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Short Communication

Liquid chromatographic determination of 2-hydroxy-3-aminophenazine and 2,3-diaminophenazine as impurities in pesticide formulations containing benomyl or carbendazim

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ABSTRACT

Highly mutagenic 2-hydroxy-3-aminophenazine (HAP) and 2,3-diaminophenazine (DAP) are possible impurities present in technical samples of the pesticides carbendazim and benomyl A liquid chromatographic method with electrochemical detection was developed for the determination of HAP and DAP in technical or formulated samples of these pesticides The method involves sample clean-up by strong cation-exchange solid-phase extraction prior to high-performance liquid chromatographic analysis The method is relatively simple and can be used for analytical control of the contents of HAP and DAP impurities in pesticide products

INTRODUCTION

Carbendazim and benomyl (Fig 1, **I** and **II**, respectively) are fungicides that are used as active ingredients in pesticide formulations The structures of these compounds are closely related and benomyl is, in fact, thought to act biologically as a precursor of carbendazim [1] For some years it has been known that the mutagenic effects displayed by carbendazim and benomyl [2,3] are caused by the presence of phenazine impurities in the technical active ingredients, namely 2-hydroxy-3-aminophenazine (HAP) and 2,3-diaminophenazine (DAP) (Fig 1, **III** and **IV**, respectively) [4,5]

According to information provided by DuPont, which produces the active ingredients, the Ames test for mutagenic effect is negative if the total content

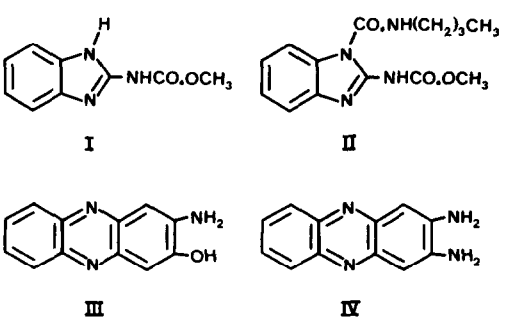

Fig 1 Structures of the active ingredients carbendazim (**I**) and benomyl (**II**) and those of the two impurities 2-hydroxy-3-aminophenazine (**III**) and 2,3-diaminophenazine (**IV**)

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of HAP and DAP impurities in the active ingredients is below 3 5 ppm For authority control of the pesticide products available in Denmark, a method was needed by which the content of these two impurities in carbendazim and benomyl formulations could be determined The method should have a detection limit below 3 5 ppm relative to the content of active ingredient (benomyl or carbendazim) in the formulation The only published method for the determination of HAP and DAP in technical carbendazim and carbendazim-containing formulations is that of Van Damme *et al* [6], which is a normal-phase high-performance liquid chromatographic (HPLC) method with UV–VIS absorption detection at 453 nm The method proposed in this paper is a reversed-phase HPLC method with electrochemical detection, which offers increased sensitivity compared with absorption detection Further, the method is also applicable to formulations containing benomyl as the active ingredient

EXPERIMENTAL

Reagents

Phosphoric acid (85%) (Merck, Germany), sodium hydroxide (Merck) and triethylamine (Fluka, Switzerland) were of analytical-reagent grade and methanol (Merck) was of gradient grade All chemicals were used as received Water was purified with a Milli-Q purification system (Millipore, USA) 2,3-Diaminophenazine and 2-hydroxy-3-aminophenazine were received as a gift from Dupont

Apparatus and chromatographic conditions

A Bransonic 52 ultrasonic bath (Branson), an Omnifuge centrifuge (Heraeus-Christ), 0 5-μm pore-size Fluoropore filters (Millipore) and Bond Elut strong cation-exchange (SCX) solid-phase extraction columns (50 mg) (Analytichem) were used

The HPLC system consisted of a Waters Model 510 pump equipped with a Waters Model 712 WISP autosampler, a BAS LC-4 electrochemical detector (Bioanalytical Systems) and a D-2000 Chromato-Integrator (Hitachi) The applied detector voltage was +0 75 V *vs* Ag/AgCl reference electrode The analytical column was a Novapak C_{18} (15 cm × 3 9 mm I D), 4-μm particle size (Waters) The column temperature was maintained at 30 0 ± 0 1°C using a laboratory-made LC column thermostat The mobile phase was 0 050 *M* phosphoric acid–0 025 *M* triethylamine, adjusted to pH 6 0 with 2 *M* NaOH–methanol (68 32, v/v), at a flow-rate of 1 0 ml/min

Preparation of standards

Standards were protected from light during preparation and analysis Stock solutions of HAP and DAP were prepared separately by dissolving 5 0 mg of the compound in 50 ml of methanol Volumes of 1 0 ml each of the HAP and DAP stock solutions were mixed and made up to 10 ml with methanol The stock solutions and mixed stock solutions were stable for at least 2 months when stored at 4°C A calibration standard solution, that was used for HPLC analysis, was prepared by diluting 100 μl of the mixed stock solution to 50 ml with mobile phase A fresh calibration standard solution was prepared each day

Preparation of samples

Samples were protected from light during preparation and analysis An amount of sample corresponding to 300 mg of active ingredient was weighed and extracted with 10 0 ml of methanol by ultrasonic treatment for 15 min Following centrifugation (*ca* 1000 *g*, 10 min) a 3 0-ml aliquot of the supernatant was diluted with 6 0 ml of 0 2 *M* phosphoric acid If necessary the mixture was filtered and 6 0 ml of the filtrate were applied to a Bond Elut SCX column, pre-equilibrated with 3 0 ml of 0 2 *M* phosphoric acid The column was washed with 3 x 3 0 ml of 0 2 *M* phosphoric acid followed by 3 0 ml of water The column was then eluted with 9 0 ml of 0 15 *M* sodium phosphate buffer (pH 9 0)–methanol (65 35) The eluate was made up to 10 0 ml with mobile phase and analysed by HPLC

RESULTS AND DISCUSSION

In order to select the optimum detection conditions for the HPLC of HAP and DAP, a comparison between UV–VIS absorbance detection and electrochemical detection was made Absorption spectra (200–600 nm) and response *vs* applied voltage curves for HAP and DAP were obtained and are shown in Fig 2 Under optimum detection conditions (electrochemical detection at +0 75 V applied voltage and absorbance detection at 254 nm), the signal-to-noise ratio was 10–15 times higher us-

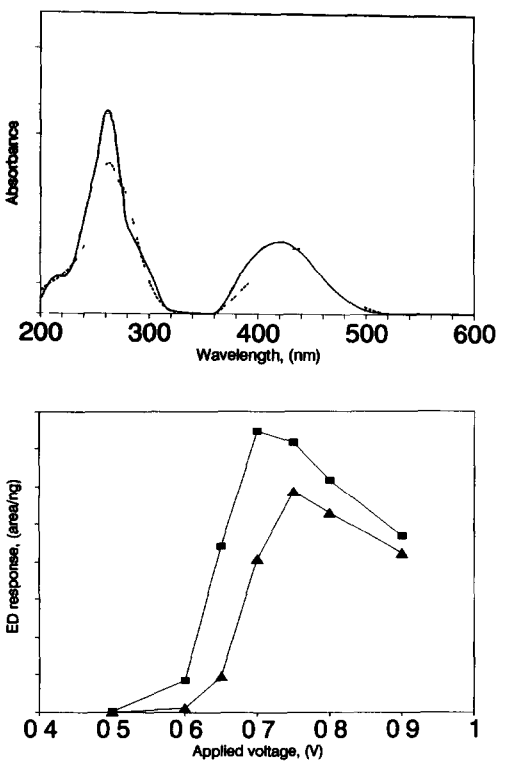

Fig 2 Top UV–VIS absorption spectra of (dashed line) HAP and (solid line) DAP, obtained on solutions of HAP and DAP in the HPLC mobile phase Bottom electrochemical detection response of (■) HAP and (▲) DAP as a function of applied voltage Other HPLC conditions as described in the text

ing electrochemical detection than using absorbance detection Hence electrochemical detection was preferred

HAP and DAP are very soluble in polar solvents such as water and methanol, but less soluble in less polar organic solvents such as dichloromethane and benzene [6,7] Methanol was chosen as the extraction solvent because preliminary experiments indicated that the stability of HAP and DAP was better in methanolic than in aqueous solution It was found that HAP and DAP degraded when they were exposed to light, and that degradation was faster in aqueous than in methanolic solution, in agreement with the findings of Van Damme *et al* [6] In order to prevent degradation of HAP and DAP, the samples were protected from light during sample preparation and analysis

A clean-up step before HPLC analysis was found to be necessary, because sample matrix components (especially when analysing suspension formulations) interfered with the electrochemical detector signal of the analytes Two different types of solid-phase extraction columns (C_{18} and SCX) were investigated Allthough C_{18} columns showed an acceptable retention of the analytes, the interfering matrix components were also retained on the column and it was not possible to find a water–methanol composition of the eluting solvent that selectively eluted the analytes

Strong-cation exchange columns were preferred because selective retention of the analytes (in the protonated ionic form) was achieved, with the possibility of washing sample matrix interferences through the column The sample solution was therefore acidified with phoshoric acid prior to application to the SCX clean-up column Elution of the analytes from the column was done by changing to an eluting solvent of increased pH (9 0) and containing 35% methanol Addition of methanol to the eluting solvent was found to be necessary to optimize the elution effiency, probably because of some non-ionic adsorption of HAP and DAP on the column

Linearity, precision and detection limits

Linearity was investigated by analysing a series of dilutions (six concentrations) of HAP and DAP standards The relationship between peak area and HAP and DAP concentration was linear (correlation coefficients 0 9999 and 0 9997, respectively) over the concentration range 10–200 ng/ml for both compounds

The precision of the method was evaluated by triplicate analysis of several different technical samples (TC), suspension concentrate (SC) and wettable powder (WP) formulation samples The results are shown in Table I As no authentic samples were available that contained the phenazine impurities, all the samples used for evaluation of the precision of the method were spiked samples A typical chromatogram from the analysis of a spiked sample is shown in Fig 3 The precision of the method was 5–7% for determination of both HAP and DAP in the various types of samples

Detection limits (calculated as injected amount of analyte with a signal-to-noise ratio of *ca* 3) was

TABLE I

PRECISION OF THE METHOD

Sample type[a]	Active ingredient	Analyte	Content[b], ppm	R S D[c] (%)	
				HAP	DAP
TC	Carbendazim	HAP	7 8	6 8	
		DAP	9 4		1 2
TC	Benomyl	HAP	6 3	6 6	
		DAP	6 7		3 8
SC	Carbendazim	HAP	1 6	3 2	
		DAP	1 8		9 4
SC	Carbendazim	HAP	2 1	11 1	
		DAP	2 3		1 9
WP	Benomyl	HAP	2 8	5 3	
		DAP	2 2		9 6
Mean				6 6	4 5

[a] TC = technical, SC = suspension concentrate, WP = wettable powder
[b] Mean of triplicate analyses
[c] Relative standard deviation

0 10 ng for both HAP and DAP, corresponding to a content of 0 3 ppm relative to the content of active ingredient in a typical sample (containing 50% of active ingredient) Detection limits reported by Van Damme *et al* [6] were 0 4 and 0 8 ppm, respectively

Recovery

The recovery of the method was evaluated by analysing samples before and after addition of known amounts of HAP and DAP Two spike levels of each of the different sample types were investigated and the results are shown in Table II The recoveries ranged between 62% and 112% with a mean recovery for both compounds of *ca* 80% The low and variable recovery for the method may be ascribed to two different parameters non-ionic adsorption of the two analytes on the SCX cartridge column, in spite of the optimized elution conditions, and the inherent limited photostability of the analytes Significant variation of the recovery within a sample type was seen, which might be explained as resulting from variations in the adsorption characteristics of the solid-phase extraction columns Despite the low recovery, the method was considered acceptable for the purpose of analytical control of the contents of HAP and DAP in pesticide formulations in relation to given limits

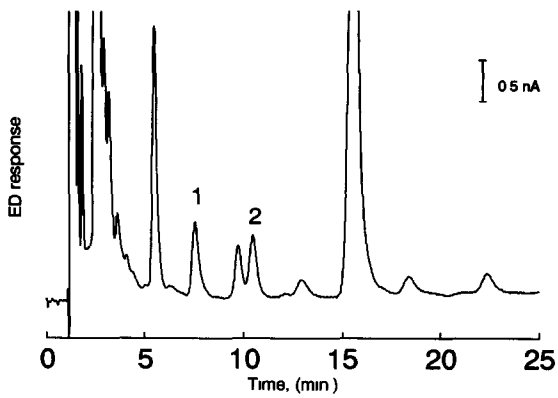

Fig 3 Typical chromatogram of a sample (suspension concentrate formulation containing carbendazim as active ingredient) spiked with HAP (1) and DAP (2) The contents of HAP and DAP in the spiked sample were 1 56 and 1 84 ppm, respectively

Application

The method has been used as a part of a authority control programme monitoring pesticide products available in Denmark Seven different formulation samples (five containing carbendazim and two containing benomyl as the active ingredient) were

TABLE II

RECOVERY DATA

Sample type[a]	Active ingredient	Spike level (ppm)		Recovery[b] (%)	
		HAP	DAP	HAP	DAP
TC	Carbendazim	5 0	5 0	90	74
		50	50	94	86
TC	Benomyl	5 0	5 0	79	83
		50	50	65	65
SC	Carbendazim	2 5	2 5	65	74
		25	25	93	107
SC	Carbendazim	2 5	2 5	87	88
		25	25	78	88
WP	Benomyl	2 5	2 5	90	72
		25	25	63	71
WP	Benomyl	2 5	2 5	112	95
		25	25	62	71
Mean				82	81

[a] TC = technical, SC = suspension concentrate, WP = wettable powder
[b] Means of duplicate analyses

analysed In all of the samples the contents of HAP and DAP were below the detection limit of the method Two plausible explanations for this can be given the contents of the synthesis impurities HAP and DAP in the active ingredients were initially very low or the impurities had degraded during the period between synthesis of the active ingredient and analysis of the commercial formulation As discussed by van Damme *et al* [6], who also were unable to detect HAP and DAP in newer formulations, it is very likely that any HAP or DAP initially present in the technical active ingredient will degrade during formulation production or storage, especially when formulations (such as SC formulations) contain water

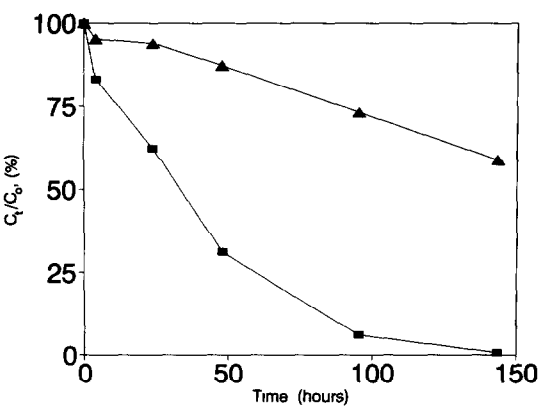

Fig 4 Stability of (■) HAP and (▲) DAP A sample of a suspension concentrate formulation containing carbendazim as the active ingredient was spiked with HAP and DAP (initial concentrations C_0 13 1 and 16 0 ppm, respectively) and stored at room temperature protected from light At different times during storage, the concentrations (C_t) of HAP and DAP in subsamples were determined (single determinations) as described in the text

This is demonstrated by the results shown in Fig 4 A sample of an SC formulation was spiked with HAP and DAP and stored at room temperature protected from light At different times during storage subsamples were analysed for their contents of HAP and DAP As can be seen from Fig 4, the stability of HAP and DAP (at least in an SC formulation) is very limited

CONCLUSIONS

The method presented here is relatively simple and useful for the determination of HAP and DAP

impurities in pesticide formulations containing carbendazim or benomyl as the active ingredient The precision and accuracy of the method are acceptable for use in authority pesticide formulation control

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