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Improving the quality of biomarker candidates in untargeted metabolomics via peak table-based alignment of comprehensive two-dimensional gas chromatography-mass spectrometry data

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Abstract

The potential of high-resolution analytical technologies like GC×GC/TOF MS in untargeted metabolomics and biomarker discovery has been limited by the development of fully automated software that can efficiently align and extract information from multiple chromatographic data sets. In this work we report the first investigation on a peak-by-peak basis of the chromatographic factors that impact GC×GC data alignment. A representative set of 16 compounds of different chromatographic characteristics were followed through the alignment of 63 GC×GC chromatograms. We found that varying the mass spectral match parameter had a significant influence on the alignment for poorly- resolved peaks, especially those at the extremes of the detector linear range, and no influence on well- chromatographed peaks. Therefore, optimized chromatography is required for proper GC×GC data alignment. Based on these observations, a workflow is presented for the conservative selection of biomarker candidates from untargeted metabolomics analyses.

Keywords

Comprehensive two-dimensional gas chromatography; GC×GC-MS; data alignment; untargeted metabolomics; biomarker

1. Introduction

Samples of biological origin typically contain hundreds to thousands of compounds that range widely in chemical properties and concentrations, making separation and characterization of the mixtures challenging. Multidimensional chromatography (MDC) techniques, such as comprehensive two-dimensional gas chromatography (GC×GC), are powerful analytical tools that are well suited for the analysis of biological mixtures due to the enhanced peak capacity afforded by the additional chromatographic dimensions [1, 2]. The utility of GC×GC for targeted analyses of biological samples as well as compound discovery in complex matrices, including untargeted metabolomics [3, 4], is well recognized and applicable to many areas of inquiry. For example, Hartman, *et al.* report using GC×GC

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in targeted analyses to quantify 3,4-methylenedioxymethamphetamine and three of its metabolites in blood and serum [5], and in an untargeted metabolomics analysis, Cordero and colleagues applied GC×GC to the detection of previously unidentified volatile metabolites from the leaves of *Mentha* species [6]. Turning the large data sets that are generated by MDC methods into information (e.g., quantifiable peaks and compound identities) is becoming less time-intensive and more reliable through advancements in commercial software packages (e.g., ChromaTOF, GCImage, and ChromSquare), as well as new unsupervised data processing and statistical analysis methods that are being developed or retooled for MDC (recently reviewed in [7–12]). In addition, a number of chromatographic alignment methods for comparing data between multiple GC×GC analyses, a cornerstone of biomarker discovery, have been described and employed with great success [13–19]. However, many of these algorithms have been developed and validated for targeted analyses, which only requires local alignment of the peaks of interest [20], and importantly, commercial or public availability of these programs are lagging [21].

The aim of untargeted biomarker analysis is to identify robust and predictive differences between sample classes, e.g., between diseased and healthy individuals, without *a priori* knowledge of the metabolism [22]. MDC separations provide a significant advantage in the search for biomarkers by increasing the number of identifiable and quantifiable metabolites by approximately 10-fold [23, 24], but the resulting large volumes of new data has now shifted the burden of biomarker discovery to the data processing and statistical analysis steps [21]. One common approach for identifying putative biomarkers is to calculate Fisher ratios – an assessment of the between-class vs. within-class variance of each compound in the samples – where the compounds with higher Fisher ratios are pursued as candidate biomarkers [25]. Accurate variance calculations rely upon the alignment of each chromatographic peak across every sample [25, 26], but the inherent variance within biological sample classes requires the analysis of a large number of samples, thus making alignment challenging [16, 26]. The importance of good chromatography (i.e., optimized peak resolution) for targeted and untargeted MDC analyses is well understood [9]; Kim and Zhang have reported on the influence of peak density on GC×GC peak-table-based alignment algorithms [27]. However, the influence of peak intensity and resolution on the outcomes of chromatographic alignment has not been reported on a peak-by-peak basis, which has significant implications for the development of unsupervised data processing and statistical methods for biomarker discovery from MDC analyses.

In this study, we assess how chromatographic peak characteristics influence the success of peak table-based alignment. Employing a commonly-used column set [3], we performed 63 GC×GC-TOFMS analyses on the volatile metabolites of closely-related bacterial isolates, and aligned the chromatograms using ChromaTOF Statistical Compare (LECO Corp.). We varied the mass spectral match parameter in the alignment algorithm to look at the interplay between peak intensity and resolution as they affect alignment, and evaluated the success of the alignment by following 16 peaks with chemical and chromatographic characteristics (e.g., area, S/N, resolution, and retention times) that are representative of the diversity of peaks observed in these complex biological samples. Based on our observations, we propose a workflow to identify high-quality biomarker candidates from less-than-perfect separations

of complex samples, the concepts of which can be applied to the alignment of MDC data collected on any software platform.

2. Experimental

2.1 Sample preparation and volatiles collection

Thirty five clinical isolates of *Pseudomonas aeruginosa* were used for this study. Between 1 and 3 biological replicates were analyzed for each isolate, yielding 63 samples. For bacterial volatiles analysis, 10 mL of spent media from stationary phase cultures and a stir bar were sealed into 20 mL glass vials with PTFE/silicone caps. The volatile metabolites of the bacteria were sampled from the headspace using solid-phase microextraction (SPME; divinylbenzene/carboxen/ polydimethylsiloxane, 50/30 μm ; Supelco/Sigma–Aldrich, St. Louis, MO), as described previously [1].

2.2 Chromatography and mass spectrometry

Two-dimensional gas chromatography–time-of-flight mass spectrometry (GC $\times$ GC–TOFMS) was performed using a LECO Pegasus 4D (St. Joseph, MI). The instrument was fitted with a two-dimensional column set consisting of a DB-5MS (5% diphenyl/95% dimethyl polysiloxane; 30 m $\times$ 0.25 mm $\times$ 0.25 μm (length $\times$ internal diameter $\times$ film thickness); Agilent Technologies) as the first dimension (^1D) column, and a ZB-50 (50% diphenyl/50% dimethyl polysiloxane; 2 m $\times$ 0.1 mm $\times$ 0.1 μm ; Phenomenex) as the second dimension (^2D) column, joined by a press-fit connection. The columns were heated independently; the ^1D column was initiated at 35 $^{\circ}\text{C}$ (0.2 min hold), then heated at 15 $^{\circ}\text{C}/\text{min}$ to 230 $^{\circ}\text{C}$ (0.8 min hold); the ^2D column was heated with a +5 $^{\circ}\text{C}$ offset relative to the primary oven. A quad-jet modulator was used with a 4 s modulation period (0.4 s hot, 1.6 s cold pulses) and a +25 $^{\circ}\text{C}$ temperature offset relative to the secondary oven. The helium carrier gas flow rate was 1 mL/min. A 10:1, 30 s pulsed split injection was used. The inlet and transfer line temperatures were 250 $^{\circ}\text{C}$. Mass spectra were acquired at 200 Hz over the range of m/z = 25–500. Data acquisition was performed using ChromaTOF software (LECO Corp.), v.4.22.

2.3 Data processing and chromatographic alignment

Data processing and chromatographic alignment were performed using the Statistical Compare package of ChromaTOF v.4.50. The baseline was drawn through the middle of the noise and the signal-to-noise (S/N) cutoff for peak finding was set to 10 for a minimum of 2 apexing masses. The ^1D and ^2D peak widths were set to 8 s and 0.15 s, respectively, based on the observed widths in the chromatograms for non-saturated peaks. ChromaTOF combined subpeaks across multiple injections into the secondary column when the second dimension retention time ($^2t_{\text{R}}$) shift was $\leq$ 100 ms early for subsequent modulation periods, and the mass spectral match was $\geq$ 600. Peaks were identified by a forward search of the NIST 08 Mass Spectral Library.

Chromatographic alignment was performed using ChromaTOF Statistical Compare v.4.50. For a peak to be identified as the same compound across chromatograms, both the retention times and the mass spectra had to meet minimum match criteria. For alignment, the first dimension retention time ($^1t_{\text{R}}$) could not vary more than 4 s (1 modulation period) from

chromatogram to chromatogram and the second dimension retention time (2t_R) could not vary more than 100 ms, based on the observed maximum variability in 1t_R and 2t_R for quinolone, an exogenous retention time marker added to each sample ($^1t_R = 744$ s, $^2t_R = 2.04$ s to 2.12 s). The mass spectrum for aligned peaks had to meet a minimum inter-chromatogram match threshold, which was varied for each analysis from 100 – 900, in increments of 100, to evaluate the influence of spectral match on alignment success. Alignment data for 16 peaks (Table 1) were evaluated for each of the nine experiments, yielding 144 experimental results. A peak was deemed to be aligned if all occurrences of the peak from the 63 individual chromatograms had been grouped into a single peak table entry in the alignment results, and the peak could be aligned using at least two mass spectral match score minima. As an additional measure of alignment, Fisher ratios were calculated for each set of aligned peaks using two sample groups. A minimum of two peaks in each group were required for the calculation, otherwise the Fisher ratio was reported as “Undefined”. Chromatographic alignment for a peak across multiple mass spectral match scores yields the same Fisher ratios for each alignment result.

3. Results and discussion

3.1 Chromatography: characteristics and alignment

The headspace of *P. aeruginosa* bacterial cultures contain hundreds of volatile metabolites, which are highly varied in chemical class [1] and concentration, making good chromatography (i.e., Gaussian peak shapes, high peak resolution) of every peak challenging. Using a common non-polar/semi-polar column combination for the 1D and 2D columns, respectively, and a 10:1 split ratio to enhance the detection of trace compounds, we obtained good separations for the *P. aeruginosa* volatiles, except for the low-boiling point compounds, where the chromatographic space was congested ($^1t_R < 420$ s, Fig. 1). Peak deconvolution algorithms, such as the one employed by ChromaTOF, are able to find many imperfectly-resolved peaks by reconstructing mass spectra from the apexes of individual ions, which significantly enhances compound detection and discovery in targeted and untargeted analyses, respectively. However, alignment of poorly-resolved peaks across many samples cannot be rescued entirely by peak deconvolution, which has significant consequences for biomarker discovery efforts.

The ChromaTOF Statistical Compare software algorithm for chromatographic alignment is peak-table based, matching three parameters for each peak: the retention times in the first and second dimensions (1t_R , 2t_R) and the mass spectrum (MS). All three parameters are influenced by peak resolution and peak shape/intensity. We hypothesized that varying the minimum MS match requirement from low match (relying on t_R alone for alignment) to high match (requiring both t_R and MS matching for alignment) would strongly influence the alignment of poorly-chromatographed peaks, but have little effect on well-resolved peaks with good peak shapes. We tested the influence of MS match scores on chromatographic alignment by analyzing 63 similar – but not identical – samples of bacterial volatile metabolites, and aligned them using minimum MS match scores varying from 100 (low) – 900 (high). To evaluate the success of the alignments, we selected 16 compounds of different chromatographic characteristics (Table 1, Fig. 2) and tracked how well these peaks

were aligned using each of the nine MS match score minima. The peaks we analyzed ranged widely in peak intensity (i.e., peak area) as well as signal-to-noise ratio (S/N), which is influenced by peak resolution (Table 1). Among the peaks were very closely-eluting compounds (3-heptanone and 2-heptanone), high-intensity peaks with average peak areas $> 10^7$ (e.g., toluene) and low intensity peaks with areas $< 10^4$ (e.g., 4-propylbenzaldehyde). All 16 of the peaks had high NIST library match scores for a subset of their deconvoluted mass spectra, allowing us to track them with confidence when they are low intensity or poorly-resolved in some chromatograms. Varying the match score criteria for alignment, we found that it was possible to align 9 of the 16 peaks under two or more MS match parameters, yielding 50 aligned peak sets out of a total of 144 experimental results (Tables 2, S1–16). Five peaks could be aligned using a broad range of MS match score minima (2-heptanone, heptanal, 4-methyl-2-heptanone, benzaldehyde, and acetophenone), two peaks were aligned only under a narrow range of moderate MS match scores (2-nonanone and benzothiazole), and 7 peaks could not be aligned (2-ethoxy-2-methylpropane, 2-pentanone, toluene, 2-hexanone, 3-heptanone, 2-undecanone, 4-propylbenzaldehyde; Table 2).

Statistical analyses that are used to calculate differences between sample classes, such as Fisher ratios, are reliant upon chromatographic alignment. Peaks that are not properly aligned across all chromatograms are split into two or more clusters in the peak table, with each cluster containing peak data from a subset of the chromatograms (e.g., 2-ethoxy-2-methylpropane, Table S1). The distribution of peaks from different treatment groups into alignment clusters can create artificially high Fisher ratios (Table S1), generating false leads as well as excluding candidate peaks for biomarker discovery. For the 16 peaks tracked in this study, we observed that the Fisher ratios were strongly dependent on the alignment results, and varied widely from one match score to another when alignment failed (Tables S1–16). For instance, the Fisher ratios for 2-hexanone ranged from 0.11, to 9.27, to undefined for MS match scores 900, 300, and 400, respectively (Tables 3 and S4). In addition, for alignments using MS match scores 700, 600, 200, and 100, the 2-hexanone peaks were divided into multiple subgroups, each with their own widely-varying Fisher ratios. Conversely, for any peak that was aligned using multiple MS match score minima, such as acetophenone, the Fisher ratios were identical between successful alignment experiments (Tables 3 and S12). Overall, we obtained the highest proportion of aligned peaks with reproducible Fisher ratios when using moderate MS match score minima (i.e., 500 – 700; Table 2), with the ChromaTOF Statistical Compare default setting of 600 yielding the best results (9 of 16 aligned peaks).

3.2 The influence of peak intensity and resolution on alignment

We obtained a variety of results for the 16 peaks we tracked, ranging from alignment across all 9 experiments, independent of the MS match score, to never aligned using any MS match score minimum (Tables 2, S1–16), which led us to investigate how peak resolution and intensity influence alignment using ChromaTOF Statistical Compare. First, we assessed the influence of peak intensity on alignment. Of the 7 peaks that could not be aligned, 2-ethoxy-2-methylpropane was the highest-intensity peak and had large t_R variability (i.e., more than ± 1 modulation period) due to column saturation (Table 1), which distorts peak shapes and leads to the misidentification of the peak apex. The correlated mass spectrometer

detector saturation also creates problems for alignment. For instance, the subpeaks of the 2-ethoxy-2-methylpropane and 2-pentanone peaks could not be properly recombined within every individual chromatogram, in part due to poor MS matching across the subpeaks (Table 1), which contributes to the appearance of multiple peak table entries after alignment (Tables S1 and S2). On the other hand, the toluene peaks did not saturate the column and had reproducible retention times throughout the 63 chromatograms (Table 1), but in some samples the toluene ions saturated the MS detector, which disrupted alignment by impeding the matching of the MS spectra from one chromatogram to another. Therefore, an alignment algorithm based on t_R and MS matching will often fail for peaks that saturate the column and/or detector. The results tables for the Statistical Compare alignments flagged these peaks in the “concerns” column as having saturated masses (SM; Table 2), which is useful quality-control information for evaluating the success of chromatographic alignment for individual peaks. Very low peak intensities also negatively impacted alignment, exemplified by 4-propylbenzaldehyde (Table S16). In this case 1t_R and 2t_R are stable from chromatogram-to-chromatogram, but the matching of the mass spectra suffers for low-intensity peaks. However, we observed that moderate MS match scores permitted peak alignment across more chromatograms than very high or very low MS match scores for low-intensity peaks, such as 4-propylbenzaldehyde (Table S16).

Peak resolution also plays a significant role in the successful alignment of peaks, and high peak resolution can compensate for saturated masses during alignment. In spite of the saturated masses for 4-methyl-2-heptanone (Tables 2, S8), this peak could be aligned using match scores ranging from 600–100, because, unlike 2-ethoxy-2-methylpropane, 2-pentanone, or toluene, this peak is well-resolved. Specifically, throughout the 63 chromatograms, the 4-methyl-2-heptanone peaks do not have shared apexes with neighboring peaks, and have S/N ranging from $10^3 - 10^4$ (Table S8). The high resolution of these peaks makes it possible to align them using t_R matching alone, without consideration for MS matching. The other four peaks that could be aligned largely independent of MS match score (2-heptanone, heptanal, benzaldehyde, and acetophenone; Table 2) also eluted in regions of the chromatograms with good peak resolution (Fig. 2) and had unique mass S/Ns that ranged from $10^2 - 10^4$ in all 63 chromatograms (Tables S6, 7, 10, 12). Conversely, the three peaks that had S/Ns ranging as low as 10^0 , i.e., 2-pentanone, 2-hexanone, and 2-undecanone (Tables S2, 4, 15), could not be aligned with reproducible Fisher ratios in any of the 9 experiments. Two peaks, 2-nonanone and nonanal, had some samples with shared apexes, but when using moderate MS match score minima (e.g., 600), we were able to align these peaks and calculate reproducible Fisher ratios. Within the group of 16 peaks we also included two closely-eluting isomers – 3-heptanone and 2-heptanone (Table 1) – to determine how well these peaks could be deconvoluted and subsequently aligned. While 2-heptanone was aligned in all 9 of the experiments, 3-heptanone was aligned in none, owing to the fact that its peak area was as much as 100-fold lower and its S/N was 10-fold lower than 2-heptanone in some chromatograms. Overall, we found that peak deconvolution can often reconstruct mass spectra for co-eluting compounds in individual chromatograms and can enhance alignment for well-resolved peaks, but its power is diminished for poorly-resolved peaks, especially those with very high (i.e., saturated masses) or very low peak intensities.

3.3 Identifying high quality biomarker candidates from less-than-perfect chromatography

High-resolution chromatography is required for proper data alignment in GC×GC analyses, whether using peak-table or pixel-based alignment methods. However, highly complex samples (e.g., samples with a broad range of compound classes and concentrations) cannot be perfectly chromatographed – and therefore cannot be perfectly aligned [27] – using a single injection. To optimally separate the majority of the compounds, we estimate that at least four injections would be required, using high and low split ratio injections on two different column sets. Due to limited time, sample, and/or funding, this is usually an unrealistic analysis plan. Therefore, drawing upon our results and the work of others, we propose the following approach for the discovery of high-confidence biomarker candidates from complex samples using the fewest possible rounds of sample analysis (Fig. 3):

3.3.1 Chromatography—The most important step in biomarker discovery is to perform the best chromatography possible, optimizing the column selection, temperature profile, and split ratio for the set of analyzed samples (recently reviewed in [28]). This will ensure that the greatest proportion of compounds in your sample will be well resolved, and therefore, more likely to be aligned across samples in the first round of chromatographic analysis, even when the peak shapes and/or mass spectra are compromised by very high or low peak intensities. While there are some tools available for evaluating chromatography quality and for optimizing chromatographic conditions (reviewed in [9, 10]), optimal chromatography typically needs to be determined empirically.

3.3.2 Data processing—There are several steps that can be taken during data processing that will improve the quality of chromatographic alignment. First, be conservative with peak picking in the data processing method. For example, use higher S/N cutoffs (e.g., $S/N > 100$) for a minimum of three apexing masses. Statistical Compare permits initial peak finding with a high S/N, then, using the resulting peak tables as a guide, searches the chromatograms again during alignment for the same list of peaks, but with a reduced S/N threshold in the second pass. Approaching peak finding in this manner ensures that the alignment is begun with a reliable list of high-quality peaks, while allowing the addition of less well-resolved or trace-level compounds to the aligned peak table. Second, exclude problem regions in the chromatogram from the alignment step (e.g., column bleed and solvent front), which can be accomplished using the Classification feature in ChromaTOF. Third, use a moderate MS match score for alignment, such as the Statistical Compare default value of 600, which was found to be the optimum value for aligning well-resolved peaks. Fourth, exclude peak table entries that are found in a small proportion of your samples, which will help to eliminate alignment artifacts, such as the ones we observed for phenol or acetophenone at MS match scores 500 and 400, respectively (Tables S9 and 12). And finally, utilizing the data collated in the “concerns” column in the Statistical Compare peak table, exclude peaks that are flagged for saturated masses in the post-hoc analysis of the aligned data as the majority of these compounds will not be correctly aligned and will generate spurious results in subsequent statistical analyses, e.g., artificially high Fisher ratios, creating false leads in the search for biomarkers.

3.3.3 Identify and evaluate candidate biomarkers—Using the conservative data set generated from the data processing described above, identify candidate biomarkers through a combination of statistical tools that have different biases (e.g., a combination of supervised and unsupervised methods; recently reviewed in [7]) and then pursue consensus biomarkers that are high-ranking candidates across several methods. Next, evaluate the candidate biomarkers using cross-validation methods and/or independent samples [22]. If vetted, the biomarkers can then be moved into the next phase of implementation (i.e., identification of the compounds using authentic standards [29]). However, if the biomarkers do not provide sufficient selectivity and specificity in the classification of unknown samples, two avenues are available to add aligned peaks to the statistical analyses in order to revise the initial candidate biomarker list. First, if enough sample material is available, another round of analysis could be performed using different chromatographic parameters (e.g., split ratio) to pick up additional candidates to add to your first list (Fig. 3a). For example, if the first analysis was performed using a low split ratio to enhance the detection of trace compounds (Fig. 3b), you can repeat the analysis using a high split ratio to obtain better peak shapes for compounds that were excluded in the previous analysis due to saturated masses (Fig. 3c). A second option, if the samples are limited, is to perform additional rounds of data processing and alignment to identify more compounds to include in subsequent statistical analyses (Fig. 3d), but at the cost of increased post-hoc analysis of the peak table data. For instance, we observed that 8 of the 9 peaks that could be aligned at MS match score 600 could also be aligned at 500 or 700, and yielded matching Fisher ratios for the aligned data (Table 2); peaks that are not aligned generate different Fisher ratios for each alignment result (e.g., 2-pentanone; Table S2). Therefore, by performing Statistical Compare chromatographic alignment several times using a range of moderate MS match scores (e.g., 500, 600, and 700; Fig. 3e), we were able to use the Fisher ratios to confirm that 4-methyl-2-heptanone, an SM-flagged peak, is aligned at MS match score 600 and can be included in subsequent statistical analyses (Fig. 3f).

Conclusions

Proper peak alignment across all of the GC×GC chromatograms in an untargeted metabolomics study is required for identifying putative biomarkers, and good chromatography is required for peak alignment. Therefore, it is necessary to perform the best chromatography possible. Currently, there are limited tools available for building chromatographic methods *de novo*, especially for column selection, which is largely based on empirical data; much more work needs to be done in characterizing column combinations in order to streamline the process. Even with optimized chromatography methods, it isn't possible to perfectly separate every compound in a complex sample, and the data processing method must account for these imperfections. The conservative approach we propose for peak picking and alignment will significantly reduce the resulting candidate biomarker list, but improve its quality. With limited resources (sample, time, and/or budget), it is preferable to obtain low quantities of high-quality biomarker candidates versus high quantities of low-quality targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Chromatographic factors affecting peak alignment in untargeted metabolomics by GC×GC/TOF MS.
- The impact of mass spectrum matching on peak alignment across 63 chromatograms was analyzed.
- Peak resolution has a large impact on alignment, especially for very high- or low-intensity peaks.
- A workflow is proposed for the selection of viable biomarkers in untargeted metabolomics projects.

Fig. 1.

Two-dimensional gas chromatogram (GC $\times$ GC) contour plot of the headspace volatiles of a representative *P. aeruginosa* clinical isolate. The plot is displayed at 0 – 8 % of the normalized signal intensity to facilitate visualization of trace components, and regions containing only the void volume ($^1t_R < 276$) or column bleed ($^2t_R < 1.5$) have been excluded for clarity.

Fig. 2.
Bubble plot of a representative GCxGC chromatogram, with the 16 peaks of interest displayed in red and the 309 peaks with a S/N > 200 shown in gray.

Fig. 3. Workflow for the identification of high-quality candidate biomarkers from the chromatography of complex samples. The steps on the left, in solid boxes shaded in green, are the recommended steps for the first round of data collection and analysis. The steps on the right, in dotted boxes shaded in blue, are recommended if additional biomarker candidates need to be identified.

Characteristics across 63 chromatograms of the peaks that were tracked through the alignment experiments.

Table 1

Compound	# Samples ^a	# Peak Markers ^b	1st Dimension Time (s)			2nd Dimension Time (s)			Area of Apexing Masses			S/N of Unique Masses		
			Avg.	Max.	Min.	Avg.	Max.	Min.	Avg.	Max.	Min.	Avg.	Max.	Min.
2-Ethoxy-2-methylpropane	63	134	330	340	324	1.62	1.77	1.39	225295393	846653033	5227	25441	130515	10
2-Pentanone	62	86	360	364	356	1.70	1.73	1.68	129271760	422491010	1174108	20890	57841	81
Toluene	61	72	408	408	408	1.72	1.74	1.70	73383676	509237712	27106324	38392	65578	1826
2-Hexanone	40	40	417	420	416	1.73	1.76	1.70	6914029	30395986	176757	1513	6931	171
3-Heptanone	48	49	479	480	476	1.76	1.79	1.73	986968	2194113	360360	405	1133	41
2-Heptanone	63	63	480	484	480	1.77	1.80	1.75	6830803	24755823	2420585	1939	7154	702
Heptanal	61	61	488	488	488	1.77	1.80	1.75	3175430	9747566	227314	435	1531	106
4-Methyl-2-heptanone	61	62	512	512	508	1.77	1.80	1.75	102301518	175889249	9817718	16273	34464	295
Phenol	61	69	536	540	536	1.91	1.94	1.88	1331672	5659228	115905	1663	10210	228
Benzaldehyde	60	68	536	536	532	1.94	1.98	1.92	5878846	23306316	1002522	3497	18316	930
2-Nonanone	61	63	588	588	584	1.78	1.81	1.76	4020880	8839023	1225808	651	1525	221
Acetophenone	63	66	601	604	600	1.97	2.01	1.94	1598284	3816788	550218	1040	2989	433
Nonanal	61	64	616	616	616	1.79	1.82	1.77	5748128	22174146	25213	362	1386	40
Benzothiazole	59	60	704	704	700	2.11	2.15	2.08	279824	1700704	44983	479	2206	137
2-Undecanone	47	47	724	724	724	1.80	1.83	1.78	8858025	31048590	582652	3215	12188	98
4-Propylbenzaldehyde	44	44	724	724	724	1.95	1.99	1.94	181916	1661072	2557	192	1466	22

^aNumber of chromatograms that the peak was found in during initial peak picking, prior to alignment.

^bNumber of total peak markers across all chromatograms, with some chromatograms containing several peak markers per compound

Table 2

Alignment results for each peak as a function of mass spectral (MS) match scores

Compound	MS Match Score ^a										Statistical Compare Concerns ^b
	900	800	700	600	500	400	300	200	100		
2-Ethoxy-2-methylpropane										SM, shared apex	
2-Pentanone										SM, shared apex	
Toluene										SM	
2-Hexanone										shared apex	
3-Heptanone											
2-Heptanone											
Heptanal											
4-Methyl-2-heptanone										SM	
Phenol											
Benzaldehyde											
2-Nonanone										shared apex	
Acetophenone											
Nonanal										shared apex	
Benzothiazole											
2-Undecanone										shared apex	
4-Propylbenzaldehyde											

^a Shaded blocks indicate that the compound was aligned for the indicated minimum MS match scores. Full alignment results are provided in Tables S1–16.

^b Concerns reported in the Statistical Compare alignment peak table for MS match score 600; SM = saturated mass.

Table 3
Fisher ratios calculated after chromatographic alignment using nine different mass spectral (MS) match score minima.

MS Match Score	Unique Mass	Fisher Ratio ^a	Peak Count		
			Group 1	Group 2	Total
2-hexanone					
900	58	0.11	21	13	34
800	58	0.37	25	21	46
700	85	U	7	0	7
	58	U	0	4	4
600	58	0.90	2	12	14
	72	4.21	12	11	23
	58	U	4	0	4
500	58	4.36	2	14	16
	58	U	4	0	4
400	58	4.71	3	11	14
300	58	9.27	3	12	15
200	58	U	9	1	10
	58	4.21	2	11	13
	58	3.84	3	3	6
100	58	3.75	2	10	12
	58	U	1	4	5
	58	0.63	8	2	10
Acetophenone					
900	105	0.96	35	28	63
800	105	1.31	35	28	63

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Peak Count				
MS Match Score	Unique Mass	Fisher Ratio ^a	Group 1	Group 2 Total
700	105	1.31	35	28 63
600	105	1.31	35	28 63
500	105	1.31	35	28 63
400	105	1.31	35	28 63
300	105	1.31	35	28 63
200	105	1.31	35	28 63
100	105	1.31	35	28 63

^a_U = Undefined