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Journal of Chromatography A, 841 (1999) 147–154

JOURNAL OF CHROMATOGRAPHY A

Characterisation of π–π interactions which determine retention of aromatic compounds in reversed-phase liquid chromatography

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Received 11 November 1998; received in revised form 13 January 1999; accepted 17 February 1999

Abstract

This paper presents the influence of π–π interactions on the retention behaviour of aromate-rich and aromate-poor analytes separated on various chromatographic sorbents. The presence of π–π interactions between the analytes and a polymer based sorbent can be very useful for separating compounds with similar retention behaviour on silica based reversed-phase sorbents (where this type of interaction is absent). Various C_4 and C_{18} modified silica sorbents (5 μm and 15 μm) are compared with some poly(styrene-divinyl benzene) (PS-DVB) sorbents. For this purpose five aromates and four non-aromates were selected. The retention times of the nine uncharged test compounds (hydrocortisone, hydrocortisone acetate, testosterone, testosterone propionate, nitrobenzene, anisole, toluene, valerophenone, cumene) increase with increasing hydrophobicity (log P). On silica based reversed-phase sorbents this relationship is the same for aromates and non-aromates. On PS-DVB sorbents this relationship is different: the aromatic compounds need more acetonitrile to elute from the column than the non-aromatic compounds with a similar hydrophobicity: at log P 2.7 the difference in acetonitrile concentration is approximately 18–20% for all tested polymer matrices which have no groups covalently bound to their PS-DVB moieties (Vydac™, Polymer Laboratories, Amersham Pharmacia Biotech). This difference is probably due to the presence of an π–π interaction between the sorbent and the analyte. The importance of the π–π interaction decreases when more acetonitrile is needed to elute an aromatic analyte from the column. The retention of these mono-aromates largely depends on hydrophobic interactions and π–π interactions. The higher the log P of the analyte (containing an aromatic group), the more important the hydrophobic interaction becomes. Although covalent coupling of functional groups to the PS-DVB sorbent (with a phenyl, isopropyl or ether linked via a $-CH_2CHOHCH_2O-$ spacer) leads to a more hydrophilic sorbent, it does not lead to a decrease in π–π interaction since the surface still accessible for the compounds tested. A comparison of the retention behaviour of the aromatic test compounds with the non-aromatic test compounds on silica based reversed-phase sorbents with that on PS-DVB based sorbents shows the importance of determining π-π interactions.
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Keywords: π–π interactions; Stationary phases LC; Aromatic compounds

1. Introduction

Hydrophobicity [1,2], electrostatic interactions [3,4], π–π interactions [5–8], hydrogen bonding [9], dipole–dipole interactions, steric hindrance as well as a combination of them [10,11] are, among the most important interactions that determine the retention behaviour of substances in reversed-phase high-performance liquid chromatography (RP-HPLC). Although a large amount of literature is

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PII: S0021-9673(99)00253-8

available on the determination of these interactions, little information is available on the characterisation of π–π interactions. This type of interaction can be defined as the interaction between π-electrons of the chromatographic material and those of the solute species [7,8,11,12]. An interaction between π-electron containing compounds is favoured when one of the compounds (e.g. the stationary phase) is electron-rich and one is electron-poor (e.g. the solute) and vice versa [5,7,13]. In these systems the stationary phase can act as donor (soft Lewis base), while the solute can act as recipient (soft Lewis acid) of electrons [14,15]. The stability of the π-donor/π-recipient complex is determined by the energy levels of the Highest Occupied Molecular Orbital (HOMO) and the Lowest Unoccupied Molecular Orbital (LUMO) of the donor and recipient respectively [7]. π–π interactions do not contribute to the retention behaviour as much as hydrophobic interactions or electrostatic interactions do. However, π–π interactions can successfully be utilised in the separation of closely related compounds like metabolites or degradation products (finely tuned separation). This feature is used in the separation of chiral compounds [5,6,12,16–18]. These findings are usually based on specific π–π interactions between modified silica or methacrylate sorbents, with aromatic moieties, and aromatic solutes. However, on PS-DVB sorbents, the π–π interaction is usually stronger due to the high content of aromates on the surface of the sorbents. To estimate the contribution of aromate-aromate interactions to the overall retention behaviour, it is important to measure the strength of this type of interaction. This article presents a validated method which can be used to determine the influence and contribution of π–π interactions to the retention of aromatic compounds on PS-DVB and silica based reversed-phase sorbents from different manufacturers.

2. Experimental

2.1. Chemicals and columns

All chemicals used were of analytical grade. The test substances anisole, cumene and toluene were obtained from MERCK (Darmstadt, Germany), nitrobenzene was obtained from AnalaR (Poole, England), valerophenone, hydrocortisone and hydrocortisone acetate were obtained from Sigma (St. Louis, MO, USA). Testosterone and testosterone propionate were obtained from Fluka (Oslo, Norway). Table 1 shows the chemical structures and log P [19,20] values of the compounds tested.

For this study the following columns and system were used: Five μm particles in 4.6×250 mm steel columns: SOURCE™5RPC particles were packed in a Supelco steel column (Bellefonte, PA, USA). Vydac Protein & Peptide C_{18}(Hesperia, CA, USA), Biorad HiPore RP (Hercules, CA, USA), Serva Si–C_{18} (Heidelberg, Germany). Polymer Laboratories PLRP-S (Teknolab A/S, Drøbak, Norway). Five μm particles in 4.6×150 mm steel columns Hypersil 100 Å C_{18} (Runcorn, UK), Polymer Laboratories PLRP-S, Vydac Polymer RP. SOURCE 5RPC particles were packed in a 4.6×150 mm Supelco steel column. Fifteen μm particles in HR5/5 columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden): SOURCE™15RPC, SOURCE™15PHE, SOURCE™15ETH, SOURCE™15ISO (Amersham Pharmacia Biotech AB, Uppsala, Sweden), Kromasil® Si–C_4, Kromasil Si–C_{18} (Akzo Nobel, Bohus, Sweden) and Polymer Laboratories PLRP-S (15–20 μm).

2.2. HPLC equipment and methods

Two HPLC systems were used:

(1) A HPLC system consisted of: Two Waters 510 HPLC pumps, Waters 715 Ultra Wisp autoinjector, Waters 996 PDA detector, Waters System Interface Module, Millennium 2010 data acquisition software (Millipore Inc., Milford, USA) and a LKB High Pressure Mixer. Dwell volume is approximately 1.0 ml.

(2) An ÄKTA™ explorer 10XT with UNICORN 2.20 data acquisition (Amersham Pharmacia Biotech AB). Dwell volume is approximately 0.70 ml. In all cases gradient elution was carried out. Mobile phase A consisted of 5% (w/w) acetonitrile in MilliQ-water (Millipore Inc.), mobile phase B consisted of 95% (w/w) acetonitrile in MilliQ-water. Gradient: 0–100% B in 40 min, 100% B isocratic for five min., back to 0% B in one min. Each column was

Table 1
Chemical structure and log P value of the test compounds used

Compound	Abbreviation	Structure	Log P[a]
Hydrocortisone	H		1.61
Hydrocortisone acetate	H-Ac		2.30
Testosterone	T		2.72
Testosterone propionate	TPr		3.72
Nitrobenzene	N	NO_2	1.85
Anisole	A	OMe	2.11
Toluene	To	CH_3	2.73
Valerophenone	V	$CO(CH_2)_3CH_3$	3.26
Cumene	C		3.53

[a] Log P values are experimentally determined [19,20].

equilibrated with 15 column volumes at start conditions. Injection volume 10 μl, flow rate 1 ml/min, for photo diode array detection (system 1), the detection range was from 300 to 200 nm for multiple wavelength detection (system 2), 205 nm, 240 nm and 263 nm were chosen as detection wavelengths. The sample was a mixture of the five aromates (5 m*M*) and four steroids (2.5 m*M*) in methanol. Retention times are corrected by subtracting the entire system volume from the observed retention time. In this way the retention times of both systems were comparable. The entire system volume is

defined as the dwell volume added to the column volume and the volume of the peek tubing from the injector to column and the column to the detector.

3. Results and discussion

3.1. Choice of test compounds and method validation

To be able to characterise the strength of the π–π interactions between aromatic analytes and the surface of the PS-DVB sorbents, nine uncharged test compounds, with an increasing hydrophobicity (log P), were chosen [19,20]: five aromates and four non-aromatic compounds. Steroids were chosen as non-aromatic compounds because they are detectable with UV (240 nm). The method as described in the experimental section (system 2) was validated on HR5/5 columns with respect to the retention time (response variable) [21–23]. The solute concentration had no significant influence on the retention time

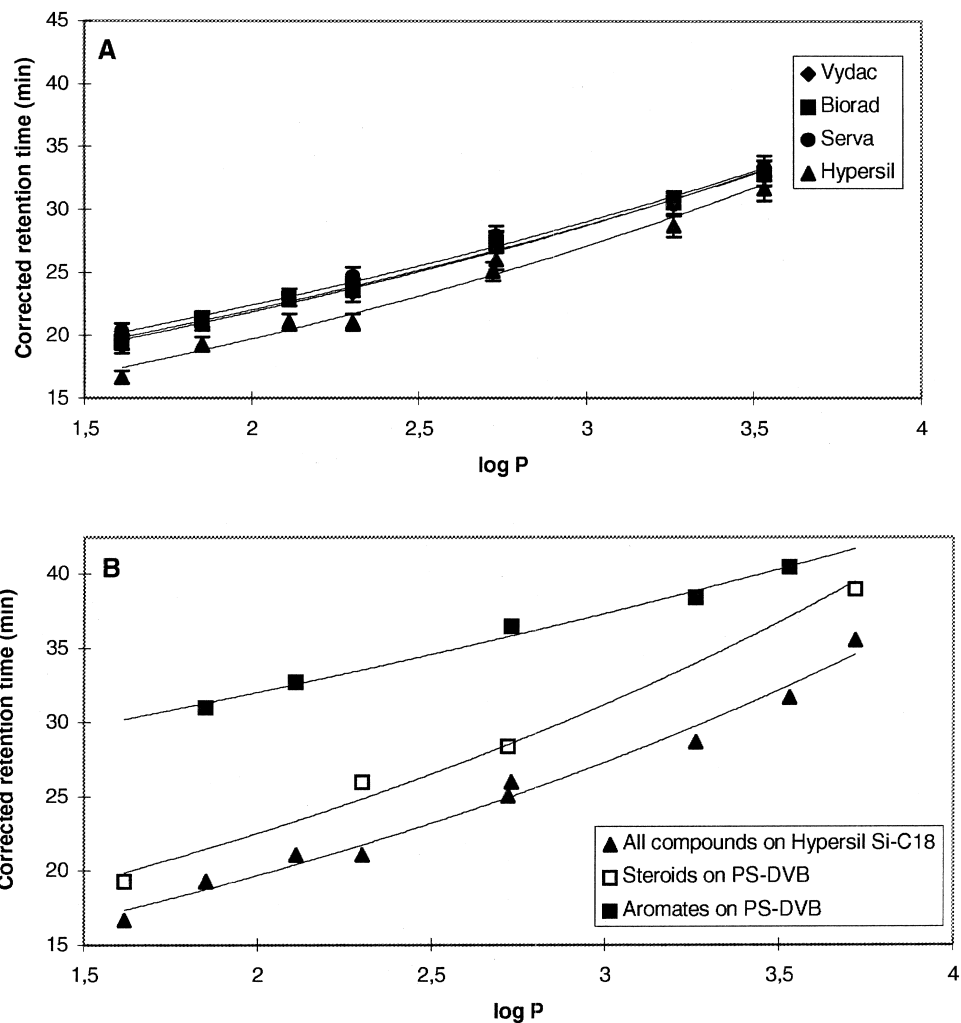

Fig. 1. (A) Corrected retention time plotted against log P. Chromatography carried out on system 1. Columns tested: $Si–C_{18}$; Vydac, Biorad, Serva 4.6×250 mm. Hypersil 4.6×150 mm. (B) Corrected retention time plotted against log P. Chromatography carried out on system 1. Columns tested: Hypersil $Si–C_{18}$ and SOURCE 5RPC, both 5 μm and packed in 4.6×150 mm (steel).

in the concentration range 1–10 m*M* for all the solutes. The relative standard deviations for the intraday precision for the retention times of all compounds are lower than 0.5%. The relative standard deviation for the interday precision varies between 1 and 3%.

3.2. Retention order/peak identification on Si–C_{18} and PS-DVB columns

Peak identification was carried out by injecting all compounds separately and by using photo diode array detection. In this way, the retention times of all compounds could be determined in a single run.

When the corrected retention times of the compounds on the 5 μm silica based reversed phase particles are plotted against the log P value, a good correlation is obtained ($r>0.99$, Fig. 1A).

When a similar experiment is carried out on a SOURCE 5RPC sorbent, this plot (corrected retention times versus log P) looks different. Fig. 1B shows the comparison between the SOURCE 5RPC sorbent and a Hypersil ODS reversed-phase sorbent. The SOURCE 5RPC sorbent appears to be more hydrophobic than the Hypersil ODS reversed-phase sorbent since a higher acetonitrile concentration is needed to elute the test compounds from the column. Another, more important, observation is that the relationship between corrected retention time and log P is disturbed: compared with the steroids, the aromatic test compounds need a relatively higher concentration of acetonitrile to elute from the column: there is a large difference in retention time (~eight min.) between testosterone (log P=2.72, $t_{corr.}$=28.39 min) and toluene (log P=2,73, $t_{corr.}$= 36.48 min). In comparison with Kromasil Si–C_{18}, these compounds elute much closer to each other ($\Delta t_{corr.}$=0.9 min). This difference might be explained by the interaction between the aromate-rich PS-DVB surface and aromatic analytes. This interaction is absent for the steroids on PS-DVB and absent for all the solutes on Si–C_{18}.

3.3. Comparison of different column materials

Both 5 μm and 15–20 μm particles were used to compare the different column materials.

Table 2 shows the corrected retention times of all analytes on two different columns (4.6×150 mm and 4.6×250 mm) and three different polymer sorbents. In all cases the aromates need a relatively higher acetonitrile concentration than the steroids to elute from the columns, considering their log P. The difference in retention between toluene and testosterone varies from 8.1 min (SOURCE 5RPC) to 8.8 min (Vydac and PLRP-S). This indicates that there is a stronger π–π interaction on the Vydac and PLRP-S sorbent than on the SOURCE 5RPC sorbent. Fig. 2 shows the chromatograms from the separation of the test compounds on 5 μm Vydac (A), PLRP-S (B) and SOURCE 5RPC (C) matrices. The highest resolution and the narrowest peaks were obtained on the SOURCE 5RPC sorbent ([Fig. 2 (C)]. On the other sorbents testosterone propionate is not separated

Table 2
Corrected retention time of the test compounds on five different sorbents (5 μm) in steel columns

Column	Corrected retention time								
	Hydrocortisone	Hydrocortisone acetate	Testosterone	Testosterone propionate	Nitrobenzene	Anisole	Toluene	Valerophenone	Cumene
PLRP-S 4.6×150 mm	10.91	15.81	17.48	28.99	20.95	22.44	26.08	27.43	30.42
Vydac 4.6×150 mm	10.72	15.53	17.63	28.69	21.54	22.97	26.46	27.66	29.89
PLRP-S 4.6×250 mm	13.72	19.49	21.94	35.16	25.37	27.07	30.80	31.90	34.79
SOURCE 5RPC 4.6×250 mm	14.09	21.28	24.29	38.67	27.07	28.80	32.44	34.07	36.03
SOURCE 5RPC 4.6×150 mm	19.29	25.99	28.39	39.00	30.99	32.70	36.48	38.43	40.49

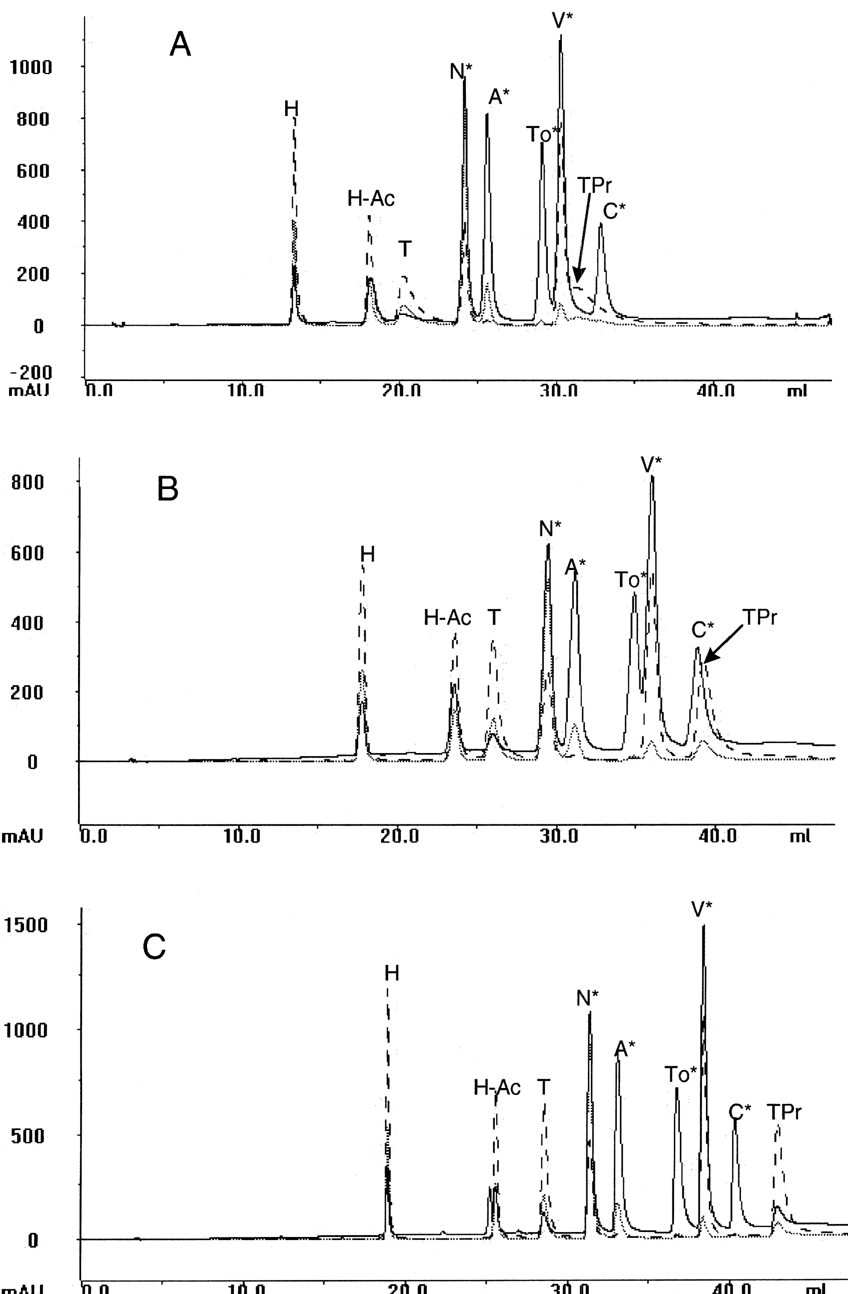

Fig. 2. Chromatograms of the nine test compounds analysed on system 2. For abbreviations see Table 1, aromatic compounds are marked with an '*'. (—) 205 nm, (---) 240 nm, (. . .) 263 nm. (A) Vydac 5μm in 4.6×150 mm, (B) PLRP-S 5 μm in 4.6×250 mm, (C) SOURCE 5 RPC 5 μm in 4.6×250 mm.

from valerophenone and/or cumene. This is probably due to the stronger π–π interaction on these sorbents: stronger π–π interaction result in longer retention of the aromates.

The bigger particles (15–20 μm) were all packed in HR5/5 glass columns. The test compounds were analysed on five PS-DVB sorbents (PLRP-S and SOURCE, Table 3) and compared with the analyses

Table 3
Sorbents used in the comparison experiments

Sorbents used in the comparison experiments

Sorbent	Column size	Particle size	Surface chemistry
Kromasil Si-C_{18}	HR5/5 : 5X50 mm	16 μm	$-O-Si-(CH_2)_{17}-CH_3$
Kromasil Si-C_4	HR5/5 : 5X50 mm	16 μm	$-O-Si-(CH_2)_3-CH_3$
PLRP-S	HR5/5 : 5X50 mm	15 -20 μm	polystyrene cross-linked with divinylbenzene
SOURCE 15RPC	HR5/5 : 5X50 mm	15 μm	polystyrene cross-linked with divinylbenzene
SOURCE 15PHE	HR5/5 : 5X50 mm	15 μm	
SOURCE 15ETH	HR5/5 : 5X50 mm	15 μm	
SOURCE 15ISO	HR5/5 : 5X50 mm	15 μm	

on two silica based reversed-phase sorbents (Kromasil Si–C_4, Kromasil Si–C_{18}, Table 3). All results are summarised in Table 4. Although the resolution is much better on the 5 μm particles due to better kinetics, the retention order is the same: The PLRP-S sorbent does not separate from cumene and testosterone propionate, whereas the SOURCE 15RPC sorbent does.

From Table 4 it can be seen that Kromasil Si–C_4 is less hydrophobic than Kromasil Si–C_{18}. This is due to the shorter chain length of the Kromasil Si–C_4 sorbent. For the polymer media, SOURCE 15RPC is the most hydrophobic one. SOURCE media modified with PHE, ISO and ETH are less hydrophobic than the silica based RPC media and the PS-DVB media. However, these polar functionalities do not influence the strength of the π–π interaction. The difference between the retention times of toluene and testosterone varies between 5 and 5.7 min which is in the same range as with PLRP-S and SOURCE RPC. Another interesting observation is that the trend lines of the aromates ($t_{corr.}$ versus log P) and steroids ($t_{corr.}$ versus log P) tend to cross (when extrapolated). This might be explained by the increase of the alkyl portion in the solute structure which will result in the greater contribution of hydrophobic interactions compared to the constant π–π interaction.

Introducing functional groups, other than PS-DVB on the surface, does not lead to a large decrease in π–π interaction. This is probably due to the hydrophilic nature of the spacer which is between the functional group and the PS-DVB surface. The hydrophobic surface forces the hydrophilic spacers to stretch, partly uncovering the PS-DVB surface. In this way the small test compounds can easily interact with the uncovered surface of the sorbent. This indicates that although the surface is chemically

Table 4
Corrected retention time of the test compounds on seven different sorbents (15–20 μm) in HR5/5 glass columns

Column	Corrected retention time								
	Hydrocortisone	Hydrocortisone acetate	Testosterone	Testosterone propionate	Nitrobenzene	Anisole	Toluene	Valerophenone	Cumene
Kromasil Si–C_{18}	11.33	15.34	18.00	28.09	13.56	15.32	19.59	22.14	24.98
Kromasil Si–C_4	10.61	14.15	14.65	23.38	10.62	11.58	15.06	18.50	20.42
PLRP-S	8.46	12.70	14.26	24.98	15.44	16.67	20.01	21.90	24.63
SOURCE 15RPC	10.33	15.06	16.66	25.72	17.10	18.24	21.63	23.74	25.66
SOURCE 15PHE	4.94	9.66	12.05	17.59	13.70	13.84	17.01	17.72	19.70
SOURCE 15ETH	5.96	9.13	11.49	17.14	13.31	13.66	16.84	17.43	19.46
SOURCE 15ISO	5.19	8.41	11.07	16.44	13.22	13.62	16.78	17.33	19.37

modified, the π–π interaction still can be used to separate aromate-rich and aromate-poor analytes with similar hydrophobicities. In general, the introduction of functional groups, other than PS-DVB, on SOURCE leads to a more hydrophilic sorbent.

4. Conclusion

The chromatographic method described yields acceptable data with respect to its validation results: No significant influence of analyte concentration (1–10 m*M*) on the retention time (response variable) was found, the relative standard deviation for intraday experiments was <0.5% and for interday experiments was 1–3%.

The retention time of the aromatic and non-aromatic test compounds increases with increasing log P. For aromates and steroids this relationship is the same on silica based reversed-phase sorbents (5 μm and 15 μm), but different on PS-DVB sorbents: the aromatic compounds need a higher concentration of acetonitrile than the non-aromatic compounds to elute from the column. This could be explained by a presence of an π–π interaction between the sorbent and the analyte thus having an additional retentive site. The difference in retention time between toluene (log P=2.73) and testosterone (log P=2.72) can be considered a measure of the strength of the π–π interaction. On silica based reversed-phase sorbents these two compounds almost co-elute, while on all PS-DVB sorbents toluene is retained longer than testosterone: toluene elutes approximately 8–9 min later. The difference in concentration of acetonitrile needed to elute an aromatic and a non-aromatic compound with similar log P tends to decrease as their log P value increases. Comparing various PS-DVB particles, the highest resolutions are obtained on SOURCE 15RPC (for 15 μm) and the SOURCE 5RPC (for 5 μm). The introduction of functional groups to the PS-DVB sorbent does not result in a large decrease in π–π interaction since the surface still is good accessible. However, it does lead to a more hydrophilic sorbent.

Acknowledgements

The authors would like to thank Dr. Geir Fonnum from Amersham Pharmacia Biotech AS (Lillestrøm, Norway) for his valuable comments.

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