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Evaluation of immunoaffinity chromatography as a replacement for organic solvent clean-up of plant extracts for the determination of triazine herbicides by liquid chromatography

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Abstract

A method employing immunoaffinity chromatography for sample clean-up has been developed for a number of triazine herbicides. The approach eliminates the requirement for solvents such as hexane, dichloromethane, acetone and other solvents commonly used for organic–aqueous partitions or adsorption chromatographic clean-up of sample extracts. The only solvent used was methanol, (the least toxic, most environmentally friendly and least expensive of those mentioned) for the initial sample extraction and clean-up. Acetonitrile was used in the liquid chromatographic mobile phase, although methanol proved to be satisfactory for all triazines except for atrazine which coeluted with simetryn and simazine which coeluted with metribuzin. An immunoaffinity cartridge employing immobilized polyclonal antibodies generated against atrazine but which cross-reacted with several other triazines, was used for the clean-up of extracts of apple, carrot, celery, corn, potato and peas. The triazines atrazine, cyanazine, simazine, propazine, terbutylazine, simetryn and prometone were studied. The method involved extraction of the triazines from the plant material with methanol followed by concentration of an aliquot of the extract with subsequent dilution in aqueous phosphate buffered saline (PBS). The PBS mixture was cleaned up sequentially using a strong anion-exchange solid phase extraction cartridge (SPE-SAX) followed by the immunoaffinity cartridge. Quantitation was carried out using reversed-phase liquid chromatography with UV absorption detection at 220 nm. Atrazine, simazine, propazine and terbutylazine were recovered from extracts consistently above 80% at a spiking level of 25 ng/g each. The average recovery for cyanazine was 71% ($n=9$), for prometone it was 72% ($n=7$) and for simetryn it was 65% ($n=9$). Detection limits were estimated to be 2–10 ng/g depending upon the triazine and the plant tissue analyzed. One immunoaffinity cartridge was used for 49 plant extracts with no loss of activity or effectiveness for extract clean-up. A combination of two immunoaffinity cartridges (one not recognizing the triazines and one recognizing them) was evaluated for the selective clean-up of several of the plant extracts. This approach compared well with the SPE-SAX–immunoaffinity cartridge combination. Spiked (25 ng/g of each triazine) extracts of celery and corn were readily confirmed by GC–mass spectrometry.

Keywords: Immunoaffinity chromatography; Triazines; Pesticides; Atrazine; Cyanazine; Simazine; Propazine; Terbutylazine; Symetryn; Prometone

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1. Introduction

Organic solvents are extensively used for the extraction and clean-up of environmental samples prior to the determination of chemical contaminants. Typical methods, for example for pesticides, natural toxins and veterinary drug residues in plant or animal tissues employ organic solvents for extraction, for aqueous–organic partitions and for some type of adsorption chromatographic step for extract purification prior to qualitative and quantitative determination by gas or liquid chromatography. However, in the past few years there has been a growing trend towards a reduction in organic solvent use for several reasons. The recent bans on production of ozone-depleting substances such as chlorofluorocarbons, halons, carbon tetrachloride and 1,1,1-trichloroethane have focused attention on the general use of organic solvents in organic analytical chemistry. Several publications have recently addressed the issues [1–4].

In efforts to reduce the use of organic solvents in organic trace analysis, many researchers have developed miniature methods requiring less sample and thus less extracting solvent to carry out their analyses. In addition, new approaches such as solid-phase extraction (SPE), supercritical fluid extraction (SFE) employing CO_2, accelerated solvent extraction (ASE, which uses hot organic solvents to minimize the amount required for extraction) and solid-phase microextraction (SPME) which makes use of polymer-coated fibres, have been investigated and show much promise in environmental analysis [1–4].

Another approach which is gaining in popularity for sample clean-up is immunoaffinity chromatography [5,6]. The focus of this work has been primarily to offer a very selective means of isolating analytes from sample extracts. However, because of the selectivity and the fact that immunoaffinity chromatography employs predominantly aqueous-based solvent systems, it offers an excellent means of carrying out environmental analyses with minimal requirement for organic solvents. The immunochemical approach for pesticides including triazine herbicides has been evaluated in the past but mainly in the form of immunoassays [7–10]. Recently, a report was published on the application of immunoaffinity chromatography for the selective trace enrichment of triazine and phenylurea herbicides from environmental water samples [11]. The work demonstrated the selectivity and effectiveness of using the technique for isolating sub ng/g concentrations of triazines for either on-line or off-line analysis by liquid chromatography. The immunosorbent used for the phenylurea herbicides was recently successfully applied to the selective clean-up of these compounds in food samples [12] using methanol as the only organic solvent with detection limits in the 5–10 ng/g range. The success of this work has prompted us to evaluate immunoaffinity chromatography as a means to selectively isolate triazine herbicides from plant extracts without the normal requirement for liquid–liquid partition or adsorption chromatography, both of which require organic solvents.

2. Experimental

2.1. Reagents

All chemicals and reagents were materials of analytical grade. Methanol (LC grade) was the only organic solvent employed in the sample extraction and clean-up. Doubly deionized water was used throughout. The triazine herbicides (and metabolites) were obtained as analytical standards from the Food Research Division, Bureau of Chemical Safety at the author's address. Stock solutions were prepared in methanol and diluted as required with 20% methanol (MeOH) (v/v) in H_2O. Phosphate buffered saline (PBS) was prepared by dissolving 2.68 g of Na_2HPO_4 and 8.76 g of sodium chloride in 1 l of H_2O. The pH was adjusted to pH 7.4 with 0.1 *M* phosphoric acid. The immunoaffinity cartridges were prepared using 0.5 g of silica-immobilized antibody (generated against atrazine in the rabbit) exactly as described elsewhere [11]. The cartridge was washed and stored in PBS at 4°C when not in use. For storage of the cartridge for longer than one week, sodium azide (0.02% w/v) was added to the PBS solution to prevent mold and bacterial growth. The samples included in the study were fresh apples, carrots, celery and potatoes and frozen corn and peas, all purchased locally.

2.2. Apparatus

The liquid chromatography system (Beckman) consisted of two single-piston pumps, a gradient controller, a solvent mixer and an autoinjector (Gynkotek). A diode-arrray, UV detector (Model 1040A, Hewlett Packard) set to 220 nm was used to monitor the mobile phase. The triazines were separated using a base deactivated Symmetry C_{18} column (15 cm× 3.9 mm I.D., 5 μm) (Waters), with a linear gradient from 20–80% acetonitrile (v/v) in water over 32 min at a flow-rate of 1 ml/min. Normally, 100-μl injections were made. The injection port was automatically flushed with 50% MeOH (v/v) in water between injections.

2.3. Sample extraction

A 5-g quantity of chopped plant tissue sample was mixed with 20 ml of MeOH and homogenized in a 100-ml beaker for 2 min with a Polytron homogenizer. The homogenate was transferred to a 50-ml centrifuge tube and centrifuged at 1500 *g* for 10 min. The supernatant was removed to a 25-ml centrifuge tube. A 5-ml aliquot (equivalent to 1 g of tissue) was transferred to a 10-ml centrifuge tube and the contents were evaporated to 0.2–0.3 ml at 50°C under a stream of nitrogen. The concentrate was diluted to 8 ml with PBS solution for clean-up using SPE-SAX ion-exchange chromatography. Spiked samples were prepared by adding the herbicides to the sample homogenate to yield concentrations ranging from 25–100 ng/g in the samples.

2.4. Ion-exchange chromatography

A strong anion-exchange solid phase extraction cartridge (SPE-SAX; 2.8 ml volume, 500 mg adsorbent, Chromatographic Specialities, Canada) was conditioned with 10 ml of MeOH followed by 3 ml of H_2O and 3 ml of PBS. The sample extract (8 ml in PBS) was applied to the cartridge and the eluent was discarded. The cartridge was rinsed with 4 ml of H_2O and the rinsings were discarded. A 5-ml volume of 75% MeOH (v/v) in H_2O was added to the cartridge and the eluent containing the triazines was collected and concentrated to 1 ml under a stream of nitrogen at 50°C. The concentrate was then diluted to 5 ml with PBS (adjusted to pH 6.0 with phosphoric acid) and filtered through a 0.45-μm Acrodisc syringe filter (Gelman Sciences).

2.5. Immunoaffinity chromatography

The atrazine immunoaffinity cartridge was rinsed with 10 ml of PBS (pH 6.0). The 5 ml solution from the SPE-SAX clean-up was added to the cartridge and permitted to flow by gravity. The eluent was discarded. The cartridge was rinsed with 10 ml of PBS (pH 6.0) followed by 3 ml of H_2O, all of which were discarded. The triazines were eluted with 5 ml of 70% MeOH (v/v) in H_2O and collected in a 5-ml centrifuge tube. The contents were then evaporated to 0.5 ml using a nitrogen stream at 50°C and 100 μl were injected into the LC system. The cartridge was rinsed with 5 ml of H_2O followed by 5 ml of PBS (pH 7.4) and stored at 4°C until further use.

3. Results and discussion

3.1. Immunoaffinity chromatography

The immunoaffinity cartridge used in this work had a binding capacity equivalent to about 500 ng of atrazine. Therefore, for routine applications, the total quantity of triazines in a single extract that was passed through the cartridge was kept below 500 ng. This small quantity did not create problems with the chromatographic analyses, since only about 5–10 ng of each herbicide were required for injection into the LC system for reliable quantitation. Although the antibodies used to prepare the immunoaffinity cartridge were generated against atrazine, they displayed sufficient cross-reactivity with other triazines to permit the use of the cartridge for multiresidue applications.

A number of triazines were initially studied in aqueous solutions including metribuzin, hydroxyatrazine and desethylatrazine in addition to the seven triazines mentioned in Section 2. Metribuzin, a nonsymmetrical triazine, was not retained by the antibodies as was expected. Hydroxyatrazine was retained by the cartridge but could not be eluted.

perhaps due to very strong non-specific binding to the silica support. The performance of the cartridge for the other triazines was not affected by this.

The sample application and elution conditions for the immunoaffinity cartridge as described in Section 2 were obtained after many trials with different combinations of solvent mixtures and pHs. We found that for application of the PBS-diluted sample extract to the cartridge, pH 6.0 yielded slightly cleaner chromatograms than pH 7.4 which was initially evaluated. A washing step using 5 or 10% MeOH in PBS before final elution of the triazines was initially evaluated, but under these conditions some of the more weakly retained compounds, such as cyanazine and simetryn, were eluted causing decreased recoveries in the 70% MeOH–H_2O elution fraction. This washing step was deleted from the procedure. An elution mixture of 70% MeOH in H_2O was found to be optimum for complete elution of the triazines. Under these conditions the immunoaffinity cartridge could be readily regenerated and used repeatedly with minimal loss of antibody activity.

The recovery of the triazines spiked in 5 ml of deionized water, through the immunoaffinity cartridge clean-up step were; atrazine (94%), simazine (90%), propazine (90%), terbutylazine (93%), prometone (87%), cyanazine (78%) and simetryn (70%). The lower recoveries for cyanazine and simetryn were due to the low affinity of the immobilized antibodies for these analytes and therefore they were not as effectively removed from the aqueous sample solution.

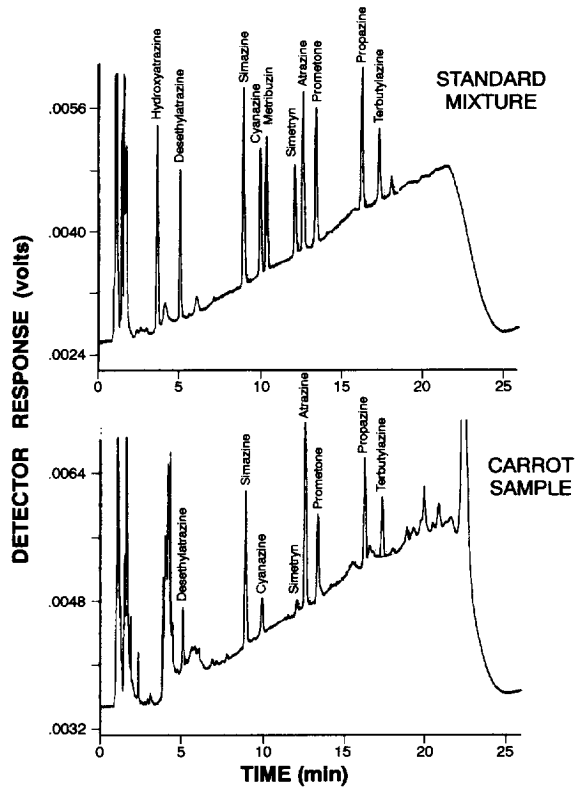

Fig. 1. Upper chromatogram: Standard mixture of triazines, 5 ng of each injected except for simetryn (2.5 ng), terbutylazine (2.5 ng), hydroxyatrazine (10 ng) and metribuzin (16 ng). Lower chromatogram: Carrot extract (spiked to contain 100 ng/g of each triazine except for simetryn (56 ng/g) and terbutylazine (64 ng/g). Conditions used are as described in Section 2.

3.2. Sample analyses

The extraction of triazine herbicides from plant tissue is usually carried out with solvents such as methanol or acetonitrile or aqueous solutions of these [13–15]. Methanol was chosen for the present work. Fig. 1 shows chromatograms of a standard mixture of triazines as well as an extract of carrots (spiked at 100 ng/g of each herbicide, except for simetryn and terbutylazine which were spiked at 50 ng/g each) that had been cleaned up using only the immunoaffinity cartridge. All triazines were detectable in the carrot extract although the recoveries varied. Atrazine, simazine, propazine, terbutylazine and prometone were recovered at greater than 80%, while desethylatrazine (29%), cyanazine (41%) and simetryn (21%) were lower.

As found in earlier work with immunoaffinity chromatographic clean-up of plant extracts for the determination of urea herbicides, the immobilized antibodies alone did not provide sufficient clean-up for all plant materials analyzed. Although, as shown in Fig. 1, acceptable results were obtained with the majority of the triazines examined in carrot samples at the Canadian regulatory level of 100 ng/g for each herbicide, additional sample clean-up was required for most other plant materials examined. While the immunoaffinity cartridge contains sites that are specific for the triazines, there are also present many other non-specific sites to which coextractives may adsorb. These include sites on the silica sorbent and

on the antibody itself where both proteinaceous and carbohydrate surfaces may exist. It is the binding of coextractives to these non-specific sites that can create problems during the elution of the triazines from the antibodies. Most earlier clean-up methods for triazines included liquid–liquid partition and or adsorption chromatographic steps in the sample purification process [13–15]. However, since these procedures employ organic solvents such as dichloromethane, acetonitrile, hexane or acetone, alternative approaches were studied. The strong anion-exchange (SPE-SAX) cartridge procedure employed for urea herbicides [12] was found to be effective in removing coextractives from the sample extracts for triazine determination. Fig. 2 compares results obtained with and without SPE-SAX or immunoaffinity chromatographic clean-up, for an apple extract spiked to contain 22 ng/g of each herbicide. As can be seen, the combination of the two cartridges yielded very clean chromatograms, enabling the detection of low ng/g levels of the triazines in the extract. The immunoaffinity cartridge alone provides sufficient clean-up to allow detection of all of the triazines except desethylatrazine, simazine and cyanazine. However, for routine use, the two cartridge combination was employed. Using the two cartridges, as low as 5–10 ng/g of each triazine (except desethylatrazine) could be detected in most of the plant extracts analyzed. It was found that under the gradient conditions employed for the parent triazines, desethylatrazine eluted too early and was often obscured by coextractive peaks in most sample extracts. Fig. 3 shows typical results for peas spiked at 25 ng/g of each triazine (14 ng/g simetryn

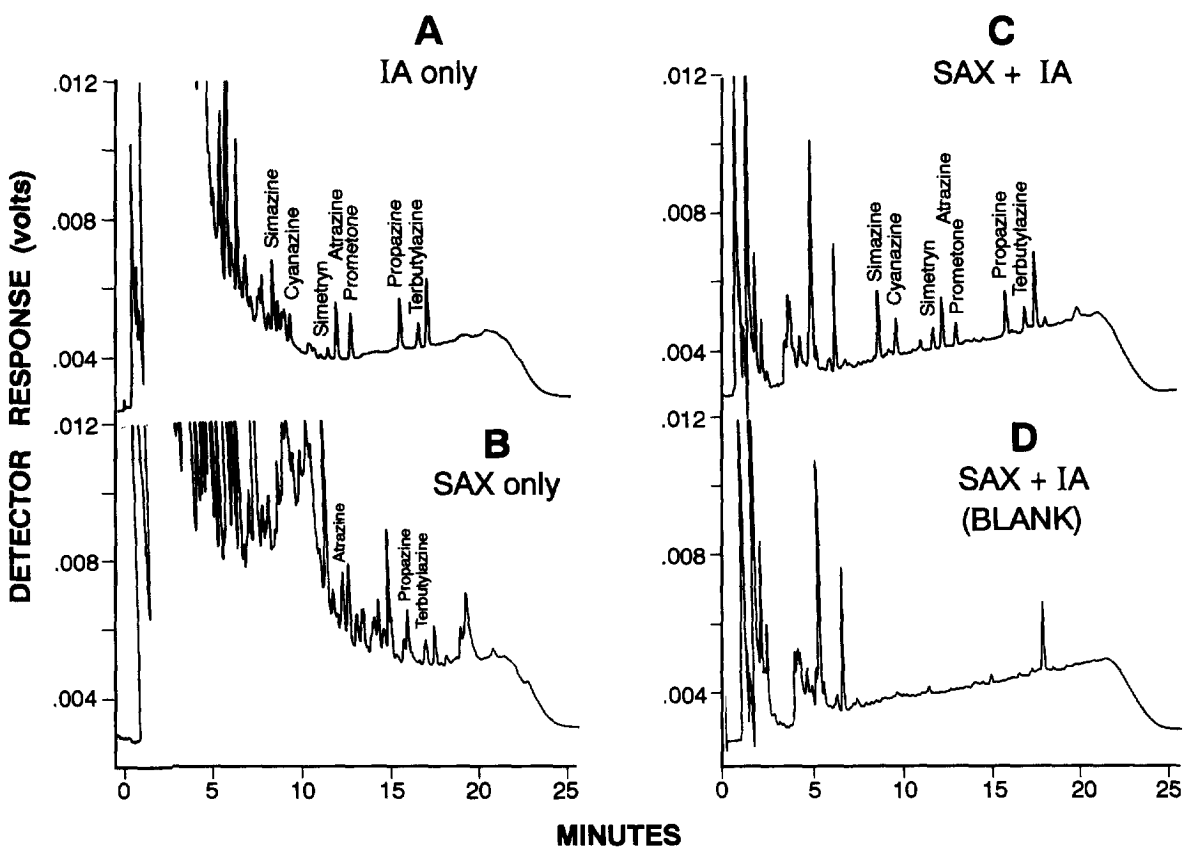

Fig. 2. Chromatograms of spiked apple extracts [containing 22 ng/g of each triazine, except simetryn (13 ng/g) and terbutylazine (14 ng/g)] cleaned up using SPE-SAX (SAX) and immunoaffinity (IA) cartridges. (A) Immunoaffinity clean-up only. (B) SPE-SAX only. (C) SPE-SAX and immunoaffinity clean-up. (D) Blank extract cleaned up by both SPE-SAX and immunoaffinity chromatography.

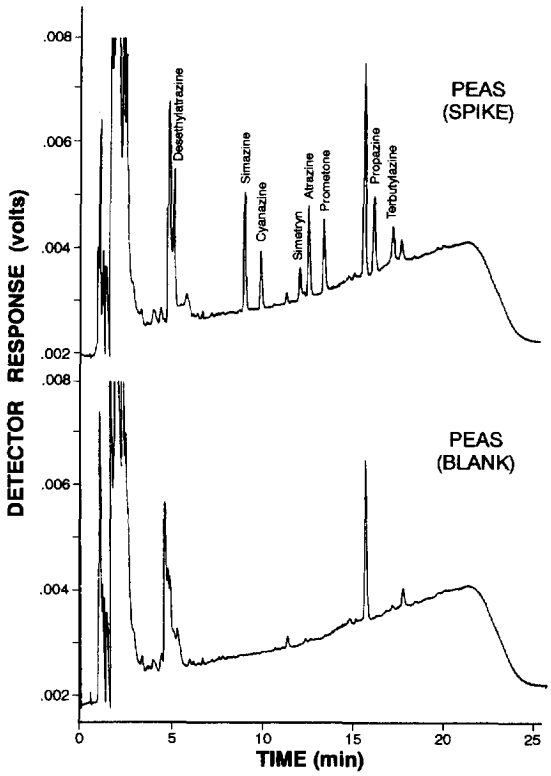

Fig. 3. Chromatograms of an extract of peas spiked at 25 ng/g (14 ng/g simetryn and 16 ng/g terbutylazine) along with a blank extract. Conditions as described in the text, clean-up by combined SPE-SAX–immunoaffinity chromatography.

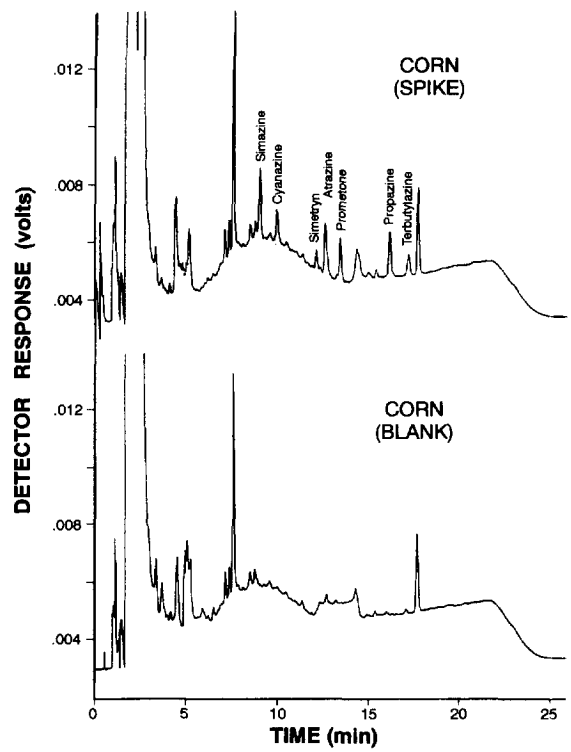

Fig. 4. Chromatograms of a corn extract spiked at 100 ng/g of each triazine (56 ng/g simetryn and 64 ng/g terbutylazine) along with a blank extract. Clean-up by combined SPE-SAX and immunoaffinity chromatography. Only 0.3 g of equivalent sample taken for clean-up. Conditions are as described in Section 2.

and 13 ng/g terbutylazine). The method worked well for apples, carrots, celery, peas and potatoes. However, some difficulties were encountered with frozen corn kernels. A significant amount of coextractives passed through the clean-up procedure, making detection of the triazines at the 5–10 ng/g level difficult. Fig. 4 shows results obtained for a corn extract spiked to contain 100 ng/g of the triazines (56 ng/g simetryn and 52 ng/g terbutylazine). The equivalent of 0.3 g of sample was passed through the clean-up cartridges whereas for the other plant materials 1 g of equivalent tissue was used. As can be seen, a significant amount of coextractives are present. Many attempts at fine-tuning the washing and elution conditions to improve the clean-up proved to be unsuccessful. In spite of this, the triazines were readily detected at the guideline level of 100 ng/g. It is possible that the addition of water [15] or a filter aid such as Celite 545 [13] to the initial extraction solvent could remove some of these coextractives. However, these were not evaluated.

In an attempt to improve the clean-up of the corn extract using immunoaffinity chromatography, we evaluated the combination of two immunoaffinity cartridges in series, the first cartridge containing antibodies (produced against phenylurea herbicides) that do not recognize the triazines, while the second cartridge contained antibodies that recognized them. Both cartridges were prepared from antibodies generated in the rabbit and immobilized under identical conditions to the same silica sorbent material [12]. The only difference in the cartridges was that one contained specific sites recognizing the triazines while the other did not. It was hoped that the coextractives that were retained by the triazine immunoaffinity cartridge would be removed using

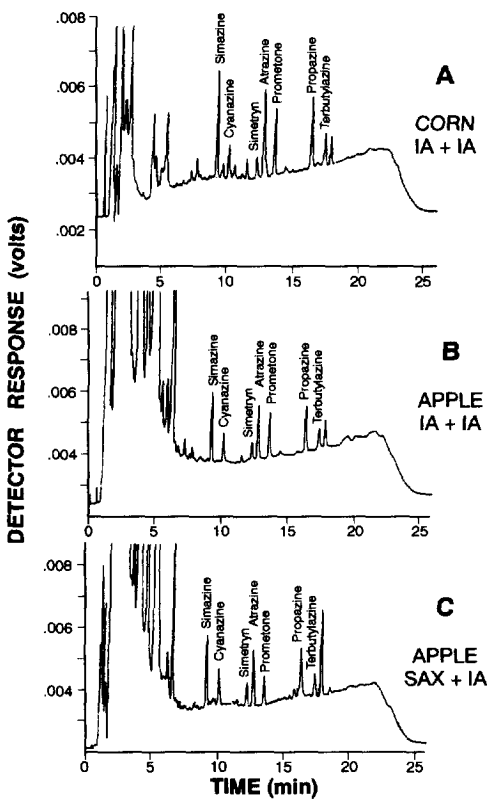

Fig. 5. Chromatograms of (A) a spiked corn extract containing 100 ng/g of each triazine (56 ng/g simetryn and 64 ng/g terbutylazine). Clean-up involved the use of two immunoaffinity cartridges as described in Section 2. (B) A spiked apple extract containing 25 ng/g of each triazine (14 ng/g simetryn and 16 ng/g terbutylazine) cleaned up as in (A) above. (C) A spiked apple extract as in (B) above but cleaned up using SPE-SAX and immunoaffinity chromatography as described in Section 2.

the first cartridge, while the triazines themselves, not being recognized by the antibodies, would pass through and be retained by the second cartridge. Fig. 5A shows results obtained for the same corn extract as shown in Fig. 4. As can be seen, the chromatogram is improved particularly in the area of simazine and cyanazine. However, it was found that using more than 0.3 g of corn in the clean-up led to relatively dirtier chromatograms, indicating that the capacity of the non-triazine immunoaffinity cartridge was exceeded. Fig. 5B and Fig. 5C compare chromatographic results of a spiked apple extract (different from Fig. 2) after dual immunoaffinity cartridge clean-up compared to that obtained after SPE-SAX–immunoaffinity clean-up. The results are very similar, indicating that the dual immunoaffinity cartridge approach is effective in cleaning up these sample extracts for detection at low ng/g concentrations. Although this particular approach was not evaluated for any of the other plant extracts in this study, in general it appears promising. One advantage is that both cartridges can be used repeatedly.

Recoveries of the triazines, through the SPE-SAX–immunoaffinity cartridge clean-up procedure for the plant samples analyzed, are shown in Table 1. Overall average recoveries for simazine, atrazine, propazine and terbutylazine were greater than 80%, while for cyanazine, simetryn and prometone they ranged from 65–72%. These recoveries reflect the ability of the immobilized antibodies to remove the triazines from the plant extracts (see recovery of standards by the immunoaffinity cartridge, above). It

Table 1
Percentage recoveries of triazines from plant samples

Triazine	Spike level (ng/g)	Sample						
		Apple	Carrot	Celery	Corn[a]	Pea	Potato	Mean (all values)
Simazine	26	96, 97	82	95	82, 109	94	95, 96	94
Cyanazine	25	84, 83	64	55	65, 69	65	78, 72	71
Simetryn	14	59, 83	63	64	58, 60	60	70, 69	65
Atrazine	24	77, 98	74	90	91, 100	89	93, 88	89
Prometone	25	41, 63	53	83	86, 93	86	int[b], int	72
Propazine	29	76, 101	61	87	88, 103	87	90, 85	86
Terbutylazine	16	76, 96	60	71	86, 89	81	73, 99	81

[a] Corn sample spiked at 100 ng/g of each triazine (except simetryn, 56 ng/g, and terbutylazine, 64 ng/g); 0.3 g of equivalent corn sample was used for sample clean-up.
[b] Interference.

appears that even with the SPE-SAX clean-up before the immunoaffinity cartridge, sample coextractives have an effect on triazine recovery.

For all of the above work only a single immunoaffinity cartridge was used. After employing it for the clean-up of 30 sample extracts, the ability of the immobilized antibodies to bind the triazines did not diminish. Recoveries of a standard mixture in water were virtually identical to those obtained before any plant extract was passed through the cartridge. These studies were carried out over a period of six months and no significant loss in activity of the antibodies was observed over that time.

Several spiked extracts [celery (25 ng/g) and corn (100 and 25 ng/g)] were analysed by capillary gas chromatography–mass spectrometry (30 m DB-5 column, electron impact 40 eV, resolution, 1000, VG-7070EQ) with selected-ion monitoring. All triazines were readily confirmed, including desethylatrazine which could not be quantitated by HPLC due to interferences, as mentioned above.

The only organic solvent other than methanol employed in this work was acetonitrile used in the HPLC mobile phase, as has been reported by others [11,14,16–18]. We have recently evaluated methanol as a replacement for acetonitrile for the LC separation of the triazines. Although atrazine and simetryn could not be separated (as well as simazine and metribuzin), a water–methanol gradient functioned satisfactorily for the separation of the remainder of the compounds.

The results of these studies indicate that immunoaffinity chromatography with or without SPE-SAX clean-up offers several advantages over classical techniques for the clean-up of sample extracts for the determination of triazine herbicides. Perhaps the most significant aspect is that no organic solvent other than methanol is required in the procedure. The resulting extracts are clean enough, for the most part, to be analyzed directly by rather simple means (HPLC with UV detection at 220 nm) at concentrations as low as 5–10 ng/g. Also, the immunoaffinity cartridges are robust enough to be regenerated many times and to be stored for relatively long periods of time (6–12 months or longer) without significant loss in activity. Similar advantages have already been demonstrated for the immunoaffinity chromatographic clean-up of plant extracts for the determination of urea herbicides [12].

Acknowledgments

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