Document No. 1
Volume 2 of 3

433283-02

STUDY TITLE

SUPPLEMENTAL ENZYME HYDROLYSIS METHOD FINAL REPORT
Determination of the Magnitude of the Residues of the Fungicide Thiabendazole in Wheat Treated with Mertect DF

DATA REQUIREMENT

Pesticide Assessment Guidelines Subdivision O
Guideline Reference No. 171-4

STUDY DIRECTOR

Jack A. Norton, Ph.D.

STUDY COMPLETION DATE

August 2, 1994

PERFORMING LABORATORY

ABC Laboratories, Inc.
Analytical Chemistry and Field Studies
7200 E. ABC Lane
Columbia, Missouri 65202

PROJECT IDENTIFICATION NUMBER

618-360-93020

Total Number of Pages: 34
Page 1 of 34

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1)(A), (B), or (C).

Company: Merck & Co., Inc.

Company Agent: Patricia A. Sheehy Date: 8/4/94

Title: Manager, Regulatory Affairs Coordination and Planning

Signature

These data are the property of Agricultural Research and Development, Merck & Co., Inc., and as such, are considered to be confidential for all purposes other than compliance with FIFRA §10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality which may exist under any other statute or in any other country.

STUDY COMPLIANCE STATEMENT

Study Compliance Statement for ABC Laboratories' Supplemental Enzyme Hydrolysis Method Final Report #39296E, "Determination of the Magnitude of the Residues of the Fungicide Thiabendazole in Wheat Treated with Mertect DF," for Merck Research Laboratories, Three Bridges, New Jersey.

ABC Laboratories' principal analytical investigator for the above test herein confirms that the study, as conducted by ABC Laboratories, was conducted in compliance with the U.S. EPA Good Laboratory Practice Standards; Pesticide Programs (40 CFR 160).

All original raw data produced by ABC Laboratories have been sent to Merck Research Laboratories with the final report. A copy of the report text and raw data has been retained at ABC Laboratories, Inc.

7/19/94

Jim Fieser — Date
ABC Laboratories'
Principal Analytical Investigator

Patricia A. Sheehy — Date
Submitter/Sponsor

Manager, Regulatory Affairs
Coordination and Planning
Agricultural Research and Development
Merck Research Laboratories
Merck & Co., Inc.

7/21/94

Jack A. Norton, Ph.D. — Date
Study Director
Agricultural Research and Development
Merck Research Laboratories
Merck & Co., Inc.

ATTACHMENT G

COVERSHEET FOR DATA SUBMITTED IN RESPONSE TO A DATA CALL-IN NOTICE

EPA Registration No(s): 616-67

Registrant's Name: Merck & Co. Inc.

Name of active ingredient: Thiabendazole

Type of Submission:

____ Commitment to submit data in the future

_X_ Original submission of data (If checked, complete certification below)

____ Duplicate submission of data (If checked, complete certification below)

Type of Study (identify applicable data requirements):
Pesticide Assessment Guidelines Subdivision O. GLR: 171-4
Residue analytical method.

Title of Study: Supplemental Enzyme Hydrolysis Method. Final Report. Determination of the Magnitude of the Residues of the Fungicide Thiabendazole in Wheat Treated with Mertect DF

Name and address of laboratory(s) or individual(s) who performed _X_, are performing ___, or will perform ___ the study. ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202

Numbers used by registrant or laboratory to identify the study:

Date when study was completed (if applicable) ~~7/14/94~~ 8/24/94 or date by which the study will be submitted to EPA (if it is not enclosed) _____.

Date when you expect study (in life portion) to be initiated by the laboratory _____ and date when you expect in life portion to be completed_____. In future progress reports, indicate when the study (in life portion) was actually initiated and completed.

CERTIFICATION: The signature below indicates I certify that this study meets all the requirements pertaining to the conditions for submittal of existing data outlined in Sec. III-C3a-d and -e, if applicable, of the Data Call-In Notice and I have attached the needed supporting information to this sheet. Also I certify that I have determined that this study fills the following data requirements (use same terminology as on DCI summary sheets):

Subdivision O, 171-4, Residue Analytical method

8/24/94
Date

Signature of Registrant's Representative

Submittal Instruction: When submitting a study, submit this coversheet behind the "Statement of Confidentiality Claims" as described on p. 17 of PR 86-5. Add to administrative materials when not submitting a study.

Note: You may receive requests from other EPA offices for added information on this study. When you respond to these requests, you do not need to provide the information to the Data Call In program.

004

618-360-93020

7200 E. ABC Lane, Columbia, MO 65202

Ph: 314/474-8579 Fax 314/443-9033

"Working for You"

STUDY TITLE

SUPPLEMENTAL ENZYME HYDROLYSIS METHOD FINAL REPORT

Determination of the Magnitude of the Residues of the Fungicide Thiabendazole in Wheat Treated with Mertect DF

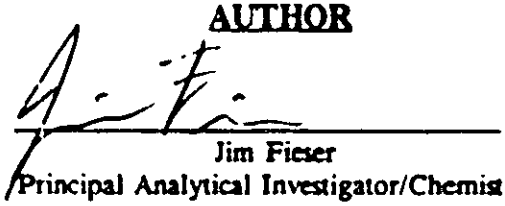
DATA REQUIREMENT

Guideline 171-4

TESTING LABORATORY

ABC Laboratories, Inc.
Analytical Chemistry and Field Studies
7200 E. ABC Lane,
Columbia, Missouri 65202

AUTHOR



Date: 7/19/94

Jim Fieser
Principal Analytical Investigator/Chemist II
ABC Laboratories, Inc.

MERCK STUDY NO. 93020

ABC LABORATORIES STUDY NO. 39296

Experimental Start Date: June 13, 1994

Experimental Termination Date: June 23, 1994

QUALITY ASSURANCE STATEMENT

ABC Laboratories' Quality Assurance Unit reviewed ABC Laboratories' Supplemental Enzyme Hydrolysis Method Final Report #39296E, "Determination of the Magnitude of the Residues of the Fungicide Thiabendazole in Wheat Treated with Mertect DF," for Merck Research Laboratories, Three Bridges, New Jersey. The following inspections/audits were conducted on this study.

Date of Inspection*	Phase Inspected	Date Reported To Study Director	Date Reported To Management
06/03/94	Enzyme Hydrolysis	06/23/94	06/23/94
06/21/94	Analysis for 4-MUF	06/23/94	06/23/94
06/28/94	Draft Report	Not Applicable	07/01/94**
07/19/94	Final Report	Not Applicable	07/19/94**

*Findings were reported to the principal analytical investigator.

**Reported to ABC Laboratories management.

The undersigned conducted the report audits. The audits indicate the report is an accurate reflection as it was conducted by ABC Laboratories, Inc.

Greg Veltri 7-19-94

Greg Veltri — Date
Quality Assurance Officer II

ABC Laboratories No. 39296E-5

APPROVAL

Submitted by:

ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202
~~314~~-474-8579
573-

Prepared by:

J. Fieser 7/19/94

Jim Fieser — Date
Principal Analytical Investigator
Chemist II, Analytical Chemistry and Field Studies

Approved by:

Greg Veltri 7-19-94

Greg Veltri — Date
Quality Assurance Officer II

Floyd E. Kaiser 7/19/94

Floyd Kaiser — Date
Manager, Analytical Chemistry and Field Studies

Jack O. Norton 8/2/94

Dr. Jack Norton — Date
Study Director, Merck Research Laboratories

:bb

PROJECT PERSONNEL

The principal analytical investigator for this project at ABC Laboratories, Inc., was Jim Fieser, Chemist II, Analytical Chemistry and Field Studies. The study director was Jack Norton, Ph.D., Director, Agricultural Research & Development, Merck Research Laboratories. The following ABC Laboratories' personnel were associated with various phases of the study.

Name	Title
Stephanie Agan	Analyst II
Jim Fieser	Chemist II
Larry Lucas	Chemist III

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ABSTRACT

A residue enforcement method for the determination of total BNZ in wheat grain and straw and residue data on selected samples of wheat grain, straw, and by-products treated with TBZ are reported in response to the EPA Phase 5 - Reregistration Review Residue Chemistry for MRID #42718401 on July 1, 1993. Additional data had been requested for the determination of the levels of conjugates of benzimidazole in wheat grain, straw, and wheat processing products from the magnitude of the residue study for thiabendazole in wheat (Merck Study No. 93020). The addition of an enzyme hydrolysis step in the method of this report is to fill the request for additional data.

Based on current residue data from selected samples from 4 of the 14 field trials and 2 processing studies, residues of total benzimidazole in wheat grain, straw, and grain processing products were <0.10 ppm.

INTRODUCTION

Nature of the Residue

In previous Merck petitions (MRID #41872901, #41872902, and #41872903), studies were reported in which wheat, treated with foliar applications of thiabendazole, were found to contain unchanged thiabendazole (TBZ) and the plant metabolite benzimidazole (BNZ) in mature wheat grain and straw. As a part of those petitions, ABC Study No. 37724 reported approximately one third of the residue in mature wheat straw as "BNZ-related" which released BNZ upon treatment with β-glucosidase[1].

Components of the Residue Report

This report, ABC Laboratories No. 39296E, details a residue enforcement method for total BNZ which employs the enzyme β-glucosidase to free BNZ from BNZ-related compounds. It is a supplement to the original report(s) of ABC Study No. 39296 issued March 9, 1993. In those reports, ABC Laboratories reported the results of the analysis of wheat grain, straw, and grain processing products from various field trials and processing studies for residue levels of thiabendazole and unconjugated benzimidazole. Results of the original analysis of field samples are found in the report ABC Laboratories No. 39296A. Analytical methods developed for the analyses of field samples using organic solvent extraction and/or a basic reflux (without enzyme hydrolysis) are described in detail in the Analytical Method report, ABC Laboratories No. 39296M. Summaries of stability data on wheat grain, bran, flour, and straw are included and discussed in the Freezer Stability report, ABC Laboratories No. 39296S. The raw data for all original analyses are included in ABC Laboratories No. 39296R.

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[1]ABC Laboratories Report No. 37724, page 13, Table; "Summary of ^{14}C TBZ Wheat Metabolism Results"

ANALYTICAL METHOD

Principle

The metabolite benzimidazole (and BNZ-related compounds[2]) are extracted from wheat grain, straw, and wheat processing products by shaking with methanol and refluxing with 3 N KOH/methanol. The extracts are filtered and combined in a flat bottom flask. Concentrated hydrochloric acid is added to precipitate potassium chloride. The salt is filtered and the filtrate rotary evaporated to a small volume of water (~ 20 mL). A buffer is added as well as KOH to adjust the pH of each extract to 5. An aliquot of a buffered solution of β-glucosidase is added and the extracts are incubated at 37 °C for at least two hours. (Activity of the enzyme is verified and is discussed later in this text.)

The residues are acidified with HCl and the aqueous solution is transferred to a separatory funnel. Ethyl acetate is added and the lipophilic compounds that partition into the organic layer are discarded. The aqueous portion is adjusted to pH 9-12 (with the addition of KOH and a buffer), extracted three times with ethyl acetate, and the combined extracts are evaporated to dryness. The residue is reconstituted with 1% ammonium acetate in water and analyzed for benzimidazole by HPLC using fluorescence detection.

MATERIALS

Equipment

1. Flat bottom flasks, 500 mL and 250 mL, with reflux condenser
2. Linear shaker
3. Heating mantle, 200 watt with variable transformer
4. Büchner funnel
5. Rotary evaporator
6. Separatory funnel, 500 mL
7. Incubator (water bath or equivalent)
8. Powder funnels
9. Mettler PM200 electronic balance (or equivalent)

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[2]Based on the metabolism report ABC Study No. 37724.

10. HPLC equipment (see Instrumentation section)

Reagents

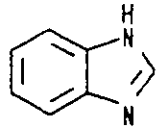
11. Methanol — HPLC grade, Burdick & Jackson
12. Acetone — Pesticide grade, Burdick & Jackson
13. Ethyl Acetate — Pesticide grade, Burdick & Jackson
14. Acetic Acid — Reagent grade, Fisher
15. Potassium Hydroxide — Reagent grade, J.T. Baker
16. Ammonium Acetate — HPLC grade, J.T. Baker
17. Sodium Carbonate — Reagent grade, Fisher
18. Hydrochloric Acid — Reagent grade, Fisher
19. Reagent Grade Water — LABCONCO Water Purification
20. Buffer Solution for pH Meter Calibration — pH=7
21. Buffer Solution for pH Meter Calibration — pH=4
22. Cotton Balls — Generic
23. Boiling Chips
24. Whatman filter papers; 2V, #4, and #40
25. β-glucosidase (Sigma Cat. # G-0395, 5.5 units per mg)
26. Potassium Phosphate (monobasic) — Reagent grade, J.T. Baker

Analytical Standards

27. Reference Standard

Chemical Name:	1,3-benzodiazole
Common Name:	benzimidazole
CAS No.:	51-17-2
Lot#:	0280JT
Purity:	98 %
Supplier:	Aldrich Chemical Company

Chemical Structure:



28. Standards for Assessment of Enzyme Activity

Chemical Name:	7-Hydroxy-4-methylcoumarin
Common Name:	4-methylumbelliferone
CAS No.:	90-33-5
Lot #:	13H0722
Purity:	Not Given
Supplier:	Sigma Chemical Company

Chemical Name:	4-methylumbelliferyl β-D-glucoside
Common Name:	4-methylumbelliferyl β-D-glucopyranoside
CAS No.:	18997-57-4
Lot #:	120H5002
Purity:	Not Given
Supplier:	Sigma Chemical Company

PREPARATION OF STANDARD SOLUTIONS

Preparation of Reference Standard Solutions

Weigh accurately 51.4 mg (corrected for purity) of benzimidazole (BNZ) reference standard into a 50-mL volumetric flask and dilute to volume with methanol. The solution contains 1.03 mg benzimidazole/mL. Label the solution, "BNZ STOCK SOLUTION 1.03 mg/mL".

Preparation of Benzimidazole Fortification Solutions

1. Transfer 0.97 mL of "BNZ STOCK SOLUTION 1.03 mg/mL" to a 100-mL volumetric flask. Dilute to the 100-mL mark with methanol. The solution contains 10 mcg/mL of BNZ. Label the solution, "BNZ STOCK SOLUTION 10 mcg/mL."

2. Transfer 10 mL of "BNZ STOCK SOLUTION 10 mcg/mL" to a 100-mL volumetric flask. Dilute to the 100-mL mark with methanol. The solution contains 1 mcg/mL of BNZ. Label the solution, "BNZ STOCK SOLUTION 1 mcg/mL."

Preparation of BNZ HPLC Calibration Standard Solutions

Transfer 5-, 2.5-, 1-, and 0.5-mL aliquots of "BNZ STOCK SOLUTION 10 mcg/mL" to separate 100-mL volumetric flasks and dilute each flask to the mark with 1% ammonium acetate in water. The solutions contain 500, 250, 100, and 50 ng/mL of BNZ. Label the solutions appropriately. Transfer a 5-mL aliquot of the "BNZ STOCK SOLUTION 10 mcg/mL" to a 50 mL volumetric flask and dilute to volume with 1% ammonium acetate for a 1,000 ng/mL standard.

[Note 1. Stock and HPLC solutions were stored in the refrigerator when not in use.]

ANALYTICAL PROCEDURE

Method Chronology

The analytical methodology used for the residue analysis of the thiabendazole plant metabolite benzimidazole (BNZ) in wheat grain, straw, and wheat processing products was developed by ABC Laboratories, Columbia, Missouri. Initial extraction conditions were optimized based on the results of the plant metabolism study for thiabendazole in wheat (MRID #41872901, #41872902, and #41872903). Cleanup and analysis techniques were based on the original residue report (MRID #42718401). The enzyme hydrolysis step was included in the current work to free BNZ from BNZ-related compounds. The analytical method was used to determine total benzimidazole residues in and on wheat grain, straw, and wheat processing products at residue levels as low as 0.10 ppm.

Sample Preparation

1. The wheat grain, straw, and wheat grain processing products are ground and store frozen at approximately - 20 °C prior to processing and analysis.

Extraction

2. Weigh 10.0 g of sample into a 500-mL flat bottom flask (for extraction and reflux). Method recovery check samples are fortified at the 0.1-2 ppm level with benzimidazole at this time.

3. Add 100 mL of methanol to each flat bottom flask (reflux flask) and place a stopper in the opening. The flasks are placed on a linear shaker with padding between them and shaken at approximately 150 excursions per minute for about 1 hour.

4. Filter the extract into a 250-mL flat bottom flask via a Whatman 2V fluted filter and funnel, retaining the bulk of the solid residue in the extraction/reflux flask. When most of the extract has filtered through the filter, shake or scrape the filtered particles back into the reflux flask.

5. Rinse the remaining solid residue from the fluted filter into the reflux flask with 100 mL 3 N KOH (300 meq.) in methanol and add boiling stones to the reflux flask. Connect a reflux condenser to the mouth of the reflux flask (use a Teflon sleeve to protect the ground glass connection) and place it on a 200 watt heating mantle with a variable transformer set to approximately 50 volts. A sufficient flow of coolant is provided to the reflux condenser jacket and the reflux maintained for approximately 16 hours.

6. Turn off the power to the heating mantle and rinse the condenser with a maximum of 5 mL methanol into the reflux flask. Filter the extract through a Büchner funnel containing an 11-cm Whatman #4 filter (wet with <5 mL methanol) into the 250-mL flat bottom flask containing the filtrate from step 3. Rinse the contents of the reflux flask with <5 mL methanol and pass the rinse over the reflux residue into the 250-mL flask.

7. Slowly add 25 mL of concentrated HCl (~300 meq.) to the flask, precipitating potassium chloride. Allow the sample to cool and filter (as in step 6 with a Whatman #40) into a clean 500-mL flat bottom flask, rinsing with <10 mL methanol. Rotary-evaporate the contents of the second flask until ~ 20 mL remains.

Cleanup

8. Add 25 mL 0.02 M KH_2PO_4 to the flask and adjust the pH to 5 with 5 N and/or 2 N KOH. Add 1 mL of a 1% solution of β-glucosidase (Sigma Cat. # G-0395, 5.5 units per mg). Swirl the flask to mix. (**Optional**: Fortification of quality control samples with 1 micromole of 4-MUF-GLU to ensure enzyme activity is made here. Fortify one quality control sample omitting the addition of enzyme.) Incubate at 37 °C for at least 2 hours.

9. When samples are fortified with 4-MUF-GLU, pour the contents of one control per matrix and the 4-MUF-GLU fortified samples into graduated mixing cylinders and fill to the 100 mL mark with D.I. water. Transfer < 1 mL to a injection vial and analyze by HPLC vs. a 10 micromole solution of 4-MUF to determine the activity of the enzyme.

10. Pour the aqueous extract into a 500-mL separatory funnel. Add 5 mL of 1 N HCl to the separatory funnel to change the pH to <3. Rinse the flask with 100 mL ethyl acetate and pour that rinse into the separatory funnel. The separatory funnel is shaken for 1 minute and the layers allowed to separate.

11. Drain the acidic aqueous (lower) portion into the flat bottom flask and empty the organic portion to waste. Return the contents of the flat bottom flask to the separatory funnel. Add 4 mL of 2 N KOH to the separatory funnel and swirl to mix. Add 25 mL of 2 N Na_2CO_3 to buffer the aqueous extract.

12. Add 100 mL of ethyl acetate to the separatory funnel; cap and shake for approximately one minute and allow the phases to separate.

13. Drain the basic aqueous (lower) layer (and any interphase emulsion) into the flat bottom flask. Pass the clear organic partition through a cotton pledget in a filter funnel into a clean 500-mL flat bottom flask.

14. Return the aqueous solution to the separatory funnel and repeat steps 11 and 12 two more times, combining the organic portions for a total of three 100-mL partitions.

15. Rotary evaporate the organic portion to dryness.

16. Reconstitute the residue with 10 mL of 1% ammonium acetate.

17. Transfer the reconstituted extract to a culture tube. Dilutions are made with 1% ammonium acetate if necessary. The extracts are now ready for quantitation by reversed phase HPLC using fluorescence detection.

INSTRUMENTATION FOR BENZIMIDAZOLE AND 4-MUF ANALYSES

HPLC Equipment and Conditions

(Equipment from other manufacturers may be used if they are shown to be functionally equivalent.)

1. HPLC System: Shimadzu 6A HPLC system equipped with an autosampler, controller, and pump.

2. HPLC Column: Supelco LC-8-DB, 25 cm x 4.6 mm, 5-micron particle size

3. Detector: Fluorescence, dual monochrometer, Varian Model 2070

   BNZ — excitation wavelength 261 nm
   — emission wavelength 300 nm

   4-MUF — excitation wavelength 335 nm
   — emission wavelength 435 nm

4. Recorder/Integrator: MULTICHROM chromatography data system run on a DEC MicroVax 3800 computer

5.	Mobile Phase:	BNZ — 750 mL water:250 mL methanol:1 g ammonium acetate 4-MUF — 600 mL water:400 mL methanol:1 g ammonium acetate
6.	Flow Rate:	2.0 mL/min
7.	Injection Volume:	100 μL
8.	Column Temp.:	Ambient

INTERFERENCES

Sample Matrices and Reagents

Interferences from the matrices for control samples subjected to the analytical procedure were <0.01 ppm apparent benzimidazole residues.

CONFIRMATORY TECHNIQUES

HPLC Retention/Fluorometric Detection of Analytes

The residues are identified by comparing the chromatographic retention time of the peak in the final sample extract with the chromatographic retention time of benzimidazole reference standards. The specificity of the dual monochrometer fluorometer is such that no 4-MUF peak is apparent when the instrument is optimized for BNZ and vise versa. Thus, the reverse phase HPLC with fluorometric detection is a specific method for benzimidazole.

Confirmation of Enzyme Activity

The fortification and hydrolysis of a glucose conjugate of 4-methylumbelliferone (4-methylumbelliferone β-D-glucoside or 4-MUF-GLU) and analysis of that hydrolysis product (4-methylumbelliferone or 4-MUF) confirms the activity of the enzyme in extracts. The lack of recovery of the hydrolysis product in a sample to which no enzyme has been added is a further indication that hydrolysis occurs due to the activity of the enzyme.

METHOD OF CALCULATION

Chromatographic Acquisition

MULTICHROM is a computer program purchased from VG systems and is run on a Digital Equipment Corporation MicroVax 3800 computer. The program allows for data acquisition, data analysis, and

reporting of results. The chromatographic signals from the detector are digitized in VG Chromatography Servers and are downloaded to the computer via a fiberoptic Ethernet network.

The MULTICHROM program measures chromatographic peak areas for standards and samples and then uses the standard concentrations versus peak areas to calculate a regression curve. The analyte concentration in each sample extract is interpolated from the regression curve. The concentration is then converted to parts per billion (ppb) of analyte in the sample using the following equation after entering the final volume, dilution factor, and sample weight into the MULTICHROM system.

$$\text{ppb analyte in sample} = \frac{C \times V \times DF}{W}$$

where:

C = concentration of analyte in final HPLC assay solution in ng/mL
V = final volume of HPLC assay solution in mL
DF = final dilution factor
W = weight of sample in g

Spreadsheet Calculations

The ppb levels of analyte in the sample derived from the MULTICHROM data system were converted to ppm and entered into a spreadsheet program (Excel) on a personal computer to calculate the recovery of analyte from fortified samples and to provide correction for recoveries to authentic samples. The percent recovery of the analyte from fortified samples corrected for background was calculated as follows:

$$\text{Corrected \% Recovery (Method Recovery Samples)} = \frac{[\text{ppm found} - \text{ppm in control}]}{\text{ppm added}} \times 100$$

The corrected percent recoveries for the method recovery samples run on the same day as the treated samples are used to correct the ppm of analyte in treated samples. No corrections are made if the percent recovery of analyte from the method recovery sample is equal to or greater than 100%. The residue is corrected for percent recovery as follows:

$$\text{ppm analyte in treated sample (corrected for procedural recovery)} = \frac{[\text{ppm analyte found in treated sample}]}{\text{average corrected \% recovery (Method Recovery Sample)}} \times 100$$

RESULTS AND DISCUSSION

Method Validation

The method was validated with two controls (one in each matrix; straw and grain) and fortified controls (one in each matrix) for each of the following levels: 0.1 ppm, 0.5 ppm, and 2 ppm. One additional fortified control for the low and intermediate levels were included for each matrix in order to provide statistical data in the event that a matrix bias exists.

Table I summarizes the wheat grain method validation. The overall average for 5 fortified quality controls in grain is 84% with a standard deviation of 4.8%. Table II summarizes the wheat straw method validation. The overall average for 5 fortified quality controls in straw is 73% with a standard deviation of 2.6%. A significant bias in recovery is shown to exist between the matrices.

Example chromatograms of the BNZ analysis are included in Figures 1 through 4.

Precision

The precision of the analytical method is expressed as the relative standard deviation of the test results. The overall precision (coefficient of variation or CV) of the analytical method is calculated from the standard deviation and the mean value of the percent recovery of thiabendazole from fortified control samples using the following equation:

$$CV = \frac{\sigma \text{ (Standard Deviation of the \% Recoveries)}}{\text{Mean \% Recovery}} \times 100$$

The results of concurrent recovery samples for all three matrices are tabulated in Table III. These recoveries (combined with the method validation recoveries in Tables I and II) and their statistics are summarized below:

Accuracy and Precision of Analytical Method				
Matrix	N	Mean Percent Recovery	SD	Precision (CV)
Grain	6	86%	5.1%	5.9%
Proc. Prod.	1	92%	-	-
Straw	6	73%	2.7%	3.7%

Where n = the number of fortified controls included in the statistics.

Linearity

The standard calibration curve for the quantitation of BNZ consisted of five concentrations of the analytes and was injected on the HPLC during the analysis of samples. The areas for the analyte peaks were shown to be approximately linear (quadratic with a low x^2 coefficient) over the concentration range of sample solutions. Subjected to quadratic regression analysis, the correlation coefficient for every set was >0.995.

Limits of Detection and Quantitation

The limit of detection is equal to the ppm equivalent of the lowest standard injected with the set. The limit of quantitation is the lowest benzimidazole fortification level for which recovery data are deemed acceptable. The limit of quantitation and detection of the analytical method was 0.1 and 0.05 ppm, respectively, for benzimidazole in wheat grain, straw, and processing products.

Specificity

The analytical method is highly specific for detecting benzimidazole residues in wheat grain, straw, and processing products. Since the method uses fluorescence detection as a means of quantitating benzimidazole residues, other pesticides or fungicides which do not have inherent fluorescence are not expected to interfere with the analysis for benzimidazole.

Enzyme Activity

One sample in each matrix on each analysis day was fortified with 1 micromole of 4-MUF-GLU per 10 g matrix (~ 34 ppm) prior to the 2 hour incubation with enzyme. The results of these analyses are tabulated in Table IV. Examples chromatograms of the 4-MUF analysis are included in Figure 5.

The straw recoveries of 4-MUF (avg. 66%) are significantly below the recoveries of the other matrices. Additional enzyme and a significantly longer incubation time did not significantly increase the recovery for sample # B 3.2. After two times the standard amount of enzyme and 24 hours of incubation, the recovery of 4-MUF for that sample was still only 68%.

Sample No. B 21 was fortified with 4-MUF-GLU without the addition of enzyme. Only 8.7% of the glucose conjugate was hydrolyzed to 4-MUF indicating that the enzyme was necessary for significant hydrolysis to occur.

Results of Analysis

All benzimidazole residue levels in treated wheat grain, straw, and processing products in actual field samples were below the limit of quantitation (< 0.10 ppm) and well below the current 1 ppm tolerance for grain and straw, and 3 ppm for processing products. The treated field samples analyzed are as follows:

Grain	001-90-3002R05 and 001-90-3006R06
Processing Products	001-90-3014R14 (Red Dog) and 001-90-3015R11 (Bran)
Straw	001-90-3008R14 and 001-90-3010R15

The results of these sample analyses are detailed in Table III.

TABLES

Table I
Benzimidazole Method Validation
Matrix: Wheat Grain

ABC LAB #	Sample ID	Fortification Level BNZ ppm	Date Extracted	Date Injected	File Name	Inj.	Percent Recovery
B7.2	Control	0	6/13/94	6/16/94	BGMV	3	-
B8.2	Control +	0.1	6/13/94	6/16/94	BGMV	4	90%
B9.2	Control +	0.1	6/13/94	6/16/94	BGMV	5	79%
					AVG.		85%
B10.2	Control +	0.5	6/13/94	6/16/94	BGMV	7	80%
B11.2	Control +	0.5	6/13/94	6/16/94	BGMV	8	88%
					AVG.		84%
B12.2	Control +	2	6/13/94	6/16/94	BGMV	9	85%
					Overall Average		84%
					SD		4.8%

Table II
Benzimidazole Method Validation
Matrix: Wheat Straw

ABC LAB #	Sample ID	Fortification Level BNZ ppm	Date Extracted	Date Injected	File Name	Inj.	Percent Recovery
B1.2	Control	0	6/13/94	6/17/94	BSMV1	3	-
B2.2	Control +	0.1	6/13/94	6/17/94	BSMV1	4	71%
B3.2	Control +	0.1	6/13/94	6/17/94	BSMV1	5	70%
					AVG.		71%
B4.2	Control +	0.5	6/13/94	6/17/94	BSMV1	7	74%
B5.2	Control +	0.5	6/13/94	6/17/94	BSMV1	8	76%
					AVG.		75%
B6.2	Control +	2	6/13/94	6/17/94	BSMV1	9	75%
					Overall Average		73%
					SD		2.6%

Table III
Total Benzimidazole Analysis Results

ABC Lab #	Sample ID	Fortification Level BNZ ppm	Date Extracted	Date Injected	File Name	Inj.	Percent Recovery	ppm Corr. for % Recovery
Wheat Straw								
B13	001-90-3003R9, 10, 11, 12	0	6/20/94	6/23/94	BSAM1	3	-	-
B14	001-90-3003R9, 10, 11, 12 +	0.1	6/20/94	6/23/94	BSAM1	4	70%	-
B15	001-90 3008R14	0	6/20/94	6/23/94	BSAM1	5	-	< 0.10
B16	001-90-3010R15	0	6/20/94	6/23/94	BSAM1	6	-	< 0.10
Wheat Processing Products								
B17	001-90-3014R6 Red Dog	0	6/20/94	6/23/94	BSAM1	8	-	-
B18	001-90-3014R6 Red Dog +	0.1	6/20/94	6/23/94	BSAM1	9	92%	-
B19	001-90-3014R14 Red Dog	0	6/20/94	6/23/94	BSAM1	10	-	< 0.10
B20	001-90-3015R11 Bran	0	6/20/94	6/23/94	BSAM1	11	-	< 0.10
Wheat Grain								
B21	001-90-3013R1, 2, 3, 4 001-90-3006R06	0	6/20/94	6/23/94	BSAM1	13	-	-
B22	001-90-3013R1, 2, 3, 4 +	0.1	6/20/94	6/23/94	BSAM1	14	91%	-
B23	001-90-3002R05	0	6/20/94	6/23/94	BSAM1	15	-	< 0.10
B24		0	6/20/94	6/23/94	BSAM1	16	-	< 0.10

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Table IV
Enzyme Activity

ABC Lab #	Matrix	Percent Recovery 4-MUF from 4-MUF-GLU
B 3.2	Straw	64%
B 9.2	Grain	88%
B 14	Straw	67%
B 18	Red Dog (Proc. Prds.)	88%
B 21	Grain (No Enzyme)	8.7%
B 22	Grain	89%

FIGURES

Figure 1

Chromatogram of a Typical Benzimidazole Calibration Standard

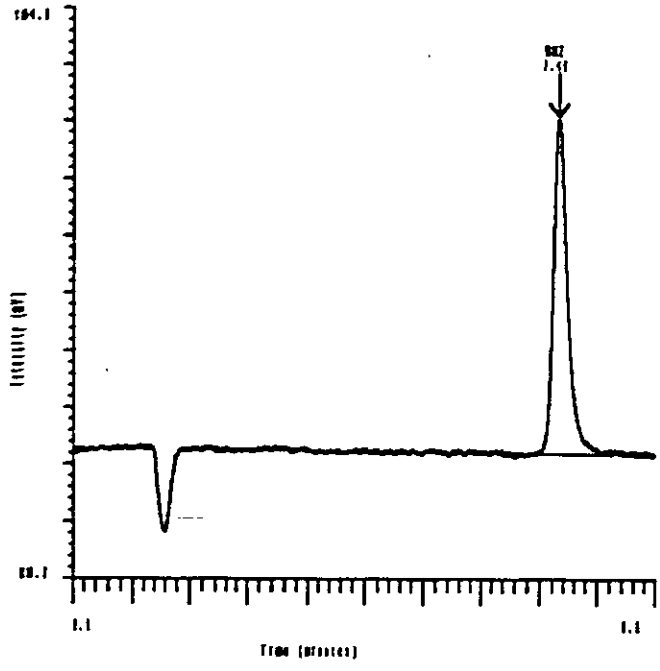

Figure 1 The 50 ng/mL BNZ standard is the lowest HPLC calibration standard (approximately equivalent to a sample containing 0.1 ppm of TBZ in which 100% of the residue converted to BNZ).

Figure 2

Wheat Straw Control and Fortified Control Chromatograms

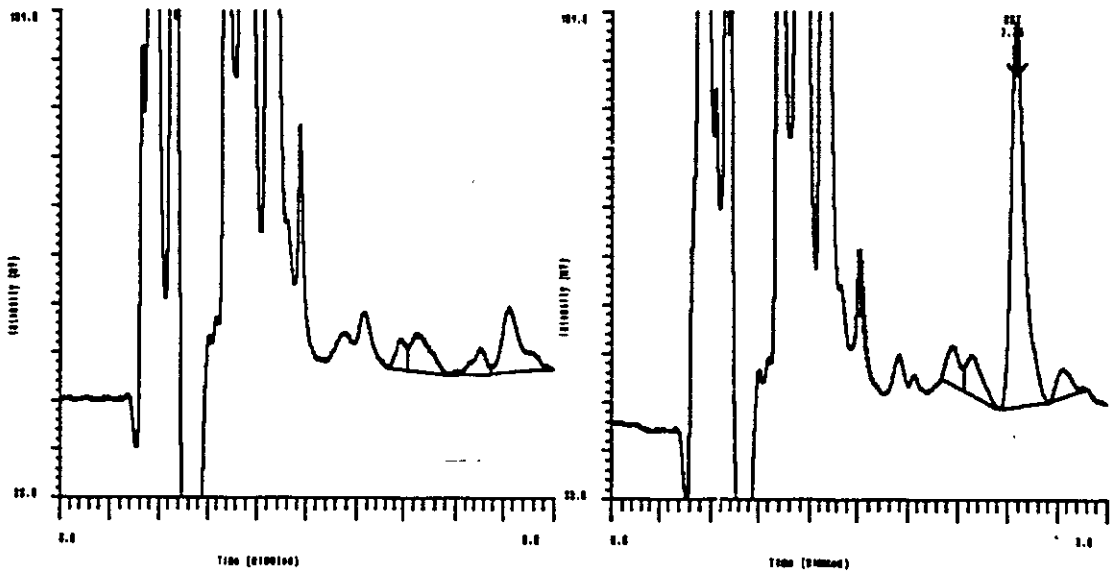

Figure 2 The chromatogram on the left is the control and the chromatogram on the right is the quality control fortified at 0.1 ppm.

Figure 3
Wheat Grain Control and Fortified Control Chromatograms

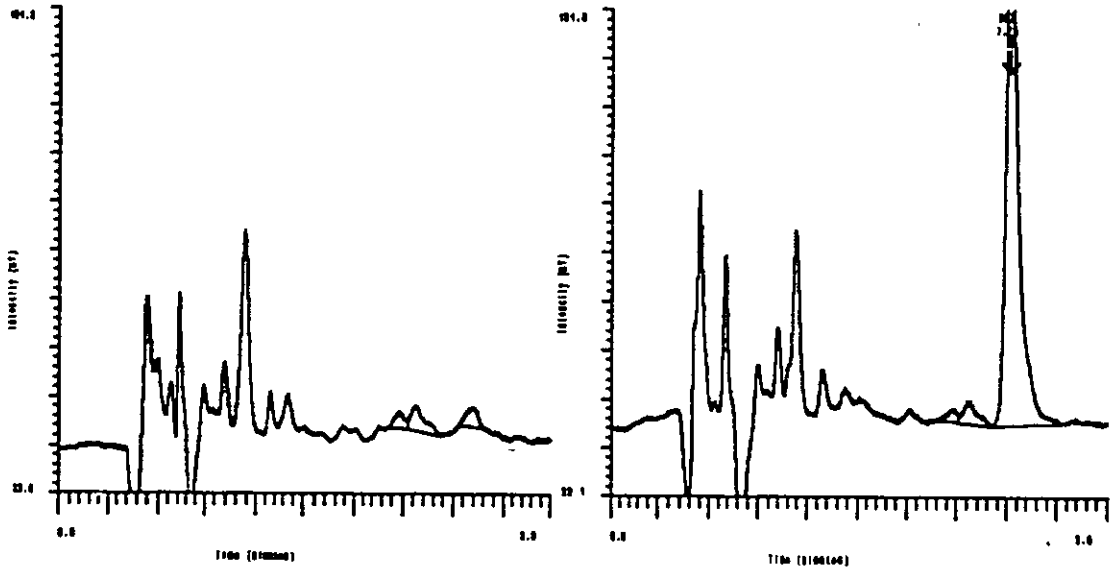

Figure 3 The chromatogram on the left is the control and the chromatogram on the right is the quality control fortified at 0.1 ppm.

Figure 4

Wheat Processing Products (Red Dog) Control and Fortified Control Chromatograms

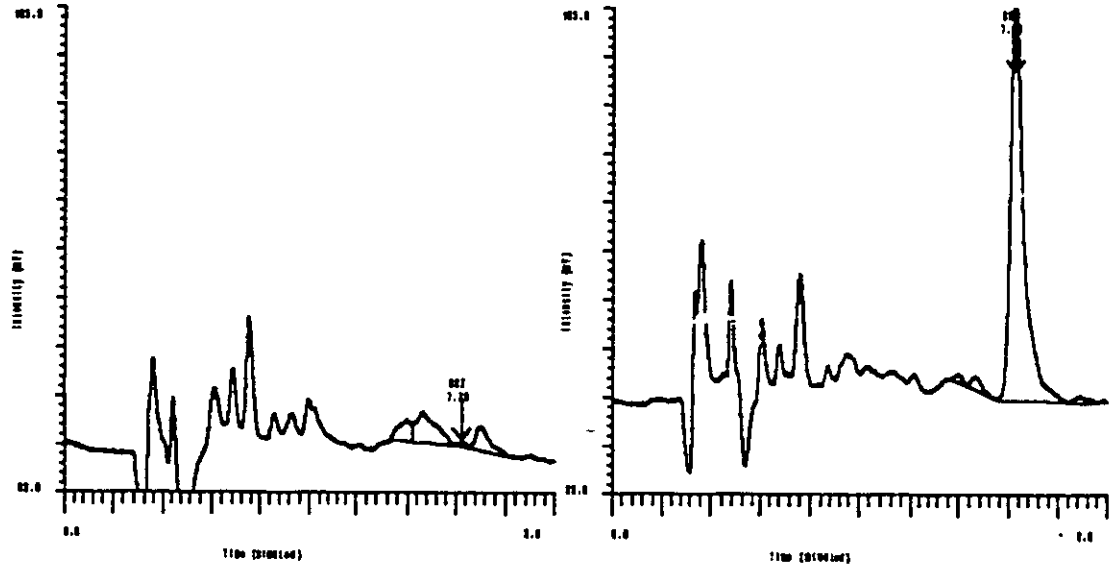

Figure 4 The chromatogram on the left is the control and the chromatogram on the right is the quality control fortified at 0.1 ppm.

Figure 5

Typical Standard, Control, and Fortified Control 4-MUF Chromatograms

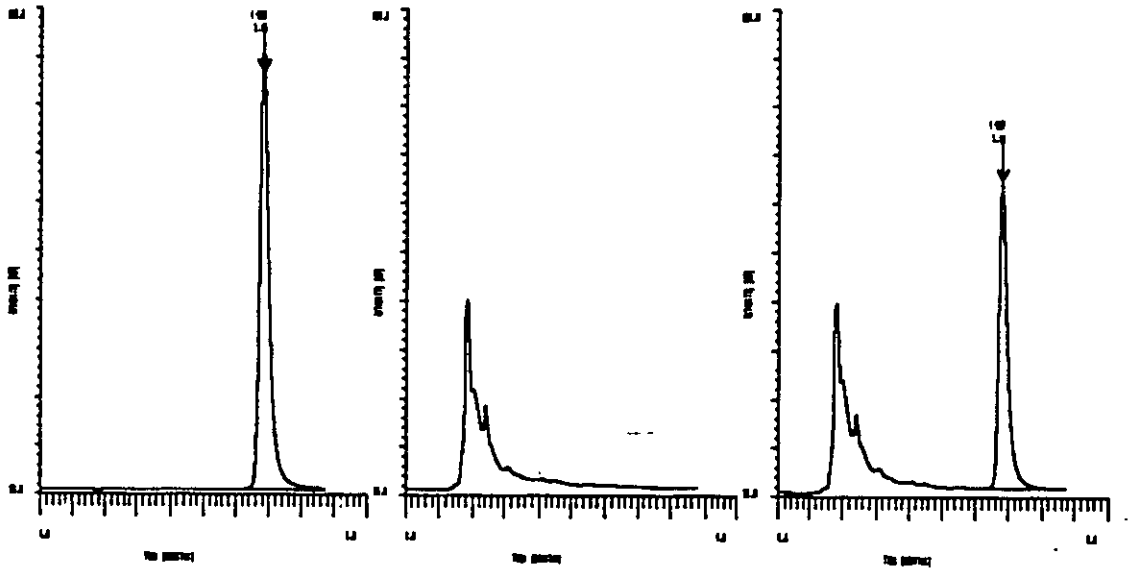

Figure 5 The chromatogram on the left is the 10 μM calibration standard, the center chromatogram is the wheat straw control, and on the right is the wheat straw quality control fortified at 10 μM.

Note: The quality control was originally fortified at 34 ppm (1 millimole in 10 grams of matrix) with 4-MUF-GLU. After hydrolysis with β-glucosidase and dilution to 100 mL, the theoretical concentration of 4-MUF is 10 μM.