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Methyl Salicylate-Based Vortex-Assisted Surfactant-Enhanced Emulsification Microextraction and HPLC for Determination of Fungicides in Honey Samples

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Abstract

Methyl salicylate based vortex-assisted surfactant-enhanced emulsification microextraction (MeSA-VASEME) has been developed and applied for rapid preconcentration of fungicides (i.e., carbendazim, thiabendazole, and fluberidazole) in honey samples followed by high performance liquid chromatographic analysis. MeSA was used as an extraction solvent, while surfactant was used to enhance the extraction performance under the dispersion by vortex agitation. The optimum MeSA-VASEME conditions were 100 µL MeSA, 2.0 mmol L^{-1} sodium dodecyl sulfate, and vortex agitation at 1200 rpm for 90 s. Preconcentration factors were obtained in the range of 32–40. The limit of detection in the studied honey samples was 0.5 µg L^{-1}. The recovery of the spiked target fungicides at 20, 50, and 100 µg L^{-1} were 81.5–116.8 % with the relative standard deviation below 11%. The proposed method is simple, sensitive, less organic solvent consuming, inexpensive, and a rapid procedure for the residue analysis of fungicides in honey samples.

Keywords: Methyl salicylate, VASEME, HPLC, Fungicide, Honey

1. Introduction

Bee products including honey are natural products that are rich in minerals, antioxidants, and simple sugars.[1] Honey is found to be used as enzymatic and nonenzymatic antioxidant to prevent deteriorative oxidation reactions in foods such as the browning of fruit and vegetables, lipid oxidation in meat, and to inhibit the growth of food borne pathogens and microorganisms leading to food spoilage.[1] In addition, honey has potential therapeutic properties in infections, wound healing, and cancer.[1] However, bee products can also be a source of toxic substances, such as heavy metals, radioactive isotopes, organic pollutants, and pesticides (e.g., insecticides, fungicides, herbicides, and bactericides) due to environmental pollution and misuse of beekeeping practices.[1,2] Indirect contamination of honey by pesticides can be found during the pesticide applications in agriculture through soil, water, air, and flowers and then bees come into contact with the pesticides and collect nectar to produce the honey.[3] Pesticide residues (e.g., organohalogens, organophosphates, organonitrogen, pyrethroids, and carbamates) in honey samples have been reported in the range of 0.05–4310 µg kg^{-1} and were found in many countries.[2] According to the legislations of maximum residue limits (MRLs) set by the European Union (EU) and Official of Brazil, it should be below 50 µg kg^{-1} for most pesticides.[3]

Benzimidazole fungicides are widely used in agriculture for pre- and post-harvest treatment to control and kill fungi or fungal spores in order to prevent the spoilage of crops.[4–6] The active benzimidazole fungicides include benomyl (BN), carbendazim (CBZ), thiabendazole

(TBZ), fuberidazole (FuBZ), thiophanate (TP), and thiophanate-methyl (TPM). Most of the fungicides are normally used to control various diseases in various fruits and vegetables. They are directly applied to soil or sprayed over the crop fields.[4,7] Hence, the studied fungicides may contaminate natural honey after bees come into contact with contaminated plants. From the literature, it was found that CBZ at the level of 1.62 μg kg^{-1} was detected in the honey sample.[8] There are several toxic effects from this fungicide exposure including teratogenicity, congenital malformations, polyploidy, diarrhea, anemia, pulmonary edemas, or necrotic lymphoadenopathy.[5,9] Therefore, the development of highly sensitive techniques for trace residue analyses of fungicides in various sample matrices (e.g. food and enverionmental samples) has been increasingly important for the environment and health protection.

The simultaneous residue determinations of benzimidazole fungicides using micellar electrokinetic chromatography (MEKC),[10] and high performance liquid chromatography (HPLC)[11–14] have been popularly employed. Recently, the solvent-microextraction technique based on the application of vortex agitation, namely vortex-assisted liquid-liquid microextraction (VALLME) has been reviewed.[15] Furthermore, surfactants (as emulsifiers) are used instead of disperser solvents (used in DLLME). This technique is named vortex-assisted surfactant-enhanced emulsification microextraction (VASEME). It was found that VALLME overcomes the disadvantage of DLLME (required disperser solvents), while surfactants used in VASEME assist extraction solvents to better disperse into a sample solution.[16] The combination of vortex agitation and surfactant has also been widely applied to improve the extraction performance and used for the analysis of various compounds.[16–21] In VASEME, extraction solvents/surfactants such as carbon tetrachloride/Triton X-100, toluene/CTAB, 1-octanol/SDS+CTAB, trichloromethane/ammonium perfluorooctanoate, 1-undecanol/Triton X-100, and methyl benzene/Tween 20 can be used.[16–21] As mentioned above,[16–21] it was found that a toxic extraction solvent (e.g. carbon tetrachloride) was used. Meanwhile, the use of lighter density solvents (e.g. toluene, octanol, undecanol) proved difficult to separate and collect the upper extract phase and normally needed special devices to accomplish the phase separation. To overcome these limitations, an alternative extraction solvent such as methyl salicylate (MeSA) seems to be interesting for the extraction and preconcentration of organic compounds, such as fungicides. MeSA has some important characteristics such as (1) high density (1.17 g mL^{-1}), (2) clear liquid solution at room temperature, (3) low water solubility (700 mg L^{-1}), and (4) low cost.[22] As our previous work demonstrated, MeSA was used in quite a large volume (250 μL) and extraction was carried out in the presence of salt in the extraction solution.[22] However, it seems to be suitable for non-polar compounds, except CBZ. Thus, the further development of preconcentration based on MeSA is of interest. The use of MeSA and surfactant (as emulsifier) instead disperser solvent and salt could maybe improve the performance of extraction of target fungicides, especially CBZ. The application of the proposed VASEME using MeSA as an extraction solvent has not been used for the analysis of fungicides in honey samples.

This work is aimed at the development and extension of our previous work using a method named methyl salicylate based vortex-assisted surfactant-enhanced emulsification microextraction (MeSA-VASEME) coupled with HPLC for the simultaneous analysis of target benzimidazole fungicides (e.g. CBZ, TBZ, and FuBZ) in honey samples. The variables affecting MeSA-VASEME procedure were investigated, and analytical performances as well as method validation were also evaluated.

2. Experimental

2. 1. Chemicals and Reagents

The chemicals and reagents used in this study are of AR grade or higher. The analytical standards of fungicides were purchased from Sigma-Aldrich including CBZ (Munich, Germany), TBZ (Milan, Italy), and FuBZ (Munich, Germany). The stock solutions of each fungicide were prepared at 1,000 mg L^{-1} by dissolving an appropriate amount in a small volume (~500 μL) of formic acid and further dilution with methanol (MeOH). Methyl salicylate was obtained from Sigma-Aldrich (Shanghai, China). MeOH, ethanol (EtOH), formic acid, and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was purchased from BDH Prolab (Leuven, Belgium). Triton X-100 was purchased from Sigma-Aldrich (MO, USA), while Tergitol® TMN-10 (Sigma-Aldrich, MO, USA) and cetyl trimethylammonium bromide (CTAB) (Sigma-Aldrich, Bangalore, India) were also purchased. The solutions of SDS (100 mmol L^{-1}), CTAB (100 mmol L^{-1}), Triton X-100 (25%, *w/v*), and Tergitol TMN-10 (25%, *w/v*) were prepared in deionized water before use. Sodium chloride (NaCl) (Ajax Finechem, Auckland, New Zealand), anhydrous sodium sulfate (anh. Na_2SO_4) (Carlo Erba, Val de Reuil, France), sodium carbonate (Na_2CO_3) (RFCL Limited, New Delhi, India), and anhydrous sodium acetate (anh. NaOAc) (Carlo Erba, Val de Reuil, France) were used. All aqueous solutions were prepared in deionized water with resistivity of 18.2 MΩ cm from RiO_s^{TM} Type I Simplicity 185 (Millipore water, MA, USA).

2. 2. Instruments

The HPLC coupled with a photo-diode array detector (PDA) (Shimadzu Corporation, Kyoto, Japan) was used. Data analysis and acquisition of the system were controlled using LCsolution software (Shimadzu). An In-

ertsil C8 column (4.6 × 150 mm, 5.0 μm) connected to a guard C8 column (4.0×10 mm, 5.0 μm) (GL Science, Tokyo, Japan) was used as the separation column for target fungicides. Centrifuge NF200 model (Nüve Inc., Ankara, Turkey) and a vortex mixer Genie-2 model (Scientific Industries Inc., NY, USA) were also used.

2. 3. HPLC Separation Conditions

The reversed-phase HPLC was used for complete separation of the studied fungicides under the gradient elution of ACN and 0.1% (*v/v*) formic acid as an optimal mobile phase. A flow rate of 1.0 mL/min was performed throughout the separation process. The chromatographic separation was performed at 25 °C. The detection of the target analytes was performed at 280 nm for CBZ, and at 311 nm for TBZ and FuBZ. The column gradient program[12,22] consisted of 0–2.0 min 15% ACN, 2.0–4.0 min ramped linearly from 15 to 45% ACN, and then 4.0–6.0 min ramped linearly to 75% ACN. After the composition was further kept constant at 75% ACN for 3 min, ACN was linearly decreased to 45% and 15%, respectively. When the pressure reached its initial value, the next separation process could be performed.

2. 4. Sample Analysis

Honey samples were purchased from a supermarket in Khon Kaen province, Thailand. Accurate weight (1.00 g) of sample was dissolved in 10 mL water. Then, the 10 mL sample solution was extracted using the MeSA-VASEME procedure and analyzed by HPLC. To evaluate the accuracy, the studied honey samples were fortified with the standard fungicides at various concentration levels of 20, 50, and 100 μg L^{-1} prior to the preconcentration.

2. 5. MeSA-VASEME Procedure

Methyl salicylate (100 μL) and SDS (2 mmol L^{-1}) were injected into the 15 mL conical tube containing a standard or sample solution (10.00 mL). Then, the solution was manually shaken for 15 s before vortex agitation at 1200 rpm for 90 s. After centrifugation at 3000 rpm for 1 min, the extract phase was obtained (at the bottom of the tube). The aqueous phase was then removed by microsyringe. Subsequently, the extract rich phase was mixed with MeOH (100 μL) before subjecting it (20 μL) to HPLC for the analysis.

2. 6. Calculation of Preconcentration Factor and Extraction Recovery

Preconcentration factor (PF) and extraction recovery (ER) were used to evaluate the performance of the extraction method and were calculated using the following equations:

$$PF = \frac{C_{ext}}{C_0} \quad (1)$$

$$ER(\%) = \frac{C_{ext}}{C_0} \times \frac{V_{ext}}{V_0} \times 100 = PF \times \frac{V_{ext}}{V_0} \times 100 \quad (2)$$

where C_{ext} is defined as target compound concentration in the collected phase, while C_0 is the initial analyte concentration. The calculation of C_{ext} was conducted from the standard calibration curves obtained from the direct analysis (without preconcentration). V_{ext} and V_0 are the volume of the collected phase and initial aqueous sample solution (10 mL), respectively.

3. Results and Discussion

The parameters affecting extraction recovery of target fungicides were investigated including surfactant, extraction solvent, salt additive, solution pH, vortex agitation and centrifugation. One-parameter-at-a-time was used for optimizing extraction conditions, while the other remaining factors were kept constant.

3. 1. Effect of Surfactants

By trial and error, three different extraction compositions including (a) MeSA in the presence of sodium acetate, (b) MeSA containing MeOH (as disperser solvent) and sodium acetate, and (c) MeSA in the presence of SDS, were studied. As the results shown in Figure S1 indicate, the chromatograms obtained from the conditions (a) and (b) are similar. This means that the extraction performance of the methods for three studied compounds is insignificantly different under the presence of disperser solvent and/or salt. Meanwhile, MeSA in the presence of SDS gave the highest peak height especially for CBZ. This behavior indicated that SDS can be used as a good emulsifier for improving the extraction of polar analytes. Therefore, the effect of SDS in comparison with other surfactants on the extraction performance of the target fungicides was further investigated.

Theoretically, surfactant was used as an emulsifier in various microextraction methods to accelerate the emulsification of water-immiscible solvent in the aqueous sample solution.[23–25] It has been proven that the addition of surfactant can improve the penetration of different target hydrophobic compounds due to its hydrophobic and hydrophilic groups within the molecule.[26] In this study, surfactants included SDS (at 2.00 mmol L^{-1}), CTAB (at 0.50 mmol L^{-1}), Tergitol TMN-10 (at 2.71 mmol L^{-1}), and Triton X-100 (0.12 mmol L^{-1}), while the concentration tested was lower than the critical micelle concentration (CMC) for each surfactant. The CMCs of SDS, CTAB, Tergitol TMN-10, and Triton X-100 were 8, 0.92, 5.7, and 0.24 mmol L^{-1}, respectively. The results (Figure 1a) show that SDS (anionic surfactant) provided the highest extraction

recovery in comparison to no surfactant addition, cationic (e.g. CTAB), and non-ionic (e.g. Tergitol TMN-10 and Triton X-100) surfactants. Is may be assumed that the target fungicides (pK_a~5-6) were in the positive charge[5] under the acidic conditions studied (pH 4) and consequently favorably penetrated and were strongly attracted to SDS molecules. Meanwhile, less interaction between positively charged analytes and cationic or non-ionic surfactant was expected. In addition, it has been reported that good emulsification process was obtained when the concentration of surfactant was lower than CMC.[16] Thus, SDS was then selected for further investigation.

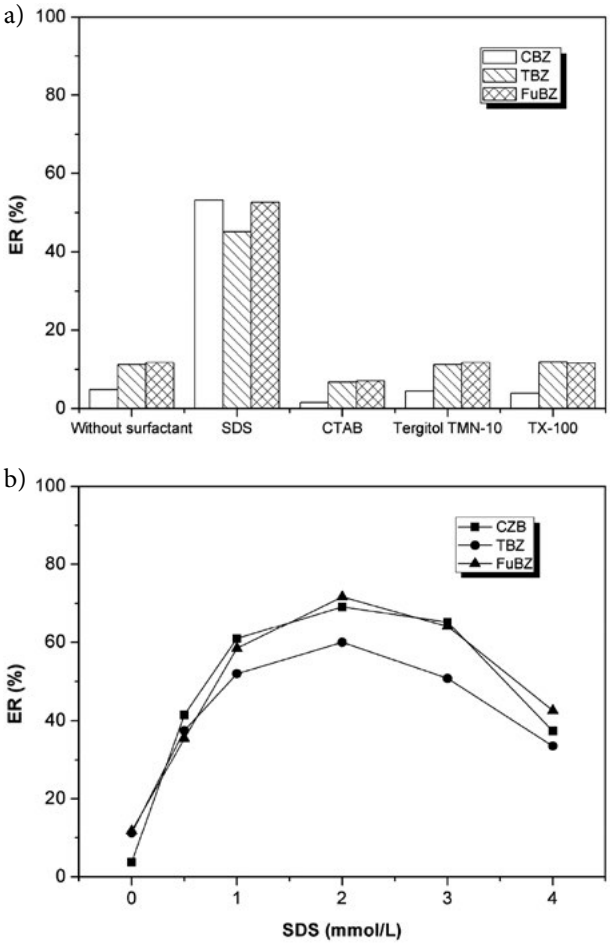

Figure 1: Effects of (a) surfactant and (b) SDS on the extraction recovery of the target analytes

The influence of SDS concentration on the extraction recovery of target fungicides was evaluated in the range of 0–4 mmol L^{-1} (see Figure 1b). Increase in the extraction recovery for most target fungicides when concentration of SDS increased was observed up to 2 mmol L^{-1}. High concentrations of SDS did not promote better extraction recovery of the target analytes. This behavior may be because of strong self interaction of SDS molecules and the analytes more favorably dissolve in the aqueous phase resulting in decreased extraction recoveries. In this study, 2 mmol L^{-1} SDS was chosen.

3. 2. Selection of Extraction Solvent and its Volume

Methyl salicylate was used as an extraction solvent in this study. Based on the logK_{ow} (related to hydrophobicity of the analytes) of target fungicides (1.5–2.7) and MeSA (2.55), MeSA seems to be good for extraction of the hydrophobic target fungicides, especially for TBZ and FuBZ. For CZB, the combination of MeSA and SDS could be used for improving the extraction performance of the method. Good characteristics of MeSA as an extraction solvent include (i) the presence of the extract phase at the bottom of the bulk solution (due to its density >1.0 g mL^{-1}), (ii) highly solubility in the organic mobile phase, and (iii) no interference of the excess MeSA with the target fungicide peaks.

In this study, the volume of MeSA on the extraction recovery was further studied by varying the volume in the

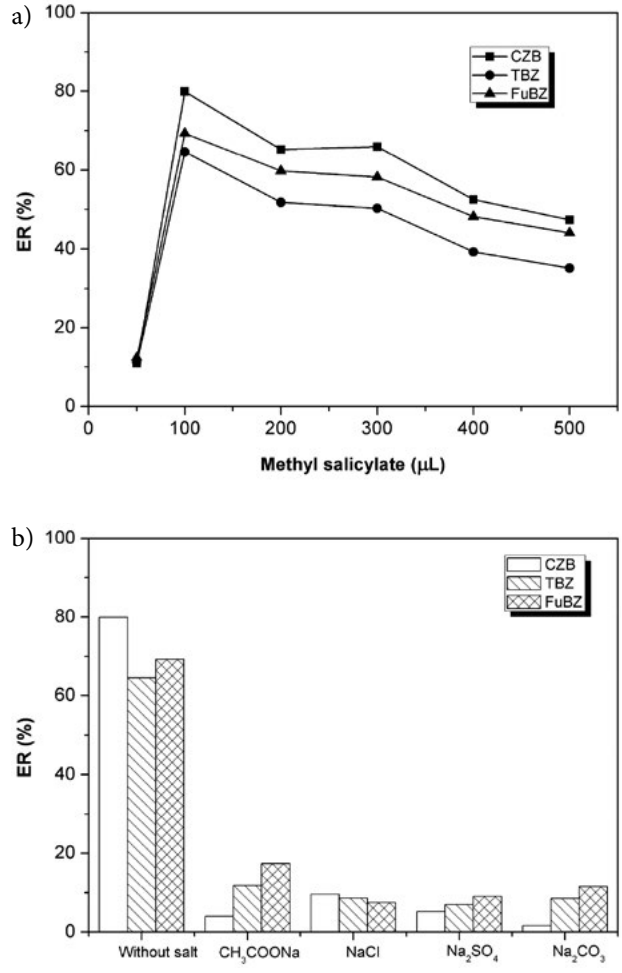

Figure 2: Effects of (a) volume of methyl salicylate and (b) salt addition on the extraction recovery of the target analytes (250 μg L^{-1} each).

range of 50–500 μL (Figure 2a). The highest extraction recovery was observed at the MeSA volume of 100 μL and decrease in the extraction performance afterwards. It may be due to the dilution of the target fungicides in higher volume of extract MeSA phase. Thus, 100 μL MeSA was chosen as the optimal value.

3. 3. Effect of Salt Addition

In general, the addition of salt into the aqueous sample solution can enhance the extraction recovery of target analytes by decreasing the solubility of the analytes in the aqueous phase and increasing mass transfer toward the organic phase.[15] The decrease in water solubility of target compounds in bulk aqueous phase was expected, resulting in increasing mass transfer of target compounds towards the extract phase. In this investigation, CH_3COONa, NaCl, Na_2SO_4, and Na_2CO_3 were studied at the equal molar concentration (~1.71 mmol L^{-1}) and compared to with and without salt addition (Figure 2b). It is clearly seen that the addition of salt could not help to improve the extraction recovery of target analytes when compared to the process without salt addition. This may be explained by (i) charge interaction between SDS and counter ions of salts resulting in decreased capability of SDS in the solution, and (ii) salt addition leading to increased viscosity of the bulk aqueous phase. In this study, salt addition was not required throughout the experiments.

3. 4. Effect of Solution pH

The effect of sample pH on the extraction recovery of the target fungicides was investigated in the range of 2.0–8.0 (data not shown). It can be found that the pH value strongly affected the extraction efficiency of MsSA-VASEME for fungicide analytes. The highest extraction recovery was found at the pH 4.0 (as the original pH value, without pH adjustment), while higher pHs decreased the extraction efficiency for most analytes. The reason may be the fact that the analytes (pK_{a1} ~4-5) are in the cationic form (i.e. positive charge) under the acidic pHs and favorably attract SDS molecules (negative charge). On the other hand, the analytes present in the neutral form or negatively charged form result in less interaction between the analytes and SDS. From the investigation, the original pH of the solution of about 4.0 was chosen for the extraction of target fungicides.

3. 5. Effect of Vortex Agitation (Extraction Time)

Vortex agitation (speed and time) is one of the important factors in vortex-based microextraction method because it affects the extraction equilibrium (e.g. emulsification and distribution process) of target analytes, and consequently influences the extraction efficiency.[12,17,27] The vortex agitation time was investigated between 30 and 150 s at speed of 1200 rpm, while the agitation speed was studied in the range of 600–2100 rpm. The results are shown in Figure S2 (a & b), which reveals that appropriate speed and time for the vortex agitation can improve extraction efficiency of the method. In this study, the highest extraction recoveries were found at 90 s at 1200 rpm. A higher speed rate (> 1200 rpm) and longer time (> 90 s) decreased the extraction recovery of the target fungicides. Thus, the vortex agitation was chosen at 1200 rpm for 90 s for further evaluation.

3. 6. Effect of Centrifugation Time and Speed

Centrifugation speed and time were also investigated in this study because they affect the phase separation of a sample solution. In our previous work,[22] it was reported that a low centrifugation speed (e.g. < 2500 rpm) could not cause complete phase separation, while the decreased extraction recoveries were observed at high centrifugation speed (i.e. 4000 rpm). In this study, the centrifugation speed (2000–3500 rpm) and time (0–5 min) were investigated (see Figure S3 (a & b)). It is clearly seen that the best extraction recoveries were obtained at a speed of 3000 rpm as the optimum speed for obtaining complete phase separation, and there was a decrease in extraction recoveries afterwards. Meanwhile, the highest extraction recoveries were also observed at the appropriate centrifugation time of 1 min. Therefore, centrifugation at 3000 rpm for 1 min was selected.

3. 7. Analytical Performance of the Method

The analytical performance and method validation of the proposed MeSA-VASEME were investigated in two sample matrices (i.e. ultrapure water and honey). The studied parameters were linear dynamic range, coefficient of determination (R^2), limits of detection (LODs), limits of quantitation (LOQs) and precision (intra-day and inter-day measurements). LODs were defined as the concentration of the target analytes giving the signal-to-noise ratio of 3 (S/N = 3), while LOQs were defined as the S/N = 10.

In ultrapure water medium, the linearity was found in the range of 0.1–100 μg L^{-1} with R^2 greater than 0.995. LODs were obtained between 0.01 and 0.05 μg L^{-1}, while LOQs were in the range of 0.1–0.2 μg L^{-1}. On the other hand, the LODs obtained from the method without preconcentration were found to be 3 μg L^{-1} for the studied analytes. The intra-day (n = 6) and inter-day (n = 6 × 3 days) precisions were also investigated by replicate injections of the certain concentration of 100 μg L^{-1} in a day and over several days. The relative standard deviations (RSDs) in terms of peak area and retention time were calculated. It was found that the RSDs below 8.3% for peak area and retention time were obtained. Under the optimal

conditions, preconcentration factors and extraction recoveries were obtained in the range of 32–40, and 64–79%, respectively.

For the investigation in honey samples, the analytical features and method validations were studied in real honey samples. Matrix-matched calibration was performed in this study. The results are summarized in Table 1. The linear dynamic range was in the range of 2–200 μg L^{-1} with $\underline{R}^2$ higher than 0.995. The calibrations obtained in each sample are listed in Table 2. LODs and LOQs in honey sample (Brand#1 as a representative sample) were 0.5 and 2 μg L^{-1}, respectively. Precisions in terms of intra-day (n = 6) and inter-day (n = 3 × 3 days) were also studied and expressed as the relative standard deviations (RSDs) of the studied target fungicides at a certain concentration each. High precisions with RSDs below 12% were accepted.

where C_{detect} is the detected concentration of analytes after the addition of known amount of standard to real sample, C_{real} is the concentration of the target analytes found in real sample, and C_{add} is the concentration of the spiked known amount of standard solution in the real sample.

The chromatograms obtained from the spiked samples (see Figure 3) and recovery results (Table 3) are shown. Good relative recoveries of the target fungicides in honey samples were found in the range of 81.5–116.8% with the relative standard deviation below 11%. Intra-day precision (n = 6) and intermediate precision (n = 3 × 3 days) of the proposed method were also studied in the spiked honey sample (Brand#1 as a representative sample) at 100 μg L^{-1} of each fungicide. The studied precisions provided the RSD below 12%. The obtained recoveries and %RSD were in good agreement with the acceptable values of 70–120%

Table 1: Figures of merit of the proposed method for the determination of the benzimidazole fungicides in honey samples

Analyte	Linearity (μg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})	Intra-day precision[a] (n = 6), %RSD	Inter-day precision[a] (n = 3 × 3 days), %RSD
CBZ	2–200	0.5	2	7.7	11.7
TBZ	2–200	0.5	2	8.4	10.0
FuBZ	2–200	0.5	2	8.6	7.2

[a] Precisions were evaluated at the concentration of 100 μg/L for each fungicide spiked in honey brand # 1 (as a representative sample).

Table 2: Calibrations obtained by the proposed method for the determination of the benzimidazole fungicides in honey samples

Analyte	Honey Brand#1		Honey Brand#2		Honey Brand#3		Honey Brand#4	
	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2
CBZ	y = 532x + 3385	0.996	y = 805x – 3714	0.996	y = 508x – 1717	0.998	y = 438x + 1506	0.997
TBZ	y = 708x + 8048	0.995	y = 1069x – 4201	0.997	y = 791x – 537	0.999	y = 573x + 3776	0.999
FuBZ	y = 1068x + 1600	0.996	y = 1865x – 8732	0.997	y = 1249x + 796	0.997	y = 940x + 6855	0.997

3. 8. Application to Real Samples

The proposed method was then evaluated in different commercial brands of honey samples (4 brands). The matrix-matched calibration was used in this study to avoid endogenous interferences effect on the analysis. The identification and confirmation of the target peaks of the analytes were performed using comparison of retention time of the standard analytes and their absorption spectra data obtained from PDA. An example of honey sample blank is demonstrated in Figure 3. It was found that contamination by the studied fungicides in the studied honey samples was not detected. Accuracy in terms of relative recovery (RR) test at different concentrations spiked (e.g. 20, 50, and 100 μg L^{-1}) in real honey samples was also investigated. The RR(%) was used for the evaluation of real honey sample analyses. The calculation of RR(%) is as follows:

$$RR(\%) = \frac{C_{detect} - C_{real}}{C_{add}} \times 100 \quad (3)$$

with RSD less than of 20%, at the concentrations spiked in the range of 10–100 μg L^{-1}.[28] According to the results obtained, the proposed method was effective and reliable for the determination of target fungicides in honey samples.

3. 9. Comparison of the Proposed Method to Other Relevant Strategies

The developed MeSA-VASEME method and other related strategies coupled to HPLC for the analysis of benzimidazole fungicides in various samples (e.g. water) are compared and summarized in Table 4. The SPME[4] and MISPE[6] are promising methods but SPME is expensive and requires a long incubation time (~40 min), high temperature, and high electrolyte salt. Meanwhile, MISPE is a tedious procedure and requires a long synthesis time for the sorbent. Micellar extractions (or cloud-point extractions) using surfactants are normally performed at high incubation temperature (e.g. 90 °C) for a long time.[29] The

Table 3: Recovery obtained from the determination of benzimidazole fungicides in honey samples (n = 3)

Analyte	Spiked (μg L^{-1})	Honey brand #1			Honey brand #2			Honey brand #3			Honey brand #4		
		Detected (μg L^{-1})	RR (%)	RSD (%)	Detected (μg L^{-1})	RR (%)	RSD (%)	Detected (μg L^{-1})	RSD (%)	RR (%)	Detected (μg L^{-1})	RR (%)	RSD (%)
CBZ	0	ND	–	–	ND	–	–	ND	–	–	ND	–	–
	20	22.97	114.8	3.7	17.47	87.3	4.8	21.15	105.8	5.7	17.99	90.0	1.8
	50	52.81	105.6	1.4	57.55	115.1	7.1	47.60	95.2	3.2	52.88	105.8	9.2
	100	111.62	111.6	10.8	115.05	115.0	3.3	85.56	85.6	4.4	109.89	109.9	9.1
TBZ	0	ND	–	–	ND	–	–	ND	–	–	ND	–	–
	20	16.77	83.8	3.9	17.77	88.9	4.1	19.10	95.5	8.1	16.55	82.7	2.4
	50	46.82	93.6	6.9	53.41	106.8	6.9	50.99	102.0	5.9	44.05	88.1	9.4
	100	89.27	89.3	6.7	115.22	115.2	1.6	90.30	90.3	5.9	111.12	111.1	6.9
FuBZ	0	ND	–	–	ND	–	–	ND	–	–	ND	–	–
	20	19.90	99.5	8.0	16.34	81.7	4.3	20.97	104.9	6.0	16.58	82.9	2.4
	50	50.70	101.4	0.6	55.18	110.4	6.8	47.27	94.5	5.9	40.76	81.5	10.0
	100	103.05	103.0	5.7	116.81	116.8	2.1	90.19	90.2	8.7	107.20	107.2	8.5

ND: Not detected RR: Relative recovery

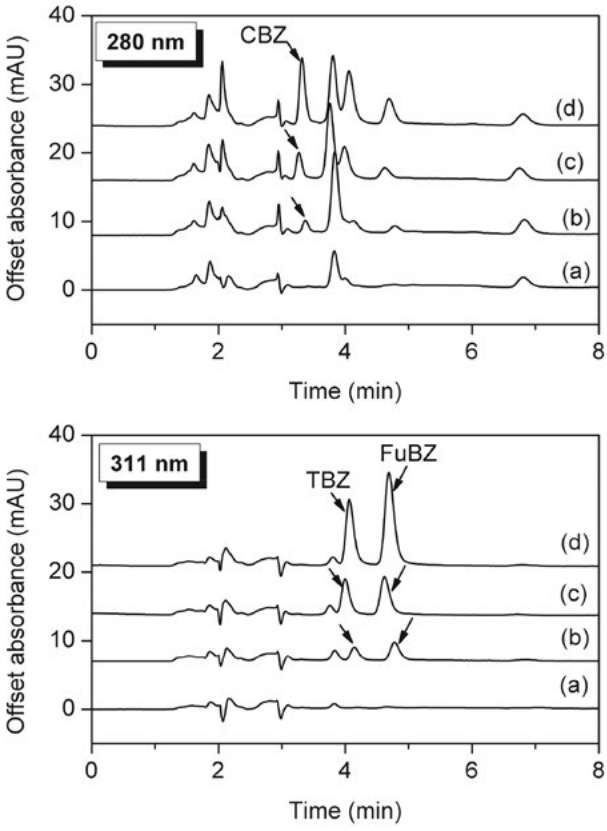

Figure 3: Overlaid chromatograms of honey samples (Brand#1) with (a) honey sample blank and (b–d) spiked at various concentrations of target fungicides (20, 50, and 100 μg L^{-1}, respectively), evaluated at 280 nm and 311 nm.

conventional DLLME uses toxic chlorinated solvent (e.g. chloroform) and needs disperser solvent for emulsification.[30] It was difficult to withdraw the upper extract rich phase and remove the aqueous lower phase in SALLME.[11] It is clearly seen that the proposed MeSA-VASEME provides comparable results such as LODs and recovery. The developed method is useful for the quantification and qualification of the target fungicides at trace levels in the studied samples. The advantages of the method are a simple procedure, short extraction time, short analysis time, and low cost. The proposed MeSA-VASEME can also be used as an alternative powerful method to the other our previous works demonstrated.[22]

4. Conclusions

A simple and fast procedure for preconcentration and analysis using MeSA-VASEME and HPLC-PDA has been successfully developed for target fungicides in honey samples. The preconcentration based MeSA in the presence of surfactant (e.g. SDS) has also been proven to improve the extraction efficiency of target compounds, especially polar analytes. Good extraction efficiency, recovery, and reproducibility were achieved. Low limits of detection at 0.5 μg L^{-1} in honey samples were also obtained. Less consumption of solvents used for the preconcentration step (< 500 μL), short extraction time (< 10 min), and short separation time (< 5 min) are the advantages of the developed method. The proposed method can be used as an alternative method for trace residue analysis of target fungicides in the studied sample and other related matrices.

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Table 4: Comparison of the HPLC technique with different preconcentration methods for the determination of benzimidazole fungicides in various sample matrices

Analyte	Extraction/ clean-up	Extraction condition	Sample matrix	LOD (μg L^{-1})	Recovery (%)	Ref.
BN, CBZ, TBZ, FuBZ	SPME	• Carboxen-polydimethylsiloxane 75 μm fiber • Heated at 60 °C for 40 min and stirred at 600 rpm • Desorbed by MeOH for 10 min	Water	0.03–1.30	80.9–119.6	4
BN, CBZ, TBZ, FuBZ	Micellar extraction	• Genapol X-080 or POLE (4%, *v/v*), 4% (*w/v*) NaCl, 20-min at 90 °C	Water	0.008-6.4 (Genapol X-080), 0.004–5.9 (POLE)	74–92 (Genapol X-080), 72–91 (POLE)	29
CBZ, TBZ	DLLME	• Chloroform (extraction solvent) + tetrahydrofuran (disperser solvent) + 10% (*w/v*) NaCl	Water	0.5–1.0	84.0–94.0	30
BN, CBZ, TBZ, FuBZ, FluBZ, FBZ, ABZ	MISPE	• Molecularly imprinted polymer-divinylbenzene • Eluted by MeOH/acetic acid (50/50, *v/v*) • Evaporated and re-dissolved in ACN	Water	0.002–0.012	90–106	6
CBZ, FuBZ, TPM, TP	SALLE	• ACN (2 mL) + NaH_2PO_4 (0.1 M) + NaCl (5.0 M) • Dried extract phase and re-dissolved with ACN (70%, *v/v*)	Water	0.14–0.38	60.4–99.1	11
CBZ, TBZ, FuBZ	VA-DLLME	• 250 μL methyl benzoate + 300 μL EtOH + NaOAc (1.0%, *w/v*)	Water	0.01–0.05	77.4–110.9	12
CBZ, TBZ, FuBZ	MeSA-DLLME	• 250 μL methyl salicylate + NaOAc (1.0%, *w/v*)	Water	0.03–0.05	74.1–118.4	22
CBZ, TBZ, FuBZ	MeSA-VASEME	• 100 μL methyl salicylate + 0.2 mM SDS	Honey	0.5	81.5–116.8	Proposed method

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Povzetek

Za hitro predkoncentracijo fungicidov (karbendazim, tiabendazol, fluberidazol) iz vzorcev medu smo uporabili emulzifikacijsko ekstrakcijo s pomočjo surfaktanta in z uporabo vorteksa s topilom metil salicilatom (MeSA-VASEME), ki ji je sledila analiza z visokozmogljivo tekočinsko kromatografijo. Metil salicilat (MeSA) smo uporabili kot ekstrakcijsko topilo, medtem ko je surfaktant izboljšal ekstrakcijo ob disperziji z vorteks mešanjem. Optimalni MeSA-VASEME pogoji so bili: 100 µL MeSA, 2,0 mmol L^{-1} natrijevega dodecil sulfata in mešanje z vorteksom pri 1200 rpm za 90 s. Dobili smo predkoncentracijske faktorje v območju 32–40. Meja zaznave v preiskovanih vzorcih medu je bila 0,5 µg L^{-1}. Izkoristek dodanih tarčnih fungicidov pri 20, 50 in 100 µg L^{-1} je bil 81,5–116,8 % z relativnim standardnim odklonom pod 11 %. Predlagana metoda je preprosta, občutljiva, porabi manj organskega topila, ni draga, je hiter postopek za analizo preostankov fungicidov v vzorcih medu.