ELSEVIER

Journal of Chromatography A, 919 (2001) 51–58

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

On-line coupling of sol–gel-generated immunoaffinity columns with high-performance liquid chromatography

M. Cichna[a,*], P. Markl[a], D. Knopp[b], R. Niessner[b]

[a]*Institute of Analytical Chemistry, University of Vienna, Währinger Strasse 38, A-1090 Vienna, Austria*
[b]*Institute of Hydrochemistry, Technical University Munich, Marchioninistrasse 17, D-81377 Munich, Germany*

Received 9 August 2000; received in revised form 19 March 2001; accepted 29 March 2001

Abstract

The paper demonstrates the possibility to use sol–gel-generated immunoaffinity columns as selective sample preparation step in on-line combination with HPLC. In the past sol–gel-generated immunoaffinity columns have only been included in off-line sample preparation schemes. Compared with conventional RP-materials on-line coupling of sol–gel-generated silica matrices with a pore structure designed to retain antibodies poses additional problems caused by their lower pressure tolerance and by the necessity to match the mobile phases not only to take into account the chromatographic properties but also the conformational stability of the antibodies. These problems have been overcome by an on-line system which can be regarded as a prototype for similar systems which exploit the selectivity of sol–gel immunoaffinity columns. The system consists of a sol–gel-generated immunoaffinity column coupled to an RP enrichment column and an analytical column. The practicality of such systems is demonstrated using the example of anti-pyrene immunoaffinity columns applied for the determination of pyrene in aqueous solutions.

Keywords: Sol–gel; Immunosorbents; Water analysis; Sample preparation; HPLC, on-line coupling; Pyrene

1. Introduction

In order to prepare samples for HPLC analysis it is often necessary to remove interfering matrix components and/or enrich the analytes of interest. Solid-phase extraction on alkyl-bonded silicas is most frequently used for these purposes (see [1] for a recently published review). For many matrices, however, the selectivity of this method is insufficient to remove all interfering matrix compounds.

Immunoaffinity chromatography which takes advantage of the highly selective interaction between antigens and antibodies is one of the most selective methods. In this technique the analytes are selectively retained in columns packed with a support material containing immobilised antibodies which were raised against the analyte. (The basic principles of affinity chromatography and its applications in clinical [2] and environmental analysis [3] have been reviewed recently).

Until now most of the packing materials for immunoaffinity columns have been prepared by covalently binding the antibodies to a solid support surface. However, this immobilisation technique

*Corresponding author. Tel.: +43-1-4277-52354; fax: +43-1-4277-9523.

E-mail address: margit.cichna@univie.ac.at (M. Cichna).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00798-1

frequently suffers from a loss of functional antibodies caused by the immobilisation procedure. This can largely be avoided by the sol–gel method, which is a mild immobilisation technique for synthesising immunoaffinity column packing materials by encapsulating antibodies in the pores of a silica glass matrix.

Immunoaffinity columns can be applied for sample preparation prior to HPLC analysis by operating them either in the off-line or on-line mode.

Off-line methods can, however, rarely achieve the detection limits needed without additional time-consuming solvent evaporation and/or solvent adjustment steps which also cause additional analyte losses. The on-line combination of three columns — an immunoaffinity column followed by an RP enrichment column and the analytical column — makes it possible to achieve selective enrichment in a minimum of time.

In the past on-line-coupled immunoaffinity chromatography–HPLC systems have frequently been applied in residue analysis [4–8] but it was only in the last years that this technique was also used in environmental chemistry [9–15]. None of these columns used a sol–gel-synthesised packing material. Although the sol–gel method offers many advantages compared to other immobilisation techniques [16–19] sol–gel-generated immunoaffinity columns have so far only been used for off-line sample preparation [18–24].

Using the determination of pyrene in aqueous samples as an example the following paper demonstrates the potential of immunoaffinity separations using columns packed with sol–gel-generated immunosorbents for on-line coupling with HPLC.

2. Experimental

2.1. Reagents and solutions

Acetonitrile hypergrade was purchased from E. Merck (Darmstadt, Germany). Tetramethoxysilane (TMOS) was obtained from FLUKA (Buchs, Switzerland). Pyrene puriss. was purchased from Supelco (Gland, Switzerland).

Pyrene stock solutions were prepared by dissolving 25 mg of pyrene in 100 ml of acetonitrile and stored at 4°C until used. Working solutions were prepared in the range from 0.004 to 0.12 ng/ml by diluting the stock solution to acetonitrile–water (10:90, v/v).

Phosphate-buffered saline (PBS) was prepared by adding 12.46 g Na_2HPO_4 $2H_2O$, 1.56 g NaH_2PO_4 $2H_2O$ and 8.5 g NaCl to 1 l doubly distilled water and adjusting the pH to 7.6.

The polyclonal anti-pyrene antiserum was produced by immunising a rabbit according to [25].

2.2. Apparatus

The set-up of the HPLC system used is schematically shown in Fig. 1. The HPLC system consisted of a Model L 6200 Merck Hitachi gradient pump (E. Merck, Darmstadt, Germany) for delivering the mobile phase to the analytical column, an isocratic pump Model System Gold (Beckman, San Ramon, CA, USA) for sample handling, a six-port injection valve Model 7161 (Rheodyne, Cotati, USA) equipped with a 5.5 ml stainless steel injection loop,

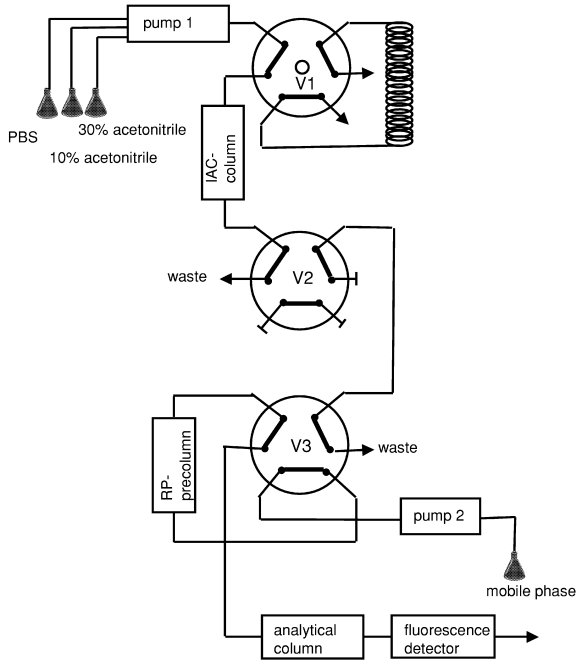

Fig. 1. Set-up of the analysis system. Valves V1–V3 are all shown in position A (for the schedule of the analytical procedure refer to Table 1).

Table 1
Schedule of the analytical procedure

Step	Event	Flow-rate (ml/min)	Valve position[a]		
			Valve 1	Valve 2	Valve 3
1	Equilibration of the IAC-column with 20 ml of acetonitrile–water (10:90, v/v)	1.0	B	A	A
2	Application of the sample and flushing of the IAC column with 16 ml of acetonitrile–water (10:90, v/v)	0.5	B	A	A
3	Elution of the analyte from the IAC column with 15 ml of acetonitrile–water (30:70, v/v) onto the precolumn	1.0	A	B	B
4	Elution of the analyte from the precolumn onto the analytical column	0.4	A	A	A
5	Regeneration of the IAC column with 20 ml of PBS buffer	1.0	B	A	A

[a] For each valve, position A corresponds to the position shown in Fig. 1.

two high-pressure six-port switching valves Model 7000 (Rheodyne), a Merck Hitachi fluorescence detector Model F 1050 (E. Merck) set at 270/380 nm and a HP integrator Model HP 3396 A (Hewlett-Packard, Little Falls, DE, USA).

Three different columns were used in the column switching system. The immunoaffinity column (IAC-column) was a self-packed 25×4 mm I.D. column prepared by immobilising the IgG fraction of a polyclonal anti-pyrene antiserum by the sol–gel method. Isolation of the IgG fraction from rabbit antiserum was carried out according to McKinney et al. [26]. The procedure of encapsulating the immunoglobulins into the porous sol–gel glass matrix was described earlier [18]. The glass monolith containing the entrapped anti-pyrene antibodies was ground in a mortar. In order to stop the condensation reactions and to let the ground glass swell before packing it into a column it was stored in about 10 ml of PBS buffer at 4°C overnight. The next day the PBS buffer was removed from the swollen glass by filtering it through a Gooch funnel. The immunosorbent was packed in a stainless steel column and flushed with PBS at a flow-rate of 0.1 ml/min for 10 min followed by a slow stepwise increase of the flow-rate up to 1 ml/min in 0.1 ml/min steps applied every third minute.

The analyte eluted from the immunoaffinity column was concentrated on a Merck 30×4 mm I.D. LiChrosorb RP-8 (7 μm) precolumn. Analytical separations were achieved on a HP 250×4 mm I.D. LiChrospher PAH-column. Mobile phase A consisted of acetonitrile–water (40:60, v/v), mobile phase B of 100% acetonitrile. The gradient program used is summarised in Table 2.

2.3. Analytical procedure

Table 1 gives an outline of the analytical procedure. It starts with preconditioning of the immunoaffinity column with acetonitrile–water (10:90, v/v) followed by the introduction of a sample aliquot via the injection valve. The immunoaffinity column is then flushed with acetonitrile–water (10:90, v/v) to remove non-retained and/or non-specifically retained sample components. After on-line switching the effluent to the head of the RP-8 precolumn specifically retained compounds are eluted from the immunoaffinity column with acetonitrile–water (30:70, v/v). With this mobile phase these compounds are strongly adsorbed and thereby concentrated in the head section of the precolumn. Column switching is then used to introduce a second pump which transfers the analyte to the analytical column by back-flushing. Remaining matrix components are separated in the analytical column by a segmented linear gradient. After switching the immunoaffinity column off-line it is regenerated by flushing with

Table 2
HPLC gradient program

Time (min)	Acetonitrile concentration (%, v/v)	Flow-rate (ml/min)
0–2	40	0.4
2–5	40–52	0.4
5–10	52	0.4
10	52	0.5
10–15	52	0.5
15–30	52–76	0.5
35–60	76–100	0.5
60–70	100–40 (reconditioning)	0.5

PBS buffer solution while the final separation is carried out on the analytical column.

All chromatographic steps were performed at ambient temperature. When not in use the immunoaffinity column was stored in PBS at room temperature.

2.4. Sampling and sample pretreatment

River samples were taken from the Danube at Klosterneuburg, a town near Vienna. Samples were filtered through a glass fibre filter GF55 (Schleicher & Schüll, Dassel, Germany) and diluted with acetonitrile to 10% acetonitrile in order to avoid nonspecific adsorption of pyrene on glass surfaces.

The concentration of pyrene in the river samples was calculated from the calibration curve obtained with pyrene calibration standards in the range from 0.004 to 0.12 ng/ml.

3. Results and discussion

3.1. Pressure stability of sol–gel immunosorbents

Pressure stability is a critical parameter for on-line coupling of sol–gel immunoaffinity columns to HPLC. Changes of the column bed during operation had not been a problem when operating the columns with the low pressure needed for off-line experiments [18–22]. However, when immunoaffinity columns become an integral part of an on-line system high pressure pumps must be used. Compression or differences in swelling when operating the columns with different mobile phases can result in changes of the bed volume or destroy the bed structure or — in the worst case — clogg the column.

The immunoadsorbent has been prepared by the method described [18]. In this method the ''aging'' of the silica gel is a condensation process liberating water and volatile methanol resulting in a weight loss which indicates the degree of condensation and thus the pore structure achieved. The silicate matrix encapsulating the anti-pyrene antibodies was manually ground in a mortar after stopping the aging process.

An attempt has, however, been made to modify the last step of this procedure to achieve a more uniform particle size by replacing manual grinding by the more reproducible grinding in a mechanical mortar Model MM 2000 (Retsch, Haan, Germany) and to collect particles of a defined particle size by sieving with a sieving machine Model AS 200 basic (Retsch).

However, dry grinding of the sol–gel glass with a mechanical mortar turned out to be impossible since the sol–gel glass was still too wet at the moment when further condensation of the silica matrix had to be stopped to guarantee the pore structure desired. Mechanical grinding of such material did not result in small particles but led to the formation of a sol–gel film on the inner surface of the agate beaker.

Sieving the manually ground sol–gel glass using a sieving machine appeared to be problematic too. Attempts to wet sieve the sol–gel glass yielded bulky particles whereas dry sieving led to a dramatic weight loss since shaking the glass during the sieving accelerated the aging process.

The mechanical stability of the column bed was investigated using the pure sol–gel silica matrix (not containing encapsulated antibodies). Pressure was

measured while repeating a cycle consisting of steps 1, 2, 3 and 5 of the analytical procedure of Table 1. The cycle was repeated 35 times. The maximum pressure acting on the sol–gel column built up in step 3 when it was operated on-line with the RP precolumn. At the beginning pressure was about 40 bar and stayed nearly constant during the first cycles. After 15 cycles the pressure began to increase — presumably by a restructuring of the bed caused by a change in the initial stationary state. After 20 cycles pressure achieved a maximum of 80 bar and stayed at the higher level with slight fluctuations. The initial low pressure value of about 40 bar could again be achieved when the sol–gel column was removed from the system and stored at room temperature for 2 weeks. Operating immunoaffinity columns as described never led to clogging of the columns.

Desorption of the immunoaffinity column is often carried out in the backflush mode. Making use of backflush desorption for our sol–gel columns yielded higher pressure values already after 8 cycles. Thus equilibration, sample loading and desorption from the immunoaffinity column in the same direction was preferred.

3.2. Selection of mobile phases and determination of sample application flow-rate

Our previous off-line experiments with sol–gel anti-pyrene immunoaffinity columns [19] have shown that the columns could be loaded with samples containing 10% acetonitrile and eluted with acetonitrile–water (40:60, v/v) yielding a quantitative recovery of pyrene without causing an irreversible damage of the antibodies.

In order to investigate the range of acetonitrile concentrations suitable for enriching the analyte on the RP-precolumn on-line coupled with the analytical column, 5.5 ml of a 0.04 ng/ml pyrene solution in acetonitrile–water (10:90, v/v) were directly injected into the RP-precolumn before flushing the precolumn with increasing volumes of the mobile phase containing 20, 30 or 40% (v/v) acetonitrile. After separation the recovery was calculated from a calibration curve constructed by injecting pyrene solutions directly onto the analytical column. With acetonitrile–water (40:60, v/v) quantitative retention of pyrene was only obtained with elution volumes

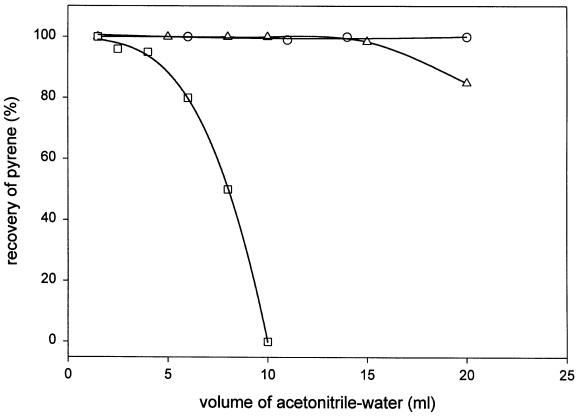

Fig. 2. Recovery of pyrene in the RP-precolumn as a function of various concentrations of acetonitrile in water. □, acetonitrile–water (40:60, v/v); △, acetonitrile–water (30:70, v/v); ○ acetonitrile–water (20:80, v/v). Sample: 5.5 ml acetonitrile–water (10:90, v/v) containing 0.04 ng/ml pyrene.

smaller than 4 ml, larger volumes led to a dramatic decrease in the recovery of pyrene due to breakthrough on the RP-precolumn (see Fig. 2). Flushing with up to 15 ml of acetonitrile–water (30:70, v/v) yielded quantitative recovery of pyrene, but a volume of 20 ml already caused analyte losses of 20%. Up to 20 ml no losses were observed for acetonitrile–water (20:80, v/v).

Another series of experiments was carried out in order to investigate if pyrene could be quantitatively eluted from the immunoaffinity column using up to 20 ml of acetonitrile–water (30:70, v/v) as eluent. After injecting 5.5 ml of a 0.04 ng/ml pyrene solution in acetonitrile–water (10:90, v/v) the immunoaffinity column was flushed with volumes from 10 to 20 ml of acetonitrile–water (30:70, v/v). After reconcentration in the RP-precolumn and separation in the analytical column the recovery was calculated. Elution volumes smaller than 15 ml did not elute pyrene quantitatively. Eluting with more than 18 ml reduced pyrene recovery due to a breakthrough of pyrene in the RP-precolumn. Using 15 ml of eluent results in both a quantitative elution of pyrene from the immunoaffinity column and quantitative retention of the analyte in the RP-precolumn.

Since antigen–antibody interactions proceed with rather slow reaction kinetics [27] experiments were carried out to determine the maximum sample application flow-rate compatible with quantitative analyte

recovery. The experiments showed that flow-rates up to 1.0 ml/min resulted in quantitative retention of pyrene, flow-rates of 1.5, 2.0 or 3.0 ml/min yielded 97, 91 and 73% recovery, respectively.

3.3. Immunoaffinity column characteristics

The selectivity of the anti-pyrene antiserum used and the extent of non-specific adsorption of pyrene and other PAHs on the sol–gel glass matrix were investigated in previous papers [19,20].

The capacity of the columns for pyrene was determined by measuring their breakthrough curves [19]. The affinity columns used in the present paper contained 0.33 g of sol–gel glass corresponding to 0.66 mg of the originally added immunoglobulins and had a breakthrough capacity of 25 ng of pyrene. Their capacity did not decrease although they were stored at room temperature. Column performance

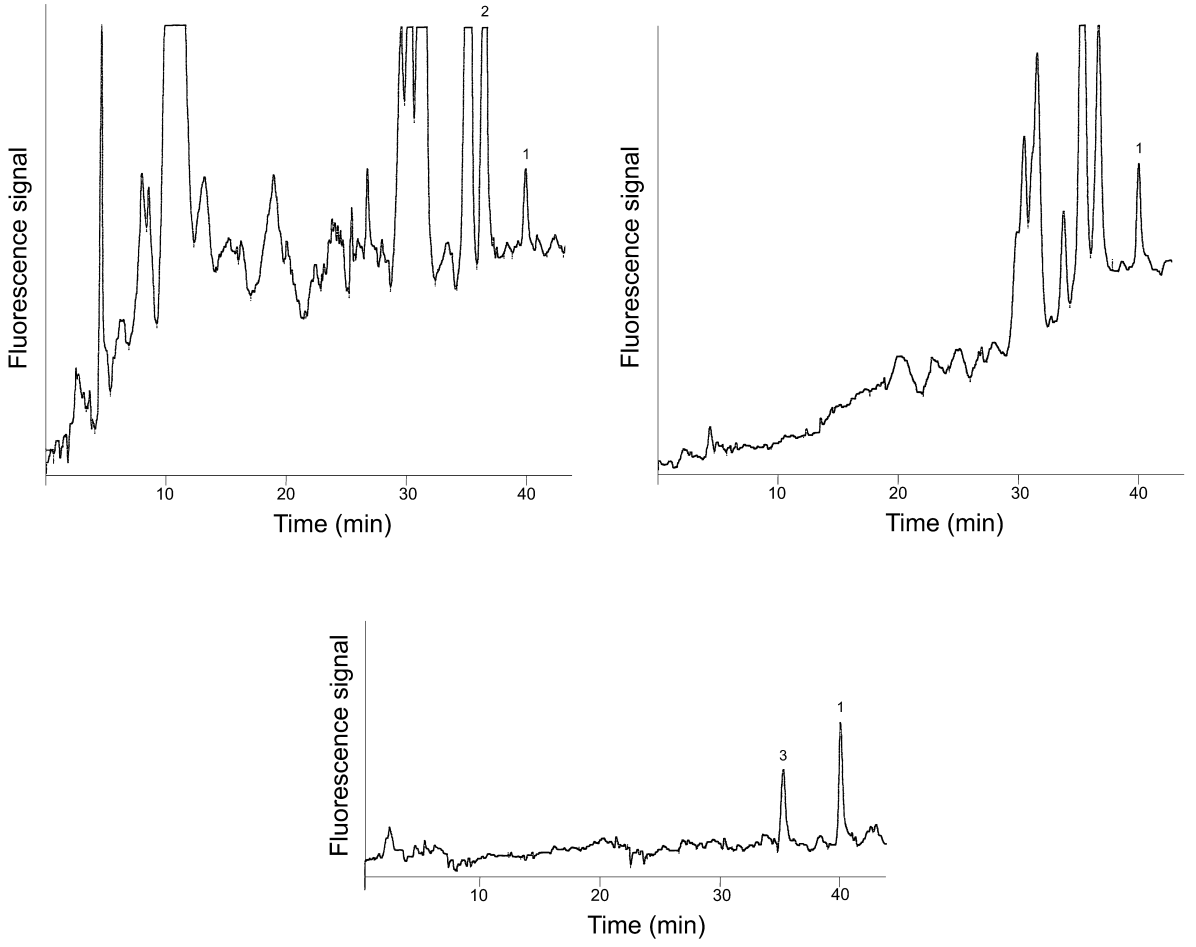

Fig. 3. Chromatograms of a Danube river sample diluted with acetonitrile to 10% (v/v) acetonitrile and a standard solution of pyrene (0.06 ng/ml in acetonitrile–water 10:90, v/v). (A) The Danube river sample was analysed without immunoaffinity column. (B) The same Danube river sample was analysed after adding the immunoaffinity column to the system. (C) A pyrene standard solution was analysed by including the immunoaffinity column. Peak 1, pyrene; peak 2, phenanthrene; peak 3, unidentified contaminant of the solvent. For further conditions, see the section on the HPLC procedure.

characteristics did not change while running 30 analysis cycles.

3.4. Analytical data

The calibration curve was calculated from the peak areas of five pyrene standard solutions in the range from 0.004 to 0.12 ng/ml. In this concentration range the curve showed good linearity: a slope of 80 828 829 (s=1 659 970), an intercept of 480 926 (s=112 133), a correlation coefficient of 0.99937 and a standard error of the mean of 156 397. The detection limit estimated from the standard deviation of the base line fluctuations observed was about 15 pg/ml (S/N=3). The average recovery of pyrene was 99% (n=7, s=3%).

3.5. Analysis of river samples

In order to demonstrate the practicality of sol–gel-generated immunoaffinity columns coupled on-line to an HPLC system and the additional selectivity thereby introduced, the present method was applied to determine pyrene in river water.

Fig. 3A shows the chromatogram of a river sample analysed without including the immunoaffinity column. Fig. 3B is a chromatogram of the same sample obtained after adding the immunoaffinity column to the system. It shows the increased selectivity provided by the immunoaffinity column which removes matrix components especially at the beginning of the chromatogram almost completely and thus illustrates the practicality and potential of sol–gel-generated immunoaffinity columns. The example is, however, not ideal since the selectivity of the immunoaffinity column cannot improve the selectivity provided by the RP column and the fluorescence detection for the determination of pyrene. In addition to the pyrene peak (peak 1) the chromatogram shows a peak for phenanthrene (peak 2) which belongs to the polyaromatic hydrocarbons cross-reacting with the anti-pyrene antiserum immobilised [19]. Fig. 3C is the chromatogram of the pyrene standard used. Peak 3 does not originate from the standard but from an unidentified contaminant of the solvent since it was also observed when running the whole procedure with only injecting 10% acetonitrile as a ‘‘blank’’.

The average concentration of pyrene in the five river water samples was 0.06 ng/ml (s=0.02).

4. Conclusion

The paper shows that sol–gel-generated immunoaffinity materials can resist the pressure needed to operate in on-line HPLC systems. The sol–gel immunoaffinity column could be used for more than 30 analysis cycles without losing its operational characteristics. The practicality of this approach to exploit the selectivity of antibodies or antibody mixtures in on-line coupled HPLC systems was demonstrated by on-line coupling of an anti-pyrene immunoaffinity column to an RP-precolumn and an analytical PAH-column. With a fluorescence detector this system allowed the determination of pyrene in 5.5 ml river samples with a detection limit of 15 pg/ml.

References

[1] M.-C. Hennion, J. Chromatogr. A 856 (1999) 3.
[2] D.S. Hage, Clin. Chem. 45 (1999) 593.
[3] V. Pichon, M. Bouzige, C. Miège, M.-C. Hennion, Trends Anal. Chem. 18 (1999) 219.
[4] E. Reh, J. Chromatogr. 433 (1988) 119.
[5] A. Farjam, G.J. De Jong, R.W. Frei, U.A.Th. Brinkman, W. Haasnoot, A.R.M. Hamers, R. Schilt, F.A. Huf, J. Chromatogr. 452 (1988) 419.
[6] W. Haasnoot, M.E. Ploum, R.J.A. Paulussen, R. Schilt, F.A. Huf, J. Chromatogr. 519 (1990) 323.
[7] A. Farjam, A.E. Brugman, H. Lingeman, U.A.Th. Brinkman, Analyst 116 (1991) 891.
[8] A. Farjam, A.E. Brugman, A. Soldaat, P. Timmerman, H. Lingeman, G.J. De Jong, R.W. Frei, U.A.Th. Brinkman, Chromatographia 31 (1991) 469.
[9] V. Pichon, L. Chen, M.-C. Hennion, Anal. Chim. Acta 311 (1995) 429.
[10] V. Pichon, L. Chen, N. Durand, F. Le Goffic, M.-C. Hennion, J. Chromatogr. A 725 (1996) 107.
[11] J.G. Rollag, M. Beck-Westermeyer, D.S. Hage, Anal. Chem. 68 (1996) 3631.
[12] M. Bouzige, P. Legeay, V. Pichon, M.-C. Hennion, J. Chromatogr. A 846 (1999) 317.
[13] C. Miège, M. Bouzige, S. Nicol, J. Dugay, V. Pichon, M.C. Hennion, J. Chromatogr. A 859 (1999) 29.
[14] M. Bouzige, V. Pichon, M.-C. Hennion, Environ. Sci. Technol. 33 (1999) 1916.

[15] C. Rivasseau, M.-C. Hennion, Anal. Chim. Acta 399 (1999) 75.
[16] L.M. Ellerby, C.R. Nishida, F. Nishida, S.A. Yamanaka, B. Dunn, S.J. Valentine, J.I. Zink, Science 255 (1992) 1113.
[17] B.C. Dave, B. Dunn, J.S. Valentine, J.I. Zink, Anal. Chem. 66 (1994) 1120A.
[18] J. Zuehlke, D. Knopp, R. Niessner, Fresenius J. Anal. Chem. 352 (1995) 654.
[19] M. Cichna, D. Knopp, R. Niessner, Anal. Chim. Acta 339 (1997) 241.
[20] M. Cichna, P. Markl, D. Knopp, R. Niessner, Chem. Mater. 9 (1997) 2640.
[21] T. Scharnweber, D. Knopp, R. Niessner, Field Anal. Chem. Technol. 4 (2000) 43.
[22] B. Spitzer, M. Cichna, P. Markl, G. Sontag, D. Knopp, R. Niessner, J. Chromatogr. A 880 (2000) 113.
[23] A. Turniansky, D. Avnir, A. Bronshtein, N. Aharonson, M. Altstein, J. Sol–Gel Sci. Technol. 7 (1996) 135.
[24] A. Bronshtein, N. Aharonson, D. Avnir, A. Turniansky, M. Altstein, Chem. Mater. 9 (1997) 2632.
[25] K. Meisenecker, D. Knopp, R. Niessner, Anal. Methods Instrum. 1 (1993) 114.
[26] M.M. McKinney, A. Parkinson, J. Immunol. Methods 96 (1987) 271.
[27] R. Karlsson, H. Roos, in: C.P. Price, D.J. Newman (Eds.), Principles and Practice of Immunoassay, 2nd ed., MacMillan Reference, London, 1997, p. 99.