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PAPER IN FOREFRONT

# Solid-phase microextraction of benzimidazole fungicides in environmental liquid samples and HPLC–fluorescence determination

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**Abstract** Solid-phase microextraction (SPME) coupled with high-performance liquid chromatography (HPLC) with fluorescence detection was optimized for extraction and determination of four benzimidazole fungicides (benomyl, carbendazim, thiabendazole, and fuberidazole) in water. We studied extraction and desorption conditions, for example fiber type, extraction time, ionic strength, extraction temperature, and desorption time to achieve the maximum efficiency in the extraction. Results indicate that SPME using a Carboxen–polydimethylsiloxane 75 μm (CAR–PDMS) fiber is suitable for extraction of these types of compound. Final analysis of benzimidazole fungicides was performed by HPLC with fluorescence detection. Recoveries ranged from 80.6 to 119.6 with RSDs below 9% and limits of detection between 0.03 and 1.30 ng $mL^{-1}$ for the different analytes. The optimized procedure was applied successfully to the determination of benzimidazole fungicides mixtures in environmental water samples (sea, sewage, and ground water).

**Keywords** Solid-phase microextraction · Benzimidazole fungicides · High-performance liquid chromatography · Water analysis

## Introduction

Because of the indiscriminate use of pesticides for different applications, important environmental problems are emerging which are a risk to plant, animal, and human health. Fungicides are one group of these pesticides which are used primarily to control spoilage of crops as a result of fungal attack. They represent approximately 20–25% of pesticides used [1]. Benzimidazole fungicides are systemic pesticides, widely used in agriculture for pre and postharvest treatment for control of a wide range of pathogens. These substances are applied directly to the soil or they are sprayed over crop fields and hence released to the environment [2]. They readily penetrate plants through the roots and leaves and can directly enter natural waters by drainage from agricultural land. Most of these compounds persist in the environment after their application; some even remain for many years. Some of the main compounds of the benzimidazole family are benomyl (BN), carbendazim (MBC), thiabendazole (TBZ) and fuberidazole (FB); they are the active components of different commercial formulations [3] and are effective and widely used.

Benomyl is applied to the soil to control a variety of fruit diseases. It rapidly degrades to carbendazim, its main degradation product. It is the most widely used of the benzimidazole carbamate class of fungicide [4]. Carbendazim has both protective and curative activity against a wide range of fungal diseases. It is toxic to humans, animals, and plants and also is very persistent in water, wastewater, soil, crops, and food. Thiabendazole is used to control fruit and vegetable diseases such as mold, rot, blight, and stain. It is also used medicinally as a chelating agent to bind metals. Fuberidazole is the most important benzimidazole used in agriculture.

There are few data about their presence in marine water, although laboratory experiments have shown that some are very toxic to the sea organisms [5, 6].

The methods most frequently used to determine these benzimidazole fungicides are fluorescence spectroscopy

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[7–9] and HPLC with UV, fluorescence, or mass spectrometric detection [10, 11]. In all these techniques benomyl is readily converted during the analytical procedure into MBC, which is then used for its determination.

The extraction and clean-up procedures usually used are solid-phase extraction (SPE) [9, 10], liquid–liquid extraction (LLE) [11, 12], cloud point extraction (CPE) [13], on line supported liquid membrane (SLM) and microporous membrane LLE techniques [14], and supercritical-fluid extraction (SFE) [15]. Benzimidazoles have been determined in different matrices, including wine [16], liver [17], apiarian samples [18], milk [19], fruits and vegetables [20, 21].

Solid-phase microextraction (SPME) is a new type of extraction technique which has been used for analysis of organic compounds in environmental samples [22]. SPME enables simultaneous extraction and preconcentration of analytes from a sample. This technique requires a first step of partitioning of the analytes between the sample and the fused-silica fiber, and a second step of desorption. SPME has many applications in pesticides analysis [23–25] followed by GC or HPLC [26–29]. Because of the thermal instability of the benzimidazoles, they cannot be analyzed by gas chromatography unless they are transformed into thermally-stable derivatives [15]; for this reason, HPLC with fluorescence detection is used instead for their analysis.

This study was undertaken to establish a suitable and sensitive method for simultaneous determination of selected fungicides in water samples. We have evaluated the efficiency of extraction of these compounds on different kinds of fiber and have optimized several SPME conditions—extraction time, ionic strength, extraction temperature, and desorption time. The optimized SPME procedure has been used for determination of these compounds in different environmental water samples (sea, sewage, and ground waters) by employing HPLC with fluorescence detection.

## Experimental

### Reagents

Carbendazim (methylbenzimidazol-2-yl carbamate), benomyl (methyl 1-butylcarbamoylbenzimidazol-2-yl carbamate), thiabendazole (2-thiazol-4-yl-benzimidazole), and fuberidazole (2,2′-furylbenzimidazole) standards of purity ≥99% were obtained from Riedel–de Haën (Seelze, Germany). The compounds studied are listed in Table 1 (the numbers and abbreviations are used to identify the compounds in the figures) and their chemical structures are shown in Fig. 1.

**Table 1** The benzimidazole fungicides, their excitation/emission wavelengths, and their retention times

| Abbreviation | Compound | Excitation (nm) | Emission (nm) | $t_R$ (min) |
|---|---|---|---|---|
| MBC | Carbendazim | 280 | 320 | 3.5 |
| BN | Benomyl | 280 | 320 | 3.5 |
| TBZ | Thiabendazole | 300 | 350 | 5.3 |
| FB | Fuberidazole | 300 | 350 | 5.9 |

Individual stock solutions of the fungicides at a concentration of 100 μg $mL^{-1}$ were prepared in methanol and stored in amber bottles at 4 °C. Ultrapure Milli-Q water (Millipore, Spain) was used to prepare working aqueous standard solutions (200 ng $mL^{-1}$ MBC and BN, 50 ng $mL^{-1}$ TBZ and 1 ng $mL^{-1}$ of FB). Methanol used to dissolve standards, prepare the mobile phase, and desorb the targets was HPLC-grade from Scharlau (Barcelona, Spain). Mobile phase was filtered through a 0.22-μm cellulose acetate membrane filter and sonicated before use.

### SPME fibers

Polydimethylsiloxane–divinylbenzene (PDMS–DVB) 65 μm, Carboxen–PDMS 75 μm (CAR–PDMS), and polyacrylate PA 85 μm fibers were purchased from Supelco (Bellafonte, PA, USA). Fiber conditioning is necessary to ensure good selectivity and sensitivity. The fibers were

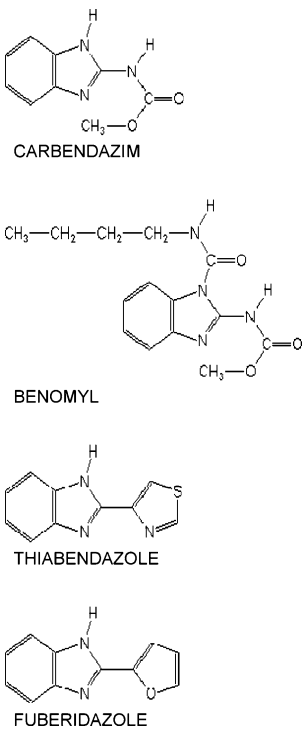


**Fig. 1** The chemical structures of the benzimidazole fungicides studied

conditioned with methanol according to the supplier's instructions.

### Instrumentation and chromatographic separation

Chromatography was performed with a Varian pump fitted with a Varian 410 autosampler with a volume selector, a column valve module with an internal oven, and a Varian scanning fluorescence detector. The system and the data management were controlled by Star software from Varian (Madrid, Spain). The separation column was a 3.9 mm× 150 mm, 8-μm particle diameter, Symmetry C-18. The analytical column was inserted in the column module and thermostatted at 30±0.2 °C.

The isocratic mobile phase, optimized for a good separation and determination of the benzimidazole fungicides, was methanol–water, 45:55 (*v*/*v*), at a flow rate of 1.0 mL $min^{-1}$. Because benomyl has the same physicochemical and chromatographic characteristics as carbendazim their quantification had to be performed as carbendazim (MBC/BN peak).

Table 1 shows the retention times ($t_R$) and excitation/emission wavelengths used for determination of the benzimidazole fungicides.

### Solid-phase microextraction procedure

All extractions were performed with 4 mL an aqueous solutions of MBC (200 ng $mL^{-1}$), BN (200 ng $mL^{-1}$), TBZ (1000 ng $mL^{-1}$), and FB (1 ng $mL^{-1}$) in screw-cap vials. Salt concentration was adjusted by adding 15% NaCl. The samples were heated and stirred at a constant speed of 600 rpm during extraction. The SPME fiber was immersed in the aqueous sample for 40 min at 60 °C. The compounds were desorbed into a 200-μL glass vial containing 50 μL methanol, for 10 min. All studies were performed in duplicate and average values were calculated.

After each analysis, the fiber was cleaned with Milli-Q water to avoid damage because of the use of salt. The fiber was then submerged in methanol for 15 min and dried before starting the next extraction.

### Solid-phase extraction

$C_{18}$ extraction cartridges were conditioned by successive elution with 6 mL methanol and 6 mL water (from a Milli-Q system). A water sample (50 mL) containing MBC (200 ng $mL^{-1}$), BN (200 ng $mL^{-1}$), TBZ (1000 ng $mL^{-1}$) and FB (1 ng $mL^{-1}$) was then aspirated through the cartridge. Before elution of the fungicides the cartridge was dried by passage of air. The fungicides were eluted with 5 mL methanol and 20 μL of the extract was injected into the chromatographic system.

### Statistical analysis

The experimental design for SPME optimization was performed using Statgraphics Plus Software, version 5.1 (Manugistic, Rockville, MD, USA). Partial and bivariate correlations were done using SPSS 11.0 (Chicago, IL, USA).

## Results and discussion

Optimization of different conditions is necessary for determination of benzimidazole fungicides in water samples by SPME. To ensure highly efficient extraction of the analytes from the samples we optimized conditions having a major effect on the extraction, for example fiber type, ionic strength, extraction time, temperature, and desorption time.

### Optimization of extraction process

A study was performed to choose the best fiber for the extraction of benzimidazole fungicides by measuring the peak area response to the pesticides where to each fungicide for three types of fiber: 65-μm polydimethylsiloxane–divinylbenzene (PDMS–DVB), 75-μm Carboxen–polydimethylsiloxane (CAR–PDMS), and 85-μm polyacrylate (PA). Extraction was performed at room temperature for 40 min. The fibers were conditioned as described in the Experimental section. Table 2 shows the areas obtained for each fiber. The best results were obtained with the CAR–PDMS fiber. This coating was therefore selected for further studies.

### Optimization of the absorption process

The effect of absorption temperature, absorption time, and addition of salt on the SPME process were optimized to obtain the best conditions for extraction of the benzimidazole fungicides. To study these conditions we used a multivariable factorial design [30, 31]. Initially the varia-

**Table 2** Responses obtained after use of fibers with different coatings to extract benzimidazole fungicides[a]

| Fiber | Area | | |
|---|---|---|---|
| | MBC/BN | TBZ | FB |
| Carboxen–PDMS 75 μm | 384305 | 1885692 | 3780452 |
| PDMS–DVB 60 μm | 66245 | 95256 | 232833 |
| PA 85 μm | nd[b] | nd[b] | nd[b] |

[a] 200 ng $mL^{-1}$ MBC, 200 ng $mL^{-1}$ BN, 100 ng $mL^{-1}$ TBZ, and 1 ng $mL^{-1}$ FB

[b] nd: not detected

bles were studied with a $2^3$ factorial design to determine the effect of each variable on the extraction and the correlations between the variables.

To ensure efficient extraction of the analytes from a sample one of the most important steps in the development of an SPME method is to determine the time necessary to reach the equilibrium between the sample matrix and the coating of the fiber. Extraction temperature is important in the absorption process, because it affects the rate of mass transfer and the partition equilibrium [32]. With regard to the effect of NaCl, it has been shown that addition of an inert salt improves the efficiency of extraction by increasing the ionic strength and reducing analytes solubility. These variables were studied in two $3^2$ factorial designs—absorption time with temperature and absorption time with salt. This involved nine experiments, each performed in duplicate; other variables possibly involved in the extraction process, for example agitation (600 rpm) and desorption time (10 min) were kept constant. Partial and bivariate correlations obtained in the screening design are shown in Table 3. Temperature (°C) and salt addition (%NaCl) are the variables which have most effect on recovery, and absorption time (min) is highly correlated with the salt addition.

To optimize these conditions we studied the efficiency of extraction of the target analytes for extraction times from 20 to 60 min and temperature s from 20 to 60 °C. In this study the salt concentration was kept constant at 30% (*w*/*v*) NaCl. Figure 2 shows the relative response surface for these two variables for TBZ. Extraction efficiency increased rapidly with increasing absorption time up to 40 min and then increased more slowly. For this reason we chose 40 min as the optimum time. Extraction efficiency increased with increasing temperature, with maximum areas at 60 °C. The results were similar for the other target compounds.

We therefore chose an extraction time of 40 min and an extraction temperature of 60 °C as a reasonable compromise between good sensitivity and acceptable analysis time for benzimidazole fungicides.

The effect of ionic strength is of great importance in SPME. It was tested by comparing different extraction times (20–60 min) and NaCl concentrations from 0–30%

**Table 3** Relevant conditions and correlation between them[a]

| Correlation | MBC/BN | TBZ | FB |
|---|---|---|---|
| Absorption time | 0.177 | 0.399 | 0.563 |
| Temperature | 0.325 | 0.507 | 0.598 |
| NaCl concentration | 0.862 | 0.648 | 0.347 |
| Absorption time×temperature | −0.062 | −0.256 | −0.495 |
| Absorption time×NaCl | −0.305 | −0.375 | −0.246 |
| Temperature×NaCl | −0.583 | −0.500 | −0.276 |

[a] The maxima correlations are +1 and −1

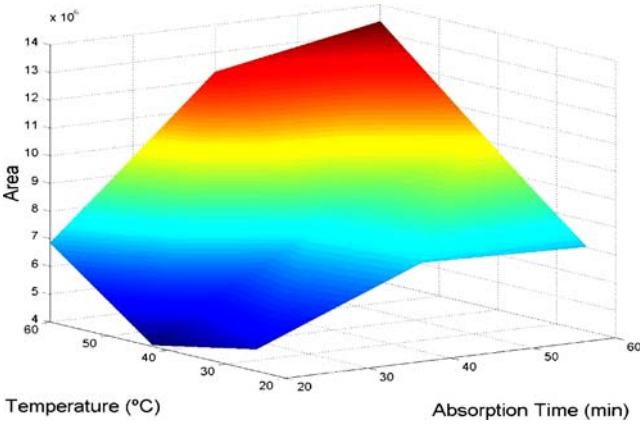


**Fig. 2** Absorption time and temperature response surface for extraction of TBZ (200 ng $mL^{-1}$) by SPME

(*w*/*v*) and studying the peak areas obtained. The results indicated peak area increased in the presence of sodium chloride, the maximum being reached in the range 15–30% (*w*/*v*) NaCl. We chose 15% (*w*/*v*) NaCl and 40 min extraction time, because higher salt concentrations did not result in significantly increased peak area and can damage the fiber. Figure 3 shows the response surface for the effect of absorption time and NaCl concentration for TBZ. The behavior was similar for the other fungicides studied.

## Optimization of desorption

It is important to study the time necessary for analyte desorption and the solvent is used for desorption. In this study we chose methanol as desorption solvent because it is compatible with the mobile phase. After absorption from the sample solutions under the optimum conditions (40 min, 60 °C, and 15% (*w*/*v*) NaCl), desorption was optimized by immersing the CAR–PDMS fiber in 50 μL methanol in a glass vial for times in the range 4–12 min. This volume was selected because it is sufficient to desorb the analytes and to achieve good preconcentration. If desorption of the analytes is poor an increase of the desorp-

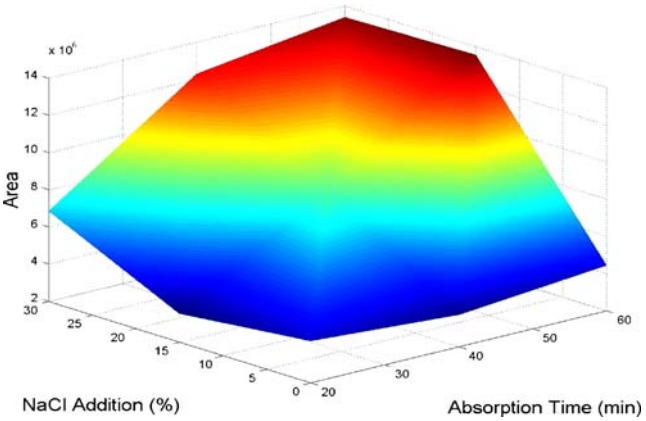


**Fig. 3** Absorption time and NaCl concentration response surface for extraction of TBZ (200 ng $mL^{-1}$) by SPME

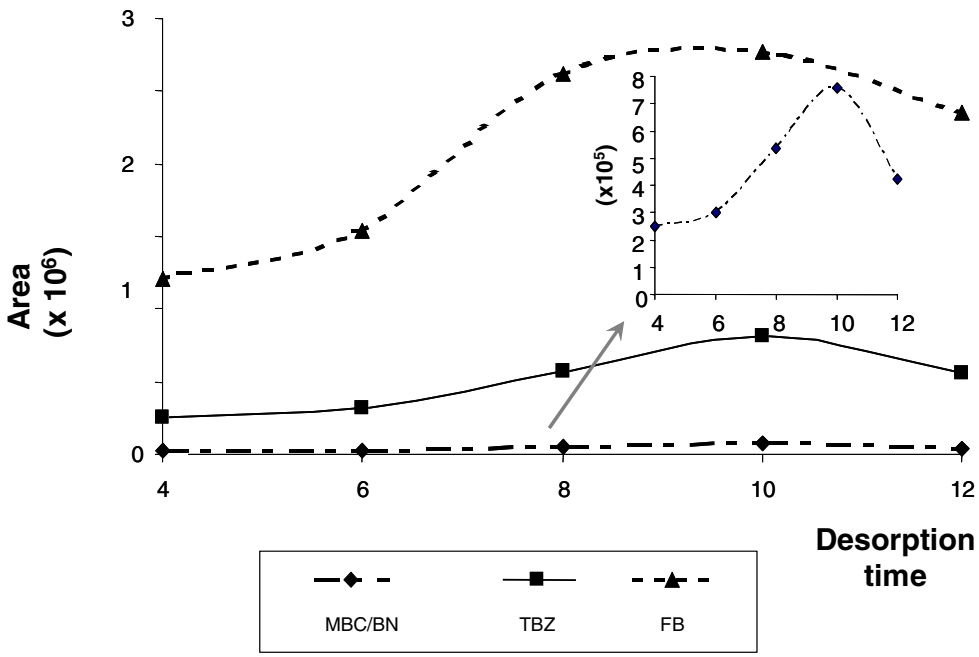


**Fig. 4** Optimization of desorption time for MBC/BN, TBZ, and FB. MBC/BN is also shown in the *inset*

tion period can enhance the relative responses of the analytes. Figure 4 shows the results obtained for the target compounds in the time-range studied. It was found that analyte peak areas increased with desorption times up to 10 min and then did not increase significantly. A desorption time of 10 min was therefore selected as optimum.

In summary, the optimum extraction conditions for these benzimidazole fungicides were: 40 min absorption at 60 °C, using 15% (*w*/*v*) NaCl and desorption with methanol for 10 min.

## Analytical data

The linearity of the calibration plot was investigated over the range 2–300 ng $mL^{-1}$ MBC/BN, 0.5–300 ng $mL^{-1}$ TBZ, and 0.05–5 ng $mL^{-1}$ FB. Each point on the calibration plot was the mean from two area measurements. All correlation coefficients ($R^2$) were >0.992 (Table 4). Reproducibility, expressed as relative standard deviation (RSD), was obtained by analyzing six replicate samples containing 200 ng $mL^{-1}$ MBC, 200 ng $mL^{-1}$ BN, 100 ng $mL^{-1}$ TBZ, and 1 ng $mL^{-1}$ FB. RSDs values ranged from 7.9% to 9.0%. Detection limits were calculated from the signal to noise ratio of the individual peaks, assuming a minimum detectable signal-to-noise level of 3 [33]. The detection limits (LODs) were 1.3 ng $mL^{-1}$ for MBC/BN, 0.04 ng $mL^{-1}$ for TBZ, and 0.03 ng $mL^{-1}$ for FB. The quantification limits (LOQs) were 4.3 ng $mL^{-1}$ for MBC/BN, 0.13 ng $mL^{-1}$ for TBZ, and 0.10 ng $mL^{-1}$ of FB (Table 4).

**Table 4** Analytical data for determination of benzimidazole fungicides by SPME–HPLC

| Compound | $R^2$ [a] | LOD (ng $mL^{-1}$)[b] | LOQ (ng $mL^{-1}$)[c] | RSD (%)[d] |
|---|---|---|---|---|
| MBC/BN | 0.992 | 1.30 | 4.3 | 9.0 |
| TBZ | 0.999 | 0.04 | 0.13 | 6.6 |
| FB | 0.994 | 0.03 | 0.10 | 7.9 |

[a] The calibration range is given in the text
[b] Detection limits are calculated for a signal-to-noise ratio of three
[c] Quantification limits are calculated for a signal-to-noise ratio of ten
[d] $n=6$

## Validation of the method

To prove the validity of the method the results obtained by use of SPME were compared with those obtained by use of SPE with a $C_{18}$ cartridge. Figure 5 shows the results obtained for a seawater sample (Las Canteras). It is apparent

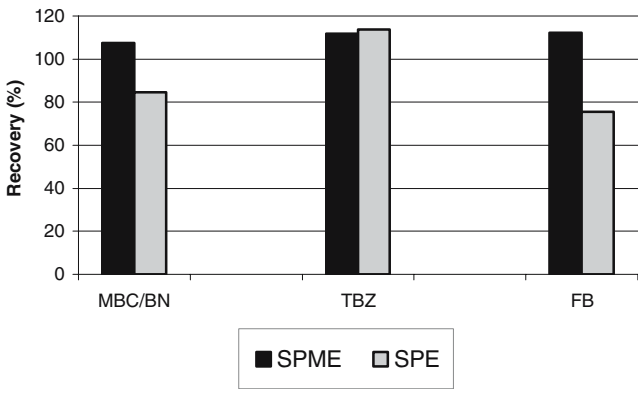


**Fig. 5** Comparison of results obtained from application of SPE and SPME for analysis of the benzimidazole fungicides (MBC (200 ng $mL^{-1}$), BN (200 ng $mL^{-1}$), TBZ (100 ng $mL^{-1}$) and FB (1 ng $mL^{-1}$)) in a spiked seawater sample from Las Canteras

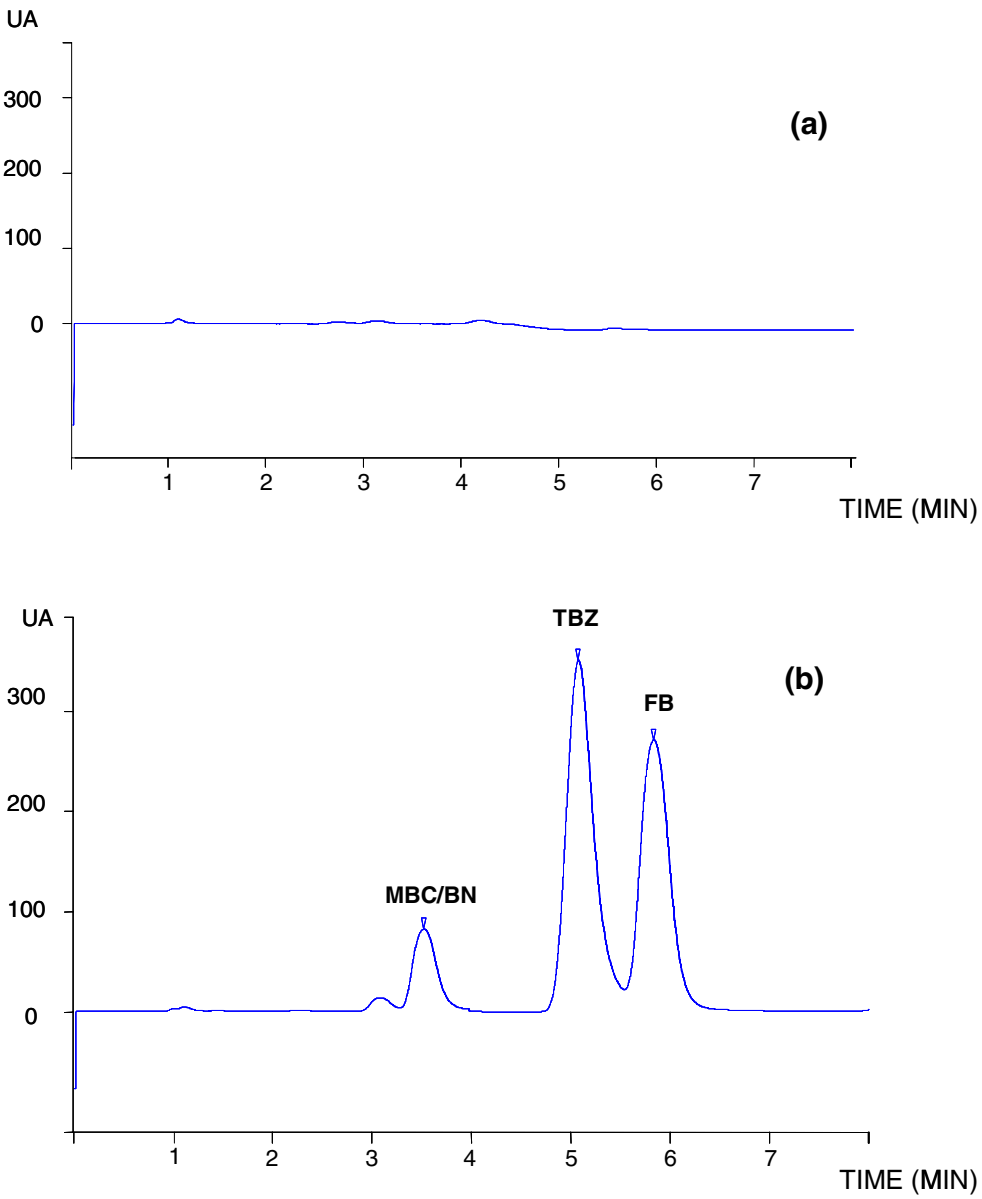


**Fig. 6** Chromatograms obtained, under the optimum conditions, from (**a**) an extract of a seawater blank (San Felipe, Gran Canaria) and (**b**) seawater spiked with MBC (200 ng $mL^{-1}$), BN (200 ng $mL^{-1}$), TBZ (100 ng $mL^{-1}$), and FB (1 ng $mL^{-1}$)

the results obtained by use of both procedures are comparable. For FB, however, the recovery obtained by SPE (~76%) was lower than expected.

## Application to water samples

SPME–HPLC with fluorescence detection was used to determine benzimidazole fungicides in six spiked water samples of different types to confirm the practicability and feasibility of our method. We analyzed four seawater samples taken from different points off the coast of Gran Canaria island: San Felipe (1) and Las Canteras (2) off the North of the island and Taliarte (3) and Castillo del Romeral (4) in the East. We also analyzed a ground water sample (5) and a sample from a wastewater pre-treatment plant (6). Samples were collected in 1-L glass bottles,

**Table 5** Recoveries and RSDs obtained for different spiked water samples using SPME–HPLC[a]

| Compound | San Felipe (1) | Las Canteras (2) | Taliarte (3) | Castillo del Romeral (4) | Ground water (5) | Wastewater (6) |
|---|---|---|---|---|---|---|
| MBC/BN | 106.9±2.9 | 107.5±0.1 | 102.4±3.1 | 115.5±0.6 | 80.9±6.0 | 88.0±5.8 |
| TBZ | 115.7±1.5 | 111.9±3.3 | 108.1±1.1 | 103.0±0.2 | 96.9±2.9 | 106.7±0.9 |
| FB | 118.9±5.4 | 112.2±1.9 | 119.6±1.0 | 119.5±6.3 | 96.8±1.4 | 82.8±2.8 |

[a] Mean from two determinations

filtered through a 0.45 μm cellulose acetate filter, and stored in the dark until analysis. First, a blank of the real samples was run to verify the absence of peaks at the retention times of the compounds under study. In general, no interfering peaks appeared in the blank chromatograms. The samples were then spiked with 200 ng $mL^{-1}$ MBC, 200 ng $mL^{-1}$ BN, 100 ng $mL^{-1}$ TBZ, and 1 ng $mL^{-1}$ FB to investigate the effect of the matrix on the method. Figure 6 shows the chromatograms obtained from the extracts of a blank sample (a) and of a spiked seawater sample (b) analyzed by the method described.

Recovery of the target compounds from the spiked environmental water samples is summarized in Table 5. The values are ratios of the amount extracted (calculated from the calibration plots) to the amount added to the sample. All values were obtained from measurements in duplicate.

The recoveries obtained for seawater samples were slightly higher than expected, probably because of the presence of different types of salt (in addition to NaCl) not included in our studies that could affect the absorption process. Recoveries from ground water and waste water samples were very satisfactory. This shows that our method based on SPME–HPLC with fluorescence detection is a reliable means of determination of benzimidazole fungicides.

## Conclusions

The potential of SPME–HPLC with fluorescence detection for analysis of benzimidazole fungicides in environmental water samples has been demonstrated. The CAR–PDMS fiber was the optimum coating for extraction of these targets. A simple calibration plot method can be used, and good reproducibility and detection limits were obtained for all the fungicides. The procedure developed enables rapid, simple, precise, and sensitive simultaneous determination of MBC/BN, TBZ, and FB in different kinds of water sample.

This method could be used for monitoring and screening of benzimidazole fungicides in contaminated environmental water samples, as a good alternative to conventional extraction and preconcentration procedures.

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