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Experimental design as a tool when evaluating stationary phases for the capillary electrochromatographic separation of basic peptides

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Abstract

Two different capillary electrochromatography (CEC) stationary phases, Hypersil phenyl and Hypersil C_{18}, have been characterised with respect to their ability to separate the four basic peptides H-Tyr-(D)Ala-Phe-Phe-NH_2 (TAPP), H-Tyr-(D)Ala-Phe-NH_2 (TAP), H-Phe-Phe-NH_2 (PP) and H-Phe-NH_2 (P). Optimal separation conditions were first established separately for the two phases by applying experimental design in a stepwise procedure. The first step comprised a study to acquire basic knowledge about the variables, their influence on the response and their respective experimental domains for each of the two stationary phases. The second step was screening the significant variables and the third step was an optimisation with response surface modelling (RSM) to locate the optimum separation conditions for each stationary phase. The experimental procedure was identical for both stationary phases, but their respective experimental domains were different. The response functions were peak resolution and peak efficiency. This procedure enables specific optimal experimental conditions to be identified for each of the two stationary phases. The optimal conditions identified for the separation on the phenyl stationary phase were to use 50% ACN, 20% 50 mM Tris(hydroxymethyl)aminomethane (TRIS) pH 7.5, 30% H_2O as BGE, operating at 20 °C and 20 kV high voltage. For the C_{18} stationary phase optimal separation was achieved using a BGE with 80% ACN, 20% 30 mM TRIS pH 8.5, again operating at 20 °C and 20 kV high voltage. Results show that the phenyl stationary phase is better suited for the separation of basic, hydrophilic peptides.

Keywords: Capillary electrochromatography; Peptides; Experimental design

1. Introduction

Peptides play a major role in the control and regulation of many vitally important processes of living organisms. They are attractive drug candidates for many reasons, including their natural abundance and their high specificity. However, similar peptides often have very similar behaviour patterns in most separation systems. Consequently, advanced methods are required to separate, prepare, characterize, and determine peptides. Capillary electrophoresis (CE), and capillary electrochromatography (CEC), have recently become recognized as excellent supplemental techniques to high-performance liquid chromatography (HPLC), for peptide separations. However, HPLC is still the most frequently used analytical technique in this context [1].

CEC may be considered to be a hybrid technique between HPLC and CE, which (at least theoretically) combines the selectivity of HPLC with the high efficiency offered by CE. Various types of CEC can be distinguished: packed-column CEC (PC-CEC) [2,3], open-tubular CEC (OT-CEC) [2,4] and, the most recently developed, CEC based on monolithic or continuous beds [5–7].

CEC has been shown to be suitable for the separation of both acidic and neutral compounds [8–10]. However, problems may appear when analyzing basic compounds, since the stationary phases normally used are not end-capped, so electrostatic interactions may occur between the residual silanol groups and the basic compounds [7].

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Several reviews covering the basic principles of CEC [11–15], the separation of basic compounds with CEC [8,10,16], peptide analysis with CEC [1,8,9,17–20] and variables influencing separation in CEC [19,21,22] have recently been published.

Experimental design is today a well-established technique for optimising chemical processes that are affected by variables, many of which are controllable [23,24]. When comparing the separation capabilities of different phases in CEC it is important to ensure that each stationary phase is tested under its own optimal experimental conditions, since the various phases will almost certainly differ in important respects, e.g. hydrophobicity. Therefore, comparing different stationary phases under identical experimental permissions will give results that are biased in favour of some phases at the expense of others that may perform much better, say at a different pH or temperature.

The approach taken in the study reported here was to optimise the separation conditions for two different stationary phases separately, thereby comparing their inherent and actual separation capability in an objective way. The experimental designs applied were similar for both stationary phases and the same response functions were chosen, but the respective experimental domains differed. The separation capability of the respective phases was studied by monitoring the separation of the four basic peptides H-Tyr-(D)Ala-Phe-Phe-NH_2 (TAPP), H-Tyr-(D)Ala-Phe-NH_2 (TAP), H-Phe-Phe-NH_2 (PP) and H-Phe-NH_2 (P). The two stationary phases used in the investigation were compared in terms of the peak resolution and peak efficiency obtained for the four test peptides.

2. Experimental

2.1. Chemicals

The peptides H-Tyr-(D)Ala-Phe-Phe-NH_2 and H-Tyr-(D)Ala-Phe-NH_2 were kindly provided by Biochem Immunosystems (Montreal, Canada), while H-Phe-Phe-NH_2 was purchased from Bachem (Bubendorf, Switzerland) and H-Phe-NH_2 was supplied by Sigma (St. Louis, USA). Phosphoric acid, acetonitrile (ACN), methanol, Tris(hydroxymethyl)aminomethane (TRIS) and HCl (37%) were obtained from Merck (Darmstadt, Germany) and were of analytical grade. Purified water was obtained from a Waters Milli-Q system (Watford, Herts, UK).

2.2. Solutions

TRIS buffers were prepared by dissolving appropriate amounts of TRIS in water and adjusting to the desired pH using 5 M HCl. To obtain the desired background electrolyte (BGE) composition, the respective buffer solution was mixed with appropriate volumes of the organic solute (modifier) and water. The organic solute used was either ACN or methanol. The BGE was then degassed by passing helium through it or treatment in an ultrasonic bath for 10 min. BGE solutions were freshly prepared before use.

For each peptide a 1 mg/ml stock solution was prepared by dissolving and diluting 10 mg of it in 10 ml water. Sample solutions were then prepared by diluting the stock solution to the desired concentration, 0.1 mg/ml, with water, if not otherwise stated.

2.3. Instrumentation

All experiments were performed using an $HP^{3D}CE$ instrument (Agilent Technologies) equipped with a diode array detector and Chemstation software (version A.05.02) for data handling. All capillaries, obtained from Agilent Technologies, had an effective length (l_e) of 25 cm and a total length (l_t) of 33.5 cm, an inner diameter of 100 μm and an outer diameter of 350 μm. Before use, the dry, or partially dry, capillary was conditioned by passing the mobile phase through it with the aid of an HPLC-pump, at a pressure of 25–30 bars for at least 4 h. Prior to all runs the capillary was preconditioned with the appropriate mobile phase for about 30–120 min and between every injection there was a 5 min preconditioning period with BGE. The temperature of the capillary cartridge was varied within the range 15–40 °C. The operating voltage (10–30 kV) was ramped up over a time interval of 1 min, thereby inducing electrophoretic motion towards the cathode. All samples were electrokinetically injected, at 5 kV for 10 s. A pressure of 8 bars was maintained over each mobile phase vial to avoid cavities forming inside the capillary during the analysis. The eluting peptides were monitored by direct UV detection at the cathodic side. Spectra were collected at wavelengths between 210 and 600 nm, and the responses were evaluated at 210 nm. All compounds were analyzed both separately and together in a mixture to verify the identification. Spectra of the compounds were also used to identify the peptides when analyzing the mixture.

The chemometric design and model evaluation were done using the MODDE 6.0 software package (Umetrics, Umeå, Sweden). The pK_a and the log D values were calculated using the software ACD/pK_a DB, version 7.00 (Advanced Chemistry Development Inc., Toronto, Canada).

2.4. Capillaries

Two different stationary phases were investigated, Hypersil C_{18} and Hypersil phenyl, both commercially available from Agilent Technologies. These phases have different properties due to the differences in the substituents on the surface of their silica particles. The silica particles in the Hypersil phenyl stationary phase have been reacted with chlorodimethylphenylsilane, thereby providing the silica surface with phenyl groups. In the Hypersil C_{18} stationary phase the silica particles have been reacted with chlorodimethyloctadecylsilane. This type of substitution gives greater hydrophobicity in comparison to the phenyl stationary phase.

2.5. Evaluation procedure

The resolution (R_s) and efficiency (N) were selected as response functions when evaluating the performance of the separation system. The basic requirements were that these response functions should provide measures of baseline separation and peak breadth for the four peptides. In addition, three chromatographic response functions were utilized: the chromatographic optimisation function, COF [25,26], the chromatographic response function, CRF_3 [26–29], and the resolution product, R_p [30].

2.5.1. Resolution

Peaks were integrated and the resolution was calculated using Chemstation software according to

$$R_s = 1.18 \times \frac{t_2 - t_1}{w_{0.5,1} + w_{0.5,2}} \quad (1)$$

where t_1 and t_2 are the migration times, and $w_{0.5,1}$ and $w_{0.5,2}$ are the peak widths at half peak heights. In this study four different peptides were separated, and the resolution between each pair of peaks was calculated, giving a total of six responses.

In this study a resolution of at least 1.5 between two peaks was considered to give baseline separation, and the aim was to get a resolution of at least 1.5 between all peak pairs.

2.5.2. Efficiency

The efficiency was calculated using the Chemstation software, according to:

$$N = 5.54\left(\frac{t}{w_{0.5}}\right)^2 \quad (2)$$

where N is the number of theoretical plates, t the migration time of the respective peak and $w_{0.5}$ is its temporal width at half its height.

2.5.3. Chromatographic functions

When several peaks are to be separated, a chromatographic function describing all the resolutions in a chromatogram or electropherogram in a single value can be useful. Many different chromatographic functions have been described in the literature [25–34], however, only the chromatographic optimisation function, COF, the chromatographic response function, CRF_3 (modified according to [29]), and the resolution product, R_p were used here. The chromatographic functions were calculated according to equations (3)–(5):

$$\mathrm{COF} = \sum_{i=1}^{n} A_i \ln(R_i/R_{id}) + B(t_m - t_n) \quad (3)$$

where R_i is the resolution of the ith pair and R_{id} is the desired resolution (1.5), t_m is the desired maximum analysis time and t_n the time of the last eluting peak. A_i and B are weights chosen by the operator [25]. In this paper the constants A_i and B were set to 2 and 0, respectively, thereby excluding the time factor. Harang et al. [35] have shown that the time factor is sometimes far too dominant in the response function, despite the weighting of the resolution factor. This results in models that poorly describe the experimental data with poor ability to predict optimum conditions. By modifying the chromatographic functions, and excluding the time factor from the equation, they obtained better models [35]. Therefore, we set the time-factor in the COF to 0.

$$\mathrm{CRF}_3 = a\sum \ln(R_{so}/R_s) + b\sum \ln(R_s/R_{so}) \quad (4)$$

where R_{so} is the optimum resolution (1.5), and R_s is the resolution between two neighbouring peaks. The weighting factors, a (the excess resolution factor) and b (the overlap degradation factor), were selected according to [28,29], and set to 5 and 50, respectively. This procedure gives more weight to the resolution (second) term. Only peaks with $R_s > 2$ were included in the first term and only peaks with resolution <1.5 were included in the last term.

$$R_p = \prod_{i=1}^{n-1} R_{si,i+1} \quad (5)$$

where R_s is the resolution between two neighbouring peaks.

2.6. Modelling

The data obtained from the statistical experimental design was fitted by means of multiple linear regression (MLR) and partial least squares (PLS) analysis. The responses could then be described by a Taylor polynomial function [36]. The main difference between MLR and PLS is that PLS fits a model with all responses simultaneously, while MLR fits all responses separately. MLR cannot fit data to a model if there are any missing data, whereas PLS can tolerate a small amount of missing data (<10%). The MODDE software calculates the fractions of variation of the response that can be explained by the model (R^2), and predicted by the model (Q^2). R^2 and Q^2 should be as close to 1 as possible in a good model [37].

The responses were differentiated into subgroups during the evaluation procedure, since the responses considered are very different and may be difficult to fit in the same model. Furthermore, a large number of responses may be difficult to interpret. One group contained the resolutions (six responses), the second group the peak efficiency data (four responses) and the third group contained the results from the chromatographic functions (three responses).

3. Results and discussion

The investigation involved three steps. The first comprised experiments designed to acquire basic knowledge about the variables and to choose the appropriate dimensions of the experimental domain. The second step was a screening study to evaluate which variables influenced the separation the most.

The third and last step was an optimisation with response surface modelling (RSM).

3.1. Step 1: Initial studies

During the initial studies some of the experimental variables and their respective experimental domains were determined for each stationary phase individually. These variables were the type of buffer in the BGE and the type and amount of organic modifier in the BGE. The amount of ACN was tested in the range from 50 to 80%.

The obtained results indicate that the C_{18} stationary phase, which is a hydrophobic and thus strongly retarding phase, requires a relatively high content of organic modifier in the BGE. The separation of the analytes was unsatisfactory and the peak shapes were poor when low contents of ACN were present in the mobile phase, see Fig. 1. Additional experiments were performed in which ACN was replaced with methanol in the BGE, and/or triethanolamine was used as the buffer in the BGE instead of TRIS. However, none of these efforts improved the resolution.

For the less hydrophobic phenyl stationary phase, the amount of ACN in the BGE was not as critical as for the C_{18} phase. Even at low ACN levels some separation was achieved between the peptides. The replacement of ACN with methanol did not result in any improvement with respect to the separation for either of the stationary phases.

The conclusion drawn from these sets of experiments was that the BGE should consist of the TRIS buffer at various concentrations, with ACN as an organic modifier. However, for the C_{18} stationary phase the amount of ACN added was kept constant at 80% in all subsequent experiments.

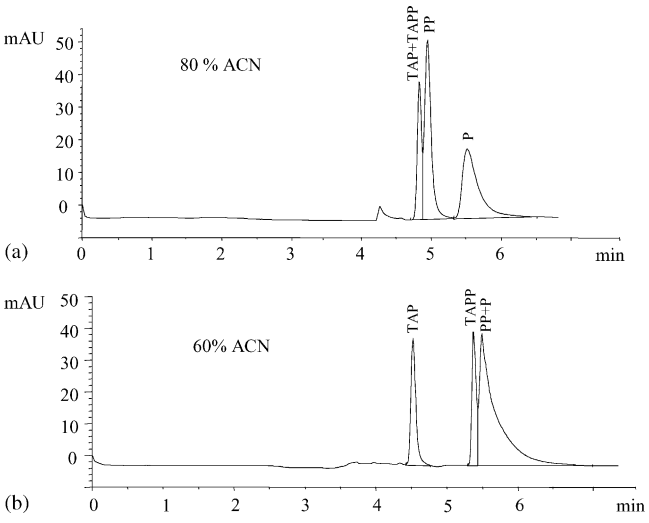

Fig. 1. Electropherograms showing the change in selectivity for the analytes separated on a C_{18} stationary phase as a function of the (v/v) amount of acetonitrile (ACN) in the mobile phase. (a) Mobile phase: 80% ACN, 20% 50 mM TRIS pH 8.0, (b) mobile phase: 60% ACN, 20% 50 mM TRIS pH 8.0, 20% H_2O. Experimental parameters: 20 °C and 20 kV; electrokinetic injection at 5 kV for 10 s.

Table 1
The experimental domains in the screening study

Variable	Experimental domains	
	Low	High
Acetonitrile (%)[a]	50	80
pH	7.5	8.5
Buffer concentration (mM)	10	50
Temperature	15	40
Applied voltage (kV)	15	30

[a] Only varied for the phenyl stationary phase.

3.2. Step 2: Screening

After the initial experiments the variables and their respective experimental domains for the two stationary phases were chosen, see Table 1.

In the screening study a Plackett–Burman design was applied for both stationary phases, comprising eight experiments and three additional centre points, resulting in a total of 11 experiments. For the phenyl stationary phase five variables were varied at two levels, and for the C_{18} phase there were four variables, varied at two levels. The worksheets are presented in Tables 2 and 3. Data were fitted with both MLR and PLS, but the MLR models had very low descriptive (R^2)

Table 2
Worksheet for the Plackett–Burman design on the phenyl stationary phase

Experiment number	Acetonitrile (%)	Buffer concentration (mM)	pH	Temperature (°C)	High voltage (kV)
1	50	10	8.5	40	10
2	80	10	8.5	15	30
3	80	50	8.5	15	10
4	80	50	7.5	40	10
5	50	50	8.5	40	30
6	80	10	7.5	40	30
7	50	50	7.5	15	30
8	50	10	7.5	15	10
9	65	30	8.0	27.5	20
10	65	30	8.0	27.5	20
11	65	30	8.0	27.5	20

Table 3
Worksheet for the Plackett–Burman design on the C_{18} stationary phase

Experiment number	Buffer concentration (mM)	pH	Temperature (°C)	High voltage (kV)
1	50	7.5	15	30
2	50	8.5	15	10
3	50	8.5	40	10
4	10	8.5	40	30
5	50	7.5	40	30
6	10	8.5	15	30
7	10	7.5	40	10
8	10	7.5	15	10
9	30	8	27.5	20
10	30	8	27.5	20
11	30	8	27.5	20

and predictive ability (Q^2) so the PLS models were chosen and exclusively used in all further evaluation of the variables.

For the phenyl phase the pH and buffer concentration of the BGE were significant when using any response function, and the amount of acetonitrile was significant for the efficiency. These three variables were further investigated in the optimisation modelling. Significant variables for the resolution obtained with the C_{18} stationary phase were the buffer concentration and the pH of the BGE, but the only significant variable for the efficiency was the buffer concentration. Optimisation on the C_{18} phase was therefore continued with the variables buffer concentration and pH of the BGE.

3.3. Step 3: Optimisation with RSM

The optimisation step was carried out according to a central composite faced (CCF)-design for each stationary phase. This allows interaction and quadratic terms to be investigated in order to detect curvature within the experimental domain. The CCF-design for the phenyl phase with three variables comprised 17 experiments (including three centre points). For the C_{18} phase only two variables were varied, so the CCF-design contained a total of 11 experiments (including three centre points). The worksheets for the phenyl and C_{18} stationary phases are presented in Tables 4 and 5, respectively.

Chromatographic response functions were introduced into the analysis at this stage of the investigation to improve the ability of the models to predict optimum separation conditions. This could have been counter-productive at earlier stages since the risk of ambiguity is obvious, and the results may be difficult to interpret when there are as many as six responses to consider.

The responses were fitted by means of both MLR and PLS using the MODDE software, but for all responses the PLS models gave better fits to the data according to the measures of their descriptive (R^2) and predictive ability (Q^2).

Table 4
Worksheet for the CCF-design on the phenyl stationary phase

Experiment number	Acetonitrile (%)	Buffer concentration (mM)	pH
1	50	10	7.5
2	70	10	7.5
3	50	50	7.5
4	70	50	7.5
5	50	10	8.5
6	70	10	8.5
7	50	50	8.5
8	70	50	8.5
9	50	30	8
10	70	30	8
11	60	10	8
12	60	50	8
13	60	30	7.5
14	60	30	8.5
15	60	30	8
16	60	30	8
17	60	30	8

Table 5
Worksheet for the CCF-design on the C_{18} stationary phase

Experiment number	Buffer concentration (%)	pH
1	10	7.5
2	50	7.5
3	10	8.5
4	50	8.5
5	10	8
6	50	8
7	30	7.5
8	30	8.5
9	30	8
10	30	8
11	30	8

Response surface plots were produced in MODDE for all the different responses and the optimum conditions were visually determined from these plots. The results obtained using all of the response functions gave consistent indications concerning the approximate locations in the response surface plots of the optimum separation conditions. The best-fitted model (with highest values of R^2 and Q^2) was found for the chromatographic function COF for both stationary phases. The response surfaces for the COF response for the two stationary phases are shown in Fig. 2a and b.

From the response surfaces in Fig. 2a, it can be clearly seen that separation of the peptides is optimal (within this tested domain) on the phenyl stationary phase when the BGE consists of 50% acetonitrile: 20% 50 mM TRIS at pH 7.5: 30% H_2O, operating at 20 kV and 20 °C. An electropherogram showing this separation is presented in Fig. 3a.

Similarly, the optimal analytical system for the separation of the peptides TAPP, TAP, PP and P on the C_{18} stationary phase can be determined from the response surface in Fig. 2b. The optimal separation conditions (within this tested domain) include a BGE composition of 80% acetonitrile: 20% 30 mM TRIS at pH 8.5, operating at 20 kV and 20 °C. An electropherogram illustrating this separation is presented in Fig. 3b. The additional peak seen in Fig. 3b is a system artefact.

A comparison of the chromatographic parameters for the two phases separating the peptides under their respective optimal conditions is presented in Table 6. As can be seen from both the electropherograms in Fig. 3a and b and the data listed in Table 6, the phenyl stationary phase provides the best separation of the tested peptides under the tested conditions, with respect to both resolution and efficiency. The C_{18} stationary phase did not have the ability to fully separate (with a resolution >1.5) all the peptides, and the peaks (except P) have significantly lower plate numbers than those obtained with the phenyl stationary phase. The phenyl stationary phase is the more polar phase of the two compared in this study, which helps explain the experimental results from the peptide analysis. Calculations of the log D and pK_a values (see Table 6) of the peptides show that they are rather polar at pH 8, and thus the more polar phenyl stationary phase is better suited for this separation.

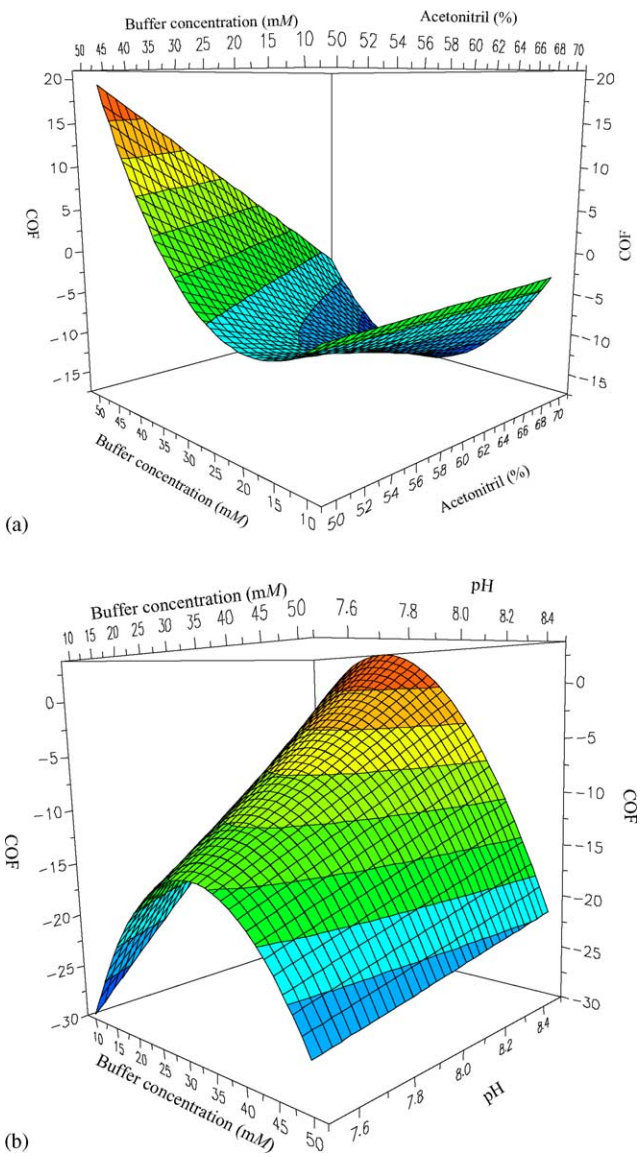

Fig. 2. Response surfaces based on the COF response function values obtained with (a) the phenyl stationary phase (pH 7.5, 20 °C and 20 kV) and (b) the C_{18} stationary phase (80% ACN, 20 °C and 20 kV).

Table 6
Chromatographic parameters for the two stationary phases

Elution order	Resolution, R_s	Efficiency (plates/m)	pK_a values	Log D value at pH 8
Phenyl phase				
P	–	10800	7.5[a]	0.06
TAP	5.38	218700	7.6[a]/10.1[b]	0.65
PP	6.04	76600	7.7[a]	1.95
TAPP	5.06	44700	7.2[a]/9.8[b]	2.22
C_{18} phase				
PP	–	47600	7.7[a]	1.95
TAPP	1.12	29000	7.2[a]/9.8[b]	2.22
TAP	0.67	33000	7.6[a]/10.1[b]	0.65
P	1.92	11800	7.5[a]	0.06

[a] pK_a value at the N-terminus amine.
[b] pK_a value at the tyrosine hydroxyl group.

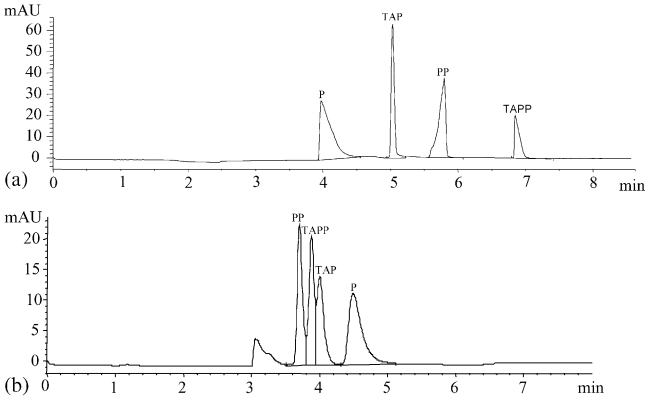

Fig. 3. Electropherograms obtained under optimal conditions for separating the four basic determinants using (a) the phenyl stationary phase with BGE: 50% ACN, 20% 50 mM TRIS pH 7.5, 30% H_2O; and (b) the C_{18} stationary phase with BGE 80% ACN, 20% 30 mM TRIS pH 8.5; 20 °C and 20 kV; electrokinetic injection at 5 kV for 10 s; capillary dimensions, effective length 25.0 (total length 33.5) cm × 100 μm.

4. Conclusions

This work shows that the stationary phases used in CEC can be swiftly and systematically characterised by applying a three-step development strategy based on experimental design. Optimal separation conditions can be developed for each phase individually and independently. The separation capabilities for the stationary phases are preferably evaluated and compared with respect to resolution and peak efficiency. For the four selected basic peptides TAPP, TAP, PP and P a phenyl phase seems to be better suited for the separation than a C_{18} stationary phase. Characterisation and comparison of other stationary phases for the separation of basic compounds in CEC according to the strategy applied in this paper is ongoing.

References

[1] V. Kašička, Electrophoresis 24 (2003) 4013.
[2] L.A. Colon, G. Burgos, T.D. Maloney, J.M. Cintron, R.L. Rodriguez, Electrophoresis 21 (2000) 3965.
[3] M. Pursh, L.C. Sander, J Chromatogr. A 887 (2000) 313.
[4] K. Jinno, H. Sawada, Trends Anal. Chem. 19 (2000) 664.
[5] F. Svec, E.C. Peters, D. Sykora, J.M.J. Frechet, J. Chromatogr. A 887 (2000) 3.
[6] C. Legido-Quigley, N.D. Marlin, V. Melin, A. Mantz, N.W. Smith, Electrophoresis 24 (2003) 917.
[7] J.J. Pesek, M.T. Matyska, G.B. Dawson, J. Chen-Chen, R.I. Boysen, M.T.W. Hearn, Anal. Chem. 76 (2004) 23.
[8] G. Vanhoenacker, T. Van den Bosch, G. Rozing, P. Sandra, Electrophoresis 22 (2001) 4064.
[9] V.T. Remcho, S.L. Clark, A. Doneau, G.S. Chirica, in: S.A. Rathore, A. Guttman (Eds.), Electrokinetic Phenomena, Marcel Dekker, New York, 2004, p. 345.
[10] C.M. Johnson, A.P. McKeown, M.R. Euerby, J. Chromatogr. Library 62 (2001) 87.
[11] R. Stol, W.T. Kok, in: S.A. Rathore, A. Guttman (Eds.), Electrokinetic Phenomena, Marcel Dekker, New York, 2004, p. 189.

[12] E. Wen, A.S. Rathore, C. Horvath, in: S.A. Rathore, A. Guttman (Eds.), Electrokinetic Phenomena, Marcel Dekker, New York, 2004, p. 141.
[13] K. Mistry, I. Krull, N. Grinberg, J. Sep. Sci. 25 (2002) 935.
[14] A.S. Rathore, Electrophoresis 23 (2002) 3827.
[15] U. Pyell, Adv. Chromatogr. 41 (2001) 1.
[16] A.-M. Enlund, G. Hagman, R. Isaksson, D. Westerlund, Trends Anal. Chem. 21 (2002) 412.
[17] K. Walhagen, K.K. Unger, M.T.W. Hearn, J. Chromatogr. A 887 (2000) 165.
[18] S. Eeltink, G.P. Rozing, W.T. Kok, Electrophoresis 24 (2003) 3935.
[19] K. Walhagen, M.I. Huber, T.P. Hennessy, M.T.W. Hearn, Biopolymers 71 (2003) 429.
[20] H. Fu, X. Huamg, W. Jin, H. Zou, Curr. Opin. Biotechnol. 14 (2003) 96.
[21] K.D. Bartle, P. Meyers, Electrokinetic Phenomena, in: S.A. Rathore, A. Guttman (Eds.), Marcel Dekker, New York, 2004, p. 167.
[22] K.D. Bartle, R.A. Carney, A. Cavazza, M.G. Cikalo, P. Myers, M.M. Robson, S.C.P. Roulin, K. Sealey, J. Chromatogr. A (2000) 892.
[23] R.G. Brereton, Chemometrics, Data Analysis for the Laboratory and Chemical Plant, John Wiley & Sons Ltd., England, 2003.
[24] R. Carlsson, Design and Optimization in Organic Synthesis, Elsevier, Amsterdam, 1992.
[25] J.L. Glajch, J.J. Kirkland, K.M. Squire, J.M. Minor, J. Chromatogr. 199 (1980) 57.
[26] A.M. Siouffi, R. Phan-Tan-Luu, J. Chromatogr. A 892 (2000) 75.
[27] J.C. Berridge, J. Chromatogr. 244 (1982) 1.
[28] M.A. Stebbins, J.B. Davis, B.K. Clark, M.J. Sepaniak, J. Microcolumn Sep. 8 (7) (1996) 485.
[29] J.P. Schaepper, S.B. Fox, M.J. Sepaniak, J. Chrom. Sci. 39 (2001) 411.
[30] E.J. Klein, S.L. Rivera, J. Liq. Chrom. Rel. Technol. 23 (14) (2000) 2097.
[31] T.D. Schlabach, J.L. Excoffier, J. Chromatogr. 493 (1988) 173.
[32] V.M. Morris, C. Hargreaves, K. Overall, P.J. Marriott, J.G. Hughes, J. Chromatogr. A 766 (1997) 245.
[33] V.M. Morris, J.G. Hughes, P.J. Marriott, J. Chromatogr. A 755 (1996) 235.
[34] S. Mikaeli, G. Thorsén, B. Karlberg, J. Chromatogr. A 907 (2001) 267.
[35] V. Harang, S.P. Jacobsson, D. Westerlund, Electrophoresis 25 (2004) 1792.
[36] G.E.P. Box, W.G. Hunter, Statistics for Experimenters, John Wiley & Sons, New York, 1987.
[37] User guide and tutorial for Modde software 6.0, Umetrics, Umeå, Sweden.