[IMP2 04] Stacking and sweeping in micellar electrokinetic chromatography (MEKC) for on-column sample preconcentration of fungicides

Na'emah A'ubid, W. Aini W. Ibrahim

Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Skudai, Johor.

Introduction

Modern agricultural production depends heavily on pesticides in order to protect the crops from pests and to ensure they get the maximum products. Large amount of pesticides and its degradation products that are used in the agricultural production are potential hazard to aquatic life and human health. The residues can be found in drinking water, rivers and soils. The used of pesticides has caused a rise in public concern. Many social sectors are interested in controlling the pesticides contain in foods and drinking water and many analysis methods have been developed and implemented for many years.

Capillary gas chromatography (GC) in conjunction with selective detectors such as nitrogen-phosphorus detector or thermionic (NPD), electron capture (ECD), flame photometric (FPD) and mass spectrometry (MS) is the most common technique for the determination of environmental pesticides samples (Vassilakis *et al*., 1998; Columé *et al*., 2000). The low detection limits, high selectivity and affordability of GC instrumentation are rather appealing to most laboratories involved in pesticides residue analysis (Cairns *et al.*, 1992).

Liquid chromatography (LC) used for environmental pesticide analysis has been extensively reviewed (Arenas, *et al.*, 1996; Blasco *et al.*, 2002). The increasing use of LC is chiefly the result of its suitability for thermally labile and polar pesticides that require derivatization prior to GC analysis. UV detectors are the most common choice for pesticides analysis (Cairns *et al.*, 1992). High performance liquid chromatography (HPLC) also has been used to analyse the pesticides (Tellier *et al.*, 2002).

Capillary electrophoresis (CE) is a relatively new technique for the separation and analysis of chemical compounds. It has been proven to be a powerful analytical tool for separation. CE offers several advantages compared to HPLC including high efficient and fast separations, relatively inexpensive and long lasting capillary columns, small sample size requirements and low reagent consumption. It can also be used for the analysis of polar ionic, nonpolar ionic and nonpolar non-ionic compounds, as well as high molecular weight biomolecules and chiral compounds (Baker, 1995). CE offers different modes of separations for different type of samples. The most often used modes are capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC) (Quirino *et al*., 2000; Rodríguez *et al.*, 2001) capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and capillary isotachophoresis (CITP).

The most widely used detector in CE is the UV detector because many solutes have UV absorption and it is easily set up and not too expensive. However, the drawback of UV detection is the poor concentration sensitivity resulting from minute injection volumes needed to maintain high efficiency and a short optical pathlength equal to capillary diameter (Kim *et al.*, 2003). To improve detection sensitivity in MEKC, two online sample preconcentration technique viz. sample stacking and sweeping were developed in this study.

Materials and Methods

Chemicals and reagents

Carbendazim, vinclozolin and propiconazole were purchased from Dr. Ehrenstorfer GmbH laboratory (Germany), thiabendazole from Sigma Chemical Co (Canada), HPLC grade methanol from Caledon Laboratories Ltd (England) and acetonitrile from Merck (Germany), ammonium formate from BDH Chemicals Ltd (Canada), sodium cholate from Anatrace (USA), β-cyclodextrin and γ-cyclodextrin from Fluka Chemica (Switzerland). Aqueous solutions were made with deionised water (18 MΩ) prepared with a NANOpure Barnsted water system (USA).

Standard solutions of each fungicides were prepared at 10 000 ppm in methanol. Working standards for normal run were prepared by diluting the corresponding stock solutions in methanol. All standard solutions were stored at 4 °C in the refrigerator and were filtered through a 0.45 µm membrane filter prior to use.

Apparatus

CE analysis was performed on a CE-L1 capillary system purchased from CE Resources Pte. Ltd., Singapore equipped with a UV detector (SPD- 10 AVP model). UV detection was set at 210 nm. CSW 1.7 software programme was used for the control of instrument settings, data acquisition and analysis. Separations were carried out at 25 ± 1 ºC. Uncoated fused silica capillaries of 50 µm inner diameter from SGE (Victoria, Australia) were used. Total capillary length was 85 cm and effective length was 42 cm. A positive voltage of 25 kV was employed in all separations or else indicated elsewhere.

Procedure

When a new capillary was used, the capillary was conditioned by rinsing with 0.1 M NaOH for 30 min followed by deionized water for 30 min. Prior to the first run on each day, the capillary was rinsed with 0.1M NaOH for 10 min followed by deionized water for 10 min. The capillary was prewashed with running buffer for 3 min before each injection and postwashed for 5 min with deionised water. At the end of the day, the capillary was rinsed with deionized water for 15 minutes and air for 2 minutes.

For normal MEKC separation, sample injections were made in hydrodynamic injection mode at a pressure of 0.41 p.s.i for 5 s. In stacking and sweeping, sample introductions were performeds by injecting sample solutions for a much longer time compared to the usual hydrodynamic injection.

Results and Discussion

Fungicides separation in normal MEKC

These fungicidal compounds are mainly neutral compounds (Figure 1), hence MEKC is suitable for the study. The separation conditions including buffer concentration and pH, surfactant concentration, separation voltage, percentage of organic solvents and concentration of organic modifiers were varied in order to achieve the optimum separation conditions for the four fungicides selected.

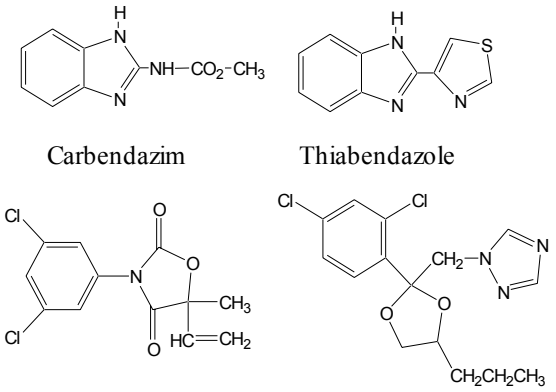

FIGURE 1 Structures of the studied fungicides

The four fungicides were successfully separated within 15 min, except propiconazole with two stereoisomer peaks. Electropherogram for theseparation of fungicides is depicted in Figure 2.

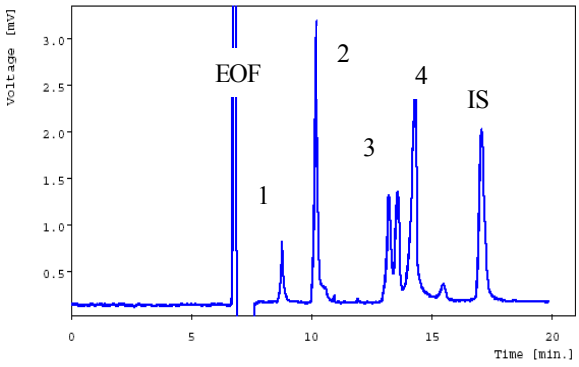

FIGURE 2 Electropherogram for separation of fungicides. Condition: 20 mM formate buffer pH 7, 60 mM sodium cholate, 5 mM β-cyclodextrin, 25 kV of separation voltage. Peaks identification: (1) Carbendazim, (2) thiabendazole, (3) propiconazole and (4) vinclozolin. IS = phenantherene.

Formate concentration was varied from 4 – 20 mM. In the range of 4 – 12 mM, slight decreased of the migration time was observed, whereas above 12 mM an increased in the migration time was noted. The resolutions of the peaks were greatly increased by the increased in the run buffer concentration. A 20

mM formate was selected for routine MEKC because it provided good resolution although it takes more time to separate the fungicides.

Migration time of the compounds increased with an increased concentration of sodium cholate. To study the effect of surfactant concentration, sodium cholate concentration was varied from 30 – 90 mM. In the range of 30 – 45 mM, the fungicides were not well separated. Above 45 mM, fungicides were well separated with an increased in the resolution of the peaks. However, 60 mM of sodium cholate was chosen for the next optimization step because it can separate the mixture in less than 15 min while it takes more than 15 min when greater than 60 mM was used.

Effect of buffer pH on migration time and peak resolution was also studied. The formate pH was varied from 5 – 9. It was found that increased in buffer pH increased the migration time. However, migration time started to decrease when buffer pH 9 was used. In the pH range of 5 – 7, a slight increased in resolution was observed and above pH 7, a decreased in peak resolution was noted. Hence, formate with pH 7 was chosen for next optimization.

Increasing the voltage from 10 to 30 kV reduced the analysis time from 42 to 12 min. Peak resolution was found to increase when 15 kV was used and slightly decrease when separation voltage was above 15 kV. As a result, 25 kV was chosen for next step because it gives shorter analysis time and peak resolution between peaks was quite good (Rs > 0.73).

When cyclodextrin was added to the run buffer, water insoluble solutes distribute themselves between the micelle and the cyclodextrin and spend no time in aqueous phase. In this study, 3 - 8 mM β-cyclodextrin was added to the run buffer. The migration time slightly increased when β-cyclodextrin concentration was increased. 5 mM β-cyclodextrin was found to give higher peak resolution compared to other concentration.

20 mM borate pH 7, 60 mM sodium cholate, 25 kV separation voltage and 5 mM β-cyclodextrin was found to give optimum separation condition for this study. This conditions will be also used for the next sample stacking and sweeping studies. Using these optimum conditions, the linearity of the separation method was tested over a concentration range of 50 - 300 ppm. Regression lines were calculated using Microsoft Excel. Calibration graphs were linear in the range tested with good correlation coefficients. The regression equation, correlation coefficient, limit of detection and repeatability (n=5) for the separation of all the fungicides are illustrated in Table 1.

Hydrodynamic Injection Stacking MEKC

To examine the influence of sample matrix, sample analytes were dissolved in aqueous solution containing various concentrations of formate buffer in the range of 0 to 50 mM. Conditions used in this experiments was obtained from optimum condition as mentioned above. Sample analytes were injected for 25 s. Peaks of four fungicides obtained without the addition of formate buffer in the sample matrix are rather broad and poorly resolved especially for propiconazole and vinclozolin peaks. Peaks were also not resolve when 4 mM and 20 mM formate buffer in sample matrix was used.

TABLE 1 The correlation coefficient, regression equation, limit of detection and repeatability of the migration time (t_m) and peak area of the fungicides for normal MEKC.

Fungicides	**Regression Equation**	**Correlation Coefficient**	**L.O.D (ppm)**	**RSD (%, n=5)**	
				t_m	**Peak area**
Carbendazim	y = 0.0038x + 0.0058	0.9663	51.54	3.52	6.19
Thiabendazole	y = 0.004x + 0.0784	0.9714	46.89	3.91	1.46
Propiconazole i	y = 0.0021x + 0.0224	0.9843	39.28	4.39	11.63
Propiconazole ii	y= 0.0017x – 0.0723	0.9894	29.23	4.45	8.4
Vinclozolin	y = 0.0069x –0.4503	0.9733	45.32	4.62	5.66

However, peaks were separated when 50 mM formate buffer in sample matrix was used. Peak height of fungicides increased with an increased concentration of formate buffer in sample matrix. Stacking enhancement factor in terms of peak height (SEF_{ht}) for 50 mM formate buffer in sample matrix was found to give the highest value compared to other concentrations. Table 2 shows the SEF for peak height and peak area. SEF value was calculated using the equation below, where the numerator is the peak height or peak area obtained with stacking and the dominator is the peak height or peak area obtained with usual MEKC injection (2 s injection).

$$SEF = \frac{H_{stack}}{H}$$

TABLE 2 Stacking enhancement factor for peak height and peak area for sample matrix studies on hydrodynamic injection stacking MEKC

Fungicides	SEF_{ht}	SEF_a
Carbendazim	52	73
Thiabendazole	99	82
Propiconazole i	51	83
Propiconazole ii	9	82
Vinclozolin	22	51

Injection time of sample analytes containing 50 mM formate buffer was varied from 20 to 40 s. Although peak height and peak area increased when more analytes were injected, but the peak shape deteriorated and an unidentified peak was observed. 20 s injection time was found to give better result, with SEF_{ht} values in the range of 26 to 72.

Using the optimum condition (sample analytes dissolved in 50 mM formate and injected for 20 s), the linearity of the separation method was tested over a concentration range of 2 - 25 ppm. Table 3 summarized the regression equation, correlation coefficient, limit of detection (LOD) and repeatability (RSD) of migration time and peak area for the fungicides. By comparing normal MEKC and hydrodynamic injection stacking MEKC, limit of detection (defined as signal-to-ratio ratio of 3) of fungicides were reduced by more than 20 times fold. The lowest limit of detection attainable is for carbendazim (0.32 ppm) and the other fungicides limit of detection is in the sub-ppm range.

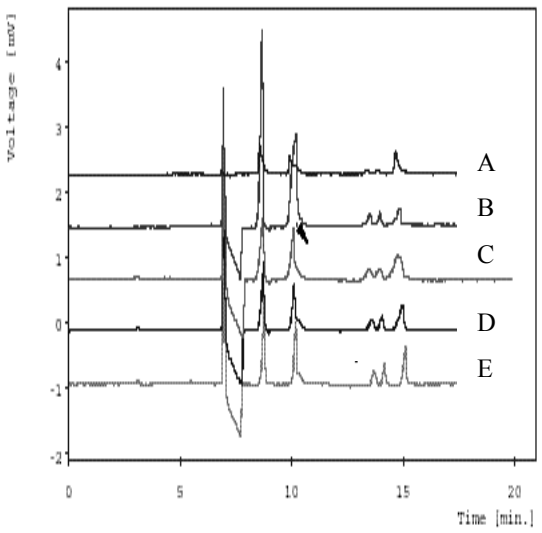

FIGURE 3 Electropherograms for separation of fungicides with different sample matrix concentration. Condition: 20 mM formate buffer pH 7, 60 mM sodium cholate, 25 kV of separation voltage and 5 kV of inection voltage. Peaks identification as in Figure 2. Samples dissolved in (A) methanol, 250 ppm, injected for 2 s (B) 2 mM formate, 25 ppm, injected for 25s (C) 20 mM, injected for 25s (D) 50 mM formate, 25 ppm, injected for 25 s.

Electrokinetic Injection Stacking MEKC

Sample stacking with electrokinetic injection was explored for the optimization of the stacking condition. Parameters studied were sample matrix, injection time and injection voltage. Samples were dissolved in different concentration of formate buffer and it was injected for 25 s at 5 kV injection voltage. Formate concentration was varied from 2 to 50 mM. Peak height increased when samples were dissolved in higher formate concentration. Resolution for propiconazole and vinclozolin peaks were good when higher concentration of sample matrix was added and depicted in Figure 3. As a result, 50 mM formate buffer in sample analytes was found to give higher SEF_{ht} value (18 to 54) and peak resolution was good.

Effect of injection time on peak height and resolution was then studied. Injection voltage was varied from 1 to 15 kV. Peaks broadening were noted when samples were injected at high voltage although it showed an increased an in peak height. Hence, 5kV injection voltage was found to give better peak resolution with good SEF_{ht} above 20.

Samples were injected into the capillary at different times between 25 to 100 s. As more samples were injected, peak height and area increased but peak resolution decreased and showed broadening. 50 s injection was chosen as the optimum condition since it gave good separation and good SEF_{ht} value.

Using the optimum condition (sample analytes dissolved in 50 mM formate and electrokinetically injected for 50 s at 5 kV), the limit of detection, repeatability of migration time and peak area for fungicides were determined. Limit of detection determined for these fungicides at 50 s injection times ranged from 2.2 – 2.5 ppm. Table 4 summarize the regression equation, correlation coefficient, limit of detection and repeatability of migration time and peak area for the fungicides separation using electrokinetic injection staking MEKC. By comparing normal MEKC and electrokinetic injection stacking MEKC, limit of detection of fungicides were reduced by between 12 – 40 fold.

Sweeping MEKC

At first, samples were hydrodynamically injected into the capillary for different times. When more samples were loaded onto the capillary, carbendazim and thiabendazole peaks become broader but it was not applicable to vinclozolin and propiconazole peaks. Vinclozolin and propiconazole peaks resolution was good until it was injected for 150 s while carbendazim and thiabendazole peaks started to broaden when sample was injected for 20 s. Sweeping is good when it is used with hydrophobic analytes, therefore carbendazim and thiabendazole was omitted.

TABLE 3 The correlation coefficient, regression equation, limit of detection and repeatability of the migration time and peak area of the fungicides for hydrodynamic injection stacking MEKC.

Fungicides	**Regression Equation**	**Correlation Coefficient**	**L.O.D (ppm)**	**RSD (%, n=5)**	
				t_m	**Peak area**
Carbendazim	y=0.5549x+0.8114	0.9991	0.32	1.36	3.19
Thiabendazole	y=0.6080x+0.2065	0.9989	1.07	1.64	2.31
Propiconazole i	y=0.0964x+0.0672	0.9947	2.01	1.68	3.35
Propiconazole ii	y=0.0910x+0.1907	0.9929	2.31	1.68	3.36
Vinclozolin	y=0.7067x+1.2746	0.9649	2.09	1.76	5.09

TABLE 4 The correlation coefficient, regression equation, limit of detection and repeatability of the migration time and peak area of the fungicides for electrokinetic injection stacking MEKC

Fungicides	**Regression Equation**	**Correlation Coefficient**	**L.O.D (ppm)**	**RSD (%, n=5)**	
				t_m	**Peak area**
Carbendazim	y=1.158x-0.4785	0.9568	2.33	0.80	1.20
Thiabendazole	y=1.13x+0.0442	0.9605	2.22	0.84	2.11
Propiconazole i	y=0.2736x+0.0835	0.9783	2.45	0.95	2.11
Propiconazole ii	y=0.2771x+0.0165	0.9785	2.43	0.97	1.38
Vinclozolin	y=1.7107x-0.4315	0.9519	2.46	1.05	10.76

TABLE 5 The correlation coefficient, regression equation, limit of detection and repeatability of the migration time and peak area of the fungicides for sweeping MEKC.

Fungicides	**Regression Equation**	**Correlation Coefficient**	**L.O.D (ppm)**	**RSD (%, n=5)**	
				t_m	**Peak area**
Propiconazole i	y=1.2162x+1.098	0.9761	1.53	0.98	4.25
Propiconazole ii	y=1.1007x+1.975	0.9106	3.06	1.03	4.86
Vinclozolin	y=3.4896x+15.361	0.957	1.66	1.12	2.76

Experiments were continued with propiconazole and vinclozolin as the sample. Injection time of the samples was varied from 2 to 200 s. Peak height and area increased with an increase in injection time and peak resolution was good until sample was injected for 200 s where vinclozolin peak started to split into two. The optimum injection time for sample sweeping was 120 s.

Limit of detection as well as repeatability of migration times and peak area was determined (Table 5). By comparing normal MEKC and sweeping, limit of detection of fungicides were reduced by more than 10 - 20 fold.

Between the three types of sample preconcentration technique, hydrodynamic injection stacking was found to be the best. It provided good linearity, good repeatability and lower limit of detection compared to the other two for the fungicide studied.

The hydrodynamic stacking MEKC method developed for the analysis and separation of fungicides is an attractive method and if coupled with off-line concentration method should yield a much lower limit of detection.

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