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Application of Comprehensive Two-Dimensional Gas Chromatography (GC × GC) to the Qualitative Analysis of Essential Oils

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Summary

This paper investigates the separation of moderately complex samples by comprehensive two-dimensional gas chromatography (GC × GC). The analysis of peppermint (*Mentha piperita*) and spearmint (*Mentha spicata*) essential oil components, including acetates, alcohols, furans, ketones, sesquiterpenes, and terpenes, was achieved by one-dimensional gas chromatography with quadrupole mass spectrometry detection (GC/MSD) and GC × GC with flame ionization detection. Peppermint essential oil was found to contain 89 identifiable peaks by GC × GC compared to 30 peaks in the GC/MSD chromatogram. Likewise, 68 peaks were found in the GC × GC chromatogram of spearmint (compared to 28 in GC/MSD). Plots of the first dimension *versus* second dimension retention times provided a fingerprint of the two essential oils, which revealed 52 similar compounds between the two essential oils as opposed to 18 matches by 1D GC.

1 Introduction

Essential oils are complex mixtures usually steam distilled, solvent extracted, or physically pressed out of plant material [1]. Their isolated constituents are extensively utilized for their flavor, fragrance, anti-microbial, pharmacological, and medicinal properties [2–4]. In total, about 1,200 compounds, including terpenes and their corresponding aldehydes, ketones and alcohols, phenylpropanoids, hydrocarbons, esters, oxides, and sulfur compounds have been identified in essential oils [1, 2]. Several of these essential oil constituents have been found to exhibit toxic (hepatotoxic, pneumotoxic, carcinogenic, *etc.*) properties [5, 6]. For instance, some mint essential oils contain terpene alcohols that irreversibly destroy detoxifying enzymes (cytochrome P_{450}) in rat liver microsomes [4, 7]. Oral doses of the pulegone (a terpene ketone) have also been shown to deplete glutathione levels [8], which allows toxic metabolites such as menthofuran to accumulate to levels that produce liver and lung tissue damage [9]. Issues concerning essential oil adulteration and the substitution of expensive oils with those of lesser value have also been investigated [10, 11].

Capillary one-dimensional gas chromatography (1D GC) has routinely been used to analyze the volatile constituents of essential oils. However, the complex nature of these samples result in extended GC run times. Extensive peak co-elution also presents a challenge for complete qualitative analysis even with the use of definitive confirmation technologies such as mass spectrometry. Essential oils have also been separated by multi-dimensional gas chromatographic (MDGC) heart-cutting methods involving valved and valveless flow switching interfaces [12–15]. These methods extend the separation power of capillary GC and clearly resolve the selected constituents in a GC chromatogram, but are limited to the analysis of only a few discrete, critical regions of the chromatogram, and analysis time is even further extended.

Comprehensive 'two-dimensional gas chromatography (GC × GC) is a multi-dimensional GC technique that uses a valve-less on-column interface between the two GC columns and a high speed second dimension to combine the advantages of increased separation power with reduced analysis time [16–20]. In the GCxGC system, the first dimension provides a separation comparable to that obtained in 1D GC. A modulator at the junction between the two columns accumulates and re-injects the effluent from the primary column into the secondary column. The modulator produces concentration pulses at regular time intervals and the second dimension column separates each concentration pulse into its components, thus giving a secondary chromatogram. During the development of each secondary chromatogram the modulator accumulates sample for the next concentration pulse. Every sample component is thus subjected to both separation dimensions. The second dimension chromatograms are essentially a series of high-speed chromatograms which each generate a "snapshot" of the second separation dimension. These snapshots are taken at a frequency that allows for continuous monitoring of the separation in both dimensions, and three dimensional data (first and second dimension retention time, signal intensity) are recorded that provide a means of viewing the peak profiles in either dimension or both dimensions simultaneously.

To date, GC × GC has been used primarily to separate petroleum products and related environmental compounds [21–25]. These compounds are complex in terms of the number of sample components (generally over 100) and have been good models in the early development of GC × GC. The fact that these studies have focused almost exclusively to petroleum samples has contributed to the unfortunate perception that GC × GC is truly necessary only for the analysis of highly complex samples. There is a need to investigate the "GC × GC advantage" in samples that may not be complex in terms of the number of compounds, but yet exhibit other forms of chemical complexity. The only account of such an application to be found in the literature was published by Liu *et al.* for the analysis of pesticides in human serum [26]. In

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this paper we present a comparison of one-dimensional GC and GC × GC for the qualitative analysis of two mint essential oils. These mixtures are moderately complex (less than 100 sample components) when compared to petroleum samples but pose some challenging problems for 1D GC methods because of their sample dimensionality [27] which can be expressed by the number of different kinds of chemical classes present, and their wide volatility range. The goal of this paper is to examine the potential of GC × GC for the qualitative analysis and characterization of essential oils.

2 Experimental

2.1 GC/MS Analysis

GC/MS analyses were performed using a HP 6890 GC (Hewlett Packard, Wilmington, DE) coupled to a HP 5973 mass selective detector (MSD) (Hewlett Packard, Avondale, PA). All injections were made with a HP series 6890 autosampler. The GC inlet port was fitted with a narrow-bore (75 mm i.d.) injection sleeve (Supelco, Bellefonte, PA) and maintained at 290 °C in split mode (10:1). High purity grade (99.997%) helium (Air Products, Allentown, PA) with a flow rate of 9.8 mL/min, was the column carrier gas. The GC oven, fitted with a 30 meter DB-1 column (J & W Scientific, Folsom, CA) was programmed as follows: the oven was held at an initial temperature of 50 °C for 1 min, followed by a first temperature ramp to 165 °C at 3 K/min. A second temperature ramp (40 K/min) brought the oven to its final temperature of 270 °C. The GC-MSD transfer line was maintained at 280 °C, and the MSD quadrupole and source heaters were held at 110 °C and 230 °C respectively. The MSD was calibrated using a routine (Autotune) provided in the HP ChemStation software (version A.03.00). Mass spectra were searched against the NIST '98 (National Institute of Standards and Technology, Gaithersburg, MD) mass spectral library.

2.2 GC × GC Analysis

GC × GC analyses were performed using a HP 6890 GC (Hewlett Packard, Wilmington, DE) retrofitted with a thermal modulation unit (Zoex Corporation, Lincoln, NE), as shown in **Figure 1** (a top view diagram of the thermal modulator is also shown in **Figure 2**). Hydrogen gas was used as carrier gas through the GC × GC system. A flame ionization detector (FID) was operated at 275 °C, with air, hydrogen and nitrogen flow rates of 350, 40, and 30 mL/min, respectively. Samples were injected manually (1 mL) into a split/splitless inlet port that was fitted with a 4 mm injection liner (Hewlett Packard), which was maintained at 235 °C in the split mode (50:1). The column set used for this research was a DB-1 type stationary phase column (1 m column length; 100 µm i.d.; 3.5 µm film thickness) connected to a OV-1701 type stationary phase column (2 m column length; 100 µm i.d.; 0.5 µm film thickness), both purchased from Quadrex (New Haven, CT). The

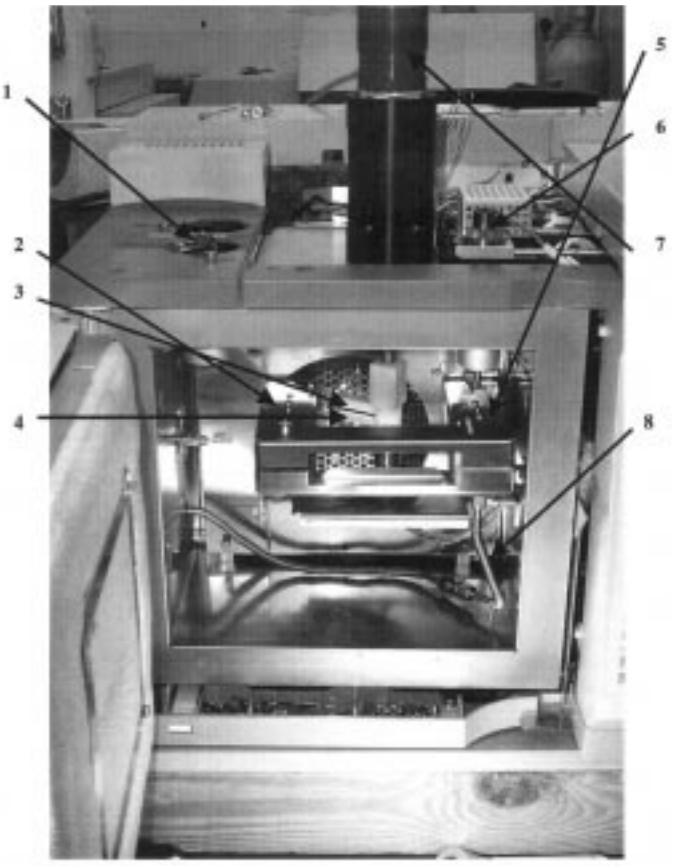

Figure 1. Photograph of the GC × GC unit. 1) Injector port; 2) 1st dimension column arm of the column cartridge; 3) thermal desorption rotating heater ("sweeper"): the heater is slotted for more efficient heat transfer onto the column modulator tube; 4) modulator tube; 5) 2nd dimension column arm of the column cartridge; 6) flame ionization detector; 7) stepper motor; 8) compressed air cooling line.

connection between the two columns (phase termination) was sleeved in a low dead volume home-made 250 mm i.d. fused silica open tubular capillary press-fit. The interface between the two columns, called the modulator tube, consisted of the last 10 cm of the first column and was placed in the path of the rotating heater (see Figure 2.A). The columns were housed in the aluminum column cartridge assembly. The primary column was heated by the HP 6890 GC oven at several different temperature programs that ranged between 30 °C and 210 °C, with ramp rates in the 3 K/min to 15 K/min range. The secondary column was heated at temperature programs that followed the temperature program rate of the first dimension column, but at temperatures that were typically between 30 °C and 90 °C higher in order to speed up the second dimension separation. A heater was placed in the second dimension column arm of the column cartridge, which was insulated from the rest of the GC oven, as shown in Figure 1. A line of compressed air was connected to this heater for fast cooling of the second dimension column at the end of each run. The rotating heater was powered through a 0–140 V variable AC transformer (Staco Energy Products, Dayton,

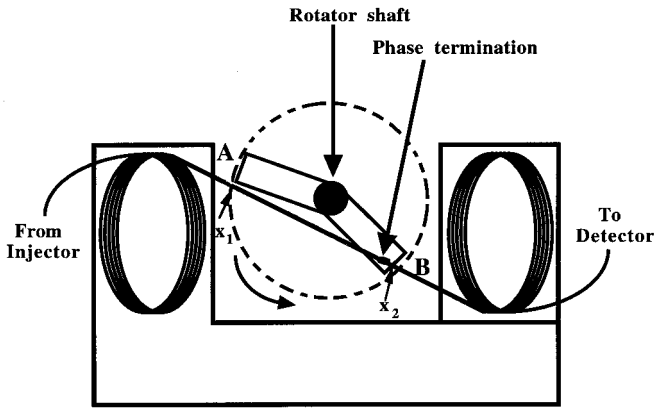

Figure 2. Top-view diagram of the thermal modulator. The arrows marked x_1 and x_2 indicate the length of the modulator tube. The rotating heater is in position A prior to each modulation cycle. Its counter-clockwise movement (from position A to position B) over the modulator tube produces the thermal modulation. See text for details.

OH) at a temperature that was 80 °C to 100 °C higher than the oven temperature. The modulation period was 4 s.

The operating process of the GC × GC system is shown in Figure 2 and **Figure 3**. The on-column interface "slices" the entire first dimension eluate into a series of sample concentration pulses that are loaded onto the head of the second dimension column. The thermal modulation cycle is a 3-stage process [28] that involves sample accumulation, sample focusing and sample acceleration. During the accumulation part of the cycle, the rotated heater is off the column and sample flows in the modulator tube. During the focusing part of the cycle, the rotating heater proceeds counterclockwise from its initial position (point A in Figure 2) to its final position (point B) at a given rotation speed (0.15 rev/s), and imparts its heat to the modulator tube. The sample in the path of the heater rapidly moves downstream because of the relative high temperature that is applied. The section ahead of the heater is relatively cool, however, and retainable substances are slowed down (focused) to form a sharp concentration pulse. As the heater moves off the modulator tube, the uncovered portions rapidly cool down (because of the low thermal mass of the fused silica tube) and begin to accumulate retainable substances in the carrier stream for the next thermal modulation cycle. The acceleration phase involves the acceleration of the rotating heater speed (typical acceleration rate: 120 rev/s^2) during the focusing phase to sharpen the pulse before it exits the modulator tube. At the end of the acceleration phase the rotating heater is briefly immobilized (0.5 s) on top of the phase termination (point B) to insure complete transfer of all the analyte accumulated during the cycle to the second dimension column. The modulation cycle is finally completed by moving the rotating heater (counter-clockwise) back to its initial position (point A).

The resulting detector signal trace is subsequently segmented into individual second-dimension chromatograms (Figure 3.A), the length of which is determined by the modulation period. The ensemble of second dimension chromatograms is then reassembled to form the GC × GC chromatogram. The GC × GC data can be viewed as a three-dimensional surface plot (Figure 3.B) or a two-dimensional contour plot (Figure 3.C).

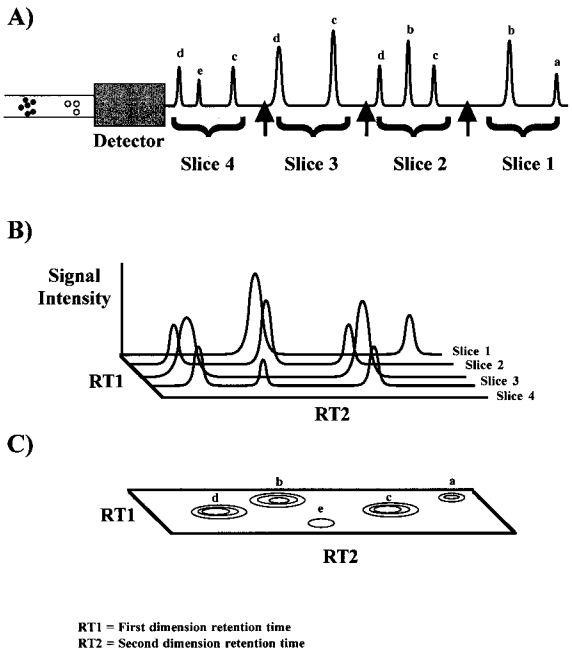

Figure 3. Construction of a GC × GC chromatogram. (A) Detector point of view: the output of the second column is simply a long continuous data stream. The same substance will appear in the sample stream at several different times in several different second dimension chromatograms (slices). The slices are determined by the modulation period. (B) Three-dimensional surface plot. Slices are assembled in increasing slice number, and peaks project from the baseplane, much like a topographic model. (C) Two-dimensional surface plot. Signal intensity is collapsed in the baseplane to form a contour plot in which signal intensity increases with the number of concentric bands.

Data processing and visualization software (GCVis 1.0) was written in-house using IDL 4.0 (Research Systems Incorporated, Boulder, CO).

2.3 Samples and Reagents

Essential oils (spearmint and peppermint) were obtained from Aromaland (Santa Fe, NM). Neat solutions were diluted 1/300 in ethanol (Sigma, St. Louis, MO).

3 Results and Discussion

3.1 GC/MSD Analysis

GC/MSD chromatograms of the two essential oils were obtained to provide a list of the essential oil constituents and their identification by one-dimensional GC means prior to GC × GC analysis. **Table 1** is a list of the compounds identi-

Table 1. Constituents, retention time, relative peak abundances of peppermint and spearmint essential oil constituents. The purity of all mass spectra was greater than 90% unless noted otherwise.

Peak #	Constituents[a]	RT[b] (min)	Relative peak abundances (%)[c]	
			Peppermint	Spearmint
1	α-Pinene	10.90	0.5	0.6
2	Sabinene	12.44	0.5	0.3
3	β-Pinene	12.58	0.7	0.7
4	3-Octanol	13.22	0.2	0.6
5	Myrcene	13.29	0.1	0.3
6	α-Terpinene	14.38	0.2	–[d]
7	Cymene[e]	14.50	0.3	tr[f]
8	Eucalyptol	14.83	4.8	0.3
9	Limonene	14.96	1.1	15.0
10	β-Ocimene	15.35	0.2	–
11	γ-Terpinene	16.28	0.4	–
12	*trans*-Sabinene hydrate	16.41	0.8	–
13	Terinolene	17.66	0.1	tr
14	Linalool	17.99	0.3[g]	–
15	Isopulegol	19.96	0.1	0.4
16	Menthone	20.11	22.1	9.7
17	Isomenthone	20.51	3.4	4.4
18	Menthofuran	20.80	3.2	–
19	Neomenthol	20.96	3.6	2.4
20	Lavandulol	21.08	–	0.4
21	Menthol	21.35	40.3	16.19
22	Neoiso(iso)pulegol	21.43	–	Coeluting
23	Terpinen-4-ol	21.49	1.0	–
24	Neoisomenthol	21.77	0.8	–
25	Dihydrocarvone[e]	21.87	–	1.1
26	α-Terpineol	21.97	0.4	0.4
27	Neodihydrocarveol	22.17	–	tr
28	*trans*-Carveol	23.20	–	0.4
29	Pulegone	23.85	1.6	Coeluting
30	Carvone	23.98	–	37.7
31	Piperitone	24.38	0.7	0.8
32	Neomenthyl acetate	26.14	0.3[g]	–
33	Menthyl acetate	26.90	6.6	1.4
34	Isomenthyl acetate	27.51	0.6[g]	–
35	Dihydrocarvyl acetate[e]	28.29	–	0.5
36	β-Bourbonene	31.31	0.4	0.6
37	β-Elemene	31.58	–	0.2[g]
38	β-Caryophyllene	32.70	1.9	0.9

[a] = Constituents were identified using NIST '98 Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, MD); a mass spectral library of essential oil constituents provided by Delaware State University and R.P. Adams, *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*, Allured Publishing Company, Carol Stream, IL, 1995; [b] = Retention Time (if component present in both oils, retention times were averaged; all retention times were within ±0.05 min agreement); [c] Peak Abundance is calculated as peak area of analyte divided by peak area of total ion chromatogram times 100, values greater than 2% are in bold; [d] Detected; [e] exact isomer (*cis*-, *trans*-) not determined; [f] tr = trace; [g] spectral purity less than 90% but greater than 80%.

fied in peppermint and spearmint, with their retention times and relative abundances. The chromatogram of peppermint essential oil is characterized by four large peaks for menthol, menthone, menthyl acetate, and eucalyptol, with smaller abundances of menthofuran, isomenthone, neomenthol, limonene, and pulegone. The chromatogram for spearmint is characterized primarily by four large peaks for carvone, menthol, limonene, and menthone, with smaller abundances of isomenthone, neomenthol, and *cis*-dihydrocarvone.

Qualitatively, several differences were observed between the two oils. Peppermint lacked the presence of detectable levels of linalool and several carvyl compounds, such as dihydrocarvone (*cis* and *trans*), carveol, and dihydrocarvyl acetate, which are found in spearmint, whereas menthofuran was detected in peppermint but not spearmint. Using area percent as a semi-quantitative means of comparing the relative amounts of essential oil constituents, both menthol and menthone were found to be about twice as abundant in peppermint as spearmint. In addition, menthyl acetate in peppermint was about four times greater than in spearmint. Carvone, on the other hand, was about 350 times higher in spearmint than in peppermint. Another striking difference involves the ratio between limonene and eucalyptol. The ratio in peppermint was 1:2.5, whereas in spearmint it was almost 50:1. These characteristics provide means of differentiating the two oils.

3.2 GC × GC Analysis

Retention times in the GC/MSD and GC × GC runs were not identical, and peak matching was done by identifying similar regions in the 1D and 2D chromatograms. A sample of this process is shown in **Figure 4**. Figure 4.A is a portion of the GC × GC peppermint chromatogram. Figure 4.B is the reconstructed 1D chromatogram of the data shown in Figure 4.A, and the equivalent GC/MSD chromatogram segment is shown in Figure 4.C. The reconstructed chromatogram was obtained by collapsing each second dimension chromatogram to a single point that represents all the data collected in that time period. This chromatogram is essentially the equivalent of a low resolution 1D chromatogram, because the signal is collected at the modulation frequency. It espouses the profile of the first dimension chromatogram, however, because the first column provides the principal separation mechanism. The first dimension column that was used in the GC × GC column set was non-polar and the profile it generated could be expected to be reasonably similar to the separation produced by the non-polar column used in the GC/MSD separation.

Several characteristics of the "GC × GC advantage" are observable in Figure 4.A. First, the amount of available baseplane is evident, and accounts for the increased resolution. The GC/MSD chromatogram shows 7 peaks in this region. The GC × GC chromatogram shows 3 additional peaks (peaks A, B, and C in Figure 4.A). These peaks are clearly identifi-

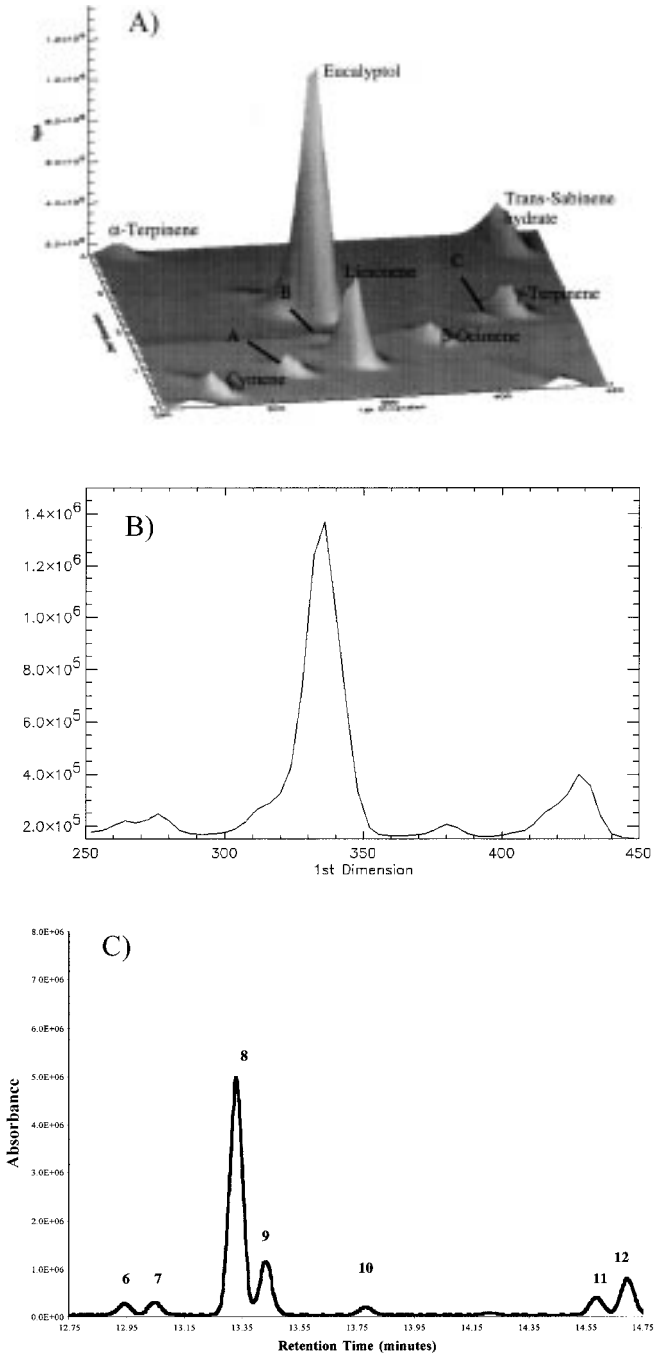

Figure 4. (A) Three-dimensional surface plot of a portion of the GC × GC chromatogram for peppermint. First and second dimensions retention times are in seconds; (B) Reconstructed 1D chromatogram of the 2D chromatogram in Figure 4.A; (C) GC/MSD signal of the equivalent chromatographic segment.

able, even though their exact identity requires mass spectral analysis. Peaks A and B are shadowed by the intensity of the eucalyptol peak in the 1D chromatogram, and peak C becomes visible in the 2D plane because it is sharpened by the effect of the mid-column thermal modulation.

Another interesting observation is with regards to the structure of the GC × GC chromatogram. The monoterpene hydrocarbons (α-terpinene, cymene, limonene, β-ocimene, and γ-terpinene) are lined up in the 2D plane because their properties as a chemical class are correlated. Even though the α-terpinene peak maximum appears to be at the top of the 2D chromatographic plane, a closer look shows that its tail is at the bottom of the chromatogram and part of the same compound class. The reason for the wrap-around of the α-terpinene peak is the fact that the modulation period and the second dimension chromatogram period are out of phase. This "aliasing" effect, which has been observed and previously discussed in the literature by Beens *et al.* [23], does not affect the overall inter-relationship between consecutive second dimension chromatograms. It is therefore possible to recognize structural trends that exist in the sample. The ability to separate compounds based on chemical class is characteristic of GC × GC, and is related to the dimensionality (or number of independent chemical properties) of the sample. The ability to structure the chromatogram into chemical compound "bands" is important, because it is now possible, without mass spectral identification, to use chemical logic to qualitatively assign the nature of the chemical class of identifiable (but not yet identified) compounds in the chromatogram. For example, it is very likely that MS identification will recognize peaks A and C in Figure 4.A as monoterperne hydrocarbons, as they fall in line with the other compounds of that class. By contrast, eucalyptol (terpinoid) and *trans*-sabinene hydrate (alcohol) are separated in different regions of the chromatogram. The possibility of using chemical class standards within a run for qualitative analysis is very exciting indeed.

The separation shown in Figure 4.A is not optimized. The monoterpene hydrocarbons shown in Figure 4.A are separated in the first dimension by their volatility, which ranges from 174 °C for α-terpinene to 183 °C for γ-terpinene. The similarity in the polarities of this set of compounds suggests that the relatively steep slope of the correlation line that can be drawn through this compound class could be improved. The optimization of GC × GC separations is beyond the scope of this paper, but it is evident that optimized conditions would lead to faster separations because the available peak capacity could be "tuned" and thus used more efficiently. Optimization of the second dimension temperature program can be done in a similar manner to the tuning of a 1D temperature program to produce equidistant retention time segments for a homologous retention index series.

A qualitative comparison between the GC/MSD and GC × GC/FID chromatograms is summarized in the "scatterplot" **Figure 5**. The GC × GC runs for these comparative studies were considerably slower because they were aimed at producing reconstructive 1D chromatograms that were fairly similar to the GC/MSD chromatograms (in terms of retention time) in order to facilitate the pairing of the GC × GC peaks

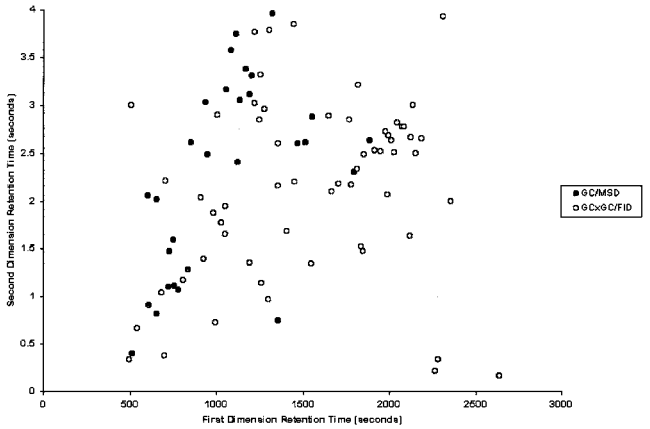

Figure 5. Overlay of GC/MSD and GC × GC/FID peak maxima for peppermint. Every GC/MSD peak is a GC × GC peak as well. See text for details.

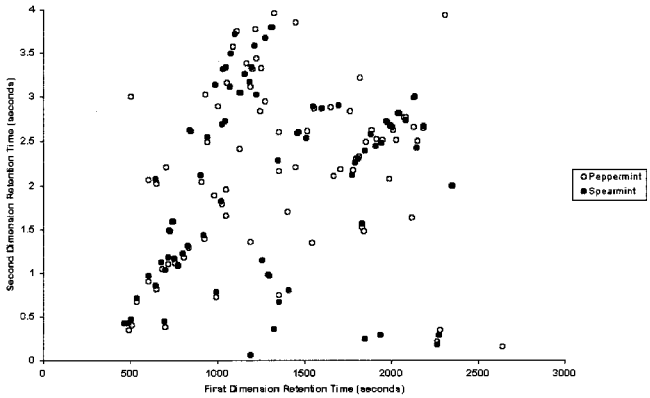

Figure 6. Overlay of peak maxima retention data for single GC × GC peppermint and spearmint runs

with their corresponding GC/MSD peaks. The data processing software included a peak finder subroutine that returned several values for each identified peak in the GC × GC chromatogram (first and second dimension retention times, peak height, peak volume, and relative peak abundance). The "scatterplot" of a complete GC × GC run of peppermint was thus obtained that marked the position of each identified peak in terms of its retention time coordinates (first and second dimension retention time) at peak maximum. This data was then compared to the GC/MSD data in Table 1, and each GC/MSD peak was assigned a position in the GC × GC scatterplot based on relative first dimension retention time, peak height and relative abundance. The GC/MSD peaks identified in Table 1 were thus matched with their corresponding GC × GC peaks in the scatterplot. The main advantage of displaying the GC × GC chromatogram in terms of its scatterplot is the fact that dynamic range problems can be avoided by reducing the contour plot chromatogram to its peak maxima, which results in a more convenient display of the data for comparison purposes.

Figure 5 illustrates the increased separation power of GC × GC over 1D GC. Peppermint is shown to have 89 GC × GC peaks, as opposed to the 30 peaks that were identified in the GC/MSD run. A closer look at the scatterplot also shows that the number of unmatched GC × GC identifiable peaks increases with the first dimension retention time. The effect of thermal modulation on the sharpening of smaller, broader peaks from the first dimension column accounts for the increased sensitivity. The amount of empty baseplane allows for a reduction of the chromatographic run time without a significant reduction in separation power. A faster GC × GC run produced a 900-second compressed scatterplot (a 67% reduction in analysis time from the run shown in Figure 5) that still showed all 89 peppermint components. A similar time-compression of the GC/MSD chromatogram significantly reduced the number of identifiable compounds, as expected. In terms of resolving speed (number of resolved peaks per minute), GC × GC averaged 5.9 peaks per minute compared to 0.9 peaks per minute for GC/MSD.

Another significant advantage of GC × GC is that it inherently provides two simultaneously measured retentions for each sample substance. **Figure 6** shows a qualitative comparison of the two essential oils that is based solely on the retention data. This substance identification power is superior to that of 1D GC because even though retention time sets could also be determined by two independent 1D chromatograms the assignment of retention time pairs for each substance is a more time-consuming task. In addition, the retention data provides polarity information for sample components, since volatility is tuned out on the second dimension.

Qualitative comparisons of the two essential oils were done on the basis of the retention time data set. Figure 6 confirms the substantial similarities between peppermint and spearmint, while providing a clearer picture of the differences between the compounds as well. In the 1D GC runs there are 18 peak matches between the two essential oils, which translates to 60% (18 of 30) of the peppermint peaks. The GC × GC data reveals 52 matched peaks, or 58% of the total GC × GC peppermint peaks. The GC × GC retention data constitutes more solid proof of the peak matches because of the matching in two separate dimensions, and can be thought of as a more accurate estimation (coarse vs. fine reading) of the true extent of compound similarity between peppermint and spearmint. Figure 6 also shows a measure of the reproducibility of the retention times, as the deviation between runs varied between 0 and 0.2 seconds in the second dimension retention data. The compounds that are unique to each sample stand out as single peaks in the retention data plane. These plots therefore provide excellent fingerprints of the sample constituents even without the use of signal intensity information.

4 Conclusion

We have shown a 2 to 3 fold increase in the separation power of GC × GC over 1D GC for the analysis of mint essential

oils. This increased separation power is a result of the "GC × GC advantage" which includes the following characteristics: increased peak capacity, improved analysis speed and sensitivity, and ordered separations based on chemical class. Future work in this laboratory will feature time-of-flight (TOF) MS detection, which is rapidly emerging as the mass spectrometer of choice for high speed chromatographic separations [29]. The addition of an MS identification dimension to the GC × GC separation would tremendously enhance the qualitative power of the analytical method. Systematic studies of GC × GC separation parameters will also be undertaken in order to optimize analysis time and resolution power.

A wide array of applications of this methodology for essential oil analysis could include the detection of products of essential oil adulteration, the identification of sources of poisoning, and the use of pattern recognition to further analyze peak pattern differences in the GC × GC chromatograms of samples. These peak patterns could prove valuable in the fingerprinting of essential oils.

Acknowledgement

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