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Short communication

Simplified reversed-phase conditions for the determination of benzimidazole fungicides in fruits by high-performance liquid chromatography with UV detection

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Abstract

An analytical method was developed for determination of benzimidazole fungicides (carbendazim and thiabendazole) in fruits. Analyses were performed by HPLC with simple operating conditions. The use of a Kromasil new-generation silica-based stationary phase needed neither pH regulators nor competing compounds, usually added to the mobile phase to analyse basic compounds on reversed stationary phase. Validation studies proved that the chromatographic method had good repeatability, reproducibility and limit of detection. Sample preparation involved extraction with acetone followed by solvent partitioning with dichloromethane and petroleum ether. Extracts were purified through selective diol-bonded silica cartridges, replacing the usual laborious liquid–liquid partitioning procedure. Validation studies proved that the global method had satisfactory repeatability and recovery. Limits of detection were about 0.06 mg/kg. © 1997 Elsevier Science B.V.

Keywords: Fruits; Food analysis; Environmental analysis; Pesticides; Carbendazim; Thiabendazole; Benzimidazoles

1. Introduction

Benzimidazole fungicides are systemic pesticides widely used in agriculture for pre- and postharvest protection of crops against fungal diseases. Therefore, they are some of the most detected pesticides during monitoring programmes and it is crucial to assess consumers' exposure to those fungicides through foods.

The main compounds of the benzimidazolic family are carbendazim (MBC) and thiabendazole (TBZ). The structure is presented in Fig. 1. Their thermal instability does not permit their analysis by gas chromatography, unless they are transformed into thermally-stable derivatives [1,2]. Therefore, the most common analytical method for analysis of carbendazim and thiabendazole is liquid chromatography coupled with UV and/or fluorescence detection. However, the reported HPLC conditions require mobile phase modifiers such as competing amine and pH buffer in normal-phase separation [3], or ion-pairing reagent [4] in reversed-phase separation [5,6] to improve the peak shape and the resolution. A polymeric-phase column could also be used to improve the quality of the separation and simplify the HPLC conditions [7,8]. Nevertheless, this kind of column is more expensive and has less applicability in other analyses than a silica-based column.

Fruit extracts are usually purified by acid–base

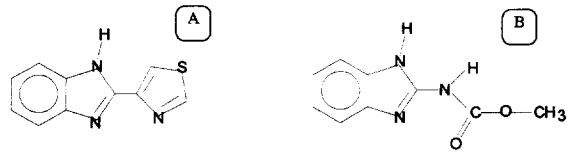

Fig. 1. Thiabendazole (A) and carbendazim (B) structures.

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liquid–liquid partitioning [1]. However, this procedure is laborious, time-consuming, difficult to automate and requires use of large volumes of usually-toxic organic solvents. Solid-phase extraction (SPE) cartridges with chemically bonded silica sorbents have become an alternative to liquid–liquid partitioning. So, Hiemstra et al. [8] developed a fast SPE cleanup procedure using diol-bonded silica cartridge (Bond-Elut) for determination of benzimidazole fungicides in various crops, Chromatographic separations were performed with a polymeric stationary phase column.

The aim of this study was to develop simplified conditions for the RP-HPLC analysis. The chromatographic method developed was utilised in a inexpensive and effective procedure to determine residues of benzimidazole fungicides in fruit, based on the method by Hiemstra et al. However a diol-bonded silica cartridge supplied by another manufacturer was adapted in our method being necessary to modify the preconditioning conditions.

2. Experimental

2.1. Reagents and material

Methanol was of HPLC grade and purchased from Carlo Erba Reagenti (Milan, Italy). Acetone, dichloromethane and light petroleum (analytical grade) were supplied by Romil (Milan, Italy). Milli-Q water was obtained with a Milli-Q system from Millipore (Milford, USA). Phosphoric acid and sodium hydroxide are supplied by Sharlau (Barcelona, Spain). Isolute diol-bonded silica cartridges (500 mg) were supplied by International Sorbent Technology, I.S.T. (Hengoed, UK). Standard of carbendazim was obtained from Riedel-de Haën (Seeize, Germany) and thiabendazole from Dr. Ehrenstorfer (Augsburg, German). Stock solutions (200 μg/ml) of carbendazim and thiabendazole were prepared in methanol (or acetone in order to fortify the samples) and stored at −25°C. Working standard solutions were prepared immediately before injection diluting stock solutions with methanol/water (50:50, v/v) (mobile phase).

Vacuum manifold Vac Master-10 from I.S.T., was used to perform the SPE.

2.2. Instruments

A Kromasil stationary phase (octadecyl silane) was made in Akzo Nobel (Bohus, Sweden) and was packed by Tecknokroma (St. Cugat, Barcelona, Spain). Kromasil-100-C_{18} column (150×4 mm I.D., 5 μm particle diameter) and a Kromasil precolumn (10×3 mm I.D., 5 μm particle diameter) were used. The chromatographic system consisted of a Merck–Hitachi L-6200 Intelligent Pump with a Rheodyne injector mod. 7125 (Cotati, USA) and of a Merck T-6300 Column Thermostat. UV detection was performed by a Merck–Hitachi L-4000 UV detector. Data were collected and integrated on a Merck–Hitachi D-2500 Chromato-Integrator.

2.3. Chromatographic conditions

Methanol–water (50:50, v/v) was used as isocratic mobile phase at a flow-rate of 1 ml/min. The analytical column was thermostated at 55°C. The UV detection was performed at 285 nm.

2.4. Procedure

2.4.1. Sample preparation

Weigh about 10 g of a well-mixed chopped fruit sample into a 100 ml high-rim beaker. Add 20 ml of acetone and homogenize mixture for 1 min with a blender. Add 20 ml of dichloromethane and 20 ml of petroleum ether, and homogenize another 1 min with the blender. Transfer solution into a 70 ml centrifuge tube and centrifuge for 4 min at 2000 *g*. Adjust organic phase volume to 50 ml, adding dichloromethane or evaporating solvent under gentle nitrogen stream. Transfer 2.5 ml of organic solution into a sample vial and evaporate to dryness in a water bath at 60°C and redissolve the residue in 2 ml of methanol.

2.4.2. Cleanup

Precondition SPE cartridge by passing 2 ml methanol and 2 ml methanol–0.1 *M* phosphoric acid (50:50, v/v), using a vacuum manifold. Apply 1 ml of methanol extract from sample vial to SPE cartridge. Wash SPE cartridge with 1 ml methanol and allow solid-phase to dry by passing air stream. Elute SPE cartridge with 2 ml methanol–0.1 *M* phosphoric

acid (50:50, v/v) and collect eluate in a tared collecting tube, draining solid-phase by passing air stream during 30 s. Weigh collecting tube in order to calculate the volume of the eluate (methanol–phosphoric acid solution) using their density previously obtained (0.9323 g/ml). Add 100 μl 1 *M* sodium hydroxide to collecting tube. Mix solution and inject 20 μl into HPLC system.

3. Results and discussion

3.1. Optimisation of chromatographic conditions

3.1.1. Stationary phase

As said in the introduction, usual RP-HPLC conditions to analyse benzimidazole fungicides need mobile-phase modifiers to improve the peak shape and the column efficiency. That was checked using a conventional octadecyl silane-stationary phase with our chromatographic conditions without modifier. The analysis of a 0.25 μg/ml solution of MBC and TBZ in methanol–water (50:50) confirmed the expected poor chromatographic performance: broad and tailing peaks (Fig. 2a). Compound adsorption on stationary phase is due to interaction between nitrogen atoms of the benzimidazole molecules (weak base) and the acid residual silanol groups of the silica surface. The greater broadness of the TBZ peak is due to the greater basicity of the compound. Therefore, adding a competing amine to the mobile phase can improve the peak shape. However, chromatographic conditions become more complicated since it is necessary to use pH buffer to maintain pH<B to preserve silica chemical stability.

Another way, checked in our laboratory, to improve chromatographic performance is to use a new-generation silica-based stationary phase: Kromasil. Special treatment allowed elimination of acid heavy metals from silica surface (Al, Na, Fe<25 ppm) and to functionalise residual silanol groups, reducing their activity towards basic compounds. As shown in Fig. 2b, it was possible to analyse a solution of MBC and TBZ with that special octadecyl stationary phase, without adding modifiers to the mobile phase.

3.1.2. Temperature

Influence of column temperature upon separation efficiency and compound adsorption was studied. Increase of temperature reduced compound retention times, maintaining efficient separation and improved weak peak shape. Temperature of 55°C was appropriate for ensuring minimum adsorption and good resolution.

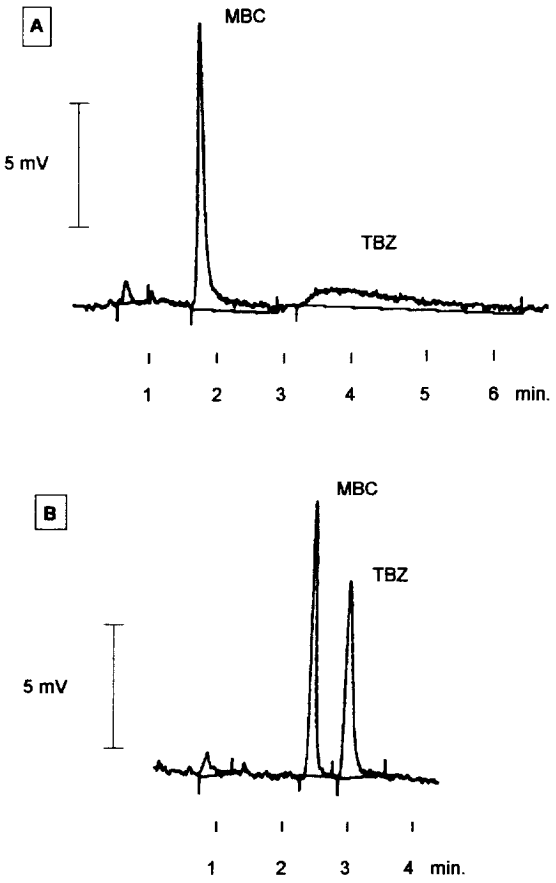

Fig. 2. Chromatograms (Att=3) of a 0.25 μg/ml solution of MBC and TBZ analysis on Picotag C_{18} column (A) from Waters (150× 3.9 mm I.D., 4 μm particle diameter) and on Kromasil C_{18} column (B) from Tecknokroma (150×4 mm I.D., 5 μm particle diameter). Chromatographic conditions: Methanol–water (50:50, v/v); 1 ml/min; 55°C; 285 nm.

3.1.3. Mobile phase and injection solvent composition

Different mobile phase compositions of water–methanol were tested in the range 50:50–70:30. Increase of water content involved increase of retention time, peak broadness and resolution. How-

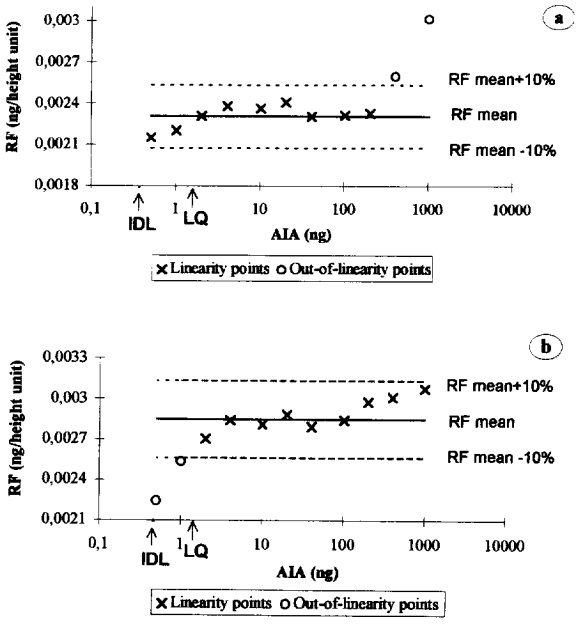

Fig. 3. Response factor (RF) vs. absolute injected amount (AIA) for MBC (a) and TBZ (b). LQ=Limit of quantification.

ever, peak shape remained symmetric. Thus, increasing water content could be useful for separating fungicide peaks from potential interfering matrix peaks, usually situated at the beginning of chromatograms. As the cleanup procedure reduced interfering peaks, 50% water content was selected, ensuring good resolution and better sensibility.

Besides, it was checked that injecting a solution of the same composition as the mobile phase brought about the best resolution, sensibility and peak shape.

3.2. Validation of chromatographic method

3.2.1. Linearity and instrumental detection limit

Linearity was studied for both MBC and TBZ standard solutions in the range 0.5–1000 ng absolute injected amounts (AIAs). Linearity ranges were determined using response-factor (RF) vs. AIA graphs, where RF is defined as the ratio AIA to peak height. Out-of-linearity points (open circles in Fig. 3) are those that get out of the zone delimited by the horizontal lines $RF_{mean} \pm 10\%$. Linearity ranges were 0.4–200 ng AIA for MBC and 2–1000 ng AIA for TBZ.

Calibration graphs – peak height vs. AIA – were constructed for both compounds in the previously-defined linearity ranges. MBC experimental points were perfectly represented by a single regression line in the whole linearity range. However two regression lines were necessary to represent TBZ experimental points in low and high AIAs (Table 1) due to the response-factor showed a slight decrease when the concentration diminished.

Instrumental detection limits (IDL) were calculated as the AIAs based on a signal-to-noise ratio 3:1. Mean of nine calculations determined carbendazim IDL at 0.45 ng AIA and thiabendazole IDL at 0.53 ng AIA. Limits of quantification (LOQs) for both compounds based on a signal-to-noise ratio 9:1 were included into linearity ranges.

3.2.2. Repeatability and reproducibility

Repeatability and reproducibility were studied with a 0.5 μg/ml solution of MBC and TBZ. Repeatability study allows checking of the precision of the chromatographic system. Eleven replicate injections were carried out within a day and relative standard deviation (R.S.D. %) were calculated for retention times, peak areas, peak heights and resolutions (Table 2). Results showed good repeatability of the chromatographic system. Higher value of TBZ peak-area R.S.D. was due to greater adsorption of TBZ (more basic than MBC) on stationary phase and slightly-tailing peaks had effect on peak integration. Thus, it would be preferable to quantify peaks through heights.

Reproducibility study allows checking of the

Table 1
Linear regression for MBC and TBZ in the linearity ranges (Height=$A+B\times$AIA)

Compound	AIA range (ng)	A	B	r
MBC	0.4–200	−21.11	430.30	1.0000
TBZ	0.5–20	54.11	345.07	0.9998
	20–1000	968.43	325.50	0. 9999

Table 2
Repeatability and reproducibility of the chromatographic method (R.S.D.%)

Parameter	Repeatability ($n=11$)		Reproducibility ($n=16$)	
	MBC	TBZ	MBC	TBZ
Retention time	0.13	0.18	1.20	1.66
Peak height	1.44	1.78	10.35	14.47
Peak area	1.91	4.01	2.20	3.08
Factor of symmetry	–	–	9.1	15.6
Resolution	5.95		4.23	

precision of the chromatographic system between days. Each day of the study ($n=16$), the same solution was injected three times. Between-run R.S.D.s (%) were calculated from the three-injection means of retention times, peak areas, peak heights, peak factors of symmetry and resolutions (Table 2). Results showed good reproducibility of the chromatographic system. Higher values of TBZ R.S.D.s (%) were also due to slightly tailing peaks, varying peak integration. However, for both compounds, peak height R.S.D.s (%) were higher than peak area R.S.D.s (%). Thus, it would be better to quantify between-day peaks through areas. Nevertheless, as chromatogram duration was short (<5 min), it was possible to inject lots of samples and standard solutions within a day.

3.3. Control charts

Control charts allow checking of the reliability and good performance of the chromatographic system. They were built for retention times, peak areas, peak heights, peak factors of symmetry and resolutions. Each day of the study ($n=16$), the same solution was injected three times. A resolution control chart is presented in Fig. 4 as an example.

3.4. Sample preparation

Sample extraction and cleanup procedure were adapted from Hiemstra [8] to our laboratory possibilities. Sample preparation presents the advantage of making extraction and liquid partitioning in organic phase in a same step. The use of a blender allows intensive mixing, creating microdroplets, which increase exchange surface. Thus, laborious conventional liquid–liquid partitioning can be substituted by a rapid technique, since a few minutes are enough to obtain the final organic phase. Moreover, the procedure only needs 60 ml organic solvents, which is economic and reduces manipulations of heavy volumes of toxic compounds.

Cleanup procedure was tested with standard solution of MBC and TBZ and with Isolute diol-bonded silica SPE cartridges (not utilised previously in this type of application), using the procedure described by Hiemstra that was recommended for Bond-Elut cartridges [8] (single preconditioning step of 2 ml methanol). Results were bad since recoveries were <10%. Fungicides were found in the washing fraction. To improve compound adsorption on solid-phase, a preconditioning step of 2 ml elution solution (methanol–0.1 *M* phosphoric acid, 50:50) was added before applying fruit extracts. Fortified orange extracts were used in order to check the performance of the SPE procedure. Recoveries were higher than 90% and repeatabilities were lower than 5% (Table 3). Probably, in dry solid-phase, the diol-bonded linear chains are very interlaced, permitting hydroxyl groups of diol function to interact with residual silanol groups of silica base. Acid seems to help

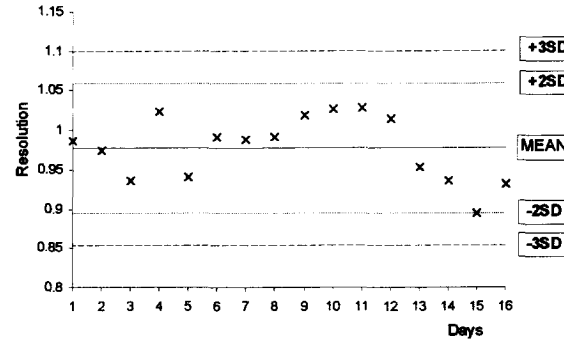

Fig. 4. Resolution control chart

Table 3
SPE performance applying fortified orange extracts[a]

Parameter	MBC	TBZ
Recovery (%)	103.1	104.5
Repeatability (R.S.D.%)	4.8	3.8

[a] Spiked level: 0.4 μg/ml. Six replicates.

weaken those polar bonds and to disentangle aliphatic chains.

Besides, it was checked that compounds remained adsorbed on the solid-phase with 3 ml of methanol during a washing step. However, the utilisation of volumes higher than 1 ml did not remove further unwanted matrix coextractives with orange and grape samples.

Diol-bonded silica SPE cartridges were used to purify orange and grape extracts. Fig. 5A shows orange extract before the cleanup procedure. It reveals matrix peaks able to interfere with benzimidazole peaks. Fig. 5B shows the same extract after the cleanup procedure, proving the great cleanup efficiency. The cleanup procedure is very selective for benzimidazole fungicides as shown in Fig. 5. Chromatograms obtained with grape samples confirm the good performance of the method. (Fig. 6).

3.5. Method validation

The whole procedure was validated for orange and grape samples fortified at levels 0.4 and 1.2 mg/kg.

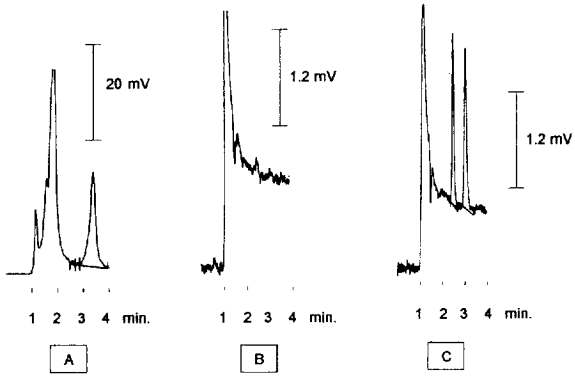

Fig. 5. Liquid chromatograms of an orange blank sample before (A, Att=5) and after (B, Att=1) SPE cleanup procedure and a fortified sample at 1.2 mg/kg of MBC and TBZ (C, Att=1).

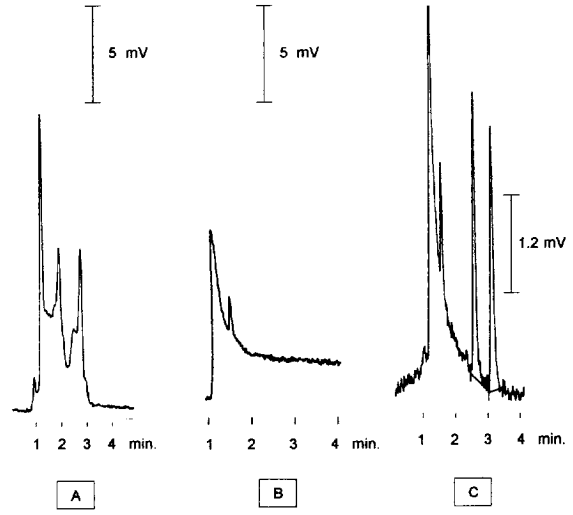

Fig. 6. Liquid chromatograms of a grape blank sample before (A, Att=3) and after (B, Att=3) SPE cleanup procedure and a spiked sample at 1.2 mg/kg of MBC and TBZ (C, Att=1).

Therefore, homogenised fruit samples were spiked with adequate acetone solution volume before extraction in total 20 ml of acetone. Up to six replicate analyses were run at both fortification levels and for both matrixes tested. Overall average recoveries were >75% for MBC and >80% for TBZ. R.S.D.s (%) for MBC and TBZ were 2.6–11.6% and 2.0–9.9% respectively (Table 4). Method detection limit were about 0.06 mg/kg and was calculated as the concentration resulting in a response equivalent to three times the noise detected. This values are lower than Spanish tolerance limits for MBC and TBZ in fruit. These detection limits of the procedure also allow an analysis of residues according to maximum European limits in vegetables and fruits.

Benomyl is totally converted into MBC applying the proposed method and their quantification has to be performed as MBC.

4. Conclusions

An analytical method was developed for determination of MBC and TBZ at low-ppm levels in fruit. The laborious procedure containing conventional liquid–liquid partitioning was replaced by selective and fast SPE cleanup procedure using Isolute diol-

Table 4
Average recoveries, method detection limit (MDL), Spanish (STL) and European (ETL) tolerance limits for MBC and TBZ

	MBC		TBZ	
	1.2 mg/kg (n=6)	0.4 mg/kg (n=3)	1.2 mg/kg (n=6)	0.4 mg/kg (n=3)
Orange				
Recovery (%)	75.5	101	85.9	89.2
Repeatability (R.S.D.%)	3.6	7.8	5.1	9.9
Limit of detection (mg/kg)	0.057		0.068	
Tolerance limits (mg/kg)	STL and ETL: 5		STL and ETL: 6.0	
Grape	n=4	n=3	n=4	n=3
Recovery (%)	75.1	77.1	81.9	95. 7
Repeatability (R.S.D.%)	2.6	11.6	2.0	6.4
Limit of detection (mg/kg)	0.052		0.065	
Tolerance limits (mg/kg)	STL: 5/ETL: 3		STL: 2.0/ETL: 0.1	

bonded silica cartridges. Benzimidazole fungicides could have been analysed by RP-HPLC without adding modifiers into mobile phase, thanks to the Kromasil stationary phase never reported in previous studies about benzimidazol fungicide analysis. Recoveries were satisfactory (>73%) and method limits of detection were about 0.06 mg/kg, lower than legal tolerance limits.

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