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BASF CORPORATION
AGRICULTURAL PRODUCTS GROUP
P. O. Box 13528
Research Triangle Park, NC 27709-3528

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Method for Determination of BAS 125 W and its Metabolite in Animal Tissues (Liver, Kidney, Fat and Muscle), Milk, and Apple Commodities.

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Authors:

Samy Abdel-Baky
Stephan Baumann

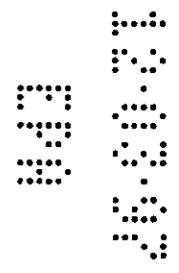
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Testing/Performing Laboratory:

BASF Corporation
Agricultural Products Center
P.O. Box 13528
26 Davis Drive
Research Triangle Park, NC 27709-3528

BATTELLE
Agrochemical Product Development
505 King Avenue
Columbus, Ohio 43201-2693



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This report contains an amendment to Method D9608

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Company: BASF Corporation, Agricultural Products
P.O. Box 13528
Research Triangle Park, NC 27709-3528

Company Agent: Edward G. Jordan, Ph.D.

Date: October 30, 1997

Registration Scientist
Title

Edward G. Jordan
Signature

Good Laboratory Practice Compliance Statement

The study meets the requirements of 40 CFR Part 160, Good Laboratory Practices.

Submitter:

Edward H. Jordan

Sponsor:

[Signature]

Study Director:

Samy Abdel-Baky

Samy Abdel-Baky, Ph.D.
BASF Corporation
Agricultural Products Groups
P.O. Box 13528
Research Triangle Park, NC 27709-3528

BASF CORPORATION
Agricultural Products Group
Agricultural Research Center
Post Office Box 13528
Research Triangle Park, NC 27709-3528

Method for Determination of BAS 125 W and its Metabolite in Animal Tissues (Liver, Kidney, Fat and Muscle), Milk, and Apple Commodities.

AUTHOR: Sammy Abdel-Baky (919) 547-2695
SUPERVISORY PERSONNEL: Laura Sears (919) 547-2646
ANALYSES DONE BY: Stephan Baumann, Dan Perry, David Broadwell, Amit Singh, and Dan Wilkinson
BATTELLE LABS: John Powell

ABSTRACT:

Analytical Method Number D9608 was developed for the analysis of BAS 125 W and its despropanoyl metabolite BAS 125-5376 in Animal Tissues (Liver, Kidney, Fat and Muscle), Milk, and Apple Commodities. Method development was carried out at BASF Corporation, Research Triangle Park, NC, using representative control animal and plant tissues. Independent Laboratory Validation was carried out at Maxim Technologies, Inc., Middleport, NY, using representative control cow kidney and milk samples. *This method report is amended here to reflect a correction to Table 9.*

Prohexadione (BAS 125 W) and its despropanoyl metabolite (BW 125-5376) are extracted from the animal tissues, apple and milk using an acetonitrile/1.5 M H₂SO₄ solution (9:1 v/v). This solvent mixture was demonstrated to effectively extract weathered residues through radiovalidation work. After filtration to remove solid materials, an aliquot is taken either through the parent method to determine concentration of BAS 125 W or through the metabolite method to determine the concentration of BW 125-5376.

A 3 % aliquot (8 % for Milk) taken for the determination of BAS 125 W is purified by mini-isolute ENV +™ column; the eluent is methylated by MeOH/H₂SO₄, mixed with water and applied to a second mini-isolute ENV +™ column. The eluent from the column (acetone-0.01% oxalic acid) is injected directly into GC-MSD for determination of BW 125-M7.

Despropanoyl is only analyzed in liver and kidney matrix; it is not a significant metabolite in other matrices. A 10 % aliquot is evaporated to remove the acetonitrile and then hydrolyzed in the presence of HCl. Purification is done by mini-SAX, followed by mini-Carbopack B columns. The final purification step is achieved by mini-isolute ENV +™ column chromatography. The eluent is evaporated and redissolved in deionized water. A high pressure liquid chromatography (HPLC) system with column switching and an ultraviolet (UV) detector is used for the final determination. The validation studies have shown that the Analytical Method Number D9608 is suitable for measuring residues of BAS 125 W in Animal Tissues (Liver, Kidney, Fat and Muscle), Milk, and Apple Commodities down to a quantitation limit of 0.05 ppm. The average recovery of BAS 125 W in all matrices was 81 ± 8% (n = 56). D9608 is suitable for measuring residues of BW 125-5376 in Animal Tissues (Liver, Kidney, Fat and Muscle) down to a quantitation limit of 0.05 ppm. The average recovery of BW 125-5376 in all matrices was 111 ± 16% (n = 15).

QAU STATEMENT

Study Initiation Date: October 23, 1996

The quality assurance unit of the testing facility at the APC has audited the protocol, the analytical portion (including the raw data) and the report for this study and reported its findings to the study director and to management.

Date of Inspection	Report to study director and to management
10/21/96	10/21/96
12/4/96	12/4/96
1/15/97	1/15/97
2/27/97	3/4/97
3/3/97	3/7/97
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3/7/97	3/7/97
9/29/97	9/29/97

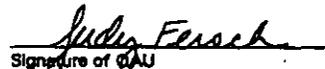

Signature of QAU

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1. Introduction and Summary

1.1 Scope and Source of the Method

1.1.1 Scope

BAS 125 W (prohexadione) is a plant growth regulator active in a variety of crops. This report describes the analytical method developed by BASF to determine residues of BAS 125 W and its metabolite BW 125-5376 in animal tissues (liver, kidney, fat and muscle), milk and apple commodities. The parent compound has been determined to be the major residue of concern (metabolite BW 125-5376 is a minor component in kidney) from a lactating goat metabolism study.

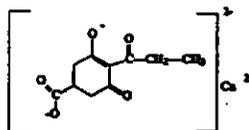
1.1.2 Source

This method was developed at the BASF Agricultural Products Center in Research Triangle Park, North Carolina. It is a modification of BASF method D9601 for Prohexadione in Peanuts (Reference:1).

1.2 Test/Fortification Substance

1.2.1 Test Substance

Common Name:	Prohexadione calcium
BAS Number:	BW 125-CA (or BW 9054-CA or BX-112)
Chemical Name:	Calcium 3-oxido-4-propionyl-5-oxo-3-cyclohexene carboxylate
CAS Name:	127277-83-8
Empirical Formula:	C ₁₀ H ₁₀ O ₅ Ca
Molecular Weight:	250.268 g/mole
Melting point:	Greater than 300°C



Solubility in organic solvent at 20°C

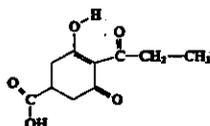
Methanol	1.1 mg/L
Hexane	Less than 0.003 mg/L
Acetone	0.038 mg/L
Toluene	0.004 mg/L

Solubility in water at 20°C

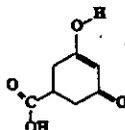
pH = 5	1602 mg/L
pH = 7	785.7 mg/L
pH = 9	685.4 mg/L

1.2.2 Fortification Substances

Common Name: Prohexadione carboxylic acid
BAS Number: BAS 125 W (or BAS 9054 W or KI-2817)
Chemical Name: 3-oxido-4-propionyl-5-oxo-3-cyclohexene carboxylic acid
Empirical Formula: $C_{10}H_{12}O_5$
Molecular Weight: 212.2 g/mole

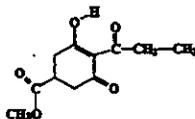


Common Name: Despropanoyl prohexadione carboxylic acid
BAS Number: BW 125-5376 (or BW 9054-5376)
Chemical Name: 3-hydroxy-5-oxo-3-cyclohexene-1-carboxylic acid
Empirical Formula: $C_7H_8O_4$
Molecular Weight: 156.1 g/mole

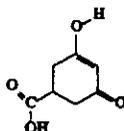


1.3 Standard Analyses

Common Name: Methyl prohexadione carboxylate
BAS Number: BW 125-M7 (or BW 9054-M7 or BX-112-M7)
Chemical Name: Methyl 3,5-dioxo-4-propionyl-1-cyclohexene carboxylate
Empirical Formula: $C_{12}H_{14}O_5$
Molecular Weight: 226.2 g/mole



Common Name: Despropanoyl prohexadione carboxylic acid
BAS Number: BW 125-5376 (or BW 9054-5376)
Chemical Name: 3-hydroxy-5-oxo-3-cyclohexene-1-carboxylic acid
Empirical Formula: $C_7H_8O_4$
Molecular Weight: 156.1 g/mole



1.4 Principle of the Method

The extraction step for the analysis of BAS 125 W and for the analysis of BW 125-5376 is the same. However, the subsequent purification steps are unique to the two methods. The final determination of the parent (BAS 125 W) will be done by GC-MSD while the final determination of the metabolite (BW 125 - 5376) will be done by HPLC. Prohexadione (BAS 125 W) and its despropanoyl metabolite (BW 125-5376) are extracted from the animal tissues, apple and milk using an acetonitrile/1.5 M H_2SO_4 solution (9:1 v/v). This solvent mixture was demonstrated to effectively extract weathered residues through redissolution work. After filtration to remove solid materials, an aliquot is taken either through the parent method to determine concentration of BAS 125 W or through the metabolite method to determine the concentration of BW 125-5376.

The aliquot taken for the determination of BAS 125 W is purified by mini-isolute ENV +™ column; the eluent is methylated by MeOH/ H_2SO_4 , mixed with water and applied to a second mini-isolute ENV +™ column. The eluent from the column (acetone-0.01% oxalic acid) is injected directly into GC-MSD for determination of BW 125-M7. A conversion factor will be used to convert back to parent acid concentrations (BAS 125 W).

To analyze for despropanoyl (BW 125-5376) a new extract can be made (same procedure as parent) or a second aliquot can be taken from the initial extraction. Despropanoyl will only be analyzed in liver and kidney matrices. The aliquot is evaporated to remove the acetonitrile and then hydrolyzed in the presence of HCl to free any bound residues. Purification is done by mini-SAX, followed by mini-Carbopack B columns. The final purification step is achieved by mini-isolute ENV +™ column chromatography. The eluent is evaporated and redissolved in deionized water. A high pressure liquid chromatography (HPLC) system with a ultraviolet (UV) detector is used for the final determination.

2. MATERIALS

2.1 Equipment-Suggested Sizes/Manufacturer

Flat-bottom flask, 24/40	50, 125, 250, 500 mL
Buchner funnel	11 cm diameter
Funnel, long stem	75 mm diameter, 150 mm stem
Funnel, short stem	75 mm diameter, 75 mm stem
Volumetric flask	10-500 mL
Volumetric pipette	0.5-10, 20 and 50 mL
Erlenmeyer flask	250 and 500 mL
Filter flask	500 mL
Glass SPE column 8 mL	J.T. Baker, Item No. 7328-08
Glass or Plastic Reservoir for SPE, 80 mL	Burdick & Jackson or equivalent
Filter paper	Whatman No. 2 or equivalent
Filter paper frits for glass columns	VWR, Grade 417 cat. No. 28313-057
Pasteur pipettes, disposable	23 cm long
Autosampler vials 1.5 mL	Sun Brokers, Inc. or equivalent
Autosampler caps 11 mm	Sun Brokers, Inc. or equivalent (snap caps) e.g. sterile
Glass wool	Fisher Scientific or equivalent
Vortex mixer	Branson 1200 or equivalent
Ultrasonic bath	N-Evap Organomation Associates, Inc. or equivalent
Nitrogen stream evaporator	Corning or equivalent
Stirring hot plate	Mettler or equivalent
Balance (with at least one-tenth of a gram capability)	Brinkmann Instruments or equivalent
Balance (with 0.0001 g)	Supelco, Inc. or equivalent
Polytron homogenizer	Buchi or equivalent
SPE manifold	Fisher Scientific, part number K520210-0124 or equivalent
Rotary evaporator	Buchi or equivalent
Rotary evaporator traps, 100 mL	Eirik Systems IPM Inc. or equivalent (Kortas, or equivalent)
Water bath	
Vacuum system for rotavap	
Reflux Condenser	

Note: Equivalent equipment may be used

2.2 Reagents and Chemicals - Source/Preparation

<u>Reagents and Chemicals</u>	<u>Source/Preparation</u>
Acetone, Toluene, Methanol	Distilled, high purity (Burdick & Jackson)
Acetonitrile,	Millipore water purification system or equivalent
Ultra pure water	Distilled, high purity
(18 Megohm-cm resistivity)	Jones Chromatography, part #8915-0100
Isolute ENV +™	Supelco Chromatography Products, Cat. # 5-7203
LC-SAX	Supelco Chromatography Products, Custom # 4525
Carbopack B*	

*Acid or neutral washed Carbopeck B was used.

1,3-Cyclohexanedione
(Hydrochloric Acid
Sulfuric acid
Oxalic acid
(Celite[®]-545)

Fluka, Cat.# 29059
Aldrich Chemical Co., Inc.
Fisher Scientific
Aldrich Chemical Co., Inc.
C.J.T. Baker, Cat.# 3371-06

Note: Equivalent reagents and chemicals from other suppliers may be used

2.2.1 Standard Solutions for Fortifications

Note: These standard concentrations are suggested. A different concentration scheme may be used and additional standards may be prepared as needed.

Standard solutions should be stored in amber bottles. Any standard stock solutions (made from the solid analyte) with a concentration of 1 mg/ mL or greater should be stored for a maximum of three months. Any standard solutions prepared from the stock solution should be stored for a maximum of one month.

BAS 125 W (Parent Acid)

- a) Prepare 1.0 mg/ mL stock solution of BAS 125 W by weighing an appropriate amount of standard into a volumetric flask. Dissolve with methanol and dilute to the mark. For example, to prepare 25 mL stock solutions, dissolve 25.0 mg of BAS 125 W in 25 mL volumetric flask. Dilute to the mark with methanol.
- b) Prepare a 100 µg/ mL standard solution by transferring an appropriate amount of the 1.0 mg/ mL stock solution from the previous step with a volumetric pipet to a volumetric flask (typically 5 mL of each of the 1.0 mg/ mL stock solution into 50 mL volumetric flask). Dilute to the mark with methanol. Other serial dilution can be made in a similar manner.

BW 125-5376 (Metabolite)

- a) Prepare 1.0 mg/ mL stock solution of BW 125-5376 by weighing an appropriate amount of standard into a volumetric flask. Dissolve with methanol and dilute to the mark. For example, to prepare 10 mL stock solutions, dissolve 10.0 mg of BW 125-5376 in a 10 mL volumetric flask. Dilute to the mark with methanol.
- b) Prepare a 100 µg/ mL standard solution by transferring an appropriate amount of the 1.0 mg/ mL stock solution from the previous step with a volumetric pipet to a volumetric flask (typically 5 mL of each of the 1.0 mg/ mL stock solution into 50 mL volumetric flask). Dilute to the mark with methanol. Other serial dilution can be made in a similar manner.

2.2.2 BW 125-M7 Standard Solutions for GC/ MS Analysis

Note: These standard concentrations are suggested. A different concentration scheme may be used and additional standards may be prepared as needed.

Note: Amber bottles should be used as storage containers for the standard solutions. Any standard stock solution with a concentration of 1 mg/ mL or greater can be stored for a maximum of three months. Any standard solution prepared from the stock solution should be stored for a maximum of one month.

Note: The recommended standard solutions for GC-MSD with BW 125-M7 (parent method) are: 1.875, 3.75, 7.5 and 15 ng/mL. Other concentrations may be used as appropriate. The presence of 0.01% oxalic acid as an additive to all solutions is to be used on the GC-MS to improve the peak shape.

- a) Prepare 1.0 mg/mL stock solution of BW 125-M7 by weighing an appropriate amount of the standard into a volumetric flask. Dissolve with acetone and dilute to the mark. For example, dissolve 25.0 mg of BW 125-M7 in a 25 mL volumetric flask. Dilute to the mark with acetone containing 0.01% oxalic acid.
- b) Prepare a 50 µg/mL standard GC solution by transferring an appropriate amount of the 1.0 mg/mL stock solution from the previous step with a volumetric pipet to a volumetric flask (typically 5 mL of the 1.0 mg/mL stock solutions into 100 mL volumetric flask). Dilute to the mark with acetone containing 0.01% oxalic acid. Subsequent serial dilutions can be made in a similar manner.

2.2.3 BW 125-5376 Standard Solutions for HPLC Analysis

Note: These standard concentrations are suggested. A different concentration scheme may be used and additional standards may be prepared as needed.

Note: The recommended standard solutions for HPLC with BW 125-5376 (metabolite method) are: 5, 10, 20, and 50 ng/mL. Other concentrations may be used as appropriate. The standard solutions are diluted in mobile phase (1% acetic acid in deionized water).

- a) Prepare 1.0 mg/mL stock solution of BW 125-5376 by weighing an appropriate amount of the standard into a volumetric flask. Dissolve with mobile phase and dilute to the mark. For example, dissolve 10.0 mg of BW 125-5376 in a 10 mL volumetric flask. Dilute to the mark with mobile phase.
- b) Prepare a 50 ng/mL HPLC standard solution by transferring an appropriate amount of the 1.0 mg/mL stock solution from the previous step with a volumetric pipet to a volumetric flask (typically 5 mL of the 1.0 mg/mL stock solutions into 100 mL volumetric flask). Dilute to the mark with mobile phase. Serial dilutions can then be made from this standard.

3. ANALYTICAL PROCEDURE

The following procedures are for animal tissues (kidney, liver, fat and muscle), milk, and apple commodities. Flow charts for the analytical method are presented in Figures 1-3.

3.1 Parent Method for Kidney, Liver, Muscles, Fat, and Milk

3.1.1 Preparation of Samples

Homogenize the samples thoroughly before subsampling and weighing.

3.1.2 Extraction

a) Kidney, Liver, Muscles, and Fat

Note: The glassware should be clean with no inside scratches.

1. Weigh 25 g (± 0.2 g) of the homogenized tissue sample into a 400 mL glass beaker or other wide mouth container. Fortify controls to be used as procedural recovery samples with appropriate concentrations of the standards of BAS 125 W, and BW 125-5376 for kidney and liver (e.g. for 0.05 ppm add 1mL of 1.25 $\mu\text{g}/\text{mL}$ of the standard into 25 grams muscle).

Note: No conversion of BAS 125 W to BW 125-5376 has been observed during extraction.

2. Add 120 mL of acetonitrile/ 1.5M H_2SO_4 (8:1, v/v) to the beaker and macerate the sample for approximately 1 minutes with a polytron. Decant the extracted solution into a vacuum filter with a Buchner funnel containing a sheet of Whatman No. 2 filter paper covered with a thin layer of celite (about 5 g, weight is not crucial, but also should not be excessive) into a 500 mL filter flask.

Note: Use one sheet of filter paper (No. 2) to cover the base of the Buchner funnel, (wetted with about 3-5 mL of water) and a layer of celite to prevent the small particles from clogging the filter paper. If the celite becomes hard, a spatula may be used to stir the mixture so that the sample will filter.

Note: Depending on the sensitivity of the GC-MSD detector and the GC injection volume, different aliquot sizes may be used as needed to determine BAS 126-M7.

3. Rinse the polytron blade with water. Collect the rinses in the beaker.

Note: Use forceps to clean the polytron generator from the matrix, if necessary.

4. Repeat extraction from step 3.1.2.2
5. Rinse the polytron blade with water. Collect the rinses in the beaker and filter through the Buchner funnel as before.
6. Transfer the filtrate to a 500 mL volumetric flask. Adjust to the mark by adding water.
7. Go to section 3.1.3 .

b) Milk/Cream

1. Weigh 50 g (± 0.5 g) of the milk or cream into a 400 mL glass beaker or other wide mouth container. Fortify controls to be used as procedural recovery samples with appropriate concentrations of the standards of BAS 125 W (e.g. for 0.01 ppm add 0.5 μ g/mL of the standard into 50 g milk).
2. Add 120 mL of acetonitrile/ 1.5 M H₂SO₄ (9:1, v/v) to the beaker and macerate the sample for approximately one minute with a polytron. Filter the solution into a vacuum filter with a Buchner funnel containing a sheet of Whatman No. 2 filter paper covered with a layer of celite (about 5 g, weight is not crucial, but also should not be excessive) into a 500 mL volumetric flask.
3. Rinse the celite with 120 mL acetonitrile/ 1.5 M H₂SO₄ (9:1, v/v). Collect the rinses with the original extract.
4. Transfer the filtrate to a 500 mL volumetric flask. Adjust to the mark by adding water.
5. Go to section 3.1.3.

3.1.3 Mini-isolute ENV+™ Column Chromatography No. 1

A mini-isolute ENV+™ column clean-up step will be used before the methylation step. The following procedure is used for the ENV+™ cleanup:

Note: Elution profiles for ENV+™ sorbent material should be done in the presence of matrix to match the conditions of use as closely as possible. It would also help to use a high concentration of BAS 125 W. Using a sample spiked with 5 ppm of BAS 125 W there should be no problems with detection or interference from matrix.

a) Column Preparation

1. Weigh out 0.50 g ± 0.05 gram ENV+™ sorbent. Transfer the sorbent into an 8 mL glass column with 2 filter paper frits, (grade 417 from VVR) at the bottom and place a glass wool plug (about 0.05 g; the weight is not crucial, but also should not be excessive) at the top of the ENV+™ sorbent.

Note: For the glass columns use two filter paper frits (grade 417 from VVR Cat. No. 28313-057) that fit the bottom of the glass columns. The correct size of the filter papers could be made by hole puncher.

2. Use an SPE vacuum manifold (aspirator) to perform all the steps for ENV+™ column. A vacuum reading of about 15 inches Hg is adequate (solvent flow rate is about 15 mL/min).

Note: The flow rate may change depending on the type of matrix. In this case keep the solvent flow as close as possible to 15 mL/min. Manifold should be able to hold four 125 mL flat-bottom flasks for highest efficiency in sample processing.

3. Condition the mini- ENV+™ column by passing 5 mL acetonitrile-1.5 M H₂SO₄ (9:1, v/v), then 5 mL of methanol, followed by 10 mL water, without allowing the column to go dry.

Note: A reservoir may be used with the mini-column.

b) Sample Load

The sample load is different for milk/ cream and animal tissues.

For animal tissues: Pipette 15 mL of the extract from step 3.1.2.a.6 and place in a 500 mL flask. Add 200 mL of water. Date, label and store the remaining extract. This extract may be used for metabolite determination in animal tissues (Section 3.2). Swirl the aliquot flask to dissolve any residues left on the wall of the sample flask. Transfer this solution to the conditioned mini-ENV +™ column. Collect the eluant in a waste container (e.g. 500 mL beaker). Do not allow the column to go to dryness.

For milk/ cream: Pipette (or use a graduated cylinder) 40 mL of the extract from step 3.1.2.b.4 and place in a 500 mL flask. Add 350 mL of water. Date, label and store the remaining extract. Swirl the aliquot flask to dissolve any residues left on the wall of the sample flask. Transfer this solution to the conditioned mini-ENV +™ column. Collect the eluant in a waste container (e.g. 500 mL beaker).

c) Column Wash

Wash the sample container with 35 mL of deionized water, add to the column, and dry for about 1 minute. Then wash the column with 1 mL of methanol and dry it for about 5 minutes. Collect the eluant in the waste container.

Note: It is very important to dry the column well before elution to minimize the water content before methylation. Strong vacuum (~20 mm/ Hg) may be used to dry the column. One way to dry the column is to apply a vacuum for 1-2 min. Then open to atmospheric pressure. Then reapply the vacuum. Repeating this process for 2-3 time should dry the column sufficiently.

d) Analyte Elution

Elute BAS 125 W with 25 mL of methanol. Collect the elution solvent in a 125 mL flat bottom flask.

Note: The volume of methanol may change depending on the lot number of the ENV +™ sorbent. Elution profiles must be established for each lot of ENV +™ sorbent.

3.1.4 Methylation Using MeOH/ Conc. H₂SO₄

Add 100 µL concentrated H₂SO₄ to the reaction flask from the previous step 3.1.3.d, attach a reflux condenser and reflux for 30 minutes on a hot plate. Boiling stones may be used. After methylation, the sample is allowed to cool to room temperature before going to step 3.1.5.

3.1.5 Mini-isolute ENV +™ Column Chromatography No. 2

A mini-isolute ENV +™ column clean-up step will be used before the final GC determination. The following procedure is used for the ENV +™ cleanup:

Note: Elution profiles for ENV +™ sorbent material should be done in the presence of matrix to match the conditions of use as closely as possible. It would also help to use a high concentration of BW 125-M7. Using a control sample that has been run through all prior steps and is spiked with 5 ppm of BW 125-M7, there should be no problems with detection or interference from matrix.

a) Column Preparation

1. Weigh out 0.50 g \pm 0.05 gram ENV +™ sorbent. Transfer the sorbent into an 8 mL glass column with 2 filter paper frits, (grade 417 from VWR) , at the bottom and place a glass wool plug (about 0.05 g; the weight is not crucial, but also should not be excessive) at the top of the ENV +™ sorbent.

Note: For the glass columns, use two filter paper frits (grade 417 from VWR Cat. No. 28313-057) that fit the bottom of the glass columns. The correct size of the filter papers could be made by hole puncher.

2. Use an SPE vacuum manifold (aspirator) to perform all the steps for ENV +™ column. A vacuum reading of 15 inches Hg is adequate (solvent flow rate is about 15 mL/ min).
3. Condition the mini-ENV +™ column by passing acetone-0.01% oxalic acid (v/w), and methanol (5 mL each) followed by 10 mL water through, without allowing the column to go dry.

Note: A reservoir may be used with the mini-column.

b) Sample Load

The sample flask from step 3.1.4 is taken directly from methylation to this step. Swirl the flask to dissolve any residues left on the wall of the sample flask. Add 100 mL of water to the reaction flask. Transfer this solution to the conditioned mini-ENV +™ column. Collect the eluant in a waste container (for example, a 200 mL beaker).

c) Column Wash

Wash the sample container with 35 mL of water, add to the column, and dry for about 1 minute. Then wash the column with 1 mL of methanol and dry it for about 5 minutes. Collect the eluant in the waste container.

Note: It is very important to dry the column well before elution. Strong vacuum (~20 mm/Hg) may be used to dry the column. One way to dry the column is to apply a vacuum for 1-2 min. Then open to atmospheric pressure. Then reapply the vacuum. Repeating this process for 2-3 time should dry the column sufficiently.

d) Analyte Elution

Elute BW 125-M7 with 10 mL of acetone-0.01% oxalic acid (v/ w). The volume of the acetone-0.01% oxalic acid solution may change depending on the lot number of the ENV +™ sorbent. Elution profiles must be established for each lot of ENV +™ sorbent. Collect the elution solvent in a 10 mL volumetric flask. Complete the volume to 10 mL by adding acetone-0.01% oxalic acid (v/ w).

Note: Regenerated ENV +™ may be used. An elution profile does not have to be established if only one lot is used for regeneration. See appendix B for regeneration technique.

3.1.6 Preparation for final determination by gas chromatography/mass spectrometry

The solutions of the control and 0.05 ppm fortification samples from the last step of 3.1.5.d are ready for injection into the gas chromatograph/mass spectrometer (GC-MSD). Further dilution is required for the 1.0 ppm fortified sample (e.g. 1/ 10 dilution from the elution solution).

Note: Different equipment and parameters than those listed below may be substituted into the method as long as interpretable chromatography results.

a) Description of Equipment

Detector: HP 5970 MSD
Gas Chromatograph: HP 5890 series II GC
Column: Stabilwax-DA from Resteck
(30 m, 0.25 mm ID, 0.25 µm film thickness)

The instrument is automatically and manually tuned for maximum sensitivity (for ion m/z 219) using perfluorotributylamine. Detection by selected ion monitoring (SIM) at m/z 228 (M⁺). The dwell time is 500 msec. The gas chromatograph is connected to the MSD with a capillary interface kept at 250°C.

Note: A J&W DB-FFAP column (nitroterephthalic acid modified polyethylene glycol) may be used as an alternative to the Stabilwax, if resolution is not achieved using the Stabilwax column.

b) Operating Conditions

Column Parameters:

Carrier Gas: ultra-high purity He (99.999%)
Head Pressure: 15 psi
Flow Rate: 1.5 mL/min
Velocity: 45 cm/s

GC Oven Program:

80	-	80	0.5
80	70	220	4
220	20	250	.11

Note: The GC to MS interface is kept at 250 °C.

Injection Parameters:

A glass insert from Restek (4mm Cyclo Double Gooseneck, cat. # 20898) can be used. Splitless injection with solenoid valve opens after 1 minute, septum purge is 2-3 mL/min. Injector temperature is 180°C, flow of 1.5 mL/min. Injection volume depends on the sensitivity of the GC-MS (typical injection volume is 1 to 5 µL).

Note: Electronic pressure regulator may be used, (suggested conditions are: pulsed splitless, 50 psi for 0.5 min, then 15 psi for 20 min). Other glass inserts and injection conditions with similar performance can also be used.

Detector Parameters:

MSD in the "SIM" mode to monitor the molecular ion at m/z 226, (M⁺ of BW 125-M7).

- Notes: 1. The GC parameters may be varied depending on required peak resolution or specific separation problems. It is highly recommended that the temperature ramps not be shortened or deleted.
2. Condition the GC system by injecting a matrix sample and two standards

3.1.7 Method of Calculation for parent (BAS 125 W)

a) Calibration Procedures

Calculation of results is based on peak area or height measurements using a calibration curve. To obtain a standard curve, 4 µL of at least three different standard concentrations, for example 1.875, 3.75, 7.5 and 15 ng/ mL of a BW 125-M7 are injected. These correspond to 7.5, 15, 30 and 60 pg/ 4µL, respectively. The peak area or height (signal counts) is plotted versus the amount of injected standard (pg).

b) Analyte in Sample

1. Principle

Calculation of results is based on peak area or height measurements. The amount of BW 125-M7 in injected samples is determined from the calibration curve and the equation described in 3.1.7.b.3 is utilized for the determination of the residue (R). Calculation can also be made by a suitable computer program.

At least one fortification and one untreated sample (control) are run with each set of samples. The amount of BW 125-M7 for fortification trials should be on the order of magnitude of the expected residue. The recovery is determined from the fortification experiments (see 3.1.7.b.2).

2. Calculation of Residue

Residue
(ppm) = $\frac{\text{ng Analyte Found}}{\text{mg Sample Injected}} \times \text{Molecular Weight Conversion Factor (MWCF)}$

Sample Weight
Injected (mg)

= $\frac{\text{g Sample} \times \mu\text{L Injected} \times \text{Aliquot \%}}{\text{Dilution Volume (mL)} \times 100}$

ng Analyte found

= Amount of analyte read from calibration curve in ng

g Sample

= Weight in gram of sample extracted

µL Injected

= µL injected into GC-MS

Aliquot %

= Aliquot in % taken from sample extract through the method

Dilution Volume

= Final volume after all dilution steps (mL)

MWCF

= 0.938 for BW 125-M7 to BAS 125

Note: Treated samples should not correct for control contributions.

3. Calculation of Recoveries

Residue
(ppm) = $\frac{\text{ng Analyte Found}}{\text{mg Sample Injected}} \times \text{Molecular Weight Conversion Factor (MWCF)}$

Sample Weight
Injected (mg) = $\frac{\text{g Sample} \times \mu\text{L Injected} \times \text{Aliquot \%}}{\text{Dilution Volume (mL)} \times 100}$

ng Analyte found = Amount of analyte read from calibration curve in ng
g Sample = Weight in gram of sample extracted
 $\mu\text{L Injected}$ = $\mu\text{L Injected into GC-MS}$
Aliquot % = Aliquot in % taken from sample extract through the method
Dilution Volume = Final volume after all dilution steps (mL)
MWCF = 0.938 for BW 125-M7 to BAS 125

Recovery % = $\frac{\text{ppm Found in Fortified Sample} - \text{ppm Found in Control}}{\text{ppm Added to Fortified Sample}} \times 100$

Note: Correction of fortification recoveries for control residues is optional. Treated sample are not corrected for control contributions.

3.1.8 Interferences

If interfering peak(s) from the matrix occur in the chromatogram, alter the GC oven program or column flow rate. Other types of GC columns may be used.

(a) Sample Matrices

None observed to date.

(b) Other Sources

Solvents: None observed to date.
Lab Ware: None observed to date.
Other Pesticides: Refer to section 3.8 of BASF Registration Document No. 96/5223 for the determination of interferences with compounds registered for use on peanut, apple, meat and milk.

3.1.9 Confirmatory Techniques

GC-MS is used as a confirmatory technique to confirm the residue of BW 125-M7 by monitoring three ions at m/z 228 (M⁺), 195 and 165 (base peak). The relative abundance of these ions are: 100%, 10% and 58% for m/z 165, 195 and 228 respectively (Figure 21). A different column, DB-5 or DB-1 or DB-17 (30 m, 0.32 mm, 0.5 mm), can be used as an alternative column for BAS 125 W residue analysis.

3.1.10 Time Required for Analysis

The time required for a set of 5 samples, 2 recoveries and 1 control is 8 hours, plus GC analysis and calculation times that can be automated and unattended, provided that no special problems arise.

It is recommended that the work-up be completed in one day, without any stopping points. If it is necessary to stop the set, complete the methylation reaction, and keep the reaction flasks in the freezer (~ -20°C).

3.1.11 Potential Problems

- a) During large analytical sets, the detector sensitivity can vary due to matrix effects. It is recommended to condition the column by injecting matrix extract followed by two standards before starting to inject samples.
 - b) Make column cuts for both mini-ENV +™ column conditions for each new lot number received.
 - c) Before analyte elution from the first mini-ENV +™ column, make sure the column is dry. The presence of water may affect the yield of the methylation step.
 - d) Before analyte elution from the second mini-ENV +™ column, make sure the column is dry. The presence of water may hydrolyze the ester in the injection port.
-

3.2 Despropanoyl Method for Kidney and Liver

3.2.1 Preparation of the Sample

Homogenize the samples thoroughly before subsampling and weighing.

3.2.2 Extraction of Kidney and Liver

Note: The glassware should be clean with no inside scratches.

Note: The extract from step 3.1.2.a.6 may be used if it has not been stored for more than a few hours at room temperature. The extraction of kidney (or liver) to determine BAS 125 W or despropanoyl is identical. Therefore, after extraction of the kidney (or liver) two different aliquots can be taken to different methods. One for the parent method (BAS 125 W) and the other for the despropanoyl method (BW 125-5376).

Note: No conversion of BAS 125 W to BW 125-5376 has been observed during extraction.
a) Weigh 25 g (+0.2 g) of the homogenized tissue sample into 400 mL glass beaker or other wide mouth container. Fortify controls to be used as procedural recovery samples with appropriate concentration of the standards of BW 125-5376 for kidney and liver (e.g. for 0.05 ppm add 1 mL of the 1.25 µg/ mL standard into 25 g kidney).

b) Add 120 mL of acetonitrile/ 1.5 M H₂SO₄ (9:1, v/ v) to the beaker and macerate the sample for approximately 1 minutes with a polytron. Decant the extracted solution (leave the solid behind) into vacuum filter with a Buchner funnel containing a sheet of Whatman No. 2 filter paper covered with a layer of celite (about 5 g, weight is not crucial, but also should not be excessive) into a 500 mL filter flask.

c) Rinse the polytron blade with deionized water. Collect the rinses in the beaker.

d) Repeat extraction step 3.2.2.b

Note: Use forceps to clean the polytron generator from the matrix if necessary.

e) Rinse the polytron blade with deionized water. Collect the rinses in the beaker and filter through the Buchner funnel as before.

f) Transfer the filtrate to a 500 mL volumetric flask. Adjust to the mark by adding deionized water.

Note: Use one sheet of filter paper (No. 2) to cover the base of the Buchner funnel, (wetted with about 3-5 mL of water) and a layer of celite to prevent the small particles from clogging the filter paper. If the celite clogs during extraction, a spatula can be used to stir up the marc.

g) Quantitatively remove 50 mL of the animal matrices extract from the previous step and place into a 125 mL, 24/ 40 standard flat-bottom flask. Date, label and store the remaining extract.

h) Reduce the aliquot volume to less than 10 mL by using rotary Evaporator.

Note: Depending on the sensitivity of the HPLC-UV detector and the injection volume, different aliquot sizes may be used as needed.

3.2.3 Acid Hydrolysis

Add 100 μ L 6N-HCl to the solution from step 3.2.2.h, heat at 80° C for 30 minutes on a water bath or on a hot plate. After hydrolysis, the sample is allowed to cool to room temperature before proceeding with next step.

3.2.4 Mini-SAX Column

A mini-SAX column clean-up step will be used before the Mini-Carbopack B Column. The following procedure is used for the SAX clean-up:

Note: Elution profiles for SAX sorbent material should be done in the presence of matrix to match the conditions of use as closely as possible. It would also help to use a high concentration of BW 125-5376. Using a control sample spiked that has been run through all prior steps and spiking with 5 ppm of BW 125-5376, there should be no problems with detection or interference from matrix.

a) Column Preparation

1. Weigh out 1.0 g \pm 0.05 gram of SAX sorbent. Transfer the sorbent into an 8 mL glass column with two filter paper frits (grade 417 VWR) at the bottom. Place a glass wool plug (about 0.05 g; the weight is not crucial, but also should not be excessive) on top of the SAX sorbent.
2. Use an SPE vacuum manifold (aspirator) to perform all the steps for the mini-SAX column. A low vacuum should be used for elution (solvent flow rate < 6 mL/min).
3. Condition the mini-SAX column by passing 5 mL of methanol followed by 20 mL of pH=2 water, adjusted with HCl without allowing the column to go dry.

Note: For the glass columns, use two filter paper frits (grade 417 from VWR Cat. No 28313-057) that fit the bottom of the glass columns. The correct size of the filter paper frits could be made by a hole puncher.

Note: A reservoir may be used with the mini-column.

b) Sample Load and Wash

Note: BW 125-5376 will be in the load and water wash.

1. Transfer the solution from the hydrolysis step 3.2.3 to the conditioned mini-SAX column with a low vacuum (solvent flow rate < 6 mL/min). Collect the eluant in an appropriate container (e.g. 125 mL flat bottom flask or 150 mL beaker).
2. Wash the remaining BW 125-5376 off the mini-SAX column with 20 mL of pH=2 water adjusted with HCl. Collect the eluant in the same container. A strong vacuum (20 mm Hg) may be used to remove the remaining water.

3.2.5 Mini-Carbopack B Column Chromatography

A mini-Carbopack B column clean-up step will be used after the mini-SAX clean-up step. The following procedure is used for the Carbopack B cleanup:

Note: Elution profiles for Carbopack B sorbent material should be done in the presence of matrix to match the conditions of use as closely as possible. It would also help to use a high concentration of BW 125-5376. Using a control sample spiked that has been run through all prior steps and spiking with 5 ppm of BW 125-5376, there should be no problems with detection or interference from matrix.

a) Column Preparation

1. Weigh out $1.0 \text{ g} \pm 0.05$ gram of Carbopack B sorbent. Transfer the sorbent into an 8 mL glass column with two filter paper frits (grade 417 from VWR) at the bottom. Place a glass wool plug (about 0.05 g; the weight is not crucial, but also should not be excessive) on top of the Carbon Black sorbent.

2. Use an SPE vacuum manifold (aspirator) to perform all the steps for mini-Carbopack B column.

Note: The flow rate may change depending on the type of matrix. In this case keep the flow rate of 4-6 mL/min. The manifold should be able to hold four 125 mL flat-bottom flasks for highest efficiency in sample processing.

3. Condition the mini-Carbopack B column by passing 5 mL of 0.1 N NaOH, 5 mL of methanol followed by 10 mL water, without allowing the column to go dry.

Note: For the glass columns, use two filter paper frits (grade 417 from the VWR Cat. No. 28313-057) that fit the bottom of the glass columns. The correct size of the filter paper frits could be made by a hole puncher.

Note: Manifold should be able to hold four 125 mL flat-bottom flasks for highest efficiency in sample processing.

Note: A reservoir may be used with the mini-column.

b) Sample Load

Transfer the eluant from step 3.2.4.b to the conditioned mini-Carbopack B column with NO VACUUM. Collect the eluant in a waste container (e.g. 500 mL beaker). Dry the column using as SPE vacuum manifold (aspirator).

Note: Dry the column well before elution. A strong vacuum (20 mm/ Hg) should be used to dry the column. One way to dry the column is to apply a strong vacuum for several minutes. Release the vacuum, allowing the pressure to increase to atmospheric pressure. Then reapply the strong vacuum. Repeating this procedure 2-3 times should dry the column sufficiently.

c) Analyte Elution

Elute BW 125-5376 with 20 mL of 0.1 N NaOH, a solvent flow rate of 4-6 mL/min should be used for elution. The volume of 0.1 N NaOH may change depending on the lot number of the Carbopack B sorbent. Elution profiles must be established for each lot of Carbopack B sorbent. The elution profile should be made in the presence of matrix

so that the profile conditions match the conditions of the column. Collect the elution solvent in an appropriate sized flask or beaker.

Note: Carboxpack B particles may leak from the column into the elution solvent. This is not a problem. If it happens, load the solution (including Carboxpack B) onto the next mini-ENV+™ column.

3.2.6 Mini-Isolute ENV+™ Column Chromatography

A mini-Isolute ENV+™ column clean-up step will be used before the final HPLC determination. The following procedure is used for the ENV+™ cleanup:

Note: Elution profiles for ENV+™ sorbent material should be done in the presence of matrix to match the conditions of use as closely as possible. It would also help to use a high concentration of BW 125-5376. Using a control sample spiked that has been run through all prior steps and spiking with 5 ppm of BW 125-5376, there should be no problems with detection or interference from matrix.

a) Column Preparation

1. Weigh out 0.50 g \pm 0.05 gram ENV+™ sorbent. Transfer the sorbent into an 8 mL glass column with 2 filter paper frits, (grade 417 from VWR) at the bottom and place a glass wool plug (about 0.05 g; the weight is not crucial, but also should not be excessive) at the top of the ENV+™ sorbent.

Note: For the glass columns, use two filter paper frits (grade 417 from VWR Cat. No. 28313-057) that fit the bottom of the glass columns. The correct size of the filter papers could be made by hole puncher.

Note: Manifold should be able to hold four 125 mL flat-bottom flasks for highest efficiency in sample processing.

2. Use an SPE vacuum manifold (aspirator) to perform all the steps for ENV+™ column.
3. Condition the mini-ENV+™ column by passing acetone and methanol (5 mL each), 20 mL of 0.04% 1,3-cyclohexanedione (w/v in pH=2 water adjusted with HCl) followed by 10 mL water without allowing the column to go dry.

Note: A reservoir may be used with the mini-column.

Note: 1,3 Cyclohexanedione is an analog for BW 125-5376. It is used to cover the active sites in the ENV+™ sorbent.

b) Sample Load

The sample flask from step 3.2.5.c is taken from mini-Carboxpack B clean-up, 100 μ L of H₂SO₄ is added to reduce the pH < 2, and the sample is ready to load onto the ENV+™ mini-column. Swirl the flask to dissolve any residues left on the wall of the sample flask. Transfer this solution to the conditioned mini-ENV+™ column. No vacuum should be used. Collect the eluant in a waste container (e.g. 200 mL beaker).

c) Column Wash

Wash the container 35 mL of water, add to the column. No vacuum should be used to load the wash. Dry the column under vacuum for about 5 minutes. Collect the eluant in the waste container.

Note: A strong vacuum (20mm/Hg) should be used to dry the column. One way to dry the column is to apply a vacuum for 1-2 min. Then open to atmospheric pressure. Then reapply the vacuum. Repeating this process for 2-3 times should dry the column sufficiently.

d) Analyte Elution

Elute BW 125-5376 with 10 mL of acetone. The volume of the acetone solvent may change depending on the lot number of the ENV +™ sorbent. Elution profiles must be established for each lot of ENV +™ sorbent. Collect the elution solvent in a flat bottom flask (e.g. 50 mL). A moderate vacuum may be used. A flow rate of about 5 mL/ min is typical.

Note: Regenerated ENV +™ may be used (see appendix B).

3.2.7 Preparation for Final Determination by HPLC-Column Switching

The solutions from step 3.2.6.d are evaporated by rotary evaporator at $45 \pm 5^\circ \text{C}$ to near dryness. Apply N_2 stream until sample is completely dry. Add 10 mL deionized water, this dilution is adequate for samples ranging from the LOQ (0.05 ppm) to 0.2 ppm. Samples with residues exceeding 0.2 ppm will require appropriate dilutions.

a) Description of HPLC Instrumentation

A model 1050 High Performance Liquid Chromatography (HPLC) from Hewlett Packard is used. The instrument includes an autosampler, a UV detector from Applied Biosystems, and a Column Switching Port from Rheodyne. Two columns are used for column switching. The first column is a Hypersil C18, 3 micron, 100 X 4.6 mm from Phenomenex. The Analytical Column is a ODS-AQ, 5 micron, 120 A pore size, 250 X 4.6 mm from YMC. The HPLC conditions are as follows:

First Column:	100 X 4.6 mm, Hypersil C18, 3 micron (Phenomenex: OOD-0145-EO)
First Column Flow:	1.0 mL/ min
Mobile Phase:	99:1 Water/Acetic Acid
Injection Volume:	500 μL
Column Cut:	2 min, from 7 to 9 minutes
Second Column:	250 X 4.6 mm, ODS-AQ, 5 micron 120 A pore size (YMC: AQ12S052546WT)
Second Column Flow:	1.0 mL/ min
Mobile Phase:	3:96:1 Acetonitrile/Water/HOAC
Wavelength:	265 nm
Retention time:	~18 min

Typical Calibration Standards: 5, 10, 20, 50 ng/ mL

The first column is flushed with acetonitrile and re-equilibrated with the mobile phase during each injection. This occurs after the column cut (7-9 min), when the analyte is switched from the first to the second column. Here is a description of that procedure:

After the analyte is switched onto the second column and the column switching window is closed, the first column is washed with 100% ACN for 4 minutes at flow rate of 2 mL/ min. Then the first column is flushed with 99:1 water/ HOAC for an additional 4 minutes at flow rate of 2 mL/ min. The column flow rate is then changed to 1 mL/ min to equilibrate the flow and pressure prior to the next injection.

This column flushing procedure helps reduce column drift on the first column and carryover of late eluting compounds onto the second column.

b) Sample injection

Before injecting a new set into HPLC, the columns should be washed with Acetonitrile (ACN) and conditioned with Mobile Phase. Turn both columns around and flush the columns with 100% ACN for at least half an hour. Turn the columns back to the normal positions and condition the columns with mobile phase for at least a half hour. Inject a standard to check the precolumn window. After columns conditioned, the HPLC system is ready for analyzing samples.

3.2.8 Method of Calculation for Despropionyl

a) Calibration Procedures

Calculation of results is based on peak area or height measurements using a calibration curve. To obtain a standard curve, 500 μ L of at least three different standard concentrations, for example 5, 10, 20 and 50 ng/ mL of BW 125-5376 are injected. These correspond to 2.5, 5, 10 and 25 ng/ 500 μ L, respectively. The peak area or height (signal counts) is plotted versus the amount of injected standard (μ g).

b) Analyte in Sample

1. Principle

Calculation of results is based on peak area or height measurements. The amount of BW 125-5376 in injected samples is determined from the calibration curve and the equation described in 3.2.8.b.3 is utilized for the determination of the residue (R). Calculation can also be made by a suitable computer program.

It is recommended that at least one fortification and one untreated sample (control) are run with each set of samples. The amount of BW 125-5376 for fortification trial should be on the order of magnitude of the expected residue. The recovery is determined from the fortification experiments (see 3.2.8.b.2).

2. Calculation of Residues

$$\text{Residue (ppm)} = \frac{\text{ng Analyte Found}}{\text{mg Sample Injected}}$$

$$\text{Sample Weight Injected (mg)} = \frac{\text{g Sample} \times \text{mL Injected} \times \text{Aliquot \%}}{\text{Dilution Volume (mL)} \quad 100}$$

ng Analyte found = Amount of analyte read from calibration curve in ng
g Sample = Weight in gram of sample extracted
mL Injected = mL injected into the HPLC
Aliquot % = Aliquot in % taken from sample extract through the method
Dilution Volume = Final volume after all dilution steps (mL)

Note: Treated samples should not be corrected for residues in the control.

3. Calculation of Recoveries

$$\text{Residue (ppm)} = \frac{\text{ng Analyte Found}}{\text{mg Sample Injected}}$$

$$\text{Sample Weight Injected (mg)} = \frac{\text{g Sample} \times \text{mL Injected} \times \text{Aliquot \%}}{\text{Dilution Volume (mL)} \quad 100}$$

ng Analyte found = Amount of analyte read from calibration curve in ng
g Sample = Weight in gram of sample extracted
mL injected = mL injected into HPLC-UV
Aliquot % = Aliquot in % taken from sample extract through the method
Dilution Volume = Final volume after all dilution steps (mL)

$$\text{Recovery \%} = \frac{\text{ppm Found in Fortified Sample} - \text{ppm Found in Control}}{\text{ppm Added to Fortified Sample}} \times 100$$

Note: Correction of fortification recoveries for control residues is optional. Treated sample are not corrected for control residues.

3.2.9 Interferences

If interfering peak(s) from the matrix occur in the chromatogram, alter the type of HPLC columns used.

a) Sample Matrices

None observed to date.

b) Other Sources

Solvents: None observed to date.
Lab Ware: None observed to date.

Other Pesticides:

The pesticides that are currently registered for use on meat (kidney and liver) are potentially present in these tissues. Standard solutions of these pesticides were prepared in methanol at a concentration equal or higher than the tolerance. All the standards were hydrolyzed with 2N HCl for 30 minutes, followed by elution from SAX, Carbo-Pack and ENV+™ mini-isolute columns, as described in the method. The elutions from the ENV+™ mini-isolute columns were evaporated and redissolved in deionized water. Injections into a HPLC-UV were done under the conditions used for despropinoyl analysis to determine if any of the compounds interfere with the determination of the analytes (retention time \pm 0.2 min). None of the pesticides interfered with BW 125-5376 (see Table 13).

3.2.10 Confirmatory Techniques

A HPLC-MS confirmatory technique has been developed. It is outlined in Appendix D.

3.2.11 Time Required for Analysis

The time required for a set of 5 samples, 2 recoveries and 1 control is 8 hours, plus HPLC analysis and calculation times that can be automated and unattended, provided that no special problems arise.

It is recommended that the work-up be completed in one day, without any stopping points. If necessary to stop the set, elute from mini-Carbopack B column, and keep the elution in the freezer (-20°C).

3.2.12 Potential Problems

- a) The HPLC column cut window may shift. It is recommended to establish the window for the column cut by injecting standard before injecting the whole set. Condition the system by injecting matrix extract followed by two standards before starting to inject samples.
 - b) Make column cuts for SAX, Carbo-pack B (in matrix), and mini-ENV+™ columns for each new lot number received. The profile conditions should match the conditions of the columns.
-

3.3 Parent Method for Apple

3.3.1 Preparation of Samples

Homogenize the samples thoroughly before subsampling and weighing.

3.3.2 Extraction

a) Apple and Wet Pomace

Note: The glassware should be clean with no inside scratches.

1. Weigh 25 g (± 0.2 g) of the homogenized apple (or wet pomace) into a 400 mL glass beaker of other wide mouth container. Fortify controls to be used as procedural recovery samples with appropriate concentrations of the standard into 25 g wet pomace apple).
2. Add 120 mL of acetonitrile/ 1.5 M H₂SO₄ (9:1, v/ v) to the beaker and macerate the sample for approximately 1 minutes with a polytron. Decant the extracted solution into a vacuum filter with a Buchner funnel containing a sheet of Whatman No. 2 filter paper. Cover with a layer of celite (about 5 g, weight is not crucial, but also should not be excessive) into a 500 mL filter flask.

Note: Use forceps to clean matrix from the polytron generator, if necessary.

3. Rinse the polytron blade with water. Collect the rinses in the beaker.
4. Repeat extraction step 3.3.2.a.2.
5. Rinse the polytron blade with water. Collect the rinses in the beaker and filter through the Buchner funnel as before.
6. Transfer the filtrate to a 500 mL volumetric flask. Adjust to the mark by adding water.

Note: Use one sheet of filter paper (Whatman No. 2) to cover the base of the Buchner funnel, (wetted with about 3-5 mL of acetonitrile/ 1.5 M H₂SO₄) and a layer of celite to prevent small particles from clogging the filter paper.

b) Apple Juice

1. Weigh 25 g (± 0.2 g) of the apple juice into 400 mL glass beaker or other wide mouth container. Fortify controls to be used as procedural recovery samples with appropriate concentration of the standards of BAS 125 W (e.g. for 0.05 ppm add 1 mL of 1.25 μ g/mL of the standard into 25g of juice).
2. Add 120 mL of acetonitrile/ 1.5 M H₂SO₄ (9:1, v/ v) to the beaker and mix the sample for approximately 0.5 minute with a stirring rod. Filter the solution into a vacuum filter with a Buchner funnel containing a sheet of Whatman No. 2 filter paper covered with a layer of celite (about 5 g, weight is not crucial, but also should not be excessive) into a 500mL filter flask.
3. Rinse the celite with 120 mL acetonitrile/ 1.5 M H₂SO₄ (9:1, v/ v). Collect the rinses with the original extract.

4. Transfer the filtrate to a 500 mL volumetric flask. Adjust to the mark by adding water.

3.3.3 Mini-isolute ENV +™ Column Chromatography No. 1

A mini-isolute ENV +™ column clean-up will be used before the methylation step. The following procedure is used for the ENV +™ cleanup:

Note: Elution profiles for ENV +™ sorbent material should be done in the presence of matrix to match the conditions of use as closely as possible. It would also help to use a high concentration of BAS 125/W. Using a sample spiked with 5 ppm of BAS 125/W there should be no problems with detection or interference from matrix.

a) Column Preparation

1. Weight out 0.50 g \pm 0.05 gram ENV +™ sorbent. Transfer the sorbent into an 8 mL glass column with 2 filter paper frits, (grade 417 from VWR) at the bottom and place a glass wool plug (about 0.05 g; the weight is not crucial, but also should not be excessive) at the top of the ENV +™ sorbent.

Note: For the glass columns, use two filter paper frits (grade 417 from VWR Cat. No. 28313-057) that fit the bottom of the glass columns. The correct size of the filter papers could be made by hole puncher.

2. Use an SPE vacuum manifold (aspirator) to perform all the steps for ENV +™ column. A solvent flow rate between 30 - 35 mL/ min is usually adequate.

Note: The flow rate may change depending on the type of matrix. In this case keep the solvent flow as close as possible to 30 mL/ min. Manifold should be able to hold four 125 mL flat-bottom flasks for highest efficiency in sample processing.

Note: A reservoir may be used with the mini-column.

3. Condition the ENV +™ mini-column by passing 5 mL ACN-1.5 M H₂SO₄ (9:1, v/ v), 5 mL of methanol followed by 10 mL water through, without allowing the column to go dry.

b) Sample Load

Quantitatively remove 15 mL of the apple extract from the previous step and place into an approximately 500 mL flask or container. Add 200 mL water. Date, label and store the remaining extract.

Swirl the flask to dissolve any residues left on the wall of the sample flask. Transfer this solution to the conditioned mini-ENV +™ column. Collect the eluant in a waste container (e.g. 500 mL beaker).

Note: Depending on the sensitivity of the GC-MSD detector, and the injection volume, different aliquot sizes may be used as needed.

c) Column Wash

Wash the container with 35 mL of water. Transfer the wash to the column. Then thoroughly dry the column. Collect the eluant in the waste container.

Note: A strong vacuum (20mm/ Hg) should be used to dry the column. One way to dry the column is to apply a vacuum for 1-2 min. Then open to atmospheric pressure. Then reapply the vacuum. Repeating this process for 2-3 times should dry the column sufficiently.

d) Analyte Elution

Elute BAS 125 W with 25 mL of methanol. The volume of methanol may change depending on the lot number of the ENV +™ sorbent. Elution profiles must be established for each lot of ENV +™ sorbent. Collect the elution solvent in a 125 mL flat bottom flask.

3.3.4 Methylation Using MeOH/ Conc. H₂SO₄

Add 100 µL H₂SO₄ to the reaction flask from the previous step 3.3.3.d, attach a condenser and reflux for 60 minutes on a hot plate. Boiling stones may be used. After methylation, the sample is allowed to cool to room temperature. Proceed with the next step.

3.3.5 Mini-Isolute ENV +™ Column Chromatography No. 2

An ENV +™ mini-column will be used for additional clean-up after the methylation step. The following procedure is used for the ENV +™ clean-up:

Note: Elution profiles for ENV +™ sorbent material should be done in the presence of matrix to match the conditions of use as closely as possible. It would also help to use a high concentration of BW 125-M7. Using a control sample that has been run through all prior steps and spiked with 5 ppm of BW 125-M7, there should be no problems with detection or interference from matrix.

a) Column Preparation

1. Weigh out 0.50 g ± 0.05 gram ENV +™ sorbent. Transfer the sorbent into an 8 mL glass column with 2 filter paper frits, (grade 417 from VWR) at the bottom and place a glass wool plug (about 0.05 g; the weight is not crucial, but also should not be excessive) at the top of the ENV +™ sorbent.

Note: For the glass columns, use two filter paper frits (grade 417 from VWR Cat. No. 28313-057) that fit the bottom of the glass columns. The correct size of the filter papers could be made by hole puncher.

2. Use an SPE vacuum manifold (aspirator) to perform all the steps for ENV +™ column. A solvent flow rate between 30 - 35 mL/ min is usually adequate.

Note: The flow rate may change depending on the type of matrix. In this case keep the solvent flow as close as possible to 30 mL/ min. Manifold should be able to hold four 125 mL flat-bottom flasks for highest efficiency in sample processing.

3. Condition the mini-ENV +™ column by passing acetone-0.01% oxalic acid (v/w), and methanol (5 mL each) followed by 10 mL water through, without allowing the column to go dry.

Note: A reservoir may be used with the mini-column.¹⁾

b) Sample Load

To the sample from step 3.2.4 is added 100 mL of deionized water. Swirl the flask to dissolve any residues left on the wall of the sample flask. Transfer this solution to the conditioned ENV +™ mini-column. Collect the eluant in a waste container (e.g. 200 mL beaker).

c) Column Wash

Wash the container with 35 mL of water, add to the column, and dry for about 5 minutes. A strong vacuum (-20 mm/Hg) may be used to dry the column. Collect the eluant in the waste container.

d) Analyte Elution

Elute BW 125-M7 with 10 mL of acetone-0.01% oxalic acid (v/w). The volume of the acetone-0.01% oxalic acid solution may change depending on the lot number of the ENV +™ sorbent. Elution profiles must be established for each lot of ENV +™ sorbent. Collect the elution solvent in a 10 mL measuring flask. Complete the volume to 10 ml by adding acetone-0.01% oxalic acid (v/w).

Note: Regenerated ENV +™ may be used. An elution profile does not have to be established if only one lot is used for regeneration. (See appendix B).

3.3.6 Preparation for Final Determination by Gas Chromatography/Mass Spectrometry

The solutions of the control and 0.05 ppm fortification samples from the last step of 3.3.5 are ready for injection into the gas chromatograph/ mass spectrometer (GC-MSD). Further dilution is required for the 1.0 ppm fortified sample (e.g. 1/ 10 dilution from the elution solution).

Note: Different equipment and parameters than those listed below may be substituted into the method as long as interpretable chromatography results.

a) Description of Equipment

Detector: HP 5970 MSD
Gas Chromatograph: HP 5890 series II GC
Column: Stabilwax-DA from Resteck
(30 m, 0.25 mm ID, 0.25 µm film thickness)

The instrument is automatically and manually tuned for maximum sensitivity (for ion m/z 219) using perfluorotributylamine. Detection by selected ion monitoring (SIM) at m/z 228 (M⁺). The dwell time is 500 msec. The gas chromatograph is connected to the MSD with a capillary interface kept at 250°C.

Note: A J&W DB-FFAP column (nitroterephthalic acid modified polyethylene glycol) may be used as an alternative to the Stabilwax, if resolution is not achieved using the Stabilwax column.

b) Operating Conditions

Column Parameters:

Carrier Gas: ultra-high purity He (99.999%)
Head Pressure: 15 psi
Flow Rate: 1.5 mL/ min
Velocity: 45 cm/s

GC Oven Program:

Beginning Temperature (°C)	Rate of Change (°C/min)	Final Temperature (°C)	Oven Hold Time (min)
80	-	80	1
80	70	220	3
220	70	250	3

Note: The GC to MS interface is kept at 250 °C.)

Injection Parameters:

A glass insert from Restek (4mm Gooseneck splitless sleeve part number 20799) is used. Splitless injection with solenoid valve opens after 1 minute, septum purge is 2-3 mL/ min. Injector temperature is 190°C, flow of 1.5 mL/ min. Injection volume depends on the sensitivity of the GC-MS (typical injection volume is 1 to 5 µL).

Note: Electronic pressure regulator may be used, (suggested conditions are: pulsed splitless, 50 psi for 0.5 min, then 15 psi for 20 min). Other glass inserts and injection conditions with similar performance can also be used.

Detector Parameters:

MSD in the "SIM" mode to monitor the molecular ion at m/z 226, (M⁺ of BW 125-M7).

- Notes: 1. The GC parameters may be varied depending on required peak resolution or specific separation problems. It is highly recommended that the temperature ramps not be shortened or deleted.
2. Condition the GC system by injecting a control and two standards

3.3.7 METHODS OF CALCULATION

a) Calibration Procedures

Calculation of results is based on peak area or height measurements using a calibration curve. To obtain a standard curve, 4 µL of at least three different standard concentrations, for example 1.875, 3.75 and 15 ng/ mL of a BW 125-M7 are injected. These correspond to 7.5, 15 and 60 pg/ 4µL, respectively. The peak area or height (signal counts) is plotted versus the amount of injected standard (pg).

b) Analyte in Sample

1. Principle

Calculation of results is based on peak area or height measurements. The amount of BW 125-M7 in injected samples is determined from the calibration curve and the equation

described in 3.3.8.b.3 is utilized for the determination of the residue (R). Calculation can also be made by a suitable computer program.

At least one fortification and one untreated sample (control) are run with each set of samples. The amount of BW 125-M7 for fortification trials should be on the order of magnitude of the expected residue. The recovery is determined from the fortification experiments (see 3.3.8.b.2).

2. Calculation of Residues

$$\text{Residue (ppm)} = \frac{\text{ng Analyte Found}}{\text{mg Sample Injected}} \times \text{Molecular Weight Conversion Factor (MWCF)}$$

$$\text{Sample Weight Injected (mg)} = \frac{\text{g Sample} \times \mu\text{L Injected} \times \text{Aliquot \%}}{\text{Dilution Volume (mL)} \times 100}$$

$$\text{ng Analyte found} = \text{Amount of analyte read from calibration curve in ng}$$

$$\text{g Sample} = \text{Weight in gram of sample extracted}$$

$$\mu\text{L Injected} = \mu\text{L Injected into GC-MS}$$

$$\text{Aliquot \%} = \text{Aliquot in \% taken from sample extract through the method}$$

$$\text{Dilution Volume} = \text{Final volume after all dilution steps (mL)}$$

$$\text{MWCF} = 0.938 \text{ for BW 125-M7 to BAS 125}$$

Note: Treated samples should not correct for control contributions.

3. Calculation of Recoveries

$$\text{Residue (ppm)} = \frac{\text{ng Analyte Found}}{\text{mg Sample Injected}} \times \text{Molecular Weight Conversion Factor (MWCF)}$$

$$\text{Sample Weight Injected (mg)} = \frac{\text{g Sample} \times \mu\text{L Injected} \times \text{Aliquot \%}}{\text{Dilution Volume (mL)} \times 100}$$

$$\text{ng Analyte found} = \text{Amount of analyte read from calibration curve in ng}$$

$$\text{g Sample} = \text{Weight in gram of sample extracted}$$

$$\mu\text{L Injected} = \mu\text{L Injected into GC-MS}$$

$$\text{Aliquot \%} = \text{Aliquot in \% taken from sample extract through the method}$$

$$\text{Dilution Volume} = \text{Final volume after all dilution steps (mL)}$$

$$\text{MWCF} = 0.938 \text{ for BW 125-M7 to BAS 125}$$

$$\text{Recovery \%} = \frac{\text{ppm Found in Fortified Sample} - \text{ppm Found in Control}}{\text{ppm Added to Fortified Sample}} \times 100$$

Note: Correction of fortification recoveries for control residues is optional. Treated sample are not corrected for control contributions.

3.3.8 Interferences

If interfering peak(s) from the matrix occur in the chromatogram, alter the GC oven program or column flow rate. Other types of GC columns may be used.

a) Sample Matrices

None observed to date.

b) Other Sources

Solvents:	None observed to date.
Lab Ware:	None observed to date.
Other Pesticides:	Refer to section 3.8 of BASF Registration Document No. 96/5223 for the determination of interferences with compounds registered for use on peanut, apple, meat and milk.

3.3.9 Confirmatory Techniques

GC-MS is used as a confirmatory technique to confirm the residue of BW 125-M7 by monitoring three ions at m/z 226 (M^+), 195 and 165 (base peak). The relative abundance of these ions are: 100%, 10% and 58% for m/z 165, 195 and 226 respectively (Figure 21). A different column, DB-5 or DB-1 or DB-17 (30 m, 0.32 mm, 0.5 mm), can be used as an alternative column for BAS 125 W residue analysis.

3.3.10 Time Required for Analysis

The time required for a set of 5 samples, 2 recoveries and 1 control is 8 hours, plus GC analysis and calculation times that can be automated and unattended, provided that no special problems arise.

It is recommended that the work-up be completed in one day, without any stopping points. If it is necessary to stop the set, complete the methylation reaction, and keep the reaction flasks in the freezer ($\sim -20^\circ\text{C}$).

3.3.11 Potential Problems

- During large analytical sets, the detector sensitivity can vary due to matrix effects. It is recommended to condition the column by injecting matrix extract followed by two standards before starting to inject samples.
 - Make column cuts for both ENV +TM mini-column conditions for each new lot number received.
 - Before analyte elution from the first ENV +TM mini-column, make sure the column is dry. The presence of water may affect the yield of the methylation step.
 - Before analyte elution from the second ENV +TM mini-column, make sure the column is dry. The presence of water may hydrolyze the ester in the injection port.
-

3.4 ¹⁴C Accountability

3.4.1 Introduction

The accountability of this method for incurred residues was demonstrated using a goat kidney sample (BASF Sample No: 1153-2-12) from a ¹⁴C lactating goat metabolism study (Reference 3). The sample was harvested on July 19, 1994.

The metabolism work by Battelle Labs included a profile of the goat kidney sample at 2 different times. The first extraction conducted as part of the metabolism study (Reference 3) was within 2 months of harvest of the kidney sample. It demonstrated 32% TRR as prohexadione and 21% TRR as the despropanoyl metabolite. The second extraction and profile conducted under the accountability protocol was conducted after 28 months of frozen storage, around the time of the accountability study. A significant decrease in the amount of prohexadione was observed after 28 months of storage, down 7% TRR. The first profile served to identify parent and despropanoyl as the major residues in fresh kidney, while the second profile quantitates the parent for comparison to the accountability study.

The apple method is very similar to the peanut method reported in BASF Method No. D9601 (Reference 1). An accountability of the method for peanuts is reported there, so no further accountability work was done for apples.

The accountability experiment consisted of analyzing the goat kidney sample by BASF Method D9608. A summary of individual recovery measurements is given in Table 8. A comparison of the radioactive distribution between the accountability and metabolism work is shown in Tables 9 and 10. The detailed recoveries of radioactivity in each method step is shown in Tables 11 and 12.

3.4.2 Sample Preparation and Extraction

Kidney sample # 1-53-096-00 from the goat metabolism study was homogenized using a polytron with liquid nitrogen as the solvent. After homogenization, the nitrogen was allowed to evaporate in BASF Freezer # 723. The specific activity of the ¹⁴C prohexadione used as test substance was 29,300 dpm/μg. Three sets of samples were extracted; two using 10 gram aliquots of kidney and one using a 15 gram aliquot of kidney. The extraction procedure consisted of two extractions with 120 mL of acetonitrile/1.5M H₂SO₄ (9:1, V:V). The filtrate was then brought to 500 mL prior to taking two 3% aliquots for the determination of BAS 125 W residues and two 10% aliquots for the determination of BW 125-5378 residues. In addition to the radioactive goat kidney, control cow kidney and fortified control cow kidney were also analyzed. The fortification samples were fortified at the 0.2 and 3.0 ppm levels with unlabeled (cold) BAS 125 W for the determination of prohexadione in the 10 gram sample experiment and 0.05 and 1.0 ppm in the 15 gram sample experiment sample. The fortification samples were fortified at the 0.2 and 1.0 ppm levels with unlabeled (cold) BW 125-5378 for the determination of the despropanoyl metabolite in the 10 gram sample experiment and 0.1 and 1.0 ppm in the 15 gram ¹⁴C sample experiment. Extraction and analyses were performed on January 15, 1997 for the two 10 g/ sample experiments and on January 20, 1997 for the 15 gram sample experiment. Radioassays for the total radioactive residues (TRR) in the starting material as well as the nonextractable residues (RRR) remaining in the marc after extraction were determined by combustion using an oxidizer and counting the evolved CO₂ with a liquid scintillation counter (LSC). The combustion analysis of the starting material was 82,782 dpm/gram (2.83 ppm). The extraction yielded 69 % of TRR. The RRR (marc) incorporated 38 % bound radioactivity for the treated sample. These results correlated

well with the results of the metabolism experiment by Battelle that was done after 28 months of storage. In this case the extraction yielded 63 % TRR and the post extraction solids yielded 41 % bound radioactivity for the RRR of the treated sample.

In order to determine the distribution of radioactivity, aliquots were taken at various stages of the method. The dpms counted at each step are summarized in Tables 11 and 12. The TRR at each step is reported in Tables 9 and 10.

3.4.3 Determination of residues for BAS 125 W (Master Sheet # 98135-24)

Note: Only Master Sheet # 98135-24 was used for TRR calculation. Master sheets # 98135-24, -26, and -28 were used for ppm determination.

a) Mini-Isolute ENV+™ Column #1

The samples were applied to 500 mg ENV+ mini-columns that had been washed with Acetonitrile-1.5M H₂SO₄ (9:1, V:V), methanol, and water prior to loading with the samples. After loading the samples, the columns were washed with water and methanol. The columns were eluted with 25 mL of methanol. Most of the radioactivity added to the column was accounted for in either the load/wash, or the eluate. Averaging the three extractions, the load/ wash contained 44 % of the TRR and the eluate contained 19 % of the TRR.

b) Methylation

The elution from the previous step was refluxed in presence of concentrated H₂SO₄ for 30 minutes. Recoveries from both samples indicated that there is no losses of radioactivity through methylation. The TRR was an average of 19 % for the samples after methylation.

c) Mini-Isolute ENV+™ Column #2

The samples were applied to 500 mg ENV+™ mini-columns that had been washed with acetone, methanol, and water prior to loading with the samples. After loading the samples, the columns were washed with water and a small quantity of methanol. The columns were eluted with acetone-0.01% Oxalic Acid. Most of the radioactivity added to the column was accounted for in the eluate. The eluate contained 14 % of the TRR. At this stage the samples were ready for GC injection, no additional handling was required.

d) GC Injection

The samples were injected on a GC-MS using the reported conditions. Due to the increased Mass-to-Charge Ratio of ¹⁴C labeled BW 9054-M7, the treated samples were analyzed at an m/z of 228 while the control and fortified samples were quantitated at the m/z of 226. The recoveries for the procedural spikes (cold BAS 125 W) were 85 % ± 20 (see Table 8 for details). The residue of ¹⁴C labeled BAS 125 W in goat kidney was calculated to be 0.132 ppm (4.5% TRR). An additional quantitation of the sample was also done by the HPLC/ β-rm.

After 28 months of storage, the goat kidney had been re-extracted to check for degradation of the test by Battelle lab substance. BAS 125 W was found to account for 7 % of the TRR. A similar extraction had been done at BASF. This extraction accounted for 10 % of the TRR as BAS 125 W by radiometric detection using HPLC and a β-rm detector.

3.4.4 Determination of residues for BW 125-5376 (Master Sheet # 96135-25, -27)

Note: Master Sheet # 96135-25 and -27 were used for TRR calculation and ppm determination.

a) Rotoevaporation and acid hydrolysis

Aliquots were taken from the sample extracts of the goat kidney and rotoevaporated to less than 10 mL. This minimized the concentration of organic solvent in the samples. Then 100 μ L of 6N HCl was added to each sample and then they were heated at 60° C for 30 minutes. After hydrolysis, the sample were allowed to cool to room temperature before they were applied onto a SAX mini-column. LSC reading showed no losses during these steps (68 % TRR).

b) SAX mini-column

The samples were applied to 1.0 g SAX mini-columns that had been prepared with methanol and pH=2 water prior to sample loading. The samples were loaded at a flow rate of less than 6 mL/min. The samples were then eluted with 20 mL of pH=2 water. All of the radioactivity added to the column was accounted for in the load/ elute. The load/ elute contained 69 % of the TRR.

c) Carbon black mini-column

The samples were applied to 1.0 g carbon black mini-columns that had been prepared with 5 mL of 0.1 N NaOH, 5 mL of methanol, followed by 10 mL of water. The samples were loaded with no vacuum before drying under a strong vacuum. The samples were then eluted with 20 mL of 0.1 N NaOH. The elute contained 41 % of the TRR.

d) ENV+™ mini-column

The samples were applied to 500 mg ENV+™ mini-columns that had been washed with 5 mL of acetone, 5 mL of methanol, 20 mL of 0.04 % 1,3-cyclohexanedione, followed by 10 mL of water. The samples were then washed with 35 mL of water before drying the columns under vacuum. The samples were then eluted with 10 mL of acetone. The elute contained 25 % of the TRR. This compares favorably with the 21 % TRR found in the Metabolism Study (Betteile Study no. N002583A). The samples were then rotoevaporated to near dryness before drying completely under a stream of nitrogen. At this stage the samples are redissolved in mobile phase and injected on a HPLC.

e) HPLC Injection

The samples were injected on a HPLC/ UV using the reported conditions. The residue of ¹⁴C labeled BW 125-5376 in goat kidney was 0.22 \pm 0.03 ppm (n= 4), compared to the 0.643 ppm concentration reported in the metabolism study. The discrepancy is probably due to the 28 month storage interval between the metabolism work and the accountability work. There had not been enough sample remaining to reanalyse for BAS 125 W and for BW 125-5376 degradation, so reanalysis for degradation was done only for parent (BAS 125 W). The recoveries for the procedural spikes (cold BW 125-5376) were 119 \pm 3 % (see Table 8 for details).

4. RESULTS AND DISCUSSION

4.1 Accuracy and Precision of Validation Results

Subsamples of control liver, kidney, fat, muscle, milk, and apple commodities were fortified at levels of 0.05 ppm and 0.5 ppm with BAS 125 W and BW 125-5376 and were analyzed by Method D9608.

The mean recoveries for BAS 125 W were: liver: $80 \pm 6\%$ (n=8), kidney: $87 \pm 9\%$ (n=8), fat: $80 \pm 7\%$ (n=8), muscle: $79 \pm 3\%$ (n=8), milk: $89 \pm 5\%$ (n=8), wet pomace: $78 \pm 7\%$ (n=8), and apple juice: $77 \pm 6\%$ (n=8). The average recoveries of BAS 125 W in all matrices at all levels were $81 \pm 8\%$ (n=56). A summary of the BAS 125 W recovery data is given in Table 1. Individual recovery data for BAS 125 W is shown in Tables 3 and 4, and standard responses are shown in Table 6. Example chromatograms are shown in Appendix A.

The mean recoveries for BW 125-5376 were for liver: $112 \pm 16\%$ (n=7) and kidney: $110 \pm 14\%$ (n=8). The average recoveries of BW 125-5376 in all matrices and at all levels were $110 \pm 13\%$ (n=15). A summary of the BW 125-5376 recovery data is given in Table 2. Individual recovery data for BW 125-5376 is shown in Table 5, and standard responses are shown in Table 7. Example chromatograms are shown in Appendix A.

4.2 Quantitation Limit

The quantitation limit for BAS 125 W residues in animal tissues, and apple commodities using Method D9608 is 0.05 ppm. The quantitation limit for BAS 125 W residues in milk is 0.01 ppm. At these levels, control samples are relatively clean and good recoveries are obtainable. These are the lowest levels which are supported by recovery data. The limit of detection (LOD) for BAS 125 W in animal tissues and in apple commodities is 0.02 ppm, based on the lowest standard to which response could be detected. The limit of detection (LOD) for BAS 125 W in milk is 0.004 ppm.

The quantitation limit for BW 125-5376 residues in liver and kidney using Method D9608 is 0.05 ppm. At this level, control samples are relatively clean and good recoveries are obtainable. This is the lowest level which is supported by recovery data. The limit of detection (LOD) for BW 125-5376 in liver and kidney is 0.02 ppm, based on the lowest standard to which response could be detected.

4.3 Ruggedness Testing

Five analysts executed 14 analysis sets of Animal Tissues (Liver, Kidney, Fat, and Muscle), Milk and Apple Commodities for BAS 125 W residues. The analysts executed 4 analysis sets of liver and kidney for BW 125-5376 residues. At least two sample sets were run for each matrix. Each matrix other than milk contained a control and duplicate analyses of control fortified with test substance at 0.05 and 1.0 ppm levels. Milk was fortified at the 0.01 ppm and the 1.0 ppm levels of BAS 125 W. The results of these analyses are reported in Section 4.1. An independent lab validation (Reference 2) of this method has been done successfully in the first trial for both kidney and milk.

4.4 Limitations

None known to date.

5. SAFETY AND HEALTH CONSIDERATIONS

5.1 General

Use personal protective equipment such as lab coats, safety glasses and gloves (nitrile/latex gloves are recommended) when performing the operations described in this method. Conduct all filtrations, nitrogen-stream evaporations and SPE procedures in a well-ventilated hood. Guard vacuum equipment, such as rotovaps, to minimize the possibility of injury caused by flying broken glass. Dispose of hazardous wastes in an environmentally acceptable manner, in compliance with applicable laws and regulations.

5.2 Solvents and Reagents

Review the Material Safety Data Sheets (MSDSs) for all solvents and reagents used in this method.

6. CONCLUSIONS

This study has shown that Analytical Method Number D9608 is suitable for measuring residues of BAS 125 W (prohexadione) in animal tissues and in apple commodities down to 0.05 ppm. The method measures residues of BAS 125 W (prohexadione) in milk down to 0.01 ppm. The average recoveries in all matrices were $81 \pm 8\%$ ($n = 56$). The analytical method number D9608 for BAS 125 W is a GC/MS method and is highly specific for the determination of prohexadione (BAS 125 W).

Analytical Method Number D9608 is suitable for measuring residues of BW 125-5376 (despropanoyl) in liver and kidney down to 0.05 ppm. The average recoveries in these matrices were $111 \pm 15\%$ ($n = 15$). The analytical method number D9608 for BW 125-5376 is a HPLC method and is specific for the determination of despropanoyl (BW 125-5376).

Statistical treatment of the validation data included determination of an average and standard deviation. Good recoveries were obtained for BAS 125 W for the fortified matrices at the 0.05 and 1.0 ppm levels in animal tissues and in apple commodities. Good recoveries were obtained for the fortified milk matrix at the 0.01 and the 1.0 ppm levels. Generally, good recoveries for BW 125-5376 were obtained for the fortified matrices at the 0.05 and 1.0 ppm levels in kidney and liver.

The raw data and final method pertaining to this study are maintained in the BASF Corporation Agricultural Products Center Archives.

7. REFERENCES

1. Abdel-Baky, S., Baumann, S. "Method for Determination of BAS 125 W (Prohexadione) Residues in Peanut RAC (Nutmeat and Hay), and Peanut Process Fractions (Meal and Refined Oil) by GC-MS." BASF Method No. D9601. BASF Registration Document Number 96/5223. November 1996.
 2. Fomenko, John. Independent Laboratory Validation of BASF Analytical Method D9608 for Prohexadione Carboxylic Acid (BAS 125 W) and its Metabolite in Animal Tissues, Milk, and Apple Commodities. BASF Registration Document Number 97/5300. August 1997.
 3. Steginsky, C., Chang, J., Watts, J. "Metabolism of ¹⁴C-BAS 9054 W (Prohexadione-Calcium) in Lactating Goat." BASF Registration Document Number 97/5330. September 1997.
-

8. SIGNATURES

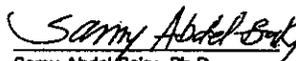
We, the undersigned, hereby declare that this study was performed under our supervision according to the procedures described herein, and that this report provides a true and accurate record of the results obtained.

Author/Analytical
Project Director:


Stephan Baumann
Associate Chemist

Date: 9-30-97

Author/Study
Director:


Samy Abdel-Baky, Ph.D.
Senior Research Associate

Date: 9-30-97

Approved By:


Laura Sears
Technical Center Leader

Date: 9-30-97

This report was amended in order to make corrections to Table 9.

Amended By:


Samy Abdel-Baky, Ph.D.
Study Director

Date: 10-13-97

Approved By:


Laura Sears
Sponsor

Date: 10/13/97

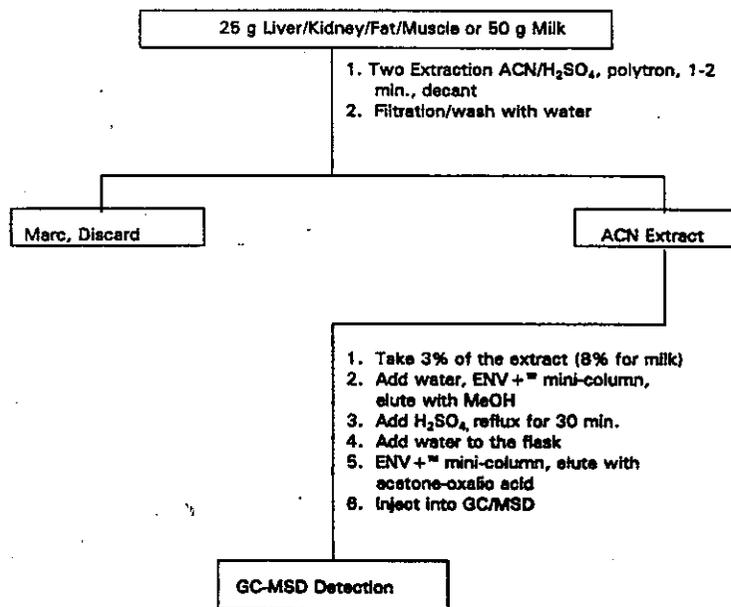


Figure 1: Flow Chart for BAS 125 W Parent Method for Kidney, Liver, Fat, Muscle and Milk.

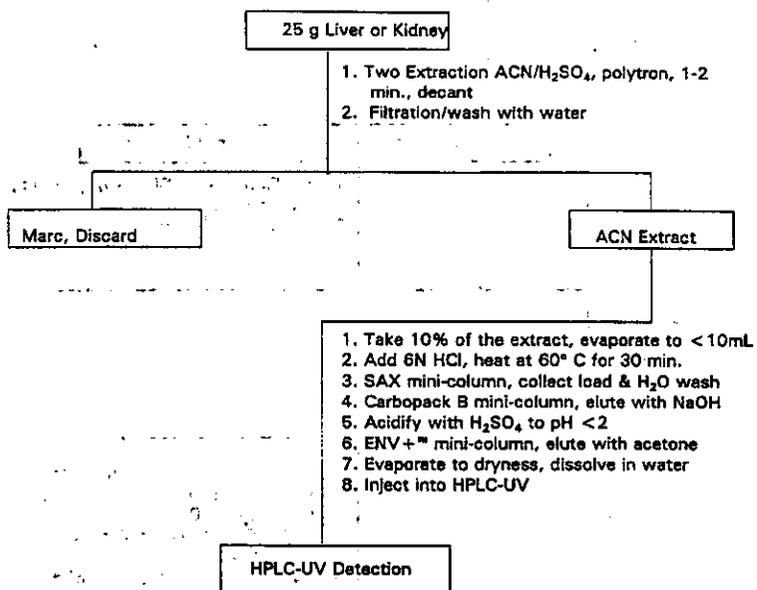


Figure 2: Flow Chart for BW 125-5376 Metabolite Method for Kidney and Liver.

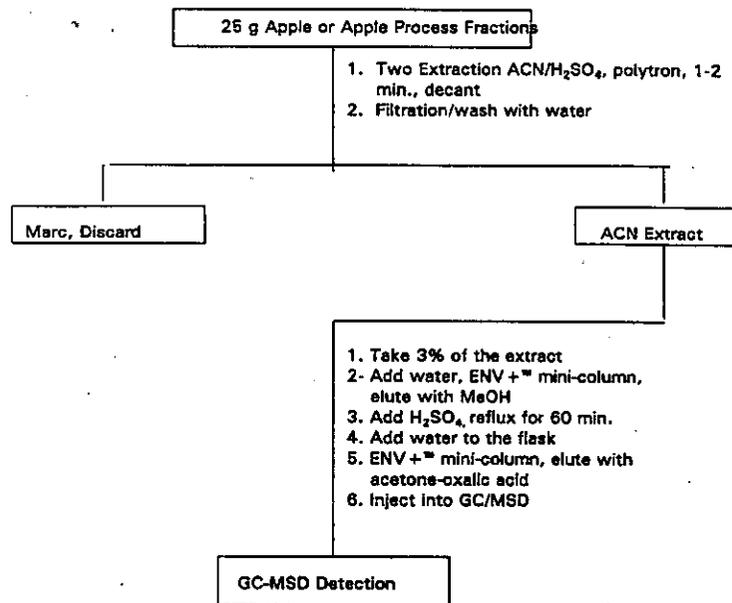


Figure 3: Flow Chart for BAS 125 W Parent Method for Apple and its Process Fractions.

Figure 4. Sample calculations for control kidney fortified with 1.0 ppm of BAS 125 W.
Sample number 96184-3122 (vial number 9) from master sheet 96135-11.

BW 125-M7 (pg) interpolated from standard curve:

$$\text{Standard curve: pg (BW 125-M7)} = \frac{\text{Peak area or height} - \text{Intercept}}{\text{Slope}}$$

$$\begin{aligned} \text{Peak Area :} &= 723788 \\ \text{Slope:} &= 2.33 \times 10^4 \\ \text{Intercept:} &= 4.89 \times 10^4 \end{aligned}$$

$$\text{pg (BW 125-M7)} = \frac{723788 - 48900}{23300} = 28.97 \text{ pg} = 0.02897 \text{ ng (BW 125-M7)}$$

$$\text{Residue (ppm)} = \frac{\text{BW 125-M7 from Curve (ng)} \times \text{Final Dilution Vol (mL)} \times 100}{\text{Sample Weight (g)} \times \text{Injection Vol (\mu L)} \times \text{Aliquot}} \times \text{MWCF}$$

$$\text{MWCF} = 0.938 \text{ from BW 125-M7 to BAS 125 W}$$

$$\text{Recovery \%} = \frac{\text{Residue in Fort Sample (ppm)} - \text{Residue in Control (ppm)}}{\text{Amount Fortified (ppm)}}$$

$$\begin{aligned} \text{BW 125-M7 from Curve} &= 0.02897 \text{ ng} \\ \text{Sample Weight} &= 25 \text{ g} \\ \text{Final Dilution Volume} &= 100 \text{ mL} \\ \text{Injection Volume} &= 4 \text{ \mu L} \\ \text{Aliquot (\%)} &= 3\% \\ \text{Amount Fortified} &= 1.0 \\ \text{Residue in Control} &= 0.00 \end{aligned}$$

$$\text{BW 125-M7 Residue (ppm)} = \frac{0.02897 \text{ ng} \times 100 \text{ mL} \times 100}{25.0 \text{ g} \times 4 \text{ \mu L} \times 3} \times 0.938 = 0.906 \text{ ppm}$$

$$\text{Recovery \%} = \frac{0.906 \text{ ppm} - 0.00}{1.0 \text{ ppm}} \times 100 = 90.6\%$$

Use full computer/calculator precision in any intermediate calculations. Round only the final value.

Figure 5. Sample calculations for control kidney fortified with 0.05 ppm of BW 125-5376.
Sample number 96184-3122 (vial number 4) from master sheet 96135-09.

BW 125-5376 (ng) interpolated from standard curve:

$$\text{Standard curve: ng (BW 125-5376)} = \frac{\text{Peak Height} - \text{Intercept}}{\text{Slope}}$$

$$\begin{aligned} \text{Peak Height :} &= 1026 \\ \text{Slope:} &= 1.17032 \times 10^2 \\ \text{Intercept:} &= -9.82348 \end{aligned}$$

$$\text{ng (BW 125-5376)} = \frac{1026 + 9.82348}{1.17032 \times 10^2} = 8.851 \text{ ng (BW 125-5376)}$$

$$\text{Residue (ppm)} = \frac{\text{BW 125-5376 from Curve (ng)} \times \text{Final Dilution Vol (mL)} \times 100}{\text{Sample Weight (g)} \times \text{Injection Vol (mL)} \times \text{Aliquot} \times 1000}$$

$$\text{Recovery \%} = \frac{\text{Residue in Fort Sample (ppb)} - \text{Residue in Control (ppb)}}{\text{Amount Fortified (ppb)}}$$

$$\begin{aligned} \text{BW 125-5376 from Curve} &= 8.851 \text{ ng} \\ \text{Sample Weight} &= 25 \text{ g} \\ \text{Final Dilution Volume} &= 10 \text{ mL} \\ \text{Injection Volume} &= 500 \mu\text{L} \\ \text{Aliquot (\%)} &= 10\% \\ \text{Amount Fortified} &= 0.05 \\ \text{Residue in Control} &= 0.00624 \end{aligned}$$

$$\text{BW 125-5376 Residue (ppm)} = \frac{8.851 \text{ ng} \times 10 \text{ mL} \times 100}{25.0 \text{ g} \times 0.5 \text{ mL} \times 10 \times 1,000} = 0.070806 \text{ ppm}$$

$$\text{Recovery \%} = \frac{0.070806 \text{ ppm} - 0.0062413 \text{ ppm} \times 100}{0.05 \text{ ppm}} = 129\%$$

Use full computer/calculator precision in any intermediate calculations. Round only the final value.

Table 1. Summary of Recovery Data of Fortified Parent Samples Using GC-MS

Matrix	Fortification Level (ppm)	Individual Recovery (%)	Average \pm SD (n=4)	Average \pm SD/Matrix (n=4)
Fat	0.05	76,88,87,78	78 \pm 7	80 \pm 7
	1.0	77,81,93,80	83 \pm 7	
Muscle	0.05	78,91,77,80	82 \pm 6	76 \pm 3
	1.0	72,73,79,78	76 \pm 4	
Kidney	0.05	77,88,76,92	83 \pm 8	87 \pm 9
	1.0	90,105,85,85	91 \pm 9	
Liver	0.05	75,78,72,80	76 \pm 4	80 \pm 6
	1.0	88,89,82,77	84 \pm 6	
Milk	0.01	84,91,89,95	80 \pm 4	89 \pm 5
	1.0	93,83,95,83	88 \pm 6	
Wet Pomace	0.05	72,70,78,80	75 \pm 6	78 \pm 7
	1.0	79,71,89,86	81 \pm 8	
Apple Juice	0.05	72,76,76,72	74 \pm 2	77 \pm 6
	1.0	71,82,80,87	80 \pm 7	
Overall Recovery (n=56)			81 \pm 8	

Table 2. Summary of Recovery Data of Fortified Despropionyl Samples Using HPLC

Matrix	Fortification Level ppm	Individual Recovery (%)	Average \pm SD (n=4)	Average \pm SD/ Matrix (n=4)
Kidney	0.05	129, 123, 91, 100	111 \pm 18	110 \pm 14
	1.0	120, 122, 100, 100	111 \pm 12	
Liver	0.05	124, 125, 102	117 \pm 13	112 \pm 16
	1.0	123, 125, 84, 100	108 \pm 20	
Overall Recovery (n=15)			110 \pm 15	

Table 3. Individual Recovery Data of the Fortified BAS 125 W in Animal matrices using GC-MS.

Fortified Level ppm, Matrix (Vial Number) ¹	Master Sheet Number 96135-2	Extract. Date	Injection Date	Final Volume (mL) ³	Peak Area (Count X10 ⁴) ⁴	Residua. ppm ⁵	Recovery %
Control, Fat, (6)	03	12-3-96	12-3-96	10	ND	<0.05	-
0.05, Fat (7)	03			10	7.08	0.038	76
0.05, Fat (8)	03			10	6.38	0.035	69
1.0, Fat(9)	03			100	14.78	0.772	77
1.0, Fat(10)	03			100	15.52	0.809	81
Control Muscle (16)	05	12-3-96	12-3-96	10	1.64	0.006	-
0.05, Muscle (17)	05			10	9.72	0.039	78%
0.05, Muscle (18)	05			10	10.98	0.045	91%
1.0, Muscle (19)	05			100	15.25	0.716	72%
1.0, Muscle(20)	05			100	15.53	0.730	73%
Control Kidney (6)	11	12-15-96	12-15-96	10	ND	<0.06	-
0.05, Kidney (7)	11			10	33.64	0.038	77%
0.05, Kidney (8)	11			10	37.64	0.043	88%
1.0, Kidney (9)	11			100	72.37	0.903	90%
1.0, Kidney (10)	11			100	83.10	1.048	105%
Control Liver(11)	12	12-15-96	12-15-96	10	ND	<0.05	-
0.05, Liver(12)	12			10	33.18	0.037	75%
0.05, Liver (13)	12			10	34.35	0.039	78%
1.0, Liver (14)	12			100	71.42	0.876	88%
1.0, Liver (15)	12			100	72.70	0.893	89%
Control, Kidney (6)	13	12-16-96	12-16-96	10	ND	<0.05	-
0.05, Kidney (7)	13			10	7.16	0.038	76%
0.05, Kidney (8)	13			10	8.54	0.046	92%
1.0, Kidney (9)	13			100	15.94	0.847	85%
1.0, Kidney(10)	13			100	16.05	0.853	85%
Control, Liver, (6)	14	12-16-96	12-16-96	10	ND	<0.05	-
0.05, Liver (7)	14			10	30.88	0.038	72%
0.05 Liver (8)	14			10	33.59	0.040	80%
1.0, Liver (9)	14			100	64.20	0.820	82%
1.0, Liver (10)	14			100	60.42	0.768	77%
Control, Fat, (6)	17	12-17-96	12-17-96	10	ND	<0.05	-
0.05, Fat (7)	17			10	8.85	0.043	87%
0.05, Fat (8)	17			10	8.00	0.039	78%
1.0, Fat (9)	17			100	18.87	0.827	93%
1.0, Fat(10)	17			100	16.30	0.800	80%
Control, Muscle, (6)	18	12-17-96	12-17-96	10	ND	<0.05	-
0.05, Muscle(7)	18			10	31.63	0.038	77%
0.05, Muscle (8)	18			10	32.79	0.040	80%
1.0, Muscle (9)	18			100	60.39	0.788	79%
1.0, Muscle (10)	18			100	59.95	0.782	78%

Continuation
of Table 3. Individual Recovery Data of the Fortified BAS 125 W in Animal matrices using GC-MS.

Fortified Level ppm, Matrix (Vial Number) ¹	Master Sheet Number 96135- ²	Extract. Date	Injection Date	Final Volume (mL) ³	Peak Area (Count X10 ⁴) ⁴	Residue ppm ⁵	Recovery %
Control, Milk, (8)	21	12-18-96	12-18-96	10	ND	<0.01	--
0.01, Milk(7)	21			10	35.10	0.008	84%
0.01, Milk (8)	21			10	38.10	0.009	91%
1.0, Milk(9)	21			100	74.37	0.928	93%
1.0, Milk(10)	21			100	66.98	0.830	83%
Control, Milk, (8)	22	12-18-96	12-18-96	10	ND	<0.01	--
0.01, Milk(7)	22			10	6.38	0.009	89%
0.01, Milk (8)	22			10	6.88	0.009	95%
1.0, Milk(9)	22			100	13.82	0.948	95%
1.0, Milk(10)	22			100	11.91	0.828	83%

FOOTNOTES

¹Vial numbers were assigned to distinguish between separate analyses of the same RCN, but different sample number, within the sample set.

²Master sheet number identifies specific analysis sets and consists of Phase number (A or B), BASF study (96135) followed by a sequential analysis set number

³Final dilution volume.

⁴Peak area from GC-MS. Values in parentheses are below the limit of Detection (LOD) of 0.02 ppm. The LOD for milk is 0.004 ppm. If no signal was detected "ND" is listed in the table.

⁵See Figure 4 for an example calculation of the residue. The value of BAS 125 W is as parent equivalent.

The following values were constant for all analyses:

- a) Sample size = 25.0 g for kidney, liver, muscle, fat
= 50.0 g for milk
- b) Injection volume = 4µL
- c) Aliquot = 3% for kidney, liver, muscle, fat
= 8% for milk

Table 4. Individual Recovery Data of the Fortified BAS 125 W in Apple matrices using GC-MS

Fortified Level ppm, Matrix (Vial Number) ¹	Master Sheet Number 96135 ²	Extract. Date	Injection Date	Final Volume (mL) ³	Peak Area (Count X10 ⁴) ⁴	Residue ppm ⁵	Recovery %
Control, Wet Pomace, (6)	01	12-3-96	12-3-96	10	ND	<0.05	--
0.05, Wet Pomace (7)	01			10	24.93	0.036	72%
0.05, Wet Pomace (8)	01			10	24.31	0.035	70%
1.0, Wet Pomace (9)	01			100	50.57	0.786	79%
1.0, Wet Pomace (10)	01			100	46.17	0.714	71%
Control Apple Juice (6)	02	12-4-96	12-4-96	10	ND	<0.05	--
0.05, Apple Juice (7)	02			10	7.26	0.036	72%
0.05, Apple Juice (8)	02			10	7.79	0.038	76%
1.0, Apple Juice (9)	02			100	14.46	0.711	71%
1.0, Apple Juice (10)	02			100	16.66	0.818	82%
Control Wet Pomace (11)	19	12-18-96	12-18-96	10	ND	<0.05	--
0.05, Wet Pomace (12)	19			10	5.95	0.039	78%
0.05, Wet Pomace (13)	19			10	6.11	0.040	80%
1.0, Wet Pomace (14)	19			100	13.78	0.867	89%
1.0, Wet Pomace, (15)	19			100	13.34	0.859	86%
Control Apple Juice (11)	20	12-18-96	12-18-96	10	ND	<0.05	--
0.05, Apple Juice (12)	20			10	29.60	0.038	76%
0.05, Apple Juice (13)	20			10	28.51	0.036	72%
1.0, Apple Juice (14)	20			100	56.52	0.804	80%
1.0, Apple Juice (15)	20			100	60.51	0.866	87%

FOOTNOTES

¹Vial numbers were assigned to distinguish between separate analyses of the same RCN, but different sample number, within the sample set.

²Master sheet number identifies specific analysis sets and consists of BASF study (96135) followed by a sequential analysis set number

³Final dilution volume.

⁴Peak area from GC-MS. Values in parentheses are below the limit of Detection (LOD) of 0.025 ppm. If no signal was detected "ND" is listed in the table.

⁵The value of BAS 125 W is as parent equivalent.

The following values were constant for all analyses:

a) Sample size = 25.0 g for apple juice, wet pomace

b) Injection volume = 4µL

c) Aliquot = 3% for apple juice, wet pomace

Table 5. Individual Recovery Data of the Fortified BW 125-5376 in Animal matrices by HPLC

Fortified Level ppm, Matrix (Vial Number) ¹	Master Sheet Number 96135- ²	Extract. Date	Injection Date	Final Volume (mL) ³	Peak Height ⁴	Residue ppm ⁵	Recovery %
Control, Kidney(2)	09	12-15-96	12-15-96	10	(81)	<0.05	--
0.05, Kidney(4)	09			10	1026	0.0846	129%
0.05, Kidney (6)	09			10	981	0.0615	123%
1.0, Kidney (8)	09			100	1760	1.204	120%
1.0, Kidney (10)	09			100	1783	1.219	122%
Control, Liver (2)	10	12-15-96	12-15-96	10	ND	<0.05	--
0.05, Liver (4)	10			10	891	0.062	124%
0.05, Liver (8)	10			10	901	0.063	125%
1.0, Liver (8)	10			100	1770	1.231	123%
1.0, Liver(10)	10			100	1799	1.252	125%
Control, Kidney (3)	15	12-16-96	12-16-96	10	ND	<0.05	--
0.05, Kidney (5)	15			10	667	0.0453	91%
0.05, Kidney (7)	15			10	737	0.0502	100%
1.0, Kidney (9)	15			100	1480	1.0043	100%
1.0, Kidney (11)	15			100	1458	1.0029	100%
Control, Liver (3)	16	12-16-96	12-16-96	10	ND	<0.05	--
0.05, Liver (5)	16			10	lost sample	--	--
0.05, Liver (7)	16			10	711	0.0512	102%
1.0, Liver (9)	16			100	1157	0.8362	84%
1.0, Liver (11)	16			100	1384	1.0005	100%

FOOTNOTES

¹Vial numbers were assigned to distinguish between separate analyses of the same RCN, but different sample number, within the sample set.

²Master sheet number identifies specific analysis sets and consists of BASF study (96135) followed by a sequential analysis set number

³Final dilution volume.

⁴Peak height from HPLC. Values in parentheses are below the limit of Detection (LOD) of 0.025 ppm. If no signal was detected "ND" is listed in the table.

⁵See Figure 5 for an example calculation of the residue.

The following values were constant for all analyses:

- a) Sample size = 25.0 g for Kidney, Liver
- b) Injection volume = 500 µL
- c) Aliquot = 10% for Kidney, Liver

Table 6. Summary of the Standard Data for BW 125-M7 in Animal Tissues, Milk, and Apple Commodities by GC-MS.

Master Sheet No. 96135-	Peak Area (Count x 10 ⁴)/Injection				Calibration Curve Data ²	
	7.5 pg	15 pg	30 pg	60 pg	Slope x 10 ⁴	Intercept x 10 ⁴
1	14.89	30.10	59.82	122.31	1.90	2.80
	17.63	33.45	61.08	110.54		
2	4.99	9.60	18.72	37.90	0.641	-0.106
	4.48	9.21	19.31	38.91		
3	4.48	8.71	17.58	36.09	0.617	-0.453
	4.44	8.59	18.01	37.31		
5	4.98	10.42	18.88	39.33	0.644	0.358
	5.55	9.90	19.82	38.96		
11	20.10	38.90	74.32	139.38	2.33	4.89
	21.90	41.78	79.11	148.85		
12	21.35	39.72	79.31	148.85	2.38	4.73
				144.87		
13	4.06	8.60	17.29	34.62	0.591	-0.081
	4.38	9.25	18.15	36.11		
14	19.56	39.64	74.56	134.75	2.26	-4.80
	21.31	38.38	74.86	144.78		
17	4.73	9.48	19.02	37.38	0.633	0.0815
	4.69	9.80	19.40	38.67		
18	19.09	38.05	70.80	136.04	2.23	4.06
	20.24	39.01	72.54	139.38		
19	3.60	7.16	14.23	28.96	0.492	-0.187
	3.30	7.62	14.72	29.82		
20	20.97	35.11	64.40	126.38	1.99	5.44
	23.38	31.87	64.70	124.03		
21	18.28	38.93	73.50	141.08	2.26	2.86
	19.10	34.81	71.94	134.68		
22	2.34	5.77	13.10	28.86	0.422	-0.0140
	2.92	6.93	13.93	23.03		

¹Master sheet numbers consist of the BASF study number (96135) followed by a sequential analysis set number. Injection dates for each Master Sheet are shown in Table 3 and 4.

²The standard curves were constructed using the following equation:

$$(\text{pg}) \text{ BW 125-M7} = \frac{\text{Peak area or height} - \text{Intercept}}{\text{Slope}}$$

Table 7. Summary of the Standard Data for BW 125-5376 In Animal Tissues by HPLC.

Master ¹ Sheet No. 98135-	Peak Height/ Injection				Calibration Curve Data ²	
	2.5 ng	5 ng	10 ng	25 ng	Slope x 10 ²	Intercept
9	295	587	1102	2919	1.170	-9.823
	301	574	1161	2932		
10	301	583	1146	2892	1.147	4.334
	288	582	1136	2855		
15	307	603	1177	2949	1.151	15.27
	305	585	1139	2842		
16	285	565	1095	2762	1.103	4.824
	278	566	1091	2768		

¹Master sheet numbers consist of the BASF study number (98135) followed by a sequential analysis set number. Injection dates for each Master Sheet are shown in Table 5.

²The standard curves were constructed using the following equation:

$$(\text{ng}) \text{ BW 125-5376} = \frac{\text{Peak Height} - \text{Intercept}}{\text{Slope}}$$

Table 8. A Summary of the Individual Recovery Measurements from Accountability sets.

Compound	Master Sheet Number	Fortification Level (ppm)	Percent Recovery	
BAS 125 W	96135-24	0.2	69	85 ± 20 %
		3.0	69	
BAS 125 W	96135-26	0.05	116	
		1.0	92	
BAS 125 W	96135-28	0.2	81	
BW 125-5376	96135-25	0.2	115	119 ± 3 %
		1.0	121	
BW 125-5376	96135-27	0.1	118	
		1.0	120	

Table 8: Radioactivity Distribution in BASF Accountability and in Metabolism Study for BAS 125 W (parent acid) from Kidney Master Sheet# 86135-24.

Step	Accountability Results ¹		Step	Metabolism Results ² (2 months storage)		Metabolism Results (28 months storage)	
	ppm	% TRR		ppm	% TRR	ppm	% TRR
Start Material	2.83	100	Start Material	3.09	100	2.83	100
Extraction	1.95	69	Extraction	2.74	88.7	1.79	63
Macro	1.08	38	Post Extraction Solid	0.681	18.8	1.17	41
ENV +™ mini-column	0.54	19	CH ₂ Cl ₂ Partition: Aqueous Fraction	1.45	46.9	1.13	40
Methylation	0.54	19	CH ₂ Cl ₂ Fraction	1.30	41.9	0.48	17
ENV +™ mini-column	0.40	14					
HPLC Quantitation	0.28	10	HPLC Quantitation	1.16	37.4	0.2	7
GC/MS Quantitation	0.132	4.5					

¹ Average of duplicate samples.

² Values from Reference 2.

Table 10: Radioactivity Distribution in Accountability and in Metabolism Study for BW 125-5376 (despropanoyl metabolite) in Kidney from Master Sheet# 96135-25,-27

Step	Accountability Results ¹		Step	Metabolism Results ²				
	ppm	%TRR		ppm	%TRR			
Start Material	2.83	100	Start Material	3.09	100			
Extraction	1.95	69	Extraction	2.74	88.7			
Marc	1.08	38	Post Extraction Solid	0.58	18.9			
Rotoevap /Hydrolysis	1.92	68	CH ₂ Cl ₂ Partition	1.3	41.9			
SAX	1.95	69				Aqueous	1.45	46.9
Carbon Black	1.16	41						
ENV+™	0.71	25						
Evaporation	0.68	24	HPLC Quantitation ⁴	0.64	21			
Pre HPLC (LSC)	0.68	24						
HPLC Quantitation ³	0.21	7						

¹Average of duplicate samples.

²Values from Reference 2.

³Quantitation of the despropanoyl acid (BW 125-5376) was done by HPLC in the accountability phase after 28 months storage.

⁴Quantitation of the despropanoyl acid (BW 125-5376) was done by HPLC for the metabolism study after 12 months storage.

Table 11. Individual Radioactivity Measurement in Accountability Phase for Kidney, Master Sheet number: A96135-24

Method Steps ¹	Sample Number ²	Sample ID Number ²	Total Amount	Alliq ³	dpm in Aliq	Sample wt (g) /Step	Total dpm in Sample ⁴
Extraction	1153-4-11	1153-6-8	500 mL	1 mL	1123.4	10	561,710
	1153-4-11	1153-6-19	500 mL	1 mL	1142.2	10	571,095
Marc	1153-7-5	1153-7-14	18.36g	0.2556 g	4057.0	10	300,453
		1153-7-15		0.2699 g	4474.5		
		1153-7-16		0.2633 g	4270.2		
		1153-7-17		0.2986 g	5186.9		
		1153-7-18		0.2642 g	4170.0		
Pre ENV + ¹⁴ C #1	Sample #1	1153-10-9	214 mL	2 mL	154.8	0.3	16,564
	Sample #2	1153-10-20	214 mL	2 mL	160.9	0.3	17,216
ENV + ¹⁴ C #1	Sample #1	1153-10-10	248 mL	2 mL	88.9	0.3	11,024
	Sample #2	1153-10-21	248 mL	2 mL	85.1	0.3	10,552
Load/ wash	Sample #1	1153-10-11	24 mL	1 mL	204.5	0.3	4,808
	Sample #2	1153-10-22	24 mL	1 mL	179.4	0.3	4,306
ENV + ¹⁴ C #1	Sample #1	1153-10-12	47.5 mL	2 mL	206.9	0.3	4,914
	Sample #2	1153-10-23	47.5 mL	2 mL	174.0	0.3	4,133
Methylation	Sample #1	1153-10-13	152 mL	2 mL	8.2	0.3	623
	Sample #2	1153-10-24	152 mL	2 mL	1.9	0.3	144
ENV + ¹⁴ C #2	Sample #1	1153-10-14	10 mL	1 mL	343.4	0.3	3,434
	Sample #2	1153-10-25	10 mL	1 mL	306.2	0.3	3,082

¹Steps involved in the analytical method

²Sample number is indicated by notebook #, followed by page and line numbers (e.g. 1153-4-11).

³Alliquot taken for radioactivity count by LSC

⁴Total radioactivity in the sample can be calculated as in the following examples:

Extraction Step:

Radioactivity	=	1,123.4 dpm
Sample Volume	=	500 mL
Alliquot Volume	=	1 mL
Total dpm	=	$1,123.4 \times 500 \text{ mL} / 1 \text{ mL} = 561,710 \text{ dpm}$

Extraction Marc:

Radioactivity	=	4,270.2 dpm
Sample Weight	=	18.36 g
Alliquot Weight	=	0.2633 g
Radioactivity	=	$4,270.2 \times 18.36 \text{ g} / 0.2633 \text{ g} = 297,763 \text{ dpm}$

Table 13: Results from the Specificity Experiment for BW 125-5376

40 CFR 180	CHEMICAL	Tolerance in Meat (K&L)	PPM of Material
.142	2,4-D	2.0	Not Detected
.169	CARBARYL	1.0	Not Detected
.205	PARAQUAT	0.3	Not Detected
.292	PICLORAM	5.0	Not Detected
.317	PRONAMIDE	0.2	Not Detected
.321	SEC-BUTYLAMINE	3.0	Not Detected
.364	GLYPHOSATE	0.5	Not Detected
.368	METOLACHLOR	0.2	Not Detected
.371	THIOPHANATE METHYL	0.2	Not Detected
.383	ACIFLUORFEN	0.02	Not Detected
.399	IPIRODIONE	3.0	Not Detected
.408	METALAXYL	0.4	Not Detected
.409	PIRIMIPHOSMETHYL	2.0	Not Detected
.413	IMAZALIL	0.5	Not Detected
.417	TRICLOPYR	0.5	Not Detected
.420	FLURIDONE	0.1	Not Detected
.421	FENARIMOL	0.1	Not Detected
.428	METSULFURONMETHYL	0.5	Not Detected
.431	CLOPYRALID	12.0	Not Detected
.434	PROPICONAZOLE	2.0	Not Detected
.443	MYCLOBUTANIL	0.3	Not Detected
.446	CLOFENTEZINE	0.4	Not Detected

Appendix A

Amendments and Additions to Protocol 96135:

1. Addition of new control samples for the method validation with animal tissues. Sections were added for the despropanoyl and the apple methods. The study title was changed to reflect these additions.

Reason: To increase the scope of the study.

2. Addition of new control sample for the method validation of fat tissues. A typographical error was also corrected.

Reason: To increase the scope of the study and to correct a typographical error in the protocol.

3. Addition of new control samples for the method validation of milk.

Reason: To increase the scope of the study.

4. Addition of a specificity test for the despropanoyl metabolite (BW 125-5376).

Reason: No specificity had been done on the metabolite.

5. Addition of an accountability phase.

Reason: No accountability had been done for method D9608.

6. Amendment to the accountability phase so that the ¹⁴C labeled kidney sample could be sent back to Battelle Labs for reanalysis.

Reason: The accountability indicated losses of BAS 125 W. The sample was reanalyzed by Battelle to determine if the sample had degraded during storage or if method D9608 was responsible for the losses.

Appendix B

Description

- Figure 1 Typical HPLC parameters from master sheet number 96135-09.
- Figure 2 Typical chromatogram of a 2.5 ng standard of BW 125-5376 from master sheet number 96135-09. Data from this standard can be found in Table 7.
- Figure 3 Typical chromatogram of a 5.0 ng standard of BW 125-5376 from master sheet number 96135-09. Data from this standard can be found in Table 7.
- Figure 4 Typical chromatogram of a 10 ng standard of BW 125-5376 from master sheet number 96135-09. Data from this standard can be found in Table 7.
- Figure 5 Typical chromatogram of a 25 ng standard of BW 125-5376 from master sheet number 96135-09. Data from this standard can be found in Table 7.
- Figure 6 Typical standard curve for 2.5, 5, 10 and 25 ng amounts of BW 125-5376 from master sheet number 96135-09. Data from these standards can be found in Table 7.
- Figure 7 Typical chromatogram of a control kidney sample. Sample number 96184-3122 (vial number 2) from master sheet number 96135-09. Data for this sample can be found in Table 5.
- Figure 8 Typical chromatogram of a control kidney sample fortified with 0.05 ppm (quantitation limit) of BW 125-5376. Sample number 96184-3122 (vial number 4) from master sheet number 96135-09. Data for this sample can be found in Table 5. Recovery 129%.
- Figure 9 Typical chromatogram of a control kidney sample fortified with 1.0 ppm of BW 125-5376. Sample number 99184-3122 (vial number 10) from master sheet number 96135-09. Data for this sample can be found in Table 5. Recovery 122%.
- Figure 10 Typical chromatogram of a control liver sample. Sample number 96184-3124 (vial number 2) from master sheet number 96135-10. Data for this sample can be found in Table 5.
- Figure 11 Typical chromatogram of a control liver sample fortified with 0.05 ppm (quantitation limit) of BW 125-5376. Sample number 96184-3124 (vial number 4) from master sheet number 96135-10. Data for this sample can be found in Table 5. Recovery 124%.
- Figure 12 Typical chromatogram of a control liver sample fortified with 1.0 ppm of BW 125-5376. Sample number 99184-3124 (vial number 8) from master sheet number 96135-10. Data for this sample can be found in Table 5. Recovery 123%.
-

- Figure 13 Typical GC-MS parameters from master sheet number 96135-11.
- Figure 14 Typical chromatogram of a 7.5 pg standard of BW 125-M7 from master sheet number 96135-11. Data from this standard can be found in Table 6.
- Figure 15 Typical chromatogram of a 15 pg standard of BW 125-M7 from master sheet number 96135-11. Data from this standard can be found in Table 6.
- Figure 16 Typical chromatogram of a 30 pg standard of BW 125-M7 from master sheet number 96135-11. Data from this standard can be found in Table 6.
- Figure 17 Typical chromatogram of a 60 pg standard of BW 125-M7 from master sheet number 96135-11. Data from this standard can be found in Table 6.
- Figure 18 Typical standard Curve for 7.5, 15, 30, and 60 pg amounts of BW 125-M7 from master sheet number 96135-11. Data from these standards can be found in Table 6.
- Figure 19 Typical chromatogram of a control kidney sample. Sample number 96184-3122 (vial number 8) from master sheet number 96135-11. Data for this sample can be found in Table 3.
- Figure 20 Typical chromatogram of a control kidney sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96184-3122 (vial number 8) from master sheet number 96135-11. Data for this sample can be found in Table 3. Recovery 88%.
- Figure 21 Typical chromatogram of a control kidney sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-3122 (vial number 9) from master sheet number 96135-11. Data for this sample can be found in Table 3. Recovery 90%.
- Figure 22 Typical chromatogram of a control liver sample. Sample number 96184-3124 (vial number 11) from master sheet number 96135-12. Data for this sample can be found in Table 3.
- Figure 23 Typical chromatogram of a control liver sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96184-3124 (vial number 13) from master sheet number 96135-12. Data for this sample can be found in Table 3. Recovery 78%.
- Figure 24 Typical chromatogram of a control liver sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-3124 (vial number 15) from master sheet number 96135-12. Data for this sample can be found in Table 3. Recovery 89%.
- Figure 25 Typical chromatogram of a control fat sample. Sample number 96184-5000 (vial number 6) from master sheet number 96135-3. Data for this sample can be found in Table 3.
- Figure 26 Typical chromatogram of a control fat sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96184-5000 (vial number 7) from master sheet number 96135-3. Data for this sample can be found in Table 3. Recovery 76%.
- Figure 27 Typical chromatogram of a control fat sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-5000 (vial number 10) from master sheet number 96135-3. Data for this sample can be found in Table 3. Recovery 81%.
-

- Figure 28 Typical chromatogram of a control muscle sample. Sample number 96184-3116 (vial number 6) from master sheet number 96135-18. Data for this sample can be found in Table 3.
- Figure 29 Typical chromatogram of a control muscle sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96184-3116 (vial number 7) from master sheet number 96135-18. Data for this sample can be found in Table 3. Recovery 77%.
- Figure 30 Typical chromatogram of a control muscle sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-3118 (vial number 10) from master sheet number 96135-18. Data for this sample can be found in Table 3. Recovery 78%.
- Figure 31 Typical chromatogram of a control wet pomace sample. Sample number 96072-13 (vial number 11) from master sheet number 96135-19. Data for this sample can be found in Table 4.
- Figure 32 Typical chromatogram of a control wet pomace sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96072-13 (vial number 13) from master sheet number 96135-19. Data for this sample can be found in Table 4. Recovery 80%.
- Figure 33 Typical chromatogram of a control wet pomace sample fortified with 1.0 ppm of BAS 125 W. Sample number 96072-13 (vial number 15) from master sheet number 96135-19. Data for this sample can be found in Table 4. Recovery 88%.
- Figure 34 Typical chromatogram of a control apple juice sample. Sample number 96072-17 (vial number 11) from master sheet number 96135-20. Data for this sample can be found in Table 4.
- Figure 35 Typical chromatogram of a control apple juice sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96072-17 (vial number 12) from master sheet number 96135-20. Data for this sample can be found in Table 4. Recovery 76%.
- Figure 36 Typical chromatogram of a control apple juice sample fortified with 1.0 ppm of BAS 125 W. Sample number 96072-17 (vial number 15) from master sheet number 96135-20. Data for this sample can be found in Table 4. Recovery 87%.
- Figure 37 Typical chromatogram of a control milk sample. Sample number 96184-504 (vial number 6) from master sheet number 96135-21. Data for this sample can be found in Table 3.
- Figure 38 Typical chromatogram of a control milk sample fortified with 0.01 ppm (quantitation limit) of BAS 125 W. Sample number 96184-504 (vial number 8) from master sheet number 96135-21. Data for this sample can be found in Table 3. Recovery 91%.
- Figure 39 Typical chromatogram of a control milk sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-504 (vial number 9) from master sheet number 96135-21. Data for this sample can be found in Table 3. Recovery 93%.

Figure 1. Typical HPLC parameters from master sheet number 96135-09.

Instrument Parameters HPLC

Date: 4.30.97 Initials: SAB
Study No: 96135 Master Sheet No: 96135.9

HPLC System Information:

Instrument No: 1231

Equipment: HP 1050 HPLC, Varian 2510 HPLC Pump, Rheodyne Column Switching Valve, and a Applied Biosystems 785 A Detector.

Detector Type: UV-Vis Wavelength: 268nm

System Temperature: Ambient

Injection Volume: 500 μ L

Precolumn: 100 x 4.5 mm, Hypersil C18, micron
(Phenomenex OOD-0148-ED)
188414
Serial No:
Precolumn Flow: 1.0 mL/min
Mobile Phase: 99:1 Water/Acetic Acid

Column Cut: 2 min, from 7 to 8 minutes

Analytical Column: 250 x 4.6 mm, ODS-AQ
(YMC: AQ125052546WT)
Serial No: 150919003
Analytical Column Flow: 1.0 mL/min
Mobile Phase: 3:97:1 Acetonitrile/Water/HOAC

Retention time: -18 min

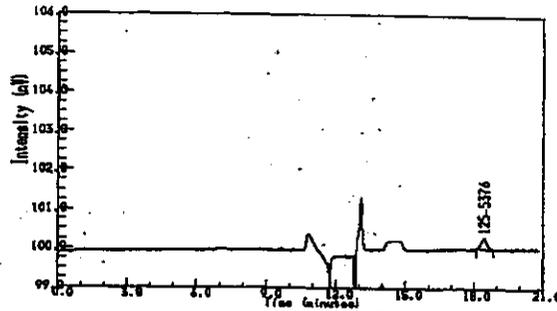
All conditions are isocratic. However, after the column switching window is closed the precolumn is washed with 100% ACN for 4 minutes at flow rate of 2 mL/min. Then the precolumn is flushed with 99:1 water/HOAC for an additional 4 minutes at flow rate of 2 mL/min. The column flow rate changed to 1 mL/min to equilibrate the flow and pressure prior to the next injection. This is done to maintain consistent column performance.

Figure 2. Typical chromatogram of a 2.5 ng standard of BW 125-5376 from master sheet number 96135-09. Data from this standard can be found in Table 7.

[96096] 11 121595,1,1
Reported on 13-JAN-1997 at 14:16

SAC
113-97

Acquired on 15-DEC-1996 at 18:49



BASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Lims Id :
Comment :
Method Title : 125-5376
Sample Name : 2.5 NG/ 5ML STD
Sample Id :
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

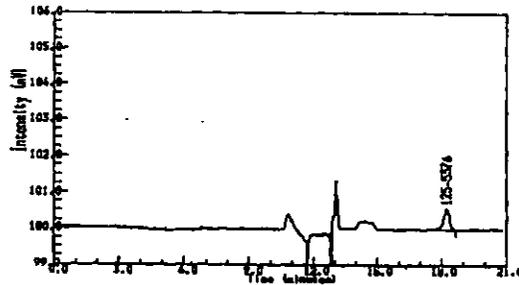
RT min	Height uV	Area uVn	Area %	ppb	Peak name
18.471	295	5206	100.00	2.6005	125-5376
Totals					
Unknowns	0	0	0.00	N/A	
	295	5206	100.00	2.6005	
	295	5206	100.00	2.6005	

Figure 3. Typical chromatogram of a 5.0 ng standard of BW 125-5376 from master sheet number 96135-09. Data from this standard can be found in Table 7.

[96096] 11 121596.3.1
Reported on 13-JAN-1997 at 14:16

516
1-13-97

Acquired on 15-DEC-1996 at 19:40



BASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Liss Id :
Comment :
Method Title : 125-5376
Sample Name : 5 NG/.SPL STD
Sample Id :
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

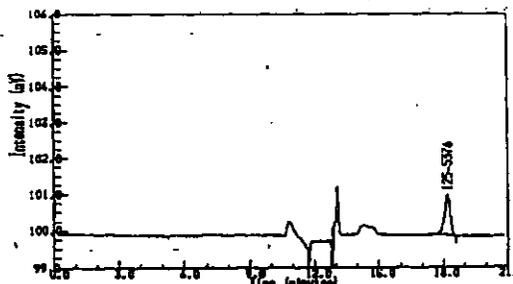
RT min	Height uV	Area uVs	Area %	prob	Peak name
10.128	587	10731	100.00	5.1028	125-5376
Totals					
Unknowns	0	0	0.00		N/A
	587	10731	100.00	5.1028	
	587	10731	100.00	5.1028	

Figure 4. Typical chromatogram of a 10 ng standard of BW 9054-5376 from master sheet number 96135-09. Data from this standard can be found in Table 7.

(96096) 11 121596.5.1
Reported on 13-JAN-1997 at 14:16

SK
11.3.97

Acquired on 15-DEC-1996 at 20:31



BASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Lims Id :
Comment :
Method Title : 125-5376
Sample Name : 10 NG/.SML STD
Sample Id :
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

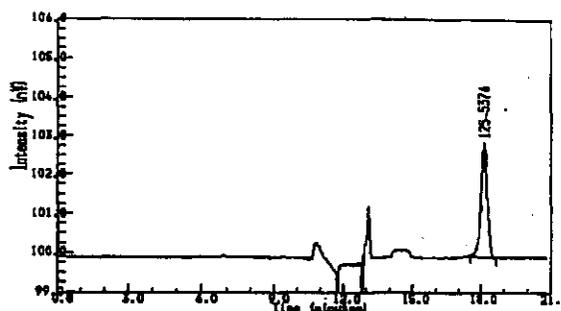
RT min	Height uV	Area uVs	Area %	Wpk	Peak name
18.204	1102	20485	100.00	9.8017	125-5376
Totals					
Unknowns	0	0	0.00	N/A	
	1102	20485	100.00	9.8017	
	1102	20485	100.00	9.8017	

Figure 5. Typical chromatogram of a 25 ng standard of BW 125-5376 from master sheet number 96135-09. Data from this standard can be found in Table 7.

[96096] 11 121596.13.1
Reported on 13-JAN-1997 at 14:17

508
1.1.77

Acquired on 15-DEC-1996 at 23:53



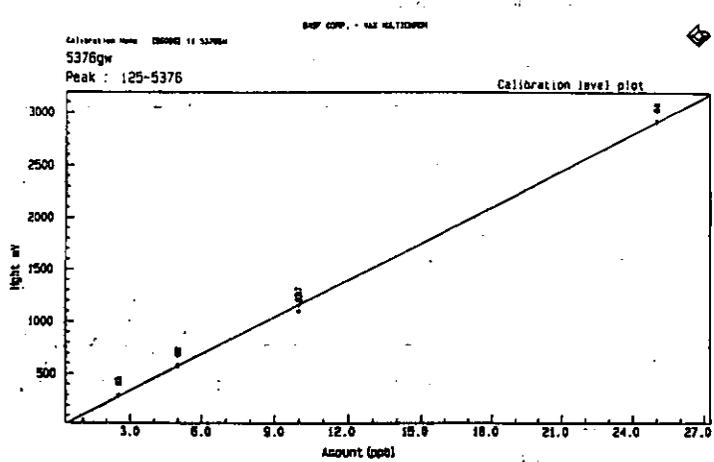
BASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Lims Id :
Comment :
Method Title : 125-5376
Sample Name : 25 NG/.5ML STD
Sample ID :
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

RT min	Height nV	Area nVs	Area %	cpb	Peak name
18.173	2932	54728	100.00	25.1331	125-5376
<u>Totals</u>					
Unknowns	0	0	0.00	N/A	
	2932	54728	100.00	25.1331	
	2932	54728	100.00	25.1331	

Figure 6. Typical standard curve for 2.5, 5, 10, 25 ng amounts of BW 125-5376 from master sheet number 96135-09. Data from these standards can be found in Table 7.



Constant : -9.82348
1st degree : 1.17032E+2

Curve fit : Linear
Correlation coefficient : 0.99975
Standard error : 2.66235E+1
Reported on 13-JAN-1997 at 14:15

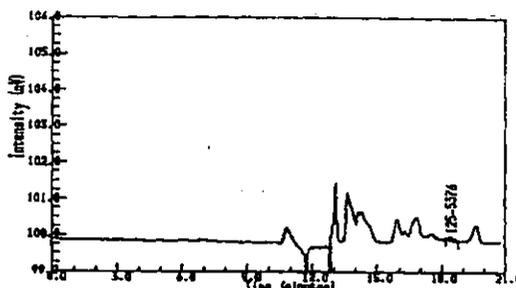
BASF
MCD
E-5376-1
5376

Figure 7. Typical chromatogram of a control kidney sample. Sample number 96184-3122 (vial number 2) from master sheet number 96135-09. Data for this sample can be found in Table 5.

(96096) 11 121596.2.1
Reported on 13-JAN-1997 at 14:16

516
1-16-97

Acquired on 15-DEC-1996 at 19:15



BASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Lims Id :
Comment :
Method Title : 125-5376
Sample Name : SAB Control Kidney (96184-3122)
Sample Id : 96184
Sample Type : Recovery Amount=1.00000
Bottle No : 1

PEAK INFORMATION

RT min	Retrt. uV	Area uVs	Area %	ppb	Peak name
18.440	81	1451	100.00	6.2413	125-5376
Totals					
Unknowns	0	0	0.00	N/A	
	81	1451	100.00	6.2413	
	81	1451	100.00	6.2413	

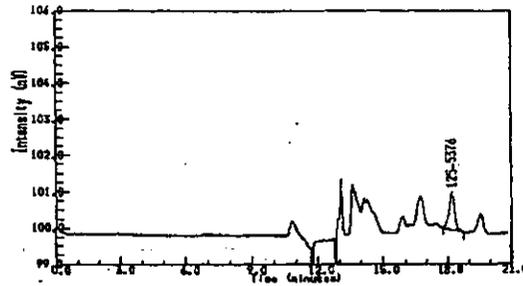
Figure 8.

Typical chromatogram of a control kidney fortified with 0.05 ppm (quantitation limit) of BW 125-5376. Sample number 96184-3122 (vial number 4) from master sheet number 96135-09. Data for this sample can be found in Table 5. Recovery 129%.

[96096] 11 121596.4.1
Reported on 13-JAN-1997 at 14:16

96
1.13.97

Acquired on 15-DEC-1996 at 20:05



BASF CORP. - VAX MULTICROM

Analyst Name : Stephan
Lims Id :
Comment :
Method Title : 125-5376
Sample Name : 0.05 ppm Port #1
Sample Id : 96184
Sample Type : Recovery Amount=1.00000
Bottle No : 1

PEAK INFORMATION

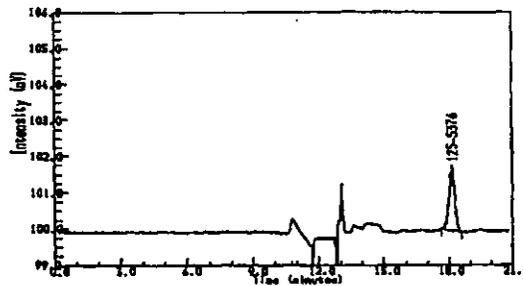
RT min	Hght uV	Area uVs	Area %	prob	Peak name
18.204	1026	18214	100.00	70.8382	125-5376
Totals					
Unknowns	0	0	0.00	N/A	
	1026	18214	100.00	70.8382	
	1026	18214	100.00	70.8382	

Figure 9. Typical chromatogram of a control kidney sample fortified with 1.0 ppm of BW 125-5376. Sample number 99184-3122 (vial number 10) from master sheet number 96135-09. Data for this sample can be found in Table 5. Recovery 122%.

[96096] 11 121596.10.1
Reported on 13-JAN-1997 at 14:16

SAG
1-17-97

Acquired on 15-DEC-1996 at 22:37



SASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Lims Id :
Concent :
Method Title : 125-5376
Sample Name : 1.0 ppm Fort #2
Sample Id : 96184
Sample Type : Recovery Amount=1.00000
Bottle No : 1

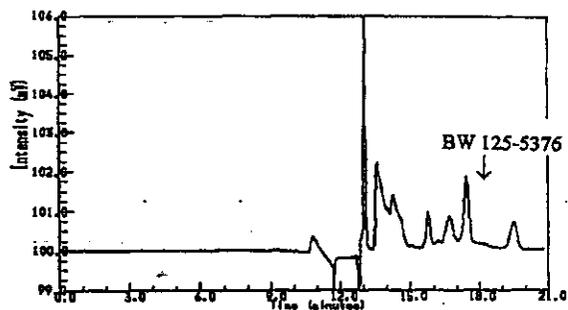
PEAK INFORMATION

RT mins	Height	Area	Area %	Conc	Peak name
12.178	1783	32658	100.00	1225.6361	125-5376
Totals					
Unknowns	0	0	0.00	W/A	
	1783	32658	100.00	1225.6361	
	1783	32658	100.00	1225.6361	

Figure 10. Typical chromatogram of a control liver sample. Sample number 96184-3124 (vial number 2) from master sheet number 96135-10. Data for this sample can be found in Table 5.

(96096) 11 121596A.2.1
Reported on 13-JAN-1997 at 13:34 *DSB*
Modified on 13-JAN-1997 at 11:30

Acquired on 16-DEC-1996 at 00:44



BASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Lims Id :
Comment :
Method Title : 125-5376
Sample Name : SAB Control Liver (96184-3124)
Sample Id : 96184
Sample Type : Recovery Amount=1.00000
Bottle No : 1

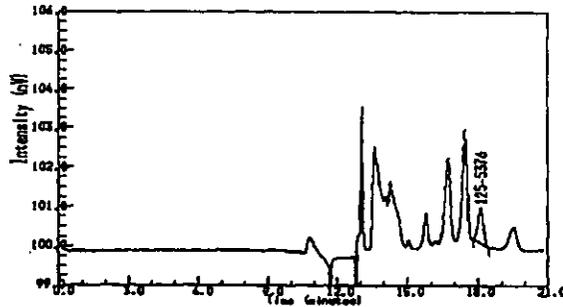
PEAK INFORMATION

No peaks detected

Figure 11. Typical chromatogram of a control liver sample fortified with 0.05 ppm (quantitation limit) of BW 125-5376. Sample number 96184-3124 (Vial number 4) from master sheet number 96135-10. Data for this sample can be found in Table 5. Recovery 124%.

[96096] 11 121596A.4.1
Reported on 13-JAN-1997 at 13:34 *DSS*
Modified on 13-JAN-1997 at 11:30

Acquired on 16-DEC-1996 at 01:34



BASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Line Id :
Comment :
Method Title : 125-5376
Sample Name : 0.05 ppm Port #1
Sample Id : 96184
Sample Type : Recovery Amount=1.00000
Bottle No : 1

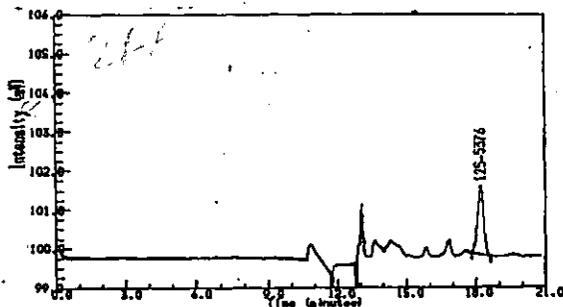
PEAK INFORMATION

RT mins	Height uV	Area uVs	Area %	ppb	Peak name
18.151	891	14451	100.00	61.8759	125-5376
Totals					
Unknowns	0	0	0.00	N/A	
	891	14451	100.00	61.8759	
	891	14451	100.00	61.8759	

Figure 12. Typical chromatogram of a control liver sample fortified with 1.0 ppm of BW 125-5376. Sample number 99184-3124 (vial number 8) from master sheet number 96135-10. Data for this sample can be found in Table 5. Recovery 123%.

[96096] 11 121596A,8,1
Reported on 13-JAN-1997 at 13:35
Modified on 13-JAN-1997 at 11:30 *DSB*

Acquired on 16-DEC-1996 at 03:15



BASF CORP. - VAX MULTICHROM

Analyst Name : Stephan
Lims Id :
Comment :
Method Title : 125-5376
Sample Name : 1.0 ppm Port #1
Sample Id : 96184
Sample Type : Recovery Amount=1.00000
Bottle No : 1

PEAK INFORMATION

RT min	Hght uV	Area uVs	Area %	cpb	Peak name
18.253	1770	31811	100.00	1231.7104	125-5376
Totals					
Unknowns	0	0	0.00	N/A	
	1770	31811	100.00	1231.7104	
	1770	31811	100.00	1231.7104	

Figure 13. Typical GC-MS parameters from master sheet number 96135-11.

TOPLEVEL PARAMETERS

Method Information For: C:\HPCHEM\1\METHODS\P121596V.M

Method Sections To Run:

- () Save Copy of Method With Data
- () Pre-Run Cmd/Macro =
- (X) Data Acquisition
- (X) Data Analysis
- () Post-Run Cmd/Macro =

Method Comments:

SPLITLESS INJECTION FOR PARENT M7; GCMSD # 11

END OF TOPLEVEL PARAMETERS

ACQUISITION PARAMETERS

General Information

Inlet : GC
Tune File : 110696.U
Acquisition Mode : Sim

MS Information

Solvent Delay : 4.00 min
EM Absolute : False
EMV Offset : 400.0
Resulting Voltage : 2400.0

[Sim Parameters]

GROUP 1
Group ID : Group 1
Dwell Per Ion : 500 msec.
Low Resolution : Yes
Group Start Time : 4.00
Ions In Group : 226.00

[Real Time Plot Parameters]

Method: P121596V.M

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Figure 13. Typical GC-MS parameters from master sheet number 96135-11. "Continued"

Time Window : 15 min
Iconize Real Time Display : False
Plot 1 type : Total Ion
Scale minimum : 0
Scale maximum : 200000
Plot 2 type : No plot

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GC Inlet Information

[Inlet A Temperature Program Information]

Oven Track : Off
Initial Temp. : 180 C
Initial Time : 480.00 min

Level	Rate (C/min)	Final Temp. (C)	Final Time (min)
1	0		

Total Program Time: 480.00 min

[Inlet B Temperature Program Information]

Oven Track : On

[Inlet A Pressure Program Information]

Constant Flow : Off
Initial Pres. : 50.0 psi
Initial Time : 0.50 min

Level	Rate (psi/min)	Final Pres. (psi)	Final Time (min)
1	99.00	10.0	15.00
2	99.00	10.0	5.00
3	0		

Total Program Time: 20.90 min
Pressure Units : psi

[Inlet A Flow Settings]

Column length : 30.00 m
Column diameter : 0.250 mm
Gas : He
Vacuum compensation : On
Pressure : 50.0 psi
Flow : 3.7 ml/min
Linear velocity : 74.2 cm/sec

[Inlet B Pressure Program Information]

Constant Flow : Off
Initial Pres. : 0.0 psi
Initial Time : 30.00 min

Figure 13. Typical GC-MS parameters from master sheet number 96135-11. "Continued"

[Auxiliary Channel, P Information]

Comment:

Assure Program:
Initial Pres. : 0.0 psi
Initial Time : 480.00 min

Level	Rate (psi/min)	Final Pres. (psi)	Final Time (min)
1	0		

Total Program Time: 480.00 min

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GC Temperature Information

[GC Zone Temperatures]

Inj. A : 180 C
Inj. B : 100 C Off
Det. A : 50 C Off
Det. B : 250 C
Aux. : 50 C Off

[Oven Parameters]

Soak Equib Time : 0.50 min
Oven Max : 250 C
Oven : On
Cryo : Off
Ambient : 25 C
Cryo Blast : Off

[Oven Program]

Initial Temp. : 80 C
Initial Time : 0.50 min

Level	Rate (C/min)	Final Temp. (C)	Final Time (min)
1	70.00	220	4.00
2	20.00	250	11.00
3	0.00		

Next Run Time : 19.00 min

Injector Information

Injection Source : Auto
Injection Location : Front

Sample Washes : 1
Sample Pumps : 2
Sample Volume : 4 stop(s)

Method: F121596V.M

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Figure 13. Typical GC-MS parameters from master sheet number 96135-11. "Continued"

Viscosity Delay : 0 sec
Solvent A Washes : 3
Solvent B Washes : 2
On Column : No

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[Purge Information]

Purge A/B	Init. Value	On Time	Off Time
A	Off	1.00	0.00
B	Off	1.00	0.00

Timed MS Detector Entries

time (min)	State (MS on/off)
5.00	On
14.00	Off

END OF ACQUISITION PARAMETERS

DATA ANALYSIS PARAMETERS

Method Name: C:\HPCHEM\1\METHODS\F121596V.M

Percent Report Settings

Sort By: Signal

Output Destination

Screen: No
Printer: Yes
File: No

Integration Events: 090495.E

Generate Report During Run Method: No

Signal Correlation Window: 0.020

Qualitative Report Settings

Method: F121596V.M

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Figure 13. Typical GC-MS parameters from master sheet number 96135-11. "Continued"

Peak Location of Unknown: Apex
Library to Search Minimum Quality
C:\DATABASE\NBS75K.L 0
Integration Events: AutoIntegrate
Report Type: Summary
Output Destination
Screen: Yes
Printer: Yes
File: No
Generate Report During Run Method: No

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VD

Quantitative Report Settings

Report Type: Detailed (single compound 1)
Output Destination
Screen: No
Printer: Yes
File: No
Generate Report During Run Method: Yes

WET POMACE TEST
Calibration Last Updated: Mon Dec 16 06:49:08 1996

Reference Window: 10.00 Percent
Non-Reference Window: 5.00 Percent
Correlation Window: 0.02 minutes
Default Multiplier: 1.00
Default Sample Concentration: 0.00

Compound Information

1) BAS125W N7 ()

Ret. Time 8.90 min., Extract & Integrate from 8.72 to 9.10 min.

Signal Rel. Resp. Pct. Unc. (rel) Integration
Tgt 225.00 090496.E

Peak ID	Conc (PG)	Response
1B	7.500	200997
2A	15.000	218933
2B	15.000	388994
3A	30.000	417817
		743166

Method: F121596V.M

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Figure 13. Typical GC-MS parameters from master sheet number 96135-11. "Continued"

3B	30.000	791083
4A	60.000	1393773
4B	60.000	1488524

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Amplifier Peak Analysis OFF
Curve Fit: Linear

END OF DATA ANALYSIS PARAMETERS

Figure 14. Typical chromatogram of a 7.5 µg standard of BW 125-M7 from master sheet number 96135-11. Data from this standard can be found in Table 6.

File : C:\HPCHEM\1\DATA\125M\121596\0101003.D
 Operator :
 Acquired : 25 Dec 96 4:21 pm using AcqMethod P121596V
 Sample Name: 7.5 µg / 4 µL M7 STANDARD
 Misc Info :
 Vial Number: 1
 CurrentMeth: C:\HPCHEM\1\METHODS\P121596V.M
 Compound: BAS125M M7
 Ret Time: 8.92
 Concentration: 6.76 µg
 Pk # and Type: 1

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Signal	Ratio	Limits	RT	Limits	Resp	Integ	Type
Tot	226.00	100.0%	8.92	8.68	286738	890498	
Q1	0.00	0.0	0.0	0.00	0	0	auto
Q2	0.00	0.0	0.0	0.00	0	0	auto
Q3	0.00	0.0	0.0	0.00	0	0	auto

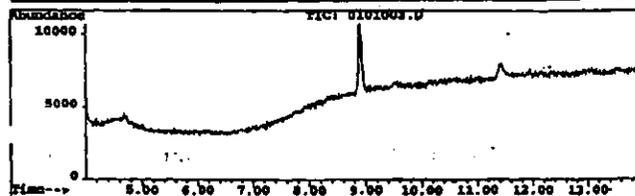
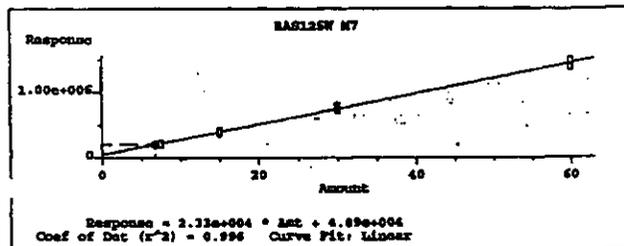
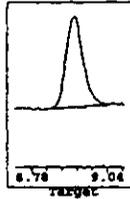


Figure 15. Typical chromatogram of a 15 pg standard of BW 125-M7 from master sheet number 96135-11. Data from this standard can be found in Table 6.

File : C:\RPCHEM\1\DATA\125M\121596\0201004.D
 Operator :
 Acquired : 15 Dec 96 4:44 pm using AcqMethod P121596V
 Sample Name: 15 PG/ 4 UL M7 STANDARD
 Misc Info :
 Vial Number: 2
 CurrentMeth: C:\RPCHEM\1\METHODS\P121596V.M
 Compound: BAS125W M7
 Ret Time: 8.93
 Concentration: 14.57 PG
 PK # and Type: 1

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	Signal	Ratio	Limits	RT	Limits	Resp	Integ	Type
Tot	228.00	100.0%		8.93	8.68	388994	090456	
Q1	0.00	0.0	0.0- 0.0	0.00	to	0	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	9.12	0	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	0	auto

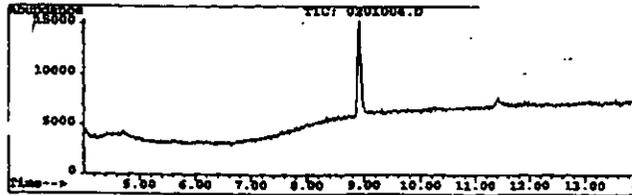
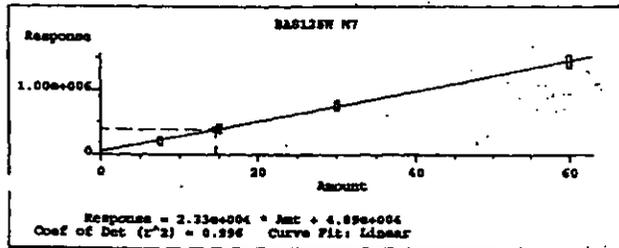
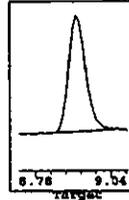


Figure 16. Typical chromatogram of a 30 pg standard of BW 125-M7 from master sheet number 96135-11. Data from this standard can be found in Table 6.

File : C:\HPCHEM\1\DATA\125W\121596\0301007.D
 Operator :
 Acquired : 15 Dec 96 5:53 pm using AcqMethod F121596V
 Sample Name: 30 PG/ 4 UL M7 STANDARD
 Misc Info :
 Vial Number: 1
 CurrentMeth: C:\HPCHEM\1\METHODS\F121596V.M
 Compound: BAS125W M7
 Ret Time: 8.93
 Concentration: 29.74 PG
 PK # and Type: 1

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 YD



	Signal	Ratio	Limits		RT	Limits	Resp	Integ Type
Tot	226.00	100.04			8.93	8.68	743165	090496
Q1	0.00	0.0	0.0	0.0	0.00	to	0	auto
Q2	0.00	0.0	0.0	0.0	0.00	9.12	0	auto
Q3	0.00	0.0	0.0	0.0	0.00		0	auto

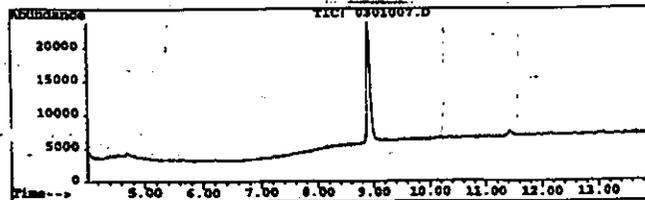
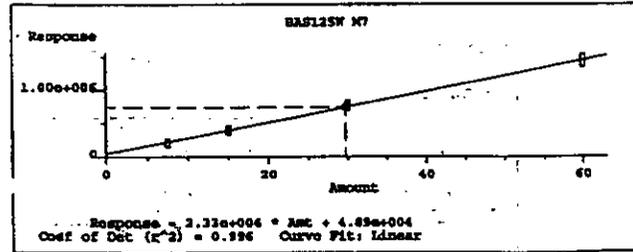
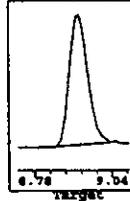


Figure 17. Typical chromatogram of a 60 pg standard of BW 125-M7 from master sheet number 96135-11. Data from this standard can be found in Table 6.

File : C:\HPCHEM\1\DATA\125W\121596\0401009.D
 Operator :
 Acquired : 15 Dec 96 6:39 pm using AcqMethod P121596V
 Sample Name: 60 PG/ 4 UL M7 STANDARD
 Misc Info :
 Vial Number: 4
 CurrentMeth: C:\HPCHEM\1\METHODS\P121596V.M

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 YD

Compound: BAS125W M7
 Ret Time: 8.93
 Concentration: 57.61 PG
 Pk # and Type: 1



	Signal	Ratio	Limits	RT	Limits	Resp	Integ Type
Tot	228.00	100.00		8.93	8.68	13537721090496	
Q1	0.00	0.0	0.0- 0.0	0.00	0.0	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	9.12	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	auto

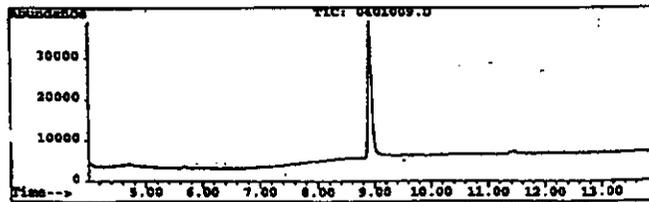
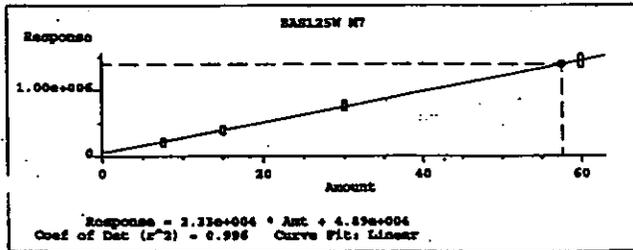
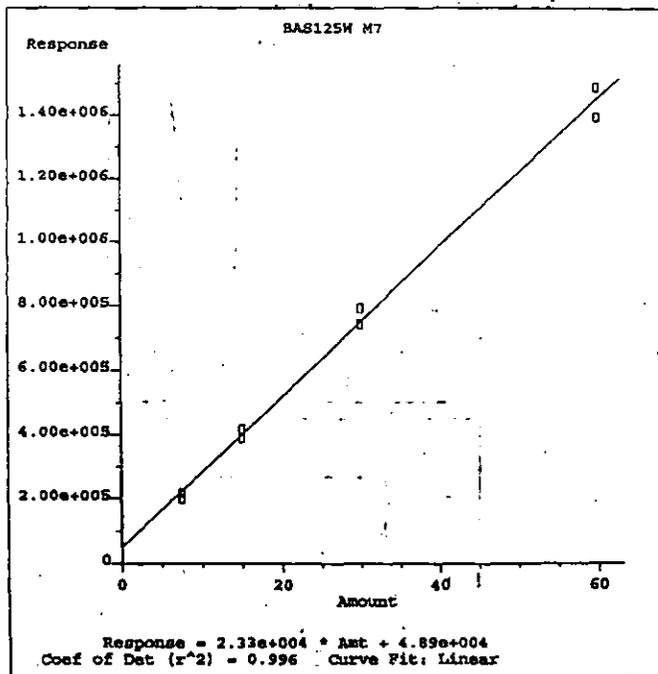


Figure 18. Typical standard curve for 7.5, 15, 30, and 60 µg amounts of BW 125-M7 from master sheet number 86135-11. Data from these standards can be found in Table 6.

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Method Name: C:\HPCHEM\1\METHODS\F121596V.M
Calibration Table Last Updated: Mon Dec 16 06:49:08 1996

Figure 19. Typical chromatogram of a control kidney sample. Sample number 98184-3122 (vial number 6) from master sheet number 98135-11. Data for this sample can be found in Table 3.

File : C:\HPCHEM\1\DATA\125W\121596\0601005.D
Operator :
Acquired : 15 Dec 96 5:07 pm using AcqMethod P121596V
Sample Name: CONTROL KIDNEY 98184-3122
Misc Info :
Vial Number: 6
CurrentMeth: C:\HPCHEM\1\METHODS\P121596V.M
Compound: BAS125W M7
Ret Time:
Concentration:
PK # and Type: 1
***** NOT FOUND *****

12-10-96
YD

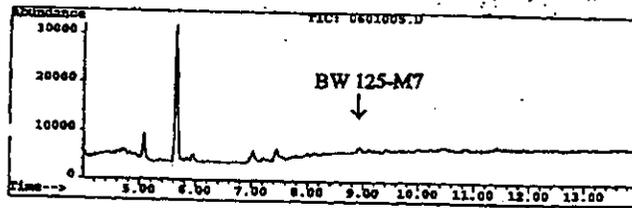
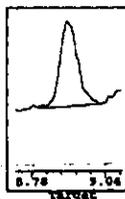


Figure 20. Typical chromatogram of a control kidney sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 98184-3122 (vial number 8) from master sheet number 96135-11. Data for this sample can be found in Table 3. Recovery 88%.

File : C:\NFCHEM\1\DATA\125W\121596\0801010.D
 Operator :
 Acquired : 15 Dec 96 7:03 pm using AcqMethod P121596V
 Sample Name: 0.05B 9PM KID 98184-3122
 Misc Info : 1-399-02D G-M-L-4-4
 Vial Number: 8
 CurrentMeth: C:\NFCHEM\1\METHODS\P121596V.M

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 YD

Compound: BAS125W M7
 Ref Time: 8.91
 Concentration: 14.03 PG
 Pk # and Type: 1



	Signal	Ratio	Limite	RT	Limite	Resp	Integ Type
Typ	224.00	180.04		8.91	8.68	374487	030456
Q1	0.00	0.0	0.0- 0.0	0.00	0.0		auto
Q2	0.00	0.0	0.0- 0.0	0.00	9.12		auto
Q3	0.00	0.0	0.0- 0.0	0.00			auto

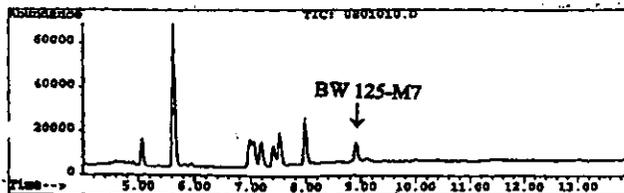
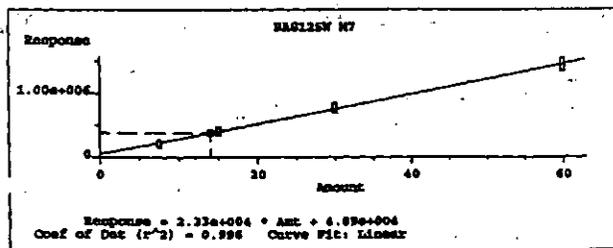
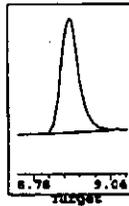


Figure 21. Typical chromatogram of a control kidney sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-3122 (vial number 9) from master sheet number 96135-11. Data for this sample can be found in Table 3. Recovery 90%.

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File : C:\MPCKEN\1\DATA\125W\121596\0901012.D
 Operator :
 Acquired : 15 Dec 96 7:49 pm using AcqMethod F121596V
 Sample Name: 1.0A PPM KID 96184-3122
 Misc Info :
 Vial Number: 9
 CurrentMeth: C:\MPCKEN\1\METHODS\F121596V.M
 Compound: BAS125W M7
 Ret Time: 8.91
 Concentration: 28.91 PPB
 Pk # and Type: 1



	Signal	Ratio	Limits	WT	Limits	Resp	Integ	Type
Tot	228.00	100.0%		8.91	8.68	721778	090496	
Q1	0.00	0.0	0.0- 0.0	0.00	to	0	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	9.12	0	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	0	auto

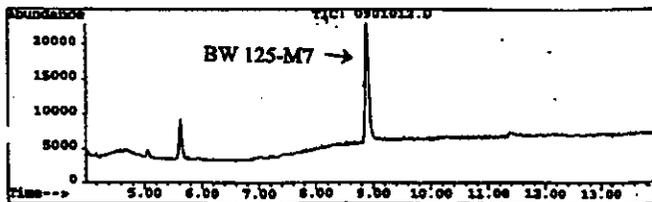
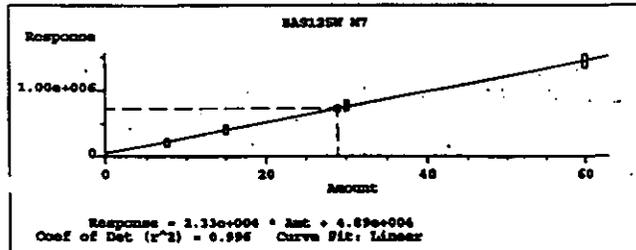


Figure 22. Typical chromatogram of a control liver sample. Sample number 96184-3124 (vial number 11) from master sheet number 96135-12. Data for this sample can be found in Table 3.

File : C:\RPCHEM\1\DATA\125M\121596\1101017.D
Operator :
Acquired : 15 Dec 96 9:49 pm using AcqMethod P121596V
Sample Name: CONTROL LIV 96184-3124
Misc Info :
Vial Number: 11
CurrentMeth: C:\RPCHEM\1\METHODS\P121596V.M

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YD

Compound: BAS125M M7
Ret Time:
Concentration:
PK # and Type: 1
***** NOT FOUND *****

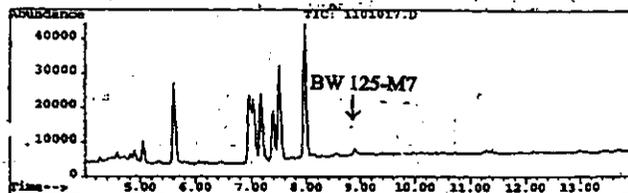
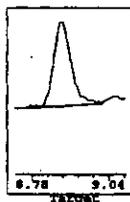


Figure 23. Typical chromatogram of a control liver sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96184-3124 (vial number 13) from master sheet number 96135-12. Data for this sample can be found in Table 3. Recovery 78%.

File : C:\RPCHEM\1\DATA\125W\121596\1301021.D
 Operator :
 Acquired : 15 Dec 96 11:18 pm using AcqMethod P121596V
 Sample Name : 0.05B PPM LIV 96184-3124
 Misc Info :
 Vial Number: 13
 CurrentMeth: C:\RPCHEM\1\METHODS\P121596V.M
 Compound: BAS125W M7
 Ret Time: 8.88
 Concentration: 12.45 PG
 Pk # and Type: 1

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	Signal	Ratio	Limits	RT	Limits	Resp	Inceq	Type
Tot	124.00	100.04		8.88	8.88	343583	090496	
Q1	0.00	0.0	0.0- 0.0	0.00	0.00	0	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	9.12	0	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	0	auto

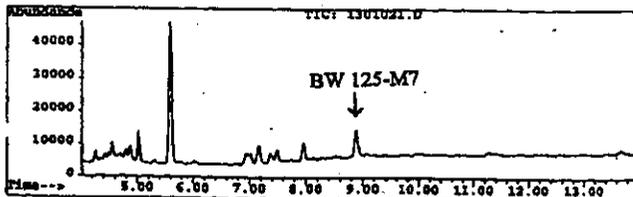
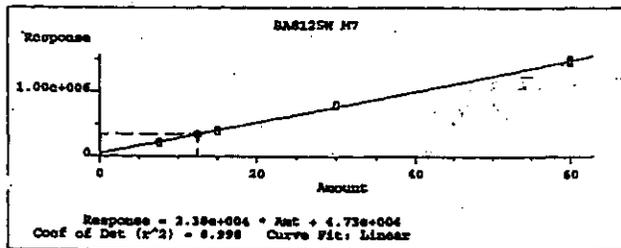
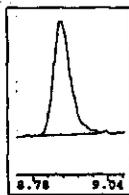


Figure 24. Typical chromatogram of a control liver sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-3124 (vial number 15) from master sheet number 96135-12. Data for this sample can be found in Table 3 *Summary only*

File : C:\HPCHEM\1\DATA\125W\121596\1501025.D
 Operator :
 Acquired : 16 Dec 96 12:51 pm using AcqMethod P121596V
 Sample Name: 1.0B PPM LIV 96184-3124
 Misc Info :
 Vial Number: 15
 CurrentMeth: C:\HPCHEM\1\METHODS\P121596V.M
 Compound: BAS125W M7
 Ret Time: 8.88
 Concentration: 28.57 PG
 PK # and Type: 1

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	Signal	Ratio	Limits	RT	Limits	Resp	Integ Type
Tot	226.00	100.00		8.88	8.88	727009	090486
Q1	0.00	0.0	0.0- 0.0	0.00	to	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	9.12	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	auto

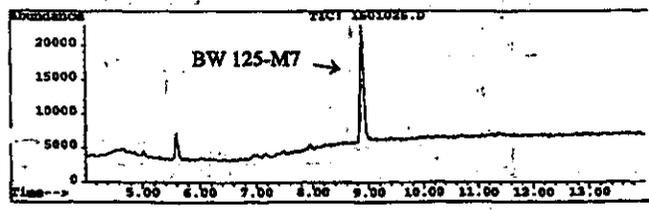
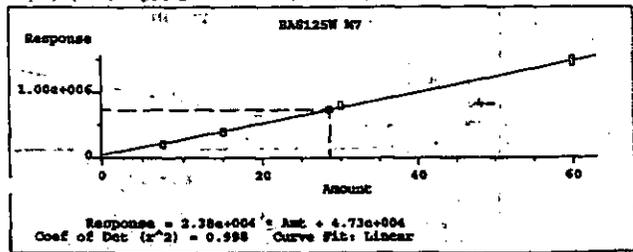
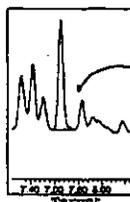


Figure 25. Typical chromatogram of a control fat sample. Sample number 98184-5000 (vial number 6) from master sheet number 98135-3. Data for this sample can be found in Table 3.

File : C:\HPCHEM\1\DATA\125M\120696\0601004.D
 Operator :
 Acquired : 6 Dec 96 10:13 am using AcqMethod P120596
 Sample Name: CONTROL FAT
 Misc Info :
 Vial Number: 6
 CurrentMeth: C:\HPCHEM\1\METHODS\P120596.M

Compound: BAS9054 M7
 Ret Time: 7.66
 Concentration: 17.46
 Pk # and Type: 1



	Signal	Ratio	Limits	RT	Limits	Resp	Integ Type
Tot	226.00	100.00		7.66	7.56	103220	091796
Q1	0.00	0.0	0.0- 0.0	0.00	to	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	7.94	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	auto

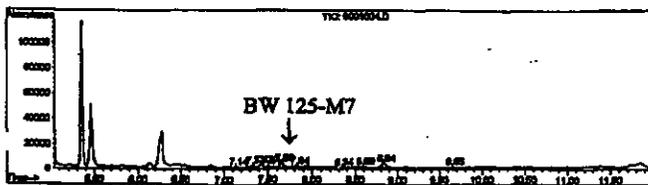
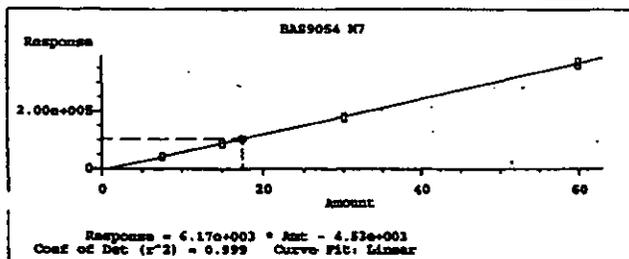
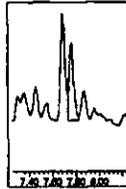


Figure 26. Typical chromatogram of a control fat sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96184-5000 (vial number 7) from master sheet number 96135-3. Data for this sample can be found in Table 3. Recovery 76%.

File : C:\HPCHEM\1\DATA\125W\120696\0701007.D
Operator :
Acquired : 6 Dec 96 11:13 am using AcqMethod P120596
Sample Name: 0.05A FPM FAT
Misc Info :
Vial Number: 7
CurrentMeth: C:\HPCHEM\1\METHODS\P120596.M

Compound: BAS9054 M7
Ret Time: 7.76
Concentration: 12.22
Pk # and Type: 1



	Signal	Ratio	Limits	RT	Limits	Resp	Integ Type
Tot	226.00	100.04		7.76	7.56	70853	091794
Q1	0.00	0.0	0.0- 0.0	0.00	0.00	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	7.94	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00	0.00	0	auto

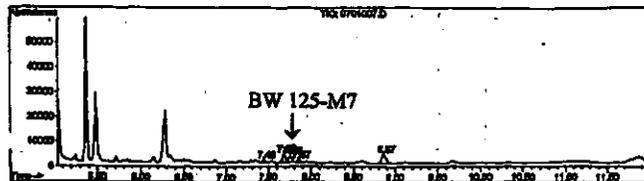
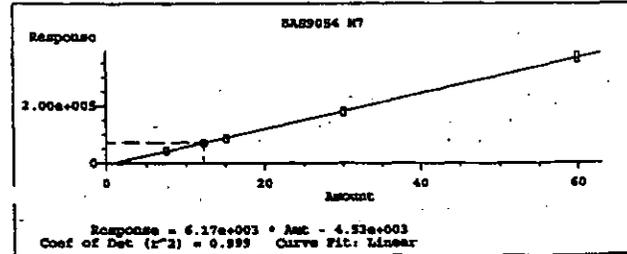
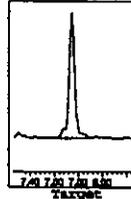


Figure 27. Typical chromatogram of a control fat sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-5000 (vial number 10) from master sheet number 96135-3. Data for this sample can be found in Table 3. Recovery 81%.

File : C:\HPCHEM\1\DATA\125W\120696\1001013.D
 Operator :
 Acquired : 6 Dec 96 1:17 pm using AcqMethod 9120596
 Sample Name: 1.0B PPM FAT
 Misc Info :
 Vial Number: 10
 CurrentPath: C:\HPCHEM\1\METHODS\9120596.M
 Compound: BAS9054 M7
 Ret Time: 7.75
 Concentration: 15.89
 Pk # and type: 1

288
11.12.96



	Signal	Ratio	Limits	RT	Limits	Resp	Integ Type
Typ	124.00	100.0%		7.75	7.56	155223	091796
Q1	0.00	0.0	0.0- 0.0	0.00	0.0	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	7.94	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	auto

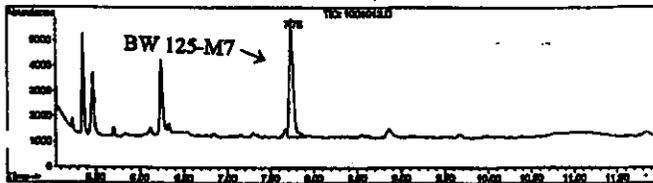
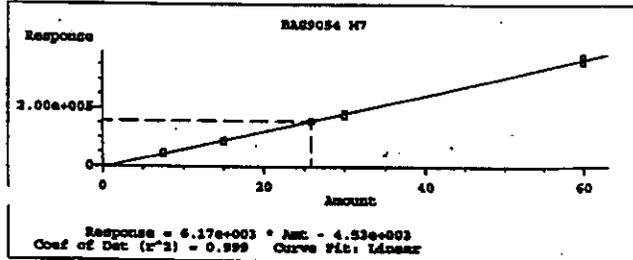
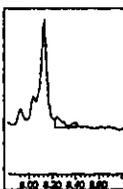


Figure 28. Typical chromatogram of a control muscle sample. Sample number 94101-2860 (vial number 16) from master sheet number 98135-5. Data for this sample can be found in Table 3.

File : C:\NPCHEM\1\DATA\125W\120496\1601031.D
 Operator :
 Acquired : 5 Dec 96 3:02 am using AcqMethod PL120496M
 Sample Name: CONTROL MUSCLE 94101-2860
 Misc Info :
 Vial Number: 16
 CurrentMeth: C:\NPCHEM\1\METHODS\PL120496M.M
 Compound: BAS9054 M7
 Ret Time: 8.34
 Concentration: 7.00
 Pk # and Type: 1'

215
 11.0.1



Typ	Signal	Ratio	Limits	RT	Limits	Resp.	Integ Type
Q1	125.00	100.00	0.0 - 0.0	8.34	8.11	16465	091796 auto
Q2	0.00	0.0	0.0 - 0.0	0.00	0.0	0	0 auto
Q3	0.00	0.0	0.0 - 0.0	0.00	8.53	0	0 auto
Q4	0.00	0.0	0.0 - 0.0	0.00		0	0 auto

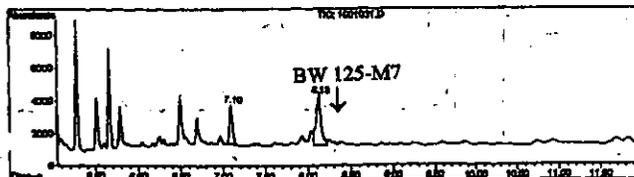
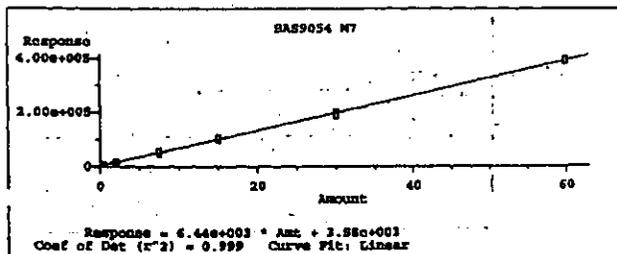


Figure 29. Typical chromatogram of a control muscle sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 94101-2860 (vial number 17) from master sheet number 96135-6. Data for this sample can be found in Table 3. Recovery 78%.

File : C:\EPCHEN\1\DATA\125W\120496\1701034.D
 Operator :
 Acquired : 5 Dec 96 4:00 am using AcqMethod P120496M
 Sample Name: 0.05A PPM MUSCLE 94101-2860
 Misc Info :
 Vial Number: 17
 CurrentMeth: C:\EPCHEN\1\METHODS\P120496.M

Compound: BAS9054 M7
 Ret Time: 8.34
 Concentration: 14.54
 Pk # and Type: 1



PK#	Signal	Ratio	Limits	RT	Limit	Resp	Integ Type
01	226.00	100.00	0.0- 0.0	8.34	8.11	97280	091736
02	0.00	0.0	0.0- 0.0	0.00	0.0	0	auto
03	0.00	0.0	0.0- 0.0	0.00	8.53	0	auto
03	0.00	0.0	0.0- 0.0	0.00	0.00	0	auto

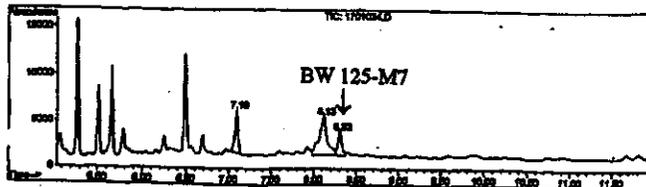
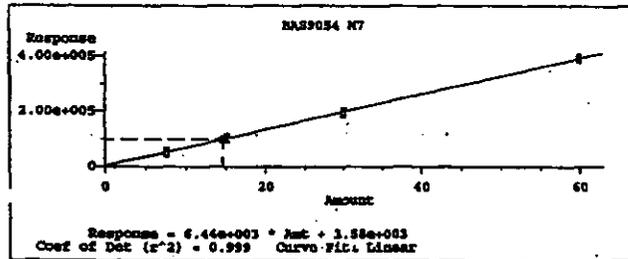
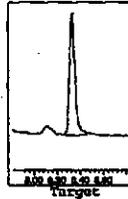


Figure 30.

Typical chromatogram of a control muscle sample fortified with 1.0 ppm of BAS 125 W. Sample number 94101-2860 (vial number 20) from master sheet number 96135-5. Data for this sample can be found in Table 3. Recovery 73%.

File : C:\HPCHEM\1\DATA\125W\120496\2001040.D
 Operator :
 Acquired : 5 Dec 96 5:57 am using AcqMethod P120496M
 Sample Name : 1.0B PPM MUSCLE 94101-2860
 Misc Info :
 Vial Number : 20
 CurrentMeth: C:\HPCHEM\1\METHODS\P120496M.M
 Compound: BAS9054 M7
 Ret Time: 8.36
 Concentration: 33.55
 Pk # and Type: 1



Qty	Signal	Ratio	Limits	RT	Limits	Resp	Integ Type
1	225.00	100.0%	0.0- 0.0	8.36	8.11	155290	091796
Q1	0.00	0.0	0.0- 0.0	0.00	0.00	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	8.53	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00	0.00	0	auto

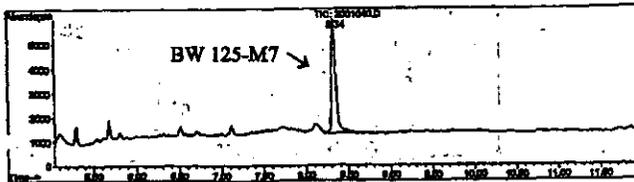
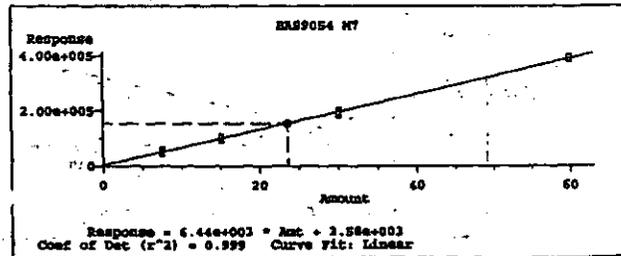
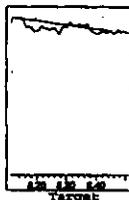


Figure 31. Typical chromatogram of a control wet pomace sample. Sample number 96072-13 (vial number 11) from master shoot number 96135-19. Data for this sample can be found in Table 4.

125W
 File : C:\HPCHEM\1\DATA\125W\121896\1101018.D
 Operator :
 Acquired : 18 Dec 96 10:57 pm using AcqMethod F121896W
 Sample Name: DSB; WET POMACE; CTRL
 Misc Info :
 Vial Number: 11
 CurrentMeth: C:\HPCHEM\1\METHODS\F121896W.M

DEC 17 1996
 DEC 19 1996
 DSB

Compound: SA9054 M7
 Ret Time: 8.30
 Concentration: 0.52
 Pk # and Type: 1



	Signal	Ratio	Limite	RT	Limite	Resp	Integ Type
Tot	226.00	100.0%		8.30	8.10	-432	091796
Q1	0.00	0.0	0.0-	0.0	0.00	0	auto
Q2	0.00	0.0	0.0-	0.0	0.00	0	auto
Q3	0.00	0.0	0.0-	0.0	0.00	0	auto

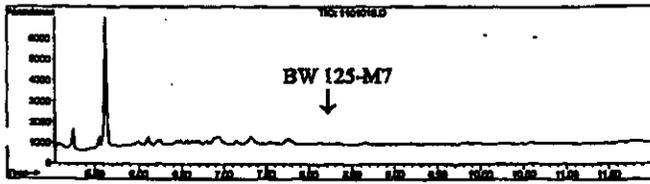
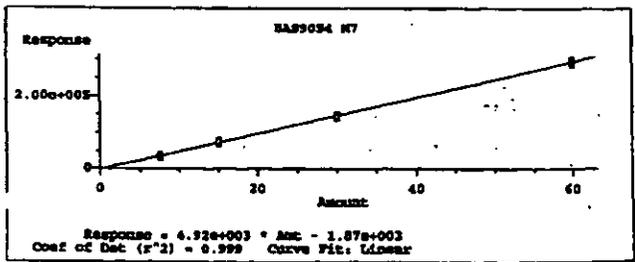


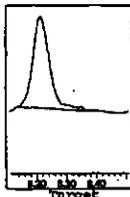
Figure 32. Typical chromatogram of a control wet pomace sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96072-13 (vial number 13) from master sheet number 86135-19. Data for this sample can be found in Table 4. Recovery 80%.

125W

File : C:\HPCHEM\1\DATA\125W\121896\1301022.D
 Operator :
 Acquired : 19 Dec 96 12:15 pm using AcqMethod P121896W
 Sample Name : OES; NET POMACE; 0.05PPM B
 Misc Info :
 Vial Number : 13
 CurrentMeth: C:\HPCHEM\1\METHODS\P121896W.M

DEC 19 1996

Compound: BAS9054 M7
 Ret Time: 8.30
 Concentration: 12.80
 Pk # and Type: 1



	Signal	Ratio	Limits	IR	Limits	Resp	Integ Type
Tot	224.00	100.00		8.30	8.10	41142	091798
Q1	0.00	0.0	0.0- 0.0	0.00	to	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	8.52	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	auto

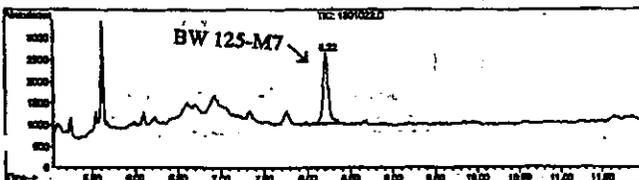
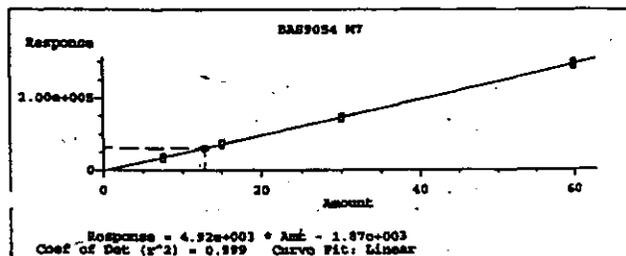


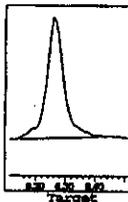
Figure 33. Typical chromatogram of a control wet pomace sample fortified with 1.0 ppm of BAS 125 W. Sample number 96072-13 (vial number 15) from master sheet number 96135-19. Data for this sample can be found in Table 4. Recovery 86%.

125W

File : C:\HPCHEM\1\DATA\125W\121896\1501026.D
 Operator :
 Acquired : 19 Dec 96 1:33 am using AcqMethod P121896W.C
 Sample Name: DSB; WET POMACE; 1.0PPM B
 Misc Info :
 Vial Number: 15
 CurrentMeth: C:\HPCHEM\1\METHODS\P121896W.M

DEC 19 1996

Compound: BAS9054 M7
 Ret Time: 8.10
 Concentration: 27.47
 PK # and Type: 1



	Signal	Ratio	Limit	RT	Limit	Resp	Integ	Type
Tot	238.00	100.0%		8.10	8.10	133410	091796	
Q1	0.00	0.0	0.0- 0.0	0.00	to	0	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	8.52	0	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	0	auto

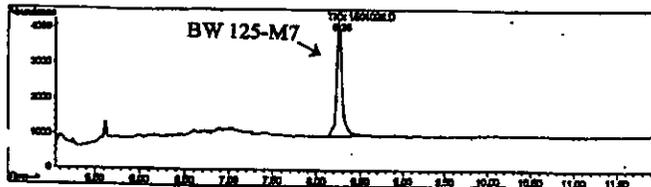
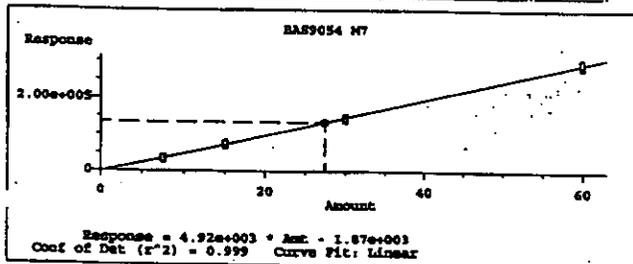


Figure 34. Typical chromatogram of a control apple juice sample. Sample number 96072-17 (vial number 11) from master sheet number 96135-20. Data for this sample can be found in Table 4.

File : C:\APCHM\1\DATA\125W\121896\1101019.D
Operator :
Acquired : 19 Dec 95 12:28 am using AcqMethod F121896J
Sample Name: CONTROL A.J. 96072-17
Misc Info :
Vial Number: 11
CurrentMeth: C:\APCHM\1\METHODS\F121896J.M

12-19-95

Compound: BAS125W M7
Ret Time:
Concentration:
PK # and Type: 1
***** NOT FOUND *****

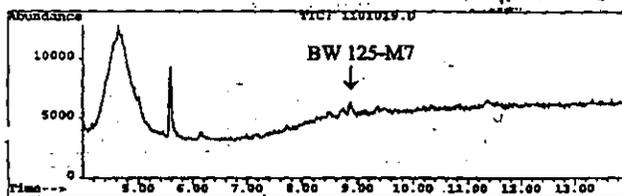
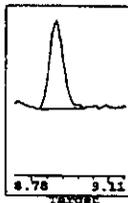


Figure 35. Typical chromatogram of a control apple juice sample fortified with 0.05 ppm (quantitation limit) of BAS 125 W. Sample number 96072-17 (vial number 12) from master sheet number 96135-20. Data for this sample can be found in Table 4. Recovery 76%.

File : C:\HPCHEN\1\DATA\125W\121896\1201022.D
 Operator :
 Acquired : 18 Dec 96 1:38 am using AcqMethod P121896J
 Sample Name: 0.05A PPM AJ 96072-17
 Misc Info :
 Vial Number: 12
 CurrentMeth: C:\HPCHEN\1\METHODS\P121896J.M
 Compound: BAS125W M7
 Ret Time: 8.89
 Concentration: 12.16 PG
 Pk # and Type: 1

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 12-19-96



	Signal	Ratio	Limits	RT	Limits	Resp	Integ	Type
Tot	226.00	100.0%		8.89	8.96	296078	090496	
Q1	0.00	0.0	0.0- 0.0	0.00	to		0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	9.10		0	auto
Q3	0.00	0.0	0.0- 0.0	0.00			0	auto

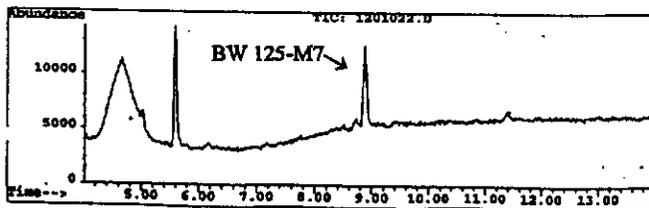
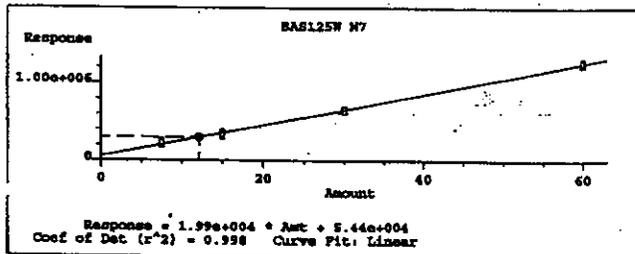
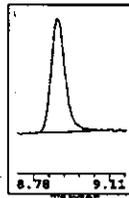


Figure 36. Typical chromatogram of a control apple juice sample fortified with 1.0 ppm of BAS 125 W. Sample number 96072-17 (vial number 15) from master sheet number 96135-20. Data for this sample can be found in Table 4. Recovery 87%.

File : C:\NFCHEM\1\DATA\125\121896\1501029.D
 Operator :
 Acquired : 19 Dec 96 4:20 am using AcqMethod P121896J
 Sample Name: 1.0B PPM AJ 96072-17
 Misc Info :
 Vial Number: 15
 CurrentMeth: C:\NFCHEM\1\METHODS\P121896J.M
 Compound: BAS125W M7
 Ret Time: 8.88
 Concentration: 27.70 PG
 Pk # and Type: 1

12-19-96



Tyt	Signal	Ratio	Limits	ET	Limits	Resp	Integ Type
01	226.00	100.00	0.0- 0.0	8.88	8.66	605123	090498
02	0.00	0.0	0.0- 0.0	0.00	to	0	auto
03	0.00	0.0	0.0- 0.0	0.00	9.10	0	auto
03	0.00	0.0	0.0- 0.0	0.00		0	auto

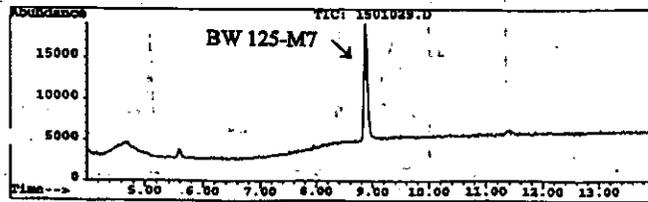
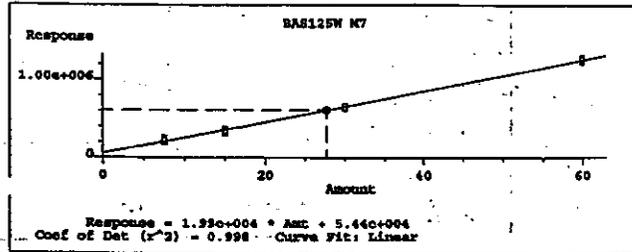


Figure 37. Typical chromatogram of a control milk sample. Sample number 96184-604 (Vial number 6) from master sheet number 96135-21. Data for this sample can be found in Table 3.

File : C:\HPCHEM\1\DATA\125W\121856\0601005.D
Operator :
Acquired : 18 Dec 96 7:03 pm using AcqMethod F121896M
Sample Name: CONTROL MILK 96184-504
Misc Info :
Vial Number: 6
CurrentMeth: C:\HPCHEM\1\METHODS\F121896M.M

12-19-

Compound: BAS125W M7
Ret Time:
Concentration:
Pk # and Type: 1
***** NOT FOUND ****

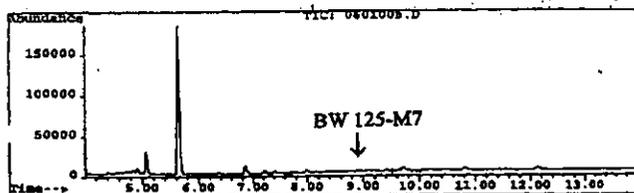
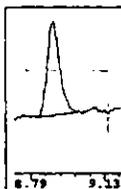


Figure 38. Typical chromatogram of a control milk sample fortified with 0.01 ppm (quantitation limit) of BAS 125 W. Sample number 96184-504 (vial number 8) from master sheet number 96135-21. Data for this sample can be found in Table 3. Recovery 91%.

File : C:\NPCHEM\1\DATA\125w\121896\0801010.D
 Operator :
 Acquired : 18 Dec 96 8:59 pm using AcqMethod P121896M
 Sample Name : 0.01 PPM MILK 96184-504
 Misc Info : 0.01
 Vial Number : 8
 CurrentMeth: C:\NPCHEM\1\METHODS\P121896M.M

UC
 12-19-96

Compound: BAS125W M7
 Ret Time: 8.90
 Concentration: 15.57 PG
 Pk # and Type: 1



Typ	Signal	Ratio	Limits	RT	Limits	Resp	Integ Type
Q1	224.00	180.04	0.0- 0.0	8.90	8.68	151010	090496
Q2	0.00	0.0	0.0- 0.0	0.00	9.13	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00	0.00	0	auto

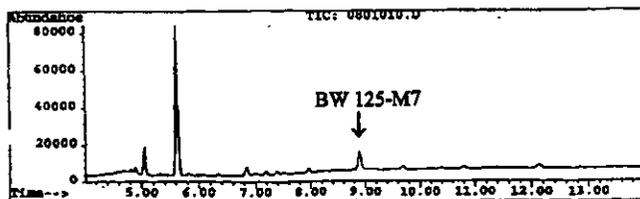
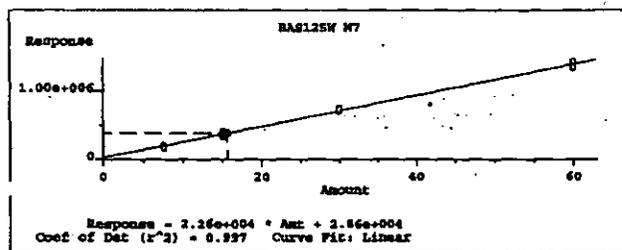
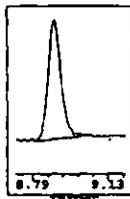
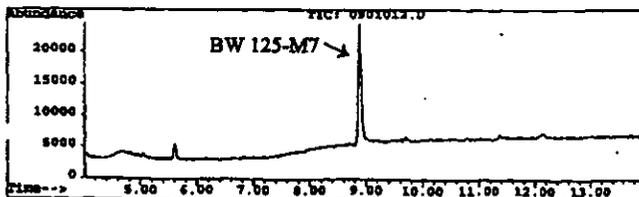
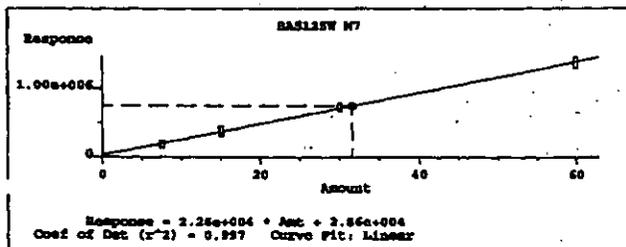


Figure 39. Typical chromatogram of a control milk sample fortified with 1.0 ppm of BAS 125 W. Sample number 96184-804 (vial number 9) from master sheet number 98135-21. Data for this sample can be found in Table 3. Recovery 93%.

File : C:\HPCHEM\1\DATA\125\121896\0901012.D
 Operator :
 Acquired : 18 Dec 98 9:48 pm using AcqMethod P121896W
 Sample Name: 1.0A PPM MILK 96184-804
 Misc Info :
 Vial Number: 9
 CurrentMeth: C:\HPCHEM\1\METHODS\P121896W.M
 Compound: BAS125W M7
 Ret Time: 8.89
 Concentration: 31.59 PG
 Pk # and Type: 1



	Signal	Ratio	Limits	RT	Limits	Resp	Integ	Type
Tgt	228.00	100.0%		8.89	8.88	743776	098498	
Q1	0.00	0.0	0.0- 0.0	0.00	0.00	0	0	auto
Q2	0.00	0.0	0.0- 0.0	0.00	9.12	0	0	auto
Q3	0.00	0.0	0.0- 0.0	0.00		0	0	auto



Appendix C

ENV +[™] Regeneration: Optional Procedure for re-use of ENV +[™] sorbent

Place the used ENV +[™] into a beaker (ENV +[™] should not fill more than 1/4 of the beaker) and add MeOH to cover the sorbent. Heat the mixture at 80°C for 10 minutes with stirring. Decant the MeOH and replace with THF. Heat and stir the THF mixture for 10 minutes. Decant the THF. Reduce the temperature to near ambient. Add DCM to cover the sorbent. Allow the mixture to stir for 10 min before transferring to a Bucher funnel with a sheet of filter paper (No. 2). Vacuum filter and rinse the marc thoroughly with DCM. Then place the cleaned ENV +[™] sorbent in a pan (in the hood) until completely dry.

Note: ENV +[™] material can be re-generated more than once. Consistent performance of the ENV +[™] sorbent has been observed after 5 re-generation cycles.

Appendix D

BASF

Analytical Services Report - ASRP97014

Study Name : Confirmation of BW 125-5376

Submitted by : Stephan Baumann

Date of Submission : 3/1/97

Experimental End Date : 9/25/97

Date of Report : 9/29/97

Analyst(s) : G. Walt *G. Walt 9/29/97*

Reviewed by : *Stephen C. Horne 9/29/97*

Description of Mass Spectral Study

Development of LC/MS/MS method with detection limit of 5pg/ul.

Summary of Results

Calibration curve was obtained with 2, 5, 10, 20 pg/ul.

Instruments

Mass Spectrometer Used :		Chromatograph Used :	
<input checked="" type="checkbox"/>	SCIEX API III + BASF#1015	<input checked="" type="checkbox"/>	P4000 BASF# 0041 Thermo Separations, San Jose, CA
<input type="checkbox"/>	SCIEX API 300#1 BASF# 789	<input type="checkbox"/>	Varian 9010 BASF# 119 Varian Chromatography, Walnut Creek, CA
<input type="checkbox"/>	SCIEX API 300#2 BASF# 784 Solex Corp, Thornhill, ON, Canada	<input type="checkbox"/>	Shimadzu Dual LC-10AD / SPD-10A BASF# 583, #584, #312
<input type="checkbox"/>	Finnigan LCQ BASF# 1083	<input type="checkbox"/>	Shimadzu Dual LC-10AD / SPD-10A BASF# 585, #586, #313 Shimadzu Scientific, Columbia, MD
<input type="checkbox"/>	Finnigan GCQ Finnigan-MAT, San Jose, CA		
<input type="checkbox"/>	HP-GC/AED Hewlett Packard, Avondale, PA		

Autosampler Used :		Radioactivity Detector Used	
<input checked="" type="checkbox"/>	AS3000 BASF# 192 Thermo Separations, San Jose CA	<input type="checkbox"/>	INUS β -Ram #1 BASF#
<input type="checkbox"/>	PE Series 200#1 BASF# 1305	<input type="checkbox"/>	INUS β -Ram #2 BASF# 465 INUS Systems, Tampa, FL
<input type="checkbox"/>	PE Series 200#2 BASF# 1309 Perkin-Elmer, Norwich CT		

Inlet Type :

HPLC : FIA "Flow Injection Analysis"
Infusion GC Direct Probe

HP-GC/AED Analysis:

Elements Monitored:

¹²C ¹⁴C N Cl S F H Other

Ionization Type Used :

API IonSpray Cl
API Turbo IonSpray Temperature: 450 C
AP/CI Heated Nebulizer Temperature: C
ElectroSpray

Mass Spec Mode of Operation :

Full Scan Range : 100 - 1200
SIM Ions Monitored :

MS/MS

Product Range: see results
Precursor Range:
Constant Neutral Loss Range: _____

MRM :
Transitions Monitored : 165 -> 111

HPLC Parameters

Injection Volume : 25 μ L

Column : MetaChem Metasil Basic 3 μ 50 mm X 2 mm

Column Serial # : 9527521

Mobile Phase :

Buffer A: 5 mM Ammonium Acetate; 0.1% Formic acid in
100% water

Buffer B: 5 mM Ammonium Acetate 0.1% Formic acid in
10% water; 90% Methanol

Gradient:

Time	Percent B
0	0
1	30
5	30
5.1	0
10	0

LC Detector: N/A

Flow Rate : 300 μ L/min

Split (Y/N) : No

Comments

Conclusions

A confirmation method was developed with required sensitivity.

Attached are chromatograms (2, 5, 10, 20 pg/ μ l), calibration curve, and result table.

MacQam, version 1.8
Printed: Mon, Sep 29, 1997 08:24
Calculation File: CalcFile SAS Path: BRAS3 DATA\LP Data\19970929
Comment: No comments

Page 4 of 5

BW 125-5378
No Internal Standard
185.0 -> 111.0
Linear
Intercept = -202.015
Slope = 13.403
Correlation Coeff. = 0.999
Use Area

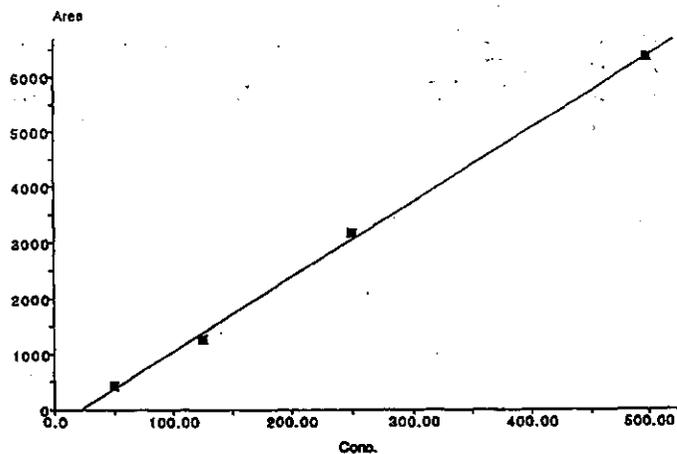
Element	Element	Sample Name	Conc.	Calc. Conc.	Height	Area
A870824208	Standard	20pp/t	200.000	487.864	1880	6388
A870824209	Standard	10pp/t	280.000	257.800	698	3106
A870824210	Standard	5pp/t	188.000	118.028	263	1263
A870824211	Standard	2pp/t	80.000	93.087	104	420
A870824212	Blank	Control	0.0	n / a	n / a	n / a

MacQuan, version 1.5
Printed: Mon, Sep 29, 1997 08:16
Calibration File: CalcFile.SAS Path: B:\MS DATA\GLP Data\199709-Sopt-1997.092(97)
Comments: No comments

Page 1 of 1

BW 125-5376 155.0->111.0 No Internal Standard
Linear

Intercept = -292.015
Slope = 13.403
Correlation Coeff. = 0.999



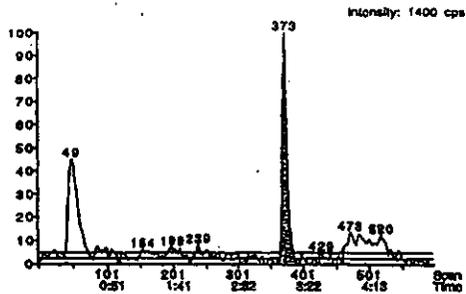
MacQuant, version 1.5
Printed: Mon, Sep 29, 1997 06:24
Calibration File: CalbFile 6AD Path: GEMAS DATA:GLP Data:1997-09-29-1997-09-24:87.
Comments: No comments

Page 1 of 3

A970924209 20pg/ul Wed, Sep 24, 1997 12:04
No Comment

5:01 in 1 period
BW 125-6376
No Internal Standard
Use Area

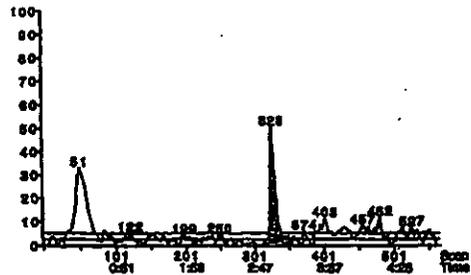
1: 490 MRM, 500 scans
155.0-111.0
Noise Thres. 4.0
Count Thres. 2.0
Min. Width 6
Max. Width 2
Base. Width 100
RT Wtd. (secs) 20
Smooth 8
Expected RT 3:08
Area 6383
Height 1890
Start Time 2:04
End Time 3:16
Integration Width 0:12.2
Retention Time 3:08
Integration Type A-08



A970924209 10pg/ul Wed, Sep 24, 1997 12:14
No Comment

5:01 in 1 period
BW 125-6376
No Internal Standard
Use Area

1: 490 MRM, 500 scans
155.0-111.0
Noise Thres. 4.0
Count Thres. 2.0
Min. Width 6
Max. Width 2
Base. Width 100
RT Wtd. (secs) 20
Smooth 8
Expected RT 2:05
Area 8168
Height 498
Start Time 2:57
End Time 3:29
Integration Width 0:12.2
Retention Time 2:50
Integration Type A-08

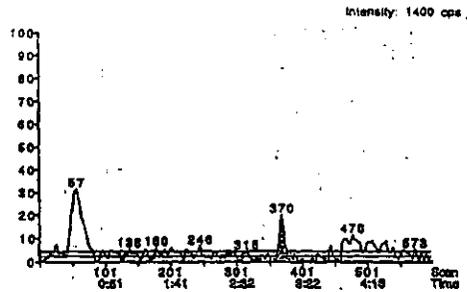


MacQuin, version 1.5
Printed: Mon, Sep 29, 1997 08:24
Calibration File: CalibFile.SAB Path: BILMS DATA:GLP Date:1997-09-24-1997-09-24:97
Comments: No comments

Page 2 of 3

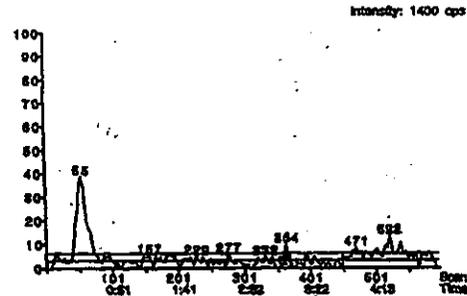
A670924210 6pg/ul Wed, Sep 24, 1997 12:25
No Comment

5:01 in 1 period
BW 125-5376
No Internal Standard
Use Area
1: 5:00 MRM, 600 scans
155.0-111.0
Noise Thresh. 4.0
Count Thresh. 2.0
Min. Width 6
Mult. Width 2
Base. Width 100
RT Wrt. (sec) 20
Smooth 5
Expected RT 3:08
Area 1258
Height 288
Start Time 3:02
End Time 3:11
Integration Width 0:09.1
Retention Time 3:08
Integration Type A-B



A670924211 2pg/ul Wed, Sep 24, 1997 12:38
No Comment

5:01 in 1 period
BW 125-5376
No Internal Standard
Use Area
1: 4:50 MRM, 600 scans
155.0-111.0
Noise Thresh. 4.0
Count Thresh. 2.0
Min. Width 6
Mult. Width 2
Base. Width 100
RT Wrt. (sec) 20
Smooth 5
Expected RT 3:08
Area 420
Height 104
Start Time 3:01
End Time 3:08
Integration Width 0:07.6
Retention Time 3:04
Integration Type M



MacQam, version 1.5
Printed: Mon, Sep 29, 1997 08:24
Calibration File: CalFile.SAB Path: 58543 DATA\GLP Data:1997-09-Sep-1997:092497:
Comments: No comments

Page 3 of 3

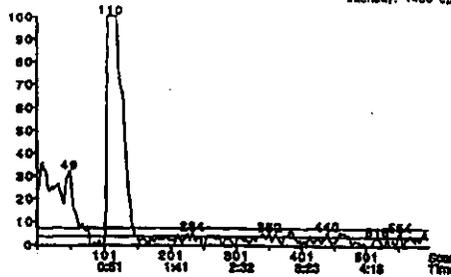
M. Watt

A870824212 Control Wed, Sep 24, 1997 18:14
No Comment

501 in 1 period
BW 128-6378
No Internal Standard
Use Area

Intensity: 1400 cps

1: 5:00 MRM, 098 scans
185.0->111.0
Noise Thresh 4.0
Quant Thresh 2.0
Min. Width 6
Mult. Width 2
Scan. Width 100
RT Wn. (sec) 20
Smooth 5
Exposed RT 3:08
Area 0
Height 0
Start Time 0:00.0
End Time 0:00.0
Integration Width 0:00.0
Retention Time 0:00.0
Integration Type



EPA ADDENDUM

PP#8F04941

BASF Method D9601 on Peanut Nutmeat
for BAS 125 W Residues
and

BASF Method D9608 on Apples
and Kidneys for BAS 125 W Residues

ACB successfully used teflon frits on the bottom and top of the glass columns for mini-isolute ENV + TM column chromatography in place of the 417 grade paper frits and glass wool cited in the analytical methods. If the petitioner wishes, this substitution can be added to the method as an alternate to the paper frits.

ACB used a Restek 4mm cyclo double gooseneck glass insert (cat. # 20896) in the GC inlet for all analyses. The ILV made this same substitution and noted that it increased their sensitivity to the BW 125-M7 analyte. Even with this substitution, ACB found that it was still necessary to use 4 uL injection volumes to achieve adequate sensitivity for the lowest fortification levels while monitoring three ions. ACB also extended the GC run times to prevent late eluting sample co-extractives from being carried over into subsequent injections.

ACB monitored three ions during the MSD analysis of all samples; m/z 226 (M+ of BW 125-M7, quantitation ion), 195 and 165 (base peak). Ion ratios were then calculated ($m/z\ 226\ \text{response} / m/z\ 165\ \text{response}$ and $m/z\ 195\ \text{response} / m/z\ 165\ \text{response}$) for confirmation of BW 125-M7 residues by comparison of calibration standard ion ratios to fortified sample ion ratios. ACB could not "confirm" residues of BW 125-M7 in beef kidney since ion ratios in these samples did not match expected ion ratios from calibration standards due to chromatographic interferences. The unfortified kidney control samples contained similar interferences in the BW 125-M7 retention time window for both m/z 195 and 165 ions. No interferences were detected in the m/z 226 ion, however, which enabled the analytical quantitation of BW 125-M7 residues in beef kidney.