

# Rapid Biorecognition Assay for Herbicides in Biological Matrices

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**A rapid assay capable of detecting several commonly used herbicides at nanogram per milliliter concentrations in biological fluids is described. The assay is based on inhibition of photosynthetic electron transport in spinach thylakoids by the target compounds with colorimetric detection using a redox dye. Using a microtiter plate format, high throughput assays of water, urine, and homogenized tissue were performed in minutes with minimal sample preparation. Detection limits of 3 ng/mL for atrazine, 3 ng/mL for diuron, and 1 ng/mL for terbutryn were observed.**

## INTRODUCTION

Many regulatory agencies carry out monitoring programs intended to detect the presence of chemical residues at levels above established thresholds in food and water supplies. These programs require analysis of hundreds of thousands of samples per year, the vast majority of which are free of detectable residues. Analytical procedures generally used are HPLC, TLC, or GC, and samples must usually be shipped to central laboratories equipped with the sophisticated apparatus and highly trained personnel necessary for these analyses. There is considerable interest in the development of rapid, inexpensive assays to screen for the presence of suspected contaminants. Only samples giving positive results in the screening test would require expensive laboratory analysis, and overall cost per sample would be significantly reduced. Such screening tests must produce results quickly so that contaminated samples can be identified and quarantined before entering the food/water supply. Detection levels of the target compounds must be low (ng/mL), and time-consuming sample cleanup steps must be minimized or eliminated. Reagents must be inexpensive, and apparatus simple and robust. It is desirable that the screening test detect a class of related compounds, e.g.,  $\beta$ -lactam antibiotics, since it is impractical to individually test for every possible analyte. At the same time, great specificity is required in order to discriminate against the enormous number of compounds present in untreated samples.

Analyses based on biorecognition are very promising for this application. Immunoassays are available for many pesticides and antibiotics, and development of new assays continues to be an area of active research. There are drawbacks to immunoassays, however, particularly for small molecules (haptens). Development of new assays is time-consuming and requires considerable expertise in synthetic

organic chemistry and immunology. Most immunoassay formats require multiple reagent additions, washes, and equilibration periods and are therefore relatively slow and labor-intensive. While very impressive detection limits can be achieved in simple matrices such as groundwater, detection of haptens at trace levels in foods often requires sample cleanup procedures comparable to those used for chromatographic analysis.<sup>1,2</sup>

Another biorecognition system suitable for detection of residues is based on the binding of certain herbicides to the photosynthetic reaction center of plants and microorganisms. Herbicide binding results in inhibition of the light-driven electron transport which provides energy for photosynthesis.<sup>3</sup> Binding may be detected indirectly as an effect on growth or metabolism of the organism, but bioassays based on this approach are too slow and cumbersome for use in screening applications. Approaches for direct detection of binding based on the fluorescence, oxygen evolution, and redox chemistry of the reaction center<sup>4,5</sup> have been developed and used by plant physiologists for many years. Although these direct approaches provide simple, rapid, and sensitive detection of herbicide binding, they have been used analytically in only a few cases, such as detection of herbicides on TLC plates<sup>6</sup> and in water.<sup>7</sup> The limited use of photosynthetic reaction centers for herbicide detection may be due to lack of information on the ability of such assays to operate in biological matrices, and to the very limited shelf life of the biorecognition element.<sup>7</sup> We report here studies which demonstrate that an assay based on inhibition of photosynthetic electron transport provides an effective means for detection of herbicide residues in complex matrices. Use of the assay in a microtiter plate format for residue screening and approaches to enhancing the shelf life of the bioreagent are discussed.

## EXPERIMENTAL SECTION

**Apparatus.** Homogenizations were performed with an Ultra Turrax T25 homogenizer (IKA Works, Cincinnati, OH). Cheese-

(1) Calverley, R. A.; Jackman, R.; Pembroke-Hattersley, J. J. In *Immunoassays for Veterinary and Food Analysis-1*; Morris, B. A., Clifford, M. N., Jackman, R., Eds.; Elsevier Applied Science: London, 1988; pp 93-108.

(2) Gee, S. J.; Miyamaot, T.; Goodrow, M. H.; Buster, D.; Hammock, B. D. *J. Agric. Food Chem.* 1986, 36, 863-870.

(3) Izawa, S.; Good, N. E. *Methods Enzymol.* 1972, 24, 355-377.

(4) Pfister, K.; Steinback, K. E.; Gardner, G.; Arntzen, C. J. *Proc. Natl. Acad. Sci. U.S.A.* 1981, 78, 981-985.

(5) Pfister, K.; Radosevich, S. R.; Arntzen, C. J. *Plant Physiol.* 1979, 64, 995-999.

(6) Kovac, J.; Henselova, M. *J. Chromatogr.* 1977, 133, 420-422.

(7) Rawson, D. M.; Willmer, A. J. *Biosensors* 1989, 4, 299-311.

cloth (Thomas Scientific, Swedesboro, NJ), 0.45-mm syringe filters (Cole Parmer Instrument Co., Chicago, IL), and 96-well microtiter plates (Sarstedt, Newton, NC) were used as received. An EL 312 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by a Macintosh IIcx computer (Apple Computer Inc., Cupertino, CA) running  $\Delta$ Soft (BioMetallics Inc., Princeton, NJ) was used to mix and read absorbance of samples at 595 nm. Sorvall RC28S (DuPont, Wilmington, DE) and IEC Centra-8R (International Equipment Co., Needham Heights, MA) centrifuges were used as indicated below. A locally constructed lightbox containing two 18-W U-shaped fluorescent bulbs with a  $9.5 \times 13$  cm lighted area was used to illuminate samples.

**Reagents.** Dichlorophenolindophenol (DPIP), diphenylcarbazide (DPC), tricine, and sorbitol were from Sigma (St. Louis, MO) and used as received. DPC was stored at  $-10^\circ\text{C}$  and solutions were prepared each week or whenever a pink coloration was observed. Terbutryn, atrazine, trifluralin, and diuron were from Supelco, Inc. (Bellefonte, PA). Aqueous solutions were prepared with house-deionized water processed with a Nanopure water treatment system (Barnstead, Dubuque, IA).

**Solutions.** All buffers were adjusted to pH 7.8 with hydrochloric acid or sodium hydroxide. The homogenization buffer contained 100 mM tricine, 0.4 M sorbitol, and 10 mM NaCl. The resuspension buffer contained 10 mM tricine and 10 mM NaCl. The analysis buffer contained 0.1 M sorbitol, 10 mM  $\text{MgCl}_2$ , 10 mM NaCl, 10 mM tricine, and 1 mM  $\text{NH}_4\text{Cl}$ . The meat grinding buffer contained 0.1 M sorbitol, 10 mM  $\text{MgCl}_2$ , 10 mM NaCl, 100 mM tricine, and 1 mM  $\text{NH}_4\text{Cl}$ . The DPIP stock contained 0.2–0.3 mM DPIP in analysis buffer. The DPC stock contained 2 mM DPC in analysis buffer. For the herbicide stock, herbicides were dissolved in methanol to give stock solutions containing approximately millimolar levels of herbicide. These concentrates were diluted with analysis buffer to yield standard solutions containing either  $4 \times 10^{-7}$ ,  $4 \times 10^{-6}$ ,  $4 \times 10^{-5}$ , and  $4 \times 10^{-4}$  M or 8, 80, 800, 8000, and 80 000 ng/mL herbicide. Methanol concentration in the final solutions was less than 2%. For the thylakoid stock concentrated thylakoid suspension (5 mg/mL chlorophyll) was diluted with analysis buffer to yield the desired final concentration (8–40  $\mu\text{g}/\text{mL}$ ). Spinach, milk, and ground beef tissue (hamburger) were purchased locally.

**Thylakoid Membrane Isolation.** All manipulations were carried out with ice cold solutions and centrifugations were performed at  $4^\circ\text{C}$  with precooled centrifuge heads and tubes. Spinach leaves were cut into small (5 mm square) pieces with scissors and veins were removed. Homogenization buffer, 80 mL in a 150-mL beaker, was cooled in an ice/methanol bath until partially frozen (slush). Leaf tissue (16 g) was added, and the mixture was homogenized by several 3-s bursts at the highest homogenizer speed (24 000 rpm). The resulting suspension was filtered through two layers of cheesecloth and then through four layers of cheesecloth into 50-mL conical centrifuge tubes. The mixture was centrifuged at 1000g for 10 min (IEC Centra-8R,  $45^\circ$  angled rotor), and the supernatant was discarded. Each pellet was mixed with 20 mL of resuspension buffer, transferred to a 25-mL round-bottom centrifuge tube, and centrifuged at 3000g for 5 min (Sorvall RC28S, swinging bucket rotor). The supernatant was discarded and the pellet again mixed with 20 mL of resuspension buffer and centrifuged at 3000g for 5 min. The supernatant was discarded and the pellet suspended in 10 mL of analysis buffer to give purified “stroma free” thylakoid concentrate suspension.

**Thylakoid Storage.** After final pelleting, thylakoids to be stored were resuspended in storage buffer at a concentration of  $\sim 600 \mu\text{g}/\text{mL}$  and small aliquots were placed in 1.5-mL screw-cap polyethylene tubes. The contents were rapidly frozen by immersing the tubes in liquid nitrogen and transferred to a freezer held at the desired temperature. Freeze-drying was performed by placing open tubes containing frozen thylakoids in the freeze-drying apparatus for 24 h, with dry-ice cooling of the vacuum vessel during the last 8 h to ensure that the thylakoids were not exposed to elevated temperatures.

**Chlorophyll Determination.** A 40- $\mu\text{L}$  aliquot of thylakoid suspension was diluted to 2 mL with 80% acetone and filtered through a 0.45- $\mu\text{m}$  syringe filter, and the absorbance was measured at 645 and 663 nm. Total chlorophyll concentration was

determined<sup>8</sup> from the formula  $20.2A_{645} + 8.02A_{663}$ . On the basis of the average of three determinations, the thylakoid concentrate suspension was diluted with analysis buffer to a chlorophyll concentration of 5 mg/mL.

**Activity Determination.** Equal volumes of thylakoid stock, DPIP stock, DPC stock, and analysis buffer were combined. The mixture was illuminated for a known period (usually 5 min), and the absorbance was measured at 600 nm. The change in absorbance was used to determine the amount of DPIP reduced, and activity [ $(\mu\text{mol reduced h}^{-1})(\text{mg of chlorophyll})^{-1}$ ] was calculated based on the concentration of chlorophyll and time of illumination.

**Sample Preparation.** Serum, urine, and milk were spiked with appropriate herbicide stock solutions (except for blank samples) and adjusted to pH 7.8. Meat tissue (5.0 g) was homogenized in 20 mL of grinding buffer at 13 500 rpm and centrifuged at 1500g for 20 min to provide clear solutions in which color changes could be observed. The pink supernatant was adjusted to pH 7.8, spiked with the appropriate herbicide stock solution, and passed through a 0.45-mm syringe filter to yield beef extract. In some experiments the tissue was homogenized, spiked with herbicide (except for blank samples), stored at  $4^\circ\text{C}$  for 1 h, and then centrifuged. The pink supernatant was then adjusted to pH 7.8 and passed through a 0.45-mm syringe filter.

**Herbicide Assay.** Microtiter plates (96 well) were filled with 50  $\mu\text{L}$  each of thylakoid solution, DPC stock solution, and DPIP stock solution using a multiple-tip pipet. A 50- $\mu\text{L}$  aliquot of either sample, standard, or analysis buffer was added to six adjacent wells to give one group of wells. Unspiked matrix materials were used as blanks for the analysis of biological materials. The contents were mixed for several seconds using the mixer in the plate reader, and the initial absorbance at 595 nm was then recorded. The plate was removed from the reader and illuminated for 1–5 min with the lightbox, and a second absorbance reading was taken. In some experiments readings were taken after each 1-min illumination interval, while in others, a single 5-min illumination interval was used. Absorbance data were transferred to an Excel (Microsoft, Redmond, WA) spreadsheet for subtraction of initial absorbance and calculations of  $\Delta A$  values, theoretical curves, and standard deviations. Plots were generated with CricketGraph (Cricket Software, Malvern, PA). Parameters for the theoretical curves were generated by fitting the data to the Hill equation<sup>9</sup> using a nonlinear least squares program.<sup>9</sup> All results are means of six adjacent wells unless otherwise noted.

## RESULTS AND DISCUSSION

**Basis of the Assay.** In plants and algae, the light reactions of photosynthesis occur at two distinct but coupled centers, photosystem I (PS I) and photosystem II (PS II). Both photosystems are found in membrane vesicles (thylakoids) located within the chloroplasts.<sup>10</sup> PS II is a membrane-bound complex containing the reaction center proteins D1 and D2, as well as eight or more additional protein subunits.<sup>11</sup> Excitation of the PS II reaction center by a photon (either directly absorbed by the reaction center or transferred from the antenna pigments) drives the transfer of an electron from water to a plastoquinone (PQ) molecule bound to the D1 protein at a site termed  $\text{Q}_\text{B}$ . The overall reaction is  $2\text{H}_2\text{O} + 2\text{PQ} + 4 h\nu \rightarrow \text{O}_2 + 2\text{PQH}_2$ . The electron acceptor PQ binds fairly strongly to the D1 protein in its oxidized state, but has little affinity for the binding site when it is fully reduced. After accepting two electrons and two protons, reduced plastoquinone ( $\text{PQH}_2$ ) detaches from D1 and is replaced by another molecule from the pool of plastoquinones associated with the complex. Free  $\text{PQH}_2$  then transfers its electrons to

(8) Arnon, D. I. *Plant Physiol.* 1949, 24, 1–15.

(9) Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes*; Cambridge University Press: Cambridge, UK, 1986; pp 521–528.

(10) Lehninger, A. L. *Biochemistry*, 2nd ed.; Worth Publishers: New York, 1975; pp 587–615.

(11) Draber, W.; Tietjen, K.; Kluth, J. F.; Trebst, A. *Angew. Chem., Int. Ed. Engl.* 1991, 30, 1621–1633.

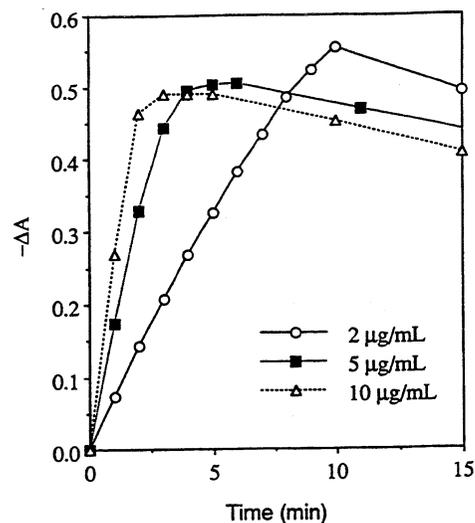
the next carrier in the electron-transport chain (cytochrome *b<sub>6</sub>/f* complex) and returns to the plastoquinone pool. Several classes of herbicides, representing ~30% of commercial sales, compete with plastoquinone for binding to the *Q<sub>B</sub>* site.<sup>11</sup> When the binding site is blocked, electron transfer can no longer occur, and oxygen evolution and PQH<sub>2</sub> production stop. In addition, fluorescence emission from PS II increases dramatically, since the normal electron-transfer route for quenching of the reaction center excited state is not available. The modulation of electron transfer and fluorescence provides a sensitive, built-in mechanism for signaling herbicide binding.

Electron-transfer activity is detected by illuminating thylakoids in the presence of an artificial electron acceptor such as DPIP or ferricyanide and monitoring absorbance changes caused by reduction of the acceptor (Hill reaction).<sup>12</sup> In uninhibited thylakoids, the blue DPIP is reduced to the colorless leuco dye in minutes, while inhibited thylakoid suspensions remain blue. The Hill reaction depends upon the water-splitting/oxygen-evolving complex of PS II to supply electrons to the reaction center.<sup>12</sup> Because this functionality is quite labile, and loss of water-splitting activity is indistinguishable from herbicide-induced inhibition of electron transport, an artificial electron donor such as DPC is used in the assay.

**Scope of the Assay.** Examples of widely used herbicides (and their chemical class) which inhibit photosystem II activity include atrazine and terbutryn (triazine), diuron (phenylurea), bromacil (uracil), phenmedipham (carbamate), ioxynil (phenolic), dinoseb (nitrophenol), and metribuzin (triazinone).<sup>11,13</sup> To determine the feasibility of detecting these herbicides directly in complex matrices, we investigated atrazine, terbutryn, and diuron. It is expected that results obtained with these three analytes are representative of the other compounds listed above, but this has not yet been confirmed experimentally. Water, urine, homogenized beef tissue, and milk spiked with the three analytes were studied as model matrices.

**Thylakoid Preparation.** The general approach to thylakoid preparation involves gentle disruption of spinach cells, removal of cellular debris by filtration through fabric mesh, and isolation of intact chloroplasts by low-speed centrifugation. The intact chloroplasts are ruptured in low ionic strength medium, and the resulting thylakoid membrane fragments separated from stromal components by centrifugation. The literature contains numerous procedures employing relatively wide variations in buffer composition, pH, and centrifugation conditions. We have generally followed the procedures of Pfister et al.<sup>4,5</sup> for thylakoid preparation and activity measurement. The yield and, to a lesser extent, the activity of the isolated thylakoids varies considerably from preparation to preparation. The most important variable appears to be the spinach quality, with fresh, young plants providing the best results.

**Assay Conditions.** The composition of the assay medium is not critical. Uncoupled thylakoids are reported to have a broad pH optimum for electron transfer ranging from pH 7.2 to 8.3.<sup>12</sup> It was necessary to adjust the pH of urine, milk, and tissue samples prior to assay to ensure that the final pH was in this range. DPIP concentration was selected to give an initial absorbance of 0.5–1 AU. DPC concentration was ~10-fold higher than DPIP to ensure rapid reduction of PS II centers. DPC has been reported to inhibit herbicide binding at concentrations above 1 mM,<sup>14</sup> but the concentrations used here do not appear to interfere with herbicide detection. The use of a microtiter plate format provides the ability to assay



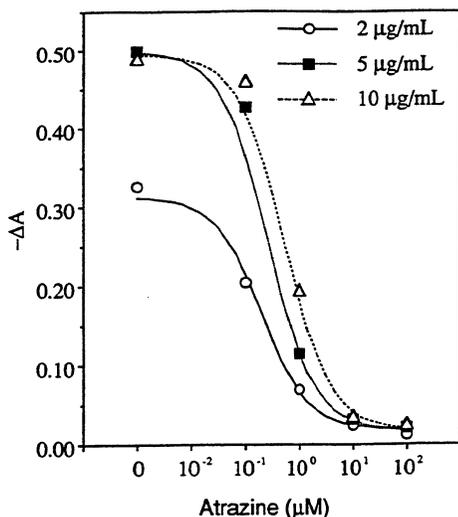
**Figure 1.** Rate of DPIP photoreduction for various chlorophyll concentrations. Each sample contained 0.5 mM DPC and 60 μM DPIP.

20 samples in triplicate (along with blanks and standards) in the time required to analyze a single sample using test tubes and a spectrophotometer. Since the samples, standards, and blanks utilize the same reagents, and receive the same illumination and temperature exposures, good precision can be obtained even with wide variations in reagent activity and assay conditions.

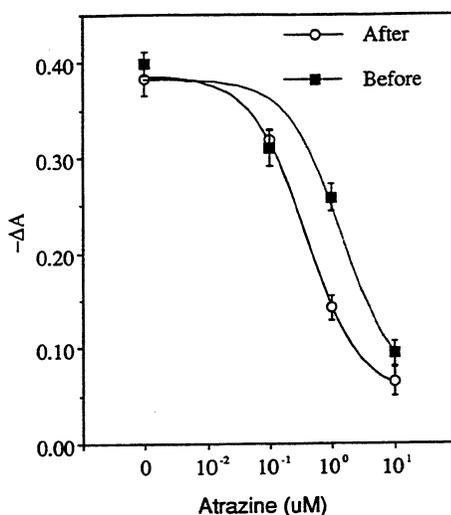
**Effect of Chlorophyll Concentration.** Figure 1 shows the effect of chlorophyll concentration on the time course of absorbance in a microtiter plate assay. The quantity plotted on the ordinate,  $-\Delta A$ , is the change in absorbance due to reduction of DPIP to produce the colorless leuco dye. The rate of DPIP reduction varied with chlorophyll concentration, as expected. With 10 μg/mL chlorophyll, reduction of the available DPIP was completed in ~3 min. As illumination was continued after the disappearance of DPIP, the pale green solution became yellow, and absorbance at 595 nm increased. It was not determined whether this was due to a spectral change or increased turbidity of the sample. When illumination was stopped (not shown), absorbance slowly increased (~0.002 AU/min) due to reoxidation of DPIP. Illumination was typically continued until the absorbance change for the blank was near maximum, and absorbance readings were taken immediately after illumination was terminated. Assay results for the three thylakoid concentrations with 5-min illumination are shown in Figure 2. The theoretical curves in Figures 2–5 are fits to a three-parameter equation using only four or five data points. They are intended as visual guides to the expected sigmoidal response curve and not as accurate descriptions of the inhibition response.

**Sample Preparation Effects.** It was necessary to centrifuge the homogenized beef tissue in order to obtain clear solutions in which the colorimetric reaction could be observed. Virtually all the fat in the sample was discarded during centrifugation, raising the possibility of poor analyte recovery. To determine the extent of analyte loss during sample preparation, homogenized beef tissue was spiked with atrazine both before and after centrifugation. The results are shown in Figure 3. The sample that was centrifuged after spiking contained approximately one-fifth the level of atrazine found in the sample that was spiked after centrifugation. It is clear that the majority of the analyte adsorbs or partitions into the insoluble fraction of the sample and is lost on centrifugation. For analysis of samples containing incurred residues, extraction of analyte from the insoluble material or a means of accounting for analyte loss would be required.

**Matrix Effects and Interferences.** Matrix components may interfere with herbicide binding or with other aspects

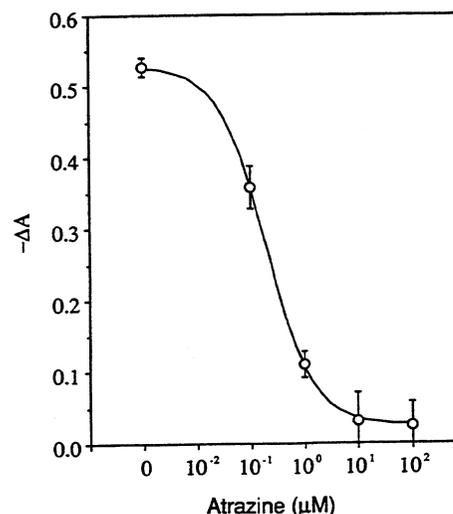
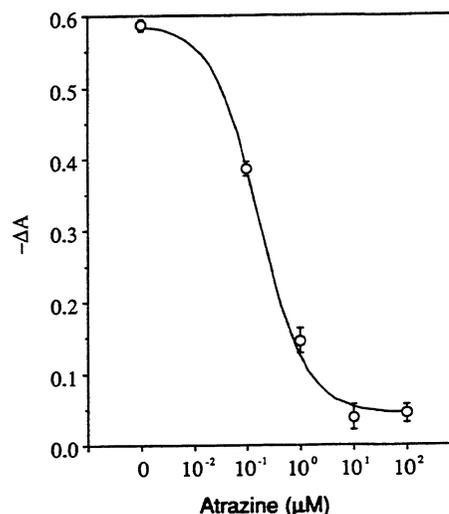
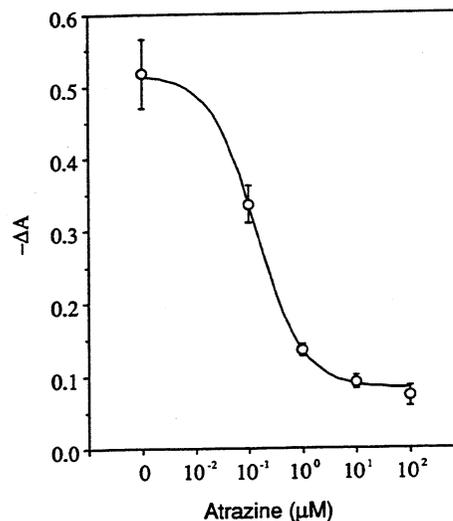


**Figure 2.** Assay response for various chlorophyll concentrations. Each sample contained 0.5 mM DPC, 60  $\mu$ M DPIP, and the indicated concentration of atrazine. Illumination time, 5 min.



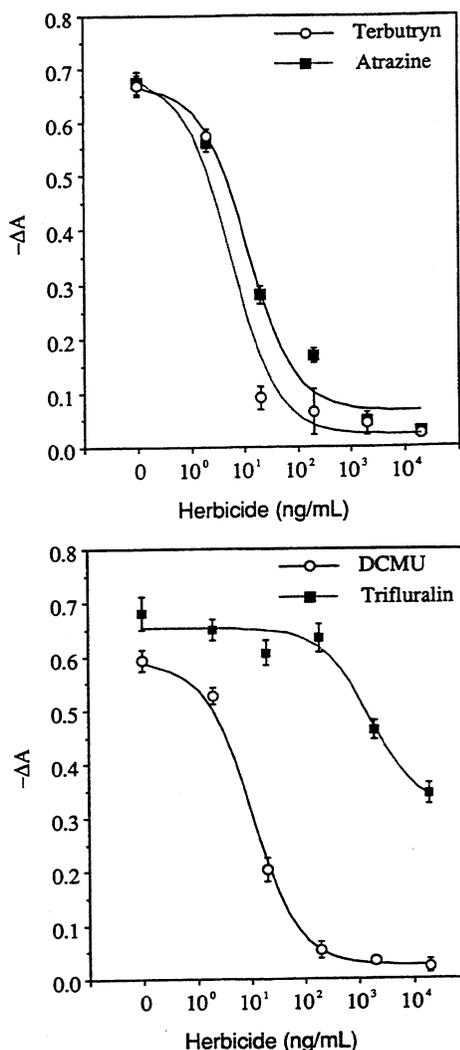
**Figure 3.** Variation in assay response with sample preparation for beef extract spiked with atrazine: (O) beef extract spiked after centrifugation; (■) beef extract spiked before centrifugation. Each sample contained 5  $\mu$ g/mL chlorophyll, 0.5 mM DPC, 60  $\mu$ M DPIP, and the indicated concentration of atrazine. Illumination time, 5 min. Error bars represent 1 standard deviation about the mean of six replicates.

of photosynthetic electron transfer, resulting in a shift in response for the biological matrices relative to water. As seen in Figure 4, no such shift was observed. The fact that virtually identical calibration curves were obtained with buffer, urine, and beef extract permitted the use of stable aqueous standards for calibration regardless of the sample matrix. This is an important advantage over immunoassay systems, which often exhibit large variations in response with different matrices.<sup>15</sup> Matrix components may also interfere with the colorimetric reaction (DPIP reduction) used to detect herbicide binding. Such indirect interference was in fact observed with beef extract and milk. With beef extract, a nearly instantaneous reaction occurred when DPIP was added to the sample, resulting in  $\sim$ 30% reduction in DPIP absorbance. This was compensated for by use of a slightly more concentrated DPIP solution in some beef extract assays. A similar reaction occurred with milk, but in this case resulted in complete bleaching of DPIP. It was therefore impossible to detect herbicides in milk and no data were generated for this matrix.



**Figure 4.** Assay response for various matrices: (A, top) water; (B, middle) urine; (C, bottom) beef extract. Each sample contained 5  $\mu$ g/mL chlorophyll, 0.5 mM DPC, 60  $\mu$ M DPIP, and the indicated concentration of atrazine. Illumination time, 5 min. Error bars represent 1 standard deviation about the mean of six replicates.

**Dynamic Range, Precision, and Detection Limit.** Binding of herbicide to PS II centers is described by the Hill equation,  $\theta/(1 - \theta) = KL^n$ , where  $\theta$  is the fraction of binding sites occupied,  $K$  is the binding constant,  $L$  is the concentration of herbicide, and  $n$  is the Hill coefficient.<sup>5,16</sup> Calibration curves are therefore expected to have sigmoidal shapes similar to those obtained in immunoassays. There is a relatively narrow



**Figure 5.** Assay response for various herbicides in water: A (top) and B (bottom). Each sample contained 5  $\mu\text{g/mL}$  chlorophyll, 0.5 mM DPC, 80  $\mu\text{M}$  DPII, and the indicated concentration of herbicide. Illumination time, 5 min. Error bars represent 1 standard deviation about the mean of six replicates.

window over which absorbance varies significantly with analyte concentration and hence an inherently limited dynamic range. In these assays, a maximum slope of  $\sim 0.5$   $\Delta\text{AU}$  per log concentration unit and a standard deviation in absorbance of  $\sim 0.02$   $\Delta\text{AU}$  for six replicates was typically observed. This resulted in an uncertainty of  $\sim 0.2$  log unit in concentration at the 95% confidence limit, i.e., an uncertainty of almost 2-fold in concentration. Due to the fairly large uncertainty and limited dynamic range, the assay is at present unsuitable for quantitative determination of herbicide concentrations in unknowns. However, for screening purposes it is only necessary to determine whether the analyte concentration exceeds a given threshold, and the assay in its present form is well suited to this application. Small variations in analyte concentration can be distinguished

readily in the region of the calibration curve where  $\Delta A$  changes rapidly with concentration, and samples can be diluted to bring the analyte concentration into this region if necessary. It is believed that improved precision can be obtained in the future with refinements in pipeting technique, reagent mixing, elimination of bubbles, and uniformity of illumination.

The detection limit or least detectable dose (LDD) in immunoassays is typically defined as the concentration of analyte giving a response 2 standard deviations away from the mean of the zero standard.<sup>17</sup> We define here a somewhat more conservative detection limit as the concentration giving an absorbance change 0.10 AU lower than the absorbance of the blank, as estimated from the fitted calibration curve. Referring to Figure 5, detection limits are estimated as 3 ng/mL for atrazine, 3 ng/mL for diuron, 1 ng/mL for terbutryn, and 800 ng/mL for trifluralin. Trifluralin is not a PS II inhibiting herbicide and was included as a test of the specificity of the assay. Terbutryn exhibited a very steep increase in inhibition between 2 and 20 ng/mL which did not fit well to the Hill equation with a Hill coefficient of unity. The cause of this behavior is not presently understood.

**Thylakoid Storage and Stability.** Activity of freshly prepared thylakoids stored at 4  $^{\circ}\text{C}$  declined continuously after preparation, reaching  $\sim 50\%$  of initial activity after 24 h, and 20% or less after 48-h storage. Concentrated solutions showed greater stability than dilute solutions, and therefore, dilute thylakoid solutions (8–40  $\mu\text{g/mL}$ ) were prepared shortly before use by dilution of concentrates. Practical application of this technique would require reagents with shelf lives of weeks or months. We therefore investigated several approaches to extend storage life of spinach thylakoids, including freezing at  $-70$  and  $-10$   $^{\circ}\text{C}$ , freeze-drying, and immobilization in a biogel. Successful storage of frozen spinach thylakoids at liquid nitrogen temperatures has been reported recently,<sup>18</sup> and we employed similar conditions (high sugar concentration) in this work. Thylakoids stored at  $-10$   $^{\circ}\text{C}$  (normal freezer temperature) showed only slightly greater stability than those stored at 4  $^{\circ}\text{C}$ . Thylakoids stored at  $-70$   $^{\circ}\text{C}$  exhibited an initial decrease in activity of  $\sim 60\%$ , but retained this level of activity for months. After thawing, activity of frozen thylakoids decayed at a rate similar to that of freshly prepared material. Freeze-dried thylakoids displayed activity similar to those frozen at  $-70$   $^{\circ}\text{C}$ , at least during the first several weeks of storage. We are unaware of previous studies on freeze-drying of thylakoids or chloroplasts. Preliminary experiments with thylakoids immobilized in calcium alginate gels<sup>19</sup> indicate that this approach yields active material with a shelf life of at least several weeks under refrigeration at 4  $^{\circ}\text{C}$ . Details of these storage studies will be reported elsewhere.

## CONCLUSIONS

Several major classes of photosystem II inhibiting herbicides can be detected at trace levels in water, urine, and meat tissue using a simple biorecognition assay. The assay utilizes safe, inexpensive reagents and is readily carried out in microtiter plates. This format permits large numbers of standards and samples to be processed in parallel, providing high throughput and minimizing effects of variations in reagent activity. Selectivity between different classes of herbicides is limited with this system, although higher plants<sup>5,20</sup> and bacteria<sup>21</sup> with resistance to various herbicide classes are known and could be employed to produce class-selective detection systems. Efforts are now underway to improve reagent shelf life and eliminate the colorimetric interferences encountered with milk.