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Comprehensive two-dimensional separations of complex mixtures using reversed-phase reversed-phase liquid chromatography

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Abstract

A comprehensive two-dimensional reversed-phase reversed-phase liquid chromatographic system for the separation of a complex mixture of oligostyrenes was developed using results from a previous theoretical assessment of the informational similarity, percent synentropy, orthogonality and peak capacity of hypothetically coupled systems. The degree of sample attribute order in the first separation dimension was also used in the development of the experimental two-dimensional system. A C18(methanol)/CCZ(acetonitrile) two-dimensional system was chosen for the comprehensive analysis of the oligostyrene mixtures because this system had the lowest solute crowding, highest orthogonality and was observed to have order with respect to a sample attribute in the first separation dimension. The separations achieved were in full agreement with the results from information theory and (a geometric approach to) factor analysis assessments. High sampling rates in the first liquid chromatographic dimension were shown to be impossible or inefficient when the peak capacity and separation time of the second dimension was high or when the aim of the exercise was to isolate individual sample constituents in high yield.

Keywords: Comprehensive two-dimensional separations; Liquid chromatography, comprehensive two-dimensional; Information theory; Factor analysis; Oligostyrenes

1. Introduction

Two-dimensional high performance liquid chromatography (2DHPLC) has become a common analytical and isolation tool. The ease with which standard HPLC equipment can be configured to allow two-dimensional chromatography to be undertaken has driven the development and increased the application of this chromatographic technique.

The most basic form of two-dimensional chromatography is cross-fractionation [1–5]. This technique involves the manual [1–4] or automatic [5] collection of segments of eluent from one chromatographic separation, followed by re-injection of individual fractions onto a separate chromatographic system. Automated two-dimensional systems have gained popularity in recent years, as they are substantially less labour intensive than the aforementioned cross-fractionation procedure. The two general schemes of 2DHPLC are heart-cutting and comprehensive modes. Heart-cutting chromatography involves the transport of one or more segments of the eluent of the first separation step to the second separation step [6–10]. This technique is useful when the analyst is attempting to resolve overlapping peaks from the first chromatographic step. In comparison, the comprehensive mode of operation involves the transfer of the entire first dimension separation to the second separation dimension [11–22].

Since the advent of two-dimensional chromatographic techniques, it has become clear that each separation step should ideally provide different selectivity [14,23–25] as this maximises the gain in the practical peak capacity and hence the number of chromatographically resolvable components [24]. However, combining two very different modes of liquid chromatography can be a difficult process since the eluent from the first separation dimension must be compatible with the eluent of the second separation dimension. Furthermore, the solvent plug from the first chromatographic dimension should not considerably alter the selectivity of the second dimension. Such considerations are not important

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in the gas phase, but considerably complicate the liquid phase.

With the ready availability of electronically actuated two-position switching valves, the chromatographer can configure a wide variety of two-dimensional chromatographic systems. These systems can usually be tailored to meet the requirements of the separation task. This is evident with the large number of configurations found in the literature [6–22,26–32] and basic solutions provided by valve manufacturers [33,34]. Sample isolation devices between chromatographic dimensions form an integral part of the chromatographic process. Sections of eluent from the first chromatographic dimension must be isolated before transportation to the second dimension. Sample isolation devices such as storage loops and intermediate trapping columns are sometimes required to allow the timing of the transportation of segments from the first chromatographic dimension to the second so that consecutive segments do not overlap or 'wrap-around' [35].

A design that is commonly employed in comprehensive liquid chromatographic separations incorporates a single, two-position-eight port switching valve [11–17], with two sample isolation loops. Eluent from the first chromatographic separation can be diverted alternately to each sample isolation loop. While one loop is loaded, the contents of the other can be injected into the second chromatographic dimension. A variation on this scheme [18–22] involves the use of a number of switching valves and a second dimension that consists of two 'identical' chromatographic columns. Commonly the first dimension is size exclusion or ion exchange chromatography, and the second dimension is reversed-phased HPLC. This scheme takes advantage of solute solubility in the second dimension where pre-concentration at the head of the column prevents band broadening associated with diffusion. While separation is being conducted on one of the columns in the second dimension, the other is being loaded with eluent from the first separation dimension. Using this mode of operation, gradient elution can be employed in the second dimension with re-equilibration occurring while separation is occurring on the other column.

Regardless of the system configuration, it is generally accepted that the second separation dimension be fast relative to the first chromatographic dimension. Murphy et al. [17] have shown that to achieve maximal two-dimensional resolution [17], the effective sampling rate of the first dimension requires at least five samples across a given peak in the first dimension. To preserve the first dimension resolution, the sampling rate into the second dimension should be equal to or greater than three samples per peak from the 1st dimension. However, this places stringent requirements on the second dimension run time and subsequently implies that the second dimension must have a low peak capacity. This type of approach in which the first separation dimension has been divided into minute slices has been extensively used in fingerprinting and profiling experiments. This method of operation, which finds it origins in GC × GC, does not always work in practice because a two-dimensional chromatographic separation usually should be carried out in a specific order [9]. For instance, a two-dimensional system incorporating size-exclusion chromatography (SEC) and adsorption chromatography in the first and second dimensions, respectively, will often not work in the reverse order. As such, a second dimension that has a long separation time, and a high peak capacity [9] is often incorporated in a two-dimensional chromatographic system. Under such circumstances, multiple slices across a band in the first dimension will not be feasible. Furthermore, the extent to which a band in the first dimension must be resolved before injection into the second dimension depends on the desired outcome. For example, multiple slices across a band in the first dimension provide resolution in terms of information, but not physical isolation of individual components. If our aim is to obtain the physical 'in-hand' isolation of sample constituents, then slicing multiple sections across a peak in the first dimension serves to decrease the productivity of the result. As chromatographers we often face these two different challenges, both of which may be important, but require very different strategies.

In the development of a comprehensive two-dimensional chromatographic separation, a complete assessment of the options available for the separation task must be undertaken [36]. A two-dimensional chromatographic separation must be configured so that ideally each separation dimension is selective to a particular sample attribute [23]. Moreover attention should also be focused on the appropriate mobile phases, since this can yield vast differences in selectivity of any given stationary phase [37]. In some cases, maximal one-dimensional resolutions might not be necessary. Each one-dimensional resolution can be a lower resolution but yet achieve together an adequate two-dimensional resolution that meets the requirements of the sample's composition. The literature is replete with examples depicting this situation in techniques other than chromatography, such as in two-dimensional NMR [38].

In previous communications [39,40], following a selectivity study [41], we determined the orthogonality and separation quality of a number of combinations of two-dimensional reversed-phase reversed-phase liquid chromatographic systems incorporating a C18 column in the first dimension and a carbon clad zirconia column (CCZ) in the second dimension. These combinations 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).

Orthogonality in two-dimensional chromatography is a measure of the difference in selectivity or separation ability of a given chromatographic system [24,25]. As mentioned previously this is important since coupling two orthogonal chromatographic systems together will produce a 2DHPLC system that can extract a large amount of chromatographic

information on a particular sample [23]. The determination of orthogonality using information theory [25] and a geometric approach to factor analysis [24] allowed us to determine the optimal 2DHPLC combinations for the separation of a group of 32 highly similar oligostyrene structural and stereoisomers [39] as well as a variety of molecular weights constituting a group of 58 oligostyrenes [40]. Following this procedure we tested these combinations, practically, using a heart-cutting technique [9] and a 'semi-comprehensive' method [35] to determine whether the theoretically highest quality, most orthogonal 2DHPLC combinations were possible in practice with real two-dimensional systems.

The results of our previous study [39] indicated that theoretically between 26 and 28 of the 32 oligostyrene structural and stereoisomers may be separated [9]. However, when a heart-cutting approach was employed only two of these systems were useful and realised their theoretical potential. These systems were the C18(methanol)/CCZ(acetonitrile) and the C18(methanol)/CCZ(methanol) combinations. The other two combinations examined, which utilised acetonitrile mobile phases in the first dimension did not realise their theoretical expectations [9]. This result was in part expected since the C18(acetonitrile)/CCZ(acetonitrile) and C18(acetonitrile)/CCZ(methanol) systems displayed high solute crowding, relatively low orthogonality and disordered band arrangement in the first separation dimension. Hence from a practical standpoint, coupling these systems was expected to be difficult. Ultimately, we concluded that the optimal two-dimensional chromatographic system was the C18(methanol)/CCZ(acetonitrile) system [9].

In this communication we will illustrate the separation of the oligostyrene isomer mixture in a comprehensive separation. The results of the development process we have followed after theoretical assessment and heart-cutting and 'semi-comprehensive' analyses in the separation of a group of 32 oligostyrene structural and stereoisomers will be shown. The comprehensive separation illustrated will show that it is not always possible to conduct multiple cuts across a given peak when the second dimension run time is long relative to the first dimension. To further test the power of the two-dimensional liquid chromatographic system developed, a third sample attribute, namely molecular weight was introduced to increase the sample complexity [40].

2. Experimental

2.1. Chemicals

HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Australia. Polystyrene standards with molecular weights of 580 (*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 [42]. The $n = 2$–5 oligomer from each of these polystyrene standards was isolated by fractionation using methods previously described [41]. The *sec*-butyl polystyrene standard did not contain a $n = 2$ oligomer. 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 [43]. 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 4 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

All oligostyrene standards 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 for the first dimension were either 0.1, 0.7, or 1.0 ml/min as indicated in the text, while in the second dimension the flow rate was 2.0 ml/min. The column in the second dimension was thermostated at 30 °C. Injection volumes were 10 μl. UV detection was at 262 nm.

3. Results and discussion

Comprehensive two-dimensional separation systems are currently the most efficient and least labour intensive processes used to analyse or separate groups of complex mixtures. In the first instance we focus on the comprehensive separation of a group of 32 oligostyrene structural and stereoisomers of five configurational repeating units. This sample base can be described in terms of two dimensions, the first being the variation in the butyl end groups and the second in terms of a variation in the stereochemistry of each of the structural isomer classes. Examples of these oligostyrenes are shown in Fig. 1. We then expand our study to include a third sample attribute, which is molecular weight. The number of components in the sample increased from 32 to 58 by the inclusion of $n = 2$–5 oligomers [40]. Each molecular weight group contains a variation in structural isomerism and stereochemistry [40]. The 2DHPLC system used in this study incorporated a C18 column in the first dimension for the separation of the structural isomer classes (i.e. end group selectivity) and a CCZ column in the second dimension for the stereoisomer selectivity.

Previously, following a selectivity study, we examined four reversed-phase reversed-phase 2DHPLC systems using information theory and a geometric approach to factor analysis to theoretically determine the two-dimensional orthogonality and separation quality [39]. These 2DHPLC systems are listed in the introduction. We determined that theoretically [39] and practically [35] a C18(methanol)/CCZ(acetonitrile) system was the optimal chromatographic combination for the separation of the $n = 5$ oligostyrene isomer sample.

Table 1 details the important results of an analysis using information theory and geometric approach to factor analysis in the theoretical evaluation of the four combinations of chromatographic systems listed in the Introduction for the oligostyrene samples with 32 and 58 components [39,40]. Using information theory [25] the informational similarity, a measure of solute crowding on a normalised two-dimensional retention plane, can be determined. Values range between zero and one with a value of one indicating total solute crowded (hence no separation) and a value of zero, no solute crowding. Secondly, using information theory [25], the percent synentropy can be determined. This is a measure of retention mechanism equivalency, with values ranging from 0 to 100%. A value of 100% indicates that the retention mechanisms of the two chromatographic dimensions are 100% equivalent. Using factor analysis [24] the orthogonality of the two-dimensional chromatographic system can be determined using retention correlation coefficients. Values range from zero, indicating total orthogonality, to one, indicating that the separation steps of a two-dimensional system are identical. The peak spreading angle is also determined using this method [24]. This is a relative measure of the theoretical two-dimensional peak capacity utilised practically. Values range between 0 and 90°. A spreading angle of 0° indicates that none of the theoretical two-dimensional peak capacity is used practically, while a value of 90° indicates that all of the theoretical two-dimensional peak capacity is utilised. Also determined is the 'percentage usage' of the theoretical two-dimensional peak capacity and is determined using the practical two-dimensional peak capacity.

From this analysis the C18(methanol)/CCZ(acetonitrile) system was predicted to be the optimal two-dimensional combination for the separation of the 32 oligostyrene isomer mixture. The dimensions in this system were highly orthogonal, the system had the lowest solute crowding and utilised the majority of the two-dimensional retention plane. Moreover, the experimental application of this system was validated as the most practical and most successful when operated in a heart-cutting mode [9]. As such this communication will focus on the comprehensive reversed phase-reversed phase separation of the complex oligomeric mixture using the C18(methanol)/CCZ(acetonitrile) system.

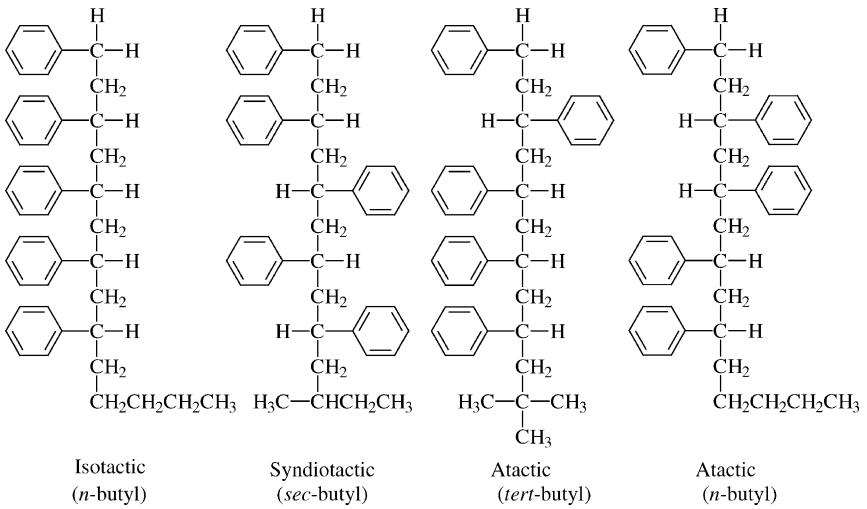

Fig. 1. Diagrammatic representations of $n = 5$ oligostyrenes showing structural and stereochemical variation.

Table 1
System attributes used to determine the measure of 2D orthogonality for each of the 2D RP–RP systems evaluated [39,41]

Attribute	Two-dimensional chromatographic combinations							
	C18M/CCZA system 1		C18M/CCZM system 2		C18A/CCZA system 3		C18A/CCZM system 4	
Oligostyrene sample	32	58	32	58	32	58	32	58
Informational similarity	0.56	0.74	0.62	0.78	0.92	0.92	0.93	0.93
Percent synentropy	3.3	1.9	3.3	1.9	3.5	1.9	3.4	1.8
Peak spreading angle	75	64	70	58	42	56	37	50
Practical peak capacity (N_p)	54	206	57	219	156	499	160	516
Correlation (c)	0.26	0.45	0.34	0.53	0.75	0.57	0.79	0.64
% Usage	90	78	87	74	56	69	52	64
Resolved components (/32)	26/32	47/58	28/32	46/58	26/32	48/58	26/32	49/58

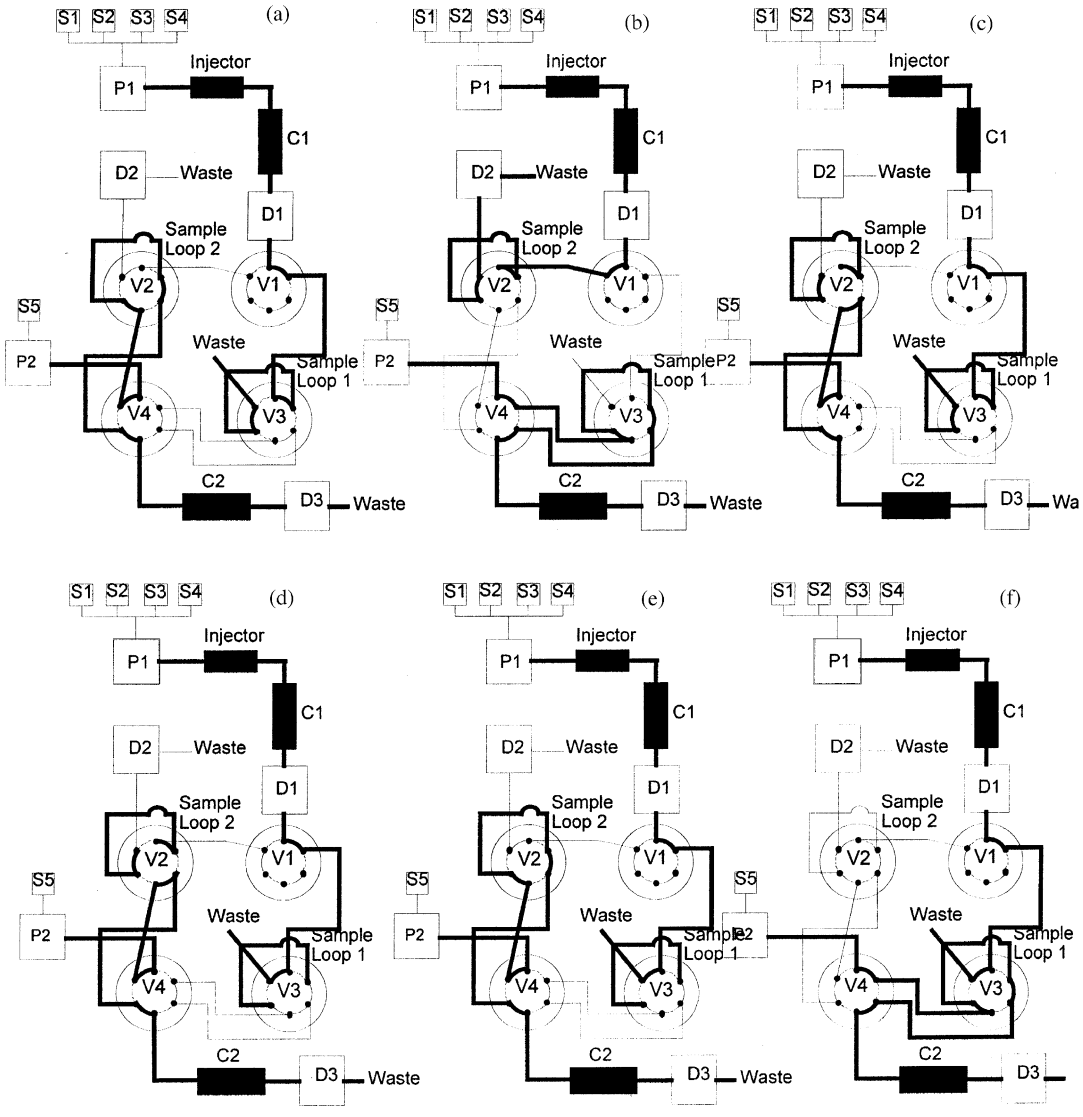

Fig. 2. Schematic diagram of the 2DHPLC system. (P1–P2) Solvent delivery systems; (V1–V4) 6-port 2-position switching valves; (C1) column in first separation dimension; (C2) column in second separation dimension. (a) System configuration for the elution of contents from C1 into sample loop 1. (b) System configuration for the elution of contents from C1 into sample loop 2 and injection of sample loop 1 contents onto C2. (c) System configuration for the elution of contents from C1 into sample loop 1 and isolation of sample loop 2. (d) System configuration for the isolation of sample loops 1 and 2. (e) System configuration for the injection of sample loop 2 contents onto C2, sample loop 1 isolated. (f) System configuration for the injection of sample loop 1 contents onto C2.

The comprehensive 2DHPLC system used in this experiment is shown in Fig. 2. The system consists of four six port two-position switching valves labelled 1–4. Operation of this system can be described in a number of steps outlined in Fig. 2. When the system is in the position shown in Fig. 2a, solute may be transported to one of the sample loops, in this instance, located on the valve 3. Switching the system to the configuration shown in Fig. 2b allows transportation of the first fraction isolated in the sample loop to the second dimension and at the same time collection of a second fraction from the first dimension into loop 2 located on valve 2. Since the separation time of the first fraction is greater than the time required to fill loop 2, the system switches to the configuration shown in Fig. 2c. Here the contents of loop 2 are held while loop 1 is refilled with a third fraction cut from the first dimension. The system is then switched to the position shown in Fig. 2d, isolating sections two and three in loops 2 and 1, respectively until the completion of the separation of the first fraction in the second dimension. At this time the system switches to the configuration shown in Fig. 2e allowing the injection of section two onto the second dimension. Finally the system switches to the configuration shown in Fig. 2f, which completes the comprehensive two-dimensional separation.

Fig. 3 illustrates the separation of the 32-oligostyrene isomer mixture on a C18 column with a methanol mobile phase. This chromatogram may be divided into three sections corresponding to the resolution of each of the structural isomer classes. Retention of the oligostyrenes increases in the order of *tert*-butyl, *sec*-butyl and *n*-butyl oligostyrenes. There is substantial overlap between the *tert*- and *sec*-butyl end group oligomers, but the *n*-butyl oligomers are well resolved. Fig. 4 is a normalised two-dimensional retention plane of the two-dimensional separation when the C18(methanol) system is utilised in the first dimension. In this system the

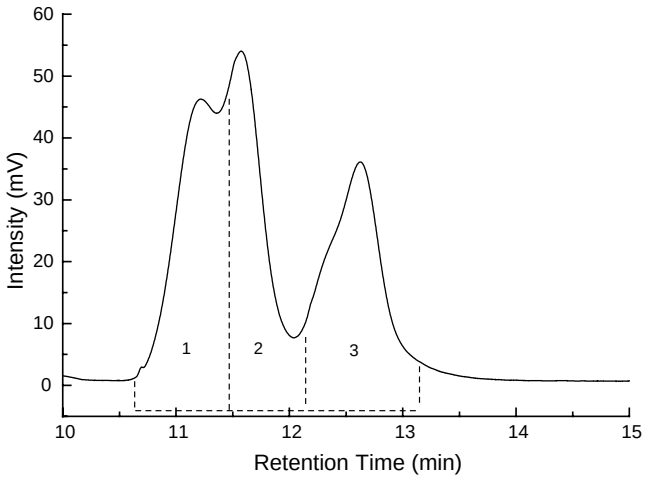

Fig. 3. Chromatogram illustrating the separation of the 32 isomers on a C18 column. Each section, labelled 1–3, represents the *tert*-butyl, *sec*-butyl and *n*-butyl oligostyrene sections transported to C2. Mobile phase 100% methanol, flow rate 1.0 ml/min, detection 262 nm, injection volume 10 μl.

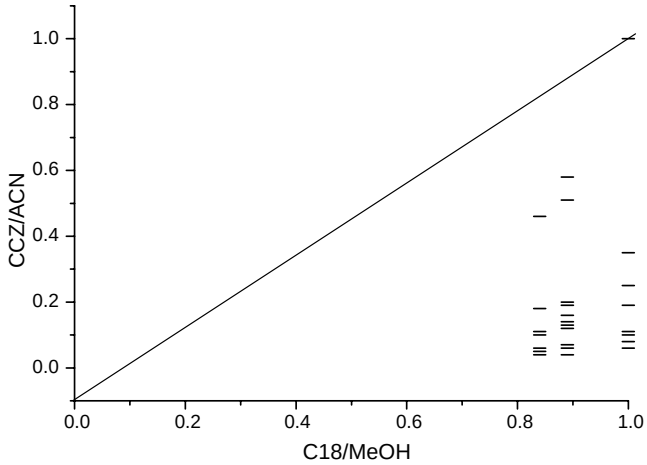

Fig. 4. Normalised two-dimensional retention plot for a C18(methanol)/CCZ(acetonitrile) theoretical two-dimension chromatographic system in the separation of the 32 oligostyrene isomer mixture.

C18 dimension resolves the oligostyrenes solely on the basis of their end groups or by structural isomer classes with no stereoisomer resolution. As such, three cuts were made across the C18 dimension as indicated in Fig. 3 and noted earlier.

Completion of the comprehensive two-dimensional separation is shown in Fig. 5, where following solute transportation to the second dimension, resolution of the diastereoisomers is achieved. In the second dimension, the dominating aspect of retention is orientated towards molecular shape and hence the diastereoisomers resolve. Fig. 5b–d are expanded sections of this chromatogram, illustrating the high-resolution separation of the highly related isomers. In total 27 (out of the 32) isomers were resolved in this separation, which was in agreement with our theoretical predictions [39]. The isomers that were not resolved were one *tert*-butyl isomer (Fig. 5b), and four *sec*-butyl isomers (Fig. 5c). All *n*-butyl isomers were resolved (Fig. 5d). We should note, however, that more than 27 bands are observed in Fig. 5a, which is due to the duplication of isomers transported as a result of overlap between the *tert*- and *sec*-butyl components as shown in Fig. 3. These bands are indicated with an asterisk and will be the focus of a subsequent communication. Such an overlap poses some problems for quantitative analysis, and to a certain extent, larger scale preparative separations if such a method was employed.

To further test the separation power of the C18(methanol)/CCZ(acetonitrile) system a comprehensive analysis of the 58 component oligostyrene sample described earlier was conducted. The separation of this sample on a C18 column with methanol mobile phase is shown in Fig. 6a. The C18(methanol) system separates the sample based according to molecular weight and within each molecular weight group there is a subgroup according to the oligostyrene end-group. Numbered sections (1–11) on the chromatogram in Fig. 6a represent the portions of the

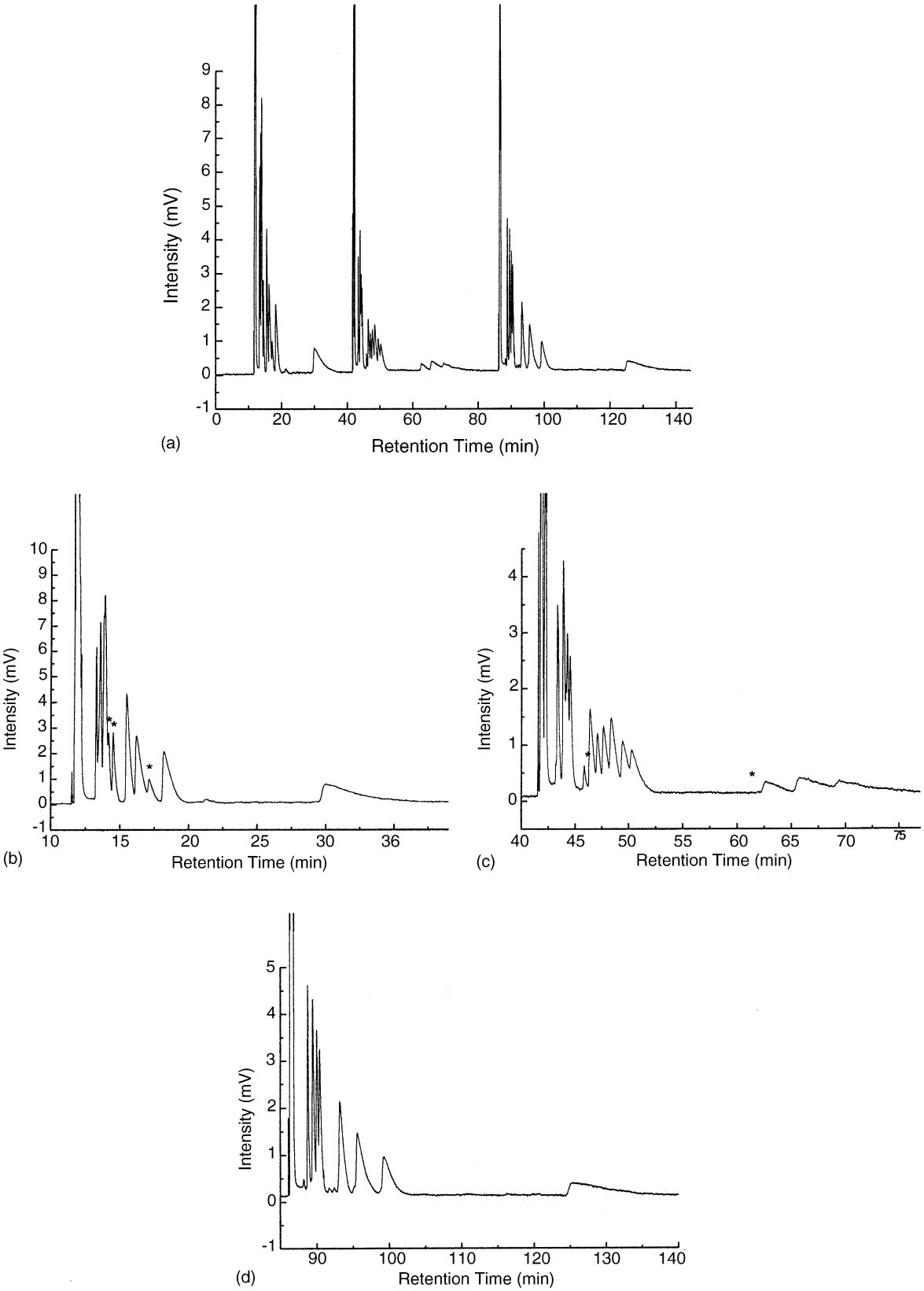

Fig. 5. Chromatogram of the 32 oligostyrene isomer mixture following separation on the C18(methanol)/CCZ(acetonitrile) chromatographic system. C1 mobile phase 100% methanol, flow rate 1.0 ml/min, injection volume 10 μl. C2 mobile phase 100% acetonitrile, flow rate 2.0 ml/min, thermostated at 30 °C. (a) Chromatogram following D2 for the separation of the 32 *tert*-butyl, *sec*-butyl and *n*-butyl oligostyrene isomers. (b–d) Expanded sections of (a).

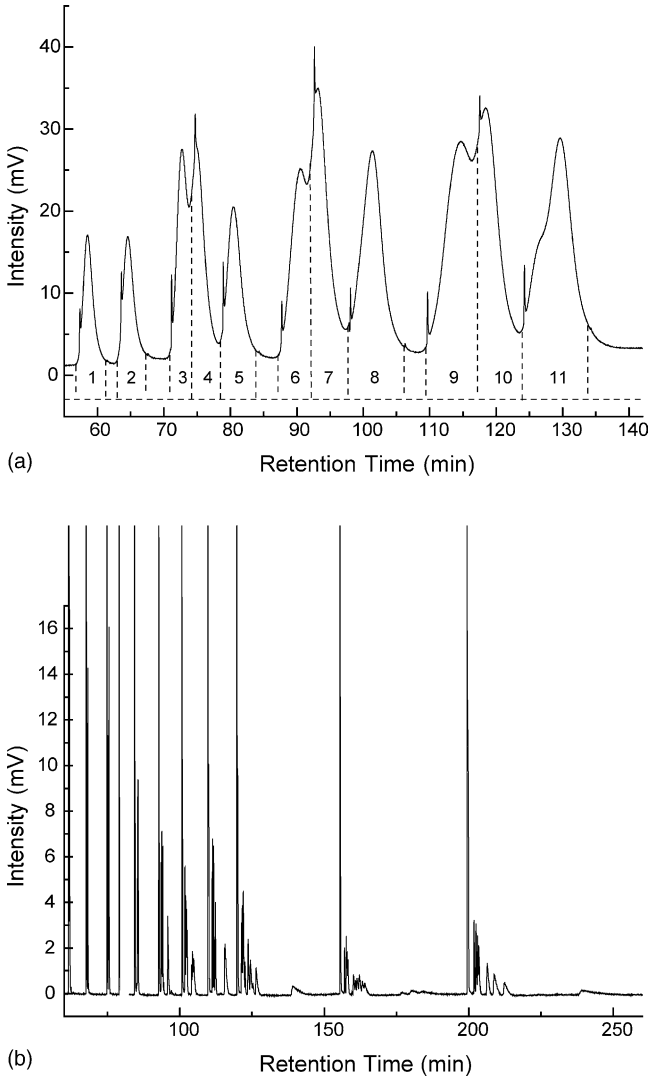

Fig. 6. (a) Chromatogram illustrating the separation of the 58 isomers on a C18 column. Each section, labelled 1–11, represents a *tert*-butyl, *sec*-butyl or *n*-butyl oligostyrene section transported to C2. Mobile phase 100% methanol, flow rate 0.1 ml/min, detection 262 nm, injection volume 10 μl. (b) Chromatogram of the 58 oligostyrene isomer mixture following separation on the C18(methanol)/CCZ(acetonitrile) chromatographic system. C1 mobile phase 100% methanol, flow rate 0.1 ml/min, injection volume 10 μl. C2 mobile phase 100% acetonitrile, flow rate 2.0 ml/min, thermostated at 30 °C.

chromatographic eluent transported to the second dimension. These sections are divided according to the molecular weight and end-group separation on the C18(methanol) system. Completion of the two-dimensional separation of the 58 component oligostyrene sample is shown in Fig. 6b. This chromatogram shows the separation of 50 components which is slightly more than that achieved according to the theoretical results presented in Table 1.

There are several methods of comprehensive analysis in liquid chromatography that undertake a process of multiple cuts per band (usually defined as a minimum of 4) in order to gain a greater understanding of the two dimensional band displacement. While these methods do undoubtedly yield a more precise chemical signature, speed in the second dimension to allow successive sample loading from the first dimension, without the wrap-around effect is paramount to its success. Operation in such a mode is highly dependent upon the nature of the retention process. In the present study, several of the diastereoisomers display very significant retention and there is a substantial range of retention factors throughout the sample, making isocratic methods more difficult. Gradient methods (either solvent or temperature) in the second dimension would decrease the retention of the strongly retained species, but re-equilibration would be necessary, hindering the attainment of an appropriate speed in the second dimension. With this in mind we attempted to undertake a comprehensive mode of separation in which we doubled the number (halved the volume) of sections cut

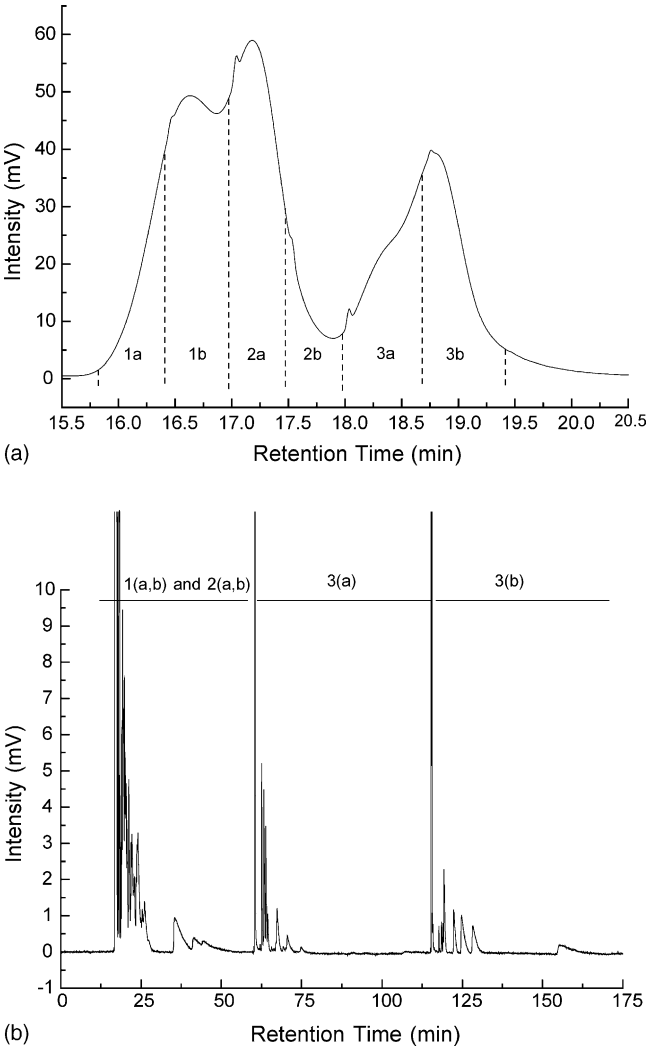

Fig. 7. Chromatogram of the 32 oligostyrene isomer mixture separation on the C18(methanol)/CCZ(acetonitrile) chromatographic system at two-cuts per section. Conditions as for Fig. 5 (exceptions noted): (a) chromatogram illustrating the separation of the 32 isomers on a C18 column. C1 flow rate 0.7 ml/min. (b) Chromatogram illustrating the separation of the 32 isomers on a CCZ column.

into the second dimension. This resulted in severe sample wrap around and a loss in resolution of the previously resolved components. The chromatograms in Fig. 7, for example, illustrate a comparison between a separation of the 32 oligostyrene isomer sample in which six cuts (Fig. 7a) were made and transported to the second dimension (Fig. 7b) as opposed to three cuts (Figs. 3 and 5). Even reducing the first dimension flow rate to 0.1 ml/min did not avoid overlap of the first four transported sections. The information gained regarding the relative positions of the stereoisomers eluting in the first dimension on the C18 column did not contribute significantly to the information as a whole. In fact the higher sampling rate in this case made the process of quantification more difficult. Furthermore, if the aim of the exercise were to isolate components then the higher sampling rate would be impractical. Also increasing the sampling rate in the first dimension would significantly increase the separation time of the overall sample mixture. Analysis time would approximately double for a sampling rate of two and triple for a sampling rate of three samples per section. It is suggested in this case at least, the extra information gained by increasing the sampling rate for these particular separations does not justify the increased separation time, system complexity, and analysis time.

4. Conclusion

We have illustrated the separation of 27 of a group of 32 oligostyrene structural and stereoisomers and 50 out of a group of 58 oligostyrenes using a comprehensive reversed-phase reversed-phase chromatographic system comprising in the first dimension a C18 column with a methanol mobile phase and in the second dimension a CCZ column with an acetonitrile mobile phase. The success of the comprehensive two-dimensional separations illustrated in this study is a direct result from preliminary work that investigated selectivity and established the orthogonality and solute crowding in potential two-dimensional systems. The results of the practical separation were in perfect agreement with the findings from our orthogonality study, which verifies the importance of the preliminary orthogonality exercise prior to coupling systems for two-dimensional separations.

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