A range of ten perfluorophenyl and perfluoroalkyl stationary phases has been evaluated using standard chromatographic tests and probes. Principal Component Analysis of the data has indicated that the phases can be divided into distinct groupings. Extending the dataset to include standard alkyl and phenyl phases provided further data interpretation to support the orthogonal selectivity claims made for perfluorinated phases. The analysis of a range of basic analytes showed an unusual extended retention of hydrophilic basic analytes with perfluorophases. Furthermore, a non-linear relationship between the amount of organic modifier and the logarithm of the retention factor was observed, for the hydrophilic bases, which could not be modelled with LC prediction softwares. This was in sharp contrast to the alkyl and phenyl phases examined. Basic analyte retention on perfluoroalkyl phases could be modelled adequately for the lipophilic bases. Exploration of the retention mechanism of these perfluoro phases indicated that silanol interactions were important in retention and selectivity. Using a rapid, isocratic, high organic modifier methodology, it was possible to analyse a mixture containing a lipophilic steroid, hydrophilic base and an internal standard in <4 minutes with a perfluorophenyl phase. This had previously only been achievable with an alkyl phase under gradient elution conditions.

**Key Words:** Perfluorinated stationary phases; Chromatographic classification; Principal Component Analysis; RP-LC

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**1 Introduction**

Perfluorinated stationary phases are becoming more widely used as an alternative to traditional C18 and C8 phases in reversed-phase liquid chromatography due to their potential for providing unique separation selectivity. As a consequence, most stationary phase manufacturers now possess one in their portfolio of phases. Perfluorinated phases have been shown to exhibit enhanced retention and selectivity in the analysis of halogenated compounds and shape selectivity for positional/geometric isomers [1–3]. The selectivity of these perfluorophases has been shown to be further enhanced by the use of fluorinated alcohols as the organic component in the mobile phase when compared to traditional C8 and C18 phases with MeOH, THF or ACN [4, 5]. In contrast to C8 and C18 phases the C-F bonds in perfluorinated phases are thought to confer a greater dipole character, which increases the phase’s interaction with polar and halogenated compounds. The combination of additional analyte interactions with the C-F dipole of the stationary phase, π-π interactions and the enhanced shape selectivity provided by the extra rigidity of the phase may provide the chromatographer with unique selectivities not obtainable with the ubiquitous alkyl phases [6].

Perfluorophases have also been found to be useful in the analysis of non-halogenated compounds such as natural product (i.e. steroids [7], carotenoids, flavonoids [1], polyphenols [1], catechins, taxanes [8], phospholipids [7], cephalosporins, and alkaloids), positional isomers of pharmaceutical starting materials, herbicides and non-ionic surfactants [9].

Recently there has been much interest in using perfluorinated stationary phases with high percentage organic content mobile phases due to the increased retention of basic analytes and the enhanced LC/MS spray characteristics afford by this approach [10, 11].

Principal Component Analysis (PCA) has previously been employed with great effect in the characterisation of sta-
tionary phases and assisting in the development of a better understanding of the underlying retention mechanisms [12–21].

The goal of this work was to characterise a range of alkyl and phenyl perfluorinated silica based stationary phases in terms of hydrophobicity, shape selectivity, hydrogen bonding capacity, and ion exchange capacity at pH 7.6 and 2.7 and compare their retention behaviour to conventional phenyl and alkyl phases. The chromatographic properties of the phases have been compared by PCA and their similarities and differences discussed in terms of the base silica and bonding technology employed in their preparation.

The fluorophases have additionally been evaluated for their chromatographic performance against a range of basic analytes of widely differing lipophilicities and pK_a values.

The chromatographic parameters of these perfluorinated and non-fluorinated phases have been used in an attempt to explain the high retentivity of these phases when used in high percentage organic content mobile phases as this approach is becoming increasingly favoured for LC/MS studies.

2 Experimental

2.1 Chemicals and reagents

All solvents used were of at least HPLC grade supplied by Romil Ltd (Cambridgeshire, UK) except for the water, which was provided by a Milli-Q-plus 185 ultra pure water system (Molsheim, France). Benzyamine hydrochloride, n-pentylbenzene, n-butylbenzene, triphenylene, o-terphenyl, caffeine, and thiourea were all supplied by Sigma-Aldrich Company (Dorset, UK). Phenol, KH_2PO_4, H_3PO_4, nicotine (free base) and procainamide hydrochloride were all supplied by Sigma-Aldrich (Dorset, UK), and four AstraZeneca compound bank – all at 0.3 mg/mL in water (with the exception of AR-C68397 which was dissolved in 20 mM KH_2PO_4 pH 2.7 buffer). The hydrophilic test mixture consisted of 100 μL each of nicotine, benzyamine hydrochloride, procainamide hydrochloride, AR-D080301, salbutamol sulphate, and phenol solutions plus 500 μL of water. The lipophilic test mixture consisted of 100 μL each of AR-C68397, AR-R12924, AR-R12495, nortriptyline, diphenhydramine, and phenol solutions plus 500 μL of water. The structures, pK_a and log P values of the basic analytes are given in Figure 1.

2.2 Instrumentation

HPLC separations were performed on an Agilent Technologies series 1100 liquid chromatograph (Agilent Technologies, Cheddle, Cheshire, UK) equipped with column switching valves (Jones Chromatography, Mid. Glamorgan, UK) and a Mistral column oven (Spark Holland B.V., Emmen, The Netherlands). The MS work was performed on an Agilent Technologies series 1100 LC/MSD equipped with an electrospray interface (Agilent Technologies, Waldbronn, Germany).

2.3 Liquid chromatography

All columns were new as supplied by the manufacturer/supplier. Table 1 highlights the manufacturers/published data available on the phases which have been evaluated.

The chromatographic conditions for the HPLC characterisation of the phases were as reported previously [12–14]. The first disturbance of the baseline on the injection of methanol was used as dead time marker.

Six variables reflecting different chromatographic properties were used for the characterisation. Each variable is briefly described below. Flow rates and injection volumes have been scaled to correspond to a 150 x 4.6 mm ID column.

Retention factor for pentylbenzene, k_{PB}: Reflects the surface area and surface coverage (ligand density). Chromatographic conditions: 8:2 v/v MeOH:H_2O, 1.2 mL/min, 40°C, individual 5 μL injections of pentylbenzene (0.6 μg/mL in MeOH).

Hydrophobicity or hydrophobic selectivity, \( \alpha_{\text{CH}_3} \): Retention factor ratio between pentylbenzene and butylbenzene, \( \alpha_{\text{CH}_3} = k_{PB}/k_{BB} \). This is a measure of the surface coverage of the phase as the selectivity between alkylbenzenes differentiated by one methylene group is dependent on the ligand density. Chromatographic conditions: 8:2 v/v MeOH:H_2O, 1.2 mL/min, 40°C, individual 5 μL injections of pentylbenzene (0.6 μg/mL) and butylbenzene (0.3 μg/mL in MeOH).

Shape selectivity, \( \alpha_{\text{O}} \): Retention factor ratio between triphenylene and o-terphenyl, \( \alpha_{\text{O}} = k_{T0}/k_{O} \). This descriptor is a measure of the shape selectivity and the functionality of the silylating reagent. Chromatographic conditions: mobile phase as above for hydrophobicity, individual 5 μL injections of o-terphenyl and triphenylene both at 0.05 μg/mL in MeOH.
Hydrogen bonding capacity, $a_{CP}$: Retention factor ratio between caffeine and phenol, $a_{CP} = k_C/k_P$. This descriptor is a measure of the number of available silanol groups and the degree of endcapping. Chromatographic conditions: 3:7 v/v MeOH:H₂O, 1.2 mL/min, 40 °C, individual 5 μL injections of phenol and caffeine (1 and 0.5 mg/mL respectively in 3:7 v/v MeOH:H₂O).

Total ion exchange capacity, $a_{IEP}$ pH 7.6: The retention factor ratio between benzylamine and phenol, $a_{IEP}$ pH 7.6 = $k_B/k_P$. This is an estimate of the total silanol activity. Chromatographic conditions: 20 mM KH₂PO₄, pH 7.6 in 3:7 v/v MeOH:H₂O, 1.2 mL/min, 40 °C, individual 5 μL injections of phenol and benzylamine·HCl both at 0.5 mg/mL in the above mobile phase.
Acidic ion exchange capacity, $a_{B/P} \text{pH} 2.7$: The retention factor ratio between benzylamine and phenol, $a_{B/P} \text{pH} 2.7 = k_B/k_P$. This is a measure of the acidic activity of the silanol groups. Chromatographic conditions: All conditions as for total ion-exchange determinations above, but using a pH 2.7 KH$_2$PO$_4$ buffer.

Hydrophilic base analysis. Chromatographic conditions: 20 mM KH$_2$PO$_4$, pH 2.7 in 3.3:96.7 v/v MeOH:H$_2$O, 1.0 mL/min, 60°C, 5 μL injection of the hydrophilic base test mixture, detection at 210 nm.

Lipophilic base analysis. Chromatographic conditions: 20 mM KH$_2$PO$_4$, pH 2.7 in 45.5:54.5 v/v MeOH:H$_2$O, 1.0 mL/min, 60°C, 5 μL injection of the lipophilic base test mixture, detection at 210 nm.

2.4 Software packages

2.4.1 Principal Component Analysis

Principal Component Analysis (PCA) was performed using Simca-P 8.1 software (Umetrics, Umeå, Sweden). All six variables from the column characterisation were included in the analysis.

2.4.2 LC computer prediction software

Drylab$^\text{m}$ 2000 (LCResource Inc. Walnut Creek, USA) was used for the LC predictions which was based on two gradients (20 and 60 minutes).

2.4.3 Log $P$ and $pK_a$ predictions

Predictions of $pK_a$ and log $P$ were calculated using Advanced Chemistry Development software programmes (Toronto, Canada).

2.5 LC/MS

LC/MS was performed using a Fluophase PFP column (5 μm, 150 × 3 mm ID) with 10 mM NH$_4$HCO$_3$H, pH 2.7 in 6:4 v/v ACN:H$_2$O mobile phase composition at a flow of 0.8 mL/min and a thermostatted column temperature of 20°C; single ion monitoring (SIM) using positive electrospray ionisation (ESI) mass spectrometry (MS) was performed using the following operational parameters: drying gas temperature of 325°C, drying gas flow rate of 12 L/min, nebuliser gas pressure of 345 kPa, a capillary voltage of 3 kV, and a fragmentor voltage of 70 V.

3 Results and discussion

3.1 Principal Component Analysis

The results of the column characterisation procedures, which have been described previously [12–14], can be found in Table 2. The numbering convention for the columns is derived from our larger column database of 135 differing stationary phases; the findings of this larger study will be reported at a later date [22]. PCA is a general tool for interpretation of large data tables [23]. The number of variables (the dimensionality of
the data – in this case the six column characterisation parameters) is reduced by a projection of the objects (stationary phases) onto a smaller number of new variables termed principal components (PC), which are orientated so that the first PC describes as much as possible of the original variation between the objects. The second PC is orientated in an orthogonal manner to the first PC and is directed to describe as much as possible of the remaining variation.

The projection of objects onto a PC is called scores – by plotting the scores for two PCs it is possible to graphically find similarities and differences between objects (stationary phases). The distance between objects in a score plot show if they are similar or different.

How much of each of the original variables that are included in a PC is described by so called loadings, one for each variable.

By plotting the loadings for two PCs, it is possible to see which of the original variables are most important (longest distance from the origin) and if any variables are correlated (the same or opposite directions on a straight line through the origin).

The reason to why two objects are different can easily be determined with a so-called contribution plot. This type of plot shows which variables that cause a difference between two objects or, alternatively, one object and the average object.

It can be concluded that the PC1–PC2 model for the ten perfluorophases describes 84% of the chromatographic variability within these phases. The PC1–PC2 score plot highlights that the ten perfluorinated phases can be categorised into three sub-groups (see Figure 2.a).

Group A contains the perfluorophenyl phases (column nos. 14, 20, 26, 61, and 71), whereas Group B contains the perfluorohexyl end-capped and perfluorooctyl phases (columns 24, 27, and 28) and Group C the perfluorohexyl non-end-capped and propyl phases (columns 25 and 72).

The loading plot (see Figure 2.b) highlights that the subgroups A–C possess the following dominant chromatographic properties: Group A, high surface area/hydrophobicity, high shape selectivity (positively correlated with the \(k_{PB}, a_{CH2}\) and \(a_{T/O}\) parameters) and low ion exchange and hydrogen bonding activity (negatively correlated with the \(a_{C/P}, a_{B/P}\) parameters); Group B, low surface coverage/hydrophobicity, low shape selectivity, (negatively correlated with the \(k_{PB}, a_{CH2}\), and \(a_{T/O}\) parameters) and high ion exchange (positively correlated with the \(a_{B/P}\) parameters); Group C, low surface coverage/hydrophobicity, low shape selectivity, low ion exchange activity (negatively correlated with the \(k_{PB}, a_{CH2}, a_{T/O},\) and \(a_{B/P}\) parameters) and high hydrogen bonding capacity (positively correlated with the \(a_{C/P}\) parameter).

These findings are in agreement with the PC1–PC2 contribution plot (Figure 3) between the FluoroSep-RP Phenyl HS phase (Group A, column no. 71) and the Fluofix end-capped phase (Group B, column no. 24) which highlights that the Group A phases exhibit increased retention (due to their high surface area), high shape selectivity (phenyl compared to an alkyl phase), low hydrogen bonding and ion exchange capacity (due to higher ligand bonding density or hindered steric accessibility) compared to the perfluoroalkyl phases.

In comparison, Figure 4 highlights the PC1–PC2 contribution plot between the FluoroSep-RP Octyl (Group B,
column 28) and the Fluofix non-end-capped phases (Group C, column 25). The main difference between the two phases appears to be that the former appears to possess a greater acidic silanol and a lower hydrogen bonding capacity compared to the latter phase.

In contrast to the PCA analysis of C18 phases in which the chromatographic parameters $a_{CP}$ and $a_{BP} pH 7.6$ showed a high degree of correlation [14, 15], the same terms did not correlate for the perfluoro phases examined in our database.

In order to compare the chromatographic properties of these perfluoro and non-perfluoro phases, two phenyl and three alkyl phases were added to the database. Unfortunately, the non-perfluoro versions of the phases, based on the same base silica as used for the perfluoro phases, were not available. It is known that most, if not all, of the perfluoro phases are based on high purity silica, hence the phenyl (XTerra and ACE) and alkyl (HyPURITY range) comparator phases, which are based on pure silica, were selected.

With the increased dataset, the PC1–PC2 score plot (see Figure 5.a) accounted for 75% of the variation of the data and yielded two distinct groups for the perfluoroalkyl and perfluorophenyl phases. The perfluoroalkyl phases exhibited low retentivity, shape selectivity (i.e. negatively correlated to $u_{LO}$, $u_{CH2}$, and $k_B$ parameters), and enhanced hydrogen bonding and ion exchange capacity (positively correlated to the $u_{CP}$ and $a_{BP} pH 2.7 & 7.6$ parameters) in
comparison to the perfluorophenyl phases, which exhibited greater retention and enhanced shape selectivity (i.e. positively correlated to $a_{T/O}$, $a_{CH_2}$, and $k_B$ parameters) (see Figure 5.b). The two-phenyl comparator phases were shown to be distinct in their chromatographic properties from the perfluorophenyl phases. The PCA contribution plot for the Monochrom MS (column no. 61, a typical perfluorophenyl phase) against the XTerra or ACE phenyl (column nos. 117 and 4) (see Figure 6.a for the XTerra results) highlighted the enhanced shape selectivity of perfluorophenyl compared to the phenyl phases, this may be attributed to the extra rigidity of the perfluorophases and may partially explain the high selectivity of these phases towards positional/geometric isomers [1–3]. Among the 135 RPLC columns that have been characterised by our group [22] the perfluorophenyl phases displayed the highest values for shape selectivity ($a_{T/O} > 2.5$) whereas the perfluoroalkyl phases displayed the lowest values ($a_{T/O} < 0.7$), i.e. these phases display a different elution order for triphenylene and $\sigma$-terphenyl. Other RPLC phases typically display a $a_{T/O}$ value between 1 and 2.

3.2 Evaluation of perfluorophases in the analysis of basic analytes

Evaluation of these perfluorinated phases (plus the two phenyl and a C18 phase for comparison purposes) in the analysis of a range of basic analytes of varying lipophilicity and $pK_a$ was performed using 20 mM KH$_2$PO$_4$ pH 2.7 in varying proportions of MeOH/water. Mobile phases compromising of 3:3: 96.7 and 45.5: 54.5 v/v MeOH/water
were employed for the hydrophilic and lipophilic bases respectively and phenol was included in each mixture to act as a control marker.

The perfluorophenyl phases (as exemplified by Figure 7.a) exhibited enhanced retention of hydrophilic bases compared to the non-perfluorophenyl and C18 phases (see Figure 7.b and Figure 10.a). The hydrophilic base salbutamol (compound 5) failed to elute within 30 minutes on the Fluophase PFP column (see Figure 7.a). Not surprisingly, the lower alkyl chain perfluoro phases (see Figure 7.c, Figure 7.d, Figure 7.e, and Figure 7.f) were not as retentive as the perfluorophenyl phases. It was observed that the Drylab\textsuperscript{2} 2000 LC predication software failed to correctly predict the elution order of these hydrophilic bases plus phenol from 20 and 60 minute gradient LC experiments on the perfluorinated phases. This was in contrast to that observed for the HyPURITY C18 and ACE Phenyl phases where the simulations matched the experimental values very closely. The lack of modelling with the perfluorophases became apparent when a graph of log $k$ versus percentage methanol was constructed for the Discovery F5 HS perfluorophenyl phase (ionic strength constant) in which the bases failed to exhibit a linear relationship between log $k$ and percentage methanol (for example see Figure 9). In contrast to the bases, the neutral analyte, phenol, exhibited the expected linear relationship suggesting that partitioning does not solely control the retention mechanism of the hydrophilic bases. Figure 9 highlights the interesting properties that these phases possess for controlling and exploiting differences in selectivity as exemplified by the crossover of peaks in the figure. If these perfluorophenyl phases are employed in stationary phase screening programmes for column selection using gradient LC conditions then non-standard LC prediction models may have to be employed.

Using the Fluofix end-capped and the FluoroSep-RP Octyl phases, log $k$ versus percentage methanol plots appear to be linear for the lipophilic bases as there is good agreement between the simulated and experimental retention times. It was not possible to assess this with the perfluoro-
phenyl phases from the two gradient approach as the bases failed to elute in the linear part of the gradient.

In all cases for alkyl and phenyl phases the bases all eluted before the neutral analyte phenol [23], however, this certainly was not the case with the perfluorophases examined (see Figure 9). The difference in slope in the log \( k \) versus percentage methanol in the mobile phase further supports the difference in retention mechanism between the protonated bases and the neutral phenol.

The analytes nicotine and procainamide (compounds 1 & 4) appear to be very susceptible to silanol interactions in that on comparison of the non-endcapped and endcapped Fluofix phases (col. No. 25 & 24 respectively, see Figure 7.e and Figure 7.f) these analytes are retained longer on the non-endcapped phase compared to the other analytes. Extremely broad and retained peaks are also observed for nicotine and procainamide on the FluoroSep-RP Octyl phase (see Figure 7.c). The fluorophases provide orthogonal selectivity compared to the phenyl and alkyl phases, especially in the analysis of hydrophilic bases, which is extremely desirable in drug purity screening. Comparison of the perfluorophenyl (column no. 26) and the XTerra phenyl stationary phases (column no. 117) clearly highlights the orthogonality of the retention mechanism as exemplified by the separation of the hydrophilic (compounds 3, 4, and 5, see Figure 7.a and Figure 7.b respectively) and the lipophilic basic analytes.
Similar conclusions can be drawn by comparing the different elution orders obtained on the perfluoroalkyl phases (for example the Fluofix end-capped phase, column no. 24) and the conventional C18 phase (HyPURITY C18, column no. 42) for the hydrophilic (see Figure 7.e and Figure 10.a) and lipophilic basic analytes (see Figure 8.f and Figure 10.b). The perfluorophenyl phases were shown to be considerably more retentive towards basic analytes than the phenyl or alkyl comparator phases studied (see Figure 8.a, Figure 8.b, and Figure 10.b). In fact the lipophilic nortriptyline (compound 11) failed to elute within 30 minutes on the Fluophase PFP column (see Figure 8.a).

3.3 Investigation into the retention mechanism of perfluorophases using high organic solvent containing mobile phases

The ability of the perfluorinated phases to demonstrate high retentivity of basic analytes in mobile phases containing high proportions of organic solvent is extremely attractive for chromatographers. LC/MS of hydrophilic bases can be problematic with traditional C8 or C18 reversed-phase materials in that high aqueous/low organic content mobile phases are required in order to retain the bases. These mobile phase conditions result in reduced MS sensitivity due to poor spraying and the increased surface tension of the aqueous droplets [24]. In comparison, the use of high organic content mobile phases coupled with the use of volatile buffers, such as ammonium formate, facilitates the desolvation process in LC/MS, thereby enhancing sensitivity [25–28].

Another potential problem is that if the early eluting peaks cannot be drawn away from the solvent front then there may be MS signal suppression as a result of co-eluting matrix components.

In order to gain a better understanding of the retention mechanism on these perfluorophases a plot of the logarithm of the retention factor of a range of basic analytes, of differing log \( P \) and \( pK_a \) values, versus percentage acetonitrile in the mobile phase was constructed. This showed a typical reversed-phase mechanism (see Figure 11). The hydrophobic bases exhibited a decrease in retention as a function of increasing percentage acetonitrile whereas the hydrophilic bases did not follow this trend as they eluted close to the void volume. This is in contrast to a similar perfluorophenyl phase (i.e. Monochrom MS) which when used with mobile phases containing in excess of 80\% acetonitrile exhibited extremely high retention factors for propranolol and amitriptyline [10]. The reason for this can be easily explained when one examines the retention of the bases as a function of the reciprocal of buffer concentration (see Figure 12). Figure 12 displays a two phase mechanism; at high buffer concentrations (i.e. > 0.5 mM) a typical reversed-phase behaviour is observed, whereas <0.5 mM an ion exchange mechanism predominates.
This suggests that when one increases the percentage acetonitrile without keeping the buffer concentration constant an ion exchange mechanism will predominate over a reversed-phase one and hence the retention of the bases will increase.

If this is the case then it could be expected that traditional reversed-phase materials based on acidic silica such as the Hypersil ODS would also exhibit high retention of bases when high percentage acetonitrile and low ionic strengths were employed. Figure 13 confirms the high retention of two basic analytes on a Hypersil ODS phase using high organic and low buffer concentration LC conditions. Hence the retention mechanism of these perfluorophases with high organic content mobile phases is proposed to be similar to that of Hydrophilic Interaction Chromatography (HILIC, [29, 30]).

4 Concluding remarks

It can be concluded that the chemometric approach of PCA is an extremely efficient tool for displaying the similarities and differences of ten perfluorinated phases, in terms of their chromatographic properties. This is particularly desirable as there is very little information available either from the manufacturers or in the published domain relating to the chromatographic properties of these perfluorophases. This paper provides an unbiased evaluation of these phases’ chromatographic properties using a stan-
standardised testing protocol. The PCA highlighted that the main difference in these perfluorophases compared to conventional alkyl and phenyl phases appears to be their high discriminating power towards shape selectivity. PCA contribution plots where shown to be an extremely visual approach to highlighting the differences between the phases. The orthogonality of the perfluorophases compared to the conventional phases was established for the hydrophilic and lipophilic bases. The basic analytes were considerably more retained, whereas the neutral analytes were less retained, on the perfluorophases compared to the non-fluorophases. The high retentivity of bases towards these perfluorophases in mobile phases containing high organic modifier and low buffer concentration appears to be controlled by a HILIC mechanism. The applicability of using these perfluorophases in the rapid LC/MS analysis of a mixture containing a lipophilic steroid and a hydrophilic base plus a basic internal standard has been successfully highlighted.

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