# University of Alberta

Mixed-Mode Retention on a Hypercrosslinked Silica-Based Column

by

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**ABSTRACT**

Column stability in Reversed Phase Liquid Chromatography (RPLC) is crucial for obtaining reproducible separations. Under acidic conditions RPLC columns may exhibit stationary phase loss. To address this problem various concepts for improving column acid resistance have been proposed. Carr and co-workers introduced a class of acid stable silica-based RPLC stationary phases based on extensive bonded phase crosslinking. This technology results in the formation of a surface-confined hypercrosslinked polymeric network. One aspect of the column preparation that was not detailed in the literature and is rather perplexing is the column acid conditioning.

This thesis explores the synthesis and chromatographic performance of a toluene-derivatized hypercrosslinked silica-based stationary phase (HC-T). This work focuses on the long-term chemical stability and chromatographic behaviour of HC-T after continuous exposure to acid and temperature extremes. It has been demonstrated that the acid treatment results in the formation of cationic functionalities on the hypercrosslinked bonded phase.

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## LIST OF SYMBOLS AND ABBREVIATIONS

| Symbol | Parameter |
|---|---|
| ***Abs*** | absorbance |
| ***Å*** | Angstrom |
| ***A*** | *A*-term in the Van Deempter equation |
| ***[A]*** | analyte concentration |
| ***[$A^-$]*** | analyte anion concentration |
| ***$A_c$*** | atomic weight of carbon |
| ***ACN*** | acetonitrile |
| ***alp*** | alprenolol |
| ***ami*** | amitriptyline |
| ***$A_s$*** | asymmetry factor |
| ***AU*** | absorbance units |
| ***B*** | *B*-term in Van Deempter equation |
| ***b*** | proportionality constant |
| ***C*** | *C*-term in the Van Deempter equation |
| ***c*** | concentration |

| | |
|---|---|
| $^{\circ}C$ | degree Celsius |
| $C_8$ | octyl alkyl chain |
| $C_{18}$ | octadecyl alkyl chain |
| $C_m$ | resistance to mass transfer in the mobile phase |
| $C_s$ | resistance to mass transfer in the stationary phase |
| $d_c$ | stationary phase channels diameter |
| ***DCE*** | dichloroethane |
| ***DCM*** | dichloromethane |
| $d_f$ | stationary phase thickness |
| $D_{IE}$ | ion-exchange distribution constant |
| $D_m$ | diffusion coefficient in the mobile phase |
| $d_p$ | particle diameter |
| $D_s$ | diffusion coefficient in the stationary phase |
| ***[E]*** | eluent concentration |
| $[E^-]$ | eluent anion concentration |
| ***EDTA*** | ethylenediaminetetracetic acid |
| ***eq*** | equilibrium |

| | |
|---|---|
| ***HPLC*** | Reversed-Phase Liquid Chromatography |
| ***HTLC*** | High Temperature Liquid Chromatography |
| $I_0$ | Intensity of incident light |
| $I$ | Intensity of transmitted light |
| ***ICP-AES*** | Inductively-Coupled Plasma Atomic Emission Spectroscopy |
| ***IEC*** | Ion-Exchange Chromatography |
| $H$ | theoretical plate height |
| ***HA*** | acid |
| ***HC*** | hypercrosslinked |
| $HC\text{-}C_8$ | hypercrosslinked octyl-derivatized stationary phase |
| ***HC-T*** | hypercrosslinked toluene-derivatized stationary phase |
| $h_p$ | peak height |
| ***ID*** | inner diameter |
| $k$ | retention factor |
| $K_a$ | dissociation constant |
| $K_{IE}$ | ion-exchange equilibrium constant |
| $l$ | light path |

| | |
|---|---|
| $L$ | column length |
| $NPLC$ | Normal-Phase Liquid Chromatography |
| $N$ | efficiency |
| $N_c$ | number of carbon atoms |
| $N_{sys}$ | efficiency of an exponentially modified peak |
| $nor$ | nortrityline |
| $PAHs$ | polycyclic aromatic hydrocarbons |
| $pK_a$ | negative logarithm of the acid dissociation constant |
| $ppm$ | parts per million |
| $r^2$ | correlation coefficient |
| $RPLC$ | Reversed-Phase Liquid Chromatography |
| $R$ | aliphatic substituent |
| $R_s$ | resolution |
| $psi$ | pounds per square inch |
| $PEEK$ | Polyether ether ketone |
| $SAM$ | self-assembled monolayer |
| $t_0$ | column dead time |

| | |
|---|---|
| $t_R$ | retention time |
| ***TFA*** | trifluoroacetic acid |
| $u$ | linear velocity |
| ***UV*** | ultraviolet |
| ***(v/v)*** | volume per volume |
| $w_b$ | peak width at baseline |
| $w_{0.1}$ | peak width at 10% of the peak height |
| $X^+$ | cationic anion-exchange sites |
| ***XA*** | salt |
| ***XPS*** | X-ray Photoelectron Spectroscopy |
| $\alpha$ | selectivity (relative retention) |
| $\lambda$ | packing factor of the stationary phase bed |
| $\psi$ | obstruction factor |
| $\varepsilon$ | molar extinction coefficient |

# CHAPTER ONE: Introduction

## 1.1 Motivation

Reversed phase liquid chromatography (RPLC) is the most widely used analytical technique for the separation of complex chemical and biological mixtures. The reasons for its popularity are numerous, such as compatibility with aqueous samples, high separation efficiency, specific selectivity, small amount of sample to perform the analysis and relatively low cost. RPLC is also preferred for the analysis of temperature labile biological molecules where the use of Gas Chromatography is limited. However, its most valuable characteristic from the perspective of its applications in the industry is the reproducibility of the analysis.

The most essential element of any chromatographic system is the chromatographic column. It is the part where the actual separation takes place but it is also the part that is most susceptible to wear during use. The changes that the column may undergo during its use depend on the type of the stationary phase and the structural support it is bound to. If the sample contains compounds that have high affinity for the stationary phase, irreversible adsorption may result. If the mobile phase reacts chemically with the stationary phase support, mechanical damage may take place [1]. Alternatively, if the mobile phase is capable of breaking the linkage between the bonded phase and the stationary phase support, phase loss may result [2].

Bonded phase loss is a serious problem with silica-based columns, as it not only causes continuous decrease in retention [2] but also may change the selectivity of the column [3]. Since the majority of the commercially available columns are silica-based, the topic of improving their stability continues to be of high interest [4]. The challenges to the stability of the silica-based columns are extreme pH and elevated temperature, conditions that favour the separation of positively charged analytes such as basic peptides and pharmaceuticals [5]. In alkaline medium the silica support dissolves [6], while under acidic conditions loss of bonded phase may result due to hydrolysis [2]. To overcome the limitations associated with high pH, hybrid silica materials with methylene bridges incorporated into the silica matrix were patented by Unger [7] and later commercialized by Waters Corporation under the trade name XTerra [8-10].

To address the problem with the hydrolytic stability of RPLC columns at low pH, several approaches have been developed. Kirkland and co-workers introduced the sterically protected bonded phases, where bulky alkyl groups impede the hydrolytic cleavage of the siloxane bonds that link the bonded phase to the silica support [11-13]. Another type of silica-based packings is the bidentate phases where the bonded phase ligands are anchored to the silica support via two points of attachment [13]. In the late 1980's Sandoval and Pesek developed the silicone hydride columns where the bonded phase is attached through silicon-carbon bonds which are more acid stable than the siloxane bonds [14, 15].

A conceptually different strategy for improving the acid and thermal stability of silica-based RPLC phases has been employed by Carr and co-workers. In their

research they crosslinked bonded organosilane ligands to one another to form a polymeric network on the silica surface such that the bonded phase is not lost as a result of the siloxane bonds hydrolysis [16, 17]. A series of studies showed that the hypercrosslinked silica phases are more resistant to phase loss than commercially available acid stable phases [16-22].

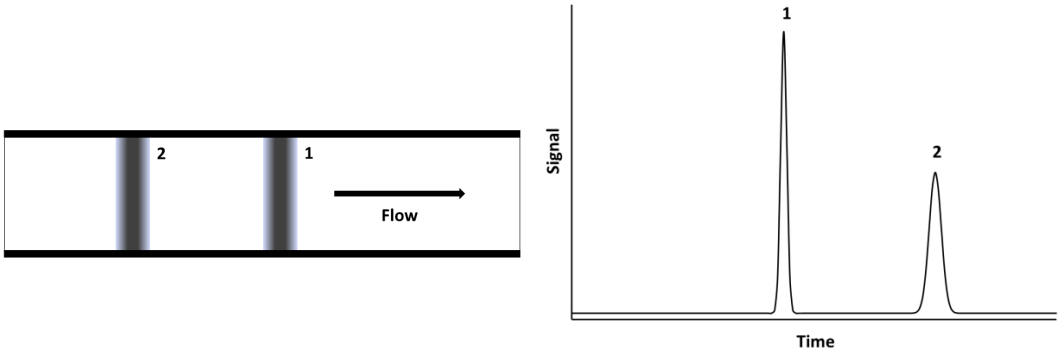
In this thesis we study the behaviour of an acid stable hypercrosslinked stationary phase after prolonged exposure to pH and temperature extremes, i.e. conditions that the stationary phase is designed for. The results of our studies reveal the limitations of the hypercrosslinked phases and can further be used to design column packings with improved properties.

## 1.2 Introduction to liquid chromatography

Liquid chromatography is a powerful technique for separating the components of a mixture for the purposes of isolation and purification (preparative chromatography) or identification and quantification (High Performance Liquid Chromatography). The driving force for the separation is the different affinity of the compounds in a mixture towards two immiscible phases, one of which is mobile and the other stationary. In modern liquid chromatography the stationary phase is packed into a column and the mobile phase passed through a packed bed of particles. As chromatography evolved, the particle size of the packings became smaller to give faster equilibration and better mass transfer. However, the more tightly packed the column, the more difficult it is to push the mobile phase

through. To meet these higher demands to the equipment Horvath and co-workers introduced new pumps capable of withstanding higher pressure. Because of the superior efficiency of the newly designed systems the technique became known as High Performance Liquid Chromatography (HPLC) [23].

The sample in HPLC is introduced at the inlet of the column and is being separated as it travels along the column while carried by the mobile phase. The higher the affinity of a given compound to the stationary phase, the more time it spends immobile and the later it comes out of the column. Figure 1-1 illustrates the column separation process and the resultant chromatogram.




bands in the column at $0 < t < t_R$ peaks in the chromatogram

**Figure 1-1.** Column separation and the resultant chromatogram

In HPLC the mobile phase plays an active role in the separation as it solubilizes analyte molecules. Thus, the retention as well as the selectivity can be controlled not only by changing the stationary phase but also by varying the mobile phase

composition. In contrast, in Gas Chromatography the mobile phase is largely inert and it serves mainly as a carrier of the sample.

Depending on the mechanism of interaction between the solute and the stationary phase there are several modes of liquid chromatography. Historically, the first packing materials used as a stationary phase were polar which determined polar interactions with the analytes [3]. This mode of liquid chromatography is called Normal Phase Liquid Chromatography (NPLC). Later, with the advent of the bonded phase technology it became possible to modify the surface of silica packings with non-polar functionalities. Such stationary phases are based on a hydrophobic mechanism of interaction. To distinguish between the two modes, the latter is referred to as Reversed Phase Liquid Chromatography (RPLC). Despite its name, RPLC is currently the most widely used liquid chromatographic technique [3].

Other mechanisms of separation include ion-exchange, ion-exclusion, size-exclusion and chelation [3, 24, 25]. However, it is important to note that in any real chromatographic separation more than one mechanism can contribute to the retention. As the focus of this thesis is RPLC, the majority of the introduction will focus on this mode.

### 1.3 Chromatographic terms

Two major theories were developed to explain the separation process in chromatography. One is the plate theory which employs the terms of the

distillation process and the other is the rate theory which is based on the principles of hydrodynamics and molecular kinetics. Although the rate theory is the one that is widely accepted today, some of the terms introduced by the plate theory are still used to describe the chromatographic performance.

One of the basic chromatographic terms is the retention time ($t_R$). It is the time since injection that a given analyte takes to cross the entire length of the column. The retention time is the total of the time the analyte spends immobile in the stationary phase and the time it spends in the mobile phase travelling along the column. If a compound is not retained it will stay in the mobile phase only and will elute once it has passed the void volume of the column. The time it takes for an unretained compound to come out of the column is referred to as the dead time ($t_0$).

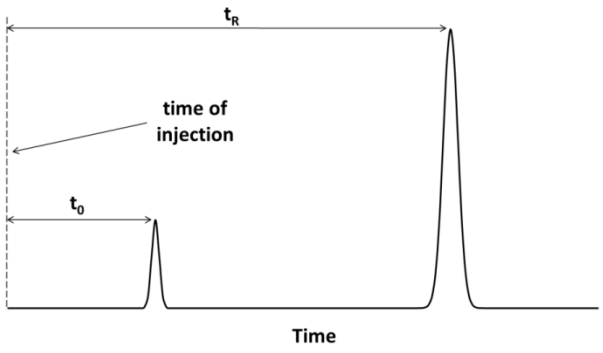



**Figure 1-2.** Illustration of retention time ($t_R$) and dead time ($t_0$)

As shown in Figure 1-2, $t_R$ and $t_0$ can be determined graphically by measuring the distance between the time of injection and the apex of the peak.

The retention factor (*k*) is another parameter that provides more consistent information about the retentive properties of the column, as both $t_R$ and $t_0$ depend on the mobile phase velocity, (i.e., the flow rate). The physical meaning of the retention factor is of a distribution constant that characterizes the equilibrium concentration of a solute between two immiscible phases (stationary and mobile):

$$k = \frac{t_R - t_0}{t_0} \tag{1-1}$$

The use of the retention factor compensates for differences in column dimensions, fluctuations in the mobile phase flow rate and variations in the extracolumn volume of the system.

The number of theoretical plates (*N*) is a measure of the efficiency of the column, i.e. its ability to minimize band broadening and produce narrow peaks. For a Gaussian peak, *N* can be calculated from the peak retention time and the width at the baseline:

$$N = 16 \left(\frac{t_R}{w_b}\right)^2 \tag{1-2}$$

Assuming a Gaussian peak tends to overestimate column efficiency since in practice peak shapes are often distorted due to tailing or fronting. To provide a more realistic measure of the efficiency Foley and Dorsey proposed the use of an empirical equation based on exponentially modified Gaussian peaks [26]:

$$N_{sys} = \frac{41.7(\frac{t_R}{w_{0.1}})}{\left(\frac{B}{A}\right) + 1.25} \tag{1-3}$$

where $t_R$ is the retention time and $w_{0.1}$ is the peak width at 10% peak height.

The asymmetry factor ($A_s$) represented in Eq. 1-3 as (*B/A*) can be determined graphically by drawing a vertical line through the peak apex and calculating the ratio of the rear to the front portion of the peak width at 10% of the peak height (*0.1* $h_p$) (Figure 1-3).

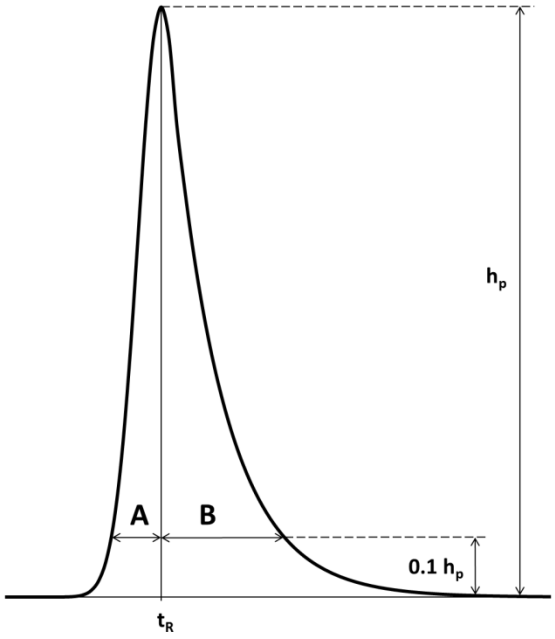



**Figure 1-3.** Graphical determination of the asymmetry factor (B/A) for a tailing peak

The number of theoretical plates or the efficiency (*N*) is related to the length of the column (*L*) and the theoretical plate height (*H*) by the following equation:

$$N = \frac{L}{H} \qquad (1\text{-}4)$$

According to Eq. 1-4, longer columns and smaller theoretical plate heights give higher efficiency. However the use of longer columns results in longer analysis

times. Longer analysis times can be shortened by increasing the flow rate but doing so would also cause a larger pressure drop and possibly poorer efficiency due to mass transfer effects (below). For a fixed column length, a higher efficiency (larger number of theoretical plates) corresponds to smaller theoretical plate height. Preferably, the chromatographic separations should be performed at such conditions that minimize *H*. In his pioneering work on the rate theory, Van Deempter et al. [27] derived the fundamental equation that relates the theoretical plate height (*H*) to the mobile phase linear velocity (*u*):

$$H = A + \frac{B}{u} + (C_s u + C_m u) \qquad (1\text{-}5)$$

At the time of injection the analytes occupy the volume of the sample and are distributed along the column as a narrow band. It is desirable that in the course of the separation the individual analyte bands remain narrow so that they not overlap with adjacent bands. However, in reality, analyte bands broaden as a result of multiple paths in the column bed (*A-term*), longitudinal diffusion in the mobile phase (*B-term*) and resistance to mass transfer in both stationary and mobile phase (*C-term*).

The *A* term in Eq. 1-5 accounts for the different pathlengths the analyte molecules travel through the column. It is also known as Eddy diffusion and is illustrated in Figure 1-4. As can be seen from Figure 1-4, the molecules that travel a shorter distance will elute earlier than those going through longer pathways.

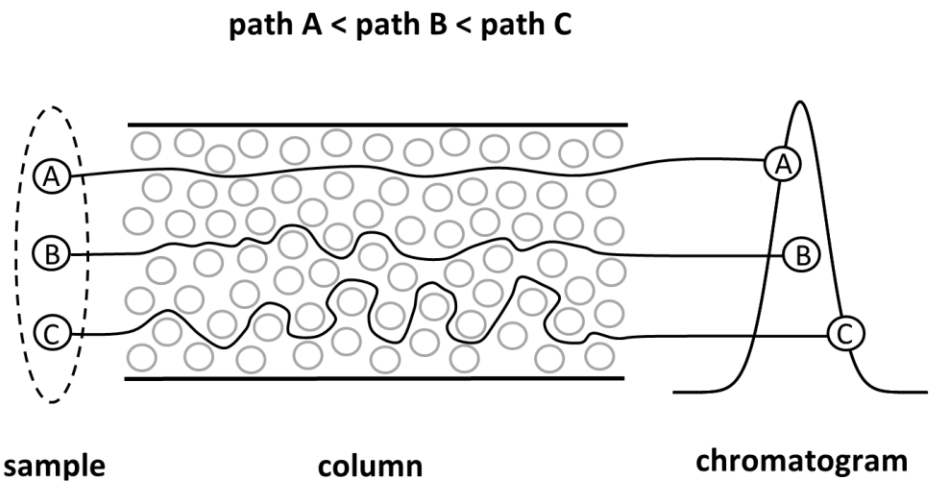



**Figure 1-4.** Schematic representation of multiple path band broadening (adapted from [24])

As a result the molecules of the same analyte will be spread in both space and time. Eddy diffusion is described by:

$$A = 2\lambda d_p \qquad (1\text{-}6)$$

where $\lambda$ is the packing factor and $d_p$ the particle diameter. The $A$ term can be minimized with uniformly and tightly packed columns using monodisperse particles (yielding lower $\lambda$) with smaller diameter.

The B-term accounts for the longitudinal diffusion of the analyte in the mobile phase due to concentration gradient along the axis of the column.

$$B = 2\psi D_m \tag{1-7}$$

where $\psi$ is the obstruction factor which depends on the quality of the packing and $D_m$ is the diffusion coefficient of the solute in the mobile phase.

It follows from Eq. 1-5 that higher mobile phase velocities reduce the longitudinal band broadening by allowing less time for diffusion. Nevertheless, in practice the contribution of the longitudinal diffusion to band broadening is negligible in HPLC columns packed with conventional particle sizes such as those used in this thesis.

$C_s$ and $C_m$ terms represent the resistance to mass transfer in the stationary and the mobile phase respectively. Slower diffusion kinetics of analyte molecules in either stationary or mobile phase determine higher resistance to mass transfer. In other words, if analyte diffusion is slow it will take more time to the analyte molecules to move through the medium in which they are dissolved. Greater resistance to mass transfer results in broader peaks as it takes more time to analyte molecules to reach the interface between the two phases. Thus, the analyte molecules in the mobile phase will be carried forward by the flow and moved away from the molecules in the stationary phase. Increasing the mobile phase velocity will worsen the situation as there will be less time to reach equilibrium.

The resistance to mass transfer in a thin film of stationary phase such as a hydrophobic bonded phase is given by:

$$C_S = \frac{8}{\pi^2}\frac{k}{(1+k)^2}\frac{d_f^2}{D_s} \qquad (1\text{-}8)$$

where $d_f$ is the thickness of the stationary phase and $D_s$ is the diffusion coefficient in the stationary phase. It can be seen from the above equation that a thinner stationary phase film with better diffusion properties is favourable for reducing the $C_s$ term contribution.

The resistance to mass transfer in the mobile phase is described by the Golay equation:

$$C_M = \frac{(1+6k+11k^2)d_c^2}{96\,(1+k)^2\,D_M} \qquad (1\text{-}9)$$

where $d_c$ is the diameter of the channels in the column packing occupied by the mobile phase and $D_M$ is the diffusion coefficient in the mobile phase.

A tightly and uniformly packed column would have smaller interstitial volume occupied by the mobile phase. Thus, the analyte molecules would cross shorter distances while moving between the mobile and the stationary phase. The result would be faster equilibration and reduced band broadening. Similar to the discussion of the stationary phase, greater mobile phase diffusion coefficients would facilitate analyte mass transfer and reduce its contribution to band broadening.

Minimizing peak broadening is very important but even a column with excellent efficiency may not be able to produce a separation if it cannot distinguish between the different analytes. If two or more analytes interact similarly with the stationary phase, the resultant chromatogram will consist of overlapping peaks. The chromatographic term that characterizes the difference in the affinity of the column towards different analytes is called selectivity or relative retention ($\alpha$):

$$\alpha = \frac{k_2}{k_1} \tag{1-10}$$

where $k_1$ and $k_2$ are the retention factors of two adjacent peaks, with $k_2$ being the retention of the more retained compound.

Selectivity is a useful indicator of the discriminating ability of the column but it does not account for the peak broadening. A more comprehensive measure of the separation power of the column is the resolution ($R_s$). It is expressed mathematically as:

$$R_s = \frac{2(t_{R2} - t_{R1})}{(w_{b1} + w_{b2})} \tag{1-11}$$

where $t_{R1}$ and $t_{R2}$ are the retention times of two adjacent peaks, and $w_{b1}$ and $w_{b2}$ are the corresponding peak widths at baseline. The analysis of Eq. 1-11 shows that higher resolution can be achieved when there is a greater difference in the retention times between two adjacent peaks and each has a smaller peak width. A pair of peaks is considered to be baseline resolved if its $R_s$ value is greater or equal to 1.5 [24].

The expression that relates the resolution ($R_s$), the efficiency ($N$), the selectivity ($\alpha$) and the retention factor ($k$) can be derived by making the necessary substitutions and rearrangements in Eq. 1-11:

$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{k_2 + 1}\right) \qquad (1\text{-}12)$$

By examining Eq. 1-12 one can see that enhancing column efficiency is not that effective in improving the resolution. $R_s$ is proportional to the square root of $N$ which makes resolution relatively insensitive to changes in efficiency. The most powerful means of manipulating $R_s$ is therefore to achieve better $\alpha$ and $k_2$ (retention factor of the more retained analyte). It should be pointed out that the latter two depend on the chemistry of the stationary and the mobile phase rather than the column dimensions. Therefore, the choice of a suitable column combined with appropriate method development is of primary importance for achieving successful separation.

## 1.4 Chromatographic system and detection

The basic elements of any HPLC system, as shown schematically in Figure 1-5, include solvent reservoirs, a high pressure pump, injector, chromatographic column, detector and data processing system. The mobile phase is prepared by mixing the solvents in specific proportion. Mixing can be done manually or performed by a mixer that is usually coupled with the pump. The high pressure pump drives the mobile phase throughout the HPLC system at a constant flow

rate and pressures up to 6000 psi (400 bar). Typical flow rates are 0.2 - 1.0 ml/min for conventional sized columns (2.1 – 4.6 mm inner diameter).

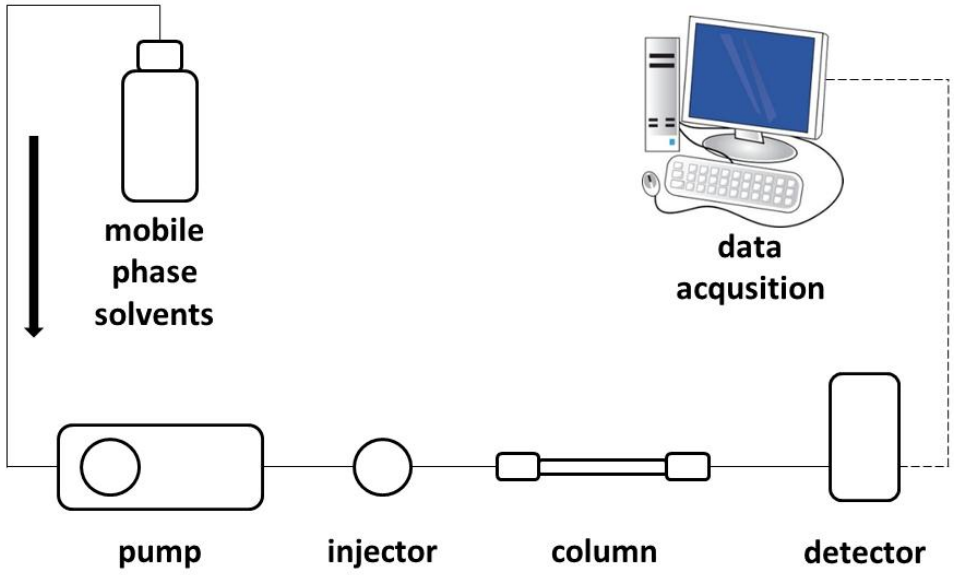



**Figure 1-5.** Basic elements of an HPLC system

The mobile phase is first passed through the injector where the sample is introduced and then loaded onto the column. After the column, the analytes, separated into individual bands travel to an on-line detector where they generate signals proportional to their concentration. Typical HPLC detectors use ultraviolet/visible absorbance, conductivity and mass spectrometry detection. Absorbance detection is used exclusively in this thesis. The signal from the detector is transmitted to the data acquisition which converts the analog electronic

signal into a digital signal. The system then records the intensity of the signals as a function of time. As a result a sequence of peaks is produced corresponding to the individual analytes. This sequence is called chromatogram and is shown in Figure 1-2.

To provide the system with continuous flow of mobile phase the HPLC components are linked by connecting tubing. It is desirable that the length of the tubing after the injector is as short as possible to minimize the extra column band broadening [28]. When making the physical connections care should be taken to avoid the formation of void volumes [29] which can also cause band broadening of the sample.

Connecting tubing is usually made of stainless steel which can withstand the high pressure used in HPLC. However, in ion-exchange chromatography or in applications where pH extremes are used, polymeric materials such as polyether ether ketone (PEEK) are preferred. In this thesis, the connecting tubing after the injector was 30 cm with an inner diameter of 0.013 mm.

The chromatographic analysis of an unknown mixture requires effective separation. However, a good separation alone without consequent identification of mixture components cannot provide meaningful information about the sample composition. This is why every analytical technique needs to be combined with a suitable detection method.

The choice of detector depends primarily on the properties of the analytes, namely their ability to produce a detection signal. Additional considerations in selecting a

detector may include appropriate sensitivity, lower limit of detection, reduced interference with mobile phase solvents and buffers components as well as capability to provide structural information about the analytes.

At present the most commonly used detection method in RPLC is ultraviolet (UV) light absorbance. The UV detector is composed of a light source (UV lamp), a flow cell through which the separated analytes are passed and a transducer that converts the light intensity into an electronic signal. The use of a UV detector requires that the analytes absorb in the UV region of the light spectrum. UV-active molecules usually contain conjugated π-electronic systems (unsaturated aliphatic hydrocarbons, phenyl rings, carbonyl groups etc.).

The absorbance (*A*) is defined as the logarithm of the ratio between the intensity of the incident light ($I_0$) and that of the transmitted light (*I*):

$$A = log\ \frac{I_0}{I} \qquad (1\text{-}13)$$

According to the Beer's law the absorption is proportional to the length of the light path (*l*), the molar extinction coefficient ($\varepsilon$) and the molar concentration of the absorbing species (*c*):

$$A = \varepsilon lc \qquad (1\text{-}14)$$

## 1.5 Modes of HPLC

### 1.5.1 Normal Phase Liquid Chromatography (NPLC)

In NPLC the stationary phase is polar and the mobile phase non-polar. Traditionally, bare silica or alumina were used as packing materials for normal phase columns [3, 24, 25]. However, to obtain the desired selectivity silica can be modified with a bonded phases possessing specific functionalities such as amino-, diol-, cyano- or nitro- groups. The predominant retention mechanism in NPLC is adsorption to specific sites on the stationary phase characterized by displacement between the analyte (*A*) and the mobile phase eluent (*E*) molecules:

$$A_m + xE_s \leftrightarrow A_s + xE_m \tag{1-15}$$

where the subscript *m* refers to the mobile phase and *s* refers to an adsorption site on the stationary phase. NPLC is suitable for the separation of polar analytes, which can be retained on the stationary phase by dipole-dipole, dipole-induced dipole interactions or hydrogen bonding [24, 25]. The technique is primarily used for the separation of polar analytes but can also be applied in the analysis of polar large-molecular weight compounds as those found in petroleum which are otherwise hard to analyze by Gas Chromatography due to their high boiling temperatures.

### 1.5.2 Reversed Phase Liquid Chromatography (RPLC)

RPLC is characterized by a non-polar stationary phase and a more polar mobile phase. It is applied for the separation of non-polar analytes, where the less

hydrophobic species elute earlier and the more hydrophobic ones elute later. Some polymeric materials such as polydivinylbenzene and polystyrene are used as stationary phases in RPLC [3]. However, the most popular type of reversed-phase columns are based on inorganic stationary phase supports such as silica that are chemically modified with hydrophobic bonded phases. The majority of the commercially available reversed-phase columns are packed with silica that is functionalized with organosilanes having long hydrophobic chains like octadecyl- ($C_{18}$) and octyl- ($C_8$), shown in Figure 1-6.

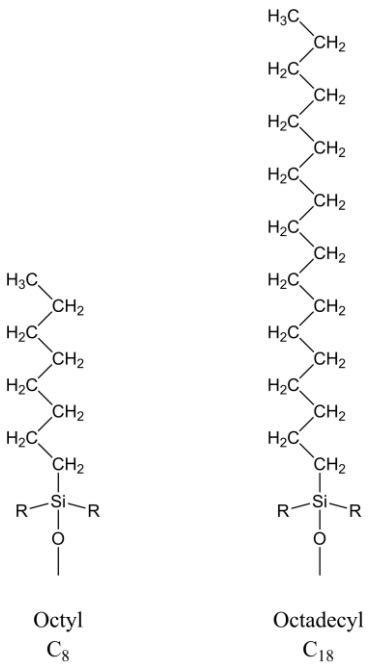



**Figure 1-6.** Hydrophobic alkyl chains used as bonded phases in RPLC

The major separation mechanism in RPLC is partitioning of the solute between the stationary and the mobile phase rather than adsorption as in NPLC. The molecular interactions in RPLC are based mainly on London dispersion forces,

caused by the formation of momentary dipoles [24, 25]. However, in the presence of aromatic or unsaturated aliphatic analytes, charge transfer interactions involving π-electrons may also be significant [30]. Typical RPLC mobile phases are composed of aqueous mixtures of organic solvents such as acetonitrile, methanol or tetrahydrofuran. Water is polar which determines its weak elution properties in RPLC. The mobile phase strength can then be adjusted by varying the amount of the organic solvent. The larger the portion of the organic solvent, the stronger would be the affinity of the mobile phase to the hydrophobic analytes, hence earlier would they come out of the column.

### 1.5.3 Ion-Exchange Chromatography (IEC)

Ion-exchange chromatography gained ground as an analytical technique after the introduction of the conductivity detector by Small *et al*. in 1975 [31]. IEC finds application in the analysis of inorganic ions that are not retained or have limited retention in other chromatographic modes. The mechanism of retention in IEC is adsorption of ionic solutes to a charged stationary phase, containing ionized or ionisable groups. IEC separations are based on displacement of the analyte from the stationary phase by mobile phase ions bearing charge of the same sign. The elution strength of the mobile phase, i.e. its ability to cause analyte desorption, can be adjusted by varying the co-ion concentration of the buffer. The type of ion exchange depends on the charge of the stationary phase. If the stationary phase is negatively charged it can interact with positively charged ions through cation

exchange. Alternatively, a positively charged stationary phase acts under anion exchange mechanism. The anion-exchange process can be described by the following equilibrium:

$$xE_s^{y-} + yA_m^{x-} \leftrightarrow yA_s^{x-} + xE_m^{y-} \quad (1\text{-}16)$$

where $A^{x-}$ denotes the analyte and $E^{y-}$ eluent anions. The subscripts *s* and *m* refer to the stationary and the mobile phase respectively, *x* and *y* are the stoichiometry coefficients of the equilibrium and $x^-$ and $y^-$ are the charges of the analyte and eluent anions.

The anion exchange process is characterized by an equilibrium constant:

$$K_{IE} = \frac{[A_s^{x-}]^y [E_m^{y-}]^x}{[E_s^{y-}]^x [A_m^{x-}]^y} \quad (1\text{-}17)$$

where $[A_s^{x-}]$, $[A_m^{x-}]$, $[E_s^{y-}]$ $and$ $[E_m^{y-}]$ are the molar concentrations of the analyte and eluent ions in the stationary and the mobile phase. It follows from Eq. 1-17 that the greater the value of $K_{IE}$, the larger the concentration of the analyte ions in the stationary phase, which translates into greater retention.

### 1.5.4 Ion-Exclusion Chromatography

Ion-exclusion chromatography is a separation technique predominantly used in the separation of carboxylic acids whose retention is controlled by the analyte degree of ionization. Purely polymeric materials whose surface is modified with

charged functional groups are used as column packings. In this chromatographic mode three phases can be distinguished: eluent phase, occluded liquid phase and polymer phase [32, 33]. The eluent phase is the mobile phase that flows through the interstitial volume of the column. The occluded liquid phase is the eluent trapped into the pores that acts as a stationary phase and the polymer phase is the actual packing material. Due to electrostatic repulsion the charged surface of the polymer phase serves as a barrier (Donnan membrane) that prevents analytes with the same charge as that on the surface from entering the pores of the packing. Thus depending on their degree of ionization, the analytes may have full, partial or no access to the pores. Those analyte molecules that can enter the pores will travel longer distance and will eluted later. Conversely, those that are excluded from the pores will cross only the void volume of the packed stationary phase bed and accordingly will elute earlier.

## 1.6 Traditional RPLC Stationary phases

### 1.6.1 Silica-based stationary phases

Silica gel is the most commonly used stationary phase support in HPLC. Due to its good mechanical strength it is capable of handling the high pressures used in HPLC without being damaged or deformed [3, 34, 35]. Owing to its highly porous structure silica has a very large specific surface area. It is this property that determines the larger sample loading capacities of silica-based packing. For chromatographic purposes silica is made either in the form of spherical micro particles that are packed into a column bed or in the form of solid porous blocks

(silica monoliths). The main physical characteristics of particulate silica are particle size, specific surface area and pore size. Particle sizes in modern HPLC columns range from 5 µm to less than 2 µm. Smaller particle sizes allow the stationary phase bed to be packed more tightly. This boosts column efficiency by minimizing the interstitial volume and reducing band broadening due to resistance to mass transfer in the mobile phase but also increases the pressure drop across the column. Typical pore sizes vary from 50 to 200 Å. Pore sizes of 50-100 Å allow free diffusion of small molecules and high surface area. Larger pore sizes are preferred in the analysis of large bio-molecules to avoid pore blockage and allow free access to the pores. It is worth to point out that the pore size is inversely proportional to the surface area. However smaller surface area dictates smaller mass of the injected sample. The surface area of silica HPLC particles vary from 100 to 500 $m^2/g$ [36].

Silica is covered with silicon-hydroxyl groups (silanols) whose surface concentration varies with the silica type and degree of hydroxylation. A maximum value of 8 ±1 $\mu mol/m^2$ can be achieved with fully hydroxylated silica [36]. Silanol groups can be easily reacted with organic chloro- or alkoxysilanes to modify the silica surface with specific functionalities. In this way, the selectivity of the column can be altered to provide the desired mechanism of separation. Figure 1-7 illustrates the chemical reaction called *silanization* that results in the formation of covalent siloxane bonds that attach the organic ligands to the silica surface. The stationary phases produced in this manner are known as *silica bonded phases* [3].

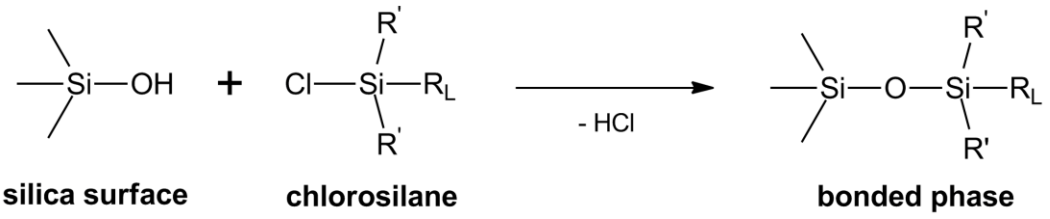



**Figure 1-7.** Silanization of silica producing a bonded stationary phase

where $R^{'}$ is an aliphatic group and $R_L$ is a specific organic ligand. Typical organic ligands for RPLC are octyl and octadecyl aliphatic chains as shown in Figure 1-6.

The surface coverage of the bonded phases is lower for larger ligands and higher for smaller ligands due to steric factors. Even with carefully optimized conditions and small bonded phase molecules the surface coverage rarely exceeds 4 μmol/$m^2$ [34-36]. Thus, only half of all silanols on the bare silica surface can be reacted. The presence of the remaining silanols can be problematic if they interact strongly and non-specifically with the analytes. Such non-specific interactions may become comparable or even dominate the desired separation mechanism which can ultimately compromise the analysis. An example for such detrimental interactions is the ion-exchange between the positively charged analytes and the deprotonated negatively charged silanols on the silica surface [5, 25]. One way to go around this problem is to lower the pH of the mobile phase so as to protonate the silanols and turn them into their neutral form. However, surface silanols are not identical and their tendency to lose or gain a proton is different. As depicted in Figure 1-8, three types of silanols can be identified on the silica surface [34-36].

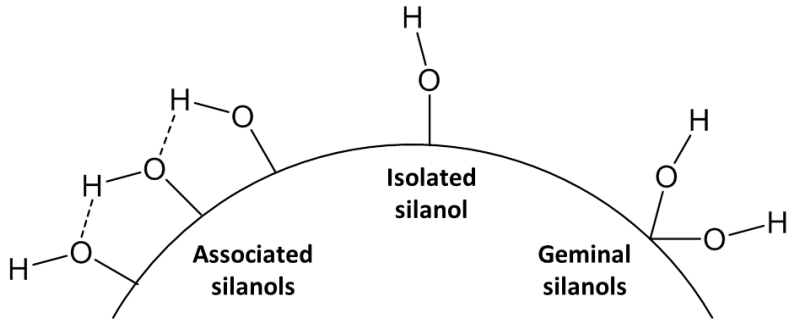



**Figure 1-8.** Types of silanols on the silica surface

Geminal silanols are the most acidic, followed by the isolated silanols and the least acidic associated silanols [36]. The lower acidity of the associated silanols is due to hydrogen bonding between adjacent silanols involving two or more silanol groups. As a result a network of hydrogen bonds is created that stabilizes the silanols. Heating silica above 200 °C leads to the condensation of adjacent silanols into siloxanes, resulting in a decrease in the silanol concentration [37]. Silica surface will thus become scarcely populated, with isolated silanols, each located away from the others and thus not capable of hydrogen bonding with one another. As a result the apparent acidity of the silica will increase. The reverse process, known as rehydroxylation, is possible. Treatment of silica with dilute acidic solutions increases the total number of silanols [38, 39]. Consequently this leads to the formation of more associated silanols resulting in a decrease in acidity.

As discussed above the extent of hydroxylation of the silica surface has an impact on the cation-exchange properties of the packing. However, the silanol acidity can

be influenced even more significantly by the presence of metals in the silica matrix [34, 35, 40]. Metal contamination can be introduced during the manufacture of silica but can also originate from the mobile phase and equipment hardware. The concentration of trace metals in chromatographic silica can be determined directly by Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES). However, a more relevant and presumably more sensitive test is to use chelating compounds as probes and monitor the peak shape they produce. The extent of peak tailing of chelating compounds can serve as a measure of the degree of metal contamination [41]. Depending on the metal content silica columns are classified as type A silica with higher metal content and type B with lower metal content [5]. The older generation type A silica has largely been replaced by purer type B packings characterized by reduced cation-exchange properties.

### 1.6.2 Polymeric stationary phases

Polymeric column packings can be made synthetically as microparticulate beads with a well developed porous structure. The porosity of the beads can be controlled by the degree of crosslinking of the polymer as well as the size of the monomer molecules. Among the polymers that have found application as RPLC stationary phase materials are polymethylmethacrylate, polydivinylbenzene and polystyrene [3]. The major advantage of the polymeric phases over silica-based packings is their stability in alkaline media which allows the pH of the mobile

phase to be varied widely in the pH range from 0 to 14. This property of the polymeric phases is particularly valuable in Ion-Exchange Chromatography where pH extremes are common but can also be beneficial in RPLC for the separation of organic bases at pH above analytes' $pK_a$ values [42]. A serious drawback of the polymeric phases is that they cannot withstand high pressures. This means that the efficiency of the column cannot be increased by reducing the size of the stationary phase particles. As a result, columns packed with polymeric particles tend to be longer with longer analysis times that cannot be compensated with faster mobile phase flow rates because of the same backpressure limitations. Polymeric phases are also sensitive to changes in the mobile phase composition as they swell or shrink upon changing the amount or the type of the organic solvent [3, 25]. This effect becomes obvious when gradient elution is used.

Similarly to silica, the surface of the polymeric phases can be modified chemically to alter their original selectivity. Some polymeric phases are designed to be free from polar functional groups. An example is the hypercrosslinked polystyrene phase developed by Davankov [43-45] and commercialized by Purolite Ltd., Llantrisant, UK. The hypercrosslinked polystyrene phase has been reported to be compatible with a wide range of mobile phase solvents with varying polarities and can be used in both reversed and normal phase mode [46, 47]. The use of hypercrosslinked polystyrene in normal phase mode is quite unique because of the absence of polar groups which normally determine the retention mechanism in NPLC.

## 1.7 Acid-stable stationary phases

The acidity of the mobile phase (pH) can be used as a powerful tool to control the relative retention and thus the elution order of ionisable analytes. Changing the selectivity can resolve overlapping peaks or improve the separation of closely eluting ones. Low pH can also be used to suppress the undesirable ion-exchange interactions between basic analytes and deprotonated silanols on the surface of the silica based columns [5]. The problem with the deprotonated silanols is further complicated by the presence of silanols with varying acidity. To ensure that even the most acidic silanols are protonated, highly acidic mobile phases (pH ~ 2) are used in the separation of cationic analytes. On the other hand low pH conditions favour the hydrolytic cleavage of the siloxane linkages that connect the bonded phase to the silica support (Figure 1-9). Such hydrolytic cleavage causes phase loss which ultimately results in decreased retention and irreproducible separations.

The most popular acid stable columns available commercially are the sterically protected bonded phases developed by Kirkland *et al.* [13]. They are designed to impede the proton-catalyzed hydrolysis by surrounding the siloxane bonds with bulky substituents located in the bonded ligand (Figure 1-9). The silanes used in the preparation of the sterically protected phases contain two chained aliphatic groups such as isopropyl- or isobutyl-, situated close to the point of attachment to the silica support [13]. These bulky substituents act as a barrier that prevents the proton catalyzed hydrolysis of the siloxane bonds.

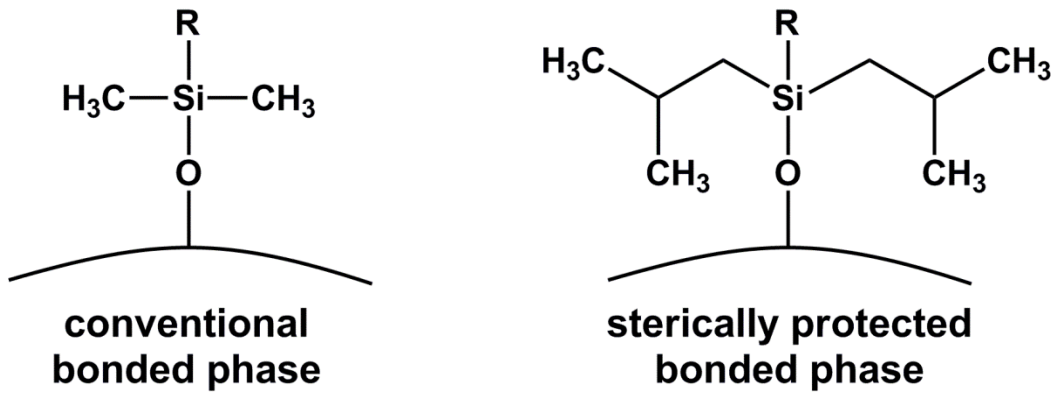



**Figure 1-9.** Chemical structure of conventional and sterically protected bonded phases

The sterically protected phases proved to be very successful for low pH and high temperature applications. Because of the bulky protective groups the surface coverage of the sterically protected bonded phase is relatively low [3].

An alternative way to improve the stability of the bonded phases is to create multiple points of attachment with the silica support by the use of polyfunctional silanes with more than one reactive group, such as two chlorosilanes linked covalently at the silicon atom by a short aliphatic bridge [48]. Another group of acid stable packings is the horizontally polymerized bonded phases [49-51]. They are prepared by crosslinking the bonded phase through a polymerization reaction involving the chlorine at the silicone atom in the silane and traces of water adsorbed on the silica surface [50, 51]. The major drawback of these materials is the poor control over the thickness of the bonded phase which results in pore blockage and poor mass transfer [52].

Some new chromatographic applications, like the two-dimensional liquid chromatography require temperatures exceeding 100 °C and the use of very acidic eluents [53]. Such conditions are beyond the limits of even the sterically protected phases. In response to the demands for more acid stable columns Carr and co-workers proposed a new synthesis strategy that combines the conventional silane modification of silica with the synthesis procedure developed by Davankov for purely polymeric hypercrosslinked polystyrene phases [43-45]. In this way the bonded ligands on the silica surface are crosslinked into an ultra-thin (2 nm) hypercrosslinked surface-confined monolayer. Due to the exceptionally low thickness of the bonded phase layer, the hypercrosslinked materials exhibit excellent mass transfer characteristics [20, 54]. The bonded phase layer does not cause pore blockage and the initial surface area before silica modification is preserved. Owing to its high degree of crosslinking the bonded phase surrounds the entire surface of the silica particles, thus preventing phase loss. Further modification of the bonded phase layer is possible through the same reaction chemistry as the one used in the crosslinking. This allows a series of hypercrosslinked phases with various selectivities to be produced. These include a purely reversed phase octylsilane column [16, 20, 21], a reduced hydrophobicity reversed phase column with incorporated hydroxyl groups [55], hydrophobically assisted cation exchange column [18] and a toluene derivatized column [54].

## 1.8 Summary and thesis overview

This thesis studies the robustness of an acid-stable toluene-derivatized hypercrosslinked stationary phase (HC-T). The stability of the HC-T packed column is assessed as a function of its continuous exposure to acid and temperature extremes. The synthesis of the HC-T phase is discussed in Chapter 2 where two batches of HC-T prepared herein are compared with the literature. Chapter 3 investigates the long-term effects of the extreme conditions that the HC-T column is designed for. Chapter 4 summarizes the results of this study and outlines future work.

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# CHAPTER TWO: Synthesis of Silica-Based Hypercrosslinked Stationary Phases

## 2.1 Introduction

As Reversed Phase Liquid Chromatography (RPLC) grew in popularity, substantial research was invested in the development of polymer-modified silica-based columns. The motivation for their development has been driven by factors such as specific selectivity, superior hydrolytic stability and the ability of these materials to shield surface silanols, responsible for the deleterious secondary interactions with cationic solutes in RPLC [1, 2]. Despite the numerous synthetic schemes used in the preparation of silica polymeric phases, several modification strategies can be identified [3]. One of them uses deposition of polymer on the silica surface by evaporation or precipitation of the coating solution [4, 5]. A serious drawback of the deposited phases is their limited stability due to solubility of the polymer in the eluent. Another approach consists of covalently bonding monomeric substrates which simultaneously polymerize into a stable polymeric network. An example is the horizontally polymerized polysiloxane stationary phases prepared through partial hydrolysis of trichlorosilane monomers [6-8]. A rather different strategy was employed by Collins and co-workers where short-chain polymer molecules, such as polyalkoxysiloxane, were first physically adsorbed onto the silica surface and then crosslinked by thermal or radiation initiation [9, 10]. Due to the presence of siloxane bonds within the chemical structure of the above polymeric packings, they are all susceptible to hydrolysis in acidic medium.

A quite original synthesis strategy combining some of the elements of the above approaches was proposed by Carr and co-workers [11-13]. The resultant new class of silica-based hypercrosslinked stationary phases displays remarkable acid and thermal stability [11-13]. The synthesis of the hypercrosslinked phases consists of conventional silanization of the silica support, followed by crosslinking of the attached functionalities with a multivalent reagent. What is unique about this type of crosslinking is that the reaction proceeds under electrophilic substitution of aromatic systems with alkylhalides in the presence of a metal halide catalyst (Friedel-Crafts chemistry) [14]. The Friedel-Crafts alkylation mechanism is shown in Figure 2-1:

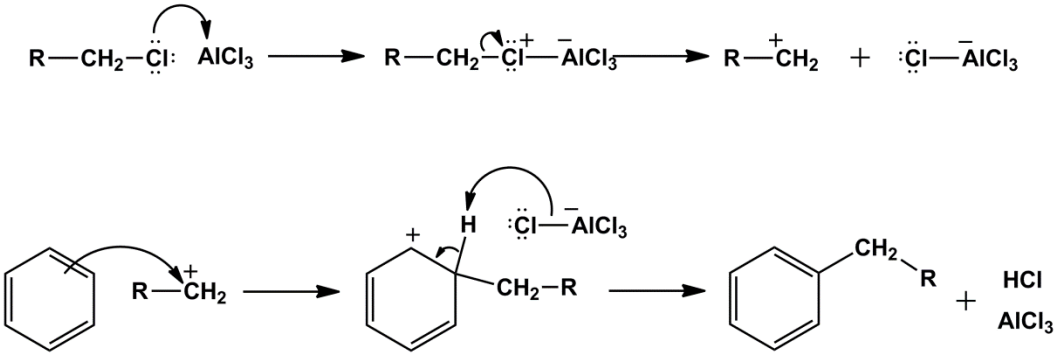


**Figure 2-1.** Friedel-Crafts alkylation mechanism

The product of the crosslinking reaction is an aromatic system interconnected with aliphatic bridges that lack polar groups. In fact, it is the absence of polar linkages that imparts the exceptional acid stability of the hypercrosslinked phases as compared to polymers crosslinked with siloxane bonds [11-13]. In addition, the mechanism of the crosslinking reaction does not allow the polymer layer to grow

in thickness. This electrophilic aromatic substitution of the benzene rings needs alkylhalide groups which are present only in the bonded benzylchloride ligands on the silica surface, and not in the crosslinker. In this way the crosslinking molecules cannot self-crosslink and can only react with the bonded ligands to form thin monolayer confined to the silica surface. In other words, the crosslinking can only proceed horizontally but not vertically from the silica surface.

As the knowledge of the hypercrosslinked phases was becoming better and in an effort to improve the column performance, the original synthetic procedure was modified which resulted in the development of three generations of hypercrosslinked stationary phases.

### 2.1.1 First generation hypercrosslinked silica-based stationary phases

The first stationary phase of the hypercrosslinked family was synthesized using chloromethyl-phenylethyltrichlorosilane as silanization agent (Figure 2-2 top). A self-assembled monolayer was formed on the silica surface through horizontal polymerization of the bonded silane, initiated by partial hydrolysis of the silane on a pre-humidified silica surface [13]. To improve the chemical stability of the bonded phase, the self-assembled monolayer was subsequently crosslinked with either triphenylmethane or styrene heptamer in a Friedel-Crafts alkylation using aluminium chloride as catalyst (Figure 2-2).

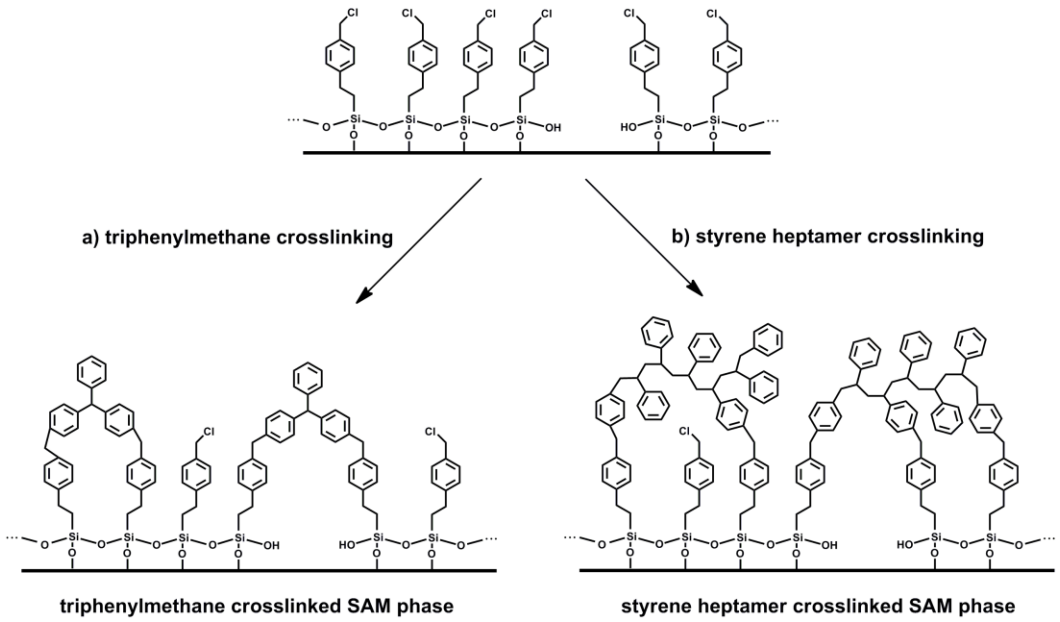


**Figure 2-2**. Crosslinking of self-assembled monolayer stationary phases

To assess its acid stability the stationary phase was packed into a column and washed with an extremely aggressive eluent containing 5 % trifluoroacetic acid at 150 °C [11]. The decreased retention of hexadecanophenone served as a measure of the stationary phase changes caused by the acid treatment. To evaluate the relative stability of the crosslinked self-assembled monolayer (SAM) phases, they were compared with one of the most acid stable commercial silica-based columns - Stable Bond $C_{18}$ [13]. The acid aging experiments revealed that the crosslinked SAM phases are significantly more stable than the commercial column. After 1500 column volumes, Stable Bond $C_{18}$ lost about half of its initial retention, while the triphenylmethane and styrene crosslinked columns lost only 15% and 10% of their initial retention, respectively [13]. To verify that the superior acid stability of the SAM phases was not achieved at the expense of degraded

separation properties the authors studied the chromatographic performance of the columns. They proved by inverse size-exclusion chromatography that the crosslinked self-assembled monolayer did not cause silica support pore plugging [13]. However, their Van Deempter curves indicated that the mass transfer in the styrene crosslinked SAM phase was poorer than that of the Stable Bond $C_{18}$, which was attributed to the greater carbon load of the former [13].

Further studies of the crosslinked self-assembled monolayer phases revealed poor peak shapes for basic analytes, due to strong silanophilic interactions stemming from defects in the surface confined monolayer [13]. Some modifications in the synthesis procedure were therefore needed to eliminate these defects. A different silanization agent was selected and an additional crosslinking step was included in the synthetic scheme [11]. This led to the development of the second generation highly crosslinked phases, referred to as hypercrosslinked silica phases (Figure 2-3).

### 2.1.2 Second generation hypercrosslinked silica-based stationary phases

The trichlorosilane used in the first generation crosslinked phase was replaced with a monochlorosilane (chloromethyl-phenylethyl-dimethylchlorosilane). After silanization, the bonded phase ligands were crosslinked with polystyrene heptamers as with the SAM phase (Figure 2-2b). Secondary crosslinking with chloromethylmethylether was then carried out to further interconnect the polymer network and improve its chemical stability. The chloromethyl groups introduced

with the secondary crosslinker that remained unreacted served as anchors for the covalent attachment of additional aromatic species. This allowed further modifications of the polymer layer to be performed involving derivatization with various functionalities which is a powerful means to alter the selectivity of the stationary phase. For example the attachment of octylbenzene groups produced a reversed phase column - HC-$C_8$ [11, 12, 15]. Controlled sulphonation of the octylbenzene groups with diluted sulphuric acid at low temperature resulted in a mixed-mode column with cation exchange properties [16]. Similarly, a column with weaker cation-exchange character was prepared by derivatization with ethylphenyl acetate which was consequently hydrolyzed and converted to carboxyl groups [17]. In an alternative procedure, a phase with a reduced hydrophobicity was obtained by hydrolyzing the chloromethyl groups, introduced with the secondary crosslinker, to hydroxyls, instead of reacting them with a derivatization reagent [18, 19].

A major synthesis issue which had to be addressed was contamination of the hypercrosslinked phases by the catalyst. Since the crosslinking and the derivatization reactions used $AlCl_3$ as catalyst, some aluminium contamination was introduced. Even traces of metals in chromatographic silica can activate surface silanols and make them more acidic [20-22]. This in turn can give rise to analyte band spreading resulting in broader and tailing peaks. In an attempt to reduce the silanophilicity of the hypercrosslinked phases, the $AlCl_3$ catalyst was replaced with $SnCl_4$ which is a weaker Lewis acid, hence a weaker silanol activating agent. In a follow-up study the authors demonstrated that the use of

$SnCl_4$ results in improved column performance for cationic analytes [15]. They also showed that the less active $SnCl_4$ provided a comparable degree of crosslinking to that obtained with the $AlCl_3$ catalyst [15].

Further characterization of the $SnCl_4$ catalyzed HC-$C_8$ column using basic peptide standards indicated elevated silanol activity as compared to a commercial $C_{18}$ column [23]. This, in combination with the observed affinity of the column to chelating compounds suggested elevated metal concentration, whose primary source was assumed to be the tin catalyst [24]. A new optimization study resulted in a synthesis protocol that minimized tin contamination without degrading the stationary phase chromatographic properties and chemical stability [25]. The time of exposure to the catalyst was reduced which limited the extent of tin contamination.

### 2.1.3 Third generation hypercrosslinked silica-based stationary phases

The optimization studies also explored the suitability of new crosslinking and derivatization reagents with the potential to produce a better structured and more reproducible hypercrosslinked layer. The optimization study used triphenylmethane as the primary crosslinker, 2,4,6-tris-(bromomethyl)-mesitylene as the secondary crosslinker, and toluene as the derivatization reagent. The newly designed phase was referred to as the third generation hypercrosslinked silica phase and was designated as HC-T (Figure 2-3).

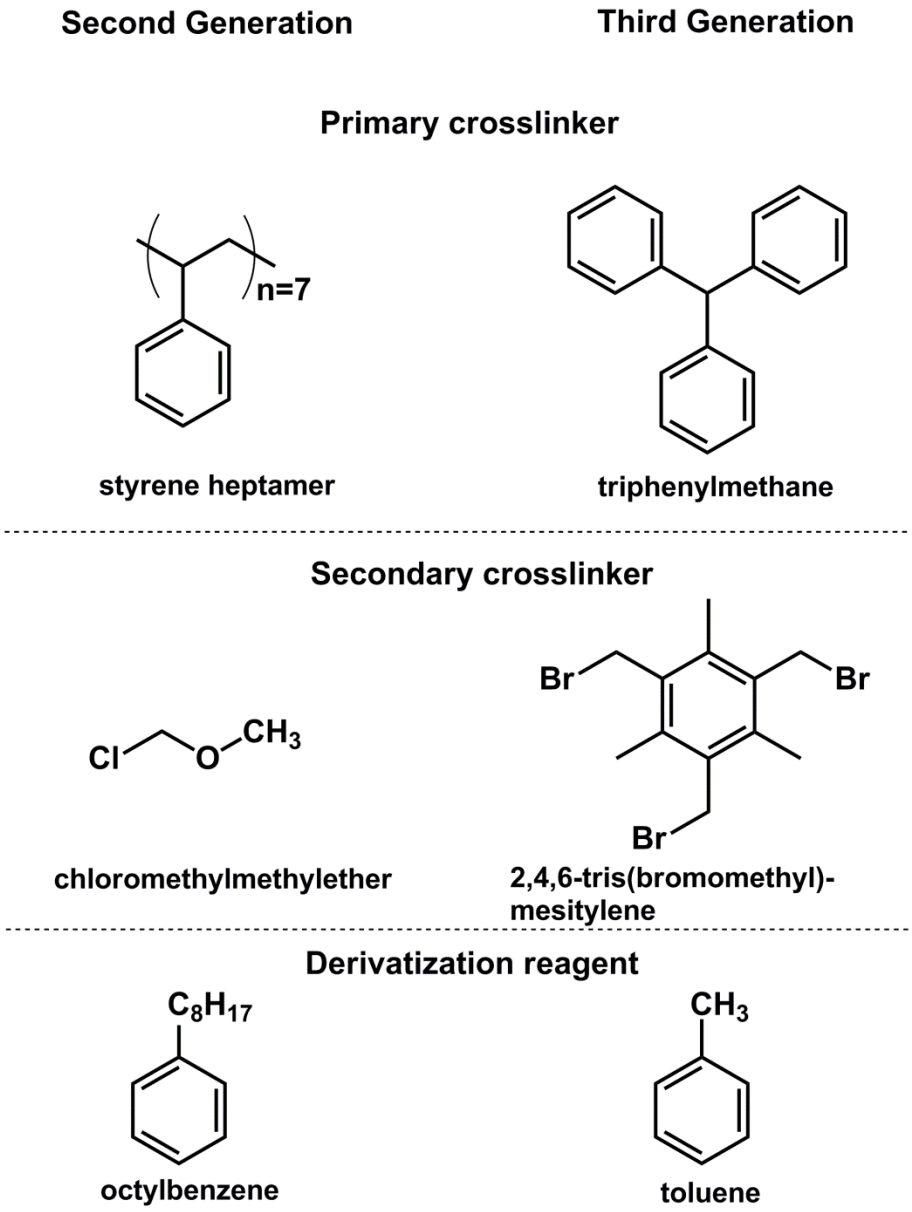


**Figure 2-3** Crosslinking and derivation reagents used in the second and third generation hypercrosslinked silica phases

To compare the second and third generation hypercrosslinked columns (Figure 2-3), optimization experiments with the newly proposed reagents were carried out in

parallel with the reagents used in the synthesis of the second generation hypercrosslinked phases.

The loading density of the secondary crosslinker and the derivatization reagent expressed as μmol/$m^2$ was reported to be lower for the HC-T phase compared to the second generation HC-$C_8$ [25]. Nevertheless, the two types of columns were indistinguishable with regard to their acid stability, as measured by the retention loss of hexadecanophenone upon acid washing the columns [25]. At the same time, the HC-T packed column showed more than 30% greater efficiency for basic analytes and the same plate number for neutral analytes compared to the HC-$C_8$ column [25]. This indicates that the reduced time of exposure to the $SnCl_4$ catalyst did indeed reduce the silanophilicity of HC-T.

### 2.1.4 Acid treatment

The hypercrosslinked silica phases are intended for use under extreme pH and temperature. Under such conditions, the siloxane bonds between the silica and the bonded phase hydrolyse. Also any unreacted bromomethyl groups that were introduced with the secondary crosslinker could be converted into hydroxyls (Figure 2-4e). To prevent the acid initiated changes in the column properties during its routine use, the column preparation protocol involves conditioning of the column with a highly acidic eluent at an elevated temperature [12, 15, 18, 19, 25]. This aggressive acid treatment is intended to break the siloxane bonds between the silica support and the bonded phase that are prone to hydrolysis and

to convert the unreacted bromomethyl groups into hydroxyls. In this way the extremely aggressive acid conditioning would prevent potential changes in the hypercrosslinked columns that might otherwise occur during routine use. In this way, the acid conditioning improves the robustness of the hypercrosslinked phases in terms of retention reproducibility, peak shape and efficiency.

With a conventional acid stable column (Section 1.7) hydrolysis of the siloxane bonds by such an aggressive acid conditioning would cause loss of the bonded phase. In contrast, no phase loss is expected with the hypercrosslinked phases since the hypercrosslinked layer is so extensively interconnected that it "encapsulates" the silica particles including the pores. As a result, even if all linkages with the silica surface are cleaved, the hypercrosslinked polymeric layer will not be removed from the silica surface. The resilience of the polymer layer was evidenced by the preserved spherical shape of the hypercrosslinked layer after the complete removal of the silica support by digestion in hydrofluoric acid [11, 25].

In addition to stabilizing the column, the acid washing is believed to reduce the metal contamination caused by the Friedel-Crafts catalyst used in the synthesis of the stationary phase. Lower metal content will then be expected to reduce the silanol activity and to decrease the acidity of the silica support.

## 2.2 Objectives of this work

Since the synthesis of the hypercrosslinked phases involves multiple reaction steps, the reproducibility of the product may be a concern. In this chapter the reproducibility of the third generation HC-T phase was assessed by comparing the synthesis results obtained herein and the literature. In addition the effect of the acid aging on the stability of the stationary phase was evaluated. This chapter also investigates the effectiveness of the continuous acid treatment in removing the tin residues introduced with the Friedel-Crafts catalyst during the HC-T synthesis.

## 2.3 Experimental

### 2.3.1 Reagents and materials

High purity reagents were used in the synthesis of the toluene derivatized hypercrosslinked silica phase (HC-T). Chloromethyl-dimethylphenylethyl-chlorosilane was obtained from Gelest Inc. (Tullytown, PA, USA). Triphenylmethane, 2,4,6-tris-(bromomethyl)mesitylene, diisopropylethylamine and tin tetrachloride were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The silica particles used as a stationary phase support were Zorbax RX-Sil, Type-B, 5 µm diameter, 80 Å pore size, 180 $m^2/g$ surface area (Agilent Technologies Inc., Wilmington, DE, USA).

All solvents used in the synthesis of HC-T were HPLC grade and were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

### 2.3.2 Synthesis and column preparation

The synthesis of the stationary phase (Figure 2-4) was carried out as in reference [25]. Before each synthesis step, the silica particles were slurried in a 100 ml three neck round bottom flask and sonicated for 15 min under static vacuum to allow the solvent to wet the pores. The flask was filled with argon which continued to flow throughout the reaction. The condenser was capped with an activated aluminium column to avoid water contamination. Evaporation losses were compensated by addition of solvent.

#### 2.3.2.1 Silanization (Figure 2-4a)

Five grams of silica particles were dried in a vacuum oven at 160°C for 12 h. The silica was slurried in 25 ml of dichloromethane in a 100 ml round bottom flask as described above. 0.75 g of diisopropylethylamine was added as a base and also to capture HCl released by the reaction. 3.6 g of dimethyl-chloromethylphenylethyl-chlorosilane was loaded into a sealed roundbottom flask in a glovebox, and added to the reaction mixture to initiate the silanization. The reaction mixture was refluxed for 4 h at 45 °C using an oil bath. The modified silica particles were filtered under argon with a 2 µm with sintered glass filter and washed sequentially with 200 ml portions of dichloromethane, tetrahydrofuran, tetrahydrofuran/water, methanol and acetone. Silica particles were then dried under vacuum at room temperature before the next step was performed.

#### 2.3.2.2 Primary crosslinking

The silanized silica was slurried in 50 ml of dichloroethane as described above. 4.0 g of triphenylmethane was added as the primary crosslinker. The reaction was initiated with 3.2 g of $SnCl_4$ catalyst, and the mixture was refluxed for 15 min at 80°C in an oil bath. The modified silica particles were filtered under argon with a 2 µm sintered glass filter and washed sequentially with 200 ml portions of dichloromethane, tetrahydrofuran, tetrahydrofuran/10% concentrated HCl, methanol and acetone. The silica particles were then dried under vacuum at room temperature before the next step was performed.

#### 2.3.2.3 Secondary crosslinking

The modified silica from Section 2.3.2.2 was slurried in 50 ml of dichloroethane as described above. 4.6 g of 2,4,6-tris-(bromomethyl)-mesitylene was added as secondary crosslinker. The crosslinking reaction was initiated with 3.2 g of $SnCl_4$ catalyst, and the mixture was refluxed for 45 min at 50 °C in an oil bath. The modified silica particles were filtered under argon with a 2 µm sintered glass filter and washed as in Section 2.3.2.2. The silica particles were then dried under vacuum at room temperature before the next step was performed.

#### 2.3.2.4 Derivatization

The modified silica from Section 2.3.2.3 was slurried in 50 ml of toluene as described above. No other reagents were added as the solvent itself served as the derivatization agent. The reaction was initiated with 3.2 g of $SnCl_4$ catalyst and

the mixture was refluxed for 10 min at 80°C in an oil bath. The modified silica particles were filtered under argon with a 2 µm sintered glass filter and washed as in Section 2.3.2.2. The silica particles were then dried under vacuum before the next step was performed.

#### 2.3.2.5 Column packing

The stationary phase was packed into a 0.46 cm x 5 cm stainless steel column fitted with 0.2 µm stainless steel frits (Grace Davison Discovery Sciences, Deerfield, IL, USA)

One gram of the hypercrosslinked silica particles were slurried in 10 ml isopropanol and sonicated for 15 min to fully wet the pores. The slurry was loaded into a 10 ml stainless steel reservoir (Lab Alliance, State College, PA, USA). Downward slurry packing was employed, using a Haskel nitrogen-driven fluid pump (Burbank, CA, USA). The packing pressure of 6 000 psi was reached in 30 s and was maintained until 200 ml of the driving solvent was collected.

#### 2.3.2.6 Acid aging set up

A hypercrosslinked silica packed column was acid washed *in situ* at 150°C using a gradient between two mobile phases (A and B), as described previously in [15, 17]. The column was connected to the HPLC system whose high-pressure pump delivered the acidic eluent. The temperature of the acid washing was maintained at 150°C with an Eppendorf CH 30 Column Heater (Eppendorf AG, Hamburg,

Germany). The composition of the mobile phase A was 7.5% isopropanol/87.5% $H_2O$/5.0% trifluoroacetic acid (v/v/v) and that of B was 87.5% isopropanol/7.5% $H_2O$/5.0% trifluoroacetic acid (v/v/v). Each gradient acid wash cycle had the following profile: 0-5 min (100 % A), 5-20 min (100 % A – 100 % B), 20-25 min (100 % B). The HC-T column was conditioned with 8 gradient acid wash cycles each 25 min in duration at 1 ml/min [15, 17]. For clarity, the duration of the acid conditioning is hereafter expressed as the number of column volumes of the acidic eluent flowed through the column. For example the 8 gradient acid wash cycles carried out in the acid conditioning correspond to 500 column volumes of acid washing or 200 min in duration.

#### 2.3.2.7 Elemental analysis

After the completion of each synthesis step, samples of the hypercrosslinked silica were vacuum dried at room temperature overnight and sent for elemental analysis. The carbon and hydrogen load were determined by combustion analysis on a Carlo Erba EA 1108 Elemental Analyzer at the Analytical and Instrumentation Laboratory, University of Alberta.

The metal content of the HC-T phase was determined by Inductively Coupled Plasma coupled with Mass Spectrometry detection on a Perkin Elmer Elan 6000 Quadrupole ICP-MS instrument (Waltham, MA, USA) at the Radiogenic Isotope Facility, University of Alberta. Bare silica particles were used as a blank to determine the background contamination of the starting material. The sample

preparation involved digestion of the bare silica and hypercrosslinked silica particles with 50 % $HF/HNO_3$ (v/v) mixture.

## 2.4 Results and Discussion

In this thesis two batches of the third generation hypercrosslinked silica phase were synthesized under the optimized reaction conditions described by Carr and co-workers [25]. Figure 2-4 summarizes the synthesis scheme which is detailed in Section 2.3.2. In this work two batches of HC-T were synthesized, depicted as *batch I* and *batch II*. *Batch I* was used for preliminary investigations of the chromatographic performance of the hypercrosslinked phase, while *batch II* was used for the systematic studies of the HC-T chromatographic performance which are detailed in Chapter 3.

### 2.4.1 Reproducibility the HC-T Synthesis

After each modification step the increase in the carbon and hydrogen content was measured to estimate the loading density of each of the reagents. Table 2-1 summarizes the results obtained with the HC-T phase synthesized herein and those obtained in the original HC-T study [25].

The first synthesis step is the silica silanization (Figure 2-4a). In this work the silanization reaction yielded bonded silane density that is consistent between the two batches. However, the silane density obtained herein was lower than that in

the original study [25]. The reason for that could be variations in the silanol surface concentration of the starting silica material.

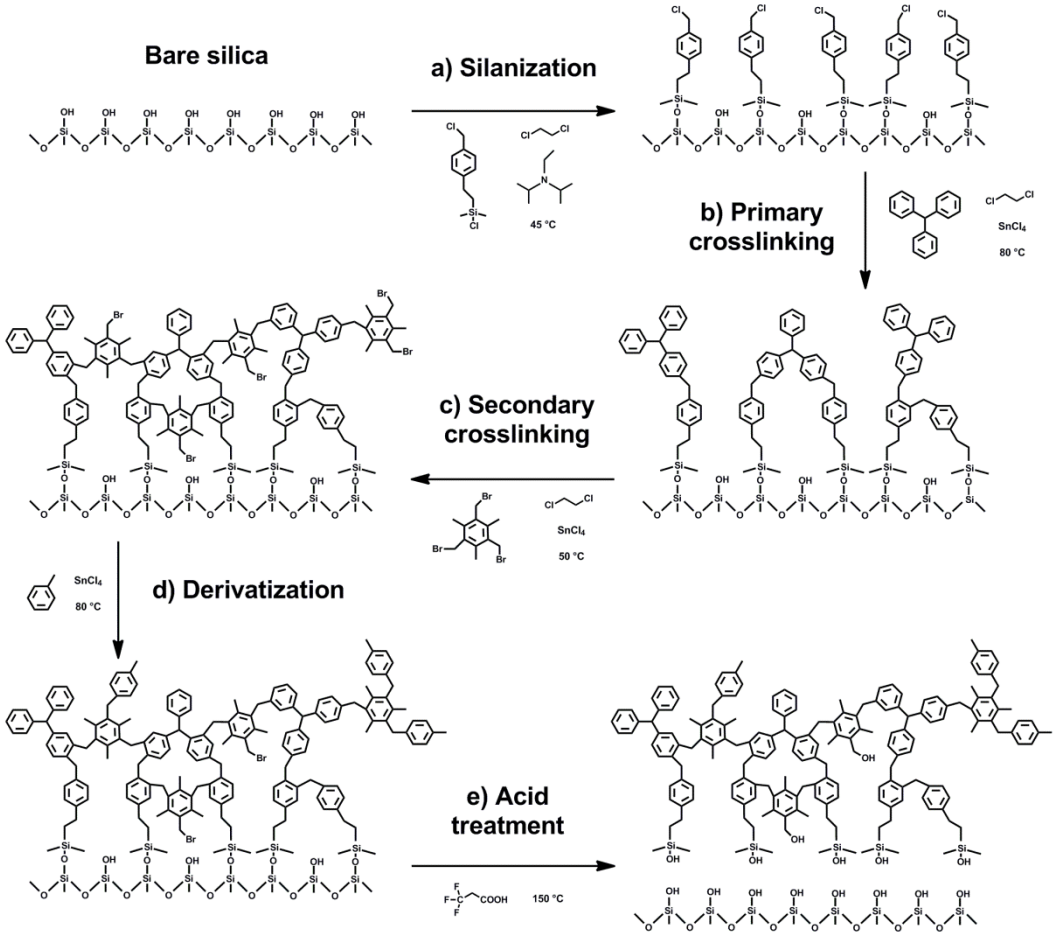


**Figure 2-4.** Synthesis of toluene derivatized hypercrosslinked silica-based stationary phase (adapted from [25]).

The overall growth of the stationary phase can be represented by the cumulative percentage of carbon and hydrogen introduced during the synthesis (Table 2.1). However, this representation does not give a direct estimate of the amount of the different crosslinkers added at each synthesis step.

**Table 2-1.** Elemental analysis of the product at each synthesis step [(a)]

| | HC-T (batch 1)[(a)] | | HC-T (batch 2)[(a)] | | HC-T, reference [25] | |
|---|---|---|---|---|---|---|
| | % C | % H | C % | H % | C % | H % |
| **Silanization** | **5.57** ±0.01 | **0.79** ±0.08 | **5.50** ±0.05 | **0.79** ±0.01 | **6.61** | **0.92** |
| **Primary crosslinking** | **6.66** ±0.05 | **0.91** ±0.02 | **6.51** ±0.02 | **0.83** ±0.00 | **7.53** | **0.94** |
| **Secondary crosslinking** | **8.32** ±0.12[(b)] | **1.01** ±0.02 | **7.73** ±0.07 | **1.01** ±0.01 | **8.73** | **1.04** |
| **Derivatization** | **9.29** ±0.10 | **1.13** ±0.01 | **8.44** ±0.14 | **0.99** ±0.01 | **9.32** | **1.07** |

*(a) The values are the average of duplicate analyses with the corresponding range*

*(b) Temperature fluctuations due to equipment malfunctioning*

Therefore, we will continue the discussion using the number of moles of the crosslinker introduced at each synthesis step. This amount may be calculated using Eq. 2-1 [17]:

$$\mu mol/m^2 = \frac{\left(\%\ ^2C - \%\ ^1C\right) x\ 10^6}{A_c\ x\ N_c\ x\left(100 - \%\ ^2C - \%\ ^2H - \%\ ^2Cl\right)}\ x\ \frac{1}{S} \qquad (2\text{-}1)$$

where $\%^1C$ is the weight percent carbon before the synthesis step, $\%^2C$ is the weight percent carbon after the synthesis step, $\%^2H$ is the weight percent hydrogen after the synthesis step, $\%^2Cl$ is the weight percent chlorine after the synthesis step, $A_c$ is the atomic weight of carbon, $N_c$ is the number of carbon atoms in the crosslinker and $S$ is the specific surface area of the silica support in $m^2/g$. The calculations using Eq. 2-1 are summarized in Table 2-2.

**Table 2-2** Amount of crosslinker and derivatization reagent introduced in each synthesis step

| | ***HC-T Batch I*** ***µmol/m²*** | ***HC-T Batch II*** ***µmol/m²*** | ***HC-T reference [25]*** ***µmol/m²*** |
|---|---|---|---|
| ***Silanization*** | ***2.34*** | ***2.31*** | ***2.78*** |
| ***Primary crosslinking*** | ***0.29*** | ***0.27*** | ***0.25*** |
| ***Secondary crosslinking*** | ***0.71[(a)]*** | ***0.52*** | ***0.52*** |
| ***Derivatization*** | ***0.72*** | ***0.52*** | ***0.44*** |

*(a) Temperature fluctuations due to equipment malfunctioning*

As can be seen from Table 2-2 the differences in the amount of the primary crosslinker (Figure 2-4b) between the three batches are relatively small, with our results being slightly higher. Despite the smaller number of silane ligands on the HC-T batches synthesized herein, slightly larger amount of the primary crosslinked was reacted. This suggests that the crosslinking in our experiments was more extensive. Otherwise, with the same degree of crosslinking as in the original synthesis we would expect a lower amount of the primary crosslinker.

In the secondary crosslinking (Figure 2-4c), *batch I* synthesized herein showed substantially higher crosslinking than *batch II* and reference [25]. Possibly the larger crosslinker load on *batch I* is related to the temperature fluctuations during this particular crosslinking step. However, the amount of the secondary crosslinker in *batch II* has the same value as that reported in the literature [25]. Therefore, *batch II* was chosen for the chromatographic characterization of HC-T described in Chapter 3.

Further analyses of our results indicate that during the derivatization step (Figure 2-4d) larger amounts of toluene were reacted with our two batches compared to reference [25]. With *batch I*, the larger toluene load that was obtained can be

attributed to the greater amount of the secondary crosslinker added in the previous synthesis step. Greater amount of the secondary crosslinker translates into larger concentration of the methylbromide groups with which the derivatization agent (toluene) reacts. With regard to *batch II*, the carbon load from toluene better agrees with that reported in reference [25].

To summarize, *batch I* showed lower silane density. However, during its synthesis larger amounts of the crosslinkers and toluene were added. As a result, its final carbon load was comparable to that of the original HC-T. With regard to *batch II*, our results indicate that its synthesis follows closely that of the original HC-T [25]. Accordingly, this batch was used for the systematic chromatographic studies detailed in Chapter 3.

### 2.4.2 Acid washing of HC-T

#### 2.4.2.1 Stationary phase stability

In Section 2.2 it was pointed out that the hypercrosslinked columns were subjected to 500 column volumes of acid treatment intended to condition the column before use (Figure 2-4e). In an attempt to identify the limits of the hypercrosslinked phases we explored the effect of the prolonged exposure of our two HC-T batches to the acidic eluent used in the conditioning procedure. As expected the acid washing caused only small phase loss measured by the decrease in the carbon content. Table 2-3 shows that after passing the first 500 column volumes of the acid aging eluent, *batch I* and *batch II* lost 8% and 9% of their

carbon load respectively. These results can be explained by loss of non-crosslinked bonded ligands rather that rupture of the hypercrosslinked polymeric layer.

Further, an additional set of acid washing consisting of 1500 column volumes was performed following the initial 500 column volumes of acid conditioning. During this additional acid treatment the rate of phase loss for *batch I and batch II* was greatly reduced. The stationary phase loss during the additional 1500 column volumes was just 4% or 1.3% percent per 500 column volumes. This indicates that in fact the HC-T phase became more stable as a result of the initial acid conditioning. This also suggests that the majority of the siloxane bonds between the stationary phase and the silica support were hydrolysed during the initial 500 column volumes. Thus, most of the non-crosslinked ligands were removed during the initial acid conditioning. During the subsequent 1500 columns volumes some of the remaining siloxanes continued to hydrolyse but due to their much smaller number the phase loss at this stage was minimal.

**Table 2-3.** Carbon and hydrogen content as a function of HC-T acid washing

| | ***HC-T (batch 1)*** | | ***HC-T (batch 2)*** | |
|---|---|---|---|---|
| | ***% C*** | ***% H*** | ***C %*** | ***H %*** |
| ***Non-acid washed column*** | ***9.29*** *±0.10* | ***1.13*** *±0.01* | ***8.44*** *±0.14* | ***0.99*** *±0.01* |
| ***500 column volumes of acid washing*** | ***8.56*** *±0.24* | ***1.04*** *±0.05* | ***7.71*** *±0.09* | ***0.91*** *±0.03* |
| ***2000 column volumes of acid washing*** | ***8.23*** *±0.05* | ***1.00*** *±0.01* | ***7.39*** *±0.12* | ***0.88*** *±0.02* |

### 2.4.2.2 Metal impurities content

In addition to stabilizing the column, the acid washing was also expected to reduce the tin contamination introduced with the catalyst during the stationary phase synthesis (Section 2.2). Figure 2-5 illustrates the decrease in the tin concentration as a function of the number of column volumes of acid washing that *batch I* was subjected to.

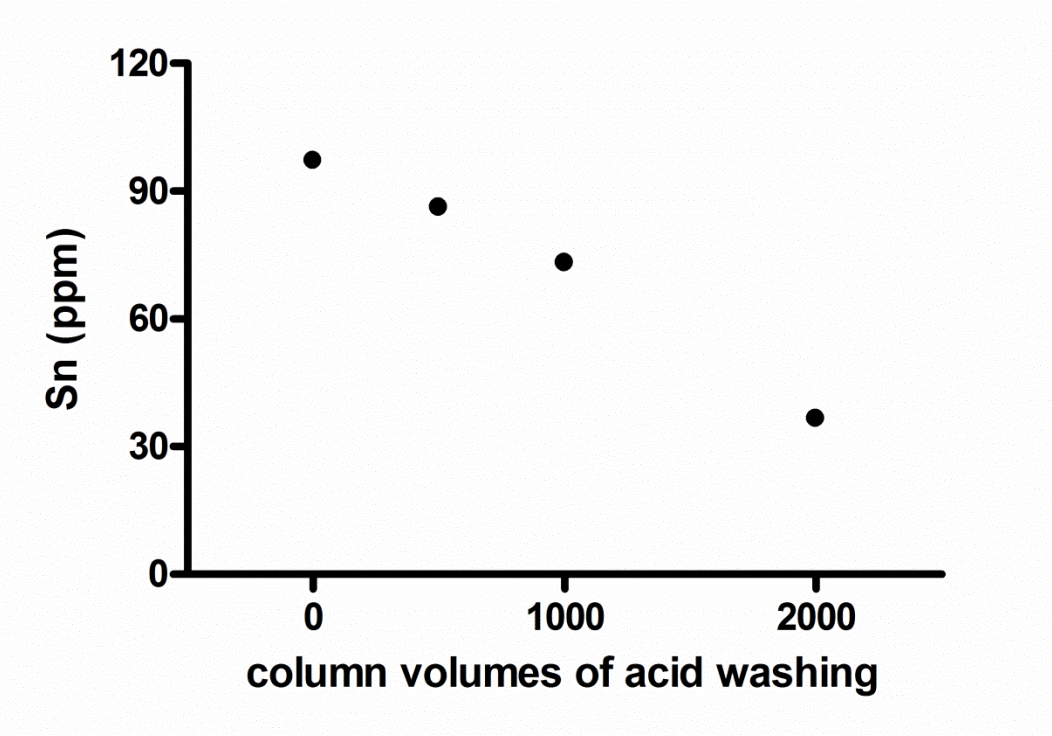


**Figure 2-5.** Tin content in the stationary phase as a function of the number of acid wash cycles of HC-T *batch I*. No replicates were run.

The first 500 column volumes removed only 11% of the initial tin content which kept decreasing steadily with the acid washing. However, even after 2000 column volumes of acid washing, which is four times longer than the initial acid

conditioning the tin content was still significant at 37 ppm or 38% percent of its initial value before any acid treatment. The tin content in the bare silica starting material was determined to be 0.17 ppm (w/w) by running a blank. This amount of tin The limit of detection of the method for tin was 0.06 ppm.

The effect of the acid treatment of silica was studied by Barrett *et al.* [26]. The authors compared the metal content in silica before and after its acid treatment using Inductively-Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) and X-Ray Photoelectron Spectroscopy (XPS). Following the acid treatment, XPS which is a surface characterization technique indicated much larger decrease in the metal content compared to ICP-AES results which measured the total metal concentration. Based on the discrepancies in the bulk and the surface metal concentration the authors concluded that the acid treatment removes metals predominantly from the silica surface and to a lesser extent from its interior.

## 2.5 Conclusions

Two batches of the toluene-derivatized hypercrosslinked (HC-T) stationary phase have been prepared in a multiple-step silica derivatization. The reproducibility of the synthesis has been evaluated based on the amount of stationary phase added during each synthesis step. A comparison between the two batches revealed that *batch I* was more extensively crosslinked than *batch II*. These differences were attributed to temperature fluctuations during the secondary crosslinking of *batch I*. Further, more derivatization reagent was loaded on *batch I* which was attributed

to the larger concentration of methylene bromide groups introduced with the secondary crosslinker. The two batches synthesized herein were also compared with the literature. This comparison showed that the results with *batch II* are consistent with the HC-T optimization studies published previously. Therefore, *batch II* was selected for the chromatographic characterization of the HC-T phase described in Chapter 3.

In addition to synthesis reproducibility, the acid stability and catalyst contamination of the two batches were also evaluated. The acid aging experiments demonstrated that the stationary phase loss upon exposure of the column to the extremely aggressive acid and temperature conditions is minimal. After the initial acid conditioning intended to stabilize the column, the rate of stationary phase loss was further reduced. It was also shown that the acid washing of the column reduces the tin contamination introduced with the Friedel-Crafts catalyst. However, even after prolonged acid treatment the amount of tin is still significant.

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# CHAPTER THREE: Chemical Stability and Chromatographic Properties of a Toluene-Derivatized Hypercrosslinked Stationary Phase

## 3.1 Introduction

In the HPLC analysis of samples containing basic peptides and other biologically active compounds, low pH conditions are usually used to suppress the undesired silanophilic interactions on silica based stationary phases [1]. As pointed out in the previous chapters of this thesis, conventional silica-based columns gradually degrade under acidic conditions due to phase loss caused by the hydrolytic cleavage of the siloxane bonds that link the bonded stationary phase to the silica support [2]. This hydrolytic cleavage is accelerated in techniques such as High-Temperature Liquid Chromatography (HTLC) where temperatures as high as 150 – 200 °C are used for faster and more efficient HPLC separations [3-6]. Due to the combined effect of the high temperature and low pH of the mobile phase in HTLC, the commercially available silica-based acid stable columns are not sufficiently resistant to siloxane hydrolysis [7, 8]. In response to the higher demands to the acid and thermal stability of reversed phase columns Carr and co-workers introduced the hypercrosslinked silica-based stationary phases [7-12]. These materials were shown to possess much better resistance to stationary phase loss under highly acidic conditions at elevated temperatures (150 – 200 °C) compared to commercial acid stable columns [7-12].

However, an essential and yet perplexing part of the synthetic strategy for the hypercrosslinked phases (Figure 2-4) is the acid conditioning of the column (Figure 2-4e). In this thesis the robustness of a toluene derivatized

hypercrosslinked stationary phase (HC-Tol) is explored by monitoring its chromatographic behaviour over the continuous exposure of the column to acid and temperature extremes (pH 0.5, temp. 150 °C). Although such low pH is not practically applicable, subjecting the column to such conditions is designed to serve as acid aging test that can reveal potential long-term changes that take place during the routine use of the column.

It should be pointed out that all chromatographic separations on the HC-T column were carried out at temperatures not exceeding 40 °C. Only the acid aging tests were performed at a temperature typical for HTLC (150 °C). The objective was to determine whether the stationary phase performance can be maintained after exposure to the temperature and pH extremes of the aggressive acid aging.

## 3.2 Experimental

### 3.2.1 Reagents and materials

The solvents used in the chromatographic separations were HPLC grade and were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid and trifluoroacetic acid were of HPLC grade and were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Phosphoric acid was of reagent grade and was obtained from Anachemia Canada Inc. (Montréal, QC, Canada).

All chemical used as analytes were of analytical grade and were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

### 3.2.2 Eluent preparation

All eluents were prepared manually with deionized water from a Barnstead Nanopure System (Dubuque, IA, USA) and filtered through 0.2 µm nylon filter (Magna, Aurora, ON, Canada). Upon mixing organic solvents with water non-additive volumes result. Therefore, to ensure consistency in the eluent preparation the necessary amounts of the organic solvent and water were measured separately and then mixed together, rather than dispensing the organic solvent and then diluting to volume. This procedure better reflects the manner in which eluents are prepared by solvent mixers in automated chromatographic systems.

Formate buffered eluents were prepared by adding formic acid to 29% acetonitrile (ACN)/water (v/v) to a final buffer concentration of 0.1% (v/v). No conjugate base was added to the buffer. Thus, the only source of formate anions was the dissociation of the formic acid.

Five phosphate buffers with constant pH and varying concentrations evenly distributed in the range of 1.0 – 20 mM were prepared according to the procedure described by Loeser [13]. The amount of the acid and its conjugated base were calculated for a constant pH of 3.0 in 30% ACN/water, as described in [14] using Eq. 3-1.

$$[H^+] = \frac{-(K_a + HA) + \sqrt{(K_a + XA)^2 + 4K_a(HA)}}{2} \qquad (3\text{-}1)$$

where *HA* and *XA* are the initial concentrations in (*mol/L*) of the acid and its conjugated base respectively, and $K_a$ is the dissociation constant of phosphoric

acid in 30% ACN/water [15]. Table 3-1 summarizes the calculations of the phosphate buffer concentration and pH:

**Table 3-1.** Calculated values of buffer anion concentrations

| $[H_3PO_4]_{init.}$ | $[Na^+H_2PO_4^-]_{init.}$ | $[H_2PO_4^-]_{eq.}$ | $[H^+]_{(calc)}$ | $pH_{(calc)}$ |
|---|---|---|---|---|
| *16.8 mM* | *18.9 mM* | *19.9 mM* | *0.999 x $10^{-3}$* | *3.00* |
| *8.94 mM* | *9.00 mM* | *10.0 mM* | *1.000 x $10^{-3}$* | *3.00* |
| *5.00 mM* | *4.04 mM* | *5.04 mM* | *1.000 x $10^{-3}$* | *3.00* |
| *2.58 mM* | *0.99 mM* | *1.99 mM* | *1.000 x $10^{-3}$* | *3.00* |
| *1.89 mM* | *0.12 mM* | *1.22 mM* | *1.000 x $10^{-3}$* | *3.00* |

In the above table $[H_3PO_4]_{init.}$ and $[Na^+H_2PO_4^-]_{init.}$ are the initial concentrations of phosphoric acid and sodium phosphate and $[H_2PO_4^-]_{eq.}$ is the equilibrium concentration of the monobasic phosphate anion. The necessary initial amounts of phosphoric acid and sodium phosphate monobasic in 30% ACN/water (v/v) were dispensed with a burette, and then made up to volume using 30% ACN/water (v/v).

### 3.2.3 Chromatographic system

All separations used a model 600 Multisolvent Delivery System (Waters, Milford, MA, USA) and model 7124 Rheodyne injection valve, (Rheodyne, Berkeley, CA, USA) fit with a 0.5 µL injection loop. The stationary phase was packed into 0.46

cm ID x 5 cm stainless steel column (Grace Davison Discovery Sciences, Deerfield, IL, USA). The detection was performed with a Model 481 Lambda-Max LC Spectrophotometer detector (Waters, Milford, MA, USA). Data acquisition was performed with PeakNet 5.2 Chromatography Workstation (Dionex Co., Sunnyvale, CA, USA) coupled with Dionex Advanced Computer Interface. Column temperature of 40 °C was maintained with an Eppendorf CH 30 Column Heater (Eppendorf AG, Hamburg, Germany). The samples to be injected were prepared by diluting stock solutions of the analytes with the mobile phase.

### 3.2.4 Chromatographic column and procedures

HC-T column was acid washed *in situ* at 150°C using a gradient between two mobile phases (A and B) [6, 9]. The composition of A was 7.5% isopropanol/87.5% $H_2O$/5.0% trifluoroacetic acid (v/v/v) and that of B was 87.5% isopropanol/7.5% $H_2O$/5.0% trifluoroacetic acid (v/v/v). Each gradient acid wash cycle followed the profile: 0-5 min (100 % A), 5-20 min (100 % A – 100 % B), 20-25 min (100 % B). For example the initial acid conditioning of the HC-T column consisted of 8 gradient acid wash cycles each 25 min in duration at 1 ml/min with 5% trifluoroacetic acid [6, 9]. For clarity, the duration of the acid treatment is hereafter expressed as the number of column volumes of the acidic eluent flowed through the column. For example the 8 acid wash cycles used in the acid conditioning correspond to 500 column volumes (or 200 min in duration).

In Section 3.3.7.1 the EDTA treatment of the HC-T column was carried out to determine whether the metal contamination was contributing to the retention. The EDTA treatment was conducted *in situ* for 1 h at 1 ml/min, 40 °C using a formate buffered eluent (Section 3.2.2) supplemented with 5 mM EDTA. For the dimensions of the HC-T column used, flowing the eluent for 1 h at 1 ml/min corresponds to 150 column volumes. The void volume of the column was determined with uracil as a dead time marker. Peak asymmetry factors were calculated as depicted in Figure 1-3. A duplicate or more chromatographic runs was performed with each analyte. The reproducibility of the dead time and analytes' retention times were excellent between the separations within the same day. The error bars were smaller in size than graph marks at the scale used and accordingly were not included.

## 3.3 Results and Discussion

### 3.3.1 Retention characteristics

As stated in Chapter 2, the hypercrosslinked stationary phases were treated with highly acidic eluent at high temperature to condition the column before use (Figure 2-4e). This acid conditioning is intended to hydrolyze the siloxane bonds prone to hydrolysis, to convert the unreacted methylene chloride groups into hydroxyls and to reduce the tin contamination introduced with the catalyst during the synthesis [7-9, 11, 16, 17]. After the initial extremely aggressive acid

treatment the column is not expected to undergo further acid initiated changes and thus would be more stable during routine use.

To assess the reproducibility of the HC-T synthesis the performance of the HC-T column synthesized herein is compared with the literature [7] using the same column dimensions, packing procedure, separation conditions, and degree of acid washing. As in reference [7], column performance was assessed using as analytes three basic drugs (alprenolol, nortriptyline and amitriptyline) [7, 9] and also a neutral compound (acetophenone). Figure 3-1 shows the chemical structures of the basic drugs.

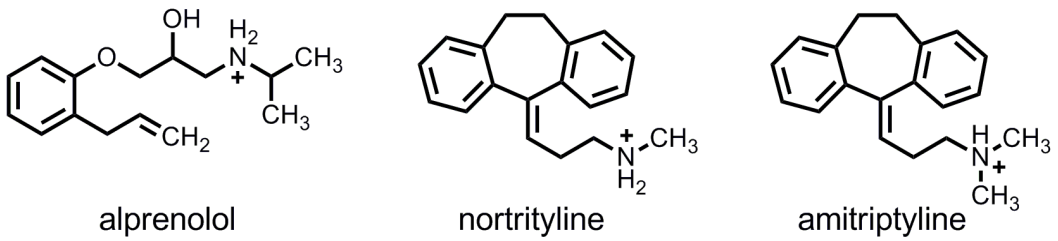


**Figure 3-1**. Chemical structures of the basic test analytes.

Figure 3-2a shows the separation of the analytes on the HC-T column prior to acid washing. Symmetrical peaks are observed for acetophenone, but tailing is evident for the basic drugs. The degree of tailing decreases with lowering of the injected amount of the basic analytes (Table 3-2).

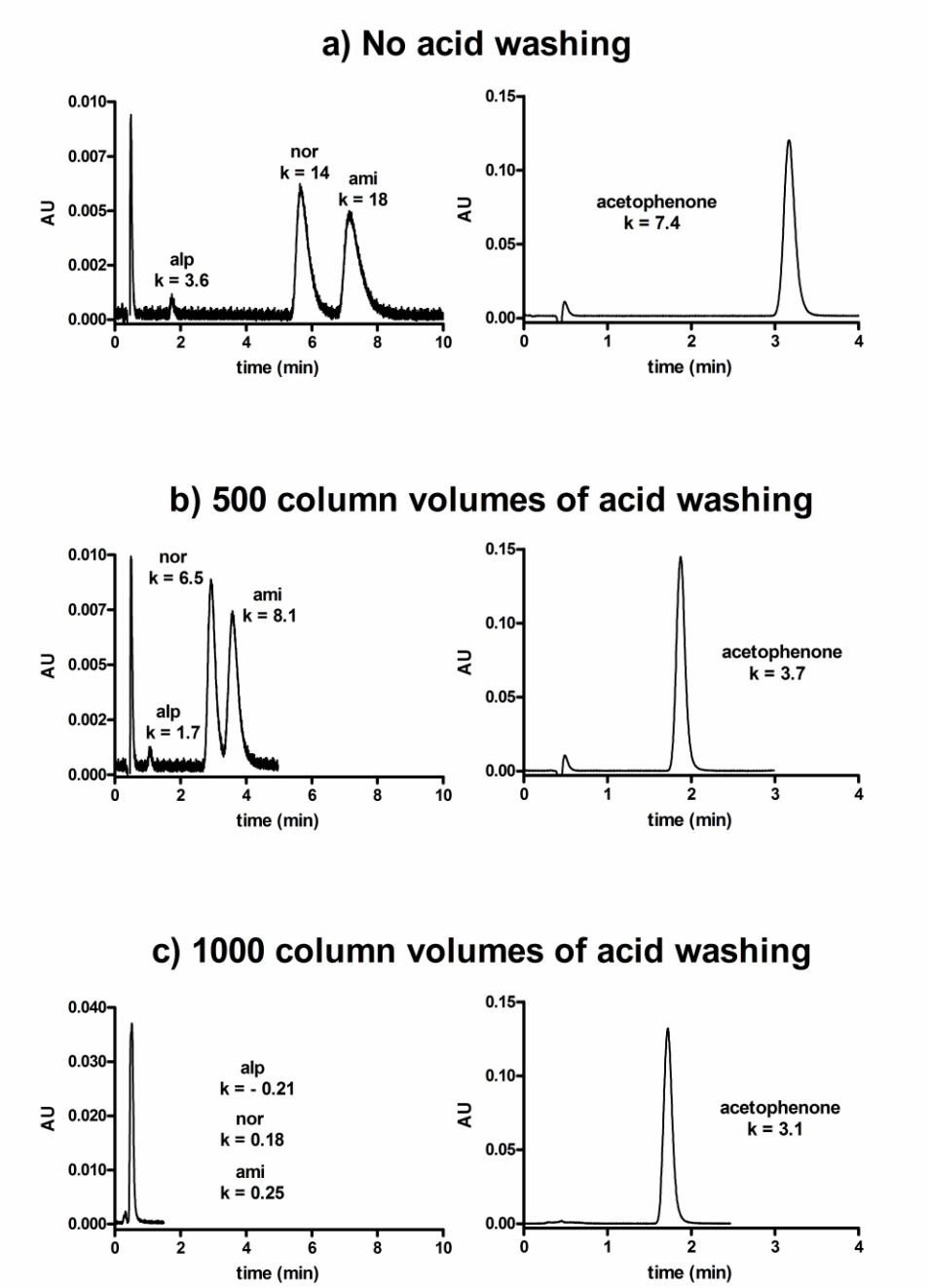


**Figure 3-2**. Effect of the acid washing on the retention of basic and neutral compounds on the HC-T column. Conditions: isocratic elution, 29 % acetonitrile in water, buffered with 0.1% formic acid, pH 3.0; 1.0 ml/min; 40 °C, 0.025 nmol amount of each basic drug and 0.2 nmol of acetophenone; 0.5 µL injection; detection at 254 nm. The HC-T column was acid treated as detailed in Section 3.2.4.

**Table 3-2.** Asymmetry factors of basic drugs as a function of the injected amount on non-acid treated HC-T. Conditions: isocratic elution, 29 % acetonitrile in water, buffered with 0.1% formic acid, pH 3.0; 1.0 ml/min; 40 °C; 0.5 µL injection; detection at 254 nm.

| ***amount injected*** *(nmol)* | ***alprenolol*** $A_s$ | ***nortriptyline*** $A_s$ | ***amitriptyline*** $A_s$ |
|---|---|---|---|
| *0.020* | *2.4* | *3.3* | *3.8* |
| *0.010* | *2.4* | *2.8* | *3.3* |
| *0.005* | *2.3* | *2.5* | *2.9* |
| *0.0025* | *1.9* | *2.4* | *2.6* |

This peak tailing is indicative of overload of the RPLC phase by the charged analytes [18, 19]. Mutual repulsion between the positively charged analyte molecules in the mobile phase and those in the stationary phase effectively reduces the capacity of the stationary phase relative to that achievable with neutral analytes such as acetophenone.

Further studies used 0.025 nmol injections. While overload is still observed with this amount, it is the best compromise between minimizing the overload effect and obtaining acceptable signal-to-noise ratio. While the overload does decrease the observed peak efficiencies (*N*), it does not significantly affect studies of the retention factor or selectivity which are the focus of this chapter.

Figure 3-2 also illustrates the effect of the acid treatment on the HC-T column. The retention of the basic and the neutral analytes decreased 50% after the first 500 column volumes of acid washing (Figure 3-2a to 3.2b) of the HC-T synthesized herein. Carr and co-workers [6, 9] reported that the same acid

treatment of an HC-$C_8$ phase resulted in a 5% retention decrease for the neutral hexadecanophenone. Similarly a comparable acid conditioning of HC-T with 47.5% acetonitrile/47.5% water/5% TFA caused 10% retention decrease for hexadecanophenone [7]. These decreased retentions can be explained by loss of small bonded phase fragments that were not completely crosslinked with the entire polymeric network [9, 11]. However the reduction in retention observed herein and by Carr *et al.* are greater than would be expected based solely on the loss in the carbon content (9% observed in Section 2.4.2 and 4% in reference [7]). The additional reduction in retention was attributed to attenuation of the hydrophobicity of the stationary phase due to the formation of polar groups within the hypercrosslinked layer [9, 11]. These polar groups formed as a result of the hydrolytic cleavage of the siloxane bonds producing silanols on both the silica surface and the hypercrosslinked polymer (Figure 2-4e). In addition, any unreacted methylene chloride groups introduced with the secondary crosslinker are hydrolyzed and converted to hydroxyls [9, 11].

With regard to the basic drugs, comparable selectivity was observed with the column synthesized herein (Figure 3-2b) to that in reference [7] after the initial 500 column volumes of acid washing: $\alpha_{(alp\text{-}nor)} = 3.8$ and $\alpha_{(nor\text{-}ami)} = 1.3$ in this work vs. $\alpha_{(alp\text{-}nor)} = 3.5$ and $\alpha_{(alp\text{-}nor)} = 1.2$ in [7]. However greater retention (59% for alprenolol, 73% for nortriptyline, 80% for amitriptyline) was observed herein. As will be discussed below, the retention of the basic drugs is strongly dependent on the extent of the acid treatment of HC-T. Thus, the retention differences between

HC-T synthesized herein (Figure 3-2b) and reference [7] may be due to slight differences in the temperature or duration of the acid conditioning.

Further column acid washing results in a continuing retention decrease for the basic analytes. After 1000 column volumes of acid washing nortriptyline and amitriptyline are very weakly retained (k of only 0.18 and 0.25, respectively (Figure 3-2c)). In addition, alprenolol, which is the least hydrophobic of the basic drugs, eluted before the dead time marker (k = -0.21). In contrast, the retention of the neutral acetophenone decreased only 16% (k = 3.7 to 3.1 in Figure 3-2c). This is a much smaller retention loss than observed during the initial 500 column volumes (Figure 3-2a vs. 3-2b). The slowing rate of hydrophobicity attenuation is consistent with the expectation that here would be a much smaller number of siloxane and methylene bromide groups left for hydrolysis after the initial acid treatment. Nevertheless, it is clear that even after the initial 500 column volumes of acid conditioning, the stationary phase continues to undergo changes. These changes and the underlying chemical cause are the primary focus of this study.

To determine if such acid initiated changes will continue, the HC-T column was challenged with further acid washing (for a total of 2000 column volumes). Figure 3-3 shows the HC-T retention behaviour as a function of the number of column volumes of acid washing.

After 1000 column volumes of acid treatment the retention for both charged and neutral analytes levelled off. At this stage acetophenone preserved 76% of the retention it had after the 500 column volumes of acid conditioning, whereas the

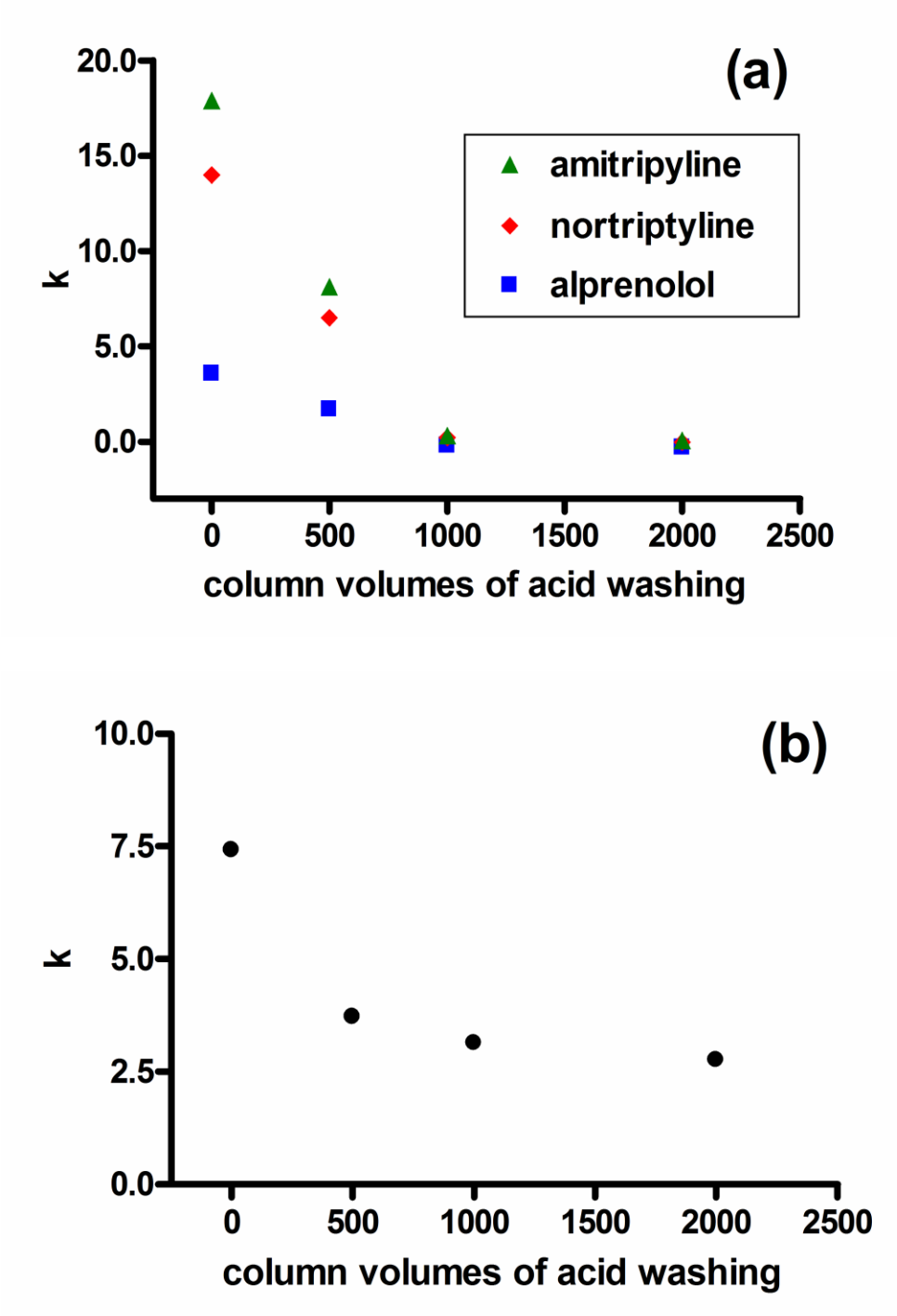


**Figure 3-3.** Retention of basic (a) and neutral analytes (b) on HC-T as a function of the acid washing. Conditions as in Figure 3-2. Error bars are smaller than the graph marks.

basic drugs became essentially unretained. Indeed, after 2000 column volumes of acid washing, alprenolol and nortriptyline eluted before the dead time of the column ($k_{alp}$ = - 0.28 and $k_{nor}$ = -0.02).

These differences in the retention patterns of the basic analytes and the neutral acetophenone and particularly the negative *k* value for alprenolol and nortriptyline suggest that the basic drugs are being excluded from the pores in the stationary phase. Due to the small size of the analyte molecules, size exclusion is unlikely. On the other hand the three basic drugs are protonated at the eluent pH in Figure 3-2 and Figure 3-3.

Therefore, the exclusion from the pores may be due to electrostatic repulsion between the positively charged analytes and some type of cationic functionality in the stationary phase [20]. This functionality could have been formed during the acid washing and this hypothesis is further explored in Section 3.3.2.

### 3.3.2 Normal Phase Separations on HC-T

In a previous study conducted in our group HC-T was used for the normal phase separation of model nitrogen heterocyclic compounds found in petroleum [21]. A mixture of standards was separated on the HC-T column according to their group type (polycyclic aromatic hydrocarbons (PAHs), pyrroles and pyridines). Figure 3-4 compares the chromatograms obtained with the HC-T column synthesized herein (Figure 3-4a) and the HC-T synthesized in [7] (Figure 3-4b).

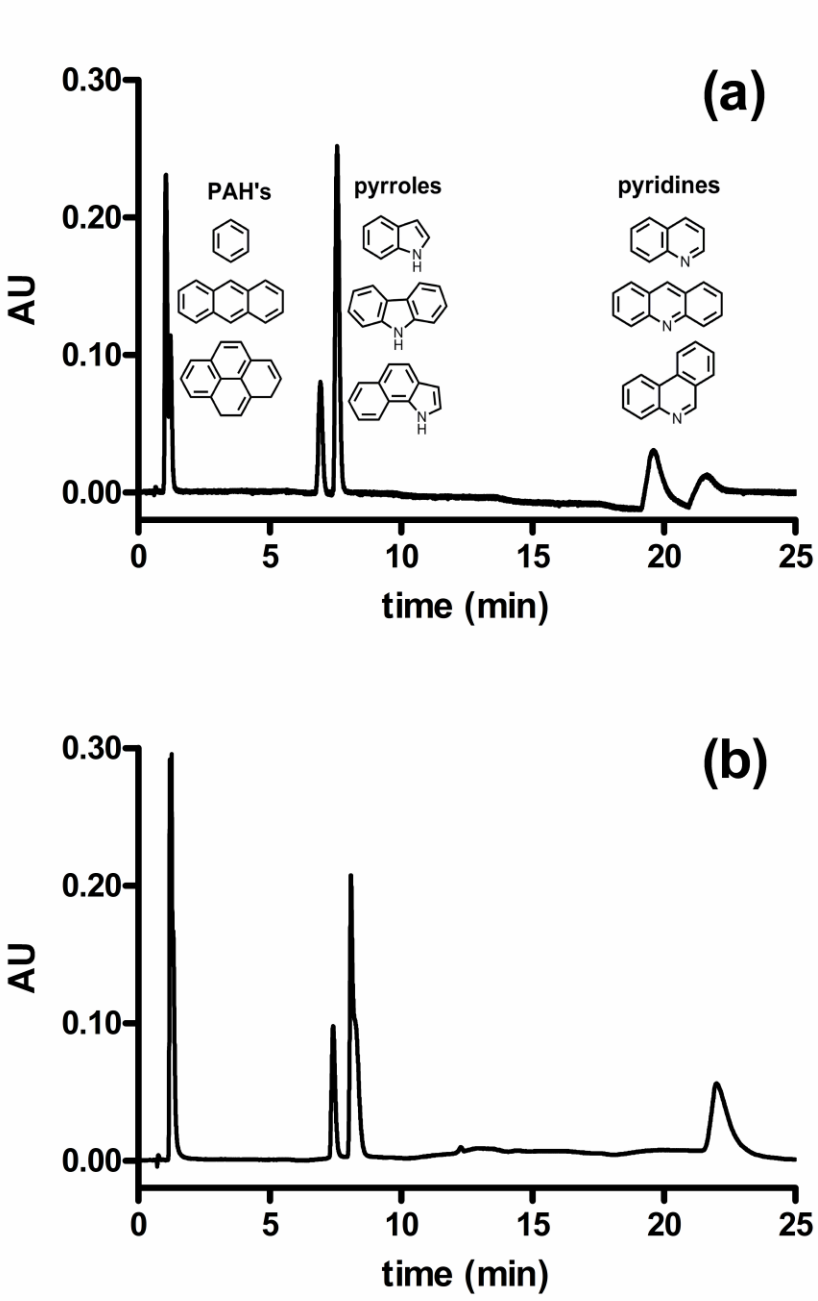


**Figure 3-4.** Group-type separation of nitrogen aromatic heterocycles on (a) HC-T synthesized herein and (b) HC-T in reference [7]. Conditions: 5.0 x 0.46 ID mm HC-T column subjected to 500 column volumes of acid washing; elution, 20 min step gradient starting at 5% dichloromethane (DCM) in pure hexane and increasing by 20% DCM every 4 min; 1 ml/min; 40 °C; detection at 254 nm.

Figure 3-4 shows that comparable retention times were obtained on the two columns in NPLC mode under the same chromatographic conditions on the same instrument. In comparison, the retention differences between the two columns in reversed-phase mode were more pronounced (Section 3.3.1). This indicates that the acid treatment of the HC-T phase affects the reversed phase character of the column but not the normal phase character.

### 3.3.3 Anion-exchange properties of HC-T

More than two decades ago Secreast observed retention of inorganic anions on Zorbax Rx $C_8$ and Nucleosil $C_8$ commercial RPLC columns [22]. Anion-exchange interactions on reversed phase packings were later reported for Symmetry $C_{18}$ [23, 24] and Gemini $C_{18}$ [13] columns. Thus there are precedents for RPLC phases possessing positively charged sites.

In this section the anion-exchange ability of the HC-T column was tested before and after the acid treatment. The experiments were performed under the same chromatographic conditions used in the separations of the basic drugs in Figure 3-2. Benzenesulphonic acid was used as a probe since its low $pK_a$ of 0.7 [25] ensures that it is negatively charged at the pH of the experiments (pH 3.0). Figure 3-5 shows the retention of benzenesulphonate on HC-T as a function of the acid treatment.

Before the acid conditioning intended to stabilize the stationary phase, no anion-exchange character was evident on the HC-T column. Indeed benzenesulphonate

had a retention factor of -0.03, suggesting that the untreated column actually possesses a net negative charge. This is consistent with previous reports that hypercrosslinked silica phases exhibit cation-exchange properties due to metal contamination by the Friedel-Crafts catalyst used in their synthesis [9-11]. After the first 500 column volumes of acid washing only negligible changes in the benzenesulphonate retention were observed.

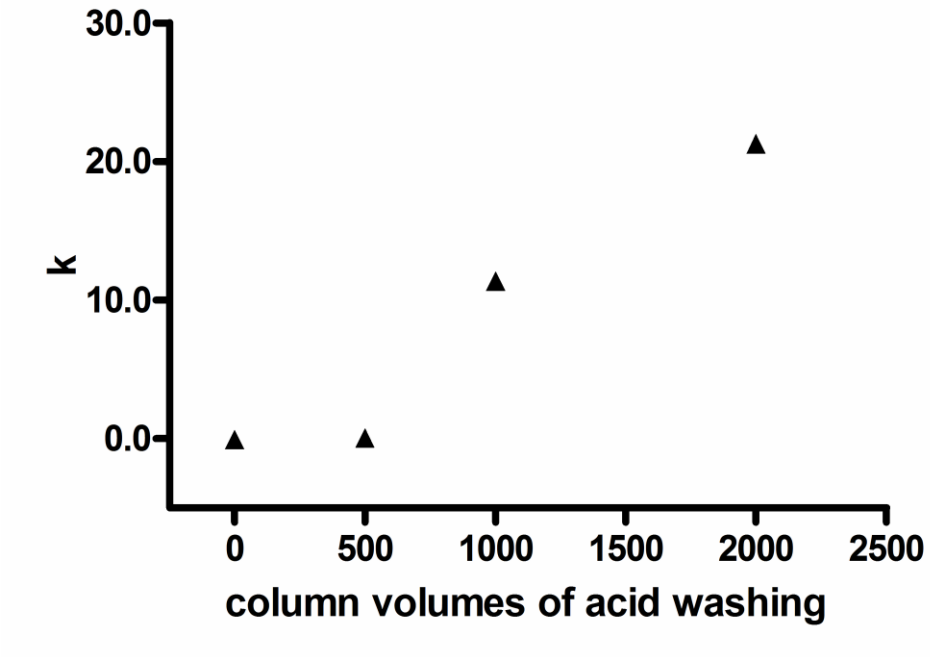


**Figure 3-5.** Retention of benzenesulphonate as a function of the HC-T column acid treatment. Conditions as in Figure 3-2. Error bars are smaller than the graph marks.

However, after 1000 column volumes, benzenesulphonate experienced a drastic increase in retention, indicating that the column had acquired anion exchange characteristics. This sudden increase in the anion retention correlates with the

sharp decrease in the retention of the basic drugs (Figure 3-2). At the same time, the retention of the neutral acetophenone did not change significantly.

The retention improvement for the anionic benzenesulphonate is consistent with the hypothesis that the acid treatment produces cationic sites that further reduce the retention for the basic analytes.

### 3.3.4 Effect of buffer on the ion-exchange properties of RPLC column

The ion-exchange properties of RPLC stationary phases can be a concern in LC applications with mass spectrometry detection where volatile organic acids are preferred as buffers [26]. Due to the partial dissociation of the organic acids, they are weaker anion exchange eluents than inorganic buffers. Thus, under such conditions, the ion-exchange properties of the column would be more evident [23].

An example of strong ionic interactions in RPLC is the cation-exchange between basic analytes and the deprotonated silanols on the silica surface. In this case an increase in the mobile phase buffer concentration yields improved peak shapes and reduced retention [27].

Section 3.3.2. demonstrated that the HC-T column develops anion-exchange properties during the acid treatment. To identify the dominant retention mechanism at the different stages of the acid treatment the ionic strength of the eluent was varied while maintaining a constant pH and organic modifier content.

In the presence of positively charged sites ($X^+$) the following anion-exchange equilibrium exists between the buffer ($E^-$) and the analyte ($A^-$) anions:

$$X^+E^- + A^- \leftrightarrows X^+A^- + E^- \tag{3-2}$$

The corresponding equilibrium constant ($K_{IE}$) is:

$$K_{IE} = \frac{[X^+A^-]_s[E^-]_m}{[X^+E^-]_s[A^-]_m} \tag{3-3}$$

where the subscripts *s* and *m* refer to the stationary and the mobile phase respectively.

As only the deprotonated form of benzenesulphonate is present, the distribution constant ($D_{IE}$) of the analyte between the stationary and the mobile phase is given by:

$$D_{IE} = \frac{[X^+A^-]_s}{[A^-]_m} \tag{3-4}$$

where $[A^-]_m$ represents the concentration of the ionized form of the analyte in the mobile phase. Substituting equation 3-3 into 3-4 yields [28]:

$$D_{IE} = K_{IE}\frac{[X^+E^-]_s}{[E^-]_m} \tag{3-5}$$

Since the pH of the mobile phase was held constant the population of the positively charged sites on the surface ($X^+$) does not change, therefore $[X^+E^-]_s$ should be constant. Then the constants $K_{IE}$ and $[X^+E^-]_s$ in Eq. 3-5 can be combined into a single constant *b*:

$$D_{IE} = b\frac{1}{[E^-]_m} \quad (3\text{-}6)$$

In chromatography the phase ratio of the column is constant, so the retention factor *(k)* is proportional to the distribution constant. Therefore:

$$k = b'\frac{1}{[E^-]_m} \quad (3\text{-}7)$$

Based on Eq. 3-7 analyte retention under a purely anion-exchange mechanism is inversely proportional to the concentration of the buffer anions.

### 3.3.5 Retention characteristics of a non-acid washed HC-T column

The retention of the three basic drugs, the neutral acetophenone and the anionic benzenesulphonate were monitored as a function of the reciprocal of the phosphate buffer concentration (Figure 3-6).

In Figure 3-6 lower buffer concentrations (larger values of $1/[H_2PO_4^-]$) result in slightly increased retention for the basic drugs. These results suggest that before the acid treatment the basic drugs experience cation-exchange retention on HC-T even at the low pH of the experiments (pH 3.00). Higher buffer concentrations reduce the cation retention, as would be expected from the greater concentration of $Na^+$ that competes with the positively charged analytes for the negatively charged sites on the stationary phase surface. The variations in retention as a function of the buffer concentration are more evident for the more hydrophobic nortriptyline and amitriptyline. This is due to the "multiplicative reversed-phase

ion-exchange interaction mechanism" where the synergetic effect of the hydrophobic and cation-exchange contributions enhances retention [29].

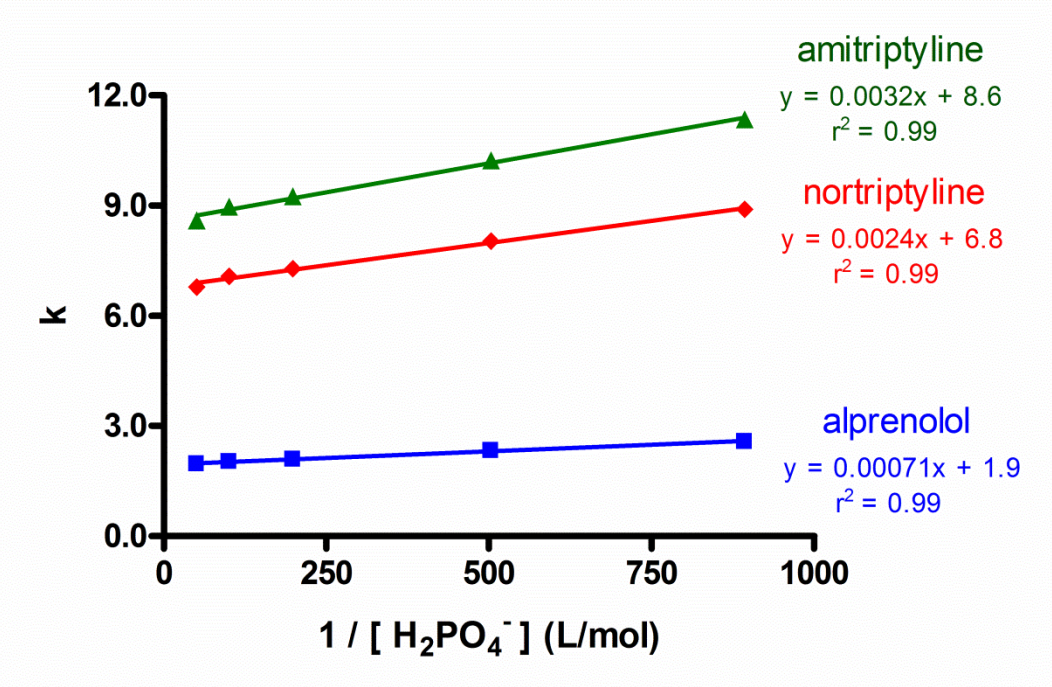


**Figure 3-6.** Retention of the basic drugs on HC-T before acid treatment. Conditions: 30 % ACN/water, phosphate buffer pH 3.0; injection volume 0.5 µL; amount injected 0.025 nmol; temp. 40 °C; detection at 254 nm. Error bars are smaller than the graph marks.

Figure 3-7 shows a chromatogram of the anionic benzenesulphonate on HC-T before acid treatment as a function of the phosphate buffer concentration. At low buffer concentration benzenesulphonate elutes before the dead time ($t_0$), which is consistent with ion-exclusion from the stationary phase pores due to the presence of negatively charged silanols. Increasing the buffer concentration results in increased retention for benzenesulphonate, due to ion-screening of the negative charge on the stationary phase [13, 23]. Figure 3-6 and 3-7 clearly demonstrate

that the HC-T possesses a net anionic charge prior to the acid treatment of the column.

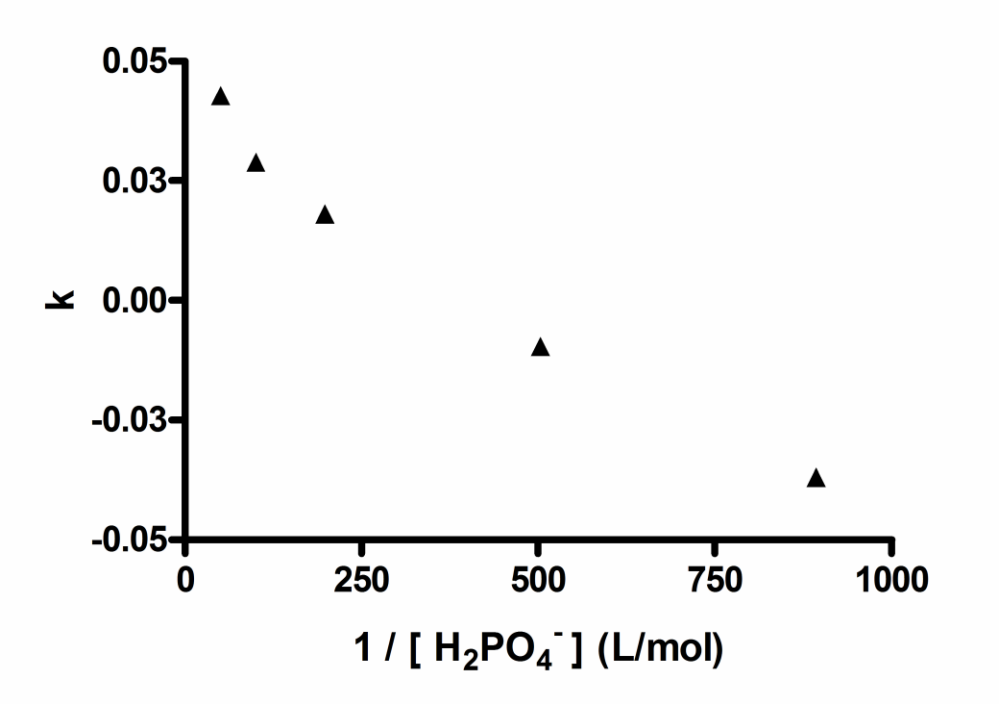


**Figure 3-7.** Retention of benzenesulphonate on HC-T before acid treatment. Conditions: 30 % ACN/water, phosphate buffer pH 3.0; injection volume 0.5 µL; amount injected 0.025 nmol; column temperature, 40 °C; detection at 214 nm. Error bars are smaller than the graph marks.

This charge may arise from either the hypercrosslinked polymer or the underlying silica. Chen *et al.* recently reported that both hypercrosslinked poly(styrene-co-vinylbenzylchloride-co-divinylbenzene) and poly(4-ethylstyrene-co-vinylbenzylchloride-co-divinyl benzene) monolithic columns exhibited a negative charge in pH 7.0 phosphate buffer/ACN [28]. They speculated that the charge was due to adsorption of phosphate onto the hypercrosslinked polymer. Alternately the cation exchange evident in Figure 3-6 may be due to deprotonated silanols on

the underlying silica support. Silica acidity is characterized by its *point of zero charge (pzc),* which is the pH at which the silica surface is neutral. Typical pzc values for silica are in the range of pH 2 - 4 but varies with the type of silica material, its degree of hydroxylation and metal content [30]. As mentioned previously tin impurities introduced with the Friedel-Crafts catalyst can activate surface silanols to make them more acidic [9-11].

However, regardless of its origin, this initial cation exchange character for HC-T disappears upon acid treatment, and is replaced by an anion exchange character. This surprising anion exchange character is the main focus of this study, and is discussed below.

### 3.3.6 Anion retention on HC-T column after 500 column volumes of acid washing

Figure 3-8 shows the effect of phosphate buffer concentration on the retention of the basic drugs on HC-T after the initial 500 column volumes of acid treatment. The effect of the buffer concentration is evident in Figure 3-8 where the concentration dependence of the retention is inverted compared to that before acid washing (Figure 3-6). On the acid-treated HC-T, higher buffer concentration yields greater retention of the basic analytes, and vice versa. A contributing factor to the observed behaviour could be ion-pairing between the buffer anions and the cationic analyte. However, as the phosphate monobasic anion has weak ion pairing abilities [31], this effect is not believed to be significant.

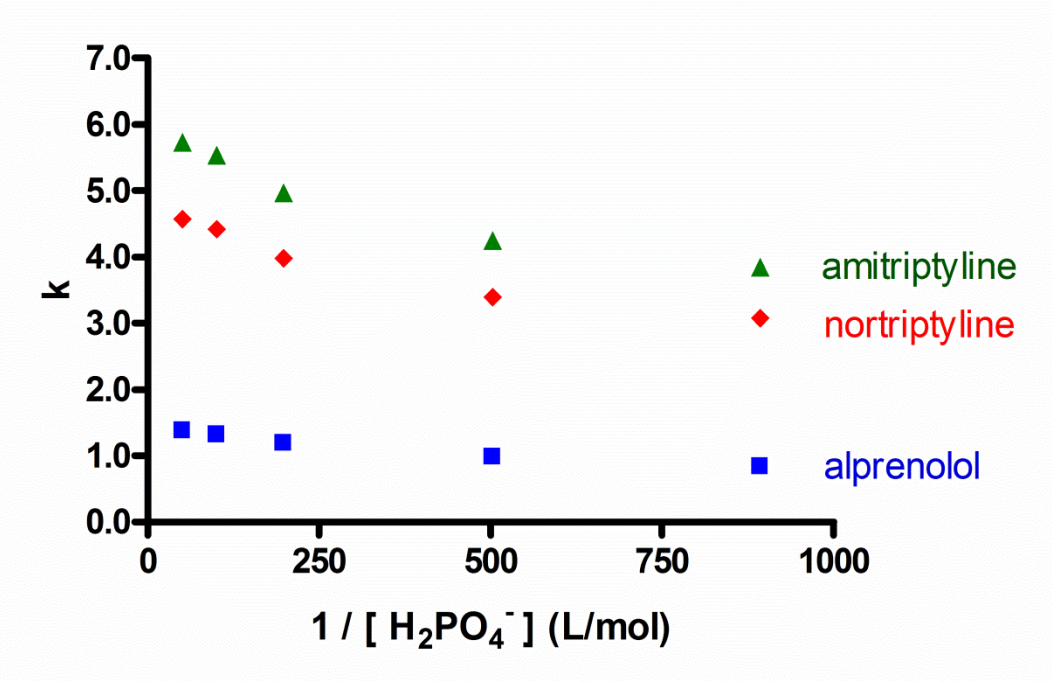


**Figure 3-8.** Retention of basic drugs on HC-T after 500 column volumes of acid treatment. Conditions as in Figure 3-6, except that the column has been conditioned as described in Section 3.2.4. Error bars are smaller than the graph marks.

The observed behavior in Figure 3-8 suggests that a positively charged functionality is being formed on the HC-T column during the acid conditioning. If such a cationic functionality were present on the column, it would be expected that higher buffer concentration would result in increased retention of the basic analytes due to a better screening of the positive charge responsible for the cation repulsion. By extension, if a positive charge is now present on the HC-T, the column should exhibit anion exchange properties.

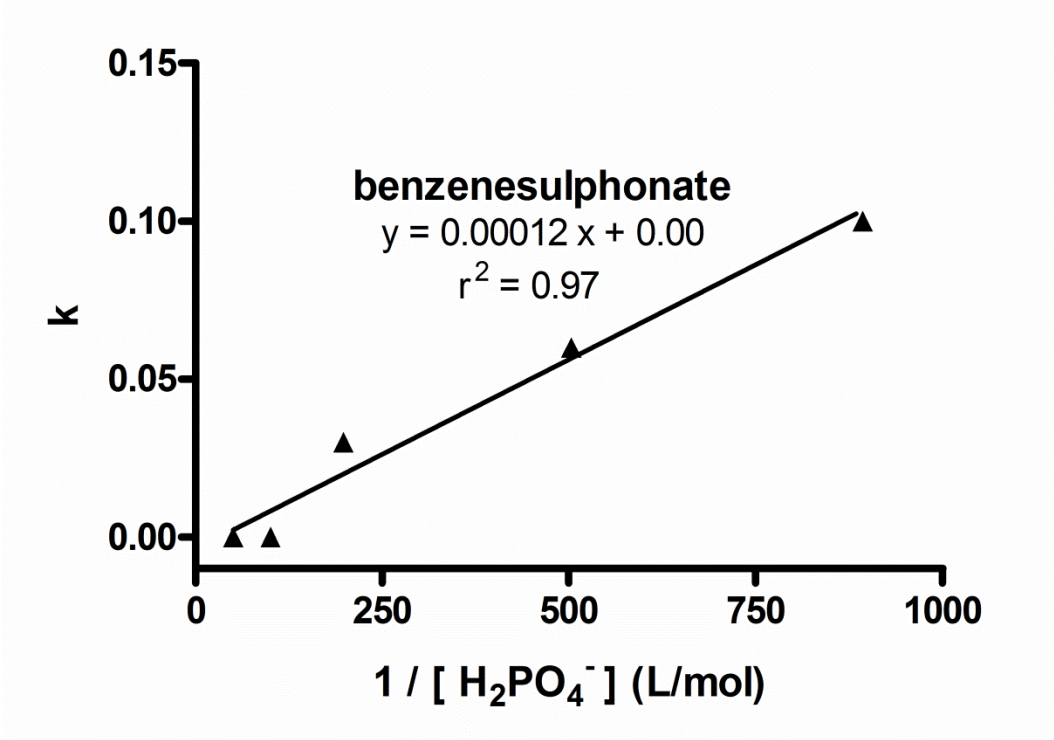


**Figure 3-9.** Retention of benzenesulphonate on HC-T after 500 column volumes of acid treatment. Conditions as in Figure 3-6. Error bars are smaller than the graph marks.

Figure 3-8 clearly demonstrates that benzenesulphonate is undergoing anion exchange on the acid washed HC-T column. At higher buffer concentrations the larger number of buffer anions compete with the analyte for the anion-exchange sites which results in weakened electrostatic retention of benzenesulphonate. Alternatively, at lower buffer concentration the anion-exchange sites are more available and benzenesulphonate is more retained. The weak anion retention in Figure 3-8 indicates that the population of the positively charged groups on the stationary phase is relatively low. Despite that, a good correlation with the

theoretical ion-exchange model was observed ($r^2$ = 0.97). The scatter of the data at low retention reflects the error in determining *k* for retention times close to $t_0$.

### 3.3.7 Anion retention characteristics on HC-T after 1000 and 2000 column volumes of acid washing

Section 3.3.3 showed that the HC-T column continues to undergo acid induced changes even after the initial 500 column volumes of the acid treatment advocated for stabilization of the column [7]. Here the retention behaviour of the HC-T column is explored in more detail as a function of the buffer concentration. Figure 3-10 illustrates the retention of the basic drugs versus the phosphate buffer concentration after subjecting the column to 1000 and 2000 column volumes of acid washing. Lowering the buffer concentration in Figure 3-8 leads to rapid loss of retention for the basic drugs. This rapid change in retention is more pronounced after 1000 and 2000 column volumes compared to that after 500 column volumes. This is an indication that the HC-T column continues to undergo acid initiated changes at this stage. Lower buffer concentration would cause less ionic screening, which would lead to enhanced repulsion between the protonated basic analytes and the positive charge on the surface, and thus exclusion from the pores of the stationary phase. Indeed at 2 mM phosphate concentration and below, the basic drugs elute prior to the dead time (*i.e.*, negative k) indicating that these analytes are excluded from the pores.

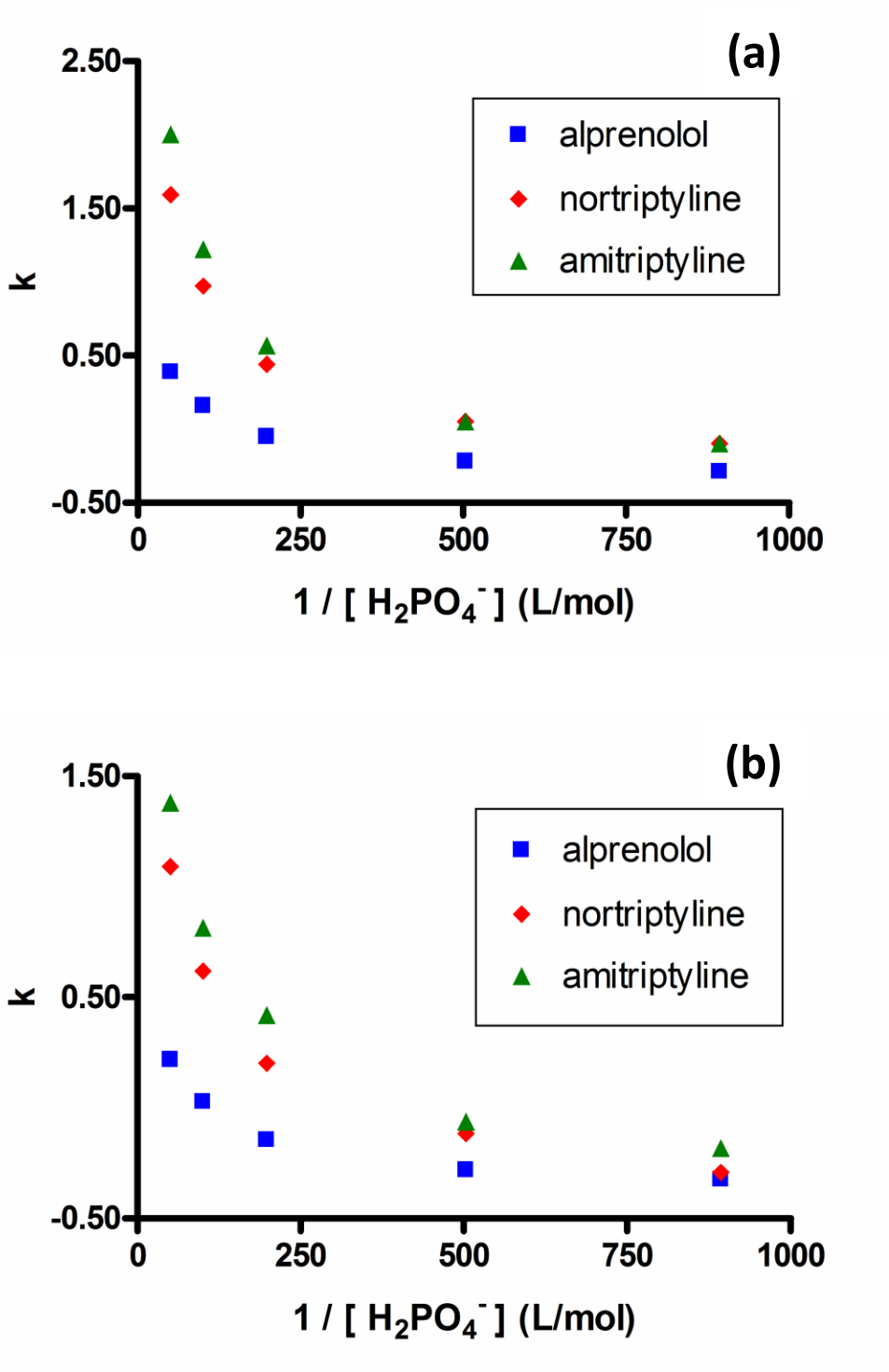


**Figure 3-10**. Retention of the basic drugs on the HC-T column; (a) after 1000 column volumes and (b) after 2000 column volumes of acid treatment. Conditions as in Figure 3-6, except for the extent of HC-T acid treatment. Error bars are smaller than the graph marks.

Conversely, at higher buffer concentrations the cation exclusion is suppressed and the basic analytes have access to the pores of the stationary phase. In the pores they are retained via a reversed phase mechanism due to their hydrophobicity. Accordingly, their retention is significantly enhanced at higher phosphate concentrations.

The ion exchange model discussed in Section 3.3.2 was next applied to the retention of anionic analytes on HC-T after 1000 and 2000 column volumes of acid treatment. In addition to anion-exchange, benzenesulphonate may be involved in reversed phase interactions with its hydrophobic part. To eliminate any hydrophobic retention contributions, nitrate was selected as a second anion-exchange probe. Figure 3-11 shows the plots of the anion retention versus the phosphate buffer concentration.

An excellent linear fit ($r^2$ = 1.0) with the theoretical anion exchange model (Eq. 3.7) was observed for both analytes in Figure 3-11. This indicates anion-exchange retention for both analytes. The steeper slope of benzenesulphonate compared to that of nitrate is consistent with the "multiplicative reversed-phase ion-exchange interaction mechanism" [29]. Further analysis of the plot shows that the 150 fold increase in the benzenesulphonate slope after 1000 column volumes (0.018 in Figure 3-11a) compared to the slope after 500 column volumes (0.00012 in Figure 3-9) correlates with the sudden appearance of anion-exchange after 1000 column volumes (Section 3.3.1).

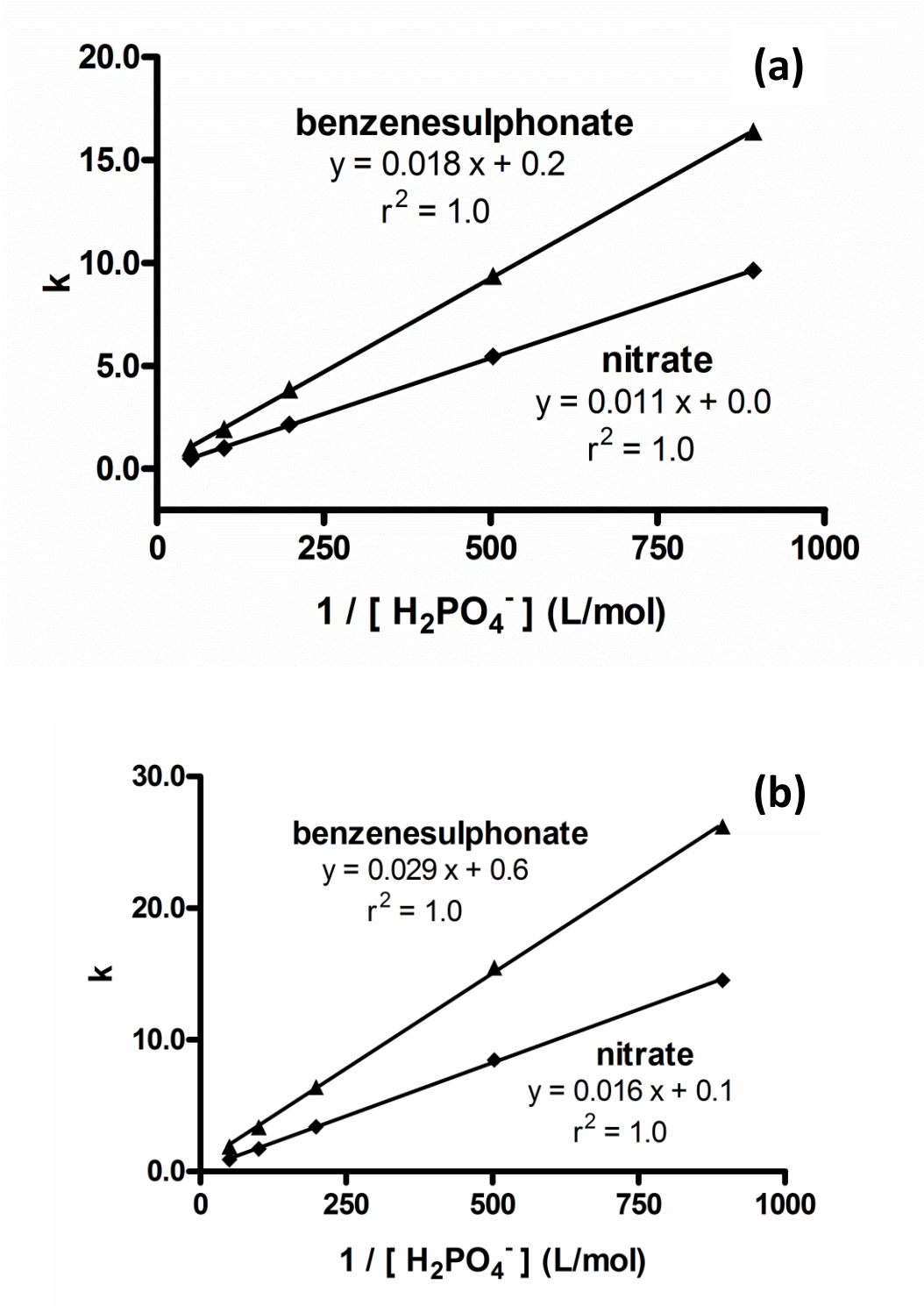


**Figure 3-11.** Retention of benzenesulphonate on HC-T; (a) after 1000 column volumes and (b) after 2000 column volumes of acid treatment. Conditions as in Figure 3-7, except for the extent of HC-T acid treatment. Error bars are smaller than the graph marks.

In addition, after 1000 column volumes, the slope of HC-T is of the same magnitude as that obtained with two commercial RPLC columns (Gemini $C_{18}$ and Symmetry $C_{18}$) under analogous chromatographic conditions using nitrate as analyte [13]. In this thesis the nitrate slope after 1000 column volumes was 10.9 x $10^{-3}$ compared with 3.01 x $10^{-3}$ and 2.18 x $10^{-3}$ on Gemini $C_{18}$ and Symmetry $C_{18}$ where the columns were not acid treated [13].

The steeper slopes of benzenesulphonate and nitrate after 2000 column volumes compared with the slopes after 1000 column volumes confirm the observation made in Section 3.3.1 that the build-up of anion-exchange sites continues. According to Eq. 3-4 a greater surface population of the anion-exchange sites on the stationary phase ($X^+$) would shift the distribution equilibrium to a higher ratio of the analyte in the stationary phase. This would result in greater distribution constant ($K_{IE}$) which translates into a steeper slopes in Figure 3-11 and greater anion retention.

### 3.3.8 Origin of the anion-exchange sites

Secreast proposed three hypotheses to explain the observed anion retention on RPLC columns [22]: metal impurities in the silica support; presence of a protonated amine in the stationary phase; or doubly protonated surface silanols.

In another study, Carr and co-workers investigated the effect of the aggressive acid treatment on the stability of a commercial RPLC column (*Zorbax StableBond $C_{18}$*) [32]. They found that the column acquired anion exchange character after

being acid washed with eluents containing iron and other metal nitrates [32]. Thus, the observed anion retention properties were attributed to metal ions acting as anion-absorption sites.

The chelating compound hinokitol (Figure 3-12) has been proposed as a test analyte for the presence of metal impurities in RPLC phases [33]. If metal ions are present in the stationary phase they may act as strong adsorption sites for the chelating test analyte which would result in tailing peaks. On the other hand treatment with ethylenediaminetetraacetic acid (EDTA) have been used to remove metal impurities from chromatographic silica [34]. To assess the effect of the EDTA treatment we compared the retention of hinokitol before and after flushing the HC-T column with EDTA. For simplicity, we will focus the discussion on the results obtained after subjecting the HC-T column to 1000 column volumes of acid washing. It should be noted that EDTA was only used in the column treatment procedure and the eluent used in the chromatographic experiments did not contain any EDTA.

Prior to flushing the HC-T column with EDTA, hinokitol eluted as a severely tailing peak (Figure 3-12a) whereas after the EDTA treatment, the same analyte produced a sharp peak (Figure 3-12b). The improved peak shape for hinokitol in Figure 3-12b treatment indicates that the metal impurities on the HC-T column were significantly reduced. Further, the peak shape and retention of hinokitol remained stable until the next acid washing, after which the peak shape degraded again. This suggests that either the acid washing re-contaminated the column or the EDTA molecules that coated the metal impurities were flushed away.

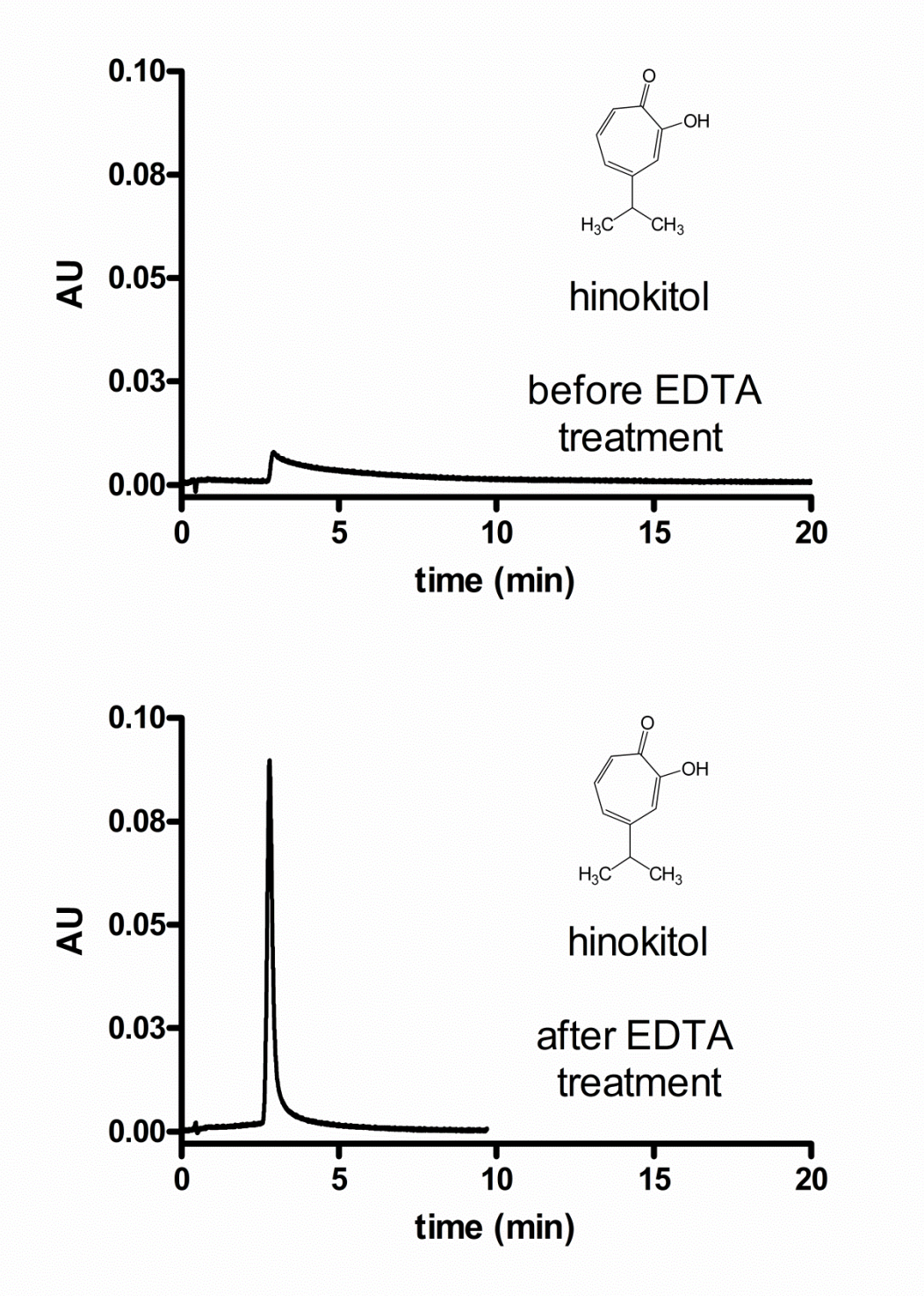


**Figure 3-12.** Effect of the EDTA treatment on the chelating properties of the HC-T column. Conditions as in Figure 3-2c.

If the metal impurities introduced by the acid treatment were responsible for the anion interactions with HC-T, the anion retention would be greatly reduced by the EDTA treatment. Surprisingly, the retention times within a given acid washing step were not affected by the EDTA treatment. For example, after 1000 column volumes of acid washing the retention for benzenesulphonate and nitrate remained stable after the EDTA treatment. This indicates that while metal impurities are introduced during the acid treatment, they are not the source of the anion exchange sites that develop on the HC-T stationary phase.

Anion retention has also been observed on *Symmetry* $C_{18}$ which is a commercial RPLC column [24]. The anion-exchange properties of that column were attributed to residues of the amine used in the synthesis of the stationary phase. In another study, [22] a similar hypothesis was proposed to explain the positive charge observed on *Zorbax Rx* $C_8$ and *Nucleosil* $C_8$.

The synthesis of the HC-T stationary phase involves a silanization step that uses diisopropylethylamine as base (Figure 2-4 step a). A column packed with silanized silica (*i.e.*, without any crosslinking) did not show any anion exchange character (data not shown) either before or after 1000 column volumes of acid treatment. Therefore, it is not likely that an amine is the source of the anion-exchange discussed above. This conclusion is also supported by the observed anion-exchange properties on a purely polymeric hypercrosslinked polystyrene phase whose synthesis did not use any amine [35, 36].

Hypercrosslinked polystyrene polymers were introduced by Davankov [37] and are the predecessor to the silica-based hypercrosslinked phases developed by Carr and co-workers [7-12, 16, 17]. The polymeric structures of these materials are similar and they also share the same residual methylenehalide groups. Since the purely polymeric phases exhibit similar anion-exchange character to that observed for HC-T, we believe that the surface charge originates with the polymer itself and not with amine impurities.

Penner and Nesterenko reported that the purely polymeric hypercrosslinked polystyrene phase discussed above exhibits anion-exchange properties in the pH range of 2.6 – 4.3 [35, 36]. In addition, the positive zeta potential [38] of this material suggests that the anion-exchange may be due to a positively charge functionality on its surface. Based on the elemental analysis of the hypercrosslinked polystyrene which showed carbon, hydrogen, oxygen and chlorine, Penner and Nesterenko concluded that the source of the anion-exchange is an organic functional group. They attributed the observed anion-exchange behaviour to protonated carbonyls incorporated into the stationary phase. However no evidence for the existence of such functionalities was provided, nor was a mechanism for their formation proposed. With respect to HC-T it is not believed that protonated carbonyls are responsible for the observed anion-exchange. If the carbonyl group were protonated at the pH of our experiments then acetophenone as a ketone would be protonated and would experience varying retention as a function of the anionic strength of the buffer similar to the basic analytes. Contrary to that, our chromatographic results show that the retention of

acetophenone remained constant with varying phosphate anion concentration in the range of 1.0 to 20 mM.

At this stage the experiments clearly establish the formation of positively charged functionalities in the course of the acid treatment of the HC-T phase. However the chemical nature of these functionalities has not been identified.

## 3.4 Conclusions

The toluene-derivatized hypercrosslinked phase (HC-T) synthesized in Chapter 2 exhibits comparable chromatographic behavior to that published in the literature in both normal and reversed-phase mode. The final step of the column synthesis involves an aggressive acid treatment that is intended to condition the column and prevent further alterations in its properties under routine use. Nevertheless, our results indicate that the HC-T column continues to undergo changes in its chromatographic behaviour even after the initial acid conditioning. The attenuated retention on HC-T as a result of its continuous exposure to the extremely acidic conditions and high temperature caused chemical modifications to the stationary phase rather than phase loss as is the case with conventional RPLC bonded phases. The decrease in retention as a function of the acid treatment was more pronounced for cationic analytes than for neutral analytes. These retention changes are due to a build-up of a cationic functionality in the stationary phase during the acid treatment. The positive charge on the column has been confirmed by the anion-exchange mechanism of retention of selected anionic analytes.

Excellent correlation has been observed between the column retention behaviour and an anion-exchange model, but the chemical nature of the cationic sites generated during the acid washing remains unknown.

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# CHAPTER FOUR: Conclusions and Future Work

## 4.1 Conclusions

This thesis explored the stability of a silica-based hypercrosslinked stationary phase intended for use the under the extremely aggressive conditions of the High Temperature Liquid Chromatography (HTLC). The toluene-derivatized hypercrosslinked phase (HC-T) used in this study is designed to be resistant to bonded phase loss at low pH and elevated temperatures.

In Chapter 2 the reproducibility of the HC-T synthesis was estimated by comparing the results obtained herein with the literature. Two batches of the HC-T phase were prepared in this study and their elemental composition was monitored during the synthesis. The first HC-T batch showed comparable results with those previously published while the second batch demonstrated even better consistency with the literature. Further, the results of the accelerated acid aging tests indicated that the HC-T column that had been acid-conditioned became more resistant to phase loss. Upon exposure to an additional set of 1500 column volumes of acid treatment the conditioned column lost only 4% of its carbon content. It should be noted that the acidity in the acid aging tests far exceeds the acidity under practically relevant conditions. Therefore, the stationary phase loss during the routine use of the column is expected to be negligible. The acid treatment has also been shown to reduce the tin contamination introduced by the catalyst during the stationary phase synthesis. However, even after a prolonged

exposure of the HC-T column to the extremely low pH and high temperature the tin impurities remained significant for chromatographic silica.

Chapter 3 briefly demonstrated that the HC-T phase synthesized herein exhibits the unique selectivity in Normal Phase Liquid Chromatography (NPLC) that had been observed on columns synthesized by Carr and co-workers. HC-T phases prepared herein have been used in our group for solvatochromic characterization of HC-T under NPLC conditions.

The primary focus of this work was to characterize the performance of the HC-T column in Reversed-Phase Liquid Chromatography (RPLC) after its prolonged exposure to acid and temperature extremes. Chapter 3 showed that the chromatographic performance of the HC-T column in RPLC is strongly dependent on the extent of the acid treatment. The attenuated retention for neutral analytes was attributed to decreased phase hydrophobicity caused by the hydrolysis of the siloxane bonds into silanols and the conversion of the methylene bromide groups into hydroxyls, and to a lesser extent to phase loss. These two factors also played a role in the decreased retention for cations. However, the more pronounced decrease in the cation retention was a consequence of the formation of a positive charge in the stationary phase during the acid aging. Due to mutual repulsion between the cationic analytes and the positively charged sites in the stationary phase the cations were partially or fully excluded from the pores of the stationary phase. The presence of a positive charge in the stationary phase has been confirmed by the chromatographic behaviour of anionic analytes whose retention pattern followed an established anion-exchange model.

In an attempt to identify the origin of the positively charged functionalities on the HC-T phase several hypotheses previously expressed in the literature were reviewed. The one that proposes adsorption of anions to metal contaminated silica has been found to be invalid for the HC-T phase. Our experiments have demonstrated that flushing the HC-T column with EDTA removes the metal impurities from the stationary phase. This was evidenced by the significantly reduced peak tailing of a chelating test compound. Under analogous conditions, no noticeable difference in the anion retention was observed after the EDTA treatment. This indicates that the source of the anion-exchange interactions is other than metal contamination.

Another hypothesis proposed that the origin of the positive charge is an amine introduced with the catalyst during the silanization reaction. However, similar anion-exchange character was observed in the literature for a purely polymeric hypercrosslinked phase whose synthesis did not use any amines. The syntheses of HC-T and the purely polymeric phase involve analogous crosslinking reagents which determine the similar chemical structure of these two materials. Therefore it is not believed that an amine is involved in the anion retention observed on HC-T. The similarities in the chemical structure and chromatographic behaviour for these two materials suggest that the positively charged sites are located in the polymer layer on the HC-T rather than on the silica support.

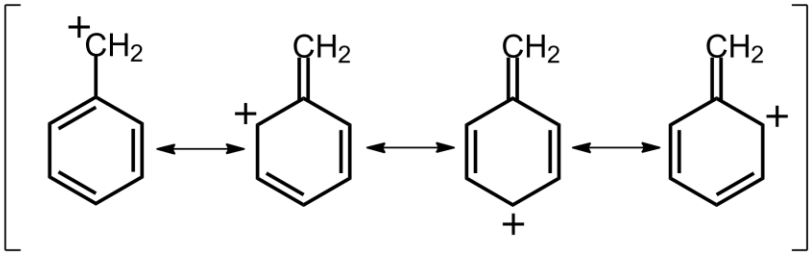
In an attempt to identify the chemical nature of the positively charged sites on the purely polymeric phase Penner and Nesterenko proposed that protonated carbonyl groups are responsible for the anion-exchange properties of this material.

However, no mechanism of the carbonyl formation has been suggested and no evidence has been presented in support of this hypothesis. Also, if the presumed carbonyl groups were protonated then the ketone used in our chromatographic experiments would have been protonated too and would have behaved similarly to the cationic analytes. On the contrary the retention of acetophenone was found to be independent of the buffer ionic strength, unlike the cationic analytes. In Chapter 3 it has been demonstrated that the buffer ionic strength has a significant effect on the retention of cations due to ionic screening and competition for the anion-exchange sites.

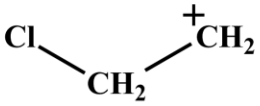
## 4.2 Future work

The key objective of Chapter 2 was to reproduce the synthesis of the HC-T stationary phase. However, some aspects of the synthesis as described in the literature warrant comment. As specified in Section 2.3.2.2 and 2.3.2.3 the solvent used in the primary and the secondary crosslinking reactions was dichloroethane (DCE). The use of DCE as solvent can be a concern since this compound may also act as an alkylating agent for aromatic systems through the same Friedel-Crafts reaction mechanism as the main crosslinking. DCE was used by Rabek *et al.* as both reagent and solvent to crosslink polystyrene at 50 °C for 1 h [1]. In comparison, the primary crosslinking of HC-T was conducted at 80 °C for 15 min (Section 2.3.2.2). In an earlier study, dichloromethane (DCM) which is poorer alkylating agent than DCE was successfully used for benzene crosslinking [2].

Similar to the above, DCE can react with and crosslink triphenylmethane used in the primary crosslinking of HC-T. This reaction can take place simultaneously with the main crosslinking between triphenylmethane and the benzylchloride ligands on the silica surface. Thus triphenylmethane, which is designed to crosslink the bonded ligands, may itself be crosslinked by the DCE solvent. This side reaction is less favourable due to the formation of the less stable primary carbocation intermediate compared to the resonance stabilized benzyl carbocation formed in the course of the main reaction (Figure 3-1).



benzyl carbocation formed in the main crosslinking reaction
(stabilized by charge delocalization)



ethylenechloride carbocation formed in the side crosslinking reaction
(less stable)

**Figure 4-1.** Carbocation intermediates formed in the course of the main and the side crosslinking reactions

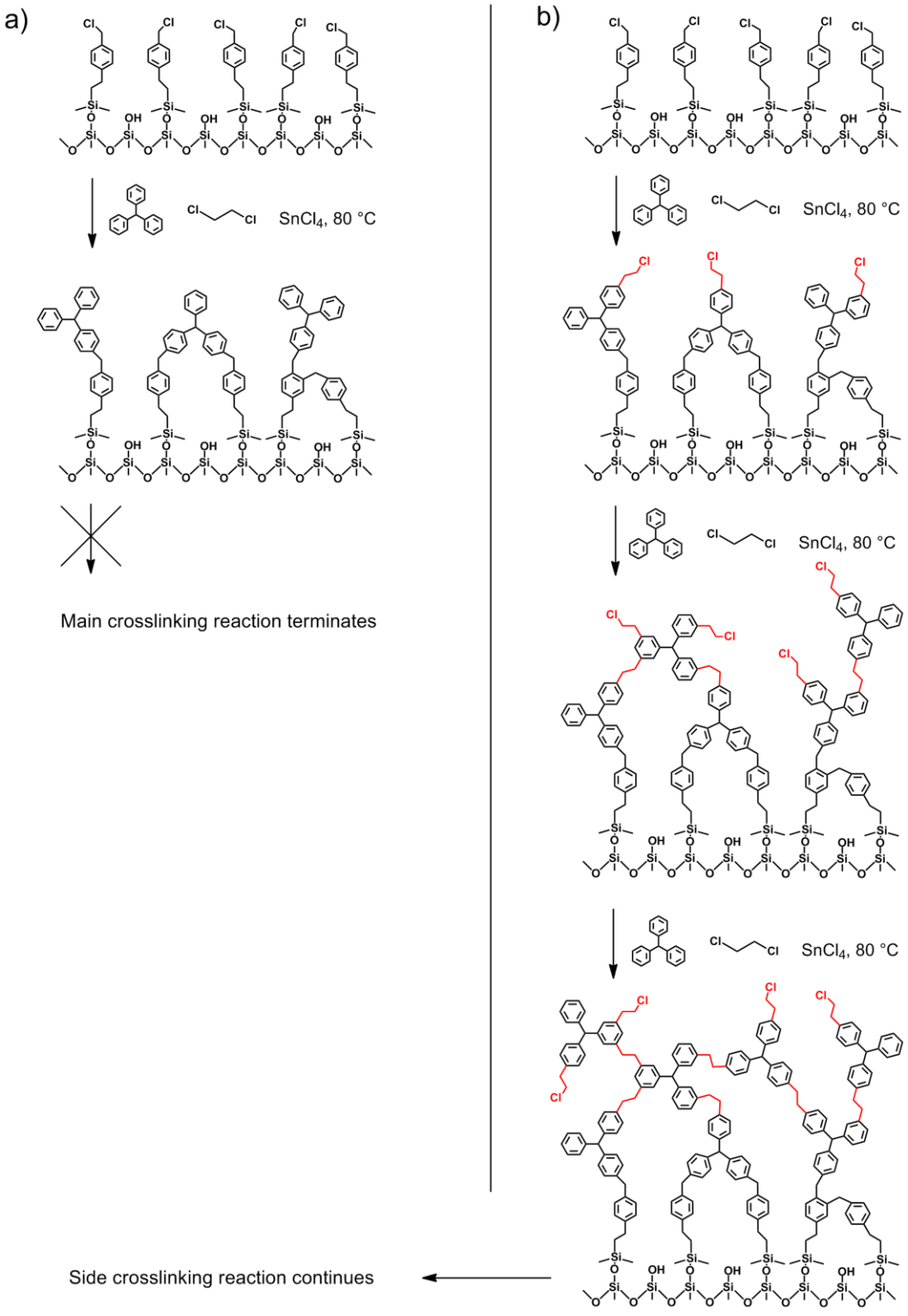



**Figure 4-2.** Products of the main crosslinking reaction (a) and the side crosslinking reaction (b)

The amount of the benzylchloride ligands available for crosslinking is 12.5x $10^{-3}$ mmol *vs.* 32 mmol triphenylmethane and 1300 mmol DCE. Given this molar disparity, the product of the side reaction, although less favourable, may become comparable or even exceed that of the main reaction. In addition, the elevated reaction temperature (80 °C) may facilitate the undesirable triphenylmethane crosslinking with DCE. As a result of these two reactions occurring simultaneously, the stationary phase may grow in thickness by repeatedly adding new chloroethyl groups to the stationary phase, followed by the attachment of new triphenylmethane molecules through the same Friedel-Crafts reaction mechanism as illustrated in Figure 3-2.

Alternatively, some triphenylmethane molecules can first be crosslinked with the solvent in the bulk solution to form oligomers (Figure 3-3). These oligomers might consequently be attached to the bonded phase:

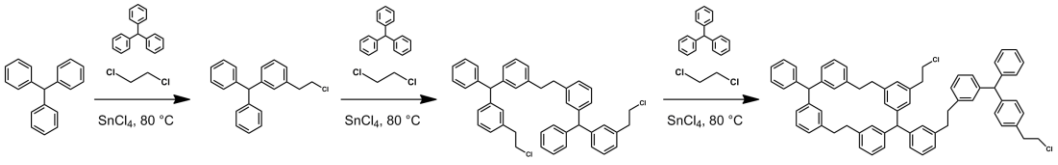



**Figure 4-3.** Crosslinking of triphenylmethane with dichloroethane in the bulk solution

Poor control over the synthesis of the bonded phase may produce a thicker hypercrosslinked polymer layer which can ultimately increase stationary phase resistance to mass transfer. Whether or not these considerations have an impact on the performance of the hypercrosslinked phases remains to be determined by

performing the primary crosslinking reaction in a different solvent. Nevertheless, the side reaction is not expected to have a dramatic effect on the stationary phase properties in RPLC. As demonstrated previously, the HC-T column exhibits comparable efficiency with commercial reversed-phase columns [3]. In addition the synthesis of the hypercrosslinked polymeric layer was shown to not cause pore plugging [4].

A future project may explore further modification of the HC-T aimed at improving its chromatographic properties and robustness. The residual silanols on the silica surface in HC-T can be converted to silicon hydrides similar to the modification performed by Sandoval and Pesek (Figure 4-4). Silanols can first be reacted with thionyl chloride ($SOCl_2$) and converted into silicon chlorides which can consequently be reduced with $LiAlH_4$ to silicon hydrides. In addition, the methyl hydroxyl groups that are produced as a result of the hydrolysis of the residual methylene bromides as well as the silanols formed in the hypercrosslinked layer following the hydrolysis of the siloxane bonds are also expected to be reacted in the same manner as in the above reaction. If successful this modification can restore the hydrophobicity of the HC-T and can also eliminate the undesirable analyte interactions with the silanols. Owing to the remarkable stability of the hypercrosslinked polymeric layer it is not expected that treatment with such aggressive chemicals as $SOCl_2$ and $LiAlH_4$ will damage the stationary phase.

Another idea that might also be explored is the formation of carbocations, incorporated into the HC-T hypercrosslinked layer.

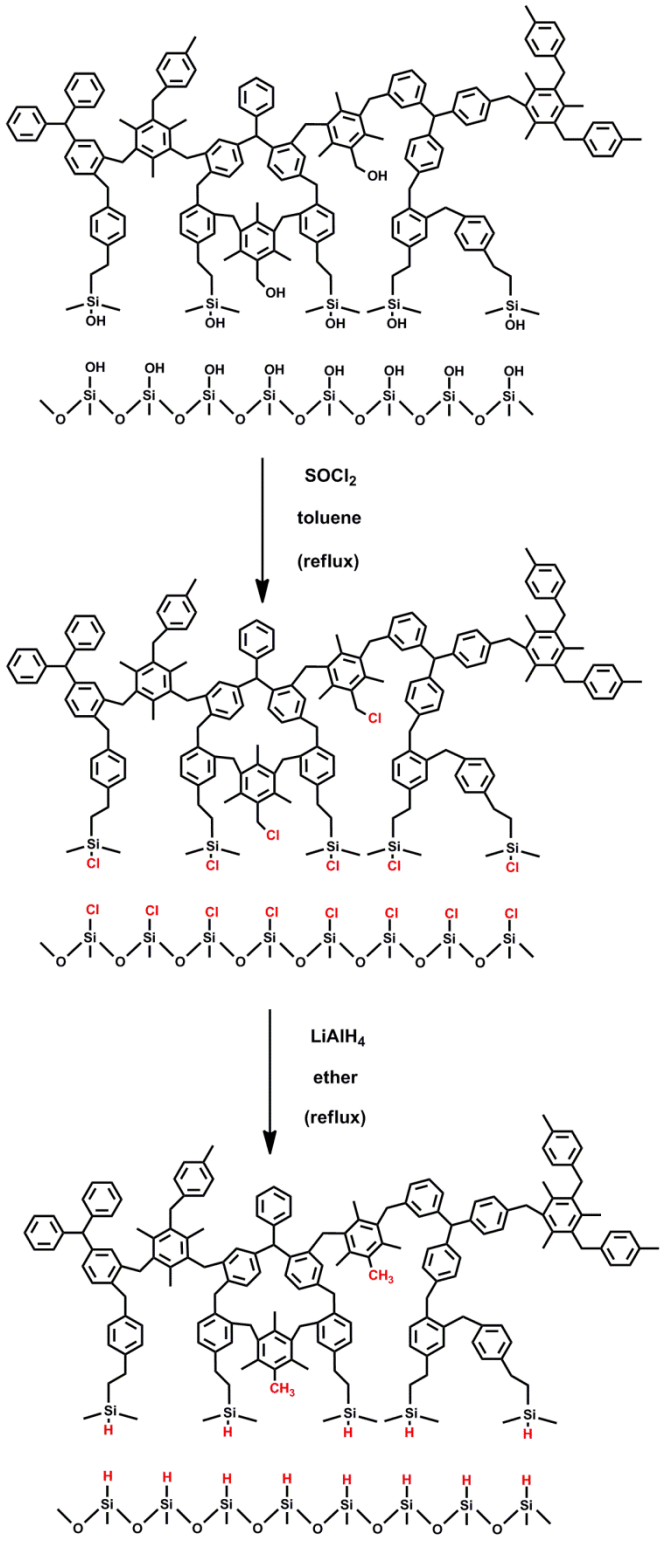



**Figure 4-4.** HC-T modification to remove residual polar groups

Future studies based on this hypothesis will focus on the presence of the trityl cation (Figure 4-5). Owing to its very high stability this tertiary carbocation exists as a salt like compound that can possibly act as an anion exchanger. This chemical species is suspected to be present in the stationary phase due to its structural similarities to triphenylmethane which was used as primary crosslinker in the HC-T synthesis. Thus, triphenylmethane can be considered as a precursor for the generation of the trityl cation. However, at present no mechanism for its formation can be proposed.

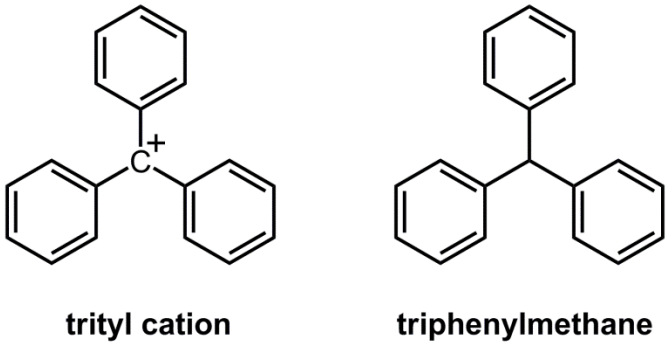



**Figure 4-5.** Chemical structures of the trityl cation and triphenylmethane

## 4.3 References

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