Journal of Chromatography A, 1218 (2011) 1147–1152

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Transfer-volume effects in two-dimensional chromatography: Adsorption-phenomena in second-dimension size-exclusion chromatography

Eva Reingruber[a,*], Jeroen J. Jansen[b], Wolfgang Buchberger[a], Peter Schoenmakers[c]

[a] Institute of Analytical Chemistry, Johannes Kepler University, 4040 Linz, Austria
[b] Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
[c] Van 't Hoff Institute for Molecular Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 11 August 2010
Received in revised form
13 December 2010
Accepted 17 December 2010
Available online 28 December 2010

Keywords:
Simulation
Peak splitting
Polymers

ABSTRACT

Gradient-elution LC × LC is a valuable technique for the characterization of complex biological samples as well as for synthetic polymers. Breakthrough and viscous fingering may yield misleading information on the sample characteristics or deteriorate separation. In LC × SEC another phenomenon may jeopardize the separation. If the analytes adsorb on the SEC column under the injection-plug conditions, peak splitting may occur. In LC × LC the effluent from the first column is the sample solvent for the analytes injected into the second dimension. If a gradient-elution LC × SEC setup is used (i.e. if reversed-phase gradient-elution LC is coupled to organic SEC and if normal-phase gradient-elution LC is coupled to SEC with a polar solvent), the percentage of weak solvent may be significant, especially at short analysis times. In this case adsorption in the first-dimension-effluent zone on the second-dimension SEC column can become an issue and two peaks – the first eluting in size-exclusion mode and the second undergoing adsorption – can be obtained. The work presented in this paper documents peak splitting in LC × SEC of polymers. The adsorption of the polymer on the size-exclusion column was proven in one-dimensional isocratic runs. The observed effects were modeled and visualized through simulation. Studies on the influence of the transfer volume were carried out. Keeping the transfer volume as small as possible helped to minimize peak splitting due to adsorption.

1. Introduction

In polymer chromatography there are several pitfalls which may lead to misleading information on the sample characteristics or deteriorate the separation. In interaction chromatography (i.e. when the elution volume of the analytes exceeds the hold-up volume of the column) even narrow polymer standards may elute as two separated peaks. This phenomenon is referred to as breakthrough [1]. Jiang et al. [2] found that breakthrough was observed when part of the polymer molecules traveled with the injection band through the column without interacting with the stationary phase. The sample solvent and the polymer concentration, the injection volume, the (initial) composition of the mobile phase, and the column temperature all had an effect on whether or not breakthrough occurred.

When a fluid pushes another miscible liquid with a higher viscosity, the interface may become unstable and a finger-like pattern may be observed. This phenomenon is called viscous fingering [3]. Broyles et al. [4] visualized the effect of viscous fingering on chromatographic separations. Viscous fingering was found to distort the peak shape significantly [4–7].

Basic strategies to avoid breakthrough and viscous fingering are described in the papers cited above. In one-dimensional LC those strategies are straightforward to implement. To minimize breakthrough, a weaker sample solvent should be chosen and the injected volume should be minimized [2]. To minimize peak distortion due to viscous fingering, a viscosity mismatch between the sample solvent and the eluent has to be avoided. It has been suggested that additives may help to fine-tune the viscosity of the fluids. Furthermore, increasing the retention of the analytes and reducing the flow rate and the injection volume can help to limit viscous fingering [4,5].

However, synthetic polymers show multiple distributions, such as a molecular-weight distribution, a chemical-composition distribution, a sequence distribution, and a block-length distribution, possibly all at the same time [8]. Thus, their accurate characterization calls for multi-dimensional techniques. Comprehensive two-dimensional liquid chromatography (LC × LC) outranks one-dimensional separation systems in terms of its resolving power [9], which makes it a valuable technique for analyzing such multiply-distributed samples (e.g. [8,10]). van der Horst et al. [11] developed

* Corresponding author. Tel.: +43 6507770181.
E-mail addresses: eva.reingruber@jku.at, evareingruber@hotmail.com (E. Reingruber).

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doi:10.1016/j.chroma.2010.12.080

a two-dimensional setup, which is easy to implement and allows truly comprehensive analysis. Several approaches to analyze synthetic polymers with LC × LC were successfully implemented (e.g. [11–13]). However, conditions where breakthrough and viscous fingering become relevant may be hard to avoid in LC × LC [14–16].

Gradient-elution LC and SEC are a powerful combination in LC × LC of polymers. The need for a sufficiently fast separation in the second dimension (^{2}D) makes it attractive to use an isocratic separation, such as SEC. The isocratic conditions in the ^{2}D also render detectors applicable (e.g. refractive index detector) which cannot be used with gradient-elution. Im et al. [17] demonstrated the potential of gradient-elution LC × SEC for the characterization of synthetic polymers. If SEC is used in the ^{2}D breakthrough is not an issue in this dimension. Peak distortion by viscous fingering has to be taken into account, but can be limited with adequate method optimization.

However, separation may be jeopardized if the analytes adsorb on the SEC column under the injection-plug (i.e. transfer volume) conditions. For one-dimensional SEC separations, polymers are usually dissolved in a strong solvent (often in the mobile phase). Therefore, peak splitting due to adsorption within the injection plug is not an issue in one-dimensional SEC. In LC × LC, however, the effluent from the first dimension (^{1}D) is the sample solvent for the analytes injected into the ^{2}D. If a gradient-elution LC × SEC setup is used, the percentage of weak solvent in the transferred volume may be significant. If reversed-phase LC is coupled to organic SEC and normal-phase LC to SEC with polar solvents, this is the case at short analysis times. This situation is similar to what is encountered in liquid chromatography under limiting conditions of desorption [18]. Here, a binary polymeric blend is dissolved in a sample solvent under which one polymer will be adsorbed on the chromatographic column. The used eluent allows size-exclusion for both components. In this way, one polymer elutes under size-exclusion conditions, while the other component elutes behind the sample–solvent plug that induces adsorption for this polymer. Accordingly, adsorption on the ^{2}D size-exclusion column within the transfer-volume plug will shift the analyte peak to a retention volume slightly higher than that of the transfer-volume plug. If the concentration profile of methanol in the transfer-volume plug is such that part of the polymer experiences size-exclusion conditions upon injection, peak splitting will occur. The work presented in this paper documents peak splitting in LC × SEC of polymers. The adsorption of the polymer on the size-exclusion column was proven in one-dimensional isocratic runs. Furthermore, the influence of the transfer volume on the described phenomenon was studied. The observed effects were modeled and visualized through simulation.

2. Experimental

2.1. Samples and chemicals

A copolymer with an ester group at every backbone carbon atom (poly(EA-*grad*-BnA)) was prepared from ethyl and benzyl diazoacetate. The strategy of synthesis as well as a basic characterization of poly(EA-*grad*-BnA) are given elsewhere [19]. The molar ratio of ethyl diazoacetate:benzyl diazoacetate was about 1:1 (determined with NMR), the molecular weight (against PS standards) was 40 kDa and the polydispersity index was 2.9.

Chloroform (HPLC grade, containing 1% of ethanol for stabilization) and methanol (HPLC grade, absolute; both from Biosolve, Valkenswaard, The Netherlands) were used for the separations.

2.2. Instrumentation

Analyses were carried out using an autosampler (SIL-9A), a column oven (CTO-10A VP), a controller (CBM-20A), and three LC pumps (LC-10AD VP), all from Shimadzu (Kyoto, Japan), as well as a two-position ten-port switching valve (Valco VICI International, Schenkon, Switzerland) with two 100-μL sample loops. The loops were connected to the valve according to the symmetrical arrangement proposed by van der Horst et al. [11]. The strong and the weak solvent were mixed directly in front of the column i.e. after the injector in a 150-μL T-piece mixer (Analytical Scientific Instruments, El Sobratne, CA, USA). In this way, there was no injection plug arriving on the column and breakthrough was avoided [20]. A charged-aerosol detector (esa CAD plus; ESA, Chelmsford, MA, USA) was used for detection.

Matlab (version R2007b, Mathworks, Natick, MA, USA) was used for implementing the simulations and for generating the experimental gradient-elution LC × SEC chromatograms.

2.3. Chromatographic conditions

All separations were performed at 30 °C. SEC experiments were carried out on a PLgel 5-μm Mix-C column (100 mm × 4.6 mm I.D., Polymer Labs/Varian, Church Stretton, Shropshire, UK). In one-dimensional experiments the flow was 1 mL/min and the injection volume was 10 μL.

For gradient-elution LC × SEC separations, the injection volume was increased to 50 μL. A column (150 mm × 4.6 mm I.D.) was packed with non-porous 40-μm glass beads. Glass beads were suspended in methanol and brought into the empty column while vacuum was being applied. Initial ^{1}D run conditions were 2% chloroform and 98% methanol. As mentioned in Section 2.2, a 150-μL mixer was used to mix the strong and the weak solvent. To ensure that all the samples were immobilized at the top of the column the mixer had to be flushed with several times its volume. In order to keep analysis time low this was done at 0.5 mL/min. After 15 min, the flow was reduced to 0.05 mL/min and the gradient program was started: the concentration of chloroform was increased from 2% up to 100% at a rate of 1%/min. The final conditions were held for 35 min. Subsequently the column was flushed with 2% chloroform and 98% methanol at a flow of 0.5 mL/min for 2 min. The switching valve was actuated with different intervals in order to examine the effects of different transfer volumes. The ^{2}D SEC runs were carried out with chloroform as the mobile phase at 1 mL/min.

3. Results and discussion

3.1. Chromatographic examinations

Gradient-elution LC was carried out on a glass-bead packed column. This column was not expected to show neither any SEC effects nor any adsorptive interactions. The observed separation in the first dimension was therefore resulting exclusively from precipitation/redissolution (controlled by the mobile-phase composition) [21]. Thus, every fraction eluted (respectively was transferred to the ^{2}D) close to its solubility point and polymer-coil contraction caused by the weak solvent was similar for all fractions. SEC was used to gain insight in the molecular-weight distribution of the fractions eluting from gradient-elution LC.

In a gradient-elution LC × SEC chromatogram of poly(EA-*grad*-BnA) (switching-valve modulation time 2 min) three distinct peaks were observed as shown in Fig. 1 a minor peak eluted at a retention time between 65 and 80 min in gradient-elution LC and between 0.9 and 1.0 min in SEC. This peak consisted of homopolymeric material; its presence could be explained by the synthesis strategy of this polymer [19]. A bimodal molecular-weight distribution – as suggested by the ^{2}D SEC results – was, however, not expected. The size-exclusion results from the LC × SEC clearly contradicted those obtained from one-dimensional SEC runs (see Fig. 2).

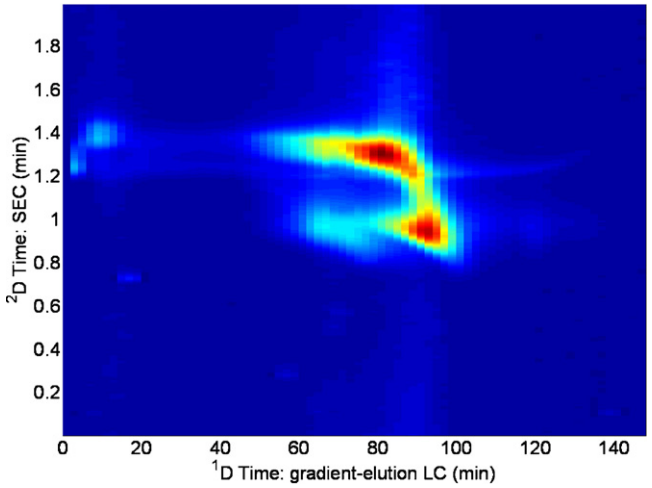

Fig. 1. Gradient-elution LC × SEC chromatogram of a poly(EA-*grad*-BnA) yielding three different peaks. For chromatographic conditions see Section 2.3.

The retention time for an analyte fully penetrating the pores of the size-exclusion column was about 1.25 min for the one-dimensional SEC as well as for ^{2}D SEC. This implies that analytes with retention times higher than 1.25 min are eluting under adsorption conditions rather than in size-exclusion mode.

The presence of adsorptive interactions between the polymer and the size-exclusion column was subsequently examined in isocratic one-dimensional experiments with pure chloroform and chloroform:methanol 60:40 as the mobile phase (see Fig. 3). While the polymer eluted in SEC mode with pure chloroform as the mobile phase, significant adsorption took place in chloroform/methanol 60:40 (v/v).

With this knowledge the peak splitting in the LC × SEC chromatogram can be interpreted. If wall friction, molecular diffusion and flow distortion would be negligible in the injection loop and tubing, an ideal transferred plug with a rectangular shape would be obtained. In reality the transferred plug experiences some mixing and arrives with a somewhat dispersed concentration profile at the column. At the front end of the transferred plug, the concentration of weak solvent in the overall mixture is relatively low so that adsorption is not significant and solutes elute in SEC mode.

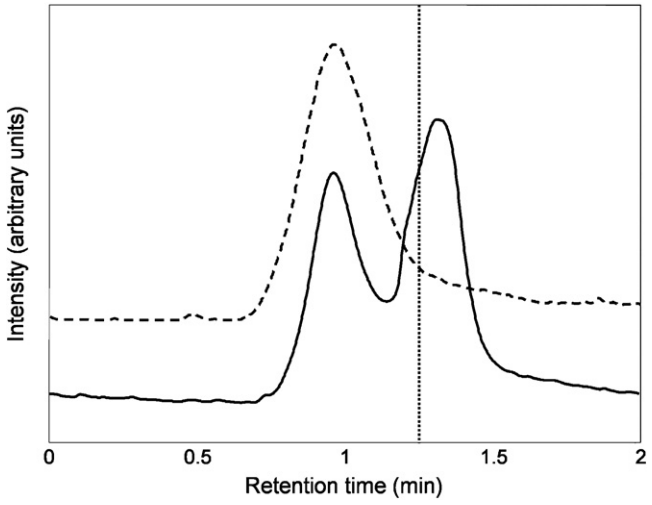

Fig. 2. Comparison of the SEC results for the examined copolymer. Eluent is chloroform in both cases. The vertical line marks t_0. The projection of the 2D chromatogram (solid line) shows a bimodal distribution, while in one-dimensional SEC (dashed line) only one peak is observed. For further chromatographic conditions see Section 2.3.

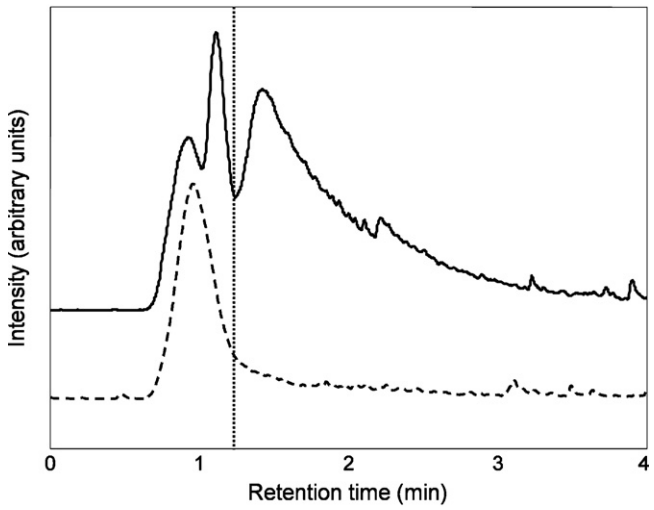

Fig. 3. Comparison of SEC with different mobile phases. The vertical line marks t_0. With chloroform analytes elute in SEC mode (dashed line), while adsorption becomes significant with chloroform/methanol 60:40 (v/v) (solid line). For further chromatographic conditions see Section 2.3.

At a certain point within the injection plug the concentration of weak solvent becomes so high that adsorption becomes significant and analytes fall behind. This mechanism may result in two distinct peaks.

The influence of the transferred volume was studied by varying the frequency of the valve switching. At a small transfer-volume of 40 μL adsorption did not affect the separation. However, the chromatograms became more and more distorted with increasing transfer-volume (see Fig. 4). Thus in order to avoid adsorption effects, the transfer volume should be kept as low as possible. Peak distortion started at short ^{1}D retention times, i.e. where the ^{1}D effluent contained high concentrations of weak solvent. Accordingly, keeping the concentration of weak solvent in the transfer volume as low as possible also reduces the risk of peak splitting. This can be achieved by using highly retentive ^{1}D columns.

3.2. Simulation of the adsorption effects

To investigate whether the observed peak splitting may indeed result from the proposed adsorption effects we simulated how polymer analytes may be retained in the presence of an injection plug (see Supplementary Material, Text Box 1).

In LC, computer simulations are frequently used to predict retention behavior (e.g. [22–27]). Furthermore, the applicability of different retention models can be evaluated (e.g. [28–30]). Besides, simulations help to understand and correct for instrumental peak broadening (e.g. [31–33]). However, in this work a model as simple as possible is created to investigate peak splitting. The present model consists of a size-exclusion column operated using a typical strong solvent (no adsorption) and an injection plug traveling through it. The injection plug contains methanol (weak solvent) and polymer-analyte molecules. The polymer analyte is retained according to the reversed-phase model [34–36]. Fitzpatrick et al. [37] demonstrated that the reversed-phase model is applicable to polymers eluted with mixtures of organic solvents. Chromatographic band broadening is disregarded.

In this simulation, the inputs are a vector defining the shape and length of the injection plug, the concentration of methanol and of polymer analyte within the plug, the number of segments of the chromatographic column, and two parameters describing the retention of the polymer analyte (slope and intercept of logarithmic

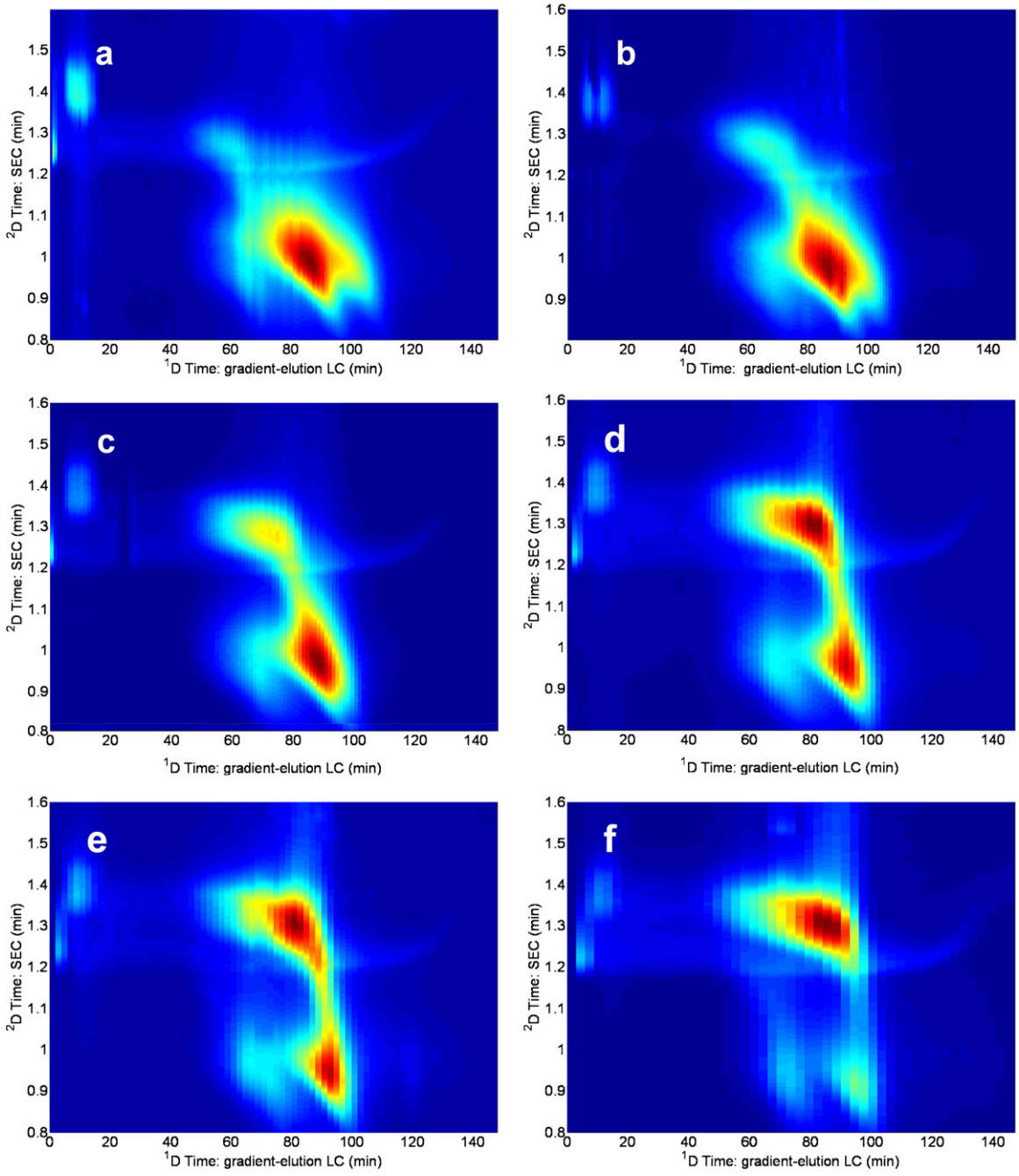

Fig. 4. Effect of transfer volume on peak splitting in gradient-elution LC × SEC. Different transfer volumes were obtained with different modulation time of the switching valve: (a) 0.8 min, (b) 1.0 min, (c) 1.25 min, (d) 1.75 min, (e) 2.0 min, and (f) 3 min. For further chromatographic conditions see Section 2.3.

retention *vs.* mobile-phase composition; see below). Initially the column is filled with the size-exclusion mobile phase. Therefore the concentration of methanol and polymer analyte is zero in all column segments. Then the injection plug enters. Methanol moves with the speed of the mobile phase through the column which is one segment (i.e. vector position) per iteration step. The methanol concentration in column segments outside the injection plug (i.e. where there is only mobile phase) remains zero. Analyte molecules are moving through the column in a retained fashion, according to the reversed-phase model [34–36]. This model implies that the natural logarithm of the retention factor (k) is linearly correlated to the volume fraction of strong solvent in the mobile phase. For each column segment, k (and subsequently the concentration of adsorbed and non-adsorbed polymer analyte) is calculated from the local concentration of methanol in the mobile phase. Adsorbed polymer analyte remains at the same column segment (i.e. same position in the vector) at the next iteration. The non-adsorbed analyte moves two segments per iteration step, which corresponds to the assumption that it is totally excluded from the pores (strongest possible SEC effect).

The output of the simulation is a movie depicting the movement of the methanol and the analyte molecules through the column. Snapshots of this video can be seen in Fig. 5. At $t = 0$, the column is empty. Then the injection plug containing methanol and the polymer analyte enters and travels through the column ($t = 1$ until 5). Methanol is not retained. The retention factor k of the polymer analyte, however, shifts to higher values with increasing methanol concentration. Therefore, the analyte plug splits into

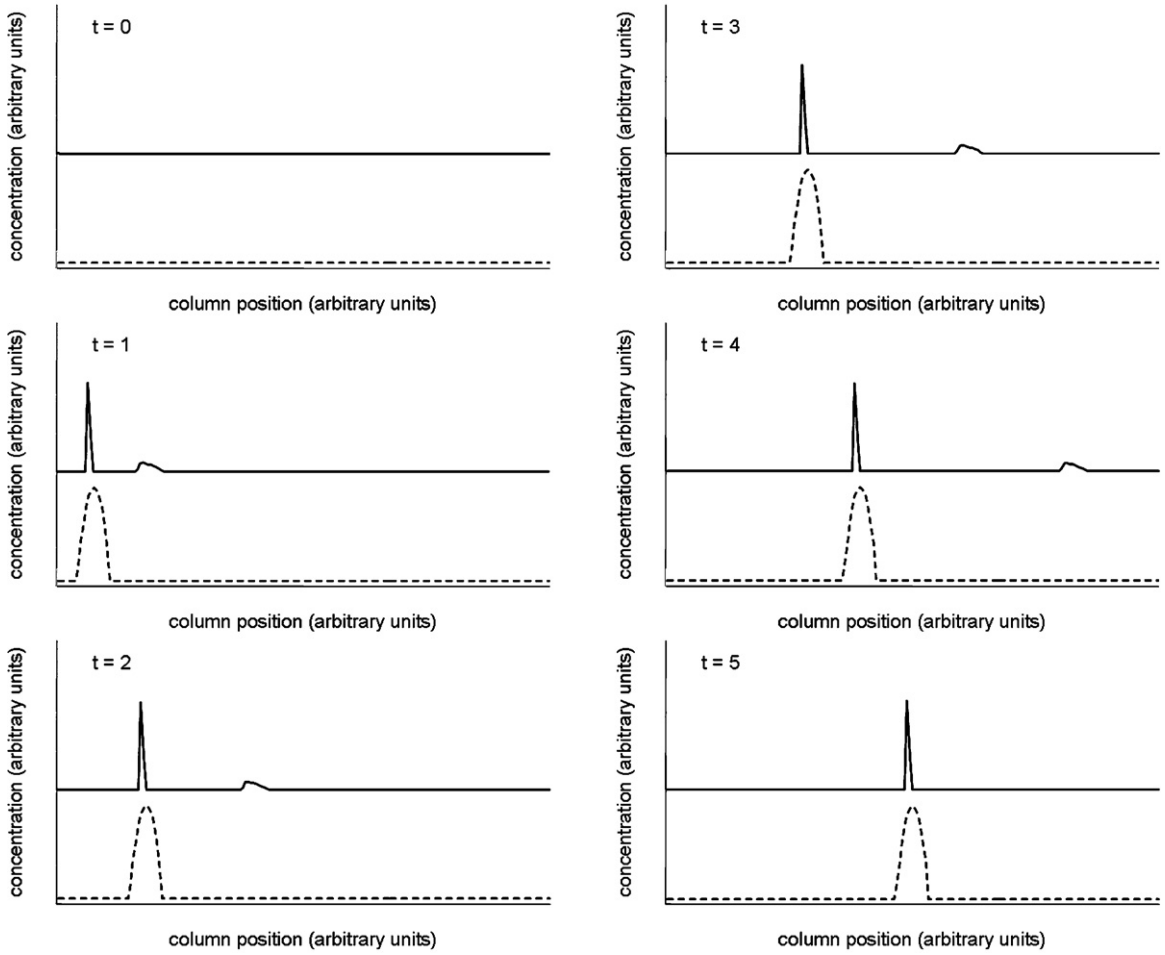

Fig. 5. Simulation of adsorption-induced peak splitting on a SEC column. Solid lines demonstrate the movement of the analyte through the column, whereas dashed lines show the movement of methanol. Analyte molecules at the front side of the plug, where the concentration of methanol does not effect adsorption, elute according to size-exclusion. At a certain concentration of methanol (i.e. at a certain position within the transfer-volume plug) adsorption becomes significant. Analyte molecules cannot overcome this barrier of high methanol concentration and thus elute behind it. Therefore, peak splitting occurs.

non-adsorbed analytes which elute in the SEC mode and adsorbed analytes which fall behind. When they reach column segments with lower methanol concentrations, these molecules will experience size-exclusion. As a result, the band of adsorbed analytes focuses at the back side of the methanol plug. In the simulated chromatogram (Fig. 6) this results in two distinct peaks, just as observed in the size-exclusion dimension of the two-dimensional chromatogram (compare Fig. 1).

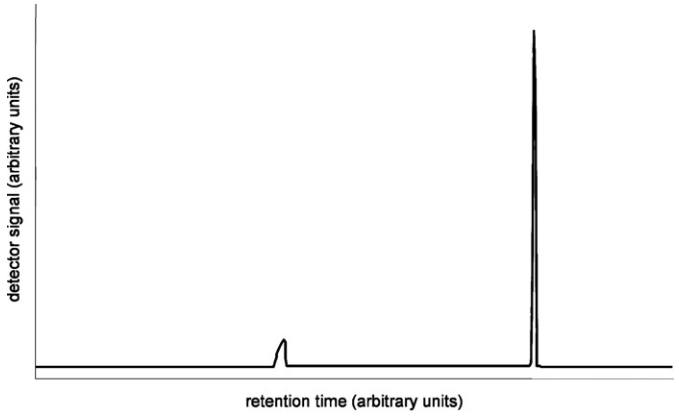

Fig. 6. Chromatogram obtained by simulating adsorption-induced peak splitting on a SEC column.

4. Conclusions

Gradient-elution LC × SEC is a powerful technique for characterizing complex biological samples as well as polymers. In this work, a pitfall associated with the use of SEC as a second-dimension stage in LC × LC has been identified. If there is a high concentration of weak solvent in the transferred plug, analytes may be adsorbed on the stationary phase of the size-exclusion column. This may lead to peak distortion or even peak splitting. In the latter case, the first peak corresponds to the fraction of the polymer eluting in size-exclusion mode and the second peak corresponds to the fraction undergoing adsorption.

To avoid peak splitting due to adsorption, the transfer volume and the concentration of weak solvent within the transfer volume should be kept as low as possible. The latter can be achieved by using highly retentive ^{1}D columns. During method development it should be verified whether the weak solvent of the designated ^{1}D gradient gives rise to adsorption effects on the ^{2}D SEC column. If possible, such weak solvents should be avoided. Furthermore, comparing the one-dimensional chromatograms with the projected chromatograms of the LC × SEC helps to diagnose the presence of any unwanted transfer-volume effects.

Acknowledgements

The authors would like to thank Prof. Michel Martin (ESPCI, Paris, France) and Prof. Sjoerd van der Wal (University of Amsterdam, The Netherlands) for fruitful discussions on injection-plug profiles.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.12.080.

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