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# Application of Open Tubular Electrochromatography for Acidic and Basic Analytes

S. Dube<sup>a</sup> and R. M. Smith<sup>b</sup>

<sup>a</sup> *Department of Applied Chemistry, National University of Science and Technology, P.O. Box AC  
939, Ascot, Bulawayo, Zimbabwe*

<sup>b</sup> *Department of Chemistry, Loughborough University, Loughborough, Leicestershire, LE11 3TU,  
UK*

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## Summary

The separation of charged analytes by electrochromatography has been examined on porous-layer open tubular capillaries prepared using the sol gel method. An electroosmotic flow of about  $2.10 \times 10^{-4} \text{ cm}^2/\text{Vs}$  was obtained using between 10 and 30% acetonitrile in the mobile phase. Acidic diuretic drug compounds were successfully separated at high pH as were the N-alkylanilines in their basic and neutral forms. The limitation of open tubular columns was observed on separating some basic pharmaceutical drugs. These components showed severe peak tailing and were not resolved on a  $20 \mu\text{m}$  i.d. porous silica layer open tubular column. Strongly acidic components could not be detected on these columns due to their higher counter electromobilities. The successful separation of neutral aryl alkyl ketones with an efficiency of 101,533 plates/m for butyrophenone was an indication of the improved phase ratio on this type of open tubular columns.

## Introduction

The inherent technical problems associated with the production and stability of packed capillary electrochromatography (CEC) columns have instigated the development of open tubular electrochromatography (OTCEC), in which the stationary phase is immobilised as a coating on the inner walls of the capillary column. Open tubular (OT) columns promise to be more robust than packed columns since they do not require frits to retain the stationary phase and hence alleviate the problems of bubble formation, fragility, band broadening and retention time variability associated with the frits. The first generation of open tubular columns used for CEC work were wall-coated open tubular (WCOT) columns prepared using the wall coating procedures employed in gas chromatography [1-3]. The limiting factor for these OT columns is that they have a very low phase ratio compared to their packed counterparts thus compromising loadability and efficiency for both neutral and ionised analytes. Crego et al. [4] pointed out that even with the best reaction conditions, dynamically wall coated capillaries gave phase ratios around 350 times smaller than those of commercial reversed phase high performance liquid chromatography (HPLC) packing materials. Attempts to improve the phase ratio by employing capillaries with smaller internal diameters (10 and 5  $\mu\text{m}$ ) have the disadvantage of reducing sensitivity, which is already limited in capillary techniques. However, the flow velocity in an OT column is expected to be higher than in a packed column since there is no resistance to flow associated with the packing material whereas in a packed column the flow is also reduced by the non-alignment of flow channels with the electric field [5].

New developments in CEC column technology and new column fabrication methods are aimed at increasing the phase ratio to approach those for packed columns [4,6-11]. Various coating methods have been suggested, which include etching the inner walls of the capillary with ammonium hydrogen difluoride [9,10]. An organic moiety is then immobilised onto the inner walls

to facilitate chromatographic retention. It is estimated that the etching process increases the surface area of the capillary by 1000-fold [12].

Sol gelling is also becoming a popular method to improve the surface area and phase ratio in open tubular columns for CEC use [6,7]. A porous silica layer (PSL) is formed through the precipitation of polyethoxysiloxane (PES) inside a capillary treated with potassium hydroxide followed by hydrochloric acid [8]. Acid catalysed hydrolysis and condensation of tetraethoxysilane (TEOS), followed by subsequent functionalisation with n-octyltriethoxysilane has also been reported [4]. The sol-gel is usually bonded with a C<sub>18</sub> chain or any other organic moiety. Using these new column fabrication methods, capillaries with internal diameters of up to 75 µm have been formed for use in CEC [13]. It has been possible to use such relatively wider internal diameters because of the plug-like flow profile exhibited by capillary electrochromatography.

The reported applications of open tubular capillary electrochromatography have largely been limited to neutral compounds, including polycyclic aromatic hydrocarbons (PAHs) [1,6,7,14-16] and alkyl parabens [11]. However, very little work has been reported on the application of open tubular CEC for charged analytes. This limited interest is probable linked to the complications and confusing results reported for charged analytes in packed column CEC [17,18]. In typical studies Pesek et al. [10] compared the separation of tetracyclines on bare and hydride etched capillary columns. They also separated a mixture of cytochromes from chicken, horse, bovine and tuna using an etched C18 modified capillary column. Benzodiazepines have also been successfully separated on etched cholesteryl modified capillary columns [19]. Schurig et al. [20] showed the potential advantages of using OTCEC when comparing different open tubular chromatographic methods (GC, SFC, OT-LC and OTCEC) for the separation of enantiomers of hexobarbital.

For open tubular columns to be recognised and accepted as robust alternatives to packed columns in CEC more work is still required, particularly on the separation of charged analytes which are of great interest and importance to the pharmaceutical and biological industry. This work therefore set out to investigate the applications of open tubular column for charged analytes. Strongly basic drugs were candidate analytes, since they had faced problems on packed column CEC with inconsistent results being reported [17,18]. It is hoped that the peak shapes of basic compounds would be improved with the use of open tubular columns due to the fewer number of ion-exchange sites than on packed columns. The separation of both weak and strongly acidic components was also investigated.

## **Experimental**

### ***Materials***

Fused silica capillary tubing of internal diameters ranging from 20  $\mu\text{m}$  to 50  $\mu\text{m}$  and outside diameter of 375 $\mu\text{m}$  was purchased from Composite Metal services (The Chase, Hallow, UK). Octadecyltrichlorosilane (ODS) and tetraethyl orthosilicate (TEOS) used for coating the open tubular columns were supplied by Sigma (Poole, UK).

Acetonitrile (HPLC grade), ammonium hydroxide, sodium hydroxide, and sodium dihydrogen phosphate were supplied by Fischer Scientific, (Loughborough, UK). Deionised water used for preparing the buffers was purified to 18.2 M $\Omega$  using Elga Pure Water (Elga, High Wycombe, UK) equipment. Di-sodium hydrogen phosphate was purchased from Aldrich (Poole, UK). All buffers used in this work were of analytical reagent grade.

Alkyl aryl ketones, benzoic acid, ibuprofen, and N-alkylanilines were purchased from Sigma (UK)

Acidic diuretics and basic drugs including ephedrine HCl, morphine HCl, pseudoephedrine HCl, chloroprocaine and pholcodine were donated by Home Office Forensic Science Services (Aldermaston, UK). The test compounds were examined as 0.444 mg/ml solutions in 50% acetonitrile-buffer.

## ***Instrumentation***

CEC experiments were carried out on a Beckman P/ACE System 2050 (High Wycombe, USA). Coated open tubular capillary columns were mounted onto a standard Beckman cartridge and were thermostatted to 25 °C using a liquid coolant. The Beckman CE instrument was equipped with a filter photometer at 214 nm and data collection was achieved using System Gold Software, version 8.0.

## ***Preparation of a Porous Silica Layer OTC***

Capillaries of 20 µm i.d. were coated according to the method described by Tock et al. [8] and Crego et al.[4]. The packing reservoir was filled with ammonium hydroxide (pH 9.0) solution and one end was connected to a fused silica capillary tubing and the solution was slowly forced into the capillary using nitrogen flow at a pressure of about 83 psi. The capillary was then capped at both ends using Teflon tubing and placed in an oven at 80 °C for 12 hrs. After this preconditioning step the capillary was washed with methanol for two hours and dried by purging with nitrogen at 200 °C overnight.

The pre-gelling mixture consisted of 800 µl of methanol, 600 µl of tetraethyl orthosilicate (TEOS) and 300 µl of ammonium hydroxide solution, pH 9.0. This mixture was stirred for 10 minutes, degassed using an ultrasonic bath for 2 minutes and filtered through a 0.2 µm Millipore filter

membrane. The capillary was then filled with the pre-gelling solution. Both ends were sealed and the reaction was allowed to proceed at 80 °C for 6 hr after which the capillary was emptied using a nitrogen gas flow. The porous silica layer was stabilised by heating the column at 100 °C for 12 hr. while nitrogen gas was purged through the capillary.

The column was allowed to cool to ambient temperature and it was then washed with water for 2 hrs and dried at 200 °C for a further 2 hrs. A C<sub>18</sub> moiety was then attached to the silica layer by filling the capillary with a 10% w/v of octadecyltrichlorosilane in xylene, sealing the capillary and allowing the reaction to proceed for 12 hrs at 120 °C. Finally the capillary column was allowed to cool to room temperature prior to equilibrating it with acetone and methanol. For a 50 µm i.d capillary the reaction times and temperatures were adjusted accordingly. Coated columns were coupled with a Teflon sleeve to a 30 µm i.d. untreated capillary carrying a detector window.

## Results and Discussion

### ***Column Performance- Effect of acetonitrile content on the EOF***

The dependency of the electroosmotic flow on the amount of acetonitrile in the mobile phase was determined using a 20 µm i.d. PSL C<sub>18</sub> open tubular column and the results were compared to those from a wall-coated capillary column (Figure 1). The PSL column showed a slight increase in the EOF as the acetonitrile content was varied from 0 to 10% and then remained constant between 10 and 30% acetonitrile in the mobile phase. However, by analogy with packed columns [21,22] the EOF was expected to increase on increasing the acetonitrile content. Crego et al. [16], has also reported an increase in the EOF with increasing the ACN concentration from 50% to 65% on a 10 µm i.d. PSL columns. The WCOT column, on the other hand, displayed a decrease in the

electroosmotic flow with increase in % of acetonitrile, a trend usually associated with the bare fused silica capillaries.

The PSL columns showed very good stability lasting for more than two months even after being exposed to the organo-buffer mobile phases. Clogging of the column was the only problem encountered. This occurred because of the poor solubility of the analytes. However, the column performance was easily rejuvenated by rinsing with high concentrations of acetonitrile.

### ***Separation of aryl alkyl ketones on OTCEC***

The effect of acetonitrile content on the retention factors of the alkyl aryl ketones as model neutral compounds (Figure 2) was studied by varying the proportion of organic solvent on the 20  $\mu\text{m}$  i.d. PSL column. For example, the retention of valerophenone, decreased from  $k = 2.77$  at 10% ACN to 0.22 at 30% ACN and these changes confirmed that the ketones were being separated by a chromatographic partition mechanism. For each of the ketones there was a linear decrease in  $\ln k_{\text{CEC}}$  as the concentration of the organic modifier was increased from 10-30% acetonitrile, which typifies a reversed-phase selectivity. At 30% acetonitrile the homologues were still resolved. Short retentions are desirable as in open tubular CEC the smaller the retention factor the higher the efficiency [23] and generally sharper peaks were obtained as the concentration of acetonitrile in the mobile phase was increased. The higher acetonitrile concentrations also prevented precipitation of the analyte in the mobile phase (<5% acetonitrile). Loss of resolution was observed at > 40% acetonitrile concentrations.

### ***Column Efficiency***

Theory predicts that the use of open tubular columns substantially increases the column efficiency



because they effectively eliminate the contribution of flow inequalities to band broadening (the A term) [23, 24]. Ideally, open tubular capillary electrochromatography should be able to generate higher efficiencies than those achieved with the packed capillary format. In this work efficiencies measured at 25% acetonitrile, on a 20  $\mu\text{m}$  i.d. PSL C18 open tubular column were 72,060, 66,630, 77,989 and 40,244 plates/m for thiourea, propiophenone ( $k_{\text{CEC}} = 0.06$ ), butyrophenone ( $k_{\text{CEC}} = 0.16$ ) and valerophenone ( $k_{\text{CEC}} = 0.41$ ), respectively (Table 1). These increased nearly two fold with 30% acetonitrile, for example, butyrophenone 101533 plates/ m. Usually higher efficiencies have been reported for less polar compounds, with 500 000 plates/m being achieved for PAHs [6,7], when significantly high concentrations of the organic modifier were used in the mobile phase. However, very low efficiencies (10 000 plates/meter) were reported for strongly interacting analytes on a 75  $\mu\text{m}$  i.d OT column which were [13] attributed to the wider bore.

### ***Separation of N- Alkylanilines at Various pH***

As the PSL open tubular columns demonstrated a reasonable performance for neutral compounds, the N-alkylanilines were investigated on a 50  $\mu\text{m}$  internal diameter PSL C18 OT column at different pH values (ure 3). Because of the relatively low phase ratio of this column the N-alkylanilines were separated using a fully aqueous buffer. The best separation was achieved at pH 2.0 where the compounds were fully ionised and were eluted primarily by electromigration (Figure 3a). Sharp and efficient peaks were observed for aniline, N-ethylaniline and N-propylaniline. Since the silanol groups were not significantly ionised at this low pH, there would be insignificant interaction between the analytes and the stationary phase. As expected the EOF was quite low under these conditions as shown by the long migration time of the unretained component, thiourea (11.40 min).

At pH 3.0 the compounds still possessed a high electrophoretic mobility and hence eluted ahead of the unretained compound (Figure 3b). However, N-ethylaniline and N-propylaniline co-eluted as a broad peak. An increase in the electroosmotic flow and a decrease in the migration time were also observed at this pH. Peak tailing particularly for N-ethylaniline was possibly due to interactions with the negatively charged stationary phase since the solutes were still protonated at pH 3.0.

A poorer separation characterised by severe peak tailing and loss of resolution (Figure 3c), was observed at pH 4.0. Since the compounds were 50% ionised under these conditions, both chromatographic retention and electromigration determined the separation. Between pH 6.0 and 8.0 the N-alkylanilines ( $pK_a < 4.2$ ) were eluted by chromatographic retention alone since they were uncharged under these conditions. Although complete resolution was attained at pH 6.0, the bulkier compounds, N-ethylaniline and N-propylaniline, showed severe band broadening apparently due to poor mass transfer. However, the increased electroosmotic flow at pH 7.0 and 8.0 appeared to have the effect of improving the peak shapes of the compounds (Figure 3d and 3e). The coating on the column was therefore sufficient to resolve the neutral and uncharged components.

An improved separation was obtained on a narrower 20  $\mu\text{m}$  i.d PSL C18 OT column using 15% acetonitrile in the phosphate buffer at pH 8.22 (Figure 4). Increasing the acetonitrile concentration to 20% led to a loss in resolution. The resolution of the N-alkylanilines on a 20  $\mu\text{m}$  i.d. PSL C18 open tubular column was an indication of chromatographic selectivity since no resolution could be achieved using a capillary electrophoresis system on an uncoated capillary under similar conditions.

### ***Separation of Acidic Compounds on OTCEC***

In packed column CEC the separation of strongly acidic analytes at high buffer pHs poses a problem because analytes cannot be loaded onto the column using electrokinetic injection [25,26]. The separation of both strong and weak acidic analytes in an open tubular column was therefore attempted at pH 8.2 with the hope that the electroosmotic flow would be stronger than in a packed column and thus circumvent the sample loading problem. OT mode also permits pressure injection in the conventional CE instruments.

### ***Separation of Diuretic Drug Compounds***

Euerby et al. [18] and Taylor et al. [27] both reported the successful separations of diuretic drugs on packed column CEC at pH 2.5, where the analytes were uncharged. However, the problem of analysing acids in their ion-suppressed mode is the need for a low-pH mobile phase, which results in a reduced EOF and ultimately to long analysis times.

In this work, four diuretics, chlorothiazide, methyclothiazide, hydroflumethiazide and benzthiazide were investigated on a 20  $\mu$ m i.d. PSL C18 open tubular column using a 12.5 mM phosphate buffer at pH 8.2 with 20% (a) and 25% (b) acetonitrile respectively (Figure 5). Increasing the concentration of acetonitrile improved the peak shapes of the diuretics significantly, especially for the later eluting compounds, chlorothiazide and benzthiazide (Figure 5b). However, the first two compounds, methyclothiazide and hydroflumethiazide were incompletely resolved in both eluents. This separation illustrates the ability to load the diuretics onto the column despite their partial charge as the EOF generated in the column is strong enough to carry the compounds to the detector. The separation could also be performed at a lower pH where the diuretics would be uncharged and hence eluted by chromatographic retention.

### ***Separation of Strongly Acidic Analytes***

Attempts were then made to exploit the significantly high EOF in an open tubular column to electrokinetically load the stronger acids, benzoic acid and ibuprofen, which are a challenge to inject onto a packed CEC column [26,27].

A test mixture of thiourea, ibuprofen and benzoic acid was examined on a 20  $\mu\text{m}$  i.d. C<sub>18</sub> WCOT column and a 50  $\mu\text{m}$  i.d. PSL C<sub>18</sub> column. At pH 8.2 both benzoic acid ( $\text{pK}_a = 4.19$ ) and ibuprofen ( $\text{pK}_a = 4.24$ ) were fully ionised and were expected to be eluted predominately by electromigration. A striking difference was observed in the separation of these two acids on the WCOT and PSL columns. Although three peaks were seen on the WCOT column, only two peaks thiourea and ibuprofen, were observed on the PSL column. Benzoic acid could not be eluted even when the electroosmotic flow was increased by reducing the acetonitrile concentration to 0%. This was an indication that the electroosmotic flow generated on the PSL C<sub>18</sub> column was lower than on the WCOT column. Thus benzoic acid could not be eluted because its electrophoretic mobility was greater than the electroosmotic flow and it was therefore migrating away from the detector. This behaviour was similar to that displayed by highly deprotonated (> 96%) sulfonamides when they were separated on a CEC packed column [26].

### ***Effect of Acetonitrile on the Electrophoretic Mobility of Acids***

The amount of acetonitrile in the mobile phase was then varied from 0% to 40% on the C<sub>18</sub> WCOT column and the electroosmotic flow and the electrophoretic mobilities of benzoic acid and ibuprofen were determined (Figure 6). The electroosmotic flow decreased with the increase in the volume fraction of acetonitrile, a trend similar to that observed when using a CZE uncoated capillary column. Increasing the acetonitrile content, however, did not have a drastic effect on the electrophoretic mobilities of either ibuprofen or benzoic acid in the acetonitrile concentration of 0%

to 15% but a significant change in the electrophoretic mobility of ibuprofen from  $-1.76 \times 10^{-4}$  cm<sup>2</sup>/Vs at 15% acetonitrile to  $-1.23 \times 10^{-4}$  cm<sup>2</sup>/Vs at 40% acetonitrile was noted.

The peak representing benzoic acid disappeared at 40% acetonitrile due to a reduced electroosmotic flow. Since there was not much change in the electrophoretic mobility of benzoic acid it was assumed that at 40% acetonitrile, the electroosmotic flow had dropped lower than the reverse mobility of this analyte.

### ***Separation of Pharmaceutical Basic Drugs on Open Tubular CEC***

The basic pharmaceutical drugs, ephedrine hydrochloride (pK<sub>a</sub> = 9.96), pseudoephedrine hydrochloride (pK<sub>a</sub> = 9.22), morphine hydrochloride (pK<sub>a</sub> = 8.2, 9.3), pholcodine (pK<sub>a</sub> = 9.3) and chlorprocaine (pK<sub>a</sub> ( 9.0), were separated on a 20 µm i.d. PSL C18 open tubular column at a pH buffer of 8.22. At pH 8.22 ephedrine hydrochloride was 98% protonated and hence exhibited a strongly electrophoretic character, which was demonstrated by its migration well ahead of thiourea, the unretained compound. However, severe peak tailing was observed which was attributed to electrostatic interactions with ionised silanol groups on the column. Some chromatographic retention might also be present which was tested by varying the concentration of the organic modifier content from 30% to 50% acetonitrile (Figure 7) which resulted in reduced retention and better peak shapes.

The three basic compounds, chlorprocaine, morphine and pholcodine were resolved at 30% acetonitrile in a 20 µm i.d. PSL C18 column at pH 8.22. (Figure 8a). The shoulder observed after morphine was thought to be due to an impurity in the sample. Pholcodine and chlorprocaine with pK<sub>a</sub> values of 9.3 and ca. 9.0, respectively, eluted as broad, tailing peaks. Both compounds were highly ionised at pH 8.22 and the poor peak shapes observed were attributed to the solute-silanol group electrostatic interactions. This effect was however not pronounced for morphine (pK<sub>a</sub> = 8.2,

9.3), which was also expected to be highly protonated under these conditions. Ephedrine and pseudoephedrine hydrochlorides co-eluted as a broad peak at 2.22 minutes and also showed peak tailing like pholcodine and chlorprocaine.

Increasing the concentration of acetonitrile to 50% resulted in the loss of resolution between pholcodine and morphine (Figure 8b). However, chlorprocaine was completely resolved from the rest of the compounds. A slight improvement in resolution was also observed between ephedrine and pseudoephedrine hydrochloride. This was thought to have been due to the reduction in the chromatographic retention of the two analytes. It was also noted that baseline drift was also reduced at 50% acetonitrile.

## **Conclusions**

These results indicate that it is possible to separate charged compounds on open tubular columns as demonstrated by the separation of acidic diuretics and N-alkylanilines as basic compounds at low pH. However, the poor separations observed for the basic pharmaceutical drugs led us to conclude that porous silica layer open tubular columns modified with ODS have enough residual silanols to interact with protonated basic components. It might be possible to add competing amines or anionic surfactants into the mobile phase.

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Figure 1. Relationship between the organic modifier content and the electroosmotic flow on a WCOT C18 50  $\mu\text{m}$  i.d, PLS C18 20  $\mu\text{m}$  i.d x 310(377) mm columns. CEC conditions 12.5 mM phosphate buffer, pH 8.14; electrokinetic injection at 3 kV for 3 secs; applied voltage, 20 kV; temperature, 25°C; UV detection at 214 nm.

Figure 2. Effect of acetonitrile content on the separation of alkyl aryl ketones on a 20  $\mu\text{m}$  i.d. PSL C18 column. CEC conditions: injection at 3 kV for 3 sec; applied voltage, 20 kV; temperature, 25°C, UV detection at 214 nm; peak identification; (1) thiourea, (2) propiophenone, (3) butyrophenone, (4) valerophenone

Figure 3. Separation of N-alkylanilines at different pH. CEC conditions: column, 289(358) mm x 50  $\mu\text{m}$  i.d. PSL C18 OTC; buffer, 12.5 mM phosphate (a) pH 2.0, (b) pH 3.0, (c) pH 4.0; injection, electrokinetic at 3kV for 3s; applied voltage, 15 kV; temperature, 25°C; detection, UV at 214 nm; peak identification: (1) aniline, (2) N-ethylaniline, (3) N-propylaniline (4) thiourea

Figure 4. Separation of N-alkylaniline on a 20  $\mu\text{m}$  i.d. PSL C18 OTC. CEC conditions: mobile phase, acetonitrile/ 12.5 mM phosphate buffer, pH 8.22, (15/85, v/v); applied voltage, 20 kV; injection, electrokinetic at 3 kV for 3 secs; peak identification, (1) aniline, (2) N-methylaniline, (3) N-ethylaniline, (4) N-propylaniline.

Figure 5. Separation of diuretics at (a) 20% and (b) 25% acetonitrile. CEC conditions: column, PSL C18 OTC: mobile phase, acetonitrile/12.5 mM phosphate buffer pH 8.2. Other conditions as in

Figure 4. Peak identification, (1) methylclothiazide, (2) hydroflumethiazide, (3) benzthiazide, (4) chlorothiazide.

Figure 6. Effect of the acetonitrile content of the mobile phase on the electrophoretic mobilities of ibuprofen and benzoic acid. Other conditions as in Figure 6.

Figure 7. Separation of ephedrine hydrochloride at (a) 30% and (b) 50% acetonitrile in the mobile phase. CEC conditions: column, 20  $\mu$ m i.d PSL C18 open tubular column; other conditions as in Figure 5.

Figure 8. Open tubular CEC of basic pharmaceutical drugs. CEC conditions: mobile phase, (a) 30% and (b) 50% acetonitrile/12.5 mM phosphate buffer, pH 8.22; peak identification, (1) ephedrine hydrochloride, (2) pseudoephedrine hydrochloride, (3) chlorprocaine, (4) morphine, (5) pholcodine; other conditions as in Figure 5.

**Table I.** Theoretical number of plates achieved for neutral compounds on a 20  $\mu\text{m}$  PSL column at 25 and 30% acetonitrile. CEC conditions as in Figure 1.

Compound.	Efficiency (N/m)	
	25% ACN	30% ACN
Thiourea	72,060	112,339
Propiophenone	66,630 ( $k_{\text{CEC}} = 0.06$ )	92,162 ( $k_{\text{CEC}} = 0.04$ )
Butyrophenone	77,989 ( $k_{\text{CEC}} = 0.16$ )	101,533 ( $k_{\text{CEC}} = 0.09$ )
Valerophenone	40,244 ( $k_{\text{CEC}} = 0.41$ )	81,242 ( $k_{\text{CEC}} = 0.22$ )

Figure 1.

*S. Dube and R.M. Smith*

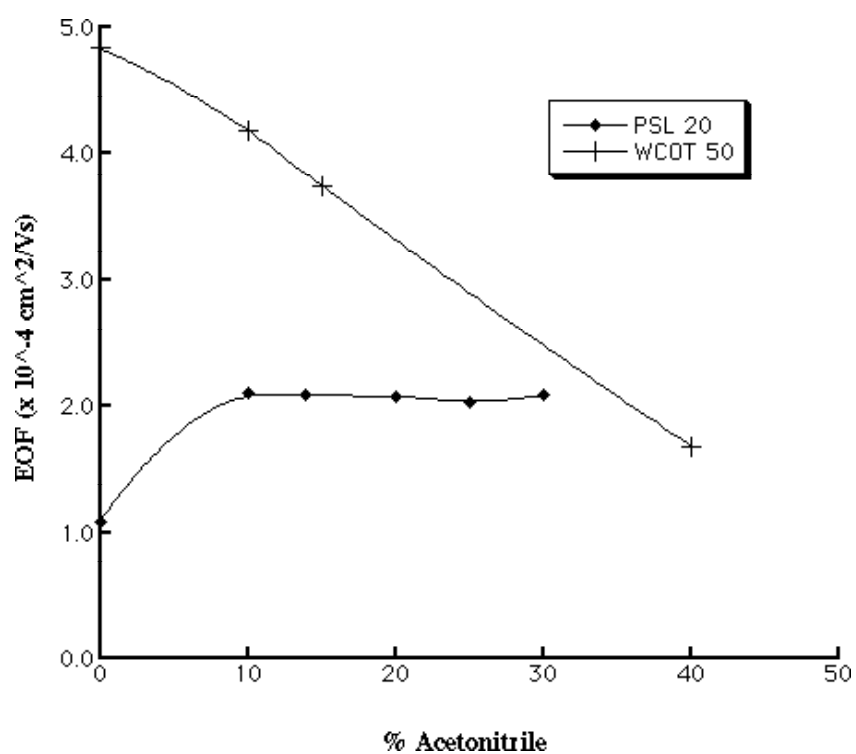


Figure 2.

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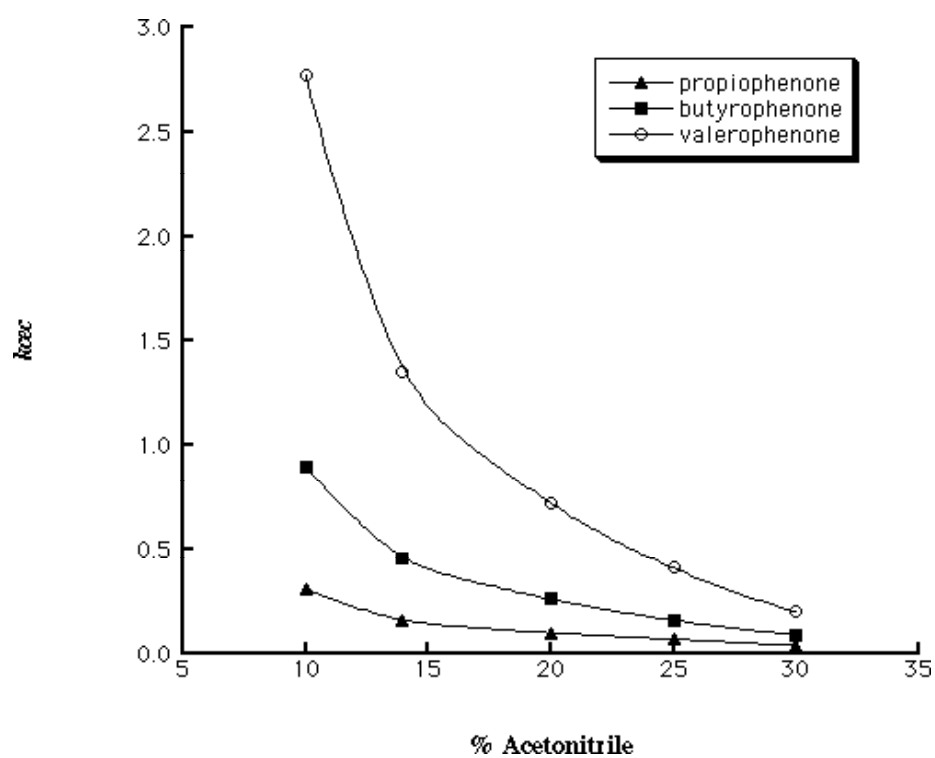




Figure 3a

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Figure 3b

*S. Dube and R.M. Smith*

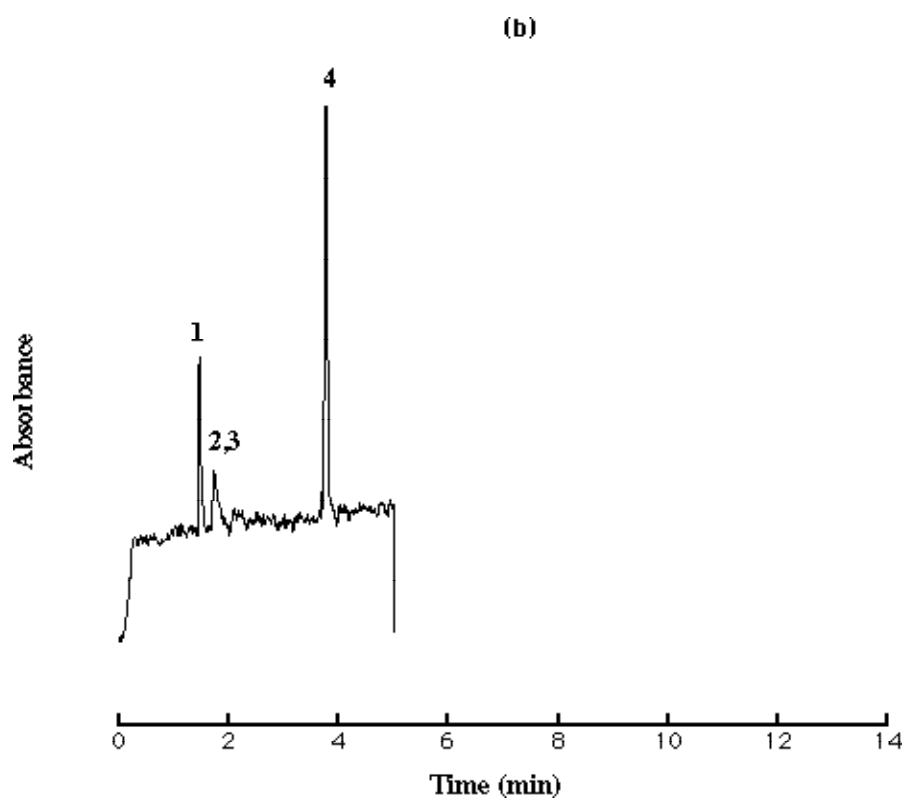




Figure 3c

*S. Dube and R.M. Smith*

Figure 3d.

*S. Dube and R.M. Smith*

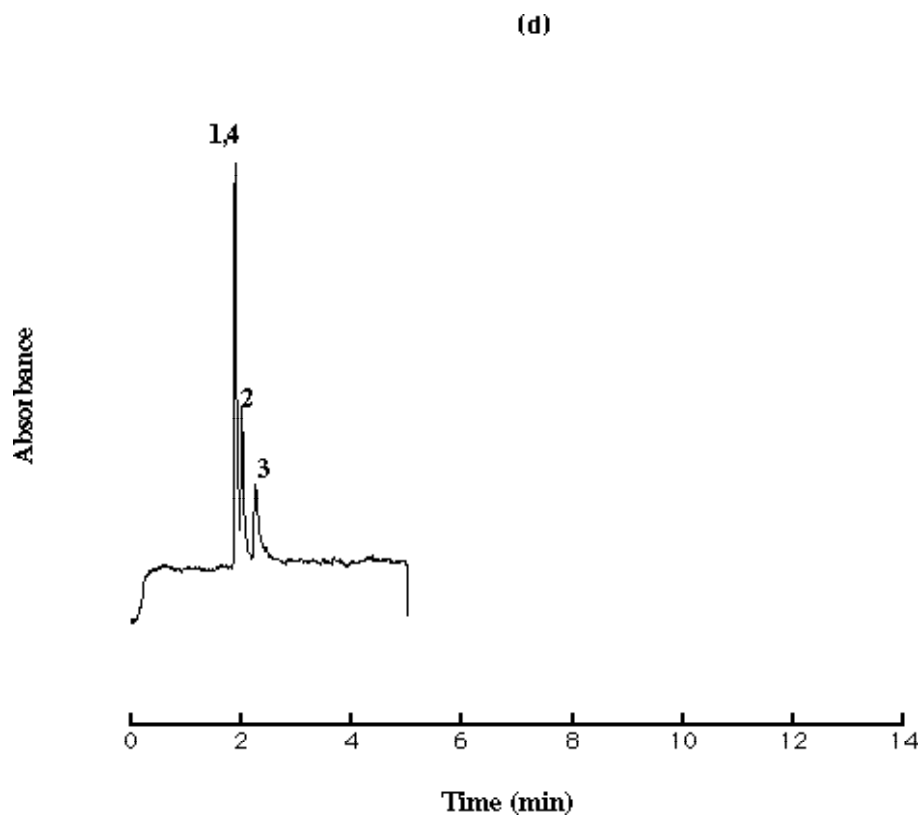


Figure3e

*S. Dube and R.M. Smith*

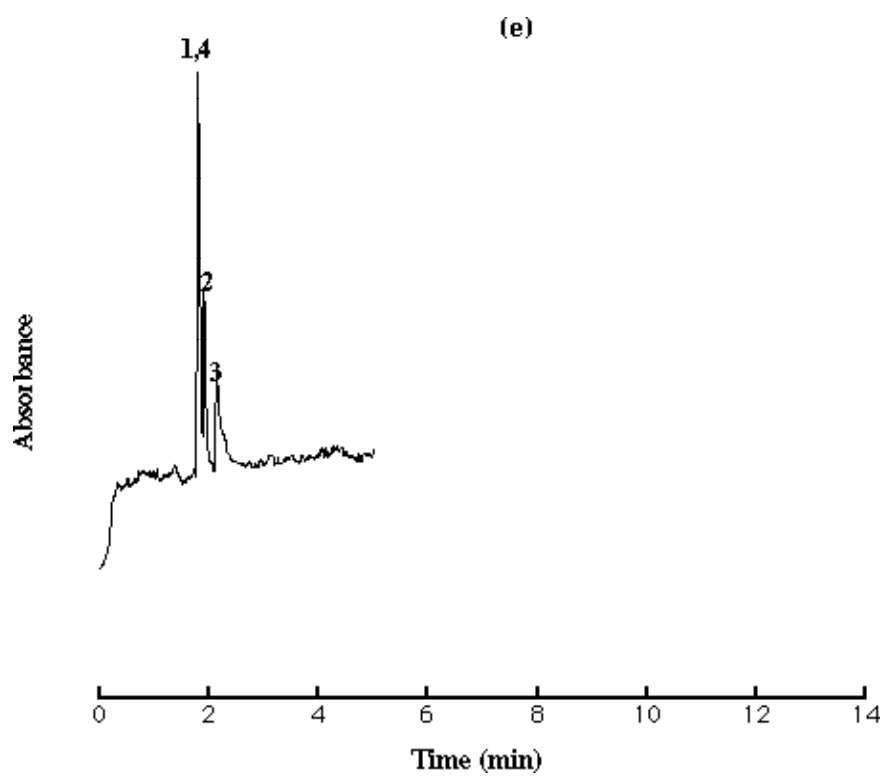


Figure 4.

*S. Dube and R.M. Smith*

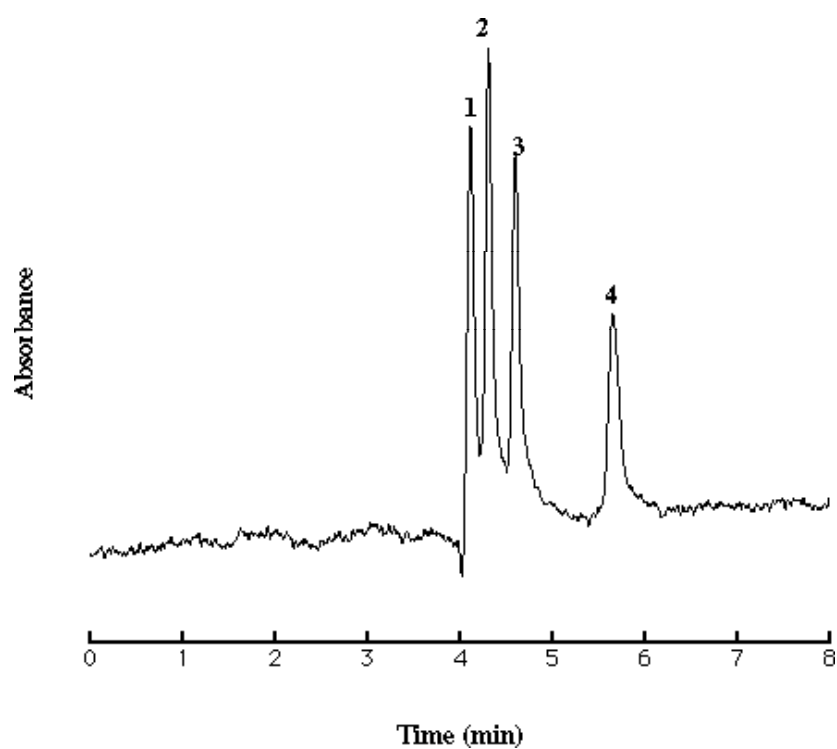


Figure 5a

*S. Dube and R.M. Smith*

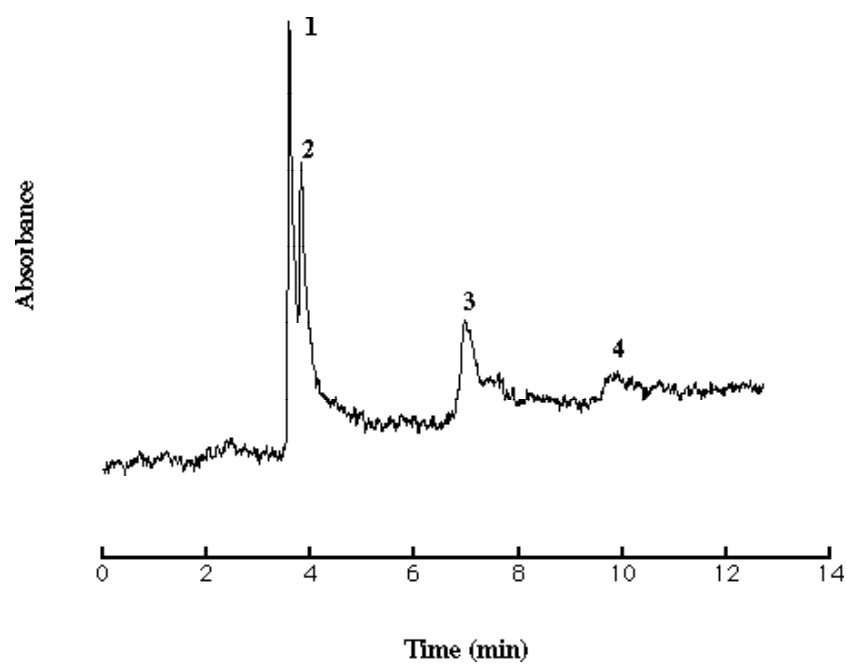


Figure 5b.

*S. Dube and R.M. Smith*

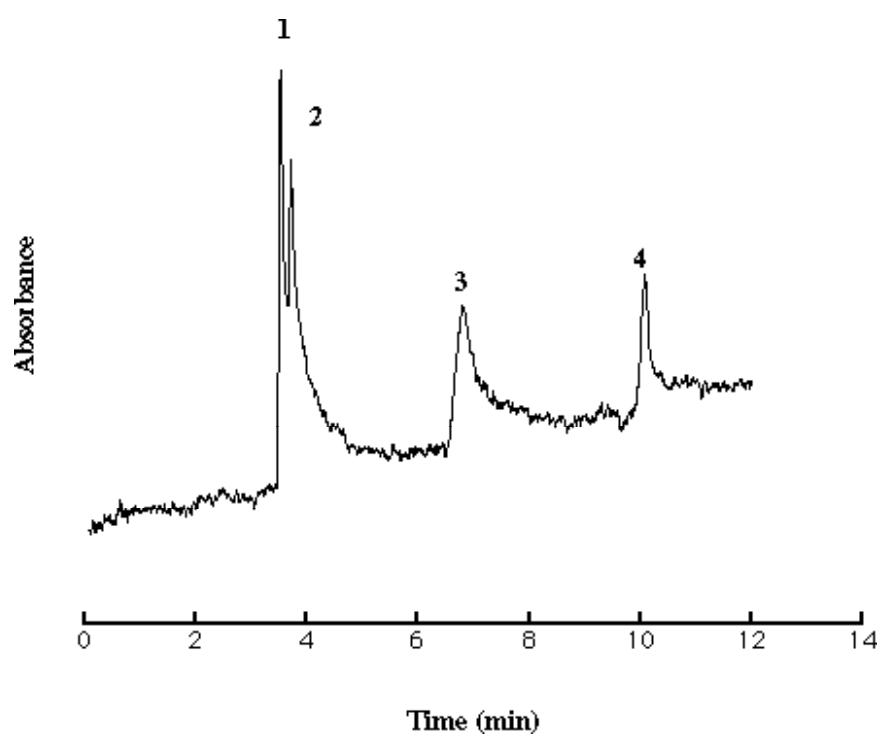


Figure 6

*S. Dube and R.M. Smith*

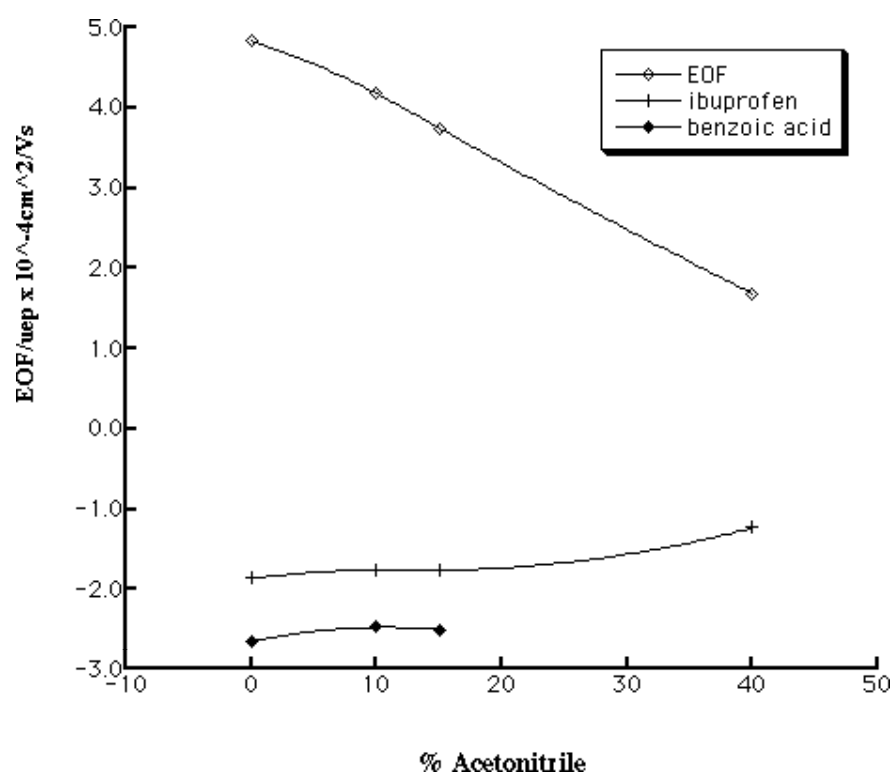


Figure 7a

*S. Dube and R.M. Smith*

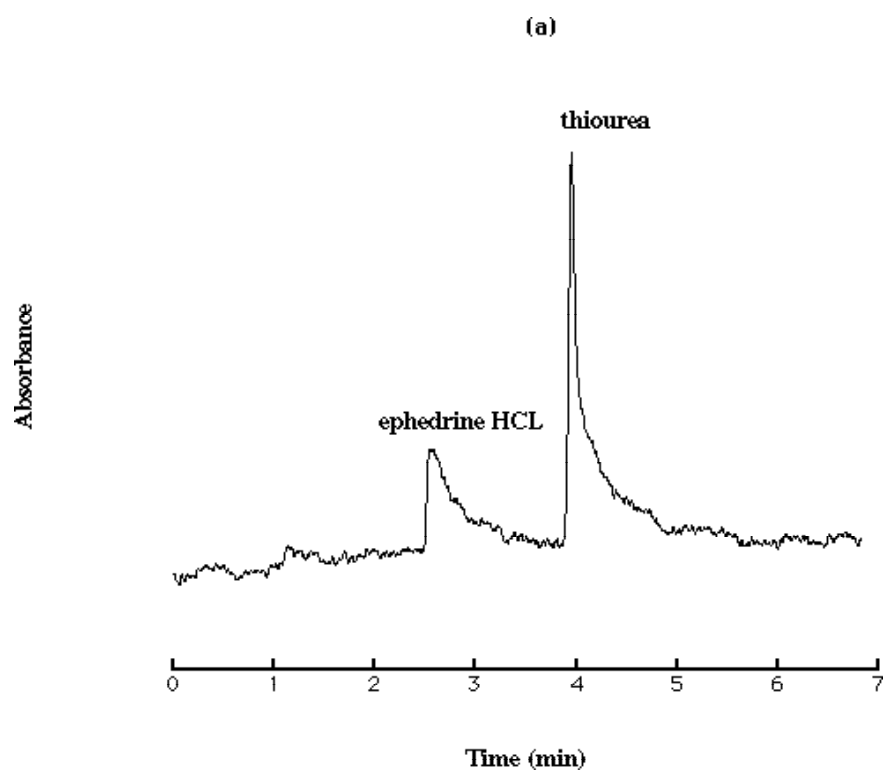




Figure 7b.

*S. Dube and R.M. Smith*

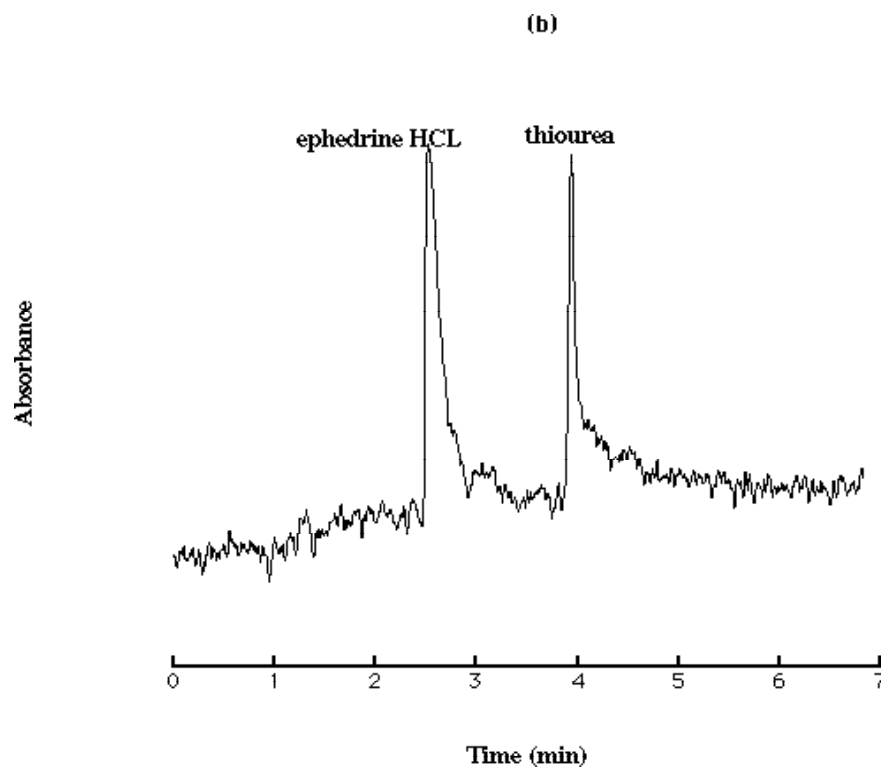


Figure 8a

*S. Dube and R.M. Smith*

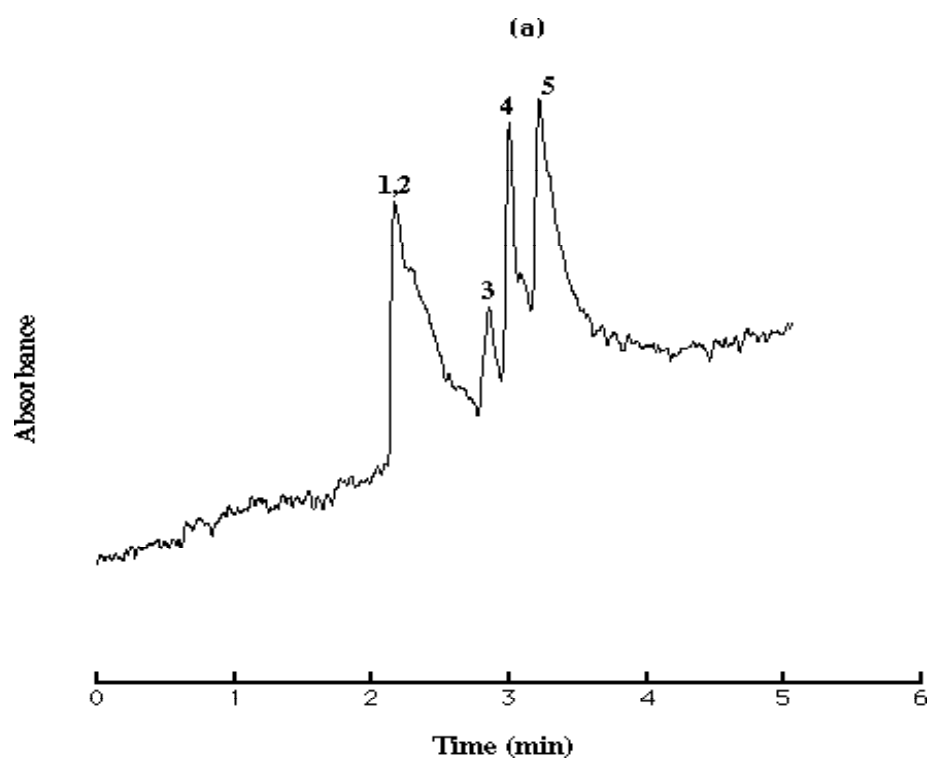


Figure 8b

*S. Dube and R.M. Smith*

