

TRADE SECRET

Study Title

**INDEPENDENT LABORATORY VALIDATION OF DUPONT-5367,
"ANALYTICAL ENFORCEMENT METHOD FOR THE DETERMINATION OF
THIFENSULFURON METHYL, METSULFURON METHYL, CHLORSULFURON,
TRIBENURON METHYL, AND FLUPYRSULFURON METHYL IN CEREALS
(WHEAT GRAIN, FORAGE AND STRAW)" IN WHEAT GRAIN, BARLEY
GRAIN, CORN GRAIN, AND TOMATO**

Test Guideline

U.S. EPA Pesticide Assessment Guidelines
OPPTS 860.1340
PR Notice 96-1

European Commission, Directorate General Health and Consumer Protection.
"Guidance Document on Residue Analytical Methods", Document No.
SANCO/825/00 rev. 6, 20/06/00

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Testing Facility

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Project Identification

DuPont-8054

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GRAIN, CORN GRAIN, AND TOMATO**

Anne M. Pentz and Frederick Q. Bramble, Jr.

1.0 ABSTRACT

The purpose of this study was to independently validate the analytical method DuPont-5367 for the determination of thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl in wheat grain, barley grain, corn grain, and tomato by liquid chromatography with tandem mass spectrometer detection (LC/MS/MS). Matrix samples were fortified with fortification solutions containing a mixture of all the test analytes. Fortifications were made at the Limit of Quantitation (LOQ) of 0.010 ppm (mg/kg) and 0.10 ppm (10×LOQ).

This method successfully passed independent laboratory validation (ILV) on the second attempt for wheat and barley grain. The ILV for corn grain and tomato was successful on the first attempt with the exception of tribenuron methyl on corn grain where recoveries were consistently low in range of 51-61%*. For each matrix, a full set consisting of 2 untreated controls, 5 fortifications at 0.010 ppm, and 5 fortifications at 0.10 ppm (as defined in the guidelines for this study) were analyzed. The method was performed as written, with the exception of minor modifications to the extract purification procedure to reduce analyte degradation and use of tandem MS detection for quantitative analysis.

The average recoveries for each analyte determined in wheat and barley grain follow.

Matrix	ppm mg/kg	Thifensulfuron Methyl (M6316)			Metsulfuron Methyl (T6376)			Chlorsulfuron (W4189)			Tribenuron Methyl (L5300)			Flupyrsulfuron Methyl (KE459)			n
		Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	
Wheat Grain	0.010	94%	4%	5%	103%	4%	4%	92%	5%	6%	78%	8%	11%	93%	13%	14%	5
	0.10	93%	4%	4%	99%	5%	5%	87%	4%	4%	76%	9%	11%	96%	7%	8%	5
	All	93%	4%	4%	101%	5%	5%	89%	5%	6%	77%	8%	10%	94%	10%	11%	10
Barley Grain	0.010	97%	11%	11%	90%	3%	3%	96%	14%	14%	79%	6%	7%	90%	6%	7%	5
	0.10	94%	8%	9%	94%	6%	6%	89%	6%	7%	85%	4%	5%	109%	6%	5%	5
	All	96%	9%	10%	92%	5%	5%	92%	11%	12%	82%	6%	7%	100%	12%	12%	10
Combined Grain	0.010	96%	8%	8%	96%	7%	7%	94%	10%	11%	78%	7%	9%	91%	9%	10%	10
	0.10	94%	6%	7%	97%	6%	6%	88%	5%	6%	81%	8%	10%	103%	9%	9%	10
	All	95%	7%	8%	97%	6%	7%	91%	8%	9%	79%	7%	9%	97%	11%	11%	20

Avg: average, SD: standard deviation, %RSD: percent relative SD, n: number of samples

The average recoveries for each analyte determined in corn grain and tomato follow.

* Since tribenuron methyl products are not registered for corn or oily crops and the results were consistent, these findings do not alter the successful outcome of this method ILV.

Matrix	ppm mg/kg	Thifensulfuron Methyl (M6316)			Metsulfuron Methyl (T6376)			Chlorsulfuron (W4189)			Tribenuron Methyl (L5300)			Flupyr sulfuron Methyl (KE459)			n
		Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	
Corn Grain	0.010	91%	9%	10%	96%	2%	2%	87%	3%	4%	58%	3%	5%	97%	4%	4%	5
	0.10	91%	5%	6%	97%	1%	2%	85%	2%	3%	53%	2%	4%	96%	3%	3%	5
	All	91%	7%	8%	97%	2%	2%	86%	3%	3%	55%	3%	6%	97%	3%	3%	10
Tomato	0.010	96%	5%	5%	94%	4%	5%	89%	5%	5%	70%	4%	6%	98%	4%	4%	5
	0.10	106%	6%	5%	100%	4%	4%	94%	6%	6%	72%	2%	3%	101%	5%	5%	5
	All	101%	7%	7%	97%	5%	5%	92%	6%	6%	71%	3%	5%	99%	4%	4%	10

Avg: average, SD: standard deviation, %RSD: percent relative SD, n: number of samples

Approximately 6 person-hours were required to prepare a set of 6 samples for this analysis. The automated LC/MS/MS analysis required approximately one hour per sample or standard injected. The HPLC analysis was run automated overnight.

2.0 INTRODUCTION

The EPA (References 1 and 2) and EU (Reference 3) require independent laboratory validation of MRL enforcement methods. The work undertaken in this study was done to comply with this requirement. The method reported in Reference 4 was validated for thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyr sulfuron methyl in wheat and barley grain, corn grain, and tomato. Fortifications were made at 0.010 ppm (LOQ) and at 0.10 ppm (10×LOQ), as called for in the EU guidelines (Reference 3). The EU MRLs for thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyr sulfuron methyl in cereal grains are in the range of 0.010–0.050 mg/kg.

Thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyr sulfuron methyl are sulfonyleurea herbicides used for control of broadleaf weeds and selected grasses in cereal crops. The method validated in this study involves extraction, extract purification, and quantitation of these analytes in cereal grain, forage, and straw. Wheat and barley grains were evaluated as being representative of cereal grains. Corn grain was evaluated as being representative of high fat/oily crop. Tomato was evaluated as being representative of high water and acid crops. Samples of each matrix were extracted using a pH 6 buffered aqueous solution of potassium phosphate. Sample extracts were purified and concentrated by solid-phase extraction using disposable Envi™-Carb solid phase extraction columns. Instrumental analysis of all matrices was performed by reversed-phase HPLC coupled to a mass spectrometer by electrospray interface (ESI) operating in positive mode with tandem mass spectrometry (MS/MS) detection.

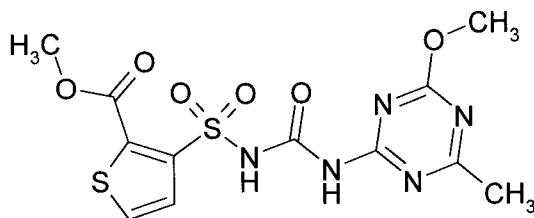
The method was performed as written, with minor modifications.

3.0 MATERIALS AND METHODS

3.1 Test Substances

Thifensulfuron Methyl

Structure



DuPont Code: DPX-M6316

Trivial Name: thifensulfuron methyl

IUPAC Chemical Name: 2-thiophenecarboxylic acid, 3-[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-,methyl ester

CAS Chemical Name:

methyl 3-[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate

CAS Registry Number: 79277-27-3

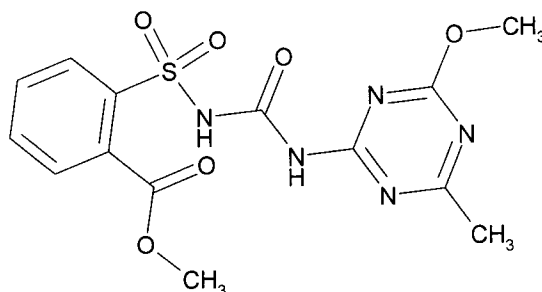
Molecular Weight: 387.40 g/mole

Monoisotopic Mass: 387.03 g/mole

pKa: 4.0

Metsulfuron Methyl

Structure



DuPont Code: DPX-T6376

Trivial Name: metsulfuron methyl

IUPAC Chemical Name: not available

CAS Chemical Name:

methyl 2-[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate

CAS Registry Number: 74223-64-6

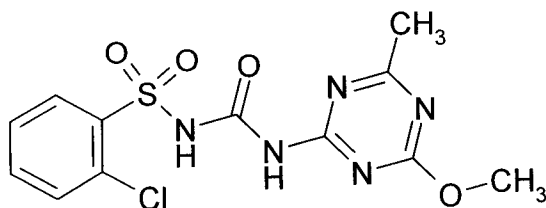
Molecular Weight: 381.37g/mole

Monoisotopic Mass: 381.07 g/mole

pKa: 3.3

Chlorsulfuron

Structure



DuPont Code: DPX-W4189

Trivial Name: chlorsulfuron

IUPAC Chemical Name: 1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl) urea

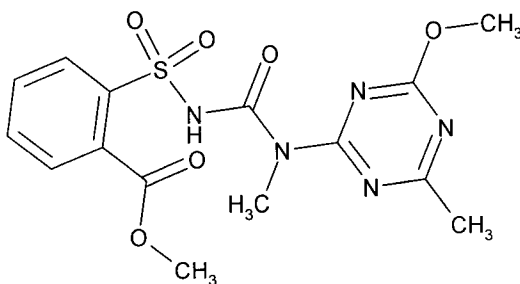
CAS Chemical Name: 2-chloro-N[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide

CAS Registry Number: 64902-72-3

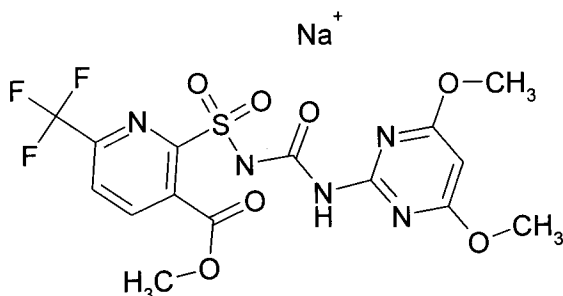
Molecular Weight: 357.78 g/mole

Monoisotopic Mass: 357.03 g/mole

pKa: 3.6

Tribenuron MethylStructure**DuPont Code:** DPX-L5300**Trivial Name:** tribenuron methyl**IUPAC Chemical Name:** 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoylsulfamoyl] benzoic acid**CAS Chemical Name:**

methyl 2-[[[N-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)methylamino]carbonyl]amino]sulfonyl]-benzoate

CAS Registry Number: 101200-48-0**Molecular Weight:** 395.39 g/mole**Monoisotopic Mass:** 395.09 g/mole**pKa:** 5.0**Flupyr-sulfuron Methyl**Structure**DuPont Code:** DPX-KE459**Trivial Name:** flupyr-sulfuron methyl**IUPAC Chemical Name:** methyl 2-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]-6-(trifluoromethyl)nicotinate, monosodium salt**CAS Chemical Name:** methyl-2-[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonylamino]sulfonyl]-6-(trifluoromethyl)-3-pyridinecarboxylate sodium salt**CAS Registry Number:** 144740-54-5**Molecular Weight:** 464.36 + 22.99 g/moleacid form in solution **465.36 g/mole****Monoisotopic Mass:** 464.05 + 22.99 g/moleacid form in solution **465.06 g/mole****pKa:** 4.9**3.2 Test System**

The test system for this study consisted of wheat grain, barley grain, corn grain, and tomato. All samples were maintained under frozen conditions prior to and during the study.

DuPont Crop Protection supplied control wheat grain, corn grain, and tomato. The wheat grain sample was obtained from the control plot of a magnitude of residue (MOR) study (DuPont Study No. AMR-2570-93) conducted in 1993 at Garden City, KS. Husks and hulls were removed, and the sample was ground into coarse flour with a Quaker mill. The corn grain sample was obtained from the control plot of a MOR study (DuPont Study No. AMR 4336-97) conducted in 1998 at Paynesville, MN. The sample composite was homogenized with dry ice using a Quaker mill. The tomato sample was obtained from the control plot of a MOR study (DuPont Study No. AMR 3734-96) conducted in 1996 at Madera, CA. The sample composite was homogenized with dry ice using a Hobart processor.

Control barley grain (variety – Nomini, whole grain in hull) was obtained from Calvert Farms in Rising Sun, MD. The grain was grown at Summer Creek Farm, Frederick, MD under certified organic growing conditions and was harvested in June, 2000. Preprocessing was done at Corn grainerstone Grain Processing in Robeson, PA, and was limited to removal of husks and pieces of straw. It was maintained at ambient conditions until purchased by the Study Director on December 22, 2000.

3.3 *Equipment*

The equipment used in this validation was identical or equivalent to that specified in the original method. Exceptions were as follows:

HPLC: As in the analytical method, an Agilent HP1100 was used, but the autosampler system was temperature controlled using a model G.A.F.4 chiller tray (GIRA SA, Morlass, France).

Mass Spectrometer: A Micromass Quattro II triple quadrupole mass spectrometer using an electrospray interface (ESI) and MassLynx NT Version 3.1 software (Waters Corporation, Milford, Mass.) with Valco zero-dead volume 3-port connector for 1/10 splitflow to mass spectrometer.

pH meter: Corn graining Model 430, Serial No. 009240 (Fisher Scientific)

Balances: AE163 – Serial No. C24118 and PM400 – Serial No. F08690 (Mettler Scientific)

Ultrasonic Bath: Branson 2200[®] Ultrasonic Cleaner, 0.75 gal. cap. (Branson Ultrasonics Corp.)

3.4 *Reagents and Standards*

Reagents which were not identical to those specified in DuPont-5367 are listed below:

Acetic Acid: OmniTrace[®], 99%, Catalog No. AX077-1 (EM Science). This reagent was not included in *Section 3.2 Reagents and Standards* section of DuPont-5367 report.

Hydrochloric Acid: GR, 500 mL, Catalog No. HX0603-4 (EM Science). This reagent was not included in *Section 3.2 Reagents and Standards* section of DuPont-5367 report.

Formic Acid: GR, ACS, 98%, Catalog No. FX0440-11 (EM Science)

Ammonium Hydroxide: GR 28-30%, Catalog No. AX1303-13 (EM Science)

Phosphoric acid is included under Section 3.2 Reagents and Standards in DuPont-5367 report, but is not used in the method.

3.5 *Principles of the Analytical Method*

Barley grain, wheat grain, corn grain, and tomato samples are extracted twice by homogenization in a 20 mM potassium phosphate pH 6 buffered solution. A 10-mL aliquot of the extract is purified and concentrated by solid-phase extraction using disposable 1-gram Envi[™]-Carb SPE columns. An aqueous sample extract aliquot is applied to the ENVI[™]-Carb SPE column and the eluate discarded. The Envi[™]-Carb SPE column is washed with distilled, deionized water followed by methanol. The five analytes are eluted from the column with 0.1 M formic acid in 10% methanol/90% methylene chloride solution. This final eluate is neutralized with

NH₄OH, evaporated to dryness, and reconstituted in a 10% acetonitrile/90% aqueous 50 mM ammonium acetate solution in preparation for LC/MS/MS analysis†.

3.6 *Modifications, Interpretations, and Critical Steps*

The method was followed as written, with minor modifications.

- DuPont-5367, Section 4.2.2: (1) 0.01 M Acetic Acid solution was replaced with aqueous 50 mM ammonium acetate buffered to pH 6.2. Solution was prepared by dissolving 3.854 g of ammonium acetate in 1 L of distilled, deionized water and adjusting to pH 6.2 using 1% solution of NH₄OH (28-30% concentrate). (2) 10% acetonitrile/90% 0.01 M acetic acid solution was replaced with 10% acetonitrile/90% aqueous 50 mM ammonium acetate. Reason: The acidity of the original solution contributed to rapid degradation of tribenuron methyl (DPX-L5300).
- DuPont-5367, Section 4.2.3: Stock Standard Solutions were prepared at 100-µg/mL concentrations instead of 1000-µg/mL concentration (10 mg of standard dissolved in 100 mL of acetonitrile using volumetric flask). This was not a critical modification.
- DuPont-5367, Section 4.2.5: Chromatographic standards were prepared using 10% acetonitrile/90% aqueous 50 mM ammonium acetate (see DuPont-5367, Section 4.2.2 comment above) in place of 10% acetonitrile/90% 0.01 M acetic acid solution. Reason: The acidity of the original solution contributed to rapid degradation of tribenuron methyl (DPX-L5300).
- DuPont-5367, Section 4.2.10, point 4: 10 mL instead of 5 mL methanol was added to the SPE cartridge. Reason: Additional methanol added to insure water was removed from SPE cartridge prior to elution of analytes in acidic organic solution.
- DuPont-5367, Section 4.2.10, point 5: The SPE drying time under full vacuum was extended from approximately 2 minutes to at least 30 minutes. Reason: Increase drying time to insure water is removed from SPE cartridge prior to elution of analytes in an acidic organic solution.
- DuPont-5367, Section 4.2.10, point 6: The acid content of the 10% methanol/90% methylene chloride/0.1 M formic acid elution solution containing the extracted analytes was adjusted to near neutral by the addition of 60 µL of concentrated ammonium hydroxide prior to evaporation to dryness. Reason: Minimize acidic hydrolysis of tribenuron methyl during evaporation.
- DuPont-5367, Section 4.2.10, point 8: Samples were reconstituted in 10% acetonitrile/90% aqueous 50 mM ammonium acetate (see DuPont-5367, Section 4.2.2 comment above) in place of 10% acetonitrile/90% 0.01 M acetic

† Adjusting pH with NH₄OH and sample reconstitution using aqueous 50 mM ammonium acetate instead of 0.01M acetic acid are modifications to the original method made to reduce degradation of tribenuron methyl (DPX-L5300) under more acidic conditions.

acid solution. Reason: The acidity of the original solution contributed to rapid degradation of tribenuron methyl (DPX-L5300).

- DuPont-5367, Section 4.2.10, point 9: Final extract samples were filtered using 0.45 μm PTFE syringe filter. Although a 0.2 μm PTFE filter is listed in the Materials Section of the DuPont-5367, no final extract filtration is described in the sample preparation for instrument analysis. Reason: Removing particulates from samples is necessary to prevent problems in the chromatographic system.
- DuPont-5367, Section 4.3.1: The aqueous 0.1M acetic acid mobile phase (A) was replaced with aqueous 2 mM aqueous acetic acid mobile phase, and the column temperature was lowered from 35°C to 25°C. Reason: Minimize acidic hydrolysis of tribenuron methyl during on-column elution. No change in chromatographic elution times were observed with these changes.
- DuPont-5367, Section 4.3.1: Typo in Conditions: should be 10% A and 90% B at 45 minutes.
- DuPont-5367, Section 4.3.2: DuPont-5367 provided for ESI-LC/MS analysis using a HP LC/MSD instrument operating in selected ion monitoring (SIM) mode. At request of Sponsor, the independent validation was performed by ESI-LC/MS/MS using a MicroMass Quattro II triple-quad mass spectrometer.

3.7 *Instrumentation*

Method validation data reported in this study were generated using an Agilent HP1100 liquid chromatograph with a chilled autosampler, coupled to a Micromass Quattro II triple-quad mass spectrometer using an electrospray interface (ESI) in positive ion tandem MS (MS/MS) mode. HPLC and MS operating conditions used are presented in Tables 1 and 2, respectively. Specific operating parameters for the LC/MS/MS system are provided in Appendix 1. Two separate molecular ion MS/MS transitions were monitored for each analyte and the Total Ion Chromatograms (TIC) were used to determine quantitative results. Calibration was accomplished using external standards to generate an average response factor (RF_{avg}) from all calibration standards in each set of samples analyzed. Calibration standards of 0.0010, 0.0025, 0.0050, 0.015, and 0.030 ppm ($\mu\text{g}/\text{mL}$) were analyzed with each set. A calibration standard was injected at the beginning and end of each sequence of samples. The injection sequence was organized from lowest to highest expected analyte concentrations. Calibration standard runs were intermixed with the test samples and analyzed before and after every 1–2 samples.

3.8 *Calculations*

Thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl residues were measured as ppm (mg/kg) in wheat grain, barley grain, corn grain, and tomato. As described in the Analytical Method report, DuPont-5367, quantitation was based on analyte response in Calibration Standard and Sample Extract analyses using an average response factor determined from the appropriate Calibration Standards. Recoveries were determined as ppm (mg/kg) residue

concentrations for the calculation of percent recovery values (%Rec) in fortified samples.

Thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl residues found at or above the LOQ are reported to 2 significant figures. Detected residues equal to or above the limit of detection (LOD) but below the LOQ are reported to 1 significant figure. Recoveries for fortified samples are reported to the nearest whole number percentage (%).

The calculation to determine ppm (mg/kg) found in grain test samples by average response factor analysis follows:

$$\text{ppm found} = \frac{(\text{Analyte Peak Area} * / \text{RF}_{\text{avg}})(\text{FV})(\text{DF})}{\text{SW}}$$

* Analyte Peak Area was corrected for interference or contamination detected in control sample by subtracting Peak Area detected in control from Peak Area detected in sample.

where,

RF_{avg.} is average response factor (peak area/μg/mL) for analyte

FV is Final Extract volume (0.002 L) for sample analysis,

DF is Dilution Factor of 20 (200 mL extract/10 mL extract aliquot purified for analysis = 20), and

SW is Sample Weight (10.0 g) of grain extracted.

The % Recovery for fortified grain samples was determined as follows:

$$\% \text{ Recovery} = (\text{ppm found, not rounded}) \times 100 / (\text{ppm applied})$$

Example Calculations

Wheat Grain–0.010 ppm fortification–thifensulfuron methyl (M6316)

Sample ID: Wh-2-010302, References: Appendix 3, Table 1, Figure 3

$$\text{ppm found} = \frac{((3116 - 381 \text{ area}) / 1145213 \text{ area}/\mu\text{g/mL})(2 \text{ mL})(20)}{10.0 \text{ g}} = 0.00955 \mu\text{g/g} = 0.010 \text{ ppm}$$

$$\% \text{ Recovery} = 0.00955 \text{ ppm} \times 100 / 0.010 \text{ ppm} = 96\%$$

Barley Grain–0.10 ppm fortification– metsulfuron methyl (T6376)

Sample ID: Ba-010402-6, References: Appendix 3, Table 1, Figure 4

$$\text{ppm found} = \frac{(15417 / 640393 \text{ area}/\mu\text{g/mL})(2 \text{ mL})(20)}{10.0 \text{ g}} = 0.0963 \mu\text{g/g} = 0.096 \text{ ppm}$$

$$\% \text{ Recovery} = 0.0963 \text{ ppm} \times 100 / 0.10 \text{ ppm} = 96\%$$

4.0 RESULTS AND DISCUSSION

4.1 *Independent Laboratory Validation (ILV) Results*

An ILV was carried out for the determination of thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl residues in wheat grain, barley grain, corn grain, and tomato by LC/MS/MS analysis. The ILV was successful on the second attempt for wheat and barley grain. In first attempt, significant degradation of tribenuron methyl was observed‡. The method procedures were modified (see *Section 3.6*) to correct this problem. The ILV was successful on the first attempt for corn grain and tomato with the exception of tribenuron methyl on corn grain where recoveries were consistently low in range of 51-61%.

4.1.2 *Calibration Information*

Representative ion chromatograms of calibration standards are presented in Figure 1. The linearity of calibration standards was demonstrated by <20 %RSD for response factors (RF) and >0.99 calibration range r-squared (RSQ) value for each analyte in all validation sets. The individual set %RSD and RSQ data are provided in Validation Results Worksheets in Appendix 2.

4.1.3 *Controls*

Typical ion chromatograms for untreated control samples of wheat and barley grain are presented in Figure 2. Typical ion chromatograms for untreated control samples of corn grain and tomato are presented in Figure 3.

Matrix peaks (0.001–0.003 ppm), most likely resulting from contamination, were observed at the approximate retention times of thifensulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl in control samples. Peak areas determined in control samples were subtracted from peak areas in fortified samples to correct for interference when an analyte was detected at a level in the control that would change the recovery of the 0.010 ppm fortified samples.

A matrix peak (0.004 ppm) was observed at the retention time of metsulfuron methyl in 1 of 2 wheat control samples and not in barley control samples. Since this observation was isolated to a single sample, recoveries for this analyte were not adjusted to response observed in the control sample.

4.1.4 *Recoveries (Accuracy and Precision)*

Typical ion chromatograms of wheat grain, barley grain, corn grain, and tomato control samples fortified with thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl are presented in Figures 4–7, respectively. The wheat and barley grain results of the individual trials for each matrix are presented in Table 3. The corn grain and tomato results of the individual trials for each matrix are presented in Table 4.

‡ Alternative chromatography was attempted with the initial validation that yielded a matrix enhanced response for all analytes except tribenuron methyl. The chromatography conditions described in the method report were used in subsequent validation set analyses.

For the wheat and barley grain matrices evaluated in this study, the average recovery results for thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl at 0.010 ppm (LOQ) and 0.10 ppm (10×LOQ) are summarized in the following table:

Matrix	ppm mg/kg	Thifensulfuron Methyl (M6316)			Metsulfuron Methyl (T6376)			Chlorsulfuron (W4189)			Tribenuron Methyl (L5300)			Flupyrsulfuron Methyl (KE459)			n
		Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	
Wheat Grain	0.010	94%	4%	5%	103%	4%	4%	92%	5%	6%	78%	8%	11%	93%	13%	14%	5
	0.10	93%	4%	4%	99%	5%	5%	87%	4%	4%	76%	9%	11%	96%	7%	8%	5
	All	93%	4%	4%	101%	5%	5%	89%	5%	6%	77%	8%	10%	94%	10%	11%	10
Barley Grain	0.010	97%	11%	11%	90%	3%	3%	96%	14%	14%	79%	6%	7%	90%	6%	7%	5
	0.10	94%	8%	9%	94%	6%	6%	89%	6%	7%	85%	4%	5%	109%	6%	5%	5
	All	96%	9%	10%	92%	5%	5%	92%	11%	12%	82%	6%	7%	100%	12%	12%	10
Combined Grain	0.010	96%	8%	8%	96%	7%	7%	94%	10%	11%	78%	7%	9%	91%	9%	10%	10
	0.10	94%	6%	7%	97%	6%	6%	88%	5%	6%	81%	8%	10%	103%	9%	9%	10
	All	95%	7%	8%	97%	6%	7%	91%	8%	9%	79%	7%	9%	97%	11%	11%	20

Avg: average, SD: standard deviation, %RSD: percent relative SD, n: number of samples

For the corn grain and tomato matrices evaluated in this study, the average recovery results for thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl at 0.010 ppm (LOQ) and 0.10 ppm (10×LOQ) are summarized in the following table:

Matrix	ppm mg/kg	Thifensulfuron Methyl (M6316)			Metsulfuron Methyl (T6376)			Chlorsulfuron (W4189)			Tribenuron Methyl (L5300)			Flupyrsulfuron Methyl (KE459)			n
		Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	Avg	SD	%RSD	
Corn Grain	0.010	91%	9%	10%	96%	2%	2%	87%	3%	4%	58%	3%	5%	97%	4%	4%	5
	0.10	91%	5%	6%	97%	1%	2%	85%	2%	3%	53%	2%	4%	96%	3%	3%	5
	All	91%	7%	8%	97%	2%	2%	86%	3%	3%	55%	3%	6%	97%	3%	3%	10
Tomato	0.010	96%	5%	5%	94%	4%	5%	89%	5%	5%	70%	4%	6%	98%	4%	4%	5
	0.10	106%	6%	5%	100%	4%	4%	94%	6%	6%	72%	2%	3%	101%	5%	5%	5
	All	101%	7%	7%	97%	5%	5%	92%	6%	6%	71%	3%	5%	99%	4%	4%	10

Avg: average, SD: standard deviation, %RSD: percent relative SD, n: number of samples

Method accuracy is considered acceptable if the average recoveries (overall and per fortification level) are in the range of 70–110%. All results obtained for each analyte examined using the method specified in Reference 4, except for tribenuron methyl in corn grain (55% average recovery) were within this range.

Method precision results should be less than 20% RSD over the range covered. When the method specified in Reference 4 was used, all precision data obtained were within these limits.

In this ILV, a triple quadrupole mass spectrometer in MS/MS mode was used in place of a single quadrupole mass spectrometer in SIM mode described in the original method (DuPont-5367) for quantitative analysis. The increase in selectivity with MS/MS detection improved the signal-to-noise response of analytes and reduced matrix response in the ion chromatograms for each analyte.

4.1.5 *Limit of Quantitation*

The minimum quantifiable concentration of chlorsulfuron, the limiting analyte in the method, in/on wheat grain, barley grain, corn grain, and tomato was 0.010 ppm (mg/kg). This was the lowest level validated in this study for all analytes.

4.2 *Communications*

4.3 *Time Requirements*

During this study, one set of six samples required approximately 6 person hours to prepare for HPLC analysis. The automated LC/MS/MS analysis was made overnight and required approximately one hour per sample or standard injected. LC/MS/MS data was processed the next day, requiring approximately 30 minutes.

5.0 CONCLUSIONS

- The independent laboratory validation (ILV) was successful for thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl in wheat grain, barley grain, and tomato matrices. The ILV was successful for thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, and flupyrsulfuron methyl in corn grain.
- The ILV results for tribenuron methyl in corn grain were consistent ($55\pm 3\%$), but below the acceptability range of 70–110%. Since tribenuron methyl products are not registered for corn or oily crops and the results were consistent, these findings do not alter the successful outcome of this method ILV.
- Modifications to the method were necessary for acceptable recoveries of tribenuron methyl.
- The LOQ stated in the method, 0.010 ppm (mg/kg), was demonstrated in the independent method validation of thifensulfuron methyl, metsulfuron methyl, chlorsulfuron, tribenuron methyl, and flupyrsulfuron methyl in wheat grain, barley grain, corn grain, and tomato. The LOQ is less than or equal to the EU MRL values for each analyte.

6.0 RETENTION OF RECORDS

7.0 REFERENCES

DUPONT-5367 (MODIFIED PROCEDURE BASED ON ILV)

3.0 MATERIALS

3.1 *Equipment*

Equivalent equipment or materials may be substituted unless otherwise specified. Note any specification in the following descriptions before making substitutions. Substitutions should be made only if equivalency/suitability has been verified with acceptable control and fortification recovery data.

Note: *To prevent trace contamination, do not re-use any equipment that is so noted. This equipment must be disposed of after each use.*

Balance

Mettler BB300 balance, Serial No. N53683 (Mettler Instruments Co., Hightstown, N.J.)

Mettler AG104 balance, Serial No. 1114493927 (Mettler Instruments Co., Hightstown, N.J.)

pH Meter

Beckman Model PHI 11, Serial No. 177001 (Beckman Instruments, Inc., Fullerton, Calif.)

Filtration Units for Buffer Solutions

ZapCap® disposable bottle-top filter units, 0.45-um Nylon® filter membrane, #28152-176 (VWR Scientific, Bridgeport, NJ) - **Do Not Re-use**

Electronic Pipettors

Rainin 1000-μL, 2500-μL, and 10-mL pipettors with pipet tips (Rainin, Walnut Creek, Calif.)

Centrifuge Bottles - Do Not Substitute or Re-use

Polypropylene centrifuge bottles (PPCO) with sealing caps, 250 mL, Nalge® #3141-0250, VWR#21010-614 (VWR Scientific, Bridgeport, NJ)

Polypropylene wide mouth laboratory bottles, 250 mL, Nalge® #2105-0008, VWR#16128-028 (VWR Scientific, Bridgeport, NJ)

Homogenizer

Tekmar SDT Tissumizer® model SDT-1810 with model SDT-182EN shaft and generator (Tekmar Co., Cincinnati, Ohio)

Centrifuge

DuPont Sorvall® Model RC-5C refrigerated centrifuge (DuPont Instruments, Wilmington, Del.)

Centrifuge Rotor

DuPont Model SLA-1500 centrifuge rotor (DuPont Instruments, Wilmington, Del.)

Measuring Cylinders

Kimax® brand single metric scale graduated cylinders, 250 mL, #34795-058 (VWR Scientific, Bridgeport, NJ)

SPE Clean-up Cartridges

Supelclean™ ENVI™-Carb SPE tubes, 1 g/12 mL, #57127-U (Supelco, Bellefonte, Pa.) - **Do Not Re-use**

SPE Vacuum Manifold

Visiprep™ SPE Vacuum Manifold, 12 port model, #5-7030 (Supelco, Bellefonte, Pa.)

Flow Control Valve Liners

Disposable flow control valve liners for the Visiprep™ Vacuum Manifold, # 57059 (Supelco, Bellefonte, Pa.) - **Do Not Re-use**

Sample Collection tubes and vials

Disposable 15 mL centrifuge tubes with snap caps, Kimble®, # 21020-764 (tubes), #60869-089 (size 2 snap caps) (VWR Scientific, Bridgeport, N.J.) - **Do Not Re-use**

Borosilicate glass sample vials, 40 mL, Kimble®, 28 mm O.D. x 108 mm length, # 66012-306 (VWR Scientific, Bridgeport, N.J.) - **Do Not Re-use**

Nitrogen Evaporator

The Meyer N-Evap Analytical Evaporator, Model 111 (Organomation Associates, Inc., South Berlin, Mass.)

Ultrasonic Bath

Branson Model 2210 Ultrasonic bath, #B2210DTH (VWR Scientific, Bridgeport, N.J.)

Mixer

VWR Vortexer 2 (VWR Scientific, Bridgeport, N.J.)

HPLC Sample Vials

HPLC Vials, Target DP Amber Kit, T/S/T Septa, 100 PK, Part # 5182-0556
(Hewlett-Packard, Wilmington, DE)

3.1.1 LC/MS SystemLiquid Chromatograph

HP1100 with temperature controlled autosampler (Hewlett-Packard, Wilmington, DE)

Mass Spectrometer: A Micromass Quattro II triple quadrupole mass spectrometer using an electrospray interface (ESI) and MassLynx NT Version 3.1 software (Waters Corporation, Milford, Mass.) with Valco zero-dead volume 3-port connector for 1/10 splitflow to mass spectrometer.

HPLC Columns: Zorbax Eclipse® XDB-C8, 4.6 x 150 mm, 5- μ m Analytical Column, #993967-906 (Hewlett-Packard, Wilmington, DE)

3.2 **Reagents and Standards**

Equivalent reagents may be substituted.

Acetic Acid: OmniTrace®, 99%, Catalog No. AX077-1 (EM Science, Gibbstown, N.J.).

Acetonitrile - EM OmniSolv®, #AX0142-1 (EM Science)

Ammonium Hydroxide: GR 28-30%, Catalog No. AX1303-13 (EM Science)

Formic Acid - EM Suprapur®, 98% min. #EM-11670-1 (EM Science)

Hydrochloric Acid: GR, 500 mL, Catalog No. HX0603-4 (EM Science).

Methylene Chloride - EM OmniSolv®, #DX0831-1 (EM Science)

Methanol - EM OmniSolv®, #MX0488-1 (EM Science)

Water - EM OmniSolv®, #WX0004-1 (EM Science)

Potassium Phosphate, Monobasic, GR, crystals, #PX1565-5 (EM Science)

Potassium Hydroxide – 1 N Potassium hydroxide solution, #VW5049-4 (VWR Scientific, Bridgeport, N.J.)

Reference standards (DuPont Crop Protection, E.I. du Pont de Nemours and Company, Wilmington, Del.):

Compound	Lot Number	% Purity
Thifensulfuron methyl	DPX-M6316-186	99.7
Metsulfuron methyl	DPX-T6376-149	98.5
Chlorsulfuron	DPX-W4189-158	99.4
Tribenuron methyl	DPX-L5300-143	97.9
Flupyrsulfuron methyl	DPX-KE459-14	93.4

4.2 *Analytical Procedure*

4.2.1 *Glassware and Equipment Cleaning Procedures*

Preparation and analysis of reagent blanks should demonstrate the effectiveness of any cleaning procedure used. In general, all reusable glass and plastic-ware should be washed in hot tap water with laboratory grade, non-phosphate detergent, rinsed several times with tap water and distilled water, rinsed once with acetone, and allowed to dry before use. Tissumizer® probes should be disassembled and washed in hot water with laboratory grade, non-phosphate detergent, rinsed several times with tap water and distilled water, and sonicated in acetone for approximately 15 minutes. Care should be taken to avoid working with high levels of the analyte being monitored in the same laboratory where samples are being extracted and analyzed.

4.2.2 *Preparation and Stability of Reagent Solutions*

20 mM Potassium Phosphate (KH₂PO₄) Extraction Solution

Dissolve 5.44 g of KH₂PO₄ in 2000 mL of HPLC-grade water in a 2-liter bottle. Adjust the pH of the solution to pH 6.0 using 1 N potassium hydroxide and a calibrated pH meter. Cap and store at room temperature.

SPE Conditioning solution – 0.1M HCl

Add 8.62 mL of concentrated hydrochloric acid to a clean 1-liter measuring cylinder. Add HPLC-grade water to the 1-liter mark. Transfer the mixture to a 1-liter solvent bottle. Cap, shake, and store at room temperature. This solution is used for conditioning the ENVI-carb SPE columns.

SPE Eluting Solution – 10% Methanol/90% Methylene Chloride/0.1M Formic Acid

Measure 100 mL of methanol in a 1-liter measuring cylinder. Add methylene chloride to the 1-liter mark. Transfer the mixture to a 1-liter solvent bottle. Pipet accurately 4.27 mL of formic acid into the 1-liter bottle. Cap and shake to mix. This solution is stored at room temperature and should be prepared as needed.

50 mM Ammonium Acetate Buffer (pH 6.2)

Dissolve 3.854 g of ammonium acetate in 1 L of distilled, deionized water and adjust pH to 6.2 using 1% solution of NH_4OH (28-30% concentrate). This solution is used for preparation of the sample extract reconstituting solution and is Eluent A in the LC/MS analysis.

Sample Extract Reconstituting Solution – 10% acetonitrile/90% 50 mM ammonium acetate(aq)

Add 100 mL of acetonitrile to a 1-liter measuring cylinder. Add 50 mM ammonium acetate to the 1-liter mark. Transfer the solution to a 1-liter solvent bottle. Cap and shake. This solution is stored at room temperature and should be prepared as needed. This solution is used for reconstituting sample extracts and preparing the chromatographic standards.

2 mM Acetic Acid (aq)

Pipet 115 μL of glacial acetic acid (99.6%, w/w) into a 1-L volumetric flask. Dilute with HPLC-grade water to the mark. Transfer the solution to a 1-L solvent bottle. Cap and shake. This solution is stored at room temperature and should be prepared as needed. This solution is used as Eluent A in the LC/MS analysis.

4.2.3 Stock and Intermediate Standard Preparation and Stability

1000- $\mu\text{g}/\text{mL}$ Stock Standard Solution

Weigh accurately 10 ± 0.01 mg of each analytical standard in a 10-mL volumetric flask. Record the actual weight of each standard. Add acetonitrile to the mark, cap the flask, shake and sonicate for 5 minutes to dissolve the standards to yield a homogenous solution. This stock standard solution should be stable for at least one month if kept in a freezer maintained at 0 to -20°C .

4.2.4 Fortification Standard Preparation and Stability

10- $\mu\text{g}/\text{mL}$ Fortification Standard Solution

From the 1000- $\mu\text{g}/\text{mL}$ Stock Standard Solution, prepare a 10.0- $\mu\text{g}/\text{mL}$ Fortification Standard Solution in acetonitrile. This fortification standard is used to fortify the sample matrices. This solution is stable for approximately two weeks if kept in a freezer maintained at 0 to -20°C .

1.0- $\mu\text{g}/\text{mL}$ Fortification Standard Solution

From the 1000- $\mu\text{g}/\text{mL}$ Stock Standard Solution, prepare a 1.0- $\mu\text{g}/\text{mL}$ Fortification Standard in acetonitrile. This fortification standard is used to fortify the sample matrices and to prepare the chromatographic standards. This standard is stable for approximately two weeks if kept in a freezer maintained at 0 to -20°C .

4.2.5 Chromatographic Standard Preparation and Stability

Prepare chromatographic standards ranging from 0.001 to 0.030 µg/mL (or in concentrations expected to cover the range of concentrations in the samples) in 10% acetonitrile/90% 50 mM ammonium acetate using the 1.0-µg/mL Fortification Standard Solution above. Prepare the standards in 20-mL scintillation vials by pipetting the required volumes of the 1-µg/mL Fortification Standard Solution, acetonitrile and 50 mM ammonium acetate into each vial. Cap and vortex. Store all chromatographic standards at or below 4°C following preparation. These standards should be prepared daily. The following table shows the volumes used to prepare the standards for the validation work presented in this report:

STANDARD CONC. (µg/ML)	VOLUME OF 1.0-µg/ML STANDARD USED (µL)	VOLUME OF ACETONITRILE (µL)	VOLUME OF 50 MM AMMONIUM ACETATE (ML)
0.001	10 µL	990 µL	9 mL
0.0025	25 µL	975 µL	9 mL
0.005	50 µL	950 µL	9 mL
0.015	150 µL	850 µL	9 mL
0.030	300 µL	700 µL	9 mL

4.2.6 Source of Samples

Control wheat grain was obtained from DuPont Study Number AMR 2570-93, "Magnitude of Residues of Chlorsulfuron in Wheat Following Application of Glean® FC Herbicide at Maximum Label Rates". Control wheat forage was obtained from DuPont Study No. AMR 4336-97, "Magnitude of Residues of Hexazinone in Rotational Crops Following Application of Velpar® Herbicide at Maximum Label Rates to Alfalfa". Control wheat straw was obtained from the DuPont Experimental field Station in Rochelle, Illinois.

4.2.7 Processing and Storage of Samples

All wheat matrices had been previously pre-processed and were stored frozen at approximately -20°C ± 5°C until time of sample preparation.

4.2.8 Sample Fortification Procedure

Fortifications were prepared using the 1-µg/mL and 10-µg/mL Fortification Standard Solutions. A 1000-µL pipetter was used to add 100 µL of the 1-µg/mL and 10-µg/mL Fortification Standard Solutions to 10 grams of wheat grain resulting in fortification levels of 0.01 and 0.10 ppm, respectively. In addition, 250 µL of the 1-µg/mL and 10-µg/mL Fortification Standard Solutions were added to 5 grams of wheat forage and straw resulting in fortification levels of 0.05 ppm and 0.50 ppm, respectively. See table below for fortification details:

	<u>Grain</u>		<u>Forage</u>		<u>Straw</u>	
Fortification Level (mg/kg)	0.01	0.10	0.05	0.50	0.05	0.50
Spiking standard conc. (µg/mL)	1	10	1	10	1	10
Volume of spiking standard (µL)	100	100	250	250	250	250
No. of fortification samples/level	5	5	5	5	5	5

4.2.9 Analyte Extraction Procedure

1. Weigh out 10 (± 0.1 g) grams of wheat grain or 5 (± 0.1 g) grams of wheat forage or wheat straw into 250-mL PPCO centrifuge bottles (Nalge Part # 3141-0250). Record weight of sample to the nearest 0.1 g.
2. If any sample is to be fortified, add the appropriate volume of the fortification standard in acetonitrile to the sample as described in Section 4.2.8 above. Air dry for 15 minutes to allow the acetonitrile time to evaporate from the sample matrices before proceeding.
3. Add 90 mL (± 1 mL) of 20-mM potassium phosphate, pH 6.0 solution to the bottles. Cap and shake briefly.
4. Refrigerate the bottles for 60 minutes. Shake the refrigerated bottles at 15-minute intervals and continue refrigeration for the 60 minutes.
5. Remove the sample bottles from the refrigerator. Shake briefly. Uncap the bottles and homogenize the samples using a Tissumizer® for 2 minutes at 40–50% of total motor speed or at a speed that efficiently homogenizes the sample matrix without over-heating and foaming. (**Note: A higher speed is required for straw samples and the bottles must be swirled continuously during homogenization to facilitate the process.**)
6. Centrifuge the bottles for 15 minutes at 0–5°C at 13000 rpm[§] (approximately 12,220 G) to achieve sufficient clarification of the supernatant.
7. Carefully decant the supernatants into clean, labeled 250-mL measuring cylinders.
8. Add 90 mL (± 1 mL) of 20-mM potassium phosphate solution to the pellet in the original sample bottles. Cap and shake or tap vigorously to completely re-disperse the pellet in the buffer solution.
9. Uncap the bottles and homogenize the samples using a Tissumizer® for 2 minutes at 40–50% of total motor speed or at a speed that efficiently homogenizes the sample matrix without over-heating or foaming. (**Note: A higher speed is**

§ The equation for determining RPM to achieve the proper G force is: Centrifugal Force (G) = $1.1118 \times 10^{-5} \times R \times (\text{RPM})^2$, where R is the measurement taken from the center of the centrifuge rotor to the mid-point of the centrifuge bottle cap. In our lab, R = 6.5 cm.

required for straw samples and the bottles must be swirled continuously during homogenization to facilitate the process).

10. Centrifuge for 15 minutes at 0-5°C at 13000 rpm (approximately 12,220 G) to achieve sufficient clarification of the supernatant.
11. Decant and combine the supernatants in the measuring cylinders. Bring the final volume of the supernatant in the cylinders to the 200-mL mark with purified water.
12. Transfer the supernatants to clean 250-mL polypropylene laboratory bottles (Nalge Part# 2105-0008). Cap and shake.

4.2.10 Analyte Purification Procedure

1. Condition 1-gram Envi™-Carb SPE columns by passing 10 mL of 10% methanol /90% methylene chloride/0.1 M formic acid, followed by 10 mL of methanol, followed by 10 mL of 0.1M HCl, followed by 15 mL of purified water. ***Stop all flow when approximately 2 - 5 mm of water remains above the column bed and do not allow the SPE columns to go dry at this point.***
2. Pipet or transfer 10-mL sample aliquots from step 12 above to each SPE column. Allow to pass through the SPE column at a flow rate of approximately 2 mL/min under vacuum, and discard.
3. Add 10 mL of purified water to each SPE column. Allow to pass through the SPE column at a flow rate of approximately 2 mL/min under vacuum, and discard.
4. Add 10 mL of methanol to each SPE column. Allow to pass through the SPE column at a flow rate of approximately 2 mL/min under vacuum, and discard.
5. Dry the SPE columns for approximately 2-30 minutes by pulling maximum vacuum through the columns.
6. **For grain and forage samples**, place a clean 15-mL disposable glass tube under each column. Add 10 mL of 10% methanol/90% methylene chloride/0.1 M formic acid to each SPE column. Allow to pass through the SPE columns dropwise under vacuum to elute and collect all five analytes in the 15-mL tube. Adjust the pH to near neutral IMMEDIATELY by adding 60 µL of concentrated ammonium hydroxide.
7. **For straw samples**, place a clean 40-mL sample vial under each column. Add 20 mL of 10% methanol/90% methylene chloride/0.1 M formic acid to each SPE column. Allow to pass through the SPE columns dropwise under vacuum to elute and collect all five analytes in the 40-mL vial. Adjust the pH to near neutral IMMEDIATELY by adding 60 µL of concentrated ammonium hydroxide.
8. Evaporate off the solvent in the tubes or vials to dryness in an N-Evap with the water-bath set at approximately 35°C.

9. Reconstitute the samples as follows:

<u>Sample</u>	<u>Volume of 10% Acetonitrile/90% 50 mM Ammonium Acetate Buffer</u>
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<i>Grain samples</i>	<i>2 mL</i>
----------------------	-------------

<i>Forage and straw samples</i>	<i>5 mL</i>
---------------------------------	-------------

Pipet the required volume of 10% acetonitrile/90% 50 mM ammonium acetate solution into each tube or vial. Cap, vortex, and sonicate for 5 minutes in a beaker of ice-cold water. Vortex briefly.

10. Filter an aliquot of the reconstituted sample extract using a 0.45- μ m PTFE syringe filter into amber autosampler vial for LC/ESI-MS/MS analysis.

TABLE 1 HPLC CONDITIONS FOR INDEPENDENT LABORATORY VALIDATION

Columns: Zorbax Eclipse® XDB-C8, 4.6 × 150 mm, 5 µm diameter packing
 Oven Temperature: 25°C
 Injection Volume: 100 µL
 Autosampler Temperature: 5–10°C
 Solvent A Aqueous 2 mM acetic acid
 Solvent B Acetonitrile
 Flow Rate: 1.0 mL/min

Chromatography Conditions

Time (min)	% A	% B	Explanation
0.00	80	20	Injection
10.00	80	20	
31.00	63	37	
32.00	50	50	
40.00	50	50	
40.10	10	90	Begin high organic wash of column
45.00	10	90	End high organic wash of column
45.10	80	20	Begin column re-equilibration
50.00	80	20	End column re-equilibration

Approximate Analyte Retention Times:

thifensulfuron methyl (M6316) = 23.0 min
 metsulfuron methyl (T6376) = 24.4 min
 chlorsulfuron (W4189) = 26.7 min
 tribenuron methyl (L5300) = 34.8 min
 flupyrsulfuron methyl (KE459) = 37.2 min

TABLE 2 MASS SPECTROMETER CONDITIONS FOR INDEPENDENT LABORATORY VALIDATION

Interface: electrospray (ESI)

Mode: **positive ion** MS/MS-MRM

Capillary Voltage: 4.2 kV

Detector Voltage: 750 V

Source Heater: 80°C

Gas Cell Pressure: 1.5-3 mbar

Nebulizing Gas Flow: 15 L/hr

Drying Gas Flow: 300 L/hr

MS/Waste Split Ratio: 1/10

Analytes	Ions Monitored (AMU)	Cone Voltage	Collision Energy	Dwell (Sec)
thifensulfuron	387.9 → 167.0	30.0	14.0	0.20
methyl (M6316)	387.9 → 205.0	30.0	27.0	0.20
metsulfuron	382.0 → 167.0	40.0	11.0	0.20
methyl (T6376)	382.0 → 199.0	40.0	24.0	0.20
chlorsulfuron	357.8 → 141.0	40.0	15.0	0.20
(W4189)	357.8 → 167.0	40.0	16.0	0.20
tribenuron	395.9 → 155.0	30.0	13.0	0.20
methyl (L5300)	395.9 → 181.0	30.0	20.0	0.20
flupyrsulfuron	465.9 → 138.8	44.0	31.0	0.20
methyl (KE459)	465.9 → 182.0	44.0	31.0	0.20

TABLE 3 WHEAT AND BARLEY GRAIN INDEPENDENT METHOD VALIDATION RESULTS

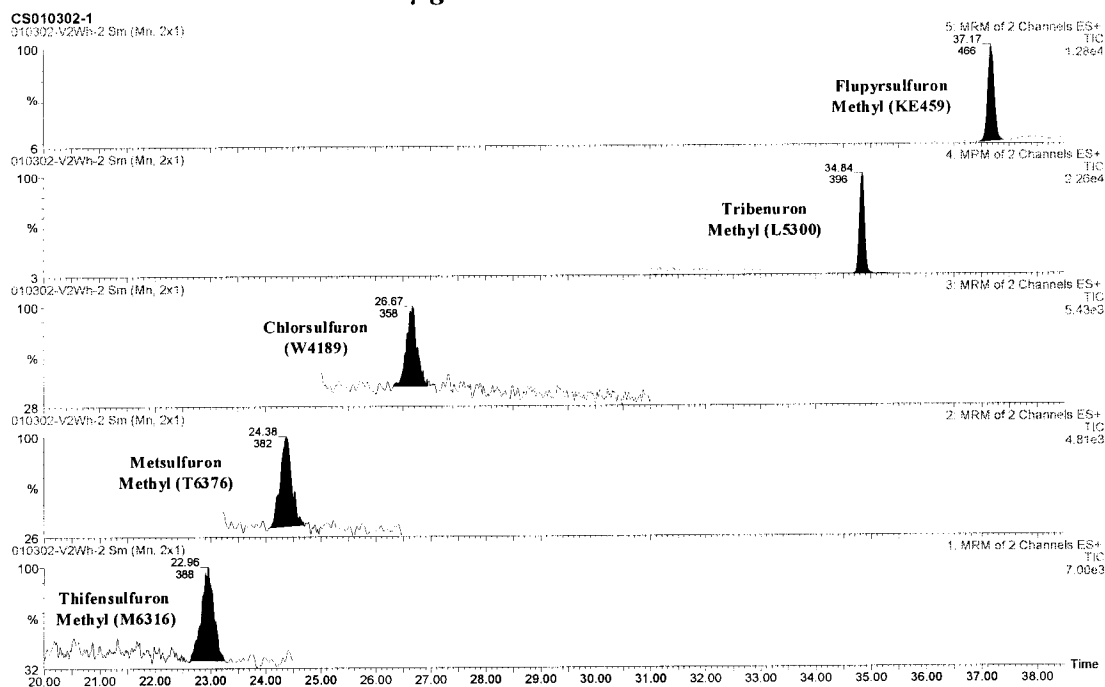
Run ID	Sample Information		SW	FV	DF	Recoveries									
	Type	ppm				Date Extracted	Thiencysulfuron Methyl (M6316)	Metsulfuron Methyl (T6376)	Chlorisulfuron (W4189)	Tribenuron Methyl (L5300)	Flupyriflurone Methyl (KE459)				
						ppm	%Rec	ppm	%Rec	ppm	%Rec	ppm	%Rec	ppm	%Rec
121901-V1bWh-3	C	0.0	19-Dec-01	10.0	2.0	20	0.001	90%	nd	105%	0.003	96%	nd	0.001	100%
121901-V1bWh-5	F	0.010	19-Dec-01	10.0	2.0	20	0.009	92%	0.010	104%	0.010	87%	0.008	0.010	102%
121901-V1bWh-6	F	0.010	19-Dec-01	10.0	2.0	20	0.009	94%	0.010	101%	0.009	89%	0.008	0.010	95%
121901-V1bWh-8	F	0.10	19-Dec-01	10.0	2.0	20	0.094	94%	0.101	101%	0.089	87%	0.077	0.095	92%
121901-V1bWh-9	F	0.10	19-Dec-01	10.0	2.0	20	0.094	94%	0.100	100%	0.092	87%	0.072	0.085	92%
121901-V1bWh-11	F	0.10	19-Dec-01	10.0	2.0	20	0.087	87%	0.091	91%	0.081	81%	0.064	0.088	88%
010302-V2Wh-3	C	0.0	3-Jan-02	10.0	2.0	20	0.001		0.004		0.002		nd	0.001	
010302-V2Wh-5	F	0.010	3-Jan-02	10.0	2.0	20	0.010	96%	0.010	98%	0.009	89%	0.009	0.010	100%
010302-V2Wh-6	F	0.010	3-Jan-02	10.0	2.0	20	0.010	100%	0.011	107%	0.010	98%	0.007	0.007	72%
010302-V2Wh-8	F	0.010	3-Jan-02	10.0	2.0	20	0.009	90%	0.010	99%	0.009	89%	0.008	0.009	89%
010302-V2Wh-9	F	0.10	3-Jan-02	10.0	2.0	20	0.093	93%	0.100	100%	0.087	87%	0.085	0.099	99%
010302-V2Wh-11	F	0.10	3-Jan-02	10.0	2.0	20	0.097	97%	0.105	105%	0.089	89%	0.084	0.107	107%
Wheat Recovery Statistics						Avg	93%	4%	101%	5%	89%	5%	77%	8%	94%
						SD	4%		5%		6%		8%		10%
						%RSD	4%		5%		6%		10%		11%
010402-V-Ba-3	C	0.0	4-Jan-02	10.0	2.0	20	0.001	102%	nd	92%	0.002	104%	nd	0.001	99%
010402-V-Ba-5	F	0.010	4-Jan-02	10.0	2.0	20	0.010	84%	0.009	90%	0.011	113%	0.008	0.010	91%
010402-V-Ba-6	F	0.010	4-Jan-02	10.0	2.0	20	0.008	93%	0.009	91%	0.009	90%	0.007	0.009	115%
010402-V-Ba-8	F	0.10	4-Jan-02	10.0	2.0	20	0.093	104%	0.091	100%	0.090	96%	0.090	0.115	116%
010402-V-Ba-9	F	0.10	4-Jan-02	10.0	2.0	20	0.104	101%	0.100	96%	0.096	93%	0.086	0.116	107%
010402-V-Ba-11	F	0.10	4-Jan-02	10.0	2.0	20	0.101	101%	0.096	96%	0.093	93%	0.079	0.107	107%
010702-V-Ba-3	C	0.0	7-Jan-02	10.0	2.0	20	nd		nd		0.003		0.000	0.002	
010702-V-Ba-5	F	0.010	7-Jan-02	10.0	2.0	20	0.011	113%	0.009	93%	0.009	92%	0.008	0.009	92%
010702-V-Ba-6	F	0.010	7-Jan-02	10.0	2.0	20	0.009	92%	0.009	91%	0.008	77%	0.009	0.009	87%
010702-V-Ba-8	F	0.010	7-Jan-02	10.0	2.0	20	0.010	96%	0.009	86%	0.009	91%	0.007	0.008	82%
010702-V-Ba-9	F	0.10	7-Jan-02	10.0	2.0	20	0.084	84%	0.086	86%	0.079	79%	0.086	0.108	108%
010702-V-Ba-11	F	0.10	7-Jan-02	10.0	2.0	20	0.089	89%	0.097	97%	0.087	87%	0.083	0.102	102%
Barley Recovery Statistics						Avg	96%	9%	92%	5%	92%	11%	82%	6%	100%
						SD	9%		5%		11%		6%		12%
						%RSD	10%		5%		12%		7%		12%

TABLE 4 CORN GRAIN AND TOMATO INDEPENDENT METHOD VALIDATION RESULTS

Run ID	Sample Information		SW	FV	DF	Recoveries									
	Type	ppm				Date Extracted	Thiensulfuron Methyl (M6316)	Metsulfuron Methyl (L6376)	Chlorisulfuron (VW4169)	Tribenuron Methyl (L5300)	Flupyrulfuron Methyl (KE459)				
						ppm	%Rec	ppm	%Rec	ppm	%Rec	ppm	%Rec	ppm	%Rec
Corn Grain															
022002-V-Corn-4	C	0.0		10.0	2.0	20	0.002	99%	0.000	0.000	0.000	0.000	61%	0.000	101%
022002-V-Corn-6	F	0.010		10.0	2.0	20	0.010	88%	0.010	0.009	0.006	0.006	59%	0.010	98%
022002-V-Corn-7	F	0.010		10.0	2.0	20	0.009	89%	0.009	0.008	0.006	0.006	56%	0.100	100%
022002-V-Corn-9	F	0.10		10.0	2.0	20	0.089	87%	0.087	0.085	0.056	0.056	53%	0.094	94%
022002-V-Corn-10	F	0.10		10.0	2.0	20	0.087	87%	0.087	0.083	0.051	0.051	51%	0.097	97%
022002-V-Corn-12	F	0.10		10.0	2.0	20	0.087	87%	0.087	0.083	0.051	0.051	51%	0.097	97%
022102-V-Corn-4	C	0.0		10.0	2.0	20	0.002	102%	0.000	0.000	0.000	0.000	55%	0.000	94%
022102-V-Corn-6	F	0.010		10.0	2.0	20	0.010	85%	0.010	0.009	0.005	0.005	56%	0.010	98%
022102-V-Corn-7	F	0.010		10.0	2.0	20	0.009	81%	0.009	0.008	0.006	0.006	57%	0.009	92%
022102-V-Corn-9	F	0.010		10.0	2.0	20	0.008	95%	0.008	0.008	0.054	0.054	54%	0.099	99%
022102-V-Corn-10	F	0.10		10.0	2.0	20	0.095	98%	0.095	0.089	0.051	0.051	51%	0.098	98%
022102-V-Corn-12	F	0.10		10.0	2.0	20	0.098	98%	0.098	0.086	0.051	0.051	55%	0.098	98%
Corn Recovery Statistics															
						Avg	91%	97%	86%	86%	55%	97%	3%	3%	3%
						SD	7%	2%	3%	3%	3%	3%	3%	3%	3%
						%RSD	8%	2%	3%	3%	6%	3%	6%	3%	3%
Tomato															
022502-V-Tom-4	C	0.0		10.0	2.0	20	0.010	99%	0.010	0.009	0.000	0.000	67%	0.000	97%
022502-V-Tom-6	F	0.010		10.0	2.0	20	0.010	99%	0.009	0.009	0.007	0.007	69%	0.010	95%
022502-V-Tom-7	F	0.010		10.0	2.0	20	0.010	101%	0.009	0.085	0.070	0.070	70%	0.098	98%
022502-V-Tom-9	F	0.10		10.0	2.0	20	0.101	108%	0.105	0.100	0.072	0.072	72%	0.099	99%
022502-V-Tom-10	F	0.10		10.0	2.0	20	0.108	102%	0.098	0.093	0.071	0.071	71%	0.099	99%
022502-V-Tom-12	F	0.10		10.0	2.0	20	0.102	102%	0.098	0.083	0.071	0.071	71%	0.099	99%
Averaged data from 2 and 27-Feb-02 analyses															
022702-V-tom-b-12	F	0.10		10.0	2.0	20	0.114	114%	0.101	0.097	0.075	0.075	75%	0.109	109%
Tomato Recovery Statistics															
						Avg	101%	97%	92%	92%	71%	99%	4%	4%	4%
						SD	7%	5%	6%	6%	3%	4%	4%	4%	4%
						%RSD	7%	5%	6%	6%	5%	5%	5%	4%	4%

FIGURE 1 REPRESENTATIVE CALIBRATION STANDARD ION CHROMATOGRAMS

1.0- μ g/L Calibration Standard



2.5- μ g/L Calibration Standard

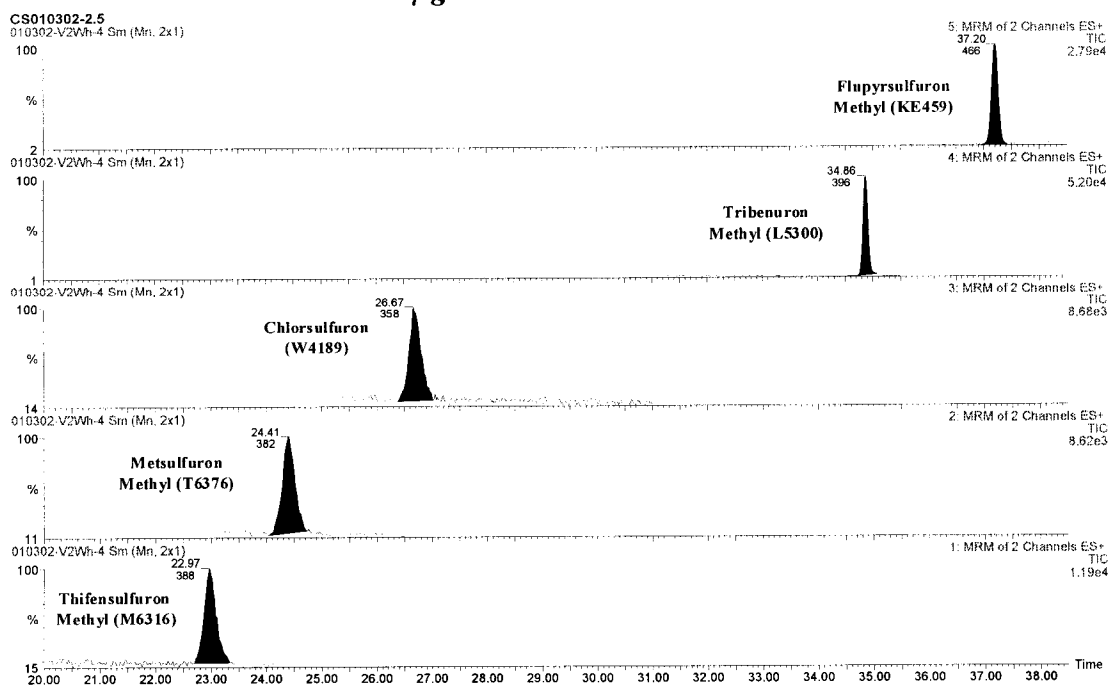


FIGURE 3 REPRESENTATIVE CONTROL CORN GRAIN AND TOMATO SAMPLE ION CHROMATOGRAMS

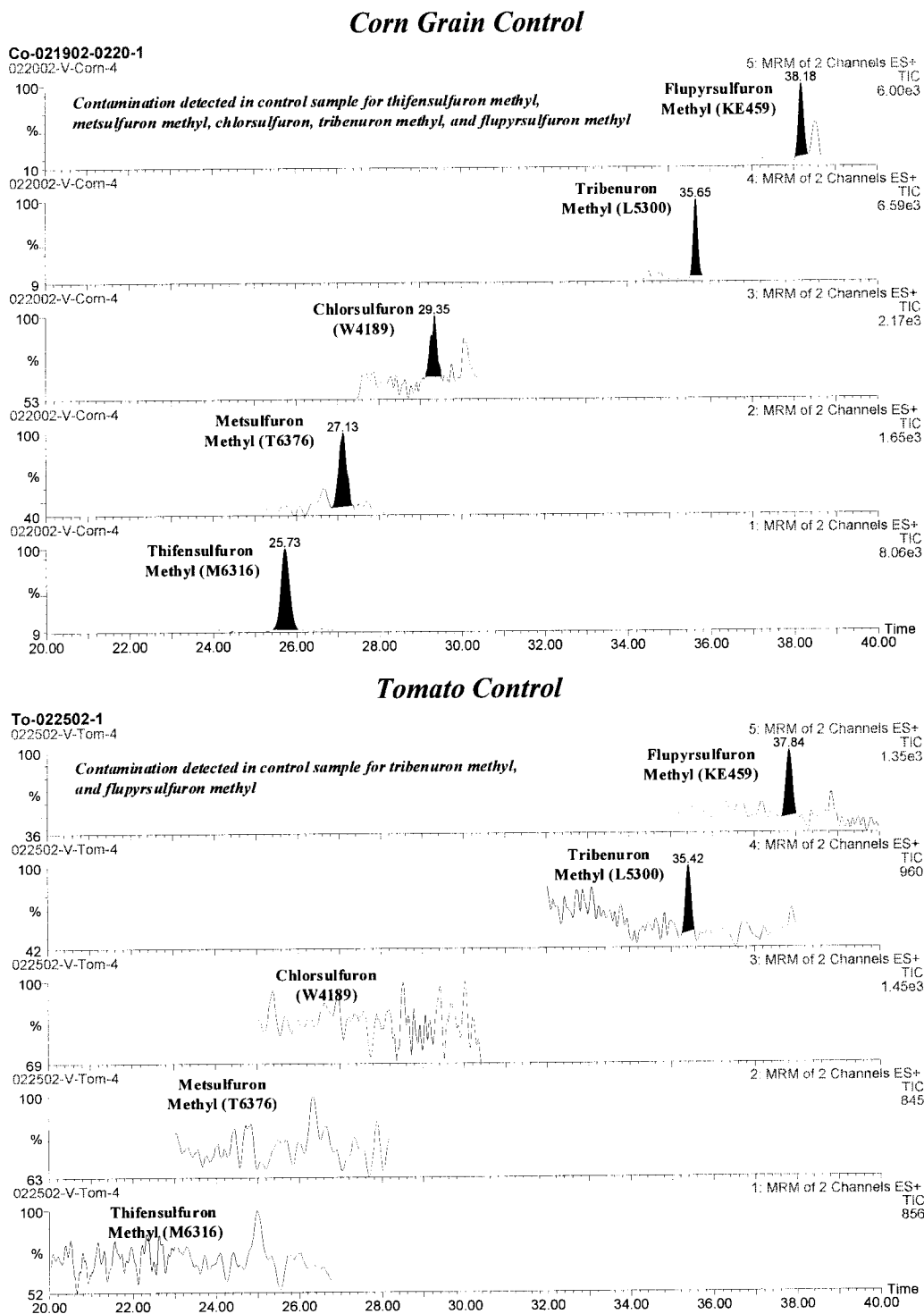
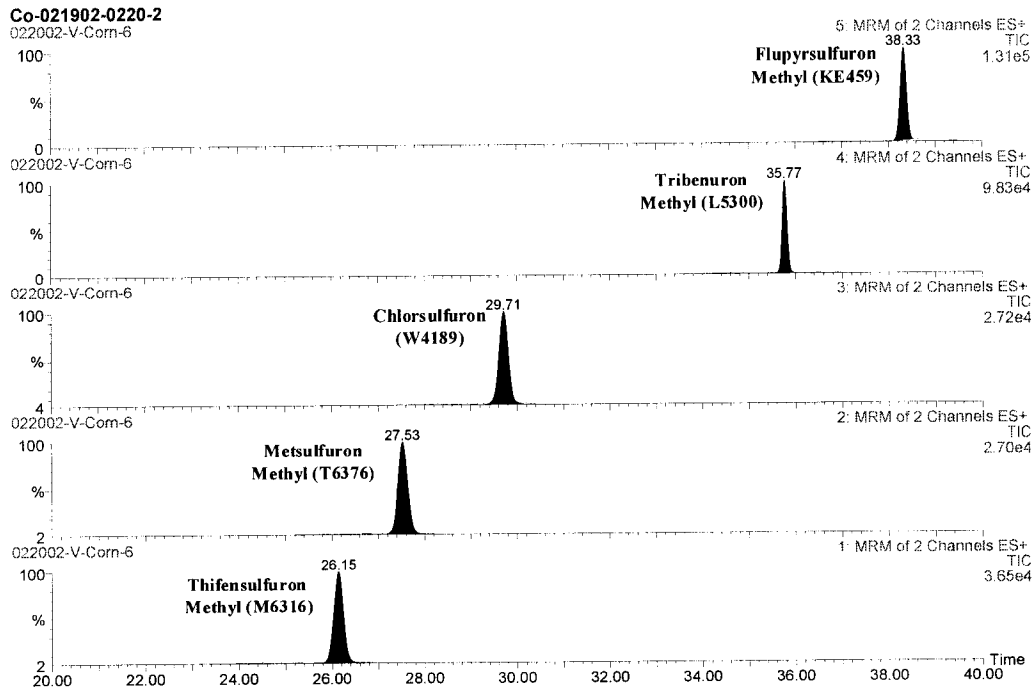


FIGURE 6 REPRESENTATIVE FORTIFIED CORN GRAIN SAMPLE ION CHROMATOGRAMS

0.010 ppm (LOQ) Fortified Control Sample



0.10 ppm (10xLOQ) Fortified Control Sample

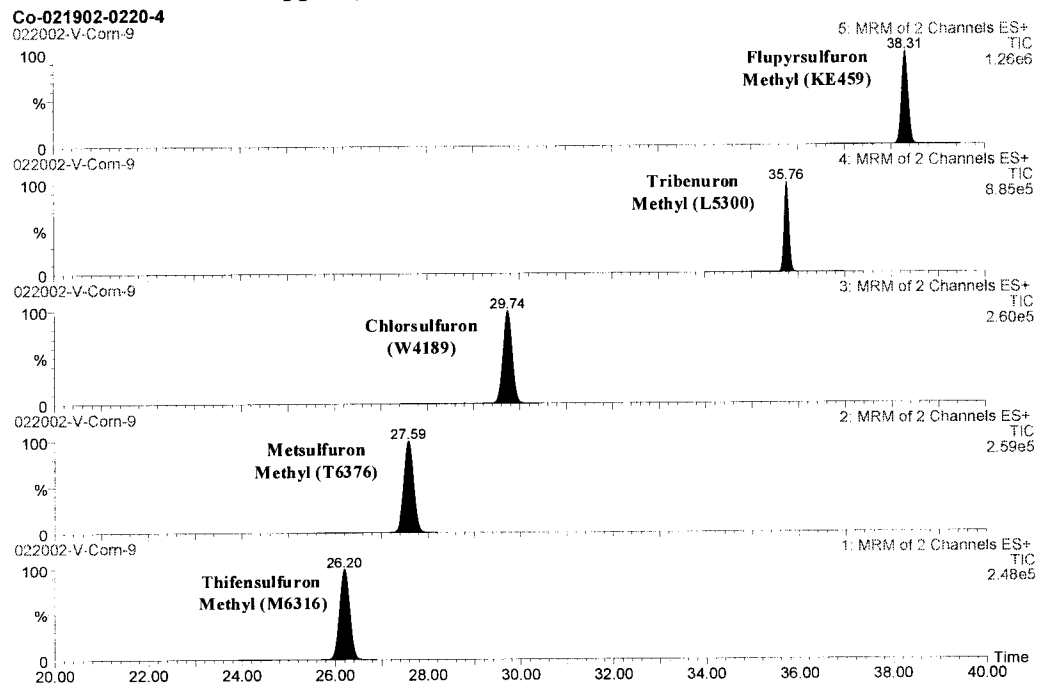
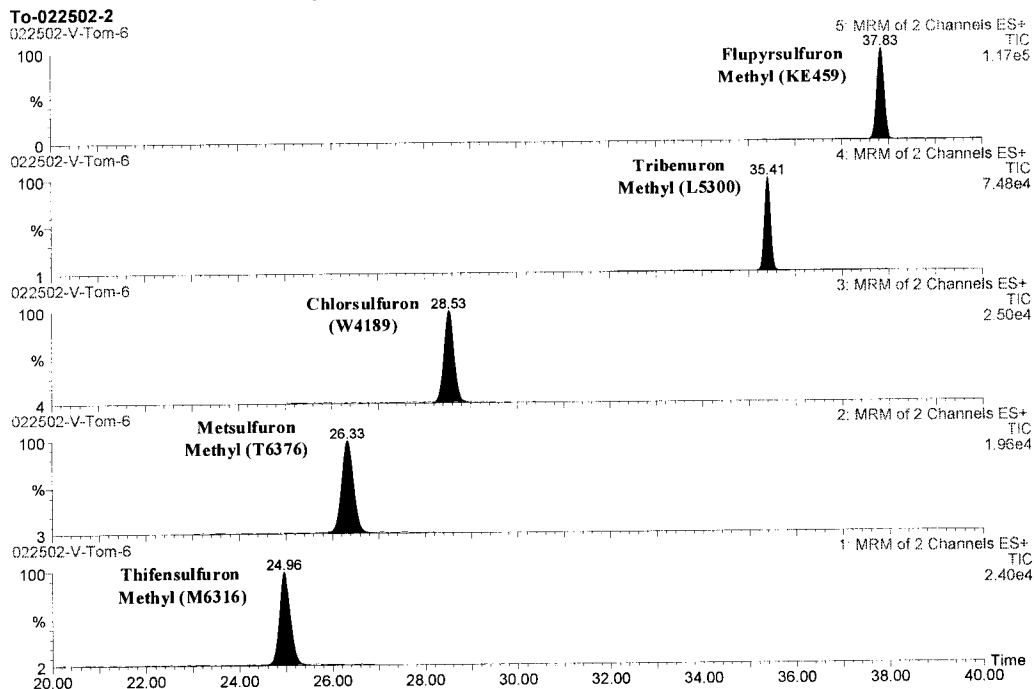
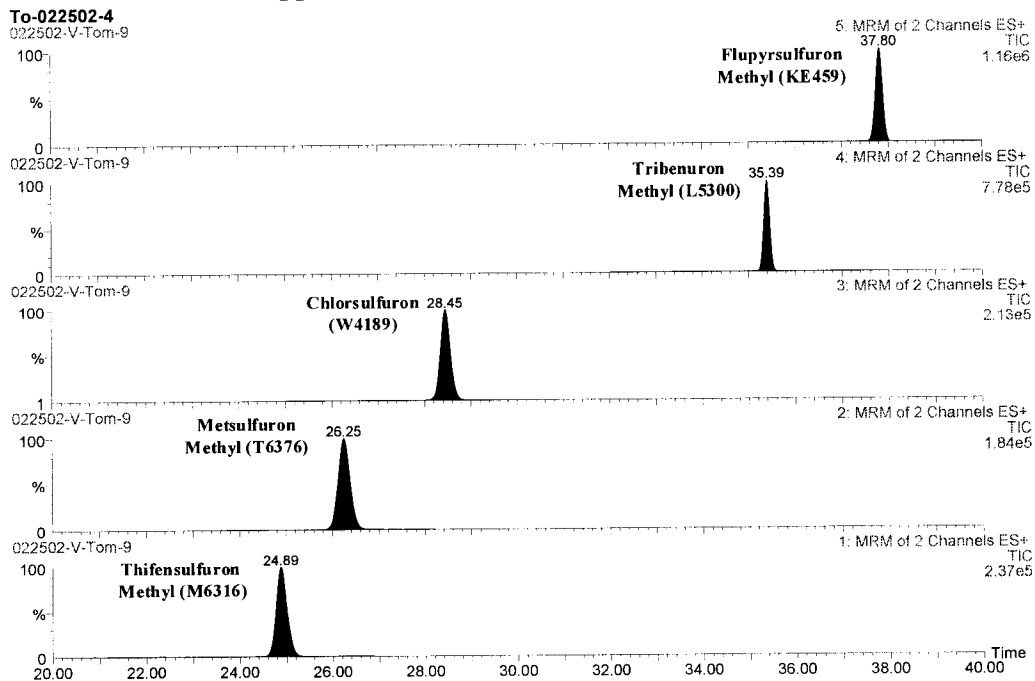


FIGURE 7 REPRESENTATIVE FORTIFIED TOMATO SAMPLE ION CHROMATOGRAMS

0.010 ppm (LOQ) Fortified Control Sample



0.10 ppm (10×LOQ) Fortified Control Sample



APPENDIX 1 LC/MS/MS CONDITIONS

Acquisition Experiment Report

File:d:\masslynx\open access.pro\data\010702-v-ba-9

Header

Acquired File Name: 010702-V-Ba-9
Acquired Date: 07-Jan-2002
Acquired Time: 22:22:02
Job code: D8054_V-Ba010702
Task code:
User Name: Administrator
Laboratory Name: Lab
Instrument: Inst
Conditions:
Submitter:
SampleID: 0.10 Fort
Bottle Number: 15
Description: Ba-010702-5

Instrument Calibration

Parameters

MS1 Static:

Mass 85 Da to 596 Da.
Resolution : 15.0/15.0
Ion Energy : 0.8
Reference File : pegnh4
Acquisition File : STATMS1

MS1 Scanning:

Mass 80 Da to 600 Da.
Resolution : 15.0/15.0
Ion Energy : 0.8
Reference File : pegnh4
Acquisition File : SCNMS1

MS1 Scan Speed:

Scan 64 to 473 amu/sec.
Resolution : 15.0/15.0
Ion Energy : 0.8
Reference File : pegnh4
Acquisition File : FASTMS1

MS2 Static:

Mass 85 Da to 596 Da.
Resolution : 15.0/15.0
Ion Energy : 0.8

APPENDIX 1 LC/MS/MS CONDITIONS (CONTINUED)

Reference File : pegnh4

Acquisition File : STATMS2

MS2 Scanning:

Mass 80 Da to 600 Da.

Resolution : 15.0/15.0

Ion Energy : 0.8

Reference File : pegnh4

Acquisition File : SCNMS2

MS2 Scan Speed:

Scan 64 to 473 amu/sec.

Resolution : 15.0/15.0

Ion Energy : 0.8

Reference File : pegnh4

Acquisition File : FASTMS2

Calibration Time: 10:20

Calibration Date: 10/31/01

Coefficients

MS1 Static: $-0.000000000023*x^4 + 0.000000036395*x^3 + -$
 $0.000021182443*x^2 + 1.005305892780*x +-0.410940902914$

MS2 Static: $-0.000000000032*x^4 + 0.000000046280*x^3 + -$
 $0.000021460490*x^2 + 1.003089283521*x +-0.039381138254$

Function 1: None

Function 2: None

Function 3: None

Function 4: None

Function 5: None

Instrument ID: OCP -v3.1_4 -QUAT2 4000

Tuning Parameters: ES+

Source Page (ESI)

Capillary: 4.20 kVolts

HV Lens: 0.10 kVolts

Cone: 30 Volts

Skimmer Offset: 8 Volts

Skimmer: 2.3 Volts

RF Lens: 0.0 Volts

Source Temp: 80 oC

APPENDIX 1 LC/MS/MS CONDITIONS (CONTINUED)

MS1

Ion Energy:	2.0	Volts
Ion Energy Ramp:	0.0	Volts
LM Resolution:	8.0	
HM Resolution:	8.0	
Lens 5:	100	Volts
Lens 6:	2	Volts
Multiplier 1:	750	Volts

MS2

Ion Energy:	2.0	Volts
Ion Energy Ramp:	0.0	Volts
LM Resolution:	8.0	
HM Resolution:	8.0	
Lens 7:	250	Volts
Lens 8:	154	Volts
Lens 9:	0	Volts
Multiplier:	750	Volts

Pressures

Analyser Vacuum:	2.5e-5	mBar
Gas Cell:	1.5e-3	mBar

Acquisition Threshold

SIR or MRM Data	
Baseline level:	1.0
General	
Ion count threshold:	0
Prescan Statistics	
Zero Level:	48
ADC zero:	81.84
ADC standard deviation:	2.23

Acquisition Threshold MS2

SIR or MRM Data	
Baseline level:	1.0
General	
Ion count threshold:	0
Prescan Statistics	
Zero Level:	45
ADC zero:	69.59
ADC standard deviation:	2.31

APPENDIX 1 LC/MS/MS CONDITIONS (CONTINUED)

ACE Experimental Record

----- Run method parameters -----

HP1100 LC Pump Initial Conditions

Solvents

A% (aqueous 2 mM HOAc)	80.0
B% (acetonitrile)	20.0
C%	0.0
D%	0.0
Flow (ml/min)	1.000
Stop Time (mins)	50.0
Min Pressure (bar)	0
Max Pressure (bar)	400
Oven Temperature Left (°C)	25.0
Oven Temperature Right (°C)	25.0

HP1100 LC Pump Gradient Timetable

The gradient Timetable contains 9 entries which are :

Time	A%	B%	C%	D%	Flow	Pressure
0.00	80.0	20.0	0.0	0.0	1.000	400
10.00	80.0	20.0	0.0	0.0	1.000	400
31.00	63.0	37.0	0.0	0.0	1.000	400
32.00	50.0	50.0	0.0	0.0	1.000	400
40.00	50.0	50.0	0.0	0.0	1.000	400
40.10	10.0	90.0	0.0	0.0	2.000	400
45.00	10.0	90.0	0.0	0.0	2.000	400
45.10	80.0	20.0	0.0	0.0	2.000	400
50.00	80.0	20.0	0.0	0.0	2.000	400

HP1100 LC Pump External Event Timetable

The Timetable contains 4 entries which are :

Time	Column	Switch	Contact 1	Contact 2	Contact 3	Contact 4
Initial	On	Off	Off	Off	Off	Off
0.00	On	Off	Off	On	Off	Off
18.00	On	Off	Off	Off	On	Off
40.00	On	On	Off	Off	Off	Off

APPENDIX 1 LC/MS/MS CONDITIONS (CONTINUED)

HP1100 Autosampler Initial Conditions

Injection Volume(μl)	100.0
Draw Speed	200.0
Eject Speed (μl/min)	200
Draw Position (mm)	0.00
Stop Time (mins)	50.00
Vial Number	15

----- oOo -----

End of experimental record.
Solvent Delay
None

Function 1

Scans in function:	499
Cycle time (secs):	0.230
Inter Channel delay (secs):	0.00
Retention window (mins):	20.000 to 24.500
Ionization mode:	ES+
Data type:	SIR or MRM data
Function type:	MRM of 2 channels
Chan Reaction	Dwell(secs) Cone Volt. Col.Energy
1 : 387.90 > 167.00	0.20 30.0 14.0
2 : 387.90 > 205.00	0.20 30.0 27.0

Function 2

Scans in function:	247
Cycle time (secs):	0.230
Inter Channel delay (secs):	0.00
Retention window (mins):	23.200 to 26.500
Ionization mode:	ES+
Data type:	SIR or MRM data
Function type:	MRM of 2 channels
Chan Reaction	Dwell(secs) Cone Volt. Col.Energy
1 : 382.00 > 167.00	0.20 40.0 11.0
2 : 382.00 > 199.00	0.20 40.0 24.0

APPENDIX 1 LC/MS/MS CONDITIONS (CONTINUED)

Function 3

Scans in function: 682
Cycle time (secs): 0.230
Inter Channel delay (secs): 0.00
Retention window (mins): 25.000 to 31.000
Ionization mode: ES+
Data type: SIR or MRM data
Function type: MRM of 2 channels
Chan Reaction Dwell(secs) Cone Volt. Col.Energy
1 : 357.80 > 141.00 0.20 40.0 15.0
2 : 357.80 > 167.00 0.20 40.0 16.0

Function 4

Scans in function: 584
Cycle time (secs): 0.230
Inter Channel delay (secs): 0.00
Retention window (mins): 31.000 to 35.500
Ionization mode: ES+
Data type: SIR or MRM data
Function type: MRM of 2 channels
Chan Reaction Dwell(secs) Cone Volt. Col.Energy
1 : 395.90 > 155.00 0.20 30.0 13.0
2 : 395.90 > 181.00 0.20 30.0 20.0

Function 5

Scans in function: 388
Cycle time (secs): 0.230
Inter Channel delay (secs): 0.00
Retention window (mins): 35.500 to 38.500
Ionization mode: ES+
Data type: SIR or MRM data
Function type: MRM of 2 channels
Chan Reaction Dwell(secs) Cone Volt. Col.Energy
1 : 465.90 > 138.80 0.20 44.0 31.0
2 : 465.90 > 182.00 0.20 44.0 31.0

APPENDIX 2 EXAMPLE OF VALIDATION RESULTS WORKSHEETS

Wheat Grain Validation Set 1a

Study: Method Development : DuPont-8054
 Extraction Date: 19-Dec-01
 Analysis Date: 19-Dec-01
 Analyst: amp
 Matrix: wheat grain
 Quantitation Ions: M6316: m/z 141.167, T6376: m/z 141.167, 199.350; KE459: m/z 182.359
 Analysis Method: 5SUjsa.meth

Run ID	Sample Information			Peak Areas			Recoveries						
	Type	mg/kg or ppm	Identification	M6316 pos. ion	T6376 pos. ion	W4189 pos. ion	L5300 pos. ion	KE459 pos. ion	M6316 %Rec	T6376 %Rec	W4189 %Rec	L5300 %Rec	KE459 %Rec
121901-V1bWh-2	s	0.001	CS-121901	1243	951	1019	2400	1642	0.001		0.003		0.001
121901-V1bWh-3	f	0	Wh-1-121901	477	nd	587	nd	255					
121901-V1bWh-4	s	0.0025	CS-121901	3659	2714	2370	6200	4313					
121901-V1bWh-5	f	0.01	Wh-2-121901	3513	2668	2839	4698	4355	0.009	90%	0.010	105%	0.010
121901-V1bWh-6	f	0.01	Wh-3-121901	3583	2656	2612	4659	4468	0.009	92%	0.010	104%	0.010
121901-V1bWh-7	s	0.005	CS-121901	6815	5149	4577	11704	8358					
121901-V1bWh-8	f	0.1	Wh-4-121901	32224	25693	21436	44293	39558	0.094	94%	0.101	101%	0.089
121901-V1bWh-9	f	0.1	Wh-5-121901	31947	20874	41393	37928	37928	0.094	94%	0.100	100%	0.087
121901-V1bWh-10	s	0.015	CS-121901	20163	15399	13444	33000	23940					
121901-V1bWh-11	f	0.1	Wh-6-121901	29571	23242	19427	37133	36274	0.087	87%	0.091	91%	0.081
121901-V1bWh-12	s	0.03	CS-121901	39201	30303	28945	63961	47918					
121901-V1bWh-13	b	0	blank	nd	nd	nd	nd	nd					

Calibration Standard Regression Data		Response Factors	
Run ID	Type	M6316	T6376
121901-V1bWh-2	s	1243	951
121901-V1bWh-4	s	3659	2714
121901-V1bWh-7	s	6815	5149
121901-V1bWh-10	s	20163	15399
121901-V1bWh-12	s	39201	30303

Calibration Range		RF %RSD	
Avg. RF	RF S/Dev	RF %RSD	RF %RSD
0.99973	81021	6%	5%
1344100	48218	5%	5%
935367	51150	6%	6%
2310567	142933	3%	3%
1646413	54356		

Calibration Range		RF %RSD	
Avg. RF	RF S/Dev	RF %RSD	RF %RSD
0.99999	0.99999	0.99974	0.99994
1020620	935367	2310567	1646413
896267	2200000	1596000	
1010100	898167	2132033	1597267

COMMENTS:
Bolded, underlined values were calculated by subtracting area value determined in control from area value determined in sample.