ELSEVIER

Journal of Chromatography A, 721 (1996) 337–344

JOURNAL OF
CHROMATOGRAPHY A

Micellar electrokinetic capillary chromatography of fungal metabolites
Resolution optimized by experimental design

Marianne S. Nielsen *, Per V. Nielsen, Jens C. Frisvad

Department of Biotechnology, Building 221, Technical University of Denmark, DK-2800 Lyngby, Denmark

First received 28 February 1995; revised manuscript received 16 July 1995; accepted 19 July 1995

Abstract

Fungal metabolites were extracted from two isolates of *Penicillium commune* and two isolates of *Aspergillus versicolor* grown on yeast extract agar (YES) and Czapek yeast extract agar (CYA). Optimized conditions for analysing the extracts by high-performance capillary electrophoresis (HPCE) were obtained by experimental designs. The following nine factors were examined by a two-level fractional factorial design: concentration of the buffer ions PO_4^{3-} and $B_4O_7^{2-}$, ionic strength of buffer, buffer pH, addition of sodium dodecyl sulphate (SDS) and sodium deoxycholate (SCD), addition of acetonitrile and methanol and voltage. Four factors significantly influenced the separation of peaks in all the fungal extracts. For optimization of the HPCE method a subsequent response-surface experiment with the important factors was made with an extract from an isolate of *Aspergillus versicolor*. Optimum separation conditions were obtained, which gave good resolution of the components in the extract.

1. Introduction

High-performance capillary electrophoresis (HPCE) is a highly efficient separation method for complex chemical mixtures [1] and is an alternative to high-performance liquid chromatography (HPLC). One of the advantages of HPCE, compared to HPLC, is the very small amount needed for injection. The volume of sample introduced onto the column is in the nanoliter range as compared to microliter range in HPLC. Another advantage of HPCE is that sample pretreatment can be reduced. Due to the rugged nature of the capillary which can be rinsed between analyses, it is possible to remove any potential interferences. Using this approach it is possible, for example, to determine directly antibiotic levels of penicillin in plasma [2].

Beside their use in separation and detection of antibiotics efficient analytical methods are needed for detection of mycotoxins in food and in fungal chemotaxonomy. These analyses are mainly based on TLC [3,4] and HPLC [5,6]. Capillary zone electrophoresis (CZE) has been reported for the separation of antibiotics [7–9]. By using micellar electrokinetic capillary chromatography (MECC), it has been possible to separate closely related antibiotics [2,10] and mycotoxins [11].

The buffer systems used in these separations

* Corresponding author.

0021-9673/96/$32.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)00801-2

consist of phosphate buffer [8] or phosphate–borate buffer [2,7,9–12]. Addition of micelles, e.g. the surfactants sodium dodecyl sulphate (SDS) and bile salts, allows separation of neutral molecules. Mixed aqueous–organic mobile phases, where e.g. acetonitrile [11,12] or methanol [13] are added to the buffer, may separate moderately hydrophobic molecules. Such solvents allow the separation of mixtures of compounds with great diversity in structures such as mycotoxins [12] by decreasing the mobile-phase polarity, and thereby extend the elution range.

In the literature factors influencing the efficiency and separation in MECC have been examined. These factors include voltage, buffer concentration, micelle concentration, column diameter [11], micelle type, mixed aqueous–organic phases and pH [12]. Because the resolution in MECC is influenced by many factors, the need for resolution optimization by use of statistical methods is obvious. A Plackett–Burman statistical design was used to obtain a HPCE method for the analysis of testosterone esters. Optimization of five factors, all related to buffer composition, only required eight experiments to obtain full resolution within an acceptable analysis time [14]. Ng et al. [9] performed a systematic optimization for the HPCE separation of sulphonamides using overlapping resolution mapping.

No reports are available on the separation of components in raw fungal extracts using HPCE for the determination of profiles of secondary metabolites for chemotaxonomy or the presence of mycotoxins. In this work experimental designs were used to develop a HPCE method for the analysis of raw fungal extracts. The important factors for the separation were determined by a fractional factorial design, optimized by response-surface modelling and verified by a full factorial design.

2. Experimental

2.1. Chemicals

Sodium dodecyl sulfate (SDS) was purchased from Serva (Germany), and sodium deoxycholate (SCD) was from Sigma (USA). The organic solvents, acetonitrile and methanol, were HPLC grade from Merck. Na_2HPO_4 and $Na_2B_4O_7$ (pro analysi) were also from Merck. For all experiments double deionized water was used.

2.2. Fungal cultures

Four fungal cultures isolated from cheese were obtained from the Fungal Culture Collection at Department of Biotechnology (IBT), Technical University of Denmark, DK-2800 Lyngby, Denmark: *Penicillium commune* IBT 10253, *Penicillium commune* IBT 10727, *Aspergillus versicolor* IBT 12384 and *Aspergillus versicolor* IBT 13736. Fungal conidia from each of the cultures were 3 point inoculated on Czapek yeast extract agar (CYA) and on yeast extract sucrose agar (YES) and incubated at 25°C for 10 days in darkness.

2.3. Extraction procedure

Extraction was made by cutting the agar cultures into small pieces (1 × 1 cm). The pieces were transferred to a 50-ml flask. 20 ml chloroform–methanol (2:1, v/v) and 0.1 ml formic acid were added and the flask was placed in a ultrasonic bath (Branson 3200, Netherlands) for 20 min. The liquid was filtered through PS filter (Whatman no. 1) into a 15-ml preparation glass. The liquid was evaporated in a steam of nitrogen and redissolved in 2 ml methanol. The final extract was passed through a 0.45-μm filter.

2.4. Apparatus

Experiments were performed with the modular capillary electrophoresis system Prince (Lauerlabs, Netherlands) with a fused-silica capillary, 75 μm I.D., 63 cm long (50 cm to the detection window) (Polymicro Technologies, Phoenix, AZ, USA). The sample was injected by pressure set at 10 mbar for 6 s. Temperature in the capillary oven was 35°C and the autosampler was maintained at 20°C. UV detection at 200 and 225 nm was obtained on a multi-wavelength detector (Linear Fast Scan Detector no. 206, Spectra-Physics, UK) using detection time setting at 0.5 s.

2.5. Experimental design

Optimization of a high-performance capillary electrophoresis (HPCE) method for the analysis of fungal metabolites was obtained by experimental designs. Important factors for separation were determined by a screening experiment and optimized by response-surface modelling (RSM). The screening experiment was carried out as a two-level fractional factorial design with nine factors with resolution IV (2^{9-4} structure, according to Box and Hunter [15]) with 3 center points giving a total of 35 runs (see Table 1). Levels of the factors were based on literature. The most important factors were selected for further optimization by a central composite response-surface modelling experiment (see Table 2). The factor levels which showed to be optimal in the screening experiment were set as centerpoint values in the RSM experiment to get a description around this area. The obtained response surface was verified by a full factorial design at two levels (2^3 structure) with factor levels close to the optimum in the RSM experi-

Table 1
Experimental design for the screening experiment

Exp. no.	PO_4^{3-} (*M*)	$B_4O_7^{2-}$ (*M*)	pH	Ionic strength (*M*)	SDS (*M*)	SDC (*M*)	MeOH (%, v/v)	CH_3CN (%, v/v)	Power (kV)
1	0	0.015	8	0.015	0	0.05	10	10	20
2	0.005	0.01	8	0.015	0	0	0	0	10
3	0.01	0.005	8	0.015	0	0	0	0	20
4	0.015	0	8	0.015	0	0.05	10	10	10
5	0	0.015	10	0.015	0	0	0	10	10
6	0.005	0.1	10	0.015	0	0.05	10	0	20
7	0.01	0.005	10	0.015	0	0.05	10	0	10
8	0.015	0	10	0.015	0	0	0	10	20
9	0	0.03	8	0.03	0	0	10	0	10
10	0.01	0.02	8	0.03	0	0.05	0	10	20
11	0.02	0.01	8	0.03	0	0.05	0	10	10
12	0.03	0	8	0.03	0	0	10	0	20
13	0	0.03	10	0.03	0	0.05	0	0	20
14	0.01	0.02	10	0.03	0	0	10	10	10
15	0.02	0.01	10	0.03	0	0	10	10	20
16	0.03	0	10	0.03	0	0.05	0	0	10
17	0	0.015	8	0.015	0.05	0.05	0	0	10
18	0.005	0.01	8	0.015	0.05	0	10	10	20
19	0.01	0.005	8	0.015	0.05	0	0	10	10
20	0.015	0	8	0.015	0.05	0.05	0	0	20
21	0	0.015	10	0.015	0.05	0	10	0	20
22	0.005	0.01	10	0.015	0.05	0.05	0	10	10
23	0.01	0.005	10	0.015	0.05	0.05	0	10	20
24	0.015	0	10	0.015	0.05	0	10	0	10
25	0	0.03	8	0.03	0.05	0	0	10	20
26	0.01	0.02	8	0.03	0.05	0.05	10	0	10
27	0.02	0.01	8	0.03	0.05	0.05	10	0	20
28	0.03	0	8	0.03	0.05	0	0	10	10
29	0	0.03	10	0.03	0.05	0.05	10	10	10
30	0.01	0.02	10	0.03	0.05	0	0	0	20
31	0.02	0.01	10	0.03	0.05	0	0	0	10
32	0.03	0	10	0.03	0.05	0.05	10	10	20
33	0.011	0.011	9	0.0225	0.025	0.025	5	5	15
34	0.011	0.011	9	0.0225	0.025	0.025	5	5	15
35	0.011	0.011	9	0.0225	0.025	0.025	5	5	15

Table 2
Experimental design for the response-surface modelling experiment

Exp. no.	pH	SDS (M)	CH_3CN (%, v/v)
1	9	0.02	5
2	11	0.02	5
3	9	0.08	5
4	11	0.08	5
5	9	0.02	15
6	11	0.02	15
7	8.3	0.08	15
8	11.7	0.08	15
9	10	0.05	10
10	10	0.05	10
11	10	0	10
12	10	0.1	10
13	10	0.05	1.5
14	10	0.05	18.5
15	10	0.05	10
16	10	0.05	10
17	10	0.05	10
18	10	0.05	10

ment (see Table 3). Statistical analysis was done using a modelling and design PC programme (MODDE version 2.0, UMETRI AB, Umeå, Sweden) running on a Zitech computer (386-40).

In the fractional factorial experiment extracts of four cultures were analysed. Only one of these extracts (*Aspergillus versicolor* IBT 12384) was used in the response-surface modelling design and the verification experiment.

Table 3
Experimental design for the verification experiment

Exp. no.	pH	SDS (M)	CH_3CN (%, v/v)
1	9.6	0.04	8
2	10.4	0.04	8
3	9.6	0.06	8
4	10.4	0.06	8
5	9.6	0.04	12
6	10.4	0.04	12
7	9.6	0.06	12
8	10.4	0.06	12
9	10.0	0.05	10
10	10.0	0.05	10
11	10.0	0.05	10

2.6. HPCE procedure

The operational parameter field strength and the electrolyte system (buffer composition and concentration, pH, micelle type and organic modifier) were altered. The instrumental arrangement was unchanged, so that the temperature and the dimensions of the capillary were the same for all experiments.

Two-level fractional factorial design: Before each run the capillary was rinsed for 10 min with 0.1 M NaOH, 1 min with H_2O and 1 min with the same buffer as in the following analysis at a pressure of 2000 mbar. Then the capillary was conditioned for 5 min at the same voltage as in the following analysis (10, 15 or 20 kV). The analysis time was set to 20, 30 and 40 min at 20, 15 and 10 kV, respectively. The experimental design is listed in Table 1. The pH was adjusted before addition of methanol and acetonitrile with concentrated NaOH, H_3PO_4 or H_3BO_3. The buffer without addition of SDS and SCD was analysed first to avoid problems with pollution by the surfactants in the capillary. Samples were made by dissolving fungal extract in center point buffer 2:1. The buffer at the outlet consisted of 0.011 M Na_2HPO_4 and 0.011 M $Na_2B_4O_7$ at pH 9.

Response surface modelling (RSM): Before each run the capillary was rinsed for 30 min with the same buffer as in the following analysis at a pressure of 1000 mbar. Then the capillary was conditioned for 5 min with voltage (15 kV). The experimental design is shown in Table 2. The pH was adjusted with concentrated NaOH, H_3PO_4 or H_3BO_3 before addition of acetonitrile. The run order was set in a way that the concentration of SDS increased during the runs to avoid problems with pollution of SDS in the capillary. Concentration of buffer ions was 0.01 M Na_2HPO_4 and 0.005 M $Na_2B_4O_7$. The power supply was set to 15 kV. The samples were prepared by dissolving fungal extract in center point buffer 2:1. The outlet buffer consisted of 0.01 M Na_2HPO_4 and 0.005 M $Na_2B_4O_7$ at pH 10.

Finally a full factorial experiment was carried out to verify the response surface near the

optimum. The experimental design with levels of the factors are shown in Table 3. The other conditions were similar to those of the RSM experiment.

For all experiments the number of baseline-separated peaks in the chromatograms was used as the response, because it was found to give the best expression for the separation of components in the extracts. The optimal detection wavelength in HPLC analysis of the fungal extract is 225 nm, but many of the components have a better absorption at 200 nm. In HPLC it is not possible to detect at 200 nm, because the baseline is too disturbed. Therefore both wavelengths were chosen for detection in HPCE–200 nm in the experiment and 225 nm as a reference to HPLC.

3. Results and discussion

Analysis of the same extracts with different buffers gave very different chromatograms with the number of separated peaks ranging from 3 to 43. There was a slight evaporation of methanol from the extract sample during the large number of runs (especially in the screening experiment with 35 runs), which could give a little variance in the number of peaks during runs.

Absorption at 200 nm gave generally better detection than absorption at 225 nm resulting in higher peaks and on average 4 additional peaks. However, baseline was less disturbed at 225 nm. In some cases in the screening experiment the baseline was so disturbed at 200 nm, that is was impossible to count the peaks. In these cases the response was excluded from the worksheet.

3.1. Result of the fractional factorial design

Multiple linear regression (MLR) analysis showed significant effects on the number of peaks in the chromatograms from pH, addition of sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC) and acetonitrile. This was valid for all four extracts. The analysis showed interaction between pH and SDS and between pH and SDC.

The statistical analysis of a model containing the important main factors, i.e. pH, SDS, SDC and acetonitrile and the two-factor interactions pH/SDS and pH/SDC gave R^2-values as shown in Table 4. The R^2-values are all above 0.8 for the analysed extracts except for the extract from *Aspergillus versicolor* IBT 12384 with detection at 225 nm ($R^2 = 0.683$) indicating acceptable regression models. The models were significant ($p \leq 0.001$) for all the experiments (see Table 4), as most of the residual variation in data (not explained by model) is due to measuring error.

3.2. Factors influencing the response

Voltage

The driving force behind the migration of ions in capillary electrophoresis (CE) is the field strength applied across the capillary, which is related to the applied voltage divided by the capillary length. The voltages used were 10–20 kV over a 63-cm long capillary giving a field strength of 150–300 V/cm. In this range of field strengths this parameter did not affect separation significantly.

In all runs the measured current was between 10 and 200 μA. Some of the runs at 20 kV gave a

Table 4
Result from multiple linear regression for detection at 200 nm and 225 nm in the screening experiment

Extract	R^2 200 nm	p 200 nm	R^2 225 nm	p 225 nm
Penicillium commune IBT 10253	0.829	0.001	0.892	0.000
Penicillium commune IBT 10727	0.855	0.000	0.850	0.000
Aspergillus versicolor IBT 12384	0.824	0.001	0.803	0.000
Aspergillus versicolor IBT 13736	0.856	0.000	0.683	0.001

value higher than 200 μA, which is the maximum allowed default value of the current for the instrument.

Increased voltage results in decreased analysis time [16], thus retention time of the last peak was less than 20 min at 20 kV and less than 40 min at 10 kV. In the following optimization experiment the voltage was set to 15 kV to avoid a too high current, while keeping the analysis time low.

Buffer composition and concentration

Neither the combination of $B_4O_7^{2-}$ and PO_4^{3-} nor the total concentration of the two ions had any significant effect on separation of compounds in the fungal extracts. A wide range of concentrations of phosphate–borate buffers has been reported as optimal condition for separation of related compounds (mycotoxins and antibiotics)–ranging from 0.006–0.05 m*M* $B_4O_7^{2-}$ and 0.01–0.05 *M* PO_4^{3-} [7–12]. Increase in buffer concentration decreases the electroosmotic flow resulting in increased analysis time [16], and it is found to decrease efficiency, due to the increased formation of Joule heating in the capillary [11]. Therefore the lowest ionic strength was chosen in the following optimization experiment (0.015 *M*) with 0.005 *M* $Na_2B_4O_7$ and 0.01 *M* Na_2HPO_4, which is close to the buffer composition used by Holland and Sepaniak in the separation of mycotoxins [12].

pH

The pH of the electrolyte solution in CE is the most important separation parameter for changing the selectivity of the system, affecting both the electroosmotic flow and the solute charge and mobility [16]. In this experiment the pH has a significant effect of the separation: increasing pH giving an increased number of peaks.

Surfactants

Addition of surfactants to generate micelles in the buffer has developed into a routine separation technique to improve the selectivity, not only for uncharged compounds but also for a large number of ionic compounds [16]. The two applied anionic surfactants in this work, SDS and SDC, showed a large significant effect on the separation. Chromatograms without addition of any of the surfactants gave very bad separations. The disadvantage of using surfactant is an increase in the analysis time [10,11,14]. For further optimization SDS was chosen, because SDS showed a larger positive effect on the number of peaks in the chromatograms than SDC. In another study–analysing fourteen active ingredients used in a cold medicine–SDC was found to give better separation compared to SDS [13].

Organic modifiers

In micellar electrokinetic capillary chromatography (MECC) addition of organic solvents such as methanol and acetonitrile influences selectivity and extends the elution range. The modifier alters the retention mechanism by changing the polarity of the aqueous phase [13,16]. In this experiment addition of acetonitrile seems to have a positive effect on separation, while methanol has no or a slightly negative effect. The better resolution obtained with addition of acetonitrile is in agreement with the work of Cole et al. [11] separating aflatoxins, but again an increase in analysis time is seen compared to the experiment without addition of acetonitrile.

3.3. Results of response-surface modelling

The three important factors from the screening experiment, pH, addition of SDS and acetonitrile, were examined by a response-surface modelling design analysing an extract of *Aspergillus versicolor* IBT 12384.

Statistical analysis using MLR showed an optimum at pH 10.0, 0.05 *M* SDS and 10% acetonitrile (Fig. 1). The parabolic shape of the response surface in Fig. 1 shows a good utilization of the experimental design. The result from the analysis of variance is shown in Table 5. The R^2-values are higher than 0.8 at both 200 and 225 nm–indicative of a good model. Both models were highly significant ($p < 0.001$).

For verification of the response-surface optimum the factor levels in the design of the verification experiment were used as predicted values in the RSM model giving confidence

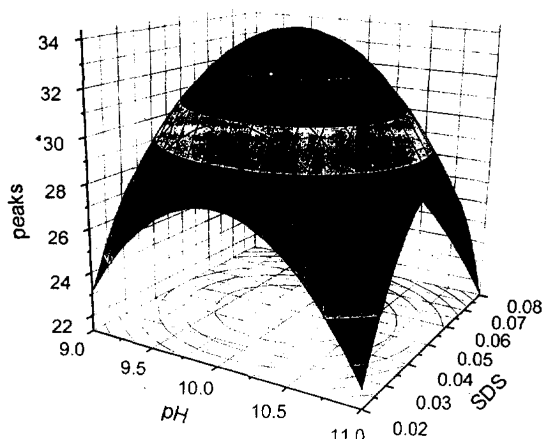

Fig. 1. Response surface showing numbers of peaks in the chromatogram at 200 nm from *Aspergillus versicolor* IBT 12384 as function of pH and SDS (concentration of acetonitrile is 10%).

intervals for the numbers of peaks for each run. The following verification experiment showed numbers of peaks lying in this interval. Thereby the optimum from the RSM experiment was verified.

The resulting response surface can be described by a second-order interaction model expressed by the three factors in the following polynomial:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

where y is the response, b_0 is the overall mean effect, b_i are the regression coefficients and x_i are the main factors (x_1, x_2 and x_3 are coated values of pH, concentration of SDS and acetonitrile, respectively). The linear and interaction terms turned out to be non-significant ($p > 0.05$)

Table 5
Result from multiple linear regression for detection at 200 nm and 225 nm in the RSM experiment

Extract	R^2 200 nm	p 200 nm	R^2 225 nm	p 225 nm
Aspergillus versicolor IBT 12384	0.936	0.000	0.903	0.000

at both wavelengths, thus the fitted data at 200 nm by MLR give the following model only consisting of the quadratic terms:

$$y = 34.4 - 5.36x_1^2 - 6.81x_2^2 - 7.26x_3^2$$

This equation can be expressed by the actual levels of the factors giving

$$y = 34.4 - 5.36(z_{pH} - 10)^2 - 7566.7(z_{SDS} - 0.05)^2 - 0.291(z_{CH3CN} - 10)^2 \Leftrightarrow$$

$$y = -549.6 - 5.36z_{pH}^2 - 7566.7z_{SDS}^2 - 0.291z_{CH3CN}^2 + 107.2z_{pH} + 756.7x_{SDS} + 5.82z_{CH3CN}$$

where z_{pH} is pH value, z_{SDS} is concentration of SDS in mol/l and z_{CH3CN} is concentration of acetonitrile in percent.

and at 225 nm:

$$y = 27.9 - 3.01x_1^2 - 5.27x_2^2 - 5.26x_3^2$$

Expressed by the actual levels of the factors giving

$$y = 27.9 - 3.01(x_{pH} - 10)^2 - 5855.6(x_{SDS} - 0.05)^2 - 0.211(x_{CH3CN} - 10)^2 \Leftrightarrow$$

$$y = -308.8 - 3.01x_{pH}^2 - 5855.6x_{SDS}^2 - 0.211x_{CH3CN}^2 + 60.2x_{pH} + 585.6x_{SDS} + 4.22x_{CH3CN}$$

Fig. 2 shows a chromatogram from the analysis of an extract from *Aspergillus versicolor* IBT 12384 using the optimal buffer conditions.

4. Conclusion

An optimal method for separating fungal secondary metabolites by HPCE has been developed by applying an experimental design strategy including screening, optimization and validation. The results showed that four different fungal extracts behaved equally and there was a good agreement between performance at the two

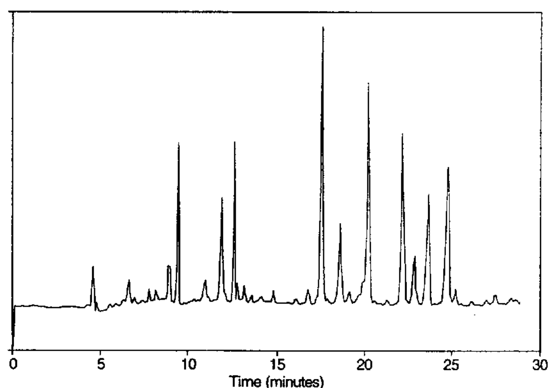

Fig. 2. Chromatogram from *Aspergillus versicolor* IBT 12384 using the optimal buffer conditions: 0.01 M Na_2HPO_4, 0.005 M NaB_40_7, 0.05 M SDS, 10% acetonitrile and pH 10.0. V = 15 kV and UV detection at 200 nm.

wavelengths, 200 and 225 nm. In general more peaks and better sensitivity were obtained at 200 nm. However at some unfavourable conditions a much more disturbed baseline and lower sensitivity were observed at 200 nm.

The optimal buffer conditions were pH 10.0, 0.05 M SDS and 10% acetonitrile with a buffer consisting of 0.01 M NaH_2PO_4 and 0.005 M $Na_2B_4O_7$.

References

[1] S.F.Y. Li, Capillary Electrophoresis: Principles, Practice and Applications, Journal of Chromatography Library, Vol. 52, Elsevier Science Publishers, Amsterdam, 1992, Ch. 7.

[2] H. Nishi, T. Fukuyama and M. Matsuo, J. Chromatogr., 515 (1990) 245–255.

[3] O. Filtenborg and J.C. Frisvad, Lebensm. Wiss. Technol., 13 (1980) 128–130.

[4] O. Filtenborg, J.C. Frisvad and J.A. Svendsen, Appl. Environ. Microbiol., 45 (1983) 581–585.

[5] J.C. Frisvad, J. Chromatogr., 392 (1987) 333–347.

[6] J.C. Frisvad, Bot. J. Lin. Soc., 99 (1989) 81–95.

[7] A.M. Hoyt and M.J. Sepaniak, Anal. Lett., 22 (1989) 861–873.

[8] C. Zhang, Z. Sun, D. Ling and Y. Zhang, J. Chromatogr., 627 (1992) 281–286.

[9] C.L. Ng, H.K. Lee and S.F.Y. Li, J. Chromatogr., 598 (1992) 133–138.

[10] H. Nishi, N. Tsumagari, T. Kakimoto and S. Terabe, J. Chromatogr., 477 (1989) 259–270.

[11] R.O. Cole, R.D. Holland and M.J. Sepaniak, Talanta, 39 (1992) 1139–1147.

[12] R.D. Holland and M.J. Sepaniak, Anal. Chem., 65 (1993) 1140–1146.

[13] H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, J. Chromatogr., 498 (1990) 313–323.

[14] J. Vindevogel and P. Sandra, Anal. Chem., 63 (1991) 1530–1536.

[15] G.E.P. Box, W.G. Hunter and J.S. Hunter, Statistics for Experimenters, John Wiley and Sons, New York, 1978.

[16] R. Kuhn and S. Hoffstetter-Kuhn, Capillary Electrophoresis: Principles and Practice, Springer Verlag, Berlin, 1993, Ch. 3.