

## Determination of the diol content of chromatographic supports by capillary electrophoresis

Anuja Chattopadhyay, David S. Hage\*

*Department of Chemistry, University of Nebraska, Lincoln, NE 68588-0304, USA*

Received 24 July 1996; revised 20 August 1996; accepted 22 August 1996

---

### Abstract

A capillary electrophoresis (CE) method was developed for determining the diol content of supports used in high-performance affinity or size exclusion chromatography. This method involved oxidizing the diol-bonded support with periodate, followed by the use of CE to separate and quantitate the iodate produced by this reaction. Both the oxidation and separation conditions were considered in optimizing this assay. The final method was performed by reacting a known amount of support with a 20-fold excess of periodate in pH 4.0, 0.5 M acetate buffer, with pyromellitic acid being used as an internal standard. After allowing 5–10 min for oxidation, the mixture was filtered and the filtrate was injected onto a 57 cm×50 μm I.D. fused-silica capillary operated at 25 kV and containing pH 4.0, 0.5 M acetate as the running buffer. The total separation time was 5 min per run and gave a detection limit of 0.1 mM iodate (or 0.1 μmol diol groups) for a 6-nl injection of a 1-ml reaction mixture. By varying the amount the support that was assayed, this method could be used with either porous or non-porous supports. This technique showed good correlation with an iodometric titration but required much less sample and time to perform.

*Keywords:* Stationary phases, LC; Diols

---

### 1. Introduction

Diol- or glycol-bonded supports are commonly used in HPLC methods for the separation of biomolecules by size exclusion or affinity chromatography [1–3]. Fig. 1 shows the basic structure for this type of support. In these materials, the support backbone (e.g., silica or a perfusion-based material) provides mechanical stability and mass transfer properties that are suitable for an HPLC system, while the diol groups create a hydrophilic surface that has low non-specific binding for most components in biological samples [1]. The diol groups

also provide a convenient means for attaching affinity ligands to these supports since the diols can be readily derivatized or converted into activated forms that can react with various side chains on these ligands (e.g., primary amines on a protein or peptide) [2,3].

Due to the importance of the hydrophilic coating on diol-bonded supports, a method for determining the diol content of these materials is needed in order to characterize and monitor the production of such matrices. In the past, this has been done by treating a diol-bonded support with periodate, followed by an iodometric titration of the resulting products [4,5]. In this approach, the periodate is first reacted with the diol groups under acidic conditions to form iodate,

---

\*Corresponding author.

formaldehyde, water and an immobilized aldehyde residue (see Fig. 1). The reaction mixture is then combined with an excess of iodide, which reacts with both periodate and iodate to form iodine but in different stoichiometric ratios. The amount of iodine produced by the sample mixture is then determined by titration with a standard thiosulfate solution. In addition, a blank titration is performed using the same reagents but in the absence of the diol-bonded support (i.e., a situation in which only periodate is present for the conversion of iodide to iodine) [4,5]. By comparing the results of the blank and sample titrations, it is possible to determine how much periodate was consumed during the oxidation of the support and, thus, the corresponding diol content of this matrix.

The iodometric titration method is inexpensive to perform but does have a number of limitations. For example, it is labor intensive and requires a fair amount time and skill to perform. The fact that this method is based on a difference titration is another disadvantage since it means that the assay has a limited dynamic range and that reagent concentrations or sample size must be chosen carefully to provide results that fall within this range. In addition, this approach requires a relatively large amount of sample (i.e., 50–150 mg for most HPLC-grade silica). Some of these problems, including the manual nature of the assay and its large sample size requirements, can be overcome by using flow-injection analysis (FIA) for endpoint detection [6]. However, this latter approach is still fairly tedious to perform and still involves a comparison between the results for samples and a reagent blank [6], thus retaining the problems associated with the appropriate selection of sample size and reagent concentrations.

This study examines the use of capillary electrophoresis (CE) as an alternative approach for measuring diol groups on chromatographic supports, using

diol-bonded silica as a model sample. In this method the diol-bonded support will again be oxidized with periodate, as shown in Fig. 1, but the resulting iodate will now be isolated and quantitated on a CE system. This is similar in some respects to an approach that has recently been used to characterize the oxidation of carbohydrates by periodate [7]. This present study will first examine the optimization of various reaction and separation conditions for the use of this technique with chromatographic supports. The performance of the final method will then be evaluated in terms of its precision, response, speed and accuracy versus a traditional iodometric titration.

## 2. Experimental

### 2.1. Reagents

Periodate (periodic acid reagent), iodate (iodic acid) and pyromellitic acid (1,2,4,5-benzene tetracarboxylic acid) were obtained from Sigma (St. Louis, MO, USA) and were used without further purification. The 7  $\mu\text{m}$  Nucleosil Si-300 (300 Å pore size), Nucleosil Si-1000 (1000 Å pore size) and Nucleosil Si-4000 (4000 Å pore size) silica supports were obtained from Alltech (Deerfield, IL, USA). The 3–10  $\mu\text{m}$  glass beads were from Polysciences (Warrington, PA, USA). All other chemicals were of the purest grades available. All solutions were prepared using water from a Nanopure water system (Barnstead, Dubuque, IA, USA).

### 2.2. Apparatus

All CE experiments were performed using a Model 3850 capillary electrophoresis system from ISCO (Lincoln, NE, USA). The capillaries were 57 cm (32 cm effective length)  $\times$  50  $\mu\text{m}$  I.D. uncoated

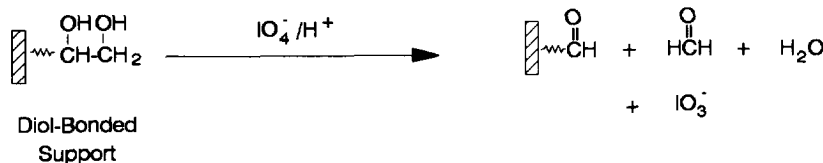


Fig. 1. Oxidation of a diol-bonded support by periodate ( $\text{IO}_4^-$ ) to form iodate ( $\text{IO}_3^-$ ) and an aldehyde-derivatized support.

fused-silica columns from Polymicro Technologies (Phoenix, AZ, USA). Sample injection was performed using the vacuum injection mode of the Model 3850 system (i.e., a 4-s injection time, or approximately 6 nl of sample). The migration of iodate, periodate and pyromellitic acid was monitored at 222 nm, according to Ref. [8]. In the final optimized procedure, the applied voltage was 25 kV (438 V/cm). The typical current obtained at this voltage with a pH 4.0, 0.5 M sodium acetate running buffer was 150  $\mu$ A.

### 2.3. Methods

The diol-bonded Nucleosil silica and diol-bonded glass beads were prepared according to previously reported procedures [6]. These samples were dried under vacuum and a known portion was weighed out for analysis either by CE or an iodometric titration [4,5]. The final CE method developed in this work involved placing an amount of the sample support that contained roughly 1  $\mu$ mol of diol groups into 1 ml of a reaction mixture containing 20 mM periodic acid (i.e., a 20-fold mole excess vs. the diol groups) and 0.1 mM pyromellitic acid (i.e., the internal standard) in pH 4.0, 0.5 M sodium acetate buffer. This solution was vortex mixed for 2 min and shaken vigorously for an additional 5 min at room temperature (i.e., a minimum total oxidation time of 7 min). The mixture was then filtered through a 0.45- $\mu$ m nylon filter from Gelman Science (Ann Arbor, MI, USA) to stop the reaction and the filtrate was injected onto the CE system for analysis. A blank sample of the initial periodic acid and pyromellitic acid reaction solution was also injected onto the CE system in order to correct for any trace amounts of iodate that were present in this solution. The areas of both the iodate and pyromellitic acid peaks were determined for each injection and the ratio of these areas was used to determine the amount of iodate that was present in samples.

Calibration standards were prepared by diluting a 50 mM stock solution of iodic acid in the running buffer to yield concentrations of 0.1–40 mM. A fixed concentration of pyromellitic acid (0.1 mM) was also present in each calibration standard. Every unknown or standard analyzed by the CE system was injected in at least triplicate, with the first run being discarded

in order to avoid potential carryover between samples. Before use, each CE capillary was conditioned by rinsing with a 0.1 M sodium hydroxide solution for 30 min. The capillary was then washed with filtered Nanopure water and pH 4.0, 0.5 M sodium acetate buffer. The solutions used on the CE system were sonicated under vacuum for 20 min and filtered using PVDF Acrodisc 0.45- $\mu$ m filters from Gelman Science before use. All studies were performed at room temperature.

## 3. Results and discussion

### 3.1. Optimization of separation conditions

Acetate buffer adjusted to the pH range of 4–5 is commonly used in the periodate oxidation of organic compounds [8], so this same buffer system was investigated for use in this work as a CE running buffer. In this case, acidic pH values were desirable since iodate and periodate were to be separated in a reversed-polarity mode, making it necessary to minimize electroosmotic flow in order to decrease the overall analysis time. For example, in going from pH 4.0 to 5.0 it was noted that there was a two- to three-fold increase in the migration times for periodate and iodate, mainly due to the change in electroosmotic flow. Because of this effect, pH 4.0 acetate buffer was used in all further work.

Along with pH, running buffer concentration was also considered in optimizing the CE method. Since electroosmotic flow decreases with increasing running buffer concentration [9], it was desirable to use a high ionic strength buffer in order to achieve a fast separation. However, the degree to which this concentration could be raised was limited by the increase in current and joule heating that also occurred at increased ionic strengths [10], particularly when high voltages were used (see Fig. 2). As a compromise between assay speed and peak broadening because of joule heating, a buffer of concentration of 0.5 M was chosen [7]. Under these conditions baseline resolution for all species of interest was obtained in a reasonable amount of time, as shown in Fig. 3.

Another factor considered was the choice of internal standard for this assay. In this study, an

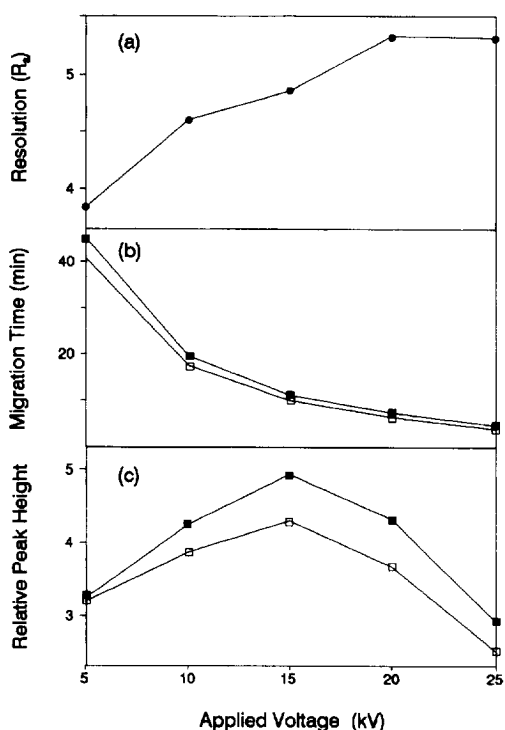


Fig. 2. Effect of applied voltage on the (a) resolution, (b) migration times and (c) peak heights for iodate (■) and pyromellitic acid (□) injected onto a CE system in pH 4.0, 0.5 M acetate buffer. The injected samples contained 2 mM iodate and 0.1 mM pyromellitic acid. All other conditions are given in Section 2.3.

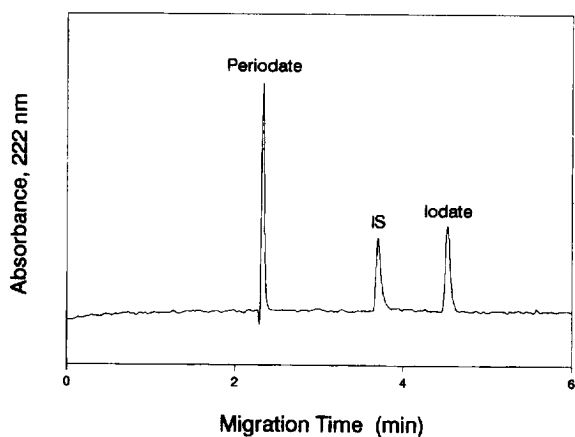


Fig. 3. Electropherogram obtained for the separation of a sample containing 0.5 mM periodate, 2 mM iodate and 0.1 mM of the internal standard (I.S., pyromellitic acid) in pH 4.0, 0.5 M acetate buffer. The operating conditions were the same as described in Section 2.2 and 2.3 for the final optimized method.

internal standard was needed that was resistant to oxidation by periodate, that had a migration time close to but distinct from that of either periodate or iodate on the CE system, and that could be monitored at the same wavelength used for detecting iodate and periodate (222 nm). Pyromellitic acid was found to be ideal for this purpose. This compound not only migrated between periodate and iodate on the CE system (see Fig. 3), but it was readily detectable and highly stable in the presence of periodate and pH 4.0 acetate buffer, thus allowing it to be added directly to samples during the oxidation step.

The final variable examined in optimizing the CE separation was the voltage applied to the capillary. As expected, there was a general increase in resolution and a decrease in analysis times as the applied voltage was increased (see Fig. 2a,b). However, an applied voltage of more than 25 kV resulted in currents that approached 200  $\mu$ A, which led to column heating and a loss of efficiency. This was particularly noticeable when examining the peak heights measured at each applied voltage (see Fig. 2c). In this case a maximum peak height was observed at an applied voltage of 15 kV, with a decrease of 12% when increasing the voltage to 20 kV or a decrease of 41% when going to 25 kV. Although the loss of peak height was noticeable at 25 kV, it was felt that the signal at this voltage was still acceptable for use in this work. Thus, 25 kV was used in all later studies in order to provide fast analyses on the CE system.

Fig. 3 shows a typical electropherogram that was obtained for this system. Under the final optimized conditions used in this study, it was possible to separate periodate, iodate and the internal standard in 5 min with baseline resolution (i.e.,  $R_s \geq 5.2$  between each set of neighboring peaks). No other peaks were detected in any of the samples analyzed during this study. The average electrophoretic mobilities for periodate, the internal standard and iodate were approximately 29, 20 and 17  $\text{cm}^2/\text{kV min}$ , respectively, with variations of  $\pm 2.6$ –4.9% (1 R.S.D.). The mobility ratios for the periodate and iodate peaks versus the internal standard (as calculated according to Ref. [11]) were 1.54 and 0.84, with precisions of  $\pm 2.0$ –2.2%.

### 3.2. Optimization of oxidation conditions

An excess of periodate was required to ensure both the rapid and quantitative oxidation of diol groups on support samples. For the oxidative treatment of most organic compounds by periodate, it is known that an increase in periodate concentration will lead to an increase in the rate of oxidation; however, too high of a periodate concentration could be detrimental since this may cause over-oxidation or other undesirable side reactions [8]. In order to determine what levels of periodate were needed, a study was carried out in which a fixed amount of diol-bonded silica was combined with various amounts of periodate (see Fig. 4a). It was found that at least a 10-fold excess of periodate versus diol groups was needed to give a maximum amount of

iodate production when using a 7-min oxidation time and a 17-mg sample of diol-bonded Nucleosil Si-1000 (i.e., approximately 1  $\mu\text{mol}$  of diol groups). A 10- to 40-fold excess of periodate gave no further increase in iodate levels, indicating that no additional side reactions that produced or consumed iodate were occurring under these conditions. All of the periodate concentrations in Fig. 4a were low enough to allow baseline resolution to be obtained between the peaks for periodate, iodate and the internal standard on the CE system. Based on these results, a periodate concentration of 20 mM was selected for use in all later experiments since this would provide an excess of 15- to 25-fold when working with most common silica supports used in high-performance affinity or size exclusion chromatography.

The time required for oxidizing diol-bonded silica was also examined (see Fig. 4b). For a 17-mg sample of diol-bonded Nucleosil Si-1000 that was reacted with a 20-fold excess of periodate, it was found that a reaction time of at least 7 min was needed to give a consistent, high level of iodate production. Similar results were obtained with other types of diol-bonded supports. From these results, 7 min was chosen as the minimum oxidation time for use in all further studies. The fact that no significant increase (or decrease) in iodate production occurred for reaction times over 7 min again suggests that no appreciable side reactions were occurring under these conditions. This agrees with the results of previous studies that have examined the reaction stoichiometry between diol-bonded silica and periodate [6].

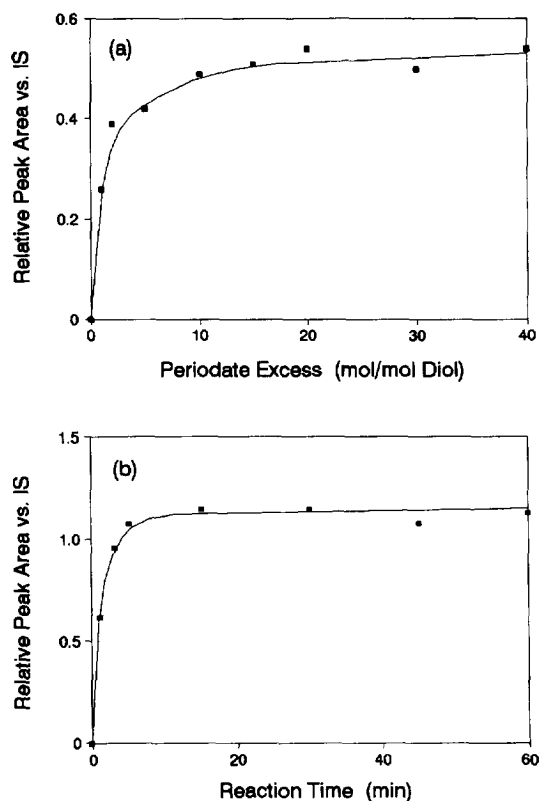


Fig. 4. Degree of diol-silica oxidation as a function of (a) mole excess of periodate versus diol groups and (b) the amount of time allowed for oxidation. The conditions are given in Section 3.2.

### 3.3. Assay performance

A typical calibration curve obtained for iodate on the CE system is shown in Fig. 5, where each point represents the average of three runs. The linear range of this curve (i.e., the region giving a response within  $\pm 5\%$  of the best-fit line) extended from 0 to 20 mM for a 6-nl sample injection. The best-fit slope and intercept over this range were 0.596 ( $\pm 0.006$ ) and  $-0.011$  ( $\pm 0.009$ ), respectively, with the numbers in parentheses representing a range of  $\pm 1$  S.D. The correlation coefficient for the best-fit line was 0.9997 ( $n=10$ ). The dynamic range extended to at

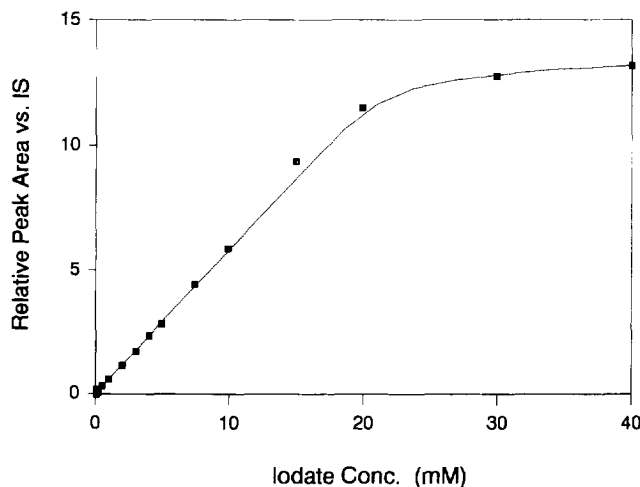


Fig. 5. Calibration curve for iodate on the CE system. The parameters for the best-fit line are given in Section 3.3.

least 40 mM and the lower limit of detection at a signal-to-noise ratio of three was 0.1 mM iodate. Given that one mole of iodate should be produced for each mole of oxidized diol groups [4–6], a detection limit of 0.1 mM for iodate would correspond to 0.1  $\mu\text{mol}$  of diol groups in a 1-ml reaction mixture.

The within-day precision of the CE method was first measured by making 20 sequential injections of standards containing either 1 or 2 mM iodate. The within-day precisions for these samples were  $\pm 1.2$ – $2.7\%$  (1 R.S.D.). A similar but smaller scale study was performed with duplicate injections of 0.1 to 5 mM iodate standards and gave within-day precision estimates of  $\pm 0.4$ – $6.3\%$ . The day-to-day precision of the assay was determined by periodically injecting 1 and 2 mM iodate samples over five consecutive days. This gave day-to-day precisions of  $\pm 2.4$ – $3.6\%$  for these samples.

The accuracy of the CE method was evaluated by comparing its results for a series of diol-bonded silica supports with those measured by a reference iodometric titration [4,5]. The results are summarized in Table 1. In comparing these two assays, it was found that the values obtained by CE varied by only 0.2–10.4% from those found by the titrations and that none of these differences were significant compared to the precision of the results (e.g., all CE and titration results overlapped at the 95% confidence level). The total analysis time per sample in the CE

method was relatively short, taking about 30 min for one complete cycle of sample oxidation and analysis. In contrast, the iodometric titration had a total analysis time of 2–3 h per cycle. Also, the observed precision for the titration method was consistently lower than that obtained with the CE assay for all samples analyzed in this study (see Table 1).

The good precision and relatively low limits of detection of the CE method made it possible to use this technique with even small quantities of porous supports. For example, the data shown in Table 1 were obtained by using a 5-mg sample for the diol-bonded Nucleosil Si-300, 17–20 mg for Nucleosil Si-1000 and 37 mg for Nucleosil Si-4000. On the other hand, analysis of the same supports by an

Table 1

Diol content of various supports, as measured by CE and an iodometric titration

Type of support	Diol content ( $\mu\text{mol/g}$ support) <sup>a</sup>	
	CE result	Titration result
Nucleosil Si-300	192 ( $\pm 2$ )	172 ( $\pm 16$ )
Nucleosil Si-1000		
Batch No. 1	57.3 ( $\pm 0.6$ )	53 ( $\pm 3$ )
Batch No. 2	54.9 ( $\pm 0.4$ )	55 ( $\pm 8$ )
Nucleosil Si-4000	24.2 ( $\pm 0.1$ )	23 ( $\pm 3$ )
Glass beads, 3–10 $\mu\text{m}$	0.41 ( $\pm 0.02$ )	Not done

<sup>a</sup> The numbers in parentheses represent a range of  $\pm 2$  S.D. of the mean for duplicate or triplicate analyses.

iodometric titration required 20 mg for diol-bonded Nucleosil Si-300 silica, 60–80 mg for Nucleosil Si-1000 and 120 mg for Nucleosil Si-4000. The low detection limits of the CE procedure also allowed the use of this method with materials that could not be easily assayed by titrations, such as diol-bonded glass beads or other non-porous materials. As an example of this, Table 1 includes results obtained when using the CE method to assay a 500-mg sample of 3–10  $\mu\text{m}$  diameter diol-bonded glass beads. In order to analyze this same support by an iodometric titration, it was estimated that 2.0–2.5 g of sample would have been required.

#### 4. Conclusion

The use of CE was examined for determining the diol content of chromatographic supports, using diol-bonded silica as a model. In this method, the diol-bonded support was first oxidized with periodate, followed by the isolation and quantitation of the resulting iodate by a CE system. Parameters considered in the development of this assay included pH, applied voltage, the time required for the periodate oxidation and the amount of periodate needed for quantitative oxidation of the diol groups. The final optimized assay gave baseline separation between iodate and pyromellitic acid (the internal standard) in a separation time of 5 min. The method required only a simple oxidation step for sample preparation and could detect as little as 1  $\mu\text{mol}$  of diol groups per sample. This method was not only found to be a

rapid and effective alternative to iodometric titrations for the determination of diol groups on porous supports, but it was also possible to use this approach with lower surface area materials, such as diol-bonded glass beads.

#### Acknowledgments

This work was supported by the National Institutes of Health under grant No. R29 GM44931.

#### References

- [1] F.E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
- [2] R.R. Walters, *Anal. Chem.*, 57 (1985) 1099A.
- [3] P.-O. Larsson, *Methods Enzymol.*, 104 (1984) 212.
- [4] S. Siggia and J.G. Hanna, *Quantitative Organic Analysis*, Wiley, New York, 1979, pp. 42–43.
- [5] A.A. Woolf, *Anal. Chem.*, 54 (1982) 2134.
- [6] P.F. Ruhn, S. Garver and D.S. Hage, *J. Chromatogr. A*, 669 (1994) 9.
- [7] S. Honda, K. Suzuki and K. Kakehi, *Anal. Biochem.*, 177 (1989) 62.
- [8] G. Dryhurst, *Periodate Oxidation of Diol and Other Functional Groups: Analytical and Structural Application*, Pergamon Press, New York, 1966, Chps. 3, 4 and 6.
- [9] J.P. Landers (Editor), *Handbook of Capillary Electrophoresis*, CRC Press, Boca Raton, FL, 1994, pp. 21–35.
- [10] E. Grushka, R.M. McCormick and J.J. Kirkland, *Anal. Chem.*, 61 (1989) 241.
- [11] J. Yang, S. Bose and D.S. Hage, *J. Chromatogr. A*, 735 (1996) 209.