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EFFECT OF THE STRUCTURE AND DENSITY OF CHEMICALLY BONDED C_{18} PHASE ON THE RECOVERY OF TRYPTOPHAN AND ITS METABOLITES FROM HUMAN URINE[a]

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SUMMARY

A series of materials with chemically bonded C_{18} phase for use as the packings in clean-up columns for solid-phase extraction were prepared. The effects of the monomeric and/or polymeric structure of the chemically bonded phase and of the porous structure of the silica gel support on the recovery of tryptophan and two of its metabolites used as test substances were considered. It appeared that the best recoveries of at least 60% of the three test substances were obtained on material of the "monomer" type containing chemically bonded C_{18} phase characterized by a high coverage density of $\alpha_{RP} \approx 3.8$ μmol/m^2. The use of a silica gel support with a larger pore size and volume permits not only the effective isolation of individual substances, *e.g.*, from urine, but also their 5-fold concentration.

INTRODUCTION

In recent years packings with chemically bonded phase (CBP) have been increasingly used. They are not only good starting materials for the production of high-performance liquid chromatographic (HPLC) columns, but are also used in the preparation of columns for solid-phase extraction This relates especially to the determination of polar substances isolated from biological materials such as urine, serum, blood and tissue[1–6]. These materials possess many advantages and are characterized by high solvolytic, mechanical and thermal resistance. These advantages permit the use of these materials on a large scale in routine clinical and biochemical analysis[1,2–4,6].

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The recovery of substances isolated from biological materials using a clean-up column for solid-phase extraction depends on many factors, the most important being the packing selectivity, the height of the packing bed and the choice of an appropriate eluent[4–6]. These factors significantly influence the reproducibility of analytical data. However, few papers have described the effect of porous structure on the recoveries obtained with off-line clean-up columns for solid-phase extraction. Studies of these effects have been related mainly to typical packings of analytical columns used in HPLC[7–10].

The detailed examination of these effects seemed to be of great interest. In this connection, compounds used in medical diagnostics and characterized by very different chemical natures[11–13] were chosen as test substances.

It is well known that if monochlorosilanes are used in the preparation of CBP packings, then packings with a strictly defined monomeric structure are obtained (a one-point covalent bond between the support surface and the modifier molecule is formed). Di- and trifunctional organosilanes give so-called "polymeric phase" packings with a cross-linked structure of the liquid organic phase[3,6,10,14–17]. The presence of such polymeric phases causes the undesirable screening of unblocked silanols, which represent specific and strong active centres on the support surface. As a result, during elution and/or extraction many effects can appear that are difficult to interpret. These effects can influence significantly efficient isolation by clean-up procedures.

Similar remarks can also be made in relation to the porous structure of CBP supports, which can play a very important role not only in the isolation of compounds but also in the concentration of substances to be determined, and this is connected in turn with the sorption capacity of these packings[6,18,19].

These problems were investigated in this study. Their explanation may permit a more precise description of the mechanism of the interactions between the solute, eluent and stationary phase.

EXPERIMENTAL

Material and reagents

Two types of silica gels were used as the support for the preparation of C_{18} chemically bonded phases used as packings for clean-up columns: Kieselgel Si 60 from Merck (Darmstadt, F.R.G.) and SG-100 prepared in the Polymer Institute, Slovak Academy of Sciences (Bratislava, Czechoslovakia)[20]. The surface characteristics of these bare materials are listed in Table I.

The following organosilanes were used as modifiers: octadecyldimethylchlo-

TABLE I

PHYSICO-CHEMICAL CHARACTERISTICS OF BARE SILICA GELS USED AS CBP SUPPORTS

Silica gel	*Fraction (μm)*	S_{BET} *(m²/g)*	*D (nm)*	V_p *(cm³/g)*
Kieselgel Si 60	40–63	348	8.6	0.88
Silica gel SG-100	50–80	196	20.0	2.10

TABLE II
CHARACTERISTICS OF THE ISOLATED AND SEPARATED SUBSTANCES

Compound	*Abbreviation*	pK_a[a]	*Standard concentration (μg/ml)*
Tryptophan	TRP	5.89	10
Serotonin	5-HT	9.8	1
5-Hydroxyindoleacetic acid	5-HIAA	4.7	2

[a] Data from ref. 23.

rosilane (ODMCS) (Petrarch System, Levittown, CA, U.S.A.), octadecylmethyldichlorosilane (ODDCS) (Dynamit Nobel, Transdorf, F.R.G.) and octadecyltrichlorosilane (ODTCS) (Dynamit Nobel). Hexamethyldisilazane (HMDS) (POCh, Gliwice, Poland) was used for secondary silanization (end-capping).

The following solvents were used: dry[21] toluene and morpholine from Reachim (Moscow, U.S.S.R) and benzene, methanol, dimethyl ether, sodium acetate and sodium hydroxide from POCh. Mobile phases used in HPLC analyses were prepared using 0.15 *M* sodium phosphate (pH 4.2) (POCh) and water purified in our laboratory. The substances listed in Table II were used as test substances. All reagents used were of analytical-reagent grade.

Chemically restitant containers of volume of 5 μl made from polyethylene (Chemical Reagents Factory, ZOCh, Lublin, Poland) were used for the construction of the clean-up columns.

Chromatographic analyses were carried out on a 250 × 4 mm I.D. column packed with 7-μm spherical particles of laboratory-made chemically bonded C_{18} phase with a high coverage density of $\alpha_{RP} \geqslant 4$ μmol/m²[6,24].

Physico-chemical measurements

The parameters characterizing the porosity of the packings, *i.e.*, specific surface area (S_{BET}), pore volume (V_p) and mean pore diameter (D) before chemical modification of these packings were determined by the low-temperature nitrogen adsorption–desorption method using a Model 1800 Sorptomatic apparatus (Carlo Erba, Milan, Italy). The degree of coverage of the surface by alkylsilyl ligands, α_{RP}, was calculated on the basis of the carbon percentage determined with a Model 185 CHN analyser (Hewlett-Packard, Palo Alto, CA, U.S.A.) using the equation

$$\alpha_{RP}\ (\mu mol/m^2) = \frac{10^6 P_c}{1200N_c - P_c(M - n)} \cdot \frac{1}{S_{BET}} \quad (1)$$

where P_c = measured carbon percentage, N_c = number of carbon atoms in the molecule of the bonded silane, M = molecular mass of the silane, S_{BET} = specific surface area (m^2/g) and n = number of functional group substituents in the silane molecule.

Chromatographic measurements were carried out using a liquid chromatograph consisting of an HPP 4001 syringe pump (Laboratorní Přístroje, Prague, Czecho-

TABLE III

CHARACTERISTICS OF THE CLEAN-UP COLUMN PACKINGS AFTER CHEMICAL MODIFICATION

No. of column	*Type of packing*[a]	*Type of CBP structure*	*Coverage density*	
			P_c (%)	α_{RP} (μmol/m^2)
1	Si–ODMCS	Monomer	10.16	1.39
2	Si–ODMCS + HMDS	Monomer	13.49	4.22
3	Si–ODMCS + A	Monomer	17.26	2.70
4	Si–ODMCS + A + HMDS	Monomer	18.66	3.80
5	Si–ODMCS + A + HMDS	Polymer	21.05	5.12
				2.68[b]
6	Si–ODTCS + A + HMDS	Polymer	21.06	4.98
				2.65[b]
7	SG-100–ODMCS + A + HMDS	Monomer	15.5	4.23
				3.87[b]

[a] A = activator (morpholine).
[b] Data obtained before end-capping with HMDS.

slovakia) and a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.). An ELDEC 102 electrochemical detector (Chromatofield, Chateauneuf-les-Martiques, France) with a glassy carbon electrode working at potentials of +0.9 V and +0.5 V *vs.* an Ag/AgCl reference electrode and a TZ-4200 linear recorder (Laboratorní Přístroje) were also used.

Synthesis of CBP

Bare silica gel samples were placed in glass reactors having an ampoule shape to avoid contact of the reagents with the environment[4–6] and then heated at 200°C under vacuum (10^{-3} Pa) for 6 h.

All packings were synthesised under the standard conditions described previously[6,24,25]. The physico-chemical characteristics of the materials prepared are listed in Table III.

Isolation procedure

Isolation of the test substances was carried out using the procedure described previously[4,6,12] but slightly modified. In the first stage, the packing bed of the clean-up column was washed with 2 ml of methanol and then with 2 ml of 0.1 *M* acetate buffer (pH 5). In the second stage, 2 ml of a mixture containing dissolved standard substances in 0.1 *M* acetate buffer (1:1, v/v) was introduced on to the column, then the bed was washed with 1 ml of water. In the third stage the sorbed substances were eluted with 1 ml of 0.1 *M* ammonia solution–methanol (3:1, v/v). A 10-μl volume of the collected eluate was injected on to the analytical column. It should be pointed out that the simultaneous analysis of serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and tryptophan (TRP) is possible only at potentials of at least to +0.9 V because TRP has a high oxidizing potential of +0.88 V; 5-HT and 5-HIAA can be determined simultaneously at a potential of +0.5 V[5,12].

RESULTS AND DISCUSSION

Table III lists the characteristics of the materials used as the packings in clean-up columns prepared under different conditions. Use of the modifiers containing 1–3-chloro substituents allowed the packings with C_{18} chemically bonded phases with "monomer" and "polymer" type structures to be prepared. According to Unger and Anspach[9], Unger[15] and Bayer *et al.*[14], the surface structures obtained can be represented schematically as shown in Fig. 1a–c.

Considering the data in Table III for packings of the "monomer" type (materials 1–4), we can conclude that depending on the synthesis conditions chemical modification leads to material of increasing coverage density, α_{RP}, and of different layer structure. The presence of an activator influences significantly (see packings 1 and 3) the C_{18} ligand contribution (α_{RP} increases by 60%)[6,24,25]. This effect confirms the opposite increase in the total α_{RP} values after secondary silanization with HMDS of the packings prepared in the first stage (packings 2 and 4). A greater difference in α_{RP} is

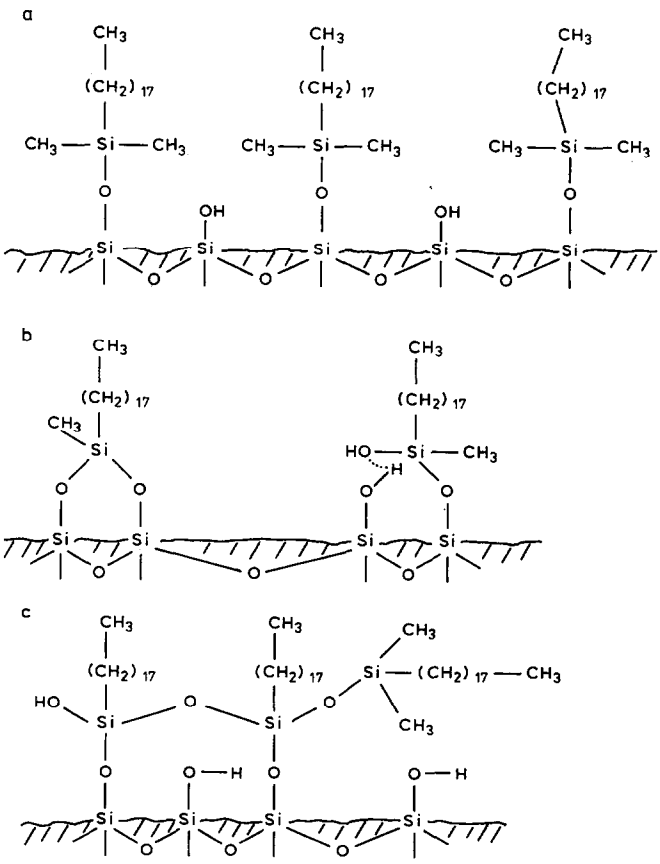

Fig. 1. Possible structures of chemically bonded C_{18} phases which can be formed, depending on the number of chlorine atoms in the modifier molecule. (a) Monochlorosilane; (b) dichlorosilane; (c) trichlorosilane.

observed for materials 1 and 2 than for 3 and 4. This is reasonable considering the greater possibility of spatial penetration of small methylsilyl molecules towards unblocked silanols. For the materials of less dense surface coverage (packing 1, $\alpha_{RP} = 1.39$ μmol/m^2) end-capping is more effective than for the materials of denser surface coverage (packing 3, $\alpha_{RP} = 2.70$ μmol/m^2)[24–26]. It seems that this penetration will be possible mainly as a result of the reduction in steric interactions between individual C_{18} ligands[26] and the change in conformation of the chain in the synthesis medium[27]. Moreover, an additional effect resulting from the porous structure of the silicagel support can be expected.

Materials 1–4 were prepared on an adsorbent characterized by a small pore size and volume (Table I). Material 4 (Table III) should permit the extraction of test substances only in the bonded organic layer without the possibility of penetration into the pores of the substances being determined[6,18]. Comparison with packings prepared under similar conditions with material of larger pore size and volume (Tables I and III, packing 7), significant differences in the α_{RP} values for both primary and secondary silanization are observed. This is in good agreement with our previous results [18].

We have mentioned many times that dense coverages of "monomer" type CBP are obtained by reaction with monochlorosilane in the presence of morpholine as activator and have described the mechanism of this synthesis[6,10,16,18,22,24–26]. Based on the investigations described in the papers cited, it would be expected that the use of di- and trifunctional silanes under conditions analogous to those for the preparation of packings 4 and 7 would permit packings with a more cross-linked structure of the support surface to be obtained (materials 5 and 6, Table III).

Comparison of the α_{RP} values shows that primary silanization by use of di- and trifunctional modifiers is not influenced significantly by the differences in the mechanism of bonding. In both instances the α_{RP} values are comparable to those obtained with a monofunctional modifier (packings 3, 5 and 6). After secondary silanization, however, significant differences appear, which suggests the formation of different CBP structures as illustrated in Fig. 1. Relatively low coverages with C_{18} alkyl ligands in the presence of an activator and their small differences independent of the functionality of the reacting silane modifier (materials 3, 5 and 6) may be explained by steric hindrance to the penetration of large C_{18} molecules into the narrow-pore Kieselgel Si 60 (the surface located outside the pores participates preferentially in the reaction).

The tests on the usefulness of the packings for solid-phase extraction were carried out using tryptophan and its metabolites serotonin and 5-hydroxyindoloacetic acid as test substances. These substances are of interest for two reasons: (i) because of the similar geometric dimensions of the molecules but their completely different chemical properties; and (ii) because of the great importance of these substances in medical diagnosis (optimization of the choice of packings for routine analyses using clean-up columns).

Table IV lists the recoveries of these three compounds after solid-phase extraction. The results indicate that the lowest recoveries of TRP and 5-HT are obtained for the non-end-capped materials synthesized by use of a monofunctional modifier (packings 1 and 3). This is probably due to the greater accessibility of unblocked silanols for the substances isolated. As a consequence, specific interactions between the substances being determined, the eluent and the packing surface occur.

TABLE IV

COMPARISON OF RECOVERIES AND COEFFICIENTS OF VARIATION (C.V.) FOR TRYPTOPHAN AND ITS METABOLITE ISOLATED FROM STANDARD SOLUTION

Off-line column No.	*Recovery (%)*					
	TRP	*C.V. (%)*	*5-HT*	*C.V. (%)*	*5-HIAA*	*C.V. (%)*
1	33.4	12.6	4.0	22.5	80.5	8.6
2	83.8	3.6	43.6	4.1	79.0	3.9
3	48.6	11.6	1.7	23.5	79.5	5.8
4	78.5	3.2	56.1	3.4	62.7	3.5
5	85.0	2.1	35.0	5.4	53.8	5.3
6	70.0	2.2	56.6	3.5	53.0	4.7
7	77.8	3.3	41.3	6.4	94.4	3.3

This supposition may be confirmed by the high values of the coefficient of variation (Table IV).

The secondary silanization (columns 2 and 4) improves the recovery of the above two substances significantly. This is probably due to limited interactions of basic solutes with the silanols, *i.e.*, leakage of free electron pairs or π-electron interactions. The possibility of an additional effect connected with the limited mobility of long C_{18} chains directed towards short trimethylsilyl groups with lower coverages should also be taken into account[25–29].

Owing to these specific interactions a change in the conformation of C_{18} ligands probably takes place, resulting in better screening of the support surface (column 2, Table IV). This may suggest that for the isolation of basic solutes and for dense CBP coverage, partial coverage with the main ligand followed by end-capping may be useful[30].

Because of the leakage of amino groups in the chemical structure of 5-HIAA, the recoveries obtained with sorbents 1–3 by use of a monofunctional silane modifier are relatively high and almost independent of the density of coverage (columns 1–3. Tables III and IV), *i.e.*, an irreversible adsorption effect does not occur. This is in good agreement with our earlier suppositions[31], confirmed recently by Bayer and Paulus[30].

Exhaustive silanization in the presence of an activator and with end-capping leads to a significant reduction in the 5-HIAA recovery (column 4), which is probably the result of blockage of the narrow pores in Si 60 silica gel[6,8,18,31]. A similar explanation may be valid for the recovery results for the three compounds obtained on materials synthesized by use of di- and trifunctional modifiers (columns 5 and 6).

In spite of the higher α_{RP} values, packings of the "polymer" type (materials 5 and 6) show some tendency for irreversible sorption of individual substances. In the examples considered, the adsorption of individual substances is often very high but elution from the surface layer is difficult in many instances (limited mass exchange, Table IV). In this connection, the lack of reproducibility of the recovery on some commercial packings for clean-up columns[4–6] is not surprising. With materials 4 and 7 (Tables III and IV) the effects of shielding of the support surface by hydrophobic ligands of the stationary phase during the isolation of the standard mixture are good. This is confirmed by the relatively small differences in recovery and the small values of

TABLE V
RECOVERY OF TRYPTOPHAN AND ITS METABOLITES ISOLATED FROM HUMAN URINE ON CLEAN-UP COLUMNS WITH DIFFERENT POROSITIES OF THE MATERIAL PACKING

Off-line column No.	*Recovery (%)*					
	TRP	*S.D.*	*5-HT*	*S.D.*	*5-HIAA*	*S.D.*
4	76.0	3.4	45.0	3.6	61.0	3.0
7	74.0	2.3	27.0	4.7	92.7	1.4

the coefficient of variation (except for 5-HT with material 7). On the other hand, material 4 appears to be more selective for the isolation of 5-HT and TRP[18,19]. This is undoubtedly due to the smaller sizes of the pores because, owing to chemical modification, almost all pores are blocked by relatively large C_{18} molecules[6,8–18]. Close packing of C_{18} ligands in narrow pores ($D \approx 6$ nm) significantly reduces the probability of interactions of unblocked silanols with the substances being determined, all the more because interactions between individual ligands (resulting from steric effects) will be preferred here[6–10,14–18,25–27]. This can undoubtedly make mass exchange difficult and also lead to lower sorption capacities[3,4,6]. For the packing 7 the larger pore size permits greater accessibility to unblocked silanols for the molecules of the substance being determined, which probably influences the lower recovery of 5-HT (Table IV). On the other hand, this effect has a very advantagenous influence on the conditions of determination of 5-HIAA, for which the recovery is high and comparable to those obtained previously[4]. The results obtained with both of these packings during the isolation of tryptopan and its metabolites from biological material (human urine, Table V) indicate that our previous suggestions are confirmed. The recovery of 5-HT with packing 4 is higher than that with packing 7, which has comparable properties. Opposite but very similar regularities are observed in the determination of 5-HIAA: in this instance packing 7 is more selective, *i.e.*, possesses a higher sample capacity. The coefficients of variation, which are low in all cases considered (Table V), appear to confirm the above conclusions.

Attempts were made to concentrate the three substances on packings 4 and 7. It appeared that using the material with a large pore size and volume (packing 7) a 5-fold concentration of substances isolated from human urine can be obtained in comparison with packing 4, which has a *ca.* 3-fold smaller pore size and volume (Table I).

The full explanation of the above phenomena is difficult. We may assume that urine contains some other active compounds that undergo preferential adsorption on the CBP surface, reducing the adsorption of the investigated solutes. A denser coverage with C_{18} alkyl ligands followed by end-capping of the wider pore material 7 should reduce such co-adsorption and allow higher recoveries of the very active 5-HT to be obtained during the desorption process.

CONCLUSIONS

For the isolation of substances in biological material (urine, serum, etc.), clean-up column packings with a monomeric structure of chemically bonded C_{18} phase in which the silanol groups are well shielded by C_{18} ligands appear to be most

suitable. End-capping significantly influences the recovery. In this instance the molar ratio of C_{18} to methylsilyl groups should be high if possible, *i.e.*, the maximum coverage with C_{18} ligands should be obtained. More reproducible results are obtained with packings prepared on the basis of a support characterized by a larger pore size and volume, which permits a more compact film of the organic phase, a denser coverage and a lower availability of remaining silanol groups for interaction with solute molecules to be obtained.

A separate question is the hydrolytic stability of end-capped materials, especially in buffer solutions. We did not investigate systematically the lifetime of the end-capped sorbents, but we noted that materials not completely covered with C_{18} monoligands and end-capped gave lower and less reproducible recoveries of 5-HT and 5-HIAA from urine[31], which may be caused by, among other things, a lower sorbent stability. Dense coverage with the use of an activator and end-capped sorbents did not result in such inconvenient phenomena.

REFERENCES

1 B. Tippins, *Int. Lab.*, 17, No. 3 (1987) 28.
2 C. K. Lim, F. Li and T. J. Peters, *Int. Lab.*, 17, No. 6 (1986) 60.
3 C. F. Poole and S. A. Schuette, *Contemporary Practice of Chromatography*, Elsevier, Amsterdam, 1984, Ch. 7, p. 429.
4 B. Buszewski, K. Šebeková, P. Božek, L. Štibranýi, J. Jenrichovský, I. Novák and D. Berek, *Chromatographia.*, 22 (1986) 299.
5 D. Sieńko, B. Buszewski and Z. Suprynowicz, *Anal. Chim Acta*, in press.
6 B. Buszewski, *Ph.D. Thesis*, Slovak Technical University, Bratislava, 1986.
7 L. C. Sander and S. A. Wise, *J. Chromatogr.*, 316 (1984) 163.
8 L. C. Sander and S. A. Wise, *Crit. Rev. Anal. Chem.*, 18 (1987) 299.
9 K. K. Unger and B. Anspach, *Trends Anal. Chem.*, 66 (1987) 121.
10 J. Nawrocki and B. Buszewski, *J. Chromatogr.*, 449 (1988) 1.
11 O. Beck, G. Polmskog and E. Hullman, *Clin. Chim. Acta*, 79 (1977) 149.
12 D. D. Koch and P. T. Kissinger, *J. Chromatogr.*, 164 (1979) 441.
13 P. Božek, O. Uličná, J. Zemek and L. Stibranyi, *Biochem. Clin. Bohemoslov.*, 15 (1986) 175.
14 E. Bayer, K. Albert, J. Reiners, M. Nieder and D. Müller, *J. Chromatogr.*, 264 (1983) 197.
15 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979.
16 B. Buszewski, K. Pilorz and Z. Suprynowicz, *J. Chromatogr.*, submitted for publication.
17 B. Buszewski, *Chem. Stosow.*, 32 (1988) 201.
18 B. Buszewski, D. Berek, J. Garaj, I. Novák and Z. Suprynowicz, *J. Chromatogr.*, 446 (1988) 191.
19 B. Buszewski, D. Berek, I. Novák and J. Garaj, *Chem. Pap.*, in press.
20 I. Novak and D. Berek, *Czech Pat.*, 235250, 1984.
21 B. Buszewski, R. Lodkowski and J. Trocewicz, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 527.
22 B. Buszewski, T. Bleha and D. Berek, *J. High Resolut, Chromatogr. Chromatogr. Commun.*, 8 (1985) 860.
23 D. D. Perrin, *Dissociation Constants of Organic Bases in Aqueous Solution*, Butterworths, London, 1965.
24 B. Buszewski, A. Jurášek, J.Garaj, L. Nondek and D. Berek, *J. Liq. Chromatogr.*, 10 (1987) 2325.
25 B. Buszewski and Z. Suprynowicz, *Anal. Chim. Acta*, 208 (1988) 263.
26 B. Buszewski and Z. Suprynowicz, *Chromatographia.*, 24 (1987) 573.
27 E. Bayer, A. Paulus, B. Peters, G. Laupp, J. Reiners and K. Albert, *J. Chromatogr.*, 364 (1986) 25.
28 S. S. Yang and R. K. Gilpin, *J. Chromatogr.*, 394 (1987) 295.
29 B. Buszewski, K. Šebeková, P. Božek and D. Berek, *J. Chromatogr.*, 367 (1986) 171.
30 E. Bayer and A. Paulus, *J. Chromatogr.*, 400 (1987) 1.
31 Z. Suprynowicz, B. Buszewski, D. Sieńko and J. Gawdzik, *J. Chromatogr.*, 456 (1988) 219.