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Environmental Analysis by On-Line Immunoextraction and Reversed-Phase Liquid Chromatography: Optimization of the Immunoextraction/RPLC Interface

MARY ANNE NELSON, EFTHIMIA PAPASTAVROS, MAUD DODLINGER, AND DAVID S. HAGE*

Chemistry Department, University of Nebraska—Lincoln, Lincoln, Nebraska 68588-0304

The use of antibodies in HPLC columns for on-line immunoextraction combined with reversed-phase liquid chromatography (RPLC) is of growing interest in environmental and agricultural analysis. This technique is typically performed by using a small RPLC precolumn to capture and concentrate analytes as they elute from the immunoextraction column; however, there is little information on the conditions required for optimizing this interface. This study examined the behavior of this interface by using 2,4-dichlorophenoxyacetic acid (2,4-D) and related herbicides as model analytes. It was found that analyte dissociation from immunoextraction columns followed first-order decay and that the elution of these analytes through the immunoextraction/RPLC interface gave an exponentially modified Gaussian profile. Computer simulations were used to see how analyte elution through the interface changed with different dissociation and retention conditions. Several guidelines were developed from this work that could be used for developing and optimizing on-line immunoextraction/RPLC systems for other chemicals of environmental or agricultural interest.

KEYWORDS: Immunoextraction; immunoaffinity chromatography; reversed-phase liquid chromatography; on-line extraction; 2,4-dichlorophenyoxyacetic acid; 2,4-D; chlorophenoxyacetic acid herbicides

INTRODUCTION

There has been growing interest in the environmental and agricultural sciences in using multidimensional techniques for the separation of chemicals. One example of such a method is on-line immunoextraction coupled with reversed-phase liquid chromatography (i.e., on-line immunoextraction/RPLC) (see examples in refs *1*−*8* and reviews in refs *9*−*11*). Immunoextraction is a method in which antibodies are covalently immobilized or adsorbed onto a chromatographic support such as silica for use in isolating a specific analyte or group of related compounds from a sample. The selectivity and strong binding of antibodies have made immunoextraction attractive as an on-line tool for the removal and concentration of chemicals from many types of complex samples. When immunoextraction is combined with RPLC, it also becomes possible to separate and analyze structurally similar chemicals that all cross-react with the given antibodies and bind to the same immunoextraction column (*6*−*8*). As shown in **Table 1**, these properties have made on-line immunoextraction/RPLC a valuable tool in many environmental and agricultural applications, including samples that range from serum, urine, and water to extracts or portions of food, sediments, and sludge (*9*−*11*).

Most applications of on-line immunoextraction/RPLC employ three columns: a column containing immobilized antibodies for immunoextraction, a small RPLC precolumn, and a RPLC analytical column. **Figure 1** shows the general design of such a system. As already indicated, the immunoextraction column is used to isolate the desired analytes from the sample, and the RPLC analytical column is used to separate these analytes from one another. The purpose of the intermediate RPLC precolumn is to act as an interface between the other two columns. This precolumn captures and concentrates analytes as they dissociate from the immunoextraction column in the presence of an aqueous elution buffer. (Note: a pH 2.5 phosphate buffer was used as the elution buffer in this particular study because it released the analytes from the immunoextraction column while also acting as a weak mobile phase for the RPLC precolumn; refs *9*−*11* describe other buffers or solvents that can be employed for this purpose.) The analytes that are captured by the RPLC precolumn are then later passed as a narrow plug through this precolumn and onto a RPLC analytical column by applying a mobile phase that contains some organic solvent (i.e., a strong mobile phase for a RPLC column) (*6*).

Although the interface between the immunoextraction column and RPLC columns is an important feature in getting this type of system to work properly, there is little information on the behavior that would be expected for such a device. Due to the wide range of samples and analytes that might be encountered in environmental or agricultural research (as illustrated in **Table 1**), such information would be quite valuable in adapting on-

* Author to whom correspondence should be addressed [e-mail dhage@unlserve.unl.edu; telephone (402) 472-2744; fax (402) 472-9402].

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Table 1. Reported Application of On-Line Immunoextraction and RPLC in the Analysis of Environmental and Agricultural Samples[a]

analyte	detection method	sample	pretreatment before immunoextraction
aflatoxin M1	fluorescence	whole milk	on-line dialysis
aflatoxins	absorbance	milk , serum, urine	preparative chromatography and concentration
atrazine and triazine herbicides	absorbance, APCI-MS	sediments, water	minimal pretreatment
atrazine metabolites	absorbance	river water, groundwater	minimal or no pretreatment
benzidine and related azo dyes	diode array	surface water, industrial effluents	filtration, addition of phosphate buffer
carbendazim	diode array, API-MS	environmental water, soil extracts	minimal for water, liquid–solid extraction for soil
carbofuran	API-MS	surface runoff water, crude potato extract	vacuum filtration of water, acid digest of potato, and filtration
chlorophenoxyacetic acids	absorbance	groundwater, surface water	filtration
E. coli	fluorescence	food samples	incubation, addition of fluorescent reagent, homogenization, dilution
isoproturon	diode array	water, soil	solvent extraction for soil samples
17β- and 17α,19-nortestosterone	absorbance	urine, bile, tissue	chromatography, incubation, extraction
phenylurea pesicides	diode array, APCI-MS	soil, sand, sediments, water, seawater	sieving, preconcentration, extraction, filtration
polycyclic aromatic hydrocarbons	fluorescence, diode array	surface water, industrial effluent, waste waters; environmental sediments, sludge	minimal pretreatment, except for extraction for sediments/sludge

[a] This list was compiled from refs *9–11.*

line immunoextraction and RPLC for new applications in these fields. The goal of this study was to obtain a better understanding of this interface, particularly with regard to environmental and agricultural applications, through the use of well-characterized analytes and chromatographic theory. The analytes used in this study were 2,4-dichlorophenoxyacetic acid (2,4-D) and related herbicides (see **Figure 2**). Information was first obtained on the dissociation rates of these analytes as they were eluted from an immunoextraction column containing anti-2,4-D antibodies. Separate data were acquired on the retention of these analytes on a RPLC precolumn. This information was then combined and used with computer modeling to study the elution of such compounds in the interface of an on-line immunoextraction/RPLC system. The results of this study were then examined to provide general guidelines that can be utilized in developing and optimizing similar systems for other analytes of interest in environmental and agricultural samples.

MATERIALS AND METHODS

Reagents. E2/G2 monoclonal anti-2,4-D antibodies (described in ref *12*) were obtained from the Vet Research Center in Brno (Czech Republic). HPLC-grade Nucleosil Si-1000 (7 μm particle diameter, 1000 Å pore size) for preparation of the immunoextraction column was obtained from P. J. Cobert (St. Louis, MO). The Platinum Extended Polar Selectivity (Platinum EPS) C_{18} silica (3 μm particle diameter, 300 Å pore size) for the RPLC precolumn was purchased from Alltech (Deerfield, IL). The 2,4-D, 2,4-dichlorophenoxyacetic methyl ester (2,4-D,Me), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 4-chloro-2-methylphenoxyacetic acid (MCPA), and 4-chlorophenoxyacetic acid (4-CPA) were from Sigma Aldrich (St. Louis, MO). When immobilized, the E2/G2 anti-2,4-D antibodies had the following association equilibrium constants and cross-reactivities at pH 7.0 and the application conditions used in this study: 7.6×10^6 M^{-1} for 2,4-D; 1.6×10^6 M^{-1} for 2,4-D,Me; and 1.2×10^6 M^{-1} for 2,4,5-T (*13*); similar results for the soluble form of these antibodies can be found in ref *12*. Reagents for the micro bicinchoninic acid (BCA) assay were from Pierce (Rockford, IL). All aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA). Other reagents were of the highest grades available.

Apparatus. The chromatographic system consisted of 10-port and 6-port Rheodyne LabPro electronically actuated valves from Alltech, 2 Knauer MicroStar Pumps, and a Knauer K-2500 UV−vis detector from Sonntek (Upper Saddle River, NJ). Data acquisition was performed using an SCB-68 NI shielded interface and a 16E series PCMCIA card from National Instruments (Austin, TX). Data were collected using a Gateway Solo 2500 laptop computer (Poway, CA) and were analyzed with a spreadsheet prepared using Excel (Microsoft, Redmond, WA). An Excel spreadsheet was also used to create a computer model of the retention of analytes on the RPLC precolumn after their elution from the immunoextraction column. A detailed description of this model and spreadsheet can be found in ref *13*.

Column Preparation. The diol-bonded silica used for antibody immobilization was prepared from Nucleosil Si-1000 according to a previous method (*14*). The coverage of diol groups on this support was found by capillary electrophoresis (*15*) to be 38 (±7) μmol of diol groups/g of silica. The E2/G2 monoclonal anti-2,4-D antibodies were covalently coupled to this support by the Schiff base method (*16*), where immobilization was carried out at 4 °C for 3 days using a 5 mL slurry that contained 1 mg of antibodies per 50 mg silica. After the immobilization step had been completed, the resulting immunoextraction support was washed several times with pH 7.0, 0.10 M potassium phosphate buffer and stored in this buffer at 4 °C until use. A small portion of the immunoextraction support was dried under vacuum at room temperature and assayed for its protein content by a micro BCA protein assay, using rabbit immunoglobulin G (Sigma Aldrich) as the standard and diol-bonded silica as the blank. The protein coverage of this particular immunoextraction support, as determined in triplicate, was 15.9 (±0.2) mg of antibodies/g of silica. The typical batch-to-batch variation for the given immobilization method was <10−20%.

The immunoextraction support was packed into a 1 cm × 2.1 mm i.d. stainless steel column according to an earlier method (*17*), employing a pressure of 3000 psi applied for 30 min and using pH 7.0, 0.10 M potassium phosphate buffer as the packing solution. This immunoextraction column had an initial binding capacity of 2.7 (±0.1) $\times 10^{-10}$ mol of 2,4-D, or 1.2 (±0.1) $\times 10^{-8}$ mol of 2,4-D/g of silica. A control column of equal dimensions was packed by the same technique; this control column contained an inert support that was prepared from Nucleosil Si-1000 in the same manner as the immunoextraction support but with no antibodies being added to the silica during the immobilization step. (Note: a control column containing immobilized species specific IgG could also be used for this purpose.) When not in use, these columns were stored at 4 °C in pH 7.0, 0.10 M potassium phosphate buffer.

Chromatographic Studies. All chromatographic studies in this paper were performed at 25 (±1) °C. The application buffer for the immunoextraction column was pH 7.0, 0.10 M potassium phosphate buffer, and the elution buffer for this column was pH 2.5, 0.10 M potassium phosphate buffer. The elution of 2,4-D and all related herbicides from the immunoextraction and RPLC columns was monitored by an on-line absorbance detector set at 223 nm.

The dissociation rate constants for analytes from the immunoextraction column were measured by first applying to the immunoextraction column a 50 ppb solution of the desired analyte in the application buffer for 4−5 min at 0.4−0.5 mL/min. A valve change was then used to pass the pH 2.5 elution buffer through this column at 0.2−0.5 mL/min, with the elution of analytes during this dissociation

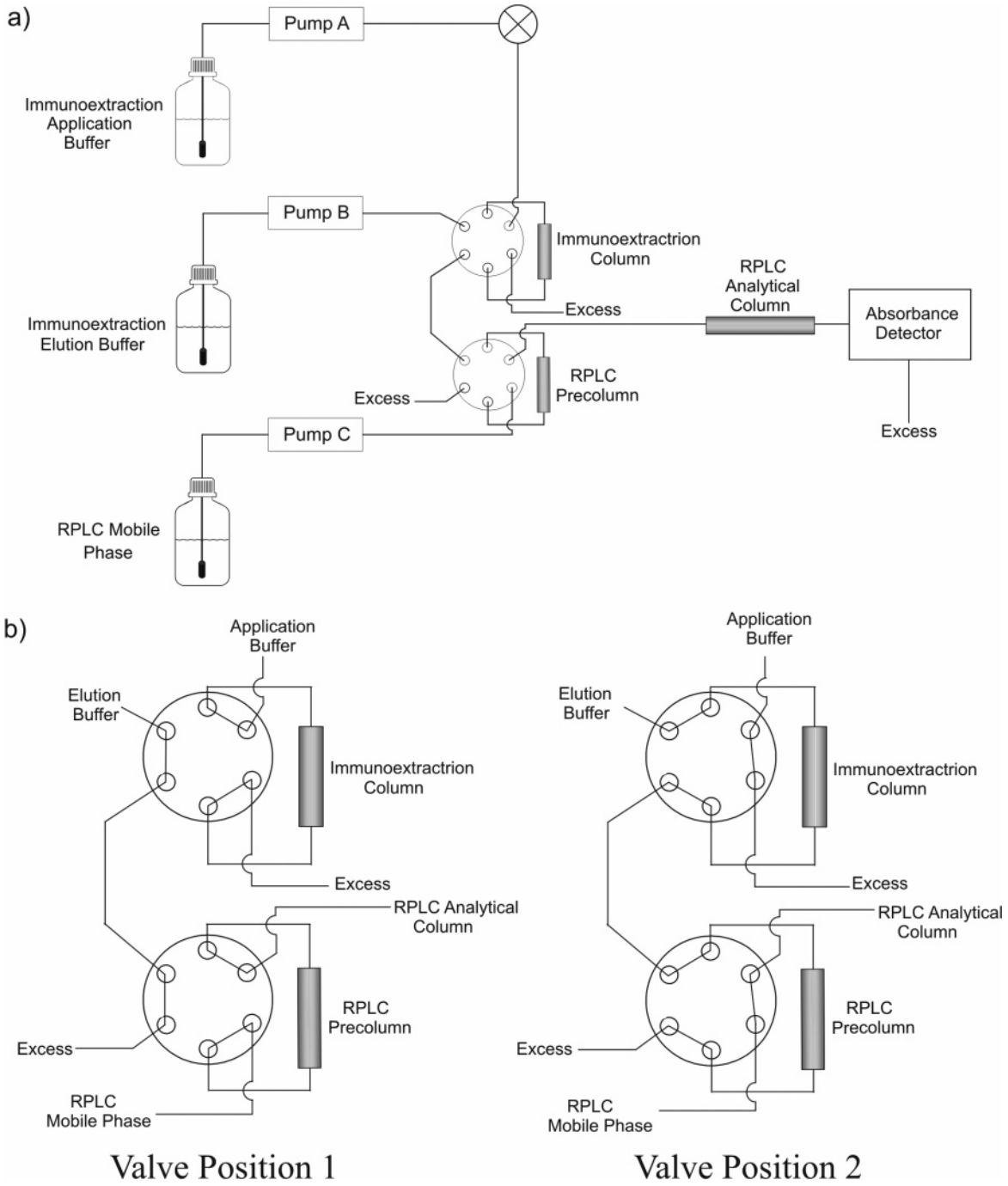

Figure 1. (**a**) Typical system used for on-line immunoextraction and RPLC and (**b**) an expanded view of the valves in this system. Valve position 1 is used during the injection of samples onto the immunoextraction column. Valve position 2 is used for the dissociation of analytes that were retained by the immunoextraction column, with these dissociated analytes then being captured by the RPLC precolumn. A switch from valve position 2 back to valve position 1 is used to place the RPLC precolumn on-line with a RPLC analytical column for separation of the captured analytes, while allowing the next sample to be injected onto the immunoextraction column.

step being monitored by an on-line absorbance detector. After the background response for the same analyte solution applied to and eluted from the control column had been subtracted out, the background-corrected elution profile was converted to a logarithmic response scale, as shown in **Figure 3**. The immunoextraction column was later regenerated by passing the pH 7.0 application buffer through this column for approximately 5 min at 0.5 mL/min. The anti-2,4-D immunoextraction column was found to be stable over several months and over hundreds of elution cycles when operated under these conditions.

A 10 cm × 2.1 mm i.d. RPLC precolumn was also used in this study. This column was packed with Platinum EPS C_{18} silica at 3500 psi using HPLC grade methanol as the packing solvent. The retention time (t_R) of each analyte from the RPLC precolumn was calculated by using the first statistical moment analysis of its peak (*18*). The retention factor (k') of each analyte was calculated by using the relationship

$$k' = (t_R - t_M)/t_M \quad (1)$$

where t_M is the void time of the column, as determined by making injections of a nonretained solute such as sodium nitrate onto the chromatographic system. The immunoextraction column and RPLC precolumn were coupled by using the general scheme shown in **Figure 1**. The *A*/*B* asymmetry ratios were calculated at $^1/_{10}$ the height for each peak eluting from the RPLC precolumn, where *A* and *B* represent the distances on the front or tailing sides of the peak versus the peak

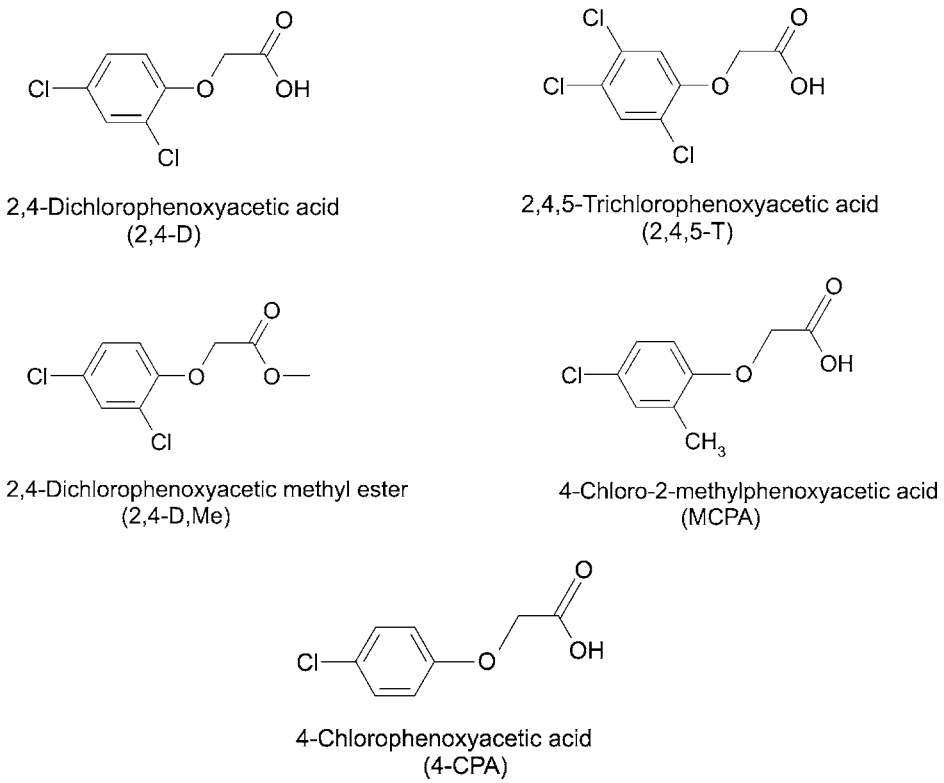

Figure 2. Structures of 2,4-D and other chlorophenoxyacetic acid herbicides that were used as model analytes in this study.

maximum (*19*). The number of theoretical plates was determined using retention time and the baseline widths of the peaks, with the latter being determined by using the distance between the points of intersection between the baseline and tangents drawn from the front and tailing sides of the given peak.

Computer Model. The movement of analytes from the immunoextraction column and through the RPLC precolumn was simulated using a modified countercurrent distribution model (*13*, *20*−*23*). This was performed using an Excel spreadsheet, in which the column was represented by a series of equal segments that each contained regions corresponding to the mobile phase and stationary phase (see ref *13* for a detail description of this spreadsheet and its use). At the beginning of each simulation, a given amount of analyte was applied to the first slice (or theoretical plate) of the column and allowed to distribute according to a two-phase equilibrium model (*13*, *24*). The amount of analyte in the mobile phase of each slice was then moved along the column by 1 unit, while additional analyte was applied to the first slice. The distribution process was again performed in each slice, followed by another movement step. As this process was repeated, the amount of analyte eluting in the mobile phase region from the last slice of the column was measured and plotted as a function of distribution/movement cycles to provide the simulated chromatogram.

The number of theoretical plates in this simulated system was controlled by varying the number of column slices that were used in the model. Retention of the analyte was varied by altering the retention factor (k') for the analyte between the mobile phase and the stationary phase in each slice, as given by the relationship

$$k' = K_D(V_S/V_M) \quad (2)$$

where K_D is the distribution constant for the analyte between the stationary phase and mobile phase and (V_S/V_M) is the relative volume of the stationary phase versus the mobile phase in any given region of the column (i.e., the phase ratio) (*19*).

The dissociation of analytes from the immunoextraction column and their entry onto the first slice of the RPLC precolumn was modeled by using a first-order decay process. In this model, the relative fraction of analyte ($f_{A,app}$) that eluted from the immunoextraction column and entered the RPLC precolumn up to time t is given by eqs 3 and 4

$$f_{A,app} = 1 - e^{-k_d t} \quad (3)$$

$$\ln(1 - f_{A,app}) = -k_d t \quad (4)$$

where k_d is the dissociation rate constant for the release of analyte from the immunoextraction column in the presence of the elution buffer.

RESULTS AND DISCUSSION

Analyte Dissociation from Immunoextraction Column. The first item considered in modeling the behavior of an on-line immunoextraction/RPLC interface was the way in which analytes are released from an immunoextraction column in the presence of an elution buffer. **Figure 4** shows the measured fraction of each analyte that was released from the anti-2,4-D immunoextraction column at various times of exposure to a pH 2.5, 0.10 M potassium phosphate elution buffer applied at 0.5 mL/min. As shown in **Figure 4a**, there was some variation in the rate of release of the tested herbicides under these particular elution conditions. At 0.5 mL/min, 2,4-D had the fastest rate of release, followed closely by 2,4,5-T. A similar rate of dissociation was noted for MCPA and 4-CPA, with 2,4-D,Me having the slowest overall rate of dissociation from the immunoextraction column. More than 95% of all these analytes were released at 0.5 mL/min within 1 min after the pH 2.5 elution buffer had been applied to the immunoextraction column. Greater than 99% was eluted within 2 min after the application of this elution buffer.

Figure 4b shows how changes in the flow rate of the pH 2.5 elution buffer altered the time needed for analyte dissociation from the immunoextraction column, using data for 2,4-D as an example. At all flow rates that were tested (0.2−0.5 mL/min), >95% of 2,4-D was recovered in <2.0 min after application of the elution buffer. However, the time required for this dissociation decreased with the elution flow rate. At 0.5 mL/min, 95%

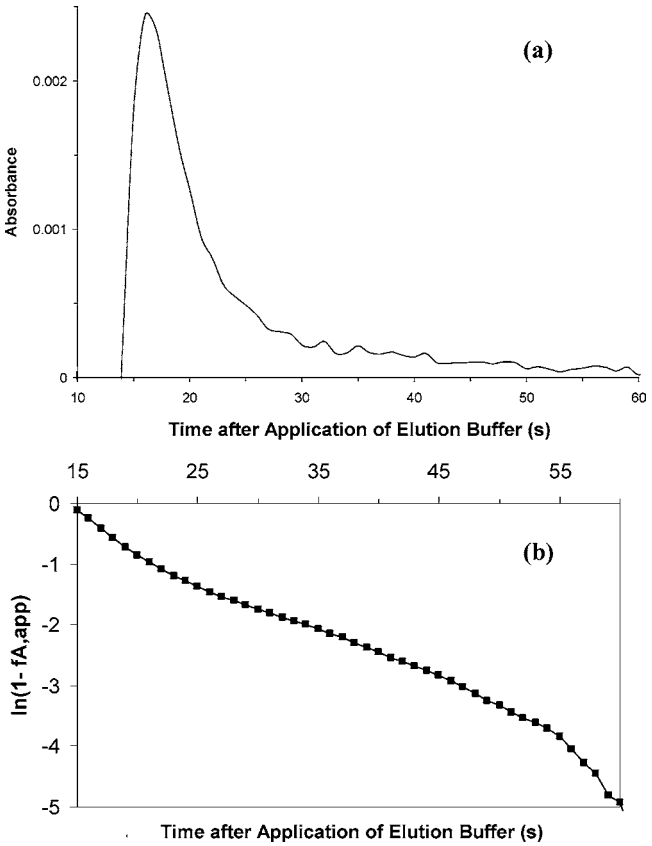

Figure 3. (**a**) Release of 2,4-D from an anti-2,4-D immunoextraction column at 0.50 mL/min using a pH 2.5, 0.10 M potassium phosphate buffer and (**b**) a graph of this same data plotted according to a first-order reaction model. A time of 0 min in these graphs represents the point at which the flow of elution buffer was begun through the column. The void time of the column (i.e., the minimum time needed for the elution buffer to pass through the column) was approximately 15 s (0.25 min) in this experiment. The results shown in this graph have been corrected for changes in the background due to column switching events by subtracting the response for 2,4-D on an inert control column from the results obtained on the immunoextraction column. The times corresponding to 0.5–4 half-lives for this dissociation occur between approximately 17 and 31 s. In the specific case of **Figure 3b**, a linear response was seen up to over 7 half-lives (over 42 s).

of 2,4-D was released from the immunoextraction column within 0.80 min, whereas 95% release occurred within 1.25, 1.45, and 1.70 min, respectively, at 0.4, 0.3, and 0.2 mL/min. Most of this difference in elution time was due to the greater speed with which the mobile phase could be changed from the application buffer to the elution buffer at the higher flow rates. However, as will be seen later, a small part of this difference was due to the slightly larger apparent dissociation rate constants that were noted for the analytes as mass transfer effects were reduced at the higher flow rates.

As noted in previous work with other immunoaffinity supports (*25*), it was found that the elution profiles in **Figure 4** could be described by a first-order exponential decay. This occurs because the elution buffer for an immunoextraction column is generally selected to promote rapid release of analytes under conditions that also avoid irreversible antibody denaturation and minimize reassociation of analytes with the immobilized antibodies. An elution profile obtained in such a process is shown in **Figure 3** for 2,4-D and the anti-2,4-D immunoextraction column. Similar first-order decay curves were noted for the other analytes considered in this study.

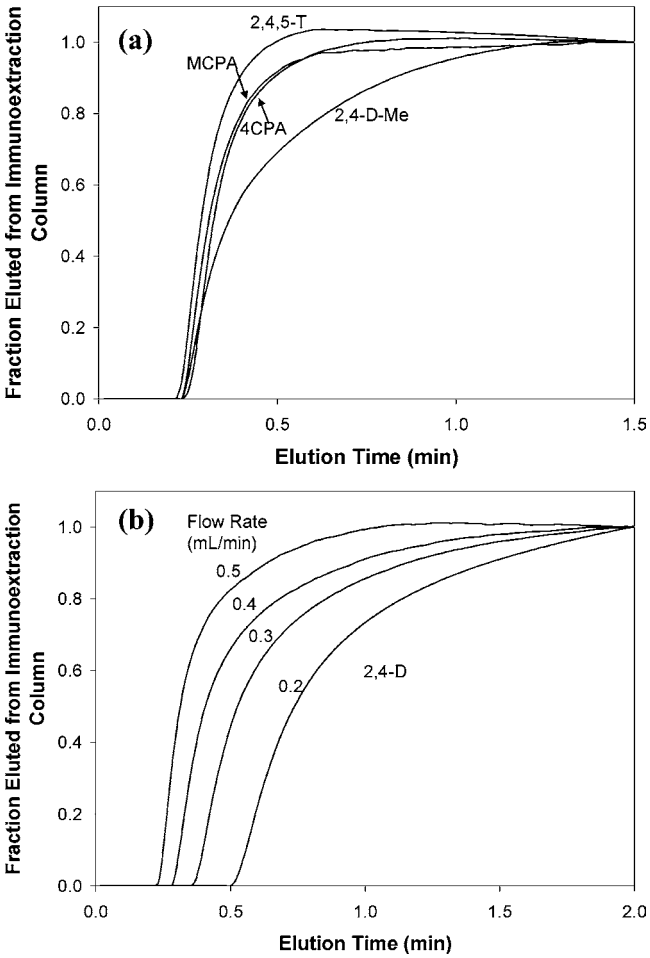

Figure 4. (**a**) Fraction of various analytes released from an anti-2,4-D immunoextraction column at 0.5 mL/min and various times when using pH 2.5, 0.1 M potassium phosphate as the elution buffer and (**b**) release of 2,4-D under the same conditions at several flow rates. The fraction of each released analyte was determined by taking the observed area for the eluted analyte up to time *t* and dividing this by the total area measured for the same eluted analyte over the entire course of the study. A time of 0.0 min in this study represents the time at which a switch was made between placing the application and elution buffers through the immunoextraction column. A slight delay between this time and the dissociation of analyte is seen in each curve due to the time necessary for the elution buffer to pass through the column (i.e., the void time). This void time was approximately 0.25 min (15 s) at a flow rate of 0.5 mL/min, but was proportionally longer at lower flow rates.

Using the linear range of logarithmic plots like the one shown in **Figure 3b**, it was possible to estimate the dissociation rate constants for analytes from the immunoextraction column in the presence of the elution buffer. This was accomplished by fitting these results to eq 4, where the linear range for a plot of $\ln(1 - f_{A,app})$ versus t should give a slope that provides k_d, the dissociation rate constant for an analyte from the immunoextraction column under the given elution conditions. All of the analytes examined in this study gave correlation coefficients that ranged from 0.92 to 0.98 when their decay profiles were fit to eq 4 for data acquired between approximately 0.5 and 4 half-lives. The dissociation rate constants that were obtained under such conditions at 0.5 mL/min are summarized in **Table 2**. These rate constants were all in the range of $0.10-0.18\ s^{-1}$ and had relative precisions of $\pm 1-30\%$.

There was some effect of flow rate on the measured dissociation rate constants as lower elution flow rates were

Table 2. Dissociation Rate Constants Measured for 2,4-D and Related Herbicides from Immobilized Anti-2,4-D Antibodies Using pH 2.5, 0.10 M Potassium Phosphate as the Elution Buffer[a]

herbicide	dissociation rate constant, k_d (s^{-1})
2,4-D	0.18 (± 0.01)
2,4-D,Me	0.10 (± 0.03)
2,4,5-T	0.15 (± 0.03)
4-CPA	0.13 (± 0.02)
MCPA	0.13 (± 0.01)

[a] These values were measured at 0.5 mL/min using the linear range of plots like that shown in **Figure 3b**. Each value in parentheses represents a range of ± 1 SD.

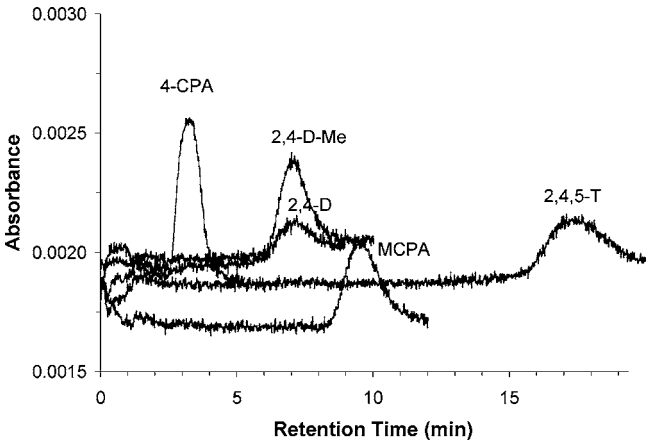

Figure 5. Elution of 2,4-D and related herbicides from a RPLC precolumn in the absence of an immunoextraction column and at 0.5 mL/min in the presence of pH 2.5, 0.10 M potassium phosphate buffer as the mobile phase.

employed. In the case of 2,4-D, the apparent values for k_d at these other flow rates were found to be 0.15 (± 0.01) s^{-1} at 0.4 mL/min, 0.09 (± 0.01) s^{-1} at 0.3 mL/min, and 0.06 (± 0.01) s^{-1} at 0.2 mL/min. A similar trend was seen for the other analytes examined in this study. This change in the apparent value of k_d indicated that mass transfer of analytes between the flow mobile phase and stagnant mobile phase regions in the support material did make a small contribution to the observed rate of analyte release from the immunoextraction column. However, the relatively consistent values for k_d that were obtained at flow rates of >0.4 mL/min indicated that the same mass transfer effects did not have a significant effect on the observed rate of analyte dissociation under these particular conditions.

Retention of Analytes on Reversed-Phase Precolumn. One assumption that is often made in the use of a RPLC precolumn with an immunoextraction column is that analytes which are retained by the precolumn will not travel any appreciable distance through this precolumn as the remainder of the same analyte dissociates from the immunoextraction column. This assumption was tested in this work by applying each of the model compounds in this study to a RPLC precolumn in the presence of the same elution buffer that was used to dissociate these analytes from the anti-2,4-D immunoextraction column. **Figure 5** shows the chromatograms that were obtained at 0.5 mL/min when using pH 2.5, 0.10 M potassium phosphate buffer on the RPLC precolumn.

An aqueous solution such as a pH 2.5, 0.10 M phosphate buffer should act as a weak mobile phase for a RPLC column, but this buffer could also act to bring about some travel of the analytes through the precolumn. It is clear from **Figure 5** that some movement through this precolumn did occur for all of the herbicides tested in this study. The herbicide that eluted through this column the most quickly was 4-CPA (t_R at 0.5 mL/min = 3.1 min). This was followed by 2,4-D,Me and 2,4-D (which coeluted around 6.9−7.1 min) and MCPA (9.5 min), with 2,4,5-T (retention time = 17.4 min) emerging last from the precolumn. This elution order agrees with a ranking of the relative polarities of these compounds based on their measured or calculated octanol−water partition ratios (*26*−*28*).

On the basis of the data in **Figure 5**, all of the tested analytes would still be on the RPLC precolumn if an elution time of only 2 min was used for dissociating these chemicals from the immunoextraction column. However, appreciable amounts of 4-CPA would pass through the RPLC precolumn if this elution time were >2.5−3.0 min. Similarly, the use of an elution time of >6.0 min would see the loss of some 2,4-D and 2,4-D,Me, whereas MCPA and 2,4,5-T would remain on the column. An elution time of >8.5 min would lead to the loss of MCPA. Only 2,4,5-T would remain on the RPLC precolumn after 12 min, but even this analyte would begin to pass through this column after approximately 15 min. This result suggests that the dissociation times employed with an immunoextraction/RPLC interface is one means by which the selectivity of the overall system can be adjusted for a given set of analytes.

The retention factors that were measured on the RPLC precolumn in the pH 2.5 elution buffer were as follows: 4-CPA, 3.2 (± 0.1); 2,4-D, 7.7 (± 0.3); MCPA, 7.8 (± 0.2); 2,4-D,Me, 10.9 (± 0.3); and 2,4,5-T, 20.6 (± 0.4). There were no significant changes in these measured retention factors at the other flow rates examined in this study, as would be expected for a system in which a local equilibrium is being reached between the stationary phase and mobile phase at the true center of each analyte peak.

Elution of 2,4-D and these other herbicides from the RPLC precolumn alone gave a reasonably good fit with a Gaussian peak profile. For example, the *A*/*B* ratios measured at $^1/_{10}$ the height for these peaks of these analytes were all between 1.1 and 1.2, with an average of 1.13 (± 0.05). (Note: an ideal Gaussian distribution would have a value of exactly 1.0.) The number of theoretical plates that were measured for these analytes at 0.5 mL/min was in the general range of 240−260, which corresponded to a plate height of roughly 0.04 cm for a 10 cm × 2.1 mm i.d. RPLC precolumn.

Coupling On-Line Immunoextraction with RPLC. The next phase of this study examined the behavior of 2,4-D and related herbicides as they passed through the combined immunoextraction column and RPLC precolumn. This was done to provide direct information on the behavior of such analytes as they pass through the interface of an immunoextraction/RPLC system. **Figure 6** summarizes the results that were obtained at 0.5 mL/min using pH 2.5, 0.10 M phosphate buffer as the mobile phase for both columns.

Figure 6a shows that the time it took each analyte to dissociate from the immunoextraction column and through the RPLC precolumn was similar to the results noted in **Figure 5** on the precolumn alone. However, in this case, each analyte gave rise to an elution profile that was slightly skewed toward longer elution times. This is illustrated in **Figure 6b**, which shows a typical chromatogram that was obtained for 4-CPA on this system. This peak had an *A*/*B* ratio of approximately 1.5 and gave a good fit to an exponentially modified Gaussian (EMG) curve. The other analytes examined in this study gave the same type of behavior on the combined immunoextraction/RPLC columns, with *A*/*B* ratios that ranged from 1.5 to 2.5 and

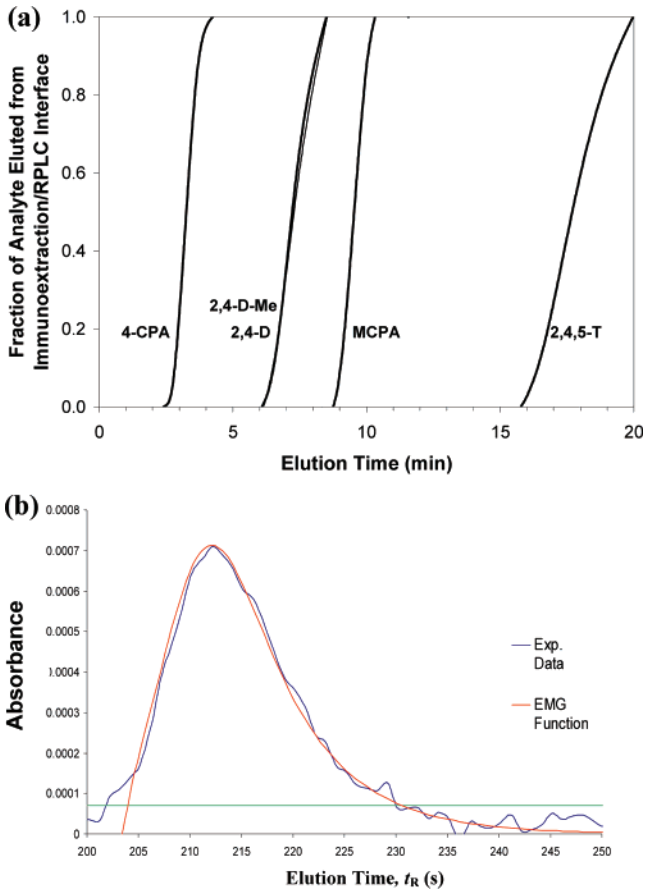

Figure 6. (**a**) Fraction of 2,4-D and related herbicides that passed through the immunoextraction/RPLC interface at 0.5 mL/min and at various times after pH 2.5, 0.1 M potassium phosphate was applied as the elution buffer and (**b**) a typical peak shape obtained for 4-CPA under these conditions. The fraction of each eluted analyte was determined by taking the observed area for the eluted analyte up to time *t* and dividing this by the total area measured for the same eluted analyte over the entire course of the study. A time of 0.0 min in this study represents the time at which a switch was made between placing the application and elution buffers through the immunoextraction column. In (**b**), the smooth line shows the best-fit response obtained for an exponentially modified Gaussian curve, whereas the rough line shows the experimental response that was obtained.

had an average of 2.0 (± 0.4). This type of behavior was expected because the dissociation of these analytes from the immunoextraction column was shown earlier to follow first-order decay. When this type of decay is combined as an input with the near Gaussian profiles that were seen on the precolumn, the result would be an EMG-type profile.

Further insights into the behavior of such a system were gained by using a countercurrent distribution model with a sample input that followed a first-order decay profile. **Figure 7** shows the results that were predicted for an immunoextraction/RPLC interface when using various retention factors for analytes on the RPLC precolumn and various dissociation rate constants for analytes on the immunoextraction columns. The types of retention that were considered were classified as "weak", "moderate", or "strong" when using retention factors of $k' = 1$, 5, or 10, respectively, as typical values. These values covered the same general range of retention factors that were seen for 2,4-D and related herbicides in this study on the RPLC precolumn. The dissociation rate constants that were used in these simulations could also be expressed in terms of the column void time (t_M), giving relative values that spanned from 0.6 to 1200 t_M^{-1}. This range covered the dissociation constants that were determined experimentally in this study, which spanned relative values of 4.4−6.0 t_M^{-1} at 0.5 mL/min when using a 10 cm × 2.1 mm i.d. RPLC precolumn.

As demonstrated at the top of **Figure 7**, the effects of the first-order dissociation of analytes from the immunoextraction column should be the most noticeable when these analytes have low retention on the RPLC precolumn (i.e., $k' = 1$ in this case). Under these conditions, a change in the dissociation rate for analytes from the immunoextraction column can create significant deviations from a Gaussian peak shape. For instance, a slowly dissociating analyte will give a severely skewed elution profile, whereas an analyte with fast dissociation will give a much more symmetrical peak. As the degree of retention for an analyte increases on the RPLC precolumn (as is shown in the middle and bottom graphs of **Figure 7**), the effects of slow dissociation from the immunoextraction column become less important. However, even under these conditions it is still possible to obtain slightly skewed peaks.

Optimization of an On-Line Immunoextraction/RPLC Interface. The experimental data in **Figure 6** and simulation results in **Figure 7** both provide several general guidelines that can be used to optimize the performance of on-line immunoextraction/RPLC. For instance, it is clear from **Figure 7** that strong retention ($k' = 5-10$ or greater) is needed on a RPLC precolumn to help focus and concentrate analytes which have slow dissociation from the immunoextraction column. For analytes that have faster dissociation, moderate or even weak retention can sometimes be used.

There is, however, a danger in losing some of the analyte from the RPLC precolumn if retention is so weak that elution from this column occurs while the analyte is still dissociating from the immunoextraction column. This was noted particularly for 4-CPA, where loss from the RPLC precolumn began after only 2.5−3.0 min following the application of elution buffer to the immunoextraction column. Thus, it is recommended in the development of a new on-line immunoextraction/RPLC system that some preliminary data be acquired regarding the retention of analytes on the RPLC precolumn and in the presence of the immunoextraction elution buffer. Following this recommendation should make it possible to identify and minimize such loss in the final immunoextraction/RPLC system.

These same results indicate that the time allowed for analyte dissociation and passage through the RPLC precolumn can be used to make the immunoextraction/RPLC system more selective for a given analyte or class of analytes. For example, it might be possible to discriminate between two analytes that have different retentions on the RPLC precolumn by varying the elution times used on the immunoextraction column. In the case of the present system, this approach could be used to create an assay that allows the selective analysis or elimination of herbicides such as 4-CPA or 2,4,5-T, which have retention factors on the RPLC precolumn that are much smaller or greater than those of other agents studied.

The data in this study suggest that it is also possible to use differences in the rate of dissociation from the immunoextraction column to improve the selectivity of an on-line immunoextraction/RPLC system. In this case, this would most easily be done by using shorter elution times for the immunoextraction column. This, in turn, would make it possible to collect most of the quickly eluting analytes (e.g., 2,4-D and 2,4,5-T), whereas some of the slower eluting analytes (e.g., 2,4-D,Me) would remain on the immunoextraction column. The presence of first-order elution would prevent the complete elimination of any given

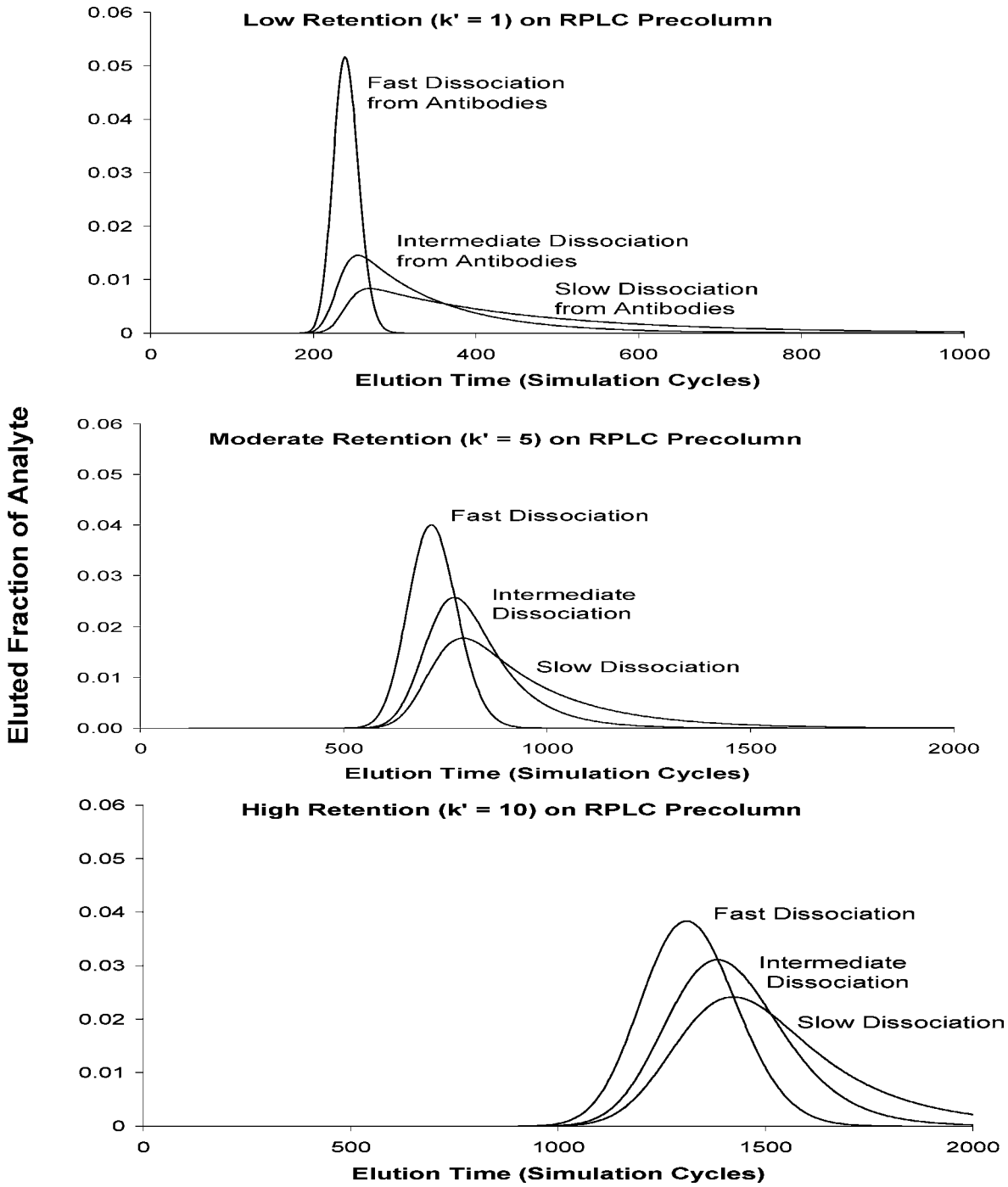

Figure 7. Simulated behavior of analytes in an on-line immunoextraction/RPLC system during the passage of an elution buffer through both the immunoextraction column and RPLC precolumn. The column was divided into 120 slices during these simulations, with the elution time being given in relative units of "simulation cycles". Retention factors (k') of 1, 5, and 10 were used to represent low, moderate, and high retention for an analyte on the RPLC precolumn. Relative dissociation rate constants of 0.005, 0.01, and 1 (with units of cycles^{-1}) were used to represent slow, moderate, and fast dissociation of analyte from the antibodies in the immunoextraction column.

analyte by this approach, but such a technique would help minimize problems with chemicals that coelute on the RPLC column (e.g., 2,4-D and 2,4-D,Me). When using this technique, some caution must be exercised because any analytes that are not allowed to pass from the immunoextraction column to the RPLC precolumn may lead to carry-over effects. This, however, can be avoided by using a separate wash step to remove such chemicals from the immunoextraction column prior to injection of the next sample.

Another way the simulations in **Figure 7** can be used is to predict the behavior that would be expected for a given analyte in an on-line immunoextraction/RPLC system. This can be accomplished by using independent measurements of (1) the dissociation rate constant for an analyte from the immunoextraction column and (2) the retention factor and plate height for the same analyte on the RPLC precolumn. These values can then be used in the simulation to predict the elution time and peak shape for the analyte as it passes through the interface between the immunoextraction column and RPLC precolumn.

An example of the latter application is shown in **Figure 8** for 4-CPA (i.e., the compound with the weakest retention in this study and the greatest chance of loss in the immunoextrac-

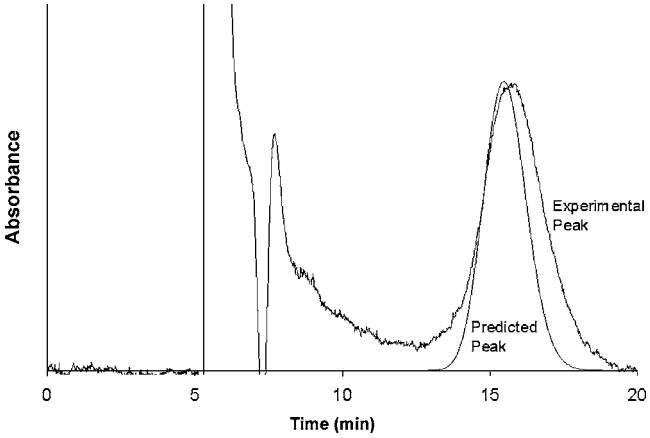

Figure 8. Comparison of predicted peak shape and actual peak shape for 4-CPA as it elutes at 0.5 mL/min in the presence of pH 2.5, 0.10 M potassium phosphate buffer from an anti-2,4-D immunoextraction column and through a RPLC precolumn. The slightly broader peak and later elution time in the experimental versus simulation data is probably due to extracolumn effects that were not considered in the current simulation method. The large peak around 7 min in the experimental data is due to the plug of pH 7.0 application buffer that remained in the immunoextraction column after a valve switch had sent the pH 2.5 elution buffer through the immunoextraction column and RPLC precolumn.

tion/RPLC interface). The simulated data in this example were generated by using a measured dissociation rate constant of 0.13 s^{-1} for 4-CPA from the immunoextraction column and a measured retention factor of 3.39 for 4-CPA on the RPLC precolumn. As shown in **Figure 8**, the simulated results obtained with this information were found to give good agreement with the experimental peak profile that was actually observed for 4-CPA when the immunoextraction column and RPLC precolumn were combined. Similar agreement was seen for the other analytes examined in this study.

These final results indicated that it was possible to use such simulations for estimating the behavior of analytes in the immunoextraction/RPLC interface. The computer model used in this study is not limited to the particular conditions that were examined in this work but can also be adapted for other conditions, such as those utilized for the analytes listed in **Table 1**. For instance, the use of a different immunoextraction column or elution buffer would be reflected by a change in the value of k_d for a given analyte. The use of a different stationary phase in the RPLC precolumn or of an alternative mobile phase for analyte passage through this precolumn would be reflected by a change in k'. Finally, a change in the efficiency of the RPLC precolumn could be made by altering the number of steps that are employed in the distribution model for analyte migration and retention. In this fashion, it should be possible to employ this modeling approach with a wide variety of analytes and immunoextraction/RPLC systems. This ability, along with the general guidelines that are provided by such simulations in the current study, should be useful in the future development of on-line immunoextraction/RPLC systems for other chemicals of interest in environmental or agricultural samples.

ABBREVIATIONS USED

BCA, bicinchoninic acid; MCPA, 4-chloro-2-methylphenoxyacetic acid; 4-CPA, 4-chlorophenoxyacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-D,Me, 2,4-dichlorophenoxyacetic methyl ester; 2,4,5-T, 2,4,5-tichlorophenoxyacetic acid; RPLC, reversed-phase liquid chromatography; $f_{A,app}$, fraction of analyte that elutes from an immunoextraction column and enters a RPLC precolumn at a given elution time; k', retention factor for the analyte on a column; K_D, distribution constant for the analyte between the stationary phase and mobile phase; k_d, dissociation rate constant for the release of an analyte from an immunoextraction column; t, time; t_R, retention time; t_M, void time; V_S, volume of the stationary phase; V_M, volume of the mobile phase.

SAFETY

Studies with 2,4-D have produced limited evidence of a carcinogenic effect for this chemical; 2,4-D is also a known teratogen, mutagen, and reproductive hazard. 2,4-D may decompose on exposure to light and should not be used with strong oxidizing agents or copper, iron, and iron salts. 2,4,-D,Me is a teratogen; contact of this chemical with acids, bases, and alcohols should be avoided. 2,4,5-T is listed as a possible carcinogen, teratogen, and mutagen; the use of strong oxidizing agents or strong bases should be avoided with 2,4,5-T. MCPA should not be used with strong oxidizing agents and is a teratogen, mutagen, and reproductive hazard. 4-CPA is a mutagen and should not be employed with strong oxidizing agents or strong bases (*29*).

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