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Retention time reproducibility in comprehensive two-dimensional gas chromatography using cryogenic modulation An intralaboratory study

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Abstract

A survey was conducted to determine the reproducibility of retention times in both the first (D1) and second dimension (D2) axes of the two-dimensional separation space, in the comprehensive two-dimensional gas chromatographic analysis of an essential oil sample using cryogenic modulation. The retention times in the two dimensions for a number of individual components comprising hydrocarbon, alcohol, ester and ketone chemical classes in a *Melaleuca alternifolia* essential oil were recorded from replicate analyses using four separate column sets and two identical gas chromatographs. Run-to-run, day-to-day, instrument-to-instrument, and column set-to-column set reproducibility were demonstrated from the experimental design. A total of 60 GC×GC analyses were conducted. The longitudinally modulated cryogenic system produced reproducible modulation start times and consistent modulation phase profiles for individual components in all experiments, and retention time variations in both dimensions were negligible. The average run-to-run reproducibility of 43 components for six replicate injections was found to be 0.12% RSD in the first dimension, and 0.74% RSD in the second dimension. Day-to-day reproducibility showed statistically ''significant'' difference (*F*-test), but this was partly ascribable to the excellent within-day reproducibility that led to apparent day-to-day differences. Confidence in absolute retention times (hence component positions) in the two-dimensional separation space is critical to component identification.

Keywords: Gas chromatography, comprehensive two-dimensional; Retention time; Cryogenic modulation; Reproducibility

1. Introduction

Comprehensive two-dimensional gas chromatography (GC×GC) is a high-resolution GC technique which has gained increasing attention over the last decade. GC×GC is unrivalled for the analysis of highly complex samples, which may contain many hundreds, and in the case of some petroleum samples, thousands of individual components. The benefits of GC×GC have been described in a number of review articles [1,2] and related publications. In the GC×GC experiment the entire sample is subjected to a chromatographic separation on two independent capillary columns. These capillary columns are comprised of different stationary phase coatings, and in a properly tuned GC×GC analysis an orthogonal two-

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dimensional separation is achieved. Thus the first advantage of GC×GC over conventional single column gas chromatography is that the peak capacity is increased from n (the peak capacity of a single column), to $n_{D1} \times n_{D2}$ (the product of the individual peak capacities of the first and second dimension columns, respectively). The resulting GC×GC chromatograms are generally presented as contour plots, where the two-dimensional (2D) separation space is defined by the range of D1 retention time and the modulation period (commonly referred to as D2 retention time). Peak intensity (height) can be represented as contour lines or as continuous coloured contour bands. These 2D chromatograms are highly organised, and identification of classes of related compounds in a sample is often possible. This presents an obvious (second) advantage in the analytical assignment of components (or groups of components) in complex samples through their location in the 2D space. The third advantage of GC×GC is an increase in signal intensity, which is a consequence of a modulation process between the two capillary columns. Modulation of the first column effluent into the second column is key to the GC×GC technique, and can be performed a number of ways. Where the modulation process utilises zone compression, increases in signal response of 10–30 times are often reported [3–5]. Numerous devices for performing modulation and hence GC×GC have been described in the literature; a brief account of the main types of modulators is given below.

1.1. Thermal modulators

Thermal modulators comprise of either heated or cryogenic operation. Both types have been reported to produce more-or-less equivalent results [6,7]. The thermal sweeper heated modulator was reported to be robust [4], and uses a rotating slot heater (maintained at ~100 °C above the oven temperature) passing over a thick film capillary column (modulator tube) positioned between the D1 and D2 columns. Solute bands are swept out of the modulation tube as the heated slot passes over the tube, resulting in a series of sharp injection bands into the D2 column. Cryogenic modulators achieve modulation by cryo-focussing the D1 effluent as it enters the D2 column [8]. The cryogenic trap is maintained at ~100 °C or more below the oven temperature [7,9]. A jet cryogenic modulator has also been described which sprays liquid CO_2 directly onto the capillary column [10]. Sharp injection bands are delivered to the D2 column in all cases by alternately cooling and heating a small section of the column, where the heat is produced by the stirred heated GC oven.

1.2. Valve/diaphragm modulators

Valve modulators employ valves, such as a six-port diaphragm valve, to direct a portion of the D1 effluent into the D2 column. The first valve modulator operates by briefly diverting the D1 effluent to the second column [11], hence only a small fraction of each component is pulsed to the D2 column [2], however it is reported to consistently deliver highly reproducible results and has been used for the development of chemometric data analysis routines for GC×GC [11]. In a later design ~80% of the injected sample reaches the detector by using differential flow modulation through a sample loop [12]. These modulators are suitable for the analysis of volatile organic compounds (VOCs), but have relatively low upper temperature limits (for instance less than 200 °C) [12]. Thermal modulators are suited to analysis of higher boiling compounds, but can also be used for VOCs, with the cryogenic type thermal modulators having higher GC operating temperature ranges than heated modulators [7].

Thus different modulators, all which should be capable of delivering similar GC×GC separation performance, are available. However the general reproducibility of a GC×GC system must be established if it is to be used for identification purposes, especially in terms of absolute analyte retention on each column in the experiment.

The present investigation used the longitudinally modulated cryogenic system (LMCS) approach [13], to perform all GC×GC analyses with the aim of quantitatively investigating the reproducibility of GC×GC peak retention times using the LMCS. Retention time consistency is important if the GC×GC data are to be used for pattern recognition, or if reliable identification of individual components is to be made on the basis of peak position in the 2D space (e.g. without the use of mass spectral data). Absolute retention times in the 2D plot require a

precise relationship between modulator start time and data conversion to matrix format. If this is not accurate, peak positions in the 2D space will vary [14]. In particular, it is not always apparent in the literature if retention alignment is used for various modulators, or if relative retentions as opposed to absolute retention are reported. Here, no adjustment is made. This investigation may also be used to gauge the suitability of GC×GC for routine complex sample analysis where known components in a standard may be used to confirm sample component identity. Note that conventional analysis often requires GC–MS authentication especially where components are incompletely resolved. The superior resolution capabilities of GC×GC can provide better quantitative data, but assignment of individual component identity is not possible if retention time variations are large. Initial characterisation of target components within the 2D separation space will be required, by injection of authentic reference standards. This paper will describe an intralaboratory comparison of four different column sets using two GCs for analysis of *M. alternifola*.

2. Experimental

GC×GC analyses were performed using two Agilent Technologies 6890 model gas chromatographs (Agilent Technologies, Burwood, Australia). Each GC system was equipped with flame ionization detection (FID; operated at 100-Hz data acquisition frequency) and Chemstation software. Both GCs were retrofitted with different Everest model LMCS units (Chromatography Concepts, Doncaster, Australia), which use a mechanical stepper motor drive for movement of the cyrotrap. A modulation frequency of 0.2 Hz (5 s cycle) was applied in all analyses and the thermostatically controlled cryogenic trap was maintained at ~0 °C. The Agilent Chemstation software is used to signal the electronic control module to commence modulation at a precise time. This module then controls the period of modulation independent of signal acquisition. Post acquisition conversion of data to two-dimensional array format uses the modulation period and detector acquisition rate.

Four identical column sets were used for the GC×GC analyses. Each set consisted of two columns, which were serially coupled by a zero-dead-volume fitting. The primary column in each column set was a low-polarity BPX5 (5% phenyl equivalent polysilphenylene-siloxane; 0.25 μm film thickness) fused silica capillary column of dimensions 30 m× 0.25 mm. The second column in each column set was a polar BP20 (polyethylene glycol; 0.10 μm film thickness) fused silica capillary column of dimensions 1.0 m×0.10 mm. All columns were from SGE International (Ringwood, Australia). The 1 m BP20 column lengths were taken from a single 10 m column.

For each analysis, the GC system was operated under temperature programmed conditions from 60 to 210 °C at 2 °C min^{-1}, then to 260 °C at 20 °C min^{-1}. Both GC systems were equipped with a split/splitless injector; an injection volume of 1.0 μl was employed using a series 7673 autosampler (Agilent Technologies), and a split ratio of ~100:1 was used. The carrier gas was hydrogen, and the column head pressure was 52 kPa. Each GC system was operated in constant pressure mode. No further steps were taken to adjust set conditions on the two GC systems.

2.1. Sample

The sample used in this investigation was a commercially available *Melaleuca alternifolia* essential oil from Thursday Plantation Labs. (Ballina, Australia). The oil was used as purchased and diluted 1:10 (v/v) with *n*-hexane prior to analysis.

2.2. Intralaboratory study

Table 1 details the range of analyses performed in

Table 1
Summary of experiments performed in the intralaboratory study

Column set	GC 1 number of replicates	GC 2 number of replicates
1	6	6
2	6	6
3	6 (×3)[a]	6
4	6	6

[a] Day-to-day experiments were performed on Friday, Saturday and Monday.

this study. In general, six replicates were performed in order to obtain adequate statistical comparisons. Each column set was used in both GC systems (therefore being also at different times), and one study involved replication of results at different times of the week.

3. Results and discussion

Data reported below were extracted from Chemstation integration results, using a program available in the laboratory that was executed using Matlab Release 12.1 (Mathworks, Natick, MA, USA). D2 retention times were determined directly from the integration results. D1 retention times were calculated based on the fitting of a normal distribution function to the pulsed peak profile of each component. Where component wrap-around occurred (i.e. a component is retained longer than 5 s on D2), it was necessary to adjust the D1 and D2 retention times (^1t_R and ^2t_R, respectively). In a manner similar to that described by Seeley et al. [15], 5.0 s was added to the apparent ^2t_R of components whose true ^2t_R was >5.0 s, and 5.0 s was subtracted from the ^1t_R of those components. Note that these values will still be absolute retention times.

3.1. Run-to-run repeatability

Using column set 1 (CS 1) and GC 1, six replicate GC×GC analyses were performed on the *M. alternifolia* essential oil sample. A total of 43 individual components from the sample were selected for comparison and reporting of ^1t_R and ^2t_R. The relative positions of the peak apexes of the 43 components are shown in Fig. 1. A wide boiling point range (given by the ^1t_R range) and also a wide range of component polarity (indicated by the ^2t_R range) is apparent. The 2D separation space scatter plot for each analysis is given in Fig. 1, and the excellent run-to-run retention time reproducibility in D1 and

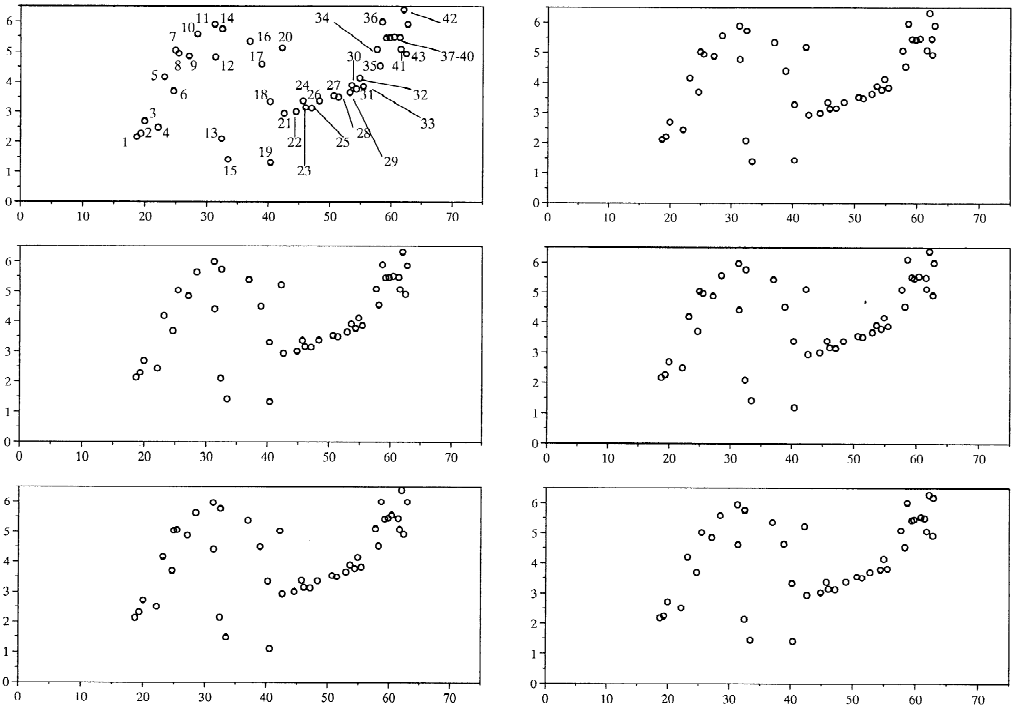

Fig. 1. 2D separation space scatter-plots showing the positions of the peak apexes of the 43 components reported in Table 1, from six replicate GC×GC analyses. Note that the D2 retention time axis is expanded to 6.5 s in order to present peaks with ''wrap-around'' (see Fig. 2 below).

D2 is demonstrated. The mean and relative standard deviation of each component retention time was calculated; for D1 the average RSD was less than 0.2%, and for D2 the average RSD was less than 1%. The calculated values for each of the 43 components are given in Table 2.

The total peak area (volume) of each component was also calculated, by summing the peak area reported by the Chemstation software of the pulsed peaks corresponding to each specific component. Generally, the deviation from the mean D1 and D2 peak apex position was greater for major compo-

Table 2
Run-to-run repeatability from six replicate analyses of *M. alternifolia* essential oil using column set 1 and GC 1

Peak number	Mean ^1t_R (min)	RSD (%)	Mean ^2t_R (s)	RSD (%)
1	18.72	0.05	2.150	1.20
2	19.38	0.08	2.268	1.67
3	19.99	0.01	2.695	0.68
4	22.17	0.03	2.478	1.26
5	23.21	0.01	4.178	0.42
6	24.68	0.01	3.688	0.32
7	25.13	0.54	5.031	0.10
8	25.48	0.01	4.992	1.09
9	27.16	0.01	4.860	0.38
10	28.50	0.01	5.589	0.63
11	31.29	0.01	5.946	0.69
12	31.40	0.02	4.569	4.08
13	32.32	0.07	7.109	0.37
14	32.46	0.01	5.749	0.30
15	33.36	0.01	6.428	0.51
16	36.98	0.03	5.367	0.68
17	38.88	0.10	4.521	1.81
18	40.26	0.08	3.330	1.14
19	40.32	0.20	6.294	1.96
20	42.19	0.11	5.147	1.50
21	42.60	0.03	2.939	0.29
22	44.60	0.34	3.007	0.68
23	46.06	0.07	3.147	0.47
24	45.67	0.10	3.371	0.44
25	47.07	0.07	3.133	0.32
26	48.41	0.49	3.370	0.37
27	50.66	0.02	3.534	0.50
28	51.42	0.06	3.503	0.53
29	52.93	0.37	3.659	0.75
30	53.60	0.03	3.897	0.28
31	54.36	0.06	3.774	0.50
32	54.85	0.07	4.136	0.51
33	55.46	0.08	3.839	0.49
34	57.70	0.05	5.083	0.35
35	58.20	0.10	4.532	0.31
36	58.63	0.10	5.990	1.16
37	59.26	0.13	5.450	0.59
38	59.78	0.10	5.541	0.28
39	60.54	0.29	5.514	0.73
40	61.62	0.60	5.470	0.42
41	61.65	0.14	5.077	0.37
42	62.07	0.07	6.342	0.66
43	62.55	0.21	4.914	0.15
Mean		0.12		0.74

nents, and such deviations can be attributed to overloading in either (or both) capillary columns. The effects of non-linear chromatographic peaks in GC×GC have been reported elsewhere [16], and the present observation is consistent with the findings presented in the former publication. Accurate co-ordinates for these components could be determined (at the expense of trace components falling below the detection limit) by repeating the analysis with a more dilute sample. The occurrence of a small number of non-linear chromatographic peaks is difficult to avoid for the complete analysis of the present sample, since the three to four major components account for more than 50% of the total sample. For sample components of intermediate concentration (non-overloaded peaks), i.e. less than ~10% of the total sample mass, the repeatability of peak positions was excellent.

A total of 19 components were chosen from those in Fig. 1 for further reporting. These components were selected such that a representative number of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes were present in the data set, ensuring that the volatility and polarity ranges of the entire sample were represented. Additionally, major components and some of low abundance were represented. The 19 components are shown in the 2D separation space scatter-plot (Fig. 2) where the clusters of class-related compounds are clearly visible.

Further experiments were performed by installing CS 1 into the second instrument (GC 2), then CS 2, CS 3, and CS 4 into each instrument (Table 1). Average RSD not greater than 0.2% was observed for $^{1}t_{R}$ and RSD not greater than 1% was observed for $^{2}t_{R}$ for the run-to-run comparison of results from any individual column set and instrument. Hence for a given column set it is possible to attain highly reproducible results.

3.2. Day-to-day repeatability

With CS 3 installed in GC 1, the between-day retention time variability was determined. Six consecutive runs were performed on Friday afternoon (when the building gas supply was under normal use), six on Saturday morning (when the building gas supply was under low use), and six on Monday morning. These times were chosen such that the maximum variation (if any) in the building gas supply could be expected; variations in laboratory temperature were also expected as the air conditioning system was not operated over the week-end. All gases are obtained from a centrally-reticulated building supply, and although instrument control of gases to the column and detector should be precise, this study should represent maximum routine variation in the central supply. It also illustrates the reproducibility of implementing analysis conditions from overnight shut-down conditions.

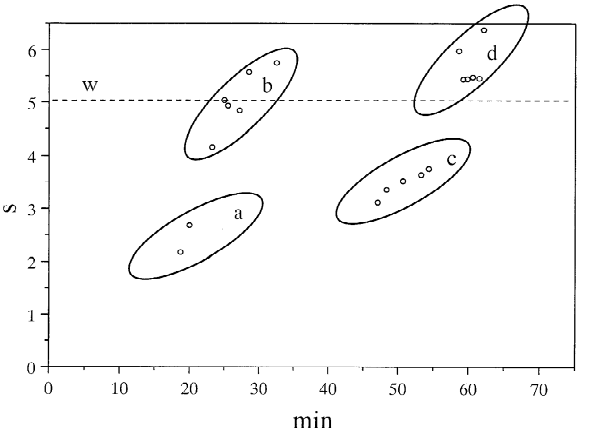

Fig. 2. 2D separation space scatter-plot showing the positions of the peak apexes of 19 *M. alternifolia* components, and the apparent clustering of class-related compounds. The dotted line (w) at 5.0 s represents the wrap-around boundary. (a) Monoterpene hydrocarbons; (b) oxygenated monoterpenes; (c) sesquiterpene hydrocarbons; (d) oxygenated sesquiterpenes.

Mean retention times (from each day) of each of the selected components were compared, to test if they differed significantly, using analysis of variance (ANOVA). These results are summarised in Table 3, revealing that many of the peak positions *do* differ significantly, in statistical terms at least, especially the D1 retention times. ANOVA simply determined that the between-day variance was greater than the within-day variance. Since the within-day variance is extremely good, the ANOVA statistic may suggest an apparently poor peak position reproducibility between days, even if the observed variation might be considered acceptable for general gas chromatographic instrumentation. Thus by overlaying either

Table 3
Mean retention times and calculated F-values for the day-to-day reproducibility experiments

Peak	F (^1t_R)	Sig. different	F (^2t_R)	Sig. different
1	2.52	×	1.59	×
3	12.08	✓	1.17	×
5	30.38	✓	4.70	✓
7	18.21	✓	1.22	×
8	25.14	✓	4.46	✓
9	12.43	✓	1.97	×
10	6.81	✓	2.43	×
14	12.08	✓	23.88	✓
25	4.20	✓	4.66	✓
26	0.58	×	1.08	×
27	45.83	✓	15.15	✓
29	11.66	✓	6.43	✓
31	3.14	×	0.56	×
36	1.20	×	6.38	✓
37	1.20	×	1.13	×
38	1.44	×	5.51	✓
39	2.10	×	2.61	×
40	0.67	×	0.87	×
42	0.39	×	3.46	×

The critical value for F is 3.682 ($P=0.05$) [22]. If the calculated value of F is greater than 3.682, then the sample means differ significantly. Note the discussion in the text for interpretation of significantly different data.

contour plots or unconverted chromatograms, the positions of the individual contour peaks and the retention times of the individual pulses from which they were derived were observed to correspond very closely, both within day and between days. Fig. 3 shows the overlay of the mean retention times of 19

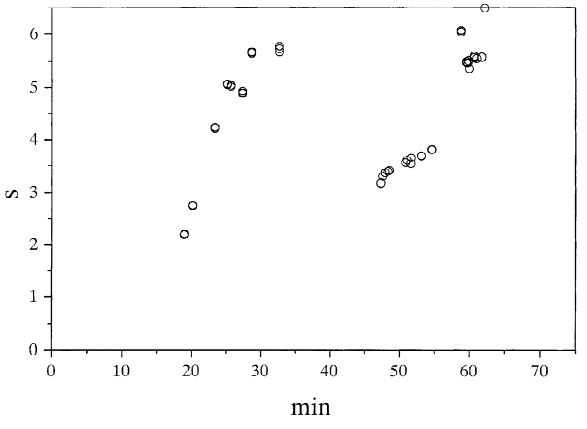

Fig. 3. Overlay plot of mean retention times illustrating between-day reproducibility (3 different days).

components from each of the three sets of experimental data. Further calculations were made to determine the absolute difference between the retention times, both within and between days. The retention time reproducibility of most component peaks was within plus or minus one modulation period in D1 (0.08 min). Peak positions differ by less than a few retention index units, which would generally be sufficient to confirm identity in single column analysis, as this is an accepted variation in index units for a component. Again the components of greater abundance were susceptible to the reporting of different retention times. Fig. 4 demonstrates that the peak pulse positions, and the pulse distribution profile are maintained for replicate analyses on different days.

The component contour positions in D2 were all reproducible within $\pm 2\sigma$ of the D2 peak width, and most were found to be reproducible within $\pm 1\sigma$ of the D2 peak width. The mean D2 peak width was 150 ms (4σ) with all D2 peak widths obtained directly from the Chemstation integration result.

3.3. Instrument-to-instrument repeatability

To investigate the repeatability between instruments, data acquired on GC 1 and CS 3 were compared directly with data acquired on GC 2 using the same column set. Fig. 5 shows the overlaid scatter plots (mean positions from six analyses) from each instrument. The excellent correlation of the two plots demonstrates that by removing and re-installing columns, very little or no problems with respect to installation and alignment of the columns in the modulating device were observed. Indeed it is the general experience of this group that columns can be quickly and easily removed and stored for later use, or installed into another instrument without affecting chromatograms. Importantly this means that new results can be compared directly with historical data.

3.4. Column set-to-column set repeatability

Finally the effect of different column sets on the position of peaks within the 2D separation space was investigated. Four different column sets were used. Fig. 6 illustrates the column set-to-column set reproducibility. Variation in ^1t_R was apparent between

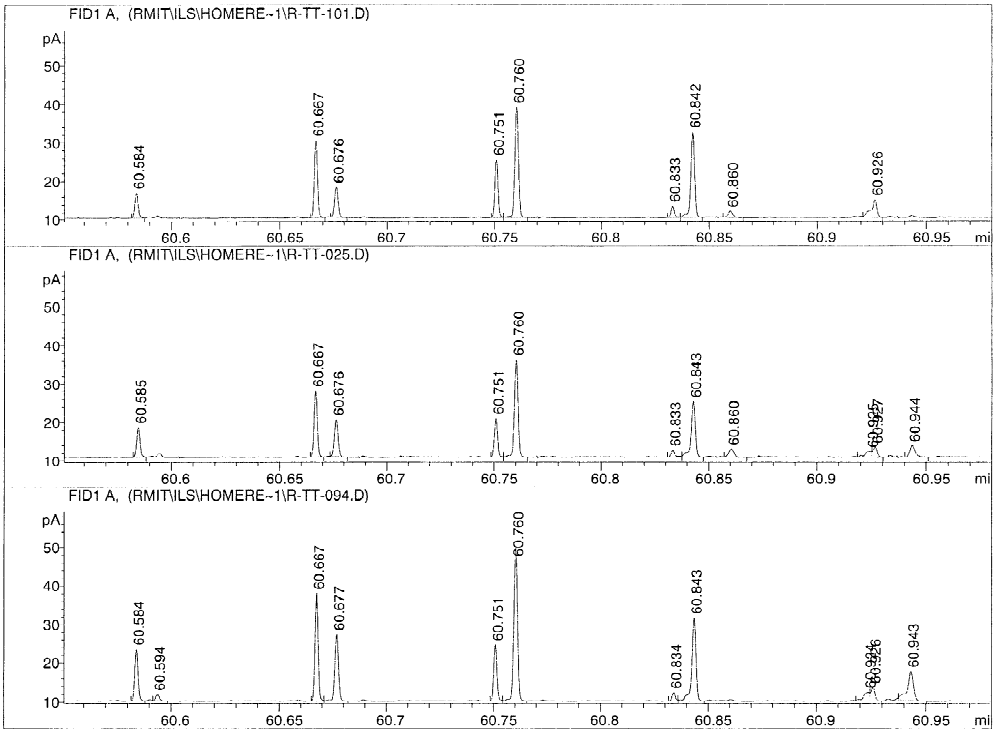

Fig. 4. Expanded peak pulses for component 39 and a co-eluting component, demonstrating consistent modulation phase profile leading to a reproducible 2D peak position. A shift in modulation phase profile or imprecision in modulation start time would result in a shift in the peak's position in the 2D plane. Data were acquired on different days.

column sets and is likely to have been caused by the columns having slightly different length, I.D., and/or phase thickness. To gauge the maximum expected variation in absolute terms, no adjustments were made to the carrier gas flow rate, i.e. the columns were not calibrated. However, the column sets can easily be calibrated by determining the retention time

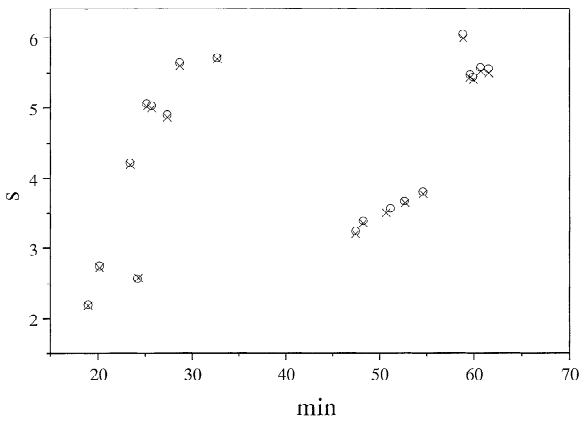

Fig. 5. Overlay plot of mean retention times illustrating instrument-to-instrument reproducibility.

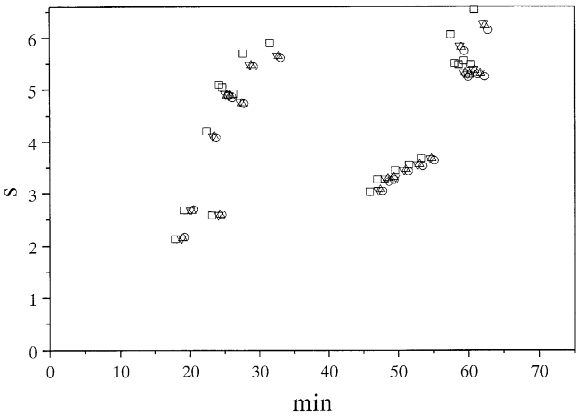

Fig. 6. Overlay plot of mean retention times illustrating column set-to-column set reproducibility. ▽, CS 1; □, CS 2; △, CS 3; ○, CS 4.

of an unretained peak (t_M) and carefully adjusting the carrier gas flow rate such that t_M is consistent for all column sets. The peak positions for the analyses performed on column sets 1, 3, and 4, all appeared within the criteria of plus or minus a few retention index units, but the ^1t_R of the CS 2 analyses (shown as squares in Fig. 6) was substantially shorter than the others. For the CS 2 results, the more polar components were observed to also consistently have higher ^2t_R. The reduced ^1t_R time on the D1 column means the elution temperature from the D1 column is lower for these components so they are retained more in D2, and so the squares plot to the upper left, compared with their analogous components on the other columns. The low polarity components are also injected into D2 at a lower analysis temperature, but the effect is less noticeable than for the polar components whose interaction with the stationary phase is stronger. By calibrating the individual column sets with respect to flow it is expected that the column set-to-column set reproducibility would be improved.

3.5. GC–MS versus GC×GC

Historically GC–MS has been the only technique available for the analysis of many truly complex samples, since in the absence of GC–MS, single column retention time is the only qualitative measure of peak identity. Some specific analyses may be performed using selective detectors which may improve identity certainty, but many established methods rely on GC–MS and the hope that overlapping components have sufficiently different mass fragmentation patterns so that the components can be de-convoluted, or unique ions allow individual component quantitation. Relying on MS data for quantitation involves a degree of uncertainty and may be tedious; quantitative results will generally be of higher quality if components can be better resolved. Davis and Samuel used statistical overlap theory to predict peak overlap in single column GC in a range of mixtures including polychlorinated biphenyls (PCBs), pyridine- and nitrogen-containing polyaromatic hydrocarbons (N-PAHs), tetrachlorodibenzo-*p*-dioxins and dibenzofurans (TCDD/Fs), fatty acid methyl esters (FAMEs), flavours and fragrances and naptha [17]. These 1D simulated chromatograms (which contained as few as 60 individual components) all exhibited severe overlap. GC×GC has been used successfully to analyse many samples containing the above classes of compounds (PCBs [18], FAMEs [19], flavours and fragrances [20], naptha [21]). This demonstrates a wide range of applications, in which GC×GC has been used to solve specific and general analysis difficulties through enhanced resolution. A definitive comparative study of GC×GC versus GC–MS is awaited. Provided that the modulation system used achieves reproducible GC×GC chromatograms over a long time period, then the discrete positions of individual components within the 2D separation space can serve as reliable markers of component identity. This can only be achieved if the modulator produces consistent modulation phase profiles run after run [14]. Consistent modulation phase profiles were observed between runs, and between days in the present investigation (Fig. 4).

4. Conclusion

The key to reproducible peak positions in GC×GC is a well-controlled and reproducible modulation event start time throughout the GC×GC analysis, which is then accurately positioned with respect to conversion of data to 2D matrix format. If this is not reproduced from run to run, then peak position varies in the 2D plane, and the use of retention alignment may be needed. However it is preferable that the absolute peak position is obtained directly from the experimental result. The LMCS was shown to produce reproducible modulation start times and consistent modulation phase profiles for individual components in all experiments.

In the present investigation, the absolute positions of major components exhibited the greatest shift between analyses (although the differences were still rather small). Despite the small peak shifts, these components are still easily characterised by overlaying the contour plots for sample and standard. Although non-linear chromatographic conditions are generally undesirable they should not present any serious problems with respect to component identification. The assignment of identity of major com-

ponents can generally be performed by conventional techniques such as GC–MS.

A more important consideration is that the peak positions of trace, minor, and intermediate concentration components were found to be extremely reproducible. Thus characterisation of a great majority of components in complex samples may also be considered to be very reliable based on individual component positions in the 2D separation plane. By using the LMCS to perform cryogenic modulation GC×GC, highly reproducible 2D chromatograms are obtained, and the positions of components within the 2D separation space and it should be possible to use peak positions with confidence for the assignment of component identity.

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