Development of an Enzyme-Linked Immunosorbent Assay for the Detection of Metosulam

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A direct enzyme-linked immunosorbent assay (ELISA) was developed to quantitate the herbicide metosulam (*N*-(2,6-dichloro-3-methylphenyl)-5,7-dimethoxy-1,2,4-triazolo(1,5a)pyrimidine-2-sulfonamide) in soil and water. The ELISA had a detection limit of 0.3 ng·mL^{-1}, a linear working range of 0.5 ng·mL^{-1} to 10 ng·mL^{-1}, and an IC_{50} value of 2.8 ng·mL^{-1}. The metosulam antisera cross-reacted with 5-OH and 7-OH metosulam (*N*-(2,6-dichloro-3-methylphenyl)-5-hydroxy-7-methoxy-1,2,4-triazolo[1,5-*a*]pyrimidine-2-sulfonamide and *N*-(2,6-dichloro-3-methylphenyl)-5-methoxy-7-hydroxy-1,2,4-triazolo[1,5-*a*]pyrimidine-2-sulfonamide, respectively) and the manufacturing intermediate XDE-564 (*N*-(2,6-dichlorophenyl)-5-ethoxy-7-fluoro[1,2,4]triazolo[1,5-*c*]pyrimidine-2-sulfonamide). The assay was used to estimate, accurately and precisely, metosulam concentrations in soil and water samples. Water samples were analyzed directly while soil sample analysis required solvent extraction and dilution prior to analysis. Extraction efficiency was 95%. The ELISA estimations in water correlated well with those from HPLC analysis ($r^2 = 0.99$; slope = 0.99), making the ELISA a cheap and reliable alternative to conventional residue analysis techniques for quantification of metosulam.

Keywords: *Metosulam; ELISA; triazolopyrimidine; herbicide; residue*

INTRODUCTION

Over the past ten years, enzyme-linked immunosorbent assay (ELISA) has become a valuable tool in residue analysis. Accuracy, speed of sample analysis, and overall cost effectiveness of ELISA when compared to chromatographic analysis (Hall et al., 1990; Hammock and Mumma, 1980) have made its use desirable for tasks such as spray-tank rinse checks, plant hygiene, fate and persistent studies, and environmental residue analysis for determination of a wide range of pesticides (Meulenberg et al., 1995).

ELISAs have been used successfully for the quantitative analysis of numerous pesticides in water matrices with little or no matrix interference (Rubio et al., 1991; Lawruk et al., 1994). However, in the past, the usefulness of immunoassays for quantitative analysis has been limited in other matrices such as soil and food extracts (Van Emon and Lopez-Avila, 1992). Assay format may have an effect on ELISA performance in these matrices. ELISAs incorporating a direct format (immobilized antibody and an enzyme conjugate) (Rubio et al., 1991; Schneider and Hammock, 1992) are generally better suited for environmental analysis than ELISAs utilizing an indirect format (Feng et al., 1990).

Metosulam (Figure 1, *N*-(2,6-dichloro-3-methylphenyl)-5,7-dimethoxy-1,2,4-triazolo[1,5-*a*]pyrimidine-2-sulfonamide) is a foliar-applied herbicide currently registered in the European Community for the post-emergent control of broadleaf weeds in corn and cereal crops (Maycock et al., 1993). It is a member of the triazolopyrimidine herbicide family and controls susceptible weeds by inhibiting the enzyme acetolactate synthase (ALS) (Maycock et al., 1993). Metosulam has a short

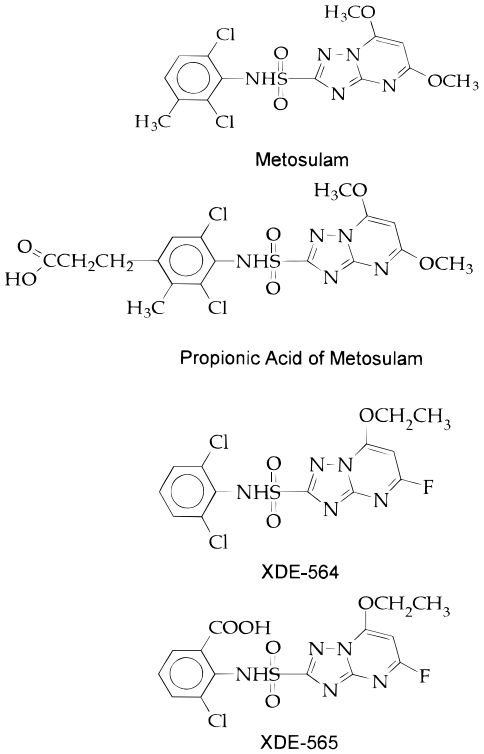

Figure 1. Structures of metosulam, the propionic acid of metosulam (hapten) used in immunogen and enzyme–conjugate synthesis, as well as the manufacturing intermediates of metosulam, XDE-564 and XDE-565, used in metosulam antisera cross reactivity testing.

to moderate half-life in soil (≈25 days) and does not migrate below the 10 cm horizon (<1.0 ng·mL^{-1}) (Maycock et al., 1993). Because the mode of action and chemistry of the triazolopyrimidines are similar to the

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sulfonylurea herbicides, persistence of soil residues may be a concern when rotating susceptible broadleaf crops on the same field. Metosulam and its metabolites are currently analyzed in soil, water, and plant material using high pressure liquid chromatography via UV detection (Maycock et al., 1993). The lowest validated level of quantification for the HPLC method is 0.05 ng·mL^{-1} following a 1000-fold concentration step. Furthermore, prior to HPLC analysis of metosulam a complicated, time-consuming sample preparation was required. Currently, there is no ELISA for the detection of metosulam. Such an assay may result in a sensitive and cost effective means of analyzing environmental samples containing metosulam when compared to the HPLC methods. In this paper, we report the quantitative performance of a direct ELISA for metosulam detection and quantification in water and soil.

MATERIALS AND METHODS

Chemicals and Instrumentation. Analytical standards of metosulam and the propionic acid of metosulam (Figure 1) were provided by DowElanco Chemical Co., Indianapolis, IN. Freund's incomplete adjuvant, 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium (ABTS) substrate tablets, horseradish peroxidase (EC 1.11.1.7), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and urea hydrogen peroxide were obtained from Sigma Chemical Co., St. Louis, MO. Protein A was obtained from Pierce, Rockford, IL. Dynatech Immulon 4 flat-bottom microtitre plates were purchased from Fisher Scientific, Don Mills, ON, Canada. ELISAs were analyzed using a Model 3550-UV microplate reader, Bio-Rad Laboratories, Richmond, CA. The HPLC used was a Shimadzu LC-10AD equipped with a SIL-10A auto injector and a Shimadzu SPD-M6A photodiode array UV-VIS detector. A Kromasil 5 μm C8 column with a length × internal diameter of 250 × 2.0 mm was used. All other chemicals were of reagent grade and obtained commercially.

Synthesis of Immunogen and Enzyme Conjugate. The method of Fleeker (1987) was used to conjugate the propionic acid of metosulam (Figure 1, *N*-[4-(carboxyethyl)-(2,6-dichloro-3-methylphenyl)-5,7-dimethoxy-1,2,4-triazolo[1,5-*a*]pyrimidine-2-sulfonamide) to bovine serum albumin (BSA) and horseradish peroxidase (HRP) to synthesize the immunogen and enzyme conjugate, respectively. Briefly, the propionic acid of metosulam (0.019 mM) and *N*-hydroxysuccinimide (NHS) (0.019 mM) were dissolved in 200 μL of 1,4-dioxane. Dicyclohexylcarbodiimide (DCC; 0.019 mM) was dissolved in 50 μL of 1,4-dioxane and added to the propionic acid of metosulam/NHS solution. The solution was mixed gently and allowed to stand covered overnight (≅ 17 h) at room temperature (22 °C). The solution was centrifuged at 1500*g* for 5 min. The supernatant was filtered through glass wool and evaporated to dryness under air at 35 °C. BSA (200 mg) or HRP (20 mg) in 300 μL of 0.1 M borate buffer (pH 9.0) was added to the tube containing the residue from the supernatant and slowly stirred for 2 h. The solution containing the conjugate was dialyzed (Spectrapor 1; 6000−8000 MW cutoff) for 24 h at 4 °C against four 1 L changes of distilled water. The solution containing the conjugate was frozen in 500-μL aliquots.

Polyclonal Antisera Production. New Zealand white rabbits were injected, intramuscularly, with the immunogen (500 μg of immunogen in 1 mL of a 10-mM phosphate buffered 15-mM NaCl (PBS) pH 7.5/Freund's incomplete adjuvant (1:1 v/v) emulsion). These primary immunizations were repeated at weekly intervals for three weeks. Following a four-week rest period, secondary immunizations (boosts) of immunogen (200 μg) in 1 mL of PBS/Freund's incomplete adjuvant (1:1 v/v) were injected intramuscularly every three weeks. The rabbits were bled and serum tested for anti-metosulam antibody titer, one week after each secondary boost.

Antisera Titer Determination. Metosulam-specific antisera titers were determined as described by Campbell (1984). Binding studies between metosulam antiserum and metosulam-HRP were used to determine their optimal dilution. The optimal dilutions were deemed to be those that resulted in an absorbance of 1.0 at 405 nm, following an incubation of 30 min at room temperature (22 °C).

Standard Curve and Sample Analysis. The ELISA was performed as described by Deschamps and Hall (1991). Immulon no. 4 flat-bottomed plates were coated with Protein A (0.2 μg·mL^{-1}; 100 μL·well^{-1}) and allowed to incubate overnight at 4 °C. The plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween) and patted dry on paper towels. Sites not containing bound protein A were blocked by adding 200 μL·well^{-1} of 0.01% gelatin in water (w/v). After a 20-min incubation, the plates were washed and dried as previously described. Anti-metosulam antibody was diluted 1/8000 with PBS (optimal dilution) and added to the plates at 100 μL·well^{-1}. The plates were incubated overnight at 4 °C before being washed with PBS-Tween and dried. Standards and/or samples were mixed 1:1 with 1/120,000 diluted enzyme conjugate (optimal dilution) prior to addition to the plates at 100 μL·well^{-1}. The plates were incubated for 1 h at room temperature (22 °C) and washed to remove any unbound metosulam and metosulam−HRP conjugate. Substrates [1 mg·mL^{-1}, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium] and 1 mg·mL^{-1} urea hydrogen peroxide in 23 mM citric acid 47 mM sodium phosphate (citrate buffer, pH 9.0) were added (100 μL·well^{-1}) and color allowed to develop until the reaction was stopped after 30 min with 0.5 M citric acid (100 μL·well^{-1}).

Standard Curve. Absorbance at 405 nm was inversely proportional to the concentration of metosulam in the standards and samples. Absorbance values were normalized by dividing by the absorbance value of a negative control (0 ng·mL^{-1} metosulam). The normalized absorbance values (A/A_o) of the metosulam standards were plotted against the log values of the metosulam concentration. The resulting standard curve was used to interpolate metosulam concentrations in the water and soil samples.

Water Analysis. Water was collected from the Makami River, near Gogama, Ontario (47°42′ lat.; 82°45′ long.). For sample analysis, 10 mL of water was filtered through a 0.45 μm-nylon filter prior to being spiked with known concentrations of metosulam and divided into 500 μL aliquots prior to analysis. Three sets of spiked samples were analyzed 10 times over the period of three days (n = 30 for each concentration).

Analysis of Soil. The soil extraction procedure was a modification of the method described by Johnson and Hall (1996). Air-dried samples (5 g) of Saskatchewan clay loam were spiked with a known concentration of metosulam dissolved in 1.0 mL of methanol. All vials were incubated for 24 h to allow the methanol to evaporate and metosulam to bind to soil colloids. After incubation, vials were shaken with 25 mL of 90% acetonitrile 10% 0.2 N HCl (v/v) for 1 h on an orbital shaker (180 rpm). Samples were left overnight (16 h) in the extraction solvent and were shaken for 1 h the following morning.

Soil extracts were filtered through a 2.0-μm glass, microfiber filter (Whatman 934-AH), and the acetonitrile was evaporated under nitrogen at 45 °C. Soil extracts were reconstituted with 15 mL of 10-mM phosphate buffered 15 mM NaCl pH 7.5 (PBS) and passed through a 0.45 μm nylon filter. Each sample was then diluted to 100 mL with PBS, adjusted to pH 7.5, and divided into 500 μL aliquots for analysis. Three sets of soil extracts for each concentration were analyzed 10 times for a total of 30 analyses per concentration. Extraction efficiency was determined using ^{14}C radiolabeled metosulam (Ag-Tracers, Dow Chemical, Midland, MI; specific activity = 23.04 mCi·mmol^{-1}).

Cross Reactivity. The manufacturing intermediates XDE-564 (*N*-(2,6-dichlorophenyl)-5-ethoxy-7-fluoro[1,2,4]triazolo[1,5-*c*]pyrimidine-2-sulfonamide) and XDE-565 (*N*-(2-carboxy-6-chlorophenyl)-5-ethoxy-7-fluoro[1,2,4]triazolo[1,5-*c*]pyrimidine-2-sulfonamide) (Figure 1), along with 5-hydroxymetosulam (*N*-(2,6-dichloro-3-methylphenyl)-5-hydroxy-7-methoxy-[1,2,4]-triazolo[1,5-*a*]pyrimidine-2-sulfonamide), 7-hydroxymetosulam

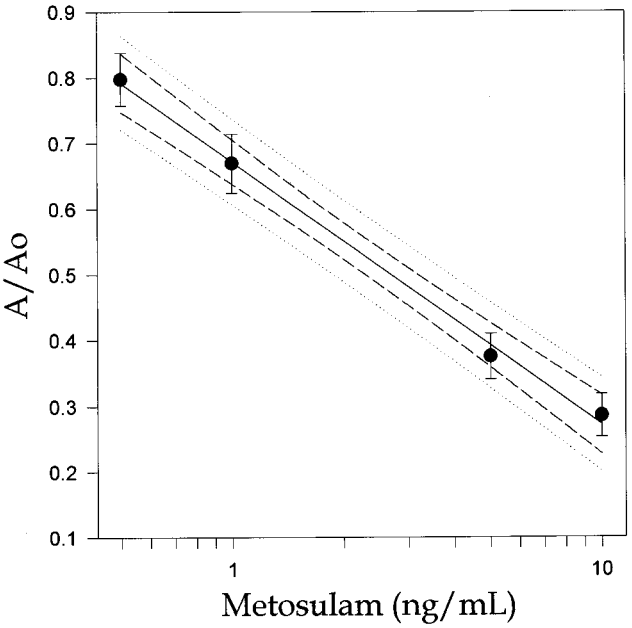

Figure 2. Plot of the cumulative standard curve of metosulam concentration versus the normalized absorbance. The standard deviation of the standards, along with 95% confidence intervals (– –) and prediction intervals (···) for the standard curve (n = 30) are shown.

(*N*-(2,6-dichloro-3-methylphenyl)-5-methoxy-7-hydroxy-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-sulfonamide) and other selected agrochemicals were tested for cross reactivity to the metosulam antisera. Standards for each chemical were prepared in PBS. Percent cross reactivity, IC_{50}, and least detectable dose (LDD) values were calculated.

HPLC Procedure. Metosulam standards were prepared in H_2O:acetonitrile (1:1, v/v) while water samples were combined with acetonitrile (1:1, v/v). The flow rate of the HPLC was set at 0.3 mL/min. Standards and/or samples were injected (50 μL) and run under isocratic conditions of 55% acetonitrile: 45% 5.1 mM phosphoric acid for 10 min. The Kromasil 5 μm C8 column was flushed between injections to remove contaminants by flushing for 12 min with 100% acetonitrile, followed by 30 min with 55% acetonitrile: 45% 5.1 mM phosphoric acid. This resulted in a total run time of 52 min per sample. Metosulam peaks were detected at a wavelength of 220 nm. Peak heights of the standards were plotted against the concentration of metosulam, and the resulting standard curve was used to interpolate metosulam concentrations in the water samples.

RESULTS AND DISCUSSION

Assay Performance. The linear working range of the ELISA was 0.5−10 ng·mL^{-1} (Figure 2). The limit of detection (LOD), defined as the concentration corresponding to 90% of the A/A_o (Midgley et al., 1969), was calculated to be 0.3 ng·mL^{-1}. The limit of quantification (LOQ), described by Fleeker (1987) as being 10 times the standard deviation of A_o from its mean value, was 0.5 ng·mL^{-1}. The IC_{50} or concentration of metosulam required for 50% inhibition of the absorbance of the positive control was 2.8 ng·mL^{-1}.

Plates used for the ELISA were coated with protein A prior to the addition of the antibody. Protein A is a cell wall component of *Staphylococcus aureus* containing four high affinity binding sites for the Fc region of antibodies, especially IgG (Hjelm et al., 1975). Wells coated with protein A orient the antibody for optimal binding while preventing passive binding of the antibody to the plates thereby decreasing the amount of antibody required for the assay (Johnson and Hall, 1996). Antibodies passively bound to a polystyrene surface may have limited antibody/antigen interaction due to random orientation or denaturation of the antibody (Butler et al., 1993; Johnson and Hall, 1996).

Table 1. Determination of Metosulam in River Water and a Clay Loam Soil Using a Direct Polyclonal-Based ELISA for Metosulam

		determined		
	added (ng·mL^{-1})	mean (ng·mL^{-1})	standard deviation (ng·mL^{-1})	coeff of variation (%)
river water	0.5	0.6	0.08	15
	1.0	1	0.2	14
	5.0	6	0.4	7
n = 30	10.0	10	0.9	10
clay loam soil	50.0	53	10	20
	100.0	89	16	17
n = 30	1000.0	1044	101	10

The propionic acid of metosulam (Figure 1) was used to synthesize both immunogen and enzyme conjugate after attempts to produce a suitable immunogen with metosulam failed. Initially, we unsuccessfully attempted to produce an immunogen that linked BSA to the $NHSO_2$ bridge group of metosulam via a glutaraldehyde linkage. By utilizing the propionic acid of metosulam and linking it to BSA through the carboxylic acid group, an immunogen was synthesized that produced antibodies with specificity for metosulam.

Soil and Water Analysis. [^{14}C]Metosulam was not used for determining recoveries in river water since there was no extraction procedure prior to analysis. Accurate estimations of metosulam concentrations were achieved through the direct analysis (without dilution) of the river water samples (Table 1).

The soil extraction method for the herbicide resulted in mean recoveries of 95% using [^{14}C]metosulam. Johnson and Hall (1996) reported that soil extracts required a 50-fold dilution in PBS to attain accurate determinations of fluroxypyr concentrations in soil using a direct ELISA for fluroxypyr. Although we were able to estimate metosulam concentrations in water samples using the assay, a 1:100 (v/v) dilution of the soil extract in PBS was required prior to analysis. Therefore, the LOD and LOQ of the ELISA were increased to 30 and 50 ng·mL^{-1}, respectively. Diluting out the effects of the coextractants from the matrix allowed for accurate determination of metosulam concentrations in the clay loam soil without a concentration step (Table 1). Maycock et al. (1995) detected metosulam levels as low as 1.0 μg·kg^{-1} using an HPLC method that involved a concentration step. Metosulam was detected at 10.0 μg·kg^{-1} of soil after the extracts were diluted 100 fold to eliminate the matrix effects.

A high-pressure-liquid chromatography (HPLC) method was used to compare and further validate the ELISA method. In previous studies, it has been shown that many ELISAs have lower quantitative ranges than conventional chromatography methods (Brady et al., 1995). This was also the case with the metosulam ELISA, which had a quantitative range 1000 times lower than the HPLC method. Samples were, therefore, spiked within the quantitative range of the HPLC and diluted 1000-fold prior to analysis by ELISA. Figure 3 illustrates how well the ELISA estimations correlated with those from the HPLC. Correlation of HPLC and ELISA estimates was r^2 = 0.99 while the slope and

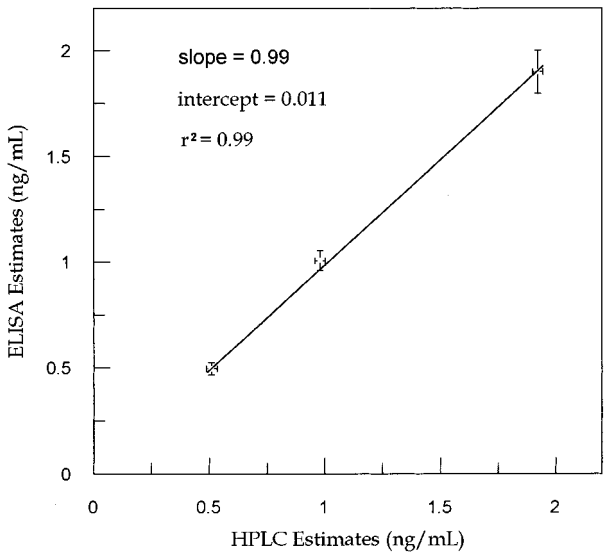

Figure 3. Correlation of metosulam concentrations in water as determined by HPLC and ELISA analysis. Each value represents the average of nine estimations and associated standard deviations. The slope and *y*-intercept were not significantly different from 1.0 and 0, respectively, as determined by a t-test ($p \geq 0.05$). Vertical and horizontal bars represent the standard deviations for ELISA and HPLC means, respectively.

y-intercept were not significantly different from 1.0 and 0, respectively, as determined by a *t*-test ($p \leq 0.05$; Figure 3). These results indicate the agreement between the two analytical methods.

Besides having a lower quantitative range than the HPLC method, the ELISA method was simpler, and samples could be analyzed in 1/6 the time it took to analyze the same samples by HPLC. The time saved can be further increased by running up to 20 samples per ELISA plate (20 samples in 1.5 h with ELISA compared to 20 samples in 24 h with the HPLC method).

Cross Reactivity. Maycock et al. (1995) found that the half-life of metosulam in soil ranged from 6−47 days. Metosulam degrades via 5-hydroxy- and 7-hydroxymetosulam to form 5-amino-*N*-(2,6-dichloro-3-methylphenyl)-1*H*-1,2,4-triazole-3-sulfonamide (ATSA) by a process of demethylation and opening of the pyrimidine ring (Maycock et al., 1995). These metabolites of metosulam have little or no phytotoxicity and, therefore, do not pose an agronomic problem to subsequent rotation crops. There was cross reactivity of the metosulam antisera with 5-hydroxy- and 7-hydroxymetosulam metabolites as well as with the manufacturing intermediate XDE-564 (Table 2). Cross reactivity to other agrochemicals was less than 0.1%. The cross reactivity to XDE-564 is not considered significant since its LDD and IC_{50} are well out of the range of the assay. Furthermore, XDE-564 is not a metabolite of metosulam and, therefore, is not likely to be found in environmental samples. The assay is not useful for quantification of the two metabolites, 5-hydroxy- and 7-hydroxymetosulam, since the IC_{50} values for the metabolites are at least 100 times greater than for metosulam. For example, if 50 g ai·ha^{-1} of metosulam (equivalent to 20 ng·g^{-1} soil) was completely metabolized, it would be equivalent to a soil extract of 100 ng·mL^{-1}, which is well below the IC_{50} of the two hydroxy metabolites.

Conclusion. An ELISA was developed for detection of metosulam in soil and water samples. It accurately estimated metosulam within its linear working range of 0.5 to 10 ng·mL^{-1} for river water samples and 50 to 1000 ng·mL^{-1} for soil samples. Furthermore there was a high correlation between the ELISA method and reverse phase (C_8) high-pressure-liquid chromatography ($r = 0.99$) in water samples. Cross reactivity was observed to the 5-hydroxy- and 7-hydroxymetosulam metabolites as well as the manufacturing intermediate XDE-564. However, the cross reactivity to XDE-564 is not considered significant since the least detectable dose does not fall within the linear working range of the assay and, therefore, will not influence the quantification of metosulam. The LDDs for 5-hydroxy- and 7-hydroxymetosulam fall within the working range of the assay (5.6 and 7.5 ng·mL^{-1}, respectively). However, the potential effect of these two metabolites on the accurate quantitation of metosulam can be eliminated by using a working range of 0.5−5.0 ng·mL^{-1}. The sensitivity of the metosulam ELISA was 1000 times greater than that of the HPLC method. The main benefits of the ELISA for metosulam were increased sensitivity, decreased time of analysis, and the requirement for less solvent.

Table 2. IC_{50}, Percent Cross Reactivity, and Least Detectable Dose for Metosulam, Its Metabolites, and Selected Agrochemicals Using Metosulam Polyclonal Antisera

compound	IC_{50} (ng·mL^{-1})	cross reactivity[a] (%)	least detectable dose[b] (ng·mL^{-1})
metosulam	2.8	100	0.3
5-OH metosulam metabolite	269	1.0	5.6
7-OH metosulam metabolite	308	0.9	7.5
XDE-564	1312	0.2	24
XDE-565	>10000	<0.1	79
metolachlor	>10000	<0.1	ND[c]
picloram	>10000	<0.1	ND
dicamba	>10000	<0.1	ND
MCPA	>10000	<0.1	ND
mecoprop	>10000	<0.1	ND
dichlorprop	>10000	<0.1	ND
clopyralid	>10000	<0.1	ND
triclopyr	>10000	<0.1	ND
2,4-dichlorophenoxyacetic acid	>10000	<0.1	ND
2,4,5-trichlorophenoxyacetic acid	>10000	<0.1	ND
atrazine	>10000	<0.1	ND

[a] Cross reactivity is IC_{50} for metosulam/IC_{50} for respective compound X 100. [b] Least detectable dose is the concentration of compound which inhibits 10% of the negative control. [c] Not detectable.

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