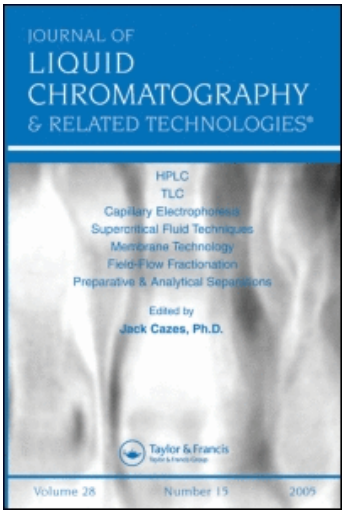

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Boguslaw Buszewski[a]; Iwona Cendrowska[b]; Katarzyna Krupczynska[a]; Renata M. Gadzala-Kopciuch[a]
[a] Department of Environmental Chemistry and Ecoanalytics, Faculty of Chemistry, Nicholas Copernicus University, Torun, Poland [b] Pharmaceutical Company GEMI, Karczew, Poland

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Bronopol as an Ingredient of a New Test Mixture for Evaluation of HPLC Columns

Boguslaw Buszewski,[1,*] Iwona Cendrowska,[2] Katarzyna Krupczynska,[1] and Renata M. Gadzala-Kopciuch[1]

[1]Department of Environmental Chemistry and Ecoanalytics, Faculty of Chemistry, Nicholas Copernicus University, Torun, Poland
[2]Pharmaceutical Company GEMI, Karczew, Poland

ABSTRACT

Different tests, including analytes or mixtures characterized by specific properties, can be used for the evaluation of chromatographic columns. High performance liquid chromatographic (HPLC) columns with chemically modified surfaces, purchased from different producers, were tested in our studies. Characteristic chromatographic parameters, such as: number of plate theoretical (N_T), reduced plate height (h), the retention factor (k), resolution (R_S), and asymmetry factor (f_{AS}) were determined. *Bronopol* (2-bromo-2-nitrpropane-1,3-diol) was suggested for column evaluation, especially as regards the homogeneity of adsorbent surface coverage by chemically

*Correspondence: Boguslaw Buszewski, Department of Environmental Chemistry and Ecoanalytics, Faculty of Chemistry, Nicholas Copernicus University, 7 Gagarin St., 87-100 Torun, Poland; E-mail: bbusz@chem.uni.torun.pl.

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bonded phase and spacing of residual silanols. This analyte was used separately, and in a mixture consisting of other analytes (uracil, acetophenone, benzene, toluene) with different physicochemical properties.

Key Words: Column evaluation; RP–HPLC; New test mixtures; Chemometry; Surface homogeneity.

INTRODUCTION

The application of high performance liquid chromatography (HPLC) for solving analytical problems guarantees high precision, speed, efficiency, and low costs of a single analysis. It is of essential significance in routine analyses, time, standardization, and validation, where the elaboration of unique procedures and standards is necessary. Column selection plays an important role in the optimisation of separation conditions. The column quality depends on a number of factors, among which the most important are: (i) homogeneity of particle packing in the column bed and (ii) homogeneity of the arrangement of chemically bonded phase on the adsorbent surface. These factors determine the reproducibility of chromatographic data and, as a consequence, resolution. The interpretation of the data obtained is difficult, sometimes impossible, because columns, even when supplied by the same manufacturer and are nominally identical, show different chromatographic properties.[1]

Advanced physicochemical techniques, such as porosimetry, elemental analysis, ^{29}Si and ^{13}C CP/MAS NMR, FTIR, differential scanning calorimetry, and others, can be applied for surface characterization,[1–9] whereas, spectroscopic techniques may be used for packing characterization. Problems arise when we have to evaluate a chromatographic column. In this case, the tests included in professional literature or those suggested by producers are used. There is no ideal procedure enabling the evaluation of columns and packings with regard to physicochemical properties. That is why proper selection of a substance used for adsorbent (column packing) testing is so important.

Many tests for determining the quality of HPLC columns are described in professional literature. They are based on empirical, statistical, and thermodynamic methods.[1] Various authors propose mixtures containing compounds of a different chemical character, depending on the packing and mobile phase.[10,11] Daldrup and co-workers[12] recommend the application of a ternary mixture of diazepam, diphenhydramine, and 5-(*p*-methylphenyl)-5-phenylhydantoine (MPPH). Plate efficiency and resolution (difference in the retention of two compounds) are fixed by using diphenhydramine and MPPH. Hydrophobicity, silanol activity, and shape selectivity were added to a test by Enhgelhardt.[13] Tanaka and co-workers[14] recommend using basic

compounds, which through interactions with the stationary phase surface, allow estimating the quantity of metal atoms built into the silica structure. These interactions can be observed by the application of analytes (theobromine, theophylline, caffeine, phenol, prokariamide, *N*-acyloprokariamide, benzyl alcohol) as a test. Toluene, lutidine, and pyridine were suggested by Buszewski and co-workers[15] as a simple test for the examination of silanols on the adsorbent surface in a normal segment system (non-aqueous conditions). Claessens et al.[16] advise the use of the QSRR method. This method, suggested by Kaliszan and co-workers,[17,18] makes it possible to investigate the quantitative structure-chromatographic retention relationships. The producers of HPLC columns recommend the employment of different test mixtures for testing their quality (Eka Nobel, Supelco, Waters, etc.).[11]

A comparison of available tests shows, that in spite of the application of various analytes, eluents, experimental conditions, and computational procedures, there are still some difficulties in the simultaneous demonstration of positive and negative column properties.[16,19,20] Despite large-scale column testing, none of the tests has been considered "the best" until now, particularly as regards routine analyses. Complex tests are very useful,[14,17,21] but we would like to propose a simple test, which may be conducted in pure aqueous conditions (close to natural) and in a traditional hydroorganic system, typical of the reversed phase; however, without any additional components of the mobile phase, e.g. salt.[22]

A test mixture, consisting of a specific analyte *Bronopol* (2-bromo-2-nitropropane-1,3-diol) in the presence of other substances: uracil, acetophenone, benzene, toluene, was applied to column evaluation, especially to determine the homogeneity of adsorbent surface coverage by chemically bonded stationary phase, and the arrangement of residual silanols. The columns were selected according to the type of the terminal functional group, type of structure of chemically bonded phase, producer, and serial number.

EXPERIMENTAL

Equipment

Chromatographic measurements were performed on a liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of a LC-10ADvp pump, a diode array detector SPD-M10Avp, a SCL-10Avp system controller, and a computer with Class vp 5.0 software for data collection and control of the process. The solutes were injected with a Rheodyne (Berkeley, CA) model 7125 sampling valve with a 20 μL sample loop.

Reagents and Materials

Methanol (HPLC-grade) was purchased from J.T. Baker–S. Witko (Lodz, Poland). Water was prepared with a Milli-Q water purification system (Milipore, El Passo, TX). The test solutes were of various origins: bronopol (Pharmaceutical Research Institute, Warsaw, Poland), uracil, acetofenon (Fluka AG, Buchs, Switzerland), benzene, toluene, phenol (E. Merck, Darmstadt, Germany). The columns used in the test are presented in Table 1.

The columns were tested in two different systems. The experimental conditions are described in Table 2.

A quality assessment of the columns was based on retention parameters, such as: the column capacity ratio (k), plate number (N_T), reduced plate height (h), resolution (R_S), peak asymmetry (f_{AS}).

RESULTS AND DISCUSSION

Bronopol is a propane derivative where two symmetrically placed —OH groups, one —Br group and one $-NO_2$ group, were introduced instead of hydrogen (Fig. 1). During the chromatographic process, the presence of these hydroxyl groups causes the "settlement" of this analyte on the neighboring free silanols, due to hydrogen interactions. Taking into consideration the fact that the length of a bronopol molecule, in optimal conformation, is equal to 10.25 Å and the shortest distance to the neighboring silanol centres is 4.5–5.0 Å, the *arcade* formed in this way can be a measure of the homogeneity of adsorbent surface coverage by the organic stationary phase. Probably $-NO_2$ groups combine, through the donor–acceptor interactions, with heteroatoms (metal impurities), which can be present on the surface of the silica adsorbent. If its surface is well covered by chains of bonded phase (hydrophobic or other alkilosilanes with polar terminal groups, e.g., —OH, —CN, $-NH_2$) we can expect an acute, symmetrical peak, corresponding to the Gaussian curve ($f_{AS} = 1.0 \pm 0.05$). When the surface is heterogeneous and there are bald heads (places without chemically bonded organic ligands), band broadening or even extra peaks can be observed (Fig. 2). The characteristic parameters obtained for new and previously used columns are listed in Table 3. The character of the bronopol band for the old column changes in a distinct way. The peak asymmetry (f_{AS}) for the new column amounted to 1.09 and for a column after 220 injections — to 5.21. Rapidly, the bronopol elution increased by about 30% in relation to the new column. The number of theoretical plates decreased by about 70%. Complementing this test with other hydrophobic non-polar compounds consisting of carboxyl, amine, and ketone groups, shows, in a better way, what kind of processes take place during

Table 1. Columns descriptions.

No	Packing	Producer	Serial number	Column dimension (mm)	Particle size (μm)
1	Supelcosil LC-18	Supelco	018604 AM	250 × 4.6	5.0
2	Supelcosil LC-18 DB		088140 AK	250 × 4.6	5.0
3	Supelcosil LC-18 DB		088148 AK	250 × 4.6	5.0
4	Supelcosil LC-18 DB		088228 AL	250 × 4.6	5.0
5	Supelcosil LC-18 DB		088964 AK	250 × 4.6	5.0
6	Supelcosil LC-18 DB		088901 AK	250 × 4.6	5.0
7	Supelcosil LC-18 DB		588297 AL	250 × 4.6	5.0
8	Ultrapack RP-18	LKB	9595	250 × 4.6	5.0
9	LiChrospher RP-18	Merck	556144	250 × 4.6	5.0
10	Kromasil ODS	Eka Nobel	3193	250 × 4.6	5.0
11	BondaPack c-18	Waters	81085	250 × 4.6	5.0
12	TSK Gel ODS-8-TM	TosoHaas	8TM2T3249	250 × 4.6	5.0
13	C18	Serva	11059/1	250 × 4.6	5.0
14	Supelcosil LC-CN	Supelco	071396 AD	250 × 4.6	5.0
15	Supelcosil LC-DP phenyl (C6)		58842C46	250 × 4.6	5.0
16	Supelcosil LC-NH2		58358C46	250 × 4.6	5.0
17	SG-AP	UMK, Toruń	#1/BB/97	250 × 4.6	5.0
18	SG-MIX	UMK, Toruń	#2/BB/97	150 × 4.6	5.0
19	Supelcosil ABZ + Plus	Supelco	323344 AB	150 × 4.6	5.0
20	Symmetry Shield TMRP8	Waters	105	150 × 4.6	5.0
21	Symmetry TMC18	Waters	129	150 × 4.6	5.0
22	Luna C18	Phenomenex		250 × 4.6	5.0

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Table 2. Conditions of columns testing.

SYSTEM I (*evaluation of chromatographic band shape*)	SYSTEM II (*evaluation of selectivity and resolution*)
Test substance: bronopol (20 μg/1 mL)	Test substance: uracil (7 μg/1 mL), bronopol (50 μg/1/mL), acetophenone (7 μg/1 mL), benzene (750 μg/1 mL), toluene (750 μg/1 mL)
Mobile phase: water	Mobile phase: methanol, water (6 : 4 v/v)
Wavelenght: 210 nm	Wavelenght: 254 nm
Flow rate: 1 mL/min	Flow rate: 1 mL/min

chromatographic elution. A new test mixture for column evaluation was proposed as a result of our own testing and literature studies.[1,16,23–25]

The characteristic parameters obtained for the same columns, but with a different serial number, are included in Table 4. The results indicate that in pure aqueous conditions, bronopol shows different retention parameters and peak asymmetry for the same type of columns, but with a different serial number. The retention ranges from about 14.1 to 16.3 min, and the flow rate is 1 mL ± 0.001 mL/min. The peak shape is also different. Only in four cases does the asymmetry factor (f_{AS}) correspond to the theoretical assumption ($f_{AS} = 0.9$–1.25); in the other cases it is too high. This suggests unrepeatable preparation of C_{18} stationary phase, rather than the presence of adsorbent

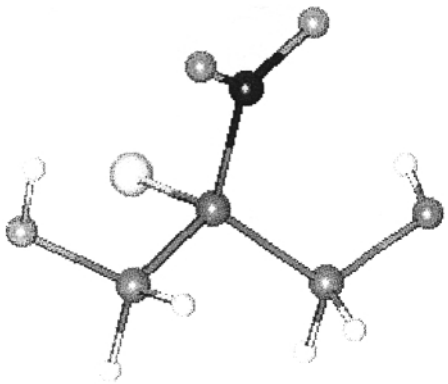

Figure 1. Model of *Bronopol* molecule.

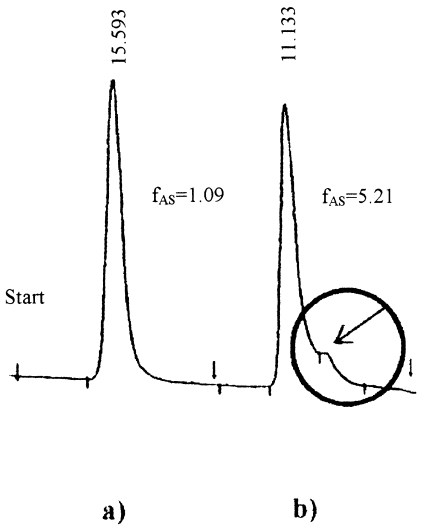

Figure 2. Chromatograms of bronopol obtained on Supelcosil LC-18 DB column: new (a), after 220 injections (b).

surface impurities (heteroatoms). The reduced plate height (h) varies from 4.63 to 9.02. The capacity ratios are normal ($k < 10$) in all cases. It is surprising that k values for the columns investigated are similar (except one), but h values are not comparable (the differences between columns reach 100%). It should be noted, that the material used for column packing was not homogenous and was characterized by low reproducibility of surface properties. Only one column

Table 3. Characteristic parameters for Supelcosil LC-18 DB (Supelcon, no 888140 AK) new and after injections.

Quantity of injections	Capacity ratio (k)	Number of theoretical plates N_T	Reduced plate height (h)	Peak asymmetry (f_{AS})
New	6.56	8520	5.87	1.09
120	5.50	6793	7.36	1.10
150	4.94	4167	11.99	1.37
200	4.94	3341	14.96	3.67
220	4.40	2460	20.32	5.21

Note: Test substance: bronopol; mobile phase: water; flow rate: 1 mL/min; detection: 210 nm; $k = t_R - t_0/t_0$, $t_0 = 2.06$ (solvent peak).

Table 4. Characteristic parameters for new Supelcosil LC-18 DB column (chromatographic conditions: System I; see Table 2).

No	Serial number	Capacity ratio (k)	Reduced plate height (h)	Peak asymmetry (f_{AS})
1	088140 AK	6.56	5.87	1.09
2	088148 AK	8.54	8.79	1.11
3	088228 AL	9.89	9.02	1.48
4	088964 AK	9.65	8.03	1.40
5	088901 AK	9.59	7.56	1.10
6	588297 AL	8.96	4.63	1.03
Average value ($\bar{X}$)		8.87	7.32	1.20
Standard deviation (δ)		1.23	1.73	0.19

(serial number 588297 AL) satisfied all requirements described by Knox and Bristow[26] for the so called good column.

The characteristic parameters obtained in system I (Table 2), and for new columns with C_{18} stationary phases, are presented in Table 5. These results show that parameters k, h, f_{AS} are different for different columns (the capacity ratios are in a range from 3.49 to 13.8, the reduced plate height is in a range from 1.7 to 14.6, and the peak asymmetry—from 0.92 to 1.35) of chromatograms in Fig. 3. This confirms, that in the case of columns purchased from the same manufacturer, (i) materials used as stationary phases are characterized by different surface properties (carbon load, coverage density, porosimetric parameters, quantity of residual silanols, concentration of metal impurities),

Table 5. Characteristic parameters for new C18 columns purchased from different producers.

No	Name of column	Capacity ratio (k)	Reduced plate height (h)	Peak asymmetry (f_{AS})
1	Supelcosil LC 18 /Supelco	4.92	4.34	1.25
2	Bondapack C 18 /Waters	13.8	1.73	1.12
3	Ultrapack RP C18 /LKB	3.49	14.6	1.35
4	LiChrospher RP-18 /Merck	7.22	7.4	1.09
5	Kromasil ODS /Eka Nobel	9.66	5.10	1.23
6	C 18 /Serva	8.90	9.10	0.92
7	TSK Gel ODS-80TM /TosoHaas	11.9	3.04	1.01

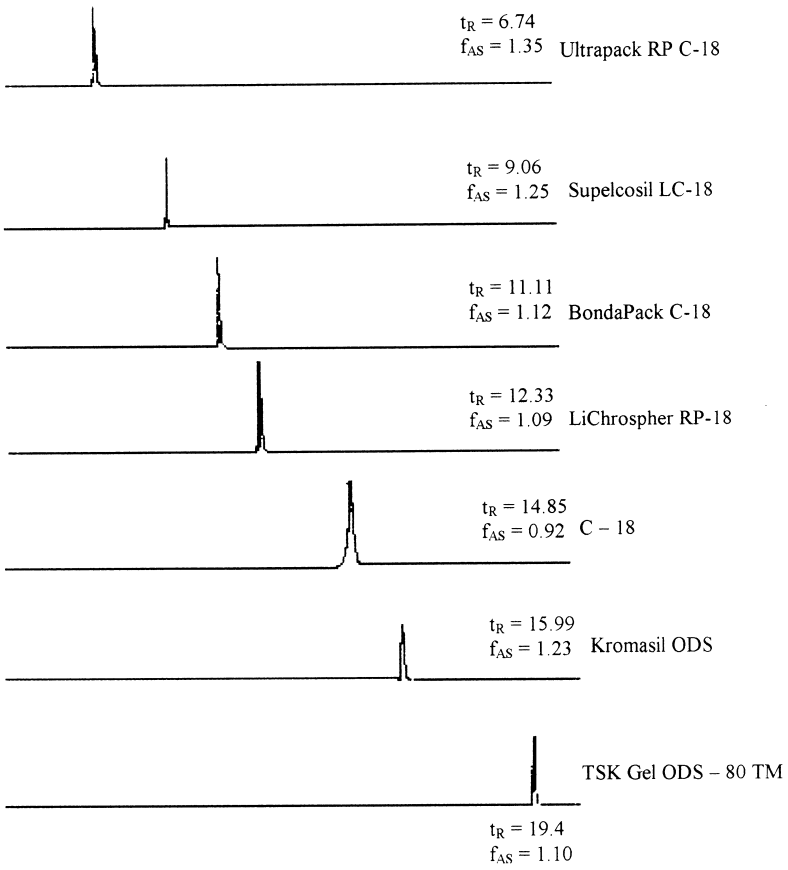

Figure 3. Chromatograms of bronopol obtained on C_{18} columns.

(ii) chemical nature of bonded ligands should be characterized more precisely, e.g., by CP/MAS NMR, FTIR.

The parameters of columns with stationary phases: C_{18}, C_{18} DB, —CN, $-NH_2$, $-C_6H_5$, AP, MIX are given in Table 6. These columns show different selectivity and behaviors in relation to various analytes, which is probably caused by different modification procedures, coverage density, and a different type of silica. The quantity of free silanols changes the character of columns, which is visible for LC-18 and LC-18 DB columns. A higher concentration of free silanols probably causes worse resolution (LC-18 column), because of non-reversible sorption of analyte and difficulties in mass transfer. The elution rapidity is one of its signs. Comparable resolution and elution rapidity for the

Table 6. Characteristic parameters for different packings (chromatographic conditions: System II; see Table 2).

No.	Type of column	Retention time (t_R)					Resolution (R_s)			
		Uracil	Bronopol	Acetophenone	Benzene	Toluene	b/u[a]	b/a[b]	b/b[c]	b/t[d]
1	Supelcosil LC18	2.94	3.15	4.56	6.18	9.06	1.33	7.92	15.2	24.19
2	Supelcosil LC18DB	2.63	3.01	5.52	9.67	16.89	2.3	12.6	26.34	37.94
3	TSK Gel ODS-80TM	3.18	4.66	10.48	17.94	28.58	4.37	14.5	25.0	35.96
4	Ultrapack RP-18	2.35	2.48	4.2	6.06	9.24	0.67	9.66	6.09	15.05
5	LiChrospher RP-18	2.32	3.34	8.91	15.56	26.49	3.33	13.4	23.09	39.15
6	Supelcosil LC-DP Phenyl (C_6)	3.7	2.65	3.7	4.03	4.03	2.69	2.69	3.67	3.67
7	Supelcosil LC-CN	3.0	3.39	3.65	3.89	3.89	1.38	1.38	2.66	3.69
8	Supelcosil LC-NH_2	2.48	2.48	2.48	2.48	2.48	0	0	0	0
9	SG-AP	1.66	1.66	2.09	3.11	3.11	0	1.99	4.52	6.53
10	SG-MIX	1.38	1.61	1.83	2.36	2.36	1.51	1.0	3.07	5.66
11	Luna C18	2.27	2.51	3.31	4.0	9.57	1.34	3.63	6.28	23.8

[a]Resolution between bronopol and uracil peak.
[b]Resolution between bronopol and acetophenone peak.
[c]Resolution between bronopol and benzene peak.
[d]Resolution between bronopol and toluene peak.

analytes tested were obtained on a TSK Gel ODS-80 and LiChrospher RP-18 column. Comparable elution rapidity, but totally different resolution and, in consequence, different selectivity was received for a Supelcosil LC-18 and Ultrapack RP-18 column. The resolution between uracil and bronopol peaks for the first column was equal to $R_S = 0.67$. Excellent separation of analytes was observed for the Supelcosil LC-18 column within a relatively short retention time. Analyte coelution took place in the case of a LC-DP C_6 column whose surface was clogged by phenyl rings. Bronopol shows the shortest retention time ($t_R = 2.65$). Good separation of compounds on a Supelcosil LC-CN column can be achieved in a short time (about 4 min), which is probably caused by π–π and donor–acceptor interactions between stationary phase and analyte. One peak was obtained for Supelcosil LC-NH2 column during 2.5 min. This was most probably caused by complete clogging of the surface by analyte molecules (a non-selective system of analyte separation). Excellent separation takes place for mix stationary phases consisting of polar and non-polar groups, such as: hydroxyl (—OH), amine ($-NH_2$), cyane (—CN), phenyl (—Ph), octadecyl ($-C_{18}$), octyl ($-C_8$). This may indicate π–π interactions and isolated participation of donor–acceptor and hydrophobic interactions.

All results testify to a different character of interactions between analytes, mobile phases, and stationary phases. They also confirm the fact that different chromatographic properties are observed, not only for columns with different packings, but even those supplied by the same producer.[1,15,16,23,24]

An analysis of the relationship presented in Fig. 4 indicates that it can be used for comparing different columns and finding similarity between the columns from Table 6. Columns no. #3 and #5, #8 and #10, #6 and #7, #1 and #4 are very similar. Probably similar properties of silica support of chemically bonded stationary phase play an important role in these cases. Columns no. #2 and #9 differ considerably from the remaining ones. Different properties of stationary phases with chemically bonded organic ligands are the reason for the differences between nominally identical columns (e.g., #1 and #5). The procedure of stationary phase synthesis, and, in consequence, coverage density, also influence the quality of packing material. This is confirmed by the results obtained for columns supplied by the same producer, but with a different serial number.

CONCLUSIONS

A new compound of the test mixture-bronopol (2-bromo-2-nitrpropane-1,3-diol), proposed for evaluating chromatographic columns, can be very useful in

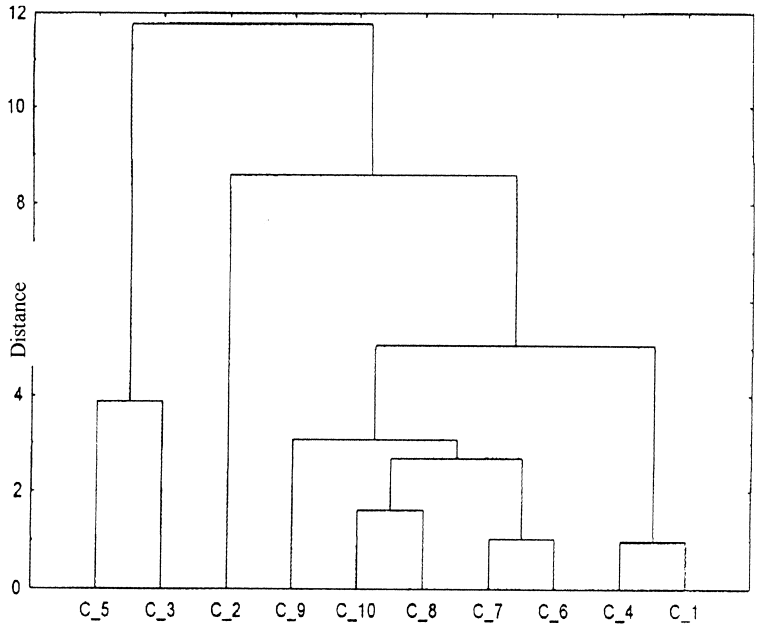

Figure 4. Relationship between the type of column and characteristic chromatographic parameters (according Table 6).

quick routine investigations.[22] Certainly, most of the known testing substances also enable a good evaluation of columns. However, their disadvantages are a long procedure and the necessity to apply properly selected mobile phase, e.g., buffer solutions, which is connected with column pre-washing and system stabilization. Bronopol is not aggressive and does not destroy the column. It can be used alone, or in a mixture, allowing estimating the column quality in a quick and simple way, which is very important in routine work at an analytical laboratory.

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