Food Chemistry 43 (1992) 283–287
Analytical Methods Section

Determination of atrazine in milk by immunoassay

Rodney J. Bushway, Lewis B. Perkins, Heather L. Hurst

Department of Food Science, University of Maine, Orono, Maine 04469, USA

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Bruce S. Ferguson

ImmunoSystems Inc., 4 Washington Avenue, Scarborough, Maine 04074, USA

(Received 7 December 1990; accepted 4 February 1991)

A polyclonal enzyme immunoassay method has been developed to determine atrazine in process milk including skim, low-fat, whole, chocolate, evaporated, non-fat dry milk, and half and half. The procedure is sufficiently simple for plant use, rapid (15 min) and sensitive (0·2 ng/ml). Atrazine concentration was linear from 0·2 ng/ml to 6·4 ng/ml. Same day and day-to-day (over a two-month period) reproducibility was excellent with most per cent coefficients of variation (%CV) below 12% even at the 0·2 ng/ml level. Cross-reactivity was such that this method could be used to determine other triazine pesticides in milk. Milk products throughout the USA and some in Czechoslovakia and Sri Lanka have been tested by this immunoassay procedure.

INTRODUCTION

Atrazine (6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine), a triazine herbicide, is used to control annual grasses and broad-leaf weeds mainly in corn, pineapple, sugarcane and macadamia orchards. This herbicide is used extensively worldwide. In the United States alone atrazine is the second most applied pesticide; approximately 79 million pounds is applied annually (Anon., 1987). Because of public perception that food supplies are unsafe, limited atrazine toxicological data (Wilson *et al.*, 1987) and contamination of European milk with triazine pesticides (Bakke *et al.*, 1971; Gajduskova, 1983; Rathouska *et al.*, 1987), there is a need for a rapid and inexpensive monitoring procedure for atrazine in milk.

Analysis of atrazine residues in food is presently performed either by chromatographic techniques (Bailey & LeBel, 1978; Majors, 1980; Roseboom & Herbold, 1980; Gajduskova, 1983; Rathouska *et al.*, 1987; Viden *et al.*, 1987; Anon., 1988; Barcario *et al.*, 1988; Tekel *et al.*, 1988*a,b*), enzyme immunoassay (EIA) (Bushway *et al.*, 1989) or capillary isotachophoresis (Krivankova *et al.*, 1989). Chromatographic and isotachophoresis methods require expensive equipment, extraction and pre-concentration steps while EIA methods use relatively inexpensive equipment and direct analysis of liquid foods such as fruit juices and soft drinks (Bushway *et al.*, 1989) with greater sensitivity.

This paper describes an enzyme immunoassay application for analyzing atrazine in several processed milk products. The method is an excellent screening procedure because of its speed, sensitivity, simplicity and low cost. Cross-reactivity with other triazines is another advantage. Furthermore, it can be quantitative if the triazine is known.

MATERIALS AND METHODS

Reagents and materials

Atrazine and all other triazine pesticides were obtained from EPA, Triangle Park, North Carolina while the metabolites of atrazine were a gift from Ciba-Geigy, Greensboro, North Carolina. Stock solutions of each pesticide were prepared by weighing 10 mg of each into a 50 ml volumetric flask and bringing to volume with methanol. Intermediate standards were obtained by

Food Chemistry 0308-8146/92/$05.00

pipetting 0·05 ml of stock solution into a 100 ml volumetric flask. The flask was brought to volume with water. Actual working standards were prepared by removing 20, 40, 80, 160, 320 and 640 μl aliquots from the intermediate standard solution and placing each aliquot into a separate 10 ml volumetric flask and bringing to volume with milk or water. This gave working standards of 0·2, 0·4, 0·8, 1·6, 3·2 and 6·4 ng/ml.

Antiserum to atrazine was prepared by derivatizing atrazine at the 2-chloro position and covalently conjugating it to bovine gamma globulin through a modified carbodiimide cross-linking procedure. The final molar ratio of hapten to globulin was 30:1. Polyclonal antibodies were prepared in multiple sub-cutaneous injection over several months. Blood was collected from the rabbits on a monthly schedule and the serum separated and stored at −10°C.

Antibodies to atrazine were coated to the walls of polystyrene test tubes by a proprietary method developed by ImmunoSystems Inc. Shelf-life of the dried and stabilized antibody-coated tubes was greater than one year at 4°C. Horseradish peroxidase was covalently bound to atrazine (the 'enzyme conjugate') also by a modified carbodiimide conjugation technique and is stable in liquid for over one year at 4°C. The substrate and chromogen were stabilized buffer preparations of hydrogen peroxide and tetramethylbenzidine (TMB), respectively.

Immunoassay method for atrazine

Milk standards and samples (undiluted) were analyzed by adding 160 μl to an antibody-coated polystyrene tube (ImmunoSystems Inc.) followed by 160 μl of enzyme conjugate. The tubes were incubated for 5 min at room temperature and then rinsed four times with tap water to remove unreacted sample and enzyme conjugate. To the rinsed tubes were added 160 μl each of substrate and chromogen. Tubes were allowed to incubate for an additional 5 min at room temperature before adding 40 μl of 2·5 M sulfuric acid to stop the reaction. The sulfuric acid converts the blue chromophore to yellow. Color intensity was measured by transferring 200 μl aliquots to the wells of microtiter strips and reading each well at 450 nm with a Bio-Tek Instruments Model EL 301 strip reader (Burlington, VT). Samples in the tubes can also be read directly without removing aliquots by using a hand-held battery-powered differential photometer from Artel Inc. (Windam, ME). The only disadvantage of the hand-held photometer is that some sensitivity is lost because water has to be added to the tubes before they can be read.

As many as six samples and a control can be analyzed simultaneously. A control (non-triazine milk) was run with each set of six tubes since its absorbance at 450 nm (A450) was used to calculate the $\%B_0$ value (which can be defined as A450 of standard or samples/A450 of the control × 100) of the standard and samples. Non-triazine milk was obtained by screening samples with the EIA; when a $\%B_0$ value of 90% or greater was obtained compared to water, then the milk was considered to be 'triazine-free'. A standard curve was run by analyzing a set of milk standards (0·2, 0·4, 0·8, 1·6, 3·2 and 6·4 ng/ml) at the beginning and end of each day with the average $\%B_0$ values used to quantify atrazine in the samples. A plot was made of $\%B_0$ values versus the logarithm of atrazine concentration (ng/ml) which was used to calculate atrazine concentration in the samples.

RESULTS AND DISCUSSION

The immunoassay showed a linear relationship (Fig. 1) from 0·2 to 6·4 ng/ml (20 to 640 pg/tube) which was observed between the logarithm of the atrazine concentration and the $\%B_0$ at 450 nm. For samples having a concentration greater than 6·4 ng/ml which is indicated by a $\%B_0$ of less than 10 a simple dilution must be performed.

For quantification of atrazine in milk by immunoassay one has to derive the standard curve from a non-triazine milk source of the same type being analyzed because of a matrix effect occurring between the assay and milk fat (Fig. 2). Figure 2 shows that the milk products (whole, chocolate, 2%, evaporated, and half and half) with the highest fat content have $\%B_0$ values greater than the non-fat milk products (skim and non-fat dry milk) for the same fortification levels of atrazine.

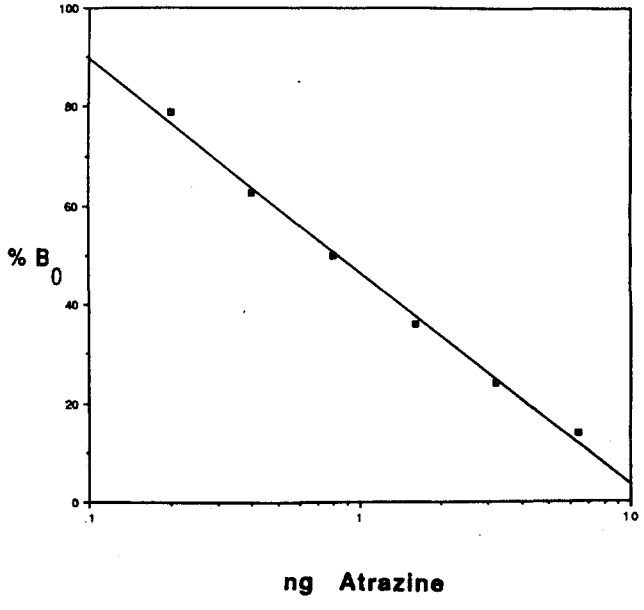

Fig. 1. Typical immunoassay standard curve of whole milk spiked with atrazine.

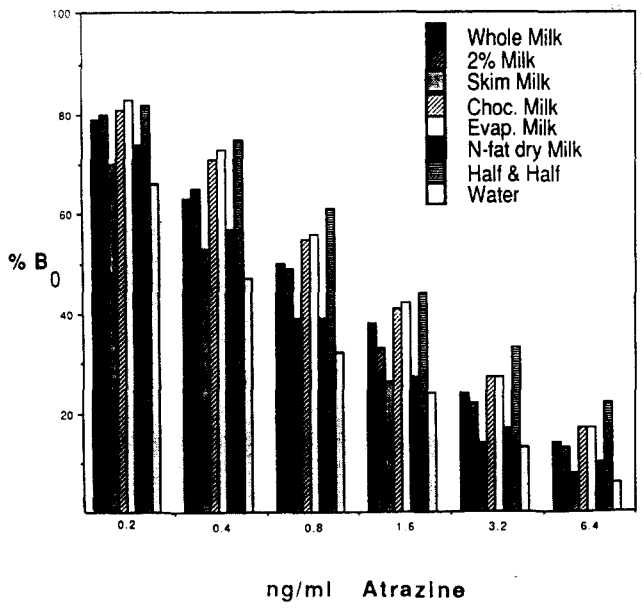

Fig. 2. Comparison of $\%B_0$ values for seven milk products and water spiked at six different concentrations of atrazine.

In fact, the $\%B_0$ values of the non-fat milk products compare with those of water. The milk product with the highest fat content (half and half) had the largest $\%B_0$ values for all spiked samples. Why the fat has such an effect has not yet been determined. One might speculate that the atrazine partitions into the fat and becomes unavailable to the antibody. Therefore, for the best quantitative and most sensitive results one should group the milk products into three categories: (1) cream type, like half and half with milk fat greater than 3·5%; (2) whole and 2% products with a fat content of 1·5–3·5%; and (3) non-fat milk products with 0·5% fat or less. To make evaporated milk the same fat content as whole milk it was necessary to dilute it 1:1. Thus, all evaporated milk was diluted before being tested.

Milk products could be diluted 1/10 which would lower the per cent fat to a level such that there was no matrix effect. However, the sensitivity was decreased from 0·2 to 2·0 ng/ml.

As with any analytical technique, consistency and accuracy on the same day as well as day to day are crucial. Reproducibility results of the atrazine in milk immunoassay can be seen in Table 1 and 2. Table 1 illustrates the same-day reproducibility of seven milk products (analyzed six times in one day) spiked with atrazine at 0·2–6·4 ng/ml. Per cent coefficients of variation (%CV) ranged from 3·7 to 17·4% with most below 12%.

Table 2 depicts consistency results obtained from analyzing six different milk products fortified with atrazine at 0·2–6·4 ng/ml over a period of two months. Per cent coefficients of variation ranged from 5·0 to 23·2%. Like Table 1, the majority of the %CVs were below 12%, but were not as low as the same-day samples. One would not expect the day-to-day %CVs to be as good as the same-day ones because of increasing variables such as time and different milk supplies. However, both sets of %CVs' were excellent for residue quantification, especially if one considers that existing chromatographic methods cannot analyze atrazine or other triazines at such low concentrations in milk even if a concentration step is employed because of interferences (Gajduskova, 1983; Rathouska *et al.*, 1987; Barcario *et al.*, 1988; Tekel *et al.*, 1988*b*).

Besides atrazine, this immunoassay can cross-react with other triazines that have 2- and 4-position diamine side chains containing the ethyl and isopropyl groups. Thus, the immunoassay is most sensitive to the following triazines and metabolites: simazine, propazine, prometryn, ametryn, simetryn, cyprazine, dipropetryn, atraton, prometon, de-ethylated atrazine and 6-hydroxy atrazine. Some of these triazines were fortified in whole milk and the results are shown in Fig. 3. As can be seen, two major metabolites of atrazine are less sensitive than the parent triazine, but are sufficiently reactive to be detected at 1 ng/ml for de-ethylated atrazine and 1·6 ng/ml for 6-hydroxy atrazine, while simazine, propazine and ametryn can be detected at 0·4, 0·4 and 0·2 ng/ml, respectively.

Table 1. Same-day reproducibility of the atrazine immunoassay for atrazine-fortified milk products

Milk type	Amount atrazine added (ng/ml): 0·2	0·4	0·8	1·6	3·2	6·4
	Per cent coefficients of variation[a]					
Whole	4·9	6·3	4·1	11·7	7·8	16·3
2%	5·0	3·7	4·4	10·6	11·9	14·1
Skim	12·6	10·0	6·4	10·6	12·5	12·3
Chocolate	8·5	3·7	10·1	10·4	6·6	11·9
Evaporated	7·2	6·0	8·5	5·7	8·6	8·9
Dry	5·6	6·0	10·8	10·8	17·4	13·8
Half and half	5·8	8·2	9·6	9·7	8·8	9·5

[a] % Coefficients of variation are based on six different $\%B_0$ determinations.

Table 2. Day-to-day reproducibility of the atrazine immunoassay for atrazine-fortified milk products

Milk type	No. analyzed	Amount atrazine added (ng/ml) 0·2	0·4	0·8	1·6	3·2	6·4
		Per cent coefficients of variation					
Whole	21	7·1	9·4	12·0	14·8	18·9	16·7
2%	10	7·8	8·2	7·0	7·2	12·2	12·5
Skim	12	7·8	9·6	13·1	14·8	16·7	23·9
Chocolate	7	7·1	7·7	11·7	12·6	12·9	23·2
Evaporated	6	5·0	7·1	5·5	8·6	6·0	13·1
Half and half	6	5·6	6·6	7·8	7·3	9·6	7·6

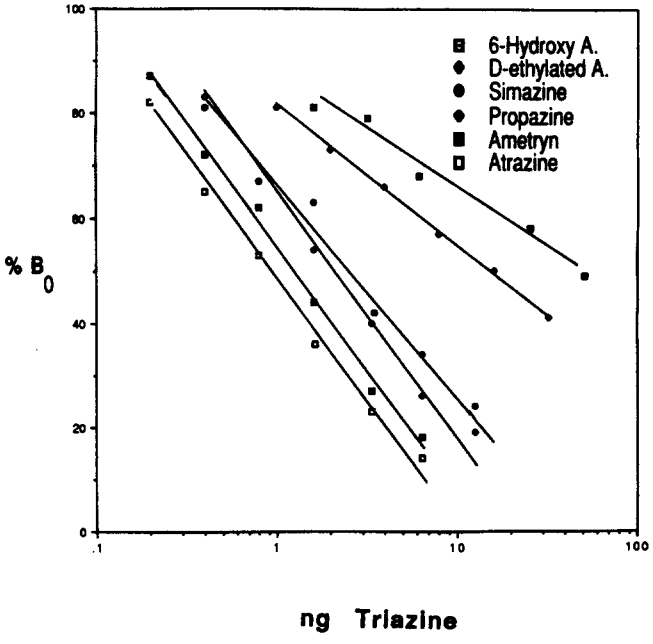

Fig. 3. Immunoassay standard curves of whole milk samples spiked with different triazines and metabolites.

Once the accuracy, reproducibility and sensitivity of the EIA were defined, the method was used to analyze milk products throughout the USA, Czechoslovakia and Sri Lanka. A list of analyzed milk products and locations is given in Table 3. Of the 114 processed milk samples analyzed from 13 different states and countries, none showed any detectable amount (0·2 ng/ml) of atrazine or other sensitive triazines and metabolites. From the authors' large sampling of the USA processed milk supply, it would seem that atrazine and other triazines are not major contaminants, although some of the metabolites like di-dealkylated atrazine could be present since the immunoassay does not react with this type of metabolite.

Immunoassays are also performed in microtiter wells. Although the well configuration takes much longer to run, it has the advantage of being able to analyze 96 samples at a time. Preliminary results have shown that the wells and tubes are in good agreement for milk analysis with the same sensitivity.

Table 3. List of milk products analyzed by atrazine immunoassay

Milk type[a]	No. analyzed	Amount atrazine found
Whole	37	ND[b]
2%	25	ND
Skim	28	ND
Chocolate	6	ND
Evaporated	7	ND
Non-fat dry	4	ND
Half and half	7	ND

[a] Samples were obtained from ME, MA, MI, CT, IA, OH, NE, FL, TX, CA, OK, Czechoslovakia and Sri Lanka.
[b] None detected at a detection limit of 0·2 ng/ml.

The triazine enzyme immunoassay method using polystyrene tubes offers an excellent procedure to determine atrazine and other triazines in milk products. The procedure is reproducible, sensitive and inexpensive compared to chromatographic techniques. In fact, the immunoassay is so simple it could be used in processing plants. Furthermore, because of the cross-reactivity of the atrazine antibody, the immunoassay has the potential to be a broad spectrum triazine test.

ACKNOWLEDGEMENT

This paper is no. 1570 of the Maine Agriculture Experiment Station.

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