# IDENTIFICATION OF COMPOUNDS RESPONSIBLE FOR THE CHARACTERISTIC "SOY SAUCE" FLAVOR OF TRADITIONAL CHINESE LIQUOR

BY

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DISSERTATION

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## Abstract

Soy sauce aroma liquor is one of the most popular types of traditional liquors in China, with the annual commercial value as high as 8.2 billion USD. The most famous soy sauce aroma liquor is the Chinese national liquor Moutai (MT), which was first developed around 206 BC. The flavor of MT is unique and highly appreciated by both experts and consumers. It received gold awards at the 1915 San Francisco "World's Fair" and at the 12$^{th}$ International Food Fair in Paris in 1986, which is testimony to the outstanding flavor and premium quality of this liquor product.

However, significant as it is economically and culturally to China, knowledge about the characteristic flavor profile of MT is quite limited. Many scientists have studied this topic for decades and raised several hypotheses with respect to the compounds that are responsible for MT's unique and characterizing flavor. However, despite much effort the compounds actually responsible for the characterizing "soy sauce" aroma of soy sauce aroma liquors are still unknown. The lack of this information has hindered the optimization of the brewing and blending technology to achieve liquors with better and more consistent aroma profiles.

This research aims to identify the key and characterizing odorants in soy sauce aroma liquor in the case of MT. Through the use of advanced separation and analytical methods, 143 odor-active components were detected. The relative potencies of the odorants were assessed by gas chromatography-olfactometry (GC-O) and aroma extract dilution analysis (AEDA). Based on the results of AEDA, the most potent odorants in MT were: acetal; ethyl 2-methylpropanoate; ethyl butanoate; ethyl 2-methylbutanoate; ethyl 3-methylbutanoate; ethyl pentanoate; ethyl 2-methylpentanoate; ethyl 4-methylpentanoate; isopentyl hexanoate; β-damascenone and ethyl phenylpropionate, 2-phenylethanol, 3-methybutanal, 2-methyl-3-furanthiol, 2-methyl-3-(methyldithio)furan, dimethyl trisulfide and 3-hydroxy-4,5-hydroxy-2(*5H*)-furanone (sotolon). Four sulfur-containing odorants which might provide "savory" top notes to the flavor of MT were identified as 2-methyl-3-furanthiol (MFT), 2-methyl-3-(methyldithio)furan (MFT-MT), 2-furfurylthiol, and *bis*(2-methyl-3-furyl) disulfide (MFT-MFT). To the best of our knowledge, MFT, MFT-MT and MFT-MFT have not been previously identified in Moutai or any other type of Chinese soy sauce aroma liquor.

Thirty-nine of the selected potent odorants were quantitated or semi-quantitated in MT. Among these, 35 were determined by stable isotope dilution analysis (SIDA)-gas chromatography-mass spectrometry (GC-MS). The concentration of each odorant was used for the calculation of its odor activity value (OAV; ratio of the concentration of an odorant to its odor detection threshold). Based on the flavor dilution (FD) factors from AEDA and OAVs, 36 potent odorants were selected for construction of an aroma recombinant model of MT. The model was created by adding 36 high purity standards at appropriate concentrations to matrix consisting of 53% alcohol by volume (ABV). Triangle difference tests were performed using 24 sensory panelists for the purpose of determining whether the aroma recombinant model differed in terms of overall aroma from the original MT liquor. According to the results, among 48 judgements, 19 answers were correct, which indicated that the aroma model and the original liquor product did not differ significantly ($P \leq 0.05$). Thus, these selected 36 odorants in the concentration determined in this study provide the typical aroma of the soy sauce aroma liquor MT.

This project provides a better understanding of the complex flavor chemistry system of Moutai. Some important are the odorants are identified herein for the first time, including several with very low odor detection thresholds which contribute savory, meaty and beefy top notes to the overall aroma profile of Moutai. The aroma recombination model achieved through this project was proven to have no detectable aroma difference from the original liquor, which indicates that the essential characteristic odorants responsible for Moutai-flavor were successfully identified and accurately quantitated.

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# Chapter 1: Introduction

Flavor is the integrated and nearly simultaneous response to the perception of taste, aroma and somatosensory (nerve) stimuli present in the oral and nasal cavities, generally as a consequence of the consumption of a food or beverage[1]. Although the peripheral sensory organs involved in the detection of taste and smell stimuli are distinct, their signals are integrated in the orbitofrontal and other areas of the cerebral cortex of the brain to generate the perception of "flavor"[2]. Most flavor researchers agree that in most instances olfaction (aroma or odor) plays the predominant and characterizing role in food flavor quality, including recognition and overall food acceptance [3]. This is true in the case of most distilled spirits, and especially for the traditional Chinese liquors including the soy sauce aroma liquors which are highly aromatic.

The various types of distilled beverages and liquors produced worldwide involve the use of different fermentation technologies, including use of different cultures, substrates, distillation methods and aging practices. These processes are often highly developed and sometimes even prescribed or regulated across the different types of spirts to achieve a consistent and characteristic flavor for each type of spirit. The potent odorants responsible for the unique flavor of a spirit or liquor are mainly derived through the fermentation and aging processes and sometimes even directly from the substrate materials. The flavor of most spirits is caused by the volatile odor-active substances; however, in some cases, e.g. whiskies, some taste-active components are extracted from the containers (e.g. oak) used for aging the spirit. Traditional Chinese distilled liquors (baijiu) differ from wood-aged spirits because they are usually aged in pottery. For this reason, the extraction or generation of taste-active components in baijiu is quite limited. That said, baijiu is not totally devoid of non-volatile substances. For example, lichenysin, a cyclooctapeptide produced by *Bacillus licheniformis*, can modulate the aroma-profile of some baijius by selectively changing the partitioning of the odorants via hydrogen bonding[4,5].

The various kinds baijius are roughly classified into 12 groups based on their typical or expected flavor attributes, which include: soy sauce aroma liquors (Moutai flavor liquors), strong aroma liquors (Luzhou-flavor liquors), Fen-flavor liquors, rice-flavor liquors, mixed-flavor liquors, Feng-flavor liquors, Fuyu-flavor liquors, Dong-flavor liquors, site-flavor liquors, Yubingshao-

flavor liquors, sesame-flavor liquors and Laobaigan-flavor liquors[6]. The grouping of these liquors is largely based on their unique and distinguishing flavors. This is possible because each baijiu type possesses a unique set of potent odorants which determines its characteristic flavor. For instance, ethyl hexanoate is the main characterizing odorant of strong aroma liquors, while ethyl acetate is responsible for the characteristic flavor of Fen-flavor liquors[7] and methional and ethyl hexanoate are important for the characteristic flavor of sesame flavor liquors[8]. Due to the limitation of expertise and technology in flavor chemistry research, not all of the 12 types of Baijiu are chemically defined with respect to their characterizing aroma component(s). This is particularly true for soy sauce aroma liquors, which have been studied by many scientists, yet the charactering odorant(s) are still unknown.

It is not possible to discuss the flavor of soy sauce aroma liquor without the mention of Moutai (MT) - the oldest and most famous among this type of baijiu and also the national liquor of China[9]. Due to the predominance of MT, the soy sauce aroma liquors are often referred to as "Moutai" flavor liquors. MT and other soy sauce aroma liquors are produced by the fermentation of sorghum through a proprietary and extremely complex fermentation, distillation and blending processes (See Appendix, **Fig. 1.1** and **Fig. 1.2**).

Because of the cultural and economic significance of MT, many scientists have tried to identify the compounds responsible for the unique and characterizing flavor constituents of this and other types of soy sauce aroma liquors. The compound 4-ethylguaiacol (4EG) was once considered to contribute to the characterizing aroma of MT as early as 1964; however, in 1976, researchers reported that the aroma of 4EG was not identical to the characterizing "soy sauce" aroma of MT, and thus, other compounds must be responsible[10]. Later, pyrazines, methylfuraneol, 5-hydroxy-5,6-dihydromaltol (DDMP), furfural and other compounds were considered as the characterizing odorants of these spirit[11]. The application of the advanced two-dimensional gas chromatography/time of flight mass spectrometry enabled the identification of 528 components in MT[12], yet these researchers were unable to indicate the specific compounds responsible for the characteristic flavor of this liquor. Thus far, all hypotheses regarding the characterizing aroma components of MT have been disproved[6,11]. Therefore, the compound(s) responsible for the characterizing aroma of soy sauce aroma liquors is still a mystery. The lack of this understanding

has hindered the optimization of the brewing technology to achieve products with better and more consistent aroma profiles. However with the accumulation of expertise in flavor chemistry and development of advanced technologies, researchers are getting closer to addressing the characterizing components in the liquor of Moutai. The central hypothesis of this study is: the existence, specific ratios and/or concentrations of these odorants is unique and responsible for typical "soy sauce" flavor of this liquor and, in turn, cause soy sauce aroma liquors to be distinguishable from other Chinese liquors.

The present study made use of advanced analytical approaches for the comprehensive analysis of MT aroma - specifically for the identification of the predominant and characterizing odorants of MT. To the best of our knowledge, this study is the first to employ solvent-assisted flavor evaporation (SAFE) for the careful and exhaustive analysis of MT aroma compounds. The use of this advanced method, considered to be the "gold standard" for flavor isolation, is significant. First, SAFE allows for generation of "clean" aroma extracts which can then be "carefully" analyzed by cool on-column injection GC analysis. This approach avoids the formation of thermally generated volatile artifacts and thus provides a more accurate determination of the actual aroma components of the product[13]. Furthermore, SAFE can be used to either cleanup the solvent extracts prepared from MT or used directly on MT to remove any nonvolatile materials or reactive intermediates/aroma precursors contained in the product, while at the same time maintaining as close as possible the original volatile composition of the product. In the latter case, SAFE could be considered a solvent-free approach to optimize sample preparation and which minimizes sampling bias caused by direct solvent extraction.

Due to the advantages of SAFE mentioned above, in the present research the odor-active components in MT were distilled by SAFE prior to analysis. The odorants components and their relative importance to the overall aroma-profile of MT were determined by gas chromatography-olfactometry (GC-O) and aroma extract dilution analysis (AEDA). In this procedure, aroma extracts were first prepared by direct solvent extraction, distilled by SAFE and then fractionated into acidic, basic and neutral classes to aid in the comprehensive determination of the aroma components in this complex flavor system of MT.

Fractionated SAFE extracts of MT were then stepwise diluted [ratio of 1:3 (v/v)] and each dilution was analyzed by GC-O. The highest dilution at which an odorant is detected is referred to as the flavor dilution (FD) factor for that odorant. FD-factors provide some measure of the relative importance or contribution of each odorant to the overall aroma profile of the product. To the best of our knowledge, AEDA has not previously been applied to the flavor analysis of MT. Thus, our study provides a more detailed accounting of the relative importance of each potent odorant in MT.

However, every coin has two sides, the disadvantages of SAFE are that it is time consuming and labor intensive. Especially when applied repeatedly for quantitation by stable isotope dilution analysis (SIDA), which is considered to be the most accurate method (gold standard) for quantitative analysis. The isotopes should be added and equilibrated in a proper amount before the SAFE extraction to guarantee the recoveries of the isotopes and the target analytes are exactly the same. For accurate quantitation of the components, the addition of isotopes should result in a mass ratio with respect to the target analytes within the linear range of the calibration curve. Thus, the "addition-SAFE extraction-adjustment" procedure must be repeated several times before a correct level of isotope addition is finally achieved.

In the case of extremely expensive products, like MT, or possibly rare or scarce materials, the qualitative and quantitative analysis of the odor-active components should be achieved by using only a minimal amount of the material. Also, in cases where products vary greatly from batch to batch or bottle to bottle, it is beneficial to be able to complete the analysis with a single batch or bottle. To achieve the above goals without compromising the accuracy and precision, an approach needs to be developed to optimize the efficiency of the aforementioned "gold standard" method – specifically, the combined use of SAFE and SIDA. In the present study, a streamlined approach was developed, which minimized the time and effort required for sample preparation and analysis - with respect to both the identification and quantitation of odor-active components in distilled spirits. In this procedure SAFE needs only be conducted only once for each liquor product. This saves time since researchers can adjust the addition of isotopes on a small scale, while at the same time SAFE isolate is suitable for the purpose of identification of odor-active components by GC-O and aroma extract dilution analysis (AEDA). The reliability, accuracy and

versatility of the proposed streamlined approach were validated by sensory difference testing, GC-FID profile comparison and GC-MS-O combined with AEDA.

Another approach to verifying or validating the completeness and accuracy of qualitative and quantitative aroma analysis is to construct an aroma (reconstitution) model, often referred to as an aroma recombinate, based on these data followed a sensory aroma comparison of the model to the original product. Sensory descriptive analysis has been wildly used to identify and quantify the differences between the reconstitution model and the original liquor product[14–16]. However, descriptive analysis generally does not provide the degree of discrimination power (sensitivity) as sensory difference testing techniques, like pair-comparison or triangle difference tests. Moreover, for descriptive analysis, panelists have to be able to define the aroma properties of the target liquor[15]. It is challenged to define the complex flavor properties of MT for which 528 aroma components have been reported[12]. Therefore, in the present study triangle difference testing which was used to assess the similarity or confusability between the reconstitution model and original MT.

MT as the national liquor of China is of great importance historically, culturally and economically. The goal of the present study was to provide further knowledge of the flavor chemistry of MT through development and application of advanced analytical techniques. This study developed and validated a novel and attractive streamlined approach for the qualitative and quantitative analysis of distilled spirits and applied the technique for the comprehensive aroma analysis of MT. To the best of our knowledge, this work is first to report on the application of AEDA for the analysis of MT, thus providing a comprehensive accounting of the odor-import compounds in MT, and was also the first report on the use of SIDA for the accurate and precise quantitation of selected odor-important aroma compounds in MT. This study also resulted in the formulation of a reconstitution model on the basis of the analytical results that successfully mimics the aroma of MT, and which to the best of our knowledge has not been previously reported in the literature.

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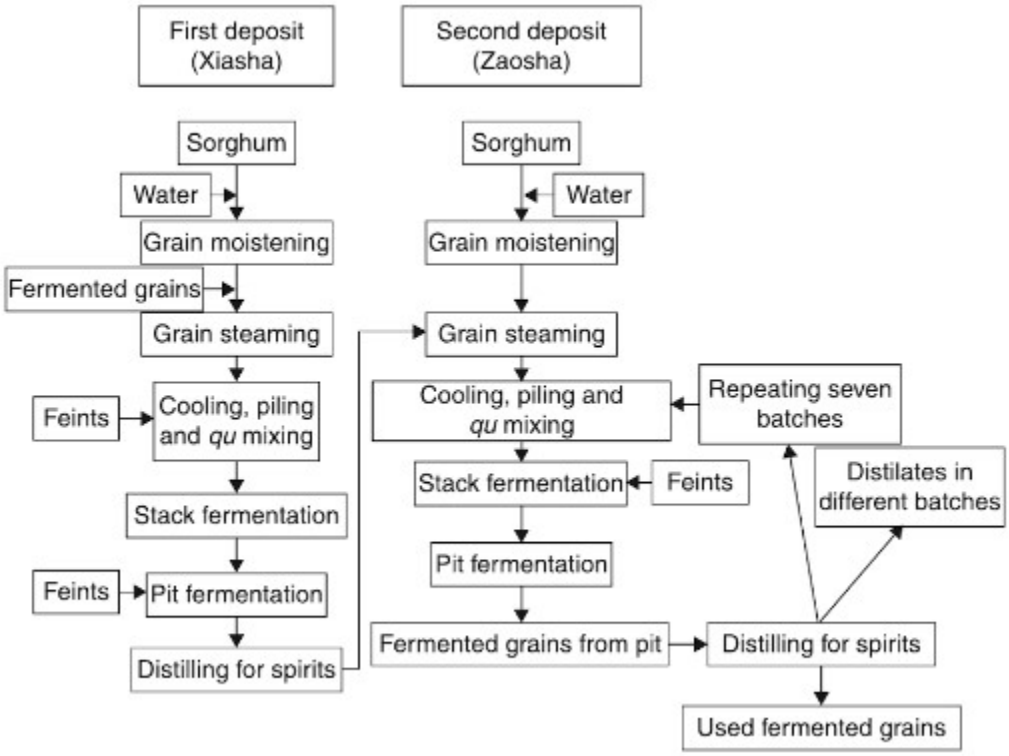


**Fig. 1.1. Process Used for the Production of Moutai (*from* Xu & Ji, 2012)**

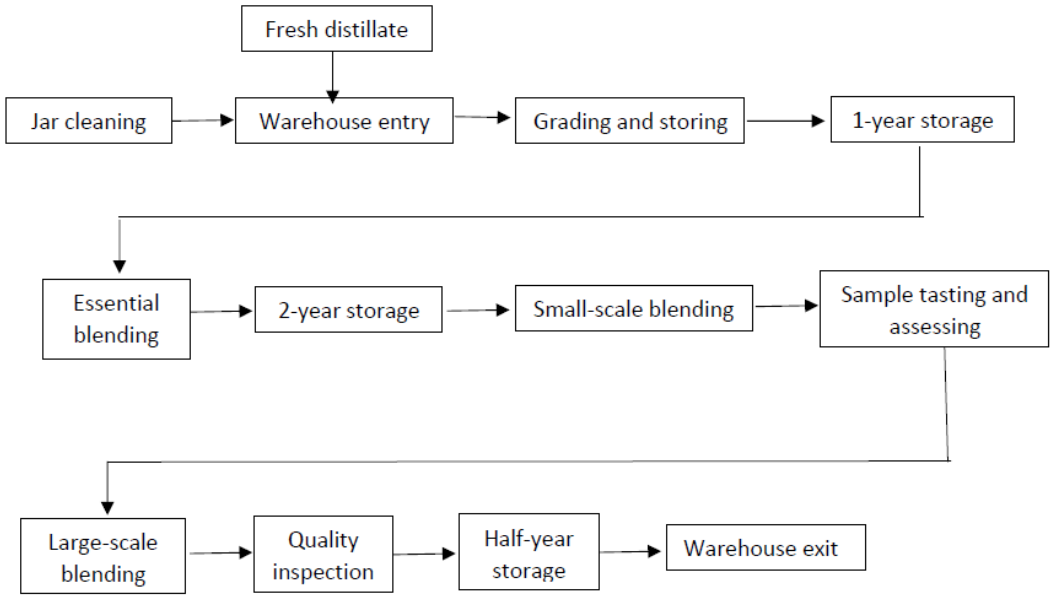


**Fig. 1.2. Blending Process of Kweichow Moutai (*from* Xu & Ji, 2012)**

# Chapter 2: Literature Review

## 2.1 Introduction

China has a history of brewing for over 7000 years[1]. Chinese distilled liquor, which is called "baijiu", is one of the oldest types of distilled liquors in the world. The annual output of Baijiu nationwide has reached 12 billion liters[2]. Obtained from cereals by solid or semi-solid state fermentation procedures using *daqv* or *xiaoqv* as starter cultures[3], baijiu has a higher alcohol content compared with most other distilled liquors such as whiskey, brandy and vodka[4].

The flavor characteristics of baijiu depends on the starting materials, microbial community, climate (including temperature, humidity etc.), fermentation cellar environment and soluble oxygen etc.[4,5]. According to the flavor characteristics of liquor products, Baijiu is roughly classified into 12 groups which include: soy sauce aroma liquors (Moutai flavor liquors), strong aroma liquors (Luzhou-flavor liquors), Fen-flavor liquors, rice-flavor liquors, mixed-flavor liquors, Feng-flavor liquors, Fuyu-flavor liquors, Dong-flavor liquors, site-flavor liquors, Yubingshao-flavor liquors, sesame-flavor liquors and Laobaigan-flavor liquors[6]. This "flavor type" based classification system of Baijiu is vague and sometimes confusing. Since the flavor of liquor products are largely determined by their aroma components, chemically defining each flavor type would provide a more exact classification and description of the flavor chemistry of each Baijiu product. However, determination of the characterizing odorant(s) of each flavor type of Baijiu is challenging. Through decades of study on the flavor chemistry of Baijiu, several flavor types have been successfully defined based on their characterizing odorants. For instance, ethyl hexanoate is considered the main characterizing odorant of strong aroma liquors, ethyl acetate is responsible for the characteristic flavor of Fen-flavor liquors[7], and methional and ethyl hexanoate are important for the characteristic flavor of sesame flavor liquors[8]. As one of the most important baijius, soy sauce aroma liquor can be differentiated by its unique aroma and flavor due to its special manufacturing procedure[3,4]. However, the odorant(s) which contributes most to this different flavor in soy sauce aroma liquor is not as obvious. Having been studied for decades, many odor-active components in soy sauce aroma liquor have been detected, identified and quantitated [3,4,9–12]; however, despite these efforts the main characterizing odorant(s) is still unknown.

There were many hypotheses proposed on the characterizing aroma component(s) of soy sauce aroma liquor[13–15]. However, they have been either disproved or too vague to clearly define the flavor of this liquor from a chemical perspective.

This review covers historical and current attempts to understand the complex flavor chemistry of soy sauce aroma liquor and to identify as best as possible its characterizing odorant(s). Although the exact characterizing odorant(s) of soy sauce aroma liquor is still a mystery, despite many efforts to find the answer, our understanding of the flavor chemistry of soy sauce aroma liquor gets deeper and deeper with more study. With the accumulation of expertise and development of analytical technology and odor-active component separation techniques, chemically defining soy sauce aroma liquor should be feasible and achievable.

### 2.2 The National Liquor of China-Moutai

China is one of the most ancient brewing countries worldwide, with a history of around 7000 years, ever since the Shen Nong Period of the New Stone Age. However, most quality liquor products were produced after alcohol yeast was discovered, which was around 1700 B.C.[1]. These liquor products have a common name in China - "baijiu". Among all the baijiu products, Moutai is possible the most outstanding example of Chinese traditional distilled liquors and is considered the national liquor of China[16].

Moutai is important not only domestically. It is also ranked as one of the world's top 3 national spirits and is equaled only by Scotch whisky and French cognac[17]. All the Moutai products in the market are produced in a small town of Moutai, from which the liquor is named. Moutai town is part of Renhuai City in the Guizhou Province. The unique local climate of Moutai town provides a suitable environment for the growth of starter culture organisms involved in Moutai production. This liquor product cannot be successfully produced in any other place in China, not even in nearby cities in the same province[17].

The production of Moutai began during the Western Han Dynasty when the production of the predecessor of Moutai –"wolfberry sauce aroma liquor" was popular in the town of Moutai and

was served as a royal tribute and offered to the emperors and kings at that time. According to historical sources, the "wolfberry sauce aroma liquor" was described as " sweet and delicious"[16].

Dating from 135 B.C. Moutai was produced by local breweries in small scale and its sale was confidential due to limited production and an undeveloped transportation system at that time[16,17]. A dramatic life-changing event happened in 1915. Moutai, which was produced by 2 private breweries – Chengyi and Ronghe breweries, was selected to showcase at the Panama World's Fair. In the beginning, since the packaging of Moutai was not stylish and charming, it was overlooked by the judging panel. However, one of the delegates dropped one bottle of Moutai accidently and its rich and elegant aroma diffused throughout the whole exhibition hall and drew the attention of the judges. After tasting, Moutai was claimed to be "the best liquor in the word " by the judging panel and awarded the gold medal[17].

Important as it is culturally, Moutai was served at the ceremony of the establishment of People's Republic of China in 1949 and is always being served at state banquets ever since then. In 1951, 3 major private breweries, Yongxing, Chengyi and Ronghe, were merged by the government into Moutai Distillery which is the predecessor of Kweichow Moutai Co., Ltd. By 1991, the output of Moutai was 2000 tons and ranked among the top 10 national brands. Kweichow Moutai developed in a dramatic way and by the year 2011 its revenues had reached $2.7 billion[17]. On April 7$^{th}$ 2017, Kweichow Moutai's market capital reached $71.5 billion surpassing Diageo, which is the world leader in alcoholic beverage sales, and thus became the top liquor company worldwide.

### 2.3 Manufacture of Moutai

The characteristic rich and tempting flavor of Moutai is contributed by several different factors which results from Moutai town being the unique origin of the liquor of Moutai, including specific materials, unique local microbiota, almost tailored climate condition to cultivate the related microorganisms that benefit the fermentation process and the special production procedure[16]. Several attempts have been undertaken to relocate the production of Moutai. Especially in 1974, trials were conducted to relocate the production of Moutai to the city of Zunyi which is only 100km away from the town of Moutai. The production in Zunyi used the

same starting materials and starter culture. Even the production water was transported to the pilot plant. Production was performed by the same technicians and guided by the same experts. However the flavor of the liquors produced in Zunyi was distinguishable from the liquor products made in the town of Moutai[18]. Thus, the liquor of Moutai cannot be produced in any other place other than the town of Moutai.

### 2.3.1 Unique Ecological Environment

Moutai is a town in the city of Renhuai which is located at southwest of China with an altitude of 450m. The special weather conditions of Moutai town are due to its special geographical location which is a basin in a plateau of Guizhou province. The weather in this town is unique for the region, the summer is long and the winter is quite short. The local temperature rages from -2 to 40°C with annual average temperature of 17.4°C. The climate is humid with relative humidity around 78% and annual average rainfall of 800-1000 mm. This climate provides an ideal environment to cultivate the relative microbiota involved in the fermentation and the formation of the distinctive flavor of Moutai[16].

### 2.3.2 Particular Materials

The materials for the production of Moutai are special in regards to both starting materials and the starter cultures involved in the fermentation. The starter is called "Qu", which is made of wheat through a wild fermentation. It is considered as the "skeleton" of the liquor which defines its significance in the flavor quality of liquor products.

"A unique characteristic of Chinese liquor making is the rise of Jiuqu (fermentation starter), which is a great invention of Chinese ancestors in brewing and also a great contribution to the world's microbial fermentation science. With the involvement of Jiuqu, Chinese liquor making has evolved a lot from low-end to high-end, from simple to complex, step by step approaching the top of brewing science and technology from rice wine to millet wine, from shaojiu to baijiu after the introduction of distillation. Chinese brewing techniques are approaching perfection day by day, all of which should be owed to the introduction of Jiuqu." – Museum of the Culture of National Liquor.

### 2.3.3 Special Production Procedure

The production of qu for Moutai is conducted annually around the Dragon Boat Festival, which is 5$^{th}$ of May on the lunar calendar[16]. At that time of the year, the indoor temperature in the town of Moutai is above 40°C which may be one cause of the difference between Moutai from other Chinese distilled liquors. Qu bricks of other Chinese distillated liquors are produced at a temperature below 40°C, while the temperature for the production of qu bricks for Moutai liquor can reach as high as 60-62°C[18]. Thus the starter for Moutai fermentation is a unique high-temperature qu in Chinese brewing industry, which contributes to the special microbiota of this starter to some extent. For qu production, wheat is mixed with water and ground/mixed after the the addition of a mother qu. Afterwards, the mixture is added to a mould and the workers involved in qu making will step on the mould with their bare feet to shape it into bricks for further fermentation, which is 40 days long[16]. At the beginning of qu production, yeasts and molds are the major organisms while bacteria comprise only a minority of the microbiota. After fermentation under high temperature, yeast and mold are eliminated. While bacteria, which are toleratant to high temperature, become the major organisms in finished qu bricks. In finished qu bricks, bacteria counts for 97.8%, mold counts for 2.2%, while yeast is below the detection limit [18,19]. Qu bricks should be loose in the center and firm at side to benefit the fermentation, and this is only achievable when made manually instead of mechanized. Thus, this ancient method for qu making is still used today. Stepping and shaping the qu bricks require highly skilled laborers. If the bricks are too tight, water cannot evaporate from the bricks. At the same time the growth of aerobic microorganisms, which are of great importance to the formation of Moutai's flavor, are depressed. If incorrectly made the qu bricks would be white color inside and with musty and ammonia off flavors[19]. Well-made qu bricks should light yellow in color and possess a strong aroma. Several factors are of significant importance of the quality of qu, which include the quality and species of wheat, the degree of grinding, the addition and quality of water and properties and quality of mother qu[19].

Even though qu is considered as the "skeleton" of the liquor, it is not the only factor that contributes to the characterizing flavor of Moutai. The water from the Red River which flows through the town of Moutai also plays an important role on the formation and production of Moutai. The importance of water has never been overlooked on the production of Chinese

distilled liquors and considered as the "soul" of the liquor. Mineral content and water quality have significant effects on the quality of liquor products.

The solid state fermentation of Moutai is operated at Double Nine Day, which is 9th September on the lunar calendar. The starting material for Moutai production is specific, which is a sorghum grown locally called "*hong ying zi*". This particular grain has thick husks, which makes it resistant to steaming during distillation. Another advantage of this red sorghum is due to its high content of starch, especially amylopectin, making its absorption of water is low and thus the gelatinization rate is relatively slow [16]. For the production of distillates, first deposit of sorghums (Xiasha) is ground and moistened by boiling water and steaming. Qu powder is mixed in after the grains are cooled down. After 2 days of stack fermentation on the ground, the mixture is moved into a stone pit for further fermentation for 30 days. Afterwards, the 1st cycle of spirit is distilled. Second deposit of sorghums (Zhaosha) is moistened and steamed with the first deposit of sorghums. Qu powder is added and mixed followed by stack fermentation, pit fermentation and distillation in the same way. The steaming, qu addition, fermentation and distillation are repeated 7 more times without sorghum addition[16]. Overall, the whole procedure includes 8 fermentation and 8 distillation processes. Distillates from different cycles are classified into 3 different categories: Jiangxiang, Chuntian and Jiaodi, which are graded into different levels according to their flavor profiles and aroma quality. After grading, fresh distillates are stored in separate jars and stored in barns[16].

Essential blending is conducted between distillates from the same cycle and of similar aroma profiles after one whole year storage. The blended distillates will endure 2 more years' storage until ready to be used for small-scale blending which is the procedure to determine the parameters for large-scale blending to achieve a consistent aroma-profile for the Moutai liquor. To ensure the aroma-profile and taste are consistent, not only base liquors from the same batch are used, but previously stored base liquors are also added. Thus the age of the liquor products is declared as the average age of the base liquors. The blending of Moutai is a significant amount of work, at least 80-100 base liquors are added to make the standard Moutai product[16]. Large-scale blended liquors will be stored for another half year before they can be bottled, packed and ready for sale.

Different from other distilled liquor products, the production of Moutai is seasonal because the fermentation processes rely upon the local microbiota, which varies during different times of the year. The whole production procedure has been determined and achieved by ancient Chinese people after countless failures and finally success after continuous exploration. Thus, the production of Moutai is the "crystal of wisdom" of ancient Chinese people and is valued as one of the non-material culture heritages of China[19].

The whole production procedure from qu making until the liquor products are ready for sale takes more than 5 years, which explains its extremely high value in the liquor market.

### 2.4 Identification and Quantitation of Odor-Active Components in Soy Sauce Aroma Liquors

The characterizing odorant(s) in Moutai liquor has been studied for decades. As early as 1953, 4-ethyl guaiacol (4-EG) was indicated as major contributor to the aroma of soy sauce. In 1964, 4-EG was again demonstrated as the characterizing odorant in MT by using paper chromatography. However, the result was later found to be inaccurate[14]. Tetramethylpyrazine, furfural, methylfuraneol (HEMF) also have been considered as characterizing odorants in MT[13,14]. However, all of these hypothesis have been disapproved. With the accumulation of expertise and the development of advanced technologies on flavor research, the study of the complex flavor system of MT has become more systematic. Many advanced technologies have been applied for the study of the flavor of soy sauce aroma liquor[3,4,11,20,21]. The complex flavor system of MT has revealed more and more complexity than previously thought.

The most commonly used methods used to study the flavor of Chinese distilled liquors are SPME and solvent extraction, other advanced technology such as stir bar sorptive extraction (SBSE ) has also been applied for the study of the flavor chemistry of soy sauce aroma liquors[4].

In 2007, by using two-dimensional gas chromatography/time of flight technology, 528 components were identified in MT including 38 organic acids, 145 esters, 112 alcohols, 94 ketones, 39 aldehydes, 10 acetals, 19 nitrogen-containing compounds and 8 lactones[11]. However, this technology did not allow the researchers to detect the odor properties of the target analytes.

It is impossible to draw any conclusion to the contribution of these components to the overall aroma profile of MT, let alone address the characterizing odorant(s) in this product.

In 2012 researchers applied direct solvent extraction followed by fractionation and identified 186 odor-active components in MT. In this study, many odorants were reported in Chinese liquor for the first time, including 2,5-dimethyl-3-butylpyrazine; 3,5-dimethyl-2-pentylpyrazine; 2-(3-methylbutyl)-6-methylpyrazine (tentatively identified); 2-methoxy-3-butylpyrazine (tentatively identified); 3-(1-methylethyl)-2-methoxypyrazine (tentatively identified); 2-furfuryl ethyl ether; 1,2-dimethoxy-3-methylbenzene; o-aminoacetophenone; (*Z*)-whiskylactone, furaneol; sotolon and 2,4,5-trimethyloxazole. Among all of the 186 odorants identified in this study, ethyl hexanoate, hexanoic acid, 3-methyl butyric acid, 3-methyl butanol, 2,3,5,6,-tetramethylpyrazine, ethyl 2-phenylaceteate; 2-phenyl acetate; ethyl 3-phenylpropanoate; 4-methylguaiacol and γ-decalactone were indicated as the most potent odorants based on their odor intensity. However, none of the above odorants was considered as the main contributor to the typical flavor of MT[10] .

SPME (solid-phase microextraction) combined with GC, GC-MS or GC-O is a solvent-free method to isolate volatile components from food matrix by using a fiber coated with different absorbents. Compare with solvent extraction methods, sample preparation for SPME methods are relatively easy and less time consuming and the method requires only a small amount of sample[22]. SPME is most suitable of detection of highly volatile components which could be lost by conventional solvent extraction methods. In addition, since it is a solvent-free method it is possible to peaks which coelute with solvent peak through liquid injection. Especially when SPME is applied in headspace analysis, the results could determine the aroma profile based on the release of odorants from food or beverage matrix which represent the real condition more accurately compared to determination of the content of those odorants[22]. Thus, SPME is commonly used for the purpose of identification and quantification of volatile components in liquor products[3,23] . Headspace solid-phase microextraction (HS-SPME) was used for the study of volatile sulfur compounds in MT and allowed for the successful identification and quantitation of many trace amount volatile sulfur components, including methanethiol, methional, dimethyl disulfide, dimethyl trisulfide, and 2-furfurylthiol which may make a significant contribution to the overall aroma profile of MT[21]. According to the quantitation results of this study,

methanethiol, dimethyl disulfide and 2-furfurylthiol were considered to be of significant importance to the overall aroma profile of MT. However, due to the selectivity of this technology, the results cannot reveal the full picture of the whole flavor system of MT.

Another advanced technology used to isolate volatile components from food matrix by using an adsorbent is stir bar sorptive extraction (SBSE). SBSE is a method with high selectivity which can achieve consistent results[24]. Since the sensitivity of SBSE is 50-250 times higher than SPME, the analysis of trace amount components is its specialty[25]. It was used for the study of volatile compounds in soy sauce aroma liquor products in 2011[4]. In this study, 76 volatile compounds were identified and quantified in 14 Chinese soy sauce aroma liquors. However, since the components were identified only by using a DB-WAX column, the results were not convincing. Besides, quantitation of all the identified components was performed by internal standard calibration, which was not as accurate as stable isotope dilution analysis (SIDA). Since the stable isotope of the target analyte have similar properties with the unlabled target analyte, their partitioning during extraction are almost the same. Thus, for accurate quantitation of aroma-active components in MT, SIDA has to be performed. However, the disadvantage of SIDA is that stable isotope is required for each target analyte, which is costly and sometimes challenging. Especially when the isotopes needed to perform SIDA are not commercially available, in which case synthesis and purification of stable isotopes would is necessary.

Flavor recombination and omission study is a typical method to address key odor-active components in the target products and has been used to study the flavor of whiskey, cola and light aroma Chinese liquor[26–29]. In 2012, odor reconstitution and omission study was conducted on MT [30] by using 52 odor-active components. However, since the reconstituted flavor model lacked of the characterizing aroma properties of MT, it cannot serve as a complete flavor model for omission study. To establish a complete model which is identical to the original product of MT advanced technologies have to be applied to provide more accurate identification and quantitation results.

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## Chapter 3: Identification of Potent Odor-Active Components in Moutai

### 3.1 Abstract

A series of experiments were conducted in order to identify the potent odorants in Moutai (MT), a famous Chinese "soy sauce aroma" type liquor. To avoid the formation of thermally generated artifacts during analysis, the volatile components were isolated by three mild and exhaustive extraction techniques: 1) direct solvent extraction of the liquor followed by solvent-assisted flavor evaporation (DSE-SAFE); 2) distillation of the liquor by SAFE without a solvent extraction step (SAFE-DIST); and 3) direct solvent extraction of a SAFE distillate (SAFE-DIST-DSE). The relative potencies of the odorants were assessed by gas chromatography-olfactometry (GC-O) and aroma extract dilution analysis (AEDA). One hundred and forty-three odorants were detected. The most potent odorants in MT included the following compounds: acetal; ethyl 2-methylpropanoate; ethyl butanoate; ethyl 2-methylbutanoate; ethyl 3-methylbutanoate; ethyl pentanoate; ethyl 2-methylpentanoate; ethyl 4-methylpentanoate; 3-methylbutyl hexanoate; β-damascenone; ethyl phenylpropionate; 2-phenylethanol; 3-methybutanal; 2-methyl-3-furanthiol; 2-methyl-3-(methyldithio)furan; dimethyl trisulfide and sotolon. Four sulfur-containing odorants, which might provide "savory" top notes to the flavor of MT, were identified as 2-methyl-3-furanthiol (MFT); 2-methyl-3-(methyldithio)furan (MFT-MT); 2-furfurylthiol; and *bis*(2-methyl-3-furyl) disulfide (MFT-MFT). To the best of our knowledge, MFT, MFT-MT and MFT-MFT have not been previously identified in Moutai or any other Chinese soy sauce aroma type liquor.

### 3.2 Introduction

For centuries Moutai has been the most influential and important soy sauce aroma liquor in China, and serves as the "gold standard" in the soy sauce aroma liquor brewing industry[1]. For this reason, soy sauce aroma liquors are also referred as "Moutai" flavor liquors[2]. For decades, scientists have studied on the special aroma profile of soy sauce aroma liquors, especially the flavor components of MT[3–8]. However, due to the limitation of available instrument and technologies and the extremely complex flavor system of MT, the characterizing odorants of MT are still unknown.

In this study, advanced analytical methods were applied for the isolation of volatile components of MT to minimize potential artifacts. The high-impact components of MT liquors were addressed through application of GC-O and AEDA to explore the uniqueness of

the MT aroma profile. Specifically, the aroma extracts MT were prepared by three types of gentle, yet exhaustive techniques, namely 1) direct solvent extraction of the liquor followed by a solvent-assisted flavor evaporation clean-up step (DSE-SAFE); 2) distillation of the liquor using SAFE without a solvent extraction step (SAFE-DIST); and 3) direct solvent extraction of a SAFE distillate (SAFE-DIST-DSE). The relative potencies of the odorants in each aroma extract were assessed by gas chromatography-olfactometry (GC-O) and aroma extract dilution analysis (AEDA).

The purpose of performing DSE-SAFE or SAFE-DIST-DSE was to isolate volatile components of the liquor from the hydro-alcoholic matrix. For this purpose, dichloromethane (DCM) was used to selectively extract the aroma compounds and to eliminate ethanol from the extract. The distillation step accomplished through SAFE was conducted to remove any nonvolatile components from the solvent extract in an effort to avoid the potential for the generation of thermally-derived artifacts during analysis[9]. The DSE-SAFE aroma extract was further fractionated into acidic, basic and neutral components and each fraction (**Figure 3.1**) was subjected to instrumental analysis by GC-mass spectrometry-olfactometry (GC-MS-O).

The relative potencies of the odorants in each aroma extract were assessed by gas chromatography-olfactometry (GC-O) and aroma extract dilution analysis (AEDA). The use of solvent extraction (i.e., in the case of DSE-SAFE and SAFE-DIST-DSE) is necessary to eliminate water and ethanol and to concentrate the volatile components for detailed analysis; however, this step can introduce an extraction bias due to selective partitioning and extraction of the different odorants. Therefore, solvent extracts may not completely represent the aroma profile of the target product. To address this potential problem, the isolate prepared by SAFE-DIST (i.e., with no solvent extraction step) was analyzed by direct injection GC-O to accomplish aroma extract dilution analysis (AEDA) using a cool on-column injection technique. In AEDA, the highest dilution at which an odorant is detected by GC-O is the flavor dilution (FD) factor for that odorant. FD-factors are used to indicate the relative importance or contribution of an odorant to the overall flavor profile of the product[10]. That is, odorants with the highest FD-factors should make the greatest contribution to the overall aroma of the product.

## 3.3 Materials and Methods

### 3.3.1 Samples

Authentic samples of MT were obtained directly from the producer, Kweichow Moutai Co. Ltd. Guizhou, China. Mention of brand name is not for advertisement or endorsement purposes and does not imply any research contract or sponsorship.

### 3.3.2 Chemicals

Dichloromethane (DCM) and anhydrous sodium sulfate were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Odorless deionized-distilled odorless water was prepared by boiling deionized-distilled water in a glass flask until it was reduced by two-thirds of its original volume.

Authentic reference standards used to confirm the identification of the detected compounds were obtained by Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Acetaldehyde and 2-methyl-1-propanol and were purchased from Fisher (Fair Lawn, NJ, USA); acetic acid; ethyl 3-phenylpropanoate; 4-ethylguaiacol; sotolon and vanillin were obtained from SAFC (St. Louis, MO, USA); ethyl cyclohexancarboxylate was obtained from Alfa Aeasar (Lancaster, UK); 2-methylbutanal was a gift from Bedoukian (Danbury CT); acetal (1,1-diethoxyethane) was purchased from Acros Organics (NJ, USA); β-damascenone was obtained from Firmenich (NJ, USA); ethyl acetate was obtained from Applied Biosystems Inc. (Foster City, CA, USA).

### 3.3.3 Preparation of Aroma Extract

**Preparation of aroma extract of Moutai liquor by direct solvent extraction followed by solvent assisted solvent extraction (DSE-SAFE)**

Liquor sample (100 mL) was diluted with 400 mL of deodorized deionized-distilled water and extracted three times with 20 mL DCM. In order to remove nonvolatile impurities, the direct solvent extract (DSE) was distilled using a modified solvent-assisted flavor evaporation (SAFE) apparatus11. SAFE was operated at 40 ºC under high vacuum ($10^{-4}$ - $10^{-5}$ Torr) and the volatile fraction was condensed in glass traps cooled with liquid nitrogen[12]. Distillation was conducted for 2 hr and then the distillate was thawed and collected. The aroma extract was then separated in to acidic, basic and neutral fractions as shown in the flow chart in **Figure 3.1**. DSE-SAFE fractions were stored at -20 ºC in 2-mL vials sealed with the PTFE-lined caps until analysis.

**Direct distillation of Moutai liquor (SAFE-DIST)**

Liquor sample (100 mL) was directly distilled using the aforementioned SAFE apparatus at 40 ºC under high vacuum ($10^{-4}$-$10^{-5}$ Torr) and the volatile fraction was condensed in glass traps cooled with liquid nitrogen. Distillation was conducted for 2 hr and then distillate was thawed and stored in an amber glass bottle equipped with a PTFE-lined cap.

**Direct solvent extraction of the distillate of Moutai prepared by SAFE (SAFE-DIST-DSE)**

Odor-active components in SAFE-DIST were extracted by direct solvent extraction. SAFE distillate (7.5 mL) was pipetted into a 50-mL glass centrifuge tube containing 40 mL of odor-free deionized-distilled water and 0.5 mL of DCM. The mixture was shaken vigorously for 5 minutes and centrifuged at 4500 rpm for 10 minutes. The extraction procedure was repeated two more times. The pooled solvent extract was frozen overnight to remove excess water, then the extract was transferred into a 2mL vial, condensed to 1 mL using a gentle stream of ultra-high purity nitrogen gas and stored at -20 ºC in 2-mL vials sealed with the PTFE-lined caps until analysis.

**3.3.4 Gas Chromatography-Mass Spectrometry-Olfactometry (GC-MS-O)**

GC-MS-O was performed using an Agilent 6890N GC/5973N mass selective detector (MSD) system (Agilent Technologies, Santa Clara, CA, USA). Analyses were performed on both polar (Stabilwax, 30m × 0.25 mm i.d., 0.25 μm film thickness; Restek Corp., Bellefonte, PA, USA) and nonpolar (Rxi-5ms, 30m × 0.25 mm i.d., 0.25 μm film thickness; Restek) columns. Aroma extracts (2μl) were injected under coldsplitless mode (50 °C initial temperature (0.1 min hold), ramped at 12 °C/s to 250 °C and held for 20 min). The carrier gas was helium at a flow rate of 1mL/min. The oven temperature was programmed from 40°C to 250°C at a ramp rate of 3°C/min with initial and final hold times of 5 and 30 min, respectively. Temperatures of MSD transfer line and olfactory port were set at 250 °C. Mass scan range was set as 33-200 amu with scan rate of 5.27 scans/s and electron energy was 70eV. GC-MS data were analyzed by ChemStation Enhanced Data Analysis Software (Aglient Technologies, Inc.). For tentative compound identifications, mass spectra of the analytes were compared against those in National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST, 2008).

### 3.3.5 On-Column Gas Chromatography-Olfactometry (GC-O)

GC-O was performed using a 6890 GC (Agilent Technologies Inc.) equipped with a flame ionization detector (FID), a sniff port (DATU Technology Transfer, Geneva, NY, USA) and a cool on-column injector (+3 ºC, oven tracking)[13]. SAFE-DIST (2µL) was injected directly into either a polar (RTX-Wax, 15m × 0.32 mm i.d., 0.5 µm film thickness; Restek, USA) or nonpolar (RTX-5, 15 m × 0.32 mm i.d., 0.5 µm film thickness; Restek, USA) column. The carrier gas was helium at a constant flow rate of 2mL/min. The oven temperature was programmed from 40°C to 250°C at a ramp rate of 10°C/min with initial and final hold times of 5 and 30 min, respectively. Column effluent was split equally between the sniff port and FID by using 0.15 mm i.d. deactivated capillary columns of equal length (1 m). FID and sniff port temperatures were maintained at 250 °C.

### 3.3.6 Aroma Extract Dilution Analysis

Relative potencies of odor-active compounds were determined by AEDA. DCM-SAFE aroma extract was diluted stepwise at a ratio of 1:3 (v/v) in DCM and each dilution was analyzed by GC-MS-O under the conditions previously described. For the SAFE-DIST aroma extract, AEDA was conducted on a 1:2 (v/v) dilution series [prepared in 53% ABV (v/v ethanol in water)] and each dilution was analyzed by cool on-column GC-O as described above. Flavor dilution (FD) factors of each odorant were determined as the highest dilution at which the odorant was last detected by GCO[14].

### 3.3.7 Compounds Identification

Retention indices (RI) were calculated based on comparing the retention times of analytes to those determined for a homologous series of n-alkanes (from C7 to C28) analyzed under the same analytical conditions[15]. Odorants were identified by comparing their retention indices (RI) on both polar and nonpolar GC columns, mass spectra and odor properties to those of authentic standards. A compound was considered positively identified if all three of the above criteria matched those of a reference standard. However, in some cases, an odorant was considered tentatively identified when one or more of the above criteria could not be met, e.g. when no mass spectrum was available due to the compound being present at a trace level - below that of the detection limit of the MSD, or whenever no authentic standard was available to confirm an RI, mass spectral or odor property match. In the latter case, the compound was considered tentatively identified when its RI, mass spectra and odor properties were in agreement with literature values or database entries (NIST, 2008).

### 3.4 Results and Discussion

Based on the comprehensive GC-O analysis of the three kinds of aroma extracts, a total of 143 odorants were detected, of which 88 were either positively or tentatively identified and 55 were unknowns (**Table 3.1-3.3**). Based on the their general odor characteristics, these odorants could be placed into 10 groups, including fruity and sweet; herbaceous, earthy, woody and smoky; waxy, plastic, metallic and solvent-like; meaty and savory; cheesy, buttery and acidic; malty, nutty, rice and cocoa; floral; cabbage and potato; green and cucumber and others.

**Fruity and Sweet:**

Nos. 4, 6, 7, 9-12, 14-17, 19-23, 27, 31, 35, 40, 48, 54, 56, 62, 74-76, 84, 113, 121, 123, 127, 132, 136 and 137.

**Herbaceous, Earthy, Woody and Smoky:**

Nos. 34, 37, 42, 44, 50, 53, 65, 66, 81, 95, 97, 107, 117, 124, 130, 138, 141 and 142.

**Waxy, Plastic, Metallic and Solvent:**

Nos. 28, 32, 41, 58, 63, 64, 73, 80, 100, 101, 103, 105, 114, 120, 126, 129 and 140.

**Meaty and Savory:**

Nos. 30, 47, 59, 71, 72, 79, 83, 85, 88, 102, 104, 116, 133 and 134.

**Cheesy, Buttery and Acidic:**

Nos. 8, 25, 87, 89, 90, 91-94, 96, 98, 99, 108, 110, 125 and 128.

**Malty, Nutty, Rice and Cocoa:**

Nos. 1, 2, 3, 5, 13, 18, 26, 29, 49, 60, 112, 115 and 135.

**Floral:**

Nos. 43, 46, 51, 57, 61, 67, 68, 69, 70, 78, 86 and 106.

**Cabbage and Potato:**

Nos. 24, 33, 52, 55, 122, 131 and 143.

**Green & Cucumber:**

Nos. 36, 38. 39, 45, 109 and 111.

**Others:**

Nos. 77, 82, 108, 118, 119 and 139.

The neutral, acidic and basic DSE-SAFE aroma extract fractions contained the greatest number of odorants, with 120 compounds detected by GC-O (**Table 3.1**). Among these, 77 were either positively or tentatively identified, while 42 compounds were unknown. Among these unknown odorants, most had FD factors $\leq 9$. Considering that the range of FD factors

was from 1 to 177147, the unknown odorants (most with FD ≤ 9) are unlikely to contribute to the characteristic aroma of MT. Eight of these unknown odorants had FD factors ≥ 9 (nos. 6, 40, 43, 48, 71, 72, 87 and 97). Among these, nos. 6 and 40 had potent fruity and berry aroma notes with FD factors of 729 and 243, respectively. Meanwhile, no. 43 with an FD factor of 81 had a floral scent, similar to that of hops or lavender. It is likely they contributed to the fruity and floral aroma of MT; however, considering their odor properties, it is unlikely that they are the characterizing components that differentiate soy sauce aroma liquor from the 11 other flavor types of traditional distilled Chinese liquors which also have floral and fruity characteristics. Odorants nos. 71 and 72 both had relatively high FD factors of 27 and possessed meaty and vitamin-like odor properties. These compounds may contribute to the savory flavor of MT to some extent. However, compared to 2-methyl-3-(methyldithio)furan (no. 47) which has a similar odor property and whose FD factor was 729, nos. 71 and 72 may not be essential for the savory and meaty aroma characteristic of MT. For the same reason, nos. 87 and 97 with FD factors of 81 and 27, respectively, might contribute to the aroma profile of MT to some extent, but may not be essential and differentiating among all the odorants in MT.

In the SAFE-DIST aroma extract prepared from MT, which was subjected to direct cool on-column injection GC-O analysis, 52 odorants were detected, among which 38 compounds were either positively or tentatively identified and 14 compounds were unknown (**Table 3.2**). Forty-one of these odorants were previously identified (above) in the DSE-SAFE fractions analyzed by cold-splitless GC-MS-O. A potential advantage of using direct cool on-column GC-O analysis is that the FD factors may be more representative of the aroma profile of the original liquor. This is because the SAFE operation eliminates non-volatile components from the extracts, thus generating a "clean" extract which is suitable for cool on-column GC-O analysis[9]. In this approach the sample is injected directly into the GC column under mild (low temperature) conditions, thus minimizing the formation of thermally generated artifacts in the inlet of the GC. Furthermore, since there was no solvent used in the procedure, solvent extraction bias was avoided[16]. For this reason, the FD factors of trace potent odorants, especially those below the limit of detection of the MSD can be used to estimate the concentration of the target analytes (results shown in Chapter 5). For this purpose, a smaller dilution factor of 1:2 (v/v) was used for AEDA. According to the AEDA results from the SAFE distillate, the most potent odorants in MT (with FD factors above 64 on either GC column phase) were: ethyl 2-methylpropanoate (no. 7); ethyl butanoate (no. 10); ethyl 2-

methylbutanoate (no.11); ethyl 3-methylbutanoate (no. 12); ethyl 4-methylpentanoate (no. 17); 2- and 3-methyl-1-butanol (no. 18); methionol (no. 52); ethyl 3-phenylpropionate (no. 68); 2-phenylethanol (no. 69); sotolon (no. 104) and an unknown compound (no. 122). Among these, ethyl 2-methylpropanoate (no. 7); ethyl butanoate (no. 10); ethyl 2-methylbutanoate (no. 11); ethyl 3-methylbutanoate (no. 12) and ethyl 4-methylpentanoate (no. 17) were responsible for fruity notes, such as berry, papaya and kiwi, in the aroma profile of MT. The compounds 2- and 3-methyl-1-butanol (no. 18) contribute to the malty, dark chocolate flavor to the overall aroma profile of MT. Ethyl 3-phenylpropionate (no. 68) and 2-phenyl ethanol (no. 69) appear to play important roles by imparting floral, rosy and honey properties to the aroma profile of MT. Methionol (no. 52) and an unknown odorant (No. 122) contributed radish and cabbage aroma notes to the flavor of MT. Sotolon (no. 104) which is a powerful odorant with spicy and curry aroma note may be an important contributor of savory flavor of MT.

It should be noted that in the case of the direct analysis of SAFE-DIST aroma extracts the concentrations of most odorants were relatively low since extraction and concentration steps were not performed. For a more detailed analysis, it was necessary to analyze more concentrated extracts prepared from the SAFE-DIST aroma extract by a DSE step. With this SAFE-DSE approach, a total of 62 odorants were detected, among which 57 were either positively or tentatively identified and 5 odorants were unknown (**Table 3.3**).

Previously, in the AEDA results from the neutral, acidic and basic fractions from DSE-SAFE, it is difficult to make direct comparisons across the various fractions or to gauge overall impact of each odorant to the overall aroma profile of MT since the additional extraction steps involved in fractionation and concentration of the aroma extracts may affect the concentration of some odorants. In contrast, the AEDA results from SAFE-DIST aroma extracts provided a better depiction of the relative significance of these odor-active components to the overall aroma profile of the liquor. Seventeen potent odorants (no. 121, 3, 7, 10-12, 15, 17, 132, 133, 24, 31, 47, 62, 68, 69 and 104) had FD factors above or equal to 243. Among these, acetal (no. 121); ethyl 2-methylpropanoate (no. 7); ethyl butanoate (no. 10); ethyl 2-methylbutanoate (no.11); ethyl 3-methylbutanoate (no. 12); ethyl pentanoate (no. 15); ethyl 4-methylpentanoate (no. 17); ethyl 2-methylpentanoate (no. 132); 3-methylbutyl hexanoate (31); β-damascenone (no. 62) and ethyl 3-phenylpropionate (no. 68) were responsible for the fruity, berry, kiwi, apple and applesauce aroma notes. 2-phenylethanol

(no. 69) had a floral and rosy scent. 3-methybutanal (no. 3) contributed malty and dark chocolate like aroma. 2-methyl-3-furanthiol (no. 133) and 2-methyl-3-(methyldithio)furan (no. 47) were responsible for meaty, beefy and savory notes. Dimethyl trisulfide (no. 24) provided a pungent cabbage-like odor, and sotolon (no. 104) contributed of spicy and curry scent.

The AEDA results of the SAFE-DIST differed to some extent from those obtained for the DSE-SAFE fractions. For example, ethyl pentanoate (no. 15) and ethyl 2-methylpentanoate (no. 132) which were among the most potent contributors in the DSE-SAFE aroma extracts did not have high FD-factors or were not detected (no. 132) in the SAFE-DIST aroma extracts. This serves as a good example of extraction bias based on selective solvent extraction/partitioning. DCM was used in the direct solvent extraction for sample preparation which is less polar than the 53% ABV matrix of the liquor. Nonpolar odorants such as esters were preferentially extracted by DCM and thus they had higher relative FD-factors than what might be expected in original liquor product. Likewise, polar components may have been under-extracted and their FD-factors thus were underestimated. Such as 2- and 3-methyl-1-butanol (no. 18), which was one of the most potent odorants in the SAFE-DIST aroma extract, was not as significant in the DSE-SAFE aroma extract.

As mentioned previously, none of the odorants marked as "unknowns" in the tables should be the critical odorants because they were determined to have relatively low FD factors. Through the application of AEDA on three types of aroma extracts of MT this study provided a comprehensive listing of the potent odorants in the complex flavor system of MT. Most of these odorants have been previously reported as volatile constituents of various kinds of distilled Chinese liquors, and in particular for soy sauce aroma liquors[4–6,17–23]. However, in the present study 4 potentially important potent odorants (nos. 47, 55, 79, 133), to the best of our knowledge were reported for the first time as aroma components of MT. Among these, dimethyl tetrasulfide (no. 55) has the sulfurous and cabbage-like odor property similar to that of dimethyl trisulfide (no. 23) which was previously reported in MT. For this reason, even it is first reported in this study, its contribution to the characterizing odor property of MT may not be significant. On the other hand, three of the newly identified odor-active components: 2-methyl-3-(methyldithio)furan (MFT-MT) (no. 47); *bis*(2-methyl-3-furyl) disulfide (MFT-MFT) (no. 79); 2-methyl-3-furanthiol (MFT) (no. 133) have odor properties distinguishable from all the other odorants previously reported. The odorants impart beefy, meaty and savory

[31] top notes to the aroma profile of MT, which may be important for differentiating soy sauce aroma liquor from the 11 other types of traditional distilled Chinese liquors.

All of these 3 potent odor-active sulfur compounds were reported to have extremely low odor detection thresholds (ODT): MFT (ODT water = 0.007 ppb[35]), MT-MFT (ODT water = 0.4 ppb[38]) and MFT-MFT (ODT water = 0.00002 ppb[35]). This may explain the reason that these compounds were overlooked or failed to be identified in this particular liquor product over the past several decades of study.

2-methyl-3-furanthiol (MFT) is a potent odorant with meaty aroma which was first identified in canned tuna[24] and later found in a multitude of products as a well-known aroma contributor in various cooked meat products and soy sauce[25–31].

Various pathways for the formation of MFT have been reported. MFT can be formed through the Maillard reaction between carbohydrates and cysteine[32–34]. It can also be generated through the degradation of thiamin[35] and via hydrolysis of thioacetates catalyzed by lipase[36]. Similar to most thiols, MFT has strong anti-oxidation properties due to its liable free thiol function group[30]. Its instability was tested by storage in diethyl either under 6 °C. After one day, 20% of MFT was dimerized into MFT-MFT, and ten days later more than 50% of MFT was dimerized. However the instability of MFT can be decreased significantly if it is stored in dichloromethane (DCM). At the same storage temperature, its decrease was only 6%[37]. MFT-MFT is also unstable at high temperature, where the dimer can be cleaved to form the monomer MFT[35].

In this study, DCM was selected as the solvent to extract and isolate odor-active components from the liquor product to eliminate alcohol, which should favor the stabilization of MFT. However, since analysis of SAFE-DIST-DSE aroma extracts were performed by cold-splitless injection mode, during which the components of the extract are heated before they enter the column, it is possible that any MFT detected is a thermally generated artifact formed in the inlet from MFT-MFT. Especially, the FD factor of MFT-MFT was 8 for the SAFE-DIST aroma extract, which was analyzed by cool on-column GC-O technology in which sample was directly injected under mild heating conditions (**Table 3.2**), compared to an FD factor of 9 in the extractive SAFE-DIST-DSE aroma extract, which was 7.5 times more condensed than the SAFE-DIST aroma extract. Therefore, it is highly likely that the MFT

detected was an artifact. For this reason, further study was conducted in Chapter 4 to determine the influence of injection methods and analysis parameters on the degradation of MFT-MFT.

MFT-MT which was also firstly identified in soy sauce aroma liquor has the similar odor property with MFT and MFT-MFT and contribute meaty, beefy and roasty aroma to the flavor profile of MT has been reported in cocoa[38], roasted sesame[39] and red wine[40]. It could either formed by Maillard reaction of ribose and cysteine or generated by MFT and methanethiol[41]. Since it has the highest FD factor among all these three sulfur compounds, it may serve as the most important contributor to the meaty, beefy note in the overall aroma profile of MT[33].

**Figure 3.1. Fractionation of the Solvent Assisted Flavor Evaporation Distillate of Moutai**

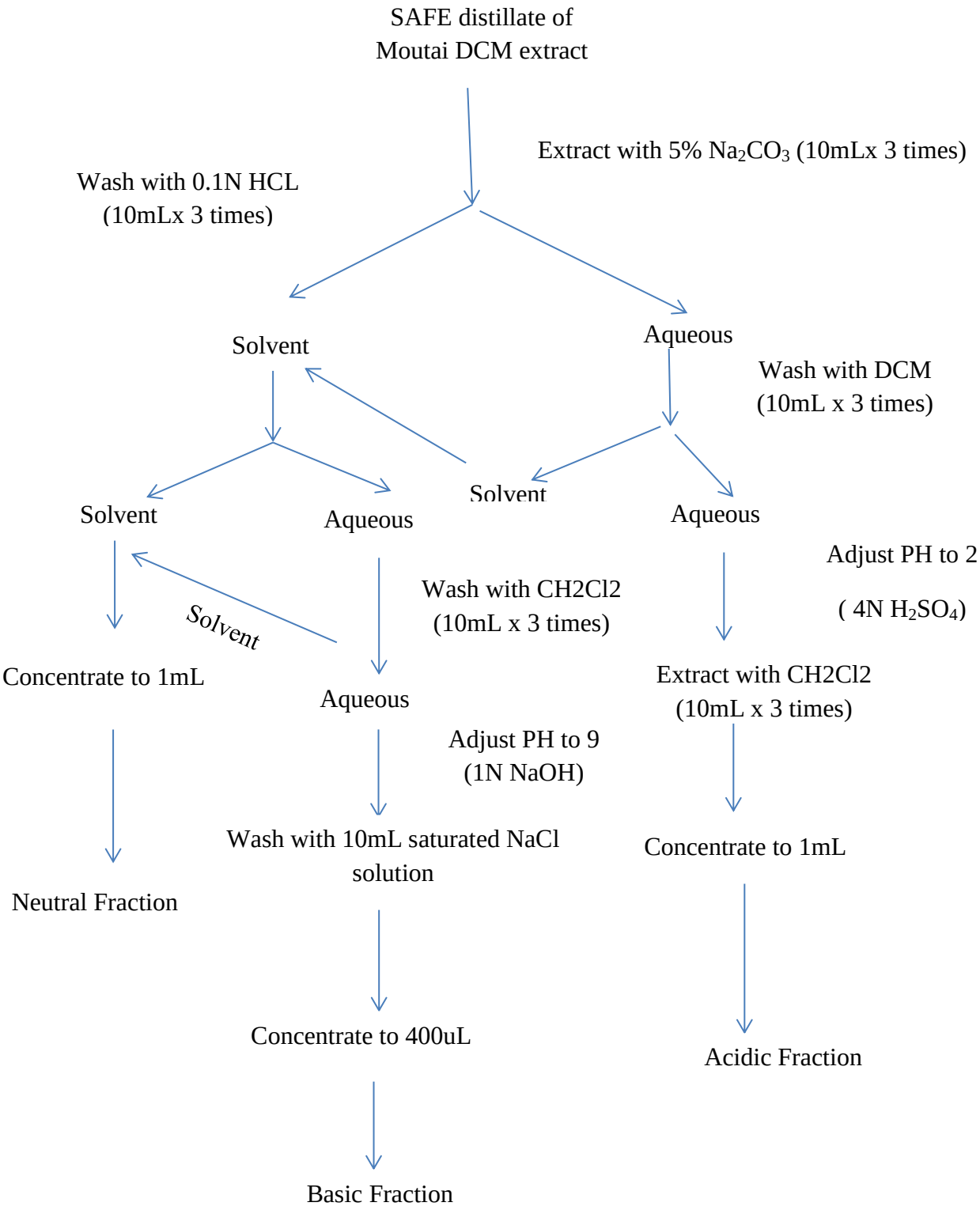

**Table 3.1 A. Odorants Detected In The Neutral Fraction of An Aroma Extract of Moutai Liquor Prepared by Direct Solvent Extraction Followed By Solvent Assisted Flavor Evaporation (DSE-SAFE)**

| *No.* | *RI Stabilwax* | *Compound* | *Odor Description* | *FD Factor* [a] | *Identification* [b] |
|---|---|---|---|---|---|
| **1** | <800 | acetaldehyde | sweet, alcohol | 9 | O |
| **2** | <800 | 2-methylpropanal | radish, cabbage | 1 | O, RI |
| **3** | 910 | 3-methybutanal | malty | 81 | O, RI |
| **4** | 957 | ethyl propionate | fruity, painty | 59049 | O, RI |
| **5** | 965 | pentanal | malty | 243 | O, RI |
| **6** | 996 | unknown | blueberry | 729 | O, RI |
| **7** | 1004 | ethyl 2-methylpropanoate | blueberry, grape | 177147 | O, RI |
| **8** | 1009 | 2,3-butanedione | buttery | 243 | O, RI |
| **9** | 1020 | 2-methylpropyl acetate | rubber, plastic | 9 | O, RI |
| **10** | 1058 | ethyl butanoate | fruity, kiwi | 6561 | O, RI, MS |
| **11** | 1069 | ethyl 2-methylbutanoate | fruity,kiwi, berry | 59049 | O, RI, MS |
| **12** | 1084 | ethyl 3-methylbutanoate | kiwi, berry | 19683 | |
| **13** | 1100 | 2-methyl-1-propanol | malty | 27 | O, RI, MS |
| **14** | 1132 | 3-methylbutyl acetate | fruity, banana | 9 | O, RI, MS |
| **15** | 1149 | ethyl pentanoate | fruity, berry, sweet | 2187 | O, RI, MS |
| **16** | 1186 | 2-methylpropyl butanoate | fruity, blueberry | 9 | O, RI |
| **17** | 1198 | ethyl 4-methylpentanoate | berry | 729 | O, RI, MS |
| **18** | 1209 | 2-/3-methyl-1-butanol | malty | 2187 | O, RI, MS |
| **19** | 1245 | ethyl hexanoate | hop, fruity, pear | 27 | O, RI, MS |
| **20** | 1292 | furfuryl ether | ether like, fruity | 27 | O, RI |
| **21** | 1290 | hexyl acetate | banana | 81 | O, RI |
| **22** | 1308 | propyl hexanoate | fruity, painty | 9 | O, RI |
| **23** | 1358 | ethyl heptanoic | pear, fruity | 9 | O, RI, MS |
| **24** | 1383 | dimethyl trisulfide | cabbage, garlic Salt | 2187 | O, RI |
| **25** | 1402 | ethyl 2-hydroxybutanoate | sweaty, sour | 9 | MS |
| **26** | 1410 | trimethylpyrazine | nutty, earthy | 9 | O, RI, MS |
| **27** | 1436 | ethyl cyclohexyl-carboxylate | berry | 81 | O, RI |
| **28** | 1437 | ethyl octanoate | plastic, soapy | 729 | O, RI, MS |
| **29** | 1439 | unknown | nutty, yogurt | 9 | MS |
| **30** | 1440 | 2-furfurylthiol | sesame, meaty | 1 | O, RI |
| **31** | 1462 | 3-methylbutyl hexanoate | fruity | 243 | O, RI |
| **32** | 1464 | unknown | ether like | 9 | |
| **33** | 1467 | methional | malty, potato, herbal, hay | 9 | O, RI |
| **34** | 1470 | ethyl 2-hydroxy-4-methylpentanoate* | hay | 9 | MS, RI |
| **35** | 1481 | unknown | berry | 3 | |
| **36** | 1486 | (*Z*)-2-nonenal | cucumber, fishy | 3 | O, RI |

***Table 3.1 A. continued***

| *No.* | *RI Stabilwax* | *Compound* | *Odor Description* | *FD Factor* [a] | *Identification* [b] |
|---|---|---|---|---|---|
| **37** | 1502 | butylmethoxypyrazine | ginseng | 3 | O, RI |
| **38** | 1507 | (*E*)-2-nonenal | O3, fishy, hay | 3 | O, RI |
| **39** | 1525 | hexyl hexanoate | pepper, green, ginseng | 27 | |
| **40** | 1533 | unknown | blueberry | 243 | |
| **41** | 1536 | unknown | plastic | 9 | |
| **42** | 1542 | ethyl 2-hydroxy-4-methylpentanoate | hay like | 9 | MS |
| **43** | 1547 | unknown | floral, lavender | 81 | |
| **44** | 1583 | unknown | minty | 1 | |
| **45** | 1585 | (*E,Z*)-2,6-nonadienal | green, cucumber, fresh | 27 | O, RI |
| **46** | 1640 | phenylcetaldehyde | rosy | 3 | O, RI |
| **47** | 1667 | 2-methyl-3-(methyldithio)furan | vitamin | 729 | O, RI |
| **48** | 1677 | unknown | guava | 27 | |
| **49** | 1700 | unknown | nutty | 1 | |
| **50** | 1716 | unknown | hay | 9 | |
| **51** | 1725 | benzyl acetate | floral | 27 | |
| **52** | 1738 | methionol | gas | 9 | O, RI |
| **53** | 1737 | 3-methylnonane-2,4-dione | hay like | 9 | O, RI |
| **54** | 1743 | unknown | citrus | 9 | |
| **55** | 1757 | dimethyl tetrasulfide | gas | 729 | O, RI |
| **56** | 1768 | unknown | fermented fruit | 3 | |
| **57** | 1783 | ethyl 2-phenylacetate | rosy | 81 | O, RI, MS |
| **58** | 1795 | unknown | plastic | 3 | |
| **59** | 1800 | furfuryl thioacetate | chicken bouillon, meaty | 27 | O, RI |
| **60** | 1807 | unknown | nutty | 3 | |
| **61** | 1809 | 2-phenylethyl acetate | peachy | 9 | O, RI, MS |
| **62** | 1816 | β-damascenone | peachy, red date | 729 | O, RI |
| **63** | 1820 | unknown | bleach | 9 | |
| **64** | 1844 | unknown | plastic | 3 | |
| **65** | 1846 | unknown | herbal | 3 | |
| **66** | 1856 | guaiacol | smoky, medicinal | 243 | O, RI |
| **67** | 1880 | ethyl 3-phenylpropanoate | rosy, floral, painty | 6561 | O, RI, MS |
| **68** | 1889 | ethyl phenyl propionate | floral, honey | 9 | O, RI, MS |
| **69** | 1906 | 2-phenylethanol | rosy, plum | 243 | O, RI |
| **70** | 1934 | β-ionone | osmanthus, violet | 1 | O, RI |
| **71** | 1965 | unknown | vitamin B | 27 | |
| **72** | 2000 | unknown | Meaty | 27 | |

***Table 3.1 A. continued***

| *No.* | *RI Stabilwax* | *Compound* | *Description* | *FD Factor* [a] | *Identification* [b] |
|---|---|---|---|---|---|
| **73** | 2010 | unknown | metallic | 9 | |
| **74** | 2031 | furaneol | marshmallow | 81 | O, RI |
| **75** | 2023 | γ-nonlactone | peachy | 27 | O, RI, MS |
| **76** | 2043 | unknown | peachy | 9 | |
| **77** | 2081 | p-cresol | fecal, animal barn | 81 | O, RI, MS |
| **78** | 2127 | ethyl cinnamate | rosy, fruity | 9 | O, RI, MS |
| **79** | 2135 | *bis*-(2-methyl-3-furyl) disulfide | meaty, vitamin | 3 | O, RI |
| **80** | 2148 | unknown | plastic, meaty | 3 | |
| **81** | 2178 | unknown | herbal, smoky | 81 | |
| **82** | 2184 | unknown | leather-like | 9 | |
| **83** | 2202 | unknown | baijiu, soy Sauce | 3 | |
| **84** | 2269 | unknown | red date | 1 | |
| **85** | 2364 | unknown | sesame | 3 | |
| **86** | 2413 | unknown | floral, cherry | 9 | |
| **87** | 2556 | unknown | sweaty | 81 | |
| **88** | 2573 | unknown | savory | 3 | |

[a] Flavor dilution factor determined on Stabilwax column. [b] Criteria for identification: O = odor property, RI = retention index, MS = mass spectrum.

**Table 3.1 B. Odorants Detected In The Acidic Fraction of An Aroma Extract of Moutai Liquor Prepared by Direct Solvent Extraction Combined With Solvent Assisted Flavor Evaporation (DSE-SAFE)**

| *No.* | *RI Stabilwax* | *Compound* | *Description* | *FD Factor* [a] | *Identification* [b] |
|---|---|---|---|---|---|
| **89** | 1439 | acetic acid | vinegar | 9 | O, RI, MS |
| **90** | 1525 | propanoic acid | fecal, cheesy | 9 | O, RI, MS |
| **91** | 1557 | 2-methylpropanoic acid | Swiss cheese | 9 | O, RI, MS |
| **92** | 1615 | butyric acid | cheesy | 6561 | O, RI, MS |
| **93** | 1660 | 2-/3-methylbutanoic acids | cheesy, Feet | 19683 | O, RI, MS |
| **94** | 1731 | pentanoic acid | cheesy | 81 | O, RI, MS |
| **95** | 1804 | unknown | smoky, herbal | 3 | |
| **96** | 1838 | hexanoic acid | body odor, sweat | 9 | O, RI, MS |
| **97** | 1869 | unknown | earthy, herbal | 27 | |
| **98** | 1925 | 2,3-dimethyl-2-pentenoic acid | sour, sweaty | 1 | MS |
| **99** | 1953 | heptanoic Acid | sour, sweaty | 1 | O, RI, MS |
| **100** | 2035 | unknown | waxy, sour, sweaty, raw peanut | 1 | |
| **- -** | 2050 | octanoic Acid | NA[c] | NA | RI, MS |
| **101** | 2115 | unknown | marine, chlorine, musty, bleach | 3 | |
| **102** | 2135 | unknown | curry, maple | 3 | |
| **- -** | 2155 | nonanoic acid | NA | NA | RI, MS |
| **103** | 2187 | unknown | chemical | 1 | |
| **104** | 2194 | sotolon | curry | 19683 | O, RI |
| **105** | 2247 | unknown | waxy, carrot, plastic | 9 | |
| **- -** | 2259 | decanoic acid | NA | NA | RI, MS |
| **106** | 2546 | phenylacetic acid | honey, waxy, sour, floral | 243 | O, RI, MS |
| **107** | 2569 | vanillin | vanilla | 9 | O, RI, MS |
| **108** | 2610 | phenylpropanoic acid | fatty, cinnamon, fermented soy | 81 | O, RI, MS |
| **- -** | 2050 | octanoic acid | NA | NA | RI, MS |

[a] Flavor dilution factor determined on Stabilwax column. [b] Criteria for identification: O = odor property, RI = retention index, MS = mass spectrum. [c] NA, not available, no odor was detected for this compound.

**Table 3.1C. Odorants Detected In The Basic Fraction of An Aroma Extract of Moutai Liquor Prepared by Direct Solvent Extraction Combined With Solvent Assisted Flavor Evaporation (DSE-SAFE)**

| *No.* | *RI Stabilwax* | *Compound* | *Description* | *Identification* [b] |
|---|---|---|---|---|
| **109** | 1203 | 2,4,5-trimethyloxazole | green | O, RI, MS |
| **- -** | 1273 | 2-methylpyrazine | NA[c] | RI, MS |
| **110** | 1292 | acetoin | creamy, buttery | O, RI, MS |
| **111** | 1299 | unknown | peanut, green | |
| **- -** | 1329 | 2,5-dimethyl pyrazine | NA | RI, MS |
| **112** | 1336 | 2-acetyl-1-pyroline | rice, popcorn | O, RI, MS |
| **- -** | 1358 | 2,3-dimethyl pyrazine | NA | RI, MS |
| **113** | 1389 | 2-ethyl-6-methylpyrazine | fruity | O, RI, MS |
| **26** | 1409 | trimethyl pyrazine | solvent like | O, RI, MS |
| **- -** | 1449 | 2-ethyl-3,6-dimethyl-pyrazine | NA | RI, MS |
| **114** | 1466 | 2-ethyl-3,5-dimethyl-pyrazine | solvent like | O, RI, MS |
| **- -** | 1469 | furfural | NA | RI, MS |
| **- -** | 1487 | tetramethyl pyrazine | NA | RI, MS |
| **115** | 1611 | 2-acetyl pyridine | rice, popcorn | O, RI, MS |
| **- -** | 1615 | diethylene glycol ethyl ether* | NA | RI, MS |
| **116** | 1635 | 2-acetyl-3-methyl pyrazine | chicken bouillon | O, RI, MS |
| **117** | 1651 | unknown | earthy | |
| **- -** | 1655 | furfuryl alcohol | NA | RI, MS |
| **118** | 2008 | unknown | paper, cardboard | |
| **119** | 2193 | unknown | rubber | |
| **120** | 2244 | unknown | plastic | |

[b] Criteria for identification: O = odor property, RI = retention index, MS = mass spectrum. [c] NA, not available, no odor was detected for this compound.

**Table 3.2. Odor-active Compounds Identified by Aroma Extract Dilution Analysis (AEDA) of a Distillate of Moutai Prepared by Solvent Assisted Flavor Evaporation (SAFE-DIST)**

| *No.* | *Compound* | *Odor Description* | *RI (RTX-wax)* | *RI (RTX-5ms)* | *FD factors in MT SAFE Distillate* | |
|---|---|---|---|---|---|---|
| | | | | | *RTX-wax* | *RTX-5ms* |
| **1** | acetaldehyde | sweet, alcohol | <700 | < 700 | 32 | 16 |
| **2** | 2-methylpropanal | malty | 802 | < 700 | 16 | 8 |
| **121** | acetal | fruity | 882 | 741 | 32 | 16 |
| **3** | 3-methybutanal | malty | 932 | < 700 | 32 | 32 |
| **122** | unknown | radish, cabbage | 975 | | 256 | - |
| **4** | ethyl propanoate | fruity, berry | 990 | 720 | 16 | 4 |
| **7** | ethyl 2-methylpropanoate | berry | 992 | 766 | 256 | 128 |
| **8** | 2,3-butanedione | buttery | 994 | < 700 | 4 | 4 |
| **123** | unknown | fruity, berry | 1054 | | 32 | - |
| **10** | ethyl butanoate | papaya, berry | 1062 | 810 | 128 | 64 |
| **11** | ethyl 2-methylbutanoate | berry candy | 1071 | 860 | 128 | 128 |
| **12** | ethyl 3-methylbutanoate | fruity, berry | 1082 | 862 | 64 | 16 |
| **13** | 2-methyl-1-propanol | malty | 1095 | | 1 | 32 |
| **15** | ethyl pentanoate | fruity | 1140 | 901 | 32 | 8 |

***Table 3.2 Continued***

| *No.* | *Compound* | *Odor Description* | *RI (RTX-lwax)* | *RI (Rtx-5ms)* | *FD factors in MT SAFE Distillate* | |
|---|---|---|---|---|---|---|
| | | | | | *RTX-wax* | *RTX-5ms* |
| **16** | 2-methylpropyl butanoate * | berry | 1176 | | 2 | - |
| **17** | ethyl 4-methylpentanoate | berry | 1183 | 976 | 64 | 16 |
| **19** | ethyl hexonoate | fruity, hop | 1215 | 1003 | 4 | 8 |
| **124** | unknown | earthy | 1240 | - | 1 | - |
| **18** | 2- & 3-methyl-1-butanol | malty | 1250 | 749 | 64 | 4 |
| **20** | furfuryl ether* | solvent | 1285 | | 32 | - |
| **125** | unknown | buttery | 1312 | | 2 | - |
| **24** | dimethyl trisulfide | cabbage | 1352 | 968 | 32 | 8 |
| **25** | ethy 2-hydroxybutanoate | fruity, berry | 1402 | | 4 | - |
| **28** | ethyl octanoate | soapy, fruity | 1420 | | 16 | - |
| **55** | dimethyl tetrasulfide | cabbage | 1430 | 1197 | - | 16 |
| **89** | acetic acid | vinegar | 1447 | - | 32 | - |
| **113** | 2-ethyl-3,5 dimethyl pyrazine | solvent | 1461 | | 8 | - |
| **126** | unknown | waxy, carrot | 1520 | | 4 | - |

***Table 3.2 Continued***

| *No.* | *Compound* | *Odor Description* | *RI (RTX-wax)* | *RI (RTX-5ms)* | *FD factors in MT SAFE Distillate* | |
|---|---|---|---|---|---|---|
| | | | | | *RTX-wax* | *RTX-5ms* |
| **40** | unknown | fruity, berry | 1530 | | 32 | - |
| **91** | 2-methyl propionic acid | sour | 1537 | 853 | 4 | 1 |
| **92** | butyric acid | stink, cheesy | 1620 | 885 | 4 | 1 |
| **46** | phenyl acetaldehyde | rosy | 1624 | 1052 | 8 | 1 |
| **47** | 2-methyl-3-(methyldithio) furan | vitamin | 1652 | 1166 | 16 | 8 |
| **93** | 3 & 2 methyl butanoic acids | stink, cheesy, feet | 1660 | - | 32 | - |
| **50** | unknown | herbal, hay | 1702 | | 2 | - |
| **52** | methionol* | cabbage | 1711 | | 256 | - |
| **62** | β - damascenone | peachy, red date | 1797 | 1374 | 32 | 8 |
| **65** | unknown | herbal medicine | 1841 | | 8 | - |
| **68** | ethyl phenyl propionate | rosy | 1864 | 1338 | 256 | 8 |
| **69** | 2-phenyl ethanol | rosy | 1893 | 1088 | 128 | 4 |
| **71** | unknown | vitamin? | 1945 | | 2 | - |

***Table 3.2 Continued***

| *No.* | *Compound* | *Odor Description* | *RI (RTX-lwax)* | *RI (RTX-5ms)* | *FD factors in MT SAFE Distillate* | |
|---|---|---|---|---|---|---|
| | | | | | *RTX-wax* | *RTX-5ms* |
| **77** | *p*-cresol | animal barn | 2065 | | 2 | - |
| **79** | *bis*-(2-methyl-3-furyl) disulfide | meaty | 2105 | 1526 | - | 8 |
| **104** | sotolone | curry | 2161 | | 64 | - |
| **107** | vanillin | vanilla | 2516 | | 2 | - |
| **106** | phenyl acetic acid | rosy | 2526 | 1251 | 2 | 4 |
| **108** | phenyl propionic acid | sweaty | 2589 | - | 2 | - |
| **127** | unknown | caramel | - | 804 | - | 1 |
| **128** | unknown | Sour dough | - | 1109 | - | 8 |
| **129** | unknown | waxy | - | 1159 | - | 2 |
| **130** | unknown | hay | - | 1309 | - | 1 |
| **131** | unknown | cabbage | - | 1440 | - | 1 |

**Table 3.3. Potent Odorants Detected by Aroma Extract Dilution Analysis (AEDA) of Direct Solvent Extracts of A Distillate of Moutai Prepared by Solvent Assisted Flavor Evaporation (SAFE-DIST-DSE)**

| *No.* | *Compound* | *Odor Description* | *RI (Stabilwax)* | *RI (Rxi-5ms)* | *FD Factor* | |
|---|---|---|---|---|---|---|
| | | | | | *Stabil-Wax* | *Rxi-5ms* |
| **2** | 2-methylpropanal | malty | <800 | <700 | 81 | 81 |
| **121** | acetal | paint | 938 | 741 | 2187 | 243 |
| **3** | 3-methybutanal | malty | 947 | <700 | 729 | 729 |
| **4** | ethyl propanoate | fruity | 972 | 720 | 243 | 81 |
| **7** | ethyl 2-methylpropanoate | berry | 979 | 768 | 19683 | 6561 |
| **8** | 2,3-butanedione | buttery, creamy | 993 | <700 | 9 | 9 |
| **9** | 2-methylpropyl acetate* | solvent | 1015 | 792 | 9 | 27 |
| **10** | ethyl butanoate | fruity | 1042 | 806 | 6561 | 729 |
| **11** | ethyl 2-methylbutanoate | fruity, berry | 1057 | 857 | 59049 | 6561 |
| **12** | ethyl 3-methylbutanoate | fruity, berry | 1075 | 862 | 6561 | 729 |
| **14** | isoamyl acetate | banana | 1129 | - | 3 | - |
| **15** | ethyl pentanoate | berry | 1142 | 902 | 729 | 243 |
| **132** | ethyl 2-methylpentanoate* | fruity | 1142 | 944 | 729 | 81 |
| **16** | propyl 2-methylbutanoate* | fruity | 1183 | - | 81 | - |
| **17** | ethyl 4-methylpentanoate | berry | 1195 | 971 | 729 | 81 |

***Table 3.3 Continued***

| *No.* | *Compound* | *Odor Description* | *RI (Stabilwax)* | *RI (Rxi-5ms)* | *FD Factor* | |
|---|---|---|---|---|---|---|
| | | | | | *Stabil-wax* | *Rxi-5ms* |
| **18** | 2-/3-methyl-1-butanol | malty | 1213 | 749 | 81 | 9 |
| **19** | ethyl hexanoate | fruity | 1240 | 999 | 243 | 81 |
| **20** | furfuryl ether * | ether like | 1296 | 831 | 27 | 9 |
| **133** | 2-methyl-3-furanthiol* [a] | beefy, vitamin | - | 872 | - | 243 |
| **134** | 1-octen-3-one* | mushroom | 1312 | 1029 | 3 | 3 |
| **23** | ethyl heptanoate | hop | 1341 | 1099 | 3 | 1 |
| **24** | dimethyl trisulfide* | cabbage | 1385 | 969 | 243 | 27 |
| **27** | ethyl cycloheanoate* | berry | 1430 | 1135 | 81 | 27 |
| **28** | ethyl octanoate | waxy, plastic | 1440 | 1197 | 27 | 9 |
| **30** | 2-furfurylthiol* | meaty | 1447 | 912 | 3 | 81 |
| **31** | 3-methylbutyl hexanoate | berry | 1463 | - | 243 | - |
| **38** | (*E*)-2-nonenal* | metallic | 1518 | 1150 | 9 | - |
| **135** | 2,3-diethyl-5-methylpyrazine | nutty, roasted cocoa | - | 1063 | - | 27 |

***Table 3.3 Continued***

| *No.* | *Compound* | *Odor Description* | *RI (Stabilwax)* | *RI (Rxi-5ms)* | *FD Factor* | |
|---|---|---|---|---|---|---|
| | | | | | *Stabil-wax* | *Rxi-5ms* |
| **40** | unknown | berry | 1544 | 962 | 81 | 3 |
| **136** | 1-octanol | citrus, waxy | 1555 | - | 3 | - |
| **91** | 2-methylpropanoic acid | cheesy, stink | 1582 | 843 | 3 | 9 |
| **45** | (*E,Z*)-2,6-nonadienal | cucumber | 1599 | 1116 | 3 | 3 |
| **92** | butyric acid | cheesy | 1637 | 872 | 27 | 1 |
| **46** | phenyl acetaldehyde | rosy | 1663 | 1032 | 27 | 9 |
| **93** | 3-/2-methylbutanoic acid | cheesy, feet | 1676 | - | 27 | - |
| **47** | 2-methyl-3-(methyldithio) furan* | vitamin | 1681 | 1174 | 243 | 27 |
| **48** | unknown | seeds | 1695 | 869 | 9 | 27 |
| **94** | pentanoic acid | cheesy | 1747 | - | 9 | - |
| **55** | dimethyl tetrasulfide* | cabbage | 1760 | 1214 | 27 | 9 |
| **57** | ethyl phenylacetate | rosy | 1801 | 1247 | 27 | 81 |
| **61** | 2-phenylethyl acetate | rosy, honey | 1829 | - | 3 | - |

***Table 3.3 Continued***

| *No.* | *Compound* | *Odor Description* | *RI (Stabilwax)* | *RI (Rxi-5ms)* | *FD Factor* | |
|---|---|---|---|---|---|---|
| | | | | | *Stabil-wax* | *Stabil-wax* |
| **62** | β-damascenone* | fruity, peachy | 1837 | 1384 | 243 | 243 |
| **137** | geraniol* | citrus | 1859 | - | 9 | - |
| **66** | guaiacol* | smoky | 1884 | 1091 | 9 | 1 |
| **68** | ethyl phenylpropionate | fruity, grape | 1898 | 1349 | 243 | 81 |
| **69** | 2-phenylethanol | rosy | 1933 | 1130 | 243 | 3 |
| **70** | β ionone | floral | 1954 | - | 3 | - |
| **138** | 4-ethylguaiacol | smoky, clove | 2055 | 1281 | 3 | - |
| **75** | γ-nonlactone | peachy | 2062 | 1380 | 9 | 1 |
| **77** | p-cresol | animal barn | 2107 | 1106 | 27 | 1 |
| **78** | ethyl cinnamate | rosy | 2159 | - | 3 | - |
| **79** | *bis*(2-methyl-3-furyl) disulfide | meaty | 2178 | 1526 | 9 | - |
| **139** | 4-ethylphenol | smoky, manure | 2205 | - | 27 | - |
| **104** | sotolone | herbal, curry | 2233 | - | 243 | - |
| **140** | unknown | plastic | 2266 | - | 9 | - |

***Table 3.3 Continued***

| ***No.*** | ***Compound*** | ***Odor Description*** | ***RI (Stabilwax)*** | ***RI (Rxi-5ms)*** | ***FD Factor*** | |
|---|---|---|---|---|---|---|
| | | | | | ***Stabil-wax*** | ***Stabil-wax*** |
| **141** | syringol | bacon, smoky | 2305 | - | 3 | - |
| **142** | unknown | herbal | 2386 | - | 3 | |
| **106** | phenylacetic acid | rosy | 2603 | 1287 | 27 | 1 |
| **107** | vanillin | vanilla | 2624 | - | 3 | - |
| **108** | 3-phenylpropanoic acid | sweaty, stink | 2657 | 1349 | 9 | - |
| **143** | unknown | cabbage | - | 1057 | - | 27 |

*Compound was tentatively identified by comparing its RI and odor properties with those of an authentic standard.
[a] Probable thermally generated artifact from *bis*(2-methyl-3-furyl)disulfide.

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# Chapter 4: A Streamlined Approach for Careful and Exhaustive Aroma Characterization of Aged Liquors

## 4.1 Abstract

An important requisite for the accurate determination of aroma compounds is their careful isolation prior to gas chromatography (GC). For this purpose, solvent-assisted flavor evaporation (SAFE) is considered to be the best overall method to produce a "clean" aroma extract which can be analyzed by cool on-column injection GC analysis and thus avoid degradation of labile aroma compounds or formation thermally-derived artifacts. However, SAFE is both time consuming and labor intensive, especially when applied repeatedly for quantitation by stable isotope dilution analysis (SIDA), which requires the addition of isotopes at specific mass ratios relative to the target analytes. In this study, a streamlined approach is described for the careful and accurate quantitation of odor-active components in various types of liquor products by use of only a single solvent assisted flavor evaporation (SAFE) operation. The quantitative results achieved by this method are nearly identical to those of the original liquors except for certain semi-volatile constituents which were not recovered well by SAFE in the brown liquors (e.g. vanillin and syringaldehyde). Furthermore, this approach also allows researchers to complete the flavor chemistry study on identification of odorants by using the same SAFE isolate which is especially suitable for GC-O dilution analysis for semi-quantitation of trace level potent odorants. In this study, 3 trace-level potent odorants (2-Methyl-3-furanthiol, 2-methyl-3-(methyldithio) furan and *bis*(2-methyl-3-furyl) disulfide) are to the best of our knowledge, identified for the first time in Moutai (MT), one of the clear liquors analyzed. Based on the results, this streamlined approach provides a simple and convenient way to expedite and streamline the study of the flavor chemistry of distilled spirits.

## 4.2 Introduction

SAFE combined with SIDA is a state-of-the-art approach for the accurate quantitative analysis of aroma components of foods and beverages. These methods represent the gold standards for extraction and quantitation, respectively. The advantage of SAFE is it can generate authentic "clean" extracts suitable for on-column GC analysis and thus avoid formation of any thermally generated odorants in the inlet of the instrument [1]. However, every coin has two sides – and thus

the main disadvantage of SAFE is it is quite labor and time intensive. Generally it takes more than 3 hours to achieve a single SAFE operation. Also, SAFE is not a method for micro-extraction. When quantitating the odor-active components of a SAFE isolate by SIDA, to guarantee the accuracy of the calibration, the ratios of all the isotopes to the target unlabeled components in the product have to be within the linear range of the calibration. Typically, isotopes are added to the product and equilibrated prior to extraction to guarantee that both the isotopes and the unlabeled target analytes undergo the same loss/concentration during the extraction process. The mixture of isotopes and unlabeled target analytes in the product is then extracted by SAFE and the peak area ratios of the isotope and target analytes are determined by GC-MS to provide guidance for the adjustment of isotope addition if needed. To establish the ratio of each isotope to its corresponding target analyte within the linear range of the standard curve, this "addition-extraction-adjustment" procedure is repeated several times before finally the correct level of isotope addition is determined. To make this procedure more efficient, generally a group of labeled internal standards are added at the same time and adjusted at the same time to eventually quantitate all the target analytes. However, if additional target analytes are included, the whole "addition-extraction-adjustment" procedures must be conducted many extra times just for quantitation of only a few target analytes. Not only is the whole procedure tedious, but also can be expensive, especially when some isotopes are very costly or the sample to be analyzed is expensive or scarce. All of these factors would be barriers to the determination of the complex flavor chemistry of the target product. In this case, optimizing the efficiency of this quantitative analysis method is necessary and essential to reduce the cost and time as well as maintain the accuracy of this state-of-the-art methodology. For this purpose, a streamlined approach is proposed.

In this proposed streamlined approach, SAFE is used to prepare a "clean" extract of the original product without altering the volatile composition. If the recovery of SAFE is complete and losses during the extraction process are negligible, there is no need to add the isotopes or internal standards before SAFE to guarantee the isotopes or internal standards undergo the same loss/concentration during extraction. Based on this rationale, a more advanced procedure based on reducing the number of SAFE operations must be conducted to reduce costs related to isotopes and samples as well as reduce analysis labor and time.

In this procedure, isotopes were added after SAFE extraction is completed instead of before any extraction procedures as in the standard protocol[2]. This adjustment makes microscale direct solvent extraction feasible which benefits quantitative analysis in multiple ways. By following the proposed procedure, it is also possible to conduct small scale direct solvent extraction using various solvents of different polarities to reach a better understanding of the aroma-profile of the target product which is not practical by the standard protocol. Besides, the SAFE isolate with no internal standards added is also suitable for other analysis such as GC-MS and GC-O dilution analysis for identification and semi-quantitative analysis purposes which allows the flavor chemistry study on the target product finished with one SAFE operation.

As mentioned earlier, the validity of the streamlined method depends on whether the aroma compositions of the SAFE isolates are identical to those of the original liquor products. In other words, volatile losses during SAFE must be negligible and thus no appreciable changes in the flavor profile should occur as result of SAFE.

To assess the feasibility of this proposed procedure and to identify potential pitfalls and limitations, a series of experiments were conducted. Since the standard protocol has limitations regarding quantitation of odor-active components of scarce or expensive products. Aged distilled liquor is a case in point. Thus, in this study the validity and limitation of the proposed streamline approach would be investigated by quantitation of odorants in aged distilled liquors. Liquor samples were compared with their counterpart SAFE isolates by using sensory and instrumental methods. To have a better assessment of the widespread potential of the proposed procedure, three clear liquors which were aged in porcelain and three brown liquors, which were aged in oak barrels, were selected to validate the proposed procedure.

## 4.3 Materials and Methods

### 4.3.1 Materials

All the liquors used in this study were commercial products (**Table 4.1**). The selected clear distilled liquors including the top soy sauce aroma liquor Moutai (MT), which is also the national liquor of China, the top sesame flavor liquor Yi Pin Jing Zhi (YPJZ) and a mid-range strong

aroma liquor Gu Jing Gong Jiu (GJGJ). These liquors represent top and medium level clear liquor products which are aged in pottery. For the brown liquors, one whiskey Evan Williams Kentucky Bourbon Whiskey (EWW), one tequila Don Julio Tequila (DJT) and one rum Appleton Estate Jamaica Rum (Aged 12 years) (AER) were selected which represent high and mid-range popular brown liquor products which are aged in oak barrel.

Mention of brand name is not for advertisement or endorsement purposes and does not imply any research contract or sponsorship.

### 4.3.2 Chemicals

All authentic reference standards were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

The following isotopically labeled compounds were purchased from the supplier listed in parentheses: [$^{2}H_5$]-propionic acid (Cambridge Isotope Laboratories, Inc., Andover, MA, USA); [1,2-$^{13}C_2$]-phenylacetic acid and [1,2-$^{13}C_2$]- butyric acid (Isotec, Miamisburg, OH, USA); [$^{2}H_9$]-pentanoic acid and [2,2-$^{2}H_2$]-3-methylbutanal (CDN Pointe-Claire, Quebec, Canada). [$^{2}H_3$]-*p*-cresol (CDN, Pointe-Claire, Quebec, Canada)

The following labeled and unlabeled compounds were synthesized according to procedures reported in the literature (in parentheses): [1,2-$^{13}C_2$]-2-phenylethanol (Schieberle, 2006) and [$^{2}H_5$]-ethyl bis(2-methyl-3-furyl) disulfide[4] (Hofmann, T.1996); $^{13}C_2$-sotolon[5] (Blank et al., 1996); 4-hydroxy-3-[$^{2}H_3$]-methoxybezaldehyde ($d_3$-vanillin) (Scheider and Rolando, 1992); [$^{2}H_4$]-β-damascenone (Kotseridis et al., 1998).

**Synthesis of 5,5,6,6-$^{2}H_4$-hexanoic acid**

5-Hexyn-1-ol was deuterated to [5,5,6,6-$^{2}H_4$]-hexan-1-ol using the method described Hausch, Lorjaroenphon, and Cadwallader (2015) [18]. The deuterated alcohol was then oxidized to the corresponding acid using potassium permanganate as described by Guth and Grosch (1994) [19] for the synthesis of [3,4-$^{2}H_2$]-3-methylbutyric acid.

**Synthesis of $^{2}H_{3}$-syringaldehyde**

$^{2}H_{3}$-Syringaldehyde was synthesized using the procedure developed by Lahne [6]. In a screw-capped test tube (PTFE-top) equipped with a stir bar 3,4- Dihydroxy-5-methoxybenzaldehyde (0.501 g; 3 mmol) was dissolved in aqueous 40% (w/v) KOH (5 mL). Then, under a gentle steam of nitrogen, over the course of 30 minutes, 0.35 mL (0.42 g, 3.2 mmol) of $d_6$-dimethylsulfate was added (5 to 6 drops every 5 minutes) to the reaction tube, after which the reaction mixture became yellow and cloudy. The vial was then capped and stirred for 2 hr. The reaction was checked for completion by removing 5-6 drops of the reaction mixture, adding it to a vial containing 1 mL aqueous 1N HCl and 0.5 mL ethyl acetate, and then analyzing the ethyl acetate layer by GC-MS. The reaction was continued, adding 0.08 mL (0.096 g, 0.73 mmol) of $d_6$-dimethylsulfate and allowing the reaction to stir overnight until nearly all starting material had been consumed. The reaction was stopped by acidifying the mixture to ~pH 1 and then it was extracted with ethyl acetate (1 x 10mL, 4 x 5 mL). The ethyl acetate layer was washed with saturated NaCl and then dried over anhydrous $Na_2SO_4$. The solution was concentrated to ~10 mL using a vigreux column and the remaining solvent was then removed under a stream of nitrogen. The final product was weighed for a final yield of 0.5734 g.

**Synthesis of $^{2}H_{5}$-ethyl esters – general procedure**

The following reagents were added to a 20-mL screw top test tube: 10 mmol of organic acid; 200 uL of $d_5$-ethanol (158 mg; 3 mmol) and 2 drops concentrated $H_2SO_4$. The test tube was sealed PTFE-lined screw cap and placed in a large bottle or beaker to protect against breakage and then incubated 100 °C oven for 2 hr. After cooling to room temperature, the mixture was diluted with pentane (10 mL) and then 5 mL of aqueous saturated $Na_2CO_3$ solution was added. The aqueous layer was removed the pentane layer was washed again with $Na_2CO_3$ (5 mL). (This step is necessary in order to remove any unreacted acid.). The pentane layer was washed with aqueous saturated NaCl (2 x 5 mL) and then dried over anhydrous $Na_2SO_4$. The solvent (pentane) was evaporated off to yield the ester, generally in high purity (>99%). If needed, the ester was further purified by flash chromatography on silica gel using 100% pentane as elution solvent.

**Synthesis of a working solution of 2-methyl-[2,3-$^{2}H_2$]-propanal and 2-methyl-[3,4-$^{2}H_2$]-butanal**

The labeled aldehydes were synthesized in two steps, beginning with the synthesis of the unsaturated alcohols followed by their oxidation to the corresponding aldehydes. 2-methyl-[2,3-$^{2}H_2$]-propan-1-ol was synthesized from 2-methyl-2-propen-1-ol (Sigma-Aldrich) using the method of Lapsongphon et al. (2015). 2-methyl-[3,4-$^{2}H_2$]-butan-1-ol was synthesized according to the method previously described for the synthesis of 3-methyl-[3,4-$^{2}H_2$]-butan-1-ol (Steinhaus and Schieberle, 2005) with slight modification, as follows: chlorotri(triphenylphosphine)-rhodium(I) (Wilkinson's catalyst, 0.15 g)(Sigma-Aldrich), 2-methyl-3-buten-1-ol (0.950 g, 11.0 mmol)(Sigma-Aldrich) were placed in a pressure reactor equipped with a stir bar and rubber septum. The reactor was flushed for 5 min with deuterium gas (40 psi; UHP grade 99.995%; isotopic enrichment 99.7%; Matheson Tri-Gas, Parsippany, NJ, USA) using a needle placed below the solution. The spent catalyst was removed by centrifugation after the reaction was complete. 2-methyl-[3,4-$^{2}H_2$]-butan-1-ol was obtained after purification by vacuum distillation: 0.470 g (49.5 % yield). MS-EI, *m/z (%)*: 58 (100), 43 (88), 42 (81), 45 (81), 57 (73), 44 (55), 41 (35), 40 (28), 39 (17), 76 (1, $M^{+}$).

2-methyl-[2,3-$^{2}H_2$]-propan-1-ol (50 mg; 0.66 mmol) and 2-methyl-[3,4-$^{2}H_2$]-butan-1-ol (50 mg; 0.55 mmol) in 2 mL of 1,2-dichloroethane (DCE)(Sigma-Aldrich) were added in one portion to a stirred solution of pyridinium chlorochromate (0.43 g; 0.002 mol) in 5 mL of DCE. After stirring for 1.5 h at room temperature the reaction mixture was passed through a column of Florisil (10 g) followed by an additional 10 mL of DCE to rinse the column. The final solution (approximately 15 mL) was used directly in SIDA. Concentrations of the labeled aldehydes were determined by GC-FID with external standard calibration using the corresponding unlabeled aldehydes as standards.

### 4.3.3 Methods

**Direct distillation of liquor (SAFE-DIST)**

Liquor sample (100 mL) was directly distilled using the aforementioned SAFE apparatus at 40 ºC under high vacuum ($10^{-4}$-$10^{-5}$ Torr) and the volatile fraction was condensed in glass traps

cooled with liquid nitrogen. Distillation was conducted for 2 hr and then distillate was thawed and stored in a glass bottle equipped with a PTFE-lined cap.

**Sensory Methodology**

Testing was approved as protocol number 17507 of the Institutional Review Board (IRB) of the University of Illinois at Urbana-Champaign. Panelists (24), ranging in age from 21-54 were selected to participate the triangle sensory testing to determine whether the overall aroma characteristics of the SAFE-DIST isolates differed from original (neat) liquor products.

SAFE isolates and liquor products (20 mL each) were transferred into 125-mL Teflon sniff bottles (Nalgene PTFE wash bottle without asiphon tube; Nalge Nunc International, Rochester, NY, USA) which were wrapped with aluminum foil to minimize visual bias and to protect the liquors from light exposure. The temperature of the room was 22-26 °C and the humidity was 12%. For triangle difference testing [7], samples were presented to panelists in two orders: one set consisted of two SAFE-DIST isolates and one original (neat) liquor product and the other consisted of one SAFE-DIST isolate and two original (neat) liquor products. The aroma of the samples was assessed by 24 experienced panelists aged from 21 to 54.

**GC-FID Analysis**

Original liquors and their counterpart SAFE isolates were analyzed using an Agilent 6890 GC-equipped with a flame-ionization detector (FID) (Agilent Technologies, Inc., Santa Clara, CA, USA). Separations were performed using an Rtx-Wax column (15m × 0.53mm i.d. ×1 μm film thickness; Restek Corp., Bellefonte, PA, USA). Analyses were conducted in triplicate to assure accurate and precise measurements. The samples were injected in hot split mode (1:10) with an inlet temperature of 250 °C. The carrier gas was helium at a flow rate of 2 mL/min. The oven temperature was programmed from 35 °C to 225 °C at a ramp rate of 10 °C/min with initial and final hold times of 5 and 20 min, respectively. Peak areas for selected compounds found in moderate to high abundance were compared across the original liquors and their counterpart SAFE isolates.

**Gas Chromatography- Mass Spectrometry-Olfactometry (GC-MS-O)**

One clear liquor (MT) and one brown liquor (EWW) were analyzed to compare the aroma profile before and after SAFE distillation. Odor-active components in liquor products and their SAFE isolates were extracted by direct solvent extraction (DSE). SAFE distillate or neat liquor (7.5 mL) was pipetted into a 50-mL glass centrifuge tube containing 40 mL of odor-free deionized-distilled water and 0.5 mL of DCM. The mixture was shaken vigorously for 5 minutes and centrifuged at 4500 rpm for 10 minutes. The extraction procedure was repeated two more times. The pooled solvent extract was frozen overnight to remove excess water, then the extract was transferred into a 2mL vial, condensed to 1 mL using a gentle stream of ultra-high purity nitrogen gas.

GC-MS-O was performed using an Agilent 6890N GC/5973N mass selective detector (MSD) system (Agilent Technologies, Santa Clara, CA, USA). Analyses were performed on both polar (Stabilwax, 30m × 0.25 mm i.d., 0.25 μm film thickness; Restek Corp., Bellefonte, PA, USA) and nonpolar (Rxi-5ms, 30m × 0.25 mm i.d., 0.25 μm film thickness; Restek Corp., Bellefonte, PA, USA) columns. Aroma extracts (2μl) were injected under colds-plitless mode (-50°C initial temperature (0.1 min hold), ramped at 12 °C/s to 250 °C and held for 20 min). The carrier gas was helium at a flow rate of 1mL/min. The oven temperature was programmed from 40°C to 250°C at a ramp rate of 3°C/min with initial and final hold times of 5 and 30 min, respectively. Temperatures of MSD transfer line and olfactory port were set at 250 °C. Mass scan range was set as 33-200 amu with scan rate of 5.27 scans/s and electron energy was 70eV. GC-MS data were analyzed by ChemStation Enhanced Data Analysis Software (Aglient Technologies, Inc.). For tentative compound identifications, mass spectra of the analytes were compared against those in National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST, 2008).

**Compound Identification**

Retention indices (RI) were calculated based on comparing the retention times of analytes to those determined for a homologous series of n-alkanes (from $C_7$ to $C_{28}$) analyzed under the same analytical conditions (van Den Dool and Kratz, 1963). Odorants were identified by comparing their retention indices (RI) on both polar and nonpolar GC columns, mass spectra and odor properties to those of authentic standards. A compound was considered positively identified if

all three of the above criteria matched those of a reference standard. However, in some cases, an odorant was considered tentatively identified when one or more of the above criteria could not be met, e.g. when no mass spectrum was available due to the compound being present at a trace level - below that of the detection limit of the MSD, or whenever no authentic standard was available to confirm an RI, mass spectral or odor property match. In the latter case, the compound was considered tentatively identified when its RI, mass spectra and odor properties were in agreement with literature values or database entries (NIST, 2008).

**Aroma Extract Dilution Analysis (AEDA)**

Relative potencies of odor-active compounds were determined by AEDA. DSE extracts were diluted stepwise at a ratio of 1:3 (v/v) in DCM and each dilution was analyzed by GC-MS-O under the conditions previously described. Flavor dilution (FD) factors of each odorant were determined as the highest dilution at which the odorant was last detected by GCO [8]. The FD factors were shown as $\log_3$ FD-factors for better evaluation and comparison between each liquor product and its respective SAFE isolate.

**Quantitation by Stable Isotope Dilution Analysis (SIDA)**

SIDA was applied to accurately evaluate the recovery of volatiles or semi-volatiles of SAFE-DIST. For this purpose, the concentrations of selected important components in MT and EWW and their respective SAFE-DIST isolates were determined by SIDA and compared.

Deuterated or carbon-13 labeled isotopes of selected analytes were dissolved in ethanol or dichloromethane and spiked into 1 mL aliquots of neat liquor products or their SAFE-DIST isolates. Sample analysis was performed in the same manner as mentioned previously by using a Stabilwax column (30m × 0.25 mm i.d., 0.25 μm film thickness; Restek Corp.) and injections were made using the cold-split mode (split ratio 10:1; -50 °C initial temperature (0.1 min hold), ramped at 12 °C/s to 250 °C and held for 20 min). Samples were analyzed under simultaneous full scan (35-300 amu) and selected ion monitoring (SIM) modes. Selected ions used for determination of peak areas of labeled and unlabeled components are listed in **Table 4.6**.

For quantitation of the Strecker aldehydes (2-methyl-1-propanal, 2-methyl-butanal and 3-methyl-butanal) in liquor samples, headspace solid-phase microextraction (HS-SPME) was applied. MT (50 μL) or EWW (500 μL) were transferred to a 20-mL SPME vial containing 0.5 g of sodium chloride and 2 mL or 1.5mL, respectively, of distilled odorless water. After spiking with a proper amount of isotope solution, samples were analyzed by a HS-SPME-GC-MS, consisting of an 6890 GC/5973N MSD (Agilent Technologies, Inc.) equipped with an MPS2 autosampler (Gerstel, Germany) and CS4 injection port (Gerstel, Germany). A three phase SPME fiber (divinylbenzene/ carboxen/ polydimethylsiloxane; Supelco, Bellefonte, PA, USA) was used for volatile extraction. Vials were equilibrated by incubation at 60 °C for 20 minutes followed by 10 minute sample headspace extraction and then injected into the GC using hot splitless mode (260°C; 4 min split valve-delay time). Separations were performed using a RTX-5ms column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Restek). Helium was used as the carrier gas at 1.7 mL/minute. Oven temperature was programmed as follows: initial temperature, 30° C (5 min hold), ramp rate 6° C/min, final temperature, 225° C (30 min hold).

**Quantitation of Sotolon**

A special case is sotolon which could be generated in the hot inlet of the GC. Due to its low threshold of 10 ppb in wine and its relatively low concentration in liquor products[9], the determination of concentration of sotolon requires extraction and fractionation. To determine whether it is an artifact and its recovery of SAFE, SIDA of sotolon was determined using 3 different methods.

1) Isotope solution was added to 10mL SAFE-DIST isolate followed by direct solvent extraction using DCM (2 mL x 3 times)
2) Isotope solution was added to 10 mL liquor product followed by direct solvent extraction by using DCM. (2 mL x 3 times)
3) Isotope solution was added to 10 mL liquor product followed by SAFE distillation (SAFE was operated as mentioned previously) and direct solvent extraction by using DCM. (2mL x 3 times)

DCM extract was washed with 3 mL 5% (W/V) sodium carbonate solution 3 times. Aqueous phase was then washed with DCM for 3 times and added back to solvent phase. Afterwards, the pH of aqueous phase was adjusted to 2 by 1N sulfuric acid solution and extracted with 2 mL DCM for 3 times. Solvent phase was condensed to 0.1mL with a gentle stream of ultra-high purity nitrogen gas. Each method was conducted in triplicate and the acid fractions were analyzed using the same method and instruments mentioned previously.

**Calibration**

The response factor (Rf) for each isotope against its unlabeled target analyte was determined by a five-point standard curve with the range of mass ratio from 1:5 to 5:1 (unlabeled : labeled see Appendix). Each point was tested in triplicate and the mass ratio of each point was plotted against its mean peak area ratio of selected ions of labeled and unlabeled target analytes.

The Rf of each labeled analyte was calculated by using the following equation:

$$\frac{mass\ (analyte)}{mass\ (labeled\ analyte)} = \frac{area\ (analyte)}{area\ (labeled\ analyte)}\ (Rf)$$

The concentration of each target analyte was calculated by using the following equation:

$$Concentration\ (analyte) = \frac{Rf \times Peak\ area\ ratio \times mass\ (labeled\ analyte)}{Sample\ volume}$$

**Determination of Potential Thermally Degradation of Disulfides**

Since some disulfides are thermally sensitive, one example is bis(2-methyl-3-furyl) disulfide (MFT-MFT) which could be formed by the thiol: 2-methyl-3-furanthiol (MFT)[10]. MFT-MFT will be degraded into thiols while being heated[11]. Two different injection methods are wildly used which are split/splitless and cool on-column injection[12]. In this case, hot or cold-splitless injection may create artifacts because samples will be heated at the inlet until the set point (250 °C) before being recondensed and enter the column. Since two disulfides [ bis(2-methyl-3-furyl) disulfide (MFT-MFT) & 2-methyl-3-(methyldithio) furan (MFT-MT) ] are identified in one of the clear liquor samples-MT, and the relative thiol (MFT) of MFT-MFT is also detected by cold splitless GC-MS-O with high FD factor (243) while not detectable by cool on-column injection which is an injection method which minimizes the artifacts formed at the inlet by allowing

samples directly go into the column. It is suspicious that the thiol is formed at the inlet by thermally degradation of MFT-MFT. For this purpose, potential artifact caused by different injection technologies are compared by using the alkanes which have the closest boiling point with the target analytes (MFT-MT & MFT-MFT) as internal standards (dodecane & hexadecane ). Boiling point of MFT-MT and MFT-MFT are 210°C and 280°C respectively while their internal standards dodecane & hexadecane have boiling points as 215-217 °C and 287 °C ( boiling points reference: Sigma official web site). The disulfides and alkanes are made as a solution in pentane, with the concentration of 10ppm MFT-MT, dodecane, MFT-MFT and 1ppm hexadecane. Samples are analyzed by the GC-MS instrument as described previously with a SAC-5 column ((30m × 0.25 mm i.d., 0.25 μm film thickness; Agilent, Santa Clara, CA, USA). Sample solution is analyzed by 3 different injection modes: cold-splitless; hot-splitless and cool on column. For cold-splitless injection, inlet was cooled to -50°C by liquid nitrogen and increased to 250 °C at the ramp of 12°C/s while the inlet was set as 200°C, 250°C and 300°C for hot-splitless injection to compare potential thermally degradation of disulfides. The inlet temperature of on-column injection is 40°C which is the same as the initial temperature of the oven. The solution was analyzed triplicated on each condition and peak areas of each disulfide were compared with its internal standard alkane for degradation assessment. The degradation was calculated by the following equation:

$$\text{Degradation (\%)} = 1 - \frac{\text{Peak area ratio } \left(\frac{\text{Disulfide}}{\text{Internal standard}}\right)}{\text{Pe}ak\ area\ ratio\ \left(\frac{Disulfide}{Interal\ standard}\right) by\ on\ column\ injection}$$

**4.4 Results and Discussion**

Sensory difference testing was conducted to assess whether perceived aroma differences existed between original liquor products and their corresponding SAFE-DIST isolates. Out of 24 judges, 7 to 11 of them, depending on the specific liquor samples, correctly picked the odd samples which is still well below the minimal correct responses needed for there to be a significant difference ($p \leq 0.05$) (**Table 4.2**). Thus, the results of sensory difference testing demonstrated that the overall aroma-profile of the original liquor products and their respective SAFE-DIST isolates did not differ ($p \leq 0.05$). Compared to the 3 brown liquors which were aged in oak

barrels, panelists had less correct responses for the 3 clear liquors which were aged in pottery (**Table 4.2**). Even though there was no chance for visual bias (the sample appearances were masked), there appeared to have been a context effect in the case of the brown liquors.

For further investigation, 9 compounds (2-methyl-1-propanol, 3-methy-1-butanol, ethyl hexanoate, acetic acid, tetra methyl pyrazine, ethyl 2-phenyl acetate, hexanoic acid, 2-phenylethanol and octanoic acid) with different function groups (including 2 fusel alcohols, 3 fatty acids, 2 esters, 1 pyrazine and 1 phenolic compound), different molecular weights and volatilities were selected evaluate the similarity between various distilled liquor products and their respective SAFE-DIST isolates (**Table.4.2**). Among all these 9 compounds, 2-methyl-1-propanol, 3-methy-1-butanol and ethyl hexanoate only have a difference below 2% between original liquor products and their SAFE isolates. The difference in peak areas of ethyl 2-phenyl acetate and 2-phenylethanol are below 5% between original liquor products and their SAFE isolates among 6 different liquors. However, for the selected 3 fatty acids, the differences between the original liquor products and their SAFE isolates are more significant in brown liquors than clear liquors. For instance, the difference in peak areas of acetic acid are below 1% in clear liquors, whereas, in brown liquors the differences are above 15%.

One clear liquor-MT and one brown liquor-EWW were selected for further study on the difference of aroma profiles before and after SAFE isolation. 61 compounds are detected through GC-MS-O analysis in MT and EWW original liquors and their SAFE isolates, 51 compounds are detected in MT and 36 compounds are detected in EWW. All the odor-active components identified in EWW have been reported[13]. Most of the odor-active components in MT were previously reported[14,15]. However, several components in MT contribute to its overall aroma-profile significantly are firstly identified, which include ethyl cyclohexylcarboxylate (No. 22); (E)-2-nonenal (No.27); 2-methyl-3-(methyldithio) furan (No.34); dimethyltetrasulfide (No.37); methyl benzenepropanoate (No. 50); bis(2-methyl-3-furyl) disulfide (No. 51).

Among these compounds, 54 compounds are identified positively or tentatively, while 7 of them were unknown. The major difference in odor-active components between MT and EWW is

during the maturation of Whiskey, some compounds are extracted from the oak barrels and contributed to its special aroma-profile, such as eugenol, syringaldehyde, etc[16].

The comparison represented the major difference in flavor chemistry between clear and brown liquors is because brown liquors are aged in oak barrels, thus some decomposition products of lignin, including those are extracted from the oak wood to the liquor products. The identified odor-active components including 18 esters (No. 4, 5, 7,-12, 14, 16, 20, 22, 23, 25, 38, 39, 43 and 50); 7 acids (No. 30, 31, 33, 36, 58 and 60); 7 sulfides (No. 18, 21, 24, 26, 34, 37, 51); 4 ketones (6, 19, 40 and 45); 5 aldehydes (No. 1, 2, 3, 27and 32); 5 phenols ( No. 42, 46, 48, 52 and 59); 4 alcohols (No.15, 41and 44); 1 pyrazine (No. 28); 1 lactone ( No. 47); 1 furanone (No. 54) and 1 ether (No. 17). The results are shown as $\log_3$ FD value to better present the similarity between original liquor products and their SAFE isolates. Among all these 61 odor-active components with different functional groups, volatilities, molecular weights and polarities, only 5 odorants in SAFE isolate of clear liquor MT are 1 dilution away from its original liquor. In the SAFE isolate of brown liquor EWW, vanillin is the only one which has smaller FD factor and is 2 dilutions away from its original liquor. The difference of vanillin is more significant than other odor-active components, which would be explained by its low volatility caused its poor recovery during SAFE distillation.

One of the noteworthy component is MFT (No. 18) which could be an artifact formed at the inlet. According to the study on potential thermally degradation of disulfides conducted in this study 16.73% MFT-MFT would be formed by cold-splitless injection due to the sample are heated at the inlet before enter the column (**Table 4.7**). Even through on-column is an ideal method to avoid this artifact (**Table 4.7**), it is not an option for comparison between extract of SAFE isolates and neat liquor products due to its little tolerance to dirty samples without SAFE distillation. Compared with hot-splitless injection method in which MFT-MFT was degraded by 20.29%, 31.72% and 42.21% with inlet temperatures of 200°C, 250°C and 300°C respectively, cold-splitless has significantly less artifacts. Therefore cold-splitless injection is used for this purpose.

The GC-MS-O AEDA provided semi-quantitative results of recovery of volatile and semi-volatile odor-active components. For an accurate evaluation of the similarity between original liquor products and their SAFE isolates, 24 components in clear (MT) or brown (EWW) liquors were selected for quantitation and comparison (**Table 4.5**). These components including 3 Strecker aldehydes, 11 esters, 7 acids, 1 alcohol and 2 semi-volatile components—vanillin and syringaldehyde. The quantitation results by SIDA show, volatile components like Strecker aldehydes, short chain fatty acids and ethyl esters from butyric acid to octanoic acid have a recovery from 98.13-100% in both clear liquor and brown liquors. However, in case of ethyl esters with acids with longer chains, the recoveries are decreased with the increase of the chains of the acids. In this study, the recovery of ethyl decanoate, dodecanoate and palmitate are 61.04%, 59.18% and 20.78% respectively. These high molecular weight esters are not very odor-active and thus do not contribute significantly to the overall aroma profile to the liquor products, that explains why even their recoveries are compromised however after extracted by SAFE the aroma profile is still not significantly different from the original liquors. However, these components may contribute to the mouth feel of a liquor product and might induce a taste difference if eliminated. The recoveries of semi-volatiles like vanillin and syringaldehyde are 17.14% and 5.24% respectively which agrees with the AEDA results of vanillin, since 2 dilutions away is roughly one ninth in concentration. The compromised recoveries of semi-volatiles may also explain why there were more correct responses in the triangle test between brown liquors and their isolates than those of clear liquors.

Based on the SIDA results, the recovery of acetic acids in brown liquor EWW is 99.2% which is much higher than the recovery determined by GC-FID. This phenomenon may be caused by the matrix change due to the SAFE procedure eliminated some semi-volatile components. This provided another reason to perform SIDA whenever possible to achieve more accurate determination of target analytes.

The quantitation results of sotolon by three different methods shows that even it could be formed at the inlet, in the case of MT, since the results of SIDA without SAFE and with SAFE (isotope added before SAFE distillation) are identical; sotolon is not an artifact but an odor-active component in this liquor. Results also show the recovery of sotolon by SAFE distillation is above

88% which could explain why the FD factors of sotolon in SAFE distillate and original liquor are the same.

To sum up, the proposed streamlined approach for quantitation of odor-active components in distilled liquors is evaluated by various technologies, including sensory tests, semi-quantitative analysis (GC-MS-O AEDA, GC-O dilution analysis) and advanced quantitation technology (SIDA).

All the assessment of the proposed streamlined approach by comparing the results of SAFE isolates with the original liquor products, the recovery of most odor-active components, including different function groups, with different polarities and of different molecular weights, through SAFE will not induce significant difference in overall-aroma profiles. The difference in concentrations of most odor-active components between original liquors and their SAFE isolates are negligible among both clear and brown liquors. There are some exceptions like semi-volatiles for example, vanillin and syringealdehyde. These components failed to be extracted exhausted by SAFE. Besides, the recovery of the esters of long chain fatty acids (contains more than 9 carbons) through SAFE is also limited and compromised. However, these esters are not odor-active and would not contribute to the overall aroma profile significantly.

Compared with the standard way to finish quantitation of compounds with the method of combination of SAFE and SIDA (**Fig. 4.1**) the "addition-extraction-adjustment" procedure to achieve reasonable addition of isotopes in this proposed streamlined approach is significantly shortened (**Fig. 4.2**). Since one distillation by using SAFE will take 1-2 hours[1], considering of setting up the whole system and guarantee the vacuum condition is excellent, generally it takes more than 3 hours to finish one SAFE operation. By following the traditional procedure, several SAFE operations are necessary to adjust the addition of isotopes within the linear range of standard curves. Especially when some quantitations of add-on compounds are performed, an extra significant amount of SAFE operations are necessary just to determine a few more compounds. However, in the proposed streamlined approach, only one SAFE operation is necessary which allows researchers adjust the addition of isotopes by micro scale extraction of the SAFE isolate and also provides the opportunities to use various solvents of different

polarities in each individual micro extraction[17] which could optimize the performance of SIDA. The researchers can always quantitate more odorants without any more SAFE operations if the SAFE isolate is properly preserved. Furthermore, the direct SAFE isolate can be used for both identification and quantitation purposes, In this study, the SAFE isolates of liquor products are also used for sample preparation of GC-MS-O AEDA and GC-O dilution analysis, it would not be feasible by following the traditional procedure due to the impurities in the internal standards may introduce artifacts to the flavor analysis. Thus this proposed approach not only allow quantitation of odor-active components with significant less time, less effort less materials, less isotopes, but also allows the whole identification and quantitation procedure to study on the flavor chemistry of liquor products of great consistency. Through various assessment of the proposed streamlined approach, among the selected 6 liquors, aroma-profile of both clear and brown liquors did not differ after SAFE distillation. Assessed by AEDA, within selected clear and brown liquor samples, most potent odor-active components have the same FD factor in the extract of liquor products and their SAFE isolates. According to the SIDA results of odor-active components before & after SAFE, the proposed approach is valid for Strecker aldehydes, short chain fatty acids and ethyl esters from butyric acid to octanoic acid. Esters with longer chains and semi-volatile components are not suitable for this stream-lined approach due to poor recoveries of SAFE distillation. It could be the explanation of there are more correct responses in triangle tests between SAFE isolates and original liquors in brown liquors than in clear liquors. Thus, for the quantitation of semi-volatiles, isotopes have to be added before SAFE distillation to guarantee accurate quantitation results. This approach could potentially be applied on flavor chemistry study on wine, beer, fruit pulps and other aqueous food products.

### 4.5 Abbreviations Used

gas chromatography-olfactometry (GCO)
flavor dilution (FD)
aroma extract dilution analysis (AEDA)
stable isotope dilution analysis (SIDA)
odor-activity value (OAV)
gas chromatography-mass spectrometry (GC-MS)
retention indices (RIs)

Response factor (Rf)

Moutai (MT)

Gu Jing Gong Jiu (GJGJ)

Yi Pin Jing Zhi (YPJZ)

EvanWilliams Kentucky Bourbon Whiskey (EWW)

Don Julio Tequila (DJT)

Appleton Estate Jamaica Rum (AER)

**Figure 4.1: Flow Chart of Traditional Quantitative Analysis**

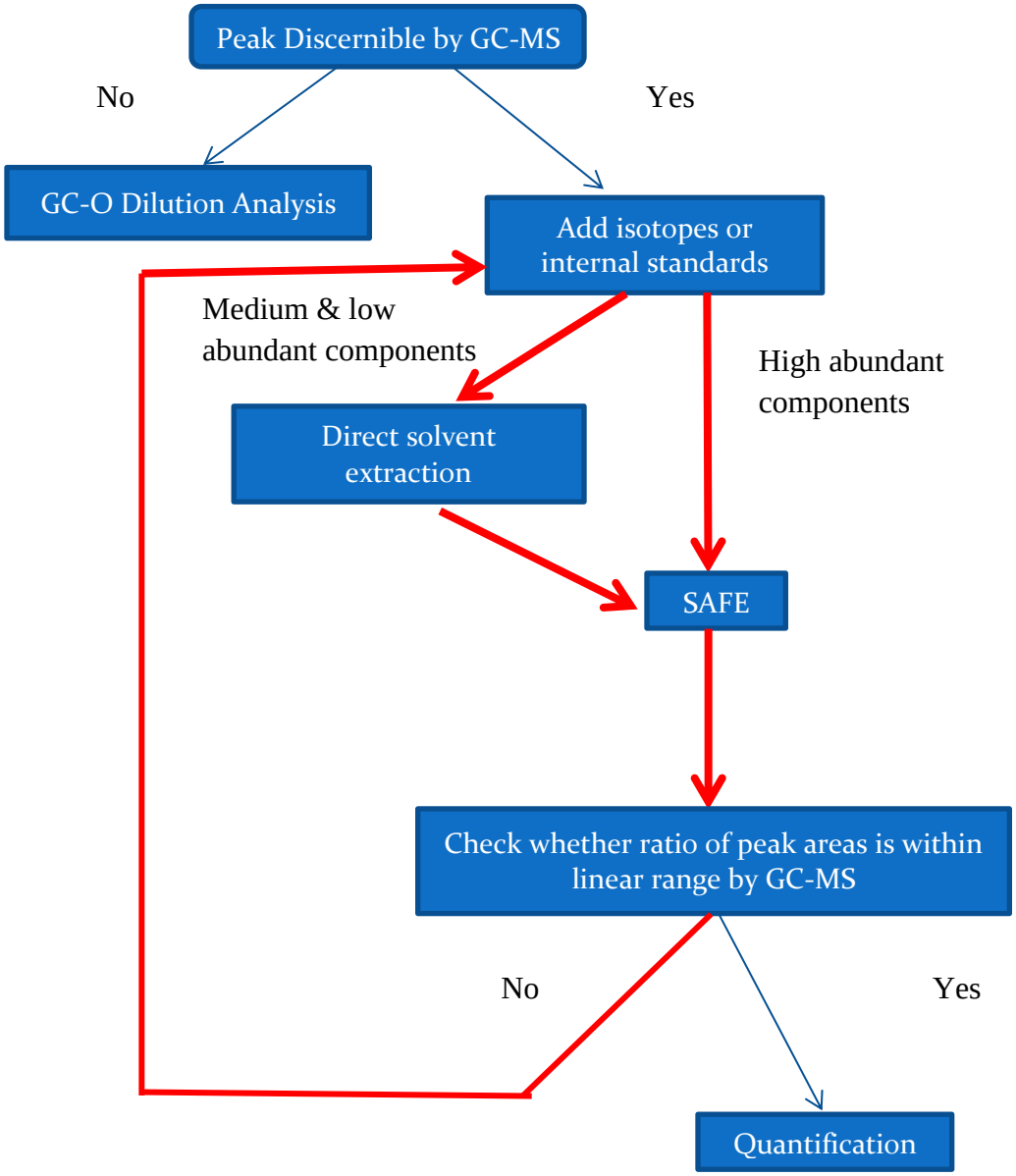


**Figure 4.2: Flow Chart of a Streamlined Approach for Quantitative Analysis Based on Hypothesis**

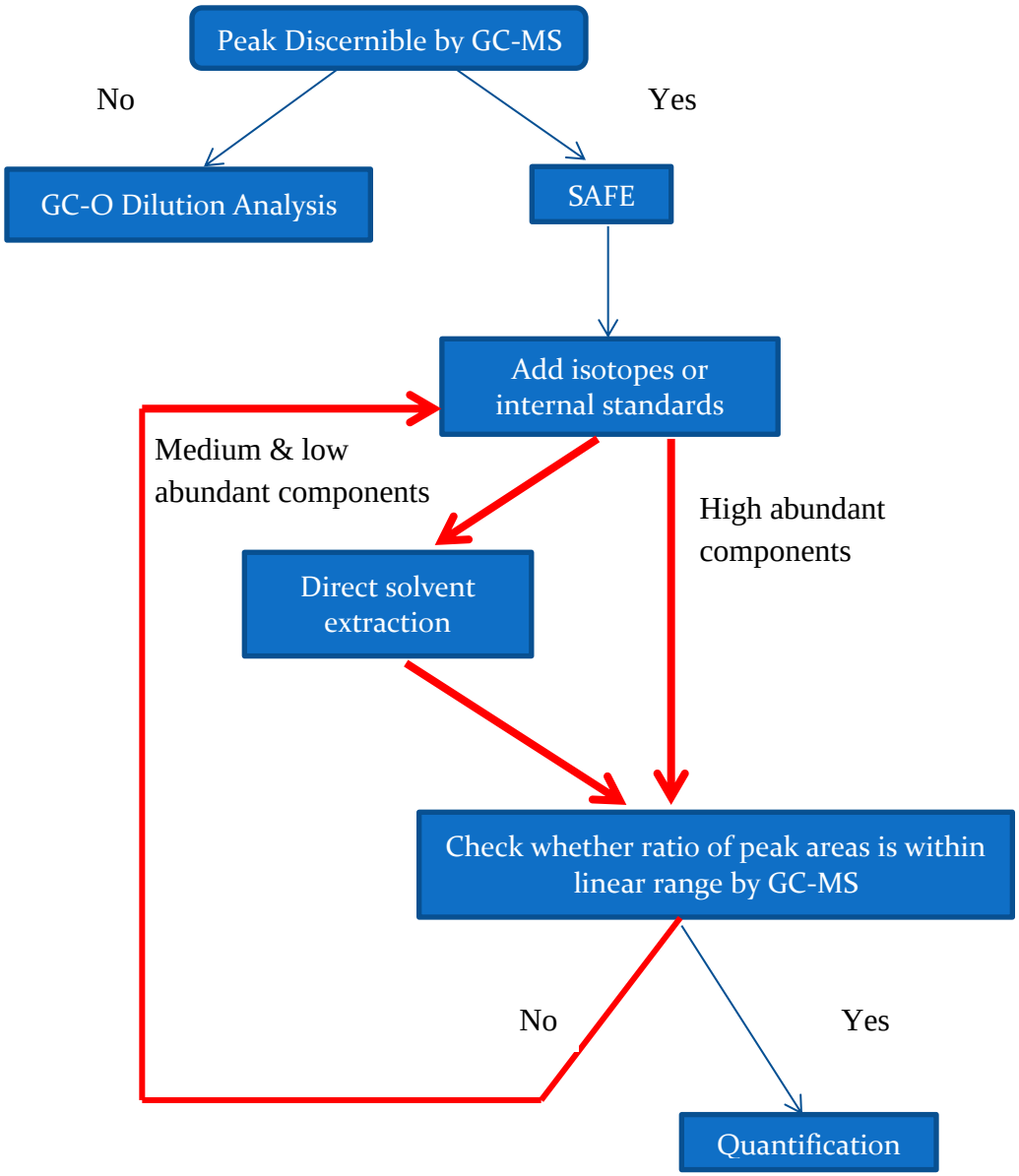


**Table 4.1. Liquor Products Evaluated in This Study**

| *Type of liquors* | *Name of liquor product* | *Producer* | *Ethanol % (V/V)* |
|---|---|---|---|
| Pottery liquors (Clear) | Moutai (MT) | Kweichow Moutai Co. Ltd. Guizhou, China | 53 |
| | Gu Jing Gong Liquor (GJGJ) | Gujinggong Liquor Co., Ltd. Bozhou, China. | 52 |
| | Yi Pin Jing Zhi (YPJZ) | Jingzhi Liquor Co.,Ltd., Shandong, China. | 46 |
| Brown liquors (Brown) | Evan Williams Kentucky Bourbon Whiskey (EWW) | Old Evan Williams Distillery, Kentucky, U.S.A. | 43 |
| | Don Julio Tequila (DJT) | Tequila Don Julio, S. A. DE C. V. Jalisco, Mexico. | 40 |
| | Appleton Estate Jamaica Rum(Aged 12 years) (AER) | J.Wray & Nephew LTD. Jamaica. | 43 |

**Table 4.2. Target Analytes, Deuterium- and $^{13}C$-labeled Isotopes, Selected Ions, and Response Factors Used for SIDA**

| *No.* | *Compound* | *No.* | *Labeled compound* | *Selected Ion (m/z)* | | $R^2$ | $R_f$ |
|---|---|---|---|---|---|---|---|
| | | | | *Unlabeled* | *Labeled* | | |
| **2** | 2-methyl-propanal | **I-2** | [$^2H_2$]-2-methylpropanal | 72 | 74 | 0.99+ | 0.60 |
| **144** | 2-methyl-butanal | **I-144** | [$^2H_2$]-2-methyl-butanal | 86 | 88 | 0.99+ | 0.52 |
| **3** | 3-methyl-butanal | **I-3** | [$^2H_2$]-3-methyl-butanal | 71 | 73 | 0.99+ | 0.58 |
| **10** | ethyl butanoate | **I-10** | [$^2H_5$]-ethylbutanoate | 88 | 93 | 0.99+ | 0.91 |
| **15** | ethyl pentanoate | **I-15** | [$^2H_5$]-ethyl pentanoate | 101 | 106 | 0.99+ | 0.60 |
| **12** | ethyl isovalerate | **I-12** | [$^2H_5$]-ethyl isovalerate | 88 | 93 | 0.99+ | 0.93 |
| **19** | ethyl hexanoate | **I-19** | [$^2H_5$]-ethyl hexanoate | 88 | 93 | 0.99+ | 0.91 |
| **23** | ethyl heptanoate | **I-23** | [$^2H_5$]-ethyl heptanoate | 88 | 93 | 0.99+ | 0.89 |
| **28** | ethyl octonoate | **I-28** | [$^2H_5$]-ethyl octanoate | 88 | 93 | 0.99+ | 0.95 |
| **145** | ethyl decanoate | **I-145** | [$^2H_5$]-ethyl decanoate | 88 | 93 | 0.99+ | 0.97 |
| **146** | ethyl dodecanoate | **I-146** | [$^2H_5$]-ethyl dodecanoate | 88 | 93 | 0.99+ | 0.97 |
| **147** | ethyl palmitate | **I-147** | [$^2H_5$]-ethyl palmitate | 88 | 93 | 0.99+ | 1.00 |
| **57** | ethyl phenylacetate | **I-57** | [$^2H_5$]-ethyl phenylacetate | 164 | 169 | 0.99+ | 0.99 |
| **68** | ethyl phenyl propionate | **I-68** | [$^2H_5$]-ethyl phenyl propionate | 178 | 183 | 0.99+ | 0.90 |
| **89** | acetic acid | **I-89** | [$^2H_3$]-acetic acid | 60 | 63 | 0.99+ | 0.50 |
| **90** | propionic acid | **I-90** | [$^2H_5$]-propionic acid | 74 | 79 | 0.99+ | 0.79 |
| **92** | butyric acid | **I-92** | [$^{13}C_2$]- butyric acid | 60 | 62 | 0.99+ | 0.83 |
| **91** | isobutyric acid | **I-91** | [$^2H_2$]-isobutyric acid | 73 | 75 | 0.99+ | 0.34 |
| **94** | pentanoic acid | **I-94** | [$^2H_9$]-pentanoic acid | 60 | 63 | 0.99+ | 0.71 |
| **96** | hexanoic acid | **I-96** | [$^2H_4$]-hexanoic acid | 87 | 91 | 0.99+ | 0.36 |
| **106** | phenylacetic acid | **I-106** | [$^{13}C_2$]-phenylacetic acid | 136 | 138 | 0.99+ | 0.93 |
| **69** | phenylethanol | **I-69** | [$^{13}C_2$]-2-phenylethanol | 122 | 124 | 0.99+ | 1.40 |
| **107** | vanillin | **I-107** | [$^2H_3$]-vanillin | 152 | 155 | 0.99+ | 1.11 |
| **148** | syringaldehyde | **I-148** | [$^2H_3$]-syringaldehyde | 182 | 185 | 0.99+ | 0.70 |
| **104** | sotolon | **I-104** | [$^{13}C_2$]-sotolon | 128 | 130 | 0.99+ | 1.16 |

**Table 4.3. Triangle Difference Test Comparison Between the Aromas of Neat Versus SAFE-DIST Isolates for Various Liquors.**

| | | *Panel response (No. Correct/No. Total Assessments)* | | | |
|---|---|---|---|---|---|
| ***Liquor Sample*** | | ***(2 SAFE – 1 Neat)*** [a] | ***(2 Neat – 1 SAFE)*** [b] | ***Total*** | ***Sign. Diff .*** [c] |
| Clear liquor products | MT [d] | 4/12 | 3/12 | 7/24 | N |
| | GJGJ [e] | 5/12 | 7/12 | 12/24 | N |
| | YPJZ [f] | 5/12 | 3/12 | 8/24 | N |
| Brown liquor products | DJT [g] | 4/12 | 6/12 | 10/24 | N |
| | EWW [h] | 2/12 | 10/12* | 12/24 | N |
| | AER [i] | 4/12 | 7/12* | 11/24 | N |

[a] 2 SAFE-1 Neat: Triangle test of 1 original liquor and 2 of their counterpart SAFE distillate.
[b] 2 Neat-1 SAFE: Triangle test of 2 original liquor and 1 of their counterpart SAFE distillate.
[c] Significantly different, $P \leq 0.05$.
[d] MT: Moutai
[e] GJGJ : Gu Jing Gong Jiu
[f] YPJZ: Yi Pin Jing Zhi
[g] DJT: Don Julio Tequila
[h] EWW: EvanWilliams Kentucky Bourbon Whiskey
[i] AER: Appleton Estate Jamaica Rum
[*] Contrast effects are evident.

**Table 4.4. Comparison of GC-FID Peak Areas for Selected Volatile Components Between Various Neat Liquor Products and Their Respective SAFE-DIST Isolates**

| | *Average peak area ($10^4$)*[a] | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***MT*** | | | ***GJGJ*** | | | ***YPJZ*** | | | ***EWW*** | | | ***DJT*** | | | ***AER*** | | |
| ***Compound*** | ***Neat*** | ***SAFE*** | ***% Diff*** | ***Neat*** | ***SAFE*** | ***% Diff*** | ***Neat*** | ***SAFE*** | ***% Diff*** | ***Neat*** | ***SAFE*** | ***% Diff*** | ***Neat*** | ***SAFE*** | ***% Diff*** | ***Neat*** | ***SAFE*** | ***% Diff*** |
| 2-methyl-1-propanol | 156 | 156 | 0.00130 | 101 | 101 | 0.303 | 172 | 171 | 0.919 | 224 | 224 | 0.0639 | 403 | 395 | 1.99[b] | 113 | 113 | 0.168 |
| 3-methy-1-butanol | 400 | 402 | 0.613 | 288 | 283 | 2.00 | 794 | 794 | 0.0604 | 1123 | 1121 | 0.127 | 736 | 729 | 0.923 | 314 | 314 | 0.0996 |
| ethyl hexanoate | 22.6 | 22.5 | 0.224 | 314 | 293 | 1.64 | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ |
| acetic acid | 433 | 433 | 0.0478 | 183 | 181 | 0.795 | 395 | 394 | 0.129 | 94.0 | 79.8 | 15.1[b] | 38.2 | 32.5 | 15.0[c] | 121 | 99.4 | 18.0[c] |
| tetramethyl pyrazine | 12.1 | 8.12 | 33.0[c] | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ |
| ethyl 2-phenyl acetate | 162 | 154 | 4.89[b] | _ | _ | _ | 44.6 | 43.5 | 2.34[c] | _ | _ | _ | _ | _ | _ | _ | _ | _ |
| hexanoic acid | 11.0 | 10.8 | 1.66 | 777 | 759 | 2.31 | 136 | 134 | 1.47 | 8.56 | 7.31 | 14.6[c] | 2.49 | 2.44 | 2.07 | _ | _ | _ |
| 2-phenylethanol | 19.4 | 18.7 | 3.48[b] | 2.98 | 2.86 | 4.12 | 40.2 | 39.0 | 3.04 | 26.2 | 25.0 | 4.36 | 6.88 | 6.78 | 1.39 | _ | _ | _ |
| octanoic acid | 1.22 | 1.22 | 0.0872 | 19.5 | 19.1 | 1.93 | 1.87 | 1.79 | 0.0425 | 2.01 | 0.964 | 52.0[c] | 2.71 | 2.70 | 0.614 | 1.04 | 1.82 | 42.8[c] |

[a] average peak areas are determined by GC-FID (n=3)
[b] peak areas of SAFE extraction is significantly different from the original liquor ($p < 0.05$).
[c] peak areas of SAFE extraction is significantly different from the original liquor ($p < 0.01$).

**Table 4.5. Comparative AEDA Results for Potent Odorants (log3FD ≥ 2) in MT and EWW Neat Liquors and Their Respective SAFE-DIST Isolates**

| *No.* | *Compound* | *Odor Description* | *RI (Stabilwax)* | *RI (Rxi-5ms)* | *Log3FD Factor of MT* | | | | *Log3FD Factor of EWW* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Stabil-Wax* | | *Rxi-5ms* | | *Stabil-Wax* | |
| | | | | | *SAFE* | *Neat* | *SAFE* | *Neat* | *SAFE* | *Neat* |
| **2** | 2-methylpropanal | malty | <800 | <700 | 4 | 4 | 4 | 4 | - | - |
| **121** | acetal | paint | 938 | 741 | 7 | 7 | 5 | 5 | 4 | 4 |
| **3** | 3-methybutanal | malty | 947 | <700 | 6 | 6 | 6 | 6 | 1 | 1 |
| **4** | ethyl propionate | fruity | 972 | 720 | 5 | 5 | 4 | 4 | 4 | 4 |
| **7** | ethyl 2-methylpropanoate | berry | 979 | 768 | 9 | 9 | 8 | 8 | - | - |
| **8** | 2,3-butanedione | buttery, creamy | 993 | <700 | 2 | 2 | 2 | 2 | - | - |
| **9** | 2-methylpropyl acetate* | solvent | 1015 | 792 | 2 | 2 | 3 | 3 | - | - |
| **10** | ethyl butanoate | fruity | 1042 | 806 | 8 | 8 | 6 | 6 | 4 | 4 |
| **11** | ethyl 2-methylbutanoate | fruity, berry | 1057 | 857 | 10 | 10 | 8 | 8 | 4 | 4 |
| **12** | ethyl isovalerate | fruity, blueberry | 1075 | 862 | 8 | 8 | 6 | 6 | 3 | 3 |
| **15** | ethyl pentanoate | berry | 1142 | 902 | 6 | 6 | 5 | 5 | 1 | 1 |
| **132** | ethyl 2-methylpentanoate* | fruity | 1142 | 944 | 6 | 6 | 4 | 4 | - | - |
| **16** | propyl 2-methylbutanoate | fruity | 1183 | - | 4 | 4 | - | - | - | - |
| **17** | ethyl 4-methylpentanoate | berry | 1195 | 971 | 6 | 6 | 4 | 4 | 0 | 0 |
| **18** | 2-/3-methyl-1-butanol | malty | 1213 | 749 | 4 | 4 | 2 | 2 | 5 | 5 |
| **19** | ethyl hexanoate | fruity | 1240 | 999 | 5 | 5 | 4 | 4 | 4 | 4 |
| **20** | furfuryl ether * | ether like | 1296 | 831 | 3 | 3 | 2 | 2 | - | - |

***Table 4.5. Continued***

| *No.* | *Compound* | *Odor Description* | *RI (Stabilwax)* | *RI (Rxi-5ms)* | *Log3FD Factor of MT* | | | | *Log3FD Factor of EWW* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Stabil-Wax* | | *Rxi-5ms* | | *Stabil-Wax* | |
| | | | | | *SAFE* | *Neat* | *SAFE* | *Neat* | *SAFE* | *Neat* |
| **133** | 2-methyl-3-furanthiol* [a] | beefy, vitamin | - | 872 | - | - | 5 | 5 | - | - |
| **23** | ethyl heptanoate | hops | 1341 | 1099 | 1 | 1 | 0 | 0 | 3 | 3 |
| **24** | dimethyl trisulfide* | cabbage | 1385 | 969 | 5 | 5 | 3 | 3 | 5 | 5 |
| **27** | ethyl cycloheanoate* | berry | 1430 | 1135 | 4 | 4 | 3 | 3 | 0 | 0 |
| **28** | ethyl octanoate | waxy, plastic | 1440 | 1197 | 3 | 3 | 2 | 2 | 3 | 3 |
| **30** | 2-furfurylthiol* | meaty | 1447 | 912 | 1 | 1 | 4 | 4 | - | - |
| **31** | 3-methylbutyl hexanoate | berry | 1463 | - | 5 | 5 | - | - | - | - |
| **33** | methional* | potato | 1475 | 907 | - | - | - | - | 4 | 4 |
| **38** | (*E*)-2-nonenal* | metallic | 1518 | 1150 | 2 | 3 | - | - | 3 | 3 |
| **135** | 2,3-dimethyl-5-ethylpyrazine | nutty, roasted cocoa | - | 1063 | - | - | 3 | 3 | - | - |
| **40** | unknown | berry | 1544 | 962 | 4 | 4 | 1 | 1 | - | - |
| **91** | 2-methylpropanoic acid | cheesy, stink | 1582 | 843 | 1 | 1 | 2 | 2 | - | - |
| **92** | butyric acid | cheesy | 1637 | 872 | 3 | 3 | 0 | 0 | - | - |
| **46** | phenylacetaldehyde | rosy | 1663 | 1032 | 3 | 3 | 2 | 2 | - | - |
| **93** | 3-/2-methyl butanoic acid | cheesy, stink | 1676 | - | 3 | 4 | - | - | 1 | 1 |
| **47** | 2-methyl-3-(methyldithio) furan* | vitamin | 1681 | 1174 | 5 | 5 | 3 | 3 | - | - |
| **48** | unknown | seeds | 1695 | 869 | 2 | 2 | 3 | 3 | - | - |
| **94** | pentanoic acid | cheesy | 1747 | - | 2 | 2 | - | - | - | - |
| **55** | dimethyltetrasulfide* | cabbage | 1760 | 1214 | 3 | 4 | 2 | 2 | - | - |
| **57** | ethyl phenylacetate | rosy | 1801 | 1247 | 3 | 3 | 4 | 4 | - | - |
| **61** | 2-phenylethyl acetate | fruity, rosy | 1829 | - | 1 | 1 | - | - | 4 | 4 |

***Table 4.5. Continued***

| *No.* | *Compound* | *Odor Description* | *RI (Stabilwax)* | *RI (Rxi-5ms)* | *Log3FD Factor of MT* | | | | *Log3FD Factor of EWW* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Stabil-Wax* | | *Rxi-5ms* | | *Stabil-Wax* | |
| | | | | | *SAFE* | *Neat* | *Neat* | *Neat* | *SAFE* | *Neat* |
| **62** | β-damascenone* | fruity, peachy | 1837 | 1384 | 5 | 5 | 5 | 5 | 4 | 4 |
| **137** | geraniol | citrus | 1859 | - | 2 | 3 | - | - | 2 | 2 |
| **66** | guaiacol* | smoky | 1884 | 1091 | 2 | 3 | 0 | 0 | 5 | 5 |
| **68** | ethyl phenyl propionate | fruity, grape | 1898 | 1349 | 5 | 5 | 4 | 4 | 2 | 2 |
| **69** | 2-phenylethanol | rosy | 1933 | 1130 | 5 | 5 | 1 | 1 | 5 | 5 |
| **70** | β-ionone | floral | 1954 | - | 1 | 1 | - | - | 2 | 2 |
| **138** | 4-ethylguaiacol | smoky, clove | 2055 | 1281 | 1 | 1 | - | - | 3 | 3 |
| **75** | γ-nonlactone | peachy | 2062 | 1380 | 2 | 3 | 0 | 0 | 1 | 1 |
| **77** | p-cresol | animal barn | 2107 | 1106 | 3 | 3 | 0 | 0 | - | - |
| **149** | unknown | hay , sweet | 2131 | - | - | - | - | - | 2 | 2 |
| **78** | ethyl cinnamate | rosy | 2159 | - | 1 | 1 | - | - | 2 | 2 |
| **79** | *bis*(2-methyl-3-furyl) disulfide | meaty | 2178 | 1526 | 2 | 2 | - | - | - | - |
| **150** | eugenol | spicy, colve | 2188 | - | - | - | - | - | 6 | 6 |
| **139** | 4-ethylphenol | manure | 2205 | - | 3 | 3 | - | - | 0 | 0 |
| **104** | sotolone | herbal, curry | 2233 | - | 5 | 5 | - | - | 7 | 7 |
| **140** | unknown | plastic | 2266 | - | 2 | 3 | - | - | 1 | 1 |
| **141** | syringol | bacon, smoky | 2305 | - | 1 | 1 | - | - | 4 | 4 |
| **142** | unknown | herbal | 2386 | - | 1 | 1 | | | 2 | 2 |
| **106** | phenylacetic acid | rosy | 2603 | 1287 | 3 | 3 | 0 | 0 | - | - |
| **107** | vanillin | vanilla | 2624 | - | 1 | 2 | - | - | 5 | 7 |
| **108** | 3-phenylpropanoic acid | sweaty, stink | 2657 | 1349 | 2 | 2 | - | - | - | - |
| **143** | unknown | cabbage | - | 1057 | - | - | 3 | 3 | - | - |

* Tentatively identified by confirming the RI and odor properties with authentic standard compounds.

[a] Probable thermally generated artifact from *bis*(2-methyl-3-furyl) disulfide.

**Table 4.6. Determination of the Potential for Thermal Degradation of Selected Odor-Important Disulfides**

| | | | | ***Hot-splitless*** | | |
|---|---|---|---|---|---|---|
| | | ***Cool on column*** | ***Cold-splitless*** | ***Inlet temperature 200°C*** | ***Inlet temperature 250°C*** | ***Inlet temperature 300°C*** |
| MFT-MFT[a] | Peak area ratio ( mean ± STD) | 6.658±0.022 | 5.544±0.027 | 5.294±0.033 | 4.547±0.038 | 3.848±0.038 |
| | Degradation (%) | 0.00 | 16.73 | 20.49 | 31.72 | 42.21 |
| MFT-MT[b] | Peak area ratio ( mean ± STD) | 0.5737±0.0022 | 0.5745±0.0012 | 0.5748±0.0025 | 0.5733±0.0015 | 0.5730±0.0037 |

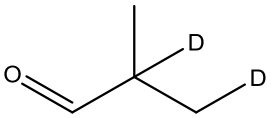
[a] MFTMFT: *bis*(2-methyl-3-furyl) disulfide
[b] MFT-MT: 2-methyl-3-(methyldithio) furan

**Table 4.7. Concentration Comparison of Selected Potent Odorants Original Neat Liquors of Moutai and Evan Williams Whiskey and Their Respective SAFE Isolates**

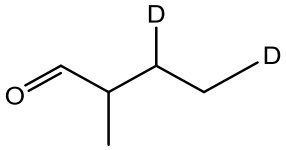
| *No.* | *Compound* | *Concentration in Original MT (mg/L) ± SD* | *Concentration in MT SAFE extract (mg/L) ± SD* | *% recovery* | *Concentration in Original EWW (mg/L) ± SD* | *Concentration in EWW SAFE extract (mg/L) ± SD* | *% recovery* |
|---|---|---|---|---|---|---|---|
| **1** | methyl-propanal | 26.12 ± 0.66 | 25.74 ± 0.40 | 98.55 | 0.920±0.022 | 0.911±0.013 | 98.91 |
| **62** | 2-methyl-butanal | 17.70 ± 0.17 | 17.67 ± 0.29 | 99.83 | 0.308±0.017 | 0.3051±0.0041 | 100.00 |
| **3** | 3-methyl-butanal | 37.11 ± 0.49 | 37.10 ± 0.46 | 99.97 | 0.3869±0.0061 | 0.3799± 0.0078 | 98.19 |
| **8** | ethyl butanoate | 57.70±0.41 | 57.36±0.36 | 99.41 | - | - | - |
| **11** | ethyl pentanoate | 4.561±0.034 | 4.539±0.037 | 99.56 | - | - | - |
| **10** | ethyl isovalerate | 11.315±0.057 | 11.315±0.038 | 99.91 | - | - | - |
| **16** | ethyl hexanoate | 17.112±0.026 | 17.109±0.083 | 100.00 | 2.691±0.020 | 2.684±0.015 | 99.63 |
| **20** | ethyl heptanoate | 1.250±0.011 | 1.235±0.013 | 98.40 | - | - | - |
| **23** | ethyl octanoate | 2.010±0.015 | 2.005±0.0054 | 99.50 | 10.356±0.064 | 10.315±0.085 | 99.61 |
| **63** | ethyl decanoate | - | - | - | 12.09±0.15 | 7.38±0.12 | 61.04 |
| **64** | ethyl dodecanoate | - | - | - | 5.61±0.19 | 3.316±0.046 | 59.18 |
| **65** | ethyl palmitate | 19.254±0.078 | 3.999±0.041 | 20.78 | - | - | - |
| **38** | ethyl phenyl acetate | 5.420±0.011 | 5.357±0.031 | 98.89 | - | - | - |
| **43** | ethyl phenyl propionate | 38.64±0.76 | 38.55±0.27 | 99.77 | - | - | - |
| **66** | acetic acid | 7257.758±0.033 | 7254.433±0.031 | 99.86 | 2.499±0.064 | 2.475±0.041 | 99.20 |
| **67** | propionic acid | 1228.9±3.6 | 1228.6±2.6 | 99.98 | - | - | - |
| **31** | butyric acid | 35.18±0.25 | 35.06±0.26 | 99.66 | - | - | - |
| **33** | isobutyric acid | 20.89±0.19 | 20.50±0.04 | 98.13 | - | - | - |
| **36** | pentanoic acid | 4.383±0.037 | 4.3841±0.0096 | 100.00 | - | - | - |
| **68** | hexanoic acid | 12.187±0.037 | 12.104±0.088 | 99.26 | - | - | - |
| **58** | phenyl acetic acid | 20.437±0.027 | 14.196±0.016 | 69.47 | - | - | - |
| **44** | phenyl ethanol | 20.77±0.15 | 19.11±0.10 | 92.01 | 28.97±0.12 | 27.05±0.19 | 93.38 |
| **59** | vanillin | - | - | - | 2.802±0.015 | 0.4819±0.0018 | 17.14 |
| **69** | syringaldehyde | - | - | - | 9.170±0.045 | 0.4775±0.0025 | 5.23 |
| **54** | sotolon | 0.0962± 0.0010[a]<br>0.097±0.015[b] | 0.08478 ±0.00050 | 88.15 | - | - | - |

[a] Concentration by addition of isotope solution to the neat liquor followed by direct solvent extraction and fractionation without SAFE.
[b] Concentration by addition of isotope solution to the neat liquor followed by direct solvent extraction, SAFE distillation and fractionation.

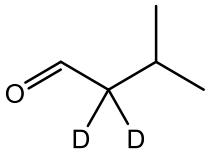
**Figure 4.1 Structures of Stable Isotopes Used in Quantitation by SIDA**



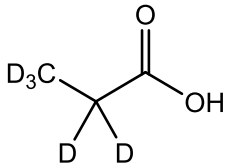

**I-2** *[$^{2}H_{2}$]*-methylpropanal



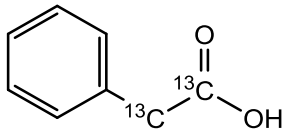

**I-144** *[$^{2}H_{2}$]*-2-methylbutanal



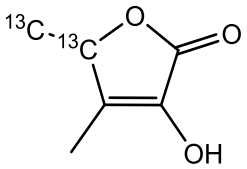

**I-3** *[$^{2}H_{2}$]*-3-methylbutanal



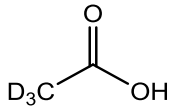

**I-90** *[$^{2}H_{5}$]*-propanoic acid



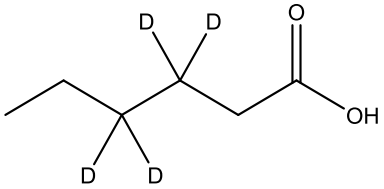

**I-106** *[$^{13}C_{2}$]*-phenylacetic acid



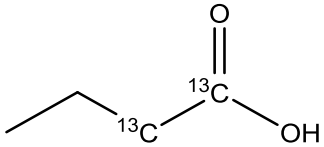

**I-104** *[$^{13}C_{2}$]*-sotolon




**I-89** *[$^{2}H_{3}$]*-acetic acid

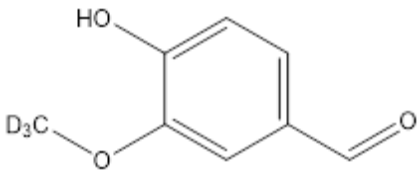



**I-96** *[$^{2}H_{4}$]*-hexanoic acid

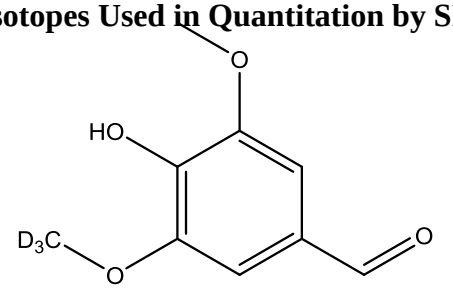



**I-92** *[$^{13}C_{2}$]*-butyric acid

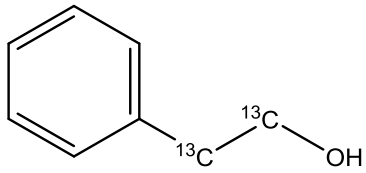
**Figure 4.1 Structures of Stable Isotopes Used in Quantitation by SIDA (continues)**



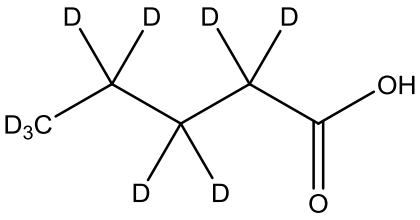

**I-107** *[$^{2}H_{3}$]*-vanillin



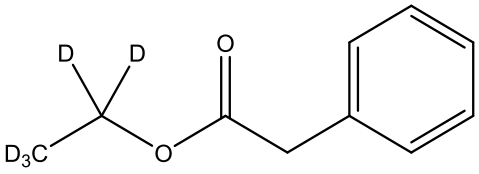

**I-148** *[$^{2}H_{3}$]*-syringaldehyde



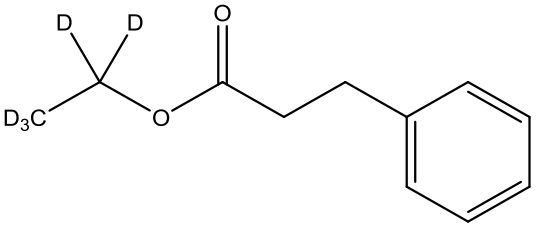

**I-69** *[$^{13}C2$]*-phenylethanol



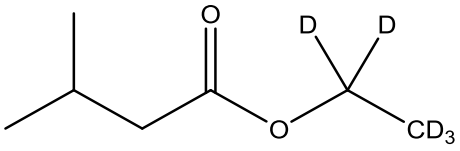

**I-94** *[$^{2}H_{9}$]*-pentanoic acid




**I-57** *[$^{2}H_{5}$]*-ethyl phenylacetate

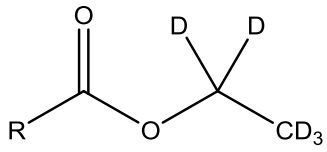



**I-68** *[$^{2}H_{5}$]*-ethyl phenylpropionate




**I-12** *[$^{2}H_{5}$]*-ethyl isovalerate

**Figure 4.1 Structures of Stable Isotopes Used in Quantitation by SIDA (continues)**




*[$^2H_5$]*-ethyl esters

**I-10:** R= - $(CH_2)_2$ $CH_3$

**I-15**: R=- $(CH_2)_3$ $CH_3$

**I-19**: R= - $(CH_2)_4$ $CH_3$

**I-23**: R= - $(CH_2)_5$ $CH_3$

**I-28**: R= - $(CH_2)_6$ $CH_3$

**I-145**: R= - $(CH_2)_8CH_3$

**I-146**: R= - $(CH_2)_{10}CH_3$

**I-147**: R= - $(CH_2)_{13}CH_3$

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# Chapter 5: Quantitation of Selected Potent Odorants in Moutai


## 5.1 Abstract

A total of 39 potent odorants were quantitated or semi-quantitated in Moutai (MT). Among these, 35 were quantitated by stable isotope dilution analysis (SIDA)-gas chromatography-mass spectrometry (GC-MS). Acetaldehyde was quantitated by external standard calibration using a GC-flame ionization detector (FID). 2,3-butanedione was quantitated after derivatization with methoxyamine and analyzed by GC-MS. Two trace level odorants, dimethyl trisulfide and 2-methyl-3-(methyldithio)furan, were semi-quantitated by GC-olfactometry (GC-O) combined with AEDA. The odor-activity value (OAV) of each odorant was calculated based on dividing its concentration by its odor detection threshold. Acetaldehyde, acetal, 2-methylbutanal, β-damascenone, ethyl butanoate, ethyl isovalerate, ethyl hexanoate and ethyl phenylpropionate had OAVs above 200 and are considered to be the main contributors to the overall aroma profile of MT. All of the above mentioned compounds were previously indicated as potent odorants in MT based on results of GC-olfactometry (GC-O) and aroma extract dilution analysis (AEDA) of MT (Chapter 3).


## 5.2 Introduction

In our previous study (detailed in Chapter 3) the potent odorants in Moutai (MT) were identified through application of three different isolation/extraction and analysis approaches, including: 1) DSE-SAFE [solvent-assisted flavor evaporation (SAFE) of a dichloromethane (DCM) extract of MT] followed by compound class fractionation and analysis by cold splitless injection GC-MS-O; 2) SAFE distillation of MT and analysis of distillate by cool on-column GC-O analysis and 3) SAFE-DSE [DCM extraction of SAFE distillate of MT] followed by analysis of the extract by cold-splitless injection GC-MS-O. The relative potency or importance of the odorants were determined by aroma extract dilution analysis (AEDA) on the basis of their flavor dilution (FD) factors, where an FD factor is equal to the highest extract dilution in which an odorant is detectable by GC-O. However, for a more comprehensive assessment of the flavor chemistry of MT, accurate quantitation of the most potent odorants is essential.

In this study, stable isotope dilution analysis (SIDA) and several other reliable techniques were applied for accurate quantitation of selected odorants in MT. In particular, SIDA is known to be the most accurate quantitation method available. In the procedure of SIDA

known amounts of stable isotopically labeled internal standards, consisting of either carbon-13 or deuterium labeled isotopologues of the target analytes, are spiked into a known mass or volume of a sample prior to analysis. The physical properties of these labeled internal standards (IS) are essentially the same as those of the unlabeled target analytes and, therefore, their partitioning and recovery during extraction are for all practical purposes also identical. However, the labeled and unlabeled isotopologues are readily distinguishable by their mass spectra (e.g. by use of GC-MS), and specifically certain mass fragment ions will differ between the two compounds. The mass ratio of the labeled/unlabeled target analyte can be calculated based on the measured GC-MS peak area ratio and the response factor ($R_f$) which can be determined by use of a calibration curve consisting of mass ratio versus area ratio for the unlabeled target analyte/ labeled IS. However, quantitation of the potent odorants in MT by SIDA is extremely challenging. First of all, generally each target analyte has to be determined by use of an appropriate isotopologue. Very few isotopes are commercially available, so in most cases synthesis of stable isotopes is required. In addition, for a very complex flavor system such as MT, the mass ions chosen to calculate the peak area ratio of the labeled/unlabeled analyte have to be exclusive; that is, without interference from other compounds in the product. All these requirements make the task of quantitation of potent odor-active components in MT highly challenging. This may explain why most other researchers have elected to use other advanced analysis methods, such as GC×GC-TOF-MS, stir bar sportive extraction (SBSE) and headspace solid-phase microextraction (HS-SPME) for the quantitative analysis of odorants in soy sauce aroma liquors and other traditional distilled Chinese liquor products[4,11,47]. To the best of our knowledge, SIDA has not been previously applied for the quantitation of potent odorants in in Chinese soy sauce aroma liquors.

In this study, potent odorants in MT liquor were selected based on their FD factors for quantitative analysis. Target analytes are quantitated by SIDA and other techniques to provide a more detailed and accurate measurement of these compounds, thus leading to a greater understanding of the flavor chemistry of MT liquor and ultimately leading to successful construction of simulation aroma model via flavor reconstitution (detailed in Chapter 6).

## 5.3 Material and Methods

### 5.3.1 Chemicals

2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All other authentic reference standards and chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The following isotopically labeled compounds were purchased from the supplier listed in parentheses: [2,2,2-$^{2}H_3$]-acetic acid (Sigma-Aldrich); [$^{2}H_5$]-propionic acid (Cambridge Isotope Laboratories, Inc., Andover, MA, USA); [1,2-$^{13}C_2$]-phenylacetic acid and [1,2-$^{13}C_2$]-butyric acid (Isotec, Miamisburg, OH, USA); [$^{2}H_9$]-pentanoic acid and [2,2-$^{2}H_2$]-3-methylbutanal (CDN Pointe-Claire, Quebec, Canada). The following labeled and unlabeled compounds were synthesized according to procedures reported in the literature (in parentheses): [1,2-$^{13}C_2$]-2-phenylethanol (Schieberle, 2006) and [$^{2}H_5$]-ethyl bis(2-methyl-3-furyl) disulfide [33](Hofmann, T. 1996); $^{13}C_2$-sotolon[34] (Blank et al., 1996).

**Synthesis of $d_3$-Syringaldehyde**

$d_3$-syringaldehyde was synthesized using the procedure developed by Lahne[37]. In a screw-capped test tube (PTFE-top) equipped with a stir bar 3,4-dihydroxy-5-methoxybenzaldehyde (0.501 g; 3 mmol) was dissolved in aqueous 40% (w/v) KOH (5 mL). Then, under a gentle steam of nitrogen, over the course of 30 minutes, 0.35 mL (0.42 g, 3.2 mmol) of $d_6$-dimethylsulfate was added (5 to 6 drops every 5 minutes) to the reaction tube, after which the reaction mixture became yellow and cloudy. The vial was then capped and stirred for 2 hr. The reaction was checked for completion by removing 5-6 drops of the reaction mixture, adding it to a vial containing 1 mL aqueous 1N HCl and 0.5 mL ethyl acetate, and then analyzing the ethyl acetate layer by GC-MS. The reaction was continued, adding 0.08 mL (0.096 g, 0.73 mmol) of $d_6$-dimethylsulfate and allowing the reaction to stir overnight until nearly all starting material had been consumed. The reaction was stopped by acidifying the mixture to ~pH 1 and then it was extracted with ethyl acetate (1 x 10mL, 4 x 5 mL). The ethyl acetate layer was washed with saturated NaCl and then dried over anhydrous $Na_2SO_4$. The solution was concentrated to ~10 mL using a vigreux column and the remaining solvent was then removed under a stream of nitrogen. The final product was weighed for a final yield of 0.5734 g.

**GC-MS Analysis**

Stock solutions of the deuterated or carbon-13 labeled internal standards, prepared in ethanol or dichloromethane, were spiked into 1 mL of MT. The spiked sample was then subjected to GC-MS analysis using a 6890 GC/5973N mass selective detector (MSD) (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with a Stabilwax column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Restek Corp., Bellefonte, PA, USA). Samples were injected in the cold-split mode [split ratio 10:1; -50 °C initial inlet temperature (0.1 min); 250 °C final temperature; ramped at 12 °C/s]. The carrier gas was helium at a constant flow rate of 1 mL/min. The oven temperature was programmed from 40°C to 250 °C at the ramp rate of 6°C /min, with initial hold and final hold times of 5 and 30 min. Samples were analyzed under simultaneous full scan (35-300 amu) / selected ion monitoring (SIM) modes. Selected ions used for determination of peak areas of labeled and unlabeled components are listed in **Table 5.1**.

Calibration curves were generated using the same analytical conditions described above. The area of the selected mass ion was integrated using Enhanced Data Analysis Software (Agilent Technologies, Inc.). The response factor (R*f*) for each isotope IS against its corresponding unlabeled target analyte was determined using a five-point standard curve with a range of mass ratios from 1:5 to 5:1 (unlabeled : labeled). Each area ratio was determined in triplicate, and each mass ratio was plotted against the mean peak area ratio of the selected ions of labeled and unlabeled target analytes.

The R*f* of each labeled analyte was calculated by using the following equation:

$$\frac{mass\ (analyte)}{mass\ (labeled\ analyte)} = \frac{area\ (analyte)}{area\ (labeled\ analyte)}\ (Rf)$$

The mass of the target analytes were calculated by using the equation as follows:

$$mass\ (target\ analyte) = Rf \times peak\ area\ (target\ analyte) \div peak\ area\ (labeled\ )$$

Samples analyses were performed triplicate. The results of concentrations determined by SIDA of selected components are shown in **Table 5.2**.

**Quantitation of acetaldehyde**

Acetaldehyde was quantitated by using external standard calibration using a 6890N GC (Agilent Technologies, Inc.) equipped with a flame ionization detector (FID). MT (2 uL) was injected using a 7683 autosampler (Agilent Technologies, Inc.) into a split/splitless inlet in the hot split mode (260° C, 5:1 split ratio). Separations were performed using a Stabiwax-DA column (30 m length x 0.32 mm i.d. x 0.5 µm film thickness; Restek). Helium was used as the carrier gas at 2.0 mL/min. FID temperature was 250°C. Oven temperature was programmed from 35 °C to 225 °C at a ramp rate of 10 °C/min, with initial and final hold times of 5 and 30 min, respectively. Calibration solutions were prepared by spiking known amounts of acetaldehyde into a 53% ABV matrix. The calibration curve for acetaldehyde can be found in the Appendix.

**Quantitation of 2,3-butanedione**

Since diacetyl coelutes with dichloromethane during GC-MS analysis it is difficult to quantitate by standard GC methods. For this reason, diacetyl was quantified after its conversion to methoxyoxime derivative, which can easily by resolved from the dichloromethane solvent peak. MT sample (10.00 mL) in a 50-mL glass centrifuge tube was spiked with 5 µL of an internal standard solution (11.9 µg/µL of 3-pentanone in methanol) followed by addition of 1.0 mL of a methoxyamine-HCl solution (10.0 mg of methoxyamine-HCl/mL in 53% ABV). The tube was capped (PTFE-lined cap), mixed thoroughly, and then incubated at 50°C for 2 hr. After cooling the solution, deodorized water (30 mL) was added along with 1.0 mL of dichloromethane (1.0 mL). The tube was capped, vigorously shaken by hand for 5 min, and then centrifuged at 7500 RMP for 5 min to separate the solvent and aqueous layers. The DCM phase was transferred to a 2 mL vial and stored at -70°C to freeze out any remaining water. The unfrozen extract portion was also dried over (~100 mg) anhydrous sodium sulfate. Extract was stored in a 2-mL vial at -20°C prior to analysis by GC-MS as described above. Triplicate determinations were performed using the same procedure.

Calibration solutions were prepared by spiking 53% ABV with the internal standard (5 µL of a 11.98 µg/µL solution of 3-pentanone in methanol) and varying levels (2, 5, 10 and 20 µL) of a solution containing 2,3-butanedione (1.58 µg/µL of 2,3-butanedione in methanol). Each solution was analyzed by GC-MS as described above and response factor (R*f*) was

determined by linear regression of a plot of peak area ratio versus mass ratio. Peak area ratios and $R_f$ values were used to calculate concentrations of 2,3-butanedione as follows:
Conc (µg/mL) = [Area (ion 144) 2,3-butanedione ÷ Area (ion 115) 3-pentanone] x 4060 µg x R*f* ÷ 10.00 mL. The Rf values for 2,3-butanedione against 3-pentanone as internal standard was 0.8167.

**Semi-quantitation of dimethyl trisulfide and 2-methyl-3-(methyldithio)furan**

GC-O dilution analysis (e.g. AEDA) can be effectively used to semi-quantitate known compounds that do not produce an instrumental detector response (i.e. when no peak is detectable by GC-MS or GC-FID) provided a pure authentic standard of the compound is available[48]. Another criterion is that it must be possible to analyze the sample or product directly without the need for an extraction step which could introduce bias. The SAFE distillate of MT has identical flavor profile with the original MT liquor as determined by triangle difference testing (see Chapter 4). Thus, the direct SAFE distillate of MT was subjected to GC-O dilution analysis.

Target analytes, dimethyl trisulfide and 2-methyl-3-(methyldithio)furan, were prepared in 53% (v/v) ethanol /water matrix (ABV), which was the same ABV as MT. A stepwise dilution series was prepared in a ratio of 1:2 (v/v). GCO analysis of the dilution series was performed on a 6890GC (Agilent Technologies, Inc.) equipped with a cool-on-column injector. This injection technique is most suitable for quantitation of thermally unstable compounds or those that might be thermally generated in a hot GC inlet (e.g. in a hot split/splitless inlet). Separations were performed using a RTX-Wax column (RTX-WAX (15m × 0.32 mm i.d., 0.5 µm film thickness; Restek). The carrier gas was helium at a constant flow rate of 2mL/min. The oven temperature was programmed 40℃ to 250℃ at the ramp rate of ºC/min with initial hold and final hold times 5 and 30 min, respectively. Column effluent was split equally between the sniff port and FID by using 0.15 mm i.d. deactivated capillary columns of equal length (1 m). FID and sniff port temperatures were maintained at 250 ℃. Each dilution was analyzed by GC-O by two experienced panelist. FD-factor was determined for each odorant in the sample (SAFE distillate) and standard dilutions series.

The FD-factors of the target analyte were used to calculate the concentration of the target compounds originally present in the liquor (SAFE distillate) by using the following equation:

$$Concentration\ of\ analyte\ in\ sample = Concentration\ of\ standard\ in\ stock\ solution \times \frac{FD\ factor\ of\ sample}{FD\ factor\ of\ stock\ solution}$$

## 5.4 Results and Discussion

In this study 39 of the odor-active components in Moutai were quantitated by either SIDA (35), direct injection GC-FID (1), derivatization-GC-MS (1) or GC-O and AEDA (2 trace constituents) and odor-activity values (OAVs) were calculated. Eight odorants were found to have OAVs above 200, including acetaldehyde; acetal; 2-methylbutanal; β-damascenone; ethyl butanoate; ethyl isovalerate; ethyl hexanoate and ethyl phenylpropionate. All of these odorants were previously indicated by AEDA (chapter 3) to be potent odorants in MT.

Six of the quantified components had OAVs below 1. These were phenylethyl acetate; heptanoic acid; nonanoic acid; decanoic acid; ethyl heptanoate and 2-phenyl ethanol. However, since the odor detection thresholds used were not determined in 53% ABV matrix, those components with OAV below 1 may not necessarily make no contribution to the overall aroma profile of MT. For the long chain fatty acids such as nonanoic acid and decanoic acid, even they are unlikely the contributors to the flavor of MT, their existence may influence the taste and mouthfeel of the liquor.

Accurate determination of odor-active components of MT is essential for the understanding of its complex flavor system and essential for the reconstitution of its aroma by flavor reconstitution – that is by establishing a flavor model based on the quantitation results (detailed in Chapter 6). On the other hand, the successful creation of a reconstitution model of MT flavor is critical to confirming the accuracy of the qualitative and quantitation results of this study.

**Table 5.1. Target analytes, deuterium- and $^{13}C$-labeled isotopes, selected ions, and response factors used for SIDA**

| *No.* | *Compound* | *No.* | *Labeled compound* | *Selected Ion (m/z)* | | $R^2$ | $R_f$ |
|---|---|---|---|---|---|---|---|
| | | | | *Unlabeled* | *Labeled* | | |
| **2** | 2-methylpropanal | **I-2** | [$^2H_2$]-2-methylpropanal | 72 | 74 | 1.00 | 0.60 |
| **144** | 2-methylbutanal | **I-** | [$^2H_2$]-2-methyl-butanal | 86 | 88 | 1.00 | 0.52 |
| **3** | 3-methylbutanal | **I-3** | [$^2H_2$]-3-methyl-butanal | 71 | 73 | 0.99+ | 0.58 |
| **10** | ethyl butanoate | **I-10** | [$^2H_5$]-ethyl butanoate | 88 | 93 | 0.99+ | 0.91 |
| **15** | ethyl pentanoate | **I-15** | [$^2H_5$]-ethyl pentanoate | 101 | 106 | 0.99+ | 0.60 |
| **12** | ethyl isovalerate | **I-** | [$^2H_5$]-ethyl isovalerate | 88 | 93 | 0.99+ | 0.93 |
| **19** | ethyl hexanoate | **I-19** | [$^2H_5$]-ethyl hexanoate | 88 | 93 | 0.99+ | 0.91 |
| **23** | ethyl heptanoate | **I-23** | [$^2H_5$]-ethyl heptanoate | 88 | 93 | 0.99+ | 0.89 |
| **28** | ethyl octonoate | **I-28** | [$^2H_5$]-ethyl octanoate | 88 | 93 | 1.00 | 0.95 |
| **145** | ethyl decanoate | **I-145** | [$^2H_5$]-ethyl decanoate | 88 | 93 | 1.00 | 0.97 |
| **146** | ethyl dodecanoate | **I-146** | [$^2H_5$]-ethyl dodecanoate | 88 | 93 | 0.99+ | 0.97 |
| **147** | ethyl palmitate | **I-147** | [$^2H_5$]-ethyl palmitate | 88 | 93 | 0.99+ | 1.00 |
| **57** | ethyl phenylacetate | **I-57** | [$^2H_5$]-ethyl phenylacetate | 164 | 169 | 1.00 | 0.99 |
| **68** | ethyl phenyl propionate | **I-68** | [$^2H_5$]-ethyl phenyl propionate | 178 | 183 | 0.99+ | 0.90 |

***Table 5.1. Continued***

| *No.* | *Compound* | *No.* | *Labeled compound* | *Selected Ion (m/z)* | | $R^2$ | $R_f$ |
|---|---|---|---|---|---|---|---|
| | | | | *Unlabeled* | *Labeled* | | |
| **89** | acetic acid | **I-89** | [$^2H_3$]-acetic acid | 60 | 63 | 0.99+ | 0.50 |
| **90** | propionic acid | **I-90** | [$^2H_5$]-propionic acid | 74 | 79 | 0.99+ | 0.79 |
| **92** | butyric acid | **I-92** | [$^{13}C_2$]- butyric acid | 60 | 62 | 0.99+ | 0.83 |
| **91** | isobutyric acid | **I-91** | [$^2H_2$]-isobutyric acids | 73 | 75 | 0.99+ | 0.34 |
| **94** | pentanoic acid | **I-94** | [$^2H_9$]-pentanoic acid | 60 | 63 | 0.99+ | 0.71 |
| **96** | hexanoic acid | **I-96** | [$^2H_4$]-hexanoic acid | 87 | 91 | 0.99+ | 0.36 |
| **106** | phenylacetic acid | **I-106** | [$^{13}C_2$]-phenylacetic acid | 136 | 138 | 0.99+ | 0.93 |
| **69** | phenylethanol | **I-69** | [$^{13}C_2$]-2-phenylethanol | 122 | 124 | 0.99+ | 1.40 |
| **104** | sotolon | **I-104** | [$^{13}C_2$]-sotolon | 128 | 130 | 0.99+ | 1.16 |
| **121** | acetal | **I-121** | [$^2H_8$]-ethyl acetate | 103 | 96 | 0.99+ | 0.16 |
| **151** | ethyl acetate | **I-151** | [$^2H_8$]-ethyl acetate | 72 | 96 | 0.99+ | 0.76 |
| **9** | isobutyl acetate | **I-9** | [$^2H_3$]- isobutyl acetate | 73 | 76 | 0.99+ | 0.75 |
| **14** | isoamyl acetate | **I-14** | [$^2H_3$]- isoamyl acetate | 87 | 90 | 0.99+ | 0.90 |
| **152** | benzaldehyde | **I-152** | [$^2H_5$]- benzaldehyde | 105 | 110 | 0.99+ | 1.50 |

***Table 5.1. Continued***

| *No.* | *Compound* | *No.* | *Labeled compound* | *Selected Ion (m/z)* | | $R^2$ | $R_f$ |
|---|---|---|---|---|---|---|---|
| | | | | *Unlabeled* | *Unlabeled* | | |
| **93a** | 3-methylbutyric acid | **I-93a** | [$^2H_3$]- 3-methylbutyric acid | 102 | 105 | 0.99+ | 1.51 |
| **93b** | 2-methylbutyric acid | **I-93b** | [$^2H_9$]- 2-methylbutyric acid | 74 | 79 | 0.99+ | 1.38 |
| **61** | phenyl ethyl acetate | **I-61** | [$^{13}C_2$]-phenyl ethyl acetate | 104 | 106 | 0.99+ | 1.08 |
| **62** | β-damascenone | **I-62** | [$^2H_4$]- β-damascenone | 190 | 194 | 0.99+ | 0.72 |
| **99** | heptanoic acid | **I-99** | [$^2H_2$]- heptanoic acid | 73 | 75 | 0.99+ | 1.13 |
| **153** | octanoic acid | **I-153** | [$^2H_4$]- octanoic acid | 73 | 75 | 0.99+ | 1.51 |
| **75** | γ-nonalactone | **I-75** | [$^2H_3$]- γ-nonalactone | 85 | 87 | 0.99+ | 0.90 |
| **77** | p-cresol | **I-77** | [$^2H_6$]- p-cresol | 107 | 115 | 0.99+ | 0.70 |
| **154** | nonanoic acid | **I-154** | [$^2H_4$]- nonanoic acid | 129 | 133 | 0.99+ | 1.29 |
| **155** | decanoic acid | **I-155** | [$^2H_4$]- decanoic acid | 143 | 145 | 0.99+ | 1.12 |
| **108** | phenyl propionic acid | **I-108** | [$^2H_2$]- phenyl propionic acid | 150 | 152 | 0.99+ | 1.01 |

**Table 5.2. Concentration of Selected Potent Odorants in the liquor of Moutai**

| *No.* | *Compound* | *Concentration (mg/L)* | *Odor Threshold (mg/L)* | *OAV* |
|---|---|---|---|---|
| **1** | acetaldehyde | 1114.000 ±11.605 | 1.200[a] | 928 |
| **151** | ethyl acetate | 1275.288 ± 5.673 | 32.600[b] | 39 |
| **121** | acetal | 418.303 ± 11.710 | 2.090[b] | 200 |
| **2** | 2-methylpropanal | 26.12 ± 0.66 | 1.300[a] | 20 |
| **144** | 2-methylbutanal | 17.70 ± 0.17 | 0.033[c] | 536 |
| **3** | 3-methylbutanal | 37.11 ± 0.49 | 0.921[a] | 40 |
| **8** | 2,3,-butanedione | 4.766 ± 2.124 | 0.100 [d] | 48 |
| **9** | isobutyl acetate | 1.265 ± 0.01125 | 0.922[b] | 1.4 |
| **14** | isoamyl acetate | 1.776 ± 0.00241 | 0.0939[b] | 19 |
| **152** | benzaldehyde | 4.720 ± 0.00702 | 4.20[b] | 1.1 |
| **93b** | 2-methylbutyric acid | 5.471 ± 0.03854 | 3.000[d] | 2 |
| **93a** | 3-methylbutyric acid | 7.950 ± 0.17079 | 1.050[b] | 1.4 |
| **61** | phenyl ethyl acetate | 0.192 ± 0.00117 | 0.909[b] | 0.21 |
| **62** | β-damascenone | 0.101 ± 0.00040 | 0.00012[b] | 842 |
| **99** | heptanoic acid | 0.982 ± 0.00100 | 13.300[a] | < 0.1 |
| **153** | octanoic acid | 2.210 ± 0.04307 | 2.700[a] | 0.82 |
| **75** | γ-nonalactone | 0.192 ± 0.00348 | 0.0907[b] | 2 |
| **77** | p-cresol | 0.073 ± 0.00476 | 0.167[b] | 0.44 |
| **154** | nonanoic acid | 0.247 ± 0.00649 | 3.560[a] | < 0.1 |
| **155** | decanoic acid | 0.323 ± 0.00274 | 13.700[a] | < 0.1 |
| **108** | phenyl propionic acid | 0.220 ± 0.00424 | 0.027[e] | 8 |
| **10** | ethyl butanoate | 57.70±0.41 | 0.0815[b] | 708 |
| **15** | ethyl pentanoate | 4.561±0.034 | 0.0268[b] | 170 |

***Table 5.2 Continued***

| *No.* | *Compound* | *Concentration (mg/L)* | *Odor Threshold (mg/L)* | *OAV* |
|---|---|---|---|---|
| **12** | ethyl isovalerate | 11.315±0.057 | 0.00689[b] | 1642 |
| **19** | ethyl hexanoate | 17.112±0.026 | 0.0553[b] | 309 |
| **23** | ethyl heptanoate | 1.250±0.011 | 13.200[b] | < 0.1 |
| **28** | ethyl octanoate | 2.010±0.015 | 0.0129[b] | 156 |
| **147** | ethyl palmitate | 19.254±0.078 | 2.000[f] | 10 |
| **57** | ethyl phenyl acetate | 5.420±0.011 | 0.407[b] | 13 |
| **68** | ethyl phenyl propionate | 38.64±0.76 | 0.125[b] | 309 |
| **89** | acetic acid | 7257.758±0.033 | 160[b] | 45 |
| **90** | propionic acid | 1228.9±3.6 | 18.200[a] | 68 |
| **92** | butyric acid | 35.18±0.25 | 0.964[b] | 36 |
| **91** | isobutyric acid | 20.89±0.19 | 1.580[b] | 13 |
| **94** | pentanoic acid | 4.383±0.037 | 0.389[b] | 11 |
| **96** | hexanoic acid | 12.187±0.037 | 2.520[b] | 5 |
| **106** | phenyl acetic acid | 20.437±0.027 | 2.650 [g] | 8 |
| **69** | phenyl ethanol | 20.77±0.15 | 28.900[b] | 0.72 |
| **135** | 2,3-diethyl-5methyl pyrazine [h] | 0.089±0.002 | 0.00014 | 636 |
| **104** | sotolon | 0.097±0.015 | 0.005[d] | 19 |

**Table 5.2** continues:

a .Wang, X.; Fan, W.; Xu, Y. Comparison on Aroma Compounds in Chinese Soy Sauce and Strong Aroma Type Liquors by Gas Chromatography–olfactometry, Chemical Quantitative and Odor Activity Values Analysis. *Eur. Food Res. Technol.* **2014**, *239* (5), 813–825.

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**Table 5.3. Concentrations GC-O dilution analysis to quantify potent trace amount odor-active components in MT**

| *No.* | *Target Analyte* | *Target compound detection threshold (ppb)* | *FD factor in MT* | *Concentration in MT (ppb)* | *Threshold (ppb)* | *OAV* |
|---|---|---|---|---|---|---|
| **24** | dimethyl trisulfide | 5.97 | 32 | 191 | 0.36[a] | 531 |
| **47** | 2-methyl-3-(methyldithio) furan | 0.164 | 16 | 2.62 | 0.4[b] | 7 |


a. Wang, X.; Fan, W.; Xu, Y. Comparison on Aroma Compounds in Chinese Soy Sauce and Strong Aroma Type Liquors by Gas Chromatography–olfactometry, Chemical Quantitative and Odor Activity Values Analysis. *Eur. Food Res. Technol.* **2014**, *239* (5), 813–825.

b. Frauendorfer, F.; Schieberle, P. Identification of the Key Aroma Compounds in Cocoa Powder Based on Molecular Sensory Correlations. *J. Agric. Food Chem.* **2006**, 54 (15), 5521–5529.

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# Chapter 6: Sensory Studies of an Aroma Recombination Model of Moutai

## 6.1 Abstract

An aroma reconstitution model of Moutai was formulated from 36 potent odorants components selected based on their flavor dilution (FD) factors (detailed in Chapter 3) and odor activity values (OAVs) (detailed in Chapters 4 and 5). Model was created by adding 36 high purity standards at appropriate concentrations to matrix consisting of a 53% ABV solution. Triangle tests were performed using 24 panelists for the purpose to assess whether the aroma reconstitution model differed in terms of overall aroma from the original MT liquor. According to the results of triangle tests, among 48 judgements, 19 answers were correct which indicated there was no detectable aroma difference ($P \leq 0.05$) between the aroma model and the original liquor product.

## 6.2 Introduction

In the previous study (Chapter 5), 39 odorants in MT were quantitated through various techniques. Their relative aroma contributions were assessed by their FD-factors (Chapters 3) and their OAVs. Based on the FD-factors and the OAV of each quantitated and semi-quantitated odorant, 36 odor-active components were selected for the formulation of the aroma recombination of MT.

Aroma model was prepared by adding high purity standard odorants in to identical food matrix[27]. In the case of MT, which contains 53% ABV, high purity standards were added into a 53% ABV base solution at the concentrations determined in the previous study (detailed in Chapters 4 & 5).

The ability to successfully formulate an aroma reconstitution model of MT aroma confirms the accuracy of identification and quantitation results in the previous studies (detailed in Chapter 3-5). Furthermore, it can also serve as the complete model for omission studies, which could address the impact of individual characterizing odorants that must present to create the typical aroma of MT[27].

## 6.3 Materials and Methods

### 6.3.1 Liquor Samples

Authentic MT samples were purchased from the producer, Kweichow Moutai Co. Ltd. Guizhou, China. Mention of brand name is not for advertisement or endorsement purpose and does not imply any research contact or sponsorship.

### 6.3.2 Chemicals

All authentic reference standards were obtained by Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned.

Acetic acid and ethanol were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA); Octanoic acid was from Bedoukian ( Bedoukian, Danburt, CT, USA). Odorless deionized-distilled water for was prepared by boiling deionized-distilled water to two-thirds of its original volume.

### 6.3.3 Constitution of the Aroma Model of Moutai

Stock solutions of high purity flavor standards were prepared individually in ethanol. Based on the quantitation results determined in the previous study (detailed in Chapters 4 & 5), the 36 selected potent odorants were spiked into 53% ABV matrix to the concentration levels shown in **Table 6.1**.

### 6.3.4 Sensory Methodology

Testing was approved as protocol number 17507 of the Institutional Review Board (IRB) of the University of Illinois at Urbana-Champaign.

Panelists (24) ranging in age from 21-54 participated the triangle sensory testing to determine whether the overall aroma characteristics of the aroma reconstitution model differed from the MT original liquor.

Reconstituted aroma model and MT (20 mL each) were transferred into 125-mL Teflon sniff bottles (Nalgene PTFE wash bottle without siphon tube; Nalge Nunc International, Rochester, NY, USA) which were wrapped with aluminum foil to minimize any visual bias and to protect the samples from light exposure. The temperature of the room was 22-26 °C and the humidity was 12%. For triangle difference testing[38], samples were presented to panelists in two orders: one set consisted of two aroma models and one original liquor

product and the other consisted of one aroma model and two original liquor products. Panelists were asked to squeeze the bottle, sniff the samples and answer the question about which sample do they think is the odd one which has different odor properties compared with the other two. Both sets were tested by the same group of people, 48 judges were collected.

### 6.4 Results and Discussion

Among 48 triangle tests, 19 answers were correct, which was below the critical value 32 for 48 judges to indicate significant differences between these 2 samples[38]($P \leq 0.05$). In the set with 2 aroma models and 1 MT, 8 answers were correct, 16 answers were wrong while in the set with 1 aroma model and 2 MT, 11 answers were correct, 13 answers were wrong. According to the triangle tests, there was no significant difference between the reconstituted model based on the identification and quantitation results determined in our previous study ($P \leq 0.05$). The aroma model of MT created in this study provided accurate match to aroma of MT. However, to address the characterizing odorants whose exist provide the typical flavor of MT, further research is necessary, e.g. omission study or other types of mixture studies. The aroma sample created in this study could serve as the complete sample for the omission studies in the future.

The reconstituted model created in this study was only tested with respect to its aroma. The aroma model of MT only included odor-active components in the product of MT. However some components with high odor thresholds which may not contribute to the aroma of MT could impact the taste or retronasal aroma of the liquor product by affecting the flavor/aroma release or partitioning. Thus, the taste or retronasal aroma of the model created in this study may differ from the original liquor of MT. To achieve an aroma model identical in terms of both flavor and taste further research should be done.

**Table 6.1 Concentration of High Purity Standard Compounds Used In Construction of Moutai Aroma Model**

| *No.* | *Compound* | *Concentration(mg/L)* | *Purity (%)* |
|---|---|---|---|
| **2** | 2-methylpropanal | 26.12 | 98.14 |
| **3** | 3-methybutanal | 37.11 | 98.59 |
| **14** | isoamyl acetate | 1.84 | 99.69 |
| **10** | ethyl butanoate | 57.70 | 99.94 |
| **11** | ethyl 3-methylbutanoate | 11.32 | 99.98 |
| **15** | ethyl pentanoate | 4.56 | 99.99 |
| **19** | ethyl hexanoate | 17.11 | 99.99 |
| **24** | dimethyl trisulfide | 0.19 | 99.53 |
| **28** | ethyl octanoate | 2.01 | 99.99 |
| **135** | 2,3-diethyl-5-methylpyrazine | 0.01 | 98.97 |
| **89** | acetic acid | 7257.76 | 98.44 |
| **92** | butyric acid | 35.18 | 99.99 |
| **152** | benzaldehyde | 5.84 | 87.44 |
| **93b** | 2-methyl butanoic acid | 5.47 | 99.98 |
| **93a** | 3-methyl butanoic acid | 7.95 | 99.67 |
| **47** | 2-methyl-3-(methyldithio) furan | 0.0026 | 96.35 |
| **94** | pentanoic acid | 4.38 | 99.84 |
| **96** | hexanoic acid | 12.19 | 99.88 |
| **153** | octanoic acid | 2.27 | 99.81 |
| **57** | ethyl phenylacetate | 5.42 | 99.75 |

***Table 6.1 Continued***

| *No.* | *Compound* | *Concentration (mg/L)* | *Purity (%)* |
|---|---|---|---|
| **68** | ethyl phenyl propionate | 38.64 | 98.94 |
| **62** | β-damascenone | 0.101 | 91.10 |
| **69** | 2-phenylethanol | 20.8 | 99.71 |
| **75** | γ-nonlactone | 0.212 | 99.93 |
| **77** | p-cresol | 0.0740 | 99.51 |
| **104** | sotolon | 0.097 | 95.44 |
| **106** | phenyl acetic acid | 20.4 | 99.99 |
| **8** | 2,3-butanedione | 4.73 | 85.07 |
| **26** | 2,3,5-trimethylpyrazine[a] | 0.474 | 99.97 |
| **121** | acetal | 418 | 98.64 |
| **151** | ethyl acetate | 1280 | 99.98 |
| **1** | acetaldehyde[b] | 0.870 | 96.65 |
| **9** | 2-methyl propyl acetate | 1.28 | 99.65 |
| **99** | heptanoic acid | 0.99 | 99.81 |
| **23** | ethyl heptanoate | 1.25 | 99.99 |
| **90** | propionic acid | 1230 | 99.84 |

a. concentration was from literature: Fan, W.; Xu, Y.; Zhang, Y. Characterization of Pyrazines in Some Chinese Liquors and Their Approximate Concentrations. *J. Agric. Food Chem.* **2007**, *55* (24), 9956–9962.

b. Concentration in μL/L converts to mg/L using density

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## Chapter 7: Aroma Chemistry of Moutai: Summary and Future Recommendations

Chinese distilled liquor products are primarily composed of water and alcohol (98%), with the remaining 2% being composed other volatile components[1]. However, this 2% plays an important and essential role with respect to the aroma profile and determines the flavor type of the Chinese liquor. The complex aroma system of Moutai (MT) liquor is determined by its unique and special manufacturing practice[2]. As a traditional solid-state fermentation product, the production of MT involves four essential processes: production of Daqu (starter), stack fermentation, pit fermentation and distillation[3]. Compared to other traditional Chinese distilled liquors, the fermentation process in MT production is done at a higher temperature[2]. Under this condition, a strain of *Bacillus licheniformis* plays an important role in the formation of Moutai-flavor. This species of *Bacillus* is also responsible for the fermentation of other solid-state fermented food products such as natto, thua-nao and others,[4] and is responsible for the formation of the typical flavor of these food products. This organism's contribution to the flavor of these fermented products is achieved mainly through the formation of volatile pyrazines, aldehydes, ketones and alcohols [5]. In the study of the aroma profile of wheat bran culture of *Bacillus licheniformis*, 2,3-butendione, pyrazines, volatile acids and phenolic compounds were formed during fermentation[2]. Compared to other *Bacillus licheniformis* strains, the one responsible for the fermentation in MT grows faster at high temperature (55 °C), and especially in the solid-state fermentation they produce 22-34 times more acids and 5 times more C4 compounds (2,3-butendione, 3-hydroxy-2-butanone and 2,3-butanediol) compared to when they are grown in submerge fermentation[2]. The fact that *Bacillus licheniformis* produces more acids during the fermentation of MT[6] might explain the extremely low pH value of Moutai, which is around 3.7, and which is lower than other distilled liquor products such as whiskey[7] and tequila[8]. Higher content of acids not only contributes to the "cheesy, sweaty and sour" odor properties to the aroma profile of MT, they determined the low pH environment for the whole flavor system of MT which affects the partition and odor impacts of some other odor-active components. Such as pyrazines and pyridines are suppressed at low pH because of their high pKa values; while aroma of acids are enhanced because of their low pKa values.

The acids formed by *Bacillus licheniformis* also serve as the materials for the esterification and produce ethyl esters. Most esters were formed through esterification of the alcohol and acid

during the fermentation and storage of liquor products[9]. Among all these acids, the most abundant acid in the product of MT is acetic acid, which concentration was determined as 7.26g/L by using SIDA. It is more than 2000 times higher compared to Evan Williams Whiskey (detailed in Chapter 4). Thus, through esterification, significant amount of acetates were formed. Ethyl esters and acetates accounts to 25 of all the odor-active components identified in this study and responsible to the fruity, berry, kiwi fruit like odor properties of MT. Among all these esters, ethyl cyclohexanoate has the lowest threshold which is around 1ng/L[10] and its occurrence is first reported in this study according to the best of our knowledge. However since it is also found in wine and rum products[11–13] and its flavor property is berry like, it is not the critical component which differentiates MT from all other liquor products.

Among odor-active components identified and tentatively identified, 5 compounds are responsible for savory, beefy, meaty odor properties of MT which including sotolon (No. 104), 2-furfurylthiol (FFT) (No. 30), 2-methyl-3-furanthiol (MFT) (No. 133), 2-methyl-3-(methyldithio) furan (MFT-MT) (No. 47) and *bis*(2-methyl-3-furyl) disulfide (MFT-MFT) (No. 79). Sotolon can be formed in various pathways. It can be formed from α-ketobutyric acid and acetaldehyde[14] or condensation of 2,3-butendione and hydroxyacetaldehyde[15] . As an odor-active component in wine products, concentration of sotolon tends to increase during aging[15,16]. Its concentration during the aging of MT may also contribute to the flavor change in aged MT products.

2-Furfurylthiol (No. 30), 2-methyl-3-furanthiol (No. 133), 2-methyl-3-(methyldithio) furan (No. 47) and *bis*(2-methyl-3-furyl) disulfide (No. 79) are all volatile sulfur compounds. Their odor thresholds (ODT) are extremely low [ODT of FFT (46% ABV)= 0.1ppb[17], ODT of MFT (12% ABV)=0.005ppb[18], ODT of MFT-MT (water)=0.4ppb[19]and ODT of MFT-MFT (water)=0.00002ppb[20]] which made the identification and quantitation of these components very challenging. Thus only until recently was FFT identified and quantified in Chinese liquors[17,21]. The concentration of FFT in MT is 39 ppb[21]whereas its content in sesame flavor type liquor, which is another traditional distilled Chinese liquor product, is 118ppb. In this case, even it contributed to the aroma profile of MT, therefore its existence in MT is not exclusive. Since the

odor activity value (OAV) in sesame flavor type liquor is much higher than that in MT, it is highly unlikely that FFT is a characterizing odorant in the target product of our study.

To the best of our knowledge, MFT, MFT-MT and MFT-MFT were first reported in this study. They are known to contribute beefy, meaty and savory top notes in various cooked meat products [22–27]. The odor properties of these 3 compounds are quite similar, thus only MFT-MT was selected in the reconstituted flavor model of MT.

Various pathways for the formation of MFT have been reported. MFT can be formed through the Maillard reaction between carbohydrates and cysteine[28–30]. It can also be generated through the degradation of thiamin[20] and via hydrolysis of thioacetates catalyzed by lipase[31]. Due to its instability, MFT tends to readily dimerize into MFT-MFT, e.g., 20% of MFT will dimerize within one day when dissolved in diethyl either under 6°C[32]. Under high temperature, MFT-MFT will be cleaved to (re)form the monomer MFT[20].

MFT-MT which is also a flavor contributor in red wine[33] could either be formed by Maillard reaction of ribose and cysteine or generated by reaction of MFT and methanethiol[34]. It has the highest FD factor among all these firstly identified three volatile sulfur compounds, it may serve as the most important contributor to the meaty, beefy note in the overall aroma profile of MT. For this reason it was selected in the reconstituted MT flavor model in this study. The addition of MFT-MT was based on gas chromatography-olfactometry (GC-O) dilution analysis, which only provided semi-quantitative results of the target analyte. In the future, for better understanding and evaluation of the aroma contribution of these 3 potent volatile sulfur compounds in the product of MT, their quantitation by using stable isotope dilution analysis (SIDA) is necessary.

An interesting case in the flavor chemistry study of MT is tetramethylpyrazine which was produced by *Bacillus licheniformis*[35]. It serves as volatile component which could be used differentiate MT (soy sauce aroma liquor) form other liquor products (detailed in Chapter 4). It was considered of great importance and was previously reported to have highest aroma intensity in basic volatile fraction of MT[36], however, its content in MT is 440ppb, which is much lower than its odor threshold 1000ppb[37] . It is highly improbable that tetramethylpyrazine is a major

flavor contributor in the aroma system of MT, even if it is a unique and differentiation volatile component in this liquor product.

Based on the identification and quantitation results, MT flavor (aroma) was successfully reconstituted using 36 potent odorants, which indicates these 36 components in the concentration determined in this study are sufficient to provide typical MT aroma in 53% ABV matrix. However, for further research to narrow down the characterizing component(s) in the product of MT, omission studies should be considered. Such studies address the key components in the aroma model by evaluating mixtures in which certain odorants are omitted. have been used in many beverage products such as whiskey[38], Cola[39]and even in the product of MT[40]. In the previous reported MT flavor model created by Lingling Wang in 2012, 52 odor-active components were included[40]. However, because the reconstituted flavor model already lacked the characterizing aroma properties of MT, it cannot serve as a complete flavor for omission studies. Since none of these 5 components which contribute savory, meaty and beefy odor properties were included in that model. It is possible these 5 compounds play important roles in the complex aroma chemistry system of MT and serve as major contributors to the characterizing or differentiating flavor of soy sauce aroma liquor products. The MT flavor model created in the present study could serve as the complete model and further omission study based on this recipe could address the most essential flavor contributor in the product of MT.

This study was mainly focused on the volatile components in the product of MT which contribute to its overall aroma profile. However, nonvolatile components also might play an important role due to their impacts on the partitioning of the odor-active components. A case in point is lichenysin[41–43] which is a cyclic lipopeptide produced by *Bacillus licheniformis*. Its content in MT is around 29 ppb [41]. At the concentration of 160ppb, lichenysin can suppress the volatility of phenolic compounds by 36-48% and hexanoic acid by 50% by forming hydrogen bonding networks between the hydroxyl group and the carboxyl group of lichenysin[41]. However no date is available to support that at the concentration it is found in MT whether its impact on the volatility of odor-active components is significant. Therefore, its impact on the overall aroma profile of MT is still not clear.

The reconstituted MT flavor model created in this study was only tested with respect to its aroma. Since the MT flavor model only included odor-active components in the product of MT. However some components with high odor thresholds which may not contribute to the aroma of MT may impact the taste or retronasal aroma of the liquor product by affecting the flavor/aroma release or partitioning. Thus, the taste or retronasal aroma of the model created in this study may differ from the original liquor of MT. To achieve an aroma model identical in terms of both flavor and taste, further research should to be done.

It is also noteworthy, the present study was only targeted on one product of Moutai which was the most popular and general one. However, the aroma profile various during aging and the aroma components would be different in different Moutai products.

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