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ของสารระเหยง่ายในไข่ไก่

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APPLICATION OF HEADSPACE-GAS CHROMATOGRAPHY-

MASS SPECTROMETRY FOR CHARACTERIZATION OF VOLATILE COMPOUNDS IN

CHICKEN EGGS

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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Thesis Title	APPLICATION OF HEADSPACE-GAS CHROMATOGRAPHY-MASS SPECTROMETRY FOR CHARACTERIZATION OF VOLATILE COMPOUNDS IN CHICKEN EGGS
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Field of Study	Chemistry
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ฮานาน เจะมะ : การประยุกต์เฮดสเปซ-แก๊สโครมาโทกราฟี-แมสสเปกโทรเมตรีสำหรับการพิสูจน์เอกลักษณ์ของสารระเหยง่ายในไข่ไก่ (APPLICATION OF HEADSPACE-GAS CHROMATOGRAPHY-MASS SPECTROMETRY FOR CHARACTERIZATION OF VOLATILE COMPOUNDS IN CHICKEN EGGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ธรรมนูญ หนูจักร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. พนิตา งามเชื้อชิต, 86 หน้า.

พิสูจน์ทราบสารระเหยง่ายในไข่ไก่ที่ผ่านระบบห่วงโซ่ความเย็นและไม่ผ่านระบบห่วงโซ่ความเย็น ด้วยเทคนิคเฮดซเปส-โซลิดเฟสไมโครเอ็กแทรคชัน-แก๊สโครมาโทกราฟี-แมสสเปกโทรเมตรี (HS-SPME-GC-MS) ร่วมกับเคโมเมทริกซ์ จากการออกแบบการทดลองแบบเซ็นทรัลคอมโพซิต ได้สภาวะที่เหมาะสมในการการสกัดด้วยเทคนิค HS-SPME ดังนี้: อุณหภูมิการสกัดที่ 65 °C เวลาสกัด 45 นาที และปริมาณเกลือโซเดียมคลอไรด์ 0.4 กรัม ในการเปรียบเทียบแมสสเปกตรัมและค่ารีเทนชั่นอินเด็กซ์เชิงเส้นตรงที่ได้จากการวิเคราะห์ด้วยเทคนิค HS-SPME-GC-MS พบว่าสารระเหยง่ายหลักที่พบในตัวอย่างไข่ไก่ประกอบด้วยโทลูอีน เฮกซะนาล โนนานาล และสารระเหยง่ายรองประกอบด้วยเฮปทานาล ออกทานาล เดคะนาล เบนซัลดีไฮด์ 1-ออกเทน-3-ออล และ 2-เอทิล-1-เฮกซะนอล ในจำนวนชนิดและปริมาณของสารระเหยง่ายตามลำดับดังนี้ พบในระบบ ไม่ผ่านห่วงโซ่ความเย็นมากกว่าระบบห่วงโซ่ความเย็น ในไข่แดงมากกว่าในไข่ขาว และในไข่ดิบมากกว่าไข่ลวก การวิเคราะห์ด้วย PCA กับตัวอย่างไข่ทั้งหมดตลอดทั้งสี่สัปดาห์ ชุดข้อมูลทั้งหมดของไข่ไก่ที่ผ่านระบบห่วงโซ่ความเย็นแยกกลุ่มกันไม่ชัดเจน แสดงให้เห็นว่าการเปลี่ยนแปลงสารระเหยง่ายในไข่ไก่ไม่มีนัยสำคัญตลอดการเก็บทั้งสี่สัปดาห์ นอกจากนี้ไข่ไก่ที่ไม่ผ่านระบบห่วงโซ่ความเย็นมีการเปลี่ยนแปลงปริมาณสารระเหยง่ายเพิ่มขึ้นตลอดการเก็บทั้งสี่สัปดาห์ วิธีเฮดซเปส-โซลิดเฟสไมโครเอ็กแทรคชัน-แก๊สโครมาโทกราฟี-แมสสเปกโทรเมตรี ยังสามารถตรวจวัดสารระเหยง่ายในตัวอย่างไข่ไก่ทั่วไปและไข่ไก่ที่มีความผิดปกติ

ภาควิชา	เคมี	ลายมือชื่อนิสิต	....
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HANAN JEHMA: APPLICATION OF HEADSPACE-GAS CHROMATOGRAPHY-MASS SPECTROMETRY FOR CHARACTERIZATION OF VOLATILE COMPOUNDS IN CHICKEN EGGS. ADVISOR: ASSOC. PROF. THUMNOON NHUJAK, Ph.D., CO-ADVISOR: PANITA NGAMCHUACHIT, Ph.D., 86 pp.

Volatile compounds in chicken eggs under the cold chain system and non-cold chain system were characterized by headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) combined with chemometrics. According to a central composite design, the following optimum HS-SPME extraction conditions were obtained: extraction temperature of 65 $^{\circ}$C, extraction time of 45 min and the amount of NaCl of 0.4 g. In comparison of their mass spectrum and linear retention index from HS-SPME-GC-MS analysis, major volatile compounds found in egg samples included toluene, hexanal, nonanal and other minor volatile compounds were heptanal, octanal, decanal, benzaldehyde, 1-octen-3-ol and 2-ethyl-1-hexanol, with the types and amounts of these volatile compounds in order: the non-cold chain system > cold chain system, the egg yolk > the egg white and the raw egg > soft boil egg. Using PCA analysis of all egg samples at 0-4 weeks, unclearly separated datasets obtained from eggs under the cold chain system imply that non-significant change in the amounts of volatile compounds in the eggs stored at 0-4 weeks. In addition, the non-cold chain system increase the amounts of volatile compounds in the eggs stored at 0-4 weeks. The HS-SPME-GC-MS method was also applied to investigate volatile compounds in commercial eggs and unpleasant eggs.

Department: Chemistry   Student's Signature ....

Field of Study: Chemistry   Advisor's Signature ....

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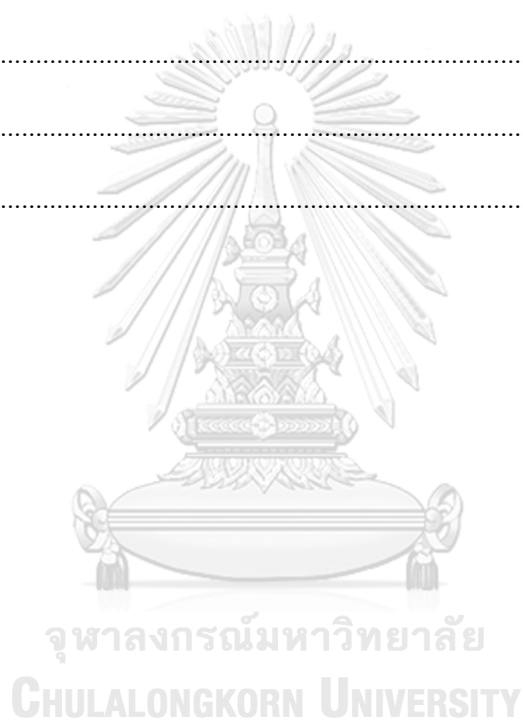
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LIST OF ABBREVIATION

β	phase ratio
CC	cold chain system
CCD	central composite design
C_G	gas phase concentration
CID	collision induced dissociation
cm	centimeter
C_S	sample phase concentration
°C	degree delsius
DOE	design of experimental
DVB	divinylbenzene
EI	Electron ionization
Exp	experimental
g	gram
GC	gas chromatography
HS	headspace
HU	haugh unit
IS	internal standard
k	mass ratio
K	partition coefficient
Lit.	literature
LRI	linear retention index
m/z	mass to charge ratios
mg	milligram

min	minute
mL	milliliter
MS	mass spectrometry
NCC	non-cold chain system
P	loading
PA_{ratio}	ratio of peak area of analyte/internal standard
PCA	principal component analysis
PDMS	polydimethylsiloxane
QMS	quadrupole mass spectrometer
QqQ	triple-quadrupole
RSD	relative standard deviation
RT	retention time
SD	standard deviation
SPME	solid phase microextraction
T	scores
V_G	volume of gas phase
V_S	volume of liquid phase
μL	microliter

CHAPTER I

INTRODUCTION

1.1 Problem Definition

Chicken eggs are one of the popular foods consumed worldwide. According to Food and Agriculture Organization of United Nation: (FAO) from 2000 to 2010, the 2.3% increase of egg production occurred every year [1]. In Thailand from 2012 to 2016, egg products were increased by 5.98% per year according to the needs of consumers [2].

Chicken eggs are inexpensive, easy to find, and high in proteins, lipids, vitamins and miner [3]. Besides, the fatty-acid consumption of egg yolks can be modified into omega-3, which can protect against coronary heart disease; especially high content when egg-laying chickens are fed with diets consist of flaxseed and fish oil [4]. In food industry, such as breads, crackers, ice creams, and cakes industry, etc., chicken eggs are considered as one of the most important ingredients. This is due to chicken eggs' properties, including gelling, forming, and emulsifing [5].

People consume chicken egg not only because of its low price, but also its chemical and physical properties. Chicken eggs' properties can change if they are contaminated, whether in storage, transportation, or distribution.

Techniques to preserve and control chicken eggs' properties like mineral coating, chitosan coating , super chilling, stored refrigerated temperature, and cold chain system, are needed. Among these techniques, the cold chain system is one of the interesting methods.

The cold chain system is controlled under 5 $^{\circ}$C or below, from collecting samples, storage, transportation, and distribution. The control temperature can prevent any changes of the properties in chicken eggs, and the growth of microorganism like *Salmonella spp.* which can cause diseases [6].

There are many methods to measure chicken eggs' quality, such as, the physical methods like egg shell quality, Haugh unit (HU) Analysis, Albumin forming [7], and the chemical methods - which is separated into non-volatile compounds and volatile compounds - like the analysis of antioxidant capacity in hen eggs [8].

This research focused on volatile compounds since a change in volatile compounds in chicken eggs can be one of the important factors affecting chicken eggs' quality. When the chemical properties in chicken eggs change, the volatile compounds will eventually arise.

Headspace-solid phase microextraction (HS-SPME) is one of the several analytical techniques that is widely used for sample preparation of volatile compounds. This is because it is easy, and fast techniques, and can directly extract volatile compounds from the liquid or solid sample without solvent. In addition, HS-SPME can be combined with gas chromatography-mass spectrometry (GC-MS) in order to measure and identify volatile compounds by comparing their mass spectra with those in library databases. In the case of a large number of compounds detected, chemometrics are used for data analysis to interpret the complex results, using an unsupervised pattern recognition method such as principal component analysis, in order to classify and identify the similarities as well as the differences of the dataset.

1.2 Literature review

The chemical components such as albumin, fatty acid, vitamin, and the physical compositions such as freshness, color of egg yolk and air cells within the egg; can last longer or change less or more partially depending on the time duration and the method of egg storage. In previous study on the age of chickens, time, and temperature of egg storage results showed that only time and temperature can affect the changes of egg quality, measured by Haugh Unit (HU) [9]. Corresponding with the study of foaming properties in albumin by considering the age of chickens and storage time, it was found that temperature for storage at 4°C during 4-90 days had the least effect on the changes of foaming properties in albumin [10]. Moreover, the pre-storage processing of chicken egg was one of the main factors in egg quality control. According to the research on washed and unwashed eggs before storage, was found that the percentage of S-ovalbumin in unwashed eggs before storage had been increasing rapidly from 30% to 70% within 2 weeks, and up to 95% within 4 weeks. The escalation of S-ovalbumin was caused an increase in pH value, leading to the decomposition of

albumin [11]. Since the storage method is very important for maintaining the food quality; therefore, the storage using the cold chain system is being interested as it can control the temperature to be below 5°C throughout the process, from harvesting, storing, and transporting [6]. With this storage temperature, the low temperature can also inhibit the growth of microorganisms; as a result, the changes of the food condition and compositions were minimized [12]. Moreover, it can also inhibit the growth of *Salmonella* spp., which is often contaminated in chicken eggs, especially when the eggs are not cleaned after collecting from the coop or before consuming. With this reason, it can adversely affect the consumers as well [13, 14].

Many researchers have been studied on volatile compounds in chicken eggs. There was also the study on volatile compounds in egg yolks obtained from the chickens that were fed with different kinds of feed. Different feeding resulted in different profile of volatile compounds found in egg yolks. However, the main volatile compounds found in egg yolks from the chickens being fed differently were alcohols, including 1-octen-3-ol, and sulfites, including dipropyl trisulfide [15]. Meanwhile, the study on the boiled eggs found that there were several types of volatile compounds up to 61 types which can be classified based on the chemical structure of compounds as follows: alcohols, aldehydes, ketones, esters, aromatic acids, organic acids, sulfide compounds and etc. The volatile compounds derived from the decomposition of chemical elements include amino acids obtained from the degradation of microbial and unsaturated fatty acids from oxidation reaction [16]. Besides, there were also the studies on volatile compounds in egg yolks [EY], Whey protein isolated [WPI], EY-WPI. There were up to 29 volatile compounds, including carbonyl compounds, alcohols, carbonic acids, esters, and for the other types including 2-pentylfuran and limonene [17]. In addition, there was also the study on volatile compounds in scramble eggs, and it was found that there were 38 volatile compounds, with aldehydes are most abundant [18].

There are several extraction techniques of volatile compounds in egg samples: solvent extraction such as soxhlet extraction [15] and non-solvent extraction such as Purge and Trap: PT [19], Sorbent Tube: ST [15], and HS-SPME. For HS-SPME, it is a very popular and widely used for extraction of volatile compounds. Especially for the food

samples with complex components, they can be extracted volatile compounds directly without preparation. This can reduce preparation time and speed up the samples extraction.

Moreover, HS-SPME can be used in quantitative and qualitative analysis because the limit of detection: LOD is low, and the percentage of recovery values and variances are acceptable [20, 21]. Likewise, HS-SPME is also commonly used for investigation of volatile compounds in chicken eggs [20-23] as it can help transfer sample compounds to GC directly, which can reduce the loss of sample [24, 25]. GC is often used with a mass spectrometry (MS) detector. This is because the MS database of various types of compounds compiled with the software used in the process, such as NIST (Wiley). Therefore, this makes it possible to quickly identify the types of compounds in the sample compared to the database   [26, 27]. With the large and complex data obtained from the sample analysis due to several types of compounds and sample groups, it is quite difficult to discuss and interpret the data. As a result, Chemometrics is widely used in the result analysis so as to solve such a problem. The Chemometrics is a method that relies in statistical methods with multiple variables, including unsupervised pattern recognition. This is a method for formatting data into graphs so as to be easily interpreted. The data is grouped into cluster in which the data of similar samples will be grouped into the same groups; whereas, the data of different samples will be grouped separately. With the clustering, it can help analyze the data similarities and dissimilarities more clearly than considering from the quantitative data of the directly-analyzed compounds. For example, the Hierarchical Cluster Analysis (HCA) and the Principal Component Analysis (PCA) that are used for grouping and identifying the similarities and dissimilarities of the samples [28]. Another example is the supervised pattern recognition. This technique can recognize the pattern of the similarities and dissimilarities of the referenced data, then use it to formulate the mathematical models so as to predict the data of samples such as Linear Discriminant Analysis (LDA) [29].

1.3 Aim, scope and expected benefits of this work

Most previous work on analysis of volatile compounds in eggs involved raw egg yolk [15, 17, 19], raw whole egg [29] and hard-boiled egg [16]. Factor affecting volatile compounds in egg sample include temperature storage, time storage, feed, egg coating. However all of this previous work reported on egg samples obtained from the non-cold chain system, the cold chain system is focused in our present work.

Accordingly, soft boiled eggs demands have been rising and become popular food consumption as an increase of production brands, abundant selling at convenience stores. Moreover, these can be an alternative choice to whom do not eat raw egg since due to the risk of bacterial contamination, *Salmonella* spp., or well-cooked egg which proteins maybe easily degrade from heating. However, egg consuming depends directly on consumers' preferring: some people prefer whole egg but some enjoy eating only egg white or egg yolk. Likewise industrial section, egg yolks are separated from the whites for utmost utilization; for example, in bakery industry mostly use egg yolks but in health and beauty industry use egg whites to produce whey protein and soap orderly. This indicates that eggs are beneficial in various angles. Therefore, it is intensity in this work to study the volatile compositions in egg yolk and egg white to examine what the differences are.

Therefore, using HS-GC-MS along with chemometrics analysis, the aims of this work are to identify and compare volatile compounds in chicken eggs under the cold chain and non-cold chain with the different edible egg parts: egg yolk & egg white, and also egg states: raw egg & soft boiled egg.

Firstly, egg whites are measured and compared with HU value to indicate internal quality. Secondly, the optimal conditions for GC-MS (such as temperature injection, time desorption and injection mode and the study of HS-SPME extraction for volatile compounds through specific experimental design (central composite design) are all required. In addition, the parameters affecting the results must be studied such as extraction temperature, extraction time and the amount of NaCl. Thirdly, the obtained conditions both for GC-MS and HS-SPME are used for determination of volatile compounds among different egg types and egg states under cold chain and

non-cold chain system. Finally, all datasets are interpreted by PCA analysis and then compared the volatile compounds by using the ratio of peak area of the volatile compounds with internal standard.

According to our hypothesis that the cold chain system for eggs produces less volatile compounds than does the non-cold chain volatile compounds, it is expected in this research to obtain scientific evidence of the profiles of volatile compounds in egg samples in order to prove this. In addition, the information of the comparative amounts of volatile compounds in egg yolk and egg white will be useful for consumers. Moreover, this developed HS-SPME-GC-ME can be used as an alternative method for quality control of egg from laying to distribution on the shelf and examine unpleasant egg odor that may be claimed from the customer.

CHAPTER II

THEORY

2.1 Volatile compounds

Eggs are inexpensive and highly nutritious food, containing essential compounds such as 18 vitamins and minerals [30]. They consist of three main parts which are egg shell (the shell membranes between albumen and inner shell surface), egg white and yolk. These parts correspond to 9.5% (w/w), 63%, and 27.5% of eggs, respectively. The general compositions of eggs are shown in Table 1. An egg is composed of ∼75% water, 12% proteins and lipids, ~1% carbohydrates and minerals. Proteins are mostly found in egg white and yolk, contributing to 50% and 44%, respectively, [31].

Table 2.1   Nutritional components of chicken eggs obtained from reference

Component (Unit)	Amount	Component (Unit)	Amount
Egg shell (%)	10.5	Calcium (mg)	56.0
Egg yolk (%)	31	Magnesium (mg)	12.0
Egg white (%)	58.5	Iron (mg)	2.1
Water (g)	74.5	Phosphorus (μg)	180.0
Energy (Kcal)	162	Zinc (mg)	1.44
Protein (g)	12.1	Thiamine (mg)	0.09
Carbohydrates (g)	0.68	Riboflavin (mg)	0.3
Lipids (g)	12.1	Niacin (mg)	0.1
Saturated fatty acids (g)	3.3	Folic acid (μg)	65.0
Monounsaturated fatty acids (g)	4.9	Cyanocobalamin (μg)	66.0
Polyunsaturated fatty acids (g)	1.8	Pyridoxine (mg)	0.12
Cholesterol (mg)	410	Retinol equivalents (μg)	227.0
Iodine (μg)	12.7	Potassium (mg)	147
Tocopherols (μg)	1.93	Carotenoids (μg)	10
Selenium (μg)	10	Cholecalciferol (μg)	1.8

Egg white contains several functionally important proteins such as ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), and lysozyme (3.5%) Abeyrathne [32], whilst; egg yolk is composed of 51% water, 16% proteins, 32.6% lipids,1.7% minerals, and 0.6% carbohydrates [8].

2.2 Cold chain system

A cold chain is a supply chain system applied for temperature-controlling and monitoring. An unbroken cold chain is a special type of cold chain which contains an uninterrupted series of refrigerated production, storage and distribution activities as shown in Figure 2.1. The system also contains other equipment and logistics, functioning at low-temperature range. It is applied for preservation and extension of the product shelf life. The examples include fresh agricultural product, seafood frozen food, photographic film, chemicals, and pharmaceutical drugs. Such products, during transport and when in transient storage, are sometimes called cool cargo. [33]. The distance to transfer food from has increased over the last two decades. The performance of the cold chain in the aspect of maintenance of safety, quality of foods and shelf life depends on the capability to control product temperature in every step. [34]
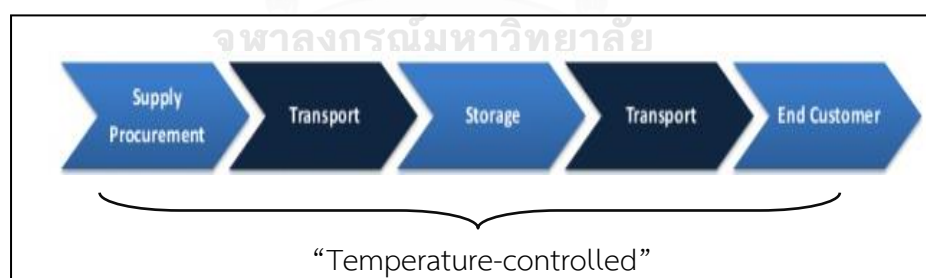
Figure 2.1   Cold chain system flow [35]

2.3 Extraction

Extraction in this study is a process to transfer target chemical components dissolved in one liquid phase (A) into another phase (B), given that the two phases are immiscible. Phase B can be solid, liquid, gas, or supercritical fluid. Distribution of the components between the two phases occurs. The extracted analytes are then recovered from the phase B prior to subsequent extraction or instrumental analysis. This process can be explained according to the theory of chemical equilibrium involving the reversible distribution reaction as

$$XA \rightleftharpoons XB$$
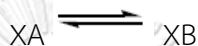
Biological samples contain a wide range of compounds with different physical properties such as molecular weight, polarity, and volatility. For complex sample analysis, efficient extraction method such as headspace sampling is generally performed prior to high performance separation technique with highly selective detection system, herein with the application of gas chromatography hyphenated with mass spectrometry (GC-MS).

2.3.1 Headspace (HS)

Headspace sampling is an attractive sample preparation technique providing fast and clean approach for analysis of volatile organic compounds in complex samples. Volatiles may be extracted from heavier or more polar sample matrices [36]. Conventionally, sample is prepared in a vial as s solid, solution with or without matrix modifier or gas. Volatiles in a complex sample mixture can be extracted in the headspace of the sample being isolated from the non-volatile components in the sample vial. A vapor aliquot in the headspace is then transferred to a gas chromatographic system for separation of the volatiles.

The theory of head space can be explained with the example of a liquid sample in a closed vial, as shown in Figure 2.2 the volatiles in this sample will evaporate into the gas phase. In equilibrium, the concentrations in both phases sample phases concentration (C_S) and gas-phase concentration (C_G) remain constant. The ratio

of concentration in each phase is captured by an equilibrium constant specifically called as the partition coefficient (K). This parameter is the product of two terms: mass ratio (k) and the phase ratio (β) [37].

$$K = \frac{C_S}{C_G} = \frac{W_S}{W_G} \cdot \frac{V_G}{V_S} = k \cdot \beta$$

$$\beta = V_G / V_S$$

$$k = W_S / W_G$$
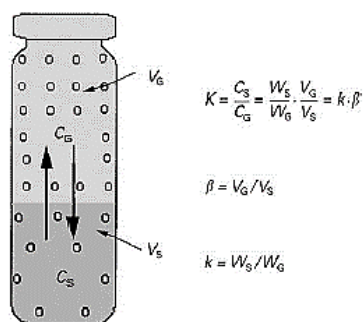
Figure 2.2   A vial containing a liquid sample with a volatile compound in the headspace, where C_G and C_S are concentrations of the compound in the gas and liquid phases, respectively. W_G and W_S are masses of the compound in in the gas and liquid phases. V_G and V_S are volumes of the compound in gas and liquid phases [37].

2.3.2 Headspace-solid phase microextraction (HS-SPME)

Solid-phase microextraction (SPME, introduced by Arthur and Pawliszyn) is a technique that can be applied to extract and concentrate compounds from sample headspace. SPME is an ideal example for a simplified extraction technique since the technique can be performed without gases or plumbing It also provides analyte pre-concentration with selectivity towards many analyte. Unlike several other techniques, SPME is very effective to eliminate solvent, such as water, which may enter the column.

Figure 2.3 shows both external and internal views of a manual holder consisting of a needle guide depth gauge. The guide can be screwed up or down to control the depth that the needle goes inside either an injection port or a vial as well as supporting the needle and reduces breakage. The manual holder also has a z-slot to fix the fiber position onto the exposed position. With the plunger unlocked, the fiber is retracted into the needle for a manual assembly [38].
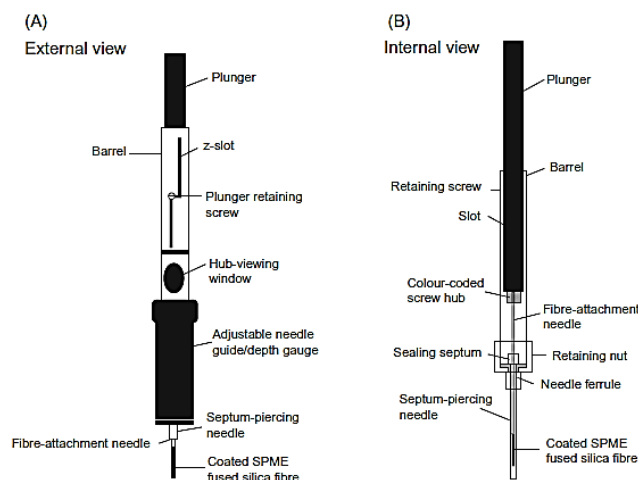
Figure 2.3 Schematic view of SPME manual fibre assembly holder (external and internal view) [39].

2.3.2.1 Sorbents

SPME sorbents (phases) are most commonly coated onto either the outside surface of fused silica fibers or the internal surface of a capillary tube. The phases are generally not covalently bonded onto the core of silica fiber except for the fiber with the polydimethylsiloxane coating thickness of 7 mm. Other phase materials are cross-linked to improve stability (prevent swelling effect) in organic solvents. Multiple-component phases were also developed to broaden selectivity in SPME. Commercially available phases for SPME analysis contain either divinylbenzene (DVB) and/or Carboxen particles suspended in polydimethylsiloxane (PDMS, a nonpolar phase) or Carbowax (CW, moderately polar) . The solid particles are suspended in a liquid phase prior to coating onto the fiber.

An example for multiple-component bipolar phase is DVB/Carboxen-PDMS which contains DVB-PDMS (50 mm) layer over the layer of Carboxen-PDMS (30 mm). This phase provides analysis with a wider range of molecular weight, since larger analytes are extracted in the meso- and macropores of the outer DVB layer, while the micropores of the inner Carboxen layer retain smaller analytes. The dual-layered phase has been applied for extraction of odor compounds and volatile and semivolatile flavor compounds prior to analysis with GC analysis. In

addition, DVB sorbents have a high affinity for small amines. The Multiple-component phase is thus a suitable choice for extraction of isopropylamine [40].

2.3.2.2 Steps of SPME extraction

HS sampling by SPME can be a 3-phase system, including gas (HS), liquid (sample) and solid (SPME) phases. The HS of the sample will interact with the SPME phase. Two thermodynamic systems simultaneously occurs which are liquid-gas and gas-solid systems. Thus two partition coefficients involve in order to achieve a final equilibration in the system [36].

There are two steps in SPME analysis Figure 2.4: (a) the sorbent is initially exposed to the sample for a certain period of time; (b) the sorbent is transferred into a device interfacing with analytical instrument for thermal desorption such as GC for gas desorption or LC for solvent desorption.
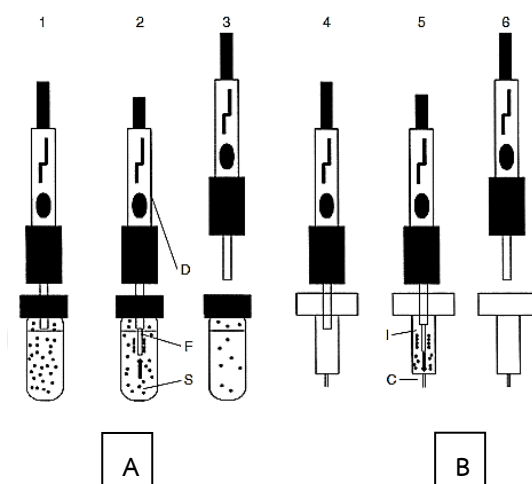
Figure 2.4   Principle of SPME:

1) Introduction of syringe needle of the SPME device (D) into the sample vial and close to the sample (S),

2) Moving the fiber (F) into the position outside the syringe and into the sample (extraction),

3) Moving the fiber back into the syringe needle and subsequent transfer of the device to the GC injector port (1) and capillary head (C),

4) Penetration of the septum with syringe needle,

5) Moving the fiber into the position outside the syringe (desorption),

6) Moving the fiber back into the syringe needle and withdrawing the needle [40].

SPME integrates sampling, extraction, concentration and sample introduction into a single step. Analytes in the sample are directly extracted and concentrated onto the extraction fibre. The method can be performed within a short period of time and disposal costs with improved detection limits. It has been routinely applied in combination with GC or GC-MS. The technique has been successfully applied to analyze a wide range of compounds, especially for volatile and semi-volatile organic compounds from many samples including environmental, biological and food samples [41].

2.4 Experimental design

Experimental design (commonly referred to as DOE) is a useful approach to generate "structured" data tables, i.e. data tables containing an important amount of structured variation. Such underlying structure can be used to support multivariate data analysis (discussed below) as a basis for multivariate approach, which will guarantee stability and robustness of the modeling [42].

2.4.1 Central composite design

Central composite design (CCD) is a type of experimental design which is frequently applied together with response models. CCD consists of three different design points: edge points as in two level designs (±1), star points at ±α, $\alpha \leq 1$ contributing to quadratic effects and centre points. The number of center points governs the design direction as well as certain properties required for the design. The α value depends on the number of analyses in the factorial portion of the central composite design [43] α = [number of factorial runs]$^{1/4}$ If the factorial is a full factorial, then α = [2^k]$^{1/4}$

Table 2.2 illustrates some typical values of α as a function of the number of factors.

Number of Factors	Factorial Portion	Scaled Value for α Relative to ±1
2	2^2	1.414
3	2^3	1.682
4	2^4	2.000
5	2^5	2.378
6	2^6	2.828
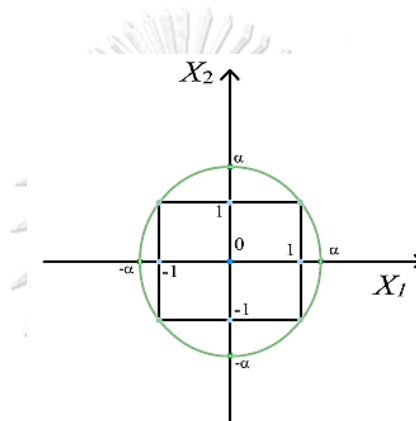
Figure 2.5   Example of central composite design for 2 factors with α >1 [43]

For example, the first order model was found to be inadequate for the region near the optimum. Once the experimenter realized that the first order model was not suitable (for the region with a reaction temperature of 350 F and reaction time of 165 minutes), it was decided to augment the experiment with axial runs. This is to be able to complete a central composite design and curve fitting (a second order model) can then be performed to predict the response. The advantage of a central composite design is simplicity. The experimenter only had to add the axial runs to the design with center point runs without the need to perform a new experiment. The experimenter decided to use α =1.41 to get a rotatable design [44].

Table 2.3   Example of Design of experiment with CCD 2 factors

Factor levels			
code		Actual	
-1	-1	345	155
1	-1	355	155
-1	1	345	175
1	1	355	175
-1.414	0	342.9	165
1.414	0	357.1	165
0	-1.414	350	150.9
0	1.414	350	179.1
0	0	350	165
0	0	350	165
0	0	350	165
0	0	350	165
0	0	350	165

2.5 Gas chromatography-Mass spectrometry

Gas chromatography hyphenated with mass spectrometry (GC-MS) is a highly effective analytical technique for the identification and quantitation of organic volatiles in complex sample matrices. This technique is indispensable in the fields of environmental science, forensics, health care, medical and biological research, health and safety, the flavor and fragrances industry, food safety, packaging, and many others. GC-MS is the combination of two powerful microanalytical techniques. Gas chromatograph approach separates components in a mixture according to their different retention time prior to analysis with mass spectrometer providing structural information for identification of each component. Such combination provides several

advantages. Firstly, the technique provides separation of components in a complex mixture which results in mass spectra of individual compounds which is useful for qualitative purposes. Secondly, it can provide quantitative information for the analytes. Figure 2.6 showed a schematic representation of a gas chromatograph-mass spectrometer (GC-MS).
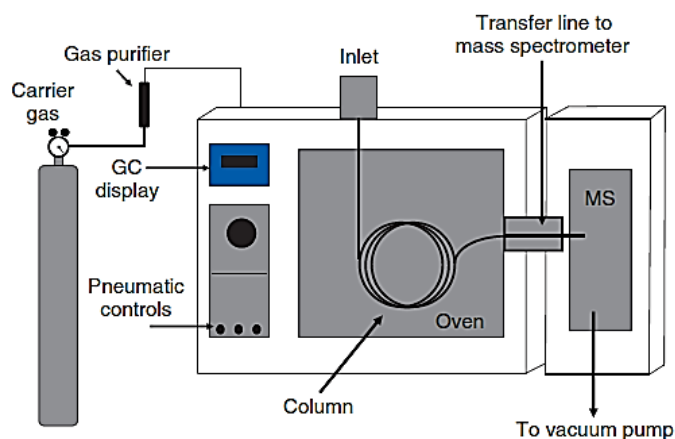
Figure 2.6   Schematic of a typical simple GC-MS [45].

2.5.1 Gas Chromatography

Gas chromatography (GC) is a high resolution separation technique for analysis of multi-component samples. The technique provides qualitative and quantitative information of analytes involving their molecular compositions and amounts. The experimental outcomes from this technique include a chromatogram (a graphical image of a detector output, signal vs time), heights and the areas of the resolved (adequately separated) peaks in a chromatogram or their boiling points, etc.

The important parts of a gas chromatograph are a supplier of gas as the mobile phase, an inlet heating the compounds and delivering them to a column, the column separating the compounds, an oven as a thermostat for the column, a detector to detect the presence of a chemical from the column effluent, and a data system recording and displaying the chromatograms [46].
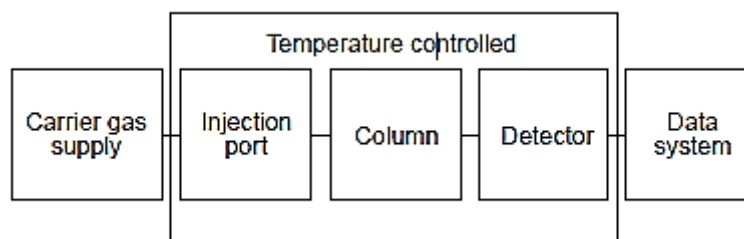
Figure 2.7　　Diagram illustrating of gas chromatograph [46].

2.5.1.1 Carrier gas

The carrier gas is mobile phase in GC. The choice of a practical carrier gas can be nitrogen or helium. Hydrogen can also be used as the carrier gas with special attention on safety issues. Common sources for nitrogen or helium are the pressurized cylinders, which are steel tanks with two-stage pressure regulators. Alternatively, gas generators for nitrogen, which are also commercially available, have advantages in safety and can be used for long period without replacement. Apart from the gas source, the purity of tubing connecting the source to the gas chromatograph. Lifetimes of most GC columns can be shortened with the presence of moisture and oxygen in the carrier gas especially when they are operated at temperatures >100 °C, which results in poor reproducibility in separation. Thus, the carrier gas should be filtered over molecular sieve beds in order to reduce moisture. In addition, specialized traps can be incorporated into the carrier gas line system to reduce or remove hydrocarbons and oxygen in the carrier gas [46].
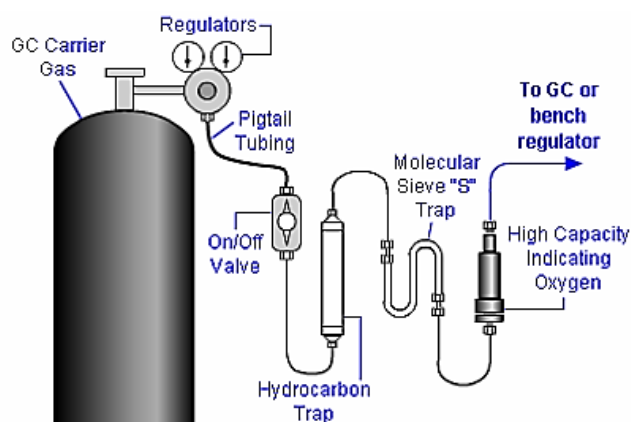
Figure 2.8　　Schematic of a component of carrier gas [47].

2.5.1.2 Injection

Sample introduction process in GC involves transfer of the components in the mixture as a narrow band (as narrow as possible) into the column. The oldest and most commonly used type applied with capillary columns is the heated split/splitless injector which can operate in split and splitless modes. Selection of the injection mode depends on analyte concentrations in samples. Figure 2.9 is a schematic diagram representing of a typical split/splitless injector.

Both split and splitless injection modes are considered as hot isothermal injection techniques with the injector temperature being high enough to vaporize the solvent and the analytes in samples. This injection temperature is constant throughout the GC run. Split injection is used for neat samples (not dissolved in a solvent) or samples with relatively high concentrations. The splitless mode is applied for samples containing analytes at trace levels.

- Split mode

In the split mode, the sample is injected and vaporized into the carrier gas stream. Only a small portion of the sample and solvent is transferred onto the inlet of the GC column. The remainder of the sample is vented going to waste (Figure 2.9). Typical split ratios can range from 10:1 to 400:1 (with the less amount of sample at higher split ratio) and can be calculated according to [45]

$$\text{Split ratio} = \frac{\text{Column flow} + \text{Vent flow}}{\text{Column flow}}$$

- Splitless mode

In the splitless mode, the sample is injected with the splitter vent closed. With sample discrimination during injection, this allows all the injected amount of the sample and solvent onto the GC column inlet. This technique is recommended for trace analysis of samples containing compounds with a fairly narrow boiling-point range. Splitless mode is not suitable for injection of thermally labile compounds.
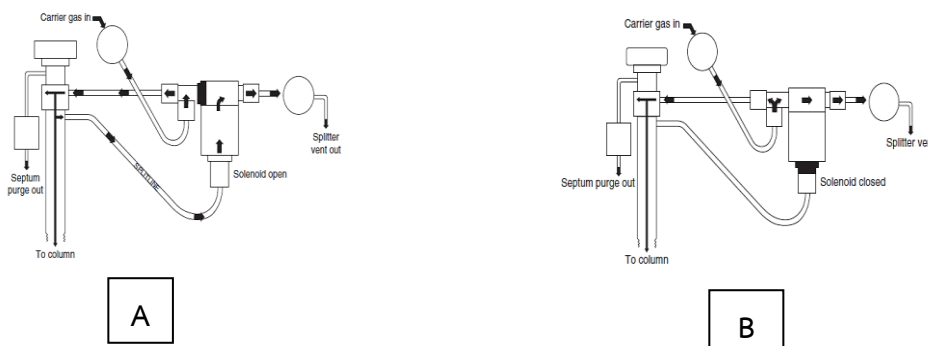
Figure 2.9   The flow path of the carrier gas in [A] the split mode and [B] the splitless mode [45].

2.5.1.3 Column

The heart of the GC is the columns separating individual components in a gas mixture which is based on some physical characteristics. For separation of most hydrocarbon, "boiling point" columns are applied to separate the components according to their individual boiling points or vapor pressures. However, other applications may use molecular size (molecular sieve columns) or polarity differences to achieve different goals in separation [48].
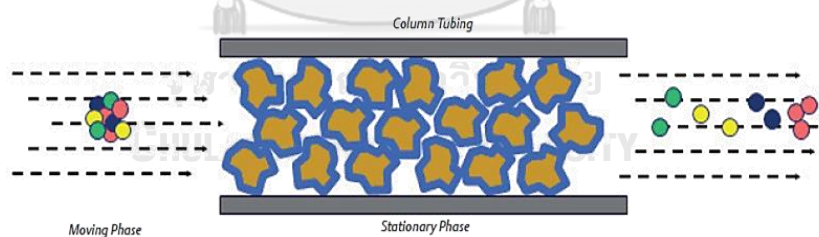
Figure 2.10   Diagram showing the stationary and moving phases [48]

The sample is separated into its constituent components in the column as shown in Figure 2.10. Columns can be packed or open tubular with different lengths and internal diameters (i.d.) depending on types of application. Packed columns (typical dimension of 1.5 m x 4 mm i.d.) are packed with a solid support coated with immobilized liquid stationary phase material. This is also known as gas liquid chromatography (GLC). Open tubular capillary columns (with typical dimension

of 30 m x 0.32 mm i.d. x 0.1 mm film thickness) are long hollow silica tubes with the inner wall of the capillary coated with immobilized liquid stationary phase material [49].
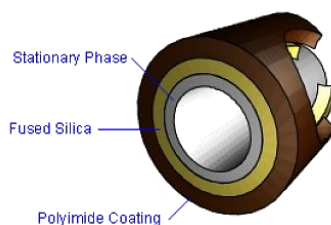
Figure 2.11   Cross section of open tubular of capillary column [49]

2.5.1.4 Detector

A detector responds to a physicochemical property of the analyte. The respond is amplified, and the detector then generates an electronic signal which will be recorded with time resulting in a chromatogram. There are many types of detectors and their application depends on analyte chemistry and required sensitivity as well as the analysis aim (quantitative or qualitative). Examples of detectors applied in GC include flame ionization (FID), electron capture (ECD), flame photometric (FPD), nitrogen phosphorous (NPD), thermal conductivity (TCD), and mass spectrometer (MS) [49].

2.5.2 Mass spectrometry

Mass spectrometry is a powerful analytical technique with several functions such as quantification of known materials, identification of unknown compounds in a sample or elucidation of structures and chemical properties of different molecules. The complete process for detection with MS involves the conversion of the sample into ions in gas phase either with or without fragmentation. These ions are then analyzed based on their mass to charge ratios (m/z) and relative abundances.

The MS instrument consists of three major parts:
- Ion source: producing ions in gas phase from the substance being studied.

- Mass analyzer: resolving the ions into their characteristics mass components according to their *m/z* ratio.

- Detector system: detecting the ions and recording the relative abundance of each of resolved ionic species.
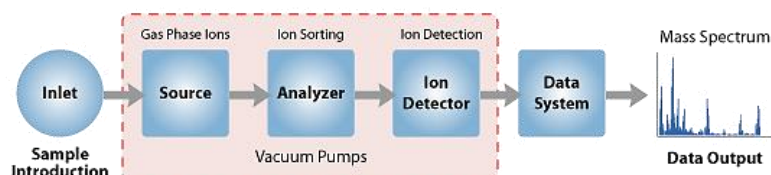
Figure 2.12   Diagram showing components of a Mass Spectrometer [50]

2.5.2.1 Ionization

Electron ionization (EI) is the most widely used type in GC/MS. EI is a very energetic process where analyte molecules are shot with the electron beam resulting in molecular ions (M+•) with a large amount of internal energy. These ions will fragment into smaller ions resulting multiple-peak mass spectra. EI involves interaction of a low-pressure ($\sim 10^{-1}$ Pa) gas with electrons accelerated through a 70-V electric field (corresponding to the energy of -70 eV) which is conventionally applied in routine analysis. [Gas Chromatography and Mass Spectrometry] As shown in Figure 2.13, EI source contains a heated filament producing electrons which are accelerated towards an anode and collide with the analyte molecules in gas phase of the analysed sample injected into the source. Compounds with high vapor pressure are introduced directly into the source [51].
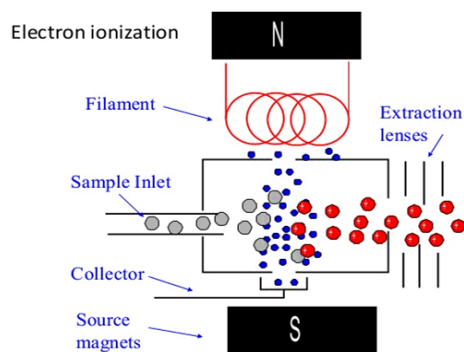
Figure 2.13   Diagram showing an electron ionization source [52]

2.5.2.2 Mass analyzer

After ions are generated in the source region, they are accelerated into the mass analyzer under the influence of an electric field. The mass analyzer separates these ions based on their *m/z* values. The selection of a mass analyzer depends upon the analysis goal with different focuses, *e.g.* on resolution, mass range, scan rate and detection limits.

Quadrupole mass spectrometer (QMS) is the most common mass analyzer. Its desirable features such as compact size, fast scan rate, high transmission efficiency, and modest vacuum requirements are ideal for analysis with small and inexpensive instruments. Most quadrupole instruments are limited to unit *m/z* resolution and have a mass range up to *m/z* 1000.

The Q analyzer contains four rods or electrodes arranged across from and in parallel to each other. As ions from the ion source travel through the Q, they are filtered according to their *m/z* value so that only a single *m/z* value ion can pass the Q and strike the detector. The *m/z* value transmitted by the Q is determined by the Radio Frequency (RF) and Direct Current (DC) voltages applied to the electrodes. Applications of these voltages result in an oscillating electric field functioning as a bandpass filter to transmit the selected *m/z* value [53].

Figure 2.14   Quadrupole Mass Analyzer [53].

- Triple-quadrupole

Triple-quadrupole (QqQ) systems are ideal for quantitative work, allowing linear, highly sensitive, simultaneous multi-component analysis. QqQ instruments consist of the first quadrupole (Q1) is used to select a precursor (parent) ion. Collision induced dissociation (CID) occurs in the second

quadrupole (Q2), which is called the collision cell. The structural information can be obtained based on specific fragmentation pathways of analyte ions colliding with neutral molecules such as Ar or N_2 (collision gas). Application of voltages adds extra energy to the analyte ions, enhancing collisionally induced fragmentation. The third quadrupole (Q3) generates a spectrum of the resulting product (daughter) ions.
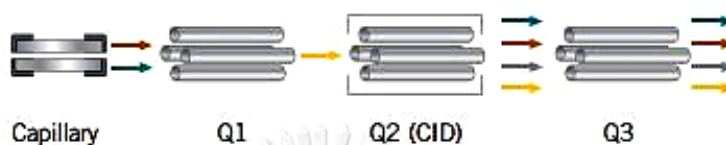
Figure 2.15   Triple quadrupole instrument [54].

2.5.2.3 Detector

Ions from mass analyzer are detected based upon their charge or momentum which is converted into current signal. For large signals a faraday cup is used to collect ions and support measurement. Older instrumental approaches employ photographic plates to detect the ion abundance with different mass to charge ratios.

Most detectors currently apply a collector to amplify the ion signal which is similar to a photomultiplier tube. Such detectors include: electron multipliers, channeltrons and multichannel plates. The signal gain can be adjusted by changing the high voltage that is applied to the detector. Performance of a detector depends on its speed, dynamic range, gain, and geometry. Some highly sensitive detectors are able to detect a single ion [53].

2.6 Principal component analysis

Principal component analysis (PCA) is a multivariate technique for analysis of data with the observations described by several inter-correlated (and quantitative) dependent variables. The technique can be applied to extract the important information from the data, to represent them as a set of new orthogonal variables

(principal components), and to display the pattern of similarity of the observations and of the variables as points in plots [55].

In general, the goals of PCA are to extract the most important information from the data removing the other information (compress the data size). Simple description of the remaining data set is then established and the simplified structure of the observations and the variables is then presented.

PCA analysis starts with differentiation of data matrix (X) with the number of samples of $I \times J$ variable parameters. After the analysis, X will be separated into two parts called scores (T) and loadings (P) according to the relationship, $X = T\,P + E$, see Figure 2.16.
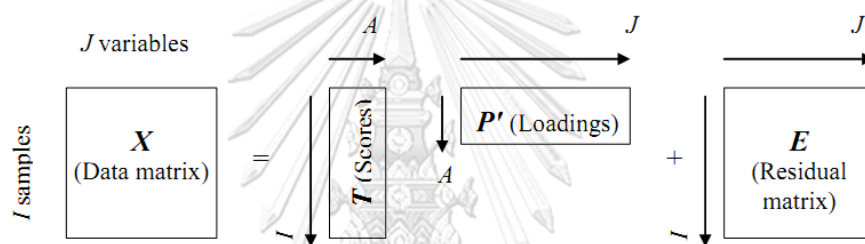
Figure 2.16   diagram illustrating separation of data matrix (X) into Score (T) and loading (P) according to PCA analysis [56].

PCA is applied to investigate variation of data compared with the origin (which may be the average of the whole data). The first PC will provide the most variation with the highest Eigenvalue. The values decrease with later PC. Mean centering approach and the set of starting parameters critically affect the analysis outcome and reliability in PCA analysis. Without comparison with the origin, the analysis can lead to false conclusion.
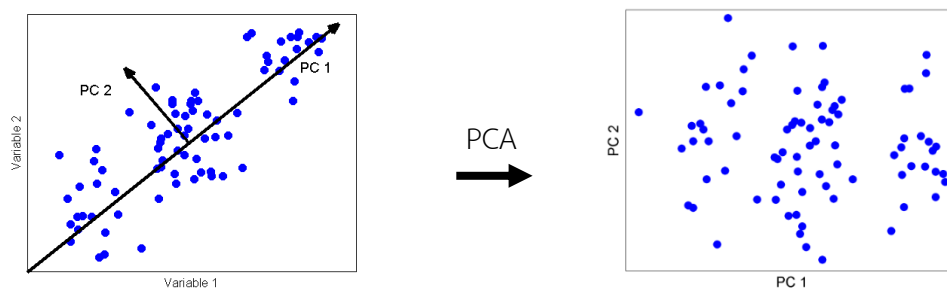
Figure 2.17   (left) normal data (right) after using PCA plot [56]

PCA is selected as the data analysis approach in this thesis since this technique is informative and simple which was applied to find the directions that best explain the variance (m/z, retention time) of the data sets in this study. The number of variables are compressed into much fewer variables which are weighted average of the original variables (herein the compounds). The PCA plots were then obtained clustering samples into different groups [56].

CHAPTER III

EXPERIMENTAL

3.1 Instruments and apparatus

3.1.1   Gas Chromatography Agilent 7890A , Mass spectrometry Agilent 7000 Triple Quadrupole

3.1.2   Capillary column HP-5MS (30 m × 0.25 mm id. with 0.25 μm film thickness), Agilent Technologies.

3.1.3   SPME Holder, SUPELCO.

3.1.4   SPME Fiber Assembly, SUPELCO (50/30 μm DVB/CAR/PDMS)

3.1.5   GC Headspace Vial, 10 mL, Agilent Technologies, USA

3.1.6   20 mm Aluminium Crimp caps, Agilent Technologies, USA

3.1.7   Milli-Q pure water system, Merck (Germany)

3.1.8   Balance (4 digits), Sartorius Model AC211S-00MS (Germany)

3.1.9   Micropipettes 20-200, 100-1000 Eppendorf (Germany)

3.1.10 Vortex mixer, Scientific industries

3.1.11 GC Headspace, 10 mL, Agilent Technologies, USA

3.1.12 Beaker 10, 50, 100, 1000 mL

3.1.13 Volumetric flask 250 mL

3.1.14 Glass syringes 10 mL

3.1.15 Thermometer

3.1.16 Spatula

3.1.17 Droppers

3.2 Chemicals

3.2.1   Sodium chloride was purchased from Carlo erba (France).

3.2.2   Methanol was purchased from Merck (Germany), HPLC grade.

3.2.3   Ethyl benzoate 99% was obtained from Fluka (Maxico), GC grade.

3.2.4   Butyl benzoate 98% was obtained from Fluka (Maxico), GC grade.

3.3 Egg Sample

All fresh raw eggs were obtained from a company A on the same day of egg harvest and were equally divided into two groups. One was stored under cold chain (temperature below 5 $^{\circ}$C), while non-cold chain for the other set. Each group were randomly selected on the 0, 1, 2, 3 and 4 weeks. The under cold chain eggs were placed in the laboratory for 4 hours before analysis at room temperature to slowly increase temperature to room temperature.

3.4 Haugh units measurements

.   The two groups of chicken eggs were determine of haugh units with egg multi tester model: EMT 7300 from 0 to 4 weeks.

3.5 Optimization of gas chromatography-mass spectrometry (GC-MS)

In this study, egg yolks were used for optimization of gas chromatography separation and mass spectrometric detection. Five grams of egg yolk were placed in a 20-mL glass vial, the vial was covered with a teflon septum and with an aluminium crimp cap and placed in a 70°C water bath and the DVB/Carboxen/PDMS fiber of SPME syringe was exposed to the headspace for at 60 min in order to extract the volatile compounds (VOCs) to the fiber. The major parameters of GC separations were optimized: desorption temperature, desorption time and mode of injection.

3.6 Optimization of HS-SPME

The HS-SPME conditions were optimized using a central composite design (CCD, with α= 1.68). The variables chosen for HS-SPME optimization were the extraction time, extraction temp and the salt addition. The factor levels and experimental domain are shown in Table 3.1.

Table 3.1   Factor levels and experimental domain applied to optimized the   HS-SPME Experimental conditions

Factor	Symbol X_i	Code for experimental domain (x)					Linear equations for code and real value
		-1.68	-1	0	1	1.68	
Extraction Temp (C^o)	X_1	43.2	50	60	70	76.8	$X_i = 10x + 60$
Extraction Time (min)	X_2	23.2	30	40	50	56.8	$X_i = 10x + 40$
NaCl (g)	X_3	0.036	0.1	0.3	0.5	0.64	$X_i = 0.2x + 0.3$

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Table 3.2   Design of experiment with experimental design

Experimental run	Code level			parameter		
	X_1	X_2	X_3	Extraction Temp (T_{ex} °C)	Extraction Time (t_{ex} min)	NaCl (g)
1	1	1	1	70	50	0.5
2	1	1	-1	70	50	0.1
3	1	-1	1	70	30	0.5
4	1	-1	-1	70	30	0.1
5	-1	1	1	50	50	0.5
6	-1	1	-1	50	50	0.1
7	-1	-1	1	50	30	0.5
8	-1	-1	-1	50	30	0.1
9	1.68	0	0	76.8	40	0.3
10	-1.68	0	0	43.2	40	0.3
11	0	1.68	0	60	56.8	0.3
12	0	-1.68	0	60	23.2	0.3
13	0	0	1.68	60	40	0.640
14	0	0	-1.68	60	40	0.036
15	0	0	0	60	40	0.3
16	0	0	0	60	40	0.3
17	0	0	0	60	40	0.3
18	0	0	0	60	40	0.3

3.7 Preparation of internal standard solution (IS)

    3.7.1   Preparation of stock internal standard solution

        3.7.1.1. Ethyl benzoate

        Stock internal standard solution 1,000 mg/L of ethyl benzoate was prepared by pipetting of 96.6 µL of Ethyl benzoate and dissolving in methanol and made up to 100 mL in volumetric flask. This solution was stored in a refrigerator at 4 °C, until required for analysis.

        3.7.1.2. Butyl benzoate

        Stock internal standard solution 1,000 mg/L of Butyl benzoate was prepared by pipetting of 101.6 µL of Butyl benzoate and dissolving in methanol and made up to 100 mL in volumetric flask. This solution was stored in a refrigerator at 4 °C, until required for analysis.

    3.7.2   Preparation of working internal standard solution

        The working internal standard solution of 3.0 µg/mL of ethyl benzoate and 5.0 µg/mL of butyl benzoate were prepared by pipetting 150 µL and 250 µL of each stock internal standard solution into 50 mL volumetric flask 1 and 2, respectively. then made up volume with milli-Q water.

3.8 Analysis of volatile compounds in raw eggs and soft boiled eggs under cold chain and non-cold chain

    3.8.1 Raw eggs

        The cold chain eggs were placed in the laboratory for 4 hours before analysis at room temperature to slowly increase temperature to room temperature. For each sample three eggs were broken and the egg yolk and egg white was separated manually and combined for HS-SPME-GC-MS analysis.

        Five grams of both raw eggs (egg yolk and egg white) were placed in a 20-mL glass vial, and then 0.4 grams NaCl and 20 µL of the working IS solution were added into the vial. After that, the vial was covered with a teflon septum and with an aluminium crimp cap. After vortexing for 1 min, the vial of all matrices was placed in a 65°C water bath and the DVB/Carboxen/PDMS fiber of SPME syringe was exposed to the headspace for 45 min in order to extract the volatile compounds to the fiber.

Weight raw egg approximately 5.0 g and add NaCl 0.4 and pipetting IS (3µg/mL)

Vortexing 1 min

Placed in a 65°C water bath, 45 min

Figure 3.1   Flow diagram of the extraction of volatile compounds in raw egg.

3.8.2 Soft boiled egg

For prepared soft boiled egg, distillation water was boiled up to 100° C for 5 minutes, and five fresh raw eggs from each group (cold chain and non-cold chain) were placed in the beaker, with water up to 3-5 cm above the eggs, then close the heater and leave for 6 minutes. After that, soft boiled eggs were placed in the laboratory before analysis for reduce temperature to room temperature.

Five grams of both soft boiled eggs (egg yolk and egg white) were placed in a 20-mL glass vial, and then 0.4 grams NaCl and 20 µL of the working IS solution were added into the vial. After that, the vial was covered with a Teflon septum and with an aluminium crimp cap. After vortexing for 1 min, the vial of all matrices was placed in a 65°C water bath and the DVB/Carboxen/PDMS fiber of SPME syringe was exposed to the headspace for at 45 min in order to extract the volatile compounds to the fiber.

Weight of soft boiled egg approximately 5.0 g
and add NaCl 0.4 and pipetting IS (3 µg/mL) 20

Vortexing 1 min

Placed in a 65°C water bath, 45 min

Figure 3.2 Flow diagram of the extraction of volatile compound in soft boiled egg.

3.8.3 Analysis of volatile compounds

The volatile compounds adsorbed on the SPME fiber were thermally desorbed by inserting the fiber to a GC injector port at temperature of 200°C prior to GC-MS analysis using the following conditions: an Agilent 7890A GC coupled to an Agilent 7000 Triple quadrupole mass spectrometer, an HP-5ms capillary column (30m × 0.25mm i.d. with 0.25μm film thickness), high-purity helium (99.999%) as a carrier gas, split mode with 2.5:1 ratio, column temperature for GC separation starting from 40°C for 10 min with programming at a rate of 10°C/min up to 150°C, and a rate of 30°C/min up to 270°C and with finally holding at this temperature for 6.3 min, scanned mass in the range of m/z 35-300, electron impact mode of 70 eV, and ion source temperature of 200°C.

The volatile compounds obtained from the headspace were identified by comparing their mass spectra with those of the libraries of the NIST mass spectral database 0.8 version. The linear retention index (LRI) values of the detected volatile compounds were also calculated using their GC-MS retention times and a series of n-alkane (C8-C20) as external references.

Linear Retention Index (LRI) were calculated using n-alkanes (C8- C20) (Sigma–Aldrich) as reference compounds using the following expression [57, 58].

$$LRI(x) = 100 \times z + 100 \times \frac{RT\,(x) - RT\,(z)}{RT\,(z+1) - RT\,(z)}$$

Where LRI (x) is the retention index of the unknown compound x

RT (x) is the retention time of the unknown compound x

RT (z) the retention time of the n-alkane eluted before the unknown compound x

RT (z + 1) n-alkane eluted after the unknown compound x

3.9 Statistic analysis

In this study, principal component analysis (PCA) was used to present and visualize the data in two dimension. The multivariate statistical analyses were performed with XLSTAT and response surface plot was used MATHLAB version 7.11 software (Math works, Natick, MA, USA).

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Haugh Unit

Figure 4.1 shows the internal quality of cold chain and non-cold chain eggs by comparing the albumin Haugh Unit (HU) using the experiment as mention in Section 3.4. The higher the value of HU, the better the quality of the egg. This indicates fresher, higher quality eggs with thicker albumin or whites, where HU of 72 or higher refers to the highest rating of AA, 60-71 for A, and 31-59 for B. From Table 4.1 , HU of the cold chain egg decreased from 90.0 to 77.4 by storage for 1 week and then to 71.8 by storage 4 weeks, along with non-significant difference in HU by storage for 1-4 week. In addition, the cold chain egg with storage for 4 week remained its fresh quality of AA rating, Implying small impact on the albumin forming propereties change albuminn like the cold chain egg, non-cold chain egg shows a slight decrese in HU from 64.4 to 43.1 by storage for 1 to 4 weeks, indicating the fresh quality of A for 1 week, and B for 2-4 weeks. These results are in agreement with previous work where a non-significant decrease in HU by egg storage for 3 to 14 days at 4 $^{\circ}$C, but a significant decrease in HU by egg storage for 3 to 14 days at 20 $^{\circ}$C [9], which are consistent with the egg storage within 10 days had a decrease in the Haugh unit value from 91.4 to 76.3 at 5 $^{\circ}$C, to 53.7 at 21 $^{\circ}$C and to 40.6 at 29 $^{\circ}$C [59].

Table 4.1   HU of albumin quality under cold chain and non-cold chain eggs

Storage type	HU at storage time (week)				
	0	1	2	3	4
cold chain		77.4 ± 4.2	75.0 ± 4.7	73.4 ± 0.9	71.8±7.1
non-cold chain	90.0 ± 0.7	64.4 ± 1.2	58.4 ± 3.2	49.9 ± 9.6	43.0±14.0
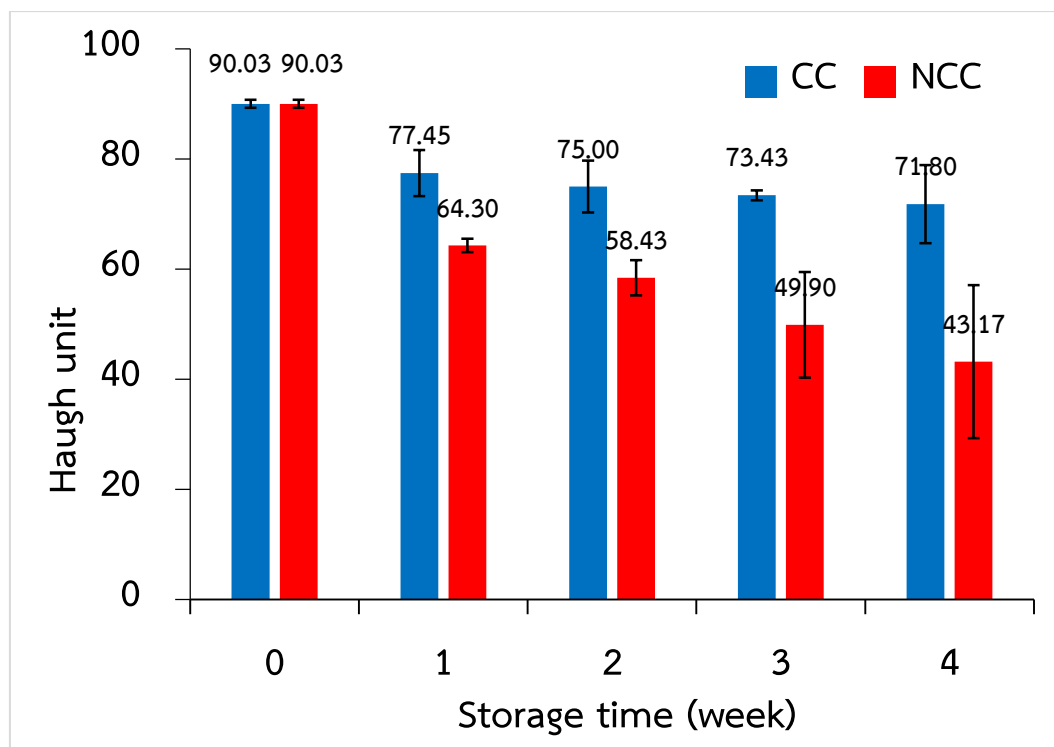
Figure 4.1   Change in Haugh unit content of albumin quality

CC: Cold chain and NCC: Non-Cold chain

4.2 GC-MS optimization

Along with MS conditions given in Table 4.2, the following GC-MS conditions were optimized as given in section 3.5: the desorption temperatures of 200 to 270 °C, desorption time of 5 to10 min, the injection modes of split and splitless mode. As shown in appendix A-1 and A-2, the desorption temperature and desorption time were not significant of shape and separation of peak area. Therefore, the 200 °C of desorption temperature was chosen because of low temperature can be extend lifespan of fiber. The 5 minutes of desorption time was picked due to decrease of analysis time. In part of injection modes as shown in A-3 the split ratio of 2.5:1 was selected for further analysis, providing good peak symmetry and sufficient detection. The suitable results obtained are summarized in Table 4.3.

Table 4.*2*   Mass spectrometer parameters used determination of volatile compounds in chicken eggs under cold chain and non-cold chain

Mass parameter	Mass condition
MS type	Single quadrupole
Ionization source	Electron impact (EI) with -70 eV
MS operating mode	Full scan
Mass range	35-300
MS source temperature	230 °C
Solvent delay time	1 min

Table 4.3   The GC conditions used for determination of volatile compounds in chicken eggs under cold chain and non-cold chain

GC Parameter	GC condition
Analytical column	Capillary column HP-5MS (30 m × 0.25 mm, 0.25 μm film thickness)
Temperature program	40 °C (hold for 10 min), to 150 °C with 10 °C/min, to 270 °C with 30 °C/min (hold for 6 min)
Injection mode	Split mode with ratio 2.5 : 1
Desorption temperature	200 °C
Desorption time	5 min

4.3 HS-SPME Optimization

In prelimary HS-SPME GC-MS analysis of eggs using a PDMS/CAR/DVB fiber, the greater amounts of volatile compounds in egg yolk than those in egg white were observed. Therefore, the egg yolk was used for HS-SPME optimization. Using a central composite design with 18 runs as shown in Table 4.4, the following three factors were optimized: extraction temperature of 50-70 °C, extaction time of 30-50 min and the NaCl amount of 0.1-0.5 g. A multiple regression model (MLR) was shown in this equation:

$$Y = 23.6048 + 4.3153\,X_1 + 1.9326\,X_2 + 0.7891X_3 - 3.9413\,X_1^2 - 1.9793\,X_2^2$$

$$- 0.9911\,X_3^2 + 2.7594\,X_1X_2 + 2.3618\,X_1X_3 - 0.1470\,X_2X_3$$

Three first-order effects X_1 X_2 and X_3 are linear terms corresponding of extraction temperature, extraction time and amount of NaCl. The three second-order effects X_1^2, X_2^2, X_3^2 and three interaction effects X_1X_2, X_1X_3 and X_2X_3. The value and sign of coefficients (positive and negative) in linear terms indicated that the magnitude and the direction of the each effect parameters. In this study all parameters showed positive effect to response value. In order to interpret optimum HS-SPME condition from multi parameters, the 3D plots by the response surface method was constructed as shown in Figure 4.2, using the total peak area as Y-axis and another two from three factor as X-axis and Z-axis. It should be noted that all values on X-axis and Z-axis for these plots are code values, (-1.68 to 1.68) designed to relate to real values as also given in Table 4.4. Taking into account for the optimum condition from response surface at the top or from a score plot with the circle area at the middile, Table 4.6 shows the optimum conditions of score plot and real value. It can be conclueded that optimum HS-SPME condition were obtained at extraction time of 45 min (score plot of 0.5), the NaCl amount of 0.4 g (score plot 0.5) and extraction temperature of 65-75 °C (score plot 0.5-1.5). Howewer, the extraction temperature of 65 °C was chosen in order to preserve the egg in the raw state.

Table 4.4   Experimental condition and response values (total peak area) of the Central Composite Design (CCD) used for the HS-SPME optimization

Run	Extraction Temp (T_{ex} °C)	Extraction Time (t_{ex} min)	NaCl (g)	Response value (Total peak area, 10^7)
1	70	50	0.5	32.2
2	70	50	0.1	24.7
3	70	30	0.5	25.7
4	70	30	0.1	15.5
5	50	50	0.5	9.3
6	50	50	0.1	9.2
7	50	30	0.5	11.8
8	50	30	0.1	13.2
9	76.8	40	0.3	12.3
10	43.2	40	0.3	9.8
11	60	56.8	0.3	21.7
12	60	23.2	0.3	11.5
13	60	40	0.640	17.7
14	60	40	0.036	21.1
15	60	40	0.3	25.7
16	60	40	0.3	22.9
17	60	40	0.3	23.2
18	60	40	0.3	23.2

Table 4.5 Factor levels and experimental domain applied to optimized the HS-SPME Experimental conditions

Factor	Symbol X_i	Code for experimental domain (x)					Linear equations for code and real value
		-1.68	-1	0	1	1.68	
Extraction Temp (T_{ex} °C)	X_1	43.2	50	60	70	76.8	$X_i = 10x + 60$
Extraction time (t_{ex} min)	X_2	23.2	30	40	50	56.8	$X_i = 10x + 40$
NaCl (g)	X_3	0.036	0.1	0.3	0.5	0.640	$X_i = 0.2x + 0.3$

Table 4.6 Shows the the HS-SPME optimum coditions of score plot and real value

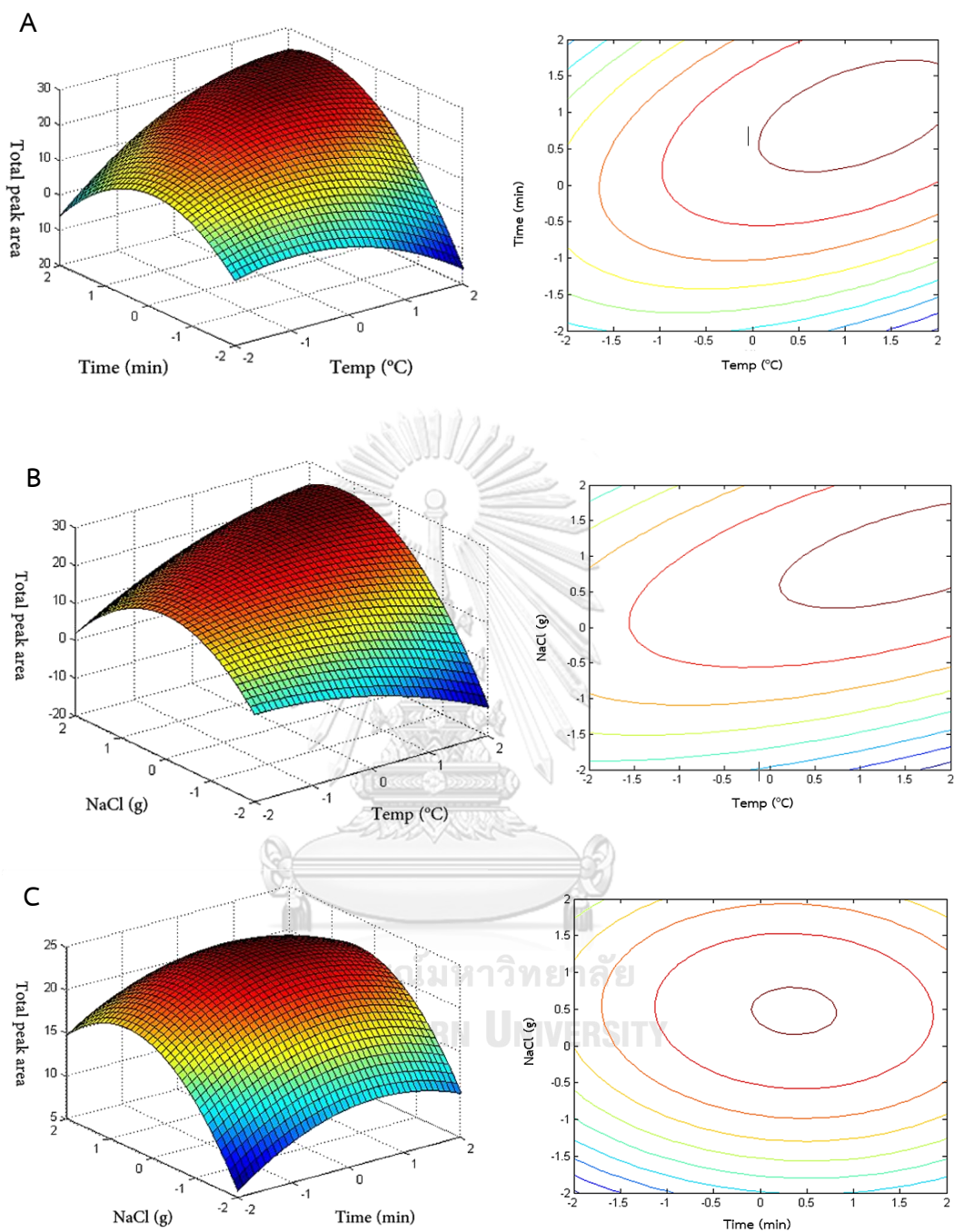| Figure	Factor	Score plot range	Real value range
[A]	Temp (°C)	0.5-1.5	65-75
	time (min)	0.5-1.5	45-55
[B]	Temp (°C)	0.5-1.5	65-75
	NaCl (g)	0.5-1.5	0.4-0.6
[C]	time (min)	0-0.5	40-45
	NaCl (g)	0.5	0.4

Figure 4.2 Response surface and score plot for total peak area of all volatile compounds vs.

(A) Extraction Temp (T_{ex} C°) and Extraction Time (t_{ex} min)

(B) Extraction Temp (T_{ex} C°) and NaCl (g)

(C) Extraction Time (t_{ex} min) and NaCl (g)

4.4 Method precision

In order to evaluate method precision for intra-day and inter-day, ethyl benzoate (3 μg/mL) and butyl benzoate (5 μg/mL) at known amounts of 20 μL each, used as internal standards (IS), were spiked to an HS-SPME vial containing the weighted raw egg prior to HS-SPME extraction, using three days and each day for five replicate samples. Figure 4.3 shows an example of GC-MS chromatogram of this study. Taking into account for a ratio of peak area of butyl benzoate (IS2) to that of ethyl benzoate (IS1), the standard deviation (SD) of a ratio of peak area was calculated as shown in Table 4.7. For intra-day and inter-day precision, the relative standard deviation (RSD) in the ratio of peak area was found to be not over 10%. It should be noted that, using an analysis of varience (ANOVA): single factor analysis at 95% confidence level, non-significant difference in intra-day SD values with P-value of 0.18 which is more than 0.05, and therefore SD and RSD values for interday precision were obtained from a set of all data with n = 15.
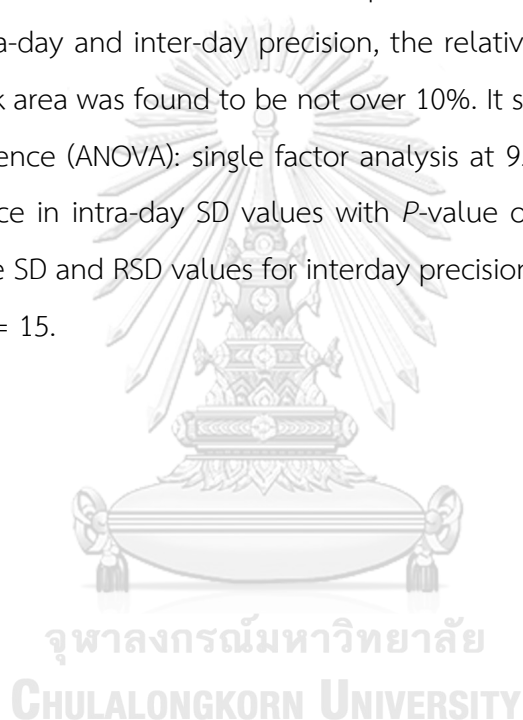
Table 4.7   Inter-day and intra-day precision studies of internal standard ethyl benzoate and butyl benzoate

Experimental run	Peak area ratio (IS2/IS1)		
	Day 1	Day 2	Day 3
1	0.642	0.724	0.814
2	0.695	0.761	0.769
3	0.695	0.650	0.825
4	0.747	0.689	0.756
5	0.783	0.689	0.664
Average (each day)	0.712	0.702	0.766
SD (each day)	0.054	0.042	0.064
%RSD (each day)	7.6	6.0	8.4
Average (Intra-day Inter-day)	0.727		
SD (Intra-day Inter-day)	0.058 (with *P*-value of 0.18, indicating non-significant difference in intra-day SD)		
%RSD (Intra-day Inter-day)	7.9		

IS1 = Ethyl benzoate 3 µg/mL   IS2 = Butyl benzoate 5 µg/mL

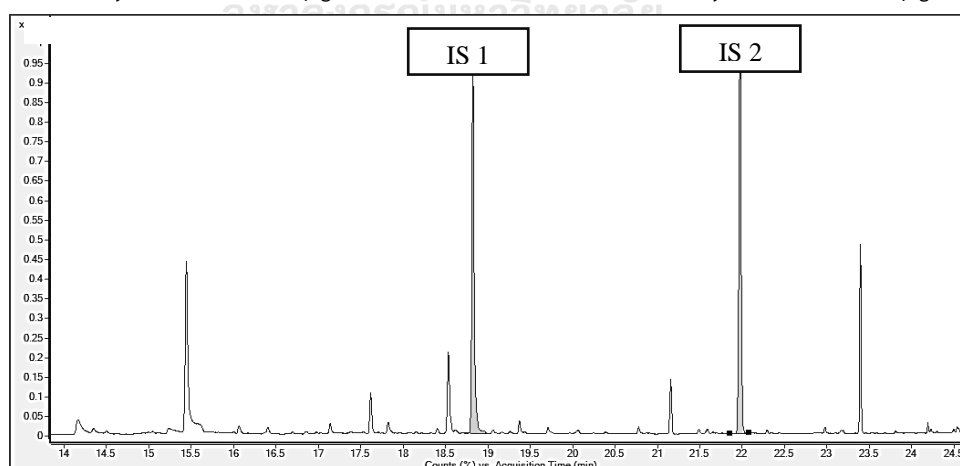**Figure 4.3**   An example of the GC-MS chromatogram of ethyl benzoate (IS1) at 3 µg/mL and butyl benzoate (IS2) at 5 µg/mL spiked with known amount of 20 µL each in egg sample.

4.5 Determination of volatile compounds in raw eggs and soft boiled eggs under cold chain and non-cold chain

4.5.1 Identification of volatile compounds in egg sample

Figure 4.4 shows an example of a GC-MS chromatogram of volatile compounds in the raw egg yolk sample obtained from a non-cold chain system at 3th week storage. In this study, the volatile compounds obtained from each peak in the GC-MS chromatogram were identified by comparing their mass spectra with NIST 0.8 version library and also comparing their experimental values of linear retention index (LRI) with the average calculated values from literature reported in NIST chemistry web book by taking into account for experimental values falling within ±SD of the literature reported. Table 4.8 shows a list of the identified volatile components. It should be noted that some peaks are alkyl siloxanes from SPME fiber, which is typically observed with SPME analysis [60].

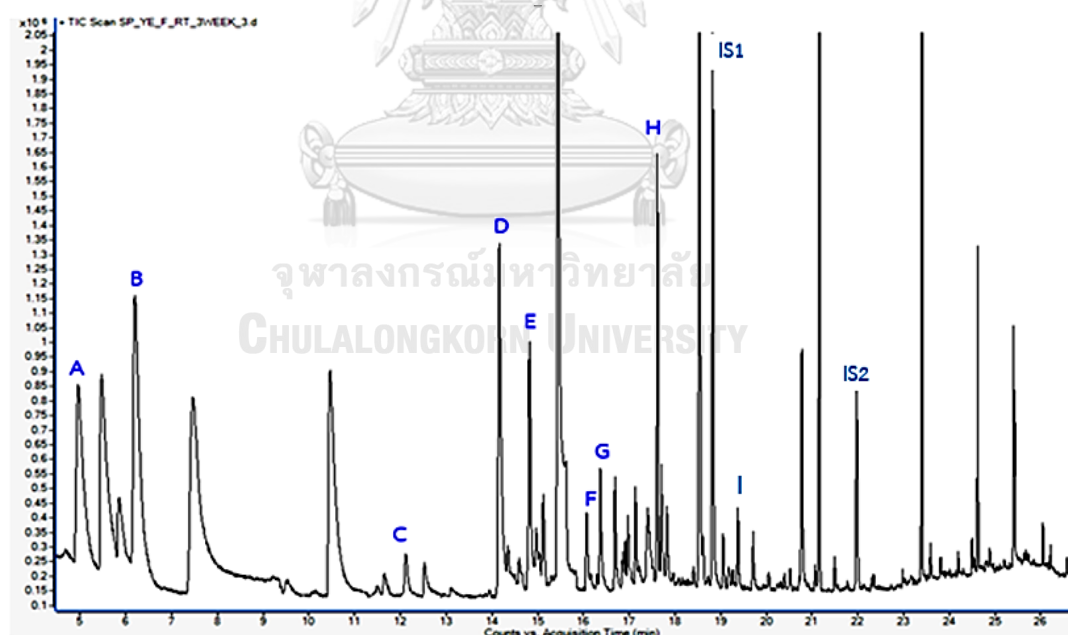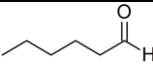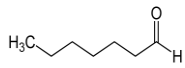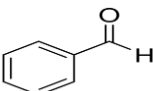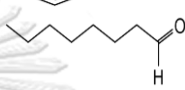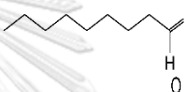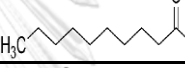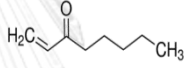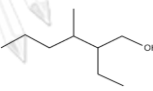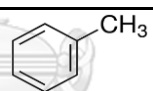**Figure 4.4**   GC-MS chromatogram of HS-SPME volatile compounds in raw egg yolk, A=toluene, B=hexanal, C=heptanal, D= benzaldehyde, E=1-octen-3-ol, F=2-ethyl-1-hexanol, G=octanal, H= nonanal, I=decanal, IS1=ethyl benzoate, and IS2=butyl benzoate.

As seen in Table 4.8, the tentative nine volatile compounds detected in raw egg yolk with the non-cold chain system include six aldehydes (C6-C10 alkyl aldehydes and benzaldehyde, two alcohols (1-octen-3-ol and 2-ethyl-1-hexanol) and one aromatic hydrocarbon (toluene). Previous work reported that the following VOCs in egg yolk were potentially derived from the oxidative decomposition of unsaturated fatty acids for aldehydes [15], nonanal from oleic acid oxidation, two alcohols from the enzymatic degradation of linoleic acid and lipid degradation [15, 17] and toluene from degradation of amino acids with the phenyl group [61]. It should be noted that some or all of these nine volatile compounds were also detected in raw egg yolk with the cold chain system and egg white with the cold chain and non-cold chain system, but different amounts which are discussed in further sections.

Table 4.8   Tentative volatile compounds in chicken egg under cold chain and non-cold chain

Classification	Label	Compound name	m/z	Structure	Identification method		
					LRI[a] (Exp.)	LRI[b] (Lit.)	MS
Aldehydes	B	Hexanal	100		802	803±7(20)	✓
	C	Heptanal	114		904	900±8(28)	✓
	D	Benzaldehyde	100		968	961±9(43)	✓
	G	Octanal	128		1005	1002±5(43)	✓
	H	Nonanal	142		1106	1103±3(71)	✓
	I	Decanal	156		1208	1204±5(51)	✓
Alcohols	E	1-Octen-3-ol	128		979	979±3(36)	✓
	F	2-ethyl-1-hexanol	130		1030	1026±12(3)	✓
Aromatic	A	Toluene	92		770	771±11(11)	✓

a: Experimental LRI on HP5-ms capillary column

b: Literatured LRI on HP5 and HP5-ms capillary columns reported as mean ± standard deviation (number of data)

4.5.2 Volatile compounds in raw eggs under cold chain and non-cold chain

From preliminary study in Section 4.5.1 different types and amounts of volatile compounds were found in raw egg yolk and raw egg white with either cold chain or non-cold chain systems. Therefore, these five sample groups were investigated for comparative amounts of volatile compounds with egg storage at 0, 1, 2, 3, 4 weeks, by measuring PA_{ratio}, where the PA_{ratio} refers to the ratio of peak area of particular volatile compound to that of ethyl benzoate (IS1). Result are summarized in Appendix Table B.1-1 to B.1-4. The principal component analysis (PCA), a chemometrics procedure, was used to interpret the differences in the comparative amounts of volatile compounds in eggs with the same sample group but various storages and different sample groups as shown in Sections 4.5.2.1 and 4.5.2.2.

4.5.2.1 Raw egg yolk

Using 9 variables according to volatile compounds in Table 4.8 and experimental data of PA_{ratio}, as shown in Table A.1, PCA analysis for raw egg yolk samples at five storages (0, 1, 2, 3, 4 weeks) is shown in Figure 4.5 A for a cold chain system with the PCA score plot (A1) and biplot (A2), and Figure 4.5 B for a non-cold chain system with the PCA score plot (B1) and biplot (B2). It should be noted that the PCA score plot is used to predict samples, while the biplot is used to predict volatile compounds. In comparison of the PCA score plots in A1 and B1, It is seen B1 that each set of all weeks can be clearly distinguished from the other set, whilexz the data aggregation for all set is obtained for A1. These indicated that a significant change in types and amounts of volatile compounds in raw egg yolk samples with the non-cold chain system (B1), while a slight change in those with the cold chain (A1). As also can be seen in B2, a significant change of volatile compounds in the non-cold chain system for 1-week storage can be defined by benzaldehyde, while that for 4-week storage can be defined by toluene, hexanal, heptanal, nonanal, 1-octen-3-ol and 2-ethyl-1-hexanol. Due to a slightly change in A1, A2 for the cold chain system cannot be defined by any types of volatile compounds.

Figure 4.5 Score plot and Biplot of principal component analysis of the volatile compounds in raw egg yolk. [A1 and A2] Score plot and Biplot of raw egg yolk under cold chain (CC) [B1 and B2] Score plot and Biplot of raw egg yolk non-cold chain (NCC).

4.5.2.2 Raw egg white

Using the previous concept in Section 4.5.2.1, PCA analysis for raw egg white samples at five storages (0, 1, 2, 3, 4 weeks) is shown in Figure 4.6C for a cold chain system with the PCA score plot (C1) and biplot (C2), and Figure 4.6D for a non-cold chain system with the PCA score plot (D1) and biplot (D2). Similar to B1, it is seen in D1 that each set all weeks can be clearly distinguished from the other set, while and the data aggregation for all set is obtained for B1 with similar to D1. These indicated that a significant change in types and amounts of volatile compounds in raw egg white samples with the non-cold chain system (D1), while a slight change in those with the cold chain (C1). As also can be seen in D2, a significant change of volatile compounds in the non-cold chain system for 3-weeks can be characterized by heptanal and 2-ethyl-1-hexanol, whereas those for 4-week storage can be characterized by octanal and 1-octen-3-ol.

According to previous work on a comparison of whole eggs stored at 4 °C and room temperature, PCA analysis was also obtained that the room temperature storage gave a set of data clearly separated from the other set (0-4 weeks), while 4°C storage resulted in the clustering dataset except for 4-week storage that was separate from the other set [62].
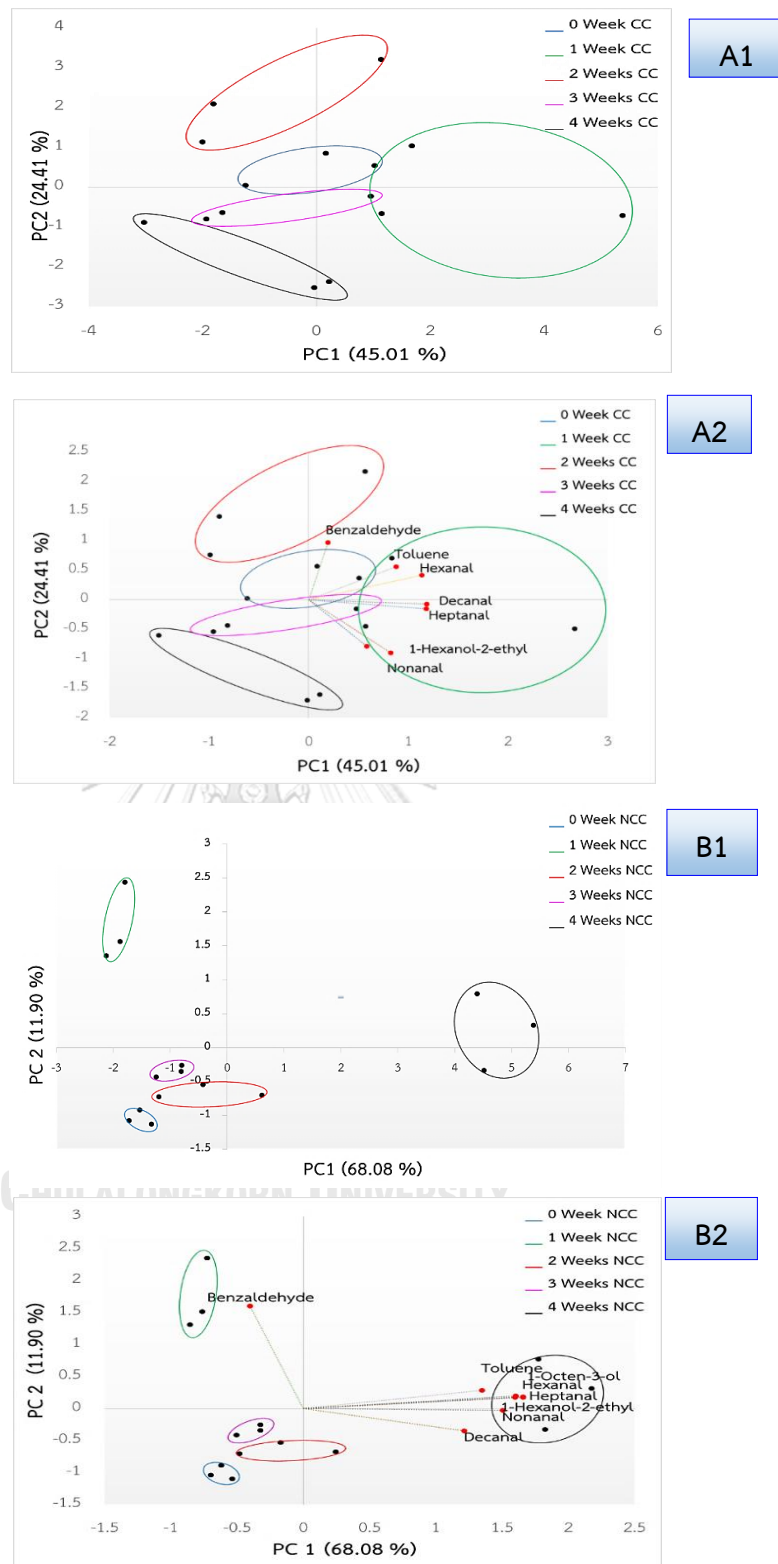
Figure 4.6 Score plot and Biplot of principal component analysis of the volatile compounds in raw egg white. [C1 and C2] Score plot and Biplot of raw egg white under cold chain (CC) [D1 and D2] Score plot and Biplot of raw egg white non-cold chain (NCC).

4.5.3 Volatile compounds in soft boiled eggs under cold chain and non-cold chain

4.5.3.1 Soft boiled egg yolk

Using the previous concept in Section 4.5.2.2, PCA analysis for soft boiled egg yolk samples at five storages (0, 1, 2, 3, 4 weeks) is shown in Figure 4.7E for a cold chain system with the PCA score plot (E1) and biplot (E2), and Figure 4.7F for a non-cold chain system with the PCA score plot (F1) and biplot (F2). As seen in F1 with similar results of B1 and D1, the datasets are clearly separated from each other, and the significant change for 4-weeks storage as shown in F2 can be defined by hexanal, heptanal, nonanal, 2-ethyl-1-hexanol and 1-octen-3-ol. Unlike the cold chain system A1 and C1 for raw egg yolk and white, respectively, the datasets for soft boiled egg yolk with the cold chain system in E1 are partially overlapping at 0 and 1-week storage, but clearly separated at 2, 3 and 4-week storage. This significant change for 2-week storage is characterized by toluene, hexanal, heptanal, benzaldehyde, octanal, nonanal, decanal and 2-ethyl-1-hexanol, while that for 3-week storage is characterized by 1-octen-3-ol.

Figure 4.7   Score plot and Biplot of principal component analysis of the volatile compounds in softed boiled egg yolk. [E1 and E2] Score plot and Biplot of soft boiled egg yolk under cold chain (CC) [F1 and F2] Score plot and Biplot of soft boiled egg yolk non-cold chain (NCC).

4.5.3.2 Soft boiled egg white

Also the same concept with Section 4.5.3.1, PCA analysis for soft boiled egg white samples at five storages (0, 1, 2, 3, 4 weeks) is shown in Figure 4.8G for a cold chain system with the PCA score plot (G1) and biplot (G2), and Figure 4.8H for a non-cold chain system with the PCA score plot (H1) and biplot (H2). Both G1 and H1 show that the all datasets are clearly separated from each other, but difference in the type of volatile compounds used to characterize for the significant change. For example, the cold chain system for 2-week storage can be characterized by benzaldehyde nonanal, decanal, 1-octen-3-ol and 2-ethyl-1-hexanol, but non-cold chain system for 2-week storage can be characterized by hexanal, heptanal and nonanal.
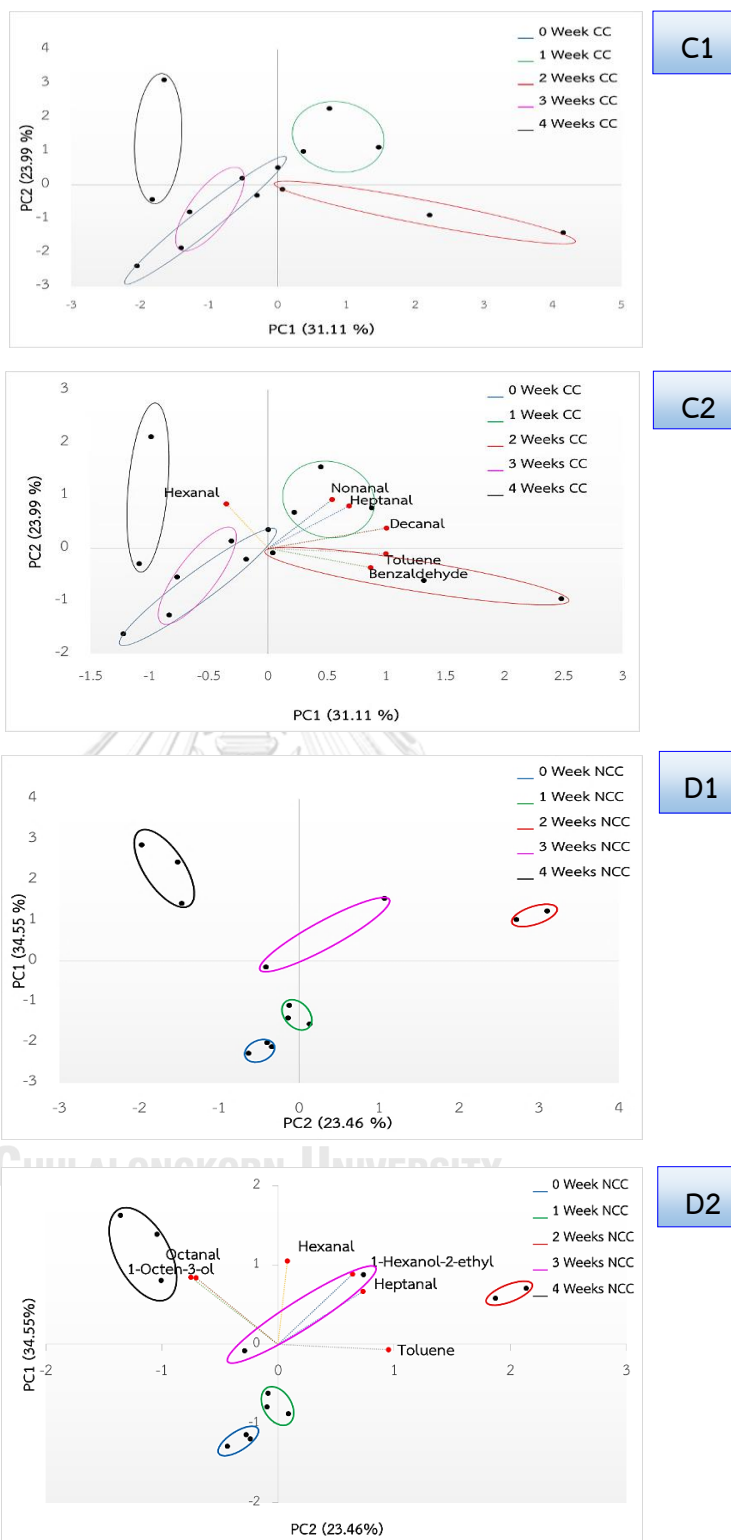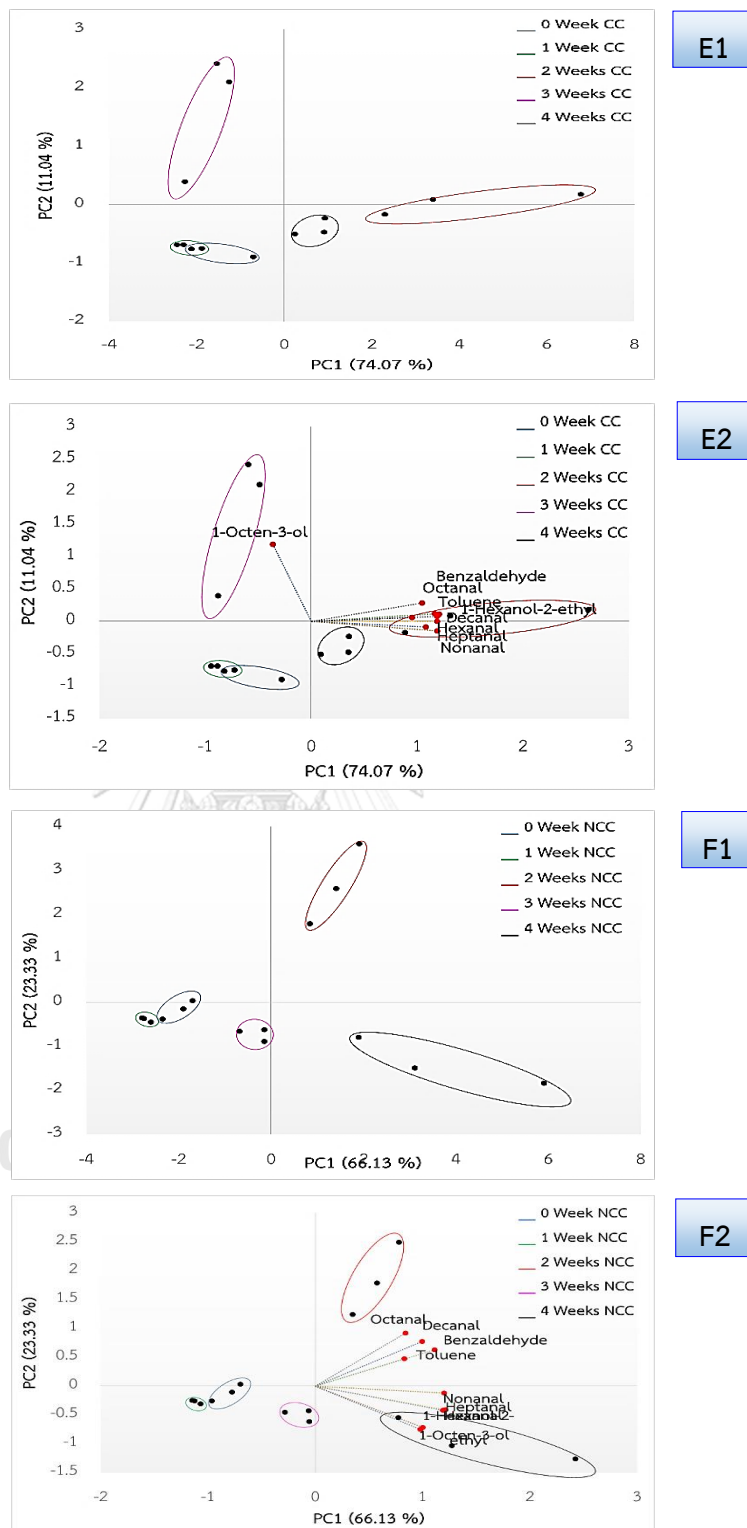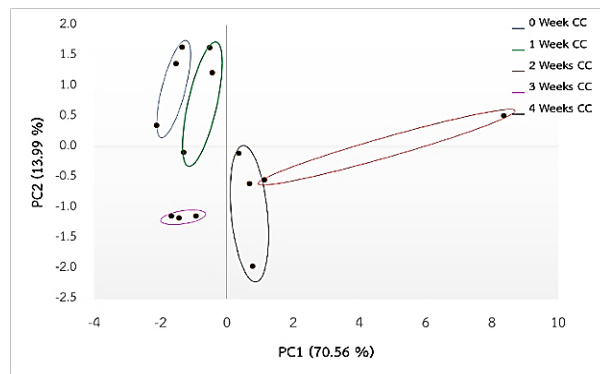
จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY
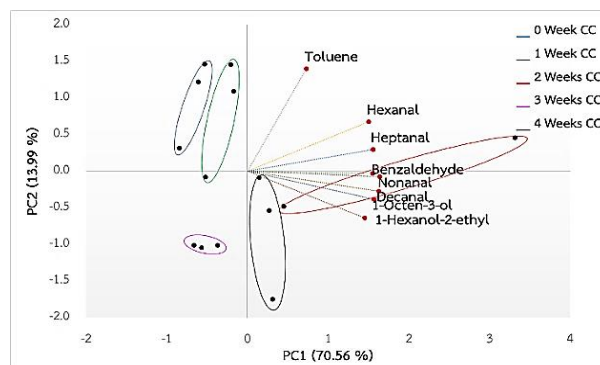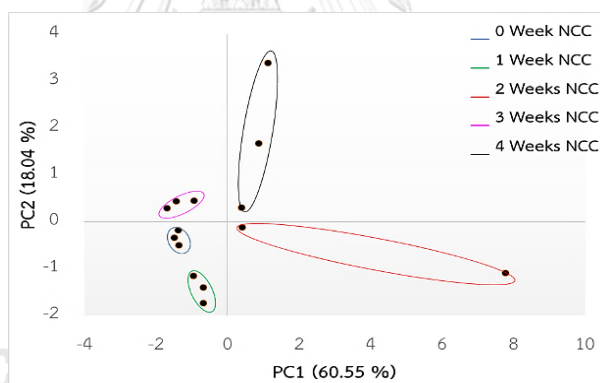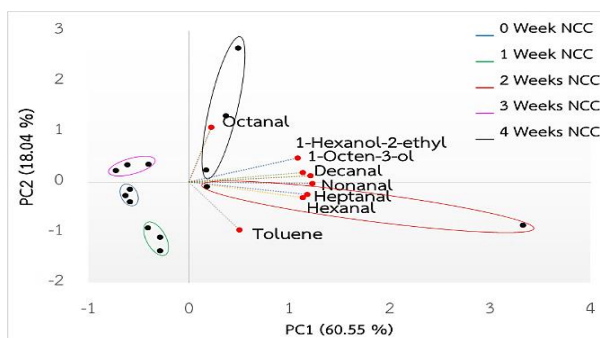
Figure 4.8   Score plot and biplot of principal component analysis of the volatile compounds in soft boiled egg white. [G1 and G2] Score plot and biplot of soft boiled egg white under cold chain (CC) [H1 and H2] Score plot and biplot of soft boiled egg white non-cold chain (NCC).

4.5.4 Summary of PCA analysis of eggs

Table 4.9 shows a comparison of the PCA analysis of eggs according to type, state and process. For the cold chain system, non-separated dataset for either raw egg yolk or raw egg white indicates that low temperature by the cold chain system starting results in a small change in volatile compounds emitted from raw eggs after their storage in the refigerator below 5°c for 0 to 4 weeks. On the otherhand, clearly separated dataset for all egg types and states from the non-cold chain system indicates that room temperature by the non-cold chain system results in a sinificant change of volatile compounds emitted from eggs. However, partially and clearly separated dataset for soft boiled eggs implies that high temperature from the soft boiling proocess may result in a significant change of volatile compounds emitted from eggs under either the cold chain or non-cold chain system.

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Table 4.9   Summary of PCA analysis of eggs

Egg state	Egg type	Procees	PCA analysis of Dataset for 0-4 week strage	
			Score plot	Loading plot from Biplot
Raw egg	yolk	CC	Non-seperated dataset	Cannot be characterized
		NCC	Clearly seperated dataset	Characterized by benzaldehyde at 1 week, and toluene, hexanal, heptanal, nonanal, 1-octen-3-ol and 2-ethyl-1-hexanol at 4-week.
	white	CC	Non-seperated dataset	Cannot be characterized
		NCC	Clearly seperated dataset	Characterized by heptanal and 2-ethyl-1-hexanol at 3-week and octanal and 1-octen-3-ol at 4-week
Soft boiled egg	yolk	CC	Partially seperated dataset	Characterized by toluene, hexanal, heptanal, benzaldehyde, octanal, nonanal, decanal and 2-ethyl-1-hexanol, at 2-week and 1-octen-3-ol at 3-week
		NCC	Clearly seperated dataset	Characterized by hexanal, heptanal, nonanal, 2-ethyl-1-hexanol and 1-octen-3-ol at 4-week
	white	CC	Clearly seperated dataset	Characterized by toluene, hexanal, heptanal, benzaldehyde, octanal, nonanal, decanal and 2-ethyl-1-hexanol at 2-week.
		NCC	Clearly seperated dataset	Characterized by hexanal, heptanal and nonanal at 2- week

CC= cold-chain   NCC = non-cold chain

4.6 A comparison of volatile compounds found in chicken eggs

Typically, the chicken eggs sold in the general market would normally expire within 2 weeks for room temperature storage. However, the longer expire may be extended upto 4 weeks for the eggs stored at the temperature of 5 $^{\circ}$C or below [63, 64]. In addition, results of PCA analysis in Section 4.5 showed a significant change of volatile compounds emitted from eggs at 2- and 4-week storage. Therefore, Figure 4.9 shows a comparison of volatile compounds found in chicken eggs stored for 2- and 4-week.

It can be seen from Figure 4.9 and 4.10, all nine volatile compounds was found in all eggs with the comparative amounts in order: non-cold chain > cold chain, egg yolk > egg white and raw egg > soft boiled egg. The first case is due to low temperature in a process of the cold chain system results in a decrease of chemical decomposition. In the second case, egg yolk has a larger amount of lipids and proteins that are sources to produce volatile compounds as previously mentioned in Section 3.5. For the last case, some volatiles compound may release from the egg during a cooking process prior to headspace extraction.

For 2-week storage, six to eight volatile compounds were found in the eggs, where non-detection of octanal in egg white either raw or boiled state, non-detection of 2-ethyl-1-hexanol was not observed in egg yolk with the non-cold chain system either raw or boiled state, non detection of 1-octen-3-ol in all eggs except for soft boiled egg white with the non-cold chain system, and non-detectioon of toluene in raw egg white with the non-cold chain system.

Figure 4.9 Volatile compounds in chicken egg under cold chain (CC) and non-cold chain (NCC) 2-week and 4-week storage [A] raw egg yolk [B] raw egg white A= toluene, B= hexanal, C= heptanal, D= benzaldehyde, G= octanal H= nonanal, I= decanal, E= 1-octen-3-ol, F= 2-ethyl-1-hexanol.

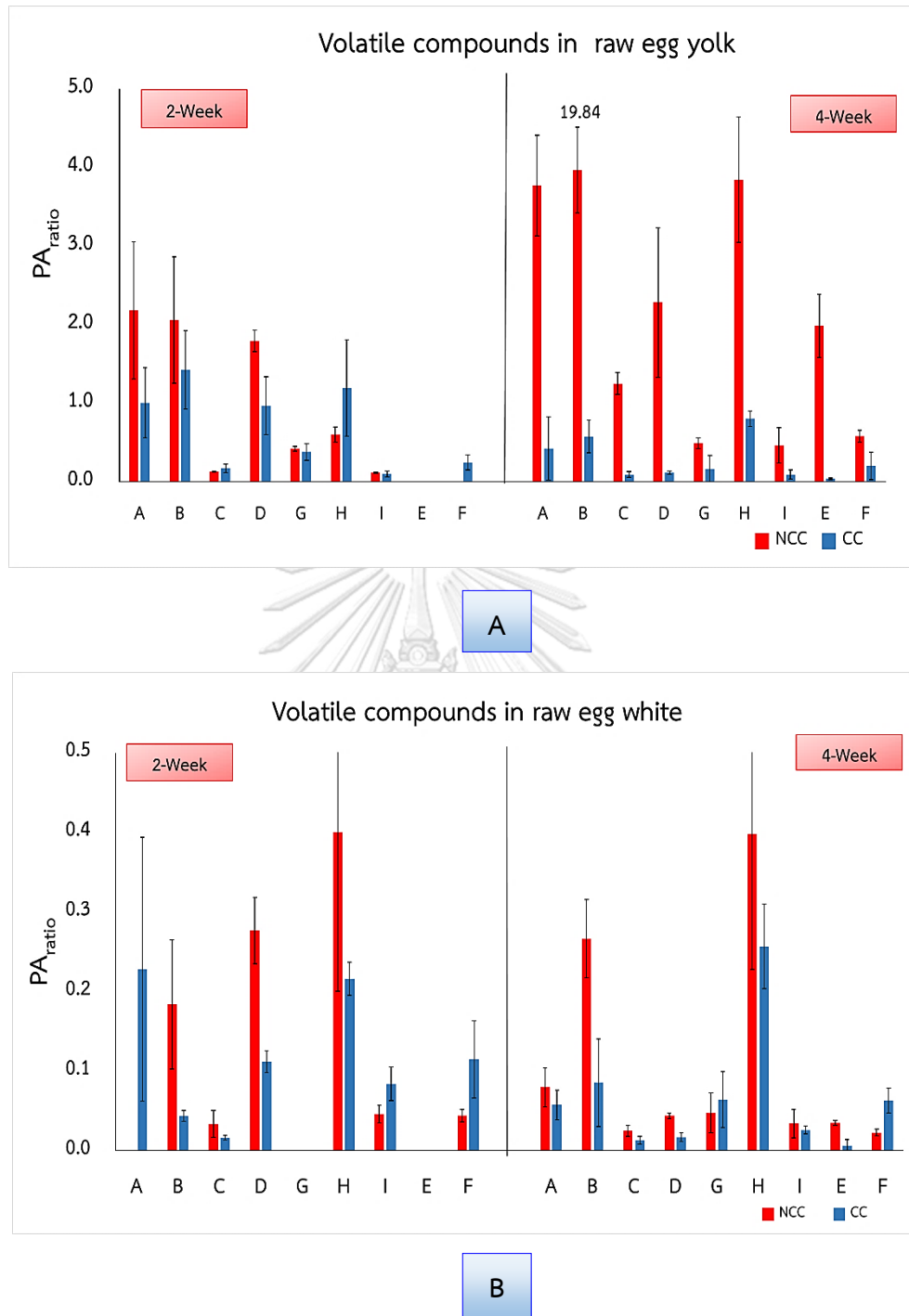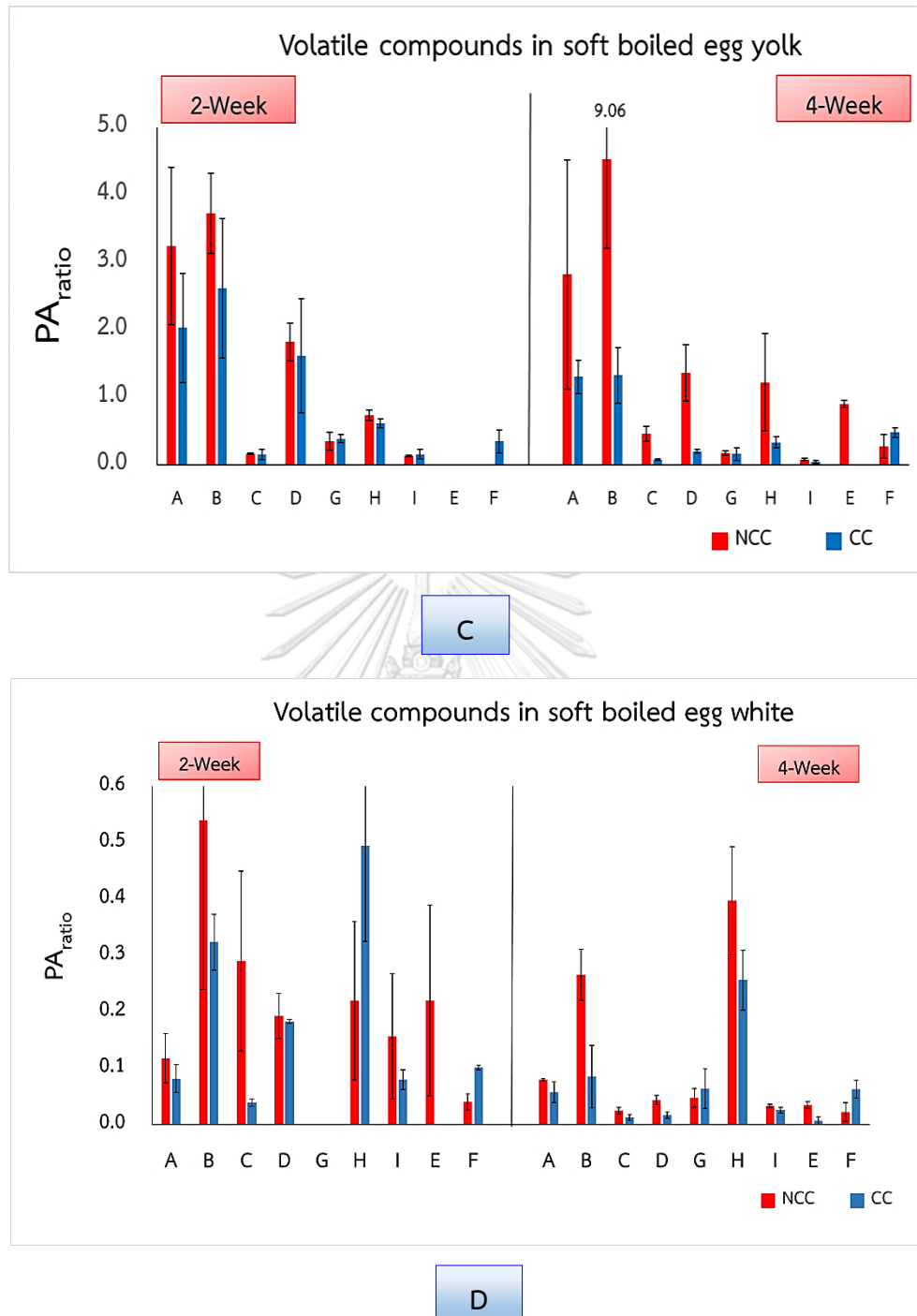Figure 4.9 Volatile compounds in chicken egg under cold chain (CC) and non-cold chain (NCC) 2-week and 4-week storage [C] soft boiled egg yolk [D] soft boiled egg white A= toluene, B= hexanal, C= heptanal, D= benzaldehyde, G= octanal H= nonanal, I= decanal, E= 1-octen-3-ol, F= 2-ethyl-1-hexanol (continued)

4.7 Application: Study of volatile compounds in commercial raw egg yolk

From the experimental method of determine of volatile compounds in egg samples under cold chain and non-cold chain system in Section 3.8.1. it was used to apply for volatile compounds determination in commercial raw egg yolk which were stored at different temperature storage: room temperature and 4 $^{\circ}$C.

Figure 4.10 shows an example of a GC-MS chromatogram of volatile compounds in egg yolk stored at room temperature for 4-week. The identified volatile components in each sample were summarized in Table 4.10.

From the Table 4.10, the tentative volatile compounds were detected in both raw egg yolk at room temperature and 4°C storage. The major aldehydes found in the headspace of egg yolk were hexanal, heptanal, nonanal and decanal. Hexanal and nonanal were found in raw egg yolk at both temperatures throughout 4 weeks of storage, in contrast to heptanal and decanal. Heptanal was found in raw egg yolk only at room temperature throughout 4 weeks, while decanal was found in both temperatures: 2-week and 3-week for room temperature and 3-week for 4°C. Two alcohols, namely 1-octen-3-ol and 2-ethyl-1-hexanol, were found in raw egg yolk both at room temperature and 4°C stored, as well as toluene.
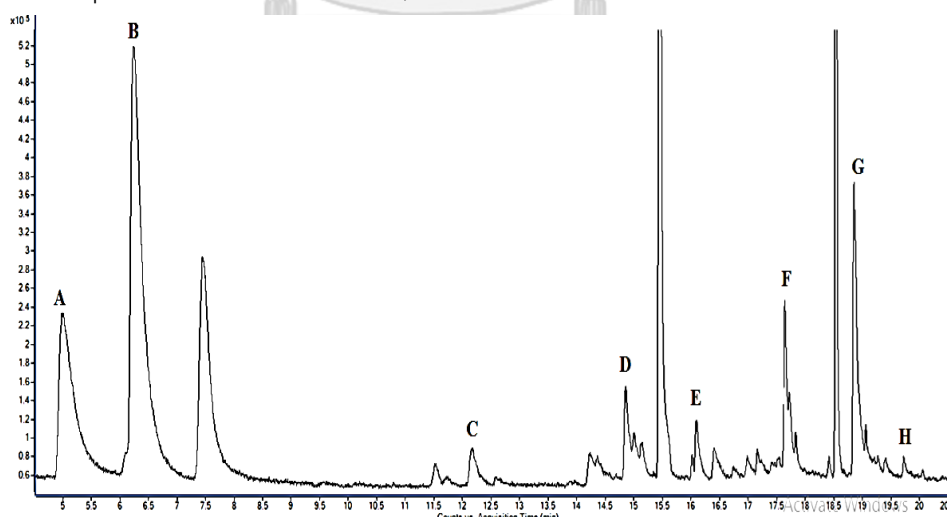
Figure 4.10   GC-MS chromatogram of HS-SPME volatile compounds of commercial raw egg yolk (A=toluene, B=hexanal, C=heptanal, D=1-octen-3-ol, E=2-ethyl-1-hexanol, F=nonanal, G=internal standard, H=decanal

Table 4.10   Tentative volatile compounds in raw egg yolk

Compound	LRI[a] (Exp.)	LRI[b] (Lit.)	Room temperature					4°C				
			0 W	1W	2W	3W	4 W	0 W	1W	2W	3W	4 W
Aldehyde												
Hexanal	802	803 ± 7 (20)	×	×	×	×	×	×	×	×	×	×
Heptanal	899	900 ± 8 (28)	-	-	×	×	×	-	-	-	-	-
Nonanal	1106	1103 ± 3 (71)	×	×	×	×	×	×	×	×	×	×
Decanal	1208	1204 ± 5 (51)	-	-	×	×	-	-	-	-	×	-
Alcohol												
1-Octen-3-ol	979	979 ± 3 (36)	×	×	×	×	×	×	×	×	×	×
2-ethyl-1-hexanol	1024	1026 ± 12 (3)	×	×	×	×	×	×	×	×	×	×
Aromatic												
Toluene	762	771 ± 11 (11)	×	×	×	×	×	×	×	×	×	×

a: Experimental LRI on HP5-ms capillary column

b: Literatured LRI on HP5 and HP5-ms capillary columns reported as mean ±

standard deviation (number of data)

symbol: × detected, - not detected

4.7.1 Effects of storage time and temperature on volatile compound in commercial raw egg yolk

According to Table 4.10, the following five volatile compounds found throughout 4-week for both room temperature and 4 $^{\circ}$C were chosen for quantitative comparison of volatile compounds in egg yolk at two different temperatures: hexanal, nonanal, 1-octen-3-ol, 2-ethyl-1-hexanol and toluene.

From Figure 4.11, using a ratio of peak area of the analyte to that of IS for quantitative comparison, the amounts of five volatile compounds increased from 0 to 4 weeks, especially dramatic change after 2 weeks storage. At 2-week, the higher amount of hexanal in egg yolk at room temperature than that at 4°C was found, while, the lower amounts of 1-octen-3-ol, 2-ethyl-1-hexanol in egg yolk at room temperature than that at 4°C. However, the slightly different amounts of hexanal in egg yolk at both temperatures within 2 weeks and that of toluene throughout 4 weeks. At 3 to 4 weeks the much larger amounts of hexanal, nonanal, 1-octen-3-ol, 2-ethyl-1-hexanol in egg yolk at room temperature than those at 4°C.

Throughout storage of chicken eggs at room temperature and 4°C up to 4 weeks, the longer the storage time, the higher the amounts of the intensity of those volatile compounds. At 3 to 4 weeks of storage, the amounts of these most volatile compounds in raw egg yolk at room temperature was higher than at 4°C. However, toluene had no changes throughout storage at both room temperature and 4°C storage.

Figure 4. 11   Volatile compounds in commercial raw egg yolk stored at room

temperature and 4 °C (A) hexanal (B) nonanal (C) toluene
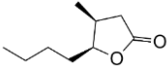
(D) 1-octen-3-ol (E) 2-ethyl-1-hexanol.

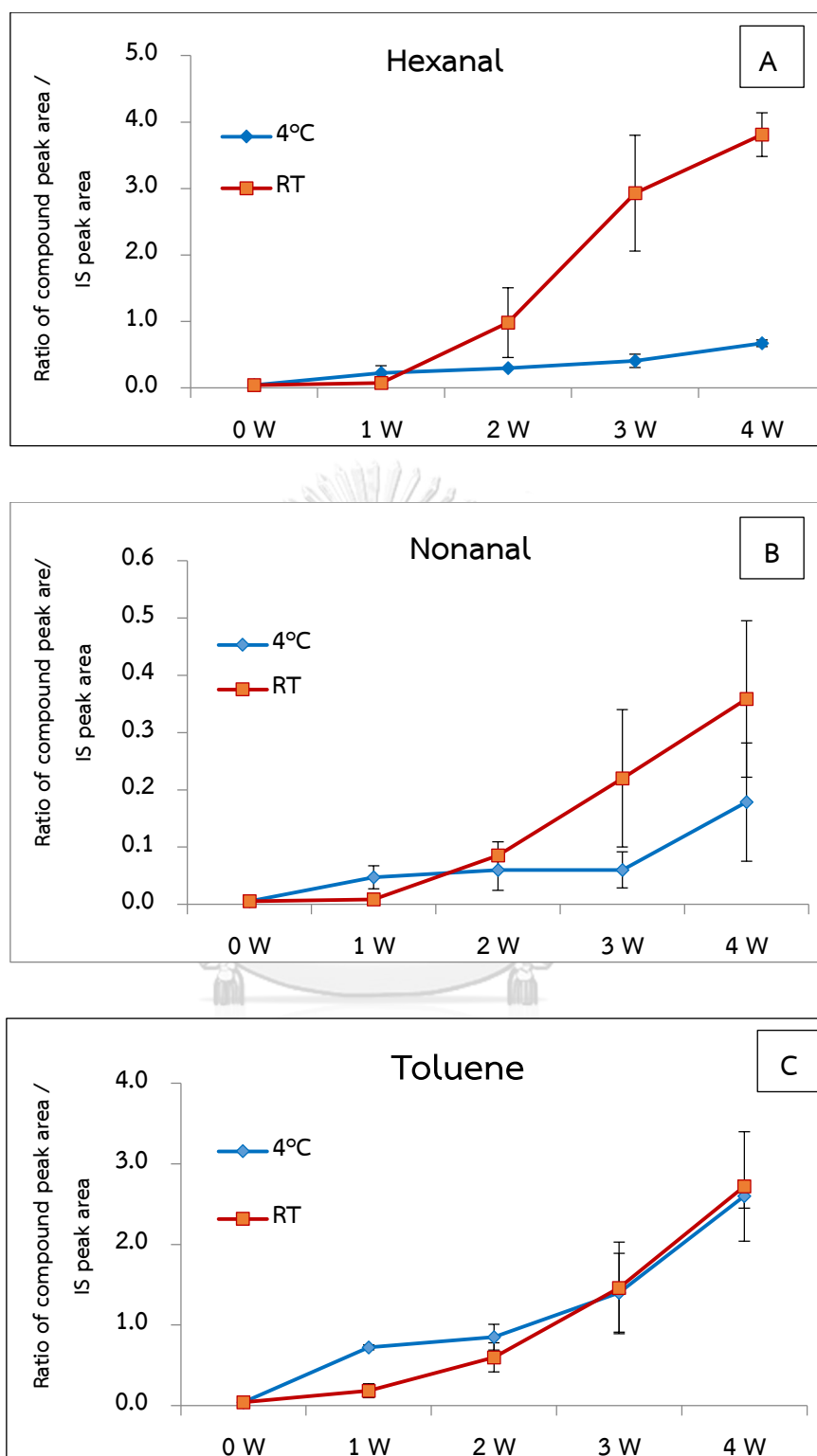**Figure 4.11**   Volatile compounds in commercial raw egg yolk stored at room temparature and 4 °C (A) hexanal (B) nonanal (C) toluene (D) 1-octen-3-ol (E) 2-ethyl-1-hexanol (continued).
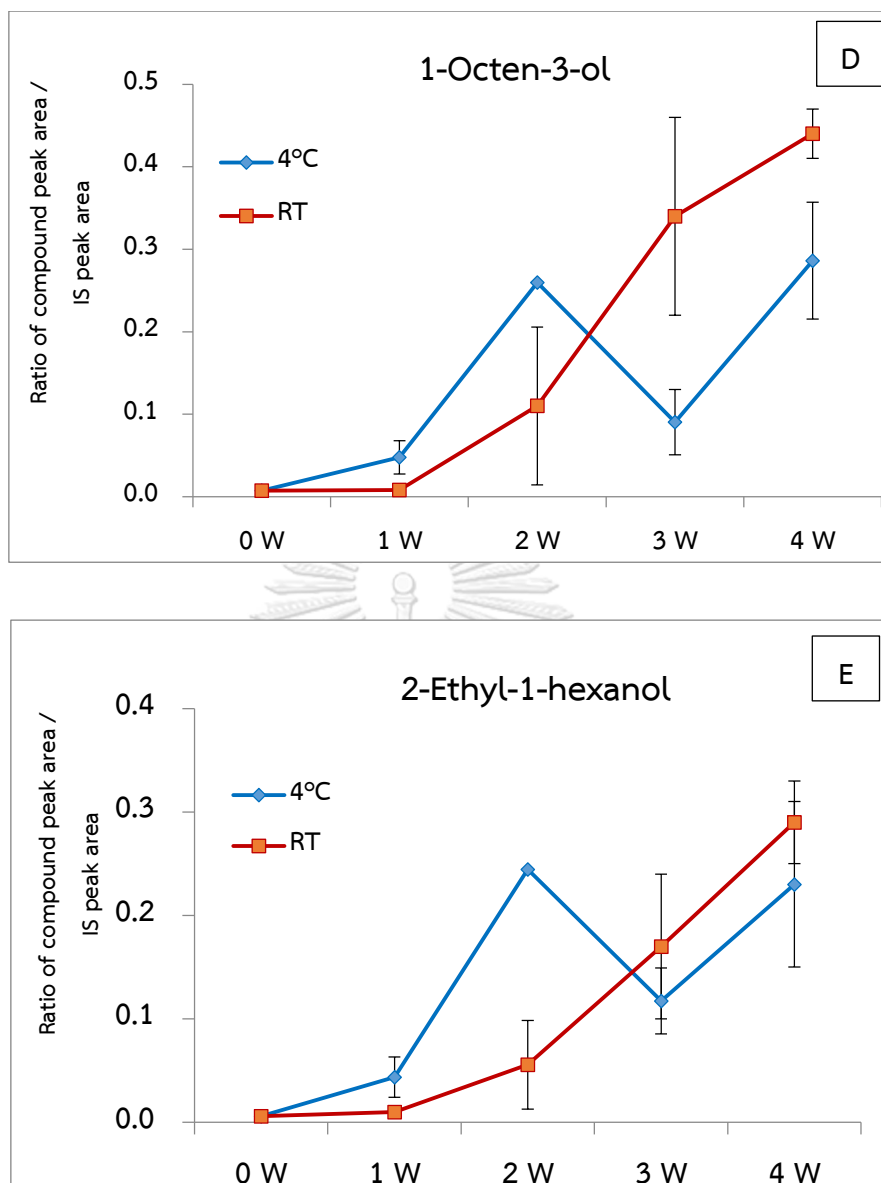
4.8 Case study

On a real case happened, the undisclosed hotel restaurant was claimed from a customer that a hard boiled egg served on a dish emitted unpleasant odor, while the rest eggs had normal odor. In fact, after boiling, all eggs were kept in the refrigerator before serving to customer. This unpleasant odored egg was sent to our lab in order to identify volatile compounds that might produce unpleasant odor.

According to experiment procedure in Section 3.8.1, volatile compounds in the unpleasant and normal egg were compared using HS-SPME-GC-MS as their chromatograms shown in Figure 4.12, as can be seen that the chromatogram of the unpleasant egg appears the outstanding unknown peak at retention time (RT) of 7.49 min, while no peak at this RT for the normal egg. In comparison of its mass spectrum and literature mass spectra in NIST database, this compound at RT of 7.49 min may be cis-3-methyl-4-octanolide with 156 mass and its stucture as shown in Table 4.11. This compound is commonly found in alcoholic beverages and exists woody aroma [65] and wine like aroma [66]. However, the origin of this compound in the unplesant egg was not known in this case. It is interesting that HS-SPME-GC-MS can be used to identify this volatile compound in the egg that may cause unplesent odor for this case heppened.

Table4.11 volatiles compounds in unplesant egg

RT	Compound Name	Structure	Chemical formular	Mass
7.49	cis-3-methyl-4-octanolide		$C_9H_{16}O_2$	156

Figure 4.12   GC-MS chromatogram of HS-SPME volatile compounds of boiled egg
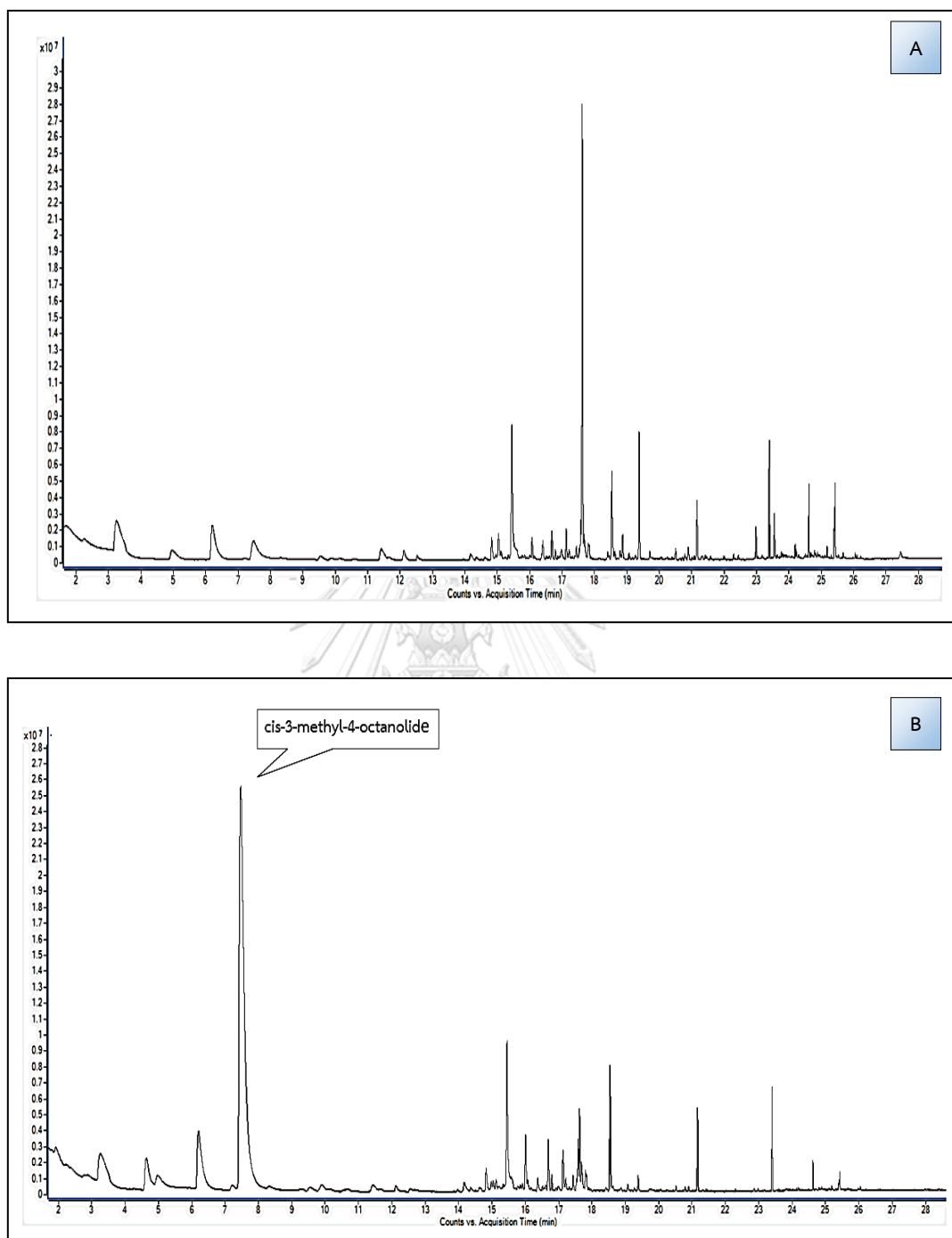white [A] Controlled egg [B] Consumer's egg

CHAPTER V

CONCLUSION

In this work, volatile compounds in eggs via the cold chain and non-cold chain system were determined and compared for different egg types: egg yolk & egg white, egg state: raw egg & soft boiled egg, and different storage duration. From initial study on the internal quality of raw egg white or albumin to classify the freshness grade of the eggs, results of the measured Haugh Unit (HU) values of albumin showed that the cold chain system remained the eggs with the highest freshness grade of AA throughout four weeks studied, while non-cold chain resulted in a decrease in the fresh grade AA at 0-week to grade A at 1-week and grade B at 2- to 4-weeks. This implies that the cold chain system can provide the better fresher eggs.

The volatile compounds in eggs were determined by HS-SPME-GC-MS using the following conditions: a 50/30 μm DVB/CAR/PDMS fiber, SPME insertion to a GC injection port at 200 °C with desorption time of 5 min and spilt ratio of 2.5:1, HP-5MS (30 m x 0.25 mm id. X 0.25 μm film thickness) capillary column programmed from 40 °C (10 min) to 150 °C with ramp rate of 10 °C/min, MS electron impact ionization at -70 eV. According to a central composite design (CCD) for three factors for HS-SPME optimization, the following conditions were obtained: extraction temperature 65oC, extraction time of 45 min and NaCl of 0.4 g. Using ethyl benzoate and butyl benzoate as the internal standards spiked into egg yolk samples for measuring method precision in the ratio of their peak area obtained from HS-SPME-GC-MS chromatograms with five replicates each day for three days, acceptable method precision for intraday and interday was obtained with relative standard deviation in this ratio of less than 10%.

.   Using raw egg yolk from a non-cold chain system at 3-week, nine volatile compounds were identified, including hexanal, heptanal, octanal, nonanal, decanal, benzaldehyde, 1-octen-3-ol, 2-ethyl-1-hexanol and toluene, based on the comparison of mass spectra with NIST library as well as experimental and literature linear retention index data. The first five aldehydes may be derived from oxidative decomposition of

unsaturated fatty acid, two alcohols from enzymatic degradation of linoleic and lipid degradation, toluene from degradation of amino acids and benzaldehyde from oxidation of toluene.

The PCA score plot and biplot of volatile compounds in raw egg (both egg yolk and egg white) under the cold chain system revealed that the datasets were not clearly separated throughout 4 weeks, while the non-cold chain system provided clearly separated groups. This implies that the cold chain results in non different in composition in term of types and amounts of volatile compounds in eggs, which is consistent with the internal quality of albumin giving freshness of grade AA throughout 4-week. For the PCA score plot of soft boiled egg from either non-cold chain or cold chain system, all datasets were partially to clearly separated. Indicating significant in the types and amounts of volatile compounds found in each groups. In comparison of volatile compounds in all samples, the non-cold chain egg samples had a larger change in the amount of the volatile compounds than did the cold chain egg samples, regarding to both raw eggs and soft boiled eggs.

Our HS-SPME-GC-MS was also used to commercial eggs from the market, where the eggs are expected from the non-cold chain system. By keeping the eggs for Group I at 4 oC and Group II at room temperature for 0-4-weeks, Group II gave a larger change in the amount of the volatile compounds in raw egg yolk. This implies that eggs stored at low temperature or under the cold chain system can slowly release volatile compounds.

In addition, this HS-SPME-GC-MS method was also applied to detect volatile compounds in unpleasant egg samples obtained from the real casern. By comparing HS-SPME-GC-MS chromatograms of normal and unpleasant egg samples along with mass spectrum of an extra peak in the unpleasant egg with the NIST library, this extra peak may be cis-3-methyl-4-octanolide.

In the future work, this proposed HS-SPME-GC-MS can be used for quality control of egg industry. Moreover, HS-SPME-GC-MS combined with chemometrics may be applied for volatile compound analysis in food ingredients.

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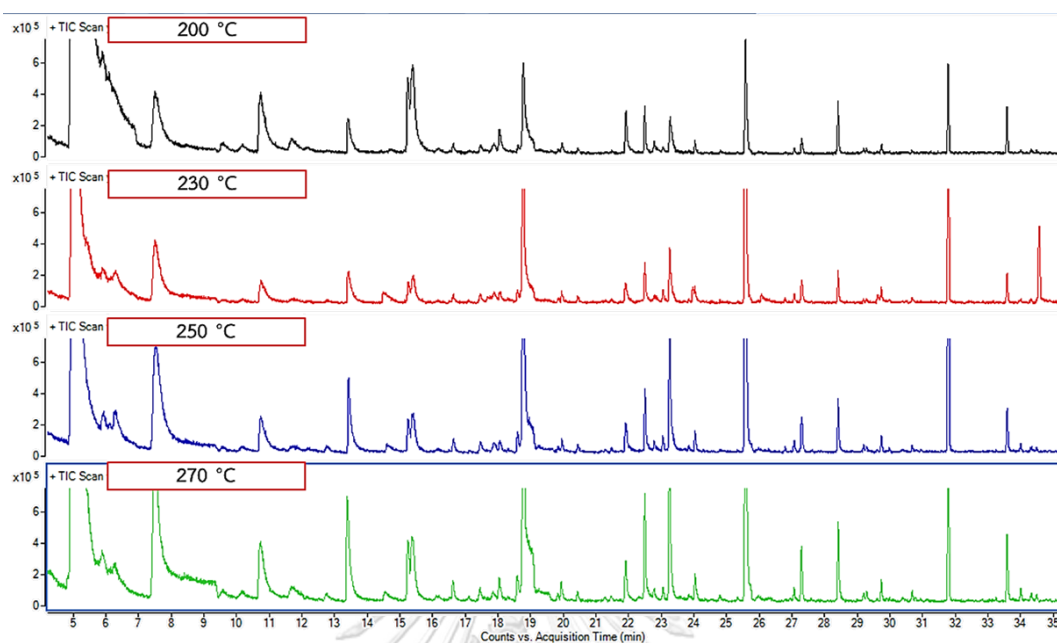APPENDIXS

APPENDIX A

Figure A-1.1   GC-MS chromatogram of desorption temperature at 200 °C, 230 °C, 250 °C and 270 °C
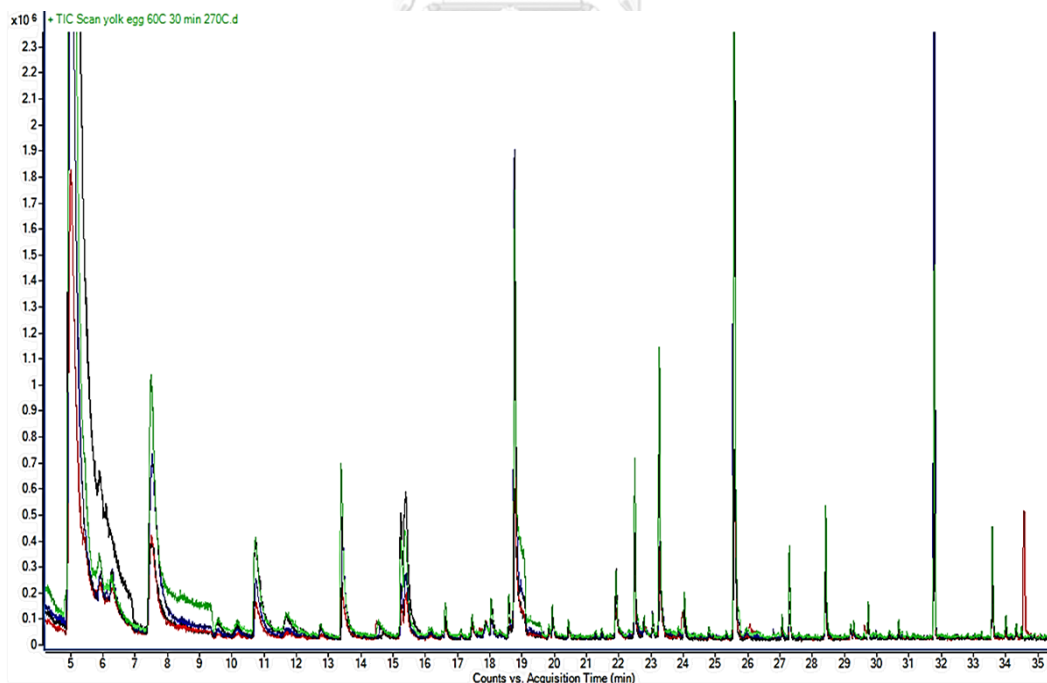

Figure A-1.2   GC-MS chromatogram overlaid of desorption temperature at 200 °C, 230 °C, 250 °C and 270 °C
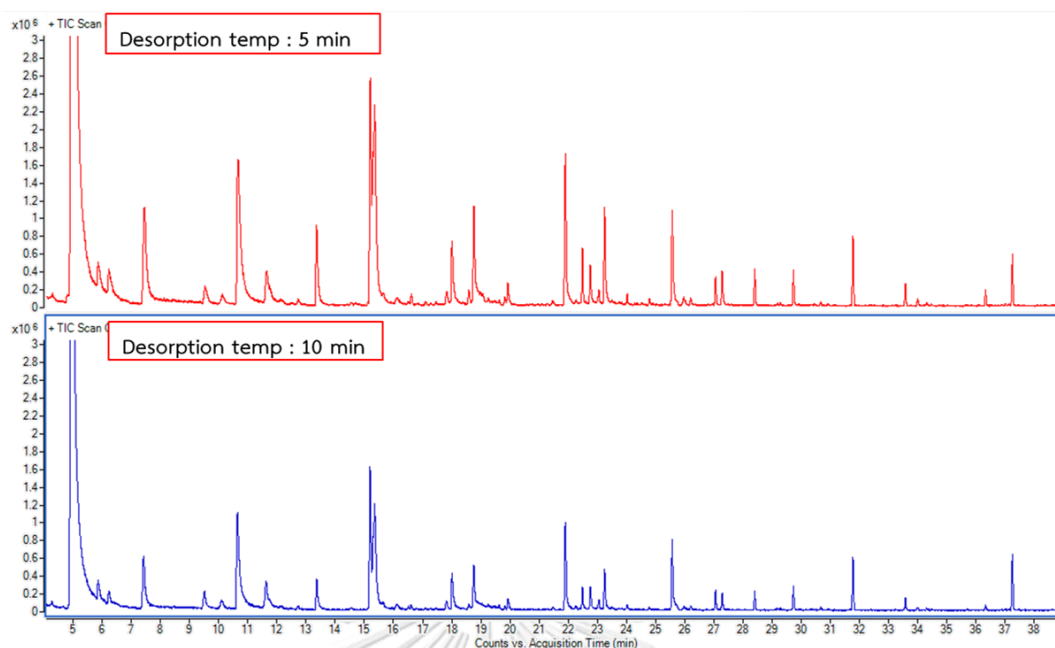
Figure A-2.1   GC-MS chromatogram of desorption time at 5 min and 10 min
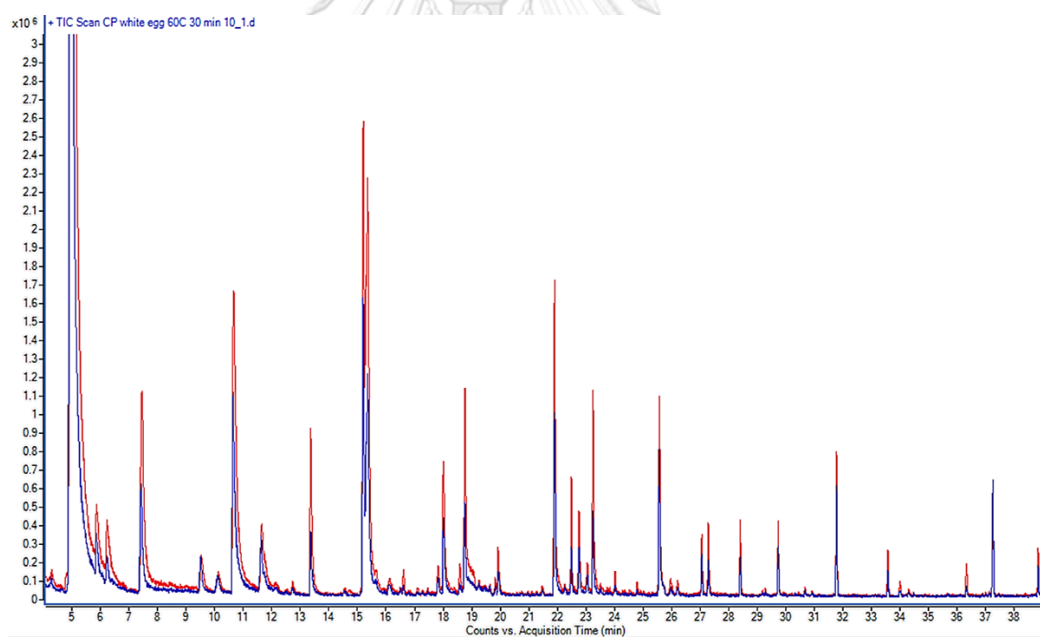
Figure A-2.2   GC-MS chromatogram overlaid of desorption time at 5 min and 10 min
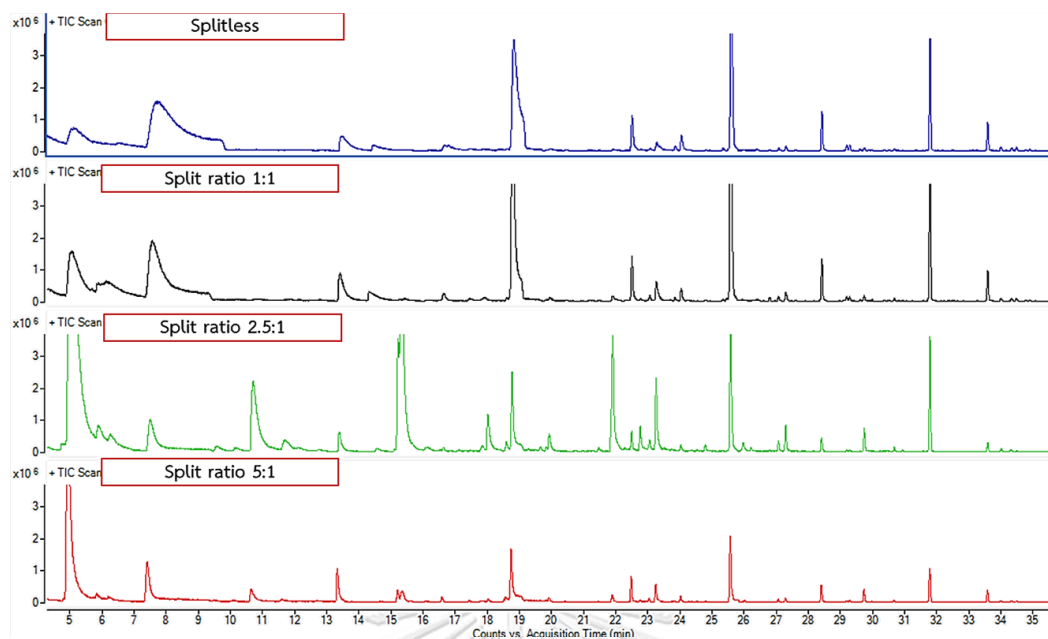
Figure A-3.1   GC-MS chromatogram of injection mode: splitless and spilt ratio (1:1, 2.5:1, and 5:1)

Figure A-3.2   GC-MS chromatogram overlaid of injection mode: splitless and spilt ratio (1:1, 2.5:1, and 5:1)

Figure A-4.1   GC-MS chromatogram of blank and egg sample (egg yolk)

Figure A-4.2   GC-MS chromatogram overlaid: red line = blank and black line = egg sample (egg yolk)

Table B-1.1 Volatile compounds in raw egg yolk for 4 weeks
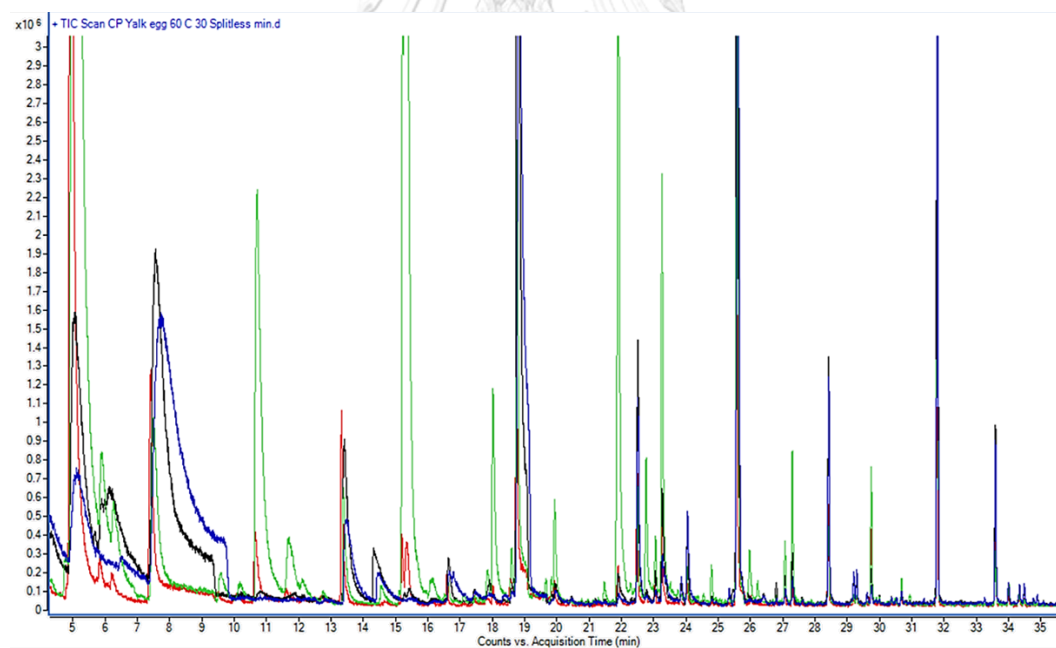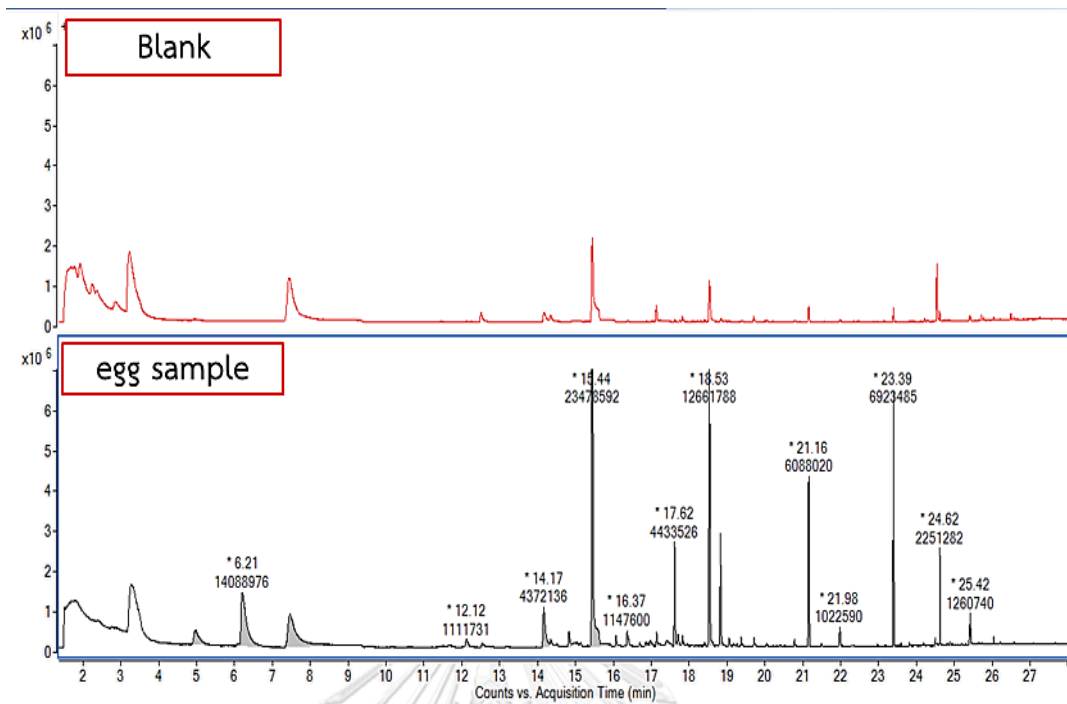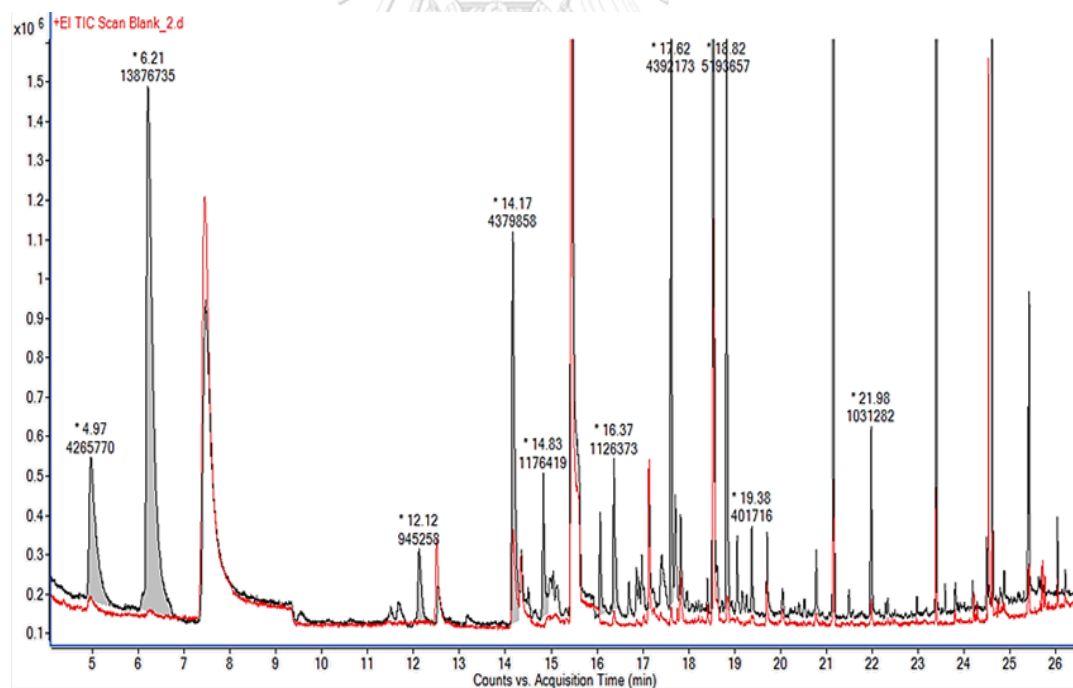
Compounds name	Peak area ratio (volatile compounds/IS1) Mean ± SD									
	Non-cold chain					Cold chain				
	0-week	1-week	2-week	3-week	4-week	0-week	1-week	2-week	3-week	4-week
Toluene	0.793 ± 0.090	1.377 ± 0.879	2.179 ± 0.872	1.027 ± 0.420	3.768 ± 0.644	0.793 ± 0.09	0.769 ± 0.378	1.003 ± 0.449	0.409 ± 0.165	0.422 ± 0.403
Hexanal	0.829 ± 0.313	0.600 ± 0.159	2.056 ± 0.803	3.074 ± 1.273	19.837 ± 2.721	0.829 ± 0.313	0.853 ± 0.639	1.422 ± 0.498	0.635 ± 0.589	0.574 ± 0.206
Heptanal	0.155 ± 0.040	0.064 ± 0.014	0.017 ± 0.005	0.251 ± 0.103	1.250 ± 0.137	0.155 ± 0.04	0.071 ± 0.044	0.168 ± 0.054	0.086 ± 0.029	0.09 ± 0.039
Benzaldehyde	ND	13.377 ± 6.495	1.791 ± 0.134	1.099 ± 0.189	2.281 ± 0.952	ND	3.312 ± 2.065	0.968 ± 0.367	0.387 ± 0.291	0.114 ± 0.018
Octanal	0.606 ± 0.397	0.192 ± 0.108	0.419 ± 0.031	0.281 ± 0.076	0.486 ± 0.068	0.606 ± 0.397	0.207 ± 0.123	0.377 ± 0.104	0.09 ± 0.035	0.161 ± 0.169
Nonanal	1.043 ± 0.902	0.339 ± 0.297	0.600 ± 0.094	0.865 ± 0.241	3.843 ± 1.682	1.043 ± 0.902	0.22 ± 0.158	1.191 ± 0.615	0.906 ± 0.187	0.994 ± 0.614
Decanal	0.18 ± 0.069	0.085 ± 0.017	0.115 ± 0.006	0.106 ± 0.026	0.461 ± 0.225	0.18 ± 0.069	0.098 ± 0.034	0.098 ± 0.034	0.109 ± 0.08	0.089 ± 0.059
1-Octen-3-ol	ND	ND	ND	0.426 ± 0.204	1.985 ± 0.402	ND	ND	ND	ND	0.038 ± 0.01
2-ethyl-1-hexanol	ND	ND	ND	0.148 ± 0.031	0.578 ± 0.075	ND	ND	0.244 ± 0.095	0.169 ± 0.034	0.203 ± 0.174

Table B-1.2 Volatile compounds in raw egg white for 4 weeks storage

Compounds name	Peak area ratio (volatile compounds/IS1) Mean ± SD									
	Non-cold chain					Cold chain				
	0-week	1-week	2-week	3-week	4-week	0-week	1-week	2-week	3-week	4-week
Toluene	0.117 ± 0.050	0.116 ± 0.030	ND	ND	0.079 ± 0.024	0.117 ± 0.050	0.090 ± 0.016	0.228 ± 0.166	0.028 ± 0.004	0.057 ± 0.019
Hexanal	0.073 ± 0.030	0.081 ± 0.057	0.184 ± 0.081	0.195 ± 0.091	0.266 ± 0.049	0.073 ± 0.030	0.072 ± 0.039	0.043 ± 0.007	0.060 ± 0.046	0.085 ± 0.055
Heptanal	0.018 ± 0.003	0.018 ± 0.011	0.033 ± 0.017	0.035 ± 0.030	0.025 ± 0.007	0.018 ± 0.003	0.014 ± 0.009	0.016 ± 0.003	0.012 ± 0.004	0.013 ± 0.005
Benzaldehyde	ND	2.599 ± 1.124	0.277 ± 0.042	0.09 ± 0.005	0.044 ± 0.004	ND	ND	0.112 ± 0.014	0.023 ± 0.019	0.017 ± 0.005
Octanal	ND	ND	ND	ND	0.047 ± 0.025	ND	ND	ND	ND	0.064 ± 0.035
Nonanal	0.364 ± 0.058	0.09 ± 0.040	0.400 ± 0.200	0.918 ± 0.040	0.397 ± 0.170	0.364 ± 0.058	0.124 ± 0.076	0.216 ± 0.021	0.161 ± 0.106	0.256 ± 0.053
Decanal	0.077 ± 0.024	0.014 ± 0.006	0.046 ± 0.011	0.027 ± 0.016	0.034 ± 0.018	0.077 ± 0.024	0.021 ± 0.013	0.084 ± 0.021	0.022 ± 0.009	0.026 ± 0.005
1-Octen-3-ol	ND	ND	ND	ND	0.035 ± 0.003	ND	ND	ND	ND	0.007 ± 0.006
1-Hexanol-2-ethyl	0.017 ± 0.002	ND	0.044 ± 0.008	0.016 ± 0.003	0.023 ± 0.004	0.017 ± 0.002	0.047 ± 0.028	0.115 ± 0.048	0.046 ± 0.006	0.063 ± 0.016

Table B-1.3 Volatile compounds in soft boiled egg yolk for 4 weeks storage

Compounds name	Peak area ratio (volatile compounds/IS1) Mean ± SD									
	Non-cold chain					Cold chain				
	0-week	1-week	2-week	3-week	4-week	0-week	1-week	2-week	3-week	4-week
Toluene	0.414 ± 0.049	0.078 ± 0.053	3.241 ± 1.165	1.235 ± 0.322	2.822 ± 1.696	0.414 ± 0.049	0.082 ± 0.040	2.030 ± 0.804	0.645 ± 0.408	1.304 ± 0.250
Hexanal	0.687 ± 0.529	0.120 ± 0.068	3.724 ± 0.596	4.274 ± 0.378	9.063 ± 2.632	0.687 ± 0.529	0.203 ± 0.108	2.614 ± 1.034	0.654 ± 0.273	1.326 ± 0.412
Heptanal	ND	0.010 ± 0.001	0.166 ± 0.015	0.190 ± 0.040	0.464 ± 0.112	ND	0.021 ± 0.003	0.154 ± 0.079	0.030 ± 0.013	0.080 ± 0.008
Benzaldehyde	ND	2.848 ± 0.056	1.822 ± 0.281	0.470 ± 0.101	1.368 ± 0.417	ND	ND	1.612 ± 0.847	0.130 ± 0.066	0.201 ± 0.026
Octanal	0.086 ± 0.024	ND	0.351 ± 0.128	0.087 ± 0.025	0.176 ± 0.031	0.086 ± 0.024	ND	0.388 ± 0.063	0.098 ± 0.077	0.161 ± 0.095
Nonanal	0.112 ± 0.052	0.086 ± 0.035	0.739 ± 0.075	0.332 ± 0.053	1.225 ± 0.721	0.112 ± 0.052	0.245 ± 0.050	0.612 ± 0.066	0.118 ± 0.001	0.332 ± 0.082
Decanal	ND	ND	0.134 ± 0.014	0.037 ± 0.003	0.080 ± 0.017	ND	0.028 ± 0.005	0.159 ± 0.074	0.015 ± 0.002	0.042 ± 0.025
1-Octen-3-ol	ND	0.022 ± 0.008	ND	0.318 ± 0.059	0.897 ± 0.056	ND	ND	ND	0.042 ± 0.019	ND
2-ethyl-1-Hexanol	ND	ND	ND	0.119 ± 0.031	0.273 ± 0.177	ND	ND	0.351 ± 0.170	0.104 ± 0.054	0.482 ± 0.069

Table B-1.4 Volatile compounds in soft boiled egg white for 4 weeks

Compounds name	Peak area ratio (volatile compounds/IS1) Mean ± SD									
	Non-cold chain					Cold chain				
	0-week	1-week	2-week	3-week	4-week	0-week	1-week	2-week	3-week	4-week
Toluene	0.113 ± 0.044	0.078 ± 0.053	0.118 ± 0.044	0.025 ± 0.005	0.079 ± 0.024	0.113 ± 0.044	0.082 ± 0.040	0.082 ± 0.041	0.027 ± 0.002	0.057 ± 0.019
Hexanal	0.269 ± 0.146	0.120 ± 0.068	0.544 ± 0.434	0.080 ± 0.048	0.266 ± 0.049	0.269 ± 0.146	0.203 ± 0.108	0.324 ± 0.282	0.064 ± 0.045	0.085 ± 0.055
Heptanal	0.028 ± 0.006	0.010 ± 0.001	0.290 ± 0.160	0.008 ± 0.003	0.025 ± 0.007	0.028 ± 0.006	0.021 ± 0.003	0.039 ± 0.025	0.010 ± 0.006	0.013 ± 0.005
Benzaldehyde	0.036 ± 0.016	2.848 ± 3.609	0.193 ± 0.04	0.016 ± 0.005	0.044 ± 0.004	0.036 ± 0.016	ND	0.183 ± 0.052	0.016 ± 0.008	0.017 ± 0.005
Octanal	ND	ND	ND	0.003 ± 0.000	0.047 ± 0.025	ND	ND	ND	ND	0.064 ± 0.035
Nonanal	0.121 ± 0.04	0.086 ± 0.035	0.220 ± 0.14	0.114 ± 0.024	0.397 ± 0.170	0.121 ± 0.040	0.245 ± 0.050	0.495 ± 0.247	0.152 ± 0.096	0.256 ± 0.053
Decanal	0.063 ± 0.042	ND	0.157 ± 0.111	0.009 ± 0.002	0.034 ± 0.018	0.063 ± 0.042	0.028 ± 0.005	0.080 ± 0.036	0.009 ± 0.003	0.026 ± 0.005
1-Octen-3-ol	ND	0.022 ± 0.008	0.220 ± 0.169	0.010 ± 0.005	0.035 ± 0.003	ND	ND	ND	0.007 ± 0.006	0.006 ± 0.007
2-ethyl-1-Hexaol	ND	ND	0.041 ± 0.015	0.009 ± 0.003	0.023 ± 0.004	ND	ND	0.102 ± 0.015	0.023 ± 0.017	0.063 ± 0.016

VITA

Miss Hanan jehma was born on July 12, 1989 in Taif, Saudiarabia. She graduated with high school diploma from Chongraksat wittaya School, Pattani, Thailand, 2007. She graduated with a Bachler's degree of Science and Technology from Prince of Songkla University in 2011. After completing her Bachler's degree. She work as assistant researcher at Prince of Songkla University with Asst. Prof. Dr. Wilairat Cheewasedtham. In the year of 2012-2016, she had worked on Chemist at the Special lab Envi and Consultant co., Ltd. In 2013, she then continued her education for Master degree at Department of Chemistry, Faculty of Science, Chulalongkorn university. Since July 2016 until present she has been working as a Product analyst at Interthai Pharmaceutical Manufacturing Ltd.

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