ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1996) 233–239

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Multivariate analysis of capillary electrophoresis separation conditions for $Z-E$ isomers of clomiphene

Daniel K. Bempong*, Irwin L. Honigberg[1]

Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66047, USA

Received for review 16 June 1995; revised manuscript received 1 May 1996

Abstract

Plackett–Burman (P–B) experimental design has been used to optimize the factors affecting separation of Z and E isomers of clomiphene (zuclomiphene and enclomiphene respectively) using capillary electrophoresis. The P–B design was used to simultaneously investigate the following five factors: buffer ionic strength, buffer pH, heptakis (2,3,6-tri-*o*-methyl) β-cyclodextrin (TMCD) concentration, methanol concentration and injection time, each at three levels. In addition to these, a dummy variable was added to estimate the variability of the system. Effects on resolution and analysis time were calculated.

Based on the information gained from the P–B design, the following set of conditions was chosen: 100 mM phosphate buffer pH 2.3, 5 mM TMCD, 5% methanol, and 1.7 s hydrodynamic injection time. These conditions gave well-resolved peaks for zuclomiphene and enclomiphene.

Keywords: Capillary electrophoresis; Enclomiphene; Plackett–Burman design; Zuclomiphene

1. Introduction

Clomiphene is a non-steroidal compound which has both estrogenic and antiestrogenic effect. Clomiphene citrate is a mixture of the Z isomer (zuclomiphene) and the E isomer (enclomiphene) (Fig. 1) and contains not less than 30% and not more than 50% of the Z isomer [1]. Clomiphene is primarily used for the treatment of anovulatory infertility [2]. It has also been used in the treatment of male infertility [3], pubertal gynecomastia [4] and seizure disorders [5]. Other uses of clomiphene are: as an adjuct in in-vitro fertilization, embryo transfer and intrauterine insemination [6]. Clomiphene exerts its therapeutic effects by increasing the output of pituitary gonadotrophic hormones by blocking the binding of endogenous estrogen to hypothalamic and pituitary estrogen receptors [7].

HPLC methods have been reported for the analysis of zuclomiphene and enclomiphene [8–

* Corresponding author. E-mail: dbempong@smissman.hbc.ukans.edu.

[1] Previous address: College of Pharmacy, Dept. of Medicinal Chemistry, University of Georgia, Athens, GA 30602, USA.

PII S0731-7085(96)01853-5

Table 1
Levels of the factors used in the P–B design

Factor level	Buffer pH	Buffer ionic conc. (mM)	TMCD conc. (mM)	Methanol conc. (%)	Injection time (s)
Low	2.2	30	9	5	1.5
Intermediate	3.12	50	12	10	2.5
High	4.12	70	15	15	3.5

11]. NMR has also been reported for the analysis of these isomers [12]. Capillary electrophoresis (CE) has several advantages which make it a method of choice for such analysis. These advantages include rapid separation, high resolution and high mass sensitivity, small sample volumes (1–50 nl) and small mobile phase requirement. There are several reports in the literature which show the use of CE to resolve isomers. These include separation of enantiomers [13,14], positional substitution isomers [15] and *Z–E* isomers [16]. In CE analysis many factors can be varied to influence separation. These factors include buffer composition, ionic strength, pH, applied voltage and capillary dimensions. In addition, buffer additives such as organic solvents, cyclodextrins and surfactants (MECC - micellar electrokinetic capillary chromatography) can be used to effect separation. Given the many parameters which can be varied, the usual "one-factor-at-a-time" method may not give an optimum set of conditions. Factorial design, which is usually used to determine the relative importance of factors and their interactions, would require too many experiments. For example, investigation of five of these factors at three levels would require 3^5 (i.e. 243) experiments. Fractional factorial design, which can be used to determine the main effects while ignoring interaction effects, is ideal for such analysis. The Plackett–Burman (P–B) design [17] is an example of this latter experimental design. The P–B design has been used to optimize the silation of silicas for RP-HPLC [18], to optimize resolution in MECC [19], and in chiral analysis with CE [20].

In this paper, the P–B experimental design is used to investigate the separation conditions for CE analysis of zuclomiphene and enclomiphene. With the use of the information from the P–B design a set of conditions were obained for the analysis of the clomiphene isomers.

2. Experimental

2.1. Chemicals and reagents

Zuclomiphene (MDL 16,312F) and enclomiphene (MDL 16,289F) were supplied by Marion Merrel Dow Research Institute, Marion Merrel Dow Inc. (Cincinnati, OH). Sodium dihydrogen phosphate, methanol and phosphoric acid were purchases from Baker (Phillipsburg, NJ). Heptakis (2,3,6-tri-*o*-methyl)β-cyclodextrin (TMCD) was obtained from Sigma (St. Louis, MO).

2.2. CE system

An analytical CE system model 270A (ABI, San Jose, CA) was used. A fused silica capillary of dimensions 50 μm × 72 cm (50 cm to the detector) was employed. On-column UV detection at 254 nm was used for all analyses. Samples were injected hydrodynamically and a constant voltage of 30 kV and a constant temperature of 30°C were used throughout this work. Migration times and peak areas were measured by a Hewlett Packard hp 3392A integrator.

2.3. Methods

Phosphate buffers were prepared by adjusting the pH of sodium dihydrogen phosphate solution with phosphoric acid solution of the same concentration to the desired pH. The modified buffer solutions were made by adding the re-

Table 2
The P–B design

Experiment	Buffer pH	Buffer conc (mM)	TMCD conc (mM)	Methanol conc (mM)	Injection time (s)	Dummy
1	2.2	50	12	5	2.5	−0
2	2.2	30	12	10	1.5	0
3	2.2	30	9	10	2.5	−0
4	3.12	30	9	5	2.5	0
5	2.2	50	9	5	1.5	0
6	3.12	30	12	5	1.5	−0
7	3.12	50	9	10	1.5	−0
8	3.12	50	12	10	2.5	0
9	3.12	50	15	10	3.5	+0
10	3.12	70	12	15	3.5	+0
11	4.12	50	15	15	3.5	0
12	3.12	70	15	15	2.5	0
13	4.12	70	15	10	2.5	0
14	4.12	70	12	10	3.5	+0
15	4.12	50	12	15	2.5	+0

quired amount of the modifers (TMCD and methanol) to the prepared buffer solution. Modified buffers were vaccum-filtered used a 0.2 μm membrane filter to remove particles and to degas the solutions. Analyte solution (5 μg ml^{-1}) was made in water–methanol (50:50) solution because limited amounts of the modified buffer components were available at the time. The capillary was washed with 0.1 M NaOH for 4 min followed by another 4 min wash with the modified buffer solution before starting each run. The

Table 3
Results of the P–B design for resolution and analysis time

Experiment	Resolution	Analysis time (min)[a]
1	1.15	13.70
2	0.46	11.53
3	0.69	11.38
4	1.70	9.70
5	1.50	13.30
6	1.31	8.90
7	1.89	15.28
8	1.29	15.47
9	0.89	15.91
10	1.49	17.60
11	0.36	6.83
12	1.37	15.82
13	0.44	5.94
14	0.62	5.81
15	0.34	5.18

[a] Analysis time is taken as the migration time of the later-eluting isomer.

Table 4
P–B effects (E)[a]: (a) low vs. intermediate level; (b) intermediate vs. high level

Factor	Resolution	Analysis time
(a)		
Dummy	−0.02	0.19
pH	0.60	−0.07
Buffer ionic conc.	0.42	4.06
TMCD conc.	−0.39	−0.02
Methanol conc.	−0.33	2.02
Injection time	−0.08	0.31
Range[b]	1.43	5.77
(b)		
Dummy	−0.03	0.11
pH	−0.82	−10.26
Buffer ionic conc.	0.26	0.45
TMCD conc.	−0.17	0.11
Methanol conc.	−0.08	0.58
Injection time	−0.03	0.52
Range[b]	1.15	12.42

[a] E is calculated as the difference between the means of the responses at low and high levels of a particular factor (see Eq. (2)).
[b] Difference between highest and lowest values of resolution or analysis time calculated from Table 3.

Table 5
Results of the P–B t-test: (a) low vs. intermediate; (b) intermediate vs. high

Factor	Resolution	Analysis time
(a)		
Dummy	–	–
pH	30.0	(–)[a] 0.4
Buffer ionic conc.	21.0	21.4
TMCD conc.	(–) 19.5	(–) 0.1
Methanol conc.	(–) 16.5	10.63
Injection time	(–) 4.0	1.6
(b)		
Dummy	–	–
pH	(–) 27.3	(–) 93.3
Buffer ionic conc.	8.7	4.1
TMCD conc.	(–) 5.7	1.0
Methanol conc.	(–) 2.7	5.3
Injection time	(–) 1.0	4.7

[a] (–) Indicates reduction in resolution or analysis time. Critical values are: 3.08 (80% CL), 6.31 (90% CL), 12.71 (95% CL) and 63.66 (99% CL).

washing step was found to significantly improve migration time reproducibility.

2.4. The P–B design

Table 1 shows the levels of the five factors—buffer pH, buffer ionic concentration, TMCD concentration, methanol concentration and hydrodynamic injection time [20]—which were used in the P–B design. With regard to the injection time, the essence was to determine the optimum volume of the analyte solution that could be introduced into the capillary without adversely affecting the resolution of the two isomers. In order to maintain the same buffer composition, phosphate buffer was used for the "high" level of pH in spite of the fact that phosphate has reduced buffer capacity at this pH. The intermediate levels of all the factors (Table 1) were based on results of preliminary investigations done on separation of these isomers. A "dummy" variable was added to these factors. There is no physical difference between the three levels of this variable so any determined effects will be an estimate of the variance of the system. Hence the effect calculated (see Eq. (2)) for the dummy can be used in statistical determinations of the significance of the other variables.

The P–B design is shown in Table 2. The design is used in this case to investigate the effect of increasing or decreasing each of the five factors (from the intermediate level) on resolution of the isomers and on the analysis time measured as the migration time of the later-eluting peak. Resolution (R_s) in this case is calculated using the following formula:

$$R_s = \frac{2(t_2 - t_1)}{(w_2 + w_1)} \quad (1)$$

where t_1 and t_2 are the migration times of the first and second peaks respectively, and w_1 and w_2 are the respective peak widths (measured at the base). The effect (E) of increasing a particular factor from a lower level to a higher level is given by the difference between the average result (resolution or analysis time) at the higher level and the average result at the lower level:

$$E = \sum \frac{R(\mathrm{H})}{n} - \sum \frac{R(\mathrm{L})}{n} \quad (2)$$

where R(H) and R(L) are the results at high and low levels respectively and n is the number of experiments in which the factor is at a low level (or at a high level). The significance of a factor may be determined using the t-test. An observed effect for any factor is said to be significant if

$$t_{\mathrm{calc}} \geqslant t_{\mathrm{ldf},X\%\ \mathrm{CL}}$$

where $t_{\mathrm{ldf},X\%\ \mathrm{CL}}$ is the critical value of the t-test and X is the chosen confidence level. The critical values are: 3.08 (80% CL), 6.31 (90% CL), 12.71 (95% CL), and 63.66 (99% CL).

$$t_{\mathrm{calc}} = \frac{|E|}{\sigma} \quad (3)$$

where $\sigma = E_{\mathrm{dummy}}$. E_{dummy} is the effect (E) calculated for the dummy variable using Eq. (2).

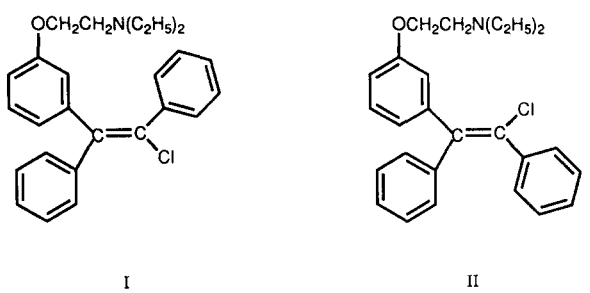
3. Results and discussion

Fig. 2 shows examples of electropherograms

from the P–B design experiments. Even though some of the conditions gave separated peaks for the two isomers, peak shapes are distorted and hence none of the conditions used in the design are adequate for the analysis of the isomers. However, information gained from analyzing the effects of each of the factors is valuable for determining suitable separation conditions. The effects of the factors on the analysis time and resolution (Table 3) are discussed below.

3.1. Analysis time

Analysis time, measured as the migration time of the later-eluting peak should preferably be short without compromising resolution of the isomers. Tables 4(a) and 4(b) show the effects of the factors used in the design on resolution and analysis time. These effects were statistically analyzed (using the t-test) to determine the factors which significantly affect resolution and/or analysis time. The results of the t-test are shown in Tables 5(a) and 5(b). Buffer ionic concentration, pH and methanol concentration are the most important factors affecting analysis time. Increasing ionic concentration or methanol concentration result in increased analysis time. This is due to the decrease in electroosmotic flow (EOF). This is explained by the fact that increasing concentration of electrolyte decreases the zeta potential which is directly related to EOF [21]. Low pH values do not have a significant effect on analysis time (at 80% CL), but a dramatic reduction in analysis time results if pH is increased above the intermediate level used in the P–B design. This is also related to the effect of pH on the ionization of the silanol groups on the surface of the fused silica capillary. The increased ionization of these silanol groups at high pH results in increased EOF and hence in the reduction in analysis time.



I II

Fig. 1. Structures of enclomiphene (I) and zuclomiphene (II).

3.2. Resolution

Resolution of the two isomers of clomiphene is very important in analytical determination of clomiphene since the isomeric content of the drug is regulated. From Tables 4 and 5, the factors found to significantly affect resolution (at 80% CL) are pH, buffer ionic concentration and TMCD concentration. Also methanol in low concentrations ($<10\%$) and injection time have a significant effect on resolution at this level of confidence. As shown in Table 5, pH had the most significant effect on resolution. An increase in pH from a low to an intermediate level improves resolution but a further increase to the higher pH value results in a dramatic loss in resolution. The latter observation may be due to incomplete ionization of the clomiphene isomers. This is further confirmed by the peak distortion and tailing observed at the higher pH. Again the sharp decrease in migration time (increase in EOF) at the higher pH may also explain the observed effect on resolution. High buffer ionic concentration signficantly enhances resolution of the isomers. This may also be the result of the long analysis time (decrease in EOF) observed under these conditions. The slow EOF allows adequate time for analyte–TMCD interaction. This is based on the fact that formation of the TMCD–clomiphene complex requires some time, the magnitude of which depends on such factors as the size of the molecule and the extent of ionization [22]. High concentrations of TMCD have a negative effect on the resolution of the isomers. This is expected as resolution is expected to go through the maximum as the TMCD concentration is increased [20].

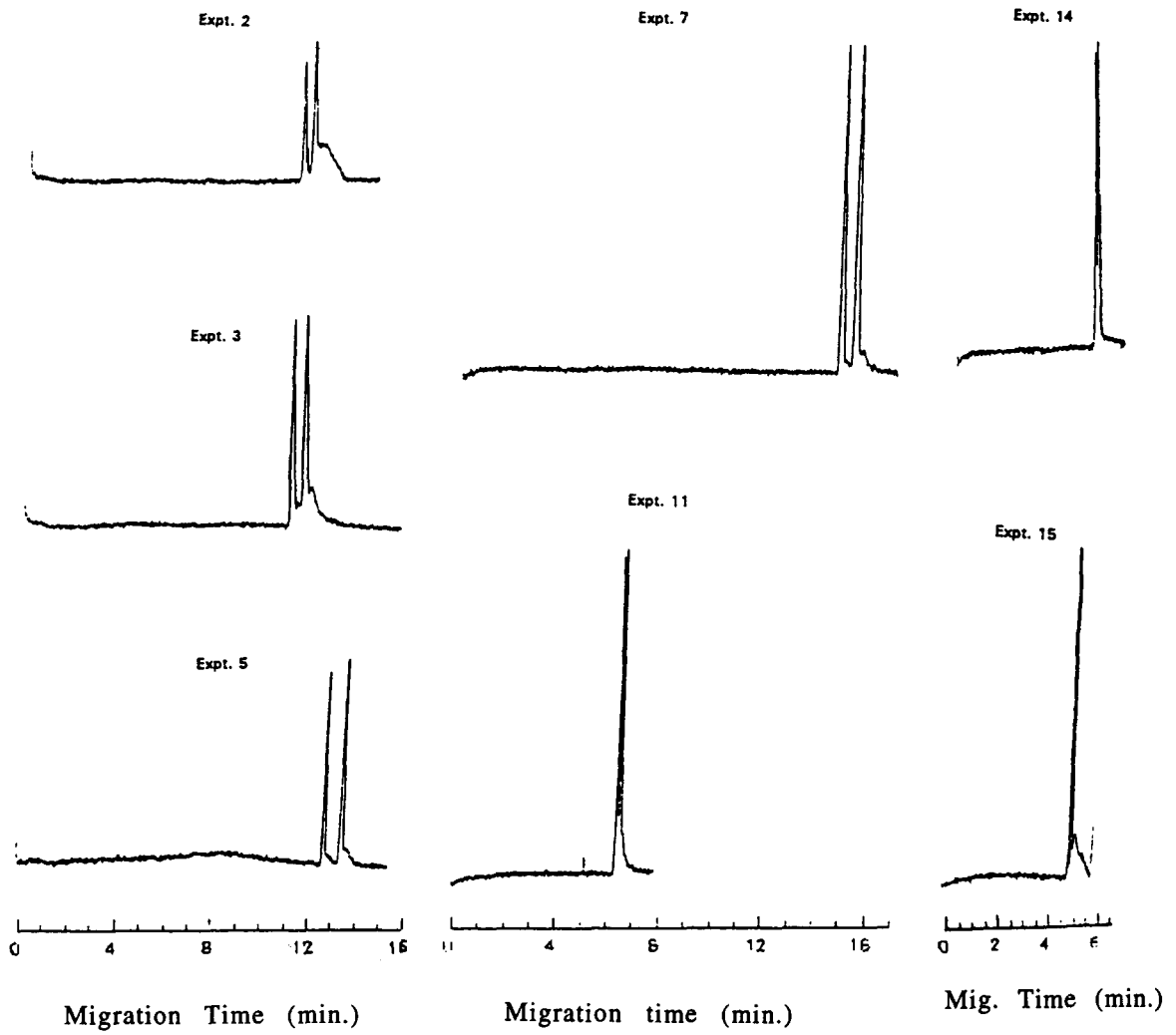


Fig. 2. Typical electropherograms from the P–B design experiments.

The following factors and ranges were selected based on the results of the P–B design experiments: TMCD 5 mM, pH 2.3 and buffer ionic concentration 100 mM. Injection time and methanol concentration have the most signficant effects on analysis time (from Table 5). These factors increase analysis time so their levels were kept low at 1.7 s and 5% respectively. The electropherogram obtained under these conditions is shown in Fig. 3(a). It must be mentioned that even though a low concentration of cyclodextrin is required, buffer solutions containing no TMCD gave zero resolution of the two isomers as shown in Fig. 3(b).

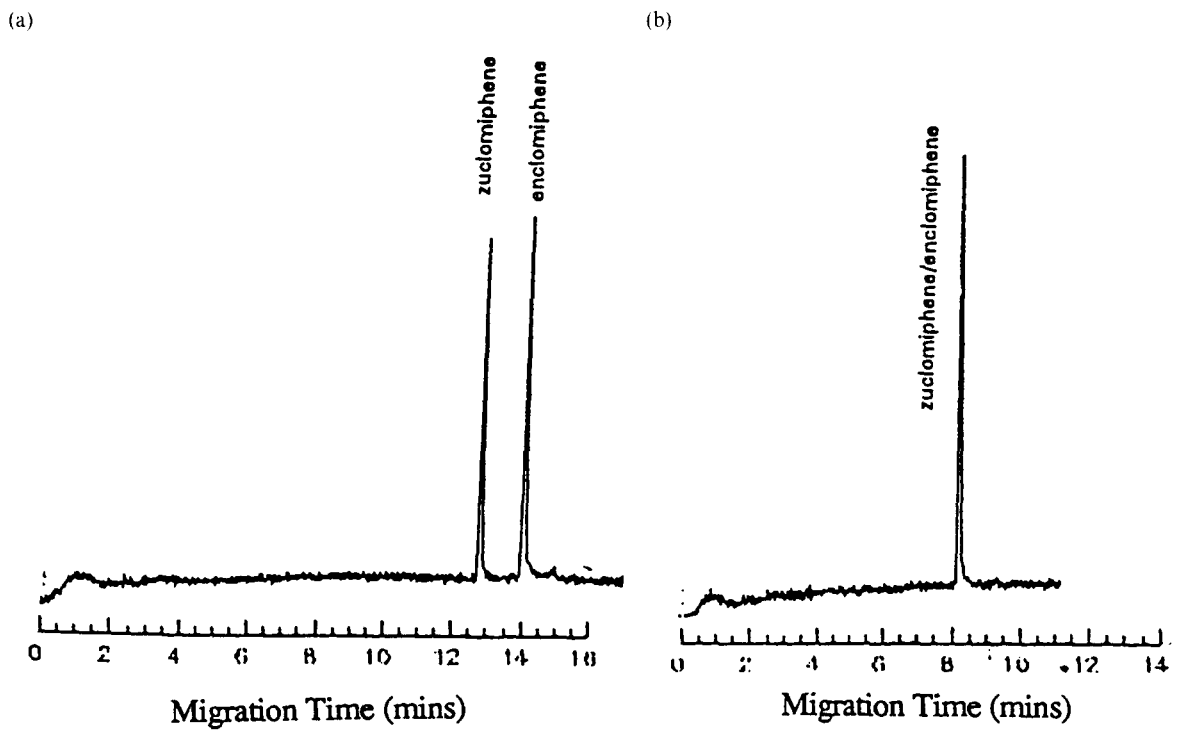


Fig. 3. (a) Electropherograms of zuclomiphene and enclomiphene obtained under the following conditions: 100 mM phosphate buffer, pH 2.3 and 5 mM TMCD. Other conditions: 72 cm × 50 μm capillary (50 cm to detector), UV detection at 254 nm, +30 kV applied voltage, 1.7 s hydrodynamic injection time and temperature 30°C. (b) Electropherogram showing zero resolution of zuclomiphene and enclomiphene when no TMCD is added. Other conditions are as given in (a).

References

[1] The United States Pharmacopeia (USP XX), United States Pharmacopeial Convention, Inc., Rockville, MD, 1980, p. 158.
[2] P.M.F. Bishop, Br. Med. Bull., 26 (1970) 22–25.
[3] P.J. Sorbie and R. Perez-Marrero, J. Urol., 131 (1984) 425–429.
[4] D. Le Roith, R. Sobel and S.M. Glick, Acta Endocrinol. (Copenhagen) 95 (1984) 177.
[5] A.G. Herzog, Arch. Neurol. (Chicago), 45 (1988) 209–210.
[6] E. Loumaye, J.M. Billion, J.M. Mine, I. Psalti, M. Pensis and K. Thomas, Fertil. Steril., 53 (1990) 295–301.
[7] C.F. Wang, B.L. Lasley and S.S. Yen, J. Clin. Endocrinol, 41 (1975) 41–43.
[8] C.L. Baustian and T.J. Mikkelson, J. Pharm. Biomed. Anal., 4 (1986) 237–246.
[9] M.S.F. Ross and H. Judelman, J. Chromatogr., 298 (1984) 172–174.
[10] R.G. Frith and G. Phillipou, J. Chromatogr., 367 (1986) 260–266.
[11] I. Urmos, S.M. Benko and I. Klebovich, J. Chromatogr., 617 (1993) 168–172.
[12] B. Lindgreen and J.R. Martin, Pharmeuropa, 5 (1993) 51–54.
[13] S. Fanali, J. Chromatogr., 545 (1991) 437–444.
[14] J. Prunonosa, R. Obach, A. Diez-Cascon and L. Gouesclou, J. Chromatogr., 574 (1992) 127–133.
[15] S. Terabe, H. Ozaki, K. Otsuka and T. Ando, J. Chromatogr. 332 (1985) 211–217.
[16] D.K. Bempong, I.L. Honigberg and N.M. Meltzer, J. Pharm. Biomed. Anal., 11 (1993) 829–833.
[17] R.L. Plackett and J.P. Burman, Biometrika, 23 (1946) 305–325.
[18] K. Jones, J. Chromatogr., 392 (1987) 1–16.
[19] J. Vindevogel and P. Sandra, Anal. Chem., 63 (1991) 1530–1536.
[20] M.M. Rogan, K.D. Altria and D.M. Goodall, Chromatographia, 38 (1994) 723–729.
[21] Introduction to Capillary Electrophoresis, Beckman Instruments, Inc., Fullerton CA, 1991, p. 4.
[22] J. Szejtli, Cyclodextrins and their Inclusion Complexes, Akademiai Kiado, Budapest, 1982 pp. 95–140.