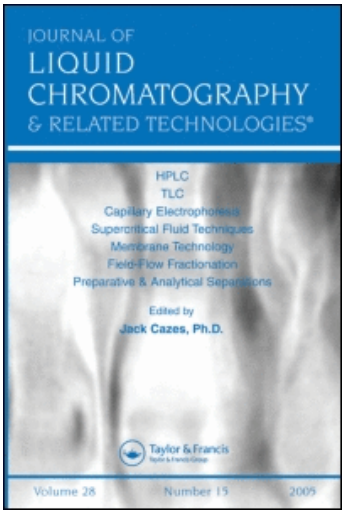

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A MODIFIED HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THIABENDAZOLE IN FRUITS AND VEGETABLES WITH ELISA CONFIRMATION

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ABSTRACT

A rapid modified high performance liquid chromatographic (HPLC) method was developed for the analysis of the benzimidazole fungicide thiabendazole (TBZ) in fresh produce. Changes were in the extraction technique and HPLC mobile phase. TBZ was extracted from the produce by either polytroning 10 g in 20 mL of methanol for 3 minutes or shaking, by hand, a 5 or 10 g sample in 20 mL of methanol for 10 minutes. A 50 μL aliquot was injected directly into an isocratic fluorescent HPLC system. Total analysis time ranged from 8 to 15 minutes per sample depending upon the extraction procedure employed. Percent recoveries averaged 90% with excellent reproducibility (%CVs from 0 to 18). For enzyme linked immunosorbent assay (ELISA) confirmation a 0.1 mL aliquot of the sample was removed and evaporated to dryness under air. The residue was dissolved in water before performing the ELISA.

INTRODUCTION

Thiabendazole (TBZ) is a pre-and postemergence benzimidazole fungicide that is used on numerous fruits and vegetables to protect against *Fusarium roseum, Collectotrichum musae, Verticullum, theobromae, Thielaviopsis paradoxa, Botryodiplodia theobromae, Deightoniella and Nigrospora spp.*[1,2]

In recent years, the main interest in quantifying TBZ residues has come about because of three reasons. First, certain countries have put restrictions on TBZ levels of imported foods. Second, the effect of TBZ on infants and children are unknown. Finally, produce in storage must be checked periodically for their effective levels.[3,4,5]

The two leading techniques for analyzing TBZ residues in food are HPLC[5,6,7,.8.9] and enzyme linked immunosorbent assay (ELISA)[10] since they are rapid, accurate, and cost effective. In this paper a modified HPLC method is described that is even quicker along with the use of ELISA as a confirmation technique.

MATERIALS AND METHODS

Materials and Reagents

All food samples were purchased from local markets in the Bangor, Maine area.

Thiabendazole (99% pure) was obtained from Crescent Chemical (New York, NY). All solvents were HPLC grade bought from EM Science (Gibbstown, NJ). A stock solution of TBZ was prepared at a concentration of 0.84 mg/mL in acetonitrile. From the stock standard working standards of 2.6, 5.2, 10.3, 20.6, 41.3, and 82.6 ppb were prepared in 80/20 methanol/water.

Liquid Chromatography System

The HPLC consisted of a Waters 510 pump (Waters Associates, Milford, MA), a Valco pneumatic injector (VICI Instruments, Houston, TX) containing a 50 μL loop, a Waters 470 fluorescence detector, and a Hewlett- Packard 3396 integrator (Avondale, PA).

ELISA System

Monoclonal antibody 448 was bound to polystyrene tubes (SDI, Newark, DE) and the conjugate was horseradish peroxidase (HRP) conjugate of 5-succinamido-TBZ. The substrate was a one-component formulation of tetramethylbenzidine (ELISA Technologies, Lexington, KY) and 1 N HCI was employed as stop solution.

Extraction Methods

The polytron procedure consisted of weighing a 10 g sample of food into a 50 mL polypropylene centrifuge tube followed by the addition of 20 mL of HPLC methanol. This mixture was polytroned for 3 minutes at high speed and then centrifuged at 5,000 x g for 10 minutes.

A shaking method employed weighing 5 or 10 g of food into a 30 mL polypropylene bottle. Next 5 ball bearings and 20 mL of HPLC methanol were added before shaking for 10 minutes. A 1 mL aliquot was centrifuged at 5,000x g for 10 minutes.

The partition extraction technique used was previously described by Bushway et al.[5] Basically, TBZ was partitioned into methylene chloride by using a high pH buffer.

HPLC Analysis of TBZ

A 50 μL sample from any extraction procedure was injected into the HPLC system under the following operating conditions: column, Ultracarb 30 ODS; mobile phase, acetonitrile: water: methanol: monoethanolamine (200 + 275 + 75 + 0.1 or 260 + 500 + 70 + 0.1); flowrate, 1 mL/min; detector, fluorescence; wavelength, 305 nm excitation and 345 nm emission.

ELISA Analysis of TBZ

A 100 μL aliquot from the samples were evaporated to dryness and the residue was dissolved in 1 mL of water. Two hundred μL of standards or samples were added to 10 polystyrene tubes followed by 200 μL of enzyme conjugate. The tubes were incubated for 15 minutes before rinsing 4 times under tap water and blotted dry.

Table 1

Recovery of TBZ from Selected Produce by Hand Shaking

Sample	TBZ Added-ppb	TBZ Found-ppb	% Recovery
Apple	36	32	90
	72	70	95
	1000	810	81
	5000	4833	97
	10000	9200	92
Grapefruit	36	36	100
	72	63	87
	1000	850	85
	5000	4850	97
	10000	9200	92
Potato	36	28	92
	72	67	93
	1000	780	78
	5000	4500	90
	10000	9500	95

Next 500 μL of substrate was added to each tube before incubating for 10 minutes. Finally, 300 μL of 1 N HCl was added to each tube before reading the optical density at 450 nm.

Recovery Studies

A small sampling of fruits and vegetables were fortified for testing the recovery of TBZ. Also, a comparison study of extraction techniques was performed using actual samples known to contain TBZ.

Reproducibility Study and Confirmation by ELISA

Several fruits and vegetables along with the standards were analyzed several times on the same day and different days to determine the intrassay and interassay variation of the HPLC method. All samples found positive for TBZ were analyzed by ELISA.

Table 2

Comparison of Extraction Methods for TBZ in Produce

	----------ppb TBZ----------		
Sample	**Partition**	**Hand Shake**	**Polytron**
Lime Peel	895	827	856
Lime Flesh	55	45	51
Grapefruit Peel	5990	5760	6111
Orange Flesh	98	68	68
Orange Peel	3590	3480	3480
Tangerine Flesh	55	33	41
Potato	1290	1197	1140
Apple Flesh	19	20	----
Pear	----	629	699
Apple	55	62	----

RESULTS AND DISCUSSION

This HPLC method for TBZ in foods is a modification of a previous method that was developed in our lab.[5] The changes are two-fold. First, the extraction procedure has been simplified. Instead of using a partitioning step which is very time consuming and uses a very unpleasant solvent, methylene chloride, methanol was employed as the extraction solvent with either polytroning the food for 3 minutes or hand shaking with ball bearings for 10 minutes.

Since the extraction procedure was changed, a recovery study had to be preformed. Table 1 shows the results of the hand shaking fortification study of apples, grapefruit, and potatoes. Raw produce spiked from 36 to 10,000 ppb yielded percent recoveries ranging from 78 to 100 with an overall average of 91.

If produce samples were allowed to set after being shaken for 4 hours and then reshaken for 5 minutes, a slight increase in TBZ levels was observed. The limit of quantitation was determined to be 6 ppb TBZ for produce.

A preliminary study on the recovery of TBZ by polytron extraction also showed results that were not significantly different from hand shaking. However, fortification studies can be misleading because the compound of

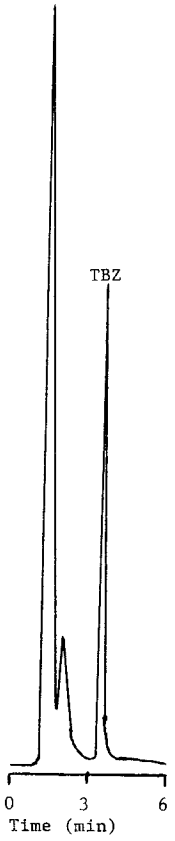

Figure 1. HPLC chromatogram of a potato sample containing TBZ.

interest is not incorporated into the matrix. Therefore, a comparison of the two new extraction methods with a well established partition procedure was performed using real samples known to contain TBZ. These results are given in Table 2.

The polytron and hand shake extraction techniques are very comparable to the methylene chloride partition except for citrus flesh which yields only about 70% as compared to the partition technique which means one would have to use a correction factor in the calculation.

Table 3

Reproducibility of HPLC Method for TBZ in Produce

Sample	TBZ-ppb	Intraassay-%CV	Interassay-%CV
Standard 1	2.6	7.0	0.0
Standard 2	5.2	5.4	6.1
Standard 3	10.3	2.5	6.3
Standard 4	20.6	3.8	3.5
Standard 5	41.6	3.9	2.3
Standard 6	82.6	2.6	1.3
Potato 1	12.5	8.9	18
Potato 2	219	4.8	8.7
Potato 3	2740	4.3	11
Apple Peel	1690	10	----
Apple Flesh	74	6.8	----
Apple	750	2.2	----
Orange	169	3.1	----
Grapefruit	1473	5.8	----

All %CVs for standards based on 6 determinations except the intraassay for 41.3 ppb which was 5. All produce %CVs based on 4 determinations.

These results point out the possible error in using fortified samples versus actual samples when determining percent recovery. Grapefruit fortified TBZ yielded over 90% recovery (Table 1) while actual samples containing TBZ demonstrated 70% recovery. (Table 2). For other kinds of produce the two extraction techniques were very comparable to the results obtained from the partition method. The partition technique was used as a bench mark for the other two extraction procedures since it was shown in a previous study to be excellent with almost 100% recoveries for all produce[5]

The second modification was the mobile phase which was changed so the TBZ would elute quicker (2.2 minutes faster). The total elution time was 3.3 minutes (Figure 1). In all produce except the citrus flesh this can be done without problems from interferences. In the case of citrus flesh, the mobile phase, containing the most water, had to be used.[5]

As with any analytical method, the reproducibility is important. Table 3 demonstrates these results both with standards and samples. The %CVs for the interaassay are all 10 or lower while for the interaassay only 2 of the %CVs

Table 4

Comparison of HPLC & ELISA Methods for TBZ in Produce

Sample	HPLC TBZ-ppb	ELISA TBZ-ppb
Fiji Apple Peel	417	368
Gala Apple Peel	350	352
Golden Delicious Peel	620	592
Red Delicious Peel	231	236
Braeburn Apple Peel	2900	2444
Potato 1	440	483
Potato 2	780	980
Fiji Apple Flesh	20	29
Fiji Apple	62	51
Gala Apple	59	47
Red Delicious Apple	31	29
Braeburn Apple	430	426
Golden Delicious	75	63
Fiji Apple Peel 2	584	584
Potato 2	1140	1100
Potato 3	1080	1080
Potato 4	917	1010
Potato 5	1130	1100
Potato 6	1150	1200
Potato 7	300	311
Potato 8	1150	1440
Potato 9	1150	1440
Potato 10	157	144
Potato 11	603	718
Orange Peel	3430	3430
Orange Flesh	67	65
Clementine Peel	1660	2030
Clementine Flesh	23	20
Grapefruit Peel	5990	6120
Grapefruit Flesh	149	165
Tangerine Peel	41	49
Lime Peel	852	900
Lime Flesh	45	63
Potato 12	326	374
Potato 13	7	14
Potato 14	149	244

Table 4 (continued)

Sample	HPLC TBZ-ppb	ELISA TBZ-ppb
Potato 15	23	25
Potato 16	811	930
Pear	699	624
Pear Peel	3370	4520
Apple 1	126	226
Pear Flesh	142	146
Apple 2	194	207
Pear 1	1670	1210
Apple 3	730	930
Apple 4	790	760
Apple 5	1680	1490
Apple 6	1250	1350
Apple 7	1060	1180
Pear 2	850	930
Pear 3	860	750
Pear 4	860	1040
Pear 5	580	1730

Equation: $y = 1.4X + 6.21$ n=53.

were greater than 10. It is always good to employ a confirmation technique when analyzing pesticides residues in food. In this case, it was decided to look at an ELISA technique since it was very specific. In fact, the only major cross-reactant was 5-OH TBZ which is not common in plants. The results from this study are given in Table 4.

As can be seen, the correlation coefficient was 0.9837 for 53 samples varying in TBZ concentrations. Thus, ELISA can be employed as a confirmation technique for TBZ in produce.

CONCLUSION

These modifications in the HPLC method for analyzing TBZ in food will make for a quicker and even safer procedure using methanol in the place of methylene chloride. Also, ELISA adds another dimension as a confirmation technique.

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