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Journal of Chromatography A, 732 (1996) 277–281

JOURNAL OF CHROMATOGRAPHY A

Use of immunoaffinity chromatography as a simplified cleanup technique for the liquid chromatographic determination of phenylurea herbicides in plant material

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Received 18 July 1995; revised 9 November 1995; accepted 15 November 1995

Abstract

Immobilized polyclonal antibodies were evaluated for the cleanup of extracts of several food samples (carrots, celery, corn, grapes, onions, potatoes and strawberries). The antibodies were generated in the rabbit after inoculations with an antigen prepared from the urea herbicide, isoproturon. The antibodies were immobilized onto the surface of activated silica particles and packed into disposable plastic syringe barrels and used in the same general manner as cartridges for standard solid-phase extraction. They displayed substantial cross-reactivity with six other urea herbicides which permitted them to be used as a multi-urea herbicide cleanup procedure. Reversed-phase liquid chromatography with UV detection at 244 nm was the only equipment required for the quantitation. Methanolic extracts of the plant tissue samples were concentrated and then diluted with water before passage through the immunoaffinity (IA) cartridge. The cartridge was washed and the herbicides eluted with 70% methanol in water for analysis by HPLC. The cleanup provided by the IA cartridge enabled the direct detection and quantitation of the herbicides at a concentration level of 25 ng/g in potatoes and carrots. An additional cleanup step using a strong anion-exchange solid-phase extraction cartridge (SPE-SAX) was required for determination of the herbicides in grape, onion, celery, corn and strawberries at levels of 25 ng/g. With the combined SPE-SAX and IA cleanup, the detection limits in the plant material examined were about 2–5 ng/g depending upon the herbicide. At 25 ng/g spiking levels, recoveries through the complete procedure for monuron, chlortoluron, isoproturon and durion averaged 103±10% (n=6 for each herbicide); for chloroxuron (80±5%, n=6); chlorbromuron (65±12%, n=6); linuron (37±15%, n=6). The only organic solvent used was methanol mainly for the initial sample extraction and in the LC mobile phase. No organic–aqueous partitions or adsorption chromatography employing organic solvents were required.

Keywords: Cleanup methods; Immunoaffinity chromatography; Food analysis; Pesticides; Phenylureas

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0021-9673/96/$15.00
SSDI 0021-9673(95)01274-5

1. Introduction

Phenylurea herbicides are widely used pesticides registered for use on a large number of crops. Although many different analytical methods have been developed most of these have focussed on only one or two compounds. A few multi-residue methods for phenylureas have been reported in the literature [1–6]. These methods generally employ an organic solvent extraction followed by an organic–aqueous partition and adsorption chromatographic cleanup. This general cleanup approach is common to many methods for pesticide residue analysis. In spite of these efforts at sample cleanup, often special instrumental techniques are required for detection at low ng/g concentration levels. The most recent multi-residue method developed for urea herbicides is that reported by Luchtfield [7,8], which employs the standard extraction and cleanup approach mentioned above. This method uses LC with a selective post-column reaction followed by fluorescence detection. It has been applied to the determination of a number of phenylurea herbicides in crops at concentrations in the low ng/g range.

Immunoaffinity chromatography (IAC) has been shown to be very effective in cleaning up sample extracts for trace concentrations of, for example, environmental contaminants such as aflatoxins [9], ochratoxin [10] and triazine herbicides [11] and veterinary drug residues [12] before determination by standard GC or LC procedures. The technique can offer a simple and selective means of purifying extracts before analysis. In addition, it offers the potential of reducing the use of organic solvents normally used in purifying extracts of complex matrices such as foods. Recently, an immunoaffinity (IA) sorbent was prepared and evaluated for the determination of phenylurea herbicides in natural water samples [13]. The procedure was combined with on-line preconcentration and LC with UV detection. The resulting method was capable of easily detecting 0.1 ng/g of selected phenylurea herbicides. The success of this IA sorbent prompted us to evaluate it for the application to complex matrices such as foods. The aims of the work were two-fold. Firstly, to simplify methodology for the determination of urea herbicide residues in foods with detection limits well below the regulatory guideline of 0.1 μg/g (in Canada) and secondly to try to eliminate as much as possible the use of organic solvents in the sample preparation.

2. Experimental

2.1. Reagents

Doubly de-ionized water and LC-grade methanol were used as solvents. All other chemicals were analytical grade materials and were used as received from the suppliers. The phenylurea herbicides, chlorbromuron, chlortoluron, chloroxuron, diuron, isoproturon, linuron and monuron were obtained as analytical standards from the Food Research Division, Bureau of Chemical Safety, Health Canada, Ottawa, Canada. Stock solutions were prepared at 1 mg/ml in methanol and diluted as required (to about 50 ng/ml) with water. Phosphate-buffered saline (PBS) solution was prepared by dissolving 2.68 g sodium phosphate (dibasic) and 8.76 g sodium chloride in 1 l of water. The pH was adjusted to pH 7.4 with 0.1 *M* phosphoric acid. The IA cartridge was prepared using 1 g of silica-immobilized antibody (generated against isoproturon in the rabbit) exactly as described elsewhere [13]. The cartridge was washed and stored in PBS at 4°C when not in use. For long-term storage of the IA cartridge (greater than 1 week) sodium azide was added to the PBS solution (0.02% w/v) to prevent mold and bacterial growth.

2.2. Liquid chromatography

The LC system consisted of two Model 114M (Beckman) pumps with a gradient controller (Beckman, Model 421A), a solvent mixer (Beckman) and an autoinjector (Gynkotek). A diode-array UV detector (Model 1040A, Hewlett-Packard) set to 244 nm was used for detection of the herbicides. The herbicides were separatead on a C_{18} column (15 cm× 4.6 mm I.D., 5 μm, Supelco) with a linear gradient from 40 to 80% methanol (v/v) in water over 30 min at a 1.0 ml/min flow-rate.

2.3. Sample extraction

A 5-g portion of homogenized (Polytron) crop sample was placed into a 50-ml centrifuge tube. A 20-ml volume of methanol was added and the

contents mixed for 1 min on a Vortex mixer. The mixture was centrifuged at 2500 rpm (1500 *g*) for 10 min. The supernatant was transferred to a 25-ml centrifuge tube. A 5-ml volume (equivalent to 1 g of crop tissue) was removed to a 10-ml centrifuge tube and the contents evaporated to 0.2–0.3 ml at 50°C under a stream of nitrogen. The concentrated solution was diluted to 10 ml with PBS solution. Spiked samples (100 ng/g and 25 ng/g) were prepared by adding the herbicides (dissolved in methanol) to the tissue homogenate and mixing before extractions.

2.4. Ion-exchange chromatography

A strong anion-exchange solid-phase extraction (SPE-SAX) cartridge (2.8 ml volume, 500 mg adsorbent, Chromatographic Specialties, Canada) was conditioned with 3 ml methanol followed by 3 ml water and 3 ml PBS. The 10 ml of sample extract, in PBS, was applied to the cartridge and the effluent discarded. The cartridge was rinsed with 4 ml water and 5 ml 10% methanol (v/v) in 0.1 *M* ammonium acetate, pH 6 (pH adjusted with acetic acid). The rinsings were discarded. A 5-ml volume of 70% methanol (v/v) in water was added to the cartridge and the effluent containing the herbicides collected and evaporated under nitrogen at 50°C to 0.2–0.3 ml. The solution was then diluted to 10 ml with PBS adjusted to pH 6 with phosphoric acid.

2.5. Immunoaffinity chromatography

The IA cartridge was rinsed with 10 ml PBS (pH 6). The 10-ml sample solution from the SPE-SAX cartridge was added to the IA cartridge and permitted to flow through the cartridge by gravity. The cartridge was rinsed with 10 ml water. The herbicides were eluted from the cartridge with 5 ml 70% methanol (v/v) in water and collected in a 5-ml centrifuge tube. The eluate was evaporated to 1 ml (under nitrogen at 50°C) and 200 μl injected onto the LC system for determination of the herbicides. The IA cartridge was rinsed with 5 ml water followed by 5 ml PBS (pH 7.4) and stored (4°C) until further use.

3. Results and discussion

From earlier work [13], the phenylurea IA cartridge was found to have a capacity equivalent to about 3 μg of isoproturon. In the present work, the total quantity of herbicides passed through the cartridge was always less than 1 μg (usually 100 ng or less of each phenylurea). The affinity of the immobilized antibodies under the conditions described in the experimental section was the greatest for monuron, chlortoluron, isoproturon, diuron and chloroxuron. These were essentially completely retained from aqueous solution and recovered (>90%) using 70% methanol in H_2O. Chlorbromuron (recovery, 75%) and linuron (recovery, 42%) were less retained by the IA cartridge. The losses were due to partial elution in the wash fractions. These elution patterns are similar to those reported earlier for the determination of phenylureas in natural water samples and reflects the weaker affinity of the antibodies for these compounds [13].

Fig. 1 shows chromatographic results obtained without SPE-SAX cleanup for the herbicides spiked

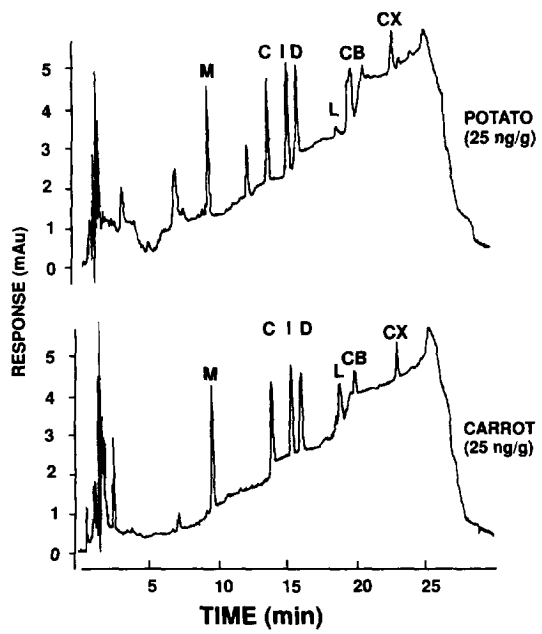

Fig. 1. Upper chromatogram: potato extract containing 25 ng/g of each herbicide cleaned up using IA chromatography. Lower chromatogram: carrot extract containing 25 ng/g of each herbicide cleaned up using IA chromatography. Quantity of each compound injected, 10 ng. M=monuron, C=chlortoluron, I=isoproturon, D=diuron, L=linuron, CB=chlorbromuron, CX=chloroxuron. Reversed-phase chromatography with linear gradient elution (40–80% methanol in water over 30 min). The rising baseline is due to the gradient conditions and detector sensitivity employed.

in a carrot and potato sample at 25 ng/g each. All herbicides were easily detected at that level. Recoveries for monuron, chlortoluron, isoproturon and diuron were consistently above 80% for these samples. However, lower recoveries were observed for linuron (11%, 41%), chlorbromuron (37%, 29%) and chloroxuron (56%, 49%) in potatoes and carrots, respectively, at the 25 ng/g level. It appears that the sample matrix has an inhibitory effect on the retention by the antibodies of these three phenylureas. This is not surprising since they exhibit a lower affinity for the antibodies and binding may be more influenced by sample coextractives. Recoveries for these compounds improved when the sample extracts were cleaned up before passage through the IA cartridge as described below.

Although the IA cartridge functioned satisfactorily for the cleanup of potato and carrot extracts, it was found that for other types of plant extracts the use of the IA cartridge alone was not sufficient for eliminating interferences from coextractives. Fig. 2 (upper chromatogram) shows results obtained for an extract of onions using only the IA cartridge for cleanup. As can be seen, large peaks due to coextractives prevent the determination of the herbicides. Coextractives also interfered in the determination of the phenylureas in red grapes and to a lesser extent in celery. These interferences appear to be due to non-specific binding of the coextractives to the antibody-sorbent matrix and not to the urea herbicide specific sites on the antibodies. In order to improve the cleanup for low ng/g detection of the herbicides, a number of parameters were studied including more extensive washing of the IA cartridge, changes in pH and the use of SPE-SAX cartridges. Lowering the pH to pH 6 slightly improved the cleanup. However, it was found that the SPE-SAX treatment removed the major interfering coextractives from the plant extracts. The middle chromatogram in Fig. 2 demonstrates the effectiveness of the SPE-SAX treatment, although it alone as a cleanup was not sufficient for quantitating the herbicides routinely at the 25 ng/g concentration level. The lower chromatogram shows results obtained with both cartridges. The chromatogram is very clean. As a result, the SPE-SAX step was included in the procedure for all sample extracts. Fig. 3 shows typical results obtained with other food samples spiked at 25 ng/g and carried through the combined SPE-SAX–IA cleanup procedure.

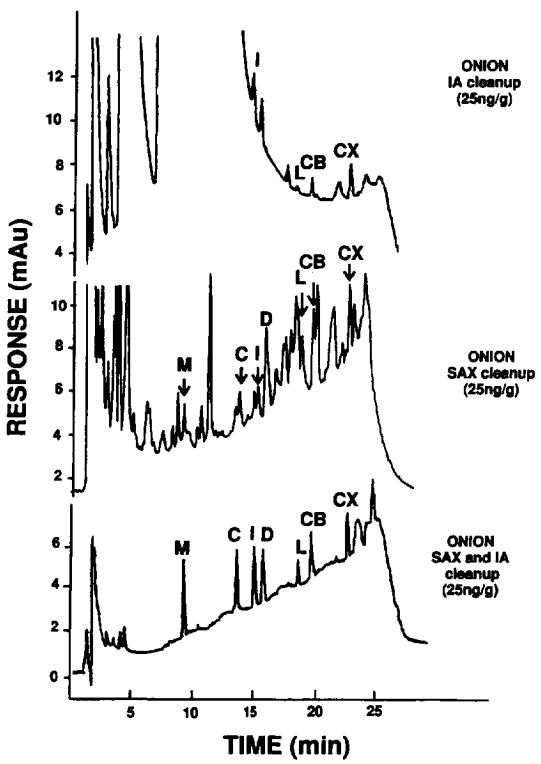

Fig. 2. Cleanup of an onion extract containing 25 ng/g of each herbicide: upper chromatogram, IA only; middle chromatogram, SPE-SAX only; lower chromatogram, SPE-SAX and IA. Chromatography conditions and peak identification as in Fig. 1.

Recoveries at 25 ng/g through the complete procedure for the compounds studied were: monuron (102±8% R.S.D., n=6), chlortoluron (110±9%, n=6), isoproturon (101±9%, n=6), diuron (101±10%, n=6), linuron (37±15%, n=6), chlorbromuron (65±12%, n=6) and chloroxuron (80±5%, n=6). The detection limits were estimated to range from 2 to 5 ng/g in the plant tissues analysed. Attempts to improve the detection limits by using larger quantities of sample extract or injecting more final extract into the LC system were not carried out since the present procedure is much more than adequate to quantitate the herbicides at the Canadian regulatory level of 100 ng/g. The linear range (in samples) was found to extend from 25 ng/g (the lowest concentration studied) to 250 ng/g for each herbicide. Higher concentrations were not evaluated since the capacity of the IA cartridge would be approached which may lead to decreased recovery. For the

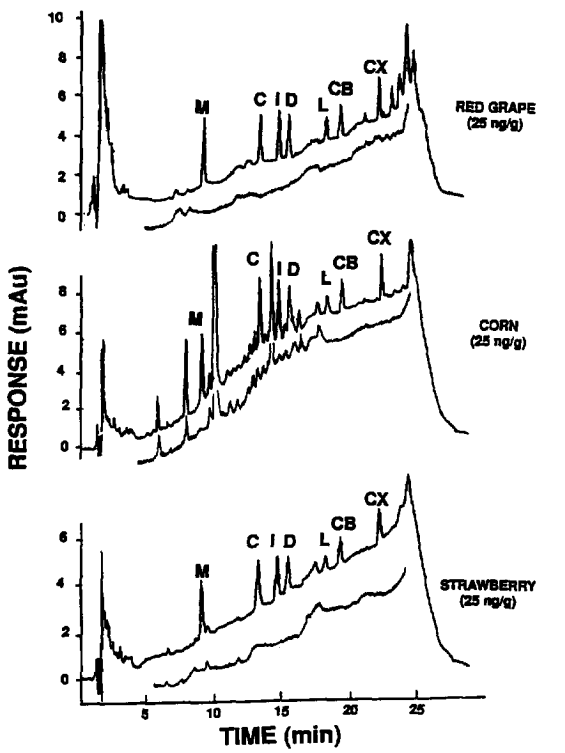

Fig. 3. Typical chromatograms of spiked (25 ng/g each herbicide, upper) and blank (lower) extracts of red grape, corn and strawberry carried through the SPE-SAX–IA cleanup procedure. Chromatography conditions and peak identification as in Fig. 1. Results obtained on different days.

determination of concentrations of phenylureas greater than the cartridge capacity, smaller quantities of sample extract would be required to ensure high recovery.

The IA cartridge was employed for the analysis of 35 plant tissue samples with minimal loss in efficiency. A standard mixture of the herbicides was passed through the cartridge to determine any loss in binding capacity due to repeated plant tissue analysis. The recoveries for a mixture of herbicide standards (50 ng each) through the IA cartridge were greater than 90% for all compounds except linuron (42%) and chlorbromuron (72%). These results are virtually identical to results obtained with standard mixtures before the cartridge was used for sample extract cleanup. This shows the usefulness of the IA cartridge for repeated use and makes the approach economically attractive. It should be noted that great care was taken to ensure that the integrity of the antibodies was maintained. Thus, PBS was used as much as possible to keep the antibodies in an environment closely resembling physiological conditions. Extremes of pH or 100% methanol were not used at all since such conditions may lead to decreased effectiveness of the IA cartridge with extended use.

The method described here using immunoaffinity cartridges in the cleanup of urea herbicides in plant samples has several advantages. The antibodies provide a high degree of selectivity enabling the detection of a number of phenylurea herbicides at low ng/g levels in plant extracts. The extracts are clean enough to permit quantitative determination using LC with UV detection, unlike the method developed by Luchtfield [8] where a post-column reaction system was required. Perhaps most importantly, the method described herein employs no organic solvent other than methanol, compared to methanol, methylene chloride, hexane, acetone, acetonitrile and isopropanol used elsewhere for urea herbicides [8] and, generally, for the determination of pesticides and other organic contaminants in plant tissues. The use of immunoaffinity chromatography has the potential for greatly reducing and even eliminating much of the use of organic solvents in the determination of organic contaminants in a wide variety of environmental samples.

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