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# Separation of oligostyrene isomers in a complex mixture using two-dimensional heart-cutting reversed-phased liquid chromatography

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**Abstract**

The development of a two-dimensional liquid chromatographic system requires a process of assessment that can yield an optimum performing system with minimal experimental evaluation. Information Theory and a geometric approach to Factor Analysis are two techniques that when used in combination, provide important information on the expected two-dimensional performance. In the present study, we compare the predicted separation performance of two-dimensional systems that have been subjected to analysis by Information Theory and Factor Analysis to that of actual chromatographic separation performance. Our test separation comprised a mixture of 32 oligostyrene structural isomers and stereoisomers. The optimal combination as determined by Information Theory and Factor Analysis consisted of a C18 column with a methanol mobile phase in the first dimension and a carbon clad zirconia column with an acetonitrile mobile phase in the second dimension. This system was also shown to be the most successful practical system when a heart-cutting approach was employed. The practical results were in total agreement with the results from Information Theory and Factor Analysis. The number of isomers resolved using this system was 27. A second system, namely one comprising of a C18 column and methanol mobile phase in the first dimension and a carbon clad zirconia column with a methanol mobile phase in the second dimension was also predicted to be a system with high separation potential. However, practical assessment of this system did not realise the theoretical predictions, largely due to the long separation times required in the second dimension. Furthermore, all combinations that employed a C18 column with an acetonitrile mobile phase in the first dimension failed to realise the theoretical separation potential due to high solute crowding, low orthogonality and a disordered arrangement of bands along the first separation axis. This was also predicted by the theoretical assessment.


*Keywords:* Information Theory; Factor Analysis; Heart-cutting technique; Liquid chromatography, two-dimensional; Oligostyrene isomers

## 1. Introduction

Two-dimensional high-performance liquid chromatography (2D HPLC) of complex mixtures in heart-cutting [1] and comprehensive modes [2] has become a common tool in the separation scientist's arsenal. Advances in technologies [3–7] and a better understanding of the requirements of two-dimensional systems [7] have been paramount in the development of the technique and will aid in making 2D HPLC a mainstream separation tool.

The incorporation of switching valves and mobile phase flow diversion techniques in HPLC has provided fresh ways to overcome difficult separation problems. The chromatographer can in many instances tailor a two-dimensional chromatographic system to tackle specific separation tasks. For example, the two-dimensional system configuration [8,9] that was utilised by Opiteck et al. [8] has been employed in a number of studies, primarily in the analysis of proteins [2] and peptides from protein digests [8]. In this system [8] the size exclusion column arrangement in the first dimension, C1, was connected to two 'identical' non-porous reversed phased C18 columns (run in parallel), denoted as C2A and C2B, in the second dimension using two four port switching valves. The eluent from the first separation dimension was diverted alternately to each of the C18 columns in the second dimension allowing successive band cuts to be loaded

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onto these columns. While the separation was underway on C2A, a second heart cut fraction would be loaded onto C2B. Once the separation on C2A was complete, eluent would be diverted to C2B, while at the same time a new cut would be diverted from C1 to C2A. This process would continue allowing the two-dimensional analysis of all sample constituents, hence the system functioned in a comprehensive manner. The main disadvantage of the technique was that multiple C18 columns were employed in parallel as the second dimension. Sweeney and Wyllie [10], Sweeney [11] and Sweeney and Shalliker [12] and Gray et al. [13] also have developed a number of two-dimensional systems for use in reversed phase-reversed phase HPLC. These systems incorporated either a sample loop for storage of cut sample fractions prior to transfer to the second dimension, or a sample trapping column for preconcentration [12] of cut sample components prior to transfer to the second dimension. In the first instance separations could be conducted either in a heart-cutting mode [10,11] or a comprehensive mode [13]. In the latter instance [12], the system was potentially useful for preparative scale separations and as a hyphenation technique coupled to NMR.

In heart-cutting chromatography, only a section of the first separation dimension is transported to the second dimension [1,7]. An advantage of this technique is that only the components of interest need be analysed, speeding the overall separation. Heart-cutting is also essential in method development for comprehensive two-dimensional systems because the elution of the components in the second dimension can be assessed in a simplified system without the complication of peak overlap or wrap-around effects [14]. Furthermore, in some instances, such as those where peak wrap around effects cannot be avoided, a heart-cutting approach is the only process that can be applied to a two-dimensional separation. Also, when an isolation process is required to obtain sample 'in-hand' a heart-cutting approach may prove to be the most appropriate process, especially in separation systems that display a high degree of chaotic band displacement. Under such circumstances, solute crowding and disordered solute displacement may make it difficult to firstly identify the component of interest and secondly separate the species in a high degree of purity and recovery. Application of a comprehensive process under such circumstances may prove to be too complicated.

The most useful 2D HPLC system is one in which there is a high degree of orthogonality [15,16] between each separation dimension. This is achieved when the stationary phase and mobile phase contributions of each separation dimension provide different selectivities. If the retention behaviours of both separation dimensions are equivalent, little if any gain in separation will be seen in a two-dimensional system over that of a single-step separation [17]. However, the more different the dimensions' retention behaviours are, higher separation potentials [17] and greater peak capacities of the system will result. The most obvious way to increase orthogonality is to utilise stationary phases that are considerably different. In the past we have shown that the C18 stationary phase in combination with carbon clad zirconia (CCZ) provided for orthogonal separation dimensions [18,19]. Orthogonality may also be achieved by varying the mobile phase or temperature in each separation dimension.

In a previous communication [18] we used Information Theory [16] and Factor Analysis [17] to theoretically evaluate the orthogonality and separation quality of a 2D HPLC system consisting of a C18 column and a CCZ column in the first and second dimensions, respectively. The number of mobile phases that provided the best separation of oligostyrenes and the isomers contained within this sample was limited to two; methanol and acetonitrile [20], each of which in combination with either C18 or CCZ stationary phases yielded varying degrees of orthogonality and separation potential [18]. In general, each of the systems could theoretically separate between 26 and 28 of the 32 isomers in the sample mixture. Although the reported calculations provided evidence for orthogonality within reversed phased liquid chromatography, they failed to take into account experimental factors such as band broadening, and the physical limitations in the operation of switching valves and dead volumes of chromatographic systems. If the physical limitations of a coupled system limit the separation power, then the theoretical peak capacity and separation quality will deviate from that predicted by theory. This was certainly the case in the verification of the separation qualities using a comprehensive system designed for the separation of these isomers [14]. Of the four systems studied, only one was successful (C18 methanol/CCZ acetonitrile), despite predictions to the contrary [18]. This system enabled 27 of the 32 isomers to be separated [14]. The success of this system was attributed to the structural isomer selectivity and the limited stereoisomer selectivity on the C18 column, complemented by a high degree of stereoisomer selectivity on the CCZ surface [18]. The three remaining chromatographic combinations did not resolve the projected number of isomers. However, interpretation of the results of Information Theory and Factor Analysis and also band order in the first separation dimension easily accounted for these deviations [14].

In this communication, we compare the results of theoretical predictions, previously determined, against the results of practical two-dimensional heart-cutting HPLC separations. The 2D HPLC separations in the present study were conducted using a heart-cutting technique employing four combinations of two-dimensional chromatographic systems. The heart-cutting technique allows transportation of discrete sections of sample from the first dimension to the second dimension [1,7]. The sample was a 32 component $n = 5$ oligostyrene isomeric mixture, which was composed of a group of oligostyrenes, containing *n*-, *sec*-, and *tert*-butyl endgroups. This represents a variation in structural isomerism within the sample base. Within each of these structural isomer groups is a variation in the stereochemistry. In total there are eight stereoisomers within the *n*- and *tert*-butyl groups and 16 *sec*-butyl stereoisomers due to a

site of stereochemistry on the *sec*-butyl end group [21]. The results of practical experimentation were compared to the results of theoretical predictions based on Information Theory and Factor Analysis by comparing the number of components physically separated to the number of components theoretically separated.

## 2. Experimental

### 2.1. Chemicals

HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Australia. Polystyrene standards with molecular weights of 580 Da (*n*-butyl) and 760 Da (*sec*-butyl) were purchased from Polymer Laboratories and Aldrich Chemical Company, respectively. *tert*-Butyl polystyrene (molecular weight ∼ 580 Da) was synthesised using anionic polymerisation of styrene initiated with *tert*-butyl lithium. The molecular weights of the members of the oligomer series were determined using mass spectroscopy [22]. The $n = 5$ oligomer from each of these polystyrene standards was isolated by fractionation using methods previously described [20]. An Activon (manufacturer no longer trading) C18 (250 mm × 4.6 mm, 5 μm particle diameter) column was used in the first separation dimension. Carbon clad zirconia (3 μm particle diameter), which was used as the stationary phase in the second dimension was purchased from ZirChrom Separations, Inc., (Anoka, MN, USA) and packed into columns (30 mm × 4.6 mm) using methods previously described [21]. The stationary phase material was used as supplied from the manufacturer.

### 2.2. Equipment

All chromatographic experiments were conducted using a Shimadzu LC system (Shimadzu Scientific Instruments, Rydalmere, NSW, Australia) incorporating a LC-10AT*VP* pumping system, SIL-10ADVP auto injector, SPD-10AVP UV detector, SCL-10AVP system controller and Shimadzu Class-VP version 5.03 software on a Pentium II 266 MHz PC. Column switching was achieved using six-port two-position switching valves fitted with micro-electric two position valve actuators (Valco Instruments Co., Inc., Houston, TX, USA). Valve switching was controlled using Shimadzu SCL-10AVP system controller and Shimadzu Class-VP version 5.03 software. Two additional UV-Vis detectors (Waters 286, Waters Associates, Milford, MA) were employed to record chromatographic information in the first and second dimensions. A HP 1050 pump (Agilent Technologies, Palo Alto, CA) was used to control flow in the second dimension. 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 either 2, 5 or 10 Hz (Lawson Labs Inc., Malvern, PA, USA). Columns were packed using a Haskel air driven fluid pump (Haskel International, Burbank, CA, USA).

### 2.3. Chromatographic separations

Oligostyrene standards ($n = 5$) were dissolved in methanol. All separations were conducted using mobile phases as described in the text. Mobile phases were sparged continuously with helium and/or degassed for 10–15 min under vacuum with sonication. Flow rates are noted in the appropriate sections of the text, but in general the first dimension flow rate was 1.0 ml/min while in the second dimension the flow rate was 2.0 ml/min. Injection volumes were either 5, 10 or 20 μl as noted in the appropriate text. UV detection was at 262 nm.

### 2.4. Results and discussion

A simple 2D HPLC system was constructed that consisted of two electronically operated six-port two-position switching valves, sample isolation loop and two independently operated chromatographic columns. The first dimension consisted of a C18 column, C1, while the second dimension consisted of a carbon clad zirconia column, C2. Operation of this system is described in Fig. 1a–c. This system was also used in another study that detailed the separation of this isomeric mixture in a comprehensive mode of operation [14]. In Fig. 1a, the eluent from C1 was sent directly to waste. In Fig. 1b, the system switches allowing the eluent from C1 to be directed to the sample loop. Switching the system to position 3 (Fig. 1c) allows the contents of the sample loop to be injected into C2 and hence complete the two-dimensional separation. In a previous communication, we evaluated four combinations of chromatographic systems using Factor Analysis and Information Theory [18]. In this communication, we evaluate these systems experimentally. The systems were:

System 1: C18 (methanol)/CCZ (acetonitrile).
System 2: C18 (methanol)/CCZ (methanol).
System 3: C18 (acetonitrile)/CCZ (acetonitrile).
System 4: C18 (acetonitrile)/CCZ (methanol).

A brief summary of the results of the orthogonality and separation quality analysis are shown in Table 1. A full discussion of these results has been discussed previously [18]. Using Information Theory [16], the informational similarity can be determined, which is a measure of the degree of solute crowding on a normalised retention plane. Values range between zero and one (one represents total crowding on the normalised retention plane, zero indicates no solute crowding) [16]. Using Factor Analysis [17], the correlation between any two separation dimensions can be assessed using retention correlation coefficients. Values range between zero and one with a correlation coefficient of one representing high correlation between the two separation dimensions. A correlation coefficient of zero indicates orthogonal

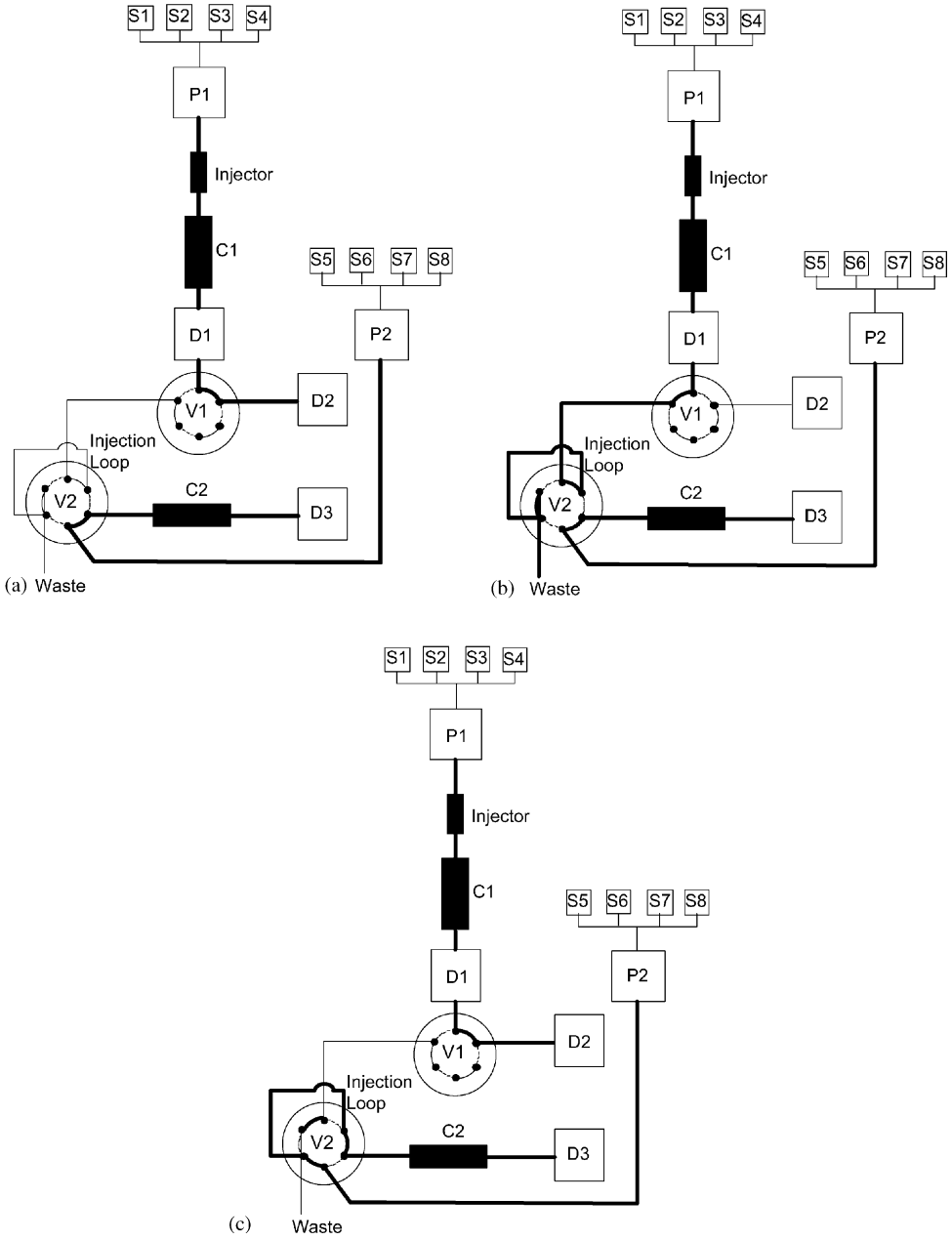


Fig. 1. Schematic diagram of the 2D HPLC column switching system. P1–P2: Low pressure quaternary solvent delivery systems; V1–V2: six-port two-position switching valves; C1: column in first separation dimension; C2: column in second separation dimension. (a) System configuration for elution of C1 mobile phase to waste and sample loop isolated. (b) System configuration for the elution of a band from C1 onto sample loop. (c) System configuration for loading the contents of sample loop onto C2.

Table 1
System attributes used to determine the measure of 2D orthogonality for each of the 2D RP–RP systems evaluated [17]

| Attribute | Two-dimensional chromatographic combinations | | | |
|---|---|---|---|---|
| | C18M/CCZA System 1 | C18M/CCZM System 2 | C18A/CCZA System 3 | C18A/CCZM System 4 |
| Informational similarity | 0.56 | 0.62 | 0.92 | 0.93 |
| Peak spreading angle | 75 | 70 | 42 | 37 |
| Practical peak capacity ($N_p$) | 54 | 57 | 156 | 160 |
| Correlation ($c$) | 0.26 | 0.34 | 0.75 | 0.79 |
| Usage (%) | 90 | 87 | 56 | 52 |
| Resolved components (/32) | 26 | 28 | 26 | 26 |

separation dimensions. Factor Analysis also allows the determination of the practical two-dimensional peak capacity and the percentage usage of the theoretical two-dimensional peak capacity [17].

In our previous communication [14] that focused on the experimental separation of a 32 component oligostyrene isomer mixture using a comprehensive mode of analysis, the system that gave excellent agreement with theoretical predictions was a C18 (methanol)/CCZ (acetonitrile) system. These separation dimensions were coupled according to the configuration shown in Fig. 1. Even though the number of separated components equalled that predicted by theory this was only achieved using a "wrap around" technique. Two important factors that enabled the successful isolation of 27 of the 32 components were: (1) a vacancy in the chromatogram in the second dimension that allowed the wrap around effect to be successful, without significant detrimental effects; and (2) the separation in the first dimension displayed a high degree of order. These factors allowed the separate transportation of the structural isomer components (with minimal overlap of the *tert*- and *sec*-butyl isomers) which minimised coelution problems [14]. With this mode of operation [14] systems 2 and 4 (in the present text) were not practical since the analysis times in the second dimension (systems 2 and 4, in the present text, employed methanol in the second dimension) were too long, not allowing a wrap around effect to be implemented. The experimental results that were obtained using system 3 in the present text (C18 (acetonitrile)/CCZ (acetonitrile)) deviated significantly from the theoretical predictions that were based on Factor Analysis and Information Theory. The number of resolved components was approximately 12 as opposed to the 26 predicted. The deviations were attributed to a high degree of solute crowding, which made the experimental realisation almost impossible. Partial resolution of stereoisomers in the first separation dimension (C18) resulted in a high degree of overlap between the structural isomer components and reduced resolution of components on the CCZ column.

Separation of the $n = 5$ oligostyrene isomer mixture on a C18 column with a methanol mobile phase is shown in Fig. 2a. Separation was dependent on the end group (retention increasing in the order *tert*-, *sec*-, and *n*-butyl) and all stereoisomers coeluted according to their respective end groups. In comparison, some stereoisomer selectivity was apparent when the mobile phase was changed to acetonitrile (Fig. 2b). This resulted in an increase in band heterogeneity as there was an increase in the overlap of the structural isomer retention windows. The separation process of these oligostyrenes on carbon clad zirconia stationary phases is dominated by stereochemistry, more so than the by structural isomeric aspects. The chromatograms shown in Fig. 2c and d illustrate the stereoisomer separations for the $n = 5$ *tert*-butyl oligomer on carbon clad zirconia. In Fig. 2c, a methanol mobile phase was employed, while in Fig. 2d, an acetonitrile mobile phase was employed. Clearly the separation on the carbon clad zirconia column in combination with an acetonitrile mobile phase was superior in terms of both the separation time and the degree of band broadening, although the chromatographic profiles were quite similar. With this in mind the two-dimensional separations of this sample can be divided into two distinct groups. These groups depend on the separation observed in the first dimension and are determined by the extent of the stereoisomer resolution.

The system that employed a C18 column with a methanol mobile phase (first dimension) and a carbon clad zirconia column with an acetonitrile mobile phase (second dimension) was examined first. This is because the heart-cutting efficiency of a system where no significant stereoisomer selectivity on occurs on C1 may be examined. In this system, three heart-cut sections in the first dimension were deemed appropriate, each cut corresponded to one of the three different end groups that made up the sample mixture. The regions corresponding to the *tert*-, *sec*- and *n*-butyl end groups are labelled accordingly as 1–3, respectively, in Fig. 2a. The heart-cut of region 1 from the C18 column is shown in Fig. 3a. The resulting diastereoisomer separation observed in the second dimension of this heart-cut is shown in Fig. 3b. Seven *tert*-butyl stereoisomers were resolved, however, the partial overlap between the *sec*- and *tert*-butyl oligostyrenes in the first separation dimension resulted in the appearance of bands that contained oligostyrene diastereoisomers with *sec*-butyl end groups. These components are labelled with an asterisk. Heart-cutting of the second section 2 (Fig. 3c) resulted in the separation of 12 *sec*-butyl stereoisomers (Fig. 3d). Similarly, there was evidence of *tert*-butyl isomers in this separation. All eight *n*-butyl stereoisomers were resolved following the heart-cutting of section 3 (Fig. 3e) to the carbon clad zirconia column, and because the *n*-butyl isomers in the first dimension were fully resolved from the *sec*-butyl isomers in the first dimension, there was no contamination in this separation (Fig. 3f). In total, 27 of the 32 isomers were resolved, which is one more than that predicted by theory [18]. The difference can be attributed to different batches of carbon clad zirconia stationary phases [23] used in the current experiments compared to the previous experiment from which our theoretical predictions were based [18]. These results are in good agreement with the results of the theoretical predictions of Information Theory and Factor Analysis.

Changing the mobile phase from acetonitrile to methanol in the second dimension had little effect on the overall number of components that could be resolved. However, the overall separation time was three–four times longer when methanol was the mobile phase for the second dimension. Band broadening and band resolution (in general) were also worse for system 2 than for system 1. There was, however eight bands apparent for the *tert*-butyl stereoisomers instead of seven, but the sample carry over due to the overlap of the *tert*- and *sec*-butyl end groups was of course still apparent (Fig. 4a and b). Heart-cutting and transport of

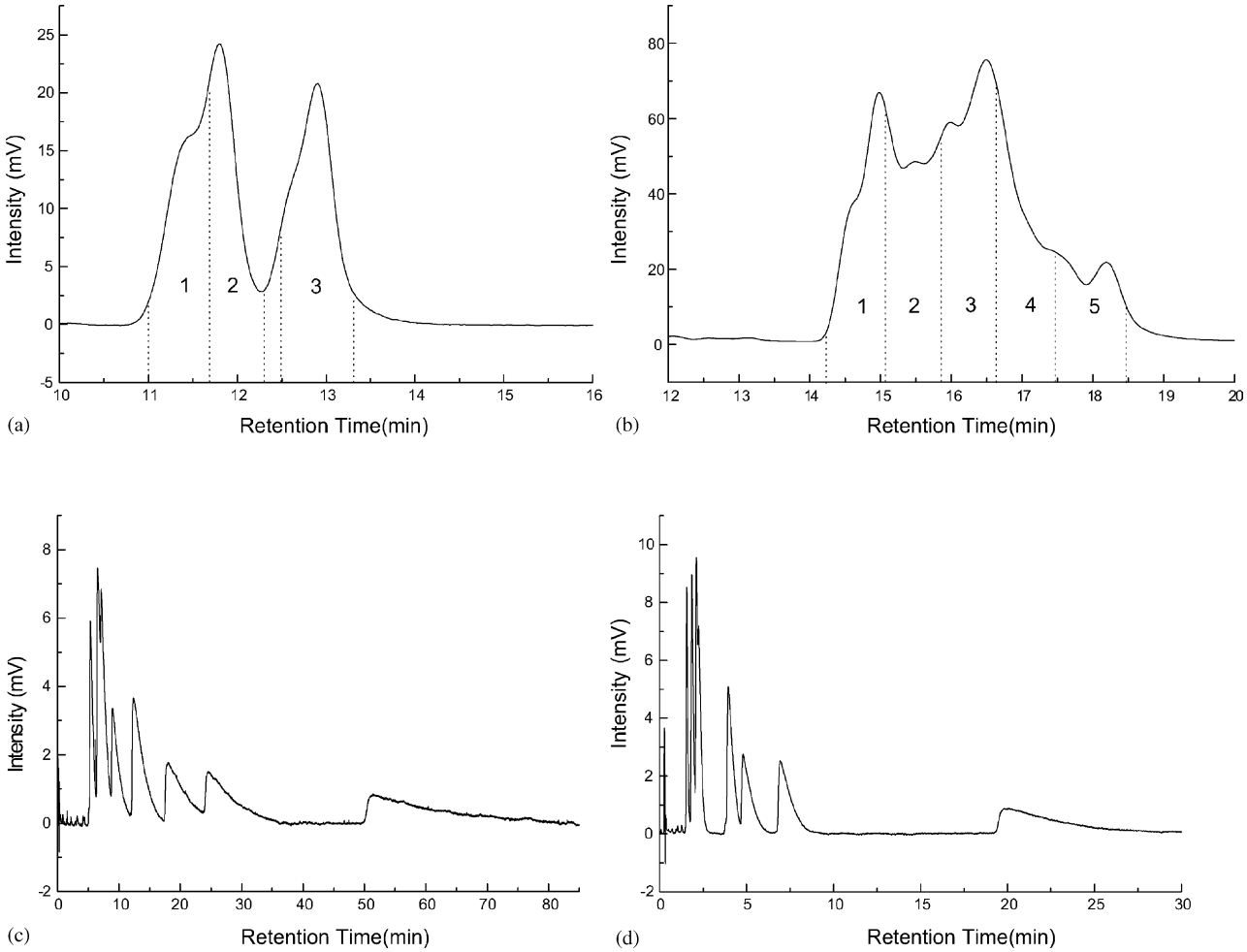


Fig. 2. Chromatograms of a $n = 5$ butyl oligostyrene samples: (a) *n*-, *sec*-, *tert*-butyl oligostyrene sample on a C18 column (250 mm × 4.6 mm). Mobile phase 100% methanol (20 μl injection). Peaks: (1) *tert*-butyl; (2) *sec*-butyl; and (3) *n*-butyl. (b) *n*-, *sec*-, *tert*-butyl oligostyrene sample on a C18 column (250 mm × 4.6 mm). Mobile phase 100% acetonitrile (20 μl injection). Sections labelled 1–5 correspond to the cut fractions referred to in the text. (c) *tert*-Butyl oligostyrene sample on a CCZ column (30 mm × 4.6 mm). Mobile phase 100% methanol (20 μl injection). (d) *tert*-Butyl oligostyrene sample on a CCZ column (30 mm × 4.6 mm). Mobile phase 100% acetonitrile (5 μl injection).

sections 2 and 3 to the second dimension (chromatograms not shown) resulted in quite poor separations, primarily due to the long analysis time, which caused the peaks to blend into the baseline making retention time and area determinations difficult. The total separation time for section 2 on the C18 (methanol)/CCZ (methanol) system was 130 min while section 3 was 169 min. This compared unfavourably with the results of system 1, the analysis times for which were: section 1: 36 min; section 2: 44 min; and section 3: 62 min. (The total sum of these three times was approximately that for section 2 using the second system.) So, although the number of isomers resolved (27/32 isomers) and the practical two-dimensional peak capacity (57 peaks) agreed with theoretical predictions clearly the separation time, peak intensity and band broadening associated with this separation system were not favourable. Overall, even though theory predicted that the C18 (methanol)/CCZ (acetonitrile) and C18 (methanol)/CCZ (methanol) systems were very similar or essentially interchangeable, the system employing an acetonitrile mobile phase in the second dimension was experimentally far superior due to the shorter analysis time in the second dimension.

The stereoisomer selectivity that occurred on the C18 (acetonitrile) system (Fig. 2b) resulted in significant overlap of structural isomer groups and consequently in a broader separation profile. Heart cutting across this sample distribution required five cuts instead of three and is shown in Fig. 2b. The chromatograms in Fig. 5a–e illustrate the separations achieved using the C18 (acetonitrile)/CCZ (acetonitrile) system. The chromatogram following transport of section 1 to the CCZ (acetonitrile) dimension is shown in Fig. 5a, section 2 in Fig. 5b, and so forth. The total number of bands resolved or even partially resolved did not exceed 22 of the 32 oligostyrene isomers. This is an increase in the number separated in a previous study that employed a comprehensive separation mode [14]. This smaller number for the comprehensive separation was due to the continuous flow requirements necessary in the comprehensive mode of

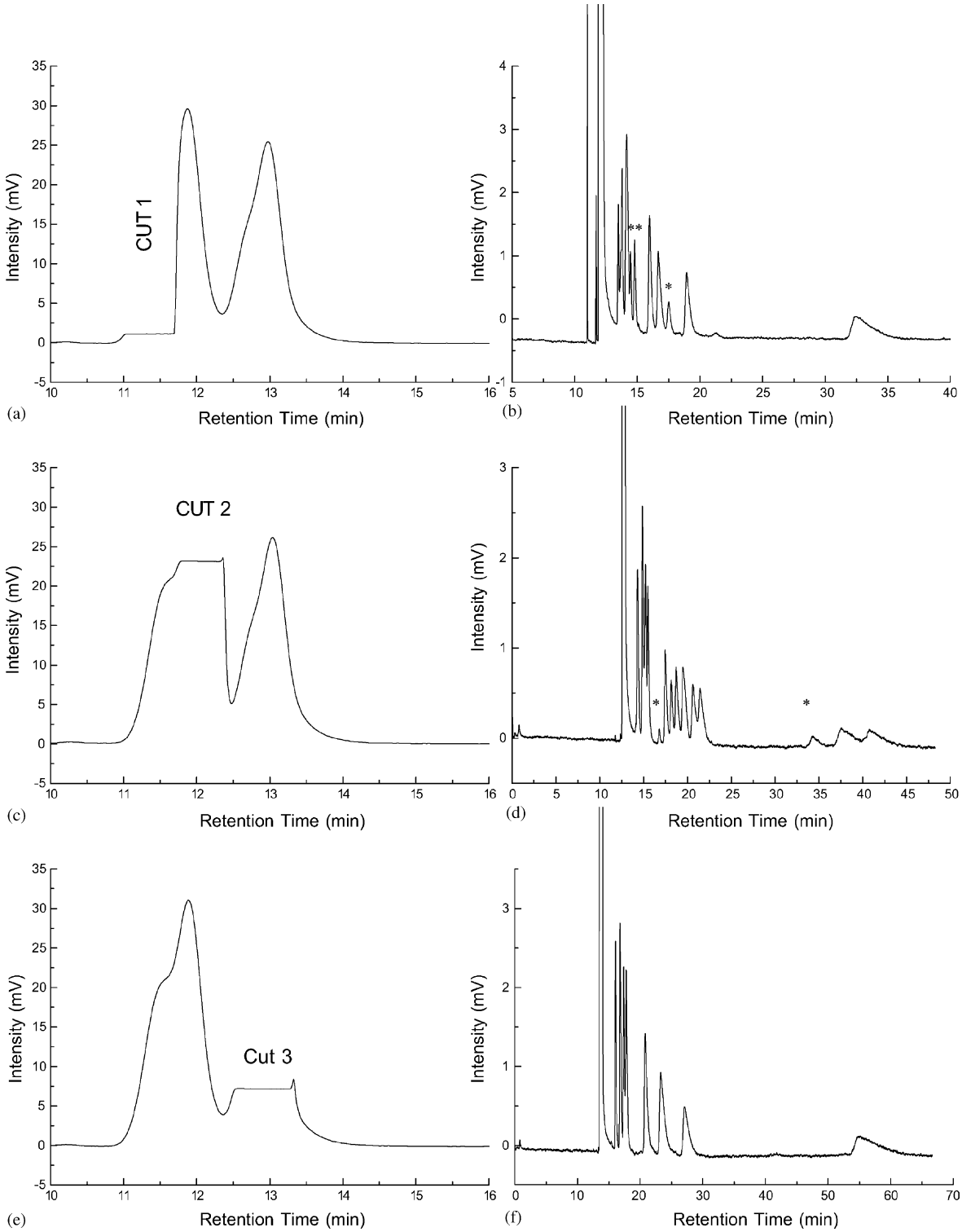


Fig. 3. Chromatograms for the heart-cutting separations of an *n*-, *sec*- and *tert*-butyl $n = 5$ oligostyrene mixture. (a) *n*-, *sec*- and *tert*-butyl oligostyrene sample on a C18 column (250 mm × 4.6 mm). Mobile phase 100% methanol (20 μl injection). Cut time = 11.00–11.70 min. Flow rate = 1.0 ml/min. (b) Transport of section 1 in (a) and injection onto a CCZ column (30 mm × 4.6 mm). Mobile phase 100% acetonitrile, flow rate = 2.0 ml/min. (c) *n*-, *sec*-, *tert*-butyl oligostyrene sample on a C18 column (250 mm × 4.6 mm). Cut time = 11.70–12.30 min; conditions as in (a). (d) Transport of section 2 in (c) and injection onto a CCZ column (30 mm × 4.6 mm); conditions as in (b). (e) *n*-, *sec*-, *tert*-butyl oligostyrene sample on a C18 column (250 mm × 4.6 mm). Cut time = 12.50–13.30 min; conditions as in (a). (f) Transport of section 3 in (e) and injection onto a CCZ column (30 mm × 4.6 mm); conditions as in (b).

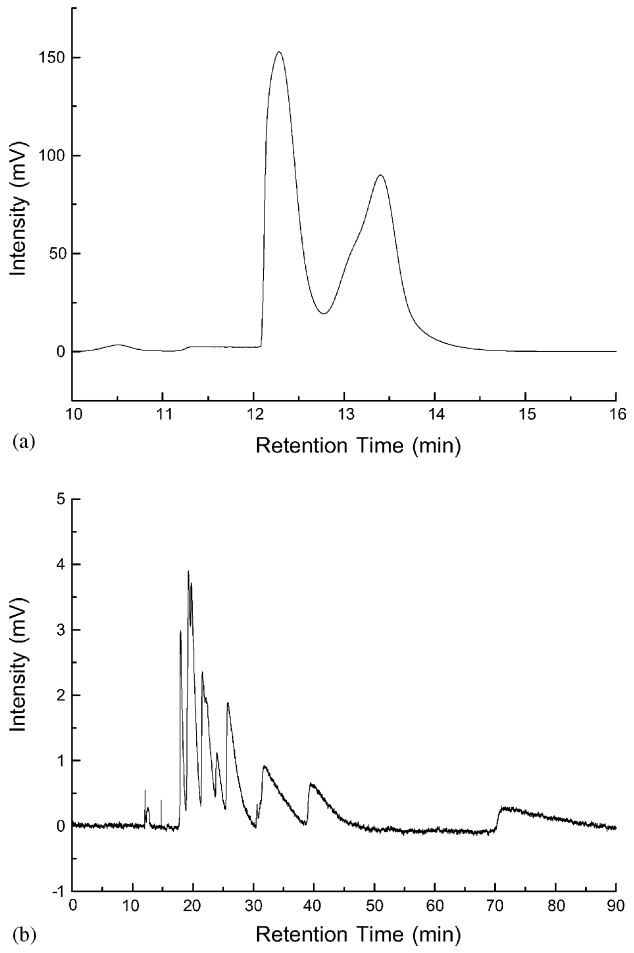


Fig. 4. Heart-cutting separation of the $n = 5$ *n*-, *sec*-, *tert*-butyl oligostyrene mixture. C18 column (250 mm × 4.6 mm). Mobile phase 100% methanol (20 μl injection). Cut time = 10.81–11.61 min. Flow rate = 1.0 ml/min. Transport of heart-cut fraction in (a) and (b) injection onto a CCZ column (30 mm × 4.6 mm). Mobile phase 100% methanol, flow rate = 2.0 ml/min.

operation. Even though 22 bands were apparent, many of these bands were poorly resolved as consequence of the increase in band heterogeneity in the first dimension and the subsequent increase in solute crowding. The number of separated components was less than the 26 predicted by Information Theory and Factor Analysis. Since separation times were relatively long, conducting more heart-cuts in order to improve the resolution and increase the number of separated components was not a practical solution. Therefore, due to the lack of order along the first separation dimension and the high solute crowding, the results obtained using real liquid chromatographic systems do not meet the expectations based on theoretical predictions for this system. Also, the extremely poor resolution observed in the first dimension of the C18 (acetonitrile)/CCZ (acetonitrile) system reduces the actual performance compared to systems where methanol was employed in the C18 dimension. These statements should not be taken as an indication that the theoretical predictions have failed in this instance, but rather all of the information gathered from theoretical predictions must be assessed in order to determine whether a given two-dimensional separation is possible to realise full expectations.

Table 2
Heart-cut times from the separation of the $n = 5$ isomeric mixture on a C18 column with a methanol mobile phase

| Figure number | Heart-cut start (min) | Heart-cut finish (min) |
|---|---|---|
| 6a | 11.40 | 12.00 |
| 6b | 11.50 | 12.10 |
| 6c | 11.60 | 12.20 |
| 6d | 11.70 | 12.30 |
| 6e | 11.90 | 12.50 |
| 6f | 12.05 | 12.65 |

The results from the experimental validation of the C18 (acetonitrile)/CCZ (methanol) system were quite similar to the C18 (acetonitrile)/CCZ (acetonitrile) system except retention of oligostyrenes was much longer in the second dimension when methanol was employed. Again the number of isomers separated did not meet the number theoretically predicted due to the high solute crowding in the first dimension.

In order to assess the effect solute crowding and band heterogeneity may have on the resulting two-dimensional separation we conducted a series of experiments where the region that was heart cut from the first dimension was systematically moved across a heterogeneous band in the first dimension. The heterogeneous band evaluated is that of the *tert*- and *sec*-butyl oligomers shown in Fig. 2a. In order to study the effect of heterogeneity the heart-cut times across the first separation dimension (C18/methanol) were changed systematically. The corresponding heart-cut times are given in Table 2. Shifting the heart-cut time, in most instances, by 6 s systematically resulted in an increase in the amount of the *sec*-butyl isomers observed in the second dimension (see Fig. 6). Many of these *sec*-butyl isomers overlapped *tert*-butyl isomers in the second dimension, resulting in increasingly impure bands and a reduction in separation power of the system. The most obvious of these, discounting the group of early eluting peaks, is indicated by an asterisk in Fig. 6d–f. Likewise the reverse was observed as the heart-cut time was systematically decreased from the right hand side of the band towards the left hand side, and an increasing amount of *tert*-butyl end group oligomers were observed to co-elute with the *sec*-butyl endgroup oligomers (Fig. 6d–f). Consequently, as the solute crowding increased (which was highest in the region corresponding to the overlap of the *tert*- and *sec*- bands) the ability to perform the separation successfully became more difficult. In this particular instance, resolution in the second dimension would only be achieved if the components of interest had different retention mechanisms in the second dimension.

An important finding from this study is that heart-cutting techniques allow for a greater degree of versatility in the experimental optimisation of two-dimensional separations

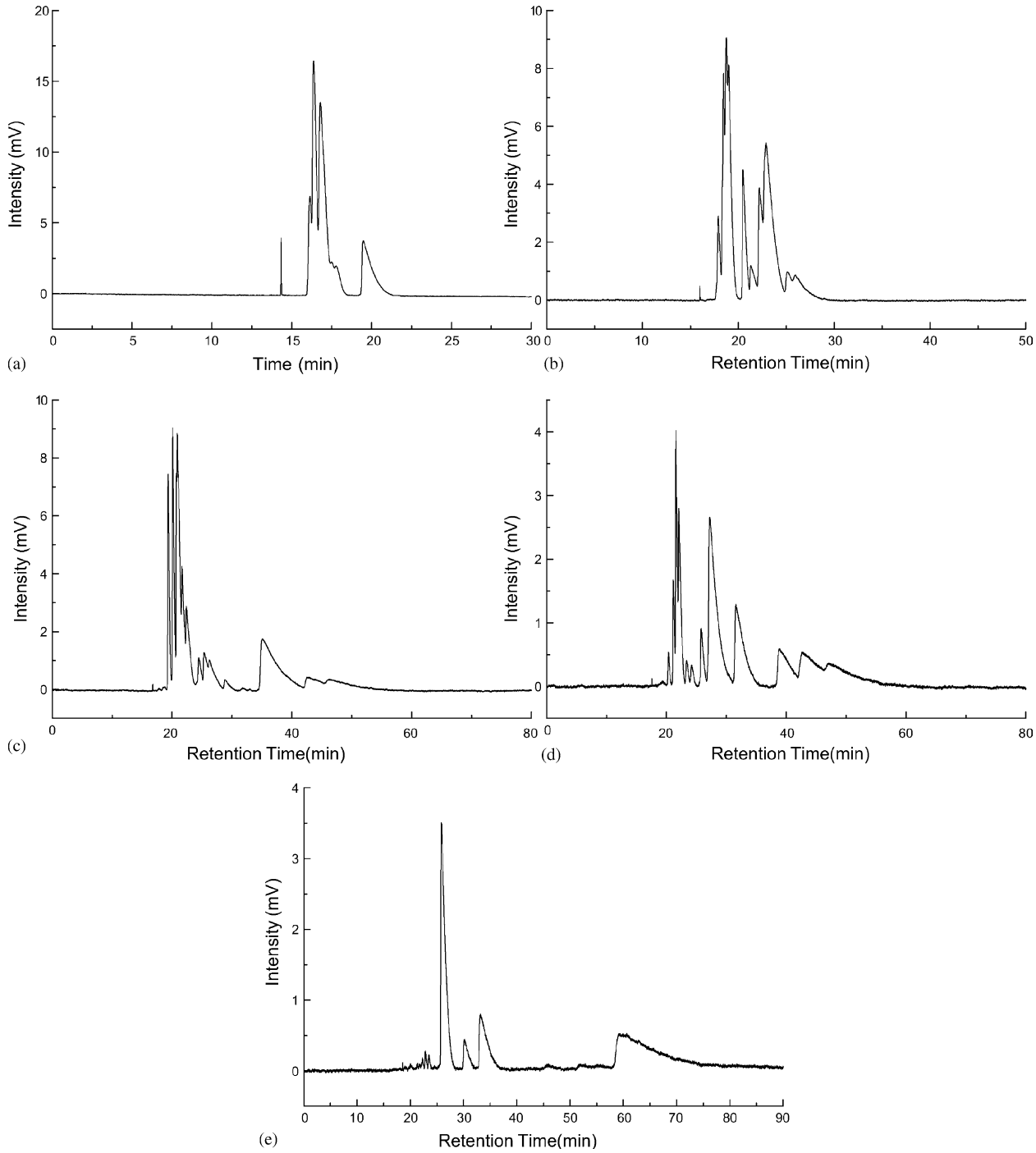


Fig. 5. Chromatograms for the heart-cutting separations of an *n*-, *sec*- and *tert*-butyl $n = 5$ oligostyrene mixture on a C18 column with acetonitrile mobile phase and a CCZ column with acetonitrile mobile phase. C1 chromatographic conditions as for Fig. 3a and C2 chromatographic conditions as for Fig. 3b. (a) Separation of heart-cut 1 (Fig. 2b) on a CCZ column; (b) separation of heart-cut 2 (Fig. 2b) on a CCZ column; (c) separation of heart-cut 3 (Fig. 2b) on a CCZ column; (d) separation of heart-cut 4 (Fig. 2b) on a CCZ column; (e) separation of heart-cut 5 (Fig. 2b) on a CCZ column.

than comprehensive mode separations. For example, when the analysis time in the second dimension is long and consequently prevents (or limits) the application of a comprehensive mode of operation, a heart-cutting approach may still be feasible. This is particularly important since the successful application of two-dimensional separations is very dependent upon solvent selection. Factors such as solvation effects and perhaps even viscous fingering [24] may ultimately determine which solvent combinations can be employed and in which dimension. The versatility of the heart-cutting mode, therefore, improves the possibility of success, albeit at the expense of analysis time.

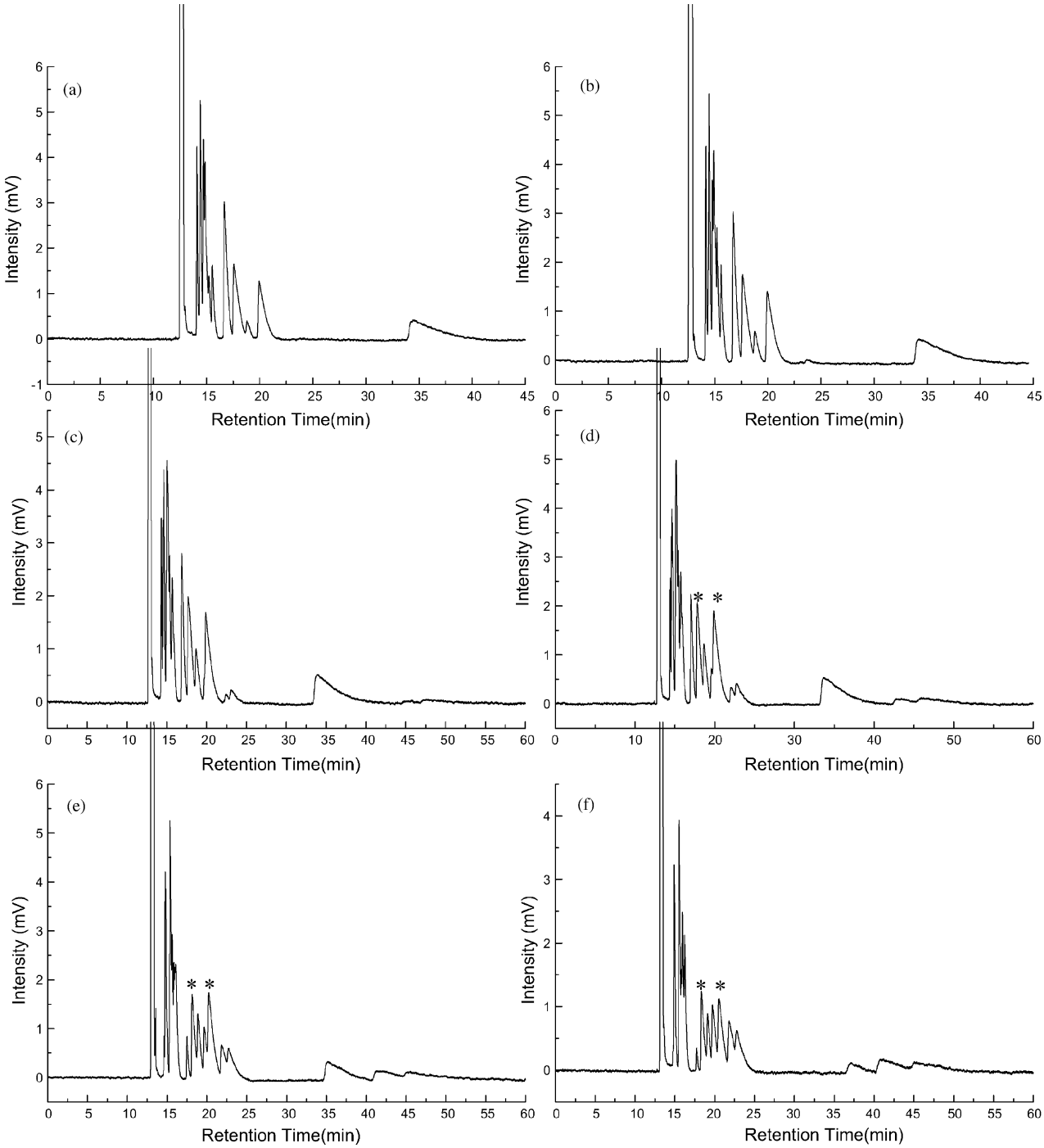


Fig. 6. Chromatograms for time shifted heart-cut separations of the $n = 5$ *n*-, *sec*- and *tert*-butyl oligostyrene mixture on a CCZ column (30 mm × 4.6 mm). See Table 2 for heart-cut times. C1 chromatographic conditions as for Fig. 3a (10 μl injection) and C2 chromatographic conditions as for Fig. 3b. (a) Separation of heart-cut: section 1; (b) separation of heart-cut: section 2; (c) separation of heart-cut: section 3; (d) separation of heart-cut: section 4; (e) separation of heart-cut: section 5; (f) separation of heart-cut: section 6.

## 3. Conclusion

The separation of 27 of the 32 highly similar structural and stereoisomers was achieved by heart-cutting using a two-dimensional reversed phased liquid chromatographic system incorporating a C18 column in the first dimension and a carbon clad zirconia column in the second dimension. The number of isomers separated was approximately equal to the number predicted by theory when solute crowding was moderate and if order was observed along the first separation dimension. Theoretical predictions of the number of isomers separated were not attained in practical experiments when

solute crowding was high and when disorder was apparent. Coupling this with the limitations of real chromatographic systems made for poor two-dimensional separations.

Having said that, however, the heart-cutting approach was more successful in resolving components from within this complex sample mixture than was a comparative comprehensive separation approach [14]. The heart-cutting approach offered more options in terms of mobile phase combinations than the corresponding comprehensive mode. As a result, the ability to perform the isolation successfully will be more probable in a heart-cutting mode than in the comprehensive mode. This is especially important if mobile phase compatibility issues limit system performance.

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