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Application of an Enzyme-Linked Immunosorbent Assay (ELISA) to Determine Paraquat Residues in Milk, Beef, and Potatoes

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There are several opportunities for the widely used herbicide paraquat to enter the food supply. For instance, when paraquat is used as a preharvest desiccant for potatoes, there is a possibility that the herbicide can be translocated to the potato tuber from the treated foliage. Residues in potatoes of 130 ppb have been reported following an application rate of 0.5-2 lb/acre (Summers 1980). Paraquat is also used for old pasture renovation, for the desiccation of silage grass, and for killing aquatic plants in drainage ditches. Through such uses, livestock could become exposed to the herbicide. Feeding studies with cows using methyl-C^{14}-labeled paraquat have shown that low levels of the compound can be secreted in the milk (Summers 1980). The federal tolerance for residues of the herbicide in meat, fat and meat by-products of cattle, hogs, goats, horses, and sheep is 10 ppb (Anon. 1972). Paraquat has been described in a Government Accounting Office report as a potential harmful residue in raw meat (Anon. 1979). The USDA Food Safety and Inspection Service (FSIS) has included paraquat on its list of compounds to be considered for monitoring in foods. However, present methods do not easily accommodate the processing of large numbers of samples, thus limiting routine monitoring of the compound. The conventional method, based on spectrophotometry of reduced paraquat solutions, requires time-consuming sample preparation. Samples are extracted with hot H_2SO_4 and then concentrated and purified by cation exchange chromatography (Anon. 1975 and 1976; Pack 1967). The method has been applied to paraquat residues in many food crops and gives an average recovery of 75% from a variety of crops with a detection limit of 10 ppb based on a 50 g sample (Calderbank and Yuen 1965). With slight modifications the method has been applied to milk and animal tissue. A limiting

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factor in the accuracy of the method is the degree of linearity of the background absorption. A gas chromatographic procedure has also been reported for crops. The method is based on extraction with H_2SO_4, catalytic hydrogenation of the acid extract, with cleanup on an alumina column (Khan 1975). The lower detection limit of the method is in the 50-ppb range.

Although the advantages of immunoassays for pesticide residue analysis have been pointed out (Hammock and Mumma 1980, Van Emon et al. 1985), the reported immunoassays for paraquat have only been applied to cases of clinical poisoning (Factori and Hunter 1980, Levitt 1977, Niewola et al. 1983) or human exposure assessment (Van Emon et al. 1986). In this study, spiked milk, potato, and beef were analyzed directly, without prior cleanup, by an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

The anti-paraquat antibodies were obtained from New Zealand white rabbits (Van Emon et al. 1986). A modified solid phase indirect competitive ELISA (Voller et al. 1976) as applied to paraquat (Van Emon et al. 1986) was used throughout the study. Whole (3.5% fat) and low-fat milk (2% fat) were diluted with a phosphate buffer saline solution (pH 7.4) containing 0.5% Tween-20 and 0.02% sodium azide (PBS-tween) to a concentration (v/v) of 75%. The diluted milk had an average pH of 6.0 and was adjusted to a pH of 7.4 before spiking and final dilution. The 75% milk was diluted with PBS-tween and a 1/2000 dilution of antibody in PBS-tween to concentrations (v/v) of 50%, 25%, and 10%, yielding an antibody dilution of 1/5000. Each milk sample was spiked at 0.111, 0.333, 1.0, 3.0, 9.0, and 27.0 ng of paraquat/mL. The antibody was incubated overnight with the samples at 21°C before analysis by ELISA.

Three replicate sample concentrations were routinely analyzed per plate. Each experiment was repeated three times on three different days. The inhibition values expressed are the average of each determination. The relative standard deviation for each matrix was determined to be 7% for whole and low-fat milk, 8% for potato samples, and 5% for aqueous standards which included residue from evaporated 6N HCl.

Potatoes, which had been shredded with dry ice, and ground beef were stored at -20°C. The same sample preparation procedure previously reported for glass fiber filters (Van Emon et al. 1986) was applied to potato and ground beef. Briefly, samples were placed in 15-mL polystyrene test tubes, covered with 2-5 mL of 6N HCl, spiked with paraquat and sonicated for 30 min. Aliquots of the acid extract were removed, evaporated to dryness with a speed vacuum concentrator (Savant Instruments, Inc.) and analyzed by ELISA. To determine background interference in the ELISA, aliquots of 6N HCl and of potato acid extracts were evaporated and included in an aqueous standard curve. The extraction efficiency of the 6N HCl was determined by using methyl-C^{14} paraquat (3.33)Ci/)mole) generously provided by Professor R.I. Krieger,

University of Idaho. Samples of potato (0.5 g) were spiked in triplicate at 0.8, 1.0, 2.0, and 15 ppb with determination by scintillation counting. To correlate ELISA with scintillation counting, 0.5 g samples of potato were spiked at 4 different levels (0.5, 0.8, 1.0, and 2.0 ppb) with methyl-C^{14} paraquat and kept at 21°C overnight before analysis by both methods. Determination of extraction efficiency and correlation of methods were done with ground beef (15-18% fat) which was spiked with methyl-C^{14} paraquat at 2.5, 5.0, 10, and 13 ppb, and allowed to stand for 2 days at 21°C before analysis by scintillation counting and ELISA. The sample was "aged" so that the paraquat could become effectively "bound" to the beef. Unlabeled and methyl-C^{14} paraquat were analyzed in the ELISA for comparison.

RESULTS AND DISCUSSION

Immunoassays can be optimized for either sensitivity or speed. An assay that is both highly sensitive and reproducible may require a longer analysis time than a simple qualitative assay. The ELISA reported here was optimized for precise quantitation. When the 18 h incubation of sample with antibody was reduced to 3.5 h, sensitivity was essentially unchanged but assay variability was slightly increased. Much shorter assay times are possible as the other incubations could also be reduced. By changing the parameters of time and temperature, the analyst can control the precision and sensitivity of the assay.

To evaluate effectively the sensitivity and applicability of an antiserum, it is essential that the analyte in the sample respond similarly to the analyte in the solution used to generate the standard curve. Both the standards and unknowns must be prepared in the same buffer, since possible interferences of the antigen-antibody reaction may occur due to differences in the diluent. This is particularly critical when analyzing samples that have not undergone rigorous cleanup. Different concentrations of PBS-tween were used in the milk samples so that each dilution of milk had the equivalent buffer content. Failure to adjust the buffer concentration in this manner resulted in complete inhibition of antibody in the assay.

The ELISA was able to detect less than 1 ppb of paraquat in whole milk, based on an average density of 1.02 g/mL. There was no difference in the assay sensitivity or linear range for the different dilutions of milk (Figure 1). The sensitivity of the ELISA in milk (I_{50} 0.3 ng/mL) was comparable to an aqueous system (I_{50} 0.5 ng/mL). Similar results were seen when low-fat milk with an average density of 0.99 g/mL containing 2% fat was analyzed. The standard curves for the different dilutions of low-fat milk were also identical and gave an average I_{50} value of 0.5 ng/mL (Figure 1). The assay would thus be capable of screening milk samples for paraquat contamination at or below the EPA tolerance level (10 ppb). The spectrophotometric determination of paraquat in milk requires a longer analysis time than does the ELISA and

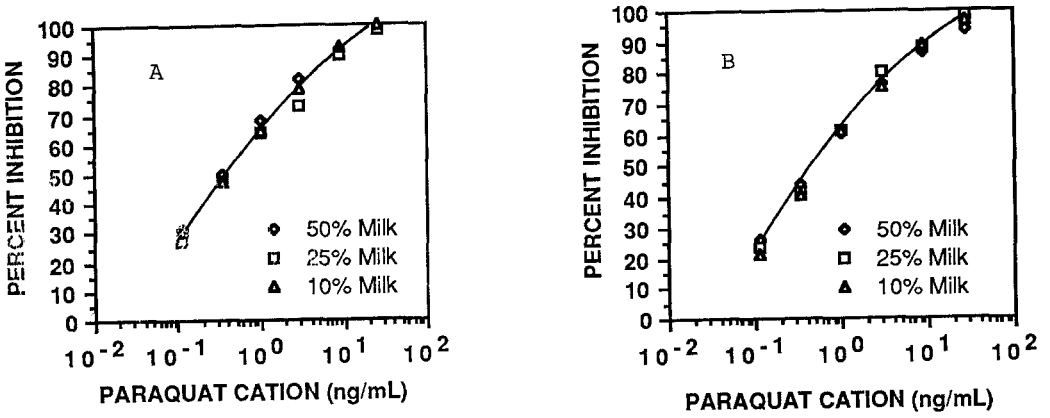

Figure 1. ELISA standard curves for whole and low-fat milk diluted with PBS-tween and spiked with paraquat. Standard deviation is less than 7% of the reported values. The correlation coefficients (r^2), for data points at 0.1, 0.33, and 1.0 ng/ml are as follows: A) Whole milk: 50%, 0.999; 25%, 0.997; 10%, 0.999. B) Low-fat milk: 50%, 0.995; 25%, 0.999; 10%, 0.999.

provides a limit of detection of only 10 ppb, based on a 100-300 mL sample with recoveries between 65-80% (Calderbank 1968).

The efficiency of 6N HCl for extracting potato and ground beef was determined by using methyl-C^{14} paraquat. Potato samples spiked at 0.8, 1.0, 2.0, and 15 ppb yielded procedural recoveries of 63, 68, 70, and 73%, respectively. Ground beef samples spiked at 2.5, 5.0, 10, and 13 ppb gave procedural recoveries of 60, 66, 70 and 74%, respectively. Since paraquat is known to bind tightly to many matrices, recoveries for both matrices were determined after the spiked samples were stored for several days at 21°C. Thus, sonicating with 6N HCl appeared relatively efficient at extracting the paraquat and may afford procedural advantages over the conventional method of boiling the crop material with sulfuric acid.

The extraction and cleanup steps used for the more conventional detection methods can be used as a starting point in devising sample preparations for the coresponding immunoassay. However, as immunoassays are performed in aqueous media, modifications may result in a reduction of sample preparation. The use of a volatile acid such as HCL allowed for concentration of the sample extract by evaporation. Due to the high affinity of the paraquat antibodies, the cleanup step could be omitted. The samples were ready for analysis after merely resuspending the dried residue in buffer. However, the use of H_2SO_4 for extraction requires the time-consuming cleanup step of cation-exchange chromatography.

Both unlabeled and methyl-C^{14} paraquat were analyzed in the ELISA in an effort to calibrate the two methods. Using the average of 3 determinations per spiking level for both unlabeled and labeled paraquat, 1 cpm was determined to equal 3.22×10^{-2} ng.

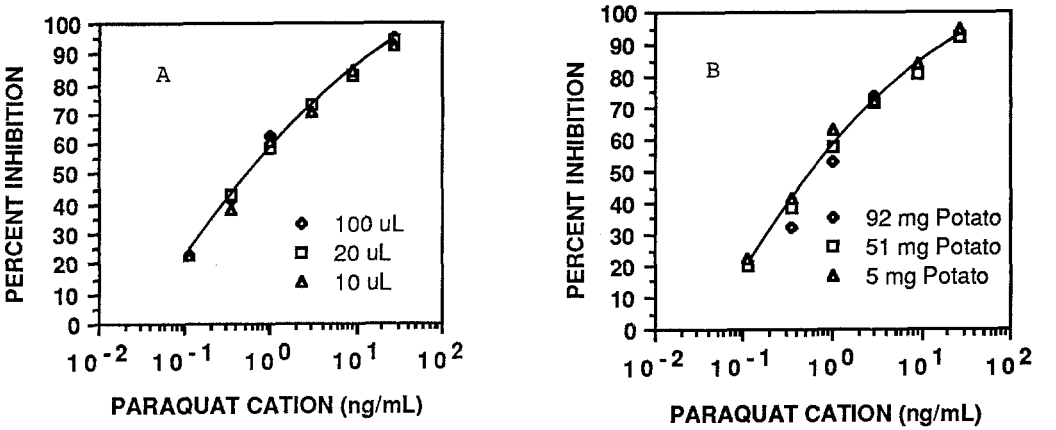

Figure 2. ELISA standard curves in the presence of aliquots of acid extracts from three quantities of potato. Standard deviation is less than 8% of the reported values. The correlation coefficients (r^2) for data points at 0.1, 0.33 and 1.0 ng/ml are as follows: A) Three different amounts of dried acid extract from a 5 mg potato sample: 100)L extract, 0.999; 20)L extract, 0.999; 10)L extract, 0.999. B) Three different quantities of potato sample: 92 mg potato, 0.999; 51 mg, 0.999; 5.0 mg, 0.990.

Calculations based on the radiolabel of 3.33)Ci/)mole yielded 3.49×10^{-2} ng/cpm. This agreement between the mass of paraquat predicted by the ELISA and the mass indicated by the tracer study provides further evidence for the validity of the ELISA.

The utility of any analytical method depends on the absence of interferences derived from reagents and the matrix. The interference question must be addressed by running appropriate blanks as controls. In this regard, ELISA is not different from other detection methods. In order to assess the reagent- and matrix-derived interferences several experiments were run (below). It is understood that such interferences may vary with reagent batches and matrix sources, and thus must be checked frequently by a combination of running appropriate blanks, and by confirming positive samples by an alternate analytical method. The latter is crucial to using any assay method, including ELISA, for monitoring samples of unknown origin when corresponding field blanks are not available.

Toward this end, the maximum amount of 6N HCl that could be accomodated by the assay was determined. No interference was detected when up to 100)L of 6N HCl was first evaporated to dryness and then included in an aqueous standard curve. However, reduction in assay sensitivity was observed when the dried residue from 300)L of 6N HCl was included in the standard curve. The volume of control acidic potato extract that could be analyzed without interference was also determined. Aliquots of 10, 20, and 100)L of acidic extract from a 5.0 mg potato sample were evaporated and included in an aqueous standard curve for paraquat. The resulting curves were essentially identical, with less than 10% difference in the inhibition values (Figure 2). The

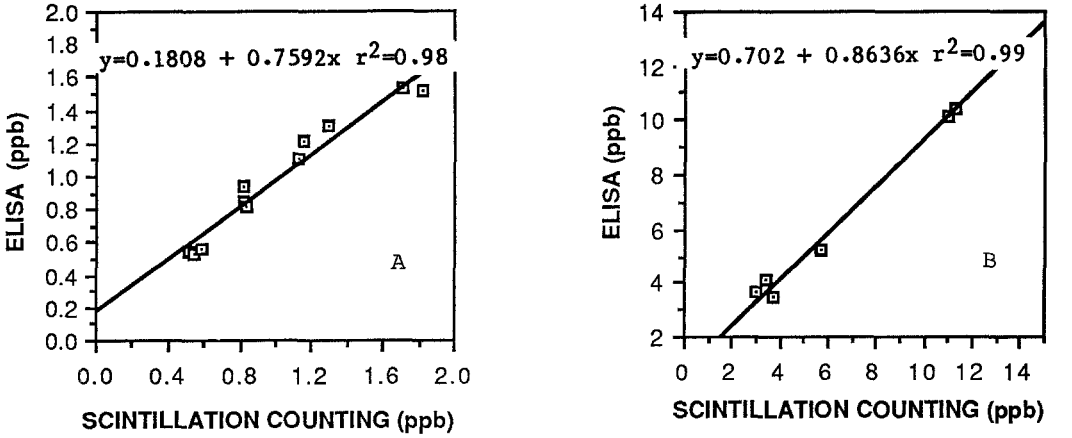

Figure 3. Correlation of ELISA and liquid scintillation counting for paraquat residues in potato and beef samples following extraction with 6N HCl. Each point represents the average of 3 determinations for each method. A) Potato: standard deviation is less that 8% of the reported values. B) Beef: standard deviation is less that 7% of the reported values.

potato extract did not affect assay sensitivity as the average I_{50} value obtained when the extract was included was 0.6 ng/mL.

To determine the interference from differing amounts of potato extract, evaporated acid extracts from 5, 51, and 92 mg of potato were included in aqueous standard curves for paraquat. Each curve was similar in shape, yielding I_{50} values of 0.50, 0.64, and 0.85 ng/mL, respectively (Figure 2). For the 92 mg sample this corresponded to a detection limit of 1.4 ppb. Because the tolerance for paraquat in potatoes is 50 ppb, the assay easily has enough sensitivity to be applied to field samples. When the spectrophotometric procedure is applied to potatoes, recoveries average 70-85% for a 250-g sample with a limit of detection of 10 ppb (Calderbank 1968).

Potato and ground beef samples spiked with methyl-C^{14} paraquat were split and analyzed by both ELISA and scintillation counting. The two detection methods correlated well for both matrices. Based on 3 determinations for each spiking level, the correlation coefficients obtained were r=0.97 for potato (Figure 3), and r=0.99 for ground beef (Figure 3). Assay sensitivity for beef was 2.5 ppb based on the lowest datum point on the standard curve. A limit of detection of 10 ppb has been reported for animal tissue by using the conventional procedure of extraction with H_2SO_4 followed by reduction and spectrophotometric assay (Calderbank 1968).

Our findings show that a simple indirect ELISA can be applied to the determination of paraquat in foodstuffs. The ELISA has

potential for application to screening of large numbers of food commodities. The relatively simple extraction and ELISA procedures described here provided lower detection limits and comparable recoveries to reported procedures used for food crops. Further testing of the applicability of ELISA to foodstuff analysis, by a comparison of ELISA with methods in current use employing field-incurred residues, is warranted.

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