AUTHENTICITY ANALYSIS OF CITRUS ESSENTIAL OILS BY MEANS OF HPLC-UV-MS ON OXYGENATED HETEROCYCLIC COMPONENTS

By

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ABSTRACT OF THE DISSERTATION

Authenticity Analysis of Citrus Essential oils by Means of HPLC-UV-MS on Oxygenated Heterocyclic Components

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Professor Chi-Tang Ho, Ph.D.

Essential oils have been the key natural raw material in flavor and fragrance industry since its inception (Dugo *et al.*, 2002). Citrus essential oil, as the largest essential oil group, comprises 70% of imported oils in US (Dugo *et al.*, 2002). Their unique sensory properties have been widely accepted and applied in beverage, confectionary, perfume, house care and other fields.

However, due to relatively simple chemical composition and tremendous price differences among citrus species, unscrupulous players have been tempted to practice adulteration in citrus oils for a long time. Addition of key aromatic chemicals into low-grade stripped oil or oil fractions is one way to lower the oil cost. Extending high quality citrus oil with oil fractions from a cheaper source is also taking place on regular basis. As they become increasingly sophisticated, perpetrators are capable of making blends that are almost indistinguishable from authentic oils through conventional GC analysis. As a consequence, essential oil industry is demanding definitive, sensitive, and efficient approaches to grade commercial citrus oils and keep adulterated oil from entering the finished products.

A reversed-phase HPLC method was developed for compositional study of essential oils from major citrus species (i.e., orange, mandarin, tangerine, lemon, lime, and grapefruit). Majority of the oxygenated heterocyclic components in citrus oils were identified by HPLC-MS and confirmed by previous literatures. Two hydroxylated PMFs (polymethoxyflavone) have been identified from cold pressed orange and tangerine oils for the first time.

A comprehensive database of major PMF compounds is built with data collected from a large pool of industrial orange, tangerine and mandarin oil samples. Numerical ranges of PMF compounds for sample approval were extrapolated from the database. Meanwhile, principle components analysis (PCA) was carried out for sample classification. Based on the numerical limits and statistical analysis results, detailed information regarding the origin and history of oil samples can be revealed.

Similar study will be performed on lemon, lime and grapefruit oil samples as well. The author is hoping this analysis procedure will be serving as a routine quality control test for authenticity evaluation and adulteration detection in citrus essential oils.

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I. INTRODUCTION

Citrus essential oils have been gradually gaining popularity for the past century in flavor and fragrance industry. Such popularity rise is due to their globally accepted flavor profile and consumers' craving for naturalness. Regional flavor preference study showed that citrus flavor was the leading flavor group in most parts of the world (Wright, 2011). It is reasonable to anticipate that citrus flavor will continue to be the driving force in flavor industry's growth. Citrus essential oils, as the backbone of citrus flavors, will be in great demand judging by current trend. As a result, to ensure decent and consistent quality of citrus oils is a great challenge to quality control groups in flavor companies.

Unfortunately, unscrupulous players have been tempering citrus essential oils for long. It is not an easy task to bring forth an analytical approach which is effective in adulteration detection, given the fact that the perpetrators are equipped with as much knowledge as we are.

The non-volatile faction of citrus essential oils has been overlooked for quite a long while due to its insignificant contribution to the flavor profile of citrus oils. Recently there is a rise in the interest of polymethoxyflavones found in the non-volatile fraction of orange oil due to their proposed anti-inflammatory and anti-carcinogenic effects (Manthey *et al.*, 2001, Murakami *et al.*, 2004, Manthey *et al.*, 2002, Li *et al*., 2009, and Ho *et al*., 2012). Such components are regarded as non-volatile because regular GC conditions are insufficient to vaporize them. To the author's view, these non-volatile components can be the key in the development of an ideal analytical approach that the flavor industry has been demanding.

Unlike most other citrus oil studies in which samples were extracted in laboratories, this study focused on the industrial situation. All the samples in this study were of industrial origin and the sample pool was large. The goal of this study is to establish an efficient, sensitive and economical procedure which can be serving as a QC routine test for incoming citrus essential oils.

II. LITERATURE REVIEW

A. Essential Oil Overview

1. General Information

Chemically speaking, essential oils are concentrated hydrophobic liquids which are recovered from various elements of the named plants. The reason for them being "essential" is that they carry distinctive aroma essence from their starting plants. The "oil" in the name simply implies their hydrophobicity. Unlike fixed oils which are mainly composed of acyl-glycerols, essential oils do not significantly contribute to nutritional value.

Function of essential oils is still largely unclear: some may act as insect attractant for pollination purpose, others may protect the plant from parasites or herbivores. Prevailing opinion suggests that they are merely metabolism waste products with functional value rather coincidental (Reineccius, 2006).

Essential oil can be recovered from virtually any parts of the plant, often the one that carries most of the plant's aroma essence (Table 1). It is not unusual that more than one type of essential oils are recovered from different parts of a given plant (Figure 1). Essential oil classifications are commonly based on their odor profiles as shown in Table 2. There are no strict boundaries between scent types since essential oils are well-balanced flavors created by nature and cannot be defined accurately by one single descriptor.

Table 1. Representative essential oils from various elements of plants

Plant part	**Representatives**
Fruit	Orange oil, Nutmeg oil
Leave	Pine needle oil, Peppermint oil
Flower	Jasmine oil, Rose oil
Stem	Cinnamon bark oil, Cedarwood oil
Root	Ginger oil, Garlic oil
Bud	Clove bud oil
Seed	Carrot seed oil, Celery seed oil

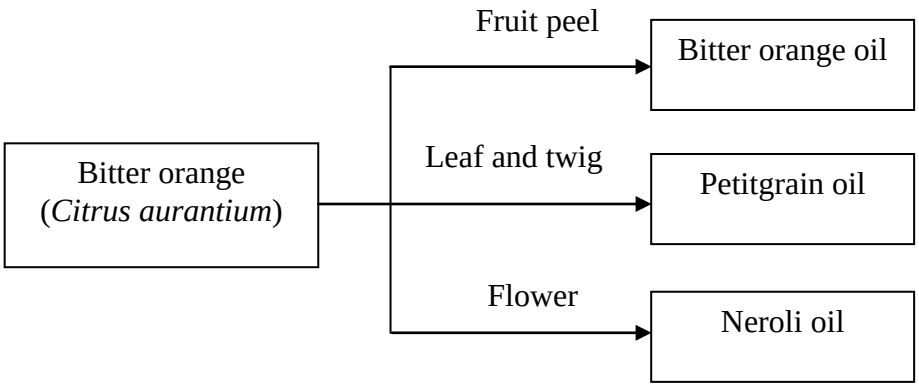


Figure 1. Three types of essential oils obtained from bitter orange plant

Table 2. Essential oil classification
(Information obtained from www.thegoodscentscompany.com)

Scent type	**Representatives**
Citrus	Orange oil, Lemon oil
Floral	Lavender oil, Jasmine oil
Herbaceous	Basil oil, Rosemary oil
Spicy	Nutmeg oil, Cumin oil
Woody	Cedarwood oil, Guaiacwood oil
Earthy	Patchouli oil, Ginger oil
Camphoraceous	Eucalyptus oil, Tea tree oil

2. Essential Oil Production Techniques

1) Distillation

Most essential oils from spices and herbs are obtained from distillation process thus should be nearly colorless. Due to the diversity of essential oil containing plants, it is impossible to generalize optimum distillation parameters for all. Depending on the nature of starting plant material, the technique of choice can be water distillation, water and steam distillation, or steam distillation (Reineccius, 2006). Vacuum is generally applied to lower the boiling points of volatile components to suppress deleterious heat induced chemical reactions.

2) Cold Expression

Citrus essential oils, which are different from spice or herb oils, are produced through cold mechanical pressing process therefore also known as “cold expressed oil”. Distillation is not chosen because of the low essential oil yield from citrus fruits and their large production volume. The mechanical pressing process results in a significant non-volatile fraction in citrus oils which is rarely seen in distilled oils. Such non-volatile fraction is important for the physical and chemical properties of citrus oils for the pigments and antioxidants they contain. Winterization is generally applied to citrus oil in order to remove excess wax content (Dugo *et al.*, 2002).

3) Solvent Extraction

Another common practice for obtaining essential oil is through solvent extraction. This method is advantageous when the starting plant material is too delicate to withstand high-temperature treatment (i.e., flower petal), or when a full, rounded profile that emphasizes on taste is required (i.e., ginger oil). Two types of solvent are generally used: polar solvents (mainly alcohol and water) and nonpolar solvents (mainly hexane and chlorinated hydrocarbons). The solvent of choice is determined by the components that are desired in the extract. Solvent removal process is usually carried out after extraction. Those prepared by non-polar solvents are known as oleoresins and those prepared by polar solvents are known as absolutes (Wright, 2011).

B. Citrus Essential Oil Overview

1. General Information

The global popularity of citrus fruits is resulted from their aroma, flavor, and nutritional value. Citrus fruits are well known as nature source of vitamin C. The

characteristic color of the fruits and their essential oils is derived from flavonoids and carotenoids. Their unique odor is due to the essential oils exist in the peel. The flavor of their juice is determined by the ratio of sugar to organic acids (mainly citric acid), and modified by the presence of aroma chemicals (Reineccius, 2006).

It is commonly acknowledged that the primitive citrus genetic pool was originated in South-Eastern Asia. Most famous citrus species known today are hybrids from their less known parent species (Table 3). Citrus fruits spread from Asia to the rest of the world following the path of civilization. Citrus trees were brought to America by the Spanish and Portuguese Conquistadores in the 16th century (Dugo *et al.*, 2002).

Table 3. Possible parent species for modern citrus species
(Dugo *et al.*, 2002)

Modern species	**Parent species**
Sweet orange (*Citrus sinesis*)	Pummelo x Mandarin
Bitter orange (*Citrus aurantium*)	Pummelo x Mandarin
Lime (*Citrus aurantifolia*)	Citron x Papeda
Lemon (*Citrus limon*)	Citron x Papeda
Kumquat (*Fortunella spp*.)	Mandarin
Grapefruit (*Citrus paradise*)	Pummelo

Citrus essential oils are less expensive than most other essential oils due to the low cost to grow and harvest the fruit. Among essential oils, the production volume of citrus oils has always been the greatest worldwide. Essential oils with production

volumes over 1000 tonnes were listed in Table 4. Production figures for individual citrus species were shown in Table 5.

Table 4. Annual production volumes of major essential oils
(Perfumer & Flavorist, 2009)

Essential Oil	**Tonnes**
Sweet orange	51000
Cornmint	32000
Lemon	9200
Eucalyptus	4000
Peppermint	3300
Clove leaf	1800
Citronella	1800
Spearmint	1800
Cedarwood	1650
Litsea cubeba	1200
Patchouli	1200
Lavadin Grosso	1100
Corymbia Citriodora	1000

Table 5. Annual production volumes of major citrus oils
(Wright, 2011)

Citrus Oil	**Tonnes**
Sweet orange	51000
Lemon	9200
Lime Distilled	1800
Grapefruit	700
Mandarin	460
Tangerine	300
Bergamot	200
Bitter orange	30

2. Application in Flavor Industry

Citrus essential oils form the backbone of their respective flavors. Unlike other fruit flavors which could be achieved solely from chemical blending, it is almost impossible to create a promising citrus flavor without referring to its essential oil or oil fraction. The essential oil base offers fresh note and rounded profile to citrus flavor, which would otherwise be perceived as spiky or unsophisticated. Citrus oils and most of their fractions are regarded as natural and Kosher therefore make their derived citrus flavors regulatory friendly and sell in every region of the world.

Citrus flavor has been and will continue to be the leading flavor group in most parts of the world. Table 6 (Wright, 2011) lists the top fifteen regional welcomed flavors in representative countries from different global regions (North America, South America, Asia, Europe, and Mid East). Despite the huge cultural difference among regions, citrus flavor (bolded in the Table 6) took the lead in all five countries. Regional preference within citrus group was self-explanatory from the table and might serve as a starting point for flavor companies who are tying to enter those foreign markets.

Table 6. Top fifteen flavors in major countries over the world in 2011 (Wright, 2011)

Rank	**USA**	**Argentina**	**China**	**Italy**	**Israel**
1	Vanilla	**Orange**	**Orange**	**Orange**	Vanilla
2	Strawberry	**Lemon**	Milk	Vanilla	Chocolate
3	**Orange**	Chocolate	Strawberry	**Lemon**	Cheese
4	Cream	Strawberry	**Lemon**	Cheese	Strawberry
5	Chocolate	Cheese	Mint	Mint	Cream
6	**Lemon**	Cream	Tea	Cream	**Lemon**
7	Raspberry	Vanilla	Chicken	Strawberry	Coffee
8	Cheese	Apple	Chocolate	Chocolate	Mint
9	Cherry	Peach	Apple	Butter	Chicken
10	Chicken	**Grapefruit**	Beef	Apple	Peach
11	Butter	Pineapple	Vanilla	Peach	Caramel
12	Mint	Mint	Peach	Raspberry	**Orange**
13	Apple	Raspberry	Pineapple	Chicken	Raspberry
14	Peach	Banana	Coffee	Cherry	Beef
15	**Lime**	Butter	Mango	Onion	Banana

Citrus essential oils find their biggest application in beverages. In United States more than 80% of soft drinks are citrus flavored (Dugo *et al.*, 2002). Citrus flavor became the most welcomed beverage flavor due to their "fresh juicy sweet" note that is appreciated across the globe. Based on lime distilled oil and lemon oil, cola flavor is an important branch of citrus flavor which is more appreciated domestically. Globally orange flavor takes the lead among citrus flavors (Table 8).

Table 7. World usage of flavor and fragrance materials of natural origin (US$ million) (Weiss, 1997)

Aromatic materials	**1984**	**1994**
Fragrance compounds	2000	3000
Flavor compounds	2000	3000
Aromatic chemicals	1000	1600
Essential oils	1000	1400
Total	6000	9000

Table 8. Estimated annual usage of citrus oil in beverages (tonnes) (Weiss, 1997)

Oil	**USA**	**International**
Lemon/Lime	850	1350
Orange	400	1900
Cola	1040	900
Others	250	750
Total	2540	4900

Most of the citrus beverages are in the form of cloudy emulsion due to the dominating amount of hydrophobic terpenes in citrus oils. Some clear citrus drinks can be found on market such as lemonades, which are made from deterpenized oils. Detailed information on terpene removal from citrus oil is discussed elsewhere and is not the focus of this study.

The second most important application of citrus essential oils is in confectionary. Approximately 20% of the confectionary is citrus flavored (Dugo *et al.*, 2002). Citrus flavored hard candies and gummy candies are enjoyed by both children and adults.

Citrus oils are utilized in other food applications such as ice cream, chewing gum, bakery and sauce. Meanwhile they are valuable raw materials in fragrance as well. Citrus oils are routinely used to scent skin, hair and facial care products, perfumes and colognes, toiletries, and also in aromatherapies.

3. Major Citrus Species

1) Sweet Orange Oil (*Citrus sinensis*)

Sweet orange oil is commonly referred to as orange oil since the literally opposite bitter orange oil (*Citrus aurantium*) is less known and consumed. Within the citrus genus, sweet orange oil has the greatest production volume and popularity worldwide. It has an annual production volume of around 51000 tonnes with Brazil and the United States as the leading producers (Wright, 2011).

Decanal (0.4% of volatile fraction), linalool (0.5% of volatile fraction) and valencene (0.05% of volatile fraction) are important markers in determining the grade of orange oil. The first two chemicals can be easily obtained with minimal cost therefore

were frequently added into low grade orange oils to boost up their values. Same as most other essential oils from citrus genus, *d*-limonene is the most abundant component which accounts for over 95% of the volatile fraction.

In order to improve the aqueous solubility of orange oils, they are frequently washed or folded to form the backbone of water soluble orange flavors. *D*-limonene, as the major byproduct of these processes, has important applications outside the flavor industry (Xu *et al.*, 2004).

2) Bergamot Oil (*Citrus bergamia*)

Bergamot essential oil is the most expensive one among citrus oils and as a result it has been frequently adulterated. Only 200 tonnes are produced annually, mainly in Italy and the Ivory Coast. It is the main component of top quality Earl Grey tea flavors and can be helpful as a minor components in some natural fruit flavors. Most bergamot oil is consumed in fragrances, especially eau de cologne types (Wright, 2011).

Linalyl acetate (30%-35% of the volatile fraction) is the key component that distinguishes bergamot oil from the rest citrus oils. Bergamot oil is a skin sensitizer because it contains 0.2% of bergaptene. Distillation is the common practice carried out to remove the bergaptene fraction which violates mainstream fragrance regulations.

3) Mandarin Oil (*Citrus reticulata*)

World production is around 460 tonnes, mainly from Italy and Brazil (Wright, 2011). Depending on the origin and harvest season, the appearance of this oil ranges from green to yellow to red. Mandarin oil is widely used alone or in combination with orange oil in beverages and confectionary.

The existence of methyl *N*-methyl anthranilate (dimethyl anthranilate or DMA, 0.8% of volatile fraction) and thymol (0.1% of volatile fraction) distinguishes mandarin oil from sweet orange oil. Comparing to sweet orange oil, mandarin oil contains a much higher level of non-volatile faction with tangeretin as the major constituent. Like orange oil, concentrated or terpeneless mandarin oils are often produced and used in beverages.

Mandarin oil is sometimes confused with tangerine oil whose odor and GC profiles are much closer to sweet orange oil. Tangerine oil is not as popular as mandarin oil in that the blend of one part of mandarin oil with nine parts of sweet orange oil often offers a better effect with less flavor cost.

4) Lemon Oil (*Citrus limon*)

Among citrus oil, lemon oil has the second largest production volume (9200 tonnes annually) with Argentina as the main producers followed by United States and Italy (Wright, 2011). Lemon oil is widely used in lemon and other natural flavors. It blends well with other citrus oil, especially lime oil to form the backbone of cola flavor.

Citral (2.5% of volatile fraction) is the characteristic compound in lemon oil. The quality of winter oils are better than those produced in summer and demand higher prices. Lemon oil is extreme prone to oxidation. The level of *para*-cymene (oxidation product of γ-terpinene) is an important indicator of the oxidation stress a lemon oil sample has undergone.

5.) Lime Oil (*Citrus aurantifolia*)

Key Lime oil (*Citrus aurantifolia*) has an annual production volume of 160 tonnes mainly from Brazil (Wright, 2011). Most of lime oil is used in lemon-lime soft drinks, where it offers a distinctive fresh character. Persian lime oil (*Citrus latifolia*), also known as Tahiti lime oil, is close to key lime oil but with a unique spicy, fragrant aroma due to its higher citral and γ-terpinene level.

Citral (5% of volatile fraction) is the major characteristic compound in key lime oil. γ-Terpinene (8% of volatile fraction) and a much higher level of non-volatile faction make lime oil different from lemon oil.

Lime distilled oil is significantly different from cold-pressed oil because of the different process. Much of the citral is lost during distillation and as a result the peely character is replaced by a piney/lilac character (*α*-terpineol, 7% of volatile fraction; borneol, 0.5% of volatile fraction; 1, 4-cineole, 3% of volatile faction; 1, 8-cineol, 2% of volatile faction) which is typically recognized as lime. Lime distilled oil has an annual production of over 1800 tonnes mainly from Mexico, Peru and Haiti (Wright, 2011).

The major application of lime distilled oil is in cola and lemon-lime drinks. However it is gradually being replaced by cold-pressed lime oil for a fresher, less piney effect.

6) Grapefruit Oil (*Citrus paradisi*)

Brazil and the United States are main producers that together give a 700 tonnes annual production volume (Wright, 2011). The main use of the oil is in grapefruit flavored soft drinks and confectionery. Like orange and mandarin oil, grapefruit oil is often concentrated to improved stability and solubility.

Nootkatone (0.2% of volatile fraction) and *para*-1-methene-8-thiol (trace) are the components that distinguish grapefruit oil from sweet orange oil. The pricing of grapefruit oil is generally proportionally to the level of nootkatone and low nootkatone oil often smells distressingly like sweet orange oil. Due to the high cost of authentic grapefruit oil, combination of sweet orange oil and grapefruit oil is often used to reduce the cost.

7) Tangerine Oil (*Citrus tangerina*)

Brazil is the largest producer of the world's 300 tonnes annual production volume (Wright, 2011). This oil is frequently concentrated for improved solubility and stability. It is used in a wide range of natural flavors from orange to mango.

Decanal (0.4% of volatile fraction), α-sinensal (0.05% of volatile fraction) and thymol (0.3% of volatile fraction) are the characteristic components in tangerine oil. The quality of tangerine oil varies drastically and some low grade oils are extremely close to sweet orange oil. Despite the higher price, mandarin oil is often a much better raw material for natural flavors.

8) Bitter Orange Oil (*Citrus aurantium*)

Around 30 tonnes are produced annually, mainly in the West Indies and Brazil (Wright, 2011). Characteristic components are decanal (0.2% of volatile fraction) and linalyl acetate (0.2% of volatile fraction). This oil is generally used to modify sweet orange flavors and as a key ingredient in "Indian Tonic" beverage flavors. It is also seen in other natural flavors such as mango and peach.

C. Citrus Essential Oil Production

1. Production Theory and Overview

Citrus essential oils are industrially cold-extracted from the peel of sweet orange, lemon, grapefruit, mandarin, tangerine, bitter orange, bergamot, and clementine by mechanical systems. Lime essential oil, as an exception, is often extracted from whole lime fruits (Reineccius, 2006). The extraction of essential oil by squeezing the peel dates back to 1700's (Dugo *et al.*, 2002). As opposed to other types of fruit whose homogeneity facilitate their industrial use, citrus fruits suffered from slow development of machineries specialized in producing their derivatives. This was mainly due to the complexity and variability in different layers of structure observed in citrus fruits.

For all citrus fruits, the essential oil is contained within numerous oval, balloon-shaped oil glands that buried just below the colored portion of the peel (flavedo). The white-colored albedo does not contain any oil, but does carry bitter glycoside compounds such as hesperidin in orange, lemon, or naringin in grapefruit (Reineccius, 2006).

With the exception of lime oil, the citrus peel oils are obtained by mechanical extraction which varies depending on the nature of the fruit to be handled. Lime oil is generally obtained as a by-product of lime juice and is recovered by distillation the acid liquors from crushing the whole fruit (Reineccius, 2006). Cold-pressed lime oil is also produced which possesses a fresher quality than distilled oil.

Other parts of citrus plant also give essential oil when distilled. Petitgrain oil (from citrus leaves and twigs) and neroli oil (from citrus flowers) are of particular importance in fragrance applications. The most widely used oils from this category are

derived from bitter orange tree (*Citrus aurantium*) and are designated “bigarade”, i.e. “oil of peptitgrain bigarade” or “oil of neroli bigarade” (Reineccius, 2006).

The cross-section view of a typical citrus fruit can be seen in Fig 2. The oil glands containing essential oil are found beneath the flavedo. These glands are not walled but surrounded by cells containing an aqueous solution rich in salts and sugar. When the peel comes into contact with water, these cells absorb water and become distended, putting their vicinal oil glands under pressure. At this moment, if the peel is subjected to a mechanical process such as pressing or grinding, these oil glands rupture and as a consequence essential oil spurts out. Because the tissues around oil glands are spongy and ready to reabsorb freed essential oil, the mechanical process is always carried out under strong jets of water to wash away oil right away before it gets reabsorbed by the peel (Dugo *et al.*, 2002).

None of the existing extraction methods are capable of recovering the total quantity of oil. The maximum attainable yield of oil depends on the extraction methods as well as the nature of the starting fruits. The thickness of the peel is a major determinant in that thick-skinned fruits yield less oil than thin-skinned fruits, due to the absorption of the liberated oil by the spongy albedo. The time interval between harvesting and processing is also important as flaccid peel yields less oil (Reineccius, 2006). The typical average yields from major citrus fruits are shown as following in Table 9:

Table 9. Typical yields of citrus essential oils from their fruits
(Reineccius, 2006)

Fruit	Lemon	Orange	Grapefruit	Lime	Tangerine
Yield (lb./ton)	2-7	1-8	1-2	0.1-0.3	1-2

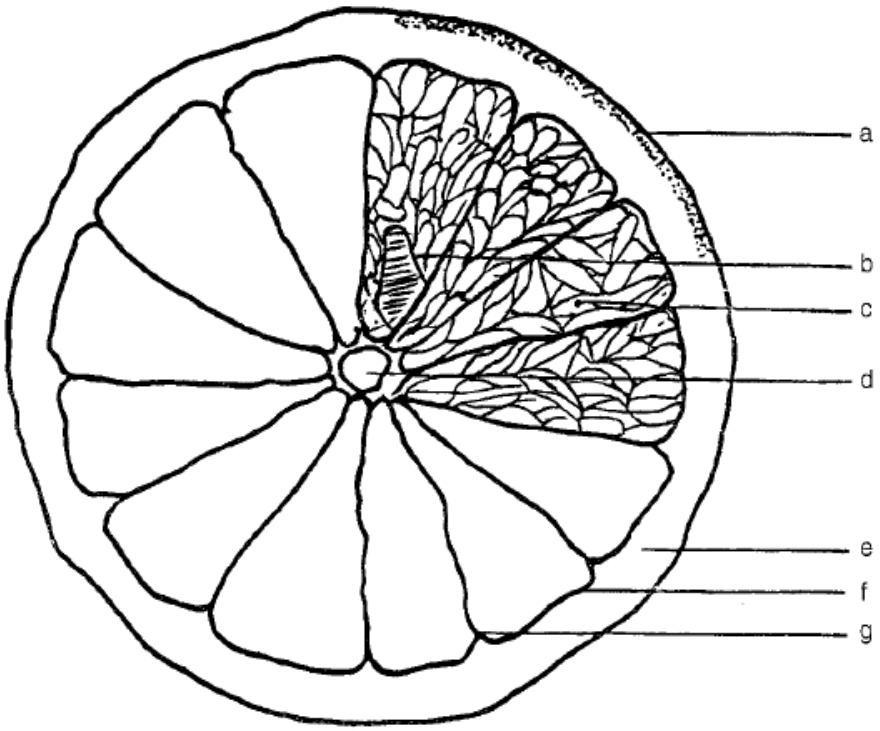


Figure 2. Cross section of a citrus fruit (a) flavedo (b) seeds (c) juice vesicles (d) central axis (e) albedo (f) segment (g) segment membrane
(Dugo, G., and Giacomo, A. *Citrus – the Genus Citrus*. P114)

After being harvested by trained personnel, the citrus fruits reach processing plant and a representative sample from each batch is send to the laboratory to assess the yield for both essential oil and juice. The fruits are then pre-washed by sprays of germicidal solution contains 220 ppm chlorine. A final wash using water is followed to eliminate contaminants. Right before extraction, all fruits must be sized in order to obtain maximum efficiency of the extractor and an optimum yield (Dugo *et al.*, 2002).

2. Processing

The mechanical cold expression of citrus peel consists of three fundamental steps, regardless the technology used (Dugo *et al.*, 2002):

1. Mechanical action on the peel, in order to cause oil gland breakage and the consequent release of the oil

2. The oil is carried by streams of water, which is often recycled

3. Separation of the oil from aqueous emulsion by means of centrifugation

Based on the order in which juice and oil get extracted, the first step can be branched into three variations:

1. Essential oil extraction from the whole fruit, preceding juice extraction

2. Juice extraction preceding that of essential oil

3. Extraction of essential oil and juice take place simultaneously

Extraction machineries built under the first two principles can still be seen worldwide, especially in small scale plants where high grade oils are expected. In United States, the most representative machinery applied for oil and juice extraction is FMC

system (FMC Food Machinery, San Jose, CA, USA), which employs the whole fruit extraction principle and separates various parts of fruit simultaneously. This simultaneous extraction is considered to be economically advantageous in that it allows a seamless, continuous production flow, at a small cost of oil quality.

FMC whole fruit extractor was introduced in 1947. It does not only extract the juice, but simultaneous squeezes oil emulsion from the peel, without juice and essential oil coming into contact. In the FMC system the juice and the oil emulsion are conveyed separately in the in-line process, with seeds and pulp pass down a spiral channel. This extractor has been under continuous mechanical improvement. In 1983 the American Society of Mechanical Engineers named this extractor “An international historical engineering landmark” (Dugo *et al.*, 2002).

During the extraction process the peel is disrupted which causes the oil glands to burst and release oil inside. Water is continuously introduced though specially designed sprays ring to wash away liberated oil together with small pieces of peel to form an emulsion called oil slurry. It is important that oil released from oil glands be washed away to prevent the oil from being reabsorbed by the peel (Dugo *et al.*, 2002). A cross section view of the separator in FMC was shown in Figure 3.

The recovery of essential oil from the emulsion obtained from the extraction is a crucial step in the processing cycle. Centrifugation is employed in FMC process for its high yield and speedy separation. The water-essential oil emulsion sequentially goes through a vibrating screen to filter the discharge emulsion, a centrifugal clarifier to concentrate the emulsion, and a centrifugal polisher to achieve a complete separation of

the essential oil from aqueous phase. The latter is then recycled into circulation and re-sprayed through the sprays ring to form another emulsion (Dugo *et al.*, 2002). An overall flow chart on the operation of FMC extractor is provided in Figure 4.

3. Preservation and Storage

Atmospheric oxygen, heat, light, and traces of moisture, acidity, and metallic ions are known to cause deleterious alteration to citrus oil. Oxygen has direct impact on citrus oil in terms of oxidation of terpenes, metallic ions can serve as catalyst of auto-oxidation, light affects the color, water causes hydrolysis of certain components, and heat normally accelerates all the processes above (Dugo *et al.*, 2002). Therefore appropriate preservation methods and storage conditions must be established for citrus oils.

First and foremost, all citrus oil must be stored in full-filled steel (or aluminum) drums or containers in a cool and dry place. Polymer-coated drums are not recommended for the presence of terpenes. Water and acids must be removed by anhydrous sodium sulfate and sodium bicarbonate before storage. It is also a common practice to remove excess wax from citrus oils by winterization. Addition of antioxidants may be considered but the regulatory restrictions of the destiny country must be consulted first.

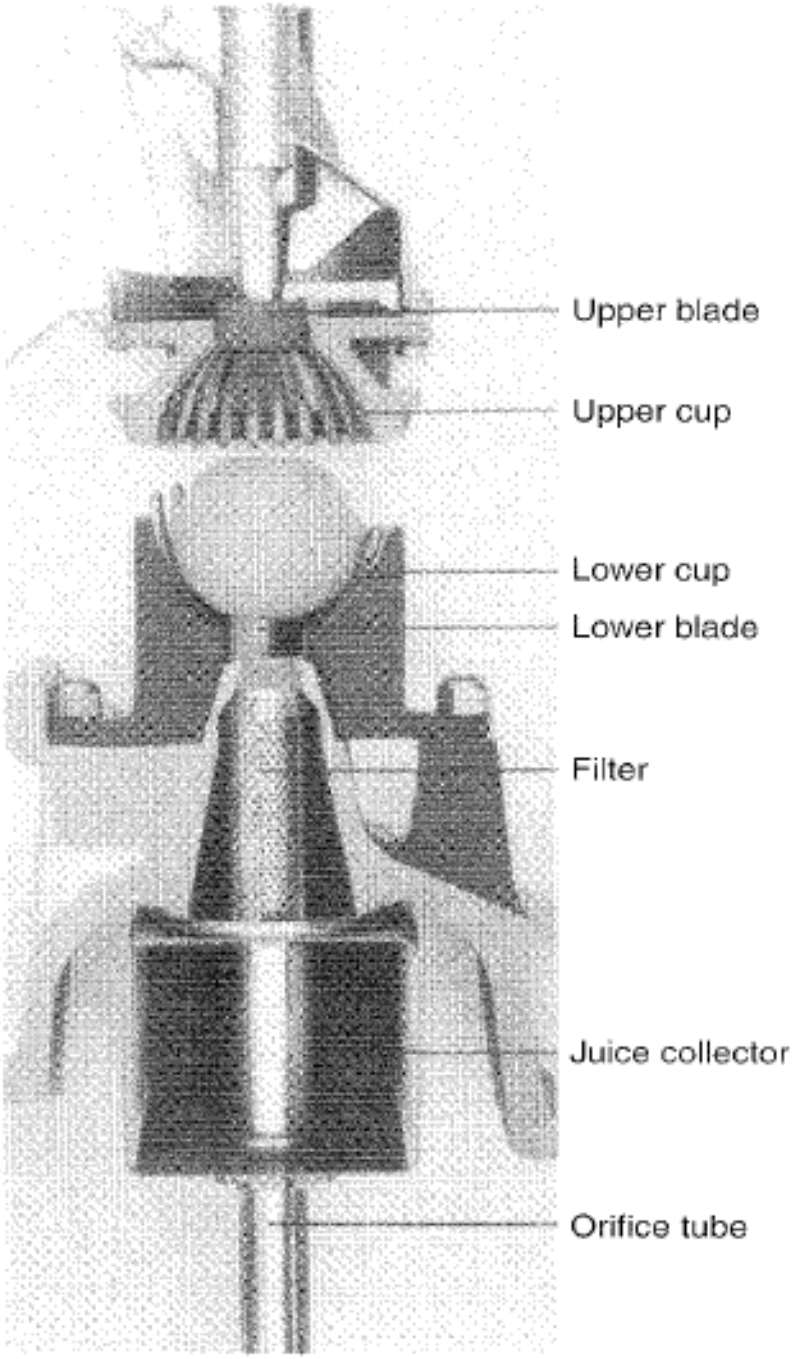


Figure 3. Cross section view of FMC kernel part
(Dugo, G., and Giacomo, A. *Citrus – the Genus Citrus*. P131)

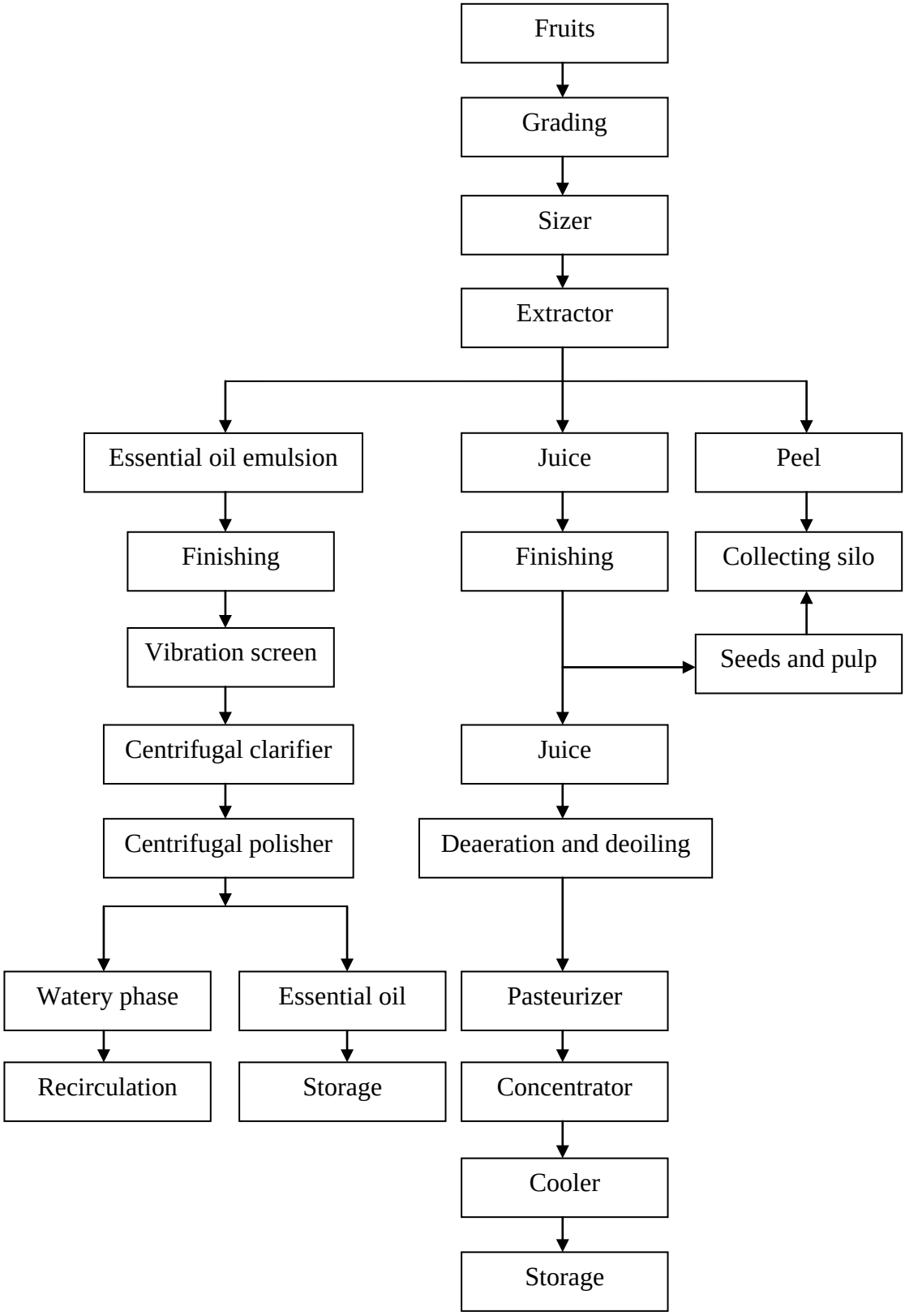


Figure 4. FMC operational flow chart

4. Factors that Influence Oil Quality

There are a number of factors to be taken into consideration in order to maintain a consistent and premium quality of citrus essential oils. Not only the quality of the starting fruit, but also processing factors determine the grade of citrus oils.

1) Variety, Maturity and Storage of the Fruits

The genetic differences within cultivars naturally lead to composition differences in the resulting essential oils. The maturity stage of the fruit can be reflected in its essential oil composition in that a good number of the components have glycosidic origins therefore the levels are heavily regulated by metabolism. Post-harvest condition also affects the quality of the essential oils due to a range of physical changes and chemical reactions that can be induced by storage parameters.

2) Treatment of the Peel

The essential oil composition can be profoundly influenced by the treatment of the peel after juice extraction. If the juice extraction takes place first, then the peel is commonly soaked in a solution of milk of lime for several hours. Such soaking treatment, if allowed to go for too long, can cause poor yield of the essential oil and hydration of sabinene to form terpinen-4-ol (Dugo *et al.*, 2002). In FMC process where the juice and essential oil are extracted simultaneously, such influence is minimized.

3) Extraction Technique

Comparing to a light extraction method, an exhaustive extraction method usually gives a larger quantity of high-boiling components, which causes the resulting oil to have higher non-volatile residue and specific gravity.

Water is applied in the extraction process to wash the oil away from the peel surface. Water also plays as a solvent to the oxygenated compounds (mainly alcohols, aldehydes, and esters) which directly contribute to the aroma of the oil. The hydrophilic moieties in these compounds increase their partition ratio in the aqueous phase when emulsion separation takes place (Reineccius, 2006). As a result, the amount of water that circulates during oil extraction can largely affect the composition of oil. In order to produce citrus oil with high aldehyde content, the amount of water should be minimized to just necessary to wash away the essential oil liberated.

D. Chemical Composition of Citrus Essential Oil

Citrus essential oils are complex natural mixtures of a wide range of compounds from diverse chemical groups. To date more than 200 components have been successfully identified from citrus essential oils (Dugo *et al.*, 2002) and this number is still growing. All these components can be divided into two subgroups: a volatile fraction and a non-volatile fraction. The criterion for their volatilities is under regular GC conditions (i.e., max 320 °C). Figure 5 showed the two main fractions of citrus oil and their sub groups.

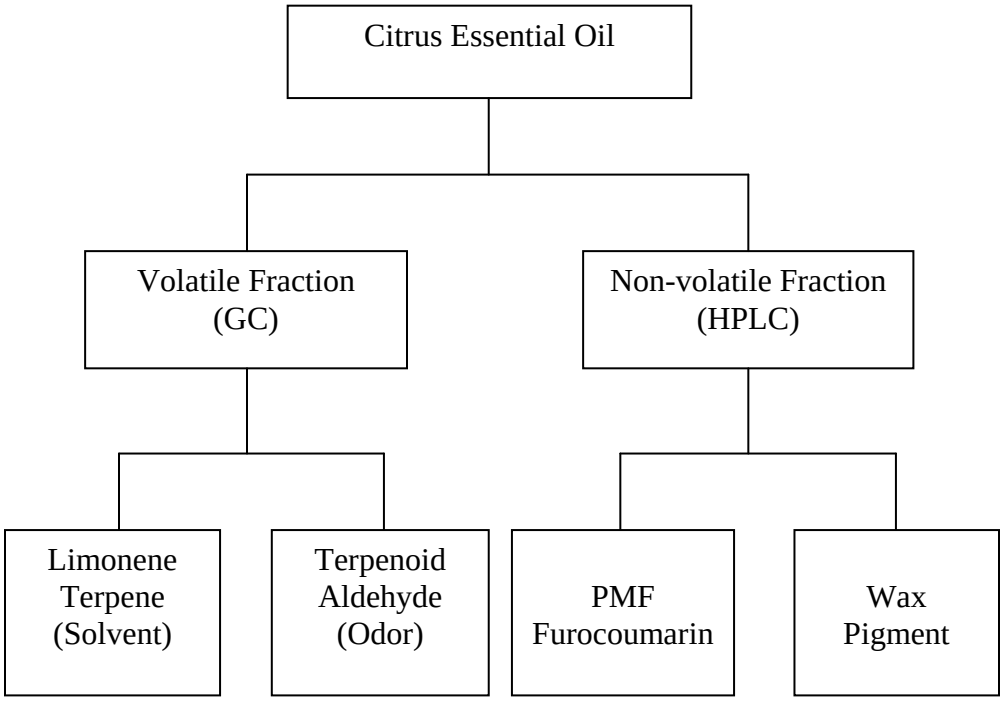


Figure 5. Typical citrus oil composition

1. Volatile Terpene and Terpenoid Fraction

The volatile fraction is responsible for 85-99% of the whole oil on weight basis. The majority of this fraction is short-chain alcohols, aldehydes, esters, acids, terpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), and their corresponding terpenoids (oxygen containing derivatives). Representative component in this group were listed in Table 10. There are also trace levels of sulfur and nitrogen containing compounds which contribute to the aroma character of citrus oils. It was the development of GC and capillary column technique that make comprehensive compositional analysis possible. Because of the complex natural of the citrus oils, it is often difficult to fully resolve every peak in a single GC analysis. As a result pre-fractionation before GC analysis and multidimensional GC analysis became the methods of choice if detailed compositional information is required.

Majority of the identified volatile compounds are common among citrus species. Also there are a few “marker compounds” in this fraction which was found to exist in certain species exclusively. Their appearance in other species might be indicative of inter-species adulteration. One example is that presence of significant level of δ-3-carene in lemon oil suggests orange fraction has been blended in (Dugo *et al.*, 2002).

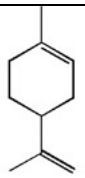
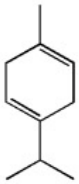
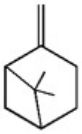
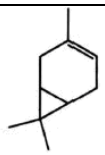
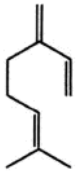
For the majority of the volatile components, their odor contributions are not proportional to their relative abundance. Hydrocarbon compounds constitute an overwhelming proportion in citrus oils, yet they contribute very little to the “citrus note” as one might expect. Citrus terpenes give a refreshing, clean perception which makes them ideal in cleaning products and toiletries. Because of their extremely hydrophobic

nature, terpenes are responsible for the poor aqueous solubility observed in citrus oils. Terpenes are unsaturated hydrocarbons that are prone to oxidation, which could lead to shortened shelf life of citrus oils if stored inappropriately. Functionally speaking terpenes serve as natural solvent that dissolves the rest of the components which have greater contribution to the odor profile. Other functions of the terpenes are yet to be discovered.

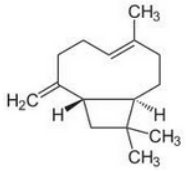
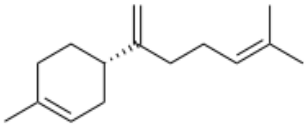
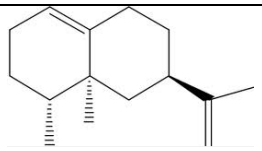
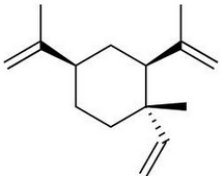
Efforts have been made to remove the hydrocarbon fraction from citrus oils for improved solubility and stability. Terpene reduction can be achieved by fractional distillation, solvent washing, and chromatography fractionation. The concentrated oils are known as folded oils (partial terpene removal) or terpeneless oils (nearly complete terpene removal). Such oils generally find much wider application especially in food industry comparing to unfolded oils. The major drawback is that these concentrated oils tend to lost their freshness and develop somewhat unbalance profiles, due to the removal of *d*-limonene and other low boiling volatiles (Fleisher, 1994).

The aroma of citrus oil is mainly characterized by aldehydes, alcohols, esters, and trace amounts of sulfur or nitrogen containing compounds. Such trait distinguishes citrus oils from non-citrus essential oils whose flavor profiles are usually defined by their abundant components (such as of *tr*.-cinnamic aldehyde in cassia oil, or anethole in sweet fennel oil). In citrus oils some of the aroma chemicals exist in such trivial quantities (sub ppb level) and pose great challenge to researchers trying to identify and isolate them. It is also these less abundant components that define the unique flavor profiles of each citrus species. During terpene removal process most of these components get enriched and makes the concentrated oils possess increased flavor strength.

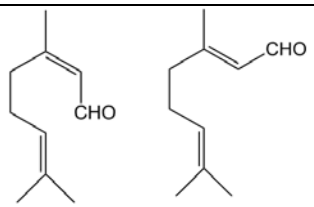
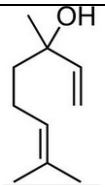
Table 10. Representative volatile components in citrus essential oils

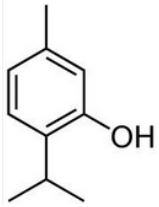
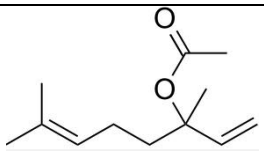
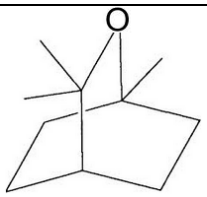
Compound	Structure	Major sources
d-limonene		All kinds of citrus oils
γ-Terpinene		Lemon oil Lime oil Mandarin oil
β-Pinene		All kinds of citrus oils
δ-3-carene		Orange oil
Myrcene		Orange oil Grapefruit oil

Terpenes $C_{10}H_{16}$

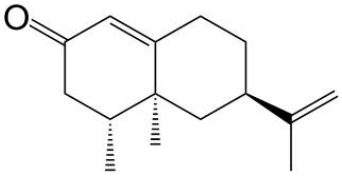
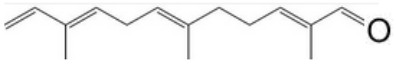
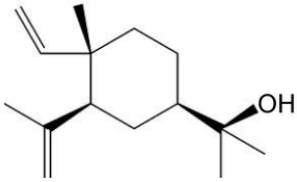
Compound	Structure	Major sources
β-Caryophyllene		Orange oil Grapefruit oil
β-Bisabolene		Lemon oil Lime oil
Valencene		Orange Oil
β-Elemene		Grapefruit Oil

Sesquiterpenes $C_{15}H_{24}$

Compound	Structure	Major sources
Citral		Lemon oil Lime oil
Linalool		All kinds of citrus oils

Thymol		Mandarin oil
Linalyl acetate		Bitter orange oil Bergamot oil
1,8-Cineole		Lime oil

Terpenoids

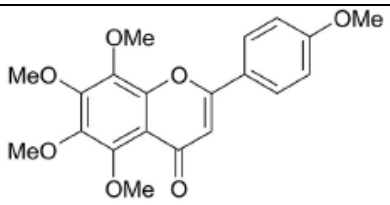
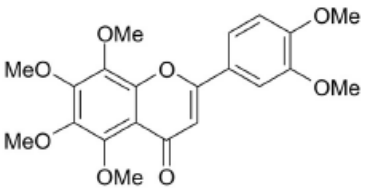
Compound	**Structure**	**Major sources**
Nootkatone		Grapefruit oil Orange oil
α-Sinensal		Orange oil Tangerine oil
Elemol		Grapefruit oil Orange oil

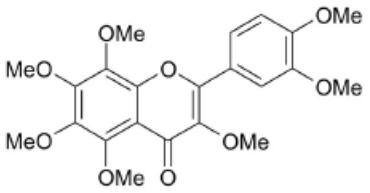
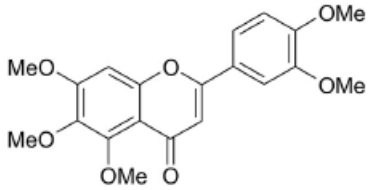
Sesquiterpenoids

2. Non-volatile Oxygenated Heterocyclic Compounds Fraction

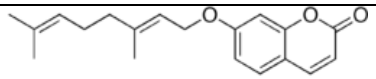
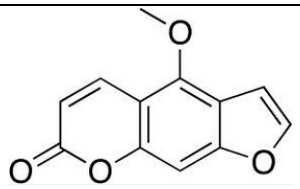
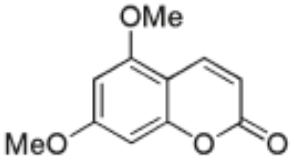
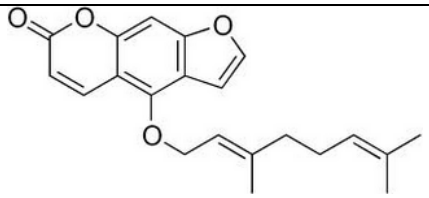
It has been frequently observed that cold-press citrus oils, especially under refrigerated condition, can form crystallized sedimentation during prolonged storage. This is direct evidence that citrus oils contain non-volatile components beyond the volatile fraction. The non-volatile fraction in citrus essential oils ranges between 1% in some sweet orange oil, and 15% in key lime expressed oil. This fraction is composed of long chain hydrocarbons, fatty acids, sterols, carotenoids, and oxygenated heterocyclic compounds (furocoumarins and polymethoxyflavones). Comparing to the volatile fraction, the non-volatile faction in citrus oils was much less explored for multiple reasons. However, the oxygenated heterocyclic components are gradually gaining attention due to their biological activity and role in authenticity analysis. Representative components in this group were outlined in Table 11.

Table 11. Representative non-volatile components found in citrus essential oils

Compound	Structure	Major sources
Tangeretin		Orange oil Mandarin oil Tangerine oil
Nobiletin		Orange oil Mandarin oil Tangerine oil

3,5,6,7,8,3',4'-Heptamethoxy flavone		Orange oil Mandarin oil Tangerine oil
Sinensetin		Orange oil Mandarin oil Tangerine oil

PMF

Compound	Structure	Major sources
Aurapten		Grapefruit oil
Bergapten		Bergamot oil
Citropten		Lemon oil Lime oil
Bergamottin		Lemon oil Lime oil

Coumarins and Furocoumarins

Because of their non-volatile nature, studies on oxygenated heterocyclic compounds are usually carried out by normal or reversed phase HPLC with ultraviolet or fluorescence detectors. Recently the development of MS interface technology has allowed on-line coupling of LC and MS, and affords a very powerful technique for the identification of natural components.

Opposed to the similar patterns of volatile components from different citrus oils, the non-volatile components are much more species-specific. Orange, mandarin, and tangerine oil contain exclusively PMFs while lemon and lime oil solely comprise of furocoumarins. In grapefruit oil and bitter orange oil, both PMFs and furocoumarins have been identified (Figure 6). Therefore oxygenated heterocyclic components can serve as markers in revealing inter-species adulteration. Table 12 listed important non-volatile components from major citrus species.

Table 12. Crucial non-volatile compounds from various citrus species

	Binominal name	**Major non-volatiles**
Sweet Orange Oil	*Citrus sinensis*	Tangeretin, Hepta PMF
Bergamot Oil	*Citrus bergamia*	Bergamottin, Bergapten
Mandarin Oil	*Citrus deliciosa*	Tangeretin, Nobiletin
Lemon Oil	*Citrus limon*	Citropten, Bergamottin, 5-Geranyloxy-7-methoxycoumarin
Lime Oil	*Citrus aurantifolia*	Citropten, Bergamottin, 8-Geranyloxypsoralen
Grapefruit Oil	*Citrus paradisi*	Aurapten, Epoxybergamottin

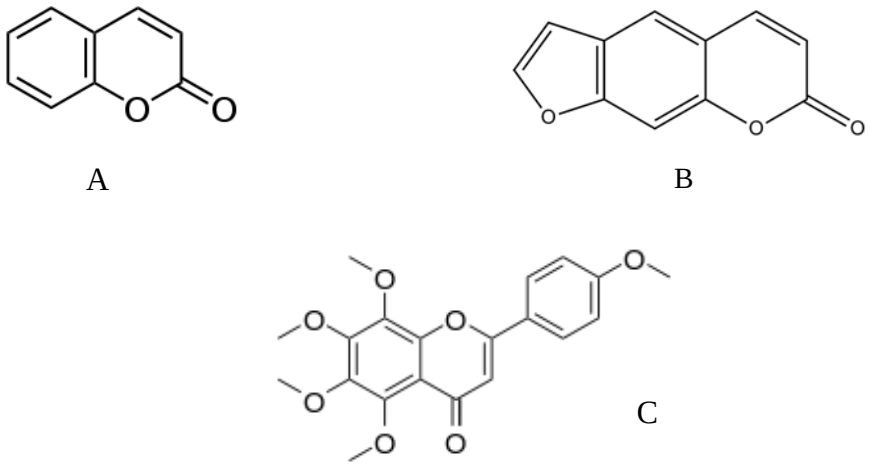


Figure 6. Crucial non-volatile compounds in essential oils
A: Coumarin; B: Furocoumarin; C: Polymethoxyflavone

3. Comparison between GC and HPLC in Adulteration Study

The development of GC technique has been serving as a two-edged sword to the industry: On one hand, buyers are able to protect themselves against falsified oil products with improved knowledge. On the other hand, such knowledge could also be taken advantage of by perpetrators in development of more sophisticated practices. One practice that has long been carried out is to extend genuine oil with washed oil or terpene fractions from the same or cheaper sources, followed by adding key aroma chemicals (citral, linalool, DMA, *etc*.) back to the blend to compensate for their dilution losses. The trivial costs of aroma chemicals nowadays have exacerbated such situation. A sophisticated producer is able to make oil blends that conform to the specification of genuine oil in terms of GC profile and physical properties while still keep an enticing profit. As a result, though GC is still used to screen citrus oils in flavor houses, it has been proved insufficient in assessing authenticity of essential oils as adulterators become increasingly skillful.

From a practical point of view, HPLC analysis bears several advantages over GC analysis when employed for citrus oil adulteration studies.

First and foremost, citrus oils are more “prone” to volatile fraction adulteration than non-volatile fraction adulteration. Citrus oils are valued due to their unique aroma, which is mainly conferred by their volatile fractions. The non-volatile factions, due to their low volatilities, can only contribute moderately to the taste of the citrus oils. It is obvious that adulterators would choose not to invest their time and resources on non-volatile fraction which has little impact on the odor of citrus oils.

Secondly, the GC profiles of citrus oils have long been established and are readily accessible to both buyers and producers. Most of the crucial volatile components are well studied and can be purchased from chemical plants or flavor houses at only a fraction of the oil price. Such fact has unintentionally encouraged perpetrators to reconstitute their diluted or extended oils with aroma chemicals in order to pass through GC screen. The non-volatile components, on the contrary, are structurally more complicated therefore difficult to synthesize. As a result, the oxygenated heterocyclic compounds are either commercially unavailable or can only be purchased at formidable prices compared to citrus oils themselves. Thus it is not possible or economically feasible for adulterators to perform the same reconstitution trick on the non-volatile fraction as they are doing to the volatile fraction.

Lastly, as discussed before, comparing to the distribution of volatile fractions among species, the distribution of non-volatile fractions is much more species-specific. As a result HPLC analysis has a much lower tolerance to inter-species adulteration in

citrus oils. Table 13 illustrated the huge price differences among citrus species from the past few years. Inter species adulteration favors similarity in composition profiles, and non-volatile fraction analysis can be the right tool to stop it.

Table 13. Price comparison among citrus oils ($/KG)
(Data obtained from Flavor Materials International)

	2011	**2012**	**2013**
Bergamot	125.00	185.00	185.00
Lemon	6.20	4.10	3.30
Lime	26.25	23.50	13.00
Grapefruit	3.40	4.0	7.00
Orange	1.80	3.50	3.50

E. Literature Review on Oxygenated Heterocyclic Compounds from Citrus

Researcher first realized the presence of the non-volatile fraction in citrus oil from the residue left on distillation and from the precipitation at the bottom of citrus oil when stored refrigerated. It was the isolation of crystalline bergapten from bergamot oil (Pomerantz, 1891) and citropten from lime oil (Tilden *et al.*, 1902) that indicated the oxygenate heterocyclic nature of these compounds. For the following years studies had confirmed that coumarins, furocoumarins, and polymethoxyflavones were the major components in the non-volatile fraction. Over 70 of these compounds had been reported to occur in citrus oils and this number is still growing (Murray *et al.*, 1982).

Stanley and Vannier (1957) noticed the UV maxima difference between lemon oil and citropten. They concluded that the UV maximum of lemon oil was not contributed by a single component, but by a mixture of structurally related compounds. D'Amore and Calapaj (1965) applied thin layer chromatography (TLC) on the essential oils of lemon, bergamot, mandarin, and orange. They had found that these oils contained a range of substances that possessed strong UV fluorescence. Further examination of individual bands suggested that these substances belonged to coumarin, furocoumarins, and polymethoxyflavone families.

The non-volatile nature of oxygenated heterocyclic compounds had made HPLC the method of choice in their identification and quantification. It was the advancement in high-resolution HPLC column and highly sensitive detectors that had made HPLC analysis on citrus oils a popular subject in the past forty years (Dugo *et al.*, 2002). Comparing to UV or fluorescence detectors, the MS detector when on-line coupled with HPLC offered a superior means in structure identification.

Dugo (2000) had provided the identification of major oxygenated heterocyclic compounds in citrus oils from sweet orange, bitter orange, mandarin, grapefruit, and bergamot using HPLC/API/MS system. Later Dugo and his colleagues (2009) developed a single HPLC method that resolved most oxygenated heterocyclic compounds found in major citrus oils and also provided quantitative data of these compounds from both essential oils and commercial juice products. The book Citrus – The Genus Citrus from the same research group is highly recommended for a better understanding of citrus essential oils (Dugo *et al.*, 2002). Desmortreux in 2009 introduced supercritical fluid chromatography into the investigation of lemon residue and successfully identified 16

coumarins and furocoumarins with a typical run time of around 10 minutes. Weber and his colleagues (Weber *et al.*, 2006) chose HPLC/MS and HPLC/NMR as complementary analytical techniques and identified eight polymethoxyflavones and hydroxylated polymethoxyflavones from orange residue from molecular distillation. Ho research group (Li *et al.*, 2006) studied orange residue from supercritical fluid extraction and successfully isolated and confirmed the structures of 18 PMFs, hydroxylated PMFs, polymethoxyflavanones, and polymethoxychalcones by MS and NMR analyses.

Till today the majority of oxygenated heterocyclic compounds found in citrus oils have been identified by HPLC-MS or NMR techniques. However, there is little published quantitative information on these compounds. Quantitative data on oxygenated heterocyclic compounds are poor and often limited by the size of sample pool. Moreover, citrus oils prepared at lab scale are not comparable to industrial oils due to different extraction machinery, technique and fruit quality. On the other hand, data from industrial citrus oils are scarce due to the reluctance of essential oil industry in revealing information that they regard as proprietary. From the industry's point of view withholding information might help keep essential oils from fraudulent practices, but it certainly has negatively affected the development of essential oil research.

F. Literature Review on Citrus Oil Adulteration

From the beginning of essential oil trading, unscrupulous producers and traders have been carrying out fraudulent practices for improved profitability (Dugo *et al.*, 2002). The practices that have been applied to extend or cheapen citrus oils are concealed with great effort to maximize their effectiveness. It is therefore at the end users' discretion to

evaluate doubtful oils as well as to establish reliable analytical procedures. One must beware that essential oil adulteration is not isolated incident, but schematic common practice which is taking place in a large scale.

In citrus oil adulteration, the adulterants can be generally divided into non-citrus derived adulterants and citrus derived adulterants. From a practical point of view, the shift and preference among adulterants are largely depended on the cost of adulterants, the cost of starting oil, and the knowledge of the mainstream buyers within a particular time period. None of these factors are static and always subject to change.

Comparing to compositional study, research that emphasized on authenticity of citrus oils is less frequently seen. Research groups who have such interest are frequently discouraged by limited access to industrial samples. Large flavor companies who have R&D capabilities are reluctant to publish their results. Fortunately there are a few active research groups who have access to industrial samples. Some of their representative studies are listed below:

1. Early Studies

During the early years of citrus oil industry, non-citrus derived adulterants such as mineral oil and turpentine were broadly used because of their availability, affordability, and inadequacy of knowledge from the buyers' side. Few physical or analytical methods were implemented around that time. It was likely that essential oils back then were only assessed by appearance and organoleptic properties which are subjective and skill-dependent. One of the earliest references on citrus oil adulteration was attributed to De Domenico (1854) who mentioned that bergamot oil was adulterated in many ways when

discussing its medical benefits. He pointed out that mixtures of turpentine and stripped oil from lemon, orange, or windfall bergamot were used to extend genuine oils.

Such practices had slowly run out of favor since the development of physical analytical methods such as specific gravity, refractive index, and specific rotation. There was always a lagging phase between the advent of a new analytical approach and their wild acceptance and application. The differences in refractive index and optical rotation between citrus oil and turpentine had been discovered by Hooke in 1665 and Biot in 1815. However, commercial refractometers and polarimeters did not come out until the end of 19^{th} century. Generally speaking, the addition of turpentine decreases specific gravity and specific rotation in that α-pinene, the major component of turpentine, has a lower density and specific rotation value than normal citrus essential oils (Dugo *et al.*, 2002).

Chemical analyses were also developed to detect petroleum components in citrus oils. Petroleum adulterated citrus oil contains saturated paraffinic hydrocarbons that are inert to fuming sulfuric acid. On the other hand, hydrocarbons that exist in citrus oils are mostly unsaturated terpenes and sesquiterpenes which can be oxidized by fuming sulfuric acid. For such reason oil showing unreacted residue after treatment with fuming sulfuric acid can be regarded as doubtful (Dugo *et al.*, 2002). All these early test methods, however, were at best qualitative. They can hardly shed light on the identification of adulterants.

2. GC Studies

It is obvious that the real challenging adulterants to deal with are those derived from citrus fruits. In this context citrus-derived adulterants can be construed as any

cheap alternative oils, oil fractions, by-products, and natural or synthetic chemicals that are confirmed to be present in citrus oils. With the advent of Gas Chromatography and capillary column technique, knowledge on the volatile fraction of citrus essential oil had advanced rapidly. GC analysis is capable of exposing any significant foreign volatile adulterants and further revealing their identifications with quantitative data given the appropriate detector. It was the introduction of GC that greatly discouraged the usage of non-citrus derived adulterants. Table 14 showed important volatile components from major citrus species. Macleod (1964) analyzed a commercial lemon oil sample and confirmed the presence of benzyl ether, which was added for greater specific gravity and refractive index. Calvarano and Di Giacomo (1970), with the help of capillary GC column, spotted trace amount of linalyl acetate from some lemon oil samples and suggested that those samples had probably been contaminated by bergamot oil. Verzera (1987) had development a procedure using GC to detection orange fractions in lemon oil by monitoring the δ-3-carene/α-pinene ratio. As a result of all these efforts, adulterators were forced to seek alternatives to maintain their profitability.

Table 14. Crucial non-volatile compounds from various citrus species

	Binominal name	**Major volatiles**
Sweet Orange Oil	*Citrus sinensis*	Decanal, Linalool
Bergamot Oil	*Citrus bergamia*	Linalyl acetate
Mandarin Oil	*Citrus deliciosa*	DMA, Thymol
Lemon Oil	*Citrus limon*	Citral
Lime Oil	*Citrus aurantifolia*	1,8-Cineole, 1,4-Cineole
Grapefruit Oil	*Citrus paradisi*	Nootkatone

3. HPLC Studies

During the first few decades non-volatile analysis was bottlenecked by underdeveloped instrumentation and suffered from lack of precision and reliability. However, just as GC to volatile fraction analysis, non-volatile fraction analysis was propelled by the flourish of HPLC technique in the last 40 years.

McHale and Sheridan (1989) looked into the non-volatile components in citrus oil and discovered that two coumarins, auraptene and epoxyauraptene appeared to be unique to grapefruit oil. They were able to detect grapefruit oil fraction in bitter orange oil with HPLC-UV. The same authors (McHale and Sheridan (1988)) had also reported that coumarins were added to lemon oils in order to enhance their CD value, including 7-methoxycoumarin and 5, 7-dipropyloxycoumarin. The presence of significant amount of those coumarins in lemon oils should be treated with caution.

P. Dugo, G. Dugo and their colleagues in Italy had published a range of papers in 1980's and 1990's on the genuineness of citrus essential oils. Over the years they had delved in to different cultivars of orange, lemon, lime, bergamot, and other citrus species over the world and tried to generalize the typical values of major volatile and non-volatile components from genuine oils. They had applied various analytical techniques (GC, HPLC, UV, *etc*.) to compare lab extracted oil, industrial samples, and commercial products. Most of their oxygenated heterocyclic standards are obtained in house through preparative HPLC or chemical synthesis (Dugo *et al*., 1999 and 2009). In their studies factors such as extraction techniques, harvest season, cultivars had been taken into consideration. Their accomplishment was invaluable to the industry and citrus essential oil research. However, comparing to GC analysis, there was few published quantitative data on the oxygenated heterocyclic compounds in citrus essential oils. Moreover, those data were often related to a limited number of samples whose origins were either unidentified or lab scale. Data from the essential oil industry were almost blank.

4. Studies with Other Techniques

Other analytical methods had also been utilized in screening citrus oils for authenticity. Unlike GC or HPLC, most of these methods are non-compositional analyses. However, they have been proven effective against certain adulteration practices. They have been and will be serving as important weapons in adulteration studies.

1) UV Studies

Ultra-violet absorption method was wildly applied as a qualitative non-volatile fraction analysis in the industry before the flourish of HPLC. It have been established that

in lemon oil non-volatile fraction is responsible for the UV spectrum with a maximum around 315 nm (Dugo *et al.*, 2002). For obvious reason neither the steam-stripped oils nor distilled terpene fractions possess such UV absorption. Oils with UV absorption value well below norm can be reasonably questioned as being adulterated with light fractions. There are two ways that adulterators have been cheating on UV detection: firstly, addition of a calculated amount of light fraction into a good quality starting oil that keeps the final UV absorbance within specification; secondly, UV absorbance enhancer (such as coumarins, ethyl *p*-dimethylaminobenzoate) can be added to bring up the UV absorbance.

2) Specific Rotation Studies

Specific rotation test is among the first few developed test methods, and is still carried out today for its accuracy and simplicity in determination of chirality and natural status. The theory is that a great number of botanical products show different chiral properties from their synthetic counterparts. The synthesis enzymes found in plants are mostly chiral-selective which result in enrichment of one enantiomer over the other, enzymes from different botanicals may vary and lead to unique chirality configuration of one chemical in some species. On the other hand, synthetic or petroleum-based chemicals never undergo such enrichment process thus are possibly racemic. One good example is to differentiate botanical-derived linalool from synthetic one. Linalool is a significant component in a range of essential oils and naturally occurring linalool from most plants exists overwhelmingly in (R) (-) enantiomer form. The synthetic linalool, on the other hand, is racemic (Dugo *et al.*, 2002). The limitation of specific rotation is obvious: it can only give a gross reading. It is effective in evaluation of chemicals but insufficient when

dealing with oil blends, particularly blends that have been manipulated by skillful producers.

3) Chirality Studies

It is worth mentioning that neither conventional GC nor HPLC is capable of differentiating between natural and synthetic chemicals or between enantiomers, because there is no chemical or physical difference between these pairs. Unfortunately, this information can be extremely helpful when tracing the history of essential oils, particularly for oils which have been reconstituted with synthetic chemicals or natural chemicals from a cheaper source. In order to cope with the ever-evolving adulteration practices, chiral GC columns were developed to determine the enantiomeric distribution of individual volatile components in citrus oils. By coupling non-chiral column with chiral column using Multidimensional Gas Chromatography (MDGC or heart cutting) technique, detection of small amount of synthetic chemical in a complicated oil blend becomes possible. Naturally occurring aroma chemicals are generally one enantiomer predominate over the other or even virtually exclusive. Mosandl and Juchelka (1997) and Cotroneo (1992) studied bergamot oil and concluded that the genuine oil comprised essentially 100% (R) (-) –linalool and (R) (-) –linalyl acetate. Thus the presence of any significant amount of the (+) enantiomers by MDGC is indicative of adulteration.

4) Stable Isotope Studies

The state of the art anti-adulteration technique is stable isotope analysis, which is unsurpassable in investigation of natural status comparing to the rest techniques. Briefly speaking, the stable isotope pattern of any organic compound is determined by that of its

precursors and the isotope shift accompanying the synthesis process. The changes involved in the synthetic process are distinct and expressed in the form of a shift (δ value) of the isotope ratio from that of an international standard (Dugo *et al.*, 2002). Stable isotope analysis has found its application in origin determination of bitter almond oil (benzaldehyde), cassia oil (cinnamic aldehyde), and vanilla extract (vanillin). Stable isotope analysis suffers from large sample volume requirement, lengthy turnaround time and affordability.

Stable isotope analysis is the most advanced and trusted analytical means in adulteration detection, and it is a powerful weapon when applied to citrus oils as well. The isotope ratio of the hydrocarbons from citrus oils, unlike compositional studies, is not influenced by harvest season, extraction technique, storage conditions, or other common variations. Only the genealogy and geological origin of the citrus fruit, and isotope effect can alter the isotope ratio of their monoterpene components ($C_{10}H_{16}$) (Sawamura, 2010). The isotope ratio is calculated from the molecular peak (m/z=136) and its isotope peak (m/z=137) of monoterpene hydrocarbon. The calculated ratio gives information about both carbon isotopes and hydrogen isotopes of essential oil components. Theses value can be used to fingerprint citrus species and their geographic origin.

Sawamura, M and Satake, A (2001) calculated the MS fragment ratios of monoterpenes from three species of oils and the data was subjected to PCA study as shown in Figure 7. Three species lemon, lime, and yuzu were clearly distinguished from one another with lemon distinguished itself in PC1 and lime distinguished itself in PC2. Cluster analysis was carried out to the same dataset and result was shown in Figure 8.

The genetic distances between different geographical origins were clearly demonstrated in the chart.

Bonaccorsi and her colleagues (2011) combined ennantiometic MDGC with combustion-isotopic ratio mass spectrometry (GC-C-IRMS) to investigate the genuineness of lime oils. They claimed that such technique combination was synergistic and was capable of revealing extremely subtle adulteration.

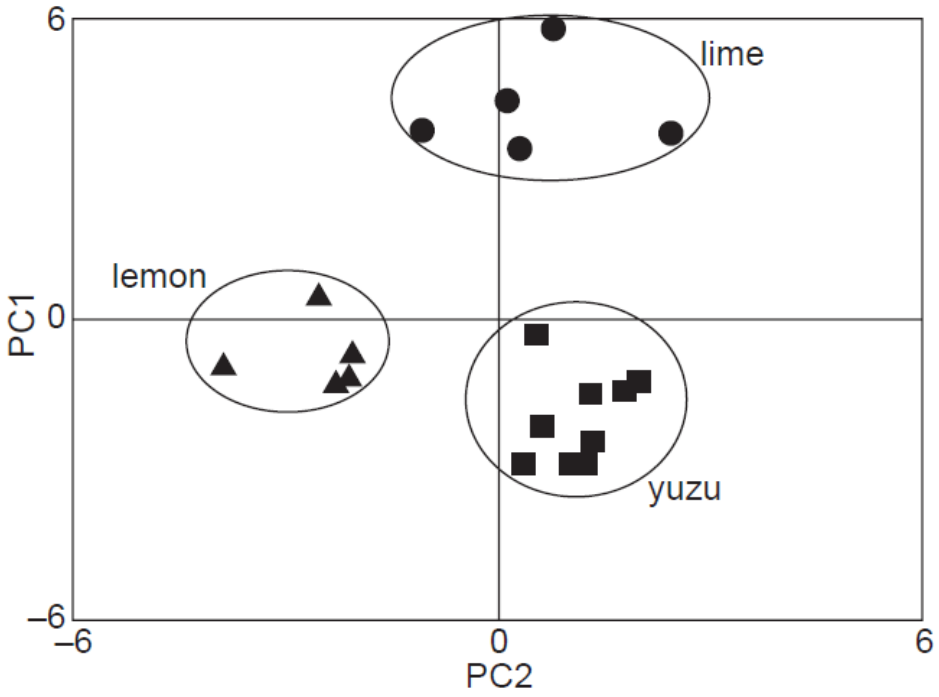


Figure 7. PCA two-dimensional projection chart
(Sawamura *et al*., 2001)

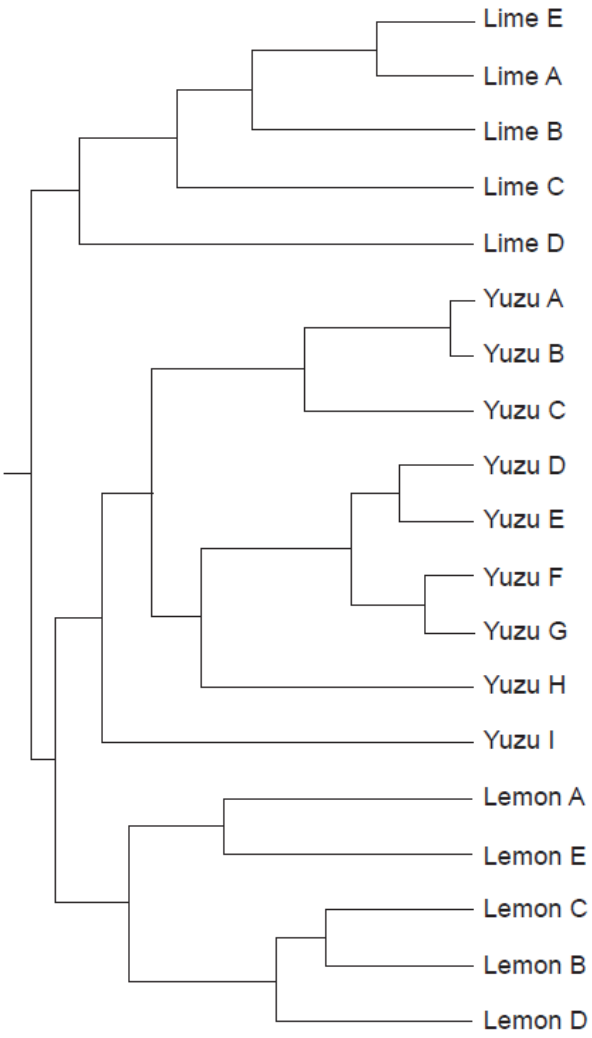


Figure 8. Cluster chart of lemon, lime, and yuzu oil samples (Sawamura *et al*., 2001)

III. HYPOTHESIS AND OBJECTIVES

A. Hypothesis

Qualitative and quantitative analysis of oxygenated heterocyclic components by HPLC can be a more effective and conclusive approach in authenticity evaluation of citrus essential oil, comparing to volatile fraction study with the aid of GC.

B. Objectives

Develop a universal HPLC method that can resolve most peaks in major species of citrus oils.

Identify major peaks in citrus essential oils via HPLC-MS.

Statistically analyze a large volume of samples to set up the tolerance limits of key non-volatile compounds from industrial essential oils

Applied acquired data to routine quality control for adulteration detection.

IV. EXPERIMENTAL

A. Sample Pool

A pool of over 300 citrus oil samples of industrial origins from the past five years (2008 to 2012) was collected and analyzed in this study. These samples were purchased or requested from a number of domestic or international vendors and kept in optimum condition to suppress oxidation and other deterioration. Few samples that were exhibiting seriously abnormal sensory properties were excluded from this study to ensure representative outcomes. Each of the chosen samples was evaluated by GC and HPLC analysis. The analysis results were combined and used for establishment of HPLC database using statistical approach (PCA study).

This study did not take cultivars or geographical origins into consideration. In another word, citrus oils from the same species are treated equally. Two main reasons have led to such decision: (1) most of the oils in this study are from large vendors or brokers in the citrus oil business. Attempt to trace down the initial source of the starting fruits for every single oil sample can be a formidable task. (2) For those samples which country names or cultivar names are given in their description, such information has to be treated with caution. From the author's experience toward the industry, sometimes the information on the sample description was given only as an implication. Component adjustment is not rare in the industry samples.

B. GC parameters

Phenomenex® ZB-1MS phase capillary column (100% dimethylpolysiloxane, 20 m x 0.10 mm x 0.10 µm, Phenomenex, Torrance, CA, USA) was used in GC analysis. Citrus oil samples were directly injected without any pre-treatment. Injection volume was 0.1 µL. Carrier gas (hydrogen) flow rate was 0.33 ml/min. Temperature range was 70 °C to 300 °C with the ramp described as below:

- 70 °C hold for 1 min
- 70-110 °C with 5 °C/min ramp for 8 min
- 110-300 °C with 25 °C/min ramp for 7.6 min
- 300 °C hold for 3.4 min

Total run time was 20 min. This temperature program is faster that most of existing methods in analyzing citrus essential oils. Baseline separation can be achieved to most vicinal peaks in this method except for some pairs (i.e., β-phellandrene and *d*-limonene) which are equally challenging other methods with similar GC column phase. PerkinElmer® XL Autosystem GC with build-in FID (PerkinElmer, Waltham, MA, USA) detector, autosampler, and Totalchrom® software was used in this study.

C. HPLC parameters

Among the several HPLC columns that were evaluated and compared, Phenomenex® Luna 3 µm PFP(2), LC Column 150 x 4.6 mm w/guard column (Phenomenex) was chosen for its outstanding capability in resolving oxygenated heterocyclic compounds. The pentafluorophenyl propyl group (Figure 9) bound to silica surface offers unique aromatic selectivity due to high electronegative fluorine atoms on

the peripheral of the phenyl ring, which makes PFP(2) column ideal in separating oxygenated heterocyclic compounds. Guard column was applied in this study to prevent terpene accumulation onto the hydrophobic stationary phase which might lead to pressure buildup.

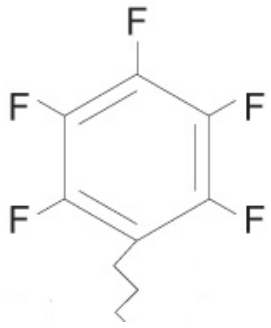


Figure 9. Pentafluorophenyl propyl group in PFP(2) column

A binary (methanol and water) solvent method was optimized for citrus essential oil analysis as below:

- 75-80% Methanol in 10 min
- 80-95% Methanol in 12 min
- 95-100% Methanol in 1 min
- Hold for 2min

Dionex® Ultimate 3000 HPLC with autosampler and UV detector (Dionex, CA, USA) was used in this study. UV Absorbance was recorded at 315 nm.

Comparing to other documented HPLC methods carried out on citrus oil analysis., this method has the following advantages:

1. Simplicity: binary solvent water + methanol

2. Low toxicity: no acetonitrile or other highly toxic solvents.

3. Efficiency: 25 min run time.

D. MS Conditions

Hewlett-Packard® 1100 series LC/MSD System (Agilent Technologies, Waldbronn, Germany) was applied in this study. The LC/MSD system was equipped with autosampler, quaternary pump system, DAD detector, degasser, MSD trap with an electrospray ion source (ESI), and HP Chemstation® software. Nebulizer (He) = 40 psi; Dry gas (N_2) = 8.0 L/min. Positive ion mode was selected in this study. HPLC conditions in HPLC-MS study were the same as previously described in section C HPLC parameters.

E. SPE Fractionation Conditions

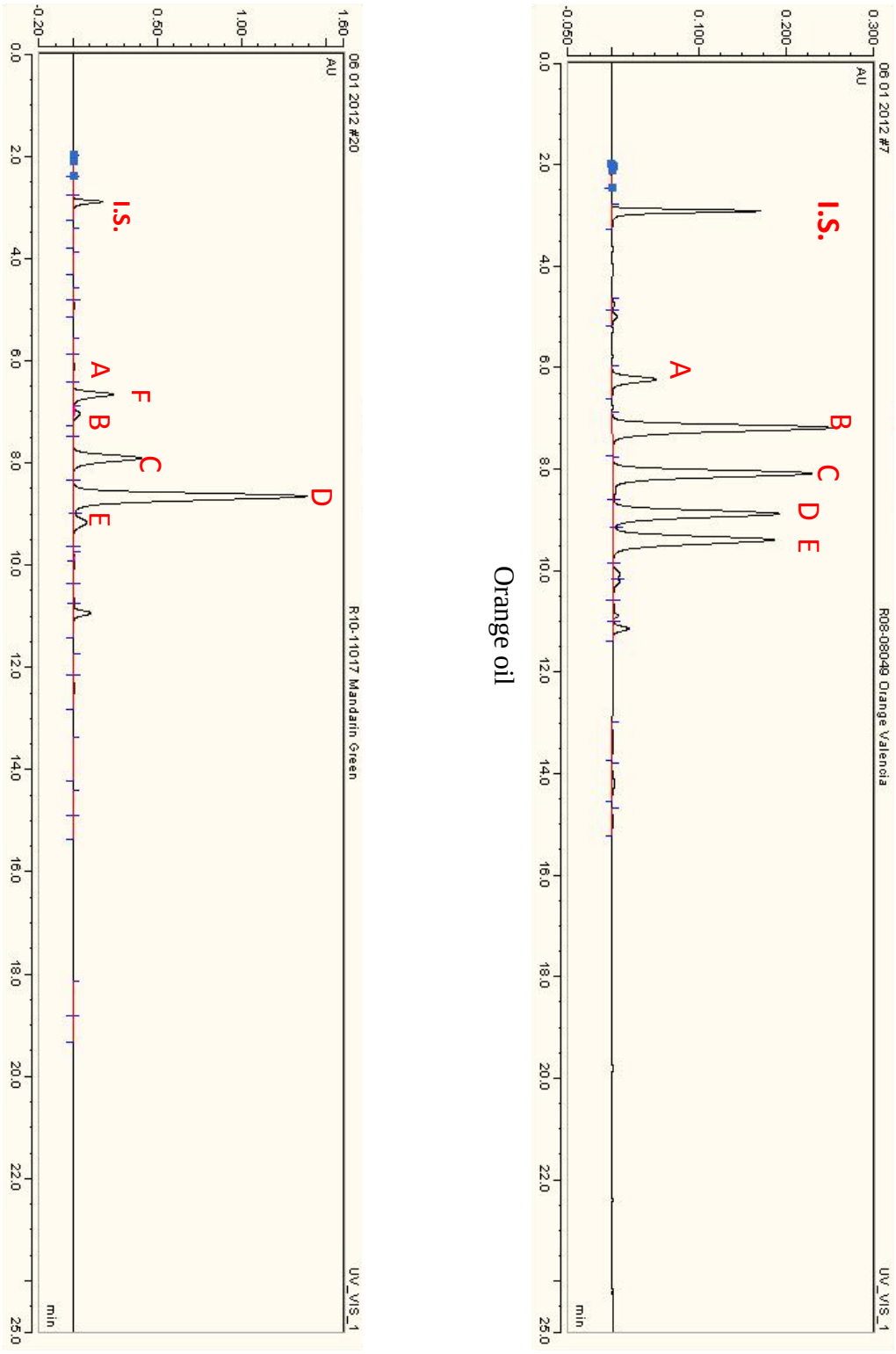
Varian® C-18 3 mL solid phase extraction (SPE) column (Varian, Cranford, NJ, USA) was applied in this study for fractionation purpose. For each fractionation batch 20 µL of citrus oil was loaded onto the pre-equilibrated SPE column. Mixture of water and methanol at 1:9 was chosen as eluent. For each fraction 1 mL of eluent was collected under gravity.

V. RESULTS AND DISCUSSION

A. Major Non-volatile Peak Identification in Citrus Oils

Figure 10 showed typical HPLC grams of citrus oils from six major species (orange, mandarin, tangerine, grapefruit, lemon, and lime). Coumarin was chosen as internal standard for quantification for two reasons: its structure is similar to oxygenated heterocyclic compounds found in citrus oils, and it does not occur naturally in citrus oils. The injected oils were from trusted sources thus genuineness is without question. It was obvious that orange oil, mandarin oil, and tangerine oil contain totally different peaks comparing to lemon and lime oil. Most of the peaks were well resolved in all six chromatograms.

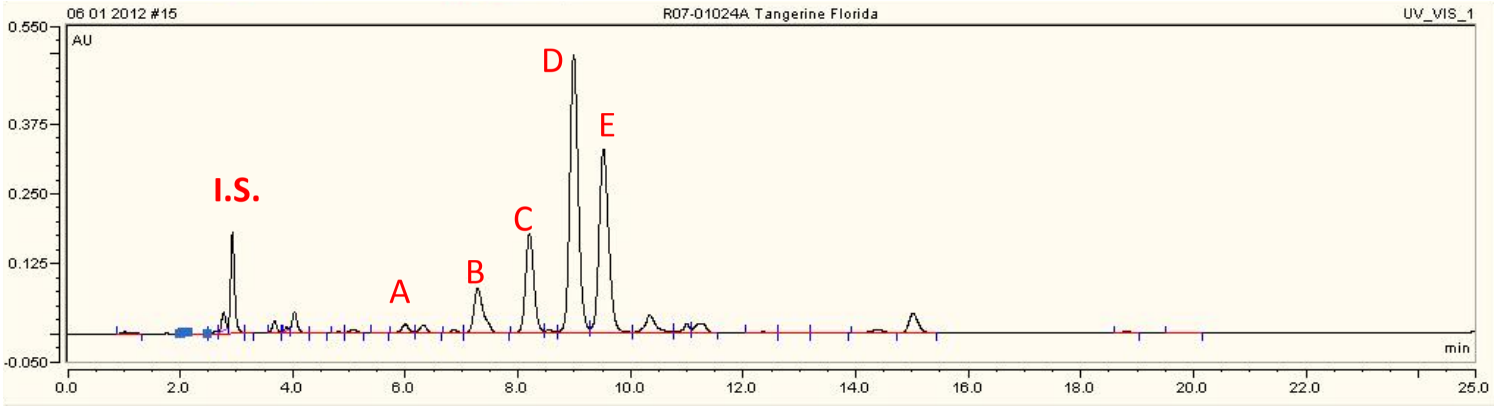
The identification of major peaks in six citrus oils had been successfully confirmed by HPLC-MS as shown in Table 15.



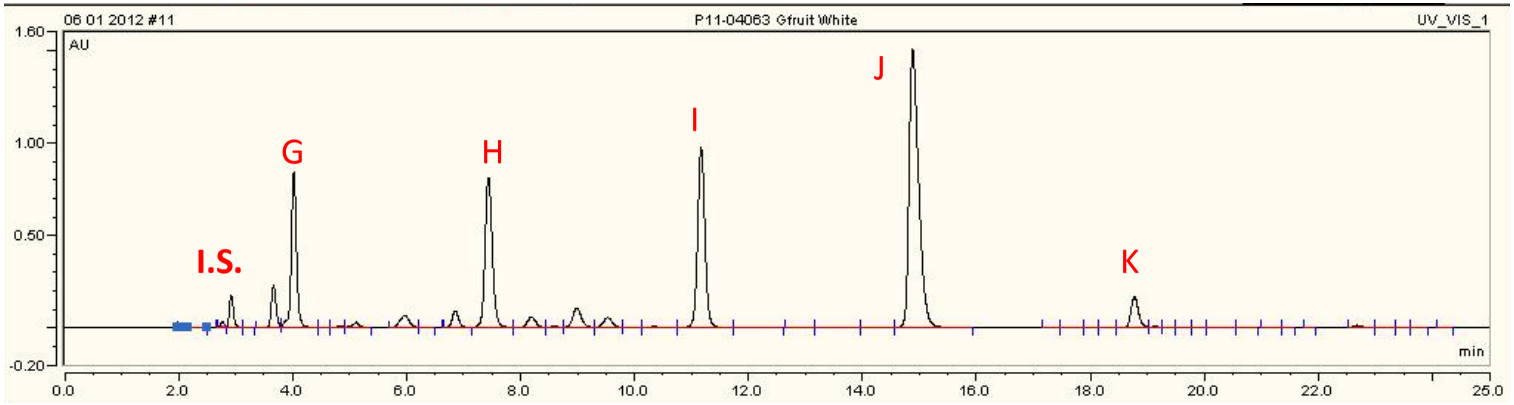
Orange oil

Mandarin oil

Figure 10. HPLC profiles of six citrus oils

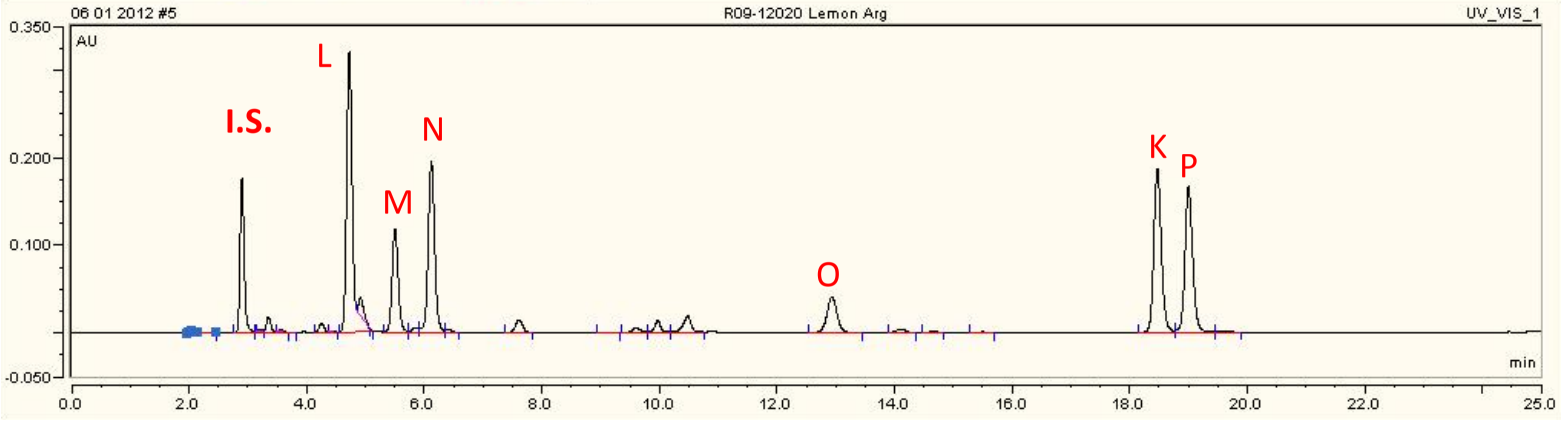


Tangerine oil

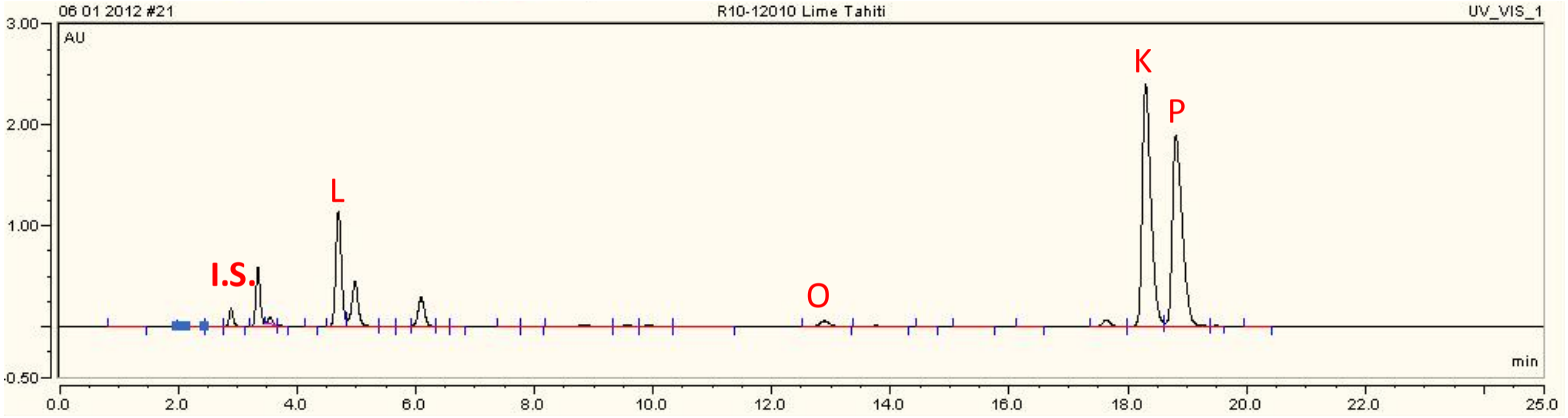


Grapefruit oil

Figure 10. HPLC profiles of six citrus oils



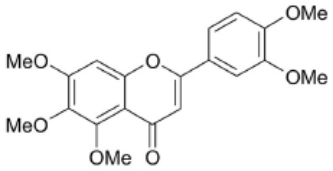
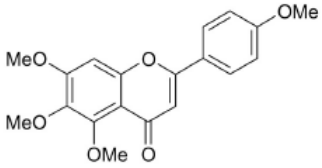
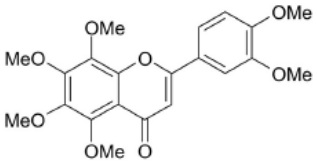
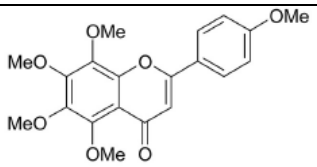
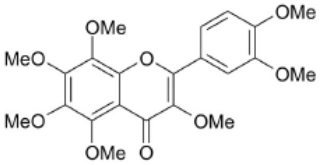
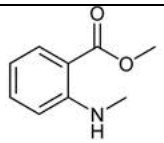
Lemon oil

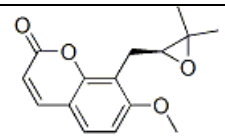
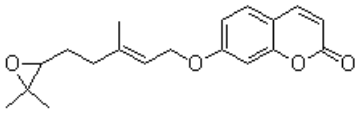
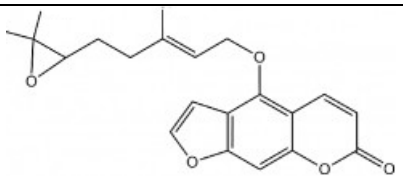
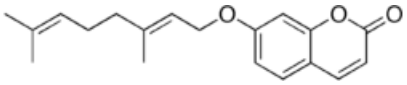
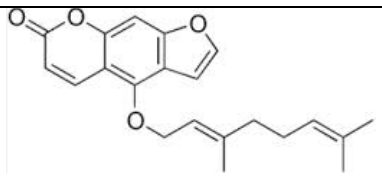
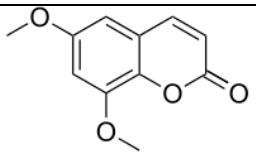
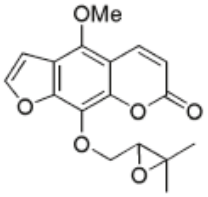


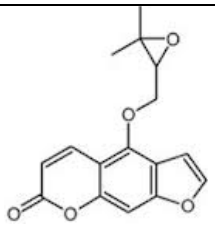
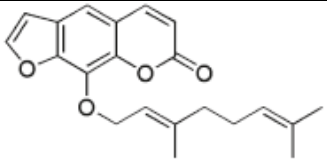
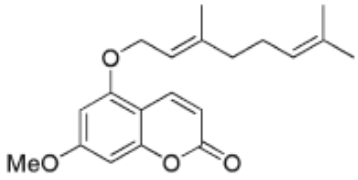
Lime oil

Figure 10. HPLC profiles of six citrus oils

Table 15. Major non-volatile peak identification in citrus oils

	Compound	Structure
A	Sinensetin	
B	5,6,7,4’-Tetramethoxyflavone	
C	Nobiletin	
D	Tangeretin	
E	3,5,6,7,8,3’,4’- Heptamethoxyflavone	
F	Dimethyl Anthranilate	

G	Meranzin	
H	Epoxyaurapten	
I	Epoxybergamottin	
J	Aurapten	
K	Bergamottin	
L	Citropten	
M	Byakangelicol	

N	Oxypeucedanin	
O	8-Geranyloxypsoralen	
P	5-Geranyloxy-7-methoxycoumarin	

B. Fractionation of Orange Oil

The terpenes do not show themselves on HPLC-UV gram due to the weak UV absorption around 315 nm as oxygenated heterocyclic compounds do. However, this dominating terpene fraction does appear in HPLC-MS gram and convolutes the subsequent peak interpretation task or even contaminates the ion source inside the MS detector. Therefore it is crucial to remove the terpene fraction from citrus oils prior to any HPLC-MS analysis.

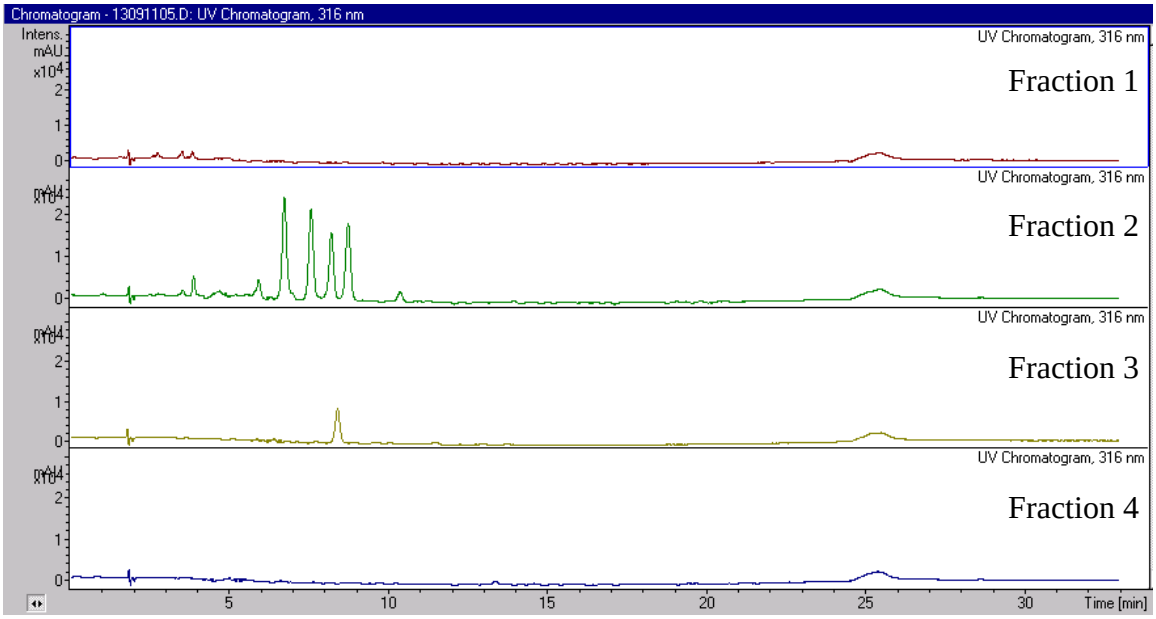


Figure 11. Fractionation of PMFs in orange oil by C-18 reverse SPE column

Different eluting ratios between methanol and water were tried to achieve the best fractionation between terpenes and oxygenated heterocyclic compounds. Methanol: water at 9:1 appeared to give the most satisfactory outcome as shown in Figure 11. The PMFs were completely eluted out in fractions 2 and 3 while all the terpenes were retained on the column (GC data not shown) after the fourth fraction. Such fractionation procedure was applied to all citrus oil samples that were subjected to HPLC-MS analysis.

C. Identification of Two Hydroxylated PMFs

The five major PMFs (sinensetin, 5,6,7,4'-tetramethoxyflavone, nobiletin, tangeretin, and 3,5,6,7,8,3',4'-heptamethoxyflavone) were already identified from orange oil and isolated from previous studies. In this study two hydroxylated PMFs have been identified from orange and tangerine oils for the first time. Peak X and Y in Figure 12

were confirmed to be 5-demethylnobiletin and 5-demethylheptamethoxyflavone by analyzing the MS data and their retention times (standards for both peaks were obtained from previous study Li *et al.*, 2006).

The mechanism of the formation of hydroxylated PMFs was postulated to be demethylation process from their corresponding PMFs (Figure 13). 5-position demethylation is the most preferred in that 5-postion hydroxyl group is able to form intra-molecular hydrogen bonding with the vicinal carbonyl oxygen atom (on 4- position) and results in a stable 6-membered ring structure (Li *et al*., 2006). Demethylation process of methoxyl groups on 3-postion were also reported for similar reason (Li *et al*., 2006). Demethylation reactions on methoxyl groups other than 3-position and 5-postion are energetically unfavored.

Hydroxylated PMFs had been identified in orange peel extracts obtained from supercritical carbon dioxide extraction in previous studies (Li *et al.*, 2006). It was generally believed that post-harvest storage triggered the onset of demethylation process. To the best of the author's knowledge, this is the first time that hydroxylated PMFs were identified from cold-pressed citrus essential oils.

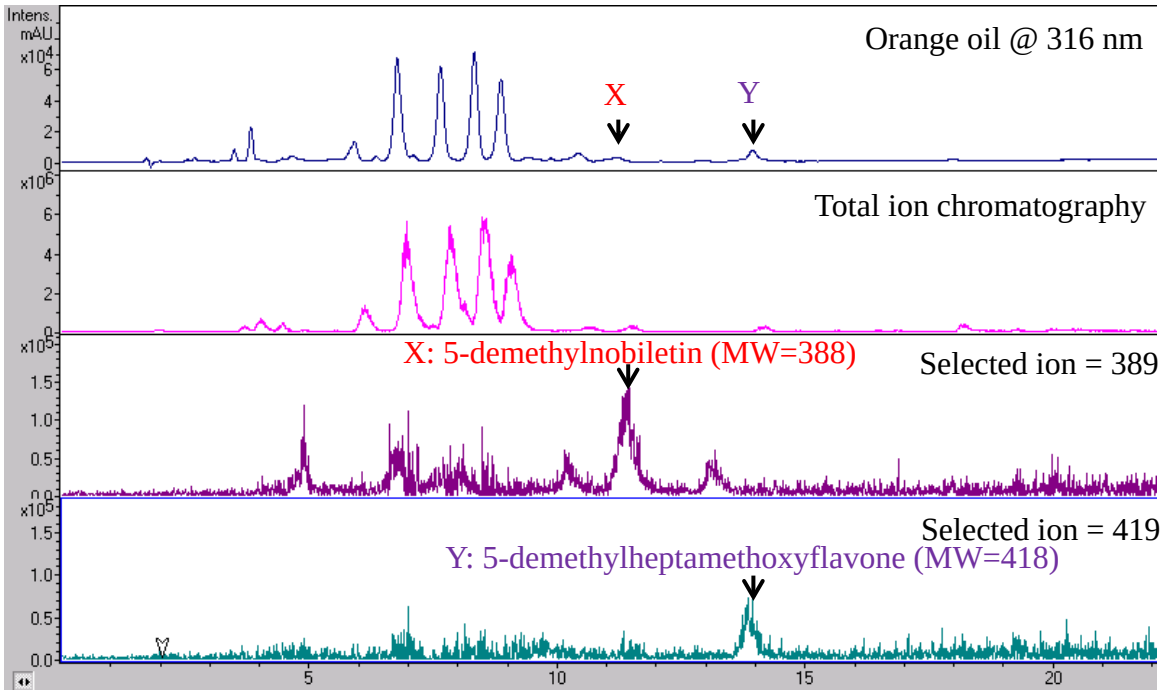


Figure 12. Proof of two hydroxylated PMFs in orange oil

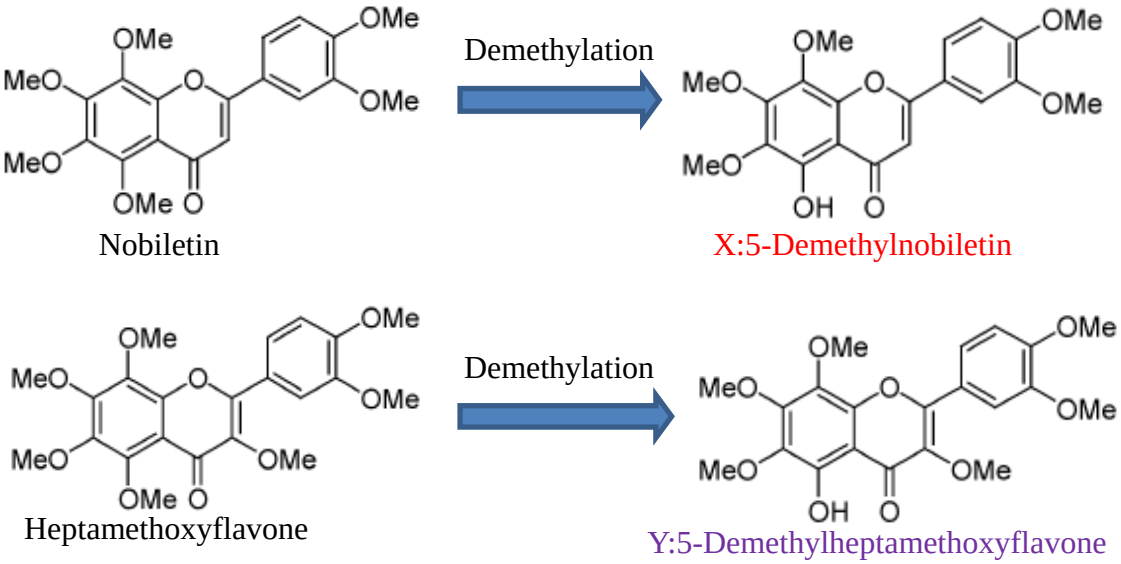


Figure 13. Proposed formation pathway of hydroxylated PMFs

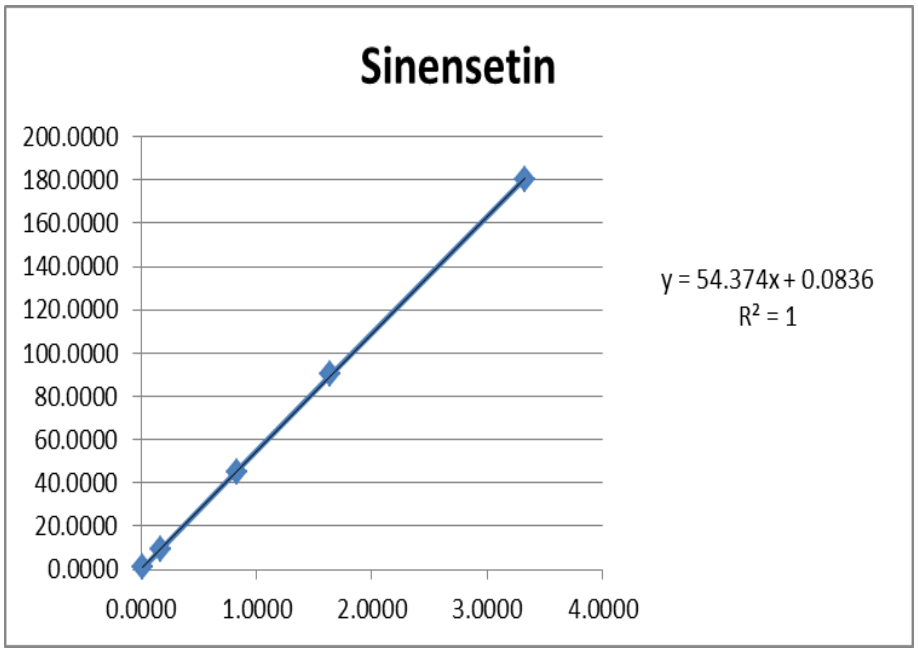
D. Quantification of PMFs

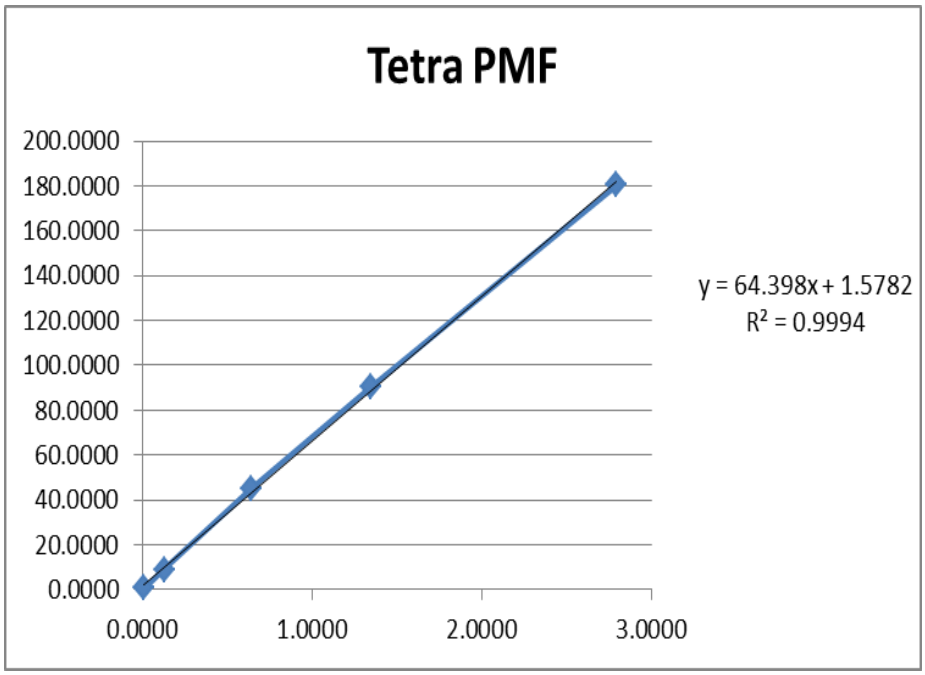
A stock solution of 0.1% (w/w) coumarin (internal standard) in ethyl alcohol was prepared. Citrus essential oils were analyzed without any filtration or fractionation. 100 mg of oil (approximately 118 µL) was accurately weighed and diluted in 800 µL of ethyl alcohol. Before HPLC analysis, 100 µL of coumarin stock solution was added.

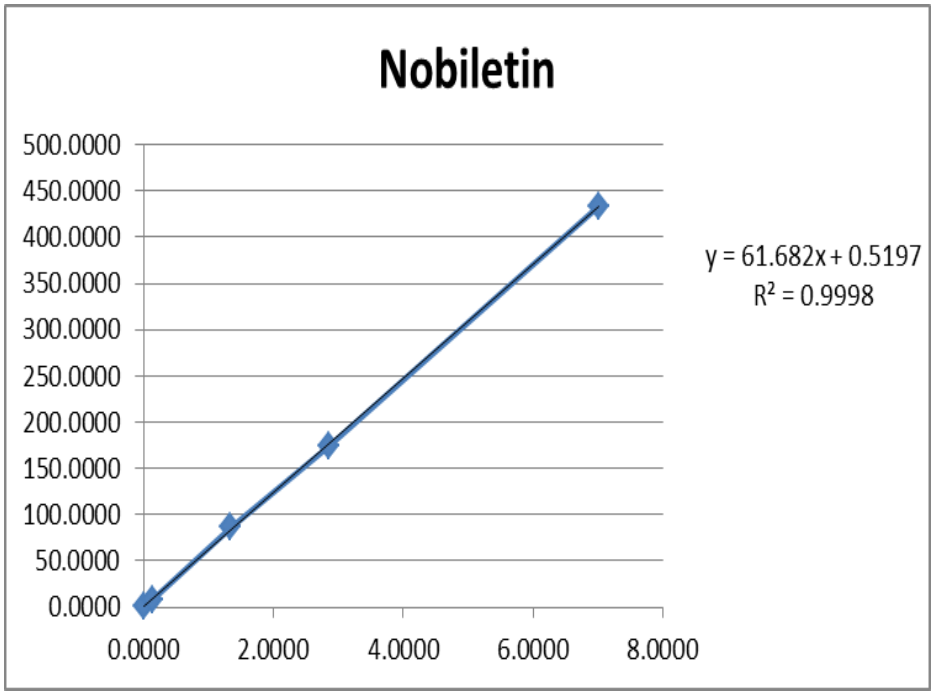
All five PMF standards for quantification were from preparative HPLC separation from citrus peel extracts (Li *et al.*, 2006). Linearity of the detector response was determined based on the calibration graphs. Regression analysis was used to assess the linearity of the analytical method. Five different concentration of each analyte were prepared using ethyl alcohol as solvent. Before injection, 100 µL of coumarin stock solution was added to 918 µL of each standard solution. Each standard solution was injected in triplicates and mean values were used to construct calibration curves as in Table 16 and Figure 14: x stood for the peak area ratio between each analyte and coumarin, y stood for the concentration of each analyte in standard solution after addition of internal standard (µg/ml).

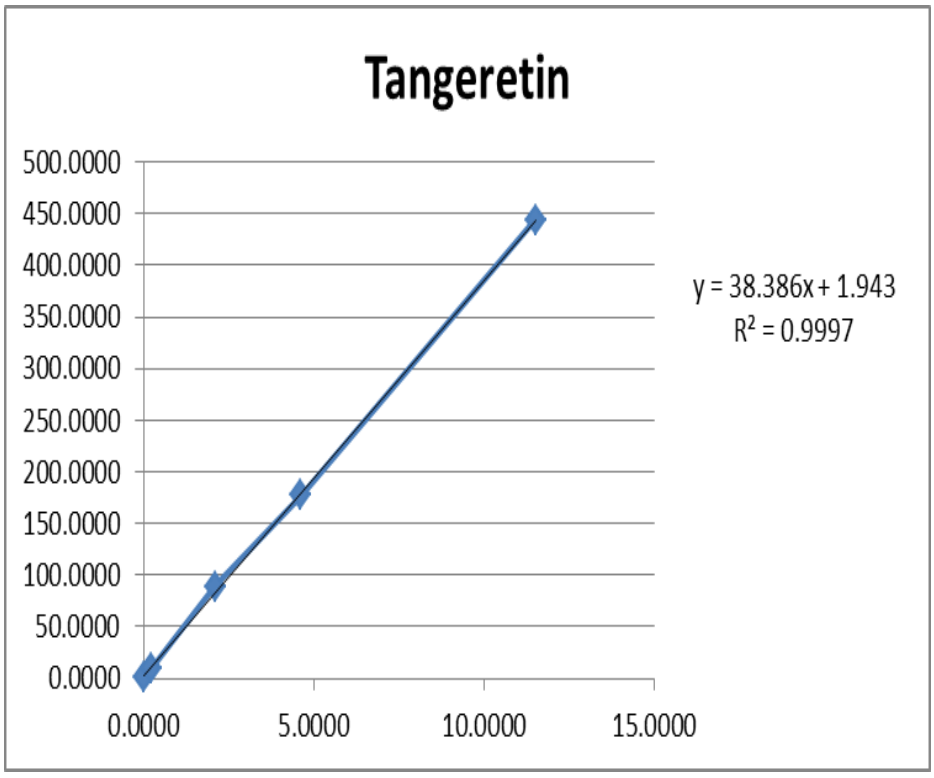
Table 16. Calibration curves for six analytes

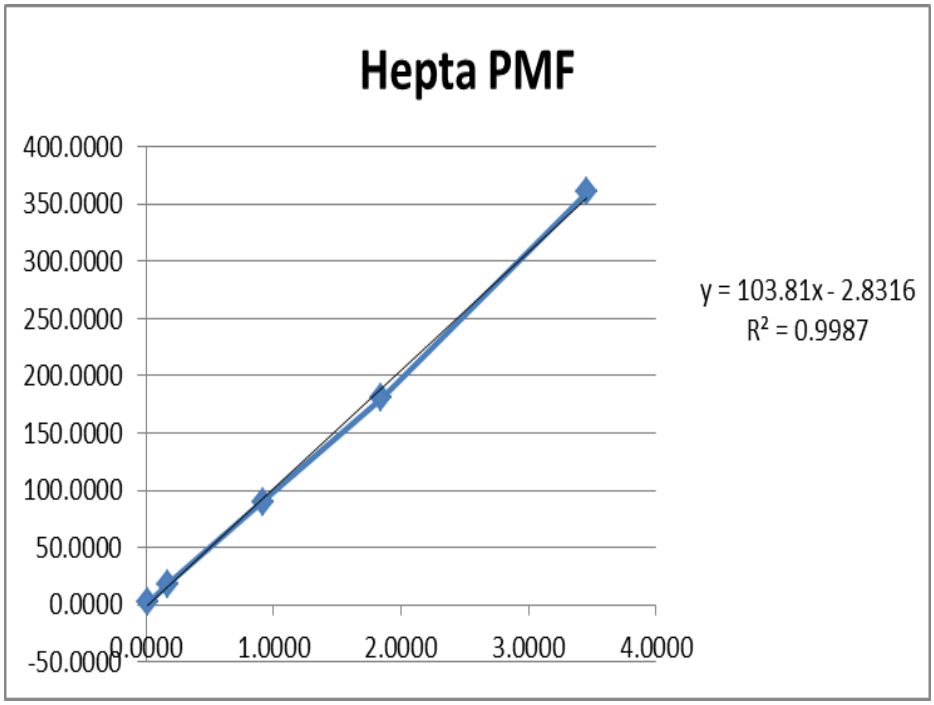
Analyte	Concentrations (µg/mL)	Linear regression equation	r^2
Sinensetin	1, 10, 50, 100, 200	y = 54.374x + 0.0836	1.0000
Tetra PMF	1, 10, 50, 100, 200	y = 64.398x + 1.5782	0.9994
Nobiletin	1, 10, 100, 200, 500	y = 61.682x + 0.5197	0.9998
Tangeretin	1, 10, 100, 200, 500	y = 38.386x + 1.943	0.9997
Hepta PMF	2, 20, 100, 200, 400	y = 103.81x - 2.8316	0.9987
Dimethyl anthranilate	4, 40, 200, 1000, 2000	y = 359.35x + 7.4793	0.9992


Sinensetin
200.0000
180.0000
160.0000
140.0000
120.0000
100.0000
80.0000
60.0000
40.0000
20.0000
0.0000
0.0000
1.0000
2.0000
3.0000
4.0000
y = 54.374x + 0.0836
R² = 1


Tetra PMF
200.0000
180.0000
160.0000
140.0000
120.0000
100.0000
80.0000
60.0000
40.0000
20.0000
0.0000
0.0000
1.0000
2.0000
3.0000
y = 64.398x + 1.5782
R² = 0.9994


Nobiletin
500.0000
450.0000
400.0000
350.0000
300.0000
250.0000
200.0000
150.0000
100.0000
50.0000
0.0000
0.0000
2.0000
4.0000
6.0000
8.0000
y = 61.682x + 0.5197
R² = 0.9998


Tangeretin
500.0000
450.0000
400.0000
350.0000
300.0000
250.0000
200.0000
150.0000
100.0000
50.0000
0.0000
0.0000
5.0000
10.0000
15.0000
y = 38.386x + 1.943
R² = 0.9997



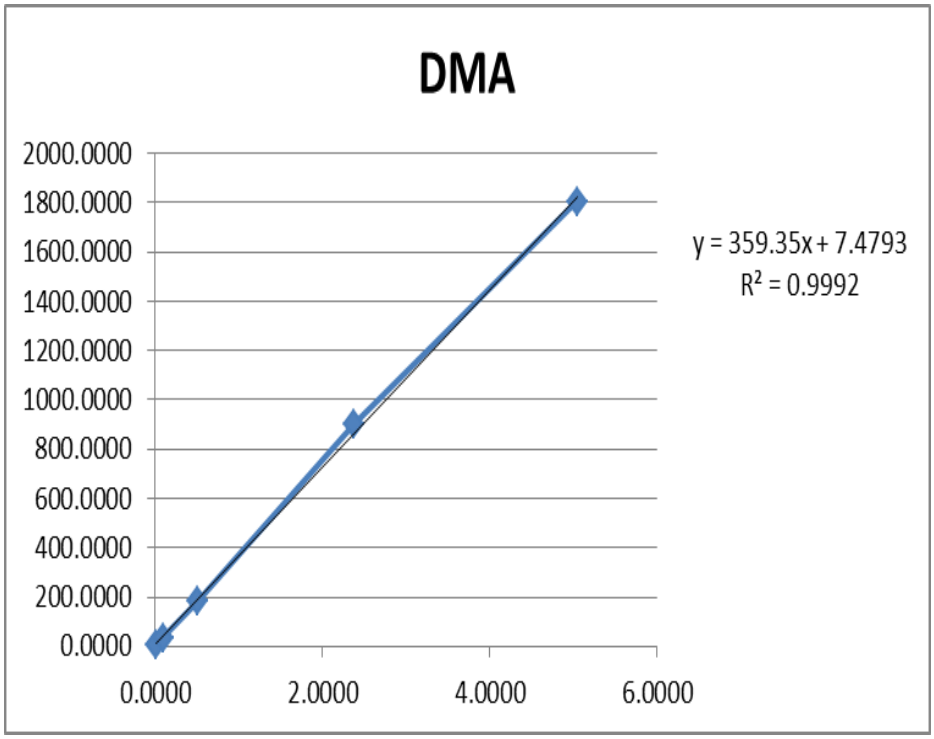


Figure 14. Calibration curves for six analytes
X axis: peak area ratio between analyte and coumarin
Y axis: concentration (µg/ml) of analyte

A total of 87 orange oils, 16 tangerine oils, and 20 mandarin oils of industrial origins were analyzed by GC and HPLC with aforementioned methods. The acquired data will be used statistically to evaluate their genuineness.

Figure 15 compared the levels (expressed in parts per million, ppm) of the five major PMFs in orange, tangerine, and mandarin oils. The calibration curves obtained previously were applied to collect quantitative data of PMFs in each oil sample. Mean values and standard deviations for each of the five PMFs were calculated and expressed in bar graph.

It was clear that the levels of sinensetin, tetra PMF, and hepta PMF were higher in orange oils than those in tangerine and mandarin oils. On the other hand, the levels of nobiletin and tangeretin were lower in orange oils than those in tangerine and mandarin oils. It was also suggested that the level of PMFs in tangerine oils were usually in between orange oils and mandarin oils. The standard deviations of PMF levels in orange oils were much smaller than those in tangerine and mandarin oils, which might suggest that orange oils, for their low prices, were less vulnerable to adulteration comparing to expensive citrus oils such as tangerine or mandarin oils.

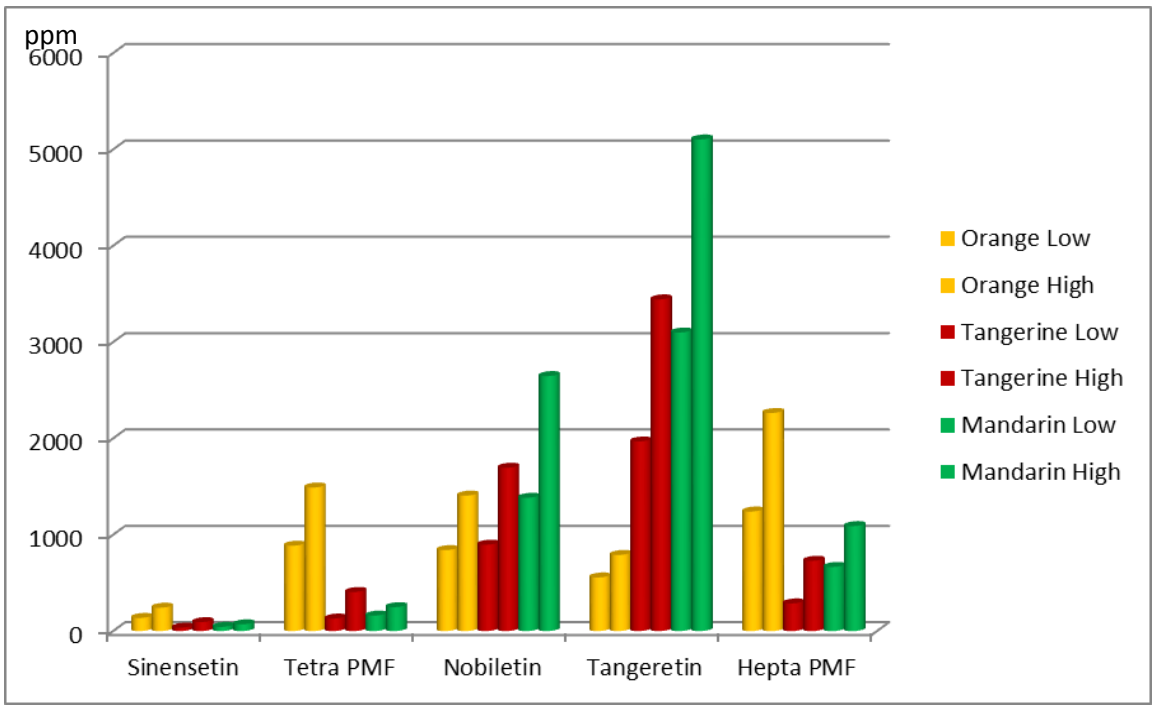


Figure 15. Calculated PMF limits in orange, tangerine, and mandarin oils
Mean value +/- standard deviation

The numeric value ranges of PMFs derived from all 123 samples were listed in Table 17. These values can be very helpful in assessing the genuineness of oil samples. Sample that displays outlying PMF level(s) can be marked as doubtful and further analysis should be carried out. Cares must be taken when applying these data: (1) Single outlying PMF value does not necessarily lead to sample rejection. Citrus oils are natural products therefore considerable natural variation can be expected. Cultivar, season, extraction method, and storage condition may all influence their PMF levels. Abnormal PMF value raises a question mark to the sample's history, but information from other analyses is imperative. After all, HPLC alone is insufficient for making the final decision. (2) High outlying values are normally not regarded as harmful. Unlike GC grams, The

HPLC-UV grams do not show the full spectra of non-volatile components from citrus oil because UV detectors are not universal. Therefore an abnormally high reading in one PMF does not necessarily result in lower readings to the rest components. It is those oils in which most of the major PMFs are low or the PMF patterns implies presence of other citrus species that should really be rejected.

Table 17. Deduced ranges for PMFs from orange, tangerine, and mandarin oils

	Orange Oil (ppm)	Tangerine Oil (ppm)	Mandarin Oil (ppm)
Sinensetin	136 – 243	35 – 93	44 – 70
Tetra PMF	887 – 1490	130 – 405	161 – 248
Nobiletin	841 – 1407	897 – 1695	1384 – 2647
Tangeretin	557 – 790	1968 – 3444	3099 – 5103
Hepta PMF	1241 – 2264	287 - 730	665 – 1090

E. Adulteration Case Study

Among all the citrus essential oils that had been analyzed, the following three cases were chosen and presented in this section to illustrate the superiority of HPLC analysis over GC analysis against certain adulteration practices. All of these cases had led

to final rejection of the pre-ship oil samples therefore prevented the company from purchasing low-grade essential oils.

1. Case I – Orange Oil

Orange oil sample R1 was purchased directly from trusted grower therefore its authenticity can be guaranteed. Orange Oil sample X was purchased from a vendor and its authenticity was yet to be confirmed. Both GC and HPLC analyses were carried out to both samples. The GC profiles of the two samples were strikingly similar (Figure 16A). The levels of key compounds such as linalool and decanal were very close between the two samples (Table 18A).

However HPLC results had suggested otherwise. For all five major PMFs that were found in orange oil, their levels in sample X were only approximately 50% of those in target sample R1 (Figure 16B). Natural variation itself cannot account for such huge difference between non-volatile fractions of the two samples. It is very likely that sample X has been adulteration with orange terpene fraction which lacks PMF components, and then was reconstituted by added important aroma chemicals such as linalool and decanal in order to give an acceptable GC profile.

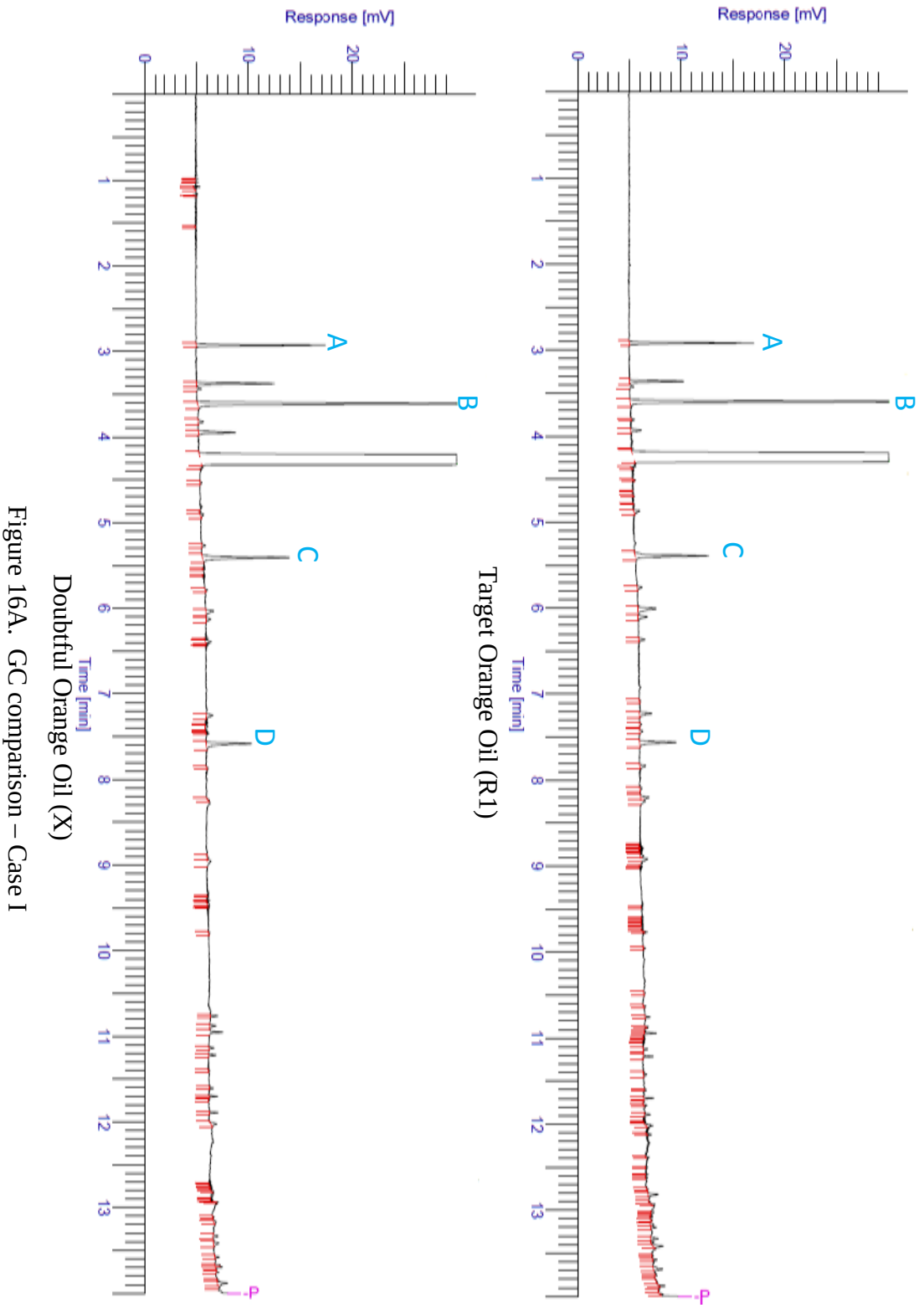
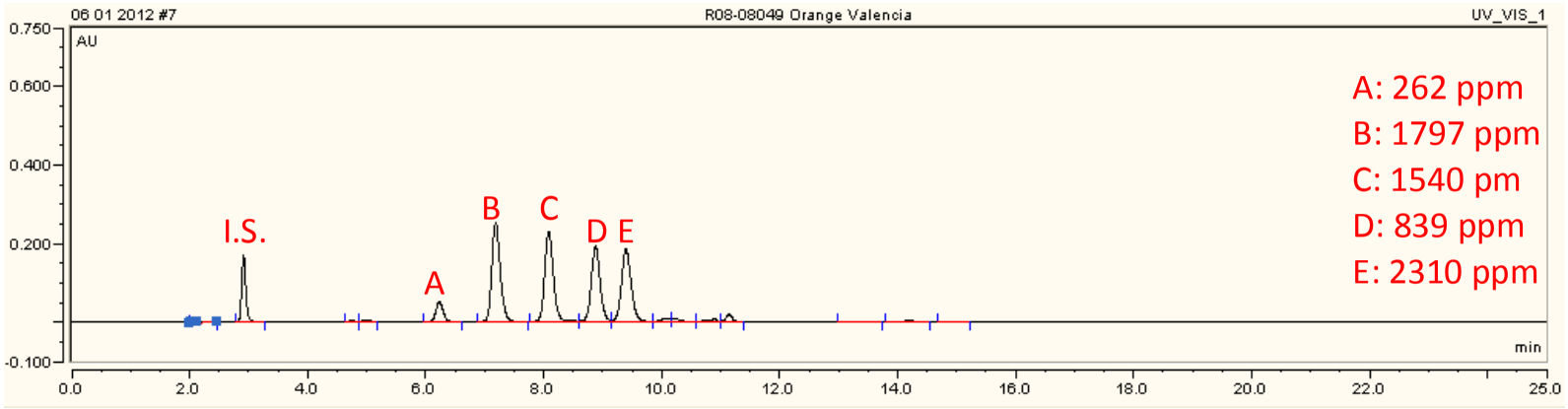
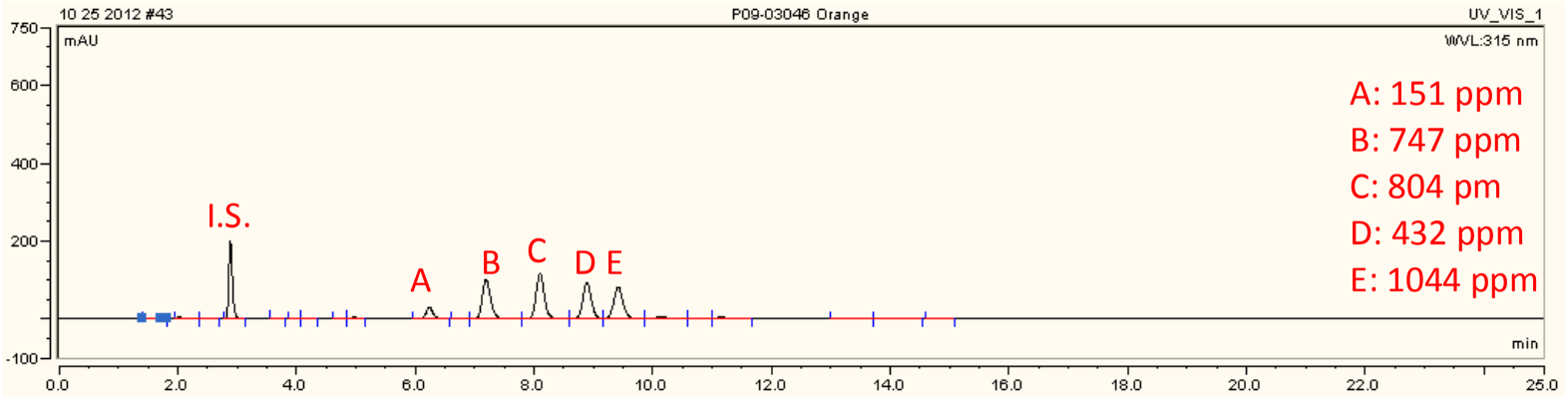


Figure 16A. GC comparison – Case I



Target Orange Oil (R1)



Doubtful Orange Oil (X)

Fig 16B. HPLC comparison – Case I

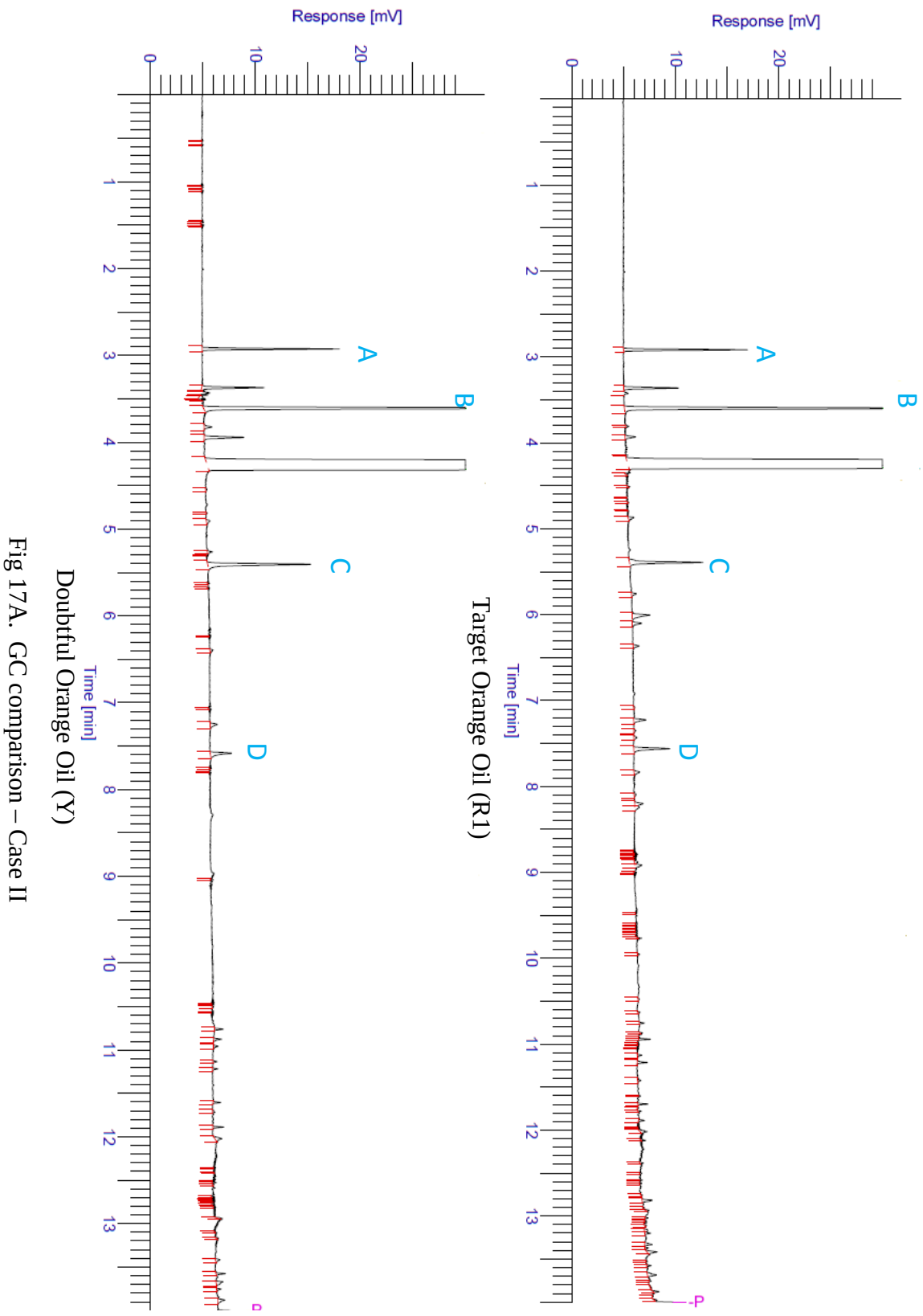
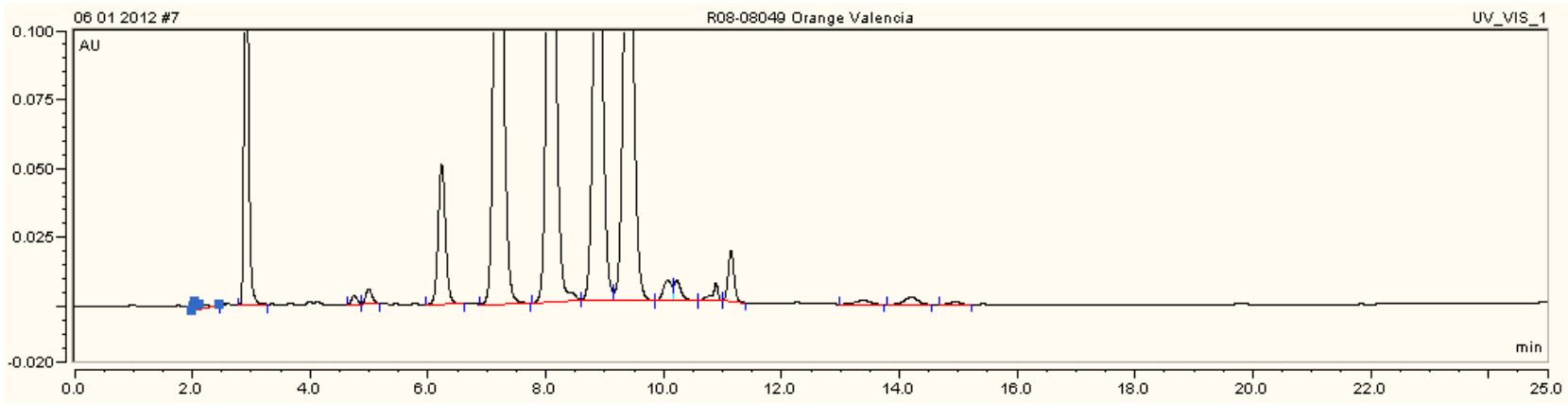
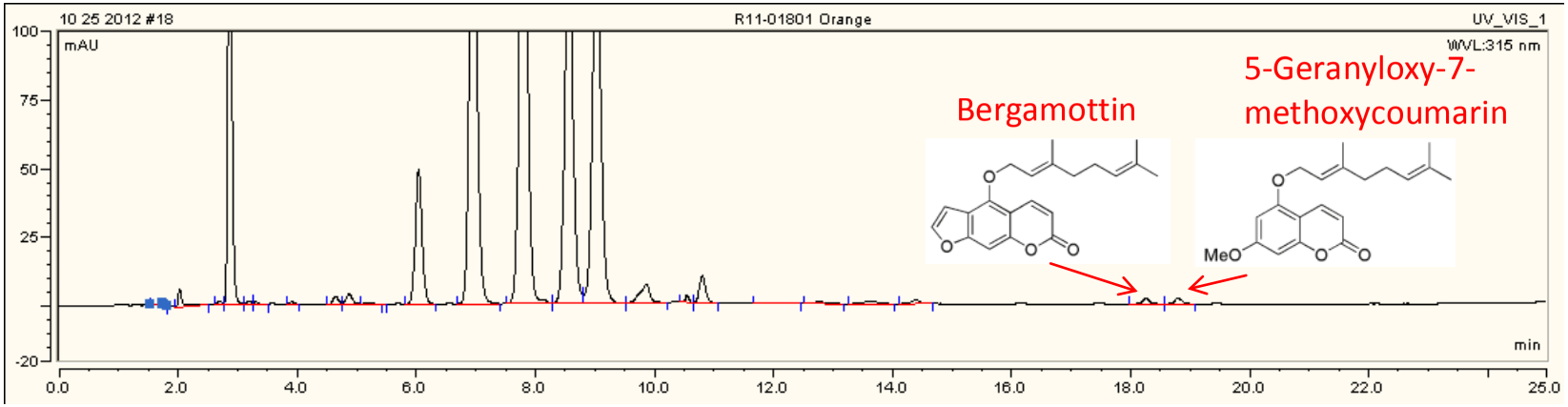


Fig 17A. GC comparison – Case II



Target Orange Oil (R1)



Doubtful Orange Oil (Y)

Fig 17B. HPLC comparison – Case II

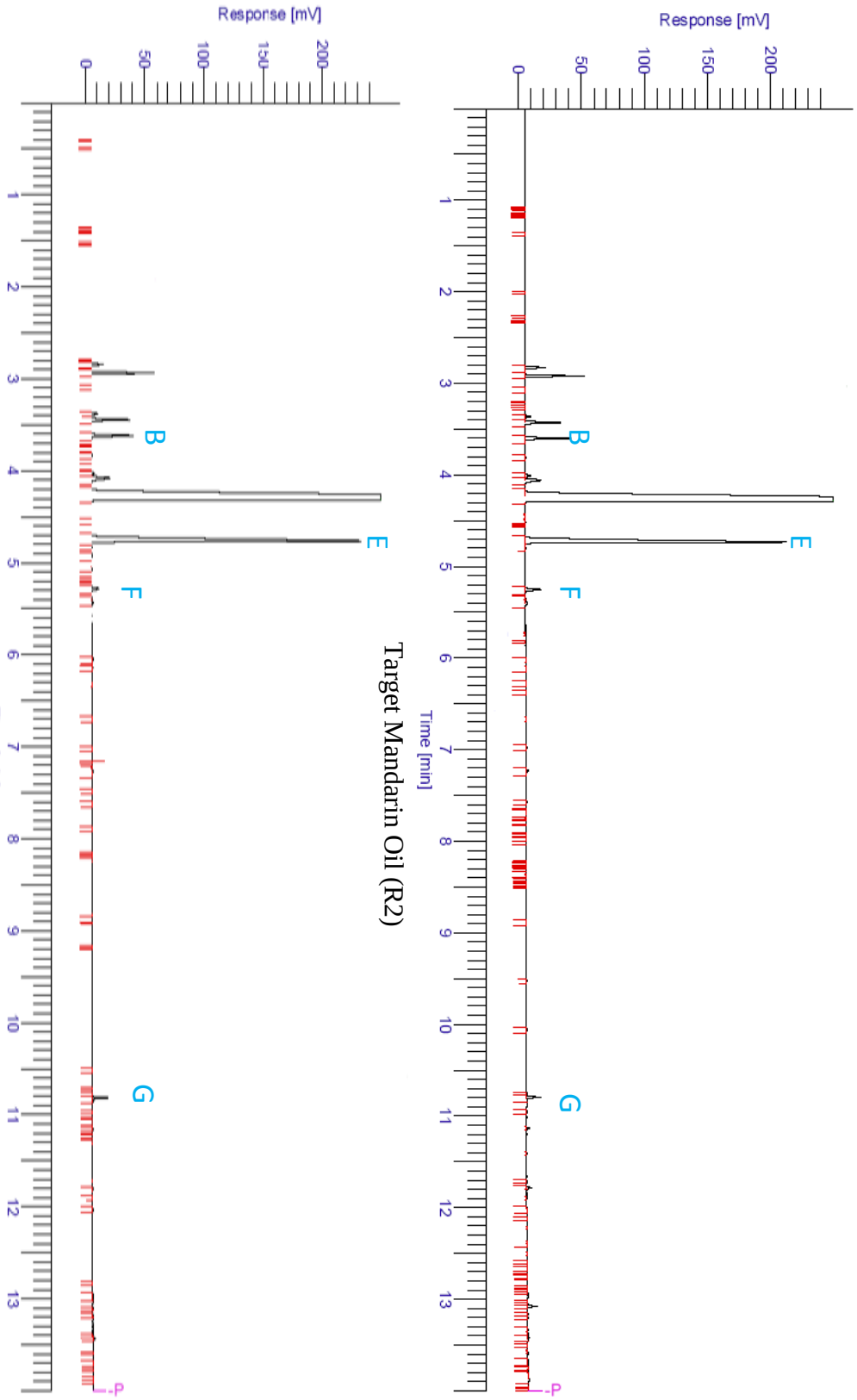
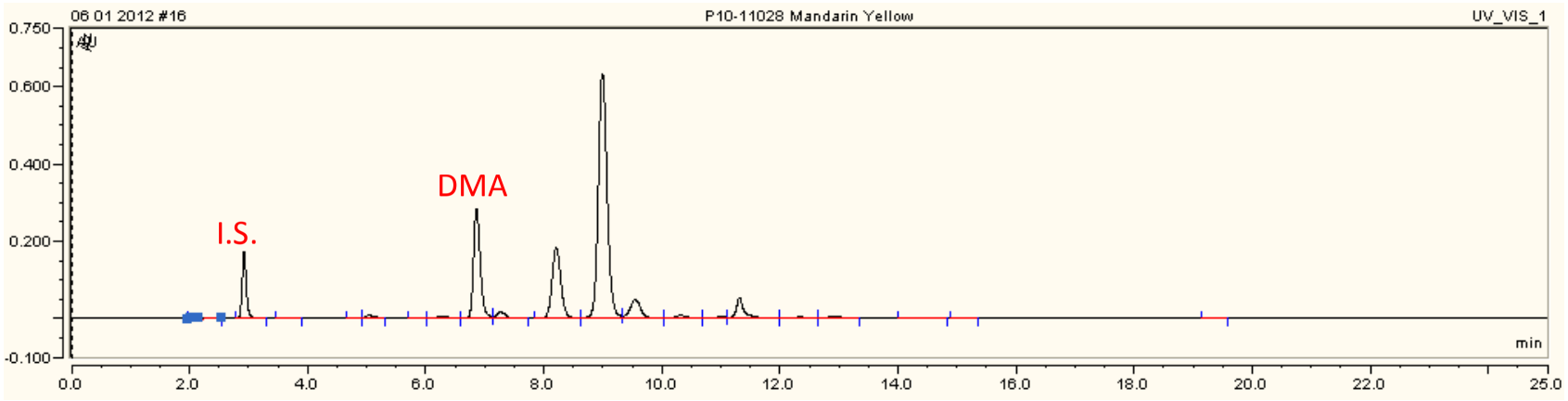
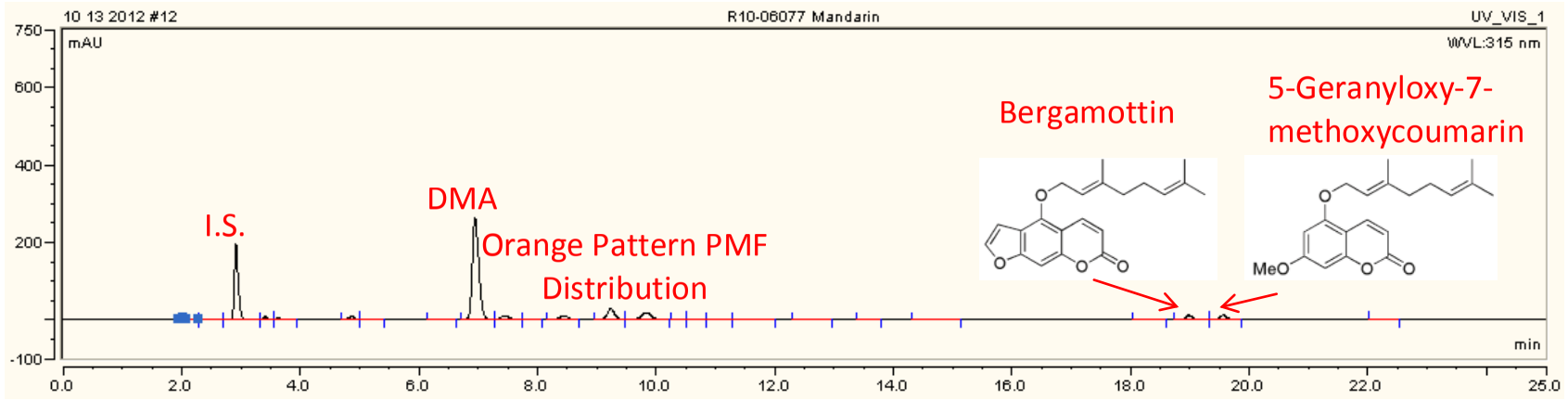


Fig 18A. GC comparison – Case III



Target Mandarin Oil (R2)



Doubtful Mandarin Oil (Z)

Fig 18B. HPLC comparison – Case III

Table 18A. GC peak ID and relative area percentage – Case I

	Peak ID	Sample R1	Sample X
A%	α-Pinene	0.53	0.48
B%	Myrcene	1.85	1.95
C%	Linalool	0.45	0.46
D%	Decanal	0.23	0.27

Table 18B. GC peak ID and relative area percentage – Case II

	Peak ID	Sample R1	Sample Y
A%	α-Pinene	0.53	0.49
B%	Myrcene	1.85	1.98
C%	Linalool	0.45	0.54
D%	Decanal	0.23	0.14

Table 18C. GC peak ID and relative area percentage – Case III

	Peak ID	Sample R2	Sample Z
B%	Myrcene	1.82	1.56
E%	γ-Terpinene	16.46	17.02
F%	Terpinolene	0.69	0.34
G%	DMA	0.46	0.47

2. Case II – Orange Oil

Another orange oil sample Y was analyzed and compared against the same target orange sample R1. The GC profile of sample Y was close to that of target sample with a significantly higher linalool level (Figure 17A and Table 18B).

However, HPLC analysis had shown more details. The two small peaks at the end of HPLC gram of sample Y were confirmed to be bergamottin and 5-geranyloxy-7-methoxycoumarin (Figure 17B), which belong to furocoumarin family and are not supposed to presence in orange oil. Bergamottin and 5-geranyloxy-7-methoxycoumarin are found at significant amounts in both lemon and lime essential oils (Fig 10) and their presence in orange oil had clearly suggested inter-species adulteration. Since the price of lemon and lime oil are generally higher that orange oil, orange sample Y might had been adulterated by cheap lemon fractions such as lemon washed oil and then its GC profile was adjusted by blending in important aroma chemicals.

3. Case III – Mandarin Oil

Mandarin oil sample R2 was from trusted grower thus authenticity was out of question. Mandarin oil sample Z was from a vendor and its authenticity was yet to be confirmed. GC and HPLC analyses were carried out like before. The GC profile of sample Z seemed to be acceptable (Figure 18A and Table 18C).

Detailed HPLC analysis had shown that this sample Z contained furocoumarins (bergamottin and 5-geranyloxy-7-methoxycoumarin) as sample Y did, which suggested presence of adulterants of lemon/lime origin. On the other hand, the peak areas of PMFs in sample Z were too evenly distributed (Figure 18B), which was not typically seen in

mandarin oil where tangeretin should always dominate. The relative ratio between major PMFs in sample Z suggested an orange origin rather than mandarin origin. Putting all the findings together, sample Z might have been heavily adulterated with or even made from orange fractions (considering the low PMF levels), then blended with lemon washed oil, and finally its volatile faction was reconstituted by adjusting chemical levels to achieve an unsuspicious GC profile.

From previous discussion, it is obvious that HPLC analysis bears advantages over GC analysis in citrus oil screening due to the nature of citrus oils and the demand of essential oil industry. The three low-grade oil samples discussed previously were representative throughout the industry based on the author's experience. It is common that sample evaluators discover doubtful citrus oils on regular basis. With the right knowledge and analytical approaches, capital losses caused by adulteration should be minimized.

The volatile fraction of citrus oils has been well studied and comprehensive GC database is available nowadays in most flavor companies. Outlying peak percentage results of key aromatic chemicals will trigger a deeper investigation on authenticity. To the author's view, it is urgently necessary to establish similar HPLC database for citrus essential oils in which important oxygenated heterocyclic compounds are quantified. Such database will be applied to routine quality control tasks for incoming oil samples. To achieve this goal a sufficiently large volume of citrus oil samples from industry is definitely required.

F. PCA Study on Orange, Mandarin and Tangerine Oils

Orange oils, mandarin oils, and tangerine oils were chosen for statistical analysis because these oils are taxonomically and compositionally closed to one another. This is also the very reason which led to frequent inter-species adulteration among these three groups. Data suggested that for the past few years, the amount of mandarin oil consumed was considerably larger than its actual production figure. Such fact led us to question the authenticity of the industrial mandarin oil on the market.

Table 19. Agronomic groups of various citrus fruits
(Dugo *et al.*, 2002)

Species	**Variations**
Sweet orange	Common orange, Navel orange, Blood orange, Acidless orange
Mandarin	Satsuma mandarin, Tangerine, Clementine, Mediterranean mandarin
Lemon	Eureka, Lisbon, Verna, Femminello
Grapefruit	Marsh, Redblush, Star Ruby
Lime	Mexican lime, Tahiti lime
Bitter orange	Seville, Granito, Chinotto, Bergamot
Citrons	Fingered citron, *Citrus medica*

1. Taxonomical Information

Orange (*Citrus sinensis*) is believed to be a hybrid species from mandarin and pummelo (Table 3). The color of its essential oil ranges from yellow to orange to reddish orange depending on the starting fruit. Mainly four groups exist in this species:

1. Common orange: also known as white orange. Famous cultivars include: “Valencia” from USA, “Pera” from Brazil, “Cadenera” from Spain, and “Jaffa” from Isarel.

2. Navel orange: name derived from the characteristic navel at the stylar end of the fruit. Famous cultivars are “ Washington” , “ Navelina” and “ Thompson”

3. Blood orange: the juice of the fruit is intense red due to the presence of anthocyanin in the fruit. Famous cultivars are “ Moro” , “Tarocco” and “Double fina”

4. Acidless orange: also known as sugar orange because of the low acidity of its juice. Two cultivars are “Imperial” and “Succari”

Describing mandarin (*Citrus reticulalta*) oil is difficult compared to other citrus oils. Botanically speaking mandarin group is very complex in that it is composed of several subgroups and a number of hybrids. The difference between mandarin and tangerine is blurry and the dominating taxonomical view regards tangerine (*Citrus tangerina*) as a subspecies of mandarin.

1. Satsuma group (*Citrus unsbiu*): this is the main citrus grown in Japan. Cultivars include “Oawri” and “Okitsu”.

2. Tangerine (*Citrus tangerina*) and clementine (*Citrus clementina*) group: the color of the rind is deep in this group. “Dancy” is the most famous tangerine cultivar while in clementine cultivar “Fina” and “Monreal” are well known.

3. Mediterranean mandarin (Citrus *deliciosa*): common mandarin grown in Mediterranean basin. Some cultivars are “Mediterranean”, “Willowleaf” and “Avana”.

4. Other mandarins: other species and hybrids such as King mandarin (*Citrus nobilis*) and Temple mandarin (*Citrus temple*).

Despite the taxonomical classification, the industry regards tangerine oil and mandarin oil separately as two independent commodities. From the industry’s point of view mandarin oil and tangerine oil have different pricings, profiles, and applications. Therefore it is advisable to treat industrial orange oil, mandarin oil, and tangerine oil as three independent groups in statistical analysis.

2. Detailed Analysis

IBM® SPSS® Statistics 20 was utilized for PCA study. 87 orange oils, 16 tangerine oils and 20 mandarin oils were subjected to analysis. Six variables, namely content of sinensetin (ppm), content of tetra PMF (ppm), content of nobiletin (ppm), content of tangeretin (ppm), content of hepta PMF (ppm), and ratio between hepta PMF and tetra PMF (expressed as HoT for short), were chosen to classify all 123 oil samples.

The correlation coefficient matrix was shown in Table 20. A very positive correlation (0.909) was observed between sinensetin and tetra PMF. Positive correlations were also found between sinensetin and hepta PMF (0.783), and between tetra PMF and

hepta PMF (0.866). Negative correlations were observed between tangeretin and sinensetin (-0.536), tetra PMF (-0.606), and hepta PMF (-0.419). HoT was observed to have positive correlations with nobiletin (0.318) and tangeretin (0.601), and negative correlations with sinensetin (-0.561), tetra PMF (-0.612), and hepta PMF (-0.240). Correlation that greater than 0.3 is indicative of possible cluster from group of variables. The KMO value was a bit low at 0.603, however Bartlett's test of sphericity gave an associated P value (Sig. in the table) of <0.001 (Table 21). All of the results above suggested that a valid PCA can be performed.

Table 20 Correlations between variables

	Sinensetin	TetraPMF	Nobiletin	Tangeretin	HeptaPMF	HoT
Sinensetin	1.000	.909	.095	-.536	.783	-.561
TetraPMF	.909	1.000	-.007	-.606	.866	-.612
Nobiletin	.095	-.007	1.000	.745	.148	.318
Tangeretin	-.536	-.606	.745	1.000	-.419	.601
HeptaPMF	.783	.866	.148	-.419	1.000	-.240
HoT	-.561	-.612	.318	.601	-.240	1.000

Table 21 KMO and Bartlett's test result

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		.603
Bartlett's Test of Sphericity	Approx. Chi-Square	885.041
	Df	15
	Sig.	.000

Two principle components were extracted from the six sets of variables as shown in Table 22. Scree plot had also confirmed that only two components had eigenvalues greater than one and the steepest drop in eigenvalue took place between the first two components (Figure 19). The first principle component (accounts for 58.998% of variance) and the second principle component (accounts for 27.482% of variance) produced a total of 86.48% cumulative variance of the whole data set. This indicated that majority of variability had survived the dimension reduction process.

Table 22 Variances of six components

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	3.540	58.998	58.998	3.540	58.998	58.998
2	1.649	27.482	86.480	1.649	27.482	86.480
3	.599	9.983	96.463			
4	.143	2.386	98.849			
5	.037	.609	99.458			
6	.033	.542	100.000			

Extraction Method: Principal Component Analysis.

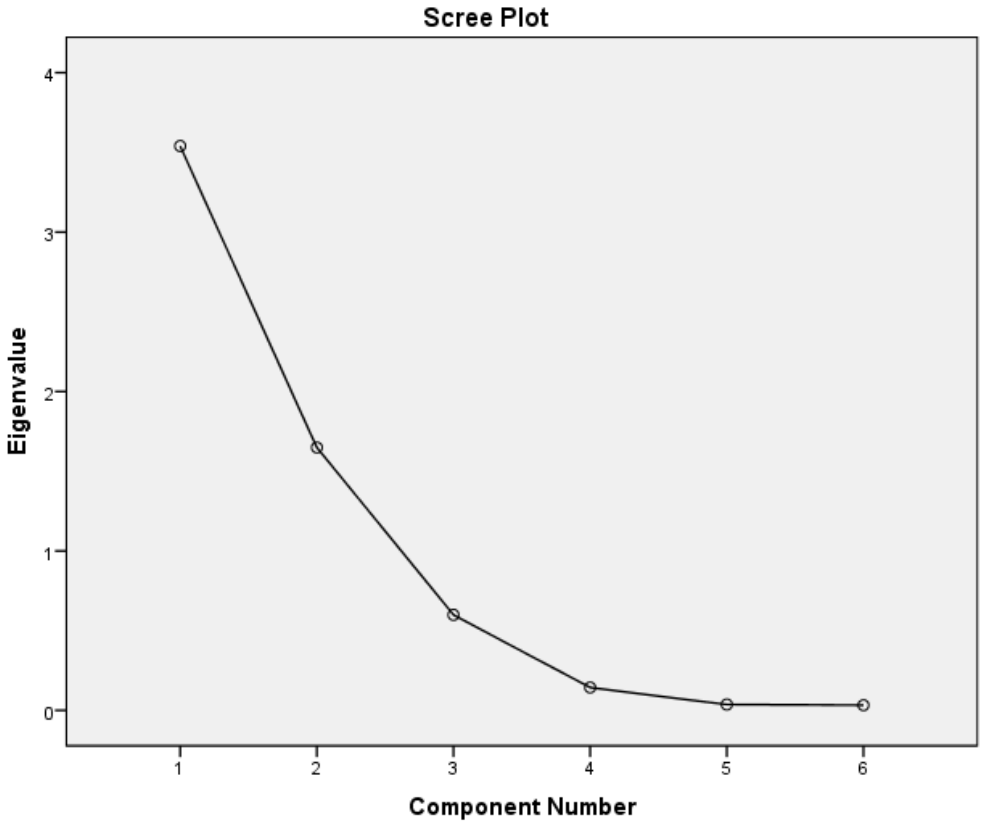


Figure 19. Scree plot of all six components

Standard direct Oblimin with Kaiser Normalization method was applied in the rotation process. The projection of six variables on the first two principle components was shown in Figure 20. It was obvious that sinensetin, tetra PMF and hepta PMF formed a cluster which loaded heavily on the first principle component (and low loadings on the second) while nobiletin and tangeretin loaded primarily on the second principle component (and moderate loadings on the first). HoT variable had a negative loading (-0.455) on the first principle component and a positive (0.543) on the second principle component (Table 23).

Table 23. Component scores for six variables

	Component	
	1	2
Sinensetin	.951	-.018
TetraPMF	.957	-.106
Nobiletin	.309	.984
Tangeretin	-.368	.819
HeptaPMF	.931	.156
HoT	-.455	.543

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.

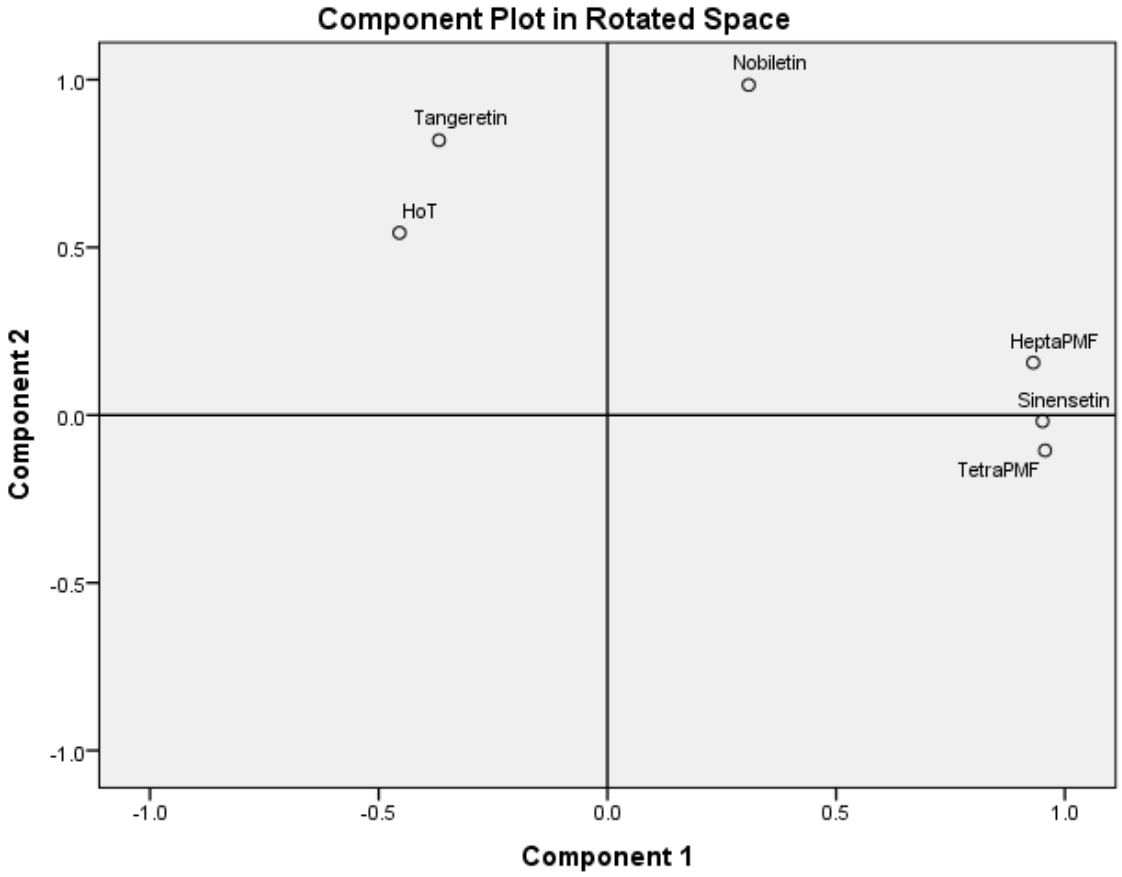


Figure 20 Two-dimensional projection chart of the six variables

Table 24. Component score coefficients for the six variables

	Component	
	1	2
Sinensetin	.306	.025
TetraPMF	.305	-.020
Nobiletin	.135	.514
Tangeretin	-.090	.406
HeptaPMF	.306	.113
HoT	-.127	.262

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.

The components score coefficients of the six variables were shown in Table 24. The individual component score values (CS) can be calculated as followed:

$$CS_1 = (0.306)Z_{(sinensetin)} + (0.305)Z_{(tetraPMF)} + (0.135)Z_{(Nobiletin)} + (-0.090)Z_{(tangeretin)} + (0.306)Z_{(heptaPMF)} + (-0.127)Z_{(HoT)}$$

$$CS_2 = (0.025)Z_{(sinensetin)} + (-0.020)Z_{(tetraPMF)} + (0.514)Z_{(Nobiletin)} + (0.406)Z_{(tangeretin)} + (0.113)Z_{(heptaPMF)} + (0.262)Z_{(HoT)}$$

Where $Z_{(variable)}$ is the standardized variable score and can be expressed as:

$Z_{(variable)}$ = (individual variable value - mean value of the population)/standard deviation of the population

Plot individual component scores of all 123 samples on the first two principle components we got Figure 21: squares represented orange oils, triangles represented tangerine oils, and circles represented mandarin oils. For all 123 citrus oils, blue color indicated samples with normal PMF values and red color indicated outlying samples. Samples with PMF values significantly deviated from ranges established in Table 17 were regarded as “outliers” and discussed separately later.

It can be observed from Figure 21 that orange oil group, mandarin oil group, and tangerine oil group (the tree oval regions in the Figure 21) can be conspicuously outlined by their respective regular samples. Most of the outlying samples were clearly separated from the oval regions which indicated acceptable PMF ranges. The tangerine group located geographically in-between orange group and mandarin group, which was supported by the fact that the PMF levels in tangerine oils are usually higher than those in orange oils and lower than those in mandarin oils. Comparing to tangerine and mandarin groups, the orange group was more positively loaded on the first principle component which emphasize on sinensetin, tetra PMF, and hepta PMF (as shown in Figure 20). Such observation was supported by the fact that orange oil contains higher levels of these three PMFs (as shown in Figure 15). Similarly, the mandarin group was primarily loaded on the second principle component which emphasize on nobiletin and tangeretin (as shown in Figure 20). This was in line with the fact that mandarin oils contain highest levels of these two PMFs (as shown in Figure 15).

3. Individual Sample Discussion

Some of the outlying samples or seemingly regular samples were of particular interests to the author. Six out of them were chosen and discussed in detail below:

1) Orange Oil Sample A (coordinate 2.465, 0.720)

	Sinensetin	Tetra PMF	Nobiletin	Tangeretin	Hepta PMF
Sample A	272 ppm	1827 ppm	1603 ppm	859 ppm	2360 ppm

This orange oil was located on the top-right corner of the orange group in Figure 15. All five of its PMF levels had exceeded the ranges outlined in Table 17. Its abnormally high PMF values had triggered the interest of the author to understand its history.

It was unlikely that this oil had been adulterated by oil fractions from another species. The relative ratios among five PMFs gave a perfect orange pattern. No oxygenated heterocyclic components that indicate inter-species adulteration (furocoumarins) were observed in the HPLC-UV gram of sample A. It was also unlikely that this oil has been diluted by orange fraction since such practice definitely decreases PMF levels instead of raising them.

To the best of the author's knowledge, this oil might be extracted from a unique cultivar of orange which is characterized by very high PMF levels in its peel. Another explanation is that this oil had undergone an exhaustive extraction. As a result larger

amount of PMFs in the peel was carried over into the oil. Unfortunately the author did not have adequate information to further trace down the source of this oil.

2) Mandarin Oil Sample B (coordinate -1.272, 0.330)

	Sinensetin	Tetra PMF	Nobiletin	Tangeretin	Hepta PMF
Sample B	42 ppm	227 ppm	1169 ppm	2009 ppm	728 ppm

This mandarin oil sample was found located within the tangerine group. Its PMF values conformed to ranges for tangerine oil (Table 17). The oxygenated heterocyclic study clearly suggested a tangerine origin of this sample, in spite of the fact that sample description stated “Mandarin oil green”.

One interesting finding was that this sample contained a very high level of DMA (data not shown). DMA is a characteristic compound found in mandarin oil. DMA level in tangerine oil is usually negligible. The presence of DMA had ruled out the possibility of inadvertent mislabeling. It was very likely that the manufacture of the oil had added high dose of DMA into tangerine oil hoping to achieve an acceptable mandarin GC profile. However analysis on PMF levels had unveiled the true identify of this oil.

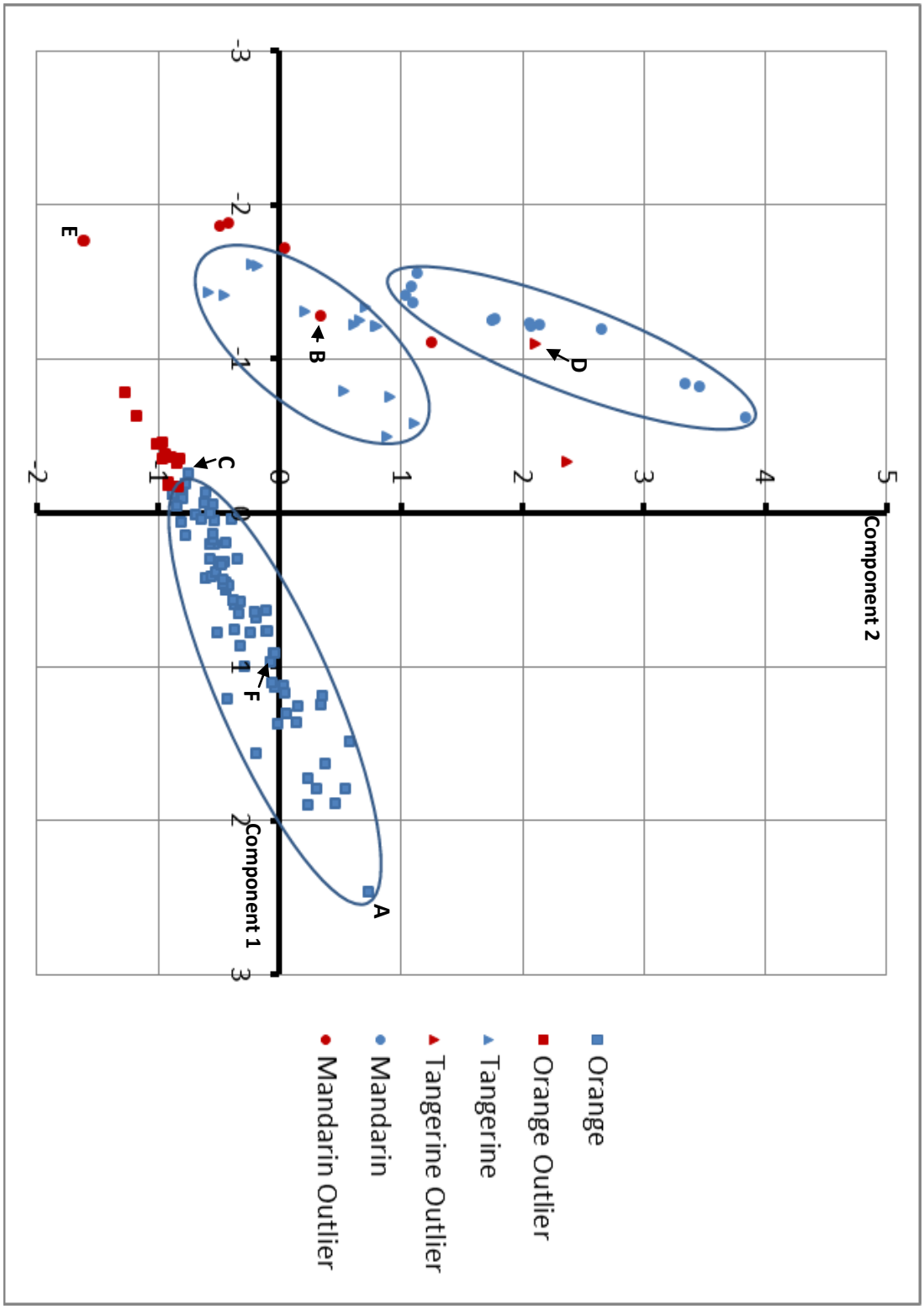


Figure 21. Projection of all samples on the first two principle components

3) Orange Oil Sample C (coordinate -0.249, -0.757)

	Sinensetin	Tetra PMF	Nobiletin	Tangeretin	Hepta PMF
Sample C	88 ppm	847 ppm	805 ppm	510 ppm	1339 ppm

Comparing to the acceptable ranges in Table 17, this oil had marginal levels of tetra PMF, nobiletin, tangeretin, a low level of sinensetin, and a normal level of hepta PMF. Sample C was not marked as an outlier during the initial screening because of the ambiguity in its PMF values. However, on the graphical projection figure sample C was clearly located outside of the normal range of orange group. Other analytical approaches were required for sample approval/rejection.

From this case it was clear that graphical assistance from Figure 21 is necessary in determining an outlying sample. Numerical comparison (Table 17) sometimes can be inconclusive and misleading. Graphical projection chart provided a much more direct idea on marginal samples than numerical comparison, especially in multi-dimensional variable cases such as PMF values in citrus oils.

4) Tangerine Oil Sample D (coordinate -1.095, 2.106)

	Sinensetin	Tetra PMF	Nobiletin	Tangeretin	Hepta PMF
Sample D	89 ppm	206 ppm	1493 ppm	2358 ppm	1787 ppm

This tangerine oil was regarded as outlying during the initial screening due to its abnormally high hepta PMF value (Table 17). On the projection plot (Figure 21) this sample had unexpectedly fallen within the mandarin group. However, the PMF values of sample D did not agree with mandarin PMF ranges as well.

The abnormally high level of hepta PMF had ruled out the possibility of dilution with citrus oil fractions. No indication of inter-species adulteration was given by HPLC-UV analysis. It was likely that this sample was extracted from an unusual cultivar of tangerine. As discussed before the tangerine group is a very complex one with many known or unknown cultivars.

From this case it can be concluded that the graphical projection figure has its own limitations as well. If we rely solely on Figure 21 without referring to the numerical values, sample D might be misidentified as mandarin oil. This limitation was intrinsically derived from the PCA process. PCA is essentially a dimension reduction process while trying to maintain as much variability as possible. In this case five sets of variables have been reduced to two principle components with 86.48% of the variability retained (Table 22). The other 13.52% of variability was lost during PCA process. PCA helps to resolve complicated multi-variable dataset by reducing the number of dimensions but minor variability lost is inevitable.

It can be concluded from sample C and sample D that both numerical ranges (Table 17) and graphical projection chart (Figure 21) should be referred to before making a decision. Numerical ranges, although indirect and laborious, represent the complete dataset and contain all the information. Graphical projection chart, which is favored for

its convenience and straightforwardness, suffers from incomplete variability and might be misleading.

5) Mandarin Oil Sample E (coordinate -1.768, -1.617)

	Sinensetin	Tetra PMF	Nobiletin	Tangeretin	Hepta PMF
Sample E	20 ppm	89 ppm	76 ppm	127 ppm	220 ppm

This mandarin oil sample was the very one that had been discussed in previous section as case III. It contained extremely low levels of PMF and its PMF distribution pattern followed neither mandarin oil nor orange oil. However, this sample had a roughly acceptable GC profile with decent level of DMA and α-sinensal (Figure 18A).

The abnormally low PMF levels clearly suggested that the sample had been vastly diluted with terpenes or distilled fractions which lacked oxygenated heterocyclic components. Its PMF pattern (high hepta PMF) suggested that the diluent or part of the diluent was from orange derivative. Figure 18B in case study III also suggested that part of the diluent had lemon/lime origin due to the presence of furocoumarins.

With the information gathered from non-volatile components study, it can be concluded that mandarin sample E was obtained from dilution of mandarin oil with large amount of terpenes or distilled fractions from orange and lemon. Then its GC profile was restored by adding calculated amount of DMA and other characteristic mandarin peaks. The level of α-sinensal in sample E suggested that the quality of starting mandarin oil

must be decent since high purity α-sinensal is difficult to obtain with any reasonable pricing.

6) Orange Oil Sample F (coordinate 0.971, -0.085)

	Sinensetin	Tetra PMF	Nobiletin	Tangeretin	Hepta PMF
Sample F	225 ppm	1215 ppm	1391 ppm	623 ppm	1628 ppm

This orange oil sample is the very one that had been discussed previously in case study II. In Figure 21 its coordinate was located on the center of orange region. The levels of its five PMFs conformed to the ranges in Table 17 very well. Based on PMF level study sample F seemed to be genuine.

However, detailed HPLC-UV chromatogram had suggested otherwise (Figure 17B). The presence of bergamottin and 5-geranyloxy-7-methoxycoumarin had proved that sample F contained fractions of lemon/lime origin. In spite of its perfect PMF values, sample F was confirmed as an adulterated one. Like mandarin sample E, the starting orange oil for sample F is likely to be of decent quality to give medium PMF values even after dilution.

It can be concluded from sample E and F that it is dangerous to rely solely on PMF levels when assessing the non-volatile fraction of orange, mandarin, and tangerine oil samples. In HPLC-UV gram interpretation, the first thing analyzer should check is the presence of foreign peaks. As was illustrated in sample E and F, the presence of peaks that were exclusively derived from other citrus species was conclusive and led to

automatic sample rejection. The PMF value study should be carried out after confirmation of the identities of all the peaks. Results that entirely based on PMF values can be inefficient (sample E) or misleading (sample F).

VI. CONCLUSION

This study focused on the adulteration practices that have been plaguing the essential oil industry for years. Different analytical approaches established to assess citrus oil quality, whether crude or sophisticated, were reviewed and compared against each other in this study. Among all these approaches GC and HPLC studies were discussed in details and their advantages and limitations were illustrated. Due to its low odor contribution, diversified pattern, high stability, and limited accessibility, the non-volatile fraction (oxygenated heterocyclic components in the sense of citrus oil) is normally left unattended during most adulteration practices. Therefore by studying non-volatile fraction researchers are able to real the adulterations that have been imposed on the oil samples. On the other hand, the volatile fraction of citrus oils is much easier to manipulate thus inappropriate for citrus oil fingerprinting. In conclusion, HPLC analysis has its intrinsic advantages over GC analysis in authenticity study of citrus oils.

Care must be taken when interpreting the oxygenated heterocyclic components from HPLC-UV grams. Apart from illegal practices, other factor might also influence the distribution of these components. These factors, if not taken into consideration, might lead to false negative results. Firstly the extraction technique can affect oxygenated components levels. Exhaustive extraction usually results in higher levels of oxygenated heterocyclic components. Secondly, storage condition can also affect the level of these components in that these oxygenated heterocyclic components tend to fall out under cold temperatures. Lastly, the distribution of oxygenated heterocyclic components is essentially genetically determined. Less known cultivars or hybrids can give seemingly abnormal oxygenated heterocyclic components profile. To sum up, samples that show

abnormally low oxygenated heterocyclic levels should be marked as suspicious; samples with high oxygenated heterocyclic levels might be resulted from difference extraction techniques or novel cultivars, which are rarely seen in large scale standardized industrial samples.

To the best of the author's knowledge, this study has for the first time outlined the acceptable level of the five major polymethoxyflavones from industrial orange, mandarin, and tangerine oils that appeared in the United States in the past five years. The author believes the data presented in this study can properly represent citrus oils that are currently circulating in the US market. By publishing this research the author is hoping that the data and conclusion presented in this study could help the industry protect itself from poor grade oils and academy better understand the nature of citrus essential oils.

This study has given detailed statistical study on PMF levels in orange, mandarin, and tangerine oil samples. Furocoumarin study in lemon, lime, and grapefruit oil samples will be completed in near future. However, it is not advisable to perform PMF level study at the early stage of sample evaluation. The appearance (color, haziness, sedimentation, *etc*.) and organoleptic properties (odor and taste in sugar/acid solution) of an incoming sample should always be evaluated first because they are the most important attributes of citrus oils and usually takes little time to complete. Then GC (volatile fraction) and HPLC (non-volatile fraction) analysis can be carried out simultaneously to arrive at a complete idea regarding the composition of the oil sample. Qualitative assessment is more conclusive in sample rejection than quantitative assessment thus should be carried out first. PMF level study (Table 17 and Figure 21) should be performed at later stage in

sample evaluation when appearance, organoleptic, physical properties (specific gravity, refractive index, and specific rotation), and GC tests all have given the green lights.

An ideal QC flow chart in the author's mind was outlined in Figure 22. The author is hoping the data and procedure presented in this study can help the essential oil industry have a better understanding about the citrus oils on their potential purchase list.

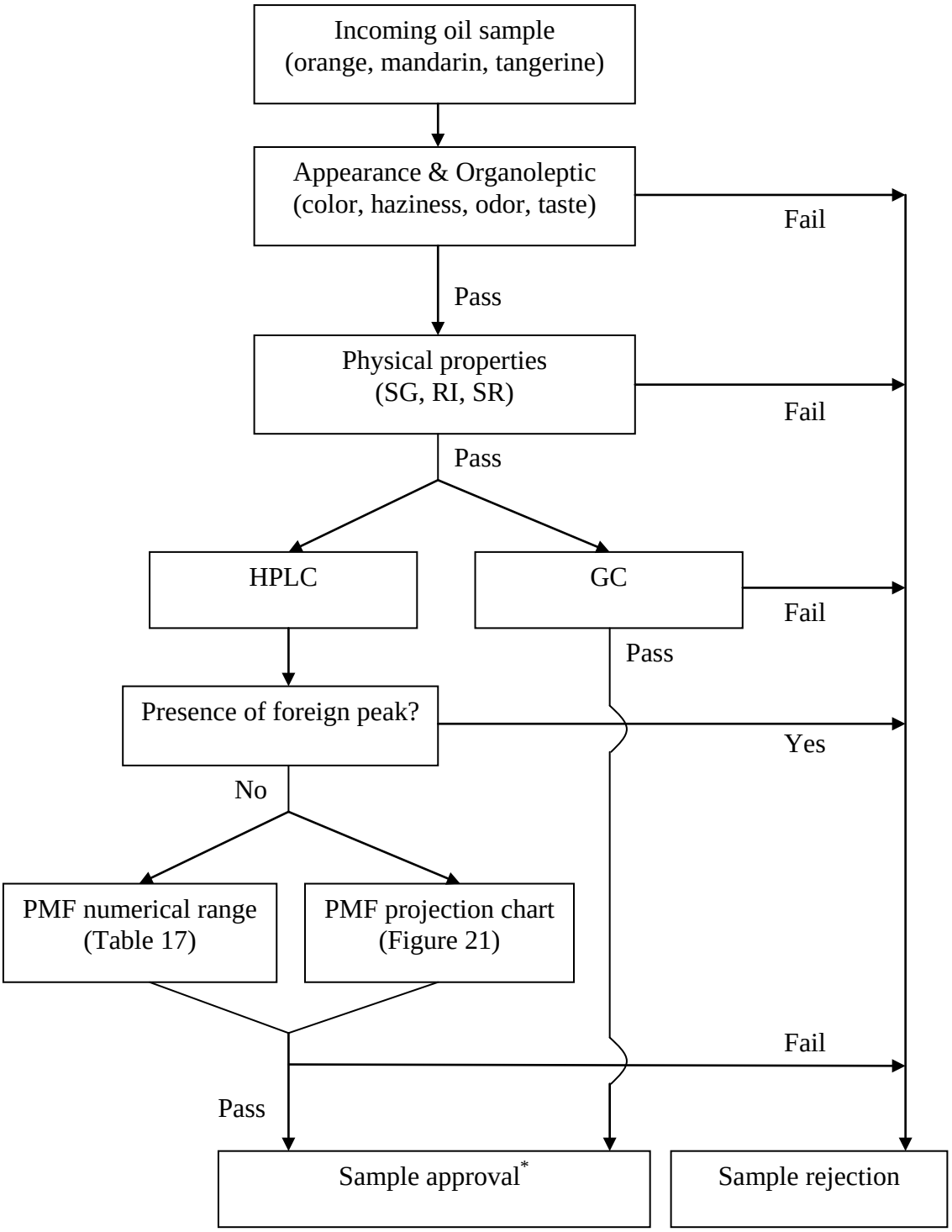


Figure 22. Proposed QC flow chart for citrus oil evaluation
*: only sample that passes both GC and HPLC test will be approved

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