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Comparative study of test methods for reversed-phase columns for high-performance liquid chromatography

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Abstract

Column selection in reversed-phase liquid chromatography (RPLC) is still not a straightforward process. A number of tests to characterise and classify RPLC columns have been suggested. Several tests are already applied in laboratory practice, while others are under development. The results of the various tests, however, are not always qualified to describe the properties of columns for RPLC. In this study different tests for RPLC-columns are studied and compared, viz. the Engelhardt, Tanaka, Galushko and Walters tests. The column descriptors hydrophobicity and silanol activity are investigated in particular. The tests are studied using approximately 20 silica, alumina and polymer based C_8- and C_{18}-columns. Hydrophobicity data from the tests generally were good and interchangeable between the tests resulting in a column classification that is independent of the applied test. It appears that buffering of the eluent is mandatory for adequate testing of column silanol activity. In contrast with the high-quality hydrophobicity data, the silanol activity results of the various tests differ significantly. As a consequence column classification with respect to silanol activity depends considerably on the applied test method.

Keywords: Stationary phases, LC; Reversed-phase columns; Column classification

1. Introduction

The continuous growth in the use of reversed-phase liquid chromatography (RPLC) techniques in many different fields has furthered the need for new generations of RPLC-phases offering better chemical stability, improved selectivity and efficiency. This is in fact one of the major driving forces of the continuous efforts of academic and manufacturers to synthesize generations of RPLC-phases that meet these requirements.

The wide variety of the presently available RPLC-phases often differs in its ligand types and the way these are bonded to the substrate. More importantly, however, the polar and ionic properties of RPLC-phases are responsible for secondary interaction mechanisms and often determine the unique character of an RPLC-phase. The present situation can be characterized by the availability of a substantial number of RPLC-phases, that may differ greatly in their selectivity and other chromatographic properties. This, fortunately, facilitates the solution of various different separation problems via stationary phase selection. By contrast, this large number of

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potential candidate RPLC-columns often leaves the analyst with the difficult problem of a proper column selection for a specific problem. This situation is further complicated by the fact that many of these RPLC-phases are nominally identical, suggesting that they may have similar chromatographic properties. Gonnet [1] and more recently Sandi [2], Barrett [3] and Cruz [4] have shown the great differences in chromatographic properties that may occur between RPLC-columns. At the same time the study of Cruz also showed the partial similarity in these properties between specific groups of RPLC-phases. In most cases the available technical information is not sufficient to objectively select the optimum column for a particular separation. Furthermore, since manufacturers use different tests and evaluation parameters too for their columns, their product claims are difficult to compare.

The necessity to distinguish between the chromatographic properties of RPLC-columns to make a proper column selection has prompted many researchers to work on evaluation methods for RPLC-phases. From the beginning of the development of RPLC-phases, extensive research was done on evaluation methods, resulting in a substantial number of books and papers on this issue [4–10]. In addition, lively debates are still ongoing regarding the improvement of existing and the development of new testing methods for RPLC-phases.

The presently available evaluation methods for RPLC-phases can be subdivided into several groups:

1. Determination of physico–chemical properties of the bulk stationary phase.
2. Spectroscopic techniques, like infrared (IR) and solid-state nuclear magnetic resonance (NMR) spectroscopy.
3. Evaluation of chromatographic data using statistical methods.
4. Thermodynamic measurements, e.g. Van't Hoff plots.
5. Chromatographic test methods.

The physical properties of substrates and stationary phases for RPLC are dominant in determining column efficiency and retentivity. Therefore, for the synthesis of well-defined and reproducible RPLC-phases these properties must be known and properly controlled during the production of these materials. In many papers and books these aspects and methods to determine the most important physical properties have appeared, viz. particle size and shape, specific surface area, pore size and porosity, and particle strength [5,6,11–15].

Amongst the spectroscopic methods, especially ^{29}Si and ^{13}C solid-state nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy have significantly advanced the development of RPLC-phases. With infrared (IR) spectroscopy, specific information can be obtained on the occurrence of isolated and bonded or associated silanol groups in silica substrates and on bonded phases as well. IR-spectroscopy techniques provide rather simple procedures for the study of reactions and reaction kinetics in the synthesis of RPLC-phases [16–18]. ^{29}Si and ^{13}C NMR techniques provide detailed information on the different groups present on substrate and chemically modified surfaces. In contrast to IR-spectroscopy, where isolated and geminal silanols absorb at nearly the same wavenumber, NMR techniques can distinguish between different types of silanol groups. Furthermore, the latter techniques can also provide detailed information about the nature of ligand bonding to the surface. Therefore, NMR techniques have become indispensable tools in the study of the synthesis of RPLC-phases and the fate of RPLC-phases under chromatographic conditions [7,12,19–21].

Statistical methods can be useful, e.g. to cluster groups of RPLC-phases of similar chromatographic properties. Such methods can effectively facilitate column classification and selection [4,22].

Plotting the log retention factor of a compound versus the reciprocal absolute temperature results in a so-called Van't Hoff plot. Such plots provide information on the thermodynamic driving forces in chromatographic separations as a function of the experimental conditions, e.g. eluent composition [23–25].

In a number of studies several attempts have been made to correlate and to predict chromatographic column properties from the data of the characterisation methods 1–4 [2,4,10,16,22,26,27].

From these studies it has become obvious that until now none of these methods has been able to distinguish between the often subtle but decisive differences in chromatographic properties of RPLC-phases in any detail. From numerous application

examples it can be learned that such apparently minor differences in the chromatographic properties between RPLC-phases very often are decisive for the success or failure in the development of a separation method.

In spite of the great benefits of the techniques categorized in the characterisation groups 1–4 in the development and characterisation of RPLC-phases, it is clear that an adequate column selection must be made using chromatographic characterisation methods. A number of such evaluation methods have been suggested during the last decades. Chromatographic evaluation methods can roughly be subdivided into two groups.

i : Empirically based evaluation methods. These methods have in common that the obtained chromatographic information depends on rather arbitrarily selected test compounds, which are supposed to reflect a specific column property, e.g. silanol activity. Important representatives of this group stem from methods developed by Tanaka [10], Engelhardt [28,29], Eyman [30], Walters [31], Daldrup [32], and also the use of retention indices [33] and many so-called 'in-house' methods.

ii : Model-based evaluation methods. The methods of this group share the fact that they are based on a specific model, e.g. the silanol scavenging model of Horvàth [34], the interaction indices model of Jandera [33], the solvatic computational model of Galushko [35] and the Quantitative Structure Retention Relationships (QSRR) model applied by Abraham, Carr, Bolliet and Kaliszan [26,27,36–39] among others.

Till now however, none of these evaluation methods has been widely accepted as a uniform method for RPLC-phases. Furthermore, no general consensus exists either regarding eluents and test substances or the experimental conditions and calculation procedures in column test protocols. This lack of uniformity severely hampers the objective comparison and classification of RPLC-columns. A further severe constraint also contributing to this problem lies in the many different application areas, where RPLC-columns are applied and where substances of very different chemical nature and size are separated. The majority of the presently available evaluation methods for RPLC-columns has been developed specifically for narrow pore phases using small molecular test solutes. Some studies have shown that column characteristics obtained from small molecular test compounds do not necessarily provide the required information for a proper column selection for the separation of larger molecules [8,9].

In sum, the present number of test methods for RPLC-columns applying different test compounds, eluents, experimental conditions and calculation procedures, does not contribute to more uniform and validated test protocols, which is not in the interest of objective column characterisation and classification.

This paper seeks to compare a number of test methods for RPLC-columns. Our goal was to compare the information from the different tests and to discriminate between these data. The objective was to find out to what extent the tests may provide similar, overlapping or contradicting information. In this study we included a number of tests that are already routinely applied in laboratories. Our selection of test methods was intentionally limited by excluding test methods, of which the interpretation is based exclusively on visual inspection of chromatograms. The study was performed on a large number of different RPLC-phases, silica-based and alumina and polymer-based phases as well, representing a substantial part of the currently used spectrum of RPLC-columns.

2. Summary of column tests

The investigated test procedures and output used in this study are summarised below.

With the exception of the Engelhardt test, the testing protocols were strictly followed. In opposition to what was formulated in the original Engelhardt test, in this study the asymmetry for *p*-ethylaniline was measured at 5% peak height (calculated from USP-tailing factor). This is because peak asymmetry detection is more sensitive at lower peak heights and the calculation algorithm is available in many software programs. If no testing temperature was specified, tests were performed under constant arbitrarily selected temperature conditions of 40°C.

For further detailed information on the different tests the reader is referred to the literature.

1a. Engelhardt test, (E test) [28,29].

i. Eluent: methanol/water 49:51 (w/w) or 55:45 (v.v). Temperature 40°C.

Test compounds: uracil (t_0), aniline, phenol, *N*,*N*-dimethyl-aniline, *p*-ethylaniline, toluene and ethylbenzene.

Output:

Hydrophobicity=$k_{\text{ethylbenzene}}/k_{\text{toluene}}$

Silanol activity=asymmetry of *p*-ethylaniline at 5% of peak height

k=retention factor

ii. Eluent: methanol/water, 75:25 (w/w) or 79:21 (v/v). Temperature 40°C

Test compounds: uracil (t_0), triphenylene and *o*-terphenyl.

Output:

Shape selectivity=$k_{\text{triphenylene}}/k_{o\text{-terphenyl}}$

1b. Modified Engelhardt test, (E_m)

Eluent: methanol/aqueous 0.02 *M* phosphate buffer, pH=7.0, 49:51 (v/v) or 55:45 (w/w).

Temperature: 40°C

Output:

Silanol activity=asymmetry of *p*-ethylaniline at 5% of peak height.

2. Walters test, (W test) [31].

Hydrophobicity test

Eluent: acetonitrile/water 65:35 (v/v). Temperature 40°C.

Test compounds: uracil (t_0), benzene, and anthracene.

Silanol activity test

Eluent: acetonitrile. Temperature 40°C.

Test compounds: *N*,*N*-diethyl-*m*-toluamide (DETA) and anthracene.

Output:

Hydrophobicity=$k_{\text{anthracene}}/k_{\text{benzene}}$

Silanol activity=$k_{N,N\text{-diethyltoluamide}}/k_{\text{anthracene}}$

3. Tanaka test, (T test) [10].

Eluent 1: methanol/water: 80:20 (v/v)

Eluent 2: methanol/water: 30:70 (v/v)

Eluent 3: methanol/aqueous 0.02 *M* phosphate buffer pH=7.6, 30:70 (v/v)

Eluent 4: methanol/aqueous 0.02 *M* phosphate buffer pH=2.7, 20:70 (v/v)

Temperature 40°C.

Test compounds: uracil (t_0), thiourea (t_0), amylbenzene, butylbenzene, triphenylene, *o*-terphenyl, caffeine, phenol and benzylamine.

Output:

Hydrophobicity=$k_{\text{amylbenzene}}/k_{\text{butylbenzene}}$, (Eluent 1).

Amount of alkyl chains=$k_{\text{amylbenzene}}$, (Eluent 1).

Steric selectivity=$k_{\text{triphenylene}}/k_{o\text{-terphenyl}}$, (Eluent 1).

Hydrogen bonding capacity=$k_{\text{caffeine}}/k_{\text{phenol}}$,=$\alpha_{\text{c,p}}$ (Eluent 2).

ion-exchange capacity (IEC) at pH>7=$k_{\text{benzylamine}}/k_{\text{phenol}}$,=$\alpha_{\text{a,p}}$ (Eluent 3).

IEC at pH<3=$k_{\text{benzylamine}}/k_{\text{phenol}}$,=$\alpha_{\text{a,p}}$ (Eluent 4).

4. Galushko test, (G test) [35].

Eluent: methanol/water 60:40 (v/v). Temperature 30°C.

Test compounds: uracil (t_0), aniline, phenol, benzene, toluene.

Output:

Hydrophobicity=$(k_{\text{toluene}}+k_{\text{benzene}})/2$

Hydrophobic selectivity: calculated from the phenol, toluene and benzene retention data.

Silanol activity=$1+3\ [(k_{\text{aniline}}/k_{\text{phenol}})-1]$

Size selectivity: calculated from the retention data of benzene, phenol and toluene.

5. Column hydrophobicity by log k_w-measurements

Eluents: methanol/water mixtures in the range of 20% to 95% methanol (v/v). Temperature: 40°C.

Test compound: hexylbenzene.

By linear extrapolation of the k-values measured at several modifier concentrations on the different columns, the log retention of the test compound in pure water (log k_w) can be obtained [40,41].

3. Experimental

3.1. Equipment

Chromatographic measurements were performed on two HP 1100 liquid chromatographs (Hewlett Packard, Waldbron, Germany), operating at the same temperature (21°C) in a conditioned laboratory. These automated instruments were equipped with

Table 1
List of tested C_{18}-columns and their physico–chemical properties

Column	RX	XC18	Puro	Hyper	HyPUR	Sym18	Poly	NuC18	Krom	All	TPW	TTS
Particle size (μm)	5.2	5	5.8	4.5–5	4.5	4.95	5.2	5.4	6.2	6.18	5	5
Pore size (Å)	80	80	120	120	180	93	x	115	x	111.9	125	80
Pore volume (ml g^{-1})	0.45	0.4	1.0	0.6	1.0	0.66	0.85	1.15	0.91	0.88	x	x
Surface area (m^2 g^{-1})	180	180	350	170	200	332	350	340	349	316	x	198
Carbon loading (%)	12	10.3	18	9.5	13	19.4	x	21.0	21.4	16.22	x	15
Surface coverage (μmol m^{-2})	3.3	3.5	3.2	x	x	3.21	x	3.60	3.45	x	x	x
Bulk density (g ml^{-1})	1.0	1.0	0.4	x	x	x	x	0.36	x	x	ca.1	x
Bonded chemistry	dimethyl -C_{18}	dimethyl- -C_{18}	tri-functio-nal	tri-functio-nal	mono-functio-nal	x	not purely mono meric	mono-meric	mono functio-nal	poly meric	mono-meric	mono-meric
Endcapping	no	double	yes	yes	yes	x	x	yes	yes	double	no	yes
Silica	RX-sil	RX-sil							poly-ester silica		meth-acrylat co-polymer	high purity

x: data not available.

diode-array detectors and an HP ChemStation for process control and data handling. Injection of 1 μl of the test solutions were made by the automated injection devices. Detection was performed at 254 nm using standard detector cells of 13 μl (optical path length 10 mm). To find out whether systematic errors might obscure our measurements in either of the applied instruments initial tests were performed. Mutual comparison by testing several columns on both instruments under similar experimental conditions did not reveal any significant deviation in the performance (plate number, peak asymmetry, retention factor) of either instrument.

3.2. Chemicals and solutions

Methanol and acetonitrile (supra-gradient quality) were from Biosolve (Bio–Lab, Jerusalem, Israel). Water was prepared with a Milli-Q water purification system (Millipore, Milford, MA, USA). Buffers were prepared with disodiumhydrogen phosphate, phosphoric acid and sodiumhydroxide from Merck (Merck, Darmstadt, Germany). To perform all tests uniformly and to obtain detection signals in between 10 and 100 mAufs at injection volumes of 1 μl, solutes were dissolved in concentrations presented in parenthesis in the next sentence. Uracil (0.2 mg ml^{-1}), thiourea (0.2 mg ml^{-1}), *n*-hexylbenzene (1 mg ml^{-1}), anthracene (0.1 mg ml^{-1}) and *N*,*N*-diethyl-*m*-toluamide (0.05 mg ml^{-1}) were from Fluka (Fluka Chemie AG, Buchs, Switzerland). Aniline (1 mg ml^{-1}), *p*-ethylaniline (2 mg ml^{-1}), *N*,*N*-dimethylaniline (0.4 mg ml^{-1}), and phenol (2 mg ml^{-1}) were from Merck. Caffeine (0.5 mg ml^{-1}), benzene (10 mg ml^{-1}), toluene (10 mg ml^{-1}), ethylbenzene (10 mg ml^{-1}), butylbenzene (5 mg ml^{-1}), amylbenzene (5 mg ml^{-1}), *o*-terphenyl (0.2 mg ml^{-1}) and triphenylene (0.2 mg ml^{-1}) were from Aldrich (Aldrich Chemical Comp. Inc., Milwaukee, WI, USA) and benzylamine (1 mg ml^{-1}) was from Janssen (Janssen Pharmaceuticals, Beerse, Belgium).

3.3. Columns

The columns used in these tests were kindly provided by the manufacturers and are summarised in Tables 1 and 2 for the C_{18}-and C_8-phases, respectively, together with some of their physico–chemical properties. For practical reasons the polybutadiene coated column (Alu) is treated in the group of C_8-columns. Table 3 lists the manufacturers of the columns, column dimensions, abbreviations and numbers used in this work.

3.4. Calculations

All column characteristics were calculated following the definitions in the various tests. The results of the Galushko test were obtained using the software program Chromlife (Merck, Darmstadt, Germany). Regression calculations were made using the soft-

Table 2
List of tested C_8-columns and their physico–chemical properties

Column	XC8	SelB	Alu	Sym8	Nova	NuC8
Particle size (μm)	5	5.5	5	5.07	4	5.4
Pore size (Å)	80	90	100	89	75	115
Pore volume (ml g^{-1})	0.4	0.9	0.5	0.65	0.30	1.15
Surface area (m^2 g^{-1})	180	360	170	343	120	340
Carbon loading (%)	7.2	11.5	7	14.4	4.0	8.0
Surface coverage (μmol m^{-2})	3.7	3.5	coated	3.35	x	2.60
Bulk density (g ml^{-1})	1.0	0.4	0.45	x	x	0.36
Bonded chemistry	dimethyl C_8	bi-functional	poly butadiene	x	x	mono-meric
Endcapping	double	no	no	x	yes	no
Silica	RX-sil					

x: data not available.

Table 3
List of column manufacturers and abbreviations

Column	Manufacturer	Dimensions L x I.D. (mm×mm)	Abbr.	No.
C_{18}-Columns				
Zorbax RX-C18	Hewlett–Packard, Newport, DE, USA	150×4.6	RX	1
Polygosil-60-5-C18	Macherey–NagelGmbH and Co., Düren, Germany	125×4.6	Poly	2
Hypersil HyPURITY C18	Shandon HPLC, Runcorn, UK	150×4.6	HyPUR	3
Hypersil ODS	Shandon HPLC, Runcorn, UK	125×4.6	Hyper	4
Symmetry C18	Waters Assoc., Milford, MA, USA	150×4.6	Sym18	5
Purospher RP-18 e	Merck, Darmstadt, Germany	125×4	Puro	6
Kromasil KR100-5C18	Eka Nobel, Bohus, Sweden	150×4.6	Krom	7
Alltima C18 5U	Alltech Assoc.,Deerfield, IL, USA	150×4.6	All	8
TSKgel OD-2PW	TosoHaas GmbH, Stuttgard, Germany	150×4.6	TPW	9
TSKgel ODS-80TS	TosoHaas GmbH, Stuttgard, Germany	150×4.6	TTS	10
Eclipse XDB-C18	Hewlett Packard, Newport, DE, USA	150×4.6	XC18	11
Nucleosil 100-5 C18 HD	Macherey-NagelGmbH and Co., Düren, Germany	150×4	NuC18	12
C_8-Columns				
Eclipse XDB-C8	Hewlett–Packard, Newport, DE, USA	150×4.6	XC8	13
SymmetryShield RP8	Waters Assoc., Milford, MA, USA	150×4.6	Sym8	14
LiChrospher RP-Select B	Merck, Darmstadt, Germany	125×4	SelB	15
Aluspher RP-Select B	Merck, Darmstadt, Germany	125×4	Alu	16
Nucleosil 100-5 C8	Macherey–Nagel GmbH and Co., Düren, Germany	150×4	NuC8	17
Nova-Pak C8	Waters Assoc., Milford, MA, USA	150×3.9	Nova	18

ware program ‘SlideWrite Plus for Windows’, version 4.0 (Advanced Graphics Software Inc., Carlsbad, CA, USA).

4. Results and discussion

4.1. Hydrophobicity and hydrophobic selectivity

In the Engelhardt (E), Tanaka (T) and Walters (W) tests column hydrophobicity is defined and calculated from the separation factor, α, of (*ethylbenzene*/*toluene*); (*amylbenzene*/*butylbenzene*) *and* (*anthracene*/*benzene*), respectively. In fact these values represent selectivities for specific molecular increments. Tanaka [10] reported a linear dependence of CH_2-selectivity vs. percentage carbon on one particular silica substrate (Develosil). In contrast, Engelhardt [28] found a partly non-linear relationship between the CH_2-selectivity and the carbon load on silica substrates from several manufacturers. Based on these findings both authors suggest the use of the hydrophobic selectivity as a measure for column hydrophobicity. In the Galushko (G) test hydrophobicity is defined and calculated from the average retention factors (k) of toluene and benzene. In this latter test the hydrophobic selectivity (methylene selectivity) of a column is calculated too. In Fig. 1a and b the hydrophobicities are plotted (calculated as hydrophobic selectivities), as defined in the E, T and W tests together with the hydrophobic selectivity of the G-test are plotted for the C_{18}- and C_8-columns, respectively. With a few exceptions of the results of the W-test (especially the Alu column), the curves obtained from E, T and W tests are rather parallel, suggesting a constant hydrophobicity for all columns. Furthermore, these lines are rather parallel to the hydrophobic selectivity curve obtained in the G-test too. Leaving out both the non-silica based columns Alu and TPW, the correlation coefficients especially between the G, E and T tests are larger than 0.93. The W-test uses

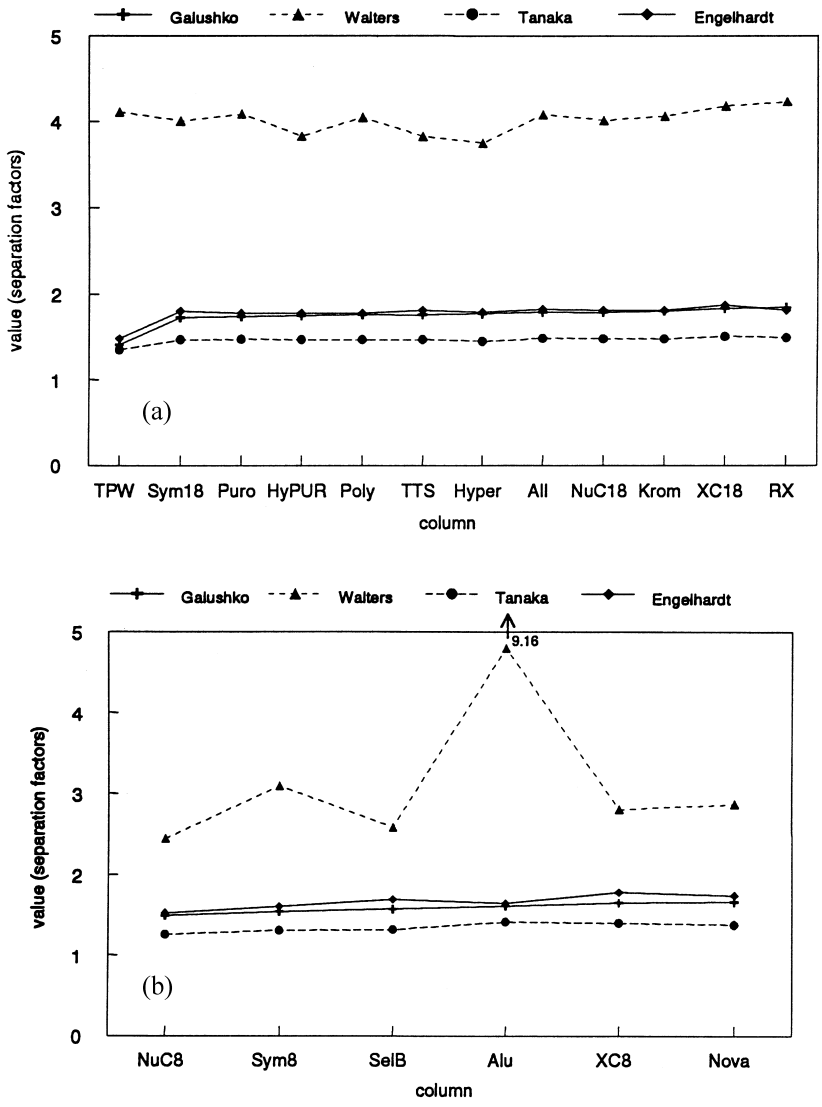

Fig. 1. (a) Hydrophobicity (calculated as hydrophobic selectivity) of the W, T, and E-tests together with the hydrophobic selectivity from the G-test for the C_{18}-columns (for conditions see text). (b) Hydrophobicity (calculated as hydrophobic selectivity) of the W, T, and E-tests together with the hydrophobic selectivity from the G-test for the C_8-columns (for conditions see text).

benzene and anthracene to measure column hydrophobicity.

Since the π-electrons of these aromatic hydrocarbons are Lewis bases, they can easily interact with the Lewis acidic sites formed by the Al^{3+} atoms of the substrate. We speculate that the occurrence of these Lewis acid/base type interactions is responsible for the high value found for the Alu column [5,6].

For silica-based RPLC-phases of similar ligand lengths, selectivity of specific increments (e.g. CH_2) for homologous series is rather constant under fixed conditions. This value may vary, however, with the portion of organic modifier in the mobile phase [33,42,43]. This explains why the CH_2-selectivity results of the E and G-tests are nearly identical, since these tests are performed in mobile phases containing 55 and 60% methanol, respectively.

In agreement with ref. [43], due to the much higher (80%) percentage of methanol, the CH_2-selectivity in the T-test is substantially lower. Keeping in mind that in the W-test the anthracene - benzene selectivity is actually measured and opposite to the other tests acetonitrile instead of methanol is used as an organic modifier, this explains the increased selectivity-values compared to the methylene values. Furthermore, we speculate that the less parallel behaviour of this latter curve must be ascribed to shape selectivity effects for that increment, which may vary more between the different phases as compared to a methylene group. This is discussed in detail in the section 'Shape and Size selectivity'.

The absolute differences in the CH_2 and (anthracene–benzene) selectivity between the C_8- and C_{18}-columns must be attributed to the different ligand chain lengths, resulting in different penetration of the test substances in between the surface ligands [44,45].

These results would suggest a rather constant hydrophobicity over the set of tested columns, which is not very likely considering their different physico–chemical properties, and especially the % carbon load and specific surface (Tables 1 and 2).

Similar results of rather constant (1.26–1.52) hydrophobicity (measured as CH_2-selectivity) for a large set of RPLC columns were recently reported by Cruz et al. [4]. In this study the authors found, however, a much larger variety (0.73–13.39) of the amount of 'alkyl ligands' as defined in the same test. Unfortunately, the authors did not report the physico–chemical properties of their columns. It seems likely, however, that a substantial variety must exist in their column population between the parameters that determine hydrophobicity. Hydrophobicity of RPLC-columns can be understood as the retentivity for apolar test compounds and is determined by surface area, % carbon load, ligand chain length, and the applied bonding chemistry and eventual endcapping of an RPLC-phase [6]. Furthermore, as discussed intensively by Antle [46] and Ying [47], differences in column hydrophobicity mostly originate from the various phase ratios rather than from different distribution coefficients of the test compounds involved. In addition, phase ratio values are largely determined by the surface area of the base support material and the carbon content of the stationary phase [47].

Both the results of the study of Cruz et al. and our findings in the present study suggest that column hydrophobicity must vary considerably between RPLC-columns of different sources. Furthermore, this column property is inadequately described by hydrophobic selectivity.

Therefore, in our opinion column hydrophobicity is better represented by absolute *k*-values rather than by hydrophobic selectivity, especially when different substrate sources are compared.

Following this and also the suggestions of Galushko [35] and Neue [6], i.e. that hydrophobicity is proportional to the retentivity of an apolar compound, the absolute retention factors (*k*) obtained in the W ($k_{\text{anthracene}}$), the E ($k_{\text{ethylbenzene}}$) and the T-test ($k_{\text{amylbenzene}}$), together with the hydrophobicity parameter [35] obtained from the G-test are plotted (Fig. 2a and b) in increasing order versus the columns. From lipophylicity studies it is well-known that log k_w-values of compounds correlate well to column hydrophobicity [41]. In Table 4 these absolute retention values from the W, E, T-tests, the hydrophobicity parameter from the G-test, and the log k_w-data for hexylbenzene are presented and columns are ranked according to hydrophobicity.

With a few exceptions and bearing in mind the different test compounds and experimental conditions, inspection of the retention data in Fig. 2a and b reveals that the various tests follow a similar trend. For instance the T and W results for all C_{18}-columns are more or less the same. As mentioned under hydrophobic selectivity, we speculate that the results may be obscured by size effects of the test compound anthracene. Furthermore, the steepness of the different lines connecting two consecutive columns is not always the same suggesting different sensitivities of the tests towards column hydrophobicity.

To obtain more insight into the (dis)similarities of the different tests all hydrophobicity data of the C_8-and C_{18}-columns were subjected to regression analysis (experimental errors in all tests were similar).

As an example in Fig. 3a and b, the *k*-values from the Engelhardt vs. Tanaka and Engelhardt vs. Galushko tests are regressed. The results of all regression calculations are provided in Table 5. From this table

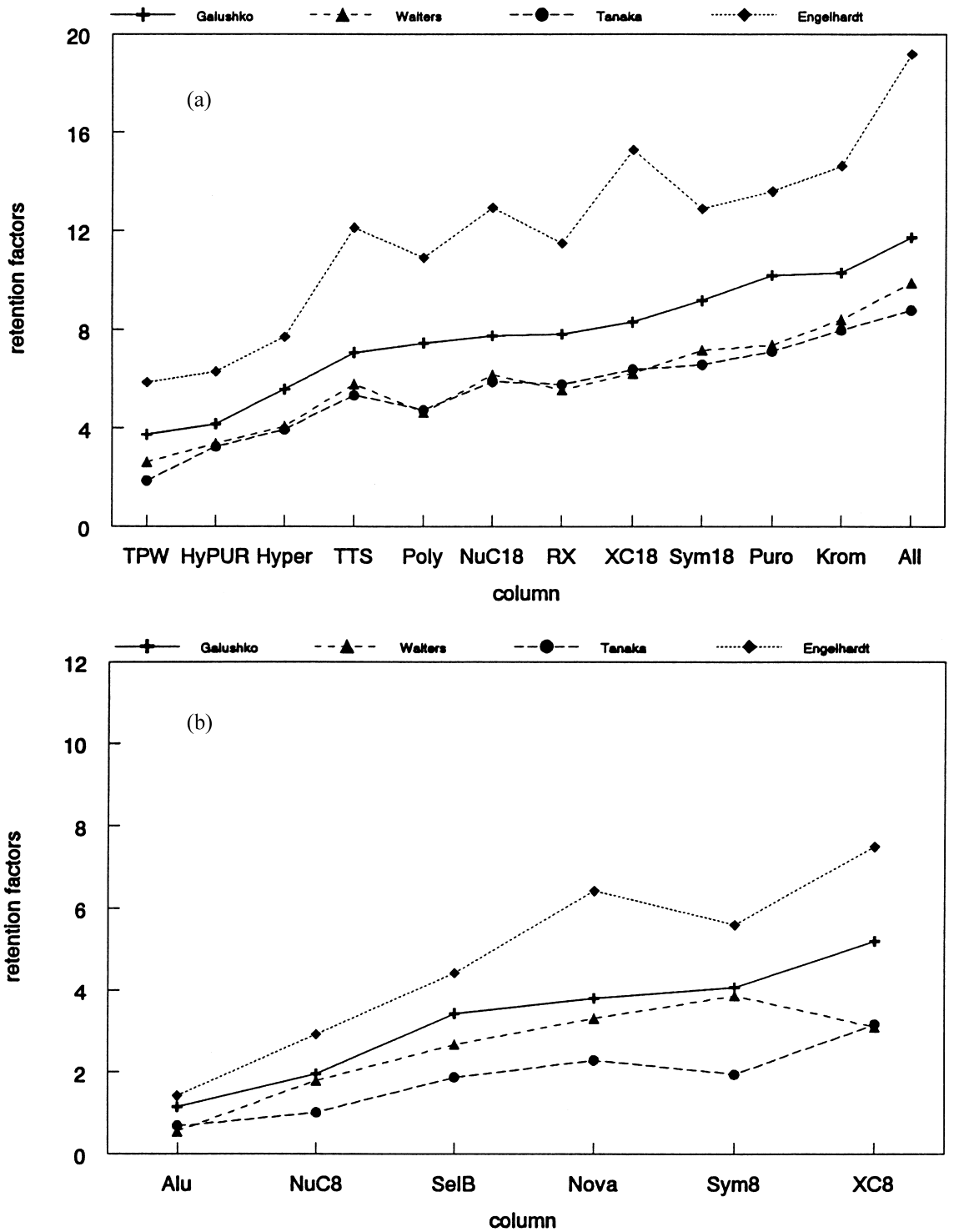

Fig. 2. (a) Retention factors from the T ($k_{amylbenzene}$), W ($k_{anthracene}$) and E-tests ($k_{ethylbenzene}$), together with the hydrophobicity parameter from the G-test for the C_{18}-columns. (b) Retention factors from the T ($k_{amylbenzene}$), W ($k_{anthracene}$) and E-tests ($k_{ethylbenzene}$), together with the hydrophobicity parameter from the G-test for the C_8-columns.

Table 4
Column ranking according to log k_w (hexylbenzene), their *k*-values from the W, E, and T-tests and the hydrophobicity parameter from the G-test

G	T	W	E	log k_w
1.15 [16]	0.690 [16]	0.561 [16]	1.414 [16]	3.9645 [17]
1.95 [17]	1.011 [17]	1.791 [17]	2.921 [17]	4.1126 [16]
3.43 [15]	1.867 [9]	2.614 [9]	4.425 [15]	4.4738 [14]
3.74 [9]	1.871 [15]	2.671 [15]	5.599 [14]	4.5554 [9]
3.81 [18]	1.939 [14]	3.106 [13]	5.862 [9]	4.6373 [15]
4.08 [14]	2.287 [18]	3.320 [18]	6.295 [3]	4.7107 [3]
4.17 [3]	3.172 [13]	3.372 [3]	6.416 [18]	4.7745 [18]
5.20 [13]	3.243 [3]	3.873 [14]	7.490 [13]	4.9374 [13]
5.57 [4]	3.934 [4]	4.064 [4]	7.701 [4]	5.0981 [4]
7.06 [10]	4.715 [2]	4.629 [2]	10.894 [2]	5.1209 [10]
7.44 [2]	5.330 [10]	5.539 [1]	11.481 [1]	5.1271 [12]
7.74 [12]	5.771 [1]	5.779 [10]	12.107 [10]	5.1909 [5]
7.81 [1]	5.881 [12]	6.155 [12]	12.890 [5]	5.1998 [2]
8.29 [11]	6.377 [11]	6.219 [11]	12.927 [12]	5.2190 [7]
9.16 [5]	6.573 [5]	7.150 [5]	13.593 [6]	5.2338 [1]
10.19 [6]	7.114 [6]	7.371 [6]	14.638 [7]	5.3069 [6]
10.29 [7]	7.978 [7]	8.407 [7]	15.277 [11]	5.3558 [11]
11.72 [8]	8.771 [8]	9.873 [8]	19.179 [8]	5.4057 [8]

Smaller values correspond to lower hydrophobicity.
[ ]=column label.

and Fig. 3a and b it can be seen that the results of the different hydrophobicity results are in fair agreement with each other. The good correlations found between the G, W, E and T tests strongly suggest that in spite of the very different conditions applied in the tests the *k*-values reflect similar column hydrophobicity properties. Further evidence that these *k*-data fairly reflect column hydrophobicity is found in the log k_w results, which correlate fairly well with the other four tests (Table 5).

From Table 4 it can be concluded that the hydrophobicity rankings of the columns according to the various tests are in fair agreement within certain bandwidths and also agree with the log k_w-data. Thus, absolute *k*-data better represent column hydrophobicity than hydrophobic selectivity. Furthermore, comparing the Tables 1 and 2, and Table 4, it can also be seen that no simple relationship exists between column hydrophobicity and its % carbon load as suggested earlier [10,28]. For instance, in the series of C_{18}-columns, opposite to what is expected, the NuC18 column (21%) has a lower hydrophobicity compared to the All-column (16.2%). Similar to that in the C_8-group, the XC8 and Alu columns have similar carbon loads (±7%), but very different hydrophobicities. The same observation can be made for the Nova (4%) and Sel B (11.5%) columns, where contrary to what was expected the latter column shows significantly lower hydrophobicity.

Summarizing, we conclude that column hydrophobicity as defined in the E, T and W tests can better be replaced by the expression 'hydrophobic selectivity' for a specific increment (e.g. CH_2). Furthermore, the regression results of the different tests combined with the log k_w-data show that column hydrophobicity is more accurately calculated from absolute retention values of neutral test substances similar to those in the G-test. Finally, the hydrophobicity and hydrophobic selectivity results of all four tests are interchangeable and column classification by one of these methods will provide similar patterns.

4.2. Silanol activity

Silanol activity, apart from purely ionic interactions, comprises a number of stationary phase–solute interactions, usually indicated as Van der Waals forces. These interactions may include ion–ion (ion-exchange), ion–dipole, dipole–dipole (e.g. hydrogen bonding), dipole–induced dipole, and induced dipole–induced dipole (London) forces. The latter two interaction types particularly depend on the polarisability of the involved solutes and stationary phase. In addition, in the case of buffered eluents, salting-out effects may also be involved in these interactions. Furthermore, it is emphasized that these intermolecular interactions may range from several hundreds of kilojoules per mol (ion–ion) to below 1 for London forces. The differences in silanol activity between RPLC-phases originate from the nature of the substrate (e.g., different silanol types), pretreatment steps (especially rehydroxylation) and the applied bonding chemistry (functionality, surface coverage, endcapping and alkyl side-group types) [5,12]. As recently discussed and reviewed by Nawrocki [12], it is obvious that silanol activity comprises several types of interaction, of which ion–ion and hydrogen bonding activity are probably the most important in RPLC [48].

Furthermore, it is clear that these interactions can act simultaneously and seriously obscure separations

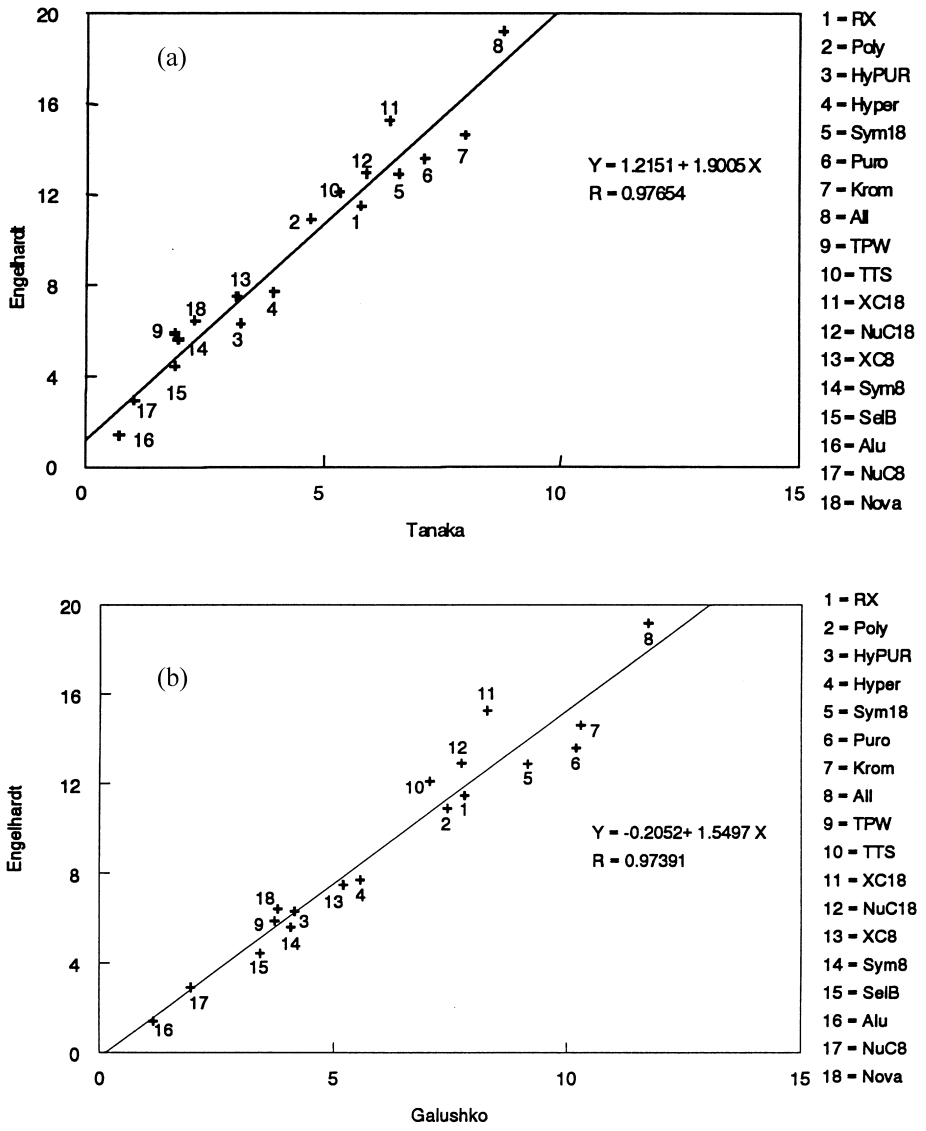

Fig. 3. (a) Regression of the retention data for the E ($k_{ethylbenzene}$) versus *T* ($k_{amylbenzene}$)-test for all C_8 and C_{18} columns. (b) Regression of the retention data for the E ($k_{ethylbenzene}$) versus G ($k_{(toluene+benzene)^{1/2}}$)-test for all C_8 and C_{18} columns.

[49]. As previously mentioned, especially the ionic and Van der Waals interactions greatly determine the unique character of RPLC-stationary phases. These activities have a tremendous effect on the separation of polar, ionic and especially basic compounds in particular.

Therefore, methods to overcome the difficulties connected to the separation of such compounds by RPLC are still the subject of ongoing discussions too [6,12,16,49–54]. Consequently, chromatographers are highly interested in the determination of silanol activity in its various aspects and also in methods to control and suppress these effects. To demonstrate the influence of eluent buffering on the silanol activity of RPLC-columns, we additionally applied a modified Engelhardt (E_m) test. In Table 6 the silanol activity data are summarised as defined from the G, W, E and E_m tests together with the hydrogen

Table 5
Coefficients of the mutual regression of hydrophobicity results of the E, T, W, G and log k_w-tests for all columns

Y	X	a_0	a_1	r
E	G	−0.2052	1.5497	0.97391
T	G	−0.7011	0.8080	0.98828
W	G	−0.1170	0.7855	0.97704
E	T	1.2152	1.9905	0.97654
E	W	0.3297	1.9097	0.96483
T	W	−0.3992	0.9909	0.97437
G	log k_w	4.1144	0.1274	0.90887
T	log k_w	4.2401	0.1541	0.89930
W	log k_w	4.2034	0.1476	0.84664
E	log k_w	4.1544	0.0797	0.90561

$Y=a_0+a_1\ X$; r=correlation coefficient.

bonding capacity from the T-test and they are ranked according to increasing activity. Furthermore, as with the hydrophobicity tests the data of the tests were also mutually regressed (Table 7), to find out whether the various tests respond to different or similar types of silanol activity interactions. Leaving out the results from the non-silica based columns did not improve the correlations (results not shown). First inspection of the Tables 6 and 7 immediately reveals that the silanol activity and hydrogen bonding data of the G, W, E, E_m and T-tests are much more scattered and generally poorly correlate compared to the hydrophobicity results. The results of all tests for buffered and non-buffered eluents are also poorly correlated. There is the exception of the T versus G and W versus G tests, where correlations of 0.63 were found, while for the T versus W tests a correlation of 0.72 was calculated. Note that in the G-test the data are claimed to represent silanol activity, while in the T-test these data are assumed to account for hydrogen bonding activity. This example indicates another problem with these tests, the confusion in nomenclature on which type of silanol activity is claimed in a specific test.

Table 6
Ranking of all columns according to silanol activity as defined in the G, W, E, and E_m-tests and hydrogen bonding capacity in the T-test

G	T	W	E	E_m
−0.18 [14]	0.114 [9]	0.403 [9]	130 [13]	99 [13]
−0.15 [16]	0.160 [16]	0.489 [11]	140 [16]	100 [15]
0.12 [13]	0.259 [14]	0.492 [5]	159 [6]	101 [1]
0.17 [7]	0.302 [13]	0.500 [6]	165 [14]	102 [11]
0.19 [5]	0.348 [5]	0.508 [7]	178 [5]	103 [2]
0.20 [3]	0.349 [3]	0.513 [3]	214 [15]	121 [7]
0.22 [11]	0.358 [7]	0.515 [12]	232 [12]	121 [8]
0.25 [12]	0.398 [11]	0.565 [10]	235 [10]	133 [6]
0.31 [10]	0.405 [18]	0.577 [1]	302 [17]	134 [12]
0.42 [6]	0.421 [4]	0.580 [8]	347 [11]	136 [3]
0.52 [4]	0.431 [6]	0.608 [14]	367 [3]	142 [10]
0.60 [8]	0.432 [12]	0.711 [4]	388 [9]	145 [18]
0.63 [18]	0.464 [8]	0.727 [13]	496 [1]	153 [17]
0.63 [9]	0.478 [10]	0.818 [18]	505 [18]	155 [4]
0.68 [1]	0.567 [2]	1.142 [16]	571 [2]	162 [14]
0.98 [15]	0.625 [1]	1.171 [2]	619 [7]	177 [5]
2.02 [17]	0.768 [15]	1.230 [15]	645 [8]	212 [9]
3.11 [2]	1.264 [17]	1.750 [17]	undefined [4]	407 [16]

Smaller values correspond to lower silanol activity.
[ ]=column label.

Table 7
Coefficients of the mutual regression of the silanophilic activity results of the E, E_m, G and W-tests and the hydrogen bonding capacity results from the T-test

Y	X	a_0	a_1	r
E	G	283.73	85.258	0.39877
E_m	G	165.46	−25.687	0.28862
T	G	0.3332	0.2001	0.62645
W	G	0.5701	0.2833	0.63167
E	T	302.99	70.205	0.10489
E_m	T	196.97	103.45	0.37139
E	W	361.87	−36.453	0.07647
E_m	W	124.18	35.174	0.17724
T	W	0.0755	0.5101	0.71599
E_m	E	197.06	−0.1409	0.33840

$Y=a_0+a_1\ X$; r=correlation coefficient.

The T-test further claims the validity of the hydrogen bonding activity test from the linearity of a series of measurements of hydrophobic (CH_2 selectivity) versus the (caffeine/phenol) selectivity, $\alpha_{c,p}$ on endcapped and non-endcapped columns, but only for one specific silica substrate (Develosil) [10]. Our results obtained on different silica substrates from several manufacturers do not confirm this finding. Leaving out the non-silica based phases from our test set, the correlation between CH_2-selectivity and $\alpha_{c,p}$ was 0.38 ($n=10$) for the endcapped columns. Thus, it is doubtful whether this hydrogen bonding activity test is applicable for the comparison of silica substrates originating from different sources. Another indication that hydrogen bonding activity measurements can be obscured by other effects is illustrated by the poor correlation (0.28) of the plot of $\alpha_{c,p}$ versus the silanol activity results at pH=2.7 from the

T-test. As suggested by Tanaka [10] and others [12,55] at this pH silanols are undissociated and therefore would only account for hydrogen bonding activity. Consequently a higher correlation might have been expected.

The assumption, however, that all silanols are completely undissociated at pH 2.7 should be considered with caution. As reviewed by Nawrocki [12,56] and also extensively discussed by others [16,49,57,58], it is postulated that a small but highly acidic silanol population of less than 1% is able to interact very strongly with polar solutes. This may explain the poor separation of such components observed on certain RPLC-phases. This issue is also related to eventual contamination of the silica substrate by traces of metals. Such impurities are known

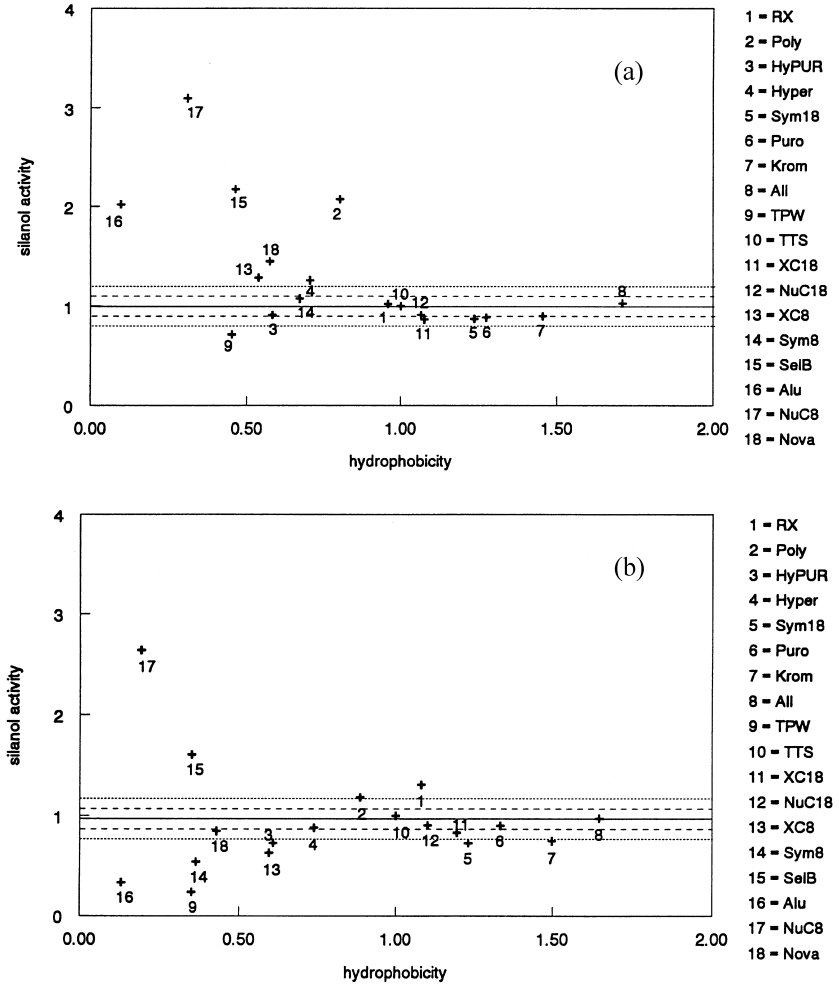

Fig. 4. (a) Normalized hydrophobicity versus silanol activity plot of the Walters test; TTS column as reference; straight line=normalized silanol activity line; −−−=±10% and dotted line=±20% deviation lines. (b) Normalized hydrophobicity versus silanol activity plot of the Tanaka test; TTS column as reference; straight line=normalized silanol activity line; −−−=±10% and dotted line=±20% deviation lines. (c) Normalized hydrophobicity versus silanol activity plot of the Galushko test; TTS column as reference; straight line=normalized silanol activity line; −−−=±10% and dotted line=±20% deviation lines. (d) Normalized hydrophobicity versus silanol activity plot of the Engelhardt test; TTS column as reference; straight line=normalized silanol activity line; −−−=±10% and dotted line=±20% deviation lines. (e) Normalized hydrophobicity versus silanol activity plot of the modified Engelhardt test; TTS column as reference; straight line=normalized silanol activity line; −−−=±10% and dotted line=±20% deviation lines.

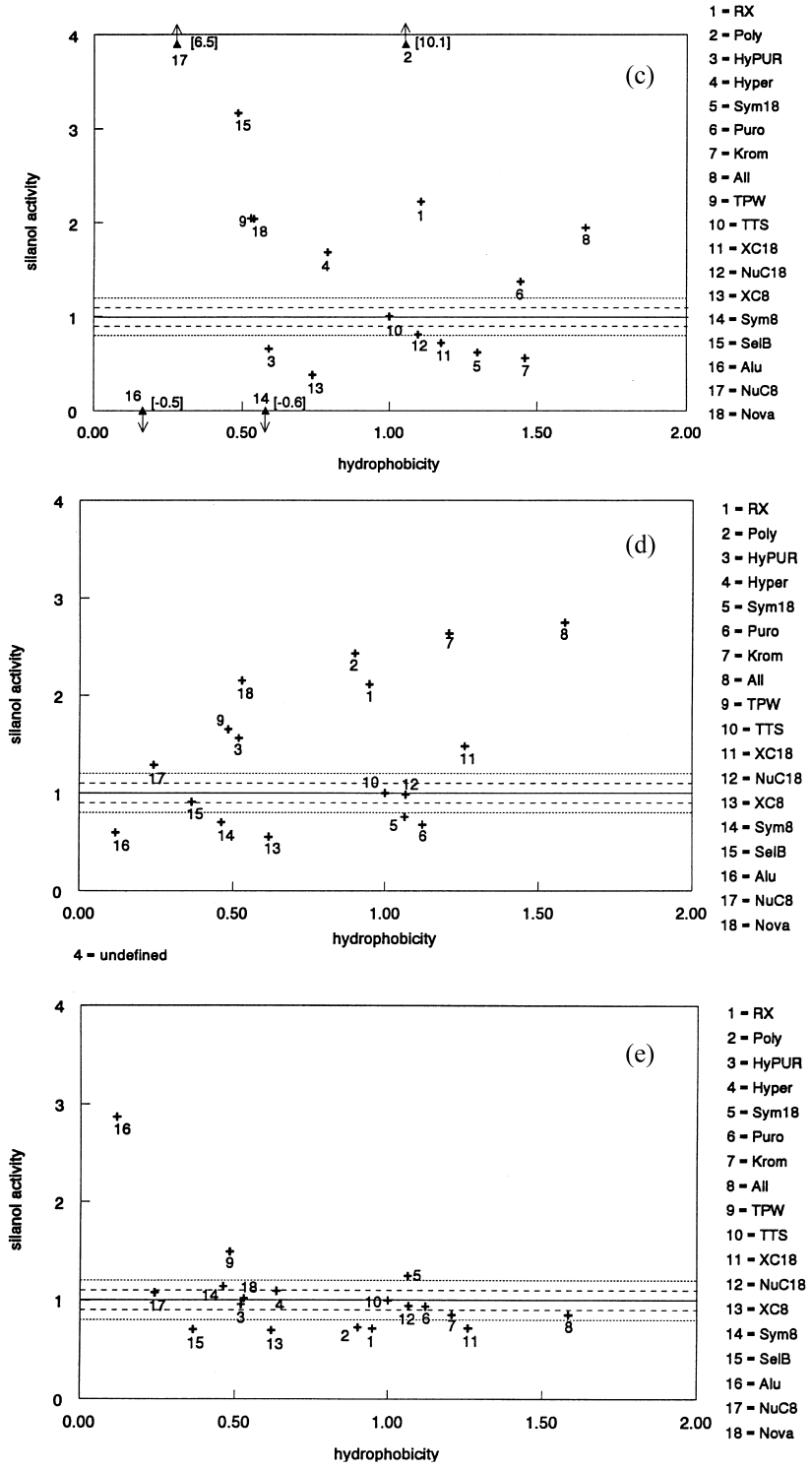

Fig. 4. (*continued*)

to enhance adsorption properties and silanol acidity of silica substrates [56].

To further illustrate the (dis)similarities between the tests in Fig. 4a–e, hydrophobicity versus silanol activity plots for the C_8- and C_{18}-columns obtained in the various tests are plotted. To facilitate comparison all values were normalized by arbitrarily taking the TTS column as reference. In addition, in an attempt to classify groups of columns of the more or less similar silanol and/or hydrogen bonding activity in these figures arbitrarily ± 10 and $\pm 20\%$ deviation lines towards the normalized line of the TTS column are drawn too.

A key issue in the ongoing discussions [6,10,12,59] on silanol activity measurements concerns the use of buffered or non-buffered eluent conditions. This important question comes down to whether one would like to show a column in its complete and maybe worst state of silanol activity or to make 'bad' columns 'good'. Considering that the majority of RPLC-separations is performed using buffered eluents and also for reasons of objective column comparison and classification, these authors believe that silanol activity should be tested under defined buffered eluent conditions.

Furthermore, Cruz [4] and McCalley [53] showed that column ranking may change depending on the actual pH of the eluent under the test conditions. Therefore, in our opinion future protocols should include the testing of RPLC-columns at more than one pH-value of the eluent.

Comparing the (non-buffered) E-plots, in Fig. 4d and the (buffered) E_m plots, in Fig. 4e obviously significant differences and shifts in silanol activity can be observed. With an exception for column 16, generally silanol activity of the columns decreases upon eluent buffering and shifts to a smaller deviation line zone, but not to the same extent. This can be seen in Fig. 4d and e and, more particularly, in Table 6 where tremendous differences and changes in mutual rankings of silanol activity of the columns under buffered versus non-buffered conditions are found. Some columns show a moderate or low influence in silanol activity by buffering of the eluent at pH=7.0, e.g. Sym18 (<1%), Puro (18%), Sym8 (<1%), XC8 (24%). In contrast with other columns these effects are much larger, e.g. Nova (71%), All (81%) and Poly (82%). The four former columns have become commercially available recently, while from the latter three columns the Nova and Poly columns have been available for many years already. We speculate therefore that the reduced silanol activity of the SymC8, SymC18, Puro and XC8 and other columns not investigated in this particular study reflects the improved technology (e.g. bonding chemistry, endcapping) used in the manufacturing process of these new generations of phases. It is noteworthy to mention that in contrast with all other columns the silanol activity of the Alu column is increased by 190%. This effect can be ascribed to the amphoteric properties of the alumina substrate.

Furthermore, the methacrylate copolymer based column TPW shows considerable 'silanol activity' too and reduction of it by 45% upon buffering. This behavior must be attributed to the alcoholic hydroxyl group and ether bonds in such packings. In addition, the finding of silanol activity on this non-silica based polymeric packing material also indicates the complexity of this phenomenon as discussed earlier in the beginning of this section. In both the E and the E_m-test silanol activity is calculated from the peak tailing of *p*-ethylaniline. In our opinion, since peak tailing may also originate from other sources (e.g. extra column band broadening), this makes these tests vulnerable to other tailing effects rather than silanol activity effects of RPLC-phases.

In Fig. 4a–e for the columns not occurring in the 10 or 20% deviation line zones, inspection of the silanol activity data clearly reveals significant differences in silanol activity found in the various tests, however without a clear pattern. For instance column 3 (Hypur) has similar scores in the G, T and W-tests. Its silanol activity, however, is lower in the G and T-tests as compared to column 6 (Puro) and higher towards this column in the W-test. Furthermore, column 18 (Nova) shows higher silanol activity in the G and E-tests compared to column 12 (NuC18), but lower in the T-test. In addition, for the W-test column 2 (Poly) and 15 (SelB) show nearly the same silanol activity. Except for the E_m-test, however, the other tests show larger differences in silanol activity between these latter two columns.

In Table 8 the columns are classified depending on their occurrence in the 10 and 20% deviation line zones of normalized silanol activity (TTS-column as reference). Column 12 (NuC18) occurs in the T, W,

Table 8
Occurrence of the C_8 and C_{18} columns in the ±10% and ±20% deviation line zones of the silanol activity results of the various tests

Test	T		W		G		E		E_m	
Zone	10%	20%	10%	20%	10%	20%	10%	20%	10%	20%
	6	2	1	5	–	12	12	–	3	7
	8	4	3	6			15		4	8
	12	11	7	11					6	14
		18	8						12	
			12						17	
			14						18	

For experimental conditions see text.

E and E_m-tests in the ±10% zone, and in the 20% zone of the G-test. In addition, the columns 6 (Puro) and 8 (All) occur three and two times in the 10% zone, respectively. In the 20% zone some other columns 18 (Nova), 11 (XC18), 3 (HyPur), 4 (Hyper) and 7 (Krom) occur two times. From Table 8 it is also clear that the various tests generally provide significantly different information with respect to the silanol activity of a specific column.

It must be emphasized here that the occurrence of columns even in the relatively narrow zone of ±10% not at all indicates that these columns behave chromatographically similar towards polar substances. It is important to note too that columns occurring outside that 10% zone should not be considered at all as columns of lower quality. As stated earlier the unique chromatographic properties of an RPLC column are greatly determined by its silanol activity, making it specifically suitable for certain application areas.

Theoretically pure silica has a pK_a-value of about 7.1 [12,58]. For commercially available silica substrates, however, variations in these values from 1.5 up to 10 have been reported [[58], and references therein]. Since present generations of silica are of high purity, it seems reasonable to assume that pK_a-values of these materials are less extreme.

In the E_m-test silanol activity is measured at pH=7.0, while in the T-test the ion-exchange capacity (IEC) is measured at a similar pH of 7.6 as $(k_{\text{benzalamine}}/k_{\text{phenol}})=\alpha_{a,p}$.

Therefore, at these pH-values silanols are not necessarily completely dissociated. Since, however, silanols are dissociated at a constant rate in both these buffered eluents, intuitively one might expect a certain correlation between the results of both these tests.

A correlation coefficient of 0.87 was found for the regression of the results of both these two tests for the whole set of columns. This strongly suggests that a similar type of silanol activity type is measured in these tests.

In addition, different amounts of methanol influence the actual pH of an eluent [54]. Therefore, the low correlation between these tests may be at least partly explained by the different amounts of methanol (49% and 30% in the E_m- and T-tests, respectively) used in these tests.

However, it further illustrates the problems in the nomenclature with respect to silanol activity; in this case silanol activity versus ion-exchange capacity (IEC).

Summarizing: opposite to the findings for the hydrophobicity results, the different silanol activity tests results are generally not in mutual agreement and not interchangeable. There is an exception for the silanol activity results of the E_m-test versus the IEC (pH=7.6) (T-test) data, where comparable results are obtained. Furthermore, it is not always clear which type of silanol activity is claimed to be measured in the various tests. Moreover, it is also obvious that column classification on silanol activity greatly depend on which test method is applied for these measurements. In this laboratory studies are underway in which these silanol activity test results are compared to the separation performance for selected test samples, e.g. well-defined basic compounds on these columns. This will be done to elucidate which test can best correlate a column's silanol activity and its separation performance.

4.3. Shape selectivity

The shape selectivities obtained from the E and T-tests together with the size selectivity from the G-test as defined in these tests are presented in Table 9. In both the E and T-tests shape selectivities are determined from the same test substances viz. triphenylene and *o*-terphenyl. Considering that nearly the same portion of methanol as organic modifier (79 and 80%) is used in both tests, the shape selectivity results of both tests are nearly the same (coefficient of correlation 0.9995).

Size selectivity in the G-test is calculated from the retention data of benzene, phenol and toluene. The G-test claims 'size selectivity is a column capability to separate solutes of similar polarity, but of different hydrophobic surfaces' [12,55]. Obviously the shape selectivity information obtained from the E and T-tests compared to the size selectivity data from the G-test are very different and show poor correlation ($r=0.53$).

Sander and Wise [60] reviewed the effects of shape selectivity of RPLC-phases thoroughly and could explain differences in shape selectivity by the 'slot model'.

Table 9
Shape selectivity of the T and E tests and size selectivity of the G-test

T	E	G
0.928 [13]	92 [13]	0.1597 [9]
0.957 [18]	95 [18]	0.1864 [17]
1.232 [15]	124 [10]	0.2032 [14]
1.251 [10]	127 [15]	0.2112 [15]
1.287 [11]	128 [11]	0.2232 [16]
1.307 [4]	129 [4]	0.2350 [13]
1.409 [12]	140 [8]	0.2374 [18]
1.414 [8]	140 [12]	0.2546 [5]
1.532 [7]	154 [7]	0.2568 [6]
1.572 [5]	158 [3]	0.2604 [3]
1.595 [3]	158 [5]	0.2628 [2]
1.610 [17]	161 [2]	0.2640 [10]
1.621 [2]	161 [17]	0.2661 [4]
1.637 [1]	166 [1]	0.2710 [8]
1.867 [14]	187 [6]	0.2715 [12]
1.875 [6]	187 [14]	0.2749 [7]
2.596 [16]	255 [16]	0.2833 [11]
3.086 [9]	306 [9]	0.2846 [1]

Columns are ranked in increasing order (top to bottom)
[ ]=column label.

In the E and T-test triphenylene (TRI) and *o*-terphenyl (*o*-TER) are used as the test substances. Both substances are of nearly the same molecular weight and of approximately the same size (length-to-width ratio). However, they strongly differ in spatial conformation, since TRI is planar, while *o*-TER is twisted out of plane.

In a recent study, Engelhardt et al. [61] compared the use of the TRI/*o*-TER selectivity, α(TRI/*o*-TER) in the T-test with the well-known shape selectivity test of Sander and Wise based on selectivity measurements between benzo(a)pyrene (BaP) and dibenzo[g,p]chrysene [60]. The former study shows that both tests correlate well in their ability to distinguish between 'monomeric', 'intermediate' and 'polymeric' phases in terms of shape selectivity. In the study of Engelhardt it was further concluded that for α(TRI/*o*-TER)-values larger than three the T-test columns have a 'polymeric'-like nature and show shape selectivity. Based on the applied bonding chemistry (di- and trifunctional modifications) and carbon loadings in our test set (Tables 1 and 2) one might expect a number of columns to be of a 'polymeric' nature and to show shape selectivity. In the same study Engelhardt also pointed out that besides carbon loading and a certain degree of polycondensation of silanes at the surface the accessibility of these groups is also of considerable importance to obtain shape selectivity. The authors state that 'the combination of high group density and wide pore diameter seems to be essential for stationary phases prepared for shape recognition'. Note that the majority of the columns in this study has pore sizes between 80 and 120 Å (Tables 1 and 2). With a few exceptions this may explain the relatively small differences in shape selectivity obtained in our column test set, classifying most of them as of a 'monomeric' or 'intermediate' nature in shape selectivity terms. Both the Nova and XC8 columns are octyl modified and have α-values <1. In the range of α-values of 1 to 1.5 we find the TTS, Sel B, XC18, Hyper, NuC18 and All. The columns in this group are C_{18}-modified, with the exception of Sel B, which is a C_8-modified stationary phase. The shape selectivity range $\alpha=1.5$ to 2.0 includes the columns Krom, Sym18, Hyper, NuC8, Poly, RX, Sym8 and Puro. Note that in this group two phases, viz Sym8 and Nu8 are C_8-modified too.

Table 10
Hydrogen bonding capacity $\alpha_{c,p}$ and ion-exchange capacity $\alpha_{a,p}$ at pH=2.7 and 7.6 of all columns from the T-test

Column	Number	$\alpha_{c,p}$	$\alpha_{a,p}$ pH<3	$\alpha_{a,p}$ pH>7
RX	[1]	0.625	0.073	3.127
Poly	[2]	0.567	0.817	1.479
HyPUR	[3]	0.349	0.101	0.290
Hyper	[4]	0.421	1.509	0.676
Sym18	[5]	0.348	0.068	0.332
Puro	[6]	0.431	0.073	0.347
Krom	[7]	0.358	0.089	0.291
All	[8]	0.464	0.084	0.595
TPW	[9]	0.114	0.006	0.631
TTS	[10]	0.478	0.076	0.372
XC18	[11]	0.398	0.067	0.359
NuC18	[12]	0.432	0.083	0.366
XC8	[13]	0.302	0.078	0.346
SymC8	[14]	0.259	1.185	0.219
SelB	[15]	0.768	0.112	1.099
Alu	[16]	0.160	3.252	20.960
NuC8	[17]	1.264	0.147	3.412
Nova	[18]	0.405	0.098	0.427

Also note that only the column TPW meets the criterion of α(TRI/*o*-TER)≥3, followed by the Alu column (α(TRI/*o*-TER)≥2.6). Since this latter column is polybutadiene-coated, we speculate that shape selectivity in this case must be attributed to interactions between the aromatic π-electrons of the test compounds and the Lewis acid sites of the alumina substrate [62].

In contrast to the T-test, the G-test accounts far more for differences in hydrophobic surfaces of smaller solutes of the same polarity. Obviously the choice of which of these tests (T, and E versus G) should be used, depends on the type of information one wants to obtain from a specific column.

4.4. Ion-exchange capacity

In Table 10 the $\alpha_{c,p}$ values together with the ion-exchange capacities measured as α(benzylamine/phenol); $\alpha_{a,p}$ at pH=2.7 and pH=7.6 are presented. In Fig. 5 the different $\alpha_{a,p}$-values

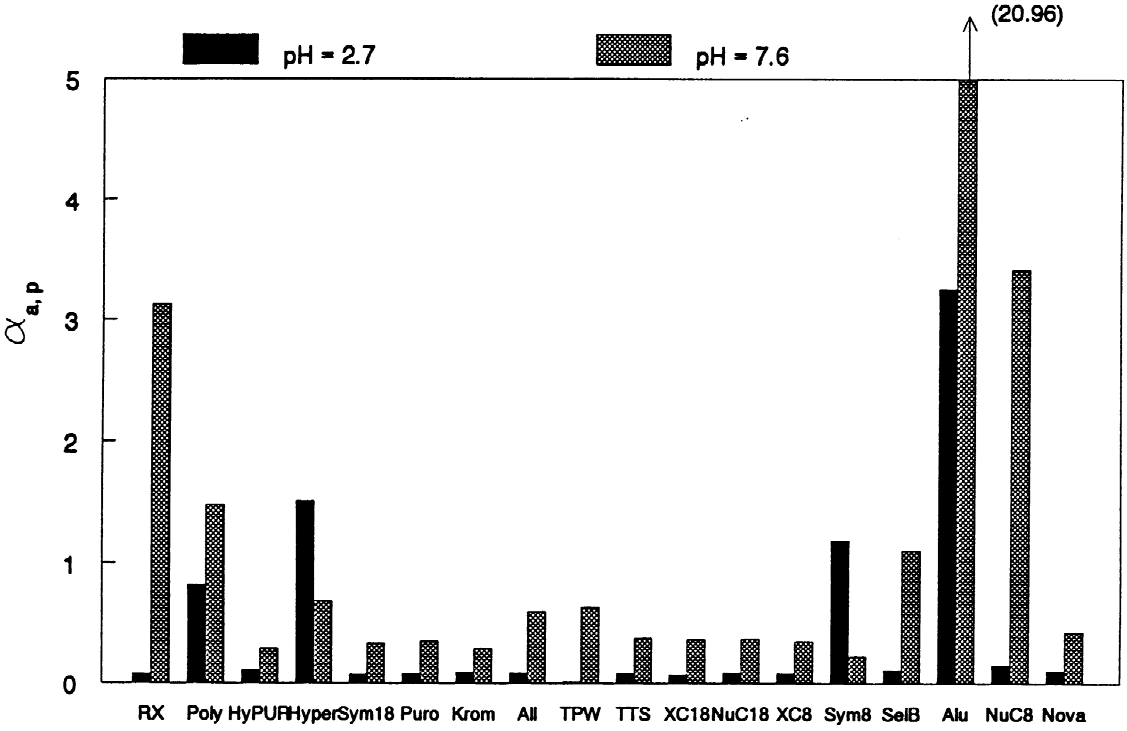

Fig. 5. Ion-exchange capacity measured as α (benzylamine/phenol), $\alpha_{a,p}$, at pH=2.7 and 7.6 from the T-test.

at both pH-values are further illustrated. The results from Table 10 confirm earlier findings of Tanaka [10] that columns showing large $\alpha_{a,p}$ values at pH=7.6 show either high $\alpha_{a,p}$ (pH=2.7) or large $\alpha_{c,p}$ values.

In this latter study this effect is ascribed to strong acidic silanols still active at pH=2.7 or silanol groups, which undergo dissociation at the neutral pH applied in the $\alpha_{c,p}$-test. More specifically, this effect can be observed for the RX, Poly, Sel B and NuC18 columns. Since these columns (except for the Poly column (unknown)), were non-endcapped (Tables 1 and 2), the results suggest that these effects must be attributed to limited shielding of silanols at the surface. A number of stationary phases from our test set show significantly larger $\alpha_{a,p}$-values upon increased pH=7.6 eluent. In contrast other columns did not show this strong tendency.

4.5. Metal activity

The major goal of this study was to compare a number of established tests for RPLC-columns. Metal activity, however, is not an integral part of any of these tests. It is emphasised that metal activity may influence the properties of RPLC-phases drastically [6,12,56–58]. Metal contamination may enhance silanol acidity, polarity and chelate formation potential of these phases. Especially for larger molecules, more particularly biomolecules, separation can be seriously obscured by such effects [8]. Well-known tests on metal activity comprise the procedures described by Verzele [63] and Euerby et al. [64]. Till now it has been unclear whether these tests are providing similar and comparable information. Furthermore, it is also questionable whether such tests are obscured by metal contamination originating from an HPLC instrument.

5. Conclusions

From the present work the following conclusions can be drawn:

1. The hydrophobic selectivity results especially from the E, G and T-tests are highly correlated.
2. The hydrophobic selectivity parameter that is used in the W, E and T-tests to measure column hydrophobicity reflects that magnitude insufficiently. As with the G-test, absolute retention of apolar substances measures column hydrophobicity much better.
3. The hydrophobicity results measured as absolute retentions in the various tests are in good agreement and are interchangeable, resulting in a column classification that is independent of the applied test.
4. Silanol activity comprises several ionic and polar effects and its terminology is confusing.
5. Buffering of the eluent greatly influences silanol activity test results. Furthermore, for the sake of objective column comparison and ranking, buffering of the eluent for such tests is mandatory.
6. The various eluent mixtures, test compounds and further experimental conditions suggested in the studied silanol activity tests also contribute significantly to difficulties in the interpretation of column silanol activity.
7. As opposed to the hydrophobicity tests, the results of silanol activity measurements are generally not in agreement and not interchangeable as a consequence. There is an exception for the E_m and IEC (pH=7.6) results from the T-test, where a fair correlation (≈0.9) was found. Column classification with respect to silanol activity greatly depends on the applied test.
8. The shape selectivity results from the T-tests reveal that the majority of the tested columns has ‘monomeric’ or ‘intermediate’-like properties in terms of shape recognition. The size selectivity parameter from the G-test represents a different column parameter as compared to shape selectivity.
9. Significant differences in IEC-values at pH=2.7 and pH=7.6 are observed over the tested set of columns and are related to endcapping effects.

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