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Complementation of direct-injection high-performance liquid chromatography and enzyme-linked immunosorbent assay for the analysis of thiabendazole in fruit juices and concentrates

Rodney J. Bushway

Department of Food Science, 5736 Holmes Hall, University of Maine, Orono, ME 04469-5736, USA

Abstract

The fungicide thiabendazole was quantified in fruit juices and their concentrates (bulk and store bought) without clean-up by simply injecting 50 μl of dissolved sample into an HPLC. This novel method used a mobile phase consisting of acetonitrile–methanol–water–ethanolamine (37:11:52:0.02) and was passed through a C_{18} column at 1 ml/min. Detection was accomplished by fluorescence at 305 nm excitation and 345 nm emission. Using peak height thiabendazole was linear from 0.032 to 2.05 ng injected. The limit of quantitation was 5 ppb for juices and store concentrates (2.0 ppb for the limit of detection), 10 ppb for bulk apple concentrates and 25 ppb for other bulk concentrates (5 and 10 ppb, respectively for the limit of detection). Intra- and interassay percent relative standard deviations for standards and samples were mostly below 7% with none above 9%. Eighty-four juices and concentrates out of 200 analyzed were found to contain thiabendazole (2–2560 ppb) by HPLC and all were shown to be thiabendazole positive by enzyme-linked immunosorbent assay (ELISA). The HPLC vs. ELISA correlation coefficient was 0.984.

Keywords: Fruit juice; Environmental analysis; Food analysis; Thiabendazole; Pesticides

1. Introduction

Thiabendazole (TBZ) is a benzimidazole fungicide that is widely used as a post-harvest pesticide on fruits to prevent rot from numerous fungi from the following geneses: *Fusarium, Collectotrichum, Verticullium, Thielaviopsis, Botryodiplodia, Deightoniella* and *Nigrospora* [1]. US Environmental Protection Agency (EPA) tolerances for TBZ on fruit range from 10 to 25 ppm. Because of TBZ's extensive use on fruit and because children consume a lot of juice, there has been much interest in being able to quantify TBZ levels in juices and concentrates [2].

Methods for analyzing TBZ residues in foods have focused on spectrophotometric, immunoassay, and chromatographic techniques. Spectrophotometric procedures have used either UV or fluorescence [3–6] but require clean-up and do not have the specificity of chromatographic techniques.

The most recent procedure to be developed for TBZ residues in foods has been immunoassay [7–9]. This technique offers quickness and is inexpensive. One major disadvantage can be matrix effects, from certain crops or products, that reduce the specificity and sensitivity of immunoassay [9]. However, if no matrix effects are observed, immunoassay can be an excellent pesticide quantitation method.

Chromatography (thin-layer, gas, or liquid) is still the most widely used method for analyzing TBZ residues in foods [1,3,6,11,12]. Of these, gas or liquid are the best since they can offer specificity and sensitivity when coupled to certain detectors like nitrogen or fluorescence. Even with these detectors

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however, in order to obtain the best results one needs to employ one or more clean-up steps such as liquid–liquid partition or solid-phase extraction.

This paper describes two novel and very simple and efficient methods developed for quantifying TBZ in juices and concentrates which complement each other. The first is an immunoassay method that can be used for the initial analysis followed by the direct injection HPLC procedure for final quantitation. By employing both techniques a laboratory can do numerous samples a day and each method can act to confirm the other.

2. Experimental

2.1. Materials

Juices and store bought concentrates were purchased from local stores in the Bangor, ME, USA area and included all major brand names. Bulk juice concentrates were obtained from Coca-Cola Foods (Auburndale, FL, USA) and Ocean Spray (Lakeville-Middleboro, MA, USA).

All solvents were HPLC grade (VWR, Boston, MA, USA), including the solvents for dissolving the standard, juices and concentrates. TBZ standard with a purity of 99% was obtained from Cresent Chemical (Newark, NJ, USA).

2.2. Preparation of standards and samples for HPLC and enzyme-linked immunosorbent assay (ELISA)

Standards for HPLC analyses were prepared by weighing accurately approximately 26 mg of TBZ into a 50 ml volumetric flask which was brought to volume with methanol. 10 μl were removed and placed into a 10 ml volumetric flask before bringing to volume with methanol. This intermediate standard was used to prepare calibration standards of 0.75, 1.25, 2.5, 5.0, 10.0 and 20.0 ppb in methanol–water (75:25).

Immunoassay standards were also prepared using the initial stock solution of 26 mg TBZ/50 ml. To a 50 ml volumetric flask was added 386 μl of TBZ stock followed by a sufficient amount of water to bring to volume. From this intermediate standard, calibration solutions of 0.25, 0.50, 1.0, 2.0, and 4.0 ppb were made using a proprietary diluent (Millipore, Bedford, MA, USA).

Samples of juices and concentrates (1.25 g) for HPLC analysis were dissolved into 3.75 ml of methanol–water (75:25). Any juice or concentrate that had pulp in it was centrifuged for 5 min at 10 000 *g*.

Immunoassay sample preparation consisted of placing 1 g of the juice, store juice concentrate or bulk juice concentrate into a 20 ml glass scintillation vial followed by 9 ml of the proprietary diluent. If further dilutions were needed because of matrix effects or high concentrations of TBZ, the proprietary diluent was always used.

2.3. HPLC

Juice and concentrates were analyzed for TBZ employing the following HPLC system: a Waters 510 pump (Waters Associates, Milford, MA, USA), Valco pneumatic injector (Vici Instruments, Houston, TX, USA), Waters 470 fluorescence detector, and a Hewlett-Packard 3396A integrator (Avondale, PA, USA).

Operating conditions were as follows: injection volume, 50 μl; flow-rate, 1.0 ml/min; column, Ultracarb 30 ODS 5 μm stainless-steel 15 cm×4.6 mm I.D. (Phenomenex, Torrance, CA, USA); fluorescence, excitation at 305 nm and emission at 345 nm; attenuation, 8; gain, 100; filter, 1.5 s.

2.4. Immunoassay

EnviroGard (Millipore) plate monoclonal immunoassay kits were used. The procedure consisted of adding 100 μl of sample followed by 100 μl of enzyme conjugate to each microtiter well. The plate was allowed to incubate at room temperature for 1 h before it was rinsed under tap water 4 times. After blotting the plate dry, 100 μl of K-blue were added to each well incubated for 30 min. After the incubation, 100 μl of 1 *M* HCl was added to each well to stop the reaction. The wells were read at 450 nm using a plate reader to obtain the absorbance (*A*). From *A*, $\%B_0$ was calculated and used along with a standard curve to quantitate the level of TBZ by immunoassay.

2.5. Quantitation

For HPLC quantitation of TBZ in juice and concentrates peak height was used. TBZ calibrators were injected at the beginning and end of each day with the average used to calculate TBZ in samples.

For immunoaassay quantitation, $\%B_0$ ($\%B_0$ is defined as A of sample or standard/A of control) was employed to make a standard curve. Again calibrators were run at the beginning and end of the day and the average used for determining TBZ in samples.

2.6. Fortifications

Samples of juices and concentrates shown to contain no detectable amounts of TBZ were used for fortification studies to determine the limit of quantitation and limit of detection [13].

2.7. Reproducibility

Actual samples containing TBZ were used to determine intra- and interassay precision of the HPLC method.

3. Results and discussion

A typical chromatogram of a TBZ standard (a) and a sample (b) are shown in Fig. 1. Retention time was short, taking only 3.3 min. Furthermore, preparation time was just as quick since samples were dissolved in a mixture of methanol–water and injected directly. Even though the TBZ retention time was short interfering peaks were not a problem for juices and store concentrates. However with bulk concentrates especially with citrus, interferences can sometimes be a problem. To decrease the effects of interferences, samples were diluted further, thus lowering the sensitivity. For juice and store concentrates the limit of quantitation was ascertained to be 5 ppb with a detection limit of 2 ppb. However, with bulk concentrates the limit of quantitation was determined to be 25 ppb except apple concentrate which was 10 ppb with a detection limit of 5 ppb for apple and 10 ppb for other concentrates.

As mentioned above for the most part interferences were not a problem. This was confirmed by injecting organic juices and concentrates. Futhermore, all samples analyzed by HPLC were also quantified by immunoassay. The correlation coefficient based on 84 positive samples analyzed by both techniques was 0.984 and the regression equation was $y=1.0x+11.8$. The slope is 1 indicating no bias by the ELISA. Thus showing excellent agreement. Also, out of the 200 samples analyzed by ELISA there were no false positives or negatives, which was not surprising since this monoclonal antibody is very

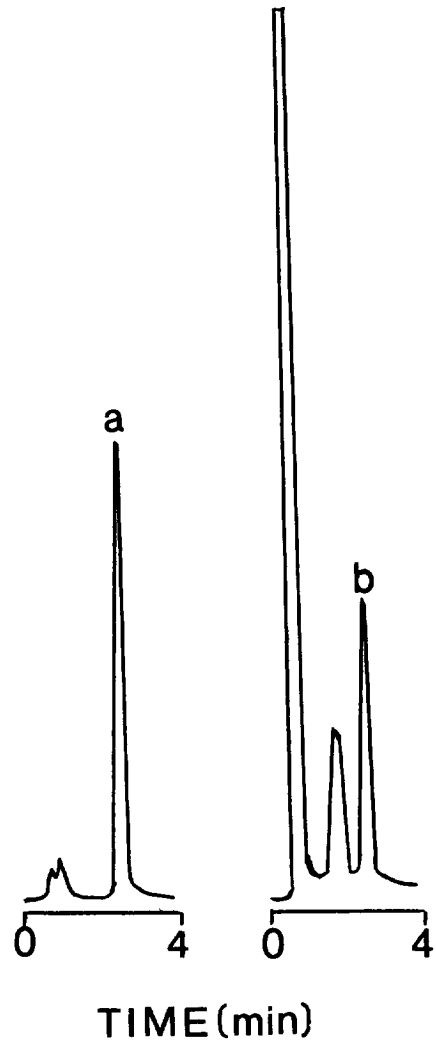

Fig. 1. HPLC chromatogram of TBZ standard (a) and grapefruit concentrate (b).

specific. It only cross-reacts with 5-OH-TBZ (an animal metabolite).

For quantifying TBZ, peak height was used. It was observed that TBZ was linear from 0.032 to 2.05 ng injected and the regression line yielded a correlation coefficient of 0.9994. Because of the linearity it would have been possible to use one standard, but we chose to use all calibrators twice a day. We felt, since we were dealing with small peak heights, that using all calibrators was a better approach.

In developing any analytical method one must determine the reproducibility of both standards and samples. Standard precision is shown in Table 1. Both intra- and inter-assay relative standard deviation (R.S.D.) percentages were excellent. They ranged from 2.5 to 7% and 3.4 to 6.3%, respectively, indicating excellent reproducibility especially if one considers that peak height was used for quantitation, with the lowest standard concentration yielding a peak height of only 0.29 cm. Also, the interassay results were based on an entire months worth of data, demonstrating how precise the standard curve is from day to day.

Actual juices and concentrates contaminated with TBZ were used to determine sample reproducibility from day to day and between days. The results are shown in Table 2 and Table 3. Intra-assay R.S.D. values for juices ranged from 0 to 3.3% and the inter-assay R.S.D. values varied from 3.7 to 5.2% (Table 2). Six concentrates were employed in this study with intra-assay R.S.D. values ranging from 3.1 to 8.0% and inter-assay values of 2.1 to 8.9%. Only two R.S.D. values were greater than 7% with most less than 6%, indicating that the direct HPLC method for analyzing TBZ is very consistent (Table 3).

Table 1
Reproducibility of the TBZ HPLC method for standards

TBZ (ppb)	R.S.D. (%)	
	Intra-assay[a]	Inter-assay[b]
2.6	7.0	3.6
5.2	5.4	4.0
10.3	2.5	3.4
20.6	3.8	4.3
41.3	3.9	5.4
82.6	2.6	3.2

[a] Based on 6 determinations in 1 day except standard 41.3 ppb which was 5 determinations.
[b] Based on 6 determinations in 6 different days.

Table 2
Reproducibility of the TBZ HPLC method for juices

Sample	TBZ (ppb)	R.S.D. (%)	
		Intra-assay[a]	Inter-assay[b]
1	398	3.3	4.5
2	272	1.8	4.1
3	80	0.0	4.6
4	5.2	0.0	3.7
5	7.1	2.2	5.2
6	17.0	3.3	4.9

[a] Based on 6 determinations in 1 day except samples 1 and 2 which were 5.
[b] Based on 6 determinations in 6 different days except samples 4 and 6 which were 5.

Fortification studies were employed not only to determine the limits of quantitation and detection but also the accuracy of the technique. In this case spiking should not be a problem since TBZ is not extracted from a sample but just injected directly. Indeed this was the case. TBZ free (at least below the detection limit of the method) juice and concentrates were fortified at 5, 10 and 25 ppb. Recoveries were excellent averaging 108% with an R.S.D. of 8.1%. Thus, the accuracy was good.

As mentioned earlier, 200 juice and concentrate samples were analyzed by both the HPLC and ELISA methods. Of these 84 or 42% were TBZ positive ranging from 2 to 2560 ppb by HPLC or 2.5 to 2400 ppb by ELISA. These samples were divided into three categories; juices, store bought concentrates and bulk concentrates. The correlation co-

Table 3
Reproducibility of the TBZ HPLC method for concentrates

Sample	TBZ (ppb)	R.S.D. (%)	
		Intra-assay[a]	Interassay[b]
1	12	3.7	2.1
2	36	5.4	8.9
3	256	8.0	4.5
4	158	3.1	6.5
5	711	3.9	5.1
6	51	4.9	6.6

[a] Based on 6 determinations in 1 day except for sample 6 which was 5.
[b] Based on 6 determinations in 6 different days except for sample 4 which was 5.

efficients for all three types of samples ranged from 0.977 to 0.995 with an overall coefficient of 0.984, which shows that all three kinds of samples yielded almost identical results by either ELISA and HPLC. These results demonstrate that for TBZ analysis of juice and concentrates ELISA and HPLC can complement each other. ELISA can be employed as the initial test and backed up by HPLC. Such a combination can be beneficial in the quantitation of pesticides in food because of the number of samples that can be analyzed in a cost-effective way.

Furthermore, this is the first time that a HPLC method has been developed to analyze TBZ in juices and concentrates directly, without employing one or more clean-up steps. This unique method will enable industrial and governmnent laboratories to be able to analyze samples quickly and more cost-effectively, thus insuring a safer food supply. Also, less organic solvents will be used which are very expensive to purchase and to remove.

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