Journal of Chromatography A, 1218 (2011) 1509–1518

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fast and efficient size-based separations of polymers using ultra-high-pressure liquid chromatography

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ARTICLE INFO

Article history:
Received 26 November 2010
Received in revised form 13 January 2011
Accepted 17 January 2011
Available online 22 January 2011

Keywords:
Ultra-high-pressure liquid chromatography
Fast size-exclusion chromatography
Hydrodynamic chromatography
Polystyrene standards
Slalom chromatography
Deborah number

ABSTRACT

Ultra-high-pressure liquid chromatography (UHPLC) has great potential for the separations of both small molecules and polymers. However, the implementation of UHPLC for the analysis of macromolecules invokes several problems. First, to provide information on the molecular-weight distribution of a polymer, size-exclusion (SEC) columns with specific pore sizes are needed. Development of packing materials with large pore diameters and pore volumes which are mechanically stable at ultra-high-pressures is a technological challenge. Additionally, narrow-bore columns are typically used in UHPLC to minimize the problem of heat dissipation. Such columns pose stringent requirements on the extra-column dispersion, especially for large (slowly diffusing) molecules. Finally, UHPLC conditions generate high shear rates, which may affect polymer chains. The possibilities and limitations of UHPLC for size-based separations of polymers are addressed in the present study. We demonstrate the feasibility of conducting efficient and very fast size-based separations of polymers using conventional and wide-bore (4.6 mm I.D.) UHPLC columns. The wider columns allow minimization of the extra-column contribution to the observed peak widths down to an insignificant level. Reliable SEC separations of polymers with molecular weights up to *ca.* 50 kDa are achieved within less than 1 min at pressures of about 66 MPa. Due to the small particles used in UHPLC it is possible to separate high-molecular-weight polymers (50 kDa $\leq M_r \leq$ 1–3 MDa, upper limit depends on the flow rate) in the hydrodynamic-chromatography (HDC) mode. Very fast and efficient HDC separations are presented. For very large polymer molecules (typically larger than several MDa, depending on the flow rate) two chromatographic peaks are observed. This is attributed to the onset of molecular deformation at high shear rates and the simultaneous actions of hydrodynamic and slalom chromatography.

1. Introduction

The term "ultra-high pressure liquid chromatography, UHPLC" was introduced by MacNair et al. for liquid chromatographic separations performed at pressures above 400 MPa and using columns packed with 1.5-μm [1] and 1.0-μm particles [2]. The same term was used by Lippert and co-workers for the system developed in their laboratory, which was able to operate at pressures up to 360 MPa [3]. They used stationary phase with 1.5-μm particles. Such systems are very complex and are not commercially available. Liquid chromatography with pressures above 40 MPa (400 bar) was first commercialized by Waters, who introduced the abbreviation UPLC. Some researchers have used the term UPLC in peer-reviewed literature [4]. However, ultra-high pressure liquid chromatography or UHPLC has become the accepted terminology for separations performed using sub-2-μm particles at pressures exceeding the 40 MPa limit of high-performance liquid chromatography (HPLC) [5–7]. Another term used less commonly for the same technique is "very high pressure liquid chromatography" [8,9]. In this work we will use the term "ultra-high pressure liquid chromatography (UHPLC)" for the separations performed using 1.7-μm particles and an HPLC system capable of generating pressures up to 100 MPa.

UHPLC is an increasingly important technique in many analytical laboratories. The proliferation of UHPLC during the last five years has been spurred by the advantages it offers for separations of complex samples. Higher pressure limits make it possible to use columns packed with smaller (sub-2-μm) particles under optimum conditions. This leads to lower theoretical plate heights at higher linear velocities and thus to faster and more-efficient separations.

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doi:10.1016/j.chroma.2011.01.053

However, several problems arise from the use of small particles at ultra-high pressures. The problem of heat dissipation in HPLC has been studied by various groups during several decades [10–13]. The problem is strongly aggravated at UHPLC conditions [14–16]. Heat is generated by friction when the mobile phase is forced through a packed bed. The viscous heat dissipation leads to temperature gradients across the column in both the axial and the radial directions. Especially radial temperature gradients may greatly increase the chromatographic band broadening and may jeopardize the column efficiency. The loss of efficiency due to heat dissipation necessitates the use of narrow-bore columns in UHPLC [17]. The internal diameter (I.D.) of columns used for UHPLC separations is typically 3 mm or less. Columns with sub-2-μm particles and conventional diameters (4.6 mm I.D.) are rarely offered by manufacturers.

Narrow-bore columns packed with small particles produce very sharp and fast-eluting peaks, which pose stringent requirements on the extra-column dispersion. In UHPLC the extra-column contribution to the total peak width is more critical than in conventional HPLC separations and should be dramatically reduced. The peak variance due to extra-column contributions is of the order of $10\,\mu L^2$ for UHPLC, while for HPLC it is typically above $40\,\mu L^2$ [4]. UHPLC peaks also require fast detectors. Thus, detectors used for UHPLC separations should feature higher sampling rates and greatly reduced dead volumes.

To a large extent the challenges associated with the application of UHPLC have been overcome. Contemporary UHPLC systems are robust, efficient and easy-to-operate. UHPLC is commonly used for pharmaceutical, environmental and food analyses, as well as in the life sciences. However, little is still published about the potential of UHPLC for the separation of polymers.

Size-exclusion chromatography (SEC) is by far the most common technique for polymer separations. It provides information on various molecular-weight averages and on the molecular-weight distribution of polymer samples. SEC separations at ultra-high pressures are of great potential interest. Faster and more-efficient SEC analyses would be highly attractive, because contemporary applications of SEC often feature long analysis times (sometimes up to several hours). UHPLC may also facilitate high-throughput SEC experiments, which are very appealing for many industrial analyses. Very fast and efficient SEC separations would also constitute a great improvement for the analyses of polymers by comprehensive two-dimensional liquid chromatography, for which there are many applications [18–22]. Despite the higher optimal linear velocities, the use of narrow-bore columns in UHPLC allows to use flow rates that are compatible with mass-spectrometry (MS) [23]. Such columns also lead to a drastic reduction in the volumes of (organic) solvents used for SEC separations. Thus, ultra-high-pressure size-exclusion chromatography (UHPSEC) would represent a significant step forward.

The introduction of UHPSEC is impeded by a number of factors of both technological and fundamental nature. First of all, SEC analysis requires sample solutions, while most polymers are hard-to-dissolve materials. Many types of polymers (*e.g.* polyesters, polyamides) require the use of strong or even aggressive solvents, such as hexafluoroisopropanol or concentrated acids. These solvents pose great demands on a chromatographic system. The parts of the equipment that are subjected to very high pressures are especially sensitive to corrosion, while any polymeric parts are clearly under threat from solvents intended to dissolve engineering plastics.

The second problem is associated with the requirements for UHPLC stationary phases. To provide information on the molecular-weight distribution of a polymer sample one would need to use size-exclusion columns with specific pore-sizes. In UHPLC the pore size is limited by the stability of the packing material at ultra-high pressures. So far, there are no UHPLC SEC columns with large pore diameters and large pore volumes available for characterization of polymers. The UHPLC columns present on the market are mostly packed with porous silica-based materials with different surface chemistries. The pore sizes range from 70 to 300 Å, depending on the column type and manufacturer.

The effects of extra-column dispersion in size-based polymer separations are potentially much greater than in conventional applications of UHPLC. This is associated with several factors. Firstly, the elution volumes are smaller than in interaction liquid chromatography. Secondly, pre-concentration of analytes at the top of the column does not occur, which adds the extra-column dispersion that takes place before the column to the total dispersion. Finally, slow diffusion of large molecules leads to higher dispersion. The temperature dependence of size-based separations is usually negligible (no retention). This makes such separations less prone to the detrimental effects of axial heat gradients. However, the effects of radial temperature gradients may be greater, again due to the slow diffusion of large molecules. Because the optimal conditions (the minimum theoretical plate height) are attained at much lower linear velocities for polymers than for small molecules, the amount of heat generated will be reduced and the axial and radial temperature gradients will be less significant. The final problem is of a fundamental importance in the chromatography of large molecules. UHPLC particles and pressures generate high shear rates, which may lead to the deformation and/or degradation of macromolecules [24].

All these complications have led some of the major manufacturers of SEC columns to take a highly critical perspective on the possibilities of SEC at ultra-high pressures and to question the future of UHPSEC [25]. In the present work we attempt to overcome the limitations of UHPLC for the analysis of polymers. We explore the possibilities of ultra-high-pressure separations based on size-exclusion and hydrodynamic mechanisms. We try to specify the useful range of conditions for the application of ultra-high-pressure size-based separations and show their advantages over conventional size-exclusion and hydrodynamic chromatography.

2. Theory

2.1. Size-exclusion and hydrodynamic chromatography

Size-exclusion chromatography is a type of liquid chromatography in which separation is based on partial exclusion of the solutes from the pores of stationary phase. The larger molecules elute earlier than the smaller molecules, because they penetrate into a smaller fraction of the pores of the packing material. All molecules that are very small in comparison with the pore size will spend equal time on the column, irrespective of their actual size. They all elute at the total permeation volume of the column (V_i). Larger analyte molecules elute ahead of the solvent peak in SEC. All molecules which are too large to penetrate into any of the pores will also elute at the same time (total exclusion volume, V_0) [26]. Depending on the pore size of the packing material, polymers of different hydrodynamic volumes, usually related to different molecular weights may be separated by SEC.

The separation in hydrodynamic chromatography (HDC) is based on the laminar flow profile of solvent between packed particles or in open channels. The selectivity is determined by unequal steric exclusion of solute molecules of different sizes from the walls (or particle surfaces). Smaller molecules are able to come closer to the walls, where the solvent velocity is lower. Thus, they travel more slowly through the column than larger molecules. In order for this process to be sufficiently selective, the size of the molecules should not be too small in comparison with the channel diameter [27–29]. Similarly to SEC, HDC allows

determination of the molecular-weight distribution of a polymer sample.

Both techniques, SEC and HDC, are based on separation according to molecular size rather than molecular weight. The molecular size can be related to the molecular weight using a calibration procedure [26]. In solution polymer molecules are usually present in a coiled shape. The size (hydrodynamic radius, R_h) of polystyrene molecules in pure THF can be estimated from Eq. (1) [30].

$$R_h = (0.024 \pm 0.002) M_w^{0.518\pm0.05} \quad (1)$$

where M_w is the weight-average molecular weight of polystyrene.

2.2. Deborah numbers and slalom chromatography

Shear forces arise during (pressure-driven) chromatographic analyses. The shear rate depends on the flow rate and the column diameter, on the particle size, the porosity of the packing material and on the mobile-phase viscosity [24]. Because UHPLC features smaller particles and higher linear velocities the shear rates are higher than in conventional HPLC. Above a certain molecular weight the shear rates generated in an LC system become large enough to deform the polymer molecules. The molecules no longer have a coiled shape (spherical for polystyrene), but they are stretched in the direction of the flow. Such stretched molecules may travel trough a channel faster than coiled molecules and smaller stretched molecules elute faster than larger ones. Thus, the elution order at these conditions is opposite to the elution order in SEC and HDC separations. The chromatography of stretched molecules is known as slalom chromatography (SC) [31,32].

The onset of the molecular deformation depends on the experimental conditions, as well as on the polymer radius of gyration and, thus, molecular weight. It may be predicted using the Deborah number (*De*), which is the ratio of hydrodynamic forces to Brownian forces [33,34]. For flow in a packed bed this may be defined by [35].

$$De = k_{PB}\left(\frac{\bar{v}}{d_p}\right)\frac{6.12\Phi\eta r_G^3}{RT} \quad (2)$$

where k_{PB} is a constant which depends on the structure of the packed bed (k_{PB} = 9.1 for a randomly packed bed [36]). $\bar{v}$ is the superficial flow velocity, *i.e.* flow rate per unit area of the empty column ($m\,s^{-1}$); d_p is the particle diameter (μm), η the solvent viscosity (Pa s), Φ the Flory–Fox parameter ($\approx 2.5 \times 10^{23}\ mol^{-1}$); R the gas constant ($R = 8.314\ J\,mol^{-1}\,K^{-1}$); T the absolute temperature (K) and r_G is the radius of gyration of the polymer molecule (μm). For polystyrene in THF the relation $r_G = 1.39 \times 10^{-5}\ M_w^{0.588}$ was obtained from light-scattering measurements [37].

Bird et al. arrived at a theoretical value of $De = 0.5$ above which deformation of macromolecules was suggested to occur [33]. The experimental results, also by other researchers [35,38–41] showed that a transition from coiled to stretched molecules does not occur suddenly, but takes place across a range of *De* numbers from around 0.1 to 1.

Stationary phases used in UHPLC contain small (sub-2-μm) porous particles with relatively small pore sizes, usually up to 200 Å (with one exception of columns for protein separations with pore sizes of 300 Å [42]). The presence of these pores offers the possibility of SEC-type separations of polymers in a specific size range under conditions where no interactions occur that would cause retention of the analytes. For very small sub-2-μm UHPLC particles (with correspondingly narrow interstitial channels) we may also expect separations based on hydrodynamic effects for polymers of higher molecular weight.

3. Experimental

3.1. Chemicals

The solvent used in this work as a mobile phase for UHPLC was unstabilized THF of ULC/MS grade (Biosolve, Valkenswaard, The Netherlands). The same THF was used as a needle-wash solvent to avoid sample precipitation in the injector. A freshly opened bottle of THF was used for no longer than two days to avoid the formation of significant amounts of degradation products, which would result in an elevated baseline.

Polymer samples used for experiments were polystyrene standards (PS) from Polymer Laboratories/Agilent Technologies (Church Stretton, Shropshire, UK). Molecular weight and polymer dispersity indices (PDI) of the standards as specified by the manufacturer are shown in Table 1. Toluene from Sigma Aldrich (St. Louis, MO, USA) was used as a low-molecular-weight (total permeation) marker. Samples of toluene and polystyrene standards with a concentration of around 2 mg/mL were prepared in unstabilized THF.

3.2. Instrumentation and operating conditions

The experiments were performed using a Waters Acquity UPLC® system (Waters, Milford, MA, USA). The system was equipped with a tetrahydrofuran/hexane compatibility kit to extend its applicability to these solvents. The system contains a Binary Solvent Manager, which can supply solvents at pressures up to 103 MPa at flow rates up to 1 mL/min. The maximum pressure is lower at higher flow rates (62 MPa at the maximum flow rate of 2 mL/min). The injection mode was "partial loop with needle overfill". An injection volume of 0.8 μL was used with a 2 μL loop. The connecting tubing between the pumps, injector, columns and detector was made from stainless steel and had an internal diameter of 127 μm. The system was equipped with a four-column Column Manager (Waters), which allows simultaneous installation and temperature control (if desirable) of up to four columns. The experiments were conducted at a constant oven temperature of 30 °C, unless otherwise

Table 1
Characteristics of polystyrene samples specified by manufacturer (polymer laboratories) and corresponding hydrodynamic diameter (calculated from the Eq. (1) [30]).

Molecular weight (Da)	Polydispersity	Hydrodynamic diameter (Å)
980	1.1	17
1990	1.05	25
2970	1.04	30
4920	1.03	39
7000	1.03	47
9920	1.02	56
13,880	1.02	67
19,880	1.02	81
30,320	1.01	101
52,400	1.02	133
70,950	1.03	156
96,000	1.03	183
197,300	1.02	266
299,400	1.02	330
523,000	1.03	440
735,000	1.02	525
1,112,000	1.03	650
2,061,000[a]	1.05	–
3,053,000[a]	1.03	–
3,742,000[a]	1.04	–
7,450,000[a]	1.07	–
13,200,000[a]	1.13	–

[a] The Eq. (1) was developed based on experimental data for polystyrene molecular weights up to 1,850,000 Da. The hydrodynamic diameters for polymers with molecular weights exceeding 1.8 MDa may not be correct and, thus, are not shown.

stated. Absorbance was measured at 221 nm using an Acquity UPLC Photodiode Array (PDA) detector (Waters) with a sampling rate of 40 Hz and the noise filter turned off to avoid a possible deterioration of the widths of very narrow peaks. The data were collected and processed with Empower 2 software (Waters).

3.3. Columns

Acquity UPLC C18 columns (Waters) were used for all the measurements. The columns were packed with 1.7 μm BEH C18 particles. The average pore size of the particles was 130 Å. The column dimensions were 50 × 2.1, 100 × 2.1 and 150 × 4.6 mm. The wide-bore (4.6 mm I.D.) UPLC columns were specifically designed for the purpose and generously supplied by Waters.

All series of measurements were repeated three times (and all experiments were repeated several times within a series). Retention times (as reflected in the calibration curves) were repeatable within 3% RSD. Peak widths and peak shapes were also highly repeatable (<3% RSD). Two sets of 4.6-mm I.D. columns from different batches were used and no significant difference was observed between the results obtained on these two sets. The columns proved to be sufficiently stable for the described applications. More than 2000 injections of polymer samples were performed without significant increase in column backpressure (less than 3% compared to the backpressure of the new columns). However, it is important to note that the mentioned columns were mainly used for the separations of polymer standards. In this case there was no need for using an in-line pre-column filter to prevent column clogging.

3.4. Extra-column-band-broadening measurements

The extra-column band broadening was measured by connecting the injector to the detector using a zero-dead-volume union instead of a column. Contributions of extra-column band broadening to the total peak width for PS standards of various molecular weights were estimated using different system configurations (*e.g.* with and without the Column Manager) and different column dimensions. To ensure comparable results the measurements were performed at equal linear velocities for columns of different internal diameters (flow rates of 0.25 mL/min for 2.1 mm I.D. columns and 1.2 mL/min for 4.6 mm I.D. columns). Because the peaks were typically non-Gaussian in shape, the variances were calculated from first and second centralized moments using a home-made Matlab (Natick, MA, USA) routine. The measured values of extra-column dispersion (variance) were in the order of 7–15 μL^2 for polystyrene standards with molecular weights below *ca.* 50 kDa, which is in agreement with the data obtained by other researchers for low-molecular-weight compounds [4,7,43]. For larger polystyrene standards the observed extra-column peak variances were found to be higher (up to 20–25 μL^2), possibly due to the lower diffusion coefficients of such molecules.

3.5. Flow-rate-accuracy measurements

To assure correct values of retention volumes the actual flow rate (F_{actual}) was measured at different set values (F_{set}). The measurements were performed by collecting the eluent (THF) at the exit of the system in a previously weighted dry volumetric flask. The flask was covered with Parafilm tape to minimize evaporation. The collection time was 20 min at all flow rates. The flask with the effluent was weighted and the volume of collected solvent was calculated. The obtained flow rates were compared to the set values and the relative error was calculated as

$$\text{error}(\%) = 100 \times \frac{F_{set} - F_{actual}}{F_{set}} \quad (3)$$

4. Results and discussion

4.1. Size separations at UHPLC conditions

Toluene and polystyrene standards of different molecular weights were injected individually on the 2.1 × 50 mm reversed-phase C18 Acquity UPLC column. As expected, the standards eluted before the total permeation volume (V_0) of the column (0.27 min under the conditions of Fig. 1). Thus, there was no indication of polystyrene retention when THF was used as mobile phase and sample solvent. We observed some selectivity and the elution order corresponded to that in SEC or HDC (smaller molecules eluted later). However, the peaks were relatively broad and tailing. The asymmetry of the peaks did not change with concentration or with the amount of sample injected. Hence, it was not caused by overloading effects. The tailing of the peaks may indicate a large extra-column contribution to the peak width [44]. Thus, such size-based separations need to be further optimized.

In Fig. 2 a calibration curve is shown that was obtained by injecting polystyrene standards with molecular weights ranging from 92 Da (toluene) to 13 MDa (see Table 1.). We can distinguish three regions in such a calibration curve, in which the separation mechanisms are different.

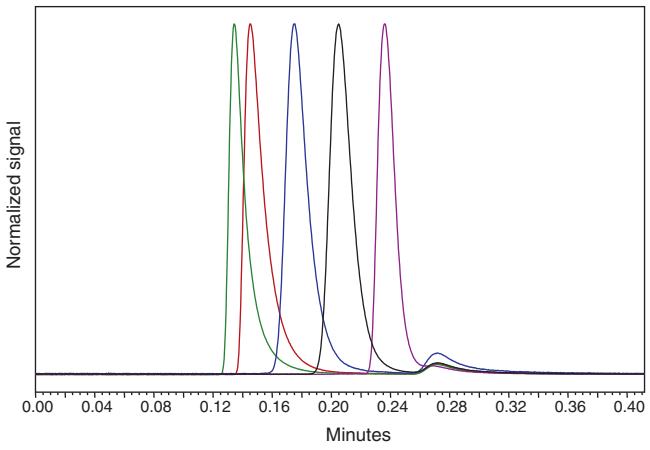

Fig. 1. Chromatograms observed for polystyrene standards under (non-optimized) UHPLC conditions. Polystyrene molecular weights (from right to left): 92 (toluene); 1990; 9920; 52,400; 523,000 Da. Acquity UPLC BEH C18 column, 50 × 2.1 mm I.D. Mobile phase: THF. Flow rate: 0.5 mL/min. Pressure 25 MPa.

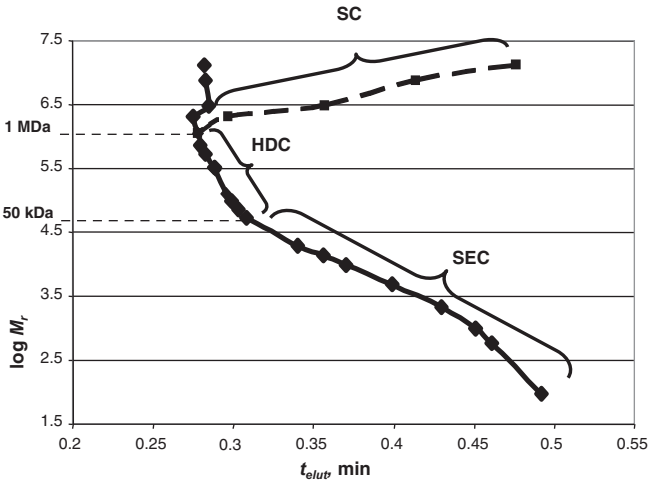

Fig. 2. Calibration curve for polystyrene standards under UHPLC conditions. Acquity UPLC BEH C18 column, 100 × 2.1 mm I.D. Mobile phase: THF. Flow rate: 0.5 mL/min. Pressure 50 MPa. — peak 1; - - - peak 2 (for explanation see text).

The lower range (M_r lower than *ca.* 50 kDa, t_{elut} above 0.31 min) is a SEC region, where separation is based on a size-exclusion mechanism. At the point where the molecular weight of PS reaches about 50 kDa the slope of the calibration curve changes.

The size (hydrodynamic diameter) of polystyrene molecules in THF solution can be calculated from equation (1) [30] and the results are listed in Table 1. A PS standard with molecular weight of 50 kDa has an estimated size in solution of about 133 Å, which is close to the average pore diameter of the particles of this particular stationary phase (130 Å, as specified by the manufacturer). This represents the upper limit up to which a separation based on the SEC mechanism is possible.

The selectivity for the separation of higher-molecular-weight polystyrenes (from *ca.* 50 kDa to 1 MDa in Fig. 2) is somewhat lower, but it is still significant. In this molecular-weight range the size of the molecules is comparable to the size of the interparticle channels, which leads to a separation based on hydrodynamic effects. Because the pores of the packing material are distributed in diameter, the transition from the SEC to the HDC mechanism does not occur suddenly, but takes place over a (narrow) range of molecular weights. Similar changes in the separation mechanism were theoretically predicted and observed in practice by Stegeman et al. for 3-μm porous particles [45]. However, columns packed with sub-2-μm particles are more attractive for HDC separations, because they show a much greater selectivity in the range of soluble polymers than columns packed with larger particles (*e.g.* 3 μm). The larger particles would be preferred for the analysis of very-high-molecular-weight polymers (>1 MDa), for which we approach the onset of molecular deformation using sub-2-μm particles at UHPLC pressures.

At a certain set of conditions (flow rate and polystyrene molecular weight) we again observe a transition in the separation mechanism. This time it is accompanied by a change in the elution order. The transition may be explained by the onset of molecular deformation. Deformed polymers elute in the slalom chromatography mode. For each polymer size there is a specific set of experimental conditions around which deformation starts to take place. Under the present conditions (see Fig. 2) the onset of molecular deformation occurs at a polystyrene molecular weight of around 1 MDa.

The situation is complicated by the dispersity of each individual polymer sample. Every sample contains a range of molecular masses. For molecules larger than a certain critical size (R_{crit}) deformation occurs. The fraction of the sample affected by molecular deformation ($R > R_{crit}$) elutes in the SC mode (larger molecules elute later); molecules of lower molecular weight ($R < R_{crit}$) are present as random coils and they elute in the HDC mode (larger molecules elute earlier). The combination of these two effects implies that molecules with $R \approx R_{crit}$ elute first, followed by a co-elution of larger stretched molecules and smaller coiled molecules. This may result in the presence of several peaks in a chromatogram [41]. The calibration curve is seen to split in this region. This phenomenon is reflected in Fig. 2. Two peaks are observed in chromatograms of PS standards with $M_r > 1$ MDa. An example of such a chromatogram is shown in Fig. 3.

The degradation of macromolecules may also play a role in the appearance of several peaks in a chromatogram. For the 13 MDa polystyrene standard at flow rates of 0.5, 0.7 and 1 mL/min (conditions of Figs. 4 and 5) more than two peaks appear in the chromatogram. This may be an indication of degradation of this polymer. Thus, the experimental points corresponding to the retention volumes of the second peak for this standard are not shown in Figs. 4a and 5. From the experimental data obtained, we believe that no polymer degradation is observed at other experimental conditions used in this work. The subject of degradation is important in analysis of polymers [41], especially when using UHPLC. This subject will be addressed in detail elsewhere [E. Uliyanchenko et al., in preparation].

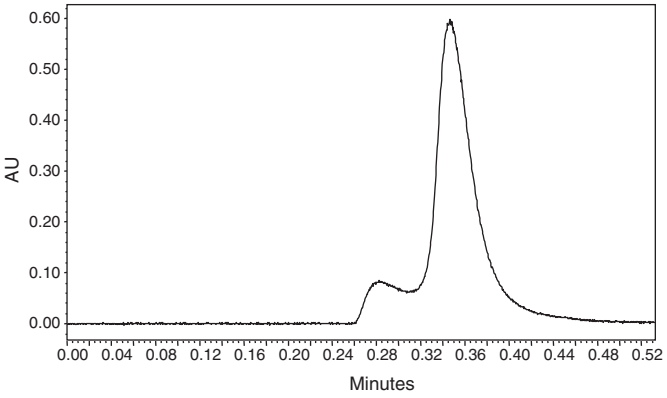

Fig. 3. Splitting of the chromatographic peak for the 3 MDa PS standard into two peaks due to a combination of SC and HDC effects. Chromatographic conditions as in Fig. 2.

4.2. Extra-column volume and its influence on the separation efficiency

To ensure the reliable characterization of polymers using the methods described above the observed peak width should be largely determined by the polymer dispersity of the sample, while the extra-column contributions to peak variance should be minimized. The extra-column band broadening may be different for different samples, depending on the nature and molecular weight of the polymer. Extra-column band broadening is more significant for polymers than for small molecules, because of the low diffusion coefficients.

We measured the extra-column variance by replacing the column by a union. The extra-column contribution to the total peak width was calculated using the following equation

$$rc_{\text{extra-column}} = \frac{\sigma^2_{\text{extra-column}}}{\sigma^2_{\text{observed}}} \cdot 100\% \quad (4)$$

where $rc_{\text{extra-column}}$ is the relative extra-column contribution to the total peak width, $\sigma^2{}_{\text{extra-column}}$ is the extra-column peak variance and $\sigma^2{}_{\text{observed}}$ is the observed (total) peak variance.

The extra-column contribution was measured for different system configurations and columns. The initial configuration included a standard narrow-bore Acquity UPLC column (50 × 2.1 mm I.D.) in a UPLC system equipped with a Column Manager. The Column Manager allows four different columns to be installed, with switching between the columns being performed using two switching valves. The valves and connecting tubing add extra volume to the system. We found that using this configuration the total peak width was mainly determined by the extra-column band broadening (Table 2). Reliable characterization of the molecular weight distribution of a sample cannot be performed at such conditions. The most effective way to reduce the extra-column volume is to decrease the diameter of the connecting tubing, as the band-broadening in the tubing is proportional to the square of the tubing diameter [4]. However, reducing the tubing I.D. below 120 μm is not recommended as it may easily lead to clogging [46], especially when working with polymer samples. The other way to somewhat decrease the extra-column dispersion is to reduce the tubing length, which will provide a proportional decrease in the band-broadening. We reduced the length of the tubing by connecting the injection valve directly to the column inlet and the column outlet directly to the detector, using short pieces of stainless steel tubing of 127 μm I.D.

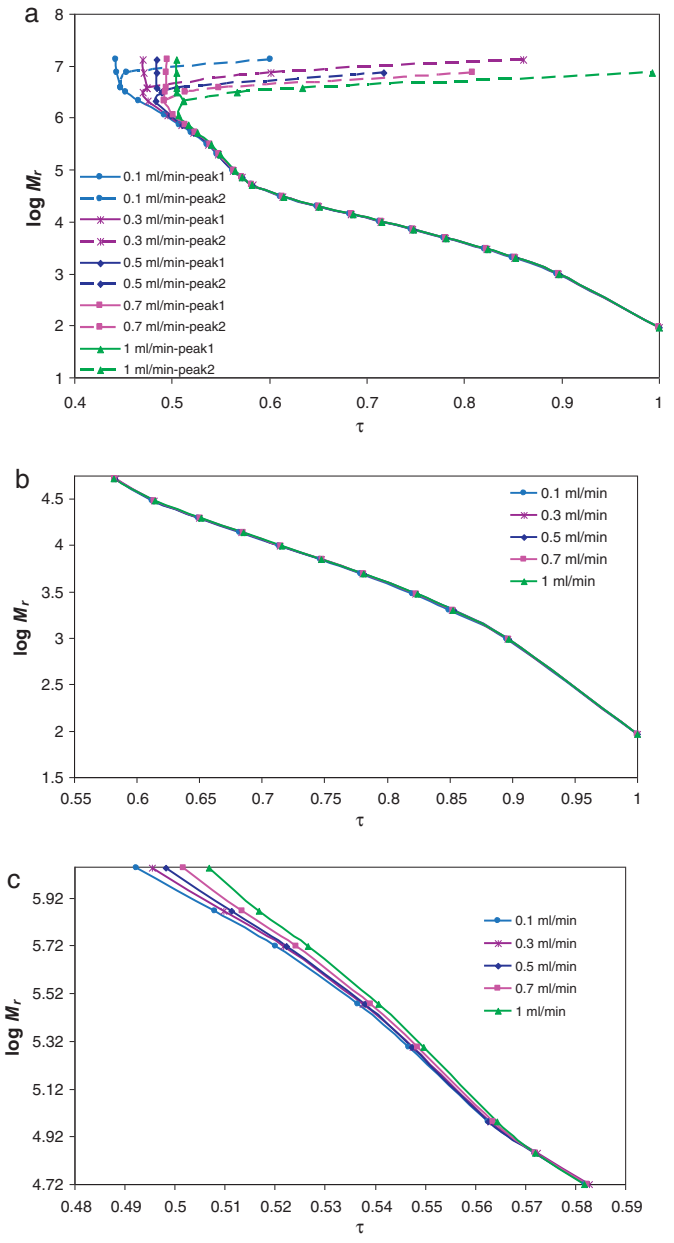

Fig. 4. (a) Calibration curves for polystyrene standards at different flow rates plotted using reduced elution volumes (τ). Two 150 × 4.6 mm I.D. Acquity UPLC C18 columns connected in series. Mobile phase: THF. Pressures: 7 MPa at 0.1 mL/min; 20 MPa at 0.3 mL/min; 34 MPa at 0.5 mL/min; 50 MPa at 0.7 mL/min; 79 MPa at 1 mL/min. (b) Enlarged SEC region. (c) Enlarged HDC region.

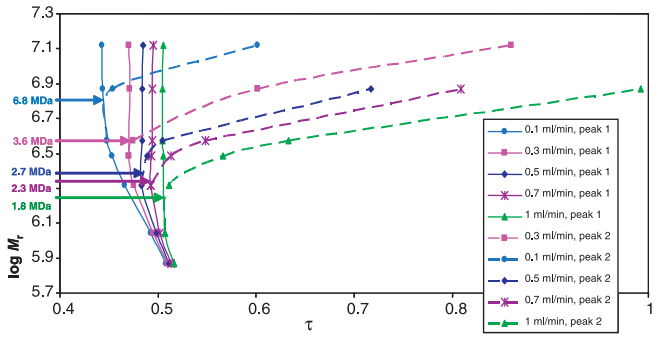

Fig. 5. Enlarged SC region of the calibration curves for PS standards at different flow rates. Two 150 × 4.6 mm I.D. Acquity UPLC C18 columns connected in series. On the *y*-axes the molecular weights corresponding to the conditions where $De = 0.5$ are indicated for different flow rates (see text for more explanation).

in either case. The total tubing length was 40.5 cm. In this way we circumvented both valves of the Column Manager, which in this case was used simply as a column oven. This configuration was used with the same short narrow column. This allowed us to decrease the extra-column contribution to the total peak width by only about 5% (see Table 2). The next step was to increase the column volume, which simultaneously increases two other contributions to the peak width (chromatographic and dispersity contributions) and, thus, decreases the relative extra-column contribution. Using a longer column (150 mm instead of 50 mm) in the previous set-up (Column Manager used only as a column oven) we could further reduce the relative extra-column band broadening, as shown in Table 2. A more-efficient way to increase column volume is by increasing the column I.D. There is a limited choice of wide-bore (>2.1 mm I.D.) UHPLC columns available because of the problems with heat dissipation. Wide-bore (4.6 mm I.D.) columns with the same packing material as used for our previous separations were specially made for this study by the manufacturer. Using one 150 × 4.6 mm I.D. BEH UPLC column in combination with reduced lengths of tubing we could significantly decrease the extra-column contribution to the total peak width. Using two such columns in series allowed reducing the extra-column contribution to an insignificant (or barely significant, for high-molecular-weight standards) level. Note, that the extra-column contribution is larger for toluene than for low-molecular-weight polystyrene standards due to the absence of a dispersity contribution to the peak width for toluene. The relative extra-column contribution increases again for high-molecular-weight polystyrenes, because the dispersity contribution to band broadening is smaller (due to the reduced selectivity), while the extra-column band broadening is larger due to slow diffusion. The last two configurations (see last two columns in Table 2) were used in our further experiments.

4.3. Calibration curves – a closer look

We obtained calibration curves for polystyrene standards with molecular weights ranging from 92 Da (toluene) to 13 MDa measured at different flow rates using two 150 × 4.6 mm I.D. Acquity UPLC C18 columns connected in series. When we plotted the logarithm of the molecular weight against the elution volume (V_{elut})

Table 2
Relative extra-column contribution (%) for different system configurations and columns.

PS molecular weight (Da)	50 × 2.1 mm I.D. column, using Column Manager (CM)	50 × 2.1 mm I.D. column, tubing length reduced (bypassing CM)	150 × 2.1 mm I.D. column, tubing length reduced (bypassing CM)	150 × 4.6 mm I.D. column, tubing length reduced (bypassing CM)	Two 150 × 4.6 mm I.D. columns, tubing length reduced (bypassing CM)
92 (toluene)	56	54	23	7	4
1,990	66	63	21	2	1
30,320	76	72	31	3	1
52,400	78	75	40	5	2
523,000	85	77	52	12	4
1,112,000	85	82	47	12	3

Table 3
Deborah numbers for some PS standards in the HDC and SC regions at different flow rates. Other conditions as in Fig. 4.

PS molecular weight (Da)	Flow rate (mL/min)				
	0.1	0.3	0.5	0.7	1
52,400	0.00009	0.0003	0.0005	0.0006	0.0009
197,300	0.001	0.003	0.005	0.007	0.01
523,000	0.005	0.002	0.03	0.04	0.05
735,000	0.01	0.03	0.05	0.07	0.10
1,112,000	0.02	0.06	0.10	0.14	0.20
2,061,000	0.06	0.18	0.31	0.43	**0.61**
3,053,000	0.12	0.37	**0.61**	**0.86**	**1.22**
3,742,000	0.18	**0.53**	**0.88**	**1.23**	**1.75**
7,450,000	**0.59**	**1.77**	**2.95**	**4.13**	**5.90**
13,200,000	**1.61**	**4.85**	**8.09**	**11.33**	**16.18**

we observed slight shifts in the curves with flow rate in both the HDC and SEC regions (not shown). To explain the slight shift in the SEC region when plotting the observed V_{elut} on the horizontal axis we measured the actual system flow rate as described in Section 3.5. Even though the deviations between the actual and the set flow rates were relatively small (0.5–2% as calculated using Eq. (3)), they could explain the shift of the curves in the SEC region. After correction for the actual flow rate the calibration curves for the different flow rates coincided in the SEC region.

The correction for variations in the flow rate may be carried out much more easily by plotting reduced volumes as in Fig. 4a ($\tau = V_{elut,PS}/V_{elut,toluene}$). After this normalization the curves in the SEC region coincide completely (Fig. 4b). In other words, the relationship between τ and M_r is independent of the flow rate.

The shift of the calibration curves with flow rate in the HDC region is more pronounced and it does not disappear upon correcting the retention volumes for the actual flow rate by using toluene as a marker (Fig. 4c). Therefore, it needs a different explanation. When flow rate increases, the retention volumes shift to higher values. Similar phenomena were observed by Stegeman et al. [29]. They suggested that this observation could be explained by the onset of molecular deformation, which would allow large molecules to travel through the column more rapidly. The *De* numbers were calculated for PS standards at the conditions of our experiments in the HDC region. The values are shown in Table 3. Almost all values for polymers in the HDC region ($M_r \leq 1$ MDa) do not exceed *De* = 0.1. Only the *De* numbers at flow rates of 0.7 mL/min and 1 mL/min for the 1 MDa polymer are somewhat higher. Thus, it is unlikely that the deformation of the macromolecules contributes to the shift of the calibration curves with flow rate observed in the molecular-weight range from *ca.* 50 kDa to 1 MDa.

Another observation for polystyrene peaks in the HDC region is the non-Gaussian (tailing) peak profile. The peak asymmetry increases with flow rate. We calculated the retention volumes and dimensionless retention volumes using the first normalized peak moments. Although, the scatter of the data was significant, we could conclude that the calibration curves for different flow rates in the HDC region coincided (not shown).

The SC region of the calibration curves is not yet fully understood. Molecular deformation followed by molecular degradation may possibly contribute to the shift of the calibration curves in this region. Both of these effects increase with molecular size and with flow rate (linear velocity) and would result in a shift of the calibration curves towards higher elution volumes. The critical molecular weight above which SC occurs varies with the flow rate and may be correlated with critical Deborah numbers. The calculated *De* numbers for very-high-molecular-weight polystyrene standards eluting in the SC region ($M_r \geq 1$ MDa) are shown in Table 3. The *De* numbers exceeding the critical value of 0.5 are shown in bold. It is interesting to note that the calculated critical molecular weight (corresponding to the critical *De*) at which the transition from HDC to SC separation mechanism occurs for each flow rate is close to the molecular weight of the smallest PS standard that is visibly split. The critical value of *De* = 0.5 corresponds well to the value obtained by other researchers [36,40,41]. In Fig. 5 the enlarged SC region is shown for different flow rates. For each flow rate the molecular weight which corresponds to *De* = 0.5 is indicated on the y-axis. In the region where M_r exceeds the critical value polymer molecules are thought to be elongated and they elute in the SC mode. This effect could possibly be useful for the separation of polymers of different topology, but more research is needed on this subject.

4.4. Experimental polymer separations by ultra-high-pressure SEC and HDC

4.4.1. Fast and efficient size-based polymer separations

Ultra-high pressures and small particles allow conducting fast and efficient separations of polymers. The separation selectivity in the SEC region is higher than in the HDC region. However, the molecular-weight range which can be separated in the SEC region is limited by the pore size of UHPLC stationary phases (see Section 4.1). With the packing material used in this study we can perform efficient SEC separations of polymers with M_r up to *ca.* 50 kDa. In Fig. 6 an example is shown of the separation of five PS standards and toluene. Almost baseline separation could be achieved for these standards in less than 1 min. To indicate the repeatability of the injections an overlay of three injections is shown. All three chromatograms coincide.

Separation of higher-molecular-weight polymers can be performed in the HDC region of the calibration curve. Separations in this mode are more challenging than in the SEC region, due to the lower selectivity and the greater extra-column dispersion (because of lower diffusion coefficients of large molecules). However, HDC separations can be performed very rapidly (*e.g.* in only 0.5 min in Fig. 7). The effective separation window in which all peaks are eluted is only 0.1 min wide. This is the relevant analysis time in case of a series of successive HDC chromatograms, as encountered in, for example, comprehensive two-dimensional liquid chromatography.

UHPLC offers great potential not only for separations of synthetic polymers, but also for biopolymers. However, several issues have to be taken into account. The onset of deformation and especially degradation of polymers is dependent on the nature of the polymer. To perform efficient SEC and HDC of biopolymers the con-

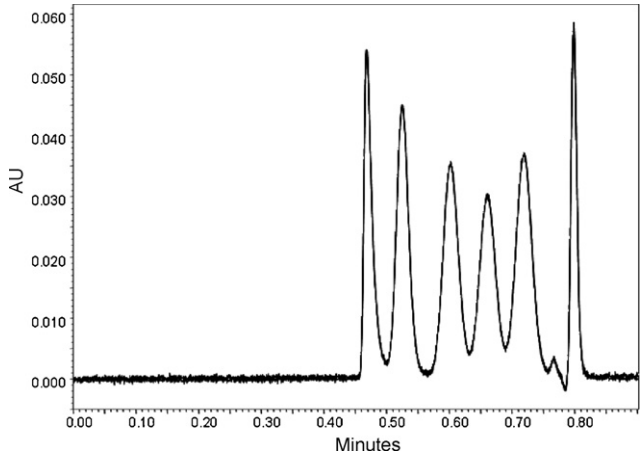

Fig. 6. Separation of polystyrene standards and toluene in the SEC region of the calibration curve (overlay of three highly repeatable injections). Molecular weight: 92 (toluene); 980; 2970; 7000; 19,880; 52,400 Da. Acquity UPLC C18 column, 150 × 4.6 mm I.D. Mobile phase: THF. Flow rate: 1.85 mL/min. Pressure 66 MPa (system limit at this flow rate).

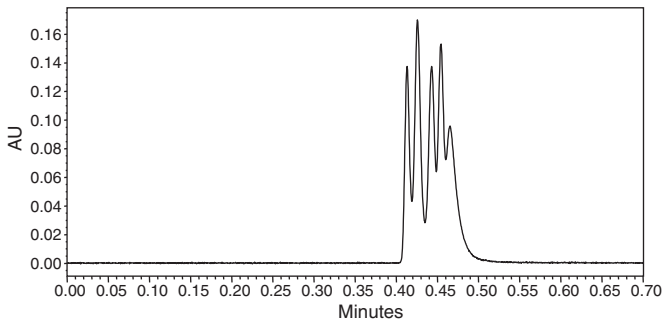

Fig. 7. Fast HDC separation of PS standards. Molecular weights: 52,400; 96,000; 197,300; 523,000; 1,112,000 Da. Acquity UPLC C18 column, 150 × 4.6 mm I.D. Mobile phase: THF. Flow rate: 1.85 mL/min. Pressure 66 MPa (system limit at this flow rate).

ditions where the onset of molecular deformation occurs have to be determined for each type of the polymers. Flow and temperature should be selected such that deformation (and consequently, degradation) of the molecules can be avoided. In some cases deformation of the biomacromolecules during chromatographic separations may be desirable. Slalom chromatography (SC) performed on conventional SEC [47] or reversed-phase [48] columns has proven to be an effective method for DNA separations. The SC region of the calibration curve at UHPLC conditions is extended to lower-molecular-weight polymers and, thus, may be attractive for the separations of other biomacromolecules. The other difficulty for size-based UHPLC separations of biopolymers is the need to avoid interactions with the stationary phase. Adsorption is a common problem in SEC separations of proteins. This becomes even more critical in UHPSEC, because of the limited choice of suitable columns on the market. The new type of UPLC columns introduced by Waters [49], which are designed to suppress interaction of proteins with the stationary phase, may be more suited for separation of proteins (and perhaps other biopolymers) at ultra-high pressures.

4.4.2. Influence of column length and flow rate on the separation efficiency

Increasing the column length could result in improved separations at the expense of an increase in analysis time. In Fig. 8 a separation of the whole range of PS standards is shown. Polymers with molecular weights up to 1 MDa may be efficiently separated in only 2.5 min (t_0 of the column), while the separation window has a duration of less than 1.5 min.

The high pressure limits of UHPLC systems allow increasing column lengths and flow rates simultaneously. Thus, the separation efficiency can be somewhat improved while maintaining the analysis time constant.

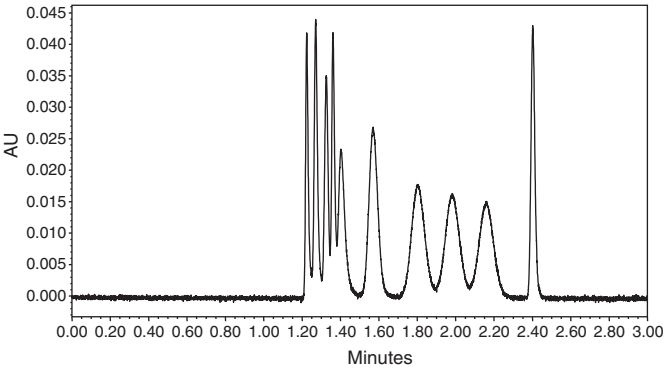

Fig. 8. Separation of PS standards by combination of SEC and HDC. PS molecular weights: 92 (toluene); 980; 2970; 7000; 19,880; 52,400; 96,000; 197,300; 523,000; 1,112,000 Da. (The concentrations of PS standards are lower than those used to record chromatograms in Figs. 6 and 7.) Two 150 × 4.6 mm I.D. Acquity UPLC C18 columns connected in series. Flow rate: 1.2 mL/min. Mobile phase: THF. Pressure 95 MPa.

It is interesting to compare size-based separations obtained using UHPLC with other methods for fast polymer separations found in literature. The following methods were evaluated for the separation of polystyrene standards: SEC using short and wide columns [50], high-temperature SEC [51,52] and HDC using 1-μm non-porous particles [53]. Because the actual dispersities of the PS samples used in each work are not known, it is impossible to compare the separation efficiencies of the methods. However, assuming that the polymer dispersities for PS standards of equal molecular weights are equal in every method, we can obtain comparable values for resolution between two PS peaks. This assumption is reasonable, as very narrow polystyrene standards (PDI ≤ 1.05) were used in all studies. In this case the variations in actual PDI values for PS samples of equal molecular weight obtained from different manufacturers are expected to be minor. The resolution was estimated in two regions of the calibration curves, *viz.* between the 7-kDa and 20-kDa PS peaks, and between the 200-kDa and 500-kDa PS peaks. The retention volumes of the PS peaks were calculated based on the experimental calibration curves. The peak width of the experimental PS peak with closest molecular weight was used to calculate the resolution. The results are shown in Table 4. SEC and HDC at UHPLC conditions of Figs. 6 and 7 provide better resolution for both low- and high-molecular-weight PS in comparison with SEC using short and wide columns and SEC at 110 °C. For high-molecular-weight polymers SEC at 150 °C provides resolution comparable to UHPLC in a shorter separation time (0.5 min). However, it does not offer a better resolution than UHPLC in the region of lower molecular weights.

The resolution of UHPLC separations may be further improved by increasing the column length (as in Fig. 8). The separation under these conditions offers both a shorter analysis time (2.5 min) and a better resolution than fast (3 min) HPLC SEC separations (see Table 4). A superior resolution may be achieved for high-molecular-weight PS by HDC using 1-μm non-porous particles at HPLC pressures. However, the analysis time increases twofold in comparison with the UHPLC conditions of Fig. 8 and more than five times in comparison with UHPLC under the conditions of Figs. 6 and 7.

4.4.3. Influence of temperature on the separation

Size-based UHPLC separations may be slightly improved by increasing the temperature. In Fig. 9 the influence of temperature on the separation of several PS standards in the HDC region

Table 4
Comparison of different methods for fast size-based separations of polymers.

Method		Estimated resolution between PS 7 kDa and 20 kDa	Estimated resolution between PS 200 kDa and 500 kDa	Separation time, min
UHPLC	UHPLC SEC and HDC, conditions of Figs. 6 and 7	1.4	1	0.9
	UHPLC SEC and HDC, conditions of Fig. 8	1.6	1.2	2.5
HPLC	Fast SEC (50 × 7.5 mm I.D. column), Ref. [50]	0.6	0.3	0.85
	Fast SEC (50 × 7.5 mm I.D. column) Ref. [50]	0.7	1	3
	High-temperature SEC, 110 °C, Ref. [51]	0.5	0.8	1.5
	High-temperature SEC, 150 °C, ref. [52]	0.4	0.8	0.5
	HDC with 1-μm particles, Ref. [53]	1.1	2	5

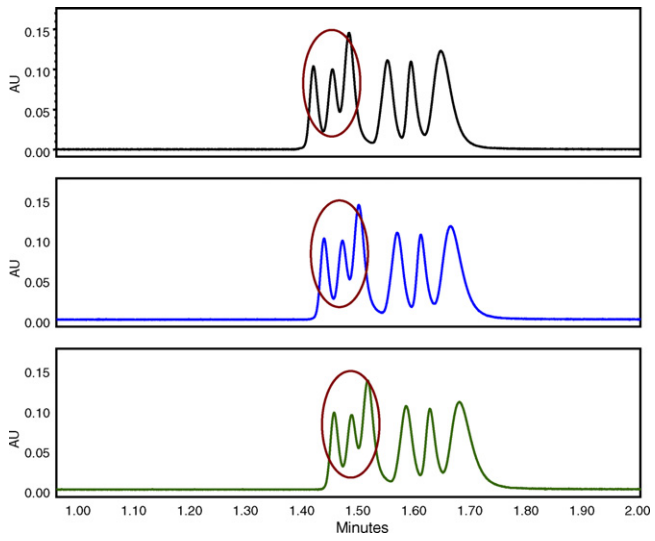

Fig. 9. HDC separation of PS standards at different temperatures. Temperatures (chromatograms from top to bottom) 60 °C, 45 °C, 30 °C. PS molecular weights: 52,400; 96,000; 523,000; 735,000; 1,112,000 Da. Two 150 × 4.6 mm I.D. Acquity UPLC C18 columns connected in series. Mobile phase: THF. Flow rate: 1 mL/min. Pressures 71 MPa at 30 °C, 65 MPa at 45 °C and 57 MPa at 60 °C.

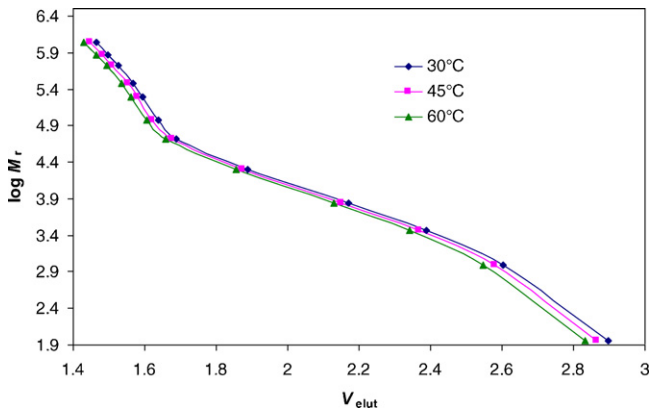

Fig. 10. Calibration curves for PS standards illustrating changes in the apparent retention volumes with temperature. Standard molecular weights from 92 (toluene) to 1 MDa. Two 150 × 4.6 mm I.D. Acquity UPLC C18 columns connected in series. Mobile phase: THF. Flow rate: 1 mL/min. Pressures 71 MPa at 30 °C, 65 MPa at 45 °C and 57 MPa at 60 °C.

is shown. When the temperature is increased from 30 to 60 °C the resolution between PS peaks of 735 and 1112 kDa improves from 0.82 to 1. This effect may be explained by a decrease in solvent viscosity and an increase in polymer diffusivity. One of the results is a decrease in the extra-column contributions to the peak dispersion, which makes the observed peaks somewhat narrower. Another effect of the decrease in viscosity is a decrease in column backpressure. This allows using higher flow rates and conducting separations even faster. The increase of temperatures above 60 °C is feasible because the boiling point of THF increases significantly with pressure. Conducting the separations at temperatures above 100 °C would certainly further improve the resolution between PS peaks. However, such experiments would need an explosion proof system configuration [51].

The separation improvement is most noticeable for very high molecular-weight polymers. The effect is almost not visible in the SEC region. The extra-column dispersion for the samples in this region is less pronounced than in the HDC region (see values in Section 3.4). Moreover, the selectivity in the SEC region is higher and the peak widths are largely determined by polymer dispersity contributions. Thus, the small decrease in the total peak width due to a decrease in the extra-column dispersion at higher temperatures is less pronounced in the SEC region.

In Fig. 9 we observe a slight shift of elution volumes with temperature. In Fig. 10 calibration curves at different temperatures are shown. The shift is clearly visible in both the SEC and HDC regions of the curves. The shift to lower elution volumes with increasing temperature is sometimes observed in SEC and can be caused by a decrease in adsorptive interactions with temperature [26]. At the conditions where adsorption of a monomer unit occurs, large molecules would be attached to the surface with more units and thus, more strongly, than small molecules [54]. The effect of increased adsorption with molecular weight is exponential (the so-called Martin rule in chromatography), so that it will easily outweigh a possible decrease in the surface area available for adsorption. Consequently, a decrease in adsorption at higher temperatures would lead to a change in the slopes of the calibration curves. However, we did not observe significant differences in selectivity with temperature (see Fig. 10). This indicates that adsorption is unlikely to be a reason of the shift of the calibration curves with temperature.

A temperature increase leads to an expansion of the mobile phase and, hence, to a decrease in the density. Thus, the volumetric flow rate supplied by a pump operating at room temperature will be different from the actual volumetric flow rate through the heated column [55]. The effect is complicated by the compression of the solvent at the high pressures used during separations and pressure changes with temperature. We corrected the set volumetric flow rates for both these effects. The THF densities at different temperatures were obtained from Ref. [56]. The values from Ref. [57] were used to account for the volume changes of THF with pressure at different temperatures. After these two corrections we found excellent agreement (less than 1% relative error) between the actual elution volumes at different temperatures.

5. Conclusions

1. UHPLC was successfully applied for the separations of polystyrene (PS) standards of different molecular weights. Polymers of molecular weight up to *ca.* 50 kDa could be efficiently separated under UHPLC conditions in less than 1 min. The molecular weight limit of SEC separations is determined by the pore size of the stationary phase. Larger polymer molecules may be separated by HDC on conventional UHPLC columns with somewhat lower selectivity in comparison with the SEC region. The upper molecular weight of such HDC separations is determined by the onset of molecular deformation at high shear rates.
2. Apart of the fundamental issues (deformation and degradation of macromolecules) there are several technological limitations which impede the use of ultra-high pressure liquid chromatography for separations of polymers. These include a lack of size-exclusion UHPLC columns on the market, a limited solvent compatibility of (first-generation) UHPLC systems and, most importantly, the need to adequately minimize system dead volume.
3. Splitting of PS standard peaks into two peaks was observed at conditions where the shear rates generated in the chromatographic system were large enough to deform macromolecules. The appearance of the second peak in the chromatogram was attributed to a combination of hydrodynamic chromatography and slalom chromatography. The transition from randomly coiled to stretched polymers in solution was correlated with a critical value of 0.5 for the Deborah numbers for different flow rates.
4. Extra-column dispersion was measured on a UPLC system for PS standards of different molecular weights. It was found to be

significantly larger for high molecular-weight polymers than for smaller analytes, due to slower diffusion. The use of wide-bore (4.6 mm I.D.) UPLC column(s) and short pieces of narrow-bore connection tubing allowed us to successfully overcome the extra-column dispersion and perform reliable size-based separations of polymers.

5. The influence of several parameters on the separation efficiency for ultra-high-pressure SEC and HDC separations was studied. The high UHPLC pressure limits permit a simultaneous increase of column length and flow rate, which allows some improvement in the resolution, while maintaining short analysis times. Increasing the temperature leads to slightly improved separations of high-molecular-weight polymers especially in the HDC region.

Acknowledgements

This work forms a part of the research programme of the Dutch Polymer Institute (project #597). The authors thank Waters Corporation (Milford, MA, USA), in particularly Jim Willis, Ed Bouvier and the Chemistry Operations group for their generous support, as well as for fruitful discussions. The authors especially appreciate useful comments and suggestions by Ed Bouvier that led to significant improvements of the present manuscript. Linda Steeneken is acknowledged for assistance with Matlab calculations.

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