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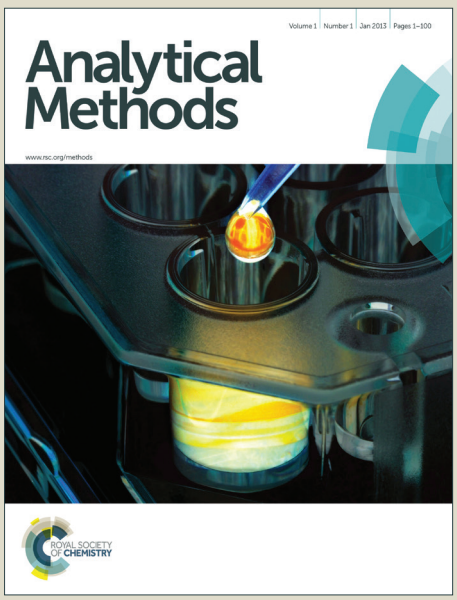

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Graphical Abstract

A novel poly (2-hydroxypropyl methacrylate-ethylene dimethacrylate) (HPMA-EDMA) monolithic capillary column was synthesized and selected as the extraction medium for polymer monolith microextraction (PMME).

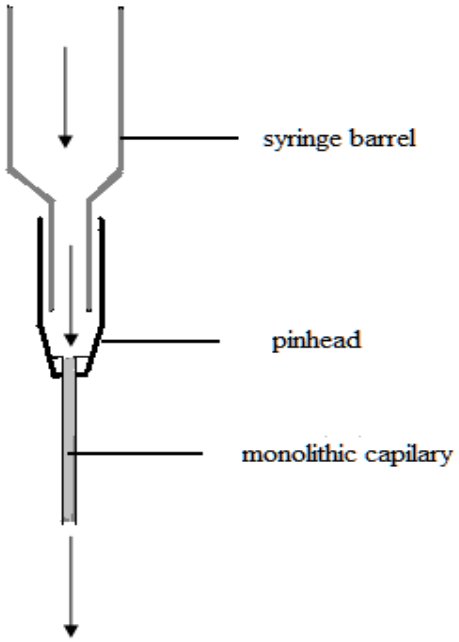

Fig 1. Scheme of the PMME

Determination of trace fungicides in environmental water samples using poly (HPMA-EDMA) monolith microextraction coupled to high performance liquid chromatography

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Abstract

A simple, rapid and sensitive strategy has been presented for the simultaneous determination of three fungicides (azoxystrobin, diethofencarb and pyrimethanil) in water samples by coupling polymer monolith microextraction (PMME) to high performance liquid chromatography. A novel poly (2-hydroxypropyl methacrylate-ethylene dimethacrylate) (HPMA-EDMA) monolithic capillary column was synthesized and selected as the extraction medium for PMME. To achieve optimum extraction performance, the conditions of PMME including sample flow rate, sample pH, eluent volume, eluent flow rate, sample volume and salt effect have been investigated. Under the optimum conditions, the limits of detection of azoxystrobin, diethofencarb and pyrimethanil are 0.19，0.22 and 0.65 μg L^{-1}, respectively. The reproducibility of the method was obtained with intra-day and inter-day relative standard deviations less than 3.1 % and 6.3 %, respectively. The proposed method has been successfully applied to the determination of the three fungicides in environmental water samples with a recovery range of 80.2-115.6 % in all the samples.

Keywords: Polymer monolith microextraction； High performance liquid chromatography； Fungicide； Environmental water

Introduction

Fungicides are a group of chemicals which are used primarily to control spoilage of crops through fungal attack. Azoxystrobin is a strobilurin fungicide, which is used for treating downy and powdery mildews and widely employed in cereals growing. Diethofencarb is a very effective fungicide for controlling various fungal species, such as Botrytis spp., Cercospora spp. and Venturia spp., that are resistant to benzimidazole fungicides.[1] Pyrimethanil is used for the control of grey mould and leaf scab on grape, strawberry, tomato, fruit, vegetables and ornamentals.[2] Besides, research shows that the mixed use of diethofencarb and pyrimethanil can effectively control gray mold disease because of their different mechanism of action.[3]

Due to their widespread use in agricultural areas, these fungicides may entered into the environment by all kinds of ways such as spraying, soil and storage, as well as the discharge of wastewater. Many fungicides are highly toxic and may have a consequent potential impact on the environment and public health.[4] Therefore, the evaluation and monitoring of trace levels of these fungicides in water are imperative for human health protection and environmental control.

Owing to the complexity of environmental matrices and the relative low concentration of the target analytes in samples, sample pretreatment and enrichment procedure is a crucial step in an analytical process to obtain accurate and sensitive results. Conventional extraction methods, such as liquid–liquid extraction (LLE)[5] and solid-phase extraction (SPE)[6-8] are the most commonly used techniques for preconcentration and cleanup of fungicide residues. However, these methods involving multistep procedures that are complicate, labor-intensive and time-consuming. Besides, LLE requires the use of large amounts of organic solvents, which cause the pollution problem accompanied risk for health.

Recent research activities are oriented towards the development of simplification, miniaturization, rapidity, and environment-friendly sample preparation techniques that could greatly reduce the organic solvent consumptions.

[9,10] As a result, new microextraction techniques such as solid-phase microextraction (SPME) and liquid phase microextraction (LPME) have been developed. Solid-phase microextraction (SPME) [11-13] has been applied to the determination of fungicides, belonging to different chemical classes, in wine using gas chromatography (GC), [14-16] liquid chromatography (LC) [17] and even capillary electrophoresis. [18] SPME is a solvent-free extraction technique that integrates sample extraction, concentration and sample introduction into a single procedure. But SPME fibers are generally fragile, expensive, have a limited lifetime, and can also suffer from analyte carryover. [19] As a further alternative to SPME, a method termed polymer monolith microextraction (PMME) based on the use of a capillary monolithic column was introduced in 2006. [20] Compared with traditional in-tube SPME, [21] PMME has shown several attractive features including frit-free construction, easy preparation with good control of porosity and diverse surface chemistry. Furthermore, it has advantages in convenience, flexibility, and easy operation. So far, poly (methacrylic acid-ethylene dimethacrylate) (MAA-EDMA) [22-24] and poly (methyl methacrylate-ethylene dimethacrylate) (MMA-EDMA) [25] have been employed for the preparation of polymer monolithic column, Moreover, most polymer monolithic capillary columns whose monomers are methyl acrylate with different alkyl substitutes are reported to show hydrophobic properties, [26, 27] however, 2-hydroxypropyl methacrylate itself carries an extra hydroxy, so poly (2-hydroxypropyl methacrylate-ethylene dimethacrylate) (HPMA-EDMA) monolithic capillary column may be used to extract somewhat polar analytes，and its combination with HPLC has not yet been reported.

The objective of the present work is to propose a novel method based on poly (HPMA-EDMA) monolith microextraction combined with HPLC for the simultaneous determination of three fungicides (azoxystrobin，diethofencarb and pyrimethanil) in environmental water samples. Several important parameters affecting the extraction efficiency such as sample flow rate, sample pH, eluent volume, eluent flow rate, sample volume and salt effect have been carefully optimized. Under the experimental conditions, the proposed method is validated for the

quantitative analysis and applications for tap water, rain lake water, field water, pool water and reservior water samples have been illustrated.

Experimental

Chemicals and materials

Azoxystrobin, diethofencarb and pyrimethanil were purchased from Sigma-Aldirich Chemical Company (St. Louis, MO, USA). 2-Hydroxypropyl methacrylate (HPMA), ethylene dimethacrylate (EDMA) and γ-methacryloxypropyltrimethoxysilane (γ-MAPS) were purchased from Acros (New Jersey, USA). Azobisisobutyronitrile (AIBN), toluene, dodecanol, sodium chloride, sodium hydroxide and hydrochloric acid were obtained from Tianjin Kermel chemical reagents development centre (Tianjin, China). Methanol, acetonitrile and ethanol were ordered from Tedia (USA). All chemical reagents were chromatographic or analytical grade. Ultrapure water was purified on a Mill-Q water purification system (Millipore, Billerica, MA, USA). Fused silica capillaries with 530 μm i.d. were purchased from Yongnian Optical Fiber Factory (Hebei, China).

The stock standard solutions of 20 μg mL^{-1} of each compound were prepared in methanol. A series of standard solutions were daily prepared by appropriate diluting from stock solutions with methanol. All solutions prepared were maintained at 4 ˚C protected against daylight.

Instrumentation

Chromatographic analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a quaternary pump and degasser, a thermostated autosampler (4 ˚C) and column compartment

(40 ˚C), a DAD detector and ChemStation software. A reverse phase Agilent HC-C18 column (250 mm × 4. 6 mm i.d., 5 μm) was used for separation of the analytes. The mobile phase was methanol-water (70:30, v/v) at a flow rate of 1.0 mL min^{-1}. The column temperature was 40 ˚C and the detection wavelength was set at 254 nm. The injection volume was 5 μL. Ultrasonic instrument KQ-100DE was purchased from Kunshan Ultrasonic Instrument Co., Ltd. (Jiangsu, China) and a pHS-3C digital pH meter (Shanghai Rex Instruments Factory, China) was employed for pH measurements.

Sample preparation

Tap water was collected from our laboratory and lake water was obtained from South Lake (Wuhan, China) and Sha Lake (Wuhan, China) Field water was obtained from a vegetable field in the outskirt of Wuhan (Hubei, China). Pool water was collected from a swimming pool in Wuhan city (Hubei, China) near a pesticide plant. Reservior water for irrigation was collected from a reservior near a big vineyard also in Wuhan city. All the samples were analyzed after filtering with a 0.45 μm micropore membrane, and stored at the temperature of 4 ˚C after collection.

Preparation of poly (HPMA-EDMA) monolithic capillary

The poly (HPMA-EDMA) monolith was synthesized inside a fused silica capillary (10 cm×530 μm i.d.) by a heat initiated polymerization method. The polymerization method was described in detail previously. [28] At first, the fused silica capillary was cleaned and activated by 1 mol L^{-1} NaOH, H_2O, 0.1 mol L^{-1} HCl, H_2O for one hour, successively. After it was dried by nitrogen gas, the capillary was filled with silanization solution containing 50% (v/v) r-MAPS in methanol, sealed with rubber and then thermostatted at 40 ˚C for 24 h. After silanization, the capillary was flushed with 50 column-volumes of methanol and dried by the purge of nitrogen gas.

The pre-polymerization mixture solution consisting of monomer HPMA (30 mg), cross-linker EDMA (214 mg), porogenic solvents toluene (55 mg) and dodecanol (436 mg), and initiator AIBN (2.75 mg) was completely mixed ultrasonically into a homogenous solution. Subsequently, the mixture solution was purged with N_2 to remove the oxygen and filled into the pretreated capillary. Immediately, the capillary was sealed with silicon rubber at both ends, and then the reaction was initiated at 60 ˚C for 36 h. Following polymerization, the capillary was washed with methanol to remove the unreacted components and porogenic solvents.

PMME apparatus and procedure

The PMME apparatus was composed of a plastic syringe (2 mL), a poly (HPMA-EDMA) monolithic capillary tube (530 μm i.d. × 3 cm) and an extraction pinhead. The syringe barrel was coupled seamlessly to one end of the pinhead, while on the other end of the pinhead, the metallic needles were removed and replaced by a 3 cm monolithic capillary tube with adhesive. [25]

A syringe infusion pump (SN-50C6, Shengnuo Medical Equipment Co., Ltd., Shenzhen, China) was employed for the delivery of solutions in the whole extraction process including precondition, sample loading, washing, and desorption. For precondition, 0.3 ml methanol was introduced into the syringe and expelled to pass through the monolithic capillary tube at a speed of 0.06 mL min^{-1}. For the sorption, 2.0 mL of sample solution was pushed through the capillary at 0.2 mL min^{-1}, and then 0.2 mL of Milli-Q water was pumped through at 0.1 mL min^{-1} to eliminate the residual matrix for avoiding the interference of separation and detection. Then the residual solution in the pinhead and monolithic capillary tube was pushed out with an empty and clean syringe to avoid polluting the eluate. In the desorption step, ethanol was injected via the monolithic capillary at 0.04 mL $min^{-1}$for 1.5 min and the eluate was collected into a vial for the subsequent analysis by HPLC.

Results and discussion

Optimization of the PMME method

To achieve the best extraction efficiency of the poly (HPMA-EDMA) monolithic capillary towards target analytes, various parameters affecting the extraction efficiency such as sample flow rate, sample volume, sample pH, eluent volume, eluent flow rate and salt effect have been optimized. The peak area of analyte as the HPLC response was used to evaluate the extraction efficiency under the various conditions.

Effect of extraction flow rate

The flow rate of the sample solution is an important parameter affecting the PMME process, which not only affects the recoveries of the analytes, but also controls the time of analysis. The flow rate of the sample solution was optimized in the range of 0.05-0.3 mL min^{-1}. As shown in Fig. 1, changing the flow rate had no significant influence on the extraction efficiency in the investigated range. Therefore, the flow rate of 0.2 mL min^{-1} was selected considering the extraction time and the pressure of monolithic capillary column.

Effect of pH value of the sample matrix

Sample pH plays an important role on the extraction efficiency for analytes. It not only influences the molecule form of the analytes but also relates closely to the interactions between analytes and the extraction phase. In order to evaluate the effect of sample pH, the standard solutions containing 0.2 μg mL^{-1} azoxystrobin, diethofencarb and

pyrimethanil have been loaded onto the poly (HPMA-EDMA) monolithic capillary after pH adjustment using H_3PO_4 or NaOH solutions. The effect of sample pH within the range of 2.0-10.0 was shown in Fig. 2. The result exhibited that as the pH value increased, the extraction efficiency increased firstly, and then decreased, with the maximum value at pH 7.0. The explanation might be based on the fact that three fungicides are extracted by the monolithic column mostly by hydrogen bond interaction, which arise from the polymer bone structure and its carboxyl and hydroxyl. In the acidic or alkaline matrix, the hydrogen bond interaction between HPMA-EDMA and the target analytes would be influenced and decreased, thus a slow decrease was observed in the high or low pH value range. As a result, the analytical samples were adjusted to pH 7.0 in the microextraction process.

Effect of desorption solvent type

The desorption procedure has been carefully optimized to achieve an accurate quantitative analysis of the three fungicides. The comparison study among methanol, mobile phase (methanol-H_2O, 70:30, v/v) and ethanol was performed, and the results indicated that ethanol gave the highest extraction efficiency .Therefore, ethanol was selected as the eluent for the desorption of the three fungicides.

Effect of desorption volume

In order to determine the required volume of ethanol to elute the analytes from the monolithic capillary, the effect of eluent volumes has been investigated. After sample extraction, 0.06 mL ethanol was used to elute the analytes. The same procedure was repeated twice, and then each of the 0.06 mL eluates was collected for detection. The results showed that the first 0.06 mL of ethanol could elute more than 90 % extracted analytes from the monolithic

capillary, which was enough for quantitative analysis. Further, increasing the methanol volume would lower the detection sensitivity. Therefore, 0.06 mL ethanol was employed to desorb the analytes.

Effect of desorption flow rate

The flow rate of the desorption solution has been optimized in the range of 0.02-0.1 mL min^{-1} as seen in Fig. 3, and the flow rate of 0.04 mL min^{-1} was found to be suitable to attain faster desorption and satisfactory desorption efficiency.

Effect of extraction volume

The extraction equilibrium profile was monitored by increasing the volume of the analyte solution extracted from 0.5 to 3.0 mL at a constant flow rate of 0.2 mL min^{-1}. The results were shown in Fig. 4, the yield of the three fungicides extracted increased with increasing volume of the extracted sample, indicating that the monolithic capillary exhibited remarkable extraction capacity towards the three fungicides. Although increasing the sample volume might improve the sensitivity for the analytes, the sample volume should be chosen according to the required sensitivity and the time acceptable for a whole analysis. To achieve sufficient sensitivity within a short time, 2.0 mL was chosen as the sample volume for subsequent analysis.

Effect of salt addition

In general, addition of salt into the sample solutions could lead to the salting-out effect, and more analyte

molecules would be extracted onto the extraction phase. Meanwhile, the viscosity of sample solution became high and the diffusion rate of solute decreased, which decreased the extraction efficiency. So the effect of inorganic salt concentration of the sample matrix on the extraction efficiency has also been investigated. Sodium chloride (NaCl) at a concentration from 0 % to 20 % (w/v) was added to sample solution to study its influence on extraction efficiency. The obtained results revealed that salt concentration had no obvious influence on the extraction efficiency. Hence, PMME was performed without salt addition to the sample solutions.

Evaluation of the PMME method

Stability of poly (HPMA-EDMA) monolithic capillary

As an extraction media, the stability of the monolithic capillary is one of the most important factors for the evaluation of the PMME process. In order to evaluate the stability of the poly (HPMA-EDMA) monolithic capillary under the experimental conditions, the reusability of the capillary has been investigated. The sample monolithic column could be used for more than 200 times without any decrease in extraction efficiency, indicating its stability for practical use. Besides, the interbatch precision of the relative peak areas were 8.0% for 10 μg L^{-1} spiked sample solutions.

Validation of the proposed method

In order to evaluate the efficiency of the proposed method, calibration curves have been constructed with a series of standard samples under the optimal experimental conditions. The results are listed in Table 1. It can be seen that good linearities for all compounds were obtained with the correlation coefficient (r) were always greater than

0.9991. The limits of detection (LODs) were studied for low concentrations and calculated at a signal-to-noise ratio (S/N) of 3. The LODs of the three fungicides were in the range of 0.19 -0.65 μg L^{-1}.

The utility of this method was examined using recovery studies by adding three fungicides in blank water samples at different concentration levels. The recoveries and relative standard deviations (RSD) are summarized in Table 2; mean recoveries are in the range of 80.2-115.6 %.

The reproducibility of the developed method was assessed by the intra-day and inter-day precisions that were expressed as the relative standard deviation (RSD). The intra-day relative standard deviations (RSD) were evaluated on the peak areas of 0.2 μg mL^{-1} standard solutions using six replicates over a day .The inter-day precision was similarly evaluated on six successive days. As shown in Table 1, excellent method reproducibility was found by intra-day and inter-day precisions, yielding the RSD less than 3.1 and 6.3 %, respectively.

Application in real samples

Under the optimized conditions, the proposed PMME and HPLC-UV detection method has been applied for the simultaneous determination of three fungicides (azoxystrobin, diethofencarb and pyrimethanil) in tap water, lake water, field water, pool water and reservior water samples. The chromatograms obtained after PMME and direct injecion under optimal experimental conditions are shown in Fig. 5. In comparison with the chromatogram of direct injection, a dramatic enhancement of the peak height was observed, indicating the remarkable preconcentration capbility of the monolithic capillary to the three fungicides. The result indicates that the proposed method is effective for the determination of the three fungicides in water samples.

Comparison of PMME with other extraction methods

In order to evaluate the feasibility of the proposed method, the comparison of PMME with other extraction methods for the determination of three fungicides (azoxystrobin, diethofencarb and pyrimethanil) has been investigated. As can be seen from table 3, inspite of comparable LODs could be achieved using other extraction methods, sample volume consumed in PMME using poly (HPMA-EDMA)monolith was much less. Besides, other existing methods such as SPE usually need more organic solvent to redissolve target analytes, however, only small volumes of organic solvent was needed for desorption of analytes in PMME due to the small caliber of the capillary. What's more, PMME showed higher extraction capacity because of its unique porous structure, so the method showed greater enrichment of analytes and subsequent higher sensitivity. Though mass spectra detection has higher sensitivity than UV detection, the pretreatment method in this paper had better enrichment capacity for the three fungicides than some existing pretreatment methods. In conclusion, the method developed from this work using HPLC-UV is more sensitive than some existing methods using mass detection.The results demonstrated that PMME using poly (HPMA-EDMA) monolith was simple, fast, sensitive, cheap, environmental friendly and can be used for the trace residue analysis of three fungicides from water samples.

Conclusions

The proposed novel PMME using a poly (HPMA-EDMA) monolith with HPLC has been developed and validated for the analysis of trace three fungicides (azoxystrobin, diethofencarb and pyrimethanil) in tap water, rain water and lake water samples. Effects of sample flow rate, sample pH, eluent volume, eluent flow rate, sample volume and salt effect were investigated to obtain the optimum experimental conditions. The stability, linearity, trueness

and precision have been investigated. In conclusion, this proposed PMME has been advocated as a simple, sensitive, inexpensive, environmentally friendly and rapid sample preparation technique, which can be used as an alternative tool for monitoring the three fungicides in water samples.

Acknowledgements

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Figure captions

Fig.1 Effect of extraction flow rate on the PMME. Fungicides concentration =0.2 μg mL^{-1}, sample volume = 1.0 mL, sample pH 7.0, eluent flow rate = 0.6 mL min^{-1}, microextraction conditions and HPLC conditions are outlined in Section 2.5.

Fig.2 Effect of pH value of the sample matrix. Fungicides concentration = 0.2 μg mL^{-1}, sample flow rate = 0.2 mL min^{-1}, sample volume = 1.0 mL, eluent flow rate = 0.6 mL min^{-1}, microextraction conditions and HPLC conditions are outlined in Section 2.5.

Fig.3 Effect of desorption flow rate on the PMME. Fungicides concentration = 0.2 μg mL^{-1}, sample flow rate = 0.2 mL min^{-1}, sample volume = 1.0 mL, sample pH 7.0, microextraction conditions and HPLC conditions are outlined in Section 2.5.

Fig.4 Effect of extraction volume on the PMME. Fungicides concentration = 0.2 μg mL^{-1}, sample flow rate = 0.2 mL min^{-1}, sample pH 7.0, eluent flow rate = 0.6 mL min^{-1}， microextraction conditions and HPLC conditions are outlined in Section 2.5.

Fig.5 Chromatograms of three fungicides obtained by direct injection (a) and PMME (b). Peaks: 1. azoxystrobin, 2. diethofencarb, 3. pyrimethanil, spiking level was 5.0 μg L^{-1}. Optimal microextraction conditions and HPLC conditions are outlined in Section 2.5.

Table captions

Table 1 Quantitative results of azoxystrobin, diethofencarb and pyrimethanil from water samples.

Analyte	Linear range (μg L^{-1})	r	LODs (μg L^{-1})	LOQs (μg L^{-1})	Intra-day Repeatability (%)	Inter-day Repeatability (%)
Azoxystrobin	3-1000	0.9991	0.19	0.63	2.4	4.9
Diethofencarb	3-1000	0.9993	0.22	0.73	3.1	5.8
Pyrimethanil	3-1000	0.9992	0.65	2.15	2.6	6.3

Table 2 Analytical results and recoveries of three fungicides in real water samples.

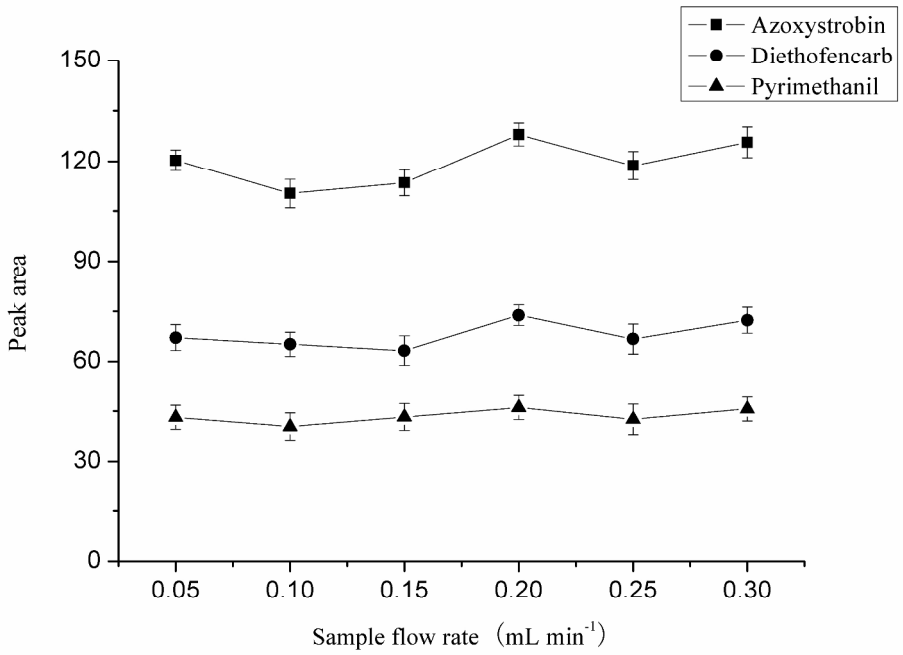
[a] N.D.: not-detected.

Analytes	Sample	Real ($\mu g\ L^{-1}$)	Added ($\mu g\ L^{-1}$)	Relative recovery ($\mu g\ L^{-1}$)	RSD (n=3) (%)
Azoxystrobin	Tap water	ND[a]	1	80.2	3.5
			10	85.9	2.3
	South lake water	ND[a]	1	115.6	2.8
			10	110.1	0.6
	Field water	1.34	1	98.2	4.2
			10	93.7	3.1
	Pool water	0.84	1	85.4	3.6
			10	82.1	2.4
	Reservior water	0.21	1	93.1	2.8
			10	87.4	2.1
	Sha lake	0.43	1	90.8	2.8
			10	91.2	2.6
Diethofencarb	Tap water	ND[a]	1	94.2	2.8
			10	99.0	2.2
	South lake water	ND[a]	1	100.1	4.2
			10	107.6	3.7
	Field water	ND[a]	1	90.6	3.2
			10	96.5	2.8
	Pool water	0.25	1	81.2	3.4
			10	87.4	2.8
	Reservior water	0.38	1	81.8	3.8
			10	90.6	3.2
	Sha lake	0.26	1	89.2	3.1
			10	95.4	2.4
Pyrimethanil	Tap water	ND[a]	1	85.6	4.1
			10	94.9	3.3
	South lake water	ND[a]	1	99.8	3.9
			10	105.4	3.2
	Field water	ND[a]	1	88.5	2.8
			10	93.5	2.4
	Pool water	1.64	1	88.6	3.6
			10	96.4	2.8
	Reservior water	0.75	1	90.2	3.9
			10	99.6	3.4
	Sha lake	ND[a]	1	81.6	3.6
			10	89.4	2.9

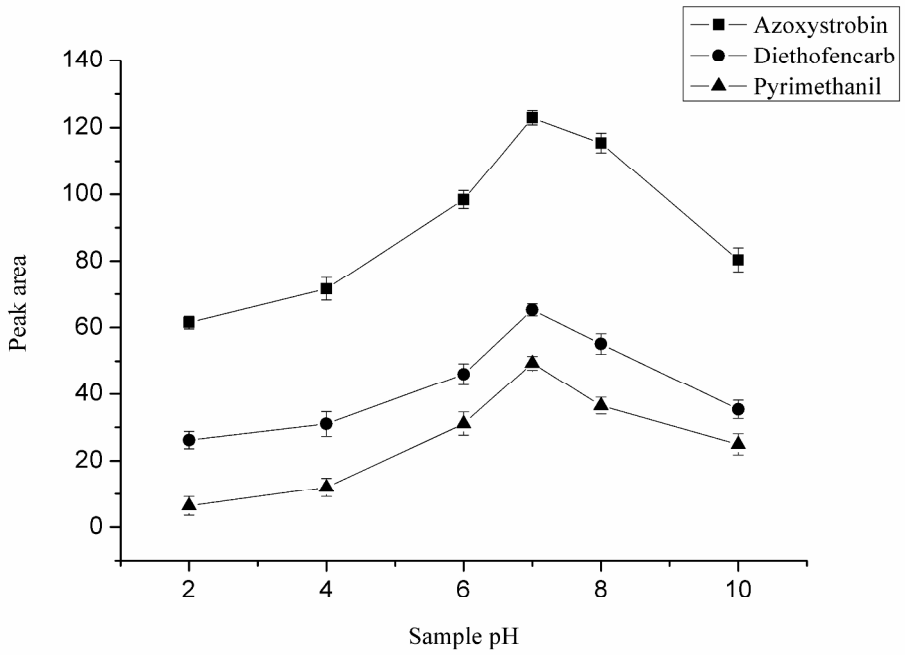
[a] N.D.: not-detected

Table 3 Comparison of PMME with other extraction methods.

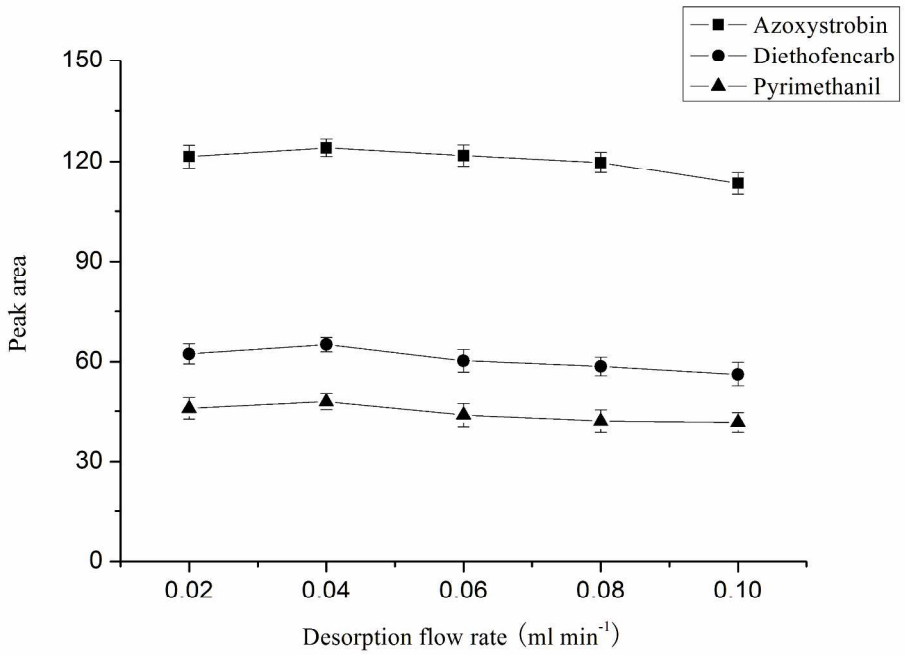
Analytes	Methods	LOD ($\mu g\ L^{-1}$)	Sample volume (mL)	References
Azoxystrobin	SPE-HPLC-UV	9.7 $\mu g\ L^{-1}$	5 mL	[8]
	LLE-LC-DAD	200 $\mu g\ L^{-1}$	5 mL	[5]
	PMME-HPLC-DAD	0.19 $\mu g\ L^{-1}$	2 mL	This work
Diethofencarb	HF-LPME-UHPLC–MS/MS	0.5 $\mu g\ L^{-1}$	15 mL	[29]
	DLLME-SFO-HPLC-DAD	0.24 $\mu g\ L^{-1}$	5 mL	[30]
	PMME-HPLC-DAD	0.22 $\mu g\ L^{-1}$	2 mL	This work
Pyrimethanil	SPE-LC-MS	5.0 $\mu g\ g^{-1}$	25 mL	[6]
	SPE-LC-MS	1.8 $\mu g\ L^{-1}$	400 mL	[7]
	PMME-LC-DAD	0.65 $\mu g\ L^{-1}$	2 mL	This work



287x215mm (300 x 300 DPI)



287x215mm (300 x 300 DPI)



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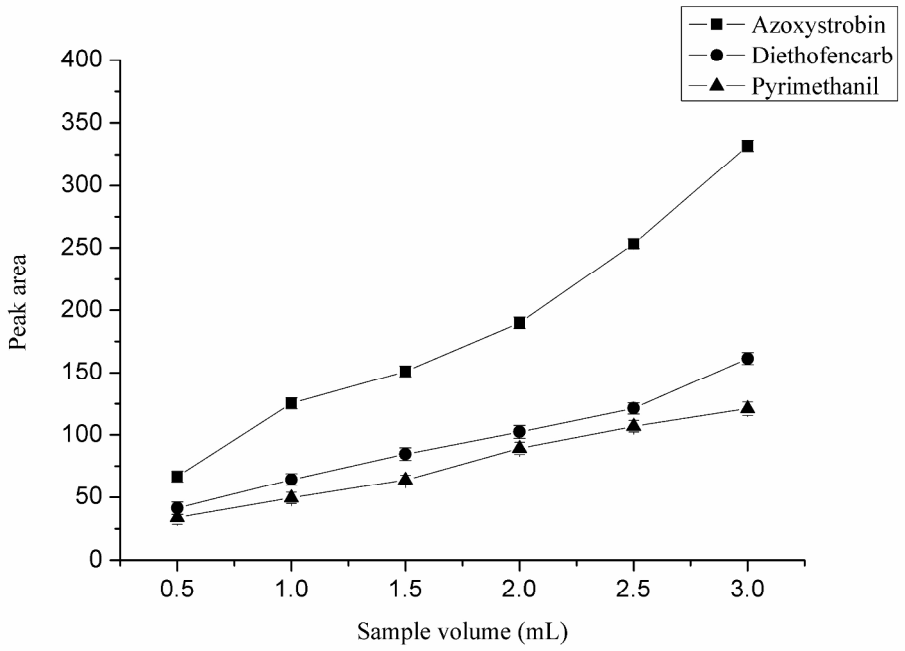
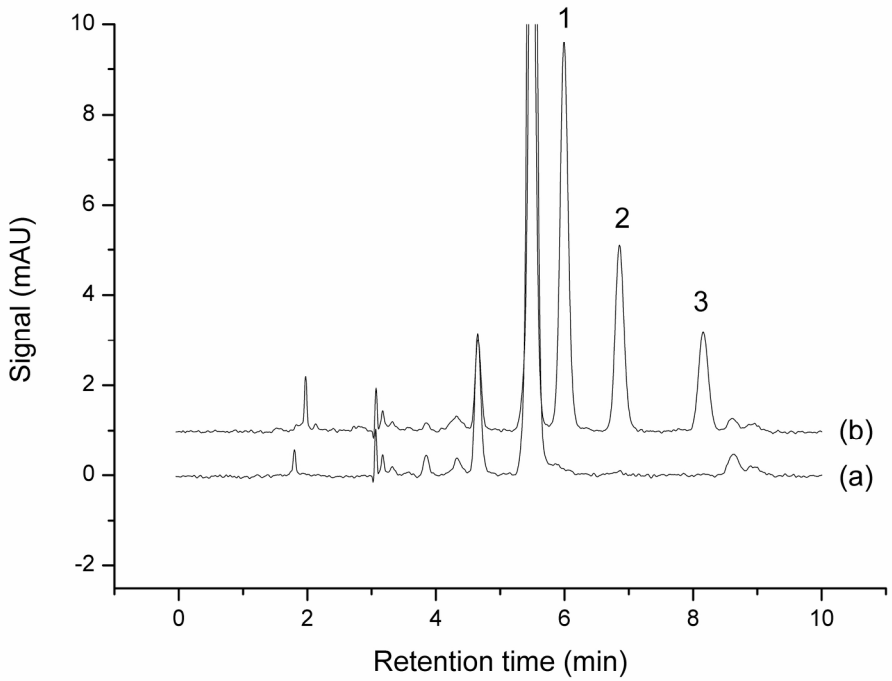


287x213mm (300 x 300 DPI)



202x160mm (300 x 300 DPI)