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Understanding and manipulating the separation in hydrophilic interaction liquid chromatography-a review.

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Highlights:

- Hydrophilic interaction chromatography is reviewed over the past 10 years.
- Parameters controlling retention are considered.
- Stationary phases, classification, and evidence for a surface water layer examined.
- Importance of organic solvent, buffer pH and strength, temperature also assessed.
- Injection and detection techniques in HILIC appraised.

Abstract

Hydrophilic interaction liquid chromatography (HILIC) has emerged as a valuable complimentary technique to reversed-phase (RP), being especially suited for the analysis of polar and ionised solutes, which are difficult to retain in RP. For solutes amenable to both separation mechanisms, HILIC provides a different selectivity to RP, and also offers possibilities as an orthogonal mechanism for 2-dimensional LC when combined with RP. HILIC has further advantages of lower column back pressures, and increased sensitivity with mobile phase evaporative detectors such as electrospray mass spectrometry. This review covers progress in our understanding of the HILIC technique, principally over the last ten years, including the classification of columns, the factors that control retention and selectivity, and attempts to model the separation process and its kinetics.

Keywords: hydrophilic interaction chromatography; HILIC; retention; selectivity; additives.

1. Introduction

It is now over 25 years since Alpert introduced the term “hydrophilic interaction chromatography” (HILIC) to describe a liquid chromatographic technique where polar or ionised solutes can be separated on a polar stationary phase with polar solvents containing water as a minor constituent of the mobile phase [1]. Although HILIC or HILIC-like analyses had been performed for many years previously [2], Alpert suggested clearly that the mechanism of these separations involved partitioning of solutes between the bulk mobile phase and a layer enriched with water and partially immobilised on the stationary phase surface. He also recognised that superposition of ionic effects could occur, depending on the solute, stationary phase and mobile phase. HILIC has undergone an upsurge in interest since the landmark review of the technique by Irgum in 2006, in part due to its numerous applications for the analysis of solutes of pharmaceutical, biomedical and clinical analysis, for which the technique is often suitable [3]. The advantages of HILIC include the ability to retain polar and ionic solutes that elute too readily in reversed-phase (RP) analysis, and the often completely different selectivity that is obtained in comparison with RP-LC. For instance, a mixture of peptides when analysed by the two techniques showed almost no correlation of retention, indicating the useful orthogonality of the methods (see Fig. 1, [4]). This difference in selectivity is also useful for separations using comprehensive 2- dimensional liquid chromatography when combined on-line with RP [5]. HILIC mobile phases typically contain high concentrations of acetonitrile (60-97%) and low concentrations of water (3-40 %), resulting in the advantages of low viscosity and small back pressures, even with relatively long columns [6]. In some cases, improved peak shape can be obtained for basic solutes analysed by HILIC compared with RP. This advantage may be connected with improved loading properties of some types of HILIC stationary phase [7] . Furthermore, improved detection sensitivity can be obtained in systems where evaporation of the mobile phase is employed.

The purpose of the present paper is to review current understanding of the mechanism of HILIC separations, those parameters that can be adjusted in order to manipulate the selectivity, and the various approaches that have been taken to modelling the separation. It will concentrate on work carried out over the last 10 years, approximately the period since the publication of the excellent and comprehensive Irgum review. As applications of HILIC in pharmaceutical analysis have recently been reviewed [8], and its application to amino acids peptides and proteins [9], proteomics [10] and in metabolomics [11] have also been covered, the numerous applications of the technique will not be

detailed again here. Other more fundamental reviews in the last 10 years have concentrated on aspects of HILIC such as mechanisms [12, 13], stationary and mobile phases [14, 15], column efficiency [16], and the effect of temperature [17]. The considerable advances that have been made in understanding the fundamental mechanisms operative in HILIC will be discussed; these advances have helped to improve and widen its application to the solution of a number of separation problems.

2. The Column

2.1 Stationary phase substrates; particulate and monolith columns; broad groupings of phases according to chemical structure.

Silica, and silica-based materials, remain by far the most commonly used in HILIC separations. Nevertheless, a number of new substrates have been proposed as suitable materials for HILIC columns. Porous graphitic carbon (PGC) is potentially a suitable material due to its chemical and thermal stability and unique selectivity, which is different to that of silica. Carbon nanoparticles can alternatively be deposited on the surface of silica [18]. PGC is strongly hydrophobic and can give excessive retention in some cases, so various ligands have been bonded to the material to alter the surface polarity [19, 20]. A synthetic diamond phase has been developed for HILIC [21]. Titania also has the advantage of pH stability over a much wider range than silica. It acts as an anion exchanger at low pH and a cation exchanger at higher pH with isoelectric point at pH 5. Ti (IV) atoms have strong Lewis acid sites and can undergo interactions with anions such as borate, carboxylate, sulfate and organophosphate groups of solutes such as nucleotides [22]. The separation of N-methylated xanthenes on a commercial native titania column was reported using ACN-ammonium acetate [23]. The plot of retention factor vs volume fraction of water was a U shaped curve, with increased retention at both low (typical HILIC conditions) and high concentrations of water (see Fig. 2). In the latter case, it is likely that some kind of hydrophobic retention occurs on the stationary phase surface, a phenomenon that also occurs with bare silica [7, 24, 25]. The efficiency of the titania column was found to be inferior for the separation of xanthenes in the HILIC mode to that obtained on silica. The same authors previously reported the separation of xanthenes on a zirconia coated silica monolith column, although peak shapes for some of the solutes were rather poor. While these alternative substrates present some interesting selectivity differences, and have pH stability advantages, silica-based materials often yield better peak shapes, and benefit from a more extensive understanding of their properties. It

therefore seems likely that silica columns will dominate HILIC separations for the foreseeable future.

The majority of studies in HILIC have used particulate- based columns, presumably as the advantage of low back pressure of monoliths, due to their large through pores, is of less importance when considering the low viscosity of HILIC mobile phases, which generate relatively low pressures even in quite small particle diameter columns. Separations using both polymer and silica monoliths in the HILIC mode were reviewed in by Ikegami et al. [16]. Tanaka notably used a long (4 m) capillary HILIC monolith, with the stationary phase modified with a urea functionality, to separate peptides, generating an efficiency of 300,000 plates, exploiting the combined effect of low pressure operation of monoliths and low HILIC mobile phase viscosity. It was claimed that the efficiency per unit length of the monolith was about the same as that of a commercial zwitterionic column packed with 3 μm particles [26]. There is clear potential for the application of long capillary monoliths to effect difficult separations requiring high column efficiencies.

A great variety of ligands have been bonded to silica, the structure and properties of the resulting stationary phases have been extensively reviewed by Jandera [14] and by Guo and Gaiki [15]. HILIC stationary phase that have been developed in the last 5 years, together with their applications, have also been listed recently by Xu and co-workers [27]. Thus, the considerable number variety of these chemically bonded phases will not be documented again in detail here. However, HILIC phases can be divided intuitively into broad groups based on their chemical structure, which include neutral (e.g. amide, cyano, diol), positively charged (e.g. amino, imidazole, triazole), negatively charged (e.g. polyaspartic acid, bare silica) and zwitterionic (e.g. sulfobetaine or peptide) [15]. The structure of some of these common bonded phases as detailed by Guo and Gaiki is shown in Fig.3. While bare silica demonstrates cation exchange properties due to the dissociation of silanols, detailed classification studies (see below) often consider it as a separate class of stationary phase [28]. Of the neutral columns, it was stated [15] that cyano and diol columns are much less useful due to lack of retention, which can be partially attributed to lack of hydrogen bond donator capability and reduced hydrophilicity in the former case. The most popular zwitterionic phase is the sulfobetaine type with a quaternary ammonium group (proximal position) separated by a short carbon chain from a sulfonic acid group (distal position) in equal quantity, leading to weaker ionic interactions than conventional ion exchangers. However, sulfobetaine phases have been found to possess a slight overall negative charge due, probably due to the accessibility of the sulfonic group at the distal end [29]. Alternatively, phosphocholine phases have been prepared which have the

positively charged ammonium group at the distal end giving alternative selectivity to the sulfobetaine phase, although these apparently did not show a net positive charge, as might have been expected.

2.2 Characterisation of HILIC stationary phases

The detailed characterisation of HILIC phases has been a goal of many researchers in the field of HILIC separations. Many studies (e.g. [30]) show beyond doubt that the stationary phase does not act as an inert receptacle to hold water for a partition mechanism, but itself plays an active role in the separation. For example, adsorptive and ionic interactions between the solute and the stationary phase can occur. Fig. 4 indicates the considerable selectivity difference of the separation of a mixture of acidic, basic, and neutral solutes on 5 different stationary phases run under the same conditions.

Solutes with different physico-chemical properties have been used to probe the various interactions that can be undergone in HILIC. The main mechanisms involved could be considered as adsorption on polar column groups; solute partition between the water layer and bulk mobile phase; and cation / anion exchange interactions. Ikegami and co-workers [31] attempted to classify 14 columns according to a comprehensive scheme involving the testing of a number of discrete interactions using 2 mM ammonium acetate buffer pH 4.7 in 90 % ACN, including: a) *tests of methylene selectivity* using alpha $k_{\text{uridine}}/k_{\text{5-Methyluridine}}$ The latter probe has an additional methyl group substituted in the pyrimidine ring making it less hydrophilic and less retained. b) *hydrophilic selectivity* with alpha uridine/2-deoxyuridine, with the former probe having an additional –OH group making it more hydrophilic and more retained c) *Discrimination of regio and configurational –OH isomers* with alpha vidarabine/ adenosine (isomers differing in the position of the –OH group to the ring d) *Shape selectivity* with alpha 4-nitrophenol α -D glucopyranoside/ 4-nitrophenol β -D glucopyranoside (axial or equatorial attachment of 4-nitrophenol to the sugar ring structure). e) *cation exchange selectivity* with alpha trimethylphenylammonium (quaternary cation) / uridine (neutral) f) *anion exchange selectivity* with alpha p-toluenesulfonate (strong acid anion)/ uridine. g) *stationary phase pH* with alpha theophylline/theobromine. This final test is interesting, arising from the work of Lämmerhofer et al, who tested a variety of different HILIC stationary phases with the xanthenes caffeine, theobromine (tb) and theophylline (tp) using ammonium acetate in 95% ACN [32]. It was noted that on amino phases, values of the separation factor $\alpha (k_{\text{tb}}/k_{\text{tp}})$ were less than 1 (i.e. tb emerges before tp) whereas on the zwitterionic phase ZIC-HILIC

and silica phases, the value was close to, or greater than one (ie no separation, or t_b emerges after t_p). The pK_a of t_b and t_p are 9.9 and 8.6 respectively [33], implying that t_p is an appreciably stronger acid, and will give up a proton to become a negatively charged anion more readily (at a lower pH). It was argued that this test indicates the surface acidity or basicity of a stationary phase. For example an amino phase could deprotonate t_p allowing formation of the complex $-\text{NH}_3^+ \dots t_p^-$, thus increasing the retention of t_p . A feature of this test is that the equilibration of stationary phases containing amino groups is slow, giving gradually decreased retention of t_p (but constant retention of t_b) over a period of 12 hours, when using an amino column with 90% ACN containing 2 mM ammonium acetate pH 4.7. The test was proposed as a measure of the equilibration state of the column. An alternative explanation might be that the amino phase generates an alkaline mobile phase in the microenvironment of the stationary phase, overcoming the rather weak buffering capacity of the mobile phase. This change in pH could cause the formation of negative ions only from the more acidic theophylline.

While the Ikegami procedure is a well-considered and comprehensive scheme, it is noticeable that the discrimination ability for some of the tests is small. For instance the range of alpha values for test C (region and conformational isomers) was 1.16 to 1.51 with 10 of the 14 columns in the range 1.16 to 1.36. The surface acidity test clearly distinguished amino columns from the rest, but for 13 other phases ranged from only 1.00-1.39. As with all tests of this kind, it is also difficult to select probes which test only a single property of the stationary phase. For instance, t_p and t_b are isomers which might experience different interactions in the system apart from probing acid-base properties.

McCalley used similar probes to estimate a more limited range of properties in measurements of the hydrophilic, anionic and cationic selectivity of various stationary phases [34]. Polymeric zwitterionic and amide phases such as ZIC –HILIC, TSK amide and BEH amide showed the highest hydrophilic selectivity, whereas bare silica phases the lowest. These results were in line with estimations of the different thickness of the water layer on various stationary phases published by Irgum [35] (see below). The cation exchange selectivity of a bare silica column (Waters Atlantis) was found to be much higher than that of most other phases, indicating that a silica phase will retain protonated bases to a much greater extent than neutral solutes of similar hydrophilicity, due to attractive interactions with negatively charged silanol groups. However, the cationic selectivity of hybrid organic-inorganic silica phases such as the Waters bridged ethyl hybrid (BEH) was much lower, due to reduction of the number of silanols in its structure. Indeed, a bonded Waters BEH amide phase based on this hybrid silica was found to have the lowest cationic

selectivity of all those tested [36]. The anionic selectivity of the phases was broadly the reverse, with zwitterionic and diol phases having the greatest anionic selectivity and bare silica the least, the latter due to repulsion from ionised silanols. However, phases synthesised with cationic groups were not tested in this study, and would be expected to have considerably greater anionic selectivity.

Irgum [28] characterised a number of HILIC stationary phases in terms of hydrophilic, hydrophobic, electrostatic, dipole-dipole, π - π , and shape selectivity interactions using principal components analysis (PCA) to evaluate the data. The method was based on the selection of pairs of test compounds which supposedly differed only in a single interaction mode, otherwise behaving identically, although it was acknowledged that finding such ideal pairs is virtually impossible. Solutes were also chosen so as to have low (preferably negative) logarithm of the octanol-water partitioning coefficient ($\log K_{ow}$), so that they had reasonable retention. The tests were all carried out in 80 % ACN containing 25 mM ammonium acetate pH 6.8. 1-ethylimidazole and 1-methylimidazole were chosen as the probes of methylene (hydrophobic) selectivity, as the diazole ring is polar enough to afford sufficient retention in HILIC and the compounds have similar pK_a . Hydrogen bonding was probed by the 3 solute pairs dimethylformamide/dihydroxyacetone, adenosine/adenine and dihydroxyacetone/methylglycolate, which all differ in the number and type of potential H bonds. For example, adenosine and adenine differ in the presence of the H-bonding possibilities of the ribose ring. Quaternary ammonium compounds were again used as probes of cationic interaction, as they remain charged irrespective of pH. The strong acid benzenesulfonic acid, and the weaker acids benzoic acid and sorbic acid were used to probe anion exchange. Cytosine was used as the reference for the electrostatic probes, because it is neutral under the test conditions while having a reasonable structural similarity to the ionogenic probes. Vinyl and ethylimidazole were used as probes of π - π interaction as they differ only by the double bond in the substituent attached to the ring. Dipole-dipole interactions were probed with the planar uncharged cis- and trans-diamminedichloroplatinum complexes, the former being the only isomer with a dipole moment. Shape selectivity was determined using sorbic acid/benzoic acid (differing in the size and shape of the hydrophobic part) and methylglycolate / α -hydroxy gamma butyrolactone (differing in the hydrophilic part of the molecule). A simplified score and loading plot from the PCA evaluation is shown in Fig. 5, based on the separation factors of all the test substance pairs but elimination of all but one of each of the anion and cation exchange markers. The columns fall into distinct groups: neutral phases such as diol (e.g. column numbers 10,11 in Fig. 5) and amide (7,8); amino

columns (19,20,21); bare silica phases (13-18) and zwitterionic phases (1-4). The columns were further divided into 4 slightly different groups according to their main selectivity a) Cation exchange characterised by unmodified silica; b) anion exchange exhibited by amino phases; c) dipole-dipole and multipoint H bonding exhibited by polymeric sulfobetaine and poly(2-sulfoethylaspartamide) and d) low specific interactions, exhibited by diol and amide phases. Furthermore, it was suggested that the selectivity of bare silica columns rely mainly on adsorptive rather than partitioning interactions, while the reverse was true for zwitterionic columns. Again, this finding suggests a correlation with the extent of the water layer on the phase surface in accord with the subsequent findings of Irgum [35] and those of Jandera [37] (see below).

Lucy and co-workers [38] attempted to simplify these comprehensive but rather complex classification schemes by plotting hydrophilic selectivity (measured from alpha cytosine/uracil) against (cat) ionic interaction (measured from alpha benzyltrimethylammonium cation/uridine). These two tests were considered to probe the most definitive properties of a HILIC stationary phase. It was stated that cytosine and uracil were both hydrophilic and structurally similar neutral solutes, but showed different HILIC retention. Although cytosine is a weak base (w^w $pK_a = 4.6$) it was argued that it would be an even weaker base (pK_a 1 or more units lower) in HILIC mobile phases which might contain e.g. 80 % ACN. Studies of the UV spectrum of cytosine over w^w pH values of 3.7-6.8 apparently showed no changes, indicating that this solute remained uncharged using the mobile phase conditions in the study. The authors found that the selectivity behaviour of most HILIC columns was dominated by silanol activity, and that minimal changes in selectivity were generally observed when varying the w^w pH between 5 and 6.8. Increasing the buffer concentration in this pH range reduced ionic interaction, as expected. At a pH below 5, decreasing cation exchange activity was observed due to reduction in the number of ionised silanols.

In general, these classification schemes have validated the intuitive division of stationary phases according to their chemical structure, in addition allowing some quantitative assessment of the selectivity differences between them.

2.3 The water layer on the column surface-does it really exist? Aqueous normal phase or hydrophilic interaction chromatography?

Most applications in HILIC use at least 2-3% water in the mobile phase. This situation is different from conventional normal phase (NP) separations where vigorous attempts are

often made to exclude water, as it is a powerful displacer in NP, and can give irreproducible retention of solutes results from variable trace quantities in the mobile phase. McCalley and Neue demonstrated the existence of the water layer indirectly by measuring its effect on the exclusion of simple hydrophobic compounds such as benzene and toluene [34]. As these compounds have limited solubility in water, it can be assumed that they partition almost entirely into the bulk mobile phase and do not penetrate the water layer. The difference between the retention volume of the probe in pure ACN and in an aqueous-ACN mobile phase can be used to estimate the proportion of the pore volume occupied by water. Tallarek used molecular simulation dynamics to explore the surface region of a silica phase in contact with ACN-water mixtures of varying composition. This treatment identified three regions close to a silica surface. These are the immediate surface region at a distance up to ~ 0.5 nm which contains almost exclusively water molecules hydrogen bonded to surface silanols and only a few ACN molecules; an interface region extending up to 1.5 nm from the surface where there is initially a high density of water molecules that relaxes gradually into the third region beyond 1.5nm, whose density profile matches that of the bulk liquid [39] (see Fig. 6).

Irgum and co-workers [35] measured the uptake of water directly on 12 different porous silica stationary phases by suspending the dried particles in ACN: water mixes from 70-99.9 % ACN for an hour with shaking, and measuring the loss in water in the supernatant liquid using the Karl-Fischer titration method (see Fig.7). Neat silicas and monomeric grafted silicas showed water uptake (in mg/m^2 of material) that indicated formation of a monolayer was complete at about 5% water in ACN, and of the subsequent multilayers at about 20% water in ACN. Conversely, polymeric grafted phases were not saturated until the mobile phase water concentration was in the range 25-30 % water in ACN. It might be that the resulting increase in water content of the stationary phase in the latter case is mostly due to increased swelling in the hydrogel layer than being due to the more limited involvement of silanol groups. The estimated pore occupancy by water when suspended in a mobile phase of 80% ACN in water containing overall 5mM ammonium acetate pH 6.8 ranged from ~ 7 -9 % for bare silica columns, $\sim 12\%$ and 14.5% for monomeric diol and amine columns respectively, to 20-25% for polymeric amide, zwitterionic and sulfoethyl columns. Soukup and Jandera [37] also showed that the uptake of water strongly depends on the polarity and type of stationary phase. They determined the excess water uptake from ACN rich mobile phases by frontal analysis followed by coulometric Karl-Fischer determination of water in the column effluent. The authors stressed that there can be no strict boundary between a water layer and the bulk mobile

phase, as ACN is infinitely miscible with water. Very little water was adsorbed on silica hydride phases (so-called "Type C" silica) or pentafluorophenyl phases and relatively small volumes were observed on bare silica or hybrid silica. For this reason, the mechanism of interaction of silica hydride phases has sometimes been termed "aqueous normal phase chromatography" (ANP) [40-42]. ANP may not be a discrete separation mechanism. The term may possibly reflect a different balance of the various contributions such as adsorption, partition and ionic retention, with a greater contribution of the adsorption mechanism. The greatest water uptake was observed on a zwitterionic stationary phase. Columns with bonded hydroxyl and diol ligands showed greater water adsorption compared with bare silica (see Fig. 8). Results were in broad agreement with those previously published by Irgum. Significant correlations between water uptake and the selectivity of the various phases were shown.

3. The mobile phase

3.1 *The organic modifier*

Although some authors have studied alternative organic modifiers (see below), ACN is by far the most widely used in HILIC separations. ACN also has the advantage of good UV transparency, even at low wavelengths. Decreasing the concentration of water in the mobile phase gives considerable increases in retention, attributable to the decreased partition of hydrophilic solutes into the mobile phase and/or increased adsorptive interactions between the solute and the stationary phase. Some changes in selectivity have been reported when the organic modifier concentration is changed. For example, changing the concentration of ACN from 85% to 95% with ammonium formate buffer pH 3.0 gave correlations for the analysis of a mixture of acidic, basic and neutral test compounds of between 0.8-0.9 [36]. It is possible that these selectivity differences result from a change in the relative contribution of adsorption (which is likely to be greater in water-lean mobile phases) and partition (likely to be greater in water rich mobile phases). However, changes in the s° pH of the mobile phase (its true thermodynamic pH measured in the aqueous-organic mobile phase) due to the change in the ACN concentration might also contribute to differences in selectivity, at least for ionisable solutes.

A number of authors have investigated the effect of the use of alcohols such as methanol, ethanol and isopropanol as full or partial substitutes either for the ACN or the water content of the mobile phase. Methanol is clearly a stronger solvent than ACN in admixtures with water. Alternatively, alcohols have weaker elution strength than water in

HILIC, thus their substitution for water should result in increased solute retention. Tallarek and co-workers extended previous modelling studies to investigate the effect of methanol on the water layer on silica surfaces carrying diol functionalised alkyl chains and residual silanol groups [43]. In contrast to water–ACN mixtures, it was shown that methanol-water mixtures fail in HILIC because the similar affinity of a silica surface for water and methanol prevents preferential adsorption and formation of a water layer [44]. Lindner and co-workers showed low retention and no separation for uracil/uridine on an oxidised thioglycerol phase with an eluent of methanol/20 mM aqueous ammonium acetate (95:5, v/v), whereas good retention and separation was obtained by switching to ACN [45]. Hao et al reported that when multiple polar functional groups contribute to analyte polarity, hydrogen bonding will be important in most HILIC columns, and this hydrogen bonding capability can be weakened by switching from ACN to methanol [17, 46]. They showed that the retention and separation of epirubicin and its analogues on a bare silica column increased in the order:

methanol < isopropanol < tetrahydrofuran < ACN

with ACN as the weakest solvent.

For the separation of tetracyclines on an amino-bonded silica column, Li et al. [47] reported that retention times increased in the order

tetrahydrofuran < methanol < isopropanol < ACN.

Using a set of nucleobases, nucleosides and deoxynucleosides with ACN-rich mobile phases (90 % v/v) containing 5mM ammonium acetate, Lindner [48] exchanged (all of) the water content of the mobile phase for various alcohols, thus decreasing the eluotropic strength of the eluent. The gain in retention largely followed the order:

water < 1,2-ethanediol < methanol < ethanol

and was accompanied by distinct effects on chromatographic selectivity.

Irgum and co-workers showed that the best overall performance from a HILIC-ICP-MS system was achieved with 1,4-dioxane as the organic component of the eluent, being a compromise between lower carbon deposition in the plasma but at the expense of a 50% drop in column efficiency [49].

McCalley and co-workers [36] compared the retention of a solute mixture in ACN-MeOH-ammonium formate pH 3 buffer (85:10:5v/v) and ACN-buffer (85:15) i.e. 10 % of the water concentration was substituted by methanol in the former case. The retention of solutes was indeed increased, particularly for basic solutes, whereas neutrals were much less affected, and small selectivity changes resulted. Again however, changes in the s^s pH of the mobile phase may be a contributory factor.

The use of detectors other than UV has allowed some investigations of the suitability of other potential solvents such as acetone. For instance, using a charged aerosol detector (CAD) acetone was shown to have a higher eluotropic strength than ACN [50], which was attributed to the better hydrogen bonding ability of this solvent. Using the same detector, Hutchinson et al. [51] investigated acetone, isopropanol (IPA), ethanol and methanol as alternative organic solvents in the analysis of sugars by HILIC using a polymeric amino phase column. The availability of high pressure instrumentation allowed the use of higher viscosity solvents like IPA. Judging by the volume fraction of organic solvent necessary to produce roughly equal retention of the test compounds, retention increased in the order:

methanol < ethanol < IPA < acetone < ACN

These results were mostly in agreement with those of the other studies above. Column efficiencies were generally inferior when using these alternative solvents in place of ACN. For example, the number of theoretical plates for maltose in ACN was about twice that for acetone, which produced the next highest efficiencies.

In summary, while the use of alternative solvents can give some changes in both solvent strength and selectivity, aqueous ACN mixtures remain the mobile phase of choice in HILIC.

3.2 Buffers and pH of the mobile phase.

Buffers are used in HILIC to stabilise the charge of ionogenic groups on the stationary phase as well as on the analyte. The majority of separations are carried out using ammonium formate and ammonium acetate buffers. These salts cover at least part of the usable pH range of most HILC columns, are soluble even in high concentrations of ACN, and are volatile for use in ESI-MS interfaces. Simple acids like formic acid, acetic acid and even phosphoric acid (which surprisingly is soluble at 0.1 % v/v concentration in aqueous ACN) act as reasonable buffers in aqueous solution and have also been used in HILIC,

although these do not always have sufficient ionic strength in high concentrations of ACN to give good peak shape (see below) [7, 52]. Li et al [47] compared the use of sodium formate-formic acid, sodium phosphate-phosphoric acid, sodium oxalate-oxalic acid and ammonium citrate–citric acid for the separation of tetracyclines at 6.7 mM concentration and w^w pH 3.0 in 85 % ACN. The nature of the buffer was found to alter the retention of the compounds but the selectivity of the separation of these solutes remained broadly unchanged. Heaton et al. showed that the use of citrate buffers gave improved peak shape for the separation of catecholamines, as it possibly reduces the detrimental effect of metals in the system by their preferential complexation [53].

The effect of pH change over the range w^w pH 3.0 to w^w pH 6.0 was investigated by McCalley and co-workers [36], using ammonium formate and ammonium acetate buffers at 5mM concentration and a number of different silica-based stationary phases. Above pH 6, it is possible that dissolution of silica might occur, especially in bare silica columns, whose surface is not protected by the presence of bonded ligands. As the pH was increased from 3 to 6, the cationic selectivity (relative retention of quaternary cations to neutrals) approximately doubled on all columns, attributable to increasing ionising of underlying silanols as the pH was raised. A much steeper increase was recorded over the pH range 4.5-6.0 compared with pH 3.0-4.5, probably indicative of the onset of more substantial silanol ionisation. In contrast to this behaviour shown by quaternary salts and strong bases, the retention of weak bases generally decreased as the pH was raised, attributable to decrease in attractive ionic interactions with the stationary phase as the solute is deprotonated at higher pH. The retention of ionised strong acids decreased as the mobile phase pH was raised, attributable to increased repulsion of these negatively charged acids from ionised silanols. For weak acids, the effect on retention as the pH is raised appears to be a combination of the increased retention of solutes as they become more ionised (and more hydrophilic) at higher pH, together with increased repulsion from the stationary phase silanols. However, the retention of acids as a function of pH deserves further study. As expected, the retention of neutral compounds was much less affected by pH change. Nevertheless, changes in the ionisation of bonded groups or underlying silanols could affect the thickness of the water layer on the column surface and thus solute retention.

More recently, retention studies have been carried out at lower pH using trifluoroacetic (TFA), heptafluorobutyric acid (HFBA) and methane sulfonic acid (MSA) as additives to the mobile phase [54, 55]. Using 0.1 % TFA or HFBA in the aqueous ACN mobile phase gave apparent anion exchange behaviour of bare silica, hybrid silica and

amide bonded columns, which contrasted with the cation exchange properties generally shown with ammonium salt buffers. Thus, the retention of strongly acidic solutes was increased relative to that of strongly basic solutes, some of the latter even being excluded from the stationary phase (retention times lower than the column void volume). Reduction of the retention of bases might be expected due to ion pairing of bases with the acid anions, which might reduce solute hydrophilicity and ionic retention, but not produce exclusion. The differences in selectivity between an Agilent glycan (amide) column operated in ammonium formate w^w pH 3.0 and in 0.1 % TFA are illustrated in Fig. 9. The same results were obtained on stationary phases from different manufacturers, so it does not seem likely that the behaviour results from some unusual method of column preparation. It is possible that the silica surface becomes positively charged at the much lower pH of TFA and HFBA solutions when measured in the aqueous- organic mobile phase (w^s pH) or when corrected to the true thermodynamic s^s pH [56] (see Fig. 10), giving ionic attraction of acidic solutes but repulsion of bases. It was shown that the positive charge is unlikely to originate from the presence of metals in the system [55] or from protonation of the stationary phase ligands. The point of zero charge of silica has been reported as pH 2-3 [57], which is in the region of the pH of TFA solutions even in high concentrations of ACN (Fig. 10). It is possible that hydronium ions are immobilised in the water layer or that the positive charge resides elsewhere on the stationary phase surface. However, much more evidence is necessary to establish the true cause of this effect. For example, operation of the same columns in aqueous ACN solutions containing MSA (which gives rise to a lower s^s pH) do not show this anion exchange effect. It is feasible that the higher ionic strength of MSA solutions could explain this behaviour, although other explanations are possible. For example, acid hydrolysis of ACN by TFA could produce charged artefacts that may somehow contribute to the retention of acidic solutes [58]. Furthermore, there could be some unexplained interactions occurring between TFA and the acidic probes used.

Some columns, such as the Waters hybrid amide phase, are claimed to be stable at higher pH than is attainable with ammonium formate or acetate buffers. Use of this column at w^w pH 9 resulted in changes in selectivity compared with the normal pH range [54]. Quaternary ammonium compounds showed high retention under these conditions, presumably due to increased ionic interactions due to further ionisation of silanol groups. Moderately strong bases such as nortriptyline, diphenhydramine and procainamide showed reduced retention compared with at w^w pH 6, presumably due to decreased solute ionisation. The retention of strong acids decreased further at w^w pH 9, due to repulsive

interactions. Reduced retention of weak acids such as 4-hydroxybenzoic acid was also shown, presumably because these are already mostly ionised at w^w pH 6, leaving ionic repulsion to affect retention.

Clearly, the mobile phase buffer and its pH can have a profound effect on the selectivity of the separation in HILIC.

3.3 Buffer concentration and ionic strength

McCalley and co-workers investigated the effects of increasing the ammonium formate buffer (w^w pH 3) concentration over the range 5-20 mM (overall) in 85 % ACN on the retention of a set of varied probe compounds using a variety of stationary phases. The retention of strongly basic solutes and quaternary compounds decreased substantially on all the silica-based columns (including neutral columns), suggesting that ionisation of silanols on the underlying material was at least partially responsible. Solute such as nortriptyline, which is relatively hydrophobic, showed a particularly strong effect, as its retention is governed mainly by these ionic processes [36]. The retention of strongly and weakly acidic compounds increased, attributed to screening of repulsion effects from negatively charged silanols. Clearly, ionic interaction effects (retention and repulsion) could be minimised at higher buffer concentrations, although they can give rise to useful selectivity effects. Neutral solutes showed increasing retention at higher buffer concentration, which can be attributed to the salt increasing the volume of the aqueous layer on the stationary phase. This effect can be demonstrated by using solutes such as toluene, which cannot penetrate the water layer and thus is increasingly excluded from the pores of the stationary phase as the buffer concentration is increased [34, 59].

West investigated the effect of buffer concentration over the range 2 to 20 mM on the retention of 76 probe analytes on zwitterionic columns using w^w pH 4 ammonium acetate in 80 % ACN [60]. Less polar neutral analytes showed a slow decrease in retention with increasing buffer strength, whereas more polar neutrals showed a continuous retention increase. Weak acids such as salicylic acid showed a similar increase in retention, whereas the retention of cations decreased rapidly up to 10 mM buffer concentration, then levelling off. The retention decrease of cations can be explained by the competitive electrostatic interactions between the more influential stationary phase distal sulfonic acid groups (and ionised residual silanols) and the buffer cations/analytes. As the buffer concentration increases further, hydrophilic partitioning becomes increasingly important. Anionic solutes face a continuous increase in retention with increasing buffer strength due

to the combined effect of reduced repulsive interactions with sulfonic acid groups and increased hydrophilic partitioning. Zwitterionic solutes were said to combine both effects.

The importance of the maintenance of sufficient ionic strength in the mobile phase was emphasised by McCalley and co-workers [52]. Peak shapes for ionised solutes in high concentrations of ACN were considerably worse when using formic acid as mobile phase additive at the usual concentration of 0.1 % (v/v) compared with ammonium formate buffers (e.g. at 5 mM). This result does not seem entirely attributable to the possible deactivation effect on ionised silanols of the ammonium ion, as peak shapes continued to be rather poor even when using 0.1 % phosphoric acid instead of formic acid, which has a lower pH that should be more effective in suppressing silanol ionisation. Instead, the poor results may be attributable to the low ionic strength of solutions of formic acid in high ACN concentrations (see Fig. 10). Note that 5 mM ammonium formate at w^w pH 3.0 has an ionic strength at least equivalent to the salt concentration at 5 mM/L [54].

3.4 Temperature

The effect of temperature and other variables in HILIC was reviewed by Hao et al. [17]. Increase in temperature generally decreases retention in HILIC (positive slopes of van't Hoff plots of $\ln k$ vs $1/T$), however these and other authors reported an increase in retention with increased temperature for some basic solutes on a bare silica phase (negative slopes of the van't Hoff plots [61]). It seems that attractive ionic interactions are involved in the interpretation of these observations, as negative slopes of the plots were also observed with acidic analytes and a basic aminopropyl column. Kumar et al [36] investigated the effect of temperature over the range 30-50 °C using a test set of acidic, neutral and basic solutes on 6 different HILIC columns. The results confirmed previous findings that neutral compounds and acids showed almost exclusively reduced retention at elevated temperature (although the study did not include an amino phase). Reduction in retention ranged from 8 % for a bare silica phase to 29 % for a diol phase. Increases in k for bases were found that ranged from ~ 3% on a zwitterionic column to ~16 % on a bare silica phase. No convincing explanation for the increase in retention of bases with temperature has been proposed. Temperature will have a complex effect on the ionisation of buffer components, silanol groups and basic analytes-and a similar effects on amino bonded phases with acidic solutes. Nevertheless, while changes in the relative retention of particular solutes indeed occurred with temperature change (e.g. in the Kumar study),

relative small differences in selectivity resulted over the temperature range investigated at least for the set of probe compounds employed [36].

3.5 Gradient elution and electrostatic repulsion hydrophilic interaction chromatography (ERLIC).

Gradient elution can be utilised in HILIC in the same way as it can in other modes of liquid chromatography-to reduce the retention window for a complex mixture of analytes [62]. Typically, the water content of the mobile phase is increased with time (which is clearly the opposite to that use in RP separations) in order to increase its polarity and reduce analyte retention. As in RP chromatography, peak capacity gains are proportional to the square root of the column efficiency. Thus, for the separation of glycans, transferring the method at constant gradient slope from 5 to 10 to 15 cm amide column produced modest increases in peak capacity of 41% and 73% [62]. HILIC is known to suffer from longer column equilibration times than RP; how this may impact on the reproducibility of gradient elution separations has not been studied in detail. In place of using gradients for peptide separations, Alpert proposed in 2008 [63] that an ion exchange column with charge of the same sign as the analytes could be used, where the mobile phase buffer concentration serves to moderate the repulsion that occurs between stationary phase and solute. Fig. 11a shows the simultaneous separation of basic and acidic peptides on a neutral PolyHydroxyethyl A column, (which as is typically found), gives much stronger retention of the basic peptides (e.g. peaks 17 and 15). Alternatively, Fig. 11b shows the same separation on PolyWAX LP, a weak anion exchange material that gives repulsion of the positively charged basic peptides, reducing their retention to similar values to the acidic peptides. The repulsion is moderated by the buffer salt. This technique can be generally used for tryptic peptides from protein digests, which at low pH are positively charged, as they are mostly uncharged at the carboxyl end. These peptides are repelled from anion exchange columns. However, peptides with phosphate groups or glycopeptides with sialic acid residues that retain negative charge under these conditions can be retained selectively. Important to note is that if a classical anion exchange column was used, the presence of a single phosphate group still produces low retention of the peptide, due to repulsion of the positive ends. However, with the superimposed HILIC mechanism, retention of such compounds can be achieved. The chromatography of phosphopeptides has been further studied [64] : numerous applications of the ERLIC technique in proteomics have been published [65-67].

4. Ranking the factors that influence retention in HILIC.

Using a consideration of the various parameters that can affect retention and selectivity in HILIC, a number of studies have sought to determine those factors which are most influential in manipulating a HILIC separation. McCalley investigated the effect of changing the stationary phase, the buffer pH (over the range w^w pH 3-6) and its concentration (5-20 mM), the column temperature (30-50 °C), organic solvent and its concentration on the selectivity of the separation of a set of neutral, acidic and basic solutes. The aim of the work was to provide a practical guide to aid in the selection of suitable conditions to establish a separation. Selectivity differences were followed from the correlation of k vs k' plots of the different conditions [36]. The magnitude of the effect of changes produced was estimated as:

stationary phase > mobile phase pH > organic solvent concentration > buffer concentration > column temperature.

While the study was performed with roughly equivalent numbers of basic, acid and neutral solutes, clearly the influence of the various factors will be dependent to some degree on the test set. For instance, varying the pH has relatively little effect on the retention of neutral solutes. In terms of the selectivity of the stationary phase, results in the study [36] were in good agreement with those of Irgum [28]. For instance, a bare silica phase (Waters Atlantis) showed poor correlation of retention factors with most of the other phases, due principally to high retention of bases and reduced retention of acids. Thus a silica column should be a choice for a "tool box" of different stationary phases which could be investigated sequentially for their ability to produce a separation with different selectivity. The retention of diol and amide columns was found to be well correlated, reflecting their common neutral ligands. An amide column might be preferred in the toolbox due to increased absolute retention compared with the diol phase investigated, as noted also by Irgum. Columns with polymeric bonded amide ligands appear to give good retention of polar neutral compounds (high hydrophilic selectivity) with reduced interaction effects for ionised solutes, The same argument is broadly true for (polymeric bonded) zwitterionic columns, an example of which could also be included in the toolbox. These also have good hydrophilic selectivity but some ionic interaction effects, giving rise to

somewhat different selectivity from amide phases. Finally, a column showing good retention of acids (such as an amino bonded phase) could be added to the toolbox.

Choice of pH is another rather complex but strongly influential factor. The retention of weak acids and bases, whose ionisation is affected considerably by mobile phase pH, can be manipulated according to the principles set out in section 3.2. The retention of stronger acidic and basic solutes, whose ionisation is less affected by pH, is mostly determined by coulombic attraction and repulsion with charged stationary phase groups. Furthermore, it appears that all silica-based phases display effects caused by ionisation of silanol groups, which give rise to superimposed cation exchange properties (except in the case of TFA or HFBA additives-see above).

In a similar study aimed at screening the most relevant parameters that affect retention, Guillarme and co-workers used a training set of 82 pharmaceutical compounds and 5 different stationary phases to provide guidelines for HILIC method development [68]. Again, the stationary phase was found to be the most important parameter governing selectivity, attributed to the very different types of interaction that could be shown. In this regard, zwitterionic and bare silica phases were found to be the most useful. Mobile phase pH was confirmed as being another important parameter, with buffer strength and nature of the organic modifier being of secondary importance.

5. Modelling the retention process in HILIC

5.1 Correlation of retention with log *D* values.

A number of authors have attempted correlation of log *D* values (usually those obtained from predictive programs) with retention. *D* is the distribution ratio of solute between octanol (which traditionally represents lipid solubility, or in HILIC the solubility in the bulk aqueous-organic phase) and water. Note that the concentration of both neutral and ionised solute is taken into account in both octanol and water phases for log *D* values, whereas only the concentration of neutral solute is accounted for in log *P*_{ow} values, the latter are therefore independent of pH. Kadar [69] investigated a set of 30 unspecified probe pharmaceutical compounds, obtaining correlation coefficients of 0.751, 0.696 and 0.689 for plots of log *k* vs log *D* pH 3.0, when using a mobile phase of 10 mM overall ammonium formate buffer pH 3.0 in 85, 90 and 95 % ACN in conjunction with a bare silica column. An empirical equation was deduced from the data relating the retention factor to log *D*. For example in 95 % ACN mobile phase buffered at _w^w pH 3.0 the equation:

$$\log k = -0.139(\log D) - 0.008 \quad (1)$$

was fitted to the experimental data.

The correlation of similar plots has been investigated by others, for example McCalley examined the correlation of plots of k vs $\log D_{\text{pH } 3.0}$ for a set of 29 probe solutes comprising acids, bases and neutrals on 6 different HILIC phases 5 mM ammonium formate pH 3.0 in 85 % ACN [36], see Fig.12. Poor correlation was found for a bare silica phase (Waters Atlantis, $R = 0.42$) which was attributed to the superimposition on the hydrophilic retention of attraction between protonated bases on ionised silanols and repulsion of strongly acidic solutes. Indeed, nortriptyline which has a $\log D_{\text{pH } 3.0}$ value of $\sim +1$ had relatively high retention ($k > 7$) in a similar mobile phase, except using 95 % ACN, indicating that ionic attraction was the main element in the retention of this relatively hydrophobic solute. The correlation of the plot for TSK amide ($R=0.83$) and a zwitterionic phase ($R = 0.65$) was considerably higher and attributed to the screening effect of polymeric phase layers on the silanols and/or the low concentration of silanols. A problem noted in this investigation was the inconsistency between $\log D$ calculated from different programs, due to the different algorithms used, which has been recognised in unrelated studies [70]. Instead, the values of $\log D$ were taken as the average of 3 different software programs (Marvin, ACD laboratories, MedChem designer) in an attempt to improve the accuracy of the estimations [36].

West and co-workers [71] obtained R^2 values of 0.70 and 0.87 for plots of $\log k$ vs $\log D$ at pH 6.2 using two zwitterionic phases with 80 % ACN containing 20 mM ammonium acetate at the same pH. With these columns and conditions it was stated that no particular group of compounds was responsible for the poor correlations, as neutrals, anions, cations and zwitterions all scattered in a homogeneous fashion about the regression line. It is likely that the polymeric nature of these phases and the relatively high buffer concentration acted to suppress ionic interactions of the solutes with the stationary phase.

Zhang and co-workers [72] found that ideal solutes selected through their hydrophilic subtraction model (see below, neutral solutes such as uridine, uracil, thymine, inosine, guanosine, salicylamide, benzamide) showed $\log k$ was inversely correlated to $\log D$ with high correlation coefficients ($R > 0.96$).

In summary, $\log D$ values have a considerable influence on retention in HILIC, and can be used as a rough guide to predict solutes suitable for separation by this mechanism. However, there are inconsistencies in these values estimated by the various commercially

available programs, which in addition, use water-based pH and solute pK_a values to predict log D. Clearly, log D values will differ under HILIC conditions, as the pK_a of solutes and the pH of the mobile phase both change in solvent mixtures containing a high proportion of acetonitrile [56]. Thus, some caution is necessary in their use. Furthermore, log D values alone will be less successful in predicting retention for solutes which can undergo other interactions (e.g. ionic repulsion and attraction) or strong specific adsorption.

5.2 More sophisticated modelling approaches.

The question of whether the retention mechanism in HILIC, (especially that part related to neutral interactions) is principally an adsorption or a partition process has vexed many authors [3, 30, 32, 48]. Retention in adsorption chromatography can be described by the Snyder-Soczewinski equation:

$$\text{Log } k = \log k_B - n \log X_B \quad (2)$$

where X_B is the mole fraction of the strong solvent B (in the case of HILIC, water) in the mobile phase, k_B is the retention factor with pure B as the eluent (when $X_B = 1$, $\log X_B = 0$) and n is the number of B solvent molecules displaced by the solute (approximately equivalent to the number of polar substituent groups in the solute molecule; typically for small molecules n ranges from 1 to 2). In some cases, the mole fraction is approximated by the volume fraction ϕ which is given by $0.01 \times \%B$. Thus a plot of $\log k$ vs \log (mol fraction water) should give a straight line for an adsorption mechanism. Alternatively for a partition mechanism

$$\log k = \log k_{\text{weak}} - S\phi \quad (3)$$

where k_{weak} is the retention factor when pure weak solvent (e.g. ACN) is used as the mobile phase and ϕ is the volume fraction of the strong solvent (e.g. water). Thus a plot of $\log k$ vs the volume fraction of water in the mobile phase should be a straight line for a partition mechanism. Use of these plots to determine the mechanism has proved rather inconclusive, with some indicating principally an adsorption mechanism and others a partition mechanism. It may be that the principal mechanism indeed varies dependent on the solute, column and mobile phase conditions. For instance, silica columns operated

with mobile phases of low water content are likely to act principally by adsorption (see above). However, it may also be that these equations are too simplistic to describe the HILIC mechanism. Other authors have used more complex empirical models to give approximate predictions of retention in HILIC [73]. Euerby and co-workers [74] used the equation:

$$\text{Log } k = a + b \log x + c (\log x)^2 \quad (4)$$

where a-c were system and analyte specific constants and x the “fraction” of strong solvent in the mobile phase. This equation was claimed to predict HILIC retention as a function of ACN concentration, buffer concentration and pH. However, it seemed that alternative equations were necessary to predict the effect of other variables (e.g. temperature). Furthermore, it appeared that the use of gradient modelling to predict HILIC isocratic conditions and vice versa was unsuccessful.

Tyteca et al investigated the possibility of making isocratic and gradient retention predictions using a limited number runs, comparing a number of different mathematical models. They found this approach mostly suitable for isocratic work: acceptable gradient predictions were difficult to obtain [75]. Nevertheless, they later reported the optimisation of the HILIC separation of nucleobases and nucleotides using a limited number of experiments, with prediction based on the empirical Neue-Keuss model [76].

$$\ln k = \ln k_w + 2 \ln(1+S_2\phi) - (S_1\phi / 1+ S_2\phi) \quad (5)$$

where ϕ is the fraction of water, k_w is the extrapolated value of k for $\phi=0$ (i.e. pure ACN) and S is the solvent strength parameter which is constant for a given solute and organic solvent, S_1 is the slope of the non-linear models, S_2 the curvature coefficient [77].

Cesla et al. [78] used a mixed mode model for the retention of maltooligosaccharides which aims to model the adsorption and partition process in HILIC:

$$\ln k = a + b \ln \phi_{H_2O} + c \phi_{H_2O} \quad (6)$$

where a is a parameter relating to the interaction energy between solutes and stationary/mobile phase, b is a coefficient related to the direct analyte-stationary phase interaction and c is a coefficient related to the interaction energy between solutes and

solvents. They found this model gave better fits to the experimental data than the simple adsorption and partition models (equations 2 and 3 above) or the Neue-Keuss model. They investigated the addition of a further term to the mixed mode model describing the contribution of the oligomeric glucose unit to retention. A later paper studied the application of the mixed mode equation and its extended variant to give predictions of the gradient retention time of the same solutes [79].

Other authors have employed a quantitative structure-retention relationship (QSRR) approach in order to predict retention [73]. West [71] studied two zwitterionic phases containing a sulfobetaine bonded ligand, using a mobile phase of ammonium acetate buffer w^w pH 4 (overall concentration 20 mM, w^s pH 6.2) in ACN. They used the equation

$$\text{Log } k = c + eE + sS + aA + bB + vV + d^-D^- + d^+D^+ \quad (7)$$

Capital letters represent the solute descriptors, while lower case letters represent the system constants. The Abraham descriptors E, S, A, B and V were obtained from a software package (ACD laboratories). c is the model intercept term, dominated by the phase ratio. E is the excess molar fraction, modelling polarizability from n and π electrons. S is solute dipolarity/polarizability. A and B are solute hydrogen bond acidity and basicity. V is the McGowan characteristic volume. Two new descriptors were introduced in this work: D^- represents the negative charge carried by anionic and zwitterionic solutes, and D^+ the corresponding positive charge where

$$D^- = 10^{(\text{pH}^* - \text{pK}^*)} / (1 + 10^{(\text{pH}^* - \text{pK}^*)}) \quad (8)$$

$$D^+ = 10^{(\text{pK}^* - \text{pH}^*)} / (1 + 10^{(\text{pK}^* - \text{pH}^*)}) \quad (9)$$

pH^* and pK_a^* are the true thermodynamic pH and pK_a in the aqueous-organic mobile phase ($s^s \text{pH}/s^s \text{pK}_a$), equivalent to that measured in the aqueous mobile phase (electrode calibrated in aqueous –organic buffers of known pH). For purposes of simplicity however, these workers used the $w^s \text{pH}$ of the mobile phase (electrode calibrated in aqueous buffers) and the $w^w \text{pK}_a$ values. Clearly, this simplification could cause errors in the procedure, but the availability of $s^s \text{pK}_a$ data is extremely limited. Neutral solutes have a D value of zero, whereas acids and bases have a value which approaches 1, depending on their ionisation state. The system constants (lower case) are obtained through multiple linear regression of the retention data for solutes of known descriptors. If a particular

coefficient is numerically large, then any solute having the complimentary property will interact very strongly with the mobile phase (if the coefficient is negative) or with the stationary phase if the coefficient is positive. The set of probe compounds to determine the system coefficients should be sufficiently large, and might contain at least 4 solutes per variable, containing solutes of different diversity, if the results are to be used to predict retention from compounds from families other than those in the test set. It was found that on both columns, good correlation was obtained between experimental $\log k$ and predicted $\log k$ values.

Schuster and Lindner [80] studied the retention of 68 probe solutes of different structure on 22 HILIC stationary phases with neutral, basic, acidic and zwitterionic surface modification in order to generate retention models based on the QSRR(LSER) approach of West [71]. The mobile phase was 90 % ACN containing overall 10 mM ammonium formate buffer w^w pH 3. While the usefulness of the D^- and D^+ descriptors was acknowledged, it seemed that the general approach could not be confirmed when these different operating conditions were employed. The use of appropriate pH scales appeared to give important differences in the results, and these authors opted for w^w pH values. Indeed, as a water layer is present in the stationary phase, it could be preferable to use this scale, although the authors recognised that it might still not reflect the exact conditions at the interaction site. The authors recognised the difficulties of pH and pK_a measurement in HILIC mobile phases as posing a fundamental problem for such models. It was found that system coefficients determined for the Nucleosil HILIC column in their study differed from those for the same column in the West study. Overall, they considered that it was not possible to use the regression equation to predict solute retention for the rather broad range of columns studied (much larger than the West study which was confined to sulfobetaine phases). A recommendation of the work was that three HILIC column types should be employed when developing a new separation (acidic, basic and neutral) in order to gain a wide selectivity and application range, with an amide (neutral) or zwitterionic (quasi-neutral column) as the first choice. These recommendations are similar to those in Section 4 above. Amide and zwitterionic columns were preferred to diol phases due to their wider retention window. In a follow-up study [81], the authors changed the buffer to ammonium acetate w^w pH 5 from ammonium formate w^w pH 3.0. However, the ability of the model to predict retention was somewhat worse. It seemed that under enhanced electrostatic conditions (more of the acidic solutes were ionised, while most basic compounds remained positively charged) the unadjusted solute descriptors used in the model, generally computed for the neutral species, are inadequate. For example, the

hydrogen bonding acidity and basicity of the solute is changing, which is important if adsorption is one of the dominant retention mechanisms. A change from formate to acetate was found to elevate the hydrogen bond basicity of the HILIC system. Nevertheless, it was stated that the partition mechanism seemed dominant on many columns.

Haddad and co-workers [82] developed QSSR models to predict the retention of analytes on 5 HILIC stationary phases (bare silica, amine, amide, diol, zwitterionic). Six beta-adrenic antagonists were used as target analytes. Molecular descriptors were calculated based on chemical structures optimised using density functional theory. An algorithm was then used to select the most relevant molecular descriptors for each stationary phase using partial least squares regression. The model was then used to predict the retention of the test set of target analytes. Mean error values in retention time prediction were 13-25 s on four of the stationary phases, with a higher error (50 s) for the zwitterionic phase. The descriptors were based on important physicochemical properties which established a strong connection between retention and meaningful chemical properties, which explained the high levels of accuracy of the predictions. The QSSR model was further combined with a design of experiment model (DoE, relating analyte retention to mobile phase pH, ACN and salt concentration) to predict the retention times of analytes outside those of a test set, under new chromatographic conditions [83]. An Acclaim HILIC-10 amide column was used in this study.

Zhang and co-workers [72] proposed a hydrophilic subtraction model for HILIC, analogous to the hydrophobic subtraction model for RP separations proposed by Snyder and co-workers [84]. The HILIC model was based on 41 solutes and 8 representative stationary phases. It models retention according to a linear solvation energy relationship of the form:

$$\text{Log } \alpha = \text{log } k - \text{log } k_{\text{ref}} = hH + aA + bB + cC + dD \quad (10)$$

where capital letters represent the solute descriptor and lower case letter s the system constants. H represents solute hydrophilic partitioning ability, A and B are solute hydrogen bond acidity and basicity respectively, C and D are solute cation and anion exchange properties. The system constants represent the magnitude of difference in the particular interactions between stationary and mobile phase. $\text{log } k_{\text{ref}}$ refers to the retention factor of uracil which is neutral under the experimental conditions employed (ACN-100 mM ammonium formate pH 3.3, 85:15 v/v). TSK gel amide is a neutral stationary phase and

was selected as the reference column. So called “ideal” solutes (neutral under the conditions of the study) were selected that were assumed to be retained solely by hydrophilic partitioning.

$$\log \alpha \sim hH \quad (11)$$

For two columns a and b

$$\log \alpha_b / \log \alpha_a = h_b / h_a \quad (12)$$

Column a is designed as the reference column with value $h_a = 1$.

For ideal solutes h_b is the slope of the plot of $\log \alpha_b$ vs $\log a_a$. This procedure allows the contribution of hydrophilic partitioning to be subtracted from the overall retention and for the other contributions to retention to be assigned. Test solutes could be grouped as weak acids, stronger acids, weak bases and stronger bases. Columns could be grouped into neutral stationary phases (e.g. amide, diol), anion exchangers (e.g. amino phases), cation exchangers (e.g. bare silica, polysulfoethyl A) and zwitterionic phases (e.g. sulfobetaine). The hydrophilic subtraction model differs from the approach taken by the West group, in that it uses empirically determined solute descriptors rather than the Abraham descriptors.

Clearly, much progress has been made in modelling HILIC separations. It remains to be seen how successful these models are when prediction of the retention of random solutes of structure unrelated to those in a test set is required. The accurate specification of the state of ionisation of ionogenic species in the aqueous-organic mobile phase seems to be a particular difficulty.

6. The kinetics of HILIC and comparison with RP.

HILIC separations are performed in low viscosity mobile phases in which solute diffusion should be enhanced. Cabooter and co-workers published an extensive database of diffusion coefficients obtained by the Taylor-Aris procedure, in which peak broadening of the solute is monitored in a flow of liquid through a long open capillary tube. Diffusion coefficients of a range of solutes in ACN-water mixtures from 0-97 % (v/v) were given [85]. Increases of solute diffusivity by a factor of two or more were reported when using typical HILIC mobile phases (95 % ACN) compared with pure water. Jorgenson and co-workers developed a capillary time of flight instrument that enable measurement of viscosity and

diffusion coefficients at pressures of up to 2000 bar [86, 87]. This method is based on the Stokes-Einstein equation:

$$D = kT / 6\pi\eta r_H$$

which indicates that the product of diffusion coefficient (D) and the viscosity (η) should be constant as long as no change in the hydrodynamic radius r_H is observed.

Due to this enhanced solute diffusivity, HILIC is expected to maintain higher efficiency at high mobile phase flow rates, in the C term region of the van Deemter curve. Conversely, at low flow rates, longitudinal diffusion is expected to be more important in HILIC than RP, leading to somewhat poorer performance in the B term region. In a practical study, it was shown that HILIC B coefficients obtained from simple non-reduced van Deemter plots were indeed higher for the same solute (nortriptyline) measured on RP and HILIC columns obtained from the same manufacturer whereas C coefficients were smaller (see Fig.13) [88]. The non-reduced curves obtained on 1.8 μm and 3.5 μm columns of the same stationary phase were similar, indicating the lack of influence of frictional heating or pressure effects on the results, which might have been highlighted in the performance of the smaller particle column. Comparison of Fig 13a and 13c shows similar behaviour with both particle size columns. However, comparison of the behaviour of basic and neutral solutes in reduced coefficients on both columns (Figs 13b and 13d) showed that b coefficients were smaller and c coefficients larger in HILIC than RP. Note the considerably flatter curves shown by naphthalene and nortriptyline in the RP mode on both particle size columns (Fig 13b and 13d). These results show that a simple interpretation of the data based on bulk diffusion coefficients of the solutes in the mobile phase is inadequate. An explanation of these results is that in RP, surface diffusion can take place due to the non-localised retention mechanism, facilitated by the layer of ACN on the surface of the stationary phase, thus increasing the b coefficient. In contrast, in HILIC, localised adsorption or ionic retention may contribute to the retention mechanism. Surface diffusion in the water layer on the stationary phase also seems less likely. The smaller c terms could also be explained by slower adsorption-desorption kinetics in the water layer [89, 90]. The results can additionally be predicted from molecular dynamics simulations [91]. It was also suggested that border effects (sample introduction into the column and sample collection before detection) affect the HETP values in HILIC at all flow rates [89]. This result is possibly explained by the fact that transverse diffusion coefficients in HILIC are not large enough to equilibrate the sample concentration across the column diameter

during migration from the inlet to the outlet. As a result, the performance of HILIC columns is expected to be very sensitive to the sample distribution at the inlet, and its collection at the outlet. An interesting observation of the practical comparison study [88] was the decrease in k at high flow rates under HILIC conditions compared with low flow, although significant changes in k were observed for all analytes in both separation modes. The effects were much more pronounced for 1.8 μm particle columns than 3.5 μm or 5 μm columns. As flow rate is increased, pressure and frictional heating increase. In the RP mode, increased pressure alone causes increased retention, whereas heating alone typically reduces retention [92, 93]. Thus these factors are generally opposed, leading to only small changes in k as a function of flow rate. However, in HILIC, increased pressure and heating both produce decreases in k , so the factors act in the same direction [94]. Thus decreases in k approaching 50 % were obtained for some solutes in HILIC when changing the flow rate from 0.025 mL/min to 2.0 mL/min on the 50 x 2.1 mm 1.8 μm column. While this variation did not influence the conclusions of the van Deemter study (especially as the results were verified on a larger particle size column), it may have implications for method transfer in HILIC.

In a similar practical study, Cabooter and co-workers [95] compared the performance of RP and HILIC columns (again including a pair from the same manufacturer, based on the same silica) with test mixes of similar analytes, run with low organic modifier concentrations in RP (~ 2% v/v) and high concentration in HILIC (~90 % v/v). The authors preferred to make deductions based on the interstitial velocity (u_i) of the mobile phase

$$u_i = F / \varepsilon_e \pi r^2$$

where F is the flow rate, ε_e is the external porosity. This approach was taken to avoid errors in the estimation of the void volume of the columns using “unretained” solutes. The authors again found that efficiency in HILIC was somewhat lower than in RP at high values of the interstitial velocity. However, kinetic plots using the interstitial time t_i where

$$t_i = L/u_i$$

revealed that the overall kinetic performance in both modes was in general very similar. The lower than expected longitudinal diffusion term of the HILIC column, and the low

viscosity of the mobile phase allows the successful operation of long columns where high column efficiency can be obtained using low flow rates to limit the pressure. A later study by the same group compared the performance differences between HILIC and RP under conditions of identical packing quality [96]. This was done studying a column first under RP conditions, and then stripping the C18 stationary phase using 3% trifluoroacetic acid in water at 60 °C for 120 minutes. It was shown that the external porosity of the column remained unaltered by the stripping procedure, and furthermore that the backpressure required to operate the column under particular mobile phase composition and flow was unaltered. Thus it was concluded that the column was undamaged by the stripping procedure. It was impossible to select exactly the same test mixture for use in HILIC and RP, but compounds with similar characteristics (nucleobases and nucleotides) were chosen. In agreement with previous results [88], plots of plate height vs (interstitial) velocity showed larger B terms and smaller C terms in the HILIC mode, in line with the bulk diffusion coefficients determined by the Taylor-Aris procedure. However, as before, plots of the reduced plate height against the reduced interstitial velocity ($u_i d_p / D_m$) showed lower b coefficients and larger c coefficients in HILIC. Again these results were explained in terms of smaller surface diffusion and a more localised retention mechanism in HILIC. A further deduction was that eddy dispersion was greater under HILIC conditions compared with RP.

An earlier practical study demonstrated the kinetic advantages of superficially porous (“shell”) columns compared with totally porous columns [6]. Fig. 14 shows the improved efficiency of a single 15 cm shell column packed with 2.7 μm silica particles compared with that of a totally porous 3.0 μm particle column of the same dimensions. The low back pressures in HILIC also allow the coupling of 3x 15 cm shell columns to give over 100,000 theoretical plates in a reasonable analysis time, even on a conventional HPLC instrument at only ~280 bar in total system pressure. Furthermore, Fig. 15 shows a moderately fast (unoptimised) separation on a single 15 cm shell column again run on a conventional instrument, where over 30,000 theoretical plates was achieved for each peak. The excellent peak symmetry for the basic compounds in Figs 14 and 15 is also notable, as it is difficult to achieve for similar compounds when analysed in the RP mode [7] Later studies confirmed the improved kinetic performance in HILIC of shell columns compared with totally porous columns, all of sub- 2 μm particle size [97]. The improved performance was attributed (as has been done for RP shell columns [98]) to the superior bed homogeneity of shell columns and possibly also to the improved thermal conductivity of these materials, which reduces the flow heterogeneity across the column radius, and

thus the van Deemter C term at high flow velocity. Nevertheless, the minimum reduced plate height was found to be larger for these smaller shell particle columns packed in 2.1mm format compared with sub 3 μm phases packed in 4.6 mm format. In this study, the reduced van Deemter b coefficient was found to vary between different analytes on the same column. For example, hydrophobic bases showed considerably greater diffusion inside the particle than hydrophilic bases, which may be due to their retention in a more acetonitrile rich region inside the pores.

In conclusion, kinetic effects are somewhat more complex than might be expected on the basis of the supposition of enhanced solute diffusion in HILIC mobile phase. However, these effects do not pose a serious barrier in practice for experimentalists who wish to use long columns to produce high efficiency, or short/standard length columns for fast analysis.

7. Sample Injection and detection procedures.

A systematic investigation of the effect of sample diluent on peak shape was performed using low MW analytes (<1000 Da) as well as peptides with MW 1000-6000 Da [4]. For small MW compounds, the best results were found for injection in pure ACN, which is a very weak eluent in HILIC. However, sample injection can be a problematic area in HILIC due to difficulties in solubilising the analytes in high concentrations of ACN. As the concentration of water in the injection solvent is increased, loss of column efficiency can occur due to the presence of a plug of stronger eluent introduced into the mobile phase. The effect increases in severity dependent on the difference in the water concentration of the injection solvent and the mobile phase [53]. However, the problem can be reduced by reducing the injection volume of the solvent. Fig. 16 demonstrates the increasing detrimental effect on column efficiency of injection solvents containing increasing amounts of water (10 % and 20 %) on a mobile phase with aqueous concentration 5% (v/v). However, for small volume injections (1 μL) the effect can be minimised. An alternative approach when solubility in high concentrations of ACN is problematic is to employ other organic solvents. For small MW compounds, isopropyl alcohol (IPA) or a mixture of ACN/IPA (50:50, v/v) was recommended [4]. For drug discovery applications, dimethylsulfoxide (DMSO) could be employed if at least 80 % ACN was included. For peptide analysis, pure ethanol or IPA were recommended to limit denaturation issues.

Guillarme and co-workers [99] showed rather surprisingly that characterisation of protein biopharmaceuticals such as insulins, interferon and trastuzumab was possible in

HILIC using mobile phases with concentrations of ACN of 65-80 % with 0.1 % TFA. Whereas precipitation was a problem when injecting in solvents containing high ACN concentration, injection in water gave satisfactory results as long as the volume of the injection was decreased to 0.1-0.2 % of the column volume. It is uncertain in these studies as to the extent of denaturation of the solutes, but for characterisation procedures, this may not be an important consideration. Later, the same group showed the application of HILIC to the comparison of originator and biosimilar therapeutic monoclonal antibodies. The intact mAb was digested to yield F(ab)₂ and Fc/2 subunits followed by reduction of the F(ab)₂ subunit to yield Fd' and C subunits. The final sample contained fragments of about 25 kDa each [100]. Due to the solubility problem in high concentrations of ACN, an aqueous sample was injected, but the strong elutropic strength of the diluent was counterbalanced by a fast initial gradient ramp (85-75 % ACN in 0.2 min.) followed by a slower ramp (in around 10 min.) to a lower ACN concentration. When combined with a small injection volume (1 µL into a 2.1mm x 150 mm column), satisfactory peak shapes were obtained.

The response of several different kinds of detector in which the mobile phase is removed by evaporation has been shown to be enhanced in HILIC mobile phases. Mitchell and co-workers [101] measured the responses of 12 polar hydrophilic solutes in RP and HILIC mode using an evaporative light scattering detector (ELSD), charged aerosol detector (CAD) and ESI MS. The Phenomenex Luna HILIC column was operated isocratically with 10 % ammonium formate pH 3 in 90% ACN, and the (RP) Waters T3 Atlantis column with 5% ACN in the same buffer. For ELSD, the HILIC mode was reported as marginally more sensitive; for CAD ~ 10 times more sensitive and ESI-MS was 5-10 times more sensitive. A later study showed how the decrease in sensitivity caused by running a gradient of increasing water content (as is normal in HILIC) could be compensated for by a reverse gradient introduced post column [51]. Russell et al. performed a detailed comparison of improvements in sensitivity using CAD with HILIC mobile phases compared with RP [102] (see Fig. 17). Sensitivity comparisons were performed by flow injection analysis (without a column) to eliminate any effects of chromatographic peak shape or irreversible solute adsorption on the results-the improvements in HILIC are shown to be considerable, .

In an early review of electrospray mass spectrometry detection (ESI) in combination with HILIC, Nguyen and Schug considered the various stages in the process and how these would be affected by the difference in the composition of the mobile phase compared with that used typically in RP separations [103]. Although the authors stressed

the great complexity of the processes involved, some relevant factors could be considered. For example, the magnitude of the electric field required for droplet formation is proportional to the square root of the surface tension of the mobile phase, which is significantly less for ACN than water. Thus droplets are formed more easily from typical HILIC mobile phases. The conversion of charged droplets to gas phase ions is again facilitated by the low surface tension of HILIC mobile phases. Additionally, the rate at which solvent evaporates from the droplet surface is improved using solvents of higher volatility. Guilleme and co-workers [104], compared the ESI sensitivity using HILIC and RP mobile phases with gradient elution separations of mixtures of solutes using either technique with an appropriate column in place. Initial studies used a Waters TQD triple quadrupole MS, comparing signal/noise ratios as an indicator of the MS response. The average gain in sensitivity was 7-10 times, but some compounds apparently showed increases of 100-800 times [105]. In later studies, it was shown that the design of the ESI source greatly influenced the relative sensitivity in the two modes. Much work has been performed recently in the design of electrospray interfaces for use in MS to improve the efficiency of the evaporation and desolvation processes involved, especially when RP solvents containing higher proportions of water are utilised. Using more recent instruments than the TQD, equipped with these better-designed interfaces, the gain in sensitivity was less significant in HILIC [106]. A further interesting study from the same group compared matrix effects (ME) in ESI-MS for plasma and urine using 38 pharmaceutical compounds and 40 doping agents in HILIC/RP analysed on 3 different columns with different mobile phase pH [107]. They also compared ME with either simple sample pretreatment (protein precipitation) or solid phase extraction (SPE). The compounds influenced by ME were different in RP and HILIC, which adds to the complimentary nature of the techniques. While it did not appear that ME were a serious problem in either technique, it was found that the use of a more thorough cleanup (SPE) in RP was less necessary than in HILIC. This finding was related to the facile elimination of polar endogenous compounds in the early part of the RP analysis.

9. Conclusions.

HILIC provides a method for the separation of polar and ionised compounds that cannot be retained by traditional RP approaches; the two techniques can also give orthogonal selectivity. HILIC offers advantages as a result of the low viscosity of typical mobile phases, although its supposed kinetic advantages over RP are less clear due to the

presence of the water layer on the stationary phase surface, which may adversely affect solute diffusivity and mass transfer. Detection appears to be improved in ESI-mass spectrometry due to improvements in droplet formation and solvent evaporation; this improvement is also shown with other detection systems involving solvent evaporation, such as the charged aerosol detector.

Although its mechanism is considerably less well understood than RP, much progress has been made in the last 10 years in appreciating the fundamental processes that govern solute retention in HILIC. Detailed information is now available on how to manipulate a HILIC separation, and which parameters produce the greatest effect. Major changes in selectivity can be achieved by the use of alternative stationary phase chemistries. Changing the organic solvent (almost invariably ACN) concentration, has a major effect on retention but relatively little influence on selectivity. Changing the pH of the mobile phase also has a very marked effect on the selectivity of the retention of ionisable solutes, while buffer concentration and temperature appear to have smaller effects. The intuitive division of columns into those with bonded neutral, positively charged, negatively charged, or zwitterionic ligands has been validated by rigorous column classification schemes. Probe solutes are used in these schemes to estimate the contribution of the various mechanisms to retention and selectivity. Particularly significant properties appear to be hydrophilic and cation/anion exchange selectivity, although probes for isomeric, hydrophobic and surface pH have also been proposed. Considerable progress has been made in modelling studies, allowing prediction of retention through much more sophisticated approaches than merely linking retention to log D values. However, the log D approach remains a convenient rule of thumb for estimating the suitability of HILIC for analysis of a given solute. A problem in modelling studies is the absence of data that describe the charge on ionisable compounds in the presence of high concentrations of organic solvent in the mobile phase. A more fundamental difficulty is that solutes may reside in regions of differing water content in the vicinity of the stationary phase surface dependent on their particular structure and hydrophilicity, making estimation of solute charge even more difficult.

A number of other problem areas remain for HILIC, which include the limited solubility of some solutes in high concentrations of acetonitrile that are necessary to retain those that are relatively weakly hydrophilic. Increasing the mobile phase water content of the injection solvent above that of the mobile phase can lead to peak distortion. Furthermore, achieving sufficient retention of solutes that are only moderately hydrophilic can be difficult, which means that a considerable number of organic compounds are not

suitable for application of this technique. Other problems include the longer equilibration times of the system compared with those experienced in RP separations.

11. References

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10. Legend to Figures

Fig 1 Analysis of peptides 1= lysine vasopressin; 2= arginine vasopressin; 3- peptide D; 4=triptolrelin; 5= peptide A; 6 = insulin; 7= peptide B; 8 = peptide E; 9= peptide C. Impurities marked with a star. Injection solvent was water for RP and IPA for HILIC. RP column Acquity BEH C18, 10-90 % ACN in 20 min; HILIC column Amide 90 % ACN for 3 min, then 90-62 % ACN in 9 min. Based on [4]

Fig. 2 Effect of water concentration on retention of xanthenes on native titania. Mobile phase ACN-ammonium acetate (1mM/L). Reprinted with permission from [23].

Fig. 3 Types of bonded HILIC stationary phases (based on reference [15] reprinted with permission).

Fig. 4 Chromatograms of probe compounds on different HILIC phases. Detection : UV at 215 nm, temperature 30 °C. Peaks: 1,4 neutrals (phenol, caffeine, green numbers): Peaks 2,3 acids (2-naphalene sulfonate, p-xylene sulfonate, red); Peaks 5,6,7,8 bases (nortriptyline, diphenhydramine, benzylamine, procainamide, blue). Mobile phase 90% ACN overall 5mM amm. form. pH 3.0. Based on [30]

Fig. 5 Principal components analysis showing grouping of amino, neutral, silica and zwitterionic columns according to Irgum. Reprinted with permission from [28].

Fig. 6 Molecular dynamics simulation of radial density profiles for water (oxygen atoms) and ACN molecules (central carbon atom) as a function of distance from the surface inside the silica pore for water: ACN mixtures. Based on [39].

Fig 7. Water uptake isotherms of HILIC stationary phases obtained by equilibration of water-ACN mixtures followed by Karl Fischer titration. Filled black symbols are polymeric phases, filled gray symbols monomeric bonded phases and open symbols are bare silica Reprinted with permission from [35].

Fig.8 Comparison of the equivalent number of monomolecular water layers N_w inside the pores of the adsorbent surface at full saturation capacity on different stationary phases. Reprinted with permission from [37].

Fig. 9 Chromatograms of a mixture of acidic, basic and neutral test compounds on an Agilent glycan (amide) column using 95% ACN containing either 0.1 % TFA or overall 5mM ammonium formate pH 3.0. Peak identities: 1 p-xylenesulfonic acid; 2= naphthalene-

2-sulfonic acid; 3= thiourea; 4= uracil; 5= nortriptyline; 6 = procainamide; 7= 4-hydroxybenzoic acid; 8 = cytosine. Acids (red numbers); bases (blue numbers); neutrals (green numbers) . Based on [54].

Fig 10 (top) s^s pH of solutions of different additives in ACN as a function of organic solvent concentration; (bottom) Ionic strength of solutions of different acids as a function of organic solvent concentration. Based on [54] .

Fig. 11 HILIC vs ERLIC for the separation of peptide standards. HILIC column PolyHydroxylethyl A. Mobile phase :20 M NaMePO₄, pH 2.0 with 63% ACN. ERLIC column PolyWAX LP. Mobile phase 20 M NaMePO₄, pH 2.0 with 63% ACN. Based on [63]

Fig. 12 Plots of average log D (from 3 calculation programs) vs log k on 3 columns. Mobile phase = 5 mM ammonium formate pH 3.0 in 85 % ACN. Blue circles = basic solutes; black squares= acids; red triangles = neutrals. Based on [36].

Fig. 13 Non-reduced and reduced van Deemter plots for HILIC (Zorbax HILIC plus) and RP (Zorbax Eclipse Plus C18) phases; 1.8 μ m columns (a) and (b) and 3.5 μ m columns (c) and (d). Mobile phase for HILIC solutes ~ 95% ACN; for RP 33 and 55% ACN. Ammonium formate buffer pH 3 overall concentration ~5 mM; exact mobile phase composition adjusted to give $k \sim 5.5$ at optimum flow velocity. Based on [88].

Fig 14 Comparative chromatograms of test mix on (top) 1 x 15 cm 3 μ m porous particle silica column (system pressure = 81 bar); (middle) 1 x15 cm 2.7 μ m shell column (95 bar); (bottom) 3 coupled 15 cm 2.7 μ m shell columns (280 bar). All columns 4.6 mm i.d., flow rate 1 mL/min, 15 mM w^w pH 3.0 ammonium formate buffer in 85 % ACN. Plate numbers are shown in parentheses. Based on [6].

Fig. 15 Fast separation of pyridine and aniline derivatives on a 15 x 0.46 cm 2.7 μ m bare silica shell column.(1) 2-ethylaniline; (2) aniline; (3) 3-butylpyridine; (4) pyridine; (5) 3-methylpyridine (6) 4-ethylpyridine; (7) 4-methylpyridine; (8) 3,4-dimethylpyridine; (9) 2,4-dimethylpyridine; (10) 2,6-dimethylpyridine. Total system pressure ~200bar. Mobile phase 80% ACN containing 20mM ammonium formate pH 3.0, flow 2 mL/min. Column efficiency ~ 32,000 plates per column. Based on [6]

Fig. 16 Plots of column efficiency (N) vs injection volume for Waters BEH HILIC column (100 x 21 mm, 1.7 μ m particle size). Mobile phase 95 % ACN containing 5 mM overall

ammonium formate pH 3.0. Injection solvent a) buffer in 95 % ACN. b) buffer in 90 % ACN. C) buffer in 80 % ACN. Based on [53].

Fig. 17 Effect of organic solvent concentration in the mobile phase on signal/noise measurements using the charged aerosol detector for 21 hydrophilic compounds measured in the flow injection analysis mode. Mobile phases contained 5 mM ammonium formate pH 3.0 in each case. Based on [102].

Fig. 1

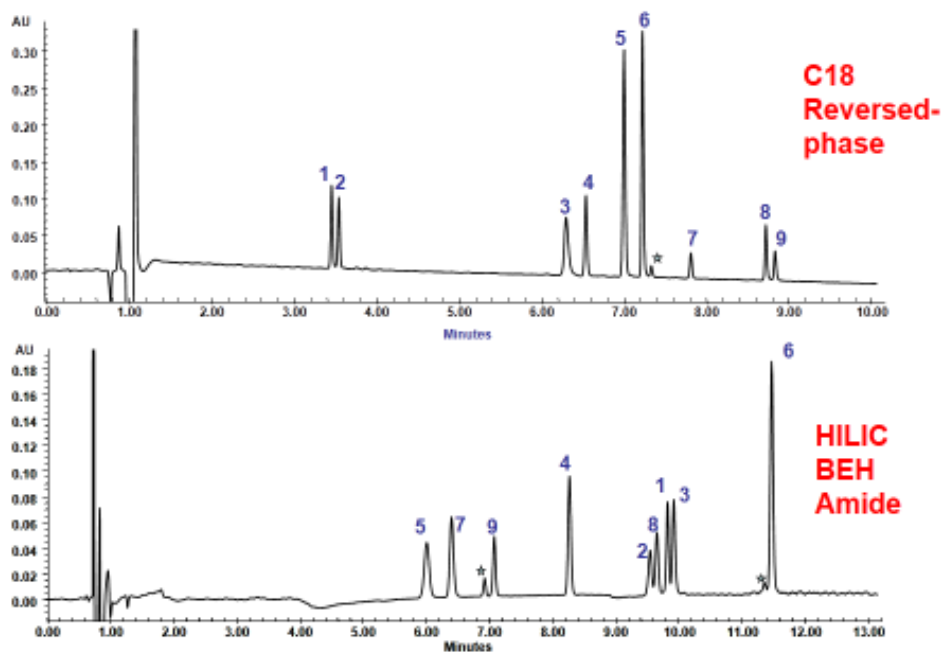
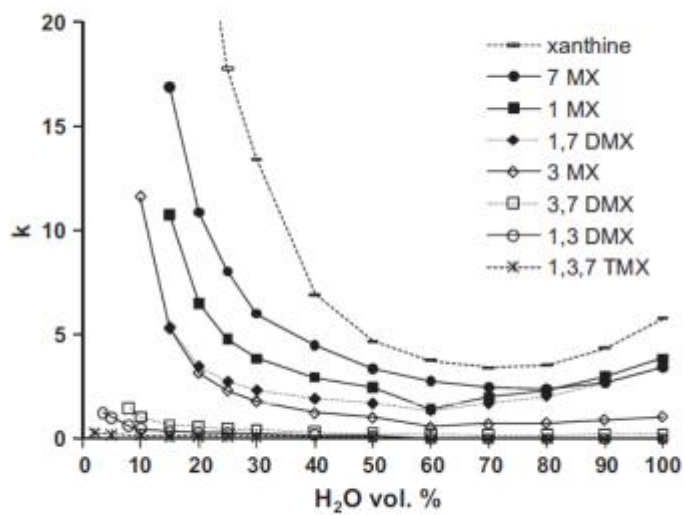


Fig. 2



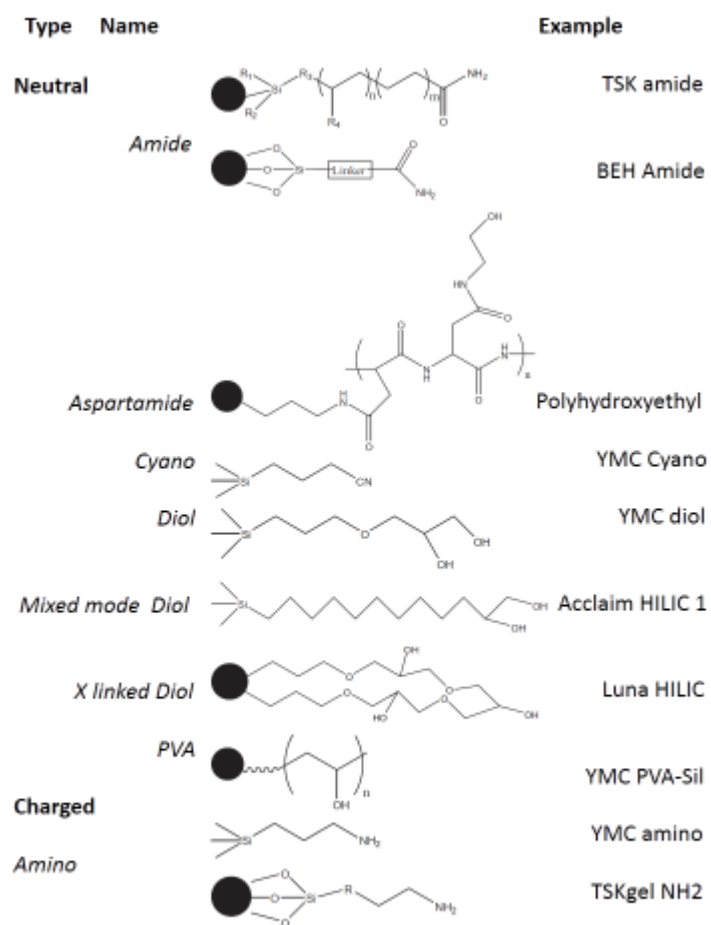


Fig. 3

Type	Name	Example
Charged	Polyamine	Luna NH2
	Imidazole	Sepax Imidazole
	Triazole	Cosmosil HILIC
	Poly(2-sulfoethyl)	Polysulfoethyl A
	Poly(aspartic acid)	Poly CAT A
	Sulfobetaine	ZIC HILIC
	Phosphocholine	Shiseido PC HILIC
Obelisc N	SIELC Obelisc N	

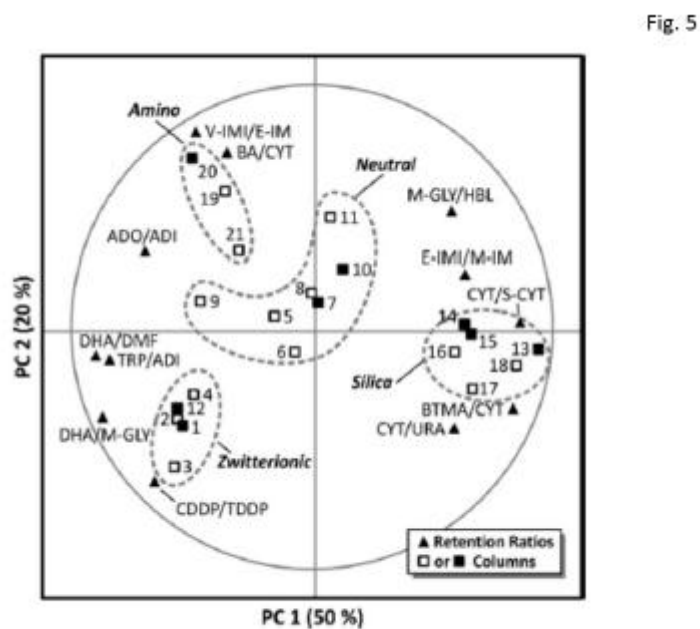
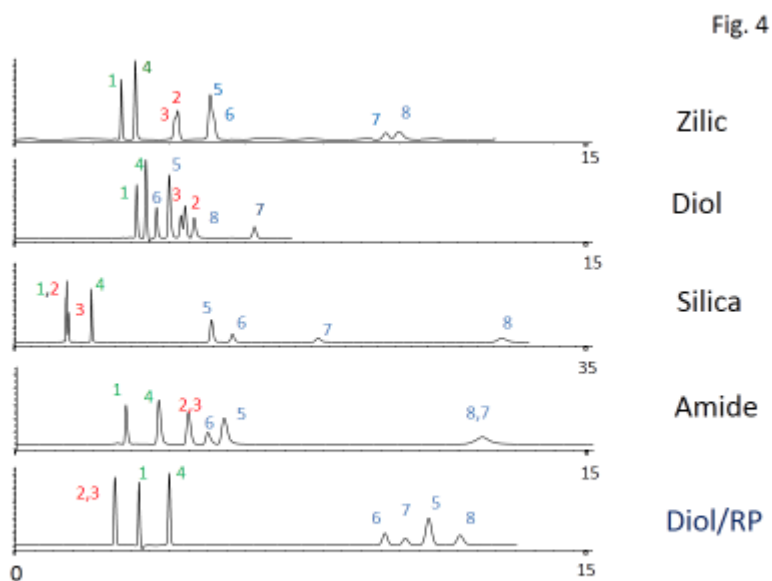


Fig.6

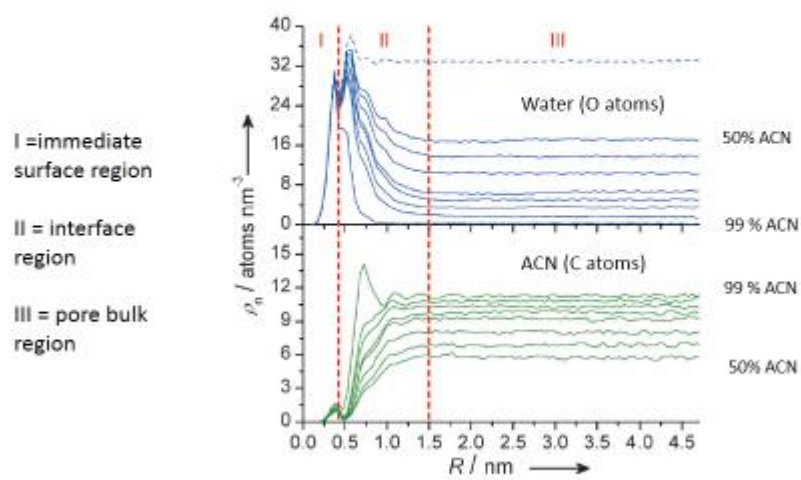


Fig 7

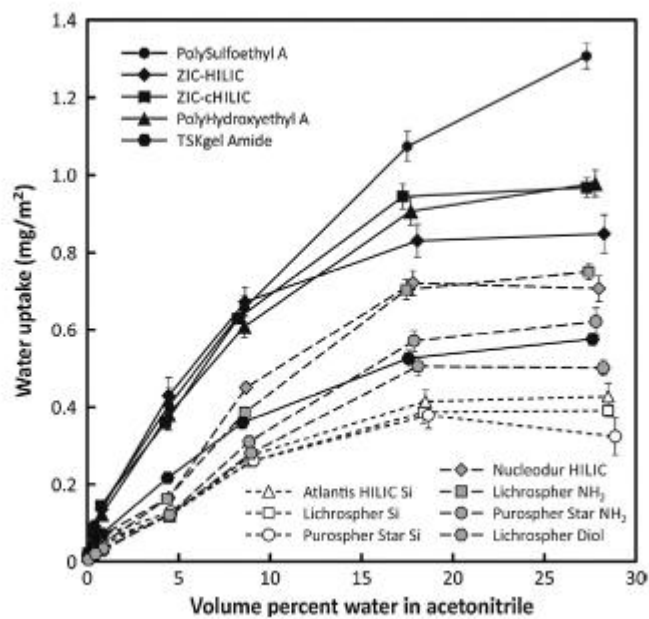


Fig. 8

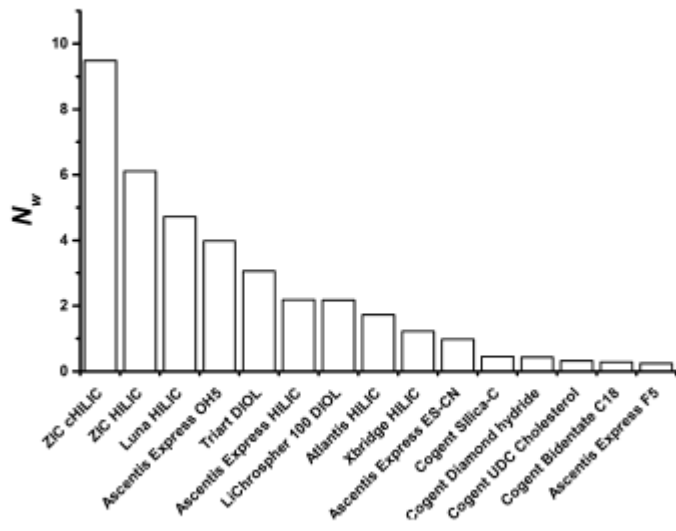


Fig. 9

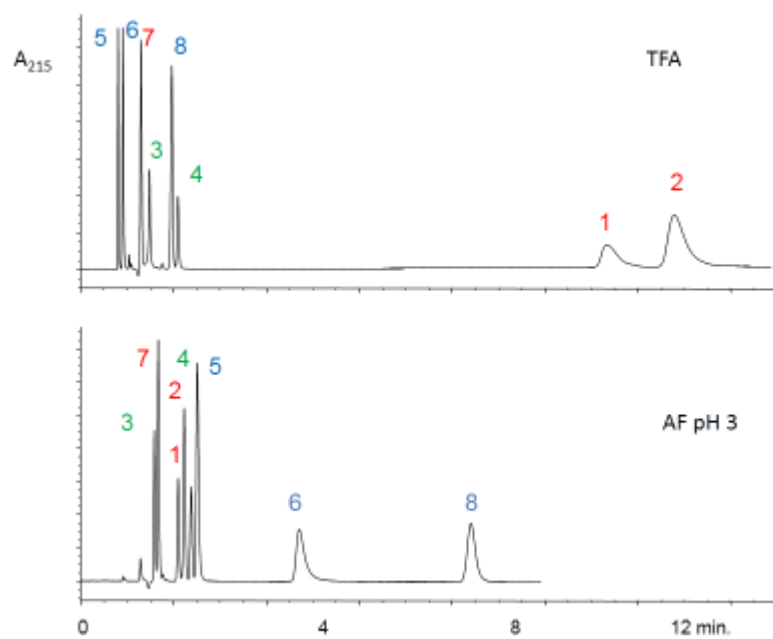


Fig. 10

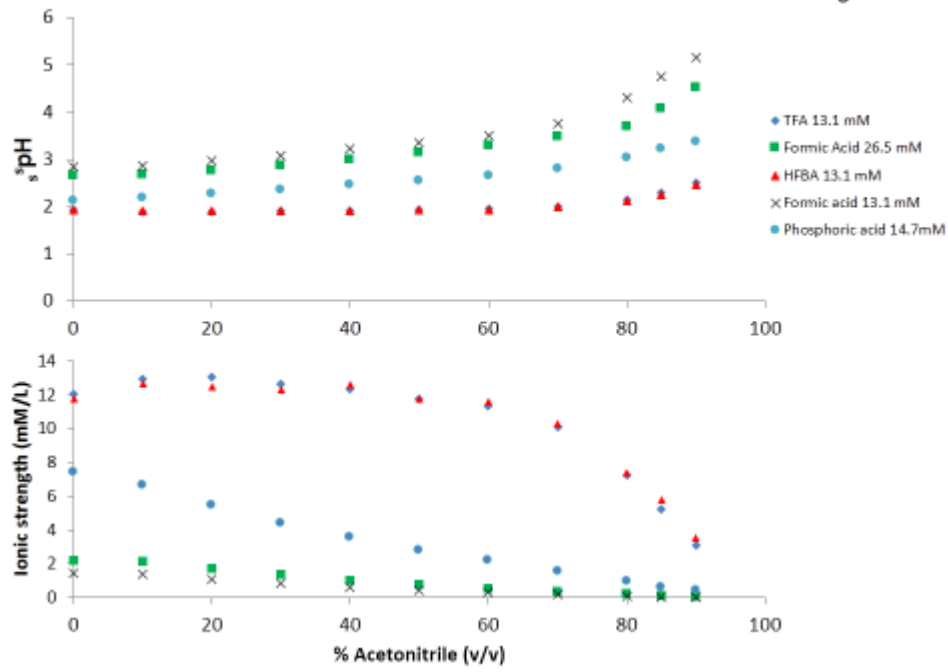


Fig. 11

HILIC vs ERLIC Separation of Peptide Standards

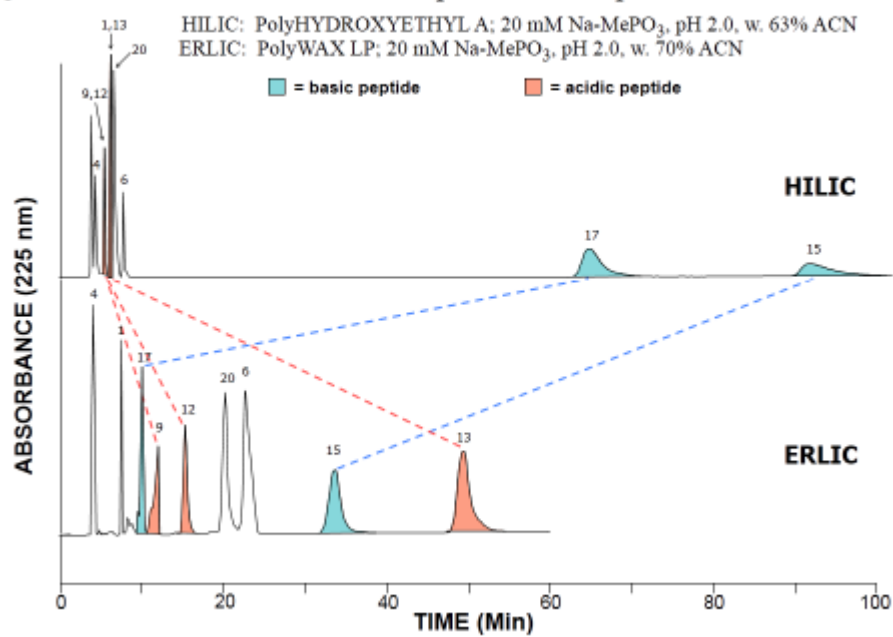


Fig. 12

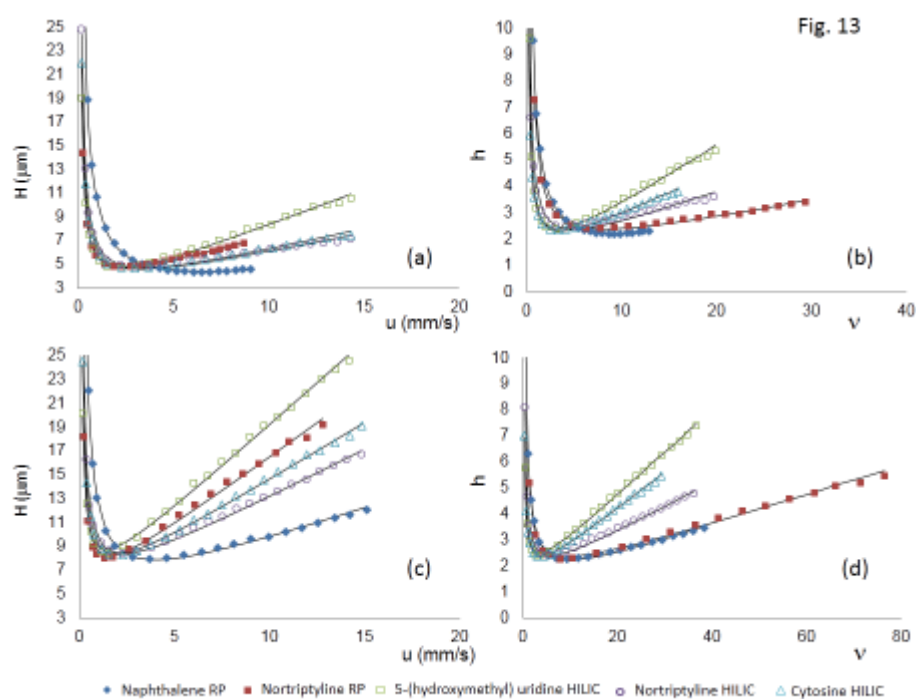
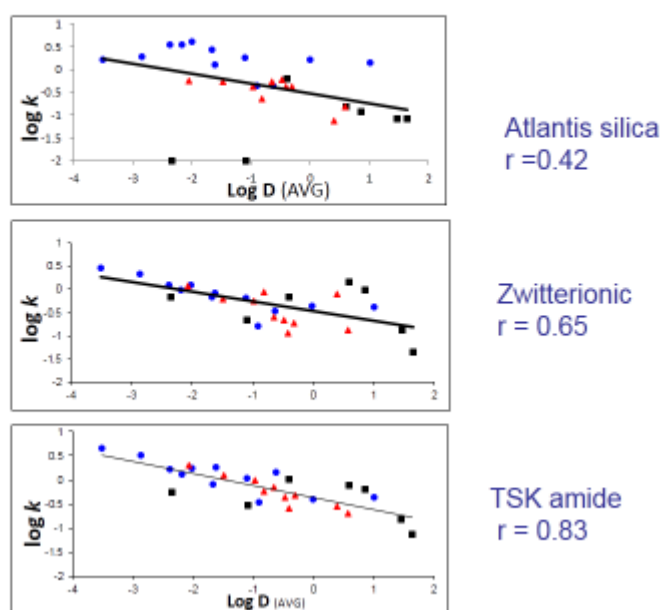


Fig. 14

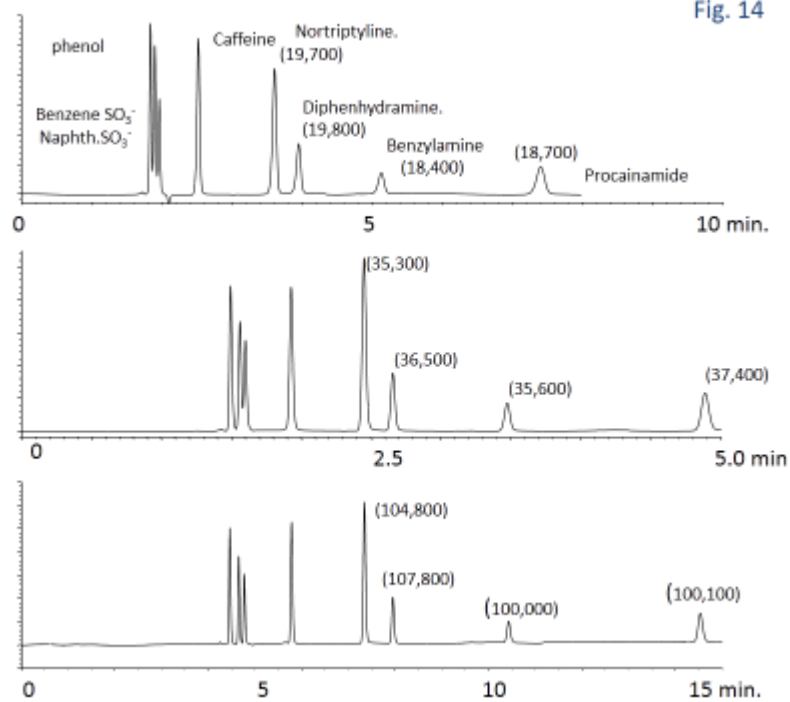
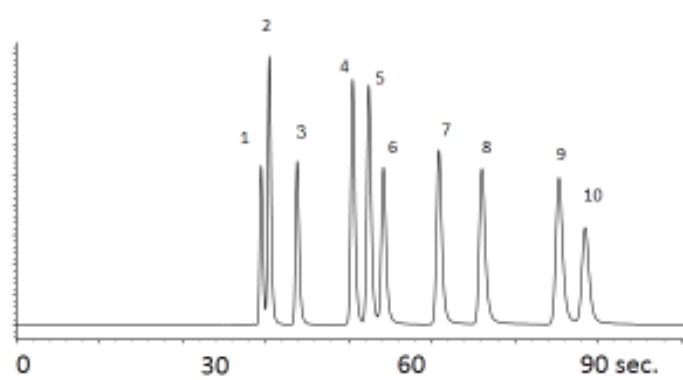


Fig 15



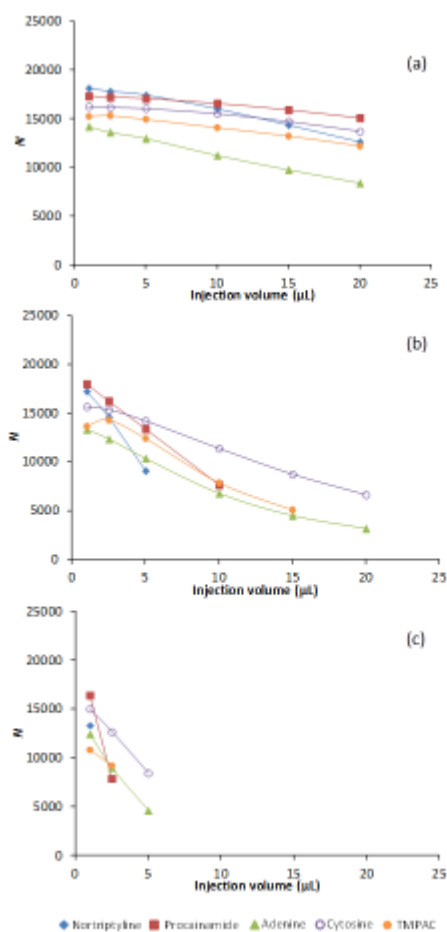


Fig. 17

