DOI:10.1021/jf9017614

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Preparation of Acetochlor Antibody and Its Application on Immunoaffinity Chromatography Cleanup for Residue Determination in Peanuts

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The preparation of an antibody against acetochlor and its application on immunoaffinity chromatography (IAC) cleanup was investigated. The hapten was synthesized by reacting acetochlor with 3-mercaptopropionic acid and then linked with a carrier protein by the carbodiimide method. After immunizing a rabbit with hapten−bovine serum albumin (BSA) conjugate, the obtained antiserum showed an IC_{50} value of 0.2 ng/mL. By coupling the purified polyclonal antibody with CNBr-activated Sepharose 4B, an IAC column was prepared. The dynamic column capacity was 5560 ng/mL gel. The IAC was then used to clean up a peanut sample solution, followed by high-performance liquid chromatography (HPLC) separation and ultraviolet (UV) detection. The recoveries of acetochlor from spiked peanut samples at levels of 5−100 μg/kg ranged from 94.7 to 102.5%, with a variation coefficient of 2.6−8.0%, and the limits of detection and quantification were 1.5 and 5 μg/kg, respectively.

KEYWORDS: Acetochlor; polyclonal antibody; IAC; HPLC; peanut

INTRODUCTION

Acetochlor is a selective and good pre-emergent chloroacetanilide herbicide (*1*). It is widely used for the control of annual grasses and some broad-leaved weeds in field crops, such as peanut, corn, bean, and sugar cane. Because of its carcinogenicity, acetochlor has been classified as B-2 carcinogen by the United States Environmental Protection Agency (U.S. EPA) (*1*), and the maximum residue limit (MRL) in food crops is set by Japan as low as 0.02−0.1 mg/kg (*2*); thus, it is necessary to develop a robust method for the detection of the acetochlor residue.

Several analytical methods, such as immunoassays (*3*−*5*), gas chromatography−mass spectrometry (GC−MS) (*6*), and liquid chromatography−tandem mass spectrometry (LC−MS/MS) (*7*), have been reported for determination of acetochlor residues in different samples. Immunoassay is generally used as a screening method because of its susceptibility to environmental factors, and the suspected noncompliant results detected by the immunoassay should be further validated by instrumental methods. For instrumental methods, these approaches usually used solid-phase extraction or liquid−liquid extraction to purify the samples and concentrate the analytes of interest (*6*, *7*). They have the pitfall of requiring a lot of organic solvent and elaborate sample pretreatment. In contrast, immunoaffinity chromatography (IAC) cleanup is a good alternative method to cleanup samples. It is based on the specific interaction of antigen−antibody and can provide a simple and selective means to purify extracts and reduce the use of organic solvent. At present, the IAC technique has been widely used in sample preparation for residue analysis of pesticides (*8*, *9*), veterinary drugs (*10*, *11*), and other contaminants (*12*, *13*). However, no method has been published to determine the chloroacetanilide herbicide residue using IAC as a cleanup procedure. The aim of this study was to develop an IAC cleanup procedure for the detection of the acetochlor residue in peanuts.

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MATERIALS AND METHODS

Chemicals and Materials. Bovine serum albumin (BSA), ovalbumin (OVA), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), goat anti-rabbit IgG−horseradish peroxidase (HRP) conjugate, acetochlor, 3-mercaptopropionic acid, *N*-hydroxysuccinimide (NHS), and dicyclohexyl carbodiimide (DCC) were purchased from Sigma-Aldrich (St. Louis, MO). CNBr-activated Sepharose 4B was purchased from Pharmacia Corporation (Uppsala, Sweden). High-performance liquid chromatography (HPLC)-grade acetonitrile (MeCN) and methanol (MeOH) were obtained from Fisher Scientific, Inc. (Pittsburgh, PA). All other chemicals and solvents were of analytical grade or better and were obtained from Beijing Chemical Reagent Co. (Beijing, China). Milli-Q water was prepared using a Millipore water purification system (Millipore, Bedford, MA).

Apparatus. Polystyrene microtiter plates were purchased from Costar Group, Inc. (Bethesda, MD). The enzyme-linked immunosorbent assay (ELISA) plate reader was obtained from Tecan, Inc. (Durham, NC). The ultraviolet−visible (UV/vis) spectrometer was obtained from Shanghai Analytical Instrument (Shanghai, China). The vortex mixer was from Fischer Scientific (Norcross, GA), and the centrifuge was purchased from Hettich Co. (Kirchlengern, Germany).

 Published on Web 08/14/2009

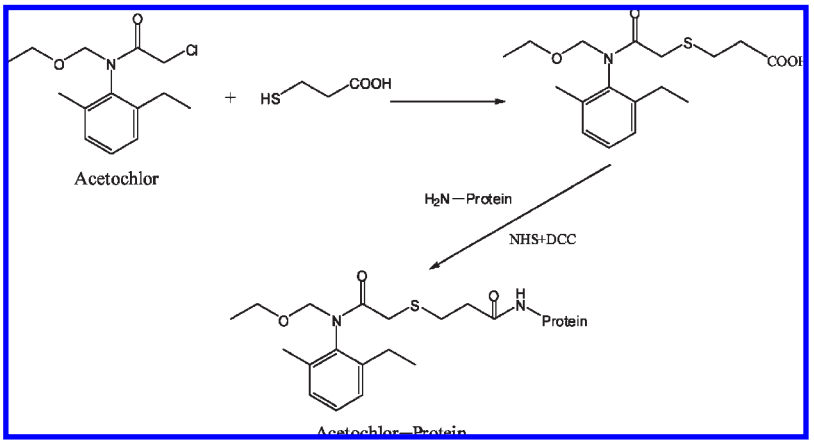


Figure 1. Synthetic route to acetochlor−protein conjugate.

Hapten Synthesis. The synthesized route of hapten was shown in **Figure 1** and referred to the method as described by Yakovleva et al. (*3*). Briefly, acetochlor (1 mmol), 3-mercaptopropionic acid (1 mmol), and potassium hydroxide (2 mmol) were added and mixed in 20 mL of ethanol. After the solution was boiled for 4 h, it was filtered and dried by rotary evaporation. The product was then redissolved in 10 mL of 5% $NaHCO_3$ solution and filtered. The pH value of the filtrate was adjusted to 3.0 with 6 mol/L HCl, and this solution was subsequently extracted by 20 mL of ethyl acetate. The extraction solution was concentrated to about 0.5 mL by rotary evaporation. The concentrated solution was further purified with thin-layer chromatography using dichloromethane/acetic ether (50:1) as a developing solvent. The band (R_f = 0.61) was scraped, and the final product was dissolved in acetic ether and dried under nitrogen gas.

Preparation of Hapten−Protein Conjugates. The hapten was covalently linked to BSA or OVA by the active ester method (**Figure 1**). A total of 20 mg of hapten, 20.07 mg of NHS, and 39.12 mg of DCC were dissolved in 2 mL of *N*,*N*-dimethylformamide (DMF). The mixed solution was stirred at room temperature for 3 h. Following centrifugation at 438*g* for 5 min, the obtained supernatant was added to 10 mL of BSA or OVA solution (6 mg/mL) dropwise. After the reaction at 4 °C for 3 h, the reaction product was dialyzed (over 2 days at 4 °C) against phosphate-buffered saline (PBS).

Production of Antiserum. Four female New Zealand white rabbits were used to prepare antisera. For each rabbit, 0.4 mg of acetochlor−BSA was dissolved in 1 mL of 0.01 M PBS (pH 7.4) and emulsified with 1 mL of CFA. The emulsion was injected intracutaneously in the primary immunization. For booster immunizations, 0.2 mg of immunogen was dissolved in 0.5 mL of the above buffer and emulsified with 0.5 mL of IFA. The emulsion was then injected subcutaneously. The booster immunizations were repeated every 3 weeks. The rabbits were bled through an ear vein 1 week after each booster injection. To obtain antiserum, blood samples were left to coagulate for 1 h at room temperature and overnight at 4 °C, followed by centrifugation at 4000*g* for 10 min. The supernatants were carefully collected and stored at −20 °C until use. Purification of the polyclonal antibody (Pab) was achieved by saturated ammonium sulfate precipitation followed by affinity chromatography on a protein G column (*14*).

ELISA Procedure. The ELISA protocol for acetochlor was carried out as follows: A polystyrene microtiter plate was coated with 100 μL/well of coating antigen (acetochlor−OVA) in 0.05 M carbonate buffer (pH 9.6) by 37 °C incubation for 2 h. After coating, wells were blocked for 2 h at 37 °C with 200 μL/well blocking solution (0.01 M PBS, containing 0.5% casein). The solution was subsequently emptied, and plates were washed 3 times with washing solution (0.01 M PBS, containing 0.05% Tween 20). Afterward, 50 μL/well analyte (prepared in 0.01 M PBS/MeOH, 9:1) followed by 50 μL/well antibody solution (prepared in 0.01 M PBS/MeOH, 9:1) were added and incubated for 1 h at 37 °C. After another washing step, 100 μL/well HRP-labeled goat anti-rabbit IgG (1:4000, prepared in 0.01 M PBS) was added and the plates were incubated for 1 h at 37 °C. After a washing procedure, the color development was initiated by adding 100 μL of the substrate/chromogen solution (TMB/H_2O_2 in acetate buffer at pH 5.5). The solution was incubated for 15 min at 37 °C before the enzymatic reaction was stopped by adding 2 M H_2SO_4 (50 μL/well). The optical density (OD) of each well was measured at 450 nm by an enzyme immunoassay reader. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentrations (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 ng/mL), which were fitted to a four-parameter logistic equation. The IC_{50} value represents the analyte concentrations with 50% inhibition and was expressed as the sensitivity of ELISA. Cross-reactivity (CR) was calculated as follows: percent cross-reactivity = (IC_{50} of acetochlor/IC_{50} of analytes × 100%).

IAC Column Preparation. The immunosorbent was produced as described by the instructions of the manfacturer. CNBr-activated Sepharose 4B (3 g) was added to 15 mL of 0.001 M HCl and poured over a sintered glass funnel (40−60 μm). After the gel was washed with 200 mL of HCl (0.001 M) and 400 mL of $NaHCO_3$ solution (0.1 M), it was mixed with 85 mg of Pab (dissolved in 5 mL of 0.1 M $NaHCO_3$ solution) and stirred gently at 4 °C for 24 h. The mixture was then washed with 50 mL of PBS to remove the uncombined Pabs. The eluant was collected to determine the antibody amount and to calculate the coupling efficiency. The mixture was subsequently transferred to 10 mL of Tris-HCl buffer (0.1 M, pH 8.0) to block the unreacted sites on CNBr-activated Sepharose 4B. After the sites were blocked at 4 °C for 2 h, the gel was washed with 3 cycles of 20 mL of acetate buffer (0.1 M, pH 4.0) and 20 mL of Tris-HCl buffer (0.1 M, pH 8.0). Finally, 1 mL of gel was transferred to the glass column (10 × 0.8 mm inner diameter) and stored in PBS containing 0.01% (w/v) sodium azide at 4 °C.

Column Capacity Determination. A total of 8 mg of acetochlor was dissloved with 40 mL of PBS containing 10% MeOH. The solutions were then transferred into the IAC column (preconditioned with 10 mL of PBS) with the flow rate of 1 mL/min. The saturated column was washed with 20 mL of PBS and 20 mL of water. At last, 5 mL of MeOH was used to elute the analytes. The eluate was evaporated to dryness by N_2 at 50 °C. The obtained residue was reconstituted in 1 mL of MeOH. After the solution was filtered through a 0.2 μm PTFE filter (Jinteng Ltd., Tianjin, China), 50 μL of the solution was injected into the LC system. The column was regenerated by equilibrating with 10 mL of water and 10 mL of PBS and stored in PBS (containing 0.01% sodium azide) at 4 °C.

Sample Preparation with IAC Column. A total of 5 g (±0.01 g) of comminuted peanut sample was added into a 50 mL polypropylene centrifuge tube. A total of 15 mL of MeOH was added, and the sample tube was then vortexed for 2 min and kept for 10 min. The supernatant was filtrated through a glass cellulose membrane. The sample residue was repeatedly extracted with 15 mL of MeOH for 1 time, followed by vortex and filtration as before. After the solution was combined and vortexed for 10 s, 25 mL of the extraction solution was concentrated to about 1 mL by rotary evaporation and 9 mL of PBS was added. The mixture was subsequently loaded on the IAC column with a flow rate of 1 mL/min. The column was then washed with 5 mL of PBS/MeOH (90:10) and eluted with 1 mL of MeOH. Finally, 50 μL of the eluent was injected into HPLC for analysis.

Table 1. Calibration Curve Parameters of the Polyclonal Antibodies

antibody	A_{max}	slope	IC_{50} (ng/mL)	A_{min}
1	1.89	0.98	0.2	0.08
2	1.91	0.93	0.9	0.11
3	1.92	0.84	5	0.15
4	1.87	0.81	8	0.20

HPLC Analysis. The LC system comprised a DGU-12A degasifier, a LC-10ATvp pump, and a 7725 sample injector fitted with a 100 μL loop (Shimadzu, Japan). The separation of acetochlor from the matrix was achieved on a Cloversil C_{18} column (150 × 4.6 mm inner diameter, 5 μm, Cloversil Sciences, Irving, TX) with an mobile phase of acetonitrile/water (75:25) at a flow rate of 1 mL/min. An isocratic elution mode was employed, and the retention time for acetochlor was 5.4 min under these conditions. A UV detector was used for the detection of acetochlor with the wavelength of 215 nm. For each analysis, 50 μL of the sample solution was injected.

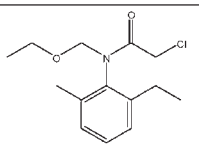
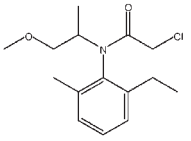
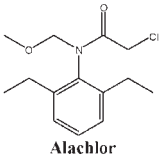
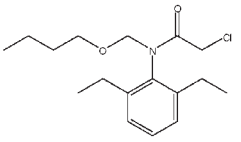
RESULTS AND DISCUSSION

Preparation of Hapten−Protein Conjugates. Because the acetochlor molecule has no amine or carboxyl groups, it cannot link directly with the protein. To introduce the carboxyl group in the acetochlor molecule, 3-mercaptopropionic acid was used to react with the molecule. After purification by thin-layer chromatography, the hapten and acetochlor could be well-separated, with the R_f values of 0.61 and 0.79, respectively. Further, thin-layer chromatography separation of hapten under different conditions indicates that the hapten has high purity. As a zero-length cross-linker, DCC could link the carboxyl groups of the hapten and the amines of BSA and OVA to form an amide linkage without leaving a spacer molecule (*15*). Because this reaction was reversible, the addition of NHS could further increase the yield of hapten−protein. The UV scanning spectrum demonstrates that the synthesis of the two conjugates is successful.

Antibody Production and Characterization. The titers and IC_{50} values of antisera from various bleeds of rabbits remained slightly changed until the fifth immunization; therefore, final bleeding was carried out after the sixth immunization. The calibration curve parameters of the obtained polyclonal antibodies are demonstrated in **Table 1**. The IC_{50} values of the four Pabs were 0.2, 0.9, 5, and 8 ng/mL, respectively. The Pab that has the best sensitivity was selected for the subsequent experiment. The sensitivity of the Pab were comparable to that of previous studies (*3*). The cross-reactivity study showed that the antibody was very specific to acetochlor and had little cross-reactivity to other chloroacetanilide herbicides, such as metolachlor, alachlor, and butachlor (**Table 2**). The high specificity of the antibody implied that the recognition site was an ethoxymethyl side chain of acetochlor. A slight modification in this region could result in a significant reduction in cross-reactivity (**Table 2**).

Preparation of IAC. In this study, CNBr-activated Sepharose 4B was employed as a coupling matrix because it is water-insoluble but hydrophilic and could be easily activated (*10*). To obtain the best binding capacity, several amounts of antibodies (4, 8, and 16 mg/mL gel) were separately conjugated to Sepharose 4B and the corresponding column capacities were measured according to the procedures as described in the Column Capacity Determination section. When the amounts of free IgG were compared before and after conjugation, the coupling efficiencies were all determined to be more than 93%. The corresponding dynamic column capacities were determined to be 2543, 5560, and 4940 ng/mL gel, respectively; thus, 8 mg of antibody/mL of gel was used for conjugation. In the case of 16 mg of antibody coupling, the CNBr-activated gel may be saturated and an excessive amount of antibody may introduce steric hindrance to antigen/antibody recognition and thus lead to the reduction of column capacity. To demonstrate that acetochlor specifically binds to the immobilized Pab and that there is no non-specific adsorption, the control column (without coupled antibodies) was used to measure the capacity, as described above. No acetochlor was found to adsorb on the control Sepharose 4B column.

Table 2. Cross-reactivity of Acetochlor Pab with Other Chloroacetanilide Herbicides

Chloroacetanilide herbicide	IC_{50} (nM)	Cross-reactivity (%)
 Acetochlor	7.4×10^{-7}	100
 Metolachlor	$>7.0\times10^{-4}$	< 0.1
 Alachlor	$>7.4\times10^{-4}$	< 0.1
 Butachlor	$>6.4\times10^{-4}$	< 0.1

Evaluation of the IAC Conditions. Optimization of loading, washing, and elution conditions is necessary to IAC cleanup because these conditions would have a strong influence on the association and dissociation of the antigen−antibody complex (*11*).

To investigate whether loading buffer containing a different ratio of MeOH has an effect on the recovery, 500 ng of acetochlor was loaded in 10 mL of PBS/MeOH (95:5), PBS/MeOH (90:10), and PBS/MeOH (80:20), respectively. After these solutions were loaded, the columns were washed by 10 mL of PBS followed by elution with 5 mL of MeOH and detection by HPLC−UV. The results indicated that the recovery did not change significantly when the ratio of MeOH increased from 5 to 10% and has slightly declined from 99.5 to 97.1% when the ratio increased up to 20%. Considering the solubility of acetochlor in PBS/MeOH, PBS/MeOH (90:10) rather than PBS/MeOH (95:5) was selected as a loading medium.

In IAC cleanup, the target analyte in samples could be selectively captured by specific antibodies immobilized on gel and, at the same time, the interfering substance may also be retained because of non-specific absorption. These interferents could be largely removed by the washing procedure. Generally, washing buffer consist of PBS and organic solvent, such as MeOH. By trial and error, 5 mL of PBS/MeOH (90:10) was found to be sufficient to eliminate the interferents; thus, it was used as the washing buffer in this study.

After the washing procedure, the trapped analyte could then be released from the IAC column by dissociating the antibody−analyte complex with elution buffer. In this study, PBS/MeOH (20:80), PBS/MeOH (10:90), and pure MeOH were tested to elute the analyte, with the volumes for complete elution of acetochlor measured to be about 3, 2, and 1 mL, respectively. Although pure

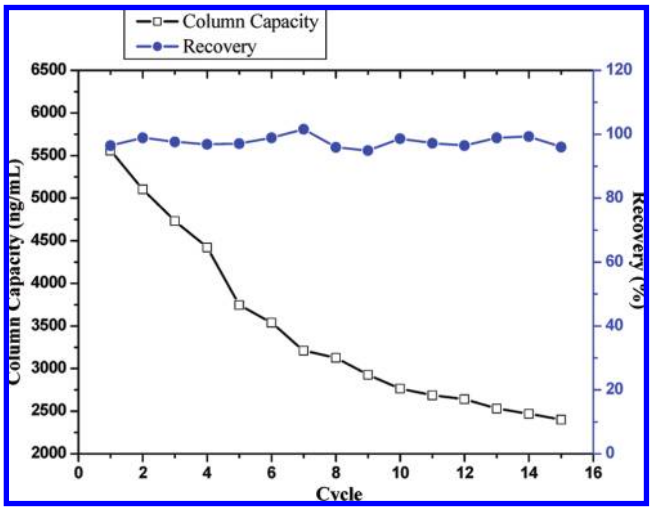


Figure 2. Capacity and recovery variation curves of the acetochlor IAC column during 15 cycles.

Table 3. Recoveries and CVs of Acetochlor from Peanut

	intraday (n = 6)		interday (n = 3)	
spiked level (μg/kg)	recovery (%)	CV (%)	recovery (%)	CV (%)
5	102.5	7.1	99.1	8.0
10	96.7	7.5	101.3	6.4
20	98.1	2..6	94.7	4.3
100	96.9	3.4	97.4	7.0

organic solvent may damage the activity of the antibody, the elution volume is the smallest and further concentration of the elution solution is not necessary; thus, 1 mL of MeOH was chosen as the elution solution.

The reusability of IAC was subsequently evaluated. For each use, more than 60 min for antibody revival is needed. The curves of column capacity and recovery after 15 cycles of sample cleanup were showed in **Figure 2**. Although the column capacity would gradually decrease to about 50% as the cycles increased, which may be attributed to the use of pure MeOH as elution solution, the recoveries of acetochlor were observed without any loss after 15 cycles.

Method Validation. The standard calibration curve was constructed by plotting the peak area versus the concentration and was used to determine the concentration of acetochlor in all subsequent analyses. It shows that the calibration curve was linear in the range of 10−500 ng/mL with satisfactory correlation coefficients (r^2) of 0.9969. The limits of detection (LOD) and quantification (LOQ) for acetochlor in peanut samples, which were defined as signal/noise ratios of 3:1 and 10:1, respectively, were determined to be 1.5 and 5 μg/kg. The average recovery of 18 replicate blank tissues fortified at the LOQ level was 99.1% with a coefficient of variation (CV) of 8.0%.

The accuracy and precision of the method was evaluated at four levels according to the recovery and coefficient of variability. When the blank peanut samples were spiked at concentrations of 5, 10, 20, and 100 μg/kg, the recoveries of acetochlor from fortified samples ranged from 94.7 to 102.5% with intraday CVs of 2.6−7.1% and interday CVs of 4.3−8.0% (**Table 3**).

In this study, we prepared a polyclonal antibody with high specificity and affinity to acetochlor. The antibody was applied for the IAC cleanup, followed by HPLC separation and UV detection. The LOD and LOQ of the method were below current MRLs established by Japan, the accuracy and precision also met the requirements for quantitative analysis; thus, the IAC−HPLC method could be used as a routine method for determination of acetochlor in peanuts.

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Received May 25, 2009. Revised manuscript received July 19, 2009. Accepted July 29, 2009. This work is supported by the Science and Technology Project of Shandong Exit and Entry Inspection and Quarantine Bureau of China (SK200826).