Review

Study on Retention in Liquid Chromatography

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Abstract

After joining the faculty at Toyohashi University of Technology (TUT) in 1978, the author has found two areas in separation sciences where microcolumn liquid chromatography (micro-LC) can be beneficial. One is the hyphenated techniques between many spectroscopic methods such as mass (MS), infrared (FT-IR) and atomic emission (ICP), and micro-LC. The other one is rather difficult, but basic and theoretical approach which deals with retention mechanism in LC. The latter project has especially been giving him a lot of scientific funs and honors in the last 20 years. On the occasion of being awarded by The Society for Chromatographic Sciences the author would like to summarize his contributions to this topic which asks us "What Is Chromatographic Retention ?".

Keywords: liquid chromatography, QSRR, retention mechanism, retention prediction, molecular recognition, design of novel stationary phases

1. Introduction

As you know chromatography, in any system can only give us information on retention, sometimes it is a retention time, or a retention volume and sometimes it is a so-called retention factor. This retention information unfortunately cannot be useful as an accurate identification tool, but it just gives us quantitative information which is defined by the peak area in the output format which is so-called chromatogram. It also gives the analyst rough qualitative information by the confirmation using some analytes which can give the same retention to those of the standard solutes. Therefore, chromatography in itself is not an accurate analytical technique, but it is rather a separation technique. The process of identification of the components resolved by chromatography requires the use of spectroscopic methods. In order to improve this situation of chromatography one has to explain the details about the retention mechanism and then to use this idea for chromatography to the tool for accurate identification.

2. QSRR Approach

Based on the mentioned idea, the work to define the retention in reversed-phase LC has been started in 1981. The approach selected is **QSRR** (**Q**uantitative **S**tructure-**R**etention **R**elationships) [1]. QSRR in LC is the method to identify the properties of the solutes that control their retention by correlating the retention factors (*k*) measured at certain separation conditions in LC and solutes' properties (*Pi*, descriptors) such as physicochemical, topological or geometrical factors, using multiple regression analysis.

Generally retention factor k obtained at certain LC conditions can be described by *Pi* by the following basic function:

$$k = f(Pi) \qquad [1]$$

If *Pi* that can give us the highest correlation coefficient for the equation [1] is found, one can know the most important factor to control the retention and then the retention mechanism can be better understood.

Many different compounds were chosen as test probes that are aromatic compounds, polycyclic aromatic hydrocarbons (PAHs), amino acids, amino acid derivatives, oligopeptides, drugs, pesticides, polymer additives and so on. For *Pi* there are so many factors which can be related to the retention, based on the idea of **Q**uantitative **S**tructure-**A**ctivity **R**elationships (**QSAR**) for drug design. Various parameters of *Pi* selected are as follows: physicochemical descriptors; hydrophobicity, log P (partition coefficient between 1-octanol and water) and hydrophobic substitution constant π [2,3], geometrical descriptors; van der Waals volume (Vw), van der Waals surface area (Aw) [4] and shape parameter[5] or length to breadth ratio (L/B)[6], topological descriptors; connectivity index χ

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[7] and correlation factor (F) [8], electronic descriptors; Hammett's constant σ, HD and HA which are proton donating and proton accepting properties, respectively. By the multiple regression analyses of these retention data with the above mentioned descriptors the most dominant properties are found by the highest regression coefficients. The results suggest that the most important descriptor in reversed phase LC separations is hydrophobicity [9,10]. The second dominant descriptors are the shape and size of the molecules for non-polar compounds and electronic descriptors for polar compounds. The most typical case for PAHs are described in details. The retention data of PAHs with four different LC systems were measured as seen in Table 1, and then the regression coefficients between k and the descriptors were calculated by using a computer. The results are summarized in Table 2. High regression coefficients were obtained for several descriptors except L/B which can give low regression coefficients alone. However, as seen in Table-3, the multi-descriptors equations with F and L/B for four different separation systems of C2, Cp, C8 and C18 stationary phases gave the highest regression coefficients, and this means that if one can generate such equations to describe the retention, like equations seen in Table 3, we can predict retention of other PAHs if F and L/B values for those compounds are available. Based on this concept the retention factor values of 16 PAHs were predicted and compared to those observed by the experiments in Table 4. It clearly appears that prediction accuracy of this method is very good.

3. Construction of Computer Assisted Separation System

Based on the facts found by the basic QSRR studies, the retention can be described by several descriptors as a function of separation conditions such as

Table 1. Retention factor values and various descriptors of PAHs on four reversed-phase columns.

	Retention factor at 20˚C					Value of descriptor			
Solute	C_2	C_p	C_8	C_{18}	log *P*	χ	Vw	L / B	F
Benzene	1.89	2.00	1.48	1.05	2.16	2.000	48.36	1.10	3.0
Indene	2.77	3.08	1.95	1.45	2.99	3.211	68.07	1.22	4.5
Indan	3.52	3.69	2.38	1.92	3.33	3.534	72.41	1.16	5.5
Naphthalene	3.01	3.55	2.22	1.67	3.18	3.405	73.96	1.24	5.0
Acenaphthylene	3.35	4.24	2.49	2.03	3.48	4.149	83.44	1.08	5.5
Acenaphthene	4.13	4.99	2.81	2.44	3.82	4.445	87.78	1.06	6.5
Biphenyl	4.11	4.94	2.69	2.10	3.90	4.071	90.08	1.72	6.0
Fluorene	4.40	5.29	2.89	2.44	4.01	4.612	93.67	1.57	6.5
Anthracene	4.81	6.27	3.36	3.20	4.20	4.809	99.56	1.57	7.0
Phenanthrene	4.49	5.86	3.14	2.99	4.20	4.815	99.56	1.46	7.0
Pyrene	5.56	7.47	3.96	4.45	4.50	5.559	109.04	1.27	8.0
Fluoranthene	5.59	7.42	3.80	3.97	4.50	5.565	109.04	1.22	8.0
Benzo[*b*]fluorene	7.36	9.50	4.48	4.91	5.03	6.017	119.27	1.78	8.5
Naphthacene	8.28	11.4	5.27	7.18	5.22	6.214	125.16	1.89	9.0
Chrysene	7.13	10.4	4.88	5.93	5.22	6.226	125.16	1.72	9.0
Triphenylene	6.45	9.34	4.67	5.48	5.22	6.232	125.16	1.12	9.0
Benz[*a*]anthracene	7.68	10.6	5.02	5.94	5.22	6.220	125.16	1.58	9.0
Perylene	8.27	12.4	6.39	8.92	5.52	6.976	134.64	1.27	10.0
Benzo[*a*]pyrene	9.06	13.8	6.75	9.86	5.52	6.970	134.64	1.50	10.0
Benzo[*b*]fluoranthene	9.15	12.7	6.28	8.70	5.52	6.976	134.64	1.40	10.0
Benzo[*j*]fluoranthene	8.78	12.4	6.05	8.20	5.52	6.976	134.64	1.39	10.0
Benzo[*k*]fluoranthene	9.74	13.4	6.12	8.98	5.52	6.970	134.64	1.48	10.0
Dibenz[*a,c*]anthracene	11.6	17.3	7.93	11.4	6.24	7.637	150.76	1.24	11.0
Dibenz[*a,h*]anthracene	12.9	19.1	7.69	12.5	6.24	7.631	150.76	1.79	11.0
Dibenzo[*b,def*]chrysene	16.1	- [a)]	10.3	- [a)]	6.54	8.381	160.24	1.73	12.0
Coronene	14.3	19.6	11.5	- [a)]	6.12	8.464	153.60	1.00	12.0
Mobile phase (CH_3CN / H_2O)	55/45	50/50	55/45	65/35					

[a)] Not detected. They might have very large retention.

Table 2. Correlation coefficients between log k and descriptors for PAHs.

Column	Correlation coefficient				
	log *P*	χ	Vw	F	L / B
C_{18}	0.9857	0.9912	0.9888	0.9931	0.4108
C_8	0.9831	0.9944	0.9882	0.9949	0.2879
C_P	0.9957	0.9926	0.9967	0.9943	0.3245
C_2	0.9911	0.9905	0.9882	0.9926	0.3796

Table 3. Relationships between log k of PAHs and the descriptors.

Column	Equation	n[a)]	Correl. coeff.
C18	$\log k = 0.1408 \cdot F + 0.0169 \cdot L/B - 0.5050$	24	0.9932
C8	$\log k = 0.0953 \cdot F - 0.0142 \cdot L/B - 0.1296$	26	0.9950
CP	$\log k = 0.1131 \cdot F + 0.0915 \cdot L/B - 0.1436$	25	0.9979
C2	$\log k = 0.0953 \cdot F + 0.0769 \cdot L/B - 0.0963$	26	0.9959

[a)] number of compounds used in establishing the equations.

Table 4. Comparison of predicted and measured retention data for 16 PAHs under isocratic condition.
mobile phase : acetonitrile / water = 70 / 30, stationary phase : C_{18}

Compound	Logarithm of the retention factor		
	Observed	Predicted	err[a)]
Naphthalene	0.724	0.743	2.5
Acenaphthylene	0.806	0.824	2.2
Acenaphthene	0.990	0.994	0.4
Fluorene	0.990	1.006	1.7
Phenanthrene	1.034	1.089	5.3
Anthracene	1.075	1.092	1.6
Fluoranthene	1.183	1.254	6.0
Pyrene	1.219	1.255	3.0
Chrysene	1.391	1.437	3.3
Benz[*a*]anthracene	1.412	1.433	1.5
Benzo[*b*]fluoranthene	1.581	1.599	1.2
Benzo[*k*]fluoranthene	1.602	1.601	0.0
Benzo[*a*]pyrene	1.615	1.602	0.8
Benzo[*ghi*]perylene	1.804	1.763	2.2
Dibenz[*a,h*]anthracene	1.790	1.779	0.6
Indeno[*1,2,3-cd*]pyrene	1.804	1.770	1.9

a) err : [(log kobs - log kpred) / log kobs] × 100.

$$\log k = f(Pi, Xi) \qquad [2]$$

where *Xi* indicates the separation conditions such as mobile phase composition or column temperature.

To generalize the retention descriptions for n different separation conditions, the following *n* equations should be obtained by the same procedure with multiple regression analysis. The relationships for system can be described in general form as follows:

$$\begin{aligned} X=X_1 \quad & \log k_1 = a_1P_1 + b_1P_2 + c_1 \\ X=X_2 \quad & \log k_2 = a_2P_1 + b_2P_2 + c_2 \\ \quad . \quad & \quad . \\ \quad . \quad & \quad . \\ X=X_n \quad & \log k_n = a_nP_1 + b_nP_2 + c_n \end{aligned} \qquad [3]$$

where *X* is, for example, the volume fraction of organic modifier in

the mobile phase and a and b are the coefficients corresponding to descriptors P_1 and P_2, respectively, c is the intercept, and n is the number of experimental conditions examined.

If a, b and c can be expressed as functions of *X*, namely *X*-a, *X*-b and *X*-c are highly correlated, the following equation [4] can be solved by the multiple regression analysis:

$$\log k = f_1(X)P_1 + f_2(X)P_2 + f_3(X) \qquad [4]$$

This equation means that, if *X*, the concentration of organic modifier in the mobile phase, and P_1 and P_2, descriptors of a compound are given, the logarithm of the retention factor, log *k* can be determined for any chromatographic conditions. Based on this concept, the following three functions can be generated:

1) retention of any solutes can be predicted under a certain separation condition [11,12]
2) optimization of the separation for selected solutes can be made based on the retention prediction [13]
3) identification of the peaks appeared on the chromatogram measured at a certain separation condition can be done by the properties estimated by the experimentally observed retention factor, matching to those in the database of such properties of the compounds groups. [14,15]

The computer assisted system so-called **M**icrocomputer **A**ssisted **S**eparation **S**ystem (**MCASYST**) that can be useful in the following three analytical areas has been constructed [16]:

a) analytical system for PAHs in diesel engine particulate matter[14];
b) analytical system of toxic drugs in human fluids for clinical, toxicological and forensic analytical problems[17,18]; and
c) estimation of amino acid sequencing of oligopeptides by LC retention information[19,20].

In order to show the applicability of the above systems one practical example, a system for drug analysis in human fluids in toxicological and forensic situations is presented in this review. The basic structure of **MCASYST** for this drug analysis is shown in Figure 1.

The actual analytical processes performed in this example are as follows:

The human fluid specimen was obtained from a patient who was brought to the emergency room of Nippon Medical School Hospital in downtown Tokyo. After some sample preparation procedures including solid phase extraction (SPE) had been performed, the sample was prepared as ready for LC injection. Inputting the desired analysis time and the desired column for the analysis into MCASYST, the system was set up with the optimum separation condition calculated, and the analysis was started. After the chromatogram had been recorded, the peaks were searched on the

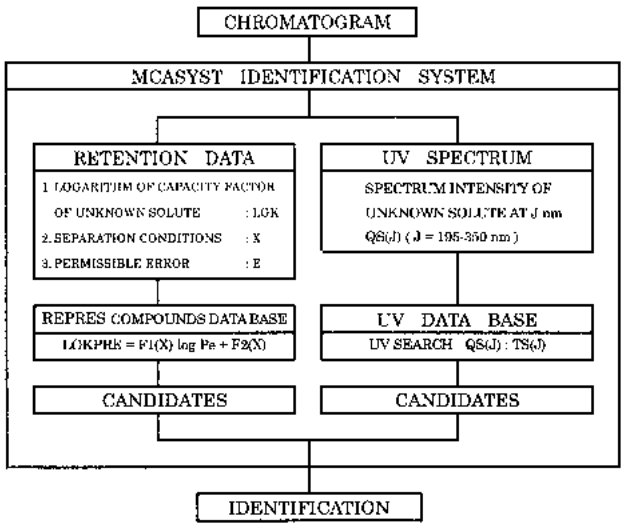

Figure 1. Flow of Identification Process for Toxic Drug Compounds by MCASYST.[18]

display and the calculated retention factors for each individual peak can be brought into the retention prediction function in the system. In another step the spectral data of each peak was measured by the photo-diode array detector assembled in this system, recorded and searched through the UV spectral library. In the first process, input of the retention factors of each peak to the equation [5] can give calculated log *Pe*, which is the estimated hydrophobicity of the analytes that appeared as peaks in the chromatogram:

$$\log k = f_1(X) \log Pe + f_2(X) \qquad [5]$$

where *X* is the separation condition, i.e.. the mobile phase composition in this case. The system's database of log *Pe* stores the log *Pe* numerical values of many compounds. The compounds in this database that have a similar log *Pe* values to those estimated by the measured retention factor were listed in their order of matching as possible candidates. Again the measured UV spectra of the peaks were searched to those in the UV spectral library and the best candidates were listed in their order of matching as possible ones. Combining the candidates from the retention search and the spectral search, the most promising candidates for the peaks in the chromatogram were selected. Two matching processes to the database systems can confirm the accurate identification for the compounds that eluted as peaks in the chromatogram. Typical chromatographic information in this example is seen in Figure 2. Identification of the peak No. 4 in the three dimensional chromatogram was tried. The output of this process is demonstrated in Figure 3. By the retention matching process the most promising candidate for identification was flunitrazepam, and by the UV spectral matching process again the most promising candidate was

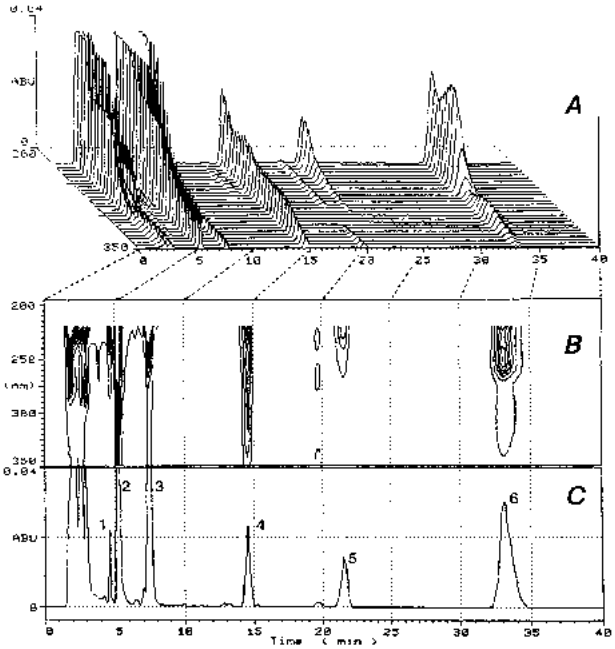

Figure 2. Typical Example of Toxic Drug Analysis for a Patient Gastric Content by MCASYST.[18]

PEAK NO. 4 TR = 14.52 MIN.

UV SPECTRAL SEARCH

NO.	COMPOUND	CORRELATION
1	FLUNITRAZEPAM	1.000
2	CLOCAPRAMINE	0.972
3	CLOMIPRAMINE	0.970
4	NIMETAZEPAM	0.968
5	DESIPRAMINE	0.965

RETENTION SEARCH (ERROR < 10%)

NO.	COMPOUND	log Pe	CORRELATION
1	FLUNITRAZEPAM	2.24	0.667
2	DIAZEPAM	2.14	0.586
3	ESTAZOLAM	2.11	0.329
4	FLURAZEPAM	2.29	0.321
5	NIMETAZEPAM	2.09	0.172

SYSTEM OUTPUT

NO.	COMPOUND	CORRELATION	BAR GRAPH
1	FLUNITRAZEPAM	0.816	********
2	NIMETAZEPAM	0.407	****

Figure 3. An Example for Identification of Peak No. 4.[18]

Table 5. Identified Drugs and Prescribed Drugs in Gastric Fluid

Drugs measured	Drugs prescribed
Bromazepam*	Bromazepam
Pentobarbital	Pentobarbital
Nitrazepam*	Nitrazepam
Flunitrazepam	Flunitrazepam
Triazolam	Triazolam
Trihexyphenidyl*	Trihexyphenidyl
Levomepromazine	Levomepromazine
	Thioridazine**
	Timiperone**
	Clofedanol**

* Identified only by retention search.
** Drugs not included in the library.

flunitrazepam. Based on these two procedures the other five peaks in the chromatogram had been identified. The results are listed in Table 5 where the identification by this system are compared to the drug names prescribed by her doctor. One can conclude that the reason for the toxic condition of this patient had been induced by some accidental or suicidal condition, where she consumed large amount of medicines prescribed by her doctor. This example clearly indicates the potential of this computerized system for practical applications.

4. Tailored Phases Based on Molecular Recognition Mechanism

Further extension of the mentioned research above has progressed to a little different but much important area of separation science, which is how to investigate the retention mechanism in liquid chromatography based on the interpretation of molecular level interactions.

Chromatography is a technique which is used to separate various components from complex mixtures based on differences at the molecular level of the interactions among the solutes, solid or liquid, the stationary phase and carrier or mobile phase. Chromatographic separations are generally based on the elution time difference depending on the strength of the interactions between the solute and the stationary phase in gas chromatography (GC) because the mobile phase is an inert gas. However, in LC, the interactions

among the solute, the stationary phase and the mobile phase are all dominant factors to control the retention. In order to understand the contribution of each molecular level interaction, solute-stationary phase, solute-mobile phase, stationary phase-mobile phase, various theoretical and experimental approaches have been applied. For the interpretation of the stationary phase structures, spectroscopic instruments such as solid state nuclear magnetic resonance spectroscopy (NMR) are used [21,22]. For the interpretation of the solute structures computer calculations for molecular modeling is usually used [23,24]. For the contribution of the mobile phase to the solute and the stationary phase structures several spectroscopic and computer methods have been proposed [25,26]. Compilation and analysis of the information related to chromatographic retention mechanism will help in understanding the separation process.

In LC the retention mechanisms are generally not known yet in details. However, to design the best separation system for a particular analytical problem, the mechanism should be known and the interactions at the molecular level be interpreted. To understand and interpret such mechanisms is a hard task, particularly in LC systems, because the interactions are so complex and some of the interactions are sometimes dominant and at other times non-dominant in controlling the retention. In such case one creates a model for the interaction in chromatographic separation process by using information about solutes and stationary phase structures. Molecular recognition mechanism is such a model in separation process. The separation process can be induced by different molecular-level interactions between the stationary phase and the solute molecules that have a different shape and size from each other. This mechanism induces the enhanced selectivity for the separation that is the most important aspect in recent separation sciences.

The design of new stationary phases that can offer higher selectivity for particular compounds groups is a chemistry-oriented approach. One gets enhanced selectivity if one can produce molecular-molecular interaction field in the separation process. For this kind of research micro-LC is the most versatile technique, since it requires very small amount of stationary phase materials and mobile phase solvents. This feature makes the evaluation procedures very effective, economical and environmentally favorable. Micro-LC permits the small-scale synthesis of stationary phase materials for the evaluation of their chromatographic performance, since it requires about 100 times smaller amount of materials and organic solvents compared to conventional LC.

One of the examples described here is the separation of fullerenes. The target compounds, fullerenes, have recently attracted attention from materials scientists, chemists and other fields scientists, since the shape of the molecules is very unique and very interesting from the chemistry point of view. The molecules are bulky and have different sizes depending on their molecular

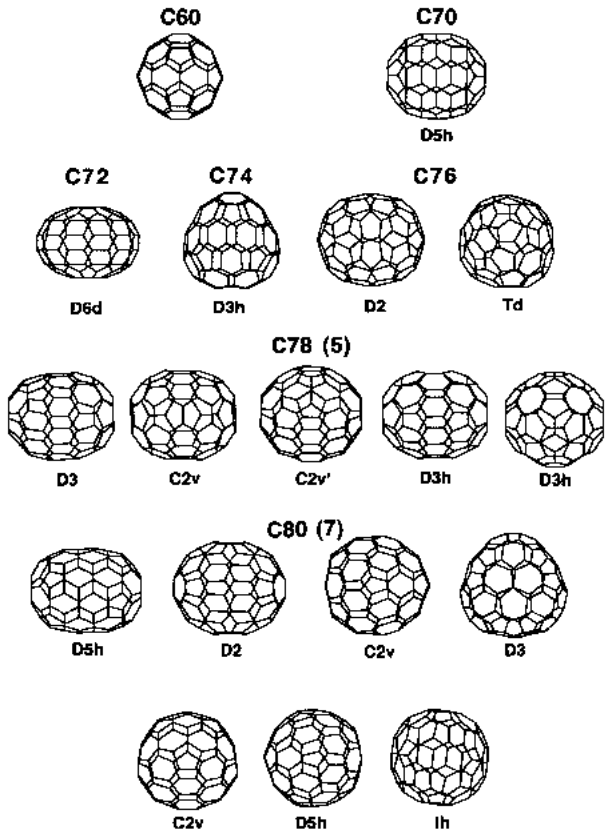

Figure 4. Example of the structure of fullerenes cited from http://shachi.cochem2.tutkie.tut.ac.jp/Fuller/Fuller.html

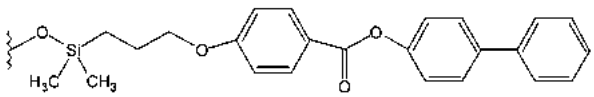

Figure 5. Chemical Structure of a Liquid-Crystal Bonded Silica Stationary Phase.

weight (carbon atom numbers which are contained in the molecules) and also the cage of the molecules can have metal elements inside that might produce a different chemistry compared to that based on elements. Separations of such molecules are one of the most interesting and difficult analytical problems.

The structures of typical small fullerenes are shown in Figure 4. The key of molecular recognition mechanism for fullerenes separation is the shape and size of the molecules. Once the shape and size of these molecules are known, the stationary phases are then tailored for their separation. In order to pursue this purpose the evaluation of typical stationary phases in LC has been performed before synthesizing new stationary phases based on molecular recognition mechanism. Typical ODS phases with different functionalities that are polymeric or monomeric were evaluated [27,28]. The results have indicated that the polymeric ODS phases have better recognition

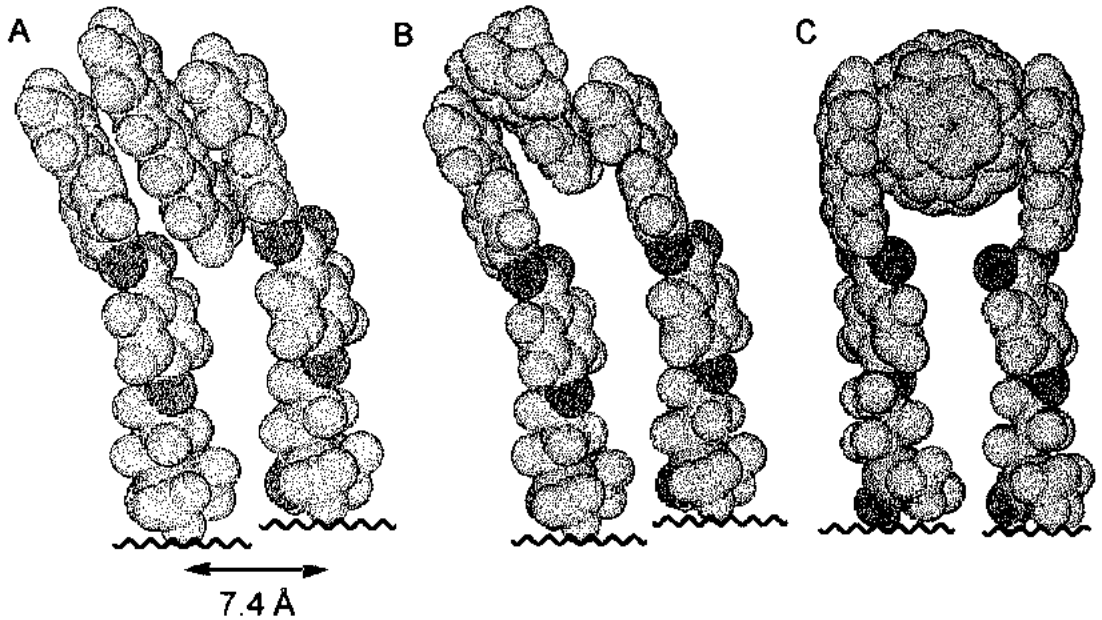

Figure 6. Molecular-Molecular Interactions Between A Liquid-Crystal Bonded Silica stationary Phase and Some Solutes Such as Triphenylene(planar), o-Terphenyl(non-planar) and C_{60}(bulky) Molecules.[29]

capability than the monomeric ODS phases for the shape and size of fullerenes. Decreasing the temperature enhances such recognition power. It has been found that the most important parameter is not the functionality of the ODS phases, but the distance between the bonded phase ligands on the silica surface and the rigidity of these ligands. To get better selectivity the distance between each bonded phase should be the similar interval to that of the size of fullerenes (i.e. diameter of fullerenes). Using this information, one can synthesize rigid phases that have an interval between the bonded moieties similar to the size of the desired solutes. The liquid-crystal bonded phase, of which chemical structure is shown in Figure 5, is the most suitable one for this purpose [29]. The interval is not the correct size for the solutes' size, but the distance could be expanded by increasing the temperature as shown in Figure 6. The molecular model clearly shows that this phase can retain C_{60} at higher temperature where the molecular movement of the liquid-crystal moiety is more freely movable than that at lower temperature, at which the ligands have a more restricted movement. At the latter case the phase can exclude fullerenes from the chromatographic interaction, since the distance between the bonded moieties is not enough to allow fullerenes to penetrate into the space. The selectivity for these two fullerenes, C_{60} and C_{70}, with the liquid-crystal bonded silica phase increases with increasing the temperature as seen in several chromatograms at different temperatures demonstrated in Figure 7. This behavior is not a typical in LC where the selectivity and the retention at high temperature are generally lower than those at lower temperature.

Similar discussions will be made regarding other stationary phases that have been designed and synthesized for the selectivity enhancement of fullerenes based on the molecular-molecular interaction concept [30]. One of the products of such discussions is the possibility to use C_{60} fullerene as a stationary phase in LC separations. The bulky structure and curvature shape of the fullerene would produce uncommon retention characteristics for some compounds. Various C_{60} bonded silica stationary phases were synthesized and evaluated their performance for the separation of polycyclic aromatic hydrocarbons (PAHs) and other compounds [31-33]. As an example one of the C_{60} bonded silicas of which structure is shown in Figure 8(A) was used to enhance the selectivity of the separation of calixarenes, since the different sizes of the cavity of calixarenes will produce different degree of interactions between the solutes and the stationary phase [34]. For the typical separation of t-butyl calix[4]arene, [6]arene and [8]arene the C_{60} phase can give much higher selectivity than a typical ODS phase, as seen in Figure 8(B). Such selectivity enhancement can be explained by the interaction model between the C_{60} bonded moiety and calixarenes as demonstrated in Figure 8(C).

One can conclude the above basic investigations, by stating that the concept of tailored stationary phases is the most important thought for designing analytical systems, since sample preparation processes can also be designed in a similar fashion, whereby selective extraction can be performed prior to selective separation based on this concept [35,36].

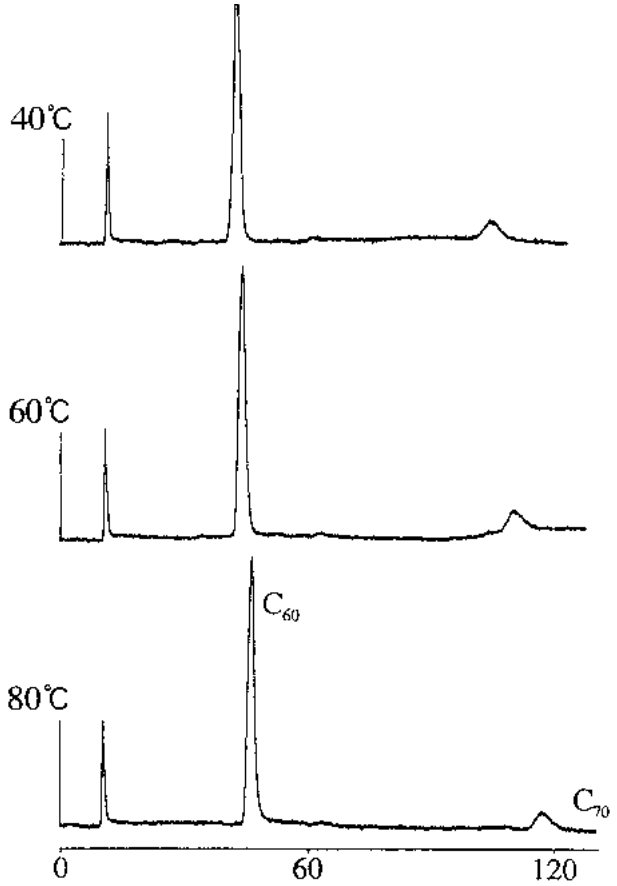

Figure 7. Separation of C_{60} and C_{70} with a Liquid-Crystal Bonded Silica Stationary Phase at Various Temperatures.

5. Conclusion

The 20 years research works on LC retention could give us a lot of knowledge on the separation mechanism, although it is still far away to understand it completely. However, based on such new knowledge one can construct analytical systems that are applied to solve practical problems in environmental, clinical, toxicological and forensic analyses. The concept of tailored phase will be the key in analytical sciences including sample preparation, separation methods and their miniaturization toward the 21st century. Before concluding this article the author should acknowledge many people who have contributed to his research projects, academic life in TUT, scientist's life in many scientific societies, and personal life, since without their valuable contributions and friendships he could not arrive at the stage he is now.

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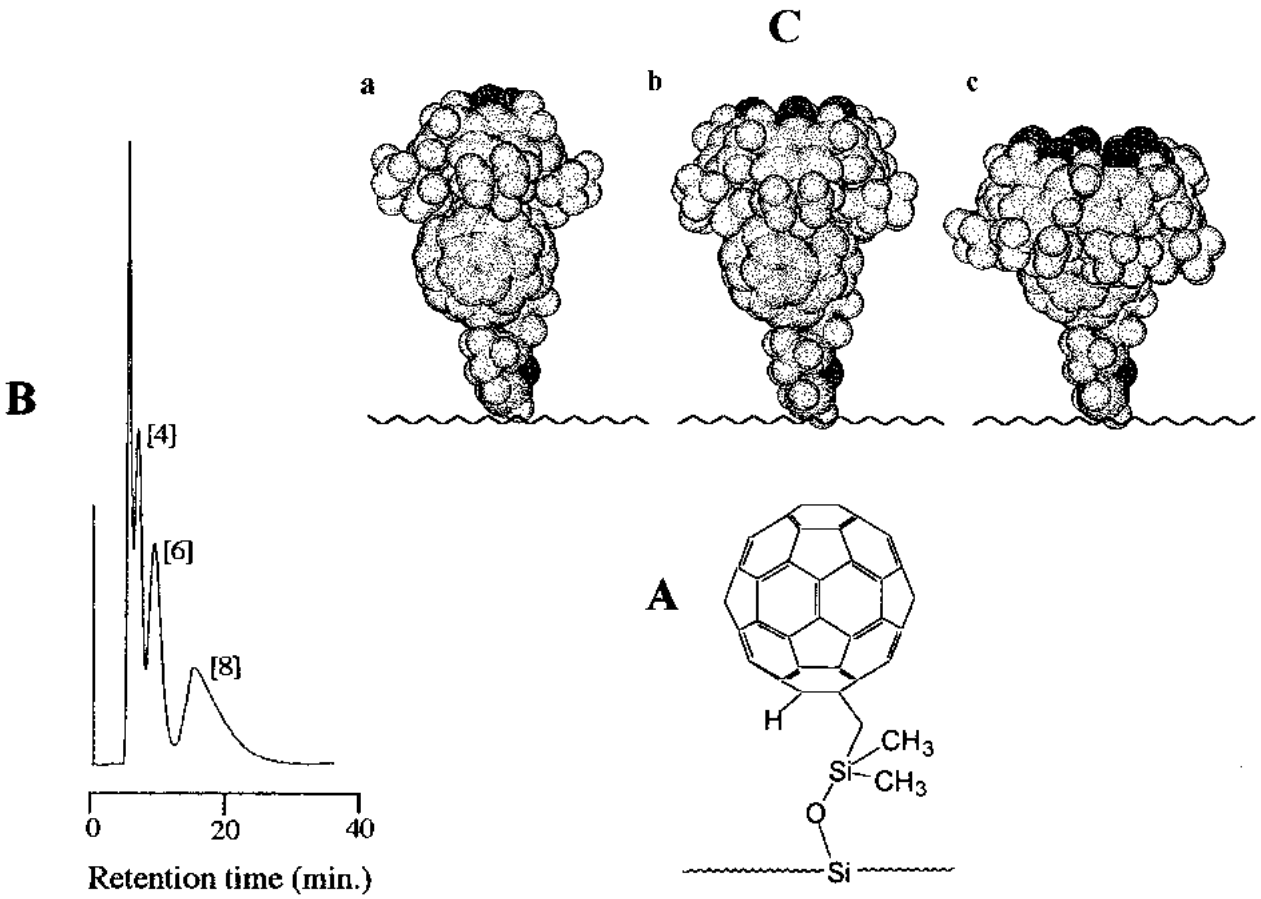

Figure 8. (A) Structure of C_{60} Bonded Silica Stationary Phase.
(B) Typical Chromatogram for the Separation of Calixarenes with the C_{60} Bonded Silica Stationary Phase.
(C) Molecular Modelling Scheme of the Proposed Interaction Between Three Calixarenes and C_{60} Bonded Silica Stationary Phase by a Space Filling Model.
(a)t-butyl calix[4]arene, (b)t-butyl calix[6]arene, (c)t-butyl calix[8]arene with C_{60} Phase.(34)

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