Published in final edited form as:

J Chromatogr A. 2018 January 12; 1532: 161–174. doi:10.1016/j.chroma.2017.12.007.

Aqueous size-exclusion chromatography of polyelectrolytes on reversed-phase and hydrophilic interaction chromatography columns

Anna M. Caltabiano^{a,b,*}, Joe P. Foley^b, and André M. Striegel^c

^aAnalytical Sciences and Development, GlaxoSmithKline, 1250 S. Collegeville Rd., Collegeville, PA, 19426, USA

^bDepartment of Chemistry, Drexel University, 3141 Chestnut St., Philadelphia, PA, 19104, USA

^cChemical Sciences Division, National Institute of Standards and Technology (NIST), 100 Bureau Drive, MS 8392, Gaithersburg, MD, 20899-8392, USA

Abstract

The size-exclusion separation of a water-soluble polyelectrolyte polymer, sodium polystyrene sulfonate (NaPSS), was demonstrated on common reversed-phase (C₁₈, C₄, phenyl, and cyano) and hydrophilic interaction chromatography (HILIC) columns. The effect of common solvents – acetonitrile (ACN), tetrahydrofuran (THF), and methanol (MeOH), used as mobile phase modifiers – on the elution of NaPSS and the effect of column temperature (within a relatively narrow range corresponding to typical chromatographic conditions, *i.e.*, 10 °C–60 °C) on the partition coefficient, K_{SEC} , were also investigated. Non-size-exclusion chromatography (non-SEC) effects can be minimized by the addition of an electrolyte and an organic modifier to the mobile phase, and by increasing the column temperature (*e.g.*, to 50 °C or 60 °C). Strong solvents such as THF and ACN are more successful in the reduction of such effects than is the weaker solvent MeOH. The best performance is seen on medium polarity and polar stationary phases, such as cyanopropyl- and diol-modified silica (HILIC), where the elution of the NaPSS polyelectrolyte is by a near-ideal SEC mechanism. Hydrophobic stationary phases, such as C₁₈, C₄, and phenyl, require a higher concentration of a strong solvent modifier (THF) in the mobile phase to reduce non-SEC interactions of the solute with the stationary phase.

Keywords

Size-exclusion chromatography; SEC; Polymer; Sodium polystyrene sulfonate; Polyelectrolyte; NaPSS

*Corresponding author at: Analytical Sciences and Development, GlaxoSmithKline, 1250 S. Collegeville Rd., Collegeville, PA, 19426, USA., caltanna@netscape.net (A.M. Caltabiano).

Publisher's Disclaimer: Disclaimer

Commercial products are identified to specify adequately the experimental procedure. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

1. Introduction

Originally, aqueous size-exclusion chromatography (SEC) was known as gel filtration chromatography (GFC). It was used for separating water-soluble macromolecules, employing columns packed with cross-linked polydextran gels and using aqueous mobile phases [1]. Because of the low mechanical strength of gels consisting of cross-linked carbohydrates (dextrans), these gels could not be used in high performance liquid chromatography (HPLC). Attempts to use porous glass or silica packings, which are much stronger mechanically than soft gels, were unsuccessful because of issues related to irreversible adsorption and/or denaturation of polymers. Bare silica was not recommended for aqueous SEC due to its finite solubility in aqueous buffers and the presence of silanols [2].

There are two main types of packings used for aqueous SEC: (*i*) silica with hydrophilic surface modification; and (*ii*) hydrophilic or ionic cross-linked polymeric packings [2,3]. Cross-linked dextran gels are still used today, however in a different form. These gels are stable in most common buffers, salt additives, organic modifiers, and extreme pH [4]. For non-ionic polymers, polymer-based packings are preferred because they are much more mechanically robust than the dextran-based packings and less susceptible to adsorption effects than are either unmodified or chemically modified silica packings [4]. Anionic polymers can be analyzed on unmodified porous-glass or silica-gel packings, while cationic polymers can be separated on silica gel modified with cationic groups, such as 3-aminopropyl [3].

For aqueous SEC, hydrophilic silica packings modified with 1,2- propanediol functional groups are the most common [2]. Such modifications neutralize the surface of the packing by blocking or reacting with many of the acidic silanol groups. Diol-modified stationary phases are ideal for SEC separation of biopolymers and synthetic water-soluble polymers, but can be problematic if polymers are charged. Polyelectrolytes can ion-exchange onto residual acidic silanols on bare or even diol-modified silica, if they are cationic, or be ion-excluded from negatively charged pores, if they are anionic.

There are several hydrophilic cross-linked polymeric packings that are used for aqueous SEC. Most of them are proprietary hydroxylated derivatives of cross-linked polymethacrylates. Specialty packings available on the market, such as sulfonated cross-linked polystyrene, polydivinylbenzene derivatized with glucose or anion-exchange groups, a polyamide polymer, and cross-linked agarose, are considered “unusual” [2].

The silica-based ethylene-bridged hybrid inorganic-organic (BEH) packing, introduced in the early 2000's, is a mixture of silica and organosiloxanes that form poly-ethoxypolysilane polymers, provides not only improved chemical stability and reduced silanol activity, but can be manufactured with a large pore size [2]. Diol-modified BEH particles provide a significant reduction in silanol activity, which is favorable for biopolymer separations [5].

There are several limitations of aqueous SEC columns, but the major one is that eluent selection may be limited with respect to pH and type/concentration of organic modifier that can be tolerated by the column. To select a column, the manufacturers' column

specifications should be consulted for eluent compatibility. Care should be taken regarding the use of organic solvents when using polymeric packings designed for aqueous mobile phases, as employing a solvent incompatible with the column packing material can irreparably damage the latter [6]. Hydrophilic polymer gels shrink in organic solvents and usually only 10%–20% (v/v) of organic modifier can be added to the mobile phase [3], a quantity which may not be sufficient to suppress undesired interactions of the solutes with the stationary phase.

Reversed-phase (RP) column packings, on the other hand, are rigid and compatible with a large variety of organic solvents and water, and therefore can be advantageous for use in SEC. While an interest in performing *non-aqueous* SEC using C₁₈ bonded phases has grown over the past few years [7–14], it is beneficial to investigate the applicability of contemporary RP columns for *aqueous* SEC. Starting from the 1970s, it was recognized that modification of the silica support offers an advantageous minimization of non-SEC effects. However, RP stationary phases were regarded as of limited use due to strong sorption properties with organic solutes if water or aqueous mixtures are used as eluents [15]. Nevertheless, bonded polar functional groups, producing hydrophilic phases, were considered promising. Typically, aqueous SEC analysis is challenging to the polymer characterization chemist, due to a variety of non-SEC effects which can potentially plague the separation. Unwanted interactions of solutes with the stationary phase, such as ion exchange, ion exclusion, ion inclusion, intramolecular electrostatic interactions, and adsorption are more commonly observed in aqueous SEC than when employing neat organic solvents as the mobile phase [3].

A successful separation of biopolymers has recently been demonstrated on a hydrophilic interaction chromatography (HILIC) column [12]. In the present study, aqueous SEC on RP (*e.g.*, C₁₈, C₄, phenyl, and cyano) and HILIC columns is explored using the polyelectrolyte sodium polystyrene sulfonate (NaPSS), a linear synthetic polymer, of various molar masses. For the analysis of polyelectrolytes, one must be cognizant of their charge and adsorptive interactions with the column stationary phase that can affect their accurate molar mass determination. NaPSS polymer is both ionic (highly anionic) and relatively hydrophobic. Mori [16] proposed that the elution of NaPSS is governed by the compromise among three effects: size-exclusion, ion-exclusion, and hydrophobic interactions. Ionic interactions can be suppressed by the addition of an electrolyte to the eluent. Equally, the addition of an organic solvent to the mobile phase is required to analyze polymers with aromatic rings in their side chain, to reduce hydrophobic interaction with the particle matrix. To reduce electrostatic effects, sodium sulfate (Na₂SO₄) was used in this study as an electrolyte in the aqueous component of the mobile phase, while to reduce hydrogen bonding and hydrophobic effects, tetrahydrofuran (THF), acetonitrile (ACN), and methanol (MeOH) were utilized as the organic component of the mobile phase, and their effect on the elution of NaPSS polymers was investigated. Also studied by evaluation of partition coefficients, K_{SEC} , of the NaPSS analytes was the effectiveness of increasing column temperature on reducing non-SEC effects during the separations.

2. Experimental

2.1. Materials

HPLC-grade solvents: ACN was purchased from EMD Millipore Chemicals (Billerica, MA, USA), unstabilized THF and MeOH from Sigma-Aldrich (St. Louis, MO, USA). Deionized (DI) water was “in-house” from Barnstead Nanopure Diamond, D11911. Sodium sulfate was purchased from Sigma-Aldrich (St. Louis, MO, USA) and uracil (Acros Organics) from Thermo Fisher Scientific (Pittsburgh, PA, USA). NaPSS standards were purchased from Phenomenex (Torrance, CA, USA). Molar mass dispersities of all NaPSS standards were 1.20, and molar mass values given here correspond to the peak average molar mass (average molar mass at peak apex, M_p); both values were supplied by the vendor.

2.2. HPLC/UHPLC of NaPSS polymers

The liquid chromatographic system, an Agilent Series 1290 (Agilent Technologies, Santa Clara, CA, USA), consisted of a binary pump with an integrated high efficiency degasser (G4220A) able to handle a maximum backpressure of 1000 bar, a column thermostat (G1316C), an autosampler (G4226A) with thermostat (G1330B), and an ultraviolet–visible diode array detector (UV DAD) (G4212A) set at 258 nm for uracil detection and at 224 nm for NaPSS detection. Chromatographic data for Fig. 1 (A through C only) and Fig. 3 (C₁₈ only) were generated on an Agilent Series 1260 (Agilent Technologies, Santa Clara, CA, USA) that consisted of a degasser (G1322A), a column thermostat (G1316C), a binary pump (G1312B) able to handle a maximum backpressure of 600 bar, an autosampler (G1367E), and an UV DAD (G4212B). One microliter (1 μ L) volumes of a 0.2 mg mL⁻¹ uracil solution in water and NaPSS standard solutions were injected into the chromatographic systems while the autosampler temperature was held at 5 °C, and eluted isocratically on the columns listed in Table 1. Note that the columns (stationary phases) described in the text as “phenyl” and “cyano” were actually propyl-phenyl and propyl-cyano derivatized silica, respectively. The HILIC column/phase was a diol-modified silica. The columns were operated at seven discrete temperatures (10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C; in view of the fact that temperatures above 60 °C may reduce column lifetime, depending upon the mobile phase composition, the study at 70 °C was conducted as quickly as possible). The mobile phase consisted of aqueous 0.2 mol L⁻¹ sodium sulfate mixed online with THF, ACN, or MeOH in the ratios specified in the text. Separations were carried out at a flow rate of 0.30 mL min⁻¹. The chromatographic system was controlled and the data were acquired through a PC running 2010 Pro Empower 3 software, version 7.20.00.00 (Waters, Milford, MA, USA).

2.3. NaPSS standards preparation

All NaPSS standards were prepared in water at a nominal concentration of 1 mg mL⁻¹ by dissolving approximately 10 mg of NaPSS in 10.0 mL of water. Three standard solutions were prepared as shown in Table 2. The solutions were shaken manually and allowed to equilibrate overnight.

3. Results and discussion

3.1. Solvent modifier effect on elution of NaPSS

The initial analysis of NaPSS standard solutions was performed on a HILIC column (Table 1), using 0.2 mol L⁻¹ sodium sulfate (Na₂SO₄) solution in water as the mobile phase at a flow rate of 0.30 mL min⁻¹. One microliter (1 µL) NaPSS solutions (Table 2) were injected into the chromatographic system, while the column was held at 30 °C. Fig. 1 (A) demonstrates a good separation of NaPSS standards by size, using different combinations of three molar mass standards (numbers denote M_p values, in g mol⁻¹): black trace (3420; 15,800; and 152,000; NaPSS 1 in Table 2); blue trace (891; 29,500; and 470,000; NaPSS 2 in Table 2); and green trace (6430; 65,400; and 976,000; NaPSS 3 in Table 2). However, the M_p 891 standard did not elute from the column; it is a “missing” peak from the blue trace in chromatogram (A). NaPSS M_p 891 apparently adsorbs irreversibly onto the stationary phase, and therefore does not elute, while the other NaPSS polymers of higher molar mass (M_p 3420 through 976,000) elute by an SEC mechanism, as demonstrated in Section 3.3.

NaPSS polymer is a macromolecule with ionizable groups and a hydrophobic backbone. In polar solvents such as aqueous solutions, the ionizable groups dissociate, leaving negatively charged chains while releasing sodium counterions into the solution [17]. The electrostatic interactions of the ionized groups on polyelectrolyte chains and the interactions of the polymer backbone with the surrounding solvent determine the conformation of the polyelectrolyte [18,19]. In a poor solvent for the polymer backbone, such as water is for NaPSS, the polyelectrolyte chain size is determined by the balance of chain elasticity and electrostatic repulsion between charged monomeric units and strongly depends on the degree of polymerization (for linear polymers chain size increases as a function of increasing degree of polymerization) [20]. The chain becomes non-uniformly stretched and experiences stronger deformation in the middle than at its ends. In poor solvents for its backbone, NaPSS adopts a necklace-like structure of pearls connected by narrow strings or a rod-like structure, depending on the degree of sulfonation, in an attempt to balance electrostatic and hydrophobic interactions between monomeric units [20,21]. Upon addition of a salt, the chains contract and assume a random coil conformation [22]. Based on this discussion, it can be assumed that M_p 3420 through 976,000 NaPSS polymers are in random coil conformations and their charged sulfonate groups are effectively shielded at the experimental conditions described above, thereby preventing non-SEC interactions with the stationary phase. The chains of NaPSS M_p 891, on the other hand, are of an insufficient length to adopt a coiled conformation. Their rod-like conformation has sulfonate groups exposed and available for electrostatic interactions with the diol-modified silica surface, and/or for hydrogen bonding with residual silanols and the bonded phase of the column packing. Additionally, end group effects tend to be much greater in oligomers than in polymers [23,24]. If these end groups are somehow providing the most dominant analyte-stationary phase interaction, then oligomers will interact more strongly with the stationary phase than will polymers.

Fig. 1 (B) demonstrates that upon addition of methanol to the mobile phase (20 (v/v) %), the non-SEC interactions of M_p 891 (indicated by an arrow) with the stationary phase are still

significant but reduced, compared to the condition without the organic solvent modifier (Fig. 1A); M_p 891 elutes as a broad, multimodal peak. The peak shape of M_p 3420 is also improved by the MeOH-containing mobile phase, compared to the peak shape obtained using modifier-free mobile phase (Fig. 1A). This is an indication that M_p 3420 also experienced non-SEC interactions with the stationary phase in the absence of modifier in the eluent (Fig. 1A). Elution of the higher molar mass NaPSS analytes is unaffected by the presence of MeOH modifier. Compared to MeOH, the same percentage of ACN (20%) in the mobile phase further reduces non-SEC interactions of M_p 891 with the stationary phase; however, its peak shape remains broad and multimodal (Fig. 1C).

The fact that a series of peaks are observed for the NaPSS M_p 891 oligomer, rather than a single peak, may also suggest that this standard contains a collection of oligomers that differ from each other in degree of polymerization, degree of sulfonation, or both. When the ACN in the mobile phase is replaced by THF at the same percentage (20%), the peak shape of M_p 891 is changed, where all multiple peaks collapse into almost a single peak, and its non-SEC interactions with the stationary phase are, if not eliminated completely, then minimized to a large extent (Fig. 1D). A similar result can be achieved by increasing the ACN percentage to 30% as seen in Fig. 2, where chromatograms generated with 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF and 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN are compared. This effect of THF on the elution of M_p 891 suggests that the ionized sulfonate groups of this oligomer might engage in hydrogen bonding with the nonionized residual silanols of the stationary phase, where the latter are disrupted by THF more efficiently than they are by MeOH and ACN due to the stronger hydrogen ion acceptor capability of THF. Weakly polar or non-polar solvents (*e.g.*, THF) do not possess sufficient polarity to cause ion pair dissociation of a polyelectrolyte, and therefore, the interaction is controlled by aggregation of ion pairs, similar to the effect of the presence of salt [22]. A polyelectrolyte chain can adopt a coil-like conformation in a good solvent for the polymer backbone, depending on the magnitude of the charge [17].

3.2. Effect of stationary phase chemistry on elution of NaPSS

Because of residual silanol groups, some of which ionize, hydrophobic RP columns (such as C₁₈, C₄, phenyl, and cyano) are subject to electrostatic interactions and hence to ion-exclusion phenomena, and possibly to hydrogen bonding, with the sulfonate groups of NaPSS. Additionally, RP ligands are subject to hydrophobic interactions with the NaPSS polymer backbone. As was seen in the previous section, organic solvent mobile phase modifiers can be ranked as THF > ACN > MeOH with respect to their ability to minimize non-SEC interactions of NaPSS polymers with a diol-modified HILIC stationary phase (*i.e.*, ranking is with respect to solvent strength and assumes equal concentrations of each modifier). Because THF was successful in eliminating both hydrophobic and electrostatic interactions, separation of NaPSS polymers was evaluated on RP columns (C₁₈, C₄, and phenyl) using 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF as the mobile phase. Fig. 3 demonstrates an SEC separation of NaPSS standard peaks by an SEC mechanism on C₁₈, C₄, and phenyl columns, except for the case of M_p 891 on the C₁₈ column, which exhibits significant non-SEC interactions based on the fact that the majority of this oligomer elutes after uracil. As previously observed with the HILIC column, the elution of NaPSS M_p 891

on RP columns evinces a collection of multiple, closely-related oligomers. Calibration plots, constructed for NaPSS standards (M_p 3420; 6430; 15,800; 29,500; 65,400; 152,000; 470,000; and 976,000) from triplicate data points (injections) for each column (C₁₈, C₄, and phenyl) are typical with good correlation coefficients (R^2 0.9985, n 18) for third-order polynomial fits (Fig. 4).

Fig. 5 demonstrates the separation of NaPSS solutes on phenyl, cyano, and HILIC columns. As the polarity of the stationary phase increases, phenyl < cyano < HILIC, the interaction of M_p 891 oligomer with it decreases. The other NaPSS solutes of higher molar masses elute by an SEC mechanism on all columns, and their near-ideal SEC elution is supported by the temperature study in the following section. However, the near-ideal SEC elution of all NaPSS analytes (including M_p 891) is seen only on the HILIC column. To minimize non-SEC interactions of M_p 891 NaPSS with the stationary phase, the less polar cyano stationary phase requires a higher concentration of organic solvent in the mobile phase than does the more polar HILIC column. The solid trace chromatogram in Fig. 6 demonstrates a significant change in the M_p 891 peak shape on the cyano column using 65/35 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase, compared to the blue dashed trace using 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase. The peak shape of M_p 891 on the cyano column with 65/35 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase (Fig. 6) is similar to that on the HILIC column with 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase (Fig. 5).

Apparently, the resolution of M_p 891 oligomers (2-mer, 3-mer, 4-mer, *etc.*), and, as a consequence, the peak shape/width of M_p 891, depends strictly on the type of non-SEC polymer-column packing interaction that is predominant for a specific stationary phase - mobile phase combination (*e.g.*, electrostatic for HILIC; hydrophobic for C₁₈; hydrogen bonding with unionized residual silanols for all columns, *etc.*) within a total non-SEC effect from all contributing non-SEC interactions. As seen in Figs. 1–3, 5 and 6, the resolution of the M_p 891 oligomers is significantly decreased when non-SEC interactions are reduced, and as a result the peak shape of M_p 891 can change from multimodal to almost unimodal. Earlier studies by Jandera et al. [25,26] demonstrated that the interaction of strong acids containing sulfonic acid groups with the stationary phase depends strongly on the degree of sulfonation. The higher the dipole moment of the acid, the longer is the retention. Moreover, stationary phase properties such as type of packing, chemistry, and bonded ligand density strongly affect the retention, selectivity, and the band shape of the eluting acids. The degree of sulfonation of all NaPSS standards used in this work was provided by the manufacturer as “Degree of sulfonation >90%; For calculation: Assumption: Degree of sulfonation is 95%”. Therefore, this 5% difference in degree of sulfonation could have a stronger effect on the elution of oligomers (and specifically on the elution of NaPSS M_p 891), rather than on that of larger molar mass polymers. While the degree of sulfonation, which is an average value, does not provide any information on its dispersity across the molar mass distribution (MMD) of the polymers, it can be expected that this dispersity, as a function of molar mass, is not substantial for the narrow MMD standards used in this study.

3.3. Temperature effect on elution and partition coefficients of NaPSS

To investigate the effect of column temperature on the elution of NaPSS and its partition coefficient, K_{SEC} , the elution volumes of uracil (used to measure the total permeation volume) and the NaPSS polymer calibrants (g mol^{-1}): 891; 3420; 6430; 15,800; 29,500; 65,400; 152,000 (used to measure total exclusion volume on the HILIC column); 470,000 (used to measure total exclusion volume on C_{18} , C_4 , phenyl, and cyano columns) and 976,000 were measured. The mobile phases used for this study were: 70/30 (v/v) aqueous $0.2 \text{ mol L}^{-1} \text{ Na}_2\text{SO}_4/\text{THF}$ (on the C_{18} , C_4 , and phenyl columns); 80/20 (v/v) aqueous $0.2 \text{ mol L}^{-1} \text{ Na}_2\text{SO}_4/\text{THF}$, and 70/30 (v/v) aqueous $0.2 \text{ mol L}^{-1} \text{ Na}_2\text{SO}_4/\text{ACN}$ (on the cyano and HILIC columns); and 65/35 (v/v) aqueous $0.2 \text{ mol L}^{-1} \text{ Na}_2\text{SO}_4/\text{ACN}$ (on the cyano column). The column temperatures examined were 10°C , 20°C , 30°C , 40°C , 50°C , and 60°C . The choice of the organic modifier content in the mobile phase for each column was related to an attempt to reduce, if not completely eliminate, non-SEC interactions of M_p 891 with the column packing. The distribution coefficient, K_{SEC} , was calculated using Eq. (1), below.

$$K_{SEC} = \frac{V_r - V_0}{V_t - V_0} \quad (1)$$

where V_r is the elution volume (or retention volume) of a solute; V_0 is the elution volume of an excluded peak, which is equal to the interstitial volume between the stationary phase particles; and V_t is the total mobile phase volume (total permeation volume) [3]. Because enthalpic interactions generally decrease with increasing temperature, K_{SEC} determined at 60°C on the cyano and HILIC columns, and at 50°C on the C_{18} , C_4 , and phenyl columns, were used as the reference to calculate % K_{SEC} (the percent change in K_{SEC} between each temperature and the reference temperature) for K_{SEC} determined at column temperatures set to 50°C , 40°C , 30°C , 20°C , and 10°C . The values of K_{SEC} and % K_{SEC} are shown in Tables 3 and 4 for NaPSS standards on each column in each mobile phase.

While there are no strict rules for the assessment of a partition coefficient's dependency on column temperature, it will be regarded here that if % K_{SEC} is below 10% (and certainly below 5%), then solutes are eluting by a near-ideal SEC mechanism, which is virtually devoid of enthalpic interactions; conversely, if % K_{SEC} is 10% or greater, then the elution is by a non-ideal SEC mechanism, where the enthalpy change contributes appreciably to the separation of solutes.

The reason for the upper temperature limit being lower on the C_{18} , C_4 , and phenyl columns than on the cyano and HILIC columns (50°C on the former three columns *versus* 60°C on the latter two) is that when a mobile phase of 70/30 (v/v) aqueous $0.2 \text{ mol L}^{-1} \text{ Na}_2\text{SO}_4/\text{THF}$ was used at a column temperature of 60°C , all the NaPSS peaks collapsed into one peak, eluting at an retention volume lower than the exclusion volume of the column and exhibiting significant tailing (note that even uracil experienced a significant peak shape distortion and a retention time shift to a lower value, approximately 1 min less). This could possibly be due to Na_2SO_4 salt precipitation from the mobile phase onto the column at high

concentration of THF solvent and at higher temperature (*e.g.*, 60 °C), since the solubility of Na₂SO₄ decreases slightly at temperatures higher than 32.38 °C [27]. The decrease in solubility might also be enhanced by the presence of a medium polarity solvent at high concentration (*e.g.*, 30%).

From Table 3, the calculated % K_{SEC} values indicate that the elution of NaPSS standards with 70/30 (v/v) aqueous 0.2 mol L⁻¹ Na₂SO₄/THF mobile phase on the C₄ and phenyl columns is by a non-ideal SEC mechanism and is influenced by non-SEC interactions at all temperatures (10 °C through 50 °C). The oligomer M_p 891 elutes after the total permeation volume at a column temperature of 10 °C, and its peak shape is a collection of multiple peaks on both the C₄ and phenyl columns (chromatograms not shown). However, this peak elutes before the total permeation volume, and its width decreases, when the column temperature is raised to 50 °C (M_p 891 is marked with an arrow in Fig. 3).

In contrast, the elution of higher molar mass NaPSS standards (3420; 6430; 15,800; 29,500; and 65,400) with 70/30 (v/v) aqueous mol L⁻¹ Na₂SO₄/THF on the C₁₈ column is by a predominantly SEC mechanism (Table 3), where enthalpic contributions are essentially absent within the range of 20 °C–50 °C. The exception is the elution of a higher molar mass NaPSS solute (152,000) for which the temperature range of near-ideal SEC is only 40 °C–50 °C. However, while the width of the oligomer M_p 891 peak significantly decreases when the column temperature is raised from 10 °C to 50 °C, this peak still elutes after the total permeation volume at 50 °C as a collection of multiple peaks (red arrow in Fig. 3 indicates M_p 891).

The HILIC column, on the other hand, provides the most inert surface for the present analyses. Values of % K_{SEC} in Table 4 demonstrate a near-ideal SEC elution of NaPSS standards on the HILIC column with 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF and 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phases over the entire temperature range investigated (10 °C–60 °C). The oligomer standard M_p 891 elutes before the total permeation volume (before the uracil peak), and its nearly unimodal peak shape changes only slightly with temperature (M_p 891 is marked with a red arrow in Figs. 7 and 8).

The cyano column also demonstrates a near-ideal SEC elution of NaPSS standards with 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF mobile phase, but only over the temperature range of 40 °C–60 °C (Table 4). As previously observed when employing the C₁₈ column with 30% of THF in the mobile phase, the elution of NaPSS M_p 891 on the cyano column with 20% THF in the mobile phase is only slightly improved when temperature is increased to 60 °C. This assembly of oligomers still elutes as a collection of multiple peaks spread around the total permeation volume (chromatogram not shown). For 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase, the cyano column demonstrates a near-ideal SEC elution of NaPSS standards over the entire temperature range investigated (10 °C–60 °C), with the exception of M_p 152,000 at 60 °C where % K_{SEC} = 10 (Table 4). As seen in Fig. 9, the peak width of M_p 891 solute decreases with increasing column temperature. Also, Fig. 9 demonstrates poor peak shapes of high molar mass NaPSS standards (M_p 976,000; 470,000; and 152,000) at 10 °C conditions. This was not observed on the HILIC column with 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase in Fig. 8. When the ACN

content is increased in the mobile phase (65/35 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN), in an attempt to improve the elution of M_p 891 (*i.e.*, to decrease its non-SEC interactions with the column packing), the temperature range of near-ideal SEC conditions is limited to the 30 °C–60 °C range (Table 4). However, the elution of M_p 891 shifts to earlier volumes and its peak width significantly decreases, especially when the column temperature is raised above 60 °C (*e.g.*, to 70 °C as in Fig. 10). Values of % K_{SEC} , calculated from the reference temperature of 70 °C in Table 5, demonstrate the nearly-ideal SEC elution of NaPSS standards over the range of 30 °C–60 °C.

Similarly to what was observed for column-mobile phase combinations in Section 3.2, temperature may have a significant impact on the resolution of an assembly of M_p 891 oligomers, and thus on the peak shape/width of M_p 891. From Fig. 9 and 10, it can be seen that a column temperature increase changes the elution mechanism of M_p 891 oligomer from non-SEC, or non-ideal SEC, to near-ideal SEC, and therefore this temperature increase significantly reduces the resolution of this assembly of oligomers, and its peak shape changes from multimodal to unimodal (Fig. 10). If an increase in column temperature does not change the elution mechanism of M_p 891 oligomer (*i.e.*, remains strictly as near-ideal SEC), then the temperature impact on the resolution and peak shape/width of this assembly of oligomers is minimal (Figs. 7 and 8).

4. Conclusions

Silica-based RP and HILIC columns are very attractive for aqueous SEC separations because they are compatible with all organic solvents and water, stable at low and moderately high pH, require short equilibration times, provide excellent efficiency (due to small particle sizes), and are relatively inexpensive. Particle pore sizes of 300 Å in RP columns can accommodate a wide range of polymer molar masses (up to approximately 500,000 g mol⁻¹ for NaPSS). Chromatographic resolution of solutes can be enhanced by extending the length of a column, thereby providing a plate number increase. Almost all RP columns are available in 250 mm lengths, as opposed to the 150 mm columns used in this study. However, a wide acceptance of RP columns for SEC will depend on the availability of columns with large pore sizes to cover high (greater than 500,000 g mol⁻¹) or even ultra-high (greater than 1,000,000 g mol⁻¹) ranges of molar masses. A further development in RP column technology that would target significantly higher column phase ratios is necessary.

Non-SEC effects on silica-based RP and HILIC columns can be minimized by the addition of an electrolyte and/or an organic modifier, such as ACN or THF, to the mobile phase, and by increasing column temperature (*e.g.*, to 50 °C or 60 °C). The best performance is seen when employing polar, medium polarity and even long-ligand non-polar stationary phases, such as diol-(HILIC), cyano- and C₁₈-modified silica, where the elution of the NaPSS polyelectrolyte is by a near-ideal SEC mechanism. Short-ligand non-polar hydrophobic stationary phases, such as C₄, and phenyl, are prone to non-SEC interactions and therefore NaPSS elutes by a non-ideal SEC mechanism.

Low molar mass oligomers (<3000 g mol⁻¹) of water-soluble polyelectrolytes may experience electrostatic, hydrophobic, hydrogen bond, or other types of non-SEC

interactions with the stationary phase of RP and HILIC columns. These interactions are possibly the result of a combination of differences in structural conformation and degree of sulfonation, as well as end-group effects, compared to their polymeric counterparts.

Acknowledgements

The authors would like to gratefully acknowledge GlaxoSmithKline for providing the facilities where this work was performed and permitting access to the instruments and all necessary supplies.

References

- [1]. Porath J, Flodin P, Gel filtration: a method for desalting and group separation, *Nature* 183 (1959) 1657–1659. [PubMed: 13666849]
- [2]. Saunders GD, Barth HG, The state of the art and future trends of size exclusion chromatography packings and columns, *LCGC N. Am* 30 (2012) 544–563.
- [3]. Mori S, Barth HG, *Size Exclusion Chromatography*, Springer-Verlag, Berlin, 1999.
- [4]. Striegel AM, Yau WW, Kirkland JJ, Bly DD, *Modern Size-Exclusion Liquid Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography*, 2nd ed., Wiley, Hoboken, New Jersey, 2009.
- [5]. Hong P, Koza S, Bouvier ESP, Size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates, *J. Liq. Chromatogr. Relat. Technol* 35 (2012) 2923–2950. [PubMed: 23378719]
- [6]. Meehan E, Semirigid polymer gels for size exclusion chromatography, in: Wu C.-s. (Ed.), *Handbook of Size Exclusion Chromatography and Related Techniques*, Marcel Dekker, Inc, New York, 2004.
- [7]. Uliyanchenko E, Schoenmakers PJ, Wal Svd., Fast and efficient size-based separations of polymers using ultra-high-pressure liquid chromatography, *J. Chromatogr. A* 1218 (2011) 1509–1518. [PubMed: 21300362]
- [8]. Uliyanchenko E, Wal Svd., Schoenmakers PJ, Challenges in polymer analysis by liquid chromatography, *Polym. Chem* 3 (2012) 2313–2335.
- [9]. Uliyanchenko E, Wal Svd., Schoenmakers PJ, Deformation and degradation of polymers in ultra-high-pressure liquid chromatography, *J. Chromatogr. A* 1218 (2011) 6930–6942. [PubMed: 21875706]
- [10]. Uliyanchenko E, Cools PJCH, Wal Svd., Schoenmakers PJ, Comprehensive two-dimensional ultrahigh-pressure liquid chromatography for separations of polymers, *Anal. Chem* 84 (2012) 7802–7809. [PubMed: 22928586]
- [11]. Janco M, Alexander JN, Bouvier ESP, Morrison D, Ultra-high performance size-exclusion chromatography of synthetic polymers, *J. Sep. Sci* 36 (2013) 2718–2727. [PubMed: 23821608]
- [12]. Caltabiano AM, Foley JP, Barth HG, Size exclusion chromatography of synthetic polymers and biopolymers on common reversed phase and hydrophilic interaction chromatography columns, *J. Chromatogr. A* 1437 (2016) 74–87. [PubMed: 26877177]
- [13]. Pirok BWJ, Breuer P, Hoppe SJM, Chitty M, Welch E, Farkas T, Wal Svd., Peters R, Schoenmakers PJ, Size-exclusion chromatography using core-shell particles, *J. Chromatogr. A* 1486 (2017) 96–102. [PubMed: 27993396]
- [14]. Caltabiano AM, Foley JP, Striegel AM, Organic solvent modifier and temperature effects in non-aqueous size-exclusion chromatography on reversed-phase columns, *J. Chromatogr. A* 1531C (2018) 76–96.
- [15]. Engelhardt H, Mathes D, Chemically bonded stationary phases for aqueous high-performance exclusion chromatography, *J. Chromatogr* 142 (1977) 311–320.
- [16]. Mori S, Effect of the sulfonation of sodium poly(styrenesulfonate) compounds on the elution behavior in aqueous size exclusion chromatography, *J. Liq. Chromatogr. Relat. Technol* 21 (1998) 2935–2945.

- [17]. Terentjev EM, Weitz DA, The Oxford Handbook of Soft Condensed Matter, Oxford University Press, Oxford, United Kingdom, 2015.
- [18]. Pethrick RA, Polymer Yearbook 17, Harwood Academic Publishers, The Netherlands, 2000.
- [19]. Carrillo J-MY, Dobrynin AV, Detailed molecular dynamics simulations of a sodel NaPSS in water, J. Phys. Chem. B 114 (2010) 9391–9399. [PubMed: 20604505]
- [20]. Dobrynin AV, Rubinstein M, Theory of polyelectrolytes in solutions and at surfaces, Prog. Polym. Sci 30 (2005) 1049–1118.
- [21]. Mantha S, Yethiraj A, Conformational properties of sodium polystyrenesulfonate in water: insights from a coarse-grained model with explicit solvent, J. Phys. Chem. B 119 (2015) 11010–11018. [PubMed: 26047770]
- [22]. Nyström B, Roots J, Dynamics of polystyrene sulfonate ionomers in solution. A neutron spin-echo study, J. Polym. Sci. Part C: Polym. Lett 24 (1986) 273–281.
- [23]. Kostanski LK, Keller DM, Hamielec AE, Size-exclusion chromatography - a review of calibration methodologies, J. Biochem. Biophys. Methods 58 (2004) 159–186. [PubMed: 14980789]
- [24]. Striegel AM, There's plenty of gloom at the bottom: the many challenges of accurate quantitation in size-based oligomeric separations, Anal. Bioanal. Chem 405 (2013) 8959–8967. [PubMed: 23887277]
- [25]. Jandera P, Bunceková S, Halama M, Novotná K, Nepras M, Naphthalene sulphonic acids - new test compounds for characterization of the columns for reversed-phase chromatography, J. Chromatogr. A 1059 (2004) 61–72. [PubMed: 15628125]
- [26]. Jandera P, Bocian S, Molikova M, Buszewski B, Characterization of the properties of stationary phases for liquid chromatography in aqueous mobile phases using aromatic sulphonic acids as the test compounds, J. Chromatogr. A 1216 (2009) 237–248. [PubMed: 19081105]
- [27]. Bharmoria P, Gehlot PS, Gupta H, Kumar A, Temperature-dependent solubility transition of Na₂SO₄ in water and the effect of NaCl therein: solution structures and salt water dynamics, J. Phys. Chem. B 118 (2014) 12734–12742. [PubMed: 25313635]

Fig. 1.

Separation of NaPSS standards from Table 2 (NaPSS 1 – black trace; NaPSS 2 – blue trace; and NaPSS 3 – green trace; red arrow points to M_p 891 peak) on a HILIC column. Mobile phases used: 0.2 mol L⁻¹ Na₂SO₄ in water (A); 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/MeOH (B); 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN (C); and 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF (D). Instrumentation: Agilent 1260 with a diode array detector set to 224 nm (A, B, and C); Agilent 1290 with a diode array detector set to 224 nm (D). Other conditions: injection volume = 1.0 µL; flow rate = 0.30 mL min⁻¹; column temperature = 30 °C; autosampler temperature = 5 °C. Note: standard solutions in A, B, and C were prepared on the same day, and solutions in D on a different day, and therefore, concentrations of standards in D differ from those in A, B and C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Comparison of NaPSS chromatograms generated using 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF (black solid trace) and 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN (blue dashed trace) on a HILIC column. Instrumentation: Agilent 1290 with a diode array detector set to 224 nm for NaPSS and to 258 nm for uracil. Other conditions: injection volume = 1.0 µL; flow rate = 0.30 mL min⁻¹; column temperature = 50 °C; autosampler temperature = 5 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Comparison of separation of uracil (black trace) and NaPSS standards from Table 2 (NaPSS 1 – dark blue trace; NaPSS 2 – green trace; and NaPSS 3 – light blue trace; red arrow points to M_p 891 peak) on C₁₈, C₄, and phenyl columns using 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF mobile phase. Instrumentation: Agilent 1260 with a diode array detector with the C₁₈ column and Agilent 1290 with a diode array detector with C₄ and phenyl columns, set to 224 nm for NaPSS and to 258 nm for uracil. Other conditions: injection volume = 1.0 µL; flow rate = 0.30 mL min⁻¹; column temperature = 50 °C; autosampler temperature = 5 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Calibration plot for a C₁₈ (A), C₄ (B) and phenyl (C) columns using uracil (green data point) and NaPSS standards (M_p 3420; 6430; 15,800; 29,500; 65,400; 152,000; 470,000 and 976,000) (blue data points) using a 70/30 (v/v) aqueous 0.2 mol L⁻¹ Na₂SO₄/THF mobile phase. Third-order polynomial curves are fitted only for the NaPSS range between M_p 3420 and 152,000 (blue data points outlined in red). Data used are from triplicate injections. Instrumentation and other condition are as in Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Separation of uracil (black trace) and NaPSS calibrants from Table 2 (NaPSS 1 – dark blue trace; NaPSS 2 – green trace; and NaPSS 3 – light blue trace) on phenyl, cyano and HILIC columns using 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase.

Instrumentation: Agilent 1290 with a diode array detector set to 224 nm for NaPSS and to 258 nm for uracil. Other conditions: injection volume = 1.0 µL; flow rate = 0.30 mL min⁻¹; column temperature = 60 °C; autosampler temperature = 5 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Comparison of uracil and NaPSS chromatograms generated using 65/35 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN (black solid trace) and 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN (blue dashed trace) on a cyano column. Instrumentation and other conditions are as in Fig. 5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Comparison of uracil and NaPSS chromatograms (M_p 976,000; 470,000; 152,000; 65,400; 29,500; 15,800; 6430; 3420; and 891 – marked by a red arrow) generated on a HILIC column with 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF mobile phase at 10 °C, 30 °C, and 60 °C column temperatures. Instrumentation: Agilent 1290 with a diode array detector, set to 224 nm for NaPSS and to 258 nm for uracil. Other conditions: injection volume = 1.0 μ L; flow rate = 0.30 mL min⁻¹; column temperature = 10 °C–60 °C; autosampler temperature = 5 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8.

Comparison of uracil and NaPSS chromatograms generated on a HILIC column with 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase at 10 °C, 30 °C, and 60 °C column temperatures. NaPSS standards, instrumentation, and other conditions as in Fig. 7. (For interpretation of the references to colour in text, the reader is referred to the web version of this article.)

Fig. 9. Comparison of uracil and NaPSS chromatograms generated on a cyano column with 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase at 10 °C, 30 °C, and 60 °C column temperatures. NaPSS standards, instrumentation and other conditions as in Fig. 7.

Fig. 10.

Comparison of uracil and NaPSS chromatograms generated on a cyano column with 65/35 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase at 10 °C, 30 °C, and 60 °C column temperatures. NaPSS standards, instrumentation and other conditions as in Fig. 7.

Table 1

Column Parameters. Column length and internal diameter (mm) 150×4.6 .

Column ID	Particle Size (μm)	Pore Size ($\text{\AA}$)
Luna HILIC ^a	3	200
ACE 3 C ₁₈ -300 ^b	3	300
ACE 3 C ₄ -300 ^b	3	300
ACE 3 Phenyl-300 ^b	3	300
ACE 3 CN-300 ^b	3	300

^aManufacturer: Phenomenex.

^bDistributor: MAC-MOD.

Table 2

Molar mass values for NaPSS standards reported on Phenomenex certificate of analysis.

Standard ID	Mp (GPC/SEC)	Mw/Mn
NaPSS 1	3420	<1.20
	15800	<1.20
	152000	<1.20
NaPSS 2	891	<1.20
	29500	<1.20
	470000	<1.20
NaPSS 3	6430	<1.20
	65400	<1.20
	976000	<1.20

Table 3

Calculated K_{sec} and % K_{sec} (from the reference K_{sec} at 50 °C) from elution volumes obtained for NaPSS standards (Mp 152,000; 65,400; 29,500; 15,800; 6430; 3420) on C₁₈, C₄ and phenyl columns with 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF mobile phase. Triplicate injections are made at 30–50 °C; single injections are made at 10 and 20 °C. Instrumentation: Agilent 1260 with a diode array detector with the C₁₈ column and Agilent 1290 with a diode array detector with C₄ and phenyl columns, set to 224 nm for NaPSS and to 258 nm for uracil. Other conditions: injection volume = 1.0 µL; flow rate = 0.30 mL min⁻¹; column temperature = 10–50 °C; autosampler temperature = 5 °C. Temperature

Column ID	Mobile Phase	NaPSS Mp	Temperature											
			10° C			20° C			30° C			40° C		
			K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}
Phenyl	70/30 (v/v) 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/THF	152000	0.091	97.8	0.069	50.0	0.066	43.5	0.059	28.3	0.046			
		65400	0.210	59.1	0.198	50.0	0.186	40.9	0.166	25.8	0.132			
		29500	0.380	46.7	0.365	40.9	0.342	32.0	0.309	19.3	0.259			
		15800	0.508	47.7	0.488	41.9	0.464	34.9	0.425	23.5	0.344			
		6430	0.630	34.9	0.613	31.3	0.590	26.3	0.549	17.6	0.467			
C ₄	70/30 (v/v) 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/THF	3420	0.704	32.1	0.686	28.7	0.665	24.8	0.625	17.3	0.533			
		152000	0.068	54.5	0.063	43.2	0.061	38.6	0.054	22.7	0.044			
		65400	0.197	84.1	0.186	73.8	0.173	61.7	0.150	40.2	0.107			
		29500	0.361	77.0	0.344	68.6	0.321	57.4	0.281	37.7	0.204			
		15800	0.490	70.7	0.469	63.4	0.441	53.7	0.391	36.2	0.287			
C ₁₈	70/30 (v/v) 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/THF	6430	0.622	61.1	0.602	56.0	0.573	48.4	0.518	34.2	0.386			
		3420	0.696	51.0	0.677	46.9	0.650	41.0	0.597	29.5	0.461			
		152000	0.050	-20.6	0.076	20.6	0.070	11.1	0.068	7.9	0.063			
		65400	0.254	33.0	0.207	8.4	0.201	5.2	0.192	0.5	0.191			
		29500	0.429	17.9	0.389	6.9	0.371	1.9	0.360	-1.1	0.364			
		15800	0.541	10.2	0.538	9.6	0.509	3.7	0.499	1.6	0.491			
		6430	0.778	19.1	0.690	5.7	0.657	0.6	0.641	1.8	0.653			
		3420	0.812	13.1	0.770	7.2	0.730	1.7	0.719	0.1	0.718			

Table 4

Calculated K_{sec} and % K_{sec} (from the reference K_{sec} at 60 °C) from elution volumes obtained for NaPSS standards (Mp 152,000; 65,400; 29,500; 15,800; 6430; 3420; and 891) on HILIC and cyano columns with 80/20 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/THF and 70/30 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phases, and on cyano column with 65/35 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase. Triplicate injections are made at 30–60 °C; single injections are made at 10 and 20 °C. Instrumentation: Agilent 1290 with a diode array detector, set to 224 nm for NaPSS and to 258 nm for uracil. Other conditions: injection volume = 1.0 µL; flow rate = 0.30 mL min⁻¹; column temperature = 10–60 °C; autosampler temperature = 5 °C. Mp 470,000 was used to measure exclusion volume of cyano column; Mp 152,000 was used to measure exclusion of HILIC column due to its small pore size (200 Å).

Column ID	Mobile Phase	NaPSS Mp	Temperature											
			10 °C			20 °C			30 °C			40 °C		
			K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}
HILIC	70/30 (v/v) 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/ACN	152000	–	–	–	–	–	–	–	–	–	–	–	–
		65400	0.081	9.5	0.078	5.4	0.077	4.1	0.079	6.8	0.075	1.4	0.074	0.074
		29500	0.242	5.7	0.237	3.5	0.235	2.6	0.237	3.5	0.231	0.9	0.229	0.229
		15800	0.403	4.1	0.400	3.4	0.396	2.3	0.393	1.6	0.389	0.5	0.387	0.387
		6430	0.583	3.2	0.579	2.5	0.576	1.9	0.577	2.1	0.568	0.5	0.565	0.565
		3420	0.687	2.4	0.685	2.1	0.682	1.6	0.678	1.0	0.673	0.3	0.671	0.671
		891	0.854	1.1	0.855	1.2	0.855	1.2	0.858	1.5	0.849	0.5	0.845	0.845
Cyano	80/20 (v/v) 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/THF	152000	–	–	–	–	–	–	–	–	–	–	–	–
		65400	0.070	9.4	0.067	4.7	0.067	4.7	0.066	3.1	0.066	3.1	0.064	0.064
		29500	0.217	9.0	0.212	6.5	0.208	4.5	0.206	3.5	0.203	2.0	0.199	0.199
		15800	0.367	6.4	0.362	4.9	0.358	3.8	0.354	2.6	0.350	1.4	0.345	0.345
		6430	0.542	4.0	0.535	2.7	0.533	2.3	0.530	1.7	0.526	1.0	0.521	0.521
		3420	0.645	2.5	0.642	2.1	0.639	1.6	0.636	1.1	0.633	0.6	0.629	0.629
		891	0.812	0.4	0.835	3.2	0.808	–0.1	0.809	0.0	0.810	0.1	0.809	0.809
	70/30 (v/v) 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/ACN	152000	0.081	3.8	0.070	–10.3	0.074	–5.1	0.076	–2.6	0.077	–1.3	0.078	0.078
		65400	0.189	–8.7	0.194	–6.3	0.199	–3.9	0.202	–2.4	0.205	–1.0	0.207	0.207
		29500	0.352	–7.4	0.361	–5.0	0.367	–3.4	0.373	–1.8	0.377	–0.8	0.380	0.380
		15800	0.483	–6.2	0.491	–4.7	0.501	–2.7	0.507	–1.6	0.512	–0.6	0.515	0.515
		6430	0.616	–4.9	0.626	–3.4	0.634	–2.2	0.640	–1.2	0.645	–0.5	0.648	0.648
		3420	0.691	–4.0	0.700	–2.8	0.708	–1.7	0.714	–0.8	0.717	–0.4	0.720	0.720

Column ID	Mobile Phase	NaPSS Mp	Temperature											
			10 °C			20 °C			30 °C			40 °C		
			K _{sec}	%	K _{sec}	K _{sec}	%	K _{sec}	K _{sec}	%	K _{sec}	K _{sec}	%	K _{sec}
65/35 (v/v) 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/ACN		152000	0.063	-18.2	0.068	-11.7	0.071	-7.8	0.073	-5.2	0.077	0.0	0.077	0.077
		65400	0.167	-17.3	0.181	-10.4	0.188	-6.9	0.193	-4.5	0.199	-1.5	0.202	0.202
		29500	0.308	-16.1	0.334	-9.0	0.346	-5.7	0.355	-3.3	0.362	-1.4	0.367	0.367
		15800	0.426	-14.3	0.458	-7.8	0.474	-4.6	0.484	-2.6	0.492	-1.0	0.497	0.497
		6430	0.557	-11.6	0.590	-6.3	0.603	-4.3	0.615	-2.4	0.624	-1.0	0.630	0.630
		3420	0.634	-9.7	0.669	-4.7	0.682	-2.8	0.692	-1.4	0.698	-0.6	0.702	0.702
80/20 (v/v) 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/THF		891	-	-	-	-	-	-	-	-	0.823	-0.7	0.829	0.829
		152000	0.073	7.4	0.072	5.9	0.071	4.4	0.069	1.5	0.068	0.0	0.068	0.068
		65400	0.204	14.6	0.200	12.4	0.196	10.1	0.191	7.3	0.186	4.5	0.178	0.178
		29500	0.380	14.5	0.372	12.0	0.364	9.6	0.355	6.9	0.345	3.9	0.332	0.332
		15800	0.520	13.8	0.509	11.4	0.499	9.2	0.487	6.6	0.473	3.5	0.457	0.457
		6430	0.657	11.4	0.645	9.3	0.633	7.3	0.621	5.3	0.608	3.1	0.590	0.590
3420			0.734	10.5	0.720	8.4	0.709	6.8	0.696	4.8	0.681	2.6	0.664	0.664

Table 5

Calculated K_{sec} and % K_{sec} (from the reference K_{sec} at 70 °C) from elution volumes obtained for NaPSS standards (Mp 152,000; 65,400; 29,500; 15,800; 6430; 3420; and 891) on cyano column with 65/35 (v/v) 0.2 mol L⁻¹ Na₂SO₄ in water/ACN mobile phase. Triplicate injections are made at 30–70 °C. Instrumentation: Agilent 1290 with a diode array detector, set to 224 nm for NaPSS and to 258 nm for uracil. Other conditions: injection volume = 1.0 µL; flow rate = 0.30 mL min⁻¹; column temperature = 10–70 °C; autosampler temperature = 5 °C.

Column ID	Mobile Phase	NaPSS Mp	Temperature											
			30 °C			40 °C			50 °C			60 °C		
			K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}	K_{sec}	%	K_{sec}
Cyano	65/35 0.2 mol L ⁻¹ Na ₂ SO ₄ in water/acetonitrile	152000	0.071	-13.4	0.073	-11.0	0.077	-6.1	0.077	-6.1	0.081	-1.2	0.082	$K_{\text{secReference}}$
		65400	0.188	-10.5	0.193	-8.1	0.199	-5.2	0.202	-3.8	0.209	-0.5	0.210	
		29500	0.346	-8.5	0.355	-6.1	0.362	-4.2	0.367	-2.9	0.376	-0.5	0.378	
		15800	0.474	-6.7	0.484	-4.7	0.492	-3.1	0.497	-2.2	0.508	0.0	0.508	
		6430	0.603	-5.5	0.615	-3.6	0.624	-2.2	0.630	-1.3	0.637	-0.2	0.638	
		3420	0.682	-3.9	0.692	-2.5	0.698	-1.7	0.702	-1.1	0.712	0.3	0.710	
		891	–	–	–	–	–	0.823	-2.4	0.829	-1.7	0.845	0.2	0.843