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Practical implications of the "Tanaka" stationary phase characterization methodology using ultra high performance liquid chromatographic conditions

Melvin R. Euerby[a,*], Matthew James[a], Patrik Petersson[b]

[a] Hichrom Ltd, 1 The Markham Centre, Station Road, Theale, Reading, Berkshire RG7 4PE, United Kingdom
[b] Novo Nordisk, Hagedornsvej 1, DK-2820 Gentofte, Denmark

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ABSTRACT

The practical implications of performing column characterization protocols (i.e. Tanaka) and their resultant chromatographic selectivity parameters using small dimension columns (i.e. 50 × 2.1 mm I.D.) at high pressures have been critically compared to those obtained using conventional LC methodology. Retention factors should be corrected for the system extra column volume even when determined on ultra high performance liquid chromatographic (UHPLC) systems with low system volumes. An increase in pressure resulted in a general increase in the retention factor for most analytes, the degree being dependent on the physico/chemical properties of each analyte and the chromatographic conditions employed. However, analytes chromatographed at pH values close to their pK_a values exhibited a substantial decrease in retention factor. Performing the Tanaka and extended column characterization procedures at pressures that would be encountered during the characterization of small particle sizes packed into 50 × 2.1 mm I.D. column formats at a constant linear velocity according to standard protocols, resulted in comparable chromatographic selectivity parameters to those determined using standard HPLC systems and column formats. However, due to the wide structural diversity of analytes employed in other popular column characterization protocols, it is imperative to demonstrate comparable results when small columns packed with small particle sizes are chromatographed at increased pressure and compared to standard column formats – otherwise erroneous comparisons and conclusions may be made.

1. Introduction

The literature contains numerous approaches in which LC stationary phases can be chromatographically characterized with respect to chromatographic selectivity and peak shape [1–11]. To date most of these procedures have been performed using conventional HPLC instrumentation on phases of 3 or 5 μm particle size packed into column dimensions of 150 × 4.6 or 250 × 4.6 mm I.D. The chromatographic fraternity have found these unbiased and independent column characterization databases invaluable for the identification of replacement LC phases which possess very similar chromatographic selectivity, as well as for the identification of phases with different selectivities for method development strategies [12,13].

With the increasing popularity of ultra high performance liquid chromatography (UHPLC) and the development of columns, packed with small particles, designed specifically for use at high operating pressures (>400 bar), there is a need to assess the validity of column characterization results obtained under UHPLC compared to the results generated on traditional HPLC columns. This is especially important for chromatographers who wish to transfer a traditional HPLC methodology using a conventional phase to a UHPLC phase of similar chromatographic selectivity. Many of the newer UHPLC phases are only available in small column dimensions (i.e. typically 50 × 2.1 mm) packed, typically, with sub-three micron particles which ideally should only be used with fully optimized UHPLC systems (i.e. possessing low system dispersions, low dwell volumes, high detector sampling rates and that can be used at high pressures). The ability to translate between HPLC and UHPLC methodologies using the same stationary phase but with differing particle sizes and column dimensions is vital as within many analytical laboratories there will be an inevitable transition period as traditional HPLC systems are replaced by UHPLC systems (this will be especially pertinent for contract research laboratories).

It was established over 40 years ago [14,15] that pressure can change the molar volume of solutes which as a result, can affect the retention characteristics of the analyte as pressure is increased [16–20]. As a consequence of this it is possible that chromatographic selectivity may change on increasing pressure as analytes are affected by pressure to differing degrees. McCalley has recently highlighted that selectivity differences can, indeed, be seen as a function of differences in retention of certain analytes when

* Corresponding author. Tel.: +44 0118 930 3660; fax: +44 0118 932 3484.
E-mail address: meuerby@hichrom.com (M.R. Euerby).

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increased pressure is applied [21]. They also warned that the selectivity of a separation developed on a larger particle column may not be absolutely reproduced in terms of selectivity when transferred to a small particle column operating at high pressures, even if the particles were nominally the same.

The robustness of the popular and well established Tanaka column characterisation protocol [5] employing conventional HPLC columns and instrumentation has been well proven [22], and a small number of published papers have started to compare the selectivities of sub-two micron phases [23]. However, to our knowledge no one has sought to assess the validity of the Tanaka results for the same stationary phase material packed into a standard column format obtained on standard instrumentation, to those of the identical phase packed into high pressure/small column format using UHPLC instrumentation. In addition, the effect of high operating pressures in combination with increased flow rate has not been adequately assessed.

In order to assess the practical implications of increased pressure on the Tanaka column characterisation protocol, a well characterized 3 μm material was packed into a 50 × 2.1 mm I.D. column, suitable to withstand pressures on standard HPLC (400 bar) and UHPLC systems (1000 bar) operating under typical conditions.

The effects of increasing pressure alone at scaled constant linear velocity (by the incorporation of a suitable length of 25 μm PEEK tubing post column) and the combined effect of increased pressure and linear velocity (by simply increasing the flow rate) on the retention and selectivity factors were investigated. This approach was undertaken to remove any underlying effects from selectivity differences which can be observed with materials of nominally the same type which only differ in particle size [23].

This paper discusses the practical implications of performing the Tanaka characterization (plus its extended modifications [5,8–10,22–26]) using small dimension columns (i.e. 50 × 2.1 mm I.D.) when used at high pressure/linear velocity operating parameters which are favoured by many UHPLC practitioners. The results discussed are critically compared to those obtained using conventional LC methodology.

2. Experimental

2.1. Chemicals and reagents

All water and solvents used were of at least HPLC grade supplied by Lab-Scan/Poch S.A. (Gliwice, Poland). Test analytes and mobile phase chemicals were supplied by Sigma–Aldrich (Poole, UK) and Fisher Scientific (Loughborough, UK). The Tanaka column characterization and its extended modifications were performed as reported previously [5,8–10,22–26]. The basic analytes AZ1, AZ2 and AZ3 were kindly supplied by AstraZeneca R&D Charnwood (Loughborough, UK).

2.2. Principal component analysis of the column characterization parameters

Principal component analysis (PCA) was performed using Simca-P+ version 11.5 software (Umetrics, Umeå, Sweden). All six variables describing retention and selectivity differences were included in the analysis. In order to give all variables the same importance the variables were “auto scaled”, i.e. the average was subtracted from each variable and each variable was divided by its standard deviation.

2.3. Instrumentation

HPLC separations were performed on the following Agilent Technologies LC systems equipped with ChemStation version LC software as specified.

An Agilent 1200 Rapid Resolution LC (RRLC) HPLC system equipped with a binary pump model G1312B, a degasser model G1379B, an autosampler model G1367C, column oven model G1316C and a photodiode array detector model G1315C equipped with a micro flow cell (1.7 μL volume and 3 mm path length), no mixer or dampener, extra column volume = 31.6 μL (definition see Section 2.4.1) was used. The system was controlled and data collected by means of Chemstation version B.04.01 (Agilent Technologies, Waldbronn, Germany).

An Agilent 1290 UHPLC system equipped with a binary pump with integrated degasser model G4220A, an autosampler model G4226A, column oven model G1316C and a photodiode array detector model G4212A equipped with a 1 μL/10 mm path length flow cell, 35 μL Jet Weaver mixer, extra column volume = 18.4 μL (definition see Section 2.4.1) was used. The system was controlled and data collected by means of Chemstation version B.04.02 (Agilent Technologies, Waldbronn, Germany).

Injection volumes used for the individual chromatographic tests can be found in Sections 2.5.1 and 2.5.2. The Agilent 1200 RRLC and 1290 UHPLC systems were configured for minimum system dispersion (i.e. compatible with small dimension columns, small particle sizes, high linear velocities with concomitant small peak volumes) as recommended by the vendor. pK_a values were estimated using LC simulator 12.02 (ACD Labs, Toronto, Canada).

2.4. Liquid chromatography

At least 20 column volumes of the appropriate mobile phase were flushed through the column prior to commencing the testing. All columns (150 × 4.6 mm I.D. and 50 × 2.1 mm I.D. formats) were new as supplied by the manufacturer (Advanced Chromatography Technologies, Hichrom Ltd, Reading, UK) and were packed with the same batch of the 3 μm ACE C18 packing material. The mobile phase was degassed and mixed on-line using a binary pump. For the 50 × 2.1 mm I.D. column format experiments the flow rate was scaled as shown in Eq. (1) to generate a constant linear velocity (flow rate of 1.0 mL min^{-1} for a 150 × 4.6 mm I.D. column).

$$F_{new} = F_{original} \frac{d_{new}^2}{d_{original}^2} \quad (1)$$

where F is the flow rate and d the column internal diameter of the new or original method.

Injection volumes quoted in Sections 2.5.1 and 2.5.2 are for a 150 × 4.6 mm I.D. format. For the UHPLC 50 × 2.1 mm I.D. column formats the injection volumes were scaled as shown below in Eq. (2).

$$V_{inj.new} = V_{inj.original} \frac{L_{new} \cdot d_{new}^2}{L_{original} \cdot d_{original}^2} \quad (2)$$

where V_{inj} is the injection volume and L the column length of the new or original methods.

The analytes typically eluted within 30 min in all of the tests on the 150 × 4.6 mm I.D. format and testing parameters. The first disturbance of the baseline on the injection of water was used as the dead time (t_M) marker.

The effect of flow rate between 0.21 and 0.75 mL min^{-1} on the small columns was investigated.

The diode array detector was set to monitor wavelengths of 214 and 254 nm with a reference set at 360 nm. The data sampling rate was set at 0.005 min (0.1 s, 40 Hz) and >0.003 min (0.062 s,

80 Hz) for the Agilent 1200 RRLC and 1290 systems respectively. Chromatographic values reported are the mean of two replicate injections.

2.4.1. Extra column volume (V_{ext})

The LC system extra column volume (V_{ext}) was determined for each LC system: this was achieved by replacing the column with a zero dead volume connector and injecting 6 × 0.5 μL of a 1% (v/v) acetone solution in the mobile phase at 100 μL min^{-1} using a detector wavelength of 265 nm and a data sampling rate of at least 20 Hz. The system extra column volume is simply the mean of the retention time of acetone x flow rate.

2.4.2. Corrected retention factors (k_{corr})

In order to correct for the extra column volume (V_{ext}) of the LC system when small column formats are used, the extra column time (t_{ext}) was calculated as below (Eq. (3)) and used in Eq. (4) to calculate the corrected retention factors (k_{corr}) [1,19]

$$t_{ext} = \frac{V_{ext}}{F} \quad (3)$$

$$k_{corr} = \frac{(t_R^g - t_{ext}) - (t_M^g - t_{ext})}{t_M^g - t_{ext}} = \frac{t_R^g - t_M^g}{t_M^g - t_{ext}} \quad (4)$$

where t_R^g is the gross retention time (i.e. the sum of the retention time and the extra column time), t_M^g the gross dead time (i.e. the sum of the dead time and the extra column time), t_{ext} the extra column time and k_{corr} the retention factor corrected for extra column time.

2.4.3. Corrected selectivity factors (α)

The corrected selectivity factors are determined as shown in Eq. (5).

$$\alpha = \frac{k_{corr2}}{k_{corr1}} \quad (5)$$

2.5. Liquid chromatographic conditions

The chromatographic conditions for the Tanaka HPLC characterization of the phases were as follows; for full descriptions of the tests see Refs. [5,8–10,22–26].

2.5.1. Standard Tanaka column characterization parameters protocols [5,8,9]

Retention factor for pentylbenzene, k_{PB}: Chromatographic conditions: 8:2 v/v MeOH:H_2O, 40 °C, 10 μL injection of pentylbenzene (0.6 mg mL^{-1}). This reflects the surface area and surface coverage of the phase (ligand density).

Hydrophobicity or hydrophobic selectivity, $\alpha_{PB/BB}$: Chromatographic conditions: 8:2 v/v MeOH:H_2O, 40 °C, 10 μL injection of a mixture containing pentylbenzene (0.6 mg mL^{-1}) and butylbenzene (0.4 mg mL^{-1}). Retention factor ratio between *n*-pentylbenzene (*PB*) and *n*-butylbenzene (*BB*), $\alpha_{PB/BB} = k_{PB}/k_{BB}$. 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.

Shape selectivity, $\alpha_{T/O}$: Chromatographic conditions: mobile phase as above for hydrophobicity, 10 μL injection of a mixture containing *o*-terphenyl and triphenylene both at 0.5 mg mL^{-1}. Retention factor ratio between triphenylene (*T*) and *o*-terphenyl (*O*), $\alpha_{T/O} = k_T/k_O$. This descriptor is a measure of the shape selectivity, which is influenced by the spacing of the ligands and probably also the shape and functionality of the silylating reagent.

Hydrogen bonding capacity, $\alpha_{C/P}$: Chromatographic conditions: 3:7 v/v MeOH:H_2O, 40 °C, 10 μL injection of a mixture containing of phenol (1 mg mL^{-1}) and caffeine (0.5 mg mL^{-1}). Retention factor ratio between caffeine (*C*) and phenol (*P*), $\alpha_{C/P} = k_C/k_P$. This descriptor is a measure of the number of available silanol groups and the degree of endcapping.

Total cation exchange capacity, $\alpha_{B/P\ w}{}^{w}$ pH 7.6: Chromatographic conditions: 20 mM KH_2PO_4, $_w{}^w$ pH 7.6 in 3:7 v/v MeOH:H_2O, 40 °C, 5 μL injection of a mixture containing phenol and benzylamine HCl both at 0.5 mg mL^{-1}. The retention factor ratio between benzylamine (*B*) and phenol (*P*), $\alpha_{B/P\ w}{}^{w}$ pH 7.6 = k_B/k_P. This is an estimate of the total silanol activity.

Acidic cation exchange capacity, $\alpha_{B/P\ w}{}^{w}$ pH 2.7: Chromatographic conditions: 20 mM KH_2PO_4, $_w{}^w$ pH 2.7 in 3:7 v/v MeOH:H_2O, 40 °C, 5 μL injection of a mixture containing phenol and benzylamine HCl both at 0.5 mg mL^{-1}. The retention factor ratio between benzylamine and phenol, $\alpha_{B/P\ w}{}^{w}$ pH 2.7 = k_B/k_P. This is a measure of the acidic activity of the silanol groups.

2.5.2. Extended column characterization protocols [9,10,25,26]

Phenolic selectivity, $\alpha_{P/BA\ w}{}^{w}$ pH 2.7 [25]*:* Chromatographic conditions: 20 mM KH_2PO_4, $_w{}^w$ pH 2.7 in 3:7 v/v MeOH:H_2O, 40 °C, 5 μL injection of a mixture containing phenol and benzylalcohol at 0.5 and 0.3 mg mL^{-1} respectively.

Aromatic selectivity, $\alpha_{TNB/TI}$, $\alpha_{DNB/TI}$, $\alpha_{TNB/NB}$ [10]*:* Chromatographic conditions: 5:5 v/v MeOH:H_2O, 40 °C, 5 μL injection of a mixture containing 1,3,5-trinitrobenzene, 1,3-dinitrobenzene, nitrobenzene and toluene (at 0.05, 0.06, 0.06 and 0.02 mg mL^{-1} respectively).

Dipole:dipole interaction capacity, $\alpha_{1,2\text{-DNB}/1,4\text{-DNB}}$, $\alpha_{1,3\text{-DNB}/1,4\text{-DNB}}$, $\alpha_{1,2\text{-DNB}/TI}$ [26]*:* Chromatographic conditions: 4:6 v/v MeOH:H_2O, 40 °C, 5 μL injection of a mixture containing 1,2-dinitrobenzene, 1,3-dinitrobenzene, 1,4-dinitrobenzene and toluene (at 0.03, 0.04, 0.06, 0.08 mg mL^{-1} respectively).

Acid test mixture 1, $\alpha_{PP/P}$, $\alpha_{CA/HC}$, $\alpha_{BN/S}$, $\alpha_{\sigma/BN}$, $\alpha_{\sigma/\rho}$, $\alpha_{P/DMP}$ [25]*:* Chromatographic conditions: 5 mM KH_2PO_4, $_w{}^w$ pH 2.5 in 35:65 v/v MeOH:H_2O, 40 °C, 10 μL injection of a mixture containing 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, phenol, 2-hydroxybenzoic acid, benzoic acid, sorbic acid, dimethylphthalate, 3-phenylpropionic acid, cinnamic acid, 4-hydroxybenzoic acid propyl ester (all at 0.03 mg mL^{-1}).

Acid test mixture 2, $\alpha_{BSA/TI}$, $\alpha_{P/BA}$, $\alpha_{P/TI}$ [25]*:* Chromatographic conditions: 5 mM KH_2PO_4, $_w{}^w$ pH 2.5 in 65:35 v/v MeOH:H_2O, 40 °C, 10 μL injection of a mixture containing phenol, benzylalcohol, benzene sulphonic acid and toluene (all at 0.1 mg mL^{-1}).

Hydrophilic bases, $\alpha_{Nic/B}$, $\alpha_{B/PROC}$, $\alpha_{PROC/TER}$, $\alpha_{TER/SAL}$, $\alpha_{SAL/P}$ [25]*:* Chromatographic conditions: 20 mM KH_2PO_4, $_w{}^w$ pH 2.7 in 3.3:96.7 v/v MeOH:H_2O, 60 °C, 5 μL injection of a mixture containing nicotine, benzylamine HCl, procainamide HCl, terbutaline sulphate, salbutamol sulphate and phenol (all at 0.12 mg mL^{-1}).

Lipophilic bases, $\alpha_{P/AZ1}$, $\alpha_{AZ1/AZ2}$, $\alpha_{AZ2/D}$, $\alpha_{D/AZ3}$, $\alpha_{AZ3/NOR}$ [10]*:* Chromatographic conditions: 20 mM KH_2PO_4, $_w{}^w$ pH 2.7 in 45.5:54.5 v/v MeOH:H_2O, 60 °C, 5 μL injection of phenol, AZ1, AZ2, diphenhydramine HCl, AZ3 and nortriptyline HCl (all at 0.03 mg mL^{-1}).

2.5.3. Extended column characterization parameters [9,10,25,26]

Phenolic selectivity, $\alpha_{P/DMP}$, $\alpha_{P/BA}$, $\alpha_{P/TL}$, $\alpha_{P/TL}$ [25]*:* Retention factor ratio between phenol (*P*) and dimethylphthalate (*DMP*), $\alpha_{P/DMP} = k_P/k_{DMP}$, phenol (*P*) and benzylalcohol (*BA*) at $_w{}^w$ pH 2.7, $\alpha_{P/BA} = k_P/k_{BA}$ and phenol (*P*) and toluene (*TL*), $\alpha_{P/TL} = k_P/k_{TL}$. These are measures of the enhanced retention of phenol compared to non-phenolic analytes.

Hydrophobicity, $\alpha_{PP/P}$ [25]*:* Retention factor ratio between 4-hydroxybenzoic acid propyl ester (*PP*) and phenol (*P*), $\alpha_{PP/P} = k_{PP}/k_P$. The difference in the retention of the two analytes corresponds to a *n*-propyl ester moiety.

Hydrophilicity, $\alpha_{BA/TL}$ [25]*:* Retention factor ratio between benzylalcohol (*BA*) and toluene (*TL*), $\alpha_{BA/T} = k_{BA}/k_{TL}$. This is a measure of the polarity of the phase.

Shape/steric selectivity, $\alpha_{CA/HC}$, $\alpha_{BN/S}$ [25]*:* Retention factor ratio between cinnamic acid (*CA*) and 3-phenylpropionic acid (*HC*), $\alpha_{CA/HC} = k_{CA}/k_{HC}$ and benzoic acid (*BN*) and sorbic acid (*S*), $\alpha_{BN/S} = k_{BN}/k_S$. This descriptor is a measure of the shape selectivity, which is influenced by the spacing of the ligands and probably also the shape/functionality of the silylating reagent.

Anionic exchange capacity, $\alpha_{\sigma/BN}$, $\alpha_{\sigma/\rho}$, $\alpha_{BSA/TL}$ [25]*:* Retention factor ratio between 2-hydroxybenzoic acid (σ) and benzoic acid (*BN*), $\alpha_{\sigma/BN} = k_\sigma/k_{BN}$; 2-hydroxybenzoic acid (σ) and 4-hydroxybenzoic acid (ρ), $\alpha_{\sigma/\rho} = k_\sigma/k_\rho$, and benzene sulphonic acid (*BSA*) and toluene (*TL*), $\alpha_{BSA/TL} = k_{BSA}/k_{TL}$. These are measures of the anion exchange capacity of the phase as shown by the increased retention of the acidic analytes.

Aromatic selectivity (π-basicity of the phase), $\alpha_{TNB/TL}$, $\alpha_{DNT/NL}$, $\alpha_{TNB/NB}$ [10]*:* The retention factor ratios between 1,3,5-trinitrobenzene (*TNB*) and nitrobenzene (*NB*), $\alpha_{TNB/NB} = k_{TNB}/k_{NB}$, 1,3-dinitrobenzene (*DN*B) and toluene (*TL*), $\alpha_{DNB/TL} = k_{DNB}/k_{TL}$ and 1,3,5-trinitrobenzene (*TNB*) and toluene (*TL*), $\alpha_{TNB/TL} = k_{TNB}/k_{TL}$. These descriptors are believed to be measures of the aromatic selectivity, which is influenced by the density of aromatic character on the phase.

Dipole:dipole capacity, $\alpha_{1,2\text{-}DNB/1,4\text{-}DNB}$, $\alpha_{1,3\text{-}DNB/1,4\text{-}DNB}$, $\alpha_{1,2\text{-}DNB/TL}$ [26]*:* The retention factor ratios between 1,2-dinitrobenzene (1,2-*DNB*) and 1,4-dinitrobenzene (1,4-*DNB*), $\alpha_{1,2\text{-}DNB/1,4\text{-}DNB} = k_{1,2\text{-}DNB}/k_{1,4\text{-}DNB}$, 1,3-dinitrobenzene (1,3-*DNB*) and 1,4-dinitrobenzene (1,4-*DNB*), $\alpha_{1,3\text{-}DNB/1,4\text{-}DNB} = k_{1,3\text{-}DNB}/k_{1,4\text{-}DNB}$ and 1,2-dinitrobenzene (1,2-*DNB*) and toluene (*TL*), $\alpha_{1,2\text{-}DNB/TL} = k_{1,2\text{-}DNB}/k_{TL}$. These descriptors are believed to be measures of the phases ability to participate in dipole:dipole interactions with analytes.

Base selectivity, $\alpha_{NIC/B}$, $\alpha_{B/PROC}$, $\alpha_{PROC/TER}$, $\alpha_{TER/SAL}$, $\alpha_{SAL/P}$, $\alpha_{P/AZ1}$, $\alpha_{AZ1/AZ2}$, $\alpha_{AZ2/D}$, $\alpha_{D/AZ3}$, $\alpha_{AZ3/NOR}$ [9,10]*:* The retention factor ratios between nicotine (*NIC*), benzylamine (*B*), procainamide (*PRO*), terbutaline (*TER*), salbutamol (*SAL*), phenol (*P*), compound AZ1 (*AZ*1), compound AZ2 (*AZ*2), diphenhydramine (*D*), compound AZ3 (*AZ*3) and nortriptyline (*NOR*) are recorded as described in Refs. [9,10].

2.5.4. Parameters recommended for an extended column characterization protocol

The protocols described above have several measures for the same type of interaction (PCA results not shown). It is therefore suggested that primarily the following measures are used for routine column characterization work: k_{PB}, $\alpha_{PB/BB}$, $\alpha_{T/O}$, $\alpha_{C/P}$, $\alpha_{P/BA}$ at pH 2.7, $\alpha_{B/P}$ at pH 2.7, $\alpha_{B/P}$ at pH 7.6, $\alpha_{BSA/TL}$, $\alpha_{TNB/TL}$ and $\alpha_{1,2\text{-}DNB/1,4\text{-}DNB}$.

Table 1
Retention factors (uncorrected [k^g] and corrected [k_{corr}] for the extra column volume) for *n*-pentylbenzene chromatographed at constant linear velocity (mobile phase conditions MeOH:water 80:20 v/v) using ACE 3 C18 columns on the 1200 RR LC system.

Column format (mm)	t_M^g	t_R^g	k^g	k_{corr}
150 × 4.6[a]	1.597	10.304	5.45	5.56
50 × 2.1[b]	0.663	3.400	4.13	5.34

[a] t_{ext} = 31.6/1000 = 0.032 min
[b] t_{ext} = 31.6/210 = 0.150 min.

3. Results and discussion

3.1. Comparison of column characterization results performed on small dimension (50 × 2.1 mm I.D.) columns compared to typical 150 × 4.6 mm I.D. column formats

3.1.1. Retention factor correction

Inaccuracies in the measurement of extra-column volumes (i.e. the contribution of volume from the tubing of the injector, detector and connections) have been previously reported to be responsible for up to 10% inaccuracy in the determination of retention factors [1,27]. In the current study, when the retention factor of *n*-pentylbenzene was determined on a 50 × 2.1 mm I.D. column format at 0.21 mL min^{-1} an inaccuracy of 24% in the retention factor was observed compared to that obtained on a 150 × 4.6 mm I.D. column format at 1 mL min^{-1} (see Table 1). Instrumental extra column volume contribution has a proportionally greater effect on the retention factors obtained with small volume columns, than those from the larger format column.

If the measured dead time and retention times are corrected, as shown in Eq. (4) (see Section 2), for the contribution of the extra column volume (i.e. t_{ext} and V_{ext}) then comparable retention factors (k_{corr} and k^g values differ by only 4%) are obtained between the different column formats and different LC systems (see Table 1).

In the case of column characterization studies it is acceptable to have variations as long as they are "small" in comparison to the variations between the columns to be studied. Ideally, the variations should be the same or smaller than the batch to batch variations of the columns to be compared. The differences we observed are smaller than the typical batch to batch reproducibility (i.e. <4%) for the determination of the Tanaka parameter k_{PB} [22] and Kele and Guiochon's seminal work on batch to batch retention reproducibility [28–31].

UHPLC columns are typically packed at a high packing pressure in order to maintain a stable phase bed when operated at high pressures. The consequence of this is that the void volume (V_M) of these columns is substantially lower (i.e. 6%) than columns packed for standard HPLC work. This results in UHPLC columns having a higher corrected retention factor than observed for standard pressure columns while the chromatographic selectivity characteristics

Table 2
Corrected Tanaka column characterization selectivity factors calculated from corrected retention factors obtained at constant linear velocity.

Column format packed with ACE 3 C18	Pressure (bar)[c]	Corrected retention and selectivity factors						
		k_{PB}[d]	$\alpha_{PB/BB}$	$\alpha_{T/O}$	$\alpha_{C/P}$	pH 2.7		pH 7.6
						$\alpha_{P/BA}$	$\alpha_{B/P}$	$\alpha_{B/P}$
150 × 4.6 mm[a]	224	5.56	1.48	1.55	0.38	0.97	0.13	0.35
50 × 2.1 mm[a]	63	5.34	1.47	1.54	0.37	0.97	0.10	0.35
50 × 2.1 mm[b]	73	5.81	1.47	1.52	0.36	0.97	0.11	0.39

[a] Standard column format, performed on the Agilent 1200 RR LC system.
[b] High pressure column format performed on the Agilent 1290 LC system.
[c] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$) using 8:2 v/v MeOH:water conditions.
[d] Corrected retention factor.

Table 3
Corrected Tanaka retention and selectivity factors obtained at differing mobile phase flow rates on the ACE 3 C18 50 × 2.1 mm I.D. column "high pressure" format using the Agilent 1200 RR LC system.

Flow rate (mL min^{-1})	Pressure (bar)[a]	% of initial corrected k_{PB} of 5.62	Corrected selectivity factors					
			$\alpha_{PB/BB}$	$\alpha_{T/O}$	$\alpha_{C/P}$	pH 2.7		pH 7.6
						$\alpha_{P/BA}$	$\alpha_{B/P}$	$\alpha_{B/P}$
0.21	73	100	1.47	1.52	0.36	0.97	0.11	0.39
0.50	152	92	1.46	1.52	0.38	0.97	0.12	0.37
0.75	226	88	1.47	1.52	0.39	0.97	0.12	0.38

[a] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$) using 8:2 v/v MeOH:water conditions.

of the two types of columns are not markedly changed (see Table 2). This must be taken into consideration when comparing retention factors between standard and high pressure format columns.

3.2. Comparison of column characterization results generated at elevated flow rates

The Tanaka column characterization was performed at flow rates of 0.21, 0.50 and 0.75 mL min^{-1} in order to examine the robustness of the methodology at higher linear velocities and higher pressures (e.g. 226 bar at 0.75 mL min^{-1} at 80:20 v/v MeOH/water). Table 3 highlights that an increase in linear velocity (and pressure) mainly results in a reduction in retention factor (k_{corr}), explainable, in part, by an increase in frictional heating. In comparison the corrected selectivity factors were not affected by an increase in flow rate.

It is recommended that all measurements should be performed at a constant linear velocity to that of the 150 × 4.6 mm I.D. column format operating at 1 mL min^{-1} (i.e. 0.21 mL min^{-1} for a 50 × 2.1 mm I.D. column format) since this must be used to generate a valid retention factor of *n*-pentylbenzene.

3.3. Comparison of column characterization results generated at elevated back pressure

Table 4 shows the corrected retention factors obtained during the experiments at standard and elevated back pressures. Elevated pressure experiments at constant flow rate were achieved by the insertion of a suitable length of 25 μm I.D. PEEK tubing between the column outlet and the detector (i.e. a 19 cm length was added to the Agilent 1290 Infinity LC systems which corresponded to an additional volume of ≅0.09 μL, therefore, its effect on V_{ext} should be minimal). An increase in the corrected retention factor was observed at the higher pressure for all the analytes at approximately 380–460 bar (with the exception of benzylamine at $_{w}^{w}$ pH 7.6 experiment, see below). It was clearly evident from Table 4 that some of the Tanaka analytes were more affected by the pressure increase than others (i.e. triphenylene and caffeine experienced retention factor increases >10%). For the majority of the neutral analytes (butylbenzene, *n*-pentylbenzene, *σ*-terphenyl), an increase in corrected retention factor of up to 8% was observed for these hydrophobic analytes. However, the degree of retention factor increase for the analytes phenol, benzylalcohol and benzylamine were dependant on the chromatographic conditions employed (see Table 4). This was consistent with data published recently by McCalley [21] who reported an increase in retention factor of approximately 12% for a pressure increase of 500 bar when small low molecular weight (Relative Molecular Mass [RMM] < 300) neutral compounds were chromatographed.

In a more recent study, McCalley reported that small molecular weight neutral polar analytes can give rise to larger pressure induced increases in retention (i.e. up to 50% for a pressure rise of 500 bar) [32]. It has been previously reported that larger molecules (i.e. insulin) exhibited a greater increase in retention as a function of increased pressure presumably due to larger changes in the molar volume when it is transferred from the mobile to stationary phase [33,34]. The probes employed in the Tanaka protocol and its modifications utilize analytes with RMM of between 94 and 465 which extends the range evaluated by McCalley [21]. The small molecular weight analyte caffeine (RMM 196) used in the Tanaka

Table 4
Comparison of corrected retention factors obtained from the Tanaka characterization performed under standard and elevated backpressure conditions on the Agilent 1290 Infinity LC system, performed on a ACE 3 C18, 50 × 2.1 mm I.D. column "high pressure" format.

Analyte	Operating pressures (bar)[g]		Corrected retention factors		% Change in corrected retention factor
	Standard[e]	Elevated[e,f]	Standard operating pressures	Elevated operating pressures	
Pentylbenzene[a]	73	380	5.62	6.01	7
Butylbenzene[a]	73	380	3.82	4.03	5
σ-Terphenyl[a]	73	380	4.75	5.05	6
Triphenylene[a]	73	380	7.24	8.00	10
Phenol[b]	90	448	3.28	3.46	5
Caffeine[b]	90	448	1.19	1.45	22
Phenol[c]	93	460	3.25	3.69	14
Benzylamine[c]	93	460	0.35	0.42	20
Benzylalcohol[c]	93	460	3.35	3.77	12
Phenol[d]	93	463	3.70	3.93	6
Benzylamine[d]	93	463	1.44	1.26	−13
Benzylalcohol[d]	93	463	3.85	4.09	6

[a] 8:2 v/v MeOH:water mobile phase conditions.
[b] 3:7 v/v MeOH:water mobile phase conditions.
[c] 20 mM potassium phosphate ($_{w}^{w}$ pH 2.7) in 3:7 v/v MeOH:water mobile phase conditions.
[d] 20 mM potassium phosphate ($_{w}^{w}$ pH 7.6) in 3:7 v/v MeOH:water mobile phase conditions.
[e] 0.21 mL min^{-1} flow rate.
[f] Insertion of 19 cm × 25 μm I.D. PEEK tubing between column outlet and detector.
[g] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$).

Table 5
Comparison of corrected retention factors obtained using various basic analytes under standard and elevated backpressure conditions using 20 mM potassium phosphate ($_w^w$ pH 7.6) in 3:7 v/v MeOH:water on the Agilent 1290 Infinity LC system, performed on a ACE 3 C18, 50 × 2.1 mm I.D. column "high pressure" format at a flow rate of 0.21 mL min^{-1}.

Analyte	Corrected retention factors (k_{corr})				1°, 2°, 3°, 4° amino group
	Standard operating pressures (108 bar)[a]	Elevated operating pressures (362 bar)[b,c]	% Δk_{corr}	$_w^w$ pK$_a$[d]	
Benzylamine	1.35	1.19	−12	9.3	1°
Salbutamol	0.88	0.78	−11	9.3	2°
Trimethylbenzylammonium	0.58	0.64	11	None	4°
Bambuterol	36.20	33.76	−7	9.6	2°
Oxprenolol	28.98	26.32	−9	9.5	2°
n-Acetylprocainamide	4.50	4.27	−5	9.4	3°
Metoprolol	15.80	14.01	−11	9.7	2°

[a] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell}$).
[b] Insertion of 19 cm × 25 μm I.D. PEEK tubing between column outlet and detector.
[c] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$).
[d] pK$_a$ values determined by ACD software.

tests is a neutral polar analyte and, as such, exhibited the largest increase of all the analytes (i.e. a 22% increase in retention for a 360 bar pressure increase). These large increases in retention have been associated with increased changes in the molar volume of the solvated analytes under elevated pressure, as they are transferred from the mobile phase to the stationary phase. This results in these polar analytes partially losing some of their hydration layer as they enter the hydrophobic stationary phase which results in an increase retention factor [16–21,32,35].

The McCalley group have also reported [21], as seen here, that greater variations in the degree of retention factor change as a function of increased pressure were observed with ionized analytes. Table 4 highlights the fact that the basic probe, benzylamine, exhibited retention behavior that was very different at pH 2.7 and pH 7.6 under the influence of elevated pressure.

Using low pH conditions, benzylamine is fully ionized whereas the base silica employed in the current studies was pure and therefore unionized, hence no ionic interaction. The ionized benzylamine probably acts in a similar fashion to that of small molecular weight polar analytes, undergoing partial loss of its hydration layer when it enters the hydrophobic stationary phase, resulting in an approximate 20% increase in retention. It is also noteworthy that the ionized benzylamine gives a greater retention increase than benzylalcohol which has a similar RMM but is unionized under these low pH conditions.

This paper supports the mounting evidence that increased pressure generally results in an increase in retention in RP-LC [16,17,21,32,35] providing that frictional heating effects are minimized/excluded. In contrast to the effect of pressure on retention (i.e. increased pressure leads to increased retention), frictional heating results in reduced retention.

Analysis of benzylamine at $_w^w$ pH 7.6 resulted in a reduction in retention as pressure was increased. This appears to be a general observation as a range of small molecular weight primary, secondary and tertiary bases ($_w^w$ pK$_a$ range of 9.3–9.7, RMM range 107–367) exhibited the same degree of loss in retention factor (5–12% reduction) when chromatographed at $_w^w$ pH 7.6, see Table 5. However, this was not the case for the fully charged quaternary base trimethylbenzylammonium chloride which on comparison exhibited a large increase in its retention factor as a function of pressure at pH 7.6.

It is believed that in our work at $_w^w$ pH 7.6, the effects of frictional heating can be excluded as a constant flow rate was maintained for standard and elevated pressure experiments and 50 × 2.1 mm I.D. column formats were used in conjunction with 3 μm packing material. In addition, at $_w^w$ pH 7.6, the neutral/polar analytes phenol and benzylalcohol and the fully charged quaternary base – trimethylbenzylammonium chloride (when co-analysed with benzylamine) exhibited an increase in retention factor, while benzylamime exhibited a reduction in retention when increased pressure was applied.

McCalley and Tanaka [32,35] have previously reported similar reductions in the retention factor when increased pressure is applied to analytes when chromatographed at a mobile phase pH close to their pK$_a$ values. We have previously shown that benzylamine ($_w^w$ pK$_a$ = 9.45), under the Tanaka $_w^w$ pH 7.6 ion exchange conditions containing 30% MeOH in the mobile phase is between 89 and 95% ionized depending on whether the $_w^w$pH or $_w^s$ pH scale is used [36].

Ionization of analytes have been reported to be enhanced when pressure is increased [32,37], thus the pK$_a$ values of the primary, secondary and tertiary bases examined will increase. The pH of the phosphate containing mobile phase employed will decrease as the pressure is increased as a result of an increased ionization of the phosphate. These effects augment the increased degree of ionization of the primary, secondary and tertiary bases and hence result in decreased retention (as retention is predominantly via an hydrophobic mechanism and there is no significant ionized silanol/base interaction as the silica was pure/inert). The increased ionization of the bases outweighs the effect of their change in molar volume as they are transferred from the mobile phase to the stationary phase.

3.3.1. Significance of the results

Table 6 shows that the $\alpha_{PB/BB}$, $\alpha_{P/BA}$ and $\alpha_{B/P}$ at $_w^w$ pH 2.7 results were essentially unaffected (<1% change in selectivity, i.e. up to 0.02 absolute selectivity difference) by increases in pressure as the analyte pairs are affected by pressure to approximately the same extent. Conversely, $\alpha_{T/O}$, $\alpha_{C/P}$ and $\alpha_{B/P}$ at $_w^w$ pH 7.6 were affected by increases in pressure as the individual analytes of the pair were affected to differing degrees. Differences of 5, 17 and −18% (i.e. 0.07, 0.06 and −0.07 absolute selectivity differences) were observed respectively for these differing selectivity factors on the application of pressure (total pressure of 380–460 bar).

However, when the Tanaka characterization is performed using small format columns and small particle sizes at a flow rate of 0.21 mL min^{-1}, the pressure is unlikely to exceed 210 bar, hence the significance of the pressure on the selectively factors should be less marked. Four different sub two micron phases (STM, i.e. Acquity BEH C18 1.7 μm, Zorbax Eclipse C18 1.8 μm HD format, Zorbax SB C18 HD format 1.8 μm and the Hypersil GOLD 1.9 μm) in a 50 × 2.1 mm I.D. column format operated on an Agilent 1290 Infinity LC system at the maximum viscosity of the testing (i.e. MeOH/water 50:50 v/v) only generated a total operating pressure of between 156 and 206 bar at 40 °C at a flow rate of 0.21 mL min^{-1}.

Table 6
Tanaka corrected selectivity factors values obtained from the elevated and non-elevated pressure experiment on an ACE 3 C18 50 × 2.1 mm I.D. column "high pressure" format on the Agilent 1290 Infinity LC system.

Pressure (bar)[b]	Corrected retention and selectivity factors						
	k_{PB}[a]	$\alpha_{PB/BB}$	$\alpha_{T/O}$	$\alpha_{C/P}$	$_{w}^{w}$ pH 2.7		$_{w}^{w}$ pH 7.6
					$\alpha_{P/BA}$	$\alpha_{B/P}$	$\alpha_{B/P}$
73	5.62	1.47	1.52	0.36	0.97	0.11	0.39
380[c]	6.01	1.49	1.59	0.42	0.98	0.11	0.32
% Change in value	6.9	1.4	4.6	16.7	1	0.0	−17.9
Estimated % change in value for a STM material	2.1	0.4	1.4	5.0	0.3	0.0	−5.4

[a] Corrected retention factor.
[b] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$) using 8:2 v/v MeOH:water mobile phase conditions at 0.21 mL min^{-1}.
[c] Insertion of 19 cm × 25 μm I.D. PEEK tubing between column outlet and detector.

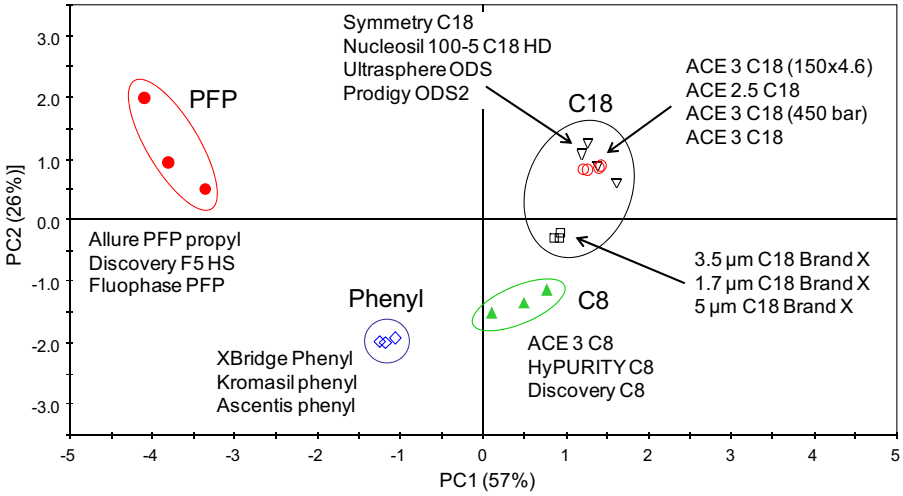

Fig. 1. PCA score plot of the 20 RP LC columns using the standard Tanaka column characterization procedure (i.e. six parameters). All columns of a 150 × 4.6 mm I.D. format except for brand X and ACE C18 columns which were 50 × 2.1 mm I.D. format unless otherwise stated. Tanaka column characterization data taken from Refs. [9,10,23,24,38], in addition to the ACE phases determined in this paper.

Our previous study [23] has shown that a well established phase with proven transferability between different particle sizes of the same material failed to show any marked difference in the Tanaka column characterization irrespective of particle size and the resultant pressure differences when performed using 50 × 2.1 mm I.D. column formats see Table 7. This can be clearly seen in the PCA score plot (see Fig. 1) where the 5, 3.5 and 1.7 μm phases are closely clustered together. Hence, the effect of pressure (up to 210 bar) on the Tanaka selectivity factors should be minimal.

The LC instrumental ($P_{LC\ instrument}$), flow cell ($P_{flow\ cell}$) and restrictor tubing ($P_{restrictor}$, inserted between the column outlet and the flow cell) pressures corresponded to 7, 3 and 307 bar respectively when a flow rate of 0.21 mL min^{-1} was employed.

Hence, the average pressure (P^{*}_{column}) in the 3 μm 50 × 2.1 mm I.D. column when the restrictor tubing is used equated to 341.5 bar (i.e. $P^{*}_{column} = [P_{column}/2] + P_{flow\ cell} + P_{restrictor}$).

Table 7
Reproducibility of the Tanaka retention and selectivity factor values as a function of particle size for a well scalable C18 phase of brand X packed in 50 × 2.1 mm I.D. columns and evaluated at 0.21 mL min^{-1} [23].

Particle size (μm)	Corrected retention and selectivity factors					
	k_{PB}	$\alpha_{PB/BB}$	$\alpha_{T/O}$	$\alpha_{C/P}$	$\alpha_{B/P}$ at	
					$_{w}^{w}$ pH 2.7	$_{w}^{w}$ pH 7.6
5	2.68	1.45	1.38	0.34	0.14	0.27
3.5	2.94	1.46	1.38	0.35	0.14	0.26
1.7	2.81	1.46	1.36	0.36	0.14	0.26
% Change in value	4.9	0.7	1.4	5.9	0.0	3.7

In contrast, for a range of four commercially available sub-2 μm UHPLC 50 × 2.1 mm I.D. columns the total pressure (P_{Total}) did not exceed 210 bar under identical conditions to those described in Table 6. Hence the pressure in the sub-2 μm UHPLC column ($P_{column} = P_{Total} - [P_{LC\ instrument} + P_{flow\ cell}]$) would correspond to approximately 200 bar. This would generate an average pressure (P^{*}_{column}) for a typical sub-2 μm UHPLC 50 × 2.1 mm I.D. column of 103 bar (i.e. $P^{*}_{column} = [P_{column}/2] + P_{flow\ cell}$).

This corresponds to approximately 30% of the pressure experienced by the analytes in the columns in this study where the pressure drop was generated by the addition of a restrictor producing 307 bar of pressure after the 3 μm column. Consequently the pressure effects shown in Tables 4–10 are approx. 3.3 times larger than what should be obtained with 50 × 2.1 mm sub-2 μm UHPLC columns under the same conditions. This estimate does not take heat of friction effects into account but still the agreement between this estimate in Table 6 and previously obtained data for 5, 3.5 and 1.7 μm columns shown in Table 7 are good, i.e. the estimated maximal deviation between HPLC and UHPLC columns is approximately 5% and thus acceptable.

Principal component analysis of 14 reverse phased LC columns (150 × 4.6 mm I.D. format – comprising of 3 × PFP, 3 × phenyl, 3 × C8, 5 × C18 columns), three phases of differing particle size (50 × 2.1 mm I.D. columns) and three 50 × 2.1 mm I.D. columns of the same C18 material (1 × 3 μm, 1 × 3 μm at elevated pressure due to the insertion of a suitable length of restrictor tubing between the outlet of the column and the flow cell and 1 × 2.5 μm) readily distinguished between the differing sub-classes of phases (see Fig. 1). All the ACE C18 phases grouped together independent of

Table 8
Corrected retention and selectivity factors for the extended column characterization parameters as described in Ref. [25] on the Agilent 1200RR LC system using a 50 × 2.1 mm I.D. high pressure column format.

Analyte	Corrected retention factors		% Change in k_{corr}
	Standard operating pressure (88 bar)[a]	Elevated operating pressure (343 bar)[a,b]	
4-Hydroxybenzoic acid	0.98	1.06	8
3-Hydroxybenzoic acid	1.44	1.55	8
Phenol	2.46	2.49	1
2-Hydroxybenzoic acid	4.00	4.16	4
Benzoic acid	4.83	4.98	3
Sorbic acid	4.83	4.98	3
Dimethylphthalate	7.24	7.67	6
3-Phenylpropionic acid	9.57	9.93	4
Cinnamic acid	11.75	12.58	7
4-Hydroxybenzoic acid propyl ester	25.76	27.95	9
Benzene sulphonic acid	0.06	0.06	0
Benzylalcohol	0.50	0.51	2
Phenol	0.50	0.51	2
Toluene	3.51	3.50	0

Operating pressure (bar)	Corrected selectivity factors								
	$\alpha_{PP/P}$	$\alpha_{CA/HC}$	$\alpha_{BN/S}$	$\alpha_{\sigma/BN}$	$\alpha_{\sigma/\rho}$	$\alpha_{BSA/TI}$	$\alpha_{P/DMP}$	$\alpha_{P/BA}$	$\alpha_{P/TI}$
88	10.47	1.23	1.00	0.83	4.08	0.02	0.34	1.00	0.14
343[a]	11.22	1.27	1.00	0.84	3.92	0.02	0.32	1.00	0.15
% Change in α value	7.0	3.0	0.0	1.0	−4.0	0.0	−6.0	0.0	7.0
Estimated % change in α value for a STM material	2.1	0.9	0.0	0.3	−1.2	0.0	−1.8	0.0	2.1

[a] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$).
[b] 10.9 cm × 25 μm PEEK tubing (≅0.05 μL) inserted between column outlet and detector.

their particle size and column format and whether the column had been tested under standard or UHPLC conditions. This highlights the fact that the Tanaka column characterization methodology can provide meaningful results even when small dimension columns are evaluated at high pressures.

When the effect of increased pressure was evaluated (by the insertion of a suitable length of restrictor tubing between the outlet of the column and the flow cell) for the extended column characterization protocol for the assessment of anionic [23,25], cationic [9,10,23–25] phenolic [10,25], dipole:dipole interactions

Table 9
Corrected retention and selectivity factors for the extended column characterization parameters as described in Refs. [9,10,23–25] on the Agilent 1200 RR LC system using a 50 × 2.1 mm I.D. high pressure column format.

Hydrophilic bases	Corrected retention factors		
	Standard operating pressure (45 bar)[a]	Elevated operating pressure (246 bar)[a,c]	% Change in k_{corr}
Nicotine	1.03	1.21	18
Benzylamine	2.42	2.87	19
Procainamide	4.02	5.15	28
Terbutaline	5.98	7.92	32
Salbutamol	8.4	11.08	32
Phenol	11.66	13.94	20
Lipophilic bases	**Standard operating pressure (67 bar)[b]**	**Elevated operating pressure (284 bar)[b,c]**	
Phenol	1.14	1.17	3
AZ1	1.66	1.88	13
AZ2	2.08	2.27	9
Diphenhydramine	2.42	2.67	10
AZ3	3.79	4.21	11
Nortriptyline	7.32	8.4	15

Operating pressure (bar)	Corrected selectivity factors				
	$\alpha_{NIC/B}$	$\alpha_{B/PROC}$	$\alpha_{PROC/TER}$	$\alpha_{TER/SAL}$	$\alpha_{SAL/P}$
45[a]	0.43	0.60	0.67	0.71	0.72
246[a,c]	0.42	0.56	0.65	0.71	0.79
% Change in α value	−2.3	−6.7	−3.0	0.0	9.7
Estimated % change in α value for a STM material	−0.7	−2.0	−0.9	0.0	2.9

Operating pressure (bar)	$\alpha_{P/AZ1}$	$\alpha_{AZ1/AZ2}$	$\alpha_{AZ2/D}$	$\alpha_{D/AZ3}$	$\alpha_{AZ3/NOR}$
67[b]	0.69	0.80	0.86	0.64	0.52
284[b,c]	0.62	0.83	0.85	0.63	0.50
% Change in α value	−10.1	3.7	−1.2	−1.6	−3.8
Estimated % change in α value for a STM material	−3.0	1.1	−0.3	−0.5	−1.2

[a] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$) using 20 mM KH_2PO_4, $_wW$ pH 2.7 in 3.3:96.7 v/v MeOH:H_2O.
[b] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$) using 20 mM KH_2PO_4, $_wW$ pH 2.7 in 45.5:54.5 v/v MeOH:H_2O.
[c] 10.9 cm × 25 μm PEEK tubing (≅0.05 μL) inserted between column outlet and detector.

Table 10
Corrected retention and selectivity factors for the extended column characterization parameters as described in Refs. [10,26] on the Agilent 1200 RR LC system using a 50 × 2.1 mm I.D. high pressure column format.

Analyte	Corrected retention factors		% Change in k_{corr}
	Standard operating pressure (85 bar)[a]	Elevated operating pressure (355 bar)[a,c]	
1,3,5-Trinitrobenzene	1.61	1.60	−1
1,3-Dinitrobenzene	2.08	2.10	1
Nitrobenzene	2.47	2.49	1
Toluene	9.50	9.31	−2
	Standard operating pressure (87 bar)[b]	Elevated operating pressure (372 bar)[b,c]	
1,2-Dinitrobenzene	3.22	3.44	7
1,3-Dinitrobenzene	3.75	3.87	3
1,4-Dinitrobenzene	3.22	3.44	7
Toluene	19.74	19.21	−3

Operating pressure (bar)	Corrected selectivity factors		
	$\alpha_{TNB/NB}$	$\alpha_{TNB/TI}$	$\alpha_{DNB/TI}$
85[a]	0.65	0.17	0.22
355[a,c]	0.64	0.17	0.23
% Change in α value	−1.5	0.0	4.5
Estimated % change in α value for a STM material	−0.5	0.0	1.4
	$\alpha_{1,2\text{-}DNB/1,4\text{-}DNB}$	$\alpha_{1,3\text{-}DNB/1,4\text{-}DNB}$	$\alpha_{1,2\text{-}DNB/TI}$
87[b]	1.00	1.16	0.16
372[b,c]	1.00	1.13	0.18
% Change in α value	0.0	−2.6	12.5
Estimated % change in α value for a STM material	0.0	−0.8	3.8

[a] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$) using 1:1 v/v MeOH:water mobile phase conditions.
[b] Total pressure of the LC system (i.e. $P_{total} = P_{column} + P_{LC\ instrument} + P_{flow\ cell} + P_{restrictor}$) using 4:6 v/v MeOH:water mobile phase conditions.
[c] 10.9 cm × 25 μm PEEK tubing (≅0.05 μL) inserted between column outlet and detector.

[26] and aromatic selectivity [10], which utilizes numerous analytes of widely differing physico/chemical properties the extent of the influence of pressure was quite variable (see Tables 8–10). For example, when a range of acidic and neutral analytes were used for the assessment of anionic [23,25] and phenolic selectivity [10,25], nine of the analytes experienced increases of <5% whereas five analytes exhibited increases in retention factor of between 5 and 9% on the application of 255 bar of pressure (total pressure 343 bar). This resulted in −6 to +7% differences in the calculated selectivity factors. When translating this to relevant conditions this corresponds to minor differences of −1 to +2%.

The basic analytes (Table 9) used for the cationic selectivity of the phase [9,10,23–25] all exhibited large retention factor increases between 9 and 32% depending on their physico/chemical properties for the application of between 201 and 217 bar of pressure (total pressure of 246 and 284 depending on the mobile phase composition). Once again this resulted in −10 to +10% differences in selectivity, which when translated to relevant conditions/pressures, correspond to only −3 to +3%.

The nitroaromatic analytes (Table 10) used to assess for aromatic selectivity and dipole:dipole interactions [10,26] only exhibited small increases in retention factor of up to 7% on the application of between 270 and 285 bar of pressure (total pressure of 355 and 372 depending on the mobile phase composition). This resulted in −3 to +13% differences in the calculated selectivity factors. Translated to relevant conditions this corresponds to −1 to +4%.

It should be stressed that none of the selectivity factors recommended from the extended column characterization test protocols described in Section 2.5.3, i.e. $\alpha_{P/BA}$, $\alpha_{BSA/TL}$, $\alpha_{TNB/TL}$ and $\alpha_{1,2\text{-}DNB/1,4\text{-}DNB}$ display any pressure effect at all.

While we have shown that the retention factor of analytes used in the Tanaka column characterization protocols and its extended tests changes markedly on the application of pressure, the resultant selectivity factors for most of the tests do not exhibit a marked deviation on the application of typical pressures, generated using small column dimensions and small particle sizes. However, the authors recommend caution when comparing selectivity factors between standard HPLC and UHPLC column format unless proven otherwise.

McCalley et al. [32] have shown that the influence of pressure on retention is also dependent on the type of bonded phase investigated as the contact area between the analyte and the stationary phase may be different for different phases. However, they have shown that the effect of pressure is more pronounced when hydrophobic C18 phases are used compared to more polar phases as the former possess a smaller contact area. Given that we have proven that Tanaka column characterization results can be successfully translated from standard HPLC formats to UHPLC for hydrophobic C18 phases, then translations to other more polar phases should be equally valid.

4. Conclusion

It is imperative that the retention factor should be corrected for the system extra column volume when column characterization work is performed using UHPLC column formats (e.g. 50 × 2.1 mm) even when determined on UHPLC systems with low system volumes in order that results comparable to those obtained using standard format columns are generated. This problem would be greatly exacerbated if small column formats are used on non-optimized HPLC equipment.

The increase in backpressure associated with the use of smaller particle size materials results in a general increase in the retention factor for most analytes. This is as a result of large changes in the molar volume of these solvated analytes as they are transferred from the mobile phase to the hydrophobic C18 stationary phase. The degree of retention factor increase on the application of increased pressure depends on the analyte's physico/chemical properties and the chromatographic conditions employed. Analytes chromatographed close to their pK_a values (i.e. bases with pK_a values close to 9 when chromatographed at $_w{}^w$ pH 7.6 in 3:7

v/v MeOH:water mobile phase) exhibited a large decrease in retention factor due to an increase in ionization on the application of increased pressure.

Performing the Tanaka and extended column characterization procedures at pressures that would be encountered during the characterization of small particle sizes (i.e. sub-two micron up to 210 bar, 2.5–2.7 μm porous and fused core materials up to 140 bar) packed into 50 × 2.1 mm I.D. column formats at a constant linear velocity according to standard protocols (0.21 mL min^{-1}), resulted in comparable corrected selectivity factors to those determined using standard HPLC systems and column formats. While increasing the linear velocity gave comparable selectivity factors, the retention factors were significantly reduced due to frictional heating effects.

Provided that the experimental conditions for the Tanaka column characterization protocol for small column formats are controlled within the above operating parameters, that a constant linear velocity is employed and that corrected retention factors are determined, then it is possible to distinguish between RP materials which possess selectivity differences larger than the typical batch to batch variability.

The extended column characterization tests generated similar results on the application of increased pressure with respect to retention and selectivity factors. The retention factors increased up to 32% depending on the physico/chemical properties of the analytes. In contrast, selectivity factors only exhibited modest changes of approximately 0.05 in absolute selectivity terms as a consequence of both analytes being affected by pressure to approximately the same extent. However, caution must be made in that this will not always be the case. For example, the phenolic selectivity factor $\alpha_{PP/P}$ gave an increase of 7% (i.e. $\alpha_{PP/P}$ = 10.47 to 11.22 on the application of an extra 255 bar as a result of 4-hydroxybenzoic acid propyl ester exhibiting a +9% change in retention factor compared to phenol which only showed a +1% increase). Due to the wide structural diversity of the many analytes employed in popular column characterization protocols, it is imperative to demonstrate comparable results when small columns packed with small particle sizes are chromatographed at increased pressure and compared to standard column formats – otherwise erroneous comparisons and conclusions may be made.

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