

## Normal Stacking as an Online Concentration in Micellar Electrokinetic Chromatography for the Separation of Different Classes of Fungicides

Wan Aini Wan Ibrahim and Na'emah A'ubid

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

Tel: 07-5534311, Fax: 07-5566162, email: wanaini@kimia.fs.utm.my

**Abstract** : Neutral analytes can be separated using micellar electrokinetic chromatography (MEKC), a method initially developed by Terabe and co-workers. MEKC offer higher efficiencies and faster analysis times than those achieved with high performance liquid chromatography (HPLC). However, one drawback of MEKC is the poor sensitivity due to a short path length in on-column UV detection and minute injection volumes (nL). In this study, an on-line concentration method called stacking with electrokinetic injection (SEKI) and stacking with hydrodynamic injection (SHDI) were explored to improve the detection sensitivity of three classes of fungicides, viz. benzimidazoles (carbendazim, thiabendazole), triazoles (propiconazole) and dicarboximide (vinclozolin). Optimized separation of these four fungicides was achieved using 20 mM ammonium formate at pH 7, 60 mM sodium cholate, 5 mM  $\beta$ -cyclodextrin (CD), 25 kV separation voltage, and on-column UV detection at 210 nm. All four fungicides were successfully separated in less than 15 minutes. It was found that SEKI at 5 kV injection voltage for 20s injection time produced the highest sensitivity enhancement factor in terms of area ( $SEF_{area}$ ) of about 40-68. A value of 10 for SEF corresponds to 1 order of magnitude improvement in concentration detection limit. The LOD of these four fungicides was found to be in the sub-ppm range (0.6-2.6 ppm).

**Keywords**: stacking, micellar electrokinetic chromatography, fungicides, ammonium formate, sodium cholate, hydrodynamic injection, electrokinetic injection.

**Abstrak** : Analit neutral boleh dipisahkan menggunakan kromatografi elektrokinetik misel, teknik yang dipelopori oleh Terabe dan rakan-rakan. MEKC memberikan kecekapan pemisahan dan masa pemisahan yang lebih pantas berbanding dengan kromatografi cecair berprestasi tinggi, HPLC. Walau bagaimanapun, satu kelemahan MEKC adalah kepekaan pengesanan yang rendah kerana jarak laluan pendek dalam pengesanan UV dan isipadu suntikan yang kecil (nL). Dalam kajian ini satu kaedah prapemekatan secara talian terus yang dikenali sebagai "penyusunan" (stacking) dengan suntikan elektrokinetik (SEKI) dan "penyusunan" dengan suntikan hidrodinamik (SHDI) telah diterokai untuk meningkatkan kepekaan penentuan tiga kelas fungisid iaitu benzimidazol (karbendazim dan tiabendazol), triazol (propikonazol) dan dikarboksimid (vinklozolin). Pemisahan optimum telah diperolehi menggunakan 20 mM ammonium format pH 7, 60 mM natrium kolat, 5 mM  $\beta$ -siklodekstrin dan voltan pemisahan 25 kV dengan pengesanan UV pada 210 nm. SEKI dengan voltan suntikan 5 kV dan masa suntikan selama 20s telah memberikan faktor peningkatan kepekaan tertinggi berdasarkan luas ( $SEF_{area}$ ) sebanyak 40-68. Nilai SEF 10 adalah bersamaan dengan peningkatan sebanyak 1 magnitud dalam pengesanan kepekatan. Had pengesanan keempat-empat fungisid adalah dalam julat sub-ppm (0.6-2.6 ppm).

**Kata kunci**: "penyusunan", kromatografi elektrokinetik misel, fungisid, ammonium format, natrium kolat, suntikan hidrodinamik, suntikan elektrokinetik.

Received : 13.09.04 ; accepted : 27.10.05

### Introduction

Since its inception in 1984 by Terabe *et al.* [1], micellar electrokinetic chromatography, MEKC is becoming a powerful analytical separation technique for various analytes [2-4]. MEKC was developed especially for the separation of neutral analytes but can also be used for charged analytes. MEKC offers a promising tool for separation of neutral analytes in that it is inherently fast, offers high efficiency and selectivity by changing composition of background

buffer (BGB) or electrolytes (BGE). One drawback of MEKC with on-column UV detection is the poor concentration sensitivity due to the short optical path defined by the column diameter and the small volume capacity (nL). Various attempts have been made to overcome this problem, which includes on-line solid phase extraction (SPE) [5] and use of powerful detectors such laser induced fluorescence [6]. In this study an on-line sample concentration technique called normal stacking (where EOF is strong) is

explored to overcome the problem of poor or low detection sensitivity associated with on-column UV detection. Two modes of injection were used, viz. electrokinetic or electromigration and hydrodynamic or pressurized injections. This stacking technique is a cheaper alternative to the use of expensive detectors or on-line SPE-CE. Stacking with electrokinetic injection (SEKI) and stacking with hydrodynamic injection (SHDI) is compared in terms of area sensitivity enhancement factor,  $SEF_{area}$  and height sensitivity enhancement factor,  $SEF_{height}$ .

In stacking, samples are prepared in low conductivity matrices such as water. In our case stock solution of samples were prepared in methanol and diluted in deionized water for stacking procedure. Stacking occurs as ions cross a boundary that separates regions of high and low electric field strength. Ions slow down when they cross this boundary of higher electrophoretic velocity (lower conductivity zone) to lower electrophoretic velocity (higher conductivity zone) resulting in a narrowing of the analyte zones. In normal polarity stacking mode (EOF strong), the neutral analytes contained within the anionic micelles migrate quickly toward the inlet end of the capillary and stack at the boundary between the sample solution and the BGE that is being drawn into the capillary by the EOF. The net migration then switches toward the detector since the electroosmotic velocity is greater than the electrophoretic velocity of the micelles in the high conductivity buffer.

In this exploratory study on normal stacking, all fungicide samples were dissolved in water. To the knowledge of the researchers, combination of separation and detection of triazole, dicarboximide and benzimidazole fungicides have not been attempted using on-line concentration technique in MEKC. Thiabendazole, a benzimidazole fungicide has been determined by HPLC with fluorescence detection [7] and four benzimidazole fungicides (benomyl, carbendazim, thiabendazole and fuberidazole) have been determined by HPLC with fluorescence detection [8]. A simultaneous determination of four different classes of pesticides (sulfonylurea, *s*-triazines, phenylurea and chlorinated acid herbicides) have been attempted using MEKC with UV detection [2]. Chiral triazole pesticides have also been separated by supercritical fluid chromatography with diode array detection [9]. A simultaneous determination of tridemorph (a morpholine fungicide), carbendazim and thiabendazole (benzimidazole fungicide), imazalil (imidazole fungicide), propiconazole and bitertanol (triazole fungicide) have been determined by LC-MS [10]. Rodriguez and co-workers [11] determined carbendazim, thiabendazole and methylthiophanate (a benzimidazole fungicide), imazalil (an imidazole fungicide), prochloraz (an amide fungicide), procymidone (a dicarboximide fungicide),

triadimefon (a triazole fungicide) and *O*-phenylphenol (a phenol fungicide) residues in vegetables using SPE-MEKC.

## Experimental

### 1. Apparatus

Capillary electrophoresis analysis was carried out using a computer controlled CE-L1 capillary system supplied from CE Resources Pte. Ltd. (Singapore) equipped with a UV detector model SPD-10 AVP (Shimadzu, Kyoto, Japan) and a CSW 1.7 software programme. It is equipped with an uncoated fused silica capillary (SGE, Australia) of 85 cm length (45 cm effective length x 50  $\mu$ m I.D x 105  $\mu$ m O.D) and an auto sampler system.

### 2. Chemicals

Carbendazim, vinclozolin and propiconazole were purchased from Dr. Ehrenstorfer GmbH laboratory (Germany) and thiabendazole from Sigma Chemical Co. (Canada), HPLC grade methanol was from Caledon Laboratories Ltd. (England) and acetonitrile from Merck (Germany), ammonium formate from BDH Chemicals Ltd. (Canada), sodium cholate from Anatrace (USA),  $\beta$ -cyclodextrin and  $\gamma$ -cyclodextrin from Fluka Chemica (Switzerland). Aqueous solutions were made with deionised water (18 M $\Omega$ ) prepared with a NANOpure Barnsted water system (USA). Stock solutions (10 000 ppm) of each of the fungicides were prepared in methanol and stored in the refrigerator at 4°C and use when needed. BGB contained 20 mM ammonium formate pH 7, 60 mM sodium cholate, and 5 mM  $\beta$ -cyclodextrin as organic modifier.

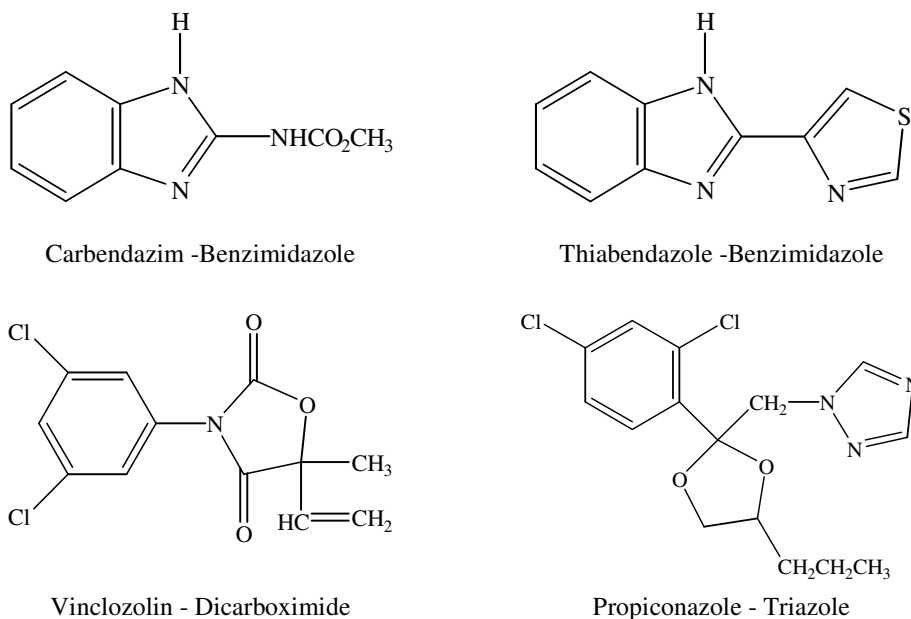
### 3. CE Stacking procedure

Detailed use of CE is described elsewhere [12]. The sample matrix contained water. All running buffers were filtered through a 0.45  $\mu$ m nylon syringe filter (Whatman, New Jersey, USA). The stacking procedure involves flushing the capillary with BGB for 5 minutes before sample injections were performed either hydrodynamically at 2.8 kPa for 5-20s injection times or electrokinetically at 5 kV for 1-50s injection times. Electropherograms were processed with a CSW 1.7 software programme from CE-L1 system.

## Results and Discussions

### 1. Optimization of the separation of fungicides

The structure of the benzimidazole, dicarboximide and triazole fungicides chosen for the study is shown in Figure 1 and some properties of these fungicides are shown in Table 1. The separation was optimized by studying the effect of the surfactant concentration, buffer concentration and pH, separation voltage, modifier concentration and

**Figure 1 :** Structure of the studied benzimidazole, dicarboximide and triazole fungicides**Table 1 :** Properties of the fungicides used in the study

Fungicides	Water Solubility at 20°C (mg/L)	Formula Weight	Calculated <sup>a</sup> Log K <sub>ow</sub>
Carbendazim	8	191.19	1.29
Thiabendazole	50	201.20	2.03
Propiconazole	110	342.2	4.16
Vinclozolin	1000	286.11	3.40

<sup>a</sup> Log Kow calculated using CS ChemOffice software

additive concentration ( $\beta$ -CD). Optimized separations of the four fungicides were achieved using 60 mM cholate, 50 mM ammonium formate pH 7, 25 kV separation voltage and 5 mM  $\beta$ -cyclodextrin [13]. All the four fungicides were separated within 15 minutes with carbendazim eluting first, followed by thiabendazole, propiconazole and vinclozolin. Two peaks were observed for isomeric propiconazole. In this separation, the migration sequence coincided with increasing order of water solubility and increasing order of hydrophobicity except for propiconazole and vinclozolin.

## 2. Stacking with hydrodynamic injections (SHDI) and stacking with electrokinetic injections (SEKI)

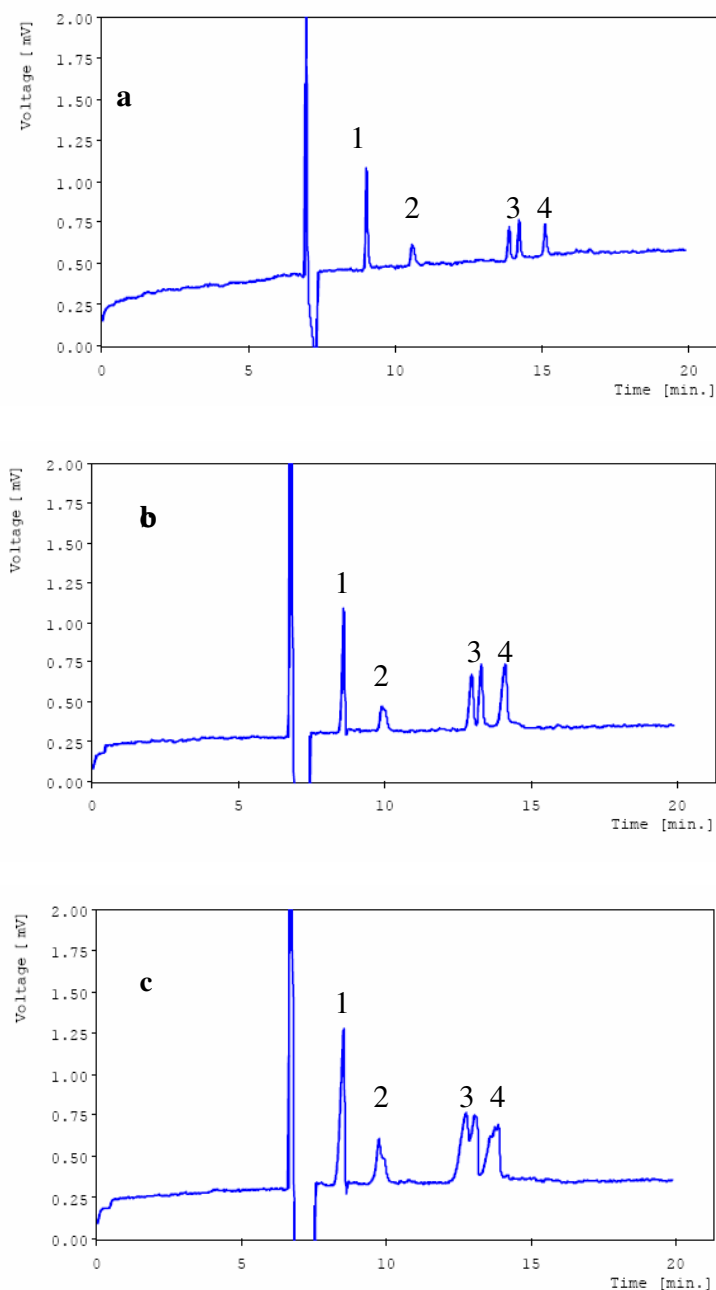
Electropherogram of the four separated fungicides in water showing the effect of hydrodynamic injection and electrokinetic injections at various times on the peaks is depicted in Figure 2 and Figure 3 respectively. SHDI of up to 10s

produced a base line separation of the propiconazole isomers but at 15s injections the peak start to be unresolved. When sample in water was injected for 20s or more the peaks start to broaden and hump start to form. 15s injection times was taken as the optimum time. SEKI of up to 20s injection times produced peaks, which are well separated. Injections for 40 and 50s produced peaks, which are biforked and humped. 20s injection time with EKI was taken as the optimum.

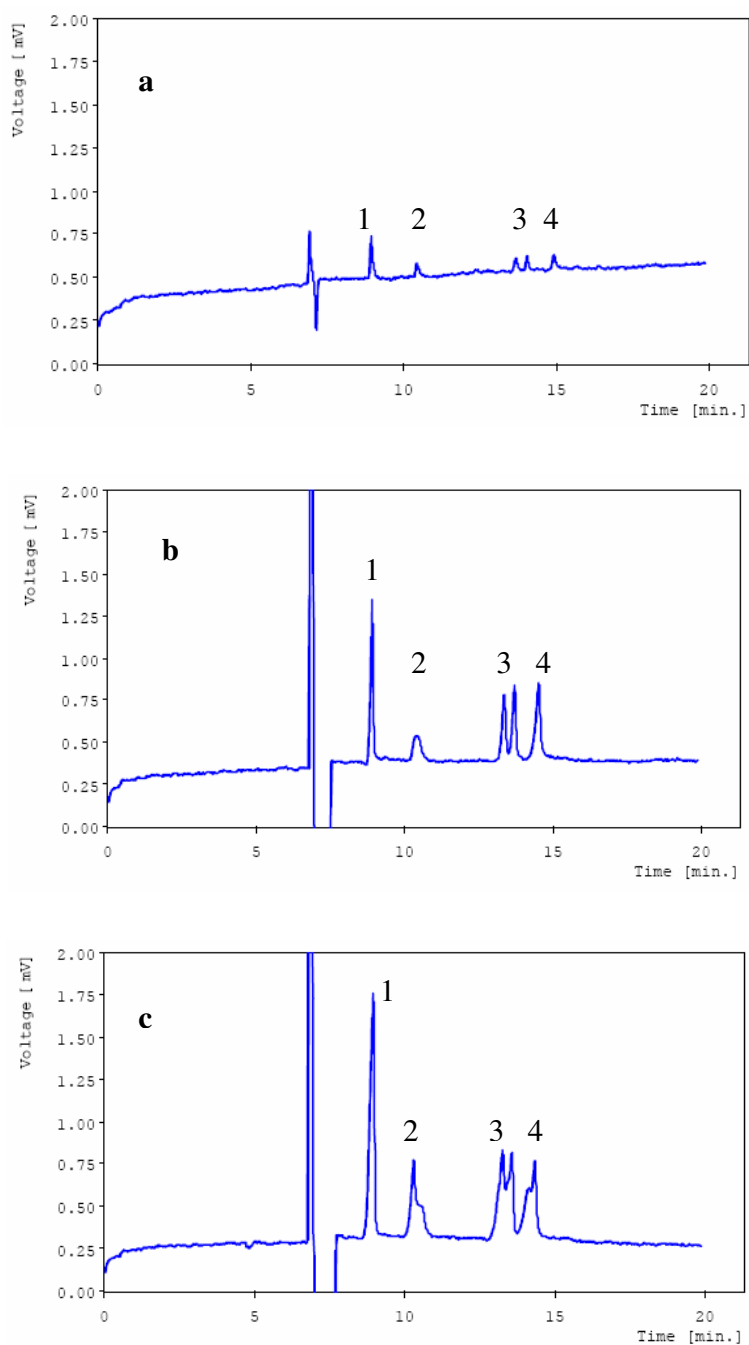
Table 2 shows the sensitivity enhancement factor attainable with SEKI and SHDI. As can be seen from the table, SEKI showed a much better improvement in the sensitivity enhancement factor, SEF. SEF<sub>area</sub> and SEF<sub>height</sub> for SHDI are all less than 10 but for SEKI the SEF values are all greater than 10 with SEF<sub>area</sub> ranging from 41-68 while SEF<sub>height</sub> ranging from 39-51 except for SEF<sub>height</sub> for thiabendazole. A value of 10 for SEF corresponds to one order of magnitude improvement in the concentration detection limit.

The SEF value obtained in the current study corresponds to LODs in the sub-ppm range (0.6-2.6 ppm). Normal MEKC produced LODs in the range of 29.2 – 51.5 ppm [13]. We are trying to improve the detection sensitivity to higher order by using sodium dodecyl sulphate, SDS so that stacking using reverse

migrating micelles (SRMM) at acidic pH of up to 2 is possible. It has been reported that SRMM produced better SEF [14]. Combination of on-line concentration and off-line concentration techniques such as SPE has been shown to improve the LOD to ppb [15, 16].



**Figure 2 :** Electropherogram of four fungicides. Conditions: 20 mM ammonium formate pH 7, 60 mM sodium cholate, 25 kV separation voltage, 5 mM  $\beta$ -CD, hydrodynamic injections for (a) 1s undiluted in MeOH (b) 10s (c) 20s, UV detection at 210 nm. Peaks identification (1) carbendazim, (2) thiabendazole (3) propiconazole (4) vinclozolin. Sample for (a) 200 ppm thiabendazole and other three fungicides are 250 ppm in methanol. Sample for (b)-(c) is 10x dilution of sample (a) in water.



**Figure 3 :** Electropherogram of four fungicides. Conditions: 20 mM ammonium formate pH 7, 60 mM sodium cholate, 25 kV separation voltage, 5 kV electrokinetic injection for (a) 1s sample undiluted in MeOH (b) 20s 10x dilution. (c) 50s 10x dilution. UV detection at 210 nm. Peaks identification (1) carbendazim, (2) thiabendazole (3) propiconazole (4) vinclozolin. Sample for (a) 200 ppm thiabendazole and other three fungicides are 250 ppm in methanol. Sample for (b)-(c) is 10x dilution of sample (a) in water. All diluted samples are in water

**Table 2** : Stacking enhancement factor (SEF) obtained with SEKI (20s injection time, 5kV injection) and SHDI (15s injection time at 2.8kPa)

Fungicides	SEKI		SHDI	
	SEF <sub>height</sub>	SEF <sub>area</sub>	SEF <sub>area</sub>	SEF <sub>height</sub>
Carbendazim	39	42	3	4
Thiabendazole	18	41	3	4
Propiconazole i	49	52	3	4
Propiconazole ii	51	68	4	5
Vinclozolin	51	68	7	6

$$SEF_{\text{height}} = \frac{\text{Height of diluted peak from stacking}}{\text{Height of undiluted peak using 1s injection}} \times \text{dilution factor}$$

$$SEF_{\text{area}} = \frac{\text{Area of diluted peak from stacking}}{\text{Area of undiluted peak using 1s injection}} \times \text{dilution factor}$$

### Conclusions

Optimized separations of four fungicides from three different classes were successfully achieved using 60 mM sodium cholate, 20 mM ammonium formate pH 7, 5 mM  $\beta$ -CD and 25 kV separation voltage. Normal stacking with electrokinetic injection (SEKI) for 20s at 5 kV was found to be successful for the simultaneous separation and detection of three different classes of fungicides. The stacking SEF in terms of area and height achieved was more than 10 for these four fungicides and these correspond to one order magnitude improvement in sensitivity detection (sub-ppm level). Work is in progress to further reduce the limit of detection attainable to ppb level using the combination of other on-line concentration techniques such as sweeping, stacking with reverse migrating micelles (SRMM) and stacking with reverse migrating micelles with a water plug (SRW) and off-line concentration using solid-phase extraction.

### Acknowledgements

We are grateful to Ministry of Science, Technology and Innovation (MOSTI) for the financial support provided through IRPA grant (project number 09-02-06-0035 EA 158) to the research vote number 74045. Financial support under National Science Fellowship provided by MOSTI to NA is gratefully acknowledged.

### References

1. Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A. and Ando, T. (1984) *Anal. Chem.*, **58**:111-113.
2. Sakulashvili, N., Revia, R., Steiner, F. and Engelhardt, H. (2004) *Chromatographia*, **60**:145-150.
3. Tripodi, V. P., Lucangioli, S. E., Scioscia, S. L. and Carducci, C. N. (2003) *J. Chromatogr. B. Analytical Technologies in the Biomedical and Life Sciences*, **785**:147-155.
4. He, Y. and Lee, H. K. (1998) *J. Chromatogr. A.*, **793**:331-340
5. Jimenez, J. J., Bernal, J. L., de Nozl, M. J., Toribio, L., Arias, E. (2001) *J. Chromatogr. A.*, **919**:147-156.
6. Penmetsa, K. V., Leidy, R. B. and Shea, D. (1996) *J. Chromatogr. A.*, **745**:201-208.
7. Arenas, R. V., Rahman, H. and Johnson, N., A. (1996) *J. AOAC Int.*, **79**:579-581.
8. Halko, R., Padro Sanz, C., Sosa Ferrera, Z., and Santana Rodriguez, J. J. (2004) *Chromatographia*, **60**:151-156.
9. Toribio, L., del Nozal, M. J., Bernall, J. L., Jimenez, J. J. and Alonso, C. (2004) *J. Chromatogr. A.*, **1046**:249-253
10. Zamora, T., Pozo, O. J., Lopez, F. J. and Hernandez, F. (2004) *J. Chromatogr. A.*, **1045**:137-143.
11. Rodriguez, R., Pico, Y. and Manes, G., *J. Chromatogr. A.*, **924**:387-396.
12. Wan Ibrahim, Wan Aini and A'ubid, N. (2003) *Analytical Chemistry: Application and Current Issues*. Sarawak: UNIMAS and ANALIS. **22-29**.
13. Wan Ibrahim, Wan Aini and 'Aubid, N., (2005) *J. Teknologi C.* (resubmitted after correction).
14. Quirino, J. P. and Terabe, S. (1998) *Anal. Chem.*, **70**:149-157.
15. Susse, H. and Muller, H. (1996) *J. Chromatogr. A.*, **730**:337-343.
16. Quirino, J. P., Inoue, N. and Terabe, S. (2000) *J. Chromatogr. A.*, **892**:187-194.

