Rapid Magnetic Particle-Based ELISA Assay Compared with Gas Chromatography-Nitrogen Phosphorus Detection for Determining Atrazine in Freeze-Dried Water Samples

J. Gascón / D. Barceló
Department of Environmental Chemistry, CID-CSIC, c/Jordi Girona, 18–26, 08034 Barcelona, Spain

Key Words
Gas chromatography
Nitrogen phosphorus detection
Atrazine and simazine
Freeze-dried water analysis
ELISA

Summary
The determination of atrazine in freeze-dried water samples containing simazine, 0.6 % glycine as stabilizer and other pesticides has been compared using three different techniques: (i) direct rapid-magnetic particle-based ELISA, (ii) dichloromethane liquid-liquid extraction (LLE) and Florisil column clean-up prior to ELISA determination and (iii) LLE, Florisil column clean-up and gas chromatography with nitrogen phosphorus detection (GC-NPD). The methodology developed in this paper has shown the advantages of the introduction of a clean-up step prior to ELISA determination and its correlation with GC-NPD determinations. Atrazine could be determined at levels between 0.1 to 51 ug L⁻¹ in water samples using the different methods described in this paper. The cross-reactivity problems found in the ELISA test associated with the presence of simazine are also discussed.

Introduction
The ubiquitous presence of atrazine and other chlorotriazine herbicides in environmental waters of the EEC and other countries has prompted the need for sensitive, specific methods of analysis. Official methods of analysis usually involve the use of robust techniques, such as dichloromethane liquid-liquid extraction (LLE) followed by gas chromatography-nitrogen phosphorus detection (GC-NPD), (US EPA Method 507) [1]. Recently introduced, batch immunoassays methods such as ELISA appear to be sensitive, reliable, simple, cost-effective, and which provide rapid results. Limitations of ELISA may arise for screening complex environmental samples. In this respect false positive results may be caused by interference from structurally similar compounds or by inadequate clean-up steps [2]. These problems may be reduced by the introduction of an efficient clean-up step, e.g., solid-phase extraction (SPE) or Florisil clean-up before ELISA. In a recent paper [3] SPE and ELISA were combined and compared with GC techniques for the determination of atrazine and alachlor. Although in this case no simazine was present in the sample the introduction of SPE permitted avoidance of 28 % of false positive results in the determination of alachlor [3]. The rapid-magnetic based ELISA has advantages over conventional ELISA since results can be obtained without encountering the common sources of variability (e.g. coating variability, antibody leaching off), observed when using coated tubes, beads or microtitre plates as the solid-phase [4]. Greater precision is obtained when using magnetic particles because of faster reaction kinetics from a larger surface area-to-volume ratio when compared to the microtitre plate, which uses a plane surface for immunochemical reaction. In this respect, a shorter analysis time than the microtitre plate-based ELISA can be helpful [3] and can be easily applied when carrying out monitoring studies, since large numbers of samples need to be processed in a relatively short time.

The present work is devoted to the determination of atrazine in freeze-dried water samples using rapid-magnetic based ELISA. These samples were prepared in collaboration with the Community Bureau of Reference of the Commission of the European Communities (BCR-CEC) with the objective of obtaining a candidate reference material containing atrazine, simazine and other pesticides. Such samples were of interest in that they were obtained from real drinking water samples where 0.6 % of glycine had been added as stabilizer. In this respect the aim of the present work will be the determination of atrazine in the presence of simazine in these samples by:
(i) direct ELISA, (ii) LLE and Florisil clean-up and ELISA and (iii) LLE-Florisil-GC-NPD according to EPA method 507. The present work will provide more objective information about the ELISA test, since atrazine will be determined in the presence of simazine,
which reflects a real environmental situation [1]. This research follows prior work form our group devoted to the comparison of various analytical techniques for the determination of pesticides in freeze-dried water samples [5].

**Experimental**

**Chemicals**

Pesticide grade solvents, ethyl acetate, n-hexane, di-ethyl ether and dichloromethane, were from SDS (Peypin, France). Florisil (100–200 mesh) was from Merck (Darmstadt, Germany). Pesticide standards were purchased through Promochem (Wesel, Germany). The rapid-magnetic particle-based ELISA assay from Ohmicron (Newton, PA, USA) was purchased through J. T. Baker (Deventer, The Netherlands).

**Sample Collection and Preparation of Freeze-Dried Water**

The preparation and stability studies of two batches of freeze-dried water samples containing pesticides has been reported in a previous paper [5]. The samples contained atrazine, simazine, fenitrothion, fenamiphos, parathion-ethyl, carbaryl, linuron and propanil with final concentrations varying from 0.02 to 7 μg l\(^{-1}\). Moreover, 0.6 % glycine was added as stabilizer.

**Sample Extraction and Clean-up**

Pesticides were extracted from 1.0 l water using liquid-liquid extraction (LLE) into n-hexane (100 ml) and dichloromethane (2 x 100 ml). After concentration in a rotary evaporator (35 °C) the extract was carefully evaporated to dryness. The extract was dissolved in 0.5 mL ethyl acetate for the clean-up which was carried out with glass column (150 x 5 mm i.d.) filled with ca. 1 g Florisil. Florisil had been activated overnight at 300 °C, cooled and deactivated with 2 % water. After packing, the column was rinsed with n-hexane and ether. The water extracts were placed on top of the column and eluted using 6 mL of a mixture of ethyl ether in n-hexane (50:50). The fractions were evaporated just to dryness and the residue dissolved in 0.5 ml ethyl acetate for GC-NPD analysis or 1.0 ml methanol to yield a final volume of 1.0 l water for immunoassay analysis.

**Gas Chromatographic Analysis by GC-NPD**

Following Florisil clean-up, the extracts were injected onto the column of a GC 5300 Mega Series gas chromatograph (Carlo Erba, Milan, Italy) equipped with NPD. A 15 m x 0.25 mm I.D. fused-silica capillary column with a 0.15 μm film of chemically-bonded cyanopropylphenyl DB 225 (J & W Scientific, Folsom, CA, USA) was used. Hydrogen was employed as carrier gas at 50 cm s\(^{-1}\) and helium as the make-up gas at 30 ml min\(^{-1}\). The injector and detector were held at 270 °C. The column was programmed from 60 to 90 °C at 10 °C min\(^{-1}\) and from 90 to 220 °C at 6 °C min\(^{-1}\), with final time of 15 min. Calibration graphs for every compound were plotted using cyanazine as internal standard. The response was linear from 0.01 to 10 μg l\(^{-1}\).

**Competitive Immunoassay Procedure**

The rapid magnetic-particle-based, solid-phase, Enzyme-linked Immunosorbent Assay (ELISA) (RAPID Assays, Ohmicron Corporation) has polyclonal antibodies coated on paramagnetic beads. A total of 200 μl of the sample water to be analyzed is added to a disposable test tube, along with 250 μl atrazine hapten-horseradish peroxidase (HRP), enzyme conjugate and 500 μl rabbit anti-atrazine magnetic particles (attached covalently). Both pesticide in the sample and enzyme-labelled pesticide compete for antibody sites on the magnetic particles. Tubes were vortexed and incubated for 15 min at room temperature. The reaction mixture was magnetically separated using a specially designed magnetic rack. After separation, the magnetic particles were washed twice with 1.0 ml distilled water to remove unbound conjugate and eliminate any potential interfering substances. Pesticide and enzyme-labelled pesticide remain bound to the magnetic particles in concentrations proportional to their original concentration. The presence of labelled pesticide is detected by adding a total of 500 μl of a 1:1 mixture of a solution containing substrate and chromogen (hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB)). The tubes were vortexed to resuspend particles and incubated for another 20 min at room temperature and dark conditions to allow color development. Since the enzyme-labelled pesticide was in competition with the unlabelled pesticide (sample) for the antibody binding sites, the color developed is inversely proportional to the concentration of pesticide in the sample. The color reaction was stopped by the addition 500 μl 2 M sulphuric acid. The final concentrations of atrazine for each sample were determined using the RPA-1 RaPID Photometric Analyzer (Ohmicron) by determining the absorbance at 450 nm. The observed sample results were compared to a linear regression line using a logit standard curve prepared from calibrators containing known levels of atrazine at 0, 0.1, 1.0, and 5.0 μg l\(^{-1}\).

**Results and Discussion**

Figure 1 shows the GC-NPD chromatograms of batch A and batch B after LLE and clean-up of both batches into a Florisil column. Figure 2 illustrates the mean dose response curve for atrazine. The displacement at the 0.1 μg l\(^{-1}\) level is significant, 81 % B/Bo. It is common to report displacement in terms of a B/Bo measurement to describe color inhibition. B/Bo is defined as the absorbance at 450 nm observed for a sample or standard divided by the absorbance at the