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Simplified clean-up for the determination of benzimidazolic fungicides by high-performance liquid chromatography with UV detection

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Abstract

A method was developed that allows the determination of benomyl, carbendazim (MBC), thiophanate methyl (TFM) as carbendazim and thiabendazole (TBZ) by HPLC with UV detection. After extraction and cyclization of TFM into MBC, the conversion of benomyl into MBC is carried out by absorbing the raw extract on a ready-to-use, disposable column of a macroporous Kieselghur-type material and percolating 0.1 *M* HCl through it. Benzimidazolic residues are partitioned into the acid solution whereas most of the co-extractives are retained on the column. The final clean-up is performed on a strong cation-exchange (SCX) cartridge. The determination of MBC and TBZ is carried out by HPLC–UV detection on a polymeric reversed-phase column eluted with a water–acetonitrile (70 : 30). Recoveries of MBC and TBZ from pear, apple, orange, grape, kiwi, tomato and lettuce, spiked at levels of 0.22 and 0.88 mg/kg, were satisfactory (>70%). The main features of the method include high selectivity towards MBC and TBZ, reduced consumption of reagents and solvents, reduced handling operations, lack of emulsions and the use of disposable items.

1. Introduction

Benzimidazolic compounds are widely used in agriculture as both field and post-harvest fungicides. The main compounds in use are thiabendazole (TBZ), benomyl, carbendazim (MBC) and thiophanate methyl (TFM). As the last three compounds are intercorrelated, having MBC as common metabolite and major fungitoxic principle, a single maximum residue limit (MRL) is generally set for this group of three compounds.

The problems associated with the analysis of this class of compounds have been dealt with in two reviews [1,2].

In almost all methods, benomyl is readily converted during the analytical procedure into MBC by dilute acid treatment, either in the extraction stage [3], when hydrophilic extraction solvent is used, or later in the procedure by shaking the solvent containing the analyte with a dilute acid solution in a separating funnel.

TFM is not so easily converted into MBC and two approaches have been pursued, i.e., determination of TFM per se [3,4] or conversion into MBC with a dedicated step. Two main reactions have been reported: (i) treatment with 20% ammonia solution in dimethylformamide (DMF) at 80°C for 1 h [5] and (ii) treatment with 50% acetic acid solution and copper(II) acetate [6].

After conversion of benomyl and TFM into MBC, a clean-up for the two main species that

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remain, i.e., MBC and TBZ, has to be set up. However, irrespective of the approach followed, the methods for the four compounds consist of lengthy, complex procedures, in which, taking advantage of the ionizable structure of MBC and TBZ, the clean-up is mostly based on a series of solvent–basic solution and solvent–acidic solution partitions carried out in separating funnels [4,5,7–9].

Although TBZ can be determined as such by gas chromatography (GC) with nitrogen–phosphorus detection (NPD) on capillary columns [10] and MBC by GC with NPD after acetylation [8] or with electron-capture detection (ECD) after trifluoroacetylation [11] or pentafluorobenzylation [7], high-performance liquid chromatography (HPLC) with UV detection [6] or UV followed by fluorescence detection [3,9] is advisable for analysing ionizable species and setting a single procedure for both MBC and TBZ.

However, the reported HPLC conditions require solvent modifiers such as a buffer [12] or ion-pairing reagent [9] in reversed-phase or acid [4] or ammonia solution [3] in normal-phase operation to improve the peak shape and/or resolution.

The aim of this work was to develop a simplified procedure, in which the clean-up is shortened and uses reduced amounts of reagents and glassware and the determination is based on simpler HPLC conditions.

2. Experimental

2.1. Reagents and materials

Ethyl acetate, dimethylformamide (DMF), ammonium formate, 20% ammonia solution (0.92 g/ml) and anhydrous sodium sulphate were of analytical-reagent grade. Hydrochloric acid (0.1 *M*) and sodium hydroxide solution (1 *M*) were used. Acetonitrile, methanol and water were of HPLC grade. Ammonium formate buffer (pH 6.8) was prepared as 0.1 and 5 *M* solutions. Celite 545 was obtained from BDH (Poole, UK). An Extrelut-3 column with an exit needle was supplied by Merck (Darmstadt, Germany). Bakerbond SPE aromatic sulphonic acid was purchased from Baker (Phillipsburg, NJ, USA). Acetone-washed glass-wool was used.

Pesticide reference standards from the collection in this laboratory were kindly supplied by the main manufacturer of the pesticides and were >99% pure.

2.2. Apparatus

HPLC analyses were carried out on a Hewlett-Packard Model 1050 instrument consisting of an autosampler (set to inject 20 μl), a pumping unit, a UV spectrophotometric detector and, in series, a Hewlett-Packard Model 1046A spectrofluorimetric detector. Chromatograms were recorded on Hewlett-Packard Model 3396 II integrators.

The HPLC conditions were as follows: HPLC column Asahipak ODP-50 (125 × 4.0 mm I.D.) (Hewlett-Packard) with a precolumn (Merck) (30 × 4 mm I.D.) filled with Perisorb RP-18, 30–40 μm (Merck); eluent, water–acetonitrile (70 : 30, v/v); column oven temperature, 40 °C; UV detector, set at 280 nm; spectrofluorimetric detector, excitation at 280 nm, emission at 310 nm, cut-off filter 280 nm.

An Omni-Mixer homogenizer was obtained from Sorvall (Norwalk, CT, USA) and a rotary evaporator from Büchi (Switzerland).

2.3. Procedure

Extraction

Weigh into the homogenization vessel 50 g of vegetable and add, in this order, 5 g of Celite, 5 ml of ammonia solution and 150 ml of ethyl acetate. Mix with a glass rod and add 25 g of Na_2SO_4 in small portions while stirring. Homogenize at medium speed for 20 min. Filter through glass-wool into a 500-ml erlenmeyer flask. Repeat the extractions with ethyl acetate (2 × 75 ml), filter, combine the ethyl acetate phases and concentrate almost to dryness with a rotary evaporator.

Cyclization of TFM into MBC

Transfer the crude extract to a 50-ml round-bottomed flask, using small portions of ethyl acetate to wash the erlenmeyer flask. Concentrate to dryness, dissolve the residue in 10 ml of DMF and add 1 ml of ammonia solution. Attach a water-cooled condenser and keep in a water-bath at 80°C for 1 h. Cool to room temperature, then pour the contents into a 250-ml separating-funnel containing 10 ml of 1 *M* NaOH. Wash the flask three times with 30, 30 and 10 ml of water, collecting the washings in the separating funnel. Mix and carry out three extractions with 75, 50 and 50 ml of ethyl acetate. Wash the combined ethyl acetate phases with 10 ml of water and discard the water. Collect the ethyl acetate in a 300-ml erlenmeyer flask and concentrate almost to dryness.

Conversion of benomyl into MBC

Dissolve the crude extract resulting at the end of either the extraction or cyclization step with 3 ml of ethyl acetate. Transfer the solution on to an Extrelut-3 column, allow the solvent to be absorbed into the bed and wait for 10 min to obtain an even distribution. Pass nitrogen through the cartridge, from bottom to top, at 0.5 l/min for 20 min, then disconnect the nitrogen.

Clean-up

Precondition a sulphonic acid cartridge by passing 2 × 1-ml portions of methanol and 2 × 1-ml portions of 0.1 *M* HCl (use a vacuum manifold). Position the Extrelut-3 column over the sulphonic acid cartridge and elute the column by passing 6 × 5-ml portions of 0.1 *M* HCl, using each portion to wash the erlenmeyer flask that had contained the ethyl acetate extract. Allow the acid solution draining from the Extrelut-3 column to pass through the cartridge so as to maintain the SCX cartridge wet.

Remove the Extrelut-3 column, wash the sulphonic acid cartridge with 2 × 1 ml of water, 3 × 2 ml of CH_3OH–H_2O (75 : 25, v/v) and 3 × 2 ml of CH_3OH–0.1 *M* ammonium formate buffer (pH 6.8) (50 : 50, v/v) and discard all the washings.

Elute the SCX cartridge with 1 × 2 ml of CH_3OH–5 *M* ammonium formate buffer (pH 6.8) (75 : 25, v/v) at ca. 0.4–0.5 ml/min to recover the MBC and TBZ residues.

Determination

Inject the solution resulting from the clean-up into the HPLC apparatus. Compare the chromatographic response (peak retention times, heights or areas) with that of standard solutions of MBC and TBZ and calculate residue amount.

2.4. Recovery experiments

Recovery trials were carried out by adding known amounts of the analytes to the selected vegetables and comparing the amounts found with the amount added.

3. Results and discussion

Most of current methodologies for benzimidazolic fungicides in vegetables rely on complex procedures based on separating funnel partitions. The main aim of this work was to develop a simplified method composed of steps carried out on disposable items. The extraction and cyclization steps were taken from the work by Gnaegi et al. [5] and no attempt was made to improve them.

Steps that have been improved are the conversion of benomyl into MBC, clean-up and determination. Based on our previously reported [13] application of a solid-matrix column in the clean-up of vegetable extracts for fungicide residue determination, we have developed a procedure for the conversion of benomyl into MBC in a single step by treating with dilute acid the raw extract dispersed on a macroporous Kieselghur-type material contained in a ready-to-use, disposable glass column.

This step offers substantial advantages over the classical separating funnel partitioning of the extract between ethyl acetate and 0.1 *M* HCl. These advantages include a straightforward operation with lack of emulsions, reduced consumption of solvents, reduced handling operations and the use of disposable items. After dispersing

the raw extract solution over the Extrelut material, most of ethyl acetate is removed by passing nitrogen through the column, from bottom to top. In this way, the co-extractives adhere to the solid particles and the major part is retained on the column because the co-extracted material is poorly soluble in diluted HCl.

On the other hand, the high surface area of the material ensures a high degree of dispersion and, hence, good efficiency of transfer of MBC and TBZ into the dilute HCl. Thus, in a single step, the Extrelut-3 column performs the conversion of benomyl into MBC and effects a partial clean-up of the extract without any possibility of emulsions, such as those frequently occurring in separating funnel operations. In Fig. 1 is shown the rate of removal of ethyl acetate from the Extrelut-3 column with different nitrogen flow rates. With the parameters chosen (0.5 l/min for 20 min) ca. 20% of the ethyl acetate remains on the column.

Although with complete removal (say 1 l/min for 20 min) of ethyl acetate the recoveries of MBC and TBZ were satisfactory, we preferred to leave some ethyl acetate on the column so that a thin film of solution, rather than a thin film of hydrophobic waxy material, surrounds the solid particles and, being more wettable, offers less resistance to the partition of the analytes into the dilute HCl.

The yield of conversion of benomyl into MBC was determined by applying 2 ml of standard solution containing 5.25 μg/ml of benomyl in ethyl acetate to the Extrelut-3 column and running the method as described from conversion to clean-up and determination. The conversion yields were between 80% and 85% ($n = 3$). Recoveries of benomyl added alone to green-red tomatoes and cucumber homogenates at levels of 0.22 and 0.88 mg/kg and analysed according to the whole procedure were in the range 83–88% ($n = 3$).

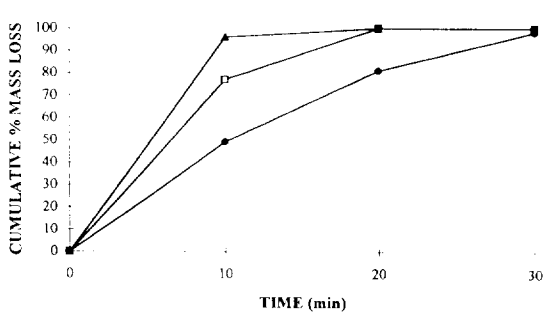

Fig. 1. Cumulative percentage mass loss of Extrelut-3 cartridges loaded with 3 ml of acetate using different nitrogen flow-rates: ● = 0.5; □ = 1.0; ◆ = 2 ml/min.

As both MBC and TBZ are in cationic form at low pH, we thought that this could offer a fairly good way to isolate the analytes of interest from the majority of other co-extracted material. We therefore used a strong cation-exchange (SCX) cartridge to reconcentrate MBC and TBZ residues from the volume of diluted acid used to elute them from Extrelut-3 column in a manner similar to that reported by Leenheers et al. [14].

As the SCX we chose is a silica–propylphenylsulphonic acid, in addition to the cation-exchange properties it also shows a mechanism of retention based on hydrophobic interaction. Hence neutral lipophilic material can be washed out of the cartridge by increasing the methanol content of water–methanol mixtures, while cationic species can be eluted by increasing the salt concentration of the buffer solution.

At least 30 ml of 0.1 *M* HCl are necessary to elute MBC and TBZ from the Extrelut-3 column. By passing this solution through the SCX cartridge and analysing the draining acid, we proved that the analytes are completely retained. After washing with water to remove the excess acid, the SCX cartridge was washed with a methanol–water (75 : 25, v/v) to remove unwanted lipophilic material.

Attempts to increase further the methanol content proved unsuccessful as the subsequent elution scheme is altered, perhaps because the ionic environment of the SCX particles is upset. We therefore applied a wash with a buffer of low ionic strength [methanol–0.1 *M* ammonium formate buffer (pH 6.8) (50 : 50, v/v)] to remove slightly retained ionic species; the content of methanol and the ionic strength were increased to the point where MBC and TBZ were still retained on the cartridge.

At a 0.1 *M* buffer concentration, MBC and TBZ start to elute on increasing the methanol

content of the eluting mixture to 75%. However, as can be seen in Fig. 2, under these conditions three 2-ml fractions are needed to elute the analytes completely. Therefore, we gradually increased the NH_4^+ concentration to 5 *M* in order to elute MBC and TBZ in only one 2-ml fraction and have better sensitivity.

To establish the performance of the described method, some vegetables, including pears, apples, oranges, grapes, kiwis, red tomatoes and lettuce, were spiked at levels of 0.22 and 0.88 mg/kg with both MBC and TBZ and processed according to the procedure. Recoveries are reported in Table 1 and are considered satisfactory.

Further, a few samples with incurred residues of benzimidazolic fungicides were analysed according to Ref. [5] (extraction with ethyl acetate, cyclization, acid–base partitioning, spectro-

Table 1
Mean recoveries values of MBC and TBZ added at different levels to different crops analysed according to the proposed method (without cyclization) and determined by HPLC–UV detection

Crop	Mean recovery (%)[a]			
	0.22 mg/kg[b]		0.88 mg/kg[b]	
	MBC	TBZ	MBC	TBZ
Pear	84	90	81	88
Apple	73	93	75	90
Orange	73	95	77	91
Grape	84	79	85	87
Kiwi	83	75	83	78
Red tomato	89	84	87	85
Lettuce	84	80	82	83

[a] $n = 3$.
[b] Spiking levels.

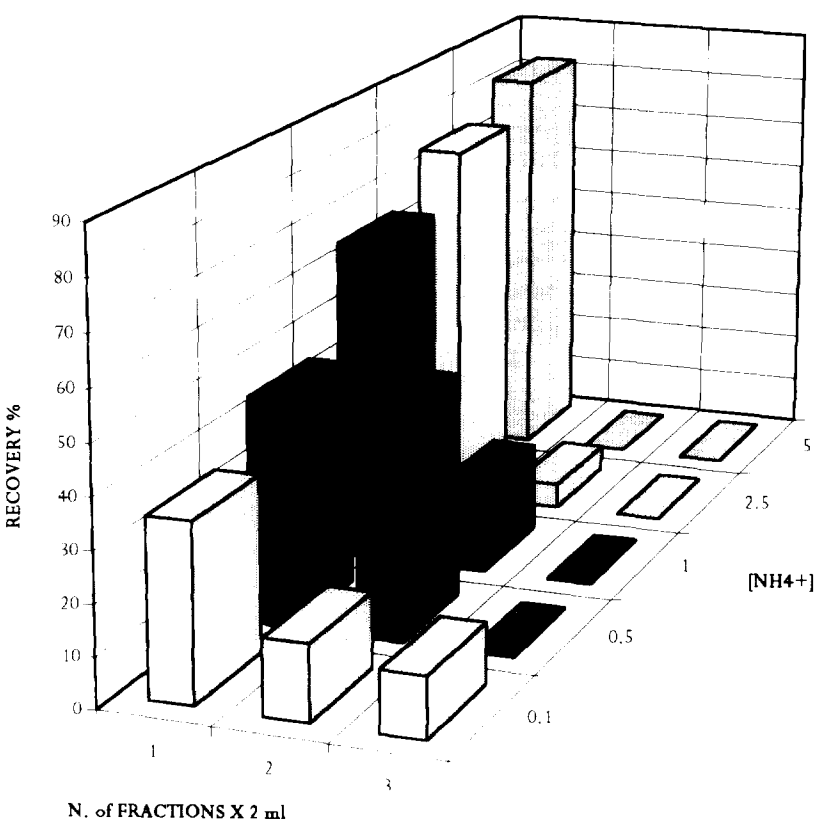

Fig. 2. Recovery of MBC from silica–aromatic sulphonic acid cartridge eluted with methanol–ammonium formate buffer (pH 6.8) (75 : 25, v/v) with increasing ionic strengths.

photometric determination, i.e., the method used previously in our laboratory) and the present HPLC–UV method. The results reported in Table 2 show reasonable agreement between the two series of determinations; where the values obtained by the present method are lower, this may be attributed to the more critical removal of the interferences in the reference method based on separating funnel partitioning and to the lower selectivity of spectrophotometry compared with HPLC–UV determination.

Although we did not determine specifically the maximum concentration levels for the different analytes to be retained on the SCX cartridge, the data reported in Table 2 show that the present method gave comparable results to the reference method up to 6 mg/kg, thus indicating that at least up to 300 μg of MBC can be retained by the SCX cartridge.

We also tried some C_{18} HPLC columns, but with unsatisfactory results for peak shape and recovery. We subsequently found that with a polymeric reversed-phase column MBC and TBZ could be eluted isocratically as symmetrical peaks with water–acetonitrile (70 : 30) without a solvent modifier, thus simplifying the chromatographic conditions. More recently we tried a Phenomenex Ultracarb 5 ODS column eluted with water–acetonitrile (50 : 50, v/v) and obtained good peak shapes similar to those reported by Lòpez et al. [15] with a Spherisorb ODS-1 column. Fig. 3 shows representative chromatograms of pear "blank", spiked pear sample and standard compounds, which show the good quality of the clean-up. In the unspiked vegetables matrices analysed, a small interfering peak was observed only with pear samples at the retention time of TBZ and at a level equivalent to ca. 0.03 mg/kg.

Table 2
Comparison of results obtained on analysing samples with incurred residues of benzimidazolic fungicides according to Ref. [5] and the present method

Crop	MBC content (mg/kg)	
	Ref. [5]	Present method
Courgette	0.36	0.21
Grape	1.20	1.10
Grape	2.90	1.80
Grape	6.00	5.40
Grape	2.40	0.60
Grape	2.00	1.70
Grape	2.90	3.00

Although the method may not appear simple, one should consider that it has the unique feature that it covers simultaneously four compounds, i.e., benomyl, MBC, TFM and TBZ. If the determination of TFM is not sought (cyclization omitted), the present method is simplified in comparison with the previous methods that mostly use time-consuming separating funnel separations. The preparation of a single sample takes ca. 4 h if cyclization is omitted. However, owing to the solid-phase operation, several samples can be processed in parallel.

Quantification was carried out via the UV detector response with reference to an external standard of comparable concentration. The HPLC–UV response was found to be linear between 0.69 and 44 μg/ml for both MBC and TBZ ($r^2 = 0.996$ and 0.995, respectively), corresponding to a range of quantification of 0.01–0.88 mg/kg. Higher concentrations were not assayed as more concentrated solutions are not readily prepared in acetonitrile. Hence, if needed, it is advisable to dilute the final extract to have the analytes in this range of concentration. Although widely different levels of the analytes can occur in field samples (say 5–6 mg/kg in the case of misuse), the most interesting range to explore is up to 1 mg/kg (i.e., the most frequently accepted MRL) in order to have the possibility of either assessing the compliance of the sample with the MRL or ruling out the presence of residues.

The spectrofluorimetric detector was added as a confirmatory technique and gave chromatograms and quantitative results similar to those with the UV detector.

As the aim of this work was to develop a clean-up procedure, the parameters for the fluorimetric detector were chosen as a compromise between MBC and TBZ and were set to give the same response as the UV detector.

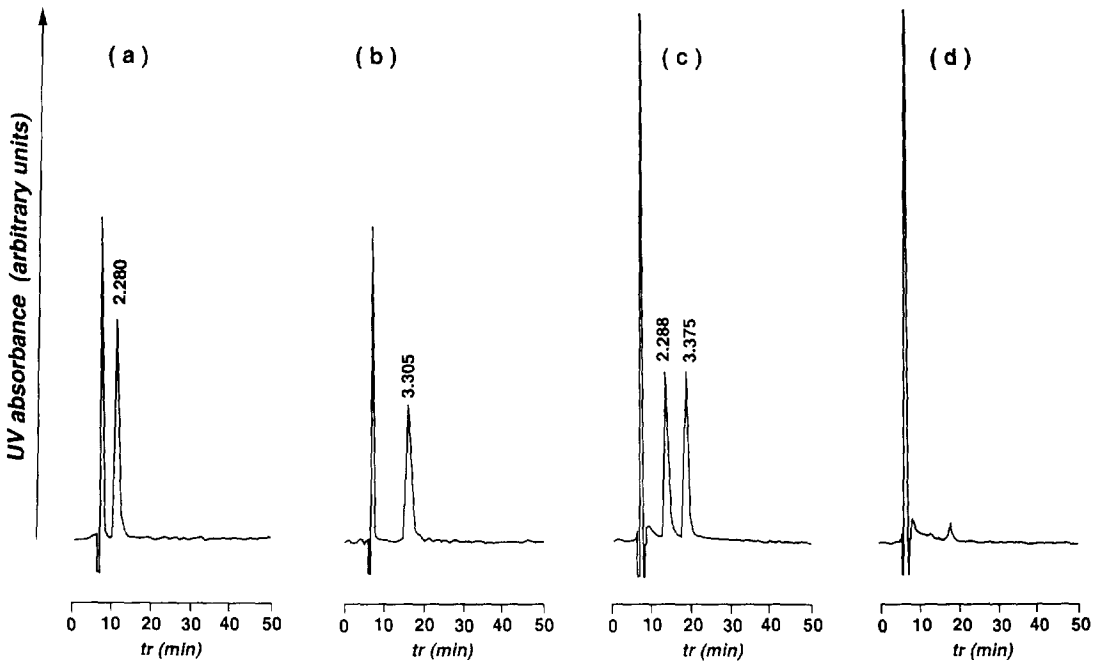

Fig. 3. Chromatograms with UV detection at 280 nm of (a) MBC standard solution, 5.5 μg/ml, t_R = 2.280 min; (b) TBZ standard solution, 5.5 μg/ml, t_R 3.305 min; (c) pear sample spiked with MBC and TBZ, 0.2 mg/kg each; (d) pear sample, same as (c) but not spiked. For conditions, see text.

Also, the UV wavelength was the optimum for MBC, not for TBZ. However, we chose this wavelength to have the same response at the same spiking level for the two compounds.

4. Conclusions

A method has been developed that allows the simultaneous determination of four benzimidazolic fungicide residues in several vegetables. The method involves ethyl acetate extraction under basic conditions, cyclization of TFM into MBC (which can be omitted if TFM is not sought), conversion of benomyl into MBC by dilute acid treatment of the raw extract adsorbed on a column of macroporous siliceous material, where also a partial clean-up occurs, a final clean-up by strong cation-exchange chromatography and HPLC–UV determination on a reversed-phase polymeric column simply eluted with water–acetonitrile (70 : 30, v/v).

The main features of the method include a clean-up based on simple operations and the use of disposable cartridges, with substantial savings of glassware, reagents and time compared with most existing methods. The clean-up is very selective towards MBC and TBZ and virtually no interfering peaks were observed in the chromatograms of the analysed crops. The recoveries values of MBC and TBZ from representative crops (pear, apple, orange, grape, kiwi, tomato and lettuce) were satisfactory (>70%) at spiking levels between 0.22 and 0.88 mg/kg.

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