

Appendix B. Diflubenzuron Analytical Method.

Diflubenzuron Analytical Method as Described in
“Diflubenzuron: Magnitude of the Residue on Small Grain”
Janine E. Rose, Ph.D., IR-4 Study No. 08024, PTRL Study No. 1129W

This method is used for the determination of diflubenzuron (DFB) in wheat and barley grain, forage, hay, straw, wheat middlings, shorts, aspirated grain, flour, bran and germ. This method has demonstrated a limit of quantitation of 0.05 ppm DFB in wheat and barley matrices.

MATERIALS AND METHODS

Equipment

Balance, Fisher XL-5000
Balance, Fisher XT top loading
Balance, Mettler AT261
Balance, Ohaus E4000D
Balance, Satorius L2200P
Beakers, various sizes
Bottles, polypropylene
Centrifuge, Sorvall RT7
Funnel, Separatory
Filter paper, GF/A grade
Flask, round bottom, various sizes
Coffee Mill/Hobart Cutter Mixer
Graduated cylinder, various sizes
Pasteur pipettes, various sizes
Solid Phase Extraction (SPE):
 C18 SPE (1g/6cc)
 Si SPE (1g/6cc)
Sonicator, Branson 2210
Syringes, microliter, various sizes

Tissuemizer, Tekmar

Vacuum evaporator, Büchi Model RE111 or RE114 with temperature controlled bath,
Brinkmann Instruments, Burlingame, CA

Vials, amber (2 mL capacity) with Teflon[®]-lined crimp cap, Chromacol, Inc.,
Trumbull, CT

Volumetric flask, various sizes

Volumetric pipette, various sizes

Reagents and Standards

- * Solvents – HPLC Grade from Fisher Scientific or VWR

Acetone

Acetonitrile

Diethyl ether

1,4-Dioxane

Hexane

Water

- * Standard Reference Substances

The diflubenzuron (DFB) reference standard was provided by Uniroyal Chemical Company, Inc. A stock solution of DFB (99.4%) was prepared at 500 µg/mL in acetone. Separate dilutions of the DFB stock standard were prepared for the fortification and linearity standards, as described below. The reference standards were concluded to be stable based on the comparison of HPLC chromatograms of the first and last analysis. As needed, new standards were prepared and compared to previously prepared standards to insure accurate concentration of the standard.

Statement of Analytical Standards

The following standard was utilized for analysis throughout the study:

Compound:	Diflubenzuron
Purity:	99.4%
Molecular Weight:	311 g/mol
Lot Number:	ARS-9438BA (PTRL West #598W-003)

Supplier: Uniroyal Chemical Company, Inc.
Date Received: July 10, 1996
Expiration Date: December, 2003
Storage Conditions: Freezer Temperature

ANALYTICAL PROCEDURES

Preparation of Sample

Organic wheat flour and wheat germ for the method validation were purchased at a local organic foods market (El Cerrito Natural, San Pablo Ave., El Cerrito, CA 94530). Wheat and barley matrices from the field sites were frozen at PTRL West, Inc and remained frozen until processed or sub-sampled. Wheat grain samples were processed in the presence of dry ice with a coffee mill and stored in Ziploc[®] storage bags. The wheat hay, forage and straw samples were frozen at PTRL West, Inc. and remained frozen until processed or sub-sampled. All wheat hay, forage and straw samples were processed in the presence of dry ice with a Hobart Cutter-Mixer and stored in Ziploc[®] storage bags. All processed samples were stored frozen (< -12°C) until the date of spiking.

Preparation of Standards

For the diflubenzuron analysis, a 500 µg/mL stock standard of DFB in acetone was prepared using the formula as described in the “Methods of Calculation” section. Dilution with acetone of the 500 µg/mL stock standard of diflubenzuron was made (10 mL into 50 mL), yielding a 100 µg/mL DFB stock standard. A portion (1 mL) of the 500 µg/mL DFB stock standard was diluted to 50 mL with acetone yield a 10 µg/mL DFB stock standard. Microliter syringes, volumetric pipettes and volumetric flasks were used throughout.

Fortification Procedure

Fortification of untreated wheat and barley matrices with DFB was performed to analyze method percent recoveries for method validation and for sample set analysis. A portion (10.0 g or 5.0 g) of untreated matrix was fortified as follows:

Fortification Level (ppm)	Sample Weight (g)	
	10.0	5.0
0.05	50 µL of 10 µg/mL	25 µL of 10 µg/mL
0.1	100 µL of 10 µg/mL	NA
0.2	200 µL of 10 µg/mL	NA
0.5	50 µL of 100 µg/mL	25 µL of 100 µg/mL
1.0	100 µL of 100 µg/mL	NA

NA = Not applicable

Preparation of Linearity Standards

Using the 500 µg/mL DFB stock standard, the following linearity standards were prepared using volumetric flasks.

DFB Standard	Dilution (in acetonitrile:water (1:1, v/v))
10.0 µg/mL =	2 mL of 500 µg/mL stock standard diluted to 100 mL or 1 mL of 500 µg/mL stock standard diluted to 50 mL
3.0 µg/mL =	30 mL of 10.0 µg/mL Linearity Standard diluted to 100 mL
2.0 µg/mL =	20 mL of 10.0 µg/mL Linearity Standard diluted to 100 mL
1.0 µg/mL =	10 mL of 10 µg/mL Linearity Standard diluted to 100 mL
0.6 µg/mL =	6.0 mL of 10.0 µg/mL Linearity Standard diluted to 100 mL
0.4 µg/mL =	4.0 mL of 10.0 µg/mL Linearity Standard diluted to 100 mL
0.2 µg/mL =	2.0 mL of 10.0 µg/mL Linearity Standard diluted to 100 mL
0.1 µg/mL =	1.0 mL of 10.0 µg/mL Linearity Standard diluted to 100 mL

A calibration curve was generated with each sample set to determine linearity and to quantitate diflubenzuron residues.

DFB Extraction Method for Small Grain Matrices

1. Weigh a 10 g processed matrix sample into 250 mL Teflon[®] or polypropylene bottle.

2. Fortify as necessary.
3. Add 50 mL of acetonitrile (ACN) and extract by blending using a Tissumizer for ~1 minute.
4. Separate solids from extract by centrifuging sample at 2,500 rpm for 10 minutes at ambient temperature.
5. Decant supernatant into 250 mL graduated cylinder.
6. Repeat extraction of matrix two times as per steps 3-5.
7. Combine supernatants and adjust volume to the nearest 10 mL increment with ACN.
8. Mix combined extract until homogeneous before proceeding.
9. Transfer an aliquot equivalent to $\frac{1}{2}$ total volume (or a measure portion) to an appropriate volume separatory funnel (typically 125 mL). Retain remaining volume of extract.
10. Add 40 mL hexane to separatory funnel and partition by shaking vigorously for ~1 minute.
11. Discard hexane (upper) layer.
12. Repeat process with a second 40 mL portion of hexane.
13. Transfer ACN layer to an appropriate size concentration flask (typically 100 mL).
14. Concentrate to near dryness by rotary evaporation under vacuum at $\sim 40^{\circ}\text{C}$.
15. Reconstitute residue in 2 mL ACN, using ultrasonication, then add 8 mL water and vortex to mix.

Clean-up

1. Condition C18 solid phase extraction (SPE) cartridge (1g/6cc) with one column volume (~ 6 mL) ACN, followed by one column volume methanol, then one column volume of water.
2. Pass entire 10 mL extract (Step 15) through pre-conditioned cartridge under vacuum or with positive pressure discarding eluate.
3. Rinse concentration flask from Step 15 with 6 mL ACN:H₂O (1:1, v/v) and elute cartridge with this eluant. Discard eluant
4. Rinse concentration flask with 5 mL ACN and elute DFB from cartridge into a 25 mL concentration flask.
5. Concentrate to dryness by rotary evaporation under vacuum at $\sim 40^{\circ}\text{C}$.
6. Reconstitute residue in 2 mL diethyl ether:hexane (1:19, v/v), using ultrasonication.
7. Condition Si SPE cartridge (1g/6cc) with one column volume (~ 6 mL) acetone, followed by one column volume of hexane.

8. Pass entire 2 mL extract (Step 6 of clean-up) through pre-conditioned Si SPE cartridge under vacuum or with positive pressure, discarding eluate.
9. Rinse concentration flask with 4 mL diethyl ether:hexane (1:19, v/v) and pass through cartridge, discarding eluate.
10. Pass an additional 4 mL diethyl ether:hexane (1:19, v/v) through cartridge, followed by 9 mL of diethyl ether:hexane (3:7, v/v) discarding both eluates.
11. Elute DFB with 5 mL acetone:hexane (1:1, v/v), collecting eluate in appropriate volume concentration flask.
12. Concentrate eluate to dryness under a stream of nitrogen at ambient temperature.
13. Reconstitute residue in 0.5 mL ACN with ultrasonication. Add 0.5 mL water and vortex to mix.
14. Transfer to crimp top vial for HPLC analysis.

Method Modifications: In the case of wheat straw, the matrix was extracted 3 times with ~100-150 mL acetonitrile in step 3, instead of 50 mL. In step 10 of Extraction, 60 mL hexane was used in straw analysis and flour validation extractions. Wheat flour validation and shorts/middlings sample extraction was conducted with 5 gram sample weights and three extractions with 50 mL ACN with 30 minutes vigorous shake on a wrist-action shaker. In this case, the entire extract for wheat flour was placed in the separatory funnel in Step 9 of the Extraction Method.

INSTRUMENTATION

Method A:

Instrument: Hewlett Packard HPLC Series 1050

Column: Zorbax C8 (250mm x 4.6 mm) with Zorbax C8 Guard column (30 mm x 4.6 mm)

Flow Rate: 1.5 mL/minute

Detector: 254 nm

Injection Volume: 20 μ L; by HP 1050 Autosampler

Solvent System:

Solvent A = Acetonitrile:Water:1,4-Dioxane (45:45:10)

Solvent B = Acetonitrile:Water:1,4-Dioxane (85:5:10)

Solvent Program:

Time (minutes)	Solvent A	Solvent B
0	100%	0%
15	100%	0%
20	0%	100%
30.5	100%	0%
40	100%	0%

Retention Time: ~8minutes

Method B:

Instrument: Hewlett Packard HPLC Series 1050

Column: Altima C18 or Zorbax C8 (250mm x 4.6 mm, 5 μ) Flow Rate: 1.0 mL/minute

Detector: 254 nm

Injection Volume: 20 μ L; by HP 1050 Autosampler

Solvent System:

Solvent A = Water

Solvent B = Acetonitrile

Solvent Program:

Time (minutes)	Solvent A	Solvent B
0	75%	25%
30	0%	100%
35	0%	100%
35.5	75%	25%
43	75%	25%

Retention Time: 21 minutes on Altima C18 or 9 minutes on Zorbax C8

Separation of the analyte from small grain matrix was achieved by high performance liquid chromatography. The analyte was identified by the coincidence of its retention time with the reference standards, and quantitated by integration of the peak area.

METHODS OF CALCULATION

Preparation of Stock Standards

$$\text{Volume of solvent (mL)} = \frac{(W) \times (P)}{(FC)}$$

where W = Milligrams of neat standard
 P = Chemical purity of neat standard
 FC = Final Concentration (mg/mL)

Recoveries

The recoveries of DFB from fortified samples were calculated as follows:

Linear regression formula from calibration curve $y = mx + b$

$$\mu\text{g/mL DFB} = \frac{y - b}{m}$$

where y = Sample peak area
 b = Calibration intercept
 m = Slope

$$\text{ppm DFB} = [(\mu\text{g/mL DFB} \times 1 \text{ mL final volume} \times \frac{\text{Init. Extract vol. (mL)}}{(\text{Aliquot vol. (mL)})}) \div 10 \text{ g}]$$

x Dilution Factor

Percent Recovery =

$$\frac{\text{Concentration of DFB Fortified Sample } (\mu\text{g/g}) - \text{Concentration of DFB Control } (\mu\text{g/g})}{\text{DFB Fortification Level } (\mu\text{g/g})} \times 100$$

To demonstrate validity of the analytical method for acceptable recovery (70-120%) of the DFB in grain, forage, hay, straw, germ and flour a control sample for each set of small grain matrix samples was fortified with DFB at 0.05 to 0.5 ppm, as described above. Residues of DFB in treated samples were calculated as shown above, with no control residues subtracted.

After spiking the samples, the samples were extracted and analyzed as previously described in the Analytical Procedures, “DFB Extraction Method for Small Grain Matrices.” Diflubenzuron standards were prepared (see Preparation of Linearity Standards) for the DFB analysis. These samples ranged in concentration from 0.1 µg/mL to 10 µg/mL and a calibration curve was generated with each sample set. The equation of the line based on the peak area of the derivatized standard versus the µg/mL concentration injected was generated by least squares linear regression calculated by the computer program, Microsoft™ Excel. The correlation coefficient (r^2) calculated for each set of standards could not be less than 0.97 for the data to be considered acceptable. Representative chromatograms of DFB and representative calibration curves are given in Appendix E. Representative chromatograms of a solvent, reagent blank, control untreated sample, fortified samples and treated samples analyzed for DFB are also given in Appendix E.

An example calculation for the recovery of DFB (0.1 ppm fortification) from wheat grain (PTRL sample no. 1129W-024) is shown below:

Linear regression analysis of the derivatized DFB standards gave a linearity with the formula $y = 22,401 + 1,792,546x$ ($r^2 = 0.9998$). The µg/mL of DFB determined by this curve was calculated as follows.

$$\mu\text{g/mL DFB} = (868,639 - 22,401) \div 1,792,546 = 0.472 \mu\text{g/mL}$$

Calculation of the ppm DFB in grain was:

$$\text{ppm DFB} = (0.472 \times 2) \div 10 \text{ g} = 0.094 \text{ ppm}$$

The percent recovery of DFB equals 0.094 ppm DFB minus control (0 ppm) DFB, divided by the 0.10 ppm fortification. The percent recovery for this sample was 94%.

Appendix C. 4-Chlorophenylurea (CPU) Analytical Method.

**4-Chlorophenylurea Analytical Method as Described in
“Diflubenzuron: Magnitude of the Residue on Small Grain”
Janine E. Rose, Ph.D., IR-4 Study No. 08024, PTRL Study No. 1129W**

This method is used for the determination of 4-chlorophenylurea (CPU) in wheat and barley grain, forage, hay, straw, wheat middlings, shorts, aspirated grain, germ, flour and bran. This method has demonstrated limit of quantitation for CPU in 0.005 ppm in wheat and barley matrices.

MATERIALS AND METHODS

Equipment

Glassware and Miscellaneous Equipment

- Balance, Fisher XL-5000
- Balance, Fisher XT top loading
- Balance, Mettler AT261
- Balance, Ohaus E4000D
- Balance, Satorius L2200P
- Beakers, various sizes
- Bottle, amber, with Teflon[®]-lined cap, various sizes
- Bottle, Teflon[®] or polypropylene, 250 mL
- Büchner funnel
- Filter paper, GF/A grade
- Flask, round bottom, 125 mL, 250 mL, 500 mL
- Graduated cylinder, various sizes
- Nitrogen Evaporator
- Pasteur pipettes, various sizes
- Solid Phase Extraction Si (5g, 20cc), Varian
- Sonicator, Branson 2210
- Suction Flask, 500 mL
- Syringes, microliter, 500 µL, 250 µL, 100 µL
- Thermometer, mercury

Vacuum evaporator, Büchi Model RE111 or RE114 with temperature controlled bath,
Brinkmann Instruments, Burlingame, CA

Vials, amber (2 mL capacity) with Teflon[®]-lined crimp cap, Chromacol, Inc.,
Trumbull, CT

Vials, glass with Teflon[®]-lined cap, 10 mL

Volumetric flask, various sizes

Volumetric pipette, various sizes

Waring[™] Blender with 1 quart cup

Reagents and Standards

- * Solvents – HPLC grade or equivalent from Fisher Scientific or VWR

Acetone

Acetonitrile

Ethanol, 200 proof, Gold Shield

Ethyl Acetate

Heptafluorobutyric anhydride (Pierce)

Hexanes

Petroleum Ether

Sodium Sulfate, anhydrous, ACS Grade, Fisher Scientific

Sodium Carbonate, ACS Grade, Fisher Scientific

Water

- * Standard Reference Substances

Uniroyal Chemical Company, Inc. provided the reference standard of 4-chlorophenylurea (CPU) with a stated purity of 99.7%. A stock solution of 4-chlorophenylurea (CPU) was prepared at 100 µg/mL in acetonitrile. Separate dilutions of the CPU stock standard was prepared for the fortification and linearity standards, as described below. Reference standards were concluded to be stable based on the comparison of chromatograms of the first and last analysis. As needed, new standards were prepared and compared to previously prepared standards to insure accurate concentration of the standard.

Statement of Analytical Standards

The following standard was utilized for analysis throughout the study:

Compound:	4-Chlorophenylurea
Purity:	99.7%
Molecular Weight:	170.59 g/mole
Lot Number:	ARS-87G27N (PTRL West #614W-002)
Supplier:	Uniroyal Chemical Company, Inc.
Date Received:	October 11, 1996
Expiration Date:	January 31, 2006
Storage Condition:	Freezer Temperature

ANALYTICAL PROCEDURES

Preparation of Sample

Organic wheat flour and wheat germ for the method validation were purchased at a local organic foods market (El Cerrito Natural, San Pablo Ave., El Cerrito, CA 94530). Wheat and barley matrices from the field sites were frozen at PTRL West, Inc and remained frozen until processed or sub-sampled. Wheat grain samples were processed in the presence of dry ice with a coffee mill and stored in Ziploc[®] storage bags. The wheat hay, forage and straw samples were frozen at PTRL West, Inc. and remained frozen until processed or sub-sampled. All wheat hay, forage and straw samples were processed in the presence of dry ice with a Hobart Cutter-Mixer and stored in Ziploc[®] storage bags. All processed samples were stored frozen (< -12°C) until the date of spiking.

Preparation of Standards

For the 4-chlorophenylurea analysis, a 100 µg/mL stock standard of CPU in acetonitrile was prepared using the formula as described in the “Methods of Calculation” section. Dilution of the 100 µg/mL stock standard of 4-chlorophenylurea with acetone or acetonitrile was made (1.0 mL into 100 mL or 0.5 mL into 50 mL), yielding 1.0 µg/mL and 5.0 µg/mL CPU fortification solutions, respectively. Dilutions of the 1.0 µg/mL CPU fortification standard were used to make the CPU linearity Solutions. Volumetric pipettes and volumetric flasks were used throughout.

Fortification Procedure

Fortification of untreated small grain matrices with CPU was performed to analyze method percent recoveries for the analyte within the validation and sample analysis sets. Portions (12.5 g or 25.0 g) of untreated matrices were fortified as follows:

Fortification Level	Sample Weight	
	12.5 g	25.0 g
0.005 ppm	625 μ L of 0.1 μ g/mL	125 μ L of 1 μ g/mL
0.01 ppm	1.25 mL of 0.1 μ g/mL	NA
0.05 ppm	625 μ L of 1 μ g/mL	250 μ L of 5 μ g/mL
0.1 ppm	1.25 mL of 1 μ g/mL	NA
0.5 ppm	NA	125 μ L of 100 μ g/mL

Preparation of Linearity Standards

Using the 100 μ g/mL CPU stock standard, the following linearity standards were prepared using, volumetric pipettes, and volumetric flasks.

<u>CPU Standard</u>	<u>Dilution (in acetonitrile)</u>
1.00 µg/mL	1.0 mL of 100 µg/mL diluted to 100 mL
0.25 µg/mL	6.25 mL of 1 µg/mL stock standard diluted to 25 mL
0.125 µg/mL	3.125 mL of 1 µg/mL stock standard diluted to 25 mL
0.0625 µg/mL	1.56 mL of 1 µg/mL stock standard diluted to 25 mL
0.025 µg/mL	0.625 mL of 1 µg/mL stock standard diluted to 25 mL or 2.5 mL of 0.25 µg/mL stock standard diluted to 25 mL
0.0125 µg/mL	2.5 mL of 0.125 µg/mL stock standard diluted to 25 mL
0.00625 µg/mL	2.5 mL of 0.0625 µg/mL stock standard diluted to 25 mL

Derivatization of the standards was carried out as per Steps 16 through 18 of the CPU Extraction Method for Small Grains. A calibration curve was generated with each sample set to determine linearity and to quantitate 4-chlorophenylurea residues.

CPU Extraction Method for Small Grains

1. Weigh 12.5 g processed matrix (25g for wheat and barley grain or 25 g for storage stability) into a beaker. Fortify samples as needed.
2. Combine sample plus 100 g anhydrous sodium sulfate and 200 mL ethyl acetate (EtOAc) in a Waring blender cup and blend for 5 minutes.
3. Filter through Whatman GF/A filter paper by vacuum filtration and collect filtrate in 500 mL filter flask. Rinse blender and filter cake with an additional 50 mL EtOAc.
4. When conducting grain samples, transfer combined filtrate to 250 mL graduate cylinder and dilute to 250 mL with EtOAc. Transfer extract back to filter flask and mix well. Use ½ or 125 mL of this extract to continue the method.

When conducting the experiment with any matrix other than small grain, continue the method with the entire extract from Step 3.

5. Transfer appropriate extract volume to 250 mL concentration flask and roto-evaporate to dryness at ~35°C.
6. Dissolve residue in 5 mL acetone plus 25 mL petroleum ether.
7. Prepare a 5g/20cc Silica SPE cartridge by pre-washing the column with 75 mL petroleum ether. Discard eluant.
8. Transfer acetone:petroleum ether sample from Step 6 to column and allow sample to elute to top of SPE cartridge before continuing.
9. Rinse concentration flask with 25 mL petroleum ether and transfer rinse to column, again elute to top of SPE cartridge before continuing.
10. Wash column with 50 mL acetone:petroleum ether (20:80, v/v) and elute to top of column. Discard eluate.
11. Elute CPU with 50 mL ethanol:petroleum ether (15:85, v/v), followed by 60 mL ethanol:petroleum ether (30:70, v/v), collecting eluant in 250 mL concentration flask.
12. Roto-evaporate to dryness at ~45°C, using nitrogen to evaporate to dryness as necessary.
13. Dissolve residue in 5 mL hexane, then roto-evaporate to dryness at ~45°C, using nitrogen to evaporate to dryness as necessary.

14. Dissolve residue in 5 mL acetonitrile, using ultrasonic bath to assure complete dissolution. Filter samples through 0.45 μ m filters, as necessary.
15. Transfer sample to 10 mL vial with Teflon[®]-lined cap.
16. Pipet 1.0 mL of sample (using volumetric pipet) into a separate vial and add 100 μ L of heptafluorobutyric anhydride (HFBA). Cap vial and mix. Allow sample to stand for ~10 minutes at ambient temperature.
17. Add 5 mL HPLC grade water, 0.5 mL saturated Na₂CO₃, plus 5 mL (volumetric pipet) of hexane. Shake for ~30 seconds then allow phases to separate.
18. Transfer a portion of the hexane layer to a GC vial for analysis.

INSTRUMENTATION

Gas Chromatography

Derivatization of CPU is known to yield the same product as derivatization of 4-chloroaniline (see PCA Method description).

Instrumentation: Model No. 5890 Hewlett Packard Gas Chromatograph (GC) equipped with Mass Spectral Detector (MSD) or electron capture detector (ECD)

Column: DB 1701 Capillary Column
30m x 0.32mm i.d. x 0.25 μ m film thickness

Flow Rate: Carrier Gas = 1 mL/minute: Helium

Injector Temperature: 250°C

Detector Temperature: 290°C

Injection Volume: 2 μ L; by Hewlett Packard 7673A Autosampler

Oven Temperature:

Initial Temperature: 80°C for 1 minute
Ramp: 80°C to 200°C at 10°C/minute
200°C for 1 minutes
200°C to 260°C at 30°C/minute

260°C for 5 minutes

Retention Time: ~ 10 minutes for derivatized CPU

Where combined peak areas of derivatized CPU ions m/z126, m/z 154 and m/z 323 or ECD peak area are used to quantitate CPU residues relative to a standard curve. A typical injection sequence for wheat sample analysis was: hexane solvent blank, 0.00625 µg/mL derivatized CPU standard, untreated wheat sample, untreated wheat sample, 0.0125 µg/mL derivatized CPU standard, fortified wheat sample, fortified wheat sample, 0.025 µg/mL derivatized CPU standard, treated wheat sample, treated wheat sample, 0.0625 µg/mL derivatized CPU standard, etc. ending with a final QC standard.

Separation of the analyte from small grain matrix was achieved by gas chromatography. The analyte was identified by the coincidence of its retention time with the reference standards, and quantitated by integration of the peak area.

METHODS OF CALCULATION

Preparation of Stock Standards

$$\text{Volume of solvent (mL)} = \frac{(W) \times (P)}{(FC)}$$

where W = Milligrams of neat standard
 P = Chemical purity of neat standard
 FC = Final Concentration (mg/mL)

Recoveries

The recoveries of CPU from fortified samples were calculated as follows:

Linear regression formula from calibration curve $y = mx + b$

$$\text{ng/mL (or } \mu\text{g/mL) CPU} = \frac{y - b}{m}$$

where y = Sample peak area
 b = Calibration intercept
 m = Slope

$$\text{ppm CPU} = \frac{\mu\text{g/mL CPU} \times \text{Final Volume (mL)} \times \text{Initial Extracted Volume (mL)}}{\text{Aliquoted Volume (mL)} \times \text{sample weight (g)}} \times \text{dilution factor}$$

Percent Recovery =

$$\frac{\text{Concentration of CPU Fortified Sample } (\mu\text{g/g}) - \text{Concentration of CPU Control } (\mu\text{g/g})}{\text{CPU Fortification Level } (\mu\text{g/g})} \times 100$$

To demonstrate validity of the analytical method for acceptable recovery (70-120%) of the CPU from wheat and barley matrices, a control sample for each set of was fortified with CPU in duplicate, as described above. Residues of CPU (ppm CPU) in treated and untreated control samples were calculated as shown above.

After spiking the samples, the samples were extracted, and analyzed as previously described in the Analytical Procedures. The linearity standards ranged in concentration from 0.00625 $\mu\text{g/mL}$ to 0.25 $\mu\text{g/mL}$ and a calibration curve was generated with each sample set. The equation of the line based on the peak area of the derivatized standard versus the $\mu\text{g/mL}$ injected was generated by least squares linear regression calculated by the computer program, Microsoft™ Excel. The correlation coefficient (r^2) calculated for each set of standards could not be less than 0.97 for the data to be considered acceptable. Representative chromatograms of CPU and representative calibration curves are given in Appendix F. Representative chromatograms of control untreated, corresponding fortified matrix samples and treated matrix samples analyzed for CPU are also given in Appendix F.

An example calculation for the recovery of CPU (0.005 ppm fortification) from barley hay method validation (PTRL sample no. 1129W-005) is shown below:

Linear regression analysis of the derivatized CPU standards gave a curve with the curve $y = 813,193x + 1349$ ($r^2 = 0.9973$). The micrograms per milliliter ($\mu\text{g/mL}$) of CPU determined by this curve was calculated as follows.

$$\mu\text{g/mL CPU injected} = (9,629 - 1,349) \div 813,193 = 0.010 \mu\text{g/mL}$$

Calculation of the ppm CPU in barley hay was as follows:

$$\text{ppm CPU} = \frac{0.010 \mu\text{g/mL} \times 5 \text{ mL} \times 250 \text{ mL}}{250 \text{ mL} \times 12.5 \text{ g}} = 0.004 \text{ ppm}$$

The percent recovery of CPU equals 0.004 ppm CPU minus control (0.000 ppm) CPU, divided by the 0.005 ppm fortification. The percent recovery for this sample was 80%.

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Appendix D. 4-Chloroaniline (PCA) Analytical Method.

**4-Chloroaniline Analytical Method as Described in
“Diflubenzuron: Magnitude of the Residue on Small Grain”
Janine E. Rose, Ph.D., IR-4 Study No. 08024, PTRL Study No. 1129W**

This method is used for the determination of 4-chloroaniline (PCA) in wheat and barley grain, forage, hay, straw, wheat middlings, shorts, aspirated grain, flour, germ and bran. This method has demonstrated a limit of quantitation of 0.005 ppm PCA in wheat and barley matrices.

MATERIALS AND METHODS

Equipment

Balance, Fisher XL-5000
Balance, Fisher XT top loading
Balance, Mettler AT261
Balance, Ohaus E4000D
Balance, Satorius L2200P
Beakers, various sizes
Bottle, amber, with Teflon[®]-lined cap, 500 mL
Büchner funnel, 11 cm
Centrifuge, Mistral[™] 3000E
Centrifuge tubes, polypropylene and Teflon[®], 250 mL and 500 mL
Filter paper, Whatman #4, 10 cm
Florisil[™], Varian[™], 6cc/1 GRM
Funnel, 5 cm diameter
Graduated cylinder, various sizes
Pasteur pipettes, 5", 9"
pH paper, Color pHast
Separatory funnel, 500 mL
Shaker Bath, Lab-Line[™] 3582
Suction flask, 500 mL
Syringes, microliter, 500 μ L, 250 μ L, 100 μ L
Thermometer, mercury

Vials, glass with Teflon[®]-lined cap, 35 mL, 50 mL
Volumetric flask, various sizes
Volumetric pipette, various sizes
Wrist-Action[™] shaker, Burrell, Model 75

Reagents and Standards

* Solvents – HPLC Grade

Acetone

Hexane

Water

Heptafluorobutyric Acid Anhydride (HFBA), Pierce

Hydrochloric Acid, Concentrated, Fisher Scientific

Sodium Carbonate, ACS Grade, Fisher Scientific

Sodium Chloride, ACS Grade, EM Science

Sodium Hydroxide, ACS Grade, EM Science

Sodium Sulfate, ACS-Residue Grade, Fisher Scientific

* Standard Reference Substances

The reference standard of ¹²C-parachloroaniline was purchased from Chem Service, while the ¹³C-parachloroaniline was provided by Uniroyal Chemical Co. (Crompton Corporation). Certification of these reference standards was conducted by Chem Service and Crompton Corp., respectively. A stock solution of parachloroaniline (¹²C-PCA) was prepared at 500 µg/mL in hexane. Additionally, a stock solution of ¹³C-PCA was prepared at 500 µg/mL in hexane. Separate dilutions of the ¹²C-PCA and ¹³C-PCA stock standards were prepared for the fortification and linearity standards, as described below. Reference standard solutions were concluded to be stable based on the comparison of gas chromatograms of the first and last analysis. As needed, new standards were prepared and compared to previously prepared standards to insure accurate concentration of the standard.

STATEMENT OF ANALYTICAL STANDARDS

The following standards were utilized for analysis throughout the study:

Compound: Parachloroaniline
Purity: 98.8%
Molecular Weight: 128 g/mole
Lot Number: 208-18-B (PTRL West #755W-022)
Supplier: Chem Service
Date Received: July 10, 1998
Expiration Date: May, 2003
Storage Condition: Freezer Temperature

Compound: Parachloroaniline
Purity: 99%
Molecular Weight: 128 g/mole
Lot Number: 298-139B (PTRL West #1129W-074)
Supplier: Chem Service
Date Received: May 29, 2003
Expiration Date: March, 2008
Storage Condition: Freezer Temperature

Compound: ¹³C-Parachloroaniline
Purity: 98%
Isotope Purity: 99%
Molecular Weight: 133 g/mole
Lot Number: P-5493 (PTRL West #810W-075)
Source: Cambridge Isotope Labs
Supplier: Uniroyal Chemical Company, Inc. (Crompton Corp.)
Date Received: March 3, 2000
Expiration Date: Not available
Storage Condition: Freezer Temperature

ANALYTICAL PROCEDURES

Preparation of Sample

Organic wheat flour and wheat germ for the method validation were purchased at a local organic foods market (El Cerrito Natural, San Pablo Ave., El Cerrito, CA 94530). Wheat and barley matrices from the field sites were frozen at PTRL West, Inc and remained frozen until processed or sub-sampled. Wheat grain samples were processed in the presence of dry ice with a coffee mill and stored in Ziploc[®] storage bags. The wheat hay,

forage and straw samples were frozen at PTRL West, Inc. and remained frozen until processed or sub-sampled. All wheat hay, forage and straw samples were processed in the presence of dry ice with a Hobart Cutter-Mixer and stored in Ziploc[®] storage bags. All processed samples were stored frozen (< -12°C) until the date of spiking.

Preparation of Standards

A stock solution of 500 µg/mL ¹²C-parachloroaniline was prepared in hexane using the formula as described under the “Methods of Calculation” section. A portion (10 mL) of the 500 µg/mL parachloroaniline stock solution was diluted (to 100 mL with hexane) to yield a 50 µg/mL parachloroaniline standard in hexane. Dilutions of the 50 µg/mL PCA standard were prepared to 0.5 µg/mL and 5.0 µg/mL ¹²C-PCA fortification standard. A stock solution of 500 µg/mL ¹³C-parachloroaniline was prepared in hexane using the formula as described under the “Methods of Calculation” section. A stock standard of ¹³C-parachloroaniline (50, 5.0 and 0.5 µg/mL) was prepared in hexane by the same method. Microliter syringes, volumetric pipettes and volumetric flasks were used throughout.

Derivatized ¹²C-PCA and ¹³C-PCA standard solutions were prepared by treating PCA stock standards (10-15 mL of 5 µg/mL or 10-20 mL of 0.5 µg/mL) with 100 µL heptafluorobutyric anhydride by the method described in Steps 26 to 29 in the Extraction Method.

Fortification Procedure

Fortification of untreated wheat and barley matrices with PCA was performed by addition of the following quantities of underivatized ¹²C-PCA stock standard and ¹³C-PCA stock standard: 0.25 mL of 0.5 µg/mL ¹²C-PCA stock standard plus 0.25 mL of 0.5 µg/mL ¹³C-PCA stock standard was added to a portion (25 g) of processed, untreated small grain matrix to yield 0.005 ppm. Similarly, fortifications at 0.05 ppm were carried out by addition of 250 µL of 5 µg/mL ¹²C-PCA stock standard plus 250 µL of 0.5 µg/mL ¹³C-PCA stock standard. Fortifications at 0.10 ppm were carried out by addition of 500 µL of 5 µg/mL ¹²C-PCA stock standard plus 250 µL of 0.5 µg/mL ¹³C-PCA stock standard. Due to the ability of the matrix to strongly adsorb PCA, the fortification standards (i.e. ¹²C-PCA and ¹³C-PCA) were premixed prior to addition to control samples.

Preparation of Linearity Standards

External Linearity Standards: External derivatized ^{12}C -PCA standards were prepared by dilutions of the derivatized standard.

^{12}C -PCA Standard	Dilutions (in hexane)
0.05 $\mu\text{g/mL}$	10 mL of 0.5 $\mu\text{g/mL}$ diluted to 100 mL
0.03 $\mu\text{g/mL}$	6 mL of 0.5 $\mu\text{g/mL}$ diluted to 100 mL
0.01 $\mu\text{g/mL}$	20 mL of 0.05 $\mu\text{g/mL}$ diluted to 50 mL
0.005 $\mu\text{g/mL}$	10 mL of 0.05 $\mu\text{g/mL}$ diluted to 100 mL

Ratio Linearity Standards:

Ratio ^{12}C -PCA: ^{13}C -PCA ^a	^{12}C -PCA Added	^{13}C -PCA Added	Hexane Added
0.5:1.0	0.25 mL of 0.025 $\mu\text{g/mL}$	0.5 mL of 0.025 $\mu\text{g/mL}$	0.25 mL
1.0:1.0	0.5 mL of 0.025 $\mu\text{g/mL}$	0.5 mL of 0.025 $\mu\text{g/mL}$	NA
5.0:1.0	0.125 mL of 0.5 $\mu\text{g/mL}$	0.5 mL of 0.025 $\mu\text{g/mL}$	0.375 mL
10.0:1.0	0.25 mL of 0.5 $\mu\text{g/mL}$	0.5 mL of 0.025 $\mu\text{g/mL}$	0.25 mL
20.0:1.0	0.5 mL of 0.5 $\mu\text{g/mL}$	0.5 mL of 0.025 $\mu\text{g/mL}$	NA

a Standards utilized for ^{12}C -PCA and ^{13}C -PCA were derivatized PCA standards.

An external linearity calibration curve and a ratio linearity curve were generated with each sample set to determine linearity of PCA analysis over the range of PCA residue concentration, as well as linear correlation of ^{12}C -PCA to ^{13}C -PCA MSD response.

PCA Extraction Method in Small Grains

1. Weigh 25.0 g. wheat or barley matrix into 250 mL Teflon[®] centrifuge tube.
2. Fortify with the appropriate amounts of ^{12}C -PCA (where applicable) and ^{13}C -PCA fortification solutions using application syringes, premixing the fortification aliquots prior to application to matrix.
3. Shake several times to mix, wait 5 minutes.
4. Add 150 mL 0.1 N HCl (aq.).
5. Place in shaker bath pre-equilibrated at 60°C.
6. Shake at medium to high speed for 30 minutes.

7. Cool sample in ice bath to approximately room temperature.
8. Centrifuge at 2500 rpm for 5 minutes.
9. Filter supernatant through Büchner funnel (11 cm) with Whatman # 4 filter paper into 500 mL filter flask, leave pellet in centrifuge tube.
10. Add 50 mL 0.1 N HCl to pellet, shake for 20 minutes on wrist-action shaker.
11. Centrifuge at 2,500 rpm for 5 minutes.
12. Filter as in step 9 using the same filter paper as above.
13. Repeat steps 10-12, once, adding the pellet to filter paper in last wash.
14. Transfer filtrate to 500 mL centrifuge tube (Teflon[®] or polypropylene), wash flask with 25 mL 0.1N HCl, adding to filtrate.
15. Add 50% NaOH (aq.) to filtrate until pH >12 (~ 2 mL), with swirling.
16. Add 100 mL hexane and 10-15 g NaCl, shake gently, or mix by swirling, for approximately one minute.
17. Centrifuge at 2500 rpm for 5 minutes.
18. Remove hexane with 25 mL pipette and place in 500 mL separatory funnel, avoiding emulsion if present.
19. Repeat hexane partition two more times, using 100 mL each time, combining hexane extracts in 500 mL separatory funnel.
20. Partition the combined hexane extracts twice with 0.1 N HCl, 25 mL then 10 mL, combining acid extracts.
21. Add 50% NaOH to acid extracts until pH > 12. (~ 0.5 mL)
22. Partition with hexane three times, 10 mL each, decanting with pipette and combining hexane extracts. Add a spatula tip full of sodium sulfate to containers, mix.
23. Put the combined hexane extracts onto 6 cc/1 GRM Florisil[™] column (Varian).
24. Elute with 1 mL 20% acetone in hexane, discard eluant.

25. Elute the analyte from the column with just under 3 mL 20% acetone in hexane, bringing the final volume to 3 mL in a 3 mL volumetric flask or graduated cylinder.
26. Transfer the eluant to a derivatizing vial.
27. Add 100 μ L HFBA, close container, mix by swirling, allow to stand at room temperature for 10 minutes.
28. Add 5 mL water and 0.5 mL saturated sodium carbonate (aq.), shake (~ 30 s.) and allow phases to separate.
29. Pipette hexane layer into GC vial, place onto GC/MS with SIM of ions 323, 329, 154, 160, 126 and 132.

INSTRUMENTATION

Instrumentation: Model No. 5890 Hewlett Packard Gas Chromatograph (GC) equipped with Mass Spectral Detector (MSD)

Column: DB-1701 Capillary Column (J & W Scientific Inc.)
30m x 0.32mm i.d. x 0.25 μ m film thickness

Flow Rate: Carrier Gas = 1 mL/minute: Helium

Injector Temperature: 250°C

Detector Temperature: 290°C

Injection Volume: 2 μ L; by Hewlett Packard 7673A Autosampler

Oven Temperature:

Initial Temperature: 80°C for 1 minute
Ramp: 80°C to 200°C at 10°C/minute
200°C for 1 minutes
200°C to 260°C at 30°C/minute
260°C for 5 minutes

Retention Time: ~ 10 minutes for derivatized PCA

Separation of the analyte was achieved by capillary gas chromatography. The analyte was identified by the coincidence of its retention time with the internal standard (¹³C-PCA), and quantitated by integration of the peak areas for ¹²C-PCA relative to peak areas

for ^{13}C -PCA. Additionally, quantitation was achieved by peak area of the ^{12}C -PCA relative to the external standard linearity curve.

A typical injection sequence for PCA samples was: hexane blank, 0.5:1.0 ^{12}C : ^{13}C -PCA derivatized ratio linearity standard, unfortified control small grain sample, 1.0:1.0 ^{12}C : ^{13}C -PCA derivatized ratio linearity standard, 0.005 ppm ^{12}C -PCA fortified small grain sample, 0.005 ppm ^{12}C -PCA fortified small grain sample, 5.0:1.0 ^{12}C : ^{13}C -PCA derivatized ratio linearity standard, unfortified control small grain sample, treated small grain sample, treated small grain sample, treated small grain sample, 10.0:1.0 ^{12}C : ^{13}C -PCA derivatized ratio linearity standard, treated small grain sample, treated small grain sample, treated small grain sample, 20.0:1.0 ^{12}C : ^{13}C -PCA derivatized ratio linearity standard, 0.005 $\mu\text{g/mL}$ derivatized ^{12}C -PCA standard, 0.01 $\mu\text{g/mL}$ derivatized ^{12}C -PCA standard, 0.03 $\mu\text{g/mL}$ derivatized ^{12}C -PCA standard, 0.05 $\mu\text{g/mL}$ derivatized ^{12}C -PCA standard, QC standard, hexane. Note that area ratio linearity standards were used to establish that the response was linear over the range of detection.

METHODS OF CALCULATION

Preparation of Stock Standards

$$\text{Volume of solvent (mL)} = \frac{(W) \times (P)}{(FC)}$$

where W = Milligrams of neat standard
 P = Chemical purity of neat standard
 FC = Final Concentration ($\mu\text{g/mL}$)

Recoveries

Analysis of the sample sets included a determination of recovery based on the level of ^{12}C -PCA spiked using peak area relative to the internal standard (^{13}C -PCA) and determination of the overall percent recovery based on an external standard linearity.

After spiking the samples with ^{12}C -PCA and ^{13}C -PCA, the fortified samples were extracted, derivatized and analyzed as previously described in the Analytical Procedures, PCA Extraction Method in Small Grain. Quantitation of the PCA was done by mass

spectral peak area relative to internal standard ¹³C-PCA. Two major ions were quantitated for each of ¹²C-PCA and ¹³C-PCA.

PPB of ¹²C-PCA =

$$\frac{(\text{PA } m/z154 + \text{PA } m/z323)}{\text{Response Factor } (160 + 329)} - \frac{(\text{control PA } m/z 154 + \text{control PA } m/z 323)}{\text{Control Response Factor } (160 + 329)}$$

where

PA = Peak area

$$\text{Response Factor } (160 + 329) = \frac{(\text{PA } m/z 160 + \text{PA } m/z 329)}{5 \text{ ppb}}$$

Control residues were only subtracted from fortified control samples. PCA residues in treated samples (ppm PCA) were calculated as shown above, without subtracting control residues. The recoveries of 4-chloroaniline from ¹²C-PCA:¹³C-PCA fortified samples were calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{concentration (PPM)} \text{ } ^{12}\text{C-PCA}}{\text{concentration (PPM)} \text{ } ^{12}\text{C-PCA fortified}} \times 100$$

An example calculation for the recovery of ¹²C-PCA (0.005 ppm fortification) from wheat grain method validation (PTRL sample no. 1129W-001) is shown below:

Twenty five grams of wheat grain were fortified with 0.005 ppm of ¹²C-PCA (or 5 ppb) and 0.005 ppm of ¹³C-PCA (or 5 ppb). The combined peak areas for the ¹²C-PCA (m/z 154 = 12,499, m/z 323 = 34,593) equaled 47,092 area units. The combined peak areas for the ¹³C-PCA (m/z 160 = 10,355, m/z 329 = 31,347) was 41,702 area units. The response factor was 41,702/5 or 8,340.4. Therefore, the ppb of ¹²C-PCA was 47,092 divided by 8,340.4 or 5.6 ppb. Subtracting the control residue of 0 ppb yields a recovered residue of 5.6 ppb or 0.0056 ppm. The recovery was:

$$\text{Recovery} = \frac{0.0056}{0.005} \times 100 = 112\%$$