Fisher Ratio feature selection by manual peak area calculations on comprehensive two-dimensional gas chromatography data using standard mixtures with variable composition, storage, and interferences

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Abstract

Comprehensive two-dimensional gas chromatography (GC×GC) is becoming increasingly more common for nontargeted characterization of complex volatile mixtures. The information gained with higher peak capacity and sensitivity provides additional sample composition information when one-dimensional GC is not adequate. GC×GC generates complex multivariate data sets when using nontargeted analysis to discover analytes. Fisher Ratio (FR) analysis is applied to discern class markers, limiting complex GC×GC profiles to the most discriminating compounds between classes. While many approaches for feature selection using FR analysis exist, FR can be calculated relatively easily directly on peak areas after any native software has performed peak detection. This study evaluated the success rates of manual FR calculation and comparison to a critical F-value for samples analyzed by GC×GC with defined concentration differences. Long term storage of samples and other spiked interferences were also investigated to examine their impact on analyzing mixtures using this FR feature selection strategy. Success rates were generally high with mostly 90-100% success rates and some instances of percentages between 80-90%. There were rare cases of false positives present and a low occurrence of false negatives. When errors were made in the selection of a compound, it was typically due to chromatographic artifacts present in chromatograms and not from the FR approach itself. This work provides foundational experimental data on the use of manual FR calculations for feature selection from GC×GC data.

Keywords

Multidimensional gas chromatography, GCxGC, volatile organic compounds (VOCs), nontargeted analysis, feature selection, chemometrics

Introduction

Comprehensive two-dimensional gas chromatography (GC×GC) has provided separation scientists with unprecedented separation power for mixtures of volatile and semi-volatile compounds since its first inception 30 years ago [1]. The increased popularity of GC×GC has resulted largely from its improvements in peak capacity for complex sample analysis, which typically yields 10 times greater magnitude in peak capacity than conventional one-dimensional gas chromatography (1D GC)[2]. In GC×GC, two columns are combined at a modulator junction, allowing the primary separation to be sliced into sections. Each subsection of effluent from the primary column undergoes a subsequent separation on a secondary column that contains a different stationary phase. These capabilities translate into significant improvements in peak detectability, sensitivity, and selectivity, which can allow the analyst to focus on the most relevant peaks in a complex sample over simply the most visible or abundant peaks that would be seen in a 1D GC analysis due to complicated coelutions. Since the secondary separation happens rapidly throughout the run, there is no additional analysis time associated with GC×GC over a translated 1D GC method.

Although substantial benefits are achieved from uncovering the wealth of data in a complex sample using GC×GC, the abundance of such data can also be a significant challenge [3]. Not only is data analysis typically more complex due to the data handling procedures and number of monitored variables, it also has the side effect of caution among new users in regards to adopting the technique for apprehension of the added complexity. A recent review on GC×GC data handling showed the multitude of processing strategies that can be considered, especially when performing non-targeted analyses [4], and these approaches are starting to be increasingly well documented and understood. The data interpretation and chemometric stages of the experimental process are crucial in presenting and understanding the results of an experiment [4]. It is becoming common to see more complex machine learning data workflows incorporated into GC×GC analysis, which adds to the perceived complexity of the technique. For comparisons on different machine learning strategies for volatile and GC×GC applications, some recent reviews provide extensive detail [5, 6]. The direction of the field indicates that the ability to handle and work with increasing complexity seems to be desirable, but there are still simpler approaches available to users that can accomplish feature selection without the added complexity.

Feature selection is a definitive and impactful stage of data analysis for GC×GC data. A large, nontargeted data set is limited in some manner to only features useful in differentiating sample groups (i.e. classes) [4]. This places focus on compound variability between groups of samples and can be extremely beneficial in nontargeted analysis. A class-based approach is considered to be a supervised strategy, since classes are defined by the analyst. After powerful chromatographic separation using GC×GC, feature selection allows extraction of the most meaningful variables in the data. For example, some features may be consistent across all samples analyzed and provide little-to-no differentiation, even if they are consistently found in high abundance. This highlights another powerful aspect of GC×GC separations, since it demonstrates the ability to physically separate abundant compounds from those of lesser abundance, in order to effectively perform class differentiation. One approach to class differentiation that has been under development for several years is the use of an F-ratio or Fisher Ratio (FR). The FR is a ratio between the class-to-class variance and the variance within a class (eq. 1.1) and it has been applied in several different ways to GC×GC data. If a group of samples have small within class variance, compared to the between class variance, the FR value is high and indicates a difference between the classes. This approach does not require matched replicates within each sample class, so it is also applicable for data sets where a replicate might have been lost due to instrument malfunction, or when dealing with biological samples where number of replicates varies from one group to another. To date, several different strategies to use FR for class differentiation have evolved.

$$FR = \frac{Between\ Class\ Variance}{Within\ Class\ Variance} \qquad eq.\ 1.1$$

This use of this statistic for GC×GC data first developed momentum in early work by Pierce et al. in 2006 [7]. Marney et al. in 2013 [8] introduced the idea of a tile-based Fisher Ratio and the current version of tile-based FR analyses are based on the foundational work of Parsons et al. in 2015 [9]. FR approaches essentially have been described in three ways: peak table-based FR [10, 11], pixel-based FR [12, 13], and tile-based FR [8, 9]. In peak table-based FR analyses, the software identifies peaks in its native algorithm, and the peak response data (e.g. peak area) can be used for FR calculation. Some software use different peak responses for calculation of FR, such as total ion current peak area, quant m/z area, or the peak area from single channel detectors. In pixel-based FR analyses, the peak responses are taken directly from pixel-level information identified by the software, rather than taking peak metrics from tabular format. In tile-based FR, a “tile” is placed in each location of the plot and shifted in several directions in order to capture the peak of interest and provide the data used in calculating FR. The tile-based approach is often beneficial because it avoids issues of retention time shifting and essentially provides a means of aligning peaks across large data sets more effectively.

Various software approaches have historically used FR in different ways. For example, LECO ChromaTOF Statistical Compare (no longer commercially available for purchase) employed a peak-table based alignment approach that calculated FR values on peak metrics from individually aligned variables. In approaches such as this, the user could sort based on descending FR but had to decide the significance of the FR value and determine their own cutoff threshold. That threshold could be an arbitrary value or could be based on a statistical significance level if desired. Other software, such as GC Image, are capable of calculating FR values for pixel-based integrated and/or aligned data, but do not incorporate a specific feature selection strategy based on this value; therefore, it is the user’s decision how to incorporate this piece of information into their decision-making process. Often when performing class comparison, the strategy is to investigate features with the highest FR and move down the list until compounds are reached that do not appear relevant (e.g. column bleed or sample introduction artifacts). This is usually referred to as supervised ranking. More recently, commercial options have been incorporated into multiple software that employ a tile-based FR approach to enable supervised ranking using FR. These software generate a hitlist that can typically be filtered based on user-defined criteria (e.g. top 50 features). Users work through these lists to remove artifacts and to identify key compounds that can be focused on in further processing strategies. In all of these approaches, the native software has decided how to perform the FR calculations, but the user's decision on where to place significance on those results can differ.

One other approach to FR that has received less attention but is still valuable and has been used in published research is to manually calculate FR values on peak area data in a spreadsheet program without employing commercial software and compare it with a critical threshold known as the critical F-value (F_{crit}) value. This makes the process similar to performing a hypothesis-based significance test. The F_{crit} used for comparison is based on the number of classes of samples, the number of samples within each class, and the significance level desired, and can be directly calculated from an Analysis of Variance (ANOVA). The ANOVA-style approach was described in 2002 [14]. The simplicity of treating GC×GC data in this manner has been veered away from as more complex approaches have been developed. This approach has continued to be used in certain applications for untargeted discovery to reveal underlying differences in samples using GC×GC for many different bioanalytical applications [7, 11, 15–23]. In addition, this can be done using any type of peak response metric, whether that data is generated from peak table information, pixel-based information or another means of generating peak data. While many studies that previously employed this approach used peak area data from mass spectrometer detection, it can also be applied to peak response data from any detector type and does not require mass channels for calculation. This may be considered an attractive option for users that are operating multiple or simultaneous detection approaches for interest in using peak area data from single channel detectors, for example, as it can be easily applied on data from any source.

The first description of Fisher Ratio compared to a critical threshold was by Johnson and Synovec in 2002 [14] and the terminology was described as an F_{crit} threshold by Brokl et al. in 2014 [11]. If the FR for a compound is less that the F_{crit} value, then that compound is not significant in class differentiation and is typically excluded from the data set. However, if the FR for a compound is greater than the F_{crit} value, then that compound is significant and worth further exploring as a class marker. Larger FRs indicate higher discriminatory power. Once the user generates peak table data, a basic spreadsheet program can be used to perform all FR calculations. This approach is different to tile-based FR approaches because an assessment of significance is provided for each individual feature, rather than returning a ranked hitlist to the user. To the authors' knowledge, no study has ever specifically addressed the success rate and limitations of the FR peak area approach by using samples where the truth of actual variation from sample to sample was known and assessed for efficacy.

The objective of this research was to use FR feature selection based on the manual calculation of FR from peak area data and comparison to F_{crit} threshold for GC×GC data to analyze volatile organic compound (VOC) mixtures with known concentration variation. This study employed the use of a GC×GC platform equipped with a reverse fill/flush modulator and dual channel detection via quadrupole mass spectrometry and flame ionization detection (GC×GC-qMS/FID); however, the workflow discussed can be applied to the output from any instrument and is platform non-specific. It is essential to understand the practical limitations of this feature selection method as it has been used widely in past research studies, but has not been directly assessed experimentally. Several experiments were carried out to evaluate the feature selection method, including across compound classes, for samples stored for different time periods, and samples spiked with interferences to simulate complexity of real samples. The success rate of the FR method was measured for all comparisons in order to understand whether compounds were included or excluded correctly, or whether incorrect assignments were made under different circumstances. This paper highlights the limitations and differences between this approach compared to a commercially available FR approach. It is the hope that this work will emphasize a relatively simple approach to variance-based feature selection for users that do not have access to sophisticated software add-ons or complex machine-learning workflows, while keeping in mind the associated limitations.

Materials & Methods

Volatiles Reference Mix (VRM) and Saturated Alkanes Mix (SAM)

The VRM was created from a combination of commercial mixes and individual compounds. Mix 1 contained 2-ethyl-1-hexanol, 1-propanol, 2-propanol, 2-butanone, cyclohexane, and 2-methylfuran, each at a nominal concentration of 1000 ppm (Restek Corporation, Bellefonte, PA, USA). Mix 2 contained styrene, 2-methylpentane, 3-methylpentane, 2,4-dimethylheptane, 2-methylhexane, naphthalene, and 1,2,3-trimethylbenzene, each at a nominal concentration of 1000 ppm (Restek Corporation). Mix 3 contained benzene, ethylbenzene, toluene, m-xylene, o-xylene, and p-xylene, each at a nominal concentration of 2000 ppm (Restek Corporation). Individual standards used were dimethyldisulfide with ≥ 98.5 % purity, dimethyltrisulfide with ≥ 98.5 % purity, heptanal with ≥ 97.0 % purity, and hexanal with ≥ 95.0 % purity (Sigma-Aldrich, St. Louis, MO, USA). The solvent used for dilution was HPLC-grade methanol (J.T. Baker, Center Valley, PA, USA).

From the commercial mixes and individual standards, a series of mixed standards were created for liquid injection. The VRM contained Mix 1, Mix 2, Mix 3, dimethyldisulfide, dimethyltrisulfide, heptanal, and hexanal. A solution of 100 ppm was made and serial dilutions were performed to make solutions with concentrations of 10 ppm, 5 ppm, and 1 ppm in HPLC-grade methanol (J.T. Baker). Solutions were prepared in 2.0 mL clear glass GC vials and then an aliquot was transferred to new 2.0 mL GC vials containing 200 µL glass inserts.

A saturated alkanes mixture (Sigma-Aldrich) with alkanes ranging from C_8 to C_{20} was also created for liquid injection. Serial dilutions were performed on the 40 ppm stock mix to make solutions with concentrations of 10 ppm, 5 ppm, and 1 ppm. The solvent used for dilution was HPLC-grade hexane (Supelco, Bellefonte, PA, USA). Solutions were prepared in 2.0 mL clear glass GC vials and then transferred to new 2.0 mL GC vials containing 200 µL glass inserts.

The Electronic Supplementary Material (Table S1) provides a collated list of each individual compound from each mix.

Analysis

Samples were injected using a Triplus RSH autosampler (Thermo Scientific, Waltham, MA, USA). A 10 µL syringe was used to inject 1.0 µL of the VRM and SAM at prepared concentrations in two separate sequences in order to set the autosampler with the correct wash solvent. For both sequences, the syringe was rinsed with 5.0 µL of solvent three times prior to injection, once with 1.0 µL of sample prior to injection, plunged three times and then collected 1.0 µL of the sample in an air gap. After injection, the syringe was rinsed with 5.0 µL of the respective solvent from the sample being injected three times. Ten replicates of each concentration were injected for both sets of mixes, and solvent blanks were also injected at the beginning, end, and between every 10 samples. Samples were compared within a concentration group (with two groups of n = 5 injections) by randomly assigning replicates to two separate groups, and between concentration groups (with two groups of n = 10 injections from each concentration comparison). This experiment was then repeated 302 days later for the VRM and 196 days later for the SAM to assess whether the long-term storage in the refrigerator of the solutions would impact the results in a negative way. Two separate experiments were later conducted to simulate real samples that contain more analytes and within different classes in order to assess potential interference of the FR feature selection results. The first experiment consisted of the VRM being spiked with a 52-component Indoor Air Standard (Supelco, 100 ppm each component in methanol:water 95:5), and the second experiment consisted of the VRM being spiked with a Fatty Acid Methyl Ester (FAME) Mix (FAME Mix #2, Restek Corporation). Spikes were performed at an equivalent concentration to the standard solution in all cases.

The highest concentration used for the indoor air standard trial was 50 ppm rather than 100 ppm due to dilution limitations. Interferences were not introduced into the alkanes mix due to the solvent incompatibility.

GC×GC - qMS/FID Method

The instrument was controlled using Chromeleon 7 version 7.2.9 (Thermo Scientific). The instrument configuration consisted of a Trace 1300 GC/FID and an ISQ 7000 Single Quadrupole Mass Spectrometer (Thermo Scientific). The inlet temperature was 250 °C, and was operated in split mode with a split flow of 10 mL/min and a purge flow of 5 mL/min. The first dimension column was an Rxi-624Sil MS column (30 m x 0.25 mm ID x 1.4 µm film thickness, Restek Corporation). The column junction was equipped with a reverse fill/flush (RFF) INSIGHT flow modulator (SepSolve Analytical Ltd., Peterborough, UK). The second dimension column was a Stabilwax column (5 m x 0.25 mm x 0.25 µm film thickness, Restek Corporation). The flow rate through the front inlet was 1.00 mL/min and the auxiliary flow rate through the second dimension was 20.00 mL/min. The bleed line of uncoated fused silica was 5 m x 0.1 mm ID. The loop dimensions were 0.53 mmID x 1133 mm, resulting in a loop volume of 25 µL. The modulation period (P_M) was 2.5 s, and the flush time was 100 ms, which was held constant throughout the full duration of the run. The calculated flow rate in the second dimension column was 17.9 mL/min using the INSIGHT flow calculator. The GC oven started at 60 ° C, was held for 3 min, ramped at the rate of 5 °C/min to a final temperature of 250 °C, and held for 5 min. The secondary column flow was split between the FID and MS using an unpurged SilFlow GC 3-port splitter (Trajan Scientific and Medical, UK) at approximately 4.5:1, which was maintained constant throughout the run. All carrier gas flow was supplied using ultra high purity helium (Airgas, Radnor, PA, USA). The total run time was 46 min. The transfer line temperature and the ion source temperature for the qMS were both set to 280 °C. The qMS was operated in electron ionization mode with a scan range from 40 - 300 *m/z*, a scan time of 0.02 s, and the total scan time of 0.0241 s. This resulted in an overall scan rate of ~41.5 scans/s. This is the maximum acquisition rate for this instrument when using this scan range. Prior publications [24, 25] provide the resulting specifications on data acquisition from this particular setup and the data processing workflow for using the qMS/FID streams simultaneously. The FID was operated with 350 mL/min ultra-zero grade air (Airgas), 40 mL/min ultra high purity nitrogen as makeup gas (Airgas), and 35 mL/min ultra high purity hydrogen (Airgas). The temperature of the FID was set at 250 °C and an acquisition rate of 120 Hz was used. Instrument control was performed using Chromeleon 7 version 7.2.9 (Thermo Scientific). This setup allows compounds to be identified using the qMS channel while using the FID as a quantitative detector due to its higher acquisition rate.

Data Processing

Data acquisition was performed for all data sets using Thermo Scientific™ Chromeleon™ V.7.2.9. GC×GC-qMS *.raw files were exported, converted into *.cdf format, and imported into ChromSpace® software V.1.4. (SepSolve Analytical Ltd) for processing. GC×GC-FID files were exported as *.cdf and imported into ChromSpace® software V.1.4. (SepSolve Analytical Ltd) for processing.

GC×GC-qMS

Dynamic baseline correction was performed on imported *.cdf files with a peak width of 0.4 s. Stencils for the peaks of interest were created by applying the curve-fitting algorithm for peak integration with a 3-point Gaussian smoothing function. The minimum peak area was 0, minimum peak height was 300,000 and minimum peak width was 0.000. Parameters for peak merging included a tolerance of 2.00 %, overlap of 2.00 %, intensity of 2.000 %, and correlation of 0.300. Subpeak apex windows for fronting and tailing were set to 2 % for both low and high P_M. Stencils were adjusted manually to ensure consistent integration for all concentration levels. A set of regions were generated using qMS data to assign compound identifications to be applied to FID data that were then used for integration to generate quantitative data.

GC×GC-FID

The generated stencil from the qMS data stream was applied to the FID stream to create a method to use for all samples in the sequencer. Top Hat baseline correction was used on imported *.cdf files using a peak width of 0.4 s. Stencils obtained from the GC×GC-qMS data processing method were transferred to FID files and the stencil was transformed to align over FID peaks. Peak detection was performed using the local regions of interest produced by these stencils with a minimum peak area of 0, a minimum peak height of 1 and a minimum peak width of 0.000. Peaks were also smoothed with a 3-point Gaussian smoothing function. The peak areas obtained from this data stream were used for application of the FR feature selection method described below.

Data Analysis

Export peak tables were transferred to a single spreadsheet database. The variance between all samples in a class comparison was calculated and divided by the mean of the variance for each individual class. The F_{crit} value was obtained by observing the generated F-value in the output for a one-way ANOVA of the data. Each FR was compared to the F_{crit} value. The F_{crit} value is dependent on the number of classes, the number of samples within each class, and the chosen significance level. The significance level for this study was $\alpha = 0.05$. Success rates were calculated based on the number of correct compounds assigned as significant out of the total compounds. For example, when comparing 1 ppm to 1 ppm, no compounds should be labelled as significant. If during an analysis between 1ppm to 1ppm, all compounds were labelled as insignificant, then the success rate of assigned compounds would be 100%. In a different scenario comparing two different solution concentrations, if 3 of 22 compounds were not labelled significant when they should have been labelled as significant, then the success rate would be 86%. Tables 1-6 throughout the manuscript display these success rates and Tables S2-S7 (see Electronic Supplementary Material) highlight the individual compounds that were incorrectly labelled.

Comparison to Commercial Options

Data from freshly prepared VRM solutions spiked with 52-component indoor air standard (prepared using the described workflow above) were compared using a commercial tile-based option. Chromcompare+ (SepSolve Analytical) was used on the same data files to provide a point of comparison to the manual method. All FID data was used for feature generation and then features were identified using the qMS data stream. Data were imported into the Chromcompare+ sequence editor as class standards and the peak detection method was performed by applying the tile sum algorithm for peak integration with a 10 s ^1t_r window width, 0.7 s ^2t_r window width, and 20 % ^1t_r and ^2t_r tile overlap. Parameters for peak merging included a tolerance of 2.00 %, overlap of 2.00 %, intensity of 2.000 %, and correlation of 0.300. Subpeak apex windows for fronting and tailing were set to 2 % for both low and high P_M. Peak filter options were set to 0 to avoid pre-filtering of features. In the Chromcompare+ project workflow application, the feature discovery was applied to report the top 50 significant features. This data was exported to a single spreadsheet and further analyzed to identify which compounds with known variance were identified in the top 50 feature hitlist.

Results and Discussion

The concept of using FRs to define important chromatographic features was first developed by Synovec et al. in 2006 [7] using a tile-based approach. The tile-based FR is intended to be used to differentiate regions within a chromatogram that distinguish class-to-class variation even when samples have high biological diversity [7]. This original tile-based approach is a combination of two pre-processing steps that often occur at discrete stages (i.e. alignment and feature selection). The manual approaches FR approach assessed herein is different because it involves the software deploying peak detection by the native algorithm. In this study, stencils were used to provide aligned peak data, though any native alignment strategy from another software could be used (i.e. peak table based or pixel based option). Once peaks were integrated, aligned, and identified, the FR was calculated on the chosen response variable (i.e. peak area) from the exported peak table data. For this reason, this approach would be considered a peak table-based approach according to past literature, but all FR calculation and assessment to the F_{crit} value was performed externally in a spreadsheet program.

Within this study, the goal was to characterize the success rate based on known ground truth about whether a compound was present, either in the same or at a different concentration between classes. Samples were created to represent these scenarios and assess whether a compound was accurately assigned as being significant. Two types of errors were of interest in this study. Type I error, also known as a false positive, exists when a compound is included as being significant when it is, in fact, not actually significant. Type II error, also known as a false negative, exists when a compound is excluded as being significant, when it is, in fact, significant. The comparison tables in Tables 1 and 2 effectively include compounds that were truly significantly variable between classes and represent the success rate of the method. Therefore, the percentage shown represents whether type I error was present when comparing two of the same concentration levels, and whether type II error was present when comparing two different concentration levels. Results are displayed separately for Table 1 and Table 2, as these were samples that were injected separately due to differences in sample solvent (i.e. volatile reference mix in methanol and saturated alkanes mix in hexane).

Table 1 Success rate of manual Fisher Ratio feature selection on compound of interest inclusion, comparing concentration levels for the volatile reference mix on freshly prepared samples with no other interferences

	1 ppm	**5 ppm**	**10 ppm**	**100 ppm**
1 ppm	100%	86%	91%	95%
5 ppm	-	100%	82%	91%
10 ppm	-	-	100%	95%
100 ppm	-	-	-	100%

Table 2 Success rate of manual based Fisher Ratio feature selection on compound of interest inclusion, comparing concentration levels for the saturated alkanes mix on freshly prepared samples with no other interferences

	1 ppm	**5 ppm**	**10 ppm**	**40 ppm**
1 ppm	100%	100%	100%	100%
5 ppm	-	100%	100%	100%
10 ppm	-	-	100%	100%
100 ppm	-	-	-	100%

Based on the data presented above, when comparing randomly assigned samples from the same concentration group (i.e. 1 ppm vs. 1 ppm, 10 ppm vs. 10 ppm), there was a 100% success rate on the volatile reference mix and saturated alkanes mix. This means that there were no instances where a compound was incorrectly classified as being significant, i.e. no type I error. This is a major success, as it demonstrates this method has a low potential for false positives. A compound list generated using this method is, therefore, not likely to list a compound that is not significantly varied between classes. When referring to studies that have applied such approaches, this can be kept in mind when looking at compound tables. The saturated alkanes mix also had a 100% success rate in including a compound when it was variable between two classes. This was true at all magnitudes of concentration variation (i.e. 1 ppm vs. 5 ppm, 1 ppm vs. 40 ppm).

Comparing different class concentration with the volatiles reference mix resulted in an 82%-95% success rate. Four individual compounds not detected as being over the F_{crit} value when they should have been (i.e. false negative) contributed to the reduced success rate. The compounds were 2,4-dimethylheptane, 2-propanol, 2-butanone, and 2-methylfuran. Tables S2-S7 (see Electronic Supplementary Material) outline the specific errors made for each comparison. It was hypothesized that these false negative scenarios were a product of the sample introduction technique and instrumental considerations of our specific instrumentation. It is important to understand these errors based on chromatographic quality, but also to understand that they may not be applicable for all instruments and scenarios. Particularly for this study, it was noted that the four compounds falsely assigned eluted in a region that appears to have a high level of background from solvent tailing in the FID data stream (Fig 1). The mass spectra for these compounds had peaks that were specific to methanol or hexane (depending on the sample solvent).

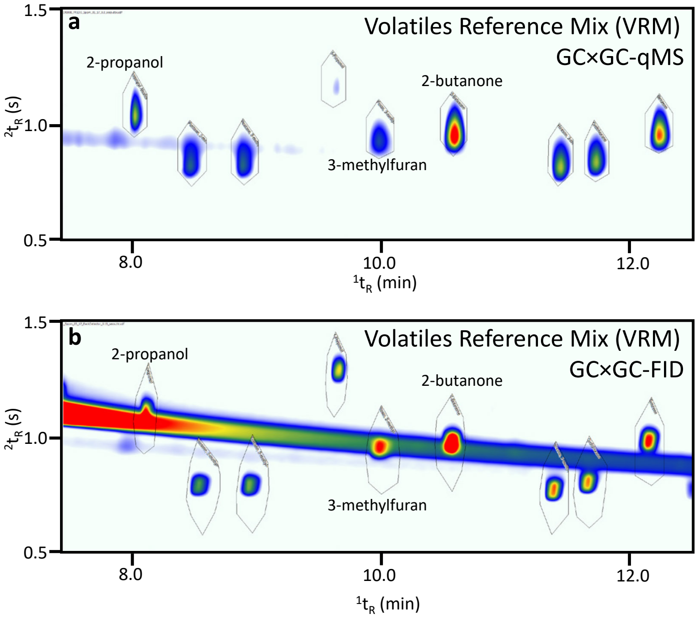

Fig 1 Comprehensive two-dimensional gas chromatography contour plots demonstrating the elution regions for 2-propanol, 2-butanone, and 2-methylfuran for **a** quadrupole mass spectrometer with lack of major impact of solvent band and **b** flame ionization detector with increased solvent interference band

If peak areas were identified from MS data, it is possible to identify this and correct for it. However, the current analytical workflow used herein [25] identified peaks using quadrupole MS data, and then integrated and assigned peak areas from the FID channel. This is typical of a dual-channel workflow but does introduce some challenges for VOCs analyzed via liquid injection. This effect may have been lessened if using a solventless injection method, such as solid-phase microextraction or sorbent tubes with thermal desorption, since these interferences would not have been observed as strongly in the FID stream. It is important to note that regardless of the chosen feature selection method, artifacts within the chromatographic separation always have the opportunity to introduce error into the resulting data processing if they interfere with the response variable being measured. This limitation is especially felt when a single-channel detector is the singular detector or part of a dual-channel workflow. The user must always take care in applying processing methods while considering how chromatographic artifacts may impact results, and this study highlighted the importance of that for compounds eluting in the lower second dimension retention window.

Storage time is an important consideration in all sample analyses. Degraded samples introduce possibilities for undesirable peak shape, poor quality spectra, or interferences from degraded components. As such, one question in this study was whether storing the samples for a long period of time would impact the VOC profile. Samples were analyzed again after several months to investigate whether there was any detrimental effect to success rate. Table 3 and Table 4 demonstrate that even when samples were stored for 10 months (volatile reference mix) and 6.5 months (saturated alkanes mix), the success rates remained relatively stable compared to the results of freshly prepared standards. Chromatographically, few changes were observed but it was important to validate this observation by measuring whether it translated to peak integration as well.

Table 3 Success rate of manual Fisher Ratio feature selection on compound of interest inclusion, comparing concentration levels for the volatile reference mix on samples stored for 302 days with no other interferences

	1 ppm	**5 ppm**	**10 ppm**	**100 ppm**
1 ppm	100%	91%	95%	91%
5 ppm	-	100%	91%	86%
10 ppm	-	-	100%	91%
100 ppm	-	-	-	100%

Table 4 Success rate of manual Fisher Ratio feature selection on compound of interest inclusion, comparing different concentration levels for the saturated alkanes mix on samples stored for 196 days with no other interferences

	1 ppm	**5 ppm**	**10 ppm**	**40 ppm**
1 ppm	100%	100%	100%	100%
5 ppm	-	100%	100%	100%
10 ppm	-	-	100%	100%
100 ppm	-	-	-	100%

In prior research applications of this FR approach, usually highly complex samples have been used. Pure standard mixtures of specific compounds, as shown herein, do not necessarily capture the complexity of those realistic samples in prior studies. As such, one aspect of this study was to further investigate the impact of adding extraneous compounds, not inherent to the mixed standards, into the samples and observe whether any effects were seen. An indoor air standard with 52 components was selected as it represented a large number of components typically encountered in a wide range of applications and therefore would represent interferences throughout the entire chromatographic space. A fatty acid methyl ester sample was also selected to represent a group of compounds that was not incorporated in the indoor air standard, and can sometimes be encountered in derivatized samples. The Electronic Supplementary Material (Fig S4) shows contour plots with the spiked VRM solution. Overall, the results of Table 5 and Table 6 indicated that these spiked interferences in the sample did not have a considerable negative effect on the success rate of the FR feature selection. This is likely due to the chromatographic resolution between compounds of interest and spiked compounds. This demonstrates the value of GC×GC in applying this type of feature selection on complex samples, where coelutions might otherwise introduce variance to a peak area in 1D GC without actually being due to the true concentration of the analyte of interest.

Table 5 Success rate of manual Fisher Ratio feature selection on compound of interest inclusion, comparing concentration levels for the volatile reference mix on freshly prepared samples spiked with an indoor air standard

	1 ppm	**5 ppm**	**10 ppm**	**50 ppm**
1 ppm	100%	91%	91%	95%
5 ppm	-	100%	91%	95%
10 ppm	-	-	100%	95%
50 ppm	-	-	-	95%

Table 6 Success rate of manual Fisher Ratio feature selection on compound of interest inclusion, comparing concentration levels for the volatile reference mix on freshly prepared samples spiked with a fatty acid methyl ester mix

	1 ppm	**5 ppm**	**10 ppm**	**40 ppm**
1 ppm	100%	91%	91%	100%
5 ppm	-	100%	91%	95%
10 ppm	-	-	100%	100%
100 ppm	-	-	-	100%

It is important to consider that the chromatographic resolution of the method likely contributed to the success of eliminating interferences from spiked compounds, since in theory, these spiked compounds should be physically separated from the target compounds being integrated. Fig 2 demonstrates a volatile reference mix standard that was spiked with the indoor air standard, where numbers 1-22 represent the VRM compounds and unlabeled peaks are those introduced by the air standard. The benefits of a multidimensional approach are emphasized in this figure, since it is clear that the spiked compounds were physically separated adequately and therefore did not impact integration of targeted compounds from the VRM. It is hypothesized there may be more of an influence on complex samples analyzed by one-dimensional GC with the application of this feature selection approach , especially in scenarios where a single channel detector is used.

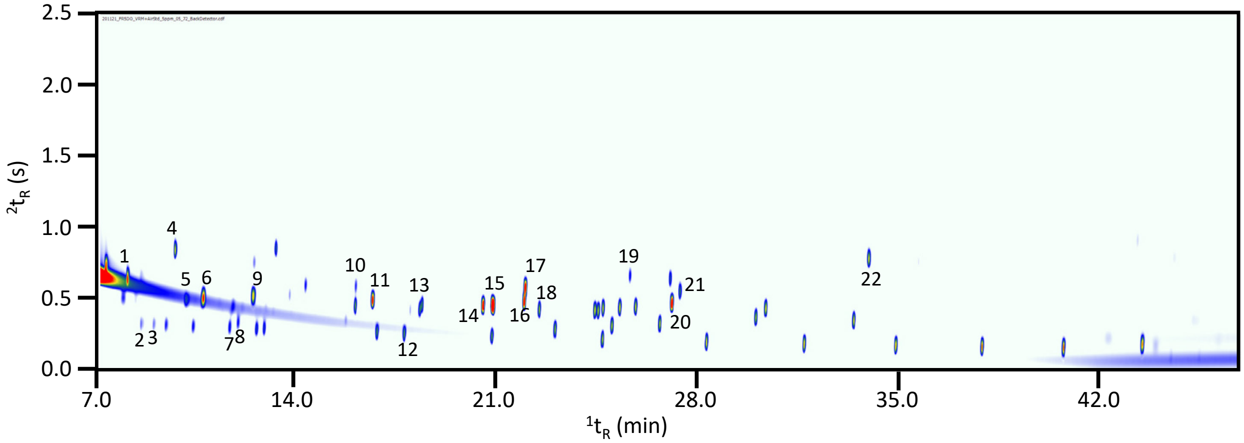

Fig 2 Comprehensive two-dimensional gas chromatography – flame ionization detection (GC×GC-FID) contour plot of the volatile reference mixture (VRM) spiked with the 52-component indoor air standard. All components had an approximate concentration of 5 ppm. Compounds highlighted with numbers are those that originated from the VRM to differentiate them from peaks that were introduced as spiked

interferences. Compound labels correspond to numbers provided in the Supplementary Information Figure S2.

The spiked VRM sample with 52-component indoor air standard was also assessed using a commercial tile-based approach for comparison (Table 7). Using this method, no errors were made when comparing samples classes with the same concentration (e.g. 1 ppm vs. 1 ppm). However, the results were not equivalent to the manual method when comparing different concentrations. Peaks that were known to be variable between classes but that were not identified within the top 50 features returned from the tile-based FR comparison are shown in the Electronic Supplementary Material (Table S8). It is possible that these compounds may have appeared lower down the list but this means there would have been significant false positives identified with that approach that were marked as being significant over the target peaks. Parameters for peak detection and comparison were manipulated with no additional improvement to the false negative rates. Since this method uses a different peak detection algorithm, it is possible that chromatographic quality of some of the FID peaks may have interfered with the identification of peaks as being significantly variable from class to class. For example, 2-methylfuran was sometimes missed using the manual approach as well and it had been noted that the proximity to solvent band may have been a factor in the integration process. However, the other peaks missing represent chromatographically resolved peaks that are defined and should have been detected using that approach due to their known variability.

Table 7 Success rate of tile-based Fisher Ratio feature selection using commercial software on compound of interest inclusion, comparing concentration levels for the volatile reference mix on freshly prepared samples spiked with an indoor air standard

	1 ppm	**5 ppm**	**10 ppm**	**50 ppm**
1 ppm	100%	65%	43%	43%
5 ppm	-	100%	70%	43%
10 ppm	-	-	100%	78%
50 ppm	-	-	-	100%

The use of tile-based commercial FR software to identify discriminating features can be helpful to save time and eliminate the need for the user to identify every peak within their sample prior to searching for discriminating features. However, in the case of direct application of both methods on this particular dataset, the method of stencil alignment and using peak area to manually calculate FR, and compare it against an FR threshold, appeared to be more successful.

This study investigated the success rate of applying a manual FR feature selection step with comparison to F_{crit} threshold to peak table data that had already undergone a detection and alignment step in the native software. The benefit of this method is that it is simple, can be performed on data exported as a spreadsheet file from any software, and does not require any add-ons or machine-learning knowledge. In collecting information for this research, it was also noted that a number of publications using peak table and pixel-based FR feature selection do not explicitly mention which peak response variable was used. It is suggested that in the future, a mention of peak area or peak volume be included if that is the variable used. With regards to the comparison of this manual approach to peak table-based and pixel-based integration options, it should be noted that this approach can be used on data generated by either method to produce a peak metric that can then be exported and handled in an external software for FR feature selection.

There are scenarios where a manual FR feature selection may be helpful to users of GC×GC, and other scenarios where more complex approaches may be warranted. Analysts should be realistic of the type of data they are dealing with and mindful of the tools available to them. It is also recommended that if using a software that employs a calculation or feature selection using FR calculations, to understand which type of approach is being used since this is not always immediately obvious to the user depending on the platform.

Limitations

This study was intended to provide a basic assessment of a previously-applied feature selection technique in research studies. Prior highlighted studies largely represent bioanalytical measurements, and therefore it is important to recognize that removing this biological complexity to perform success rate measurements on chemical standards does not provide a direct comparison of real sample variance. The additional interferences added to the solutions were meant to introduce additional complexity to represent some of the challenges that may be encountered in real samples; however, it is recognized that these do not represent all bioanalytical variability that can be encountered. The ability to investigate this feature selection method on simplified samples with known ground truth about each sample variation does provide the ability to assess the reliability of the method more closely. While this study does not evaluate all possible scenarios that could be encountered in complex bioanalytical samples, it provided a reference point of general issues that can be faced when using this approach. Some additional factors to take into account in future studies are concentration range, the incorporation of more compounds for assessment, different sample introduction techniques, and different detectors. While the authors are aware this study does not comprehensively cover all possible application scenarios of this strategy, it does provide foundational information that will have utility for users. Future work will investigate the potential to use standard reference materials where concentration amounts are strictly known but where known ground truth of concentration variation can be defined. This is challenging currently because there are few standard reference materials under development for nontargeted VOC studies. However, this is likely to change in the near future and would be a good avenue for future work investigating feature selection in GC×GC.

A major consideration of the use of Fisher Ratio feature selection as described in this study is the potential of adverse outcomes when stochastic effects are present in samples. It is common in biological samples to see stochastic fluctuations of compound presence/absence and of compound abundance between biological and technical replicates. In some cases this can increase variance within a class and in other cases it can introduce missing values across the data set. It is true that stochastic effects on peak areas between sample replicates can impact variance calculations, leading to additional false negatives (i.e. Type II error). This type of stochastic effect may not be accurately captured by the study design since samples were consistently reproduced. However, stochastic effects should not increase the false positive rate (i.e. Type I error). This is a strength of this method that should indeed be understood by those who apply it. It is more likely to miss a component that is significant than to include a component that is not significant. This means the approach could be considered conservative when stochastic effects exist. This is better than the contrary scenario in which a compound is considered significant when it is not actually significant. In other words, if error must exist, it is typically better to deal with Type II error than Type I error for nontargeted research.

An additional limitation of this type of approach is that it does not examine the correlation of variables to one another. This approach is a univariate approach, treating each compound individually in isolation from the rest of the data set to determine which peaks should be included or excluded as a compound of interest based on its variance amongst sample groupings. This tool does not capture the relationship between variables, and each component is essentially considered a separate data set. When using a nontargeted "fingerprinting" approach with complex samples, there is the possibility that a univariate approach like this misses networks of information. Two recent studies compared the effectiveness of FR feature selection with F_{crit} threshold to random forest (RF) to investigate the comparison of this method to other machine learning approaches to identify significant features on biological samples [26, 27]. Both studies [26, 27] commented that FR feature selection on peak table data using an F_{crit} threshold yielded very similar results to the RF approach. However, one study [27] commented that the use of RF may be more ultimately more robust than FR feature selection. Additional investigation of FR analysis with other machine learning approaches will be interesting in the future. A more simplified manual FR approach is still able to provide valuable information and remains a common approach within the field of GC×GC to couple an unsupervised technique (e.g. principal component analysis (PCA), hierarchical cluster analysis (HCA)) with a supervised technique (e.g. FR analysis) to understand which features impact group similarity and dissimilarity [6].

Conclusions

The increase in interest in GC×GC necessitates some simpler and more straightforward approaches to understand how to differentiate classes of highly complex mixtures from one another in a meaningful and accurate manner. It is true that no perfect approach currently exists, and that numerous approaches are available to accomplish this task. Manual FR feature selection represents an easy tool for users to implement on any existing GC×GC data set, so long as a peak table is available from each sample and the information has been collected from samples in a uniform manner. This study demonstrated that for most compounds across the range of possible VOC classes, success rates were high in classifying compounds as significant or not significant. In addition, when errors existed, they appeared to be related to chromatographic quality linked potentially to sample introduction technique and/or the use of a single channel detector and not from the FR approach itself. Storage of samples for relatively long periods did not introduce artifacts that impaired the success of this method, nor did the introduction of interfering solutions. When comparing a commercially-available tile-based FR software to the same data set, no improvement was observed. While it is possible that this data does not directly translate to the success rate on real samples due to dynamic range, chromatographic peak shape, or magnitude of variance in samples, it serves as a foundation for understanding when limitations may exist. Manual FR feature selection can serve as a starting point for data analysis when more complex tools are not available, as long as there is an understanding of the impacts, limitations, and challenges of using such a strategy. It would be unrealistic to assume that all applications of GC×GC will inherently necessitate complex data science strategies in the future, and that the adoption of GC×GC for routine industry applications should rely on ever more increasingly complex processing strategies. In the interest of adoption of the technique across more routine areas, approaches such as those suggested in this work can help make the use of GC×GC more accessible while maintaining data integrity.

Declarations

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Competing Interests

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supplementary Information

The following figures and tables are available in a single Supplementary Information file associated with this manuscript:

Fig S1 Contour plots demonstrating the comprehensive two-dimensional gas chromatography (GC×GC) output for each mixture. The volatiles reference mix (VRM) is displayed in plot **a** using the qMS detector and plot **c** using the FID detector. The saturated alkanes mix (SAM) is displayed in plot **b** using the qMS detector and plot **d** using the FID detector. All concentrations are 5 ppm.

Fig S2 Elution location for each compound in the VRM using GC×GC-FID

Fig S3 Elution location for each compound in the SAM using GC×GC-FID

Fig S4 Elution pattern for the VRM spiked with **a** indoor air standard and **b** fatty acid methyl ester mix. Both contour plots were acquired by GC×GC-FID. Numbers refer to Figure S2 and all concentrations are 5 ppm.

Table S1 List of compounds assessed for each of the volatile reference mixture and saturated alkanes mix

Table S2 Compounds incorrectly assigned using manual Fisher Ratio feature selection on compound of interest inclusion when comparing concentration levels for the volatile reference mix on freshly prepared samples with no other interferences

Table S3 Compounds incorrectly assigned using manual Fisher Ratio feature selection on compound of interest inclusion when comparing concentration levels for the saturated alkanes mix on freshly prepared samples with no other interferences

Table S4 Compounds incorrectly assigned using manual Fisher Ratio feature selection on compound of interest inclusion when comparing concentration levels for the volatile reference mix on samples stored for 302 days with no other interferences

Table S5 Compounds incorrectly assigned using manual Fisher Ratio feature selection on compound of interest inclusion when comparing concentration levels for the saturated alkanes mix on samples stored for 196 days with no other interferences

Table S6 Compounds incorrectly assigned using manual Fisher Ratio feature selection on compound of interest inclusion when comparing concentration levels for the volatile reference mix on freshly prepared samples spiked with an indoor air standard

Table S7 Compounds incorrectly assigned using manual Fisher Ratio feature selection on compound of interest inclusion when comparing concentration levels for the volatile reference mix on freshly prepared samples spiked with a fatty acid methyl ester mix

Table S8 Compounds not included in the top 50 features when applying tile-based Fisher Ratio feature selection using commercial software on compound of interest inclusion when comparing concentration levels for the volatile reference mix on freshly prepared samples spiked with an indoor air standard