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Optimisation of the separation of the dimethylpyridines by capillary electrophoresis

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Abstract

The separation of the isomers of dimethylpyridine has been studied by capillary electrophoresis. Studies at low pH where the dimethylpyridines all bear the same charge showed a partial separation probably due to size/shape effects. The charge profiles of the analytes calculated from their ionisation constants were compared with their separation between pH 2.5 and 9. The optimum resolution was found at a pH of 6.5 in the area of maximum charge difference between the dimethylpyridines.

Keywords: Capillary eleactrophoresis; Optimization; pH effects; Dimethylpyridines; Pyridines

1. Introduction

Capillary electrophoresis (CE) is a separation technique which has been successfully used to achieve the highly efficient separation of a vast range of analytes, ranging from biomolecules to smaller analytes, such as small organic molecules or inorganic ions [1]. Because the separation depends on the migration velocities of the analytes under the influence of the electrical field, it should be possible to predict the differences between the behaviour of even closely related compounds from their size and charge. By selecting a suitable buffer pH the analyst should be able to adjust the charge on partially ionised analytes to optimise the resolution of a mixture. If the ionisation constants (pK_a values) of the analytes are known then it should be also possible to predict the conditions for maximum separation.

In previous studies, Terabe and co-workers studied the separation of oxygen isotopic benzoic acids as examples of closely related compounds [2]. An optimum pH for separation was calculated to be ($pK_a - \log 2$) based on the theoretical resolution equation in CE. The effects of applied voltage and capillary length were also investigated. Optimisation of pH in the separation of the methylpyridines was studied by Wren [3], who predicted that the maximum charge difference between two species could be calculated by taking the average of their pK_a values. The relationship between the calculated charge on an analyte and its electrophoretic mobility was investigated as well as the relationship with electroosmotic mobility. Separation of the methylpyridines was improved by the use of a cationic surfactant to suppress electroosmotic flow.

Friedl and Kenndler investigated resolution as a function of the pH of the buffer for multivalent ions,

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including the benzenecarboxylic acids [4]. It was determined that resolution depended on two terms, the ratio of the electrophoretic mobilities and an efficiency term. Based on the pK_a values and the actual mobilities the resolution was calculated across the pH range and compared with experimental data. The effect of experimental parameters on the separation of *p*- and *m*-aminobenzoic acids was investigated by Nielen [5]. The pH was varied between 4.0 and 6.0 and the optimum resolution was found to be close to the pK_a values of the analytes. Because these analytes have two pK_a values, a reversal in migration order was observed at low pH with co-migration at pH 4.2. The effect of the partial charge on the migration rates has also been used to determine ionisation constant at low solute concentrations [6]. By studying the change in electrophoretic mobility across the pH range, pK_a values between 2.55 and 5.26 were determined to within 0.03 pH units for analyte concentrations less than 100 μM.

Separations due to differences in the shape of closely related analytes have been observed by several workers. The separation of *cis*-retinoic acid and *trans*-retinoic acid was investigated by Chadwick and Hsieh [7] and Korman and co-workers investigated the separation of codeine and its synthesis by-products [8]. A series of monoalkylpyridines was investigated by Rowe and coworkers and molecular descriptors were used to explain the separation [9]. A mixture of (Z) and (E) pentenylpyridines has been investigated by CE and NMR and quantified by the two techniques [10].

In this study, the resolution of a complex mixture of the six dimethylpyridine isomers has been studied by capillary electrophoresis. The mobilities of the fully charged analytes were determined at low pH and these values, together with the partial charge calculated from their ionisation constants by using the Henderson–Hasselbalch equation, were used to predict the mobility of each isomer across a wide pH range. These predictions were then compared with the experimentally determined resolution.

2. Experimental

2.1. Chemicals

Distilled water was purified to 18 MΩ using an Elga Maxima water purification system. The dimethylpyridines (DMP), benzamide and hydroxypropylmethylcellulose (Average M.N. 86 000. HPMC) were obtained from Aldrich Chemical (Gillingham, UK). Sodium dihydrogen phosphate, citric acid and orthophosphoric acid were obtained from BDH (Poole, UK). Lithium hydroxide was obtained from Fisons Scientific Apparatus (Loughborough, UK).

2.2. Apparatus

The capillary electrophoresis was carried out on a P/ACE 2050 system (Beckman Instruments, High Wycombe, UK), using a fused-silica capillary (Beckman) with an internal diameter of 75 μm, a length from inlet to detector of 50 cm and a total length of 57 cm. The capillary was thermostatted to 25°C. The electropherograms were recorded at 254 nm using a 5 Hz collection rate. An IBM 433/DX microcomputer with System Gold Personal Chromatograph (Beckman) software was used for data collection.

Buffer pH values were measured using a combination pH electrode fitted to a radiometer Copenhagen PHM 64 research pH meter.

2.3. Methods

A lithium phosphate buffer was prepared by making up a 40 m*M* solution of orthophosphoric acid and adjusting the pH to 2.5 with 1 *M* lithium hydroxide. A sodium phosphate buffer was prepared by making a 50 m*M* solution of sodium dihydrogen phosphate and adjusting the pH to 6.5 with 1 *M* hydrogen chloride. Lithium citrate buffer systems were prepared by making up a 40 m*M* citrate solution and adjusting to the required pH with 1 *M* lithium hydroxide.

The dimethylpyridines were used as received. Stock solutions of 1 mg ml^{-1} were made up in de-ionised water and samples for injection were prepared each day by taking 50 ml of the DMP stock solution and diluting it in 4.4 ml of de-ionised water. Samples were loaded onto the CE column by using a 2 s pressure injection and were separated using a voltage of 15 kV.

Electrophoretic mobilities μ_e of the analytes were determined using the equation:

$$\mu_e = \frac{lL}{Vt} - \mu_{eo} \quad (1)$$

where l is the length of the column from inlet to detector, L is the total capillary length, V is the operating voltage, t is the migration time and μ_{eo} is the electroosmotic mobility. The electroosmotic mobility was measured by using benzamide (2 mg ml^{-1}) in the phosphate buffer systems as a neutral marker. An acetone solution (5% v/v in de-ionised water) was used in the citric acid buffer systems.

3. Results and discussion

3.1. Separations at pH 2.5

A partial separation of the dimethylpyridines (DMP) could be achieved at pH 2.5 (Fig. 1a), where each of the isomers bears a full positive charge because the pH is much lower than their pK_a values. In order to improve the baseline separation the ionic strength of the background electrolyte was varied from 10 mM to 100 mM and it was concluded that an optimal separation was obtained at about 40–60 mM. At lower ionic strengths, the electroosmotic flow in the capillary increases, and so the time window for separation is decreased. At higher ionic strengths, Joule heating effects were observed, leading to band broadening of the dimethylpyridine peaks. Both these effects contributed to a loss in resolution.

The migration order at pH 2.5 was determined by spiking studies as 3,4-DMP, 3,5-DMP, 2,3-DMP≈ 2,5-DMP, 2,4-DMP, 2,6-DMP. As expected this order did not correspond to the order of pK_a values determined by potentiometric titration (Table 1) [11] confirming that all the analytes were fully charged. The electrophoretic mobility of the dimethylpyridines at low pH, $\mu_{e,q=+1}$ was calculated (Table 1). These differences in mobility must be due to small differences in the effective size and shape of the analytes caused by differences in the relative positions of the substituents compared to the position of maximum charge and are the subject of a continuing study.

By adding 0.1% hydroxypropylmethylcellulose (HPMC) to the buffer the viscosity of the background electrolyte is increased reducing the migration mobility potentially increasing the discrimination between isomers. However, no significant improvement in resolution was obtained. The temperature of the background electrolyte was then reduced from 25°C to 15°C and an increase in resolution was observed (Fig. 1b). However, 2,3-DMP and 2,5-DMP still did not separate and it was concluded that they had identical mobilities.

3.2. Prediction of mobility profile from pK_a values

The partial charge on each of the dimethylpyridines was then calculated across a broad pH range from their reported pK_a values (Table 1) [11] and the Henderson–Hasselbach equation. Since the electrophoretic mobility of each isomer should be a product of the fully charged mobility (at pH 2.5) and the partial charge, the mobility of the dimethylpyridines can be predicted across the pH range (Fig. 2). These predictions suggest that points of co-migration of isomers should occur and the order of elution will be reversed on either side of these points. The pH of maximum mobility difference, which occurs at pH 6.5, should give the optimum separation (Fig. 2). In the intermediate pH range of between 4 and 6, there should be points where the mobilities of pairs of analytes should coincide. This was confirmed by separating the mixture at a number of pH values between pH 4.5 and 5.77 (Fig. 3).

At pH 4.5, the mobility order was 3,4-DMP, 3,5-DMP≈2,3-DMP≈2,5-DMP, 2,4-DMP≈2,6-DMP which was the same order as at pH 2.5, suggesting that the separation was still determined by the shape of the analytes. As the pH was raised, the migration order becomes scrambled as the charges on the different isomers were reduced by different extents. For example at pH 4.75, the 2,3-DMP, 2,6-DMP and 3,5-DMP isomers coeluted. As the pH was raised further, the differences in the partial charge on the DMPs became the dominant factor, and at pH 5.5 the migration order was 2,6-DMP, 2,4-DMP, 2,5-DMP, 3,4-DMP, 2,3-DMP, 3,5-DMP in agreement with the order predicted from pK_a data (Fig. 2).

3.3. Separation at pH 6.5

The separation at a buffer pH 6.5 produced baseline separation of all six dimethylpyridines (Fig.

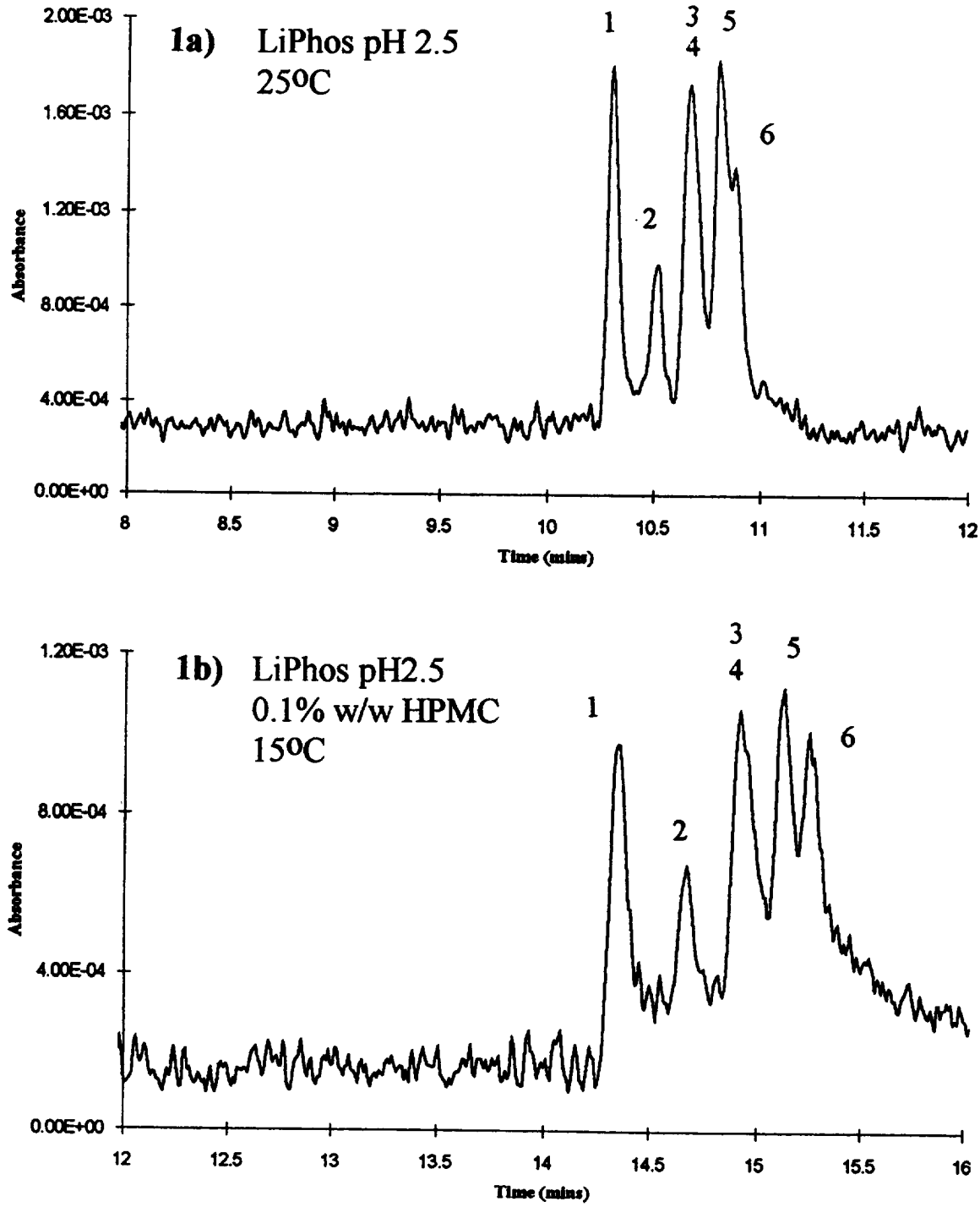

Fig. 1. Separation of the dimethylpyridines (DMP) at pH 2.5 with different buffers and temperatures: (a) 25°C 40 m*M* lithium phosphate buffer; b, 15°C, 40 m*M* lithium phosphate buffer, 0.1% w/w hydroxypropylmethylcellulose. Compounds: **1**, 3,4-DMP; **2**, 3,5-DMP; **3**, 2,3-DMP; **4**, 2,5-DMP; **5**, 2,4-DMP; **6**, 2,6-DMP.

Table 1
Electrophoretic mobilities of the fully charged dimethylpyridines at pH 2.5 and their reported pK_a values

Compound	Electrophoretic mobility ($cm^2\ V^{-1}\ s^{-1}$)	Literature pK_a values [11]
3,4-Dimethylpyridine	$3.349 \cdot 10^{-4}$	6.52
3,5-Dimethylpyridine	$3.285 \cdot 10^{-4}$	6.25
2,3-Dimethylpyridine	$3.236 \cdot 10^{-4}$	6.60
2,5-Dimethylpyridine	$3.236 \cdot 10^{-4}$	6.47
2,4-Dimethylpyridine	$3.196 \cdot 10^{-4}$	6.72
2,6-Dimethylpyridine	$3.168 \cdot 10^{-4}$	6.77

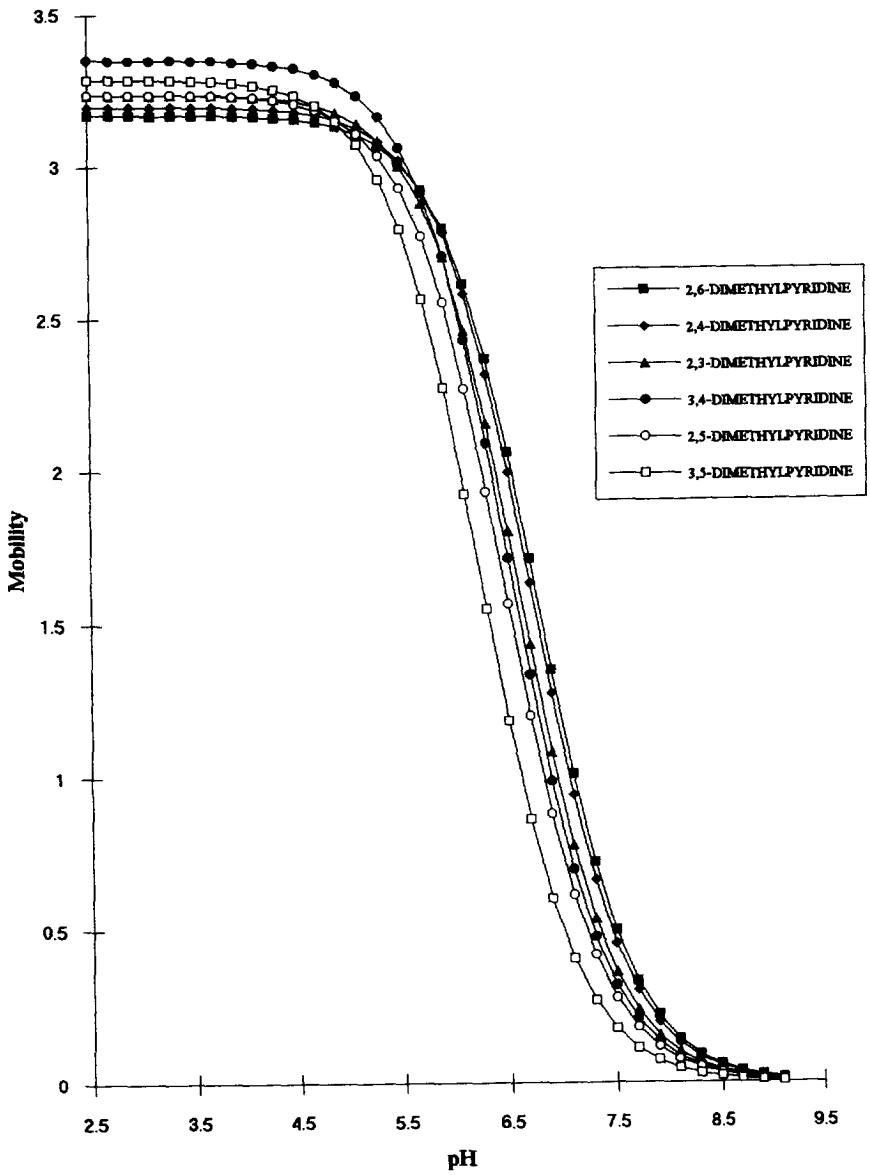

Fig. 2. Variation in predicted mobility of the dimethylpyridines with pH based on the mobilities at pH 2.5 and the partial charge on the analyte calculated from the buffer pH.

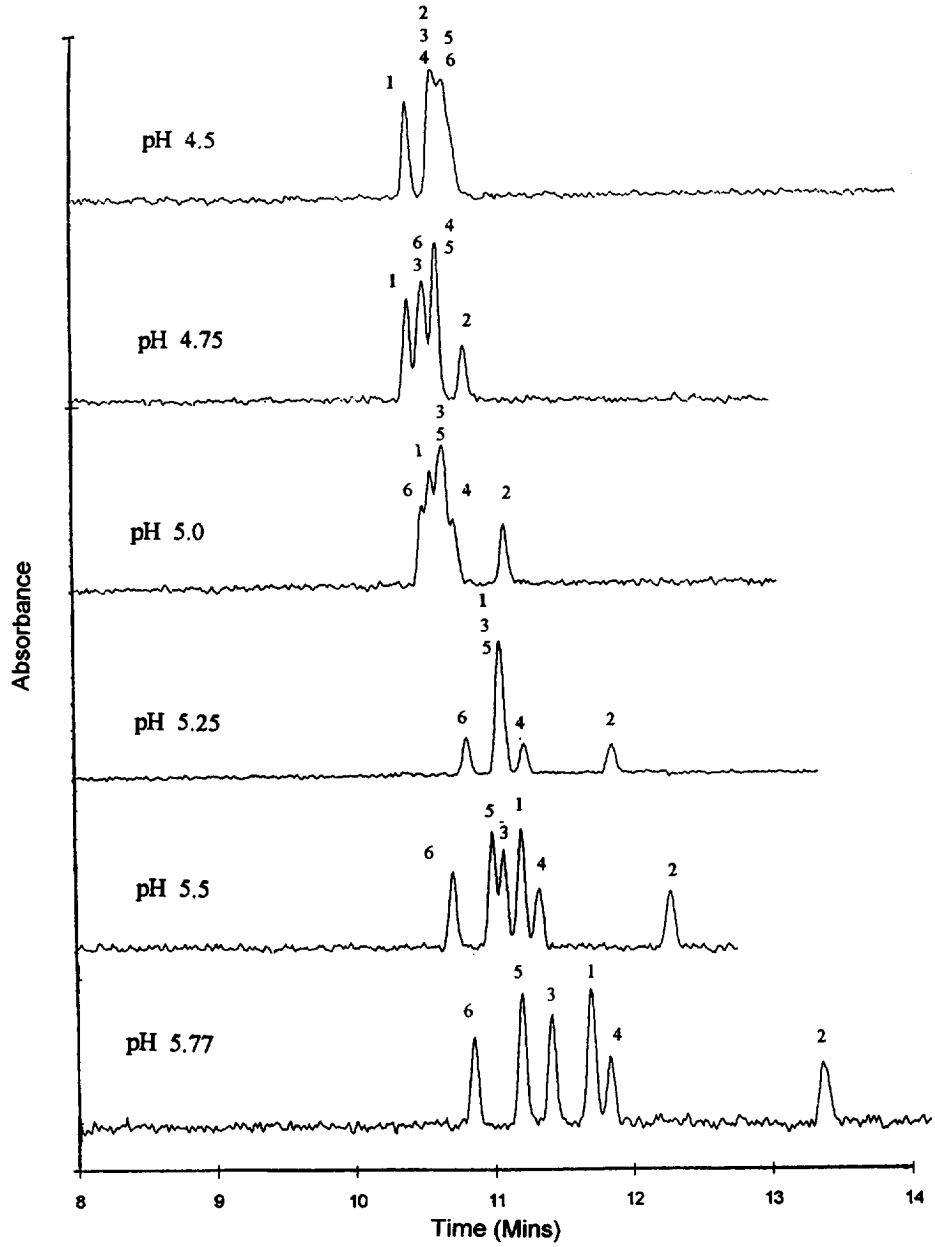

Fig. 3. Electropherograms showing the change in migration order of the dimethylpyridines at different buffer pH values. Compounds: **1**, 3,4-DMP; **2**, 3,5-DMP; **3**, 2,3-DMP; **4**, 2,5-DMP; **5**, 2,4-DMP; **6**, 2,6-DMP.

4) and the mobility order follows the predicted separation (Fig. 2). The mobilities for each isomer were calculated and were compared with the predicted values for the partial charge and electrophoretic mobility (Table 2). As can be seen the relative order of migration was predicted correctly but the absolute values of the electrophoretic mobility were all smaller than the predicted values with

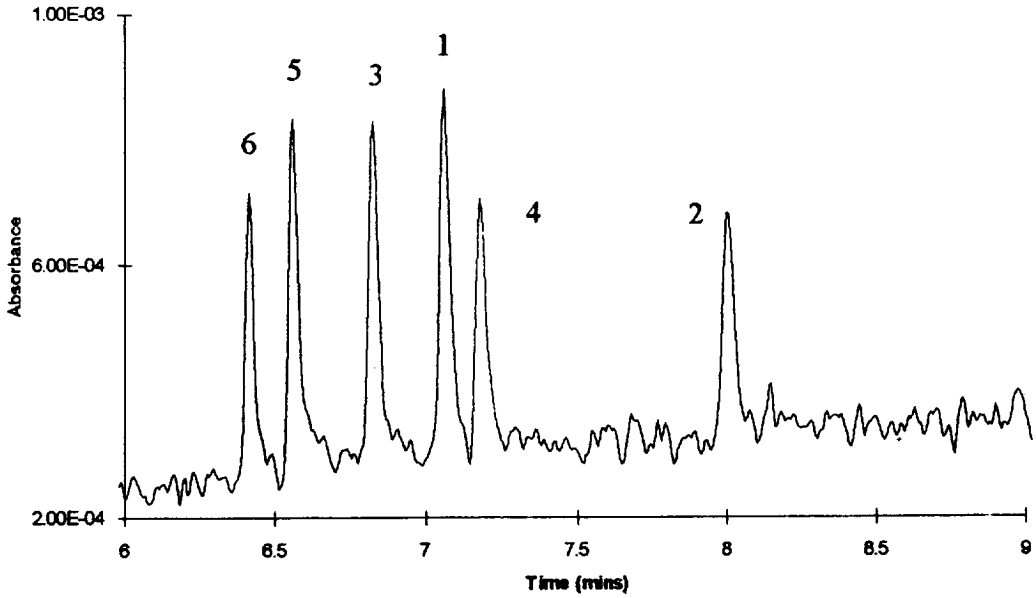

Fig. 4. Separation of the dimethylpyridines at pH 6.5. Buffer, 50 m*M* lithium phosphate buffer, 25°C. Compounds: **1**, 3,4-DMP; **2**, 3,5-DMP; **3**, 2,3-DMP; **4**, 2,5-DMP; **5**, 2,4-DMP; **6**, 2,6-DMP.

differences between 7% and 30%, with an average deviation of 15%. These differences are probably caused because the pH of the background electrolyte was slightly higher than the intended value. As can be seen in Fig. 2, at pH 6.5 the mobilities are very sensitive to pH and would change markedly with only a small discrepancy in the actual value compared to the nominal value.

4. Conclusions

The six isomeric dimethylpyridines have been separated by free-zone capillary electrophoresis. The order of elution changed with pH of the buffer and it was demonstrated that the relative order of elution and the electrophoretic mobilities over a range of pH could be predicted from the separation of the fully charged species and the calculated partial charges based on their pK_a values and the pH of the buffer.

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Table 2
Experimental and calculated mobilities and predicted partial charge on the dimethylpyridines at pH 6.5

Compound	Experimental mobility ($cm^2\ V^{-1}\ s^{-1}$)	Predicted mobility ($cm^2\ V^{-1}\ s^{-1}$)	Calculated partial charge
2,6-Dimethylpyridine	$1.947 \cdot 10^{-4}$	$2.06 \cdot 10^{-4}$	0.651
2,4-Dimethylpyridine	$1.848 \cdot 10^{-4}$	$1.99 \cdot 10^{-4}$	0.624
2,3-Dimethylpyridine	$1.630 \cdot 10^{-4}$	$1.80 \cdot 10^{-4}$	0.557
3,4-Dimethylpyridine	$1.464 \cdot 10^{-4}$	$1.71 \cdot 10^{-4}$	0.512
2,5-Dimethylpyridine	$1.386 \cdot 10^{-4}$	$1.56 \cdot 10^{-4}$	0.483
3,5-Dimethylpyridine	$0.912 \cdot 10^{-4}$	$1.18 \cdot 10^{-4}$	0.360

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