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Journal of Chromatography A, 968 (2002) 41–52

JOURNAL OF CHROMATOGRAPHY A

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Development of a two-dimensional liquid chromatography system with trapping and sample enrichment capabilities

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Received 13 February 2002; received in revised form 6 June 2002; accepted 6 June 2002

Abstract

A two-dimensional HPLC system was developed where ''heart-cutting'' chromatography, in conjunction with cold temperature trapping, was used to isolate and concentrate specific sample analytes. Low molecular mass polystyrene oligomers were used as model compounds to illustrate the operation of the instrument and evaluate the performance of the trapping system. A critical factor in the operation of the trapping system was the relative degree of retention between the first column and the trapping column. The results of this study showed that up to 32 consecutive heart-cut fractions from the first separation dimension could be stored in a trapping column with good analyte recovery and without significant loss in resolution upon elution on the second separation dimension.

Keywords: Heart-cutting; Cold temperature trapping; Two-dimensional liquid chromatography; Polystyrenes, low-molecular-mass

1. Introduction

Complete separation of all components in complex mixtures is virtually impossible in linear one-dimensional chromatography, particularly for randomly spaced bands [1]. Consequently, two-dimensional chromatographic techniques have been developed in order to increase the peak capacity of the separation system and improve the separation of complex samples. In two-dimensional chromatography components are displaced along two axes of separation [2–5], rather than a single axis as in one-dimensional chromatography. In an ideal two-dimensional chromatographic system each dimension of the separation would employ a stationary phase that would generate orthogonal retention behaviour of the solutes under study, consequently maximising the peak capacity of the system. However, in reality, most two-dimensional systems have at least some retention correlation and this decreases to a certain extent the optimum resolution and peak capacity of the system [6].

Numerous two-dimensional chromatographic systems have been described in the literature that allow full automation of two-dimensional separations [7] in the absence of manual sample transfer from one separation environment to a second. Many of these systems employ a process referred to as ''heart-cutting'', which is a term used to describe a process of analysis whereby only a portion of the sample separated in the first dimension is transported to the

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00782-3

second dimension. This is in contrast to comprehensive two-dimensional systems in which all components from within a sample mixture are subjected to two separation dimensions. However, in comprehensive two-dimensional separations any two components separated in the first dimension must remain separated when subjected to a second dimension and elution profiles from both dimensions must be preserved [8]. For two-dimensional separations heart-cutting techniques are more commonplace in liquid chromatography than in gas chromatography because the mobile phase in LC plays an active role in the separation process, in contrast to the passive role of the carrier gas in GC.

Unlike two-dimensional systems in which there is an obvious difference between the retention processes in each of the single dimensions, such as an ion-exchange separation coupled to a size exclusion separation [9], many two-dimensional separations reported in the literature are in reality simply examples of column switching processes. In these systems there is high correlation between the separation mechanisms in each of the so-called dimensions. Nevertheless, many of these column switching processes often describe techniques that offer very valuable attributes to the separation of components from within complex sample matrices. For example, these types of systems may aim to resolve a small number of components from within a given region of components that display a vast range of retention. In this case the separation can be optimised to resolve only the components of interest that are heart-cut to the second column, leaving behind the components that are of little interest. Systems may also be designed for sample pre-concentration or sample accumulation. Most of these techniques employ a sample enrichment column where the entire sample is eluted through the trapping column [10–17]. Once sufficient sample has been accumulated the enrichment column is back flushed and the analysis is completed.

In the present study we aim to describe the operation of a system designed for sample accumulation following heart-cutting from a first dimension. The accumulated heart-cut fraction is then transported to a second dimension whereby components not resolved in the first dimension are resolved in the second dimension. There are numerous advantages in using such a system for the analysis of complex samples, three of which include: (a) enhanced signal-to-noise for trace compounds; (b) the accumulation of sample which may be unstable in an environment outside the chromatographic column, and which may then be transported directly to an NMR; and (c) the process of sample enrichment and collection is automated, requiring no manual intervention. Examples of other studies where the trapping of heart-cut analytes in two-dimensional/column switching HPLC systems include coupled achiral–chiral HPLC for the separation of enantiomeric compounds using a silica guard column to trap heart-cut analytes [18,19]. The separation of components from human serum, rat plasma and rat bile using a mixed function polymeric phase in the first dimension and a C_{18} column to trap analytes of interest, prior to elution and separation on a C_{18} column in the second dimension [20,21]. In these two studies [20,21] the dominant trapping factor was the difference in retention capacity between the mixed function polymeric phase and the C_{18} phase, although the ~20 °C difference in temperature between the analytical column and the enrichment column may have been of minor benefit to the trapping efficiency [21].

In the present work low molecular mass polystyrene oligomers were used to study the operation of the trapping system. Oligostyrenes may be chromatographically considered as a multidimensional sample, with the first dimension being the variation in the molecular mass of each oligomeric unit, while the second dimension may be described according to the stereochemistry of the configurational repeating unit. Previous studies have shown that low molecular mass oligostyrenes could be separated according to molecular mass on C_{18} surfaces in methanol mobile phases, and when a carbon clad zirconia surface was employed with an acetonitrile mobile phase, the diastereoisomers within each oligomeric fraction could be separated [22–24].

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile, methanol and dichloro-

methane were obtained from Mallinckrodt, Australia. Milli-Q water was obtained in-house and filtered through a 0.2-μm filter. All mobile phases were sparged continuously with helium. A polystyrene SEC calibration standard with a molecular mass of 770 Da was purchased from Aldrich, and was produced via anionic polymerization with *sec*-butyl lithium to produce polymers with *sec*-butyl end groups. The stationary phase materials used for the preparation of columns used in this study were either Nucleosil C_{18} (3-μm particle size) (Alltech Associates Australia, Baulkham Hills, NSW Australia) or carbon clad zirconia (ZirChrom-CARB; 3-μm particle size) (ZirChrom Separations, Anoka, MN, USA). Both stationary phase materials were used as supplied by the manufacturer. A Sephasil C_4 (5-μm particle size) column (100×4.6 mm) was purchased from Amersham Pharmacia Biotech Australia and an Activon Valupak ODS (5-μm particle size) column (250×4.6 mm) was purchased from Regis Chemical.

2.2. Equipment

All chromatographic experiments were conducted using a Shimadzu LC system (Shimadzu Scientific Instruments, Rydalmere, NSW, Australia) incorporating a LC-10ATVP pumping system, SIL-10ADVP auto injector, SPD-10AVP UV detector set at 262 nm, SCL-10AVP system controller and Shimadzu Class-VP version 5.03 software on a Pentium II 266 PC. Column switching was achieved using 6-port 2-position switching valves fitted with micro-electric two position valve actuators (Valco Instruments, Houston, TX, USA). Valve switching was controlled using Shimadzu SCL-10AVP system controller and Shimadzu Class-VP version 5.03 software. Temperature regulation of the trapping column was performed using either an LKB Bromma 2219 Multitemp II Thermostatic Circulator (for 0 °C) or B. Braun Thermomix MM (Melsungen, West Germany) (for 50 °C). Data acquisition was achieved using a Lawson Labs model 203 serially interfaced 20-bit data acquisition system with a custom ±1-V gain range operated at 5 Hz (Lawson Labs, Malvern, PA, USA). Columns were packed using a Haskel air driven fluid pump (Haskel International, Burbank, CA, USA).

2.3. Chromatographic separations

The polystyrene standard with an average molecular mass of 770 Da was dissolved in 100% dichloromethane at a concentration of ~10 mg/ml). Polystyrene sample injection volumes were 5 μl. Purified oligomer fractions were collected in methanol and subsequently injected into the LC system in the same solvent in a volume of 10 μl. All flow-rates were 1.0 ml min^{-1}.

2.4. Preparation of chromatography columns

The carbon clad zirconia column and C_{18} trapping column were prepared using a procedure previously described [22,23].

3. Results and discussion

The design of a two-dimensional HPLC system incorporating a sample trap is illustrated in Fig. 1(a–f). In this system the sample is first subjected to a separation according to the mechanism afforded by the mobile phase and stationary phase in the first dimension (Fig. 1a). The sample region of interest may then be heart-cut following elution from the first column and transported to the sample trap (Fig. 1b), after which the unwanted components from the first dimension are flushed from the first column (Fig. 1a). The process may be repeated until the desired concentration of sample has been accumulated on the trap (in the present text we repeated this process up to 32 times, however, the number of trapping event processes would be entirely dependent upon the sample component concentration and the required aims of the experimental outcome). Residual solvent (90% methanol/10% water) in the solvent delivery system could be replaced with the required back-flushing solvent (Fig. 1c). The trap is then back flushed (Fig. 1d) to load sample onto the second dimension column C2 where the sample components are subjected to a further separation process according to a different retention mechanism (Fig. 1e). Of critical importance to this separation process is the successful operation of the trap in that migration of sample along the trap does not occur following successive loading of the trap from sample heart-cut

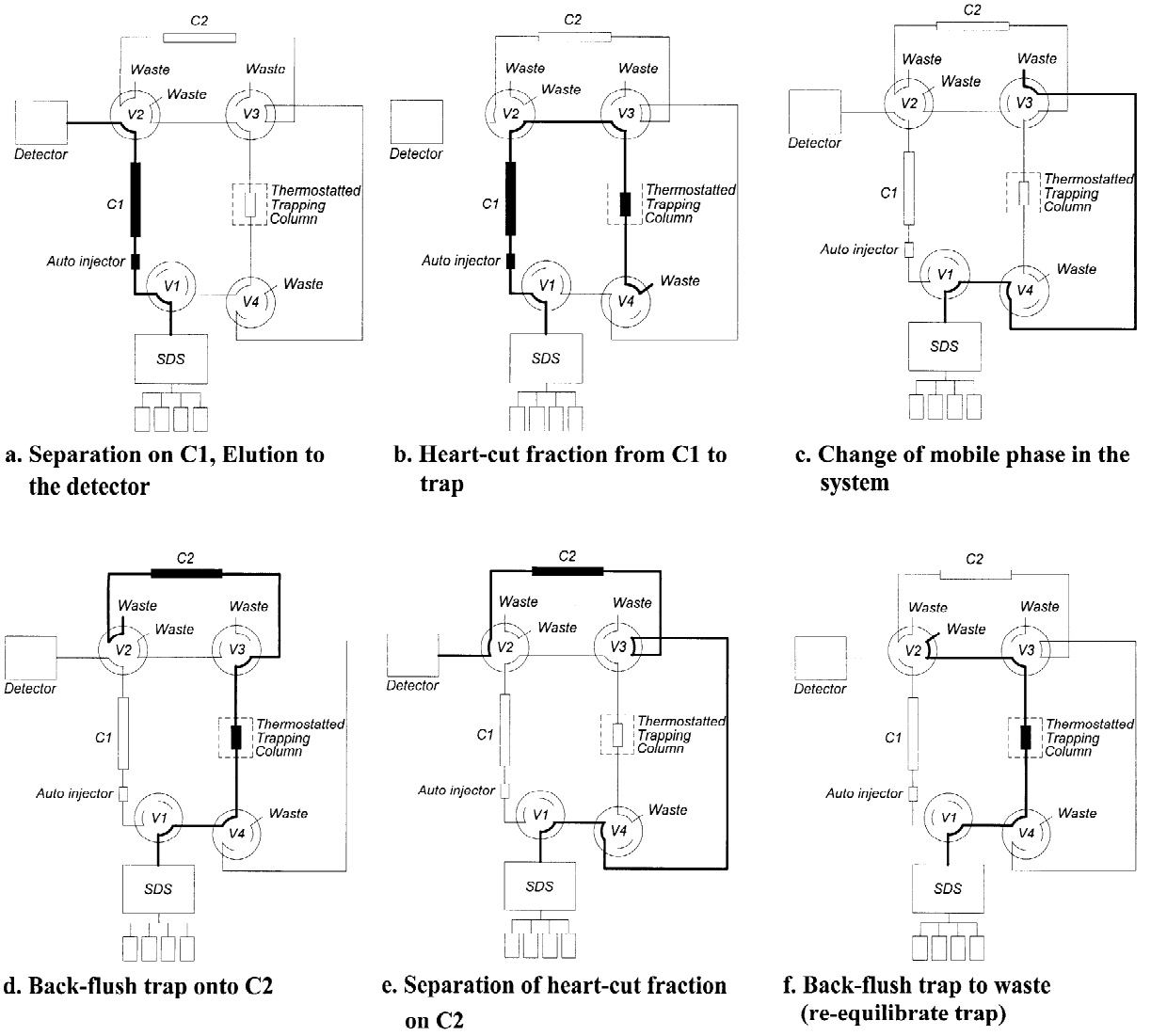

Fig. 1. Schematic diagram of two-dimensional HPLC system with cold temperature trapping. SDS: low pressure quaternary solvent delivery system; V1–V4: 2-position 6-port switching valves.

fractions from the first column. The factor that was identified as being most important for the trap operation was the relative increase in the degree of retention afforded by the stationary phase in the trap in comparison to the stationary phase in the column employed in the first dimension. A second factor, which produced a slight increase in the performance of the trap was the trap temperature, with cold temperatures slowing the rate of migration along the trap and higher temperatures improving the process of solute removal from the trap.

The design of the trapping process necessitates that the trap, at the time of sample loading, contains the same mobile phase as the first column. Therefore, in order to prevent migration along the trap, the retention capability of the trap must be greater than that of the first column. In reversed-phase systems this may simply mean that the alkyl chain length in

the trap must be sufficiently longer than that of the alkyl chain length in the first column, or that the stationary phase carbon loading of the trap be greater than that of the first column. In the present study the trap contained a C_{18} packing material, while the first column contained a C_4 packing material, hence the retention capacity of the trap was greater than that of the first column. Consequently greater retention on the trap would be apparent, especially considering that higher aqueous concentrations would be required to bring about sufficient resolution on the C_4 column and this in turn further increases the retention on the C_{18} trap. Of course not all separations would be feasible on a C_4 column in the first dimension. In such instances when say a C_{18} column is required to gain sufficient resolution in the first separation, the conditions would necessitate that the trap contain a stationary phase of longer (or denser) alkyl chain length, perhaps a C_{32} or a polymeric phase.

To illustrate the operation of the trapping process a polystyrene oligomer comprising of four configurational repeating units was allowed to migrate along the first column and then subsequently was transported to the trap. This process was repeated numerous times before the trap was back flushed and the oligomer $n=4$ was allowed to elute along a second column, which in this instance was a C_{18} column. We should note that this system was employed to illustrate the operation of the trap only and this system in its current state affords no separation value, but illustrates nicely the operation processes as shown in the chromatograms in Fig. 2. Fig. 2a–e illustrates the band profile of the $n=4$ oligomer fraction eluting from the C_{18} column (denoted as C2 in Fig. 1) following two trapping processes in Fig. 2a, four in Fig. 2b, etc., up to 32 trapping processes on Fig. 2e, which involved a corresponding number of individual separations taking place in the first dimension (C1) in each of the separations illustrated in Fig. 2. Note that even following 32 successive trapping processes from the first column to the trap, the resulting band profile on the second column remained uniform. Fig. 2f illustrates an overlay of the band shapes that were eluted on C2 following 32 and 16 trappings compared with the band profile of a single injection of the $n=4$ oligomer on C2 without any transportation to the trap. This figure shows that for the trapping of 16 fractions to the trap and subsequent elution on C2, there was little change in the band profile compared with no trapping processes. This same trend was apparent for trappings of less than 16. However, for the trapping of 32 fractions a slight increase in the band width was observed, although a single uniform band was still maintained. However, when the solvent composition in the trap and C1 was increased to 100% methanol, which increased the rate of migration of solute along the trap, substantial migration along the trap between successive heart-cut trappings was apparent. This resulted in the trapped component eluting from the second column in a series of multiple bands. This is illustrated in Fig. 3.

The effect of solute retention with changes in temperature was then evaluated on the trapping column in order to determine the viability of reduced temperature as a means of further increasing retention on the trap and then increasing temperature to assist solute removal from the trap. Fig. 4 is a plot of retention factor versus changes in temperature for a homologous series of oligostyrenes, where n represents the number of configurational repeating units of the polymer chain. The results in Fig. 4 indicate that the retentive effect of temperature is more significant for more strongly retained solutes, as shown by the greater change in the retention factor as a function of temperature. This was more apparent when the solvent strength was reduced to 90% methanol and 10% water as was employed in the trapping process illustrated in the chromatograms in Fig. 2. In reality, the reduction of the temperature to assist in trapping the solute is only a secondary process compared to that of the difference afforded by the retention capabilities of the trap and the first column, that is, the difference in the alkyl chain length of the first column stationary phase to that of the trap stationary phase. However, the increase in trap temperature at the time of back flushing the trap serves to decrease the peak volume of the trapped components, which would then ultimately lead to a more efficient second dimensional separation. Consequently, the operation of the trap is best carried out using low temperatures for solute loading and high temperatures for solute removal during back flushing.

In the examples described above all trap back flushes were carried out using a 1-ml flush volume. After the separation on the second column was

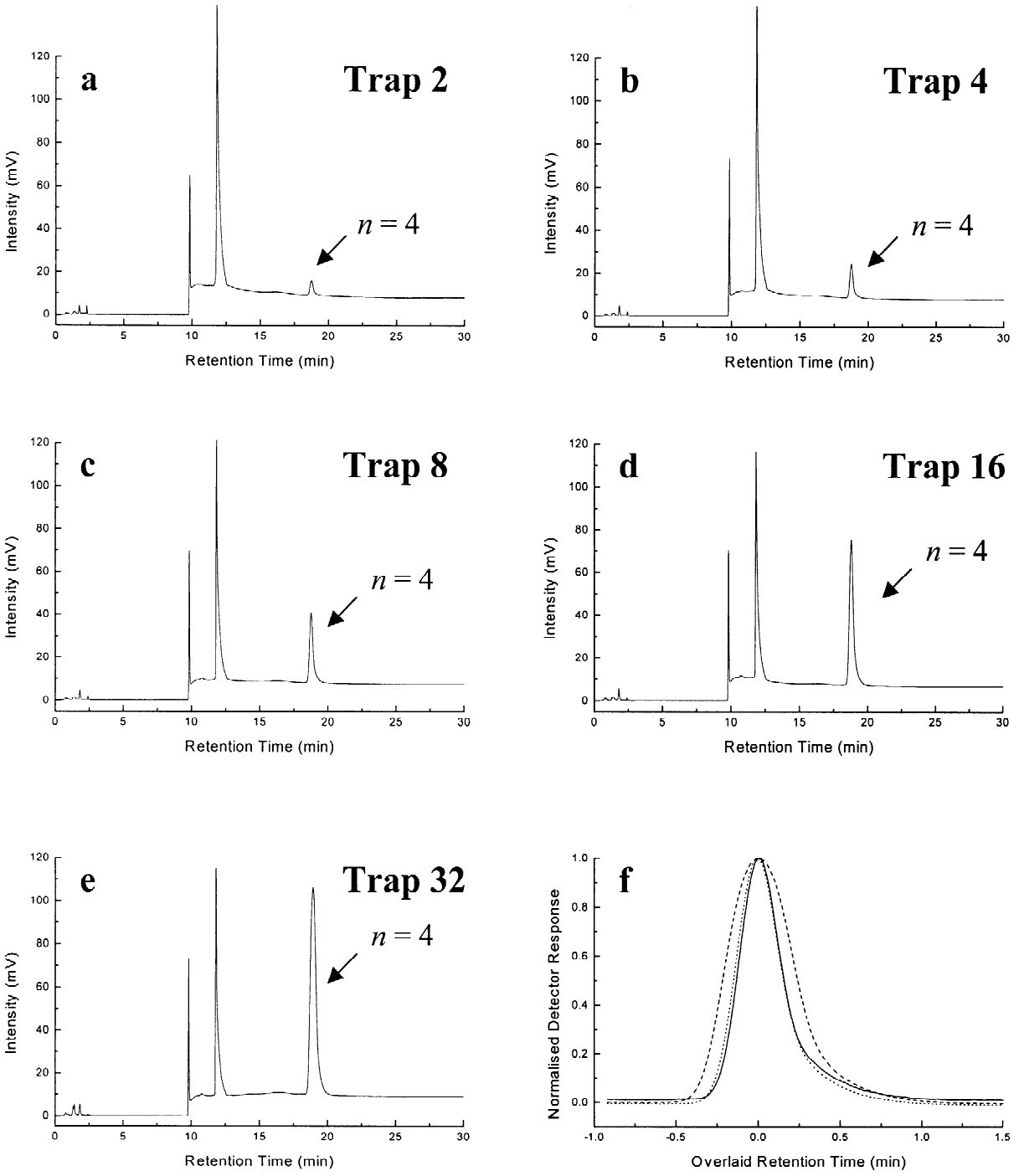

Fig. 2. Chromatograms illustrating the trapping of polystyrene oligomer $n=4$, using the system shown in Fig. 1. C1: C_4 Sephasil Peptide 5-μm particle diameter (100×4.6 mm). Trap: C_{18} Nucleosil 3-μm particle diameter (30×4.6 mm) at 0 °C. C2: C_{18} Activon Valupak 5-μm particle diameter (250×4.6 mm). Mobile phase in C1 and trap methanol:water (90:10), in C2 100% methanol. Trap back-flushed with 100% methanol (1000 μl) at 50 °C. (a) Trap 2; (b) trap 4; (c) trap 8; (d) trap 16; (e) trap 32; (f) overlay of band profiles normalised with respect to retention time following the trapping process. (—) Elution on C2 in 100% methanol with no trappings; (···) trap 16; (----) trap 32.

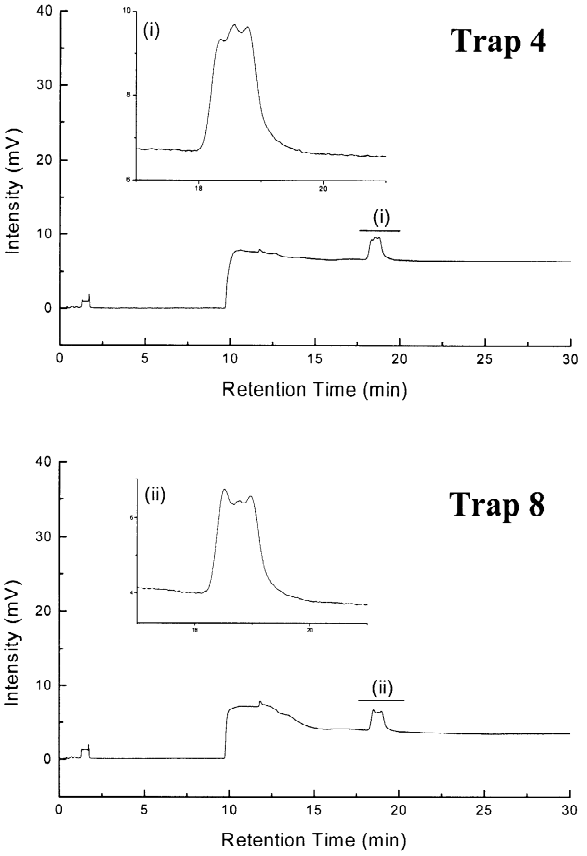

Fig. 3. Chromatograms illustrating the trapping of oligomer $n=4$, using the system shown in Fig. 1. C1: C_4 Sephasil Peptide 5-μm particle diameter (100×4.6 mm). Trap: C_{18} Nucleosil 3-μm particle diameter (30×4.6 mm) at 0 °C. C2: C_{18} Activon Valupak 5-μm particle diameter (250×4.6 mm). Mobile phase in C1, C2 and trap 100% methanol. Trap backflushed with 100% methanol (1000 μl) at 50 °C.

completed, a second 1-ml back-flush of the trap was conducted to ensure that complete removal of analyte from the trap had occurred in the first back-flushing process. Such a large back-flush volume is unimportant in the system described above as the mobile phase in each separation dimension contained a high concentration of methanol. Furthermore, the volume of the column in the second dimension was relatively large (being a 250×4.6-mm column). However, in real two-dimensional systems rarely is the mobile phase in both dimensions the same. Also, in order to maximise the number of heart-cut fractions that can be transported from the first dimensional separation

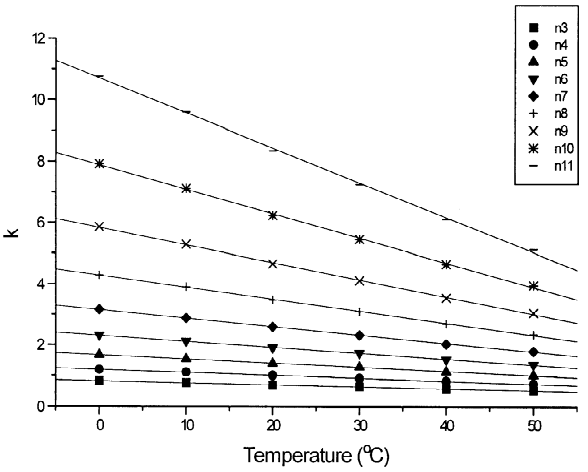

Fig. 4. *k* versus temperature for oligostyrenes with *sec*-butyl end groups on trapping column, where *n* refers to the number of configurational repeating units. Column C_{18} Nucleosil 3-μm particle diameter (30×4.6 mm). Mobile phase 100% methanol.

to the second dimension, the separation that takes place in the second dimension should be very fast. Hence, the columns employed in the second dimension are very often short with a small column volume. Consequently, transportation of a large solvent plug having a different solvent composition to that of the second dimensional mobile phase may have a severe detrimental effect on the separation in the second dimension. Therefore, minimisation of the trap back-flush volume is important. In order to evaluate the minimum volume of solvent that was required to successfully remove all of the components from the trap with a single back-flushing step, various back-flushing elution volumes were evaluated. In each instance eight heart-cut fractions of the $n=4$ oligomer were accumulated on the trap, followed by various back-flushing volumes of methanol. A second back-flush (1 ml) was then performed to ensure complete removal of sample analytes. The results in Fig. 5 show that a 400-μl or less back-flushing volume was an insufficient volume to remove all the analytes from the trapping column, with sample carry-over observed in the second back-flushing eluent. However, complete removal of the sample analyte was observed following a 600-μl back-flushing volume. The back-flush volume was, however, dependent on the number of times sample was loaded on to the trap. After 16 trapping pro-

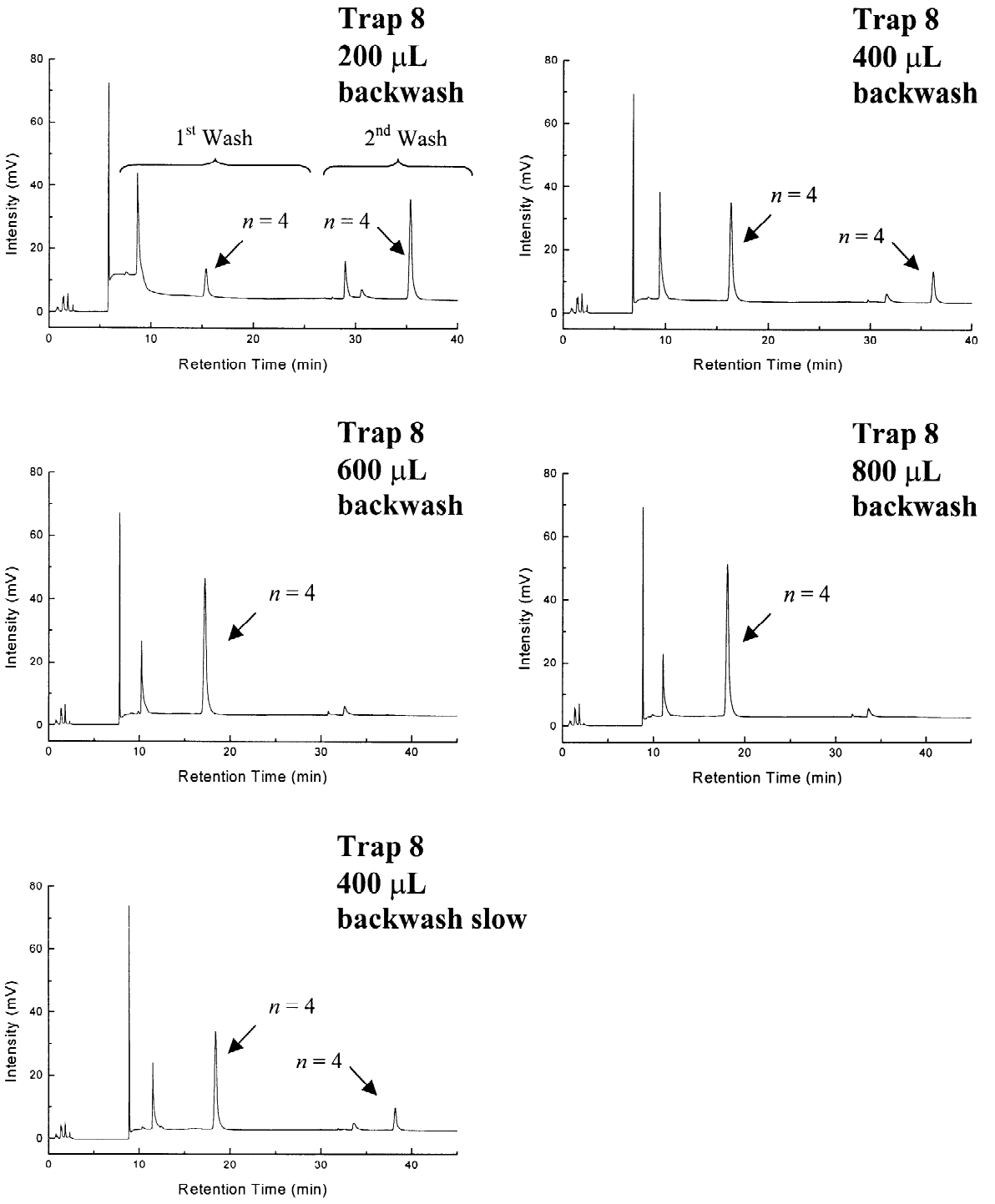

Fig. 5. Chromatograms illustrating the trapping of oligomer $n=4$, using the system shown in Fig. 1 with various back-flushing volumes. C1: C_4 Sephasil Peptide 5-μm particle diameter (100×4.6 mm). Trap: C_{18} Nucleosil 3-μm particle diameter (30×4.6 mm) at 0 °C. C2: C_{18} Activon Valupak 5-μm particle diameter (250×4.6 mm). Mobile phase in C1 and trap methanol:water (90:10), in C2 100% methanol. Trap backflushed with 100% methanol at 50 °C.

cesses, for example, a back-flush volume of greater than 600 μl was required to fully remove the trapped sample from the trap.

The rate of back-flushing was also examined by repeating the 400-μl back-flushing volume at a slower rate, to examine whether time exposure to the increased temperature was important. However, the same result was observed as for the higher back-flushing rate. It is believed that the back-flushing volume required may be further reduced with the employment of a smaller trapping column and a reduced dead volume between switching valve *V4* and the trapping column.

Suitable consideration of the conditions described above has allowed us to carry out a 32-step trapping process without loss of sample analyte. That is, 32 injections of the $n=4$ oligomer were made on the first dimensional column and following each injection, an aliquot of sample was heart cut to the trap. After these 32 heart-cut fractions were completed, the trap was back flushed and the sample eluted as a concentrated single band on the second dimension column. The peak area, width and height of the resulting $n=4$ oligostyrene that subsequently eluted from the second dimension column were measured and the results are recorded in Table 1. Fig. 6 shows a graphical plot of the peak area plotted against the number of trapping events. Perfect linearity was apparent over the entire range studied with a correlation coefficient equal to 0.999. Peak height, however, was observed to increase linearly only up to 16 trapping events, with a slight curvature resulting following 32 trapping events, as illustrated in Fig. 7. Peak width also increased monotonically (but non-linear; Fig. 8) as the number of trapping events increased, which supports the back-flush volume being a function of the number of trapping

Table 1
Comparison of peak area, peak height and peak width for various trapping event numbers

Trap event number	Peak area (arbitrary units)	Peak height (mV)	Peak width at 50% height (min)
2	114 907	6.54	0.26
4	279 123	15.34	0.26
8	614 334	32.08	0.27
16	1 451 097	66.87	0.31
32	2 875 543	95.18	0.46

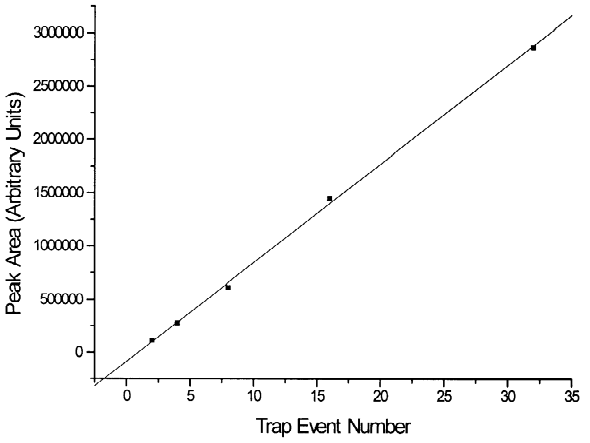

Fig. 6. Plot of peak area versus number of trapping processes for oligomer $n=4$. Conditions as per Fig. 2.

events. Consequently, from the consistent peak area we can conclude that the system described above can repetitively cut fractions from the first column and transport them to the trap, following which back flushing of the trap removes all the heart-cut analyte in a single back-flush process. However, as the number of trapping events increase (particularly 32 trapping events) the system undergoes a slight reduction in system performance as noted by the non-linearity in peak height and the increasing band width at half height. As such, we did not attempt to further demonstrate higher number of trapping

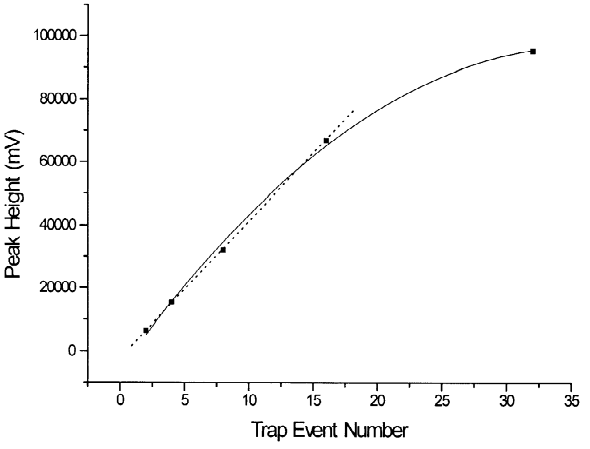

Fig. 7. Plot of peak height versus number of trapping processes for oligomer $n=4$. Conditions as per Fig. 2.

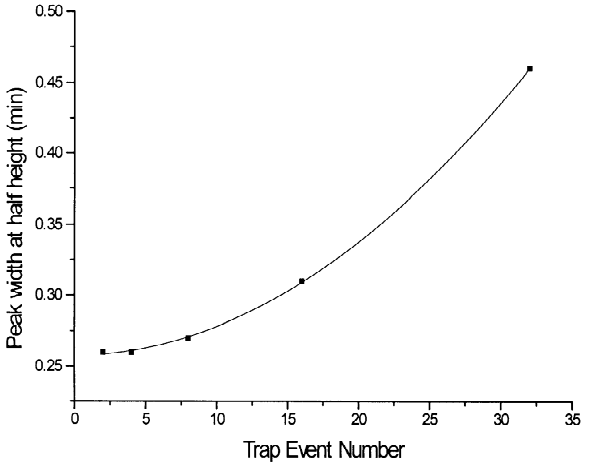

Fig. 8. Plot of peak width at half height versus number of trapping processes for oligomer $n=4$. Conditions as per Fig. 2.

events, particularly since 64 tapping events would be almost a 24-h operation.

In reality, the demands of the system described above would be substantially more rigorous for the two-dimensional analysis of real samples. As such we then employed the system to perform a truly two-dimensional separation incorporating the accumulation of sample in the trap. A polydisperse polystyrene sample was subjected to an oligomeric separation in the first dimension of the system. In this dimension the oligomers resolve on the C_4 column in a 90:10 methanol:water mobile phase. In the second dimension of the separation the stereoisomers of selected oligomers may be resolved on a carbon clad zirconia column using an acetonitrile mobile phase. The details of these stereoisomer separations have been previously reported [22–24] and are beyond the scope of the present study. Suffice to say, however, that using the $n=4$ oligostyrene as an example, we have a total of eight diastereoisomers for a polystyrene with a *sec*-butyl end group. Consequently, the concentration of each diastereoisomer is one eighth (assuming that each isomer is present in equal proportions) of the total $n=4$ oligomer peak area that elutes in the first dimension of the separation. Hence a substantial reduction in peak height and peak area is observed for each diastereoisomer eluting in the second dimension of the separation. Sample accumulation on the trap increases the concentration of the sample in the second dimension, and hence the peak detection improves.

The chromatograms in Fig. 9 illustrate the results of this trapping process. After 16 trapping processes no reduction in resolution was apparent compared to an isomeric separation of a purified $n=4$ oligomer, which was injected directly onto the carbon clad zirconia column [22]. However, a reduction in the diastereoisomer resolution was apparent following 32 trapping processes, but this was a result of overloading the carbon clad zirconia column rather than as a consequence of the trapping process. To verify this fact we performed 32 trapping processes on a dilute $n=4$ oligomer, with subsequent diastereoisomer separation on the carbon clad zirconia column with the result being a restoration of the diastereoisomer separation as shown in Fig. 9e.

One of the major advantages of the system described above may be found in hyphenated techniques, such as LC–NMR, in this case 2DLC–NMR. The online enrichment of low concentrated sample components will no doubt increase the sensitivity of the analytical method by increasing the signal-to-noise ratio of trace components, which in turn will lead to a reduction in the analysis time as the stop flow process in analysis will be reduced. Furthermore, incorporation of the automated 2D aspect into the analysis eliminates manual sample manipulations. Other uses for the system may be found in the reduction of solvent effects or for the accumulation of sample components that may be unstable outside of the chromatographic environment. Sample concentration may be increased and unwanted solvent from a first separation dimension removed in a second dimension containing a more favourable solvent environment—perhaps the removal of non-volatile solvents or the removal of organic solvents prior to application of on-line bioassays.

4. Conclusion

A two-dimensional HPLC system was developed where heart-cut analytes from a first column could be stored and accumulated on a trapping column prior to elution on a second separation column. An important factor in the operation of the trapping

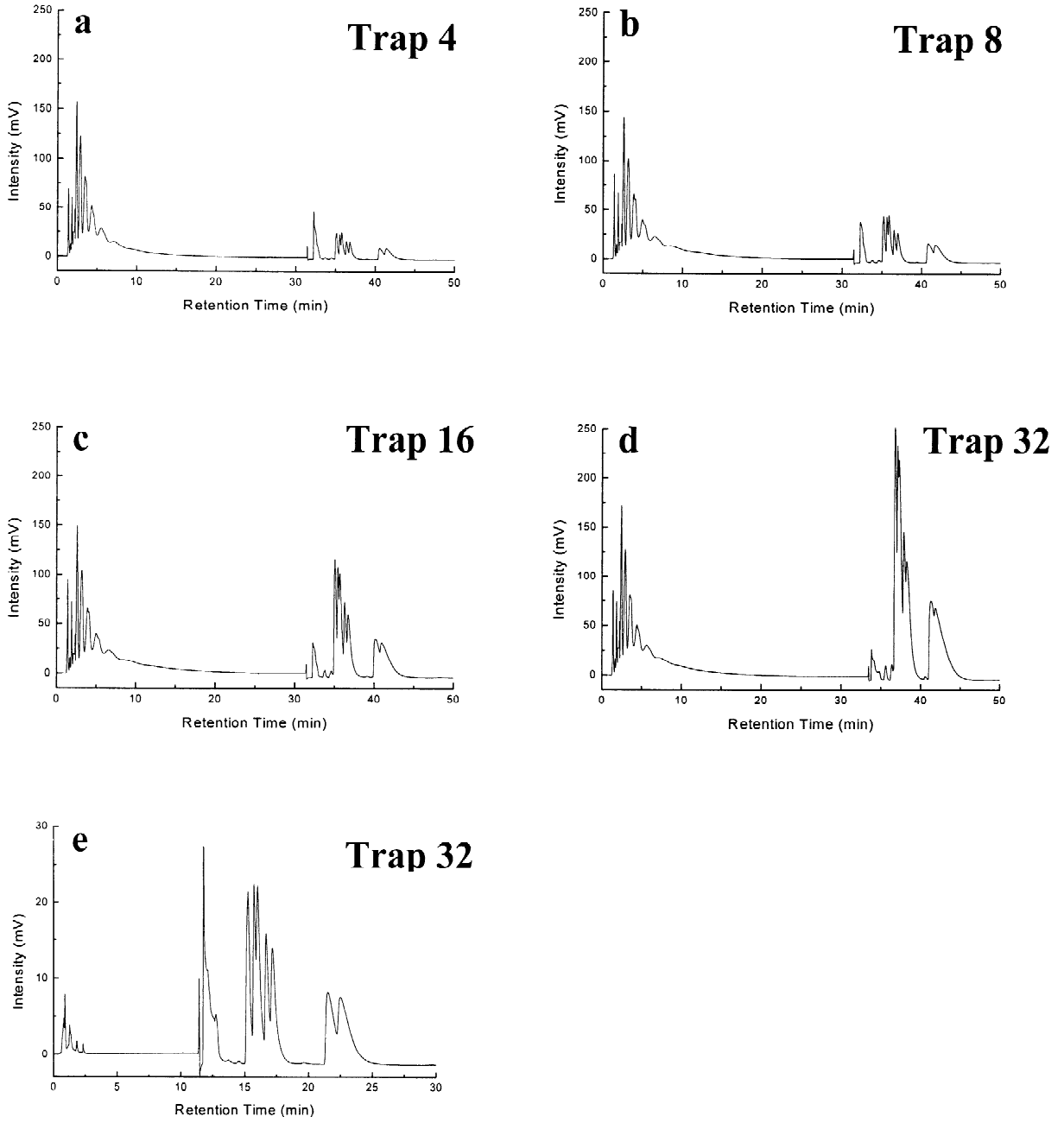

Fig. 9. Chromatograms of polystyrene oligomer ($n=4$) separation from a polystyrene sample (770 Da), with sample accumulation using trapping column. C1: C_4 Sephasil Peptide 5-μm particle diameter (100×4.6 mm). Trap: C_{18} Nucleosil 3-μm particle diameter (30×4.6 mm) at 0 °C. C2: carbon clad zirconia (ZirChrom-CARB) 3-μm particle diameter (100×4.6 mm). Mobile phase in C1 and trap methanol:water (90:10), in C2 100% acetonitrile. Trap backflushed with 100% methanol (600 and 1000 μl for trap 32) at 50 °C.

column was the relative retention between the first column and the trapping column. Variation in the solvent strength in conjunction with cold temperature trapping was employed as a means of retaining and removing analytes from the trapping column. Using low molecular mass polystyrenes as analytes in which to examine the operation of the system it was found that the system employed was able to accumulate and elute up to 32 heart-cut fractions without a substantial reduction in the resolution of the second dimensional separation.

Acknowledgements

The author A.P.S. would like to acknowledge the receipt of an Australian Postgraduate Award (APA).

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