Extraction of Thiabendazole and Carbendazim from Foods Using Pressurized Hot (Subcritical) Water for Extraction: A Feasibility Study

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Pressurized hot water was used to extract two fungicides, thiabendazole (TBZ) and carbendazim (MBC), from agricultural commodities including banana pulp, whole lemons, orange pulp, mushrooms, and rice at extraction temperatures below 100 °C and an extraction pressure of 50 atm. The extraction parameters that were studied include temperature, equilibration time, flow rate, pH, and collection volume. Liquid−liquid partitioning using ethyl acetate was then used to partition MBC and TBZ from the aqueous extractant and concentrate the analytes for final analysis and the determination of recovery. Residues of TBZ were also determined in incurred matrixes which had already been analyzed by another laboratory using validated methodology. Reversed-phase ion-pairing HPLC with UV absorbance and fluorescence detection was used to determine the recoveries of TBZ and MBC from fortified homogenates. Using an extraction temperature of 75 °C, the average recoveries of MBC and TBZ ranged 80.9−100.5% at fortification levels ranging from 0.14 ppm in fresh mushrooms to 10 ppm in whole lemon homogenates. The relative standard deviations were 10% or less.

Keywords: *Thiabendazole; carbendazim; benomyl; subcritical; water; extraction; ion-pairing HPLC*

INTRODUCTION

Analytical methods that are used for the analysis of pesticide residues in foods and environmental samples (e.g., soil and water) require an initial sample preparation step in which the analyte(s) of interest are isolated from the bulk of the matrix. Subsequent steps in a method may be needed to purify a sample extract further and concentrate the analytes for a final determinative step. Traditionally, methods for pesticide residue analysis are labor intensive and typically rely on the use of hazardous chlorinated solvents for extraction. Many techniques have been developed which attempt to streamline the process of sample extraction and eliminate the need for large volumes of potentially hazardous wastes. These techniques include solid-phase microextraction (SPME) (Louch et al., 1992; Zhang et al., 1994), pressurized liquid extraction (Richter et al., 1996; Lehotay and Lee, 1997), microwave extraction (Pylypiw et al., 1997), and supercritical fluid extraction (SFE) (Hawthorne, 1990). SFE using supercritical CO_2 and modified supercritical CO_2 are the most widely used of these methods and there are numerous applications for pesticide residue analysis in foods (Hopper et al., 1995; King et al., 1993; Lehotay et al., 1995; Lehotay and Eller, 1995; Lehotay, 1997; Khan, 1995). Because supercritical CO_2 is an inherently nonpolar solvent, SFE has its limitations, even with modification, to solubilize and extract polar analytes. Other approaches which have been investigated for enhancing the extraction of polar analytes include ion-pair SFE (Jimenez-Carmona et al., 1995) and chemical dervatization (Hawthorne et al., 1992). Even some nonpolar analytes which are tightly bound to a matrix may be difficult to extract with supercritical CO_2 (Hawthorne et al., 1994).

An extraction scheme which has recently been reported in the literature involves the use of subcritical and supercritical water as an extractant. This extraction scheme addresses the issue of replacing hazardous solvents for extraction and is shown to be effective for the extraction of polar, moderately polar, and nonpolar analytes. Water exists in a supercritical state at temperatures above 374 °C and pressures exceeding 221 atm (Holgate and Tester, 1994). Under these conditions, the solvent characteristics of water change dramatically. For instance, the dielectric constant (ϵ) of water is 80 under ambient conditions, but under supercritical conditions, $\epsilon = 5-15$; by comparison, $\epsilon = 2$ for hexane. Under supercritical conditions, therefore, water takes on the solvent characteristics of a low-polarity solvent. For routine extraction, however, supercritical water is a difficult medium to work with because it is highly corrosive and is a powerful oxidizer. One industrial application that uses supercritical water is the rapid oxidative destruction of hazardous metabolic and organic wastes (Holgate and Tester, 1994). Fortunately, a wide range of polarities can be acheived for water under much milder or subcritical conditions (Hawthorne et al., 1994; Yang et al., 1995; Hartonen et al., 1997).

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Hawthorne and co-workers (1994) demonstrated quantitative, class-selective extraction of polar organics (e.g., chlorinated phenols), low-polarity organics (e.g., PAHs), and nonpolar organics (e.g., alkanes up to C-30) from environmental solids (e.g., soil) in a single extraction by sequentially increasing the extraction temperature from 50 to 150, 250, and 400 °C. Since pressure has a small effect on the dielectric constant of water, an extraction pressure of 345 atm was arbitrarily chosen regardless of the extraction temperature used. From 50 to 400 °C, the dielectric constant of water decreased from 71 to 45, 29, and 8, respectively.

The results of Hawthorne's work were encouraging, so we decided to explore the possibility of using hot, pressurized water for the extraction of pesticide residues from foods. This initial effort focused on the extraction of thiabendazole (TBZ) and carbendazim (MBC) from a variety of agricultural commodities. TBZ and MBC are broad-spectrum fungicides; TBZ is widely used while MBC is used on fruits, vegetables, coffee, and tea outside the United States. MBC is also the main degradation product of benomyl, another fungicide which is used worldwide.

A variety of techniques have been developed for the analysis of MBC, TBZ, benomyl, and related benzimidazole fungicides (Aharonson et al., 1994; Arenas et al., 1996; Capitan-Vallvey et al., 1994).

Benomyl readily converts to MBC under neutral and acidic conditions and is recognized as the fungitoxic principle of benomyl. Benomyl is typically determined as MBC after complete conversion. MBC and TBZ are weak bases which have limited solubility in water under ambient and neutral or basic conditions. TBZ has pK_a values of 2.5 and 4.7; MBC has a pK_a value of 4.0. Both analytes are soluble in alcohol, acetone, and chlorinated hydrocarbons. Tolerance levels for MBC range from 0.13 ppm in banana pulp (0.2 ppm benomyl) to 6.58 ppm in whole citrus fruits and mushrooms (10 ppm benomyl), and 3.29 ppm in rice (5 ppm benomyl). Tolerances for TBZ range from 0.4 ppm in banana pulp to 10 ppm in whole citrus fruits and pears and to 40 ppm in mushrooms. MBC and TBZ were selected for this study because of their limited solubililty in water under neutral conditions and because current methodology described in the Food and Drug Administration's Pesticide Analytical Manual (PAM) for MBC and TBZ uses an initial extraction with methanol followed by liquid−liquid partitioning (LLE) with dichloromethane (DCM). The method was developed and described by Gilvydis and Walters (1990). The FDA method for MBC and TBZ required additional sample cleanup for citrus fruits such as whole lemon and orange (Gilvydis, 1994). This was necessary because UV absorbing and fluorescing matrix coextractants eluted for several hours after the initial injection and adversely affected subsequent injections.

Hawthorne and co-workers (1994) set a precedent by using the term "subcritical" to define the experimental conditions of their work and refers to all temperatures below 374 °C and pressures below 221 atm as subcritical. Since the extraction temperatures described in the current study are less than 100 °C and the extractions are carried out at only 50 atm (static extraction) and atmospheric pressure (dynamic extraction), we have chosen to refer to the extractant as pressurized hot water, not subcritical water. The objective of the current study was to determine the feasibility of using water to extract TBZ and MBC from foods, replacing the methanol/DCM extraction scheme of the PAM method, and to study the experimental parameters which influence the extent of analyte recovery. We also wanted to determine the possibility of using pressurized hot water to extract more problematic matrixes such as citrus fruits.

METHODS AND MATERIALS

Preparation of Standards. Standards of TBZ (CAS no. 148-79-8) and MBC (CAS no. 10605-21-7) were obtained from the Environmental Protection Agency Pesticides Repository (2 Triangle Dr., Research Triangle Park, NC). Stock solutions were prepared in acetone at concentrations of ca. 50 μg/mL. Appropriate dilutions were prepared for sample fortification.

Sample Preparation and Fortification. Mushrooms, whole lemons, and whole oranges were thoroughly washed then homogenized using a Robot Coupe 6 L batch food processor (Robot Coupe U.S.A., Inc., Jackson, MS). Orange and banana pulps were similarly homogenized. White rice was milled to pass through a 20−40 mesh seive (Retsch KG, 5657 Hahn, Germany). Aliquots of 100 and 200 g of homogenized food sample were fortified with a volume of standard stock dilution to achieve desired fortification levels of MBC and TBZ. Two gram samples of milled rice were fortified with 200 μL of an appropriate standard dilution. High moisture samples were analyzed as soon as possible after initial preparation or were frozen until use.

Water Extraction Apparatus. Water extractions were carried out using a Suprex 200A (Pittsburgh, PA) supercritical fluid chromatograph modified for SFE. Bottled water (EM Science, Gibbstown, NJ) extractant was delivered, using a syringe pump, to a 1 m preheat coil of 1/16 in., 0.030 in. i.d. stainless steel tubing positioned before the extraction cell, as described by Hawthorne, Yang, and Miller (1994). The coil and cell were mounted in the chromatograph's oven and equilibrated at selected temperatures and a pressure of 50 atm. Stainless steel tubing (outlet tubing, 1/16 in., 0.030 in. i.d.) connected the extraction cell and a two-way stem valve (no. 20180, Scientific Systems, Inc., State College, PA) mounted outside the oven; water flow through the cell was controlled with this valve.

Water Extraction. For extraction with subcritical water, 5 mL SFE extraction cells with 5 μm stainless steel frits were used (Supelco, Bellefonte, PA). The frits were backflushed with distilled water, using a wash bottle, to prevent cross-contamination and the possible accumulation of sample fines. Frits were also sonicated in methanol, for 5−10 min, when water would not flow freely during backflushing. Prior to loading a cell with sample, a 13/16 in., 20 μm polyethylene frit (Varian, Harbor City, CA) was placed against the stainless steel frit of the outlet endcap of the cell to also protect it from sample fines. Homogenized sample, 2−3 g, was loaded into the cell then mixed with a dispersant consisting of 2 g of glass beads (60/80 or 80/120 mesh, Alltech, Deerfield, IL). For fortified milled rice, 2 g was transferred to the extraction cell and mixed with approximately 1.5 g of 3 mm glass beads as the dispersant (Thomas Scientific, Swedesboro, NJ). Assembly of the extraction cell was completed with the inlet endcap. The loaded cell was positioned in the oven using a ring stand and clamp and connected to the preheat coil and outlet tubing with finger-tight HPLC fittings. For all extractions, the cell was pressurized with water to 50 atm. The cell was equilibrated (static extraction) at a selected temperature for 5−20 min. Following equilibration, the cell was vented and the extractant was allowed to flow through the cell at flow rates between 2 and 20 mL/min (dynamic extraction). The extractant was collected in 50 mL Corning polypropylene centrifuge tubes (Fisher Scientific, Pittsburgh, PA). Extractant was collected in volumes ranging 10−80 mL depending on the experiment being performed.

Several studies were conducted using fortified banana pulp. To study the effects of temperature on analyte recovery,

banana pulp, fortified at the 1 ppm level for MBC and TBZ, was extracted at 50, 60, and 75 °C; the sample size was 2 g, the equilibration time was 5 min, the collection flow rate was 2 mL/min, and the collection volume was 40 mL. In a second experiment, 2 g aliquots of the same banana pulp composite were extracted at 75 °C but were collected in four 10 mL fractions to determine the pattern of analyte elution from the sample matrix. All other experimental conditions were as described above. Finally, banana pulp fortified at levels ranging from approximately 0.3 to 3 and 4 ppm were extracted at 75 °C, with a 5 min equilibration time, a flow rate of 10 mL/min, and a collection volume of 40 mL.

For whole lemon homogenates, fortified at the 5 and 10 ppm levels, 2 g samples were extracted at 75 °C, with an equilibration time of 5 min, a flow rate of 2 mL/min, and a 40 mL collection volume. To study the effect of sample size, 2 and 4 g samples were extracted at the 10 ppm level. As previously described, the 2 g samples were mixed with 2 g of dispersant. The 4 g samples were not mixed with dispersant due to insufficient room in the extraction cell. Whole orange homogenate fortified at the 1 and 5 ppm levels for both MBC and TBZ were also extracted under the same conditions as whole lemons. Orange pulp, fortified at the 0.5 ppm level, was used to simultaneously study the effects of equilibration temperature and collection volume. Extractions of orange pulp were carried out under ambient conditions (23 °C) and at 50 and 75 °C with a 10 min equilibration time and 10 mL/min flow rate. At 23 and 50 °C, collection volumes were 40 and 80 mL; 20, 30, and 40 mL were collected for samples extracted at 75 °C. Whole orange homogenate was also extracted under ambient temperature conditions and at 50 °C.

Milled rice samples of 2 g were fortified at the ca. 1.5 ppm level and extracted at 50, 60, and 75 °C. For all temperatures, the equilibration time was 10 min, flow rate was 10 mL/min, and collection volume was 40 mL. Rice extractions were repeated at 60 °C with a collection volume of 80 mL.

The recovery of MBC and TBZ from mushrooms fortified at approximately 4 ppm was determined using homogenate, which had been stored frozen for over 1 month. Extractions were done using water and acidified water on 2 g sample aliquots. The extraction temperature was 75 °C with an equilibration time of 20 min, collection flow rates of 5, 10, and 20 mL/min, and a 40 mL collection volume. Because average recoveries were inconsistent, the extractions were repeated using 2 g aliquots of freshly prepared mushroom homogenates which were fortified at 1.10 and 0.14 ppm levels of MBC and TBZ, respectively. Samples were extracted at 75 °C for 10 min; 40 mL was collected at 10 mL/min. To study the effect of an acidified extractant on the recovery of MBC and TBZ, extractions were performed on mushrooms using 0.003 M H_3PO_4 (pH 2.7) as the extractant. The fresh homogenates were also extracted using 0.003 M H_3PO_4 with equilibration times of 10 and 20 min and a collection flow rate of 10 mL/min.

Finally, incurred samples of orange pulp, apples, pears, and banana pulp, for which the levels of TBZ and MBC had been determined by a second FDA laboratory using the established PAM/FDA method, were extracted using subcritical water followed by LLE with ethyl acetate. Since this was a blind study, the ethyl acetate extracts of 2 or 3 aliquots of each sample were combined with the intent of maximizing detection in the final determinative step. The total amount of sample used for each duplicate was 6 g. All incurred samples were extracted with subcritical water at 75 °C for 20 min. A 5 mL/min collection flow rate was used.

Liquid−Liquid Extraction (LLE). Water extracts were refrigerated for up to 3 days before LLE, if necessary. For LLE, water extracts were adjusted to pH 8−9 with 1 M NaOH. The pH was measured using EM Science ColorpHast pH strips. Rice extracts collected at 60 °C and 75 °C contained precipitate. These extracts were centrifuged for 2 min at 2500 rpm, and the supernatants were decanted into second tubes for pH adjustment. All pH adjusted extracts were then transferred to 250 mL separatory funnels. The tubes were rinsed with distilled water and the rinse added to the funnel. LLE extractions were performed using two aliquots of ethyl acetate (EM Science). The volumes of ethyl acetate depended on the volume of water extract. The 20 mL samples were partitioned with two 50 mL aliquots of ethyl acetate, 30 and 40 mL samples with 50 and 100 mL aliquots, and 80 mL samples with two 100 mL aliquots. The separatory funnels were shaken for 3 min, and the liquid phases allowed to separate for at least 10 min. The aliquots of ethyl acetate were dried through 25 g of anhydrous sodium sulfate (Mallinckrodt Chemical, Chesterfield, MO) contained in 32 mm i.d. × 230 mm Pyrex brand extraction tubes (model 3700, Lab Glass, Vineland, NJ) and combined in 250 or 300 mL round-bottomed flasks and carefully taken to dryness at 50 °C under vacuum using a rotary evaporator (Buchi Rotavapor, Brinkman, Westbury, NY). The final sample was dissolved in 1 or 2 mL of ion-pairing HPLC mobile phase. When necessary, samples were filtered with 13 mm, 0.45 μm Nylon Acrodisc filters (Gelman Sciences, Ann Arbor, MI).

Ion-Pairing HPLC. *Mobile Phase.* HPLC ion-pairing solution was prepared using a modified version of the mobile phase described by Gilvydis and Walters (1990). One gram of 1-decanesulfonate, sodium salt (Aldrich Chemical Co., Milwaukee, WI) was dissolved in ca. 200 mL of water obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA). Triethylamine (10 mL, Aldrich, Milwaukee, WI) and 7 mL of H_3PO_4 were added and the final volume brought to 1 L with purified water. The finished solution was filtered through a Millipore Type DV, 0.65 μm nylon filter. Ion-pairing solution (550 mL) was combined with 450 mL of methanol (EM Science) and thoroughly degassed with helium before equilibrating the column. Some analyses were carried out using 40% methanol and will be indicated as necessary in the figure legends.

Guard and Analytical Columns. The guard column was a 4 mm × 1.25 cm Zorbax SB-C18 cartridge column. The analytical column was a 4.6 mm × 25 cm Zorbax Stablebond SB-Cl8. Both columns were purchased from Mac-Mod Analytical Inc. (Chadds Ford, PA).

HPLC Apparatus. Mobile phase was typically delivered at a flow rate of 1.5 mL/min with an SP8800 ternary HPLC pump (Spectra-Physics, San Jose, CA). Sample injection was accomplished with an SP8880 autosampler (Spectra-Physics) equipped with a 100 μL sample loop. Sample injection volumes ranged from 25 to 75 μL. The columns were thermostated at 45 or 50 °C using a Perkin-Elmer LC-100 column oven (Norwalk, CT) and were equilibrated with flowing mobile phase for about 60 min. Detection was achieved with a diode array UV−vis absorbance detector or a programmable fluorescence detector. The diode array detector was an HP 1040A with a Chemstation, and the fluorescence detector was an HP 1046A (Hewlett-Packard, Palo Alto, CA). UV absorbance of MBC and TBZ was done at 280 nm. TBZ was also monitored at 305 nm simultaneously because its response was twice that at 280 nm. MBC was monitored using the fluorescence detector with an excitation wavelength of 280 nm and an emission wavelength of 310 nm. Detection of TBZ by fluorescence was accomplished with an excitation wavelength of 305 nm and an emission wavelength of 380 nm (Arenas et al., 1996). The detectors were usually not used in tandem because of excessive back pressure at flow rates of 1.5 mL/min. Flow rates of 1−1.25 mL/min were used during tandem operation. (Specific HPLC operating conditions are provided in the figure legends.) The signal of the fluorescence detector was recorded with a C-R6A Chromatopac integrator (Shimadzu, Tokyo, Japan).

Determination of Recoveries. Chromatographic responses (peak retention times, heights, and/or areas) of MBC and TBZ standards were compared to those of sample extracts, and recoveries were calculated.

RESULTS AND DISCUSSION

The technique described in this study consists of the following basic steps: sample preparation (e.g., homogenization and addition of dispersant), sample extraction (static and dynamic), collection of an aqueous sample

Table 1. Average Recoveries of MBC and TBZ from Banana Pulp[a]

	analyte[b]	fortification level (ppm)	flow rate (mL/min)	equilibration time (min)	av % recovery	%RSD[c]
A	MBC	0.32	5	20	80.8	5.6
	TBZ	0.29	5	20	83.6	9.8
B	MBC	0.32	10	5	74.0	33.5
	TBZ	0.29	10	5	90.3	9.3
C	MBC	1.58	10	5	83.3	1.9
	TBZ	1.44	10	5	84.7	2.3
D	MBC	3.15	10	5	78.2	3.2
	TBZ	4.06	10	5	82.2	0.7

[a] Extraction temperature was 75 °C and collection volume was 40 mL for all determinations. [b] Ethyl acetate LLE extracts for two 2 g samples were combined for sample A; the total sample size for samples B−D was 2 g. [c] $n = 4$ for samples A−D.

extract, concentration of the analytes using LLE, and final determination by HPLC. The results of this study indicate that several experimental parameters influence the extent of analyte recovery. These include the extraction temperature, equilibration time, collection flow rate, volume of extractant collected, pH, sample size, the nature of the sample, and added dispersant. In some instances, MBC and TBZ, responded very differently despite the fact that they are chemically similiar (i.e., weak bases).

Effect of Temperature and Time Using Banana Homogenates. The average recoveries ($n = 3$) of MBC fortified at the 1 ppm level in banana pulp extracted at 50, 60, and 75 °C were 86.9% (%RSD = 4.2), 98.1% (%RSD = 7.9), and 82.3% (%RSD = 5.5), respectively. For TBZ, the average recoveries ($n = 3$) were 84.2% (%RSD = 12.3), 104.5% (%RSD = 11.0%), and 84.9% (%RSD = 3.7), respectively. While these average recoveries were greater than 80% at all extraction temperatures, the reproducibility was highest for both analytes at an extraction temperature of 75 °C. On the basis of this information, subsequent recovery studies for fortified banana pulp were carried out at 75 °C. Banana pulp samples, in triplicate, were extracted at 75 °C, and the water extractant was collected in four 10 mL aliquots. Recoveries were determined for each aliquot. The average recovery of MBC and TBZ in the first 10 mL of water extractant was approximately 42% with the remaining MBC and TBZ recovered in the three additional 10 mL aliquots of water extractant. These initial studies with banana homogenate indicated that the sample collection step is also one of dilution. A collection volume of 40 mL was required to obtain average recoveries greater than 80% at the 1 ppm fortification level. The average recoveries of MBC and TBZ fortified at several levels in banana are given in Table 1. The lowest average recovery of 74% was for MBC fortified at 0.32 ppm with a relative standard deviation of 33.3% (row B). Average recoveries at fortification levels greater than approximately 0.3 ppm are higher with better reproducibility. Incurred TBZ was found in the control banana extract at the 75 ppb level; recoveries were calculated using background subtraction. In an earlier experiment (row A), the equilibration time was 4 times longer with a slower sample collection flow rate. For MBC in these samples, the average recovery was 80.8% with a significantly better relative standard deviation of 5.6%. These results suggest that the process of mass transfer or partitioning of the analytes from the sample into the aqueous extractant may require a longer equilibration time or an extraction temperature greater than 75 °C. The latter approach would ideally improve the efficiency of the extraction through an enhanced rate of transfer.

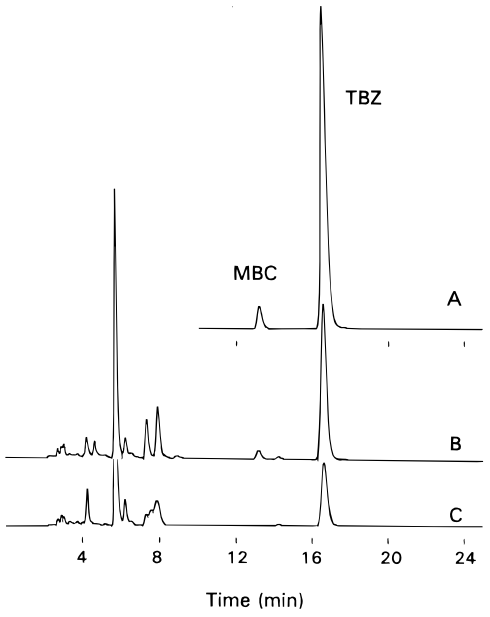

Figure 1. Fluorescence chromatograms (0.064 mAUFS) of banana pulp extracts with MBC and TBZ fortified at the 0.32 and 0.29 ppm levels, respectively. MBC (28 ng) and TBZ (25 ng) standards are shown in chromatogram A; a fortified banana extract in chromatogram B; and a control in chromatogram C. Incurred TBZ was quantitated for the control extract at 0.086 ppm. The ethyl acetate LLE extracts of two 2 g banana extracts were combined and the final sample residue dissolved in 2 mL of HPLC mobile phase. An injection volume of 25 μL was used for a sample equivalent of 50 mg injected on-column. The flow rate was 1.25 mL/min, oven temperature was 45 °C, and the mobile phase contained 40% methanol.

Although the ethyl acetate extracts of two 2 g banana water extracts were combined for these analyses, the actual amount of sample equivalent injected was the same for samples of rows B−D. Fluorescence chromatograms of banana extracts and a standard injection are shown in Figure 1.

Concentration of TBZ and MBC from Water Extractants. Because the sample collection step is also a dilution step, concentration of the analytes is necessary. In the work described by Hawthorne and coworkers (1994), concentration of the analytes was accomplished on-line by percolating the aqueous extractant through 5 mL of chloroform. Sequential extractions were carried out at 50, 150, 250, and 400 °C for 10 min each at a flow of 1 mL/min. Hence, the total volume of water extractant collected was 40 mL. Most of the extracted analytes partitioned into the solvent without additional chloroform extractions. An internal standard was added and the solvent injected onto a GC system for quantitative determination. For the concen-

Table 2. Average Recoveries of MBC and TBZ from Whole Lemon Homogenates[a]

analyte	fortification level (ppm)	sample size (g)	av % recovery	%RSD	(*n*)
MBC	5	2	95.1	5.4	(5)
TBZ	5		85.5	5.7	(5)
MBC	10	2	86.0	7.0	(3)
TBZ	10		80.9	6.9	(3)
MBC	10	4[b]	73.4	**	(2)
TBZ	10		67.4	**	(2)

[a] For all samples, extraction temperature was 75 °C; equilibration time was 5 min; flow rate was 2 mL/min; and collection volume was 40 mL. [b] Dispersant was not added to this sample.

tration of MBC and TBZ from water extractant, however, it was apparent that because of the varied nature of the matrixes in terms of moisture content and pH, for instance, and the ionizability of the analytes, an online concentration step was not attempted. Partitioning MBC and TBZ from an aqueous to an organic phase requires adjustment of the pH to approximately 8 to ensure that the analytes exist in a nonionized state. A liquid−liquid extraction using ethyl acetate (Arenas et al.,1996) was used, therefore, primarily because the determination of recovery through the LLE was easily accomplished and losses were found to be insignificant (i.e., 2%) for fortified control banana homogenates.

Ideally, a solid-phase extraction method using either a sorbent cartridge, disk, or membrane would be a preferred approach for concentrating MBC and TBZ because a much smaller volume of organic solvent(s) would be required as compared to the ethyl acetate partitioning method described here. Numerous SPE methods have been developed for the analysis of pesticide residues in water samples (Viana et al., 1996; Molina et al., 1996; Young, 1998; Junker-Buchheit and Witzenbacher, 1996). These methods may be suitable for the concentration of pesticide residues from the water extractants with only minor sample handling (e.g., filtration to remove sample fines or pH adjustment) or method modification (e.g., adjustment of rinse volumes).

Effect of Sample Size Using Whole Citrus Fruits. Table 2 shows the average recoveries of MBC and TBZ extracted from whole lemon samples. Average recoveries for 2 g samples are greater than 80% for both analytes fortified at the 5 and 10 ppm levels. Larger sample size (i.e., 4 g) with no modification of extraction conditions, however, resulted in lower average recoveries, particularly for TBZ (i.e., 67.4%). These lower recoveries suggest that the larger sample size may require a longer equilibration time to solvate the sample matrix or an insufficient volume of extractant was collected. In terms of mass transfer, a higher extraction temperature may have resulted in improved efficiency and higher recovery of the analytes. There was a concern, however, that the matrix components, which were so highly retained on the C-18 analytical column in the original FDA/PAM method (Gilvydis, 1994), believed to be oils or other nonpolar sample components of the fruit, would more likely be extracted by water at increased temperatures. A UV chromatogram of whole lemon extracts at the 5 ppm fortification level are shown in Figure 2. No interferences were encountered for MBC or TBZ and repetitive injections were made every 30 min even though coextractants were not encountered beyond 10 min. Whole oranges were also extracted

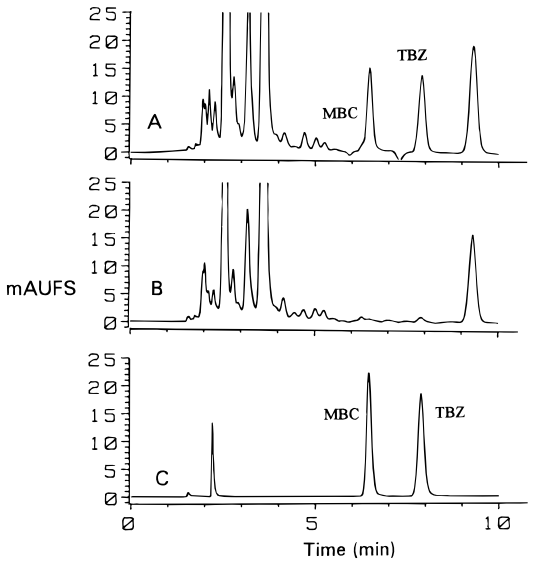

Figure 2. UV absorbance chromatograms of lemon extracts at 280 nm. Fortified lemon extract is shown in chromatogram A; a control in chromatogram B; and standards of MBC (1 47.5 ng), and TBZ (1 39.6 ng) in chromatogram C. The recoveries of MBC and TBZ for this sample were 89.57% and 81.53%, respectively. The flow rate was 1.5 mL/min, oven temperature was 45 °C, and the mobile phase contained 45% methanol. The injection volume was 25 μL for a sample equivalent of 25 mg on-column.

using water under the same conditions as lemons. Unfortunately, as encountered with the FDA/PAM method, late-eluting (e.g., >60 min) sample components made repetitive injections in a reasonable time frame difficult, although MBC and TBZ were chromatographed without interference at the 1 and 5 ppm fortification levels. Recoveries of MBC and TBZ at these levels from whole oranges could only be estimated at 85%.

Effect of Temperature and Collection Volume Using Orange Pulp. Extractions of fortified orange pulp were used to study the average recoveries of MBC and TBZ at several extraction temperatures including 23 °C (ambient conditions) and with varying collection volumes. It was anticipated that the recovery of MBC and TBZ might be enhanced because of the acidic nature of the sample matrix regardless of the extraction temperature used. The pH of homogenized orange pulp is approximately 3. At this pH, MBC and TBZ would be approximately 90% ionized and, therefore, highly water soluble. Fortification levels for both analytes were approximately 0.5 ppm. At lower extraction temperatures, it was expected that a larger volume of water would be needed to achieve adequate and reproducible recoveries. The average recoveries of MBC and TBZ extracted from orange pulp are summarized in Table 3. At all extraction temperatures and with collection volumes ranging between 20 and 80 mL, the average recovery of MBC was >90% with relative standard deviations less than 10%. For TBZ, the average recoveries decreased with lower extraction temperatures and smaller collection volumes. The highest average recoveries for TBZ were achieved at 75 °C with collection volumes ranging 20−40 mL. Under similar extraction conditions, recall that MBC and TBZ were only recovered to an extent of approximately 42% from banana homogentes in the first 10 mL of water extractant and were found at sequentially decreasing levels in the remaining 30 mL collected. Virtually quantitative recovery was achieved for MBC and TBZ in 20 mL of

Table 3. Average Recoveries of MBC and TBZ from 3 g Samples of Orange Pulp[a]

analyte	extraction temp (°C)	collection vol (mL)	% recovery	av recovery	%RSD
MBC[b]	ambient (23 °C)	40	90.4, 93.5	92.0	
	ambient (23 °C)	80	94.8, 86.2	90.5	
	50	40	90.6, 96.3	93.5	
	50	80	83.6, 99.8, 97.4	93.6	9.3
	75	20	94.1, 88.0, 106.5	96.2	6.2
	75	30	96.1, 98.9, 98.8	98.0	1.7
	75	40	86.1, 97.2, 89.6	91.0	9.8
TBZ[b]	ambient (23 °C)	40	71.2, 71.4	71.3	
	ambient (23 °C)	80	89.3, 70.6	80.0	
	50	40	77.9, 73.7	75.8	
	50	80	88.9, 92.4, 87.3	89.5	2.9
	75	20	88.3, 86.5, 97.1	90.6	6.3
	75	30	99.4, 97.8, 104.3	100.5	3.4
	75	40	85.9, 92.7, 87.0	90.0	6.9

[a] For all extractions, the equilibration time was 10 min and the collection flow rate was 10 mL/min. [b] Fortification levels of MBC and TBZ were 0.51 and 0.58 ppm, respectively.

extractant for the orange pulp samples using shorter (10 min vs 20 min) equilibration times and faster (10 vs 5 mL/min) collection flow rates. At least two conditions may account for the efficiency of the recoveries from the orange pulp samples. First, the acidic pH of the samples themselves may account for the enhanced extractability of MBC and TBZ from these samples. Second, orange pulp has a higher moisture (86%) content than banana pulp (76%). Orange pulp may therefore be more amenable to solubilization or "wetting". Water may also not flow as readily through banana pulp; upon examining sample pellets following extraction, banana pulp is clearly more densely packed than orange pulp. In the case of the banana samples, longer equilbration times, an increased amount of dispersant, a larger collection volume, acidification of the sample aliquot and/or the extractant, or extraction temperatures greater than 75 °C may all contribute to enhanced mass transfer and subsequently more efficient recoveries.

Fluorescence chromatograms of orange pulp extracts are shown in Figure 3. Sample coextractants did not interfere with the chromatography of MBC or TBZ and no peaks were observed eluting after TBZ. Incurred TBZ was found in the control extract at a level of approximately 7 ppb. Following this study, whole oranges were extracted at 23 and 50 °C. It was anticipated that the lower extraction temperature would result in a more selective extraction whereby fewer nonpolar matrix components would be extracted. While the chromatograms revealed fewer peaks resulting from sample co-extractants, late-eluting sample components were still encountered even 1 h after injection.

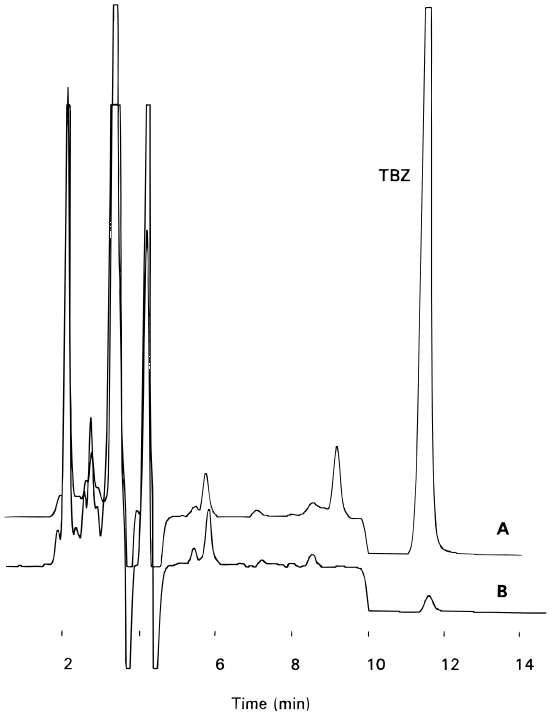

Figure 3. Fluorescence chromatograms of orange pulp extracts. Chromatogram A is of a 3.2 g aliquot of sample fortified at the 0.51 ppm level. Chromatogram B is a control showing incurred TBZ at ca. 7 ppb. The collection volume for both samples was 30 mL at an extraction temperature of 75 °C. A sample equivalent of 240 mg is represented for each. The HPLC injection volume was 75 μL, column flow rate was 1.5 mL/min, column temperature was 50 °C, and the methanol content of the mobile phase was 40%.

Effect of Temperature and Collection Volume Using Rice. The average recoveries of MBC and TBZ from milled white rice fortified at ca. 1.5 ppm are shown in Table 4. Average recoveries ranged from 60.1% for TBZ at 75 °C to 79.4% for MBC at 50 °C. Because rice is a low-moisture commodity, it was thought that these low recoveries were due either to incomplete solvation or to wetting of the matrix in the time selected for static extraction or insufficient collection volume of the sample extract. Since the highest recoveries for both analytes were achieved at 60 °C, rice extractions were repeated at this temperature with a final collection volume of 80 mL. All other extraction conditions remained the same. MBC and TBZ were fortified at the 1.02 and 1.15 ppm levels, respectively. With this collection volume, the average recoveries of MBC and TBZ were 94.0% (%RSD = 1.5%, n = 4) and 92.5% (%RSD = 3.8%, n = 4), respectively. UV absorbance HPLC chromatograms of these rice extracts are shown in Figure 4. No incurred residues were found in the control extract, no interferences of MBC or TBZ were encountered, and no matrix components were chromatographed after TBZ. Some initial extractions of milled rice were carried out at 75 °C without the addition of 3 mm glass beads. A rapid rise in back pressure to over 100 atm during sample collection was encountered with subsequent plugging of the extraction cell. With the addition of glass beads, plugging did not occur, although some rise in back pressure was encountered (e.g., <40 atm). With all other food commodities, back pressure during sample

Table 4. Average Recoveries of MBC and TBZ from 2 g of Milled Rice[a]

analyte[b]	extraction temp (°C)	av % recovery	%RSD[c]
MBC	50	79.4	9.0
	60	79.1	3.5
	75	72.3	6.2
TBZ	50	64.8	13.1
	60	79.1	10.6
	75	60.1	7.1

[a] Equilibration time was 10 min, flow rate was 10 mL/min, and the collection volume was 40 mL. [b] Fortification levels for MBC and TBZ were 1.58 and 1.44 ppm, respectively. [c] $n = 3$ for all determinations.

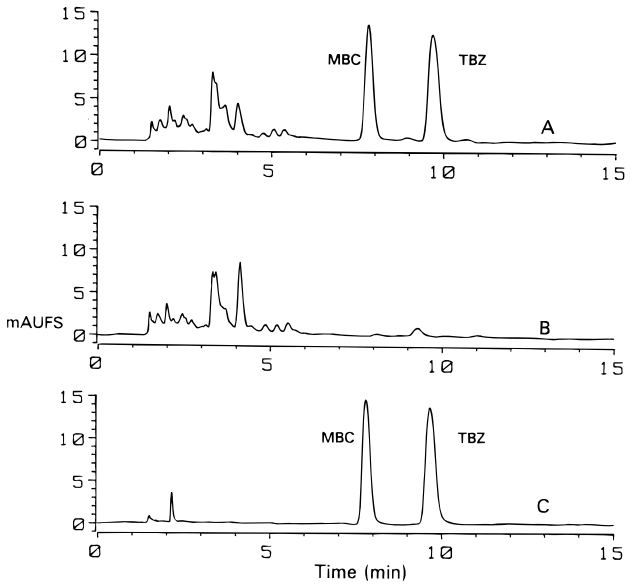

Figure 4. UV absorbance chromatograms at 280 nm of rice extracts and a standard injection. Chromatogram A is of a fortified rice sample extract which was extracted with subcritical water at 60 °C and a collection volume of 80 mL. The recoveries of MBC and TBZ were 95.08 and 94.79%, respectively. Chromatogram B is of a control extract. Standards of MBC (153.2 ng) and TBZ (172.9 ng) are shown in chromatogram C. The HPLC column flow rate was 1.5 mL/min, the oven temperature was 50 °C, and the methanol content was 45%. A sample equivalent of 150 mg is represented (75 μL injection volume).

collection was typically no higher than 3 or 4 atm and no plugging problems were encountered.

Addition of Dispersant. The addition of dispersant, fine mesh, or 3 mm glass beads, was essential to maintain flow through the cell by preventing compaction of the samples under pressure. Recoveries were also shown to be highly dependent on the presence of dispersant. The presence of a dispersant allowed more thorough contact of the sample by water resulting in enhanced mass transfer of the analytes from the sample into the aqueous extractant. During these studies, random sample aliquots were extracted without the addition of dispersant. In most cases, complete plugging of the outlet frit occurred with a rapid rise in back pressure. For those aliquots that could be extracted, recoveries were, at most, 50% of those samples extracted in the same sample set with added dispersant. The exception to this general observation occurrred with the 4 g whole lemon sample extractions (Table 2). Dispersant was not added to the extraction cell, yet the average recovery was only 13% lower than those obtained for 2 g samples with dispersant. This may be due to the acidic nature of the sample which may have enhanced the extraction of MBC and TBZ as proposed for the orange pulp samples (Table 3). In these experiments, glass beads were the only dispersants used for the water extractions. Other, perhaps more suitable, dispersants might be sand or Celite (Lehotay, 1997).

Table 5. Average Recoveries of MBC and TBZ from Fresh Mushroom Homogenates[a]

analyte	extractant	equilibration time (min)	av % recovery	%RSD[c]
MBC[b]	water	10	96.0	10.0
TBZ			90.0	7.0
MBC	0.003 M H_3PO_4	10	87.7	5.0
TBZ			49.8	9.9
MBC	0.003 M H_3PO_4	20	96.4	3.0
TBZ			45.0	11.4

[a] Extraction temperature was 75 °C, flow rate was 10 mL/min, and the collection volume was 40 mL. [b] Fortification levels for MBC and TBZ were 1.10 and 0.14 ppm, respectively. [c] $n = 3$ for all determinations.

Effect of Sample Acidification Using Mushroom Homogenate. The average recovery of MBC fortified at the 4 ppm level in a mushroom homogenate, using a collection flow rate of 5 mL/min was 45.3% ($n = 3$) with a %RSD of 10.8%. With a flow rate of 10 mL/min, the recovery was 50.1% (%RSD = 2.6%, $n = 4$). For these same samples, the average recovery of TBZ, also fortified at 4 ppm, was 84.9% ($n = 3$) with a relative standard deviation of 8.5% at 5 mL/min and 95.4% (%RSD = 2.6%, $n = 4$) at 10 mL/min. Using 0.003 M phosphoric acid as the extractant and collection flow rates of 10 and 20 mL/min, the average recoveries of MBC increased to 70.5% (%RSD = 6.0%, $n = 4$) and 68.2% (%RSD = 18.9%, $n = 3$), respectively. For TBZ, the average recoveries were 79.0% (%RSD = 6.5%, $n = 4$) and 80.2% (%RSD = 16.7%, $n = 3$), respectively. Due to concern over the integrity of the samples, the experiment was repeated using freshly prepared mushroom homogenate fortified at 1.10 and 0.14 ppm for MBC and TBZ, respectively. These average recoveries are summarized in Table 5. The average recoveries using unmodified water are approximately 90% or greater for both analytes. Recoveries of MBC using acidified water are similar, but those for TBZ are lower by approximately 50%. There is no apparent explanation for this observation especially since the acidic nature of the citrus fruit extracts may account, in part, for average recoveries greater than 80%. Chromatograms of mushroom extracts showed no interferences for MBC or TBZ using either UV absorbance or fluorescence detection.

Determination of Incurred Residues. Finally, incurred residues of TBZ were quantitated in orange pulp, apples, pears, and banana pulp. The samples were initially analyzed by another laboratory using the validated PAM procedure for MBC and TBZ then in our laboratory using the water extraction technique. Using the PAM procedure, TBZ was quantitated at the following levels: 0.04 (orange), 0.80 (apple), 0.39 (pear), and 0.01 (banana) ppm. Benomyl, determined as MBC, was also found in the apple sample at a level of 0.08 ppm. Using the water extraction technique, the levels of TBZ quantitated were 0.04, 0.86, 0.22, and 0.01 ppm, respectively; MBC was not detected in the apple extracts. The levels of TBZ found in the orange and banana pulp samples compare very closely using the two methods. The level of TBZ found in the apple samples

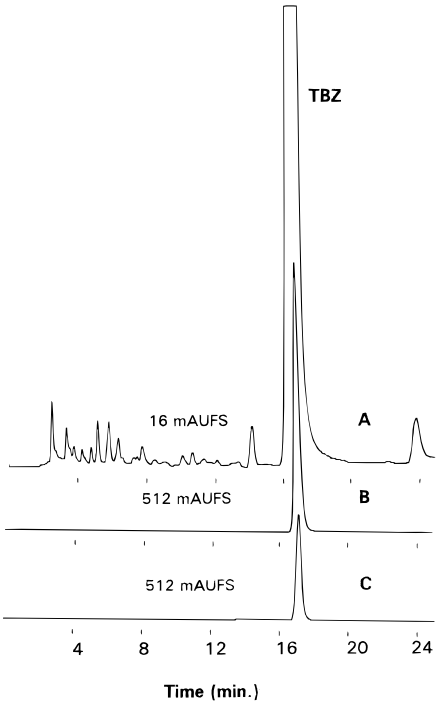

Figure 5. Fluorescence chromatograms of incurred apple sample (A and B) and a standard injection of 25 ng each MBC (not seen) and TBZ. A sample equivalent of 150 mg is represented; injection volume was 75 μL. The column temperature was 45 °C, the flow rate was 1.25 mL/min, and the methanol content of the mobile phase was 40%.

was about 7% higher using the water extraction procedure than the level determined with the PAM procedure. The level of TBZ determined for the pear sample was about 43% lower using the water extraction technique than using the PAM procedure. Despite the variablity encountered, the results of this cursory comparative study are encouraging. Fluorescence chromatograms of the apple extract and a standard injection are shown in Figure 5.

The inability to detect incurred MBC in apples at the 0.08 ppm level illustrates a significant drawback with the extraction technique as it has been described. The 5 mL extraction cell restricts sample size to no more than 3−4 g plus added dispersant. In the case of MBC, this led to a lack of detectability even with fluorescence detection. The detection of TBZ, however, was easily accomplished using a fluorescence detector even at fortification levels in the lower parts per billion range using only 2−3 g of sample. The estimated limit of detection for MBC and TBZ using fluorescence detection was 100 and 1 ppb, respectively. The most obvious solution to increasing the detectability of MBC would be to use an increased sample size. This would require that a larger extraction cell (e.g., 10 mL) be employed with subsequently larger collection volumes, for instance, if recovery is to be optimized. In the case of MBC or other analytes which may have limited detectability, it will be imperative to determine those factors which have the greatest influence on extraction efficiency if the method is to be streamlined. Using increased extraction temperatures may be a significant key to extraction efficiency. Some possible problems of this approach are sample or analyte degradation and enhanced coextraction of sample components (e.g., sugars, proteins).

For these studies, sample homogenates were fortified with TBZ and MBC. The latter is the main degradation product of benomyl which is typically determined as MBC because it is readily converted to this analyte particularly under acidic conditions. However, no provisions were made in the water extraction technique to ensure complete conversion of any benomyl which might be incurred. The PAM procedure, for instance, uses an acidified aqueous solution for LLE and simultaneous conversion of benomyl to MBC. Fortunately, all control sample extracts used in the current study were free of detectable MBC residues. Further development of the water extraction technique could include a step in which sample homogenates are acidified to achieve complete conversion of benomyl to MBC prior to extraction with water. The effect on recovery would need to be evaluated.

CONCLUSIONS

The results of this study indicate that extraction with pressurized hot (subcritical) water is a feasible technique for the extraction of pesticide residues from foods. With the notable exception of whole orange homogenates, the chromatography of MBC and TBZ was achieved for all other matrixes without interferences using both UV and fluorescence detection. Like supercritical carbon dioxide, so widely used for SFE, water is environmentally friendly and is inexpensive in terms of purchase and disposal costs. Although a limited number of extraction conditions were used in this study (i.e., temperatures below 100 °C at 50 atm), pressurized hot water can potentially be used under experimental conditions in which it demonstrates a wider range of solvent polarities making it suitable for the extraction of a variety of analytes. While the more obvious experimental parameters, such as temperature and dispersant, were shown to have a great impact on extraction efficiency, it is possible that a variety of chemistries can be employed by modifying the extractant in terms of pH, for example. Selectivity may most likely be achieved by the techniques which are employed to partition analytes of interest out of the water extractant and concentrate them for subsequent determination. SPE, for instance, has the potential to provide such selectivity.

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