Development of an Enzyme-Linked Immunosorbent Assay for Alachlor and Its Application to the Analysis of Environmental Water Samples

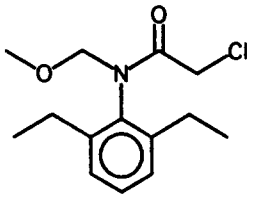
Paul C. C. Feng,* Stephen J. Wratten, Susan R. Horton, C. Ray Sharp, and Eugene W. Logusch

Monsanto Agricultural Company, A Unit of Monsanto Company, 700 Chesterfield Village Parkway, St. Louis, Missouri 63198

Alachlor was covalently attached to sheep γ-immunoglobulin (IgG), and the resulting hapten–protein conjugate was used to induce the production in rabbits of polyclonal antibodies directed toward alachlor. Cross-reactivity studies showed that these antibodies were capable of distinguishing alachlor from other structurally similar chloroacetanilide herbicides, including metolachlor, and that they showed little cross-reactivity toward the major soil metabolites of alachlor. The antibodies were used to develop an enzyme-linked immunosorbent assay (ELISA) for alachlor in water with a detection range of 0.2–8.0 ppb. The concentration of alachlor in environmental water samples was measured by ELISA, and the results were compared with those obtained by a GC/MS method.

Immunoassays offer certain advantages over conventional instrumental methods for the analysis of pesticide residues (Hammock and Mumma, 1980; Van Emon et al., 1985; Hammock et al., 1987; Wratten and Feng, 1990). Analysis of pesticides in drinking water is one example where immunoassay methods might compare favorably with other analytical methods. Immunoassays are highly efficient and cost-effective and are ideally suited for screening large numbers of water samples for low levels of specific analytes.

Alachlor (1) is the active ingredient of Lasso herbicide and several other herbicide products. It is one of the most widely used of the chloroacetanilide herbicides. We report herein on the development of an inhibition ELISA method for detecting alachlor, based on polyclonal rabbit antibodies raised against an alachlor–protein conjugate. The ELISA method was evaluated for the detection of alachlor in environmental water samples and was compared with an established GC/MS method. Our results indicate that the alachlor ELISA can be effectively utilized as a primary screen to select environmental samples for confirmatory instrumental verification of the presence of alachlor at low ppb levels.



1 ALACHLOR

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), sheep γ-immunoglobulin (IgG), and *o*-phenylenediamine (PDA) were purchased from Sigma Chemical Co. *S*-Acetylmercaptosuccinic anhydride (AMSA) and *N*-acetylhomocysteine thiolactone (AHT) were obtained from Aldrich Chemical Co. Immulon-1 96-well flat-bottom microtitre plates were purchased from Dynatech. Goat anti-rabbit γ-globulin conjugated to horseradish peroxide (GAR–HRP) was obtained from Cooper Biomedical Co. Freund's complete and incomplete adjuvants were obtained from Difco Laboratories. Nonfat dry milk powder (Food Club brand) was obtained locally. Uniformly [^{14}C]phenyl-labeled alachlor was obtained from New England Nuclear and showed greater than 98% radiological and chemical purity; it was diluted to a specific activity of 0.06 mCi/mmol prior to use.

Instrumentation. Spectrophotometric absorbances of 96-well microtitre plates were recorded on a Bio-Tek EI 310 reader equipped with a 490-nm filter. The plates were washed using a Dynatech Dynawasher II. A 12-channel Titertek pipet (50–200 μL) from Flow Laboratories was used for dispensing liquids.

GC/MS Analysis of Alachlor. Quantitation of alachlor in water samples was carried out with an established GC/MS method. Deuterated alachlor, used as an internal standard, was spiked into 1.0-L samples of water. Organic soluble materials in the samples were concentrated with use of a reverse-phase (C_{18}) solid-phase extraction filter (J. T. Baker Co.) and were eluted afterward with a solvent mixture consisting of 5:45:50 ethyl acetate–isooctane–methylene chloride. Instrumental analyses were performed on a Finnigan Model 4535 quadrupole GC/MS in the electron-impact mode. Samples entered the mass spectrometer after injection onto a 15-m DB-5 capillary column (90 °C for 1 min, 90–120 °C at 10 °C/min, 120–140 °C at 2 °C/min). Alachlor was detected by selected ion monitoring of two characteristic fragment ions (m/z 160, 188) and quantitated by comparison with the corresponding deuterated ions (m/z 171, 199) arising from the internal standard. The sensitivity of this assay was established at 0.2 ppb alachlor contained in the raw water sample.

Synthesis of Alachlor–Protein Conjugates. Radiolabeled alachlor (hapten) was covalently attached to thiolated bovine serum albumin (BSA) and sheep γ-immunoglobulin (IgG). Sulfhydryl groups were introduced onto lysine residues of BSA with the thiolating agent AHT and onto the lysine residues of IgG with the thiolating agent AMSA. The protein (200 mg of BSA or IgG) and 25 molar equiv of AHT or AMSA were dissolved in water (6 mL) at 0 °C, to which alachlor (25 equiv) dissolved in dioxane (1 mL) was slowly added. Sodium carbonate–bicarbonate buffer (1.0 M, pH 11) was then added to adjust to pH 11, and the reaction mixture was stirred at 0 °C for 15 min. After 2 h of additional stirring at 50 °C, the reaction mixture was neutralized and the alachlor–protein conjugate was purified by a 24-h dialysis against running water or by Sephadex G-25 size exclusion chromatography (2 × 50 cm column using 0.2 M NaCl). Both methods effectively separated the alachlor–protein conjugates from excess alachlor and thiolating agents. The radioactivity of alachlor–protein conjugates was determined by liquid scintillation counting. The protein concentrations of BSA and IgG were calculated from their UV absorbance at 280 nm and their molar extinction coefficients (39 and 188 mM/cm for BSA and IgG, respectively). Calculations showed that 12 and 19 mol of alachlor/mol of BSA and IgG, respec-

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tively, had been conjugated to the proteins. The IgG conjugate was used as the immunization antigen in rabbits, and the BSA conjugate was used as the coating antigen in ELISA. The alachlor–protein conjugates were lyophilized and stored at −20 °C.

Antibody Generation. The IgG conjugate of alachlor (1 mg) was dissolved in 0.3 mL of phosphate-buffered saline (PBS; pH 7.4, 0.01 M phosphate and 0.15 M NaCl). The resulting solution was emulsified with Freund's complete adjuvant (1 mL) and was then injected intradermally into female New Zealand white rabbits. The animals were immunized with 1 mg of the immunogen and boosted at 4–6-week intervals with 0.1–0.5 mg of the same immunogen in Freund's incomplete adjuvant. Whole blood (25 mL) was obtained 2 weeks after each boost by bleeding from the ear vein, allowed to coagulate overnight at 4 °C, and centrifuged to generate the serum. Aliquots of the sera were stored at −80 °C.

Immunoassays. A checkerboard assay (Campbell, 1984) was initially conducted with sera obtained from different bleeds and different animals to select the serum with the highest titre of antibodies. The checkerboard assay consisted of reacting varied amounts of the coating antigen (alachlor–BSA) with varied concentrations of the antiserum to establish the most sensitive combination of serum and coating antigen concentrations to be used in the inhibition ELISA. For alachlor, this concentration was established to be 5 ng/well of the coating antigen and 3500-fold dilution of the selected serum. Plates coated with the coating antigen were stored desiccated at −20 °C and remained stable for at least 4 months.

Inhibition ELISA was conducted in the following manner. A solution of the coating antigen, consisting of 5 ng of alachlor–BSA in 0.1 mL of sodium carbonate–bicarbonate buffer (0.05 M, pH 9.6) was dispensed into each well of 96-well microtitre plates and stored for 12 h at 4 °C. The unbound coating antigen was then removed from the wells, and the plates were washed three times with PBS. The plates could be stored desiccated at −20 °C at this point. For ELISA preparation, the remaining active sites in the wells were blocked with a solution of 8% powdered milk in PBS (0.3 mL) for 1 h at 22 °C. Serum previously stored at −80 °C was freshly thawed and diluted 3500-fold with PBS–T (0.02% Tween 20 in PBS). An aqueous alachlor standard or unknown sample was preincubated with an equal volume of diluted serum at 22 °C for 1 h. This mixture (0.1 mL/well) was then dispensed into six replicate wells on the plate, which was then covered and incubated at 22 °C for 1.5 h. After triplicate washes of the wells with PBS–T, each well was treated with 0.1 mL of GAR–HRP (freshly thawed and diluted 4000-fold with 1% powdered milk in PBS). After a final cycle of four washes with PBS–T, 0.2 mL of freshly prepared PDA substrate solution (0.4 mg/mL PDA and 0.01% H_2O_2 in 0.05 M citric acid containing 0.15 M sodium dibasic phosphate, pH 5.0) was dispensed into each of the wells and incubated in the dark at 22 °C for 30–60 min. Sulfuric acid (4 N, 50 μL) was added to each of the wells to stop the reaction, and the final absorbance of each well (490 nm) was recorded. The presence of free alachlor in samples inhibited the binding of the antibody to alachlor–BSA, resulting in an inhibition of the development of absorbance at 490 nm. The amount of free alachlor was thus inversely proportional to the intensity of color produced. The level of alachlor in unknown water samples was calculated based on alachlor standards that had been analyzed simultaneously on each plate. Alachlor standards (0, 0.2, 0.5, 1.0, 3.0, 5.0, and 8.0 ppb in deionized water) were stored at −20 °C in 1-mL portions and were freshly thawed for each assay.

Six wells on each plate not coated with the coating antigen served as background wells. Seven alachlor standards and eight samples were each analyzed in six replicate wells per plate. The absorbances of the replicate wells measured by the Bio-Tek reader were used in the following calculations. The median was calculated for the six replicate background wells and was then subtracted from the medians of the standards and samples. All the resulting median values of standards and samples were divided by the median of the standard without alachlor (0 ppb) to generate percentages of absorbance. The percentages of absorbance of the alachlor standards were plotted against the ppb concentrations of alachlor in the sample on a logarithmic scale.

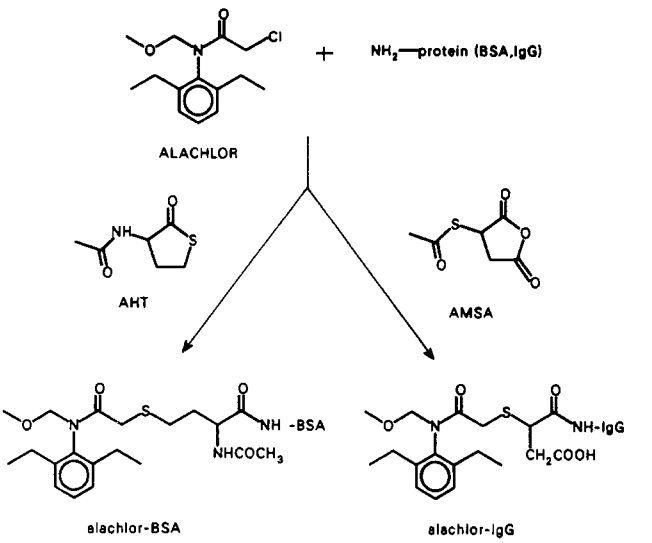

Figure 1. Covalent conjugation of alachlor to BSA with AHT and to sheep IgG with AMSA. (Alachlor–BSA was used as the coating antigen, and alachlor–IgG was used as the immunization antigen.)

A hyperbolic curve was fitted to the data to generate the standard curve, which was then used to calculate concentrations of alachlor in unknown samples.

Cross-Reactivity Studies. The reactivity of the antibodies with a series of alachlor analogues was examined. The concentration of an analyte producing a 50% inhibition in absorbance in the ELISA was defined as its IC_{50} value (50% inhibition concentration). The IC_{50} value of alachlor in picomoles per milliliters was divided by the corresponding value from the analyte and multiplied by 100 to produce the percentage cross-reactivity values. The percentage cross-reactivity of the antibodies to alachlor calculated in this way was 100%.

RESULTS AND DISCUSSION

Generation of Antibodies. Because of their small molecular weight, pesticides such as alachlor are not immunogenic. In order to render alachlor immunogenic, it was necessary to conjugate either alachlor itself or a suitable analogue to a protein carrier. In considering various approaches to hapten–protein conjugation, we thought it desirable to utilize for attachment the single functional group common to all chloroacetamide herbicides, i.e., the chloroacetamide moiety. Such an approach would in principle leave free the aromatic ring and methoxymethyl side chain, thus ensuring maximal sensitivity to these functional groups and minimal cross-reactivity with other chloroacetanilide herbicides. The chloroacetamide group would normally be expected to react readily under basic conditions with protein thiols. In order to increase the availability of such thiols and therefore increase the maximum epitope density, we employed the protein thiolating agents *S*-acetylmercaptosuccinic anhydride (AMSA; Klotz and Heiney, 1962) and *N*-acetylhomocysteine thiolactone (AHT; Singer et al., 1960). Such thiolating groups are thought to react with the ε-amino groups of lysines in proteins. Thiolation of proteins, followed by thiolate displacement of the alachlor chlorine atom, generated the alachlor–IgG immunizing antigen and the alachlor–BSA screening antigen, with proposed structures as illustrated in Figure 1. The use of ^{14}C-labeled alachlor permitted facile verification of the covalent attachment of alachlor to proteins, as well as measurement of epitope density.

Since only the alachlor moiety was common to these two hapten–protein conjugates, antibodies generated using the alachlor–IgG conjugate and reacting with the alachlor–

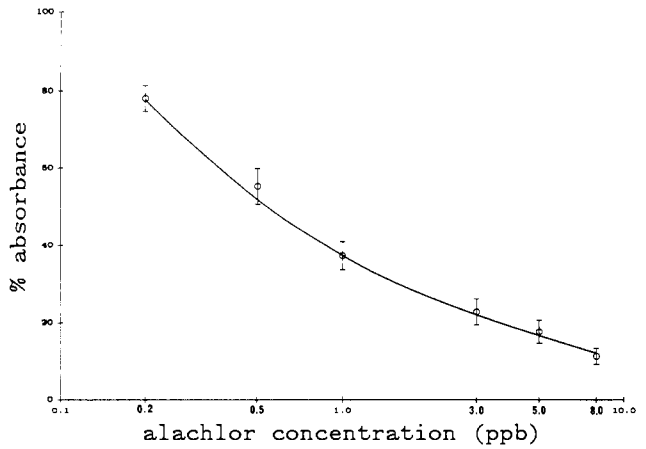

Figure 2. Inhibition ELISA analysis of alachlor in deionized water. (Means and standard deviations calculated from 20 separate analyses.)

BSA conjugate could be considered as recognizing only the alachlor moiety and not any portion of the linking reagent or the protein carrier. The checkerboard assay demonstrated the presence of antibodies in the antisera that reacted with the coating antigen (alachlor–BSA). The inhibition ELISA was subsequently employed to demonstrate that alachlor was able to inhibit the binding of the antibodies to the alachlor–BSA conjugate and to establish the affinity of the antibodies toward alachlor. The optimized alachlor inhibition ELISA was most effective in the range from 0.2 to 8.0 ppb alachlor in water, with corresponding percentages of absorbance ranging from 80% to 10%. The percentages of absorbance for the seven alachlor standards were obtained from 20 separate assays conducted on different days and on different plates. The means and standard deviations were used to generate the standard curve shown in Figure 2. The percentage coefficient of variabilities (% CV) ranged from 4.2% at 0.2 ppb to 18.6% at 8.0 ppb.

Cross-Reactivity of Antibodies. Alachlor belongs to a family of structurally similar chloroacetanilide herbicides. Specificity of the antibodies for alachlor was therefore crucial for the successful application of this assay to environmental samples. Studies were conducted to determine the specificity of the antibodies for alachlor (Figure 3). The cross-reactivity of the antibodies for alachlor was 100%. As illustrated in Table I, the reactivities of the antibodies for acetochlor (**2**) and metolachlor (**3**) were 4.4% and 1.8%, respectively. Among other chloroacetanilide herbicides such as butachlor (**4**), amidochlor (**5**), and propachlor (**6**), none showed more than 2% cross-reactivity. Furthermore, absence of the *N*-methoxymethyl side chain of alachlor, as in the secondary chloroamide **7**, resulted in a complete loss of antibody reactivity.

As mentioned previously, alachlor was conjugated to IgG via thioether linkages in the immunization antigen (Figure 1). It was therefore not surprising to find that some of the alachlor analogues showing the greatest cross-reactivity contained a thioether functional group. The greatest cross-reactivity, almost twice that of alachlor, was observed with sulfide **8** (188.0%; Table I). On the other hand, when the sulfur was oxidized as in sulfoxide **9** and sulfone **10**, the cross-reactivities were reduced to 15.0% and 9.4%, respectively. The secondary amide sulfide **11** produced no cross-reactivity. The methylthio analogues **8–10** have been reported as products of animal metabolism of alachlor (Sharp, 1988). Another commonly observed animal metabolite of alachlor is the mercapturic acid **12**, which showed 65.0% cross-reactivity with the antibodies in the present study. Two major soil metabolites of alachlor have been identified as the oxanilic and sulfonic acids **13** and **14** (Sharp, 1988). The cross-reactivities of the antibodies for these compounds were found to be 2.5% and 2.3%, respectively.

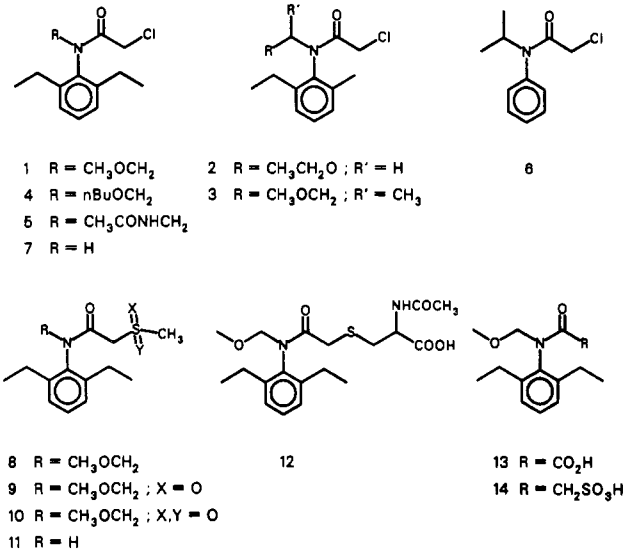

Figure 3. Structures of chloroacetanilide herbicides and alachlor metabolites.

Table I. Cross-Reactivity Data for Alachlor Analogues

no.	analyte	% cross-reactivity
1	alachlor	100.0
2	acetochlor	4.4
3	metolachlor	1.8
4	butachlor	1.2
5	amidochlor	0.6
6	propachlor	0.0
7		0.0
8		188.0
9		15.0
10		9.4
11		0.0
12		65.0
13		2.5
14		2.3

Our cross-reactivity studies demonstrated that the antibodies were sensitive to modifications in the methoxymethyl side chain of alachlor and were able to distinguish alachlor from structurally similar chloroacetanilide herbicides. Minor modifications in the *N*-methoxymethyl side chain of alachlor led to significant reductions in reactivity. The presence of either sulfur or chlorine at the acetamide 2-position of the alachlor molecule was important for the reactivity. Oxidation of the sulfur in the thioether linkage reduced the cross-reactivity substantially.

ELISA Analysis of Environmental Water Samples. The concentration of alachlor in environmental surface water is generally negligible but can vary depending on patterns of herbicide use and on geographical and environmental factors. Under worst-case conditions following planting, alachlor concentrations of less than 20 ppb have been reported for river water samples from Ohio (Baker, 1985). On the other hand, most of the water samples analyzed in the same study during the period from May to August showed nondetectable levels of alachlor.

A potential application of our ELISA method would be for the analysis of alachlor in water. To test this pos-

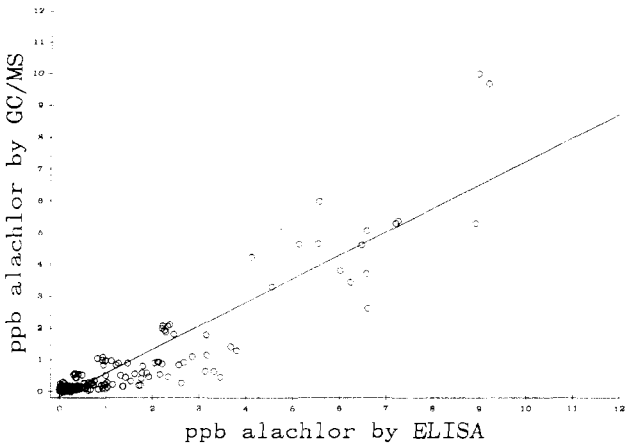

Figure 4. Analysis of alachlor in environmental water samples by ELISA and by GC/MS. (Correlation coefficient was 0.84; 208 water samples were analyzed.)

sibility, 208 water samples were collected for analysis from rivers and water treatment plants. Some of the samples were intentionally spiked with alachlor as controls. The samples were analyzed by ELISA without any pretreatment, and by an established GC/MS method. For the ELISA analysis, a sample size of 1 mL was required. The GC/MS analysis required 1.0 L of sample volume. The results of the ELISA and GC/MS analyses are presented in Figure 4. The x-axis displays ppb of alachlor as determined by ELISA, and the y-axis displays ppb of alachlor as determined by GC/MS. The correlation coefficient of the two methods was 0.84, and the slope of the regression line was 0.74.

Analysis of spiked samples showed that ELISA was generally less accurate and less precise than the GC/MS method. The percentage coefficient of variabilities for ELISA ranged from approximately 10% to 40% and were considerably higher than from the GC/MS analysis. The samples ranged from river water containing suspended soil particulates to finished water from water treatment plants. Since ELISA is conducted without any sample pretreatment, the assay is more susceptible to sample matrix effects. We suggest that the higher variability of the ELISA data contributes to the observed discrepancy between the ELISA and GC/MS methods.

However, the ELISA method can be used very effectively as a primary screen to select water samples with reference to an arbitrary threshold of alachlor concentration. A sample selected by ELISA and confirmed by GC/MS as being below a threshold can be classified as a correct negative. Correspondingly, a sample selected by ELISA and confirmed by GC/MS as being equal to or above a threshold can be classified as a correct positive. Threshold levels were chosen at 0.5, 1.0, and 5.0 ppb.

Using the 1.0 ppb threshold as an example (Table II), 145 out of 147 samples (99%) selected by ELISA to contain below 1 ppb alachlor were confirmed by GC/MS. On the other hand, only 29 out of 61 samples (48%) selected by ELISA to contain greater than or equal to 1.0 ppb alachlor were confirmed by GC/MS. The use of ELISA as a screen would thus have advanced only 29% (61/208) of the samples to GC/MS, thereby reducing the sample load for GC/MS by 71%. The use of a 5.0 ppb threshold in ELISA would have reduced the sample load for GC/MS by 94%. The very high percentage of correct negatives demonstrate that ELISA can be used reliably as a screen. However, because of the comparatively low percentage of correct positives, positive samples selected by ELISA will require confirmation by an alternative method.

Table II. ELISA as a Primary Screen for the Analysis of Alachlor in Environmental Water Samples

threshold, ppb	% correct neg[a]	% correct pos[a]	% samples to GC/MS
0.5	117/122 = 96	54/86 = 63	86/208 = 41
1.0	145/147 = 99	29/61 = 48	61/208 = 29
5.0	193/195 = 99	7/13 = 54	13/208 = 6

[a] The percentages of correct negatives and positives were calculated by dividing the number of correct GC/MS samples by the number of correct ELISA samples. A sample below the threshold of ELISA and confirmed by GC/MS is a correct negative. A sample above or equal to the threshold of ELISA and confirmed by GC/MS is a correct positive. Dividing the number of ELISA positive samples by the total number of samples produced the percentage of samples requiring confirmation by GC/MS.

In conclusion, our results demonstrate an effective application of polyclonal antibodies directed toward alachlor. These antibodies were used to develop an ELISA for alachlor in water showing a detection range from 0.2 to 8.0 ppb. The demonstrated ability of the antibodies to distinguish alachlor from other chloroacetanilide herbicides allowed the successful application of this assay to the analysis of environmental water samples. Based on the analysis of a limited number of environmental water samples, the ELISA method was shown to be less accurate and precise than GC/MS. On the other hand, ELISA offers considerable advantages over GC/MS in terms of cost, speed, sample throughput, and portability. Our results suggest that the most effective use of this ELISA would be as a primary screen, in which samples falling below a threshold level of alachlor can be rapidly and cost-effectively eliminated from further instrumental analysis.

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Registry No. Alachlor, 15972-60-8; water, 7732-18-5.

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HPLC Determination of Thiamin and Riboflavin in Soybeans and Tofu

Sharma M. Fernando and Patricia A. Murphy*

Department of Food Technology, Iowa State University, Ames, Iowa 50011

A high-performance liquid chromatographic (HPLC) methodology has been developed to determine thiamin and riboflavin in soy products. An aqueous reversed-phase C_{18} system with fluorescence detection was employed. Sample preparation included acid hydrolysis and an oxidation step to produce thiochrome from thiamin. Fluorescent chromatograms indicated the presence of many other fluorescing compounds in addition to the vitamins. Thus, vitamin contents of soy products are less than reported in the literature with use of the AOAC methods. Consistent recoveries of 84% for thiamin and 95% for riboflavin were routinely obtained in whole soy flour samples. The contents ranged from 6.26 to 6.85 and 0.92 to 1.10 $\mu g/g$ for thiamin and riboflavin, respectively, in three soybean varieties. Examination of the vitamin distribution on the processing of tofu revealed that the ranges of retention of thiamin and riboflavin were 7.6-15.7% and 11.7-21.1%, respectively. Retention of these water-soluble vitamins in tofu stored in water indicated remarkable losses due to leaching.

The growth of the soy foods industry and the advent of labeling requirements have necessitated the quantitation of the vitamins thiamin and riboflavin in soy products. Attempts to use the standard Association of Official Analytical Chemists (AOAC) method (AOAC, 1984) for the quantitation of riboflavin were not successful, especially because of the interference caused by nonvitamin compounds. Not only did the sample analyzed fluorometrically have a high background fluorescence but the fluorescence also kept changing and did not reach an equilibrium in a reasonable time. The standard AOAC method for the analysis of thiamin is lengthy and involves the use of an enzyme hydrolysis step. Extraction into organic phase and cation-exchange column purification lengthens the procedure and may impart inaccuracy (Pippen and Potter, 1975). Enzymes such as clarase and takadiastase impart fluorescing interferences to the analysis of riboflavin in the same sample extract. Compounds coeluting and cofluorescing with riboflavin are reported to be present in these enzymes (Egberg and Potter, 1975; Edijala, 1979).

Vitamins thiamin and riboflavin are quantitated in food samples by ultraviolet (UV) absorption of thiamin and riboflavin and the fluorescence of thiochrome and riboflavin. The high content of proteins and phospholipids (Smith and Circle, 1978) in soy systems ruled out the use of UV detection because of its low selectivity and sensitivity. Using UV detection techniques, Ayi et al. (1985) were not able to detect significant levels of thiamin in unfortified soy products. The use of manual and automated nonchromatographic methods for thiamin and riboflavin was not promising because of their inability to isolate the vitamin from other interfering components before fluorometric observation. Thus, development of a rapid, accurate, and adequately sensitive method for the analysis of these vitamins, thiamin and riboflavin, in soy products was necessitated.

The method presented in this paper was used to examine the vitamin distribution in the processing of soybeans to tofu. The stability of these vitamins on refrigerated storage of tofu in water was examined. Attempts were made to account for the observed loss of thiamin and riboflavin from tofu on storage. USDA Agriculture Handbook 456 (Adams, 1975) reports the thiamin and riboflvain content of whole soyflour, soy milk, and tofu. The handbook data reported were the average vitamin contents of two samples of unknown soybean varieties. The possible varietal differences in the content of these two vitamins were examined in our study.

MATERIALS AND METHODS

Soybeans. Three soybean varieties (Prize, Vinton, Weber) grown in 1985 in Hudson, IA, were donated by Strayer Seed Co., Hudson, IA. Soybeans were ground in a coffee and spice mill to pass a No. 60 sieve and used in the vitamin analysis.

Tofu Manufacture. The traditional method of tofu manufacture was adapted from Johnson (1984). Soybeans (900 g) were soaked and ground with 6 L of tap water in a Cherry-Burrell vibroreactor. The slurry was cooked at 95 °C for 7 min together with 1 L of water in a steam-jacketed kettle. The cooked slurry was filtered through two cloth sacks. The residue (okara) was washed with 1 L of water. A 20-mL aliquot of filtrate was removed to measure the solids content by using the light-scattering technique of Johnson and Snyder (1978). Upon adjustment of the solids content to 5%, the combined filtrates were reheated to 85 °C and calcium sulfate was added at a concentration of 0.018 N for coagulation. After a 5-min coagulation, the coagulum was cut and poured into a cheesecloth-lined, stainless steel box with perforations on all sides. The coagulum was pressed for 15 min. The resulting tofu was stored at 4 °C in a

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