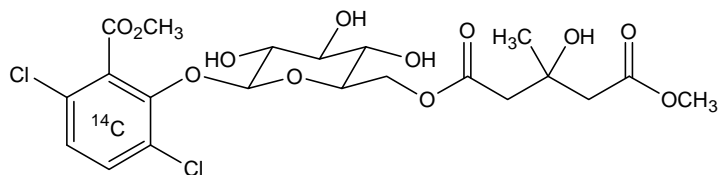
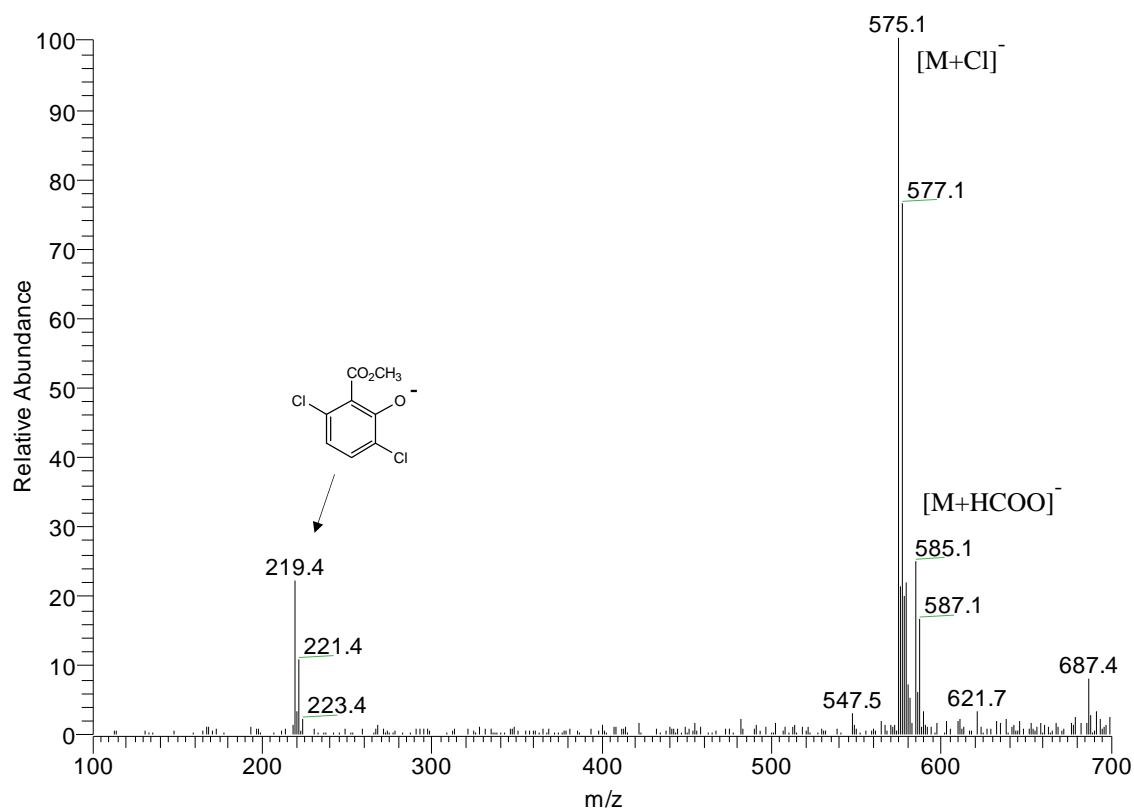


Figure 81. Positive Ion Electrospray Mass Spectrum of Methylated Peak 11 from POE-T Forage



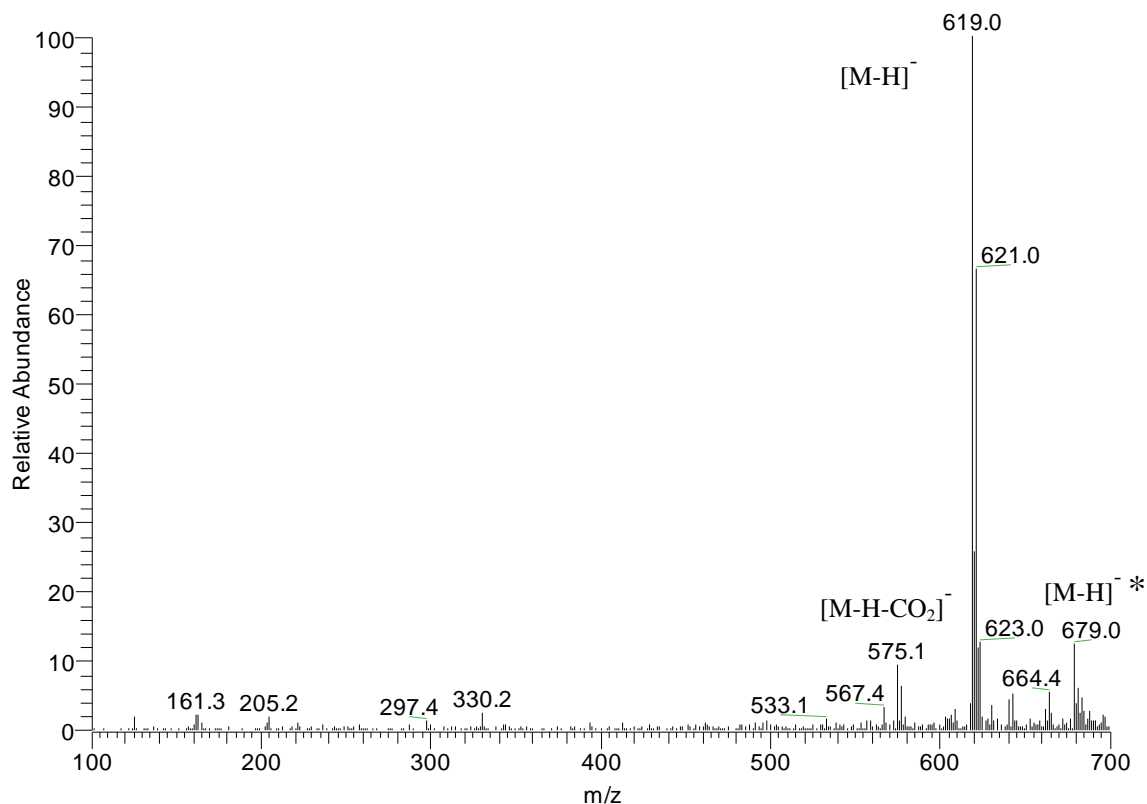
DCSA HMGglucoside, Dimethyl Ester
Nominal Mass 540

Figure 82. Negative Ion Electrospray Mass Spectrum of Methylated Peak 11 from POE-T Forage

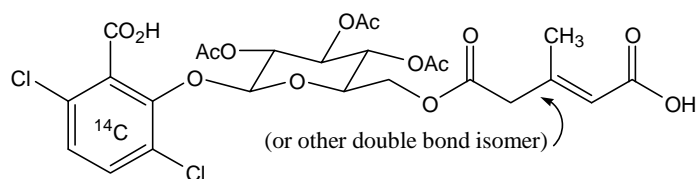


DCSA HMGglucoside, Dimethyl Ester
Nominal Mass 540

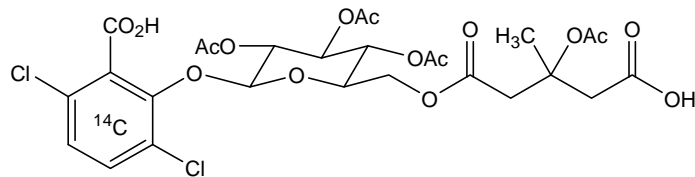
Figure 83. Negative Ion Electrospray Mass Spectrum of Acetylated Peak 11 from POE-T Forage



* parent ion of minor tetraacetate product



Eliminated DCSA HMGglucoside Triacetate (major)
Nominal Mass 620



DCSA HMGglucoside Tetraacetate (minor)
Nominal Mass 680

Figure 84. HPLC/RAD Analysis of Methylated and Acetylated Peak 11 from POE-T Forage Using HPLC Method B

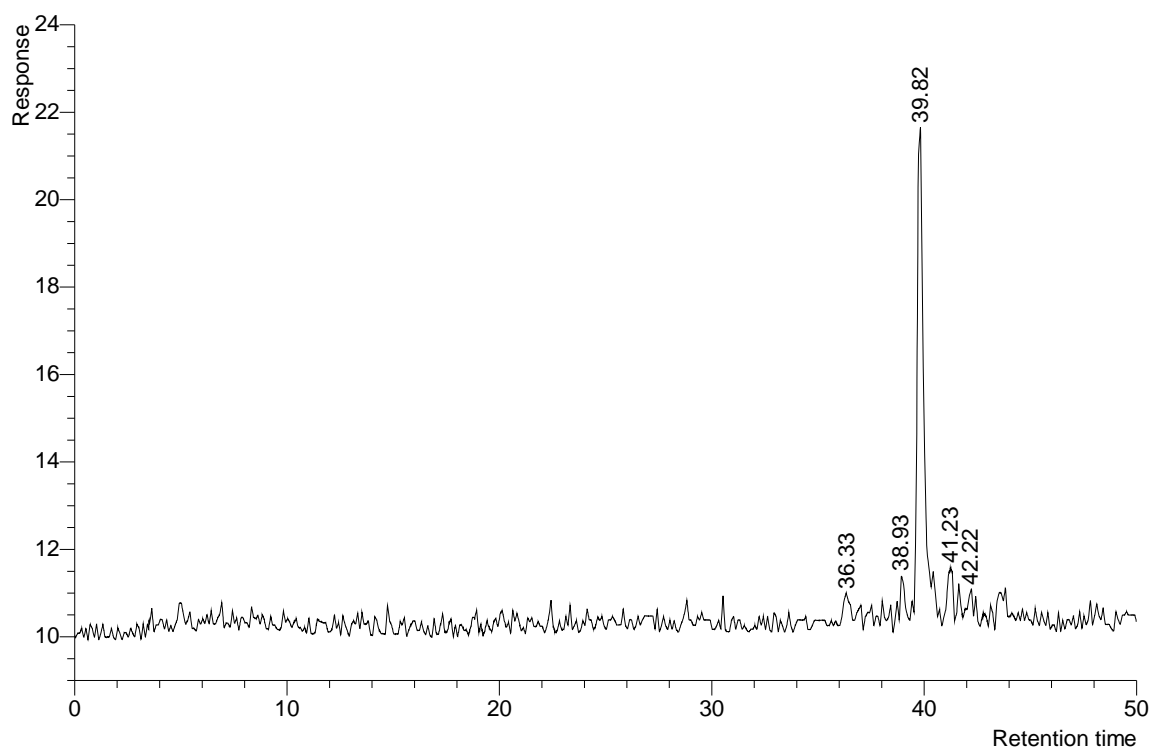
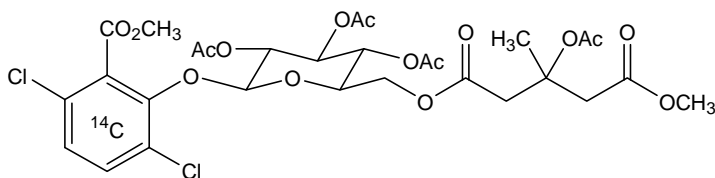
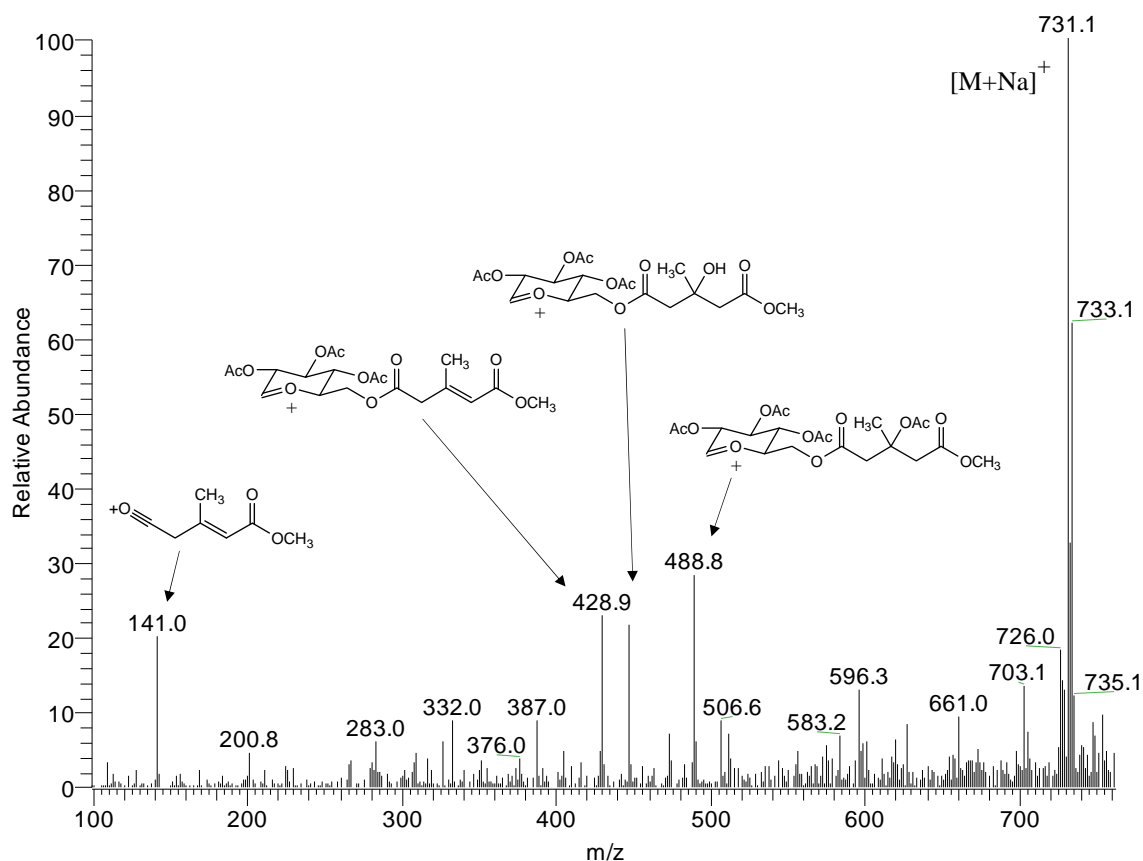
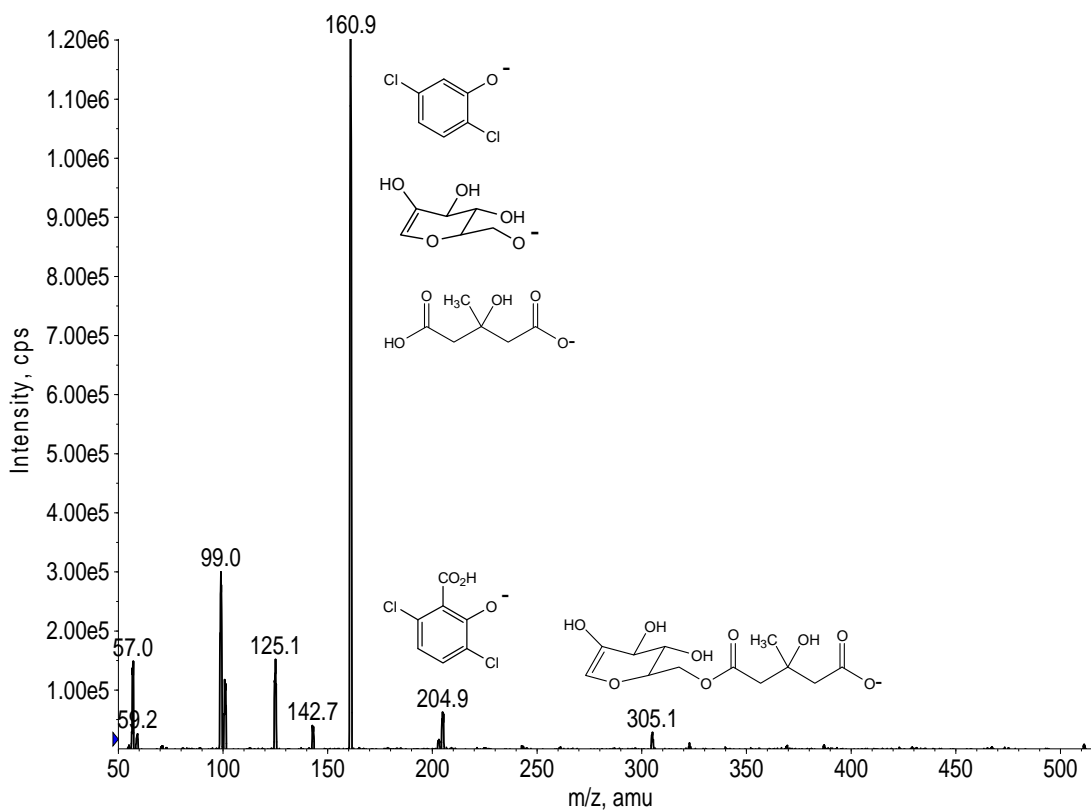


Figure 85. Positive Ion Electrospray Mass Spectrum of Methylated and Acetylated Peak 11 from POE-T Forage

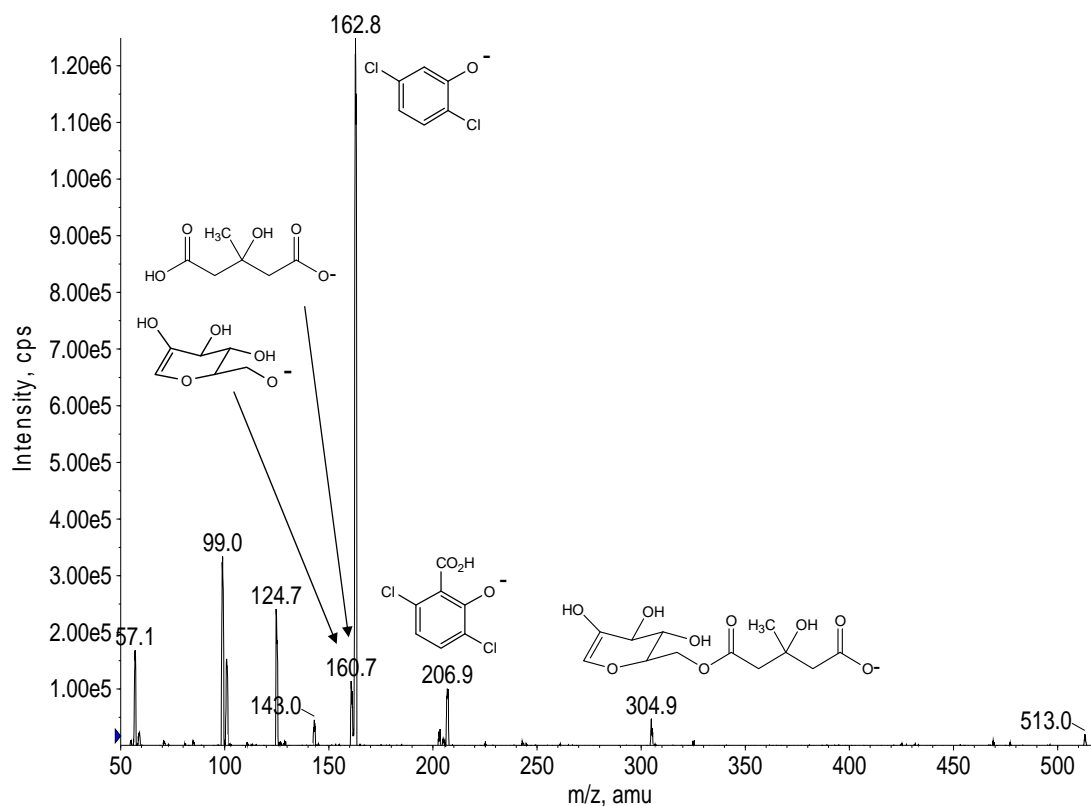


DCSA HMGglucoside Tetraacetate, Dimethyl Ester
Nominal Mass 708

Figure 86. Negative Ion IonSpray Tandem (MS/MS) Mass Spectrum of the Parent Ion (m/z 511) of Peak 11 from POE-T Forage

Mass Peak (m/z)	Proposed Structure	
305.1	[M-DCSA] ⁻	
204.9	[DCSA-H] ⁻	
160.9	[DCSA-H-CO ₂] ⁻ + [Anhydroglucose] ⁻ + [HMGA] ⁻	
142.7		[Anydroglucose-H ₂ O] ⁻
125.1		[Anydroglucose-2H ₂ O] ⁻
101		
99.0		[Anydroglucose-2H ₂ O-HC≡CH] ⁻
59.2		
57.0		

Figure 87. Negative Ion IonSpray Tandem (MS/MS) Mass Spectrum of the Parent Ion (m/z 513) of Peak 11 from POE-T Forage



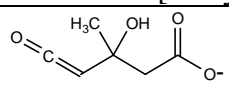
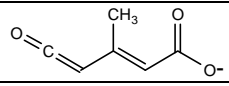
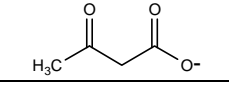
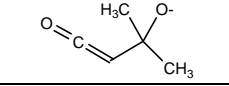
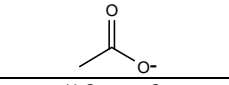
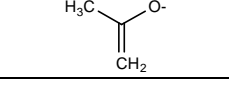
Mass Peak (m/z)	Proposed Structure	
304.9	[M-DCSA] ⁻	
206.9	[DCSA-H] ⁻	
162.8	[DCSA-H-CO ₂] ⁻	
160.7	[Anhydroglucose] ⁻ + [HMGA] ⁻	
143.0		[Anhydroglucose-H ₂ O] ⁻
124.7		[Anhydroglucose-2H ₂ O] ⁻
101		
99.0		[Anhydroglucose-2H ₂ O-HC≡CH] ⁻
59		
57.1		

Figure 88. High Resolution Negative Ion IonSpray Time-of-Flight Mass Spectrum of Peak 11 from POE-T Forage with Fexofenadine, MRFA and Taurocholic Acid Reference Standards

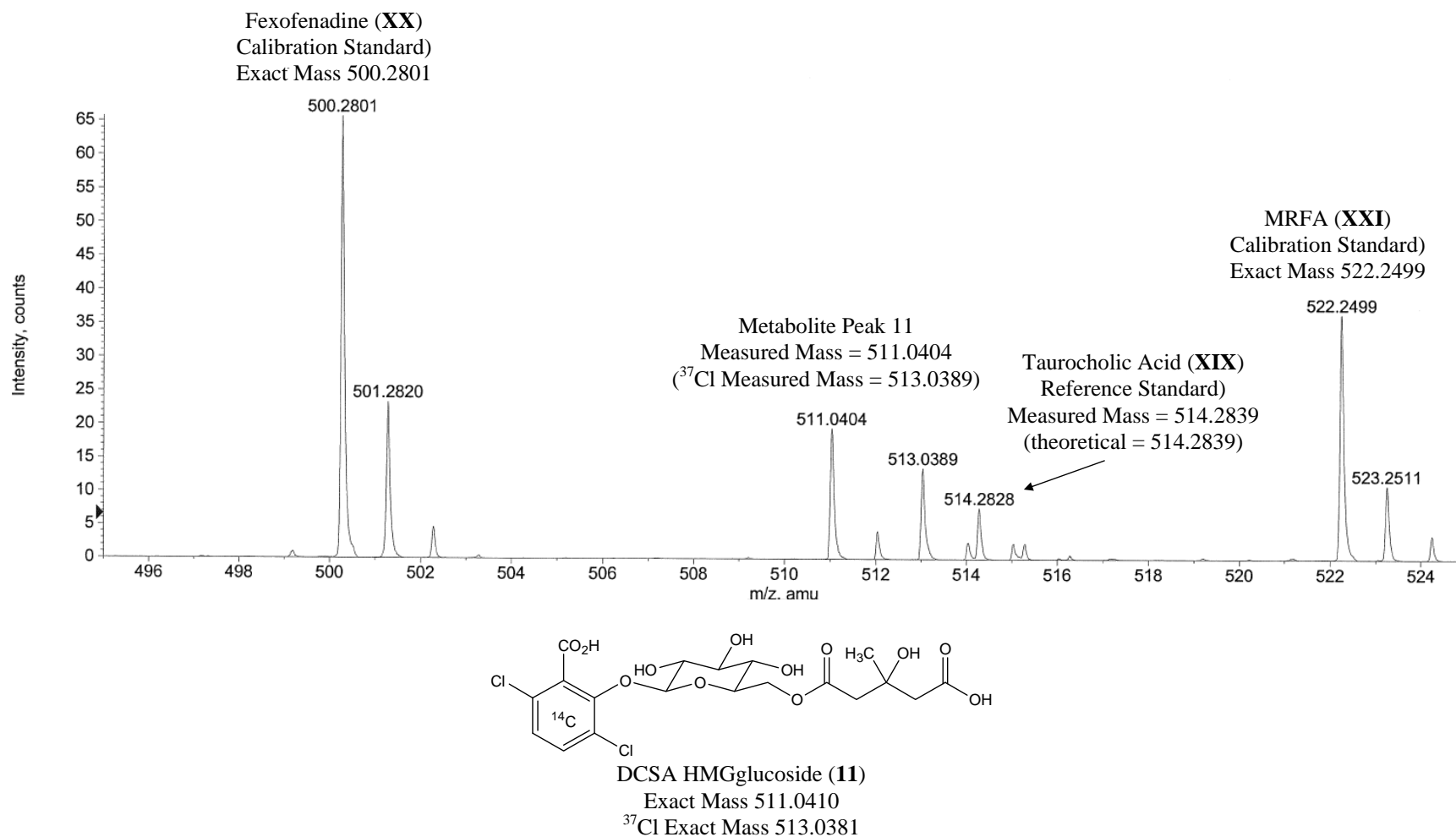


Figure 89. Proton NMR Spectrum of Peak 11 from POE-T Forage

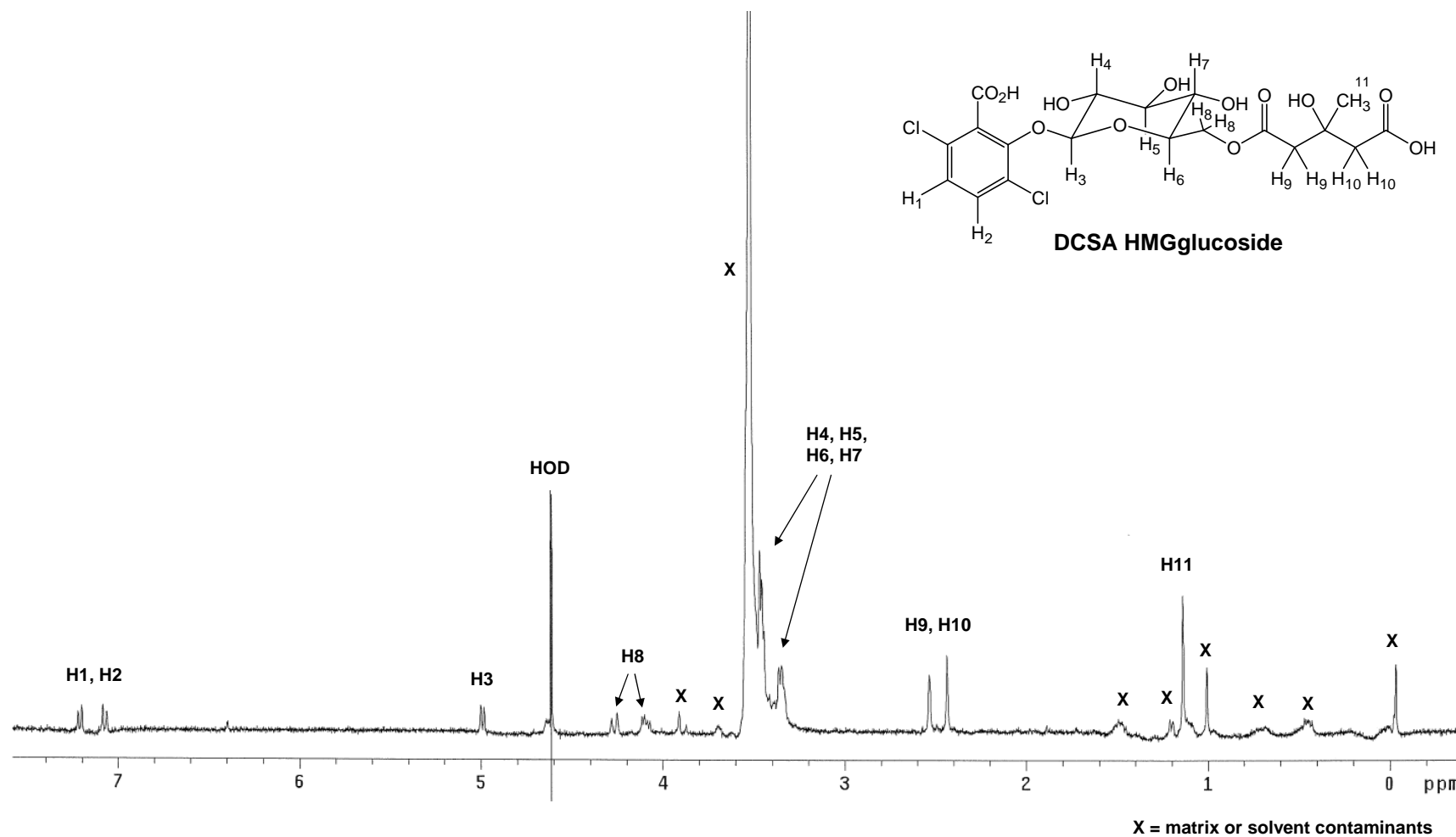


Figure 90. Positive Ion Electron Ionization (GC/EI) Extracted Ion Chromatogram (m/z 363) of the Silylated Mild Base Hydrolysate of Peak 11 from POE-T Forage (top) with Tris(trimethylsilyl)HMGA Reference Standard (Positive Control, middle) and Negative Control (bottom)

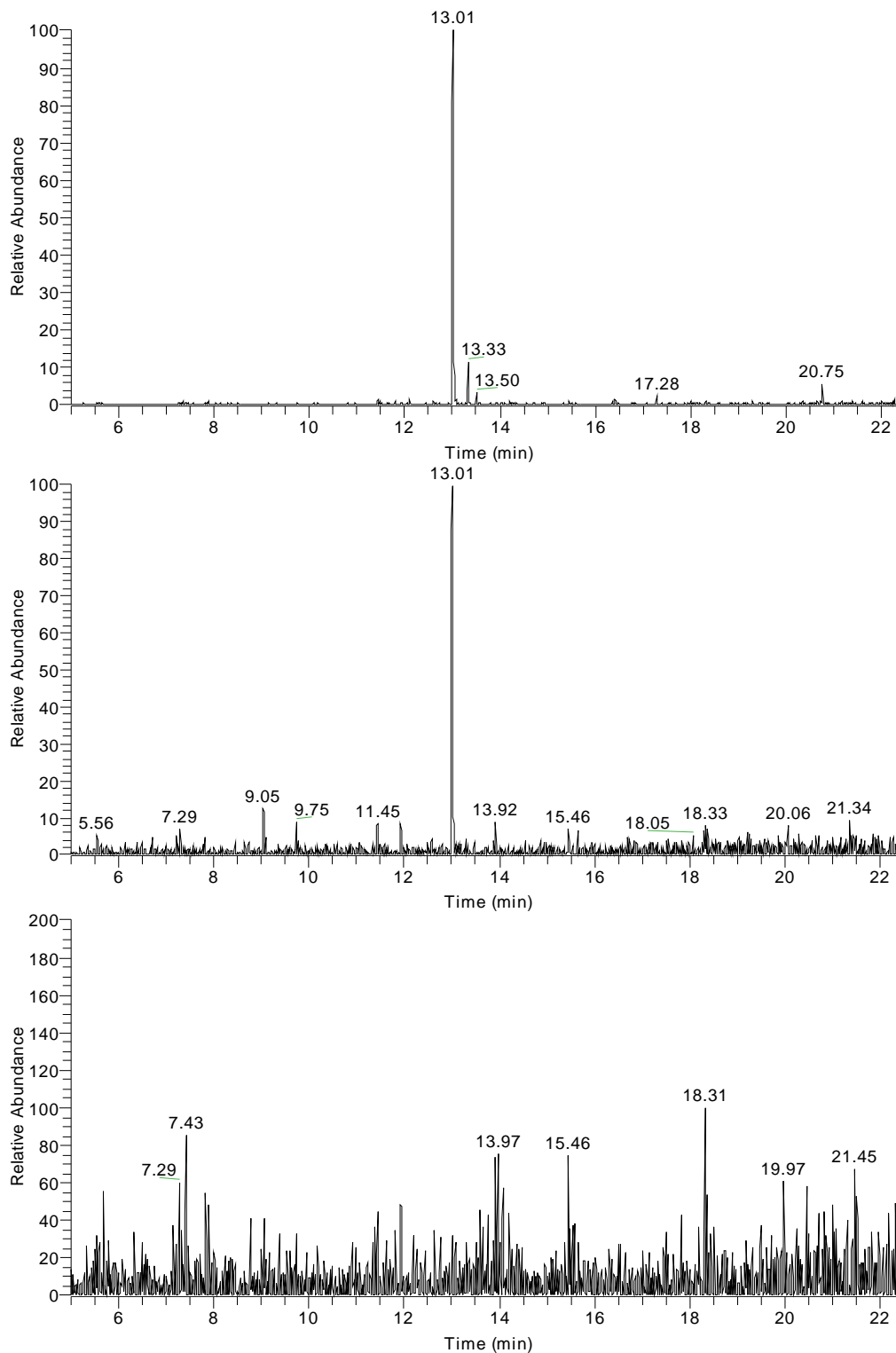


Figure 91. Positive Ion GC/EI Mass Spectrum of the Silylated Mild Base Hydrolysate of Peak 11 from POE-T Forage (top) with Tris(trimethylsilyl)HMGA Reference Standard (Positive Control, middle) and Negative Control (bottom)

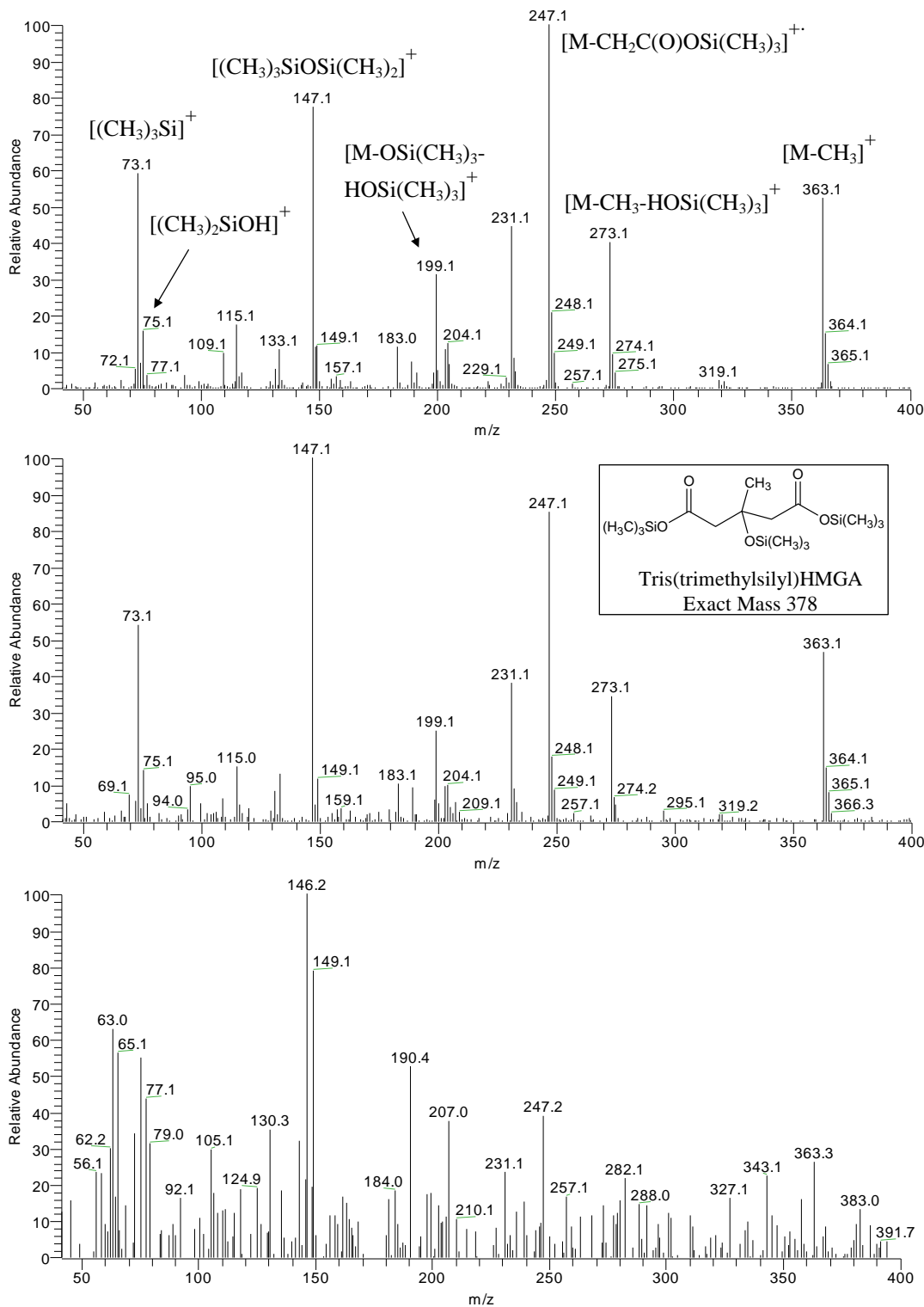


Figure 92. HPLC Coinjection Analysis of Peak 14 from POE-T Forage and 5-Hydroxydicamba Reference Standard Using HPLC Method B

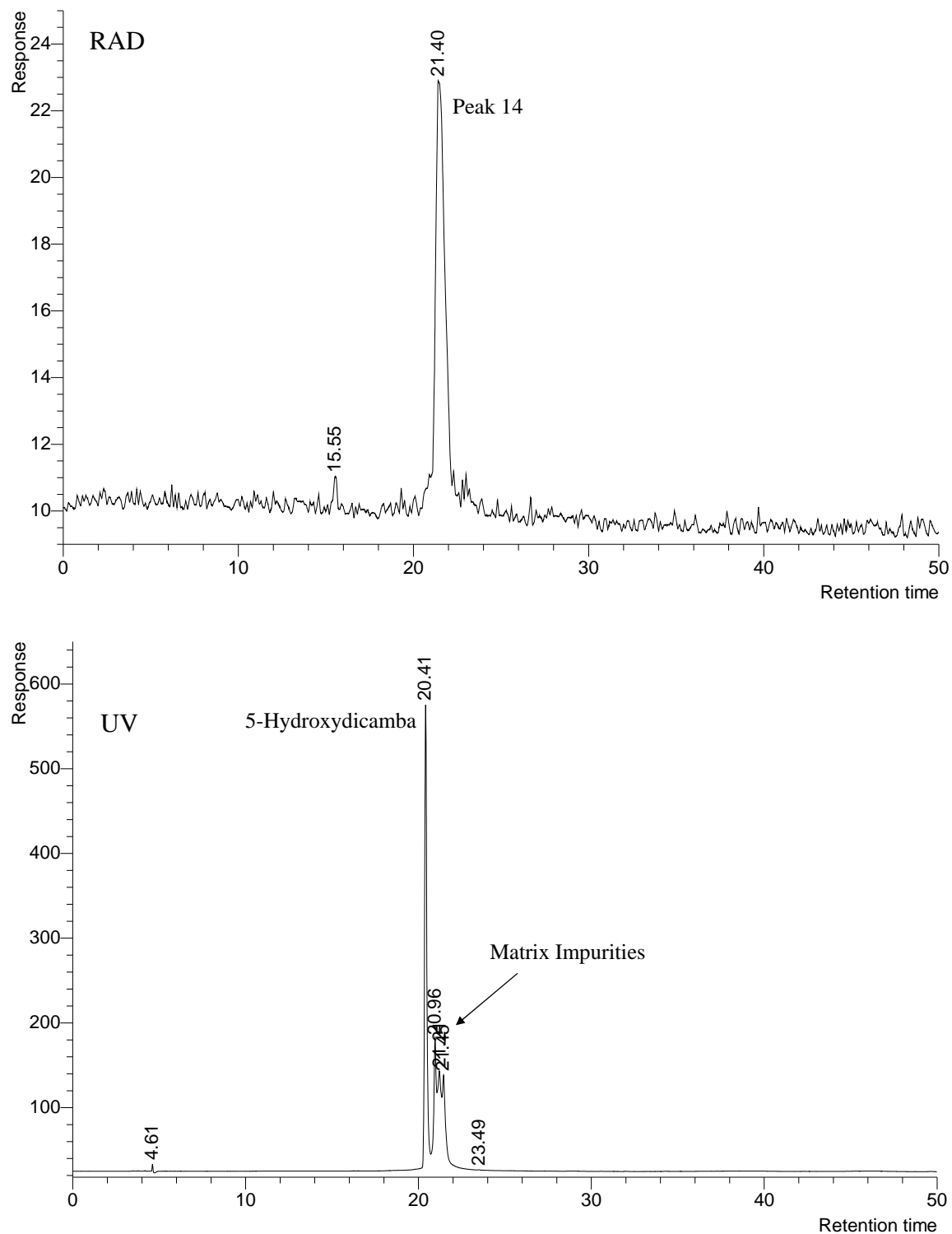


Figure 93. HPLC/RAD Analysis of Peak 14 from POE-T Forage Using HPLC Method C

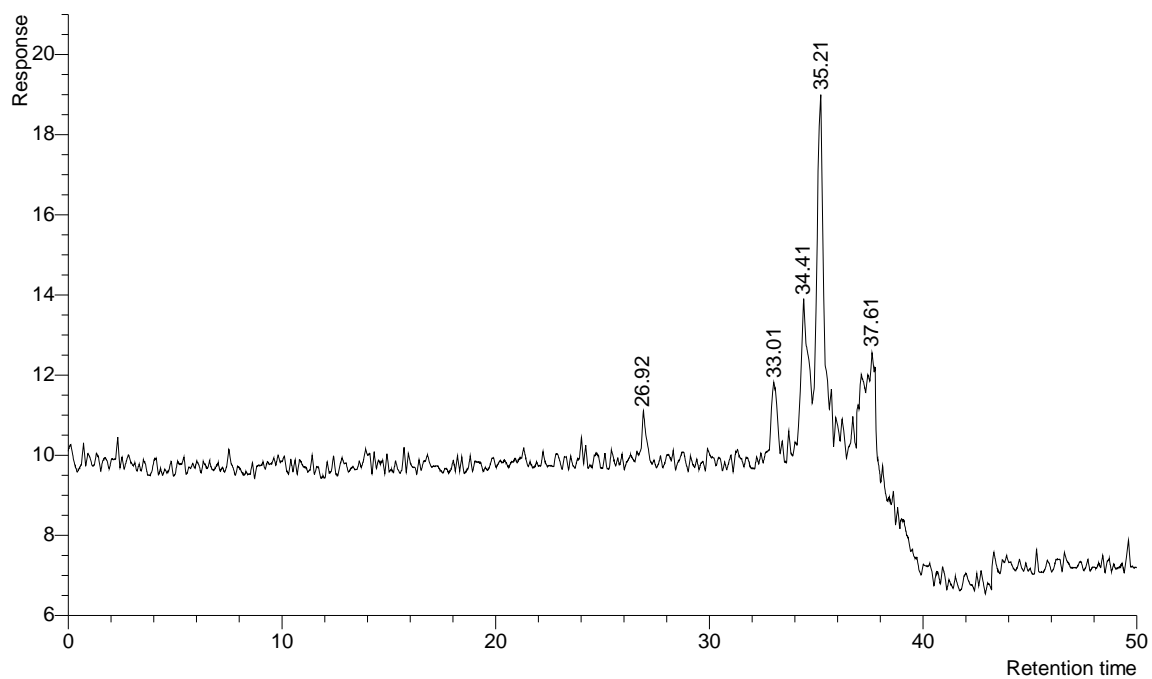


Figure 94. HPLC/RAD Analysis of Peak 14 from POE-T Hay Using HPLC Method C

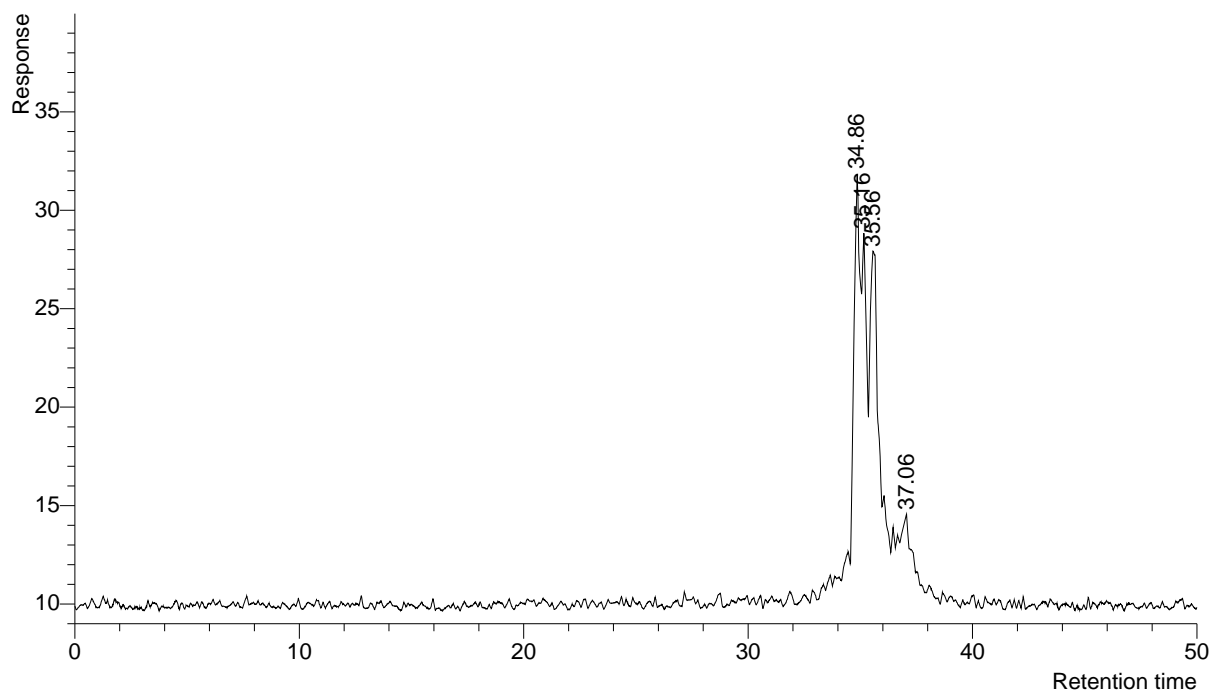


Figure 95. HPLC/RAD Analysis of the Acid Hydrolysate of Peak 14 from POE-T Hay Using HPLC Method B

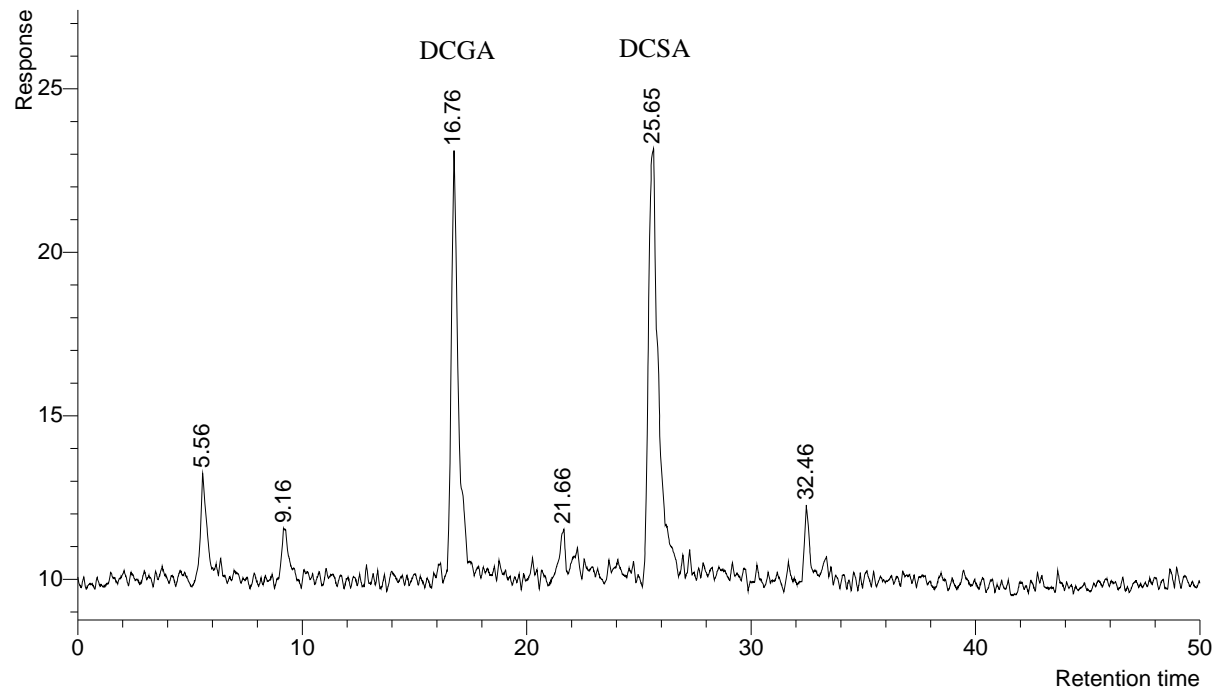


Figure 96. Negative Ion Electrospray Mass Spectrum (top) and m/z 543 Selected Ion Chromatogram (bottom) of Peak 17 Isolate from POE-T Hay

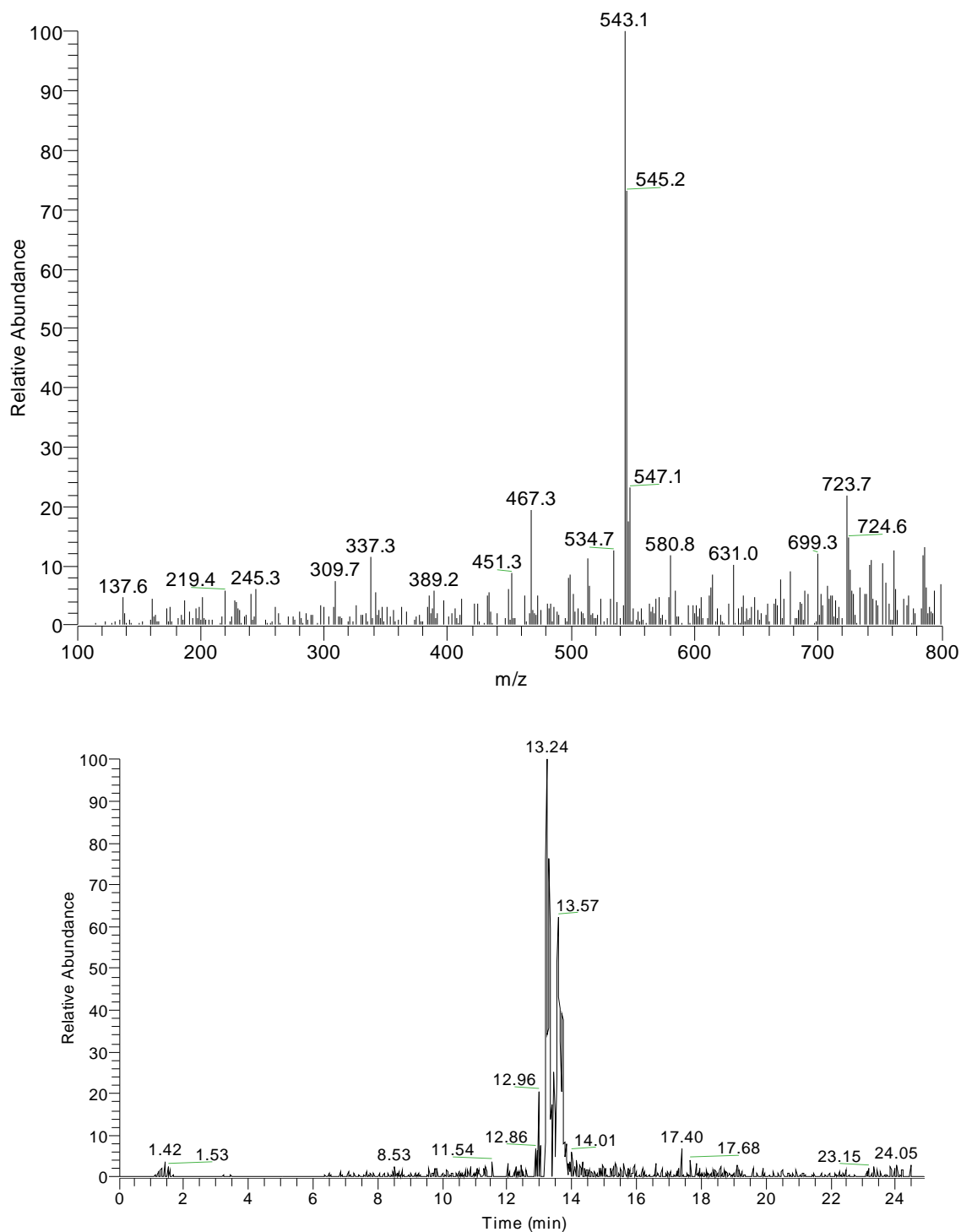


Figure 97. HPLC/RAD Analysis of the Organic Phase of the Ethyl Acetate Partition of POE-T Hay Extract Concentrate Using HPLC Method B (Expanded Scale)

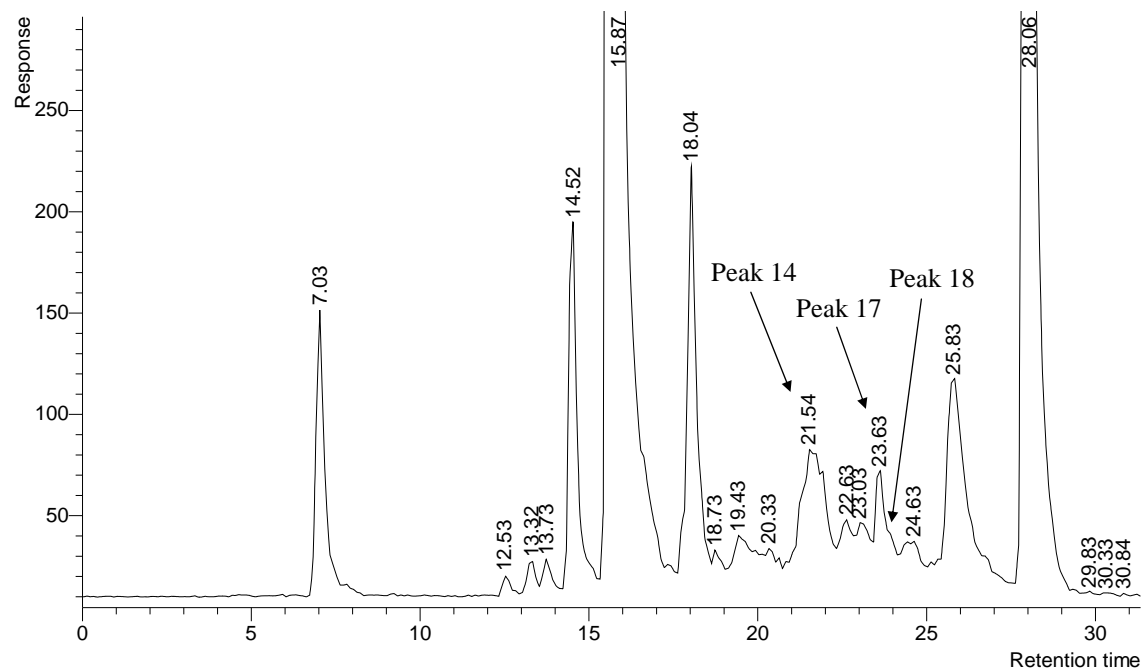


Figure 98. HPLC/RAD Analysis of the Acid Hydrolysate (top) and Mild Base Hydrolysate (bottom) of Peak 18 from POE-T Forage Using HPLC Method B

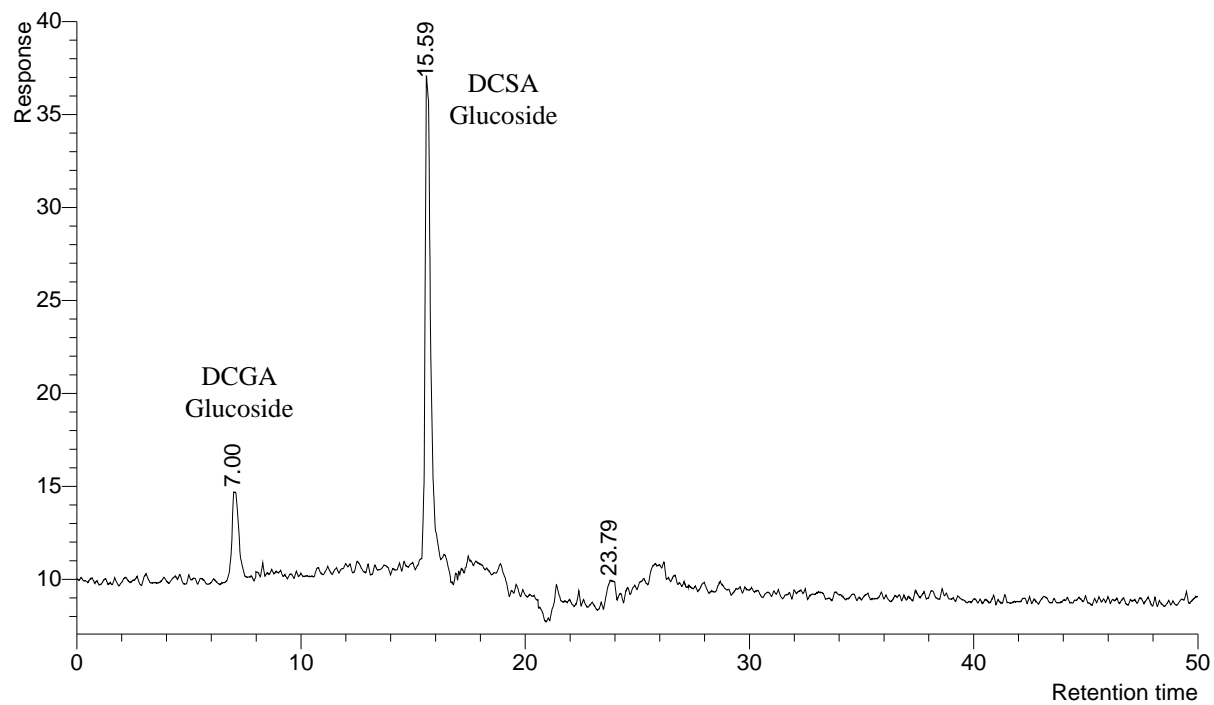
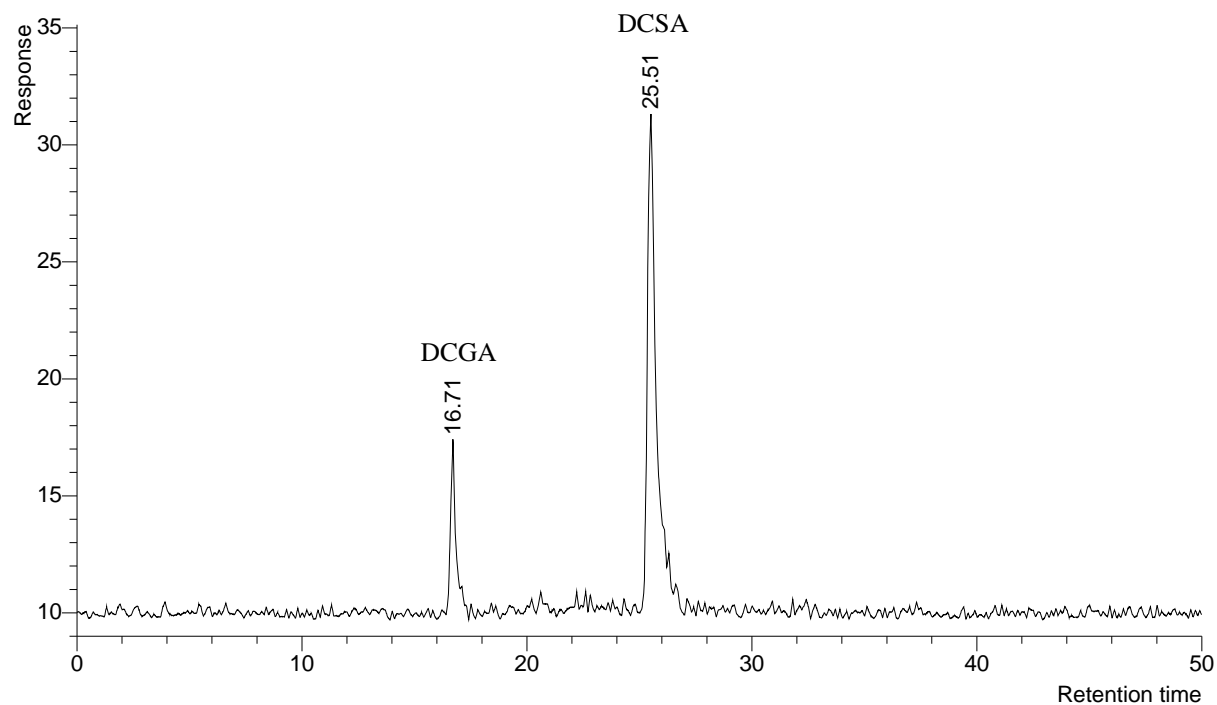


Figure 99. Negative Ion Electrospray Mass Spectrum (top) and m/z 543 Selected Ion Chromatogram (bottom) of Peak 18 from POE-T Forage

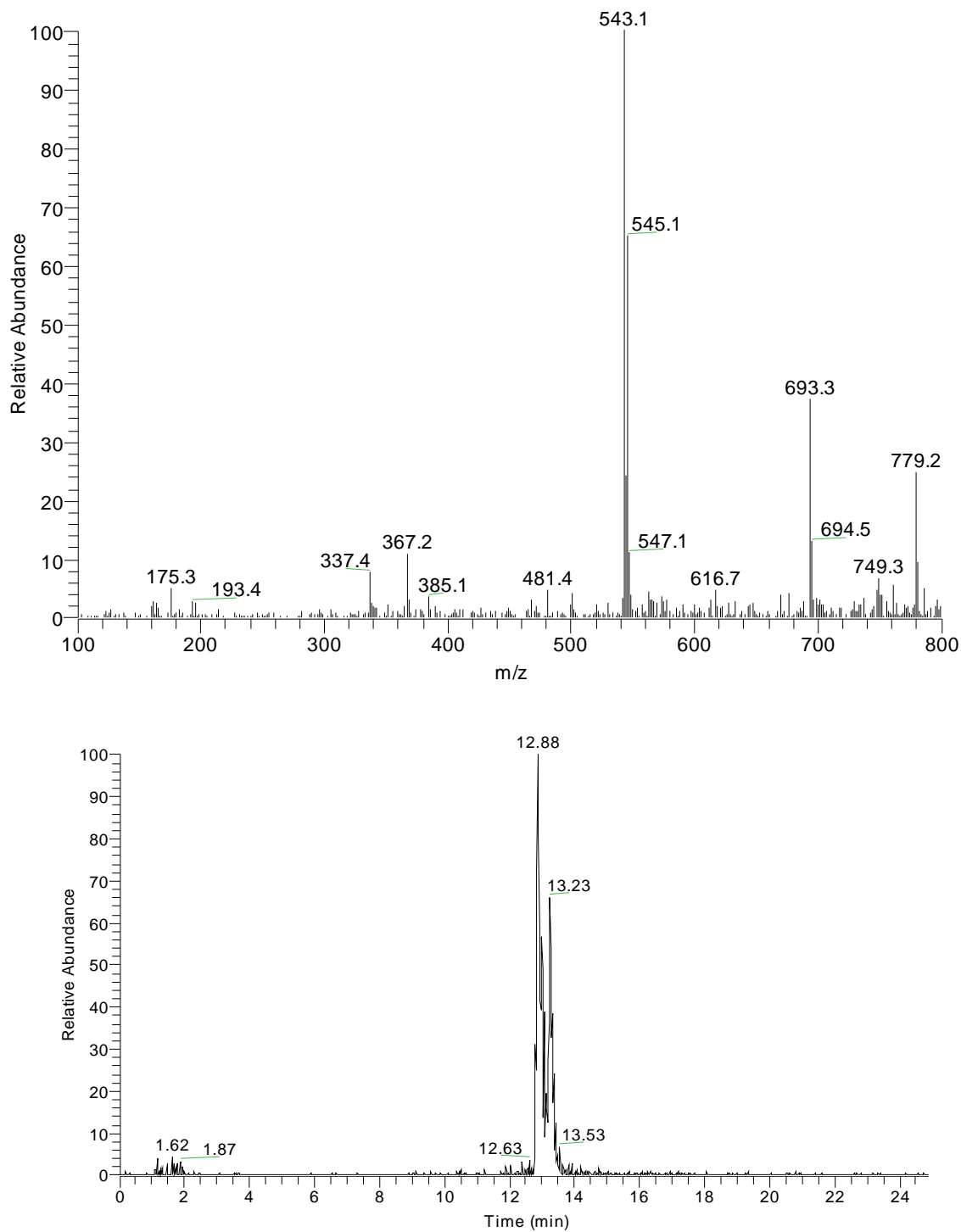


Figure 100. HPLC Coinjection Analysis of Peak 22 from POE-T Forage and DCSA Reference Standard Using HPLC Method B

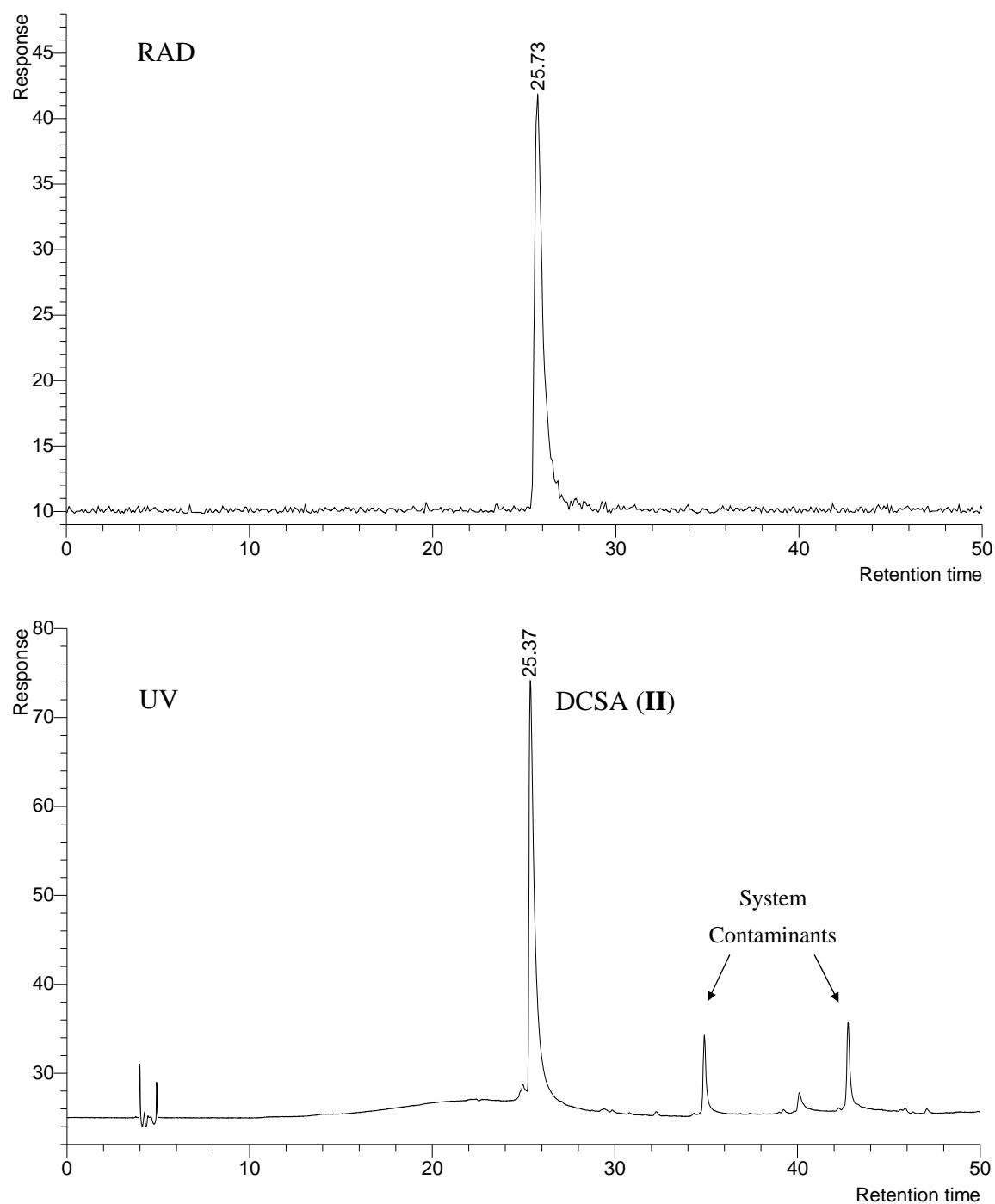
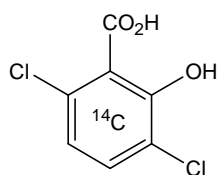
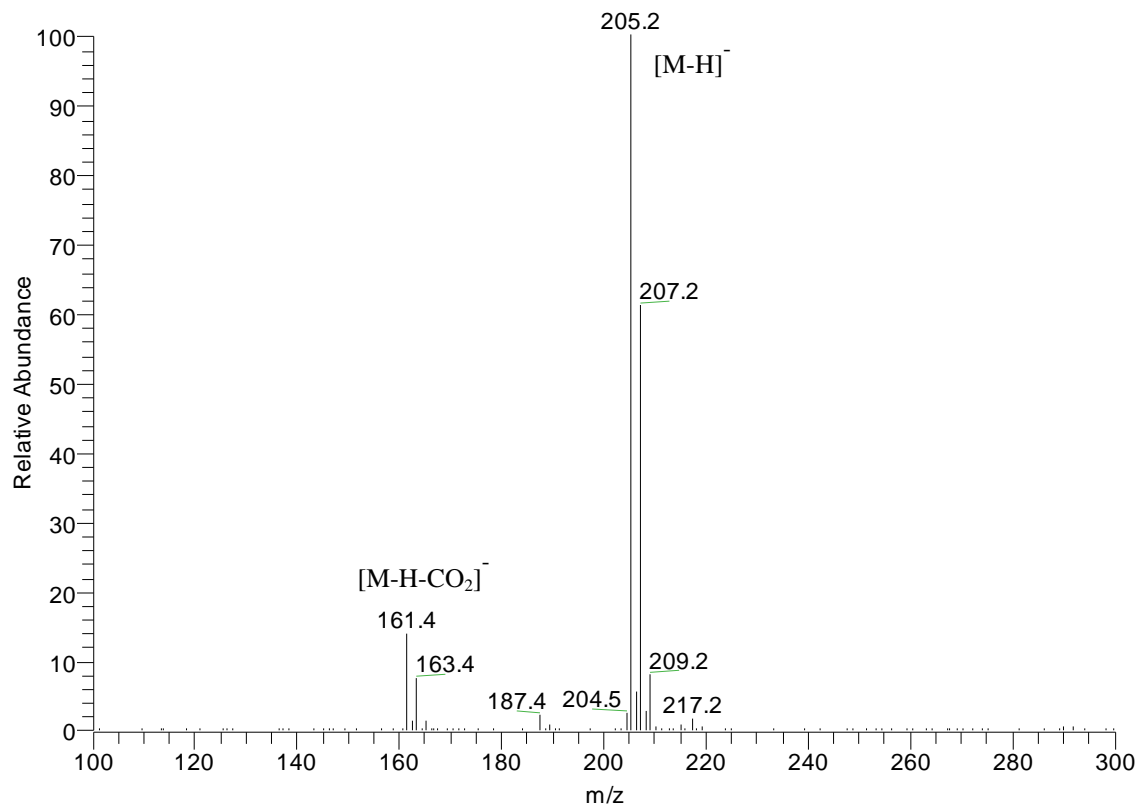
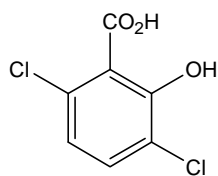
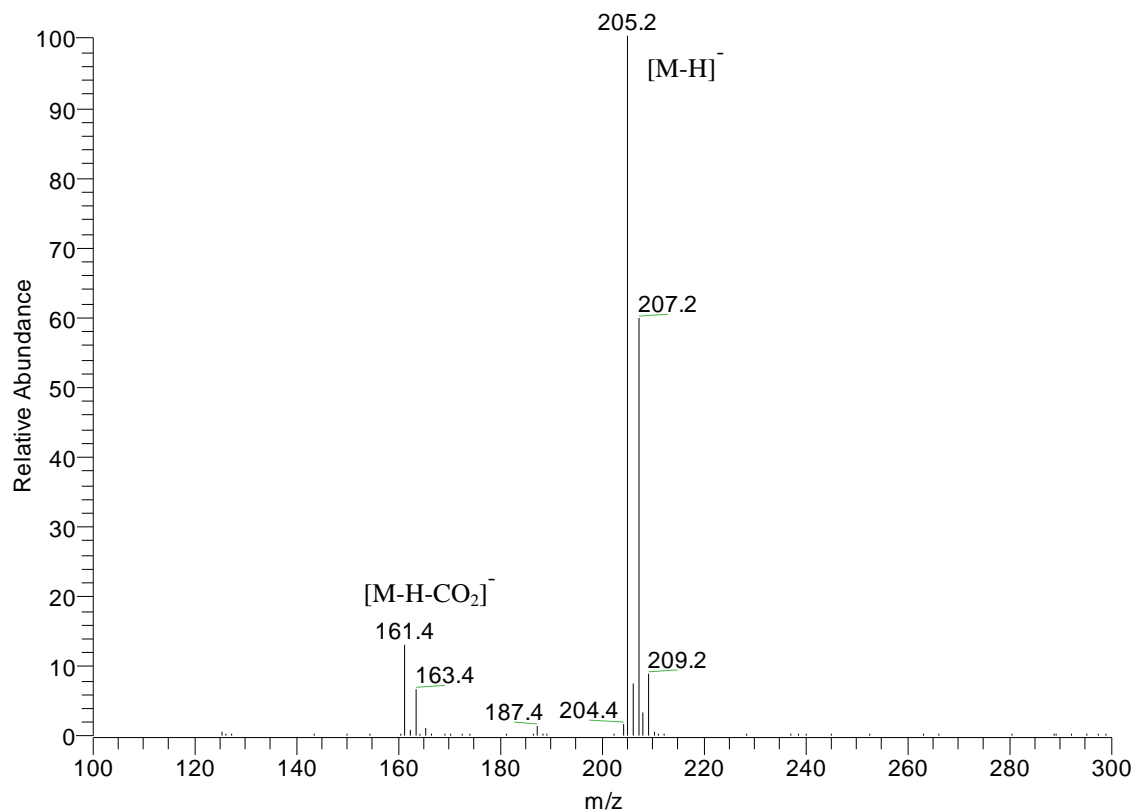


Figure 101. Negative Ion Electrospray Mass Spectrum of Peak 22 from POE-T Forage



DCSA (22)
Nominal Mass 206

Figure 102. Negative Ion Electrospray Mass Spectrum of DCSA Reference Standard



DCSA (II)
Nominal Mass 206

Figure 103. HPLC Coinjection Analysis of Peak 22 from POE-T Hay with Reference Standards Using HPLC Method C

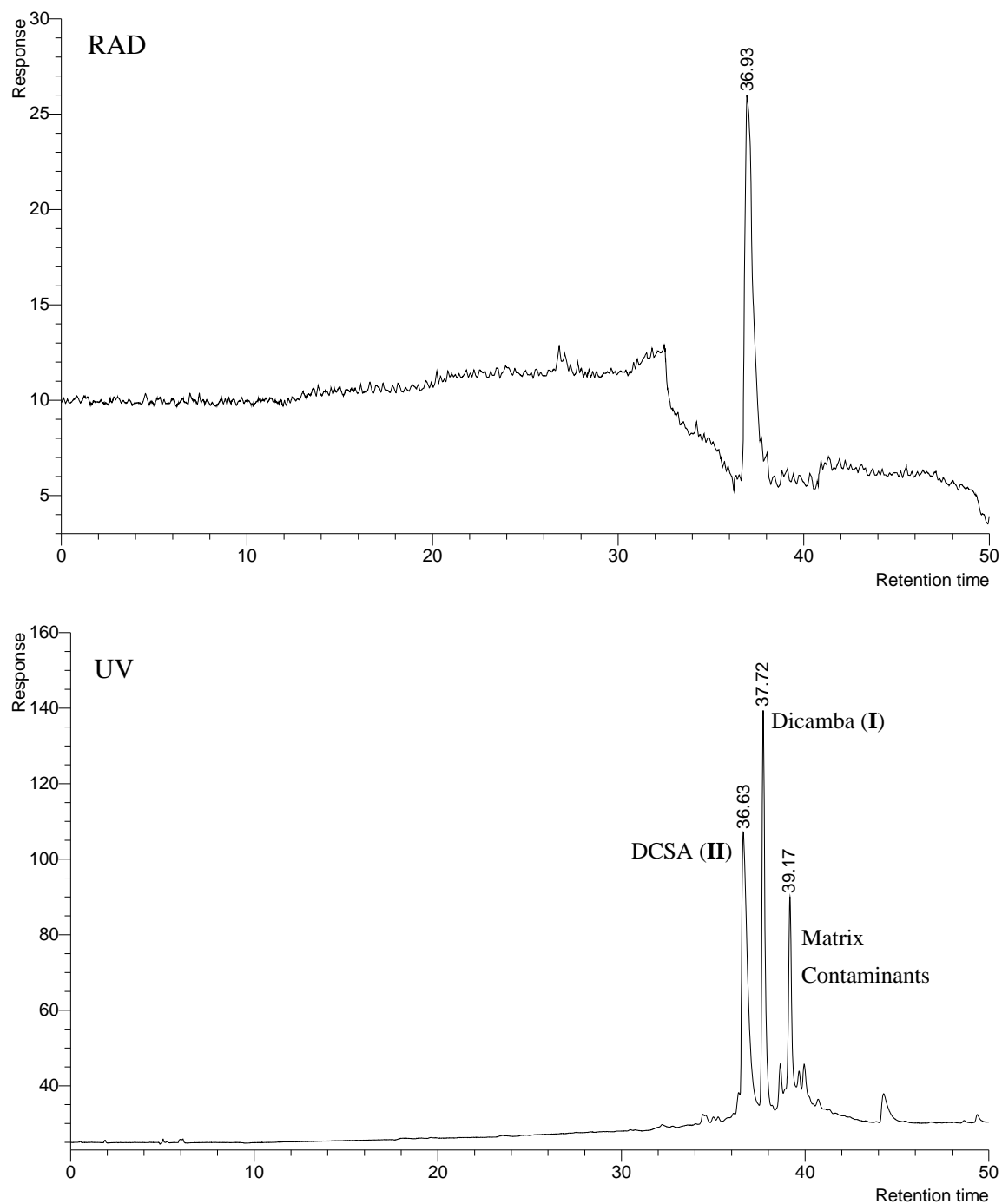
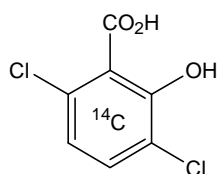
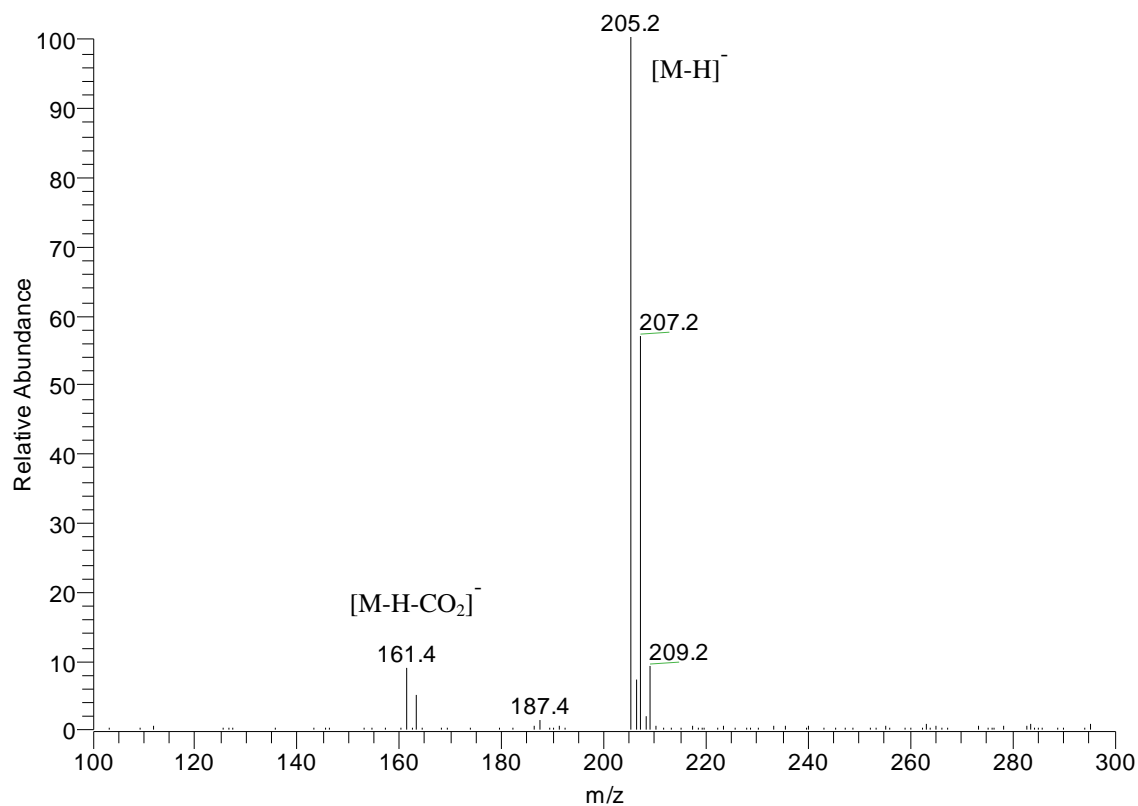


Figure 104. Negative Ion Electrospray Mass Spectrum of Peak 22 from POE-T Hay



DCSA (22)
Nominal Mass 206

Figure 105. HPLC Coinjection Analysis of Peak 23 from POE-T Forage with Dicamba Reference Standard Using HPLC Method B

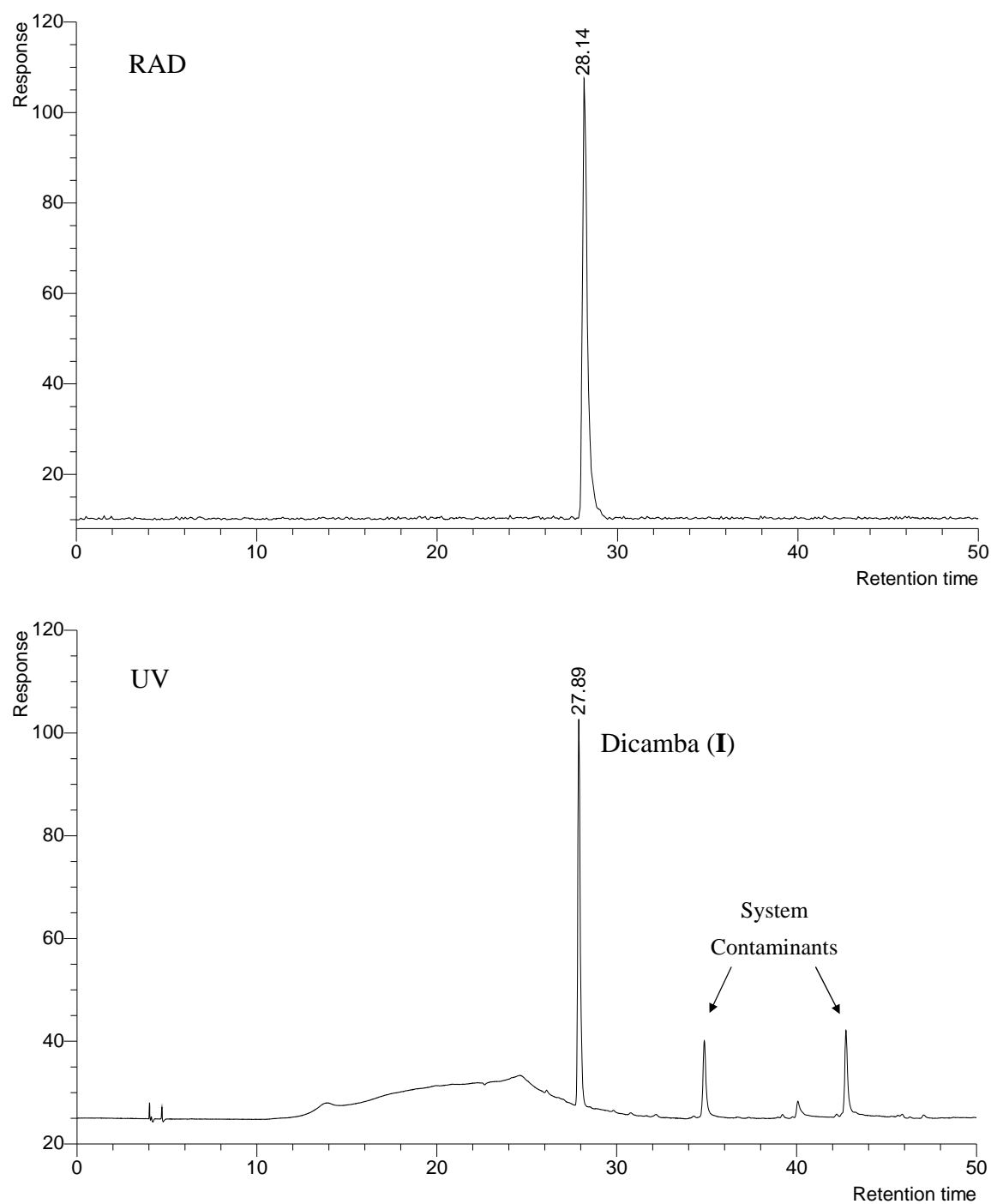
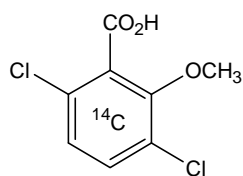
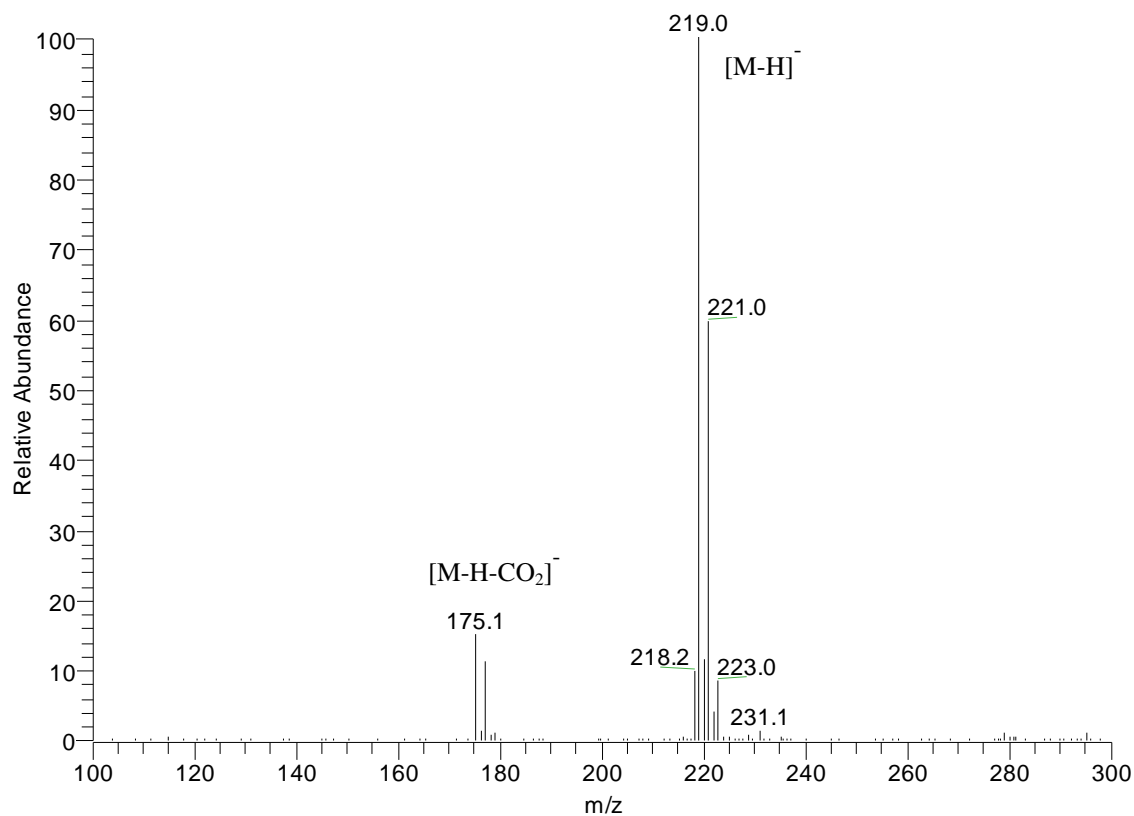
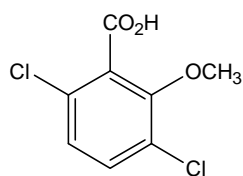
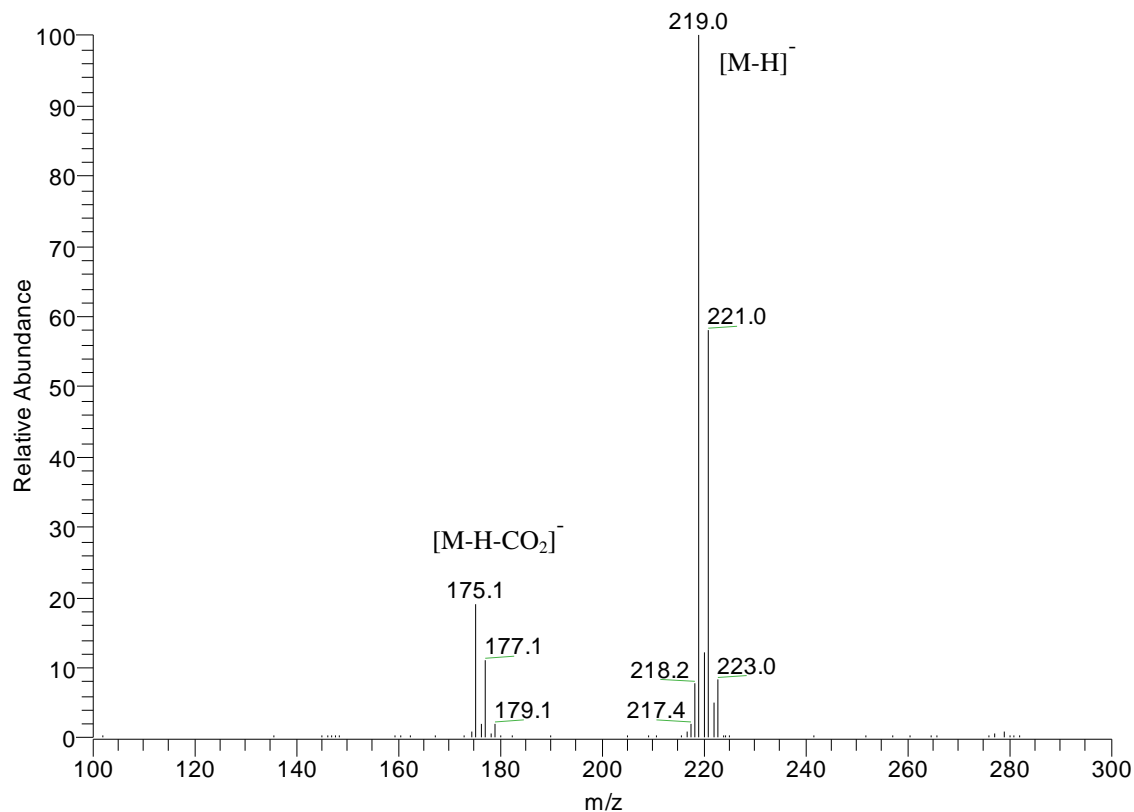


Figure 106. Negative Ion Electrospray Mass Spectrum of Peak 23 from POE-T Forage



Dicamba (**23**)
Nominal Mass 220

Figure 107. Negative Ion Electrospray Mass Spectrum of Dicamba Reference Standard



Dicamba (I)
Nominal Mass 220

Figure 108. HPLC Stability Profiles of PRE-T Forage Using HPLC Method B – PTRL Initial (top), Monsanto Initial (middle) and Final (bottom)

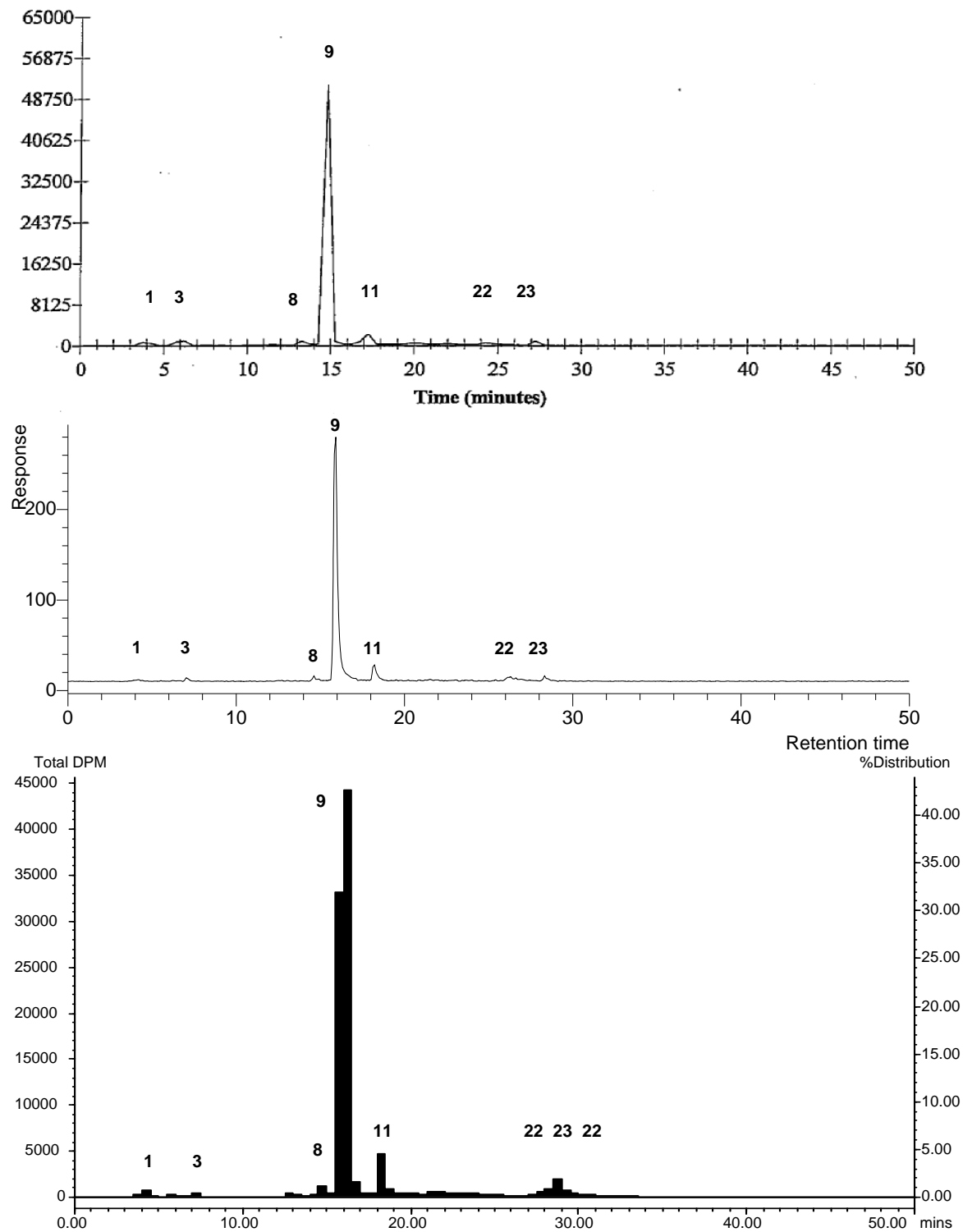


Figure 109. HPLC Stability Profiles of PRE-T Hay Using HPLC Method B – Initial (top) and Final (bottom)

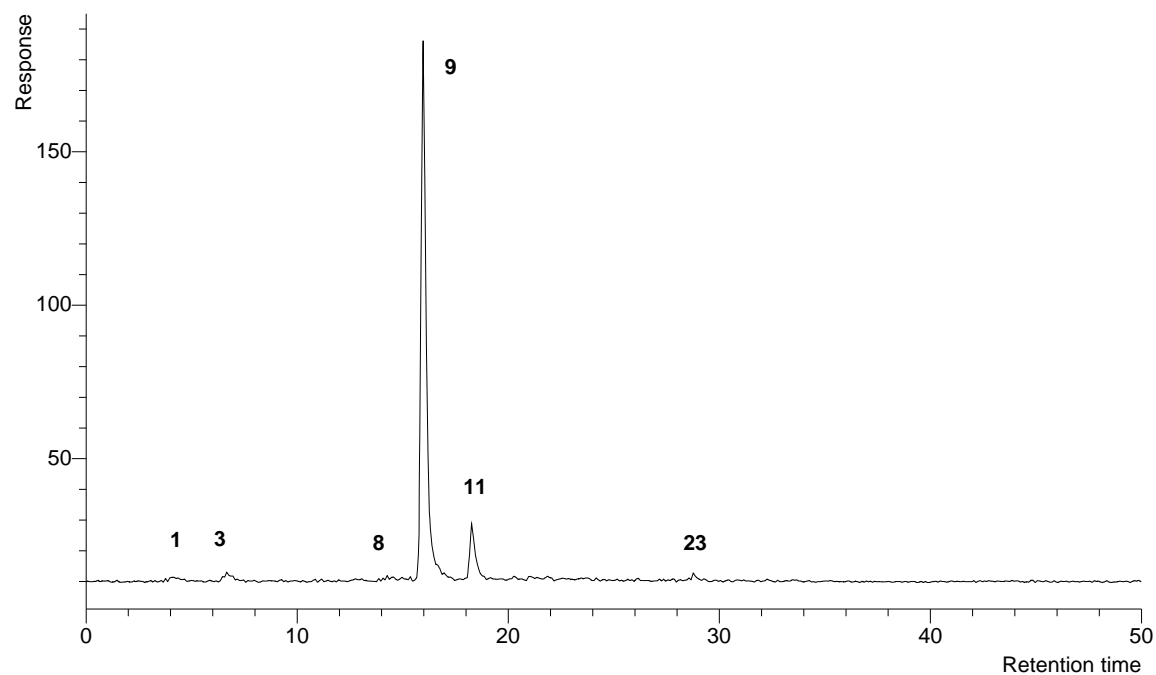
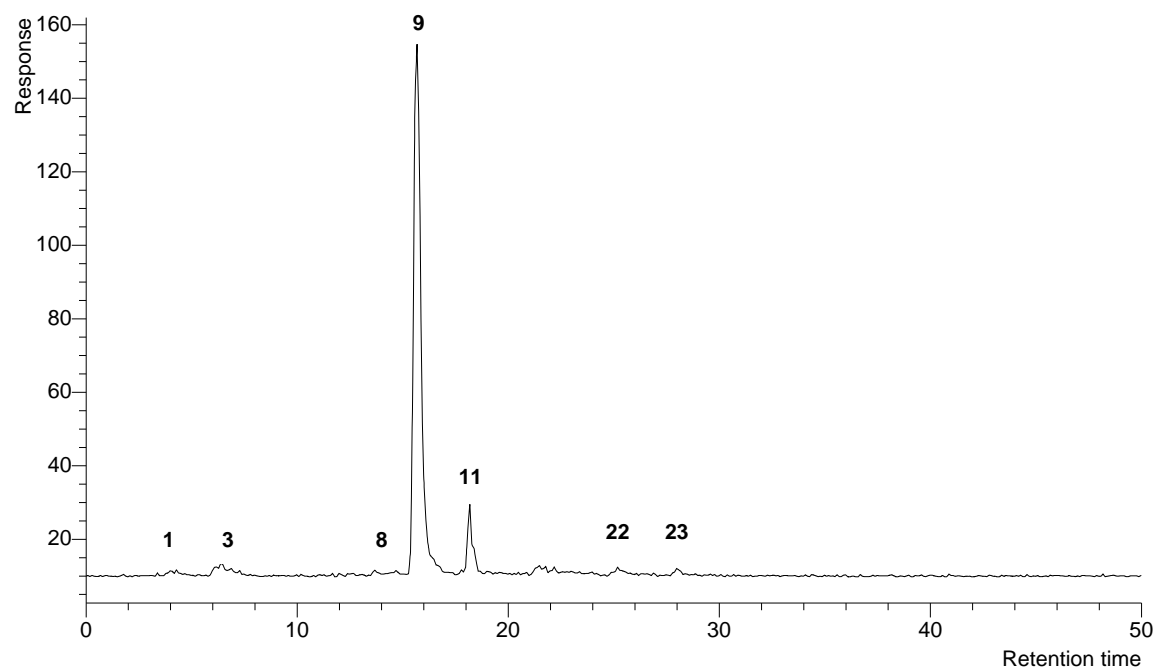


Figure 110. HPLC Stability Profiles of POE-T Hay Using HPLC Method B – Initial (top) and Final (bottom)

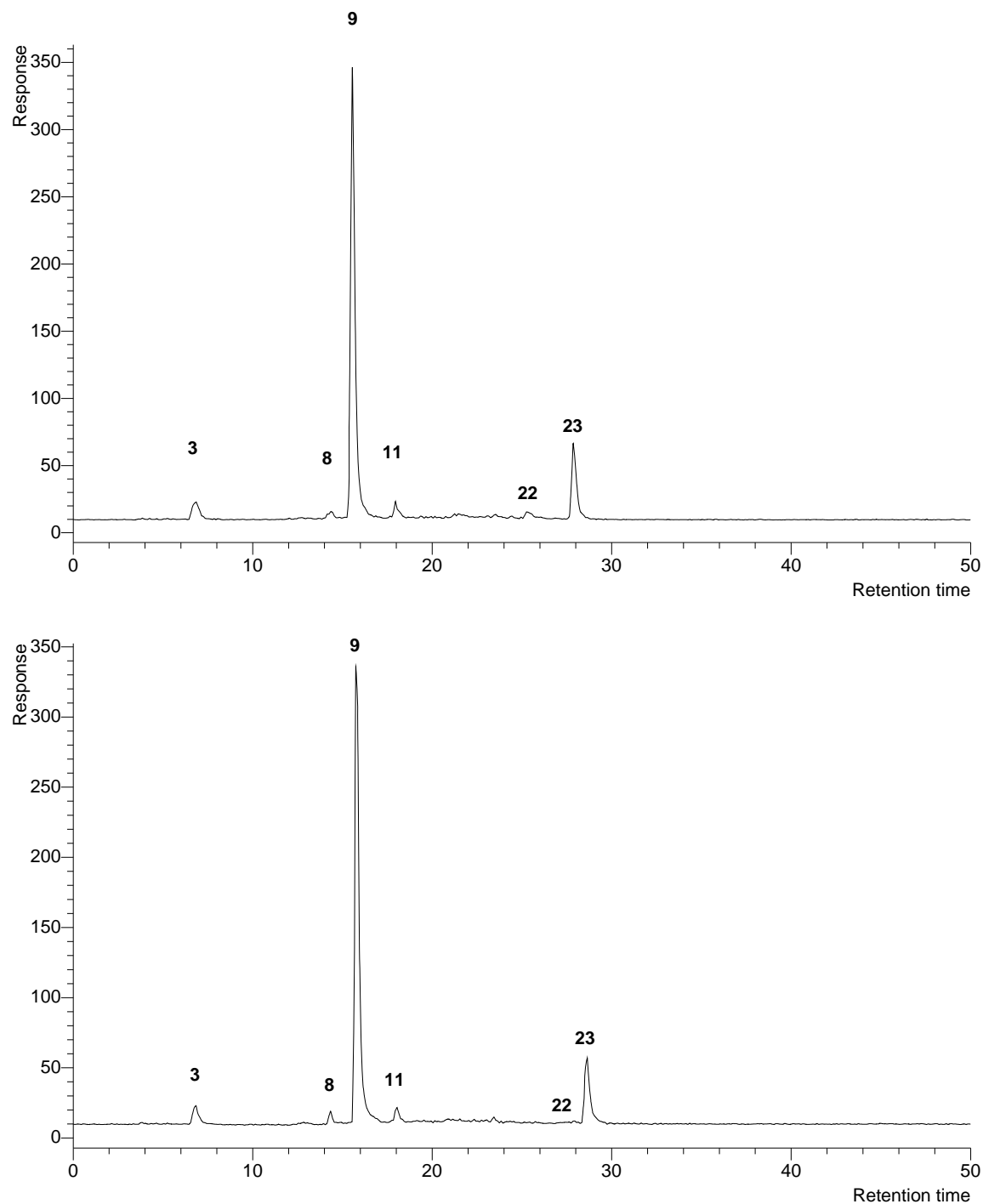


Figure 111. HPLC Stability Profiles of POE-T Seed Using HPLC Method B – PTRL Initial (top), Monsanto Initial (middle) and Final (bottom)

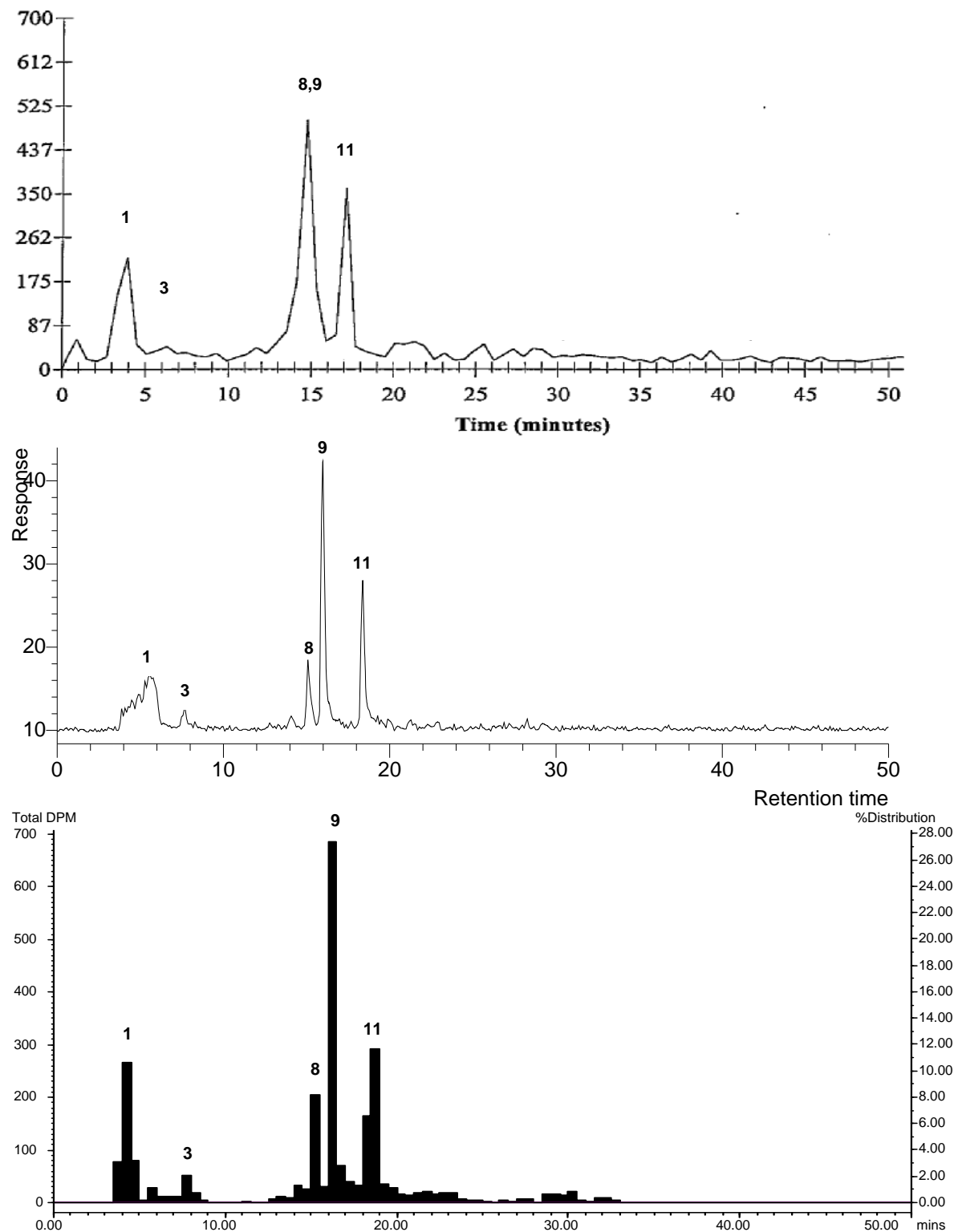
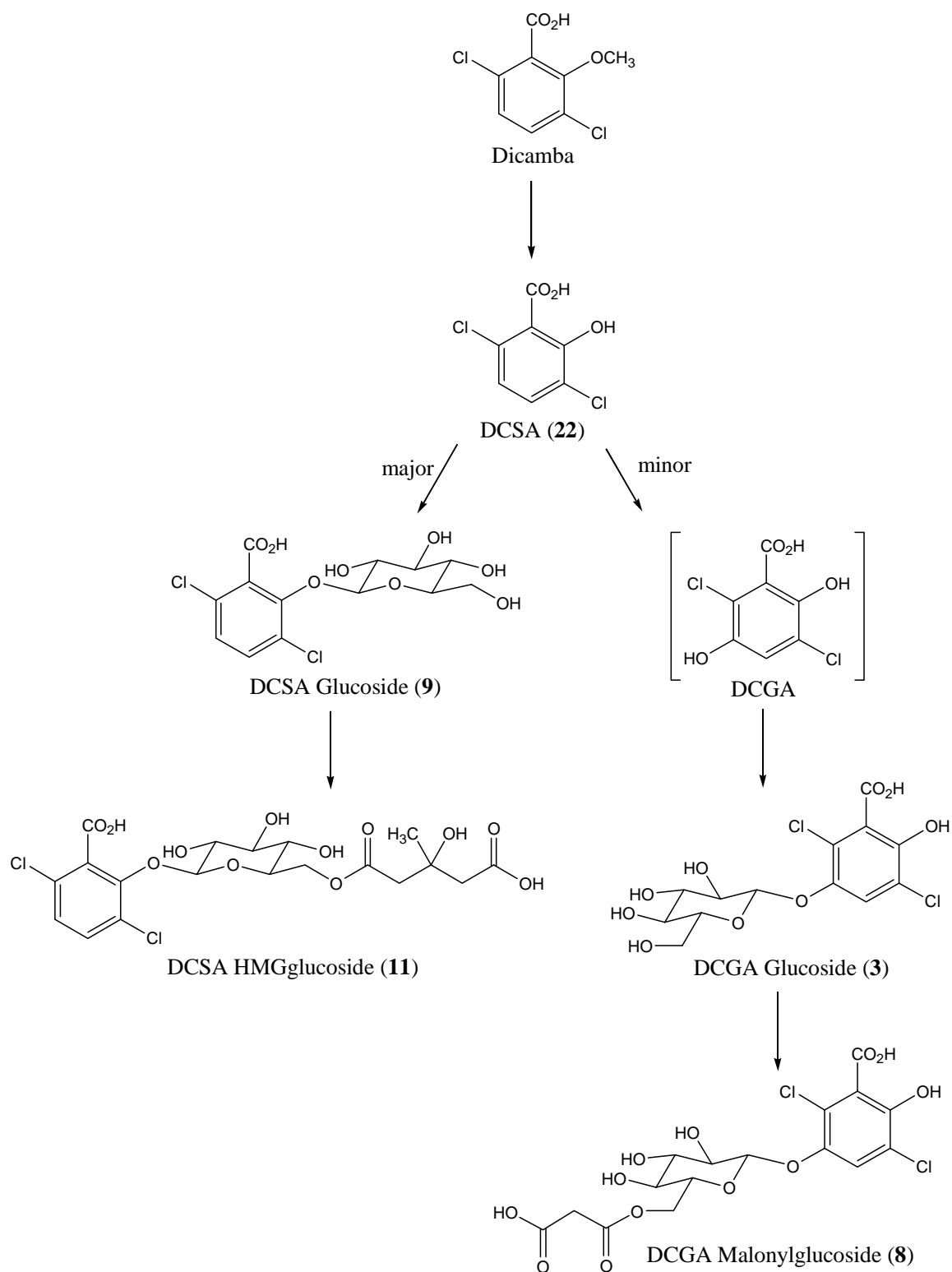


Figure 112. Proposed Pathway for the Metabolism of Dicamba in Dicamba-Tolerant Soybean



7 Other Information

7.1 Notebook References

G-807801-99, G-815801-99, G822901-99, G-828901-99 and G-839301-80

7.2 Study Chronology

Study start date (date Study Director signed the Protocol): May 25, 2006

Experimental Start Date (Test Substance Application): June 01, 2006

Experimental Completion Date (In-life Phase): September 21, 2006 (seed harvest)

Experimental Completion Date (Analysis Phase): September 02, 2009 (extended storage stability)

Study completion date (Final Report): December 23, 2009

Amended report date: March 30, 2010

7.3 Acknowledgements

The authors wish to thank Tracy Whitehead for data QC review, Mitch Kurtzweil for report review and assistance in protocol development, Dayong Sun for assistance in obtaining the high resolution mass spectra of Peak 11 on the PerkinElmer Sciex QSTAR mass spectrometer and James Foster for obtaining MS/MS spectra of Peak 11 on the ABI/Sciex API 5000 mass spectrometer.

8 References

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- 10) Cai, Y., Sun, M. and Corke, H., *J. Agric. Food Chem.*, **49**(4), 1971-1978 (2001), Identification and Distribution of Simple and Acylated Betacyanins in the *Amaranthaceae*
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b) Struijs, K., Vincken, J.-P., Verhoef, R., Voragen, A.G.J., Gruppen, H., *Phytochemistry*, **69**, 1250-1260 (2008), Hydroxycinnamic Acids are Ester-Linked to Glucosyl Moieties within the Lignan Macromolecule from Flaxseed Hulls

- 12) Roberts, T.R., *et al.*, ed., *Metabolic Pathways of Agrochemicals, Part 1: Herbicides and Plant Growth Regulators*, The Royal Society of Chemistry (1998), pp 148-151
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- 15) Residue Analytical Method AM-0691B-0593-3, Sandoz Agro, Inc. (1993), Determination of Dicamba and 5-hydroxydicamba Residues in Barley, Corn, Cotton, Cotton Processed Fractions, Pasture Grass, Peanut, Sorghum, Soybean, Sugar Cane, Tomato, Tomato Processed Fractions, Wheat and Wheat Processed Fractions (GC)

9 Appendix A – Study Protocol and Amendments

The following is an exact representation of the study protocol except that the study number and page numbers originally included in the header have been moved to the footer to avoid overlap with the main report header.

STUDY PROTOCOL

Study Title

Metabolism of Dicamba in Dicamba-Tolerant Soybeans

Year

2006

Guideline

Nature of the Residue – Plants, Livestock, US EPA OPPTS 860.1300

Study Number

Monsanto Study Number: 06-98-M-1
PTRL West, Inc. Study Number: 1491W

Sponsor

Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167

1 STUDY INFORMATION

1.1 Study Title

Metabolism of Dicamba in Dicamba-Tolerant Soybeans

1.2 Project Numbers

Monsanto Study Number: 06-98-M-1
PTRL West, Inc. Study Number: 1491W

1.3 Sponsor

Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167
Contact: Annette M. Kirk

1.4 Testing Facility

Monsanto Company
Environmental Sciences Technology Center
800 N. Lindbergh Blvd.
St. Louis, MO 63167

1.5 Testing Facility Management

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800 N. Lindbergh Blvd.
St. Louis, MO 63167
(314) 694-8299

1.6 Study Director

Michael J. Miller
Monsanto Company - V2C
800 N. Lindbergh Blvd.
St. Louis, MO 63167
Phone: (314) 694-8269
Fax: (314) 694-8774
email: michael.j.miller@monsanto.com

1.7 Performing Laboratories and Study Participants

Test Site: PTRL West, Inc.
625-B Alfred Nobel Drive
Hercules, CA 94547
Principal Investigator: Alex V. Bautista
Telephone: (510) 741-3000, ext 255
Fax: (510) 741-3030
e-mail: abautista@ptrlwest.com

Field Test Site: Research For Hire
1696 S. Leggett St.
Porterville, CA 93257
Principal Investigator: Heather Holmes
Telephone: (559) 784-5787
Fax: 559-784-4351
e-mail: hholmes@research4hire.com

Analytical Phase: Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167
Study Director: Michael J. Miller
Telephone: (314) 694-8269
Fax: (314) 694-8774
e-mail: michael.j.miller@monsanto.com

Soil Characterization: AGVISE Laboratories
P. O. Box 510, Hwy. 15
Northwood, ND 58267

1.8 Proposed Study Dates

Proposed experimental start date (application): May 2006
Proposed greenhouse phase completion date (harvest): September 2006
Proposed experimental completion date (analysis phase): September 2007
Proposed study termination date: December 2007

1.9 Relevant Testing Guidelines

The study will be conducted in accordance with the requirements of EPA residue chemistry test guideline OPPTS 860.1300, "Nature of the Residue – Plants, Livestock".

2 PURPOSE

The purpose of this study is the determination of the nature of residues found in/on agricultural commodities of dicamba-tolerant soybeans treated with [¹⁴C]dicamba.

Specific objectives of the study are the following: 1) determination of the total

radioactive residues (TRR) in soybeans following treatment with radiolabeled dicamba; 2) determination of the efficiency of extraction of the residue components; 3) identification and quantification of the major components of the terminal residue, and delineation of the major routes of metabolism of dicamba in soybeans.

3 CONFIDENTIALITY

All information regarding the identity of the test substance, samples, test system, and data must be kept strictly confidential.

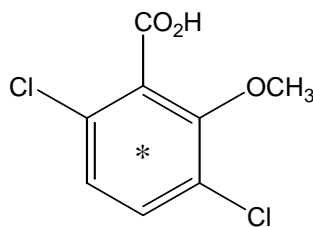
Test areas are considered restricted access areas, and measures should be in place to exclude unauthorized persons from the test area. No designation in or around the test area will be made that identifies the test substance or Sponsor.

No raw data, worksheets, data or information summaries, reports, or other information related to this study may be revealed or released to any third party without prior notification and authorization of Monsanto Company.

4 TEST SUBSTANCE

4.1 Identification

The test substance for this study is dicamba. Dicamba is a solid, which is soluble in organic solvents and sparingly soluble in water (0.5 g per 100 mL at 25 °C). The test substance consists of a mixture of dicamba uniformly labeled in the phenyl ring with carbon-14 and unlabeled dicamba. The amounts of labeled and unlabeled dicamba have been adjusted to give the desired specific activity. The test substance will be applied as its diglycolamine (DGA) salt, which has a solubility of 107 g per 100 mL of water.



dicamba

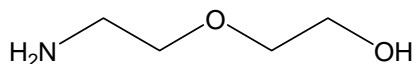
* designates uniform ¹⁴C-labeling

Chemical Name: 3,6-dichloro-2-methoxybenzoic acid (CAS)
3,6-dichloro-*o*-anisic acid (IUPAC)

Molecular Formula: C₈H₆Cl₂O₃

Molecular Weight: A MW of 221.0 g/mol will be used for all calculations.

CAS Number: 1918-00-9 (free acid, unlabeled)



diglycolamine (DGA)

Chemical Name: 2-(2-aminoethoxy)ethanol (CAS and IUPAC)

Molecular Formula: C₄H₁₁NO₂

Molecular Weight: A MW of 105.14 g/mol will be used for all calculations.

CAS Number: 929-06-6

4.2 Specific Activity

The target specific activity of the test substance will be 5.4 mCi/mmol (approximately 54000 dpm/μg). This will allow determination of TRR levels down to approximately 0.005 mg/kg (ppm). At a residue level of 0.005 mg/kg, a typical combustion sample weighing 0.25 g will contain a radioactivity level of approximately 70 dpm, which can be measured with good statistical significance by combustion and liquid scintillation counting (LSC). The total amount of radioactivity to be applied in the study based on the target specific activity will be approximately 28 mCi. The actual specific activity of the test substance will be provided by the Sponsor prior to conducting any of the applications.

4.3 Characterization and Purity

The test substance will be characterized under GLP at Monsanto prior to use in the study. The radiochemical purity will be determined and an assessment of the chemical purity will be made by HPLC prior to shipment to PTRL West, Inc. The radiochemical purity will be ≥ 97%. Retain samples of the test substance will be archived by the Sponsor.

4.4 Stability

The stability of the test substance during shipment, formulation and application will be assessed by PTRL West, Inc. through analysis by HPLC with radioactivity detection. The analytical method for the radiochemical analysis will be provided by the Sponsor. These radiochemical purity determinations will be compared with the original purity determination in order to determine stability. The test substance will be considered to be stable during shipment and formulation if the radiochemical purity from the analysis of an aliquot prior to application is within 5% of the radiochemical purity of the value obtained by Monsanto prior to shipment. The test substance will be considered to be stable during application if the radiochemical purity of the test substance after application is within 5% of the radiochemical purity of an aliquot prior to application.

4.5 Shipment and Storage

The neat test substance (preweighed at Monsanto) will be sent to PTRL West, Inc. on dry ice in two separate shipments; one for each of the treatments. Each shipment will contain

an excess (ca. 5-10%) of test substance required for the respective treatment groups. Monsanto will also provide the neat DGA required for the formulation. The test substance will be stored in a freezer (<-10 °C) when not in use. The DGA will be stored at ambient laboratory temperature.

4.6 Safety Precautions

Appropriate safety precautions will be observed as typical for use of pesticides and radiolabeled materials. All mandated safety practices for handling and use of ¹⁴C-containing radioactive isotopes are required. In addition, dicamba is slightly volatile, which may present additional radioactivity and toxicity issue considerations. Precautions will be in place to avoid any ¹⁴C-contamination of the immediate study area. An MSDS is available for dicamba and will be distributed to appropriate study personnel by the Study Director. The greenhouses containing the study plants will be located at Research For Hire. Research For Hire SOP's will be followed for security and identification of radioactive plant material.

5 REFERENCE AND CONTROL SUBSTANCES

5.1 Reference Substances

Reference substances may be employed as aids in metabolite analysis and identification, especially as HPLC retention time standards or as mass spectral standards.

The following reference standards may be utilized:

Dicamba, 3,6-dichloro-2-methoxybenzoic acid, CAS Registry Number 1918-00-9

DCSA (acronym for 3,6-dichlorosalicylic acid), 3,6-dichloro-2-hydroxybenzoic acid, CAS Registry Number 3401-80-7

5-Hydroxydicamba, 2,5-dichloro-3-hydroxy-6-methoxybenzoic acid, CAS Registry Number 7600-50-2

The need for additional reference standards may arise during the study, and these will be identified in the final report.

5.2 Reference Substance Characterization

The reference substances will be characterized under GLP at Monsanto prior to their use in the study. Retain samples of the reference substances will be archived by the Sponsor.

5.3 Control Substances

The use of control substances is not anticipated in this study.

6 TEST SYSTEM

6.1 Test System Description and Justification

The soybean (*Glycine max* L.) test system used in this study will be GM_A90617, which contains the dicamba monooxygenase (DMO) gene isolated from *Pseudomonas maltophilia*. The expression of DMO confers dicamba tolerance to GM_A90617. Dicamba is intended for use as a herbicide in dicamba-tolerant soybeans to control certain weeds.

6.2 Test System Characterization

The GM_A90617 seed used in this study was obtained from Monsanto Trait Development and has been assigned a lot number of GLP-0604-17294-S. The tolerance to dicamba of plants grown from the GM_A90617 seed will be verified before the use of the seed in the study. The seed will be further characterized by the Sponsor during the course of the study to verify it contains the dicamba monooxygenase gene. These characterization data will be retained in the study file.

6.3 APHIS Regulations

The genetically modified soybean used in this study is currently regulated by the USDA Animal and Plant Health Inspection Service (APHIS). **Study participants must follow all relevant APHIS regulations and Monsanto Performance Standards.** For emphasis, the protocol includes key APHIS requirements, but study participants must refer to the separate Compliance Packet for the full detailed handling and documentation requirements. Compliance Packets will be provided to all study participants prior to the start of the study. Data collected solely to meet APHIS requirements will be retained separately from the study file and will not be included in the final report.

6.4 Shipment and Handling of Seed

The genetically modified seed used in this study will be provided to Research For Hire by the Sponsor. All shipping and handling of seed must be done following all APHIS regulations. A seed chain-of-custody form will be used to track the shipment and receipt of the seed. Seed will be shipped under double containment to prevent accidental release. The regulated seed must be stored in a secured facility (e.g. locked file or storage cabinet) separate from nonregulated seed. At least one sign must be posted on the secured facility stating that a regulated genetically modified organism (transgenic seed) is being stored and that unauthorized access is prohibited. **Any regulated seed remaining after establishment of the test system must be devitalized on site or returned to the Sponsor after approval by the Study Director.** The seed shipping container (or a photograph of the container) is to be saved for possible APHIS inspection for the duration of the greenhouse phase of the study. The seed container will be sent back to the Sponsor, incinerated, or otherwise properly disposed of after approval by the Study Director.

7 EXPERIMENTAL DESIGN

7.1 Test Site and APHIS Requirements for Containment and Identification

The test system will be planted in 12-inch diameter pots containing sandy loam soil and grown in greenhouses at Research For Hire.

The regulated soybean seed used in this study has not been approved for field release. Therefore, it must be handled and maintained at all times in a contained environment in such a way that there is no release into the environment. Such handling will require ensuring that regulated material is not accidentally mixed with nonregulated material, does not transfer genes to nonregulated material inside or outside the facility, and does not accidentally escape from the contained facility. In order to prevent accidental mixing of regulated and nonregulated material, a uniform identification scheme, such as by obvious marks, color-coding, or strict segregation of material, should be implemented when identifying the test system.

The greenhouses that contain the soybean plants must be lockable and must be posted with a sign stating **‘This Greenhouse Contains Genetically Modified Organisms’**. The temporary spray chamber in which spray applications are to be conducted will be attached to the greenhouse and enclosed with plastic on all sides, including the floor, except for an opening to allow entry from the greenhouse.

7.2 Collection and Characterization of Soil

Soil for use in the study will be collected by Research For Hire from a location on the Research For Hire Experimental Farm. It is recognized that the soil may have been treated with pesticides in prior years; however, the soil collected for use will not have had any exposure to dicamba (or similar compounds) for at least three years prior to treatment. A three-year agronomic history of the soil will be obtained from Research For Hire.

Prior to planting, a sample of the soil to be used in the study will be collected for analysis. Research For Hire will send the sample to AGVISE Laboratories who will conduct the analysis under GLP according to their SOP(s) and will include the following parameters: texture class (USDA), textural composition (percent sand, silt and clay), percent organic matter, pH, cation exchange capacity, moisture holding capacity and bulk density (disturbed soil).

7.3 Planting of Test System

There will be a total of five separate treatment groups for the study. These will include two groups that will receive applications of the test substance, and three control groups. A minimum of 80 pots will be planted as follows:

Treated Groups and Number of Pots

- PRE-T These will receive a preemergence application of 2.5 lb/acre. A minimum of 28 pots will be planted.
- POE-T These will receive a postemergence application of 2.5 lb/acre at the R1 stage. A minimum of 28 pots will be planted.

Control Groups and Number of Pots

- UNT-C Untreated control pots will be located in a separate greenhouse from any treated plants in this or any other study to avoid contamination by ^{14}C -labeled volatile compounds or $^{14}\text{CO}_2$. A minimum of 8 pots will be planted.
- PRE-C Preemergence control pots will be interspersed with the preemergence treated plants. A minimum of 8 pots will be planted.
- POE-C Postemergence control pots will be interspersed with the postemergence treated plants. A minimum of 8 pots will be planted.

Four seeds will be planted in each pot to ensure the emergence of at least one plant from each pot. All seed will be planted at the same time at a depth of 1.0 – 1.5 inches.

Following the preemergence application, the PRE-T and PRE-C groups will be maintained in a greenhouse for the ^{14}C -treated plants. No other test systems from other studies will be present in this greenhouse. The UNT-C, POE-T and POE-C groups will all initially be located in a separate greenhouse to avoid uptake of $^{14}\text{CO}_2$ and/or volatilized [^{14}C]dicamba. No other test systems treated with radiolabeled test substances will be present in this greenhouse. Following the postemergence application, the POE-T and POE-C plants will be moved to the greenhouse area for ^{14}C -treated plants. These plants will be separated from the PRE-T and PRE-C plants in an appropriate manner to minimize cross-contamination between the treated groups. The UNT-C group will remain in the separate greenhouse until the end of the study.

7.4 Test System Identification

Each treated and control group pot will be uniquely identified by a label containing the following information: PTRL West, Inc. study number, treatment group (PRE-T, POE-T, UNT-C, PRE-C, or POE-C), plant identification (soybean GM_A90617), and initials of responsible person(s). Numbering each pot is also encouraged to aid in identification.

Because the designation of a pot as being part of the POE-T or POE-C group will not be made until time of the postemergence application, these pots will initially be labeled as 'POE'. At application, a second label with either 'POE-T' or 'POE-C' will be added.

In order to distinguish the PRE-C and POE-C plants from their treated counterparts, these control plants should have different colored labels or tape.

7.5 Test System Maintenance and Observation

Pots will be placed on a surface containing an absorbent pad to allow for subirrigation of plants. Irrigation will be applied to the test containers either by subirrigation or watering of each pot as necessary to maintain healthy plant development. Care will be taken when watering to avoid getting water on plants. The plants also will be fertilized, as necessary, to promote plant vitality and growth. The source, date and amount of any water or fertilizer applied must be recorded and included in the study notebook. Plants will be staked as necessary to maintain healthy development. Greenhouses will be temperature-controlled to ensure that the temperature is appropriate for the cultivation of soybeans.

At the V1 to V2 growth stage, the PRE-T, UNT-C, and PRE-C groups will be thinned to two plants per pot. At the same time, the POE-T and POE-C groups will be thinned to only one plant per pot. Thinnings from the PRE-T and UNT-C groups will be collected, pooled and frozen as the pre-forage harvest. All other thinnings will be discarded in an appropriate manner.

A detailed description of practices used to maintain the test system must be included in the study notebook. This includes records for weeding and use of any maintenance chemicals (date, method of application and rate). Research For Hire will provide the Study Director with a list of maintenance pesticides for preapproval. Additional maintenance pesticides may be used during the study following consultation with and approval by the Study Director.

Each group of treated pots will be observed during the study for any developing problems with fertilization, watering, insect infestation, etc. Observations will be recorded and the Study Director will be notified of any abnormal findings. Appropriate means to prevent loss of the plant materials due to pests will be used as necessary.

Plants must be assessed for phytotoxicity at 14±1 days after application for both the PRE-T and POE-T groups. Plant damage must be documented and communicated to the Study Director as soon as possible. The extent of damage must be indicated and a comparison to untreated plots must be made. Observation of damage at any other time during the study must also be documented and communicated to the Study Director. Photographs will be taken in the event of plant damage. Photographs will be labeled with a least the study number, description of the photo taken and the signature/initials of the person taking the photo and the date taken.

The following data will be obtained for the duration of the study:

- Temperature data, including, if available, minimum and maximum daily temperature
- Humidity data, including, if available, minimum and maximum daily humidity
- Irrigation data, including frequency and amounts used
- Any additional events that might impact the study

7.6 Test Substance Treatment Information

7.6.1 Method of Application

The test substance will be applied as its DGA salt in an aqueous solution through spray application to two separate treatment groups to simulate potential use patterns. One group will involve a preemergence spray application at planting; the other group will involve a postemergence spray application to plants at approximately the R1 growth stage. Spray application is the proposed agricultural practice for applying dicamba to soybean. Due to limitations on the minimum volume that can be effectively sprayed on the small pot surface areas, the spray volume in this study will necessarily be higher than that used in agronomic practice. Applications to individual pots or plants within the treatment groups will be made in the following manner:

The **preemergence** applications will be made directly to the soil on the day of planting, after seed is planted.

The **postemergence** applications will be made to soybean plants when at least 10% of the plants have reached the R1 reproductive growth stage, which is defined as the stage when one open flower is observed at any node on the main stem. **Do not let more than 50% of the plants reach the R1 stage before making the applications. Plants treated postemergence do not receive a preemergence treatment.**

At the time of the postemergence treatment, the pots that were previously thinned to one plant per pot will be separated into POE-T and POE-C groups. Twenty-eight plants that most closely match the R1 growth stage will be placed in the POE-T group. The remaining plants will be placed in the POE-C group.

Because the stage at application is a critical factor, the plants must be monitored closely. The Study Director must be notified as they approach the R1 stage.

7.6.2 Treatment Rate

The target rate for both treatment groups will be 2.5 lbs/acre of dicamba acid. This rate is equivalent to the potential maximum label rate for any single or combined preemergence and postemergence application.

7.6.3 Treatment Rate Calculation

The amount of test substance required for a 2.5 lb/acre application to each treated 12-inch diameter pot will be:

$$\text{Pot size} = 3.14159 \times (0.5 \text{ ft})^2 \times 1 \text{ acre}/43560 \text{ ft}^2 = 1.803 \times 10^{-5} \text{ acre}$$

$$\begin{aligned} \text{Treatment Rate} &= 2.5 \text{ lb/acre} \times 1.803 \times 10^{-5} \text{ acre/pot} \times 453.59 \text{ g/lb} \times 10^3 \text{ mg/g} \\ &= 20.45 \text{ mg ai/pot} \end{aligned}$$

Assuming a target specific activity of 5.4 mCi/mmol for the test substance, the amount of radioactivity required for the 56 treated pots in the study will be:

$$1145.2 \text{ mg} / 221.0 \text{ mg/mmol} \times 5.4 \text{ mCi/mmol} = 28 \text{ mCi}$$

The amount of radioactivity used in the study may vary slightly depending on the actual specific activity of the test substance used in each treatment and the number of pots treated.

7.6.4 Test Substance Formulation

At the appropriate time for a treatment, the test substance formulation will be prepared by PTRL West, Inc. personnel. The test substance will be dissolved in the container sent by Monsanto by adding a solution of HPLC-grade water containing 1.05 equivalents of DGA. The target solution concentration will be 20.45 mg dicamba/10 mL DGA solution. The amount of solution to add will depend upon the weight of the test substance and will be provided by the Study Director prior to preparation of the test substance formulations. Six (6) aliquots of an appropriate volume will be taken by PTRL West, Inc. personnel for liquid scintillation counting (LSC) analysis in order to determine the solution concentration of dicamba. Values for all six aliquots must be within 10% of the average value in order to establish homogeneity of the solution. If the value for any of the aliquots differs from the average value by more than 10%, the Study Director will decide if the data are acceptable or if aliquots need to be reanalyzed.

Upon establishing solution homogeneity, an aliquot (50 µL) will be removed prior to the start of applications for a treatment group and placed in frozen storage for use in verifying the preapplication radiochemical purity of the test substance. The test substance solution will be transported to Research For Hire in a cooler with substitute ice (blue ice). At the test site, the solution will be allowed to reach ambient temperature and the solution will be checked visually for homogeneity and the absence of precipitate. An aliquot (50 µL) will be removed upon completion of the last application in a treatment group and placed in frozen storage for use in verifying the postapplication purity of the test substance. This aliquot will be returned to PTRL West, Inc. on dry ice.

The stability of the test substance during each treatment will be confirmed at PTRL West, Inc. by a radiochemical purity check using small samples of the preapplication and postapplication aliquots taken from the formulated application solutions.

7.6.5 Test Substance Application

Aliquots of the dose solution will be dispensed by PTRL West, Inc. personnel to at least twenty-eight 30-mL bottles for applications to the 28 pots in a treatment group. An extra bottle may be used in order to prime the sprayer. Additional pots may be treated if sufficient test substance remains after treatment of the 28 pots. The amount of dose solution to be added to each bottle will be approximately 10 mL, but will be adjusted to ensure the application of 20.45 mg test substance per pot or plant. At the field test site, the [¹⁴C]dicamba dose solution will be applied by Research For Hire personnel using a

small hand-held sprayer, which fits directly on the dose solution bottles. For the preemergence treatment, the soil will be sprayed to obtain coverage as evenly as possible. For the postemergence treatment, the dosing solution will be applied uniformly to the foliage of the plants and as evenly as possible to avoid excessive run-off. The entire volume of spray solution in a bottle will be applied to each pot or plant in a treated group.

All spray bottles, including the bottle used to prime the sprayer, the collected spray from priming of the sprayer, and a wash of the sprayer, will be returned to PTRL West, Inc. to determine the residual dpm levels through LSC analysis. The amount of test substance applied to each pot will be calculated as an average value by subtracting the residual dpm in the spray bottles, the dpm in the primer spray and the dpm in the sprayer wash from the total amount originally added to the bottles divided by the number of pots treated. The dose solution container with any remaining formulated test substance will be placed on dry ice and returned to PTRL West, Inc. for frozen storage. Final disposition of the dose solution will be determined by the Study Director.

The control groups will not be treated.

7.6.6 Radiosafety Measures

During application, the area surrounding a pot will be protected in an appropriate manner to avoid excessive contamination of the surrounding area.

7.7 Specimen Collection and Handling

7.7.1 Specimen Collections

The specimens listed below will be collected by Research For Hire personnel from all treatment groups in this study except where noted.

Pre-forage will be collected from thinnings at the V1 to V2 growth stage, but only from the PRE-T and UNT-C groups.

Forage will be collected 7 ± 1 days after the postemergence application.

Hay will be collected 21 ± 2 days after the collection of forage, but only from the UNT-C, PRE-T and POE-T groups.

Mature seed will be collected from plants at maturity.

The following table shows the specimens to be collected and their amounts for each treatment group. The third column indicates the total number of pots from which to sample the number of plants shown. The amounts assume the maximum number of plants that could be available at each collection based on treatment of 28 pots for each treatment. It is possible that more or fewer plants will actually be present.

Minimum Required Specimen Collections from Each Treatment Group and Matrix

Treatment Group	Matrix	Number Plants/Pots to Sample
UNT-C	Pre-forage	16/8
	Forage	4/2
	Hay	4/2
	Mature Seed	8/4
PRE-C	Forage	8/4
	Mature seed	8/4
PRE-T	Pre-forage	56/28
	Forage	10/5
	Hay	10/5
	Mature seed	36/18
POE-C	Forage	8/4
	Mature seed	8/4
POE-T	Forage	5/5
	Hay	5/5
	Mature seed	18/18

Specimens will be collected from the control groups prior to collections from the treated groups.

Weights will be recorded for all collected specimens.

7.7.2 Forage Washes

At the time of forage collection from the postemergence treatment group (POE-T), 2 plants will be taken in order to analyze for surface and absorbed residues. Plants will be washed by Research For Hire personnel in 3 consecutive vessels containing an appropriate amount of HPLC-grade water. Plants will be held above the wash vessel for an appropriate amount of time (ca. 15-30 sec) until the majority of the water has dripped off of them. Different vessels will be used for both plants. After the third wash, plants will be air-dried and then placed in separate containers and frozen. Appropriate aliquot amounts of the washes will be taken in triplicate for analysis by liquid scintillation counting at PTRL West, Inc. Samples (ca. 500 mL each) of the first washes from both plants will be transferred to separate containers and frozen. These samples will be transported to PTRL West, Inc. for HPLC analysis.

7.7.3 Specimen Pooling

All collections (pre-forage, forage, hay, and seed) will be pooled within their respective groups prior to shipment to PTRL West, Inc. except for the plants collected for forage washes from the postemergence treatment group. These plants will be kept separate from each other and will also be sent to PTRL West, Inc.

7.7.4 Specimen and Sample Labeling

Specimens and samples derived from specimens (derived samples) will be placed in plastic bags or other appropriate containers, which are labeled with at least the following information: Monsanto study number, treatment group (PRE-T, POE-T, UNT-C, PRE-C, or POE-C), sample identification (including test system and nature of the sample), date of collection or sample generation, initials of person generating the sample, and a radioactivity warning where appropriate.

7.7.5 Specimen and Sample Handling and Transport

Following collection at Research For Hire, plant specimens will be frozen on dry ice within 2 hours after collection and will be transported to PTRL West, Inc. on dry ice in separate coolers for control and treated samples. Upon arrival at PTRL West, Inc., samples will be inventoried and then placed in frozen storage until processed for analyses.

After processing and initial analyses are conducted at PTRL West, Inc., all samples will be shipped frozen on dry ice in separate containers for control and treated samples to Monsanto Company at the address below. Samples should be shipped by air freight.

Treated:

Monsanto Company
Attn: Joe Eades, RSO – S361
S-Dock Receiving
800 N. Lindbergh Blvd.
St. Louis, MO 63141
Phone: (314) 694-8517

Control:

Monsanto Company
Attn: Michael J. Miller, – V220
S-Dock Receiving
800 N. Lindbergh Blvd.
St. Louis, MO 63141
Phone: (314) 694-8269

The outside containers used for **movement of seed** between Research For Hire and PTRL West, Inc. and between PTRL West, Inc. and Monsanto must also be clearly marked with the following additional information:

USDA Notification Number
Soybean Test Samples
Monsanto Study Number: 06-98-M-1

As a sample transfer document and chain-of-custody record, all specimen and sample shipments will include an inventory list identifying each specimen or sample in the shipment, which will be signed and dated by the persons shipping and receiving the specimens or samples.

If seed specimens are not shipped on the day of collection, and are retained on site, they must be stored frozen in a secured facility separate from nonregulated specimens until shipment. At least one sign must be posted on the secured facility stating that a regulated genetically modified organism is being stored and that unauthorized access is denied. Seed samples will require a label on the outside of the regulated materials shipping container stating the contents of the container along with the appropriate APHIS notification number. Neither the forage nor the hay is viable after frozen storage; therefore, they do not require storage in a secured facility separate from noregulated specimens nor do they require APHIS labels for shipping.

7.7.6 Specimen and Sample Storage

All specimens and derived samples will be stored frozen at $< -10^{\circ}\text{C}$ at all times when not in use.

7.7.7 Specimen Processing

Soybean specimens will be prepared for analyses at PTRL West, Inc. where they will be homogenized to a fine consistency in a food processor or equivalent device with dry ice added to keep the sample cold and assist with pulverization. The processed material will be stored in an open container in a freezer overnight to allow the dry ice to sublime. For each collection, to minimize the possibility of contamination, untreated control (UNT-C) specimens will be processed prior to the preemergence and postemergence control (PRE-C and POE-C) specimens followed by processing of the preemergence treated (PRE-T) specimens, and finally the postemergence treated (POE-T) specimens.

7.8 Sample Analysis

7.8.1 Total Radioactive Residues (TRR) Determination

The TRR in processed soybean samples will be determined at PTRL West, Inc. by combustion of quintuplicate aliquots of appropriate size in a Harvey Biological Oxidizer and LSC analysis of the $^{14}\text{CO}_2$ trapping solutions. Values will be considered acceptable if each data point lies within 15% of the sample average. If any data point is outside the acceptable range, the material will be reprocessed to improve homogeneity and then re-combusted.

The efficiency of the combustion process is checked at the beginning and end of each run by spiking approximately 20 mg of cellulose with a $[^{14}\text{C}]$ mannitol standard. The efficiency must be in the range 93-103%. In addition, for each matrix, the efficiency must be checked prior to the first sample analysis using control matrix (25, 50, 100 and 250 mg) spiked with $[^{14}\text{C}]$ mannitol.

TRR levels present in combusted samples will be expressed as mg/kg (ppm) dicamba and calculated in the following manner.

$$\frac{\% \text{ Oxidizer Efficiency}}{=} = \frac{\text{Combusted } [^{14}\text{C}]\text{standard dpm} - \text{background dpm}}{\text{Non - combusted } [^{14}\text{C}]\text{standard dpm} - \text{background dpm}} \times 100$$

$$\text{Sample dpm / g} = \frac{\text{Gross dpm / aliquot} - \text{background dpm}}{\text{Oxidizer efficiency} \times \text{aliquot weight (g)}}$$

$$\text{Sample ppm} = \frac{\text{Sample dpm / g}}{\text{Specific activity (dpm / } \mu\text{g)}}$$

7.8.2 Storage Stability

For the determination of the stability of dicamba and/or its metabolites in soybean matrices during frozen storage, representative samples of the ground immature soybean plants and seeds (specifically PRE-T and POE-T forage and mature seed) will be extracted with an appropriate solvent mixture and analyzed by HPLC with radioactivity detection at PTRL West, Inc. as soon as practical after collection and processing using methods provided by the Sponsor. Additional samples will be extracted and analyzed using very similar methods at Monsanto Company near the end of the metabolite analysis phase of the study. The stability of dicamba and/or metabolites will be determined by comparison of the initial and final extractabilities and HPLC profiles. Dicamba and/or metabolites in soybean matrices will be considered stable if initial and final extractabilities are within 5% of each other and the initial and final HPLC profiles are qualitatively similar.

7.8.3 Quantitation, Characterization and Identification of Residue Components

Samples of the ground soybean matrices will be extracted and analyzed by Monsanto using HPLC with radioactivity detection to determine the distribution and quantitation of dicamba and/or its metabolites in these matrices. The extracts may be prepared, if necessary, for chromatographic analysis utilizing methods such as solvent partitioning, solid phase extraction, ultrafiltration, centrifugation or concentration under vacuum.

Metabolites constituting $\geq 10\%$ of the TRR in soybean forage, hay, or seed will be isolated, characterized at a minimum, and identified if possible. Metabolites constituting $< 10\%$ of the TRR may be characterized/identified as appropriate in order to delineate the major metabolic pathways for dicamba in soybeans. Metabolites will be isolated by techniques such as HPLC. Identification and characterization of metabolites may employ one or more of the following techniques: solvent partitioning, mass spectrometry, NMR spectrometry, electrophoresis, derivatization and degradation chemistry, and spectral or chromatographic comparison with authentic standards.

Appropriate solvents for extraction, processing of the extracts, chromatographic

conditions and metabolite isolation or identification techniques will be determined by method development experiments during the study. The specific methods utilized will be described in the final report.

7.8.4 Proposed Statistical Methods

Statistical analysis of data collected from the study will be limited to simple measures of central tendency and/or dispersion including e.g. means and standard deviations.

7.9 Methods for Control of Bias

All pots/plants within a treatment group will be treated in an identical manner. With the exception of the two washed plants of the POE-T forage collection, all specimens within a group and specific collection will be pooled for processing and analysis.

8 RECORDS AND DATA RETENTION REQUIREMENTS

8.1 Records to be Maintained

All data and information generated during the conduct of this study must be recorded directly, promptly and legibly in indelible blue or black ink. Data and information shall never be recorded in pencil. All entries will be dated on the day of entry and signed or initialed by the person entering the data. Any change in entries must be made so as not to obscure the original entry, must indicate the reason for such change and must be dated and signed (or initialed) at the time of the change.

All raw data will be entered into research notebooks or worksheets dedicated exclusively to this study or will be referenced in the notebook and maintained in supplementary records according to established policies. Data will include, but shall not be limited to: test substance data, dates and records for the in-life portion of the study including greenhouse data, sample weights, LSC data, chromatographic and spectroscopic data, percentages and relevant dates.

For this study, "raw data" is defined as any laboratory or field worksheets, records, memoranda, notes, or exact copies thereof, which are the result of original observations and activities of a study and are necessary for the reconstruction and evaluation of the report of that study. Certified exact copies or exact transcripts may be substituted for the original source as raw data.

In addition to the above, study data or documentation prepared by PTRL West, Inc. on a computer in word processing, database, or spreadsheet programs may be provided to the Sponsor in electronic form for possible further analysis or database maintenance, should the Sponsor so request. The electronic copies of the study records are not raw data.

8.2 Retention of Records

This protocol and all deviations and amendments thereto, raw data generated during the conduct of this study, and copies of correspondence related to this study shall be retained.

Upon completion of the in-life portion of the study, all study specific documentation, records and raw data generated in the in-life portion of the study, and the original in-life sub-report will be transferred to the Sponsor. Prior to completion of the study (when the Study Director signs the final report), all documentation, records, raw data and the final report will be archived in the Monsanto Regulatory archives.

Original records of the maintenance of PTRL West, Inc., Research For Hire or AGVISE equipment such as freezers, balances and liquid scintillation counters will be retained in the respective PTRL West, Inc., Research For Hire or AGVISE archives. Certified copies of these records will be transferred to the Sponsor only if specifically requested by the Study Director.

8.3 Waste Disposal

Soil and other samples containing radioactivity will be disposed of in accordance with state and local regulations covering radioactive or other hazardous waste materials and with APHIS regulations and Monsanto Performance Standards, as applicable.

Any excess test substance or reference substances not used in the study will be held in appropriate storage until acceptance of the in-life report by the Sponsor. At that time the test substance and any reference substances for the study may either be returned to the Sponsor or disposed of at the discretion of the Study Director.

8.4 Signatures

The names, signatures and initials of all individuals recording data and/or observations for this study must be supplied in the data package.

8.5 Final Report

PTRL West, Inc. will prepare a final subreport describing, at minimum, the following: dose solution preparations, overview of applications, sample processing, and sample analyses. Research For Hire will prepare a report or summary of the in-life phase of the study that will be added as an appendix to the PTRL West, Inc. subreport. This subreport will include a Quality Assurance statement per 40 CFR 160.35(b)7 and a Statement of Compliance per 40 CFR 160.12. The subreport will be included as an appendix in the Monsanto final report. Draft copies of both the PTRL West, Inc. subreport and the Research For Hire report (or summary) shall be prepared and submitted to the Study Director for review and comment prior to submission of the final report. The subreport containing the in-life report will not be signed until it has been reviewed by the Study Director.

A final report will be prepared by the Study Director and will include complete descriptions of all study methods and results, all items enumerated in 40 CFR 160.185, and items enumerated in the 'Data reporting – plant studies' section of guideline 'Nature of the Residue – Plants, Livestock' (OPPTS 860.1300).

9 PROTOCOL AMENDMENTS AND DEVIATIONS

Amendments to the approved protocol shall be dated and justified by the Study Director. The Sponsor shall be informed of the amendment and must approve it prior to implementation. If immediate changes in the protocol are required, approval by the Sponsor may be granted by telephone or other electronic means, followed by submission of a written protocol amendment. Fully executed protocol deviations and amendments are to be filed with the study protocol.

Deviations from the protocol, Good Laboratory Practice Standards or standard operating procedures will be promptly reported to the Study Director. All deviations from standard operating procedures or protocol procedures specified and/or utilized for this study must be documented in writing and signed by the Study Director.

10 QUALITY ASSURANCE

This study will be performed in compliance with the Good Laboratory Practice Standards as specified in 40 CFR 160.

Each performing laboratory or test site will have a Quality Assurance Unit (QAU), either as part of their staff, or under contract. If a performing laboratory or test site does not have a qualified QAU, study related quality assurance at that laboratory or test site will be conducted by a qualified subcontractor chosen by the Study Director or by the Sponsor's QAU. Any change in the staffing of the laboratory or test site's QAU will be communicated immediately to the Sponsor's QAU.

The responsible QAU at each performing laboratory or test site will conduct at least one (1) inspection of the appropriate phase and prepare written reports of its findings per 40 CFR 160.35(b)(3). The findings of these inspections will be reported, at a minimum, to the Study Director and the Sponsor's Testing Facility Management in a timely manner.

11 PROTOCOL APPROVED BY:

Study Director

Michael J. Miller
Michael J. Miller
Monsanto Company

5/25/06
Date

Sponsor Representative

Annette M. Kirk
Annette M. Kirk
Monsanto Company

5/25/06
Date

12 PROTOCOL REVIEWED BY:

Paula A. Price
Paula A. Price
Quality Assurance
Monsanto Regulatory

5-25-06
Date

13 PROTOCOL ACCEPTED BY:

Test Site Principal Investigator

Alex V. Bautista
Alex V. Bautista
PTRL West, Inc.

5-31-06
Date

Field Test Site Principal Investigator

Heather Holmes
Heather Holmes
Research For Hire

6/1/06
Date

PROTOCOL AMENDMENT

Monsanto Protocol Number: 06-98-M-1
PTRL West, Inc. Study Number: 1491W
Research For Hire Study Number: R330606

Amendment Number: 1

Title of Study: Metabolism of Dicamba in Dicamba-Tolerant Soybeans

Change #1

Reason for Amendment: Aliquots of the forage washes will be transferred to scintillation vials and analyzed by LSC at Research For Hire before transfer to PTRL West. This is to 1) ensure that accurate, representative aliquots are taken from the forage washes prior to freezing of the whole washes in plastic containers, and 2) ensure that liquid scintillation counting data are available in the event that sample integrity of the aliquots is compromised in shipment.

Effective date: 07/07/06 (phone and e-mail correspondence)

Change to Protocol:

Modify the following sentence of Section 7.7.2 (Forage Washes) of the protocol:

Appropriate aliquot amounts of the washes will be taken in triplicate for analysis by liquid scintillation counting at PTRL West, Inc.

To the following:

Appropriate aliquot amounts of the washes will be taken at Research For Hire in triplicate and added to scintillation vials. The vials will be analyzed by liquid scintillation counting at Research For Hire, then transferred to PTRL West, Inc. At PTRL West, Inc., the vials will be reanalyzed by liquid scintillation counting. Liquid scintillation counting data from the PTRL West, Inc. analysis will be included in the report. Liquid scintillation counting data from the Research For Hire analysis will be included in the study data file, but will not be reported unless the integrity of one or more samples is compromised in transfer from Research For Hire to PTRL West, Inc.

Change #2

Reason for Amendment: To correct an error in the specimen collection table regarding the number of plants to be harvested from the POE-C group at the forage and mature seed stages. The pots for the POE-C group were thinned to one plant per pot as specified in Section 7.5 of the protocol.

Effective date: 06/30/06

Change to Protocol:

Modify the two rows of the table in Section 7.7.1 (Specimen Collections) of the protocol that apply to the POE-C group specimen collections:

Treatment Group	Matrix	Number Plants/Pots to Sample
POE-C	Forage	8/4
	Mature seed	8/4

To the following:

Treatment Group	Matrix	Number Plants/Pots to Sample
POE-C	Forage	4/4
	Mature seed	4/4

Change #3

Reason for Amendment: Because excess amounts of the test substance formulations were available, one extra pot was treated for the PRE-T group and four extra plants/pots were treated for the POE-T group. The extra PRE-T pot will be harvested at the mature seed stage. One extra POE-T plant/pot, each, will be harvested at the forage and hay stages. The specimen collection table is adjusted to reflect these extra samplings. See Change #4 (below) for the disposition of the remaining two extra POE-T plants/pots.

Effective date: 06/30/06 (direct communication with Field Test Site personnel)

Change to Protocol:

Modify the three rows of the table in Section 7.7.1 (Specimen Collections) of the protocol that apply to the PRE-T mature seed and POE-T forage and hay specimen collections:

Treatment Group	Matrix	Number Plants/Pots to Sample
[PRE-T]	Mature seed	36/18
POE-T	Forage	5/5
	Hay	5/5

To the following:

Treatment Group	Matrix	Number Plants/Pots to Sample
[PRE-T]	Mature seed	38/19
POE-T	Forage	6/6
	Hay	6/6

Change #4

Reason for Amendment: Additional POE-T plants were treated because an excess of test substance formulation was available. The two additional plants remaining after the POE-T forage and hay collections will be harvested at the immature seed stage prior to the final harvest of mature seed. Immature seed will be collected and transferred directly to Monsanto Company for analysis in order to obtain initial estimates of seed residue levels and the nature of seed residues for non-study related decision making purposes and for potential analytical method development. Data collected on the immature seed samples will be included in the study file but will not be included in the final report.

Effective date: 09/08/06 (communication by phone and e-mail)

Changes to Protocol:

Add the following to Section 7.7.1 (Specimen Collections) of the protocol:

Immature seed will be collected from two POE-T treated plants approximately two weeks prior to the expected final harvest. The two plants will be randomly selected from the plants ensuring that the plants are relatively far along in maturity compared to all of the POE-T plants. The harvested plants (or separated pods) will be allowed to dry in the greenhouse for 2-3 days; then the dried immature seed will be collected from the pods. The seed will be frozen and shipped on dry ice directly to Monsanto Company at the address, and following the instructions and APHIS requirements, in Section 7.7.5 of this protocol. At Monsanto, the immature seed will be processed and analyzed to determine approximate radioactive residue levels and distribution of residues for decision making purposes and may be used for analytical method development. Data collected on the immature seed will be included in the study file but will not be included in the final report.

Insert the row below, applying to the immature seed collection, into the sample collection table in Section 7.7.1 (Specimen Collections) of the protocol immediately above the last row of the table:

Treatment Group	Matrix	Number Plants/Pots to Sample
[POE-T]	Immature seed	2/2

The revised specimen collection table, in Section 7.7.1 of the protocol, considering changes #2, #3 and #4 of this amendment is as follows:

Minimum Required Specimen Collections from Each Treatment Group and Matrix

Treatment Group	Matrix	Number Plants/Pots to Sample
UNT-C	Pre-forage	16/8
	Forage	4/2
	Hay	4/2
	Mature Seed	8/4
PRE-C	Forage	8/4
	Mature seed	8/4
PRE-T	Pre-forage	56/28
	Forage	10/5
	Hay	10/5
	Mature seed	38/19
POE-C	Forage	4/4
	Mature seed	4/4
POE-T	Forage	6/6
	Hay	6/6
	Immature seed	2/2
	Mature seed	18/18

Change #5

Reason for Amendment: The Field Test Site Principal Investigator, Heather Holmes, is no longer an employee of Research For Hire necessitating a change in the Field Test Site Principal Investigator from Heather Holmes to Blaine Turner.

Effective date: 11/01/06

Change to Protocol:

Modify the Field Test Site section of Section 1.7 (Performing Laboratories and Study Participants) of the protocol:

<u>Field Test Site:</u>	Research For Hire 1696 S. Leggett St. Porterville, CA 93257 Principal Investigator: Heather Holmes Telephone: (559) 784-5787 Fax: 559-784-4351 e-mail: hholmes@research4hire.com
-------------------------	---

To the following:

<u>Field Test Site:</u>	Research For Hire 1696 S. Leggett St. Porterville, CA 93257 Principal Investigator: Blaine Turner Telephone: (559) 784-5787 Fax: 559-784-4351 e-mail: btturner@research4hire.com
-------------------------	--

Approved by:

Sponsor

Representative:

Sharon J. Moran

Date: *8/12/09*

Sharon J. Moran, Monsanto Company

Study

Director:

Michael J. Miller

Date: *8/12/09*

Michael J. Miller, Monsanto Company

Reviewed by:

Quality Assurance:

Bradley A. Comstock

Date: *8/12/09*

Bradley A. Comstock, Monsanto Company

PROTOCOL AMENDMENT

Monsanto Protocol Number: 06-98-M-1
PTRL West, Inc. Study Number: 1491W
Research For Hire Study Number: R330606

Amendment Number: 2

Title of Study: Metabolism of Dicamba in Dicamba-Tolerant Soybeans

Change #1

Reason for Amendment: To provide clarification regarding the samples to be utilized for storage stability assessment. In addition to the forage and seed samples analyzed by PTRL West, Inc. shortly after collection and processing, PRE-T hay and POE-T hay samples were analyzed by the Sponsor within 30 days of collection. For the radiovalidation portion of the dicamba residue method validation (AG-ME-1321-01), PRE-T hay, POE-T hay and POE-T seed samples from this study will be utilized. For efficiency, the two hay samples and the seed sample will be included in the final storage stability analysis for this study in addition to the PRE-T forage sample.

Effective date: 11/11/08

Change to Protocol:

Change the following sentence in Section 7.8.2 (Storage Stability) of the protocol:

Original:

“Additional samples will be extracted and analyzed using very similar methods at Monsanto Company near the end of the metabolite analysis phase of the study.”

Change:

Samples of PRE-T forage, PRE-T hay, POE-T hay and POE-T seed will be extracted and analyzed at Monsanto Company near the end of the metabolite analysis phase of the study using very similar methods to those utilized in the initial analyses.

Change #2

Reason for Amendment: To provide quantitative storage stability data in support of dicamba soybean residue study REG-08-096.

Effective date: 08/24/09

Change to Protocol:

Add the following paragraph to Section 7.8.2 (Storage Stability) of the protocol:

In addition to qualitative storage stability analyses, quantitative storage stability data will be generated to demonstrate stability of dicamba and its endogenous radioactive metabolites in soybean matrices in frozen storage to support the residue study of dicamba in dicamba-tolerant soybeans (Monsanto study REG-08-096). Dicamba and its major endogenous metabolites will be quantitated in the initial and final HPLC stability profiles of PRE-T forage, PRE-T and POE-T hay, and POE-T seed. In addition, a third stability analysis (referred to as 'extended' stability) will be added to the study to provide additional stability data.

Approved by:

Sponsor
Representative: Mitchell L. Kurtzweil Date: 12-21-09
Mitchell L. Kurtzweil, Monsanto Company

Study Director: Michael J. Miller Date: 12/21/09
Michael J. Miller, Monsanto Company

Reviewed by:
Quality Assurance: [Signature] Date: 12/21/09
Steven C. Reale, Monsanto Company

10 Appendix B – In-Life Report

STUDY TITLE

Metabolism of Dicamba in Dicamba-Tolerant Soybeans

AUTHORS

Alex Bautista, B.A. and Fred C. Baker, Ph.D.

REPORT DATE

December 15, 2009

PERFORMING LABORATORY

Test Site
PTRL West, Inc.
625-B Alfred Nobel Drive, Hercules, California 94547, USA

Field Test Site
Research for Hire
1696 S. Leggett St.
Porterville, CA 93257

SPONSOR

Monsanto Company.
800 N. Lindbergh Blvd.
St. Louis, MO 63167

MONSANTO COMPANY STUDY NUMBER: 06-98-M-1


LABORATORY PROJECT NO.

PTRL Project No.1491W
PTRL Report No.1491W-2

GOOD LABORATORY PRACTICE STATEMENT

This project (PTRL Project No. 1491W), the in-life portion of Monsanto Company study 06-98-M-1 titled "Metabolism of Dicamba in Dicamba-Tolerant Soybeans", was conducted in compliance with the current EPA Good Laboratory Practice Standards as specified in 40 CFR part 160.

Principal
Investigator:

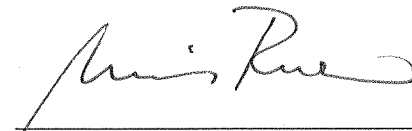


Alex V. Bautista

12-15-09

Date

PTRL West
Management:



Date

Dec 15 2009

Date

PTRL WEST, INC. QUALITY ASSURANCE UNIT STATEMENT

Compound: Dicamba

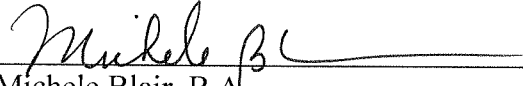
Title: Metabolism of Dicamba in Dicamba-Tolerant Soybeans

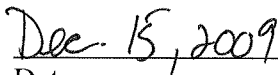
GLP QUALITY ASSURANCE INSPECTIONS:

Date of GLP Inspections	Date Reported to Management and to the Study Director	Phases of the Study Which Received a GLP Inspection by the Quality Assurance Unit
5/19/06	5/23/06	Draft Protocol
5/31/06	7/26/06	Dose Preparation Calculation
10/19/06	11/10/06	HPLC Analysis
1/8-11/07	1/17/07	Raw Data
7/29-30/09	8/11/09	Draft 1491W-1 Sub-Report
12/10/09	12/10/09	Final 1491W-2 Sub-Report

QUALITY ASSURANCE STATEMENT:

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

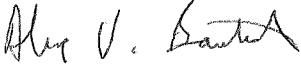

Michele Blair, B.A.
PTRL West, Inc., Quality Assurance


Date

PROJECT PERSONNEL

	Position	Name
PTRL West, Inc.	Director/Principal Scientist	Fred C. Baker, Ph.D.
	Principal Investigator	Alex Bautista, B.A.
	Senior Laboratory Technician	Gordon Glantz
	Associate Research Chemist	Sam Woodbury, B.S.

Research for Hire General Manager John Corkins
Additional Research For Hire
personnel associated with the study
are listed in the field report
(Appendix M)

Report Completed By:  12-15-89
Alex V. Bautista Date
Principal Investigator
PTRL West, Inc.

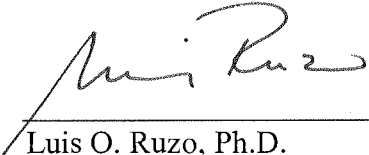
Report Reviewed By:  Dec 15, 2009
Luis O. Ruzo, Ph.D. Date
Director
PTRL West, Inc.

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SUMMARY

A metabolism study was conducted with [^{14}C]dicamba on soybeans. The objective of the study was to determine the nature of dicamba residues resulting from pre- or postemergence applications of dicamba to dicamba-tolerant soybeans. This report describes the in-life portion (PTRL West project number 1491W) of the study consisting of the field phase, test substance formulation and application, processing of harvested soybean matrices (immature foliage, forage, hay and seed), determination of total radioactive residues (TRR) and initial stability analyses for selected soybean matrices (forage and seed).

The test substance consisted of ring-labeled [^{14}C]dicamba with a final specific activity of 54,190 (preemergence treatment) or 54,560 dpm/ μg (postemergence treatment). This was formulated as an aqueous solution of the diglycolamine salt and applied by spray to the soil (preemergence) or to the plants (postemergence). The in-life portion of the study was conducted on behalf of Monsanto Company by PTRL West. Soybean plants (dicamba-tolerant GM_A90617) were grown in 12-inch pots in greenhouses located at the Research for Hire facility in Porterville, California.

One preemergence application was made to the soil of a group of plants (PRE-T) on the day of seed planting, after the seed was planted. A separate postemergence application was made to plants of a second group (POE-T) when 10-50% of the plants had reached the R1 (first flower) growth stage. Groups of untreated plants were grown in the proximity of the treated plants: PRE-C interspersed amongst the PRE-T plants and POE-C interspersed amongst the POE-T plants. The target rate for the applications was 2.5 lbs/acre for each treatment. The achieved rates were 2.55 lbs/acre (102.1%) for the preemergence application and 2.52 lbs/acre (100.9%) for the postemergence application. A group of untreated plants (UNT-C) was maintained in a separate greenhouse.

Immature foliage (pre-forage) from the PRE-T and UNT-C groups was collected, processed and combusted. Subsequently, mature forage and seed from all groups, as well as hay from the PRE-T, POE-T and UNT-C groups, were collected, processed and combusted. Total radioactive residues (TRR) are shown in Summary Table I.

Summary Table I: Distribution of Residues in Soybean Plants (TRR, parent equiv., ppm)

Matrix	Treatment:				
	PRE-T	POE-T	UNT-C	PRE-C	POE-C
Pre-forage	3.248	No harvest	0.000	No harvest	No harvest
Forage	1.433	134.147	0.000	0.080	0.280
Hay	1.056	39.149	0.001	No harvest	No harvest
Seed	0.291	0.389	0.013	0.170	0.138

Two selected individual forage plants from the POE-T group were harvested separately. These plants were rinsed at the field site with water to remove surface residues and the washes were analyzed by LSC and HPLC at PTRL West. The rinsed forage was processed and combusted: the TRR (rinse + rinsed forage) was 183.169 ppm and 123.084 ppm, respectively for the two forage plants. These values were consistent with the POE-T forage TRR value of 134.147 ppm. Approximately 33% of the forage residues were surface residues. The HPLC analysis of the rinses indicated that the surface residues were virtually exclusively (99-100%) [¹⁴C]dicamba.

Forage and seed samples were subjected to extraction with acetonitrile/water and the extracts were analyzed by HPLC to establish initial storage stability profiles. The extractions removed 81.7-91.0% of TRR (93.6-96.2% normalized extractability) from forage. Combustion of unextracted forage residues accounted for 3.6-5.6% (3.8-6.4% normalized) of TRR. Extraction of seed removed 52.9-58.6% (52.6-57.5% normalized) of TRR with 43.4-47.8% (42.5-47.4% normalized) unextracted.

The processed plant materials were shipped to Monsanto Company for conduct of the analytical phase of the study.

INTRODUCTION

Dicamba, 3,6-dichloro-2-methoxybenzoic acid, is a foliar- or soil-applied herbicide used to control broadleaf weeds.¹ Monsanto Company contracted PTRL West Inc. to conduct the in-life portion of a metabolism study (Monsanto study number 06-98-M-1) designed to determine the nature of residues resulting from application of dicamba to dicamba-tolerant soybeans. The in-life portion of the study consisted of oversight of the field phase of the study, formulation and application of the test substances, receipt and processing of the soybean specimens, determination of total radioactive residues (TRR) in the soybean matrices, extraction and HPLC profiling of soybean forage and seed as initial stability analyses, and shipment of the processed soybean specimens to the Sponsor. The in-life portion of the study was initiated May 25, 2006 and completed August 2009.

Dicamba-tolerant soybean seed (GM_A90617), provided by the Sponsor, was planted in 12-inch diameter pots. Two batches of [¹⁴C]dicamba containing ¹⁴C label in the ring (Figure 1) were supplied by the Sponsor to conduct two applications. A single application of an aqueous formulation of [¹⁴C]dicamba-diglycolamine salt was made on the day of planting (preemergence application) to the soil of a group of soybean plants designated PRE-T. A single application was made to the foliage of a separate group of soybean plants, designated POE-T when 10-50% of the plants had reached the R1 stage (postemergence treatment). A control group received no application (UNT-C) and was grown in a separate greenhouse. Two other groups of untreated plants were introduced among the treated plants (PRE-C and POE-C). The field phase was conducted in Porterville, California, in greenhouses operated by Research for Hire. The product was applied by spraying at a target rate of 2.5 lbs/acre. Immature plants (pre-forage), forage, hay and seed were sampled.

The study was conducted in accordance with US EPA Residue Chemistry Test Guidelines (OPPTS 860.1300, Nature of the Residue—Plants, Livestock). The in-life portion of the study was conducted under current Good Laboratory Practices as required by EPA.

This soybean metabolism study mimicked commercial application methods and crop harvesting methods as closely as possible except that the study was conducted indoors in greenhouses. The in-life part of the study was conducted as described in the protocol and

amendments. Applications took place on June 1 and June 30, 2006 and harvests took place June 15, August 7, August 27 and September 21, 2006.

MATERIALS AND METHODS

FIELD PHASE

Test Site

The test crop was grown in greenhouses located at Porterville, CA and operated by:

Research For Hire
1696 S. Leggett St.
Porterville, CA 93257

Test System

The test system consisted of five groups of dicamba-tolerant soybean plants (*Glycine max* L., GM_A90617, lot # GLP-0604-17294-S) grown in 12-inch diameter pots. The untreated groups each consisted of 8 pots and were designated UNT-C (untreated), PRE-C (untreated, interspersed amongst PRE-T pots) and POE-C (untreated, interspersed amongst POE-T pots). The treated groups were designated PRE-T (preemergence treatment, 29 pots) and POE-T (postemergence treatment, 32 pots).

Refer to the field report (Appendix M) for details of crop planting, maintenance and harvest, and the schedule of events for the field phase of the study.

Application of Test Substance to the Test System

Applications were conducted using a hand-held sprayer that fit directly on the spray bottles. Separate bottles containing the required amount of test substance formulation (aqueous solution of diglycolamine salt of [¹⁴C]dicamba) were used for each pot. The preemergence application was made directly to the soil of the PRE-T group pots on the day of planting, June 1, 2006, after the soybean seeds were planted. The postemergence application was made to the foliage of the plants of the POE-T group 29 days later (June 30, 2006). The target application rate was 2.5 lbs/acre, which corresponds to 20.45 mg ai/pot, and 10 mL of solution per pot.

$$\text{Pot size} = 3.14159 \times (0.5 \text{ ft})^2 \times 1 \text{ acre}/43560 \text{ ft}^2 = 1.803 \times 10^{-5} \text{ acre}$$

$$\begin{aligned} \text{Treatment Rate} &= 2.5 \text{ lb/acre} \times 1.803 \times 10^{-5} \text{ acre/pot} \times 453.59 \text{ g/lb} \times 10^3 \text{ mg/g} \\ &= 20.45 \text{ mg ai/pot (target)} \end{aligned}$$

Sample Collection

Selected immature foliage samples (pre-forage) were collected from UNT-C and PRE-T plants on June 15, 2006, 14 days after planting and the preemergence application. Mature forage samples were collected from all pots 7 days after the postemergence application and 36 days after the preemergence application. Two selected individual forage plants (plant 13 and plant 28) were collected separately from the POE-T group. Hay was collected from UNT-C, PRE-T and POE-T groups on July 27, 2006, 27 days after the postemergence treatment and 56 days after the preemergence treatment. Seed was collected from all groups on September 21, 2006, 83 days after postemergence treatment (112 days after planting and preemergence treatment).

Sample Storage and Transport

All samples were stored at <-10 °C prior to and after processing when not in use. Samples were shipped from Research For Hire to PTRL West, Inc., and from PTRL West, Inc. to Monsanto Company, overnight in coolers with dry ice. (see Table VIII for shipping dates).

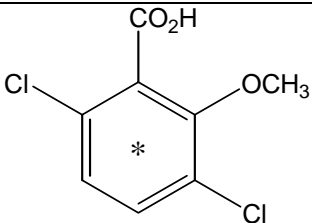
IN-LIFE ANALYTICAL PHASE

Chemicals

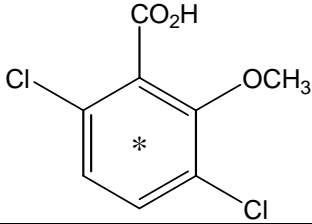
Radiolabeled Test Substances

The identification, labeling position, specific activities and purities of the ¹⁴C-test substances are shown below. Certificates of analysis are shown in Appendix A.

For preemergence application:

Structure	
Label	[ring-U- ¹⁴ C]dicamba
	* Indicates position of radiolabel
Name	3,6-dichloro-2-methoxybenzoic acid
CAS Number	1918-00-9 (unlabeled)
Specific Activity	5.39 mCi/mmol (54,190 dpm/μg)
Radiochemical Purity	99.2%
Supplier	Monsanto Company
Supplier Lot #	6103-01A

For postemergence application:

Structure	
Label	[U- ¹⁴ C]dicamba
	* Indicates position of radiolabel
Name	3,6-dichloro-2-methoxybenzoic acid
CAS Number	1918-00-9 (unlabeled)
Specific Activity	5.43 mCi/mmol (54,560 dpm/μg)
Radiochemical Purity	99.5%
Supplier	Monsanto Company
Supplier Lot #	6103-01C

Reference Substances

Three reference substances were provided by the Sponsor (unlabeled dicamba, DCSA and 5-hydroxydicamba). The structures are shown in Figure 2. The certificates of analysis are shown in Appendix A. During the study there was no indication that any of the reference standards underwent any decomposition.

Diglycolamine

A sample of diglycolamine (Sigma-Aldrich lot # 02403EC) was provided by the Sponsor for the preparation of the formulated ¹⁴C test substances. The dicamba was applied as its diglycolamine salt.

Other Chemicals

All solvents and chemicals (reagent grade or better) were obtained from Fisher Scientific or VWR. All water used was HPLC grade or purified with a Barnstead NANOPure II™ system (ASTM Type I).

Equipment

Fisher XL-5000 Balance
Fisher XT Balance
Mettler AT261 Balance
OHAUS Explorer Balance
Sartorius B310S Balance
XE-300 Toploader Balance

K-25 Food Processor
Mr. Coffee Coffee Mill
Waring Blender Food Processor
WestBend Food Processor
Harvey OX500/600 Oxidizer
Beckman Liquid Scintillation Counters LS6000/6500
Eppendorf Centrifuge 5415C
Sorvall Superspeed RC2-B Centrifuge
Sorvall RT7 Centrifuge
Burrell Wrist-Action Shaker
Fisher Touch-Mixer
Corning Stirplates
Buchi Rotavapor RE111/121 with Buchi Water Bath Model 461
Fisher 910 Circulator or Lauda RM20 Circulator
Organomation Nitrogen Evaporator
Agilent Series 1050 HPLC
Agilent Series 1100 HPLC
Thermo Separation Products SP8800 HPLC
CF-1 Fraction Collector
Cygnat Fraction Collector
Dionex Data Collection

Preparation of Application Solutions

Preemergence Application Solution

The radiolabeled test substance (643.6 mg, 15.71 mCi, 2.912 mmol) was provided by the Sponsor as a solid in a Teflon bottle. Diglycolamine (321.5 mg, 3.058 mmol, 5% excess) was weighed into a glass vial. HPLC grade water (314.7 mL) was measured into a glass beaker. The test substance bottle was placed on a magnetic stir plate and a magnetic stir bar was added. An aliquot of water (~100 mL from the 314.7 mL) was transferred to the bottle and magnetic stirring was activated. The diglycolamine was transferred to the stirred solution using ~9 rinses each with ~3 mL of the HPLC grade water. The remaining HPLC grade water was added and the solution stirred until the solids were completely dissolved (~30 minutes). Aliquots (6 x 25 µL) were taken from the stirring solution for concentration and homogeneity determinations, and an additional aliquot was taken for pre-application purity determination. The application solution was stored on blue ice in a cooler and transported to the field site where it was stored refrigerated overnight. At the field site, the application solution was allowed to warm to ambient temperature, and the solution was dispensed as 30 equal volumes (10.24 mL + 0.16 mL each) into 30 separate glass bottles. Of the 30 solutions, 29 were applied to 29 individual pots and one was used to prime the sprayer. An aliquot was taken from the test substance

remaining in the original bottle for post-application purity analysis, placed in a vial and stored in a cooler on dry ice for transport to PTRL West. After application, the bottles, spray nozzle and primer sprayate were returned to PTRL West. Rinses of the bottles and the primer sprayate were counted by LSC to determine residual (non-applied) radioactivity in order to calculate the exact amount of test substance applied. Each bottle contained, on average, 0.5070 mCi, corresponding to 20.87 mg a.i. per pot. Detailed calculations for the preparation of the application solutions are shown in Appendix B. Detailed calculations for achieved application rates are shown in Appendix C.

Postemergence Application Solution

The procedure described above was duplicated for the postemergence application. The amount of test substance supplied was 695.8 mg (17.10 mCi, 3.148 mmol). The amount of diglycolamine was 347.6 mg (3.306 mmol, 5% excess) and the volume of HPLC grade water was 340.2 mL. The diglycolamine was transferred to the test substance bottle using 4 x 4 mL aliquots of the HPLC grade water, followed by 2 x 10 mL rinses. After the solids were fully dissolved, aliquots (6 x 25 μ L) were taken from the stirring solution for concentration and homogeneity determinations, and an additional aliquot was taken for pre-application purity determination. Before transport to the field, the solution was dispensed as 33 equal volumes (9.90 mL each) into 33 separate glass bottles. The bottles were stored on blue ice in coolers and transported to the field site where they were stored refrigerated overnight. The remaining test substance was dispensed into a bottle for use in post-application purity analysis, stored on blue ice in a cooler and transported to the field for overnight refrigerator storage. At the field site, the solutions were allowed to warm to ambient temperature prior to application. Of these solutions, 32 were applied to individual plants and one was used to prime the sprayer. After application, the bottles, spray nozzle and primer sprayate were returned to PTRL West. The bottle for post-application purity analysis was stored on dry ice in a cooler for transport to PTRL West. Rinses of the bottles and the primer sprayate were counted by LSC to determine residual (non-applied) radioactivity in order to calculate the exact amount of test substance applied. Each bottle contained, on average, 0.5025 mCi, corresponding to 20.63 mg a.i. per pot. Detailed calculations for the preparation of the application solutions are shown in Appendix B. Detailed calculations for achieved application rates are shown in Appendix C.

Determination of Test Substance Pre-and Post-Application Purity

Aliquots were analyzed by HPLC Method 1 to determine the purity of test substances before and after application in order to establish the stability of the test substances during transport, formulation and application. See Table II and Figures 3-6.

Calculation of Specific Activity

Based on the calculations and weights in the certificates of analysis:

Preemergence Specific Activity

$((15.71 \text{ mCi} \div 643.6 \text{ mg}) \times 2,220,000,000 \text{ dpm/mCi}) \div 1000 \text{ } \mu\text{g/mg} = 54,189 \text{ dpm}/\mu\text{g}$, rounded to 54,190 dpm/ μg

Postemergence Specific Activity

$((17.10 \text{ mCi} \div 695.8 \text{ mg}) \times 2,220,000,000 \text{ dpm/mCi}) \div 1000 \text{ } \mu\text{g/mg} = 54,559 \text{ dpm}/\mu\text{g}$, rounded to 54,560 dpm/ μg

Sample Processing

Rinses of Select Individual Plants from POE-T Group

At the field site at the time of harvest, forage collected from individual POE-T plants #13 and #28 was rinsed (separately) in three consecutive vessels each containing 1 L of HPLC grade water. An aliquot of the first rinse was transported to PTRL West for HPLC analysis. Aliquots (3 x 1 mL) from each rinse were collected at the field site for LSC analysis. These aliquots were then transported to PTRL West for repeat LSC analysis.

Rinsed Forage from Select Individual Plants from POE-T Group

Rinsed forage from the POE-T plants #13 and #28 were homogenized (separately) in a West Bend type food processor in the presence of dry ice. The processed samples were transferred to plastic bags and placed in a freezer, where the dry ice was allowed to sublime.

Pre-Forage

Pre-forage from the UNT-C and PRE-T treatments was homogenized (separately) in a West Bend type food processor in the presence of dry ice. The processed samples were transferred to plastic bags and placed in a freezer, where the dry ice was allowed to sublime.

Forage

Forage from the UNT-C, PRE-C, POE-C, PRE-T and POE-T treatments was homogenized (separately) in a K25 food processor in the presence of dry ice. The processed samples were transferred to plastic bags and placed in a freezer, where the dry ice was allowed to sublime.

Hay

Hay from the UNT-C, PRE-T and POE-T was homogenized (separately) in a K25 food processor in the presence of dry ice. The processed samples were transferred to plastic bags and placed in a freezer, where the dry ice was allowed to sublime.

Seed

Seed from the UNT-C, PRE-C, POE-C, PRE-T and POE-T treatments was homogenized (separately) in a Waring blender type food processor in the presence of dry ice. The processed samples were transferred to plastic bags and placed in a freezer, where the dry ice was allowed to sublime.

Calculation of Total Radioactive Residues (TRR)

Residues were calculated based on specific activities of 54,190 dpm/ μ g (PRE-T) and 54,560 dpm/ μ g (POE-T) for the two batches of [14 C]dicamba utilized.

Determination of Radioactive Residues in Pre-forage, Forage, Hay and Seed

Quintuplicate aliquots (typically ~0.25 g for pre-forage, forage and hay, ~0.1 g for seed) were analyzed by combustion. Additional replicates were combusted as necessary. Combustion data for pre-forage, forage, hay and seed are presented in Appendices E, F, H and I, respectively.

Determination of Radioactive Residues in Rinses and Rinsed Forage

Triplicate aliquots (1 mL) were removed from the plant #13 and #28 POE-T forage rinses and analyzed by LSC (see below) to determine surface residues. Quintuplicate aliquots (~0.1 g) of the rinsed forage were analyzed by combustion to determine internal plant residues. The TRR was calculated as the sum of the residues (ppm) in the rinses and each rinsed forage plant, based on the weight of rinsed plant. Combustion data for the rinsed forage plants and LSC data for the rinses are presented in Appendix G.

Characterization of the Radioactive Components in Forage Rinses by HPLC

Aliquots from the plant #13 and #28 POE-T forage rinses, forage extracts and seed extracts were analyzed by HPLC method 1, described below. Where necessary, portions of these samples were filtered and/or concentrated under vacuum on a rotary evaporator or nitrogen evaporator. See Figure 7 for a representative chromatogram of the reference standards and Figures 8-9 for HPLC profiles of the forage rinses.

Storage Stability Analyses

General

Processed samples of forage and seed were extracted and the extracts were analyzed by HPLC to obtain initial storage stability profiles. Samples will be extracted and analyzed at Monsanto Company near the end of the experimental phase of the study. The stability of dicamba and its metabolites in soybean matrices, under frozen storage conditions, will be assessed by comparison of the initial and final extractabilities and HPLC profiles.

Extraction of Forage

Subsamples (~10 g) of PRE-T or POE-T forage were weighed into centrifuge bottles. Acetonitrile/water (4/6 v/v, ~50 mL) was added and the mixture was shaken for 20 minutes on a wrist-action shaker. The bottles were then centrifuged in a Sorvall RT7 centrifuge at 4,000 rpm for 10 minutes, the supernatants were decanted and the extraction of each subsample was repeated three times (~200 mL total). The extracts were weighed and aliquots were taken by weight for LSC analysis.

Quantification of radiolabel in post-extraction solids (PES) was conducted by combustion radioanalysis. Extraction data for forage are presented in Appendix J.

Extraction of Seed

Extraction of PRE-T and POE-T seed was performed using procedures identical to the extraction of forage, except that a Sorvall RC2-B Superspeed centrifuge was used (10,000 rpm for 10 minutes). These extraction procedures were performed four times for each subsample (~200 mL total). The extracts were weighed and aliquots were taken by weight for LSC analysis.

Quantification of radiolabel in post-extraction solids (PES) was conducted by combustion radioanalysis. Extraction data for seed are presented in Appendix K.

HPLC Analysis of Forage and Seed Extracts

Aliquots from the PRE-T and POE-T forage and seed extracts were analyzed by HPLC method 2, described below. Where necessary, portions of these samples were filtered and/or concentrated under vacuum on a rotary evaporator or nitrogen evaporator. HPLC profiles for the forage and seed extracts are presented in Figures 10-13.

General Analytical Methods

Liquid Scintillation Counting

Liquid samples (rinses, extracts, etc.) for liquid scintillation counting (LSC) were mixed with Safety Solve scintillation cocktail (Research Products International Corp.) in scintillation vials. Liquid scintillation counting was carried out with Beckman LS 6500 or LS 6000 IC liquid scintillation spectrometers. Computer-constructed quench curves, derived from a series of ten sealed quenched standards, automatically converted cpm to dpm. All sample count rates were corrected for background.

Combustion Analysis

Combustion analysis was carried out using a Harvey OX-500 or OX-600 Biological Oxidizer and the $^{14}\text{CO}_2$ generated was trapped with Carbon 14 Cocktail (R.J. Harvey Instrument Corporation). The ^{14}C content was determined by LSC. The radiocarbon content of the samples was corrected by dividing the count rate by the efficiency of the combustion process. The efficiency was determined by comparing the count rate obtained from samples of [^{14}C]mannitol standards, as follows.

$$\text{Combustion efficiency} = \frac{\text{combusted standard dpm} - \text{background dpm}}{\text{standard dpm} - \text{background dpm}}$$

Where the standard dpm was determined by adding the [^{14}C]mannitol directly to LSC cocktail.

HPLC

Reversed phase HPLC of test substances, sample extracts and forage rinses was performed using linear solvent gradients. Reference standards (included with the sample of interest) were detected by their absorbance at 280 nm. Radioactive components were detected by fraction collection followed by LSC of the fractions. The HPLC effluent was collected in 7 ml scintillation vials (99, 100 or 120 x 0.5/0.6 min fractions) using an

automatic fraction collector. The LSC cocktail was manually added to these vials, and the radiocarbon content determined by liquid scintillation counting. The LSC data was used to construct a radiochromatogram with the aid of a PTRL West computer program. This program is described in Appendix D.

The retention times of the [^{14}C]-containing components in samples were compared to standard compounds that were co-injected with the samples. The performance of the HPLC system suitability was verified at the start of each sample set by the injection of one or more reference standards. The following HPLC methods were used in this study.

HPLC Method 1

Method used for determination of radiochemical purity and analysis of forage rinses.

- Column: Beckmann Coulter Ultrasphere ODS
- Length: 250 mm
- Diameter: 4.6 mm
- Particle size: 5 μm
- Column temperature: Ambient
- UV detection: 280 nm
- ^{14}C detection: Fraction collection (0.3 or 0.5 min intervals)/liquid scintillation counting
- Flow rate: 1 ml/min
- Mobile phase and gradient conditions as follows:

Time (min)	% A 0.1% TFA in H_2O	% B ACN
0	90	10
5	90	10
30	0	100
35	0	100
40	90	10
50	90	10

HPLC Method 2

Method used for analysis of forage and seed extracts.

- Column: Beckmann Coulter Ultrasphere ODS
- Length: 250 mm
- Diameter: 10 mm

- Particle size: 5 μm
- Column temperature: Ambient
- UV detection: 280 nm
- ^{14}C detection: Fraction collection (0.5 or 0.6 min intervals)/liquid scintillation counting
- Flow rate: 3 ml/min
- Mobile phase and gradient conditions as follows:

Time (min)	% A 0.5% Formic Acid in H_2O	% B ACN
0	90	10
5	90	10
50	0	100
60	0	100
65	90	10
75	90	10

Statistical Methods

Statistical methods used included calculation of means and relative standard deviations.

Calculations

1) Target Application Rate:

$$\text{Pot size} = 3.14159 \times (0.5 \text{ ft})^2 \times 1 \text{ acre}/43560 \text{ ft}^2 = 1.803 \times 10^{-5} \text{ acre}$$

$$\begin{aligned} \text{Treatment Rate} &= 2.5 \text{ lb/acre} \times 1.803 \times 10^{-5} \text{ acre/pot} \times 453.59 \text{ g/lb} \times 10^3 \text{ mg/g} \\ &= 20.45 \text{ mg ai/pot (target)} \end{aligned}$$

2) Specific Activity:

Preemergence Specific Activity

$$((15.71 \text{ mCi} \div 643.6 \text{ mg}) \times 2,220,000,000 \text{ dpm/mCi}) \div 1000 \text{ } \mu\text{g/mg} = 54,189 \text{ dpm}/\mu\text{g}, \text{ rounded to } 54,190 \text{ dpm}/\mu\text{g}$$

Postemergence Specific Activity

$$((17.10 \text{ mCi} \div 695.8 \text{ mg}) \times 2,220,000,000 \text{ dpm/mCi}) \div 1000 \text{ } \mu\text{g/mg} = 54,559 \text{ dpm}/\mu\text{g}, \text{ rounded to } 54,560 \text{ dpm}/\mu\text{g}$$

3) Test Substance Volume per Pot:

Preemergence

$20.45 \text{ mg/pot} \div ((2,705,534 \text{ mean dpm} \div 0.025 \text{ mL mean aliquot}) \div 54,190,000 \text{ dpm/mg}) = 10.24 \text{ mL/pot},$

Postemergence Specific Activity

$20.45 \text{ mg/pot} \div ((2,817,098 \text{ mean dpm} \div 0.025 \text{ mL mean aliquot}) \div 54,560,000 \text{ dpm/mg}) = 9.90 \text{ mL/pot},$

4) Achieved Application Rate:

$(\text{mCi/pot (x30 for PRE-T, x33 for POE-T)} - \text{mCi in rinses}) \times 2,220,000,000 \text{ dpm/mCi} \div \text{specific activity} \\ \div \text{number of pots} = \text{Achieved Application Rate}$

5) Matrix dpm/g:

$((\text{Mean of (Oxidizer dpm} \div \text{oxidized weight)}) - \text{mean oxidizer background dpm}) \div \text{oxidizer efficiency} \\ \text{oxidizer efficiency} = \text{mean net oxidized } ^{14}\text{C-standard dpm} \div \text{mean net unoxidized } ^{14}\text{C-standard dpm}$

6) Total Radioactive Residue:

For Pre-forage, Forage, Hay and Seed:

$\text{Oxidizer TRR} = \text{Matrix dpm/g} \div \text{specific activity}$

For Individual Plants:

$\text{TRR} = \text{oxidizer ppm} + \text{rinse ppm}$

7) Normalized %Extracted (Extractability):

$\text{Normalized \% Extracted} = \text{ppm extracted} \div (\text{ppm extracted} + \text{ppm unextracted})$

Limit of Detection and Limit of Quantification

Limits of detection (and quantification) for TRR determinations were set at two times the background radiocarbon level as determined by combustion of blank samples. Combustion values that were less than 2 x background were considered non-detects. Background levels ranged from 36-71 dpm.

RESULTS AND DISCUSSION

FIELD PHASE

The preemergence application took place on June 1, 2006 at the Research For Hire site in Porterville, California. The postemergence application took place June 30, 2006.

Harvests were made 14 days after preemergence application (pre-forage), and 7 days (mature forage), 27 days (hay) and 83 days (seed) after postemergence application (36, 52 and 112 days, respectively, after planting and preemergence application).

Details of the field phase of the study are in the field report attached as Appendix M to this report.

IN-LIFE ANALYTICAL PHASE

Application of Test Substance

For the preemergence treatment, a total of 14.77 mCi was applied. This was equivalent to 20.87 mg/pot or 2.55 lbs/acre. This was 102.1% of the target (20.45 mg/pot, 2.5 lbs/acre). For the postemergence treatment, 16.22 mCi was applied. This was equivalent to 20.63 mg/pot or 2.52 lbs/acre. This equaled 100.9% of the target (20.45 mg/pot, 2.5 lbs/acre). See Table I.

Determination of Test Substance Purity and Solution Homogeneity

The purity of [^{14}C]dicamba was 99.8% prior to the preemergence application and 99.9% afterwards (see Table II and Figures 3-4). The purity of [^{14}C]dicamba was 100.0% prior to the postemergence application and 99.8% afterwards (see Table II and Figures 5-6). These results indicate that the [^{14}C]dicamba test substances were stable during formulation, transfer and application. Aliquots taken from the application solutions before application were analyzed by LSC and indicated that the solutions were homogeneous and contained the expected level of test substance (Table III).

Total Radioactive Residues

Pre-Forage

Pre-forage from the UNT-C group contained 0.000 ppm, while pre-forage from the PRE-T group contained 3.248 ppm. Pre-forage was not collected from other groups.

Forage

PRE-T forage contained 1.433 ppm and POE-T forage contained 134.147 ppm. UNT-C forage contained 0.000 ppm, PRE-C forage contained 0.080 ppm and POE-C forage contained 0.280 ppm.

The higher level in POE-T forage reflects the direct application of the test substance to the plant as opposed to the soil application to PRE-T plants. The presence of residues in PRE-C and POE-C samples suggests uptake of $^{14}\text{CO}_2$ and/or volatilized dicamba from the surrounding PRE-T or POE-T pots/plants.

The TRR in individual POE-T plant #13 forage was 183.169 ppm, of which 58.877 ppm was in the rinse (surface residues) and 124.292 ppm was in the rinsed forage. The TRR in

individual POE-T plant #28 was 123.084 ppm, of which 41.298 ppm was in the rinse and 81.786 ppm was in the rinsed forage. These TRR values are comparable to the TRR from combined POE-T forage.

Hay

The PRE-T hay TRR was 1.056 ppm, POE-T hay TRR was 39.149 ppm and UNT-C hay TRR was 0.001 ppm. These data are consistent with those obtained for forage.

Seed

Total radioactive residues were 0.291 ppm in PRE-T seed and 0.389 ppm in POE-T seed. In UNT-C seed the TRR was 0.013 ppm, while in PRE-C and POE-C seed it was 0.170 ppm and 0.138 ppm, respectively. The levels in the PRE-T, POE-T, PRE-C and POE-C seed were all roughly comparable. This suggests limited translocation of residues to the seed through plant tissues.

Total radioactive residue data are summarized in Table IV. Spreadsheets showing the combustion data are in Appendices E-I.

Extraction of Forage

Acetonitrile/water extraction removed 1.171 ppm from PRE-T forage (81.7%, 93.6% normalized) and 122.074 ppm from POE-T forage (91.0%, 96.2% normalized). Combustion of post-extraction solids accounted for 0.080 ppm in PRE-T (5.6%, 6.4% normalized) and 4.829 ppm in POE-T (3.6%, 3.8% normalized). See Table V and Appendix J for forage extraction data.

Extraction of Seed

Acetonitrile/water extraction accounted for 0.154 ppm from PRE-T seed (52.9%, 52.6% normalized) and 0.228 ppm from POE-T seed (58.7%, 57.5% normalized). Subsequent combustion of post-extraction solids totaled for 0.139 ppm in PRE-T (47.8%, 47.4% normalized) and 0.169 ppm in POE-T (43.6%, 42.5% normalized). See Table VI and Appendix K for seed extraction data.

HPLC Analysis of Dicamba and Reference Standards

Figure 7 shows the HPLC UV chromatogram from analysis of a mixture of 5-hydroxydicamba, DCSA and dicamba reference standards.

Characterization of Residues in Forage Rinses

The HPLC quantitation results for the forage rinses are presented in Table VII and the HPLC profiles are presented in Figures 8-9. The rinse of forage from POE-T plant 13 contained 99.0% [^{14}C]dicamba. The rinse from POE-T plant 28 contained 100.0% [^{14}C]dicamba.

HPLC Analysis (Initial Storage Stability Profiles) of Forage Extracts

HPLC/LSC initial storage stability profiles for the PRE-T and POE-T forage extracts are presented in Figures 10-11. In the forage extracts a major metabolite was observed with a retention time of ~15 minutes. A peak that corresponded in retention time to dicamba was observed (substantial peak in POE-T forage profile). A minor peak that corresponded in retention time to DCSA was also observed. Dicamba and DCSA were not conclusively identified. Metabolite identification and quantification, as well as final storage stability profiles, will be presented in the Monsanto Company final report.

HPLC Analysis (Initial Storage Stability Profiles) of Seed Extracts

HPLC/LSC initial storage stability profiles for the PRE-T and POE-T seed extracts are presented in Figures 12-13. As observed in the forage extracts, the seed extracts also contained a major metabolite with a retention time of ~15 minutes. An additional significant metabolite was observed in seed extracts with a retention time of ~17 minutes along with a significant polar peak eluting near the solvent front. Metabolite identification and quantification, as well as final storage stability profiles, will be presented in the Monsanto Company final report.

Sample Processing and Shipment

The schedule of events associated with the in-life phase of the study is shown in Appendix L. Processed weights of soybean matrices are shown in Table VIII. Processed samples were shipped to Monsanto Company on dry ice. Dates of shipment are shown in Table VIII.

Disposition of Remaining Test Substance and Test Substance Containers

Any remaining test substance and the original test substance containers will be retained until further instruction by the Study Director or Monsanto Company representative. All radioactive waste associated with the study was disposed of in a manner consistent with radioactive waste disposal guidelines.

Protocol Deviations

Copies of the study protocol and amendments will be provided in the Monsanto Company final report. Two protocol deviations occurred that were associated with the field phase of the study and are described in the field report (Appendix M).

The following deviations occurred during the test substance application and analysis phase of the study:

Section 7.6.4: for purity determination, a 200 µL aliquot of the preemergence application test substance formulation was taken before homogeneity aliquots, instead of a 50 µL aliquot after.

Section 7.6.4: for purity determination, the protocol stated that the aliquot of formulated test substance for purity determination was to be frozen prior to analysis. For both the preemergence and postemergence solutions the aliquot was not frozen but analyzed immediately.

Section 7.6.5: 60 mL bottles were used for the preemergence application instead of 30 mL bottles.

Section 7.6.5: the unused test substance formulation remaining after the preemergence application was transported to PTRL West on blue ice instead of dry ice.

None of these deviations had a negative impact on the outcome or interpretation of the study results.

CONCLUSIONS

Dicamba-tolerant soybean plants were grown in pots in greenhouses in Porterville, California and [¹⁴C]dicamba was applied either pre- (PRE-T) or postemergence (POE-T). The application rates were 2.55 lbs/acre for PRE-T (102.1% of target) and 2.52 lbs/acre for POE-T (100.9% of target).

Soybeans were harvested 83 days after the postemergence application (112 days after the preemergence application and planting). Good yields of soybean forage, hay and seed were obtained.

Immature foliage, forage, hay and seed were homogenized and combusted to determine radioactive residue levels. Total radioactive residues (TRR) in PRE-T pre-forage, forage, hay and seed were 3.248 ppm, 1.433 ppm, 1.056 ppm and 0.291 ppm, respectively. TRR in POE-T forage, hay and seed were 134.147 ppm, 39.149 ppm and 0.389 ppm, respectively. PRE-C forage and seed contained 0.080 ppm and 0.170 ppm, respectively. POE-C forage and seed contained 0.280 ppm and 0.138 ppm, respectively.

The TRR of forage from an individual POE-T plant was 183.169 ppm (58.877 ppm in a surface rinse and 124.292 ppm in rinsed forage). Forage from a second individual POE-T plant was 123.084 ppm (41.298 ppm in rinse and 81.786 ppm in rinsed forage).

Samples of processed forage and seed were extracted and analyzed by HPLC/LSC to establish initial storage stability profiles. Processed pre-forage, forage, hay and seed samples were shipped to Monsanto Company for metabolite identification and quantification.

ARCHIVING:

All study specific documentation, records and raw data generated in the in-life portion of the study, and the original PTRL sub-report have been transferred to the Sponsor. A copy of this sub-report will be maintained in the archives of PTRL West, Inc.

Original records of the maintenance of PTRL West, Inc. equipment such as freezers, balances and liquid scintillation counters will be retained in PTRL West, Inc. archives.

Specimens were sent to the Sponsor after processing.

REFERENCES

1. *The Pesticide Manual*, Crop Protection Publications, 13th Ed., 2003.

Table I. Application Rates.

	PRE-T	POE-T
Target (mg/plot)	20.45	20.45
AMOUNT AVAILABLE*:		
Application Bottles (mCi)	15.21	16.58
AMOUNTS REMOVED*:		
Bottle Rinses (mCi)	0.44	0.36
AMOUNT APPLIED (mCi)*:	14.77	16.22
AMOUNT APPLIED (mg):	605.1	660.0
AMOUNT APPLIED (mg/pot):	20.87	20.63
AMOUNT APPLIED (lb/ac):	2.55	2.52
% of Target	102.1	100.9

* Refer to Appendix C

Target Rate = 2.5 lb/ac

$$2.5 \text{ lb/ac} \times 1.803 \times 10^{-5} \text{ ac/pot} \times 453.59 \text{ g/lb} \times 1000 \text{ mg/g} = 20.45 \text{ mg ai/pot}$$

Table II. Pre- and Post-Application Purity Checks.

	PRE-T	POE-T
Pre-Application Purity	99.8%	100.0%
Post-Application Purity	99.9%	99.8%

Table III. Application Solution Homogeneity.

Application Solution Homogeneity	PRE-T	POE-T
Aliquot 1 DPM	2,681,279	2,811,420
Aliquot 2 DPM	2,703,583	2,808,742
Aliquot 3 DPM	2,714,258	2,813,237
Aliquot 4 DPM	2,711,538	2,825,504
Aliquot 5 DPM	2,710,686	2,822,231
Aliquot 6 DPM	2,711,862	2,821,452
Mean DPM	2,705,534	2,817,098
% Relative Standard Deviation	0.46%	0.24%

Table IV. Total Radioactive Residues (TRR) in Soybean.

Matrix	Treatment: PRE-T	POE-T	UNT-C	PRE-C	POE-C
Pre-Forage	3.248	NS	0.000	NS	NS
Forage	1.433	134.147	0.000	0.080	0.280
Hay	1.056	39.149	0.001	NS	NS
Seed	0.291	0.389	0.013	0.170	0.138

Total Radioactive Residues expressed as parts per million [^{14}C]dicamba equivalent.

Data determined by combustion radioanalysis.

NS = no sample.

Select Individual Plant TRR:

	Treatment: POE-T Plant 13 Washed Forage	POE-T Plant 28 Washed Forage
Rinses (ppm)	58.877	41.298
Combustion (ppm)	124.292	81.786
TRR	183.169	123.084

Rinse data determined by liquid scintillation counting.

Table V. Extraction of Radioactive Residues from Forage.

	PRE-T ppm	PRE-T % of TRR	PRE-T % of TRR*	POE-T ppm	POE-T % of TRR	POE-T % of TRR*
ACN/Water Extractions	1.171	81.7	93.6	122.074	91.0	96.2
PES Combustion	0.080	5.6	6.4	4.829	3.6	3.8
Extracted + Unextracted	1.251	87.3	100.0	126.903	94.6	100.0

* Normalized to total extracted + unextracted.

Table VI. Extraction of Radioactive Residues from Seed.

	PRE-T ppm	PRE-T % of TRR	PRE-T % of TRR*	POE-T ppm	POE-T % of TRR	POE-T % of TRR*
ACN/Water Extractions	0.154	52.9	52.6**	0.228	58.6	57.5
PES Combustion	0.139	47.8	47.4**	0.169	43.4	42.5
Extracted + Unextracted	0.292	100.3	100.0	0.398	102.1	100.0

* Normalized to total extracted + unextracted.

** Calculated to (Extracted + Unextracted=0.293 ppm). Difference stems from rounding in extraction spreadsheet.

Table VII. Radiolabeled Components Detected by HPLC in Rinses of Forage from Select Individual Plants.

Metabolites Detected	Individual Plant: Plant 13 Forage Rinse	Plant 28 Forage Rinse
[¹⁴ C]Dicamba Area %	99.0%	100.0%
All other Area%	1.0%	0.0%

Table VIII. Processing Weights and Dates of Shipment.

Matrix	Treatment: PRE-T	POE-T	UNT-C	PRE-C	POE-C
Pre-Forage Weights:	97.8	NS	26.1	NS	NS
Pre-Forage Date of Shipment:	June 28, 2006	NS	June 28, 2006	NS	NS
Forage Weights:	302.7	151.4	109.1	294.3	153.8
Forage Date of Shipment:	August 16, 2006	August 16, 2006	August 16, 2006	August 16, 2006	August 16, 2006
Hay Weights:	995.7	622.9	289.8	NS	NS
Hay Date of Shipment:	August 16, 2006	August 16, 2006	August 16, 2006	NS	NS
Seed Weights:	450.3	369.6	96.2	111.0	81.0
Seed Date of Shipment:	October 25, 2006	October 25, 2006	October 25, 2006	October 25, 2006	October 25, 2006

All weights in grams.

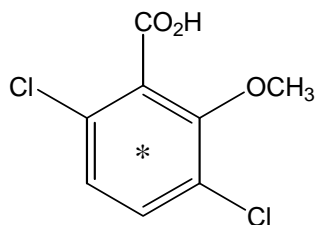
NS = no sample.

Select Individual Plant Forage:

	Treatment: POE-T Plant 13 Washed Forage	POE-T Plant 28 Washed Forage
Washed Forage Weight:	24.3	29.1
Date of Shipment:	September 23, 2008	September 23, 2008

All weights in grams.

Figure 1. Chemical Structure and ^{14}C Labeling Position of Dicamba.



dicamba

* designates uniform ^{14}C -labeling

Figure 2. Structures of Dicamba Reference Standards.

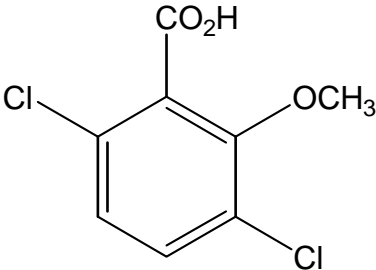
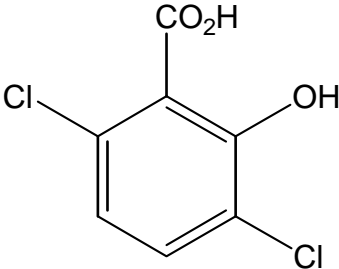
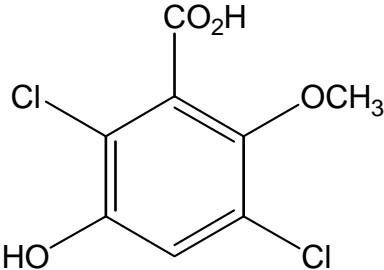
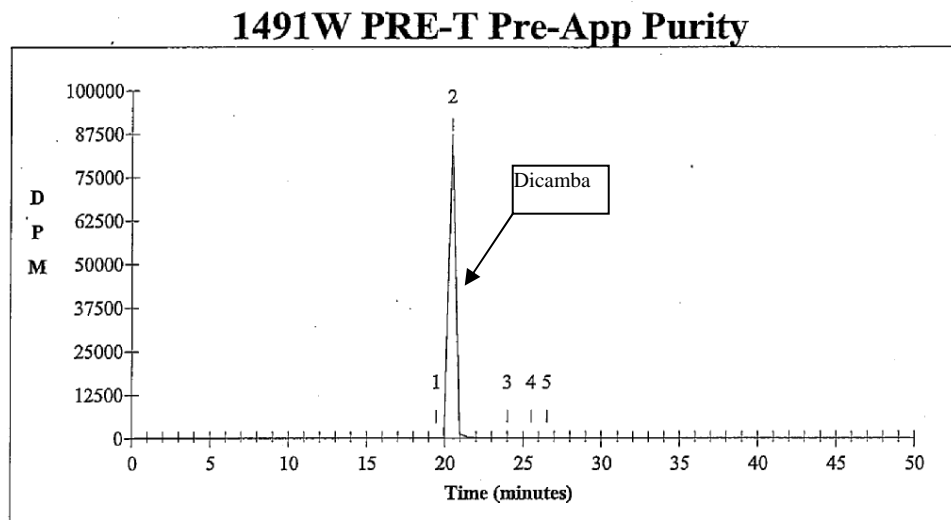
<p>Dicamba</p> <p>3,6-dichloro-2-methoxybenzoic acid</p>	
<p>DCSA (3,6-dichlorosalicylic acid)</p> <p>3,6-dichloro-2-hydroxybenzoic acid</p>	
<p>5-Hydroxydicamba</p> <p>2,5-dichloro-3-hydroxy-6-methoxybenzoic acid</p>	

Figure 3. HPLC Radiochromatogram and UV Chromatogram of [^{14}C]Dicamba Prior to Preemergence Application (HPLC Method 1).



Fraction Number: Fraction DPM

1: 27	14: 25	27: 18	40: 36	53: 60	66: 32	79: 16	92: 17
2: 20	15: 22	28: 34	41: 87357	54: 40	67: 26	80: 24	93: 19
3: 20	16: 26	29: 23	42: 1276	55: 40	68: 29	81: 28	94: 21
4: 21	17: 27	30: 36	43: 265	56: 37	69: 28	82: 17	95: 26
5: 37	18: 20	31: 26	44: 129	57: 28	70: 34	83: 9	96: 25
6: 32	19: 15	32: 29	45: 104	58: 27	71: 21	84: 24	97: 20
7: 24	20: 24	33: 23	46: 64	59: 34	72: 31	85: 30	98: 15
8: 24	21: 20	34: 18	47: 48	60: 30	73: 31	86: 26	99: 30
9: 24	22: 18	35: 50	48: 60	61: 30	74: 20	87: 19	
10: 28	23: 26	36: 31	49: 36	62: 25	75: 28	88: 26	
11: 22	24: 26	37: 36	50: 46	63: 24	76: 19	89: 24	
12: 24	25: 18	38: 25	51: 63	64: 31	77: 20	90: 15	
13: 20	26: 26	39: 62	52: 36	65: 29	78: 24	91: 26	

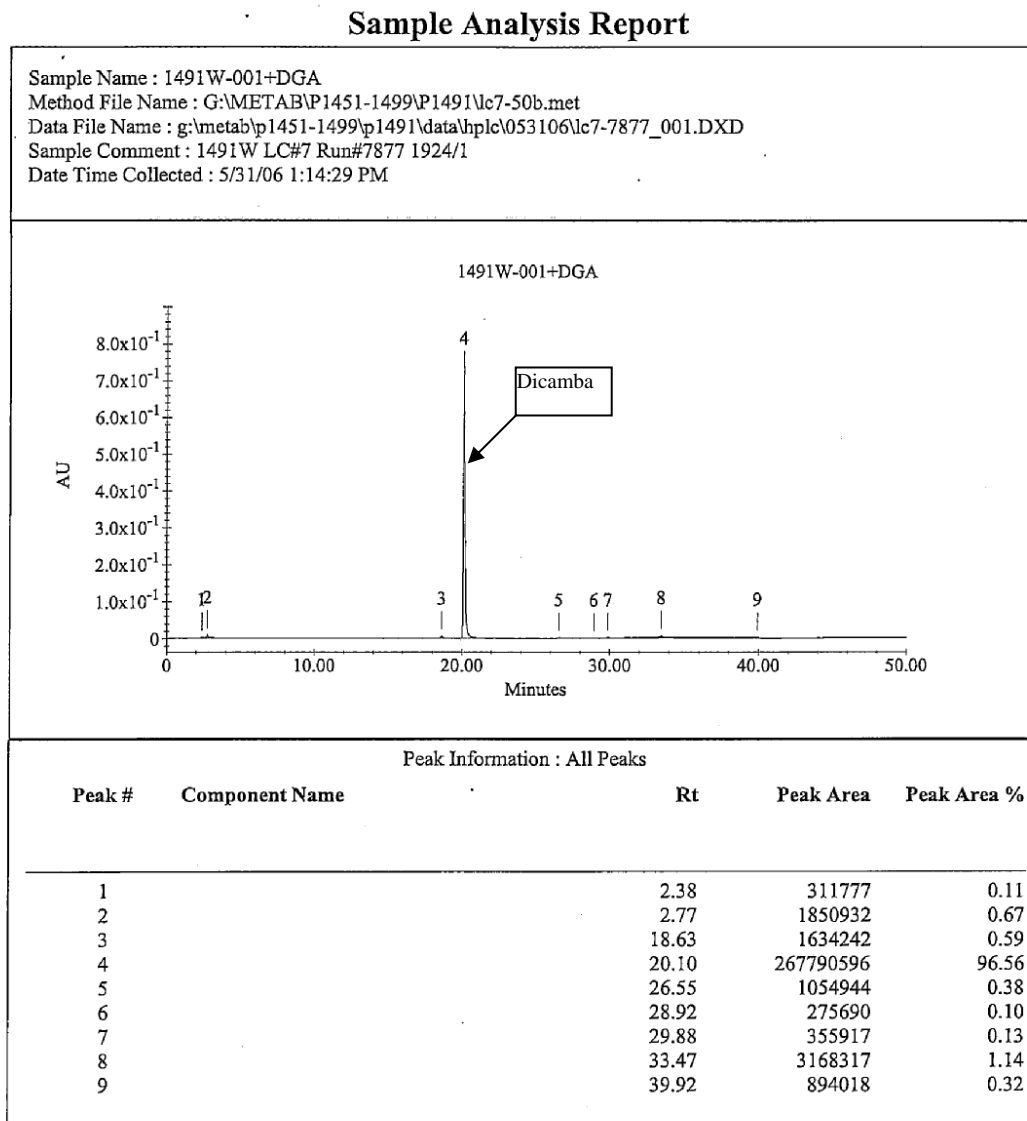
Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent
1	19.44	36	0.0%
2	20.45	89039	99.8%
3	23.99	34	0.0%
4	25.51	37	0.0%
5	26.52	34	0.0%

Average Background = 26 DPM.

Total Peak Area = 89180 DPM.

Project: 1491W HPLC #: 7 Run #: 7877
 Analysis Date: 5/31/06 NB/p: 1924/1
 Detection: Fraction Sample PPM:
 Sensitivity: 0 Max Bkg: 50
 DPM Injected: 88601 Recovery: 100.7%
 Date & Initials: 5/31/06

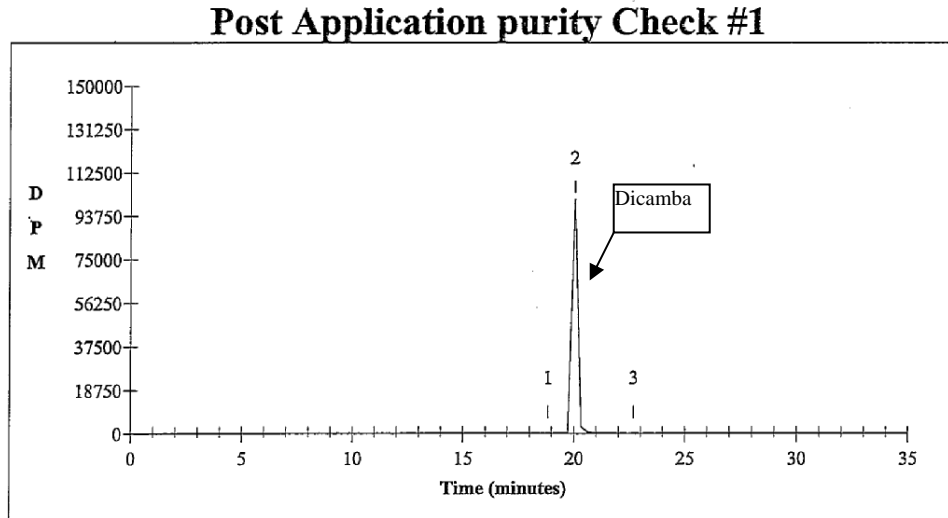
Figure 3. HPLC Radiochromatogram and UV Chromatogram of [¹⁴C]Dicamba Prior to Preemergence Application (HPLC Method 1). (cont.)



MS 5/31/06
 Current Date : 5/31/06
 Current Time : 14:04:3

1 ml 60%

Figure 4. HPLC Radiochromatogram and UV Chromatogram of Application Solution After Preemergence Application (HPLC Method 1).



Project: 1491W HPLC #: 7 Run #: 7879

Fraction Number: Fraction DPM

1: 17	16: 19	31: 21	46: 18	61: 35	76: 49	91: 23	106: 29
2: 17	17: 29	32: 9	47: 16	62: 24	77: 65	92: 35	107: 27
3: 17	18: 22	33: 18	48: 25	63: 21	78: 57	93: 29	108: 19
4: 23	19: 24	34: 9	49: 22	64: 55	79: 32	94: 34	109: 23
5: 19	20: 13	35: 14	50: 33	65: 25	80: 23	95: 37	110: 36
6: 17	21: 19	36: 25	51: 17	66: 30	81: 19	96: 22	111: 12
7: 20	22: 19	37: 17	52: 22	67: 410	82: 31	97: 21	112: 24
8: 17	23: 17	38: 21	53: 18	68: 101099	83: 21	98: 21	113: 26
9: 25	24: 14	39: 16	54: 21	69: 3000	84: 24	99: 28	114: 26
10: 14	25: 12	40: 15	55: 21	70: 652	85: 43	100: 32	115: 29
11: 23	26: 17	41: 28	56: 28	71: 244	86: 35	101: 30	116: 23
12: 19	27: 23	42: 24	57: 30	72: 130	87: 25	102: 25	117: 28
13: 14	28: 16	43: 25	58: 37	73: 85	88: 24	103: 21	118: 27
14: 13	29: 23	44: 18	59: 28	74: 85	89: 30	104: 28	
15: 19	30: 14	45: 12	60: 21	75: 71	90: 24	105: 29	

Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent
1	18.83	32	0.0%
2	20.02	105569	99.9%
3	22.69	76	0.1%

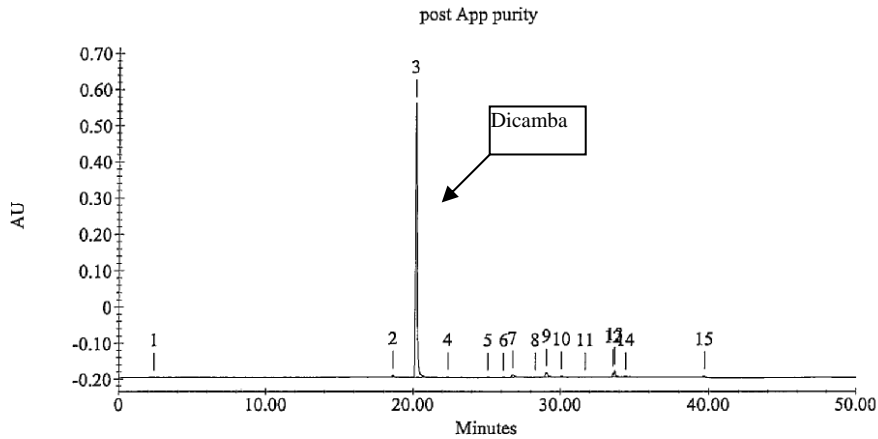
Average Background = 23 DPM.
Total Peak Area = 105676 DPM.

Analysis Date: 06/02/06 NB/p: 1924/6
Sensitivity: 0 Max Bkg: 50
DPM Injected: 104546 Recovery: 101.1%
Date & Initials: SFW 6-5-06

Figure 4. HPLC Radiochromatogram and UV Chromatogram of Application Solution After Preemergence Application (HPLC Method 1). (cont.)

Sample Analysis Report

Sample Name : post App purity
Method File Name : G:\METAB\p1451-1499\p1491\lc7-50b.met
Data File Name : g:\metab\p1451-1499\p1491\data\hplc\053106\lc7-7877_004.DXD
Sample Comment : 1491W LC#7 Run#7879 1924/6
Date Time Collected : 6/2/06 3:19:11 PM



Peak Information : All Peaks

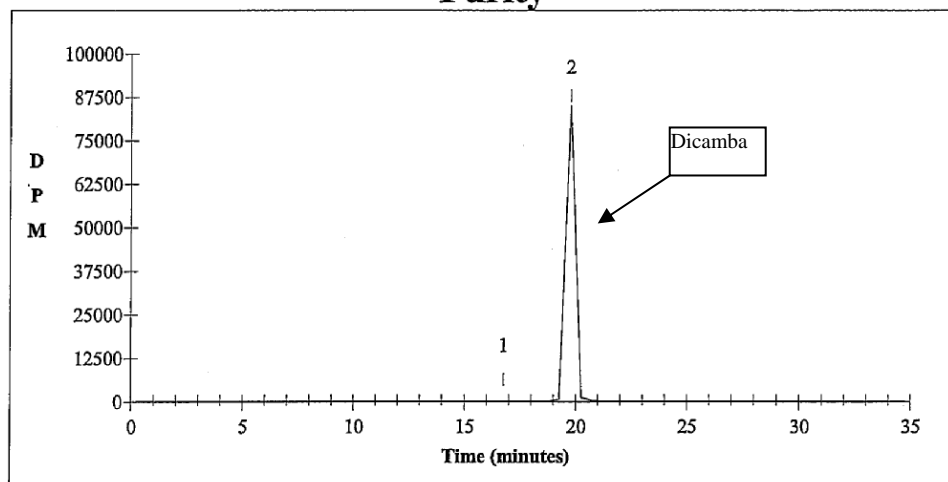
Peak #	Component Name	Rt	Peak Area	Peak Area %
1		2.40	255423	0.08
2		18.63	1895816	0.62
3		20.17	280095572	91.98
4		22.37	766057	0.25
5		25.08	205109	0.07
6		26.15	543527	0.18
7		26.75	4694926	1.54
8		28.30	249166	0.08
9		29.07	6297825	2.07
10		30.08	962207	0.32
11		31.68	280954	0.09
12		33.57	2465679	0.81
13		33.68	5221799	1.71
14		34.40	257774	0.08
15		39.70	335528	0.11



STW 6-2-06

Figure 5. HPLC Radiochromatogram and UV Chromatogram of [^{14}C]Dicamba Prior to Postemergence Application (HPLC Method 1).

**1491W-006 14-Dicamba Post-Emergence Pre-App
Purity**



Fraction Number: Fraction DPM

1: 19	10: 14	19: 24	28: 27	37: 23	46: 38	55: 31	64: 27
2: 20	11: 20	20: 12	29: 27	38: 65	47: 34	56: 31	65: 18
3: 17	12: 16	21: 18	30: 22	39: 593	48: 44	57: 27	66: 13
4: 25	13: 21	22: 16	31: 30	40: 85208	49: 31	58: 38	67: 28
5: 19	14: 26	23: 28	32: 23	41: 1222	50: 38	59: 27	68: 27
6: 12	15: 21	24: 16	33: 23	42: 286	51: 39	60: 24	69: 25
7: 27	16: 14	25: 30	34: 62	43: 135	52: 47	61: 29	70: 43
8: 19	17: 26	26: 19	35: 31	44: 72	53: 34	62: 30	
9: 19	18: 14	27: 22	36: 42	45: 68	54: 27	63: 33	

Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent
1	16.75	36	0.0%
2	19.75	87444	100.0%

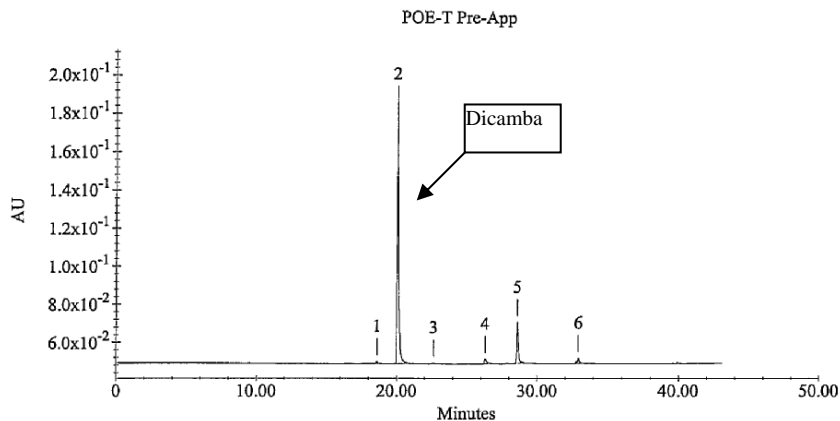
Average Background = 26 DPM.
Total Peak Area = 87480 DPM.

Project: 1491W HPLC #: 10 Run #: 5486
Analysis Date: 6/30/06 NB/p: 1924/11
Detection: Fraction Sample PPM:
Sensitivity: 0 Max Bkg: 50
DPM Injected: 93552 Recovery: 93.5%
Date & Initials: Rms 6/30/06

Figure 5. HPLC Radiochromatogram and UV Chromatogram of [^{14}C]Dicamba Prior to Postemergence Application (HPLC Method 1). (cont.)

Sample Analysis Report

Sample Name : POE-T Pre-App
Method File Name : G:\METAB\p1451-1499\p1491\data\hplc\lc7-50b.met
Data File Name : g:\metab\p1451-1499\p1491\data\hplc\062906\lc10-5486_001.DXD
Sample Comment : 1491W LCC#10 Run#5486 1924/11
Date Time Collected : 6/29/06 2:54:05 PM



Peak Information : All Peaks

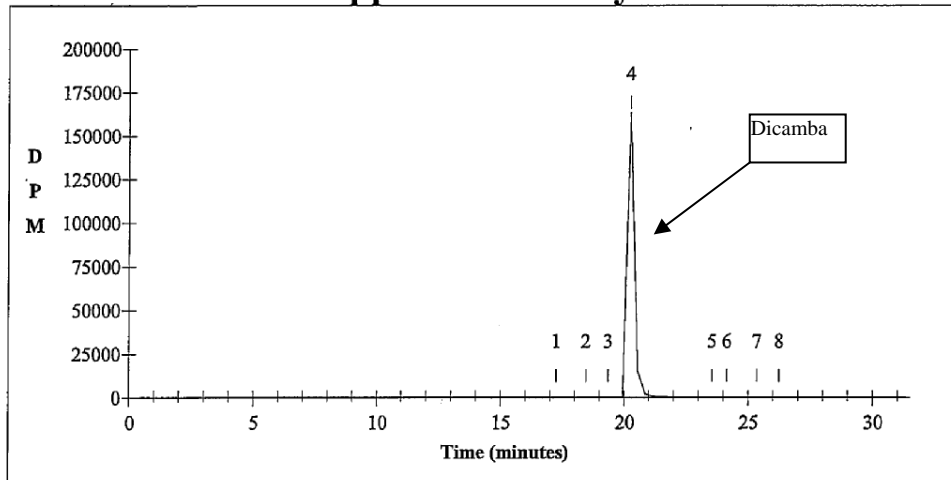
Peak #	Component Name	Rt	Peak Area	Peak Area %
1		18.60	291550	0.47
2		20.03	51101515	81.73
3		22.63	195795	0.31
4		26.30	1261655	2.02
5		28.60	8493428	13.58
6		32.93	1178097	1.88



By 6-29-06
JL

Figure 6. HPLC Radiochromatogram and UV Chromatogram of Application Solution After Postemergence Application (HPLC Method 1).

1491W-006 14C-Dicamba Post-Emergence Post-Application Purity Check



Fraction Number: Fraction DPM

1: 17	15: 22	29: 31	43: 28	57: 30	71: 734	85: 62	99: 27
2: 16	16: 20	30: 17	44: 34	58: 78	72: 366	86: 49	100: 28
3: 16	17: 20	31: 16	45: 26	59: 33	73: 206	87: 45	101: 44
4: 19	18: 18	32: 19	46: 28	60: 31	74: 152	88: 85	102: 32
5: 19	19: 21	33: 22	47: 27	61: 34	75: 141	89: 44	103: 38
6: 9	20: 22	34: 22	48: 26	62: 60	76: 103	90: 24	104: 26
7: 21	21: 26	35: 23	49: 41	63: 31	77: 98	91: 31	105: 26
8: 22	22: 18	36: 23	50: 36	64: 25	78: 64	92: 36	
9: 28	23: 26	37: 24	51: 30	65: 90	79: 78	93: 40	
10: 16	24: 27	38: 23	52: 26	66: 51	80: 59	94: 38	
11: 22	25: 16	39: 19	53: 35	67: 45	81: 66	95: 31	
12: 18	26: 23	40: 19	54: 27	68: 163521	82: 43	96: 44	
13: 22	27: 14	41: 28	55: 33	69: 15183	83: 49	97: 41	
14: 15	28: 22	42: 18	56: 33	70: 2061	84: 56	98: 32	

Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent
1	17.25	51	0.0%
2	18.45	33	0.0%
3	19.35	87	0.0%
4	20.25	182311	99.8%
5	23.55	85	0.0%
6	24.15	55	0.0%
7	25.35	64	0.0%
8	26.25	58	0.0%

Average Background = 27 DPM.

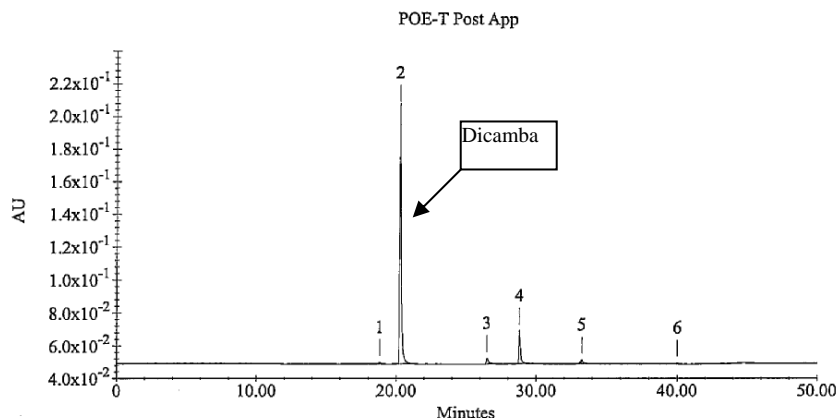
Total Peak Area = 182742 DPM.

Project: 1491W HPLC #: 10 Run #: 5488
 Analysis Date: 7/3/06 NB/p: 1924/13
 Detection: Fraction Sample PPM:
 Sensitivity: 0 Max Bkg: 50
 DPM Injected: 197606 Recovery: 92.5%
 Date & Initials: 8/3/06

Figure 6. HPLC Radiochromatogram and UV Chromatogram of Application Solution After Postemergence Application (HPLC Method 1). (cont.)

Sample Analysis Report

Sample Name : POE-T Post App
Method File Name : G:\METAB\p1451-1499\p1491\data\hplc\lc7-50b.met
Data File Name : g:\metab\p1451-1499\p1491\data\hplc\070306\lc10-5488_001.DXD
Sample Comment : 1491W LC#10 Run#5488 1924/13
Date Time Collected : 7/3/06 9:57:31 AM



Peak Information : All Peaks

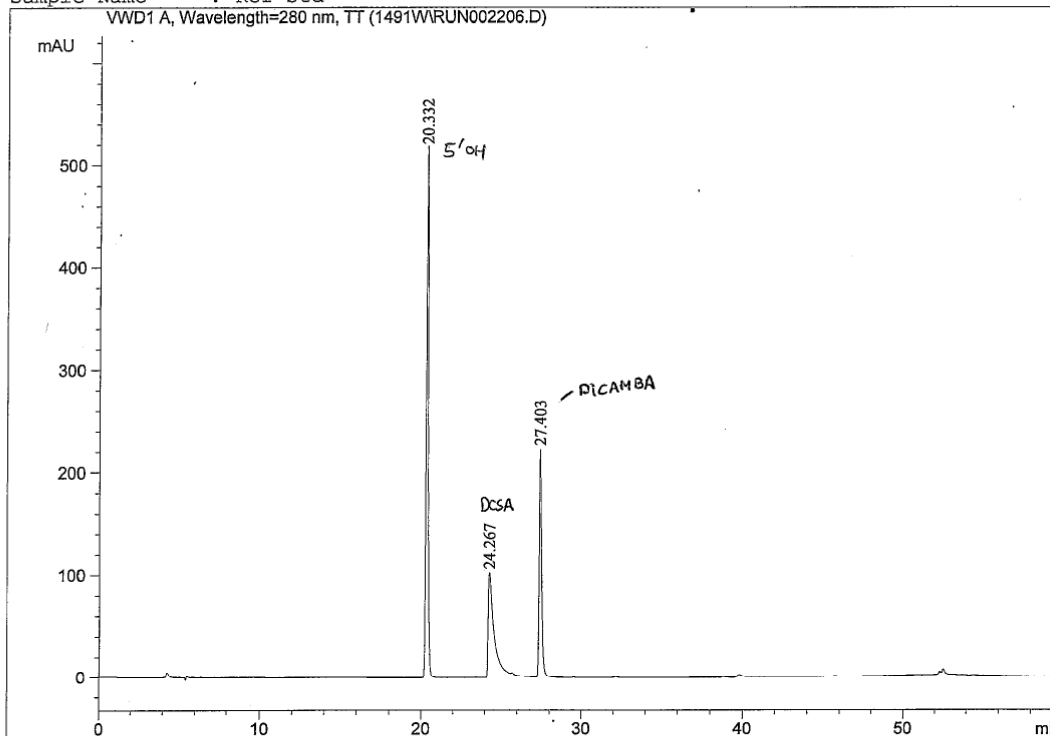
Peak #	Component Name	Rt	Peak Area	Peak Area %
1		18.82	313413	0.43
2		20.25	60369831	83.48
3		26.48	1827177	2.53
4		28.80	8442780	11.67
5		33.25	1142732	1.58
6		40.00	224158	0.31



Figure 7. Representative UV Chromatogram of Reference Standards (HPLC Method 2).

Data file : G:\CHEMSTATION\HPLC-20\DATA\1491->
Injection Date : Fri, 28. Jul. 2006 Acq Operator : AVB
Analysis Method : G:\CHEMSTATION\HPLC-20\METHODS\ ->
1491W Ref Std 1924/21 RUN # 2205

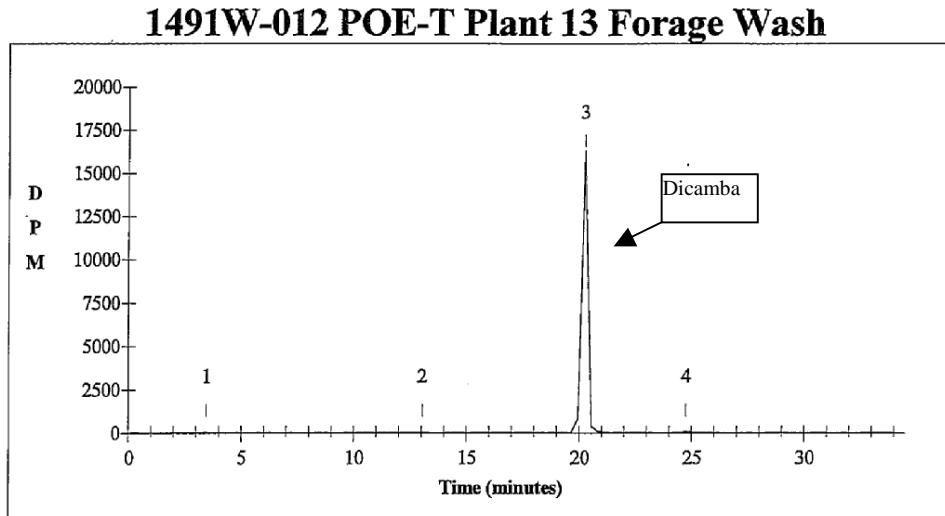
Sample Name : Ref Std



Peak #	RT [min]	Name	Area	Area %
1	20.33		4525.2	47.0
2	24.27	DCA	2740.6	28.4
3	27.40	PICAMBA	2371.4	24.6

Run #2205
3 µl each std.
AVB 7/28/06

Figure 8. HPLC Radiochromatogram and UV Chromatogram of Rinses from Forage of POE-T Plant 13 (HPLC Method 1).

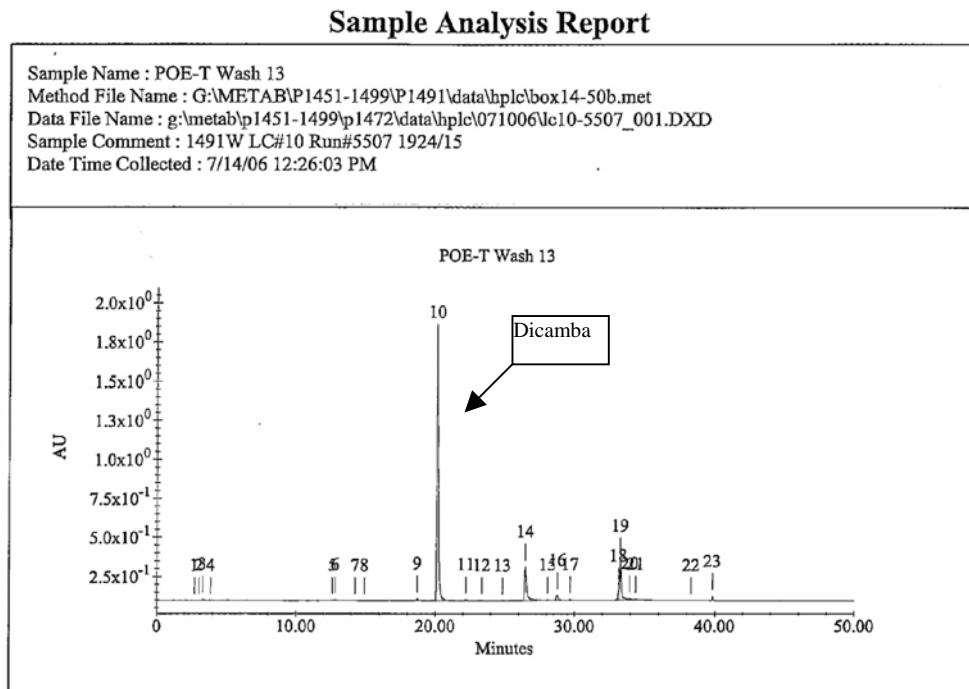


Fraction Number. Fraction DPM							
1: 17	16: 24	31: 14	46: 15	61: 27	76: 29	91: 14	106: 26
2: 27	17: 25	32: 28	47: 20	62: 25	77: 36	92: 25	107: 17
3: 11	18: 15	33: 18	48: 18	63: 33	78: 26	93: 20	108: 15
4: 28	19: 20	34: 22	49: 20	64: 18	79: 13	94: 16	109: 17
5: 12	20: 18	35: 18	50: 22	65: 28	80: 13	95: 24	110: 21
6: 11	21: 18	36: 24	51: 23	66: 25	81: 24	96: 21	111: 25
7: 17	22: 26	37: 22	52: 23	67: 837	82: 26	97: 18	112: 18
8: 31	23: 13	38: 22	53: 24	68: 16302	83: 70	98: 21	113: 22
9: 37	24: 22	39: 25	54: 22	69: 391	84: 63	99: 13	114: 26
10: 20	25: 28	40: 21	55: 20	70: 76	85: 18	100: 13	115: 15
11: 20	26: 16	41: 23	56: 27	71: 59	86: 23	101: 11	
12: 61	27: 16	42: 28	57: 20	72: 23	87: 22	102: 26	
13: 29	28: 30	43: 22	58: 37	73: 29	88: 22	103: 17	
14: 23	29: 17	44: 60	59: 23	74: 25	89: 12	104: 22	
15: 20	30: 22	45: 20	60: 17	75: 21	90: 20	105: 12	

Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent
1	3.45	40	0.2%
2	13.05	39	0.2%
3	20.25	17558	99.0%
4	24.75	90	0.5%
Average Background =			21 DPM.
Total Peak Area =			17727 DPM.

Project: 1491W HPLC #: 10 Run #: 5507
 Analysis Date: 7/14/06 NB/p: 1924/15
 Detection: Fraction Sample PPM:
 Sensitivity: 0 Max Bkg: 50
 DPM Injected: 18665 Recovery: 95.0%
 Date & Initials: 8/5 7/14/06

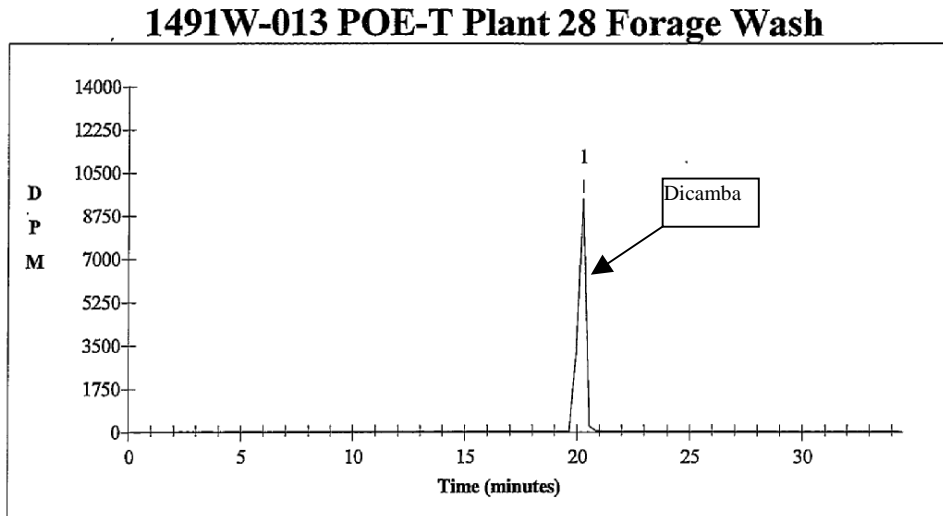
Figure 8. HPLC Radiochromatogram and UV Chromatogram of Rinses from Forage of POE-T Plant 13 (HPLC Method 1). (cont.)



Sample Analysis Report

Peak Information : All Peaks				
Peak #	Component Name	Rt	Peak Area	Peak Area %
1		2.70	1305854	0.14
2		3.02	912500	0.10
3		3.28	2683025	0.28
4		3.87	318906	0.03
5		12.63	525565	0.06
6		12.82	1478585	0.16
7		14.27	471355	0.05
8		14.93	243315	0.03
9		18.72	7454800	0.79
10		20.10	648946088	68.71
11		22.20	2319234	0.25
12		23.33	1242606	0.13
13		24.83	348825	0.04
14		26.47	114512189	12.12
15		28.07	2184925	0.23
16		28.77	17463897	1.85
17		29.68	2410531	0.26
18		33.13	16927531	1.79
19		33.27	107840499	11.42
20		33.97	4410556	0.47
21		34.37	4061049	0.43
22		38.32	469528	0.05
23		39.82	5900379	0.62

Figure 9. HPLC Radiochromatogram and UV Chromatogram of Rinses from Forage of POE-T Plant 28 (HPLC Method 1).



Fraction Number: Fraction DPM

1: 14	16: 28	31: 23	46: 25	61: 17	76: 24	91: 12	106: 16
2: 25	17: 19	32: 18	47: 24	62: 20	77: 21	92: 12	107: 15
3: 19	18: 25	33: 14	48: 17	63: 20	78: 16	93: 21	108: 19
4: 21	19: 28	34: 17	49: 20	64: 13	79: 14	94: 12	109: 16
5: 21	20: 18	35: 22	50: 21	65: 14	80: 21	95: 12	110: 16
6: 14	21: 21	36: 13	51: 23	66: 12	81: 19	96: 12	111: 18
7: 15	22: 12	37: 14	52: 17	67: 3213	82: 26	97: 21	112: 24
8: 20	23: 18	38: 12	53: 23	68: 9461	83: 20	98: 26	113: 18
9: 25	24: 19	39: 20	54: 15	69: 234	84: 15	99: 13	114: 15
10: 13	25: 30	40: 22	55: 26	70: 54	85: 19	100: 16	115: 14
11: 21	26: 13	41: 23	56: 12	71: 35	86: 21	101: 22	
12: 40	27: 14	42: 25	57: 23	72: 32	87: 20	102: 13	
13: 25	28: 15	43: 19	58: 24	73: 33	88: 20	103: 15	
14: 17	29: 31	44: 46	59: 14	74: 20	89: 21	104: 14	
15: 26	30: 25	45: 17	60: 20	75: 25	90: 19	105: 18	

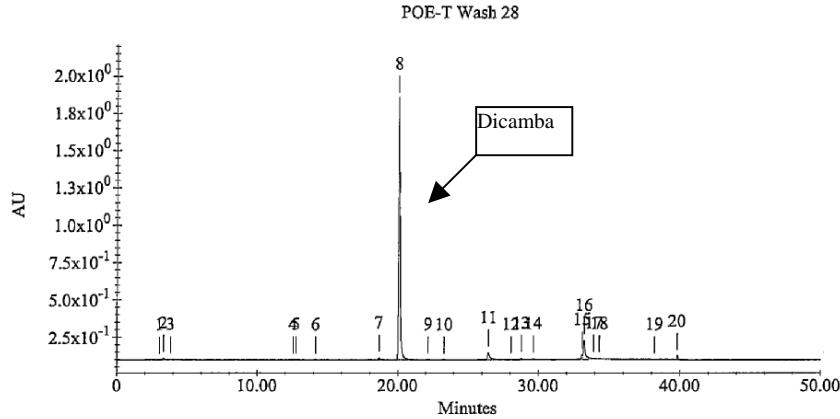
Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent
1	20.25	12883	100.0%
Average Background = 20 DPM.			
Total Peak Area = 12883 DPM.			

Project: 1491W HPLC #: 10 Run #: 5508
 Analysis Date: 7/14/06 NB/p: 1924/15
 Detection: Fraction Sample PPM: _____
 Sensitivity: 0 Max Bkg: 50
 DPM Injected: 13519 Recovery: 95.3%
 Date & Initials: P-AB 7/14/06

Figure 9. HPLC Radiochromatogram and UV Chromatogram of Rinses from Forage of POE-T Plant 28 (HPLC Method 1). (cont.)

Sample Analysis Report

Sample Name : POE-T Wash 28
Method File Name : G:\METAB\p1451-1499\p1491\data\hplc\box14-50b.met
Data File Name : g:\metab\p1451-1499\p1472\data\hplc\071006\lc10-5508_001.DXD
Sample Comment : 1491W LC#10 Run#5508 1924/15
Date Time Collected : 7/14/06 1:19:24 PM



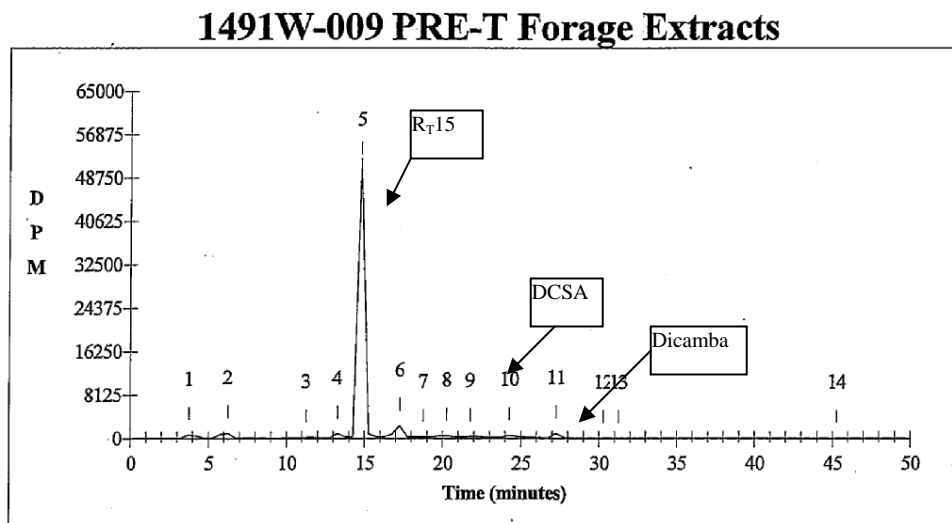
Peak Information : All Peaks

Peak #	Component Name	Rt	Peak Area	Peak Area %
1		3.03	996302	0.12
2		3.32	2658666	0.33
3		3.83	475430	0.06
4		12.57	431752	0.05
5		12.77	656210	0.08
6		14.17	364857	0.04
7		18.67	5078336	0.62
8		20.05	677355371	83.05
9		22.17	890233	0.11
10		23.30	389740	0.05
11		26.45	31970237	3.92
12		28.05	785745	0.10
13		28.78	4407464	0.54
14		29.65	2228236	0.27
15		33.10	9037609	1.11
16		33.23	65918759	8.08
17		33.92	3202720	0.39
18		34.33	2793648	0.34
19		38.18	254404	0.03
20		39.78	5749693	0.70



Handwritten signature

Figure 10. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from PRE-T Forage (HPLC Method 2).



Fraction Number: Fraction DPM

1: 22	14: 104	27: 894	40: 477	53: 124	66: 28	79: 22	92: 26
2: 18	15: 67	28: 272	41: 493	54: 95	67: 33	80: 27	93: 23
3: 23	16: 62	29: 218	42: 315	55: 938	68: 23	81: 24	94: 33
4: 35	17: 57	30: 52392	43: 242	56: 143	69: 20	82: 26	95: 23
5: 21	18: 57	31: 875	44: 365	57: 63	70: 25	83: 22	96: 29
6: 29	19: 52	32: 292	45: 343	58: 42	71: 23	84: 21	97: 29
7: 56	20: 55	33: 244	46: 170	59: 39	72: 25	85: 31	98: 17
8: 654	21: 55	34: 760	47: 165	60: 35	73: 30	86: 16	99: 27
9: 442	22: 94	35: 2437	48: 194	61: 42	74: 18	87: 19	100: 25
10: 84	23: 190	36: 275	49: 586	62: 31	75: 23	88: 21	
11: 80	24: 174	37: 222	50: 375	63: 47	76: 27	89: 26	
12: 751	25: 93	38: 254	51: 217	64: 31	77: 32	90: 29	
13: 1025	26: 75	39: 222	52: 186	65: 30	78: 19	91: 60	

Peak #	Retention Time (min)	Peak Area (DPM)	Percent	PPM	Peak #	Retention Time (min)	Peak Area (DPM)	Percent	PPM
1	3.75	1164	1.7%	0.020	8	20.25	1417	2.1%	0.024
2	6.25	1990	2.9%	0.034	9	21.75	982	1.4%	0.017
3	11.25	550	0.8%	0.009	10	24.25	1638	2.4%	0.028
4	13.25	1238	1.8%	0.021	11	27.25	1135	1.7%	0.020
5	14.75	53690	79.1%	0.926	12	30.25	17	0.0%	0.000
6	17.25	3605	5.3%	0.062	13	31.25	22	0.0%	0.000
7	18.75	426	0.6%	0.007	14	45.25	35	0.1%	0.001

Average Background = 25 DPM.

Total Peak Area = 67909 DPM.

Project: 1491W HPLC #: 20 Run #: 2207
 Analysis Date: 7/28/06 NB/p: 1924/21
 Detection: Fraction Sample PPM: 1.171
 Sensitivity: 0 Max Bkg: 35
 DPM Injected: 68093 Recovery: 99.7%
 Date & Initials: 7/28/06

Figure 10. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from PRE-T Forage (HPLC Method 2). (cont.)

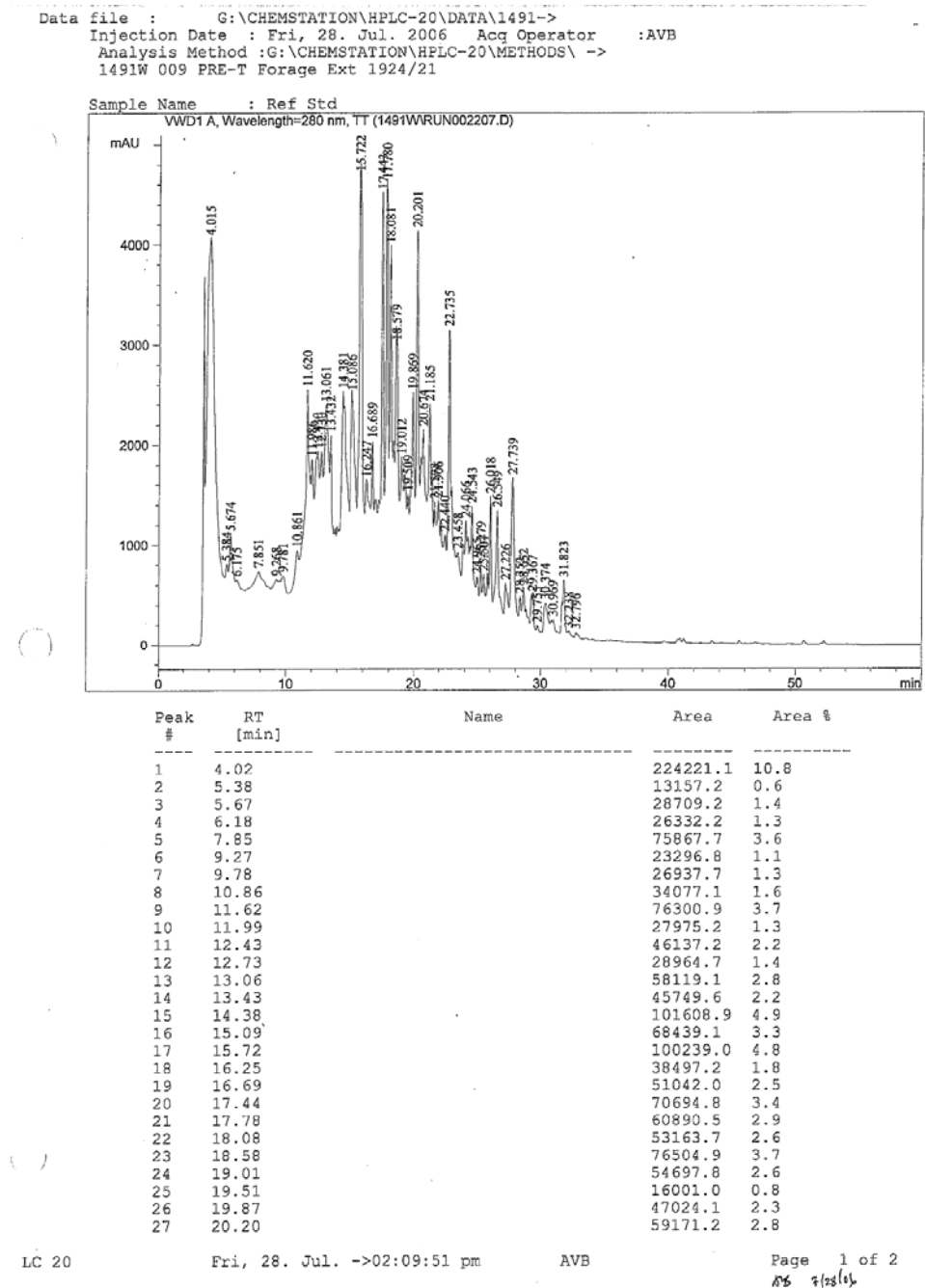
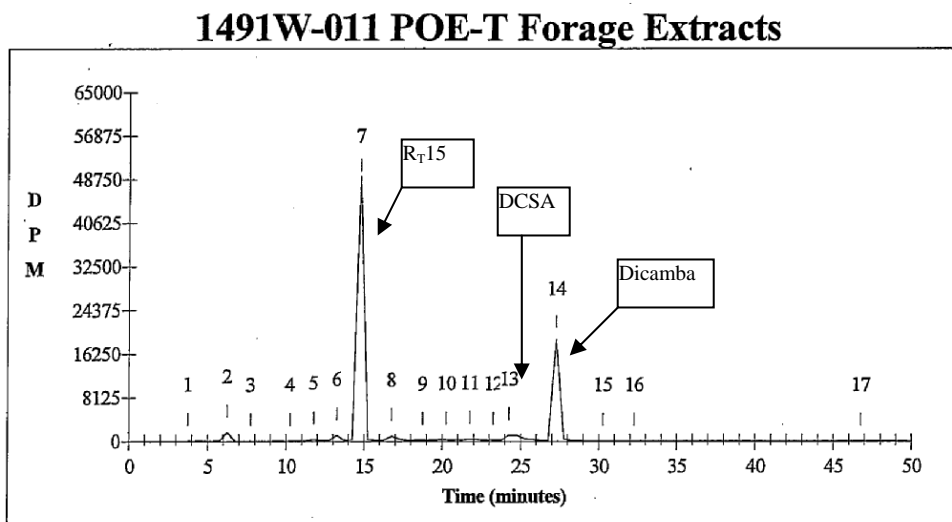


Figure 10. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from PRE-T Forage (HPLC Method 2). (cont.)

Data file : G:\CHEMSTATION\HPLC-20\DATA\1491->

Peak #	RT [min]	Name	Area	Area %
28	20.67		57192.9	2.7
29	21.18		56440.2	2.7
30	21.60		18293.3	0.9
31	21.91		38384.1	1.8
32	22.44		16815.3	0.8
33	22.73		69809.4	3.4
34	23.46		18732.3	0.9
35	24.07		36718.6	1.8
36	24.54		28150.0	1.4
37	24.96		10228.3	0.5
38	25.28		9944.4	0.5
39	25.51		8951.0	0.4
40	26.02		26034.1	1.3
41	26.55		31174.4	1.5
42	27.23		12558.1	0.6
43	27.74		34611.8	1.7
44	28.35		6076.1	0.3
45	28.65		14421.5	0.7
46	29.37		9941.1	0.5
47	29.76		3653.8	0.2
48	30.37		11387.2	0.5
49	30.97		8137.3	0.4
50	31.82		13343.4	0.6
51	32.24		3306.0	0.2
52	32.80		3739.8	0.2

Figure 11. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from POE-T Forage (HPLC Method 2).



Fraction Number, Fraction DPM

1: 25	14: 98	27: 1098	40: 277	53: 199	66: 45	79: 17	92: 27
2: 26	15: 52	28: 181	41: 306	54: 136	67: 33	80: 19	93: 28
3: 20	16: 56	29: 271	42: 233	55: 18971	68: 25	81: 17	94: 45
4: 20	17: 47	30: 49369	43: 232	56: 484	69: 29	82: 20	95: 27
5: 20	18: 44	31: 329	44: 400	57: 127	70: 29	83: 27	96: 22
6: 24	19: 26	32: 205	45: 334	58: 79	71: 28	84: 30	97: 23
7: 53	20: 43	33: 187	46: 180	59: 59	72: 26	85: 23	98: 24
8: 103	21: 55	34: 962	47: 279	60: 43	73: 22	86: 26	99: 33
9: 91	22: 38	35: 437	48: 173	61: 47	74: 25	87: 26	100: 27
10: 83	23: 67	36: 203	49: 1161	62: 45	75: 24	88: 20	
11: 106	24: 354	37: 225	50: 1092	63: 43	76: 23	89: 23	
12: 118	25: 152	38: 228	51: 465	64: 38	77: 23	90: 21	
13: 1727	26: 126	39: 209	52: 351	65: 54	78: 31	91: 18	

Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent	PPM	Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent	PPM
1	3.75	203	0.2%	0.303	10	20.25	939	1.1%	1.402
2	6.25	1994	2.4%	2.978	11	21.75	867	1.1%	1.294
3	7.75	88	0.1%	0.131	12	23.25	407	0.5%	0.607
4	10.25	56	0.1%	0.084	13	24.25	3276	4.0%	4.892
5	11.75	557	0.7%	0.832	14	27.25	19663	24.1%	29.362
6	13.25	1203	1.5%	1.796	15	30.25	78	0.1%	0.116
7	14.75	50236	61.5%	75.016	16	32.25	57	0.1%	0.085
8	16.75	1521	1.9%	2.271	17	46.75	21	0.0%	0.031
9	18.75	586	0.7%	0.875					

Average Background = 24 DPM.

Total Peak Area = 81750 DPM.

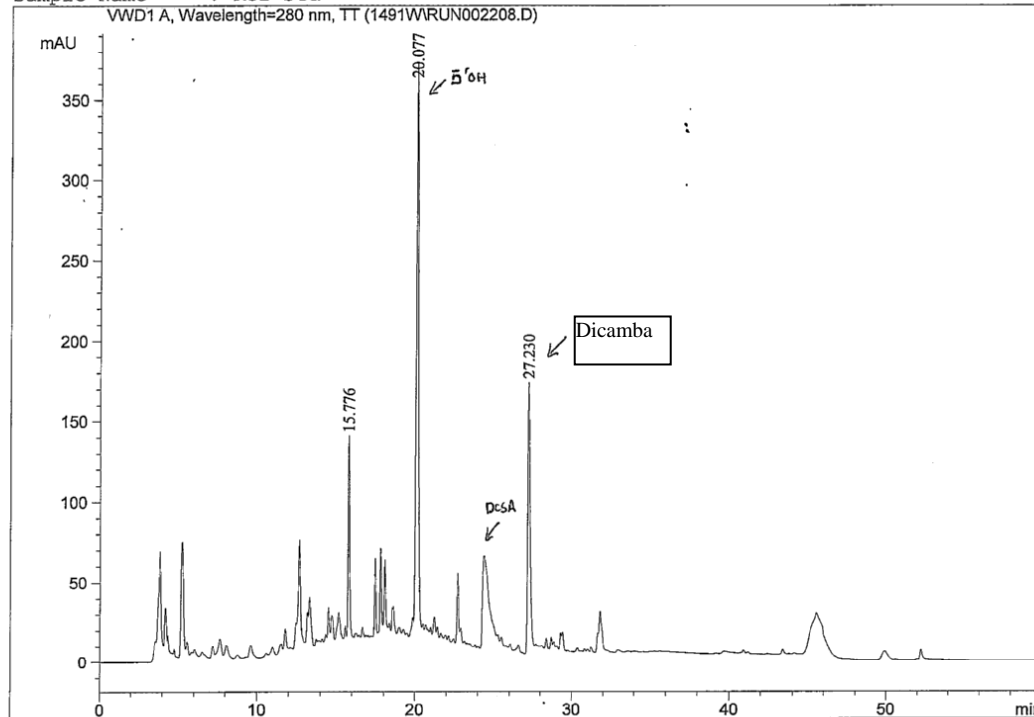
Project: 1491W HPLC #: 20 Run #: 2208
 Analysis Date: 7/28/06 NB/p: 1924/21
 Detection: Fraction Sample PPM: 122.074
 Sensitivity: 0 Max Bkg: 35
 DPM Injected: 81958 Recovery: 99.7%
 Date & Initials: *AKS* 1/16/07

Figure 11. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from POE-T Forage (HPLC Method 2). (cont.)

Data file : G:\CHEMSTATION\HPLC-20\DATA\1491->
Injection Date : Fri, 28. Jul. 2006 Acq Operator : AVB
Analysis Method : G:\CHEMSTATION\HPLC-20\METHODS\ ->
1491W 011 POE-T Forage Ext 1924/21

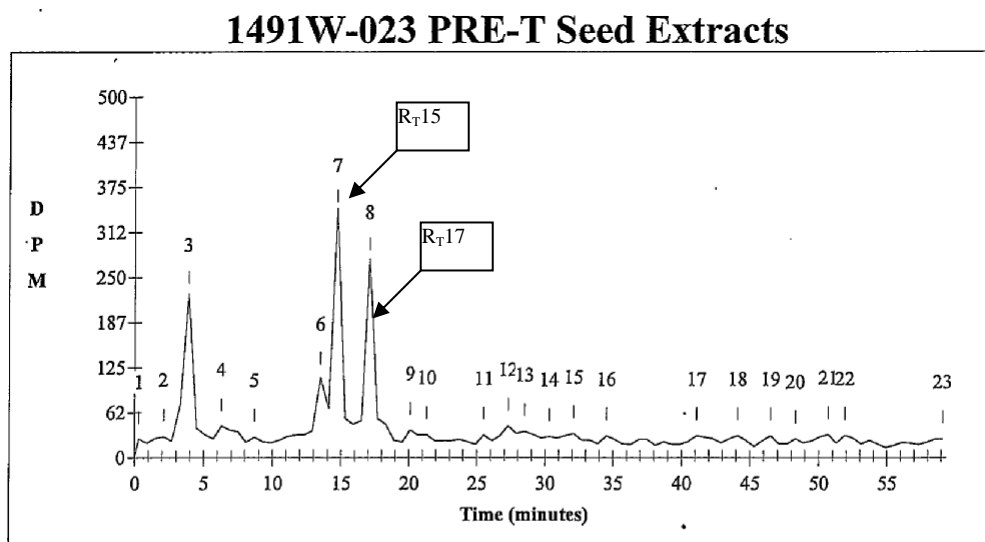
Sample Name : Ref Std

VWD1 A, Wavelength=280 nm, TT (1491WVRUN002208.D)



Peak #	RT [min]	Name	Area	Area %
1	15.78		1372.3	17.3
2	20.08		4512.0	56.7
3	27.23		2067.2	26.0

Figure 12. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from PRE-T Seed (HPLC Method 2).



Fraction Number: Fraction DPM

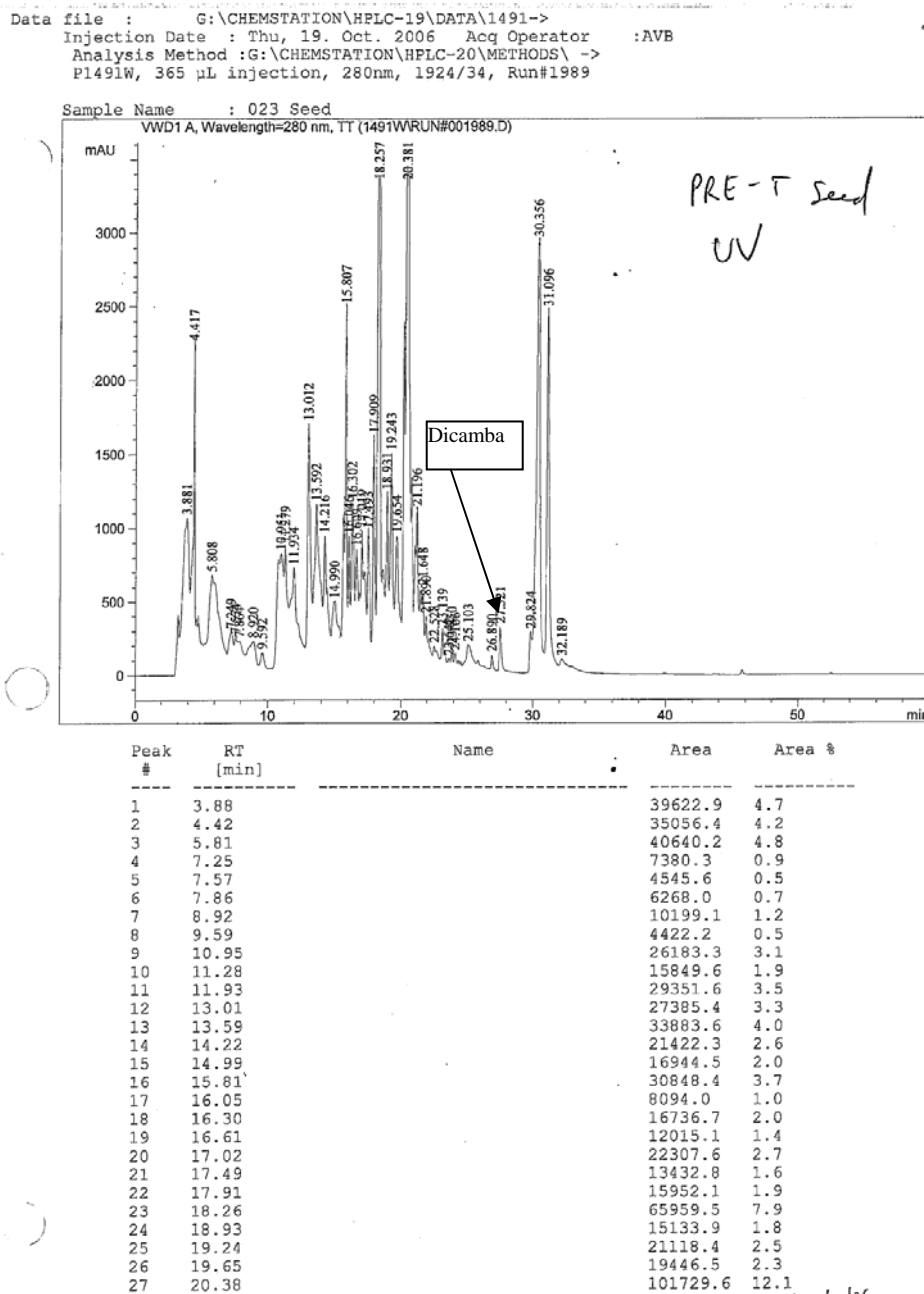
1: 26	14: 21	27: 46	40: 25	53: 30	66: 18	79: 19	92: 14
2: 20	15: 28	28: 51	41: 22	54: 33	67: 18	80: 19	93: 17
3: 27	16: 22	29: 276	42: 18	55: 24	68: 22	81: 26	94: 21
4: 29	17: 20	30: 54	43: 31	56: 24	69: 30	82: 20	95: 20
5: 23	18: 24	31: 46	44: 23	57: 19	70: 28	83: 23	96: 18
6: 74	19: 29	32: 24	45: 30	58: 30	71: 26	84: 29	97: 21
7: 228	20: 31	33: 21	46: 44	59: 25	72: 20	85: 32	98: 25
8: 41	21: 31	34: 38	47: 33	60: 19	73: 26	86: 20	99: 26
9: 32	22: 37	35: 31	48: 36	61: 18	74: 30	87: 31	
10: 26	23: 111	36: 32	49: 32	62: 25	75: 24	88: 27	
11: 44	24: 68	37: 23	50: 27	63: 25	76: 15	89: 19	
12: 38	25: 345	38: 23	51: 29	64: 17	77: 23	90: 24	
13: 36	26: 54	39: 23	52: 27	65: 22	78: 30	91: 19	

Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent	PPM	Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent	PPM
1	0.30	5	0.3%	0.001	13	28.50	36	2.3%	0.004
2	2.10	15	1.0%	0.001	14	30.30	15	1.0%	0.001
3	3.90	295	18.9%	0.029	15	32.10	25	1.6%	0.002
4	6.30	59	3.8%	0.006	16	34.50	9	0.6%	0.001
5	8.70	7	0.5%	0.001	17	41.10	22	1.4%	0.002
6	13.50	160	10.2%	0.016	18	44.10	15	1.0%	0.001
7	14.70	394	25.2%	0.039	19	46.50	9	0.6%	0.001
8	17.10	357	22.9%	0.035	20	48.30	5	0.3%	0.001
9	20.10	23	1.4%	0.002	21	50.70	20	1.3%	0.002
10	21.30	17	1.1%	0.002	22	51.90	17	1.1%	0.002
11	25.50	10	0.7%	0.001	23	59.10	5	0.3%	0.001
12	27.30	39	2.5%	0.004					

Average Background = 21 DPM.
Total Peak Area = 1562 DPM.

Project: 1491W HPLC #: 19 Run #: 1989
Analysis Date: 10/19/06 NB/p: 1924/34
Detection: Fraction Sample PPM: 0.154
Sensitivity: 0 Max Bkg: 25
DPM Injected: 1556 Recovery: 100.4%
Date & Initials: Arv 10/20/06

Figure 12. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from PRE-T Seed (HPLC Method 2). (cont.)



LC 19

Thu, 19. Oct. ->02:25:37 pm

AVB

10/19/06
Page 1 of 2
500 µL loop

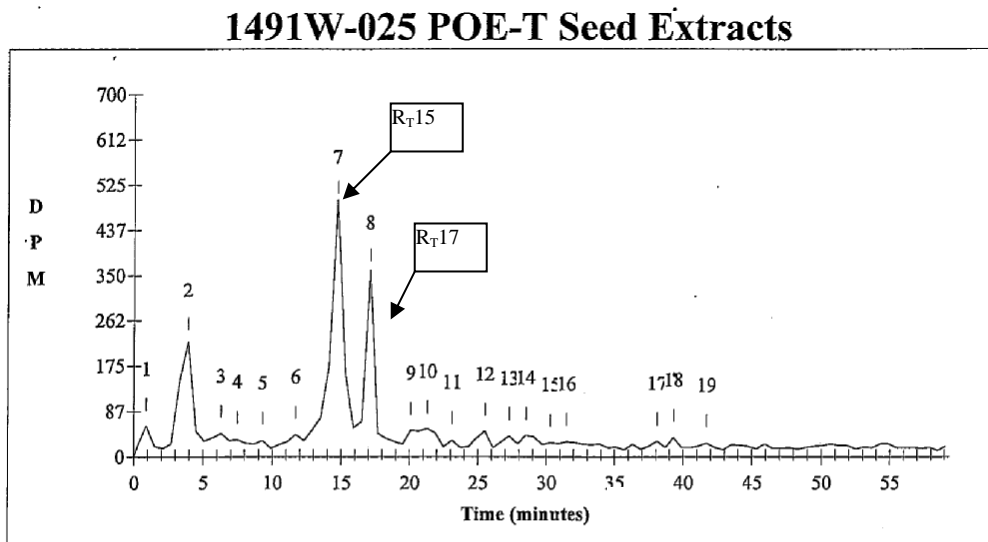
Figure 12. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from PRE-T Seed (HPLC Method 2). (cont.)

Data file : G:\CHEMSTATION\HPLC-19\DATA\1491->

Peak #	RT [min]	Name	Area	Area %
28	21.20		20086.3	2.4
29	21.65		12122.9	1.4
30	21.89		8353.3	1.0
31	22.53		4940.8	0.6
32	23.14		5275.1	0.6
33	23.64		1294.4	0.2
34	23.85		1880.1	0.2
35	24.11		2031.4	0.2
36	25.10		7744.5	0.9
37	26.89		1882.2	0.2
38	27.52		5106.4	0.6
39	29.82		3578.8	0.4
40	30.36		59532.7	7.1
41	31.10		35372.1	4.2
42	32.19		7524.0	0.9

PRE-T Seed

Figure 13. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from POE-T Seed (HPLC Method 2).



Fraction Number: Fraction DPM

1: 23	14: 26	27: 55	40: 18	53: 29	66: 37	79: 16	92: 25
2: 60	15: 24	28: 68	41: 19	54: 27	67: 18	80: 17	93: 17
3: 20	16: 31	29: 360	42: 35	55: 24	68: 17	81: 14	94: 17
4: 16	17: 16	30: 45	43: 49	56: 23	69: 20	82: 18	95: 18
5: 25	18: 23	31: 35	44: 17	57: 25	70: 26	83: 20	96: 16
6: 147	19: 29	32: 29	45: 28	58: 17	71: 18	84: 21	97: 18
7: 222	20: 43	33: 24	46: 40	59: 19	72: 13	85: 25	98: 12
8: 49	21: 31	34: 51	47: 25	60: 13	73: 23	86: 21	99: 20
9: 30	22: 51	35: 49	48: 41	61: 24	74: 22	87: 21	
10: 37	23: 75	36: 54	49: 38	62: 14	75: 20	88: 15	
11: 45	24: 172	37: 45	50: 23	63: 20	76: 15	89: 19	
12: 31	25: 496	38: 19	51: 27	64: 30	77: 24	90: 17	
13: 33	26: 160	39: 32	52: 25	65: 18	78: 16	91: 25	

Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent	PPM	Peak #	Retention Time (min)	Peak Area (DPM)	Area Percent	PPM
1	0.90	41	1.9%	0.004	11	23.10	13	0.6%	0.001
2	3.90	367	16.6%	0.038	12	25.50	47	2.1%	0.005
3	6.30	56	2.5%	0.006	13	27.30	31	1.4%	0.003
4	7.50	28	1.3%	0.003	14	28.50	42	1.9%	0.004
5	9.30	12	0.6%	0.001	15	30.30	8	0.4%	0.001
6	11.70	41	1.8%	0.004	16	31.50	19	0.8%	0.002
7	14.70	885	40.0%	0.091	17	38.10	11	0.5%	0.001
8	17.10	461	20.9%	0.048	18	39.30	18	0.8%	0.002
9	20.10	47	2.1%	0.005	19	41.70	7	0.3%	0.001
10	21.30	77	3.5%	0.008					

Average Background = 19 DPM.

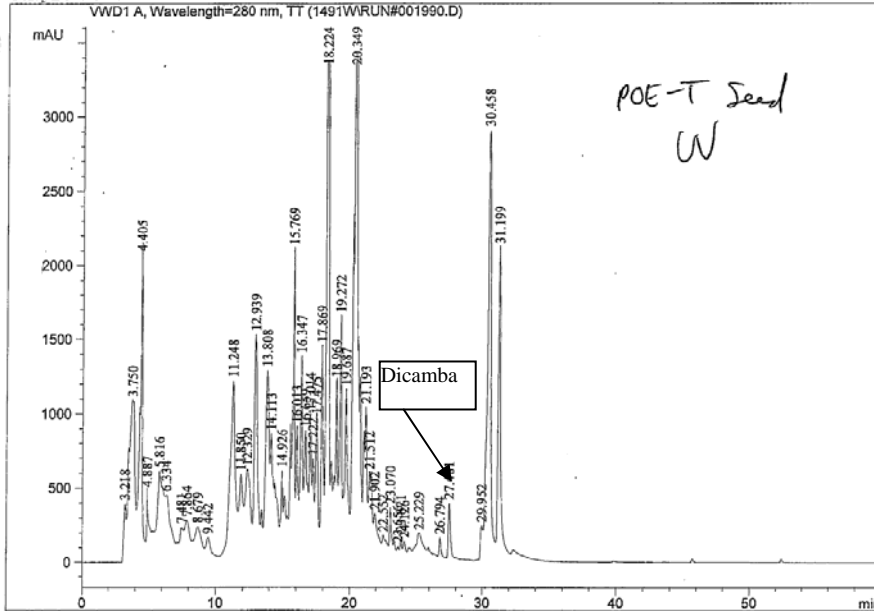
Total Peak Area = 2211 DPM.

Project: 1491W HPLC #: 19 Run #: 1990
 Analysis Date: 10/19/06 NB/p: 1924/34
 Detection: Fraction Sample PPM: 0.228
 Sensitivity: 0 Max Bkg: 25
 DPM Injected: 2578 Recovery: 85.8%
 Date & Initials: 10/24/06

Figure 13. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from POE-T Seed (HPLC Method 2). (cont.)

Data file : G:\CHEMSTATION\HPLC-19\DATA\1491->
Injection Date : Thu, 19. Oct. 2006 Acq Operator : AVB
Analysis Method : G:\CHEMSTATION\HPLC-20\METHODS\ ->
P1491W, 415 µL injection, 280nm, 1924/34, Run#1990

Sample Name : 025 Seed



Peak #	RT [min]	Name	Area	Area %
1	3.22		4438.0	0.6
2	3.75		34166.6	4.3
3	4.40		28390.6	3.6
4	4.89		7886.7	1.0
5	5.82		23132.9	2.9
6	6.33		14111.9	1.8
7	7.48		5773.0	0.7
8	7.86		8682.9	1.1
9	8.68		9331.5	1.2
10	9.44		5574.0	0.7
11	11.25		34082.2	4.3
12	11.85		12322.4	1.6
13	12.33		18123.6	2.3
14	12.94		30396.2	3.9
15	13.81		25045.7	3.2
16	14.11		22289.0	2.8
17	14.93		14458.2	1.8
18	15.77		28194.0	3.6
19	16.01		9434.2	1.2
20	16.35		16684.3	2.1
21	16.66		14026.1	1.8
22	17.01		10926.5	1.4
23	17.22		6871.3	0.9
24	17.47		13337.4	1.7
25	17.87		15104.1	1.9
26	18.22		63883.3	8.1
27	18.97		16192.3	2.1

LC 19

Thu, 19. Oct. ->03:43:33 pm

AVB

Page 1 of 2
50 µL loop

Figure 13. HPLC Radiochromatogram and UV Chromatogram of Acetonitrile/Water Extracts from POE-T Seed (HPLC Method 2). (cont.)

Data file : G:\CHEMSTATION\HPLC-19\DATA\1491-->

Peak #	RT [min]	Name	Area	Area %
28	19.27		20323.0	2.6
29	19.69		18741.8	2.4
30	20.35		97943.9	12.4
31	21.19		18751.0	2.4
32	21.51		12074.9	1.5
33	21.90		7951.0	1.0
34	22.55		4413.8	0.6
35	23.07		6239.4	0.8
36	23.66		1207.3	0.2
37	23.87		1857.6	0.2
38	24.13		2121.8	0.3
39	25.23		8228.9	1.0
40	26.79		2544.5	0.3
41	27.48		6996.6	0.9
42	29.95		3119.4	0.4
43	30.46		53489.1	6.8
44	31.20		30247.1	3.8

POE-T Seed
UV

Appendix A. Certificates of Analysis

PRE-T:

MONSANTO
imagine®



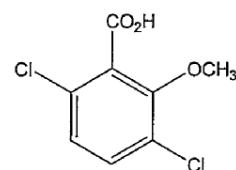
Environmental Sciences Technology Center

800 N. Lindbergh Blvd
St. Louis, MO 63167
Tel: 314-694-8269

**CERTIFICATE OF
ANALYSIS**

RADIOLABELED TEST
SUBSTANCE

Name: [Ring-U-¹⁴C]Dicamba



Chemical Name (CAS):	3,6-Dichloro-2-methoxybenzoic acid	
CAS Number:	1918-00-9 (unlabeled)	
Molecular Formula:	C ₈ H ₆ Cl ₂ O ₃	
Molecular Weight:	221.0 g/mol	
Sample Code Number:	6103-01A	
Specific Activity:	5.39 mCi/mmol	
Radiochemical Purity:	99.2% (HPLC / radioactive flow detection)	
Chemical Purity:	99.4% (HPLC / UV detection at 280 nm)	
Structure Confirmation:	Negative ion electrospray mass spectroscopy – consistent with structure	
Amount:	15.71 mCi	643.6 mg
Expiration Date:	June 23, 2006	Appearance: White solid

- Storage Conditions -

Less than 0° C (frozen)

Signature:

Michael J. Miller

Date:

May 24, 2006

POE-T:

MONSANTO
imagine



1491W-006
Updated 11/2/07

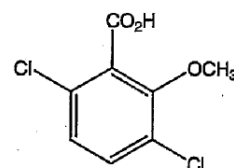
Environmental Sciences Technology Center

800 N. Lindbergh Blvd
St. Louis, MO 63167
Tel: 314-694-8269

CERTIFICATE OF ANALYSIS

RADIOLABELED TEST SUBSTANCE

Name: [Ring- ^{14}C]Dicamba



Chemical Name (CAS):	3,6-Dichloro-2-methoxybenzoic acid	
CAS Number:	1918-00-9 (unlabeled)	
Molecular Formula:	$\text{C}_8\text{H}_6\text{Cl}_2\text{O}_3$	
Molecular Weight:	221.0 g/mol	
Sample Code Number:	6103-01C	
Specific Activity:	5.43 mCi/mmol	
Radiochemical Purity:	99.5% (HPLC / radioactive flow detection)	
Chemical Purity:	98.4% (HPLC / UV detection at 280 nm)	
Structure Confirmation:	Negative ion electrospray mass spectroscopy – consistent with structure	
Amount:	17.10 mCi	695.8 mg
Expiration Date:	July 23, 2006	Appearance: White solid

- Storage Conditions -

Less than 0° C (frozen)

Signature: Michael G. Miller

Date: November 9, 2006 (revised)

*Copy of
original
rsm 5/22/06*

MONSANTO



Analytical Reference Standard Certificate of Analysis

800 N. Lindbergh Blvd.
St. Louis, MO 63167
314-694-1000

ARS Name: **Dicamba**

Class 4

Lot #: GLP-0202-12068-A

Orig. Cert. Date: 02/21/2002

Expiration Date: 02/04/2007

Purity: 99.16% Assigned by BASF Corporation

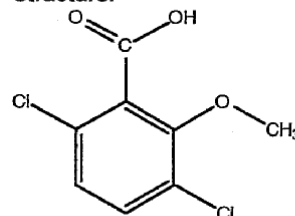
Appearance: White Granular Solid

Storage Condition: Ambient Lab Temperature

Molecular Formula: C₈H₆Cl₂O₃

Molecular Weight: 221.04

Structure:



Chemical Name: 3,6-dichloro-2-methoxybenzoic acid

Other Name(s): MON 11900

Certification Tests

H-NMR

Comments/Deviation None.

Modifications to this certification have occurred on 03/07/2002.

Data generated in the certification of this standard is in general compliance with GLPs as required by AG-PO-0522-01.

The elemental analysis was performed at a non-GLP facility

Certification Coordinator DENISE LUNDREY
(Electronic Signature)

Date: 03/07/2002

MONSANTO



Analytical Reference Standard
Certificate of Analysis
Expiration Extension

800 N. Lindbergh Blvd.
St. Louis, MO 63167
314-694-1000

ARS Name: DCSA

Class 4

Lot #: GLP-0601-16849-A

Orig. Cert. Date: 02/06/2006

Expiration Date: 02/28/2007

Purity: 95%

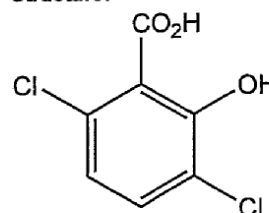
Appearance: White Crystalline Solid

Storage Condition: Refrigerate

Molecular Formula: C₇H₄Cl₂O₃

Molecular Weight: 207.01

Structure:



Chemical Name: 3,6-dichloro-2-hydroxybenzoic acid

Other Name(s):

Certification Tests

LC Area% Impurities

H-NMR

Moisture Analysis

Physical Description

Recertification Tests

H-NMR

Moisture Analysis 0.58 %

LC Area% Impurities 3.33 %

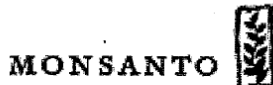
Elemental:	Theory	Found
%C	40.61	40.77
%H	1.95	1.96

Comments/Deviation None.

The (re)certification of this standard showed no significant changes since the original certification. The expiration date has been extended for any sample of the same lot, if stored at the conditions indicated. Data generated in the recertification of this standard is in general compliance with GLPs as required by AG-PO-0522-02.

Certification Coordinator Susan Horton
(Electronic Signature)

Date: 08/17/2006



Analytical Reference Standard Certificate of Analysis

800 N. Lindbergh Blvd.
St. Louis, MO 63167
314-694-1000

ARS Name: 5-Hydroxydicamba

Class 4

Lot #: GLP-0606-17478-A

Orig. Cert. Date: 07/06/2006

Expiration Date: 12/31/2006

Purity: 100%

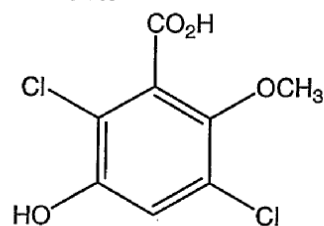
Appearance: Off-White Solid

Storage Condition: Freeze

Molecular Formula: C₈H₆Cl₂O₄

Molecular Weight: 237.04

Structure:



Chemical Name: 2,5-Dichloro-3-hydroxy-6-methoxybenzoic acid

Other Name(s):

Certification Tests

Mass Spec

Area% Impurities 0.00 %

Elemental:

Theory

Found

%C 40.54

%C 40.62

%H 2.55

%H 2.57

Comments/Deviation None.

Data generated in the certification of this standard is in general compliance with GLPs as required by AG-PO-0522-02.

The elemental analysis was performed at a non-GLP facility

Certification Coordinator

Susan Horton

(Electronic Signature)

Date: 07/06/2006

Appendix B. Preparation of Application Solutions.

PRE-T application solution preparation calculations:

1) Amount of ¹⁴C Dicamba Available:

¹⁴ C-Dicamba	15.71 mCi
	5.39 mCi/mmol
	2.91 mmol
	221.0 mg/mmol
	643.6 mg Dicamba available (from COA)

2) Amount of DGA required to prepare DGA solution:

2.912 mmol Dicamba
3.058 1.05 x equivalent mmol
105.14 mg/mmol DGA
321.5 mg DGA to be added

3) Amount of DGA solution to add to ¹⁴C Dicamba:

	643.6 mg Dicamba available
Target concentration:	20.45 mg Dicamba/10 mL DGA Solution
	314.7 mL DGA Solution to be added

4) Determination of exact ¹⁴C Dicamba concentration:

	2,705,534 Mean Net DPM determined by LSC of six aliquots
	0.025 Aliquot Volume (mL)
	108,221,360 DPM/mL
Dicamba Spec. Act.:	54,190,000 DPM/mg ((15.71 mCi x 2.22E9 DPM/mCi)/643.6 mg)
	1.997 mg/mL in DGA solution
Target concentration:	20.45 mg Dicamba per plot
	10.24 mL per plot to be applied

POE-T application solution preparation calculations:

1) Amount of ¹⁴C Dicamba Available:

14C-Dicamba	17.1 mCi
	5.43 mCi/mmol
	3.15 mmol
	221.0 mg/mmol
	695.8 mg Dicamba available (from COA)

2) Amount of DGA required to prepare DGA solution:

3.1484 mmol Dicamba
3.306 1.05 x equivalent mmol
105.14 mg/mmol DGA
347.6 mg DGA to be added

3) Amount of DGA solution to add to ¹⁴C Dicamba:

	695.8 mg Dicamba available
Target concentration:	20.45 mg Dicamba/10 mL DGA Solution
	340.2 mL DGA Solution to be added

4) Determination of exact ¹⁴C Dicamba concentration:

	2,817,098 Mean Net DPM determined by LSC of six aliquots
	0.025 Aliquot Volume (mL)
	112,683,920 DPM/mL
Dicamba Spec. Act.:	54,560,000 DPM/mg ((17.1 mCi x 2.22E9 DPM/mCi)/695.8 mg)
	2.065 mg/mL in DGA solution
Target concentration:	20.45 mg Dicamba per plot
	9.90 mL per plot to be applied

Appendix C. Calculation of Application Rates.

1491W: PRE Achieved Application Rate

Part I: Maximum mCi Available in Bottles:

10.40 mL/bottle
108,221,360 dpm/mL
1,125,502,144 Total dpm/bottle
0.5070 Total mCi/bottle
30 bottles
15.21 Total mCi

Part II: Residual mCi in Bottles and Sprayer:

Bottle#	Residual Mean Net DPM	Rinse Vol. (mL)	Aliquot Vol. (mL)	Rinse DPM	Rinse mCi
1	641,609	10	1	6,416,090	0.003
2	665,479	10	1	6,654,790	0.003
3	818,307	10	1	8,183,070	0.004
4	817,137	10	1	8,171,370	0.004
5	632,834	10	1	6,328,340	0.003
6	930,582	10	1	9,305,820	0.004
7	1,311,744	10	1	13,117,440	0.006
8	700,534	10	1	7,005,340	0.003
9	946,321	10	1	9,463,210	0.004
10	611,261	10	1	6,112,610	0.003
11	850,933	10	1	8,509,330	0.004
12	666,122	10	1	6,661,220	0.003
13	700,864	10	1	7,008,640	0.003
14	720,827	10	1	7,208,270	0.003
15	922,291	10	1	9,222,910	0.004
16	790,754	10	1	7,907,540	0.004
17	765,408	10	1	7,654,080	0.003
18	685,353	10	1	6,853,530	0.003
19	768,958	10	1	7,689,580	0.003
20	950,576	10	1	9,505,760	0.004
21	664,098	10	1	6,640,980	0.003
22	794,864	10	1	7,948,640	0.004
23	770,987	10	1	7,709,870	0.003
24	1,122,628	10	1	11,226,280	0.005
25	663,569	10	1	6,635,690	0.003
26	700,468	10	1	7,004,680	0.003
27	502,859	10	1	5,028,590	0.002
28	721,980	10	1	7,219,800	0.003
29	676,984	10	1	6,769,840	0.003
30 (primer)	519,428	10	1	5,194,280	0.002
31 (sprayate)	750,106	50	0.05	750,106,000	0.338
Total in Rinses:				980,463,590	0.442

Part III: Actual mCi Applied and Achieved Application Rate:

15.21 Total mCi in Bottles

0.44 Total mCi in Rinses

14.77 Total mCi Applied

32,789,400,000 Total DPM Applied

54,190,000 Specific Activity (DPM/mg)

605.1 Total mg Applied

29 Plots (28 + 1)

20.87 mg Applied/plot

20.45 mg/plot Target Rate

102.1% Achieved Application Rate (Percent of Target)

2.55 Achieved Application Rate (lb/ac)

1491W: POE Achieved Application Rate

Part I: Maximum mCi Available in Bottles:

9.90 mL/bottle
112,683,900 dpm/mL
1,115,571,000 Total dpm/bottle
0.5025 Total mCi/bottle
33 bottles
16.58 Total mCi

Part II: Residual mCi in Bottles and Sprayer:

Bottle#	Residual Mean Net DPM	Rinse Vol. (mL)	Aliquot Vol. (mL)	Rinse DPM	Rinse mCi
1	998,092	10	1	9,980,920	0.004
2	554,100	10	1	5,541,000	0.002
3	471,795	10	1	4,717,950	0.002
4	459,307	10	1	4,593,070	0.002
5	416,971	10	1	4,169,710	0.002
6	564,765	10	1	5,647,650	0.003
7	539,929	10	1	5,399,290	0.002
8	450,538	10	1	4,505,380	0.002
9	533,489	10	1	5,334,890	0.002
10	482,805	10	1	4,828,050	0.002
11	490,973	10	1	4,909,730	0.002
12	384,870	10	1	3,848,700	0.002
13	494,418	10	1	4,944,180	0.002
14	732,978	10	1	7,329,780	0.003
15	536,084	10	1	5,360,840	0.002
16	601,457	10	1	6,014,570	0.003
17	779,955	10	1	7,799,550	0.004
18	860,363	10	1	8,603,630	0.004
19	363,310	10	1	3,633,100	0.002
20	410,167	10	1	4,101,670	0.002
21	514,586	10	1	5,145,860	0.002
22	899,825	10	1	8,998,250	0.004
23	874,620	10	1	8,746,200	0.004
24	530,215	10	1	5,302,150	0.002
25	634,743	10	1	6,347,430	0.003
26	784,451	10	1	7,844,510	0.004
27	683,976	10	1	6,839,760	0.003
28	1,027,936	10	1	10,279,360	0.005
29	973,210	10	1	9,732,100	0.004
30	739,887	10	1	7,398,870	0.003
31	739,653	10	1	7,396,530	0.003
32	655,545	10	1	6,555,450	0.003
33 (primer)	894,707	10	1	8,947,070	0.004
34 (sprayate)	587,081	50	0.05	587,081,000	0.264
Total in Rinses:				797,878,200	0.359

Part III: Actual mCi Applied and Achieved Application Rate:

16.58 Total mCi in Bottles

0.36 Total mCi in Rinses

16.22 Total mCi Applied

36,008,400,000 Total DPM Applied

54,560,000 Specific Activity (DPM/mg)

660.0 Total mg Applied

32 Plots

20.63 mg Applied/plot

20.45 mg/plot Target Rate

100.9% Achieved Application Rate (Percent of Target)

2.52 Achieved Application Rate (lb/ac)

Appendix D. PTRL West, Inc. Radiochromatograph Program.

PTRL Radiochromatogram Program.

This program, currently written to be compatible with Windows[®] based system computers, is designed for the entry, integration, and reporting of radiochromatographic data derived from HPLC with liquid scintillation counting (LSC) of collected fractions.

The program allows for manual entry of DPM values for each fraction collected during an HPLC analysis or for direct import of files generated from the RS232 port of the Beckman LSC using an LSC Data Capture Program. Data can be edited and displayed to the computer screen reconstructed as a chromatographic trace.

This data is integrated automatically as described below:

1. Requests a maximum background threshold (in DPM).
2. Selects all points with DPM values less than the maximum background threshold as final background values, and all other points as peak values.
3. Calculates final background mean.
4. Determines, from all non-background (i.e., peak) values, the starts and ends of peaks based on valleys or intervening background values.
5. Sums counts over each peak, background corrects peak DPM-areas, calculates total peak DPM-area, calculates peak percentages of total peak DPM, and reports these values.
6. Calculation of HPLC column recoveries from the calculated total peak DPM-area (see 5 above).

1. Aliquots of the injected sample are counted by LSC and entered at the prompt to determine applied DPM.
2. Calculation:

$$\frac{\text{Total Peak Area}}{\text{Applied DPM}} \times 100 = \% \text{ Recovered DPM}$$

Appendix E. TRR Spreadsheets for Pre-Forage.

Total Radioactive Residue Determination

UNT-C Pre-Forage

Project:1491W

Test Substance: Dicamba

Specific Activity (dpm/μg):			54,190		Total Weight (g):			26.1		Sample TRR (ppm):		0.000	
Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)				
1491W-004	1	26.1	39	0.9633	39	0	0	0	0.000				
UNT-C	2	0.262	71			0							
Pre-Forage	3	0.260	34			0							
	4	0.260	65			0							
	5	0.263	48			0							

* These values were corrected for oxidizer efficiency. Gross dpm <2x BKG considered = 0.

* These values were corrected for oxidizer efficiency. Gross dpm <2x BKG considered = 0.

Oxidizer efficiency for Combustions done on Oxidizer #5

	14C		oxidizer efficiency
	cell	standard	
GG			
1924/9	12,810	13,338	28
6/28/2006	12,726	13,168	30
			39
bkg. subtracted=	12,771	13,299	41
	12,687	13,129	30
			28
			58
			54
average=	12,729	13,214	39

Total Radioactive Residue Determination

PRE-T Pre-Forage

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g):		54,190		Total Weight (g):		97.8		Sample TRR (ppm):		3.248	
Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)		
1491W-005	1	97.8	0.257	43,728	0.9633	39	176,473	176,020	3.248		
PRE-T	2	0.260	42,979				171,446	3%			
Pre- Forage	3	0.255	41,836				170,154				
	4	0.256	43,858				177,689				
	5	0.250	44,432				184,337				

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

GG	14C		BKG	oxidizer efficiency
	cell	standard		
1924/9	12,810	13,338	28	0.9633
6/28/2006	12,726	13,168	30	
			39	
bkg. subtracted=	12,771	13,299	41	
	12,687	13,129	30	
			28	
			58	
			54	
average=	12,729	13,214	39	

Appendix F. TRR Spreadsheets for Forage.

Total Radioactive Residue Determination

UNT-C Forage

Project:1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g):		54,190		Total Weight (g):		109.1		Sample TRR (ppm):		0.000	
Rep	Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)		
1491W-007	1	109.1	0.254	68	0.9681	46	0	0	0.000		
UNT-C	2		0.256	66			0				
Forage	3		0.253	71			0				
	4		0.258	64							
	5		0.251	79							

* These values were corrected for oxidizer efficiency. Gross dpm <2x BKG considered = 0.

Oxidizer efficiency for Combustions done on Oxidizer #5

GG	14C		BKG	oxidizer efficiency
	cell.	standard		
1924/14, 17	13,520	14,131	34	0.9681
7/18/2006	13,655	13,937	40	
			84	
bkg. subtracted=	13,474	14,085	63	
	13,609	13,891	36	
			43	
			36	
average=	13,542	13,988	46	

Total Radioactive Residue Determination

PRE-C Forage

Project:1491W

Test Substance: Dicamba

Specific Activity (dpm/μg) :				54,190		Total Weight (g):			294.3		Sample TRR (ppm):		0.080	
	Rep	Total		Aliquot	Gross	Oxidizer	Oxidizer	Net	dpm / g (RSD)	Total	Radioactive			
		Weight (g)	Wt. (g)									dpm	Efficiency	dpm (BKG)
1491W-008 PRE-C Forage	1	294.3	0.251	1,181	0.9741	41	4,663	4,333	1,275,202	0.080				
	2		0.256	1,151			4,451	4%						
	3		0.251	1,174			4,634							
	4		0.253	1,096	0.9681	46	4,287							
	5		0.255	1,105			4,290							
	6		0.251	996			3,910							
	7		0.258	1,069			4,096							

* These values were corrected for oxidizer efficiency. Gross dpm <2x BKG considered = 0.

Total Radioactive Residue Determination

PRE-C Forage

Project 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g):		54,190	Total Weight (g):		294.3	Sample TRR (ppm):		0.080
Oxidizer efficiency for Reps 1-3 Oxidizer #5:								

GG	14C		BKG	oxidizer efficiency
	cell.	standard		
1924/14, 17	13,746	13,989	36	0.9741
7/17/2006	13,574	14,055	33	
bkg. subtracted=	13,705	13,948	41	
	13,533	14,014	36	
			35	
			58	

average= 13,619 13,981 41

Oxidizer efficiency for Reps 4-7 Oxidizer #5:

GG	14C		BKG	oxidizer efficiency
	cell.	standard		
1924/14, 17	13,520	14,131	34	0.9681
7/18/2006	13,655	13,937	40	
bkg. subtracted=	13,474	14,085	84	
	13,609	13,891	63	
			36	
			43	
average=	13,542	13,988	35	
			46	

Total Radioactive Residue Determination

POE-C Forage

Project:1491W

Test Substance: Dicamba

Specific Activity (dpm/ug) :		54,560		Total Weight (g):		153.8		Sample TRR (ppm):		0.280	
Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BK.G)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)		
1491W-010	1	153.8	0.252	3,554	0.9681	46	14,379	15,285	2,350,833		0.280
POE-C	2		0.257	3,841			15,253	4%			
Forage	3		0.250	3,924			16,023				
	4		0.255	3,966			15,879				
	5		0.255	3,722			14,891				

* These values were corrected for oxidizer efficiency. Gross dpm <2x BK.G considered = 0.

Oxidizer efficiency for Combustions done on Oxidizer #5

GG	14C		14C		oxidizer	
	cell	standard	BK.G	efficiency		
1924/14, 17	13,520	14,131	34	0.9681		
7/18/2006	13,655	13,937	40			
			84			
bkg subtracted=	13,474	14,085	63			
	13,609	13,891	36			
			43			
			36			
average=	13,542	13,988	46			

Total Radioactive Residue Determination

PRE-T Forage

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g) :			Total Weight (g):			Sample TRR (ppm):		
54,190			302.7			1.433		
Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BK.G)	Net dpm/g*	dpm / g (RSD)	Radioactive Residue (ppm)
1491W-009	1	302.7	20,598	0.9741	41	82,436	77,635	1.433
PRE-T	2	0.255	19,141			76,894	4%	
Forage	3	0.252	18,438			74,945		
	4	0.253	18,778			76,028		
	5	0.252	19,156			77,870		

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

GG	14C		oxidizer	
	cell	standard	BKG	efficiency
1924/14	13,746	13,989	36	0.9741
7/17/2006	13,574	14,055	33	
			48	
bkg. subtracted=	13,705	13,948	41	
	13,533	14,014	36	
			35	
			58	
average=	13,619	13,981	41	

Total Radioactive Residue Determination

POE-T Forage

Project 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g) :				54,560		Total Weight (g):		151.4		Sample TRR (ppm):		134,147	
	Rep	Total Weight (g)		Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)		
1491W-011 POE-T Forage	1	151.4	0.252	1,865,275	0.9741	41	7,598,524	7,319,053	3%	1,108,104,624	134,147		
	2		0.253	1,836,903			7,453,366						
	3		0.258	1,757,328			6,992,290						
	4		0.252	1,763,872			7,185,432						
	5		0.257	1,843,986			7,365,654						

* These values were corrected for oxidizer efficiency

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

GG	14C		14C		oxidizer efficiency
	cell	standard	BKG	efficiency	
1924/14	13,746	13,989	36	0.9741	
7/17/2006	13,574	14,055	33		
			48		
blg. subtracted=	13,705	13,948	41		
	13,533	14,014	36		
			35		
			58		
average=	13,619	13,981	41		

Appendix G. TRR Spreadsheets for Forage from Select Individual POE-T Plants.

Total Radioactive Residue Determination
POE-T Washed Forage Plant 28

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g) :		54,560	Total Weight (g):	29.1	Sample TRR (ppm):	123.084
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RINSE DATA:

1491W-015

POE-T Plant 28	Rep	Rinse		Aliquot Vol. (mL)	Aliquot Net DPM	Aliquot DPM/mL	Rinse DPM
		Vol. (mL)	Vol. (mL)				
Runse 1	1	1,000	1,000	1,000	57,394	57,855	57,855,000
	2			1,000	58,102		
	3			1,000	58,068		
Runse 2	1	1,000	1,000	1,000	5,842	6,027	6,027,000
	2			1,000	6,086		
	3			1,000	6,152		
Runse 3	1	1,000	1,000	1,000	1,662	1,686	1,686,000
	2			1,000	1,659		
	3			1,000	1,736		

Total DPM in All Rinses: Rinse PPM: 41.298

COMBUSTION DATA:

	Rep	Total		Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BK.G)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)
		Weight (g)									
1491W-015 POE-T Plant 28	1	29.1	0.107	444,089	0.9611	38	4,317,979	4,462,217	6%	129,850,515	81.786
	2		0.098	422,035			4,480,379				
	3		0.098	448,137			4,757,506				
	4		0.105	413,020			4,092,354				
	5		0.102	457,149			4,662,866				

* These values were corrected for oxidizer efficiency

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5				
AVB	14C		BKG	oxidizer efficiency
	cell.	standard		
1924/25	12.695	12.999	33	0.9611
8/7/2006	12.289	12.992	31	
bkg. subtracted=	12.657	12.961	46	
			38	
	12.251	12.954	33	
			49	
average=				
	12.454	12.958	38	

Total Radioactive Residue Determination
POE-T Washed Forage Plant 13

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g):	54,560	Total Weight (g):	24.3	Sample TRR (ppm):	183,169
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RINSE DATA:

1491W-014					
POE-T					
Plant 13					
Rep	Rinse Vol. (mL)	Aliquot Vol. (mL)	Aliquot Net DPM	Aliquot DPM/mL	Rinse DPM
1	1,000	1,000	75,434	74,492	74,492,000
2		1,000	73,764		
3		1,000	74,279		
Rinse 2					
1	1,000	1,000	2,916	2,865	2,865,000
2		1,000	2,793		
3		1,000	2,887		
Rinse 3					
1	1,000	1,000	712	703	703,000
2		1,000	719		
3		1,000	677		
Total DPM in All Rinses:				78,060,000	Rinse PPM: 58.877

COMBUSTION DATA:

	Rep	Total Weight (g)		Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)
		1	2								
1491W-014	1	24.3		0.176	1,200,291	0.9722	71	7,014,433	6,781,376	164,787,437	124,292
POE-T	2			0.164	1,008,052			6,321,977	5%		
Plant 13	3			0.197	1,269,011			6,625,509			
	4			0.181	1,250,280			7,104,744			
	5			0.192	1,276,882			6,840,215			

* These values were corrected for oxidizer efficiency

Total Radioactive Residue Determination
POE-T Washed Forage Plant 13
Project: 1491W
Test Substance: Dicamba

Specific Activity (dpm/μg) :		54,560	Total Weight (g):		24.3	Sample TRR (ppm):	183,169
Oxidizer efficiency for Combustions done on Oxidizer #5							
AVB	1924/24 8/2/2006	14C		BKG	oxidizer efficiency		
		cell.	standard				
		15,360	15,724	44	0.9722		
		15,247	15,753	37			
				83			
bkg. subtracted=		15,289	15,653	82			
		15,176	15,682	56			
				44			
				114			
				72			
				141			
				40			
average=		15,233	15,668	71			

Appendix H. TRR Spreadsheets for Hay.

Total Radioactive Residue Determination

UNT-C Soybean Hay

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g) :		Total Weight (g) :		Sample TRR (ppm):		0.001			
Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BK.G)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)
1491W-018	1	289.8	0.246	90	0.9692	60	49	14,200	0.001
UNT-C	2	0.256	93			0	22.4%		
Soybean Hay	3	0.249	59			0			
	4	0.243	79			0			
	5	0.257	121			245			

* These values were corrected for oxidizer efficiency. Gross dpm <2x BK.G considered = 0.

Oxidizer efficiency for Combustions done on Oxidizer #5

AVB	14C		BKG	oxidizer		14C	efficiency
	cell	standard		efficiency	in hay		
1924/28	11,973	12,330	78	0.9692	12,688		0.9891
8/8/2006	12,361	12,770	55		12,629		
	12,443	12,641	35		12,327		
	12,305	12,893	56		12,445		
			44				
blk. subtracted=	11,913	12,270	56		12,628		
	12,301	12,710	44		12,569		
	12,383	12,581	94		12,267		
	12,245	12,833	69		12,385		
			70				
average=	12,211	12,599	60		12,462		

Total Radioactive Residue Determination

PRE-T Soybean Hay

Project:1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g) :		Total Weight (g):		Sample TRR (ppm):	
54,190		995.7		1.056	

Rep	Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BK/G)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)
1491W-019	1	995.7	15,196	0.9692	60	60,767	57,229	56,982,915	1.056
PRE-T	2	0.267	14,587			56,137	3%		
Soybean Hay	3	0.249	13,634			56,246			
	4	0.248	13,717			56,819			
	5	0.255	13,944			56,177			

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

AVB	14C		14C		oxidizer efficiency
	cell.	standard	BKG		
1924/28	11,973	12,330	78		0.9692
8/8/2006	12,361	12,770	55		
	12,443	12,641	35		
	12,305	12,893	56		
			44		
bkg. subtracted=	11,913	12,270	56		
	12,301	12,710	44		
	12,383	12,581	94		
	12,245	12,833	69		
average=	12,211	12,599	60		

Total Radioactive Residue Determination

POE-T Soybean Hay

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g) :			54,560		Total Weight (g):			622.9		Sample TRR (ppm):		39,149	
	Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)			
1491W-020	1	622.9	0.254	514,670	0.9692	60	2,090,408	2,135,966	1,330,493,221	39,149			
	2		0.249	493,298			2,043,825	6%					
Soybean Hay	3		0.245	543,722			2,289,547						
	4		0.248	485,312			2,018,842						
	5		0.253	548,641			2,237,210						
* These values were corrected for oxidizer efficiency													

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

AVB	14C		14C		oxidizer efficiency
	cell	standard	BKG		
1924/28	11,973	12,330	78		0.9692
8/8/2006	12,361	12,770	55		
	12,443	12,641	35		
	12,305	12,893	56		
			44		
blk. subtracted=	11,913	12,270	56		
	12,301	12,710	44		
	12,383	12,581	94		
	12,245	12,833	69		
average=	12,211	12,599	60		

Appendix I. TRR Spreadsheets for Seed

Total Radioactive Residue Determination

UNT-C Soybean Seed

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/ μ g) :			Total Weight (g) :			Sample TRR (ppm) :			
54,190			96.2			0.013			
Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)
1491W-021	1	96.2	113	0.9615	36	748	723	69,553	0.013
UNT-C	2	0.106	105			677	11%		
Soybean Seed	3	0.103	105			697			
	4	0.105	101			644			
	5	0.104	121			850			

* These values were corrected for oxidizer efficiency. Gross dpm <2x BKG considered = 0.

Oxidizer efficiency for Combinations done on Oxidizer #5

AV/B	14C		BKG	oxidizer efficiency	seed aliquot		efficiency in seed
	cell	standard			1	2	
1924/31	13,858	14,319	41	0.9615	25 mg 13,932	13,438	0.9380
9/29/2006	13,678	14,127	52		50 mg 13,783	13,488	
	13,945	14,644	36		100 mg 13,156	13,357	
	13,892	14,495	36		250 mg 13,516	13,377	
			36				
bkg. subtracted=	13,822	14,283	33		25 mg 13,896	13,402	
	13,642	14,091	39		50 mg 13,747	13,452	
	13,909	14,608	35		100 mg 13,120	13,321	
	13,856	14,459	30		250 mg 13,480	13,341	
			34				
			30				
average=			30				
			37				
			40				
	13,807	14,360	36			13,470	

Total Radioactive Residue Determination

PRE-C Soybean Seed

Project: 1491W

Test Substance: Dicamba:

Specific Activity (dpm/μg) :		54,190		Total Weight (g):		111.0		Sample TRR (ppm):		0.170	
Rep	Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BK.G)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)		
1491W-022	1	111.0	918	0.9615	36	9,457	9,196	1,020,756	0.170		
PRE-C	2	0.105	947			9,024	3%				
Soybean Seed	3	0.102	908			8,891					
	4	0.097	919			9,468					
	5	0.099	906			9,140					

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

AVB	14C		14C		oxidizer	
	cell	standard	BKG	efficiency		
1924/31	13,858	14,319	41	0.9615		
9/29/2006	13,678	14,127	52			
	13,945	14,644	36			
	13,892	14,495	36			
			36			
bkg. subtracted=	13,822	14,283	33			
	13,642	14,091	39			
	13,909	14,608	35			
	13,856	14,459	30			
			34			
			30			
			30			
			37			
			40			
average=	13,807	14,360	36			

Total Radioactive Residue Determination

POE-C Soybean Seed

Project: 1491W

Test Substance: Dicamb.

Specific Activity (dpm/μg):		54,560		Total Weight (g):		81.0		Sample TRR (ppm):		0.138	
Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)		
1491W-024	1	81.0	0.101	755	0.9615	36	7,404	611,064	0.138		
POE-C	2	0.100	752				7,447				
Soybean Seed	3	0.098	693				6,973				
	4	0.105	821				7,776				
	5	0.105	856				8,122				

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

14C cell		14C standard		oxidizer efficiency	
AVB	13,858	14,319	41	0.9615	
1924/31	13,678	14,127	52		
9/29/2006	13,945	14,644	36		
	13,892	14,495	36		
			36		
blk. subtracted=	13,822	14,283	33		
	13,642	14,091	39		
	13,909	14,608	35		
	13,856	14,459	30		
			34		
			30		
			30		
			37		
			40		
average=	13,807	14,360	36		

Total Radioactive Residue Determination

PRE-T Soybean Seed

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/μg):		Total Weight (g):		Sample TRR (ppm):					
54.190		450.3		0.291					
Rep	Total Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BKG)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)

1491W-023	1	450.3	0.104	1,530	0.9615	36	14,941	15,781	7,106,184	0.291
PRE-T	2		0.101	1,555			15,642	5%		
Soybean Seed	3		0.102	1,717			17,140			
	4		0.108	1,654			15,581			
	5		0.108	1,656			15,601			

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

AVB	14C		14C		oxidizer	
	cell.	standard	BKG	efficiency		
1924/31	13,858	14,319	41	0.9615		
9/29/2006	13,678	14,127	52			
	13,945	14,644	36			
	13,892	14,495	36			
			36			
bkg. subtracted=	13,822	14,283	33			
	13,642	14,091	39			
	13,909	14,608	35			
	13,856	14,459	30			
			34			
			30			
			30			
			37			
			40			

average= 13.807 14.360 36

Total Radioactive Residue Determination

POE-T Soybean Seed

Project: 1491W

Test Substance: Dicamba

Specific Activity (dpm/μg) : 54,560 Total Weight (g): 369.6 Sample TRR (ppm): 0.389

Rep	Weight (g)	Aliquot Wt. (g)	Gross dpm	Oxidizer Efficiency	Oxidizer dpm (BK G)	Net dpm/g*	dpm / g (RSD)	Total dpm	Radioactive Residue (ppm)
1491W-025	1	369.6	2,061	0.9615	36	20,251	21,244	7,851,782	0.389
POE-T	2	0.109	2,565			24,131	8%		
Soybean Seed	3	0.105	2,076			20,207			
	4	0.108	2,143			20,290			
	5	0.104	2,170			21,341			

* These values were corrected for oxidizer efficiency

Oxidizer efficiency for Combustions done on Oxidizer #5

AVB	14C cell	14C standard	BKG	oxidizer efficiency
1924/31	13,858	14,319	41	0.9615
9/29/2006	13,678	14,127	52	
	13,945	14,644	36	
	13,892	14,495	36	
			36	
blk. subtracted=	13,822	14,283	33	
	13,642	14,091	39	
	13,909	14,608	35	
	13,856	14,459	30	
			34	
			30	
			30	
			37	
			40	
average=	13,807	14,360	36	

Appendix J. Extraction Spreadsheets for Forage.

1491W: PRE-T Forage Extraction
PTRL ID: 1491W-009

Weight of		Specific Activity	Concentration of							
Sample Extracted (g)		(dpm/ μ g)	¹⁴ C in Sample (dpm/g)		(ppm)	TRR				
9.819		54,190	77,635		1,433	1,433				
Extracted with Acetonitrile/Water 2/3 v/v										
#	Available	Aliquot		Extract	Mean					
		DPM	Weight (g)		Net DPM	DPM/g (RSD)	Percent Extracted	PPM Extracted	% of TRR	
1	762,298	0.521	38.472	5,861	11,350	57.3%	0.821	57.3%		
2	762,298	0.504	45.631	5,701	3,146	18.8%	0.269	18.8%		
3	762,298	0.502	48.338	5,770	777	4.9%	0.070	4.9%		
4	762,298	0.501	45.592	1,574	106	0.6%	0.009	0.6%		
		0.501		1,572	8%					
		0.503		1,589	2%					
Totals:		622,604	81.7%	1.171	81.7%					

1491W: PRE-T Forage Extraction

PTRL ID: 1491W-009

Post Extraction Solid Combustion #1

Oxidizer Efficiency	BKG DPM	Gross DPM	Adjusted DPM	Aliquot Weight (g)	Mean		Total DPM	Percent Inextracted	PPM Inextracted	% of TRR
					DPM/g	RSD				
0.9722	71	1,304	1,268	0.212	5,981	5,880	42,536	5.6%	0.080	5.6%
PES(g) =	7.234	1,319	1,284	0.240	5,350	8.30				
		1,494	1,464	0.232	6,310					
Background and Efficiency										
AVB	Bkg									
1924/24	44									
8/1/2006	37	Combustion Efficiency		14C	14C	Oxidizer Efficiency				
	83	Cellulose	15,360	Cocktail	15,724	0.9722				
	82		15,247		15,753					
	56									
	44	Bkg Subtracted	15,289		15,653					
	114		15,176		15,682					
	72	Average	15,233		15,668					
	141									
	40									
Average	71									
Total Extracted + Unextracted = 87.3%										
1.251										
87.3%										

1491W: POE-T Forage Extraction

PTRL ID: 1491W-011

Weight of		Specific Activity		Concentration of					
Sample Extracted (g)		(dpm/μg)		¹⁴ C in Sample (dpm/g)		(ppm)		TRR	
9.879		54,560		7,319,053		134,147		134,147	
Extracted with Acetonitrile/Water 2/3 v/v									
#	Available	Aliquot		Extract	Net	Mean		Percent	PPM
		DPM	Weight			DPM/g	DPM		
			(g)	Wt. (g)	DPM	(RSD)	46,114,190	63.8%	85,586
AVB	1	72,304,925	0.051	38.597	60,232	1,194,761	3%		63.8%
1924/29			0.051		61,177				
7/27/2006			0.053		63,779				
	2	72,304,925	0.050	46.630	16,530	322,136	15,021,202	20.8%	27,903
			0.052		16,157	2%			20.8%
			0.052		16,922				
3	72,304,925	0.050		46.761	3,952	78,248	3,658,955	5.1%	6,841
		0.054			4,229	3%			5.1%
		0.053			4,104				
4	72,304,925	0.050		47.953	1,052	21,135	1,013,487	1.4%	1,878
		0.052			1,093	5%			1.4%
		0.054			1,152				
Totals:						65,807,834	91.0%	122,074	91.0%

Oxidizer	BKG	Gross	Aliquot		Mean	Total	Percent	PPM	% of TRR
			Adjusted	Weight					
Efficiency	DPM	DPM	DPM	(g)	DPM/g	DPM	Unextracted	Unextracted	
0.9722	71	80,908	83,149	0.191	435,335	427,513	2,614,242	3.6%	4.829
PES (g) =	6.115	76,601	78,718	0.187	420,952	1.70			
		85,852	88,234	0.207	426,251				
Background and Efficiency									
AVB	Bkg								
1924/24	44								
8/1/2006	37								
	83								
	82								
	56								
	44								
	114								
	72								
	141								
	40								
Average	71								
		Bkg Subtracted		15,289	15,653				
		Average		15,176	15,682				
				15,233	15,668				
				Total Extracted + Unextracted =		94.6%		126.903	
						3.6%		4.829	
								3.6%	

Appendix K. Extraction Spreadsheets for Seed.

1491W: PRE-T Seed Extraction
PTRL ID: 1491W-023

Sample Extracted (g)	Weight of	Specific Activity (dpm/ μ g)	Concentration of 14 C in Sample (dpm/g)	(ppm)	TRR
10.483		54,190	15,781	0.291	0.291

Extracted with Acetonitrile/Water 2/3 v/v

#	Available DPM	Aliquot Weight (g)	Extract Wt. (g)	Net DPM	Mean DPM/g (RSD)	DPM Extracted	Percent Extracted	PPM Extracted	% of TRR
AVB 1924/32 10/5/2006	1	165,432	0.468	741	1,578	39,821	24.1%	0.070	24.1%
			0.468	758	3%				
	2	165,432	0.468	716		27,616	16.7%	0.049	16.8%
			0.900	584	624				
			0.929	574	1%				
			0.935	568					
3	165,432	0.904	44.879	283	298	13,374	8.1%	0.024	8.2%
		0.932		249	8%				
		0.933		293					
4	165,432	0.906	44.134	134	148	6,532	3.9%	0.011	3.8%
		0.919		166	22%				
		0.917		106					
Totals:						87,343	52.8%	0.154	52.9%

1491W: PRE-T Seed Extraction

PTRL ID: 1491W-023

Post Extraction Solid Combustion #1

Oxidizer Efficiency	BKG DPM	Gross DPM	Adjusted DPM	Aliquot		Mean		Total DPM	Percent Unextracted	PPM Inextracted	% of TRR
				Weight (g)	DPM/g	DPM/g	RSD				
0.9914	48	1,469	1,433	0.579	2,475	2,740		78,964	47.7%	0.139	47.8%
		1,709	1,675	0.618	2,710	10.24					
PES(g) =	28.819	1,735	1,702	0.561	3,034						
Background and Efficiency											
Total Extracted + Unextracted = 100.5% 0.292 100.3%											
AVB	Bkg										
1924/35	65	Combustion Efficiency		14C	14C	Oxidizer					
10/24/2006	56	Cellulose	Cocktail	Efficiency							
	35	13,605	13,797	0.9914							
	38	13,667	13,711								
	58										
	42	Bkg Subtracted	13,557	13,749							
	37		13,619	13,663							
	49	Average	13,588	13,706							
Average 48											

1491W: POE-T Seed Extraction
PTRL ID: 1491W-025

Weight of		Specific Activity		Concentration of				
Sample Extracted (g)		(dpm/ug)		¹⁴ C in Sample (dpm/g)		(ppm)		
10.020		54,560		21,244		0.389		
Extracted with Acetonitrile/Water 2/3 v/v								
#	Available	Aliquot	Weight	Extract	Net	Mean		TRR
						DPM	DPM/g	
AVB 1924/32 10/5/2006	1	212,865	0.453	24.613	971	2,120	24.5%	0.095
			0.463		1,014	3%		
	2	212,865	0.470	46.550	953	947	20.7%	0.081
			0.932		916	5%		20.8%
	3	212,865	0.923	45.546	857	437	9.4%	0.037
			0.907		403	2%		9.5%
			0.930		410			
			0.925		394			
	4	212,865	0.901	45.029	138	187	4.0%	0.016
			0.924		199	18%		4.1%
			0.919		175			
	Totals:						124,587	58.5%
								58.6%

1491W: POE-T Seed Extraction
PTRL ID: 1491W-025
Post Extraction Solid Combustion #1

Oxidizer Efficiency	BKG DPM	Gross DPM	Adjusted DPM	Aliquot		Mean		Total DPM	Percent Inextracted	PPM Inextracted	% of TRR
				Weight (g)	DPM/g	DPM/g	RSD				
0.9914	48	2,125	2,095	0.575	3,643	3,480	5.79	92,589	43.5%	0.169	43.4%
PES (g) =	26.606	2,131	2,101	0.593	3,543						
		1,984	1,953	0.600	3,255						
Background and Efficiency											
AVB	Bkg										
1924/35	65										
10/24/2006	56										
	35										
	38										
	58										
	42										
	37										
	49										
		Bkg Subtracted		13,605	13,797						
		Average		13,667	13,711						
				13,636	13,754						
Total Extracted + Unextracted = 102.0%											
0.397 102.1%											
Average 48											

Appendix L. Schedule of Events - In-Life Phase.

	Harvest Date	Receipt Date	Date Processed	Combust Date	Extraction Date	Analysis Date	Lab Storage Days	Total Storage Days
Pre- Forage	6/15/2006	6/20/2006	6/27/2006	6/28/2006	NA	NA	NA	NA
Forage	7/7/2006	7/11/2006	7/13/2006	7/17/2006	7/27/2006	7/28/2006	16	20
Hay	7/27/2006	8/2/2006	8/7/2006	8/8/2006	NA	NA	NA	NA
Seed	9/21/2006	9/26/2006	9/27/2006	9/28/2006	10/5/2006	10/19/2006	9	14

Lab Storage Days = Days after receipt before extraction.

Total Storage Days = Days after harvest before extraction.

Appendix M. Field Report.

FIELD PHASE REPORT

STUDY TITLE

Metabolism of Dicamba in Dicamba-Tolerant Soybeans

DATA REQUIREMENT

US EPA OPPTS 860.1300, "Nature of the Residue – Plants, Livestock."

FIELD PHASE TEST SITE AND PRINCIPAL INVESTIGATOR

Blaine Turner
Research For Hire
1696 South Leggett Street
Porterville, California 93257

STUDY DIRECTOR AND TESTING FACILITY

Michael J. Miller
Monsanto Company
Environmental Sciences Technology Center
800 N. Lindbergh Blvd.
St. Louis, MO 63167

SPONSOR

Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167

FIELD PHASE REPORT DATE

August 25, 2009

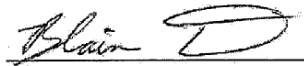
STUDY IDENTIFICATION

Monsanto Study Number: 06-98-M-1
PTRL West Study Number: 1491W
Research For Hire Study Number: R330606

REGULATORY COMPLIANCE STATEMENT

I have been directly related to the conduct and/or supervision of the field phase of study "Metabolism of Dicamba in Dicamba-Tolerant Soybeans" (Monsanto Study Number 06-98-M-1, PTRL West Study Number 1491W, Research For Hire Study Number (R330606) and do hereby certify that this phase of the study was conducted in accordance with the U.S. Environmental Protection Agency's Good Laboratory Practice Regulations (40 CFR 160), with the following exceptions:

1. No claim of compliance is made for the off-site climatic data provided by the California Irrigation Management Information System (CIMIS) and Research For Hire Experimental Farm Data Logger CR 10.
2. Test site information (crop history, pesticide history, maintenance practices and irrigation data).


Blaine Turner
Principal Field Investigator

8/25/09
Date

QUALITY ASSURANCE STATEMENT

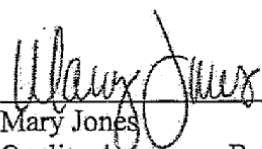
The field phase of this study was monitored by the Quality Assurance Unit of Research For Hire in accordance with the GLP Standards set forth in 40 CFR 160.35(b). The following list describes the inspections made and the dates that the findings were reported:

Summary of Inspections

Date of Inspection	Phase Inspected	Date Findings Reported to Study Director and Study Director's Management
6/1/06	Application #1 pre-emergence	6/16/06
7/7/06	Forage Sampling	7/13/06
5/11/09	Raw Data Audit and In-Life Phase Report	8/25/09

The following inspections were conducted by the Agvise quality assurance unit:

Date of Inspection	Phase Inspected	Date Findings Reported to Study Director and Study Director's Management
6/29/06	Procedures for the Determination of Ammonium Acetate Extractable Calcium, Magnesium, Sodium, and Potassium in Soil	7/31/06
7/19/06	Raw Data Audit	7/31/06



Mary Jones
Quality Assurance Representative

8/25/09

Date

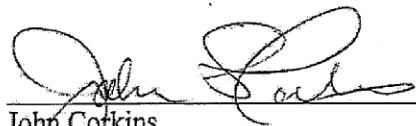
PROJECT PERSONNEL

RESEARCH FOR HIRE

<u>Personnel</u>	<u>Position</u>
Joshua Tilton	Research Technician I
Kristin Gentry	Research Assistant
Nancy Sutherland	Research Assistant
Thomas Sukut	Research Technician I
Heather Holmes	Research Biologist (Principal Field Investigator 5/25/06-11/01/06)
Emily Vossler	Research Assistant
Blaine Turner	Principal Field Investigator
Stephanie Bonilla	Office Coordinator
Elyse Spry	Research Technician II
Sandy Medina	Office Assistant

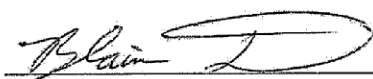
REPORT CERTIFICATION and APPROVAL

This report is an accurate and complete representation of the data and activities associated with the field phase of Monsanto study 06-98-M-1.



John Corkins
General Manager
Research For Hire

8/25/09
Date



Blaine Turner
Principal Field Investigator
Research For Hire

8/25/09
Date



Mary Jones
Quality Assurance Representative
Research For Hire

8/25/09
Date

STUDY IDENTIFICATION PAGE

Field Phase Study Site:	Research For Hire (RFH) 1696 South Leggett Street Porterville, CA 93257
Testing Facility:	Monsanto Company Environmental Sciences Technology Center 800 N. Lindbergh Blvd. St. Louis, MO 63167
Study Director:	Michael J. Miller
Monsanto Study Number:	06-98-M-1
PTRL West Study Number:	1491W
RFH Study Number:	R330606
Study Initiation Date:	May 25, 2006
RFH Experimental Start Date:	June 1, 2006
RFH Experimental End Date:	September 21, 2006
Inquiries:	Monsanto Company Environmental Sciences Technology Center 800 N. Lindbergh Blvd. St. Louis, MO 63167

All original study specific raw data and the original final report will be transferred to Monsanto Company.

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INTRODUCTION and SUMMARY

The field phase of Monsanto study 06-98-M-1 was conducted in greenhouses at the Research For Hire (RFH) facility in Porterville, CA. Dicamba-tolerant soybean seed (GM_A90617) provided by the Sponsor was planted in a loamy sand soil contained in 12-inch diameter pots on June 1, 2006. The test material, [¹⁴C]dicamba, was applied as an aqueous solution using a hand-held sprayer to the soil as a preemergence treatment for one treatment group (PRE-T) or to the soybean plants as a postemergence treatment for the other treatment group (POE-T). The preemergence application was made immediately after planting. The postemergence application was made 29 days after the seeds were planted at the R1 (first flower) growth stage. There were three control groups. One control group (PRE-C) consisted of pots that were interspersed amongst the PRE-T pots. A second control group (POE-C) consisted of pots that were interspersed amongst the POE-T pots. The third control group (UNT-C) was kept isolated from the treated pots in a separate greenhouse.

Immature soybean plants were collected on 6/15/2006 from the thinnings of the PRE-T and UNT-C groups at the V1/V2 growth stage. Forage was collected on July 7, 2006 from all groups 7 days after the postemergence treatment (approx. R2 growth stage), and hay (UNT-C, PRE-T and POE-T groups) was collected on July 27, 2006, 20 days after the forage collection. Mature seed was collected from all groups on September 21, 2006, 112 days after planting.

This report summarizes the procedures utilized and data generated during the field phase of the study.

OBJECTIVES

The objective of the field phase of the study was to generate samples necessary to meet the stated objectives of the study: the determination of the radioactive residues (TRR) in soybeans following treatment with radiolabeled dicamba; determination of the efficiency of extraction of the residue components; identification and quantification of the major components of the terminal residue, and delineation of the major routes of metabolism of dicamba in soybeans.

The field phase of Monsanto study 06-98-M-1 was conducted in compliance with the EPA Good Laboratory Practice Standards, 40 CFR 160. The study was undertaken to support data requirements under the EPA Residue Chemistry Test Guidelines OPPTS 860.1300, Nature of the Residue – Plants, Livestock.

APPARATUS

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

CONDUCT OF THE STUDY

Research For Hire conducted the field phase of this study according to Monsanto Company protocol 06-98-M-1, Metabolism of Dicamba in Dicamba-Tolerant Soybeans, and amendments. Two protocol deviations, described in Appendix B, were associated with the field phase of the study. These deviations had no effect on the results of the study. The schedule of events for the field phase is shown in Table 1.

EXPERIMENTAL FACILITIES

Research For Hire (RFH), 1696 South Leggett Street, Porterville, California 93257, conducted the field phase of the study from June 1, 2006 (planting and PRE-T application) to September 21, 2006 (final harvest). The untreated and [¹⁴C]dicamba treated pots were in secured greenhouses accessible to authorized personnel only. The maps for the test site and the diagrams showing pot arrangement are in Appendix C.

Test Material Receipt and Distribution

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

All test materials received at RFH were logged in as per RFH Standard Operating Procedures. The formulated radiolabeled test materials were received from PTRL West Inc., delivered by PTRL West personnel. Refer to PTRL West 1491W in-life report. See Table 2 for information on test material receipt and use. The test materials for application were received in good condition. Packing material was monitored with a survey meter with no above-background radiation detected.

Test Material

[Ring-U-¹⁴C]-Dicamba

Details for the test materials will be described in the final study report.

TEST CROP

Test Crop Variety

The variety of soybean planted was GM_A90617. Refer to Table 1 for the planting date. This is a genetically modified dicamba-tolerant soybean and was regulated under the USDA Animal and Plant Health Inspection Service (APHIS). APHIS compliance was maintained throughout the study.

Crop and Field History

The soil used to fill the pots was obtained from the Research For Hire Experimental Farm, Plot P-104. It had been exposed to the fungicidal chemicals Agri-Fos® (potassium salts of phosphorous acid) and Bravo (chlorothalonil) in the year prior to trial initiation. The Agri-Fos was applied at 10.0 qt/A and the Bravo was applied at 5.5 pt/A. For three years before that, the soil was fallow.

Crop Planting and Thinning

Four soybean seeds were sown in each pot at a depth of approximately 1-1.5 inches. At the V1/V2 growth stage (14 days after planting), the plants were thinned to a density of two plants per pot for the PRE-T, PRE-C and UNT-C treatment groups, and to a density of one plant per pot for the pots intended for the POE-T and POE-C treatment groups.

Maintenance Pesticides

The insecticide Capture was applied at 1.0 pt/A on July 7, 2006 and again on August 22, 2006. No other maintenance pesticides were applied during the field portion of this study.

Fertilization

The pots were fertilized with Miracle-Gro at a rate of 100 mL per pot of a 1 tablespoon per gallon mixture. They were fertilized on three occasions, June 27, 2006, July 14, 2006 and August 22, 2006.

Irrigation

The pots were watered carefully by hand, applying the water to the soil surface immediately after planting on June 1, 2006. On June 6, 2006 sub-irrigation of the pots was initiated. The pots were placed on an absorbent pad on a plastic sheet with the sides of the plastic raised approximately two inches. The amounts of water added to the pad were recorded. Refer to Table 3 for irrigation data.

Soil Type

Soil for characterization was collected from the treated pots on 05/30/06 prior to application of the test material. The soil was collected from the 0-6 inch horizon by inserting a clean spade into random locations within the pots. The composite sample was then placed into an Agvise soil bag and shipped under ambient conditions to Agvise Laboratories. Agvise Laboratories collected the soil characterization data in compliance with GLP methods. Refer to Appendix D for the results of the soil analysis.

Test Site

The test site was located at 1696 S. Leggett, Porterville, Tulare County, California. The latitude for the test site is 36°N and the longitude is 119°W.

Eighty-five pots with a 12 inch diameter were used for the study. Based on the study protocol, 80 pots were prepared/planted for the study along with several extra pots. Eight of the pots were designated as control pots. One additional (extra) pot was added to the PRE-T group at the time of the preemergence application for a total of 29 PRE-T pots. An additional four extra pots were added to the POE-T group at the time of the postemergence foliar application for a total of 32 POE-T pots. There were also 8 pots for the PRE-C group and 8 pots for the POE-C group that were randomly dispersed amongst the pots of their respective treatment groups immediately after the respective applications. The untreated control (UNT-C) pots, and the pots intended for use as POE-C and POE-T pots (including extra pots), were maintained in a separate greenhouse from that housing the treated pots. Immediately prior to the POE-T application, the POE-C and POE-T pots were moved to the greenhouse housing the treated pots. Refer to the plot maps in Appendix C for the position of the pots.

Plot Description

The enclosed treatment area (greenhouse) was posted with a radioactive caution sign, an "authorized personnel only" sign and a "This Greenhouse Contains Genetically Modified Organisms" sign. The maps for the test site and pot diagrams are in Appendix C.

Climatic Data

The California Irrigation Management Information System (CIMIS) station #33 was used to collect 10-year climatic data. It was located approximately 19.8 miles northwest of the test site. The Research For Hire Experimental Farm Weather Data Logger CR-10 collected daily climatic weather data and was located approximately 5.25 miles southwest of the test site. The air temperatures and rainfall were normal for the trial period. Because this study was conducted in greenhouses, the outdoor climatic data are included in the study file, but are not included in this report. HOBO temperature monitors were placed in each greenhouse. At the time of both applications there was no wind because

the pots were enclosed in a plastic lined spray booth attached to the greenhouse. Table 3 summarizes the greenhouse temperature data.

MATERIALS and METHODS

Test Material Preparation

PTRL West prepared the test material.

Time and Rate of Application

The application to the PRE-T group occurred at the time of planting on June 1, 2006. The application to the POE-T group was made on June 30, 2006. Details of the rates of application are provided in the PTRL West in-life report.

Method of Application

The applications were made using amber glass bottles equipped with a plastic spray trigger in a plastic spray booth attached to a side door of the greenhouse that housed the ¹⁴C-treated groups. A separate spray bottle containing a pre-dispensed aliquot of test material solution was used for each treated pot or plant. The preemergence application was applied evenly to the soil. The postemergence application was applied over the top and from the sides of the plant to obtain coverage of the plant leaf surfaces as evenly as possible. The details of the application solutions are provided in the PTRL West in-life report.

SAMPLING

Appropriate safety measures were observed during sampling (samplers wore lab coats and gloves).

Refer to Table 4 for sample weights.

Pre-Forage/Thinning Harvest

On June 15, 2006 (14 days after planting), a harvest of immature foliage (pre-forage) was collected by thinning the plants in all pots of the PRE-T and UNT-C groups. Plants were cut off just below the cotyledon (approx. 1 inch above the soil). Any soil that was on the collected samples was brushed off. The pots were thinned to two plants per pot. The collected plants were placed in tared plastic bags and weighed on balance EQP 13-1. The samples were then placed in the RFH walk-in freezer (EQP 28-2).

Forage Harvest

On July 7, 2006, soybean forage was collected from all treatment groups by cutting with scissors approximately $\frac{3}{4}$ of an inch above the soil. The harvested plants were placed into tared zip-lock bags. The samples were weighed on balance EQP 13-1 and placed in the RFH walk-in freezer (EQP 28-2). Two pots were harvested from the UNT-C group, four pots each were harvested from the PRE-C and POE-C groups, five pots were harvested from the PRE-T group, and six pots were harvested from the POE-T group.

Two plants from the POE-T group were each washed 3 times with 1,000 mL of HPLC grade water for 30 seconds each. The plants were then laid on foil and allowed to dry before being frozen. A 500 mL aliquot from each wash was collected as sample and frozen. Three 1 mL aliquots were removed from each wash and added to 10 mL of Ready Solv. The aliquots were then analyzed by LSC. These LSC analyses were conducted as a contingency in the event of breakage/loss in storage or transit because the wash aliquots could not be immediately transferred to PTRL West, Inc. Since the washes and LSC aliquots were safely transferred to PTRL West, Inc., these data were not used and LSC generated from the samples at PTRL West, Inc. were used instead.

Hay Harvest

On July 27, 2006, soybean hay was collected from the PRE-T, POE-T and UNT-C treatment groups by cutting the plants with scissors approximately $\frac{3}{4}$ of an inch above the soil and placing the collected plants into zip-lock bags. The samples were weighed on balance EQP 13-1 and then placed in the RFH walk-in freezer (EQP 28-2). Two pots were harvested from the UNT-C group, five pots were harvested from the PRE-T group, and six pots were harvested from the POE-T group.

Immature Seed Harvest

On September 8, 2006, at the request of the Study Director, two plants from the two remaining extra POE-T treated pots were harvested by cutting the plants above the soil surface. The plants were allowed to dry in the greenhouse for 3 days, then the seed was removed from the pods, frozen and shipped on dry ice directly to Monsanto Company.

Mature Seed Harvest

On September 21, 2006 the mature soybeans were harvested. The seed pods were cut from the plants and placed on foil-lined trays. Pods from each treatment group were composited on separate trays. Seeds were removed from the pods and placed in tared zip lock bags. Fresh weights were obtained using scale EQP 13-1. Seeds were then placed in cloth residue bags and placed in the RFH walk-in freezer (EQP 28-2).

Sample Handling

All sampling procedures were in compliance with the pertinent Standard Operating Procedures of Research For Hire and the Study Protocol.

Pre-shipping Storage

Samples were stored in the RFH walk-in freezer (EQP 28-2).

Sample Shipments

The soil for characterization was shipped via UPS to Agvise Laboratory at ambient temperature. All other samples, except the immature seed samples, were shipped frozen on dry ice and delivered to the following address:

Attention: Alex V. Bautista
PTRL West, Inc.
625-B Alfred Nobel Drive
Hercules, CA 94547

Table 4 summarizes the dates of sample shipment.

RESULTS and DISCUSSION

Study events are listed in Table 1. A summary of the sampling including sample weights is shown in Table 4. The crop conditions were good with the exception of the POE-T plants that had some wilting and curling of the leaves immediately following their treatment. However, the POE-T plants appeared healthy 14 days after treatment. No signs of phytotoxicity were observed for any of the other treatment groups. There were no unusual events during the in-life phase that would have affected the results of this study.

**TABLE 1: SCHEDULE OF EVENTS FOR THE FIELD PHASE OF
THE STUDY**

Date	Field Phase Events
05/30/2006	The pots were filled with soil from RFH Experimental Farm, plot P-104. The pots were double lined with 6 mil black plastic.
05/30/2006	The test pots were set up and soil samples were collected for characterization analysis.
06/01/2006	The soybeans were planted and the application was made to the PRE-T group pots. The Study Director observed the application.
06/15/2006	The plants were assessed for phytotoxicity. Plants appeared to be healthy. Pots were thinned. The pre-forage (thinning) harvest also took place.
06/19/2006	The samples from 6/15/06 were shipped to PTRL West via FedEx overnight on dry ice.
06/30/2006	The application was made to the POE-T group pots. The Study Director observed the application.
06/30/2006	Approximately 8 hours after the application, some wilting of the POE-T plants was observed. Per Study Director request the number of pots to be sampled from the POE-T group was changed - 6 each for forage and hay, and 20 for mature seed.
07/01/2006	Additional wilting with some curling of the leaves of the POE-T plants was observed.

Date	Field Phase Events
07/07/2006	The forage sampling was completed.
07/10/2006	Forage samples were shipped to PTRL West via FedExovernight on dry ice.
07/14/2006	Plants were assessed for phytotoxicity. Plants appeared to be healthy.
07/27/2006	The hay sampling was completed.
08/01/2006	The hay samples were shipped to PTRL West via FedEx overnight on dry ice.
09/08/2006	At the request of the Study Director, the two extra plants from the POE-T group were harvested and allowed to dry in the greenhouse for the early (immature) seed harvest.
09/11/2006	The immature seeds were removed from the two dried extra POE-T plants harvested as the early seed harvest and stored.
09/18/2006	The immature seeds were shipped to Monsanto via FedEx on dry ice.
09/21/2006	The mature seeds were harvested and the remaining plant material was discarded per Study Director's request.
09/25/2006	The mature seeds were shipped to PTRL West via FedEx overnight on dry ice.

TABLE 2: TEST MATERIAL RECEIPT AND DISTRIBUTION

Material	Date	Distribution	Purpose	Quantity
[Ring-U- ¹⁴ C]Dicamba	05/31/06	Receipt	Received	15.71 mCi
	06/01/06	Use	PRE-T Application	~15.71 mCi
	06/29/06	Receipt	Received	17.1 mCi
	06/30/06	Use	POE-T Application	~17.1 mCi

TABLE 3: CLIMATIC DATA

	Greenhouse 1 TRT		Greenhouse 2 UTC		
Date Range	Daily Minimum Temperature (°F)	Daily Maximum Temperature (°F)	Daily Minimum Temperature (°F)	Daily Maximum Temperature (°F)	Monthly Irrigation (inches)
6/1/06-6/30/06	57	106	58	99	~12.25
7/1/06-7/31/06	64	108	64	99	~13.00
8/1/06-8/31/06	61	101	62	95	~13.00
9/1/06-9/21/06	52	102	52	99	~02.50

Note: The greenhouse temperatures were recorded with HOBO temperature monitors at 30 minute intervals. The data will be provided to the Study Director electronically. There are no precipitation data because the study was conducted in greenhouses. The data are reported from the beginning of the test application month to the last sampling (9/21/06).

TABLE 4: SAMPLING, SHIPPING AND WEIGHT SUMMARY

SAMPLE IDENTIFICATION AND LOG

PTRL Sample Number	Treatment Group	Sample Description	Sample Event	Date Sampled	Sample Weight	Date Shipped
R330606-02	UNT-C	Pre-forage	14 days after planting	6/15/06	29 g	06/19/06
R330606-03	PRE-T	Pre-forage	14 days after PRE-T application	6/15/06	101 g	06/19/06
R330606-04	UNT-C	Forage	36 days after planting	7/07/06	113 g	07/10/06
R330606-05	PRE-C	Forage	36 days after PRE-T application	7/07/06	300 g	07/10/06
R330606-06	PRE-T	Forage	36 days after PRE-T application	7/07/06	312 g	07/10/06
R330606-07	POE-C	Forage	7 days after POE-T application	7/07/06	168 g	07/10/06
R330606-08	POE-T	Forage	7 days after POE-T application	7/07/06	157 g	07/10/06
R330606-09	POE-T	500 mL of water from forage wash	7 days after POE-T application	7/07/06	N/A	07/10/06
R330606-10	POE-T	500 mL of water from forage wash	7 days after POE-T application	7/07/06	N/A	07/10/06
R330606-11	POE-T	Forage (washed plant)	7 days after POE-T application	7/07/06	26 g	07/10/06
R330606-12	POE-T	Forage (washed plant)	7 days after POE-T application	7/07/06	31 g	07/10/06
R330606-13	UNT-C	Hay	56 days after planting	7/27/06	300 g	08/01/06
R330606-14	PRE-T	Hay	56 days after PRE-T application	7/27/06	1007 g	08/01/06
R330606-15	POE-T	Hay	27 days after POE-T application	7/27/06	648 g	08/01/06
R330606-16	UNT-C	Mature Seed	112 days after planting	9/21/06	98 g	09/25/06
R330606-17	PRE-C	Mature Seed	112 days after PRE-T application	9/21/06	112 g	09/25/06
R330606-18	PRE-T	Mature Seed	112 days after PRE-T application	9/21/06	453 g	09/25/06
R330606-19	POE-C	Mature Seed	83 days after POE-T application	9/21/06	83 g	09/25/06
R330606-20	POE-T	Mature Seed	83 days after POE-T application	9/21/06	372 g	09/25/06
R330606-21	POE-T	Immature Seed from Pot #19	70 days after POE-T application	9/08/06	26 g	09/18/06
R330606-22	POE-T	Immature Seed from Pot #5	70 days after POE-T application	9/08/06	31 g	09/18/06

**APPENDIX A: LIST OF EQUIPMENT USED FOR GENERATING
FIELD PHASE RAW DATA**

Liquid Scintillation Counter - Model LS 6500 (EQP 30-2)

Mfg: Beckman Instruments, Inc., 3500 Harbor Blvd., Fullerton, California 92634-3100
(714) 871-4848

Psychro-Dyne - (EQP 27-14)

Mfg: Environmental Tectonics Corporation, County Line Industrial Park, Southampton,
Pennsylvania 18966
(215) 355-9100

Toploading Balance - Model AB-87 (EQP 13- 1)

Mfg: Abbeon Cal, Inc., 123-21T Gray Avenue, Santa Barbara, California 93101-1895

Survey Monitor - Model AB-87 (EQP 14-1 and 14-2)

Mfg: Technical Associates, 7051 Eton Avenue, Canoga Park, CA 91303
(312) 942-2500

Lindberg Sola Basic Oxidizer - Model 55035 (EQP 15-1)

Mfg: Lindberg, 2450 W. Hubbard Street, Chicago, Illinois 60612

Microlab-P Hamilton Programmable Pipette (EQP1-1)

Freezer (walk-in) (EQP28-2)

Mfg: Kysor, McElmoyl Commercial Refrigeration, Inc. 165 W. Cross Street, Tulare,
CA 93274
(559) 686-1987

APPENDIX B: PROTOCOL DEVIATIONS

Statement in the Protocol

Section 7.7.5: Following collection at Research For Hire, plant specimens will be frozen on dry ice within 2 hours after collection and will be transported to PTRL West, Inc. on dry ice in separate coolers for control and treated samples.

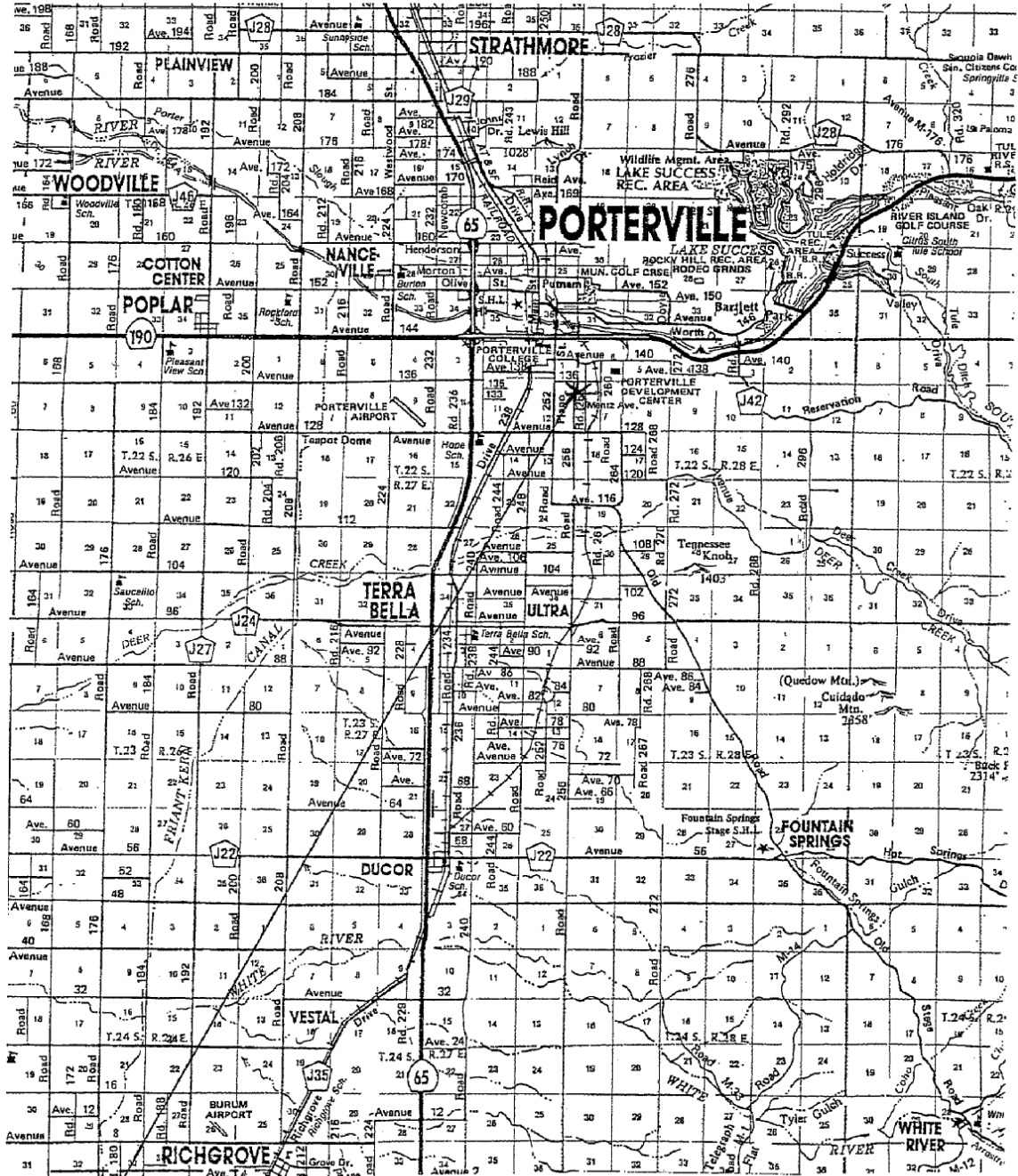
Section 7.7.3: All collections (pre-forage, forage, hay, and seed) will be pooled within their respective groups prior to shipment to PTRL West, Inc. except for the plants collected for forage washes from the postemergence treatment group.

Deviation from Protocol

Specimens were placed in coolers and stored in a walk-in freezer prior to shipment to PTRL West rather than freezing on dry ice.
Reason: Specimens were not immediately shipped to PTRL West, Inc. after collection.
Effect on study: None. Samples were frozen soon after collection and sample integrity was maintained.

The PRE-T hay specimens were collected into two separate bags rather than pooling into one sample.
Reason: Bags of sufficient size were not available.
Effect on study: None. The PRE-T hay specimens were pooled at the time of sample processing.

APPENDIX C: MAPS



Test site

HLH 5/31/06

Protocol No: 1491W

06-98-M-1

Protocol No: 1491W

Study No: 06-98-M-1

AUTHENTIC COPY
4/4 5/8/06

256 LEGGETT

LEGGETT ROAD

RE16
RW15
RW14
RW13
RW12
RW11
RW10
RW9
RW8
RW7
RW6
RW5
RW4
RW3
RW2
RW1

RE3
GRASS
MOBILE HOME
OFFICE
LAB
BUILDING
EQUIPMENT SHED
C-14 PLOT
RE21
RE22
RE23
RE24
RE25
RE26
RE27
RE28
RE29
RE30
RE31
RE3
RE4
RE5
RE6
RE7
RE8
RE9
RE10
RE11
RE12
RE13
RE14
RE15
RE16
RE17
RE18
RE19
RE20
RE32
RE33

UTC
TRT

LEGEND

- LOT BOUNDARY
- NEIGHBORLY BOUNDARY
- EXISTING SIDE OF ALLEYWAY
- EXISTING SIDE OF ALLEYWAY

0 100 FEET

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APPENDIX C: MAPS (CONTINUED)

RESEARCH FOR HIRE

1696 South Leggett Street
Porterville, California 93257

Telephone (559) 784-5787
Facsimile (559) 784-4351

PLOT MAP

Study/Protocol/Trial No. Protocol No: 1491W

06-98-M-1

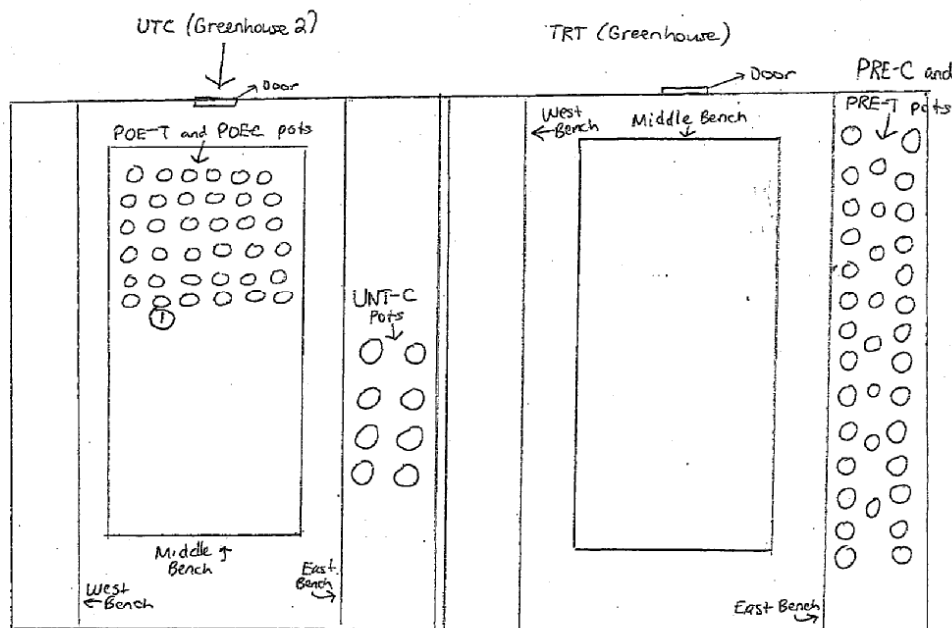
RFH No. R330606

Provide a detailed map of the plot layout including:
plot identification (treatment, replicate number);
plot dimensions; distances between plots and fixed
points of reference; direction of rows; prevailing
wind direction; direction and degrees of slope:
(Note: Map not drawn to scale)

Indicate
Direction:
(with arrow)

Prevailing
windy

↑ N → NA N ← NA
Rows: W E Downslope: W E
S S



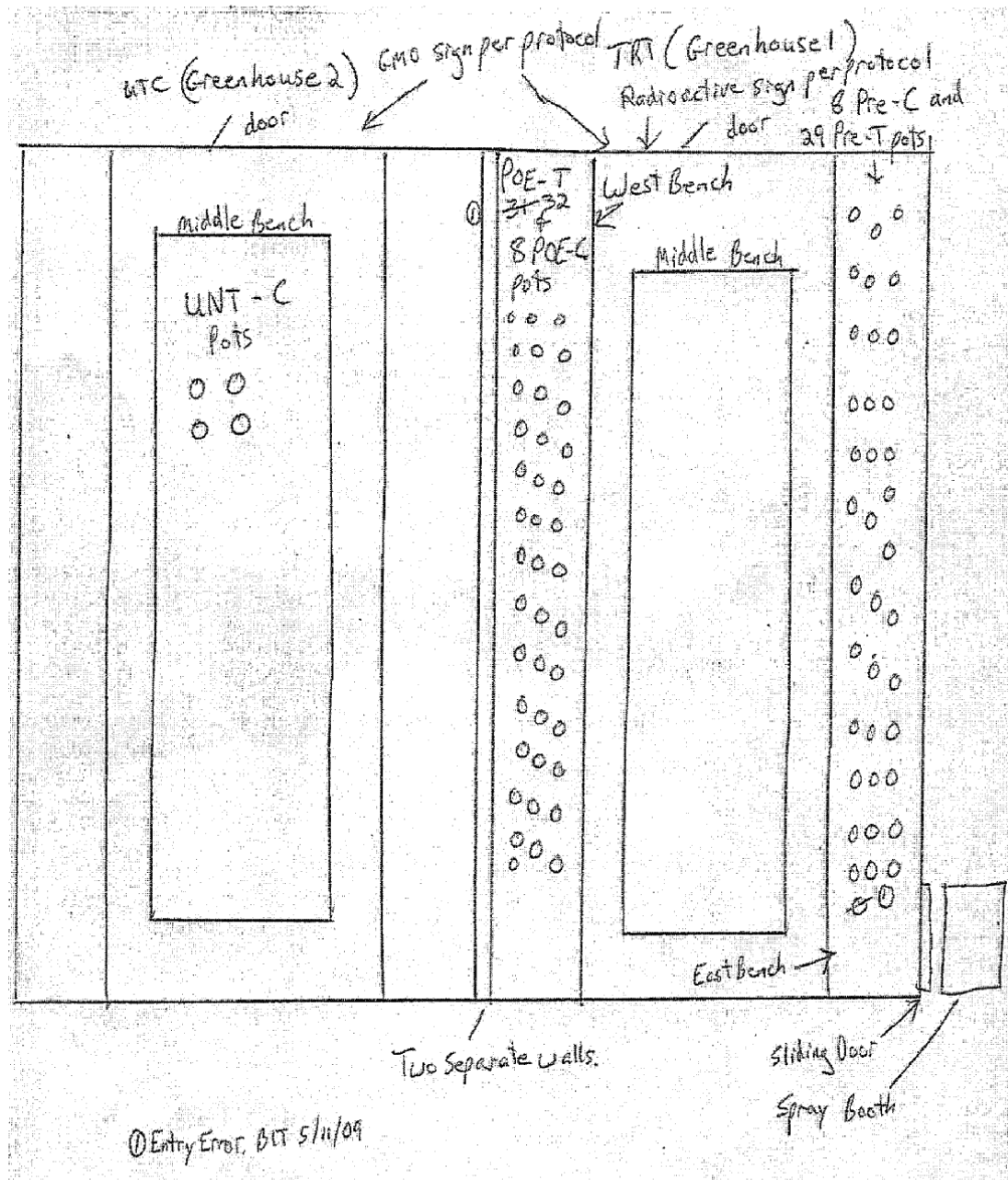
① The POE-T and POE-C pots remained in Greenhouse 2 during Application 1 when the PRE-T pots were sprayed. They will be moved to Greenhouse 1 after they are treated. They will be on the west bench. HLH 5/31/06

Signature
FORMSPLOTMAP

Heather Holmes

Date 5/31/06

APPENDIX C: MAPS (CONTINUED)



APPENDIX D: AGVISE REPORT



604 Highway 15 West
P.O. Box 510
Northwood, ND 58267
(701) 587-6010
FAX (701) 587-6013
email: agvise@polarcomm.com
Homepage: www.agvise.com

AGVISE Soil Characterization Report

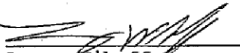
Submitting firm = RESEARCH FOR HIRE
Protocol or Study No = 06-98-M-1
Sample ID. = R330606-01 0-6"
Trial ID. = 1491W
Date Received = 6-5-06
Date Reported = 06-08-2006

AGVISE Lab No 06-964

Percent Sand	80
Percent Silt	12
Percent Clay	8
USDA Textural Class (hydrometer method)	Loamy Sand
Bulk Density (disturbed) gm/cc	1.39
Cation Exchange Capacity (meq/100 g)	4.9
% Moisture at 1/3 Bar	7.7
% Organic Matter--Walkley Black	0.4
pH in 1:1 soil:water ratio	7.1

Base Saturation Data	Percent	ppm
Cation		
Calcium	72.1	711
Magnesium	11.2	66
Sodium	1.9	21
Potassium	5.1	99
Hydrogen	9.7	5

These tests were completed in compliance of 40 CFR Part 160.


Larry Wikoff
Analytical Investigator

6/8/06
Date

RECEIVED
BY JEH DATE 6/12/06

Agricultural Testing

11 Appendix C – Quantitative Storage Stability

11.1 Summary of Quantitative Storage Stability Results

Quantitative storage stability data were generated in this study to support the residue study (Monsanto study REG-08-096) for dicamba in dicamba-tolerant soybean.¹³ For these additional quantitative storage stability analyses, metabolite HPLC profiles for PRE-T forage, PRE-T and POE-T hay, and POE-T seed that had been stored frozen at approx. -20 °C were generated at the end of the analysis phase of this metabolism study (final stability) and at one additional time point approx. 9 months later (extended stability). At each time point, the samples were combusted, extracted and analyzed by HPLC/RAD or HPLC/LSC using the methods described in Section 2.9 of this report. The results of these analyses are compared to the results of the initial analyses conducted by PTRL and Monsanto shortly after harvest of the samples. The soybean residue analytical method (AG-ME-1321-01)¹⁴ for dicamba and its metabolites that was utilized for the dicamba-tolerant soybean residue study incorporates an acid hydrolysis step which converts DCSA and DCGA conjugates to their respective chemophores DCSA and DCGA. Dicamba, DCSA, DCGA and 5-hydroxydicamba are then quantitated by LC/MS/MS in the method. For this reason, the quantitative storage stability results reported herein are presented as DCSA, DCGA and dicamba analyte values. For each quantitative HPLC profile, the amounts (as mg/kg dicamba equivalents) of DCSA glucoside **9**, DCSA HMGglucoside **11** and free DCSA **22** were summed to give the 'DCSA analyte' value. Similarly, for each profile, the amounts of DCGA glucoside **3** and DCGA malonylglucoside **8** were summed to give the 'DCGA analyte' value. The 'dicamba analyte' value is simply the quantitation of the dicamba peak (**23**) in the profiles. Combustion and extraction results, as well as dates of analyses, are summarized in Table C1. Analyte quantitation results are presented in Table C2 and the quantitative HPLC stability profiles from which these values are derived are presented in Figure C1 through Figure C12.

In the dicamba soybean residue study, the intervals between sampling and analysis were 29-177 days for seed, 119-287 days for forage and 143-283 days for hay. A few forage samples were re-extracted and analyzed in that study to verify early analysis results. The storage interval for those samples was 400 days. For the storage stability analyses conducted in this study, the initial quantitative HPLC analyses (MON Initial) were all conducted within 6 months of the date of collection. The interval between sample collection and this first analysis was 154 days for PRE-T forage, 22 days for PRE-T and POE-T hay, and 174 days for POE-T seed. The storage interval between the final (MON Final) and initial analyses was 704 days for PRE-T forage, 816 days for PRE-T and POE-T hay, and 608 days for POE-T seed. For the extended stability analyses, the storage interval between the initial and extended (MON Extended) analyses was 991 days for PRE-T forage, 1103 days for PRE-T and POE-T hay, and 895 days for POE-T seed.

The results shown in Table C1 indicate that the combustion (TRR) values and extractabilities for all matrices in frozen storage do not change substantially from the initial values determined either at PTRL or at Monsanto. While there may be some reduction in the TRR for POE-T hay at the extended time point and some reduction in extractabilities for several of the matrices at the extended time point, the differences between the initial and extended time point values are small (10% or less).

The analyte quantitation results presented in Table C2 demonstrate that DCGA and DCSA (as their endogenous metabolites), and dicamba, were stable between the initial and final analyses with the exception of DCGA in PRE-T hay. In PRE-T hay, 30% of the original DCGA-forming metabolites were lost over the 816 days of storage. It should be noted that the overall DCGA residue level in the PRE-T hay was quite low (0.046 mg/kg initially). In the POE-T hay, where the DCGA-forming metabolites represented a residue level of 2.314 mg/kg, these metabolites were stable over this time period. At the extended time point, the residue levels for all three analytes in forage and seed compared very well to their initial values. For the hay, dicamba residue levels showed very little change. DCSA metabolite residue levels in hay were slightly lower at the extended time point compared to the initial values, but the difference was less than 10% for both the PRE-T and POE-T hay. Additional degradation of DCGA metabolites in the PRE-T hay occurred between the final and extended time points. The 0.026 mg/kg value represented a loss of 43% of the original DCGA-forming metabolites in the PRE-T hay. A significant drop in the residue level of the DCGA-forming metabolites occurred in the POE-T hay between the final and extended time points. The 0.887 mg/kg value represented a loss of 62% of the DCGA-forming metabolites originally in the sample. A re-analysis (including re-extraction) confirmed this result. No concrete reason for this drop is available; however, the degradation of DCGA in this sample may be due to the fact that the sample had been thawed and re-frozen for analyses several times. This may have contributed to the loss in DCGA residues.

The results of the quantitative storage stability analyses demonstrate that dicamba in forage and hay at levels of approx. 0.01-0.02 or 4.8 mg/kg is stable for a period of 2.7-3 years (991 days for forage and 1103 days for hay). The level of dicamba in seed (0.002 mg/kg) was too low to adequately assess the stability in seed. DCSA (primarily as its endogenous DCSA-forming conjugates) also was demonstrated to be stable in forage at a level of approx. 1.0 mg/kg, hay at levels of approx. 1.0 mg/kg and 28 mg/kg, and seed at a level of approx. 0.10 mg/kg over a 2.5-3 year storage period. DCGA (as its endogenous DCGA-forming metabolites) was stable in frozen forage and seed at a level of 0.02-0.03 mg/kg for more than two years (991 days for forage, 895 days for seed). DCGA in hay was stable at a level of 2.3 mg/kg for more than two years (816 days) between the initial and final time points. However, at the low level of 0.05 mg/kg,

DCGA degraded approx. 30% (from 0.046 to 0.032 mg/kg) over this time period. Further degradation of DCGA occurred between the final and extended time points at the low level (to 0.026 mg/kg, 43% degradation from the original 0.046 mg/kg). At the high level, a significant degradation of DCGA (from 2.281 to 0.887 mg/kg) in the POE-T hay sample occurred between the final and extended time points even though no degradation had been observed between the initial and final time points. This may be due to several freeze-thaw cycles that occurred for this sample during this time period. Although some degradation of DCGA-forming metabolites occurred in hay, the degradation was minimal in the first 816 days. Therefore, degradation of DCGA metabolites in hay over the much shorter storage period (143-283 days) of the dicamba soybean residue study would be expected to be insignificant.

The initial quantitative HPLC analyses of the PRE-T and POE-T hay occurred within 30 days of the hay harvest. For forage and seed, the initial quantitative analyses occurred 5-6 months after the respective harvests. However, considering that no degradation of the analytes were observed between the initial quantitative analyses and the final or extended analyses, one can be reasonably confident that no significant degradation occurred in the first 5-6 months of storage. In addition, little or no change in the TRR values and extractabilities for these matrices occurred over the approx. 3-year period (and from the original analyses conducted at PTRL within 30 days of harvest) lending additional support to the stability of the residues in these matrices. As well, the initial quantitative HPLC profile for PRE-T forage is qualitatively identical to the original profile obtained by PTRL within 30 days of harvest (see Figure 108).

The results of the quantitative storage stability analyses described above provide assurance that the DCGA and DCSA residues in forage, hay and seed, and dicamba residues in forage and hay, were stable in the samples generated in study REG-08-096 over the storage intervals in that study.

Table C1. Summary of Quantitative Storage Stability Analyses (Combustion, Extraction and Dates of Analyses)

Matrix	TRR (mg/kg, ppm)*			
	PTRL	MON Initial	MON Final	MON Extended
PRE-T Forage	1.433	1.237	1.355	1.364
PRE-T Hay	1.056	1.098	1.087	1.057
POE-T Hay	39.149	39.030	39.342	35.107
POE-T Seed	0.389	0.388	0.383	0.402

* Dicamba equivalents

Matrix	Percent Extracted (Normalized)			
	PTRL	MON Initial	MON Final	MON Extended
PRE-T Forage	93.6	91.21	90.02	90.09
PRE-T Hay	NA	90.88	90.64	87.63
POE-T Hay	NA	95.30	95.68	91.85
POE-T Seed	57.5	57.00*	55.52	54.52

* Value is not normalized (pellet combustion was not conducted)

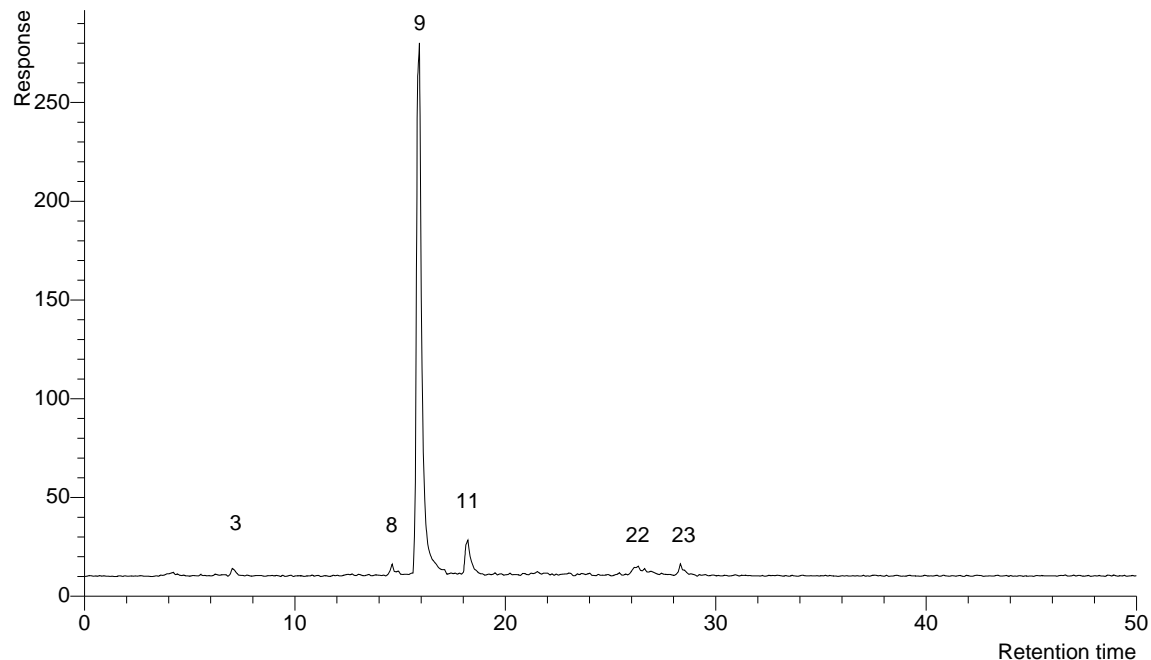
Matrix	Collection Date	Processing Date	Extraction and Analysis Date			
			PTRL	MON Initial	MON Final	MON Extended
PRE-T Forage	7/7/2006	7/13/2006	7/27-28/06	12/8-12/06	11/11-14/08	8/25-28/09
PRE-T Hay	7/27/2006	8/7/2006	NA	8/18-23/06	11/11-13/08	8/25-27/09
POE-T Hay	7/27/2006	8/7/2006	NA	8/18-24/06	11/11-13/08	8/25-27/09
POE-T Seed	9/21/2006	9/27/2006	10/5,19/06	3/14-15/07	11/11-18/08	8/25-28/09

Table C2. Summary of Quantitative Storage Stability Analyses (Analyte Quantitation)

Matrix	Analyte	Analyte mg/kg (ppm)*		
		MON Initial	MON Final	MON Extended
PRE-T Forage	DCGA	0.031	0.031	0.031
	DCSA	1.025	1.052	1.073
	Dicamba	0.020	0.023	0.021
PRE-T Hay	DCGA	0.046	0.032	0.026
	DCSA	0.868	0.862	0.822
	Dicamba	0.009	0.012	0.010
POE-T Hay	DCGA	2.314	2.281	0.887
	DCSA	27.975	28.047	25.400
	Dicamba	4.813	4.878	4.547
POE-T Seed	DCGA	0.023	0.025	0.028
	DCSA	0.093	0.099	0.115
	Dicamba	0.002	0.003	0.004

* Dicamba equivalents

Figure C1. Initial Stability HPLC/RAD Quantitation Profile of PRE-T Forage

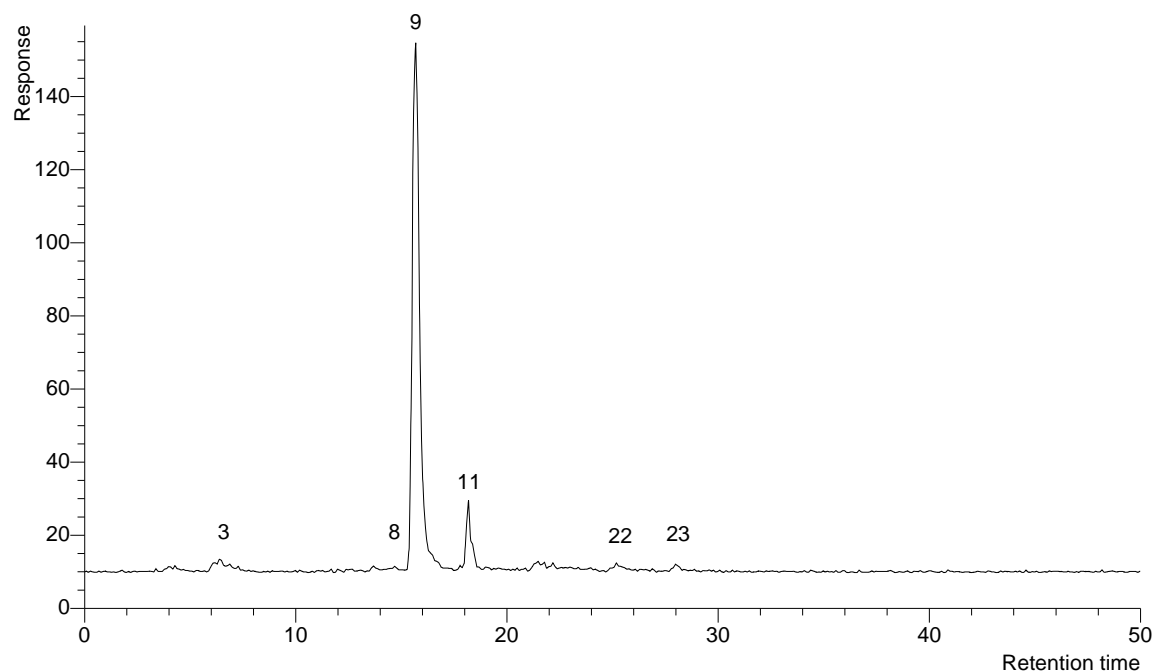


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	1.237	91.21%	98.82%	58920	100.50%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	7.0	DCGA Glucoside	1.25	1.14	0.014
8	14.6	DCGA Malonylglucoside	1.54	1.40	0.017
		Total DCGA Metabolites	2.79	2.54	0.031
9	15.9	DCSA Glucoside	81.66	74.48	0.921
11	18.2	DCSA HMGglucoside	5.71	5.21	0.064
22	26.3	DCSA	3.50	3.19	0.039
		Total DCSA Metabolites	90.87	82.88	1.025
23	28.3	Dicamba	1.77	1.61	0.020

* Dicamba equivalents

Figure C2. Initial Stability HPLC/RAD Quantitation Profile of PRE-T Hay

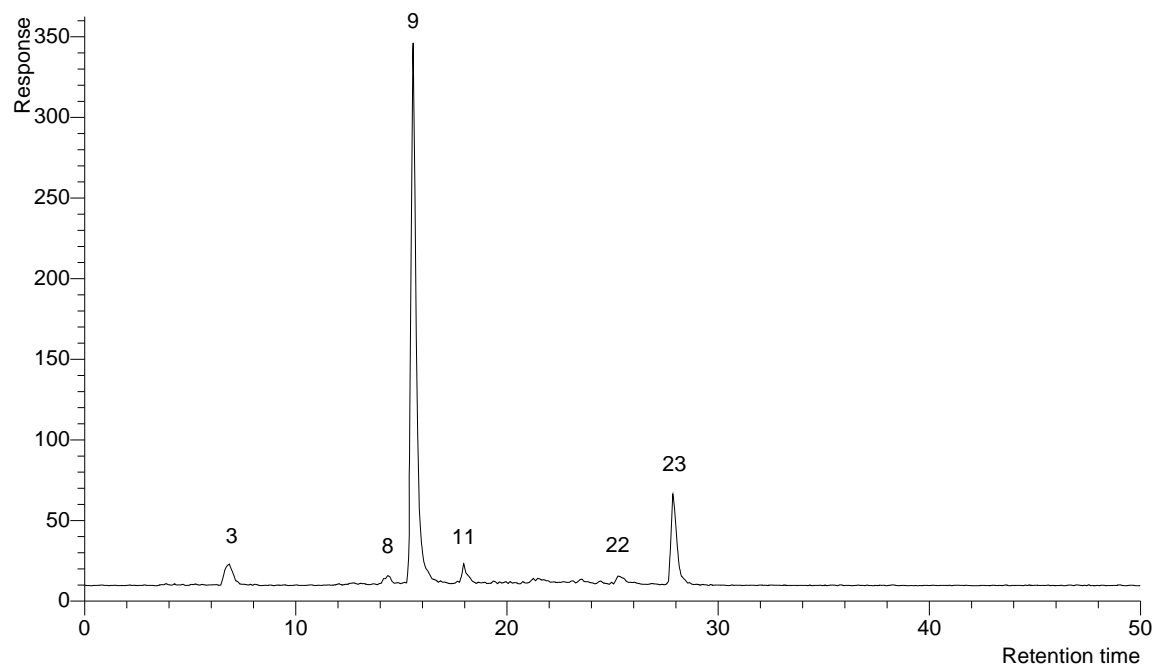


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	1.098	90.88%	100.32%	43913	98.39%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	6.4	DCGA Glucoside	3.80	3.45	0.038
8	14.7	DCGA Malonylglucoside	0.80	0.73	0.008
		Total DCGA Metabolites	4.60	4.18	0.046
9	15.7	DCSA Glucoside	77.92	70.81	0.778
11	18.2	DCSA HMGglucoside	7.34	6.67	0.073
22	25.2	DCSA	1.70	1.54	0.017
		Total DCSA Metabolites	86.96	79.03	0.868
23	28.0	Dicamba	0.94	0.85	0.009

* Dicamba equivalents

Figure C3. Initial Stability HPLC/RAD Quantitation Profile of POE-T Hay

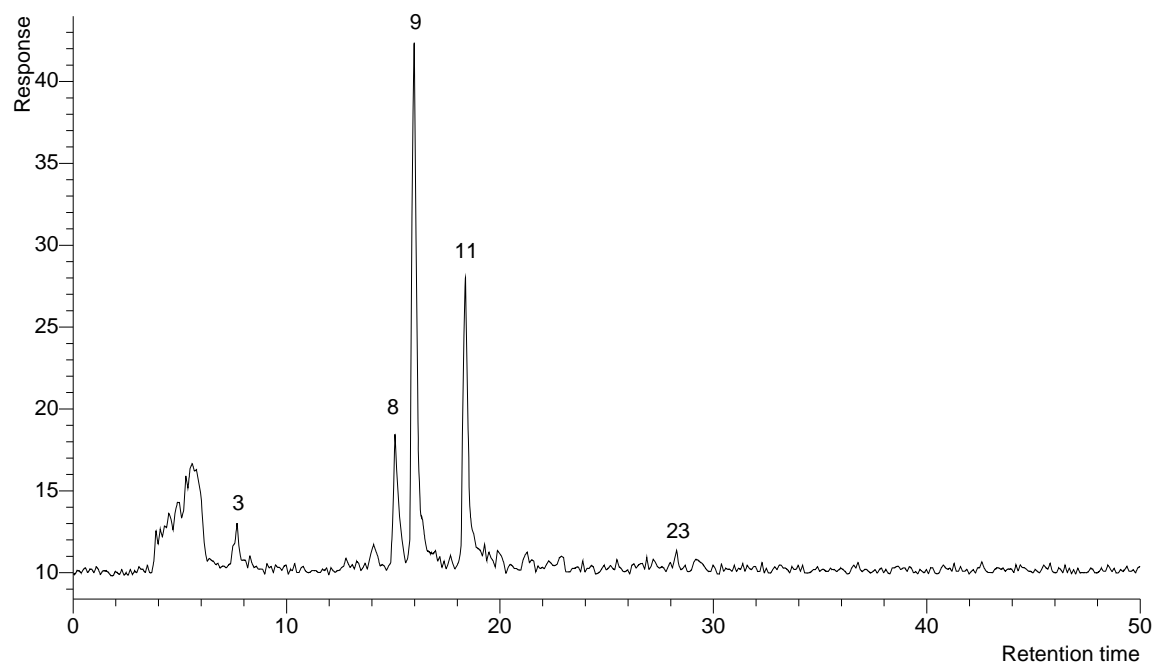


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	39.030	95.30%	99.61%	86113	98.34%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	6.9	DCGA Glucoside	4.53	4.32	1.685
8	14.4	DCGA Malonylglucoside	1.69	1.61	0.629
		Total DCGA Metabolites	6.22	5.93	2.314
9	15.6	DCSA Glucoside	70.58	67.26	26.253
11	18.0	DCSA HMGglucoside	2.60	2.48	0.967
22	25.3	DCSA	2.03	1.93	0.755
		Total DCSA Metabolites	75.21	71.68	27.975
23	27.9	Dicamba	12.94	12.33	4.813

* Dicamba equivalents

Figure C4. Initial Stability HPLC/RAD Quantitation Profile of POE-T Seed



HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Sample Preparation Recovery**	Total dpm Injected	Column Recovery
B	0.388	57.00%	88.45%	16953	99.56%

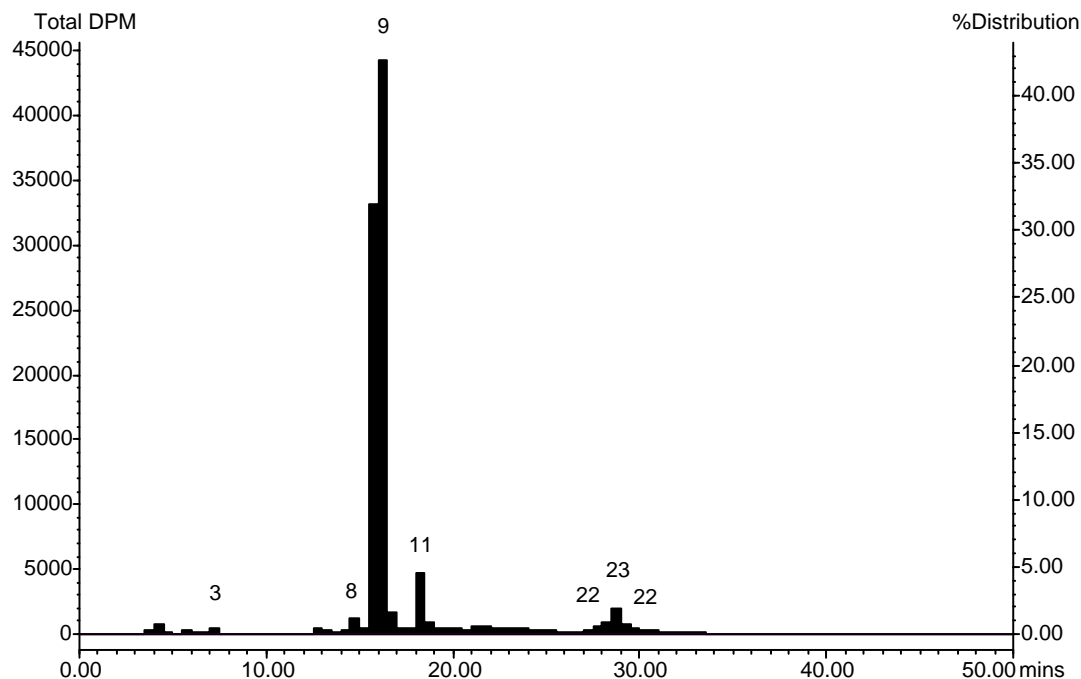
Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	7.7	DCGA Glucoside	4.23	2.12	0.008
8	15.1	DCGA Malonylglucoside	7.49	3.76	0.015
		Total DCGA Metabolites	11.72	5.88	0.023
9	16.0	DCSA Glucoside	29.31	14.71	0.057
11	18.4	DCSA HMGglucoside	18.44	9.26	0.036
		Total DCSA Metabolites	47.75	23.97	0.093
23	28.3	Dicamba	1.22	0.61	0.002

* Dicamba equivalents

** Includes extract evaporation, centrifugation, SPE and final evaporation

Extractability is not normalized (pellet combustion was not done)

Figure C5. Final Stability HPLC/LSC Quantitation Profile of PRE-T Forage

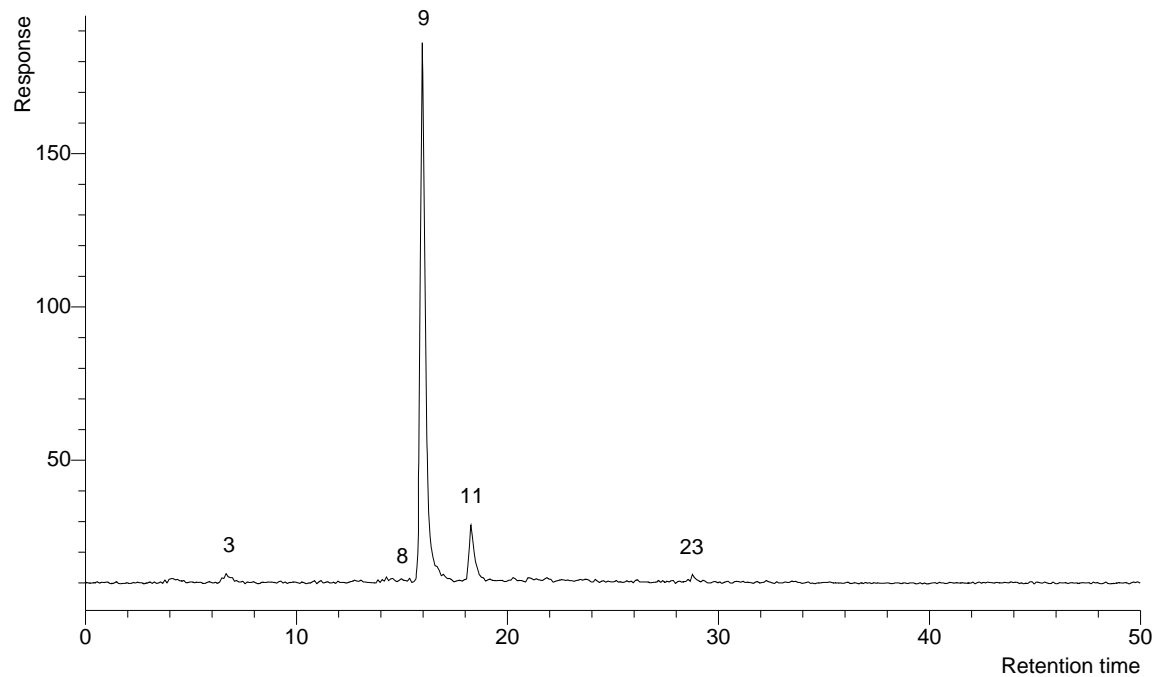


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	1.355	90.02%	94.33%	104796	99.07%

Peak Number	LSC Fractions Comprising Peak	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	14-16	7.3	DCGA Glucoside	0.65	0.59	0.008
8	29-31	14.8	DCGA Malonylglucoside	1.87	1.68	0.023
			Total DCGA Metabolites	2.52	2.27	0.031
9	32-35	16.3	DCSA Glucoside	76.66	69.01	0.935
11	36-38	18.3	DCSA HMGglucoside	5.81	5.23	0.071
22	55-57, 59-64	28.25, 29.25	DCSA	3.79	3.41	0.046
			Total DCSA Metabolites	86.26	77.65	1.052
23	58	28.8	Dicamba	1.90	1.71	0.023

*Dicamba equivalents

Figure C6. Final Stability HPLC/RAD Quantitation Profile of PRE-T Hay

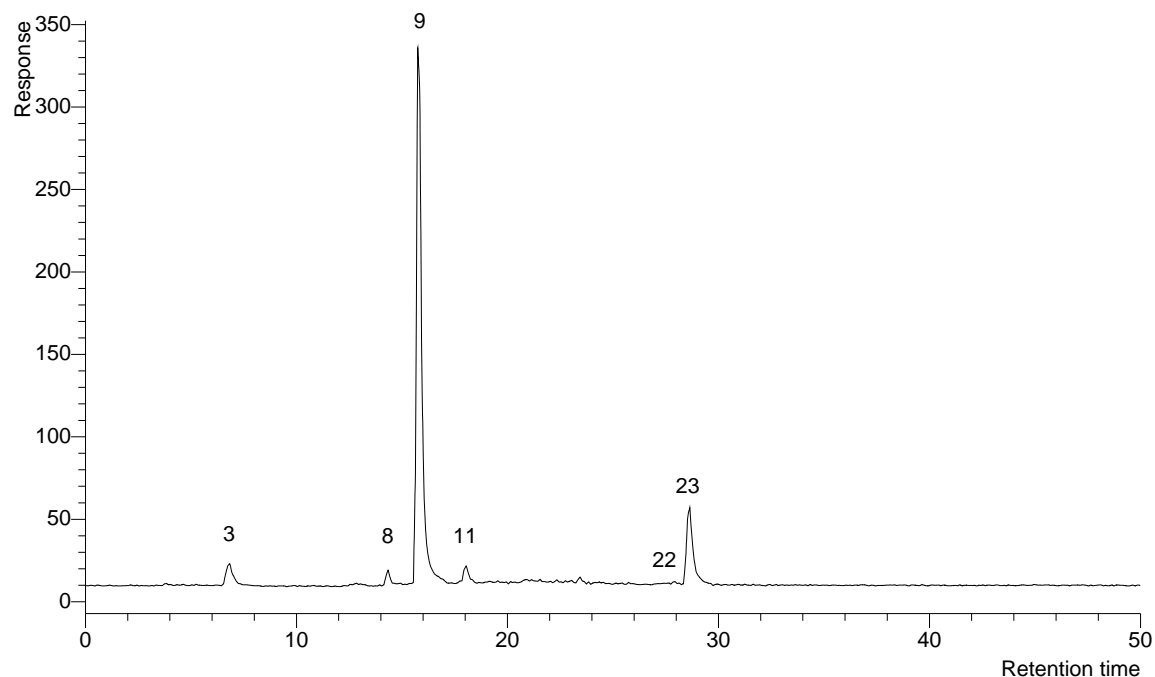


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	1.087	90.64%	101.06%	39741	99.57%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	6.7	DCGA Glucoside	2.45	2.22	0.024
8	15.4	DCGA Malonylglucoside	0.77	0.70	0.008
		Total DCGA Metabolites	3.22	2.92	0.032
9	16.0	DCSA Glucoside	78.22	70.90	0.771
11	18.3	DCSA HMGglucoside	9.25	8.38	0.091
		Total DCSA Metabolites	87.47	79.28	0.862
23	28.8	Dicamba	1.20	1.09	0.012

*Dicamba equivalents

Figure C7. Final Stability HPLC/RAD Quantitation Profile of POE-T Hay

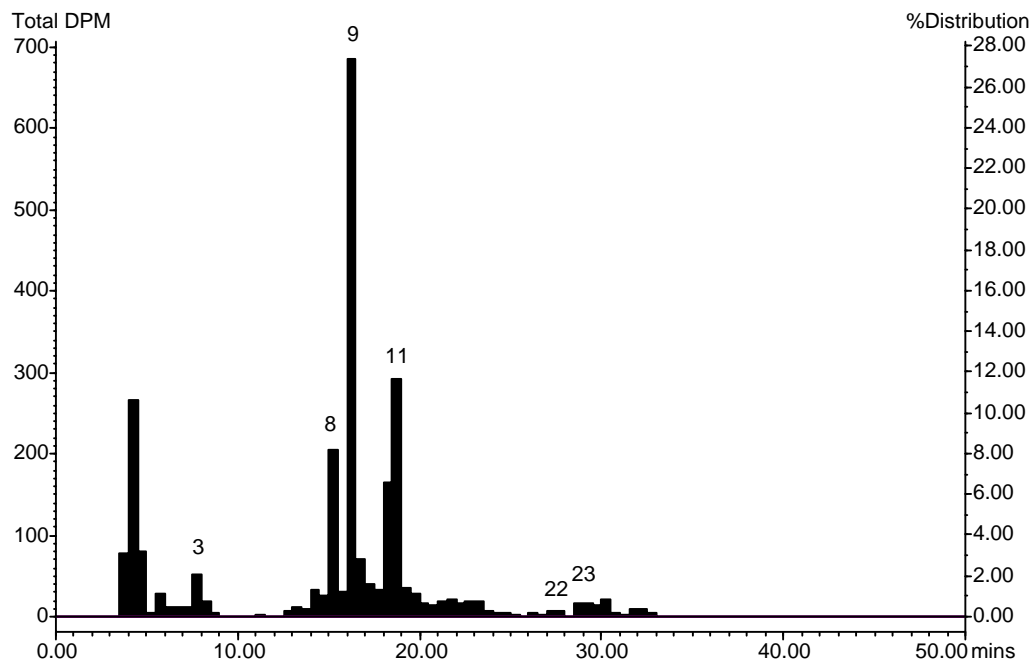


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	39.342	95.68%	100.62%	83410	100.37%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	6.8	DCGA Glucoside	4.25	4.07	1.600
8	14.3	DCGA Malonylglucoside	1.81	1.73	0.681
		Total DCGA Metabolites	6.06	5.80	2.281
9	15.8	DCSA Glucoside	71.17	68.10	26.790
11	18.0	DCSA HMGglucoside	2.40	2.30	0.903
22	27.6,27.9	DCSA	0.94	0.90	0.354
		Total DCSA Metabolites	74.51	71.29	28.047
23	28.6	Dicamba	12.96	12.40	4.878

*Dicamba equivalents

Figure C8. Final Stability HPLC/LSC Quantitation Profile of POE-T Seed

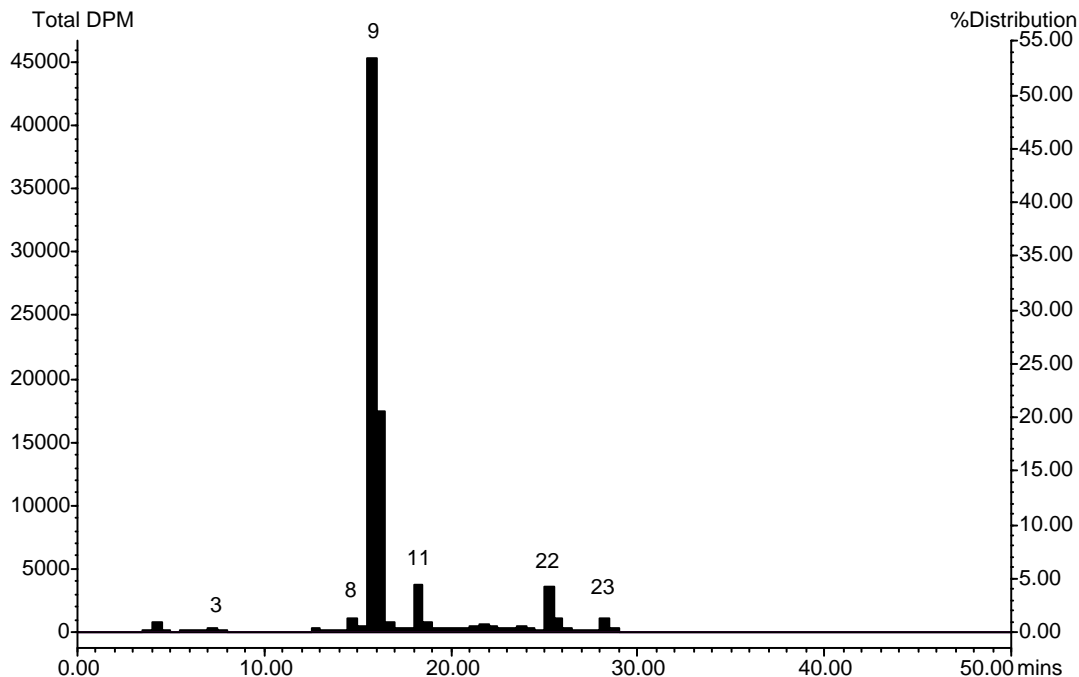


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	0.383	55.52%	97.96%	2731	91.65%

Peak Number	LSC Fractions Comprising Peak	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	15-18	7.8	DCGA Glucoside	3.43	1.71	0.007
8	30-32	15.3	DCGA Malonylglucoside	9.87	4.92	0.019
			Total DCGA Metabolites	13.30	6.63	0.025
9	33-35	16.3	DCSA Glucoside	31.78	15.84	0.061
11	37-39	18.8	DCSA HMGglucoside	19.66	9.80	0.038
22	54-56	27.3	DCSA	0.63	0.31	0.001
			Total DCSA Metabolites	52.07	25.95	0.099
23	58-60	29.3	Dicamba	1.60	0.80	0.003

*Dicamba equivalents

Figure C9. Extended Stability HPLC/LSC Quantitation Profile of PRE-T Forage

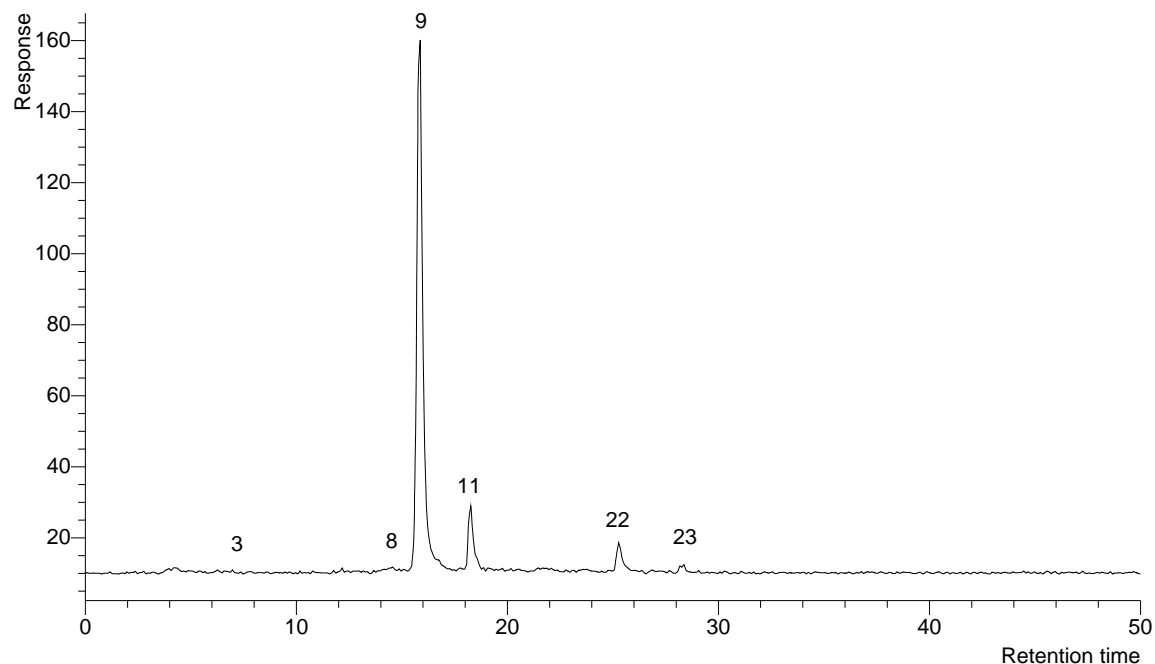


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	1.364	90.09%	101.32%	85461	99.24%

Peak Number	LSC Fractions Comprising Peak	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	14-16	7.3	DCGA Glucoside	0.61	0.55	0.007
8	29-31	14.8	DCGA Malonylglucoside	1.88	1.69	0.023
			Total DCGA Metabolites	2.49	2.24	0.031
9	32-35	15.8	DCSA Glucoside	75.31	67.85	0.925
11	36-38	18.3	DCSA HMGglucoside	5.68	5.12	0.070
22	50-54	25.3	DCSA	6.29	5.67	0.077
			Total DCSA Metabolites	87.28	78.63	1.073
23	56-59	28.3	Dicamba	1.68	1.51	0.021

*Dicamba equivalents

Figure C10. Extended Stability HPLC/RAD Quantitation Profile of PRE-T Hay

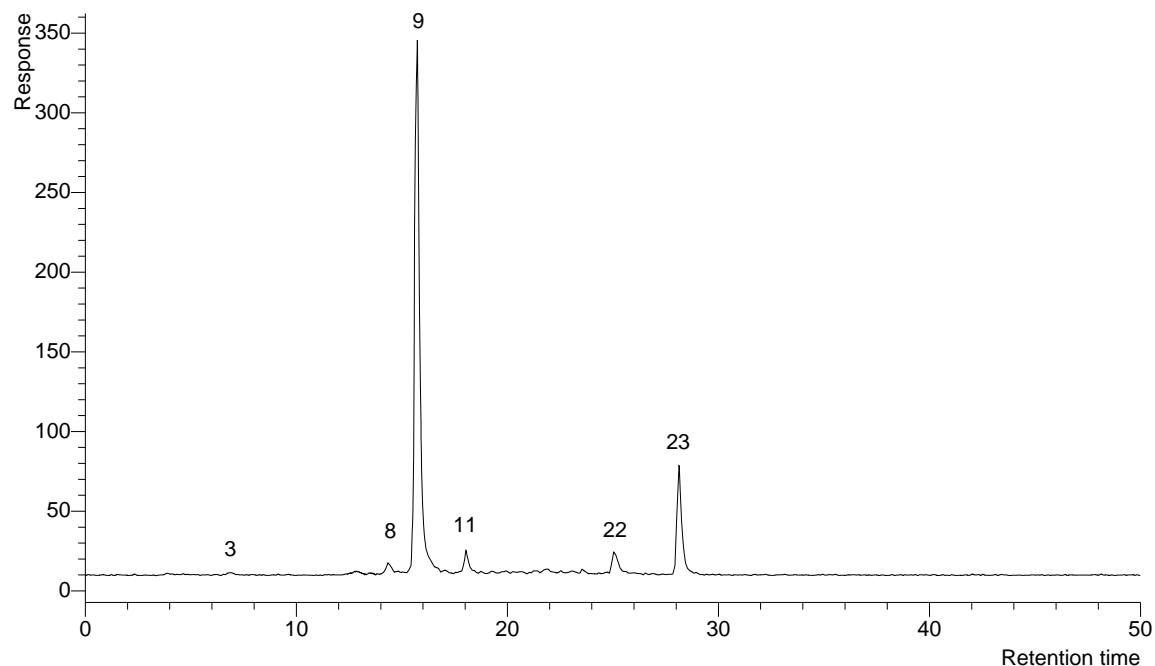


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	1.057	87.63%	100.75%	38930	102.17%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	7.0	DCGA Glucoside	1.28	1.12	0.012
8	14.6	DCGA Malonylglucoside	1.48	1.30	0.014
		Total DCGA Metabolites	2.76	2.42	0.026
9	15.9	DCSA Glucoside	75.27	65.96	0.697
11	18.3	DCSA HMGglucoside	8.89	7.79	0.082
22	25.3	DCSA	4.58	4.01	0.042
		Total DCSA Metabolites	88.74	77.76	0.822
23	28.4	Dicamba	1.06	0.93	0.010

*Dicamba equivalents

Figure C11. Extended Stability HPLC/RAD Quantitation Profile of POE-T Hay

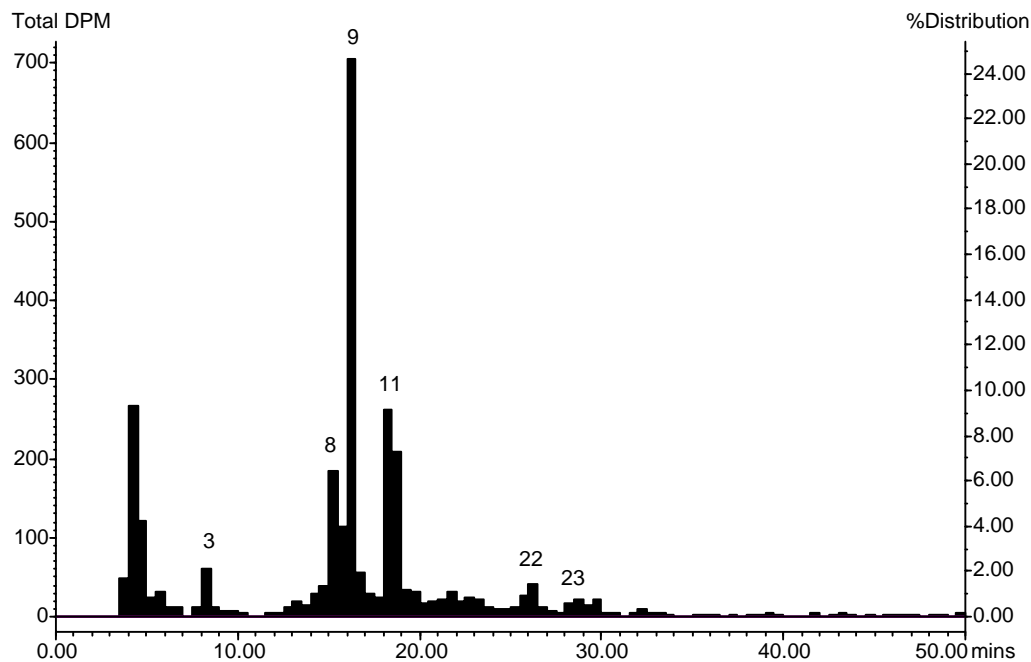


HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	35.107	91.85%	100.12%	82712	99.00%

Peak Number	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	6.8	DCGA Glucoside	0.61	0.56	0.197
8	14.3	DCGA Malonylglucoside	2.14	1.97	0.690
		Total DCGA Metabolites	2.75	2.53	0.887
9	15.7	DCSA Glucoside	72.18	66.30	23.275
11	18.0	DCSA HMGglucoside	3.07	2.82	0.990
22	25.0	DCSA	3.52	3.23	1.135
		Total DCSA Metabolites	78.77	72.35	25.400
23	28.1	Dicamba	14.10	12.95	4.547

*Dicamba equivalents

Figure C12. Extended Stability HPLC/LSC Quantitation Profile of POE-T Seed



HPLC Method	Matrix TRR (mg/kg, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
B	0.402	54.52%	97.32%	2704	105.91%

Peak Number	LSC Fractions Comprising Peak	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	16-19	8.3	DCGA Glucoside	3.17	1.78	0.007
8	30-32	15.3	DCGA Malonylglucoside	9.09	5.11	0.021
			Total DCGA Metabolites	12.26	6.89	0.028
9	32-35	16.3	DCSA Glucoside	29.63	16.65	0.067
11	37-39	18.3	DCSA HMGglucoside	17.61	9.90	0.040
22	51-55	26.3	DCSA	3.48	1.96	0.008
			Total DCSA Metabolites	50.72	28.50	0.115
23	57-59	28.8	Dicamba	1.64	0.92	0.004

*Dicamba equivalents

12 Appendix D – Report Amendment

The report for Monsanto study 06-98-M-1 is hereby amended to correct an incorrect OECD guideline number, to update references to Monsanto reports, and to correct minor typographical errors or errors of omission. These changes increased the accuracy and consistency of the report and had no impact on the study results. The following is a list of the changes in the amended report:

- 1) Page 1 (Title page) – changed “OECD 517” to “OECD 501”; added “Amended Report Date” and date of report amendment; added “Amended Report Number” and new report number (“MSL0022659”) with old report number in parentheses; added “Amendment 1”; changed total number of pages from “444” to “446”.
- 2) Pages 3-5 – provided new compliance, quality assurance and certification pages.
- 3) Page 6 (Study Information) – changed “Report Number: MSL-20277” to “Amended Report Number: MSL0022659 (original report number MSL-20277)”; added “Amended Report Date: March 19, 2010”; added amended report to records retention statement; changed “©2009” to “©2010”.
- 4) Page 9 (Table of Contents) – added this appendix (“Appendix D – Report Amendment”) and page number.
- 5) Page 12-19 (Table of Figures) – updated the table of figures to reflect changes in the titles of figures as described in points 21-24 and 27, below.
- 6) Page 20 (Abbreviations and Acronyms) – deleted superfluous entry for “dpm”.
- 7) Page 21 – changed “OECD Guideline for the Testing of Chemicals No. 517” to “OECD Guideline for the Testing of Chemicals No. 501”.
- 8) Page 26 – changed “PRE-C and POE-C, respectively” to “PRE-C and POE-C seed, respectively”.
- 9) Page 29 – information for standards **XV** and **XVI** was reversed in table; this was corrected.
- 10) Pages 32, 36, 46, 55, 78, 99, 430 – minor typographical or grammatical errors were corrected.
- 11) Page 46 – changed “Figure 18 and Figure 19” to “Figure 18 and Figure 19 for POE-T seed and Figure 97 for POE-T hay”.
- 12) Page 52 – changed “(Peak 18 was” to “(Peak 8 was”.
- 13) Page 58 – changed “PRE-T unextracted” to “PRE-T seed unextracted”.
- 14) Page 59 – changed “2.5-year” to “2-year” and “2.5 years” to “approx. 2 years”.
- 15) Page 70 – changed “*m/z* 171/173” to “*m/z* 177/179”.
- 16) Page 72 – changed “Method C gave” to “Method C (Figure 45) gave”; changed “789/791/793 [2M-H]” to “789/791/793 [2M-2H+Na]”.

- 17) Page 73 – changed “Method B (Figure 53) gave” to “Method B (not shown) gave”; added “(not shown)” to end of last paragraph on the page.
- 18) Page 75 – changed “POE-T hay” to “POE-T forage”.
- 19) Page 79 – changed “doublet at m/z 511” to “doublet at m/z 511/513”.
- 20) Page 81 – changed “spectrum gave” to “spectrum (Figure 82) gave”; changed “ m/z 219” to “ m/z 219/221”.
- 21) Page 98 – changed “Figure 12, pg 66” to “Figure 12, pg 63”.
- 22) Page 147 (Figure 19) – changed “HPLC/LSC” to “HPLC/RAD” in figure title.
- 23) Pages 147, 152-154, 188-189 (Figures 19, 24-26, 60-61) – added “Using HPLC Method B” to figure titles.
- 24) Page 179 (Figure 51) – changed “of Peak 8” to “of Methylated Peak 8” in figure title.
- 25) Page 181 (Figure 53) – changed “Methylated Mild Base and Acid Hydrolysate of Peak 8” to “Mild Base and Acid Hydrolysate of Methylated Peak 8” in figure title; changed “Peak 8” to “Hydrolysate of Methylated Peak 8” in peak label of top chromatogram; added additional explanatory annotation to chromatograms.
- 26) Pages 190, 196, 203 (Figures 62, 68, 75) – added annotation “RAD” and “UV” to the chromatograms.
- 27) Page 207 (Figure 79) – changed “Nominal Mass 367” to “Nominal Mass 368”.
- 28) Page 227 (Figure 99) – added “(top) and m/z 543 Selected Ion Chromatogram (bottom)” to figure title.
- 29) Page 241 (Other Information) – added amended report date.
- 30) Page 243 (References) – changed “MSL0022267 (2009)” to “MSL0022660 (2010)” and “Determination” to “Magnitude”; changed “MSL0022390 (2009)” to “MSL0022661 (2010)” and “Mierkowski, M.J.” to “Mierkowski, M.”.
- 31) Page 428 – changed “DCGA malonylglucoside **3** and DCGA glucoside **8**” to “DCGA glucoside **3** and DCGA malonylglucoside **8**”.
- 32) All changed pages – added “Amendment 1” to the footer.