Evaluation of the two-dimensional reversed-phase–reversed-phase separations of low-molecular mass polystyrenes

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Abstract

The resolving power of four reversed-phase–reversed-phase two-dimensional (2D) chromatographic systems was evaluated using information theory (IT) and a geometric approach to factor analysis. The first separation dimension employed a C18 column, while the second separation dimension employed a carbon clad zirconia column. Mobile phases in each dimension were either methanol or acetonitrile. The sample matrix that was employed in this study contained a mixture of 58 components, comprised of stereoisomers and structural isomers of a systematic variation in molecular weight. Each of the components were oligostyrenes, with between two and five configurational repeating units having either *n*-butyl, *sec*-butyl or *tert*-butyl end groups.

In the two-dimensional systems employed in this study, between 46 and 49 of the 58 components could be separated, depending on the mobile phase combinations—with apparent resolution that could not possibly be achieved in a single one-dimensional separation. The results from this study indicate that in order to fully evaluate the resolving power of a 2D system multiple methods of analysis that evaluate the separation potential are most appropriate. This becomes increasingly important when the sample contains components that are very closely related and the retention of solutes displays a high degree of solute crowding. © 2003 Elsevier B.V. All rights reserved.

Keywords: Factor analysis; Two-dimensional reversed-phase chromatography; Information theory; Polystyrenes, low-molecular mass; Oligostyrenes

1. Introduction

Two-dimensional chromatographic systems are becoming more frequently employed as a tool for the separation of mixtures unable to be separated by a single chromatographic step [1] or where the two-dimensional approach is less time consuming [2–4]. In some instances, two-dimensional separations are required by regulatory authorities in order to provide sufficient identification of sample components [5–7].

The benefits of two-dimensional chromatography become obvious when one considers the enormous increase in the separation space afforded in moving from one dimension to two dimensions [8–10]. The peak capacity of a two-dimensional separation is commonly regarded as simply the product of the peak

capacities of the constituent one-dimensional separations [2,11]. However, this assumption is naive since correlation between the two separation dimensions is usually observed and this correlation will reduce the separation space to a fraction of the theoretical value [12]. According to Ernie and Frei [13], using information theory (IT), keeping the synentropy (or cross-information) as low as possible will provide maximal information and considerably enhance the power of a given separation.

There are many separation processes that may be employed in a two-dimensional separation. For instance, liquid chromatographic techniques may be coupled to capillary electrophoresis techniques, which would more than likely yield a non-correlated or orthogonal two-dimensional separation since their displacement mechanisms are highly divergent [14]. Furthermore, two of the many different modes of liquid chromatography may be coupled together to provide a two-dimensional system consisting of a single separation technique, but one in which each dimension offers a different separation mechanism [15–18]. As an example, size exclusion liquid chromatography may be coupled with reversed-phase liquid chromatography [16]. Both techniques employ liquid chromatography, but their separation modes are very different. Hence the problem of deciding which combination should be chosen for a particular application, and also which of these are the most orthogonal and hence yield the greatest separation power is not trivial and requires a systematic approach to achieve optimal separations. The usual approach is to choose processes that are obviously different, however, the more divergent the displacement processes are, the more incompatible they tend to be in practice [14,19]. Coupling techniques that are similar in type, for instance, reversed-phase chromatography using a different type of stationary phase in each dimension will usually be the simplest solution, albeit with the potential for a reduction in the orthogonality of the system. Nevertheless, continued development of new stationary phases has provided for divergent retention processes even though the fundamental basis of the separation process remains essentially the same [20,21]. For example, carbon type surfaces, such as carbon clad zirconia provide for different retention mechanisms than the classical C18 reversed-phase stationary phase, even though both are reversed-phase

materials [1,22]. In the present study, we employed information theory and factor analysis to illustrate the orthogonality that would be expected when two reversed-phase materials, such as carbon clad zirconia and C18 are to be used in two-dimensional separations.

1.1. System and sample description

In the current study, we wish to examine the separation of a three-dimensional sample using a two-dimensional separation system. The sample base contains a total of 58 oligostyrene components that may be described in terms of three sample attributes: namely (1) variations in chain length (i.e. oligostyrenes with $n = 2$–5 repeating units), (2) structural isomers (i.e. oligostyrenes with *n*-butyl, *sec*-butyl and *tert*-butyl end groups) and (3) stereoisomers (resulting from the stereochemistry of configurational repeating units yielding isotactic, syndiotactic and atactic isomers) [23]. Each of these attributes can be easily visualised by evaluating the structures shown in Fig. 1. The analysis of a sample matrix containing three sample attributes expands on our previous study in which there were only two sample dimensions under consideration, namely stereoisomers and structural isomers. Consequently, the addition of the third sample attribute (molecular weight) increases the extent of solute crowding and this will ultimately influence the procedures that must be implemented in designing experimental two (or three)-dimensional chromatographic systems. This will be discussed further in Section 3 and a subsequent manuscript will describe the experimental ramifications of such a third sample attribute. The separation system chosen consisted of a conventional reversed-phase C18 column in the first dimension, which allowed discrimination between oligostyrenes with different molecular weights and oligostyrenes with *n*-butyl, *sec*-butyl and *tert*-butyl end groups. Carbon clad zirconia, which is known to be highly shape selective, was used in the second dimension for the separation of the stereoisomers of the structural isomer classes [1,24]. In this instance, since the C18 stationary phase could discriminate between molecular weight and the type of end-group [22] this potentially reduces the need for a three-dimensional system, which would theoretically be required for the best separation of a three-dimensional sample [8].

n-butyl (isotactic)
3 configurational
repeating units.

sec-butyl (syndiotactic)
4 configurational
repeating units.

tert-butyl (atactic)
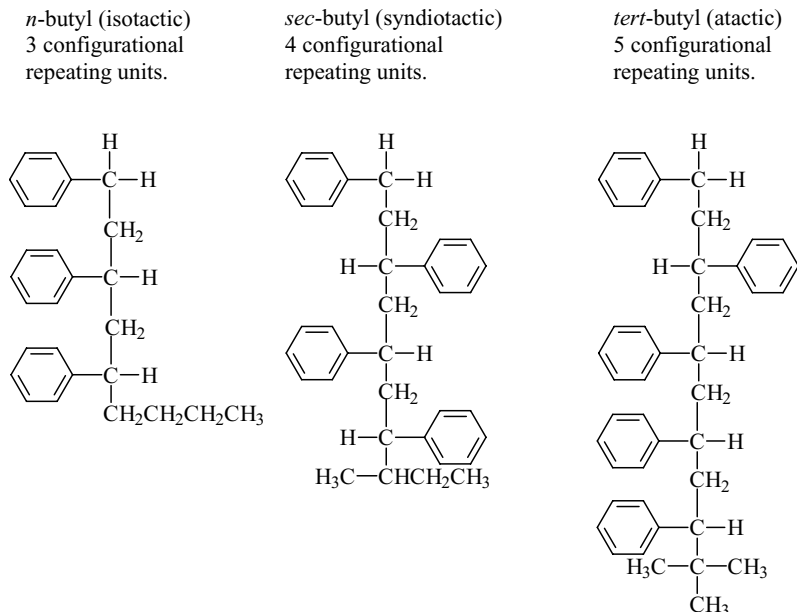5 configurational
repeating units.

Fig. 1. Molecular structures representing the variations in molecular weight, tacticity and end group.

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 [1]. The $n = 5$ oligomer from each of these polystyrene standards was isolated by fractionation using methods previously described [1].

2.2. Equipment

All chromatographic separations were performed on a Shimadzu LC system, incorporating a LC-10ATvp pumping system, SIL-10ADvp auto injector, SPD-10Avp UV detector (set at 262 nm), and Shimadzu Class-VP software on a Pentium II 266 MHz pro-

cessor. The C18 chromatography column used in conjunction with an acetonitrile mobile phase in this study was a Value-pak (Activon—no longer trading) 250 mm × 4.6 mm with 5 μm particles. A Nucleosil C18 column (100 mm × 4.6 mm, 10 μm Pd), used in conjunction with methanol mobile phases was prepared in-house using a slurry packing technique discussed that has been discussed elsewhere [1]. The carbon clad zirconia column (50 mm × 4.6 mm, 3 μm Pd) was packed in-house, using methods previously described [1] from packing material supplied by Zirchrom Separations Inc.

2.3. Chromatographic separations

Oligostyrene standards ($n = 5$) were dissolved in methanol. All separations were conducted using either a 100% methanol mobile phase or a 100% acetonitrile mobile phase as appropriate. Mobile phases were sparged continuously with helium. All flow rates were 1.0 ml/min and injection volumes were 10 μl. All chromatographic columns were thermostated at 20 °C. UV detection was set at 262 nm. Duplicate injections were performed for each sample. Void volumes were measured according to

the time associated with a first disturbance of the baseline.

Two-dimensional separations were accomplished via a fractionation–reinjection process. Fractions of the eluent from the first separation or dimension (in most cases the C18 column) were collected and separately injected onto the second dimension (in all cases the CCZ column). We should note at this point that the results of this study reflect only the theoretical two-dimensional separation of the components within this sample matrix. Factors such as band broadening and delay times associated with valve switching, etc. were not taken into consideration. Hence, retention information was derived solely from peak maxima, irrespective of peak widths or volumes. Future studies evaluating the experimentally derived data will offer comparisons to these theoretical results. Only at that time can we gauge the suitability of any of these systems for true two-dimensional separations.

3. Results and discussion

The quality and thus the power of the two-dimensional separation systems that incorporate the C18 and CCZ columns were determined using two approaches (information theory and factor analysis). Using IT, the informational similarity was determined to assess the degree of solute crowding on the two-dimensional retention plane [25]. The percent synentropy may also be determined using IT and is a measure of retention mechanism equivalency [25]. A geometric approach to factor analysis was used to determine retention correlation coefficients, which is another measure of retention orthogonality. The practical two-dimensional peak capacity was also determined using this method, allowing assessment of the potential separation space being utilised [12]. Application of both these methods required the retention data to be normalised according to Steuer et al. [26] and Lui et al. [12].

In previous work [22] on a similar but less complex sample matrix the most orthogonal two-dimensional separation systems incorporated a C18 column with a methanol mobile phase and a CCZ column with either a methanol mobile phase or an acetonitrile mobile phase. We now wish to further test the power of these separation systems with a more complex sample. Consequently, the two-dimensional chromatographic

systems that we initially investigated were:

- C18 (methanol)/CCZ (methanol)
- C18 (methanol)/CCZ (acetonitrile)

The normalised two-dimensional retention plot shown in Fig. 2 is a representation of the sample components separating into the two-dimensional separation area in which a C18 column with methanol mobile phase forms the first dimension and a CCZ column with a methanol mobile phase forms the second dimension. The normalised retention data (X_a) is derived according to Eq. (1).

$$X_a = \frac{Rt_i - Rt_0}{Rt_f - Rt_0} \qquad (1)$$

where Rt_i is the retention time of any solute i, Rt_f the retention time of the last eluting solute and Rt_0 the retention time of an unretained solute. Each circle in the plot shown in Fig. 2 represents the location of a composite or single component peak. Visualising the separation along the first dimension would reveal a typical oligomeric distribution on the C18 dimension, which also discriminates between the components according to the structural differences in their end groups with retention increasing as the degree of chain branching decreases [22,27]. We should point out, however, only two $n = 2$ oligomer components are apparent in Fig. 2, because the average molecular weight of the *sec*-butyl component was higher than both the *n*-butyl and *tert*-butyl components leading to the absence of an $n = 2$ oligomer for the *sec*-butyl oligostyrenes. We should also note that for each respective oligomeric component, retention increased as the chain branching of the end group decreased. That is, the *tert*-butyl end group oligostyrenes eluted first and the *n*-butyl oligostyrenes eluted last. In agreement with previous findings [22], no stereoisomer resolution was apparent in this first-dimensional separation step. Visualising the separation along the second dimension reveals the discrimination between stereoisomers on the CCZ column. The fact that the components are displaced in a line perpendicular to the first dimension verifies that no stereoisomer separation was occurring in the first separation dimension.

The informational similarity, a measure of solute crowding, for the C18 (methanol)/CCZ (methanol) system was 0.78, indicating a medium to high degree of solute crowding on the two-dimensional retention
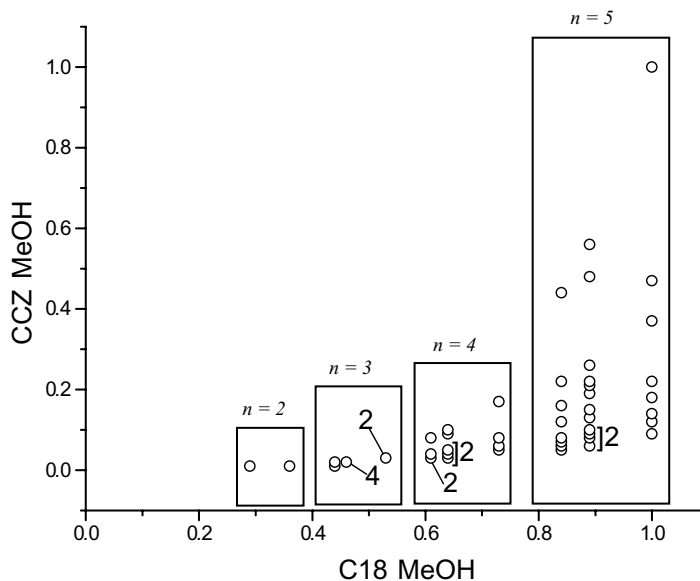
Fig. 2. Normalised two-dimensional plot of the C18 (methanol)/CCZ (methanol) system in the separation of the 58 oligostyrene isomer mix. Each boxed section represents isomeric components containing the same number of configurational repeating units. The numbers adjoining the data points indicate the number of components co-eluting.

plane. In Fig. 2, the separation on the C18 dimension appears relatively non-crowded, but the stereoisomer separation on the CCZ dimension is more crowded, leading to the overall relatively high value for the informational similarity. The percent synentropy was determined to be 1.9% which indicated that the retention mechanisms of each dimension were not equivalent for the components in this particular sample mixture. The retention correlation coefficient, which is another measure of the orthogonality of the system, was moderate with a value of 0.53, and the peak spreading angle (β) was also modest with an angle of $58°$. The interpretation of these values are that 74% of the two-dimensional theoretical peak capacity was utilised with a practical two-dimensional peak capacity of 219 peaks. The peak spreading angle and the practical two-dimensional peak capacity are shown diagrammatically on the geometric plot in Fig. 3. As a result of the moderate correlation coefficient and the ordered separation along the C18 dimension 46 oligostyrenes of the 58 oligostyrenes may be expected to be resolved.

A change in the mobile phase for the second dimension to acetonitrile (leaving the first dimension unchanged as methanol) resulted in only minor dif-

ferences between the two systems. The normalised two-dimensional retention plot of this separation is shown in Fig. 4. A similar type of separation pattern to that seen for the previous separation combination (Fig. 2) was obtained. Peak crowding in this system was slightly lower than in the previous combination with an informational similarity of 0.74. The percentage synentropy was low (1.9%) indicating again that these two separation dimensions were not equivalent. As expected, correlation across the separation dimensions was also lower than in the previous system (0.45 compared to 0.53) because, as found previously [22] the CCZ with acetonitrile as a mobile phase gave greater stereoisomer selectivity than when methanol was employed as the mobile phase. Subsequently, the peak spreading angle was marginally larger with a value of $64°$, but the practical two-dimensional peak capacity decreased to 215 peaks (because of the stronger solvent strength using acetonitrile as opposed to methanol) in the second dimension. However, this combination of chromatographic dimensions resulted in a higher efficiency separation utilising 78% of the theoretical peak capacity (not shown). The number of components separated was essentially equivalent to the previous system (47 compared to 46).
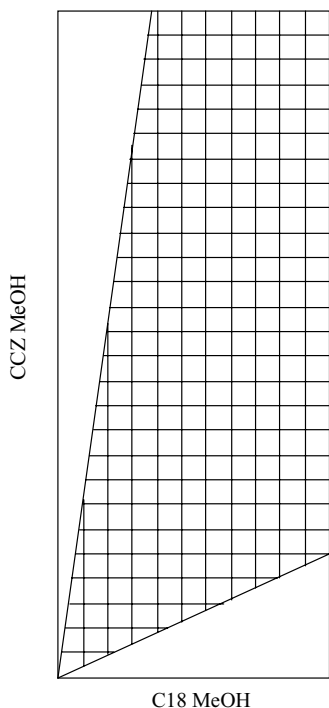
Fig. 3. Geometric plot showing the practical two-dimensional peak capacity of the C18 (methanol)/CCZ (methanol) separation system in the separation of the 58 isomer mixture.

Each of the above two systems illustrate the two-dimensional retention behaviour of systems whereby the order of the separation is maintained following transport from the first dimension to the second. In this instance, we define the order of the separation as being a function of the molecular weight and structural isomerism. The order of the separation comes about from the limited stereoisomer selectivity in the first dimension. Consequently, all isomeric components co-elute within a region defined by their molecular weight and their particular end group. For instance, each of the oligostyrenes that eluted from the C18 column are contained within columns (labelled in Fig. 2) according to their number of configurational repeating units. Note that there is no overlap between adjacent oligomeric members and that in all cases retention increases according to an increase in molecular weight with a sub unit increase in retention according to the chain branching of the end group. Such a system is extremely useful because peak integrity can be more easily maintained. Only the second dimension exhibits a degree of disorder in which there is overlap between the retention windows of polymers with different molecular weights, different end groups and different stereochemistry.
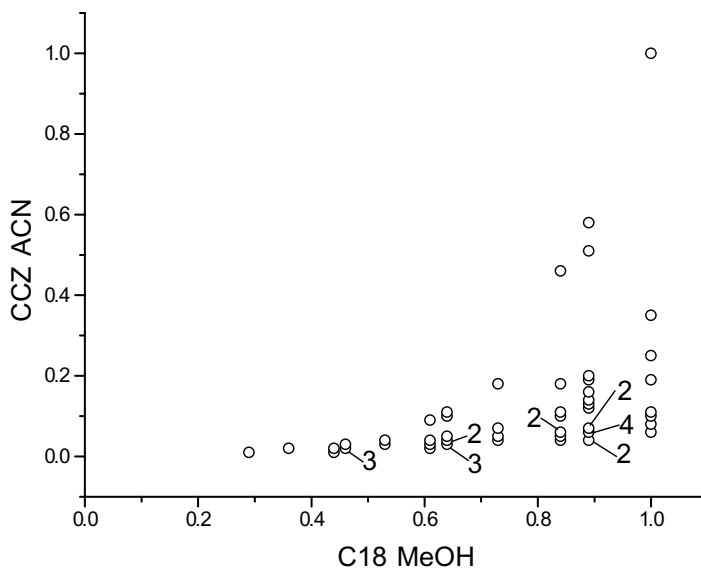

Fig. 4. Normalised two-dimensional plot of the C18 (methanol)/CCZ (acetonitrile) system in the separation of the 58 oligostyrene isomer mixture. The numbers adjoining the data points indicate the number of components co-eluting.

It is therefore interesting to evaluate the effect on the separation power when the first dimension also displays some resolution of the stereoisomers. Such a system could be attained when the C18 column was used with an acetonitrile mobile phase [22]. Consequently, the next two systems that we investigated were:

- C18 (acetonitrile)/CCZ (methanol)
- C18 (acetonitrile)/CCZ (acetonitrile)

The two-dimensional retention plot of the C18/ acetonitrile and CCZ/acetonitrile system is shown in Fig. 5. This plot shows that the separation on the
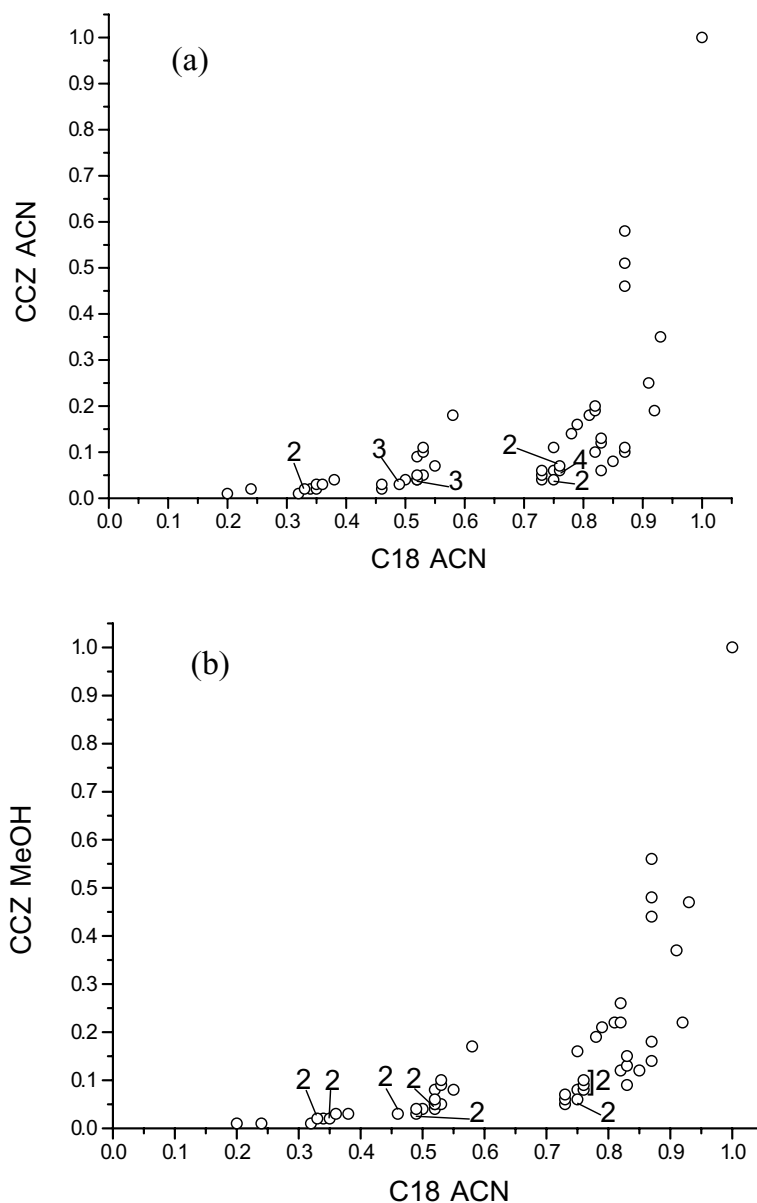

Fig. 5. Normalised two-dimensional plot of (a) C18 (acetonitrile)/CCZ (acetonitrile) and (b) C18 (acetonitrile)/CCZ (methanol) system in the separation of the 58 oligostyrene isomer mixture. The numbers adjoining the data points indicate the number of components co-eluting.

C18 column is still molecular weight dependent even though some stereoisomer selectivity is apparent. However, there is some overlap between the different end group oligostyrenes. Visualisation of the normalised retention factors in the CCZ dimension still reveals a chaotic band displacement, as has previously been reported [22]. In this system, an increase in the solute crowding was apparent and the percent synentropy indicated that the mechanisms of retention were highly different between each dimension (1.9%). In this instance the informational similarity increased to 0.92 and the correlation between the dimensions increased marginally to 0.57, largely as a result of the fact that now both separation dimensions could resolve isomeric components. The increased isomeric resolution in the first dimension, however, resulted in a dramatic increase in the practical two-dimensional peak capacity (499). But, the peak spreading angle was only $56°$ and consequently only 69% of the theoretical peak capacity was actually utilised. Similar results were found for a two-dimensional system incorporating a C18/acetonitrile mobile phase and a CCZ/methanol mobile phase (see Table 1). Theoretically, 48 and 49 components were resolved using the C18 (acetonitrile)/CCZ (acetonitrile) and C18 (acetonitrile)/CCZ (methanol) systems respectively, which is higher than the systems that employed methanol in the C18 separation dimension. However, in experimentally coupled systems, which employ acetonitrile in the C18 dimension, the number of resolved components should be substantially reduced [28] due in part to the high degree of solute crowding.

Now it is interesting to compare the findings from our previous study, in which the sample contained 32 isomers (stereo and structural), to the findings in the present study where the sample consisted of 58 components, which included various molecular weight stereoisomers and structural isomers. The peak crowding (measured by the informational similarity) was substantially higher for the equivalent 2D system combination in the present study compared to the 32 isomer mixture of our previous study [22]. This is solely due to the 58 components in the present system occupying a separation space fractionally larger than the space used by the 32 isomeric mixture. Hence, as expected, there was an increase in the solute crowding of components along the C18 separation.

The orthogonality of this two-dimensional LC system is dependent upon the stationary phase, mobile phase and the sample mixture. Consequently, since the sample population was changed to incorporate the molecular weight variable it was not surprising that the correlation coefficients for the current sample population were not the same for the same two-dimensional chromatographic environments, although the same trend was observed when the same changes were made to the mobile phases used in the second dimension. That is to say, the correlation increased when we changed the mobile phase of the CCZ dimension from acetonitrile to methanol. The orthogonality of the current separations was more correlated than for the previous study [22]. This can be gauged from the distributions of the peaks on the normalised retention plots, since a greater portion of the peaks lie closer to the main diagonal. This result is interesting since the addition of molecular weight to the sample has increased retention correlation when the two separation dimensions provide clearly different information. In the C18 dimension there was separation based predominantly on structural isomer

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

Attribute	C18M/CCZM	C18M/CCZA	C18A/CCZM	C18A/CCZA
Percentage synentropy	1.9	1.9	1.8	1.9
Informational similarity	0.78	0.74	0.93	0.92
Peak spreading angle (β) degrees	58	64	50	56
Theoretical peak capacity (N_t)	297	264	810	720
Practical peak capacity (N_p)	219	206	516	499
Correlation (c)	0.53	0.45	0.64	0.57
Usage (%)	74	78	64	69
Resolved components ($n/58$)	46	47	49	48

and molecular weight separation while in the CCZ dimension separation was based predominantly according to stereoisomer selectivity. This result could perhaps be partly attributed to the end group effect on retention in both dimensions, since in both dimensions, retention of the oligostyrenes increased in the order of *tert*-butyl, *sec*-butyl and *n*-butyl.

A summary of the results of this study may be found in Table 1. It is clear that with the current sample population, orthogonality has been achieved and maintained in two-dimensional reversed-phase liquid chromatography. The orthogonality of the separation dimensions (with particular emphasis on the C18 (methanol)/CCZ (acetonitrile) system) has led to the utilisation of the majority (78%) of the theoretical two-dimensional peak capacity. In the current systems it is obvious that the stationary phase plays a significant role in the contribution to the orthogonality of the separation dimensions and consequently the two-dimensional resolving power. In the previous study the sample was considered as complex, with 32 very similar isomers (structural and stereo). The complexity of the sample in the present study was increased to a mixture of 58 stereo and structural isomers with molecular weight variations, and it was found that 47 of the 58 compounds could potentially be resolved. Although in this study only two separation dimensions were employed to separate the components in a three-dimensional sample matrix the results have indicated that a successful outcome should be achievable in an experimental situation. That is of course provided there is a high degree of order associated with each sample attribute. In the C18 methanol/CCZ acetonitrile system for example, there was a high degree of order associated with molecular weight and structural attributes in the first dimension, and this should allow the second dimension to express separation according to the third sample attribute. We would, however, expect that if disorder were to be generated in the expression of the sample attributes in the first dimension, that is, if the different molecular weight components were randomly distributed then the success of the separation would be less likely and the assignment of peaks more difficult. In which case a system with three separation dimensions should be employed in order to maintain the order of the separation and hence peak identity.

4. Conclusion

The results have revealed that orthogonality was mainly influenced by changes in the stationary phase surface and the sample base, and was less dependant upon the mobile phases chosen. Thus by utilising different separation principles that are aimed at markedly different sample attributes, orthogonality in reversed-phase two-dimensional separations was achieved. Moreover, it was seen that greater separation potential was achieved when different separation mechanisms were utilised in each separation dimension. The power of this two-dimensional chromatographic system was shown with a high number (47 of the 58) of the very 'similar' components being potentially resolved.

The question that has yet to be addressed is how do these theoretical predictions stand up to experimental reality. There are a number of important factors that will dictate the successful transition from theory to practice. These include, the degree of solute crowding, the homogeneity of the sample bands that are to be transported from the first dimension to the second, and the time required to complete the separation in the second dimension. Each of these factors will dictate whether the two-dimensional separation can be performed comprehensively or whether the separation will be limited to a heart cutting process. Consequently, it is very important to consider carefully the findings from both of the methods that we employed in this work. For example, the factor analysis may indicate the 'efficiency' of space utilisation and the number of components that could be expected to be resolved, however, it is the informational similarity that ultimately determines the practicality of the separation in a real chromatographic system. As the extent of the solute crowding increases, irrespective of the orthogonality, the separation in a comprehensive mode will become more difficult.

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