COMPARISON OF AROMA ACTIVE COMPOUNDS IN FRESH THAI HOLY BASIL LEAVES FROM 2 VARIETIES

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น.ส.ทนัชชา วิเชียรกัลยารัตน์

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Field of Study	Food Science and Technology
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ทนัชชา วิเชียรกัลยารัตน์ : การเปรียบเทียบสารระเหยให้กลิ่นในใบกะเพราไทยสด 2 พันธุ์ . (COMPARISON OF AROMA ACTIVE COMPOUNDS IN FRESH THAI HOLY BASIL LEAVES FROM 2 VARIETIES) อ.ที่ปรึกษาหลัก : ผศ. ดร.พนิตา งามเชื้อชิต, อ.ที่ปรึกษาร่วม : ศ. ดร.สุวิมล กีรติพิบูล

กะเพรา (*Ocimum tenuiflorum* L.) เป็นพืชสมุนไพรที่นิยมนำมาเป็นวัตถุดิบในอาหารไทยหลากหลายชนิด ใบกะเพรามีกลิ่นที่เป็นเอกลักษณ์ ซึ่งให้ทั้งกลิ่นหอมฉุนและความเผ็ดร้อน ซึ่งการนำใบกะเพราสดมาเพิ่มกลิ่นให้ผลิตภัณฑ์อาหารนั้น ต้องอาศัยการควบคุมคุณภาพตั้งแต่วัตถุดิบตลอดจนระหว่างกระบวนการผลิต เพื่อให้ยังคงความเป็นเอกลักษณ์ด้านกลิ่นและรสชาติของใบกะเพราเอาไว้ได้ งานวิจัยนี้จึงมุ่งเน้นการระบุและเปรียบเทียบสารระเหยให้กลิ่นในใบกะเพราไทยสด 2 พันธุ์ (กะเพราขาว และกะเพราแดง) การศึกษาสารให้กลิ่นสำคัญของใบกะเพราสดนั้นอาศัยกระบวนวิธีทางเซนโซมิกซ์ (Sensomics approach) โดยเริ่มต้นจากการศึกษาลักษณะกลิ่นของใบกระเพราสดด้วยการทดสอบทางประสาทสัมผัสแบบพรรณนาเชิงปริมาณ (Olfactory profiling) และการศึกษาองค์ประกอบทางเคมีของสารระเหยให้กลิ่นใบกระเพราสด ซึ่งทำโดยสกัดแยกสารระเหยออกจากตัวอย่างใบกะเพราสดด้วยวิธีการสกัดด้วยตัวทำละลายอินทรีย์ร่วมกับเทคนิค SAFE (Solvent assisted flavor evaporation) เพื่อลดการสูญเสียหรือการเปลี่ยนแปลงโครงสร้างของสารระเหยจากความร้อนระหว่างกระบวนการสกัดภายใต้อุณหภูมิและความดันต่ำ จากนั้นจึงนำสารสกัดไปวิเคราะห์หาสารที่ให้กลิ่นด้วยเครื่อง gas chromatography-olfactometry/flame ionization detector (GC-O/FID) ขั้นตอนต่อมาคือการจัดลำดับกลิ่นที่มีศักยภาพเป็นสารให้กลิ่นที่สำคัญด้วยเทคนิค aroma extract dilution analysis (AEDA) จากนั้นระบุชนิดของสารระเหยด้วยการประเมินผลร่วมกับการวิเคราะห์ด้วยเครื่อง gas chromatography-mass spectrometry (GC-MS) จากการทดสอบทางประสาทสัมผัสแบบพรรณนาเชิงปริมาณ พบใบกะเพราแดงมีลักษณะโดยรวมของกลิ่นกะเพราที่แรงกว่าใบกะเพราะขาว โดยใบกะเพราแดงพบกลิ่นมิ้น กลิ่นเขียว กลิ่นฉุน กลิ่นไขมันที่สูงกว่าใบกะเพราขาว ยกเว้นกลิ่นผลไม้ตระกูลส้ม ที่พบต่ำกว่าใบกะเพราขาว ในการประเมินผลร่วมกันระหว่าง ค่าRI และ ลักษณะกลิ่นที่วิเคราะห์ได้จากเครื่อง GC-O/FID และค่ามวลของสารที่ตรวจพบจากเครื่อง GC-MS โดยพิจารณาสารระเหยที่มีศักยภาพที่จะเป็นสารให้กลิ่นสำคัญ (Potential aroma active compound) จากสารที่มีค่า Flavor dilution (FD) factor สูง พบว่า สารที่ให้กลิ่นกานพลูใบกะเพราขาว มี eugenol (FD ≥ 4096) ในขณะที่ในใบกะเพราแดงมีทั้ง eugenol (FD 2048) และ methyl eugenol (FD ≥ 4096) สารที่ให้กลิ่นดอกไม้ ได้แก่ 2-hexanol (FD 512) และ α-farnesene (FD 1024) และสารที่ให้กลิ่นยูคาลิปตัส ได้แก่ cis-sabinene hydrate (cis-4-thujanol) (FD 2048) พบเฉพาะในใบกะเพราขาว นอกไปจากนี้พบใบกะเพราแดงพบ สารที่ให้กลิ่นสมุนไพร 3 กลิ่น ได้แก่ 1-decen-3-ol (FD 1024), naphthalene (FD 2048), 1-undecen-3-ol (FD 2048) มากกว่าใบกะเพราขาวที่พบได้ 2 กลิ่น ได้แก่ trans-4,5-epoxy-(E)-2-heptenal (FD 512) และ caryophyllene oxide (FD 1024)

สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต .....
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Thanatcha Vichienkalayarat : COMPARISON OF AROMA ACTIVE COMPOUNDS IN FRESH THAI HOLY BASIL LEAVES FROM 2 VARIETIES. Advisor: Asst. Prof. Panita Ngamchuachit, Ph.D. Co-advisor: Prof. SUWIMON KEERATIPIBUL, Ph.D.

Holy Basil is an herb with a high consumption in Thailand domestically and internationally due to its unique odor. A study on the aroma active profile of holy basil would allow for better quality control and food product development. The goal of this study is to identify and compare aroma active compounds in fresh Thai holy basil leaves from 2 varieties (*Ocimum tenuiflorum* var. Rama and *Ocimum tenuiflorum* var. Shyama) using sensomics approach. First, olfactory profiling was performed by trained panelists to characterize odor attributes and their intensity. Second, volatile compounds were isolated by solvent extraction, and non-volatile constituents were removed by solvent-assisted flavor evaporation and concentrated using the Vigreux column and microdistillation. Third, extract was injected into the gas chromatography-olfactometry with flame ionization detection (GC-O/FID) to screen for odor quality associated with the retention index (RI). Potential aroma active compounds were determined through aroma extract dilution analysis (AEDA). Gas chromatography-mass spectrometry (GC-MS) enables compound identification by comparing mass spectra with RI and odor quality from GC-O/FID. From olfactory profiling, red holy basil leaves had stronger overall attributes than white holy basil leaves. Red holy basil leaves have stronger minty, green, pungent, and fatty/waxy attributes than white holy basil leaves but have lower citrus-like attribute. Among the potential aroma active compounds with high FD factors, white holy basil leaves consisted of only eugenol (FD ≥ 4096), whereas red holy basil leaves consisted of both eugenol (FD 2048) and methyl eugenol (FD ≥ 4096). In white holy basil leaves, the unique flowery odor might be contributed from 2-hexanol (FD 512) and α-farnesene (FD 1024). Moreover, the distinct eucalyptus-like odor might potentially come from cis-sabinene hydrate (cis-4-thujanol) (FD 2048). In red holy basil leaves, herbal odor was potentially come from 3 high FD factor compounds, including 1-decen-3-ol (FD 1024), naphthalene (FD 2048), and 1-undecen-3-ol (FD 2048), while only 2 compounds with lower FD factor, including trans-4,5-epoxy-(E)-2-heptenal (FD 512) and caryophyllene oxide (FD 1024), were presented in white holy basil leaves.

Field of Study:	Food Science and Technology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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Thanatcha Vichienkalayarat

TABLE OF CONTENTS

Page

List of Tables

List of Figures

Chapter I

1.1 Introduction

As a consequence of globalization, the national cuisine of several countries has gained popularity around the world. Particularly, Thai food has been listed among the top ten most popular cuisines worldwide. Currently, the global estimate of Thai food restaurants is around 14,900. As a result, herbs and spices are consumed as raw materials in Thai cuisine and are exported to many different countries. Holy basil is an herb grown in Thailand with high export potential, particularly to the European region. The Royal Project Foundation has been exporting basil, as well as other herbs and vegetables, to Europe and other overseas markets (Bung-ila, 2009). Holy basil, especially the white holy basil leaves, is a traditional herb used in Thai cuisine, preferred for enriching the flavor of dishes. Estimated domestic consumption of the holy basil is 12,816 metric tons/year or around 200 million Thai baht (กรมส่งเสริมการเกษตร, 2560). However, the holy basil has several varieties including *Ocimum tenuiflorum* var. Shyama, *Ocimum tenuiflorum* var. Rama, etc. which frequently cultivated in several countries in South and South-East Asia including Thailand (Juntachote et al., 2006; Mahajan et al., 2013). Different holy basil varieties differ in sensory characteristics as well as the usage. *Ocimum tenuiflorum* var. Shyama or red holy basil has purple stalks, and some reddish tint on the leaves with strong pungent aroma, whereas *Ocimum tenuiflorum* var. Rama or white holy basil has green stalks and leaves with moderate pungent odor. Evaluating the olfactory profiling of the white holy basil would allow for enhanced quality control, crop selection, further product development, and market expansion. The goal of this study is to identify and compare aroma active compounds in fresh Thai holy basil leaves from 2 varieties (*Ocimum tenuiflorum* var. Rama and *Ocimum tenuiflorum* var. Shyama*)* using the sensomics approach. The sensomics approach is a tool used for identifying aroma compounds involving molecular sensory and descriptive sensory science.

In previous studies, Raina et al. (2013) obtained the holy basil extract by hydro-distillation with diethyl ether to characterize the chemical compounds related to its aroma. The essential oil of the holy basil was mainly constituted by phenylpropanoids (methyl eugenol and eugenol), followed by sesquiterpene

hydrocarbons (β-caryophyllene and β-elemene). Tangpao et al. (2018) extracted the essential oil of holy basil using hydro-distillation at 150°C for 2 hr. Their sensory evaluation study revealed that the essential oil extracted from *O. tenuiflorum* var. Shyama or red holy basil, has a stronger spice and herb odor than *O. tenuiflorum* var. Rama or white holy basil. Wongpraneekul et al. (2022) analyzed volatile compounds of holy basil extract by hydro-distillation, followed by solid phase microextraction (SPME). The major constituents of the essential oils of holy basil were eugenol, (E)-caryophyllene, and β-elemene. In several studies, the extraction method for holy basil were usually done by hydro-distillation and headspace extraction such as solid phase microextraction (SPME), stir-bar sorptive extraction (SBSE), and dynamic headspace (DHS). Even though those method are quick and simple, however, the limitations of these extraction techniques are the choice for selectivity, the premature contamination and thermal degradation, and the displacement effects due to the matrix compound (Camino-Sánchez et al, 2014; Etievant, 1996).

Engel et al. (1999) developed a solvent extraction technique to extract volatile compounds called solvent-assisted flavor evaporation (SAFE), which omits the problem of selectivity and premature contamination. This technique uses low boiling solvents (diethyl ether or dichloromethane) to extract volatile constituents from food matrix under mild condition and consequently separate those volatile compounds from non-volatile residue by SAFE apparatus under low temperature and vacuum condition. The mild condition is used to avoid elevated temperature which causes artifact formation and compound degradation.

Olfactory profiling is a technique developed in response to the need for analytical and statistical analysis of qualitative profile data based on the perceptions of a group of trained panelists.

Gas chromatography-olfactometry with flame ionization detection (GC-O/FID) is a tool to screen aroma active compounds from the odorless volatile compounds. During GC-O/FID analysis, after chromatographic separation, the volatile compounds are split into FID and sniffing port. Each panelist perceive odor at the sniffing port and record the retention time and odor quality. The result of 30 – 60 odor quality is usually detected in each food sample. To rank the odor quality, aroma extract dilution analysis (AEDA) is used. The initial extract is stepwise diluted, which

each diluted sample is subjected to GC-O/FID analysis until no odor detected for each odor quality. A flavor dilution (FD) factor is assigned for each odorant. It is the highest dilution.

Structural assignment of each aroma active compound was completed by comparing 3 parameters with the respective data of structurally characterized reference compounds. Those 3 parameters are odor quality, RIs, and mass spectra. The odor quality and RIs are obtained from GC-O/FID. Mass spectra is from gas chromatography-mass spectrometry (GC-MS).

1.2 Objective

To identify and compare aroma active compounds in fresh Thai holy basil leaves from 2 varieties (*Ocimum tenuiflorum* var. Rama and *Ocimum tenuiflorum* var. Shyama*)*.

1.3 Expected Output

Aroma attributes and aroma active compounds database of fresh Thai holy basil leaves from 2 varieties including white holy basil (*Ocimum tenuiflorum* var. Rama) and red holy basil (*Ocimum tenuiflorum* var. Shyama). The obtained data and information in this study could be beneficial for quality control in terms of crop selection, production, as well as further product development.

Chapter II

LITERATURE REVIEW

2.1 Holy basil

Ocimum tenuiflorum is an herb in the family Lamiaceae, known as the holy basil. Holy basil often grown for aromatic leaves in tropical and subtropical area and cultivated in several countries of South and South-East Asia including Thailand. The holy basil plant is a small annual or short-lived perennial shrub, 1 meter height with hairy stems. The leaves are green or purple, depending on the variety.

2.1.1 White holy basil

White holy basil, known as *Ocimum tenuiflorum* var. Rama, has ovate-obovate, elliptic-oblong, surface patently hairy to clothed with soft spreading hair and green leaf (Tangpao, 2018).

2.1.2 Red holy basil

Red holy basil, known as *Ocimum tenuiflorum* var. Shyama has ovate-obovate, elliptic-oblong, surface patently hairy to clothed with soft spreading hair and green to red leaf (Tangpao, 2018).

2.2 Aroma active compounds in food

In a particular food substance (Figure 2.1), there are numerous compounds in the food constituent that are volatile or non-volatile. Aroma active compounds known as volatile compounds that are released from food into the ambient air and reach the olfactory epithelium in the nasal cavity when humans take breath (Figure 2.2). Meanwhile, volatility is an important prerequisite for an aroma active compound. On the other hands, there are volatile compounds that have odor and odorless. Odorless compounds may be due to the compounds that could not interact with odorant receptor or the amount that do not exceed the odor detection threshold concentration. Odor detection threshold is the minimum concentration of aroma compound that is perceived by the human sense of smell. Certain aroma compound has concentration of odor detection threshold that is necessary to activate the intracellular reaction cascade

of the olfactory neurons. Odor detection threshold of would depend on chemical compound including shape, polarity, partial charges, and molecular mass etc. In addition, since most aroma active compounds are nonpolar substances, this could facilitate extraction from food by using organic solvents as hydrophobic interaction plays an important role in their binding to the olfactory receptors.

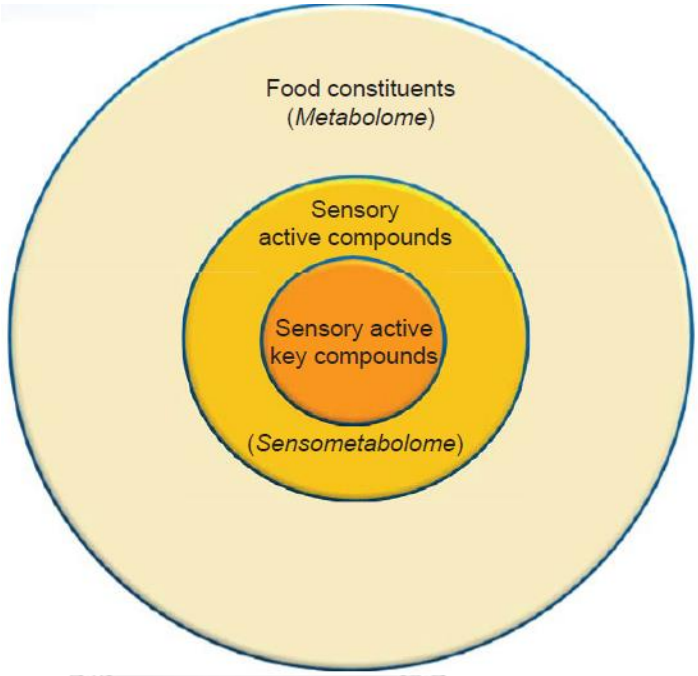

Figure 2.1 Relationship between active compounds and key compounds in food constituents (Dunkel et al., 2014)

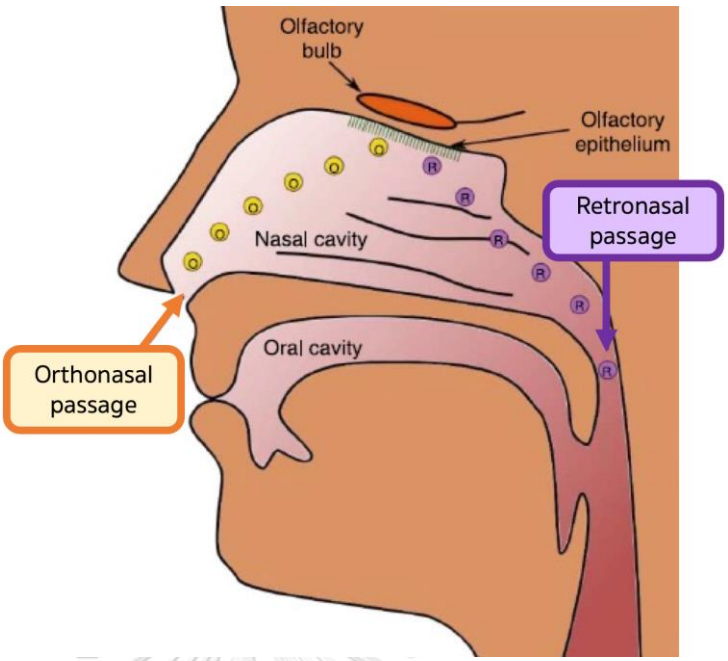

Figure 2.2 Schematic of the human olfactory system (Dietrich, 2009)

2.3 Sensomics approach

Sensomics approach is the method for elucidation of the key aroma compounds in various foods. Sensomics approach comprises of a bioactivity-guided discovery of key odorants with accurate quantitation, followed by aroma reconstitution and omission test. Such the methods covered up a comprehensive and quantitative analysis of the chemical related to the odor that represent the odor attributes of the given food. By omitting just one key odorant or applying incorrect concentrations of the volatiles in the odor blends, this will significantly affect odor attributes that represent the given food.

In sensomics approach (Figure 2.3), before starting the isolation, olfactory profiling is performed by trained panelists who involved in developing odor attributes, reference compounds of the sample, practicing and sensory evaluating by rating each odor attribute. So, olfactory profiling enables to characterize odor attributes and represents in radar chart with scale. In order to analyse the aroma active compounds, the first step is the isolation of volatile compounds of the food sample using solvent extraction with solvent assisted flavor evaporation or SAFE technique. The technique involved extraction with low boiling organic solvents such as diethyl ether or

dichloromethane and distillation of the solvent extracts in high vacuum at ambient temperature followed by gentle concentration by Vigreux column and microdistillation. The distillate still represented the typical odor attributes of the actual sample. The distillate is used for analysis of aroma active compounds using gas chromatography-olfactometry (GC-O) analysis. Screening result from GC-O analysis is a list of typically 30 to 60 odor impressions that associated retention times. The aroma active compounds are determined by aroma extract dilution analysis (AEDA). The initial extract is diluted. Each diluted sample is subjected to GC-O analysis until no aroma active compound detected. Odor quality and RIs are obtained from GC-O. Then, gas chromatography-mass spectrometry (GC-MS) enables compound identification by comparing the obtained mass spectra of the analytes. Structural assignment is achieved by comparing odor quality, RI, and mass spectra with the data of characterized reference compounds. Quantification of the compounds is performed by determining the concentration using GC-MS. Moreover, concentration then uses to calculate with odor threshold to identify odor activity value (OAV). Consequently, aroma reconstitution is performed by preparing odor blends mimicking the original food based on results from aroma active compounds, for which OAVs >1 have been calculated. Lastly, to identify such insignificant odorants, omission tests is done by omitted each of the aroma active compound from an aroma reconstitution model. Therefore, omission test helps to elucidate the aroma contribution of individual odorants.

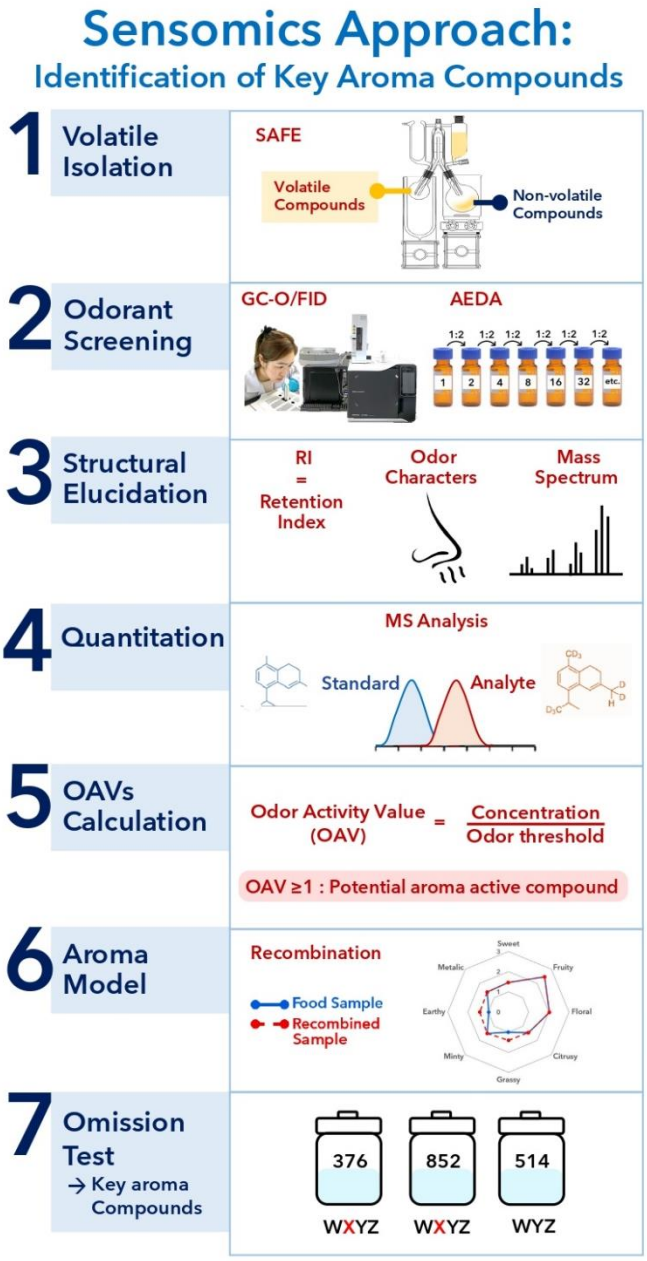

Figure 2.3 Sensomics Approach

2.4 Olfactory profiling

As yet no instrument can duplicate the sensory and psychological responses of a human being since human nose can be more sensitive than an instrumental detector. Even though instrumental methods require progressed from simple physical or chemical tests to sophisticated instrumental procedures, data obtained by such procedures should be validated against sensory data collected by the human

instrument (Noble, 1975). So, one category of sensory techniques that can be used to provide analytical and reliable information on sensory perception is the quantitative descriptive analysis known as olfactory profiling. Olfactory profiling was performed by trained panelists. The trained panelists are involved in developing odor attributes and reference compounds related to certain sample. Reference compounds are prepared as a solution in water at certain concentration above the respective odor threshold concentration (Munafo et al., 2015). Then panelists are practice and sensory evaluate by rating the odor intensity of each odor attribute presented in the samples and its relevant odor standard compound. Olfactory profiling is not only used for characterizing odor attributes of the sample, but also for quality control of the isolation and concentration steps to ensure that the extract and distillate still have the same odor quality as in the original food sample. The intensity of each attribute of the food sample is presented on the scale of radar chart.

2.5 Isolation of volatile compounds

Engel et al. (1999), has developed solvent extraction technique called solvent-assisted flavor evaporation (SAFE) which admit the problem of choice for selectivity and premature contamination. For extraction, low boiling organic solvents such as diethyl ether or dichloromethane were used to extract volatile compounds from food sample followed by distillation of the solvent extract under high vacuum and low temperature (40 °C). Then, the distillate is gently concentrated by Vigreux column and microdistillation device. To explain, non-volatile compounds will be removed from the extracts by SAFE through distillation, whereas the volatile compounds will be preserved in cold trap (Figure 2.4). Consequently, solvent will be concentrated by Vigreux column and microdistillation device. Importantly, elevated temperatures were avoided throughout the entire workup to avoid artifact formation and compound degradation (Munafo et al., 2015). Therefore, the odor compounds isolated from food sample could be preserved.

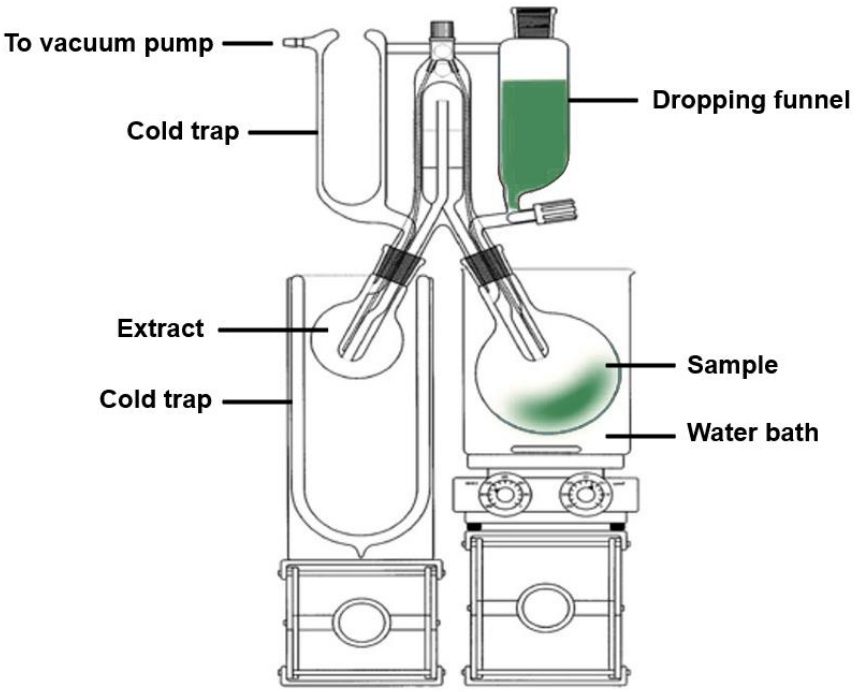

Figure 2.4 Solvent-assisted flavor evaporation (SAFE) apparatus (Munafo et al., 2015)

2.6 Determination of aroma compounds using gas chromatography-olfactometry/ flame-ionization detection (GC-O/FID)

Gas chromatography-olfactometry/ flame ionization detector (GC-O/FID) is a technique that integrates the chromatographic separation of volatile compounds using a gas chromatography and the detection of odor compounds by a flame ionization detector and a sniffing port (Figure 2.5). The volatile fraction was separated by GC column and the column effluent was split into two equal parts which are FID and sniffing port. The FID uses a flame to ionize organic compounds that contained carbon. Following separation of the sample in the GC column, each analyte passes through the flame, fuelled by hydrogen and zero air, which cause carbon atom ionization. Consequently, the ions are collected and measured as they create a current at the detector's electrodes. The current is produced as the detector collects the charged ions and then converted to an electrical signal. Odor quality perceived from sniffing port and retention time are perceived and recorded during the analysis by trained panelists.

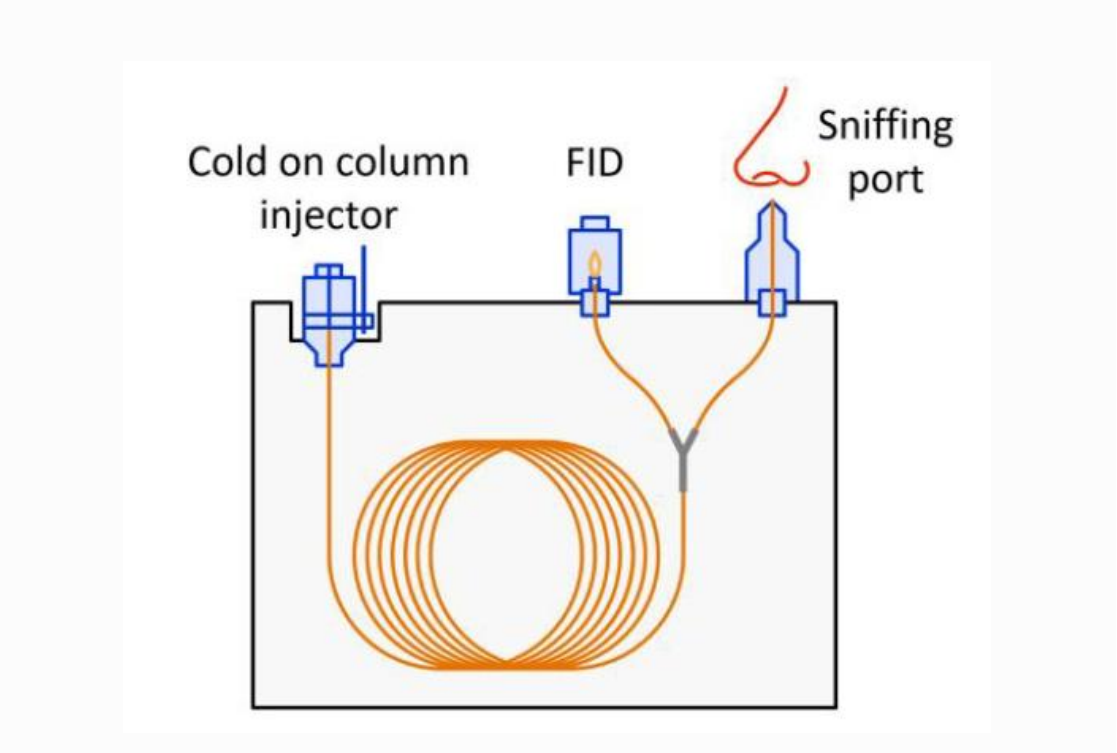

Figure 2.5 Gas chromatography-olfactometry/ flame-ionization detection (GC-O/FID) (Munafo et al., 2015)

2.7 Determination of Retention index

From GC-O analysis, retention index (RI) is calculated associated to retention time that perceived the odor from GC. To clarify, retention time (RT) is the measure of the time taken for a solute to pass through chromatography column and calculated as the time from injection to detection. Retention index (RI) is retention time which interpolated between adjacent n-alkanes (Kovats, 1958). Meanwhile, retention time vary between different chromatographic system or condition. However, retention index is independent to the chromatographic system or condition which allow comparing values measured by different chromatographic system and analytical instrument. Calculation of the retention index is shown in the equation following (Equation (2.1)).

$$I_x = 100n + 100\frac{t_x - t_n}{t_{n+1} - t_n}$$ Equation (2.1)

I_x = Retention Index

n = Number of carbon atom of n-alkane hydrocarbon eluting immediately before compound X

t_x = Retention time of compound X

t_n = Retention time of carbon atom of n-alkane hydrocarbon eluting immediately before compound X

t_{n+1} =Retention time of carbon atom of n-alkane hydrocarbon eluting immediately after compound X

2.8 Aroma extract dilution analysis (AEDA)

Aroma extract dilution analysis (AEDA) is a quantitative gas chromatography olfactometry procedure for determining the potency of aroma active compounds in food extracts. In AEDA, stepwise dilutions of the original extract are performed, and the diluted extracts are then evaluated by GC-O. The dilution was continued until no odorant could be detected by GC-O. Aroma active compound was then assigned as flavor dilution (FD) factor defined by the highest dilution at which the compound was detected (Grosch, 2001).

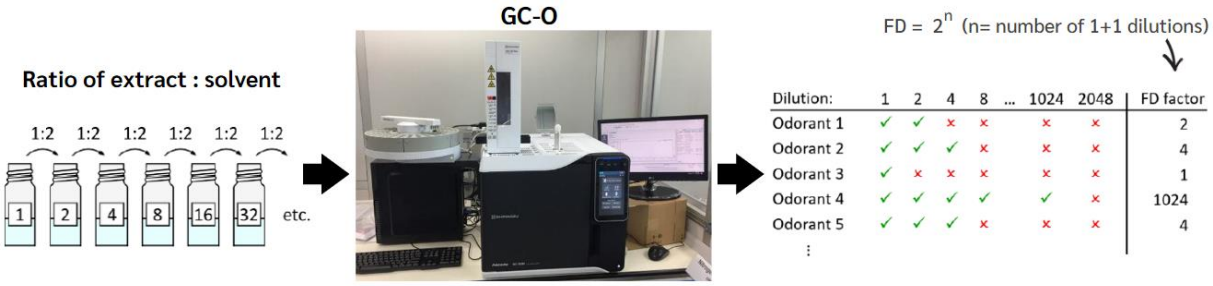

Figure 2.6 Aroma extract dilution analysis (AEDA) (Dunkel et al., 2014)

2.9 Determination of volatile compounds using gas chromatography-mass spectrometry (GC-MS)

In structural assignment, gas chromatography-mass spectrometry (GC-MS) enables compound identification by comparing the obtained mass spectra of the analytes with RI and odor quality from GC-O result (Munafo et al., 2015). To explain, mass spectra in electron ionization and chemical ionization mode are obtained from GC-MS analysis. MS is an analytical technique that measures the mass-to-charge ratio of the charged particles which used to determine the molecular weight, elemental composition, and elucidating the chemical structures of molecules. Thus, GC-MS is capable of detecting compounds based on chemical composition of the compounds.

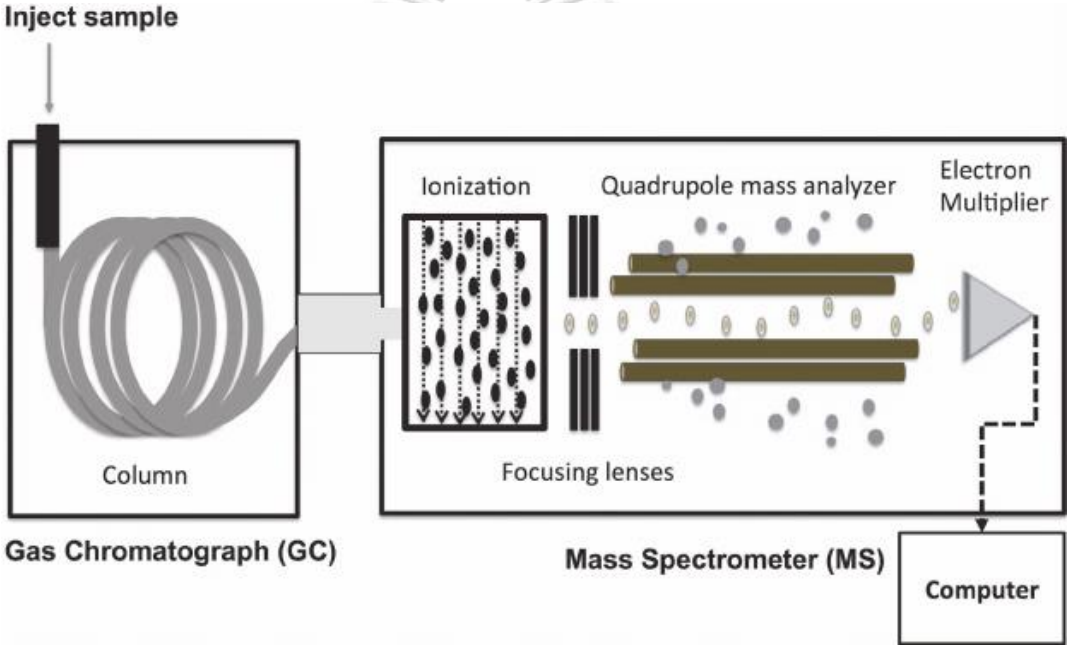

Figure 2.7 Gas chromatography-mass spectrometry (GC-MS) (Kim et al., 2016)

2.10 Comparison aroma compounds of white holy basil leaves and red holy basil leaves

For white holy basil leaves, Raina et al. (2013) obtained the white holy basil leave extract by hydro-distillation with diethyl ether to study the chemical characterization of its odor compounds, followed by identification of volatile compounds using GC-MS. The essential oil of the white holy basil was mainly constituted by methyl eugenol, eugenol, the phenylpropanoids, followed by β-caryophyllene and β-elemene as sesquiterpene hydrocarbons. Wongpraneekul et al.

(2022) analyzed volatile compounds of white holy basil leave extract by hydro-distillation, followed by solid phase microextraction (SPME) and identification of volatile compounds using GC-MS. The major constituents of the essential oils of white holy basil leave were eugenol, (E)-caryophyllene, and β-elemene. In previous studies, the extraction method for holy basil leave was usually done by hydro-distillation and headspace extraction.

For red holy basil leaves, Sriprom (2020) had study aroma active compounds of red holy basil leaves following sensomics approach. Red holy basil leaves extracted using SAFE technique. Aroma active compounds identified using GC-O/FID with AEDA techniques and GC-MS. Potential aroma active compounds with high FD factor of 256 to 1024 are anise alcohol, eugenol (4-allyl-2-methoxyphenol), 4-ethylphenol, geosmine, 3-mercapto-3-methylheptyl acetate, and butyl 3-cyclohexylpropionate.

Tangpao et al. (2018) identified aroma profile of white holy basil leaves, red holy basil leaves, and basil leaves. The leaves were extracted into essential oil using hydro-distillation under 150°C for 2 hr, followed by identification of volatile compounds using GC-MS. Their sensory evaluation study revealed that the essential oil extracted from red holy basil has a stronger spice and herb odors than white holy basil. The essential oils of white holy basil leave contained methyl eugenol, α-cubebene, α-copaene, borneol, β-elemene, eugenol, etc. The essential oils of red holy basil leave contained methyl eugenol, β-caryophyllene, α-cubebene, α-copaene, borneol, etc.

Chapter III

MATERIALS AND METHODS

Materials

Chemical reagents

- 3-hexenal, α-pinene, camphene, eugenol (4-allyl-2-methoxyphenol), trans-2-octen-1-ol, α-terpineol, 1-octen-3-ol, and (-)-carveol (Sigma-Aldrich, Taufkirchen, Germany)
- Calcium chloride ($Cacl_2$) (Merck, Germany)
- Sodium sulfate anhydrous (Na_2SO_4) (Merck, Germany)
- Dichloromethane (Merck, Germany)
- Hexane (Merck, Germany)
- n-alkane C_7-C_{30} (Sigma-Aldrich, USA)
- n-alkane C_{12} (Sigma-Aldrich, USA)
- n-alkane C_{16} (Sigma-Aldrich, USA)
- liquid nitrogen (Biologix, Thailand)

Laboratory equipments

- Laboratory Balances (Metter Toledo/ Model AB104, USA)
- Homogenizer (IKA/ T25 digital ULTRA-TURRAX, Germany)
- Magnetic stirrer (IKA/ C-MAG HS7, Germany)
- Filter paper No. 1 (Whatman, USA)
- Solvent-assisted flavor evaporation (SAFE) equipment (Glasbläsecrei Bahr/ Manching, Germany)
- Vigreux column (DN15, Pfaudler Normag Systems, Germany)
- Microdistillation column (Glasbläsecrei Bahr /Manching, Germany)
- Heating/Cooling immersion circulator (XTemp, China)
- High vacuum turbomolecular pump (TURBOVAC T 450i/Leybold, Germany)
- Laboratory chemical fume hood (GTech/Flow MAX-180A, Germany)
- GC-O/FID (GC-O 60 -2030AF, AOC-20i plus auto injection, Shimadzu, Tokyo, Japan,)

- DB-FFAP Column (Length 30 m × 0.32 mm i.d. and 0.25 µm film thickness, Agilent J&W GC column, USA)
- GC-MS (Agilent/Model 700GC/MS, Japan) (GCMS-TQ8050 NX, Shimadzu, Tokyo, Japan)
- SLB®-5ms Column (Length 30 m × 0.32 mm i.d. and 0.25 µm film thickness, Merck, Germany)

Methodology

3.1 Plant material

White holy basil leaves (*Ocimum tenuiflorum* var. Rama) were obtained from Nakhon Pathom province, Thailand. Red holy basil leaves (*Ocimum tenuiflorum* var. Shyama) were obtained from Chiang Rai province, Thailand. They were harvested at full bloom and stored at 8–10°C overnight prior to the isolation.

Figure 3.1 White holy basil leaves(left) and red holy basil leaves(right)

3.2 Olfactory profiling

The olfactory profiling was performed by eight trained panelists from the molecular sensory science research group at Chulalongkorn University. The panelists were trained in the following first steps: 1) developing odor attributes; 2) finalizing

odor attributes; and 3) developing reference compounds (Table 1). The reference compounds include 3-hexenal, α-pinene, camphene, eugenol (4-allyl-2-methoxyphenol), trans-2-octen-1-ol, α-terpineol, 1-octen-3-ol, and (-)-carveol (Sigma-Aldrich, Taufkirchen, Germany). Each odor attribute was defined (Table 1) based on the odor of a reference compound dissolved in water at a concentration of 100 - 10,000 times above the respective odor threshold concentration. Each sample and the reference compound were placed into cylindrical ground-neck glasses with lids for the evaluation. Then, in 4) practicing sensory evaluation, panelists were asked to rate the odor intensities of each odor attribute in the samples on a 7-point numerical scale with 0.5-point increments from 0 to 3 (0 = not detectable, 1 = weak, 2 = moderate, and 3 = strong). And in the final step, 5) performing the actual sensory evaluation of samples (Munafo et al., 2015).

Table 3.1 Odor attributes, definitions, and reference compounds of fresh holy basil leaves

No.	Odor Attributes	Definitions	Reference Compounds	CAS No.
1	green	odor of fresh-cut grass	3-hexenal	6789-80-6
2	pungent	odor of strong and pungent spicy	α-pinene	80-56-8
3	terpene-like	odor of terpene-like herb	camphene	79-92-5
4	clove-like	odor of clove-like herb	eugenol (4-allyl-2methoxyphenol)	97-53-0
5	fatty/waxy	odor of fat	trans-2-octen-1-ol	18409-17-1
6	citrus-like	odor of citrus fruits	α-terpineol	98-55-5
7	earthy	odor of soil	1-octen-3-ol	3391-86-4
8	minty	odor of peppermint-like	(-)-carveol	99-48-9

3.3 Isolation of volatile compounds from holy basil leaves

The volatile compounds of holy basil leaves (from 3.1) were extracted using solvent extraction. Fresh Thai holy basil leaves (30 g) were mixed with saturated calcium chloride solution (200 ml) in a 1L-beaker, and the mixture was homogenized (T25 digital Ultra-Turrax, IKA, Germany) for 1 min under cold water at 12-15°C. The homogenate was further extracted twice with dichloromethane (DCM; 300 ml and 200 ml) (Sigma-Aldrich, Taufkirchen, Germany) under cold water at 12-15°C. After the first extraction, the extracted was filtered through filter paper (Whatman No. 1),

and the residue went to the second extraction. All extracts were combined. Anhydrous sodium sulfate (100 g) was added into the combined extract to remove water, and then the extract was filtered through a filter paper (Whatman No. 1) to remove anhydrous sodium sulfate. The filtrate was subjected to SAFE to remove non-volatile constituents at 40°C and a pressure of 10^{-2} – 10^{-4} mbar and then was gently concentrated using the Vigreux column (50 × 1 cm^2) at 50°C until reaching 5 ml and the microdistillation apparatus at 50°C until reaching a final volume of 1 ml. The isolated aliquot was stored at −20°C prior to gas chromatography analysis.

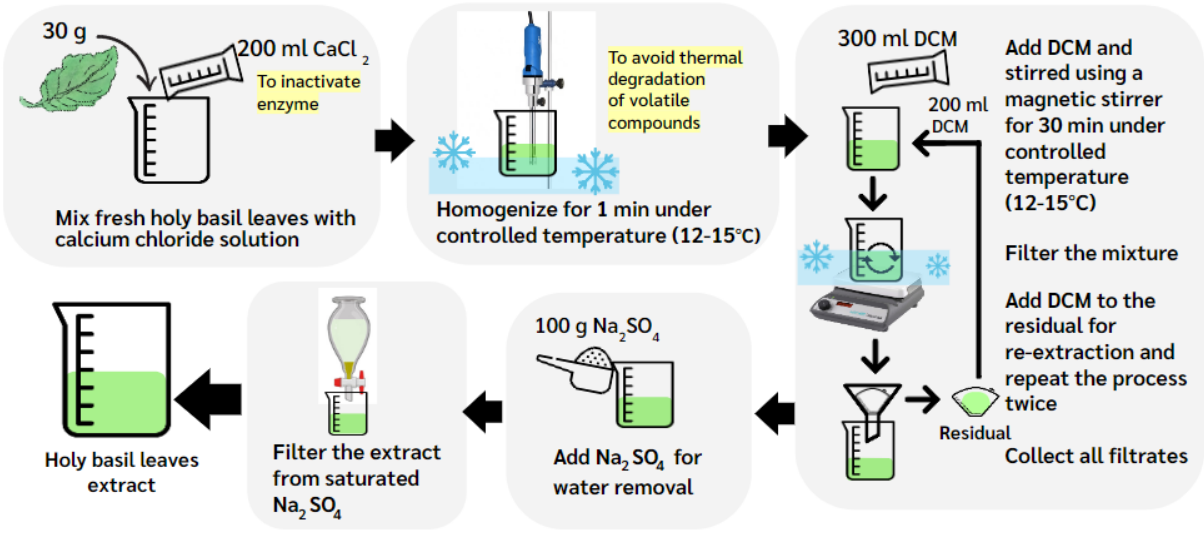

Figure 3.2 Solvent extraction from holy basil leaves

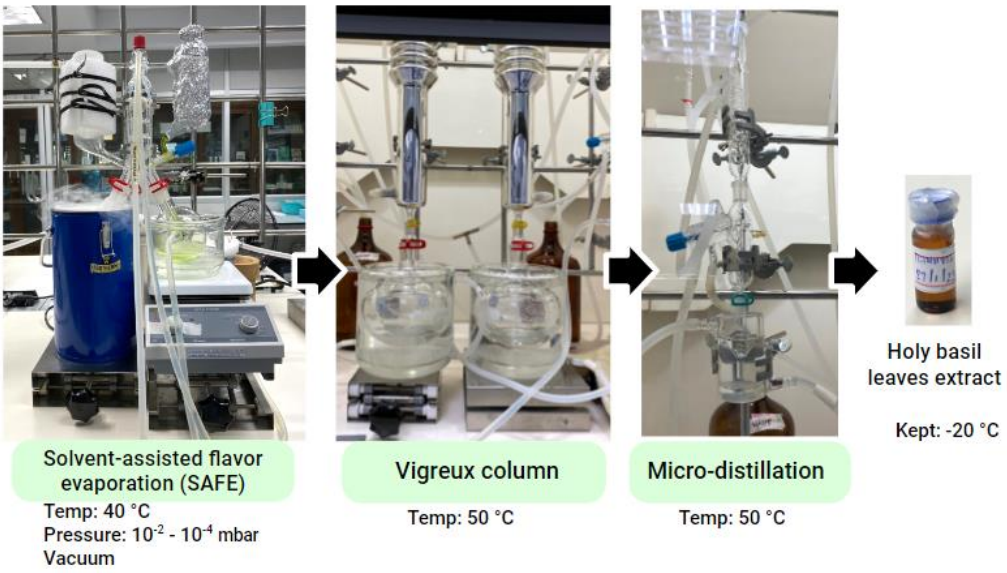

Figure 3.3 Isolation of volatile compounds from holy basil leaves

3.4 Determination of aroma compounds using gas chromatography-olfactometry/ flame-ionization detection (GC-O/FID) and aroma extract dilution analysis (AEDA)

A 1 µL of holy basil leaves extract was injected into the GC-O/FID (Shimadzu, Tokyo, Japan, Nexis GC-2030) for volatile compound determination using a cold on-column injector, fused silica DB-FFAP column (length 30 m × 0.32 mm i.d. and 0.25 µm film thickness, Agilent J&W GC column, USA). The carrier gas used was helium gas (70 kPa). The oven temperature program was started at 30°C, increased at a rate of 6°C/min to 230°C, and held at 230°C for 8 min (total time: 41.33 min). The column was connected with a Y-shape glass splitter of the deactivated fused silica capillaries (50 cm × 0.25 mm i.d.) at a ratio of 1:1, with one side connected to the FID detector and the other to the sniffing port. The odor characteristics were determined and recorded according to the retention time that the panelists detected them. The odor characteristics were further determined by identification of the retention index (RI) of the odor, which comes from the calculation of n-alkane (C7-C30) standards. The evaluation of the odor characteristics was conducted by trained panelists. Lastly, potential aroma compounds were

identified using the RI, and the odor quality was matched with the TUM database. RI shown in the equation following (Equation (3.1)).

$$I_x = 100n + 100\frac{t_x - t_n}{t_{n+1} - t_n} \qquad \text{Equation (3.1)}$$

I_x = Retention Index

n = Number of carbon atom of n-alkane hydrocarbon eluting immediately before compound X

t_x = Retention time of compound X

t_n = Retention time of carbon atom of n-alkane hydrocarbon eluting immediately before compound X

t_{n+1} =Retention time of carbon atom of n-alkane hydrocarbon eluting immediately after compound X

In AEDA, original extracts were diluted using dichloromethane. Stepwise dilutions of the original extract are performed with the ratio of original extracts and solution 1:2, 500 µL, and the diluted extracts are then evaluated by GC-O. The dilution was continued until no odorant could be detected by GC-O. Aroma active compound was then assigned as flavor dilution (FD) factor (Equation (3.2) defined by the highest dilution at which the compound was detected (Grosch, 2001).

$$\text{FD Factor} = 2^n \qquad \text{Equation (3.2)}$$

FD Factor = flavor dilution factor

n = number of dilutions

Figure 3.4 Determination of aroma compounds using gas chromatography-olfactometry/ flame-ionization detection (GC-O/FID)

3.5 Determination of volatile compounds using gas chromatography-mass spectrometry (GC-MS)

The aroma compounds of isolated holy basil leaves were determined using gas chromatography-mass spectrometry (GC-MS) (Shimadzu, Tokyo, Japan, GCMS-TQ8050 NX). The volatile extracts were injected to fused silica column SLB®-5ms (length 30 m × 0.32 mm i.d. and 0.25 μm film thickness, Merck, Germany). The condition was set similar to GC-O/FID (from 3.4). The volatile compounds were identified using retention index (RI) (Equation (3.1)), the volatiles mass spectra fragmentation patterns with the mass data of the corresponding compounds obtained from the NIST MS Search library (National Institute of Standard and Technology, Gaithersburg, MD, USA). Potential aroma compounds were identified with the RI and odor characteristics from GC-O/FID.

Figure 3.5 Determination of volatile compounds using gas chromatography-mass spectrometry (GC-MS)

Chapter IV

RESULTS AND DISCUSSION

4.1 Olfactory profiling

As yet, no instrument can duplicate the sensory and psychological responses of a human being. One category of sensory techniques that can be used to provide analytical and reliable information on sensory perception is descriptive analysis, such as providing quantitative descriptions of products based on the perceptions of a group of trained panelists through odor profile. The technique relies on determining appropriate descriptors, procedures, and panelists for the analysis of samples. The olfactory profiling was performed by eight trained panelists from the molecular sensory science research group. To explain, the trained panelists were involved in developing odor attributes, reference compounds, and sensory evaluate the sample with the reference compounds (Table 3.1).

Olfactory profiling of fresh white holy basil leaves and red holy basil leaves shown as radar chart in Figure 4.1. There were eight odor attributes of fresh holy basil including green, pungent, terpene-like, clove-like, fatty/waxy, citrus-like, earthy, and minty odor. The intensity scale of the olfactory profiling of fresh holy basil leaves was present in a 7-point scale. The olfactory profiling revealed that red holy basil leaves have stronger minty, green, pungent, and fatty/waxy attributes than white holy basil leaves but have lower in citrus-like attribute. There is no difference in terpene-like, clove-like, and earthy attributes between red and white holy basil leaves. Red holy basil leaves had stronger overall attributes than white holy basil leaves.

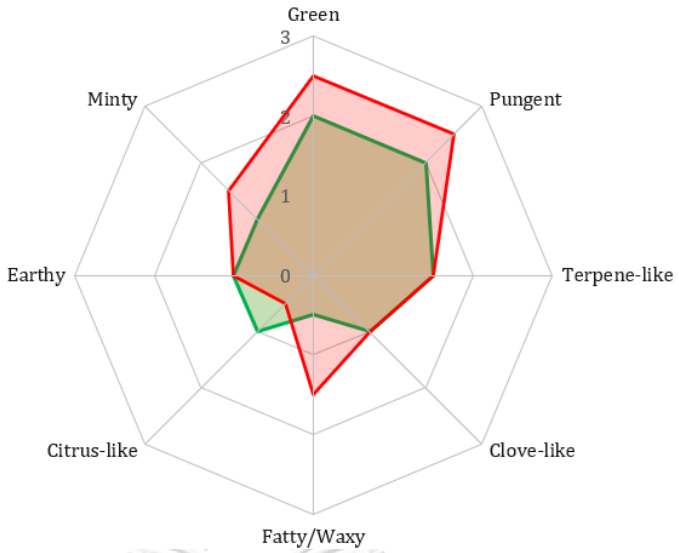

Figure 4.1 Olfactory profiling of white holy basil leaves(green) and red holy basil leaves(red) (0 = not detectable, 1 = weak, 2 = moderate, and 3 = strong)

4.2 Isolation of volatile compounds from holy basil leaves

In several studies, the extraction methods of holy basil were hydro-distillation and SPME (Raina et al., 2013; Tangpao et al., 2018; Wongpraneekul et al., 2022). However, limitations of these extraction techniques are the choice for selectivity, premature contamination of artifacts and thermal degradation, and the displacement effects due to the matrix compound (Camino-Sánchez et al., 2014; Etievant, 1996). Therefore, SAFE developed by Engel et al. (1999) is used to omit the issue of choice for selectivity and premature contamination. This technique requires low temperatures and vacuum conditions with the use of a low boiling point solvent, such as diethyl ether or dichloromethane, to facilitate the isolation of volatile compounds from different matrices, including solvent extracts, aqueous foods, food suspensions, or matrices with a high oil content. In the isolation of volatile compounds from fresh holy basil leaves, saturated calcium chloride was added before being homogenized in order to inactivate lipoxygenase and peroxidase activities available in plants that could lead to the formation of other organic compounds that could generate odors (Kubo et al., 2017; Baysal & Demirdöven, 2007). Dichloromethane which is an organic solvent was added to the solution, stirred, and filtered to get crude solvent extract under cooling bath at 12 to 15°C. The temperature during solvent extraction

must be controlled to avoid precipitation. The extraction at 9°C or below causes precipitation of saturated calcium chloride solution (Figure 4.2). This might come from an effect of the reduced solubility of the calcium chloride under a low temperature extraction.

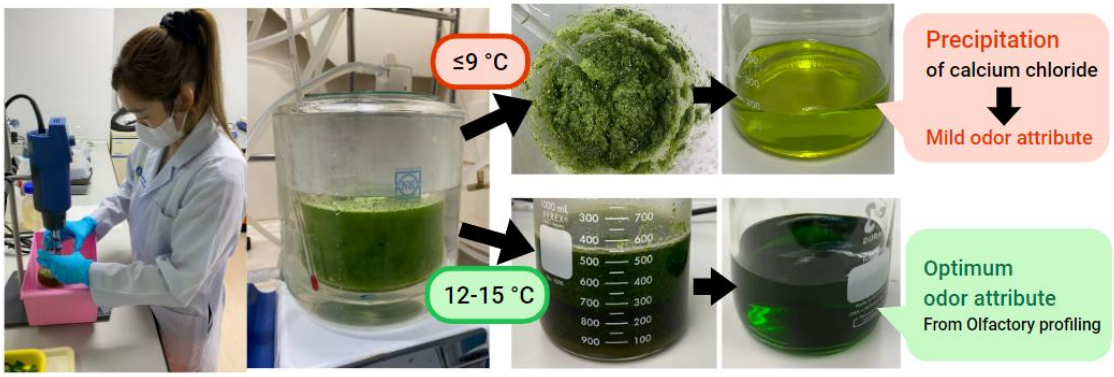

Figure 4.2 Solvent extraction from holy basil leaves

Anhydrous sodium sulphate was added for removing water from the extract for GC-O/FID analysis because an excess water injected into the gas chromatography could damage the stationary phase, polyethylene glycol (PEG), of the GC-column (Kuhn, 2002). The approximately 400 ml of the fresh holy basil (30 g) was obtained after solvent extraction and filtration. Then, the solvent assisted flavor evaporation was done with SAFE apparatus under vacuum condition at pressure of 10^{-2} – 10^{-4} mbar and temperature of 40°C. The elevated temperature was avoided throughout the process to prevent artifact formation and compound degradation. As shown in Figure 4.3, volatile and non-volatile compounds of the holy basil leaves were separated to the left side and the right side of the SAFE apparatus which the non-volatile constituents, including chlorophyll pigments, sugars, amino acids, vitamins, and minerals, were removed from the final extract.

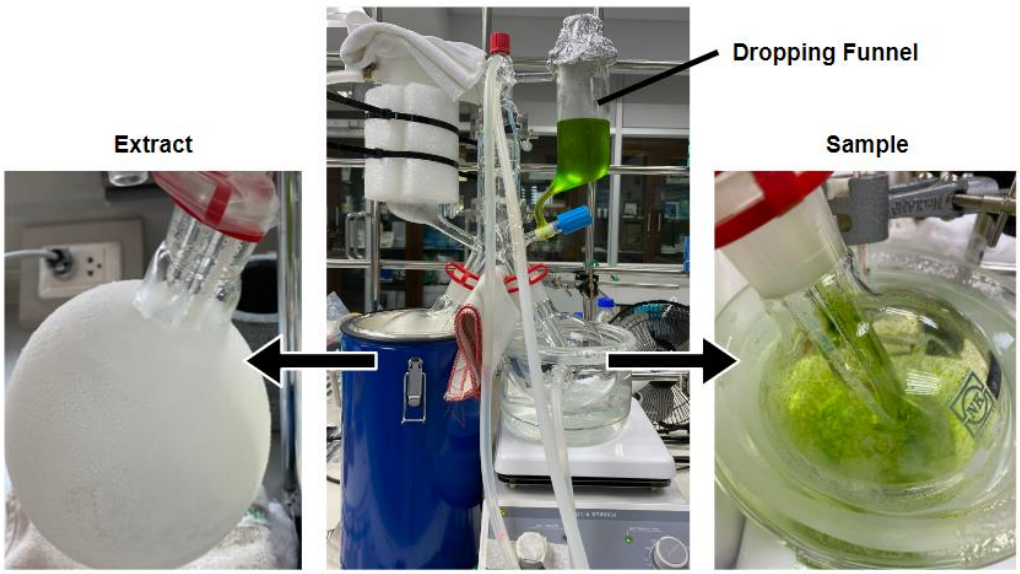

Figure 4.3 Solvent assisted flavor evaporation (SAFE) from holy basil leaves; Extract (left), SAFE apparatus (middle), Non-volatile constituent of sample (right)

The SAFE distillate, approximately 200 ml, that contain volatile compounds, was then gently concentrated, as dichloromethane was removed, to 5 ml by Vigreux column following by concentrated by microdistillation device to 1 ml and kept at −20°C for further analysis (Figure 4.4).

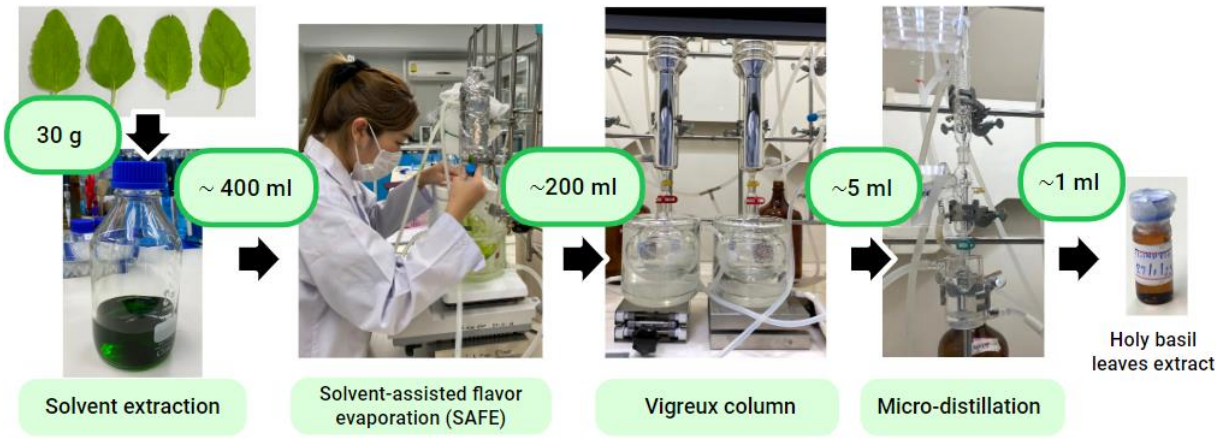

Figure 4.4 Isolation of volatile compounds from holy basil leaves

The odor attributes of the extract were checked by olfactory profiling in all isolation steps to ensure that the solvent extract and distillates still retained the typical odor attributes and olfactory profile of fresh holy basil leaves.

4.3 Determination of aroma compounds using gas chromatography-olfactometry/ flame-ionization detection (GC-O/FID) and aroma extract dilution analysis (AEDA)

GC-O/FID was used to distinguish between odor and odorless volatiles compounds. In GC-O/FID, the volatile fraction isolated from the holy basil leaves was separated by GC through different polarity and the column effluent and then each volatile compound was spitted into two parts, the first directed to flame ionization detector (FID), and the second part directed to a sniffing port (Munafo et al., 2015). The odor character of each compound was determined and recorded according to the retention time that the panelists perceived. Retention index (RI) of each odorant was calculated from its retention time, according to the n-alkane (C7-C30) standards as shown in Table 4.1 and 4.2.

Table 4.1 Retention time, retention index, odor quality, and intensity of white holy basil leaves extract

No.	RT[1]	RI[2]	Odor Quality	Intensity[3]
1	3.98	1012	pungent	1
2	4.81	1070	green	1
3	5.29	1103	terpene-like	2-3
4	5.90	1136	green	1
5	6.25	1155	green, herbal	1-2
6	6.88	1189	green, sweet	1
7	7.14	1202	eucalyptus-like	1-2
8	7.91	1240	terpene-like, earthy	2-3
9	8.25	1256	citrus-like, soapy	2-3
10	10.03	1341	flowery, green	1-2
11	10.65	1370	terpene-like	2-3
12	11.23	1397	green	1
13	11.65	1418	earthy	1
14	12.69	1468	herbal, mushroom-like	1-2
15	13.24	1494	soapy, citrus-like	2
16	14.24	1544	eucalyptus-like	2-3
17	14.48	1556	citrus-like	2
18	15.18	1591	earthy	1-2
19	16.52	1660	flowery	1-2
20	17.04	1688	earthy	2
21	17.10	1691	sweet, citrus-like	2-3
22	17.19	1695	herbal, pungent	3-5
23	18.22	1751	flowery, sweet	3
24	19.43	1818	earthy	1
25	20.00	1850	green, sweet	1-2
26	22.06	1970	herbal, soapy	2-3
27	22.73	2011	clove-like, green	2-3
28	23.46	2056	musty, green	1
29	23.81	2078	green	1
30	25.16	2165	clove-like	4-5
31	26.84	2276	musty, green	1

[1] Retention time

[2] Retention index

[3] For, intensity, 1 = lowest intensity and 5 = highest intensity.

Table 4.2 Retention time, retention index, odor quality, and intensity of red holy basil leaves extract

No.	RT[1]	RI[2]	Odor Quality	Intensity[3]
1	3.98	1012	pungent	1
2	4.72	1063	sweet	2-3
3	5.29	1104	terpene-like	2
4	5.91	1136	green	2
5	6.25	1155	green, herbal	2-3
6	6.82	1185	sweet, musty	1
7	7.77	1233	sweet	1
8	7.92	1240	terpene-like, earthy	1
9	8.81	1283	citrus-like	1-2
10	8.97	1291	pungent	1
11	9.86	1333	green, cooked rice	2-3
12	10.41	1359	sweet	4
13	10.65	1370	terpene-like	2
14	11.21	1397	green	2
15	11.66	1418	earthy	1-2
16	11.92	1431	soapy, sweet	1-2
17	12.12	1440	terpene-like	2-3
18	13.24	1494	soapy, citrus-like	1
19	14.16	1540	soapy	2-3
20	14.48	1556	citrus-like	2-3
21	15.03	1583	pungent	3
22	16.16	1642	herbal	2-3
23	17.10	1691	sweet, citrus-like	3
24	17.28	1700	herbal	2-3
25	17.65	1720	sweet	2
26	18.11	1745	herbal, musty	1
27	19.26	1808	soapy, waxy	1-2
28	19.93	1847	soapy, citrus-like	2
29	20.30	1868	sweet	1
30	22.05	1970	herbal, soapy	1-2
31	22.50	1997	green, fruity	2
32	22.74	2011	clove-like, green	4-5
33	23.64	2067	pungent, citrus-like	1
34	23.82	2078	green	2
35	25.17	2165	clove-like	2-3
36	26.81	2274	soapy, waxy	1
37	28.02	2358	herbal	1

[1] Retention time

[2] Retention index

[3] For, intensity, 1 = lowest intensity and 5 = highest intensity.

There were 31 and 37 odorants perceived from the GC-O/FID analysis of white and red holy basil leaves extracts (Table 4.1 and 4.2), respectively. In agreement with olfactory profiling, GC-O/FID result revealed that there were green, pungent, terpene-like, clove-like, fatty/waxy, citrus-like, earthy, and minty odor quality also perceived. Moreover, there were eucalyptus-like, flowery, sweet, musty odors from white holy basil leaves and herbal, musty, sweet odors from red holy basil leaves detected through GC-O/FID. The GC-O/FID result showed more numbers and qualities of odorants perceived from GC-O/FID were more than olfactory profiling (8 odor attributes in total, Figure 4.1). In addition, from GC-O/FID analysis, the comparison of the odorants between white and red holy basil leaves was shown that eucalyptus-like odor (RI 1202 and 1544) and flowery odor (RI 1341, 1660 and 1751) were presented only in white holy basil leaves.

To clarify, olfactory profiling evaluated from fresh holy basil leaves at room temperature which composed of a complex mixture of volatile compounds. The mixture of volatile compounds in food matrix could cause loss of individual perception of each compound. Moreover, the interaction between compounds could also produce a new odor percept conveying a unique odor quality which was not elicited by the single components (Munafo et al., 2015). On the other hand, GC-O/FID evaluated from holy basil leaves extract which the fresh sample were extracted, distillated, and concentrated from isolation process. The final extract was volatilized and separated through GC column. The volatile compounds were then split into FID and sniffing port where the panelists perceived the odor. Generally, from GC-O/FID analysis, 30 – 60 odor quality is usually detected in each food sample (Munafo et al., 2015).

In addition, minty odor was the only odorant that perceived from olfactory profiling but not detected from GC-O/FID analysis. This described that odorant could be a highly volatile compound that have a lower boiling point than the organic solvent. the highly volatile compounds were lost during isolation process and not be able to be perceived by panelists at the GC-O/FID. Therefore, a static headspace GC-O analysis can be used as a complementary technique for further analysis.

Aroma extract dilution analysis (AEDA) was used to find potential aroma active compounds in the holy basil leaves. After stepwise dilutions, potential aroma active compounds were then assigned flavor dilution (FD) factors. From AEDA, red holy basil leaves had more number of odorants with high FD factor (512 to ≥ 4096) than white holy basil leaves. There were 7 out of 31 odorants with high FD factor in white holy basil leaves (Figure 4.5), whereas 10 out of 37 odorants in red holy basil leaves (Figure 4.6). This AEDA result was corresponded to olfactory profiling result as fresh red holy basil leaves had stronger overall attributes than fresh white holy basil leaves. White holy basil leaves had distinct eucalyptus-like (FD 2048) and flowery odors (FD 512 and 1024) which not presented in red holy basil leaves. On the other hand, red holy basil leaves showed more herbal (FD 1024, 2048 and 2048) and clove-like odors (FD 2048 and ≥ 4096) than white holy basil leaves.

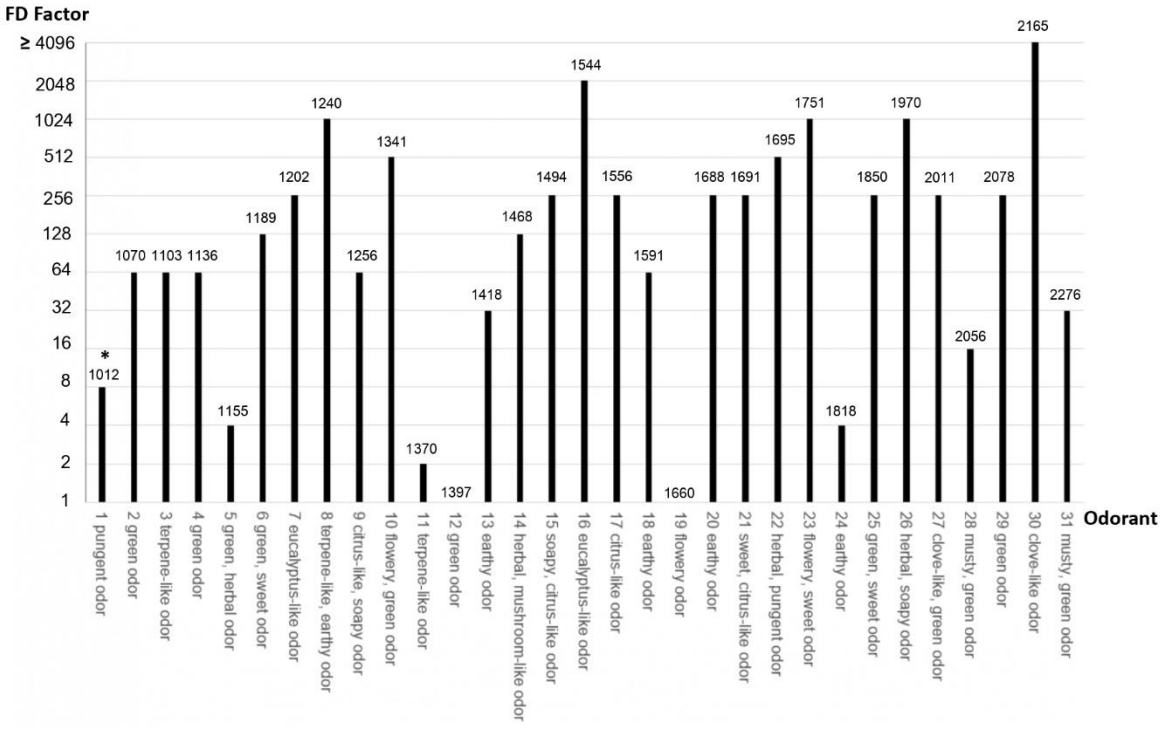

Figure 4.5 Flavor dilution (FD) Factors of white holy basil leaves extract

***= Retention index (RI) of each odor quality**

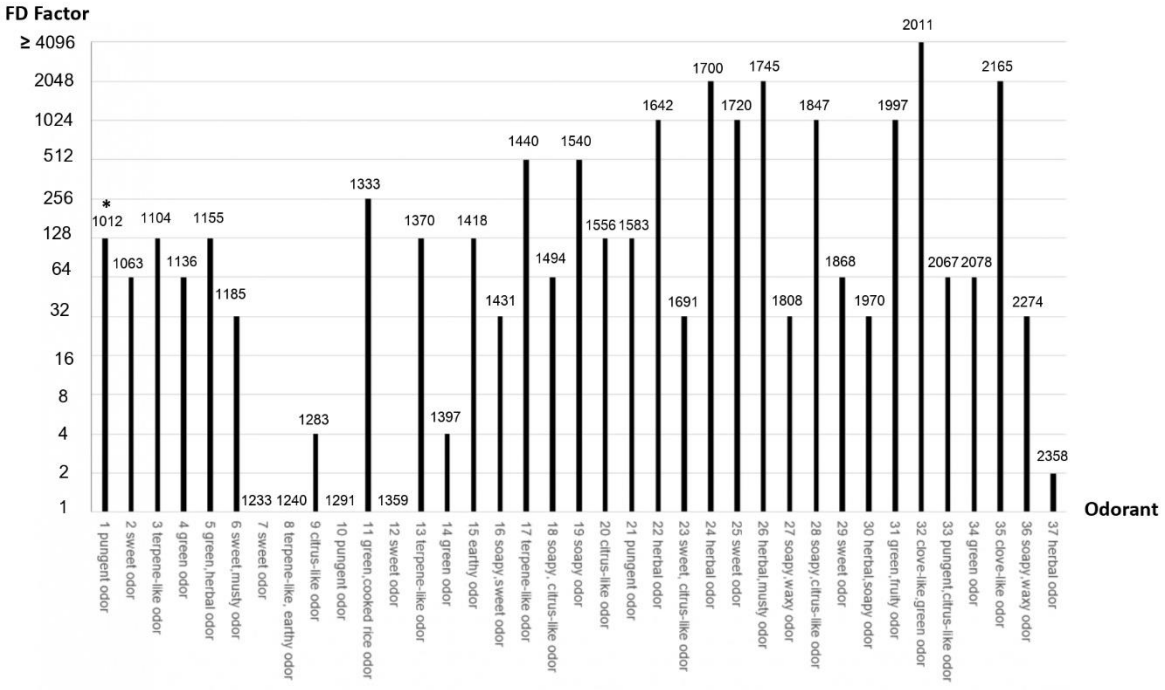

Figure 4.6 Flavor dilution (FD) Factors of red holy basil leaves extract
***= Retention index (RI) of each odor quality**

4.4 Determination of volatile compounds using gas chromatography-mass spectrometry (GC-MS)

Structural assignment of each potential aroma active compound was completed by comparing 3 parameters with the respective data of structurally characterized reference compounds. Those 3 parameters were odor quality, RIs, and mass spectra. The odor quality and RIs were obtained from GC-O/FID. Mass spectra is from gas chromatography-mass spectrometry (GC-MS).

Potential aroma active compounds in white and red holy basil leaves extract were determined, as shown in Table 4.3. In white holy basil leaves, from GC-MS result, only 11 out of 53 aroma compounds were matched with aroma compounds from GC-O/FID including α-pinene (pungent odor), β-pinene (terpene-like odor), β-myrcene (green, herbal odor), 1,8-cineole (eucalyptol) (eucalyptus-like odor), (E)-β-ocimene (citrus-like, soapy odor), 2-hexanol (flowery, green odor), (E)-3-hexen-1-ol (green odor), isocaryophyllene (citrus-like odor), α-farnesene (flowery, sweet odor), methyl eugenol (4-allyl-1,2-dimethoxybenzene) (clove-like, green odor), eugenol (4-allyl-2-methoxyphenol) (clove-like odor). To improve an structural assignment, an

increase in fresh sample for extraction or fractionation step should be done to increase concentration of the and to avoid coelution problems. From AEDA, the data showed that potential aroma active compounds of white holy basil leaves extract were eugenol (4-allyl-2-methoxyphenol) (clove-like odor) (FD ≥ 4096), cis-sabinene hydrate (cis-4-thujanol) (eucalyptus-like odor) (FD 2048), γ-terpinene (terpene-like, earthy odor) (FD 1024), α-farnesene (flowery, sweet odor) (FD 1024), caryophyllene oxide (herbal, soapy odor) (FD 1024), 2-hexanol (flowery, green odor) (FD 512), trans-4,5-epoxy-(E)-2-heptenal (herbal, pungent odor) (FD 512).

Table 4.3 Potential aroma compounds, RI, FD factor, MS in white holy basil leaves extract (W) and red holy basil leaves extract (R)

No.	Compound	Odor Quality	RI Experiment [1]		FD factor [2]		MS [3]	
			W	R	W	R	W	R
1	α-pinene	pungent	1012	1012	8	128	Y	Y
2	2-isopropyl-4,6-dimethyltetrahydro-(2H)-pyran	sweet	-	1063	-	64	-	N
3	hexanal	green	1070	-	64	-	N	-
4	β-pinene	terpene-like	1103	1103	64	128	Y	Y
5	(Z)-2-pentenal	green	1136	1136	64	64	N	Y
6	β-Myrcene	green,herbal	1155	1155	4	128	Y	Y
7	methyl hexanoate	sweet,musty	-	1185	-	32	-	N
8	(Z)-2-hexenal	green,sweet	1189	-	128	-	N	-
9	1,8-cineole (eucalyptol)	eucalyptus-like	1202	-	256	-	Y	-
10	6-methylheptanal	sweet	-	1233	-	1	-	N
11	γ-Terpinene	terpene-like, earthy	1240	1240	1024	1	N	Y
12	(E)-β-ocimene	citrus-like, soapy	1256	-	64	-	Y	-
13	octanal	citrus-like	-	1283	-	2	-	N
14	1-(ethylthio)-ethanethiol	pungent	-	1291	-	1	-	N
15	hexyl 2-methylpropanoate	green,cooked rice	-	1333	-	256	-	N
16	2-hexanol	flowery, green	1341	-	512	-	Y	-
17	2,3-dimethyl-5-methoxypyrazine	sweet	-	1359	-	1	-	N
18	p-mentha-1,3,8-triene	terpene-like	1370	1370	2	128	N	N
19	(E)-3-hexen-1-ol	green	1397	1397	1	2	Y	Y
20	3-isopropyl-2-methoxypyrazine	earthy	1418	1418	32	128	N	N
21	dihydrothiocitronellol (3,7-dimethyloctane-1-thiol)	soapy,sweet	-	1431	-	32	-	N

[1] RI experiment or retention index from experiment was calculated using Equation (1).
[2] FD Factor, flavor dilution factor obtained from d aroma extract dilution analysis (AEDA)
[3] Mass spectrum from GC-MS; Y = found signal, N = not found signal

Table 4.3 (continue). Potential aroma compounds, RI, FD factor, MS in white holy basil leaves extract (W) and red holy basil leaves extract (R)

No.	Compound	Odor Quality	RI Experiment [1]		FD factor [2]		MS [3]	
			W	R	W	R	W	R
22	3-carene	terpene-like	-	1440	-	512	-	Y
23	(Z)-1,5-nonadien-3-one	herbal, mushroom-like	1468	-	128	-	N	-
24	camphor	soapy, citrus-like	1494	1494	256	64	N	Y
25	1-(methylthio)nonane	soapy	-	1540	-	512	-	N
26	cis-sabinene hydrate (cis-4-thujanol)	eucalyptus-like	1544	-	2048	-	N	-
27	isocaryophyllene	citrus-like	1556	1556	256	128	Y	Y
28	(Z)-1,5-nonadien-3-ol	pungent	-	1583	-	128	-	N
29	1-terpinen-4-ol	earthy	1591	-	64	-	N	-
30	1-decen-3-ol	herbal	-	1642	-	1024	-	N
31	phenylacetaldehyde	flowery	1660	-	1	-	N	-
32	naphthalene	herbal	-	1687	-	2048	-	Y
33	endo-Borneol	earthy	1688	-	256	-	N	-
34	α-terpineol	sweet, citrus-like	1691	1691	256	32	N	Y
35	trans-4,5-epoxy-(E)-2-heptenal	herbal,pungent	1695	-	512	-	N	-
36	benzyl acetate	sweet	-	1720	-	1024	-	N
37	1-undecen-3-ol	herbal,musty	-	1745	-	2048	-	N
38	α-farnesene	flowery,sweet	1751	-	1024	-	Y	-
39	p-cymene-9-thiol	soapy,waxy	-	1808	-	32	-	N
40	geosmine	earthy	1818	-	4	-	N	-
41	3-mercapto-3-methylheptyl acetate	soapy,citrus-like	-	1847	-	1024	-	N
42	α-ionone	green,sweet	1850	-	256	-	N	-
43	(E,E,E)-2,4,7-decatrienal	sweet	-	1868	-	64	-	N
44	caryophyllene oxide	herbal,soapy	1970	1970	1024	32	N	Y
45	3-hydroxypropyl pentanoate	green	-	1997	-	1024	-	N
46	methyl eugenol (4-allyl-1,2-dimethoxybenzene)	clove-like,green	2011	2011	256	≥ 4096	Y	Y
47	octanoic acid	musty,green	2056	-	16	-	N	-
48	benzyl isothiocyanate	pungent,citrus-like	-	2067	-	64	-	N
49	(E)-1,5-tetradecadien-3-ol	green	2078	2078	256	64	N	N
50	eugenol (4-allyl-2-methoxyphenol)	clove-like	2165	2165	≥ 4096	2048	Y	N
51	3-mercapto-1-decanol	soapy,waxy	-	2274	-	32	-	N
52	2,4,6-tribromoanisole	musty,green	2276	-	32	-	N	-
53	undecanoic acid	herbal	-	2358	-	2	-	N

[1] RI experiment or retention index from experiment was calculated using Equation (1).

[2] FD Factor, flavor dilution factor obtained from d aroma extract dilution analysis (AEDA)

[3] Mass spectrum from GC-MS; Y = found signal, N = not found signal

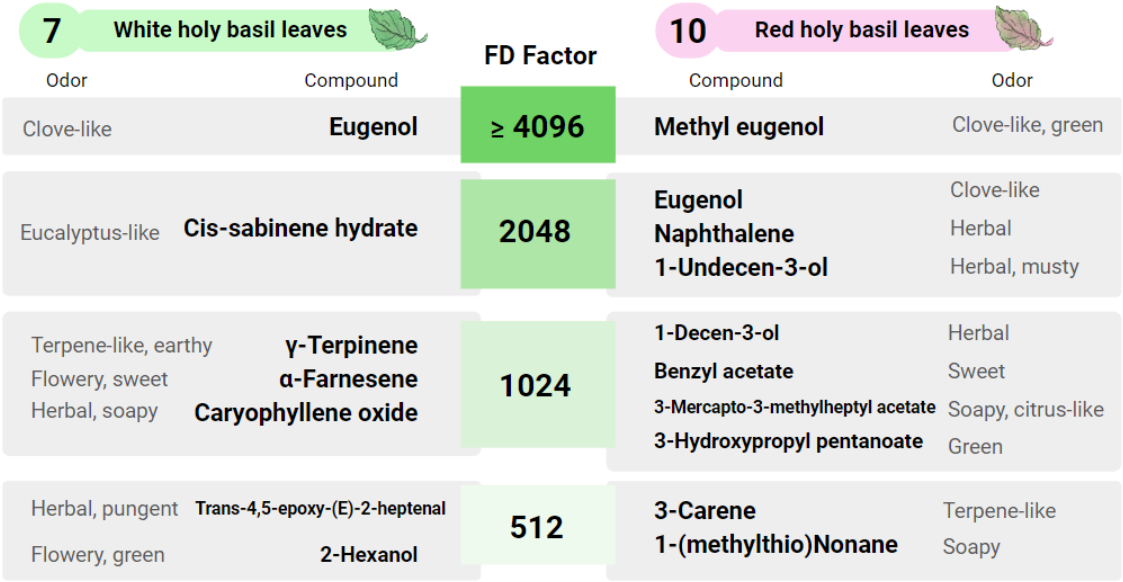

Figure 4.7 Potential aroma active compounds (FD ≥ 512) in white holy basil leaves extract (left) and red holy basil leaves extract (right)

In red holy basil leaves, from GC-MS, 13 out of 53 aroma compounds were matched with aroma compounds from GC-O/FID including α-pinene (pungent odor), β-pinene (terpene-like odor), (Z)-2-pentenal (green odor), β-Myrcene (green, herbal odor), γ-terpinene (terpene-like, earthy odor), (E)-3-hexen-1-ol (green odor), 3-carene (terpene-like odor), camphor (soapy, citrus-like odor), isocaryophyllene (citrus-like odor), α-terpineol (sweet, citrus-like odor), naphthalene (herbal odor), caryophyllene oxide (herbal, soapy odor), and methyl eugenol (4-allyl-1,2-dimethoxybenzene) (clove-like, green odor). From AEDA, the data suggested that potential aroma active compounds of red holy basil leaves extract were methyl eugenol (4-allyl-1,2-dimethoxybenzene)(clove-like, green odor) (FD ≥ 4096), naphthalene (herbal odor) (FD 2048), 1-undecen-3-ol (herbal, musty odor) (FD 2048), eugenol (4-allyl-2-methoxyphenol) (clove-like odor) (FD 2048), 1-decen-3-ol (herbal odor) (FD 1024), benzyl acetate (sweet odor) (FD 1024), 3-mercapto-3-methylheptyl acetate (soapy, citrus-like odor) (FD 1024), 3-hydroxypropyl pentanoate (green odor) (FD 1024), 3-carene (terpene-like odor) (FD 512), and 1-(methylthio)nonane (soapy odor) (FD 512).

Among 53 potential aroma compounds of white and red holy basil leaves, the result showed that there were 15 potential aroma compounds presented in both white and red holy basil leaves including α-pinene (pungent odor), β-pinene (terpene-like odor), (Z)-2-pentenal (green odor), β-Myrcene (green, herbal odor), γ-terpinene (terpene-like, earthy odor), *p*-mentha-1,3,8-triene (terpene-like odor), (E)-3-hexen-1-ol (green odor), 3-isopropyl-2-methoxypyrazine (earthy odor), camphor (soapy, citrus-like odor), isocaryophyllene (citrus-like odor), α-terpineol (sweet, citrus-like odor), caryophyllene oxide (herbal, soapy odor), methyl eugenol (4-allyl-1,2-dimethoxybenzene) (clove-like, green odor), (E)-1,5-tetradecadien-3-ol (green odor), and eugenol (4-allyl-2-methoxyphenol) (clove-like odor).

For potential aroma active compounds (FD ≥ 512) (Figure 4.7), white holy basil leaves consisted of only eugenol (FD ≥ 4096), while red holy basil leaves consisted of both eugenol (FD 2048) and methyl eugenol (FD ≥ 4096). In white holy basil leaves, the unique flowery odor might be contributed from 2-hexanol (FD 512) and α-farnesene (FD 1024). Moreover, the distinct eucalyptus-like odor might potentially come from cis-sabinene hydrate (cis-4-thujanol) (FD 2048). In red holy basil leaves, herbal odor potentially came from 3 high FD factor compounds, including 1-decen-3-ol (FD 1024), naphthalene (FD 2048), and 1-undecen-3-ol (FD 2048), while only 2 compounds with lower FD factor, including trans-4,5-epoxy-(E)-2-heptenal (FD 512) and caryophyllene oxide (FD 1024), were detected from red holy basil leaves.

In agreement with previous studies (Raina et al. (2013), Tangpao et al. (2018), and Wongpraneekul et al. (2022), Sriprom (2020)), this study also detected eugenol, methyl eugenol, endo-borneol, α-pinene, and caryophyllene oxide in white and red holy basil leaves.

3-carene presented only in red holy basil leaves, which also be reported in Tangpao et al. (2018). Moreover, eucalyptol, (E)-β-ocimene, (E)-3-hexen-1-ol, and isocaryophyllene presented only in white holy basil leaves which also reported in Wongpraneekul et al. (2022). However, β-pinene was detected in this study in both white and red holy basil leaves, whereas Tangpao et al. (2018) reported β-pinene only in white holy basil leaves.

For the application, this study reveals differences in aroma profiles between white and red holy basil leaves at molecular level. The result suggested the potential odorants that distinct between each cultivar of holy basil leaves. Odor attributes and aroma profiles of white and red holy basil leaves could be used for industrial purposes such as a crop selection and a quality control, especially raw materials' inspection. In addition, the data of the aroma active compound could also be used for further product development especially for products' flavor.

Chapter V

5.1 Conclusion

To summarize, olfactory profiling of white and red holy basil samples was performed by trained panelists to characterize odor description and their intensity. Second, volatile compounds were isolated by solvent extraction, and non-volatile constituents were removed by solvent-assisted flavor evaporation, followed by gentle concentration using the Vigreux column and microdistillation device. Third, the concentrated crude extract was injected into the GC-O/FID to screen for odor quality. The odor quality and its corresponding retention index (RI) were recorded. AEDA was used to rank the odor quality which an FD factor is assigned for each odorant. Structural assignment of each aroma active compound was completed by comparing 3 parameters with the respective data of structurally characterized reference compounds. Those 3 parameters are odor quality, RIs, and mass spectra. The odor quality and RIs are obtained from GC-O/FID. Mass spectra is from gas chromatography-mass spectrometry (GC-MS).

The olfactory profiling revealed that red holy basil leaves have stronger minty, green, pungent, and fatty/waxy attributes than white holy basil leaves but have lower in citrus-like attribute. There was no difference in terpene-like, clove-like, and earthy attributes between red and white holy basil leaves. Red holy basil leaves had stronger overall attributes than white holy basil leaves.

Among the potential aroma active compounds with high FD factors (FD 512 to ≥ 4096), white holy basil leaves consisted of only eugenol (FD ≥ 4096), while red holy basil leaves consisted of both eugenol (FD 2048) and methyl eugenol (FD ≥ 4096). In white holy basil leaves, the unique flowery odor might be contributed from 2-hexanol (FD 512) and α-farnesene (FD 1024). Moreover, the distinct eucalyptus-like odor might potentially come from cis-sabinene hydrate (cis-4-thujanol) (FD 2048). In red holy basil leaves, herbal odor was potentially come from 3 high FD factor compounds, including 1-decen-3-ol (FD 1024), naphthalene (FD 2048), and 1-undecen-3-ol (FD 2048), while only 2 compounds with lower FD factor, including trans-4,5-epoxy-(E)-2-heptenal (FD 512) and caryophyllene oxide (FD 1024), were presented in white holy basil leaves.

5.2 Recommendations for the future work

Future research would consider using a static headspace GC-O/FID as a complementary technique to cover up highly volatile odorants in the fresh sample. Since there was minty odor that perceived only from olfactory profiling but not detected from GC-O/FID analysis. This described that the highly volatile compounds may have a lower boiling point than the boiling point of the organic solvent. The highly volatile compounds were lost during isolation process and not be able to perceive by GC-O/FID.

In structural assignment, GC-O and GC-MS analysis are suggested to use at least two GC separation systems of different stationary phase polarities, typically polar and nonpolar column. However, this study uses only DB-FFAP column which is polar column to analyze aroma compound in GC-O analysis and SLB-5 column which is nonpolar column to analyze volatile compound in GC-MS due to limitation of time and technical maintenance. Thus, for further study, GC-O and GC-MS analysis should be applied both polar and nonpolar column such as DB-FFAP and DB-5 column. Moreover, to improve a structural assignment, an increase in fresh sample for extraction or fractionation step should be done to increase concentration of the and to avoid coelution problems.

For further research using sensomics approach, quantification using stable isotope dilution assay (SIDA), calculation for odor activity value (OAV), aroma reconstitution, and omission test must be made to be able to reveal key compounds of red and white holy basil leaves.

Appendix A

Table A-1 GC-O/FID Condition

Injection volume:	1 µl
Column flow:	2.3 ml/min
Purge flow:	2 ml/min
Carrier gas:	He
Makeup gas: N_2 flow	24 ml/min
H_2 flow:	32 ml/min
Air flow:	200 ml/min
Column temperature:	Initial temp.: 30^{o}C
	Ramp: 6^{o}C/min
	Final temp.: 230^{o}C (hold 8 min)
Analyse time:	41.33 min

Recording sheet

Recording Sheet of Gas Chromatography-Olfactometry (GC-O/FID) Analysis

Name-Surname:.....Time:..... Date:...../...../.....

Sample:.....Solvent.....Extraction Date:...../...../.....

Method:.....Column.....Flow:.....ml/min

Final Temp.....°C Ramp.....°C Run time.....min

No.	Time	Odor Quality	Intensity (Min 1 → 5 Max)	Remark

Figure A-1 Recording sheet of GC-O/FID analysis

Table A-2 Retention time, odor quality, intensity, retention index and FD factor of white holy basil leaves extract

No.	RT[1]	Odor Quality	Intensity[2]	RI[3]	FD Factor[4]
1	3.98	pungent	1	1012	8
2	4.81	green	1	1070	64
3	5.29	terpene-like	2-3	1103	64
4	5.90	green	1	1136	64
5	6.25	green, herbal	1-2	1155	4
6	6.88	green, sweet	1	1189	128
7	7.14	eucalyptus-like	1-2	1202	256
8	7.91	terpene-like, earthy	2-3	1240	1024
9	8.25	citrus-like, soapy	2-3	1256	64
10	10.03	flowery, green	1-2	1341	512
11	10.65	terpene-like	2-3	1370	2
12	11.23	green	1	1397	1
13	11.65	earthy	1	1418	32
14	12.69	herbal, mushroom-like	1-2	1468	128
15	13.24	soapy, citrus-like	2	1494	256
16	14.24	eucalyptus-like	2-3	1544	2048
17	14.48	citrus-like	2	1556	256
18	15.18	earthy	1-2	1591	64
19	16.52	flowery	1-2	1660	1
20	17.04	earthy	2	1688	256
21	17.10	sweet, citrus-like	2-3	1691	256
22	17.19	herbal, pungent	3-5	1695	512
23	18.22	flowery, sweet	3	1751	1024
24	19.43	earthy	1	1818	4
25	20.00	green, sweet	1-2	1850	256
26	22.06	herbal, soapy	2-3	1970	1024
27	22.73	clove-like, green	2-3	2011	256
28	23.46	musty, green	1	2056	16
29	23.81	green	1	2078	256
30	25.16	clove-like	4-5	2165	≥ 4096
31	26.84	musty, green	1	2276	32

[1] Retention time

[2] For, intensity, 1 = lowest intensity and 5 = highest intensity.

[3] Retention index

[4] FD Factor, flavor dilution factor obtained from aroma extract dilution analysis (AEDA)

Table A-3 Retention time, odor quality, intensity, and FD factor of red holy basil leaves extract

No.	RT[1]	Odor Quality	Intensity[2]	RI[3]	FD Factor[4]
1	3.98	pungent	1	1012	128
2	4.72	sweet	2-3	1063	64
3	5.29	terpene-like	2	1104	128
4	5.91	green	2	1136	64
5	6.25	green, herbal	2-3	1155	128
6	6.82	sweet, musty	1	1185	32
7	7.77	sweet	1	1233	1
8	7.92	terpene-like, earthy	1	1240	1
9	8.81	citrus-like	1-2	1283	2
10	8.97	pungent	1	1291	1
11	9.86	green, cooked rice	2-3	1333	256
12	10.41	sweet	4	1359	1
13	10.65	terpene-like	2	1370	128
14	11.21	green	2	1397	2
15	11.66	earthy	1-2	1418	128
16	11.92	soapy, sweet	1-2	1431	32
17	12.12	terpene-like	2-3	1440	512
18	13.24	soapy, citrus-like	1	1494	64
19	14.16	soapy	2-3	1540	512
20	14.48	citrus-like	2-3	1556	128
21	15.03	pungent	3	1583	128
22	16.16	herbal	2-3	1642	1024
23	17.10	sweet, citrus-like	3	1691	32
24	17.28	herbal	2-3	1700	2048
25	17.65	sweet	2	1720	1024
26	18.11	herbal, musty	1	1745	2048
27	19.26	soapy, waxy	1-2	1808	32
28	19.93	soapy, citrus-like	2	1847	1024
29	20.30	sweet	1	1868	64
30	22.05	herbal, soapy	1-2	1970	32
31	22.50	green, fruity	2	1997	1024
32	22.74	clove-like, green	4-5	2011	≥ 4096
33	23.64	pungent, citrus-like	1	2067	64
34	23.82	green	2	2078	64
35	25.17	clove-like	2-3	2165	2048
36	26.81	soapy, waxy	1	2274	32
37	28.02	herbal	1	2358	2

[1] Retention time

[2] For, intensity, 1 = lowest intensity and 5 = highest intensity.

[3] Retention index

[4] FD Factor, flavor dilution factor obtained from aroma extract dilution analysis (AEDA)

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