Analysis of Thiabendazole in Potatoes and Apples by ELISA Using Monoclonal Antibodies

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Thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole, TBZ] is used as a preharvest or postharvest fungicide on pome fruits, citrus, bananas, potatoes, and several other commodities. An enzyme-linked immunosorbent (ELISA) method was developed which uses monoclonal antibodies that bind thiabendazole. The antibodies were elicited using the hapten 5-succinamido-2-(4-thiazolyl)benzimidazole. Aqueous buffers or water is adequate for extraction of the TBZ, and the method can be completed, without access to sophisticated laboratory equipment, within 4 h. Results were validated by comparison to a method employing chromatographic fractionation with UV detection. The ELISA method using aqueous extraction of apple or potato peel has a limit of detection of <0.2 ppm. Since U.S. federal tolerances for residues of TBZ in potatoes and apples are both 10 ppm, this rapid screening test has sufficient sensitivity for regulatory monitoring of residues. In addition, the ELISA could serve as a quality control procedure for dipping or spraying operations.

INTRODUCTION

Thiabendazole (TBZ, 2-[4-thiazolyl]-benzimidazole) is a member of the benzimidazole group of fungicides, which also includes benomyl, thiophanate-methyl, thiophanate-ethyl, and methyl benzimidazolecarbamate (MBC, carbendazim). These compounds are widely used for pre- and postharvest treatment of fruits and vegetables and exert their effect when the active benzimidazole form of the fungicide binds to fungal tubulin, with subsequent disruption of cell division [reviewed by Davidse (1986) and Eckert and Ogawa (1988)]. Although this mode of action is the basis of the teratogenicity of some benzimidazoles [reviewed by Delatour and Parish (1986)], thiabendazole is relatively safe, with an acceptable daily intake (ADI) established by the World Health Organization of 0.3 mg/kg of body weight per day.

Analytical methods for thiabendazole include HPLC, using either reversed-phase [e.g., Cano et al. (1987) and Martindale (1988)] or normal-phase columns [e.g., Bicchi et al. (1989)]. Sample workup for these methods employs organic solvents, liquid–liquid extractions, and concentration steps. These steps are not eliminated by use of solid-phase cleanup procedures [e.g., Jamieson and Duncan (1989)].

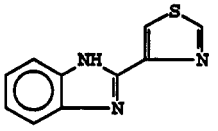
Recently, there has been increased interest in the development and implementation of rapid screening methods, including immunoassays, for residue monitoring (National Research Council, 1987; Office of Technology Assessment, 1988). Rapid screening tests could permit testing of more samples and produce greater confidence in the safety of the food supply. An immunoassay method for thiabendazole using organic solvent extraction and polyclonal antibodies was reported by Newsome and Collins (1987). Our approach has been to develop monoclonal antibodies specific for thiabendazole and sample workup procedures that eliminate the need for organic solvents and sophisticated laboratory equipment. We previously reported monoclonal antibodies which bind thiazolylbenzimidazoles including TBZ (Brandon et al., 1992) and a competitive ELISA suitable for a liver or urine matrix. Since that assay format proved to be unsuitable for some produce matrices, we now describe an alternate procedure for ELISA of TBZ in two matrices, apple and potato, using a one-step aqueous extraction.

METHODS AND MATERIALS

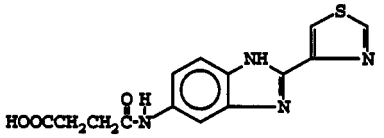
Samples and Treatment of Produce with TBZ. Red Delicious apples and russet, white, and red potatoes were obtained from local stores. Apples and potatoes, sold as "organically grown" and subsequently determined by both HPLC and ELISA to have no detectable residue of thiabendazole (<0.12 ppm in the peel), were treated by dipping them in a suspension of Mertect 340-F (MSD Agvet, Rahway, NJ), in accordance with the product label. Apples were dipped for 3 min in a suspension containing 0.57 g of TBZ/L, and potatoes were dipped for 20 s in a suspension of 1.5 g of TBZ/L. The dipped produce was then drained and stored refrigerated at 4 °C. Spiked samples were prepared by adding TBZ (kindly provided by MSD Agvet) in methanol or dimethylformamide (1.44 mg/L) to minced produce or extracts. For determination of the limit of sensitivity using treated potatoes, treated whole potatoes or peels were minced and mixed with control TBZ-negative sample to achieve various concentrations of TBZ residue prior to extraction of the sample.

Extraction of Samples for HPLC Analysis. Procedures were adapted from Tjan and Burgers (1973) and Cano et al. (1987). Chopped whole fruit or tuber (50–70 g) was transferred to a household blender (Osterizer Dual Range 10, Oster, Milwaukee, WI) and homogenized for 10 min with 150 mL of ethyl acetate. After the liquid was decanted, the residue was re-extracted with an additional 100 mL of solvent. The combined extracts were filtered through a coarse glass Büchner funnel. The filtered ethyl acetate extract was transferred to a separatory funnel and extracted three times with 50-mL portions of 0.1 N HCl. The organic phase was discarded, and the aqueous phase was washed three times, or until no emulsion formed, with 50-mL portions of ethyl acetate. The aqueous phase was then adjusted to pH 8.5–9.0 with concentrated NaOH solution and extracted three times with 100 mL of ethyl acetate. The combined ethyl acetate extracts were dried with anhydrous Na_2SO_4 and taken to dryness on a rotary evaporator at 40 °C. The residue was dissolved in 25 mL of methanol and passed through a 0.45-μm Teflon filter.

HPLC Analysis. The method of Bogan and Marriner (1980) was adapted as follows. The chromatograph consisted of a SP8700 solvent delivery system and pump module and a Spectro Monitor III UV detector (Spectra-Physics, San Jose, CA). Chromatography was performed on an Ultrasphere 5-μm C_{18} column, 250 × 10 mm (Altex, Berkeley, CA), using methanol–0.05 M ammonium carbonate (60:40) and a flow rate of 1.5 mL/min, completely resolving the TBZ peak in extracts of apple and potato.



Thiabendazole



5-Succinamidothiabendazole (I)

Figure 1. Structures of thiabendazole and hapten used for preparing conjugates.

Some analyses were performed using a smaller (250 × 4.6 mm) column (Vydac, Separations Group, Hesperia, CA). This method afforded nearly complete resolution of the TBZ peak (R_s = 1.9). Peaks were detected at 290 nm and quantitated using a SP4200 computing integrator (Spectra-Physics).

Preparation of Samples for Immunoassay. For analysis of the whole fruit or tuber, cored apples or potatoes were coarsely cut or chopped, distilled water (4 mL/g) was added, and the mixture was homogenized in a household blender (Model B, Waring Co., New York, or Dual Range 10, Oster) at high speed for 10 min. In initial experiments buffer (93 mM Tris-HCl, pH 8.1) was used as extractant, and samples were homogenized for 10, 20, or 30 min. Homogenates were clarified by passing through four layers of cheesecloth and frozen as aliquots. Peels were prepared using a conventional household peeler and represented approximately 10% of the weight of the whole potato or apple. They were extracted as above. In later experiments extracts were prepared in distilled water using high-speed blending for 1 min, followed by 9 min at low speed, to reduce heating. In addition, aliquots were centrifuged at low speed (4000g for 10 min) to remove gelatinous and particulate material. These changes had no detectable effect on the ELISA results.

Antibodies. The ELISA method employed monoclonal antibodies and the bovine serum albumin (BSA) conjugate of the hapten 5-succinamido-2-(4-thiazolyl)benzimidazole (I, Figure 1), described in detail previously (Brandon et al., 1992). The two antibodies differ in their sensitivity to substitution at the 5-position. Antibody 448 binds TBZ and 5-OH-TBZ equivalently. Antibody 430 has 50-fold reduced affinity for the 5-OH metabolite, compared to the parent molecule.

Immunoassay. Assays were conducted in the inhibition format as follows. The inner 60 wells of 96-well polystyrene immunoassay plates (Immulon II, Dynatech, Chantilly, VA) were coated with the BSA conjugate of the hapten. This conjugate contained 6 mol of hapten/mol of BSA and was coated at 10 μg/mL phosphate-buffered saline (PBS, 0.15 M NaCl, 5 mM sodium phosphate, pH 7.0), 100 μL/well, for 4 h at 20 °C or for 16 h at 4 °C. Plates were washed with PBS containing 0.05% Tween 20 (PBS–Tween) and rinsed with distilled water. Remaining protein-binding sites were blocked by incubation with PBS–Tween + 10 mg/mL BSA (200 μL/well, 1 h at room temperature), and the plates were washed and rinsed again. If the plates were not used immediately, the wells were filled with PBS + 0.01% sodium azide and the plates were stored refrigerated for up to 4 weeks.

Extracts to be analyzed were diluted in PBS–Tween so that (for positive samples) the final TBZ concentration would be within the working range of the assay. Extracts from treated samples were typically diluted 1:20 to 1:500. Extracts or dilutions were then mixed with equal volumes of purified monoclonal immunoglobulin G (3 ng/mL) and preincubated for 1 h at room temperature. The preincubated mixture was applied to assay wells (100 μL/well) and incubated for 1 h with shaking. The assay plate was washed and rinsed, and bound immunoglobulin was detected with horseradish peroxidase (HRP) labeled rabbit anti-mouse IgG (Zymed Laboratories, South San Francisco, CA). Substrate consisted of 1 mM 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) and 6.7 mM H_2O_2 in 60 mM sodium citrate buffer, pH 4.2. After 20 min, the absorbance was determined at 414 nm and the absorbance at 570 nm was subtracted for each well with a microplate reader (Vmax, Molecular Devices, Menlo Park, CA). The data obtained with standards were fit to a logistic model (Finney, 1978) using the Softmax program (Molecular Devices). In some experiments Maxisorp polystyrene ELISA plates (Nunc, Waterloo, IA) or poly(vinyl chloride) plates (Costar, Cambridge, MA) were used. Since the latter plates have smaller wells, all volumes were reduced by half.

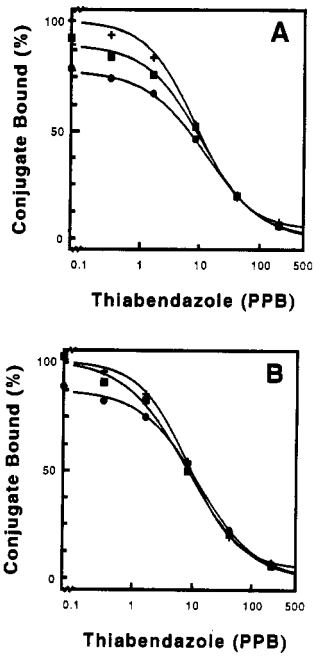


Figure 2. Standard curve for thiabendazole ELISA. Assays were performed on standard solutions of thiabendazole using assay buffer (+) or buffer containing 5% (■) or 10% (●) peel extract as diluent. (A) Apple peel extract; (B) potato peel extract.

Table I. Recovery of TBZ from Apple Peel by HPLC and ELISA

	% recovery[a]	
level of fortification, ppm	HPLC	ELISA
3	105 ± 2 (n = 3)	80 ± 10 (n = 6)
10	94 ± 10 (n = 3)	86 ± 17 (n = 6)
100	97 ± 2 (n = 3)	77 ± 12 (n = 4)

[a] Mean ± standard deviation.

RESULTS AND DISCUSSION

ELISA. Typical standard curves for the ELISA using antibody (Ab) 448 are shown in Figure 2. The TBZ concentration is that of the diluted standard, ready for assay, prior to mixing with antibody. The working range of the assay is 1–10 ppb, thus permitting the analysis of typical samples by dilution of the TBZ-containing extract. The effects of extracts of apple peel and potato peel are illustrated by the standard curves obtained in the presence of 5% or 10% extract. Unidentified components of these matrices appeared to interfere with maximal antibody binding to the BSA–thiabendazole conjugate coating the assay plates. This effect became insignificant at thiabendazole concentrations over 10 ppb or extract concentrations less than 2%. Table I illustrates the recovery of TBZ from spiked apple peel samples at three levels of fortification. The ELISA procedure produced a recovery of about 80% at each level. To determine the limit of sensitivity of the ELISA in the apple peel matrix, peels from Red Delicious apples having no detectable TBZ residue were chopped and blended and various concentrations of TBZ in dimethylformamide were added. The

Table II. TBZ in Treated Apples

method	TBZ,[a] ppm
ELISA	
expt 1[b]	
Ab 430	1.53 ± 0.18 (n = 3)
Ab 448	1.50 ± 0.16 (n = 3)
expt 2[c]	
Ab 448	1.21 ± 0.11 (n = 5)
HPLC	3.02 ± 0.01 (n = 2)

[a] Mean ± standard deviation (n > 2); average deviation from the mean (n = 2). [b] Tris-HCl buffer used to extract apple peels for 10–30 min. [c] Water extraction for 10 min.

Table III. Thiabendazole Residue in Treated Potatoes[a]

	thiabendazole, ppb			
tuber	peel	whole	pulp	% of residue in peel
1	10900	960	110	91
2	8520	800	65	93
3	8130	895	70	93

[a] Control samples contained less than 80 ppb of thiabendazole in the whole tuber and in the peel.

samples were then prepared for ELISA as described above. The results were analyzed by linear regression, and the 95% one-sided lower confidence limit was determined to be 160 ppb. Thus, analysis of peels containing as little as 160 ppb could be analyzed at 100-fold dilution and give a positive ELISA.

Analysis of Apple Samples. The analysis of apples treated with TBZ is summarized in Table II. The data obtained using antibody 448 were compared to those obtained using a second antibody (430), which has greater sensitivity to substitution in the 5-position (Brandon et al., 1992). In addition, the ELISA results were compared to the analysis by HPLC. The ELISA results obtained using both antibodies were not significantly different (p > 0.1, Student's t test). This result implies that metabolites such as 5-hydroxy-TBZ were not present in the extract, a result confirmed by HPLC. However, recovery of TBZ was slightly lower when water was substituted for buffer (p < 0.05). Compared to HPLC, the ELISA underestimated the TBZ content of the sample by 50–60%. Extracts prepared for HPLC were also analyzed by ELISA. The ELISA value for TBZ was 108% ± 27% of the HPLC value (n = 4). Recovery of the TBZ spike by the HPLC method was 107% ± 1% (n = 2). Thus, it appears that aqueous extraction was responsible for the lower recovery in the ELISA. Despite the lower recovery of TBZ using aqueous extraction, water was used as extractant in all subsequent studies because of its desirability for potential field use.

Analysis of Potato Samples. Since thiabendazole is generally applied to apples and potatoes as a brief postharvest dip or spray, it seemed likely that residues would be concentrated in the peel. We investigated the distribution of residue in potatoes using the ELISA (Table III). Over 90% of the TBZ residue was found in the peel, about 10-fold concentrated compared to the whole potato. The concentration in the pulp was about 1% of the concentration in peel. These results appear similar to those of Ben-Arie (1975), Cano et al. (1987), and Friar and Reynolds (1991), who found that in pears and apples TBZ residues are concentrated in the peel. Because of the relatively high concentration of residue in the peel, it seemed most appropriate to use peel in screening market produce.

The limit of sensitivity of the ELISA in the potato matrix was determined as described above for the apple peel matrix. The 95% one-sided lower confidence limit was determined to be 360 ppb for whole russet potatoes and 120 ppb in the red potato peel matrix. Therefore, when whole potato is analyzed, the limit of detection is <0.4 ppm, well below the U.S. tolerance (CFR 180.242) of 10 ppm. Since about 90% of the TBZ residue was found in the peel (Table III), ELISA analysis of peel could detect potatoes containing TBZ as low as 15 ppb in the whole tuber. For regulatory screening purposes, peel extracts could be analyzed at high dilution (for example, 500-fold) and presumptive positive samples flagged for further analysis. Tubers containing 2.5 ppm, corresponding to about 25 ppm in the peel, would produce extracts (5 mL/g of peel) having about 5 ppm of TBZ. This would produce a strongly positive ELISA (5 ppm ÷ 500 = 10 ppb).

Table IV. TBZ in Market Russet Potatoes

method	TBZ in peel, ppm
ELISA	9.6 ± 0.4 (n = 2)[a]
HPLC	9.3 ± 0.1 (n = 3)[b]

[a] Mean ± average deviation from the mean. [b] Mean ± standard deviation from the mean. Replicates were independent assays of a single extract.

Potato samples from retail markets were tested by analysis of the peels as described above. Of eight samples analyzed, all were negative (<0.12 ppm in the peel), except for one sample of russet potatoes. The ELISA was repeated on a second sample of russet potatoes purchased 5 days subsequently from the same store and sold under the same brand name. This sample was also analyzed by HPLC, and the data are summarized in Table IV. On the basis of the observed 10-fold higher concentration of TBZ residues in peel compared to whole potato, the expected TBZ content of the positive samples was less than 1 ppm, well below U.S. tolerance.

Potato extracts had a high level of peroxidase activity, 6 μmol min^{-1} mg^{-1} of whole potato and 30 μmol min^{-1} mg^{-1} of potato peel. To determine the effect of this endogenous peroxidase activity on the ELISA, extracts were compared before and after complete inactivation of the activity by heating at 100 °C for 2 min. The ELISA analysis was 2.99 ± 0.06 ppm of TBZ (n = 3) before inactivation and 2.95 ± 0.28 ppm (n = 3) after heat treatment, not significantly different (p > 0.1). These results imply that the peroxidase activity did not interfere with the ELISA. Presumably, the potato peroxidase does not bind to the conjugate-coated assay wells and is removed by the plate-washing procedure.

Conclusions. This simple ELISA offers a rapid test for an important postharvest fungicide in apples, potatoes, and potentially other commodities. The availability of the method may prove timely, if recent losses of postharvest registrations of benomyl lead to increased use of TBZ. For screening purposes, the assay can be interpreted by visual inspection of the ELISA plate. With visual reading of plates, the method does not depend on the use of organic solvents or sophisticated laboratory equipment and can be complete within half a day by personnel with minimal training. Matrix effects did not interfere with the assay in highly diluted aqueous extracts of apple or potato, and the between-assay coefficient of variation was approximately 10%. Further validation of this assay for specific regulatory uses should take into account the effects of varietal differences and storage conditions on migration of TBZ from peel to pulp. An additional use of this assay for quality control purposes can be envisioned. Since it has been reported that the amount and depth of TBZ penetration in pears is highly dependent on the dip concentration (Ben-Arie, 1975), it appears likely that this ELISA could be used to monitor treatment procedures

and help ensure that the TBZ level attained is adequate to control disease, while residue concentrations are minimized.

ACKNOWLEDGMENT

We are grateful to Anne H. Bates for antibody and conjugate preparation.

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Received for review March 8, 1993. Accepted March 25, 1993.