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Optimization of separation of porphyrins by micellar electrokinetic chromatography using the overlapping resolution mapping scheme

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ABSTRACT

The capillary electrophoretic separation of nine porphyrins having two to eight carboxylic acid side-chains was optimized by using a systematic optimization scheme based on the overlapping resolution mapping method. Three parameters were considered for the optimization, *viz.*, surfactant (sodium dodecyl sulphate) concentration, amount of organic modifier (N,N-dimethylformamide) and the ionic strength of the buffer. To utilize the scheme, a set of seven preplanned experiments were performed. Optimum separation conditions were determined which provided satisfactory separation of the porphyrins within an analysis time of *ca.* 30 min.

INTRODUCTION

Since its introduction in 1984 by Terabe *et al.* [1], micellar electrokinetic chromatography (MEKC) has been applied to the separation of a wide variety of compounds, both neutral and ionic, such as pharmaceutical drugs [2,3] and biomolecules [4,5]. Although MEKC was originally conceived for the electrokinetic analysis of neutral molecules, its application has been extended to the analysis of ionic compounds. For some applications, MEKC provides a better resolving power than capillary zone electrophoresis (CZE) [6,7], by taking advantage of the interaction or partition of the analytes with the micellar phase. For further enhancement of selectivity, modifiers such as cyclodextrins [8], organic solvents [9] and tetraalkylammonium salts [10] have been added to the electrophoretic media.

As the degree of complexity of the analyte mixture increases, the need for multi-parameter separation systems arises. Usually, optimization of separation conditions is achieved by varying one parameter at a time, while keeping the other parameters constant. Although such a univariate approach has been successful in obtaining satisfactory separations in many investigations, it should be stressed that owing to its trial-and-error nature, the process tends to be time consuming and tedious, and often local rather than global optima would be obtained. To date, there have been few reports on the systematic optimization of capillary electrophoretic (CE) separations. Recently, Little and Foley [11] and Ghowsi *et al.* [12] presented a theory and two fundamental equations for the optimization of resolution and resolution per unit time in MEKC. In another investigation, Vindevogel and Sandra [13] employed the Plackett–Burman statistical design for the optimization of the MEKC of testosterone esters. Five buffer parameters were optimized within eight experiments.

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Although this design is capable of optimizing several factors simultaneously, no fixed rules existed for the selection of the low and high levels and further experiments may have to be carried out based on conclusions from the optimized experimental conditions.

In our laboratory, we have developed several optimization schemes for CE separations, based on the overlapping resolution mapping (ORM) procedure [14]. In one study, two parameters, *i.e.*, buffer pH and β-cyclodextrin concentration, were optimized using a two-dimensional rectangular ORM scheme for the separation of eight sulphonamides [15]. In another investigation, the concentrations of α-, β- and γ-cyclodextrins were optimized for the CE separation of a group of plant growth hormones with a triangular ORM scheme [16]. To date, the use of a systematic optimization scheme for the CE separation of porphyrins has not been reported.

Naturally occurring porphyrins are intermediate metabolites of haem biosynthesis. Disturbances in the biosynthesis, caused by inborn or acquired defects of the corresponding enzymes, give rise to a family of diseases called porphyria. Depending on the break of the metabolic pathway, different intermediate porphyrins are subsequently formed and accumulated in body fluids and tissues. Porphyrins with different numbers of carboxylic acid groups have been conventionally separated by gradient reversed-phase high-performance liquid chromatography (HPLC) owing to their varying polarity [17]. While providing satisfactory results, the gradient HPLC procedures tended to be laborious and time consuming [17]. The ionizable carboxyl groups on these porphyrins, however, provide an advantage for separation in CE. Under appropriate conditions, they can give rise to different electrophoretic mobilities under an applied voltage [18]. Consequently, it was considered worthwhile to investigate systematic approaches which can be employed to obtain the optimum conditions for the separation of porphyrins by CE. In this investigation, the parameters chosen for the optimization study were sodium dodecyl sulphate (SDS) concentration, percentage of N,N-dimethylformamide (DMF) as modifier and the ionic strength of the buffer solution.

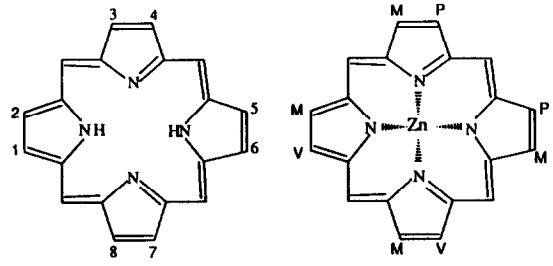
EXPERIMENTAL

Equipment

Separations were performed at ambient temperature using a CE system consisting of a Spellman (Plainview, NY, USA) RHR30 high-voltage power supply, capable of delivering up to 30 kV, and a Shimadzu (Kyoto, Japan) RF535 variable-wavelength fluorescence detector. The excitation and emission wavelengths were set at 405 and 615 nm, respectively. Electropherograms were recorded with a Hewlett-Packard (Palo Alto, CA, USA) Model 3394A integrator. Untreated fused-silica capillary tubing (50 μm I.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). A separation tube length of 54.8 cm (effective length 51 cm) was used for all experiments. Samples were injected by hydrostatic siphoning at an elevation of 10 cm for 5 s. The amount injected was estimated to be 1.3 nl.

Chemicals and reagents

The structural formulae of the compounds studied are shown in Fig. 1. Mesoporphyrin IX, deuteroporphyrin IX, pentacarboxylporphyrin I, hexacarboxylporphyrin I, heptacarboxylpor-



Zinc Protoporphyrin IX (ZP)

Porphyrins	side chain substitution pattern							
	1	2	3	4	5	6	7	8
Deuteroporphyrin IX (DP)	M	H	M	H	M	P	P	M
Mesoporphyrin IX (MP)	M	E	M	E	M	P	P	M
Protoporphyrin IX (PP)	M	V	M	V	M	P	P	M
Coproporhyrin I (CP)	M	P	M	P	M	P	M	P
Pentacarboxylporphyrin I (PeP)	M	P	M	P	A	P	M	P
Hexacarboxylporphyrin I (HxP)	M	P	A	P	A	P	M	P
Heptacarboxylporphyrin I (HtP)	A	P	A	P	A	P	M	P
Uroporphyrin I (UP)	A	P	A	P	A	P	A	P

Abbreviation for substitutions:
M = $-CH_3$, P = $-CH_2CH_2COOH$, E = $-CH_2CH_3$, V = $-CH{=}CH_2$, A = $-CH_2COOH$

Fig. 1. Structures of the porphyrins studied.

phyrin I and uroporphyrin I were purchased from Porphyrin Products (Logan, UT, USA), zinc protoporphyrin IX and coproporphyrin I from Aldrich (Milwaukee, WI, USA), protoporphyrin IX and 3-cyclohexylamino-1-propanesulphonic acid (CAPS) from Sigma (St. Louis, MO, USA), SDS and diethylene glycol monoethyl ether from Fluka (Buchs, Switzerland) and HPLC-grade N,N-dimethylformamide (DMF) from BDH (Poole, UK). All other reagents were of analytical-reagent grade. All aqueous solutions were prepared with water purified using a Milli-Q system (Millipore, Bedford, MA, USA) and filtered through 0.45-μm pore size filter membranes (Whatman, Arbor Technologies, Ann Arbor, MI, USA).

Porphyrin standards

Stock solutions of each porphyrin (about 100 μM) were prepared by dissolving the appropriate amounts of the porphyrins in DMF. The porphyrins are readily soluble in DMF. When stored at −20°C and protected from light, these stock solutions are stable for about 1 month. The concentrations of the porphyrins were determined spectrophotometrically as described in ref. 19. Further dilutions with methanol were made as required.

Rinsing of capillary tube

A new tube was first flushed with plenty of water, followed by 0.1 *M* NaOH solution. The alkaline solution was left in the tube for a day and was subsequently removed with water. With every new buffer system, the tube was similarly treated with NaOH for 30 min. To ensure a better run-to-run reproducibility, the column was rinsed with the operating buffer for 1 min between runs. With this rinsing procedure, the run-to-run relative standard deviation of the migration times for all the analytes was kept within 1% ($n = 7$).

Flow cell design

Owing to the large amount of light scattering at the cylindrical surface of the fused-silica flow cell, the flow cell design was modified according to Kurosu *et al.*'s immersed flow cell design [20]. The separation capillary with on-column detection window was passed through a conventional rectangular HPLC fluorescence flow cell. The space between the capillary and the rectangular cell was filled with a liquid having refractive index close to that of fused silica. In the present experiment, DMF (refractive index at 20°C = 1.431, refractive index of fused silica at 18°C = 1.459) was chosen as the immersing liquid. The fluorescence emission was collected by a 5.0 mm diameter, 10 mm focal length plano-convex lens (Melles Griot, Irvine, CA, USA). With this design, the minimum detectability of deuteroporphyrin was 4 nmol/ml (=5 fmol) at a signal-to-noise ratio of 4.

RESULTS AND DISCUSSION

Choice of parameters

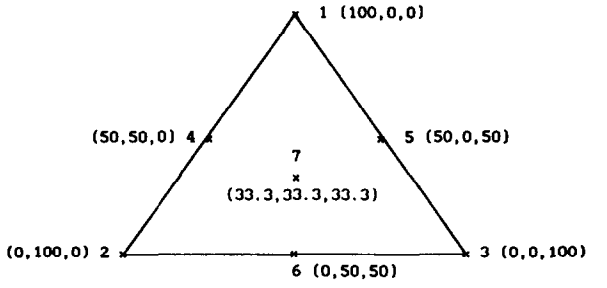
The porphyrins investigated have two to eight –COOH side-chains which are ionizable at high pH. To utilize fully the electrophoretic mobility differences as a result of this ionization, an alkaline buffer at pH 10.8, using CAPS as the buffering ion, was chosen. Preliminary experiments to separate the porphyrins in the CZE mode revealed some problems. Analytes with different numbers of –COOH groups were well resolved and their migration order was governed by their negative charges. Uroporphyrin, being the most negatively charged, migrated last. However, the four dicarboxylporphyrins migrated close to each other. They also gave comparatively smaller peak heights, probably owing to their poorer solubility in the CZE buffer. In addition, the peak shapes deteriorated with increasing number of runs, accompanied by increasing migration times, both suggestive of possible adsorption of the analytes on the capillary wall.

To overcome these problems, SDS was added, as this anionic surfactant is believed to bind to any electrostatic or hydrophobic sites on the capillary wall, reducing, if not eliminating, interaction of analytes with the bare silica wall through competitive adsorption or repulsion from the wall [18]. Peak shapes were improved slightly in the presence of SDS. SDS also contributes to the separation selectivity by solubilizing the more hydrophobic dicarboxylporphyrins. With 100 m*M* SDS, these compounds co-mi-

grated later, between penta- and hexacarboxylporphyrins. However, SDS alone does not provide sufficient selectivity and solubility and a wider migration range is necessary. To achieve this, DMF was added to the electrophoretic buffer. With increasing amounts of DMF in the buffer system, a lowering of the electroosmotic flow and an expanded migration range were expected [21]. Porphyrins are readily soluble in DMF, hence the presence of DMF in the electrophoretic buffer increased the affinity of the analytes in the aqueous phase, improving the analyte solubility and peak efficiencies. On the other hand, a high percentage of DMF in the operating buffer was found to cause the analysis time to increase substantially. To maintain the total analysis time within reasonable limits without compromising the selectivity, a third variable, the ionic strength of the buffer, was included. It has been shown that the ionic strength of the buffer has significant effects on solute mobilities and separation efficiency [22,23]. With adequate heat dissipation, improvement in resolution and reduction of electroosmotic flow have been observed with increasing buffer concentration [24].

Overlapping resolution mapping scheme

In this study, optimum separation of the porphyrins was accomplished by varying the concentrations of CAPS, SDS and DMF in the run buffer. The triangular ORM scheme was employed to determine the optimum conditions. The first step in the ORM scheme was to define the working range of each parameter. The respective ranges were chosen such that the overall migration times were kept within reasonable limits. Otsuka *et al.* [25] pointed out that migration range is an important factor in determining the final separation of compounds of interest. Although a wider migration range usually improves resolution, longer migration times also generally result in undesirable peak broadening. In this investigation, a maximum analysis time of *ca.* 30 min was chosen as a guide in setting the upper limits of the parameters. Based on these limits, seven preplanned experiments were performed at strategic locations on a triangle. The experimental design and conditions for the seven experiments are as depicted in Fig. 2. From the



Expt*	CAPS, mM	SDS, mM	DMF, %
1	80	20	4
2	20	50	4
3	20	20	10
4	50	35	4
5	50	20	7
6	20	35	7
7	40	30	6

* All experiments were performed at pH 10.8

Fig. 2. Experimental design and conditions for the seven experiments. The coordinates at the points on the triangle (x_1, x_2, x_3) denote the percentages of CAPS, SDS and DMF, respectively.

electropherograms obtained with these experiments, the resolution, R, between adjacent peaks were calculated using the equation

$$R = \frac{2\Delta t}{W_1 + W_2} \quad (1)$$

where Δt is the difference in migration times between adjacent peaks and W_1 and W_2 are peak widths at the baseline. These resolution values were then fitted into the following polynomial equation [16]:

$$R = a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{123}x_1x_2x_3 \quad (2)$$

where a_i are the coefficients and x_i are the percentages of each parameter as defined in Fig. 2. With the aid of a computer program, the coefficients were determined. Once the coefficients were known, the resolutions for every adjacent peak pairs could be calculated at any buffer composition within the triangle. In all seven experiments, hexacarboxylporphyrin, hep-

tacarboxylporphyrin and uroporphyrin were always well separated from each other and from the other porphyrins, hence the resolutions for these compounds were not computed. For the remaining six porphyrins, five resolution maps were generated. By subsequently overlapping all the resolution maps and plotting the lowest resolution amongst all the individual resolution maps, regions defining buffer compositions with which the minimum desired resolution can be achieved for all solutes in the mixture can be identified. In the final overlapped resolution map (Fig. 3), regions denoted by # represent buffer compositions which can give resolutions of at least 1.8 for all peak pairs. To verify the success of the ORM scheme, a point Y was chosen from that region with buffer containing 38 m*M* CAPS, 20 m*M* SDS and 8.2% DMF. The electropherogram obtained is shown in Fig. 4, which indicates that all peaks are baseline-resolved and the overall analysis time is within 30 min. In Fig. 4, three peaks are observed for hexacarboxylporphyrin. The smaller peaks are probably by-products of hexacarboxylporphyrin during its synthesis [18]. The experimentally determined resolutions were compared with those predicted with eqn. 2 (shown in Table I) and the agreement was found to be satisfactory, *i.e.*, all within 10% deviation.

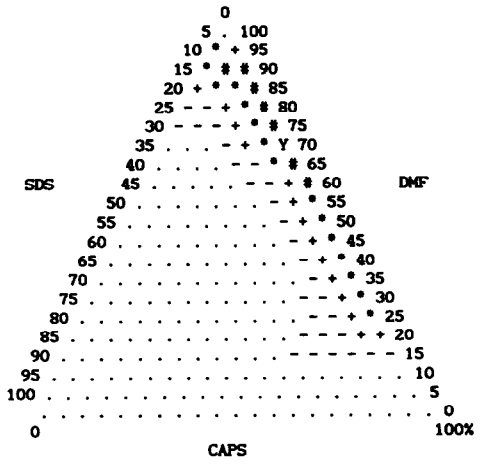


Fig. 3. Overlapped resolution map for the six peak pairs. Notation: •, $R < 0.8$; –, $0.8 \leq R < 1.2$; +, $1.2 \leq R < 1.5$; *, $1.5 \leq R < 1.8$; #, $R \geq 1.8$.

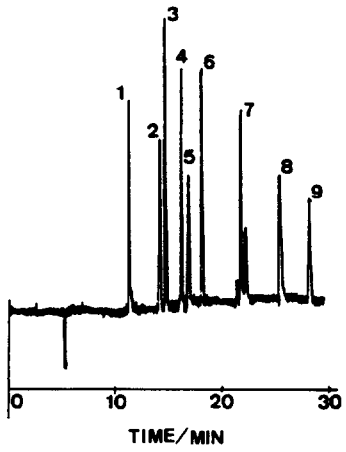


Fig. 4. Electropherogram for the nine porphyrins using the optimum electrophoretic conditions corresponding to point Y within the # region in Fig. 2: 38 m*M* CAPS (pH 10.8), 20 m*M* SDS and 8.2% DMF. Detection, excitation at 405 nm, emission at 615 nm; voltage, 18 kV, current, 18 μA. Peaks: 1 = DP; 2 = ZnPP; 3 = CP; 4 = MP; 5 = PP; 6 = PeP; 7 = HxP; 8 = HtP; 9 = UP. Abbreviations as in Fig. 1.

TABLE I

COMPARISON OF EXPERIMENTAL AND PREDICTED RESOLUTIONS OF ADJACENT PEAK PAIRS UNDER THE OPTIMUM ELECTROPHORETIC CONDITIONS

Experimental conditions as in Fig. 4.

Peak pair	Resolution	
	Experimental	Predicted[a]
1–2	12.47	11.62
2–3	2.53	2.70
3–4	4.62	4.23
4–5	2.18	2.04
5–6	4.16	4.54

[a] Values calculated according to eqn. 2.

CONCLUSIONS

The use of a systematic optimization scheme for the separation of nine porphyrin free acids has been successfully demonstrated. Three buffer parameters, *viz.*, the ionic strength of the buffer and SDS and DMF concentrations, were simultaneously optimized by using a triangular

ORM scheme. The method is straightforward and does not require a theoretical model to describe the migration behaviour, versatile, as it can be readily applied to other experimental parameters, capable of locating the global optimum, rather than a local optimum, and rapid, *i.e.*, with as few as seven preplanned experiments within the preset experimental range, optimum conditions can be determined. The results suggest that there are significant advantages in the use of ORM to optimize CE separations.

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