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Siliceous sorbents with immobilized Carbowax 20M as column packings for liquid chromatography

II. Application in high-performance liquid chromatography

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ABSTRACT

The chromatographic properties of siliceous supports with thermally immobilized Carbowax 20M as packings for high-performance liquid chromatography are described. Such materials were examined as sorbents in normal- and reversed-phase chromatographic systems and as macromolecular sieves for size-exclusion chromatography of biopolymers. Additionally, the stability of the Carbowax layer was determined.

INTRODUCTION

Carbowax 20M is one of the most popular stationary phases applied in gas chromatography (GC) [1], owing to its very high selectivity in relation to many compounds, especially to those differing only slightly in polarity or boiling temperatures. The application range of Carbowax 20M has been increased by introducing various immobilization procedures [2–10]. Such phases are mainly employed in GC and sorbents with immobilized Carbowax 20M have hardly been used in HPLC.

This paper deals with chromatographic properties of siliceous sorbents with thermally immobilized Carbowax 20M as packings for HPLC. Synthesized materials were investigated as sorbents in normal- and reversed-phase systems and as macromolecular sieves for the size-exclusion chromatography of biopolymers. In addition, the stability of packings with thermally immobilized Carbowax 20M is discussed.

EXPERIMENTAL

Materials

The preparation and synthesis of materials used in this experiment were described in Part I [11]. LiChrosorb DIOL (Merck, Darmstadt, Germany) taken additionally for the comparison (abbreviated here to Si-Diol), was characterized by the following data: S_{BET} = 229 m^2/g, V_p^{des} = 0.90 cm^3/g, V_p^{ads} = 0.88 cm^3/g, D_c^{des} = 157 Å, D_c^{ads} = 154 Å and D_{max} = 108 Å. The particle

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size of Si-Diol and other sorbents was 10 μm. The plate numbers (N) (calculated from the band width at half-height of the nitrobenzene peak using hexane as mobile phase) and the peak asymmetry factors A_s (calculated for the same peak at 5% of the peak height) were as follows:

	CPG II	CPG III	Si-100	CPG II c	CPG III C	Si-100 C	Si-Diol
N =	5600	5350	4300	4350	3900	2400	1600
A_s =	1.35	1.80	1.00	1.25	1.00	1.00	1.00

Protein standards (Kit MS II) were purchased from Serva (Heidelberg, Germany).

Methods

HPLC investigations were carried out using a Liquochrom 2010 liquid chromatograph (MIM, Budapest, Hungary) with a UV detector (254 nm).

The mobile phases used were pure hexane, methanol-2-propanol-hexane (10:30:60) and methanol-water (50:50, 40:60 and 30:70). In size-exclusion chromatography, 0.1 M NaH_2PO_4 buffer (pH 6.8) was applied. The flow-rate was 1 ml/min. Stainless-steel columns (250 × 4 mm I.D.) were packed using the balanced density slurry method.

Methods for the determination of specific surface areas, mean pore diameters and amounts of Carbowax were described in Part I [11].

RESULTS AND DISCUSSION

Immobilized Carbowax 20M in a normal-phase HPLC system

As mentioned in Part I [11], sorbents with thermally immobilized Carbowax 20M can be successfully employed in HPLC [12–14]. In order to elucidate their mechanism in a normal-phase system, the separation of aromatic hydrocarbons performed on columns filled with materials with an immobilized Carbowax layer were compared with analogous separations obtained on columns packed with pure silica sorbents.

Table I gives capacity factors (k') of aromatic hydrocarbons analysed on the sorbents CPG II, CPG III, Si-100, CPG II C, CPG III C, Si-100 C, Si-Diol (see Part I [11]). For better illustration, Fig. 1 shows related chromatograms obtained on the chosen sorbents. As can be seen, both retention times and k' values obtained on the sorbents with immobilized Carbowax 20M are much lower than those obtained on the initial materials, *i.e.*, CPG II, CPG III and Si-100. This is most evident for polar nitrobenzene. The peaks are well separated, narrow and symmetrical; however, naphthalene and diphenyl elute in a common band. A very similar separation to those obtained on the materials with immobilized Carbowax was obtained using Si-Diol as a column filling.

As was mentioned in the Introduction, sor-

TABLE I

CAPACITY FACTORS (k') OF AROMATIC HYDROCARBONS ON COLUMNS PACKED WITH THE INVESTIGATED SORBENTS

Mobile phase: hexane.

Compound	k'						
	CPG II C	CPG III C	Si-100 C	Si-Diol	CPG II	CPG III	Si-100
Benzene	0.07	0.15	0.07	0.16	0.19	0.32	0.29
Naphthalene	0.19	0.34	0.25	0.32	0.37	0.57	0.50
Diphenyl	0.19	0.34	0.25	0.32	0.56	0.84	0.69
Anthracene	0.50	0.82	0.70	0.57	0.69	1.01	0.90
Nitrobenzene	1.14	1.91	1.50	0.93	4.59	6.25	5.10

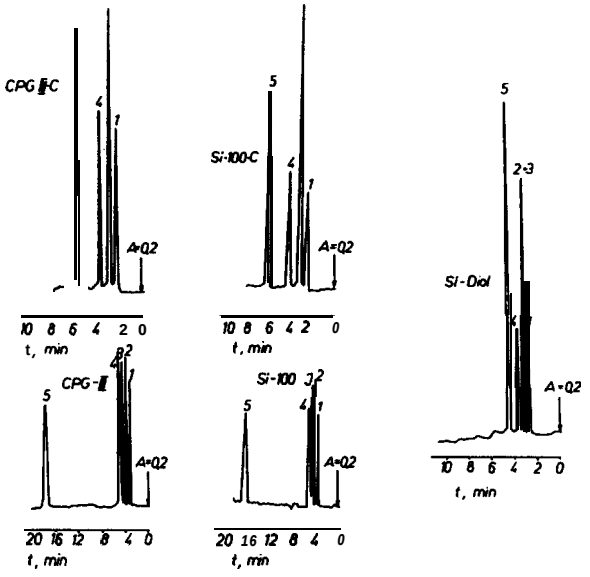

Fig. 1. Separation of aromatic hydrocarbons on sorbents CPG III, CPG III C, Si-100, Si-100 C and Si-Diol with the normal-phase system. Column, 250 × 4 mm I.D.; mobile phase, hexane; flow-rate, 1 cm^3/min. Compounds: 1 = benzene; 2 = naphthalene; 3 = diphenyl; 4 = anthracene; 5 = nitrobenzene.

bents with bonded or adhesively deposited Carbowax 20M are especially recommended for GC separations of polar compounds [1,15]. However, amino compounds are an exception. According to many publications [15,161, caution should be exercised when analysing substances with amino groups on polyethylene glycol (PEG) phases, because of the possibility of reactions of these substances with aldehyde groups that can be formed as a result of PEG chain degradation. There is a great possibility that aldehyde, carboxy1 or oxirane groups exist in the polymer layer because the sorbents used in the present work were obtained by means of thermal immobilization. Potentiometric titration (see Part I [11]) indicated the presence of a carboxyl or oxirane groups which may also be associated with the presence of aldehyde groups. Hence, the separation of amino compounds allows one to obtain some information about the probable presence of aldehyde groups.

Table II gives the capacity factors of nitroanilines, aniline, nitrobenzene and benzene separated using columns filled with CPG II C, CPG III C and Si-100 C. For comparison analogous data obtained using the columns packed with the pure siliceous materials CPG II, CPG III and Si-100 are also given. As can be seen, the retention sequences are identical for the initial supports, ***i.e.,*** for CPG II, CPG III and Si-100. o-Nitroaniline elutes before aniline and other nitroanilines. This suggests that an intra-hydrogen bond is formed between the amino and nitro groups in the ***ortho*** position. It was found that ***m-*** and p-nitroaniline elute

TABLE II

CAPACITY FACTORS (k') OF NITROANILINES, ANILINE, BENZENE AND NITROBENZENE ON COLUMNS PACKED WITH THE INVESTIGATED SORBENTS

Mobile phase: methanol-2-propanol-hexane (10:30:60).

Compound	*k'*					
	CPG II	CPG II C	CPG III	CPG III C	Si-100	Si-100 C
Benzene	0.25	0.38	0.21	0.35	0.17	0.38
Nitrobenzene	0.34	0.80	0.33	0.88	0.27	0.86
Aniline	0.59	–	0.72	1.80	0.53	1.72
o-Nitroanihne	0.39	2.02	0.42	2.07	0.33	2.21
m-Nitroaniline	0.59	4.21	0.72	4.08	0.53	4.54
p-Nitroaniline	0.59	5.45	0.72	5.33	0.53	6.14

together with aniline, which seems to show that only the amino group interacts with the silica surface and that the mesomeric effect of the nitro group does not influence protonation of hydrogen atoms connected with nitrogen. The sorbents with a bonded Carbowax layer possess entirely different properties from the pure siliceous initial materials. This is confirmed by the increase in capacity factors, by the increase in selectivities (all substances are completely separated) and by the different order of the elution (aniline elutes before o-nitroaniline and then *m*-nitroaniline and p-nitroaniline elute). It is probable that in the interaction with the PEG layer o-nitroaniline employs both functional groups. The same applies to *m*- and p-nitroaniline. The sequence of nitroanilines is related to the increasing mesomeric effect. The chromatographic properties of the sorbents with a Carbowax layer are very similar.

The lack of the aniline peak for CPG II C (even when aniline was injected alone) is of great interest; see the absence of the capacity factor for aniline on this sorbent (Table II). It is probable that CPG II C possesses much more reactive groups than the other sorbents with immobilized Carbowax 20M. It may be connected with a greater amount of immobilized Carbowax 20M relative to the support surface (w/S) in the case of the sorbent CPG II C (see Part I [11]).

The same phenomenon can be observed in the separation of toluidines. Table III gives the capacity factors of the mixture of toluidines and aniline obtained for the sorbents CPG II C, CPG III C and Si-100 C. As with nitroanilines, the separations of toluidines on CPG III C and Si-100 C are very similar (the k' values are almost identical). For CPG II C only the *o*-toluidine peak is obtained and has a k' value comparable to those on CPG III C and Si-100 C. The other toluidines and aniline "disappear". These results seem to indicate a strong interaction between amino groups of the analyte compounds with aldehyde, carboxyl or oxirane groups of the sorbents. These interactions can be weakened by the presence of other functional groups (e.g., nitro groups), decreasing the basic properties of the amino group (as with m- and p-nitroanilines) or by steric hindrance as with o-toluidine and o-nitroaniline (screening effect or intra-chelate).

TABLE III

CAPACITY FACTORS (k') OF TOLUIDINES AND ANILINE ON COLUMNS FILLED WITH CPG II C, CPG III C AND Si-100 C

Mobile phase: methanol-2-propanol-hexane (10:30:60).

Compound	k'		
	CPG II C	CPG III C	Si-100 C
o-Toluidine	1.20	1.20	1.18
m-Toluidine	–	1.42	1.38
p-Toluidine	–	1.42	1.38
Aniline	–	1.71	1.73

Immobilized Carbowax 20M in reversed-phase HPLC

As was mentioned earlier, Carbowax 20M is a medium-polarity stationary phase. However, in immobilized polyethylene glycol chains some of the ether oxygen atoms are engaged in interactions with the support surface (for silica gel with hydroxyl groups bonded with surface silica atoms, and for porous glass with boron atoms and with hydroxyl groups connected with both Si and B atoms [17]). The polarity of such a polyethylene glycol layer is lower than that of

TABLE IV

CAPACITY FACTORS (k') OF AROMATIC COMPOUNDS ON A COLUMN PACKED WITH Si-100 C AT VARIOUS METHANOL-WATER MOBILE PHASE COMPOSITIONS

Compound	k'		
	Methanol-water (50:50)	Methanol-water (40:60)	Methanol-water (30:70)
Benzene	0.24	0.37	0.50
Phenol	0.24	0.37	0.50
Toluene	0.24	0.37	0.50
Nitrobenzene	0.24	0.37	0.50
Naphthalene	0.69	0.96	1.40
Diphenyl	0.79	1.23	1.89
Anthracene	1.53	2.81	5.12

bulk PEG [18]. The aim here was to investigate whether the decrease in the polarity of Carbowax 20M is sufficient to be able to use siliceous sorbents with a PEG layer as packings for RP chromatography. A positive answer can be expected because PEGs covalently bonded to the silica gel surface (via y-glycidoxypropylsilane) have been successfully used in the high-performance hydrophobic interaction chromatography of proteins [7,19].

In order to resolve the above problem, a mixture of benzene, phenol, toluene, nitrobenzene, naphthalene, diphenyl and anthracene was separated using a column filled with Si-100 C employing methanol-water as the mobile phase.

Table IV gives the capacity factors of the analyte substances obtained with various proportions of the mobile phase components *i.e.,* methanol-water (50:50, 40:60 and 30:70). Fig. 2 shows the corresponding chromatograms. Larger amounts of methanol do not allow the separation of naphthalene from diphenyl.

As can be seen, with methanol-water (30:70) there is a complete separation of the last three eluting substances in the investigated mixture (substances which are characterized by the longest retention times), but benzene, phenol, toluene and nitrobenzene still elute in one peak. This seems to indicate that the hydrophobic properties of the immobilized Carbowax layer are much weaker than those of sorbents with chemically bonded alkyl or aryl radicals. This conclusion is in agreement with the observations of Chang and co-workers [7,19], who called Carbowax phases "soft" in contrast to "hard phases" represented by chemically bonded alkyl radicals. The present investigations (in which one of the most popular mobile phases used in RP chromatography was applied) show how different the properties of Carbowax are in relation to alkyl or aryl phases.

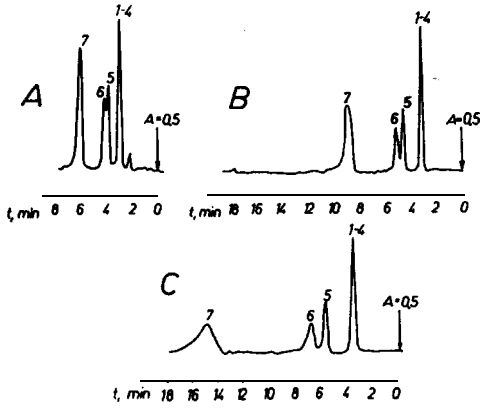

Fig. 2. Separation with the reversed-phase system on a column packed with S-100 C. Mobile phase, methanol–water: (a) 50:50; (b) 40:60; (c) 30:70 (v/v). Compounds: l-4 = benzene, phenol, toluene and nitrobenzene in a single peak; 5 = naphthalene; 6 = diphenyl; 7 = anthracene.

In order to elucidate the chromatographic possibilities of sorbents with immobilized Carbowax 20M, more detailed experiments are needed, using other test substances and mobile phases.

Silica gel with immobilized Carbowax 20M as sorbents for size-exclusion chromatography

Sorbents with adhesively deposited Carbowax 20M are commonly used as column fillings in the size-exclusion chromatography (SEC) of biopolymers [20,21]. The Carbowax layer blocks adsorption centres on the support surface, preventing adsorption of proteins and peptides. It is worth adding that polyethylene glycol is readily water-wettable, *i.e.,* by the main component of mobile phases used in SEC of biopolymers. However, the lifetime of columns with adhesively deposited PEG is short because of strong phase bleeding [21]. Darling *et al.* [22] described an attempt at thermal treatment of the adhesively deposited Carbowax 20M layer. The sorbent obtained showed adsorption properties towards proteins, so the experiment was not successful.

The procedure of Carbowax layer immobilization proposed by Aue and co-workers [9,10] is more effective and probably destroys the structure of the PEG layer to a lesser extent than the simple thermal treatment employed by Darling *et al.* Hence the problem appears to be to establish how proteins behave during elution on a column packed with siliceous materials with thermally immobilized Carbowax 20M.

Fig. 3 shows the calibration graph for protein standards (see Table V) obtained with the columns filled with Si-100 C. It also shows, for comparison, the calibration graph obtained with the column packed with Si-Diol. It should be

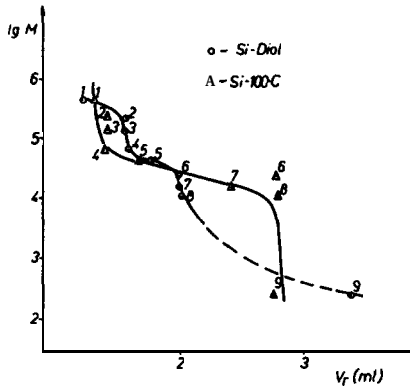

Fig. 3. Calibration graphs obtained for a column filled with Si-100 C (solid line) and Si-Diol (dashed line) using peptide standards numbered as in Table V. Mobile phase: 0.1 *M* NaH_2PO_4 (pH 6.8).

stressed that the separation conditions applied in these experiments were selected on the basis of literature data concerning the separation of biopolymers on diol phases [23,24].

A decidedly better calibration graph was obtained using Si-100 C as a column filling. Only chymotrypsinogen was an exception and eluted with a much larger elution volume than expected. This protein possesses a very high isoelectric point (p*I* = 9.5), indicating the presence of positively charged groups (primary amino groups) in the molecule. Consequently, chymotrypsinogen can interact very strongly with the negatively charged sorbent surface. However, several other proteins are also characterized by high p*I* values (that for cytochrome c is even higher), but their retention volumes seem to be only slightly enhanced. The ionic strength of the mobile phase used was 0.2 and it has been shown [24,25] that this ionic strength is high enough to prevent electrostatic interactions. There must be another explanation for the extremely high elution volume for chymotrypsinogen. Hydrophobic interactions may be responsible for such a behaviour of chymotrypsinogen. In contrast to the hydrophilic myoglobin and cytochrome c, chymotrypsinogen is a highly hydrophobic protein. Hydrophobic interactions can be diminished by addition of an organic modifier (e.g., ethylene glycol) to the mobile phase [24,25]. With a column packed with the examined sorbent, the addition of 10% of ethylene glycol to the mobile phase caused, instead of a decrease, an increase in the retention volumes of chymotrypsinogen, myoglobine and cytochrome c. The same addition of ethylene glycol to the mobile phase hardly influenced the retention of biopolymers injected onto the Si-Diol column. Surprisingly, both before and after addition of ethylene glycol, the calibration graph obtained for Si-Diol was far from the ideal calibration graphs reported for this material in other publications [23,24,26]. Increases in the retention volumes on addition of organic modifiers to the aqueous eluent were observed by De Ligny *et al.* [27] in the SEC of proteins on Sephadex and were explained in terms of adsorption and partitioning. Therefore, considering the elution of chymotrypsinogen on the investigated materials, the influence of adsorption effects or specific partition phenomenon also cannot be excluded.

TABLE V

ISOELECTRIC POINTS (p*I*) AND AVERAGE MOLECULAR MASSES ($\bar{M}$) OF PROTEINS

No.	Protein	A	p*I*
1.	Fenitin	450 000	4.4
2	Catalase	240 000	–
3	Aldolase	160 000	9.5
4	Bovine serum albumin (BSA)	67000	4.4-4.8
5	Ovalbumin	45ooo	4.7
6	Chymotrypsinogen	25 000	9.5
7	Myoglobin	17800	7.1
8	Cytochrome c	12 300	10.6
9	DNP-L-Alanine	255	

Stability of siliceous sorbents with thermally immobilized Carbowax 20M

Aue and co-workers, who developed the method for the thermal immobilization of Carbowax 20M, called the polymer layer they obtained a "non-extractable layer", because it remains on the support surface after very exhaustive extraction [8–10]. As described under Experimental, we were forced to change Aue and co-workers' method, introducing additionally a sorbent washing procedure performed in the thermostated columns. The applied washing procedure was even more effective than that

TABLE VI

PHYSICO-CHEMICAL PROPERTIES OF SORBENTS **AFTER** USE IN CHROMATOGRAPHIC COLUMNS

Symbols the same as in Tables I and II in Part I **[11].**

Sorbent	S_{BET} (m^2/g)	V_p^{des} (cm^3/g)	D_c^{ads} (A)	D_{max} (Å)	w/w (×100) (g/g)	w/S (×100) (g/m^2)	d_f (Å)
CPG III C	216	0.83	154	100	15.17	0.044	3.3
		0.81	150				
Si-100 C	277	1.20	173	140	8.45	0.027	2.0
		1.20	173				

used by Aue and co-workers. Assuming that the excess amount of Carbowax 20M was washed from the surface" and that PEG chains directly interacting with the support surface remained on the surface only, there is still the question of whether the immobilized layer is stable and whether or not it bleeds during the **chromato**-graphic process.

The most heavily used columns, *i.e.*, Si-100 C and CPG III C, were unpacked and CHN elemental analysis was performed to establish the amount of Carbowax remaining. The results are given in Table VI. When compared with Table II in Part I **[11],** it can be seen that the amount of the bonded Carbowax and conse-quently the thickness of the polymer layer had

TABLE VII

CAPACITY FACTORS (k') OF THREE GROUPS OF SUBSTANCES ON COLUMNS FILLED WITH CPG III C AND Si-100 C AT THE BEGINNING OF THE INVESTIGATION AND AFTER A LONG PERIOD (ABOUT 2 MONTHS) OF OPERATION OF THE COLUMNS

Group I substances, mobile phase = hexane; group II and III substances, mobile phase = methanol-2-propanol-hexane **(10:30:60).**

Group	Compound	*k'*			
		CPG III C		**SI-100** c	
		At the beginning	At the end	At the beginning	At the end
I	Benzene	0.15	0.18	0.07	**0.24**
	Naphthaiene	0.34	0.42	0.25	0.48
	Diphenyl	0.34	0.42	0.25	0.48
	Anthracene	0.82	0.89	0.70	0.88
	Nitrobenxene	1.91	1.70	1.50	1.61
II	Benzene	0.32	0.35	0.32	0.38
	Nitrobenxene	0.82	0.88	0.83	0.86
	Aniline	1.73	1.80	1.77	1.72
	***o*-Nitroaniline**	1.87	**2.07**	2.28	2.21
	***m*-Nitroaniline**	3.86	4.08	4.94	4.54
	***p*-Nitroaniline**	4.99	5.33	6.78	6.14
III	***o*-Toluidine**	1.21	1.21	1.18	1.24
	***m*-Toluidine**	1.42	1.42	1.38	1.41
	p-Toluidine	1.42	1.42	1.38	1.41
	Aniline	1.71	1.71	1.73	1.71

decreased threefold (for CPG III C) or fivefold (for Si-100 C). The specific surface areas and pore volumes increased and the values of D_{cyl}^{ads} and D_{cyl}^{des} decreased, which means that the narrowest pores were unblocked.

Table VII gives the capacity factors of three groups of substances measured on the columns filled with Si-100 C and CPG III C at long intervals of time. Despite the loss of Carbowax between the separated measurements, the capacity factors were fairly stable. A possible explanation of the observed phenomenon is the assumption that not all Carbowax chains are bonded with the surface in the same way or with the same strength. It is highly improbable that the bulk polymer remains in the Carbowax layer after the washing procedure. However, it is possible that some Carbowax chains are connected with the support surface all over the length of the chain and some of them are bonded only partially. The remaining parts of the latter could protrude over the layer parallel to each other and form a crystalline structure, as confirmed by X-ray diffraction (see Part I [11]). These not entirely bonded chains could be solvolysed only during the chromatographic process. The X-ray patterns measured after the chromatographic studies using CPG III C and Si-100 C did not reveal any crystalline form.

It is probable that in the Carbowax 20M layer remaining on the surface after prolonged operation of the column all chains are bonded through their lengths and they are non-extractable. The mean thickness of the layer (3.3 Å for CPG III C and 2 Å for Si-100 C) is an argument for this interpretation. Aue *et al.* [28] reported that the thickness of the Carbowax 20M layer obtained on silica gel of specific surface area 140 m^2/g is 2 A. Unfortunately they did not discuss this value, which conflicts with the value they obtained on Chromosorb W (15 A) [8]. The latter value is similar to those obtained here for materials after the washing procedure but before being used in HPLC columns.

CONCLUSIONS

Siliceous materials with immobilized Carbowax 20M can be applied in normal-phase HPLC. There are limitations connected with analyses of amines and other compounds that react with aldehyde, carboxyl or oxirane groups (as in GC analysis).

The results obtained show that the hydrophobic properties of the bonded Carbowax layer are weaker than those of typical RP sorbents. Further experiments on the determination of the optimum mobile phase composition (ensuring better selectivity of the RP system with immobilized Carbowax) seem to be desirable.

The materials with immobilized Carbowax 20M seem to be more effective than the sorbents with the diol phase for the separation of proteins.

The retention data for materials with an immobilized Carbowax layer are constant despite the partial removal of the stationary phase during operation of the column. Probably after a long period of column operation a real non-extractable layer of Carbowax remains on the support surface. It is also possible that directly after synthesis the sorbents contain Carbowax chains bonded with the surface in a different way and with various strengths. Weakly bonded PEG chains are removed from the sorbent surface during HPLC column operation. This problem requires further investigation.

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