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Improved testing procedures for comprehensive evaluation of reversed phase HPLC columns

Farid. R. Zaggout*[1], M. R. Alfarra[1] and D. Taylor[2]

(1) Chemistry Department, AL-Azhar University- Gaza, Gaza, P.O.Box 1277, PNA, Palestine. E-mail Zaggout2004@yahoo.com

(2) Chemistry Department, UMIST, Manchester M60 IQD, PO BOX 88, UK.

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Abstract: The ever increasing number of commercially available reversed phase columns with which the analyst is confronted can cause problems in columns selection, moreover these columns provide great variety in their chromatographic selectivity. These and the non standard test procedure used by the column manufactures and packing companies cause further confusion. This work describes comprehensive test procedure that was used to evaluate reversed phase columns in terms of their kinetic and thermodynamic properties. The quality of column packing process and therefore column efficiency and the suitability of HPLC system for separation were evaluated in the kinetic part of the test procedure. In the thermodynamic part reversed phase columns were evaluated for their hydrophobicity, silanophilic activity and shape selectivity in an unbuffered eluant and were evaluated for metal activity and cation exchange capacity in abuffered eluant. The work discusses the repeatability of best procedures and the effect of temperature and eluant composition evaluation. During this work a total of 38 C_8 and C_{18} reversed phase columns were used. A simple improved procedure for a comprehensive evaluation of C8 and C18 reversed phase HPLC columns has been investigated. The column evaluation process is highly reproducible and is not significantly influenced by slight changes in column temperature and mobile phase composition using the improved procedures on reversed phase columns able the analyst to characterize and classify these columns according to their efficiency and validity for the analysis of different classes of compounds.

Keywords: Reversed phase columns, Testing procedure for HPLC columns,stationary phase evaluation, HPLC, column kinetic and thermodynamic test.

* Corresponding Author, Farid R. Zaggout.

INTRODUCTION

There have been numerous attempts to characterize and classify reversed phase columns. Many different procedures have been employed, basically. These can be divided into three types, each of which possesses certain advantages and disadvantages[1]. The first approach employed non chromatographic methods in order to characterize physical and chemical parameters of the bulk Packing material such as NMR, ^{13}CNMR ,NIR and FTIR [2,4-8]. These are useful to column manufactures for controlling the quality of their columns but they are not so useful for routine testing of purchased columns. These techniques are expensive, not available in chromatography laboratories, time consuming and require adequate samples of the bulk packing. They have limited practical applicability [9,10].

The second approach is based on chemometrices and depends mainly on statistical chromatographic evaluation of selected compounds. These methods such as PCA and clusters analysis CA [11-17]. Column can be effectively classified by such methods, however little chemical knowledge is gained because complex compounds are employed where the retention behavior can be influenced by many factors. It is therefore difficult to predict the responsible mechanism for the observed chromatographic behavior and difficult to make extrapolations to other data set [1]. Lastly, there is the chemical chromatographic approach, which seeks to characterize and classify reversed phase columns according to their chemical and physical interactions with a carefully selected group of compounds. This is achieved either by visual evaluation of a test mixture or by calculating specified chromatographic parameters determined from the analysis of certain compounds. This approach allows a better understanding of the different factors that contribute to the chromatographic behaviour of column.

In general, two specific types of chemical chromatographic testing can be distinguished. The first is kinetic testing where the column performance can be evaluated through the calculation of values related to column efficiency and packing quality. Bidlingmeyer and warren compared a number of procedures for calculating N. It has been found that for gaussian peaks, all procedures provided similar plate counts, however, as peak asymmetry increases measurements based on assumed gaussian plate counts [18]. For this kind of peak, the use of Dorsey-Foley equation was introduced [16,19]. A more comprehensive approach to kinetic testing was introduced by Knox and Bristow[20]. They presented detailed procedures for evaluating column performance and packing quality based upon reduced variables. The recommended parameters are reduced plate height (h), column flow

resistance (Q), reduced velocity (v), and column impedance (E) [2,21,22].

In addition to these routine column tests, other more specific test are required to identify the chemically active sites on the packing material so that the suitability of a column for a certain types of analysis can be assessed. These kinds of tests are known as column activity tests [22,23], or thermodynamic tests, because they are related to the distribution of particular solutes between the stationary phase and the mobile phase[20]. Silanophilic activity, hydrophobicity, ion exchange capacity and residual trace metal impurities in the silica substrate are among the most important parameters that can be measured.

A variety of test mixtures for these types of procedure have been reported in the literature. Verzele and Dewaele [24] proposed a test mixture composed of acetylacetone, 1-nitronaphthalene and naphthalene with a mobile phase 60 methanol /40 water containing 0.5% sodium acetate, for the evaluation of reversed phase packing materials . Engelhardt and coworkers used a mixture of compounds including acidic, basic and neutral specific with unbuffered eluant (methanol-water 49:51 w/w) for the evaluation of RP18 and RP8 reversed phase columns [2,25,26]. Thiourea was used as a marker compound, toluene and ethyl benzene were used to demonstrate the hydrophobic interactions, phenol and benzoic acid ethyl ester for the acidic and neutral polar interaction five amino derivatives were used to trace silanophilic interactions (aniline and m-, o-, p- toluidine or ethyl aniline isomers). By observing the elution order of N, N-dimethyl aniline and phenol, it was possible to distinguish between RP18 and RP8 columns.

Mccalley has modified the Englhardt test procedure by using uracil instead of thiourea and benzene be toluene and ethyl benzene and substituting pyridine for N, N- dimethyl aniline [27]. The new test was employed for the evaluation of reversed phase columns to be used for the analysis of very basic compounds. Very recently, a number of other similar test procedures have been reported. In the first procedure an approach based on Tanaka methodology[28] and the application of various chemometric methods has been introduced [1]. The second procedure employed a gradient elution technique and chemometric methods for the evaluation of reversed phase columns of different pore sizes and containing different types of ligands [15]. Third, Eymann has established test procedures to determine hydrophobic and silanophilic interactions, as well as trace metal impurities on the packing material and the measurement of stationary phase stability and column performance at two different pH condition were also achieved .

The test procedure employed four eluant systems and four test mixtures used neutral molecules, amines and chelate forming compounds and acids[29]. A proposal for a universal column quality certificate for HPLC

columns has been presented lately[30], no new procedure were introduced, as this attempt presented three test procedures developed independently by kimata [28], Engelhardt [2,25,26] and Eymann [29]. Column selectivity for reversed phase liquid chromatography can be characterized quantitatively based on properties of solute and retention related parameter including hydrophobicity, steric selectivity, hydrogen acidity, hydrogen basisty and cation exchange [42,44-46]. Visky etal made a correlation between the pevious methods for the characterization of RPLC [58]. Ultimately, a general testing procedure for the evaluating and classification of reversed phase columns is needed for three main reasons . Firstly, a new column, whether purchased or packed in laboratory, should always be tested before use and periodically retest while in use .In this way the analyst can be sure that the column meets reasonable specification for general performance and has aveliable method to monitor changes in column properties as a function of time or the type of sample analysed[31]. Secondly the obvious need for a single very comprehensive test procedure in the literature. Lastly, users tend to have neither the time nor the resources to perform their own column comparison and determine the data themselves.

The main aim of this work was to establish an improved procedure for evaluating reversed phase columns. The use of this procedure would be useful in characterizing and classifying reversed phase columns produced by different manufacturers or in different batches by the same manufacturer. This procedure would also be useful in determing the suitability of a column for analysis of certain classes of compounds. In order to perform comprehensive evaluation, both of the kinetics and thermodynamic parameters were considered. Time factor was taken into account by using the minimum possible number of eluants.

MATERIALS AND METHODS

Equipment:

Two HPLC system were employed in carrying out this work .The first of which was a Gilson HPLC system equipped with the following parts; A model 115 UV detector, operated at 254 nm, A model 231 automatic sample injector set at 20 ml, two model 302 pumps and one model 307 pump. Solvent mixer 811, Automation was realized with Gilson 714 HPLC software, column temperature was maintained at 40^{o}C and Atrasonic T460 (CAMLAB) ultrasonic bath for mobile phase degassing. The second instrument was a Shimadzu HPLC system with the following specifications SCL-10Avp system controller, SIL-10AD autoinjector, Two LC-10AT vp pumps SPD-10AV vp UV/VIS detector, CTO-10AC vpoven class vp, version 4.2, chromatography data acquisition and analysis.

Chemicals and Solvents:

Methanol was HPLC grade from BDH (UK) and deionized water, chemicals was obtained from a variety of sources and were used as received. Uracil (UMIST), Toluidine (BDH), P-Toluidine(Aldrich), 1-Nitronaphtalene (BDH), Naphathalene (Aldrich), Diphenylmethane (Fluka), Fluorene (Lancast), n-Butyl benzene (Aldrich), n-Amyl benzene (Aldrich), phenol (Fisons), N,N-dimethyl aniline (Fisons), Quinizarine (Fluka).

Columns:

A total of 38 commercially available reversed phase C18 and C8 bonded phase columns were used in this work. Table (1) shows the list of these columns along with their specifications.

Table (1): Evaluated reversed phase columns

Column number	Packing material	Column dimensions (mm)	Particle size (μm)	Supplier
1	HyPUTIYT Elite C18	250x4.6	5	Hypersil
2	Hypersil BDS C18	250x4.6	5	Hypersil
3	Hypersil ODS C18	250x4.6	5	Hypersil
4	Luna C18 (2)	250x4.6	5	Phenomenex
5	Genesis C18	250x4.6	4	Jones
6	Lichrosorb RP18 C18	250x4.6	10	Jones
7	Spherisorb ODSI C18	250x4.6	5	Hichrom
8	Spherisorb ODS1 C18	250x4.6	5	Hichrom
9	Lichrosorb RP18 C18	250x4.6	10	Hichrom
10	Spherisorb ODS2 C18	250x4.6	5	Phase-separation
11	Lichrosorb RP18 C18	250x4.6	10	Technicol
12	Nucleosil C18	250x4.6	5	Hichrom
13	Nucleosil C18	250x4.6	5	Hichrom
14	Nucleosil C18	250x4.6	5	Hichrom
15	Nucleosil C18	250x4.6	5	Hichrom
16	Nucleosil C18	250x4.6	5	Hichrom
17	Ultrashere ODS C18	250x4.6	5	HPLCTechnology
18	Kromasil C18	250x3.8	5	Hichrom
19	Spherisorb ODS2 C18	250x4.6	5	Hichrom
20	Spherisorb ODS2 C18	250x4.6	10	Hichrom
21	Lichrosorb RP18 C18	250x4.6	5	Hichrom
22	Bondapak C18	250x4.6	10	Hichrom
23	Spherisorb ODS1 C18	250x4.6	5	UMIST
24	Spherisorb ODS2 C18	250x4.6	5	Phase-separation
25	Spherisorb ODS2 C18	250x4.6	5	Phase-separation

26	Spherisorb ODS2 C18	250x4.6	10	Hichrom
27	Spherisorb ODS2 C18	250x4.6	5	Phase-separation
28	Spherisorb ODS C18	250x4.6	5	Hichrom
29	Hypersil ODS C18	250x4.6	5	Shandon
30	Lichrosorb RP18-Select B	250x4.6	5	Merck
31	Hi-RPB 2517	250x4.6	NA	Hichrom
32	Spherisorb ODS2 C18	250x4.6	5	Hichrom
33	Lichrosorb RP18 C18	250x4.6	5	Chromex
34	Lichrosorb RP-8 C8	250x4.6	5	Technicol
35	Lichrosorb RP-8 C8	250x4.6	10	Chromex
36	Lichrosorb RP-8 C8	250x4.6	10	Hichrom
37	Lichrosorb RP-8 C8	250x4.6	10	Chromex
38	Spherisorb C8	250x4.6	5	Phase-separation

Sample preparation:

One general protocol was employed in preparing all of the samples for analysis by HPLC, the required amount of the analyte was dissolved in the chosen mobile phase, unless otherwise mentioned. In a few cases, an ultrasonic bath was used to dissolve the compound completely. Concentrations of the tested compounds ranged from 0.1-4 mg/mL

One litter phosphate buffer solution (pH=2.8) was prepared by mixing 841.5 ml of 0.1M citric acid and 158.5 ml of 0.2 M Na_2HPO_4 [47]. The pH measurement was carried out on a digital pH-meter which was first calibrated at pH=7 and pH=2.

Mobile phase preparation:

Methanol/water 65:35 (w%:w%) and methanol/buffer 70:30 (w%:w%) solution were prepared externally (by using an electronic balance) and were delivered by a single pump from a single reservoir.

Test mixtures:

Two sets of compounds have been selected to evaluate reversed phase columns. The composition of test mixtures A and B are summarised in Tables (2and3). Both test mixtures were prepared by mixing equal volumes of the included solution. Test mixture A was employed with 65% methanol: 35% water (by mass), while test mixture B was employed with 70% methanol: 30% aqueous phosphate buffer pH=2.8 (by mass). For both mixture flow rate was 1ml/min detection by UV/VIS at 254 nm and column temperature was 40^{o}C.

A column should be conditioned at 40^{o}C at 1mL/min with each mobile phase for at least 35 minutes to insure that an equilibrium state between the mobile and stationary phases has been achieved. Practically, each column

was equilibrated with the mobile phase for 40-50 minutes.

Table 2: Composition of test mixture A and evaluated column properties

Component	Conc.(mg/ml)	Property of column
Uracil	0.1	Dead volume time
m-Toluidine	0.5	Residual silanol gps and degree of activity or Endcapping status
p- Toluidine	0.5	
1-NitroNaphtalene	0.2	
Naphthalene	0.5	
Diphenyl methane	2	Shape selectivity
Fluorene	0.1	
n-Butyl benzene	4	Amount of Alkyl gps and hydrophobic selectivity
n-Amyl benzene	4	

Table 3: Composition of test mixture B and evaluated column properties

Component	**Conc. mg/ml**	**Column property**
Uracil	0.1	Dead volume time
phenol	1	Ion exchange sites
N,N-dimethyl aniline	0.5	Ion exchange sites at pH 2.8
Quinizarine	1.5	Trace metal activity

Influence of temperature on column evaluation :

The influence of temperature on column evaluation and solute's retention times was investigated on typical columns for both mixture A and mixture B.

Mixture A was analyzed at 35^{o}C and 45^{o}C on column number 1 (Hypurity Elite). The column was conditioned with the mobile phase at least for 35 minutes at each temperature before the analysis. Mixture B was evaluated for temperature dependence on column number 28 (spherisorb ODS2C18), using the same procedure as for mixture A, Duplicate injection were performed at each temperature.

Influence of eluant composition on column evaluation:

To investigate the influence of slight changes in mobile phase composition on the column evaluation, a series of mobile phase compositions for both mixture A and mixture B were made up by weight, as shown in Table 4. A typical column was chosen for each mixture and was conditioned with each mobile phase for at least 40 minutes to insure that an

equilibrium state had been achieved. Column number 1(Hypurity Elite) was selected for mixture B. Duplicate injections were carried out for each mixture with each mobile phase composition.

Table (4): Mobile phase composition for mixture A and mixture B

Mixture A		Mixture B	
CH_3OH Mass percentage	H_2O Mass percentage	CH_3OH Mass percentage	Phosphate buffer pH=2.8
68	32	73	27
67	33	72	28
66	34	71	29
64	36	69	31
63	37	68	32
62	38	67	33

Repeatability of test parameters:

The repeatability of kinetic and thermodynamic test parameters was investigated. For mixture A, Column no.1 (Hypurity Elite) was conditioned with the mobile phase CH_3OH/H_2O 65:35 (w%:w%) for 40 minutes and the test mixture was injected 10 times each for 35 min. for mixture B, column number 2 (Hypersil BDS) was conditioned with the mobile phase CH_3OH: Buffer 70:30 (w%: w%) for 50 minutes and the test mixture was injected 10 times each for 20 minutes.

RESULTS AND DISCUSSION

The major objective of the present study has been to find a general test, which should enable chromatographer to characterize his columns easily and reproducibly, in terms of kinetic performance and thermodynamic activity. Basically, evaluating the kinetic performance of a column provides information about the packing quality, mass transfer and other physical concepts of the separation process. But such information indicates nothing about the chemical structure of the column packing materials or about the potential of column to resolve particular mixtures of compounds without undue peak tailing with certain solutes. On the other hand, reported chemical activity tests are claimed to be able to evaluate the column packing materials interms of its suitability for separation certain classes of compounds. Nevertheless, such chemical tests do not provide any data on column packing quality and column efficiency. Throughout the literature, Kinetics and chemical activity tests have never been applied together in a single test procedure. In this work we combine both of them in a more

comprehensive column evaluation procedure.

Column Kinetic Test:

Reduced plate height (h), reduced eluant velocity (v), reduced flow resistance (φ), and separation impedance (E) were calculated for the naphthalene peak. The following equation were used to calculate the parameters [20,21,31].

$h=(1000/5.54)(L/dp)(W_{0.5}/t_R)^2$ (1)

$V=10^{-9}(L.dp)/t_o.D_m)$ (2)

$\varphi=100(p.t_o.d^2p/\eta.L^2)$ (3)

$E=h^2.\varphi$ (4)

Where L is column length, dp the particle size in μm, $W_{0.5}$ naphtalene peak width at half height, t_R naphthalene retention time, t_o uracil retention time in seconds (unretained compounds), p column's pressure value in bar, D_m diffusion coefficient of naphthalene in the mobile phase in m^2s^{-1}, and η is the viscosity of the mobile phase. The values of L and dp were supplied by column's manufacturer. $W_{0.5}$, t_R and t_o were calculated by using the shimadzu software (Class vp version 4.2). The pressure values of the columns were displayed on the screens of both system controller and pump. The diffusion coefficient of naphthalene in the mobile phase at 40°C was calculated by the emprical Wilke-Change equation [48, 49, 50).

Naphthalene was included in test mixture A for Kinetic performance evaluation. It was selected because it does not contain any functional groups that might have further interaction with a column packing material except the simple hydrophobic interaction. Therefore, the evaluation was mainly for kinetic performance and none of the thermodynamic interaction was expected to interfere.

From the values of the parameters h,v,φ and E as shown in Table 5 it is revealed that:

Table (5): Kinetic evaluation results of tested columns based on naphthalene peak

Column Number	Packing Material	h	V	φ	E
1	HyPUTIYT Elite C18	3.25	6.88	1023	10826
2	Hypersil BDS C18	3.19	7.58	738	7495
3	Hypersil ODS C18	3.61	7.65	738	7495
4	Luna C18 (2)	2.57	7.95	794	5260
5	Genesis C18	3.42	6.06	533	6246
6	Lichrosorb RP18 C18	3.86	15.28	1189	17718
7	Spherisorb ODSI C18	8.16	7.64	802	53406
8	Spherisorb ODS1 C18	8.75	7.67	724	55471

9	Lichrosorb RP18 C18	5.84	13.94	1242	42416
10	Spherisorb ODS2 C18	8.28	8.23	832	57067
11	Lichrosorb RP18 C18	3.97	15.17	1331	20953
12	Nucleosil C18	4.45	9.44	955	18904
13	Nucleosil C18	6.59	9.44	936	40591
14	Nucleosil C18	5.70	9.78	774	25112
15	Nucleosil C18	9.92	9.70	650	64004
16	Nucleosil C18	6.54	12.59	1100	47016
17	Ultrashere ODS C18	9.88	8.20	659	64320
18	Kromasil C18	5.49	8.37	797	24005
19	Spherisorb ODS2 C18	4.93	8.07	670	16248
20	Spherisorb ODS2 C18	2.35	17.43	1439	7971
21	Lichrosorb RP18 C18	5.59	7.11	862	26960
22	Bondapak C18	5.73	13.03	1949	63930
23	Spherisorb ODS1 C18	12.41	7.98	700	107810
24	Spherisorb ODS2 C18	11.04	7.13	733	89365
25	Spherisorb ODS2 C18	8.97	7.80	1026	82599
26	Spherisorb ODS2 C18	3.81	17.51	1450	21096
27	Spherisorb ODS2 C18	9.74	7.88	709	67281
28	Spherisorb ODS C18	4.77	8.12	635	14428
29	Hypersil ODS C18	3.76	8.10	713	10068
30	Lichrosorb RP18-Select B	18.24	9.50	987	328338
31	Hi-RPB 2517	8.09	7.72	935	61170
32	Spherisorb ODS2 C18	5.03	7.92	865	21920
33	Lichrosorb RP18 C18	6.31	6.88	1101	43810
34	Lichrosorb RP-8 C8	21.91	6.75	935	448595
35	Lichrosorb RP-8 C8	3.82	14.88	2326	33930
36	Lichrosorb RP-8 C8	NA	13.66	1689	NA
37	Lichrosorb RP-8 C8	3.16	14.62	4538	45235
38	Spherisorb C8	6.73	7.15	1160	52552

1-The value of the reduced flow resistance φ was within the acceptable range 500-1000 for 63% of the columns. 15% of columns had slightly high values ranging from 1100-1400, which was accompanied with slightly high pressure drop values. This may be because most of these columns have been in use for a long time, but does not indicate blockage of the outlet frit. Columns numbers 35 and 37 show remarkably high values of reduced flow resistance (2326 and 4538 respectively) which indicates partial blockage of a column.

2-The recorded values of v were mostly between 6 and 9 for 5 μm column packing and columns with 10μm particle sizes had values of v ranging form

13 to 17.5 (c.f. Table 5). In the region of the Konx/Van Deemter plot between v=5 and v=100, the value of h for an ideal column is determined mainly by the C term and is approximately proportional to v as shown in the following equation: h= const +Cv

where C = 0.1 and the constant is 3 and 5 at v=5 and v=100, respectively. Hence it is possible to estimate a desirable values of h, which a good column should achieve at any experimentally achieved value of v. For example if 5<v<10 then 3.5<h<4 and if 10<v<20 then 4<h<5. Applying these limits to the data in Table 5 showed that some of the tested columns have relatively ideal values of h, which indicate well packed column and high column efficiency. This was supported by the observation of narrow peaks for all of the test compounds. Figure (1) shows the shape of the peaks eluted by column number 4, for which the value of h was 2.57 at v=7.95.

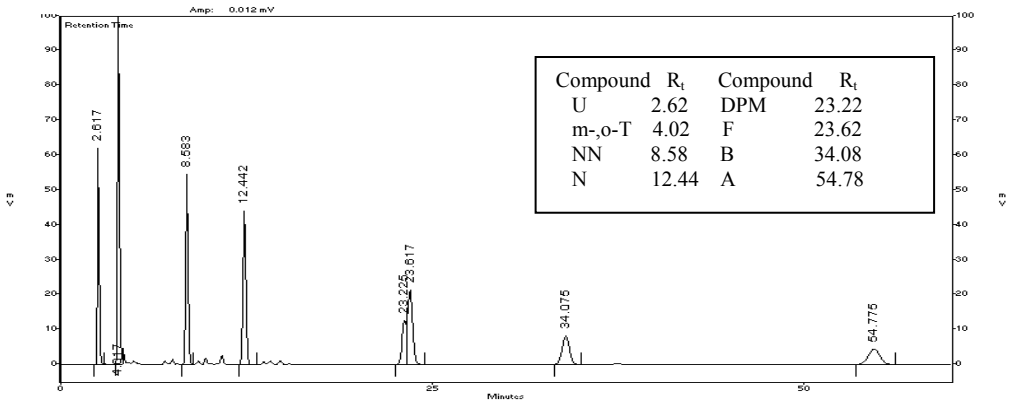

Figure 1 : Test mixture A on column number 4 (Luna C18),where h=2.57 at v=7.95

Another example is shown in Figure(2), a chromatogram obtained using column number 6 which had value of h=3.86 at v=15.28.

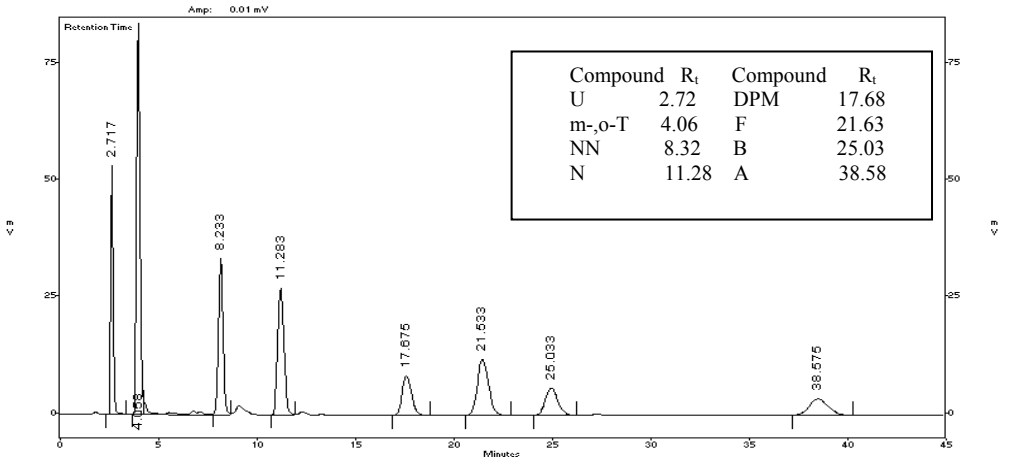

Figure 2: Test mixture A on column number 6 (Lichrosorb RP 18), where h=3.86 at v=15.28

However, the values of h were very high on some columns, which indicated very bad packing quality and therefore low efficiency. Such column (e.g. columns no. 27 and 30) produced chromatograms which displayed very broad peaks as shown in Figures (3) and (4). For column 27, h was 9.74 at v=7.88 and for column 30, h was 18.24 at v=9.5. Some columns (e.g. column 13) for which h=6.59 at v=9.44 had neither optimum nor remarkably high values of h indicating a border line quality of packing. The above classification of columns according to the values of h and v revealed that a comparison of columns from the stand point of efficiency could be carried out.

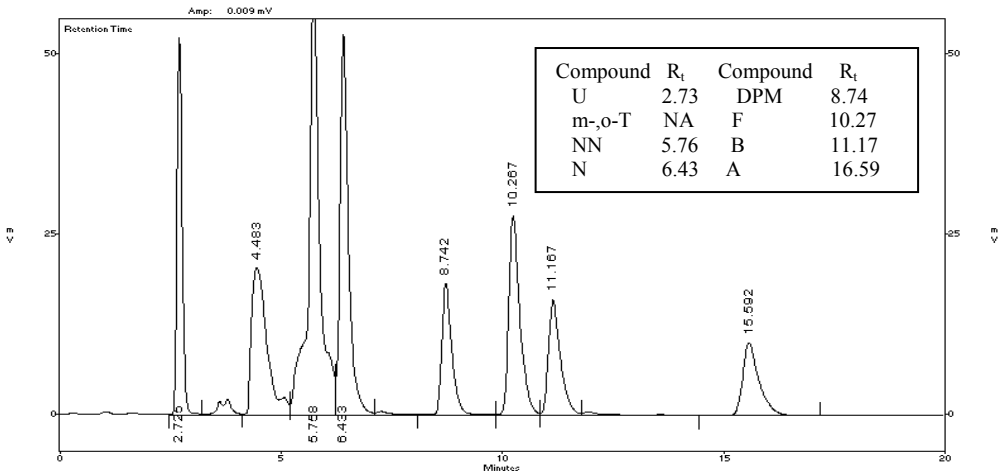

Figure 3:Test mixture A on column 7(Sherisorb ODS1 C18), where h=9.74 at v=7.88

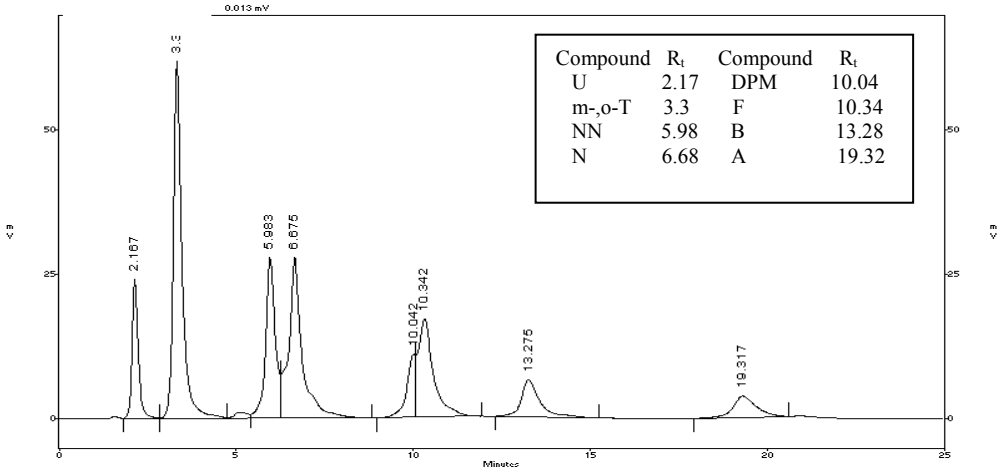

Figure 4: Test mixture A on column number 30 (Lichrosorb RP-Select B), where h=18.24 at v=9.50

3-Columns which showed good kinetic performance with respect to the values of h, v and φ were found to produce values of separation impedance E within the range 5000<E<11000 (e.g columns 1,2,3,4,5,20,23) as shown in Table 5. The corresponding range of E for 21% of the columns was 11000<E<25000 (e.g columns 6,11,12,18,19, 26,28,32). These results agree quite closely with values stated in the literature [20,21,31]. Very bad columns gave high values of E=328338 (e.g. column 30). For some other very inefficient columns, E values were in the range 30000<E<90000 (e.g. columns 8,9,10,15,19,24,27, 34,38).

The separation impedance was suggested by Knox [20] to be the major parameter for kinetic performance evaluation, because it is a combination of the other parameter h and φ. It has the advantages that it is measurable for any column structure and only the values of Δp, t_o, t_R, $W_{0.5}$ and η are required to calculate its value regardless of column dimensions or packing. On the other hand, the value of E on its own has a very poor diagnostic value, particularly if it has high value, say 10 times the optimum one, in this case we will not be able to know whether this high value is because of blockage, bad packing or bad mass transfer unless the values of φ and h are calculated individually. To sum up, the applied kinetics test parameter h,v and φ can tell us useful information about the efficiency of the column and the quality of the packing process as well as whether the column is blocked or not.

Column Activity Test:

Test mixture A:

Test mixture A was employed to evaluate four thermodynamic parameters. Hydrophobicity of a column (retention factor of n-amyl benzene K_A), hydrophobic selectivity (separation factor of n-amyl and n-butyl benzene $\alpha_{A/B}$), shape selectivity (separation factor of flourene and diphenyl methane $\alpha_{F/D}$), residual silanol groups and degree of activity or endcapping status (separation factor of naphthalene and 1-nitroNaphthalene $\alpha_{N/NN}$ and visual evaluation of the toluidine isomers peaks.

Test mixture B:

Test mixture B was employed to characterize two thermodynamic parameters. Ion exchange capacity (selectivity factor of N,N-dimethyl aniline and phenol $\alpha_{DM/P}$ and peak asymmetry of N, N-dimethyl anilline A_{SDM}). The second parameter is trace metal activity, which is based on the peak asymmetry of quinizarine A_{SQ}, and the ratio of area to height for quinizarine peak Q_A/Q_H.

Hydrophobicity and hydrophobic selectivity:

The hydrophobic character of reversed phase column represent the

hydrocarbon part of the column packing materials. Therefore, the hydrophobicity of reversed phases column increases with carbon loading and this can be increased by increasing chain length of the bonded ligand, by increasing bonding density by endcapping the phase and by increasing the surface area of the silica substrate [11].

n-butyl benzene and n-amyl benzene were used for determing the hydrophobicity of reversed phase columns which have been selected by Kimata and his co-worker[28] but with longer retention time. The values of $K`_A$ (retention factor for n-amyl benzene) and $\alpha_{A/B}$ (separation factor of n-amyl and n-butyl benzene) were measured for the 38 tested columns using a 65:35 methanol/water mixture (prepared by mass) as mobile phase. $K`_A$ is indicative of the amount of alkyl chains on the packing material and this is proportional to the surface area of silica support material and its surface coverage. Furthermore, relatively low values of $K`_A$ indicate poor surface coverage of column packing materials. In contrast, relatively high values of $K`_A$ indicate good surface coverage and non-polar compounds will be highly preferred by the column. The recorded values of $K`_A$ in Table 6 were in the range $4<K`_A<22$ for C18 columns and $3<K`_A<9$ for C8 column. This is evidence of the generally observed diversity in the chromatographic behaviour of reversed phase column.

Table (6): Column properties evaluated by test mixture A

Column Number	**Packing materials**	**K'_A**	**$\alpha_{A/B}$**	**$\alpha_{N/NN}$**	**$\alpha_{F/D}$**
1	HyPUTIYT Elite C18	9.17	1.63	1.73	1.17
2	Hypersil BDS C18	11.22	1.63	1.79	1.17
3	Hypersil ODS C18	11.24	1.62	1.62	1.07
4	Luna C18 (2)	19.90	1.66	1.65	1.02
5	Genesis C18	17.98	1.65	1.71	1.13
6	Lichrosorb RP18 C18	13.13	1.61	1.55	1.26
7	Spherisorb ODSI C18	4.72	1.52	1.22	1.25
8	Spherisorb ODS1 C18	4.82	1.51	1.23	1.33
9	Lichrosorb RP18 C18	7.94	1.57	1.45	1.28
10	Spherisorb ODS2 C18	13.44	1.63	1.56	1.26
11	Lichrosorb RP18 C18	12.14	1.59	1.48	1.27
12	Nucleosil C18	11.85	1.62	1.43	1.15
13	Nucleosil C18	9.27	1.59	1.37	1.17
14	Nucleosil C18	10.57	1.60	1.43	1.19
15	Nucleosil C18	10.35	1.60	1.43	1.19
16	Nucleosil C18	8.95	1.58	1.42	1.21
17	Ultrashere ODS C18	11.85	1.62	1.50	1.29

18	Kromasil C18	21.34	1.65	1.77	1.18
19	Spherisorb ODS2 C18	9.91	1.59	1.53	1.26
20	Spherisorb ODS2 C18	12.01	1.61	1.64	1.26
21	Lichrosorb RP18 C18	11.23	1.61	1.55	1.27
22	Bondapak C18	4.06	1.52	1.35	1.10
23	Spherisorb ODS1 C18	10.15	1.60	1.50	1.15
24	Spherisorb ODS2 C18	9.05	1.60	1.62	1.29
25	Spherisorb ODS2 C18	9.17	1.59	1.39	1.29
26	Spherisorb ODS2 C18	11.86	1.60	1.45	1.27
27	Spherisorb ODS2 C18	8.16	1.57	1.38	1.32
28	Spherisorb ODS C18	11.54	1.62	1.65	1.09
29	Hypersil ODS C18	11.44	1.63	1.81	1.20
30	Lichrosorb RP18-Select B	7.79	1.54	1.19	1.04
31	Hi-RPB 2517	12.08	1.57	1.56	1.05
32	Spherisorb ODS2 C18	5.83	1.53	1.19	1.24
33	Lichrosorb RP18 C18	9.62	1.60	1.48	1.24
34	Lichrosorb RP-8 C8	3.46	1.45	1.12	1.07
35	Lichrosorb RP-8 C8	8.38	1.50	1.39	0.95
36	Lichrosorb RP-8 C8	3.28	1.43	1.08	1.06
37	Lichrosorb RP-8 C8	8.98	1.51	1.40	0.94
38	Spherisorb C8	3.30	1.50	1.37	1.14

The selectivity factor of n-amyl benzene and n-butyl benzene is indication of column hydrophobic selectivity. It represents the column selectivity between alkyl benzene differentiated by one methylene group[30]. Like hydrophobicity, hydrophobic selectivity depends on surface coverage but the extent of the silica bonding process [31]. For C18 columns, $\alpha_{A/B}$ values were found to be in the range $1.5 < \alpha_{A/B} < 1.67$, while for C8 column the range was $1.42 < \alpha_{A/B} < 1.52$. In this work both $K`_A$ and $\alpha_{A/B}$ values were measured, because $K`_A$ gives an estimation of the absolute retention times of non polar compounds on column, whereas $\alpha_{A/B}$ provides information on the column's ability to separate hydrophobic compounds. The recorded data showed that some columns were still able to separate the two non-polar compounds fairly well, although the absolute retention times of the non-polar compounds are relatively low. For example $K`_A$ for column number 22 Figure (5) was only 4.06 while $\alpha_{A/B}$ was 1.52 and $K`_A$ for column number 32 Figure (6) was 5.83 and $\alpha_{A/B}$ 1.53. This is consistant with the findings of Kimata and his coworkers [28,34] and also consistant with studies reported in ref. [42, 57].

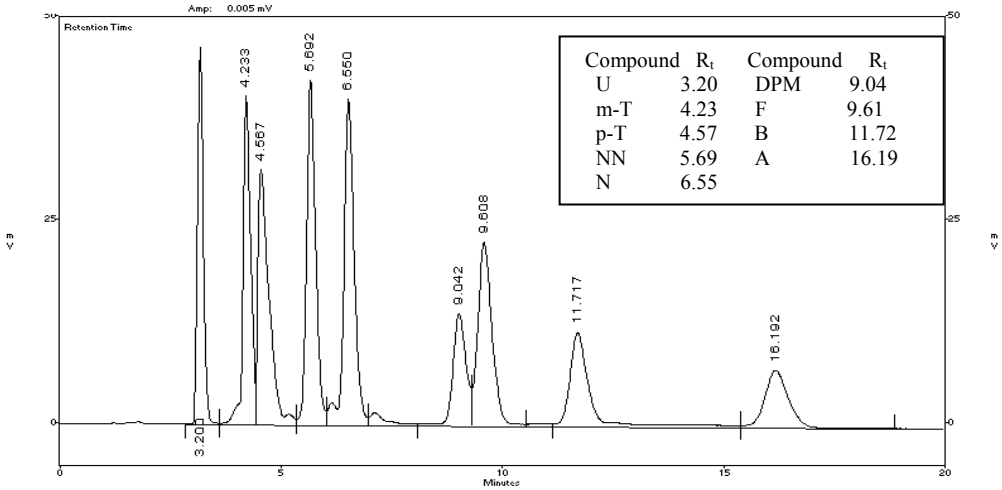

Figure 5: Test mixture A on column number 22 (Bondapak C18), where the last two peaks are for n-butyl and n-amylbenzene

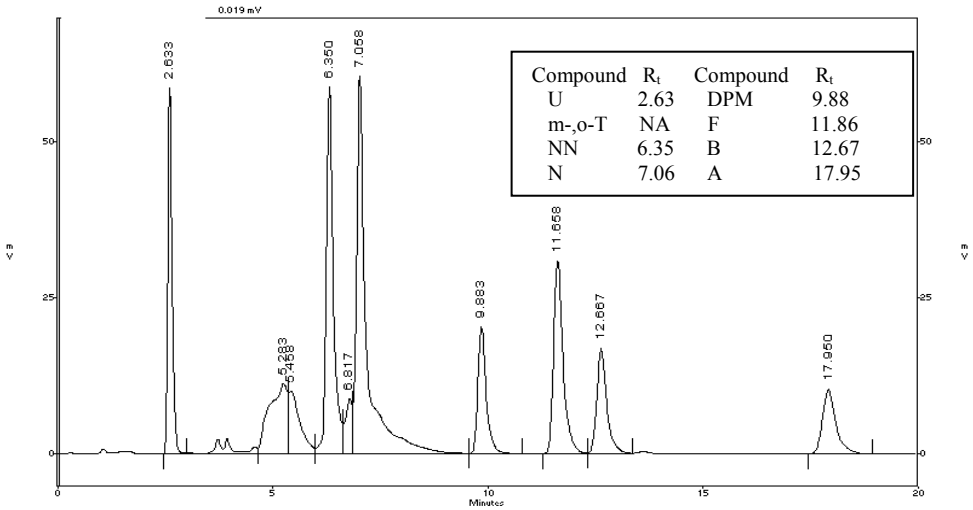

Figure 6: Test mixture A on column number 32 (Spherisorb ODS2 C18), where the last two peaks are for n-butyl and n-amylbenzene

Silanophilic Activity:

The importance of minimizing the number of unreacted accessible silanols stems from their deterious effects on the chromatographic behaviour of certain classes of compounds. The presence of residual silanol groups in reversed phase columns is associated with a number of undesirable interactions with polar solutes such as excessive peak tailing, irreducible retention times and excessively long retention times. Rabel [51] developed a simple qualitative test for silanophilic activity using aniline and phenol. Verzele and Dewale reported that the separation factor for naphthalene and

nitronaphtalene can be used to determine the success of deactivation of reversed phase columns by endcapping [52]. Engelhardt and coworkers proposed five basic compounds for the evaluation of silanophilic activity including aniline, phenol, and the m-, p- and o-isomers of toluidine with the mobile phase composed of 51% water and 49% methanol by mass. During the development of the test mixture in this work, the probes proposed by Engelhardt for evaluating silanophilic activity were first included in our test for the same purpose, because of its reported versatility and simplicity and employed in reversed phase rather than normal phase system. In our work, the mobile phase which was used by Engelhardt (55:45 methanol / water prepared by volume) was replaced by 65:35 methanol / water, in order to increase the speed of elution of the added test solutes. Measurment of the ratio of the asymmetry factor of aniline and phenol was found to be difficult when performed on some of the reversed phase columns, because the phenol aniline peaks overlapped. Therefore aniline was replaced by p-toluidine so that, in addition to the realative asymmetry factors, the relative retention of p-toluidine and phenol could be used to assess the silanophilic activity. In addition to phenol and p-toluidine, the three ethyl aniline isomers were also used for complete assesment. The evaluation is based on the fact that the asymmetry factor, as well as the retention factor of p-toluidine would increase with increasing the residual silanol group concentration, while phenol is used to normalise the hydrophobic contribution to the retention. Unfortunatly, this test could not be employed, as intended, because the problem of measuring the asymmetry factor of the p-toluidine peak, due to peak overlap, occurred again on some of the evaluated columns, particulary those which contained a relatively high concentration of residual silanol groups. On those columns the p-toluidine peak showed significant tailing and therefore it overlapped with the ethylaniline isomer's peaks. Finally, we decided to combine the two reported tests for the evaluation of the silanophilic activity and apply them together in our test mixture, One test proposed by Verzele and Rewale and involve the use of nitronaphthalene and Naphthalene. In addition the p- and m-toluidine isomers were used as proposed by Engelhardt [2,25,26]. These probes were found to elute at suitable retention times with respect to the other test probes. Nitronaphtalene interact with silanol groups via hydrogen bonding and therefore the retention of these compounds is much influenced by the presence of silanol groups. Naphthalene is not influenced by silinophillic activity because it does not contain any functional group that is able to interact with silanol groups. Because of this naphthalene was used as a reference to normalize the hydrophobic retention for nitronaphthalene. Moreover, the separation factor for naphthalene and nitronaphthalene could be used to assess the presence of residual silanol groups and therefore

evaluate the degree of activity or endcapping for a reversed phase column. According to Verzele and Dewale [24], a separation factor of least 1.4 or more is characteristic of a well-deactivated column. Lower values around 1.1-1.2 are characteristic phase without endcapping or high silanophilic activity. The toluidine isomers should be hardly separated on a good column because they possess identical hydrophobicity, but different pka values (4.73 and 5.08 for m- and p- tolnidine) respectively any residual silanol activity on the silica surface would separated these isomer's peaks.

The 38 columns evaluated in this work show widely different behaviour with respect to their silanophilic activity. These columns could be divided into four categories according to the separation factor values of naphthalene and nitronaphthalene and to their ability to separate the toluidine isomers. The first category contained columns on whish the toluidine was completely coeluted or was hardly separated, with highly symmetric peaks and for which $\alpha_{N/NN}$ values (separation factor of naphthalene and nitronaphthalene) were 1.4 or more. These columns are believed to have no accessible silanol groups on the surface and are suitable for the analysis of polar and basic compounds. Examples of these columns are columns number 1, Figure (7) and column number 5, Figure (8), where $\alpha_{N/NN}$ values 1.73 and 1.71 respectively as shown in Table 6.

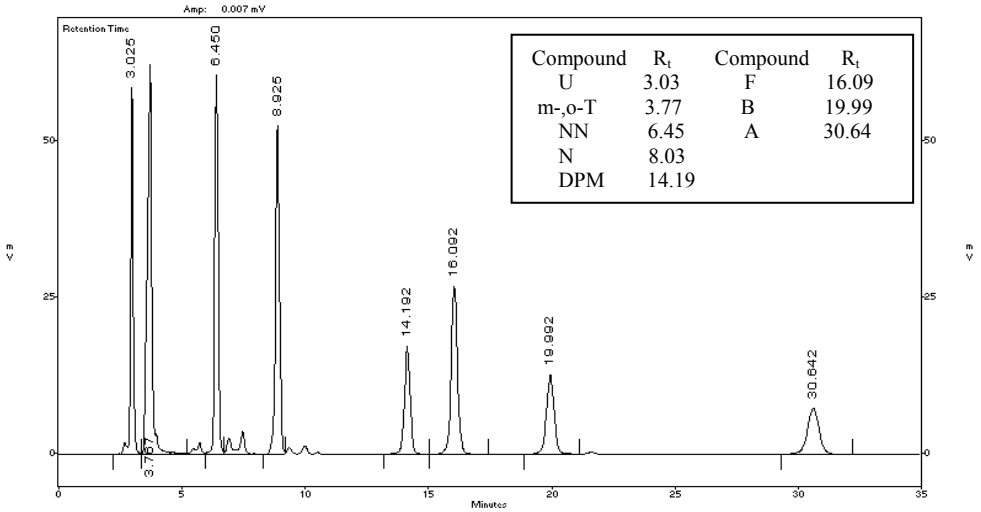

Figure 7: Test mixture A on column 1 (HyPURITY Elite C18), which shows no contribution from the residual silanol groups (first category column)

The toluidine isomers were completely separated or distorted and excessively retained, on the second category of columns. The separation factor values on naphthalene and nitronaphthalene on these column were less than 1.4 which indicated that these packing were not endcapped and as

result their content of unbonded and accessible silanol group is high. These columns can be classified as poor columns in terms of silnophilic activity and furthermore, can not be used for analysis of polar and basic compounds particularly in unbuffered mobile phases. Columns numbers 7 Figure (9) and column number 8 Figure (10) represent this category of reversed phase columns.

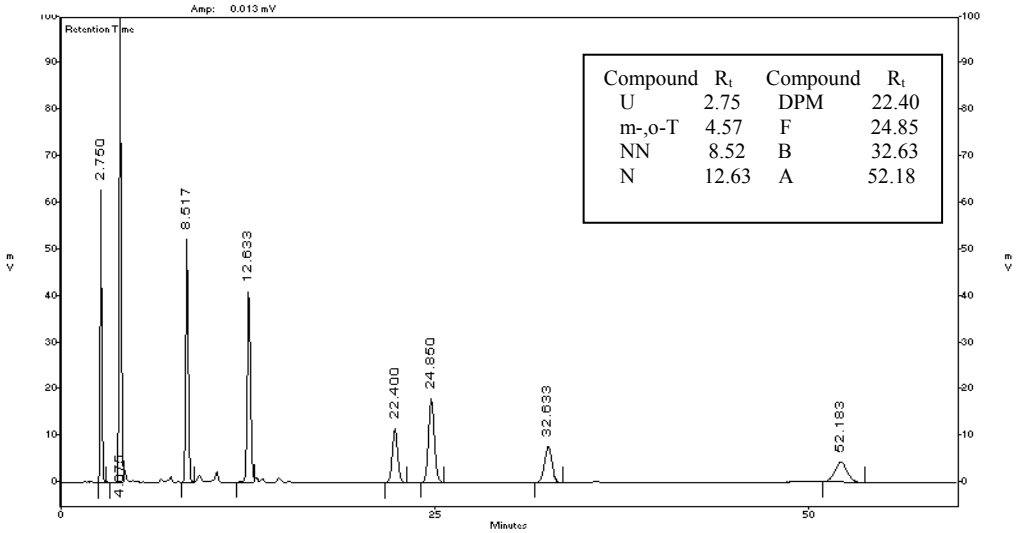

Figure 8: Test mixture A on column 5 (Genesis C18), which shows no contribution from the residual silanol groups (first category coloumn)

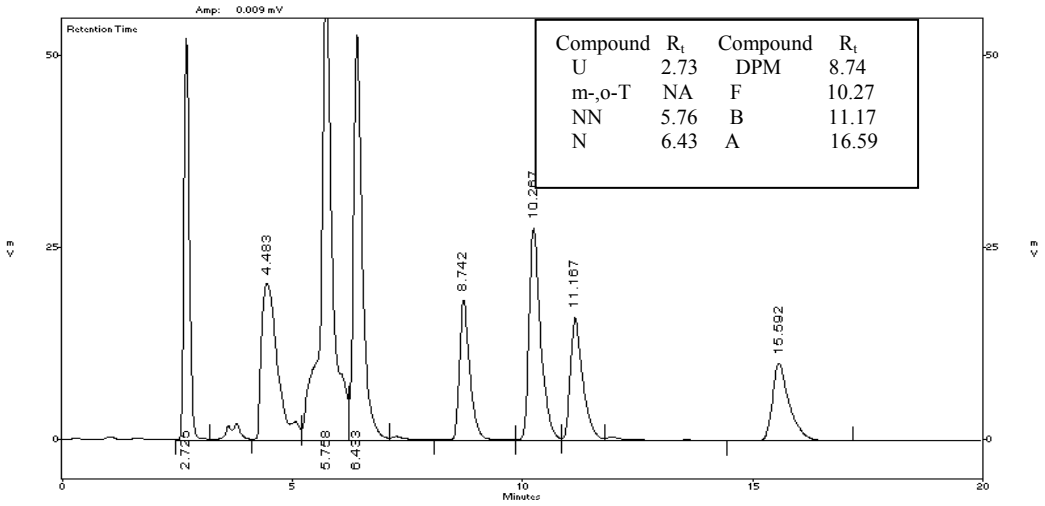

Figure 9: Test mixture A on column number 7 (Spherisorb ODS1 C18), a second category column.

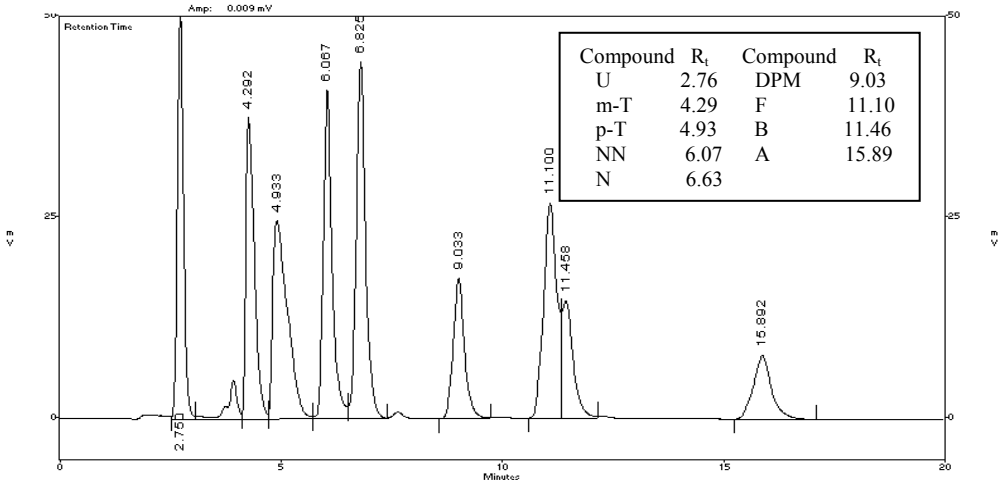

Figure 10:Test mixture A on column number 8 (Spherisorb ODS1 C18), a second category column

Although all the columns in the third category were found to be endcapped the toluidine isomers peaks varied from partially separated to completely distorted, which these columns have separation factor values around 1.5. An explanation of the activity of these columns towards basic compounds could be that there are residual silanol groups, which are sterically inaccessible to nitronaphtalene but which are accessible to the toluidines which consistent with the studies reported in ref. [42]. The different degrees of observed separation of the toluidine's peaks are thought to be proportional to the amount of these silanol groups. The possibility of trace metals contribution as lewis acid sites were excluded, according to Sadek and Car[41]. Representative examples of the third category columns are column number 2 Figure (11), column number 10 Figure (12) and column number19 Figure (13).

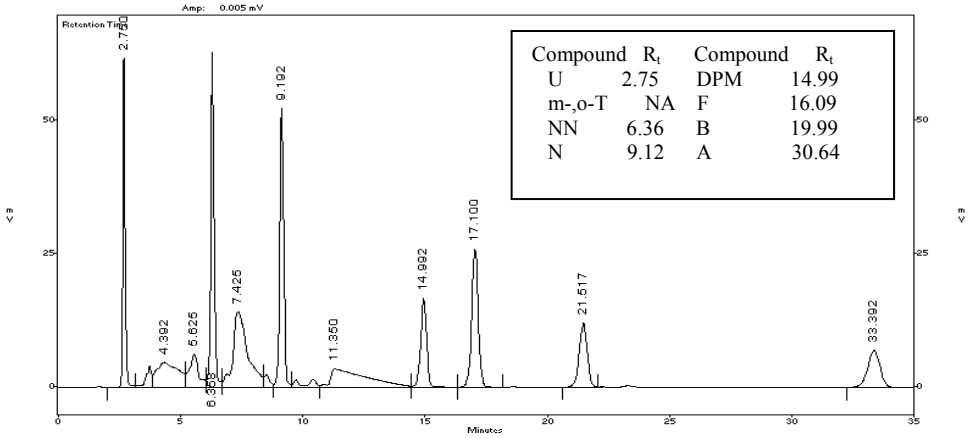

Figure 11: Test mixture A on column number 2 (Hypersil BDS C18), a third category column

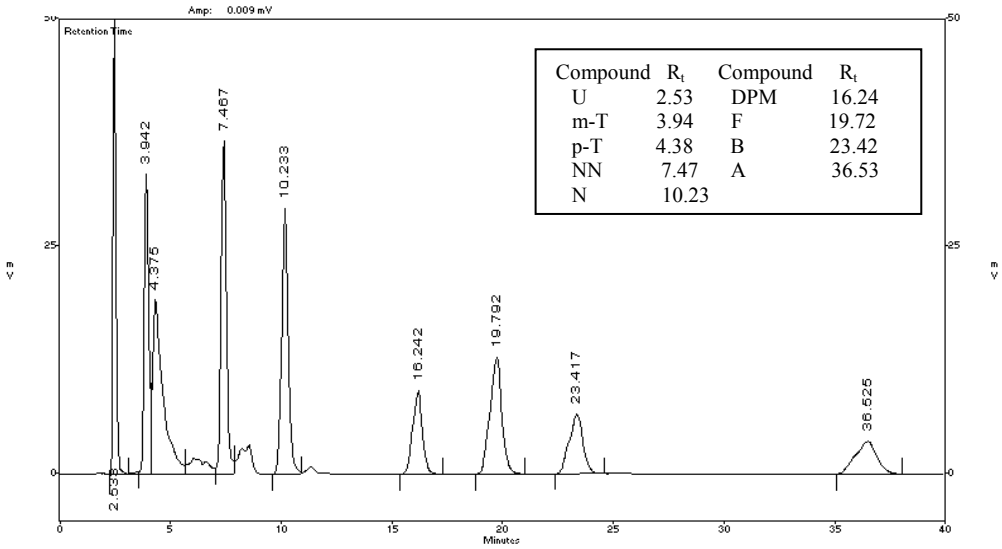

Figure 12: Test mixture A on column number 10 (Spherisorb ODS2 C18), a third category column

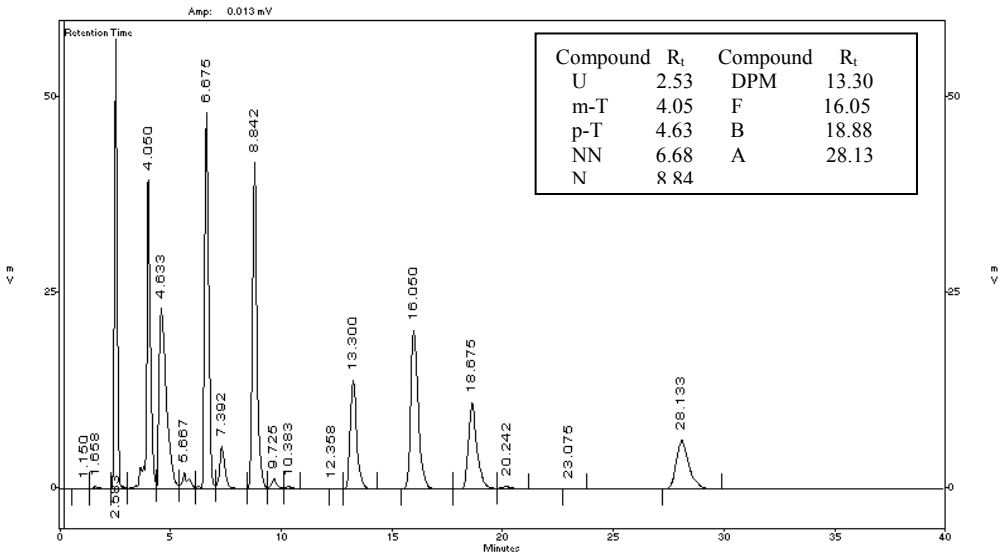

Figure 13: Test mixture A on column number 19 (Spherisorb ODS2 C18), a third category column.

The observed behaviour of columns in the first and the third categories implied that column manufactures vary in performing their endcapping processes. As the main objective of these processes is to remove all the sterically in accessible silanol groups by the non-polar ligands (e.g. C8, C18) by bonding them with a small, highly reactive silanizing reagent such

as trimethyl chlorosilane (TMS). Columns in the first and the third categories were proved to be endcapped by both the values of $\alpha_{N/NN}$ and according to column suppliers. The use of toluidine isomers revealed that although some columns undergo endcapping processes some active silanol groups remain on the packing material surface.

Columns in the fourth category, which have separation factor values on naphthalene and nitro naphthalene on these columns were around 1, showed unexpected behaviour where the values of $\alpha_{N/NN}$ implied that these columns were not endcapped yet the toluidine isomers were completely coeluted. Examples of this category are column number 30 and 34. The widely different behavior found for reversed phase in terms of silanophilic activity emphasis that column manufacturers vary significantly in preparing their reversed phase columns and therefore a column must be characterized for this aspect before performing any analysis for basic or polar compounds. The test developed in this work was efficiently able to characterize reversed phase columns for their important parameters. According to the obtained results with the 38 commercially available columns, a good column in term of silanophilic activity should fulfil two requirements, even with an unbuffered mobile phase. Firstly, the $\alpha_{N/NN}$ value should be at least 1.4 and secondly the toluidine isomers should coelute or be hardly separated with highly symmetric peak's shapes, exactly like the first category columns. Any other behaviour, like that observed in the other categories of columns described above, would restrict the possibility of using a column for the analysis of basic and polar compounds at least in unbuffered mobile phases.

Shape Selectivity:

Shape selectivity (or steric selectivity) of reversed phase column can be defined as the chromatographic discrimination of compounds on the basis of molecular structure or a column ability to separate compounds having similar hydrophobic properties but which differ in planarity [28,32]. During this work, Fluorene and diphenylmethane were used for the evaluation of shape selectivity and were found to elute at suitable retention times relative to other test solutes.

Fluorene eluted after diphenyl methane on all of the evaluated columns except on two C8 columns number 35 and 37 where diphenyl methane eluted first. The values of $\alpha_{F/D}$ (separation factor of fluorene and diphenyl methane) ranged from 1.02 to 1.33 on the tested columns and 0.95 and 0.94 on the C8 columns respectively, as shown in Table 6. The relatively low values measured for the evaluated columns implied that all of these columns were prepared by reaction of monochlorosilane reagent with the support silica and as a result all of them have a monomeric phase type. It can be concluded that these columns are not suitable for separating polyaromatic

hydrocarbons and related compounds. A column with higher $\alpha_{F/D}$ value would be more suitable for this purpose. Figure(1) and (14) show how reversed phase columns differ in their shape selectivity.

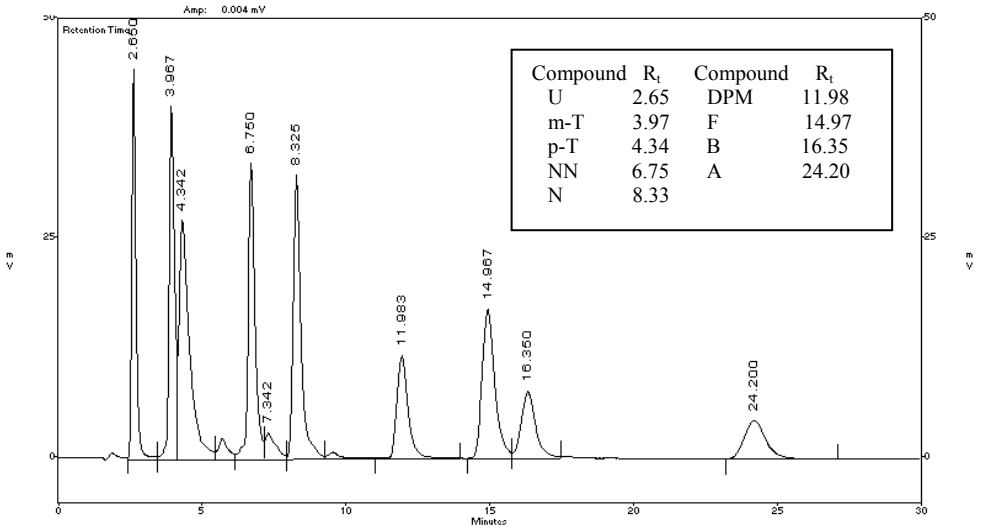

Figure14: Test mixture A on column number 27 (Spherisorb ODS2 C18), where $\alpha_{F/D}$ value is 1.32

Trace metal activity:

Conventionally manufactured silica gel are usually prepared from alkaline silicate, which contains various amounts of sodium, calcium, magnesium, iron and aluminum ions [30]. It is found that the degree of sulphoxide epimerisation of the α-ethyl sulphoxide epimers of triedane showed no direct correlation with measured chromatographic parameters for a wide range of commercially available octadecylsilyl stationary phases. The correlation was established with metal content of these phases [56]. There are no standardized procedures taken by column manufacturers to control the metal content in their column. It is therefore, of interest to evaluate reversed phase columns interms of their metal activity. In this work, selecting a compound for evaluating reversed phase column in terms of their metal activity has been achieved after trying a large number of compounds including 2,3-dihydroxy naphthalene, B-diketone, 2,4-pentadione, phthalates. Since a compound for evaluating trace metal activity could not be included in test mixture A. It was decided to include such a compound in test mixture B. This would not effect the evaluation process as investigation of trace metal activity were reported in both buffered and unbuffered mobile phase [33-35]. Since test mixture B contained only three compounds (uracil, phenol, N, N-dimethyl aniline), it was relatively easy to employ a compound for evaluating trace metal activity with a suitable elution time, 1,4-dihydroxy anthraquinone was found to be useful for this

purpose.

The presence of metal in a reversed phase column could be estimated according to the asymmetry factor and peak shape of quinizarin. The asymmetry factor should be around 0.9 to 1.3 for column prepared from pure silica, while it would increase as the trace metal impurities increase. The data obtained for the 38 columns evaluated in this work showed significant differences in their trace metal contents. As the quinizarin peak shape varied from highly symmetric to very poor peaks. The ratio of quinzarin peak area to peak height Q_A/Q_H (c.f. Table 7) was used, in addition to the asymmetry factor, to evaluate metal impurities for two reasons. Because of severe tailing, the asymmetry factor for some peaks could not be calculated and on some column quinizarin eluted with very flat peak shape resulted in high asymmetry factor, in spite of its poor diagnostic shape. The calculated values of Q_A/Q_H did not exceed 25 for good columns where asymmetry factors were around 0.9-1.3. Figure (15) shows the quinizarin peak shape on column number 2 where the value of As is 1.06 and Q_A/Q_H is 14.6 as shown in Table 7.

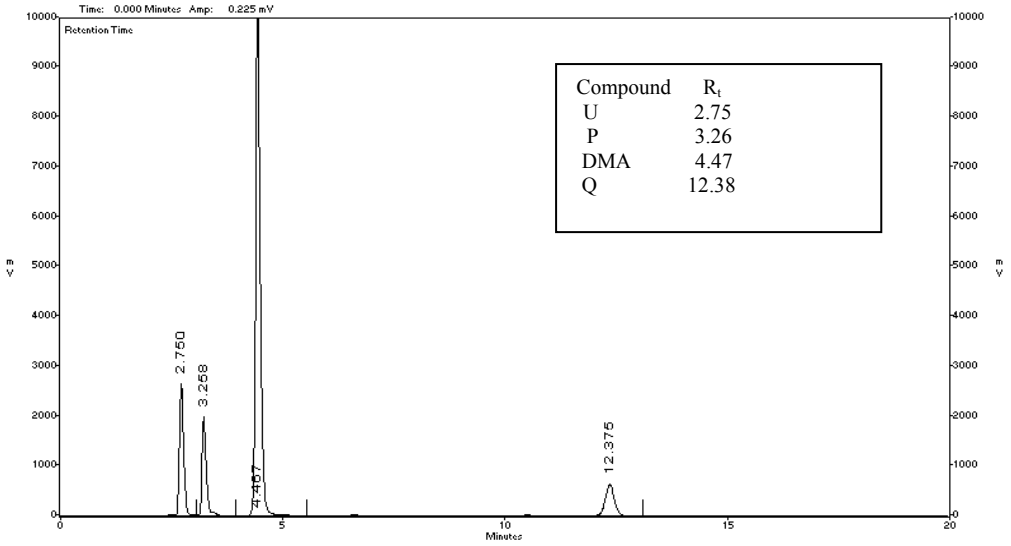

Figure 15: Test mixture B on column number 2 (Hypersil BDS C18), where the labeled peak is for quinizain

Table (7): Column properties evaluated by test mixture B

Col. No.	Packing Materials	$\alpha_{DM/P}$	A_{SDM}	A_{SQ}	Q_A/Q_H
1	HyPUTIYT Elite C18	2.91	1.19	1.14	18.49
2	Hypersil BDS C18	3.32	1.24	1.06	14.63
3	Hypersil ODS C18	12.47	8.87	NA	63.06

4	Luna C18 (2)	3.68	0.95	1.07	17.19
5	Genesis C18	3.96	1.03	1.15	20.83
6	Lichrosorb RP18 C18	4.31	2.11	2.92	52.94
7	Spherisorb ODSI C18	5.86	2.05	3.22	41.91
8	Spherisorb ODS1 C18	5.92	2.40	2.38	35.18
9	Lichrosorb RP18 C18	8.88	4.76	1.25	149.72
10	Spherisorb ODS2 C18	13.54	4.88	1.58	112.57
11	Lichrosorb RP18 C18	4.59	1.80	1.65	59.69
12	Nucleosil C18	3.51	1.31	3.46	40.39
13	Nucleosil C18	3.55	2.18	4.16	43.33
14	Nucleosil C18	3.59	1.16	1.98	48.77
15	Nucleosil C18	3.66	1.54	3.30	57.21
16	Nucleosil C18	3.69	1.58	2.48	65.72
17	Ultrashere ODS C18	8.70	4.96	3.96	154.94
18	Kromasil C18	3.54	0.76	0.78	42.44
19	Spherisorb ODS2 C18	4.72	2.69	3.75	114.82
20	Spherisorb ODS2 C18	9.27	4.86	1.09	170.64
21	Lichrosorb RP18 C18	4.28	2.52	3.66	74.53
22	Bondapak C18	3.29	1.20	1.75	22.52
23	Spherisorb ODS1 C18	7.20	3.19	2.21	49.93
24	Spherisorb ODS2 C18	6.73	2.92	6.44	111.46
25	Spherisorb ODS2 C18	5.17	2.45	3.38	61.20
26	Spherisorb ODS2 C18	7.98	2.85	2.59	122.29
27	Spherisorb ODS2 C18	7.77	4.23	3.76	57.08
28	Spherisorb ODS C18	6.85	5.51	NA	147.93
29	Hypersil ODS C18	3.47	1.47	1.16	19.49
30	Lichrosorb RP18-Select B	2.90	1.72	3.45	33.34
31	Hi-RPB 2517	2.68	1.46	1.54	26.19
32	Spherisorb ODS2 C18	4.86	2.03	4.50	62.25
33	Lichrosorb RP18 C18	3.78	0.85	1.12	45.25
34	Lichrosorb RP-8 C8	2.75	1.86	1.76	27.07
35	Lichrosorb RP-8 C8	2.31	0.95	0.90	20.37
36	Lichrosorb RP-8 C8	2.74	1.81	2.00	35.51
37	Lichrosorb RP-8 C8	2.33	1.11	1.22	24.40
38	Spherisorb C8	12.57	2.78	1.08	10.53

The value of Q_A/Q_H was found to increase with increasing metal activity as the peak height decreased with increasing the tailing effect. For example on column number 24, Figure (16). The value of As is 6.4 and the Q_A/Q_H is 111.5. The benefit of using the ratio Q_A/Q_H would be appreciated in cases like columns number 3 and number 20 where the As value could

not be measured on the first and a very symmetric flat peak obtained on the second.

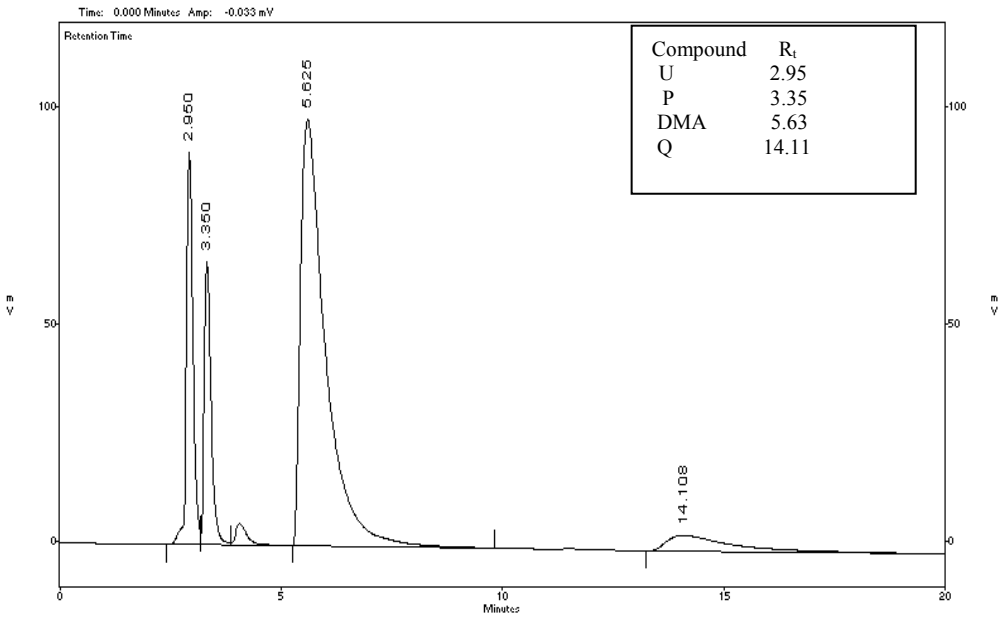

Figure 16: Test mixture B on column number 24 (Spherisorb ODS2 C18), where the labled peak is quinizain

Cation exchange capacity:

The surface silanol groups are not homogenous and small proportions of these are strongly acid sites and ionized even at low pH[31,36,37]. The small number of highly acidic sites can function as cation exchanges and are thought to be responsible for the anomalous behaviour of solutes which are ionised under the conditions of elution[28,31,37,38]. These sites are generally considered to contribute significantly to the low recovery and poor chromatographic behaviour of proteins and basic compounds in reversed phase chromatography. A general test for ion exchange capacity of reversed phase packing has been described based on the separation factor for benzyl amine/phenol (A/P) in the mobile phase buffrered to pH 2.7 [53]. At this pH normal silanol groups are undissociated and highly acidic silanol contribute to the retention of the amine by ion exchange. The value of A/P was found to vary widely for a number of column packing indicating a wide range of ion exchange capacities. Kimata found that the retention of protonated amines at acidic pH are not correlated with the amount of silanol measured at unbuffered mobile phase [53]. Stationary phases prepared from acid treated silica showed very small retention of protonated amines and good performance at acidic pH regardless of the amount of silanol, while

phases endcapped showed the absence of the hydrogen effect regardless of the extent of ion exchange effect. Other compounds have been suggested to evaluate cation exchange capacity for reversed phase columns [53,54]. These compounds are mainly amines and include anilline, N-methyl anilline, N,N-dimethyl anilline, N-procainamide and N-acetyl procainamide. In this work, N,N-dimethyl aniline has been used due to elution time consideration. Phenol was used to normalise the hydrophobic interaction of the packing material. The evaluation was based on two parameters. The separation factor of N,N-dimethyl aniline and phenol $\alpha_{DMA/P}$ and the asymmetry factor for the N,N-dimethyl aniline peak A_{SDM}. The greater these values, the greater the cation exchange capacity of a column. Since more retention of the amine on the packing material will be expected due to further interaction with this cation exchange sites. The values of these parameters were found to very from 2.3 to 13.5 for $\alpha_{DMA/P}$ and 0.85 to 8.87 for the A_{SDM} on thirty eight columns evaluated reflecting widely different cation exchange capacities. Unsurprisingly, a good correlation has been found for the values of $\alpha_{DMA/P}$ and A_{SDM} values. Another correlation could be done for the tarce metal activity indicated by the asymmetry factor of the quinizarin peak and cation exchange capacity. The value of $\alpha_{DMA/P}$ was found to increase with increasing the value of A_{SQ}. This might support the idea of metal influence on silanol groups acidity. Figures (16) and (17) show two chromatograms of columns on which the cation exchange capacity increases with increasing metal content.

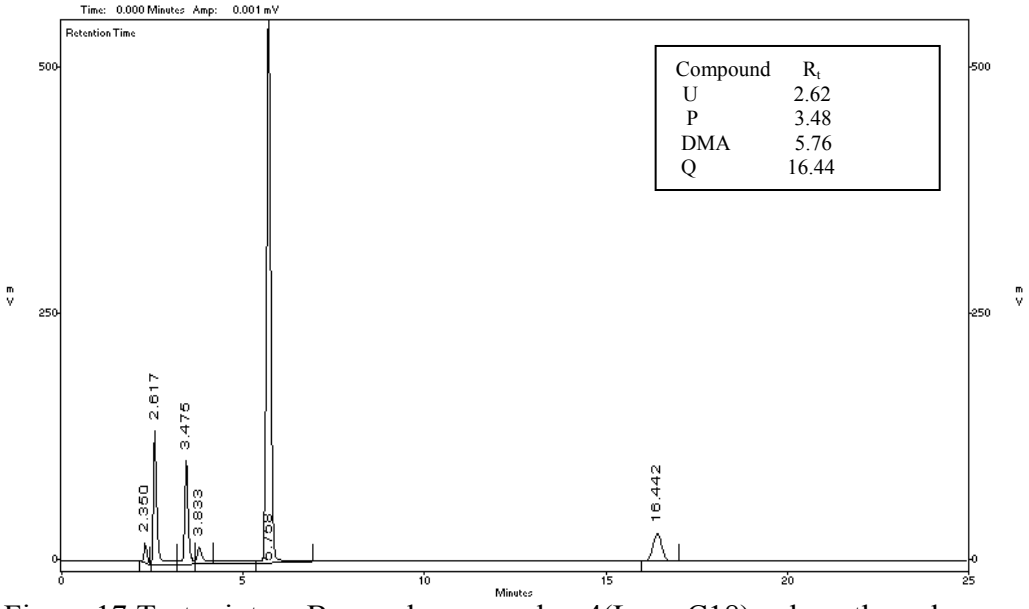

Figure 17:Test mixture B on column number 4(Luna C18), where the value of $\alpha_{DMA/P}$ is 3.68 and the value of A_{SQ} 1.07

Evaluation Procedures Conditions:

Column temperature:

Nearly all the physical parameters that play a role in liquid chromatography (LC) separations are affected by temperature. For general use of HPLC, maintaining the column temperature at constant value is very important to avoid quantitation and identification errors caused by resolution loss and variable retention times, respectively.

Effect of temperature and mobile phase composition on column evaluations:

Reversed phase separations are dependent on fluctuations in temperature and mobile phase compositions [25,26,39]. The influence of these fluctuations on column evaluation was studied by varying the temperature and the mobile phase compositions at values close to those of the test conditions.

Effect of mobile phase compositions on column evaluation:

Retention in reversed phase chromatography is strongly influenced by slight changes in the mobile phase composition. A series of mobile composition ranged from 68:32 methanol/water were prepared by mass and the same column was evaluated comprehensively at each single composition at constant conditions.

Data in Table 8 shows the influence of mobile phase composition on the kinetic performance evaluation of a column. The observed changes in the values of dimensionless parameters do not affect significantly the evaluation as the column is still within the same stated limits. This is consistant with Snyder's studies [45]. The change in E value was more obvious because it is result of multiplication of two other variables h and φ each one of them is also changed.

Table (8): Effect of mobile phase composition on column kinetic evaluation

MeOH:H_2O % by mass	**h**	**v**	**φ**	**E**
62:38	3.02	7.09	1040	9471
63:37	3.30	7.09	1057	11541
64:36	3.37	6.14	1172	13280
65:35	3.25	6.88	1023	10826
66:34	3.24	6.81	1031	10850
67:33	3.52	6.81	1032	12803
68:32	3.53	6.68	1025	12798

The effect of mobile phase composition on the parameters measured by

test mixture A is displayed in Table 9.

As expected the retention factor of n-amyl benzene is affected significantly by changing water content in the mobile phase. The $K`_A$ value varied from 6.42 to 12.46 over the employed range of mobile phase composition. Because the other parameters measured in this test are ratios of two different retention factors, the effect of the mobile phase composition on them is not significant. The slight changes of these parameters might be because the retention of every compound is affected slightly. Table 10, displays the effect of the mobile phase composition on the evaluation performed by test mixture B. The values of all the parameters measured in this test are still in the same range and therefore the column is still classified in the same categories in terms of its cation exchange capacity and trace metal activity. It is clear that a slight change in the mobile phase composition does not change significantly the column evaluation. This may be attributed to the fact that most of these parameters are relative but not absolute measurements. However, maintaining mobile phase constant, like temperature, is recommended for reproducible results.

Table (9): Effect of mobile phase composition on the parameters evaluated on the parameters evaluated by test Mixture A

MeOH:H_2O %by mass	**$K`_A$**	**$\alpha_{A/B}$**	**$\alpha_{N/NN}$**	**$\alpha_{F/D}$**
62:38	12.46	1.68	1.72	1.15
63:37	11.38	1.66	1.73	1.16
64:36	9.97	1.64	1.73	1.16
65:35	9.17	1.63	1.73	1.17
66:34	8.06	1.61	1.72	1.17
67:33	7.34	1.59	1.72	1.18
68:32	6.42	1.59	1.71	1.19

Table (10): Effect of mobile phase composition on the parameters evaluated by test mixture B

MeOH:Buffer %by mass	**$\alpha_{DM/P}$**	**A_{SDM}**	**A_{SQ}**	**Q_A/Q_H**
73:27	3.63	1.30	1.08	12.74
72:28	3.47	1.27	1.09	13.38
71:29	3.59	1.28	1.10	14.06
70:30	3.32	1.24	1.06	14.63
69:31	3.26	1.30	1.08	15.58
68:32	3.20	1.31	1.07	16.31
67:33	3.05	1.27	1.06	17.31

Effect of temperature on column evaluation:

Table 11 shows the effect of changing temperature within the range 35-45^{o}C on the kinetic performance evaluation of column. As expected, the value of reduced eluant velocity v decrease with increasing the temperature due to increasing the diffusion coefficient. The change in the reduced plate height value was not significant (1.2%) at 35^{o}C while it remained constant at 45^{o}C. Although the values of dimensionless flow resistance φ and separation impedance (E) were changed slightly the same evaluation is still valid for the column.

Table (11): Effect of temperature on column kinetic evaluation

Temp ^{o}C	**h**	**v**	**φ**	**E**
35	3.29	7.89	1039	11246
40	3.25	6.88	1041	11020
45	3.25	5.60	1095	11565

The influence of temperature on the chromatographic parameters measured by test mixture A is displayed in Table 12, The decrease in the capacity factor of n-amyl benzene as the temperature increased was expected since the retention is inversely proportional to temperature[40]. Slight changes were observed for the measured separation factors, the shape selectivity increased as the temperature decreased. This behaviour has been observed before [28,40] and was explained as the alkyl bonded phases become more rigid at low temperatures and therefore, the retention of linear and planar molecules is better than of spherical and other molecules. Despite the slight changes in the parameters measured in test mixture A, the same evaluation is still valid since the values of these parameters are still within the same limits discussed earlier.

Test mixture A:

Table (12): Effect of Temperature on the parameter evaluated by Test mixture A

Temp ^{o}C	**K`$_{A}$**	**α $_{A/B}$**	**α $_{N/NN}$**	**α $_{F/D}$**
35	9.99	1.65	1.73	1.19
40	9.17	1.63	1.73	1.17
45	8.05	1.60	1.72	1.15

Table13, shows the influence of temperature on the chromatographic parameters measured by test mixture B. Temperature had no clear effect on the parameters measured by test mixture B, but it can be stated that the same evaluation is still valid despite the difference in temperature. This indicates

that the evaluation of column kinetic performance and thermodynamic activity are not affected significantly by fluctuations in temperature. These findings are consistant with studies reported by Snyders et.al [45]. Nevertheless maintaing constant temperature is recommended for reproducible results that help in investigating the column behaviour periodically.

Test mixture B:

Table(13):Effect of Temperature on the parameter evaluated by Test mixture B

Temp ^{o}C	α_{DMP}	A_{SDM}	A_{SQ}	Q_A/Q_H
35	7.94	3.70	2.52	59.74
40	7.94	4.22	2.52	59.74
45	7.31	3.70	2.63	52.60

Repeatability of column evaluation:

The test procedure has been repeated 10 times on one column under the same conditions. The statistical calculation were carried out by using a computer based statistical package called "SPSS 7.5.1". The data obtained for the kinetic parameter test mixture A and mixture B are displayed in Table 14. The results indicate that the test parameters are highly precise. An alternative of precision is the 95% confidence interval of the main, a small intervals represents high precision.

Table (14): Repeatability of evaluation procedures

Variable X	Mean X	SD	S.E.Mean	95%confidence interval of the mean
Kinetic perforamance evaluation				
H	3.30	0.01	0.00	3.30±0.00
V	6.87	0.02	0.01	6.87±0.02
Φ	1024	3	1	1024±0.02
E	11144	47	15	11144±34
Test mixture A				
$K`_A$	9.01	0.03	0.01	9.01±0.02
$\alpha_{A/B}$	1.63	0.01	0.00	1.63±0.00
$\alpha_{N/NN}$	1.72	0.00	0.00	1.72±0.00
$\alpha_{F/D}$	1.17	0.00	0.00	1.17±0.00
Test mixture B				
$\alpha_{ADM/P}$	3.37	0.00	0.00	3.37±0.00
A_{SN}	1.25	0.02	0.01	1.25±0.02
A_{SQ}	1.04	0.01	0.00	1.04±0.00
Q_A/Q_H	14.61	0.02	0.01	14.61±0.02

CONCLUSION

A simple improved procedure for a comprehensive evaluation of C8 and C18 reversed phase HPLC columns has been investigated. A column can be characterised in terms of the kinetic performance, silnophilic activity, hydrophobicity, hydrophobic selectivity, shape selectivity, ion exchange capacity and trace metal activity. The simplicity of this work is derived from its dependence on only two chromatographic runs in two mobile phases, the use of readily available solutes and the simple calculation needed.

The column evaluation process is highly reproducible and is not significantly influenced by slight changes in column temperature and mobile phase composition. Application of these improved procedures on 38 reversed phase columns enabled us to characterise and classify these columns according to their efficiency and validity for the analysis of different classes of compounds. This procedure would be useful in charcterizing and classifying reversed phase columns and determing the suitability of a column for analysis of certain classes of compounds.

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Nomenclature

RPLC: Reversed phase liquid chromatography
h: reduced plate height
v:reduced eluant velocity
φ: reduced flow resistance
E:separat ion impedance
L:column length
dp: the particle size in μm
$W_{0.5}$: Naphthalene peak width at half height
t_R: Naphthalene retention time
η: viscosity of mobile phase
Dm: diffusion coefficient of naphthalene in mobile phase in m^2s^{-1}
t_o : Uracil retention time
P: column's pressure value in bar.
K_A : retention factor of n-amyl benzene
$\alpha_{A/B}$: hydroohobic selectivity (separation factor of n-amyl and n-butyl benzene)
$\alpha_{F/D}$: shape selectivity (separation of Flourene and diphenyl methane)
$\alpha_{N/NN}$: Separation factor of naphthalene and 1-nitronaphthalene.

$\alpha_{DM/P}$: selectivity factor of N,N-dimethyl aniline and phenol
A_{SDM} : peak asymmetry of N,N-dimethyl aniline
A_{SQ}: peak asymmetry of quinizarine
Q_A/Q_H: ratio of quinzarine peak area to peak height
R_t: retention time
As: Asymmetry factor
U: uracil
m-T:m-Toluidine
P-T: P-Toluidine
NN: nitronaphthalene
N: naphthalene
DPM: diphenyl methane
F:Fluorene
B:n-butyl benzene
A: n-Amyl benzene
P:phenol
DMA: N,N-dimethyl aniline
Q: Quinizarine

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