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Application of an Enzyme-Linked Immunosorbent Assay (ELISA) to Determine Deltamethrin Residues in Milk

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Because deltamethrin has a broad-spectrum of insecticidal activity and relatively low mammalian toxicity, this compound is being widely used for field-treatment of crops and the control of endo- and ecto-parasites on animals (Leahey 1985; Muccio et al. 1997). Although this chemical is relatively susceptible to biotransformation and excretion in mammals compared to organochlorinated and organophosphate compounds, the possibility of contamination in milk is very high due to its wide application and high lipophilicity (Akhtar et al. 1992; Venant et al. 1990). Deltamethrin is reported to cause various adverse effects in epidemiological and experimental studies (Agarwal et al. 1994; He et al. 1989; Kontreczky et al. 1997; Shukla et al. 2001). High levels of deltamethrin causes neurotoxicity with pawing and burrowing motions reported in mammals (Miyamoto et al. 1995).

The MRL for deltamethrin in milk prescribed by the FAO-Codex Alimentarius (1993) is 0.02 ppm. Many methods have been described for the determination of deltamethrin residues in milk by using various analytical techniques such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) (Akhtar 1982; Akhtar et al. 1992; Bissacot and Vassilieff 1997; Muccio et al. 1997; Venant et al.1990). Such methods are relatively expensive and skill-intensive. An immunoassay would provide a fast, sensitive, and selective approach for the detection of deltamethrin at trace levels (Lee et al. 1998; Lee et al. 2002). We developed an immunoassay for deltamethrin in a previous study (Lee et al. 2002). In this study, we apply this assay to the detection of deltamethrin in milk.

MATERIALS AND METHODS

A competitive indirect ELISA format was used as described previously (Lee et al. 2002). The optimized reagent concentrations were: 0.06 μg/mL of coating antigen, antibody at a dilution of 1:22,000, deltamethrin in 40% methanol-2 *M* PBS, pH 7.0. The stock solution consisted of 10 mg of deltamethrin dissolved in 2-mL of acetonitrile and stored at room temperature. No degradation was noted. Working standards were prepared by diluting the stock solution to the appropriate concentration in acetonitrile. This standard solution was used for the preparation of both calibration solutions and fortified samples.

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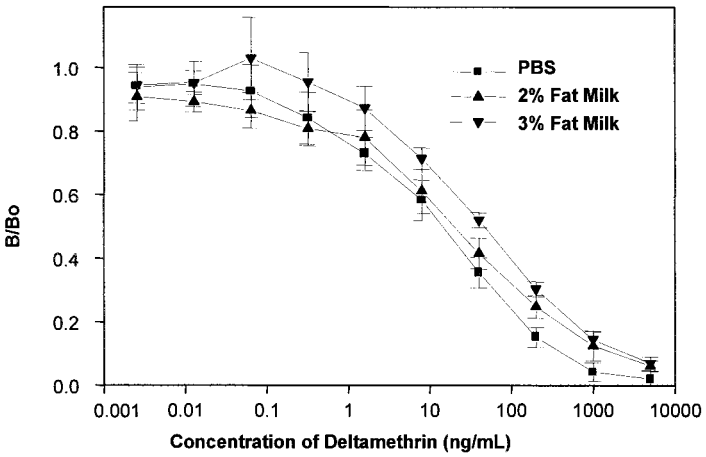
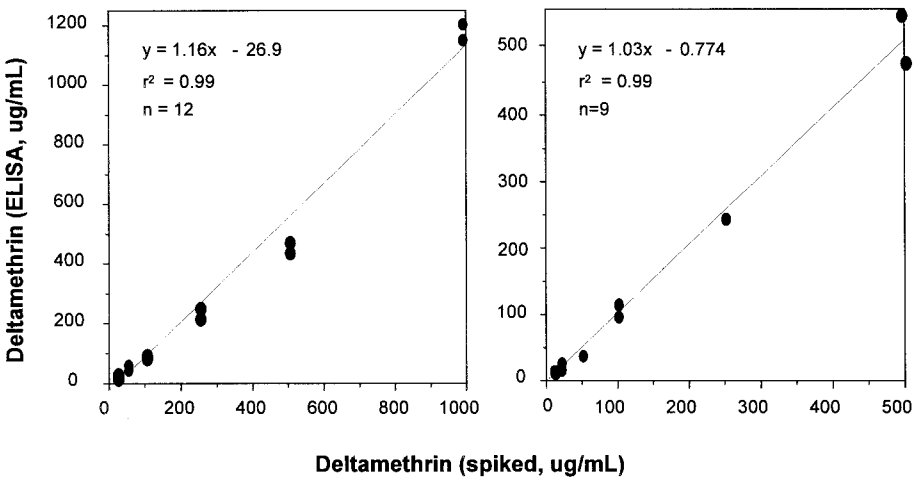


Figure 1. Milk extracts spiked with deltamethrin at various levels and analyzed by ELISA. Concentration of reagents: antiserum, 1/22,000; coating antigen, 0.06 μg/mL.

Regular-fat (3%) and low-fat (2%) milk were obtained from local supermarkets. The method of sample extraction was modified from the protocol described by Bissacot and Vassilieff (1997). A 2-mL sample of spiked milk sample was quantitatively transferred to an Erlenmeyer flask and acidified with 1.0 *N* HCl to approximately pH 4.0. Then, 5-mL of acetonitrile was added to the sample and the Erlenmeyer flask was closed and mixed mechanically for 30 min. The sample was filtered in a glass funnel with a 12.5-cm diameter No. 42 Whatman filter paper (Whatman International Ltd., Kent. U.K.), and the filtrate was collected in a separatory funnel. The residue in the paper filter was transferred to the same Erlenmeyer flask. Five ml of acetonitrile was added, and the flask was sealed and shaken mechanically for 15 min. The sample was then filtered again using the same procedure and the same filter paper. The filtrate was added to the same separatory funnel with the first filtrate. Three mL of *n*-hexane was added to the filtrate, and the mixture was shaken for about 1 min. The procedure was repeated twice. The acetonitrile phase was collected in a glass tube. To the hexane phase remaining in the separatory funnel, 9 mL of acetonitrile was added and shaken for 1 min. The hexane phase was discarded, and the acetonitrile layer was collected in a glass tube containing the first acetonitrile phase. The acetonitrile phase was evaporated under a gentle stream of nitrogen and heated to dryness at 50°C. The residue was redissolved with 1.0 mL of methanol. An aliquot was analyzed by ELISA diluted with 40% methanol in 2 *M* PBS (1 : 9). To investigate the recovery rate, each milk sample was spiked at 20, 100, and 500 ng of deltamethrin/mL. Standard curves were fitted using the four-parameter logistic equation.

Liquid chromatography-tandem mass spectrometry (LC-MS-MS) was also used to identify deltamethrin in milk samples. The LC-MS-MS system consisted of a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an atmospheric pressure ionization



(A) (B)

Figure 2. Relationship between deltamethrin spiked into milk samples (A; 2% fat milk, B; 3% fat milk) and determined by ELISA in a blind fashion.

source. The HPLC system consisted of a Waters model 2790 separations module (Waters Corporation, Milford, MA) equipped with Waters model 2487 dual λ absorbance detector (Waters Corporation, Milford, MA). The mass spectrometer was coupled to the outlet of an HPLC column. Chromatographic separation was performed using an Xterra MS C18 column (3.5 μm, 30 × 2.1 mm i.d.; Waters Corporation, Milford, MA) at ambient temperature isocratically (acetonitrile containing 0.1 % formic acid/water containing 0.1 % formic acid = 6/4(v/v)). The electrospray ionization (ESI) was performed in positive ion mode with a capillary voltage of 3.5 kV. The source and the desolvation temperature were set at 100 °C and 300 °C, respectively. The optimum cone and collision voltage was 80 V and 40 V, respectively.

RESULTS AND DISCUSSION

Food matrixes may influence immunoassay performance. Milk is a difficult matrix for all analytical methods because it contains high levels of lipids, sugars, proteins and other materials in a heterogeneous aqueous base. This matrix must often be simplified to attain the necessary sensitivity (Bennett et al. 1997). Therefore, matrix effects should be determined before an assay can be applied to samples containing the pesticide. The simplest way to minimize matrix effects is sample dilution (Rosso et al. 2000). To evaluate the sensitivity of the assay, the milk sample was directly analyzed by ELISA without any pretreatment or diluted 5, 10, 15, and 20 times with 40% methanol-2*M* PBS.

The sensitivity was very low (I_{50} >370 ng/mL) (Table 1), probably because the antibody was involved in nonspecific interactions with lipids, sugars, and proteins in milk. In order to reduce the matrix effect, the extraction method described in the Materials and Methods was applied to milk samples.

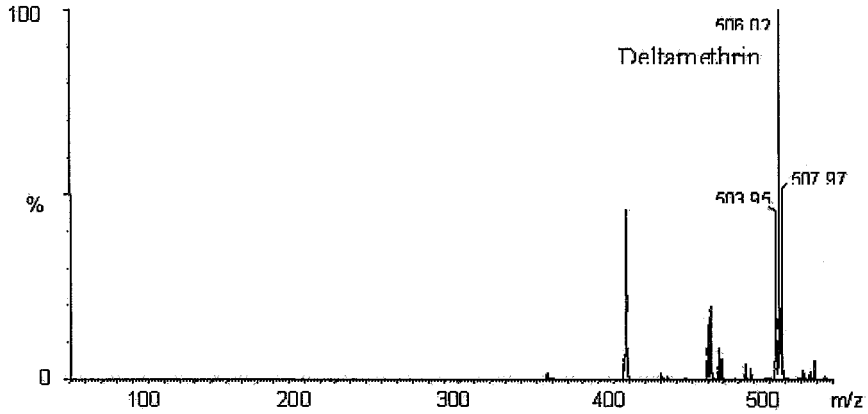


Figure 3. Mass spectrum of deltamethrin.

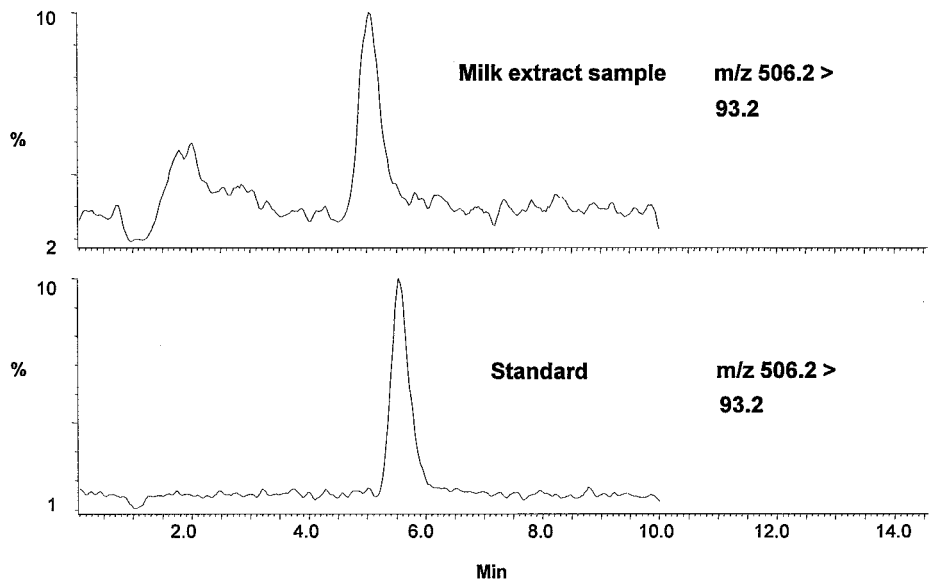


Figure 4. Multiple reaction monitoring profiles of deltamethrin standard and milk extract sample under ESI LC-MS-MS conditions.

Standard curves for the deltamethrin competitive immunoassay prepared in 40% methanol-2*M* PBS are shown in Figure 1. The I_{50} values in PBS, low-fat milk (2%), and regular-fat milk (3%) were 17.5 ± 3.6, 41.1 ± 6.8, and 48.9 ± 5.8 µg/L, respectively. Since the I_{50} value of 3% fat milk was the highest, milk fat is likely contributing to matrix interferences.

The lower detection limits (LDL) in PBS, low-fat milk (2%), and regular-fat milk (3%) were 1.05 ± 0.47, 1.37 ± 0.56, and 2.22 ± 0.99, respectively. The LDL was

Table 1. ELISA results for low-fat milk samples spiked with deltamethrin and analyzed without cleanup.

Dilution factor	A_{max}	Slope	I_{50} (*ng/mL*)	A_{min}	R^2
5	0.424	0.272	3.87×10^6	-1.50	0.936
10	0.390	0.159	1.15×10^{10}	-3.21	0.984
15	0.422	0.239	2.32×10^4	-0.45	0.971
20	0.384	0.444	371	-0.05	0.991

[a] Milk samples were diluted with 40% methanol-2*M* PBS. Each set of data represents the average of three experiments.

Table 2. Spiked recovery in milk by ELISA [a]

Spiked conc (μg/L)	2% Fat Milk		3% Fat Milk	
	Measured (ppb)	Recovery (%)	Measured (ppb)	Recovery (%)
0	<0.02		<0.02	
20	30 ± 7.4	148 ± 37.2	22 ± 5.5	112 ± 27.4
100	94 ± 6.2	94 ± 6.2	108 ± 9.4	108 ± 9.4
500	460 ± 17.2	92 ± 3.44	513 ± 36.7	103 ± 7.3

[a] Different amounts of deltamethrin (in acetonitrile) were added to milk samples to give final concentrations in milk of 20, 100 and 500 μg/L. After mixing and standing for at least 2 hr at room temperature, these samples were extracted as described above then analyzed in a blind fashion.

estimated as the concentration that corresponded to the absorbance of the control (zero concentration of analyte) minus 3 times the standard deviation of the control (Grotjan and Keel 1996). The assay would thus be capable of screening milk samples for deltamethrin contamination at or below MRLs (20 ppb) set by FAO-Codex Alimentarius (1993).

Recovery of 0, 20, 100, and 500 μg/L of deltamethrin spiked into 2% fat and 3% fat milk is shown in Table 2. All recoveries were > 90% of the spiked value.

Assay validation was conducted by spiking milk with deltamethrin concentrations ranging from 0 to 1000 ppb in a blind fashion and extracting with acetonitrile and *n*-hexane, then analyzing by ELISA. A good correlation between spiked and ELISA-measured deltamethrin was obtained from linear regression analysis (Figure 2).

To identify and/or quantify deltamethrin in milk, the optimum conditions for LC-MS-MS were investigated. As shown in Figure 3, a typical pseudo molecular ion [M+H]+ of deltamethrin was obtained with full scanning and also the isotopic form derived from Br was confirmed. For quantification, the MS-MS conditions were optimized. The precursor and product ions were carefully fixed with m/z 506.02 and m/z 93.2, respectively. Figure 4 illustrates multiple reaction monitoring profiles of a deltamethrin standard and a milk extract sample spiked with 12 μg/mL deltamethrin. Though the retention time of the milk extract sample

was faster than that of deltamethrin standard, no matrix effect was seen on the mass chromatogram. Unfortunately, the sensitivity of LC-MS-MS was not adequate for monitoring deltamethrin residues in milk, because the signal to noise ratio of deltamethrin was too high, 21 in the case of 12 μg/mL. Thus, the detection limit for deltamethrin was 1-2 μg/mL. GC-MS was also determined to have poor sensitivity for determining residual deltamethrin. These results demonstrate that, under these conditions, the ELISA is more favorable for the monitoring deltamethrin residues in milk.

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