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GC×GC-MS AND PARALLEL 2DLC-MS BASED UNTARGETED
METABOLOMICS

By
Md Aminul Islam Prodhan

A Dissertation
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville
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for the Degree of

Doctor of Philosophy in Chemistry

Department of Chemistry
University of Louisville
Louisville, Kentucky 40208

December, 2019

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METABOLOMICS

By

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A Dissertation Approved on

December 2 , 2019

by the following Dissertation Committee:

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Dr. Richard P Baldwin

Dr. Michael H Nantz

Dr. Shahid P Baba

DEDICATION

This dissertation is dedicated to my parents.

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ABSTRACT

GC×GC-MS AND PARALLEL 2DLC-MS BASED UNTARGETED METABOLOMICS

Md Aminul Islam Prodhan

December 2, 2019

Untargeted metabolomics aims to analyze as many metabolites as possible in a biological system. Due to the vast complexity of the metabolome, bioanalytical platforms have been developed for untargeted metabolomics. While comprehensive two-dimensional gas chromatography mass spectrometry (GC×GC-MS) has been used in different scientific fields, its use in untargeted metabolomics is limited due to several challenges, such as complicated data analysis and limited accuracy of metabolite identification. Two-dimensional liquid chromatography mass spectrometry (2DLC-MS) has also been employed in untargeted metabolomics, but with limited metabolite coverage.

This dissertation describes the efforts in developing new data analysis algorithms to improve the accuracy of metabolite identification in GC×GC-MS as well as integration of multiple analytical techniques for increased metabolite coverage and high confidence of metabolite quantification and pathway assignment.

This dissertation is divided into six chapters. Chapter One provides the overview of the current methodologies for untargeted metabolomics. Chapters Two, Three and Four describe the methods developed for the calculation of the second dimension retention index (2I) in GC×GC-MS. Specifically, Chapter Two introduces a surface fitting approach to

2I calculation using *n*-alkanes as reference compounds and considering the second dimension separation as in pseudo-isothermal mode. Chapter Three describes an improved method for calculation of 2I by considering the second dimension separations in its actuality, i.e., temperature-programmed mode. Chapter Four introduces a universal reference system for 2I calculation to improve retention map coverage that allows the use of any compounds as reference compounds while keeping retention scale in conventional *n*-alkane scale. Chapter Five introduces a method that integrates the GC×GC-MS and parallel 2DLC-MS platforms for untargeted metabolomics to achieve higher metabolite coverage and accurate metabolite quantification. Chapter Six summarizes the overall scientific contribution of this dissertation.

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CHAPTER 1

INTRODUCTION

1.1. Metabolomics

Metabolome is the collection of all small compounds in an organism (i.e., metabolites, with molecular weight typically under 1500 Dalton. Metabolites are the intermediates and products of all biological processes that take place in a biological system. Therefore, a metabolome is the result of a large network of metabolic reactions. The output from one enzymatic chemical reaction are input of other biochemical reactions. Comparing with genome and proteome, the metabolome is much more dynamic and complex because each metabolite is biochemically, spatially and temporally defined.^{1,2} Metabolomics is the systematic study of the metabolite formation. Inherent complexity of the metabolome in a biological sample makes the metabolomics a multidisciplinary research area that requires knowledge in Chemistry, Biology, Computer Science, Biostatistics, Biomedical Science, etc. Broadly, metabolomics methodology falls into two major categories, untargeted metabolomics and targeted metabolomics.

Untargeted metabolomics compares the difference of abundance levels of metabolites detected in two or more groups (e.g., control group and test group), to enlighten the abundance difference of metabolites that may provide the insight about relevance to the specific biological conditions. The goal of untargeted metabolomics is to detect as many metabolites as possible, and it allows the collection of data without prior knowledge about metabolites. Owing to the large number of metabolites, diverse chemical characteristics and huge concentration differences between metabolites, multiple analysis steps are involved in untargeted metabolomics at every step (i.e., sample preparation, separation methods and instrumental analysis, affects the accuracy of metabolite analysis).

Targeted metabolomics is a quantitative method that focused on a group of chemically characterized and biochemically annotated metabolites. It generally uses internal standards to make the analysis quantitative or semi-quantitative. As predefined lists of metabolites are studied, novel associations between metabolites may be illuminated in the context of specific physiological states. Targeted metabolomics exploits different mass spectrometric methods, (e.g., selected reaction monitoring (SRM) by tandem mass spectrometry, multi-reaction monitoring (MRM), parallel reaction monitoring (PRM), etc.).

1.2. Technologies Employed in Untargeted Metabolomics

Metabolites have diverse physical and chemical characteristics, as well as very large concentration difference among them in metabolome. For instance, metabolites can be polar or non-polar, as well as organic or inorganic compounds.^{3,4,5} These differences in chemical diversity have profound implications in metabolomics. As the chemical diversity of the metabolome is so broad, the analytical technologies needed to isolate and identify

thousands of chemically distinct metabolites are inherently complex. Furthermore, one does not know the number of metabolites in a biological sample such as human body fluids. It is estimated that at least thousands of metabolites should be detectable. The diverse characteristics and huge number of entities make metabolite separation challenging, in that there is not a singular instrument capable of analyzing all types of metabolites.⁶⁻¹⁰ To date, multiple analytical platforms have been developed and used in untargeted metabolomics. Among those analytical platforms, biological samples are frequently analyzed by gas chromatography mass spectrometry (GC-MS) and/or liquid chromatography mass spectrometry (LC-MS).¹¹⁻¹³

1.2.1. Gas Chromatography Mass Spectrometry

GC-MS is known to be the gold standard technique for analysis of volatile or semi-volatile metabolites. Figure 1.1 is a schematic diagram of a GC-MS instrument. In GC-MS based metabolomics, metabolites are first derivatized by a derivatization reagent to make the metabolite volatile and thermally stable. The derivatized metabolites are then separated on a GC column and the separated metabolites are further subjected to mass spectrometer for measurement. The GC column is usually 10 to 60 m

Figure 1.1. Schematic of a GC-MS.

long and the stationary phases can be polar, semi non-polar or non-polar. GC separates the metabolites by partitioning the metabolite between the stationary phase and the mobile phase inside the GC column. Specifically, inside of a GC column, a flow of mobile phase,

usually helium, percolates through an absorbent known as stationary phase. The metabolites initially adsorbed to the surface of the stationary phase followed by evaporation from the stationary phase to the open space of the column. The metabolites are then hit by carrier gas molecules and start travelling down the column. After a short distance, however, they touch the sticky surface of the stationary phase and go through another partitioning process. This process continues until the metabolites enter into mass spectrometer that mostly equipped with an electron ionization (EI) ion source. While GC-MS has an excellent resolving capacity to separate metabolites, it has limited peak capacity. Moreover, a GC-MS system requires chemical derivatization of metabolites in order to make them volatile and thermally stable.

Identification of metabolites in GC-MS: GC-MS provides two measures for each metabolite, retention time and an EI mass spectrum. A GC-MS system can detect 600 – 900 peaks and only about 200 – 300 metabolites can be identified.¹⁴ Compound identification in analysis of GC-MS data is usually achieved by matching a query spectrum, i.e., an experimental EI mass spectrum, to the mass spectra of compound standards in a reference library. A metabolite in the reference library with the highest spectral similarity measure is usually considered as the metabolite given rise to the query spectrum. This process is entitled mass spectrum matching.¹⁵ Many mass spectrum matching methods have been developed for analysis of GC-MS data, including composite similarity,¹⁶ probability-based matching system,¹⁷ cosine correlation¹⁸⁻²¹ and Hertz similarity index.²² Stein and Scott²³ examined the performance of composite measure with other measures using National Institute of Standard Technology (NIST) database, while Horai *et al.*²⁴ performed a similar study using MassBank database. Both studies demonstrated that the

composite measure performed better than other methods. Dr. Xiang Zhang's group (University of Louisville) further demonstrated that the identification accuracy of existing methods is about 80%,²⁵⁻²⁷ likely due to the significant enrichment of reference spectral library and the increased sample complexity. A large spectral library increases the chance that the mass spectrum of the true metabolite is present in the library, but it also increases the chance that highly similar mass spectra from other metabolites are also present. Multiple metabolites in a biological sample may have similar mass spectra, resulting in some metabolites in the mass spectrum library having multiple spectrum matched peaks in the experimental data, and the spectrum with the highest similarity score may not be the true match. In addition, the mass spectrum represents only partial information of the molecular structure of a metabolite. Therefore, the mass spectrum matching based identification method in GC-MS has limited identification accuracy.

Retention index to aid in metabolite identification in GC-MS. Identifying metabolites solely based on mass spectrum matching has inherent limitations. Therefore, additional information such as metabolite separation information (e.g., retention time) can be employed to increase the identification confidence. Since retention time in GC depends on experimental conditions, retention index was introduced to reduce such dependency. Retention index is normalized retention time using homologous reference series mostly *n*-alkanes. Figure 1.2 shows the elution order of two homologous reference compounds (*x* and *y*) bracketing a compound (*u*). The retention time of compound *u* can

be normalized with respect to the retention times of reference compounds x and y . This normalized retention time is known as retention index (I_u^T).

Two equations have been developed to calculate the retention index based on the operation mode of GC-MS, temperature-programmed mode or isothermal mode. For the temperature-programmed mode, retention time t_R of a compound (u) is converted into a linear retention index I_u^T using the retention times of the two bracketing n -alkanes as follows:²⁸

$$I_u^T = 100 \times \left[n + \frac{t_{R(u)} - t_{R(n)}}{t_{R(n+1)} - t_{R(n)}} \right] \dots \dots \dots (1)$$

where n is the number of carbon atoms in n -alkane, $t_{R(u)}$, $t_{R(n)}$ and $t_{R(n+1)}$ are the retention times of the compound u , the n -alkane with n carbon atoms and the n -alkane with $n+1$ carbon atoms, respectively.

For isothermal conditions, retention time t_R of a compound (u) is converted into Kovats retention index I as follows:²⁹

$$I_u = 100 \times \left[n + \frac{\log(^2t'_{R(u)}) - \log(^2t'_{R(n)})}{\log(^2t'_{R(n+1)}) - \log(^2t'_{R(n)})} \right] \dots \dots \dots (2)$$

where n is the number of carbon atoms in n -alkane, $^2t'_R$ is the adjusted second-dimension retention time, i.e., the second-dimension retention time of a chromatographic peak minus the retention time of a unretained peak 2t_M .

Both mass spectrum and retention index have been used for compound identification in GC-MS.³⁰⁻³² All of the existing methods employ retention index as a filter to remove the potential false-positive identifications generated by mass spectrum matching. Such an

analysis strategy uses the retention index and mass spectrum in two separate analysis steps. The sequential nature of the two-step analysis strategy increases the risk of introducing errors from each independent stage since there is no way to correct the errors caused by the previous step. To improve the identification accuracy, Dr. Xiaoli Wei developed a SimMR method that simultaneously evaluates the mass spectrum similarity and the retention index distance using an empirical mixture score function,³³ and her study demonstrated that the SimMR method outperforms the literature reported compound identification methods by improving the overall identification accuracy up to 1.53%.

1.2.2. Comprehensive Two-dimensional Gas Chromatography Mass Spectrometry

Due to the limited peak capacity of GC-MS,³⁴ two-dimensional gas chromatography (2DGC) was developed decades ago. Conventional 2DGC technique uses the “heart cutting” method where selected portions of analytes eluted from the first dimension (¹D) column are transferred to the second dimension (²D) column for further separation to achieve enhanced resolution for the heart-cutting zone.³⁵ This technique is unsuitable for untargeted metabolomics owing to its limited peak capacity and longer analysis time. Comprehensive two-dimensional gas chromatography mass spectrometry (GC×GC-MS) is the latest development in the 2DGC technique to enhance the GC separation power.

A GC×GC-MS system has four major components: a first dimension (¹D) column, a modulator, a second dimension (²D) column and a mass spectrometer. The two columns ¹D and ²D are connected in series by a modulator. After the sample is injected into the

system through an injector, metabolites are initially separated on the 1D column. Metabolites eluted from the 1D column within a certain period of time, termed as modulation period P_M , are collected as a fraction by the modulator and subjected to the 2D column for further separation. After the separation on the 2D column, metabolites are transferred to the mass spectrometer for measurement. This process is sequential and continuous throughout the analysis until all fractions collected from the 1D column are analyzed by the 2D column and mass spectrometer (Figure 1.3). Therefore, the GC $\times$ GC-MS offers a much increased peak capacity and superior resolution, selectivity, sensitivity, and dynamic range for the analysis of small molecules.^{36,37}

Figure 1.3. A photo of a GC $\times$ GC-MS workstation, the components of which are discussed below. (Left) The prominent parts of GC $\times$ GC-MS which includes the TOF-MS, an autosampler, and the GC oven. (Right) Two GC oven and column.

Metabolite identification in GC $\times$ GC-MS: GC $\times$ GC-MS characterizes each metabolite by its first dimension retention time (1t_R), the second dimension retention time (2t_R) and an EI mass spectrum.^{38,39} Likewise to GC-MS, identification in GC $\times$ GC-MS also is done by mass spectrum matching as described in section 1.2.1. Identification of

metabolites solely based on the mass spectrum matching also incurs the similar problem as encountered in GC-MS.

In order to increase the identification accuracy, retention index has also been used in analysis of GC×GC-MS data. GC×GC-MS has two columns. For the first dimension column, the retention time (1t_R) of a compound u is converted into linear retention index I^T using Equation (1). While Equation (1) can be used for calculation of the first dimension retention index, it cannot be used for calculation of the second dimension retention index. Several factors hinder the use of Equation (1) for the second dimension retention index calculation. First, the second dimension separation is limited by the modulation time. In order to use Equation (1), the second dimension retention time of a compound of interest must locate between the second dimension retention times of two reference compounds. That is the metabolite of interest and the two reference compounds must have the same first dimension retention times, i.e., eluting from the first dimension column in the same modulation period. However, it is not possible to always get two bracketing reference compounds in the same modulation period. Figure 1.4 shows a sample contour plot of C₇-C₃₀ *n*-alkanes analyzed by GC×GC-MS, where x-axis is the first dimension retention time and y-axis is the second dimension retention time. The unit of the both axis are in seconds. Specifically, compound U₁ eluted between

two consecutive *n*-alkanes reference compounds C₁₁ and C₁₂ wherein compound U₂ was eluted between reference compounds C₁₈ and C₁₉. Therefore, the first dimension retention index of U₁ and U₂ can be calculated using the first dimension retention times of their corresponding bracketing reference compounds employing Equation (1). However, there is no bracketing reference compounds eluted after of compounds U₁ and before U₂ in y-axis. Therefore, we cannot use Equation (1) to calculate the second dimension retention index of these compounds. In order to solve this problem, a method called “isovolatility curve” has been developed to calculate the second dimension retention index (2I) using Equation (2).^{40,41} The reason for using Equation (2) to calculate 2I is that the second dimension separation was considered as pseudo-isothermal as the second dimension column is very short and the resultant temperature change in each modulation period is very small.

The retention time of a compound in the second dimension (2t_R) column decreases with increases in elution temperature (2T_e). The relation [$^2t_R \sim ^2T_e$] of a compound can be captured by the isovolatility curve. Figure 1.5 shows an example of an isovolatility curve constructed using the GC×GC-MS data of C₈-C₂₈ even-numbered *n*-alkanes, where every curve represents one isovolatility curve corresponding to a *n*-alkane compound. Isovolatility curve can be constructed by several different ways while the main

Figure 1.5. Isovolatility curve constructed from C₈-C₂₈ even numbered carbon *n*-alkanes reference compounds.

idea is to get the retention time of reference compounds at different elution temperatures. As the second dimension separation considered as pseudo-isothermal, the hold-up time, 2t_M , of reference compounds at different temperatures must be estimated to get the adjusted retention time, ${}^2t'_R$. The ${}^2t'_R$ values of the same reference compound at different column temperatures were then used to construct the isovolatility curves of the reference compounds. The isovolatility curves of all reference compounds formed the second dimension retention index map.

After the construction of the isovolatility curves, the 2I value of a compound can be calculated using the two adjacent isovolatility curves corresponding to two reference compounds. In Figure 1.5, compound u was eluted between the isovolatility curves of reference compounds C_{12} and C_{14} . Compound u has its second dimension retention time and the corresponding second dimension elution temperature. If this elution temperature value is interpolated in the isovolatility curve equations of reference compounds C_{12} and C_{14} , two corresponding retention time values can be obtained at the elution temperature of u . Now, 2I of compound u can be calculated using Equation (2) as retention times of compound u and the reference compounds are at the isothermal condition (same x-axis value).⁴⁰

While the first dimension retention index has been used to aid in compound identification⁴², the second dimension retention index 2I has not been extensively used in aiding compound identification in GC×GC-MS due to inaccuracy of 2I calculation.

1.2.3. Liquid Chromatography Mass Spectrometry (LC-MS)

The liquid chromatography mass spectrometry (LC-MS) has also been used in untargeted metabolomics.⁴³⁻⁴⁷ In LC-MS, metabolites are separated based on their interaction with mobile phase and stationary phase when the mobile phase moves through the stationary phase in the column under pressure. Figure 1.6 depicts the instrumentation of LC-MS that consists of a pump, an injection valve, a column and a mass spectrometer.

A sample is loaded into the column through an injection valve. Pressure created by the pump forces the mobile phase to carry the metabolites to the detector through the LC column. The column is

packed with stationary phase particles. Depending on the stationary phase particles, the LC can be categorized as normal phase chromatography, reversed-phase chromatography, ion-exchange chromatography, etc.

Choices a column stationary phase and the corresponding mobile phase are the most important and challenging decision in LC-MS. Among the different liquid chromatography modes, reversed-phase chromatography (RPC) is the most widely used technique. In RPC, the stationary phase is non-polar (hydrophobic) and the mobile phase is polar. Water mixed with other solvents (i.e., methanol, isopropanol and/or acetonitrile) are used as the mobile phase wherein long-chain alkyl groups (i.e., *n*-octadecyl or C18) attached to the surface of spherically shaped silica particles are used as the stationary phase.⁴⁸ Therefore, RPC is suitable for separation of non-polar metabolites. Hydrophilic interaction chromatography

(HILIC), a form of normal phase chromatography, has also been used to separate metabolites in LC-MS based metabolomics.⁴⁹ In HILIC, mobile phase is water miscible solvents like acetonitrile, methanol, etc. mixed with water and the stationary phase is polar. Therefore, HILIC provides strong retention of metabolites that are unretained under conventional reversed-phase condition.

1.2.4. Two-dimensional Liquid Chromatography Mass Spectrometry

Due to the limited peak capacity of one-dimensional LC-MS, two-dimensional liquid chromatography mass spectrometry (2DLC-MS) has been developed to increase the peak capacity of the liquid chromatographic system. Figure 1.7 depicts a sample 2DLC-MS system, where different separation mechanisms, such as RPC and HILIC, was incorporated into the 2DLC-MS setup to provide high resolution as well as increased peak capacity.

The 2DLC is usually configured in either a heart-cutting mode or a comprehensive mode.⁵⁰ The heart-cutting analysis involves the collection of a few peaks of interest from the elution of the first dimension column, and then subjects only these peaks onto the second dimension column for further separation. This configuration can increase signal-to-noise ratio and improve sensitivity for targeted metabolomics without extensive pre-sample preparation.⁵¹ In the comprehensive configuration, all eluents from the first dimension column are collected into multiple fractions and each fraction is subjected to the second dimension column for further separation. Collection of the fractions of elution from the first column and transferring them to the second column are done by an interface called a modulator. This is the key element of a two dimensional liquid chromatography system. The modulator consists of a switching valve and two storage loops. The comprehensive

2DLC offers a higher resolution, so that more metabolite information can be obtained from the sample.⁵²

Most of the 2DLC systems are operated in an online mode, where the second dimension separation is carried out simultaneously with the first dimension separation.⁵³

Another form of 2DLC-MS gaining popularity now is called parallel 2DLC-MS, where orthogonal HILIC and RPC separation occur in one analytical run. In parallel 2DLC-MS, a sample is first delivered to two sample loops during

sample loading.⁵⁴ The two sample aliquots are then simultaneously injected onto a dual column setup, and parallel separations are performed on the two columns. The eluates from the two columns are then merged and subjected to a mass spectrometer for further analysis. This strategy is simple yet effective for coupling HILIC and RPC for the purpose of decreasing analysis time and increasing throughput.

1.3. Objectives of This Dissertation

The first objective of this dissertation was to develop methods to increase the identification accuracy of metabolites in GC×GC-MS. The second objective of this dissertation was to integrate the multiple analytical platforms, i.e., GC×GC-MS and parallel 2DLC-MS, for analysis of same biological samples. Overall, this dissertation was devoted to solve the following problems encountered in untargeted metabolomics:

Poor accuracy of metabolite identification in GC×GC-MS. The accuracy of metabolite identification in GC×GC-MS using the first dimension retention index and mass spectrum matching is about 80–85%, depending the identification algorithms. While the information of the second dimension retention time of each metabolite is readily available in GC×GC-MS data, this information is not yet widely used in metabolomics to aid metabolite identification. The lack of using the second dimension retention index contributes to the poor accuracy of metabolite identification in GC×GC-MS based metabolomics.

Large variation in calculating the second dimension retention index in GC×GC-MS. Isovolatility approach has been proposed to calculate the second dimension retention index in GC×GC-MS. This process introduces large variation in the calculated second dimension retention index and prevents the use of the second dimension retention index for accurate metabolite identification. Furthermore, construction of a retention map using *n*-alkanes as reference compounds is not suitable for every column class. For instance, *n*-alkanes are nonpolar compounds and they do not have good retention in mid-polar and polar columns. Therefore, the retention map constructed using their retention times on either of these two column classes is very small. Moreover, several other problems are also associated with the second dimension retention index calculation, including that the current isovolatility curve approach approximates the second dimension separation as under pseudo-isothermal

condition while the instrument actually operate under temperature-programmed mode; multiple data processing steps are involved in converting the second dimension retention time to the second dimension retention index; the retention index of a query compound is estimated using the isovolatility curves of two reference compounds; the public retention index database only contains limited retention index information, etc.

High rate of false positive biomarker discovery in GC×GC-MS. Current GC×GC-MS based metabolomics performs metabolite identification solely depending on comparing the similarity of the first dimension retention index and EI mass spectrum between an experimental data and the information of compound standards. With the increase of the number of metabolites in the public metabolite database, a high rate of false metabolite identification occurs owing to the lack of using the second dimension retention index.

Limited metabolites coverage. Metabolite coverage in a particular analytical platform is determined by the separation technique and the detector used. For example, metabolites have to be volatile and thermally stable in order to be analyzed by GC-MS. Chemical derivatization can be opted to increase the coverage by derivatizing the metabolites thus making them volatile and thermally stable. Still, a huge number of compounds cannot be derivatized and therefore cannot be detected by GC-MS. In contrast to GC-MS, LC-MS can analyze non-volatile metabolites. However, the ionization technique used in LC-MS such as electron spray ionization (ESI) or matrix assisted laser induced ionization (MALDI) may have very low ionization efficiency for some metabolites and result that these metabolites cannot be detected. Moreover, metabolites need to be soluble in the solvents and mobile phase in order to be analyzed by LC-MS.

In order to solve the above mentioned challenges encountered in untargeted metabolomics, several data analysis algorithms and bioanalytical methods have been developed in this dissertation. These developments have been organized in this dissertation as follows: Chapter Two introduces the development of a surface fitting method for calculating the second dimension retention index (2I). Chapter Three describes a further development in 2I calculation by removing the hold-up time calculation. Chapter Four presents a novel method of 2I calculation where mixtures of reference compounds are to allow the use of any compounds as reference compounds. Chapter Five demonstrates the analysis of metabolomic samples by multiple platforms for purposes of increased metabolite coverage, improved accuracy of metabolite quantification, and reliable metabolic pathway assignment.

CHAPTER 2

SURFACE FITTING FOR CALCULATING THE SECOND DIMENSION RETENTION INDEX IN COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY MASS SPECTROMETRY

2.1. Introduction

While mass spectrometry (MS) coupled with a one-dimensional separation technique, i.e., gas chromatography (GC) or liquid chromatography (LC), has been widely used in metabolomics, the limited separation power of one-dimensional GC or LC contributes to the poor performance of GC-MS and LC-MS in detecting low abundance compounds from complex samples, such as human body fluids and tissues. Comprehensive two-dimensional gas chromatography mass spectrometry (GC×GC-MS) uses two GC columns with different stationary phases to increase the separation power. The first dimension column, i.e., the primary column, is usually a 10 – 60 m long column while the second dimension column is a short column, about 1 – 2 m.⁵⁵⁻⁵⁹ The two columns are connected by a thermal modulator. Compounds eluted from the first dimension column within a modulation period

P_M are collected by the thermal modulator and subjected to the second dimension column for further separation at an increased temperature. Depending on the system configuration, the modulation period is usually set 2–10 s and the temperature gradient on both columns

is in a range of 5–10 °C/min.⁶⁰⁻⁶⁴ The two columns of the GC×GC-MS are operated in linear temperature gradient condition. However, the fast separation and slow temperature gradient on the second dimension column result in a small temperature change within a P_M , making a condition such that the second dimension column separation treated as pseudo-isothermal condition.⁶⁵⁻⁷¹ GC×GC-MS provides three pieces of information for each compound, the first dimension retention 1t_R , the second retention time 2t_R and electron ionization (EI) mass spectrum, if an EI source is used.^{38,39} To reduce the dependence of retention times on GC×GC-MS experimental conditions, 1t_R and 2t_R of a compound u are respectively converted into linear retention index I^T and Kovats retention index 2I .^{28,29}

During GC×GC-MS analysis, compounds eluted within the same P_M from the first dimension column are subjected to the second dimension column for further separation at a different temperature, as well as on a different stationary phase. While the separation of the compounds on the second dimension column within one P_M can be considered as in pseudo-isothermal condition, the overall temperature of the second dimension column, 2T_e , changes during GC×GC-MS analysis. The retention time 2t_R of a compound in the second dimension column decreases with the increase of column temperature, 2T_e . The relation $[^2t_R \sim ^2T_e]$ of a compound is captured by an isovolatility curve. Several methods have been proposed to construct the isovolatility curves (using reference compounds such as *n*-alkanes) including Beens *et al.*^{72,73}, Marriot *et al.*^{40,41,74,75}, and Zhao *et al.*⁷⁶ In these reported methods, the hold-up time, 2t_M , at different temperatures must be first estimated to get the adjusted retention time $^2t'_R$. The $^2t'_R$ values of the same reference compound at

different column temperatures were then used to construct the isovolatility curves of the reference compounds. The isovolatility curves of all reference compounds formed the second dimension retention index map, and the 2I value of a compound was finally calculated from the two adjacent isovolatility curves of two reference compounds.

While several methods have been developed to calculate the 2I of compounds, however 2I has not been extensively used for compound identification due to the inaccuracy of 2I calculation. It should be noted that a few papers used the second-dimension retention index to aid compound identification in analysis of GC×GC-MS data.^{39,77} However, from our experience, the variation window of the second-dimension retention index used in these papers are much smaller and the credibility of these papers is questionable. Inaccuracy of 2I calculation arise from the isovolatility curve construction step as different fitting method will provide different results. For instance, the isovolatility curve in Figure 1.5 can be constructed by polynomial fitting, logarithmic fitting, exponential fitting, etc. Different fitting will give different 2I values for a particular compound.

The objective of this study was to develop a method to calculate the 2I value of each compound without construction of an isovolatility curves. We developed a surface fitting method for the calculation of 2I values for all compounds detected by GC×GC-MS. The developed surface fitting method used *n*-alkanes as reference compounds, where the second dimension retention time, 2t_R , and the second dimension column temperature, 2T_e , formed a X-Y plane and the 2I was the Z-axis to form the 2I surface. The robustness of the surfacing fitting method was validated using 10-fold cross validation. The method

was then applied to calculate the retention indices of 152 compound standards in two commercially available mixtures, MegaMix A and MegaMix B.

2.2. Experimental

2.2.1. Chemicals

A mixture containing C₇ – C₃₀ *n*-alkanes was purchased from Sigma-Aldrich Corp., St. Louis, MO. MegaMix A (Cat. No. 8270) and MegaMix B (Cat. No. 8260) were purchased from Restek Corp., Bellefonte, PA. All of the compounds were present at a purity of between 95-99%. Two solutions were prepared from these three commercial mixtures. One solution contained only C₇ – C₃₀ *n*-alkanes, while the other contained C₇ – C₃₀ *n*-alkanes, MegaMix A and MegaMix B. These two solutions were prepared in dichloromethane at a concentration of 25 µg/mL per compound.

2.2.2. GC×GC-MS Experiment

The LECO (St. Joseph, MI) Pegasus® 4D GC×GC-MS instrument was equipped with a Gerstel MPS2 autosampler and an Agilent 6890 gas chromatograph featuring a LECO two-stage cryogenic modulator and a secondary oven. The first dimension column was a non-polar DB-5ms (phenyl arylene polymer virtually equivalent to a (5%-phenyl)-methylpolysiloxane) 60.0 m × 0.25 mm ¹*d*_c × 0.25 µm ¹*d*_f column, and the second dimension column was a medium polar BD-17ms ((50%-phenyl)-methylpolysiloxane) 1.0 m × 0.1 mm ²*d*_c × 0.1 µm ²*d*_f column. Both columns were obtained from Agilent Technologies (Santa Clara, CA), and were connected by means of a press-fit connector before the thermal modulator. The flow rate of ultra-high purity helium carrier gas

(99.999%) was set to 2.0 mL/min at a corrected constant flow via pressure ramps with an initial head pressure of 36.9 psi.

A 1 μ L sample was injected into the inlet chamber of the GC $\times$ GC-MS system and the split ratio was set at 20:1. The inlet temperature was 280 $^{\circ}$ C. The secondary oven and the thermal modulator were respectively set to +5 $^{\circ}$ C and +15 $^{\circ}$ C relative to the primary oven. The MS parameters were: MS range 45 – 800 m/z; data acquisition rate 200 spectra/s; temperature of ion source chamber 230 $^{\circ}$ C; temperature of MS transfer line 280 $^{\circ}$ C; detector voltage 1641 V; electron energy 70 eV. The acceleration voltage was turned on after a solvent delay of 220 s.

The mixture containing C₇–C₃₀ *n*-alkanes was analyzed on GC $\times$ GC-MS 46 times under isothermal conditions. Temperature of the first dimension column, 1T_e , was set at 60 $^{\circ}$ C in the first injection, and increased +5 $^{\circ}$ C in each of the following 45 injections with 1T_e = 285 $^{\circ}$ C in the last injection. The second dimension column was also set under isothermal conditions but at +5 $^{\circ}$ C with respect to the first dimension column. P_M was set to 10 s.

MegaMix A, MegaMix B and C₇–C₃₀ *n*-alkanes were mixed as one solution, and then analyzed four times in temperature programmed conditions with the first dimension column temperature initially set as 60, 80, 100, and 120 $^{\circ}$ C, respectively. The column temperature was held at the initial temperature for 1 min and then ramped at 5 $^{\circ}$ C/min to 285 $^{\circ}$ C, and kept at 285 $^{\circ}$ C for 13 min. The secondary column temperature program was identical to that of the first dimension column but temperature was set +5 $^{\circ}$ C higher than the first dimension column. P_M was set as 4 s.

2.2.3. Spectrum Deconvolution and Compound Identification

The LECO ChromaTOF software (version 4.51), equipped with the National Institute of Standards and Technology MS database (NIST 2014), was used for instrument control, spectrum deconvolution, and compound identification. Manufacturer recommended parameters for ChromaTOF were used to reduce the raw instrument data into a peak list of compounds. These parameters were: baseline offset 0.5; auto smoothing; peak width in first dimension 6 s; peak width in the second dimension 0.1 s; signal-to-noise ratio 100; match required to combine peaks 600; R.T. shift 0.08 s; minimum forward similarity match 600. The true peak spectrum was exported as part of the information for each chromatographic peak as absolute intensity values.

2.2.4. Hold-up Time Calculation

To calculate the hold-up time on the second dimension column 2t_M , *n*-alkane mixture was analyzed on GC×GC-MS at 46 different column temperatures in isothermal conditions. Hold-up time 2t_M was then calculated as follows⁷⁶

$${}^2t_M = \frac{t_{R1}t_{R3} - t_{R2}^2}{(t_{R3} - t_{R2}) - (t_{R2} - t_{R1})} \quad (3)$$

where t_{R1} , t_{R2} and t_{R3} are the continuous retention times of three homologs such as C_{n-1} , C_n and C_{n+1} alkanes.

Multiple *n*-alkanes were analyzed on the second dimension column at the same column temperature. Therefore, multiple hold-up times were obtained for each temperature using equation (3). The mean values of hold-up time at different temperatures were then fitted by a function (Figure 2.1) and the form of the function is as follows:

$${}^2t_M = -0.06000\ln({}^2T_e) + 1.194 \quad (4)$$

Figure 2.1. The second dimension hold-up time calculation. The means of hold-up times at each column temperature were fitted using a logarithm function.

where 2t_M is the fitted hold-up time in the second dimension column and 2T_e is the second dimension column temperature in °C. The fitting accuracy R^2 was 0.9843.

2.2.5. Construction of the Second Dimension Retention Index Surface

$C_7 - C_{30}$ *n*-alkanes were analyzed 46 times by GC×GC-MS in isothermal conditions.

2t_R and 2T_e were obtained for each reference compound in each analysis. The 2I value

of a reference compound with n carbon atoms was defined as $100 \times n$. Therefore, each n -alkane compound was represented by $\{ {}^2t'_R, {}^2T_e, {}^2I \}$. A second dimension retention index surface 2I_S was then constructed using the data of all n -alkane compounds, where ${}^2t'_R$ and 2T_e formed the X - Y plane and the 2I was the Z -axis to form the 2I_S . A polynomial surface model in MATLAB (Mathworks, Natick, MA) named 'polyij' was used to construct 2I_S , where i was the degree of polynomial in ${}^2t'_R$, and j was the degree of polynomial in 2T_e . The maximum for both i and j was five in the MATLAB platform. The degree of polynomial was the maximum of i and j .

2.2.6. Evaluation Criteria of Surface Fitting Method

Three measures were used to evaluate the quality of surface fitting, including R -square (R^2), degrees of freedom adjusted R -square (R^2_{adj}), and root mean squared error (RMSE). R^2 measures how successful the fit is in explaining the variation of the data, and is defined as the ratio of the sum of squares of the regression (SSR) and the total sum of squares (SST).

$$SSR = \sum_{i=1}^n w_i (\hat{y} - \bar{y})^2$$

$$SST = \sum_{i=1}^n w_i (y_i - \bar{y})^2$$

$$R^2 = \frac{SSR}{SST}$$

where y_i is the original retention index value 2I_i , $\hat{y}$ is the predicted 2I value, $\bar{y}$ is the mean of the original retention index values, and the w_i is the weight on the value 2I_i . R^2 can take on any value between 0 and 1, with a value closer to 1 indicating that a greater proportion of variance is accounted for by the model.

R_{adj}^2 adjusts R^2 based on the residual degrees of freedom v that is defined as the number of response values, n , minus the number of fitted coefficients, m , estimated from the response values. The R_{adj}^2 can take on any value less than or equal to 1, with a value closer to 1 indicating a better fit.

$$R_{adj}^2 = 1 - \frac{SSE(n-1)}{SST(v)}$$

where SSE is defined as

$$SSE = \sum_{i=1}^n w_i (y_i - \hat{y})^2$$

$RMSE$ is an estimate of the standard deviation of the random component in the data, and is defined as

$$RMSE = \sqrt{MSE} = \sqrt{\frac{SSE}{v}}$$

where MSE is the mean square error or the residual mean square. An $RMSE$ value closer to 0 indicates a fit that is more useful for prediction.

2.3. Results and Discussion

C_7 – C_{30} n -alkanes were chosen as the reference compounds to create a retention index surface, 2I_S , in this study. All n -alkanes were identified from the GC×GC-MS data. A total of 102 compounds were identified from the mixture of MegaMix A and MegaMix B.

2.3.1. GC × GC-MS Experimental Variability

The mixture of *n*-alkanes was analyzed in temperature-programmed mode 25 times in five continuous days. For the experiments performed within one day, the largest standard deviation of the second dimension retention time of *n*-alkanes is 0.007 s. Figure 2.2 displays the cumulative probability curves of the day-to-day experiments, *i.e.* 25 replicates analyzed in five days.

Figure 2.2. Cumulative probability curves of standard deviation of the second dimension retention times of *n*-alkanes.

The standard deviation of the second dimension retention time of the *n*-alkanes fluctuated from 0.001 s to 0.007 s. The standard deviation of 90% of the second dimension retention time values in all 25 experiments were less than 0.005 s. Such a small standard deviation of the second dimension retention time indicates that the instrument has a very

good reproducibility in measuring the retention times of compounds separated on the second dimension column in temperature-programmed mode.

While the *n*-alkane mixture was analyzed in temperature-programmed mode, each chromatographic run was ramped to 285 °C in the first dimension column and held at 285 °C for 13 min. By design, the second dimension column was in fact operated in isothermal mode at 290 °C during the last 13 min in each of the 25 experiments. Five *n*-alkanes, i.e. C₂₆–C₃₀ *n*-alkanes, were eluted in isothermal mode in the second dimension column at 290 °C. The standard deviation of the second dimension retention times of these five *n*-alkanes were 0.004, 0.004, 0.004, 0.004 and 0.005 s, respectively. Such small standard deviations indicate that the reproducibility of the GC × GC-MS system is also quite good in isothermal mode.

2.3.2. Construction of the Second Dimension Retention Index Surface

A total of 415 data points of C₇–C₃₀ *n*-alkanes were used to construct the second dimension retention surface, 2I_S . To obtain the optimal surface fitting, all combinations of *i* and *j* values in the polynomial surface model ‘poly*ij*’ were tested. The performance of each polynomial surface model was then evaluated Table 2.1.

Table 2.1. Selection of polynomial surface model ‘poly*ij*’ to construct the second dimension retention index surface using GC×GC-MS data of *n*-alkanes (weight ‘y’ and robust ‘LAR’)

Poly <i>ij</i>		R ²	R_{adj}^2	RMSE
I	j			
1	1	0.955	0.9547	148.7
2	1	0.9984	0.9984	27.8

3	1	0.9972	0.9972	37
4	1	0.9961	0.996	44.2
5	1	0.995	0.9949	49.8
1	2	0.999	0.999	22.4
2	2	0.9995	0.9995	16
3	2	0.9991	0.999	21.7
4	2	0.9986	0.9985	26.9
5	2	0.9982	0.9981	30.4
1	3	0.9987	0.9987	25.3
2	3	0.9997	0.9997	11.3
3	3	0.9997	0.9997	12.2
4	3	0.9995	0.9995	15.6
5	3	0.9994	0.9993	18.1
1	4	0.9984	0.9983	28.5
2	4	0.9999	0.9999	7.8
3	4	0.9995	0.9995	15.7
4	4	0.9995	0.9995	15.7
5	4	0.9998	0.9998	10.4
1	5	0.9978	0.9977	33.4
2	5	0.9999	0.9999	6.1
3	5	0.9999	0.9999	6.3
4	5	0.9999	0.9999	6.7
5	5	0.9999	0.9999	6.8

The optimal polynomial model in this study was ‘poly25’, i.e. $i = 2$ and $j = 5$. The optimal model was selected based on the three evaluation criteria. R^2 , R_{adj}^2 , and $RMSE$ of the optimal surface fitting model ‘poly25’ were 0.9999, 0.9999 and 6.1 iu, respectively. Ten-fold cross validation was performed to study how accurately the polynomial surface model ‘poly25’ would perform in fitting the n -alkane data points. The original 415 data points were randomly partitioned into 10 subsamples, where 9 subsamples had equal sample size (42 data points each) and the 10th subsample had 37 data points. Of the 10 subsamples, a single subsample was retained as the validation data for testing the model, and the

remaining 9 subsamples were used as training data. Figure 2.3 is an example showing that the validation data set was randomly distributed.

Figure 2.3. Distribution of one validation data set used in 10-fold cross validation. The filled circles are the training data set and the open circles are the validation data set.

The cross validation process was repeated 10 times, with each of the 10 subsamples used exactly once as the validation data. The 10 validation results were then averaged to produce a single estimation. Table 2.2 lists the results of the 10-fold cross validation. The R^2 , R_{adj}^2 , and $RMSE$ of the training data set were 0.9999, 0.9999 and 7.1 iu, respectively.

The average *RMSE* for the validation set of data was 16.0 iu. The 10-fold cross validation process was repeated three more time, and the Table 2.3 shows the results of the second repeated trial. Third and fourth repeated trial's average *RMSE* for the validation set of data was 15.8 iu and 15.7 iu respectively (data not shown). The similar results of the four trials demonstrate that the polynomial surface model 'poly25' was very robust in fitting the *n*-alkane data points.

Table 2.2. Results of a ten-fold cross validation of surface fitting using the second dimension retention indices of *n*-alkanes

Fold count	Goodness of fit		Goodness of validation	
	R^2	R^2_{adj}	<i>RMSE</i>	<i>RMSE</i>
1	0.9999	0.9999	6.3	17.2
2	0.9999	0.9999	6.2	15.7
3	0.9999	0.9999	6.3	17.3
4	0.9999	0.9999	6.3	17.1
5	0.9999	0.9999	10.1	16.0
6	0.9999	0.9999	6.3	16.4
7	0.9999	0.9999	10.6	14.5
8	0.9999	0.9999	6.4	17.3
9	0.9999	0.9999	6.1	17.0
10	0.9999	0.9999	6.4	11.9
Average	0.9999	0.9999	7.1	16.0

Table 2.3. Results of a ten-fold cross validation of surface fitting using the second dimension retention indices of *n*-alkanes (repeated trial)

Fold Count	Goodness of fit		Goodness of validation	
	R^2	R^2_{adj}	$RMSE$	$RMSE$
1	0.9999	0.9999	6.4	10.2
2	0.9999	0.9999	6.4	13.2
3	0.9999	0.9999	6.4	13.7
4	0.9999	0.9999	6.3	16.5
5	0.9999	0.9999	6.2	21.2
6	0.9999	0.9999	6.2	15.7
7	0.9999	0.9999	6	21.4
8	0.9999	0.9999	6.4	11.6
9	0.9999	0.9999	6.2	19.5
10	0.9999	0.9999	6.1	20.5
Average	0.9999	0.9999	6.2	16.3

Figure 2.4 (A) shows the second dimension retention index surface, 2I_S , constructed from the isothermal data of the *n*-alkanes using polynomial surface fitting model ‘poly25’. Figure 2.4(B) is the residual plot of the surface fitting. A residual from the fitted surface model was defined as the difference between the original retention index 2I of an *n*-alkane and its fitted value. The residuals had a near normal distribution (Figure 2.4(C)), suggesting that no significant bias was introduced by the polynomial surface model ‘poly25’. However, Figure 2.4. B indicates large residuals showed at the lower end of the *Y*-axis, which were mainly caused by the inaccuracy of chromatographic peak picking and the small values of 2t_R of *n*-alkanes. Small variation in determining an *n*-alkane’s 2t_R during peak picking resulted in large variation in positioning the data point of that *n*-alkane in the *X-Y* plane.

A.

B.

C.

Figure 2.4. The second dimension retention index surface constructed using the $\text{GC} \times \text{GC}$ –MS data of *n*-alkanes. The retention index surface is a function of the elution temperature of each *n*-alkane in the second dimension column 2T_e and the corresponding adjusted retention time ${}^2t'_R$. (A) is the second dimension retention index surface. (B) is the residual plot, and (C) is the distribution of the residuals.

2.3.3. Calculation of Retention Index of Compounds in MegaMix A & MegaMix B

MegaMix A, MegaMix B, and $\text{C}_7 - \text{C}_{30}$ *n*-alkanes were mixed and analyzed by $\text{GC} \times \text{GC}$ -MS in temperature programmed conditions with the first dimension column started at 4 different initial temperatures, 60, 80, 100 and 120 °C, respectively. After data processing, 68 compounds were identified from MegaMix A and 41 compounds were identified from MegaMix B. Seven compounds were identified from both MegaMix A and

MegaMix B. Pyridine is one of the 76 compounds in the Megamix A. While pyridine was identified from the experiment data, it was excluded from the retention index calculation because it is one of the carry-over compounds frequently detected in our GC×GC-MS system. Tables 2.4 and 2.5 list the 2I values of compounds identified from MegaMix A and MegaMix B, respectively. The standard deviation of 2I calculated from the four analyses ranged from 1 to 10 iu. The mean standard deviation of 2I was 1.6 iu for compounds in MegaMix A and 3.4 iu for compounds in MegaMix B. The average RSD for MegaMix A and MegaMix B was 0.10% and 0.31%, respectively.

Table 2.4. The second dimension retention indices of compounds identified from MegaMix A

Compound name	Second dimension retention index (iu) ^a				Mean (iu)	STD (iu)
	65 °C	85 °C	105 °C	125 °C		
N-Nitrosodimethylamine	983	977	977		979	3
Aniline	1250	1252	1250	1249	1250	1
Phenol	1205	1202	1198	1199	1201	3
Bis(2-chloroethyl) ether	1218	1215	1211	1208	1213	4
2-Chlorophenol	1217	1213	1216	1219	1216	2
Benzyl alcohol	1297	1299	1300		1298	1
2,2'-Oxybis(2-chloropropane)	1247	1245	1242	1241	1244	3
2-Methylphenol	1290	1290	1288	1286	1288	2
N-nitrosodipropylamine	1304	1302	1305	1295	1301	4
3-Methylphenol	1312	1313	1315	1309	1312	2
Hexachloroethane	1275	1275	1272	1277	1275	2

Isophorone	1373	1374	1373	1373	1373	1
2-Nitrophenol	1417	1420	1423	1426	1421	3
2,4-Dimethylphenol	1396	1396	1396	1394	1396	1
Bis(2-chloroethoxy)-methane	1422	1423	1422	1418	1421	2
2,4-DichloroPhenol	1435	1434	1435	1436	1435	1
4-Chloroaniline	1537	1538	1542	1548	1541	5
4-Chloro-3-methylphenol	1587	1586	1586	1590	1588	2
2-Methylnaphthalene	1608	1607	1610	1614	1610	3
1-Methylnaphthalene	1640	1642	1644	1649	1644	3
1,3-Cyclopentadiene, 1,2,3,4,5,5-hexachloro-	1573	1572	1571	1572	1572	1
2,4,6-Trichlorophenol	1665	1664	1666	1667	1666	1
2,4,5-Trichlorophenol	1673	1673	1672	1678	1674	2
2-Chloronaphthalene	1725	1726	1728	1732	1728	3
2-Nitroaniline	1834	1835	1837	1842	1837	3
1,4-Dinitrobenzene	1875	1875	1876	1879	1876	2
1,3-Dinitrobenzene	1886	1886	1887	1890	1887	2
Dimethyl phthalate	1842	1842	1842	1846	1843	2
2,6-Dinitrotoluene	1883	1882	1883	1887	1884	2
1,2-Dinitrobenzene	1931	1929	1931	1933	1931	1
Acenaphthylene	1851	1851	1852	1857	1853	2
3-Nitroaniline	1951	1950	1951	1957	1952	3
Acenaphthene	1883	1883	1884	1887	1884	2
2,4-dinitro-phenol	1944	1943	1941	1944	1943	1
Dibenzofuran	1914	1914	1914	1919	1915	2
2,4-Dinitrotoluene	1968	1966	1966	1967	1967	1
2,3,5,6-Tetrachlorophenol	1922	1922	1921	19223	1922	1
2,3,4,6-Tetrachlorophenol	1936	1935	1936	1937	1936	1

Diethyl Phthalate	1984	1982	1981	1982	1982	1
Fluorene	2007	2007	2007	2009	2008	1
4-Chlorophenyl phenyl ether	1979	1979	1978	1980	1979	1
4-Nitroaniline	2141	2141	2140	21411	2141	1
4,6-Dinitro-2-methylphenol	2050	2050	2049	2050	2050	1
Diphenylamine	2062	2061	2063	2063	2062	1
Azobenzene	2034	2034	2034	2035	2034	0
4-Bromophenyl phenyl ether	2122	2120	2121	2122	2121	1
Hexachlorobenzene	2112	2109	2112	2112	2111	1
Pentachloro-phenol	2202	2199	2201	2200	2201	1
Anthracene	2298	2298	2298	2299	2298	0
Phenanthrene	2308	2305	2308	2309	2307	1
Carbazole	2412	2412	2410	2412	2411	1
di-n-Butyl phthalate	2370	2370	2369	2368	2369	1
Fluoranthene	2676	2677	2676	2676	2676	1
Pyrene	2772	2772	2771	2773	2772	1
Benzyl butyl phthalate	2930	2931	2929	2927	2929	1
Bis(2-ethylhexyl) adipate	2642	2644	2640	2644	2643	2
Benz[a]anthracene	3198	3199	3200	3197	3199	1
Chrysene	3231	3231	3231	3230	3231	1
Bis(2-ethylhexyl) phthalate	2929	2927	2931	2931	2929	1
di-n-Octyl phthalate	3076	3081	3074	3079	3077	3

^a The blank means the compound was not detected during the experiment.

Table 2.5. The second dimension retention indices of compounds identified from MegaMix B

Compound name	Second dimension retention index (iu) ^a				Mean (iu)	STD (iu)
	65 °C	85 °C	105 °C	125 °C		
cis-1,3-Dichloro-1-Propene, (Z)	888	891			889	2
trans-1,3-Dichloro-Propene (E)	927	924			925	1
Toluene	899				899	0
1,1,2-Trichloroethane	937	941			939	2
Ethyl methacrylate	906	915			910	5
1,3-Dichloropropane	953	949	965		956	7
Dibromochloro-methane	987	984	994		988	4
Tetrachloroethene	926	931	950		936	10
1,2-Dibromoethane	1000	994	1006		1000	5
Chlorobenzene	1015	1012	1021		1016	4
1,1,1,2-Tetrachloroethane	10011	1002			1019	5
Ethylbenzene	1007	1005			1006	1
p-Xylene	1014	1015	1019		1016	2
Styrene	1072	1070	1075	1075	1073	2
o-Xylene	1054	1052	1058	1046	1052	4
Bromoform	1128	1125	1125	1140	1130	6
cis-1,4-Dichloro-2-butene, (z)-	1114	1111	1106	1117	1112	4
1,1,2,2-Tetrachloroethane	1118	1114	1110	1119	1115	4
1,2,3-Trichloropropane	1139	1136	1131	1135	1135	3
Isopropylbenzene(cumene)	1072	1070	1069	1088	1075	8
trans-1,4-Dichloro-2-butene, (E)-	1152	1149	1139	1147	1147	5
Bromobenzene	1140	1138	1135	1145	1139	4

2-Chlorotoluene	1141	1140			1141	0
n- Propylbenzene	1109	1107	1105		1107	2
4-Chlorotoluene	1142	1141	1140	1149	1143	3
1,3,5-Trimethylbenzene	1122	1120	1120	1131	1123	4
Pentachloroethane	1171	1168	1165	1181	1171	6
tert-Butylbenzene	1148	1146	1146	1151	1148	2
1,2,4-Trimethylbenzene	1163	1160	1156	1167	1162	4
sec-Butylbenzene	1167	1167	1164	1168	1166	1
p-Cymene(4-isopropyl toluene)	1182	1178	1177	1186	1181	4
n-Butylbenzene	1220	1219	1214	1217	1218	3
1,2-Dibromo-3-Chloropropane	1363	1366	1365	1368	1366	2
Benzene, 1,2,3-trichloro-	1493	1494	1497	1499	1496	2
1,3-Dichlorobenzene*	1216	1215	1214		1215	1
1,4-Dichlorobenzene*	1231	1230	1227	1213	1225	7
1,2-Dichlorobenzene*	1266	1267	1268	1269	1267	1
Nitrobenzene*	1380	1385	1388	1390	1386	4
Benzene, 1,2,4-trichloro-*	1434	1435	1436	1436	1435	1
Naphthalene*	1485	1487	1489	1495	1489	4
1,3-Butadiene, 1,1,2,3,4,4-hexachloro-*	1416	1417	1415	1415	1416	1

^a The blank means the compound was not detected during the experiment.

Compared with the compounds in MegaMix A, the relatively increased mean standard deviation of 2I of the compounds in MegaMix B may be related with the retention times of the compounds. The compounds in MegaMix A eluted in the middle to high temperature

range whereas the compounds in MegaMix B eluted in the low to mid temperature range. To an individual compound, the large standard deviation of 2I was mainly caused by the inaccuracy of peak picking, during which the second dimension retention time 2t_R of the compound was determined. For instance, the standard deviation in 2I of compound 1, 4-dichlorobenzene was 7 iu. While 1, 4-dichlorobenzene was well separated from other compounds during the analysis with the initial temperature of the first dimension column set at 60 °C, its chromatographic peak overlapped with the chromatographic peaks of other compounds during the analysis when the initial temperature of the first dimension column was at 125 °C (Figure 2.5).

Figure 2.5. GC $\times$ GC-MS separation of 1, 4-dichlorobenzene (black arrow) and other compounds in MegaMix A analyzed in temperature-programmed mode at different column temperatures. With the increase of the second dimension column temperature from 65 °C to 125 °C, the separation between 1, 4-dichlorobenzene and other compounds became worse and resulted in chromatographic peak overlapping. The overlapping chromatographic peaks introduce a high degree of variation in the retention time and

therefore the calculated retention index. (A) are the three-dimensional images of the two dimensional chromatographic peaks, and (B) are the corresponding contour images.

Inaccuracy of deconvoluting overlapping chromatographic peaks increased the chance of introducing large variation in determining a compound's second dimension retention time 2t_R and therefore its 2I value. Tables 2.6 and 2.7 list the second dimension column temperature 2T_e and corresponding adjusted second dimension retention time ${}^2t'_R$ of each identified compound in MegaMix A and MegaMix B, respectively.

The average range of elution temperature of compounds in MegaMix A is 8 °C, and 25 °C for compounds in MegaMix B. Compared with compounds in MegaMix A, compounds in MegaMix B had larger 2I variations (Table 2.5) and their elution temperature ranges were also quite broader (Table 2.7) than those of the compounds in MegaMix A (Tables 2.4 and 2.6).

Previous study demonstrated that retention index had temperature dependency, i.e. retention index of an analyte in general increases with the increase of elution temperature⁷⁸. We observed that some of the identified compounds followed this trend in this study, while other compounds did not. The reason is that temperature dependency of retention index is also related to the structure of an analyte.⁷⁸⁻⁸¹

Table 2.6. Second dimension elution temperature (2T_e) of MegaMix A compounds at different initial temperature with ramp 5 °C/min and the corresponding adjusted second dimension retention time.

Compounds name	2T_e (°C) at different initial temperature with ramp 5 °C/min				Adjusted 2t_R (s) at different initial temperature with ramp 5 °C/min			
	65 °C	85 °C	105 °C	125 °C	65 °C	85 °C	105 °C	125 °C
N-Nitrosodimethylamine	90.5	106.1	123.8		0.711	0.446	0.260	
Aniline	116.9	123.2	133.8	148.1	1.341	1.095	0.795	0.541
Phenol	116.8	122.5	133.1	147.4	1.056	0.879	0.644	0.440
Bis(2-chloroethyl) ether	117.8	123.8	134.1	148.1	1.097	0.900	0.665	0.451
2-Chlorophenol	119.2	124.8	135.1	149.1	1.043	0.865	0.660	0.461
Benzyl alcohol	125.2	129.5	138.1		1.286	1.123	0.871	
2,2'-Oxybis(2-chloropropane)	127.5	131.5	139.5	151.4	0.937	0.829	0.657	0.477
2-Methylphenol	127.5	131.2	139.1	151.1	1.147	1.023	0.807	0.582
N-nitrosodipropylamine	130.2	133.5	140.8	152.5	1.128	1.009	0.828	0.582
3-Methylphenol	130.8	133.8	140.8	152.1	1.148	1.050	0.863	0.622
Hexachloroethane	131.8	135.8	142.8	154.1	0.939	0.835	0.683	0.518
Isophorone	138.2	140.8	146.8	156.5	1.207	1.113	0.925	0.714
2-Nitrophenol	139.9	142.5	148.2	157.5	1.402	1.298	1.096	0.849
2,4-Dimethylphenol	141.5	143.5	148.2	157.1	1.203	1.129	0.981	0.759
Bis(2-chloroethoxy)-Methane	143.2	145.2	149.8	158.1	1.279	1.204	1.036	0.810
2,4-DichloroPhenol	147.5	149.2	153.5	160.1	1.175	1.111	0.983	0.820
4-Chloroaniline	150.5	151.9	155.5	162.5	1.687	1.612	1.439	1.176
4-Chloro-3-methylphenol	162.5	162.9	164.8	169.5	1.376	1.356	1.272	1.119
2-Methylnaphthalene	164.2	164.9	167.2	172.2	1.422	1.382	1.298	1.130
1-Methylnaphthalene	166.5	167.2	169.2	173.8	1.498	1.473	1.389	1.220

1,3-Cyclopentadiene,								
1,2,3,4,5,5-hexachloro-	167.8	168.5	170.5	174.8	1.103	1.078	1.014	0.906
2,4,6-Trichlorophenol	171.5	171.9	173.2	176.8	1.399	1.380	1.330	1.191
2,4,5-Trichlorophenol	172.9	173.2	174.5	177.8	1.380	1.365	1.306	1.202
2-Chloronaphthalene	175.2	175.9	177.2	180.5	1.566	1.536	1.481	1.348
2-Nitroaniline	178.3	178.6	179.9	182.6	2.182	2.162	2.072	1.918
1,4-Dinitrobenzene	181.6	181.9	182.9	185.2	2.263	2.238	2.163	2.014
1,3-Dinitrobenzene	183.6	183.6	184.6	186.9	2.199	2.194	2.129	1.975
Dimethyl phthalate	182.9	183.2	184.2	186.2	1.893	1.873	1.809	1.709
2,6-Dinitrotoluene	184.3	184.2	185.2	187.2	2.119	2.114	2.044	1.930
1,2-Dinitrobenzene	185.3	185.6	186.3	188.3	2.444	2.399	2.359	2.215
Acenaphthylene	184.9	185.2	186.2	188.5	1.824	1.804	1.749	1.640
3-Nitroaniline	188.6	188.6	189.3	190.9	2.335	2.330	2.280	2.196
Acenaphthene	189.2	189.2	190.2	192.2	1.770	1.765	1.716	1.616
2,4-dinitro-phenol	191.9	191.9	192.2	193.6	2.016	2.011	1.976	1.902
Dibenzofuran	193.2	193.5	194.2	195.9	1.727	1.707	1.667	1.602
2,4-Dinitrotoluene	193.6	193.9	194.2	195.6	2.077	2.042	2.017	1.927
2,3,5,6-Tetrachlorophenol	195.9	195.9	196.2	197.9	1.617	1.622	1.598	1.523
2,3,4,6-Tetrachlorophenol	196.9	196.9	197.5	198.5	1.648	1.638	1.608	1.563
Diethyl Phthalate	199.9	200.2	200.5	201.2	1.759	1.729	1.704	1.669
Fluorene	201.6	201.6	201.9	203.2	1.799	1.804	1.779	1.715
4-Chlorophenyl phenyl								
ether	201.9	202.2	202.5	203.5	1.614	1.599	1.574	1.535
4-Nitroaniline	203.6	203.6	204.0	204.3	2.645	2.650	2.605	2.590
4,6-Dinitro-2-								
methylphenol	203.6	203.6	203.9	204.6	1.950	1.950	1.920	1.885
Diphenylamine	204.9	204.9	205.2	206.2	1.940	1.935	1.925	1.861
Azobenzene	205.6	205.9	205.9	206.9	1.725	1.705	1.705	1.656
4-Bromophenyl phenyl								
ether	213.6	213.6	213.9	214.5	1.768	1.753	1.738	1.708
Hexachlorobenzene	214.2	214.2	214.5	215.2	1.673	1.658	1.653	1.618

Pentachloro-phenol	220.2	220.2	220.2	220.6	1.824	1.809	1.819	1.795
Anthracene	224.3	224.3	224.3	224.6	2.151	2.151	2.151	2.131
Phenanthrene	225.3	225.2	225.6	225.9	2.141	2.126	2.121	2.101
Carbazole	230.3	229.9	229.9	230.3	2.467	2.492	2.477	2.467
di-n-Butyl phthalate	239.5	239.5	239.5	239.5	1.620	1.620	1.615	1.610
Fluoranthene	252.3	252.3	252.3	252.6	2.478	2.483	2.478	2.448
Pyrene	257.6	257.6	257.6	257.6	2.659	2.659	2.654	2.669
Benzyl butyl phthalate	274.9	274.9	274.9	274.9	2.278	2.283	2.273	2.263
Bis(2-ethylhexyl) adipate	277.8	277.8	277.8	277.8	1.128	1.133	1.123	1.133
Benz[a]anthracene	286.0	286.0	286.0	286.0	2.955	2.960	2.965	2.950
Chrysene	287.0	287.0	287.0	287.0	3.070	3.070	3.070	3.060
Bis(2-ethylhexyl) phthalate	289.2	289.2	289.2	289.2	1.516	1.511	1.521	1.521
di-n-Octyl phthalate	290.0	290.0	290.0	290.0	2.011	2.031	2.001	2.026

Table 2.7. Second dimension elution temperature (2T_e) of MegaMix B compounds at different initial temperature with ramp 5 °C/min and the corresponding adjusted second dimension retention time.

Compound name	2T_e (°C) at different initial temperature with ramp 5 °C/min				Adjusted 2t_R (s) at different initial temperature with ramp 5 °C/min			
	65 °C	85 °C	105 °C	125 °C	65 °C	85 °C	105 °C	125 °C
cis-1,3-Dichloro-1-Propene, (Z)	90.8	106.4			0.401	0.251		
trans-1,3-Dichloro-Propene	92.8	107.1			0.483	0.311		
Toluene	93.1				0.403			
1,1,2-Trichloroethane	93.5	107.8			0.503	0.342		
Ethyl methacrylate	93.8	107.8			0.413	0.287		

1,3-Dichloropropane	94.5	108.1	124.8		0.539	0.357	0.230	
Dibromochloro-Methane	96.1	109.4	125.4		0.620	0.423	0.276	
Tetrachloroethene	96.4	109.4	125.8		0.435	0.303	0.196	
1,2-Dibromoethane	97.1	110.1	125.8		0.650	0.438	0.296	
Chlorobenzene	100.8	112.1	127.1		0.638	0.459	0.311	
1,1,1,2-Tetrachloroethane	101.1	112.4			0.618	0.429		
Ethylbenzene	102.1	113.1			0.588	0.430		
p-Xylene	103.1	113.8	128.1		0.594	0.445	0.297	
Styrene	105.8	115.5	129.1	145.4	0.745	0.566	0.397	0.265
o-Xylene	105.8	115.5	129.1	144.8	0.680	0.516	0.362	0.199
Bromoform	106.2	115.8	129.5	145.4	0.991	0.736	0.508	0.350
cis-1,4-Dichloro-2-butene, (z)-	107.2	116.1	129.4	145.4	0.891	0.681	0.463	0.310
1,1,2,2-Tetrachloroethane	108.8	117.5	130.1	145.8	0.867	0.667	0.463	0.310
1,2,3-Trichloropropane	109.8	118.1	130.8	146.1	0.938	0.727	0.503	0.335
Isopropylbenzene(cumene)	109.8	118.1	130.8	146.1	0.668	0.527	0.368	0.255
trans-1,4-Dichloro-2-butene, (E)-	109.8	118.1	130.5	146.1	1.003	0.772	0.528	0.355
Bromobenzene	111.2	119.5	131.5	146.8	0.903	0.708	0.504	0.345
2-Chlorotoluene	113.8	121.1			0.840	0.684		
n- Propylbenzene	113.8	121.1	132.4		0.720	0.584	0.424	
4-Chlorotoluene	114.8	121.8	133.1	147.4	0.820	0.674	0.494	0.345
1,3,5-Trimethylbenzene	115.8	122.5	133.4	147.8	0.726	0.599	0.444	0.311
Pentachloroethane	117.5	124.1	134.5	148.8	0.877	0.715	0.535	0.391
tert-Butylbenzene	119.1	125.1	135.1	148.8	0.748	0.631	0.480	0.336
1,2,4-Trimethylbenzene	119.5	125.5	135.5	149.1	0.798	0.666	0.500	0.361
p-Cymene(4-isopropyl toluene)	123.8	128.8	137.5	150.4	0.770	0.662	0.521	0.382
n-Butylbenzene	121.8	127.1	136.5	149.8	0.759	0.657	0.506	0.356
n-Butylbenzene	128.5	132.5	140.1	152.1	0.807	0.719	0.572	0.422
1,2-Dibromo-3-Chloropropane	133.5	136.8	143.8	154.5	1.344	1.221	0.979	0.738
Benzene, 1,2,3-trichloro-	146.8	148.8	153.2	161.1	1.195	1.131	0.998	0.801
1,3-Dichlorobenzene*	121.5	127.1	136.5		0.969	0.817	0.631	
1,4-Dichlorobenzene*	122.8	128.1	137.1	149.8	0.999	0.852	0.656	0.441

1,2-Dichlorobenzene*	125.5	130.2	138.8	151.5	1.091	0.948	0.747	0.537
Nitrobenzene*	133.2	136.9	143.5	154.5	1.484	1.331	1.089	0.803
Benzene, 1,2,4-trichloro-*	151.5	153.2	156.8	164.2	1.332	1.268	1.139	0.932
Naphthalene*	148.5	150.2	154.5	162.2	1.421	1.357	1.188	0.971
1,3-Butadiene, 1,1,2,3,4,4-hexachloro-*	151.5	152.8	156.5	163.5	0.967	0.933	0.839	0.697

2.3.4. Robustness of Surface Fitting to Variations of Instrumental Settings

To investigate the robustness of the developed surface fitting method in analyzing GC×GC-MS data, we analyzed the mixture containing MegaMix A and MegaMix B in different instrumental conditions, by varying the temperature offset of the secondary oven, the ramp rate of temperature gradient, and modulation period. Tables 2.8 and 2.9 list the second dimension retention indices of compounds identified from MegaMix A and MegaMix B in different instrumental conditions, respectively.

An average relative standard deviation (*RSD*) of the calculated second dimension retention index 2I for compounds in MegaMix A was 0.63% and 0.42 % for compounds in MegaMix B. The small *RSD* of calculated 2I demonstrates that the developed surface fitting method is robust in calculating the second dimension retention index of compounds analyzed in different instrumental conditions.

As we analyzed the mixture of MegaMix A and MegaMix B in different temperature-programmed modes, the same compound eluted at different column temperatures. In general, the elution temperature of compounds in either MegaMix A or MegaMix B was increased with the increase of temperature ramp rate (data not shown). The low elution temperature was corresponding to ramp rate 4 °C/min and the high elution temperature was corresponding to ramp rate 6 °C /min. Therefore, the temperature range was calculated by subtracting the elution temperature of ramp rate 4 °C /min from the elution temperature of ramp rate 6 °C /min. The average range of the elution temperature for compounds in MegaMix A is 15.2 °C and 16.2 °C for compounds in MegaMix B.

Table 2.8. The second dimension retention indices of compounds identified from MegaMix A by surface fitting.

Chromatographic conditions	Retention index of MegaMix A in different chromatographic conditions								RSD of RI (%) ^a	² T _e range (°C) ^b
Secondary oven offset (°C)	+5	+5	+5	+5	+5	+5	+5	+10		
Ramp rate (°C/min)	4	4.5	5	5	5.5	5.5	6	5		
Modulation period (s)	5	5	5	4	5	4	5	4		
N-Nitrosodimethylamine	974	975	976	979	978	978	978	979	0.19	9.5
Aniline	1233	1240	1245	1251	1250	1250	1255	1253	0.57	13.9
Phenol	1189	1193	1197	1201	1201	1200	1203	1204	0.40	13.4
Bis(2-chloroethyl) ether	1205	1208	1212	1214	1215	1215	1217	1217	0.34	13.4
2-Chlorophenol	1199	1205	1209	1217	1216	1213	1216	1218	0.50	14.0
Benzyl alcohol	1278	1285	1290	1299	1297	1296	1299	1300	0.56	14.4
2,2'-Oxybis(2-chloropropane)	1234	1237	1239	1244	1243	1240	1244	1244	0.28	14.5
2-Methylphenol	1274	1278	1282	1289	1288	1286	1290	1291	0.45	14.5
N-nitrosodipropylamine	1288	1291	1296	1302	1300	1299	1302	1303	0.41	14.7
3-Methylphenol	1295	1301	1304	1313	1310	1308	1311	1313	0.47	14.2
Hexachloroethane	1261	1266	1271	1275	1273	1273	1278	1275	0.40	15.0
Isophorone	1355	1362	1366	1374	1371	1371	1374	1375	0.49	14.7
2-Nitrophenol	1396	1404	1411	1422	1419	1419	1425	1422	0.68	15.0
2,4-Dimethylphenol	1378	1382	1387	1396	1394	1393	1396	1398	0.50	15.0
Bis(2-chloroethoxy)-Methane	1403	1409	1414	1422	1421	1420	1422	1424	0.48	14.7
2,4-DichloroPhenol	1413	1420	1425	1435	1434	1434	1437	1438	0.60	14.2
4-Chloroaniline	1511	1522	1530	1542	1540	1540	1547	1544	0.76	15.2
4-Chloro-3-methylphenol	1562	1571	1579	1588	1587	1586	1592	1591	0.62	15.7
2-Methylnaphthalene	1580	1591	1600	1611	1609	1608	1615	1614	0.71	15.9

1-Methylnaphthalene	1612	1623	1635	1644	1644	1643	1649	1648	0.75	16.4
1,3-Cyclopentadiene, 1,2,3,4,5,5-hexachloro-	1548	1557	1563	1573	1569	1568	1571	1574	0.54	16.2
2,4,6-Trichlorophenol	1637	1648	1655	1666	1663	1665	1672	1677	0.73	16.0
2,4,5-Trichlorophenol	1645	1655	1663	1675	1674	1672	1679	1678	0.68	16.2
2-Chloronaphthalene	1696	1707	1718	1729	1729	1729	1735	1733	0.75	16.4
2-Nitroaniline	1809	1819	1830	1838	1842	1841	1851	1844	0.72	16.0
1,4-Dinitrobenzene	1852	1859	1869	1877	1882	1880	1889	1883	0.63	15.7
1,3-Dinitrobenzene	1863	1871	1881	1889	1893	1892	1901	1893	0.64	16.0
Dimethyl phthalate	1817	1826	1836	1844	1849	1846	1852	1850	0.64	15.9
2,6-Dinitrotoluene	1860	1868	1877	1885	1891	1890	1898	1892	0.66	15.9
1,2-Dinitrobenzene	1908	1915	1924	1932	1937	1937	1946	1938	0.63	16.4
Acenaphthylene	1818	1831	1844	1854	1858	1857	1866	1861	0.83	16.5
3-Nitroaniline	1927	1935	1945	1954	1960	1959	1969	1962	0.69	16.5
Acenaphthene	1851	1864	1876	1885	1890	1889	1896	1893	0.79	16.7
2,4-dinitro-phenol	1916	1926	1938	1944	1951	1951	1957	1952	0.70	15.9
Dibenzofuran	1881	1894	1905	1916	1920	1920	1927	1923	0.79	16.4
2,4-Dinitrotoluene	1939	1948	1960	1968	1974	1971	1980	1975	0.68	16.2
2,3,5,6-Tetrachlorophenol	1889	1902	1913	1923	1925	1926	1929	1930	0.72	16.2
2,3,4,6-Tetrachlorophenol	1904	1916	1927	1937	1940	1940	1945	1949	0.75	16.2
Diethyl Phthalate	1954	1965	1974	1983	1985	1985	1988	1989	0.59	15.9
Fluorene	1971	1986	2000	2009	2013	2014	2020	2018	0.80	16.4
4-Chlorophenyl phenyl ether	1947	1959	1970	1980	1983	1984	1988	1987	0.70	16.2
4-Nitroaniline	2118	2126	2135	2142	2152	2152	2159	2150	0.63	16.2
4,6-Dinitro-2-methylphenol	2021	2030	2041	2051	2055	2056	2064	2058	0.69	16.5
Diphenylamine	2031	2043	2055	2063	2071	2068	2077	2071	0.71	16.2
Azobenzene	2001	2015	2026	2035	2041	2039	2044	2042	0.71	16.5
4-Bromophenyl phenyl ether	2088	2100	2112	2122	2127	2126	2132	2129	0.69	16.9
Hexachlorobenzene	2074	2089	2100	2112	2115	2116	2121	2120	0.75	16.7

Pentachloro-phenol	2166	2179	2193	2202	2209	2209	2218	2213	0.77	16.9
Anthracene	2261	2276	2290	2300	2309	2307	2319	2310	0.80	17.0
Phenanthrene	2271	2286	2299	2309	2317	2317	2329	2319	0.79	17.2
Carbazole	2380	2394	2404	2413	2424	2422	2433	2423	0.69	16.7
di-n-Butyl phthalate	2338	2348	2356	2370	2367	2370	2369	2373	0.50	16.0
Fluoranthene	2638	2652	2668	2678	2690	2690	2701	2688	0.76	17.5
Pyrene	2735	2749	2765	2774	2786	2784	2800	2785	0.73	17.7
Benzyl butyl phthalate	2892	2904	2918	2931	2940	2936	2943	2937	0.59	16.5
Bis(2-ethylhexyl) adipate	2608	2613	2615	2643	2629	2627	2617	2636	0.44	15.7
Benz[a]anthracene	3157	3172	3187	3201	3201	3208	3157	3194	0.59	13.0
Chrysene	3156	3202	3219	3233	3218	3204	3176	3213	0.73	12.7
Bis(2-ethylhexyl) phthalate	2894	2902	2908	2931	2893	2889	2849	2881	0.76	9.2
di-n-Octyl phthalate	3066	3058	3057	3077	3068	3068	3057	3076	0.24	0.0

^a Percentage (%) relative standard deviation

^b Temperature range is calculated by subtracting the temperature value corresponding to ramp 4 °C /min minus ramp 6 °C /min.

Table 2.9. The second dimension retention indices of compounds identified from MegaMix B by surface fitting.

Chromatographic conditions	Retention index of MegaMix B in different chromatographic conditions								RSD of RI (%) ^a	² T _e range (°C) ^b
Secondary oven offset (°C)	+5	+5	+5	+5	+5	+5	+5	+10		
Ramp rate (°C/min)	4	4.5	5	5	5.5	5.5	6	5		
Modulation period (s)	5	5	5	4	5	4	5	4		
cis-1,3-Dichloro-1-Propene, (Z)	879	880	893	888				887	0.57	11.5
trans-1,3-Dichloro-Propene	917	918	923	927	921	921		924	0.33	10.0

Toluene	890	893	904	899				901	0.58	10.0
1,1,2-Trichloroethane	929	930	932	937	934	935	937	933	0.32	10.2
Ethyl methacrylate	895	898	908	906				906	0.57	10.0
1,3-Dichloropropane	947	948	953	954	952	952	954	951	0.26	10.5
Dibromochloro-Methane	979	979	985	987	984	984	986	984	0.29	10.4
Tetrachloroethene	917	918	924	927				927	0.47	10.3
1,2-Dibromoethane	992	995	998	1001	996	998	1001	996	0.28	11.2
Chlorobenzene	1007	1008	1011	1016	1011	1014	1015	1012	0.29	11.9
1,1,1,2-Tetrachloroethane	992	1001	1005	1012	1007	1008	1007	1007	0.58	12.0
Ethylbenzene	997	999	1003	1008	1000	1001	1003	1003	0.32	12.0
p-Xylene	1005	1005	1008	1014	1006	1007	1014	1010	0.35	12.0
Styrene	1062	1062	1071	1072	1067	1067	1066	1070	0.34	11.5
o-Xylene	1052	1045	1053	1054	1050	1050	1052	1048	0.26	12.0
Bromoform	1118	1123	1129	1129	1126	1131	1134	1129	0.41	12.3
cis-1,4-Dichloro-2-butene, (z)-	1104	1106	1112	1114	1111	1113	1113	1112	0.30	12.4
1,1,2,2-Tetrachloroethane	1112	1112	1118	1119	1115	1116	1119	1113	0.25	12.7
1,2,3-Trichloropropane	1127	1130	1134	1140	1135	1133	1139	1137	0.36	13.0
Isopropylbenzene(cumene)	1062	1060	1069	1072	1068	1067	1072	1070	0.37	13.0
trans-1,4-Dichloro-2-butene, (E)-			1147	1153	1147	1147	1152	1149	0.19	6.4
Bromobenzene	1130	1133	1137	1140	1138	1139	1141	1137	0.30	13.2
2-Chlorotoluene	1128	1132	1137	1141	1136	1136		1140	0.38	12.0
n- Propylbenzene	1096	1100	1106	1110	1104	1104	1109	1109	0.42	13.0
4-Chlorotoluene	1133	1134	1140	1142	1139	1139	1142	1142	0.30	13.4
1,3,5-Trimethylbenzene	1112	1112	1121	1123	1120	1119	1118	1118	0.33	13.9
Pentachloroethane	1162	1164	1170	1172	1172	1172	1173	1167	0.32	13.7
tert-Butylbenzene	1133	1137	1145	1148	1142	1142	1145	1147	0.42	13.9
1,2,4-Trimethylbenzene	1151	1154	1160	1164	1158	1161		1162	0.37	12.0
sec-Butylbenzene	1155	1156	1161	1167	1162	1163	1163	1163	0.32	14.2
p-Cymene(4-isopropyl toluene)	1168	1171	1177	1182	1176	1176	1180	1179	0.37	14.2

n-Butylbenzene	1207	1208	1214	1221	1215	1215	1218	1219	0.40	14.5
1,2-Dibromo-3-Chloropropane	1343	1351	1359	1364	1362	1364	1368	1367	0.59	15.0
Benzene, 1,2,3-trichloro-	1470	1479	1488	1494	1494	1495	1502	1498	0.66	15.9
1,3-Dichlorobenzene*	1199	1204	1210	1217	1211	1212	1212	1215	0.45	14.0
1,4-Dichlorobenzene*	1215	1219	1222	1232	1227	1228	1231	1231	0.48	14.2
1,2-Dichlorobenzene*	1250	1255	1261	1267	1264	1265	1267	1269	0.49	14.5
Nitrobenzene*	1360	1368	1377	1381	1380	1383	1388	1384	0.62	15.2
Benzene, 1,2,4-trichloro-*	1413	1420	1426	1434	1432	1432	1436	1438	0.57	15.9
Naphthalene*	1461	1470	1481	1485	1487	1489	1493	1491	0.70	15.7
1,3-Butadiene, 1,1,2,3,4,4-hexachloro-*	1398	1402	1408	1416	1412	1413	1415	1418	0.47	15.2

^a Percentage (%) relative standard deviation;

^b Temperature range is calculated by subtracting the temperature value corresponding to ramp 4 °C /min minus ramp 6 °C /min.

2.3.5. Comparison with Literature Reported 2I Values

We previously identified 47 compounds from MegaMix A by GC×GC-MS, where a DB-17ms column was used as the secondary column.⁷⁶ The second dimension retention index of each identified compound was then calculated from the isovolatility curves of reference compounds *n*-alkanes. From these 47 compounds, 44 compounds were identified in this study.

Figure 2.6. Correlation of second dimension retention index of 44 compounds calculated by conventional isovolatility curves and by surface fitting approach.

Figure 2.6 shows the relation of the 2I values of the 44 compounds in MegaMix A calculated by surface fitting and by isovolatility curves. The R^2 value of the fitted curve was 0.9995, indicating a very good agreement between these two methods.

While the 2I values calculated by the conventional isovolatility curves had a good correlation with those calculated by the surface fitting methods, there are some differences between the 2I values of individual compounds. Twenty five compounds had the differences in 2I values, as calculated by the conventional isovolatility curves and by surface fitting, within 5 iu; 8 compounds within 6-10 iu; 5 compounds within 11-15; and 5 compounds within 16-25 iu; One compound had a variation of 36 iu. Benzyl butyl phthalate had the largest I difference, i.e., 36 iu. The mean 2I value of this compound calculated by the isovolatility curve was 2965 iu with standard deviation of ± 2 iu⁷⁶, while its mean 2I value calculated by the surface fitting was 2929 iu with standard deviation of ± 1 iu.

The standard deviations of the retention index of the 44 compounds calculated from the conventional isovolatility curves are in the range of 1–12 iu with an average standard deviation of 5.2 iu.⁷⁶ However, the standard deviations of the retention index of the 44 compounds calculated by the surface fitting method is in the range of 0–5 iu with an average standard deviation of 1.6 iu. While the true values of the retention index of these compounds remain unknown, the small value of average standard deviation in the calculated retention index using surface fitting method shows that the surface fitting method outperforms the conventional isovolatility curve method by introducing much decreased variation during data analysis.

2.4. Conclusions

With the increased use of GC×GC-MS for analysis of complex samples, it is crucial to have a robust method to calculate the second dimension retention index 2I for every compound to aid the accuracy of compound identification. A surface fitting method was

developed to calculate the 2I values from the second dimension retention time, 2t_R , and the secondary column temperature, 2T_e . In the surface fitting approach, the second dimension retention time, 2t_R , and second dimension column temperature, 2T_e , formed a X-Y plane and the 2I was the Z-axis to form the 2I surface. The predictability of the surface fitting method was validated using 10-fold cross validation. The average *RMSE* for the validation set of data was 16.0 iu. Analysis of the GC×GC-MS data acquired from the mixture of MegaMix A and MegaMix B showed that the mean standard deviation of 2I values calculated by the surface fitting approach was 1.6 iu for compounds in MegaMix A and 3.4 iu for compounds in MegaMix B. Compared with the literature reported results, there was a very good agreement between the 2I values of the compounds in MegaMix A calculated by the surface fitting and by the conventional isovolatility curves with R^2 value of 0.9995. However, the small value of standard deviation in the calculated retention index using surface fitting method shows that the surface fitting method outperforms the conventional isovolatility curve method by decreasing variation during data analysis.

While the developed method showed excellent reproducibility, the method was not validated by the retention index of these compounds from DB-17ms column. There is no available database containing retention index for DB-17ms column class. Therefore, it is crucial to develop a database for DB-17ms column and validate the developed method. Up until now all the 2I calculation method treated the second dimension separation as pseudo-isothermal as the second dimension analysis is very fast and column is very short. However, there is actually a temperature change in the second dimension separations though it is small. As second dimension separation was treated as pseudo-isothermal, therefore hold-up time must be calculated. Hold-up calculation methods introduce certain

degrees of variations which incur certain degree of inaccuracy in 2I calculation. It is imperative to develop a second dimension retention index calculation method which will treat the second dimension separation in its actuality and eliminate the erroneous hold-up time calculation step.

CHAPTER 3

CALCULATING THE RETENTION INDEX OF SECOND DIMENSION SEPARATION IN COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY MASS SPECTROMETRY WITHOUT INVOLVING HOLD-UP TIME CALCULATION

3.1. Introduction

Comprehensive two-dimensional gas chromatography mass spectrometry (GC×GC-MS) has become an excellent method for identification and quantification of compounds in complex samples owing to its high separation power.^{59-61,63,82} GC×GC-MS characterizes each compound by its first dimension retention time (1t_R), second dimension retention time (2t_R), and mass spectrum. Mass spectrum matching is widely used for compound identification, where a query spectrum is compared with the mass spectra recorded in a database.^{83,84} The top ranked compound whose mass spectrum has the largest spectrum similarity score is usually considered as the compound giving rise to the query spectrum. With the dramatic increase in the number of mass spectra in the database, the chance that the true compound presents in the database is increased. However, the rate of false identification is also increased owing to the increase of the number of similar mass spectra of different compounds.⁸⁵ In order to solve this problem, additional experimental

information such as retention time has been employed to aid in compound identification. Retention time is usually converted into retention index to reduce its dependency on experimental conditions, and the retention index is then used to filter out the compounds that are identified by mass spectrum matching but have a large retention index difference with that of the experimental value.^{32,33,86}

In GC×GC-MS, the two columns are usually operated in temperature gradient mode. The first dimension retention time 1t_R of a compound u is converted into a linear retention index I^T by following the equation 1.²⁸ Compounds eluted from the first dimension column during a modulation period (P_M) are subjected to the second dimension column for further separation. Compared with 1t_R , P_M is usually a very small value, e.g., 2 – 10 s. The temperature change on the second dimension column during one modulation period is small and therefore, the second dimension separation is often considered as being in pseudo-isothermal mode.⁶⁵⁻⁷¹ So 2t_R of a compound u is converted into Kováts retention index by following the equation 2.²⁹

Equation 2 has indirectly been used for 2I calculation in GC×GC-MS because the separation on the second dimension column is achieved within one P_M and the bracketing reference compounds may not be found in each P_M . For this reason, the concept of isovolatility curve mapping was proposed, and each 2I value is obtained via interpolation between two adjacent isovolatility curves.^{40,41,73-76} In chapter 2, we developed a surface fitting approach to calculate 2I using n -alkanes as references, in which we also approximated the second dimension separation as pseudo-isothermal.⁸⁷ As the second dimension separation is approximated in pseudo-isothermal mode, 2t_M must be calculated

in order to convert 2t_R to ${}^2t'_R$ for the calculation of 2I . While several mathematical models have been developed for the calculation of 2t_M , the calculation of 2t_M always introduces a certain degree of variation.⁸⁸⁻⁹² Veenaas and Haglund proposed a PEG- 2I system for the calculation of 2I , where the 2I value of each compound was defined based on predefined retention indices of polyethylene glycols (PEGs) with the 2t_R of *n*-alkanes as the reference (zero) points.⁹³ The PEG- 2I system is simple and avoided the calculation of 2t_M . However, a new retention index database must be generated for all compounds in order to use the PEG- 2I system to identify compounds from complex samples.

The objective of this work was to improve the accuracy of calculating the 2I by developing a method that treats the second dimension separation in GC×GC-MS in its actuality, i.e., temperature-programmed mode instead of the approximation approach, i.e., pseudo isothermal mode. By doing so, we can directly calculate the retention index for the second dimension separation, and do not need to approximate the second dimension separation as in pseudo-isothermal mode. This approach eliminates the hold-up time calculation. We constructed a second dimension retention index surface (2I_S) using *n*-alkanes as reference compounds, where the unadjusted second dimension retention time 2t_R and the second dimension column elution temperature 2T_e formed a *X-Y* plane and the 2I was the *Z*-axis to form 2I_S . The method was then applied to calculate the 2I of 387 compounds. The calculated 2I of those compounds were compared with their linear retention indices (I^T) calculated from GC-MS.

3.2. Experimental Section

3.2.1. Sample Preparation

Five hundred and two compounds were used in this study. Six different solutions were prepared by mixing 80 compound standards in different combinations, including a solution containing an odd number of C₅–C₂₃ saturated fatty acid methyl esters (FAMES) mixed with a solution containing C₄–C₂₄ (even number) carbon saturated FAMES, a solution containing C₅–C₂₂ saturated fatty acids, a 2-ketone mixture, a *n*-alcohol mixture, an 1-iodoalkane mixture, and an aldehyde mixture. All six mixtures were prepared in dichloromethane with a concentration of 100 µg/mL per compound. Each of MegaMix A and MegaMix B both contain 76 compounds. They were mixed in dichloromethane with a concentration of 50 µg/mL per compound. The six solutions and the mixture of MegaMix A and MegaMix B were analyzed by GC-MS and GC×GC-MS without derivatization.

Another ten solutions were prepared by mixing another 135 compound standards in different solvents including water, ethanol and HCl, depending on the solubility of those compounds. Each of those mixtures was derivatized using *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), respectively. To derivatize a sample, the sample was first freeze dried and then dissolved in 40 µL methoxamine hydrochloride pyridine solution (20 mg/mL) followed vigorously vortex-mixing for 1 min. Methoxymation was carried out by sonicating for 20 min and incubating at 60 °C for 1 h. Derivatization was conducted by adding 40 µL of MSTFA or MTBSTFA to the glass vial and incubating at 60 °C for 1 h. Each stock solution was then transferred to a GC vial for analysis. The methoxymation and derivatization were conducted prior to the GC-MS and GC×GC-MS analysis.

3.2.2. GC×GC-MS Experiments

The LECO (St. Joseph, MI) Pegasus® 4D GC×GC-MS was configured with a Gerstel MPS2 autosampler and an Agilent 6890 gas chromatograph featuring a LECO two-stage cryogenic modulator and a secondary oven. The first dimension column was a non-polar DB-5ms (phenyl arylene polymer virtually equivalent to a (5%-phenyl)-methylpolysiloxane) 60.0 m × 0.25 mm 1d_c × 0.25 μm 1d_f column, and the second dimension column was a medium polar BD-17ms ((50%-phenyl)-methylpolysiloxane) 1.0 m × 0.1 mm 2d_c × 0.1 μm 2d_f column. Both columns were obtained from Agilent Technologies (Santa Clara, CA), and were connected by means of a press-fit connector before the thermal modulator. The flow rate of ultra-high purity helium carrier gas (99.999%) was set to 2.0 mL/min at a corrected constant flow via pressure ramps with an initial head pressure of 31.9 psi. The MS parameters were as follows: MS range 29 – 800 m/z; data acquisition rate 200 spectra/s; temperature of ion source chamber 230 °C; temperature of MS transfer line 280 °C; detector voltage 1641 V; electron energy 70 eV. The acceleration voltage was turned on after a solvent delay of 220 s.

A 1 μL sample was injected into the inlet chamber of the GC×GC-MS system and the split ratio was set at 10:1. The inlet temperature was 280 °C. The thermal modulator was set to +20 °C relative to the primary oven. Initial temperature of the first dimension column 1T_e was set at 60 °C and kept for 1 min and then ramped to 285 °C. 1T_e was kept at 285 °C for 13 min to let all compounds elute off the column. Other parameters for analysis of each sample are listed in Table 3.1.

Table 3.1. GC×GC instrumental parameters

Sample name	Instrument parameters		Secondary oven offset (+°C/min)	Purpose
	Ramp rate (°C/min)	P_M (s)		
n-Alkanes	0.85, 1.00, 1.13, 1.25, 1.50, 1.65, 1.75, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 7.00	4	5	Construction of the 2I_s
Mixture of 80 compound standards	4.00, 4.50, 5.00, 5.50, 6.00	5	5	Reproducibility evaluation
MTBSTFA derivatized compounds	5.00	2-5	5	Calculation of the 2I and validation
MSTFA derivatized compounds	5.00	2-5	5	Calculation of the 2I and validation
MegaMix A & B	3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00	4-5	5-10	Robustness of surface fitting method

3.2.3. GC-MS Experiments

All in-house made mixtures, Megamix A and MegaMix B were respectively analyzed on a Thermo ITQ 1100 Ion Trap MS equipped with a Trace 1310 GC and an AI 1310 auto sampler. The column was a DB-17ms capillary column ($45.0\text{ m} \times 0.1\text{ mm}^2 d_c \times 0.1\text{ }\mu\text{m}^2 d_f$, (50%-phenyl)-methylpolysiloxane) from Agilent Technologies (Agilent Technologies

J&W, Santa Clara, CA, USA). The helium carrier gas (99.999% purity) flow rate was set to 2.0 mL/min, and the inlet temperature was 280 °C. The column temperature was programmed with an initial temperature of 60 °C for 1.0 min and then ramped at 5 °C/min to 290 °C and maintained at 290 °C for 13 min. The mass range was set as 29–800 *m/z*. The ion source chamber was 230 °C with transfer line temperature of 280 °C.

A mixture of C₇–C₃₀ *n*-alkanes at a concentration of 75 µg/mL was also analyzed by GC-MS to calculate the linear retention indices (I^T) of the compounds in all mixtures.

3.2.4. Instrument Data Preprocessing

Table 3.2. Data processing parameters used in LECO ChromaTOF software

Data processing parameters used in LECO ChromaTOF	
Baseline Offset	1
Number of Data Points Averaged for Baseline Smoothing	Auto
Peak Width for Peak Finding	GC-MS: 4, GC×GC-MS: 7.5
Minimum Number of Apexing Masses for Peak Finding	2
Minimum SNR for Peak Finding	10
Library Search Mode	Normal, Forward
Library Hits Returned per Peak	10
Minimum/Maximum Molecular Weight Allowed	29/850
Relative Mass Threshold for Library Searching	10
Minimum Similarity Match Before Name is Assigned	600
Libraries Used for Searching	In-House, NIST-14
Mass Used for Area/Height Calculation	Unique
Match Required to Combine Peaks in the Second Dimension	500
Second Dimension Peak Width	0.15
Minimum SNR for Subpeak to be Retained	6
Integration Approach	Traditional

LECO ChromaTOF software (version 4.51) equipped with the National Institute of Standards and Technology MS database (NIST 2014) was used for instrument control as

well as for spectrum deconvolution and compound identification. Manufacturer recommended parameters for ChromaTOF were used to reduce the raw instrument data into a peak list of compounds. The true peak spectrum was also exported as part of the information for each chromatographic peak as absolute intensity values. Table 3.2 contains all the parameters for GC×GC-MS data processing.

GC-MS data were collected by Thermo Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA) and converted to mzML files using our in-house software, followed by peak picking and compound identification. The retention time of each compound was then converted into I^T using *n*-alkanes as reference compounds following Van Den Dool and Kratz equation.²⁸

3.2.5. Construction of the Second Dimension Retention Index Surface

To construct the second dimension retention index surface, 2I_S , C₇–C₃₀ *n*-alkanes were analyzed by GC×GC-MS in temperature-programmed mode at sixteen ramp rates. After chromatographic peak picking and compound identification, 2t_R and 2T_e were extracted for each reference compound in each analysis. The 2I value of a reference compound with *n* carbon atoms was defined as $100 \times n$. Therefore, a *n*-alkane was represented by $\{ {}^2t_R, {}^2T_e, {}^2I \}$ in each analysis. The 2I_S was then constructed using the data of all *n*-alkanes, where 2t_R and 2T_e formed the *X-Y* plane and the 2I was the *Z*-axis to form the 2I_S . A polynomial surface model in MATLAB (Mathworks, Natick, MA) named ‘polyij’ was used to construct the 2I_S , where *i* was the degree of polynomial in 2t_R , and *j* was the degree of polynomial in 2T_e . The maximum for both *i* and *j* was five in MATLAB.

3.2.6. Selection of Surface Fitting Model

To avoid the overfitting in construction of the 2I_S , k -fold cross-validation (CV) was used to select the optimal polynomial surface fitting model. K -fold CV was done by first randomly partitioning the dataset into k subsets of roughly equal size. One subset was used as the testing data to study the performance of the polynomial surface model built by the remaining subsets, i.e., the training data. This process is repeated k times so that each subset was used for testing exactly once. The average cross-validation error was used as a performance indicator of the model. The k -fold CV was then repeated N times, and the model with the smallest estimated error was considered as the optimal model.

After selecting the optimal polynomial model, all dataset was used to construct the second dimension retention index surface 2I_S . The quality of surface fitting was then evaluated based on the distribution of residuals, R-square (R^2), degrees of freedom adjusted R-square (R^2_{adj}), and root mean squared error ($RMSE$). Briefly, the distribution of residuals can assess the random errors of surface fitting. R^2 is the measurement of successfulness of the fitting, which explains the variation of the data. R^2_{adj} adjusts R^2 based on the residual degrees of freedom⁸⁷. $RMSE$ is an estimate of the standard deviation of the random component in the data.

3.3. Results and Discussion

The six solutions prepared using the 80 compound standards and the mixture of MegaMix A and MegaMix B were not derivatized, while each of the 10 solutions prepared using 135 compound standards was derivatized using MSTFA and MTBSTFA, respectively. All 80 compounds were identified from the six solutions. One hundred and two compounds were identified from the MTBSTFA derivatized ten mixtures while 103

compounds were identified from the MSTFA derivatized ten mixtures. One hundred and two compounds were identified from the mixture of MegaMix A and MegaMix B. Together, 502 compounds were analyzed in GC×GC-MS and 387 compounds were identified.

3.3.1. Construction of the Second Dimension Retention Index Surface

C₇–C₃₀ *n*-alkanes were analyzed in temperature-programmed mode at sixteen ramp rates. After the temperature of the first dimension column reached the final temperature at 285 °C, the column temperature was kept at 285 °C for 13 min to elute all compounds. Therefore, both the first and the second dimension columns were operated in isothermal mode during the last 13 min. Figure 3.1 shows the elution trend of C₇–C₃₀ *n*-alkanes at different ramp rates, where each series represents the ²*t_R* of *n*-alkanes in a chromatographic run at a specific ramp rate.

A total of 346 data points of *n*-alkanes acquired under the temperature-programmed mode were used to construct the ²*I_S*. To select an optimal polynomial model for surface fitting, *N* times (*N* = 100) of *k*-fold (*k* = 10) CV was performed for every combination of *i* and *j* in the polynomial surface model ‘poly*ij*’, where *i* = 1,2, ...,5 and *j* = 1,2, ...,5. Table 3.3. lists the results of CVs. The optimum model was selected based on minimum validation *RMSE* that was defined as the average *RMSE* of all testing cases for a combination of *i* and *j*. From these 25 results, the optimal polynomial model was ‘poly35’, i.e., *i* = 3 and *j* = 5 with validation *RMSE* = 4.31 iu.

Figure 3.1. Two-dimensional separation space for *n*-alkanes analyzed in temperature-programmed mode using different ramp rates. The inflection point in each curve indicates the second dimension column temperature was 285 °C, from which the corresponding *n*-alkane was analyzed in isothermal mode for 13 min.

Table 3.3. Selection of polynomial surface model ‘polyij’ to construct the second dimension retention index surface using GC×GC-MS data of *n*-alkanes (robust ‘LAR’)

Poly‘ij’		R^2	R^2_{adj}	RMSE	Validation RMSE
<i>i</i>	<i>J</i>				
1	1	0.9787	0.9785	94.50	94.91
1	2	0.9931	0.9930	53.93	54.29
1	3	0.9937	0.9936	51.68	52.25
1	4	0.9942	0.9940	49.84	50.58
1	5	0.9946	0.9944	48.35	49.25
2	1	0.9927	0.9926	55.31	55.35
2	2	0.9999	0.9999	5.92	20.01
2	3	1.0000	1.0000	3.97	9.52
2	4	1.0000	1.0000	3.28	5.98

2	5	1.0000	1.0000	2.79	4.57
3	1	0.9935	0.9933	52.71	53.54
3	2	0.9999	0.9999	7.88	18.94
3	3	1.0000	1.0000	4.28	9.48
3	4	1.0000	1.0000	3.46	5.89
3	5	1.0000	1.0000	2.87	4.31
4	1	0.9937	0.9935	52.06	52.88
4	2	0.9998	0.9998	9.67	18.26
4	3	0.9999	0.9999	4.74	8.65
4	4	1.0000	1.0000	3.61	5.93
4	5	1.0000	1.0000	2.96	4.33
5	1	0.9938	0.9936	51.45	52.95
5	2	0.9997	0.9997	10.43	18.25
5	3	0.9999	0.9999	4.94	8.25
5	4	1.0000	1.0000	3.62	5.23
5	5	1.0000	1.0000	3.02	4.36

After selecting the optimal polynomial model ‘poly35’, all 346 data points were used to construct the 2I_S . Figure 3.2(A) depicts the 2I_S constructed from the temperature-programmed data of the *n*-alkanes using polynomial surface fitting model ‘poly35’. Figure 3.2(B) is the residual plot of the surface fitting. The residuals had a near normal distribution Figure 3.2(C) suggesting that no significant bias was introduced by the polynomial surface model ‘poly35’, even though a few relatively large residuals showed at the lower end of *X*-*Y* plane Figure 3.2(B).

A.

B.

C.

Figure 3.2. The second dimension retention index surface constructed using the GC×GC-MS data of *n*-alkanes. The retention index surface is a function of the elution temperature of the second dimension column 2T_e and the corresponding retention time 2t_R . (A) is the second dimension retention index surface 2I_S . (B) is the residual plot. (C) is the distribution of the residuals.

3.3.2. Calculation and Validation of the 2I of Known Compounds

For the validation of the developed method, each of the sixteen in-house made mixtures, MegaMix A, and MegaMix B were respectively analyzed by GC×GC-MS and GC-MS in temperature-programed mode using similar chromatographic conditions where ramp rate was 5 °C/min. The column in GC-MS is the same as the second dimension column in GC×GC-MS except the column length. Therefore, the 2I of a compound

calculated from the GC×GC-MS data should be identical to the linear retention index I^T of that compound calculated from its GC-MS data with a certain degree of experimental variation.

The 2I value of each compound was calculated from the 2I_S constructed in section 3.1 based on its 2t_R and 2T_e , while the I^T of that compound was calculated from its GC-MS data. A total of 387 compounds were identified from the 502 compounds in GC×GC-MS, where 322 compounds were identified from the 502 compounds in GC-MS. Figure 3.3(A) shows that the second dimension retention indices 2I calculated by the surface fitting method and the retention indices I^T obtained from the GC-MS data have excellent correlation, with $R^2 = 0.9974$ for 322 compound standards. Figure 3.3(B) depicts the deviation of calculated 2I from their corresponding I^T of the same compounds, i.e. $(I^T - {}^2I)$, where different color represent the different set of mixture of compound standards. 86% of the 322 compounds have a retention index difference less than 40 iu, while the maximum retention index difference was 79 iu. These results agree with the results from the NIST database. Our lab did a study on NIST database and found that most compounds standard deviation (STD) fall on the range within 40 iu and retention index variations is column's stationary phase dependent ⁹⁴.

Compared to the non-polar column, polar columns shows comparatively poor chromatographic reproducibility thus comparatively higher STD of retention index. For our current study, we used DB-17ms column as second dimension column which is a mid-polar column. NIST database have not registered any RI value corresponding to the DB-17ms column class.

A.

B.

C.

Figure 3.3. Validation of the second dimension retention indices 2I calculated using surface fitting method and its corresponding I^T calculated from GC-MS data. (A) Correlation between 2I of 322 compound standards with their corresponding I^T . (B) Deviation between I^T and 2I , i.e., $|I^T - ^2I|$. ($\blacktriangle$) represents the mixtures of 80 compound standards, ($\blacksquare$) represents the 135 compound standards derivatized with MTBSTFA derivatizing agent, ($\blacktriangle$) represents the 135 compound standards derivatized with MSTFA derivatizing agent, ($\blacksquare$) represents the MegaMix B compound standards, and ($\blacklozenge$) represents the MegaMix A compound standards. (C) Distribution of $I^T - ^2I$ (iu).

However, results from the NIST study showed that STD of the retention index increase with the increased polarity, therefore, the STD of the retention index for the DB-17 ms should fall between the non-polar and polar column. Figure 3.3(C) depicts the distribution of the deviation of calculated 2I from their corresponding I^T which is consistent with the NIST results.⁹⁴

Table 3.4 lists the calculated I^T , 2I and $|I^T - {}^2I|$ of the compound standards detected in the six mixtures that were not derivatized. These six mixtures showed an excellent correlation between the GC×GC-MS and GC-MS data with an average retention index difference of 5.9 iu. The very small difference of retention index between I^T and 2I can be attributed to the compound structure. These straight chain homologous compounds have similar structure to those reference series, i.e., *n*-alkanes.

Tables 3.5 and 3.6 list the calculated I^T , 2I and $|I^T - {}^2I|$ of the compound standards detected in the ten mixtures that were derivatized by both MSTFA and MTBSTFA. Figure 3.4 shows the effect of derivatization agents on $|I^T - {}^2I|$ of the detected compounds. The MTBSTFA derivatized compounds have small variation in $|I^T - {}^2I|$ with an average variation of 12.5 iu, while the MSTFA derivatized compounds have a comparatively large variation with an average of 25.5 iu. This was caused by the polarity of the derivatized compounds. In general, the MTBSTFA derivatized compounds are less polar than the MSTFA derivatized compounds owing to the MTBSTFA derivatizing group. With decreasing polarity of the derivatized compounds, the separation mechanism of a compound is similar to the separation mechanism of the non-polar reference series (i.e., *n*-alkanes), thus giving less variation.

Table 3.4. I^T , 2I and $|I^T - {}^2I|$ of the compound standards detected in the six mixtures of compound standards that were not derivatized

Compounds name	2I (iu) ^a					<i>STD</i> (iu)	<i>RSD</i> (%) ^b	I^T from GC- MS ^c	$ I^T - {}^2I $
Ramp (°C/min)	4	4.5	5	5.5	6				
Methyl butyrate	823	821	818	820	824	2	0.27		
Methyl valerate	932	935	932	933	931	1	0.13		
Methyl caproate	1041	1041	1040	1041	1040	0	0.05	1033	7
Methyl enanthate	1137	1138	1137	1138	1135	1	0.09	1130	7
Methyl caprylate	1237	1236	1238	1237	1234	1	0.11	1229	9
Methyl pelargonate	1333	1332	1332	1334	1331	1	0.09	1331	1
Methyl caprate	1434	1434	1432	1435	1430	2	0.13	1429	3
Methyl undecanoate	1531	1530	1531	1536	1527	3	0.19	1531	0
Methyl laurate	1632	1633	1629	1632	1629	2	0.09	1628	1
Methyl tridecanoate	1732	1733	1731	1732	1721	4	0.25	1733	2
Methyl myristate	1833	1832	1832	1835	1829	2	0.11	1832	0
Methyl pentadecanoate	1932	1934	1930	1936	1925	4	0.19	1935	5
Methyl palmitate	2039	2036	2028	2040	2023	7	0.33	2034	6
Methyl margarate	2135	2131	2129	2134	2122	5	0.22	2138	9
Methyl stearate	2237	2239	2227	2239	2229	5	0.23	2236	9
Methyl nonadecanoate	2333	2334	2328	2338	2326	4	0.18	2337	9
Methyl arachidate	2437	2437	2432	2442	2427	5	0.21	2440	8
Methyl heneicosanoate	2533	2535	2531	2544	2528	5	0.21	2542	11
Methyl behenate	2638	2639	2630			4	0.14	2641	11
Methyl tricosanoate	2734					0	0		

Butyric acid	903	912	896	897	900	6	0.64	905	9
Valeric acid	1005	1017	1003	1004	1006	5	0.53	996	7
Caproic acid	1105	1118	1103	1104	1105	6	0.51	1108	5
Enanthic acid	1200	1210	1202	1203	1202	3	0.28	1200	2
Caprylic acid	1301	1312	1295	1305	1301	6	0.44	1296	1
Pelargonic acid	1403	1416	1393	1406	1407	7	0.53	1393	0
Capric acid	1500	1518	1491	1504	1507	9	0.6	1492	1
Undecylic acid	1599	1619	1592	1611	1614	10	0.62	1598	6
Lauric acid	1700	1726	1692	1708	1716	12	0.7	1697	5
Tridecylic acid	1806	1822	1793	1812	1815	10	0.54	1798	5
Myristic acid	1914	1937	1898	1925	1922	13	0.67	1901	3
Pentadecylic acid	2008	2026	1994	2014	2017	11	0.53	1999	5
Palmitic acid	2112	2126	2098	2122	2120	10	0.47	2101	3
Margaric acid	2213	2228	2202	2227	2224	10	0.46	2205	3
Stearic acid	2318	2334	2302	2323	2323	10	0.45	2307	5
Nonadecylic acid	2420	2429	2407	2422	2424	7	0.3	2409	2
Arachidic acid	2521	2528	2507	2526	2527	8	0.31	2513	6
Heneicosylic acid	2622	2636	2612	2632	2613	10	0.38	2618	6
Behenic acid	2731	2733				1	0.02	2726	
2-Pentanone	809	810	801	816	795	7	0.91		
2-Hexanone	922	910	917	924	915	5	0.55		
2-Heptanone	1025	1024	1021	1027	1023	2	0.18	1008	13
2-Octanone	1125	1125	1122	1126	1121	2	0.18	1109	13
2-Nonanone	1224	1227	1220	1226	1222	3	0.22	1208	12
2-Decanone	1322	1326	1319	1326	1321	3	0.2	1308	11
2-Undecanone	1422	1427	1420	1429	1419	4	0.27	1410	10
2-Dodecanone	1525	1527	1521	1537	1521	6	0.38	1512	9
2-Tridecanone	1627	1626	1620	1629	1620	4	0.24	1612	8
2-Tetradecanone	1727	1727	1720	1733	1720	5	0.29	1714	6
2-Pentadecanone	1833	1834	1823	1831	1819	6	0.32	1816	7
2-Hexadecanone	1937	1937	1927	1943	1923	7	0.37	1918	9

2-Heptadecanone	2026	2025	2024	2028	2021	2	0.12	2017	7
2-Octadecanone	2136	2145	2130	2147	2127	8	0.38	2123	7
2-Nonadecanone	2244	2245	2232	2250	2235	7	0.3	2225	7
1-Pentanol	873	877	873	875	874	2	0.18	862	11
1-Hexanol	979	986	981	979	980	3	0.26	972	9
1-Heptanol	1080	1081	1080	1082	1077	2	0.15	1069	11
1-Octanol	1182	1185	1182	1183	1180	2	0.14	1174	8
1-Nonanol	1282	1286	1271	1276	1271	6	0.47	1275	4
1-Undecanol	1481	1480	1470	1477	1469	6	0.4	1480	10
1-Dodecanol	1576	1585	1569	1573	1567	6	0.41	1582	13
1-Iodobutane	952	956	948	954	954	3	0.28	949	1
1-Iodopentane	1063	1065	1066	1067	1069	2	0.19	1065	1
1-Iodohexane	1168	1171	1172	1173	1173	2	0.16	1168	4
1-Iodoheptane	1273	1278	1278	1279	1279	2	0.17	1273	5
1-Iodooctane	1381	1382	1380	1390	1388	3	0.24	1380	0
1-Iodononane	1488	1492	1490	1494	1500	4	0.28	1488	2
1-Iododecane	1602	1606	1598	1605	1599	3	0.2	1596	2
1-Iodoundecane	1703	1708	1705	1711	1709	3	0.15	1702	3
1-Iodododecane	1813	1816	1813	1819	1819	3	0.14	1810	3
1-Iodohexadecane	2244	2246	2243	2253	2245	3	0.16	2237	6
1-Iodooctadecane	2454	2461	2457	2468	2461	5	0.19	2449	8
Pentanal	812	809	807	813	808	2	0.29	808	1
Hexanal	923	922	922	923	921	1	0.09	919	3
Heptanal	1032	1031	1030	1033	1030	1	0.14	1016	14
Octanal	1134	1134	1131	1135	1131	1	0.13	1119	12
Nonanal	1233	1232	1232	1233	1231	1	0.07	1220	12
Decanal	1332	1331	1330	1333	1328	2	0.14	1320	10
Undecanal	1432	1428	1428	1434	1427	2	0.17	1422	6
Dodecanal	1534	1530	1526	1531	1526	3	0.19	1523	3

^a Blank cells: means either compound was not identified or it eluted at 290 °C (isothermal condition in 2T_e)

^b Percentage(%) relative standard deviation

^c Linear retention index I^T of six standards mixtures that were not derivatized calculated from GC-MS

Table 3.5. I^T , 2I and $|I^T - {}^2I|$ of the compound standards detected in the ten mixtures that were derivatized by MTBSTFA derivatized reagent.

Compound name	MTBSTFA derivatized compounds ^b			
	Derivative ^a	² <i>I</i>	<i>I</i> ^T from GC-MS ^c	$ I^T - {}^2I $
Isobutyric acid				
Benzoic acid	TBDMS	1638	1653	15
3-Hydroxybutyric acid	2TBDMS	1603	1590	13
l-Norleucine	2TBDMS	1737	1735	2
Fumaric acid	2TBDMS	1835	1838	3
Lidocaine	TBDMS	2107	2118	11
Adipic acid	2TBDMS	2049	2050	1
cis-Aconitic acid	3TBDMS	2453	2452	1
Citrulline				
Isovaleric acid				
Nicotinic acid	TBDMS	1714	1737	23
Malonic acid	2TBDMS	1711	1710	1
Maleic acid	2TBDMS	1854	1853	1
Sarcosine	2TBDMS	1619	1625	6
l-Pyroglutamic acid	2TBDMS	2106	2116	10
l-Malic acid	3TBDMS	2123	2117	6
Citric acid				

Ethylene glycol	2TBDMS	1396	1365	31
Oxalic acid	2TBDMS	1625	1625	0
3-Aminobutanoic acid	2TBDMS	1668	1686	18
Succinic acid	2TBDMS	1834	1825	9
Glutaric acid	2TBDMS	1933	1941	8
Salicylic acid	2TBDMS	2091	2104	13
Azelaic acid	2TBDMS	2362	2345	17
d-Tartaric acid	4TBDMS	2381	2404	23
Ferulic acid				
Lactic acid	2TBDMS	1502	1492	10
(r)-2-Hydroxybutyric acid	2TBDMS	1565	1564	1
dl-Norleucine	2TBDMS	1737	1735	2
γ -Aminobutyric acid	2TBDMS	1793	1782	11
α -Ketobutyric acid				
Glycerol	3TBDMS	1794	1751	43
Trans-4-Hydroxy-l-proline	3TBDMS	2165	2179	14
Palmitoleic acid	TBDMS	2340	2322	18
Glutamine				
β -Alanine	2TBDMS	1674	1656	18
l-Alanine	2TBDMS	1540	1535	5
l- α -Aminoadipic Acid	3TBDMS	2394	2366	28
l- α -Amino-n-butyric Acid	TBDMS	1612	1579	33
D,L- β -Aminoisobutyric Acid	2TBDMS	1612	1644	32
l-Asparagine	3TBDMS	2363	2374	11
l-Aspartic Acid	3TBDMS	2163	2178	15
l-Citrulline				
Cystathionine				
l-Cystine				
l-Glutamic Acid	3TBDMS	2284	2289	5
Glycine	2TBDMS	1588	1575	13

Hydroxy-l-proline	3TBDMS	2164	2152	12
l-Isoleucine	2TBDMS	1683	1696	13
l-Leucine	2TBDMS	1680	1666	14
l-Methionine	2TBDMS	2045	2045	0
l-Phenylalanine	2TBDMS	2188	2196	8
O-Phospho-l-serine				
O-Phosphoethanolamine	3TBDMS	2273	2257	16
l-Proline	2TBDMS	1816	1823	7
l-Sarcosine	2TBDMS	1619	1625	6
l-Serine	3TBDMS	1952	1946	6
Taurine	2TBDMS	2048	2061	13
l-Threonine	3TBDMS	1976	1977	1
l-Tyrosine				
Urea	2TBDMS	1774	1780	6
l-Valine	2TBDMS	1650	1639	11
γ -Amino-n-butyricAcid	2TBDMS	1793	1782	11
AmmoniumChloride				
l-Anserine				
l-Arginine				
l-Carnosine				
Creatinine				
Ethanolamine	2TBDMS	1442	1415	27
l-Histidine (ISO)				
l-Homocystine				
dl-Hydroxylysine	4TBDMS	2590	2600	10
l-Lysine	3TBDMS	2359	2343	16
l-Methyl-l-histidine	2TBDMS	2520	2548	28
3-Methyl-l-histidine(1D				
ISO)	2TBDMS	2607	2648	41
l-Ornithine	3TBDMS	2248	2216	32

l-Tryptophan (ISO)				
Hexanoic acid (C6)				
Heptanoic acid (C7)	TBDMS	1427	1416	11
Octanoic acid (C8)	TBDMS	1530	1516	14
Nonanoic acid (C9)	TBDMS	1629	1628	1
Decanoic acid (C10)	TBDMS	1732	1741	9
Undecanoic acid (C11)	TBDMS	1833	1829	4
Dodecanoic acid (C12)	TBDMS	1935	1947	12
Tridecanoic acid (C13)	TBDMS	2036	2046	10
Tetradecanoic acid (C14)	TBDMS	2135	2131	4
Pentadecanoic acid (C15)	TBDMS	2232	2211	21
Hexadecanoic acid (C16)	TBDMS	2334	2313	21
Heptadecanoic acid (C17)	TBDMS	2436	2418	18
Octadecanoic acid (C18)	TBDMS	2537	2543	6
Nonadecanoic acid (C19)				
Eicosanoic acid (C20)				
Heneicosanoic acid (C21)				
Docosanoic acid (C22)				
Tricosanoic acid (C23)				
Tetracosanoic acid (C24)				
5-Aminovaleric acid	2TBDMS	1897	1887	10
Pipecolic acid				
	2TBDMS	1895	1903	8
Itaconic acid	2TBDMS	1881	1883	2
n-Acetyl-l-glutamic acid	3TBDMS	2462	2446	16
Levulinic acid	TBDMS	1587	1590	3
4-Hydroxyphenyllactic acid	3TBDMS	2648	2648	0
l-Dihydroorotic acid	3TBDMS	2513	2520	7
Palmitolinoleic acid	TBDMS	2340	2329	11
4,methyl 2 oxovaleric acid)				

Caffeine	Und ^c	2384	2422	38
Cotinine	Und	2195	2231	36
Uridine				
Uracil	2TBDMS	1852	1851	1
n-Acetylaspartic acid	3TBDMS	2489	2489	0
α -Keto-glutarate	2TBDMS	2132	2112	20
Linoelaidic acid	TBDMS	2554	2558	4
Pyruvate	TBDMS	1332	1347	15
13-Octadecenoic acid,	TBDMS	2539	2520	19
l-Cysteine	3TBDMS	2218	2204	14
Oleic Acid	TBDMS	2530	2529	1
l-Homoserine				
Creatinine				
2,3-Butanediol				
l-Glutamic acid				
Niacinamide				
l-Lysine				
n- α -Acetyl-l-Lysine				
Methylmalonic acid				
α -Linolenic acid				
9-Octadecenoic acid,(E)				
9,12-Octadecadienoic acid (Z,Z)-				
11-Eicosenoic acid, (E)-				
Arachidonic acid				
Docosahexaenoic acid				

^a TBDMS: tert-butyl-dimethylsilane

^b Blank cells: means either compound was not identified or it eluted at 290 °C (isothermal condition in 2T_e)

^c During derivatization process compound remained underivatized

Table 3.6. I^T , 2I and $|I^T - {}^2I|$ of the compound standards detected in ten mixtures that were derivatized by MSTFA derivatized reagent

Compound name	MSTFA derivatized compounds ^b			
	Derivative ^a	2I	I^T from GC-MS ^c	$ I^T - {}^2I $
Isobutyric acid				
Benzoic acid	TMS	1391	1408	17
3-Hydroxybutyric acid	2TMS	1120	1166	46
l-Norleucine	2TMS	1336	1307	29
Fumaric acid	2TMS	1398	1379	19
Lidocaine	Undc	2246	2299	53
Adipic acid	2TMS	1600	1597	3
cis-Aconitic acid	3TMS	1833	1812	21
Citrulline				
Isovaleric acid				
Nicotinic acid	TMS	1464	1489	25
Malonic acid	2TMS	1296	1274	22
Maleic acid	2TMS	1430	1420	10
Sarcosine	2TMS	1179	1137	42
l-Pyroglutamic acid	2TMS	1698	1727	29
l-Malic acid	3TMS	1534	1499	35
Citric acid	4TMS	1852	1827	25

Ethylene glycol	2TMS	980	941	39
Oxalic acid	2TMS	1239	1226	13
3-Aminobutanoic acid	2TMS	1246	1208	38
Succinic acid	2TMS	1405	1385	20
Glutaric acid	2TMS	1493	1484	9
Salicylic acid	2TMS	1642	1660	18
Azelaic acid	2TMS	1894	1866	28
d-Tartaric acid	4TMS	1671	1622	49
Ferulic acid				
Lactic acid	2TMS	1107	1084	23
(r)-2-Hydroxybutyric acid	2TMS	1167	1145	22
dl-Norleucine	2TMS	1335	1304	31
γ -Aminobutyric acid	3TMS	1545	1511	34
α -Ketobutyric acid				
Glycerol	3TMS	1255	1199	56
Trans-4-Hydroxy-l-proline	3TMS	1542	1515	27
Palmitoleic acid	TMS	2100	2072	28
Glutamine				
β -Alanine	3TMS	1435	1398	37
l-Alanine	2TMS	1138	1098	40
l- α -Aminoadipic Acid	3TMS	1759	1704	55
l- α -Amino-n-butyric Acid	2TMS	1198	1155	43
D,L- β -Aminoisobutyric Acid	3TMS	1466	1435	31
l-Asparagine	4TMS	1870	1896	26
l-Aspartic Acid	3TMS	1564	1518	46
l-Citrulline				
Cystathionine	2TMS	2367	2387	20

l-Cystine				
l-Glutamic Acid	2TMS	1698	1713	15
Glycine	3TMS	1319	1302	17
Hydroxy-l-proline	3TMS	1540	1503	37
l-Isoleucine	2TMS	1307	1286	21
l-Leucine	2TMS	1284	1243	41
l-Methionine	2TMS	1610	1609	1
l-Phenylalanine	2TMS	1735	1744	9
O-Phospho-l-serine				
O-Phosphoethanolamine	4TMS	1843	1808	35
l-Proline	TMS	1353	1315	38
l-Sarcosine	2TMS	1179	1137	42
l-Serine	2TMS	1337	1326	11
Taurine	3TMS	1767	1756	11
l-Threonine	3TMS	1387	1350	37
l-Tyrosine	2TMS	2070	2068	2
	3TMS	2016	1983	33
Urea	2TMS	1372	1383	11
l-Valine	2TMS	1232	1203	29
γ -Amino-n-butyricAcid	3TMS	1545	1511	34
AmmoniumChloride				
l-Anserine				
l-Arginine				
l-Carnosine				
Creatinine				
Ethanolamine	3TMS	1189	1212	23
l-Histidine (ISO)				
l-Homocystine				
dl-Hydroxylysine				
l-Lysine				

l-Methyl-l-histidine				
3-Methyl-l-histidine(1D ISO)				
l-Ornithine	3TMS	1809	1774	35
l-Tryptophan (ISO)	3TMS	2414	2435	21
Hexanoic acid (C6)				
Heptanoic acid (C7)	TMS	1224		
Octanoic acid (C8)	TMS	1318	1295	23
Nonanoic acid (C9)	TMS	1412	1387	25
Decanoic acid (C10)	TMS	1506	1488	18
Undecanoic acid (C11)	TMS	1602	1589	13
Dodecanoic acid (C12)	TMS	1698	1682	16
Tridecanoic acid (C13)	TMS	1795	1785	10
Tetradecanoic acid (C14)	TMS	1893	1865	28
Pentadecanoic acid (C15)	TMS	1991	1959	32
Hexadecanoic acid (C16)	TMS	2089	2055	34
Heptadecanoic acid (C17)	TMS	2187	2153	34
Octadecanoic acid (C18)	TMS	2285	2248	37
Nonadecanoic acid (C19)	TMS	2383	2347	36
Eicosanoic acid (C20)	TMS	2483	2444	39
Heneicosanoic acid (C21)	TMS	2584	2548	36
Docosanoic acid (C22)				
Tricosanoic acid (C23)				
Tetracosanoic acid (C24)				
5-Aminovaleric acid	3TMS	1640	1602	38
Pipecolic acid	TMS	1408	1424	16

	2TMS	1413	1404	9
Itaconic acid	2TMS	1448	1430	18
n-Acetyl-l-glutamic acid	2TMS	1978	1994	16
Levulinic acid				
4-Hydroxyphenyllactic acid	3TMS	1982	1970	12
l-Dihydroorotic acid				
Palmitolinoleic acid	TMS	2100	2072	28
4,methyl 2 oxovaleric acid)	TMS	1333	1320	13
Caffeine				
Cotinine				
Uridine	3TMS	2663	2683	20
Uracil				
n-Acetylaspatic acid				
α -Keto-glutarate				
Linoelaidic acid				
Pyruvate				
13-Octadecenoic acid,				
l-Cysteine				
Oleic Acid	TMS	2297	2298	1
l-Homoserine	3TMS	1389	1411	22
	2TMS	1434	1433	1
Creatinine	3TMS	1593	1573	20
2,3-Butanediol	2TMS	1640	1675	35
l-Glutamic acid	3TMS	1664	1628	36
Niacinamide	TMS	1733	1767	34
l-Lysine	4TMS	1845	1836	9
n- α -Acetyl-l-Lysine	3TMS	1905	1892	13
Methylmalonic acid	2TMS	2013	2035	22

α -Linolenic acid	TMS	2297	2310	13
9-Octadecenoic acid,(E)	TMS	2303	2318	15
9,12-Octadecadienoic acid (Z,Z)-	TMS	2318	2346	28
11-Eicosenoic acid, (E)-	TMS	2471	2477	6
Arachidonic acid	TMS	2514	2551	37
Docosahexaenoic acid	TMS	2719	2733	14

^a TMS: Trimethylsilyl

^b Blank cells: means either compound was not identified or it eluted at 290 °C (isothermal condition in 2T_e)

A.

B.

Figure 3.4. Effect of derivatization agent on $|I^T - {}^2I|$. (A) Distribution of $|I^T - {}^2I|$ of MTBSTFA derivatized compounds vis. $|I^T - {}^2I|$ of corresponding compounds derivatized by MSTFA. The red line shows the result of linear fitting while the blue and pink lines are fitting confidence with 95% confidence interval. (B) Box plot of $|I^T - {}^2I|$ for the MTBSTFA and MSTFA derivatized compounds.

It should be noted that two active hydrogen atoms in *dl*- β -aminoisobutyric acid, taurine, and ethanolamine were respectively derivatized by MTBSTFA and three hydrogen atoms were derivatized by MSTFA, which resulted in MSTFA derivatized compounds showing slightly smaller difference between I^T and 2I than those of the MTBSTFA derivatized compounds. The steric effect hindered the ability of the derivatizing group of MTBSTFA to completely replace the three active hydrogen atoms of those compounds. In contrast, MSTFA derivatizing agent replaced all of the three active hydrogen atoms in each molecule thus making the compound less polar.

MegaMix A and MegaMix B contain compounds with a broad range of cyclization and branching. The 2I of these compounds had large deviation from their I^T with an average variation of 40.9 iu. Six compounds (i.e., anthracene, phenanthrene, carbazole, fluoranthene, pyrene, benzyl butyl phthalate) had much larger variations because of their structure and were excluded from the average retention index variation calculation. Their structure had polyaromatic ring which are very different from the structure of *n*-alkanes. From the previous study it was found that, polyaromatic hydrocarbon shows poor reproducibility of retention index calculation using *n*-alkanes as reference series. Therefore, a special reference index calculation method was developed exclusively for the

polyaromatic carbon known as Lee retention index using some polyaromatic compounds as reference series.⁹⁵

Overall, the magnitude of deviation between the calculated 2I and the corresponding I^T can be attributed to several factors, including compound polarity, structure, and elution temperature, etc. The elution temperature of compounds increases with increased ramp rate as listed in Table 3.7.

Table 3.7. Second dimension elution temperature of 80 standard compounds with different ramp rates

Compounds name	I^2 , (iu) ^a					$^2T_e\Delta^b$
Ramp (°C/min)	4	4.5	5	5.5	6	
FAME						
Methyl butyrate	84.8	86.8	89.3	91.2	93.6	8.9
Methyl valerate	92.1	94.7	97.6	100.0	102.6	10.5
Methyl caproate	102.4	106.0	108.9	111.9	115.1	12.7
Methyl enanthate	115.8	119.5	123.0	126.1	129.6	13.9
Methyl caprylate	129.8	133.8	137.6	141.2	144.7	14.9
Methyl pelargonate	144.5	148.4	152.2	155.4	159.2	14.7
Methyl caprate	158.1	162.3	166.0	169.6	173.2	15.0
Methyl undecanoate	171.5	175.4	179.3	182.9	186.7	15.2
Methyl laurate	183.8	188.1	191.8	195.8	199.2	15.4
Methyl tridecanoate	195.8	199.8	203.9	207.7	211.2	15.4
Methyl myristate	207.1	211.4	215.1	218.7	222.7	15.5
Methyl pentadecanoate	217.8	221.9	226.0	229.7	233.2	15.4
Methyl palmitate	227.8	232.0	236.4	239.8	243.7	15.9
Methyl margarate	237.8	242.1	246.0	249.9	253.7	15.9
Methyl stearate	247.1	251.1	255.1	259.0	262.7	15.5
Methyl nonadecanoate	255.8	260.1	264.3	268.2	271.7	15.9
Methyl arachidate	264.5	268.8	272.6	276.4	280.2	15.7
Methyl heneicosanoate	272.8	277.0	281.0	284.7	288.7	15.9
Methyl behenate*	280.5	284.9	288.9			
Methyl tricosanoate*	288.1					
Fatty acid						
Butyric acid	88.1	91.0	93.4	95.8	98.1	10.0
Valeric acid	97.4	100.7	103.9	106.8	109.6	12.2

Caproic acid	109.4	113.1	116.8	120.1	123.1	13.7
Enanthic acid	123.1	127.0	131.0	134.4	137.7	14.5
Caprylic acid	137.8	142.0	145.6	149.0	152.7	14.9
Pelargonic acid	150.5	154.8	158.9	161.9	165.2	14.7
Capric acid	164.1	167.9	172.2	175.2	178.7	14.5
Undecylic acid	176.5	180.6	184.7	188.0	191.7	15.2
Lauric acid	188.8	192.6	196.8	200.4	203.7	14.9
Tridecylic acid	200.1	204.3	208.5	211.8	215.2	15.0
Myristic acid	210.8	215.1	219.3	222.8	226.7	15.9
Pentadecylic acid	221.5	225.6	230.1	233.4	237.2	15.7
Palmitic acid	231.5	235.8	240.1	243.5	247.2	15.7
Margaric acid	241.1	245.1	249.7	253.1	256.7	15.5
Stearic acid	250.1	254.5	258.9	262.2	266.2	16.0
Nonadecylic acid	259.1	263.1	267.7	271.4	274.7	15.5
Arachidic acid	267.8	271.8	276.4	279.7	283.7	15.9
Heneicosylic acid*	275.8	280.0	284.3	287.9		
Behenic acid*	283.8	287.9				
Ketone						
2-Pentanone	83.1	85.3	87.2	89.4	91.6	8.5
2-Hexanone	89.1	91.7	94.3	96.8	99.1	10.0
2-Heptanone	98.4	101.9	104.7	107.8	110.6	12.2
2-Octanone	111.1	114.6	118.1	121.5	124.7	13.5
2-Nonanone	125.5	129.3	133.1	136.2	139.7	14.2
2-Decanone	139.8	143.9	147.6	151.3	154.7	14.9
2-Undecanone	154.1	158.1	162.2	165.5	169.2	15.0
2-Dodecanone	167.8	172.0	175.6	179.3	183.2	15.4
2-Tridecanone	180.5	184.8	188.5	192.1	196.2	15.7
2-Tetradecanone	192.8	196.8	201.0	204.5	208.2	15.4
2-Pentadecanone	204.1	208.4	212.6	216.4	220.2	16.0
2-Hexadecanone	215.1	219.6	223.5	227.4	231.2	16.0
2-Heptadecanone	225.8	230.1	234.3	237.9	241.7	15.9
2-Octadecanone	235.8	239.9	243.9	247.6	251.7	15.9
2-Nonadecanone	245.1	249.6	253.5	257.2	261.2	16.0
Alcohol						
1-Pentanol	87.4	89.9	92.6	94.9	97.1	9.7
1-Hexanol	96.4	99.2	102.2	105.0	108.1	11.7
1-Heptanol	108.8	112.0	115.5	119.2	122.1	13.4
1-Octanol	122.5	125.9	129.7	133.4	136.6	14.2
1-Nonanol	137.1	141.6	148.9	151.8	157.1	20.0
1-Undecanol	168.4	171.6	178.0	180.2	186.1	17.7
1-Dodecanol	186.1	185.1	192.6	196.2	200.6	14.5
Iodobutne						
1-Iodobutane	92.1	95.1	98.0	100.4	103.1	11.0
1-Iodopentane	103.1	106.7	109.7	112.8	116.2	13.0
1-Iodoheptane	117.1	120.6	124.3	127.9	131.2	14.0

1-Iodoheptane	131.8	136.0	139.7	143.5	146.7	14.9
1-Iodooctane	146.8	151.0	155.1	158.7	162.2	15.4
1-Iodononane	161.5	165.6	169.7	173.3	177.2	15.7
1-Iododecane	175.1	179.5	183.5	187.1	191.2	16.0
1-Iodoundecane	188.1	192.3	196.4	200.4	204.2	16.0
1-Iodododecane	200.5	204.6	208.9	212.7	216.2	15.7
1-Iodohexadecane	243.5	247.8	251.8	255.8	259.7	16.2
1-Iodoctadecane	261.8	266.5	270.6	274.6	278.2	16.4
Aldehyde						
Pentanal	83.8	85.7	88.0	90.3	92.1	8.4
Hexanal	90.1	92.9	95.5	98.1	100.6	10.5
Heptanal	100.1	103.4	106.8	109.6	112.6	12.5
Octanal	113.1	116.9	120.6	123.8	127.2	14.0
Nonanal	127.5	131.5	135.1	138.5	142.2	14.7
Decanal	142.1	146.1	150.1	153.6	157.2	15.0
Undecanal	156.1	160.4	164.3	167.8	171.7	15.5
Dodecanal	169.8	173.9	178.1	181.6	185.2	15.4

^a Blanks means either the compound was not identified or it eluted at 290 °C (isothermal condition 2T_e)

^b Temperature range is calculated by subtracting the temperature value corresponding to ramp 4 °C /min from the ramp 6 °C /min

In general, retention index variations increase with increased elution temperature. However, some compounds did not show this trend, as retention index also depends on the structure. Figure 3.5(A) shows the variations of retention index with increased ramp rate (increased elution temperature) for some randomly chosen compounds from MegaMix A and MegaMix B. Figure 3.5(B) shows the average variations of all compounds from MegaMix and MegaMix B at different elution temperature. Figure 3.5(B) shows that, generally, retention index variation increased with increased elution temperature i.e. increasing ramp rate. However, MegaMix B showed comparatively higher variations at ramp rate 3.5 °C/min in comparison with its adjacent ramp rate that could be due to the experimental variations. By design, the transfer line located between the secondary oven and the mass spectrometer was set at 280 °C during the entire experiment.

A.

B.

Figure 3.5. Effect of elution temperature on retention index calculation. (A) Some randomly selected compounds from MegaMix A and MegaMix B at different ramp rate. (B) Average $|I^T - {}^2I|$ (iu) for MegaMix A (red square) and MegaMix (violet triangle) B at different ramp rate.

The effect of this fixed temperature on the magnitude of 2I was different from compound to compound as each compound eluted at different temperature.

3.3.3. Robustness of Surface Fitting to Variations of Instrumental Settings

To investigate the reproducibility and the robustness of the developed method in analyzing GC×GC-MS data, we analyzed the sixteen in-house made mixtures, MegaMix A, and MegaMix B under different instrumental conditions by varying the temperature offset of the secondary oven, the ramp rate of temperature gradient, and modulation period.

Tables 3.4, 3.8 and 3.9 respectively lists the 2I of the compounds identified from the six mixtures that were not derivatized, from MegaMix A and from MegaMix B. An average *RSD* of the calculated 2I for the compound standards in the six in-house made mixtures, MegaMix A and MegaMix B was 0.28%, 0.46% and 0.34%, respectively. The small *RSD* of 2I demonstrates that the developed method is robust in calculating the second dimension retention index of compounds analyzed in different instrumental conditions.

Table 3.8. The second dimension retention indices of compounds identified from MegaMix A

Chromatographic conditions	2I (iu) ^a								I^T from GC-MS
Secondary oven offset (°C)	+5	+5	+5	+5	+5	+5	+5	+10	
Ramp rate (°C/minute)	3	3.5	4	4.5	5	5.5	6	5	
Modulation period (s)	5	5	5	5	5	5	5	4	
N-Nitrosodimethylamine	967	968	971	972	973	975	974	975	952
Phenol	1170	1175	1182	1186	1188	1191	1193	1195	1157
2-Chlorophenol	1180	1186	1192	1197	1200	1206	1205	1207	1162
Bis(2-chloroethyl) ether	1187	1192	1198	1200	1203	1206	1206	1208	1180
2,2'-Oxybis(2-chloropropane)	1215	1222	1226	1227	1228	1231	1230	1231	1210
Aniline	1215	1221	1229	1234	1238	1242	1245	1245	1203
Hexachloroethane	1238	1245	1252	1255	1259	1260	1263	1260	1215
2-Methylphenol	1255	1260	1267	1269	1272	1277	1277	1278	1242
Benzyl alcohol	1261	1267	1275	1279	1281	1286	1288	1289	1250
N-nitrosodipropylamine	1271	1275	1281	1281	1285	1288	1288	1290	1260
3-Methylphenol	1277	1281	1288	1291	1292	1297	1297	1300	1263
Isophorone	1337	1342	1348	1352	1354	1356	1359	1361	1324
2,4-Dimethylphenol	1359	1364	1370	1372	1373	1379	1380	1382	1345
2-Nitrophenol	1376	1384	1394	1398	1401	1406	1410	1409	1358
Bis(2-chloroethoxy)-Methane	1387	1393	1398	1400	1401	1406	1406	1408	1375
2,4-DichloroPhenol	1391	1397	1404	1407	1410	1417	1419	1420	1367
4-Chloroaniline	1509	1502	1511	1520	1524	1529	1532	1532	1477
1,3-Cyclopentadiene,									
1,2,3,4,5,5-hexachloro-	1526	1529	1532	1537	1541	1546	1547	1551	1492
4-Chloro-3-methylphenol	1542	1549	1555	1559	1563	1567	1570	1571	1521
2-Methylnaphthalene	1557	1567	1574	1580	1584	1590	1593	1594	1561
1-Methylnaphthalene	1588	1598	1608	1613	1620	1626	1627	1628	1581
2,4,6-Trichlorophenol	1613	1622	1629	1634	1636	1641	1647	1654	1648

2,4,5-Trichlorophenol		1628	1635	1640	1643	1651	1654	1655	
2-Chloronaphthalene	1669	1680	1691	1696	1702	1709	1711	1711	1640
2-Nitroaniline	2178 ^b	1857	1804	1812	1824	1834	1838	1835	1761
Dimethyl phthalate	1867	1805	1810	1820	1826	1834	1831	1834	1776
Acenaphthylene	1835	1798	1812	1824	1833	1841	1845	1843	1785
1,4-Dinitrobenzene	2327	1921	1851	1850	1862	1874	1877	1874	1806
Acenaphthene	1835	1831	1844	1855	1862	1871	1872	1873	
2,6-Dinitrotoluene	2087	1935	1852	1860	1870	1881	1884	1880	1823
1,3-Dinitrobenzene	2481	1946	1858	1863	1874	1884	1888	1883	1816
Dibenzofuran	1853	1861	1874	1883	1889	1899	1901	1901	1819
2,3,5,6-Tetrachlorophenol	1857	1870	1880	1887	1893	1900	1901	1905	1828
2,3,4,6-Tetrachlorophenol	1870	1883	1895	1902	1907	1915	1917	1925	1843
1,2-Dinitrobenzene	2510	2154	1939	1905	1915	1929	1936	1930	1858
2,4-dinitro-phenol		1907	1906	1918	1928	1936	1937	1936	1874
3-Nitroaniline	2589	2061	1931	1924	1936	1951	1957	1953	1910
4-Chlorophenyl phenyl ether	1913	1929	1937	1943	1948	1957	1958	1960	1890
2,4-Dinitrotoluene	2199	1999	1928	1940	1950	1961	1961	1960	1890
Diethyl Phthalate	1933	1935	1946	1954	1956	1962	1961	1965	1914
Fluorene	1948	1951	1962	1975	1984	1992	1994	1996	
Azobenzene	1968	1979	1991	2001	2006	2018	2015	2017	1941
4,6-Dinitro-2-methylphenol	2015	1999	2009	2020	2027	2036	2040	2039	1972
Diphenylamine	2048	2010	2020	2033	2041	2053	2053	2051	1979
Hexachlorobenzene	2032	2051	2063	2073	2077	2087	2090	2093	1998
4-Bromophenyl phenyl ether	2052	2064	2078	2086	2092	2102	2102	2104	2023
4-Nitroaniline		2590	2190	2118	2119	2141	2148	2138	2064
Pentachloro-phenol	2125	2140	2155	2166	2174	2184	2188	2188	
Anthracene	2359	2247	2244	2263	2277	2294	2299	2294	2174
Phenanthrene	2395	2251	2254	2274	2287	2301	2307	2301	2185
di-n-Butyl phthalate	2300	2315	2323	2327	2329	2335	2333	2340	2281
Carbazole	3228	2540	2373	2375	2391	2412	2420	2411	2300
Bis(2-ethylhexyl) adipate	2568	2573	2570	2572	2573	2585	2572	2592	2575

Fluoranthene	3090		2625	2636	2658	2680	2688	2678	
Pyrene	3973	2713	2755	2732	2752	2777	2791	2777	
Bis(2-ethylhexyl) phthalate	2859	2874	2871	2870	2872				2832
Benzyl butyl phthalate	3008	2878	2879	2898	2911	2929	2924	2924	2823

^a Blanks means either the compound was not correctly identified or it eluted at 290 °C (isothermal condition in 2T_e)

^b *Italic* values were considered as outliers and were not included in *RSD* calculation. Those compounds had a very large second dimension retention index at ramp rate 3°C/minute and 4 °C/minute compared to their retention index calculated in other ramp rates

Table 3.9. The second dimension retention indices of compounds identified from MegaMix B

Chromatographic conditions	2I (iu) ^a								I^T from GC-MS
Secondary oven offset (°C)	+5	+5	+5	+5	+5	+5	+5	+10	
Ramp rate (°C/minute)	3	3.5	4	4.5	5	5.5	6	5	
Modulation period (s)	5	5	5	5	5	5	5	4	
cis-1,3-Dichloro-1-Propene, (Z)	872	875	872	871	882				869
trans-1,3-Dichloro-Propene			913	912	915	911	912		910
Toluene	887	891	883	883	894				882
1,1,2-Trichloroethane		930	925	924	924	925	925		920
Ethyl methacrylate			888	889	897				887
1,3-Dichloropropane	942	949	943	943	946	943	943		940
Dibromochloro-Methane	973	977	975	975	979	977	977		975
Tetrachloroethene	911	915	910	909	913				909
1,2-Dibromoethane	985	990	988	990	992	990	992		987
Chlorobenzene	999	1007	1002	1002	1004	1005	1004		1002
1,1,1,2-Tetrachloroethane	997	1001	986	994	997	999	995		996

Ethylbenzene	991		991	992	995	990	990	991	
p-Xylene			999	997	999	995	1001	998	992
Styrene			1055	1055	1062	1058	1055	1060	1033
o-Xylene			1038	1037	1044	1040	1040	1036	
Bromoform	1101	1108	1110	1114	1120	1121	1123	1119	
cis-1,4-Dichloro-2-butene, , (z)-	1091	1097	1096	1098	1102	1103	1103	1102	
1,1,2,2-Tetrachloroethane	1097	1104	1103	1103	1108	1106	1108	1102	
1,2,3-Trichloropropane	1111	1118	1119	1120	1124	1123	1140	1126	
Isopropylbenzene	1055	1060	1055	1052	1059	1056	1059	1057	1025
trans-1,4-Dichloro-2-butene, (E)-					1137	1137	1127	1139	
Bromobenzene	1113	1121	1121	1124	1127	1128	1129	1126	1097
2-Chlorotoluene	1106	1121	1118	1122	1126	1125		1128	
n- Propylbenzene	1086	1093	1087	1091	1095	1092	1095	1097	1073
4-Chlorotoluene	1116	1124	1123	1124	1130	1127	1130	1130	
1,3,5-Trimethylbenzene	1102	1107	1102	1102	1110	1106	1103	1104	1090
Pentachloroethane	1145	1155	1152	1153	1159	1160	1159	1154	
tert-Butylbenzene	1125		1122	1126	1133	1129	1129	1133	1112
1,2,4-Trimethylbenzene	1136	1145	1141	1143	1148	1147		1149	
sec-Butylbenzene	1140	1147	1145	1144	1149	1149	1146	1148	
p-Cymene	1156	1162	1156	1159	1164	1161	1163	1164	1142
n-Butylbenzene	1192	1198	1194	1194	1200	1200	1200	1203	
1,2-Dibromo-3- Chloropropane	1325	1335	1338	1341	1346	1349	1351	1351	1305
Benzene, 1,2,3-trichloro-	1449	1463	1461	1466	1471	1476	1481	1479	1420
1,3-Dichlorobenzene*	1179	1184	1188	1192	1197	1198	1198	1201	1161
1,4-Dichlorobenzene*	1195	1200	1204	1207	1209	1214	1217	1217	1179
1,2-Dichlorobenzene*	1229	1234	1240	1243	1248	1251	1251	1254	1208
Nitrobenzene*	1341	1348	1358	1361	1366	1369	1371	1370	1325
Benzene, 1,2,4-trichloro-*	1392	1397	1401	1404	1409	1413	1416	1419	1364
Naphthalene*	1438	1448	1456	1460	1466	1472	1474	1473	1414

1,3-Butadiene, 1,1,2,3,4,4-hexachloro-*	1375	1379	1381	1383	1389	1393	1394	1397	1350
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^a Blank means the compound was not identified

3.3.4. Comparison with 2I Calculated by Pseudo-isothermal Condition

Chapter Two reported a surface fitting method for the calculation of 2I by adopting the conventional concept, i.e., approximating the second dimension separation in GC×GC-MS as in pseudo-isothermal condition, and demonstrated that the method can reduce the variation introduced by conventional isovolatility curve approach.⁸⁷ In that study, we analyzed the *n*-alkanes in isothermal mode at 46 different temperatures. After deducting the temperature-dependent second dimension hold-up time 2t_M from each retention time 2t_R , we obtained the adjusted ${}^2t'_R$ for each data point. We then constructed the 2I_s using the adjusted ${}^2t'_R$ and 2T_e of *n*-alkanes. In the current study, the *n*-alkanes were analyzed in temperature-programmed mode at 16 ramp rates. The 2t_R and 2T_e values of *n*-alkanes were directly used to construct the 2I_s , where the calculation adjusted retention time ${}^2t'_R$ and hold-up time 2t_M was eliminated.

Figure 3.6 shows the distribution of the deviation $|I^T - {}^2I|$ of the 322 compounds, where 2I_a was calculated using the method developed in this study and 2I_b was calculated using the method developed in our previous study.⁸⁷ The average deviation of the 322 compounds was 30.4 iu using the method developed in our previous study, where the second dimension separation was considered as in pseudo-isothermal condition. The average deviation of the 322 compound was reduced to 18.0 iu using the method developed in the current study.

A.

B.

Figure 3.6. Effect of hold-up time calculation on the deviation $|I^T - {}^2I|$. 2I_a is the second dimension retention index calculated by without involving hold-up time calculation. 2I_b is the second dimension retention index calculated by involving hold-up time calculation. (A) Distribution of $|I^T - {}^2I|$ by approximating the second dimension separation as in pseudo-isothermal mode vis. $|I^T - {}^2I|$ of considering the second dimension separation in its actuality, i.e., temperature-programmed mode. The red line shows the result of linear fitting while the blue and pink lines are fitting confidence with 95% confidence interval. (B) Box plot of $|I^T - {}^2I|$ calculated using the two methods.

In order to study the robustness of the developed method, the six mixtures that were not derivatized, the mixture of MegaMix A and MegaMix B, were respectively analyzed in different instrumental settings. We also calculated the 2I of those compounds using the method developed in our previous study. The average *RSD* of the 2I for the compounds in the six in-house made mixtures, MegaMix A and MegaMix B is 0.31%, 0.63% and 0.42%, respectively. However, the corresponding average *RSD* of the 2I calculated using the method developed in this study for the compounds in the six mixtures, MegaMix A and MegaMix B is 0.28%, 0.46% and 0.34%, respectively.

The higher deviation of the 2I from corresponding I^T , as well as the increased average *RSD* in the previous method were caused by the concept of approximating the second dimension separation as pseudo-isothermal, which therefore involved the calculation of adjusted retention time ${}^2t'_R$ and hold-up time 2t_M . While difference methods have been developed to estimate 2t_M , each of those methods introduces a certain degree of variation that was propagated to the calculation of ${}^2t'_R$ and 2I .

We also investigated the effect of the polynomial model selection for surface fitting model. In our previous study, i.e., the method including hold-up time calculation, the optimal fitting model was “poly25” but in our current study it is “poly35”. Therefore, we calculated the 2I of 80 compound standards, MSTFA and MTBSTFA derivatized compounds, MegaMix A and MegaMix B using both “poly25” and “poly35” surface fitting model, and found that the average 2I difference between these two method only 1.1 iu. Therefore, the larger variations was solely introduced during the hold-up time calculation. Furthermore, our previous method used 46 chromatographic analysis of *n*-alkanes at different temperatures in isothermal mode to calculate the 2t_M , while the current method used only 16 chromatographic runs. In summary, the method developed in this study is much simpler and outperforms our previous method as well as the conventional isovolatility curve approach for the calculation of the second dimension retention index.

3.4. Conclusions

This study was a continuation of our previous work on calculating the second dimension retention index in GC×GC-MS via surface fitting. The method developed in this study considered the second dimension separation as its actuality, i.e., in temperature-programmed mode, and directly calculated the second dimension retention index via surface fitting. This approach eliminated the concept of approximating the second dimension separation as in pseudo-isothermal condition. Therefore, the current method does not involve the calculation of temperature dependent second dimension hold-up time 2t_M , resulting in increased accuracy of the second dimension retention index calculation. Analysis of a wide range of chemicals ranging from very non-polar to polar compounds as well as biologically important compounds such as amino acids and organic

acids showed that the deviation of 2I values from their corresponding linear retention index I^T calculated from GC-MS data is within the normal deviation range, thus showing the effectiveness of the developed method. Compared with the existing approaches, the method developed is much more accurate in calculating the second dimension retention index in GC×GC-MS. Despite the unique advantage of the developed method over the existing method, the developed method has limited separation map coverage compared with the method developed in Chapter Two where isothermal modes of GC×GC-MS were used. In the current developed method, for the second dimension retention index surface construction, reference compounds were analyzed under temperature gradient mode. While very slow temperature gradient (0.85 ° C) was used, the separation map was still not sufficient to cover the separation region of some compounds. Therefore, few compounds from MegaMix A at ramp rate 3 ° C fell outside of the second dimension retention index surface and showed very high 2I deviations from their retention index measured at GC-MS. Future endeavor should focus on the development of method for increasing the second dimension retention map coverage under temperature-programmed mode of GC×GC-MS.

CHAPTER 4

UNIVERSAL REFERENCE SYSTEM FOR CALCULATING THE SECOND DIMENSION RETENTION INDEX IN COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY MASS SPECTROMETRY

4.1. Introduction

Comprehensive two-dimensional gas chromatography mass spectrometry (GC×GC-MS) is an excellent tool for chemical profiling of complex samples owing to its superior separation capacity, selectivity, sensitivity and dynamic range.^{59-61,63,82,96} While mass spectrum matching is widely used for compound identification in analyzing GC×GC-MS data^{83,84,97}, unambiguous identification of compounds is still a challenge due to the similar mass spectra of different compounds, e.g., homologous compounds or isomers.⁸⁵ Incorporating compound separation information with mass spectrum matching can significantly improve the accuracy of compound identification.⁹⁸

GC×GC-MS provides two pieces of chromatographic separation information including first dimension retention time (1t_R) and the second dimension retention time (2t_R). Retention time cannot be used directly as there is always a retention time variation between instrument to instrument, run-to run and among the different laboratory using

different kinds of instrument. In order to use compound separation information for compound identification and avoid the variations, the first dimension retention time (1t_R) and the second dimension retention time (2t_R) of a compound in GC×GC-MS must be normalized. Conventionally, analytes' retention times are converted into retention index by using a homologous reference series predominantly *n*-alkane homologous series. However, *n*-alkanes have poor separation in a polar column and result in a poor reproducibility in calculating the retention index. Therefore, some other reference series has been used as reference series. For instance, fatty acid methyl esters (FAME) homologous series have been employed to calculate the retention index using a retention index scale different from the *n*-alkanes scale. Specifically, the FAME based scale is based on the calculation of the equivalent chain length, carbon number and modified equivalent chain length.⁹⁹ Moreover, some other homologous series, i.e., 2-ketones^{100,101}, *n*-propyl ester¹⁰², alkanoate esters¹⁰³, alkanols and acetates¹⁰⁴ etc, have also been employed. Another type of retention index system was defined based on the series of polycyclic aromatic hydrocarbon (PAH) standard compounds including naphthalene, Phenanthrene, chrysene and pentacene to calculate the retention index of polyaromatic compounds.⁹⁵

In GC×GC-MS, the first dimension separation is usually operated under temperature-programmed mode and the 1t_R is converted into a linear retention index (I^T) using the Van Den Doll and Kratz equation.²⁸ The second dimension separation is often considered as being in pseudo-isothermal mode as the temperature change within a modulation cycle is negligible⁶⁵⁻⁷¹, and the 2t_R is converted into Kováts retention index 2I .²⁹ However, the calculation of 2I is not straightforward because the temperature of the second dimension column varies at each modulation period (P_M) during GC×GC-MS analysis.

Several methods have been developed to calculate the 2I from the 2t_R , such as isovolatility curve and retention index surface approaches.^{40,41,73-76,93,105} In the isovolatility curve approach, the hold-up time 2t_M at different temperatures of the second dimension column was first estimated for the calculation of adjusted retention time, ${}^2t'_R = {}^2t_R - {}^2t_M$. The ${}^2t'_R$ s of the same reference compound at different column temperatures were then used to construct the isovolatility curve of that reference compound. The isovolatility curves of all reference compounds formed the second dimension retention map (i.e., the two-dimensional separation space formed by 2t_R and 2T_e of all reference compounds), and the 2I value of a compound was finally calculated from the two adjacent isovolatility curves of two reference compounds. Our group proposed a second dimension retention index surface approach for calculation of 2I value of each compound using *n*-alkanes as reference compounds¹⁰⁵, where the adjusted second dimension retention time ${}^2t'_R$ and the second dimension column temperature 2T_e formed an X-Y plane and the 2I was the Z-axis. The second dimension retention index surface, 2I_S , was constructed using the 2I values of all reference compounds via a surface fitting model. The 2I of an analyte measured by GC×GC-MS was then calculated from 2I_S based on its ${}^2t'_R$ and 2T_e .

In order to use the two-dimensional retention index of GC×GC-MS to aid compound identification in analysis of complex samples, the second dimension retention map of reference compounds should encompass the values of 2t_R and 2T_e of all compounds in a sample. The separation of reference compounds by GC×GC is mainly determined by column chemistry and the chemical property of the reference compounds, i.e., interaction between the column stationary phase and the reference compounds. The most widely used

reference series for calculating the second dimension retention index is *n*-alkanes, which are non-polar compounds. *n*-Alkanes are the ideal reference compounds for non-polar and mid-polar compounds, but not for polar compounds, as the polar compounds elute much later than *n*-alkanes in a polar column. In that case, the second dimension retention map of *n*-alkanes is not large enough to cover the polar compounds and therefore, cannot be directly used to calculate the 2I of the polar analytes. A solution to that problem is to extrapolate the second dimension retention map of the reference compounds. However, extrapolation introduces a large degree of variation in calculation of 2I . Another approach is to use a polar reference series (e.g., alcohols) for the polar column and a non-polar reference series for the non-polar column.⁷⁵ However, this method raises a problem of retention index scaling. Alcohols generate 2I values in alcohol scale, but most of the existing retention index databases contain retention indices that use *n*-alkanes as references, i.e., in *n*-alkane retention index scale. For example, the National Institute of Standards and Technology MS database (NIST 2014) contains Kovats and LRI data for 385872 and 82337 compounds, respectively.

The objective of this work was to construct a large second dimension retention map for reference compounds in GC×GC-MS using C₄–C₂₅ fatty acid methyl esters (C₄–C₂₅ FAMES) as a reference series instead of conventional *n*-alkane based reference series that covers non-polar to polar compounds. The second objective of this work was to develop a method to calculate the 2I in second dimension separation space using C₄–C₂₅ FAMES while keeping the retention index scale in *n*-alkane scale. We developed a surface fitting method using C₄–C₂₅ FAMES as reference compounds and considered the second dimension separation in GC×GC-MS as linear temperature-programmed separation

(unpublished results. Performance of the developed method was then tested and validated by analyzing *n*-alkanes, 80 standard compounds and two commercially available mixtures (i.e., MegaMix A and MegaMix B) with their corresponding linear retention indices on the same column measured by GC-MS.

4.2. Experimental Section

4.2.1. Sample Preparation

A total of 80 compound standards and 4 commercial mixtures were purchased in this study. Six in-house made solutions were prepared by mixing the 80 compound standards in different combinations, including a solution containing odd number of C₅–C₂₃ FAMES, a solution containing C₄–C₂₄ (both even and odd number) carbon saturated FAMES, a solution containing C₅–C₂₂ saturated fatty acids, a 2-ketone mixture, a *n*-alcohol mixture, an 1-iodoalkane mixture, and an aldehyde mixture. All these six mixtures were prepared in dichloromethane with a concentration of 100 µg/mL per compound. MegaMix A and MegaMix B were prepared in dichloromethane at a concentration of 50 µg/mL per compound.

4.2.2. GC×GC-MS Experiments

The LECO (St. Joseph, MI) Pegasus® 4D GC×GC-MS was configured with a Gerstel MPS2 autosampler and an Agilent 6890 gas chromatograph featuring a LECO two-stage cryogenic modulator and a secondary oven. The first dimension column was a non-polar DB-5ms (phenyl arylene polymer virtually equivalent to a (5%-phenyl)-methylpolysiloxane) 60.0 m × 0.25 mm 1d_c × 0.25 µm 1d_f column, and the second dimension column was a medium polar BD-17ms ((50%-phenyl)-methylpolysiloxane) 1.0

m $\times$ 0.1 mm $^2d_c \times$ 0.1 μm^2d_f column. Both columns were obtained from Agilent Technologies (Santa Clara, CA), and were connected by means of a press-fit connector before the thermal modulator. The flow rate of ultra-high purity helium carrier gas (99.999%) was set to 2.0 mL/min at a corrected constant flow via pressure ramps with an initial head pressure of 31.9 psi. The MS parameters were as follows: MS range 45–800 m/z; data acquisition rate 200 spectra/s; temperature of ion source chamber 230 °C; temperature of MS transfer line 280 °C; detector voltage 1641 V; electron energy 70 eV. After a solvent delay of 245 s, the acceleration voltage was turned on.

In order to construct the second dimension retention index surface, the reference mixture containing C₄–C₂₅ FAMES were analyzed by GC $\times$ GC-MS seventeen times in temperature-programmed mode using different ramp rates, including 0.85, 1.00, 1.13, 1.25, 1.50, 1.65, 1.75, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, and 7.00 °C/min. A 1 μL sample was injected into the inlet chamber of the GC $\times$ GC-MS system and the split ratio was set at 10:1. The inlet temperature was 280 °C. The thermal modulator and the secondary oven were set to +20 °C and +5 °C relative to the primary oven, respectively. Initial temperature of the first dimension column 1T_e was set at 60 °C which was kept for 1 minute and then ramped to 285 °C using different ramp rates. 1T_e was kept at 285 °C for 13 minutes to let all compounds elute off the column. The second dimension column was also set under same conditions but at +5 °C with respect to the first dimension column. P_M was set as 6 s.

In order to validate the developed method, C₇–C₃₀ *n*-alkanes were analyzed by GC $\times$ GC-MS seventeen times in identical chromatographic conditions as used for the reference compounds, C₄–C₂₅ FAMES. To test the reproducibility of the developed method,

the six in-house made mixtures were each analyzed five times in temperature-programmed mode using different ramp rates, i.e. 4.00, 4.50, 5.00, 5.50 and 6.00 °C/min. To study the robustness of the surface fitting method, the MegaMix A and MegaMix B mixtures were respectively analyzed under different instrumental conditions by varying the temperature offset of the secondary oven from +5 to +10 °C, the ramp rate of temperature gradient from 4.0 to 6.0 °C/min, and the modulation period from 4 to 5 s. The other instrumental parameters were kept the same as described above.

4.2.3. GC-MS Experiments

All six in-house made mixtures, MegaMix A and MegaMix B were respectively analyzed on a Thermo ITQ 1100 Ion Trap MS equipped with a Trace 1310 GC and an AI 1310 auto sampler. The column was a DB-17ms capillary column (45.0 m $\times$ 0.1 mm $^2d_c \times$ 0.1 μm 2d_f , (50%-phenyl)-methylpolysiloxane) purchased from Agilent Technologies (Agilent Technologies J&W, Santa Clara, CA, USA). The helium carrier gas (99.999% purity) flow rate was set to 2.0 mL/min, and the inlet temperature was 280 °C. The column temperature was programmed with an initial temperature of 60 °C for 1.0 minute, ramped at 5 °C/min to 290°C, and finally maintained at 290 °C for 13 minute. The mass range was set as 29 – 800 m/z . The ion source chamber was 230 °C with a transfer line temperature of 280 °C.

A mixture of C₇–C₃₀ *n*-alkanes at a concentration of 75 $\mu\text{g/mL}$ was also analyzed by GC-MS and the retention times of these *n*-alkanes were used to calculate the retention indices of compounds in the six in-house made mixtures, MegaMix A and MegaMix B.

4.2.4. Instrumental Data Preprocessing

LECO ChromaTOF software (version 4.51) equipped with NIST 2014 was used for instrument control as well as for spectrum deconvolution and compound identification. Manufacturer recommended parameters for ChromaTOF were used to reduce the raw instrument data into a list of compounds. These parameters were as follows: baseline offset 0.5; auto smoothing; peak width in first dimension 6 s; peak width in the second dimension 0.1 s; signal-to-noise ratio 100; match required to combine peaks 600; R.T. shift 0.08 s; minimum forward similarity match 600. The true peak spectrum was also exported as part of the information for each chromatographic peak as absolute intensity values.

GC-MS data was collected by Thermo Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA) and converted into mzML file format for peak picking and compound identification, using an in-house developed software package. The retention time of each compound generated by in-house software was then converted into a linear retention index in Microsoft Excel (Microsoft, Redmond, WA, USA) using *n*-alkanes homologous series (C₇-C₃₀) as reference compounds following the Van Den Doll and Kratz equation.²⁸

4.2.5. Construction of the Second Dimension Retention Index Surface

We first analyzed the mixture of C₄-C₂₅ FAMES by GC-MS to get their linear retention index I_u^T for each compound using *n*-alkanes as references compounds by using Equation 1 described in Chapter 1.

To construct the 2I_S , C₄-C₂₅ FAMES were then analyzed by GC×GC-MS in temperature-programmed mode at seventeen ramp rates. After chromatographic peak picking and compound identification, 2t_R and $^2T'_e$ were extracted for each FAME compound in each GC×GC-MS analysis. The I_u^T values calculated from GC-MS data for

C₄–C₂₅ FAMES were then used as the second dimension retention indices of C₄–C₂₅ FAMES. Therefore, a compound u of the C₄–C₂₅ FAMES was represented by $\{ {}^2t_{R,u}, {}^2T_{e,u}, {}^2I_u \}$, where ${}^2I_u = I_u^T$. The 2I_S was then constructed using the data of all C₄–C₂₅ FAMES, where 2t_R and 2T_e formed the X - Y plane and the 2I was the Z -axis. A polynomial surface model in MATLAB (Mathworks, Natick, MA) named ‘polyij’ was used to construct the 2I_S , where i was the degree of polynomial in 2t_R , and j was the degree of polynomial in 2T_e . The maximum for both i and j was 5 in the MATLAB platform. The degree of polynomial was the maximum of i and j .

4.2.6. Selection of Optimal Surface Fitting Model

Multidimensional polynomial model ‘polyij’ in MATLAB was employed to fit the 2I values of all reference compounds C₄–C₂₅ FAMES into a surface, 2I_S . To prevent overfitting, cross-validation (CV) was employed to select the optimal polynomial surface fitting model. We used k -fold cross validation method that partitions data into k randomly chosen subsets (or folds) of roughly equal size. One subset was used to validate the model trained, using the remaining subsets. This process was repeated k times such that each subset was used exactly once for validation. The advantage of this method is that all observations are used for both training and validation, and each observation is used for validation exactly once. The k -fold CV was then repeated N times. In this study, k and N were set to 10 and 100, respectively.

4.2.7. Criteria for Evaluating the Surface Fitting

The criteria for evaluating the quality of surface fitting model were identical to those used in our previous work, including distribution of residuals, R-square (R^2), degrees of

freedom adjusted R-square (R_{adj}^2), and root mean squared error ($RMSE$)¹⁰⁵. Briefly, the distribution of residuals can assess the random errors of surface fitting. R^2 is the measurement of goodness of fit, which explains the variation of the data. R_{adj}^2 adjusts R^2 based on the residual degrees of freedom, with a value closer to 1 indicating a better fit. $RMSE$ is an estimate of the standard deviation of the random component in the data.

4.3. Results and Discussion

A second dimension retention index surface, 2I_S , was created by using the second dimension retention times of C₄–C₂₅ FAMES acquired at different ramp rates under temperature-programmed mode. All compounds in C₄–C₂₅ FAMES and compound standards in the six in-house made mixtures were identified from the GC×GC-MS and GC-MS data in each chromatographic run, respectively. A total of 102 compounds were identified from the mixtures of MegaMix A and MegaMix B by GC×GC-MS.

4.3.1. Construction of the Second Dimension Linear Retention Index Surface

C₄–C₂₅ FAMES were analyzed in temperature-programmed mode at seventeen different ramp rates (°C/min). By design, the first dimension column was kept at 285 °C in every chromatographic run to elute all compounds. Therefore, both the first and the second dimension columns were operated in isothermal mode during the last 13 minutes. Figure 4.1 shows the elution trends of C₄–C₂₅ FAMES at different ramp rates. A total of 350 data points were acquired under temperature-programmed mode and these data points were used to construct the second dimension retention index surface, 2I_S .

Figure 4.1. The second dimension retention map of C4-C25 FAMES analyzed in temperature-programmed mode using different ramp rates. Inflection points in the curve indicate the second dimension column temperature was 285 °C, from which the corresponding C4-C25 FAMES were analyzed in isothermal mode for 13 min.

Multidimensional polynomial model ‘polyij’ was employed to construct the 2I_S using those 350 data points. To optimize the i and j values in polyij, 10-fold CV with 100 replications was employed to evaluate the performance of each polynomial model. From those repeated 100 times of the 10-fold CV, a total of 1000 *RMSE* values were obtained and their average was denoted as *validation RMSE*, E . Table 4.1 lists the E values for all possible combinations of i and j in ‘polyij’ ($i = 1, 2, \dots, 5; j = 1, 2, \dots, 5$). The model ‘poly35’ ($i = 3, j = 5$) has the minimum E value and was selected as the optimal polynomial model to construct 2I_S .

Table 4.1. Selection of polynomial surface model ‘poly ij ’ to construct the second dimension retention index surface using GC $\times$ GC–MS data of C₄–C₂₅ FAMES

Poly‘ ij ’		R^2	R^2_{adj}	$RMSE$	E
i	j				
1	1	0.9760	0.9758	95.32	95.69
2	1	0.9922	0.9921	54.57	54.87
3	1	0.9930	0.9928	51.95	52.49
4	1	0.9933	0.9931	50.95	52.30
5	1	0.9937	0.9935	49.25	50.55
1	2	0.9891	0.9890	64.33	64.91
2	2	0.9988	0.9987	21.64	22.72
3	2	0.9989	0.9988	20.78	21.26
4	2	0.9996	0.9996	12.26	20.33
5	2	0.9996	0.9996	12.65	20.64
1	3	0.9904	0.9902	60.67	61.74
2	3	0.9998	0.9998	8.13	9.20
3	3	0.9999	0.9999	4.77	9.29
4	3	0.9999	0.9999	5.83	9.70
5	3	0.9999	0.9999	6.09	9.07
1	4	0.9910	0.9907	59.01	60.04
2	4	0.9999	0.9999	4.93	5.08
3	4	1.0000	1.0000	2.89	4.34
4	4	1.0000	1.0000	2.89	4.27
5	4	1.0000	1.0000	4.05	4.10
1	5	0.9910	0.9907	59.16	60.77
2	5	0.9999	0.9999	4.61	4.72
3	5	1.0000	1.0000	3.83	3.91
4	5	1.0000	1.0000	3.81	3.95
5	5	1.0000	1.0000	3.84	3.96

After obtaining the optimal polynomial model ‘poly35’, all 350 data points of C₄–C₂₅ FAMES were used to construct 2I_5 . The constructed 2I_5 had excellent fitting accuracy with $R^2 = 1.0$, $R^2_{adj} = 1.0$ and $RMSE = 3.8$ iu. Among the 100 times of repeated 10-fold CV, Table 4.2-4.6 lists the results of randomly selected 5 trails of the 10-fold CV.

Table 4.2. Results of one randomly selected 10-fold cross validation of surface fitting
‘poly35’ (case one)

Fold count	Goodness of fit		Goodness of validation	
	R^2	R^2_{adj}	$RMSE$	$RMSE$
1	1.0000	1.0000	3.7	4.7
2	1.0000	1.0000	3.9	3.5
3	1.0000	1.0000	3.8	4.1
4	1.0000	1.0000	3.9	3.6
5	1.0000	1.0000	3.8	3.9
6	1.0000	1.0000	3.9	2.9
7	1.0000	1.0000	3.9	3.6
8	1.0000	1.0000	3.8	4.5
9	1.0000	1.0000	3.8	4.3
10	1.0000	1.0000	3.9	3.9
Average	1.0000	1.0000	3.8	3.9

Table 4.3. Results of one randomly selected 10-fold cross validation of surface fitting
‘poly35’ (case two)

Fold count	Goodness of fit		Goodness of validation	
	R^2	R^2_{adj}	$RMSE$	$RMSE$
1	1.0000	1.0000	3.8	3.9
2	1.0000	1.0000	3.8	4.0
3	1.0000	1.0000	3.8	3.8
4	1.0000	1.0000	3.9	3.4
5	1.0000	1.0000	3.9	3.4
6	1.0000	1.0000	3.9	3.9
7	1.0000	1.0000	3.7	5.2
8	1.0000	1.0000	3.9	3.3
9	1.0000	1.0000	3.9	3.0
10	1.0000	1.0000	3.8	4.4
Average	1.0000	1.0000	3.8	3.8

Table 4.4. Results of one randomly selected 10-fold cross validation of surface fitting ‘poly35’ (case three)

Fold count	Goodness of fit		Goodness of validation	
	R^2	R^2_{adj}	$RMSE$	$RMSE$
1	1.0000	1.0000	3.8	4.4
2	1.0000	1.0000	3.9	3.2
3	1.0000	1.0000	3.7	4.5
4	1.0000	1.0000	3.8	3.7
5	1.0000	1.0000	3.7	4.6
6	1.0000	1.0000	3.9	3.5
7	1.0000	1.0000	3.9	3.0
8	1.0000	1.0000	3.9	3.9
9	1.0000	1.0000	3.8	4.1
10	1.0000	1.0000	3.9	3.9
Average	1.0000	1.0000	3.8	3.9

Table 4.5. Results of one randomly selected 10-fold cross validation of surface fitting ‘poly35’ (case four)

Fold count	Goodness of fit		Goodness of validation	
	R^2	R^2_{adj}	$RMSE$	$RMSE$
1	1.0000	1.0000	3.8	4.4
2	1.0000	1.0000	3.9	3.8
3	1.0000	1.0000	3.8	3.9
4	1.0000	1.0000	3.7	4.5
5	1.0000	1.0000	3.9	3.5
6	1.0000	1.0000	3.9	3.2
7	1.0000	1.0000	3.7	5.3
8	1.0000	1.0000	3.9	3.1
9	1.0000	1.0000	3.9	3.0
10	1.0000	1.0000	3.8	3.8
Average	1.0000	1.0000	3.8	3.8

Table 4.6. Results of one randomly selected 10-fold cross validation of surface fitting ‘poly35’ (case five)

Fold count	Goodness of fit			Goodness of validation
	R^2	R^2_{adj}	$RMSE$	$RMSE$
1	1.0000	1.0000	3.9	3.5
2	1.0000	1.0000	3.8	4.3
3	1.0000	1.0000	3.9	3.6
4	1.0000	1.0000	3.8	4.2
5	1.0000	1.0000	3.9	3.5
6	1.0000	1.0000	3.7	4.7
7	1.0000	1.0000	3.9	3.1
8	1.0000	1.0000	3.8	3.7
9	1.0000	1.0000	3.8	4.1
10	1.0000	1.0000	3.8	4.2
Average	1.0000	1.0000	3.8	3.9

The very close results of these trials demonstrate that the polynomial surface model ‘poly35’ was robust in terms of prediction. The *STD* of the 1000 (i.e., 100×10) validations *RMSE* was 0.49 iu, also demonstrating that the ‘poly35’ model is robust. Figure 4.2(A) depicts the 2I_5 constructed from the temperature-programmed data of the C₄–C₂₅ FAMES using polynomial surface fitting model ‘poly35’. Figure 4.2(B) is the residual plot of the surface fitting. The residuals are symmetrically distributed Figure 4.2(C) around the fitted plane suggesting that its variance is constant and there was no bias introduced by the polynomial surface model ‘poly35’. The residuals were in a range of 0-14 iu with an average value of 2.8 iu.

A.

B.

C.

Figure 4.2. Second dimension retention index surface constructed using the $\text{GC} \times \text{GC}$ –MS data of C_4 – C_{25} FAMES. The retention index surface is a function of the elution temperature of each C_4 – C_{25} FAMES in the second dimension column 2T_e , and the corresponding unadjusted retention time 2t_R . (A) Is the second dimension retention index surface, (B) is the residual plot and (C) is the distribution of the residuals.

Figure 4.3 shows the second dimension retention maps constructed from C_4 – C_{25} FAMES and n -alkanes data, respectively. C_4 – C_{25} FAMES data demonstrated greatly increased coverage of the second dimension separation space compared to the separation space covered by the n -alkanes data.

Figure 4.3. Second dimension retention map constructed from C₄– C₂₅ FAMES and *n*-alkanes data. Red triangles are the data points of *n*-alkanes. Green circles are the data points of C₄– C₂₅ FAMES. Blue rectangles are the data points of compounds in MegaMix A.

All compounds in MegaMix A, which were eluted beyond the retention map of *n*-alkanes, but were within the retention map of C₄–C₂₅ FAMES. Encompassing the 2I of all compounds of MegaMix A in the retention map ensures that the 2I of those compounds can be directly calculated from the 2I_S without extrapolation.

4.3.2. The Second Dimension Retention Indices of *n*-Alkanes

n-Alkanes are the most widely used reference series in calculating retention index. In this study, we used C₄–C₂₅ FAMES as a reference series to construct the 2I_S , where the Z-axis was the I^T values of C₄–C₂₅ FAMES that were calculated from GC-MS data using *n*-

alkane as references. Therefore, the 2I_S of C₄–C₂₅ FAMES are in *n*-alkane scale. We analyzed *n*-alkanes by GC×GC-MS in seventeen different ramp rates ranged from a very slow ramp rate of 0.85 °C/min to a fast ramp rate of 7.0 °C /min. A total of 364 data points were acquired for *n*-alkanes. We then calculated 2I values of these 364 data points from the 2I_S constructed using the I^T values of C₄–C₂₅ FAMES. Figure 4.4 shows that the *STD* of the calculated 2I of the *n*-alkanes among the seventeen chromatographic runs ranged from 3.6 iu to 14.0 iu, demonstrating that the developed method had an excellent reproducibility in calculating the 2I of *n*-alkanes.

Figure 4.4. Standard deviation of calculated 2I of each *n*-alkane compound among the seventeen chromatographic runs with different ramp rates.

The calculated 2I values of n -alkanes also showed an excellent agreement with their hypothetical retention index, i.e., $100 \times n$. Figure 4.5 depicts the distribution of the variation of the calculated 2I of the n -alkanes from their hypothetical values. The variation ranged from 0.0 iu to 50 iu with an average variation of 11.8 iu. Among the 364 data points of n -alkanes, 94% of data points were within 20 iu variation. The deviation of C₇-alkane was 50 iu, and that was the largest value.

Figure 4.5. Retention index variation of n -alkanes between the calculated 2I from their hypothetical value $100 \times n$. n is the number of carbon atoms in an n -alkane.

Figure 4.6 shows that all *n*-alkanes, except C₇-alkane, had near exponential decrease of 2t_R with the increase of 2T_e . This exceptional relation between temperature and retention time might contribute to the high variation of C₇-alkane. The observed variation could also be related to temperature dependency of the calculated retention index value 78,79.

Figure 4.6. The second dimension retention map of *n*-alkanes. The inset shows the relation between 2T_e and 2t_R for C₇-alkane.

A previous study of the NIST retention index database revealed that the retention index deviation is column dependent, and the variation of linear retention index increases with the increase of column polarity.¹⁰⁶ The NIST retention index database registered three types of columns, i.e., standard non-polar, semi non-polar and standard polar. The polarity of the DB-17ms column used in this study is between the standard polar column and the standard non-polar column.

The 2I variations of the *n*-alkanes, i.e., 11.8 iu, is much smaller than the variation reported in the literature concerning the NIST retention index database, demonstrating that the method developed in this work introduces much smaller variation in calculating the linear retention index for compounds separated on the second dimension column of GC×GC-MS. Therefore, the 2I values calculated from the developed method can be used to aid compound identification in analysis of GC×GC-MS data.

4.3.3. Calculating the 2I of Compound Standards

The six in-house made mixtures, MegaMix A and MegaMix B were analyzed in temperature-programmed mode by GC×GC-MS. Table 4.7 lists the calculated 2I values of the 80 compound standards using their second dimension retention times and the 2I_S constructed in Section 3.1.

Tables 4.8 and 4.9 list the calculated 2I values of compounds in MegaMix A and MegaMix B, respectively. In order to validate the 2I values calculated by the method developed in this study, we analyzed the 80 compound standards, MegaMix A and MegaMix B by GC-MS in temperature-programmed mod

Table 4.7. Second dimension retention indices 2I of the 80 compound standards

Compounds name	$I^2(\text{iu})^a$					STD (iu)	RSD (%) ^b	1I from GC-MS ^c	$\Delta \text{ } ^2T_e^d$
Ramp rate (°C/min)	4	4.5	5	5.5	6				
FAME									
Methyl butyrate	818	818	819	820	826	3	0.33		8.9
Methyl valerate	920	923	928	923	924	3	0.27		10.5
Methyl caproate	1029	1028	1029	1030	1030	1	0.06	1028	12.7
Methyl enanthate	1126	1127	1127	1127	1125	1	0.06	1129	13.9
Methyl caprylate	1228	1226	1228	1227	1225	1	0.09	1229	14.9
Methyl pelargonate	1324	1322	1327	1325	1323	2	0.14	1329	14.7
Methyl caprate	1425	1425	1428	1427	1423	2	0.11	1429	15
Methyl undecanoate	1522	1521	1530	1528	1521	4	0.24	1531	15.2
Methyl laurate	1623	1623	1630	1624	1624	3	0.17	1630	15.4
Methyl tridecanoate	1722	1723	1733	1725	1717	5	0.29	1733	15.4
Methyl myristate	1823	1822	1835	1827	1824	5	0.26	1832	15.5
Methyl pentadecanoate	1922	1924	1933	1929	1921	5	0.24	1935	15.4
Methyl palmitate	2029	2026	2037	2031	2018	6	0.31	2034	15.9
Methyl margarate	2125	2120	2138	2126	2117	7	0.33	2138	15.9
Methyl stearate	2227	2228	2244	2230	2223	7	0.32	2237	15.5
Methyl nonadecanoate	2323	2323	2335	2328	2318	6	0.24	2340	15.9
Methyl arachidate	2427	2427	2439	2432	2419	7	0.27	2440	15.7
Methyl heneicosanoate	2523	2525	2538	2533	2519	7	0.27	2545	15.9
Methyl behenate	2629	2629	2641			5	0.21	2644	
Methyl tricosanoate	2726					0	0		
Fatty acid									
Butyric acid	890	899	897	888	892	4	0.47		10
Valeric acid	990	1003	996	991	994	4	0.45		12.2
Caproic acid	1092	1105	1099	1090	1092	5	0.49	1113	13.7

Enanthic acid	1188	1197	1200	1190	1190	5	0.4	1210	14.5
Caprylic acid	1290	1301	1285	1294	1290	5	0.4	1304	14.9
Pelargonic acid	1393	1406	1404	1395	1397	5	0.36	1407	14.7
Capric acid	1491	1509	1508	1495	1498	7	0.48	1501	14.5
Undecylic acid	1591	1611	1608	1602	1606	7	0.42	1598	15.2
Lauric acid	1693	1718	1709	1699	1708	9	0.52	1697	14.9
Tridecylic acid	1798	1814	1812	1804	1807	6	0.31	1798	15
Myristic acid	1906	1929	1913	1916	1915	7	0.39	1901	15.9
Pentadecylic acid	2000	2018	2007	2005	2010	6	0.29	1999	15.7
Palmitic acid	2104	2117	2113	2113	2112	4	0.21	2101	15.7
Margaric acid	2204	2219	2215	2218	2215	5	0.24	2205	15.5
Stearic acid	2309	2325	2313	2313	2313	5	0.23	2307	16
Nonadecylic acid	2412	2419	2418	2412	2413	3	0.13	2409	15.5
Arachidic acid	2512	2519	2516	2515	2515	2	0.09	2513	15.9
Heneicosylic acid	2614	2627	2618	2621		5	0.18	2618	
Behenic acid	2724	2724				0	0		
Ketone									
2-Pentanone	805	808	802	815	800	5	0.66		8.5
2-Hexanone	909	899	907	914	907	5	0.55		10
2-Heptanone	1013	1012	1009	1015	1012	2	0.19	1008	12.2
2-Octanone	1116	1114	1111	1115	1110	2	0.2	1109	13.5
2-Nonanone	1216	1218	1209	1215	1212	3	0.25	1208	14.2
2-Decanone	1314	1316	1309	1316	1312	3	0.22	1308	14.9
2-Undecanone	1414	1418	1410	1419	1411	4	0.25	1410	15
2-Dodecanone	1517	1518	1511	1527	1514	6	0.36	1512	15.4
2-Tridecanone	1619	1617	1610	1620	1613	4	0.22	1612	15.7
2-Tetradecanone	1718	1718	1711	1724	1714	5	0.27	1714	15.4
2-Pentadecanone	1823	1824	1813	1822	1814	5	0.27	1816	16
2-Hexadecanone	1927	1927	1918	1934	1917	6	0.33	1918	16
2-Heptadecanone	2015	2015	2014	2020	2015	2	0.1	2017	15.9
2-Octadecanone	2126	2135	2120	2137	2120	7	0.35	2123	15.9

2-Nonadecanoe	2233	2234	2222	2240	2227	6	0.29	2225	16
Alcohol									
1-Pentanol	863	868	866	870	871	3	0.3		9.7
1-Hexanol	966	974	970	969	971	2	0.25		11.7
1-Heptanol	1068	1070	1068	1071	1068	1	0.11	1069	13.4
1-Octanol	1172	1175	1171	1172	1170	1	0.12	1174	14.2
1-Nonanol	1273	1276	1262	1268	1266	5	0.39	1275	20
1-Undecanol	1471	1475	1463	1471	1469	4	0.27	1480	17.7
1-Dodecanol	1567	1576	1564	1570	1570	4	0.26	1582	14.5
Iodobutane									
1-Iodobutane	939	944	937	943	944	3	0.3		11
1-Iodopentane	1052	1053	1054	1055	1058	2	0.18	1065	13
1-Iodoheptane	1159	1161	1161	1162	1162	1	0.1	1168	14
1-Iodoheptane	1265	1269	1268	1269	1269	1	0.11	1273	14.9
1-Iodooctane	1373	1374	1375	1380	1378	3	0.2	1380	15.4
1-Iodononane	1480	1483	1481	1485	1491	4	0.27	1488	15.7
1-Iododecane	1594	1597	1588	1595	1591	3	0.19	1596	16
1-Iodoundecane	1695	1698	1696	1702	1701	3	0.16	1702	16
1-Iodododecane	1804	1807	1803	1810	1811	3	0.17	1810	15.7
1-Iodohexadecane	2234	2235	2233	2243	2236	3	0.15	2237	16.2
1-Iodooctadecane	2445	2451	2446	2457	2450	4	0.17	2449	16.4
Aldehyde									
Pentanal	808	807	807	814	812	3	0.36		8.4
Hexanal	909	909	911	913	912	2	0.17		10.5
Heptanal	1018	1016	1015	1019	1017	1	0.14	1016	12.5
Octanal	1121	1120	1117	1121	1119	1	0.13	1119	14
Nonanal	1221	1219	1219	1221	1220	1	0.07	1220	14.7

Table 4.8. The second dimension linear retention indices 2I of compounds identified from MegaMix A

Chromatographic conditions	2I (iu) ^a								<i>RSD</i> (%) ^b	$\Delta ^2T_e$ (°C) ^c
Secondary oven offset (°C)	+5	+5	+5	+5	+5	+5	+5	+10		
Ramp rate (°C/minute)	4	4.5	5	5	5.5	5.5	6	5		
Modulation period (s)	5	5	5	4	5	4	5	4		
N-Nitrosodimethylamine	959	959	959	967	961	961	959	960	0.26	9.5
Aniline	1220	1225	1229	1235	1232	1232	1235	1235	0.41	13.9
Phenol	1173	1176	1177	1186	1180	1179	1180	1182	0.32	13.4
Bis(2-chloroethyl) ether	1189	1191	1193	1200	1194	1194	1194	1196	0.26	13.4
2-Chlorophenol	1183	1187	1189	1198	1194	1191	1192	1195	0.38	14
Benzyl alcohol	1264	1269	1271	1280	1277	1275	1277	1279	0.39	14.4
2,2'-Oxybis(2-chloropropane)	1215	1216	1216	1225	1218	1215	1217	1218	0.24	14.5
2-Methylphenol	1257	1260	1262	1270	1266	1263	1265	1268	0.32	14.5
N-nitrosodipropylamine	1271	1272	1275	1284	1277	1276	1276	1279	0.3	14.7
3-Methylphenol	1278	1282	1282	1292	1286	1285	1286	1289	0.33	14.2
Hexachloroethane	1242	1244	1247	1252	1247	1247	1251	1248	0.25	15
Isophorone	1338	1342	1344	1353	1347	1347	1348	1351	0.33	14.7
2-Nitrophenol	1382	1386	1391	1399	1397	1397	1401	1400	0.46	15
2,4-Dimethylphenol	1360	1362	1364	1375	1369	1367	1369	1372	0.36	15
Bis(2-chloroethoxy)-Methane	1387	1390	1392	1401	1397	1395	1396	1399	0.32	14.7
2,4-DichloroPhenol	1394	1398	1401	1412	1408	1408	1409	1411	0.44	14.2
4-Chloroaniline	1498	1506	1512	1520	1519	1519	1523	1522	0.54	15.2
4-Chloro-3-methylphenol	1543	1549	1554	1563	1559	1558	1562	1563	0.44	15.7
2-Methylnaphthalene	1561	1569	1575	1585	1582	1581	1585	1586	0.53	15.9
1-Methylnaphthalene	1594	1602	1610	1617	1618	1616	1620	1620	0.55	16.4

1,3-Cyclopentadiene,										
1,2,3,4,5,5-hexachloro-	1523	1529	1533	1544	1537	1536	1537	1542	0.41	16.2
2,4,6-Trichlorophenol	1616	1624	1628	1640	1634	1635	1640	1647	0.56	16
2,4,5-Trichlorophenol	1623	1630	1635	1647	1644	1641	1647	1647	0.51	16.2
2-Chloronaphthalene	1676	1684	1692	1701	1701	1701	1703	1704	0.57	16.4
2-Nitroaniline	1793	1801	1809	1815	1819	1818	1824	1820	0.55	16
1,4-Dinitrobenzene	1836	1840	1848	1855	1858	1856	1862	1858	0.48	15.7
1,3-Dinitrobenzene	1846	1852	1859	1866	1868	1868	1874	1868	0.48	16
Dimethyl phthalate	1798	1805	1812	1819	1822	1819	1822	1822	0.47	15.9
2,6-Dinitrotoluene	1842	1848	1854	1862	1866	1864	1870	1866	0.5	15.9
1,2-Dinitrobenzene	1890	1896	1903	1911	1914	1914	1920	1914	0.5	16.4
Acenaphthylene	1798	1809	1819	1827	1829	1829	1836	1832	0.66	16.5
3-Nitroaniline	1908	1915	1922	1930	1935	1934	1941	1936	0.56	16.5
Acenaphthene	1830	1839	1849	1858	1861	1860	1863	1863	0.63	16.7
2,4-dinitro-phenol	1895	1902	1912	1920	1923	1922	1926	1923	0.54	15.9
Dibenzofuran	1858	1868	1877	1887	1889	1889	1893	1892	0.63	16.4
2,4-Dinitrotoluene	1918	1925	1934	1944	1946	1943	1949	1946	0.54	16.2
2,3,5,6-Tetrachlorophenol	1864	1874	1883	1893	1892	1893	1894	1897	0.58	16.2
2,3,4,6-Tetrachlorophenol	1879	1889	1896	1908	1907	1907	1910	1916	0.61	16.2
Diethyl Phthalate	1930	1938	1944	1955	1952	1953	1953	1955	0.46	15.9
Fluorene	1947	1959	1970	1978	1981	1982	1985	1985	0.66	16.4
4-Chlorophenyl phenyl										
ether	1921	1929	1938	1948	1949	1949	1951	1952	0.55	16.2
4-Nitroaniline	2093	2101	2109	2117	2124	2124	2129	2122	0.56	16.2
4,6-Dinitro-2-										
methylphenol	1997	2003	2011	2022	2023	2024	2029	2026	0.55	16.5
Diphenylamine	2007	2016	2025	2034	2039	2036	2042	2038	0.58	16.2
Azobenzene	1975	1986	1994	2004	2007	2005	2007	2007	0.58	16.5
4-Bromophenyl phenyl										
ether	2061	2069	2079	2091	2091	2091	2094	2093	0.56	16.9
Hexachlorobenzene	2046	2058	2066	2080	2078	2080	2082	2084	0.62	16.7

Pentachloro-phenol	2137	2148	2159	2170	2173	2172	2179	2176	0.65	16.9
Anthracene	2233	2246	2258	2268	2276	2274	2284	2276	0.72	17
Phenanthrene	2242	2256	2267	2277	2283	2283	2292	2284	0.7	17.2
Carbazole	2350	2364	2373	2383	2392	2390	2399	2390	0.65	16.7
di-n-Butyl phthalate	2304	2312	2317	2333	2326	2329	2325	2331	0.4	16
Fluoranthene	2606	2621	2637	2647	2658	2658	2668	2656	0.74	17.5
Pyrene	2703	2718	2736	2744	2757	2755	2769	2755	0.75	17.7
Benzyl butyl phthalate	2865	2877	2890	2905	2911	2906	2911	2907	0.56	16.5
Bis(2-ethylhexyl) adipate	2562	2563	2563	2592	2574	2573	2560	2581	0.41	15.7
Benz[4.9anthracene	3138	3155	3172	3185					0.57	9
Chrysene	3137	3185	3205	3220					0.98	9.7
Bis(2-ethylhexyl) phthalate	2859	2863	2866	2890					0.42	8.4

Table 4.9. The second dimension linear retention indices 2I of compounds identified from MegaMix B

Chromatographic conditions	2I (iu) ^a								<i>RSD</i> (%) ^b	$\Delta ^2T_e$ (°C) ^c
Secondary oven offset (°C)	+5	+5	+5	+5	+5	+5	+5	+10		
Ramp rate (°C/minute)	4	4.5	5	5	5.5	5.5	6	5		
Modulation period (s)	5	5	5	4	5	4	5	4		
cis-1,3-Dichloro-1-Propene, (Z)	861	862	873	868				865	0.5	11.5
trans-1,3-Dichloro-Propene	900	899	903	907	900	900	903	901	0.27	10
Toluene	872	873	884	878				877	0.48	10
1,1,2-Trichloroethane	911	911	912	917	914	913	915	910	0.24	10.2
Ethyl methacrylate	877	878	888	885				882	0.46	10
1,3-Dichloropropane	930	930	933	934	931	931	932	929	0.19	10.5
Dibromochloro-Methane	962	961	965	968	964	964	964	963	0.21	10.4
Tetrachloroethene	898	898	903	905				903	0.33	10.3
1,2-Dibromoethane	976	977	979	982	977	976	979	975	0.22	11.2
Chlorobenzene	989	989	991	996	992	989	992	989	0.21	11.9
1,1,1,2-Tetrachloroethane	973	981	984	991	986	984	983	984	0.48	12
Ethylbenzene	978	978	982	986	978	977	979	979	0.29	12
p-Xylene	986	984	986	993	983	983	989	986	0.33	12
Styrene	1044	1043	1050	1052	1045	1045	1042	1048	0.31	11.5
o-Xylene	1026	1025	1031		1027	1027	1027	1024	0.22	12
Bromoform	1102	1107	1111	1111	1112	1106	1113	1109	0.3	12.3
cis-1,4-Dichloro-2-butene, , (z)-	1088	1089	1093	1096	1093	1091	1091	1091	0.21	12.4
1,1,2,2-Tetrachloroethane	1095	1094	1098	1099	1095	1094	1096	1090	0.24	12.7
1,2,3-Trichloropropane	1111	1112	1115	1121	1112	1110	1130	1115	0.56	13
Isopropylbenzene(cumene)	1043	1039	1047	1050	1043	1044	1047	1045	0.28	13
trans-1,4-Dichloro-2-butene, (E)-			1129	1134	1127	1127	1116	1128	0.48	6.4
Bromobenzene	1113	1115	1117	1121	1117	1117	1118	1115	0.19	13.2
2-Chlorotoluene	1110	1112	1115	1120	1114	1114		1117	0.28	12
n- Propylbenzene	1076	1079	1083	1087	1079	1079	1083	1084	0.31	13

4-Chlorotoluene	1115	1114	1119	1121	1116	1115	1118	1118	0.19	13.4
1,3,5-Trimethylbenzene	1092	1091	1098	1100	1094	1095	1091	1092	0.28	13.9
Pentachloroethane	1144	1144	1149	1150	1149	1148	1148	1142	0.23	13.7
tert-Butylbenzene	1112	1115	1121	1125	1117	1117	1118	1121	0.33	13.9
1,2,4-Trimethylbenzene	1132	1132	1137	1141	1136	1134		1137	0.26	12
sec-Butylbenzene	1135	1133	1137	1143	1138	1137	1135	1137	0.24	14.2
p-Cymene(4-isopropyl toluene	1147	1148	1152	1158	1149	1149	1152	1152	0.28	15
n-Butylbenzene	1185	1184	1189	1196	1188	1188	1189	1192	0.29	14
1,2-Dibromo-3-Chloropropane	1329	1334	1340	1345	1343	1341	1344	1345	0.41	14.5
Benzene, 1,2,3-trichloro-	1452	1459	1464	1471	1470	1469	1474	1472	0.48	15.2
1,3-Dichlorobenzene*	1181	1184	1188	1196	1189	1188	1187	1191	0.34	14.2
1,4-Dichlorobenzene*	1197	1200	1201	1211	1205	1204	1206	1207	0.34	14.5
1,2-Dichlorobenzene*	1233	1237	1241	1247	1243	1242	1242	1246	0.34	15.9
Nitrobenzene*	1347	1353	1359	1364	1363	1360	1365	1364	0.44	14.2
Benzene, 1,2,4-trichloro-*	1394	1398	1403	1411	1406	1406	1408	1412	0.4	15.2
Naphthalene*	1446	1452	1460	1465	1466	1463	1467	1467	0.51	15.9
1,3-Butadiene, 1,1,2,3,4,4-hexachloro-*	1374	1376	1381	1389	1383	1382	1384	1387	0.35	15.7

The column used in GC-MS was a DB-17ms column, which was the same as the second dimension column used in GC×GC-MS.

Figure 4.7. Correlation between the linear retention index, I^T , of compounds calculated from GC-MS data and their corresponding second dimension linear retention indices 2I calculated using the method developed in this study. The compounds include 80 compound standards and compounds in MegaMix A and MegaMix B

Figure 4.7 shows the overall relation of the retention indices calculated by the surface fitting method and the linear retention indices obtained from the GC-MS data ($R^2=0.9998$). The retention index difference between the 2I values of the 80 compound standards ranged from 0.0 iu to 19.0 iu, with an average linear retention index difference of 5.1 iu. The

MegaMix A and MegaMix B had a wide range of retention index difference with an average variation of 23.9 iu because they are composed of a wide variety of chemical structures from different chemical classes, including branching and cyclization.

The retention index difference between the linear retention index I^T calculated from GC-MS data and the 2I calculated from GC×GC-MS data can be attributed to the polarity of the molecule, instrumental variation, chemical structure of the molecule, length of the column, effect of the transfer line and column temperature.⁷⁸⁻⁸¹ Previous study demonstrated that the retention index of an analyte generally increases with the increase of elution temperature.⁷⁹ We observed that some of the identified compounds followed this trend, while other compounds did not. The reason is that temperature dependency of retention index is also related to the structure of an analyte.⁷⁸⁻⁸¹ Table 4.10 shows an example of elution temperature of the 80 compound standards, where the elution temperature of a compound was increased with the increase of temperature ramp rate. The lowest elution temperature corresponded to the ramp rate of 4 °C/min and the highest elution temperature corresponded to the ramp rate of 6 °C/min. The temperature range was calculated by subtracting the elution temperature of the ramp rate 4 °C/min from the elution temperature of the ramp rate 6 °C/min. The average range of the elution temperature for the 80 compound standards was 14.5 °C. A similar effect was also observed for compounds in MegaMix A and MegaMix B, where most of the compounds showed a trend of increasing calculated retention index with the increasing of elution temperature, with an average range of elution temperature 18.5 °C.

Table 4.10. The second dimension elution temperature 2T_e of the 80 standard compounds with different ramp rates

Compounds name	2I (iu) ^a					$\Delta {}^2T_e$ ^b
Ramp (°C/min)	4	4.5	5	5.5	6	
FAME						
Methyl butyrate	84.8	86.8	89.3	91.2	93.6	8.9
Methyl valerate	92.1	94.7	97.6	100.0	102.6	10.5
Methyl caproate	102.4	106.0	108.9	111.9	115.1	12.7
Methyl enanthate	115.8	119.5	123.0	126.1	129.6	13.9
Methyl caprylate	129.8	133.8	137.6	141.2	144.7	14.9
Methyl pelargonate	144.5	148.4	152.2	155.4	159.2	14.7
Methyl caprate	158.1	162.3	166.0	169.6	173.2	15.0
Methyl undecanoate	171.5	175.4	179.3	182.9	186.7	15.2
Methyl laurate	183.8	188.1	191.8	195.8	199.2	15.4
Methyl tridecanoate	195.8	199.8	203.9	207.7	211.2	15.4
Methyl myristate	207.1	211.4	215.1	218.7	222.7	15.5
Methyl pentadecanoate	217.8	221.9	226.0	229.7	233.2	15.4
Methyl palmitate	227.8	232.0	236.4	239.8	243.7	15.9
Methyl margarate	237.8	242.1	246.0	249.9	253.7	15.9
Methyl stearate	247.1	251.1	255.1	259.0	262.7	15.5
Methyl nonadecanoate	255.8	260.1	264.3	268.2	271.7	15.9
Methyl arachidate	264.5	268.8	272.6	276.4	280.2	15.7
Methyl heneicosanoate	272.8	277.0	281.0	284.7	288.7	15.9
Methyl behenate	280.5	284.9	288.9			
Methyl tricosanoate	288.1					
Fatty acid						
Butyric acid	88.1	91.0	93.4	95.8	98.1	10.0
Valeric acid	97.4	100.7	103.9	106.8	109.6	12.2
Caproic acid	109.4	113.1	116.8	120.1	123.1	13.7
Enanthic acid	123.1	127.0	131.0	134.4	137.7	14.5
Caprylic acid	137.8	142.0	145.6	149.0	152.7	14.9
Pelargonic acid	150.5	154.8	158.9	161.9	165.2	14.7
Capric acid	164.1	167.9	172.2	175.2	178.7	14.5
Undecylic acid	176.5	180.6	184.7	188.0	191.7	15.2
Lauric acid	188.8	192.6	196.8	200.4	203.7	14.9
Tridecylic acid	200.1	204.3	208.5	211.8	215.2	15.0
Myristic acid	210.8	215.1	219.3	222.8	226.7	15.9
Pentadecylic acid	221.5	225.6	230.1	233.4	237.2	15.7
Palmitic acid	231.5	235.8	240.1	243.5	247.2	15.7
Margaric acid	241.1	245.1	249.7	253.1	256.7	15.5

Stearic acid	250.1	254.5	258.9	262.2	266.2	16.0
Nonadecylic acid	259.1	263.1	267.7	271.4	274.7	15.5
Arachidic acid	267.8	271.8	276.4	279.7	283.7	15.9
Heneicosylic acid	275.8	280.0	284.3	287.9		
Behenic acid	283.8	287.9				
Ketone						
2-Pentanone	83.1	85.3	87.2	89.4	91.6	8.5
2-Hexanone	89.1	91.7	94.3	96.8	99.1	10.0
2-Heptanone	98.4	101.9	104.7	107.8	110.6	12.2
2-Octanone	111.1	114.6	118.1	121.5	124.7	13.5
2-Nonanone	125.5	129.3	133.1	136.2	139.7	14.2
2-Decanone	139.8	143.9	147.6	151.3	154.7	14.9
2-Undecanone	154.1	158.1	162.2	165.5	169.2	15.0
2-Dodecanone	167.8	172.0	175.6	179.3	183.2	15.4
2-Tridecanone	180.5	184.8	188.5	192.1	196.2	15.7
2-Tetradecanone	192.8	196.8	201.0	204.5	208.2	15.4
2-Pentadecanone	204.1	208.4	212.6	216.4	220.2	16.0
2-Hexadecanone	215.1	219.6	223.5	227.4	231.2	16.0
2-Heptadecanone	225.8	230.1	234.3	237.9	241.7	15.9
2-Octadecanone	235.8	239.9	243.9	247.6	251.7	15.9
2-Nonadecanone	245.1	249.6	253.5	257.2	261.2	16.0
Alcohol						
1-Pentanol	87.4	89.9	92.6	94.9	97.1	9.7
1-Hexanol	96.4	99.2	102.2	105.0	108.1	11.7
1-Heptanol	108.8	112.0	115.5	119.2	122.1	13.4
1-Octanol	122.5	125.9	129.7	133.4	136.6	14.2
1-Nonanol	137.1	141.6	148.9	151.8	157.1	20.0
1-Undecanol	168.4	171.6	178.0	180.2	186.1	17.7
1-Dodecanol	186.1	185.1	192.6	196.2	200.6	14.5
Iodobutne						
1-Iodobutane	92.1	95.1	98.0	100.4	103.1	11.0
1-Iodopentane	103.1	106.7	109.7	112.8	116.2	13.0
1-Iodoheptane	117.1	120.6	124.3	127.9	131.2	14.0
1-Iodoheptane	131.8	136.0	139.7	143.5	146.7	14.9
1-Iodoctane	146.8	151.0	155.1	158.7	162.2	15.4
1-Iodononane	161.5	165.6	169.7	173.3	177.2	15.7
1-Iododecane	175.1	179.5	183.5	187.1	191.2	16.0
1-Iodoundecane	188.1	192.3	196.4	200.4	204.2	16.0
1-Iodododecane	200.5	204.6	208.9	212.7	216.2	15.7
1-Iodoheptadecane	243.5	247.8	251.8	255.8	259.7	16.2
1-Iodoctadecane	261.8	266.5	270.6	274.6	278.2	16.4
Aldehyde						
Pentanal	83.8	85.7	88.0	90.3	92.1	8.4
Hexanal	90.1	92.9	95.5	98.1	100.6	10.5
Heptanal	100.1	103.4	106.8	109.6	112.6	12.5

Octanal	113.1	116.9	120.6	123.8	127.2	14.0
Nonanal	127.5	131.5	135.1	138.5	142.2	14.7
Decanal	142.1	146.1	150.1	153.6	157.2	15.0
Undecanal	156.1	160.4	164.3	167.8	171.7	15.5
Dodecanal	169.8	173.9	178.1	181.6	185.2	15.4

4.3.4. Reproducibility and Robustness of Surface Fitting

To study the reproducibility and robustness of the developed method, we analyzed the 80 compound standards, MegaMix A and MegaMix B in different instrumental conditions by varying the temperature ramp rate of GC×GC-MS. We first analyzed the 80 compound standards in five ramp rates. The standard deviation (*STD*) of the 2I calculated from the five analyses ranged from 0.0 to 9.0 iu. Figure 4.8 shows the distribution of the *STD* of the 80 compound standards.

Figure 4.8. Distribution of standard deviation of the calculated 2I of the 80 standard compounds among different ramp rates.

Among the 80 compound standards, the *STD* of 64 compounds were within 5 iu; 15 compounds within 6-7 iu; and 1 compound within 8-9 iu. Overall, the mean standard deviation (*mSTD*) and relative standard deviation (*RSD*) of 2I was 3.8 iu and 0.25%, respectively. An average relative standard deviation (*RSD*) of the 2I for compounds in MegaMix A and MegaMix B was 0.51% and 0.32 %, respectively. The small *RSD* of 2I demonstrates that the developed method is robust in calculating the second dimension linear retention index of compounds analyzed in different instrumental conditions.

This study is a continuation of our previous work, i.e., calculating the second dimension retention index in GC×GC-MS via surface fitting.¹⁰⁵ The method developed in this study considered the second dimension separation in temperature-programmed mode and directly calculated the linear retention index via surface fitting. Furthermore, the current method used C₄–C₂₅ FAMES as the reference series while keeping the retention index in *n*-alkane scale. This approach allowed using any compound as the reference series where needed. Therefore, the current method is not limited to any reference series and allowed much more scope to maneuver the reference series selection based on the need.

4.4. Conclusions

We developed a surface fitting method for calculating the linear retention index 2I of compounds separated by GC×GC-MS from their second dimension retention time 2t_M and the secondary column temperature 2T_e , using C₄–C₂₅ FAMES as the reference series and keeping the retention index in *n*-alkane scale. The developed method showed an excellent agreement with the *n*-alkane based method. The standard deviation of the 2I of *n*-alkanes ranged from 3.6 to 14.0 iu with an average variation of 11.8 iu. The average relative

standard deviation of the 2I for compounds in MegaMix A and MegaMix B was 0.47% and 0.33%, respectively. Although we used only C₄–C₂₅ FAMES as the reference series to create a spacious second dimension retention map, it is possible to use any compounds as references to construct the retention index surface and calculate the 2I of analytes.

A future endeavor would be the use of mixture of reference compounds having different degree of polarity and diverse structure along with some individual reference compounds in order to construct the second dimension retention index surface. In addition, besides the MATLAB based surface fitting approach, other statistical methods i.e. machine learning algorithm can be employed to establish the relationship between the second dimension elution temperate , second dimension retention time and the corresponding second dimension retention index. From this relationship, second dimension retention index of unknown analytes can be calculated.

CHAPTER 5

INTEGRATING COMPREHENSIVE TWO-DIMENSIONAL GAS
CHROMATOGRAPHY MASS SPECTROMETRY AND PARALLEL TWO-
DIMENSIONAL LIQUID CHROMATOGRAPHY MASS SPECTROMETRY FOR
UNTRARGETED METABOLOMICS

5.1. Introduction

Metabolites are the intermediates and products of all biological processes that take place in a biological system. Metabolites can be polar or non-polar, as well as organic or inorganic compounds.^{3,5,107,108} The diverse chemical characteristics and huge number of entities make metabolite separation challenging in metabolomics. Separation methods such as liquid chromatography (LC) and gas chromatography (GC) are usually coupled with mass spectrometry (MS) to increase the metabolite coverage.¹⁰⁹ Due to the limited peak capacity of a single column, one dimensional separation method using LC or GC can only resolve a limited number of metabolites in a biological sample.¹¹⁰ In order to obtain better separation and higher peak capacity, multi-dimensional separation methods have been developed and applied in metabolomics even though the multi-dimensional separation usually needs long separation time and reduces the sample throughput.¹¹¹⁻¹¹⁴

Comprehensive two-dimensional gas chromatography mass spectrometry (GC×GC-MS) uses two capillary GC columns with different stationary phases for separation of metabolites.^{60-62,115,116} The two columns are usually connected via a thermal modulator. The second column is typically much shorter than the first (i.e., 1–2 m as opposed to 30–60 m for the first column) and is generally operated at a higher temperature. In the case of metabolites that co-elute from the first column, the second column may allow for further separation due to the different stationary phases as well as the different column temperatures. Thus, GC×GC-MS provides superior chromatographic peak capacity, selectivity, and lower detection limit for analysis of metabolites.

Two-dimensional liquid chromatography (2DLC) is usually configured in either a heart-cutting mode or a comprehensive mode.^{50,117,118} The heart-cutting analysis involves the collection of a few peaks of interest from the elution of the first dimension column, and then subjecting only those peaks onto the second dimension column for further separation. This configuration can increase signal-to-noise ratio and improve sensitivity for targeted metabolites.⁵¹ In the comprehensive configuration, eluate from the first dimension column is collected into multiple fractions and each fraction is subjected to the second dimension column for further separation. The comprehensive 2DLC offers an increased resolution.⁵² Most of the 2DLC systems are operated in online mode, where the second dimension separation is carried out simultaneously with the first dimension separation.⁵³ While the online mode of 2DLC has many advantages such as improved reliability and sample throughput, shortened analysis time and minimum sample loss, it requires the second dimension analysis to be completed during the time needed to collect and transfer a fraction from the first dimension column except that the 2DLC was configured in a stop-flow

mode.¹¹⁹ Another limitation of the online 2DLC technique is that the mobile phases used in the two columns must be compatible in both miscibility and solvent strength. In addition, some metabolites may partition between the fractions collected from the first dimension column, resulting in large variation in metabolite quantification and even metabolite identification.

Klavins, *et al.* developed a parallel 2DLC system to perform orthogonal hydrophilic interaction chromatography (HILIC) and reversed-phase chromatography (RPC) in one analytical run, where a sample was first delivered to two sample loops during sample loading.⁵⁴ The two sample aliquots were then simultaneously injected onto a dual column setup, and parallel separations were performed on the two columns. The eluates from the two columns were then merged and subjected to a mass spectrometer for further analysis. This strategy is simple yet effective for coupling HILIC and RPC for the purpose of decreasing analysis time and increasing throughput. Furthermore, the parallel 2DLC-MS configuration allows the use of two long columns and gradient time to increase separation power. However, parallel 2DLC-MS has the potentials of peak overlapping which incurs resolution problem. On the other hand, unlike the comprehensive 2DLC-MD, parallel 2DLC-MS does not suffer from the peak partition and solvent miscibility and solvent strength issues.

While the effectiveness of both GC×GC-MS and 2DLC-MS for metabolomics has been separately demonstrated in multiple studies^{82,115,120-122}, analysis of biological samples on the two platforms has not yet been explored. In the current study, we aimed to integrate the GC×GC-MS and the parallel 2DLC-MS for wider metabolite coverage, high confidence of metabolite identification and quantification, as well as high confidence of metabolic

pathway assignment. The performance of developed system was tested by analyzing polar metabolites extracted from rat livers, where each metabolite extract was analyzed by GC×GC-MS and parallel 2DLC-MS, respectively. After metabolite identification and quantification, the performance of GC×GC-MS and parallel 2DLC-MS was assessed based on the number of identified metabolites, the accuracy of metabolite quantification, and the extent of their pathway coverage.

5.2. Experimental

5.2.1. Animal Treatment

Thirteen male weanling Sprague–Dawley rats (35–45 g) from the Harlan Laboratories (Indianapolis, IN) were fed (*ad lib*) a purified AIN-76 diet with a defined copper content in form of cupric carbonate. The animals were housed in stainless steel cages in a temperature and humidity controlled room with a 12:12 h light–dark cycle. The 13 rats formed two groups, Group 1 (G1, n=6) and Group 2 (G2, n=7). The rats in G1 received adequate dose of copper (6.0 ppm) with free access to deionized water. The rats in G2 received supplemental dose of copper (20 ppm) with free access to deionized water containing 30% fructose (w/v). Fructose enriched drinking water was changed twice each week. All animals were fed for 4 weeks. At the end of the feeding period, each rat was killed under anesthesia with pentobarbital (50 mg/kg I.P. injection) after overnight fasting. Portions of rat liver were fixed with 10% formalin for subsequent sectioning, while others were snap-frozen with liquid nitrogen. All studies were approved by the University of Louisville Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

5.2.2. Metabolite Sample Preparation

All samples were processed in random order to avoid systemic bias. After placing about 100 mg of liver tissue in a 1.5-mL Eppendorf tube, water was added at a ratio of 100 mg liver/mL water. After that, glass beads were added to the tube and the sample was homogenized using a Retsch MM 200 model mixer mill (Fisher Scientific, Hampton, NH, USA). To extract polar metabolites for GC×GC-MS analysis, 800 μ L methanol was added to 200 μ L homogenized liver. The mixture was vortex-mixed for 2 min and then placed on ice for 10 min. After another 2 min of vortex mixing, the sample was centrifuged at 15,000 rpm for 20 min at 4 °C. Seven hundred micro-liters of supernatant was transferred into a glass vial and dried in a SpeedVac evaporator to remove methanol, followed by lyophilization to remove water. The dried metabolite extract was then dissolved with 30 μ L of 20 mg/mL methoxyamine hydrochloride pyridine solution followed by vigorous vortex mixing for 1 min. Methoxymation was carried out by sonicating the sample for 20 min and incubating it at 60 °C for 1 h. Derivatization was conducted by adding 30 μ L of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane to the glass vial. After 1 h incubation at 60 °C, the mixture was transferred to a GC vial for analysis. A pooled sample was prepared by mixing 30 μ L of derivatized metabolite extract from each sample.

To extract polar metabolites for parallel 2DLC-MS analysis, 400 μ L methanol was added to 100 μ L homogenized liver. The mixtures was vortex-mixed and centrifuged using the same extraction protocol used for GC×GC-MS analysis. Three hundred micro-liters of supernatant was transferred into a glass vial. Methanol in the sample was removed using a SpeedVac evaporator and water was removed by lyophilization. The dried metabolite extract was reconstituted with 100 μ L 20% acetonitrile. The reconstitution was

immediately preceded the parallel 2DLC-MS analysis. A pooled sample was prepared by mixing 50 μ L of metabolite extract from every sample of the same group.

5.2.3. GC $\times$ GC-MS and Its Data Analysis

A LECO (St. Joseph, MI, USA) Pegasus GC $\times$ GC-MS instrument was coupled with an Agilent 6890 gas chromatography and a Gerstel MPS2 auto-sampler (GERSTEL Inc., Linthicum, MD, USA), featuring a LECO two-stage cryogenic modulator and a secondary oven. The primary column was a 60 m $\times$ 0.25 mm $^1d_c \times 0.25 \mu\text{m}^1d_f$ DB-5 ms capillary column (phenyl arylene polymer virtually equivalent to (5%-phenyl)-methylpolysiloxane). The secondary GC column was a 1 m $\times$ 0.25 mm $^2d_c \times 0.25 \mu\text{m}^2d_f$ DB-17 ms column ((50% phenyl)-methylpolysiloxane) that was placed inside the secondary GC oven following the thermal modulator. Both columns were obtained from Agilent Technologies (Agilent Technologies J&W, Santa Clara, CA, USA). The helium carrier gas (99.999% purity) flow rate was set to 2.0 mL/min at a corrected constant flow with pressure ramps. The inlet temperature was set to 280 $^{\circ}\text{C}$. The primary column temperature was programmed with an initial temperature of 60 $^{\circ}\text{C}$ for 0.5 min, then ramped at 5 $^{\circ}\text{C}/\text{min}$ to 270 $^{\circ}\text{C}$, and maintained at 270 $^{\circ}\text{C}$ for 15 min. The secondary column temperature program was set to an initial temperature of 70 $^{\circ}\text{C}$ for 0.5 min and then ramped at the same temperature gradient used in the first column to 280 $^{\circ}\text{C}$. The thermal modulator was +15 $^{\circ}\text{C}$ compared with the primary oven. The other instrument parameters were as: modulation period 2 s, mass range 29-800 m/z , spectrum acquisition rate 200 mass spectra per second, ion source chamber temperature 230 $^{\circ}\text{C}$, transfer line temperature 280 $^{\circ}\text{C}$, detector voltage 1420 V, electron energy 70 eV, and split ratio 20:1. The acceleration voltage was turned on after a solvent delay of 640 s.

The pooled sample was analyzed by GC×GC-MS eight times. The experiment data of the pooled sample were used to monitor the instrument variation. In addition, an aliquot of C₇–C₃₀ *n*-alkane series was analyzed for retention index calculation.

To analyze the GC×GC-MS data, LECO's instrument control software, ChromaTOF (version 4.21), was used for peak picking and tentative metabolite identification. The threshold of spectral similarity score was set as ≥ 500 with a maximum value of 1000. MetPP software was used for retention index matching, peak merging, peak list alignment, normalization and statistical significance test.^{123,124} The *p*-value threshold was set as $p \leq 0.001$ for retention index matching.

To verify the tentative identification of metabolites detected with significant abundance difference between groups, commercially available authentic standards of those compounds were analyzed by GC×GC-MS under the identical experimental conditions as those used for analyses of biological samples. A tentative metabolite assignment was considered as a correct identification only if the experimental information of the authentic metabolite agreed with the corresponding information of a chromatographic peak in the biological samples, i.e., difference of the first dimension retention time ≤ 10 s, difference of the second dimension retention time ≤ 0.06 s, and the mass spectral similarity ≥ 500 .

5.2.4. Parallel 2DLC-MS and Its Data Analysis

All samples were randomly analyzed on a Thermo Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled with a Thermo UltiMate 3000 HPLC system (Thermo Fisher Scientific, Inc., Germany). The UltiMate 3000 HPLC system was equipped with a hydrophilic interaction chromatography (HILIC) column and a reversed-phase chromatography (RPC) column that were configured in parallel mode.⁵⁴ The HILIC

column (SeQuant® ZIC®-cHILIC, 150×2.1 mm i.d., 3 µm) was purchased from EMD Millipore (Darmstadt, Germany). The RPC column (ACQUITY UPLC HSS T3, 150×2.1 mm i.d., 1.8 µm) was purchased from Waters Corp. (Milford, MA, USA). The temperature of those two columns was each set to 40 °C. The HILIC column was operated as follows: mobile phase A was 10 mM ammonium acetate (pH adjusted to 3.25 with acetate) in water and mobile phase B was 100% acetonitrile with 0.1% formic acid. The gradient was: 0.0 min, 95% B; 0.0 to 5.0 min, 95% B to 35% B; 5.0 to 6.0 min, 35% B; 6.0 to 6.1 min, 35% B to 5% B; 6.1 to 23.0 min, 5% B; 23.0 to 23.1 min, 5% B to 95% B; 23.1 to 40.0 min, 95% B. The flow rate was set to 0.3 mL/min. For the RPC column, the mobile phase A was water with 0.1% formic acid and mobile phase B was 100% acetonitrile with 0.1% formic acid. The gradient was as follows: 0.0 min, 5% B; 0.0 to 5.0 min, 5% B; 5.0 to 6.1 min, 5% B to 15% B; 6.1 to 10.0 min, 15% B to 60% B; 10.0 to 12.0 min, 60% B; 12.0 to 14.0 min, 60% B to 100% B; 14.0 to 27.0 min, 100% B; 27.0 to 27.1 min, 100% B to 5% B; 27.1 to 40.0 min, 5% B. The flow rate was 0.4 mL/min.

The metabolite extract of each biological sample or the pooled sample was analyzed by 2DLC-MS in positive mode (+) and negative mode (-), respectively. The electrospray ionization probe was fixed at level C. The parameters for the probe were set as follows: sheath gas 55 arbitrary unit, auxiliary gas 15 arbitrary unit, sweep gas 3 arbitrary unit, spray voltage 3.5 kV, capillary temperature 320 °C, S-lens RF level 65.0, and auxiliary gas heater temperature 450 °C. The method of mass spectrometer was set as follows: full scan range 50-750 *m/z*, resolution 30,000, maximum injection time 50 ms, and automatic gain control (AGC) target 1×10^6 ions for both positive and negative modes.

The pooled sample was also analyzed by 2DLC-MS/MS (-) and 2DLC-MS/MS (+), respectively, to acquire the MS/MS spectra of metabolites. The 2DLC-MS/MS method and electrospray ionization condition were the same as those used in parallel 2DLC-MS analyses. The parameters used for mass spectrometry were as follows: for full-MS scan, scan range 50-750 m/z , resolution 30,000, maximum injection time 50 ms, and AGC target 1×10^6 ions; for dd-MS² scan, resolution 15,000, maximum injection time 100 ms, AGC target 5×10^4 ions, loop count 6, isolation window 1.3 m/z , and dynamic exclusion time 1.2 s. Each pooled sample was analyzed using 3 collision energies, i.e., 20, 40 and 60 eV, respectively.

To analyze the experimental data, all 2DLC-MS (-) and 2DLC-MS (+) data were processed using MetSign software for spectrum deconvolution, metabolite assignment, cross-sample peak list alignment, normalization, pattern recognition, and statistical significance test.¹²⁵⁻¹²⁸ Metabolite identification was achieved in MetSign using the 2DLC-MS (-) and 2DLC-MS (+) data of biological samples and the 2DLC-MS/MS (-) and 2DLC-MS/MS (+) data of the pooled sample. MetSign respectively aligned the 2DLC-MS/MS (-) and 2DLC-MS/MS (+) data to the 2DLC-MS (-) and 2DLC-MS (+) data by retention time and parent ion m/z values with following thresholds: retention time variation ≤ 0.2 min and parent ion m/z variation ≤ 4 ppm. To identify the metabolites in the pooled sample, the parent ion m/z , retention time, and MS/MS spectra of a metabolite were matched to the corresponding information of 205 metabolite standards recorded in an in-house database, where the matching thresholds were set as follows: MS/MS spectral similarity ≥ 0.4 , retention time difference ≤ 0.15 min, and m/z variation ≤ 4 ppm. The 2DLC-MS/MS (-) and 2DLC-MS/MS (+) data without a match in the in-house database were further analyzed

using Compound Discoverer 2.0 software (Thermo Fisher Scientific, Inc., Germany) to match the remaining MS/MS spectra to the MS/MS spectra recorded in the Compound Discoverer database with a threshold of MS/MS spectral similarity score ≥ 20 . For the peaks detected in the 2DLC-MS (-) or 2DLC-MS (+) data of biological samples that were not matched to any metabolites by MS/MS spectral matching, the m/z values of those parent ions were then matched to the compounds recorded in the Human Metabolome Database (HMDB) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. The m/z variation window was set ≤ 4 ppm.

5.3. Results and Discussion

5.3.1. Metabolite Coverage by GC×GC-MS

Metabolite identification in analysis of GC×GC-MS data was done using the GC×GC-MS data of the pooled sample and biological samples by spectral similarity matching followed by retention index matching.^{106,124} Details of data processing parameters are listed in Table 5.1.

Table 5.1. Data processing parameters used in LECO ChromaTOF software

Data processing parameters used in LECO ChromaTOF	
Baseline Offset	1
Number of Data Points Averaged for Baseline Smoothing	Auto
Peak Width for Peak Finding	GC-MS: 4, GC×GC-MS: 7.5
Minimum Number of Apexing Masses for Peak Finding	2
Minimum SNR for Peak Finding	10
Library Search Mode	Normal, Forward
Library Hits Returned per Peak	10
Minimum/Maximum Molecular Weight Allowed	29/850
Relative Mass Threshold for Library Searching	10

Minimum Similarity Match Before Name is Assigned	600
Libraries Used for Searching	In-House, NIST-14
Mass Used for Area/Height Calculation	Unique
Match Required to Combine Peaks in the Second Dimension	500
Second Dimension Peak Width	0.15
Minimum SNR for Subpeak to be Retained	6
Integration Approach	Traditional

Table 5.2 lists the numbers of detected chromatographic peaks and their identification results from the pooled sample and the rat liver samples.

Table 5.2. Numbers of detected peaks from pooled samples and biological samples by GC × GC-MS and the results of metabolite identification using different matching methods

Sample	Analysis ID	Chromatographic peaks	Metabolite identification		
			Similarity score threshold ≥ 500	After RI matching	Unique metabolites
Pooled sample	Inj-1	3280	992	911	476
	Inj-2	3004	836	778	423
	Inj-3	3443	842	778	404
	Inj-4	3082	936	862	429
	Inj-5	3029	927	864	450
	Inj-6	3106	941	637	367
	Inj-7	3210	1037	947	447
	Inj-8	2952	712	865	477
	Average	3138±153	903±96	830±91	434±35
Biological sample	S-1	3389	1017	902	484
	S-2	3371	953	842	462
	S-3	3304	984	845	469
	S-4	3531	1003	918	507
	S-5	3873	1080	925	506
	S-6	3263	974	881	443

S-7	3062	990	898	458
S-8	3288	1075	954	480
S-9	2559	750	693	399
S-10	3508	961	859	443
S-11	3154	964	846	413
S-12	3463	1048	929	470
Average	3314±301	983±82	874±65	461±32

Out of 13 biological samples, one sample from G1 was detected as an outlier during the peaks alignment step due to very smaller number of peaks detected compared to the other samples. We believe this was caused by the injection in GC×GC-MS. Therefore, this sample was excluded in the subsequent analysis. While about $3,138 \pm 153$ chromatographic peaks were detected in the eight injections of the pooled sample, 830 ± 91 metabolites were identified. Using the same set of parameters, $3,314 \pm 301$ chromatographic peaks were detected from the biological samples, from which 874 ± 65 metabolites were identified. A metabolite might be identified from multiple distinct chromatographic peaks owing to incomplete derivatization, presence of isomers, or false identifications. After removing the redundant metabolite identifications, 434 ± 35 unique metabolites were identified from the pooled sample and 461 ± 32 unique metabolites were identified from the biological samples. In GC×GC-MS, metabolites co-eluted from the first dimension GC column might be separated by the second dimension GC column (Figure 5.1), which can increase the chance of acquiring high quality EI mass spectrum for metabolite identification.

Figure 5.1. Two compounds co-eluted from the first dimension GC but separated by the second dimension GC. (A) is a three-dimensional view, and (B) is a contour plot.

Despite the excellent instrumental capability, the identification accuracy of current mass spectrum matching method is only about 79.6%.^{33,129,130} Therefore, additional information such as retention index must be used to reduce the rate of false identifications.^{131,132} On average, retention index matching removed about 8.9% of false identifications generated by mass spectrum matching (Table 5.2). Overall, only about

13.7% chromatographic peaks detected by GC×GC-MS were assigned to metabolites in this study.

5.3.2. Metabolite Coverage by Parallel 2DLC-MS

To identify metabolites from the 2DLC-MS data, 2DLC-MS/MS (-) and 2DLC-MS/MS (+) data of the pooled sample were first respectively aligned with the 2DLC-MS (-) and 2DLC-MS (+) data of the biological samples based on the retention time and parent ion m/z of each metabolite. The aligned data were then used for metabolite identification. Table 5.3 summarizes the identification results of the 2DLC-MS data. We made fresh solvent of mobile phase for analysis of three samples by 2DLC-MS, one sample from G1 and two samples from G2. In order to avoid the potential problems of solvent change, the experiment data of these three samples were not included in Table 5.3. Details of metabolites identified in this study by MS/MS matching are listed in Tables 5.4 and 5.5.

Table 5.3. Numbers of detected peaks from pooled samples and biological samples by 2DLC-MS and the results of metabolite identification using different matching methods.

Sample	Analysis ID	2DLC-MS (-)			2DLC-MS (+)		
		Isotopic peaks	Public DB	MS/MS DB	Isotopic peaks	Public DB	MS/MS DB
Pooled sample	Inj-1	15586	2706	278	14617	4241	217
	Inj-2	14903	2526	276	14834	4215	224
	Inj-3	15381	2494	275	14823	4265	217
	Inj-4	15196	2564	267	15109	4405	229
	Inj-5	14951	2440	274	16033	4590	231
	Inj-6	14302	2201	220	16238	4621	223
	Inj-7	14959	2313	255	16341	4546	236
	Inj-8	15585	2494	270	16079	4489	236
	Average	15108±399	2467±144	264±18	15509±681	4422±153	227±7
	S-1	15112	3590	264	18128	5791	214
	S-2	14753	3363	266	17164	5561	197
	S-3	15184	3625	274	17875	5572	201
	S-4	14699	3560	262	18193	5503	205
	S-5	15075	3673	276	17922	5371	186
	S-6	14776	3514	278	17082	5457	189
	S-7	14844	3463	242	17806	5806	196
	S-8	15514	3553	279	17450	5519	217
	S-9	14888	3623	237	17010	5235	196
	S-10	14546	3550	243	17541	5788	202
Biological sample	Average	14939±269	3551±85	262±15	17617±410	5560±180	200±9

Table 5.4. Details of metabolites identified by MS/MS matching in 2DLC-MS (-) mode

Name	<i>m/z</i>	Retention time (min)	Database
(±)13-HODE	295.227	13.96	In-house DB ^a
(R)-2-Hydroxybutyric acid	103.04	2.13	In-house DB
2-Hydroxycaproic acid	131.071	2.45	CD ^b
2-Methylglutaric acid	145.051	2.17	CD
2-Oxobutyric acid	101.024	5.54	CD
2-Oxoglutaric acid	145.014	5.36	CD
2-Phosphoglycerate (2PG)	184.985	0.88	In-house DB
3-AMP	346.055	1.2	In-house DB
3-Hydroxybutyric acid	103.04	1.68	In-house DB
3-Phosphoglyceric acid	184.985	6.3	CD
4-Dodecylbenzenesulfonic acid	325.184	4.52	CD
4-Methyl-2-oxovaleric acid	129.056	7.53	In-house DB
4-Oxoproline	128.035	4.53	CD
5-CMP	322.044	0.94	In-house DB
Alanine	88.0402	0.83	In-house DB
Alpha-D-Glucose 1-phosphate	259.023	0.83	In-house DB
alpha ketoglutaric acid	145.014	1.15	In-house DB
Arabitol	151.061	5.16	CD
Asparagine	131.046	0.83	In-house DB
Aspartic Acid	132.03	0.83	In-house DB
Azelaic acid	187.097	9.05	In-house DB
Benzoic acid	121.029	0.1	CD
Citrate	191.02	1.21	In-house DB
Citrulline	174.088	0.85	In-house DB
Cystathionine	221.06	0.8	In-house DB
D-Erythrose-4-phosphate	199.001	0.88	In-house DB
D-Glucosamine-6-phosphate	258.038	5.97	In-house DB
D-Glucose (Glc)	179.056	0.88	In-house DB
D-Glucose 6-phosphate	259.022	6.2	CD
D-Glyceraldehyde 3-Phosphate (GADP)	168.991	0.91	In-house DB
Dihydroxyacetone Phosphate (DHAP)	168.991	4.96	In-house DB
DL-alpha-Glycerol phosphate	171.006	0.86	In-house DB
DL-β-Leucine	130.087	4.96	CD
Dodecanedioic acid	229.144	9.59	CD
D-Ribulose-5-phosphate	229.012	0.85	In-house DB

D-Saccharic acid	209.03	0.86	CD
Esculin	339.074	0.89	CD
Ethyl- β -D-glucuronide	221.067	1.46	CD
Fructose	179.056	5.56	CD
Fumaric acid	115.004	1.53	In-house DB
Glutamic acid	146.046	0.85	In-house DB
Glutamine	145.062	5.74	CD
Glutaric acid	131.035	2.44	In-house DB
Glutathione	306.076	1.15	In-house DB
Glycerol 3-phosphate	171.006	5.75	CD
Glycine	74.0246	0.83	In-house DB
Glycyl-L-leucine	187.109	5.25	CD
Histidine	154.062	0.79	In-house DB
Hypotaurine	108.012	5.7	CD
Hypoxanthine	135.031	1.18	CD
Inosine	267.073	1.6	In-house DB
Isobutyric acid	87.045	2.45	In-house DB
Isocitric acid	191.02	0.89	In-house DB
Isoleucine	130.087	1.75	In-house DB
Itaconic acid	129.019	6.16	CD
Lactic acid	89.0243	1.24	In-house DB
L-alpha-Aminoadipic acid	160.061	0.9	In-house DB
L-alpha-Amino-n-butyric acid	102.056	0.85	In-house DB
L-Ascorbic acid 2-sulfate	254.981	0.97	CD
Leukotriene B4	335.222	2.42	In-house DB
Levulinic acid	115.04	2.43	In-house DB
Glutathione reduced	306.076	5.62	CD
L-Glutathione oxidized	611.143	1.17	CD
Lipoxin A4	351.217	2.43	In-house DB
Lipoxin B4	351.217	10.39	In-house DB
Phenylalanine	164.072	4.93	CD
Pyroglutamic acid	128.035	1.35	In-house DB
Serine	104.035	5.96	CD
Lysine	145.098	0.75	In-house DB
Malic acid	133.014	1.04	In-house DB
Maleic acid	115.004	1.2	In-house DB
Malonic acid	103.004	1.11	In-house DB
Maltose	341.108	5.74	CD
Mannitol	181.072	5.36	CD
Mesaconic acid	129.019	0.89	CD
Methionine	148.044	1.21	In-house DB

Monobutyl phthalate	221.082	10.73	CD
N-Acetylalanine	130.051	1.65	CD
N-Acetyl-alpha-D-glucosamine 1-phosphate	300.049	0.84	In-house DB
N-Acetyl-D-Glucosamine	220.082	0.95	In-house DB
N-Acetyl-L-glutamic acid	188.056	1.22	In-house DB
N-Acetyl-L-glutamine	187.072	4.61	CD
N-Acetylneuraminic acid	308.098	0.91	CD
N-Acetyl- α -D-glucosamine 1-phosphate	300.048	5.85	CD
Nicotinic acid	122.025	1.16	In-house DB
N- α -Acetyl-L-asparagine	173.057	5.79	
O-Phosphoethanolamine	140.012	0.82	In-house DB
O-Phosphorylethanolamine	140.012	6.22	CD
Ornithine	131.082	0.78	In-house DB
Orotic acid	155.01	4.85	CD
Oxalic acid	88.9879	0.89	In-house DB
Pantothenic acid	218.103	3.97	CD
Phosphoenolpyruvate	166.975	0.9	In-house DB
Phosphoenolpyruvic acid	166.975	6.03	CD
Prostaglandin G2	367.211	2.63	In-house DB
Pyruvate	87.0086	1.07	In-house DB
Quinolinic Acid	166.015	1.19	In-house DB
Ribose	149.045	4.81	CD
Salicylic acid	137.024	9.35	In-house DB
Succinate	117.019	1.61	In-house DB
Sucrose	341.108	0.89	In-house DB
Taurine	124.007	0.85	In-house DB
Taurochenodeoxycholic acid	498.289	9.71	CD
Thiamine	263.097	0.78	In-house DB
Threonine	118.051	0.86	In-house DB
trans-4-Hydroxy-L-proline	130.051	0.89	In-house DB
trans-Ferulic acid	193.051	2.43	In-house DB
Tryptophan	203.083	5.12	CD
Tyrosine	180.067	1.2	In-house DB
UMP	323.028	1.08	In-house DB
Uracil	111.02	1.15	CD
Uric acid	167.021	1.12	CD
Uridine	243.062	4.57	CD
Valine	116.072	1.05	In-house DB
Xanthine	151.026	1.32	CD

Xanthosine	283.068	4.72	CD
α -D-Mannose 1-phosphate	259.022	6.02	CD
β -D-Glucopyranuronic acid	193.035	6.05	CD
δ -Ribono-1,4-lactone	147.03	5.91	CD
γ -Aminobutyric acid (GABA)	102.056	5.91	CD

Table 5.5. Details of metabolites identified by MS/MS matching in 2DLC-MS (+) mode

Name	<i>m/z</i>	Retention time (min)	MS/MS database
1-Methyl-L-histidine	170.092	0.75	In-house DBa
2-Aminobutyric acid	104.07	5.39	CDb
3-Aminobutanoic acid	104.07	0.87	In-house DB
3-Hydroxypyridine	96.0442	0.93	In-house DB
4-Acetamidobutanoic acid	146.081	5.38	CD
4-Guanidinobutyric acid	146.092	1.03	CD
4-Indolecarbaldehyde	146.06	5.08	CD
4-Methyl-5-thiazoleethanol	144.048	6.18	CD
5-Aminovaleric acid	118.086	0.88	In-house DB
5-Methylcytosine	126.066	1.19	CD
Alanine	90.0548	0.84	In-house DB
α -Aminoadipic acid	162.076	0.93	In-house DB
Arginine	175.119	7.18	CD
Asparagine	133.061	5.84	CD
Aspartic acid	134.045	6.23	CD
Betaine	118.086	5.06	CD
Carnitine	162.112	4.31	CD
Choline	104.107	0.83	CD
cis-4-Hydroxy-D-proline	132.066	5.57	CD
Citrulline	176.103	0.85	In-house DB
Creatine	132.077	5.85	CD
Cystathionine	223.074	0.81	In-house DB
Cystine	241.03	6.29	CD
Cytosine	112.05	6.22	CD
D-Serine	106.05	6.02	CD
Ethanolamine	62.0601	0.8	In-house DB
Glucose 1-phosphate	261.036	6.28	CD
Glutamic acid	148.06	5.88	CD
Glutamine	147.076	0.86	In-house DB

Glycine	76.0392	0.81	In-house DB
Histidine	156.077	0.79	In-house DB
Homoserine	120.066	5.74	CD
Hypotaurine	110.027	5.72	CD
Hypoxanthine	137.046	4.62	CD
Isoleucine	132.102	1.75	In-house DB
Leucine	132.102	1.87	In-house DB
Leucylproline	229.154	4.81	CD
Lysine	147.113	7.04	CD
Methionine	150.058	1.2	In-house DB
Methylimidazoleacetic acid	141.066	5.74	CD
N3,N4-Dimethyl-L-arginine	203.15	6.15	CD
N6,N6,N6-Trimethyl-L-lysine	189.16	6.31	CD
N6-Acetyl-L-lysine	189.123	1.02	CD
N-Acetyl-D-galactosamine	222.097	5.59	CD
N-Acetyl-D-Glucosamine	222.097	0.92	In-house DB
N-Acetyl-L-glutamic acid	190.071	1.44	In-house DB
N-Acetyl- α -D-glucosamine	222.097	5.23	CD
Nicotinamide	123.055	1.18	CD
Nicotinic acid	124.039	1.17	In-house DB
O-Phosphoethanolamine	142.026	0.82	In-house DB
Ornithine	133.097	0.75	In-house DB
Phenylalanine	166.086	3	In-house DB
Phosphoenolpyruvate	168.989	0.92	In-house DB
Pipecolic acid	130.086	0.75	CD
Proline	116.07	5.31	CD
Pyridoxal	168.065	0.92	CD
Pyroglutamic acid	130.05	5.74	CD
Serine	106.05	0.83	In-house DB
Stachydrine	144.102	0.91	CD
Taurine	126.022	5.61	CD
Threonine	120.065	0.85	In-house DB
Tryptophan	205.097	5.08	CD
Tyrosine	182.081	1.44	In-house DB
Uracil	113.034	4.53	CD
Urea	61.0397	0.92	In-house DB
Uric acid	169.035	5.28	CD
Urocanic acid	139.05	1.03	CD
Valine	118.086	4.33	In-house DB
γ -Aminobutyric acid (GABA)	104.07	6.11	CD

A total of $15,108 \pm 399$ and $15,509 \pm 681$ features were detected from the pooled sample by 2DLC-MS (-) and 2DLC-MS (+), respectively. Here, a feature in 2DLC-MS data was defined by retention time and isotopic peak m/z value. By parent ion m/z matching, $2,467 \pm 144$ features in 2DLC-MS (-) and $4,422 \pm 153$ features in 2DLC-MS (+) of the pooled sample were assigned to at least one metabolite in KEGG or HMDB databases. By MS/MS spectrum matching, 264 ± 18 metabolites were identified from the 2DLC-MS/MS (-) data and 227 ± 7 metabolites were identified from the 2DLC-MS/MS (+) data.

Table 5.3 also shows that $14,939 \pm 269$ and $17,617 \pm 410$ features were detected from the biological samples by 2DLC-MS (-) and 2DLC-MS (+), respectively. Among those features, $3,551 \pm 85$ and $5,560 \pm 180$ metabolites were respectively assigned to the 2DLC-MS (-) and 2DLC-MS (+) data based on parent ion m/z matching. By MS/MS spectrum matching, 262 ± 15 and 200 ± 9 metabolites were identified from the 2DLC-MS/MS (-) and 2DLC-MS/MS (+), respectively.

By parent ion m/z matching, about 23.8% to 31.6% of metabolites giving rise to the features in 2DLC-MS (-) and 2DLC-MS (+) were assigned to at least one metabolite. However, the percentage of assignment was dramatically reduced to only 1.1% to 1.8% when the MS/MS spectra were used for metabolite identification, even though we maximized the chance of acquiring high quality MS/MS spectra for each metabolite by fragmenting each parent ion using three collision energies, i.e., 20, 40 and 60 eV, respectively. The extremely low percentage of metabolite identification agrees with Silva, *et al.*'s observation that 98% of the instrumental data were not used in metabolomics.¹³³ Multiple factors contributed to those results. For instance, a fraction of the isotopic peaks detected by MS are not monoisotopic peaks and therefore cannot be matched to the

metabolites. During the experiment, the top six abundant ions were subjected to MS/MS data acquisition in the dd-MS² mode. A number of metabolites with low instrument response were not selected for MS/MS spectra acquisition even though they were detected in the full MS mode. Furthermore, a metabolite in the sample might not be present in our in-house database and the Compound Discoverer database, and therefore could not have an identification result by MS/MS spectrum matching.

5.3.3. Integrating GC×GC-MS and Parallel 2DLC-MS Data for Higher Metabolite Coverage

Figure 5.2 (A) depicts the identification results of the three analytical platforms GC×GC-MS, 2DLC-MS (-), and 2DLC-MS (+) in analyzing the polar metabolites extracted from the biological samples. A total of 3,965 peaks were assigned to metabolites from the GC×GC-MS, 2DLC-MS (-), and 2DLC-MS (+) data.

B.

Figure 5.2. Overlap of metabolite identification. Metabolites were identified from GC × GC-MS by EI mass spectrum matching and retention index matching. (A) The metabolite identification in the analysis of 2DLC-MS data was done by parent ion m/z matching. (B) The metabolite identification in the analysis of 2DLC-MS/MS data by MS/MS spectrum matching with or without retention time match.

A majority of metabolites were assigned by parent ion m/z in the 2DLC-MS (-) and 2DLC-MS (+) data, while all metabolites were identified from the GC×GC-MS data by mass spectrum matching. Therefore, the number of metabolites assigned using the 2DLC-MS (-) and 2DLC-MS (+) data are much larger than that using the GC×GC-MS data. Owing to the diverse chemical properties of the GC and LC columns, each analytical

platform favors detection of different metabolites in the biological samples. As expected, the GC×GC-MS and 2DLC-MS have much different metabolite coverage. Only 37 metabolites were commonly assigned to the GC×GC-MS and 2DLC-MS data. Of those 37 metabolites, 32 metabolites were assigned to the GC×GC-MS and the 2DLC-MS (-) data, and 28 metabolites were assigned to the GC×GC-MS and 2DLC-MS (+) data. There were only 23 metabolites detected by all three analytical platforms.

Assigning a metabolite to a peak by parent ion m/z only in the analysis of 2DLC-MS data generates a very high rate of false assignments. Figure 5.2(B) depicts the overlap of metabolite identification among GC×GC-MS, 2DLC-MS/MS (-), and 2DLC-MS/MS (+) data by MS/MS spectrum matching and other constrains, i.e., retention index filtering in analysis of GC×GC-MS data, parent ion m/z matching in analysis of 2DLC-MS/MS (-) and 2DLC-MS/MS (+) data. A total of 326 metabolites were identified by the three platforms, of which 205, 120 and 69 metabolites were identified from the GC×GC-MS, 2DLC-MS/MS (-) and 2DLC-MS/MS (+) data, respectively. Only 22 metabolites were commonly identified in all three platforms, which is only 7.0 % of the total metabolites identified by the three platforms.

In this study, we employed a parallel 2DLC-MS platform that was configured with a HILIC column and a RPC column. The downside of such a configuration is that the metabolite coverage was not dramatically increased. Furthermore, one metabolite might be detected twice and therefore increased the chance of metabolite overlapping in mass spectrometry. However, the parallel 2DLC-MS configuration does not have the problem of metabolite partition between two or more fractions that occurs in the comprehensive 2DLC configuration. In addition, we can use long columns and long gradient times to

improve the separation. Overall, each platform, i.e., GC×GC-MS, 2DLC-MS/MS (-) or 2DLC-MS/MS (+), has limited metabolite coverage. The number of metabolites commonly detected by all those platforms is very small. Therefore, it is necessary to analyze the biological samples on different platforms to increase metabolite coverage.

5.3.4. Integrating GC×GC-MS and Parallel 2DLC-MS for Accurate Metabolite Quantification

During the metabolite identification, metabolites identified by parent ion m/z matching have a high ratio of false identifications compared with the identification by MS/MS spectrum matching. In order to ensure the high degree of confidence in biomarker discovery, only metabolites identified by MS/MS spectrum matching were used for the

(A)

(B)

(C)

(D)

metabolite quantification.

Figure 5.3. PLS-DA analysis of the metabolite profiles of all samples. (A) is the clustering result of 2DLC-MS (-) data. (B) is the clustering result of 2DLC-MS(+) data. (C) is the cross validation result of the PLSDA for analysis of 2DLC-MS (-) data. The magnitudes of R^2 and Q^2 are ($R^2 = 0.91$ $Q^2 = 0.40$). (D) is the cross validation results of the PLSDA for analysis of 2DLC-MS (+) data with $R^2 = 0.72$ and $Q^2 = 0.12$.

Figure 5.3 shows clustering results of the metabolite profiles of all samples (G1 and G2) analyzed on 2DLC-MS (-) and (+) using partial least-squares discriminant analysis (PLS-DA). The two groups show significant difference in metabolite profile (Figure 5.3(A) and 5.3(B)). The cross validation also shows a good predictive ability of the PLS-DA models, with Q^2 values of 0.40 for 2DLC-MS (-) data and 0.12 for 2DLC-MS (+) data (Figures 5.3(C) and 5.3(D)).

(A)

(B)

Figure 5.4. PLSDA analysis of the metabolite profiles of all samples. (A) is the clustering result of GC×GC-MS data. (B) is the cross validation results of the PLSDA for analysis of GC×GC-MS (+) data with $R^2 = 1.0$ and $Q^2 = -0.18$.

Figure 5.4 shows clustering results of the metabolite profiles of all samples (G1 and G2) analyzed on GC×GC-MS using PLS-DA. Compared to the 2DLC-MS, GC×GC-MS data show a certain degree of separation between the G1 and the G2 with a poor predictive ability of the PLSDA models where Q^2 value is -0.18.

A pairwise two-tail t -test with equal variance was used to study the abundance change of each metabolite between G1 and G2, during which sample labels were permuted up to 1000 times. Table 5.6 shows that 41, 13 and 11 metabolites were detected with significant changes in their abundance levels between G1 and G2 in the GC×GC-MS, 2DLC-MS (-) and 2DLC-MS (+) data, respectively.

Figure 5.5. Overlap of metabolites detected with significant changes in their abundance levels between groups by three platforms.

Figure 5.5 shows the overlap of those metabolites. It is clear that each platform detected different sets of metabolites that had significant changes in their abundance levels. Among those metabolites, two were detected by all three platforms and three were detected by two of the three platforms. Integrating the results of the three platforms generated a comprehensive set of metabolites, i.e., 58 metabolites.

Table 5.6. Metabolites in rat liver detected with significant changes in their abundance levels between G1 and G2 by different platforms

Metabolite ID	Metabolite name	<i>p</i> -value	Fold-change
GC×GC-MS			
1	1,2-Benzisothiazol-3-amine	4.30E-04	0.8
2	1-Butanol	2.20E-02	0.9
3	2-Pyrrolidone-5-carboxylic acid	7.40E-04	0.5
4	3-Aminobutyric acid	1.90E-02	0.6
5	3-Hydroxy-3-phenylpropionic acid	1.50E-02	1.4
6	3-Hydroxybutyric acid	2.50E-02	0.5
7	5-chloroindoline-2,3-dione	2.60E-02	0.7
8	5-Hexen-1-ol	3.00E-02	0.7
9	Acetimidamide	3.50E-02	0.6
10	Alanylglycine	9.30E-03	1.9
11	Benzoic Acid	2.70E-02	1.3
12	cis-11,14-Eicosadienoic acid	1.20E-02	1.8
13	DL-Glyceraldehyde	1.00E-02	0.1
14	Doconexent	5.50E-04	1.4
15	Eicosapentaenoic Acid	2.10E-02	2.2
16	Ethanolamine	3.80E-03	0.5
17	Heptadecanoic acid	3.40E-04	1.8
18	Hypoxanthine	8.60E-04	0.7
19	Isobutyl alcohol	9.10E-05	0.7
20	l-Aspartic acid	5.40E-04	0.5

21	l-Glutamine	4.50E-02	0.6
22	l-Leucine	4.50E-02	0.7
23	l-Lysine	8.50E-03	2
24	l-Malic acid	5.00E-02	1.6
25	l-Ornithine	4.10E-02	0.6
26	l-Phenylalanine	2.80E-02	0.6
27	l-Pyroglutamic acid	2.90E-02	0.7
28	l-Threonine	7.30E-05	0.4
29	Maleic acid	4.10E-03	1.5
30	Methylmalonic acid	5.70E-03	0.7
31	Niacinamide	3.20E-03	0.7
32	Nonanoic acid	1.20E-04	2
33	Octadeca-9,12-diynoic acid	9.80E-03	2.6
34	O-phosphoethanolamine	1.60E-03	0.5
35	Oxalic acid	7.90E-03	2.2
36	Propylene glycol	1.60E-02	1.7
37	Pyruvate	1.30E-02	0.5
38	Stearic acid	9.70E-04	1.3
39	Taurine	4.60E-04	5.6
40	Uracil	4.40E-02	1.2
41	α -Aminobutyric acid	5.60E-04	0.4
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2DLC-MS (-)			
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1	2-Methylglutaric acid	8.70E-03	2.3
2	4-Methyl-2-oxovaleric acid	3.10E-02	0.7
3	4-Oxoproline	3.90E-05	0.6
4	5-CMP	4.00E-02	1.4
5	Hypotaurine	1.40E-03	2.2
6	l-Glutathione oxidized	1.20E-04	2
7	l-Malic acid	4.90E-02	1.3
8	l-Ornithine	4.10E-02	0.7
9	N-acetylalanine	1.10E-02	1.5
10	Pyruvic acid	4.70E-02	0.5
11	Taurine	1.70E-03	1.8
12	Taurochenodeoxycholic acid	7.80E-05	8.4
13	α -Ketoglutaric acid	9.80E-03	0.4
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2DLC-MS (+)			
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1	4-Acetamidobutanoic acid	1.10E-02	1.4
2	4-Guanidinobutyric acid	6.50E-05	2.9
3	Carnitine	1.00E-02	0.8
4	Cytosine	4.70E-02	10.9
5	Hypotaurine	1.60E-03	2.3

6	l –Ornithine	3.20E-02	0.9
7	l –Phenylalanine	2.40E-02	0.7
8	N-acetyl-D-Glucosamine	4.50E-02	1.5
9	Proline	9.20E-03	0.9
10	Serine	1.80E-02	0.9
11	Taurine	3.20E-02	1.7

Table 5.7 and Table 5.8 respectively lists the details of the abundance information of those five metabolites that were detected by more than one platform. The regulation directions of those metabolites detected in different platforms agree to each other, i.e., the fold-change of a metabolite detected in different platforms are all either larger than 1.0 or less than 1.0 (Tables 5.7 and 5.8 and Figure 5.6). Furthermore, except taurine, the fold-changes of those metabolites are almost identical, indicating the robustness and accuracy of the three platforms.

Figure 5.6. The regulation directions of metabolites that showed significant changes in their abundance levels between groups in all platforms (GC×GC-MS, 2DLC-MS (-) and 2DLC-MS (+).

Table 5.7. Quantification information (*p*-value) of metabolites that were detected by more than one platform with significant changes in their abundance levels between groups

Compound	<i>p</i> -value		
	GC×GC-MS	2DLC-MS(-)	2DLC-MS(+)
Taurine	4.60E-04	1.70E-03	3.20E-02
Ornithine	4.10E-02	4.10E-02	3.20E-02
Phenylalanine	2.80E-02	5.70E-01	2.40E-02
Malic acid	5.00E-02	4.90E-02	-
Hypotaurine	-	1.40E-03	1.60E-03

Table 5.8. Quantification information (Fold change) of metabolites that were detected by more than one platform with significant changes in their abundance levels between groups

Compound	Fold-change		
	GC×GC-MS	2DLC-MS(-)	2DLC-MS(+)
Taurine	5.6	1.8	1.7
Ornithine	0.6	0.7	0.9
Phenylalanine	0.6	0.6	0.7
Malic acid	1.6	1.3	-
Hypotaurine	-	2.2	2.3

Figures 5.6(A), 5.6(B), and 5.6(C) depict the detection of L-ornithine in different platforms. This metabolite had relatively large instrument response and good

chromatographic peak shape in all three platforms, and its chromatographic peak did not overlap with other compounds. Therefore, the quantification results of this metabolites were almost identical among the three platforms, with fold-changes of 0.6, 0.7 and 0.9 in GC×GC-MS, 2DLC-MS (-) and 2DLC-MS (+), respectively. However, the fold-change of taurine detected by GC×GC-MS had a larger variation compared to those detected by 2DLC-MS (-) and 2DLC-MS (+). Figures 5.7E and 5.7F show that taurine had a good instrument response and good peak shape in 2DLC-MS (-) and 2DLC-MS (+), respectively.

Figure 5.7. Samples of instrument response of a metabolite affecting the quantification accuracy of that metabolite. (A) Three-dimensional chromatographic peak of l-ornithine detected by GC $\times$ GC-MS. (B) Extracted ion chromatograms of l-ornithine in a randomly selected biological sample detected by 2DLC-MS (-). (C) Extracted ion chromatograms of l-ornithine in a randomly selected biological sample detected by 2DLC-MS (+). (D) Three-dimensional chromatographic peak of taurine detected by GC $\times$ GC-MS. (E) Extracted ion chromatograms of taurine in a randomly selected biological sample detected by 2DLC-MS (-). (F) Extracted ion chromatograms of taurine in a randomly selected biological sample detected by 2DLC-MS (+).

Therefore, the changes of its abundance levels between G1 and G2 detected by 2DLC-MS (-) and 2DLC-MS (+) were very similar with fold-change of 1.8 and 1.7, respectively. However, the instrument response of taurine in GC $\times$ GC-MS was low and its chromatographic peak in the second dimension GC was very poor (Figure 5.7(D)). Furthermore, the chromatographic peak of taurine overlapped with an abundant peak. For those reasons, the data analysis software ChromaTOF could not accurately quantify the abundance of taurine, and resulted in a large variation in its fold-change compared with the fold-change calculated from the 2DLC-MS (-) and 2DLC-MS (+) data.

Overall, the GC $\times$ GC-MS, 2DLC-MS (-), and 2DLC-MS (+) platforms implemented in this study are robust for metabolite quantification. Manual analysis of the data reveals that the variation in metabolite quantification was mainly induced by the poor instrument response and the limited accuracy of data analysis software.

5.3.5. Biomarker Discovery and Pathway Analysis

For metabolic pathway analysis, we first used the metabolites detected by each platform that had significant changes in their abundance levels between groups as the input of MetaboAnalyst software¹³⁴, to recognize the pathways that were affected by the treatments of rats in G1 and G2. From the 41 significant metabolites found from the GC×GC-MS data, the MetaboAnalyst software produced five pathways with $p \leq 0.05$ (Table 5.9).

Table 5.9. Pathways affected by the treatment difference between groups

Significantly impacted pathways	<i>p</i> -value	Match status
Integrated platforms		
Aminoacyl-tRNA biosynthesis	1.10E-03	8/69
Arginine and proline metabolism	2.10E-03	6/44
Valine, leucine and isoleucine biosynthesis	4.10E-03	3/11
Butanoate metabolism	4.30E-03	4/22
Alanine, aspartate and glutamate metabolism	5.90E-03	4/24
Biosynthesis of unsaturated fatty acids	9.10E-03	5/42
d-Glutamine and d-glutamate metabolism	9.30E-03	2/5
Taurine and hypotaurine metabolism	2.40E-02	2/8
GC×GC-MS		
Biosynthesis of unsaturated fatty acids	2.30E-03	5/42
Aminoacyl-tRNA biosynthesis	4.20E-03	6/69
Butanoate metabolism	1.30E-02	3/22
Alanine, aspartate and glutamate metabolism	1.70E-02	3/24
Valine, leucine and isoleucine biosynthesis	2.50E-02	2/11
2DLC-MS (-)		
Taurine and hypotaurine metabolism	1.20E-03	2/8
Valine, leucine and isoleucine biosynthesis	2.40E-03	2/11
Citrate cycle (TCA cycle)	8.00E-03	2/20
Butanoate metabolism	9.60E-03	2/22
Alanine, aspartate and glutamate metabolism	1.10E-02	2/24
D-Glutamine and D-glutamate metabolism	3.50E-02	1/5
Primary bile acid biosynthesis	3.90E-02	2/46
2DLC-MS (+)		
Arginine and proline metabolism	2.30E-04	4/44
Taurine and hypotaurine metabolism	1.50E-03	2/8
Aminoacyl-tRNA biosynthesis	1.40E-02	3/69
Phenylalanine, tyrosine and tryptophan biosynthesis	3.10E-02	1/4
Cyanoamino acid metabolism	4.60E-02	1/6

Likewise, MetaboAnalyst showed that seven pathways were statistically significant using the 13 significant metabolites detected from the 2DLC-MS (-) data, and 5 pathways using the 11 significant metabolites detected from the 2DLC-MS (+) data. We then combined all 58 significant metabolites detected from the three platforms and used them as the input of MetaboAnalyst software. By doing so, eight pathways were considered as significantly impacted pathways (Table 5.9).

Figure 5.8. Overlap of the pathways that were affected using different sets of significant metabolites for metabolic pathway analysis.

Figure 5.8 shows the overlap of the pathways using different sets of significant metabolites for metabolic pathway analysis. Compared with the results of pathway analysis using the data acquired by individual platform, the confidence of pathway assignment using the integrated data was significantly increased. For instance, 8 metabolites associated with

aminoacyl-tRNA biosynthesis pathway were detected by integrating the data of all three platforms. However, 0, 3 and 6 metabolites were detected by 2DLC-MS (-), 2DLC-MS (+) and GC×GC-MS, respectively.

Using the integrated data acquired from multiple platforms clearly increase the confidence of metabolic pathway analysis. The confidence of assigning others pathways was also increased, including arginine and proline metabolism; valine, leucine and isoleucine biosynthesis; alanine, aspartate and glutamate metabolism; D-glutamine and D-glutamate metabolism; and butanoate metabolism.

We also performed another analysis by matching all detected metabolites to the metabolic pathway regardless whether those metabolites had significant changes in their abundance levels between groups. For example, biosynthesis of unsaturated fatty acid pathway was a significantly impacted pathway as suggested by the 58 significant metabolites detected from the three platforms. Supplementary Table S5 lists the metabolites in this pathway that were detected by different platforms. Docosahexaenoic acid (DHA), linoleic acid (LA), and gamma-linolenic acid (GLA), were only detected by GC×GC-MS. Prostaglandin G2, leukotriene B4, LXA₄/LXB₄ were detected by 2DLC-MS (-), and stearidonic acid was only detected by 2DLC-MS (+). Detecting more metabolites in a pathway and knowing whether their abundance levels were affected by the biomedical treatments clearly can narrow down to the specific steps in the pathways that were affected by the treatments.

Table 5.10. Detection of metabolites from the “biosynthesis of unsaturated fatty acid pathway” by different platforms

Compound Name	GC×GC-MS	2DLC-MS(-)	2DLC-MS(+)
α -linolenic acid, ALA	x ^a	X	X
Stearidonic acid	x	X	y ^b
Arachidonic acid	y	Y	X
Eicosapentaenoic acid, EPA	y	Y	Y
Docosapentaenoic acid (DPA)	x	Y	Y
Docosahexaenoic acid, DHA	y	X	X
Linoleic acid, LA	y	X	X
Gamma-linolenic acid, GLA	y	X	X
Dihomogamma-linolenic acid, DGLA	y	X	Y
PG2 (prostaglandin G2)	x	Y	X
TXA2 (thromboxane A2)	x	X	X
leukotriene B4	x	Y	X
LXA4	x	Y	X
LXB4	x	Y	X

In summary, one analytical platform is not enough to give high metabolite coverage in a metabolic pathway, and the use of only one platform reduces the confidence of metabolic pathway assignment. Integrating the data acquired from multiple platforms not only provides high metabolite coverage, but also increases the confidence of pathway assignment and the confidence of biomarker discovery. The details of the biomarkers and pathways discovered in this study will be described in a separate report.

5.4. Conclusions

Polar metabolites extracted from rat livers were analyzed by GC×GC-MS and parallel 2DLC-MS, respectively. 903 ± 96 chromatographic peaks were detected by GC×GC-MS, and $2,467 \pm 144$ and $4,422 \pm 153$ features were respectively detected by 2DLC-MS (-) and 2DLC-MS (+). Integrating the experimental data acquired from the three platforms clearly increased the metabolite coverage. A total of 58 metabolites were detected with significant

changes in their abundance levels between groups. Three of the 58 metabolites were detected in two platforms and two in all three platforms. The agreement of metabolite regulation detected by different platforms demonstrated the robustness and accuracy of the three platforms used in the current study. Manual examination showed that the discrepancy of metabolite regulation measured by different platforms was mainly caused by the poor shape of chromatographic peaks. Pathway analysis demonstrated that integrating the results from multiple platforms increased the confidence of metabolic pathway assignment. While the developed method showed excellent results in terms of metabolites coverage and increased confidence of metabolic pathway assignment, however, several factors still need to be worked on for better identification and quantification. Metabolite identification still remains as a major challenge in untargeted metabolomics. While huge number of isotopic peaks (raw data) were generated from the instrument, most of them remained unidentified. Incompleteness of the database contributed to this problem.

Future endeavor would be the combination of all available databases to maximize the metabolite assignment and develop robust bioinformatics tools for accurate metabolite identification. Furthermore, a comparison of the parallel 2DLC-MS results with the comprehensive 2DLC-MS results would enlighten the overall capability of the multidimensional analytical technique for the untargeted metabolomics.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Metabolomics is a branch of 'omics study that is still being enriched by more instrumental development as well as the method development. While GC×GC-MS has extensively been used in different scientific fields, its use in untargeted metabolomics is limited due to the complicated data analysis especially metabolites identification. This dissertation summarizes our efforts in developing innovative methods to accurately calculate the second dimension retention index from GC×GC-MS data. We first developed a novel approach using the retention information of entire reference compounds to construct the retention surface for calculation of the second-dimension retention index for a query compound. This approach reduces the variation and increased the accuracy of the 2I calculation, which in turns greatly increase the confidence of metabolite identification. In Chapter Three, we developed a method that considered the second-dimension separation as temperature gradient mode and avoided the hold-up time calculation. In Chapter Four, we developed a universal reference method that employs multiple classes of reference compounds as reference compounds to greatly increase the coverage of retention map area, and therefore to eliminate the use of extrapolation. Our long term goal is to apply the developed methods to large scale metabolomics to aid metabolite identification in GC×GC-MS.

A majority of current metabolomics studies are carried out using a single analytical platform such as LC-MS or GC-MS, and therefore have limited metabolite coverage. While GC×GC-MS and 2DLC-MS has been separately used in metabolomics for high metabolite coverage, each of them still has limited metabolite coverage. In this dissertation, GC×GC-MS and parallel 2DLC-MS, for the first time, were used to analyze the same biological sample. Each of these analytical platforms separates metabolites using different mechanisms. Therefore, integration of the metabolomics data generated from these platforms can greatly increase the metabolite coverage. Furthermore, some metabolites can be identified and quantified by both analytical platforms. Detecting the same metabolite on multiple platforms provides a chance to validate the accuracy of metabolite quantification. The analysis results of analyzing the metabolite extract of a biological sample using GC×GC-MS and parallel 2DLC-MS demonstrated that the integrated platform not only provides high metabolite coverage and accurate metabolite quantification, but also reliable metabolic pathway assignment.

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APPENDIX
LIST OF ABBREVIATIONS

GC-MS: Gas chromatography mass spectrometry

GC×GC-MS: Two-dimensional gas chromatography mass spectrometry

GC×GC-TOF MS: Two-dimensional Gas Chromatography Time-of-flight Mass
Spectrometry

LC-MS: Liquid chromatography mass spectrometry

2DLC-MS: Two-dimensional liquid chromatography mass spectrometry

EI: Electron Impact

CI : Chemical ionization

ESI: Electro-spray ionization

t_R : Retention time

I^T : Linear retention index

I : Kovats retention index

$t_{R(u)}$: Retention times of the compound u

$t_{R(n)}$: The n -alkane with n carbon atoms

$t_{R(n+1)}$: The n -alkane with $n+1$ carbon atoms

$^2t'_R$: Adjusted second dimension retention time

KEGG: Kyoto Encyclopedia of Genes and Genomes

mL: milliliter

MS: Mass Spectrometry

RP-LC: Reverse Phase Liquid Chromatography

RSD: Relative Standard Deviation

SIR: Selected Ion Recording

SRM: Selected Reaction Monitoring

CURRICULUM VITAE

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ACADEMIC RECORDS

- **Ph.D., Chemistry, University of Louisville, Louisville, KY (Fall-2019)**
Cumulative GPA: 3.72 /4.0
Supervisor: Prof. Xiang Zhang
- **M.S., Chemistry, University of Louisville, Louisville, KY (Spring-2018)**
Cumulative GPA: 3.87 /4.0
Supervisor: Prof. Xiang Zhang
- **B.S., Applied Chemistry & Chemical Engineering, University of Dhaka, (2012)**
Cumulative GPA: 3.54/4.0

RESEARCH EXPERIENCE

- ❖ *January-2015 – December 2019, Ph.D., University of Louisville, Louisville, Kentucky*
- **Methods of calculating the second dimension retention index for GC×GC-MS based metabolomics**
 - Developed a surface fitting method for second dimension retention index calculation to increase the confidence in compound identification from GC×GC-MS
 - Developed a method to eliminate the need for calculation of the hold-up time of the second dimension column for retention index calculation
 - Developed a method for generalization of the reference system to calculate the second dimension retention index in GC×GC-MS

- Developed a universal reference system for the second dimension retention index calculation to allow the use of any compounds as reference
- **Integration of GC×GC-MS and parallel 2DLC-MS for untargeted metabolomics**
 - Developed an integrated approach for untargeted metabolomics using GC×GC-MS and parallel 2DLC-MS
 - Implemented the developed method for analysis of polar metabolites extracted from rat livers
 - Achieved high metabolite coverage and accurate metabolite quantification, and high confidence metabolite biomarker discovery & metabolic pathway assignment
- **Simultaneous quantification of straight-chain and branched-chain short chain fatty acids**
 - Participated in development of a method for simultaneous quantification of straight-chain and branched-chain SCFAs
 - Two columns with different stationary phases were hyphenated together to achieve optimal separation of the SCFAs
 - Obtained the best LOD and LOQ for detecting all SCFAs from biological samples

INDUSTRY EXPERIENCE

- One month training on “**Industrial Process Unit Operation & Process Control Technique**” in Training Institute for Chemical Industries (TICI); Polash, Narshindi, Bangladesh.
- Intern at Ghorasal Fertilizer Factory Limited (an enterprise of **Bangladesh Chemical Industry Corporation**) to complete supervision on Chemical Process reaction, Process Control, Mechanical Equipment.

TEACHING EXPERIENCE

- **CHEM 207, INTRO TO CHEM ANALYSIS I**, Summer 2015, 2016, 2017; Fall 2015, 2016; Spring 2016, 2017.
- **CHEM 208, INTRO TO CHEM ANALYSIS II**, Summer 2015, 2016, 2017; Fall 2015; Spring 2016, 2017.

PEER REVIEWED PUBLICATIONS

- **Prodhan, M.A.I.;** Song, M.; He, L.; Shi, B.; Fang, Y.; Yin, X.; McClain, C.J.; Zhang, X. Integrating comprehensive two-dimensional gas chromatography mass spectrometry and two-dimensional liquid chromatography mass spectrometry for untargeted metabolomics. *Analyst* **2019**, DOI: 10.1039/C9AN00560A.

- Dennis R. Warner, Jeffrey B. Warner, Josiah E. Hardesty, Ying L. Song, Taylor N. King, Jing X. Kang, Chih-Yu Chen, Shanfu Xie, Fang Yuan, **Md Aminul Islam Prodhhan**, Xipeng Ma, Xiang Zhang, Eric C. Rouchka, Joan Whitlock, Eric C. Li, Gary P. Wang, Craig J. McClain, and Irina A. Kirpich. Beneficial effects of decreased omega-6:omega-3 PUFA ratio on intestinal homeostasis and microbiota are linked to the improvement of liver injury associated with ethanol and LPS administration in mice. *Manuscript* in preparation.
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PRESENTATIONS

- **Prodhhan, M.A.I.**; Shi, B.; Yin, X.; Feng, W.; McClain, G.; Zhang, X. Integrating comprehensive two-dimensional gas chromatography mass spectrometry and two-

dimensional liquid chromatography mass spectrometry for metabolomics. *Pittcon 2019*, Philadelphia, PA, March 17-21, 2019. (Oral)

- **Prodhan, M.A.I.**; Shi, B.; Yin, X.; Feng, W.; McClain, G.; Zhang, X. Integrated parallel two-dimensional liquid chromatography mass spectrometry and comprehensive two-dimensional gas chromatography mass spectrometry for metabolomics. *HPLC 2018*, Washington, DC, July 29-August 2, 2018. (Oral)
- **Prodhan, M.A.I.**; Yin, X.; Kim, S.; McClain, C.J.; Zhang, X. Calculation of the Second Dimension Retention Index in GC×GC-MS Excluding Hold-up Time Calculation. *70th Midwest University Analytical Chemistry Conference (MUACC)*, Athens, OH, October 20-21, 2017. (Oral)
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- **Prodhan, M.A.I.**; Yin, X.; Kim, S.; McClain, C.J.; Zhang, X. Universal Reference System for Calculating the Second Dimension Retention Index in GC×GC-MS. *Research! Louisville- 2018*, Louisville, KY, October 8, 2018. (Poster)
- He, L.; Li, F.; Yuan, F.; Yin, X.; **Prodhan, M.A.I.**; McClain, G.; Feng, W.; Zhang, X. Relative quantification of metabolites in fecal samples using three analytical platforms and the role of cathelicidin-related antimicrobial peptide in alcoholic liver disease. *Research! Louisville*, University of Louisville, Louisville, KY, October 12, 2018. (Poster)
- **Prodhan, M.A.I.**; Yin, X.; Kim, S.; McClain, C.J.; Zhang, X. Surface Fitting for Calculating the Second Dimension Retention Index in GC×GC-MS. *70th Midwest University Analytical Chemistry Conference (MUACC)*, Athens, OH, October 20- 21, 2017. (Poster)

AWARDS AND RECOGNITIONS

- Institute for Molecular Diversity and Drug Design (IMD3) – Travel Award, University of Louisville, 2017
- Graduate Network in A&S (GNAS) Travel Award, University of Louisville, 2017
- Department of chemistry Travel Award, University of Louisville, 2019

- People's Republic of Bangladesh Government Scholarship for Outstanding result in Secondary School Certificate examination.

PROFESSIONAL MEMBERSHIPS

- American Society for Mass Spectrometry (ASMS)
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SKILLS

- **Analytical instrumentation** : HPLC, 2DLC-MS/MS (ThermoScientific QExactive- HF), GC-MS, GC×GC-MS, FT-MS, QqQ-MS, AAS
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- **Extra-curricular activities** : Ex- scout