Two dimensional reversed-phase–reversed-phase separations Isomeric separations incorporating C_{18} and carbon clad zirconia stationary phases

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Abstract

Informational theory and a geometric approach to factor analysis were employed to evaluate the degree of orthogonality of a two-dimensional reversed-phase–reversed-phase chromatographic system. The system incorporated a C_{18} column as one dimension and a carbon clad zirconia column as the second dimension. In order to study the resolving power of this system, the separation of a sample matrix containing an artificial mix of 32 isomers (structural and diastereoisomers) was evaluated. Using this system, between 25 and 28 of the 32 isomers could be separated, depending on the mobile phase combinations— with 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 are most appropriate. This becomes increasingly important when the sample contains components that are very closely related and the retention of solutes is clustered in one quadrant of the 2D space. Ultimately, the usefulness of the 2D separation is determined by the goals of analyst.
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1. Introduction

The resolving power of a modern chromatography column is far better now than those of a decade or more ago. The reduction in particle sizes and improvements in the physical nature of the stationary phase surface have led to vast improvements in the separation technique [1]. Yet despite this, the actual separation power has only marginally increased because as chromatographic columns have improved overlapped components have begun to emerge from bands previously thought to be single component peaks [2]. In fact, no realistic amount of improvement in chromatographic column technology and design will be able to bring about complete separation of random component peaks in complex samples [3,4]. For instance, Giddings predicts that approximately four million theoretical plates would

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be required to separate only 82 of 100 components randomly distributed in a one dimensional separation [4]. Clearly, production of a column with this level of performance is an unreal expectation.

The need to achieve these extreme separation conditions has driven the demand for multidimensional separation techniques. Here, multiple separation systems may be coupled together to provide a separation environment in which two or more different retention processes take place, providing a much larger physical separation space [4,5]. In fact, the separation space (or theoretical peak capacity) of the multidimensional separation system becomes the product of the individual peak capacities of each single dimensional separation technique [6–8]. Clearly, to provide maximal separation power each dimensional separation step must function according to a different retention mechanism [9]. Techniques such as liquid chromatography–capillary zone electrophoretic method (LC–CZE) [10,11] and liquid chromatography–gas chromatography (LC–GC) [12] are two examples of coupled systems that provide divergent retention behavior in each separation step, largely because of their fundamentally different retention processes. In some instances, hyphenated techniques may be considered as multidimensional if the detection process affords a degree of selectivity [9,13]. A simple example is LC with UV photo diode array detection and a more complex example is LC-ICP. In practice, multidimensional separations are restricted to two dimensions. In this study, we confine our discussion to two-dimensional systems where both dimensions are liquid chromatographic separation steps.

Coupled multidimensional separation systems have developed rapidly in the last decade, largely due to improvements in instrumentation. Nowadays, the physical uniting of otherwise independent separation processes is becoming routine; however, automated operation of even a 2D system requires precise timing of events, which are best carried out using reliable PC control. Furthermore, manipulation of the separation data often requires the use of sophisticated data handling processes, which include high-speed data acquisition for fast separations using methods such as GC–GC [14,15]. Consequently, multidimensional methods have still not reached their separation potential, and the development of such processes will continue as long as advancements are made in instrumental technologies.

Perhaps the simplest multi-dimensional separation process can be achieved by uniting separation environments that are similar in operation. That is for instance, GC–GC [16–18] or LC–LC [19,20] coupled separations. In these types of systems computer driven operation is less demanding, but more importantly, compatibility between the different separation environments is usually improved. GC–GC techniques are particularly suited to multidimensional separations because the carrier gas is compatible between each separation step. Changes in the retention mechanism can be readily achieved using different types of stationary phases, for which there are a large number of options available that provide different types of selectivities. LC–LC methods that incorporate size exclusion chromatography (SEC) for molecular mass separations, and reversed-phase (RP) [21] or normal-phase (NP) [22] methods for separations based on chemical properties, are commonly employed in the analysis of copolymers. However, coupling together different LC systems is often more demanding. For instance, the mobile phase from one separation dimension is often not compatible with the separation environment for the second dimension. For this reason, coupled two-dimensional separations that incorporate reversed-phase and normal-phase chromatography are rarely employed, even though these separation steps are commonly employed in sample prep in natural product chemistry. SEC–RP or SEC–NP and ion exclusion (IEC)–RP systems are more common because of the greater compatibility between the solvents for each separation step. Also rarely seen are RP–RP systems because gaining orthogonal separation in a coupled RP–RP system is typically more difficult as the retention mechanisms are usually highly correlated. Hence, there is little or no gain in separation power. Even so, limited separation divergence may be achieved by varying mobile phase conditions. For instance, some orthogonality may be gained by using a single column type and varying gradient profiles in consecutive separation steps or by using different mobile phase additives in each dimension [23]. Tandem column set-ups employing two different reversed-phase stationary phases provide another separation avenue to chroma-

tographers [24]. Although this method employs at least two columns, they are coupled in series. As a consequence, they are not considered to be two-dimensional since the retention factor of a given solute is the sum of the individual retention factors on these two columns [24,25]. Moreover, while separation of a number of components may be achieved on the first column, this separation may be reversed on the second column leading to an overall zero gain in separation.

In the current study we explore the concept of RP–RP two-dimensional separation processes. Our primary aim is to show that as a consequence of a new stationary phase technology, divergent retention behaviour on different types of reversed-phase materials can lead to marked differences in retention processes. This makes the separation of complex samples viable in a coupled technique with little consideration to solvent compatibility. A secondary aim in this work is to illustrate the importance of employing multiple methods of system evaluation to assess the orthogonality of the coupled systems.

1.1. Reversed phase environments

The most common stationary phase is the C_{18}. This has been the industry work-horse since its inception in the early years of modern LC. While the mechanism of retention for solutes on this stationary phase remains a contentious issue [26–29], it is clear that this surface is excellent for the separation of members in a homologous series. Alkyl benzenes, and oligomers for instance all enjoy excellent resolving power on a C_{18} column, as do structural isomers where, for instance, the location of a methyl group dictates the retention of the solute. This can be clearly seen in the change in retention of the various forms of butyl benzene [30,31]. The C_{18} column is, however, in general only a poor separator of diastereoisomers [32]. These types of compounds are better separated on stationary phases that are more selective to molecular orientation, such as carbon surfaces. Carbon clad zirconia (CCZ), for example, provides an environment that is excellent for the separation of diastereoisomers, but is not as useful for the separation of homologues [2,33,34]. Consequently, CCZ may be incorporated into a multi-

dimensional system with the expectation of divergent retention behaviour compared to that of the C_{18} column. Furthermore, the coupling of a CCZ system to a C_{18} system should be seamless because of the good solvent compatibility.

1.2. Sample description

Before we can investigate the separation behaviour of coupled C_{18} and CCZ systems, we must first define our sample. Perhaps the most difficult separation problem is one in which there is close resemblance between all compounds in the sample matrix. Under these conditions we believe multidimensional separations are at their most useful. However, if the sample can not be described according to two dimensions, then a two-dimensional separation approach is of course useless and no gain in separation would be achieved by coupling systems that offer different retention mechanisms.

In our current study, we have chosen a sample matrix that contains structural isomers and diastereoisomers of each structural isomer. Our aim was to explore the separation power of the two-dimensional C_{18}-CCZ system, so we chose structural isomers that differed by only a very subtle change in the location of a single methyl group. Furthermore, we wanted this change to be as insignificant as possible with respect to the overall molecule. Hence, we chose relatively large molecules for our sample. An oligostyrene with five configurational repeating units served perfectly as our model sample (Fig. 1). The molecular formula of these isomers is $C_{44}H_{50}$. Three types of structural isomers were obtained by using polystyrenes with either an *n*-butyl, *sec*-butyl or *tert.*-butyl end-group. The structures of these molecules are depicted in Fig. 1a–c, respectively. Because of the stereochemistry of the configurational repeating units, each of these different structural oligostyrenes contains a number of diastereoisomers [35]. In total, there are eight diastereoisomers for both the *n*- and *tert.*-butyl oligostyrenes and 16 for the *sec*-butyl oligostyrene (because of the stereochemical *sec*-butyl end group). That yields a total of 32 combined isomers, defined according to two dimensions namely (a) structural isomers and (b) diastereoisomers.
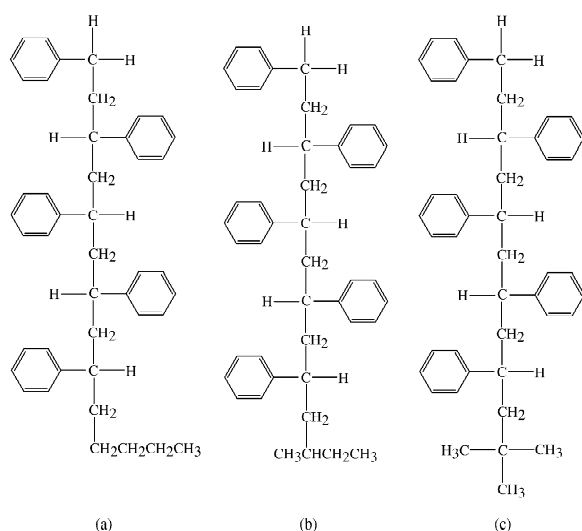
Fig. 1. Isotactic forms of the fifth ($n=5$) oligostyrene, illustrating the difference between the three end groups: (a) *n*-butyl polystyrene; (b) *sec*-butyl polystyrene; and (c) *tert*.-butyl polystyrene.

2. Experimental

2.1. Chemicals

High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were obtained from Mallinckrodt Australia. Polystyrene standards with molecular mass 580 (*n*-butyl) and 760 (*sec*-butyl) Da were purchased from Polymer Laboratories and Aldrich Chemical Company, respectively. *tert*.-Butyl polystyrene (molecular mass ~580 Da) was synthesised using anionic polymerisation of styrene initiated with *tert*.-butyl lithium. The molecular mass of the members of the oligomer series were determined using mass spectroscopy [2]. The $n=5$ oligomer from each of these polystyrene standards was isolated by fractionation using methods previously described [36].

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 processor. A Value-pak (Activon – no longer trading) (250×4.6 mm, P_d 5 μm) C_{18} column was employed for separations employing an acetonitrile mobile phase, while a Nucleosil (100×4.6 mm, P_d 10 μm) C_{18} column, which was prepared in-house [2] was employed for separations employing a methanol mobile phase. The carbon clad zirconia column (50×4.6 mm, P_d 3 μm) was packed in-house, using methods previously described [2] 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 unless stated otherwise. UV detection was set at 262 nm. Duplicate injections were performed for each sample.

Two dimensional separations were accomplished following a fractionation–reinjection process. Fractions of the eluent from the first separation or dimension (in most cases the C_{18} column) were collected and separately injected onto the second dimension (in all cases the CCZ column).

3. Results and discussion

As our aim was to study the two dimensional separation power for the chosen solutes in only reversed-phase–reversed-phase systems, the number of stationary phase and mobile phase combinations could be narrowed through simple deductive processes. A change in the length of the alkyl chain on a silica support simply reflects a change in the degree of retention rather than a change in selectivity. Correlations between retention data on C_4, C_8 and C_{18} stationary phases by Slonecker and Dorsey show that retention is highly correlated [37]. Hence, there was no reason to suggest that divergent behaviour would be observed as a function of the alkyl chain length for the solutes studied in the current work. Further, all reported studies conducted on the re-

tention behaviour of oligostyrenes on C_{18} columns in which diastereoisomers have been partially resolved have suggested that the primary mechanism of retention is dictated by the molecular mass of the oligomer [2,32]. Stereoisomers typically elute according to a secondary mechanism underlying the molecular mass separation. In addition, the retention of oligostyrenes on C_{18} columns is sensitive towards the type of end-group [38]. Since we chose to study only RP–RP systems, we can ignore solvents that elute these molecules under ''critical'' conditions. Likewise we can ignore solvents for which elution occurs in an exclusion process. Hence, for the separation process in which the stationary phase is C_{18}, either methanol or acetonitrile mobile phases would be the most suitable. Solvents such as tetrahydrofuran and dichloromethane are unsuitable since the isomers are not retained by the stationary phase under these mobile phase conditions. On the other hand, water is too weak as an eluent, but could be used as a modifier for both methanol and acetonitrile [36]. However, in reality this would not be necessary since molecules of the molecular mass chosen are adequately retained in both pure methanol and acetonitrile [36].

In the second dimension of the separation in which CCZ is employed as the stationary phase, the number of potentially suitable mobile phases has been previously evaluated [36]. On CCZ, retention behaviour has been shown to be dominated by the stereochemistry of the molecule and largely independent of the molecular mass. Typically, acetonitrile and methanol yield the best separation, while *iso*-propanol and hexane show some separation power for diastereoisomers, but not as substantial as that of either acetonitrile or methanol. The polystyrenes were unretained in tetrahydrofuran and dichloromethane [36]. Consequently, the number of 2D combinations that would be useful can be limited to four. They are:

- C_{18}(acetonitrile)/CCZ(acetonitrile)
- C_{18}(acetonitrile)/CCZ(methanol)
- C_{18}(methanol)/CCZ(acetonitrile)
- C_{18}(methanol)/CCZ(methanol)

It is important to note that two-dimensional separation systems are only useful in the separation of mixtures unable to be separated in a single chromatographic step or where a two-dimensional system is

less time consuming. The chromatogram shown in Fig. 2a illustrates the separation of the 32 oligostyrene mixture on a ValuePak C_{18} column using an acetonitrile mobile phase. Resolution of the isomers is poor. Likewise, the separation of the same isomer mixture on the carbon clad zirconia column resulted in a poor separation, as shown in Fig. 2b. Consequently, if these components in this sample matrix were to be separated, a two-dimensional approach would be required.

As a starting point to this discussion, we investigated the resolving power of a system that incorporated as the first separation dimension a C_{18} column with an acetonitrile mobile phase. The second separation dimension consisted of a carbon clad zirconia column also with acetonitrile as the mobile phase. In both dimensions, the separation is sensitive to diastereoisomer resolution, however, the second dimension affords a substantially greater degree of diastereoisomer selectivity [2,32,33]. The first dimension is also sensitive to the structural factors, whereas little selectivity with regard to structural factors is apparent in the second dimension. Using an acetonitrile mobile phase in both dimensions affords complete solvent compatibility between the dimensions. Any orthogonal behaviour will then totally depend on the properties of the stationary phase, which is an interesting prospect in a reversed-phase–reversed-phase 2D system and is akin to GC–GC.

As each dimension of the separation utilises different columns with different column formats and particle characteristics, the retention data are normalised. Normalisation according to Eq. (1) [10,37] yields scaled retention factors (X_a), which then allow direct comparisons to be made between independent systems. In Eq. (1) Rt_i is the retention time of any solute i, Rt_f is the retention time of the last eluting solute and Rt_0 is the retention time of an unretained solute. The retention time of an unretained solute was taken to be the retention time of the solvent front in all cases.

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

The plot in Fig. 3 illustrates the transformed retention data for the 2D system incorporating the C_{18} (acetonitrile) and CCZ (acetonitrile) dimensions.
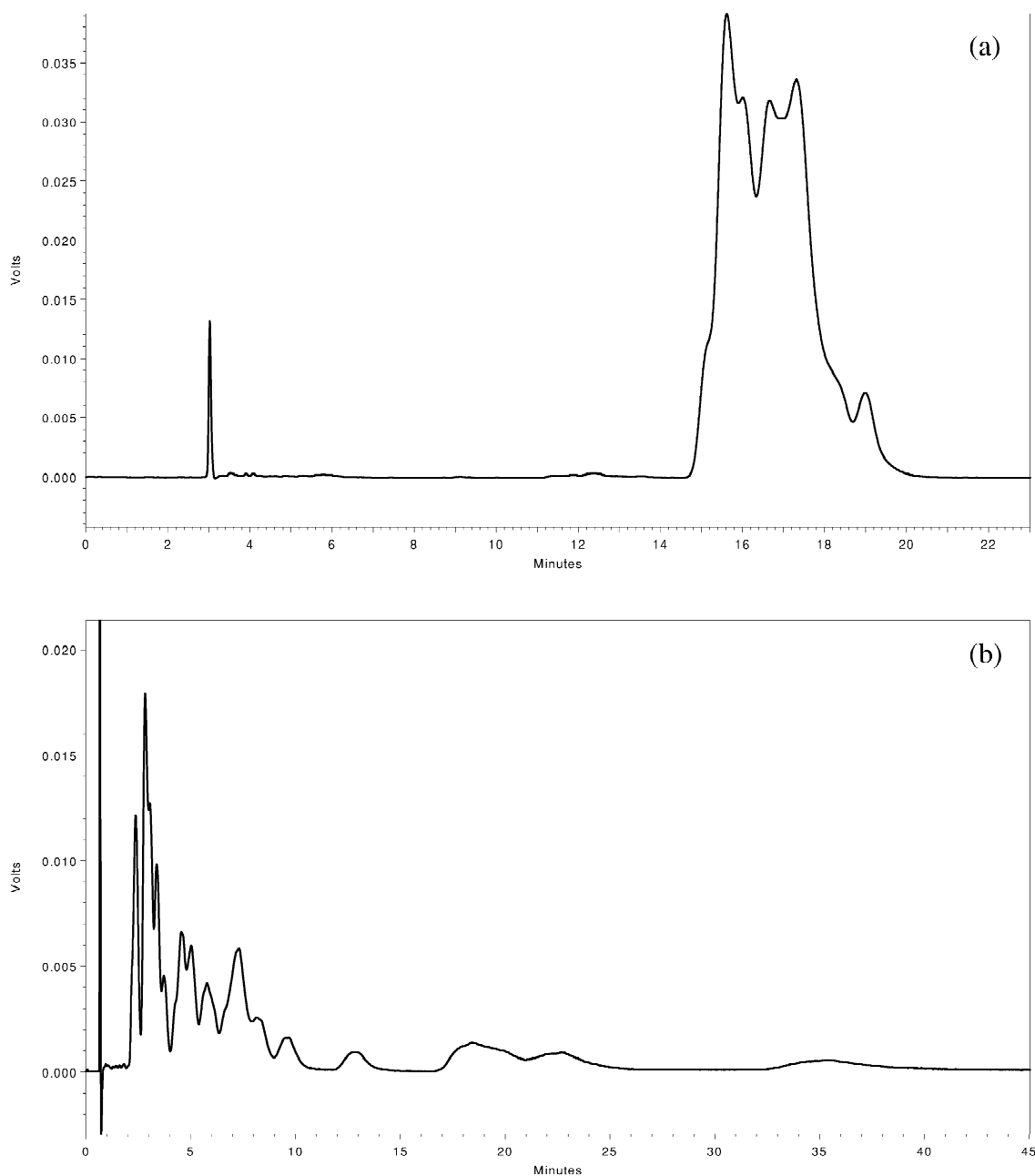
Fig. 2. Separation of the 32 isomer mixture containing the *n*-butyl, *sec*-butyl and *tert*.-butyl oligostyrenes on (a) the Valupak C_{18} column and (b) the carbon clad zirconia column. Flow rates = 1 ml/min, UV detection at 262 nm, injection volume = 10 µl, both columns thermostated at 25 °C.

Each bar represents the normalised 2D elution time of an individual isomer. A total of 26 out of the 32 isomers were resolved in this system. This represents a very high degree of resolving power when the complexity of the sample is considered.

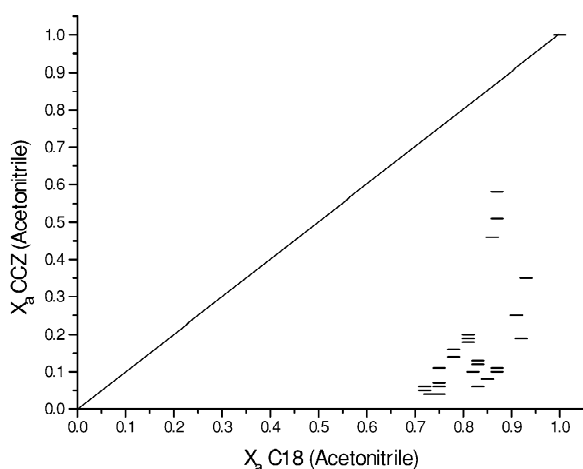Least squares and other correlation methods are

Fig. 3. Normalised two-dimensional plot of the C_{18} (acetonitrile)/ CCZ (acetonitrile) system in the separation of the 32 oligostyrene isomer mix.

primarily designed for determining the degree of data correlation, not for measuring data scatter. In contrast, tools of informational theory (IT) permit the description and quantification of data scatter through entropy calculations based on the ''bit'' unit of measure. The degree of data overlap in IT, which is known as ''informational similarity'', provides a means to measure the informational orthogonality of coupled systems. The assessment of informational orthogonality of chromatographic separation systems has been comprehensively reviewed by Slonecker et al. [37]. In this IT approach, individual one-dimensional separations of a molecular probe are evaluated, the mutual retention components between each

system are determined, and the corresponding independent retention behaviour is calculated [37]. The informational similarity of the two-dimensional separation can then be computed [37] which measures the degree of the solute crowding in the separation space. A value of unity indicates high solute crowding, while a value of zero indicates no solute crowding with a high degree of utilization of the separation space [37]. The extent of solute crowding can be visualised in the plot of normalised retention factors as shown in Fig. 3. For this 2D system the informational similarity was 0.92 (see Table 1) indicating a high degree of solute crowding, reflecting the grouping of the isomers in the lower right section of the plot. We must note that in the calculation of solute crowding, bands were deemed to be separated when their respective X_a factors differed by greater than 0.01 X_a in accordance with the work of Slonecker et al. [37].

Another tool of IT, which is used to determine retention mechanism equivalence in a multi-dimensional chromatographic system, is the percentage synentropy. The % synentropy is a measure of the percentage of 2D informational entropy that is contributed equally from each dimension. An estimation of the % synentropy may be calculated as the percentage of the normalised retention data (X_a factors) that are diagonally aligned on the normalised retention plot. The % synentropy is determined by dividing the informational entropy of the data diagonally aligned on the normalized retention plot by the total two-dimensional informational entropy. A % synentropy of 0% describes a 2D system that

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

Attribute	2D systems				
	$C_{18}A/CCZA$	$C_{18}A/CCZM$	$C_{18}M/CCZA$	$C_{18}M/CCZM$	CCZA/CCZM
Informational similarity	0.92	0.93	0.56	0.62	0.99
Percentage synentropy	3.5	3.4	3.3	3.3	86[a]
Peak spreading angle (β) degrees	41.5	37.4	74.7	70.1	8.9
Theoretical peak capacity (N_t)	280	308	60	66	440
Practical peak capacity (N_p)	156.0	159.6	53.8	57.3	64.1
Correlation (c)	0.75	0.79	0.26	0.34	0.99
(% Usage)	55.7	51.8	89.7	86.7	14.6
Resolved components (/32)	26	26	26	28	25

[a] The informational entropy for the calculation of percentage synentropy was included assuming an X_a factor of variance for correlated data equal to ±0.05 of the normalized retention factor (X_a).

has no correlation between each of the individual dimensions and thus exhibits no retention mechanism equivalency. In contrast, a % synentropy of 100% describes a 2D system in which there is complete correlation between both dimensions of the system, which thus exhibits complete retention mechanism equivalency. A practical application of the IT equivalency technique is for the comparison of retention behavior of stationary phases produced by the same and by different manufacturers [39].

In the 2D separation system described above the % synentropy was only equal to 3.5%. This indicates that the individual dimensional retention mechanisms were not equivalent with this molecular probe. If they were equivalent, the data would have been diagonally aligned. The high 2D informational similarity value of 0.92 describes a 2D chromatographic system comprised of 1D systems whose retention behavior is not orthogonal. This is entirely reasonable because less than one quadrant of the 2D separation space contains all of the scattered data. Only one data point was aligned along the main diagonal.

A different approach useful in describing the orthogonality of two-dimensional chromatographic systems is to utilize the chromatographic resolving power to estimate the divergent nature of the 2D retention behaviour. Therefore, in order to fully evaluate the extent of divergent retention behaviour a geometric approach to factor analysis, as described by Liu and Patterson [40], was applied to the data set. In this method the practical peak capacity of the separation space can be readily calculated from correlation matrices and then visualised by plots of the peak capacity in one dimension against the peak capacity in the second dimension. A rectangular plot, the axes of which correspond to the peak capacity in each dimension, illustrates the effective separation space utilized in the separation process. Fig. 4 illustrates the retention relationship for the C_{18} (acetonitrile)/CCZ (acetonitrile) 2D system. The spreading angle (β) depicted in Fig. 4 is a measure of the separation space utilization. A spreading angle of 90° indicates complete utilisation of the theoretical separation space. As the dimensions become more correlated the spreading angle decreases and the effectiveness of the two-dimensional separation decreases. For the C_{18} (acetonitrile)/CCZ (acetonitrile)
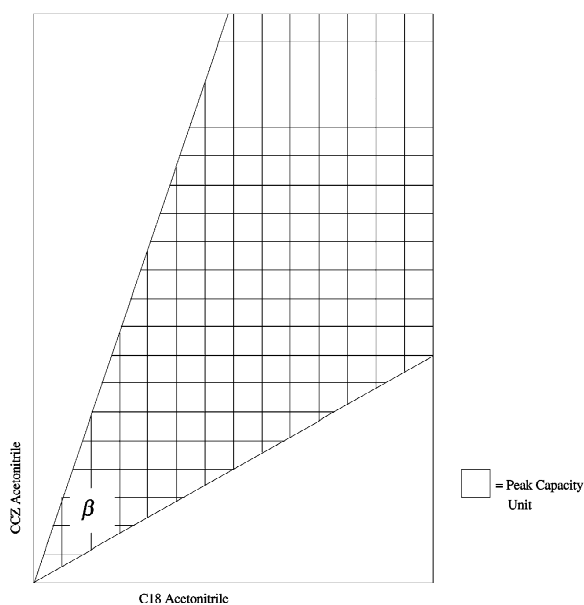

Fig. 4. Geometric plot showing the practical two-dimensional peak capacity of the C_{18}(acetonitrile)/CCZ(acetonitrile) separation system in the separation of the 32 isomer mix.

system the spreading angle was 41.5°. The peak capacity of each separation dimension was calculated from the normalised retention data where each normalised peak that was separated by a normalised retention factor of 0.01 was given a value of one peak capacity unit. Multiplication of the peak capacities of the constituent separation steps in each of the two-dimensional separations resulted in the theoretical two-dimensional peak capacity. The practical peak capacity of the system was measured to be 156, while the theoretical peak capacity was 280. This represents an overall 56% usage of the available separation space and correlation between each dimension was 0.75 (1.00 = perfect correlation, 0.00 = perfectly orthogonal) (Table 1).

The next system that was evaluated employed methanol as the mobile phase in the carbon clad zirconia dimension. Again, like the C_{18} (acetonitrile)/CCZ (acetonitrile) system, both separation dimensions of the C_{18} (acetonitrile)/CCZ (methanol) system displayed selectivity towards diastereoisomers, with the CCZ (methanol) dimension having a greater diastereoisomer selectivity. The normalised retention plot (not shown) for this sepa-

ration combination was almost identical to the retention plot shown in Fig. 3. Not surprisingly, very similar results to the C_{18} (acetonitrile)/CCZ (acetonitrile) system were observed. A total of 26 isomers were separated and the informational similarity was equal to 0.93, which signifies a high degree of data overlap and clustering. A geometric factor analysis revealed that the peak spreading angle decreased marginally to 37.4°, and the peak capacity of the system remained almost constant (160). The theoretical peak capacity of this system was 308. A total of 52% of the separation space was utilised, which was slightly less than the C_{18} (acetonitrile)/CCZ (acetonitrile) system. Correlation between each dimension was almost the same as for the previous system (0.79). The high correlation between each of the single dimensions in both these 2D systems is due to the diastereoisomer selectivity afforded in each of the single dimensions. From a practical aspect, the diastereoisomer resolution on the CCZ (acetonitrile) system was substantially greater than the C_{18} (acetonitrile) system. Nevertheless, both these 2D systems could be used almost interchangeably, but the determining factor as to which 2D system would be most appropriate as a coupled system would be

dictated by separation time and solvent compatibility. In this respect the C_{18} (acetonitrile)/CCZ (acetonitrile) 2D system would be more suitable because the total analysis time was shorter and acetonitrile was common to both columns.

A more interesting effect on the separation space of the two dimensional system resulted when the first dimension was changed from C_{18} (acetonitrile) to C_{18} (methanol). No diastereoisomer separation was observed in the C_{18} (methanol) dimension, but structural isomers were partially resolved (which is apparent in the plots shown in Fig. 6). Consequently, eight *tert*.-butyl oligostyrenes co-eluted on the C_{18} column, 16 *sec*-butyl oligostyrenes co-eluted and eight *n*-butyl oligostyrenes co-eluted. In contrast, the CCZ column employing an acetonitrile mobile phase allowed the full resolution of all diastereoisomers of the *sec*-butyl and *n*-butyl oligostyrenes, and seven of the eight *tert*.-butyl oligostyrenes (An example of the diastereoisomer separation for the *n*-butyl oligostyrenes is shown in Fig. 5). There was, however, considerable overlap in their retention time windows, which would make the separation of this mixture impossible in a single dimension as shown also in Fig. 2b. This is also depicted in the plot of X_a
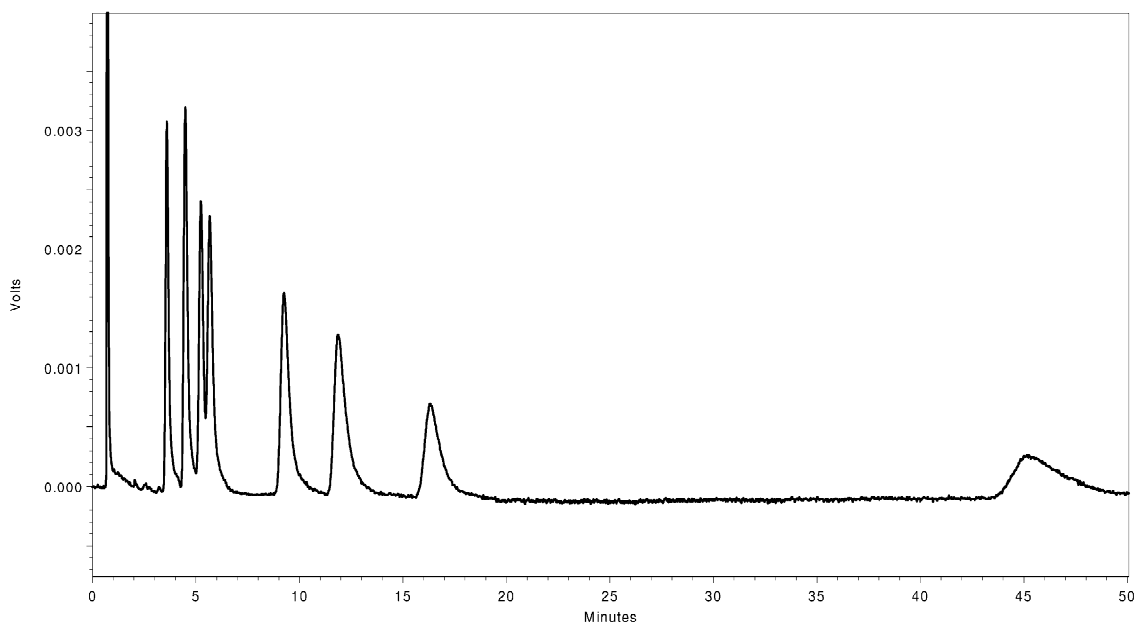

Fig. 5. Stereoisomer separation of the $n = 5$ *n*-butyl oligostyrenes on the carbon clad zirconia column. Mobile phase: 100% acetonitrile, flow-rate = 1 ml/min, UV detection at 262 nm, injection volume = 10 µl, column temperature thermostated at 20 °C.
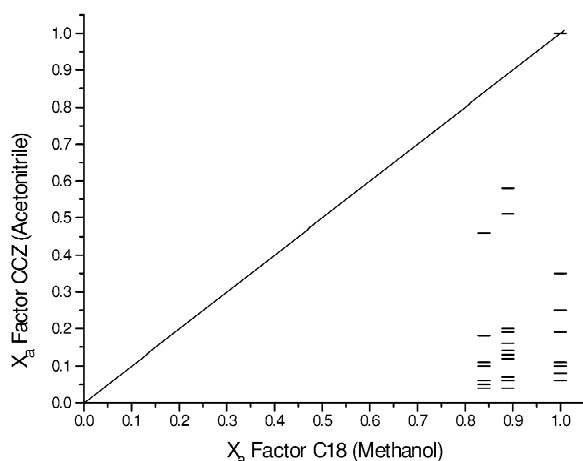
Fig. 6. Normalised two-dimensional plot of the C_{18}(methanol)/ CCZ(acetonitrile) system in the separation of the 32 oligostyrene isomer mix.

factors shown in Fig. 6 for the C_{18} (methanol) and CCZ (acetonitrile) 2D system (note the separation is less chaotic). Despite the higher degree of order within the 2D separation space, the number of isomers separated remained at 26 out of the possible 32. The increased order of the C_{18} (methanol) system decreased the degree of solute crowding in the 2D system (for this system the informational similarity was 0.56). In comparison, partial diastereoisomer separation was observed in the previous 2D systems that employed acetonitrile as the eluent for the C_{18} column. Because the elution order of the diastereoisomers on the CCZ column and the C_{18} column was different, the re-organisation of the elution order increased the solute crowding. This was not so when methanol was the eluent on the C_{18} column, so no band reordering took place, and hence a less chaotic displacement of bands occurred, which was ultimately reflected in the informational similarity. Geometric factor analysis of this 2D system revealed that the total peak capacity for this coupled system would be expected to be lower. In total, the theoretical peak capacity was 60—approximately one third of the previous two systems in which C_{18} (acetonitrile) was employed and a slight degree of diastereoisomer selectivity was apparent. However, the actual utilization of the separation space increased, with a spreading angle equal to 74.7° (see Fig. 7). The practical

peak capacity was now only 53.8, but this represented a 90% usage of the available separation space and correlation between the two individual dimensions decreased to 0.26 (Table 1). The decrease in correlation between the single dimensions reflects the fact that diastereoisomers are no longer separated in the C_{18} (methanol) system. The overall reduction in total peak capacity is due to the reduction in separation power of the C_{18} (methanol) system. It is interesting to note, however, that because of the increased order in this system the practical peak capacity almost reached the theoretical limits.

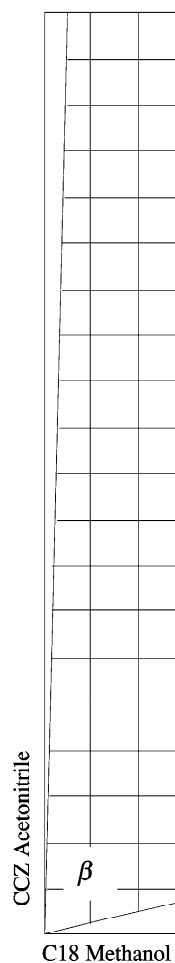Similar results were obtained in a 2D system that

Fig. 7. Geometric plot showing the practical two-dimensional peak capacity of the C_{18}(methanol)/CCZ(acetonitrile) separation system in the separation of the 32 isomer mix.

employed methanol as the mobile phase for both the C_{18} and CCZ columns. The number of separated components increased to 28 out of 32. However, despite this increase the solute crowding also increased slightly such that the informational similarity was 0.62. Geometric factor analysis revealed the peak spreading angle to be 70.1° and the practical peak capacity was 57.3, with a theoretical peak capacity equal to 66. Space utilisation was 87% and correlation between the two single dimensions was 0.34. Like the C_{18} (methanol)/CCZ (acetonitrile) combination, the resolving power approached the theoretical limit of 66 (Table 1).

The evaluation of system orthogonality is perhaps most appropriately determined using multiple methods of evaluation, particularly when the sample population contains a large number of very closely related members. In this work informational similarity was used to measure the extent of system orthogonality and the degree of global solute crowding. Informational similarity measurements of the C_{18} (acetonitrile)/CCZ (acetonitrile), C_{18} (acetonitrile)/CCZ (methanol) and CCZ (acetonitrile)/CCZ (methanol) 2D systems indicate limited potential of these systems for future coupled 2D separations because most of the data was clustered in one quadrant of 2-dimensional space. Informational similarity measurements of the C_{18} (methanol)/CCZ (acetonitrile) and C_{18} (methanol)/CCZ (methanol) 2D systems indicate a higher potential of these systems for future coupled 2D separations. Geometric factor analysis was also employed to evaluate the potential 2D resolving power. Calculation of theoretical peak capacities and practical peak capacities also gave a quantitative comparison of the resolving power of the 2D systems. The greatest utilisation of separation space was achieved when the separation theoretical peak capacities were small (i.e. C_{18} (methanol)). Then, the practical peak capacity closely approached the theoretical peak capacity, largely due to the limited peak capacity of the C_{18} (methanol) dimension. The correlation between each dimension also decreased accordingly. But, as this example demonstrated, a high utilisation of separation space does not necessarily mean that these systems would be best suited as a coupled system. The practical experimental outcome must also be considered. In that respect, the practical resolving powers of all four systems were essentially identical. Between 26 and

28 of the 32 isomers could be resolved in each system. The choice of which system would be best coupled to produce a 2D system comes down to the selection criteria. Factors that influence this decision are numerous, and should be weighted according to the analyst's preferences. They may be, for instance, analysis time considerations in which case the C_{18} (methanol)/CCZ (acetonitrile) system would be preferable. Or, preference may be given to the maintenance of elution order (as opposed to chaotic band displacement) in which case the C_{18} (methanol)/CCZ (acetonitrile) or the C_{18} (methanol)/CCZ (methanol) systems would be more suitable. A third selector may be the practical resolving power. In this case the C_{18} (methanol)/CCZ (methanol) system was the most effective because this system separated the most components in this analysis. A fourth selector may be the maximum theoretical peak capacity, in this case the C_{18} (acetonitrile)/CCZ (methanol) system. In such a system, the analysis of more complex samples may feasible, but the correlation between these separation steps is high, which would ultimately limit its potential.

It is interesting, by way of comparison, to evaluate the effective separation power of a ''2D system'' in which the retention mechanisms of each individual system are highly correlated or equivalent: For instance, a ''2D system'' that contains a CCZ column in each dimension, but the mobile phase in the first dimension is methanol and in the second the mobile phase is acetonitrile. A high % synentropy (86%) was obtained as shown by the alignment of data along the main diagonal in the X_a factor plots (see Fig. 8 and Table 1). The informational similarity increased to almost one, indicating a high degree of solute crowding, which is particularly apparent for the less retained isomers. Geometric factor analysis indicated that the peak spreading angle was very poor (8.9°) as the data were aligned essentially along the main diagonal resulting in a correlation between the two dimensions being equal to 0.99. The practical peak capacity (64) was very much less than the theoretical peak capacity (440), which represented a 14.6% utilisation of the separation space (Table 1).

4. Conclusion

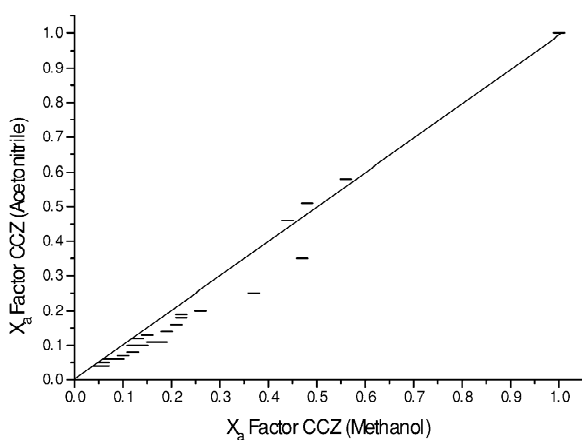We set out to evaluate the resolving power of 2D

Fig. 8. Normalised two-dimensional plot of the CCZ(methanol)/CCZ(acetonitrile) system in the separation of the 32 oligostyrene isomer mix.

reversed-phase-reversed-phase systems for the separation of an artificial sample population consisting of 32 closely related isomeric species, differing according to two factors—structural and stereochemistry. The separation problem so chosen was very complex, one that a single dimensional separation could not possibly resolve. Furthermore, resolving these components in a retention process governed solely according to reversed-phase principles is one that we considered a difficult task. However, by utilising the very different retention characteristics between the conventional C_{18} stationary phase and the recently developed CCZ stationary phase this complex separation problem in fact became almost trivial.

The results of this study reinforce two very important chromatographic concepts: (1) multidimensional separations can lead to exceptional resolving power; and (2) the development of new stationary phase surfaces is extremely important for realising the separation of complex mixtures.

Furthermore, we have shown that it is possible to describe a 2D separation in which both phases of the system incorporate fundamentally the same technique (i.e. RP–RP). This lends itself nicely to the development of separations in which solvent compatibility between each dimension of the separation proves to be an issue in the separation process.

Lastly, we illustrated the importance of employing multiple methods of data analysis for the establishment of orthogonality, and that evaluation of the

system using these methods must be made in conjunction with the ideals of the experimental outcome.

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