ANALYSIS OF FATTY ACID METHYL ESTERS BY TWO-DIMENSIONAL GAS CHROMATOGRAPHY USING CRYOGEN-FREE MODULATOR

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การวิเคราะห์เมทิลเอสเทอร์ของกรดไขมันด้วยแก๊สโครมาโทกราฟี สองมิติที่ใช้โมดูเลเตอร์แบบไม่ใช้สารหล่อเย็น

นายเอเดรียน โฮเซ่ ซาคาน

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Field of Study	Green Chemistry and Sustainability
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เอเดรีนย โฮเซ่ ซาคาน : การวิเคราะห์เมทิลเอสเทอร์ของกรดไขมันด้วยแก๊สโครมาโทกราฟี สองมิติที่ใช้โมดูเลเตอร์แบบไม่ใช้สารหล่อเย็น . (ANALYSIS OF FATTY ACID METHYL ESTERS BY TWO-DIMENSIONAL GAS CHROMATOGRAPHY USING CRYOGEN-FREE MODULATOR) อ.ที่ปรึกษาหลัก : ผศ. ดร.ชฎิล กุลสิงห์

แก๊สโครมาโตกราฟีแบบสองมิติอย่างทั่วถึงเป็นเทคนิคที่มีประสิทธิภาพสูง มีความคุ้มค่าและเป็นมิตรต่อสิ่งแวดล้อม โดยทำงานร่วมกับเครื่องตรวจวัดแมสสเปกโทรมิเตอร์ และเฟลมไอออนไซเซชันดีเทคเตอร์ โดยในงานนี้ต้องการศึกษากรดไขมันในตัวอย่างนม ดังนั้นจึงได้พัฒนาเทคนิค SNAT ที่ทำการดักจับสารแบบเทียมโดยไม่ต้องใช้น้ำแข็ง ซึ่งใช้คอลัมน์สำหรับการแยกสารทั้งแบบกึ่งไม่มีขั้ว และแบบมีขั้ว ท่อสำหรับปรับสมดุลของการไหลในระบบและดีนสวิตช์ ซึ่งเป็นอุปกรณ์สำหรับควบคุมการจับและส่งสารจากระบบโดยใช้การส่งผ่านสารแบบฮาร์ตคัตอย่างทั่วถึง โดยอุปกรณ์ที่จำเป็นจะเป็นตัวแยกแบบหลายตัวระหว่างทางออกของคอลัมน์แรกและดีนสวิตช์ ซึ่งใช้ในกระบวนการดักจับแบบเทียม ซึ่งประกอบด้วยการแยกพีคของสารอย่างเป็นระบบ การหน่วงเวลาหลังแยก การซ้อนทับกันของพีก และการฮาร์ตคัตเลือกกลุ่มสาร โดยเวลาที่ใช้ในการดักจับสารตัวอย่างและระยะเวลาในการแยกในคอลัมน์ที่สอง สามารถปรับได้ด้วยจำนวนตัวแยกที่แตกต่างกัน ซึ่งจะช่วยให้สามารถดักจับสารประกอบที่ระเหยง่ายและยากได้ภายในการฉีดครั้งเดียว โดยไม่ต้องใช้ระบบน้ำแข็งในการดักจับสาร และยังช่วยเพิ่มขีดความสามารถสูงสุดในการแยกพีคของสารในระบบแก๊สโครมาโตกราฟีแบบสองมิติ ซึ่งไม่สามารถทำได้ด้วยเทคโนโลยีทั่วไป สิ่งนี้จะถูกนำไปใช้เพื่อระบุสารประกอบในตัวอย่างนม และมีการเปรียบเทียบผลของเทคนิค SNAT และวิธีที่ต้องใช้น้ำแข็งเทียมด้วย อีกทั้งยังได้ศึกษาผลของการวิเคราะห์ซ้ำในสารละลายแบบผสม ได้ค่าเบี่ยงเบนมาตรฐานสัมพัทธ์น้อยกว่า 10

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Adrian Jose Sacan : ANALYSIS OF FATTY ACID METHYL ESTERS BY TWO-DIMENSIONAL GAS CHROMATOGRAPHY USING CRYOGEN-FREE MODULATOR . Advisor: Asst. Prof. Chadin Kulsing, Ph.D.

A simple and environmentally friendly method in two-dimensional gas chromatography coupled with mass spectrometry (GC-MS) and flame ionization detector has been developed for the analysis of fatty acids in commercial milk products. This applies splitter-based non-cryogenic artificial trapping (SNAT) modulator with unique feature imitating the cryogenic modulation process. The method involves chemical derivatization into fatty acid methyl esters (FAME) with methanolic potassium hydroxide, liquid-liquid phase extraction using hexane, and determination of the FAME composition. The GC×GC approach revealed improved separation of FAME using SNAT modulator with better separated peaks, a ten-fold higher total peak capacity and improved compound identification, compared to 1D GC-MS system. SNAT modulator also showed several areas of FAMEs mapped by longitudinally modulated cryogenic system trapped at 10 ^{0}C whereas the gas chromatography-flame ionization detector (GC-FID) using a cryogenic modulator revealed the absence of separated peaks that were previously seen in the other two GC systems. The method precisions have also been investigated with a known set of standard compounds with relative standard deviation percentages of <10%. The developed approach is considered economical without cryogen consumption.

Field of Study:	Green Chemistry and Sustainability	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Problem definition

Fatty acids presented in commercial goods such as milk and butter need to be evaluated as their consumption may cause diverse effects on health and body. Many techniques that analyze fatty acids require chemical derivatization that uses excessive solvents in the esterification and extraction process [1-4].

It is known that the success of GC x GC analysis is attributed on the modulation process of the instrument. Modulation allows the segregation of effluent fragments from the first column, and their re-injection onto the second column, throughout the analysis continuously and subsequently [1]. However, the use of the current and main modulators has known disadvantages [3]. Heat based modulators are not suitable for highly volatile compounds such as the fatty acids due to the limited thermal stability of the sorbent. Cryogenic modulators also have drawbacks due to the excessive consumption of nitrogen gas and the ineffective release of high molecular weight compounds. Flow modulators eventually become good alternatives to thermal based devices but the inapplicability of a required longer ^{2}D column and longer modulation period due to a small injection channel inside a microfluidic device or in a T-junction dead volume reduces lower ^{2}D separation power. There is also a cryogen-free H/C analysis that could address the

problems of previous modulators but its main disadvantage is a longer analysis time [5]. The limitations of the current modulators and the current chemical derivatization techniques make it difficult for FAME analysis to be considered green [1, 5].

However, a novel non-cryogenic modulator called as Splitter-based Non-cryogenic Artificial Trapping (SNAT) was made which described the use of a novel cryogen-free modulation mechanism whose unique feature imitated the peak splitting and focusing responsible for its efficiency and better selectivity and was not found in valve based and other conventional flow based modulators [4]. This breakthrough gives us a direction to propose that the application of an optimized sample preparation method in the derivatization of fatty acids in a 2D GC-MS with SNAT device will be greener and more capable of artificially modulating highly volatile compounds. It is predicted that the combination of the two will give better peak separation and identification and more importantly, it will highlight the fundamental principles of green chemistry in terms of efficiency, less consumption of harmful substances, better economic costs, and a shorter period of analysis.

1.2 Literature review

Commercial goods like milk are made of important short-chain fatty acids, complex mixture of cis and trans-unsaturated acids with 18 carbons and other compounds that form their unique matrices. The oil and fats of milk products from

toe-even four legged mammals are different compared to those found in plants and other sources. Since many of these products are known to be expensive because of the presence of their unique natural oil and fats, other products are commonly cheap due to the replacement of these compounds with cheaper analogues [6-8]. The adulteration of milk, butter and processed oil is possible but it can be monitored by identifying and quantifying the fatty acids present in a sample known as fatty acid profiling or the analysis of fatty acid methyl esters (FAMEs). The final fatty acid composition profile gives critical assistance in valuing the authenticity these products sold and consumed in the marketplace [5].

GC can successfully identify the fatty acids by employing the appropriate sample preparation method. The most common used techniques include the direct esterification of fatty acids with methanol using an acid or a base with basic catalysis to be considered faster. However, the said method regardless of the identity of the base requires strict anhydrous conditions that may affect the quality and time management of the analysis. In addition, excessive amounts of toxic solvents such as hexane and dichloromethane are used to separate the resulting fatty acid methyl esters from the aqueous part to the organic part of the solution [6-8]. Analysis of FAME by gas chromatography requires a sample preparation that uses large volumes of organic solvents that can seriously affect both the environment and human well-being. This is true when the compounds of interest are within samples

whose matrices are complex. There is definitely a need to limit the impacts of these solvents and that can only be attained when researchers push for more sustainability and greener methods [7, 8]. The use of comprehensive two-dimensional gas chromatography (GC x GC) has demonstrated excellent separation capacity for complex mixtures of chemicals ranging from fatty acid analysis and metabolite profiling [2, 6, 8-10]. The number of separated peaks could be three to ten-fold more detected in GC x GC when results are compared to one-dimensional gas chromatography analysis. The high-resolution strategies of the analysis of complex mixtures are all because of the two important components of the instrument: a.) the two separate columns, and b.) the use of a modulator. In GC x GC, the use of two columns allows further analysis of the compound of interest by subjecting the effluent of the first column to the second dimension. The typical result obtained from GC x GC is a modulated chromatogram which can be converted into a contour plot with the retention time in ^{1}D and ^{2}D separations as the x- and y-axes, respectively. This is illustrated in **Figure 1**. The peak capacity could be roughly approximated as the product of peak capacities in ^{1}D and ^{2}D separations ($^{1}n_c \times {}^{2}n_c$).

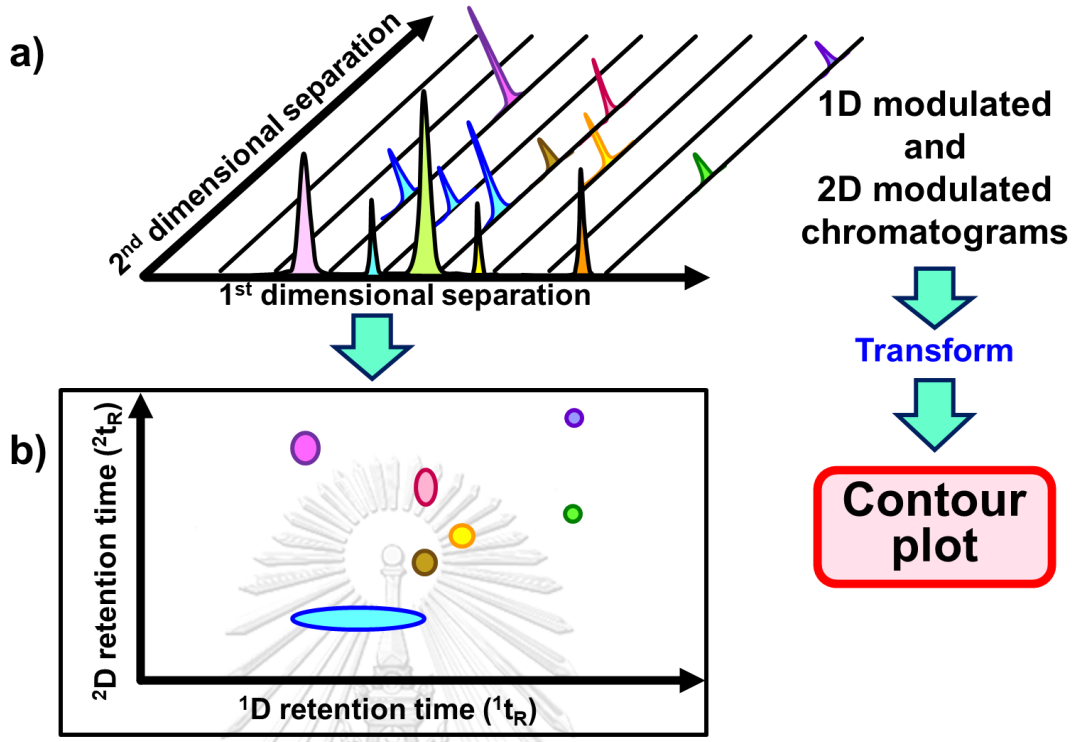

Figure 1*. a) Aligned 1D modulated chromatograms and b) contour plot.*

A modulator, an interface between the two columns is responsible for the transfer of components from ^{1}D to ^{2}D column [4]. Modulators can be classified as heat-based, cryogenic, valve-based and flow according to their functions. Heat-based modulators are not suitable for low volatility compounds such as the fatty acids and triglycerides due to the limited thermal stability of the sorbent [9, 11, 12]. Valve-based modulators also showed limited applicable temperature range. Cryogenic modulators also have drawbacks due to the excessive consumption of nitrogen gas and the ineffective release of high molecular weight compounds [4]. Flow modulators eventually become good alternatives to thermal-based devices but

the inapplicability of a longer ^{2}D column, longer modulation period due to the small injection channel inside the interface makes them less reliable and inefficient. There is also a cryogen- free H/C analysis that could address the problems of previous modulators but its main disadvantage is a longer analysis time. Studies about the right modulator for compounds of interest are still considered in progress [4, 13-16].

By considering the limitations of all the modulation approaches described above, development of a new modulation approach is still required. In 2022, the use of GC x GC system that employs semi-nonpolar/polar columns, a restrictor, and a microfluidic Dean's switch (DS) enabled periodic multiple heartcuts (H/C) and an array of splitters found between the ^{1}D column outlet and the DS. A systematic approach in GC x GC called Spliter-based non-cryogenic artificial trapping (SNAT) modulation demonstrates the use of artificial split-and-trap mechanism made of 1) a systematic pulse splitting, 2) split-pulse delay and (3) the combination of pulse before 4) the periodic selective H/C of the pulse that overlaps. The overall process is shown in **Figure 2**.

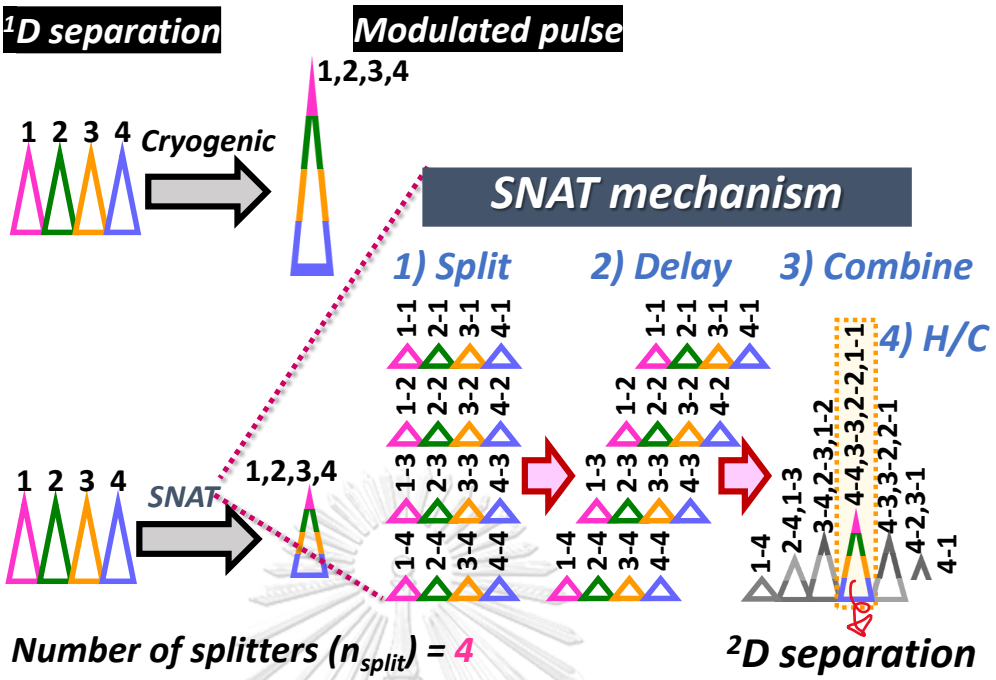

Figure 2. Diagram illustrating SNAT modulation mechanism compared with the cryogenic modulation of model peaks 1-4.

This process is similar to the mechanism in a cryogenic phenomenon albeit with the split comprehensive compound profile transferred into the ^{2}D separation. Specific components of a sample are selectively sampled or heart-cut onto the ^{2}D column and the other parts of split peaks must be delayed for the next heart-cut interval before moving to the ^{2}D column. As a result, H/C zones during the modulation periods are expected to give confirming peaks that are missed out in the previous modulated parts of a sample zone which usually occurs in 1D gas chromatography where some compounds are not trapped to give the regular detected signal [4, 13, 15]. This is the reason why GC x GC analysis is more effective when the modulator used undergoes heart-cutting all the artificially modulated

zones onto the ^{2}D column to confirm the identity and separation of peaks based on the combination of their split images [1, 3, 4, 7, 13, 15].

1.3 Aims, scope and benefit of this work

A splitter-based non-cryogenic artificial modulation technique (SNAT) in GC x GC has been created in 2022 to provide the artificial modulation of highly volatile compounds such as alcohols, alkanes and acetates in complex samples. However, the method has to established yet on its peak separation on the derivatization of triglycerides leading to the analysis of fatty acids in complex matrices using contour plot [4]. This study compares the performance of two-dimensional gas chromatography (GC x GC) hyphenated with mass spectrometry using SNAT and the multi-dimensional gas chromatography hyphenated with flame ionization detector (FID) using a cryogenic modulator in the analysis of fatty acid methyl esters (FAMEs). In order to assess the validity of the method and to achieve the best peak separation of FAMEs, this research covers samples of commercial-based liquid pasteurized milk products whose matrix is considered complex due to the presence of other compounds. This study avoids the use of massive amounts of cryogenic substances such as carbon dioxide (CO_2) and nitrogen (N_2) gases in the modulation process of highly volatile compounds, both of which are essentially needed, known to be expensive and are harmful to the environment. This novelty also proves the

flexibility of using SNAT as a cryogen-free modulator in GC x GC in the analysis of several compounds including FAMEs.

CHAPTER II

THEORY

2.1 Chemical derivatization of fatty acids

Reactions such as derivatization of sample components before GC analysis are quite common since most of the liquid-based microextraction technique require chemical derivatizations to be compatible with GC. The main goals of this method include (1) conversion of polar or nonvolatile compounds to relatively nonpolar or volatile products; (2) improvement of thermal stability of target compounds; (3) increasing the detector response by making certain functional groups inclusive which result to higher detector signals; or (4) enhancement of GC separation performance [17].

There is a wide range of derivatization reagents that are commercially available. While derivatization phase ranges to at least a couple of minutes, reactions like trans-esterification of fatty acids can be achieved within 2 minutes at room temperature for glycerolipids in order to convert undetectable fatty acids in GC into fatty acid methyl esters [18]. These methods include KOH- or NaOH-catalyzed methanolysis and the use of hazardous and corrosive chemicals like BF_3 and H_2SO_4 [19, 20]. The conventional use of base may appear rapid if time is of essence but basic methanolysis does not react with free fatty acids which are commonly present in commercially available milk. Regardless of the its disadvantage, basic catalysis is still considered better than the use of acids in catalysis because of the former's

scientific findings of not being too risky for the environment and of being economical as well. Commercial dairy products are known to have fatty acids and triglycerides from lipids. An ordinary pasteurized milk is made of 3-5% lipids and fats, 4% proteins, 6% minerals and carbohydrates and more than 85% water. The derivatization of fatty acids into FAMEs are conducted via transesterification of triglycerides using methanol whose mechanism of action is demonstrated in **Figure 3**.

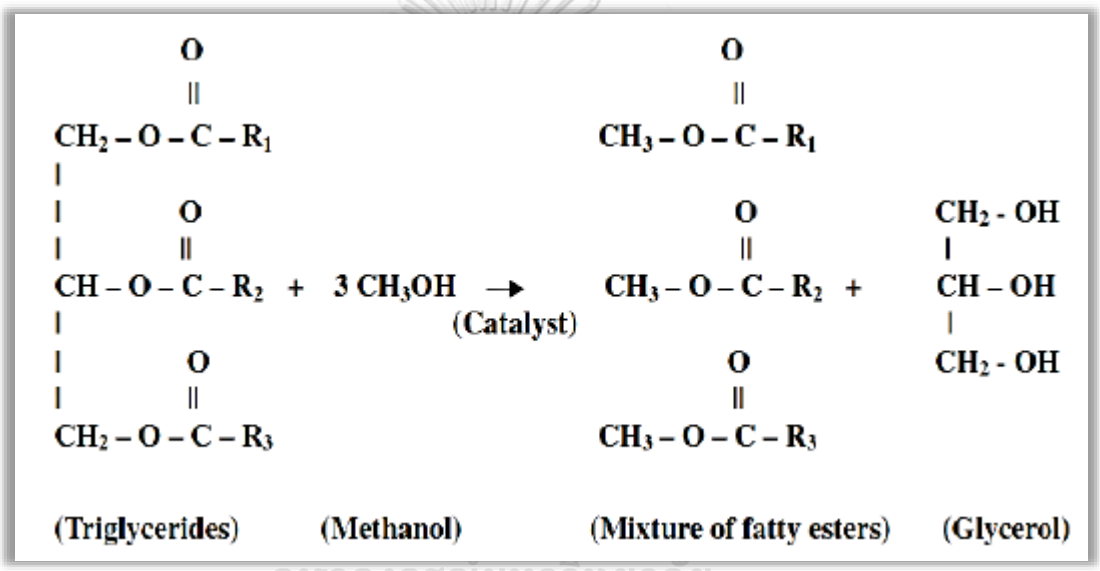

***Figure** 3. Mixtures of fatty acid methyl esters and glycerol are formed in the transesterification reaction of triglyceride using methanol.*

Chemical derivatization reactions are termed as in situ derivatization and can be performed simultaneously with extraction. A derivatizing agent is neither nor added to the sample solution or loaded into the extraction solvent. After reaction/extraction, the derivatized products are collected and analyzed. The common *in situ* approach improve analyte enrichment, and the systematic

procedure replaces the multistep operation used in conventional derivatization means [21]. Pre-extraction derivatization is recommended when the reaction requires a longer time, higher temperature, or other conditions that are not the same with simultaneous microextraction [22, 23]. Extraction is performed after the derivatization reaction is completed. The derivatized fatty acids are expected to have higher affinity with the extraction solvent after its conversion into fatty acid methyl esters which can be effectively separated and detected in the gas chromatographic analysis.

2.2 Analysis using gas chromatography

2.2.1 One-dimensional gas chromatography (1DGC)

GC is a separation technique of compounds in sample mixtures whose system is equipped with an oven consisting of a mobile phase (or carrier gas) as an inert gas, an injection port and a column classified as the stationary phase. Compound separation is due to the difference of boiling points and polarities of compounds which also includes the different strengths of interactions of the compounds with the stationary phase. Compounds with stronger affinity to the stationary phase take a longer time to move through the column while compounds with weak interactions travel faster.

Figure 4 shows the components of the one-dimensional gas chromatography hyphenated with mass spectrometry. 1DGC is only manned with one column and this causes problems especially with complex matrices.

Examples of these problems are co-eluting compounds resulting to broadening of the peaks that are difficult to be identified by NIST software and other libraries. Thus, the reliability of this method particularly in separating compounds from complex matrices is still in question. However, this method is a good baseline in determining the efficacy of the sample preparation.

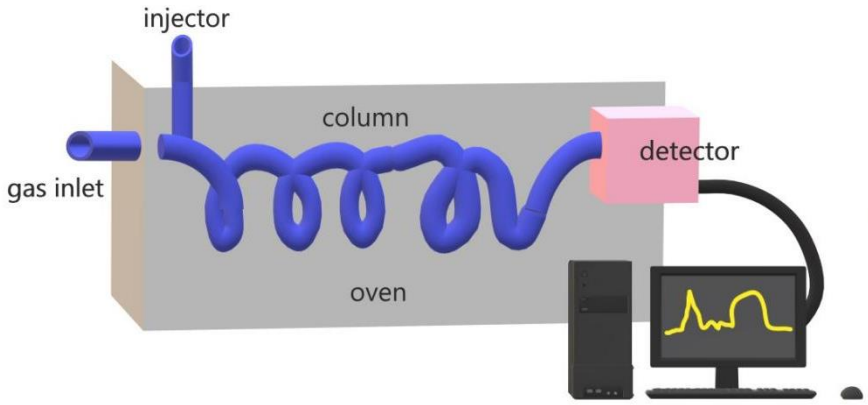

Figure 4. *One-dimensional gas chromatography and its components.*

2.2.2 Two-dimensional gas chromatography (GCxGC)

The introduction of Comprehensive Two-Dimensional Gas Chromatography (GC×GC) using resistively-heated modulation by Liu and Phillips in 1991, which fully demonstrated the concept of comprehensiveness in multidimensional separation has been recognized in the scientific community over the years. It evidently outperforms 1D GC on peak separation, providing peak capacities of >20,000, which demonstrates the

presence of superior separation performance for the analysis of complex chemical entities [24].

GC×GC is considered an extension of MDGC approaches, where multiple heart cuts are sourced out from a conventional ^{1}D column at a specific modulation period (P_M), into a short ^{2}D column for additional separation, while the analysis is on-going. An interfacing device, commonly known as a modulator, is specially designed for periodic sampling, refocusing, and re-injection of contiguous 'slices' of ^{1}D fractions immediately into the ^{2}D column. **Figure 5** shows the components of 2DGC-MS along with its components such as the modulator and the two distinct columns. The complete separation in ^{2}D should happens before the injection of the next ^{1}D fractions, thus preventing the unintentional combination of ^{1}D component separation in ^{2}D. The current modulation systems have two types. A thermal modulator has a temperature control, either using a refrigerant unit or a cryogen to trap analytes and introduce them into the ^{2}D via rapid heating. A valve-based modulator utilizes gas flow for the control and isolation of portions of the ^{1}D eluate before redirecting them for ^{2}D separation, in which case a longer ^{2}D is used.

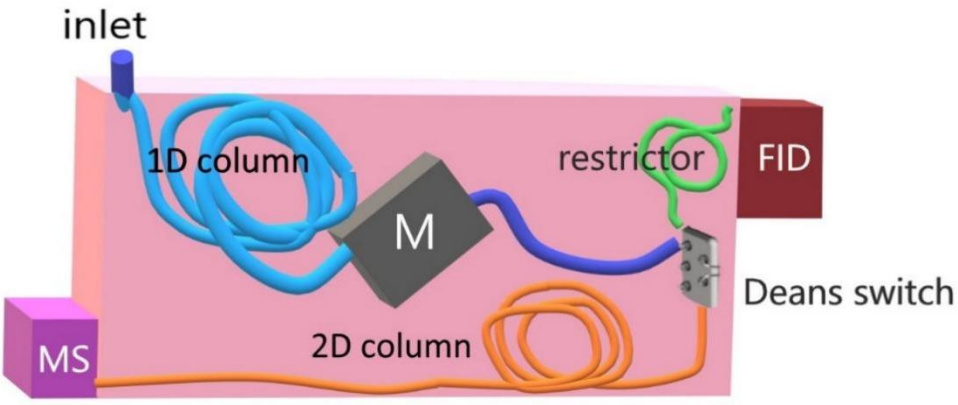

Figure 5. *Two-dimensional gas chromatography and its component including the location of the modulation system (M).*

However, thermal modulation systems become more prevalent due to a higher sensitivity enhancement and better modulation performance [24]. Unfortunately, its major disadvantage is the high consumption of cryogen (e.g., liquid nitrogen and liquid carbon dioxide) to trap and focus volatile components, as well as the bulkiness of the instrumentation. **Figure 6** shows the components of a GC x GC with a cryogen-based modulator system that uses CO_2. Recent innovations in GC×GC modulator technology are moving toward simpler and more economical devices. There are a few notable studies whose output demonstrated the practicability of cryogen-free thermal modulators that provides relatively good portability and low operational cost compared to cryogenic modulators [12]. For instance, the splitter-based non-cryogenic artificial modulation technique which mimics the thermal modulation process by the use of artificial split-and-trap mechanism made of

a systematic pulse splitting, split-pulse delay and the combination of pulse before the periodic selective H/C of the pulse that overlaps. Duty cycle, injection pulse width, $P_{M,}$ and resulting peak capacity in ^{2}D separation are among the important parameters in gauging the performance of modulation [24]. The development, advantages, and shortcomings of various modulation systems have been comprehensively reviewed and can be found elsewhere [24].

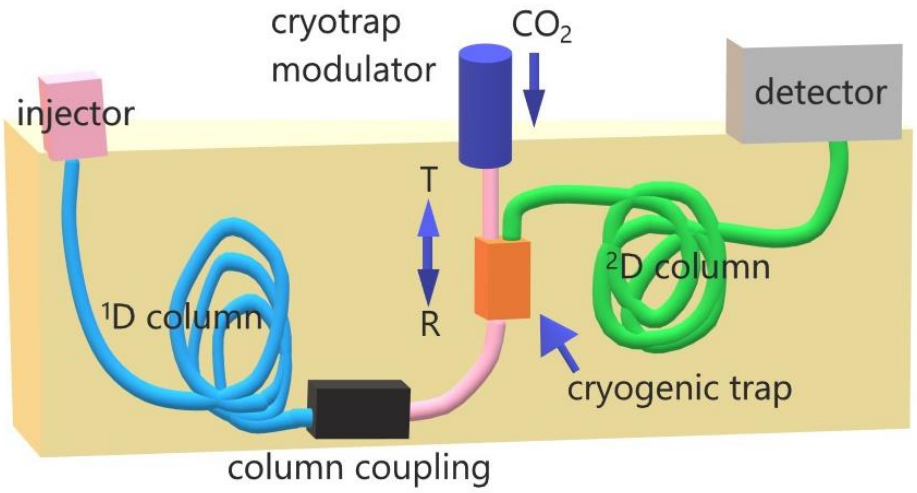

Figure 6*. A GC x GC with a cryotrap modulator using CO_2 gas.*

Two columns of different selectivity is need to execute GC × GC separation. The process involves fractionation and reinjection of the eluate coming from the first column followed by entry to the modulator then it goes through the second capillary column. A single oven serves as enough for two columns to be mounted in

it, or in two different ones, the latter option providing a higher degree of flexibility during method optimization. Separation on the second column is a rapid which is compatible to a good modulation period necessary to abide the requirement for a comprehensive analysis. Dedicated detectors, with high acquisition rates, negligible internal volumes, and rapid rise times, are needed to accurately show the narrow chromatography bands that are generated.

The entire GC × GC process may need data overhaul and characterization but basic information on the chromatographic performance can be assessed by operators using the modulated raw signal. It is also important to have a comprehensive assessment on the results that can only be achieved by a transformation process. Dedicated software packages stack 2D chromatograms side-by-side and, considering the modulation time, derive the 1D and 2D retention times for each peak. Peak areas are obtained by holistically adding the areas relative to each modulated peak, whereas signal intensity is perceived as the height of the tallest modulated peaks and can be interpreted in three ways: (1) by the means of colors (color plot), (2) by contour lines (contour plot), or (3) by a 3D plot.

CHAPTER III

EXPERIMENTAL

3.1 Instruments and apparatus

3.1.1 Gas chromatography-mass spectrometer (GC-MS), Agilent technologies with 7890A equipped with a mass spectrometer Agilent 7000 QqQMS scan mode Triple Quadrupole, GC consists of autosampler and column oven, electron ionization (EI) interface, and MassHunter processing software.

3.1.2 A 1D nonpolar HP-5 column (30 m × 0.25 mm inner diameter (ID) × 0.25 μm (film thickness); J&W Scientific, US) and a 2D polar DB-WAX column (60 m × 0.25 mm ID × 0.50 μm (film thickness); J&W Scientific, US).

3.1.3 A restrictor column (deactivated fused silica (DFS): 1.5 m × 0.1 mm, Agilent technologies Inc.) connected to flame ionization detector (FID) and MS.

3.1.4 A Deans Switch device (DS, Agilent technologies Inc.) connecting the 1D, 2D and the restrictor columns.

3.1.5 Several splitters (combinations of column restrictors connected via Y splitters or pressfits) using different number of applied splitters (n-split) and different GC inlet pressure ramp programs.

3.1.6 A Gerstel MPS 7890B-5977B MSD one-dimensional gas chromatograph (1D GC-MS system), Agilent technologies with autosampler, and a column oven coupled with a mass spectrometer operated by electron impact ionization (EI), triple quadrupoles, MassHunter software processing and a column of Agilent 19091S-433: 3144.59091 HP-5MS 5% Phenyl Methyl Silox 325 °C: 30 m x 250 µm x 0.25 µm.

3.1.7 A GC×GC–FID system consisting of two independent (GC2010) gas chromatographs, a cryogenic loop-type modulator, and a FID.

3.1.8 A GC×GC–FID system consisting of two independent (GC2010) gas chromatographs, a cryogenic loop-type modulator carried out using longitudinally modulated cryogenic system (LMCS) using liquid CO_2 as the cryogen and a FID with the frequency of 50 Hz and the temperature of 250 °C for data collection.

3.1.9 A rotary evaporator R-300, Thermofischer Scientific for the removal of at least 30% of water from samples.

3.1.10 A nitrogen gas evaporator Grey-144, Athena Technology.

3.1.11 Polytetrafluoroethylene (PTFE) microfilters at 0.45 µm, VertiClean.

3.2 Chemicals

3.2.1 Series of *n*-alkanes

A mixture of *n*-alkanes (C_8-C_{20}), Sigma Aldrich (St. Louis, MO) was used as a reference to calculate retention index (*I*) of the compounds.

3.2.2 Hexane, Sigma Aldrich (St. Louis, MO).

3.2.3 Methanol, Sigma Aldrich (St. Louis, MO).

3.2.4 Potassium hydroxide, Sigma Aldrich (St. Louis, MO).

3.2.5 Sodium sulfate anhydrous, Sigma Aldrich (St. Louis, MO).

3.2.6 Deionized water, Merck Incorporated.

3.3 Sample preparation

A sample of pasteurized milk, Dutch Mill brand was evaporated using a rotary evaporator at a temperature of 67 ^{0}C for at least one hour. The sample was loaded to sterilized vials when approximately 50% of its water content is removed.

3.4 Chemical derivatization

A volume of 1.0 mL of 1.0 M of methanolic-potassium hydroxide was used in derivatizing the fatty acids of 0.500 mL sample of pasteurized milk, Dutch Mill brand. The resulting emulsion was mixed with 5.0 mL of hexane and the derivatized sample underwent strong vortexing for 3 minutes. After settling the sample for at least one minute, the organic phase was carefully removed and placed in a clean screw-capped bottle. Another batch of 5.0 mL hexane was loaded to the remaining polar phase and the whole mixture underwent strong vortexing for the second time for 3 minutes. When the organic and polar phases became visible, the organic phase was isolated and transferred to the same clean bottle and the remaining polar phase was mixed for with another final batch of 5.0 mL of hexane. Then the mixture was

vortexed strongly and the last batch of organic phase was moved to the same clean bottle for the succeeding procedure.

3.5 Sample dehydration and solvent removal using nitrogen evaporator

The organic phase carrying fatty acid methyl esters was filtered using sodium sulfate anhydrous placed in a syringe equipped with a 0.45 µm PTFE microfilter to remove water and the other hydrophilic compounds carried by the organic phase sample. The 1.5 mL capacity GC vials were loaded with the organic phase and these vials were subjected to evaporation using nitrogen gas to reduce volume of the solvent content in it.

3.6 Peak separation using GC x GC-MS with SNAT modulator

GC x GC instrument was modified according to the right conditions where the analyte of interest would not be corrupted. The prototype design of SNAT applied restrictor columns which were made from an assembly of a pre-manufactured capillary columns connected with several connectors *(*pressfit and Y-splitters*)*. Each restrictor column was created from capillary column segments of different length and I.D. to meet SNAT key conditions. The success of this mechanism relied on splitting of a pulse of a sample from ^{1}D separation into a number of sub-pulses or peaks *(*n_{split}*)* with the same peak area and equal time difference *(*$Dt_{R,split}$ between any two adjacent split peaks of the same compound*)*. $Dt_{R,split}$ was experimentally measured *(*or approximated using flow calculator software*)* for a given experimental condition. This led to a specific region

observed within every artificial modulation period (P_{AM}) of $Dt_{R,split}{}'n_{split}$ which contained the overlapped sub-pulse profile similar to that obtained from a cryogenic modulation within this P_{AM}. All of these specific regions throughout ^{1}D separation time were selectively H/C to undergo ^{2}D separation resulting in the comprehensive profile of the sub-pulses within a single injection. This was similar to the profile of the sample obtained from the comprehensive GC′GC with cryogenic modulation using the split ratio of n_{split} to 1. The ideal process for splitting of a pulse (or peak) resulted in n_{split} split pulses each of which had the same amount (approximated herein with the same area) and DtR,split. This was obtained using $n$split splitters (restrictors) with different lengths and inner diameters. The splitting system applied restrictor columns (with 0.10 mm and 0.25 mm inner diameters) and many y-splitters connecting at both ends of each splitter which results in n_{split} = 16 split pulses. These restrictors had the same flow resistance and different void times ($t_{0,split}$) resulting in 16 split peaks with the same areas and $Dt_{R,split}$. By adjusting either ^{1}D column inlet pressure ($^1P_{in}$) or DS pressure (P_{DS}), $Dt_{R,split}$ was finely tuned to result in a target $P_{AM} = Dt_{R,split}{}'n_{split}$.

3.7 Peak separation using GC x GC-flame ionization detector (FID) with cryogenic modulator

GC×GC–FID system consisting of two independent (GC2010) gas chromatographs, a cryogenic loop-type modulator, and a FID. The injected volume

was 1 µL, at a split ratio of 10:1. The same column set, the oven temperature program and modulation period as that used with the SNAT analysis was applied. Modulation was also carried out with longitudinally modulated cryogenic system (LMCS) using a liquid CO_2 as the cryogen. The FID data were collected with the frequency of 50 Hz with the temperature of 250 °C. The data were collected using the GC Chem Station (Agilent).

3.8 Data analysis

The resulting chromatograms were integrated manually for data presentation using MassHunter Software. Each peak of interest was initially identified by comparing its experimental MS spectrum from the data obtained at the NIST library 2017 version base on MS match score of >650. The whole process had taken time but all compounds were narrowed down to the list of fatty acid methyl esters for validation. Confirmation of compound identities was done using a maximum value of 20-unit difference between experimental retention index (I) and the literature I for every suspected fatty acid methyl ester under the same stationary phase. The *I* of a peak of interest was determined based on the relative retention times of homologous series of alkanes C_8 to C_{20} (Sigma Aldrich, St. Louis, MO) done by injection of an alkane mixture under the same experimental conditions for sample separation. The experimental I was calculated based on the retention time of the alkane references under the same experimental conditions according to the van den Dool and Kratz

relationship as shown (1) [67]. Data processing alignment was done using Microsoft Excel.

$$I = 100n + 100\left(\frac{tr(i) - tr(n)}{tr(n+1) - tr(n)}\right)$$

where

$^{1}t_{r(i)}$ the retention time of the compound of interest

$^{1}t_{r(n)}$ the alkane eluting before the unknown compound with the carbon number of n

$^{1}t_{r(n+1)}$ the alkane eluting after the compound of interest with the carbon number of n + 1

CHAPTER IV

RESULTS AND DISCUSSION

4.1 1D-GC-single MS separation of fatty acid methyl esters

Milk samples were separated using DB-1 MS column hyphenated with MS detector whose conditions were optimized mentioned in **Table 1**. The results produced by 1D-GC MS is an important baseline to see if the chosen sample preparation method works. The chromatogram of milk brand A containing peaks of fatty acid methyl esters and other untargeted compounds is shown in **Figure 7**. The result revealed that several overlapping and broad peaks were seen during the time of 20-25 minutes. The period of 20-25 minutes was chosen for further study to confirm the hypothesis.

Table 1. *One-dimensional gas chromatography and its conditions.*

GC system	A Gerstel MPS 7890B-5977B
S/SL Inlet	Splitless, 325 ℃
Oven ramp program	40 ℃ for 0 min, 8 ℃/min to 300 ℃ for 30 min
Carrier gas	Nitrogen, 4.772 psi, constant
Runtime	62.5 min
Column	Agilent 19091S-433: 3144.59091 HP-5MS 5% Phenyl Methyl Silox 325 ℃: 30 m x 250 μm x 0.25 μm
Detector	MS

Data analysis showed the list of fatty acid methyl esters in **Table 2.** Using retention indices in literature, the number of FAMEs determined is only limited to approximately 10. The list of fatty acid methyl esters was validated based on their

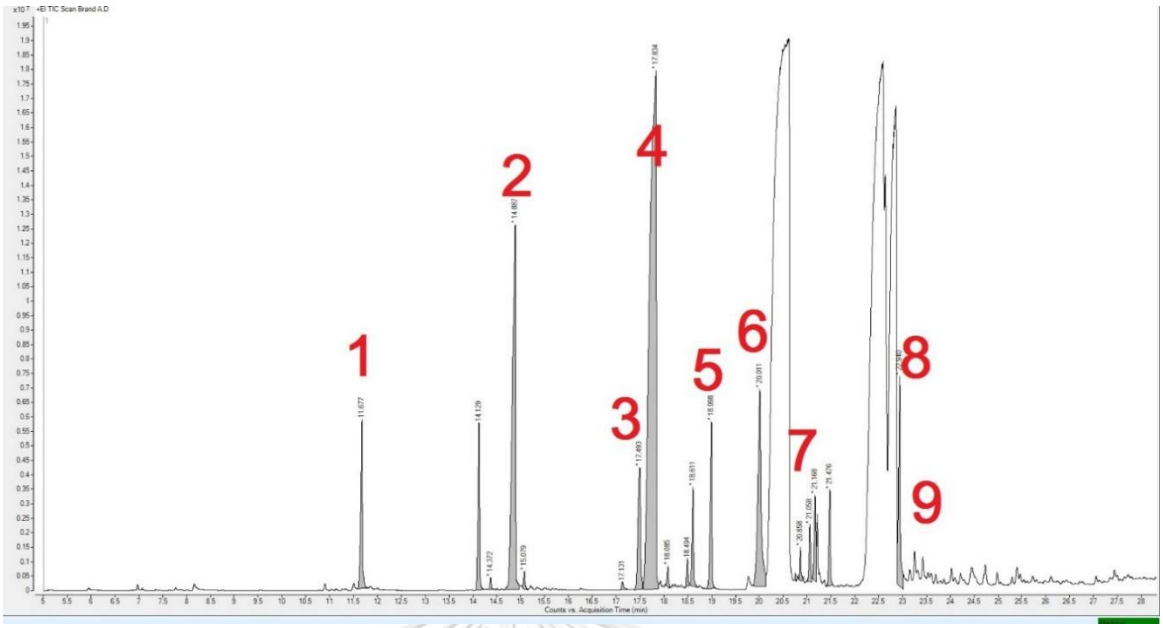

Figure 7. *A chromatogram of milk brand A sample in the analysis of FAME using 1D-GC MS.*

retention index and correlation of each compound in literature. Meanwhile, there were lesser peaks showing untargeted compounds but the more visible peaks observed to no avail could not be identified by NIST Library. These peaks were classified as broad and overlapping and they appeared in the time of 20-25 minutes. A different milk brand was used and the resulting chromatogram would suggest whether or not 1D-GC could demonstrate high peak separation within timeframes 20-25 minutes, the ideal retention times of C_{17}-C_{21} fatty acid methyl esters.

A different milk brand B was derivatized and analyzed on the same conditions using 1D-GC MS. Results revealed similar broad and overlapping peaks appearing on retention times between 20-25 minutes which a closer look on these peaks can be found in **Figure 8**.

Table 2. *List of identified fatty acid methyl esters in milk brand A sample including their retention times (RT) and retention indices (RI)*

Order	Compound	RT	RI
1	Decanoic acid, methyl ester	11.686	1325
2	Dodecanoic acid, methyl ester	14.913	1526
3	cis-9-Tetradecenoic acid, methyl ester	17.509	1715
4	Tetradecanoic acid, methyl ester	17.826	1725
5	Hexadecanoic acid, methyl ester	20.925	1926
6	9-Octadecenoic acid (Z)-, methyl ester	23.129	2091
7	n-Octadecanoic acid, methyl ester	23.213	2128
8	8,11-Octadecadienoic acid, methyl ester	23.260	2112
9	11-Eicosenoic acid, methyl ester	24.580	2297

Resulting chromatograms of both milk brands A and B suggested that 1D-GC MS cannot completely and efficiently separate peaks in the said 20–25-minute retention times. While other untargeted compounds appeared and were identified using NIST Library, specific and other important FAMEs such as methyl linoleate and methyl palmitate could not be detected. This indicates the limitations of conventional 1D separation in terms of selectivity and peak capacity. The accuracy

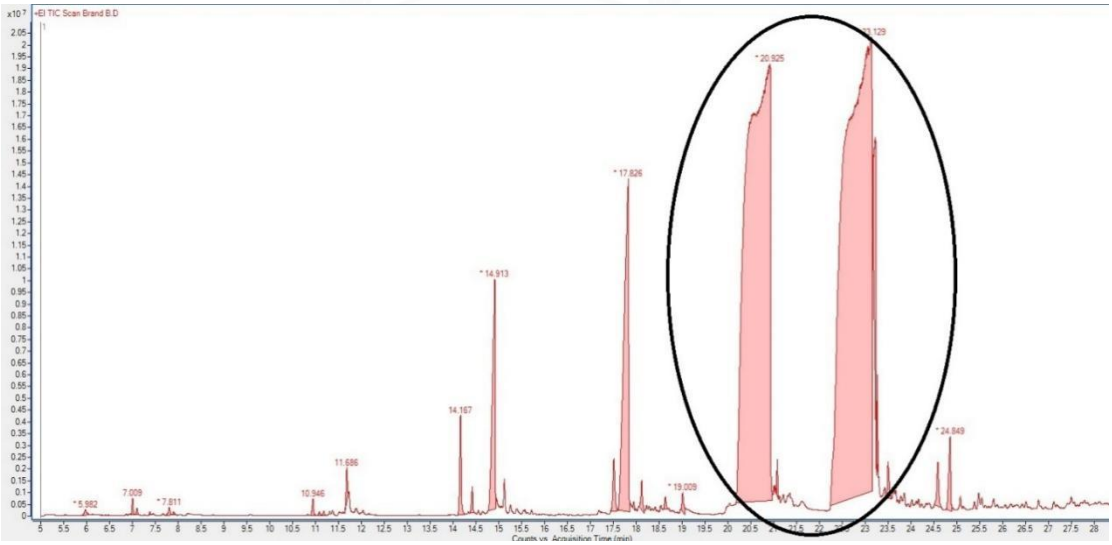

Figure 8. *A chromatogram of milk brand B in the analysis of FAME using 1D-GC MS with broad and overlapping peaks.*

and precision of this technique suffers in complex matrices (>100 volatile compounds) and/or in the presence of another compound coeluting with the target compound.

4.2 Fatty acid methyl ester analysis using two-dimensional gas chromatography

Milk samples were separated in a GC × GC-MS using 1D nonpolar HP-5 column (30 m × 0.25 mm inner diameter (ID) × 0.25 μm (film thickness); J&W Scientific, US) and a ^{2}D polar DB-WAX column (60 m × 0.25 mm ID × 0.50 μm (film thickness); J&W Scientific, US) and FID-MS detector whose conditions were optimized mentioned in **Table 3**. The results of the chromatogram showed a significant change in the appearance of the peaks which can be observed in **Figure 9** below**.** The region with broad and overlapping peaks were no longer observed and were replaced with several sharp peaks with increasing intensity which were identified successfully by NIST library software. It was a typical 1D modulated chromatogram obtained from the GC × GC analysis and was converted into a contour plot as shown in **Figure 10**.

Table 3 *Two-dimensional gas chromatography and its conditions.*

GC system	GC-MS 7890A
S/SL Inlet	Split10 (10:1), 325 °C
Oven ramp program	40 °C for 0 min, 8 °C/min to 300 °C for 30 min
Carrier gas	CO2, 4.772 psi, constant
Runtime	110 min
Column	Agilent 19091S-433: 3144.59091 HP-5MS 5% Phenyl Methyl Silox 325 °C: 30 m x 250 μm x 0.25 μm; a 2D DB-WAX column (60 m × 0.25 mm ID × 0.50 μm
Detector	FID; MS

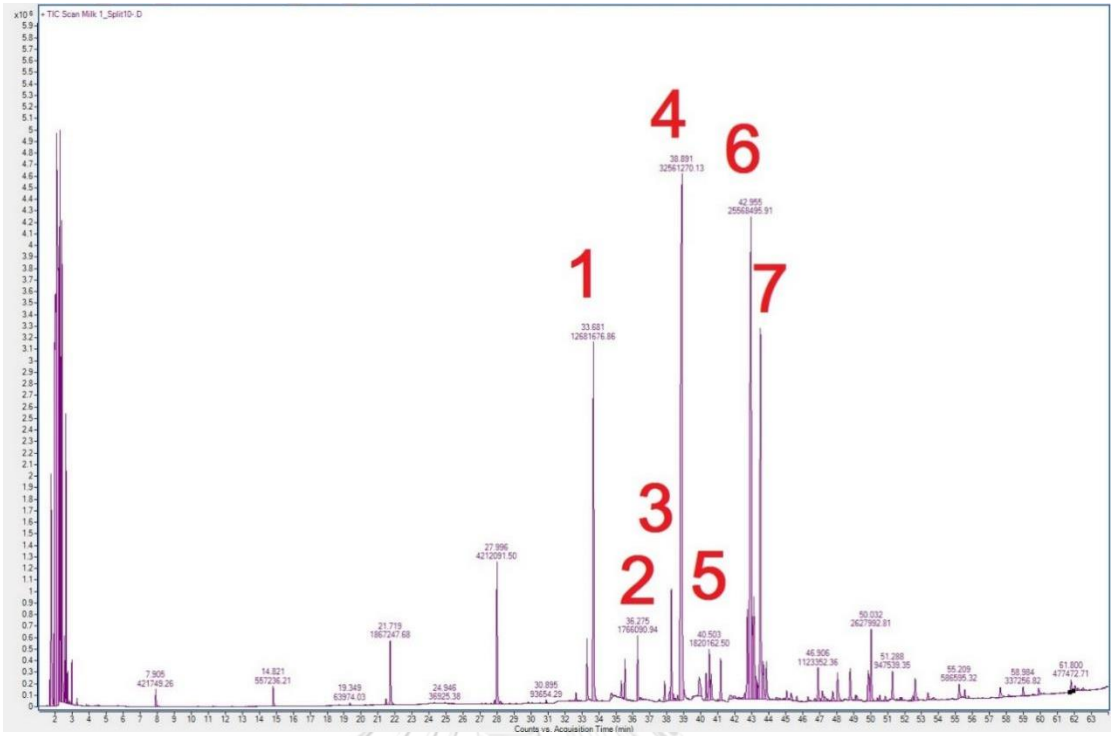

Figure 9*. A 1D modulated chromatogram of the derivatized milk sample in GC x GC-MS with SNAT modulator.*

The number of peaks increased including its peak capacity while several untargeted compounds could also be detected using NIST Library. There was also an increasing number of FAMEs observed. The list of identified fatty acid methyl esters and other compounds can be found in **Table 4**.

The previous overlapping and broad peaks in 1D-GC MS were separated in 2D-GC MS analysis with SNAT modulator. While the time it took for 1D-GC MS to detect the broad peaks was around 20-25 minutes, peak separation in this method took place within 32-44 minutes. In other words, 1D-GC MS could show the peaks in 20-25 minutes while the two-dimensional method with a SNAT modulation system needed nearly twice the time 1D-GC MS performed it. This was due to the presence of a second column which increased the amount of time needed for peak separation but

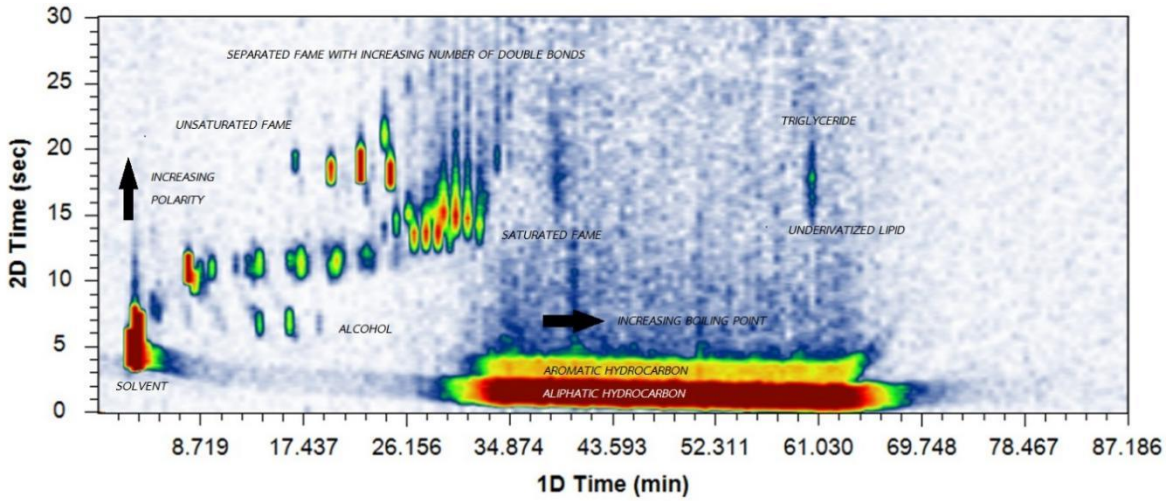

***Figure 10**. A contour plot of the derivatized milk sample in GC x GC-MS with SNAT modulator.*

at the same time, the amount of time it consumes is compensated by a higher precision, accuracy and energy efficiency. 1D-GC MS on the other hand is still functional in the analysis of fatty acid methyl ester however this method only works best in 1) samples that are not complex; 2) samples without co-eluting substances; and 3) samples with fatty acid methyl esters whose number of carbons is below 17 and is over 10.

Contour plot of the derivatized milk sample in GC x GC-MS with SNAT modulator demonstrated interesting results. To read the contour plot, the separation in the x-axis is based on the separation capacity of ^{1}D column in terms of increasing boiling points of the volatile compounds particularly the hydrocarbons. The y-axis is assessed on the separation performance of semi-polar ^{2}D column based on increasing polarity of the compounds. In the case of FAME, clusters in the upper region have increasing number of double bonds. With derivatization of milk samples, plot showed large distinct clusters well separated in the leftmost corner of the contour plot which meant successful derivatization of lipids which were well separated by GC x GC-MS with SNAT modulator while a small cluster of triglycerides in the middle right corner was the remaining underivatized lipid from the sample. The clear and distinct clusters widely distributed in the region which included

identified alcohols, the used solvent, the hydrocarbon region, the lone cluster of underivatized lipid and the identified saturated and unsaturated FAMEs proved that SNAT modulator widens the flexibility of GC x GC-MS system in compehensively visualizing the outcome of the sample preparation in this study. The contour plot alone can easily make experts deduce the character of the complex sample through this important step.

In terms of peak profiling, 1D-GC MS analysis and the NIST Library could not locate and identify the presence of any FAME. The overlapping chromatogram was assessed using GC x GC-MS with SNAT modulator and results showed additional

Table 4*. List of identified fatty acid methyl esters in areas where broad and overlapping peaks found in 1D-GC MS analysis.*

Peak		Name	RT	RI
1	C5H10O2	Methyl butyrate	3.271	722
2	C7H14O2	Methyl caproate	7.905	925
3	C9H18O2	Methyl caprylate	14.821	1126
4	C11H22O2	Methyl caprate	21.719	1325
5	C13H26O2	Methyl laurate	27.996	1526
6	C15H30O2	Methyl isomyristate	32.654	1686
7	C15H28O2	Methyl myristoleate	33.306	1715
8	C15H30O2	Methyl tetradecanoate	33.681	1725
9	C16H32O2	A1 Methyl n-pentadecanoate	35.525	1779
10	C16H32O2	A2 Methyl n-pentadecanoate	36.275	1820
11	C17H32O2	Methyl palmitoleate	38.258	1899
12	C17H34O2	Methyl palmitate	38.891	1926
13	C18H36O2	A1 Methyl 14-methylhexadecanoate	40.503	1984
14	C18H34O2	A2 Methyl 14-methylhexadecanoate	40.608	2016
15	C18H36O2	Methyl margarate	41.174	2028
16	C19H34O2	Methyl linoleate	42.738	2092
17	C19H36O2	Methyl oleate	42.955	2091
18	C19H38O2	Methyl stearate	43.513	2128
19	C21H42O2	Methyl arachisate	47.764	2329

FAMEs which were confirmed by NIST Library. The full profile of the milk component is provided in **Table 4**. Peak information and retention index calculation of the detected FAMEs were shown in this table. In terms of retention index, the difference between experimental and library indices is within the +/- acceptable gap which is less than 20 units. Match values are above 650 while areas where considered significant. Data analysis also identified other untargeted compounds with their corresponding peak information and retention index calculation.

The broad peak region also revealed additional number of FAMEs identified by NIST Library. Using 1D-GC MS analysis, the method and the NIST Library could not locate and identify the presence of the above seven FAMEs. The full profile of the milk component is provided in **Table 4**. From a list of 9 identified FAMEs using 1D-GC MS, the number increased to 19 using GC x GC-MS with SNAT modulator. Four compounds were analyzed to gather peak information and retention index calculation of the detected FAMEs were shown in this **Table 5**. In terms of retention index, the difference between experimental and library indices is within the +/- acceptable gap which is less than 20 units. Match values are above 650 while areas where considered significant. Any peak that did not meet the said standards were excluded in the list after further verification using the literature in the NIST library information system.

Table 5. *Peak information and retention index calculation of 4 selected FAMEs.*

Peak #	Compound Name	Exp RT	Retention index Calculation			Peak Info.			
			Exp	Library (selected column type)	Difference (Exp-Lib), +-20	Match	R Match	Prob (%)	Area
1	2-Hexanol	4.738	804	801	-3	681	727	3.17	210778
2	Heptane, 2,2-dimethyl-	5.269	824	816	-8	717	777	1.33	306725
3	Pentane, 3-ethyl-2,2-dimethyl-	5.565	836	824	-12	758	784	4.35	231974
4	Pentanoic acid, 3-methyl-, methyl ester	6.499	872	866	-6	734	805	17.70	177286
5	Pentanoic acid, 4-methyl-, methyl ester	6.817	885	881	-4	680	711	13.30	157701
6	Octane, 3,5-dimethyl-	8.071	926	922	-4	775	823	6.43	401771
7	3-Ethyl-3-methylheptane	8.769	948	953	6	703	725	3.55	152566
8	Hexane, 3,3,4,4-tetramethyl-	9.068	957	984	27	755	829	6.49	119173
9	Hexanoic acid, 5-methyl-, methyl ester	9.885	982	993	11	646	663	0.69	393794
10	Methyl 6-methyl heptanoate	13.234	1076	1095	19	692	780	0.20	4E+06
11	4-Octenoic acid, methyl ester, (Z)-	13.849	1093	1097	4	642	671	0.04	691158
12	Decane, 2,9-dimethyl-	15.432	1137	1126	-11	789	835	3.30	1E+06

13	5-Methyloctanoic acid, methyl ester	16.269	1161	1178	17	694	748	0.07	3E+07
14	Undecane, 2-methyl-	16.422	1165	1164	-1	793	822	1.65	3E+06
15	3-Methyloctanoic acid, methyl ester	16.537	1168	1162	-6	662	710	0.06	4E+06
16	7-Methyloctanoic acid, methyl ester	16.867	1176	1185	9	725	765	0.75	1E+06
17	7-Methyloctanoic acid, methyl ester	17.154	1184	1185	1	687	747	1.66	642393
18	Octanoic acid, 4-methyl-, methyl ester	17.482	1194	1187	-7	653	730	2.66	386088
19	Nonanoic acid, methyl ester	18.575	1226	1226	0	669	761	0.12	2E+06
20	Undecane, 3,8-dimethyl-	18.763	1231	1228	-3	803	834	1.76	4E+06
21	Dodecane, 3-methyl-	20.095	1269	1271	2	746	805	0.62	2E+06
22	Methyl 8-methyl-nonanoate	20.586	1284	1290	6	723	794	0.10	3E+07
23	Methyl 8-methyl-nonanoate	20.872	1292	1290	-2	699	819	0.30	2E+06
24	Methyl 8-methyl-nonanoate	21.26	1303	1290	-13	723	802	0.54	7E+06
25	Decanoic acid, methyl ester	21.538	1312	1326	14	717	837	0.95	5E+06
26	11-Dodecenoic acid, methyl ester	21.621	1314	1309	-5	709	765	0.02	7E+07
27	Decane, 2,3,5,8-tetramethyl-	21.755	1318	1318	0	792	829	2.79	4E+06
28	Decane, 2,3,5,8-tetramethyl-	22.417	1338	1318	-20	775	808	1.00	9E+06
29	Decanoic acid, methyl ester	22.49	1340	1326	-14	685	838	0.02	4E+08

30	Dodecane, 2,6,10-trimethyl-	22.755	1348	1366	18	786	806	1.96	5E+06
31	Undecanoic acid, methyl ester	25.136	1421	1428	7	695	807	0.04	1E+08
32	10-Undecenoic acid, methyl ester	25.869	1445	1427	-18	690	737	0.31	7E+06
33	Tetradecane, 2-methyl-	26.079	1452	1463	11	759	795	0.88	3E+06
34	Undecanoic acid, 10-methyl-, methyl ester	27.189	1488	1471	-17	705	781	1.23	6E+06
35	Dodecane, 5,8-diethyl-	29.733	1574	1572	-2	743	747	0.67	3E+06
36	Hexadecane	30.053	1585	1600	15	800	818	1.70	5E+06
37	Hexadecane, 2-methyl-	32.377	1667	1664	-3	694	812	1.18	1E+07
38	Heptadecane	33.055	1691	1700	9	780	799	1.90	3E+06
39	Methyl myristoleate	33.396	1703	1715	12	768	811	32.60	110580
40	Methyl tetradecanoate	33.662	1714	1725	11	930	932	82.00	3E+06
41	Methyl 13-methyltetradecanoate	**35.367**	1782	1779	-3	730	765	46.10	55607
42	Tetradecanoic acid, 12-methyl-, methyl ester	**35.555**	1789	1788	-1	776	882	16.20	207773

4.3 Analysis of FAME in GC x GC using a Conventional GC-FID system using LMCS

An underivatized milk sample extracted in hexane was investigated in this section in order to illustrate that the performance of the modulators towards a wider range of compounds (from hexane solvent, alcohols, fatty acids, hydrocarbons to triglycerides). The GC x GC-FID using a cryogenic modulator (longitudinally modulated

cryogenic system (LMCS) with the trap temperature of 10 ^{0}C) showed difficulty in modulation of these compounds with only a few separated clusters. An observable opaque white trace is also present in the plot which indicated a modulator problem. This alone indicated less reliability of the method due to the unstable conditions of the cryogen gas in terms of handling and in terms of its dynamics with the GC x GC-FID system. In short, the said method provided poor contour plot in a sample with underivatized lipids. On the other hand, the GC x GC with a SNAT modulator showed much greater separations results with more separated peaks as shown in Figure 11.

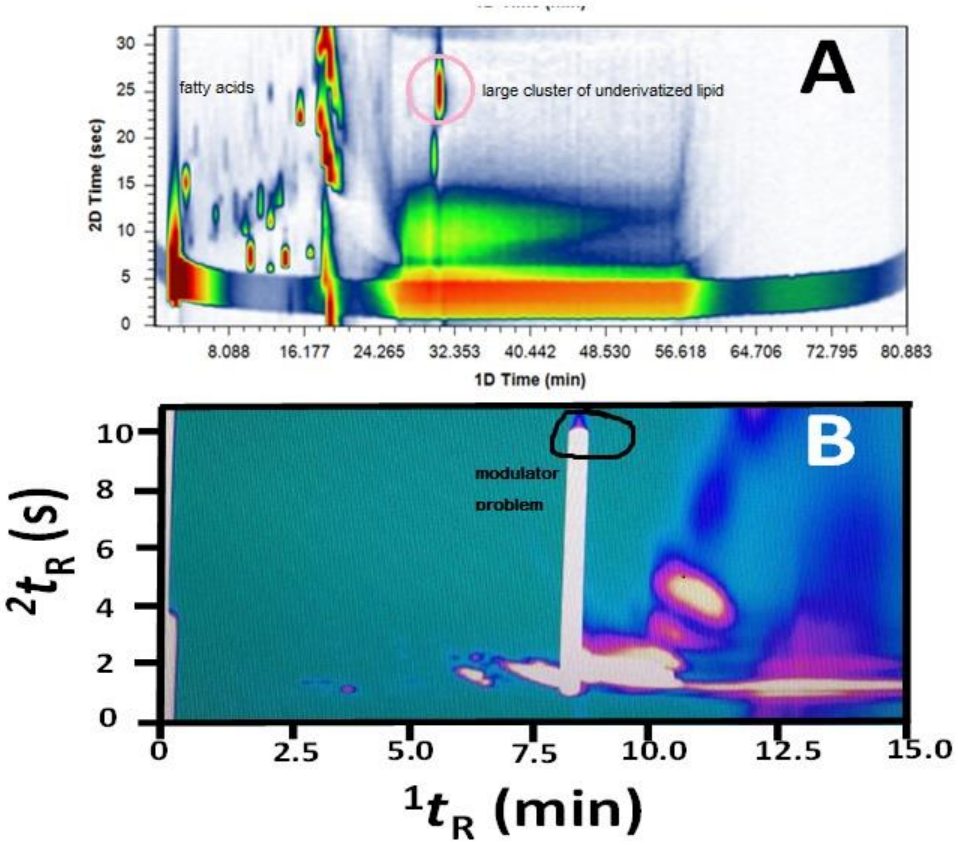

Figure 11. (A) SNAT GC x GC system and (B) Conventional GC x GC system using LMCS with the trap temperature of 10 °C for analysis of the underivatized milk sample.

Clusters of fatty acids and alcohols were observed in the left most region while a large cluster of underivatized lipid is observed in the upper middle region. The fatty acids which were not derivatized were also clearly separated by clusters according to their boiling points and polarity with respect to ^{1}D (x-axis) and ^{2}D (y-axis) separation performance. The fatty acids without methyl esters may have been separated in the contour plot but without chemical derivatization, gas chromatographic technique cannot provide proper and accurate identification of the said compounds. It is vital to subject complex samples with lipids and triglycerides to chemical derivatization if the main objective is to determine the identities and concentration of fatty acids through the analysis of fatty acid methyl esters.

CHAPTER V

Conclusion

Two-dimensional gas chromatography – mass spectrometric analysis of fatty acid methyl esters using a splitter-based non-cryogenic modulation system revealed improved separation with better separated peaks and a ten-fold higher total peak capacity as well as an improved compound identification, compared to 1D GC-MS system and GC x GC-flame ionization detector (FID) with cryogenic modulator. Using 1DGC-MS result as the baseline for a successful sample preparation, chemical derivatization plays a huge role in the analysis of FAME. The existence of a large cluster of underivatized lipid in contour plots for samples without undergoing chemical derivatization proved to be futile in this analysis. Meanwhile, contour plots also concluded to be an effective system in comparing the performance of modulators. In this case, SNAT modulator proved to be efficient in providing clear separation data in contour plots compared to cryogen-based modulator. The milk sample that only exhibited nine identified peaks in 1D-GC MS later showed 19 resolved identified FAMEs in GC x GC technique. The unresolved broad peaks from 1D-GC MS revealed at least 7 identified FAMEs in GC x GC analysis using the SNAT modulator with 10 as the number of splitters. Also, preliminary data revealed that a higher number of splitters and lower modulation interval are the most desirable conditions in peak separation in GC x GC system when using samples of complex matrices. While the analysis of FAMEs in 1D-GC MS was performed in a span of 60 minutes, the test using GC x GC would take at least 110 minutes which is considered longer but more sensitive to other compounds undetected in 1D-GC MS that has no modulation system. The developed approach is considered economical without cryogen consumption and repetition of tests caused by unresolved broad peaks.

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