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Determination of Isoxaflutole (Balance) and Its Metabolites in Water Using Solid Phase Extraction Followed by High-Performance Liquid Chromatography with Ultraviolet or Mass Spectrometry

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Balance (isoxaflutole, IXF) belongs to a new family of herbicides referred to as isoxazoles. IXF has a very short soil half-life (<24 h), degrading to a biologically active diketonitrile (DKN) metabolite that is more polar and considerably more stable. Further degradation of the DKN metabolite produces a nonbiologically active benzoic acid (BA) metabolite. Analytical methods using solid phase extraction followed by high-performance liquid chromatography−UV (HPLC-UV) or high-performance liquid chromatography−mass spectrometry (HPLC-MS) were developed for the analysis of IXF and its metabolites in distilled deionized water and ground water samples. To successfully detect and quantify the BA metabolite by HPLC-UV from ground water samples, a sequential elution scheme was necessary. Using HPLC-UV, the mean recoveries from sequential elution of the parent and its two metabolites from fortified ground water samples ranged from 68.6 to 101.4%. For HPLC-MS, solid phase extraction of ground water samples was performed using a polystyrene divinylbenzene polymer resin. The mean HPLC-MS recoveries of the three compounds from ground water samples spiked at 0.05−2 μg/L ranged from 100.9 to 110.3%. The limits of quantitation for HPLC-UV are approximately 150 ng/L for IXF, 100 ng/L for DKN, and 250 ng/L for BA. The limit of quantitation by HPLC-MS is 50 ng/L for each compound. The methods developed in this work can be applied to determine the transport and fate of Balance in the environment.

KEYWORDS: Isoxaflutole; herbicide; metabolites; chromatography; diketonitrile; benzoic acid metabolite, HPLC-MS; HPLC-UV

INTRODUCTION

Balance [isoxaflutole, IXF; 5-cyclopropyl-4-(2-methylsulfonyl-4-(trifluoromethyl)benzoyl)-5-cyclopropylisoxazole, CAS Registry No. 141112-29-0] is a herbicide manufactured by Rhône-Poulenc Agro (Research Triangle Park, NC). [Rhône-Poulenc Agro and AgrEvo (a company of Hoescht and Schering) merged in January 2000 to form Aventis CropScience.] It belongs to a new family of herbicides called isoxazoles. These herbicides inhibit 4-hydroxyphenylpyruvate dioxygenase (HPPD), thereby indirectly blocking carotenoid biosynthesis (*1*). This impairs chloroplast development and leads to bleaching symptoms of plant leaves.

IXF represents a new strategy for weed control. The parent compound is actually a precursor molecule without herbicidal activity. To be activated, IXF is abiotically hydrolyzed in soils to the more soluble and stable diketonitrile (DKN) metabolite, which is the biologically active herbicide compound (**Figure 1**) (*1*−*4*). The intended strategy of the manufacture is that under field conditions IXF would degrade to the biologically active DKN in a series of pulses of following rainfall. Because DKN is more stable than IXF in soils, residual weed control may be achieved by this so-called recharge mechanism. However, DKN is also more water soluble than IXF and, therefore, its transport to surface or ground water is a concern.

In field trials, Balance has proven to be effective against a wide spectrum of problem weeds in corn production (*5*, *6*). It appears to perform well at relatively low dosages (~11−64 g/ha active ingredient) and offers season-long pre-emergence weed control (*5*, *6*). Balance received conditional regulatory approval from the U.S. Environmental Protection Agency in 1998. It was commercially introduced for the 1999 growing season in 16 key corn-producing states. The herbicide has a very short soil

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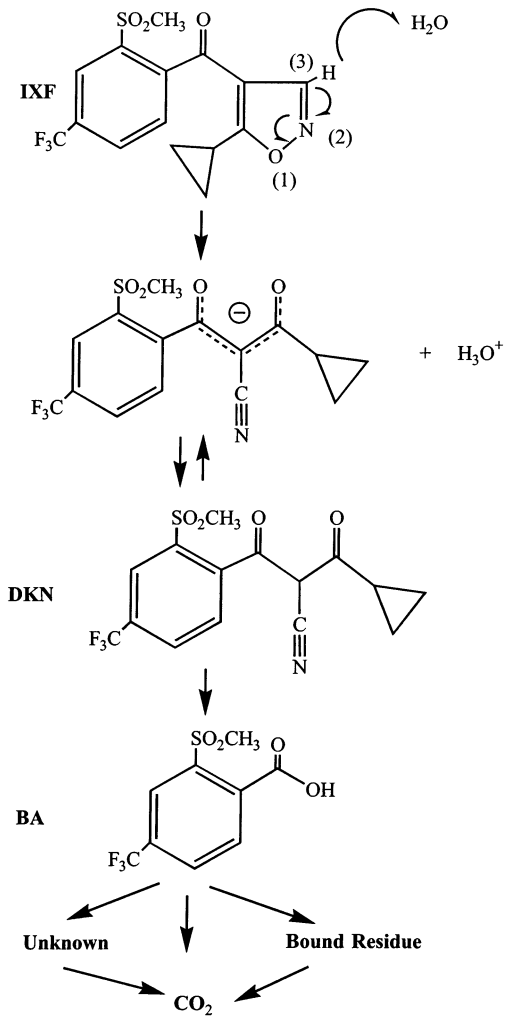

	Half-life in soil (days)	Partition coefficient K_{oc}[a]	Solubility in water (mg/L)
IXF	1.4-3	122	6.2
DKN	8-16	92	300
BA	20-60	69	8000

[a] K_{oc} – organic carbon partition coefficient.

Figure 1. Degradation pathway and chemical properties of isoxaflutole and its metabolites [Beltran et al. (*9*) and personal communication from Rhône-Poulenc, Co.].

half-life of <24 h (*3*). Degradation of IXF into DKN appears to follow second-order kinetics. Under the influence of dim light (~10 μeinstein/m^2), a second-order rate constant of 1.5×10^{-7} L/μg/s has been measured (*7*). Grünanger and Vita-Finzi (*8*) reported that the hydrolysis of many isoxazoles follows second-order kinetics. However, the degradation pathway of IXF may exhibit first-order kinetics (*9*). Degradation of DKN produces a nonbiologically active benzoic acid metabolite (BA), a highly stable water-soluble compound (personal communication from Rhône-Poulenc Agro).

The parent IXF compound does not inhibit the HPPD enzyme and is particularly sensitive to light and hydrolysis (*1*, *2*, *4*, *9*). The conversion of IXF to DKN occurs rapidly under field conditions (*10*). In a field lysimeter study, IXF was observed to be extremely unstable and DKN was the most predominant form of the herbicide detected in the leachate 30 h after application (*10*). The photolysis half-life of the parent IXF on or near the soil surface has been observed to be ~20−23 h (*11*), whereas the half-life of IXF was observed to be <9 h in an aqueous solution (250 μg/L) of pH 7.18 exposed to dim visible light (~10 μeinstein/m^2) at 25 °C (*7*). The hydrolysis half-life of IXF to DKN has been measured to be 11.1 h at pH 5, 20.1 h at pH 7, and 3.2 h at pH 9 (*12*). Under anaerobic conditions in an aquatic environment, the half-life of IXF was <2 h (*12*). The low stability of IXF is associated with its high unequal electronic distribution in the isoxazole nucleus that results in the electron deficiency of the carbon-3 on the isoxazole ring (**Figure 1**) (*9*). Due to the electron deficiency, the proton released from the carbon-3 position has a high potential to protonize a water molecule (pH 5.2−6) or hydroxide ion and lead to the opening of the isoxazole ring (*9*). Beltran et al. (*9*) reported a possible intermediate resonance stabilized enolated ion was formed before completely converting to the β-ketontrile, DKN. This high reactivity of the unsubstantiated 3-position has been observed in both alkaline and acid media (*8*, *9*).

Due to the low application rate and low stability of the compound in the field, a robust analytical procedure is required to provide an acceptable detection limit and to prevent or significantly reduce any degradation during analysis. There have been gas chromatography (GC) procedures developed for the analysis of IXF (*13*, *14*). However, the methods are limited to the detection of the total IXF (i.e., parent plus metabolites) because they involve hydrolysis of IXF to DKN followed by conversion of DKN to BA and then derivatization of BA to a methyl ester (*12*). These methods are, therefore, insufficient for determining the fate of IXF and its metabolites in environmental samples.

Procedures that combine solid phase extraction (SPE) with reversed-phase high-performance liquid chromatography (HPLC) have been suggested as being well suited for the separation and quantitation of nonpolar to moderately polar compounds that are not sufficiently volatile for analysis by GC. Octadecyl (C18) bonded silica SPE resins have been widely used for the extraction of various classes and concentrations of hydrophobic pesticides (*15*−*17*). Recently, a styrene divinylbenzene polymer SPE resin exhibited better performance for retaining medium- and high-polar pesticides (*18*−*20*). The stationary phase of this type of column consists of cross-linked polystyrene derived from the copolymerization of styrene and divinylbenzene (*21*). The degree of polymerization and, therefore, the pore structure are determined by the amount of divinylbenzene added during column formation. This polymer SPE column has advantages over the silica-based C18 SPE column (*19*, *20*, *22*). These include excellent pH stability (pH 1−13), higher percentage recoveries, and improved reproducibility. Also, many analytes are less likely to irreversibly bind to the polystyrene divinylbenzene polymer resin than to the C18 coated silica matrix (*22*, *23*).

Following sample preparation by SPE or other methods, the HPLC-UV spectrophotometric method has been successfully applied to the detection of numerous aromatic organic pesticides and their metabolites at nanograms per liter levels (*15*, *24*). HPLC coupled with an electrospray ionization interface followed by mass spectrometry has also been applied for the analysis of both polar and nonpolar compounds that can be ionized by the interface (*25*, *26*). During the ionization process, the high potential charges the emerging droplets when the analyte passes through the sprayer (*27*). As a result, highly charged desolvated ions are formed. The analyte then is detected and quantified by the facilitated fragmentation pattern of the ion as characterized by its mass to charge ratio (*m*/*z*).

Balance has the potential for widespread agronomic use and may significantly lower atrazine usage in the next decade. It has been estimated that this new pre-emergent herbicide has been applied to ~4−23% of corn-belt state farmland in corn production during the first three years of introduction (*28*). In Europe, Balance has also been approved for agricultural application in several countries, including England and The Netherlands (personal communication from TNO Research Institute, Zeist, The Netherlands). Because this is a new herbicide, its impact on surface/ground water is not fully understood. As a part of the U.S. Environmental Protection Agency's evaluation of the impact of Balance on water quality, they used the Pesticide Root Zone Model (PRZM) and the Screening Concentration in Ground Water (SCI-GROW) model to predict concentrations of IXF, DKN, and BA in surface and ground water, respectively, following field application (*13*). The model results indicated that IXF, DKN, and BA concentrations in surface water might be as high as 0.4, 2.0, and 10.0 μg/L, respectively. The estimated maximum concentrations of IXF, DKN, and BA in ground water are 0.00025, 0.23, and 6.1 μg/L, respectively. Therefore, Balance or, more correctly, its metabolites DKN and BA would be expected to appear as nonpoint source pollutants soon.

The objective of this study is to develop solid phase extraction procedures coupled with HPLC-UV and HPLC-MS [with an atmospheric pressure ionization (API) electrospray ionization interface] to unambiguously identify and quantify IXF and its two metabolites (DKN and BA) in environmentally derived water samples at sub-micrograms per liter concentrations. The developed methods will be valuable for predicting and acquiring information about the fate of this herbicide in commercial agriculture systems.

MATERIALS AND METHODS

Chemical Standards, Solvents, and Solid Phase Extraction Columns. Isoxaflutole [5-cyclopropyl-4-(2-methylsulfonyl-4-(trifluoromethyl)benzoyl)isoxazole], diketonitrile [2-cyclopropyl-3-(2-methylsulfonyl-4-(trifluoromethyl)benzoyl)-3-oxopropanenitrile], and benzoic acid derivative [2-methylsulfonyl-4-(trifluoromethyl)benzoic acid] were obtained in 95−99% purity from Rhône-Poulenc Agro Co. (Research Triangle Park, NC). All solvents used were of HPLC grade. Standard stock solutions were prepared using 100% acetonitrile (ACN). The working standards were prepared in 9:1 1.3% formic acid/ACN solution (pH 2.1). SPE cartridges used for preparing samples for HPLC-UV were a silica-based Sep Pak LRC 500-mg C_{18} cartridge (Varian, Inc., Harbor City, CA) and a Spe-ed RP-102 highly cross-linked polystyrene divinylbenzene 2 g SPE cartridge (Applied Separations Inc., Allentown, PA) modified to 500 mg. Sample preparation for HPLC-MS analysis was preformed using an Isolute Env+ 200 mg polystyrene divinylbenzene polymer SPE cartridge (International Sorbent Technology−Jones Chromatography Inc., Lakewood, CO).

HPLC-UV Analysis of Spiked Samples. *Recovery from Distilled Deionized Water*. Two sets of samples were prepared by SPE (**Figure 2A**). In the first set, IXF, DKN, and BA were mixed in distilled deionized water. The concentration of each analyte was 10 μg/L. The solution was acidified with formic acid (0.6%, pH 2.1) to stabilize IXF. Each 500 mg C_{18} SPE cartridge was conditioned with 20 mL of ACN followed by 20 mL of HPLC grade water at a flow rate of 4 mL/min. The flow rate was controlled by the use of a vacuum manifold. Samples containing 200 mL of the solution were then passed through the cartridge at a flow rate of 4 mL/min using the same vacuum system followed by washing with 10 mL of distilled deionized water. A total of 21 mL of 80% ACN was applied to elute the analytes from the SPE column using a 3 × 7 mL sequential elution scheme at a 2 mL/min flow rate. Each 7 mL fraction was collected separately and evaporated to ~50 μL under a stream of dry nitrogen gas at 40 °C. The final extracts were reconstituted in 1 mL of 9:1, 1.3% formic acid/ACN (pH 2.1) solution and then filtered through a polytetrafluoroethylene (PTFE) Acrodisc CR 0.45 μm filter (Pall Gelman Sciences, Ann Arbor, MI). For the second set of samples, IXF, DKN, and BA were mixed to concentrations of 1, 5, and 12.5 μg/L for each analyte. Preparation of analyte solutions, SPE column conditioning, and sample loading were done as noted above; however, the analytes were eluted with 21 mL of 80% ACN into a single fraction. Evaporation and reconstitution of the analytes were carried out in the same manner as for the first set of samples.

Recovery from Ground Water. The ground water was collected from 18 field lysimeters filled with a sandy loam soil with average pH of 7.0, organic content of 0.72%, and cationic exchange capacity of 3.0 mequiv/100 g. Filtered ground water samples were spiked with the IXF, DKN, and BA standards to create sample solutions containing 0.5 and 1 μg/L of each analyte (**Figure 2B**). The samples were acidified with formic acid (0.6%, pH 2.1) to stabilize the IXF. Each 500 mg C_{18} SPE cartridge was conditioned as described above for distilled deionized water. Spiked sample solutions, either 100 or 200 mL, were passed through the cartridges at a flow rate of 4 mL/min.

Analytes were eluted from the SPE column with a single volume of 80% ACN or a sequential elution scheme using two ACN solutions. The first procedure used 21 mL of 80% ACN to elute the analytes into a single fraction. This fraction was evaporated to ~50 μL under a stream of nitrogen gas at 40 °C and then reconstituted in 1 mL of 9:1, 1.3% formic acid/ACN (pH 2.1) solution. The final extract was filtered through a PTFE Acrodisc CR 0.45 μm filter. In the second procedure, the SPE cartridge was soaked with 10 mL of 13% ACN for 30 min. The cartridge was then eluted with an additional 20 mL of 13% ACN to isolate BA and DKN. This was followed by elution with 20 mL of 80% ACN to isolate IXF. Both fractions were evaporated to ~10 mL with a Savant concentrator (Savant Instruments, Farmingdale, NY) and then further evaporated to ~50 μL under a stream of nitrogen gas at 40 °C. The fractions were reconstituted with 1 mL of 9:1, 1.3% formic acid/ACN (pH 2.1) solution. The final extracts of both fractions were filtered through a PTFE Acrodisc CR 0.45 μm filter.

The utility of using a stationary phase based upon a styrene divinylbenzene SPE matrix for the preparation of the analytes was also tested. In this experiment, the same sequential elution scheme described above was applied to a 2 g Spe-ed RP-102 SPE cartridge modified in the laboratory to contain 500 mg of resin. Only one concentration (1 μg/L) of each analyte and one sample volume (200 mL) were tested.

HPLC-UV Conditions. For analyses of the spiked deionized water samples, HPLC was performed using a Beckman model 338 HPLC system (Beckman, San Ramon, CA) equipped with a model 110B solvent delivery module, a model 507 autosampler module, and a model 166 UV detector. The mobile phase for HPLC consisted of 0.1% H_3PO_4 buffer (pH 2.2) and 100% ACN with a flow rate of 1.0 mL/min and isocratic conditions of 60% H_3PO_4 buffer/40% ACN. The analytical column was a silica-based Columbus C8, 4.6 mm × 100 mm, 5 μm (Phenomenex, Torrance, CA). The column temperature was maintained at 40 °C. A 100 μL volume of final extract was injected into the HPLC system. Beckman Gold (GoldDV810) application software was used for instrument control and data processing. Prior to HPLC-UV, absorption spectra of IXF, DKN, and BA were measured in 100% ACN in order to determine optimum wavelengths for UV detection (**Figure 3**). The concentration of the analytes in these test samples was 10 mg/L. A Hewlett-Packard UV diode array spectrophotometer (8452A) was used for this purpose. On the basis of these data, UV absorption during HPLC was measured at 270 nm for both IXF and BA and at 300 nm for DKN. The concentration of each analyte following SPE and HPLC-UV was determined by external standard calibration curve over a concentration range of 5−1000 μg/L. Percentage recoveries were calculated from this information.

For ground water, chromatography was performed with the HPLC-UV system described for distilled deionized solutions. However, to avoid coelution interference and to enhance the signal to noise ratio, a longer analytical column (Columbus C8, 4.6 × 250 mm, 5 μm; Phenomenex) was used and a stepwise mobile gradient of ACN and phosphoric acid buffer (pH 2.2) was applied. The concentration of ACN was changed from 20 to 90% over 60 min and then back to 20% ACN in 30 min for a total run of 90 min. Although BA has a unique UV

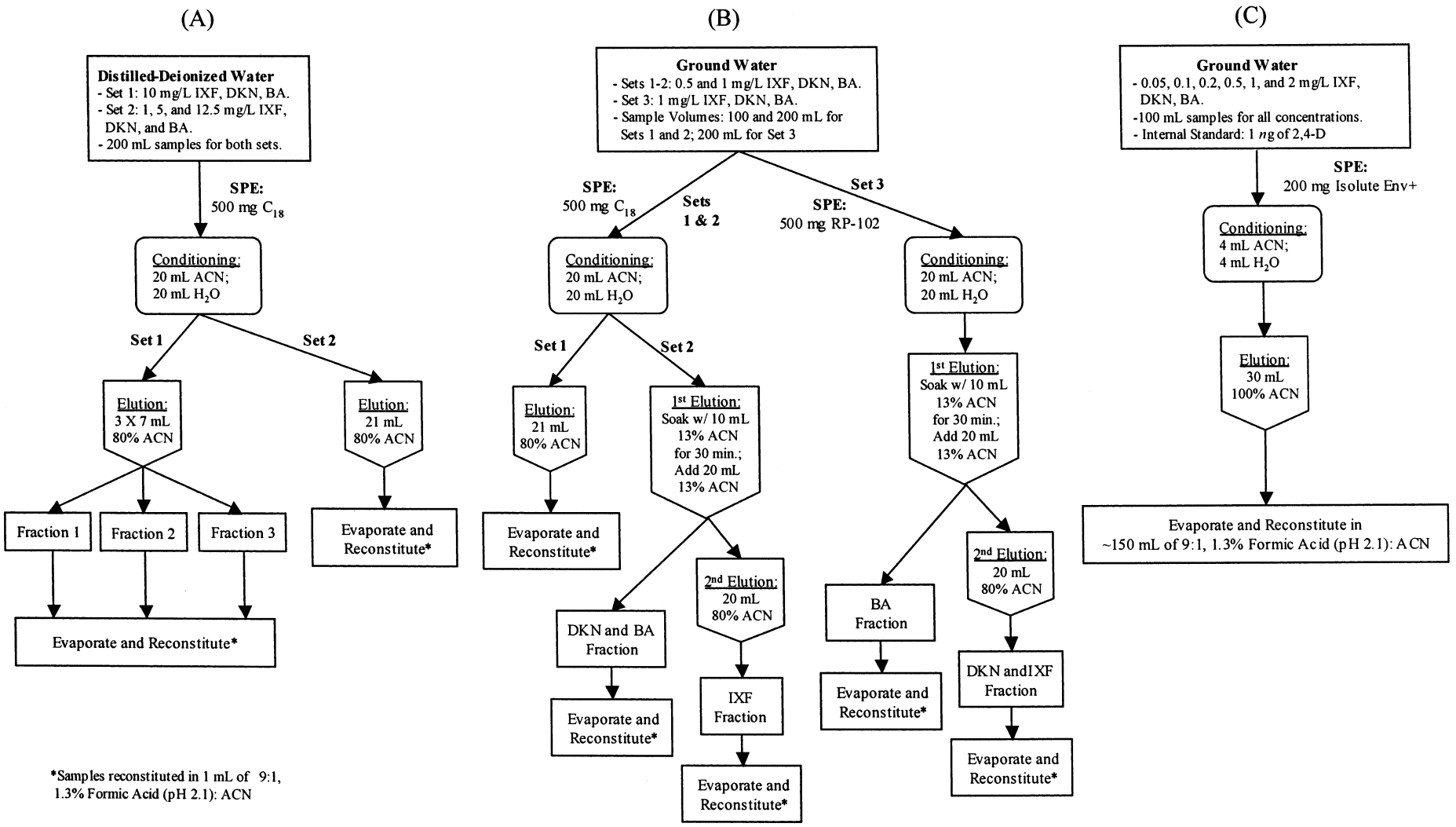

Figure 2. Procedural schemes for evaluating the effects of analyte concentration, elution conditions, and solid phase extraction type on recovery of IXF, DKN, and BA for (A) distilled–deionized water using HPLC-UV, (B) ground water using HPLC-UV, and (C) ground water using HPLC-MS.

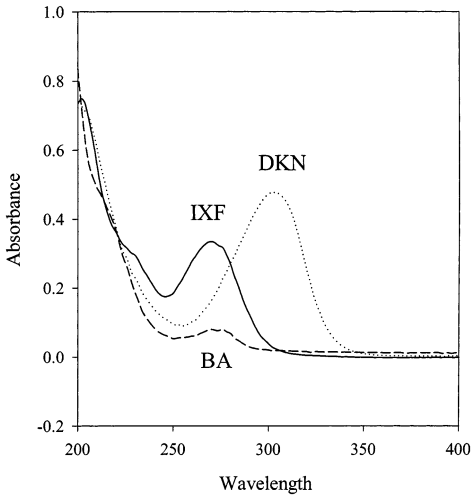

Figure 3. UV absorption spectra of IXF, DKN, and BA.

absorption peak at 270 nm, peak height is relatively small. To enhance sensitivity, absorbance measurements for BA were performed at 220 nm (**Figure 3**). Limits of quantitation were calculated on the basis of a signal to noise ratio of 10:1 (*29*).

HPLC-MS Analysis of Spiked Samples. *Recovery from Ground Water*. Ground water samples collected as described for HPLC-UV were spiked with IXF, DKN, and BA standards to make solutions containing 0.05, 0.1, 0.2, 0.5, 1.0, or 2.0 μg/L of each analyte (**Figure 2C**). The samples were acidified with formic acid (0.6%, pH 2.1) to stabilize IXF. A small volume (100 μL of 10 μg/L) of a internal standard compound, 2,4-dichlorophenoxyacetic acid (2,4-D), was spiked into the samples for final volume and recovery correction. The molecular ion of 2,4-D exhibited high selectivity and strong signal under negative ion mode. In addition, the short half-life of 2,4-D in soils and the source of water make it extremely unlikely that it would be present in the ground water samples used in this study. Polystyrene divinylbenzene polymer SPE cartridges (200 mg Isolute Env+) were used to prepare samples for HPLC-MS. The cartridges were conditioned with 4 mL of 100% ACN followed by 4 mL of distilled deionized water. The flow rate was 2 mL/min. Sample volumes of 100 mL were passed through the cartridges at a flow rate of 4 mL/min. The cartridges were then rinsed with 3 mL of distilled water and eluted with 30 mL of 100% ACN at a flow rate of 2 mL/min. The eluates were evaporated by rotovap and further concentrated to ~50 μL with nitrogen gas and reconstituted to ~150 μL with 9:1, 1.3% formic acid/ACN (pH 2.1) solution.

HPLC-MS Conditions. A 50 μL volume of reconstituted extract was injected into a Hewlett-Packard 1100 HPLC unit coupled to a Hewlett-Packard 1100 mass selective detector (MSD). This instrument contained a binary pump, a vacuum degasser, an autosampler, a temperature-controlled column compartment, an LC-MSD API nebulizer-assisted electrospray ion source, an MS quadrupole filter and a detector. The mobile phase for HPLC consisted of a programmed mixture of water with 1.5% acetic acid and 100% methanol. These were mixed with the binary pump to produce the following mobile phase conditions: 40% methanol for 1 min followed by a ramp to 80% methanol in 8 min. The flow rate was 0.8 mL/min. The analytical column was a Betasil 4.6 × 250 mm, 5 μm C_{18} silica-based column (Phenomenex). The column temperature was maintained at 40 °C. Column effluent was directly input into the capillary of the API electrospray ion source. The API ion source was operated in the negative ion mode. Nebulizer gas (nitrogen) pressure was 30 psi, and drying gas (nitrogen) flow rate was set at 9 L/min with a temperature of 350 °C. The capillary voltage was 4000 V, and the fragment voltage was 100 V. The characteristic ions used for analysis were *m*/*z* 159 for BA and *m*/*z* 358 for IXF and DKN. These diagnostic ions were selected by injecting 50 μL of 100 μg/L standard solutions of each analyte into the HPLC-MS system.

Table 1. Average Recoveries and Range of IXF, DKN, and BA from Spiked Deionized Water Using Sequential Elution of C_{18} SPE Followed by HPLC-UV Analysis

compd	elution step	recovery (%)	range ($n = 2$)
IXF[a]	first 7 mL	81.8	12.0
DKN		84.4	10.7
BA		74.8	3.53
IXF	second 7 mL	0.00	0.00
DKN		3.91	0.41
BA		0.86	0.28
IXF	third 7 mL	0.00	0.00
DKN		1.72	0.17
BA		0.52	0.17
IXF	total 21 mL	81.8	12.0
DKN		90.0	11.3
BA		76.2	3.43

[a] 2 μg (10 μg/L × 0.2 L) of IXF, DKN, and BA was loaded onto the C_{18} SPE.

Table 2. Average Recoveries and Standard Deviations of IXF, DKN, and BA from Spiked Deionized Water Using a Single Elution of C_{18} SPE Followed by HPLC-UV Analysis

	recovery (%)		
spike concn[a] (μg/L)	IXF	DKN	BA
1.0	97.6 (13.9)[b]	73.7 (7.30)	87.5 (6.80)
5.0	77.8 (5.05)	86.3 (6.49)	90.3 (3.12)
12.5	83.2 (4.65)	88.1 (8.62)	87.9 (5.41)
mean	86.2	82.7	88.5
p value for concn effect[c]	0.800	0.130	0.119

[a] Sample volume was 200 mL, and SPE cardridges were eluted with a single elution of 21 mL of 80% ACN. [b] Mean ± standard deviation. [c] *p* values determined by analysis of variance. $n = 3$ for each concentration treatment.

RESULTS AND DISCUSSION

HPLC-UV Analysis. *Recovery from C_{18} SPE: Distilled Deionized Water*. Retention times for standard solutions of BA, DKN, and IXF using HPLC-UV were 3.2, 7.7, and 12.8 min, respectively. In general, the retention times coincide with the partition coefficients and the water solubility, that is, polarity, of the analytes (**Figure 1**). The addition of formic acid stabilized the parent IXF by preventing cleavage of the N−O bond of the isoxazole ring. Additionally, the acidification of sample solution increased the hydrophobicity of DKN and BA, which led to higher affinity for the C_{18} SPE resin, and therefore less analyte breakthrough occurred during the SPE sample loading. Under neutral conditions (pH 7), the metabolites DKN and BA are expected to be in the anionic form ($pK_a \sim 2$; personal communication from Zeneca Agrochemicals, Richmond, CA).

The results of HPLC-UV analyses for these solutions showed that 75−84% of the 2 μg (10 μg/L × 0.2 L) of IXF, DKN, or BA applied to the SPE C_{18} column was eluted with the first 7 mL of 80% ACN using the 3 × 7 mL sequential elution scheme (**Table 1**). Additional elutions did not improve the recovery of IXF, but DKN recovery was improved by ~5.6% and BA recovery by ~1.4%. Elution of the analytes with a single 21 mL fraction of ACN resulted in recoveries of 73.7−97.6% (**Table 2**). Spike concentration had no significant effect on recovery of the analytes. Because recovery was ≥80% at 12.5 μg/L; at least 2.0 μg of each analyte can be retained by the 500 mg C_{18} SPE column. It should be noted that the concentration treatment of 12.5 μg/L is higher than that anticipated to be found

Table 3. Average Recoveries and Standard Deviations of IXF, DKN, and BA from Spiked Ground Water Samples Using C_{18} SPE[a] Followed by HPLC-UV

compd[b]	recovery (%)	SD ($n = 5$)
IXF	84.8	8.29
DKN	68.1	5.24
BA	coelution	coelution

[a] SPE cartridges were eluted with a single elution of 21 mL of 80% ACN. [b] Analyte application to SPE was 1 μg/L × 0.2 L.

Table 4. Average Percent Recoveries and Range of IXF, DKN, and BA from Spiked Ground Water Samples Following Sequential Elution of C_{18} SPE[a] Followed by HPLC Analysis

spike concn (μg/L)	sample vol (mL)	recovery (%)		
		IXF[b]	DKN[c]	BA[b]
0.5	100	77.6 (1.95)[d]	65.6 (3.62)	123.4 (8.98)
1.0	100	91.3 (13.8)	62.5 (3.54)	107.6 (22.9)
0.5	200	85.8 (2.12)	69.1 (5.20)	113.9 (26.7)
1.0	200	88.94 (2.12)	77.1 (0.59)	60.6 (21.9)
mean		85.9	68.6	101.4

[a] Sequential elution entailed soaking for 30 min with 10 mL of 13% ACN followed by addition of 20 mL of 13% ACN and then a second elution with 20 mL of 80% ACN. [b] No significant effect of concentration, volume, or their interaction was observed on recovery of each analyte at the 5% level of probability using analysis of variance. [c] Significant volume effect on recovery of DKN ($p = 0.025$). [d] Mean ± range.

in actual field surface or ground water samples containing Balance and its metabolites (*13*).

Recovery from C_{18} and Polymer SPE: Ground Water. Elution of the C_{18} SPE cartridge with a single 21 mL fraction of 80% ACN resulted in an 85% recovery of IXF and a 68% recovery of DKN (**Table 3**). However, the signal of BA on the chromatogram was obscured by a large, coeluting peak of unknown origin. Sequential SPE elution, a stepwise mobile gradient, and a longer HPLC column were required to successfully isolate and quantify BA using HPLC-UV analysis (**Table 4**). Retention times using the stepwise gradient were 20.8 min for BA, 47.0 min for DKN, and 54.5 min for IXF (**Figure 4**). With sequential elution, BA was isolated with the first 30 mL of 13% ACN. The 30-min presoaking with 10 mL of 13% ACN was necessary in order to elute DKN into the first fraction along with BA. The mean HPLC-UV recoveries of IXF and its two metabolites from samples spiked at 0.5 and 1 μg/L ranged from 68.6 to 101.4% (**Table 4**). There was no significant effect of concentration, volume, or their interaction on the recovery of IXF and BA. However, the effect of sample volume has a positive effect on the recovery of DKN. This positive effect may result from the higher applied mass of DKN, which led to a larger peak for more accurate integration. Comparison of ground water and distilled deionized water showed that IXF recovery was similar, but DKN and BA were quite different (**Tables 2** and **4**). DKN recoveries were lower from ground water, suggesting that matrix interferences caused greater breakthrough or impeded elution from the SPE and HPLC columns. This was verified by the broadened peak with lower signal to noise ratio for DKN observed on the chromatograms derived from ground water samples. Conversely, BA recoveries were greater from ground water, but the variability of recovery was also much greater. In general, BA recovery from ground water was >100%, indicating that the coeluting interferences

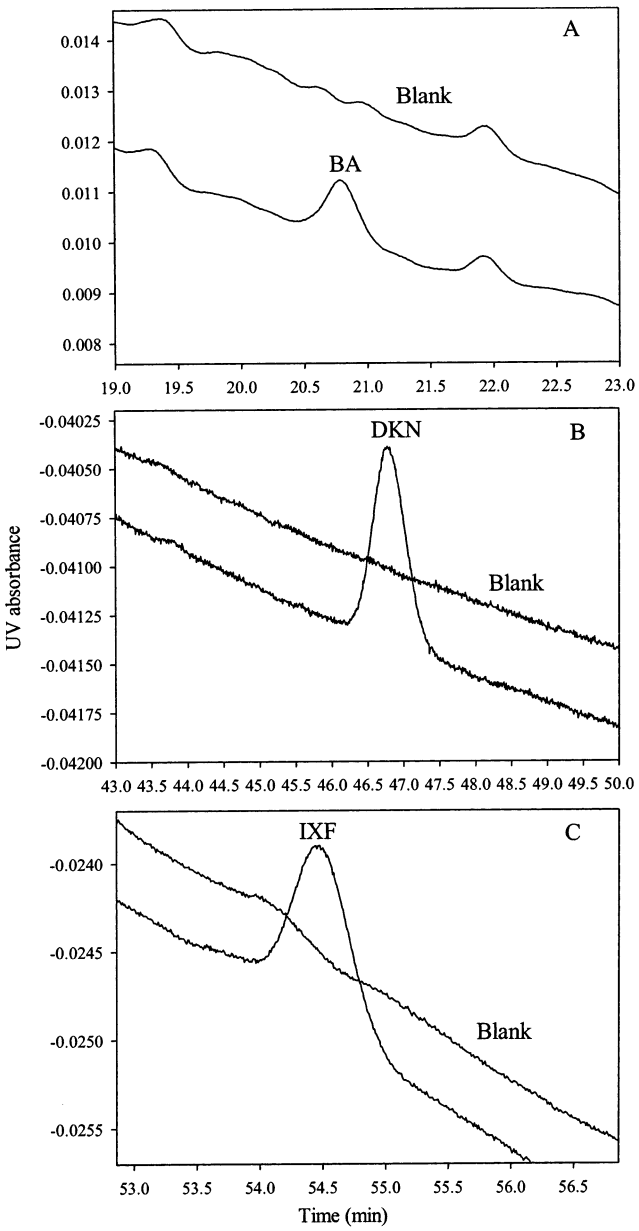

Figure 4. HPLC-UV chromatograms of blank and spiked ground water samples at 1 μg/L using sequential elution of C_{18} SPE cartridge: (A) BA; (B) DKN; (C) IXF.

during HPLC-UV analysis were resulting in an overestimation of BA recoveries.

As noted previously, BA has a unique UV absorption peak at 270 nm, but the absorbance is relatively small. Detection sensitivity was enhanced by ~5 times by measuring UV absorbance at 220 nm. Good selectivity of UV absorption by IXF at 270 nm and by DKN at 300 nm was satisfactory for quantifying the levels of each compound. Using the sequential elution scheme with samples concentrated 200-fold, the limits of quantitation are approximately 150 ng/L for IXF, 100 ng/L for DKN, and 250 ng/L for BA.

Unlike the results of C_{18} SPE, IXF and DKN were both eluted with the second fraction of 80% ACN during sequential elution of the RP-102 SPE. The recovery percentages of IXF and DKN were 81.48 ± 1.81% ($n = 2$) and 75.20 ± 15.27% ($n = 2$), respectively. It was not possible to detect BA in the first or second fraction at either 270 or 220 nm on the chromatogram due to the presence of coeluting interferences. Analysis of a

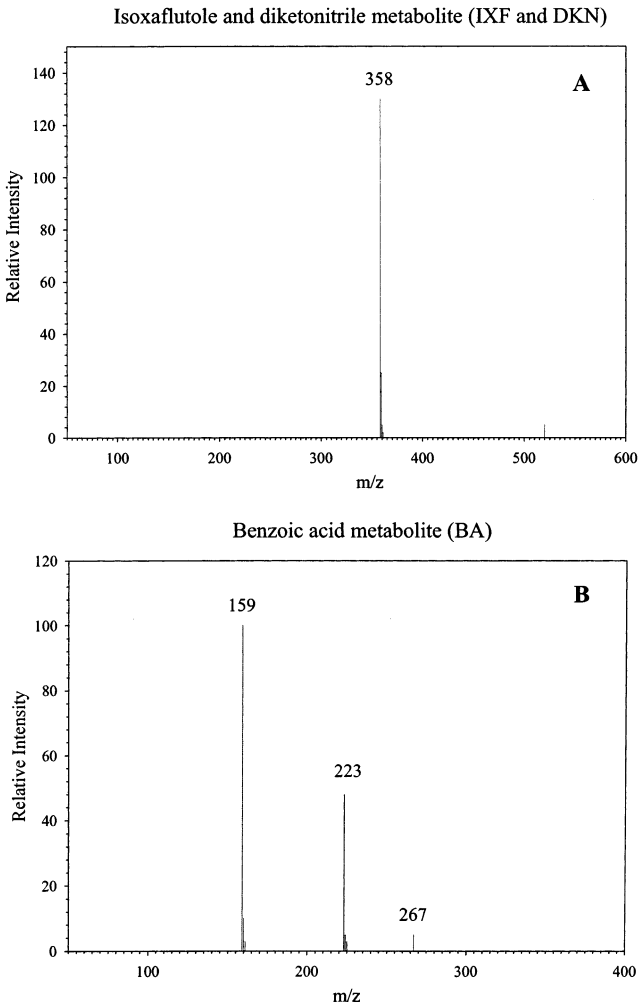

Figure 5. Full-scan mass spectra for IXF and DKN (A) and BA (B). The *m/z* of the molecular ion is 358 for both IXF and DKN.

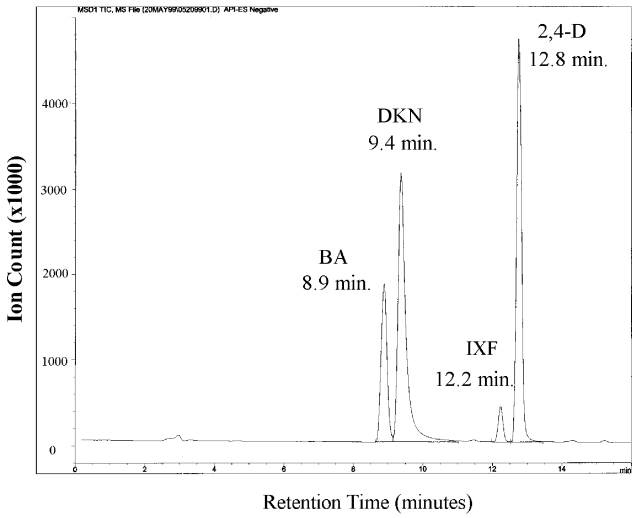

Figure 6. Total ion chromatogram of spiked IXF, DKN, and BA at 1 μg/L in ground water sample after polystyrene divinylbenzene polymer SPE.

Table 5. Average Recovery of IXF, DKN, and BA from Spiked Ground Water Samples Using Polystyrene Divinylbenzene Polymer SPE (Isolute Env+) Followed by HPLC-MS Analysis

	recovery (%)		
spike concn[a] (μg/L)	IXF	DKN	BA
0.05 ($n = 2$)	80.0 (0.0)[b] B[c]	111.0 (12.7) A	115.0 (7.1) A
0.10 ($n = 2$)	135.0 (21.2) A	110.0 (28.3) A	95.0 (35.4) A
0.20 ($n = 1$)	95.0 AB	100.0 A	110.0 A
0.50 ($n = 2$)	111.0 (9.9) AB	84.0 (2.8) A	93.0 (7.1) A
1.00 ($n = 3$)	129.3 (16.8) A	100.3 (2.3) A	102.3 (2.1) A
2.00 ($n = 2$)	111.3 (5.3) AB	100.0 (0.0) A	99.5 (0.0) A
mean	110.3	100.9	102.5
p value for concn effect	0.047	0.425	0.707

[a] Total sample volume applied was 100 mL. [b] Mean recovery ± range for $n = 2$ or standard deviation for $n = 3$. [c] Means followed by the same letter within the column do not differ significantly from each other at the 5% level of probability using Fisher's leasts ignificant difference test for concentration effect.

working standard solution of BA spiked into distilled deionized water (1 μg/L) showed that BA was eluted in the first 30 mL fraction of 13% ACN (data not shown). The polystyrene divinylbenzene SPE resin has been widely used for separation of polar compounds (*21*) and has a stronger affinity for polar molecules than the C_{18} coated silica column. Apparently, larger amounts of polar interferences were bound along with BA onto the RP-102 cartridge and then both were eluted into the first fraction during SPE.

All ground water samples displayed considerable organic interference as manifested by the broadening of peak widths and decreased signal to noise ratios. Despite this difficulty, it was possible to achieve acceptable recoveries of the IXF and DKN during single-phase elution of the C_{18} SPE cartridge and sequential elution of the RP-102 cartridge. More importantly, all analytes were successfully recovered during sequential elution of the C_{18} SPE column. During sequential elution of the C_{18} cartridge, satisfactory recovery of BA was achieved when samples were concentrated 100- and 200-fold followed by a stepwise mobile phase gradient and a longer column for HPLC analysis (**Table 4**).

HPLC-MS Analysis. *Recovery from Isolute Env+: Ground Water.* Full-scan mass spectra of IXF, DKN, and BA standards are shown in **Figure 5**. The deprotonated *m/z* 358 ion was utilized for the quantification of IXF and DKN. The predominant fragmented *m/z* 159 ion was used for quantification of BA. Retention times were 8.9 min for BA, 9.4 min for DKN, 12.2 min for IXF, and 12.8 min for the surrogate 2,4-D (**Figure 6**). The average recoveries of the three compounds during HPLC-MS from ground water samples spiked at 0.05−2 μg/L ranged from 100.9 to 110.3% (**Table 5**). With the exception of 0.1 μg/L, the ranges and standard deviations were <20%. The generally >100% recovery of IXF may result from the integration error associated with the smaller peak of IXF. Also, highly concentrated matrix in this work may have interfered with the ionization process and led to the signal enhancement. The possibility also exists that the matrix caused signal suppression of the internal standard, leading to the high recoveries of IXF. As is common in HPLC-MS (*29*), the limit of quantitation for IXF, DKN, and BA was set at the lowest spiked level, 0.05 μg/L. The molecular ion at *m/z* 358 is the deprotonated molecular ion of both IXF and DKN. Formation of the *m/z* 159 ion of BA is initiated by ejection of COO^- from the molecular ion (*m/z* 267) to form an intermediate (*m/z* 233). The further rearrangement and ejection of SO_2 from the *m/z* 233 ion results in the formation of the *m/z* 159 ion (**Figure 7**). The similar ejection of SO_2 and rearrangement reaction in RSO_2R or $ArSO_2$-OR molecules have been described by McLafferty (*30*). Because the *m/z* 358 and 159 ions were unique in the mass spectra, interferences were of no concern. The accurate quantitation of

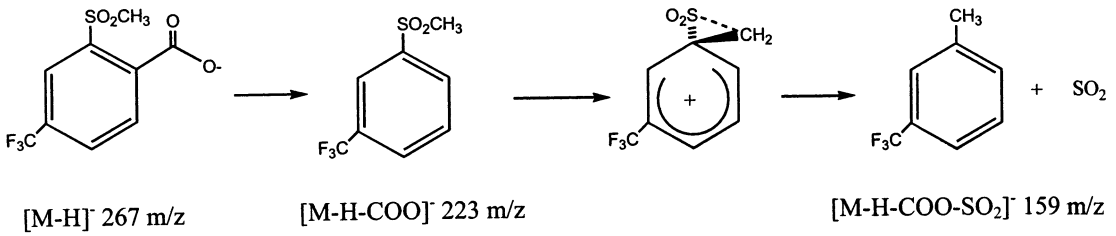

Figure 7. Formation of BA product ion *m/z* 159 during HPLC-MS using the API electrospray ion source (*30*).

these compounds by HPLC-MS, therefore, can be readily achieved. The sensitivity of the MS detector is at a minimum 80 times greater than that of the UV detector. This is based upon a comparison of the signal to noise ratios of each of the standard samples injected directly into HPLC-UV or HPLC-MS systems.

Conclusion. Solid phase extraction techniques followed by HPLC-UV and HPLC-MS can be successfully applied to quantify IXF and its metabolites, DKN and BA, in environmental water samples.

IXF, DKN, and BA can all be easily recovered from distilled deionized water samples after single-phase or sequential elution from a C_{18} SPE silica-based resin. IXF and DKN can be recovered at satisfactory levels from ground water samples during HPLC-UV after single or sequential elution of a C_{18} SPE silica-based resin. They can also be recovered after sequential elution of a polystyrene divinylbenzene polymer resin. However, HPLC-UV analysis of BA is susceptible to coeluting interferences. Only sequential elution of the C_{18} resin and a stepwise mobile phase gradient are satisfactory for the isolation and quantification of BA from ground water samples using HPLC-UV.

HPLC using an API electrospray ion source and MS detector was also successfully applied for the analysis of IXF, DKN, and BA after SPE preparation using a polystyrene divinylbenzene polymer resin. The unique product ions of Balance and its metabolites produced during HPLC-MS give the method considerably more selectivity than HPLC-UV. Additionally, the higher signal to noise ratios in the HPLC-MS method resulted in a considerably greater sensitivity than shown by HPLC-UV. Compared to the HPLC-UV method, HPLC-MS required a less difficult sample preparation scheme, and instrumental analysis time was also reduced. Overall, the polymer resin SPE combined with HPLC-MS resulted in higher recoveries and reproducibility for all analytes.

Considering the levels of Balance and its metabolites expected to be found in environmental water samples, the HPLC-MS procedures developed in this study are recommended for the analysis of IXF and its metabolites, and this method has been successfully applied to their analysis in a field lysimeter study (*7*). The results of the lysimeter study confirmed the instability of IXF and high mobility of its first degradation metabolite DKN in the field. There was only a small fraction of BA detected in the lysimeter leachate throughout the 25 days of the study.

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LITERATURE CITED

(1) Pallett, K. E.; Little, J. P.; Sheekey, M.; Veerasekaran, P. The mode of action of isoxaflutole I. Physiological effects, metabolism, and selectivity. *Pestic. Biochem. Physiol.* **1998**, *62*, 113−124.

(2) Lee, D. L.; Prisbylla, M. P.; Cromartie, T. H.; Dagarin, D. P.; Howard, S. W. The discovery and structural requirements of inhibitors of *p*-hydroxyphenylpyruvate dioxygenase. *Weed Sci.* **1997**, *45*, 601−609.

(3) Pallett, K. E. The mode of action of isoxaflutole: a case study of an emerging target site. In *Herbicides and Their Mechanisms of Action*; Cobb, A. H., Kirkwood, R. C., Eds.; Sheffield Academic Press: Sheffield, U.K., 2000; pp 215−238.

(4) Viviani, F.; Little, J. P.; Pallett, K. E. The mode of action of isoxaflutole II. Characterization of the inhibition of carrot 4-hydroxyphenylpyruvate dioxygenase by diketonitrile derivative of isoxaflutole. *Pestic. Biochem. Physiol.* **1998**, *62*, 125−134.

(5) Lazo, M.; Lopez de Medina, F.; Sardina, J. L.; Gomez-Arnau, J. *Actas-Congr., Soc. Esp. Malherbol, Lleida, Spain*; Sociedad Espanola de Maherbologia: Lleida, Spain, 1997; pp 383−387.

(6) Menendez, J.; De Prado, A.; Gomez-Arnau, J. *Actas-Congr., Soc. Esp. Malherbol, Lleida, Spain*; Sociedad Espanola de Maherbologia: Lleida, Spain, 1997; pp 89−92.

(7) Lin, C. H. Bioremdeiation Capacity of Five Forage Grasses for Atrazine, Balance (Isoxaflutole) and Nitrate Removal. Ph.D. Dissertation, University of Missouri, 2002.

(8) Grünanger, P.; Vita-Finzi, P. Chapter I: Isoxazoles. In *Isoxazoles*; Wiley-Interscience Publishers: New York, 1991; pp 1−413.

(9) Beltran, E.; Fenet, H.; Cooper, J. F.; Coste, C. M. Kinetics of abiotic hydrolysis of isoxaflutole: influence of pH and temperature in aqueous mineral buffered solutions. *J. Agric. Food Chem.* **2000**, *48*, 4399−4403.

(10) Lin, C. H.; Lerch, R. N.; Garrett, H. E.; George, M. F. *Proceedings for the International Workshop on Pesticide 2000: Harmonization of Pesticide Management, Regulation, Monitoring and Evaluation, Taichung, Taiwan*; IUPAC-TACTRI/COA: 2000; p 236.

(11) Mitra, S.; Bhowmik, P. C.; Xing, B. Sorption of isoxaflutole by five different soils varying in physical and chemical properties. *Pestic. Sci.* **1999**, *55*, 935−942.

(12) Office of Pesticide Programs. *Pesticide Fact Sheet: Isoxaflutole*; Environmental Protection Agency: Washington, DC, 1998.

(13) Office of Pesticide Program Isoxaflutole; pesticide tolerance. *Fed. Regist.* **1998**, *63*, 50773−50784.

(14) Rouchaud, J.; Neus, O.; Callens, D.; Bulcke, R. Isoxaflutole herbicide persistence and mobility in summer corn and winter wheat crops. *Bull. Environ. Contam. Toxicol.* **1998**, *60*, 577−584.

(15) Lerch, R. N.; Donald, W. W.; Li, Y. X.; Albert, E. E. Hydroxylated atrazine degradation products in small Missouri streams. *Environ. Sci. Technol.* **1995**, *29*, 2759−2768.

(16) Johnson, W. E.; Fendinger, N. J.; Plimmer, R. P. Solid phase extraction of pesticides from water: possible interferences from dissolved organic material. *Anal. Chem.* **1991**, *63*, 1510−1513.

(17) Nash, R. G. Solid-phase extraction of carbofuran, atrazine, simazine, alachlor, and cyanazine from shallow well water. *J. AOAC Int.* **1990**, *73*, 438−442.

(18) Chen, Z.; Adams, M. A. Retention behavior and simultaneous separation of carboxylic and aromatic acids using ion-exclusion chromatography. *J. Liq. Chromatogr. Relat. Technol.* **1999**, *22*, 2595−1611.
(19) Peruzzi, M.; Bartolucci, G.; Cioni, F. Determination of phenoxyalkanoic acids and other herbicides at the ng/mL level in water by solid-phase extraction with poly(divinylbenzene-*co*-*N*-vinylpyrrolidone) sorbent and high-performance liquid chromatography-diode-array detection. *J. Chromatogr.* **2000**, *867*, 169−175.
(20) Loos, R.; Niessner, R. Analysis of atrazine, terbutylazine and their N-dealkylated chloro and hydroxy metabolites by solid-phase extraction and gas chromatography−mass spectrometry and capillary electrophoresis-ultraviolet detection. *J. Chromatogr.* **1999**, *835*, 217−229.
(21) Meyer, V. R. *Practical High-Performance Liquid Chromatography*; Wiley: Chichester, U.K., 1994; p 376.
(22) Posyniak, A.; Zmudzki, J.; Ellis, R. L.; Semeniuk, S.; Niedzielska, J. Validation study for the determination of tetracycline residues in animal tissues. *J. AOAC Int.* **1999**, *82*, 862−865.
(23) Nicewonger, R. B.; Ditto, L.; Varady, L. Alternative base matrices for solid-phase quenching reagents. *Tetrahedron Lett.* **2000**, *41*, 2323−2326.
(24) Lerch, R. N.; Thurman, E. M.; Blanchard, P. E. Hydroxyatrazine in soils and sediments. *Environ. Sci. Technol.* **1984**, *18*, 2161−2168.
(25) Parriott, D. *A Pratical Guide to HPLC Detection*; Academic Press: San Diego, CA, 1993; p 293.
(26) Gaskell, S. J. Electrospray: principles and practice. *J. Mass Spectrom.* **1997**, *32*, 677−688.
(27) Niessen, W. M. A.; Greef, J. V. d. *Liquid Chromatography−Mass Spectrometry*; Dekker: Mount Laurel, NJ, 1992; p 479.
(28) U.S. Department of Agriculture. National Agricultural Statistic Service Agricultural Chemical Usage: 2000 Field Crop Summary; 2001.
(29) Snyder, L. R.; Kirkland, J. J.; Glajch, J. L. *Practical HPLC Method Development*, 2 ed.; Wiley: New York, 1997; pp 765.
(30) McLafferty, F. W. *Interpretation of Mass Spectra*; University Science Books: Mill Valley, CA, 1993; pp 135−223.

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