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Regulatory Application of ELISA: Compliance Monitoring of Bromacil in Soil

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Bromacil (DuPont; Fig. 1), the active ingredient of Hyvar®, is used as an herbicide for citrus crops and on noncrop land for vegetation control (Worthing and Hance 1991). Bromacil has been detected in ground water in agricultural areas of California (State of California 1994). As a consequence, all non-crop uses of bromacil have been prohibited in Pesticide Management Zones (PMZ) that are one-square-mile areas and are considered to be sensitive to ground water pollution (State of California 1994). Soil samples are collected by the California Department of Pesticide Regulation to monitor the compliance of bromacil prohibition. Each year, many samples are produced which must be analyzed by high performance liquid chromatography (HPLC). Analysis by HPLC requires a laborious cleanup at a substantial expense (Pease and Deye 1967). Enzyme-linked immunosorbent assays (ELISA) have been found to be simple and cost-effective alternatives to instrumental analysis when numerous samples are being analyzed (Goh et al. 1993, Linde and Goh, 1995). By using bromacil ELISA as a screen and only verifying positive samples by HPLC could reduce analytical costs.

Here we report (a) a modified version of a previous ELISA assay for bromacil, (b) the use of sodium hydroxide and methanol as extractants of bromacil from soil, and (c) a comparative study of field samples that were collected and analyzed by both HPLC and ELISA.

MATERIALS AND METHODS

This assay for bromacil is a modified version of a previously reported assay (Bekheit et al. 1993). Ninety-six well microtiter plates (Nunc Maxisorp immuno-plates, Roskilde, Denmark) were coated with 100 µL of 0.35-µg/mL coating antigen 2b-CONA (Conalbumin; Bekheit et al. 1993; Fig 1) in carbonate-bicarbonate buffer (pH 9.6). The plates were sealed with an acetate plate sealer and incubated overnight at 4°C. The following day the plates were washed five times with PBSTA (0.2 M phosphate buffer with 0.8% NaCl, 0.05% Tween 20, and 0.02% NaN_3, pH 7.5) and tapped dry. Samples were prepared and diluted on a microtiter plate (Dynatech;

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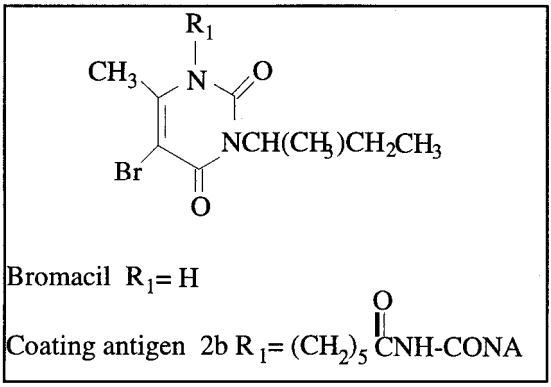

Figure 1. Structure of bromacil and coating antigen

Chantilly, VA) in PBSTA prior to addition to the coated plate. The prepared samples of 50 µL from the Dynatech plates were added to each well on the coated plate. A 50-µL aliquot of polyclonal antibody #2005 (Bekheit et al. 1991) at a dilution of 1/1000 in PBSTA was also added to each well of the plate which was then sealed and incubated for one hr at room temperature. The plate was washed five times with PBSTA and tapped dry. A 100-µL aliquot of alkaline phosphatase conjugated IgG (Sigma A-0418; St. Louis, MO), diluted at 1/2500 in PBSTA, was added and incubated for one hr at room temperature. The plate was again washed with PBSTA. A solution of 100 µL of 1 mg/mL p-nitrophenyl phosphate (Sigma; St. Louis, MO) in 10% diethanolamine buffer (pH 9.6) was added, incubated 40 min and the absorbance was read at 405 nm (UV Max Molecular Devices; Menlo Park, CA). For each sample, four well replicates were made. Samples were diluted to bring them into the range of the assay standard curve (0.05 - 15 µg/L).

Sodium hydroxide and methanol were evaluated for their efficiency of extracting bromacil from soil. The sodium hydroxide method used a 0.75% NaOH solution in water (Pease 1966, 1968). Twenty-five grams of untreated Delhi Loamy Sand (Soil Conservation Service, 1971) were placed in pint jars. Bromacil standard (provided by DuPont; Wilmington, DE) was dissolved in methanol (Optima grade, Fisher Scientific) to a concentration of 1 mg/mL. Bromacil spike levels of 10.0, 3.0, 1.0, 0.30, 0.10, 0.030, 0.010 and 0.0 mg/L were made from the standard and dissolved in 25 mL of methanol and added to the soil samples. Three replicates of each spike level were made. The samples were stored under a hood overnight at room temperature to facilitate evaporation of methanol. The following day the samples were extracted by adding 50 mL of 0.75% NaOH solution (v/v H_2O) to the sample. The samples were placed on an orbital shaker at 200 rpm for 10 min. The samples were allowed to settle before decanting the aqueous layer into a beaker. Another 50 mL of 0.75% NaOH solution were added to the soil and shaken for another 10 min. The aqueous layers were combined and 10 mL were decanted into the barrel of a 12-mL syringe. A vacuum manifold (Baker Spe-12G; J.T. Baker Inc., Phillipsburg, NJ) was used to pull the solution through a 0.22-µm, 25-mm syringe filter (MSI;

Westboro, MA) connected to the end of the syringe. Filtrates were analyzed by ELISA.

Soil extraction of bromacil by methanol is similar to the method used for 0.75% sodium hydroxide extraction. The soil samples were spiked as described above. Bromacil was extracted by adding 10-mL methanol and 15-mL de-ionized (DI) water to the sample. Then it was placed on an orbital shaker (200 rpm) for 10 min followed by a 10-min waiting period to allow for settling before decanting the methanolic layer into a beaker. An aliquot of 25 mL of DI water was added to the soil which was shaken for another 10 min. After settling for 10 min the aqueous layers were pooled, mixed and 10 mL decanted into a 12-mL syringe barrel. Vacuum filtration was used as described above. The filtrates were analyzed by ELISA.

Twenty-four field samples, collected from five sites in California's central valley, were analyzed by HPLC at the California Department of Food and Agriculture (CDFA) laboratories. Soil samples to be analyzed by ELISA were extracted using the methanol method described above. For HPLC analysis, soil samples (25 g) were extracted using 30- g sodium sulfate and 50 mL of hexane:acetone (60:40 v/v) by shaking for 2 hr at 210-rpm on a G-10 Gyrotory shaker (New Brunswick Scientific; Edison, NJ). The extract was decanted through 10-g sodium sulfate on a #l filter paper. Another 20 mL of hexane:acetone were added and shaken for 1-2 min, decanted and added to the first extract. An additional 10 mL of hexane:acetone were washed through the funnel and the final volume for the extracts was brought to 75 mL. A 15-mL aliquot of the extract was concentrated to 1 mL using a nitrogen evaporator (Myers Organomation Assoc., Inc.; South Berlin, MA) at 45°C. One mL of hexane and 0.2 g of anhydrous sodium sulfate were added. A silica Sep-Pak® (Waters; Milford, MA) was connected to a 0.2 micron Acrodisc® filter (Gelman Sciences; Ann Arbor, MI) pre-conditioned with 4-mL hexane. The extract was added to the Sep-Pak® after discarding the hexane. Another 4 mL of hexane were added, passed through and discarded. Methanol (10 mL) was added and the eluted extract was collected and concentrated down to 3 mL using the nitrogen evaporator. The sample was analyzed using a Hewlett Packard (HP)1050 HPLC and a HP1050 variable wavelength detector. The column was a Beckman ODS (C-18) 5.0 µm, 4.6 mm x 15 cm. The guard column was a Brownlee BP-18 Newguard 7 pm, 15 x 3.2 mm. The flow rate was set as 1.0 mL/min. The mobile phase contained 60% water and 40% acetonitrile (v/v). Detections were measured at 280 nm. The method detection limit (MDL) for bromacil was 0.05 mg/L. ELISA and HPLC results were compared using linear regression. Non-detect data were excluded from the linear regression comparison.

RESULTS AND DISCUSSION

The standard curve for the bromacil ELISA assay is reproducible and has a range of 0.05 to 15 µg/L with an 5, of 0.5 ug/L (n = 34). The MDL for the soil assay is 0.01 µg/mL and results below this threshold are reported as non-detect (ND). Samples were diluted in PBSTA by at least a factor of ten to ensure that the methanol and

Table 1. Soil fortification levels and mean recoveries using both a 0.75% sodium hydroxide solution and methanol as the extractants (n = 3; ND = non-detect).

Spike level (µg/mL)	10.0	3.0	1.0	0.30	0.10	0.03	0.01	0.0
Sodium Hydroxide Percent recovery	92.2	101	102	132	92.8	104	137	ND
Coefficient of variation (%)	9.4	6.3	15.4	6.3	4.8	5.7	57	
Regression equation	ln(NaOH conc.) = 0.218 + 0.972*ln(spike level conc.) R^2 = 99.1%; intercept t-ratio = 1.64, p = 0.118; slope t-ratio = 44.38, p = 0.000							
Methanol Percent recovery	107	86.6	109	91.6	88.3	92.0	89.8	ND
Coefficient of variation (%)	20.9	9.4	7.2	6.1	10.9	8.6	15.3	
Regression equation	ln(MeOH conc.) = -0.168 + 1.02*ln(spike level conc.) R^2 = 99.7%; intercept t-ratio = -2.31, p = 0.032; slope t-ratio = 86.78, p = 0.000							

sodium hydroxide concentrations would be low enough to minimize matrix effects on the ELISA plate. Data obtained from the soil extraction by methanol and sodium hydroxide were analyzed and compared by regression analysis (Table 1). Linear models describing ELISA concentration as a function of spike level were fitted to NaOH and methanol data sets. The slopes for sodium hydroxide and methanol were not significantly different from 1.0 (t = 1.36, df = 18, p = 0.15, and t = 1.66, df = 18, p = 0.11 respectively). These results indicate there is a 1:1 correspondence between ln(ELISA concentration) and ln(spike level concentration). Methanol and sodium hydroxide extraction do not differ in mean percent recovery (t-ratio = 1.88, approximate df = 24 assuming unequal variances, p = 0.072). The overall mean percent recovery is not significantly different from 100% (t-ratio = 0.52, df = 40, p = 0.6). For the sodium hydroxide method, the recovery for the 0.01 µg/mL spike level was 137%, and the coefficient of variation was 57%. This large variation is probably due to a matrix effect caused by the sodium hydroxide. Samples for this method must be diluted by a factor larger than 10 to minimize matrix effects to acceptable levels. The soil extraction of bromacil with methanol is as effective as sodium hydroxide and has lowered the assay detection limit due to a decrease in matrix effects. Methanol is also an easier extractant to use. Thus methanol is the solvent of choice for this ELISA.

Twenty-four field samples were analyzed by both ELISA and HPLC (Fig. 2). Nine of the samples analyzed showed as non-detect by both analysis methods. They are therefore not shown in Fig. 2. Regression analysis of 1n (ELISA) as a function of ln

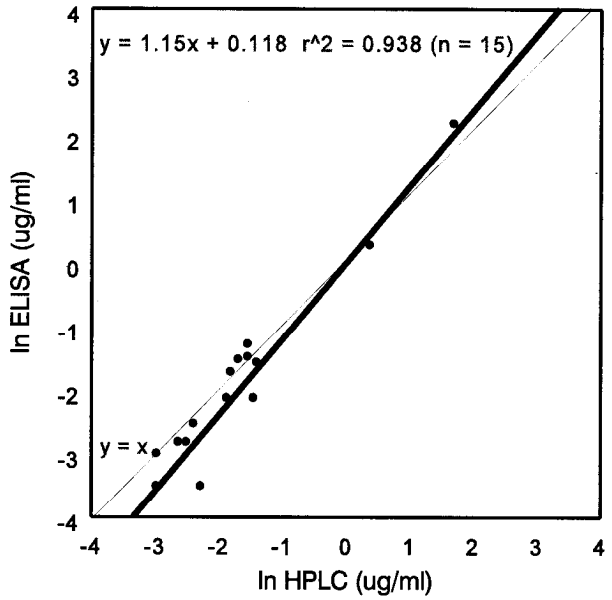

Figure 2. Comparison of bromacil analysis by ELISA and HPLC

(HPLC) yielded a slope of 1.15 and an intercept of 0.118. The slope is not different from 1.00 (t = 1.83, df = 13, p 0.08) and the intercept is not different from zero (t = 0.69, df = 13, p = 0.30). These results indicate that the concentrations measured by ELISA do not differ from the concentrations measured by HPLC.

The bromacil ELISA offers many advantages over HPLC analysis in terms of time, solvent waste, and cost. Soil extraction and preparation for HPLC analysis required eight hours whereas ELISA only needs two hours. Amount of organic solvents used by the HPLC method were about 100 mL per sample which consisted of hexane, acetonitrile, acetone and methanol. Only 20 mL of methanol were required in ELISA. Hence, ELISA generates less solvent waste and does not require the use of more hazardous solvents such as hexane and acetonitrile. A decrease in waste and analysis time translates into lower cost for ELISA analysis. ELISA for bromacil in soil offered a cost savings of over 76% per sample compared to HPLC analysis (HPLC $250, ELISA $60). This ELISA method will be used routinely for compliance monitoring purposes.

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