Rapid Detection of Pesticides in Honey by Solid-Phase Micro-extraction Coupled with Electrospray Ionization Mass Spectrometry

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Abstract

Detection of pesticide residues in food samples is important for safeguarding food quality and safety. Conventional approaches for detection of pesticides in food samples typically involve labour-intensive and time-consuming sample pre-treatment and chromatographic separation. In this study, solid phase micro-extraction fibres were used to rapidly extract and enrich pesticides in honey, a popular agricultural product with complex matrix, and then directly coupled with electrospray ionization mass spectrometry for qualitative and quantitative analysis. Three pesticides, i.e., atrazine, benalaxyl and pirimicarb, were investigated using the technique and their analytical performances were evaluated. The limits of detection and limits of quantitation of all the three pesticides could fulfil the cut-off values of the international standard. Linear calibration curves were constructed with good R^2 coefficients, and the accuracy and precision were in acceptable ranges for all the pesticides. The analysis time is much reduced, with only minimum sample preparation and no chromatographic separation involved. The technique is simple and easy to set up, and can be extended for analysis of other analytes and sample systems.

Keywords: Pesticides; honey; solid phase micro-extraction; electrospray ionization mass spectrometry; direct coupling.

1. Introduction

Pesticides have been widely used in agriculture to reduce the damage caused by pests and to increase the yield and quality of agricultural products. However, the applications of pesticides may leave residues in food products and cause food contamination. These pesticide residues may be harmful to human being as some of the pesticides are potentially toxic.[1, 2] Fast screening of pesticide residues in foodstuff is thus important for food quality and safety assurance.

Honey is one of the most popularly consumed agricultural products in the world. It is a complex substance with sugars and water as the main ingredients, and contains minor components such as amino acids, proteins, vitamins and volatile compounds.[3] Honey could be contaminated by pesticides due to improper beekeeping practices or agricultural activities. For instance, pesticide residues on the flowers of plants may be carried by honey bees to their hive, leading to contamination of the product, i.e., honey.[4] A recent study has shown that 75% of the collected 198 honey samples contained at least one insecticide and multiple contaminations were found in 45% of the same batch of samples.[5] The monitoring of pesticide residues in honey and bee products can not only ensure the quality of honey for human consumption, but also serve as an indicator of environmental pollution.[6-8] Electrospray ionization mass spectrometry (ESI-MS) is a powerful technique for identification and quantitation of various compounds. Due to its high sensitivity and high accuracy, ESI-MS has been widely used for analysis of pesticide residues in food samples including honey and bee products,[9-11] vegetable,[12] fruit juice[13] and wine.[14] However, due to the presence of high content interfering components such as sugars, extensive sample pre-treatment such as solid-phase extraction (SPE), liquid-liquid extraction and chromatographic separation are

typically required before mass spectrometric analysis and these procedures could be time-consuming and laborious.[15-17]

Solid phase micro-extraction (SPME) has been widely used to extract various compounds from gaseous, liquid and solid samples, with the processes of sampling, extraction, concentration and sample introduction combined in one step. After the extraction process, adsorbed analytes on the SPME tips can be desorbed in gas chromatography or liquid chromatography, depending on the nature of the analytes.[18, 19] Although SPME extraction of pesticides in food is robust, chromatographic separation of the extracted pesticides is normally required prior to the detection by MS. In recent years, our group has been devoted to develop electrospray ionization from solid substrates.[20-26] Herein, we introduce the direct coupling of SPME and ESI-MS (SPME-ESI-MS) for the rapid analysis of pesticide residues in honey. Honey is chosen as the food sample for the analysis as it is popularly consumed and pesticide contaminations in honey have been public concerns. Moreover, the large content of sugars in honey and the viscous texture of honey represent analytical challenges and can serve as interference models for detection of target analytes. The commercially available SPME tips were used for sample extraction and enrichment and directly connected to ESI-MS for the analysis. Our results demonstrated that rapid detection of pesticides in honey could be achieved using the technique, without the need of extensive sample pre-treatment and chromatographic separation.

2. Materials and methods

2.1. Materials

Pesticide standards of atrazine, benalaxyl, pirimicarb and pirimicarb d-6, and reagents of methanol (MeOH, HPLC grade) and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was prepared by a Milli-Q system (Millipore Laboratory, USA). The SPME fibres with carbon-18 (C18) embedded in a biocompatible polymer were purchased from Supelco (St. Louis, USA). Each fibre is coated with a layer of C18 with a thickness of 45 µm and length of 15 mm.

Po Sang Yuen Acacia Flower Honey (100% pure bee honey) was purchased from a local supermarket (Wellcome supermarket, Hong Kong) and used in this study. The honey sample was diluted with water (1:9 v/v) and centrifuged at a speed of 4000 rpm for 15 min to remove the solid particles. The supernatant was collected for further use. Sample preparation was made on the same day with the instrumental analysis.

2.2 Sample preparation

Stock solutions (1 µg mL^{-1}) of all pesticides and internal standard (IS) solution of pirimicarb d-6 (1 µg mL^{-1}) were prepared in methanol. Working solutions were prepared by diluting the stock solution in water. For the quantification of pesticides in honey by SPME-ESI-MS, 1000 µL of each sample was spiked with working solutions of pesticides to achieve concentration of 2, 3, 12.5, 25, 50, 100, 200 ng mL^{-1} and a fixed concentration of internal standard solution (10 ng mL^{-1}) was added to each solution.

2.3. Equipment and setup

The SPME fibres were used according to the guideline provided by the supplier. In brief, a SPME tip was firstly pre-washed with 1 mL of MeOH for 5 min, and then with 1 mL of H_2O for another 5 min. Then, the SPME tip was used to extract 1 mL of sample with vortex for 10 min. The tip was then quickly washed with water before the mass spectrometric analysis. After SPME-ESI-MS analysis, the tip was washed with MeOH for at least 30 min to remove the residues.

All SPME-ESI-MS experiments were performed on an Agilent 6460 triple quadrupole mass spectrometer (California, USA). A schematic diagram of the SPME-ESI-MS set-up is shown in Figure 1 (see Figure S-1 for the photo). In brief, the tip was mounted onto a microscopic slide and connected with a wire for high voltage supply (+3.5 kV). A solvent containing MeOH: H_2O: FA (90: 10: 0.1, v/v/v) was supplied by a glass syringe (1000 µL, Hamilton, USA) at a rate of 40 µL min^{-1} and a syringe pump (New Era Pump System, USA), and sprayed onto the SPME tip for eluting the adsorbed analytes. Each sample were repeatedly analyzed for three times with the signals lasted for around 10 s each time, and the three results were averaged as the data of the sample. The distance of the SPME tip to the MS inlet was set as ~ 1 cm. The detection of ions was under positive ion mode and multiple reaction monitoring (MRM) mode with selected reactions, *m/z* 216 → 174, *m/z* 326 → 148, *m/z* 239 → 72 and *m/z* 245 → 78 for the detection of atrazine, benalaxyl and pirimicarb and pirimicarb d-6, respectively. Nitrogen was used as the nebulizer gas, with the gas temperature of 150 °C, gas flow of 6 L min^{-1} and nebulizer pressure of 3 psi. Sheath gas was at 125 °C with gas flow of 3 L min^{-1}. The mass spectra collected were processed by using MassHunter Qualitative Analysis Software (Version B.07.00).

3. Results and discussion

SPME was directly coupled with ESI-MS for rapid analysis of pesticides in honey samples in this study. To perform the SPME-ESI-MS, SPME tip was firstly pre-washed and used to extract analytes from the sample with vortex. The vortex speeds up the partition and adsorption of targeted analytes onto the stationary phase, i.e., C18 coating on the SPME tip. After the quick washing step, the tip with extracted analytes was connected to the MS. A solvent was then sprayed onto the SPME tip to elute the adsorbed analytes. As the tip end of the SPME tip is sharp, it could serve as the emitter for electrospray ionisation directly. With the application of spraying solvents and a high voltage, ESI was generated and MS spectra were obtained. The position between the SPME tip end and the MS inlet, and other experimental parameters such as sample volume, extraction time, composition and flow rate of spray solvent were optimised (see Figures S-2 and S-3), and the settings are described in Materials and Method.

Three commonly used pesticides, i.e., atrazine, benalaxyl and pirimicarb, were investigated in this study and their chemical structures are shown in Figure 2. Atrazine is a widely used herbicide to kill weeds on crops such as sugarcane, corn, and is one of the most common pesticides found in soil, agricultural regions, groundwater and waterways.[27] Atrazine is well known as a potent hormone disruptor that has been associated with birth defects of animals[28] and can increase the risk of cancer in humans.[29, 30] Benalaxyl is a broad-spectrum fungicide for vegetables and fruit crops, and is moderately persistent in soil and water systems.[31] It is moderately toxic to mammals, honey bees and most aquatic organisms. Pirimicarb is an insecticide for aphid (small insects) control in a wide range of crops, and is also used in controlling parasitic mites in bee colonies which is associated with honeybee mortality.[32] Pirimicarb is shown to be potentially carcinogenic

to mammals.[33] These three pesticides have been reported to exist in honey samples and bee products,[10, 11, 34-36] and thus it is necessary to develop techniques for their detections.

A spectrum obtained by analysis of a honey sample spiked with the pesticides by conventional capillary-based ESI-MS is shown in Figure 3a. The main components of honey, i.e., sugars, were predominantly observed in the spectrum, with the signals from the pesticides significantly suppressed. The saccharides in honey were observed in sodium adduct forms such as [Glucose + Na]$^+$ at *m/z* 203, [Glucose + K]$^+$ at *m/z* 219, [Sucrose + Na]$^+$ at *m/z* 365, [Sucrose + K]$^+$ at *m/z* 381, [2Glucose + Na]$^+$ at *m/z* 383 and [2Glucose + K]$^+$ at *m/z* 399. With the SPME extraction followed by ESI-MS detection, all sugar signals were significantly reduced, and the pesticides, which were in their protonated forms, could be predominantly observed (Figure 3b). This result demonstrated that the SPME tip could be used to effectively remove the sugars in honey and extract the analytes, i.e., the pesticides, from the complex matrix, and that the SPME tip could be efficiently coupled with ESI-MS for rapid detection of the extracted analytes.

The performance of SPME-ESI-MS for measurements of the pesticides were tested. For the determination of the limit-of-detection (LOD) and limit of-quantitation (LOQ), a blank sample was prepared by spiking the internal standard only to the honey sample. Signals were observed even for the blank sample due to the chemical and electronic noises and the level of these noises varied with different SPME tips. To compensate such variations, the LOD and LOQ were determined by comparing the intensity (peak height) ratio of the analyte and internal standard of the spiked samples (i.e., the samples spiked with both the analyte and the internal standard) with that of the blank sample, i.e., $(I_{analyte}/I_{IS})_{spiked}/(I_{analyte}/I_{IS})_{blank}$. The LOD and LOQ values were determined as the quantity of analyte that could achieve a signal-to-noise ratio of three and ten,

respectively. The LODs of atrazine, benalaxyl and pirimicarb in honey were determined to be 0.6, 1 and 0.5 ng mL^{-1}, respectively, and the LOQs of atrazine, benalaxyl and pirimicarb in honey were determined to be 2, 3 and 2 ng mL^{-1}, respectively. All these data fulfilled the cut-off values which are 50 ng mL^{-1} according to the international standard.[37]

Linear calibration curves for quantitation were constructed for detection of the pesticides by SPME-ESI-MS. The calibration curves were generated using six different concentrations of the pesticides, with each solution spiked with a fixed amount of the internal standard. Each solution was measured three times and the mean peak height was used for plotting the calibration curve. Both peak height and peak area were attempted for the plotting, and it was found that there was no discrepancy between them. Peak height was chosen because it was more straightforward and its data were more convenient for processing. The calibration curves for the analysis of pesticides in honey by SPME-ESI-MS are shown in Figure 4. Good linearity was observed in all calibration curves for all three pesticides with R^2 coefficients of 0.993 – 1.000 in the range of 2 – 200 ng mL^{-1}. Among the three pesticides, pirimicarb showed the best linearity. The LODs, LOQs and linearity of the three pesticides are summarized in Table 1.

The precision and accuracy of SPME-ESI-MS were evaluated. They were measured by analyzing three different concentrations (low, medium and high concentrations within the linear range) of the pesticides, with each concentration measured by five replicates. The precision was presented as relative standard deviation (RSD) which was calculated as: (SD of the measured concentrations ÷ mean of the measured concentrations) × 100%. The accuracy was calculated as: (the measured concentration ÷ the spiked concentration) × 100%. The precision and accuracy for quantitation of

the pesticides in honey by SPME-ESI-MS are shown in Table 2. Among the three pesticides tested, pirimicarb gave the highest precision and accuracy, which are believed to be due to the best signal response of pirimicarb (see Figure 1) and the better suitability of the internal standard for pirimicarb. The accuracy of pirimicarb was determined to be 98 – 100% and its precision was determined to be 3 – 8% at the three concentrations. The accuracies of atrazine and benalaxyl were determined to be 86 – 103% and 99 – 104%, respectively, and the precisions of atrazine and benalaxyl were determined to be 14 – 20% and 9 – 26%, respectively. Although the accuracies and precisions of atrazine and benalaxyl were slightly poorer than those of pirimicarb, they were still in acceptable ranges.

4. Conclusions

In this study, we demonstrated that SPME-ESI-MS could be applied for rapid detection and quantitation of pesticides in honey samples. SPME tips allowed effective extraction and enrichment of analytes, i.e., pesticides, in honey with complex matrix, and could be directly connected to the mass spectrometer for qualitative and quantitative analysis of the analytes. SPME tips could be used as ESI emitters to directly generate ions of the absorbed analytes upon application of a high voltage. The LOD, LOQ, linearity, accuracy and precision of the technique were all in acceptable ranges. The set-up of the technique is simple and easy to assemble, and the analysis time is much shortened since only minimum sample preparation is involved and chromatographic separation is not required. This technique could be further extended for analysis of other analytes and sample systems.

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Table 1. The LOD and LOQ values, linear ranges and R^2 coefficients of the calibration curves of pesticides in honey as analyzed by SPME-ESI-MS.

Pesticide	**LOD (ng mL^{-1})**	**LOQ (ng mL^{-1})**	**Linear range (ng mL^{-1})**	**R^2**	**MRLs[37] (mg kg^{-1})**
Atrazine	0.6	2	2-200	0.993	0.05
Benalaxyl	1	3	3-200	0.994	-
Pirimicarb	0.5	2	2-200	1.000	0.05

Table 2. The precision and accuracy for quantitation of the pesticides in honey by SPME-ESI-MS.

Pesticide	**Spiked concentration (ng mL^{-1})**	**Measured concentration (ng mL^{-1}) (*n=5*) (mean±SD)**	**RSD (%)**	**Accuracy (%)**
Atrazine	25	25±5	20	99
	100	111±15	14	111
	200	208±40	19	104
Benalaxyl	25	21±5	23	86
	100	96±8	9	96
	200	207±54	26	103
Pirimicarb	25	25±1	5	98
	100	100±3	3	100
	200	200±16	8	100

Figure captions

Figure 1. A schematic diagram showing the set-up of SPME-ESI-MS.

Figure 2. The chemical structures of three pesticides: (a) Atrazine, (b) benalaxyl and (c) pirimicarb.

Figure 3. Mass spectra of honey spiked with atrazine, benalaxyl, and pirimicarb (1 μg mL^{-1} each), obtained by (a) direct infusion ESI-MS and (b) SPME-ESI-MS.

Figure 4: The calibration curves for analysis of the pesticides in honey by SPME-ESI-MS: (a) Atrazine, (b) benalaxyl, and (c) pirimicarb.

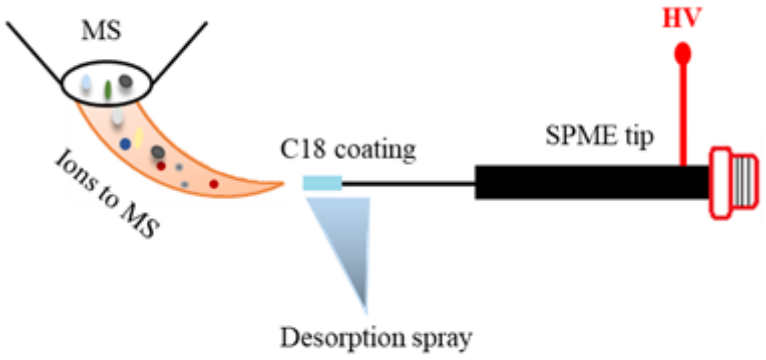
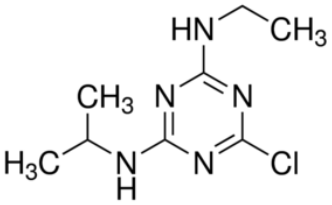
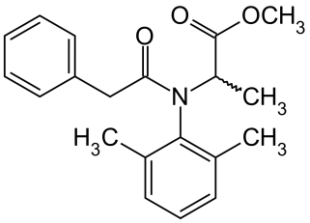


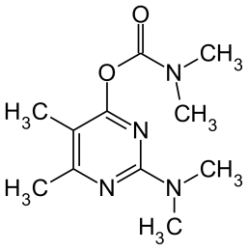
Figure 1



(a) Atrazine



(b) Benalaxyl



(c) Pirimicarb

Figure 2

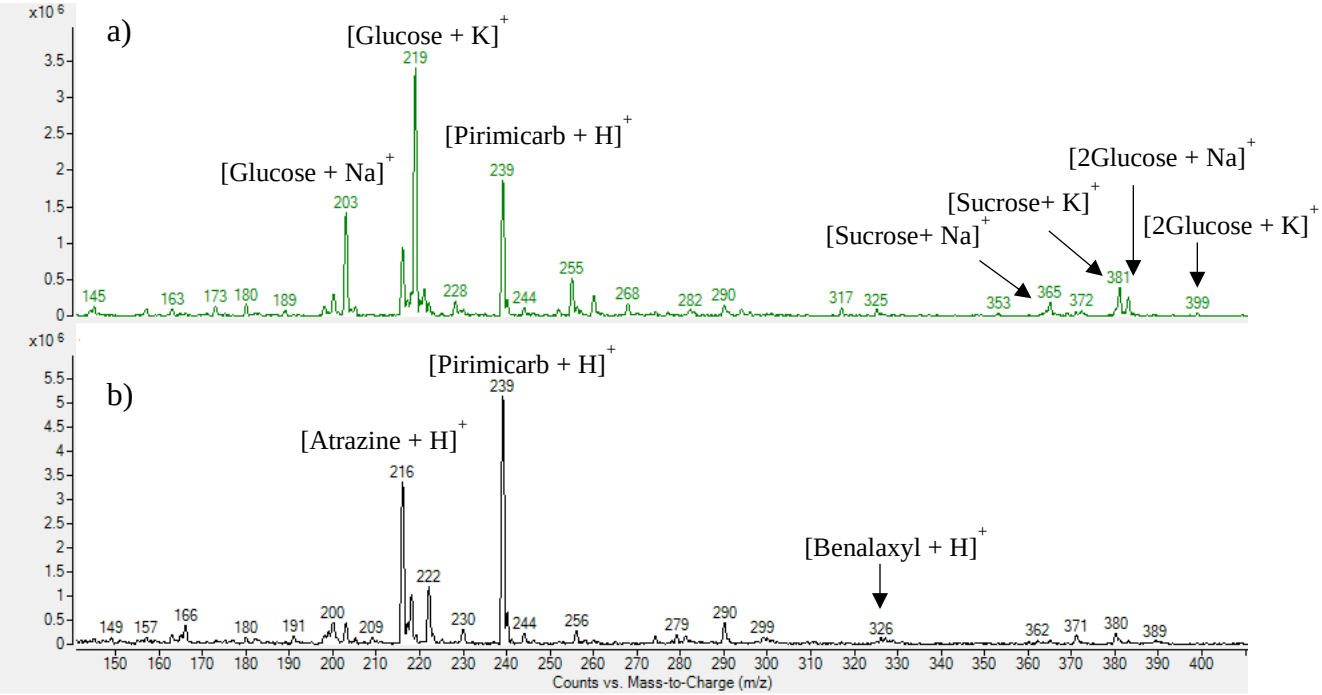

a)
[Glucose + K]+
[Pirimicarb + H]+
[Glucose + Na]+
[2Glucose + Na]+
[Sucrose+ K]+
[Sucrose+ Na]+
[2Glucose + K]+
b)
[Pirimicarb + H]+
[Atrazine + H]+
[Benalaxyl + H]+
Counts vs. Mass-to-Charge (m/z)

Figure 3

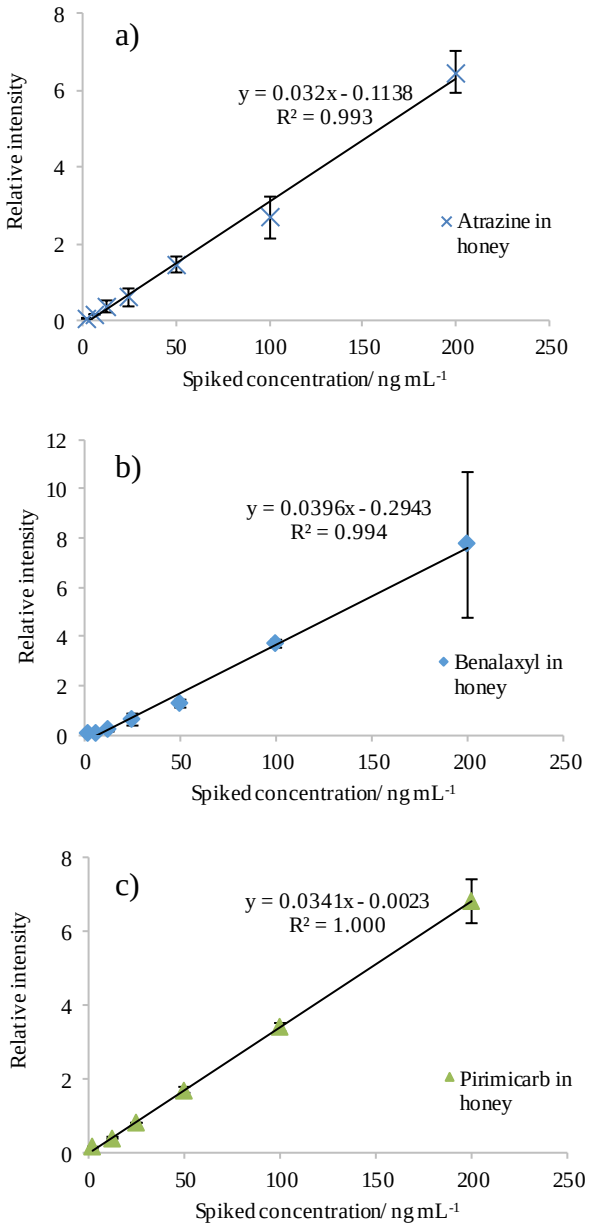

a)
y = 0.032x - 0.1138
R² = 0.993
Atrazine in honey
Relative intensity
Spiked concentration/ ng mL-1
b)
y = 0.0396x - 0.2943
R² = 0.994
Benalaxyl in honey
Relative intensity
Spiked concentration/ ng mL-1
c)
y = 0.0341x - 0.0023
R² = 1.000
Pirimicarb in honey
Relative intensity
Spiked concentration/ ng mL-1

Figure 4