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Determination of Residues of Isoxaben in Cereals, Chicory and Endives by Liquid Chromatography with Tandem Mass Spectrometry

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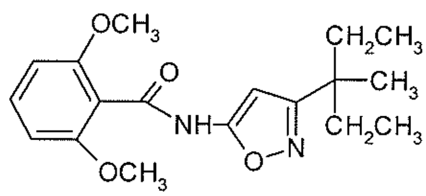
1. SCOPE

This method is applicable for the quantitative determination of isoxaben (*N*-(3-(1-ethyl-1-methylpropyl)-5-isoxazolyl)-2,6-dimethoxybenzamide) in cereals (wheat and barley), chicory (roots and leaves) and whole endives. The method was validated over the concentration range of 0.010-1.0 µg/g with a validated limit of quantitation of 0.010 µg/g.



Isoxaben
CAS Number 82558-50-7

2. PRINCIPLE

Residues of isoxaben are extracted from the crop samples by homogenizing and shaking with an acetonitrile/water solution (70:30). An aliquot is diluted with water and applied to a liquid-liquid extraction column. Isoxaben residues are partitioned from the aqueous sample extract into dichloromethane during the elution process. The eluate is evaporated to dryness and the residues reconstituted in a methanol:water (50/50) mobile phase containing 5mM ammonium acetate. The final solution is analyzed by liquid chromatography with positive-ion atmospheric pressure chemical ionization tandem mass spectrometry (LC/MS/MS).

A calibration curve resulting from the injection of eight standards demonstrated linearity with a correlation coefficient of at least 0.998. LC/MS/MS affords a highly specific method for quantitation and confirmation of isoxaben by retention time matching with standards in conjunction with monitoring a compound specific precursor-ion/product-ion transition (*m/z* 333/165).

3. SAFETY PRECAUTIONS

3.1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE MATERIAL SAFETY DATA SHEETS, LITERATURE, AND OTHER RELATED DATA. Safety information on non Dow AgroSciences LLC products should be obtained from the container label or from the supplier. Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.

3.2. Acetonitrile and methanol are flammable and volatile and should be used in well-ventilated areas away from ignition sources. It is imperative that proper eye and personal protection equipment be worn when handling these chemicals.

3.3. Dichloromethane is toxic. It is imperative that proper eye and personal protection equipment be worn when handling this reagent.

4. EQUIPMENT (Note 12.1.)

4.1. Laboratory Equipment

4.1.1. Balance, analytical, Model AE100, Mettler Instrument Corporation, Hightstown, NJ 08520.

4.1.2. Balance, analytical, Model P-1200, Mettler Instrument Corporation.

4.1.3. Evaporator, TurboVap LV, Zymark Corporation, Hopkinton, MA 01748.

4.1.4. Hammer mill, with 3/16-inch screen, Model 2001, AGVISE Laboratories, Inc., Northwood, ND 58267.

4.1.5. Homogenizer, Omni-mixer, Model ES, Omni International, Inc., Warrenton, VA 20187.

4.1.6. Homogenizer generator, Omni-mixer, catalog number 15010W, Omni International, Inc.

4.1.7. Pipetter, adjustable, Eppendorf, 10-100 µL, catalog number 05-402-48, Fisher Scientific, Pittsburgh, PA 15275.

4.1.8. Pipetter, adjustable, Eppendorf, 50-1000 µL, catalog number 21-378-83, Fisher Scientific.

4.1.9. Shaker, variable speed reciprocating with box carrier, Model 6000, Eberbach Corporation, Ann Arbor, MI 48106.

4.1.10. Ultrasonic cleaner, Model 1200, Branson Cleaning Equipment Company, Shelton, CT 06484.

4.1.11. Vacuum manifold, VacMaster-20, catalog number 121-2027, International Sorbent Technology Ltd, Hengoed, Mid Glamorgan UK and distributed by Jones Chromatography USA, Inc., Lakewood, CO 80228.

4.1.12. Vortex mixer, Model G-560, Scientific Industries, Inc., Bohemia, NY 11716.

4.2. Chromatographic Equipment (Note 12.1.)

4.2.1. Column, analytical, Diazem Pharma C18-A, 4.6 x 100 mm, 5-µm, catalog number 080C18Q-10046, Diazem Corporation, Midland, MI 48640.

4.2.2. Liquid chromatograph autosampler, Model 1100, Agilent Technologies.

4.2.3. Liquid chromatograph binary pump, Model 1100, Agilent Technologies.

4.2.4. Liquid chromatograph degasser, Model 1100, Agilent Technologies.

4.2.5. Mass spectrometer, Model API 3000, Applied Biosystems, Foster City, CA 94404.

4.2.6. Mass spectrometer data system, Analyst 1.1, Applied Biosystems.

5. GLASSWARE AND MATERIALS (Note 12.1.)

5.1. Bottle, 1.0-L media bottle, catalog number 06-423-3D, Fisher Scientific.

5.2. Bottle, 2.0-L media bottle, catalog number 06-423-3E, Fisher Scientific.

5.3. Bottle, 8-oz (237-mL), round, wide-mouth, with PTFE-lined screw cap, catalog number B7768, National Scientific Company, Lawrenceville, GA 30243.

5.4. Cartridge, HM-N, 0.3 mL capacity, catalog number 800-0040-BM, International Sorbent Technology Ltd, Hengoed, Mid Glamorgan UK and distributed by Jones Chromatography USA, Inc.

5.5. Culture tube, 12-mL (16 x 100 mm), catalog number 99449-16, Corning Products, Corning, NY 14831.

5.6. Cylinder, graduated, 1000-mL, catalog number C7000-1L, National Scientific Company.

5.7. Cylinder, graduated, 2000-mL, catalog number C7000-2L, National Scientific Company.

5.8. Flask, volumetric, 100-mL, catalog number 161-8987, National Scientific.

5.9. Pipet, 10-mL disposable seriological, catalog number, 13-666-7E, Fisher Scientific.

5.10. Pipet, 3-mL disposable transfer, catalog number, 13-711-7, Fisher Scientific.

5.11. Pipet, volumetric, 0.5-mL, catalog number 261-6010, National Scientific Company.

5.12. Pipet, volumetric, 1.0-mL, catalog number 261-6011, National Scientific Company.

5.13. Pipet, volumetric, 2.0-mL, catalog number 261-6012, National Scientific Company.

5.14. Pipet, volumetric, 3.0-mL, catalog number 261-6013, National Scientific Company.

5.15. Pipet, volumetric, 5.0-mL, catalog number 261-6015, National Scientific Company.

5.16. Pipet, volumetric, 10.0-mL, catalog number 261-6020, National Scientific Company.

5.17. Pipet, volumetric, 15.0-mL, catalog number 261-6025, National Scientific Company.

5.18. Pipet, volumetric, 20.0-mL, catalog number 261-6030, National Scientific Company.

5.19. Vial, autosampler, 2-mL, catalog number C4011-1, National Scientific Company.

5.20. Vial cap, for autosampler vial, catalog number C4011-1A, National Scientific Company.

6. <u>REAGENTS, STANDARDS, AND PREPARED SOLUTIONS</u> (Note 12.1.)

6.1. <u>Reagents</u>

6.1.1. Acetonitrile, HPLC grade, catalog number 2856, Mallinckrodt Baker, Inc., Paris, KY 40361.

6.1.2. Ammonium acetate, HPLC grade, catalog number A639-500, Fisher Scientific.

6.1.3. Dichloromethane, OmniSolv grade, catalog number DX0831-1, EM Science, Gibbstown, NJ 08027.

6.1.4. Methanol, HPLC grade, catalog number 3041, Mallinckrodt Baker Inc.

6.1.5. Nitrogen, refrigerated liquid, BOC Group Inc., Murray Hill, NJ 07974.

6.1.6. Water, HPLC grade, catalog number WX0004-1, EM Science.

6.2. <u>Standards</u>

6.2.1. Isoxaben: (*N*-(3-(1-ethyl-1-methylpropyl)-5-isoxazolyl)-2,6-dimethoxybenzamide).

Compound can be obtained from Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

6.3. <u>Prepared Solutions</u>

6.3.1. acetonitrile/water (70:30)

Measure 1400 mL of acetonitrile using a 2.0-L graduated cylinder and then transfer into a 2.0-L bottle. Measure 600 mL of HPLC water using a 1.0-L graduated cylinder and transfer to the 2.0-L bottle. Cap the bottle and mix. Allow the solution to equilibrate to room temperature before use.

6.3.2. methanol/water (50:50) containing 5 mM of ammonium acetate

Weigh 0.39 g of ammonium acetate into a 40-mL vial and quantitatively transfer with methanol into a 1.0-L graduated cylinder. Add methanol to the graduated cylinder until the 500-mL mark is reached and transfer to a 1.0-L bottle. Measure 500 mL of HPLC water using a 1.0-L graduated and transfer to the 1.0-L bottle. Cap the bottle and mix. Allow solution to equilibrate to room temperature before use.

6.3.3. methanol containing 5 mM ammonium acetate

Weigh 0.39 g of ammonium acetate into a 40-mL vial and quantitatively transfer with methanol into a 1.0-L graduated cylinder. Add methanol to achieve a final volume of 1.0 L. Transfer to a 1-L bottle. Cap the bottle and mix.

6.3.4. water containing 5 mM ammonium acetate

Weigh 0.39 g of ammonium acetate into a 40-mL vial and quantitatively transfer with water into a 1.0-L graduated cylinder. Add water to achieve a final volume of 1.0 L. Transfer to a 1-L bottle. Cap the bottle and mix.

7. <u>PREPARATION OF STANDARD SOLUTIONS</u> (Note 12.2.)

7.1. <u>Preparation of Isoxaben Spiking Solutions</u>

7.1.1. Weigh 0.100 g of isoxaben analytical standard and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with methanol to obtain a 1000-µg/mL stock solution of isoxaben.

7.1.2. Pipet 10.0 mL of the 1000-µg/mL isoxaben solution (Section 7.1.1) into a 100-mL volumetric flask. Adjust to volume with methanol to obtain a 100.0-µg/mL isoxaben spiking solution.

7.1.3. Pipet 10.0 mL of the 100.0-µg/mL solution in Section 7.1.2 into a 100-mL volumetric flask and adjust to volume with methanol to obtain a 10.0-µg/mL isoxaben spiking solution.

7.1.4. Pipet 10.0 mL of the 10.0-µg/mL solution in Section 7.1.3 into a 100-mL volumetric flask and adjust to volume with methanol to obtain a 1.0-µg/mL isoxaben spiking solution.

7.1.5. Pipet 10.0 mL of the 1.0-µg/mL solution in Section 7.1.4 into a 100-mL volumetric flask and adjust to volume with methanol to obtain a 0.1-µg/mL isoxaben spiking solution.

7.2. Preparation of isoxaben Calibration Solutions

Prepare calibration standard solutions in methanol/water (50:50) containing 5 mM of ammonium acetate from the 10.0-µg/mL mixed standard (Section 7.1.3) over the range 0.00003–0.02 µg/mL as described below.

Original Standard Concentration µg/mL	Aliquot of Original Standard mL	Final Soln. Volume mL	Calib Soln. Final Conc. µg/mL	Equivalent Sample Conc. µg/g
10.00	10.0	100	1.00	N/A
1.00	10.0	100	0.10	N/A
0.10	10.0	100	0.01	N/A
0.10	20.0	100	0.02	2.00
0.10	15.0	100	0.015	1.50
0.10	10.0	100	0.01	1.00
0.10	5.0	100	0.005	0.50
0.10	1.0	100	0.001	0.10
0.01	5.0	100	0.0005	0.05
0.01	1.0	100	0.0001	0.01
0.001	3.0	100	0.00003	0.003

8. LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

8.1. Typical Liquid Chromatography Operating Conditions (Note 12.3.)

Instrumentation: Agilent Model 1100 autosampler
Agilent Model 1100 binary pump
Agilent Model 1100 degasser
PE SCIEX API 3000 LC/MS/MS System
PE SCIEX Analyst 1.1 data system

Column: Diazem Pharma C18-A, 4.6 x 100 mm, 5-µm (Note 12.4.)

Column Temperature: 35 °C

Injection Volume: 50 µL

Run Time: 6 minutes

Mobile Phase: A – methanol with 5 mM ammonium acetate
B – water with 5 mM ammonium acetate

Flow Rate: 900 µL/min

Gradient:

Time, min	A, %	B, %
0.0	50	50
3.0	100	0
6.0	100	0

Equilibration Time: 3 minutes

8.2. Typical Mass Spectrometry Operating Conditions

API 3000:

Interface: APCI
Scan Type: MRM
Resolution: Q1 – unit, Q3 – low
Curtain Gas (CUR): 14
Collision Gas (CAD): 4.0
Temperature (TEM): 450 °C
Ion Source Gas 1 (GS1): 8
Ion Source Gas 2 (GS2): 80 psi

Period 1
Time: 6.0 minutes
Polarity: Positive
Nebulizer Current (NC) 2

Compound	Ion, m/z		Time, ms	CE, v
	Q1	Q3		
Isoxaben	333	165	150	29

8.3. Typical Mass Spectra

Typical precursor-ion and product-ion spectra of isoxaben are presented in Figure 1.

8.4. Typical Calibration Curve

A typical calibration curve for the determination of isoxaben in wheat grain is shown in Figure 2.

8.5. Typical Chromatograms

Typical chromatograms for a calibration standard, control wheat grain sample, and a wheat grain sample fortified at 0.01 µg/g (limit of quantitation) and the highest fortified level are presented in Figure 3.

9. DETERMINATION OF RECOVERY OF ISOXABEN IN CEREALS, CHICORY AND ENDIVES

9.1. Method Validation

Unless otherwise specified, a sample set should contain the following samples:

At least one reagent blank.
At least one control.
At least one control fortified at the limit of detection.
At least two controls fortified at the limit of quantitation.
At least one control fortified at a higher concentration.

9.2. Sample Preparation

Prepare the samples for analysis by freezing with liquid nitrogen and then grinding or chopping using a hammer mill with a 1/8 or 3/16 inch screen size. Prepared samples are stored deep frozen prior to analysis.

9.3. <u>Sample Analysis for Isoxaben in Cereals, Chicory and Endives</u>

9.3.1. Weigh 5.0 ± 0.05 g of the crop samples into individual 8-oz jars. For recovery samples, add appropriate aliquots of the appropriate spiking solutions to obtain concentrations ranging from 0.01 to 1.0 µg/g.

9.3.2. Add 100 mL of an acetonitrile/water (70:30) extraction solvent and homogenize the sample for approximately 1 minute. Cap the jar and shake the sample for 1 hour on a reciprocating shaker at approximately 180 excursions/minute.

9.3.3. Centrifuge the sample for 5 minutes at 2000 rpm.

9.3.4. Pipet 0.5 mL of the extraction solution into a 12-mL culture tube. Add 1.0 mL of water and vortex mix for approximately 10 seconds.

9.3.5. Purify the samples using the following procedure (Note 12.5.):

a. Transfer 0.3 mL of the sample solution from Step 9.3.4 onto an Isolute HM-N cartridge and allow to soak ***(do not use vacuum)*** for at least 5 minutes.

b. Elute the isoxaben from the cartridge ***(do not use vacuum)*** with two 1.5-mL aliquots of dichloromethane, collecting the eluate in a 12-mL culture tube.

9.3.6. Evaporate the eluate to dryness using a TurboVap evaporator set at 40 °C and 10 psi nitrogen pressure.

9.3.7. Reconstitute the samples in 0.5 mL of methanol/water (50:50) containing 5mM ammonium acetate. Vortex mix and sonicate the vials for approximately 30 seconds.

9.3.8. Dilute samples which contain isoxaben and metabolite concentrations >0.02 µg/mL (equivalent to 2.0 µg/g) with methanol/water (50:50) containing 5 mM ammonium acetate to give a concentration within the calibration range.

9.3.9. Analyze the samples along with the calibration standards using the LC/MS/MS conditions listed in Section 8. Determine the suitability of the chromatographic system using the following criteria:

a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.

b. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 3a-3d with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for the 0.0001-µg/mL calibration standard (equivalent to 0.01 µg/g of isoxaben in the crop sample).

10. CALCULATIONS

10.1. Calculation of Percent Recovery for Isoxaben

10.1.1. Inject a series of calibration standards (Section 7.2.) as described in Section 8 and determine the peak areas for isoxaben as indicated below.

isoxaben *m/z* Q1/Q3 333/165

10.1.2. Prepare a standard curve by plotting the concentration of isoxaben on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis) as shown in Figure 2. Using linear regression analysis (13.1.) with a 1/x weighting (13.2.), determine the equation for the curve with respect to the abscissa (Note 12.6.).

For example, using linear regression with the isoxaben data from Figure 2:

$$X = \left(\frac{Y - \text{intercept}}{\text{slope}}\right)$$

$$\text{isoxaben conc. } (\mu g/mL) = \left(\frac{\text{isoxaben peak area } - \text{intercept}}{1.27e8}\right)$$

$$\text{isoxaben conc. } (\mu g/mL) = \left(\frac{\text{isoxaben peak area} - 2.65e3}{1.27e8}\right)$$

10.2. Calculation of Percent Recovery for Isoxaben

10.2.1. Determine the gross concentration in each recovery sample by substituting the peak area obtained into the above equation and solving for the concentration.

For example, using the data for isoxaben from Figure 3c:

$$\text{isoxaben conc. } (\text{gross } \mu g/mL) = \left(\frac{\text{isoxaben peak area} - 2.65e3}{1.27e8}\right)$$

$$\text{isoxaben conc. (gross µg/mL)} = \left(\frac{1.50e4 - 2.65e3}{1.27e8}\right)$$

$$\text{isoxaben conc. (gross)} = 0.000097\ \mu g/mL$$

Convert the concentration of µg/mL of isoxaben found in the final sample prepared for analysis to µg/g of isoxaben in the original wheat grain sample aliquot as follows:

$$\text{isoxaben conc. (gross µg/g)} = 0.000097\ \mu g/mL \times \frac{(100/0.5) \times (1.5/0.3) \times 0.5}{5\ g}$$

$$\text{isoxaben conc. (gross)} = 0.0097\ \mu g/g$$

10.2.2. Determine the net concentration of isoxaben in each recovery sample by subtracting any isoxaben concentration found at the retention time of isoxaben in the untreated control sample from that of the gross isoxaben concentration in the recovery sample.

For example, using the data from Figures 3b and 3c:

$$\text{isoxaben conc. (net µg/g)} = \text{isoxaben conc. (gross µg/g)} - \text{isoxaben conc. (control µg/g)}$$

$$\text{isoxaben conc. (net µg/g)} = 0.0097\ \mu g/g - 0.0000\ \mu g/g$$

$$\text{isoxaben conc. (net)} = 0.0097\ \mu g/g$$

10.2.3. Determine the percent recovery by dividing the net concentration of each recovery sample by the theoretical concentration added.

$$\text{Recovery} = \frac{\text{conc. found}}{\text{conc. added}} \times 100\%$$

$$\text{Recovery} = \frac{0.0097\ \mu g/g}{0.01\ \mu g/g} \times 100\%$$

$$\text{Recovery} = 97\%$$

10.3. Determination of Isoxaben in Crop Samples

10.3.1. Determine the gross concentration of isoxaben in each crop sample by substituting the peak area obtained into the equation for the standard calibration curve, and calculating the uncorrected residue result as described in Sections 10.2.1.

10.3.2. For those samples that require correction for method recovery, use the average recovery of all the recovery samples from a given sample set to correct for method efficiency. For example, using the isoxaben data from Figure 3c and the average recovery from Table 1 for the sample analyzed on 01-Aug-2002:

$$\text{isoxaben conc. (corrected } \mu g/g) = \text{isoxaben conc. (gross } \mu g/g) \times \left(\frac{100}{\text{Average \% Recovery}}\right)$$

$$\text{isoxaben conc. (corrected } \mu g/g) = 0.0097\ \mu g/g \times \frac{100}{93}$$

$$\text{isoxaben conc. (corrected)} = 0.0104\ \mu g/g$$

11. RESULTS AND DISCUSSION

11.1. Method Validation

11.1.1. Recovery Levels and Precision

A method validation study was conducted to determine the recovery levels and the precision of the method for the determination of isoxaben in cereals, chicory and endives. The results are summarized below and listed individually in Tables 1 - 6. An overall summary is presented in Table 7.

Matrix	n	Average Recovery %	Recovery Range %	Standard Deviation %
Cereal Grain	24	91	81-104	5.3
Cereal Forage	24	88	77-93	4.1
Cereal Straw	24	90	71-103	7.3
Whole Endives	18	88	79-93	4.4
Chicory Roots	18	94	85-105	6.2
Chicory Leaves	18	94	88-108	5.2

For isoxaben analysis, all of the individual recovery samples at each fortification level were between 70 and 110% with standard deviations less than 15%.

11.1.2. Standard Curve Linearity

For the linear regression analysis, the correlation coefficients (r) were greater than 0.998 for each of the analytes for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard calibration curve.

11.1.3. Calculated Limits of Quantitation and Detection

Following established guidelines (13.3.), the limits of quantitation (LOQ) and detection (LOD) were calculated for isoxaben using the standard deviation from the 0.01-µg/g recovery results.

The LOQ was calculated as ten times the standard deviation (10*s*), and the LOD was calculated as three times the standard deviation (3*s*) of the LOQ results. The results are summarized below. Individual µg/g values are presented in Tables 1 - 6.

Matrix	Average Recovery µg/g	Standard Deviation (*s*)	Limit of Detection (3*s*)	Limit of Quantitation (10*s*)
Cereal grain	0.00915	0.0007	0.0022	0.0072
Cereal forage	0.00873	0.0005	0.0016	0.0053
Cereal straw	0.00907	0.0010	0.0029	0.0098
Endives	0.00903	0.0003	0.0008	0.0025
Chicory roots	0.00904	0.0006	0.0018	0.0060
Chicory leaves	0.00936	0.0006	0.0017	0.0057

The calculated LOQ supported the validated LOQ of 0.01 µg/g. Since the lowest level of fortification for recovery samples was 0.01 µg/g, the method LOQ is considered to be 0.01 µg/g.

The calculated LOD were 0.0016-0.0029 µg/g. In actual residue samples, numerical results should be reported as less than the LOQ (<0.01 µg/g) for residues that are greater or equal to the LOD but less than the validated LOQ. For results less than the LOD, numerical results should be reported as not detected.

11.2. <u>Confirmation of Residue Identity</u>

The presence of isoxaben is confirmed by comparing the liquid chromatography retention times of the analyte in the calibration standards with those found in the samples as well as by the MS/MS transitions monitored. Due to the highly specific nature of the MS/MS transition monitored during detection, no further confirmation technique is required.

11.3. Assay Time and Stopping Points

A typical analytical run would consist of a minimum of eight standards encompassing the expected range of sample concentrations, a reagent blank, a control (a non-fortified sample), a minimum of four fortified controls (two of which must be at the LOQ), and 30 samples. This typical analytical set can be prepared in approximately 6 hours followed by the chromatographic analysis.

Acceptable "stopping points" are after Sections 9.2.3, 9.2.5. b and 9.2.7. Samples should be stored in a refrigerator during these stopping points.

11.4. Standardization of HM-N Cartridges

11.4.1. Pipette 0.5 mL of extraction solution and 1.0 mL of water into a culture tube. Add 50-µL of a 0.1-µg/mL isoxaben standard solution. Vortex mix the tube for 5 seconds to mix.

11.4.2. Profile the HM-N cartridge using the following procedure:

a. Transfer 0.3 mL of the sample solution from Step 11.4.1 onto an Isolute HM-N cartridge and allow to soak ***(do not use vacuum)*** for at least 5 minutes.

b. Elute the isoxaben from the cartridge ***(do not use vacuum)*** with four 1.0-mL aliquots of dichloromethane, collecting the eluate in separate 12-mL culture tubes.

11.4.3. Evaporate the eluate to dryness using a TurboVap evaporator set at 40 °C and 10 psi nitrogen pressure.

11.4.4. Reconstitute the samples in 1.0 mL of methanol/water (50:50) containing 5mM ammonium acetate. Vortex mix and sonicate the vials for approximately 30 seconds.

11.4.5. Analyze the samples along with the calibration standards using the LC/MS/MS conditions listed in Section 8. A typical elution profile is illustrated in Figure 4. If the elution profile differs from that shown, adjust the volume of dichloromethane to be collected in Step 9.2.5. b.

12. NOTES

12.1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and are, therefore, not listed.

12.2. Section 7 provides suggested concentrations for calibration standard preparation. Other dilution schemes may be followed.

12.3. The data presented in this method were generated using a Sciex 3000 API 3000 in optimal condition. Operating conditions may be modified to obtain optimal separation or sensitivity. However, increasing injection volume to compensate for low instrument sensitivity may compromise method performance.

12.4. If chromatographic problems are experienced using the Diazem column, it may be substituted with an Inertsil ODS3 (5μm 100 x 4.6 mm i.d) or equivalent with no adverse effects on method performance.

12.5. Before using each lot of HM-N cartridges, determine the elution profile as described in Section 11.4.

12.6. Linear regression analysis using a quadratic curve fit may also be used.

13. REFERENCES

13.1. Freund, J. E.; Williams, F. J. *Dictionary/Outline of Basic Statistics*; Dover: New York, 1991; p 170.

13.2. Neter, J.; Kutner, M. H.; Nachtssheim, C. J. ; Wasserman, W. *Applied Linear Regression Models*; The McGraw-Hill Company: New York, 1996; p 409.

13.3. Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. *Anal. Chem.* **1983**, *55*, 2210-2218.

Table 1. Recovery of Isoxaben from Wheat and Barley Grain

Sample Number	Matrix	Date of Analysis	Isoxaben, µg/g Added	Isoxaben, µg/g Found	Recovery[a] %
40094701	Wheat Grain	01-Aug-02	0.00	0.0000	--
40097101	Wheat Grain	31-Jul-02	0.00	0.0000	--
40099801	Barley Grain	31-Jul-02	0.00	0.0000	--
40102101	Barley Grain	30-Jul-02	0.00	0.0000	--
40094701	Wheat Grain	01-Aug-02	0.003	0.00215	NA[b]
40097101	Wheat Grain	31-Jul-02	0.003	0.00262	NA
40099801	Barley Grain	31-Jul-02	0.003	0.00258	NA
40102101	Barley Grain	30-Jul-02	0.003	0.00299	NA
40094701	Wheat Grain	01-Aug-02	0.01	0.00970	97
40094701	Wheat Grain	01-Aug-02	0.01	0.00901	90
40097101	Wheat Grain	31-Jul-02	0.01	0.00939	94
40097101	Wheat Grain	31-Jul-02	0.01	0.00871	87
40099801	Barley Grain	31-Jul-02	0.01	0.00837	84
40099801	Barley Grain	31-Jul-02	0.01	0.00941	94
40102101	Barley Grain	07-Aug-02	0.01	0.01040	104
40102101	Barley Grain	30-Jul-02	0.01	0.00824	82
40094701	Wheat Grain	01-Aug-02	0.10	0.0926	93
40094701	Wheat Grain	01-Aug-02	0.10	0.0927	93
40097101	Wheat Grain	31-Jul-02	0.10	0.0893	89
40097101	Wheat Grain	31-Jul-02	0.10	0.0899	90
40099801	Barley Grain	31-Jul-02	0.10	0.0829	83
40099801	Barley Grain	31-Jul-02	0.10	0.0852	85
40102101	Barley Grain	31-Jul-02	0.10	0.0940	94
40102101	Barley Grain	30-Jul-02	0.10	0.0946	95
40094701	Wheat Grain	01-Aug-02	1.00	0.928	93
40094701	Wheat Grain	01-Aug-02	1.00	0.928	93
40097101	Wheat Grain	31-Jul-02	1.00	0.935	94
40097101	Wheat Grain	31-Jul-02	1.00	0.935	94
40099801	Barley Grain	31-Jul-02	1.00	0.925	93
40099801	Barley Grain	31-Jul-02	1.00	0.896	90
40102101	Barley Grain	31-Jul-02	1.00	0.965	97
40102101	Barley Grain	30-Jul-02	1.00	0.810	81

[a] All calculations were performed in Microsoft Excel 97 with full precision.

[b] Not applicable. Results are less than the validated limit of quantitation (0.01 µg/g).

Table 2. Recovery of Isoxaben from Wheat and Barley Forage

Sample Number	Matrix	Date of Analysis	Isoxaben, µg/g Added	Isoxaben, µg/g Found	Recovery[a] %
40096301	Wheat Forage	01-Aug-02	0.00	0.0000	--
37336201	Wheat Forage	31-Jul-02	0.00	0.0000	--
40100501	Barley Forage	31-Jul-02	0.00	0.0000	--
37337001	Barley Forage	30-Jul-02	0.00	0.0000	--
40096301	Wheat Forage	01-Aug-02	0.003	0.00286	NA[b]
37336201	Wheat Forage	31-Jul-02	0.003	0.00301	NA
40100501	Barley Forage	31-Jul-02	0.003	0.00217	NA
37337001	Barley Forage	30-Jul-02	0.003	0.00251	NA
40096301	Wheat Forage	01-Aug-02	0.01	0.00879	88
40096301	Wheat Forage	01-Aug-02	0.01	0.00909	91
37336201	Wheat Forage	31-Jul-02	0.01	0.00914	91
37336201	Wheat Forage	31-Jul-02	0.01	0.00927	93
40100501	Barley Forage	31-Jul-02	0.01	0.00899	90
40100501	Barley Forage	31-Jul-02	0.01	0.00858	86
37337001	Barley Forage	30-Jul-02	0.01	0.00834	83
37337001	Barley Forage	30-Jul-02	0.01	0.00767	77
40096301	Wheat Forage	01-Aug-02	0.10	0.0904	90
40096301	Wheat Forage	01-Aug-02	0.10	0.0860	86
37336201	Wheat Forage	31-Jul-02	0.10	0.0906	91
37336201	Wheat Forage	31-Jul-02	0.10	0.0837	84
40100501	Barley Forage	31-Jul-02	0.10	0.0877	88
40100501	Barley Forage	31-Jul-02	0.10	0.0870	87
37337001	Barley Forage	30-Jul-02	0.10	0.0899	90
37337001	Barley Forage	30-Jul-02	0.10	0.0861	86
40096301	Wheat Forage	01-Aug-02	1.00	0.887	89
40096301	Wheat Forage	01-Aug-02	1.00	0.915	92
37336201	Wheat Forage	31-Jul-02	1.00	0.883	88
37336201	Wheat Forage	31-Jul-02	1.00	0.880	88
40100501	Barley Forage	31-Jul-02	1.00	0.886	89
40100501	Barley Forage	31-Jul-02	1.00	0.880	88
37337001	Barley Forage	30-Jul-02	1.00	0.885	89
37337001	Barley Forage	30-Jul-02	1.00	0.919	92

[a] All calculations were performed in Microsoft Excel 97 with full precision.

[b] Not applicable. Results are less than the validated limit of quantitation (0.01 µg/g).

Table 3. Recovery of Isoxaben from Wheat and Barley Straw

Sample Number	Matrix	Date of Analysis	Isoxaben, µg/g		Recovery[a] %
			Added	Found	
40095501	Wheat Straw	01-Aug-02	0.00	0.0000	--
37336202	Wheat Straw	31-Jul-02	0.00	0.0000	--
40101301	Barley Straw	31-Jul-02	0.00	0.0000	--
37337002	Barley Straw	30-Jul-02	0.00	0.0000	--
40095501	Wheat Straw	01-Aug-02	0.003	0.00277	NA[b]
37336202	Wheat Straw	31-Jul-02	0.003	0.00291	NA
40101301	Barley Straw	31-Jul-02	0.003	0.00312	NA
37337002	Barley Straw	30-Jul-02	0.003	0.00226	NA
40095501	Wheat Straw	01-Aug-02	0.01	0.01000	100
40095501	Wheat Straw	01-Aug-02	0.01	0.01030	103
37336202	Wheat Straw	31-Jul-02	0.01	0.00922	92
37336202	Wheat Straw	31-Jul-02	0.01	0.00895	90
40101301	Barley Straw	31-Jul-02	0.01	0.00984	98
40101301	Barley Straw	31-Jul-02	0.01	0.00837	84
37337002	Barley Straw	30-Jul-02	0.01	0.00846	85
37337002	Barley Straw	30-Jul-02	0.01	0.00738	74
40095501	Wheat Straw	01-Aug-02	0.10	0.0938	94
40095501	Wheat Straw	01-Aug-02	0.10	0.0929	93
37336202	Wheat Straw	31-Jul-02	0.10	0.0959	96
37336202	Wheat Straw	31-Jul-02	0.10	0.0888	89
40101301	Barley Straw	31-Jul-02	0.10	0.0708	71
40101301	Barley Straw	31-Jul-02	0.10	0.0896	90
37337002	Barley Straw	30-Jul-02	0.10	0.0863	86
37337002	Barley Straw	30-Jul-02	0.10	0.0865	87
40095501	Wheat Straw	01-Aug-02	1.00	0.927	93
40095501	Wheat Straw	01-Aug-02	1.00	0.933	93
37336202	Wheat Straw	31-Jul-02	1.00	0.865	87
37336202	Wheat Straw	31-Jul-02	1.00	0.951	95
40101301	Barley Straw	31-Jul-02	1.00	0.866	87
40101301	Barley Straw	31-Jul-02	1.00	0.905	91
37337002	Barley Straw	30-Jul-02	1.00	0.869	87
37337002	Barley Straw	30-Jul-02	1.00	0.900	90

[a] All calculations were performed in Microsoft Excel 97 with full precision.

[b] Not applicable. Results are less than the validated limit of quantitation (0.01 µg/g).

Table 4. Recovery of Isoxaben from Endives

Sample Number	Matrix	Date of Analysis	Isoxaben, µg/g Added	Isoxaben, µg/g Found	Recovery[a] %
40093901	Whole Endives	07-Aug-02	0.00	0.0000	--
40093901	Whole Endives	07-Aug-02	0.003	0.00253	NA[b]
40093901	Whole Endives	07-Aug-02	0.01	0.00891	89
40093901	Whole Endives	07-Aug-02	0.01	0.00861	86
40093901	Whole Endives	07-Aug-02	0.01	0.00898	90
40093901	Whole Endives	07-Aug-02	0.01	0.00913	91
40093901	Whole Endives	07-Aug-02	0.01	0.00928	93
40093901	Whole Endives	07-Aug-02	0.01	0.00927	93
40093901	Whole Endives	07-Aug-02	0.10	0.0858	86
40093901	Whole Endives	07-Aug-02	0.10	0.0903	90
40093901	Whole Endives	07-Aug-02	0.10	0.0803	80
40093901	Whole Endives	07-Aug-02	0.10	0.0822	82
40093901	Whole Endives	07-Aug-02	0.10	0.0865	87
40093901	Whole Endives	07-Aug-02	0.10	0.0908	91
40093901	Whole Endives	07-Aug-02	1.00	0.898	90
40093901	Whole Endives	07-Aug-02	1.00	0.864	86
40093901	Whole Endives	07-Aug-02	1.00	0.851	85
40093901	Whole Endives	07-Aug-02	1.00	0.788	79
40093901	Whole Endives	07-Aug-02	1.00	0.895	90
40093901	Whole Endives	07-Aug-02	1.00	0.893	89

[a] All calculations were performed in Microsoft Excel 97 with full precision.
[b] Not applicable. Results are less than the validated limit of quantitation (0.01 µg/g).

Table 5. Recovery of Isoxaben from Chicory Roots

Sample Number	Matrix	Date of Analysis	Isoxaben, µg/g Added	Isoxaben, µg/g Found	Recovery[a] %
40111001	Chicory Root	18-Nov-02	0.00	0.0000	--
40111001	Chicory Root	18-Nov-02	0.003	0.00294	NA[b]
40111001	Chicory Root	18-Nov-02	0.003	0.00275	NA
40111001	Chicory Root	18-Nov-02	0.003	0.00273	NA
40111001	Chicory Root	18-Nov-02	0.01	0.00887	89
40111001	Chicory Root	18-Nov-02	0.01	0.00848	85
40111001	Chicory Root	18-Nov-02	0.01	0.00872	87
40111001	Chicory Root	18-Nov-02	0.01	0.00889	89
40111001	Chicory Root	18-Nov-02	0.01	0.01020	102
40111001	Chicory Root	18-Nov-02	0.01	0.00907	91
40111001	Chicory Root	18-Nov-02	0.1	0.1040	104
40111001	Chicory Root	18-Nov-02	0.1	0.0975	98
40111001	Chicory Root	18-Nov-02	0.1	0.0951	95
40111001	Chicory Root	18-Nov-02	0.1	0.0952	95
40111001	Chicory Root	18-Nov-02	0.1	0.0962	96
40111001	Chicory Root	18-Nov-02	0.1	0.0948	95
40111001	Chicory Root	18-Nov-02	1.0	0.991	99
40111001	Chicory Root	18-Nov-02	1.0	1.050	105
40111001	Chicory Root	18-Nov-02	1.0	0.950	95
40111001	Chicory Root	18-Nov-02	1.0	0.883	88
40111001	Chicory Root	18-Nov-02	1.0	0.851	85
40111001	Chicory Root	18-Nov-02	1.0	0.902	90

[a] All calculations were performed in Microsoft Excel 97 with full precision.

[b] Not applicable. Results are less than the validated limit of quantitation (0.01 µg/g).

Table 6. Recovery of Isoxaben from Chicory Leaves

Sample Number	Matrix	Date of Analysis	Isoxaben, μg/g Added	Isoxaben, μg/g Found	Recovery[a] %
40111002	Chicory Leaves	18-Nov-02	0.00	0.0027	--
40111002	Chicory Leaves	18-Nov-02	0.003	0.00297	NA[b]
40111002	Chicory Leaves	18-Nov-02	0.003	0.00312	NA
40111002	Chicory Leaves	18-Nov-02	0.003	0.00290	NA
40111002	Chicory Leaves	18-Nov-02	0.01	0.00920	92
40111002	Chicory Leaves	18-Nov-02	0.01	0.00877	88
40111002	Chicory Leaves	18-Nov-02	0.01	0.01010	101
40111002	Chicory Leaves	18-Nov-02	0.01	0.00881	88
40111002	Chicory Leaves	18-Nov-02	0.01	0.00926	93
40111002	Chicory Leaves	18-Nov-02	0.01	0.01000	100
40111002	Chicory Leaves	18-Nov-02	0.1	0.0964	96
40111002	Chicory Leaves	18-Nov-02	0.1	0.0966	97
40111002	Chicory Leaves	18-Nov-02	0.1	0.0932	93
40111002	Chicory Leaves	18-Nov-02	0.1	0.0886	89
40111002	Chicory Leaves	18-Nov-02	0.1	0.0882	88
40111002	Chicory Leaves	18-Nov-02	0.1	0.0924	92
40111002	Chicory Leaves	18-Nov-02	1.0	0.908	91
40111002	Chicory Leaves	18-Nov-02	1.0	0.940	94
40111002	Chicory Leaves	18-Nov-02	1.0	1.080	108
40111002	Chicory Leaves	18-Nov-02	1.0	0.922	92
40111002	Chicory Leaves	18-Nov-02	1.0	0.940	94
40111002	Chicory Leaves	18-Nov-02	1.0	0.943	94

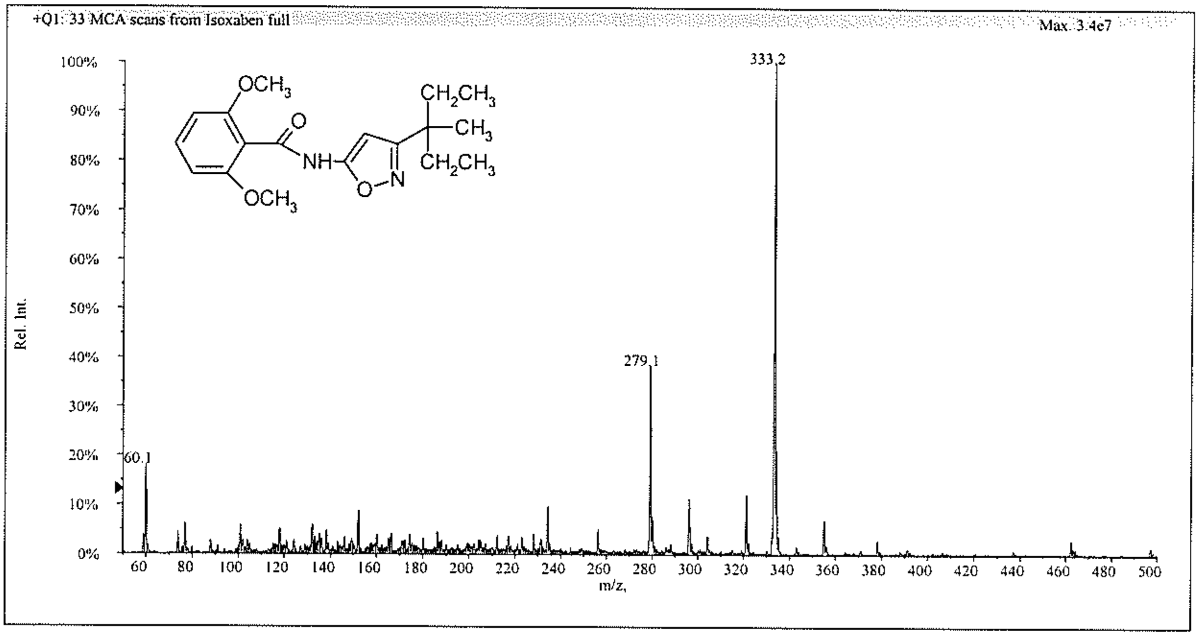
[a] All calculations were performed in Microsoft Excel 97 with full precision.

[b] Not applicable. Results are less than the validated limit of quantitation (0.01 μg/g).

Table 7. Recovery Summary of Isoxaben from Cereals, Endives and Chicory

Matrix	Fortification Rate µg/g	Average Recovery (%)	Recovery Range (%)	SD (%)	RSD (%)	n
Wheat and Barley Grain	0.01	92	82-104	7.2	7.9	8
	0.10	90	83-95	4.2	4.7	8
	1.00	92	81-97	4.6	5.1	8
	0.01-1.00	91	81-104	5.3	5.8	24
Wheat and Barley Forage	0.01	87	77-93	5.3	6.1	8
	0.10	88	84-91	2.5	2.8	8
	1.00	89	88-92	1.6	1.8	8
	0.01-1.00	88	77-93	3.6	4.1	24
Wheat and Barley Straw	0.01	91	74-103	9.8	10.8	8
	0.10	88	71-96	7.8	8.8	8
	1.00	90	87-95	3.3	3.7	8
	0.01-1.00	90	71-103	7.3	8.1	24
Whole Endives	0.01	90	86-93	2.5	2.8	6
	0.10	86	80-91	4.2	4.9	6
	1.00	86	79-90	4.2	4.9	6
	0.01-1.00	88	79-93	4.4	5.0	18
Chicory Roots	0.01	90	85-102	6.0	6.7	6
	0.10	97	95-104	3.5	3.6	6
	1.00	94	85-105	7.4	7.9	6
	0.01-1.00	94	85-105	6.2	6.6	18
Chicory Leaves	0.01	94	88-101	5.7	6.1	6
	0.10	93	88-97	3.6	3.9	6
	1.00	96	91-108	6.2	6.5	6
	0.01-1.00	94	88-108	5.2	5.5	18

(a)



(b)

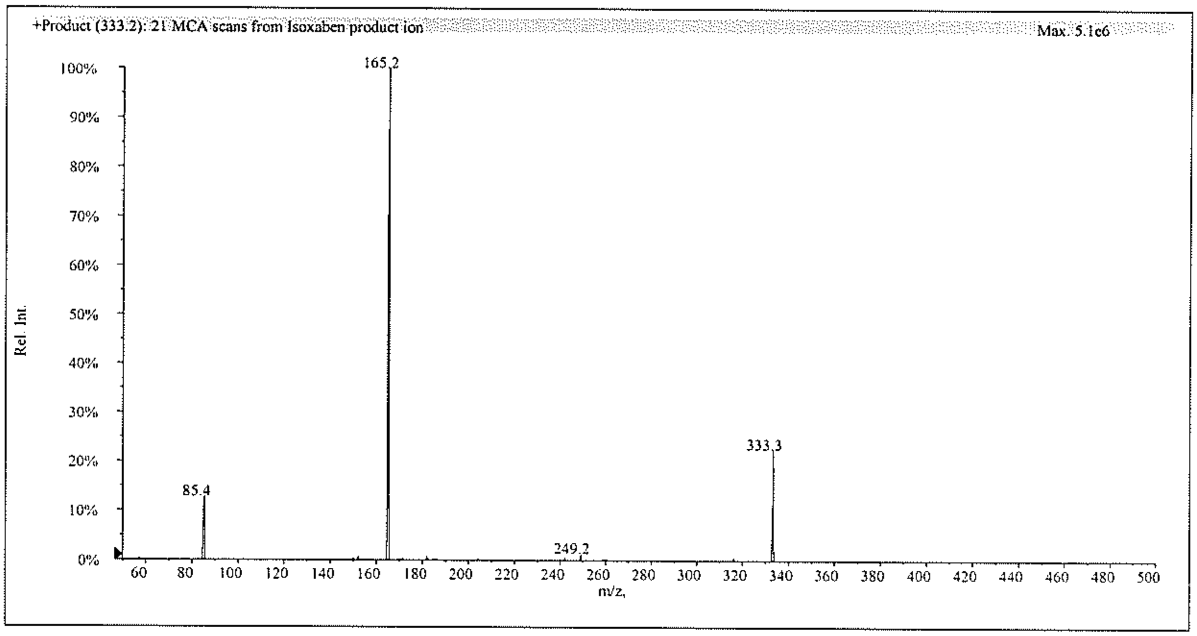
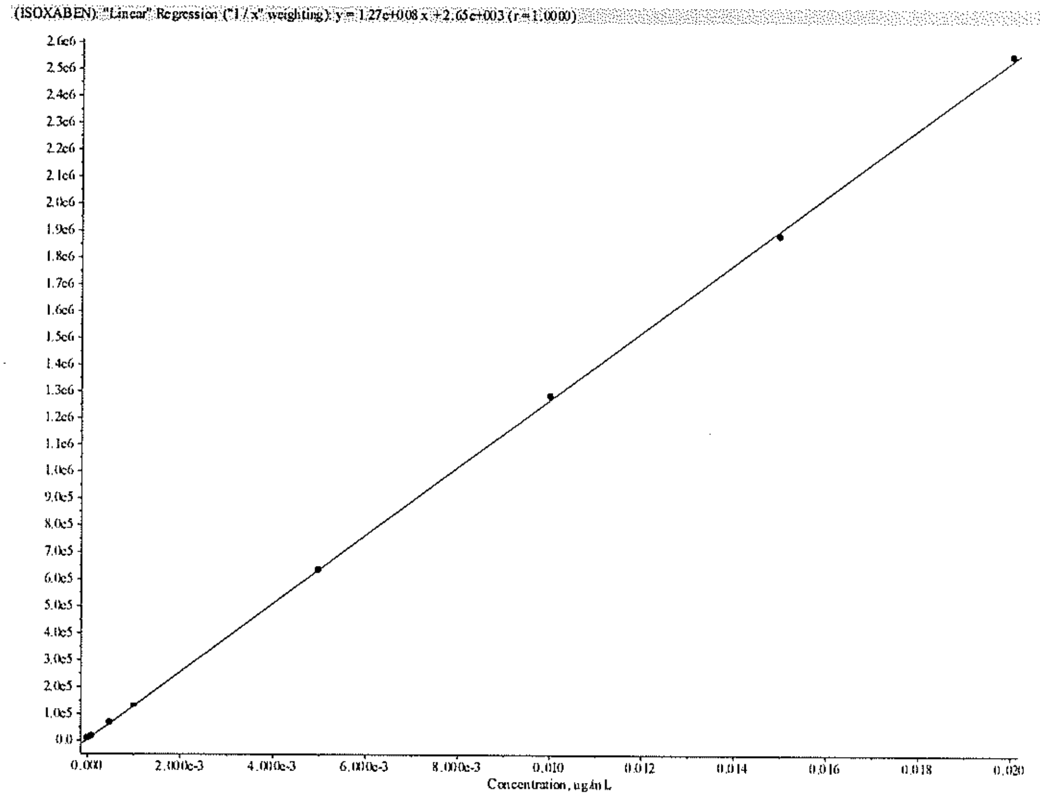


Figure 1. Mass Spectra for Isoxaben: (a) Mass Spectrum for Q1 Scan using Electrospray Positive Ionization $(M+H)^+$ at *m/z* 333 (b) Product-Ion Mass Spectrum of Isoxaben Showing Fragment Ion at *m/z* 165



Linear Equation:	Y= 1.27e8 X + 2.65e3
Correlation Coefficient (r):	1.0000
Weighting:	1/x

Isoxaben Conc. µg/mL	Equivalent Sample Conc. µg/g	Isoxaben Peak Area
0.00003	0.003	6.36e3
0.0001	0.01	1.56e4
0.0005	0.05	6.71e4
0.001	0.1	1.29e5
0.005	0.5	6.32e5
0.01	1.0	1.28e6
0.015	1.5	1.89e6
0.02	2.0	2.55e6

Figure 2. Typical Calibration Curve for the Determination of Isoxaben in Wheat

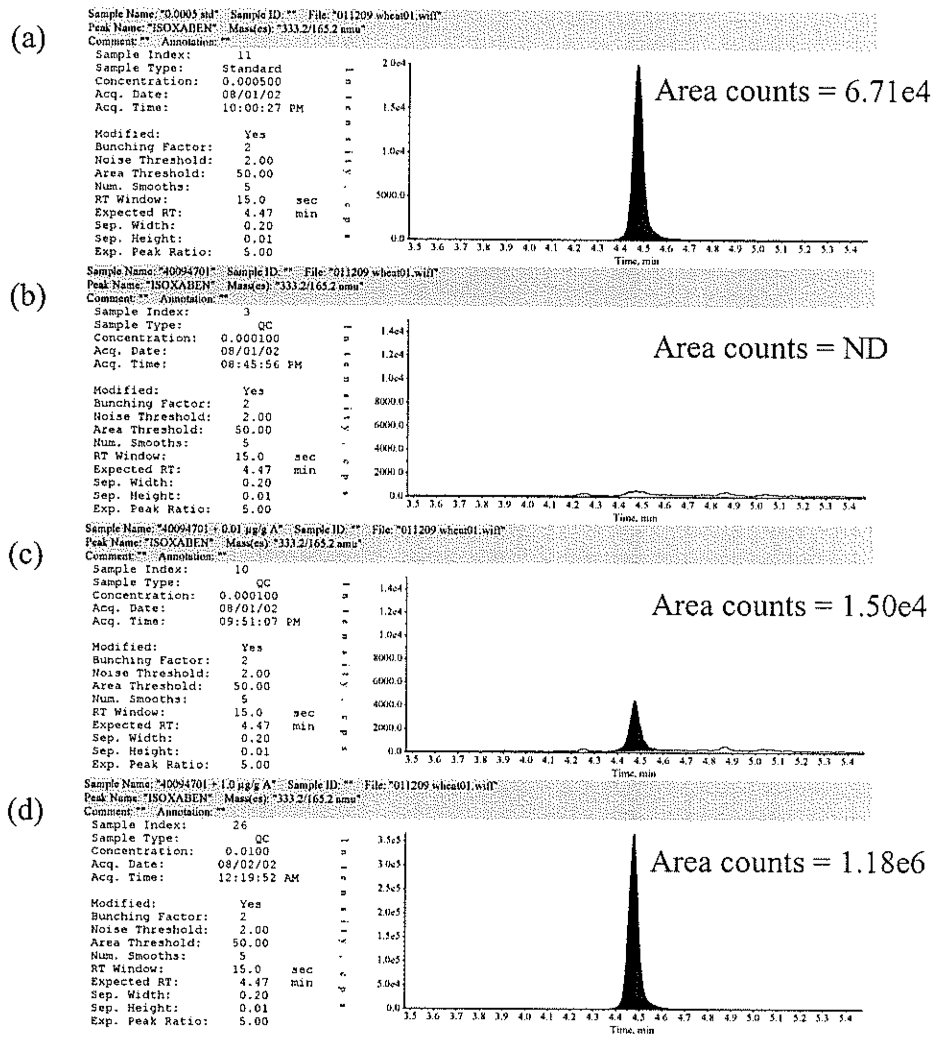


Figure 3. Typical MRM Chromatograms for the Determination of Isoxaben in Wheat Grain

(a) 0.0005-µg/mL standard. Equivalent to a sample concentration of 0.05 µg/g.
(b) Control wheat grain sample 40094701. No Detectable (ND) Residue.
(c) Control wheat grain sample 40094701. Fortified at 0.01 µg/g. 97% Recovery.
(d) Control wheat grain sample 40094701. Fortified at 1.0 µg/g. 93% Recovery.

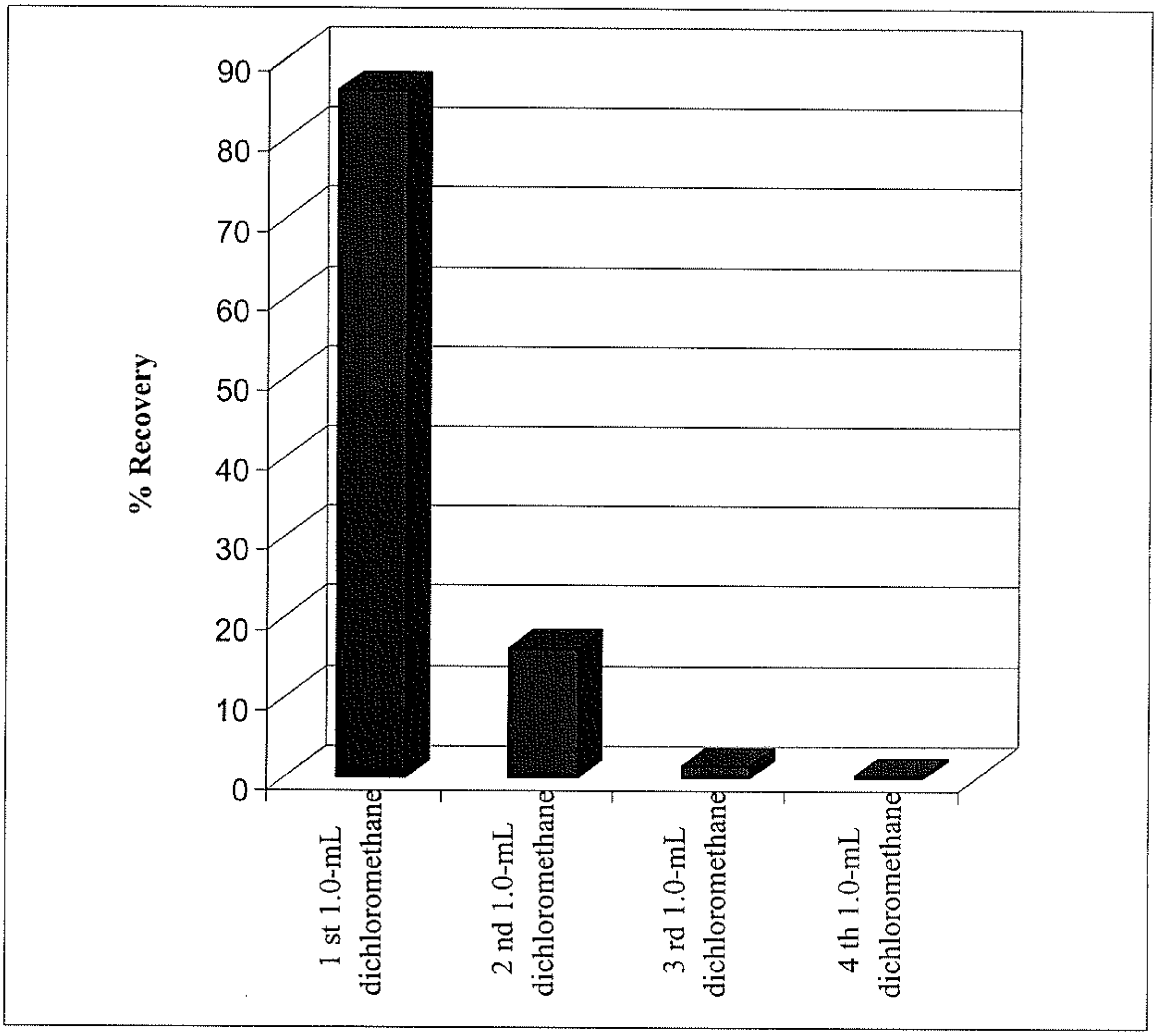


Figure 4. Typical HM-N Cartridge Profile for Isoxaben