Low-Level Immunoassay Screen for 2,4-Dichlorophenoxyacetic Acid in Apples, Grapes, Potatoes, and Oranges: Circumventing Matrix Effects

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We have been able to circumvent the variability in immunoassay data due to matrix effects by development of a standard addition ratio method. This technique was used to develop a screen for ≥10 ppb of 2,4-dichlorophenoxyacetic acid (2,4-D) in apples, grapes, oranges, peaches, and potatoes using a commercially available (Ohmicron) immunoassay. To compensate for matrix effects observed at 0.5 g produce/mL, standard is added to an aliquot of each sample extract and run alongside it, without need of a standard curve. The apparent response of each sample varies according to its matrix, but the ratio of spiked to blank sample is consistent (±4.6%) and characteristic of the quantity spiked. For a positive sample (≥5 ppb in the assay), the ratio is ∼22% higher than for a negative sample. Comparing immunoassay with gas chromatography values for 226 samples gave 2−18.7% false positives and 2.9% negatives. The immunoassay is carried out by diluting an acetonitrile extract produced in support of other analyses with aqueous buffer, avoiding a separate extraction, derivatization, and cleanup necessary for GC. The immunoassay screen can reduce analysis time from 2 days to 5 h for an 18-sample set.

Keywords: *Immunoassay; phenoxyherbicides; 2,4-dichlorophenoxyacetic acid; 2,4-D; apples; grapes; oranges; peaches; potatoes*

INTRODUCTION

Immunoassay is relatively new to the field of pesticide analysis, and there has been considerable interest in exploring its role in the residue chemist's arsenal of tools (Lee et al., 1991; Kaufmann et al., 1991; Ferguson et al., 1993). A number of papers have been published about applying immunoassay to food matrices (Brandon et al., 1993; Bushway et al., 1989; Hill et al., 1993; Itak et al., 1993; Lehotay and Argauer, 1993; Wigfield and Grant, 1992; Witmann and Hock, 1993). In this report, we describe the use of immunoassay as a low-level screen for food matrices that are mostly negative. Our objective was to develop a rapid, low-cost screen at the 10-ppb level for 2,4-dichlorophenoxyacetic acid (2,4-D) in apples, oranges, grapes, potatoes, peaches, and grapefruit. The conventional analysis for 2,4-D requires a separate extraction and derivatization with diazomethane (or other methylating agent) prior to gas chromatography (GC) analysis (Texas Department of Agriculture, 1992). We wished to decrease the amount of sample preparation and to utilize extracts already prepared for the analysis of other compounds. The greatest challenge proved to be the low detection level required. The immunoassay, previously described by Lawruk et al. (1994), can detect 0.7 ppb of 2,4-D in water, but the level of detection in food matrices is substantially above this theoretical minimum because of matrix effects. The variation in concentration values obtained from individual sample matrices compared with standards in a blank matrix of the same commodity proved so large at 10 ppb that a cutoff condition could not easily be determined. The matrices we used are representative of the variety to be expected because they come from all over the United States. Variability in calculated concentration was high for both blank matrices and matrices spiked with 10 ppb of 2,4-D. We have not determined the source of the observed matrix variability but believe it to be nonspecific; for example, substances from the matrix may bind nonspecifically to the antibody in such a way as to prevent analyte binding. To address the difficulties caused by this type of matrix effect we developed the "standard addition ratio" method.

Matrix effects have traditionally been dealt with by adding known amounts of standard and extrapolating the result to zero added standard (i.e., running a standard curve in the matrix of interest). After characterizing the dependence of observed signal on analyte concentration *in that matrix*, one can determine what analyte concentration produces the signal observed when no standard is added. The method proposed for immunoassay parallels this strategy by adding standard to individual extracts, but it also relies on an observed constancy in the relationship between relative absorbance and concentration for produce extracts. Immunoassay color development depends on the logarithm of the concentration, so adding a known amount of standard to a blank sample has a much greater effect on the absorption than adding the same amount of standard to a positive sample. Because of this, the ratio of absorbances of a "standard-added" to unaltered sample can be used to classify a sample as positive or negative. The ratio method is similar to the conventional "standard addition" method in that standard is added to each individual extract but avoids the need for a three-point standard curve for each sample. In addition, the ELISA format allows all samples and overspikes to be run simultaneously.

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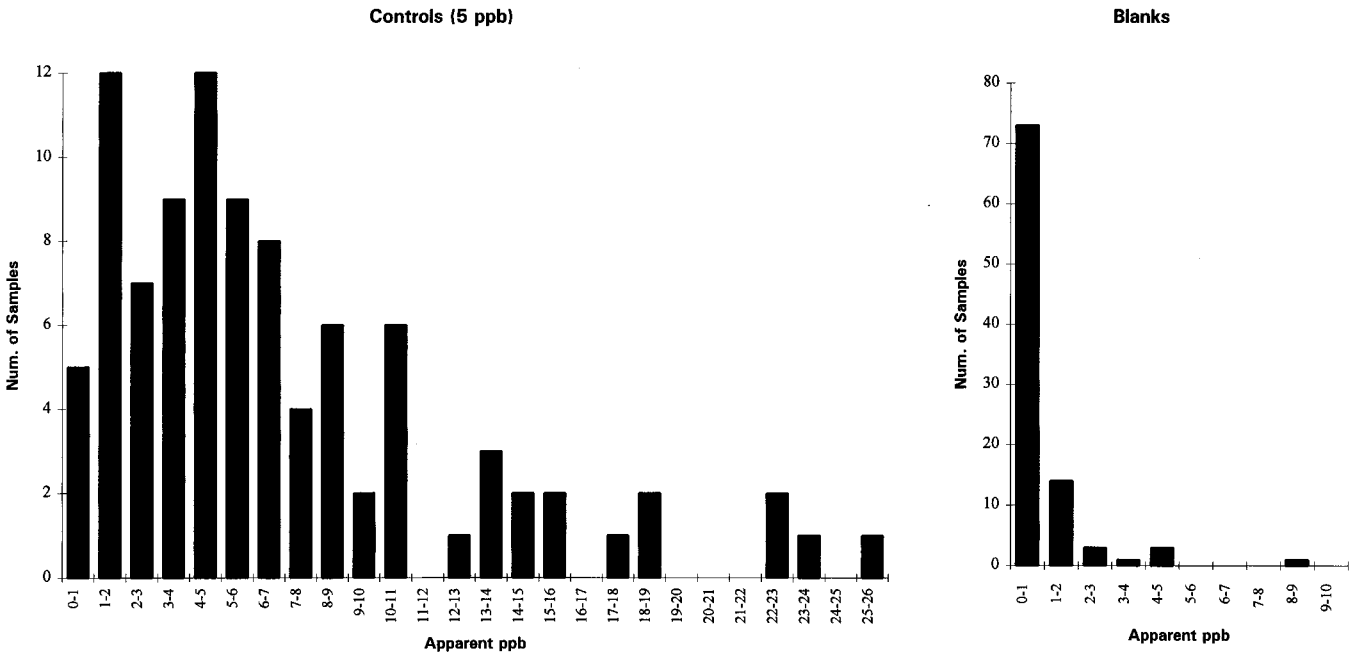

Figure 1. Distribution of values determined for apple controls (5 ppb) and apple blanks by comparison with a standard curve in apple matrix. Controls and blanks were made by diluting 95 blank apple extracts in both plain sample diluent and sample diluent spiked with 2,4-D to give a final concentration of 0.5 g of produce extract per 1 mL with 0 and 5 ppb of 2,4-D, respectively. Standards for each data set were prepared analogously from a single apple extract.

EXPERIMENTAL PROCEDURES

Materials. Residue grade methanol was purchased from EM Chemicals, and sulfuric acid, hydrochloric acid, and ethyl ether were ACS reagent grade. Diazomethane was prepared in the laboratory from diazald (Aldrich). Standard 2,4-D was obtained from Chem Service (West Chester, PA) at a purity of 99%. Standard 3,4-D was purchased from Aldrich (99%). No further validation of standard purity was performed. Immunoassay kits were purchased from Ohmicron (Newtown, PA) with additional sample diluent. The assay tubes were read on an RPA-I photometric analyzer (Ohmicron). Samples of fruit and vegetables were collected from all over the United States as part of the USDA Pesticide Data Project and represent specimens of many different botanical varieties grown under different conditions.

Preparation of Standards. *GC Determination.* 3,4-D and 2,4-D standards were made up in blank matrix extract. The standards were methylated simultaneously with the samples.

ELISA Analysis. Kit standards were used for the standard curve and were modified by addition of blank extract so that the grams of produce/milliliter was the same as for the samples (i.e., 0.5 g/mL). The volume of extract added was 2.5% of the total (i.e., 10 μL) added to 390 μL of standard so that the concentration of standard was only minimally affected. The 5 ppb "standard added" diluent was made by appropriate dilution of 2,4-D stock (100 ng/μL in acetonitrile) in sample buffer and was used in a manner analogous to the kit standards to create "5 ppb controls" that were run with each set.

Analysis by Conventional Method. This method is adapted from a procedure developed by the Texas Department of Agriculture (1992). The commodity was washed, inedible portions were removed, the commodity was finely chopped, and 50-g aliquots were weighed into jars. The internal standard 3,4-D (60 ppb) was added, followed by 100 mL of 55% methanol/45% 0.1 M KOH. The commodity was blended with an Omni-Mixer for 4 min. A portion of the mixture was then removed and centrifuged for 8−10 min at 20 000 rpm to remove solids. A 10-mL aliquot of the clarified methanolic/aqueous extract was removed and diluted with 40 mL of 1% H_2SO_4/2.5% NaCl. The diluted extract was loaded onto a conditioned C-18 cartridge under reduced pressure and the cartridge was then dried in air. The cartridge was rinsed with 2 mL of hexane, and the 2,4-D the was eluted with 10 mL of ether. The ether layer was dried with acidified Na_2SO_4 and evaporated to 0.3 mL, and analytes were methylated with diazomethane for 1−1.5 h. (It should be noted that diazomethane is a carcinogen and should be handled in a well-ventilated hood. It should also be held in glassware without ground glass surfaces or cracks to avoid danger of explosion.) Diazomethane was removed by blowing reaction mixture gently with a nitrogen stream to a final volume of 0.2−0.3 mL. The extract was diluted to a final volume of 4 mL with hexane and chromatographed on a DB-5 column (J&W Scientific) with a 5890 gas chromatograph (Hewlett-Packard) and detected with an electrolytic conductivity detector (OI Corp.)

Analysis by ELISA Method. *Preparation of ELISA Extract.* The commodity was washed, inedible portions were removed, the commodity was finely chopped, and 100-g aliquots were weighed into jars. At this point, 1 mL of a standard 2,4-D solution (1 ng/μL in acetone) was added to those samples to be spiked. To each jar was added 200 mL of acetonitrile, and the sample was blended for 3 min and filtered through a Sharkskin filter. The extract, which includes ~80 mL of water from the commodity, was passed through a C-18 cartridge to trap lipids and waxes. It was then collected and shaken with 2 mL of 1 M phosphate buffer (pH 7) and 10 g of NaCl (to neutralize the extract and separate aqueous and acetonitrile layers, respectively). A 10-mL aliquot of the acetonitrile layer was removed and dried in a stream of nitrogen (the remaining acetonitrile extract was used for other analyses). The residue was dissolved in 250 μL of acetone, and the sample was microfiltered directly into an autosampler vial. A more detailed method will be submitted in a separate publication.

Immunoassay Procedure. For immunoassay, the aforementioned extract was diluted 1:40 (10 μL extract were added to 390 μL of diluent) both in sample diluent and in 5 ppb-spiked sample diluent, and run in the assay using the manufacturer-recommended procedure as follows: 250 μL of each sample and its "standard-added" partner were added to assay tubes. To each tube was then added 250 mL of enzyme conjugate and 500 μL of antibody-coupled magnetic particles. The tubes were vortexed, and the competition mixture incubated at room temperature for 30 min. The tubes were then seated in a magnetic rack that immobilizes the magnetic particles while the tubes are washed twice with washing buffer and carefully blotted. A 500-μL aliquot of "color reagent" solution containing hydrogen peroxide and tetramethylbenzidine was added to

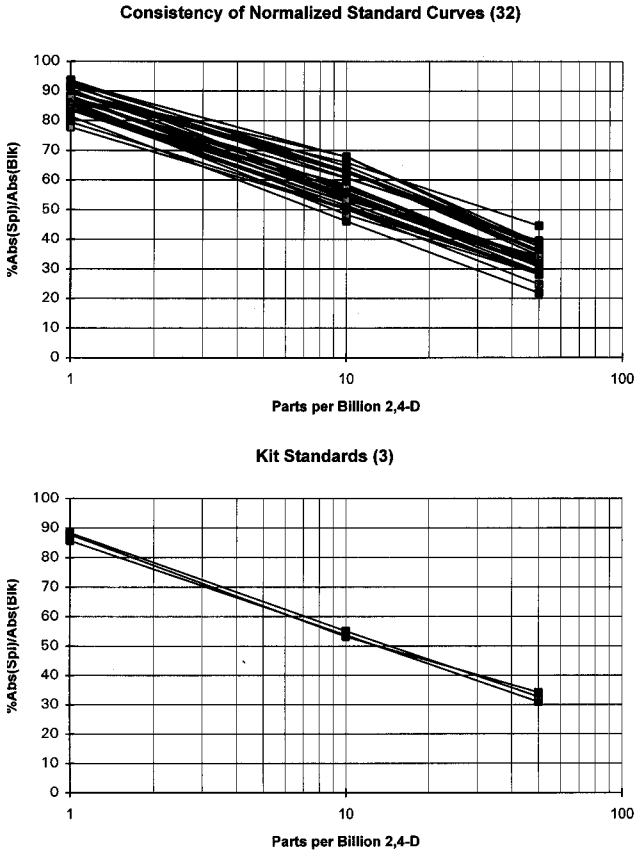

Figure 2. Consistency of normalized standard curves across several matrices. The 32 standard curves include 11 apple, five grapefruit, four grape, five potato, four orange, and three kit standards. The lower graph contains curves for kit standards alone as a comparison.

each tube, and the color development was allowed to proceed for 20 min. At this time, 500 μL of sulfuric acid stopping solution was added to each tube, and each tube was read at 450 nm in the RPA-I tube reader. The optical density obtained from the "standard-added" tube was divided by the optical density obtained from the tube containing the sample diluted in plain sample diluent. If the ratio obtained was $\geq$0.80, the sample was considered positive and should be analyzed by conventional methods. If the ratio obtained was <0.80 and the absolute optical density of the sample is >0.20, the sample is negative (contains <10 ppb of 2,4-D) and, in our protocol, requires no further testing. A sample with an optical density of <0.20 is considered to be positive even if the ratio puts it in the negative range. In this range, the absorbance is itself so small that random error could conceivably bring the ratio of standard added to plain sample into the negative range, but such samples likely either contain 2,4-D or another substance that significantly inhibits the assay.

RESULTS AND DISCUSSION

Our initial efforts to quantify 2,4-D in 95 apple samples by the conventional immunoassay comparison to a standard curve (prepared with apple matrix) gave the results shown in Figure 1. It is difficult to arrive at a cutoff condition to distinguish positives (samples containing $\geq$5 ppb of 2,4-D) from negatives (samples containing <5 ppb 2,4-D) in this figure. Apples with stronger matrix effects than the apple used for the standard curve give large ppb values for both controls and blanks, whereas apples with weaker matrix effects give low ppb values for both controls and blanks. (In this series of experiments, recovery does not play a role because extracts were spiked with 5 ppb of 2,4-D just

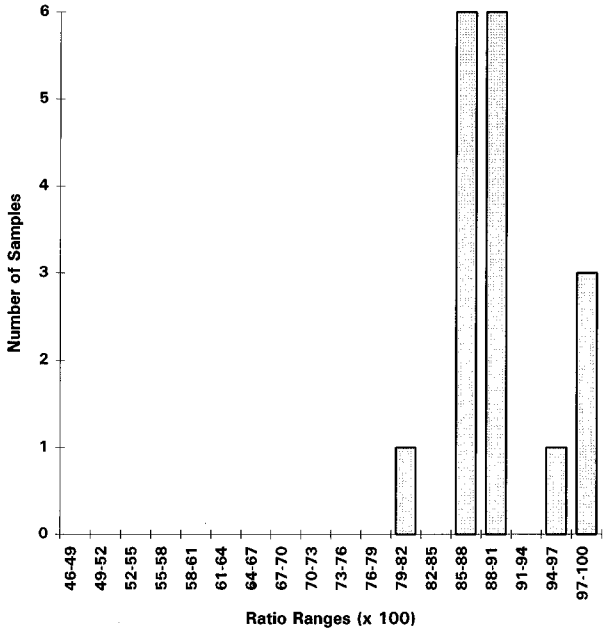

Figure 3. Distribution of cutoff ratios ($\times$ 100). The ratios were obtained by dividing the absorption of the 10-ppb standard by that of the 5-ppb control.

prior to running the assay.) Comparing absorptions to a standard curve in apple and using 2.5 ppb as a cutoff gives 21 false negatives (for 5-ppb controls spiked into apple extract) and four false positives. Lowering the cutoff to 1.0 gives five false negatives and 21 false positives. Even lowering the cutoff to the theoretical detectable limit of the assay (i.e., 0.7) gives two false negatives and 29 false positives.

We noted that in the midst of variations in the amount of signal arising from different matrices there was consistency in the percent of maximum absorbance obtained by adding a certain quantity of analyte to any blank matrix and in the slope of the line that relates relative absorbance to ln(ppb). This can be seen in Figure 2, which shows 32 standard curves, including kit standards, and standards prepared in apple, orange, grape, potato, and grapefruit extracts. In each case, the absorbances obtained for the standards have been divided by the absorbance for the corresponding blank sample. Hence, adding 5 ppb to a blank matrix extract will produce an absorbance that is characteristic of the level added and averages 65.3 $\pm$ 4.6% of the absorbance for the blank matrix:

$$B(\text{Blk} + 5\ \text{ppb})/B(\text{Blk}) = K(5\ \text{ppb}) = 65.3\% \pm \text{SD}\ 4.6 \quad (1)$$

Similarly, spiking 10 ppb will produce a signal that is characteristic of the amount spiked and averages 57.1 $\pm$ 5.9% of the absorbance for the blank matrix:

$$B(\text{Blk} + 10\ \text{ppb})/B(\text{Blk}) = K(10\ \text{ppb}) = 57.1\% \pm \text{SD}\ 5.9 \quad (2)$$

Based on eqs 1 and 2, the ratio of a 10-ppb absorbance to a 5-ppb absorbance should have a characteristic value also, which is independent of the absorbance of the blank:

$$\frac{B(\text{Blk} + 10\ \text{ppb})/\cancel{B(\text{Blk})}}{B(\text{Blk} + 5\ \text{ppb})/\cancel{B(\text{Blk})}} = \frac{K(10\ \text{ppb})}{K(5\ \text{ppb})} = \frac{57.1\% \pm \text{SD}\ 5.9}{65.3\% \pm \text{SD}\ 4.6} = 87.4\% \pm \text{SD}\ 3.7 \quad (3)$$

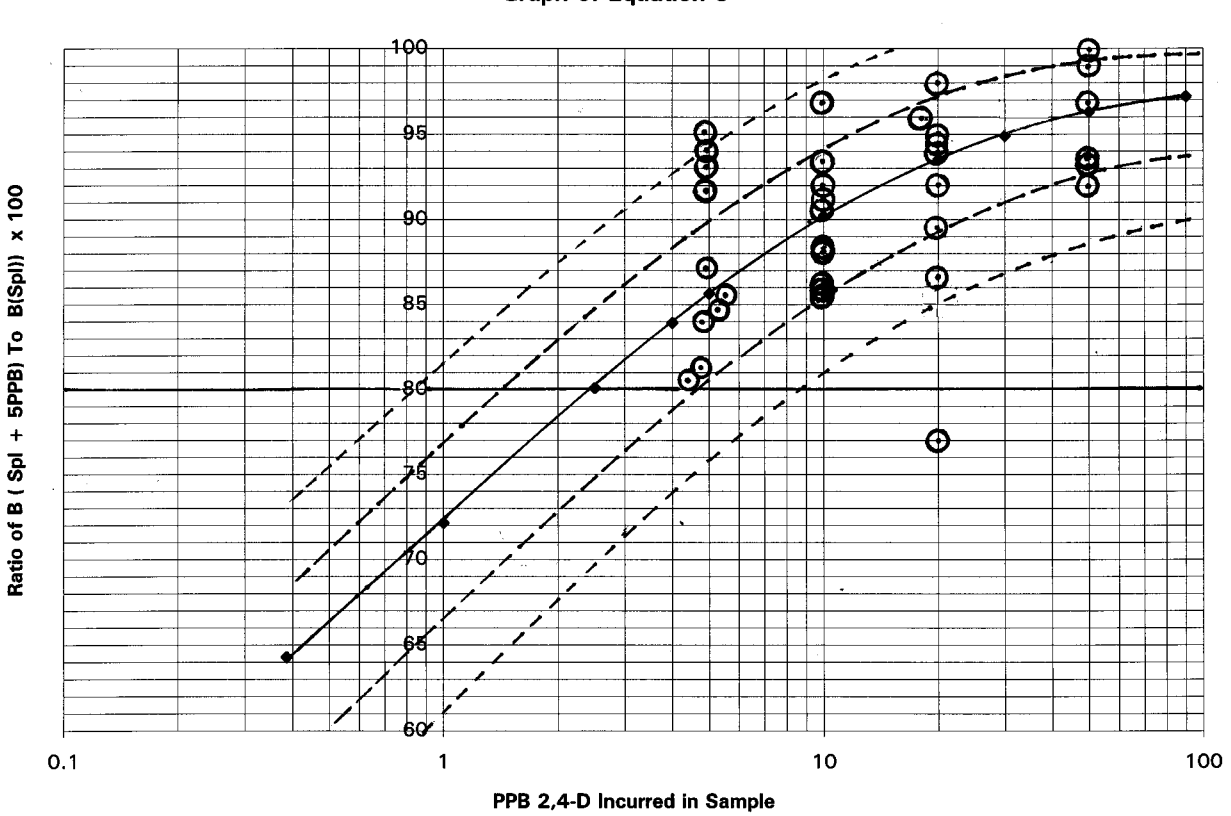

Figure 4. Graph of standard addition ratio versus concentration for 5 ppb standard addition. The function reaches the experimentally observed minimum ratio at 0.38 ppb. Ratios for 10-, 20-, 40-, and 100-ppb spikes are plotted versus their nominal spiked value in the assay (at 0.5 g/mL) or value obtained by GC where available. Spikes were generated as described in *Preparation of ELISA Extract* (i.e., 10, 20, 40, and 100 ppb of 2,4-D were added to chopped apple, orange, and potato matrices). A 35-ppb potato positive is also included. Dashed lines indicate regions of 1 and 2 standard deviations around the calculated value. The 80% cutoff line is indicated.

This value was calculated for each of the standard curves in Figure 2 and averaged 87.4% ± SD 3.7. Thus with a positive matrix (i.e., one that gave an extract containing ≥5 ppb in the assay), adding 5 ppb to it would make ≥10 ppb, and the ratio of the "standard added" to the unmodified sample would be governed by eq 3. Examination of the experimental distribution for this quantity (using the 32 standard curves) resulted in Figure 3. The center of the distribution is 0.874, but the lowest value observed for the ratio of a 10 to a 5 ppb absorbance is 0.80; this ratio was chosen as the cutoff.

The mathematical principles of the consistencies in the assay, which are usable in a practical sense, were explored. An equation that describes the average linear behavior of this particular assay as shown in Figure 2 is as follows:

$$\text{relative absorbance} = B(\text{Spl})/B(\text{Blk}) = -0.1354 \ln (\text{ppb}) + 0.871 \quad (4)$$

Other immunoassays for which the dependence of absorbance on ln [concentration] is close to linear should be able to be described this way. Sigmoidal assays can be similarly described by a normalized four-parameter logistic equation. The relative absorbance is the ratio of the absorbance for a given concentration of 2,4-D, *B* (Spl), and the absorbance of the same extract with no 2,4-D in it, *B*(Blk). The 0.871 term is the average inhibition caused by addition of 1 ppb of 2,4-D to an extract or buffer [at 1 ppb the first term drops out because ln(1) = 0]. The 0.134 factor is the average slope of the standard curves in Figure 2. Using eq 5, the more general form of eq 3, it is possible to calculate the standard addition ratio to be expected for addition of any amount *Y* to a sample containing an incurred residue *X* of the same analyte. As in eq 3, *B*(Blk) cancels out:

$$R = \frac{B(X+Y)/\cancel{B(\text{Blk})}}{B(X)/\cancel{B(\text{Blk})}} = \frac{-0.1354 \ln (X+Y) + 0.871}{-0.1354 \ln (X) + 0.871} \quad (5)$$

The level of incurred residue in the assay, *X*, and the overspike amount, *Y*, should be chosen such that the ratio obtained with a positive *X* is at least 2 SD [$B(X + Y)/B(\text{Blk})$] + 2 SD [$B(X)/B(\text{Blk})$] away from the ratio obtained when *X* is zero (or a value indistinguishable from zero). In our case, SD $B(X + Y)/B(\text{Blk}) = 5.9\%$ and SD $B(X)/B(\text{Blk}) = 4.7\%$, so the sum is $2(5.7) + 2(4.7) = 21.2\%$, which is just met by the 22.1% difference between the "zero residue" ratio of 65.3% and the "positive" ratio of 87.4%. This result will generally require that *X* be well up on the scale of the assay, but not so high that the assay is severely inhibited. In this assay, a positive *X* was chosen to be 5 ppb (about half of the IC_{50} for the assay), and *Y* was also made 5 ppb. The calculated ratio varies with the incurred quantity *X*, when a *Y* overspike of 5 is used (see Figure 4).

As the data in Figure 4 demonstrate, standard addition ratios between 0.65 and 0.95 are most sensitively related to ln(concentration). At ratios >0.95, the relationship between the ratio and the concentration flattens. For example, it would be impossible to distin-

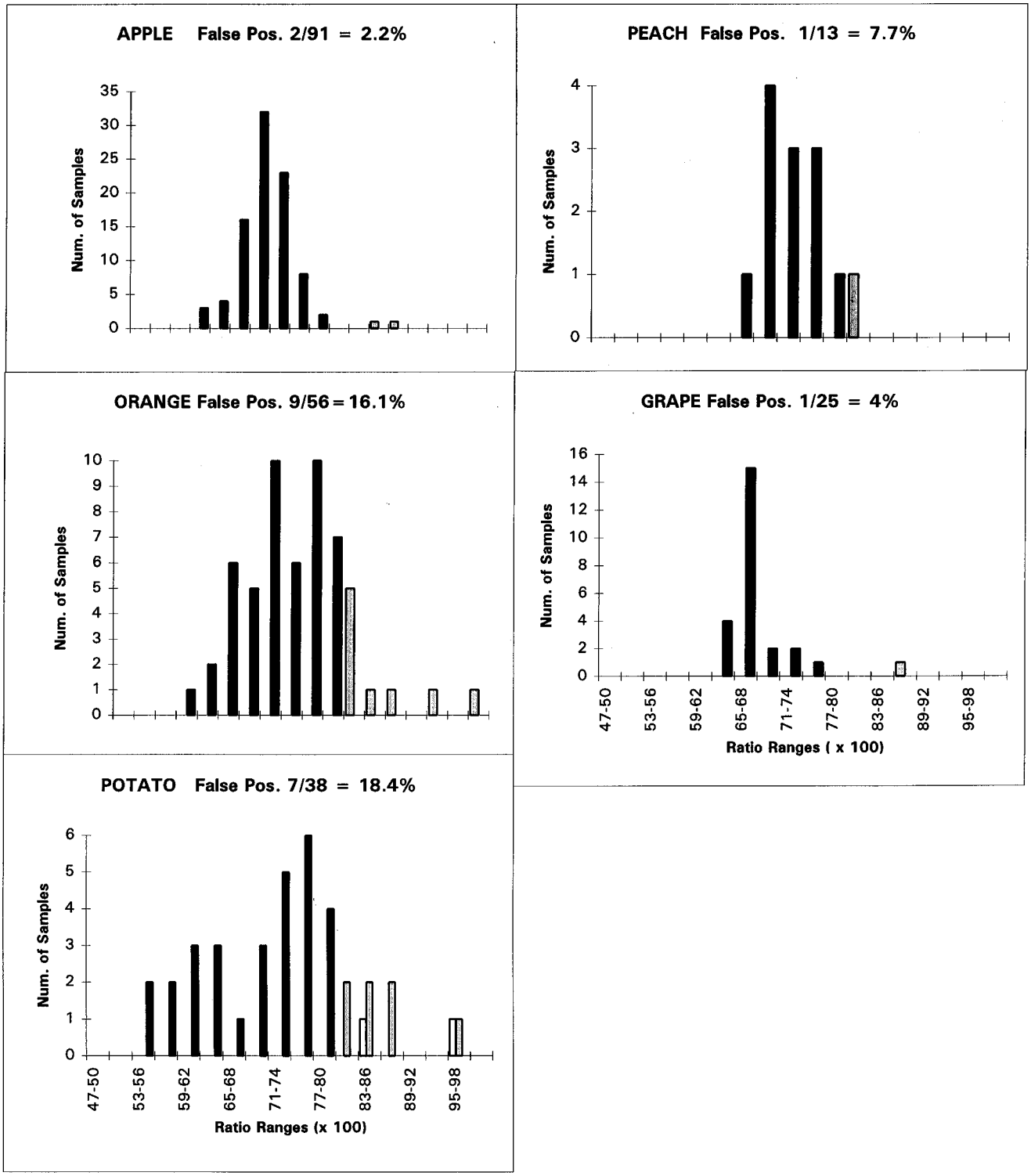

Figure 5. Distribution of standard addition ratios (× 100) for grape, orange, apple, potato, and peach samples (5 ppb of standard added). In each case, samples with ratios greater than or equal to the cutoff value of 80 are shown as gray bars. The percent of false positives is obtained by dividing the number of positives by the number of known negatives, as tested by GC. All samples were negative by GC, with the exception of the white bars in the potato profile, which correspond to a 35-ppb incurred 2,4-D residue.

guish whether a particular positive sample contained 30 or 70 ppb. Nevertheless, this sample would be identified as a high positive, and its actual residue concentration would be determined by subsequent analysis. The dotted lines represent regions of 1 standard deviation (SD) and 2 SD around the calculated line (drawn from our 32 curves). The points on the graph represent ratios obtained from 10, 20, 40, and 100 ppb spikes, which will be discussed later.

The "standard addition ratio" criterion was tested by running all our commodities by both conventional and ELISA methods for 19 sets (226 samples). During this period, conventional analysis only detected one positive of 35 ppb in potato, with all other analyses giving "none detected" results. Different commodities gave different amounts of variability in the ratios of "standard-added" to "normal" samples, as can be seen in Figure 5, with grape and apple giving sharp ratio distributions and

potato and orange evidencing broader ratio distributions. Grey bars are used for samples with ratios greater than or equal to the 80% cutoff value, allowing us to calculate the percent false positives obtained for each matrix type. False positive rates range from a minimum of 2% for apple to 18.4% for potato, which we feel makes the screen an attractive alternative to running all 2,4-D samples conventionally. All ELISA positives must be run by conventional means, but the numbers would be greatly reduced; that is, out of 226 samples we would only have had to analyze a maximum of 20 (8.8%). Grapefruit is not included in Figure 5 because it contains substances that lead to unacceptably high (~50%) levels of false positives. Further work is being pursued to develop a cleanup for grapefruit.

There were very few positive samples with incurred residues so spiked samples were used to characterize the rate of false negatives expected using standard addition ratios. Chopped samples of oranges, apples, and potatoes (35 in all) were spiked with 10, 20, 40, or 100 ppb of 2,4-D, extracted as specified earlier and subjected to immunoassay. These results are shown as data points in Figure 4. The false negative rate is 2.9% for the commodities tested (A ratio of 0.77 was obtained for one 20-ppb spike. This outlier did not reproduce upon retesting of the extract.) The spike recovery data and low false negative rate further demonstrate the efficacy of this method as a screening tool.

The extract preparation is already done in support of other analyses, so ELISA screening of an 18-sample set takes 4−5 h and costs $13.21/sample in reagents and student assistant time compared with 2 days and $200−$300/sample for conventional analysis. We feel that time and cost savings, and the elimination of a large portion of samples that do not contain any detectable 2,4-D justify the use of a rapid ELISA screening method.

We demonstrated the validity of the standard addition ratio technique for Ohmicron's 2,4-D ELISA in five food matrices at low level. The technique was helpful in lowering detection limits and false positive and negative rates in these cases. The standard addition ratio technique described is valid for assays in which the relationship between the absorbance and the logarithm of the concentration is fairly constant over time and across different matrices. The cutoff ratio obtained is dependent on the dose-response curve for the particular assay, and the particular X and Y values chosen. Any significant change in the antibody pool, assay format, choice of Y, or choice of X would require the cutoff ratio to be reexamined. Persons seeking to apply the standard addition ratio technique to their own assay are advised to thoroughly characterize the behavior of their relative absorbance versus concentration curve (in matrix) and follow a similar procedure to arrive at a workable cutoff value. Initial investigations indicate that the standard addition ratio technique is adaptable to sigmoidal assays also. Further work using different ELISAs with a variety of matrices is needed to determine the scope of the standard addition ratio technique.

LITERATURE CITED

Brandon, D. L.; Binder, R. G.; Wilson, R. E.; Montague, W. C. Analysis of thiabendazole in potatoes and apples by ELISA using monoclonal antibodies. *J. Agric. Food Chem.* **1993**, *41*, 996−999.

Bushway, R. J.; Savage, S. A. Determination of methyl 2-benzimidazolecarbamate in fruit juices by immunoassay. *Food Chem.* **1990**, *35*, 51−58.

Ferguson, B. S.; Kelsey, D. E.; Fan, T. S.; Bushway, R. J. Pesticide testing by enzyme immunoassay at trace levels in environmental and agricultural samples. *Sci. Total Environ.* **1993**, *132*, 415−428.

Hill, A. S.; McAdam, D. P., Edward, S. L.; Skerrit, J. H. Quantitation of bioresmethrin, a synthetic pyrethroid grain protectant, by enzyme immunoassay. *J. Agric. Food Chem.* **1993**, *41*, 2011−2018.

Itak, J. A.; Selisker, M. Y.; Jourdan, S. W.; Fleeker, J. R.; Herzog, D. P. Determination of Benomyl (as carbendazim) and carbendazim in water, soil, and fruit juice by a magnetic particle-based immunoassay. *J. Agric. Food Chem.* **1993**, *41*, 2329−2332.

Kaufmann, B. M.; Clower, M. Immunoassay of pesticides. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 239−247.

Lawruk, T. S.; Hottenstein, C. S.; Fleeker, J. R.; Hall, J. C.; Herzog, D. P.; Rubio, F. M. Quantification of 2,4-D and related chlorophenoxy herbicides by magnetic particle-based ELISA. *Bull. Environ. Contam. Toxicol.* **1994**, *52*, 538−545.

Lee, S. M.; Richman, S. J. Thoughts on the use of immunoassay techniques for pesticide residues. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 893.

Lehotay, S. J.; Argauer, R. J. Detection of aldicarb sulfone and carbofuran in fortified meat and liver with commercial ELISA kits after rapid extraction. *J. Agric. Food Chem.* **1993**, *41*, 2006−2010.

Nam, K.-S.; King, J. W. Supercritical fluid extraction and enzyme immunoassay for pesticide detection in meat products. *J. Agric. Food Chem.* **1994**, *42*, 1469−1474.

Texas Department of Agriculture. Acid herbicides in vegetation and soil, 1992, personal communication.

Wigfield, Y. Y.; Grant, R. Evaluation of an immunoassay kit for the detection of certain organochlorine (cyclodiene) pesticide residues in apple, tomato, and lettuce. *Bull. Environ. Contam. Toxicol.* **1992**, *49*, 342−347.

Wittmann, C.; Hock, B. Analysis of atrazine residues in food by an enzyme immunoassay. *J. Agric. Food Chem.* **1993**, *41*, 1421−1425.

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