CONDUCT OF STUDY

1 INTRODUCTION AND SUMMARY

1.1 Scope

This method describes the analysis of water samples generated from aquatic dissipation studies in rice paddies for residues of Bentazon, N-Methylbentazon and 8-Chlorobentazon. Water samples (100 mL) are prepared by C18 solid phase extraction (SPE) with methanol elution. The analysis is performed by thermospray liquid chromatography tandem mass spectrometry (TSP-LC/MS/MS). Bentazon and 8-Chlorobentazon are analyzed by negative ion TSP-LC/MS and N-Methylbentazon is analyzed by positive ion TSP-LC/MS/MS. The extraction and instrumentation methods (Method ID: BASBENW) were developed at ALTA Analytical Laboratory, El Dorado Hills, CA 95762.

2 MATERIALS

2.1 Equipment

2.1.1 Balance, Analytical, capable of weighing to the nearest 0.0001 g.

2.1.2 Balance, Toploader, capable of weighing to the nearest 0.01 g.

2.1.3 Bottle, amber, appropriate size for storage of standard solutions.

2.1.4 Rotary evaporator, Buchler 421-1409 or equivalent.

2.1.5 Test tubes, 16x125-mm, (Curtin Matheson Scientific (CMS) 251-884 or equivalent).

2.1.6 Graduated centrifuge tubes, 10-mL calibrated "To Contain", readable to 0.1-mL (CMS 253-819 or equivalent).

2.1.7 Positive displacement micropipets, 10/20 uL, 25/50 uL, 50/100 uL, 100/200 uL (Cole Parmer N-07951-10/15/20/25 or equivalent).

2.1.8 Pipets, 0.5-mL (CMS 080-465 or equivalent).

2.1.9 Pipets, 1-mL (CMS 080-507 or equivalent).

2.1.10 Pipets, 2-mL (CMS 080-515 or equivalent).

2.1.11 Pipets, 5-mL (CMS 080-523 or equivalent).

2.1.12 Pasteur pipettes (CMS 355-123 or equivalent).

2.1.13 Flask, 250-mL round bottom (CMS 096-495 or equivalent).

2.1.14 Flask, volumetric 25-mL (CMS 105-304 or equivalent).

2.1.15 Flask, volumetric 50-mL (CMS 106-138 or equivalent).

2.1.16 Flask, volumetric 100-mL (CMS 105-320 or equivalent).

2.1.17 Autosampler vials, 1-mL (Waters 78514 or equivalent).

2.1.18 Disposable syringe, 5-mL with Luer-Lok (CMS 256-851 or equivalent).

2.1.19 Syringe filter disks, Gelman Acrodisc CR 0.45-um or smaller (CMS 141-226 or equivalent).

2.1.20 Vortex-Genie 2 mixer or equivalent.

2.1.21 Solid Phase Extraction Vacuum Manifold (VWR JT7208-0 or equivalent).

2.2 Reagents and Standards

2.2.1 Water, EM Omnisolve HPLC grade (CMS MWX004-1 or equivalent).

2.2.2 C-18 Solid Phase Extraction Column (Varian 1225-6001 or equivalent).

2.2.3 Formic acid, ~88% (CMS 830-937 or equivalent).

2.2.4 Hydrochloric acid, Conc., ~37% (Mallinckrodt 2612 or equivalent).

2.2.5 Ammonium acetate, crystals, (CMS MAX 1220-1 or equivalent).

2.2.6 HPLC mobile phase, 0.2M ammonium acetate/0.1% formic acid.

2.2.7 Methanol, EM Omnisolve HPLC grade (CMS MX0488-1 or equivalent).

2.2.8 Bentazon, N-Methylbentazon and 8-Chlorobentazon, analytical standards, BASF Corporation, Agricultural Research Center, P.O. Box 13528
Research Triangle Park, North Carolina, 27709-3528.

2.3 Safety and Health

The toxicity or carcinogenicity of each reagent used in this method has not been precisely determined; however, each chemical compound must be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available.

3 PRINCIPLES

BASF Analytical Method D9303 (ALTA Analytical Method BASBENW) was developed for the analysis of water samples generated from aquatic dissipation studies in rice paddies for residues of Bentazon, N-Methylbentazon and 8-Chlorobentazon. Method development and validation was performed with water samples from rice paddies.

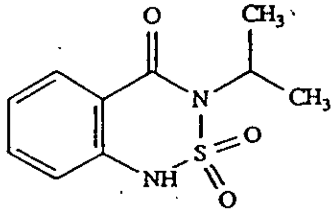
Water samples (100 mL) acidified with approximately 100 uL of 6N HCl are extracted by passing the sample through a C-18 solid phase extraction (SPE) column and eluting with 8 mL of methanol. The eluate is concentrated to approximately 0.5 mL under a stream of nitrogen and brought back to 2 mL with water. No additional clean-up or derivitization is required for this method.

Standards and sample extracts are injected onto a C-18 reverse phase HPLC system connected to a quadrupole mass spectrometer via a thermospray interface. Bentazon and 8-Chlorobentazon are analyzed by negative ion TSP-LC/MS and N-Methylbentazon is analyzed by positive ion TSP-LC/MS/MS.

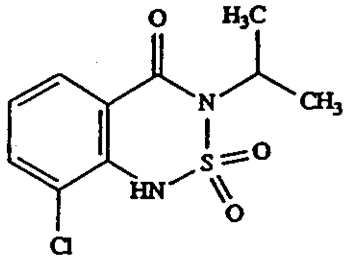
The limit of quantitation for this method was validated at 1 ppb for each compound.

Analytical standards used as the test and reference substances for the method validation were obtained from BASF Corporation, Research Triangle Park, North Carolina. Structures, chemical names, lot numbers, purity, and expiration dates for each can be found below.

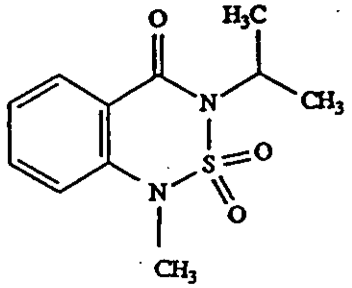
Common Name:	Bentazon
Chemical Name:	3-(1-methylethyl)-1H-2,1,3-benzothiadiazin-4(3H)-one-2,2-dioxide
ALTA ID:	R921119A
Lot Number:	CH40/204-1
Purity:	99.9%
CAS Number:	25057-89-0
Purity Statement Valid Until:	05/1996



Common Name:	8-chlorobentazon
Chemical Name:	8-chloro-3-(1-methylethyl)-1H-2,1,3-benzothiadiazin-4(3H)-one-2,2-dioxide
ALTA ID:	R921119C
Lot Number:	L30/259
Purity:	99.8%
Purity Statement Valid Until:	05/1996



Common Name:	N-methylbentazon
Chemical Name:	1-methyl-3-(1-methylethyl)-1H-2,1,3-benzothiadiazin-4(3H)-one-2,2-dioxide
ALTA ID:	R92119B
Lot Number:	L45/197
Purity:	99.4%
Purity Statement Valid Until:	05/1994



4 ANALYTICAL PROCEDURES

4.1 Preparation of Standard Solutions

The following standard concentrations are suggestions and may be modified if needed.

4.1.1 A 100-ug/mL stock solution of each analyte is prepared by dissolving 10 mg of pure material in methanol and then diluting to 100 mL with methanol. If the compound purity is certified at 96% or greater, the weight may be used without correction to calculate the concentration of the stock standard. Transfer the stock standard solution into an amber bottle and seal with teflon lined caps only. Store the standards in a freezer at -10°C or colder and protect from light when not in use. Stock standard solutions must be replaced after 1 year or sooner if comparison with check standards indicates a problem.

4.1.2 Using the 100-ug/mL stock solutions from 4.1.1, prepare a 10-ug/mL mixed standard containing all analytes in methanol.

4.1.3 Using the 10-ug/mL stock solution from 4.1.2, prepare a 1-ug/mL mixed standard containing all analytes in methanol.

4.1.4 The stock solutions 4.1.2 and 4.1.3 are to be used as fortification standards. Use the appropriate fortification standard such that no more than 1.0 mL is used for fortification.

4.1.5 A three point LC/MS/MS calibration standard curve should be prepared such that one standard is lower than the desired screening. The stock solutions are used for making the calibration standards. Dilutions are made such that the solvent has a composition of 75:25 HPLC water:methanol.

4.1.6 Example Calibration Standards

4.1.6.1 2500-ng/mL standard, dilute 2.5 mL of the 10-ug/mL stock to 10 mL with HPLC water.

4.1.6.2 250-ng/mL standard, dilute 1.0 mL of the 2500-ng/mL calibration standard to 10 mL with 75:25 HPLC water:methanol.

4.1.6.3 25-ng/ml standard, dilute 1.0 mL of the 250-ng/mL calibration standard to 10 mL with 75:25 HPLC water:methanol.

4.2 Extraction/concentration

4.2.1 Shake sample vigorously, transfer 100 g(mL) of sample (by weight difference) to an appropriate container for transfer to the SPE column.

4.2.2 If appropriate, fortify the sample at this step using positive displacement micropipets or 1-mL pipet.

4.2.3 Add ~100ul of 6N hydrochloric acid to the sample and mix well.

4.2.4 Place the SPE column on the vacuum manifold and condition by drawing 2-column volumes of methanol followed by 2-column volumes of HPLC water through the column. Release the vacuum just as the water reaches the packing. Do not let the column go dry at this point.

4.2.5 Attach a reservoir to the column and begin drawing the acidified sample through the column. Rinse the container with a small portion of HPLC water and add to the reservoir.

4.2.6 After all of the sample plus rinse has passed through the column, draw air through the column for ~3-6 seconds. Discard all eluates to this point.

4.2.7 Elute the column with 8 mL of methanol. Collect the eluate in a test tube. Concentrate the solution to ~2 mL with nitrogen and water bath at room temperature. Do not allow the extract to go to dryness.

4.2.8 Quantitatively transfer the concentrate to a 10-mL calibrated centrifuge tube using methanol as a rinse.

4.2.9 Concentrate the eluate to 0.3-0.6 mL with nitrogen and water bath at room temperature.

4.2.10 Dilute the concentrate to 2.0 mL with HPLC water. This dilution is suitable for analyte concentrations down to the Limit of Quantitation of 1 ppb for bentazon, 8-chlorobentazon and N-methylbentazon.

4.2.11 Syringe filter a portion of the final extract through 0.45-um filter into an autoinjector vial.

4.3 Instrumentation

4.3.1 Description of Operating Conditions (See Table I)

4.3.1.1 Standards and extracts are analyzed by LC/MS on a C18 reverse phase column. The analysis is performed with gradient chromatography using methanol as the organic modifier. See Table I for a description of the chromatographic conditions.

4.3.1.2 Bentazon and 8-chlorobentazon are determined by negative ion monitoring of their pseudo molecular ions $[M-H]^-$. The dwell time used for selected ion monitoring is 0.3 seconds for each analyte resulting in a total scan time of approximately 0.8 seconds/scan.

4.3.1.3 For the analysis of N-methylbentazon, the monitored product ion (m/z 213) is formed by collisionally induced fragmentation of a precursor ion in the collision cell of a triple stage quadrupole mass spectrometer (MS/MS). The product ion represents the loss of propyl from the protonated molecular ion (m/z 255). The dwell time used for selected ion monitoring is 1.2 seconds resulting in a total scan time of approximately 1.3 seconds/scan.

4.3.1.4 The analysis is initially performed in the negative ion mode. At 7.5 minutes, the analysis mode is switched to positive ion MS/MS. The approximate retention times for each analyte are listed in Table I. The analysis mode switching time may be changed to allow for approximately the same number of scans to be acquired after 8-chlorobentazon and before N-methyl bentazon.

4.3.2 Hardware Tuning and Calibration

4.3.2.1 The successful detection (S/N > 5:1) of the lowest calibration standard and the criteria described in Section 4.7.3 are used to verify that the instrument is tuned and calibrated properly. If these criteria are met, no instrument adjustments are necessary.

4.3.2.2 Generally, no day to day tuning adjustments are required unless major repairs have been made to the mass spectrometer. If tuning or calibration is required, it must be performed by a qualified instrument operator and recorded in the instrument maintenance logbook.

4.3.2.3 Tuning is based on thermospray reagent ions. For positive ion tuning, reagent ions (e.g. m/z 59, m/z 100) are used for lens optimization. For negative ion, m/z 119 is typically chosen for lens optimization.

4.3.2.4 Mass calibration is based on thermospray reagent and background ions. A low mass calibration point may be set using one of the reagent ions mentioned above. A higher mass calibration point may be set using a background ion (e.g. m/z 269). The instrument will extrapolate all other calibration points. The successful detection of the target analytes at their expected nominal masses is used to verify calibration.

4.4 Potential Interferences

There are no known interferences originating from the sample preparation, extraction, cleanup or concentration procedure.

4.5 Time Required

4.5.1 Approximately 20 samples can be prepared for analysis in an eight hour period.

4.5.2 Each instrumental analysis requires approximately 20 minutes.

4.6 Modifications or Potential Problems

The SPE columns used in this validation were 6-mL Varian Mega Bond Elut columns (#1225-6001) with C_{18} loading of 1-g. If other loading levels or columns from other manufacturers are used, the extraction and/or elution efficiencies may differ.

Prior work done by BASF and ALTA suggests that N-methylbentazon is more volatile than bentazon or 8-chlorobentazon. Therefore, N-methylbentazon could be easily lost during the nitrogen concentration steps, especially if the extract goes to complete dryness. The validation data demonstrates that if the method is followed as written, losses of N-methylbentazon can be prevented.

4.7 Methods of Calculations

4.7.1 Quantitation is performed by using the average of a three point curve bracketing the sample in an injection sequence (4.9.2). The low standard should have at least a 5:1 signal/noise ratio for each analyte.

4.7.2 Analytes should be calibrated relative to the average response factor from the two preceding standards and one standard following. Deviation from this guideline should be noted in the raw data packet. The three standards used must be of three different concentrations, encompassing the range of calibration.

4.7.3 If the relative standard deviation (RSD, 4.9.3) for the three standards used for bracketing a sample is less than 20%, then the method is considered to be linear. The calculated RSD for each bracketed sample is documented in the raw data.

4.7.4 If the RSD is greater than 20% then the affected samples must be re-injected after acceptable re-calibration has been established.

4.7.5 No more than three samples may be injected between two standards.

4.7.6 The criteria listed in 4.7.4, does not apply if the results for the associated samples are less than the reporting limit, and the low standard has a signal/noise greater than 5:1.

4.7.7 The sample extract may be diluted with 75:25 HPLC water:methanol if the analyte response exceeds the range of the calibration curve (dilution factor, df = original volume/final volume).

4.7.8 The amount of analyte injected (ng/mL) is determined by dividing the value of the chromatographic peak area by the average response factor. See section 4.9 for a complete description of all calculations.

4.7.9 Deviation from the above guidelines should be noted in the raw data packet.

4.8 Fortified Samples

4.8.1 The method is validated for each set of samples analyzed by including a control sample and one or more samples fortified prior to the extraction procedure with 1 ppb or higher.

4.8.2 Add an appropriate fortification solution (from 4.1.3) to the water sample, (Example: add 100-uL of a 1 ug/mL fortification solution to a 100 mL water sample to fortify at 1 ppb). Do not exceed 1.0 mL as a fortification volume.

4.9 Equations

4.9.1 Calculate the response factor for each standard from the following equation:

$$RF = \frac{Area_{STD}}{Conc_{STD}}$$

Where: $Area_{STD}$ = the area of response for the product ion from an injected standard

$Conc_{STD}$ = concentration of the injected standard (ng/mL)

4.9.2 Quantitation of samples will be performed based on the average response for the standards preceding and following the samples and is defined as follows:

$$RF_{AVG} = \frac{(RF_{STD1}) + (RF_{STD2}) + (RF_{STD3})}{3}$$

Where: RF_{STD1} = the response factor from the first standard

RF_{STD2} = the response factor from the second standard

RF_{STD3} = the response factor from the third standard

Normally, RF_{STD1} and RF_{STD2} will precede the samples and RF_{STD3} will follow.

4.9.3 The relative standard deviation (RSD) is derived from the coefficient of variation (CV):

$$CV = s/RF_{AVG}$$

$$RSD = CV \times 100 \text{ (percent)}$$

Where: CV = coefficient of variation
RF_{AVG} = Average RF (4.9.2)
s = standard deviation (population) of the RF_{AVG}

4.9.4 Sample calculations are done according to the following formula:

$$Amt_{SAMP} = \frac{(Area_{SAMP})\ (FV)}{(V_{SAMP})\ (RF_{AVG})\ (Df)\ (Af)}$$

Where: $\mathrm{Amt}_{\mathrm{SAMP}}$ = Final Sample Amount (ppb),

$\mathrm{Area}_{\mathrm{SAMP}}$ = Area of response for the product ion from the sample,

$\mathrm{RF}_{\mathrm{AVG}}$ = Average Response Factor (area/ng/mL),

FV = Final Volume (mL),

$\mathrm{V}_{\mathrm{SAMP}}$ = Sample volume (g=mL),

Df = Dilution factor ($\mathrm{Vol}_{\mathrm{init}}/\mathrm{Vol}_{\mathrm{fin}}$).

Af = Aliquot factor ($\mathrm{Vol}_{\mathrm{alq}}/\mathrm{Vol}_{\mathrm{total}}$)

Example: For ALTA Lab ID 12001-2-MS5, bentazon;

$$Amt_{SAMP} = \frac{(3104796)\ (2.0)}{(100.04)\ (5382)\ (0.10)\ (1.0)} = 115\ ppb$$

4.9.5 If $\mathrm{Amount}_{\mathrm{SAMP}}$ is less than the reporting limit then the results are reported as less than this amount.

4.9.6 The results for field samples, control samples, fortified control samples, and percent recoveries are reported to three significant figures.

4.9.7 If the analyte concentration in the control sample used for fortification is greater than half the low calibration standard (1/4 of the reporting limit, 1 ppb), this background will be subtracted from the result for the fortified control before percent recovery is calculated and reported.

5 Method Validation

5.1 Disposition of Study Materials and Data

Upon completion of the study, copies of all materials associated with this report will be retained in the archives at ALTA Analytical Laboratory, Inc., 5070 Robert J. Mathews Parkway, El Dorado Hills, CA 95762. The originals of the above cited materials will be archived at BASF Corporation, Agricultural Products Group, 26 Davis Drive, Research Triangle Park, NC 27706-3528.

5.2 Source and Characterization of control samples

5.2.1 The control water used for the method validation was provided as the test system by BASF. The control water was taken from RCN 92235, Mississippi, sample 0039.

5.3 Preparation of Samples

No sample preparation was required. The control water was thawed and shaken thoroughly prior to aliquoting.

5.4 Fortification

To validate this method, control water from RCN 92235, Mississippi was provided by BASF. The water sample was fortified in duplicate at each of four levels, 1 ppb, 10 ppb, 100 ppb and 250 ppb.

5.5 Sample Management

Water samples were received at ALTA and handled according to ALTA Standard Operating Procedures, Chapter 10. Samples were received at the laboratory with dry ice and placed into freezers prior to processing. Samples were stored frozen between 0 and -20° C prior to extraction. Freezer temperature records for residue samples were maintained and archived.

5.6 Protocol Changes

A change was made to Protocol 93007 that modified the operating conditions for the HPLC to the following gradient: 90% 0.2M ammonium acetate (0.1% formic acid):10% methanol with a linear gradient to 40% 0.2M ammonium acetate (0.1% formic acid):60% methanol. The mass spectrometer operating conditions have been changed to monitor bentazon and 8-chlorobentazon by negative ion at m/z 239 and m/z 273, respectively. This change allowed for the analysis of all three analytes in one run and monitoring of the pseudo molecular ions for bentazon and 8-chlorobentazon.

A change was made to Protocol 93007 that allowed for adding volumes larger than 1-mL when performing fortifications. This change also allowed the use of positive displacement pipets when adding fortification solutions.

Table I

ANALYSIS CONDITIONS FOR THE DETERMINATION OF BENTAZON, N-METHYLBENTAZON AND 8-CHLOROBENTAZON

Instrumentation:

Waters 600-MS HPLC gradient pump (or equivalent)
Waters WISP 712 autosampler (or equivalent)
Finnigan MAT TSQ-700 equipped with a TSP2 thermospray interface (or equivalent).

HPLC Operating Conditions

Column:	Waters Nova-Pak C18, 3.9 x 150 mm column
Injection Vol.	50-uL (20-100 uL)
Flow Rate	1.2 mL/min

Time (min.)	Gradient	0.2M NH_4OAc/0.1% Formic Acid	Methanol
Initial	-	90	10
0-4	Linear	40	60
9.5	Step	90	10

Abbreviated LC conditions: When diluted extracts for bentazon only are analyzed, the LC system will be re-equilibrated after the elution of bentazon to facilitate shorter run times.

Mass Spectrometer Operating Parameters:

Vaporizer Temperature:	95 degrees C (85 - 130 degrees C)
Source Temperature:	280 degrees C (220 - 320 degrees C)
Repeller Voltage:	0 volts
Collision Gas:	Argon
MS/MS Collision Energy:	-18eV

Analyte	MW	Mode	Ion Monitored	Dwell Time (sec.)	Retention Time (approx. min.)
Bentazon	240	neg. ion	m/z 239	0.3	5.5
8-Chlorobentazon	274	neg. ion	m/z 273	0.3	6.4
N-Methylbentazon	254	positive MS/MS	m/z 255 - m/z 213 (precursor) - (product)	1.2	9.5