AGRICULTURAL MATERIALS

Determination of Azadirachtin in Agricultural Matrixes and Commercial Formulations by Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay (ELISA) was developed for azadirachtin (aza), a biopesticide from the neem tree (*Azadirachta indica* A. Juss). The immunogen was synthesized by epoxidation using the furan ring in the aza molecule. Rabbits were immunized with either bovine serum albumin (BSA)-azadirachtin or ovalbumin (OA)-azadirachtin conjugate. Evaluation of the antisera by antibody capture assay showed that the antibody titer of antisera raised against OA-aza was 1:30 000. An indirect competitive ELISA was developed with BSA-azadirachtin as coating antigen and aza-specific antibodies raised against OA-aza immunogen. The immunoassay showed an inhibitory concentration (IC_{50}) value of 75 ppb, with a range of detection from 0.5 to 1000 ppb for azadirachtin [based on regression analysis, $y = 85.87 (-18.89x)$; $r^2 = -0.97$]. Cross-reactivity of the antibodies with 2 aza- derivatives (22,23-dihydro-23β-methoxy azadirachtin and 3-tigloylazadirachtol) was 33 and 29%, respectively. The indirect competitive ELISA was validated and evaluated by quantitating aza in spiked agricultural commodities and from neem formulations. Azadirachtin was spiked into 5 different agricultural commodities: tomato, brinjal, coffee, tea, and cotton seed at 500 and 1000 ppb and recovered at 62–100%. In samples drawn from 6 lots, the aza content in neem-seed kernels ranged from 0.1 to 0.15%; in commercial neem formulations the content ranged from 200 to 2000 ppm. The method developed may be applied to environmental monitoring of aza and quality assurance studies of aza-based commercial formulations.

Azadirachtin ($C_{35}H_{44}O_{16}$), a tetranortriterpenoid from the neem tree (*Azadirachta indica* A. Juss), has generated wide academic (1–3) and industrial interests (4–6). It is the most potent natural insect antifeedant with growth-disruptive activity towards a variety of insect pests (7, 8). Azadirachtin (aza) strongly interferes with molting and reproduction in several species of insects (9–11), which indicates the neuroendocrine system as the target site (12). The dual advantage of aza, as a natural insect control agent and toxic insect growth regulator, make it a viable alternative to synthetic chemical pesticides. It is also an insecticide of considerable environmental safety (13). The constant use of commercial neem formulations on crops such as tobacco, cotton, and vegetables worldwide, confirms its application as a botanical pest control agent (14). Neem extracts are being evaluated within India as a potential human contraceptive, and studies in the United States suggest that it may act as potential genotoxic carcinogen (15).

A sensitive and reliable method is needed for the quantitation of aza in neem extracts, commercial formulations, and foods and for monitoring aza both in ecotoxicological studies and various environmental samples. Current methods using liquid chromatography (LC) and UV spectrophotometry (16) are expensive. Determination by LC requires measuring absorbance at 214 nm, and the use of methanol for extraction requires a solvent exchange because methanol absorbs in this region, thereby limiting sensitivity. Further environmental monitoring of aza demands a simple, cost-effective screening method. Immunoassays, which are highly sensitive and selective analytical tools for detecting trace amounts of target compounds, offer the advantage of simple sample preparation and can be applied to numerous samples simultaneously (17).

The structure of aza is amenable to the development of simple immunoassays. Presently, only one immunoassay method has been reported for azadirachtin (18). Although aza-containing formulations are now being widely used on pulses, vegetables, and stored agricultural commodities such as groundnut, rice, and cotton crops (19), no rapid, sensitive, and specific analytical procedure is available for residue analysis of aza in foods.

The present study describes a specific and sensitive indirect competitive enzyme linked-immunosorbent assay (ELISA) for quantitating aza in different agricultural matrixes. A synthetic strategy is presented for design of an immunogen, subsequent production of polyclonal antibodies, methodology of the immunoassay, and evaluation of the

Received March 22, 2000. Accepted by WEB September 25, 2000.
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Microtiter plates were coated with 50 μL coating buffer per well containing 500 ng coating antigen BSA-aza (equivalent to 76 ng aza).

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Plate was dried overnight at 37°C in ELISA incubator. Immunogen-coated plate was washed thrice, with washing buffer.

↓

Wells were blocked for nonspecific binding with 100 μL/well blocking buffer containing 0.5% fish gelatin for 30min at 37°C.

↓

The plate was washed (3×) with washing buffer and 50 μL per well of a mixture containing antibodies pre-incubated (at 37°C for 2 h) in eppendorf tubes along with different concentrations of aza (reference standards), or sample was added and the plate was later incubated at 37°C for 2 h in an ELISA incubator.

↓

At the end of 2 h, the plate was washed (3×) and incubated at 37°C for 1 h with 50 μL per well of 1:6000 diluted horseradish peroxidase labeled anti-rabbit IgG (raised in goat) in 10mM phosphate-buffered saline (pH 7.2).

↓

After 1 h incubation, the plate was washed (3×) and 150 μL per well substrate buffer was added.

↓

The reaction was terminated after 15 min by adding 100 μL stopping reagent (1.25M H_2SO_4).

↓

Absorbance at 450 nm was recorded by using an ELISA reader along with the reagent blank.

Figure 1. Detailed procedure for indirect competitive ELISA.

method for quantitation of aza in spiked food matrixes, neem seeds, and commercial neem formulations.

Experimental

Apparatus

(**a**) *ELISA reader*.—SLT Spectra II (SLT Lab Instruments, Salzburg, Austria).

(**b**) *Microtiter plates*.—Microlon-600 flat bottom polystyrene, 96-well plates (Greiner Labortechnix, Kremsmunster, Germany).

(**c**) *Spectrophotometer*.—Beckman DU 50 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

(**d**) *Microfuge*.—Beckman TM Microfuge (Beckman Instruments).

(**e**) *Precoated thin-layer chromatographic (TLC) plates*.—Precoated polyester silica gel-G TLC plates, particle size, 2–25 μm (Sigma Chemical Co., St. Louis, MO).

(**f**) *C_{18} RP cleanup columns*.—Disposable C_{18} RP columns, containing 500 mg octadecyl silane (Waters Corp., Milford, MA).

(**g**) *Gel documentation system*.—GDS 5000 (Ultraviolet Products Ltd., Cambridge, UK).

(**h**) *ELISA incubator*.—With air circulation fan (NSW, New Delhi, India).

(**i**) *Rabbit cages*.—Plastic coated, mild steel cages ($45 \times 65 \times 40$ cm); floor space 2600 cm^2.

(**j**) *Refrigerated circulating bath*.—Cool Line, Heto-Holten A/S, Gydevang, Denmark.

(**k**) *Laminar flow ultra clean air unit*.—Micro-Filt, Pune, India.

(**l**) *High speed dry blender*.—Sumeet, Mumbai, India.

(**m**) *High speed solvent blender*.—12 000 rpm (Boss, Mumbai, India).

(**n**) *Lyophilizer*.—FTS systems, MP-Dura Dry (Stone Ridge, New York, NY).

(**o**) *Rotary flash evaporator*.—Superfit, Mumbai, India.

Chemicals and Reagents

(**a**) *Chemicals*.—Azadirachtin (98% pure) was obtained from Chem Services (West Chester, PA). Bovine serum albumin (BSA) fatty acid free; ovalbumin (OA); dimethyl sulfoxide (DMSO); Freund's adjuvants (complete and incomplete); antirabbit immunoglobulin G (IgG, whole molecule raised in goat) labeled with horseradish peroxidase; florisil; fish-gelatin; tetramethyl benzidine (TMB); β-cyclodextrin; and urea hydrogen peroxide were obtained from Sigma. *m*-Chloroperbenzoic acid (MCPBA), siliconized glass wool, and vanillin were from Merck (Munich, Germany). All other chemicals used were of analytical grade.

(**b**) *Sodium phosphate buffer*.—Sodium phosphate, 0.1M, pH 7.2.

(**c**) *Coating buffer*.—Carbonate buffer, 0.1M, pH 9.6, with 0.01% Na N_3.

(**d**) *Washing buffer*.—Sodium phosphate buffer, 0.01M, pH 7.2, with 0.05% Tween 20 (v/v), 0.85% (w/v) NaCl, and 0.01% Na N_3 (w/v).

(**e**) *Blocking buffer*.—Sodium phosphate buffer, 0.01M, pH 7.2, with 0.5% fish gelatin (v/v), 0.85% (w/v) NaCl, and 0.01% Na N_3 (w/v).

(**f**) *Diluent buffer*.—Sodium phosphate buffer, 0.02M, pH 7.2, with 1.7% NaCl (w/v), and 0.6% BSA (w/v).

(**g**) *Substrate buffer*.—*Solution A*.—TMB dissolved in dimethyl sulfoxide (10 mg/mL, w/v). *Solution B*.—Acetate buffer, 0.1M, pH 5, containing 0.25% β-cyclodextrin (w/v), and 0.015% urea hydrogen peroxide (w/v). Three parts solution A was added to 97 parts solution B to prepare substrate buffer.

(**h**) *Stopping reagent*.—H_2SO_4, 1.25M.

(**i**) *Phosphate-buffered saline (PBS)*.—Sodium phosphate 0.01M, pH 7.2, with 0.85% (w/v) NaCl.

(**j**) *Phosphate brine solution (PB)*.—Phosphate buffer, 1M, pH 7.0, containing saturated NaCl.

(**k**) *Coagulating reagent (CR).*—Ammonium chloride solution 1.25% (w/v), containing 2.5% (v/v) phosphoric acid.

(**l**) *Vanillin–H_2SO_4 reagent.*—Vanillin (3 g) and 1.5 mL concentrated H_2SO_4 dissolved in 100 mL absolute ethanol.

Neem Commercial Formulations

Commercial aza-based formulations of neem such as Bioneen, Neemitox, Amazin, and Neemark were procured locally and were coded for validation analysis.

Experimental Animals

Rabbits.—Two males (New Zealand white strain, 16 weeks old) were procured from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (Hyderabad, India). The animals were housed in plastic-coated mild steel cages and were maintained on stock diet obtained from NCLAS and provided with water ad libitum, for the production of polyclonal antibodies.

Hapten Synthesis

Azadirachtin is a low molecular weight (720.2) biorational insecticide. The complex structural features of aza have eluded immunogen synthesis for years. Aza was conjugated to BSA or OA by using the 22-23C- double bond position of the furan ring in the aza molecule to generate an epoxide in a biphasic reaction (20) which was later conjugated to carrier protein BSA or OA.

MCPBA was used as an oxidant to generate aza-epoxide (21). *m*-Chloroperbenzoic acid, 6.8 mg (60% pure, equivalent to 4.08 mg or 23.64 μmoles) in 1 mL dichloromethane (DCM) was washed with 100mM phosphate buffer (pH 7.2: 1 mL × 3). The resulting *m*-chloroperbenzoic acid solution was passed through anhydrous sodium sulfate. Azadirachtin 1 mg (1.388 μmoles) was dissolved in 150 μL dichloromethane and converted into aza-epoxide by the addition of 250 μL of the above *m*-chloroperbenzoic acid solution (equivalent to 1.02 mg MCPBA, 5.9 μmoles; mole/mole ratio of aza to MCPBA was 1:4). The reaction was carried out at 5°C for 100 min with continuous vigorous stirring in an all-glass mini-reaction chamber, connected to a refrigerated circulating

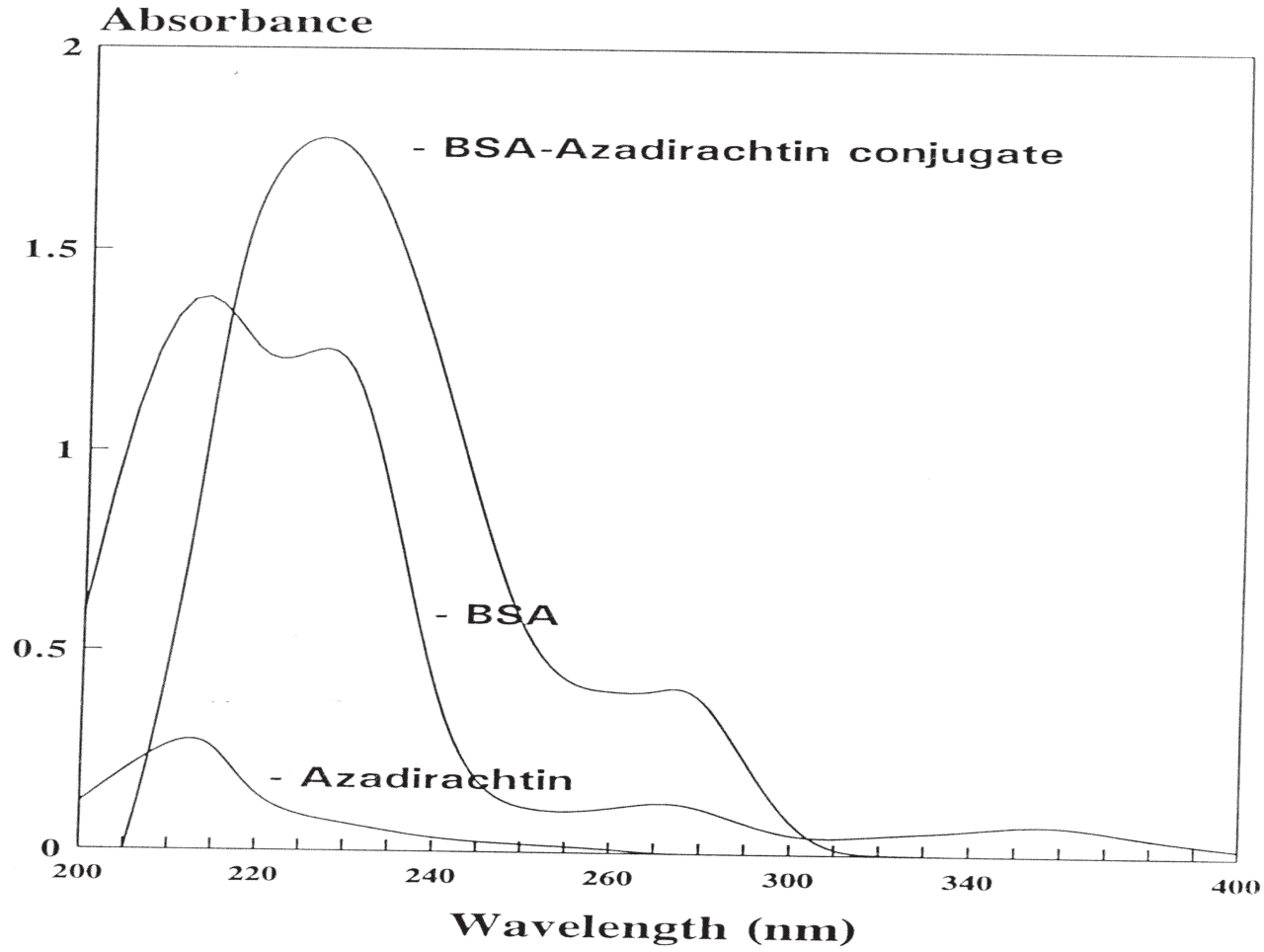

Figure 2. Absorption spectra of aza, BSA-aza, and BSA in phosphate buffer (100mM, pH 7.2).

bath. After 100 min of the reaction, a 5 μL aliquot from DCM fraction was saved for TLC analysis.

Synthesis of Immunogen

(**a**) *Bovine-serum albumin-aza conjugate*.—Aza-epoxide generated was immediately conjugated to BSA. After 100 min of reaction, BSA 2.25 mg (37.6 nmoles; mole/mole ratio of BSA to aza 1:40) in 250 μL phosphate buffer (100mM, pH 7.2) was added to the all-glass reaction chamber containing aza-epoxide in 400 μL DCM. Additionally, 150 μL phosphate buffer (100mM, pH 7.2) was added to maintain the equal ratio of organic and buffer phases. The reaction was continued for 2 h at 22°C with gentle and continuous stirring. At the end of the reaction, the mixture was centrifuged at 10 000 rpm for 5 min in a microfuge to check for any precipitation. The buffer phase and DCM phases were separated. The DCM fraction was checked for unreacted aza by TLC. The aqueous fraction (buffer phase) was processed for protein estimation (22) and for the presence of any unreacted aza.

(**b**) *Synthesis of ovalbumin-aza conjugate*.—OA-aza conjugate was synthesized in a similar way as described above. The aza-epoxide generated was conjugated to OA (3 mg, 67.2 nmoles) in 250 μL phosphate buffer (100mM, pH 7.2, mole/mole ratio of OA to aza 1:20), and an additional 150 μL phosphate buffer (100mM, pH 7.2) was added.The reaction mixture was stirred for 2 h at 22°C. At the end of the reaction, the mixture was centrifuged at 10 000 rpm for 5 min in a microfuge, and the buffer phase and DCM phases were separated. Both phases were processed for TLC analysis. The aqueous phase (buffer phase) was processed for protein estimation by the Lowry method (22).

Characterization by TLC

Polyester silica gel TLC plates were used to characterize the aza-epoxidation reaction. Normal phase analytical TLC was performed on precoated (0.25 μm) TLC plates. A 5 μL aliquot of DCM fraction at the end of 100 min reaction (hapten synthesis) and 5 μg/5 μL (in DCM) azadirachtin reference standard were spotted. Plates were developed in chloroform–acetonitrile (3 + 1) solvent system. After the chromatographic run the TLC plates were air-dried, and compounds were visualized on TLC plates after the plates were sprayed with vanillin–H_2SO_4 reagent. Later the plates were air-dried and heated in a hot-air oven at 110°C for 2–3 min

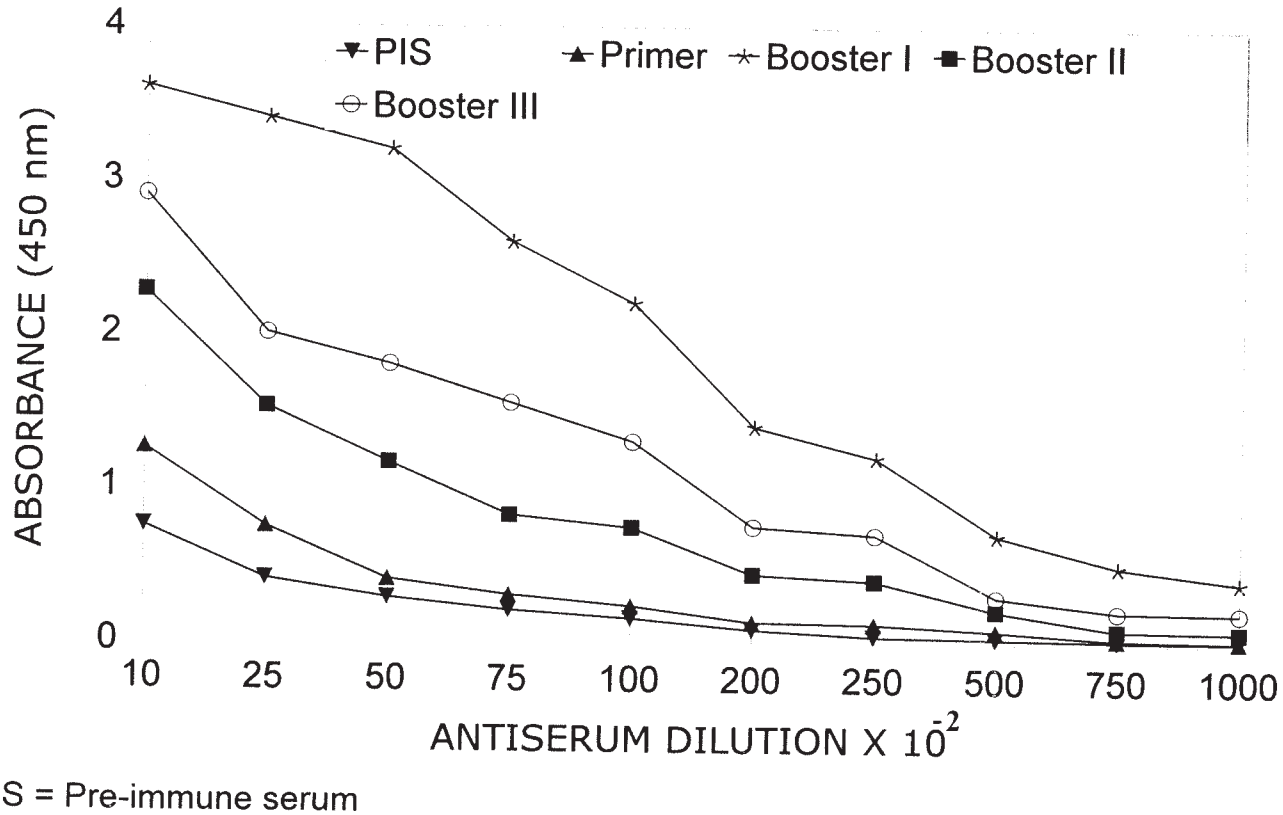

Figure 3. Antibody titer profile of Rabbit No. 2 [antisera raised against immunogen OA-aza; coating antigen BSA-aza (500 ng/well)] as determined by antibody capture assay.

(23). Similarly, aqueous phases of conjugates, namely BSA-aza and OA-aza, were also analyzed for the presence of aza. The R_f values of the compounds were recorded.

(**a**) *Chemical confirmation of conjugates (BSA-aza and OA-aza).*—Confirmation of conjugation and determination of hapten-carrier protein ratio were ascertained on the basis of estimation of free ϵ-amino groups using the trinitro benzene sulfonic acid (TNBS) assay (24). In this assay, concentrations of free ϵ-amino groups of BSA or OA were determined before and after conjugation with aza. To 1 mL BSA-aza, or OA-aza or BSA (100 μg) solution, was added 1 mL 4% $NaHCO_3$ (pH 8.5) and 1 mL 0.01% freshly made TNBS reagent. The reaction was continued at 42° ± 2°C for 2 h and followed by addition of 1 mL 10% sodiumdodecyl sulfate (SDS) and 0.5 mL 1 NHCl solution. Absorbance was monitored at 335 nm with a Beckman recording spectrophotometer.

Conjugation of aza to BSA or OA was calculated with the following equation:

$$\text{Conjugation, \%} = \frac{\text{concn. of } \varepsilon\text{ – amino groups in protein – concn. of } \varepsilon\text{ – amino groups in conjugate}}{\text{concn. of } \varepsilon\text{ – amino groups in carrier protein}} \times 100$$

(**b**) *Recovery of BSA-aza and OA-aza*.—Protein content in aqueous buffer fraction was determined to quantitate recovery of protein conjugates BSA-aza or OA-aza by the Lowry method (22). The conjugates were lyophilized and stored at –22°C until further use.

(**c**) *Spectral analysis*.—UV spectra of pure aza (250 μg/mL), protein-aza conjugate (BSA-aza; 250 μg/mL), and BSA (250 μg/mL) were taken separately, in phosphate buffer (100mM, pH 7.2) to assess the carrier protein-aza conjugation.

Immunization

Production of polyclonal antibodies against BSA-aza or OA-aza.—Two male rabbits (body mass, 2.5–3 kg) were used to produce polyclonal antiserum against BSA-aza (rabbit No. 1) or OA-aza (rabbit No. 2). Before the first injection, pre-immune serum was collected from each animal, lyophilized, and stored at –22°C. A primer dose of 150 μg equivalent of aza (equivalent to 1 mg BSA) was given by multiple site epidermal injections to rabbit No.1. The immunogen was processed under sterile conditions. It was dissolved in sterile saline and emulsified with Freund's complete adjuvant in 1:1 ratio (volume of injection 1.2 mL). For subsequent boosters, Freund's incomplete adjuvant was used. Boosters were given intramuscularly. The doses of immunogen were 75 μg equivalent aza for the first booster, 45 μg equivalent aza for the second booster and 40 μg equivalent aza for the third booster. Doses of immunogen OA-aza for rabbit No. 2 were as follows: a primer dose of 180 μg equivalent aza (equivalent to 1 mg OA) was given by multiple site epidermal injections. It was dissolved in sterile saline and emulsified with Freund's complete adjuvant in 1:1 ratio (volume of injection 1.2 mL). The subsequent doses of immunogen were 152 μg equivalent aza for the first booster, 50 μg equivalent aza for the second booster, and 40 μg equivalent aza for the third booster, respectively. Each booster was spaced by 10–12 days. On day 30 of the primer and day 10 of each booster, blood was collected with a heparinized capillary from retro-orbital plexus. At the end of the immunization schedule, the animals were killed and blood was collected by cardiac puncture. Serum was separated, lyophilized, and stored at –22°C until further use. Antibody titer was determined by antibody capture assay.

Determination of Antisera Titers by Antibody Capture Assay

Antisera titers were determined by checkerboard analysis using antibody capture assay. Antibody titer was determined for antisera (pre-immune serum, primer, booster I, booster II, and booster III) and raised against OA-aza immunogen by using BSA-aza conjugate as a coating antigen on the microtiter plates to avoid interference by antibodies specific to the carrier protein (OA). Microtiter plates were coated with 50 μL coating buffer/well containing different concentrations of BSA-aza (protein equivalent 250, 500, 750, and 1000 ng or equivalent to 38, 76, 114, and 152 ng bound aza per ELISA well). The plate was dried overnight at 37°C in an ELISA incubator. Antigen-coated (BSA-aza) plate was washed 3 times with washing buffer. Wells were blocked with 100 μL blocking buffer/well containing 0.5% gelatin for 30 min at 37°C to avoid nonspecific binding. The plate was washed 3 times with washing buffer, and 50 μL different dilutions of antisera (1:1000–1:100 000) raised against OA-aza immunogen in diluent buffer was dispensed per well. The plate was incubated at 37°C for 2 h. Later the plate was washed 3 times and incubated at 37°C for 1 h with 50 μL/well 1:6000 diluted horseradish peroxidase-labeled antirabbit IgG (raised in goat) in 10mM PBS buffer, pH 7.2, per well. After incubation, the plate was washed 3 times with washing buffer, and 150 μL substrate buffer was added per well. The reaction was terminated after 15 min by adding 100 μL stopping reagent (1.25N H_2SO_4) per well. Absorbance at 450 nm was recorded using an ELISA reader.

Development of Indirect Competitive ELISA for Azadirachtin

In competitive ELISA, antibodies were incubated for 2 h along with different concentrations of aza or sample extract in separate test tubes before being added to ELISA plates. Different concentrations of aza reference standard or aza isolated from neem kernels, food matrixes, and formulations were dissolved in 1.6% DMSO in separate eppendorf tubes to which an equal volume of 1:15 000 dilution of antisera in 2X diluent buffer was added. The test tubes were incubated for 2 h at 37°C in the dark. The rest of the procedure was similar to the antibody capture assay. Total duration of the immunoassay was <6 h. Final concentration of DMSO in the assay was 0.8%. Figure 1 gives a detailed protocol for the indirect competitive ELISA.

Cross-Reactivity Studies of Aza-Related Compounds

Cross-reactivity of antibodies was tested with naturally occurring azadirachtin related compounds in neem seeds 22,23-dihydro-23β-methoxy azadirachtin and 3-tigloylazadirachtol. These compounds were isolated by the method described by Kraus (25).

Evaluation of the Method

The ELISA methodology was evaluated by spiking studies. Food matrixes such as tomato and brinjal, beverage matrixes such as coffee and tea, and an agricultural matrix (cotton seed) were spiked at 500 and 1000 ng/g (500 and 1000 ppb) aza. The method was also used to quantitate aza in neem-seed kernels and commercial aza-based formulations.

(**a**) *Spiking studies in food matrixes: method of spiking.*—Beverage matrixes such as coffee *(Coffea arabica)* and tea (*Camellia sinensis)*; vegetable matrixes, such as tomato (*Lycopersicum esculantum)* and brinjal *(Solanum melanginum)*; and an agricultural matrix, cotton seed *(Gossipium hirsutum)*, were used for the spiking studies. Coffee, tea, tomato, brinjal, and cotton seed were procured from local markets and finely powdered in a high speed blender. Azadirachtin (dissolved in methanol) was spiked at 2500 and 1000 ppb in all the food matrixes. Tomatoes and brinjal were cut into small pieces and 10 g spiked sample was used to extract the biopesticide. Spiked aza was extracted with methanol as the extraction solvent (50 mL/10 g sample). Simultaneously, samples (without aza) were also processed as controls for ELISA analysis.

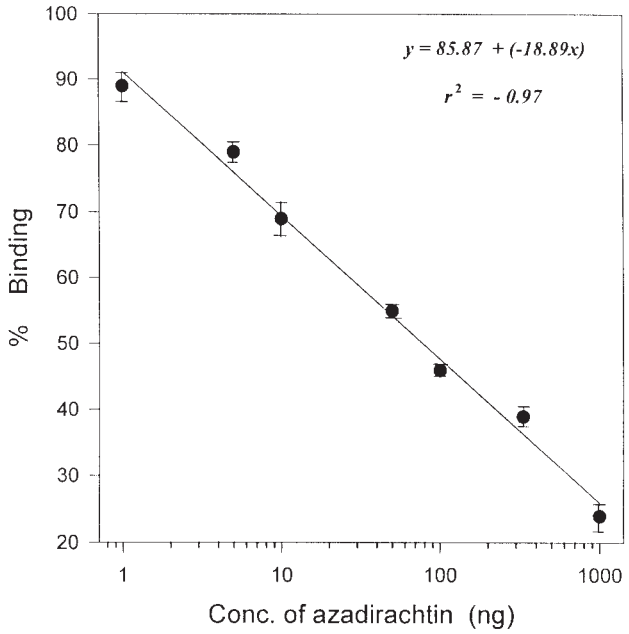

Figure 4. Standard displacement plot for azadirachtin as determined by indirect competitive ELISA [regression analysis, $y = 85.87 (–18.89x)$; $r^2 = –0.97$].

Method of sample extraction and processing.—Agricultural matrixes for extraction of biopesticide were processed according to the method described (26). Spiked food and agricultural matrixes were incubated with aza overnight for 16–18 h. Vegetable matrixes (tomato and brinjal) were extracted into methanol (10 g wet weight per 50 mL) in a high speed solvent blender for 5 min at 12 000 rpm. The solvent from the matrix was recovered by filtration. The resulting clear methanolic extract was flash evaporated and the residue was taken into methanol: H_2O (70 + 30) and partitioned with dichloromethane 3 times. The DCM layer was passed through anhydrous sodium sulfate. The resulting DCM layer was concentrated by flash evaporator and dried under N_2. The samples were kept at –22°C until further use.

Matrixes rich in phenolics (coffee, tea, and cotton seed) were extracted into acetonitrile (10 g/50 mL) in a high speed solvent blender. The acetonitrile extract was passed through siliconized glass wool and the clear extract, taken in a separating funnel, was treated with equal volume of phosphate brine and coagulating reagent, shaken vigorously and partitioned with hexane. The resulting aqueous phase was partitioned with DCM 3 times. The DCM layer was dried and stored as described above. Aza extracted from various spiked food matrixes was dissolved in DMSO to give a concentration of 1.6% DMSO. An appropriate dilution of sample was used for analysis by indirect competitive ELISA. Simultaneously, unspiked samples (without aza) were also processed and served as controls.

(**b**) *Extraction of aza from neem seeds.*—For quantitation of aza content in neem seeds, neem kernels were extracted with hexane in a soxhlet apparatus for 6 h. Ten grams of defatted kernel powder was extracted with three 50 mL portions of ethanol (27) for 6 h. The ethanol was removed by flash evaporation and subjected to 2 quick, efficient partitionings: between petroleum ether and 95% aqueous methanol, and between water and ethyl acetate. The ethyl acetate fraction was then vacuum-evaporated, and the residue was dissolved in 1.6% DMSO and estimated for azadirachtin content by indirect competitive ELISA.

(**c**) *Isolation of aza from commercial formulations*.—Commercial neem formulations were either neem oil- or extract-based. Aza from these formulations was isolated by the method of Sundaram (28). A 3 mL portion of the formulation was taken in 10 mL 95% aqueous methanol and subjected to hexane partition. The methanolic layer was flash-evaporated and subjected to a quick partition between (70 + 30) MeOH–H_2O/DCM. The DCM layer was passed through anhydrous sodium sulfate and dried under vacuum. Residue was dissolved in acetonitrile (ACN). The ACN fraction was again subjected to hexane partition (1 + 1). The ACN layer was flash-evaporated to dryness, and the residue was processed for florisil column cleanup.

Florisil column cleanup.—The extracts from formulations required column cleanup to remove the bulk of co-extractive impurities before analysis by ELISA. The glass column (15 × 1 cm) was plugged with glass wool at the bottom, and 10 g florisil was sandwiched and settled uniformly between the glass wool of anhydrous sodium sulfate (28). The column

Table 1. Cross-reactivities of antibodies raised against ovalbumin-aza conjugate with azadirachtin derivatives as determined by indirect competitive ELISA

Compound	IC_{50} value,[a] ng	Cross-reactivity, %
Azadirachtin	75	100
22,23-Dihydro-23β-methoxy azadirachtin	225	33
3-Tigloylazadirachtol	255	29

[a] IC_{50} = inhibitory concentration of the analyte at 50% antibody displacement.

was preconditioned with 10 mL ethyl acetate, loaded with an aliquot of aza extract, and eluted with 15 mL ethyl acetate. The eluate containing aza was evaporated to dryness under vacuum. Residue was reconstituted in 1 mL ethyl acetate in an eppendorf tube, dried under N_2, and stored at –22°C until further use.

Sample preparation.—All 4 commercial neem formulations were processed by the above described method. Aza isolated from each sample was dissolved in 1.6% DMSO and processed for estimation of azadirachtin content by indirect competitive ELISA. The final concentration of DMSO in the immunoassay was 0.8%.

Results and Discussion

Hapten Synthesis

The immunogen design depends on the purpose of immunoassay and the molecular structure of the hapten molecule. To produce specific antibodies, the hapten-carrier protein conjugate should be designed to preserve and enhance structural determinants such as charge, electron density, electropolority, and isomerism. Any change in these determinants may affect the specificity and affinity of the antibodies produced. In the present study, hapten aza-epoxide was synthesized by an epoxidation method. The complex structural features of azadirachtin with a densely packed array of many different oxygen functions made it difficult to determine which portion of the molecule was the most unique. In this methodology, epoxidation of the C-22/C-23 bond of the furan ring of the aza molecule was achieved by using *meta*-chloroperbenzoic acid as an oxidant (21). Because antibodies recognize best the part of hapten most distant from the conjugate linkage, we selected a group far from the characteristic groups and without any concomitant structural rearrangement in the aza molecule. Hence selecting the C-22 and C-23 double bond which is most distant from the biologically active groups for conjugation would project the whole azadirachtin molecule for good antibody response. In the earlier reported ELISA (18) for azadirachtin, hydroxyl (OH) groups were used for hapten synthesis by the hemisuccinate method. Hemisuccinate was used as a link to the carrier protein at the 3-OH position of the aza molecule. In this approach the stability of the hapten molecule was low because of the destabilizing effect of the free carboxylic group. The intrinsic acidity of the compound (aza-hemisuccinate) may result in rapid degradation. In the present study, a simple single step procedure for synthesis of hapten, aza-22, 23-epoxide used MCPBA, a stable oxidant, to generate epoxide as described earlier (21).

MCPBA was chosen as a chemical oxidant (20) in a 2-phase system. The reaction was carried out in DCM in contact with an aqueous buffer (pH 7.2) to remove the by-product *m*-chlorobenzoic acid. The completion of the epoxidation reaction (100 min) was judged by the disappearance of aza as determined by TLC.

Formation of aza-22,23-epoxide was confirmed by TLC analysis of the DCM phase. Aza in DCM before epoxidation gave a single spot (green, R_f 0.25); after epoxidation (100 min) a major spot was revealed with R_f value 0.77 (major spot >90% recovery), followed by a minor spot with R_f 0.49, in $CHCl_3$–ACN (3 + 1) solvent system.

Synthesis of Immunogens

The aza 22,23-epoxide generated was conjugated directly to ϵ-amino groups of lysine residues of BSA or OA as described earlier (21). BSA-aza and OA-aza were conjugated in a 2-phase system, and conjugates were recovered in the aqueous buffer fraction. Analysis of buffer fraction by TLC revealed no free aza, indicating the conjugation of aza to BSA or OA by its lysine residues. Spectral analysis of aza (250 μg/mL), BSA-aza (250 μg/mL), and BSA (250 μg/mL) indicated increased absorbance in BSA-aza at 228 nm (absorbance 1.73) over native BSA (absorbance 1.24) and suggested conjugation of aza to BSA. Aza showed a peak at 217 nm. Spectral analysis of aza, BSA-aza conjugate, and BSA are shown in Figure 2. Further, the molar ratio of BSA or OA to aza was established specifically by TNBS assay (24) and was 1+14 for BSA-aza;

Table 2. Percent recovery of azadirachtin spiked to beverages, cotton seed, and food matrixes as determined by indirect competitive ELISA

Sample	Amount of aza spiked (in ppb), ng/g	Amount recovered[a] (in ppb), ng/g	Recovery %
Coffee	500	356 ± 10 (2.80)	71
	1000	766 ± 12 (1.56)	77
Tea	500	336 ± 20 (5.95)	67
	1000	760 ± 10 (1.31)	76
Cotton	500	312 ± 9 (2.88)	62
	1000	685 ± 15 (2.18)	68
Tomato	500	432 ± 12 (2.77)	86
	1000	860 ± 20 (2.32)	86
Brinjal	500	466.5 ± 14 (3.00)	93
	1000	1000 ± 11 (1.10)	100

[a] Values = mean ± SD; values in parentheses are coefficients of variation; n = 3 (values are based on triplicate analysis).

Table 3. Quantitation of azadirachtin from neem-seed kernels as determined by indirect competitive ELISA

Samples	Aza content, mg/g[a]
Lot 1	1.4 ± 0.20 (14)
Lot 2	1.3 ± 0.17 (13)
Lot 3	1.5 ± 0.10 (7)
Lot 4	1.4 ± 0.10 (7)
Lot 5	1.0 ± 0.20 (20)
Lot 6	1.2 ± 0.20 (17)

[a] Values = mean ± SD; values in parentheses are coefficients of variation; $n = 3$ (values are based on triplicate analysis); values corrected for moisture content of seeds.

(14 moles aza were conjugated to 1 mole BSA). In OA-aza conjugate, 11 moles aza were conjugated to 1 mole OA. Recoveries of conjugate (BSA-aza or OA-aza) in terms of protein conjugates were 78–90% for BSA-aza and 54–56% for OA-aza. The lower recovery of OA-aza may be attributed to the difference in protein solubility.

Determination of Titer of Antisera Against BSA-Aza and OA-Aza

Titer of antisera raised against OA-aza determined by antibody capture assay showed that a dilution of 1:30 000 antisera (booster I, rabbit No. 2) at 500 ng BSA-aza/well (equivalent to 76 ng aza) as coating antigen gave absorbance of 1.2 at 450 nm when horseradish peroxidase was used as enzyme label. Antibody response of rabbit No. 2 is shown in Figure 3. Appropriate reagent and BSA blanks (coating BSA only) were simultaneously run. Conversely, antisera raised against BSA-aza showed absorbance of 0.7 at 1:3000 dilution. The antibody titer of rabbit No. 1 (raised against BSA-aza) gave a lower titer than antisera raised against OA-aza in rabbit No. 2. Thus antisera raised against OA-aza was used to develop and standardize the ELISA.

Specificity of Antibodies and Recognition of Conjugated Azadirachtin

Antibodies raised against ovalbumin conjugate (OA-aza) were tested by ELISA for binding to conjugate of the same hapten (BSA-aza). The superior binding of OA-aza antibodies to BSA-aza immunogen coated onto the ELISA plate, indicates that polyclonal antibodies are specific to aza (Figure 3). This clearly demonstrates the ability of the antibodies to recognize the azadirachtin molecule conjugated to BSA by epoxidation.

Indirect Competitive ELISA and Recognition of Free Azadirachtin

Figure 4 depicts the standard displacement curve for various concentrations of standard aza. Assay sensitivity ranged from 0.5 to 1000 ng. Under optimized conditions (OA-aza antiserum of rabbit No. 2: at a final dilution 1:30 000/well, BSA-aza 500 ng/well), the assay for azadirachtin showed a standard displacement curve [$y = 85.87$ (–18.89x); $r^2 = –0.97$] that allowed measurement of analyte from 0.5 to 1000 ng with IC_{50} value of 75 ng (Figure 4). Standard displacement curves were reproducible, as indicated by their low standard deviations between immunoassays, and the reproducibility of ELISA using synthesized conjugate BSA-aza as coating antigen for 9 months indicated the stability of the conjugate. A literature survey indicated that an indirect competitive ELISA was developed with an IC_{50} value of 100 ppb and a detection range of 10–1000 ppb (18). However, that method was not evaluated for application in the residue analysis of aza in agricultural commodities and aza-based commercial formulations.

Cross-Reactivity Studies of Aza-Related Compounds

Antibodies were tested by indirect competitive ELISA for their cross-reactivity with 2 azadirachtin-related compounds, 22,23-dihydro-23β-methoxy azadirachtin and 3-tigloylazadirachtol, which co-occur in small quantities (25). The cross-reactivity of the 2 compounds was 33 and 29%, respectively (Table 1). Although conventional LC techniques using UV detect azadirachtin down to 0.2 μg/g (29), they fail to detect bioactive degradation products of azadirachtin because of missing UV-absorbing moieties such as tigloyl or vinyl ether groups. The difference in IC_{50} between aza- derivatives and aza confirms the ability of antisera to distinguish among closely related molecules on the basis of their structure.

Evaluation of the Method

Spiking Studies in Food Matrixes

Two beverage matrixes (coffee and tea), 2 vegetable matrixes (tomato and brinjal), and an agricultural matrix (cotton seed) were processed for quantitation of aza. In the foods, recovery of aza was 62–100% as quantitated by indirect competitive ELISA. Food matrixes were spiked with aza at 500 and 1000 ppb. Recovery from brinjal and tomato was 100 and 86%, respectively (Table 2). In beverages rich in phenolics (coffee and tea), the recovery of aza was 71–77 and 67–76%, respectively. In high-density, fat-containing matrix (cotton

Table 4. Quantitation of azadirachtin from commercial neem formulations as determined by indirect competitive ELISA

Commercial formulation, Code No.	Concentration of azadirachtin	
	Conc. as recorded on product label, ppm (μg/mL)	Estimated value,[a] ppm (μg/mL)
Formulation I	300	300 ± 5 (1.66)
Formulation II	300	200 ± 3.5 (1.73)
Formulation III	3000	2000 ± 35 (1.75)
Formulation IV	3000	2000 ± 34 (1.70)

[a] Values = Mean ± SD; values in parentheses are coefficients of variation; $n = 3$ (values are based on triplicate analysis).

seed), the recovery of aza was in the range of 62–68%, which variation can be attributed to the matrix effect (26, 30). Quick monitoring of a large number of samples in different environmental matrixes demands cost-effective screening methods. Wide usage of azadirachin-based neem formulations on cotton and vegetable crops demands a suitable analytical method for detection of aza residues. The present ELISA for azadirachtin based on polyclonal antibodies is simple and cost-effective, and offers a potential analytical tool comparable to LC and other conventional methods. Levels of azadirachtin detection in environmental samples such as soil (31) or foliage (32) have been in the parts per millions (μg/g) range, whereas the present enzyme immunoassay is highly sensitive with an IC_{50} value of 75 ng. Despite poor quality control of the neem extracts in commercial formulations, neem-based insecticides hold promise for use as plant protection products.

Estimation of Aza Content in Neem Seeds

The developed ELISA was evaluated by quantitation of the aza content in neem-seed kernels by indirect competitive ELISA (Table 3). Neem seeds from different geographic regions vary considerably with respect to aza content. Aza content present in neem seeds has been reported at 0.2–0.6% (33), 0.1–0.15%, as quantitated by the present ELISA method. Geographical variations in seed content probably exist, but to our knowledge this has not been systematically investigated. Further, storage studies on neem seeds indicate that aza content decreases during prolonged storage (34).

Quantitation of Aza in Neem Commercial Formulations

Four commercial neem formulations were processed for isolation of aza and subsequent quantitation of aza by indirect competitive ELISA (Table 4). Neem oil- and extract-based formulations contain aza at 0.03–0.3% (claimed by manufacturers on product label) with surfactants and stabilizers. These interferences were removed for isolation and further purification of aza on a fluorisil column (28). Quantitative analysis of aza from formulations by ELISA indicated that aza content varied. In 2 of the commercial formulations, aza content was less than the concentrations given on the product label, which could be attributed to deterioration of aza during storage. Formulations based on neem-seed kernel extracts may contain additional constituents such as triglycerides, fatty acids, and surfactants (35, 36); hence, a fluorisil cleanup was used to remove these interferences before processing for ELISA. Estimation of aza content in these commercial formulations using LC has been reported (29, 32). These methods used reversed-phase chromatography with detection by UV absorption at 215 nm and reported a lower limit of detection of aza at 0.001% (w/w). Most of the contribution to absorbance at 215 nm is from the α,β- unsaturated carbonyl chromophore in tiglate ester and vinyl ether. Obviously these conventional techniques fail to detect other analogs and limonoids in the absence of UV moieties. The present ELISA method is sensitive enough to quantify aza in the commercial formulations at ng/g (ppb) level.

The ability of the antibody to discriminate compounds of different biological activity may facilitate quality assurance of biorational insecticidal formulations based on azadirachtins. The ELISA developed in the present study can be successfully used in the quality assurance studies of aza-based commercial formulations and residual analysis of aza. Thus, it is an inexpensive, specific, and sensitive analytical procedure for environmental monitoring of azadirachtin and may find wide application.

Acknowledgments

We thank the Australian Centre for International Agricultural Research (ACIAR; Canberra, Australia) for research funding (Grant No. 9309). We also thank John Skerritt, Deputy Director, R&D, ACIAR, for the kind gift of pure azadirachtin. N.B.R.K.Venugopal is grateful to the Council of Scientific & Industrial Research (CSIR; New Delhi) for the award of Pool Scientist-fellowship. We thank the University Grants Commission (New Delhi, India) for providing instrumentation facilities under the COSIST program in the Department of Biochemistry (Osmania University, Hyderabad, India).

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