ELSEVIER

Journal of Chromatography A, 724 (1996) 207–217

JOURNAL OF CHROMATOGRAPHY A

Determination of carbendazim in water by high-performance immunoaffinity chromatography on-line with high-performance liquid chromatography with diode-array or mass spectrometric detection

David H. Thomas[a], Viorica Lopez-Avila[a,*], L.D. Betowski[b], Jeanette Van Emon[b]

[a]*Midwest Research Institute, California Operations, 555-C Clyde Avenue, Mountain View, CA 94043, USA*

[b]*U.S. Environmental Protection Agency, National Exposure Research Laboratory, Characterization Research Division, P.O. Box 93478, Las Vegas, NV 89193–3478, USA*

Received 2 March 1995; revised 21 August 1995; accepted 21 August 1995

Abstract

An automated method for the determination of carbendazim in water that combines high-performance immunoaffinity chromatography (HPIAC), high-performance liquid chromatography (HPLC) in the reversed-phase mode, and detection by either UV-Vis diode array detector (DAD) spectroscopy or mass spectrometry (MS) is presented here. This method allows for the on-line extraction, preconcentration, and positive confirmation of carbendazim with a throughput of one sample analyzed every 10 min. The method requires minimal manual sample pretreatment, yet it is free from coextracted interferences that often occur in solid-phase extractions based on nonspecific sorbents. The linear range of the calibration curve for 200-μl injections of carbendazim is 0.025 to 100 μg/l for HPIAC–HPLC–MS and 0.075 to 100 μg/l for HPIAC–HPLC–DAD. Because the calibration curve is mass-dependent, lower detection limits could be achieved by applying larger sample volumes to the HPIAC column. The within-day precision is ±4.5% for HPIAC–HPLC–MS and ±16% for HPIAC–HPLC–DAD for samples containing 0.1 μg/l carbendazim. The results from this method correlate well with results from an ELISA.

Keywords: Immunoaffinity columns; Carbendazim

1. Introduction

The benzimidazole fungicides are highly effective, broad-spectrum, systemic fungicides that are used to protect fruits and other produce from spoiling. Carbendazim (1H-benzimidazole-2-yl carbamic acid methyl ester) is a degradation product of several benzimidazole fungicides including benomyl, which is the active ingredient in the fungicide Benlate (DuPont Agricultural Products, Wilmington, DE, USA). Carbendazim itself is a potent fungicide, and it is relatively stable and persistent in the environment. Because of the widespread application of Benlate, about 1 million kg/yr, it is desirable to monitor carbendazim in the environment [1].

Enzyme-linked immunosorbent assay (ELISA)

*Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)00917-5

techniques are gaining acceptance as a simple, low-cost approach to environmental monitoring. In these assays, a sample containing the target analyte is incubated with a known amount of analyte-binding antibodies and a known amount of an enzyme-labeled analog of the analyte. The amount of analyte in the sample is then determined by measuring how much of the enzyme-labeled analog binds to the antibodies [2].

These ELISAs have detection limits in the μg/l range, and their results correlate well with those obtained from reference methods such as gas chromatography [3]. However, manually performed ELISAs require multiple dilutions and reagent additions that lower their precision and accuracy compared to automated instrumental techniques. The accuracy of ELISAs may be further compromised by cross-reactivity of the antibodies with compounds structurally similar to the target analyte. Many commercially available ELISAs respond to the presence of not only one but a class of compounds; the inability to differentiate among the individual compounds in a class can be a disadvantage when they exhibit very different toxicological properties. For these reasons, ELISA test kits have found a niche mainly in the rapid screening of field samples [4].

A more recent approach that incorporates many of the advantages of immunochemical techniques into a format more suitable for routine analytical laboratories is high-performance immunoaffinity chromatography (HPIAC). In HPIAC, the antibody is immobilized onto a high-performance liquid chromatographic support and used as an affinity ligand to extract the target analyte from an aqueous sample injected onto the HPIAC column. Any material not specifically recognized by the antibody passes through the column to waste, while the target analyte remains bound to the immobilized antibody until the mobile phase conditions are changed to disrupt the antibody-analyte interaction. The analyte is subsequently eluted and detected. HPIAC is fast, precise, and easily automated [5–7]. Coupling HPIAC to HPLC results in an effective two-dimensional separation that combines the selectivity of immunoaffinity extraction with the high resolving power needed to separate structurally similar compounds [8–11]. The use of a specific detector ensures the unambiguous identification of the target analyte.

This work, which is part of an ongoing evaluation of new sample preparation and analysis methods conducted by the U.S. EPA in Las Vegas, NV, describes the application of HPIAC coupled on-line with either HPLC–DAD or HPLC–MS to determine the fungicide carbendazim in water samples.

We evaluated two different support materials for the HPIAC column, and optimized the application and elution conditions for the HPIAC and HPLC columns. The HPIAC technique was characterized with respect to its speed, response, accuracy, precision, and stability. Finally, the performance of the HPIAC–HPLC–MS, HPIAC–HPLC–DAD, and ELISA techniques were compared.

2. Experimental

2.1. Reagents and materials

The anti-carbendazim monoclonal antibody (cell line 72S/10.3.9.85.7.5, Lot 2) was a gift from DuPont Agricultural Products (Wilmington, DE, USA). The IgG was purified by Protein-G chromatography and stored in phosphate-buffered saline (PBS) with 0.02% sodium azide. Alachlor, benomyl, carbaryl, carbendazim, cyanazine, and 2,4-D were obtained from ChemService (West Chester, PA, USA). Atrazine was purchased from Supelco (Bellefonte, PA, USA); metolachlor from UltraScientific (North Kingstown, RI, USA); and aldicarb, captan, and carbofuran from the U.S. EPA (Research Triangle Park, NC, USA). The Nucleosil 1000-7 silica (7-μm particle diameter, 1000-Å pore size) was purchased from Alltech Associates (Deerfield, IL, USA), and the aldehyde-activated POROS support was a gift from PerSeptive BioSystems (Cambridge, MA, USA). The γ-glycidoxypropyl trimethoxysilane, periodic acid, and sodium cyanoborohydride were purchased from Aldrich Chemical (Milwaukee, WI, USA). The horse immunoglobulin G (IgG) and Micro BCA protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL, USA). The ELISA kit was a gift from Ohmicron (Newtown, PA, USA), and was used in accordance with manufacturer instructions. All other reagents were ACS-grade or better. All solutions were prepared by using

HPLC-grade solvents and water from a Millipore Milli-Q water system (Milford, MA, USA).

2.2. Standards

Carbendazim stock standards were prepared by weighing 10 mg of neat carbendazim (98% pure) into a 100-ml volumetric flask, adding about 50 ml of methanol, sonicating the solution to dissolve the solid, and then bringing the solution to volume with methanol. An intermediate standard (1 mg/l) was prepared by diluting 1 ml of this stock standard to 100 ml with methanol. The stock standards were prepared monthly, and comparison of new with old standards indicated that they were stable for several weeks when stored at 4°C. Calibration standards were prepared as needed from these stock solutions by serial dilution with appropriate volumes of 0.1 *M* phosphate buffer.

2.3. Samples

The samples used in the spike-recovery studies were collected in clean amber glass bottles from three sites around Kansas City, MO, USA during the month of August 1994.

2.4. Instrumentation

A schematic of the chromatographic system used in this study is shown in Fig. 1. The system is comprised of a Hewlett-Packard HP 1050 quaternary pump module with an added solvent degasser and column thermostat (Hewlett-Packard Co., Palo Alto, CA, USA), and a Suprex SFE-50 syringe pump module (Suprex Corporation, Pittsburgh, PA, USA). Samples of up to 250 μl were manually injected by using a Rheodyne 7126 six-port injector with an external injection loop (Cotati, CA, USA). Larger samples, up to 40 ml, were applied directly to the HPIAC column by using channel C of the quaternary pump. The column coupling was effected by using a Valco six-port, two-position switching valve (Valco Instruments, Houston, TX, USA), controlled by a Valco microelectric actuator connected via a 24 V DPDT solenoid to the HP 1050 pump module timed-events output.

The detector was either an HP UV–Vis absorbance

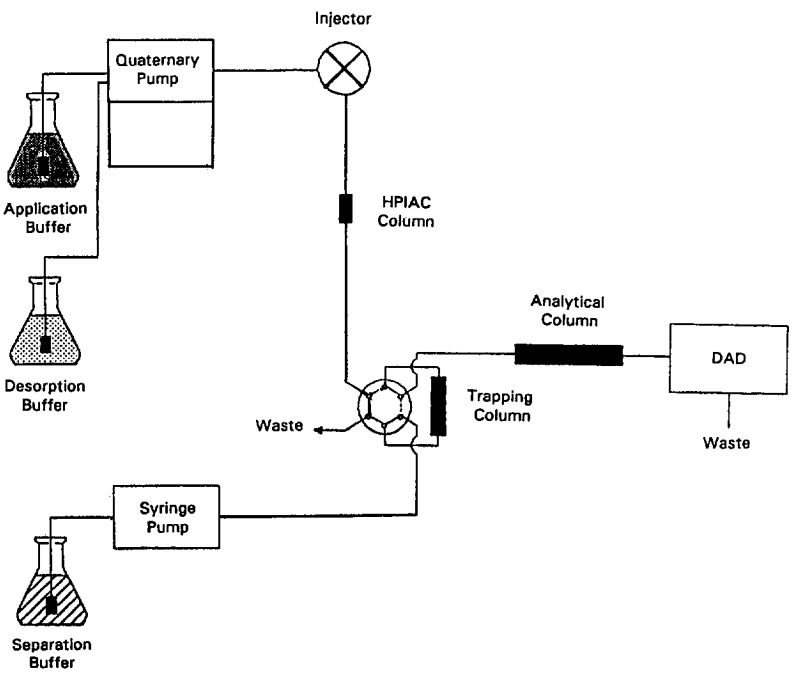

Fig. 1. Diagram of the HPIAC–HPLC–DAD system.

diode array detector (DAD), or a Fisons Quattro tandem mass spectrometer interfaced to the liquid chromatograph by an atmospheric pressure chemical ionization (APCI) interface (Fisons, Cheshire, UK). Data from the DAD were collected and analyzed on an HPLC^{3D} ChemStation chromatography data system (Hewlett-Packard). Data from the APCI-MS were collected and analyzed using MassLynx software from Fisons. The mass spectrometer was tuned for maximum sensitivity while maintaining unit resolution. The instrument operating parameters and time sequence are summarized in Table 1.

2.5. Immobilized antibody columns

In accordance with a previously published procedure [12], Nucleosil 1000-7 was converted to epoxy silica by reacting 1 g of silica with 100 μl of γ-glycidoxypropyl trimethoxysilane in 75 ml of 0.1 *M* potassium acetate solution (pH 5.5). This reaction was performed at 90°C for 6 h. The silica was then refluxed in dilute H_2SO_4 (pH 2.0) for 2 h to convert the epoxy functional group to the diol form. The diol-silica was washed with water, methanol, and diethyl ether, and dried under vacuum. A portion of this diol-silica was submitted to an iodometric titration of the diol groups [13], and determined to have a surface coverage of 43±4 μmol diol groups/g. The theoretical monolayer coverage would be about 62 μmol/g [14].

A portion of the diol-silica was converted to aldehyde silica by refluxing 0.5 g of diol-silica in 90% acetic acid with about 2 mg of periodic acid ($HIO_4 \cdot 2H_2O$) for 2 h. The aldehyde silica was washed with water, methanol, and diethyl ether, and dried under vacuum. The anti-carbendazim IgG was then immobilized onto the aldehyde silica by using the Schiff-base method [14]. A mixture of 0.5 g aldehyde-silica and 100 mg of $NaCNBH_3$ in several ml of 0.1 *M* phosphate buffer (pH 6.0) was sonicated for 15 min under aspirator vacuum to deaerate the silica. To this was added 0.9 ml of a solution containing 3 mg/ml of the IgG in PBS, and the mixture was gently shaken at 4°C. After six days, 5 ml of 1 *M* triethanolamine in 0.1 *M* phosphate buffer (pH 6.0) was added to the silica mixture and allowed to react for 2 h to block remaining aldehyde groups.

The anti-carbendazim IgG was immobilized onto

Table 1
HPLC operating conditions

Columns	HPIAC column	Trapping column	Analytical column
Material	IgG-Nucleosil	C_{18}-bonded silica	C_{18}-bonded silica
Dimensions	1.5 cm×4.6 mm I.D.	1 cm×2 mm I.D.	25 cm×2 mm I.D.
Particle size	7.5 μm	5 μm	5 μm
Back-pressure	$6.9 \cdot 10^4$ Pa at 1 ml/min	$6.9 \cdot 10^4$ Pa at 1 ml/min	$1.3 \cdot 10^7$ Pa at 0.2 ml/min
Injector	Rheodyne with 20-μl external sample loop		
Mobile phases	Sample application	Desorption	Separation
Composition	0.05 *M* Phosphate buffer (pH 7)	0.05 *M* Phosphate buffer (pH 2.5)	Methanol–water (75:25) isocratic
Flow-rate	1 ml/min	1 ml/min	0.2 ml/min
Time	4 min	5 min	Continuous
UV–Vis detector	HP Diode-array detector; 6-mm pathlength (8-μl volume) flow cell; single wavelength monitoring at 286 nm; bandwidth, 4 nm; spectra: range 190–400 nm, peak width >0.05 min.		
MS detector	Fisons Quattro MS–MS		
Interface	Fisons atmospheric pressure chemical ionization (APCI) probe		
Conditions	Ion mode: positive Bath gas: nitrogen at 450 l/h Sheath gas: nitrogen at 150 l/h Source temperature: 120°C Probe temperature: 500°C Cone voltage: 10 V SIM of $[M+H]^+$ at m/z 192; dwell time=0.25 s		

aldehyde-activated POROS support (POROS-AL) by a similar procedure. POROS-AL (0.2 g) was placed into a 10-ml test tube, 5 ml of 0.1 *M* phosphate buffer (pH 6.0) was added, and the mixture was sonicated under vacuum for 15 min. The monoclonal antibody (3 mg/ml in PBS) was thawed, 0.85 ml added into the test tube, and the mixture was gently shaken overnight at room temperature. As with the Nucleosil support, ethanolamine was added to block the remaining aldehyde groups.

A portion of the IgG-Nucleosil was washed several times with PBS, and then analyzed (Micro BCA Assay, Pierce Chemical Co.) to determine the amount of protein covalently bound to the silica by the immobilization procedure. At the same time, a portion of the IgG-POROS support was also analyzed. The analyses were performed in triplicate with horse IgG as the standard, and procedural blanks for both of the supports were included. The initial protein loading of each support, as determined by triplicate analyses, was 4.52±0.16 mg protein/g of Nucleosil support and 5.33±0.38 mg protein/g of POROS support.

2.6. Chromatography

The anti-carbendazim IgG-Nucleosil was vacuum-slurry-packed into a 1 cm×4.6 mm I.D. column of a previously published design [15]. The anti-carbendazim IgG-POROS support was downward-slurry-packed into a 1.25 cm×2.1 mm I.D. column of similar design by using a Model 1666 slurry packer (Alltech, Deerfield, IL, USA) at $2.4 \cdot 10^7$ Pa (3500 p.s.i.). When not in use, the immobilized antibody columns were stored at 4°C in 0.1 *M* phosphate buffer (pH 7.0) containing 0.02% sodium azide as biocide. All chromatography was performed at room temperature. Elution of carbendazim was monitored with the DAD at 286 nm, or with the APCI-MS detector by selected-ion monitoring of the protonated molecular ion at *m/z* 192.

Samples (up to 250 μl) were applied to the HPIAC column in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 0.5 ml/min. At 4 min after injection, the quaternary pump was switched to channel B to desorb analytes from the HPIAC column with 0.05 *M* phosphate buffer (pH 2.5) flowing at 1 ml/min. Simultaneously, the switching valve was actuated to couple the HPIAC column to the 1 cm×2.1 mm I.D. C_{18}-bonded silica trapping column (Upchurch Scientific, Oak Harbor, WA, USA), which trapped the desorbed carbendazim. After 5 min of desorption, the switching valve was returned to its original position to recouple the trapping column to the 25 cm×2.1 mm I.D. Primesphere C_{18}-bonded silica analytical column (Phenomenex, Torrance, CA, USA). This caused a 70:30 (v/v) mixture of methanol and water, pumped by the syringe pump at a flow-rate of 0.2 ml/min, to elute the analytes from the trapping column and separate them on the analytical column. During this step, 0.1 *M* phosphate buffer (pH 7.0) was applied to the HPIAC column to regenerate the immobilized antibodies prior to the next sample cycle.

The initial binding capacity of the IgG-POROS and IgG-Nucleosil HPIAC columns was estimated by making 20 injections of 20 μl of a 1 mg/l solution of carbendazim and observing when breakthrough of carbendazim from the saturated IgG occurred.

3. Results and discussion

3.1. Initial design and optimization of system

Table 2 summarizes the initial binding properties of the immobilized anti-carbendazim IgG supports (IgG-POROS and IgG-Nucleosil) used in this work. Based on these data, the total binding capacity of the immunoaffinity columns was determined to be 160 ng of carbendazim for the IgG-Nucleosil support and 60 ng of carbendazim for the IgG-POROS support. Under the conditions used in this study, it was found that a 200-μl injection of sample was sufficient to allow quantitation of carbendazim down to a concentration of about 0.1 μg/l. For a 200-μl sample injection, the IgG-Nucleosil column had sufficient capacity to handle carbendazim concentrations up to 0.8 mg/l, and the IgG-POROS column could bind up to 0.3 mg/l. At carbendazim levels of 10 μg/l, less than 3% of the column binding capacity was used per analysis.

3.2. Evaluation of IgG-POROS support

First, we evaluated the nonspecific binding of the IgG-POROS support by applying a pesticide mixture containing alachlor, aldicarb, atrazine, benomyl,

Table 2
Initial properties of the anti-carbendazim immunoaffinity supports

Property	IgG-POROS	IgG-Nucleosil 1000-7
Antibody immobilized (nmol antibody/g of support)[b]	35±2[a]	30±1[a]
Antibody coverage[c] (monolayers)	–	0.1±0.003[a]
Binding capacity (nmol carbendazim/g of support)	15.7	11.2±9[a]
Specific activity (nmol carbendazim/nmol Ab)	0.45	0.37
Amount of support (g/column)[d]	0.02	0.075

[a] Mean±S.D. for triplicate determinations.
[b] Determined by using a molecular mass of 150 000 g/mol for mouse IgG.
[c] Calculated by using a surface area of 25 m^2/g for Nucleosil 1000-7 and a Stoke's diameter of 100 Å for IgG.The surface area of the POROS support was unknown.
[d] Estimated using a packing density of 0.45 g/ml.

captan, carbaryl, carbendazim, carbofuran, cyanazine, metolachlor, and 2,4-D to the HPIAC column containing IgG-POROS, using 0.1 *M* phosphate buffer (pH 7.0) as the application mobile phase. We found that under these conditions, the IgG-POROS support extracted several pesticides other than carbendazim. This was clearly shown by the appearance of several peaks in the chromatogram, but no attempt was made to identify the peaks other than that from carbendazim.

Next, we verified that the binding of these compounds was truly nonspecific, and not the result of cross-reactivity with the immobilized antibody. This was done by repeating the injections of the pesticide mixture, while using 0.1 *M* phosphate buffer (pH 2.5) as the application mobile phase. At low pH, the IgG is not expected to actively bind carbendazim or any other specifically recognized analyte. However, peaks from several strongly retained compounds still appeared in the chromatograms.

One possible explanation for the observed retention of these compounds by the support is that the polymeric matrix of the POROS support (polystyrene–polydivinyl benzene) interacts with aromatic groups of the applied pesticides. Because carbendazim also has an aromatic group, we wondered if the binding of carbendazim to the IgG-POROS support was also nonspecific. We examined the binding of carbendazim by making sequential injections of carbendazim onto the IgG-POROS column, while directly monitoring the nonretained fraction from each injection. The results (not shown) confirmed that carbendazim, although showing some nonspecific retention, is mostly bound specifically. The first few injections of carbendazim were almost completely retained by the IgG-POROS column (i.e., no noticeable nonretained peak). Subsequent injections of carbendazim showed larger and larger nonretained peaks until a plateau was reached; this is characteristic of sequential injections of analyte to an affinity column. As the antibody binding sites become saturated by analyte, more of each injection passes through nonretained. When saturation is complete, no more analyte binds specifically, and the entire injected amount, minus the nonspecifically bound, passes through.

Once we knew that the IgG-POROS support binds carbendazim specifically, but also retains carbendazim and other compounds nonspecifically, we attempted to minimize the nonspecific retention by adding 1 to 5% ethanol to the application mobile phase; however, this did not result in a significant reduction of nonspecific retention, so we made no further attempts to evaluate the IgG-POROS support.

3.3. Evaluation of IgG-Nucleosil support

Next, we evaluated the extent of nonspecific binding to the IgG-Nucleosil support by injecting onto it the same mixed-pesticide standard used with the IgG-POROS support. In contrast to the IgG-POROS support, the IgG-Nucleosil support demon-

strated virtually no nonspecific binding to the components of this mixture (Fig. 2). Injections were made of the mixed-pesticide standard onto the trapping column alone, to obtain the chromatogram for the mixture (Fig. 2a); then onto the HPIAC column using the column-switching technique (Fig. 2b). Because the IgG-Nucleosil support exhibited such low nonspecific binding, we chose to use this support for the HPIAC–HPLC–MS system.

A second factor important to consider is the rate at which injected carbendazim binds to the immobilized antibody support. This was examined by injecting 20 μl of a 1000 μg/l carbendazim solution onto the HPIAC–HPLC–DAD system at application flow-rates ranging from 0.05 to 10 ml/min. The percent of injected carbendazim bound at each application flow-rate was determined by comparing the peak areas obtained under these conditions with the peak areas obtained when the same amount of carbendazim was injected directly onto the trapping column (bypassing the HPIAC column), then eluted through the analytical column. As shown in Fig. 3, the extraction efficiency decreases from about 75 to 60% as the application flow-rate is increased from 0.25 to 10 ml/min. This decrease in extraction efficiency as the application flow-rate increases is consistent with previous kinetic studies performed with other types of high-performance affinity columns [5,16]. At

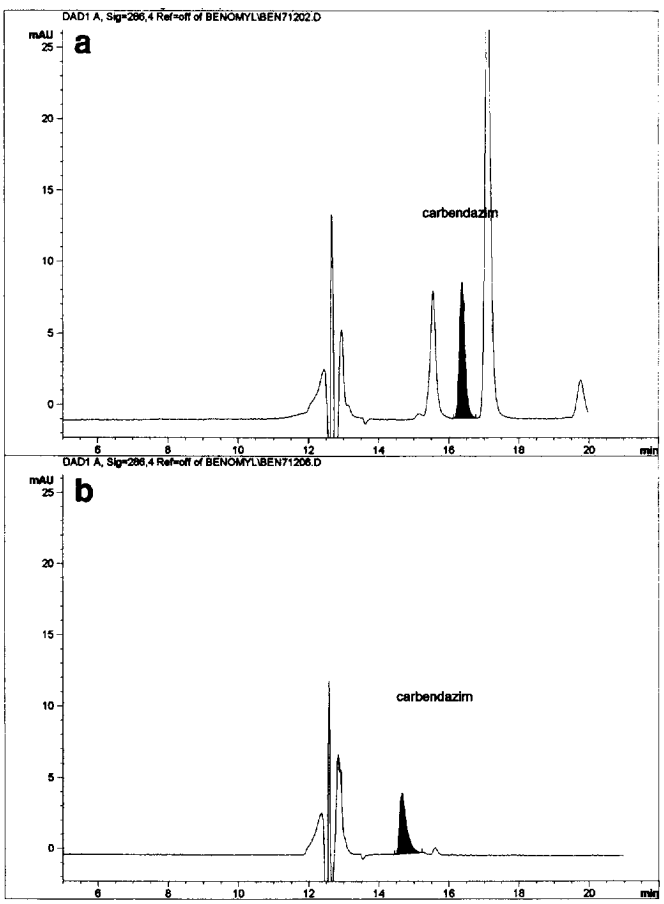

Fig. 2. (a) Pesticide mixture containing carbendazim (20 μl; concentration 10 μg/ml) injected into the trapping column with phosphate buffer (pH 2.5) and eluted with 65% methanol. (b) Pesticide mixture containing carbendazim (20 μl; concentration 10 μg/ml) injected into the IgG-Nucleosil support in phosphate buffer (pH 7.0), desorbed from the HPIAC column onto the trapping column with phosphate buffer (pH 2.5), and eluted with 65% methanol.

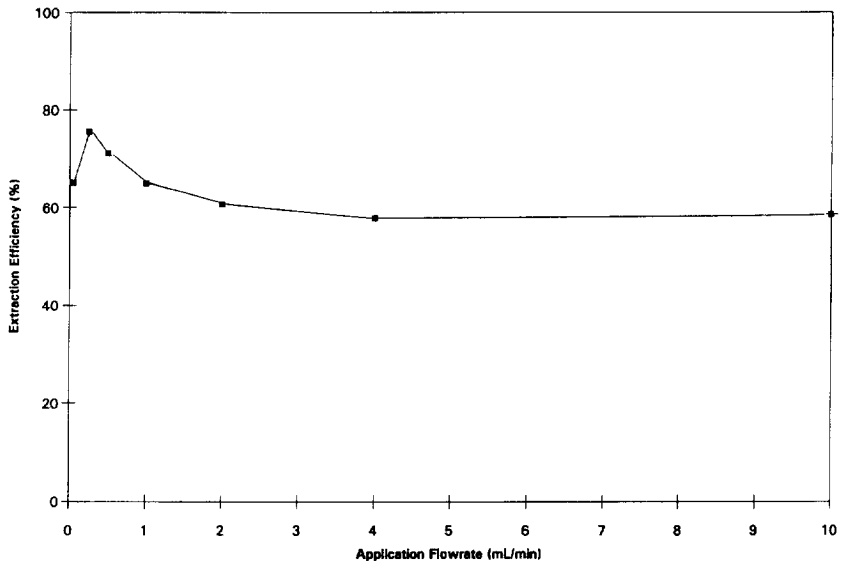

Fig. 3. Extraction efficiency as a function of the application flow-rate.

higher flow-rates, the shorter residence time of the analyte in the column does not allow as much analyte to bind to the immobilized antibody. An application flow-rate of 0.5 ml/min was selected for all further work. At this application flow-rate, about 75% of the carbendazim is extracted from the samples at carbendazim levels of 1000 μg/l or less.

The amount of time allotted to desorb carbendazim from the HPIAC column was also considered in optimizing the system. It is desirable to fully desorb the analyte from the HPIAC column, because this results in the maximum peak area and sensitivity. Furthermore, incomplete desorption of analyte from the HPIAC column will lead to carry-over from one sample to the next. However, the precolumn used to trap the desorbed analyte has a limited capacity to retain the analyte. As longer desorption times are used, the analyte will eventually elute from the trapping column, and the peak area will decrease.

The amount of time allotted to desorb carbendazim from the HPIAC column was optimized by injecting carbendazim onto the HPIAC–HPLC system, desorbing the HPIAC column at 1 ml/min for a certain length of time, and measuring the peak area of carbendazim obtained. This was repeated for several cycles, with each one allowing more time for desorbing the HPIAC column. The results are shown in Fig. 4. The maximum signal is obtained after 5 to 6 min of desorbing from the HPIAC column. At less than 5 min, the analyte has not been completely desorbed from the HPIAC column. At more than 6 min, the analyte is eluting from the trapping column. We selected a desorption time of 5 min for subsequent experiments.

3.4. Evaluation of HPIAC on-line with HPLC–DAD or HPLC–MS

Calibration curves were obtained for carbendazim by using both the HPIAC–HPLC–DAD and the HPIAC–HPLC–MS systems. The parameters of the calibration curves are summarized in Table 3. The linear range of the carbendazim curve extends from 0.075 to 100 μg/l for the HPLC–DAD technique, and from 0.025 to 100 μg/l for the HPLC–MS technique. The correlation coefficients are >0.998 over the linear portion of each curve. A plateau in the calibration curve at high carbendazim levels (data not shown) begins as the amount of injected carbendazim approaches the number of available antibody binding sites, and is not dependent on the detector used.

An important characteristic of the HPIAC method is that the response of the calibration curve is

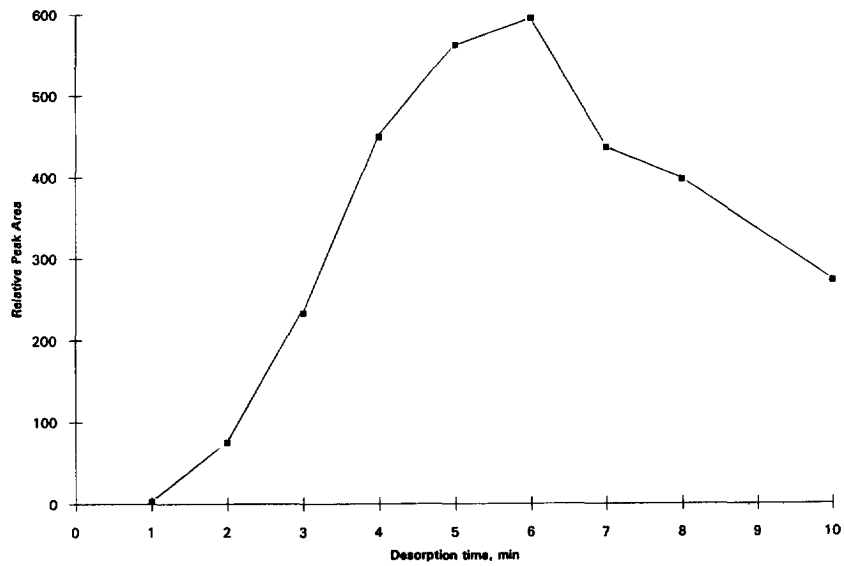

Fig. 4. Optimization of the desorption step in the HPIAC–HPLC technique. Experimental conditions are given in the text.

dependent on the mass of analyte applied, rather than the concentration of analyte applied. This is true as long as analyte dissociation from the immobilized antibody is negligible during the application process, which is often the case for immobilized antibody–antigen interactions [17]. We confirmed this for the present system by applying to the HPIAC–HPLC–MS system sample volumes ranging from 1 to 40 ml (carbendazim concentration was kept at 0.1 μg/l). The response of the system (peak area) increases linearly with the mass of carbendazim over the entire range, with the best-fit line ($n=7$, data not shown) characterized by a correlation coefficient (R^2) of 0.997, a slope of 130 and a y-intercept of 46 800. Thus, samples too dilute or too concentrated to fall within the linear range can be reanalyzed simply by applying more or less sample, as needed. An advantage of the serial processing mode used in chromatographic immunoassays, versus the parallel mode used in conventional immunoassays, is that such samples can be rerun immediately, without the need to rerun a whole new set of standards and controls.

As can be seen in Table 3, the APCI-MS detector provides approximately 3 times better sensitivity for carbendazim than the DAD, and 4 times better sensitivity than ELISA. Carbendazim is well-suited to detection by APCI-MS because it is easily protonated, somewhat volatile, and undergoes little frag-

Table 3
Method performance for HPIAC–HPLC–MS, HPIAC–HPLC–DAD, and ELISA determination of carbendazim

	HPIAC–HPLC–MS	HPIAC–HPLC–DAD	ELISA[a]
Limit of detection (μg/l)[b]	0.025	0.075	0.10
Linear range (μg/l)[c]	0.025–100	0.075–100	0.25–5.0
Correlation coefficient	0.998	0.999	0.990
Precision (%R.S.D.)[d]	4.5	16	10

[a] ELISA method performance reported by Ohmicron Corporation.
[b] Limit of detection at a signal-to-noise ratio of 3 for a sample size of 200 μl.
[c] The portion of the calibration curve showing a best-fit line with $r^2 \geq 0.998$.
[d] The number of determinations was 13 for HPIAC–HPLC–MS and 10 for HPIAC–HPLC–DAD. The concentration of carbendazim in the spiked reagent water samples was 0.1 μg/l.

mentation under the "soft" ionization that occurs in the APCI source. For compounds that have either a better chromophore or less favorable behavior in the APCI source, the DAD detector might provide greater sensitivity. In any case, because the HPIAC technique allows analyte preconcentration from variable sample volumes, the sensitivity of the detector may be less critical than its cost or ease of operation.

In addition, both the APCI-MS and the DAD detectors provide a degree of specificity beyond that of traditional ELISA methods. The APCI-MS detector provides confirmation of the analyte by its mass-to-charge ratio and fragmentation pattern, and the DAD provides confirmation of the analyte by its UV-Vis absorbance spectrum. The use of either detector to provide positive identification might be useful especially in a multi-analyte method, or if more non-specific binding of matrix compounds to the support were occurring.

The accuracy of the HPIAC–HPLC technique was evaluated by comparing it with an enzyme-linked immunosorbent assay (ELISA). Samples of reagent water, pond water, creek water, and water from an outdoor fountain were collected, and portions of each were spiked with carbendazim at 0.1, 0.5 and 1.0 μg/l. The spiked and unspiked samples were then divided, and one portion was analyzed by HPIAC–HPLC–MS or HPIAC–HPLC–DAD, while the other was analyzed by ELISA. The level of carbendazim measured in the spiked sample was compared to that in the unspiked sample to determine recovery of the added carbendazim (Table 4). Our data indicate that the HPIAC-based techniques provide results similar to ELISA over the concentration range examined.

The within-day precision of the HPIAC–HPLC technique was evaluated by making 10 injections of a spiked reagent water sample. A precision of ±4.0% R.S.D. was obtained at a carbendazim concentration of 0.1 μg/l for the HPIAC–HPLC–MS system, and 16% R.S.D. for the HPIAC–HPLC–DAD.

The IgG-Nucleosil column has been used for over two months and for more than 200 injection and desorption cycles. We observed, but did not quantitate, a gradual decrease in the activity of immobilized antibody over this time. Loss of binding capacity was not a problem because the HPIAC column originally contained a large excess of binding sites relative to the amount of carbendazim injected. Furthermore, adjustments for small decreases in activity were made automatically whenever a standard curve was prepared.

Table 4
Average recoveries and %R.S.D.s for various water samples analyzed by HPIAC–HPLC–MS, HPIAC–HPLC–DAD, and ELISA[a]

Matrix	Spike Level (μg/l)	HPIAC–HPLC–MS		HPIAC–HPLC–DAD		ELISA	
		Average recovery	%R.S.D.	Average recovery	%R.S.D.	Average recovery	%R.S.D.
Reagent water	0.1	88.7	4.0			ND[b]	
	0.5			106	2.2	126	5.8
	1.0			110	1.5	137	4.1
Pond water	0.1	87.1	0.2				
	0.5			115	2.5	102	27
	1.0			106	6.4	136	2.7
Creek water	0.1	92.0	3.4				
	0.5			113	4.5	124	6.0
	1.0			116	3.1	124	2.7
Fountain water	0.1	131	1.8				
	0.5			107	1.6	138	7.2
	1.0			118	0.5	125	2.0

[a] The number of determinations was three.
[b] ND=not detected.

4. Conclusions

This work demonstrates the feasibility of tandem HPIAC–HPLC for determining carbendazim in water. Both HPIAC–HPLC–MS and HPIAC–HPLC–DAD demonstrate high specificity in detection, and also in the immunoaffinity extraction step when appropriate chromatographic supports are used. Chromatographic automation using conventional HPLC equipment provides a throughput of one sample every 10 min, accuracy and precision that compare favorably to reference methods based on ELISA, and low detection limits simply by preconcentrating larger sample volumes on–line.

The use of HPIAC–HPLC for environmental testing yields some advantages over traditional ELISA formats. Serial sample introduction in the chromatographic system, instead of the parallel processing used in most manual or semi-automated immunoassays, allows automated quality control and reanalysis of samples. Most importantly, the chromatographic method will be preferred whenever the goal is to quantitate individual members of a related class of compounds, because the problem of cross-reactivity is solved by the subsequent reversed-phase separation of the compounds.

5. Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development (ORD), funded and collaborated in the research described here. It has been subjected to the Agency's peer review and has been approved as an EPA publication. Neither the EPA nor ORD endorses or recommends any trade names or commercial products mentioned in this article; they are noted solely for the purpose of description and clarification.

Acknowledgments

The authors wish to thank David Johnson of E.I. duPont de Nemours and Company for providing the monoclonal antibodies used in this study, and John Hoffman of PerSeptive Biosystems, for providing the POROS chromatographic support. David Robaugh, of Midwest Research Institute, provided technical support for the HPLC–MS work. Chatan Charan of Midwest Research Institute performed the ELISA determinations for the split-sample comparison.

References

[1] R.P. Singh and M. Chiba, J. Chromatogr., 643 (1993) 249.
[2] J. Van Emon and V. Lopez-Avila, Anal. Chem., 64 (1992) 78A.
[3] E.M. Thurman, M. Meyer, C. Perry and P. Schwab, Anal. Chem., 62 (1990) 2043.
[4] K. Goh, J. Hernandez, S.J. Powell, C. Garretson, J. Troiano, M. Ray and C. Greene, Bull. Environ. Contam. Toxicol., 46 (1991) 30.
[5] U. De Alwis and G.S. Wilson, Anal. Chem., 59 (1987) 2786.
[6] D.S. Hage and R.R. Walters, J. Chromatogr., 386 (1987) 37.
[7] S. Ohlson, B.-M. Gudmundsson, P. Wikstrom and P.-O. Larsson, Clin. Chem., 34 (1988) 2039.
[8] M. de Frutos and F.E. Regnier, Anal. Chem., 65 (1993) 17A.
[9] G.S. Rule, A.V. Mordehai and J. Henion, Anal. Chem., 66 (1994) 230.
[10] A. Farjam, G.J. De Jong, R.W. Frei, U.A.Th. Brinkman, W. Haasnoot, A.R.M. Hamers, R. Schilt and F.A. Huff, J. Chromatogr., 452 (1988) 419.
[11] D.H. Thomas, M. Beck-Westermeyer and D.S. Hage, Anal. Chem., 65 (1994) 3823.
[12] R.R. Walters, J. Chromatogr., 249 (1982) 19.
[13] S. Siggia and J.G. Hanna, in Qualitative Organic Analysis, Wiley, New York, 1979, p. 42.
[14] P.-O. Larsson, Methods in Enzymology, 104 (1984) 212.
[15] R.R. Walters, Anal. Chem., 55 (1983) 591.
[16] D.S. Hage and R.R. Walters, J. Chromatogr., 436 (1988) 111.
[17] S.A. Cassidy, L.A. Janis and F.E. Regnier, Anal. Chem., 64 (1992) 1973.