Cover Sheet for

ENVIRONMENTAL CHEMISTRY METHOD

Pestcide Name: Sulfometuron Methyl

MRID #: 435050-01

Matrix: Soil/Water

Analysis: Immunoassay

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An ELISA Immunoassay Method for the Determination of Residues of Sulfometuron methyl in Soil and Water

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Abstract

This analytical method was developed for the determination of Sulfometuron methyl (the active ingredient in Oust® Herbicide) residues in soil and water. Residues are quantitated using an enzyme-linked immunosorbent assay (ELISA) having a Limit of Quantitation of 0.1 ppb.

Sulfometuron methyl (SM) residues are extracted from 10-g soil samples using 90% acetone/10% phosphate-buffered saline (PBS) solution. An aliquot of the soil extract is evaporated then redissolved in pure PBS solution. Water samples are simply filtered then diluted (1:1, minimum) with PBS solution. **No sample cleanup is required, due to the high selectivity of the ELISA procedure.**

Extracts are first reacted with antibody solution. Then an aliquot of this reaction mixture is transferred to an antigen-coated microtiter plate. An enzyme-substrate reaction (alkaline phosphatase -- *p*-nitrophenylphosphate) produces an absorbance of 405-nm light that is correlated to the original SM soil residue concentration.

The average recovery (± S. D.) for 26 soil samples (from Greenville, Miss.; Rochelle, Ill.; Uvalde, Tex.; and Madera, Calif.), fortified over the range 0.10-1.00 ppb, was 111 ± 10%. The average recovery (± S. D.) for 12 water samples (from 3 local surface water sources), fortified over the range 0.025-0.200 ppb, was 107 ± 10%. The validity of this method for soil was further confirmed using LC/MS (field-aged soils from a dissipation study were analyzed using both this ELISA method and LC/MS).

This method is highly cost-effective, uses few hazardous reagents, and provides fast turn-around time to results.

INTRODUCTION

Sulfometuron methyl (methyl 2-[[[[(4,6-dimethyl-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoate) is the active ingredient in Oust® Herbicide. Sulfometuron methyl is used for vegetation management along roadsides, within railroad right-of-ways, and on industrial, noncrop sites. In addition, it can be used for selective weed control in forest site preparation and release of pines. Oust® Herbicide has been shown to control a broad range of herbaceous species (annual grasses, perennial grasses, and broadleaf weeds).

Sulfometuron methyl (SM) belongs to the sulfonylurea class of herbicides -- the properties of which are well known **(1)**. Their low toxicity combined with low application rates makes these compounds especially attractive from an environmental and human health standpoint.

Method Scope

This report describes an analytical method based on an enzyme-linked immunosorbent assay (ELISA) for the determination of sulfometuron methyl (SM) in soil with a limit of quantitation of 0.1 ppb (based on 10-g samples), or in water with a limit of quantitation of 0.025 ppb.

ELISA methods are frequently the most sensitive analytical methods available at a low cost **(2,3)**. Selectivity tends to be excellent (or at least can be readily characterized and/or controlled).

ELISA Method Principles

(1) Anti-SM polyclonal antibodies (Ab) and buffer are added to sample containing an unknown amount of SM and incubated. The antibodies bind to any SM molecules present in the sample.

(2) Aliquots of the solution are added to wells on a 96-well microtiter plate, which have been coated with an SM derivative-ovalbumin conjugate. Any excess Ab not bound to SM in the sample will bind to SM immobilized on the microwell plate. The plate is then washed to remove any Ab not bound to the plate. The amount of Ab bound to the microwell is an inverse measure of the amount of SM in the sample.

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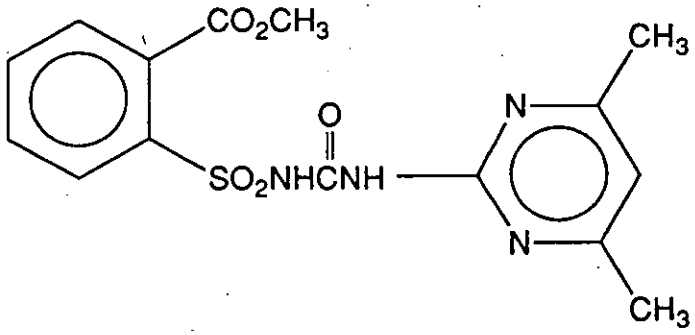
(3) To detect the Ab bound to each microwell, an anti-rabbit antibody conjugated to an enzyme, alkaline phosphatase (Ab-E), is added to each well and incubated. This Ab-E will bind to any anti-SM antibodies bound to the microwell. The microwell plate is then washed to remove any unbound Ab-E.

(4) An alkaline phosphatase substrate, paranitrophenyl-phosphate, is added to the microwells. The enzyme-substrate reaction produces a yellow color which is inversely proportional to the concentration of SM in the sample. A microtiter plate reader quantitates the absorbance in each well at 405 nm. Computer software is used to construct a standard curve from standards run on the plate, and to calculate SM concentrations for each unknown on the plate.

MATERIALS AND METHODS

Compound Identity

The *Chemical Abstracts* structure and uninverted, systematic name of sulfometuron methyl are as follows:



sulfometuron methyl

methyl 2-[[[[(4,6-dimethyl-2-pyrimidinyl)amino]carbonyl]amino]-sulfonyl]benzoate

CAS Registry No. 74222-97-2

Equipment

Disposable glassware or plasticware is recommended for all possible uses to minimize contamination. Polypropylene is preferred for plasticware, but high-density polyethylene is also acceptable. In addition, comparable equipment may be substituted for all equipment; however, note any specifications

in the following descriptions before substituting other equipment.

(1) Tubes: *Centrifuge, polypropylene; 15 and 50 mL*: Falcon Blue Max #2097 & 2098, Becton Dickinson Labware, 2 Bridgewater Lane, Lincoln Park, NJ 07035.

Assay tubes, 12 x 75 mm polypropylene culture tubes: VWR #60818.

Cube 2ubes®, polypropylene: DBM Scientific Corp., 511 Fifth Street, San Fernando, CA 91340 (818) 360-3610 Cat. No. CT-205.

Multi-Use Rack Cat. No. ACT-850

(2) Pipettes: Improvements in pipettes are continually being made. Following are some previously recommended pipettes and also a new line of pipettes from Biohit which are lighter in weight, faster, and easier to use. These pipettes are designed to avoid the types of problems one may encounter with the repetitive type of motion associated with pipetting.

Eppendorf Repeater 4780, with reservoir tips of 0.5-, 2.5-, and 5-mL capacity: Brinkman Instruments, Cantiaque Road, Westbury, NJ 11590.

edp™ motorized pipettes, 0.25, 1.0, 2.5, and 10.0 mL: Rainin Instrument Co., Cantiaque Road, Westbury, NJ 11590

Titertek 12-channel adjustable volume (0.050-0.300 mL) multipipettes with tips: Flow Labs, 7655 Old Springhouse Rd., McLean, VA 22102.

Biohit Proline® Electronic Pipettors (1.0 mL, 50 to 1000 µL, and a Multichannel (12) 25 to 250 µL; the 2.5-mL capacity is not available at this time): These are made in Finland and distributed by Vangard International, Inc., 1111-A Green Grove Rd., P.O. Box 308, Neptune, NJ 07754-0308. 1-(800)-922-0784.

(3) Reagent Reservoirs: *disposable, #4870*: Costar Corp. 206 Broadway, Cambridge, MA 02139.

(4) ELISA Microwell Plates: *NUNC-Immunoplate, IF with certificate, Cat. No. 439454*: Distributed in U.S.A. by Vangard International, Inc., 1111-A Green Grove Rd., P.O. Box 308, Neptune, NJ 07753. Other plates from this vendor (e.g., stripwell plates) or plates from other

vendors must give equivalent performance in terms of accuracy, precision, and recovery in order to be substituted.

(5) ELISA Plate Washer: *Bio-Tek EL403 Microplate Auto Washer:* Bio-Tek Instruments, Inc., Winooski, VT 05404, or *Ultrawash II:* Dynatech Laboratories, Inc., 14340 Sullfield Circle, Chantilly, VA 22021.

(6) ELISA Plate Reader: *Titertek Multiskan MCC/340 Plate Reader, equipped with 405-nm filter:* Flow Labs, address above, or *UV max Kinetic Microplate Reader,* Molecular Devices Corporation, 3180 Porter Drive, Palo Alto, CA 94304 or comparable equipment.

(7) Computer and Software:

IBM Personal System 2 Model 50, with one hard drive, one floppy drive, IBM Proprinter II printer and IBM Disc Operating System Software Version 3.30: IBM Corporation, 1001 Jefferson St., Wilmington, DE 19801.

TiterCalc Software Version 2.1: Hewlett-Packard, Rt. 41 - Starr Rd., P.O. Box 900, Avondale, PA 19311,

or

Macintosh SE/30 with Laserwriter Plus printer and Macintosh Disk operating system 6.07 or comparable equipment.

Microsoft® Mouse and Microsoft® Windows Software Version 2.0:

Microsoft Corporation, 16011 NE 36th Way, Box 97017, Redmond, WA 98073-9717.

(8) pH Meter: *Corning Model 120 pH Meter:* Corning Glass Works, Medfield, MA 02052.

(9) Balance: *Mettler PM 460 Balance*: Mettler Instrument Corp., P.O. Box 71, Hightstown, NJ 08520.

(10) Vortex Mixers and Stir Plates: *VWR brand*: distributed by VWR Scientific, P.O. Box 626, Bridgeport, NJ 08014.

(11) Filters: *Millex-HV Unit, 0.45-μm pore size, 25-mm diameter*: Millipore Products Division, Bedford, MA 01730. Other filters may only be substituted if assay performance is validated and found to be comparable to the performance with these filters.

(12) B.D. Disposable Syringes: 20-cc Plastipak without needles, VWR, Rutherford, NJ 07070.

(13) Tumbler: Design of tumbler for soil extraction (Appendix I).

(14) Sorvall RC5C Centrifuge: A comparable refrigerated model with capacity for 50-mL centrifuge tubes at 5000 RPM may be substituted. DuPont/Sorvall Marketing Services, Barley Mill Plaza, Wilmington, DE 19898.

(15) Infrared Moisture Determination Balance: Model AD-4712 or a comparable moisture determination balance. A&D Engineering, Inc., 1555 McCandless Drive, Milpitas, CA 95035.

Reagents

If the vendor and catalog number of a chemical are not specified, reagent-grade chemicals from any reputable vendor may be used. If the vendor and catalog number are specified, then substitution with material from another vendor may require method validation studies to assure quality performance.

Polyclonal antibodies specific for SM were produced in rabbits by standard immunization procedures (DuPont Medical Products, Glasgow, Del.). The immunogen used consisted of the SM Hapten conjugated to pumpkin seed globulin (PSG). The antigen used for coating plates consisted of an SM Hapten conjugated to ovalbumin. When satisfactory performance was demonstrated in antigen-coated microtiter plates, the assay was optimized and validated.

Either of two equivalent antibody formulations are possible: a liquid formulation or a lyophilized tablet. However, only the tableted formulation was used for this report.

(A) *Water*: Water quality is critical to reagent stability and assay performance. Use Milli-Q™ (Millipore Corp., Bedford, Mass.) or a comparable system. This water is deionized, carbon-filtered, and micro-organism-filtered at a pore size of 0.2 μm. It is important to maintain the system so as to keep the quality of the water high. In some cases, it may be desirable to use U.S.P. grade pyrogen-free sterile water to make up plate coating and blocking buffers.

Unless otherwise specified, "water" in this method refers exclusively to Milli-Q™ water or water of equivalent quality (i.e., HPLC-grade water).

(B) *Phosphate-Buffered Saline Solution (PBS)*:

0.12 M NaCl
2.7 mM KCl
10 mM Phosphate Buffer
pH 7.4 at 25°C
0.02% Sodium Azide (Optional)

Add one bottle Sigma Diagnostics Phosphate Buffered Saline powder (PBS, Catalog #1000-3, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178) to 1-liter water and stir until dissolved. Typically made up in polypropylene bottles. Store at nominal 2 to 8°C (maximum desirable range 1 to 10°C) for a maximum of one month. If preservative is required for longer storage, add 0.2-g sodium azide (Sigma #1000-3, or equivalent).

(C) *10 x PBS/1% BSA*:

1.2 M NaCl
27 mM KCl
100 mM Phosphate Buffer
1% BSA (Bovine Serum Albumin)
0.02% Sodium Azide (Optional)
pH 7.4 at 25°C

Add one bottle of Sigma PBS (see B) to of 70 mL of water. Stir to dissolve and then add 1 g of Bovine Serum Albumin (BSA), Fraction V, or BSA giving equivalent performance in the assay, and stir until all clearly dissolves. Add 0.02-g sodium azide if desired. Make up to 100-mL total volume and store 10-mL aliquots in 15-mL polypropylene tubes at 2 to 8°C for a maximum of one week without azide and for one month with azide. Equilibrate at room temperature with stirring before use, to dissolve crystals which may have formed.

(D) *PBS/0.5% BSA*:

0.12 M NaCl
2.7 mM KCl
10 mM Phosphate Buffer
0.5% BSA

0.02% Sodium Azide (Optional)
pH 7.4 at 25°C

1 Sp in 200 mL H2O pH.

Add one bottle of Sigma PBS (see B) to 900 mL of water and stir until dissolved. Add 5 g of Bovine Serum Albumin (see C) and stir until dissolved. Make up to 1000-mL total volume. Store at 2 to 8°C for a maximum of one week without azide and for one month with azide.

(E) *Wash Buffer (PBS-Tween)*:

0.12 M NaCl
2.7 mM KCl
10 mM Phosphate Buffer
0.05% Tween-20
pH 7.4 at 25°C

Add four bottles Sigma PBS (see B) to 4-L water. Add 2-mL Tween-20. Stir for approximately 20 minutes. Store at room temperature for a maximum of 36 hours. Make fresh daily.

(F) *Coating Antigen Master Stock Solution*:

An-ovalbumin conjugate (obtained internally from DuPont -- see beginning of this section), stored frozen at -20°C or -70°C. Details can be found in Table I.

(G) *Coating Antigen Intermediate Stock Solution*:

Prepare a dilution of an aliquot of Coating Antigen Master Stock Solution in PBS to give a concentration such that 1 mL of this dilution + 99 mL of PBS provides the desired coating antigen concentration for coating 5 plates.

For example, 1 mL of a 10-µg/mL intermediate stock solution + 99-mL PBS provides a coating antigen solution of 0.1 µg/mL.

To make 20 plates, add 4 mL of coating antigen intermediate stock solution with stirring to 396-mL PBS. Use immediately. Do not store.

Store the remainder of the dilution at -20°C.

(H) *Blocking Buffer (3% BSA)*:

2.7 mM KCl
10 mM Phosphate Buffer

3% BSA
0.02% Sodium Azide (Optional)
pH 7.4 at 25°C

Add 15-g BSA to 500-mL PBS. Stir without heat until all is clearly dissolved (about 1 hr). Use immediately. Do not store. Makes 20 plates.

Note: Blocking the plates is optional. It is not recommended unless it offers a significant advantage to the assay performance.

(I) *Anti-SM Antibody Tablets*

Each tablet delivers the equivalent amount of antibody as the liquid formulation when hydrated as described. Each tablet lot contains tris buffer, BSA, trehalose, and polyethylene glycol with approximately 1 µL of anti-SM antibody per tablet. Specific concentration is given with the specific data for SM in Table I. Hydrate the tablet in a volume of 10X PBS/1%BSA and rock gently until tablet is completely dissolved. This reagent combines the antibody with the protein buffer for a one-step addition. Add 0.1 mL to each 0.9 mL of standard or sample. Note that the titer of the antibody in the 10X PBS/1%BSA is 10X desired titer in assay.

(J) *Second Antibody-Enzyme Conjugate Stock*:

Use an affinity-purified anti-[rabbit(polyclonal) IgG (H + L)]-alkaline phosphatase conjugate. Recommended vendor is Jackson Immunoresearch Laboratories, Inc., 872 West Baltimore Pike, P.O. Box 9, West Grove, PA 19390, Catalog #315-055-003. Reconstitute each vial of lyophilized powder with 1 mL water 24 hours before use to ensure full solubilization. Rock gently for a minimum of 1 hour. Do not vortex or shake. Follow directions on insert sheet. Store hydrated stock at 2 to 8°C according to vendor's recommendation. Store lyophilized powder at 2 to 8°C for up to 1 year, or for the shelf-life specified by the vendor. Alternate vendors may be qualified.

(K) *Second Antibody-Enzyme Conjugate Reagent*:

Add 40 µL of Second Antibody-Enzyme Conjugate Stock to 40 mL of PBS/0.5% BSA. Mix gently in a

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50-mL polypropylene tube. This is enough for two plates. This gives a substrate development time of 40 to 60 minutes. The titer may need to be adjusted in order to keep the substrate development time in the optimal 40- to 60-minute range. New lots or new vendors' materials may require re-optimizing titer to obtain the desired substrate development time. Perform crossover studies between lots or vendors to ensure adequate performance in terms of substrate development time, precision, accuracy, and recovery. Most manufacturers will take back inadequate lots for exchange or credit.

(L) *Substrate Buffer Stock*:

10% Diethanolamine (DEA)
0.1 g/L $MgCl_2 \cdot 6H_2O$
0.02% Sodium Azide

Add 97-mL diethanolamine, 0.2-g sodium azide and 0.1-g magnesium chloride ($MgCl_2 \cdot 6H_2O$) to 800-mL water. Adjust the pH to 9.8 with concentrated HCl. Make up to 1000 mL with water.*

(M) *Enzyme Substrate Reagent*: 1 mg/mL *p*-nitrophenyl-phosphate (PNPP)

1 mg/mL *p*-nitrophenylphosphate (PNPP)
2% Diethanolamine 0.02 g/L $MgCl_2 \cdot 6H_2O$

Make fresh before use. For two plates, add 32 mL of water to 8 mL of Substrate Buffer Stock and mix. Add two 20-mg substrate tablets and mix until dissolved in a 50-mL disposable polypropylene tube. The 20-mg tablets in individual foil wrappers (Sigma Catalog No. N-2765), are recommended. These tablets are made to tighter specifications and designed for ELISA use. For best results, store at -20°C.

(L) + (M) *Enzyme Substrate Reagent with Buffer*:

Instead of preparing (L) and (M) separately, Sigma Chemical Co. now makes available these reagents in combined form as Sigma FAST™ Tablets (Sigma

* For our work, we obtained these ingredients in kit form (Code #77544) from New England Nuclear, 549 Albany Street, Boston, MA 02118. This kit has been discontinued, however.

Catalog No. N-2770). Each tablet yields 20 mL of the DEA buffer containing PNPP.

(N) *Soil Extraction Solvents:* 90% Acetone/10% PBS

Store at 2 to 8°C up to one month. Dispense 20 mL/10-g soil.

(O) *SM Analytical Standard (powder)*:

Analytical standards are nominally 95-99% pure (actual purity is stated) and are available from E. I. du Pont de Nemours and Company, DuPont Agricultural Products, Wilmington, DE 19880-0402.

(P) *SM Master Stock Solution*:

Following are general directions for the preparation of SM solutions and standards.

0.1 mg/mL in methanol or appropriate solvent (100,000 ng/mL): Weigh out 10.0 mg and place in a 100-mL volumetric flask. Dissolve in 100-mL, HPLC-grade solvent. Store preferably in glass vial with solvent-resistant cap or high-density polyethylene (HDPE) bottle. Seal and store in secondary container in freezer at nominal -20°C. Expiration of this solution is six months if no evaporative loss is observed, or longer if concentration is still determined to be 0.1 mg/mL (recheck at six-month intervals). Thoroughly clean stir bars with acetone. Stir bars should be reserved for exclusive use with this solution, to avoid contamination of other solutions with standard.

(Q) *SM Intermediate Stock Solutions*:

These are generally used to make further dilutions in order to make standards, but can also be used for control sample fortifications if desired.

10,000-ng/mL Stock: Add 1 mL of Master Stock Solution to 9-mL PBS and mix. Store at 2 to 8° for one month.

1000-ng/mL Stock: Add 1 mL of 10,000-ng/mL Stock to 9-mL PBS (or equivalent proportions) and mix. Store at 2 to 8°C for one month.

(R) *Spiking Stock Solutions*:

These are used to prepare standards and may also be used for fortifications. They are stored at 2 to 8°C for up to one month. They are all 100x the desired final concentration so that the spiking volume for the standards is minimal and the same at each level.

These spikes may also be used for fortified controls.

(S) Preparation of Spiking Stock Solutions:

Volume (mL) of 1000 ng/mL Intermediate Stock	Volume of PBS (mL)	Spiking Stock Concentration (ng/mL)
0.01	9.99	1.0
0.025	9.975	2.5
0.05	9.95	5.0
0.1	9.9	10.0
0.25	9.75	25.0
0.50	9.5	50.0
1.0	9.0	100
2.5	7.5	250

(2) Preparation of Standards:

Normally, a large volume (40 mL) of each standard is prepared in 50-mL polypropylene tubes. Add 400 µL of each Spiking Stock Solution to tube containing 39.6-mL PBS. Preparation of 40 mL of each standard reduces the potential for variability when making standards fresh each day. It also improves precision and accuracy by eliminating the need to pipette extremely small amounts of spiking solution for smaller volumes of standards. The 0 standard should be from the same PBS solution used to dilute samples. Standards are stable for up to one month stored at 2 to 8°C.

Spiking Solution Used (ng/mL)	Standard Concentration (ng/mL)
1.0	0.010
2.5	0.025
5.0	0.05
10.0	0.1
25.0	0.25
50.0	0.5
100	1.0
250	2.5

ANALYTICAL METHOD

Microtiter Plate Preparation

An antigen or analog of the antigen covalently conjugated to ovalbumin is used to prepare the antigen-coated 96 well, polystyrene microtiter plates. The adsorptive characteristics of the ovalbumin on polystyrene and the protein-adsorptive capacity imparted during the manufacture of the plates provides the stability of the protein adsorption to the solid phase, which is required through the several washing steps in the ELISA assay. Note that all microtiter plates from all vendors are not the same, and selection of a different manufacturer requires testing in the assay format. Plates from different vendors or from different grades from the same vendor vary in total protein absorptive capacity and in the precision of the amount of protein adsorbed per well.

Nunc-Immuno Plate, MaxiSorp F96 (or equivalent) microtiter plates are coated with the Coating Antigen Reagent (200 µL/well), dispensed from a disposable reagent reservoir with a 12-channel pipettor. Plates are covered and incubated at room temperature for a minimum of two hours or they may incubate overnight at 2 to 8°C. The following morning, the plates are washed two cycles of three, 400-µL washes each (2 x 3), turning the plate 180° between wash cycles. Note that 180° rotation of the plate on the plate washer after each wash ensures that each well is washed at least once in case of a blocked tube in the dispensing head. Plates are air-dried or

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dried in a 37°C oven for 30 minutes. Cover each plate and store in a plastic "ziploc"-type bag with dessicant packs. Store at 2 to 8°C for up to three months (longer if performance stability is demonstrated).

Water Sample Preparation

Filtering all samples through a Millipore-HV, 0.45 µm pore size, 25 mm diameter filter is recommended. These filters contain Durapore® membranes, which are made from polyvinylidine difluoride (an inert, high purity, hydrophilic polymer) and do not retain SM. A minimum dilution of 1:2 (one part sample plus one part PBS) is recommended. Since natural waters may contain a wide variety of minerals, organic substances, acids, bases, suspended solids like colloidal silica, sediment, algae, microorganisms, etc., one must be alert to the possibility of a significant matrix effect on the assay response versus the aqueous standard curve. The 1:2 recommended dilution of the sample in the assay is intended to reduce the matrix effect. If control water samples are available, it is necessary to validate the method by performing spiking/recovery studies at appropriate concentrations and dilution factors to encompass the concentrations to be measured in the unknown samples. If no control samples are available, spiking recovery studies and dilution recovery studies should be performed on selected samples, to validate the quantifiable range in that matrix.

Soil Sample Preparation

Step 1

If necessary, thaw 35 grams each of soil samples (20 to 24) overnight at 4 to 8°C in a 50-mL polypropylene centrifuge tube. If the soil is very wet, centrifuge the following morning at 4000 RPM, (3100 G) and 0°C for 30 minutes to remove excess water. Decant supernatant and proceed to next step.

Step 2

Transfer approximately 5 g (record actual exact weight) of each sample type to moisture balance to determine % Moisture and record on worksheet. This will be used to correct the soil wet weight in the final calculations. Transfer 10 g (record actual

exact weight) of each to 50-mL tubes for extraction. Carefully transfer sample I.D. At this time also set up fortified soil controls.

Step 3

Add 20-mL acetone/PBS extraction solvent to each of the 10-g soil samples. For fortified soils, add the spike and then the extraction milieu to 20-mL total volume. Vortex and place on tumbler for one-hour extraction. Design of tumbler is described in Appendix I.

Step 4

Remove samples from tumbler and centrifuge at ~ 3400 RPM, 0°C, for 30 minutes. Decant supernatant and filter it through a Millipore-HV, 0.45-µm pore size, 25-mm diameter filter into a 50-mL polypropylene tube, transferring label information onto that tube. These filters contain Durapore® membranes, which are made from polyvinylidine difluoride (an inert, high-purity, hydrophilic polymer) and do not retain SM.

Step 5

Pipette 2.5 mL of each filtered extract into a 15-mL polypropylene tube and take just to dryness under nitrogen or filtered, compressed air, 40 psi, in a 35-50°C (actual temperature not critical, within this range) water bath. Do not over dry. Reconstitute with 5 mL of PBS and then vortex each tube vigorously. Store at 2 to 8°C until analysis. CAUTION: The 2.5 mL of extract must be dispensed accurately.

The reconstituted soil extracts may be held overnight at 2 to 8°C.

Each day, up to 24 samples can be prepared for analysis the following day. It is possible that there will be some repeats each day from the previous day at different dilutions. For that reason, the reconstituted soil extracts should be retained at 2 to 8°C until reportable results are obtained.

Assay Procedure

Step 1: Reagents

Take reagents, plates, standards, and samples out of the refrigerator to allow them to warm up to room temperature before starting the assay. For 10XPBS/1% BSA, take out the 10-mL aliquot in the 15-mL polypropylene tube to prepare the antibody reagent. Inspect the tube to make sure there are no precipitates. If it is necessary to warm the tube in a 37°C water bath to solubilize any precipitates, allow the contents to equilibrate to room temperature before adding the antibody tablet(s).

Step 2: Anti-SM Antibody Tablets

Visually inspect the integrity of the tablets before use. If there are any pieces broken off, do not use that tablet. Discard and use only "perfect" tablets. Dissolve 4 tablets in 10 mL of 10XPBS/1% BSA. Rock tube gently until tablets completely dissolve. This reagent combines the antibody with the protein buffer for one-step addition. Add 0.1-mL antibody solution to each 0.9 mL of sample. This reagent is good for three working days if maintained at 2 to 8°C. Normally, it is made fresh and used up each day.

Step 3: Controls

Fortified (spiked) controls are prepared by spiking soil or water with appropriate concentrations across the quantifiable range using the Spiking Stock Solutions and then processed exactly like the samples. The usual practice is to run at least one fortified control on each microtiter plate. In each working day, include fortifications to cover the assay range. It may also be necessary to dilute one or more fortifications to accompany any samples out of range requiring a further dilution.

All samples and controls are diluted to minimize potential matrix effects.

Soil: After drying extract and reconstitution in PBS, each mL = 2.0-mL supernatant. This means that the measured ng/mL in the assay must be multiplied by 2 to determine ng/mL supernatant. For further calculations for ng/g soil sample, see Table II.

Water: For aqueous samples which are diluted with PBS in a ratio of 1:1, simply multiply measured ng/mL x 2 to equal SM concentration (ng/mL) in the sample. Again refer to Table II.

Step 4: Assay Set-Up

Set up two rows of 12 cube 2ubes® for each plate; see Figure 1 for plate design. Use of cube 2ubes® allow use of a multichannel pipette so that the 12 samples can be treated simultaneously. Standards are not diluted; dispense 0.9 mL each. Controls and samples have all been pre-diluted. Dispense 0.9 mL each of control or sample. Add antibody (0.1 mL) to each of the 12 tubes simultaneously using a reagent reservoir and the multichannel (12) pipette and mix by aspirate/dispense X 5. Change tips before adding antibody to the second row. Incubate at room temperature for 30 minutes. To allow for completion of each step in the assay and keep the time the same for each plate, a 15-minute delay is suggested between plate 1 and plate 2 throughout the assay.

Alternatively, the 12 x 75 polypropylene culture tubes may be used if preferred to the cube 2ubes®; use a sample size of 1.8 mL (vs. 0.9 mL) and 0.2 mL (vs. 0.1 mL) antibody reagent. If the assay is set up this way, the multichannel pipette cannot be used for antibody addition or plate loading. The EDP (or comparable) pipette, set for a multi-dispense mode, is used for each individual tube.

Step 5: Addition of Reaction Mixture to Microtiter plate

After a 30-minute incubation in the assay tubes, 200-µL aliquots of the standards, controls, and samples are pipetted into wells of the antigen-coated microtiter plate according to the typical plate format shown in Figure 1. Use the multichannel pipette to transfer 12 samples simultaneously (three replicate rows). Change tips or have a second multichannel pipette ready for the next set of 12 samples. Alternatively, use the EDP (or comparable) pipette set for a multi-dispense mode to dispense the three replicates. Set timer for another 30-minute incubation on the plate.

Step 6: Second Antibody-Enzyme Conjugate Addition

During the sample incubation, prepare the second antibody enzyme conjugate as described in the ***Reagent*** Section. After the plate incubation, wash the plate using 2 x 3 cycles on the plate washer and then invert, with mild force, on several thicknesses of paper towels to blot and remove any remaining droplets. Add 200-µL freshly prepared conjugate to all wells with standards, controls, samples, and Row H (about 20 mL/plate). Use a multichannel pipette and a disposable reagent reservoir. Load the plate as shown in Figure 1, starting with Row B. Row A is reserved for the substrate blank. Row H is used for the non-specific binding blank.

Incubate at room temperature for 30 minutes after the plate is loaded.

Step 7: Enzyme Substrate Addition

While the plate is incubating, prepare the enzyme substrate reagent. After the plate incubation is completed, wash plate 3 X 3 cycles on plate washer and blot any excess liquid as previously described. Add 200-µL substrate reagent to all wells in all rows. Begin with Row B, C, D, E, F, G, H, and then A. The reaction begins immediately when substrate is added, therefore, timing differences between standards and samples must be minimized. Set clock and monitor yellow color development. Note that the enzyme-substrate reaction is temperature sensitive so the reaction time may vary from day to day.

Alternatively, the substrate may be added in the following order: Row B, E, C, F, D, G, H, & A. The purpose is to minimize the differences between the standards on the top of the plate and the samples on the bottom half of the plate. This may be particularly important with soil samples and can be determined by spiking/recovery studies. It is a good idea to draw a line across the plate between Rows D and E and also to put a mark on the plate by D and E for rapid, visual orientation.

A satisfactory standard curve and sample data may be obtained when the 0 absorbance is at least 1000 ± 100 mAU if the substrate has been on the plate at least 45 minutes or the final readings can be taken when the 0 absorbance is near 2000 mAU (2.0 O.D).

CAUTION: The UVMax® microtiter plate reader has an automix function which should be turned on. Each time you read a plate, the reaction mixture in the wells is mixed which is desirable for assay precision. However, this also has the effect of accelerating the reaction kinetics. Therefore, the second plate should be read as often and at approximately the same time intervals as the first plate to minimize any differences between the two plates. It is also recommended that an initial read should be done right after substrate addition to insure good mixing.

Instrumentation

Data are collected on a microplate reader with an IBM PS/2-50 computer running Titercalc 2.1 software in Microsoft Windows, or on a Molecular Devices UVMax plate reader with a Macintosh SE/30 computer running SOFTmax version 2.01. Equivalent equipment and software can be used. In general, the following parameters apply:

Control Mode:	Computer controls reader
Assay Read Mode:	Endpoint
Wavelength:	405 nm
Standard Curve Fit:	4-parameter logit
Automix:	On
Computer Report Contents:	Run date, disc file number, plate format, plate absorbance readings, standard curve data, curve fit parameters and graph, control and/or blank data, sample data, and calculated concentration results

Data Collection and Analysis

See Appendix II for complete sample calculations.

The absorbance of each of the 96 wells of the microwell plate is read on a microplate reader equipped with a 405-nm filter and processed by a computer program which generates a standard curve based upon a four- or five-parameter logit function.

The standard four-parameter logit curve generated from this type of assay on semi-log paper is sigmoidal in shape with

absorbance (AU, mAU or O.D.) on the Y axis and the log of the concentration of the compound on the X axis. Such curves can fit the following equation:

$$y = \frac{A - D}{1 + (x/C)^B} + D$$

Initial estimates of the coefficients may be made as follows:

A = upper asymptotic absorbance, approximated by the absorbance of the "zero" standard

B = slope of the curve at the inflection point, approximated by the decrease in absorbance per 10-fold increase in analyte concentration

C = value of x at the inflection point of the curve

D = lower asymptotic absorbance, approximated by the absorbance of the highest standard

The computer program makes an iterative adjustment of A, B, C, and D until the residual sum of the squares of the differences between measured standard points and their calculated concentrations is minimized (a nonlinear regression). The computer then uses the fitted equation to calculate concentrations for the samples and controls from their absorbance readings. For more information, see your software manual for the exact equation form and iteration algorithm used.

Plate reader analysis reports are imported into Microsoft EXCEL® (Version 4.0, Microsoft Corp., Redmond, WA) spreadsheets, where the SM concentrations are actually calculated. The spreadsheet software is run on a Macintosh SE/30® computer.

Quality Control

A visual inspection of the standard curve (Figure 2 illustrates a typical curve) shows whether the line fits the points, the degree of scatter of the data, and whether a gross error such as switching two standards has occurred. A more quantitative evaluation of the curve fit is shown by the correlation coefficient (R-Sqr), standard error (St_{err}), and residuals (% difference between actual and calculated standard concentrations) and calculation of % inhibition of each of the

standards. The % inhibition[1] ($[A_O - A_{Std}]/A_O$) X 100) should be constant from plate to plate and day to day. Any significant change (>10%) suggests a problem which should be investigated. The R-Sqr correlation coefficient usually runs between 0.95 and 0.99 for a curve with good precision and fit.

Precision, as measured by % CV (% coefficient of variation = standard deviation/mean X 100), is another measure of the reliability of the assay. The absorbance precision (i.e., % CV for triplicate absorbance readings [mAU]) should be ≤10.5% (or ≤10.% for 2 significant figures).

If any set of triplicates is over 10.5% CV, inspect the data to see whether one of the triplicates is an outlier (> 2 standard deviations above or below the closest of the two remaining readings, with the standard deviation being that of the remaining two readings). Outliers may be deleted and the data recalculated, provided the % CV of the two remaining readings is <10.5%. If no outlier is found, the sample must be rerun. (Following these criteria assures that the %CV of the analyte concentration will be about 20% or less.)

The measured SM concentration in the fortified control must be within specifications (± 30% of target) in order to accept data from that plate. If more than one fortified control was run on a plate, and one does not meet specifications, then all samples with SM concentrations corresponding to that control level must be rerun.

For quality control, monitoring the substrate blank (three or more wells in Row A [See Figure 1] which received substrate only), and conjugate blank (three or more wells in Row H which received conjugate and substrate only) on each microtiter plate is recommended. A significant change in mAU for any of these blanks may indicate a problem. A change in reagent lots may also cause a shift in one or more of the blank readings. If there is an apparent problem, fresh reagents should be prepared and the samples rerun.

Duplicate samples must show SM concentrations within ±30% of each other to show reproducibility of the method.

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1 A_O is the absorbance (mean O.D.) for the 0.000-ng/mL standard, and AStd is the absorbance for the particular standard for which % inhibition is being calculated.

Limits of Detection and Quantitation

Limit of Detection (LOD): *The minimum concentration which is statistically significantly distinguishable from 0*

- This can be assessed by running multiple replicates at 0 and the LOD, and performing a *t*-test to determine whether the absorbances are statistically different to a confidence level of 95% (p-value ≤0.05). It has also been defined as the concentration at which 15% inhibition of the "0" standard absorbance reading occurs.

Limit of Quantitation (LOQ): *The minimum concentration at which the method meets the specifications for precision, accuracy, spiking and recovery*

Method Detection Limit (MDL): *Defined in Reference 4*

- In this statistics-based approach, replicate fortifications are carried out, using an estimated MDL (essentially an educated guess, such as S/N* = 2-3, or LOQ/2). The MDL is that level above which there is high confidence (99% confidence was selected for this work) that a positive finding has occurred. The analyte quantity cannot be determined as accurately at levels near the MDL compared to higher residue levels (e.g., LOQ and above); however, at (or above) the MDL, the sample can be determined to be reliably different (i.e., higher in analyte concentration) from the control. Relevant formulas and sample calculations are provided Appendix III.

Final Data Acceptability, Calculating, and Reporting

Apply the Quality Control criteria to the plate and the calculated individual sample results as described above. (See Table II and Appendix II for calculation of assay results.)

Determine whether any samples fall below the LOQ and report concentration as "<LOQ." Determine whether any samples fall above the standards range. These should be rerun at an appropriate dilution factor which can be estimated from the result, so that the rerun result will fall within the quantifiable range. **A control spiked to a similar concentration and diluted by the same factor should also be run.**

* Signal-to-noise ratio

Finally, if there is any doubt about the response in the assay attributable to anything other than the compound being measured, the test of parallelism should be applied: serial dilutions of the questionable sample should show linearity on dilution, or 100 ± 30% recovery of the calculated concentration.

RESULTS AND DISCUSSION

Antibody Cross-Reactivity

The specificity of the anti-SM antibody was evaluated for cross-reactivity. A general definition of cross-reactivity is the binding of the antibody to other closely related compounds.

The method of calculating cross-reactivity and specific cross-reactivity data are shown in Table III. These data and the context in which the SM ELISA method is run should dictate whether a confirmatory method (e.g., LC/MS) is warranted.

Method Validation: Recovery Study, Soil

This ELISA method was validated using control Greenville, Rochelle, Uvalde, and Madera soils that were available in connection with a field soil dissipation study involving sulfometuron methyl [5]. Pre-application soil (composite of all depths [0-90 cm]) from sub-plot #1 (at each field site) was used for this recovery study. See Table IV for a complete description of these soils.

Replicate soil samples were fortified with sulfometuron methyl over the range 0.1-1.0 ppb (SM concentration in soil). Table V presents the results of this work. Standard curves and raw data are provided in Appendix IV.

These recovery data are within acceptable criteria for valid residue methods (also see previous ***Quality Control*** section):

Test Site	N*	Average Recovery (%)	S.D. (%)	% CV
Greenville, Miss.	6	123	4	3
Rochelle, Ill.	6	110	9	8
Uvalde, Tex.	5	117	5	4
Madera, Calif.	9	101	9	9

* number of samples

The overall (N = 26) average recovery, S.D., and % CV were calculated to be 111, 11, and 10%, respectively. Average recoveries reflect a generally high bias (due to soil matrix background effects; this is commonly seen with ELISA methods involving sub-ppb analyte levels [3]). Standard deviations and % CVs are quite reasonable.

Limit of Quantitation (LOQ), Soil

The recovery data described in the previous section and precision, accuracy, etc. associated with the raw data (see Appendix IV) support an LOQ of 0.1 ppb for this method.

Method Detection Limit (MDL), Soil

This method was evaluated from the standpoint of MDL as defined in Reference 4 (also see previous section on ***Limits of Detection and Quantitation***). Again, the MDL is that level above which there is a high confidence (99% confidence for this work) that a positive finding has occurred.

Table VI contains the results of our MDL determination. Although calculated MDLs are all 0.03 ppb or lower, **further work should be performed, to verify/validate these results** (the lowest fortification level examined was 0.05 ppb). Nevertheless, an appropriate MDL is clearly somewhat lower than 0.05 ppb (probably around 0.02-0.03 ppb for these soils).

This is superb sensitivity for soil residues. The low MDLs reflect, in part, the excellent intra-run precision (low standard deviations for replicate samples, analyzed simultaneously on the same plate) obtained with the ELISA technique.

Method Validation: Recovery Study, Water

This ELISA method was validated using surface water obtained from three local sources: North East River (Maryland), White Clay Creek (Delaware), and Lums Pond (Delaware). Replicate water samples were fortified with sulfometuron methyl over the range 0.025-0.200 ppb (SM concentration in water). Table VII presents the results of this work. Raw data are in Appendix IV.

These recovery data are within acceptable criteria for valid residue methods (also see previous *Quality Control* section).

Test Site	N*	Average Recovery (%)	S.D. (%)	% CV
North East River	4	112	7	6
White Clay Creek	4	102	8	8
Lums Pond	4	108	14	13

* Number of Samples

The overall (N=12) average recovery, S.D., and % CV are calculated to be 107, 10, and 9%, respectively. These values are all quite reasonable.

Limit of Quantitation (LOQ), Water

The recovery data described in the previous section and precision, accuracy, etc., associated with the raw data (see Appendix IV) support a LOQ of 0.025 ppb for this method.

Method Validation: Use in Oust® Herbicide Dissipation Study

A two-year field soil dissipation study [5] with Oust® Herbicide was started by DuPont in 1991, to fulfill reregistration requirements of the U. S. EPA. In order to analyze samples with the highest available sensitivity, both this ELISA method and a method employing Liquid Chromatography/Mass Spectrometry (LC/MS) were utilized in the determination of SM soil concentrations down to 0.1 ppb.

While not the primary purpose of the dissipation study, the results of this work provided a very useful validation of the ELISA method by a completely different technique (i.e., LC/MS). This study involved numerous soil samples (from four field test locations) containing field-aged residues of SM at quantifiable levels.

Figure 3 presents a plot of immunoassay (ELISA) results versus LC/MS results for the analysis of the same soil samples† (this is sometimes referred to as a "correlation plot"). This plot shows the line that would be generated if the ELISA and LC/MS techniques had produced identical data.

† Different subsamples from the same original soil core section

This plot demonstrates an excellent correlation between the two sets of data (135 samples are represented in this plot); the data points are clearly distributed closely around the ideal correlation line. This correlation is particularly strong, given that the data have not been corrected for recovery.

These plots show ELISA results to have a slightly positive bias, relative to the LC/MS results (average percent recovery for SM by LC/MS was 93% and the average percent recovery by ELISA was 111%; for more information, see Reference 5). Therefore, the sulfometuron methyl concentrations determined by ELISA should, if anything, present a somewhat exaggerated portrayal of the "real" residue levels (i.e., false negatives are minimized).

Because ELISA is most likely employed with samples having low analyte levels, Table VIII presents the data from the soil dissipation study where SM was either determined to be present in the range 0.1-0.5 ppb (approximately) or where residues were too low to quantitate. Figure 4 graphically depicts these same data. The agreement between the two disparate methods of SM analysis is obviously excellent (again, no values were corrected for percent recovery).

Stability

Satisfactory performance of the microtiter plates was found after three months storage at 2 to 8°C. These plates were tested with freshly prepared plates run as controls. Therefore, it is recommended that up to a month's supply of microtiter plates be prepared at one time and stored at 2 to 8°C. Besides the time savings by preparing a lot of plates at one time, another advantage is the elimination of day-to-day variability in the microtiter plate preparation.

The tableted antibody is stable when desiccated at 2 to 8°C for one year or longer if no change in assay performance is observed.

Limitations

The precision of the results of the assay is dependent upon the use of reproducible techniques as described in the assay steps. This is of particular importance when adding substrate solution. The timing in each well should be similar from substrate addition to reading the absorbance. The use of a

multichannel pipette ensures that all wells in a row receive substrate solution simultaneously. However, variability in the timing from row to row may result in imprecision. This should be evaluated by the individual operators in their own labs by simply pipetting the same standard curve in each row.

Another caution to be observed in this assay concerns contamination. Since the assay is sensitive to very low levels of concentration, the handling and storage of the samples is of particular importance. This also applies to the laboratory environment. It is recommended that primary standards be prepared in another area to avoid any possible contamination and that a high level of awareness be maintained by all laboratory personnel. Disposable plasticware, glassware, pipettes, etc., are also recommended to minimize the possibility of contamination.

Timing

We routinely analyzed 8-16 soil samples (plus recoveries, QC samples, standards, etc.) during an 8-hr day, using this method. Analysis of 16 soil samples required that most solutions be prepared ahead of time. Because extraction steps were unnecessary, water samples required approximately 2 fewer hours for complete workup and analysis. By the end of the analysis day, results for SM concentrations were available, due to automated data collection and analysis.

CONCLUSIONS

A highly sensitive ELISA microtiter plate immunoassay has been developed for the detection of sulfometuron methyl in soil and water. The minimum limits of quantitation are 0.1 and 0.025 ppb for soil and water, respectively.

A recovery study validated this method across four soil and 3 water types, demonstrating good accuracy and precision. A comparison of analyses of field-aged soils where replicate samples were subjected to both ELISA and LC/MS further demonstrated the high accuracy and validity of the ELISA methodology.

There are many advantages of this technology for residue analysis. The most compelling is the sensitivity and specificity which may be obtained with an antibody assay. Another advantage is that the extensive sample preparation and clean-

up required for HPLC is significantly reduced in the ELISA assay (essentially no sample clean-up is needed in this ELISA method!). It is also a cost-effective technology, uses few hazardous chemicals and provides a fast turn-around time to results. Finally, this method is easily mastered by the typical residue laboratory analyst.

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This state-of-the-art residue method is truly the result of a team effort.