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Molar mass distributions by gradient liquid chromatography: predicting and tailoring selectivity

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Abstract

Interactive liquid chromatography (iLC) for polymer analysis is usually applied to the characterisation of distributions other than molar mass. In particular, its use for the determination of chemical-composition, functionality-type and tacticity distributions has been demonstrated. The application of iLC for the determination of molar mass distributions (MMDs), however, has not yet been fully explored. An expanded version of the reversed-phase liquid chromatography model has been developed to describe and predict how the retention behaviour of polydisperse polystyrene samples changes with molar mass. The relationship between molar mass and the parameters of the model has been investigated in some detail and non-linear correlations were found. From the model and the relationships between the model parameters and molar mass, calibration curves (retention time versus molar mass) were constructed to predict changes in chromatographic selectivity across a given molar mass range. These calibration curves were compared to experimentally obtained curves and, in most cases, excellent agreement was found. The dramatic enhancement in selectivity that can be obtained with iLC in comparison to size-exclusion chromatography (SEC) was illustrated by measuring matrix-assisted laser desorption ionisation (MALDI) MS spectra of fractions collected during a gradient-LC separation. In the low-molar mass range, essentially monodisperse fractions were obtained. Calibration curves, predicted by the model and validated experimentally using narrow-dispersity standards and MALDI-MS spectra of fractions, were used to determine the molar mass distribution of some narrowly distributed polystyrene samples. Molar mass distributions for such standards were found to be somewhat lower than the values reported by the manufacturers. The results also deviated from those obtained by MALDI-MS.

Keywords: iLC; Polydisperse samples; Molar mass distributions; Retention models; MALDI-MS

1. Introduction

1.1. Chromatographic determination of molar mass distributions (MMDs)

The chromatographic characterisation of molar mass distributions of polydisperse macromolecules is performed almost exclusively using size-exclusion chromatography (SEC) [1,2]. SEC separates macromolecules according to their hydrodynamic radii in a given (strong) solvent. Samples are separated using a packing material that has a pore size comparable to the size of the macromolecular analytes. The pores of the stationary phase act as a sort of a 'sieve' that excludes large molecules, while allowing smaller molecules to fully or partially permeate into them. The mobile phase must be strong enough to prevent any interaction of the polymer with the surface of the stationary phase. The elution profile of a sample is then related to the size distribution of the polymer. The molar mass average and its distribution are determined by constructing a calibration curve for a series of polymeric standards with different average

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masses (i.e. an elution volume versus molar mass profile) [3].

SEC is a well-established technique. It has been used routinely in polymer-analysis laboratories since its development in the late 1950s [4]. However, the technique is not without its drawbacks, which include limited resolution, significant band broadening and inflexible selectivity (i.e. selectivity is a fixed parameter of the column and cannot be influenced by the mobile phase or by any other experimental parameter) [5–7].

Interactive liquid chromatography (iLC), i.e. chromatographic separations based on the partitioning of analyte molecules between the mobile and stationary phases, has also seen many applications in the area of polymer analysis, although it remains less popular than SEC. To date, it has been used primarily for the characterisation of functionality-type (FTDs) and chemical-composition distributions (CCDs) and for the separation of oligomers [8,9]. In the case of FTDs and CCDs, it is usually required that any contribution to retention from the molar mass distribution is at least minimised, if not entirely suppressed [10,11].

1.2. Applications of iLC to separations according to molar mass

Although the application of iLC to the separation of polymers according to molar mass was reported by Van der Maeden [12] more than 25 years ago, the technique remains largely unexplored and relatively few practical applications have been published. Armstrong et al. [13] separated polystyrenes ranging in molar mass from 2350 up to 10×10^6 μ with reversed-phase gradient LC. The separation was considered to be a fractionation, controlled solely by the solubility of the sample in the mobile phase. Lochmüller et al. [14] demonstrated isocratic separations of polystyrenes for polymers with molar masses up to 2.8×10^6 μ. In order to prevent size-exclusion effects, they used stationary phase materials with large pores. Shalliker et al. [15] studied the effects of the particle size and the pore size of reversed-phase stationary phases on the separation of high-molar mass polystyrenes and concluded that both the particle size and the pore size influence resolution. Some work has also been reported on the use of temperature gradients for the separation of polymers according to molar mass. Lochmüller et al. [16] used temperature to optimise separations of polyethylene glycols. Chang and co-workers [17–19] separated polystyrenes, polyisoprenes and polymethylmethacrylates using temperature gradients. They found that temperature gradients gave better resolution than SEC methods. The resulting molar mass distributions were significantly narrower than those found using conventional SEC techniques. They also highlighted the advantages of the technique for the MMD characterisation of star-shaped and branched polymers, since separation is based on molar mass rather than size.

1.3. Optimising separation conditions

The choice of working under isocratic or gradient conditions usually depends on the molar masses of the polymers of interest. The retention of macromolecules in an iLC separation system is known to increase exponentially with the number of monomeric units on the polymer [8]. Even if there is only very slight retention of the monomeric unit, this can lead to infinitely long retention times of high-molar mass polymers. This means that the range of mobile phase compositions where there is reasonable (i.e. non-zero but finite) retention becomes narrower. For high-molar masses, the transition between the fully retained and fully unretained states can be very sharp and isocratic chromatography then becomes impracticable. For this reason, gradient chromatography tends to be the preferred technique for the characterisation of high-molar mass polydisperse macromolecules. The selectivity of iLC can be remarkably high in comparison to SEC, particularly in the low-molar mass range and oligomers can easily be separated [12]. In this range, iLC shows significant advantages over size-exclusion for separations according to molar mass.

One of the main advantages of iLC in comparison to SEC is its versatility. In an interactive-LC system, selectivity can be controlled in a way that is not possible in SEC. The strength of the mobile phase can be tailored, either isocratically or within a gradient, to influence the degree of retention of the macromolecules in a given molar mass range. By optimising the separation conditions, i.e. mobile phase composition, gradient conditions, temperature etc., selectivity can be focused on the mass range of interest.

One of the main disadvantages of iLC for the characterisation of polymers also arises from its versatility. The separating power of iLC changes significantly with the stationary and mobile phases and therefore separations need to be optimised to give the best possible results. In comparison to SEC-based characterisations, where a given combination of a stationary phase and a mobile phase has a fixed calibration curve relating molecular size to elution volume, this can be a time-consuming task.

In order to overcome this problem, we have used an expanded version of the RPLC model to optimise the separation of polydisperse samples across a range of molar masses. The model can predict retention behaviour under any isocratic or gradient conditions and it has been incorporated into an Excel spreadsheet that can automatically predict chromatograms and calibration curves that correspond to a particular separation.

1.4. Retention mechanisms in iLC

In the case of high-molar mass polymers, there is no commonly accepted interpretation of the mechanisms that govern gradient chromatography. It has been suggested that the retention of a macromolecule is solely dependent on its solubility in the mobile phase [13,20]. Polymers injected into a

weak mobile phase precipitate within the column until (during the course of a gradient program) the mobile phase becomes strong enough to redissolve the macromolecule. In this case, the stationary phase plays no significant role in the chromatographic process and acts only as a medium to contain the analyte molecules and to prevent them from moving with the mobile phase. Other research has concluded that traditional chromatographic theories, based on the partitioning of analyte molecules between the mobile and stationary phases is equally applicable to large molecules [21]. At this stage, it is generally accepted that the mechanisms involved will depend on the sample, the concentration of sample injected onto the column, on the choice of mobile phase and on the strength of the interaction between the sample and the stationary phase [22,23].

1.5. Retention models in iLC

The RPLC model i.e.:

$$\ln k = \ln k_0 - S\varphi \quad (1)$$

is based on a linear relationship between the logarithm of the retention factor ($\ln k$) and the volume fraction of strong solvent in the mobile phase (φ). It is widely used in LC optimisation methods for small molecules [24]. In previous papers [25–27], we have presented various approaches to the optimisation of the chromatographic separation of polydisperse macromolecules using an expanded version of this model.

For isocratic experiments, the change in retention with changing volume fraction of strong solvent in the mobile phase is measured for an analyte. If the model adequately describes retention, a linear relationship between $\ln k$ and φ should be obtained and the slope S and intercept $\ln k_0$ can be calculated. When gradient chromatography is required, equations must be derived depending on the shape of the applied gradient. In the case of a linear gradient, retention is controlled by the model parameters and by the slope of the gradient B. When an analyte elutes within a linear gradient, its retention time can be calculated as:

$$t_R = \frac{1}{SB} \ln\left[1 + SBk_A\left(t_m - \frac{t_D}{k_A}\right)\right] + t_m + t_D \quad (2)$$

where k_A is the retention factor in the starting mobile phase composition, t_m is the column dead time and t_D is the system dwell time. Because k_A is usually very large, the equation can be simplified to:

$$t_R \approx \frac{1}{SB} \ln(SBk_A t_m) + t_m + t_D \quad (3)$$

This allows S and k_A to be estimated from experiments where the retention time is measured as a function of the gradient slope B, once t_m and t_D have been measured. If S and k_A are known, k_0 can easily be calculated. In our approach, a number of gradient experiments (usually at least four) are run and values for S and $\ln k_0$ are calculated such that the error between the experimental and predicted retention times is minimised. The iterative solver tool in Microsoft Excel is used for this purpose. Once values of S and $\ln k_0$ are calculated for a given analyte, it is possible to predict the retention time of that analyte under any isocratic or gradient mobile phase conditions.

To expand the model to cover a *range* of molar masses, there must be some relationship between the model parameters and molar mass. It has previously been shown that there is a strong correlation between S and $\ln k_0$ for a homologous series [26,27]. It has also been reported that there are correlations between molar mass and both S and $\ln k_0$. Stadalius et al. [23] found that S was dependent on M through a power curve i.e. $S = CM^n$. This was the case for polystyrenes in a THF-water mobile phase and for proteins and peptides in an acetonitrile-water mobile phase. The Martin rule predicts a linear relationship between $\ln k$ and the number of repeat units on the macromolecule [28], although it has been reported that it can fail for both low and high masses [29]. Skovrtsov and Trathnigg have suggested that the Martin rule only holds under special conditions, related to the radius of gyration of the molecule [30].

1.6. Applying the model to polydisperse samples

Once correlations between the model parameters and the molar mass of a polymer have been established, it becomes possible to predict the retention behaviour of samples of that polymer with any molar mass (within the defined limits of the model) and any polydispersity. Unlike traditional low-molar mass analytes, synthetic homopolymers (and many natural polymers) consist of a range of different molar masses. Even in the case of a narrow polydispersity standard, there can be a large number of different masses present. For example, a typical narrow polydispersity polystyrene sample with a number-molar mass average (M_n) of 22 000 and a polydispersity index (PDI) of 1.03, will have more than 140 different types of molecules (i.e. molecules with different degrees of polymerisation) within that ‘narrow’ sample (taking the width of the distribution to be $\pm 2\sigma$). In a chromatographic system optimised for separation according to molar mass, each member of this series will behave in a different way and will have a specific retention time related to its actual mass (rather than the average mass of the sample). In practical terms, this means that a peak attributed to a single polydisperse sample will feature a varying molar mass across that peak, with a distribution of masses that is assumed to be Gaussian. In our model, we split a single polydisperse sample into (up to) 100 separate molecules and assign each one of those a particular value of S and $\ln k_0$, calculated from the correlations between the model parameters and molar mass. The retention behaviour of each separate portion of the sample is then independent of the rest of that sample. The predicted shape of the chromatographic peak depends on the change in the selectivity of the separation across the molar

mass distribution of the sample and will not necessarily be Gaussian.

2. Experimental

2.1. HPLC system

The experiments were carried out on a Waters 2690 LC system. Gradient control, data acquisition and data analysis were controlled by Waters Millennium 3.2 software. The stationary phase was Supelco Discovery C_{18}, particle size 5 μm, pore diameter 180 Å, column dimensions were 150 mm × 2.1 mm I.D. and column temperature was maintained at 25 °C. The solvents were tetrahydrofuran (THF; Biosolve, Valkenswaard, The Netherlands) and acetonitrile (ACN; Rathburn, Walkerburn, UK), both were HPLC grade. The flow rate was 0.2 mL/min. Samples consisted of low-dispersity polystyrene standards (Polymer Labs., Church Stretton, UK; Pressure Chemical, Pittsburgh, PA, USA and Polymer Standards Service, Mainz, Germany). The sample-injection volume was 10 μL and sample concentrations were 1.5 mg/mL. The mass injected onto the column was thus 15 μg. This was sufficiently low to avoid any breakthrough effects in the chromatogram. Breakthrough occurs when a portion of the injected sample remains in the solvent plug and is eluted around the dead volume of the column. This is an undesirable effect that can be minimised using a variety of parameters, such as the strength of the sample solvent, the mass of sample injected onto the column and the temperature [31].

For the calculation of the model parameters, gradient programs from 5 to 95% THF in acetonitrile were run over 20, 45, 60 and 90 min. Detection of the samples was performed with a Waters PDA 996 diode-array detection (DAD) system at 260 nm. All samples were run in duplicate. Data-modelling spreadsheets were written in Microsoft Excel 97 on a Windows NT operating system.

2.2. Matrix-assisted laser desorption ionisation (MALDI) MS experiments

MALDI time of flight (TOF) MS analysis was carried out on a Voyager DE-STR from Applied Biosystems. The matrix was *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB) which was synthesised according to Ref. [32]. Silver trifluoracetate (Aldrich, 98%) was added to the polystyrene samples as a cationic ionisation agent. The matrix was dissolved in THF (40 mg/mL). Silver trifluoracetate was added to the THF (∼1 mg/mL). All the spectra were acquired in the linear mode. For each spectrum, 1000 laser shots were accumulated. In a typical MALDI experiment, the matrix, salt and polymer solution were premixed in the ratio: 5 μL sample:5 μL matrix:0.5 μL salt. Approximately 0.5 μL of this mixture was hand spotted on the target plate.

Table 1
A comparison of the experimental and predicted retention times for a polystyrene standard (76 600 μ)

Experimental retention time (s)	Predicted retention time (s)	Time difference (s)
1008.8	1014.4	5.5
1013.3	1014.4	1.0
1738.0	1735.9	2.1
1737.4	1735.9	1.6
2164.4	2162.5	1.9
2164.0	2162.5	1.5
3005.4	3007.1	1.7
3006.1	3007.1	1.0

3. Results and discussion

3.1. Modelling retention behaviour

To construct a model to describe the chromatographic separation of polystyrene, a series of standards (differing in molar mass) were run under various gradient conditions, i.e. different gradient slopes. The retention times of the standards in each of the gradients were measured and the values were compared with values predicted using the RP model. The iterative 'solver' tool in Excel was used to calculate values of S and $\ln k_0$ that gave the smallest differences between predicted and experimental retention times. A comparison between the predicted and experimental retention times for polystyrene with an average molar mass of 76 600 μ is shown in Table 1. The fit, i.e. the difference between the experimental and predicted retention times, is presented as the sum of the squared differences (SSQ) between each of the experimental retention times and its predicted equivalent. S and $\ln k_0$ values were calculated in the same way for polystyrene standards ranging in molar masses from 1700 μ to 325 000 μ and in each case the SSQ was similarly small (Table 2). Low SSQs indicate that the model can accurately describe the retention behaviour of the standards under the gradient conditions that were applied. It is then taken that the model can accurately predict retention behaviour under *any* gradient or isocratic mobile phase conditions.

Table 2
The calculated 'best fit' values for the model parameters S and $\ln k_0$ and the sum of the squared difference between predicted and experimental retention times using these values

Molar mass (μ)	Best fit S	Best fit $\ln k_0$	SSQ (s^2)
1,700	13.98	3.64	3.12
4,000	23.08	8.26	74.09
7,000	25.96	10.07	42.87
10,900	29.55	12.12	62.27
17,600	36.10	15.64	24.58
30,000	48.97	22.19	20.39
39,200	57.22	26.40	16.00
76,600	80.46	38.11	48.16
117,000	113.73	54.55	50.03
160,000	164.42	79.39	75.99
325,000	319.87	155.96	3.41

3.2. Determination of the correlations between S, ln k_0 and molar mass

A strong linear relationship between S and ln k_0 was found for the homologous polystyrene series (equation of the line: $y = 2.0153x + 4.7476$; $R^2 = 0.999$). This relationship can be used to determine the critical point for a polymer, i.e. the point at which the monomeric units no longer influence retention. This has been demonstrated in an earlier paper [25].

Correlations between the model parameters and molar mass have also been reported. However, there is no firm agreement on the type of line that best fits these relationships (see introduction). To determine the *best* possible correlation between molar mass and the model parameters, we examined the relationship between ln k_0 and molar mass in more detail. The relationship was initially taken to be linear i.e.

$$\ln k_0 = A + B\,(\text{molar mass}) \quad (4)$$

The intercept (A) and the slope (B) were then varied incrementally and corresponding ln k_0 values (for a given mass) were calculated. From the resulting grid of ln k_0 values (250×250) *and* the established correlation between S and ln k_0, a second grid was constructed, indicating how the error in predicted retention times changed as the slope and intercept were varied. Error in the prediction was represented as the inverse of the sum of the squared differences (SSQ^{-1}) between the predicted and experimental retention times, thus, the higher the value, the lower the error. Fig. 1a shows a surface plot of the variation in the SSQ^{-1} as A and B are varied, for a PS 30 000 μ standard. The high ridge on the surface corresponds to values of A and B that give the best prediction of retention times for that standard i.e. lines that intersect at the optimum ln k_0 value. The surface is a sharply rising, flat ridge, indicating that small deviations from the optimum ln k_0 leads to a large decrease in the accuracy of the prediction. Similar plots were constructed for all of the polystyrene standards and in all cases, comparable contours were found, except that higher molar masses (above 100 000 μ) had wider ridges with shallower slopes, suggesting that there is a larger confidence interval associated with predicted ln k_0 values of high mass polymers (Fig. 1b). This has also been demonstrated in an earlier paper [27].

If it is the case that there is a single straight line to describe the ln k_0 versus molar mass relationship, then the surface plots obtained for *all* of the polystyrene standards should converge to one common point, corresponding to the slope and intercept of that 'best fit' line. By summing the contours of all standards into one grid, an overall surface plot, corresponding to the sum of the inverse SSQ's for all standards, was obtained (Fig. 2). Before summation, each grid was normalised to the highest SSQ^{-1} value in that grid, so that standards with significantly different SSQs could be compared easily.

The sharp maximum on the surface plot clearly indicates that there is a common intersection point, corresponding to one line (one slope and intercept value) that can predict retention times significantly better than any other line. However, upon closer examination, it can be seen that not all masses converge through this point. This indicates that a single straight line may not be the best way to describe the ln k_0 versus molar mass relationship.

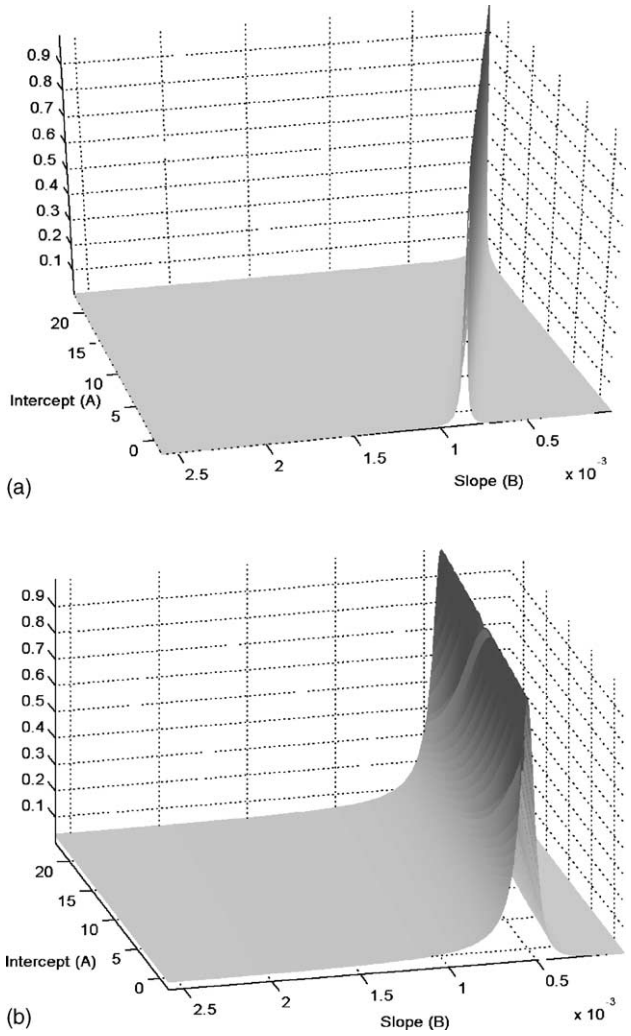

Fig. 1. Surface plot showing the error of prediction of the model (SSQ^{-1}) as a function of the slope and intercept of the ln k_0 vs. molar mass correlation. (a) PS 30 000 μ and (b) PS 160 000 μ. Both plots were normalised to the highest value on the grid.

SSQ^{-1} grids for pairs of standards were then summed, in order to determine the best line in narrower mass regions. Standards were paired according to increasing molar mass. Sharp intersection points between the summed contour lines were found, with very clear maxima for the lower molar mass standards (up to 76 600 μ) (Fig. 3a). The sharpness of this intersection decreased as molar masses increased (Fig. 3b), suggesting that as mass increased, the range of lines that could reasonably predict retention times within the range of masses covered by the two standards broadened.

The intersection point of each grid, i.e. the maximum SSQ^{-1}, then corresponded to the slope and intercept of the line that best predicted the retention times of masses in that

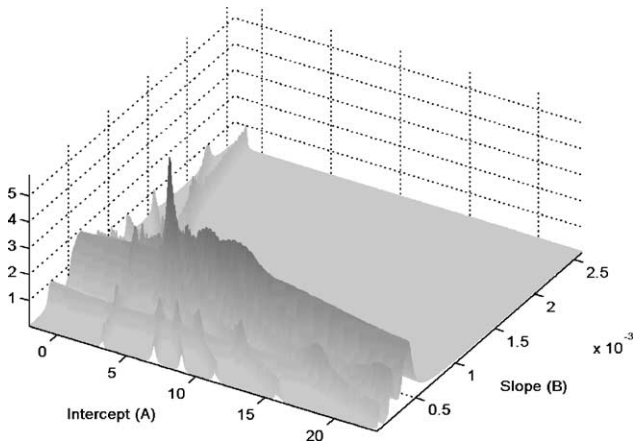

Fig. 2. Surface plot showing the error of prediction of the model (SSQ^{-1}) as a function of the slope and intercept of the $\ln k_0$ vs. molar mass correlation for *all* of the standards (normalised and summed). The peak represents the slope and intercept values that give the best prediction over the *entire* mass range.

range. By repeating this procedure across the mass range, optimal slopes and intercepts were determined as a function of mass. The overall relationship between $\ln k_0$ and molar mass was then determined as a series of straight lines all with different slopes and intercepts. By plotting each of these lines within their relevant mass ranges, the overall (non-linear) relationship between $\ln k_0$ and molar mass was determined. Fig. 4 shows the adjoining lines over the entire mass range. The true shape of the 'best fit' correlation clearly deviates from a straight line and, in fact, was best described by the power curve also shown in Fig. 4 (dashed line). The curvature of this line implies deviations from the Martin rule, which states that there should be a linear relationship between $\ln k$ and the number of repeat units in a polymer [28]. It must be stressed that the Martin rule is empirical. Deviations from this rule have previously been predicted and observed in both the high-end and the low-end mass ranges. Deviations at the low mass end are generally considered to be caused by interaction of the polymer end-groups with the packing material [29], while at the high mass end, it has been suggested that a decrease in the expected retention is caused by a collapse of the random-coil configuration of larger molecules due to hydrophobic effects [21], or from a change in the mechanism of sorption [29]. Despite the great value of the Martin rule for chromatography, it is perhaps naïve to expect it to hold for a series of polymers with such a great variation in the number of repeat units (i.e. from 15 to over 3000). Further investigation of the Martin rule across very broad ranges will be required for a more thorough understanding of all the effects.

3.3. Using the model to construct calibration curves

Once it has been established that the RP model (or any other model) adequately describes retention behaviour and a correlation between the model and molar mass has been found, the retention time of any polydisperse sample can be

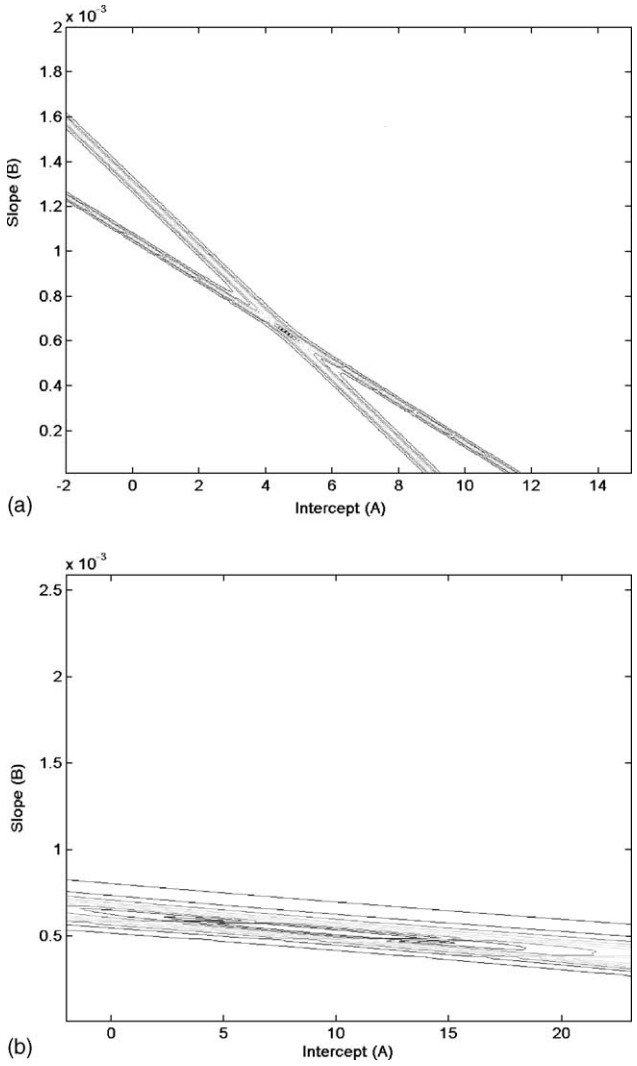

Fig. 3. Contour plots showing the intersection points in the relationship between the error of prediction of the model (SSQ^{-1}) and the slope and intercept of the $\ln k_0$ vs. molar mass correlation for two polystyrene standards (a) 7000 and 10 900 μ and (b) 76 000 and 116 000 μ.

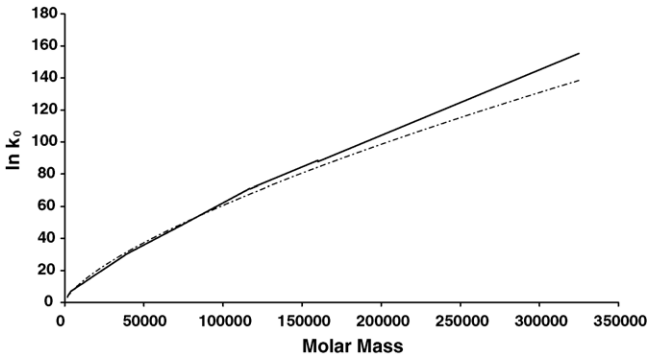

Fig. 4. Best relationship between molar mass and $\ln k_0$, determined by the range of maxima calculated using intersection points such as those in Fig. 5. The resulting line is best described by a power curve: $y = 0.019x^{0.7008}$, $R^2 = 0.9956$ (dashed line).

predicted, under any gradient or isocratic conditions. The model can then be used to construct both chromatograms and calibration curves that clearly indicate the relationship between gradient conditions and the retention time of the polymer. Calibration curves show how retention varies over the *entire* mass range. Unlike conventional SEC calibration curves, which are fixed for a given mobile/stationary phase system, these are 'tuneable' curves that change with the mobile phase conditions. Significantly, this allows the chromatographer to separate a sample according to the requirements of the analysis. For example, in some cases, it may be required that the influence of molar mass is minimised, e.g. for the characterisation of copolymers according to their chemical composition. In this case, a vertical curve (where the *x*-axis represents retention time and the *y*-axis is molar mass) is best, i.e. the polymer should elute at one time regardless of molar mass. If the molar mass distribution is to be determined, a shallower calibration curve is required. Fig. 5 shows some examples of (predicted) calibrations curves that can be obtained using iLC. The shape of each curve depends on the applied gradient conditions and can be optimised to give separations that show immense selectivity in specific mass regions. Fig. 5a is an example of a separation that spans more that an order of magnitude of mass values. Masses elute over almost the entire chromatogram and in the lower mass ranges, the level of separation is much greater than can normally be achieved using size-exclusion chromatography.

The accuracy of these predicted curves was established by measuring the retention times of a series of polystyrene standards under the relevant gradient conditions and comparing the experimentally obtained calibration curves to their predicted equivalents. In general, there was excellent agreement between the predicted and experimental calibration curves. Comparisons of experimental and predicted results are also given in Fig. 5. The accuracy of these predicted calibration curves, illustrates not only that the RP model is an appropriate descriptor of the retention behaviour of the samples in this chromatographic system, but also that the established correlations (i.e. S versus $\ln k_0$ and $\ln k_0$ versus mass) are also adequate.

3.4. Using predicted calibration curves to optimise separations

The predicted calibration curves can be tuned so that the selectivity of iLC (in comparison to SEC) is greatly enhanced, particularly for lower masses. In the low-molar mass region, peaks in iLC can be extremely broad (in our experience up to 60 min wide) and quite asymmetric in shape (Fig. 6). This broadness (and asymmetry) arises from the immense selectivity of the separation rather than from any adverse band-broadening effects. MALDI-MS spectra of fractions that were collected in the low-molar mass range of a shallow gradient separation (5–60% THF in ACN in 220 min) proved that the separation was good enough to obtain effectively *monodisperse* fractions (i.e. polydispersity indices <1.000), even up to molar masses of 18 500 μ (Fig. 7).

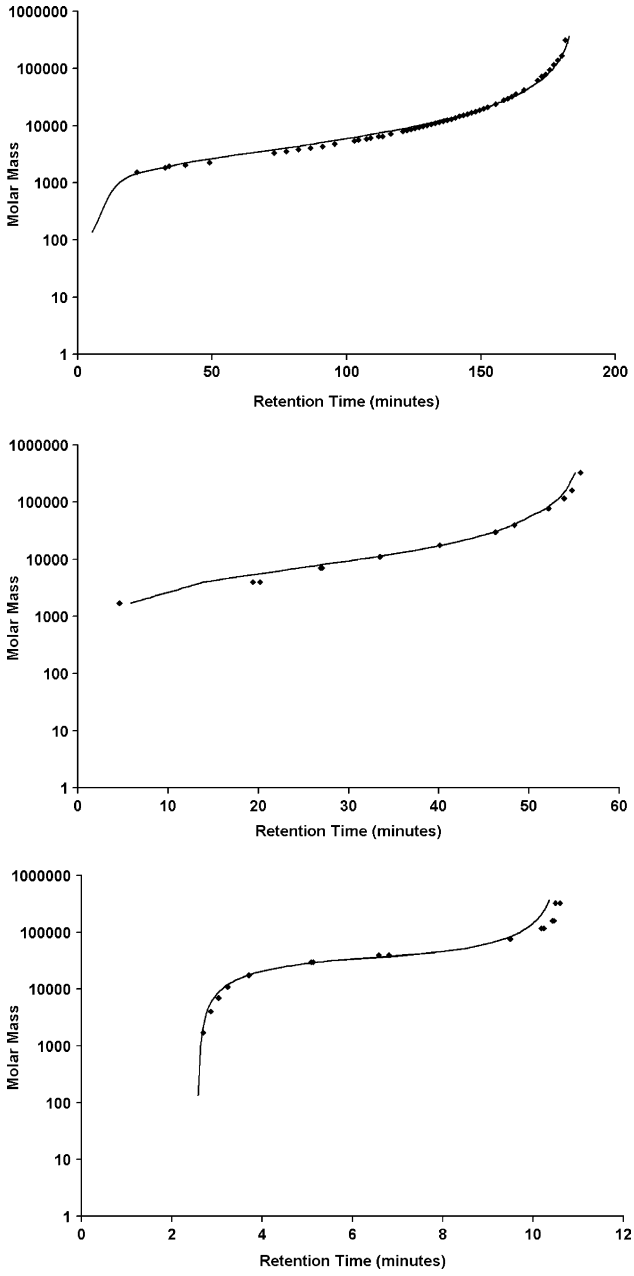

Fig. 5. Predicted and experimental calibration curves for polystyrene. Continuous lines correspond to predicted curves, points correspond to experimentally obtained retention times. (a) Gradient conditions: 5–60% THF in ACN in 220 min, experimental data points are taken from MALDI-MS fractions, (b) gradient conditions: 25–75% THF in ACN in 100 min, experimental data points are taken from chromatographic data of standards and (c) Gradient conditions: 45–60% THF in ACN in 12.5 min experimental data points taken from chromatographic data of standards.

A direct comparison of the two techniques (iLC and SEC) was made by separating the same sample (PS 10 900) in both modes. Equal fractions (0.1 mL) from each separation were collected and MALDI-MS spectra of the fractions were measured. For a typical iLC fraction, only nine oligomers were

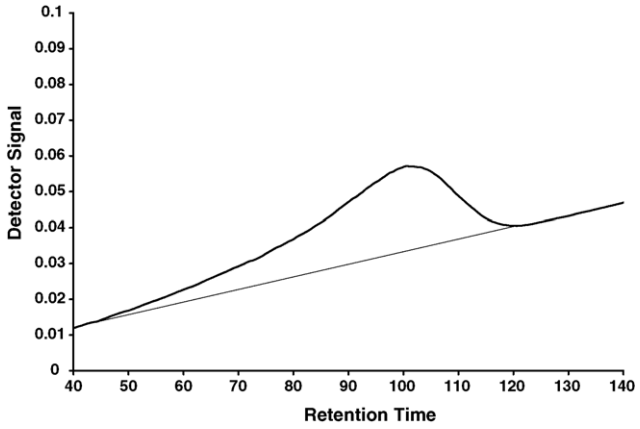

Fig. 6. iLC separation of a narrow dispersity (PDI = 1.06) polystyrene standard (4000). Gradient conditions: 5–95% THF in ACN in 220 min.

present. This compares very favourably with a SEC fraction in a similar mass range, which contained 27 separate oligomers (Fig. 8).

3.5. Understanding asymmetrical peaks and peak splitting in iLC

The shape of the calibration curve can also be used to understand and control the sometimes strange chromatographic peak shapes that are obtained for the chromatographic separation of polydisperse samples. When the mass range of a normally distributed sample is in a non-linear region of the calibration curve, then the resulting peak shape will not be Gaussian, because selectivity in that mass range is not constant. Peaks can then appear to be fronting or tailing. For example, when the selectivity of the system is higher in the low-molar mass region, then the lower mass portion of the sample will be more separated than the higher mass portion of the sample. This is seen experimentally as a 'fronting' peak such as the peak in Fig. 6. If selectivity is greater in the high-molar mass region, then the peak will have a sharp leading edge but will exhibit what would be called tailing in conventional chromatography. In some cases, a single sample can even split into two separate peaks. This occurs when the starting conditions of the gradient are strong enough to elute some of the lower masses present in the sample, either as an unretained peak eluting with the void volume or as weakly retained polymer eluting before the start of the gradient. Higher molar masses will then be eluted once the mobile phase is strong enough, resulting in two separate peaks (each containing different molar masses) for the same sample.

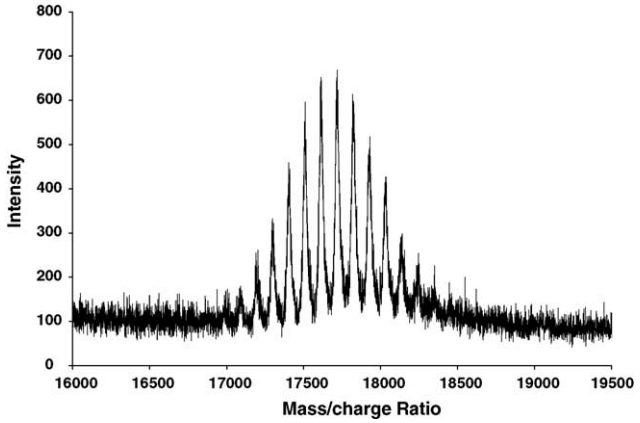

Fig. 7. MALDI-MS spectrum of a polystyrene fraction collected after an iLC separation. Gradient conditions: 5–95% THF in ACN in 220 min. Calculated polydispersity: 1.00024.

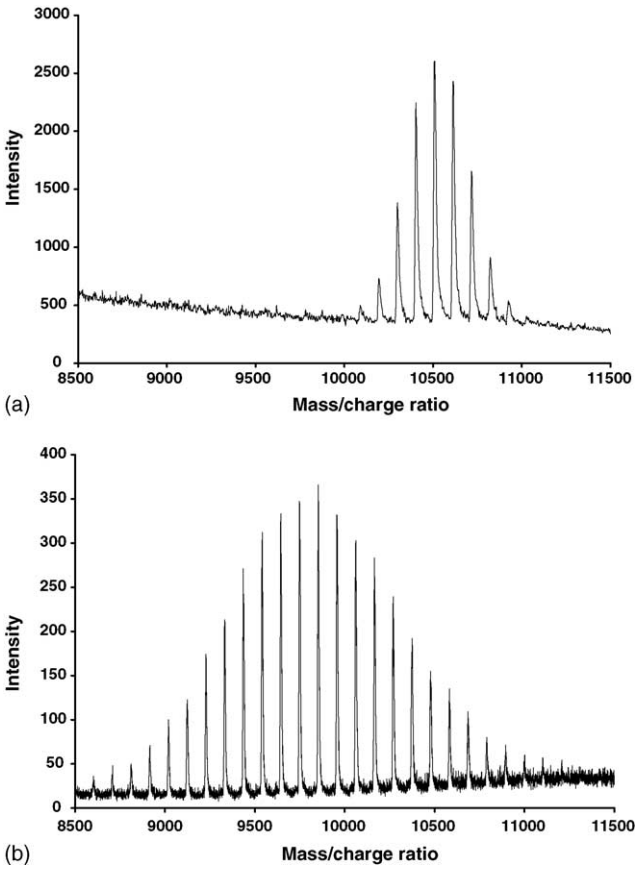

Fig. 8. A comparison of MALDI fractions (30 s in each case) from (a) an iLC and (b) a SEC separation of a polystyrene standard (10 900 μ). iLC gradient: gradient conditions: 5–95% THF in ACN in 220 min, flow rate: 0.2 mL/min. SEC column: PL Gel 10^3 Å, mobile phase: 100% THF, flow rate: 0.2 mL/min, injection volume 10 μL.

3.6. Using interactive LC for the determination of molar mass distributions

A consequence of the enhanced selectivity of iLC in comparison to SEC in the low-molar mass range, is that iLC can be more accurate for the determination of molar mass distributions. In the same way that calibration curves are used in SEC to transform retention times (or elution volumes) into molar masses, the calibration curves obtained in iLC experiments can also be used. The molar mass distribution of a

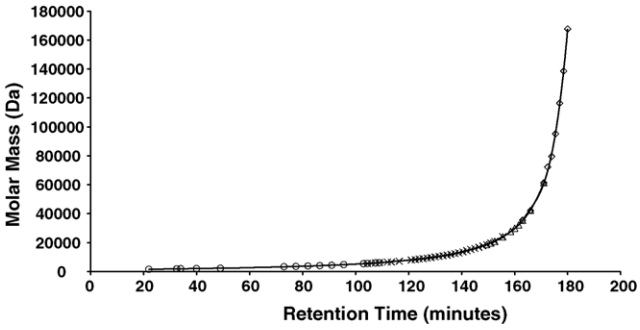

Fig. 9. Experimental data points and fitted calibration curves over the entire mass range. Equations of the line: (○) from 1500 to 6000, $y = 0.0052x^3 - 0.5497x^2 + 47.568x + 696.02$, $R^2 = 0.9994$, (×) from 5600 to 24 000, $y = 0.1405x^3 - 47.889x^2 + 5591.9x - 216391$, $R^2 = 0.9992$, (△) from 18 500 to 61 000, $y = 3.501x^3 - 1599.1x^2 + 244\,372x - 1 \times 10^7$, $R^2 = 0.9995$, (◊) from 35 000 to 160 000, $y = 29.541x^3 - 14\,677x^2 + 2 \times 10^6x - 1 \times 10^8$, $R^2 = 0.9991$.

sample can then be calculated from its peak shape, as long as the detector has a known response to concentration.

An experimental calibration curve was constructed by separating polystyrene standards using a shallow gradient (5–60% THF in ACN in 220 min; Fig. 5a). The gradient conditions were chosen to give the greatest selectivity in the molar mass region up to ~40 000 μ. The peak shapes for the various standards varied dramatically with increasing mass. Low molar masses were eluted as peaks that were extremely broad (i.e. low masses were very well separated). Higher molar masses (above ~40 000 μ) eluted as sharp peaks with much less resolution between masses. For the best fit for the experimental calibration curve, fractions were taken across each of the sample peaks (0.1 mL) and the molar mass at the peak top (M_p) of each fraction was measured using MALDI-TOF-MS. The resulting calibration line was almost identical to the predicted calibration line (Fig. 5a).

Fitting the curve to one simple formula was not possible. However, when the curve was split into different sections, excellent fits were found for specific mass ranges within the curve (a linear rather than a log mass scale was used in this case to improve the curve fit). Fig. 9 shows the experimental data points and the third order polynomial fits that were used to describe the relationship between retention and molar mass over the entire mass range.

The calibration line was used to calculate the average molar mass and the molar mass distribution of a range of polystyrene standards, using the signal from a UV detector for the concentration profile. A comparison of the calculated and quoted polydispersities and M_p values of the standards are given in Table 3, along with the calculated weight-average (M_w) and number-average (M_n) molar masses for each standard and the polydispersity values calculated using MALDI-MS. For most of the standards, the calculated polydispersity indices were significantly lower than the values quoted by the manufacturers. This is in agreement with other research that suggests that quoted polydispersities are upper limits rather than exact values [5,18,33]. Two of the standards (4 000 μ and 10 900 μ) had marginally higher polydispersities. M_p values were calculated as the mass at the peak top in the molar mass distribution.

The MMD obtained for the PS 7000 standard was fitted to both a normal and a log normal distribution. A plot of the residuals (Fig. 10) showed that there was no significant difference between the normal and the log-normal fit for the standard.

When the polydispersity values calculated using iLC were compared with values calculated using MALDI-MS, it was seen that the latter yielded significantly lower PDI values. This result was surprising. One explanation may be that the chromatographic separation was not only due to molar mass but may also have been influenced by other effects (for example many stereoisomers exist for every member of the polystyrene series). Chromatographic peak broadening may also have been a contributing factor, although the MALDI-MS spectra of the fractionated polymer showed that resolution was very high (see Figs. 7 and 8a). Mass discrimination in the MALDI, which could lead to lower perceived PDI's, may also explain the anomaly. Although it is generally accepted that for polymers with narrow molar mass distributions (such as standards), mass discrimination is not a problem [34], the accuracy of MALDI for the determination of PDI's has not yet been fully proven [35]. Some further investigations into the sources of peak broadening in iLC of polymers will be required in order to clarify this issue.

Table 3
Comparison of the quoted and calculated average masses and polydispersities for a series of polystyrene standards

Supplier*	M_p quoted	M_p calculated	M_w calculated	M_n calculated	PDI quoted	PDI calculated	
						iLC	MALDI
PL	1,700	1,822	1,913	1,838	1.06	1.040	1.022
PC	4,000	5,118	4,511	4,190	1.06	1.077	1.017
PL	7,000	7,461	7,347	7,218	1.03	1.018	1.008
PSS	10,900	10,103	9,926	9,553	1.03	1.039	1.016
PSS	17,600	16,738	16,363	16,135	1.03	1.014	1.012
PC	30,000	29,525	29,415	29,243	1.06	1.006	Not available
PSS	39,200	39,133	37,223	36,503	1.03	1.020	1.016
PL	76,600	82,892	80,386	79,390	1.03	1.013	1.004

* Suppliers: PL = Polymer Laboratories, PC = Pressure Chemical, PSS = Polymer Standards Services.

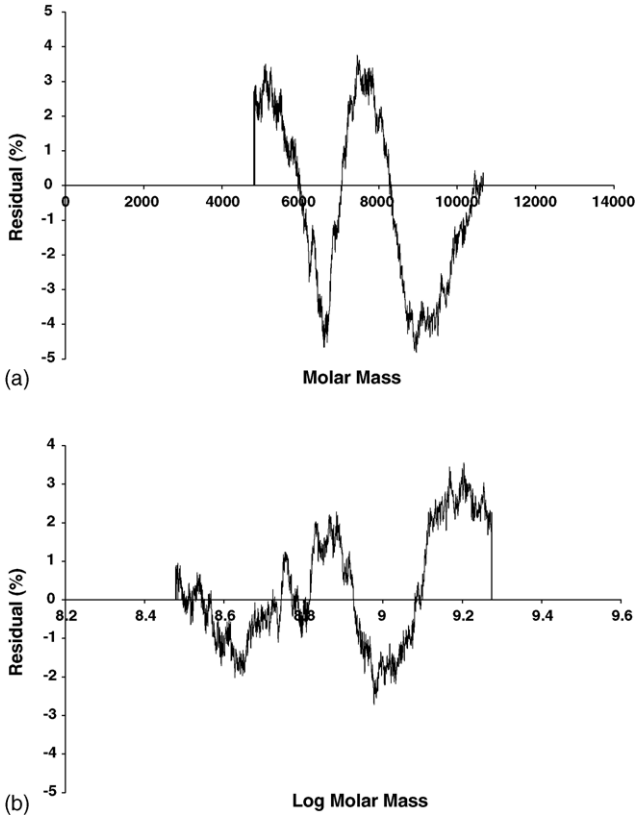

Fig. 10. Residual plots comparing (a) a normal and (b) a log-normal fit of the experimentally determined molar mass distribution.

The shape of the calibration curve dictates the upper-mass limit of iLC for determining MMDs. When there is no longer any separation according to molar mass (i.e. the vertical range of the curve), peaks are narrow and have very little contribution from polydispersity. The upper limit is partly controlled by the applied gradient conditions, but also by the steepness of the $\ln k$ versus φ relationship. For higher masses, slopes are steep (high S values in the RP model) and the change in $\ln k$ with φ hardly varies with molar mass. In this mass range, SEC becomes the more appropriate choice for MMD determinations.

4. Conclusions

iLC is a highly selective, easily tuneable separation technique, that can be used for the determination of molar mass distributions in the low-to-medium (<40 000 μ) molar mass range. Separations can be understood and predicted by applying a chromatographic model (in this case the RP model) and determining the relationship between the model parameters and molar mass. We found that the best relationship between $\ln k_0$ and molar mass for this separation was a power curve and that there was a linear relationship between $\ln k_0$ and S. Comparisons of the predicted and experimental retention times (presented in the form of calibration curves) showed that the model accurately described retention under any mobile phase conditions and over a broad range of molar masses. The shape of the calibration curve can be optimised (using the model) to best suit the requirements of a particular analysis. Extremely narrowly distributed (effectively monodisperse) mass fractions can be obtained using iLC, even up to molar masses as high as almost 20 000 μ. The technique can be used to accurately determine molar mass distributions, however some further investigation will be required to account for the differences between the polydispersity values calculated using iLC and MALDI-MS.

While it seems unlikely that iLC could replace SEC as the chromatographic technique of choice for the determination of MMDs, solvent (and temperature) gradient separations can be very valuable tools when the best possible separation of a polydisperse sample (within the appropriate mass range) is required, for instance, for the calculation of MMDs of low-dispersity samples such as standards.

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