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On-line kinetic monitoring for biochemical reactions using multi-point detection in high-performance capillary electrophoresis

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Abstract

The potential utility of high-performance capillary electrophoresis for on-line kinetic monitoring of biochemical reactions is demonstrated using a multi-point detection method. The adenosine deaminase-catalyzed deamination of adenosine to inosine is studied. The deamination speed increases with the increase in enzyme concentration in the run buffer. Degradation of heme protein, myoglobin, has been investigated by this method. Under certain electrophoretic conditions, the degradation follows first-order kinetics. The degradation rate constant increases with an increase in temperature and the electric field applied to the capillary does not appreciably effect the degradation rate.

1. Introduction

High-performance capillary electrophoresis (HPCE), as a complementary method to high-performance liquid chromatography (HPLC), has become an important analytical technique [1–5]. It has been successfully used for the separation of small ions [6,7], peptides [8,9], carbohydrates [10], polymers such as proteins and nucleic acids [11–14] and chiral molecules [15–18]. The broad application range and the high performance demonstrated by HPCE have attracted much interest. More recent research efforts involve the application of HPCE to the study of biomolecular interactions such as determination of binding constants [19–21] and enzyme activities [22–24].

However, there has been little information in literature regarding the use of HPCE for monitoring reaction rates. Karger and co-workers [25,26] reported that degradation of myoglobin appeared to occur as temperature was elevated from 20 to 50°C because of the reduction of Fe^{3+} to Fe^{2+} in myoglobin. The conversion from Fe^{3+} to Fe^{2+} was found to follow first-order kinetics by adjusting the time that myoglobin spent in the capillary using electric field variations. To the best of our knowledge, no on-line kinetic study method has been published using HPCE. Recently, Srichaiyo and Hjertémn [27] developed a simple multi-point detection method for HPCE. They used this method to study the unexpected, discontinuous change of a DNA sample during an HPCE run. This method also has been used for the dispersive study of oligonucleotides in gel filled capillaries [28]. The simple multi-point

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detection method offers a very useful tool for the on-line monitoring of analytes for HPCE.

In this paper, the feasibility of using HPCE for on-line kinetic studies is demonstrated using a multi-point detection method. Enzyme catalyzed deamination of adenosine to inosine is studied by this method. The effect of enzyme concentration on the deamination speed is investigated. The degradation of myoglobin protein is also monitored by this method. The kinetics, influence of temperature and the electric field on the reaction are also presented.

2. Experimental

2.1. Reagents and materials

Adenosine (A), inosine (I), adenosine deaminase (ADA) from calf spleen and horse heart myoglobin [type III, iron present in the ferric(Fe^{3+})state] were obtained from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Bio-Rad Labs. (Richmond, CA, USA) and boric acid from Fisher Scientific (Fair Lawn, USA NJ). Reagent-grade 3-methylphenol was obtained from Aldrich (Milwaukee, WI, USA).

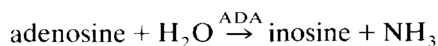
2.2. Apparatus

The experiments were performed on an HPCE apparatus which was designed and constructed in our laboratory. The instrument consisted of a laboratory-made Plexiglass box, a Spectra 100 UV detector (Thermo Separation Products, Freemont, CA, USA) a CZE 1000 PN 30 high-power supply (Spellman, Plainview, NY, USA) and a high-power supply local control (Chamonix Industries, Johnson City, NY, USA) for adjustment of the actual separation voltage. A special cell, which was fitted to the detector [28], was used for multi-point detection. The cell body was constructed by gluing two fabricated pieces of metal. There was a small hole in the center of the lower part of the cell and a ball lens was inserted. A fused-silica capillary with 150 cm total length (75 μm I.D. × 360 μm O.D.; Polymicro Technologies, Phoenix, AZ, USA) was used in this work. Four detection windows (with effective lengths of 35, 66.5, 97.5 and 129 cm) were opened on the capillary by burning off the external coating. The capillary was bent into loops with the four windows on top of each other in a slit located on the upper part of the cell. There was a securing arm crossing the slit on each side of the cell which kept the looped capillary in position and aligned in the slit. During an HPCE run, the solutes in the capillary could be detected four times as they passed through the light path at different times. The detection wavelength was 260 nm for adenosine and 214 nm for myoglobin. The detector rise time was 0.1 s. Neutral molecule, 3-methylphenol, was used as a reference standard in all the HPCE runs. The run buffer in the capillary was renewed after each run. 0.1 *M* Tris, 0.025 *M* boric acid buffer at pH 8.63 or 0.025 *M* borate buffer at pH 8.01 were used as run buffers. Temperature inside the Plexiglass box was increased by using a space heater and cooling was achieved with a fan. The electrophoregrams were processed on a SP-4400 integrator (Thermo Separation Products).

3. Results and Discussion

3.1. Enzyme-catalyzed adenosine deamination

ADA is a highly specific enzyme that catalyses the deamination of adenosine to inosine with liberation of NH_3 as follows [29]:



$$\text{adenosine} + H_2O \xrightarrow{\text{ADA}} \text{inosine} + NH_3$$

A genetic lack of adenosine activity causes an abnormality in purine nucleoside metabolism that selectively kills lymphocytes (a type of white blood cell) [30]. The consequent lack of lymphocytes mediates the immune response and can cause severe combined immunodeficiency disease. Without special protective measures, the disease is invariably fatal in infancy due to overwhelming infection. Enzyme catalyzed

deamination is an active area in biochemistry and clinical chemistry research.

Fig. 1 shows the electropherograms of an adenosine and inosine mixture sample before (a) and after (b) enzymatic reaction. It can be seen that within 10 min after the addition of ADA to the sample, all the adenosine is deaminated to inosine, since only one peak (inosine) is detected after the enzymatic reaction. To study the reaction rate and enzyme concentration influence on the reaction, different amounts of ADA have been added to run the buffer, and the looped capillary is then filled with this buffer. Fig. 2 represents the electropherograms obtained from the multi-point detection method. It can be seen that before the addition of ADA enzyme to the run buffer (Fig. 2a), no adenosine deamination happens. After ADA enzyme is added to the run buffer (Fig. 2b), adenosine starts to be deaminated to inosine.

In order to monitor the deamination of adenosine accurately, 3-methylphenol (P) has been used as a reference standard. According to

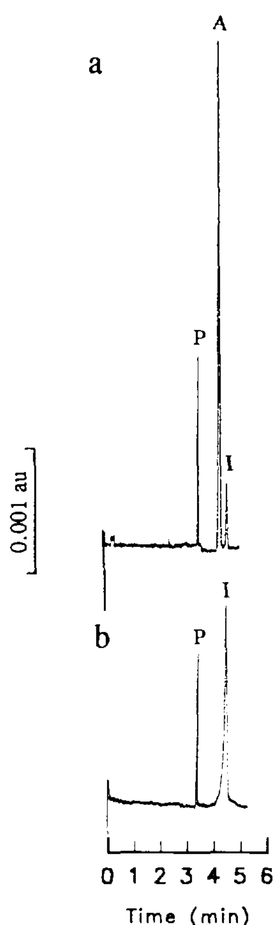


Fig. 1. Electropherograms of a mixture of adenosine (A), inosine (I) and 3-methylphenol (P). Sample was $2.76 \cdot 10^{-3}$ *M* adenosine and $4.86 \cdot 10^{-4}$ *M* inosine in water. Capillary: 75 μm I.D. × 360 μm O.D. bare capillary with 35 cm effective length. Electric field: 167 V/cm. Injection: 6 cm × 6 s. Run buffer: 0.025 *M* borate at pH 8.01. (a) Before adenosine deaminase treated; (b) after 10 min adenosine deaminase treated.

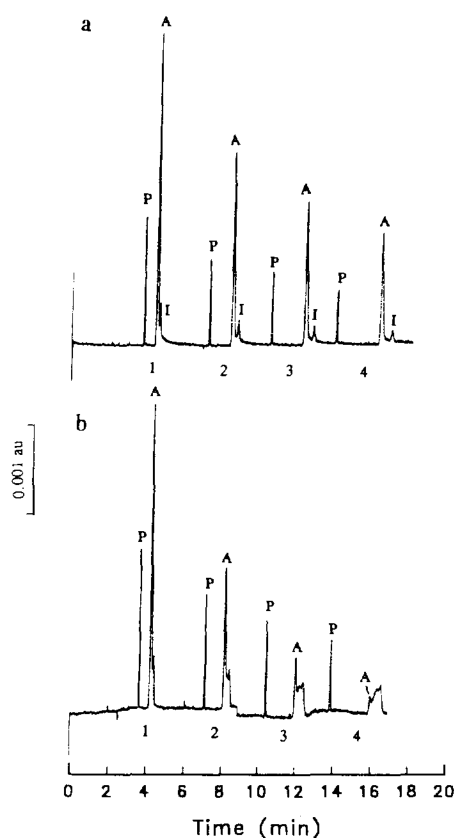


Fig. 2. Electropherograms of adenosine (A), inosine (I) and 3-methylphenol (P) mixture obtained from four consecutive detection. Capillary: 75 μm I.D. × 360 μm O.D. bare capillary with 150 cm total length. The four effective window lengths were 35, 66.5, 97.5 and 129 cm. Electric field: 167 V/cm. Injection: 6 cm × 10 s. Temperature: 25°C. Run buffer: (a) 0.025 *M* borate at pH 8.01; (b) 0.025 *M* borate with 0.04 units/ml adenosine deaminase at pH 8.01.

Beer's law, the absorbance of a specific sample solution can be expressed as:

$$A = \varepsilon b C \quad (1)$$

where A is the absorbance, ε is the molar extinction coefficient of sample, b is the light path length and C is the sample concentration. Since a ball lens is used to focus the light, the light path lengths may vary slightly at different detection windows. Consequently, the integrated absorbance areas obtained from different windows can change even if the amount of sample does not. However, this error can be corrected by using a reference standard, 3-methylphenol in this case. When samples pass through the first detection window, the absorbance of the reference standard is given by:

$$(A_P)_1 = \varepsilon_P b_1 (C_P)_1 \quad (2)$$

the absorbance of adenosine is:

$$(A_A)_1 = \varepsilon_A b_1 (C_A)_1 \quad (3)$$

So,

$$\frac{(A_A)_1}{(A_P)_1} = \frac{\varepsilon_A \cdot (C_A)_1}{\varepsilon_P \cdot (C_P)_1} \quad (4)$$

When the samples pass through the second window, the following relationship exists:

$$\frac{(A_A)_2}{(A_P)_2} = \frac{\varepsilon_A \cdot (C_A)_2}{\varepsilon_P \cdot (C_P)_2} \quad (5)$$

Since the reference standard 3-methylphenol (P) does not participate in the enzymatic reaction, the amount of 3-methylphenol stays constant as it migrates in the capillary. As a result, one can use the ratio of integrated absorbance area of adenosine (A) to that of the reference standard at different detection windows to monitor the change of adenosine reactant. Before enzyme ADA is added to the buffer, the deamination of adenosine does not occur. The amount of adenosine does not change as it migrates through the capillary. Theoretically, one should obtain the following results at four detection windows:

$$\frac{(A_A)_1}{(A_P)_1} = \frac{(A_A)_2}{(A_P)_2} = \frac{(A_A)_3}{(A_P)_3} = \frac{(A_A)_4}{(A_P)_4} = \text{constant} \quad (6)$$

Eq. 6 has been proven by the experimental data. In Fig. 3, it can be seen that before the addition of ADA enzyme to the run buffer (line 1), the ratio of integrated area of A to that of P does not change at different windows. A straight line parallel to the time axis is obtained. After the addition of ADA to the run buffer, the adenosine deamination occurs (Figs. 2 and 3).

It is known that under certain conditions, enzyme concentration can affect the rate of enzymatic reactions. The higher the enzyme concentration, the faster the rate [30]. Fig. 3 illustrates the influence of ADA concentration in the run buffer on the adenosine deamination. It can be seen that deamination of adenosine to inosine increases as the enzyme concentration in the buffer increases. When the enzyme concentration is lower than 0.04 units/ml, the amount of adenosine injected in the capillary is relatively

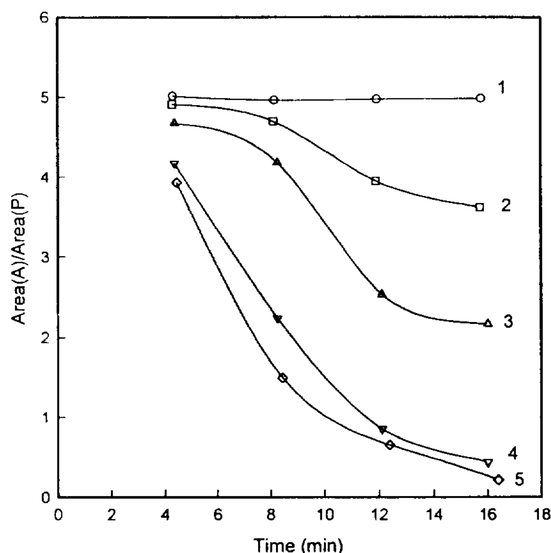


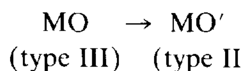
Fig. 3. Influence of adenosine deaminase (ADA) concentration in the run buffer on adenosine deamination. Electrophoretic conditions as in Fig. 2. The concentrations of ADA in the buffer were: (1) 0 units/ml; (2) 0.01 units/ml; (3) 0.02 units/ml; (4) 0.04 units/ml; (5) 0.06 units/ml.

high compared to ADA, and the reaction rate is slow at the beginning. As adenosine migrates in the capillary, the amount of adenosine decreases because of deamination, while the enzyme concentration does not change. Thus, more of the adenosine can be catalyzed by ADA, and reaction rate increases until it reaches a maximum value. After almost all of the adenosine has been deaminated, the rate decreases again. As the ADA concentration in the buffer is increased (lines 4 and 5 in Fig. 3), the deamination is faster at the beginning since the enzyme concentration becomes relatively high compared to adenosine. The reaction also slows down after most of the adenosine has been deaminated.

3.2. Kinetic study of myoglobin degradation

As has been reported before, sample degradation of myoglobin (MO) can occur with an increase in temperature [25,26]. A possible explanation for this is the reduction of Fe^{3+} (ferric) to Fe^{2+} (ferrous) metal ion coordinated to the heme group in myoglobin by either the reducing agent impurity in buffer system or autoreduction, in which an amino acid residue in myoglobin is the reducing agent [26]. Fig. 4 shows the electropherograms of a myoglobin and 3-methylphenol mixture sample obtained from four-point detection. The degradation of initial myoglobin (ferric form) occurs as temperature inside the capillary increases. Again, 3-methylphenol has been used as a reference standard. Fig. 5 demonstrates the temperature effect on the myoglobin degradation. It can be seen that the ratio of integrated absorbance of MO to that of P decreases faster at higher temperature (Fig. 5).

In order to determine the kinetic order of this degradation reaction, different functions of (A_{MO}/A_P) have been plotted against the migration time. The myoglobin degradation can be expressed by the following reaction:



if this reaction obeys first order kinetics, the following equations exist [31]:

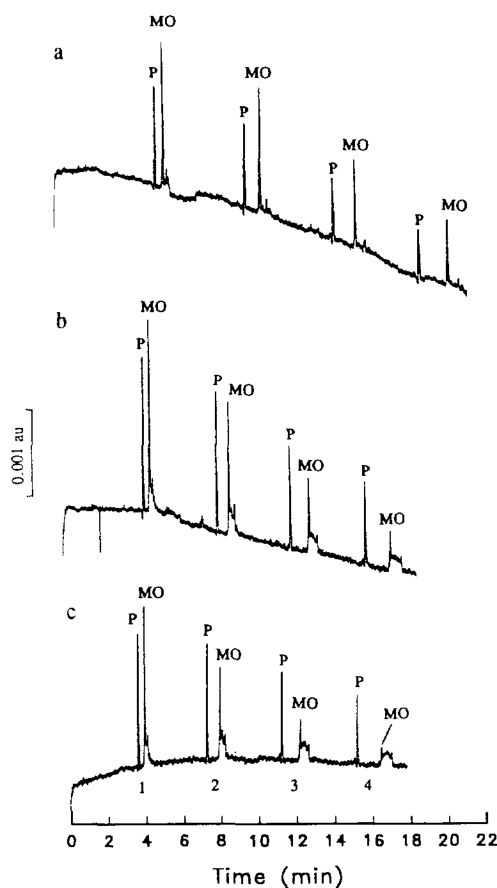


Fig. 4. Electropherograms of myoglobin (MO) and 3-methylphenol (P) obtained from four consecutive detections. Electrophoretic conditions as in Fig. 2, except run buffer was 0.1 *M* Tris, 0.025 *M* boric acid at pH 8.61. (a) 20°C; (b) 26°C; (c) 28°C.

$$-\frac{d[MO]}{dt} = k[MO] \quad (7)$$

$$\log [MO]_0 - \log [MO] = kt \quad (8)$$

where k is the rate constant. From algebraic treatment, we have:

$$\log \frac{[MO]_0}{[P]} - \log \frac{[MO]}{[P]} = kt \quad (9)$$

where P is the 3-methylphenol reference standard which does not participate in the degradation reaction. So, the amount of P remains

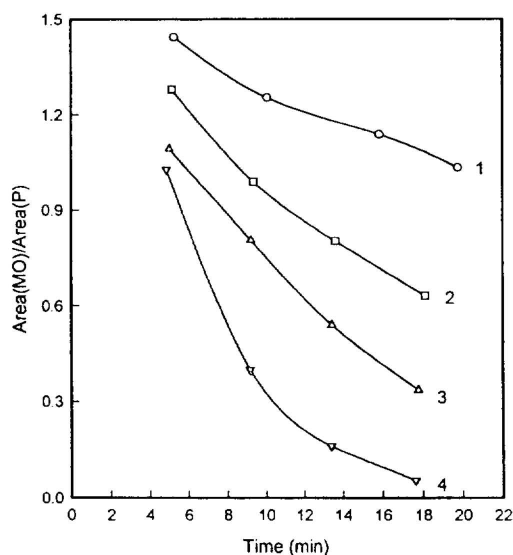


Fig. 5. Plot of the ratio of integrated absorbance area of myoglobin (MO) to that of 3-methylphenol (P) vs. time. Electrophoretic conditions as in Fig. 4. (1) 22°C; (2) 24°C; (3) 26°C (4) 30°C.

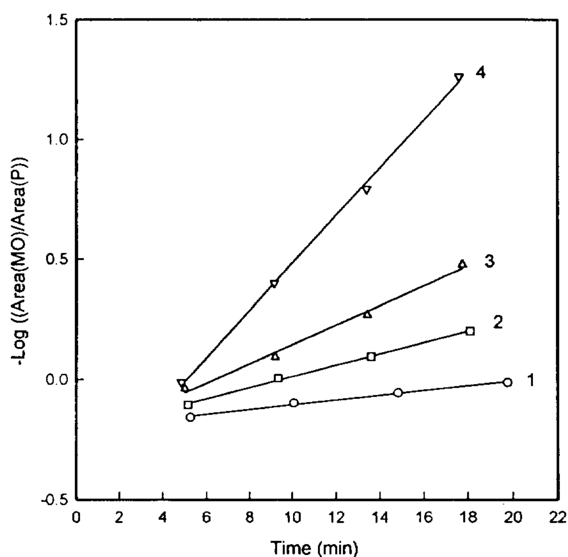


Fig. 6. Relationship of $\log(A_{MO}/A_P)$ with time. Electrophoretic conditions were the same as those of Fig. 4. (1) 22°C; (2) 24°C; (3) 26°C; (4) 30°C.

constant during a HPCE run. According to Beer's law, at one detection point the ratio of concentration of MO to that of P is proportional to the ratio of integrated absorbance area of MO to that of P. The following equation can be derived:

$$\log \frac{(A_{MO})_0}{A_P} - \log \frac{A_{MO}}{A_P} = kt \quad (10)$$

It follows that $\log(A_{MO}/A_P)$ should have a linear relationship with time (t). The slope of the straight line should be equal to the rate constant, k. From the experimental results, it is found that $\log(A_{MO}/A_P)$ has a linear relationship with run time for all the electrophoretic runs under different conditions (Fig. 6). Therefore, the degradation of myoglobin should follow first order kinetics under certain electrophoretic conditions. Similar results have been reported by Rush *et al.* [26].

The influence of temperature on myoglobin degradation was also studied. The rate constant increases with temperature (Fig. 7). Another result shows that the rate constant does not change obviously in a certain range of electric field strengths (150–200 V/cm) (Fig. 8). This result illustrates the reliability of the method for studying the kinetics of conversion from Fe^{3+} to Fe^{2+} in MO by electric field changes [26].

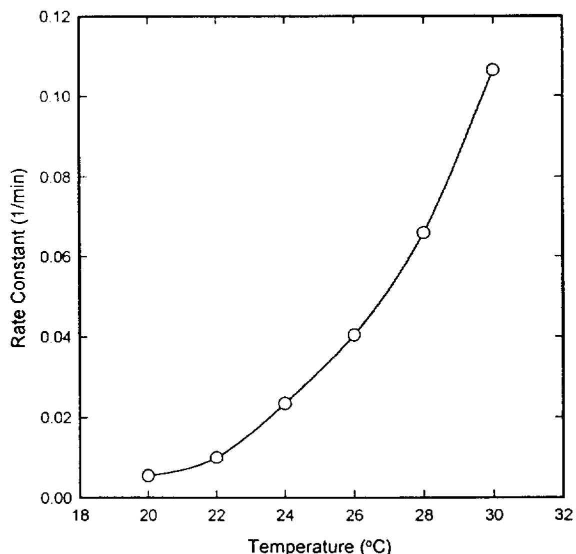


Fig. 7. Effect of temperature on myoglobin degradation rate constant.

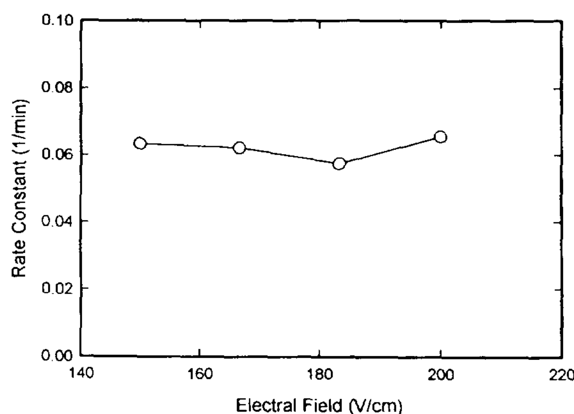


Fig. 8. Rate constant of myoglobin degradation obtained from different electric field strengths.

4. Conclusions

The results show that utilizing multi-point detection in HPCE for on-line kinetic studies is feasible. The major advantage of this method is its simplicity. It can be used to monitor the enzymatic reactions such as on-line enzyme activity studies. Kinetics of degradation of heme protein, myoglobin, has been studied by this method. Another advantage of this method is that very small amounts of agent are needed. It represents a low-cost alternative for some biochemical studies in which expensive agents are needed. It represents a low-cost alternative for some biochemical studies in which expensive agents are needed. This multi-point detection method opens a new application area in HPCE.

Acknowledgements

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References

[1] J.W. Jorgenson and K.D. Lukacs, *Anal., Chem.*, 53 (1981) 1298–1302.
[2] J.W. Jorgenson and K.D. Lukacs, *Science (Washington, D.C.)*, 222 (1983) 266–272.
[3] B.L. Karger, A.S. Cohen and A. Guttman, *J. Chromatogr.*, 492 (1989) 585–614.
[4] W. Kuhr, *Anal. Chem.*, 62 (1990) 403*R*–414*R*.
[5] W. Kuhr and C. A. Monnig, *Anal. Chem.*, 64 (1992) 398*R*–407*R*.
[6] J.W. Jorgenson and K.D. Lukacs, *J. Chromatogr.*, 218 (1981) 209–216.
[7] W.R. Jones and P. Jandik, *J. Chromatogr.*, 546 (1991) 445–458.
[8] R.M. McCormick, *Anal. Chem.*, 60 (1988) 2322–2328.
[9] P.D. Grossman, K.J. Wilson, G. Petrie and H.H. Lauer, *Anal. Biochem.*, 173 (1988) 265–270.
[10] S. Hoffstetter-Kuhn, A. Paulus, E. Gassmann and H.M. Widmer, *Anal. Chem.*, 63 (1991) 1541–1547.
[11] A.S. Cohen and B.L. Karger, *J. Chromatogr.*, 397 (1987) 409–417.
[12] J.K. Towns and F.E. Regnier, *Anal. Chem.*, 63 (1991) 1126–1132.
[13] A. Guttman, A.S. Cohen, D.N. Heiger and B.L. Karger, *Anal. Chem.*, 62 (1990) 137–141.
[14] H. Drossman, J.A. Luckey, A.J. Kostichka, J.D'Cunha and L.M. Smith, *Anal. Chem.*, 62 (1990) 900–903.
[15] E. Gassmann, J.E. Kuo and R.N. Zare, *Science (Washington, D.C.)*, 230 (1985) 813–814.
[16] S. Fanali, *J. Chromatogr.*, 474 (1989) 441–446.
[17] A. Guttman, A. Paulus, A.S. Cohen, N. Grinberg and B.L. Karger, *J. Chromatogr.*, 448 (1988) 41–53.
[18] K. Otsuka and S. Terabe, *J. Chromatogr.*, 515 (1990) 221–226.
[19] Y.-H. Chu, L.Z. Avila, H.A. Biebuyck and G.M. Whitesides, *J. Med. Chem.*, 359 (1992) 2915–2917.
[20] Y.-H. Chu and G.M. Whitesides, *J. Org. Chem.*, 57 (1992) 3524–3525.
[21] G.E. Barker, P. Russo and R.A. Hartwick, *Anal. Chem.*, 64 (1992) 3024–3028.
[22] W. Nashabeh and Z. El Rassi, *J. Chromatogr.*, 596 (1992) 251–264.
[23] J. Bao and F.E. Regnier, *J. Chromatogr.*, 608 (1992) 217–224.
[24] J.P. Landers, M.D. Schuchard, M. Subramaniam, T.P. Sismelich and T.C. Spelsberg, *J. Chromatogr.*, 603 (1992) 247–257.
[25] R.J. Nelson, A. Paulus, A.S. Cohen, A. Guttman and B.L. Karger, *J. Chromatogr.*, 480 (1989) 111–127,
[26] R.S. Rush, A.S. Cohen and B.L. Karger, *Anal. Chem.*, 63 (1991) 1346–1350.
[27] T. Srichaiyo and S. Hjertén, *J. Chromatogr.*, 604 (1992) 85–89.
[28] P. Sun and R.A. Hartwick, *J. Liq. Chromatogr.*, 17 (1994) 1861–1875.
[29] H. Mollering and H.U. Bergmeyer, in H.U. Bergmeyer (Editor), *Methods of Enzymatic Analysis*, Academic Press, New York, 1965, pp. 491–494.
[30] D. Voet and J.G. Viet, *Biochemistry*, John Wiley & Sons, New York, 1990, pp. 748–760.
[31] W.J. Moore, *Physical Chemistry*, Prentice-Hall, Englewood Cliffs, NJ, 4th ed., 1972, p. 324.