APPENDIX 1

DETERMINATION OF BENSULFURON METHYL IN NATURAL WATER BY HPLC-UV

(Method Reference: Du Pont Report AMR-232-84 (Ref. 3))
Quantitation Limit 0.5 PPB

PRECAUTIONS

1. Do not leave samples at room temperature longer than 1 day. Thaw samples no longer than 24 hours. Do not thaw samples over the weekend.

2. Process samples one at a time at extractions involving a pH change. Samples may be processed simultaneously for all other steps.

3. Use separatory funnels with teflon stopcocks. Do not use glassware with greased connections.

4. Do not use sodium sulfate at any point of the procedure.

5. Do not evaporate any portion of the extract at over 40° C. Do not evaporate to dryness until the final extract is in the calibrated test tube.

6. Evaporate the final sample solution to 0.5 to 0.2 mL and store the sealed sample tube in the freezer until analysis.

SAMPLE PREPARATION AND FORTIFICATION

1. Shake the thawed sample thoroughly and measure 100 mL in a graduated cylinder. If the sample is to be fortified for recovery determination, fortify the sample at this point in the procedure from a bensulfuron methyl standard.

2. Transfer the filtered sample to a 250-mL beaker. Cover the beaker with foil.

SAMPLE EXTRACTION

3. Process 4 samples at a time. Wash all glassware with soap and water and rinse with boiled deionized water prior to processing each sample.

4. Adjust the sample pH to 7.0 with 0.1 M HCl or 0.1 M NaOH using a pH meter that is standardized against pH 7 buffer at least once per day. Complete steps (4) through (9) before proceeding to the next sample.

5. Transfer the sample to a 250-mL separatory funnel with a teflon stopcock.

6. Extract the sample with 100 mL of high purity methylene chloride (dichloromethane). Use the 100 mL of methylene chloride to rinse the beaker before extracting the sample. The methylene chloride should not contain methanol or ethanol as a preservative.

7. Shake the extraction mixture for approximately 1 minute and swirl the separatory funnel to get a good separation. Vent the funnel through the top by releasing the cap as necessary.

8. Drain the methylene chloride (lower) layer into a 250-mL flask or beaker through a funnel with a glass wool closure. Make certain that there is no water layer or droplets in the extract.

9. Repeat the extraction of steps (6) through (8) with a second quantity of methylene chloride, and combine the two extracts.

10. Evaporate the combined extracts to 5-10 mL on a rotary evaporator or steam bath at 40° C or less. The flask should not get hot or cold.

11. Add one drop of acetic acid and continue evaporating to 1 to 2 mL. Transfer the solution to a graduated test tube using several small portions (not more than 5 mL total) of methylene chloride for rinsing.

12. Add exactly 0.05 mL of acetic acid to the test tube and evaporate to 0.5-0.2 mL. Check with general range pH paper to make certain that the solution is acidic, then immediately cap the test tube and store it in the freezer until HPLC analysis.

HPLC ANALYSIS

13. Just prior to HPLC analysis, blow dry each sample with a gentle stream of dry nitrogen and make each sample to 1.0 mL total volume with mobile phase. Analyze the samples and standards for bensulfuron methyl by HPLC using the following conditions:

DETECTOR:	Spectra Physics Model 8440, 8450, or 8490 UV detector or equivalent. (Spectra Physics, San Jose, California).	
COLUMN:	4.6 mm by 25 cm Zorbax ODS, 5-µm particle size, (MAC-MOD Analytical, Inc., Chadds Ford, Pennsylvania) or equivalent.	
COLUMN TEMPERATURE:	Ambient	
MOBILE PHASE:	Acetonitrile	415 mL
	Water	579 mL (use deionized water or equivalent)
	Acetic Acid	6 mL
FLOW RATE:	1.0 mL/min (or as appropriate)	
INJECTION VOLUME:	100 µL	

APPENDIX 2

DETERMINATION OF BENSULFURON METHYL IN SEDIMENT OR SOIL BY HPLC-PCD

(Method References: Du Pont Reports AMR-295-84 (Ref. 4) and AMR-132-83 (Ref. 5))
Quantitation Limit 1 PPB

SAMPLE PREPARATION

1. Thaw the frozen soil sample completely and mix it thoroughly with a metal rod or spatula to produce a homogeneous soil mixture.

DRY WEIGHT DETERMINATION

2. Weigh into a preweighed disposable metal or plastic pan a 10.00 g representative sample of soil. Dry the sample to constant weight at 135° C and record the sample dry weight for use in calculations.

EXTRACTION AND FORTIFICATION

3. Weigh a 10.00 g representative portion of the homogenized soil sample from Step 1 into a stability extraction jar or a 100-150 mL beaker. If the sample is to be fortified for recovery determination, fortify the sample at this point in the procedure from a bensulfuron methyl standard. Evaporate the solvent with a gentle stream of dry nitrogen.

4. Add 30 mL of HPLC grade acetonitrile and blend using a Tekmar tissumizer for one minute.

5. Decant the acetonitrile into a centrifuge bottle.

6. Repeat Steps 4 and 5 two more times. Combine the extracts in the centrifuge bottle and centrifuge (at 40 setting) for 5 minutes.

7. Decant the extract through a Whatman 2V filter paper into a 200 mL round-bottom flask. Add 3 drops of acetic acid and evaporate at 35° C to 1-3 mL.

8. Evaporate the acetonitrile with a gentle stream of dry nitrogen and add 5 mL of 0.15 M ammonium hydroxide to the sample for Bond Elut™ cleanup.

BOND ELUT™ CLEANUP

9. Condition a disposable C-18 Bond Elut™ column by flushing it with 20 mL of acetonitrile, 20 mL of boiled deionized water, and then 20 mL of 0.15 M ammonium hydroxide.

10. Apply the sample extract with a disposable pipet to the column and allow the liquid to pass through the Bond Elut™ Column at a moderate rate. Rinse the flask with 2 mL more of 0.15 M ammonium hydroxide and pass the rinse solution through the column.

11. Wash the flask with 20 mL of boiled deionized water (cooled to room temperature) and pass this wash solution completely through the column. Use differential pressure to remove traces of water from the column. Transfer the solution to a clean dry reservoir.

12. Elute bensulfuron methyl into a 125-mL round bottom flask with 10 mL with acetonitrile.

13. Add 3 drops of acetic acid and evaporate to 3-5 mL on a rotary evaporator at 35° C.

TOLUENE CLEANUP

14. Blow the sample dry and immediately dissolve the residue in 50 mL of 0.15 M ammonium hydroxide. Transfer the solution to a separatory funnel.

15. Wash the sample with 50 mL of HPLC-grade toluene, shaking for 2 minutes. Discard the toluene (upper) layer.

16. Acidify the aqueous layer (in a 250-mL beaker) to approximately pH 4.0 using 6 M HCl.

17. Transfer the solution back to the separatory funnel and extract two times with 50-mL quantities of toluene per extraction, shaking two minutes for each extraction.

18. Filter the toluene (upper) layers through glass wool into a 500-mL round bottom flask.

19. Add 3 drops of acetic acid and evaporate the solution to approximately 1 mL at 35-40° C.

20. Evaporate the solution to dryness with a gentle stream on dry nitrogen, and dissolve the residue in 10 mL of HPLC-grade chloroform for GPC cleanup.

GPC CLEANUP

GPC Cleanup is optional. If GPC Cleanup is not needed, complete step 19 and then go to step 23.

21. Prepare a GPC column 2.5 cm in diameter by 40 cm long of Bio-Beads S-X3 styrenedivinylbenzene copolymer (Bio-Rad Laboratories, Richmond, California) using chloroform as the mobile phase. Using the GPC column on the Auto Prep Model 1002 (Analytical Biochemistry Laboratories, Columbia, MO), chromatograph 5 mL of the sample through the column at 5 mL/min. On the basis of prior calibration, collect the fraction that contains the bensulfuron methyl for analysis. (Typical conditions are: dump 20 mL, collect 10 mL, and wash 10 mL.)

22. Evaporate the chloroform solution to approximately 1 mL and transfer the solution to a test tube using no more than 2 mL of acetonitrile for rinsing.

23. Evaporate the sample solution with a gentle stream of dry nitrogen to an estimated 0.2 mL. If HPLC analysis will be delayed, freeze the sample until analysis. Prior to analysis, blow the sample to dryness and dissolve the sample residue in 0.25 mL of mobile phase for HPLC analysis.

24. Analyze the samples and standards for bensulfuron methyl by HPLC using the following conditions:

DETECTOR:	Tracor Model 965 Photoconductivity (Tracor Instruments, Austin, Texas) or equivalent with mercury lamp. Ion exchange resin tube and micr. .ump should be disconnected and not used.
COLUMN:	4.6 mm by 25 cm silica, 10 µm particle size, (Alltech Associates, Deerfield, Illinois) or equivalent.
COLUMN TEMPERATURE:	Ambient
MOBILE PHASE:	Hexane 720 mL Isopropyl alcohol 110 mL Methyl alcohol 110 mL Acetonitrile 55 mL Acetic Acid 2 mL Water 1 mL (use deionized water or equivalent)
FLOW RATE:	0.5 mL/min (or as appropriate)
INJECTION VOLUME:	20 µL

PROTOCOL AMENDMENT NO. 2
ANALYSIS

A. FIELD DISSIPATION (CLOSED POND) STUDIES FOR AQUATIC USES AND AQUATIC IMPACT USES

B. FIELD ACCUMULATION (CLOSED POND) STUDIES FOR AQUATIC NON-TARGET ORGANISMS

SPONSOR:	E. I. du Pont de Nemours & Co., Inc. Agricultural Products Department Experimental Station Wilmington, DE 19880-0402
STUDY NUMBER:	AMR-1350-88
CHANGE 1:	Change item 2 on page 9 of Amendment No. 1 under the heading "Sample Preparation and Fortification" to read as follows: 2. Transfer the unfiltered sample to a 250-mL beaker. Cover the beaker with foil.
REASON:	Filtration of water samples should be consistent for the immunoassay method and for the UV-HPLC method. To make them consistent, filtration for the UV-HPLC method will be eliminated.

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