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Development and Comparison of Three Diagnostic Immunoassay Formats for the Detection of Azoxystrobin

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The currently accepted method of detection for azoxystrobin, a strobilurin fungicide, involves a labor-intensive organic solvent extraction and gas chromatography analysis. Three diagnostic assay formats, i.e., enzyme-linked immunosorbent assay (ELISA), fluorescence polarization (FP), and time-resolved fluorescence (TR-FIA), were developed and compared with regard to detection and quantification of azoxystrobin in grape extract and river, lake, and well water samples. These three assay formats require no initial sample extraction and were not affected by any of the environmental matrices tested, and each had a linear working range of 0−400 pg/mL. The polyclonal antibodies used for each of the immunoassays were specific to azoxystrobin; that is, the highest cross-reactivity to other pesticides observed was 5.7%. The limits of detection of the immunoassays were similar at 3 (ELISA), 46 (FP), and 28 (TR-FIA) pg/mL, as were the respective IC_{50} values of 306, 252, and 244 pg/mL. Each of the three immunoassays developed was less labor-intensive and approximately 100-fold more sensitive than the gas chromatographic method. While the three formats were comparable in terms of performance, the fluorescence polarization assay was the least labor-intensive and required the least time to perform.

KEYWORDS: Azoxystrobin; ELISA; fluorescence polarization; time-resolved fluorescence; immunoassay

INTRODUCTION

Azoxystrobin (**Figure 1a**) is a fungicide that is effective against all four major groups of plant pathogenic fungi (Ascomycetes, Basidiomycetes, Deuteriomycetes, and Oomycetes) including those responsible for downy mildew, powdery mildew, and black rot in grapes, stem rot and black spot in canola, and gray snow mold and brown patch in turfgrass and, other than lower aquatic life forms, is generally safe against nontarget organisms (*1*). A synthetic derivative of strobilurin A, a naturally occurring β-methoxyacrylate, azoxystrobin is known to inhibit mitochondrial respiration by binding to the ubiquinol-oxidizing site, Q_o, of cytochrome b. The cytochrome bc_1 complex, of which cytochrome b is an integral part, is located in the inner mitochondrial membrane of fungi and other eukaryotes. When azoxystrobin binds to this site, electron transfer between cytochrome b and cytochrome c_1 is blocked and ATP production is halted. This disruption of the energy cycle inhibits metabolic activities within the fungi, reducing their ability to parasitize crop plants (*1*, *2*). The strobilurins are most effective when applied prior to or in the early stages of infection since their activity is most effective against spore germination thus preventing disease development (*1*, *2*). Its widespread acceptance and use has made azoxystrobin the largest-selling fungicide worldwide (*2*). The possibility of runoff and drift of azoxystrobin from application sites into sensitive aquatic ecosystems demands controlled use and effective detection methods (*3*).

The current reported method for the detection of azoxystrobin involves an elaborate organic solvent extraction followed by nitrogen/phosphorus detection gas chromatographic analysis (*4*). Although sufficiently sensitive, the chromatographic method is time-consuming and requires expensive equipment. The required solvent extraction of environmental samples adds to the labor-intensive methodology and may often represent the most time-consuming and costly step of this technology (*5*). Furthermore, disposal of organic solvents creates a potential environmental hazard. Consequently, immunoassay formats such as enzyme-linked immunosorbent assay (ELISA), fluorescence polarization (FPIA), and time-resolved fluorescence (TR-FIA) offer easy-to-use, rapid, and sensitive methods that have been successfully utilized for the detection of small molecules other than azoxystrobin (*5*−*13*). In this paper, we report on the development of three assay formats, ELISA, FPIA, and TR-FIA technologies, and compare these assays for the detection and quantification of azoxystrobin in water and grape samples.

MATERIALS AND METHODS

Chemicals and Instrumentation. Azoxystrobin [methyl (*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate] and its acid were obtained from Syngenta (Guelph, ON, Canada). Kresoxim-methyl [(*E*)-methoxyimino[α-(*o*-tolyloxy)-*o*-tolyl]acetate], pyraclostrobin [methyl *N*-{2-[1-(4-chlorophenyl)-1*H*-pyrazol-3-yloxymethyl]-phenyl}(*N*-methoxy)carbamate], and trifloxystrobin [methyl (*E*)-methoxyimino-{(*E*)-α-[1-(α,α,α-trifluoro-*m*-tolyl)ethylideneaminooxy]-*o*-tolyl}acetate] were purchased from Chem Service (West Chester, PA). Bovine serum albumin (BSA), ovalbumin, Freund's incomplete adju-

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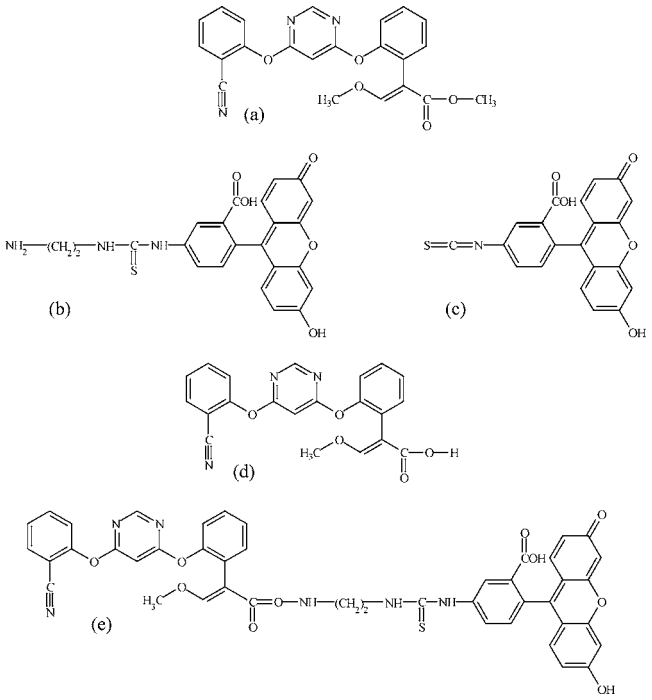

Figure 1. Structures of (**a**) azoxystrobin, (**b**) EDF, (**c**) FITC, (**d**) azoxystrobin acid, and (**e**) azoxystrobin-EDF.

vant, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, triethylamine, fluorescein isothiocyanate isomer I, and ethylenediamine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Acetone, Immulon 4 96 well flat bottom microtiter plates, Tween-20, sulfuric acid, methanol, dimethylformamide, and methylene chloride were obtained from Fisher Scientific (Don Mills, ON, Canada). Goat anti-rabbit horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Pierce Immunochemicals (Rockford, IL). Black 96 well microtiter plates, Delphia yellow 96 well microtiter plates, goat anti-rabbit europium chelate, Delphia reagent buffer, and Delphia enhancement solution were obtained from Perkin-Elmer (Woodbridge, ON, Canada). An Osterizer Galaxie Ten blender was used to homogenize the Chilean green grapes, which were purchased at a local supermarket. FP and TR-FIA were measured with a Perkin-Elmer Envision 2100 multilabel reader. ELISA results were measured with a BioRad 3550-UV microplate reader.

Synthesis of Immunogen and Coating Conjugate. The immunogen and coating conjugate, azoxystrobin conjugated to BSA and ovalbumin, respectively, were prepared using the method described by Fleeker (*14*) for conjugates of 2,4-D. Azoxystrobin acid (50 mg, 0.124 mmol) was mixed with 14 mg (0.124 mmol) of *N*-hydroxysuccinimide in 2 mL of acetone. Dicyclohexylcarbodiimide (25 mg, 0.124 mmol), in 1 mL of acetone, was added, mixed gently, and allowed to stand overnight at 22 °C in the dark. The solution was evaporated to dryness under nitrogen gas at 35 °C, and either BSA (200 mg) or ovalbumin (200 mg) in 3 mL of borate buffer (0.1 M, pH 9.0) was added dropwise to the vial with stirring. The reaction mixture was stirred for 2 h at 22 °C. The resulting conjugate was dialyzed against deionized water at 4 °C for 36 h.

Rabbit Polyclonal Antisera Production. Four New Zealand white rabbits were injected subcutaneously, at weekly intervals, for 3 weeks with a preparation of 63 μL of immunogen (32 μg/μL) in 1438 μL of phosphate-buffered saline plus 1500 μL of Freund's incomplete adjuvant (500 μg immunogen/rabbit contained in 750 μL of immunogen solution). Following this series of injections, the rabbits were subcutaneously injected monthly for 5 months with a preparation of 31 μL of immunogen (32 μg/μL) in 1469 μL of phosphate-buffered saline plus 1500 μL of Freund's incomplete adjuvant (250 μg immunogen/rabbit contained in 750 μL of immunogen solution). Serum samples of approximately 3−6 mL each were collected prior to each injection, and the final serum collection was performed by heart bleed (ca. 150 mL).

ELISA Assay Development. To determine the appropriate dilutions of the rabbit serum and coating conjugate required to optimize the final diagnostic assay, both were titrated through the use of a checkerboard assay. A standard curve showing competition between the coating conjugate and the free azoxystrobin in solution was prepared through the use of a competition assay. Azoxystrobin−ovalbumin coating conjugate (16 ng/mL PBS, 100 μL/well) was passively adsorbed overnight at 4 °C to wells of a 96 well polystyrene microtiter plate. The plate was washed three times with PBS−Tween (0.1% Tween-20) buffer and then blocked with 3% skim milk in water (200 μL/well) for 1 h at 22 °C. The rabbit anti-azoxystrobin serum was diluted appropriately, as determined by the checkerboard assay, in phosphate-buffered saline and mixed with various concentrations of azoxystrobin to derive a standard curve. The solutions of serum and pesticide were allowed to react at 22 °C for 1 h after which they were applied (100 μL/well), in triplicate, to the coated and blocked 96 well plate. The plate was again washed thrice with PBS−Tween buffer. Goat anti-rabbit horseradish peroxidase conjugate was added (100 μL/well) at a dilution of 1:4000 in phosphate-buffered saline and allowed to react for 30 min at 22 °C. Unbound conjugate was removed by washing three times with PBS−Tween wash buffer. TMB substrate was added (100 μL/well) and allowed to react for 15 min at 22 °C in the dark. Sulfuric acid (100 μL, 1 N) was added to quench the reaction, and the resulting pigmentation was observed at 450 nm.

Cross-Reactivity of Serum. The collected serum was analyzed for cross-reactivity with other small-molecule pesticides through the use of a competition ELISA. Rabbit anti-azoxystrobin serum was mixed with azoxystrobin to form a standard curve as described above. Serum was also mixed with other strobilurins, i.e., kresoxim-methyl, pyraclostrobin, or trifloxystrobin, or various unrelated pesticides, i.e., mecoprop, MCPA, metribuzin, triclopyr, 2,4-DB, 2,4-D, fluroxpyr, chlorsulfuron, chlorpyrifos, clopyralid, dichlorprop, diclofop, flumetsulam, atrazine, metolachlor, or chlorfenvinphos, to test its cross-reactivity to these compounds. These pesticides represent commonly used insecticides and herbicides, some of which contain similar chemical structures to azoxystrobin. Cross-reactivity was expressed as a percentage of inhibition observed as compared to the inhibition by azoxystrobin observed at the IC_{50} of the standard curve.

Preparation of Fluorescent Azoxystrobin Tracer. Fluorescein thiocarbamyl ethylenediamine (EDF; **Figure 1b**) was prepared as described by Nistor et al. (*15*), a modification of the method by Pourfarzaneh et al. (*16*). Triethylamine (100 μL) was added to 10 mL of methanol; hereafter, this solution is designated as solution A. Fluorescein isothiocyanate isoform I (FITC; see **Figure 1c**; 11.7 mg; 30 μmol) was dissolved in 1 mL of solution A. Ethylenediamine dihydrochloride (20 mg; 150 μmol) was dissolved in 5 mL of solution A. These two solutions were mixed and allowed to stand for 16 h at 4 °C. The resulting precipitate was filtered though a glass microfiber filter (Whatman) and allowed to dry overnight at 22 °C in the dark.

Azoxystrobin acid (**Figure 1d**) was conjugated to the prepared EDF based on a modification of the procedure for the conjugation of EDF to 4-nitrophenol carboxylic derivatives as described by Nistor et al. (*15*). Azoxystrobin acid (8 mg; 20 μmol), N-hydroxysuccinimide (4.6 mg; 40 μmol), and dicyclohexylcarbodiimide (8 mg; 40 μmol) were added to 1 mL of dimethylformamide and mixed for 4 h at 22 °C. This solution (250 μL) was added dropwise to EDF (1 mg; 2.5 μmol) and stored at 4 °C (**Figure 1e**).

Thin-Layer Chromatography. The azoxystrobin−EDF conjugate solution (50 μL; approximately 80 nM) was spotted onto LK6F silica gel plates (1000 μm, 20 cm × 20 cm; Whatman) along with samples of azoxystrobin (20 nM), azoxystrobin acid (20 nM), EDF (10 nM), and FITC (30 nM) solution, which were used as reference materials. The chromatography plates were developed in methylene chloride:methanol (4:1 v/v). Standards and products visualized at 366 nm in the azoxystrobin−EDF conjugate lane were scraped from the plate, placed in 0.5 mL of methanol overnight at 4 °C, and filtered through glass wool, and the eluant was stored at −20 °C until required.

FP Assay Development. To determine the appropriate dilution of fluorescent tracer to use in the diagnostic assay, the azoxystrobin−EDF conjugate was serially diluted in 3 mg/mL skim milk in water and analyzed by fluorescence polarization (100 μL/well) with an

excitation wavelength of 488 nm and an emission wavelength of 530 nm. The appropriate operating dilution of azoxystrobin−EDF conjugate was the dilution yielding a total fluorescence within the linear range of the above titration, nearest the lower plateau. This dilution represents the lowest concentration of azoxystrobin−EDF conjugate that is accurately measurable, thus requiring the least amount of antibody to saturate the system.

A standard curve of competition between azoxystrobin−EDF and free azoxystrobin in solution was prepared through the use of a competition assay. Rabbit anti-azoxystrobin serum was diluted 1:100 (v/v) in phosphate-buffered saline containing 0, 50, 100, 200, 300, 400, or 500 pg/mL azoxystrobin. These mixtures were allowed to react for 1 h at 22 °C after which they were applied (80 μL/well), in triplicate, to a black 96 well microtiter plate. Azoxystrobin−EDF was diluted 1:2000 in 3 mg/mL skim milk and added (20 μL/well) to the serum−azoxystrobin solution in each well. These mixtures were allowed to react for 10 min at 22 °C in the dark and were analyzed by fluorescence polarization.

TR-FIA Assay Development. To determine the appropriate dilutions at which the rabbit serum and coating conjugate should be used in a diagnostic assay, both were titrated through the use of a checkerboard assay. A standard curve of competition between bound and free azoxystrobin was prepared as described for the development of the ELISA with the exception of the following: a yellow 96 well polystyrene microtiter plate was used; goat anti-rabbit europium chelate conjugate was added (100 μL/well) at a dilution of 1:5000, as suggested by the manufacturer, in Delphia reagent buffer instead of the goat anti-rabbit horseradish peroxidase; and Delphia enhancement solution was added (100 μL/well) instead of TMB and allowed to react for 5 min at 22 °C in the dark with gentle shaking. The resulting fluorescence (615 nm) was observed after excitation at 340 nm and a delay of 400 μs.

Analysis of Azoxystrobin in Environmental Samples. Water was collected from three sources: river water from the Speed River (Guelph, ON, Canada), lake water from Fairy Lake (Acton, ON, Canada), and well water from an active residential well (Eramosa Township, ON, Canada). The samples (10 mL aliquots) were stored at −20 °C until required.

Commercially purchased Chilean green grapes (500 g) were homogenized in deionized water with an Osterizer Galaxie Ten blender for 10 min and filtered through Whatman #1 filter paper. Phosphate-buffered saline was added to yield a stock solution (500 mL) of 1 g grape/mL extract and stored at −20 °C until used.

Each of the environmental samples was used as the diluent for a 1 ng/mL stock solution of azoxystrobin. Working solutions of 100, 200, and 300 pg azoxystrobin/mL of water were prepared as dilutions of the stock solutions utilizing the respective sample matrix as diluent. All samples were analyzed using each of the competition-based immunoassays. Standard solutions of azoxystrobin at concentrations of 0, 100, 200, 300, 400, and 500 pg/mL in phosphate-buffered saline were prepared for interassay standard curve generation and direct comparison of the resulting data for each of the samples.

RESULTS

Detection of Azoxystrobin by ELISA. When absorbance/maximum absorbance observed, i.e., A/A_o, is plotted against concentration of azoxystrobin, the competition ELISA was found to have a linear working range of 0−400 pg/mL (**Figure 2**). The limit of detection (LOD), defined as the concentration of azoxystrobin that gives an observed signal three times the standard deviation of the mean blank (zero competition, A_o) (*17*), was found to be 3 pg/mL. The limit of quantitation (LOQ), defined as the concentration of azoxystrobin that gives an observed signal 10 times the standard deviation of the mean blank (zero competition, A_o) (*17*), was found to be 104 pg/mL. The IC_{50} or concentration of azoxystrobin required for 50% inhibition of the mean blank, A_o, was calculated to be 306 pg/mL.

Antiserum Cross-Reactivity. It is important to determine any cross-reactivity that an assay may have with other pesticides that may contaminate environmental samples. Little or no cross-reactivity was observed with the variety of pesticides tested in the ELISA format. Only five of the 19 compounds tested yielded cross-reactivity greater than 1%, i.e., trifloxystrobin, 5.7%; diclofop, 4.0%; pyraclostrobin, 3.4%; 2,4-DB, 2.8%; and 2,4-D, 1.5%. The structures of these five pesticides are presented in **Figure 5**. Furthermore, only two of the three strobilurins (kresoxim-methyl, pyraclostrobin, and trifloxistrobin) displayed any cross-reactivity (less than 6%).

Detection of Azoxystrobin by the FP Assay. Thin-layer chromatography of the azoxystrobin−EDF conjugate yielded three bands. When compared to the standards, two of these compounds were determined to be azoxystrobin acid ($R_f = 0.40$) and EDF ($R_f = 0.64$). The remaining compound, azoxystrobin−EDF, was the only one that demonstrated fluorescence polarization activity when mixed with the rabbit anti-azoxystrobin serum ($R_f = 0.78$).

Figure 2. Standard curve for the competitive azoxystrobin ELISA ($y = -0.0018x + 1.0501$; $r^2 = 0.9977$). Error bars represent ±1 SD; $n = 6$; $IC_{50} = 306$ pg/mL. The coating conjugate was used at 16 ng/mL, and rabbit anti-azoxystrobin serum was diluted 1:400 in PBS.

Figure 3. Standard curve for the competitive azoxystrobin fluorescence polarization assay ($y = -0.0019x + 0.979$; $r^2 = 0.9979$). Error bars represent ±1 SD; $n = 6$; $IC_{50} = 252$ pg/mL. Rabbit anti-azoxystrobin serum was diluted 1:100 in PBS.

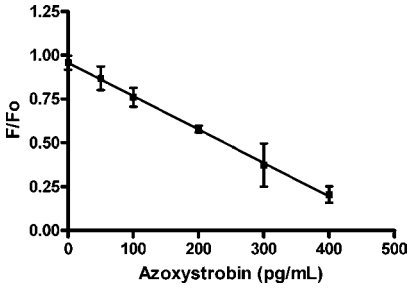

Figure 4. Standard curve for the competitive azoxystrobin time-resolved fluorescence assay ($y = -0.0019x + 0.9629$; $r^2 = 0.9988$). Error bars represent ±1 SD; $n = 6$; $IC_{50} = 244$ pg/mL. The coating conjugate was used at 16 ng/mL, and rabbit anti-azoxystrobin serum was diluted 1:400 in PBS.

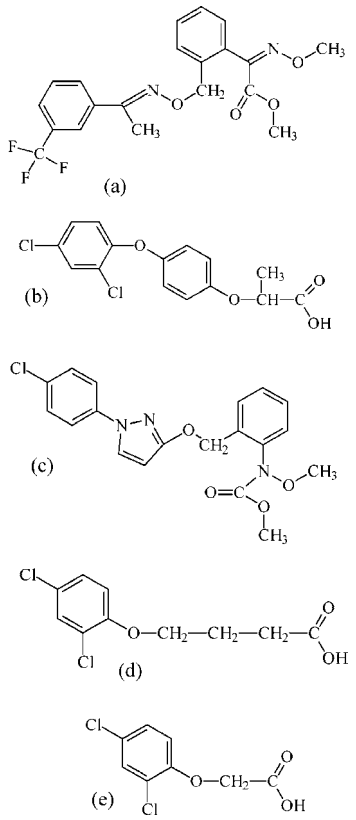

Figure 5. Structures of (**a**) trifoxystrobin, (**b**) diclofop, (**c**) pyraclostrobin, (**d**) 2,4-DB, and (**e**) 2,4-D, which resulted in more than 1% cross-reactivity as determined using the ELISA for axozystrobin.

When polarization/maximum polarization observed, i.e., P/P_o, is plotted against the concentration of azoxystrobin, the competition fluorescence polarization assay was found to have a linear working range of 0−400 pg/mL (**Figure 3**). The LOD was 46 pg/mL while the LOQ was 163 pg/mL. The IC_{50} was calculated to be 252 pg/mL.

Detection of Azoxystrobin by the TR-FIA Assay. When fluorescence/maximum fluorescence observed, i.e., F/F_o, is plotted against the concentration of azoxystrobin, the competition TR-FIA was found to be sensitive within a linear working range of 0−400 pg/mL (**Figure 4**). The LOD was 28 pg/mL while the LOQ was 114 pg/mL. The IC_{50} was calculated to be 244 pg/mL.

Analysis of Azoxystrobin in Environmental Samples. The detection of azoxystrobin in spiked grape extract and river, lake, and well waters was similar among environmental sample types and among immunoassay formats (**Tables 1** and **2**). The recovery ranges as determined by ELISA, FP, and TR-FIA assays were 71−109, 88−110, and 75−107%, respectively. The average recoveries of azoxystrobin spiked in grape extract and river, lake, and well waters were 94, 100, 99, and 92%, respectively.

DISCUSSION

The specificity of an immunoassay depends on the specificity of the antibodies used (*8*). The cross-reactivity of the rabbit anti-azoxystrobin serum was negligible with the highest value being 5.73% for the strobilurin triflozystrobin, followed by that of the herbicide diclofop (4.03%) and the strobilurin pyraclostrobin (3.40%). This limited cross-reactivity is likely due to substituent groups of the test compounds being different from those presented to the immune system by azoxystrobin. The compounds that had activity greater than 1% did have structural similarities (e.g., aromatic structures and/or ether-linked aromatic structures) to azoxystrobin; however, other pesticides tested containing these same structural components showed little to no cross-reactivity. Therefore, it is difficult to elucidate the cause of the cross-reactivity observed.

The sensitivity and working range of an assay can be optimized through the manipulation of its component reagents, i.e., altering the concentrations of antibody, altering the reaction times, etc. Once the use of the components was optimized, the three immunoassays had similar limits of quantitation (ELISA = 104 pg/mL; FPA = 163 pg/mL; TR-FIA = 114 pg/mL) and IC_{50} values (ELISA = 306 pg/mL; FPA = 252 pg/mL; TR-FIA = 244 pg/mL). According to the Pest Management Regulatory Agency of Canada (*3*), the currently accepted method for azoxystrobin residue analysis is a gas chromatography method using a nitrogen/phosphorus detector, which has a LOQ of 0.01 ppm, i.e., 10 ng/mL. The three immunoassay formats

Table 1. Azoxystrobin Recovery from Spiked River, Lake, and Well Waters Using ELISA, Fluorescence Polarization (FPIA), and Time-Resolved Florescence (TR-FIA) Assays[a]

assay	sample	spiked (pg/mL)	recovered (pg/mL)	SD[b]	% recovered	% CV
ELISA	river	300	327	10	109	3
		200	180	18	90	10
		100	103	10	103	10
	lake	300	269	42	90	15
		200	186	41	93	22
		100	105	7	105	7
	well	300	255	55	85	22
		200	165	2	82	1
		100	102	4	102	4
FPIA	river	300	292	17	97	6
		200	211	19	105	9
		100	98	17	98	17
	lake	300	284	22	95	8
		200	207	12	104	6
		100	103	21	103	20
	well	300	286	36	95	13
		200	214	13	107	6
		100	88	18	88	20
TR-FIA	river	300	288	8	96	3
		200	198	11	99	6
		100	107	14	107	13
	lake	300	280	28	93	10
		200	210	8	105	4
		100	105	10	105	10
	well	300	279	38	93	14
		200	151	6	75	4
		100	97	10	97	11

[a] For each concentration, $n = 6$. [b] Standard deviation.

Table 2. Azoxystrobin Recovery from Spiked Green Grape Extract Using ELISA, FPIA, and TR-FIA[a]

assay	spiked (pg/mg)	recovered (pg/mg)	SD[b]	% recovered	% CV
ELISA	300	309	26	103	8
	200	204	31	102	15
	100	71	5	71	7
FPIA	300	248	39	83	16
	200	220	9	110	4
	100	101	16	101	16
TR-FIA	300	236	10	79	4
	200	202	36	101	18
	100	95	11	95	11

[a] For each concentration, $n = 6$. [b] Standard deviation.

Table 3. Summary of Assay Statistics (pg/mL) of Three Competitive Inhibition Immunoassays for the Detection of Azoxystrobin

assay	LOD[a]	LOQ[b]	IC_{50}[c]
ELISA	3	104	306
FPIA	46	163	252
TR-FIA	28	114	244

[a] LOD (3× standard deviation of zero competition). [b] LOQ (10× standard deviation of zero competition). [c] Concentration at 50% inhibition.

have LOQs ranging from 104 to 163 pg/mL, approximately 2 orders of magnitude more sensitive than the established gas chromatographic method.

As defined by Christensen and Granby (*18*), an analytical method should have mean recovery, from spiked samples, between 70 and 110% for validation data to be accepted. All three assay formats described could be used to detect azoxystrobin in fortified environmental samples within this accepted range regardless of the sample type, i.e., water and grape extract. The ranges of recovery in fortified samples were 71−109% for the ELISA, 83−110% for the FP assay, and 75−107% for the TR-FIA assay. With regard to the grape extract, the range was 71−103% for the ELISA, 83−110% for the FP assay, and 79−101% for the TR-FIA assay; all of which are similar to the currently accepted gas chromatography method for azoxystrobin detection, which has a range of recovery of 80−111% with spiked grape extract (*4*).

The Pest Management Regulatory Agency of Canada (*3*) has determined the maximum environmental load (MEL) of azoxystrobin when used on canola, grapes, and turfgrass based upon a worst-case scenario, i.e., when samples are taken immediately after application of the maximum allowable field dose. The two lowest expected environmental concentrations of azoxystrobin in the Pest Management Regulatory Agency's comprehensive list are 47 μg/L in human drinking water from a small watershed and 63 μg/L in pond water, both due to runoff from a treated canola field (*3*). Each of the three immunoassay methods described here can detect azoxystrobin residues in various water samples at concentrations as low as 100 pg/mL (i.e., 100 ng/L), with negligible matrix effect; approximately 500-fold lower than the MEL accepted by PMRA. The maximum residue limit for azoxystrobin in grapes has been set at 3 ppm (i.e., 3 ng/mg) (*3*). Each of the three immunoassay methods described can be used to detect azoxystrobin residues in green grape extract at concentrations as low as 100 pg/mg (i.e., 100 ppb), 30-fold lower than the maximum residue limit accepted by PMRA.

As a direct comparison, all three immunoassay methods described are approximately 100-fold more sensitive than the currently accepted gas chromatography method and have similar percentage recoveries as determined by using fortified environmental samples. It is difficult to distinguish between these three immunoassay formats based on sensitivity (**Table 3**). However, the FP assay standard curve does show more variability than the other assays, suggesting that additional replicates may be necessary to ensure precision of azoxystrobin estimation. The development of the ELISA and TR-FIA assays required the preparation of a coating conjugate (azoxystrobin−BSA) while the FP assay required the preparation of a fluorescent conjugate. In terms of performing the assays, i.e., preparation of samples and conducting the procedure, the ELISA and TR-FIA assays required approximately 3 h to perform following incubation overnight with coating conjugate. In contrast, the FP assay required less than 90 min to perform and no coating of plates was required. While none of the immunoassays were affected by the matrix of the environmental samples at the levels tested, the FP assay may be more susceptible than the other immunoassay formats to background interference from naturally fluorescing compounds that may exist in some sample matrices. On the basis of these results, it is difficult to determine which assay is, generally, the most appropriate to use; this determination will depend on each specific situation.

In conclusion, three similarly sensitive immunoassays for the specific detection of azoxystrobin have been developed utilizing ELISA, FP, and TR-FIA technologies. Under the conditions tested, the assays differ only in time to perform, with the FP assay requiring significantly less time to obtain results than the other two formats. Each of these immunoassays would be a suitable replacement for the currently utilized, organic solvent-based gas chromatography method based on sensitivity, specificity, sample matrix interference, and labor intensity.

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