

**TITLE**

Dimilin® 25W, Dimilin® 80WG, and Dimilin® 2L in Almonds:  
Magnitude of the Residue Study

**DATA REQUIREMENT**

EPA Assessment Guidelines:  
Subdivision O, Section 171-4: Residue Analytical Method  
and  
EPA Residue Chemistry Test Guidelines:  
OPPTS 860:1500: Crop Field Trials

**AUTHOR**

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**STUDY INITIATION DATE**

January 28, 1998

**ANALYTICAL REPORT DATE**

September 13, 1999

**PERFORMING LABORATORY**

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**LABORATORY STUDY IDENTIFICATION**

Uniroyal Study No. RP-98003  
PTRL Report No. 723W-1  
PTRL Project No. 723W

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**Diflubenzuron Analytical Method as Described in  
"Dimilin<sup>®</sup> 25W, Dimilin 80<sup>®</sup> WG and Dimilin<sup>®</sup> 2L in Almonds, Magnitude of the  
Residue Study," Janine E. Rose, Ph.D., Uniroyal Study No. RP-98003, PTRL  
Study No. 723W with modifications by PTRL West, Inc.**

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This method is used for the determination of diflubenzuron in almond nutmeats and hulls. This method has demonstrated a limit of quantitation of 0.05 ppm diflubenzuron in almond matrices.

## **MATERIALS AND METHODS**

### **Equipment**

#### **\* Glassware and Miscellaneous Equipment**

Balance, Fisher XT top loading

Balance, Fisher XL-5000

Balance, Mettler AT 261

Balance, Ohaus E4000D

Beakers, various sizes

Büchner funnel, 11 cm

Chromatography column, 240 mm x 11 mm with a 200 mL reservoir

Filter paper, GF/A grade, 11 cm

Flask, round bottom, various sizes

Florasil<sup>™</sup>, 60-100 mesh, Fisher Scientific

Food Processor

Glass Wool

Graduated cylinder, various sizes

Meat Grinder

Pasteur pipettes

Separatory funnel

Sonicator

Suction flask, 500 mL

Syringes, microliter, 500  $\mu$ L, 100  $\mu$ L

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

Vials, amber (2 mL capacity) with Teflon<sup>®</sup>-lined crimp cap, Chromacol, Inc.,  
Trumbull, CT

Volumetric flask, various sizes

Volumetric pipette, various sizes

Waring<sup>™</sup> Blender with 1 quart cup

### Reagents and Standards

#### \* Standard Reference Substances

The reference standard of diflubenzuron was provided by Uniroyal Chemical Company, Inc. with a stated purity of 99.9%. A stock solution of diflubenzuron was prepared at 100 µg/mL in 1,4-dioxane. Dilutions of the diflubenzuron stock standard were prepared for the fortification and linearity standards, as described below. Standard solutions were allowed to return to room temperature prior to use. All reference standards were concluded to be stable throughout the conduct of the study based on the comparison of gas chromatograms of the first and last analysis.

### STATEMENT OF ANALYTICAL STANDARDS

The following standards were utilized for analysis throughout the study:

Compound:	Diflubenzuron
Purity:	99.9%
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:	February, 2001
Storage Conditions:	Freezer Temperature

#### \* Solvents

Acetone, Optima Grade, Fisher Scientific

Dichloromethane, Optima Grade, Fisher Scientific

1,4-Dioxane, Fisher Scientific

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Ethyl Acetate, Fisher Scientific  
Hexane, Optima Grade, Fisher Scientific  
Petroleum Ether, Optima Grade, Fisher Scientific  
Water, HPLC Grade, Fisher Scientific

## **ANALYTICAL PROCEDURES**

### **Preparation of Sample**

All samples were received frozen at PTRL West, Inc. and remained frozen until processed or sub-sampled. Almond nutmeat samples were processed by grinding with dry ice in a food processor and almond hulls were processed in the presence of dry ice with a meat grinder. Processed samples were stored in Ziploc<sup>®</sup> storage bags, allowing the dry ice to sublime at freezer temperature. The processed samples were stored frozen (< 0°C) until used for fortification and analysis.

### **Preparation of Standards**

A 100 µg/mL stock standard of diflubenzuron in 1,4-dioxane was prepared using the formula as described under the "Method of Calculations" section. Linearity standards were prepared by dilution of the 100 µg/mL stock DFB standard. Microliter syringes, volumetric pipettes and volumetric flasks were used throughout.

### **Fortification Procedure**

Fortification of untreated matrix was conducted to determine the percent recovery within each sample set. A portion (25 µL of the 100 µg/mL for the 0.05 ppm, 50 µL of the 100 µg/mL for the 0.10 ppm, and 250 µL of the 100 µg/mL for the 0.50 ppm) of the diflubenzuron fortification stock was added to 50.0 g of almond matrix using a Hamilton syringe.

### **Preparation of Linearity Standards**

A portion of the 100 µg/mL diflubenzuron standard was diluted with acetonitrile:water:1,4-dioxane (45:45:10, v:v:v) to prepare the linearity standards. All dilutions were made with volumetric flasks, volumetric pipettes, and microliter syringes.

<u>DFB Standard</u>	<u>Dilution (all dilutions made with ACN:H<sub>2</sub>O:dioxane, 45:45:10)</u>
5.0 µg/mL	5 mL of 100µg/mL stock standard diluted to 100 mL
1.0 µg/mL	20 mL of 5.0 µg/mL stock standard diluted to 100 mL
0.8 µg/mL	800 µL of 1.0 µg/mL stock standard diluted with 200 µL of solvent
0.6 µg/mL	600 µL of 1.0 µg/mL stock standard diluted with 400 µL of solvent
0.4 µg/mL	400 µL of 1.0 µg/mL stock standard diluted with 600 µL of solvent
0.2 µg/mL	200 µL of 1.0 µg/mL stock standard diluted with 800 µL of solvent

A calibration curve was generated with each sample set to determine linearity of the diflubenzuron analysis. See "Methods of Calculation" for mathematical determination of diflubenzuron residues.

#### **DFB Extraction Method for Almonds**

1. Weigh 50.0 grams almond meat into a beaker. Fortify as necessary.
2. Place sample into Waring blender cup, rinse beaker with 200 mL ethyl acetate. Add to blender cup.
3. Blend for 5 minutes.
4. Filter through a Büchner funnel fitted with Whatman GF/A paper (11 cm) into a 500 mL filter suction flask.
5. Transfer filtercake and filter paper to blender and add 200 mL ethyl acetate. Blend for ~5 minutes.
6. Repeat Step 3.
7. Transfer the combined filtrates to a round bottom flask and rotoevaporate to dryness under vacuum at ~35°C.
8. Dissolve the residue in 50 mL hexane with sonication and transfer the solution to a separatory funnel. Rinse round bottom with an additional 50 mL hexane and add to separatory funnel.
9. Rinse round bottom flask with 50 mL acetonitrile and transfer to separatory funnel.
10. Shake separatory funnel for ~2 minutes. Allow the layers to separate (5 minutes). Collect the acetonitrile layer and emulsion (lower) into a 250 mL flask. Discard hexane layer.
11. Return acetonitrile layer and emulsion to separatory funnel and wash with 50 mL hexane two (2) more times.

12. Draw off acetonitrile layer into a round bottom flask. Rotoevaporate to dryness under vacuum at  $\sim 35^{\circ}\text{C}$ .
13. Re-dissolve sample from Step 12 in 3.0 mL dichloromethane.
14. Prepare a chromatography column: Weigh 11.7 g of 5.5% deactivated Florisil. Place a glass wool plug at the bottom of a chromatography column (240 mm x 11 mm with a 200 mL reservoir). Pack column with the deactivated Florisil. Wash column with 100 mL petroleum ether.
15. Add 25 mL petroleum ether to the dichloromethane dissolved sample (Step 13).
16. Transfer the dichloromethane/petroleum ether solution to the column. Allow the sample to drain to the top of the column.
17. Add 25 mL petroleum ether to the column, drain to the top of the column.
18. Add 45 mL petroleum ether to the column, drain to the top of the column.
19. Add 3 x 10 mL of acetone:petroleum ether (1:9) solution to the column, drain to the top of the column. Discard eluants.
20. Add 10 mL acetone:petroleum ether (1:4). Drain to the top of the column. Collect this portion.
21. Elute DFB with 50 mL acetone:petroleum ether (1:4).
22. Repeat Step 21.
23. Rotoevaporate sample to dryness at  $\sim 35^{\circ}\text{C}$ . Dissolve the residue in 1.0 mL 1,4-dioxane with sonication, then add 9 mL acetonitrile:water (1:1).
24. Place  $\sim 1$  mL in GC vial for HPLC analysis.

## INSTRUMENTATION

Instrument: Hewlett Packard HPLC Series 1050  
Column: Zorbax XDB-C8 (250mm x 4.6 mm)  
Flow Rate: 1.5 mL/minute  
Detector: 254 nm  
Injection Volume: 20  $\mu\text{L}$ ; 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)

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Solvent Program: 0-15 minutes Solvent A  
15.5-20 minutes Solvent A to Solvent B  
20.5-30.5 minutes Solvent B to Solvent A

Retention Time: 8-10 minutes

Addition of the Solvent B portion of the solvent program was necessary to wash out interfering matrix components. A typical injection sequence was: solvent blank, solvent blank, 0.1 µg/mL standard, 0.2 µg/mL standard, 0.4 µg/mL standard, 0.6 µg/mL standard, 0.8 µg/mL standard, 1.0 µg/mL standard, reagent blank, control almond matrix, control almond matrix, fortified matrix sample, fortified matrix sample, treated sample, treated sample, etc.

Separation of the analyte was achieved by high performance liquid chromatography (HPLC). 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  $y = mx + b$

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

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

$$\text{ppm DFB} = \mu\text{g/mL DFB} \times \frac{\text{Final volume (mL)}}{\text{Sample weight (g)}}$$

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 from almond nutmeat or hull, a control sample for each set of almond samples was fortified with DFB at 0.05 ppm, 0.10 ppm or 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 Almonds. Diflubenzuron standards were prepared in ACN:water:1,4-dioxane (see Preparation of Linearity Standards) for the DFB analysis. These samples ranged in concentration from 0.20  $\mu\text{g/mL}$  to 5.0  $\mu\text{g/mL}$  and a calibration curve was generated with each sample set. The equation of the line based on the peak area of the standard versus the concentration ( $\mu\text{g/mL}$ ) injected was generated by least squares linear regression calculated by the computer program, Dionex. 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 mushroom, 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 almond nutmeats (PTRL sample no. 723W-057) is shown below:

Linear regression analysis of the derivatized DFB standards gave a linearity with the formula  $x = (5.204e^{-7})y - 0.005187$  ( $r^2 = 0.999230$ ). The concentration of DFB determined by this curve was calculated as follows.

$$\mu\text{g/mL DFB} = (5.205e^{-7} \times 686065) - 0.005187 = 0.352$$

Calculation of the ppm DFB in almond nutmeat is dependent on the final volume and sample weight.

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$$\text{ppm DFB} = 0.352 \mu\text{g/mL} \times \frac{10 \text{ mL}}{50 \text{ g}} = 0.070$$

The percent recovery of DFB equals 0.070 ppm DFB minus control (0 ppm) DFB, divided by the 0.1 ppm fortification. The percent recovery for this sample was 70%.

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**4-Chlorophenyl Urea Analytical Method as Described in  
"Dimilin<sup>®</sup> 25W, Dimilin 80<sup>®</sup> WG and Dimilin<sup>®</sup> 2L in Almonds, Magnitude of the  
Residue Study," Janine E. Rose, Ph.D.,  
Uniroyal Study No. RP-98003, PTRL Study No. 723W**

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This analytical method is used for the determination of 4-chlorophenyl urea (CPU) in almond nutmeats and hulls through use of LC/MS detection. The limit of quantitation for CPU in almond nutmeats is 5.0 ppb and 10.0 ppb in hulls.

### **Equipment**

#### *Glassware and Miscellaneous Equipment*

##### **Balance**

Bottle, Teflon<sup>®</sup> or polypropylene, 250 mL

Bottle, amber glass with Teflon<sup>®</sup>-lined lid

Centrifuge, Mistral 3000E

Flask, round bottom, 100 mL, 50 mL, 25 mL

Graduated cylinder, various sizes

Nitrogen evaporator

Separatory funnel, 125 mL

Solid Phase Extraction, C-18 (1g, 6 mL), Varian

Solid Phase Extraction Si (1g, 6cc), Varian

Sonicator

Syringes, microliter, various sizes

Tissumizer (Tekmar, S25N generator probe)

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

Vials, glass with crimp-top cap

Volumetric flask, various sizes

Volumetric pipette, various sizes

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### **Solvents/Reagents**

All solvents were HPLC grade unless noted

Acetone

Acetonitrile

Diethyl ether

1,4-Dioxane

Hexane

Methanol

Trifluoroacetic acid

Water

### **Standard Reference Substances**

The 4-chlorophenyl urea reference standard was provided by Uniroyal Chemical Co. with a stated purity of 99.7%. Reference standards were concluded to be stable throughout the conduct of the study based on the comparison of chromatograms of the first and last analysis.

### **STATEMENT OF ANALYTICAL STANDARDS**

The following standard was utilized for analysis throughout the study (Appendix B):

Compound:	4-Chlorophenyl Urea
Purity:	99.7%
Molecular Weight:	170 g/mole
Lot Number:	ARS-87G27N (PTRL West #614W-002)
Supplier:	Uniroyal Chemical Company, Inc.
Date Received:	October 11, 1996
Expiration Date:	March, 2001
Storage Condition:	Freezer Temperature

**ANALYTICAL PROCEDURES**

**Preparation of Sample**

All samples were received frozen at PTRL West, Inc. and remained frozen until processed or sub-sampled. Almond nutmeat samples were processed by grinding with dry ice in a food processor and almond hulls were processed in the presence of dry ice with a meat grinder. Processed samples were stored in Ziploc<sup>®</sup> storage bags, allowing the dry ice to sublime at freezer temperature. The processed samples were stored frozen (< 0°C) until used for fortification and analysis.

**Sample Storage**

Processed samples were stored frozen until used for fortification or sub-sampling.

**Preparation of Standards**

A 0.5 mg/mL solution of the 4-chlorophenyl urea (CPU) reference standard was prepared in acetonitrile as described under the “Method of Calculations” section.

<u>CPU Concentration (µg/mL)</u>	<u>Dilution (prepared with ACN)</u>
100	10 mL of 0.5 mg/mL diluted to 50 mL
0.2	100 µL of 100 µg/mL stock diluted to 50 mL

Microliter syringes, volumetric pipettes and volumetric flasks were used throughout.

**Fortification Procedure**

Fortification of untreated matrix was conducted to validate the analytical method and to determine the percent recovery within each sample set. The fortification solution was used to fortify untreated peaches before the addition of solvent. The almond samples were fortified at 5.0 and 10.0 ppb CPU to validate the method. Concurrent fortification of almond nutmeats was conducted at 5.0 ppb and hulls at 10.0 ppb CPU. Fortification of untreated samples during set analysis was conducted as follows.

<u>Fortification Level (ppm)</u>	<u>Volume of Standard in acetonitrile</u>	<u>Control Specimen (g)</u>
0.005	250 µL of a 0.2 µg/mL standard	10
0.010	500 µL of a 0.2 µg/mL standard	10

### Preparation of Linearity Standards

All dilutions made with acetonitrile:water (1:1, v:v). Microliter syringes, volumetric pipettes and volumetric flasks were used throughout.

<u>CPU Standard</u>	<u>Dilution (with acetonitrile: water, 1:1)</u>
1000 ng/mL	250 $\mu$ L of 100 $\mu$ g/mL stock diluted to 25 mL
100 ng/mL	5 mL of 1.0 $\mu$ g/mL stock diluted to 50 mL
50 ng/mL	2.5 mL of 1.0 $\mu$ g/mL stock diluted to 50 mL
40 ng/mL	2 mL of 1.0 $\mu$ g/mL standard diluted to 50 mL
30 ng/mL	1.5 mL of 1.0 $\mu$ g/mL standard diluted to 50 mL
20 ng/mL	1.0 mL of 1.0 $\mu$ g/mL standard diluted to 50 mL
15 ng/mL	750 $\mu$ L of 1.0 $\mu$ g/mL standard diluted to 50 mL

Calibration curves for CPU were generated with each sample set to determine linearity and to quantitate residues. Calibration standards were taken to be stable throughout the study by comparison of chromatograms early and near the end of the study.

### Extraction Method for CPU in Almonds

1. Weigh 10 g processed matrix into 250 mL Teflon<sup>®</sup> or polypropylene bottle.
2. Fortify appropriate samples as needed.
3. Add 50 mL of acetonitrile (ACN) and extract by blending using a tissumizer.
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 almond residue as per steps 3-5.
7. Combine supernatants and adjust volume to the nearest 10 mL increment with ACN (typically 110 or 120 mL).
8. Mix combined extract until homogenous before proceeding.
9. Transfer an aliquot equivalent to  $\frac{1}{2}$  total volume 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 approximately 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 ACN to approximately 5 mL by rotary evaporation under vacuum at ~40°C.
15. Add an approximate equal volume of hexane (~5 mL) and partition by shaking vigorously for approximately 1 minute.
16. Transfer the entire volume of ACN/hexane solution to an appropriate volume centrifuge tube (typically 15-25 mL) and allow phases to separate completely (centrifuge if necessary to facilitate separation, typically 5 minutes at 1,000 rpm).
17. Discard hexane (upper) layer.
18. Repeat partition in the centrifuge tube with a second ~5 mL portion of hexane.
19. Transfer ACN layer back into concentration flask and continue concentrating to dryness by rotary evaporation under vacuum at ~40°C.
20. Reconstitute residue in 2 mL ACN, using ultrasonication, then add 8 mL water and vortex to mix.

#### **Clean-up**

1. Condition C<sub>18</sub> solid phase extraction (SPE) cartridge (1g/6cc) with one column volume (~6 mL) ACN, followed by one column volume of methanol then one column volume of water.
2. Pass entire 10 mL extract through pre-conditioned cartridge under vacuum or with positive pressure discarding eluate.
3. Rinse concentration flask (from step 20 above) with 5 mL ACN:H<sub>2</sub>O (1:1, v:v).
4. Elute CPU from cartridge with the 7 mL ACN:H<sub>2</sub>O rinse of flask from step 3 above. Collect eluate in appropriate volume centrifuge tube (typically 15-25 mL).
5. Add an approximate equal volume of hexane (~5 mL) and partition by shaking vigorously for approximately 1 minute.
6. Discard hexane (upper) layer.
7. Repeat partition with a second ~5 mL portion of hexane.
8. Transfer ACN layer to concentration flask (typically 25 mL) and concentrate to dryness by rotary evaporation under vacuum at ~40°C.
9. Reconstitute residue in 2 mL diethyl ether/hexane 1:19 v:v, using ultrasonication.
10. Condition Si SPE cartridge (1g/6cc) with one column volume (~6 mL) acetone, followed by one column volume of hexane.

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11. Pass entire 2 mL extract through pre-conditioned cartridge under vacuum or with positive pressure discarding eluate.
12. Rinse concentration flask with 4 mL diethyl ether/hexane 1:19, v:v and pass through cartridge discarding eluate.
13. Pass an additional 4 mL diethyl ether/hexane 1:19, v:v through cartridge, followed by 9 mL diethyl ether/hexane 3:7, v:v discarding both eluates.
14. Elute with 5 mL acetone:hexane (1:1, v:v), discard eluate.
15. Elute CPU with an additional 4 mL acetone/hexane 1:1, v:v collecting eluate in concentration flask (typically 25 mL).
16. Concentrate eluate to dryness at ambient temperature under a stream of nitrogen gas.
17. Reconstitute residue in 0.5 mL ACN with ultrasonication. Add to each 0.5 mL water and vortex to mix.
18. Transfer samples to separate crimp-top vials for analysis.

## CHROMATOGRAPHY

### HPLC Unit # 14 Components:

LC Pump	ThermoSeparation Product Spectra P4000
Detector	Finnigan MAT LCQ Mass spectrometer Detector
Integrator	3396B Series II
Autosampler	ThermoSeparation Product Spectra Autosampler AS3000

A = ACN

200 µL injection volume

B = Water

Microsorb MV C18 (25 cm x 4.6 mm id)  
with pre-column

D = 0.01% TFA in ACN (used for high  
fort. validation only)

Detection: LC/MS

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Step	Time (min)	% A	% B	% D	Flow Rate (mL/min)
1	0	5	95	0	0.80
2	18	60	40	0	0.80
3	19	0	0	100	1.00
4	22	0	0	100	1.00
5	25	5	95	0	1.00
6	25.10	5	95	0	0.80
7	30	5	95	0	0.80

Flow is diverted from waste to detector from 12.50 to 16.50 minutes.

**MS detector Settings**

Segments	1	2	3	4
Duration (min)	0.10	12.40	4.00	13.50
Event	APCI Positive (165-220)	APCI low gas flow (165-220)	APCI Positive (165-220)	APCI low gas flow (165-220)

**Statistical Methods**

The residue data included the following statistical calculations: means, averages, standard deviations and linear regression analysis.

**Limit of Quantitation**

The limit of quantitation was assigned as the lowest fortification level of analyte validated by the residue method. The limit of quantitation for CPU in almond nutmeats was 5 ppb and 10.0 ppb almond hulls.

**Time Required for Analysis**

Time required for analysis of one set of 10 samples and 6 linearity standards:

Extraction time = 20 hours

Analysis time CPU = 9 hours

TOTAL time equals 29 hours or 3 calendar days.

## 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)

### Recovery

The recovery of CPU from fortified almond samples was calculated as follows:

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

where  $y$  = peak area  
 $x$  = ng/mL CPU  
 $m$  = Slope  
 $b$  = Calibration intercept

$$\text{ppb CPU} = \frac{\text{CPU concentration (ng/mL)} \times \text{final volume (mL)}}{\text{Aliquot sample weight (g)}}$$

$$\text{where aliquot sample weight} = \text{sample wt (g)} \times \frac{\text{Aliquot volume (mL)}}{\text{Extract volume (mL)}}$$

$$\% \text{ Recovery} = \frac{\text{Concentration of CPU Fortified Sample (ng/g)}}{\text{CPU Fortification Level (ng/g)}} \times 100$$

Validity of the CPU analytical method in almonds was established by acceptable recovery (70-120%) from fortified untreated control samples. Residues of CPU in treated samples were calculated as for the fortified samples, without control subtraction.

After spiking the samples, the samples were extracted, and analyzed as previously described in the "Extraction Method for CPU in Almonds". CPU standards were prepared in acetonitrile:water (1:1, v:v) (see Preparation of Linearity Standards). These standards ranged in concentration from 0.015 µg/mL to 0.050 µg/mL and a calibration curve was generated with each sample set. The equation of the line based on the peak area of the

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standard versus the CPU concentration (ng/mL) injected was generated by least squares linear regression calculated by the computer program associated with the LC/MS. 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.

An example calculation for CPU analysis in almond nutmeats (Uniroyal Sample no. 723W-047, 5.0 ppb fortification) is shown below.

Linear regression analysis of the CPU standards gave a curve with the curve  $y = -405750 + 170782 x$  ( $r^2 = 0.9992$ ). The ng/mL CPU determined by this curve was:

$$\text{ng/mL CPU injected} = (3186529.95 + 405750) \div 170782 = 21.034 \text{ ng/mL}$$

$$\text{ppb CPU} = \frac{21.034 \text{ ng/mL} \times 1.0 \text{ mL}}{5 \text{ g}} = 4.21 \text{ ppb}$$

$$\text{where aliquot sample weight} = 10.0 \text{ g} \times \frac{60 \text{ mL}}{120 \text{ mL}} = 5.0 \text{ g}$$

$$\% \text{ Recovery} = \frac{4.21 \text{ ppb}}{5.0 \text{ ppb}} \times 100 = 84\%$$

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**4-Chloroaniline Analytical Method as Described in  
"Dimilin<sup>®</sup> 25W, Dimilin<sup>®</sup> 80<sup>®</sup> WG and Dimilin<sup>®</sup> 2L in Almonds, Magnitude of the  
Residue Study," Janine E. Rose, Ph.D., Uniroyal Study No. RP-98003, PTRL  
Study No. 723W with modifications by PTRL West, Inc.**

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This analytical method is used for the determination of 4-chloroaniline (PCA) in almond nutmeats and hulls through use of <sup>13</sup>C-PCA as an internal standard. The limit of quantitation for PCA in almond nutmeats and hulls is 5.0 ppb.

## **MATERIALS AND METHODS**

### **Equipment**

- \* **Glassware and Miscellaneous Equipment**
  - Balance, Mettler AT261
  - Balance, Sartorius, L2200P
  - Balance, Fisher XL, 5000
  - Beakers, 250 mL
  - Bottle, amber, with Teflon<sup>®</sup>-lined cap, 500 mL
  - Büchner funnel, 10 cm
  - Centrifuge, Mistral 3000E, MSE
  - Centrifuge tube, Teflon<sup>®</sup>, 250 mL
  - Centrifuge tube, polypropylene, 750 mL
  - Filter paper, Whatman #4 Grade, 11 cm
  - Florisil column, MegaBond Elut, 6cc/1g, Varian
  - Food processor
  - Funnel, 5 cm diameter
  - Graduated cylinder, 25 mL, 50 mL, 100 mL
  - Meat grinder
  - Pasteur pipettes, 5"
  - Pipettes, graduated, various sizes
  - pH paper, Color pHast
  - Separatory funnel, 500 mL
  - Shaker, Wrist action, Model 75, Burrel

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Shaker bath, Lab-line Model 3582  
Suction flask, 500 mL  
Syringes, microliter, 500  $\mu$ L, 100  $\mu$ L  
Thermometer, mercury  
Vials, glass with Teflon<sup>®</sup>-lined cap, 15 mL, 35 mL  
Vials, amber (2 mL capacity) with Teflon<sup>®</sup>-lined crimp cap, Chromacol, Inc.,  
Trumbull, CT  
Volumetric flask, various sizes  
Volumetric pipette, various sizes

### Reagents and Standards

#### STATEMENT OF ANALYTICAL STANDARDS

The following standards were utilized for analysis throughout the study:

Compound:	Parachloroaniline ( <sup>12</sup> C-PCA)
Purity:	100%
Molecular Weight:	128 g/mole
Lot Number:	29023/8 (PTRL West #598W-002)
Supplier:	Uniroyal Chemical Company, Inc.
Date Received:	July 10, 1996
Expiration Date:	June 29, 2000
Storage Condition:	Freezer Temperature

Compound:	<sup>13</sup> C-Parachloroaniline ( <sup>13</sup> C-PCA)
Chemical Purity:	98%
Isotope Purity:	99%
Molecular Weight:	133.44 g/mole
Lot Number:	P-5493 (PTRL West # 613-031)
Source:	Cambridge Isotope Laboratories
Supplier:	Uniroyal Chemical Company, Inc.
Date Received:	January 9, 1997
Expiration Date:	Not available
Storage Condition:	Freezer Temperature

#### \* Standard Reference Substances

The reference standards listed below were provided by Uniroyal Chemical Company, Inc. Characterization of the reference standards was conducted by Uniroyal and Cambridge

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Isotope Laboratories. All analytes were stored frozen ( $< 0^{\circ}\text{C}$ ) until used for sample preparation. A stock solution of parachloroaniline ( $^{12}\text{C}$ -PCA) was prepared at 0.5 mg/mL in hexane. Similarly, a stock solution of  $^{13}\text{C}$ -4-chloroaniline ( $^{13}\text{C}$ -PCA) was prepared at 0.5 mg/mL in hexane. Dilutions of the  $^{12}\text{C}$ -PCA and  $^{13}\text{C}$ -PCA stock standards were prepared for the fortification and derivatized linearity standards, as described below. Stock and diluted standard solutions were stored at approximately  $0^{\circ}\text{C}$  until used for sample fortification or analysis. Standard solutions were allowed to return to room temperature prior to use. All reference substances were concluded to be stable throughout the conduct of the study based on the comparison of the gas chromatograms of the first and last analysis.

\* Solvents

Acetone, Optima Grade, Fisher Scientific  
Hexane, Optima Grade, Fisher Scientific  
Water, HPLC Grade, Fisher Scientific

\* Reagents

Heptafluorobutyric Acid Anhydride (HFBA), Pierce  
Hydrochloric Acid, Concentrated, Fisher Scientific  
Sodium Chloride, ACS Grade, Fisher Scientific  
Sodium Carbonate, ACS Grade, Fisher Scientific  
Sodium Hydroxide, ACS Grade, Fisher Scientific  
Sodium Sulfate, anhydrous, ACS Grade, Fisher Scientific

## **ANALYTICAL PROCEDURES**

### **Preparation of Sample**

All samples were received frozen at PTRL West, Inc. and remained frozen until processed or sub-sampled. Almond nutmeat samples were processed by grinding with dry ice in a food processor and almond hulls were processed in the presence of dry ice with a meat grinder. Processed samples were stored in Ziploc<sup>®</sup> storage bags, allowing the dry ice to sublime at freezer temperature. The processed samples were stored frozen ( $< 0^{\circ}\text{C}$ ) until used for fortification and analysis.

**Preparation of Standards**

A stock solution of 500 µg/mL <sup>12</sup>C-parachloroaniline was prepared in hexane using the formula as described under the “Methods of Calculation” section. A portion of the 500 µg/mL parachloroaniline stock solution was diluted to yield a 5.0 µg/mL parachloroaniline standard in hexane. Dilutions of the 5.0 µg/mL PCA standard were prepared to 0.5 µg/mL and 0.05 µg/mL <sup>12</sup>C-PCA fortification standards. A stock solution of 500 µg/mL <sup>13</sup>C-parachloroaniline was prepared in hexane using the formula as described under the “Methods of Calculation” section. A stock standard of <sup>13</sup>C-parachloroaniline (0.5 µg/mL) was prepared in hexane by the same method. Microliter syringes, volumetric pipettes and volumetric flasks were used throughout.

Derivatized <sup>12</sup>C-PCA and <sup>13</sup>C-PCA standard solutions were prepared by treating PCA stock standards (10 mL of 5.0 or 0.5 µg/mL) with 100 µL heptafluorobutyric anhydride by the method described in Steps 25 to 28 in the “Method of Extraction”.

**Preparation of Derivatized External Standards**

External derivatized <sup>12</sup>C-PCA standards were prepared by dilutions of the derivatized standard.

<u><sup>12</sup>C-PCA Standard</u>	<u>Dilutions (in hexane)</u>
0.05 µg/mL	10 mL of 0.5 µg/mL diluted to 100 mL
0.03 µg/mL	6 mL of 0.5 µg/mL diluted to 100 mL
0.01 µg/mL	20 mL of 0.05 µg/mL diluted to 100 mL
0.005 µg/mL	10 mL of 0.05 µg/mL diluted to 100 mL

External <sup>12</sup>C-PCA:<sup>13</sup>C-PCA ratio linearity standards were prepared by dilutions of the derivatized standards as follows:

<u>Ratio <sup>12</sup>C-PCA:<sup>13</sup>C-PCA</u>	<u><sup>12</sup>C-PCA</u>	<u><sup>13</sup>C-PCA</u>	<u>Hexane</u>
0.5:1.0	0.25 mL of 0.025 µg/mL	0.5 mL of 0.025 µg/mL	0.25 mL
1.0:1.0	0.5 mL of 0.025 µg/mL	0.5 mL of 0.025 µg/mL	NA
5.0:1.0	0.125 of 0.5 µg/mL	0.5 mL of 0.025 µg/mL	0.375 mL
10.0:1.0	0.25 mL of 0.5 µg/mL	0.5 mL of 0.025 µg/mL	0.25 mL

### Fortification Procedure

The almond matrices were fortified by addition of the following quantities of underivatized stock standard:

<u><math>^{12}\text{C-PCA}</math></u> <u>Fort. Level</u>	<u><math>^{12}\text{C-PCA}</math></u>	<u><math>^{13}\text{C-PCA}</math> (5 ppb)</u>
0 ppb	0	0.25 mL of 0.5 $\mu\text{g/mL}$
1.0 ppb	0.05 mL of 0.5 $\mu\text{g/mL}$	0.25 mL of 0.5 $\mu\text{g/mL}$
5.0 ppb	0.25 mL of 0.5 $\mu\text{g/mL}$	0.25 mL of 0.5 $\mu\text{g/mL}$
10.0 ppb	0.5 mL of 0.5 $\mu\text{g/mL}$	0.25 mL of 0.5 $\mu\text{g/mL}$

### Method of Extraction for PCA in Almonds

1. Weigh 25.0 g almonds into 250 mL Teflon<sup>®</sup> centrifuge tube.
2. Fortify with the appropriate amounts of  $^{12}\text{C-PCA}$  and  $^{13}\text{C-PCA}$  fortification solutions using application syringes.
3. Shake several times to mix, wait 5 minutes with caps off.
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.

- 13A. Transfer filtrate to 500 mL centrifuge tube (teflon or polypropylene), wash flask with 25 mL 0.1N HCl, adding to filtrate.
14. Add 50% NaOH (aq.) to filtrate until pH > 12 (~ 2 mL) and 10-15 g NaCl (s), mix with swirling.
15. Transfer to 500 mL separatory funnel, rinsing filter flask with 25 mL water. Add rinsate to filtrate.
16. Add 100 mL hexane and 10-15g NaCl, shake gently, or mix by swirling, for approximately 60 seconds. Centrifuge at ~2,500 rpm for 5 minutes.
17. Remove hexane with 25 mL pipette, avoiding emulsion if present. Place in a clean 500 mL separatory funnel.
18. Repeat hexane partition two more times, using 100 mL each time, combining hexane extracts into 500 mL separatory funnel.
19. Partition the combined hexane extracts twice with 0.1 N HCl, 25 mL then 10 mL, combining acid extracts.
20. Add 50% NaOH to acid extracts until pH > 12 (~ 0.5 mL).
21. 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.
22. Put the combined hexane extracts onto 6 cc/1 GRM Florisil column (Varian).
23. Elute with 1 mL 20% acetone in hexane, discard eluant.
24. 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.
25. Transfer the eluant to a derivatizing vial.
26. Add 100  $\mu$ L HFBA, close container, mix by swirling, allow to stand at room temperature for 10 minutes.
27. Add 5 mL water and 0.5 mL saturated sodium carbonate (aq.), shake (~ 30 s.) and allow phases to separate.
28. Pipette hexane layer into another vial, place onto GC/MS with SIM of ions 323, 329, 126, and 132.

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## INSTRUMENTATION - GAS CHROMATOGRAPHY

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

Column: Supelco SPB 1701 Capillary Column  
30m x 0.32mm i.d. x 0.25  $\mu$ m film thickness  
Lot # 8529-04B

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

Injector Temperature: 250°C

Detector Temperature: 270°C

Injection Volume: 2  $\mu$ L; by Hewlett Packard 7673A Autosampler

Temperature Program:

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 ( $^{13}\text{C}$ -PCA), and quantitated by integration of the peak areas for  $^{12}\text{C}$ -PCA relative to peak areas for  $^{13}\text{C}$ -PCA.

A typical injection sequence for PCA samples was: hexane solvent blank, 0.005  $\mu\text{g}/\text{mL}$  derivatized  $^{12}\text{C}$ -PCA standard, unfortified control almond sample, unfortified control almond sample, reagent blank, 0.01  $\mu\text{g}/\text{mL}$  derivatized  $^{12}\text{C}$ -PCA standard, 5.0 ppb  $^{12}\text{C}$ -PCA fortified almond sample, 5.0 ppb  $^{12}\text{C}$ -PCA fortified almond sample, 0.03  $\mu\text{g}/\text{mL}$  derivatized  $^{12}\text{C}$ -PCA standard, treated almond sample, treated almond sample, 0.05  $\mu\text{g}/\text{mL}$  derivatized  $^{12}\text{C}$ -PCA standard, treated almond sample, treated almond sample, 0.5:1.0  $^{12}\text{C}$ : $^{13}\text{C}$ -PCA derivatized ratio linearity standard, 1.0:1.0  $^{12}\text{C}$ : $^{13}\text{C}$ -PCA derivatized

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ratio linearity standard, 5.0:1.0  $^{12}\text{C}$ : $^{13}\text{C}$ -PCA derivatized ratio linearity standard, 10.0:1.0  $^{12}\text{C}$ : $^{13}\text{C}$ -PCA derivatized ratio linearity standard, hexane solvent blank.

## INTERFERENCE

Control almond matrices, as analyzed by GC/MSD, were sometimes found to yield mass spectral ions corresponding in mass to those derived from  $^{12}\text{C}$ -PCA. The ratio of the areas from the control matrix ions at m/z 126 and m/z 323 were not similar to the ratio obtained for derivatized PCA from standards and fortified samples. Therefore, the areas of the ions (m/z 126 and 323) in control samples were determined to be the results of matrix coextractives. The calculated "PCA" residue in control almonds was subtracted from the residues of the fortified samples analyzed by the internal standard method.

## CONFIRMATORY TECHNIQUE

Quantitation was also achieved by peak area of the  $^{12}\text{C}$ -PCA relative to the external standard linearity curve. The linearity standards ranged in concentration from 0.005  $\mu\text{g/mL}$  to 0.05  $\mu\text{g/mL}$  derivatized  $^{12}\text{C}$ -PCA.

## METHODS OF CALCULATION

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

### -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

After spiking the samples, the samples were extracted, derivatized and analyzed as previously described above. Quantitation of the PCA was done by mass spectral peak area

integration relative to internal standard  $^{13}\text{C}$ -PCA. Two major ions were quantitated for each of  $^{12}\text{C}$ -PCA and  $^{13}\text{C}$ -PCA.

PPB of  $^{12}\text{C}$ -PCA =

$$\frac{(\text{PA } m/z 126 + \text{PA } m/z 323)}{\text{Response Factor Sample } (132 + 329)} - \frac{(\text{control PA } m/z 126 + \text{control PA } m/z 323)}{\text{Response Factor Control } (132 + 329)}$$

where

PA = Peak area

Response Factor (132 + 329) =

$$\frac{(\text{PA } m/z 132 + \text{PA } m/z 329)}{\text{ppb of } ^{13}\text{C} - \text{PCA spike}}$$

Note that an average control residue may be subtracted from fortified sample residues. The recoveries of 4-chloroaniline from  $^{12}\text{C}$ -PCA: $^{13}\text{C}$ -PCA fortified samples were calculated as follows:

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

An example calculation for the recovery of  $^{12}\text{C}$ -PCA (5.0 ppb fortification) from almond nutmeat (Uniroyal Sample no. U-98-001NT, PTRL sample no. 723W-003A) is shown below:

Twenty five grams of almond nutmeat were fortified with 5.0 ppb of  $^{12}\text{C}$ -PCA and 5.0 ppb of  $^{13}\text{C}$ -PCA. The combined peak areas for the  $^{12}\text{C}$ -PCA (m/z 126 = 8207, m/z 323 = 15037) equaled 23244 area units. The combined peak area for the  $^{13}\text{C}$ -PCA (m/z 132 = 10612, m/z 329 = 18004) was 28616 area units. The response factor was 28616/5 or 5723.2. The ppb of  $^{12}\text{C}$ -PCA was 23244 divided by 5723.2 minus the average control ppb of  $^{12}\text{C}$ -PCA (0.53 ppb). Thus, the residue of  $^{12}\text{C}$ -PCA quantitated was 4.061 minus 0.53, or 3.53 ppb. Therefore, the recovery was:

$$\text{Recovery} = \frac{3.53}{5.0} \times 100 = 71\%$$