IDENTIFICATION OF ROTUNDONE AS AN IMPORTANT CONTRIBUTOR TO THE FLAVOR OF OAK AGED SPIRITS

BY

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DISSERTATION

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Abstract

The practice of barrel aging of spirits has been used for centuries. It began as an alternative storage and transportation method, but aging in an oak cask is now exclusively used as a means to impart flavor to the spirits. Oak wood is the wood of choice for barrel making, not only for its physical characteristics that lend itself to manufacturing a barrel, but also for its unique chemical properties that impart key flavors to aged spirits. Oak aging of spirits develops flavor in a number of different ways, all which contribute to a wide range of odor descriptions, creating the complex flavor with which we are familiar. Extensive research has been performed on oak wood and oak aged spirits; however, the identity of the component(s) responsible for the "woody/incense" flavor attribute of age spirits was, prior to this investigation, unknown. Experiments were conducted in order to unambiguously identify a compound responsible for a "woody/incense" odor note in oak aged spirits. The target compound was isolated from oak wood chips followed by several purification steps, as well as the use of a custom built GC-MS/olfactometry system equipped with a heart-cutting system/internal CryoTrap which enabled the acquisition of an interpretable electron-impact mass spectrum (EI-MS) for the compound. The EI-MS revealed that the unknown target compound possessed a molecular weight of 218. A thorough investigation of naturally occurring organic compounds having a molecular weight of 218, along with deducing the nature of the functional groups on the molecule, indicated numerous compounds as possible candidates. Most of these compounds were found to occur naturally in a number of roots, spices, oils, and herbs, which were subsequently analyzed. Results of the analyses revealed that the compound was most likely the sesquiterpene ketone 5-isopropenyl-3,8-dimethyl-3,4,5,6,7,8-hexahydro-1(2*H*)-azulenone, or

rotundone. This identification was confirmed by comparison of the compound's EI-MS and GC retention indices against those of authentic rotundone obtained by chemical synthesis. The next question addressed was whether this compound is present in oak aged spirits. Accurate quantification of this trace level target compound was done by stable isotope dilution analysis (SIDA). The presence of rotundone in different aged spirits including bourbons, rye, scotch, whiskey, rum, and tequila was demonstrated. Trends in aging were established, showing that rotundone increases with aging time; however, its quantity may also be influenced by other factors as there was a clear brand to brand variation. Interestingly, rotundone was also found in un-aged (silver) tequila, which suggests that the compound may also be present in the agave plant. Results of quantification of all potent odorant in bourbons, aged 4, 8, and 12 years and calculation of their odor activity values (OAVs) demonstrated rotundone's importance to the overall flavor of bourbon. From the quantification data some interesting trends were established that demonstrate some effects of barrel aging. OAVs are used as a gauge for potency and, generally, any compound with an OAV above 1, provides evidence of whether a compound is important. With an OAV of 42.8 to 56.6, rotundone lies well above this requirement and is among the top 10 odorants quantified in these samples. Thus, it is concluded that rotundone is an important contributor to the flavor of these aged spirits.

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My fiancé, Joseph Kreger, my inspiration for finishing.

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Chapter 1: Introduction

"Too much of anything is a bad thing, but too much of whiskey is barely enough." (Mark Twain). Distilled spirits are heavily ingrained into society and have enormous sociological, anthropological, and economical implications. Whiskey, in particular, is legally defined as a fermented mash of grain that is distilled and must be stored in oak containers (Code of Federal Regulations). The practice of oak barrel aging has been used for centuries in the production of distilled beverages and wine. Although the identity of the inventors and date of development is uncertain, the most recent evidence indicates that the Celts were the first to use the wooden barrel around 900 BC (Preet 2012). Regardless of the exact origins of the wooden barrel, to this day this practice is still the main contributor to the flavor of whiskeys and nearly all other aged spirits. The flavor characterization of alcoholic beverages has been as important to food scientists as the cultural aspect of beverage alcohol is to historians. Due to the significance of alcoholic beverages, both culturally and economically, massive grants and whole institutes have been created to generate new scientific information to understand the basis for the flavor and aroma of alcoholic beverages. One of the most important historical advances in the manufacturing of alcoholic beverages is the invention and subsequent use of the wooden barrel, or "cask". Since the advent of the wooden cask, a large majority of alcoholic beverages are identified by and appreciated for the flavor imparted to the product as a result of being aged in the cask. This step in production is notably important as many alcoholic beverages have legal standards regarding the use of the wood, the wood type, and the time of aging in the cask. Although several wood types have been used, oak is almost always the wood of choice for casks. While distilled spirits aged in oak casks rely heavily on the wood as the primary source of flavor, products like wines and beers may also undergo barrel aging to develop secondary flavorings. Focusing on spirits, the flavor of an un-aged distillate

has been extensively researched, uncovering flavors developed from the starting grain as well as those that are byproducts of the fermentation. Their flavors are considered to be important to the overall product, contributing to hot/solvent-like, fruity, green, sweet, and malty characteristics. Flavors derived from the fermentation of grains include ethanol, fusel alcohols, acetates, and esters. These are present in all spirits; however, the quantity and importance of each may vary (Cole et al. 2003).

"Aging is among the most important and most costly factors influencing the quality of distilled beverages – fundamentally contributing to the finished taste and aroma." (Mosedale et al. 1998). Statements such as these resound throughout relevant literature and consistently indicate the importance that oak has on the flavor of aged distilled spirits. Extensive research has been performed on both spirits and the oak wood itself. However, there is a noticeable lack of understanding of the connection between the woody attributes of aged spirits and specific wood-derived aroma compounds that contribute to those flavors.

Oak cask aging post distillation develops flavor in several different ways, including: 1) ethanolysis of the wood acids 2) lignin pyrolysis from charring the barrel and 3) direct extraction of volatiles from the wood. These have a wide range of odor descriptions, creating the complex flavor with which we are familiar. Originating from the wood, the oak lactones, *cis*- and *trans*-β-methyl-γ-octalactone, are particularly important, even being nicknamed the "whiskey lactones", and impart an aroma described as coconut-like. Other lactones are present, including γ-nonalactone, δ-nonalactone, γ-decalactone, δ-decalactone and *cis*-6-dodeceno-γ-lactone. Additional wood extractives known to contribute to the flavor include: eugenol, isoeugenol, β-ionone, syringol, β-damascenone, syringaldehyde, and vanillin. These provide clove, spicy, floral, smoke, apple, sweet smoke and vanilla aroma notes, respectively. Ethanolysis of the wood acids results in the formation of fruity ethyl esters like ethyl propanoate, ethyl butyrate,

ethyl hexanoate, and ethyl octanoate. (Conner et al. 1993, Conner et al. 2001, Piggott et al. 2003, Poisson et al. 2008a, Poisson et al. 2008b, Lahne 2010).

Numerous studies have been conducted to better understand the flavor of aged spirits (Otsuka et al. 1974, Nicol et al. 1989, Mosedale et al. 1998, Conner et al. 2001, MacNamara et al. 2001, Demyttenaere et al. 2003, Madrera et al. 2003, Netto et al. 2003, Piggot et al. 2003, Câmara et al. 2007, Poisson et al. 2008a, Poisson et al. 2008b, Fernanadez de Simon 2010, Lahne 2010, Pino et al. 2012). While many important odorants have been identified, there appears to be many gaps in our understanding of the flavor chemistry of these products. For example, several studies reported the presence of one or more unidentified "wood-like" odorants in whiskey and other aged spirits. Studies done on rum described the product as exhibiting vanilla, dry fruit, coconut, caramel, and wood odor qualities, with the credit for the wood notes being given to the oak lactones (Pino et al. 2012). But actually the oak lactones were found to contribute more to the coconut-like characteristic of aged spirits (Abbot et al. 1995). Caninha, a regional specific rum from Brazil, was found to have wood-like odorants which were detected at high gas chromatography (GC) retention times. These compounds were detected with high dilution flavor (FD) factors as determined by aroma extract dilution analysis-GC-olfactometry (AEDA-GC-O), thus indicating their high odor potencies in the product. However, these notes could not be attributed to any of the volatile compounds identified by GC-MS (Netto et al. 2003). These researchers concluded that wood-like volatiles were present at very low concentrations and that further investigation was warranted. Añejo tequila and whiskey share many of the same potent odorants since both products rely heavily on the oak cask for flavor (Lahne and Cadwallader 2012). Thus it is not surprising that wood-like odorants were also indicated in studies performed on tequila. Several terpenes have been identified in tequila, including α-bisabolol, a woody compound typically found in sandalwood. Other wood-like odor descriptors were noted in the GC-O data at lower dilution factors,

although theses could not be identified due to their low concentration in the extract (Benn et al. 1996). A study by Lahne and Cadwallader (2012) on añejo tequila flavor also detected an unidentified woody, incense-like odorant by GC-O. Two additional studies which evaluated the potent compounds in brandy (Caldeira et al. 2002) and scotch whiskey (Conner et al. 2001) both mentioned the presence of a wood-like attribute that could not by identified by GC-MS analysis.

The central hypothesis of this study is that knowing the identity of this/these "woody/incense" odorant(s) from oak wood will give an explanation for what causes the overall woody characteristic in oak aged spirits. Thus, the goal of the present investigation was to identify an odorant in oak wood having a characteristic wood/incense-like aroma note and determine whether it is detectable within oak aged spirits, therefore, contributing to the flavor.

This study was designed to answer three main questions: 1) what is the identity of the compound responsible for imparting wood- and incense-like flavor to oak aged spirits?; 2) at what concentration is the compound found in various oak aged spirits?; and 3) how important is the compound to overall flavor of oak-aged spirits, with special emphasis place on bourbon whiskey?

The spirits industry holds a position of enormous importance, both socially and economically. The goal of this study was to expand and strengthen current knowledge of the flavor chemistry of oak-aged spirits. Identification of a previously unknown, potent flavor compound which contributes a wood-like attribute will not only fill in gaps from past studies, but will also help frame better questions for future studies.

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Chapter 2: Literature Review

2.1 Introduction

Wooden barrels have been used for centuries to store and transport fermented and distilled beverages. Although its history is still a subject of debate, recent evidence suggests that the Celts invented the wooden barrel around 900 B.C. The Celts were not only extremely skilled metallurgists, having perfected the process of smelting rock and extracting ore, but also skilled woodcrafters. While inhabiting the Danube River valley, the Celts are credited for combining their knowledge of woodworking and metallurgy to create the first wooden barrel (Preet 2012). Wine and beer had been produced for millennia, long before the invention of the wooden barrel, with the first record of wine dating back to 6000 B.C. in Persia, (i.e. modern day Iran). Wines at that time were transported using clay vessels. Although Greek and Roman methods for growing grapes and fermenting grape juice into wine were highly regarded, their use of fragile clay jugs (amphora) was an inferior method for storage and transportation of the wine, as they had a tendency to crack, leak, and break. Modifying their methods to include use of wooden barrels not only provided secure transport, but storage in wooden barrels also serendipitously imparted a more desirable flavor and taste to their wines. When the Celts migrated to the Irish Isles, where the climate was unsuitable for growing and harvesting grapes, they turned to using honey to produce the fermented beverage mead, which was also stored in wooden barrels.

Distilled spirits did not appear in the Irish Isles until the 12th century A.D., when Irish monasteries produced the first whiskey. Whiskey was broadly defined as ethanol distilled from a number of fermented cereal grains, and subsequently stored in wooden barrels. Today whiskey is the best-selling distilled spirit in the world (Piggott et al. 2003). Aged distilled spirits, which are known worldwide, include whiskey, scotch,

bourbon, Irish whisky, rum, tequila, mescal, cachaça and brandy, as well as other regionally specific spirit varieties. Barrel-aged distilled spirits rely on the wooden barrel, or cask, as their primary source for taste and flavor. With fermented products, such as wine, which can be aged in either stainless steel or oak barrels, the use of wooden barrels is an optional step to add a more complex flavor. Oak, in particular, stands out from other woods as being particularly good for aging fermented beverages. Its physical properties allow it to be shaped into a barrel with good tensile strength, high compression strength, elasticity and hardness. In addition its unique chemical makeup gives oak wood the capacity to impart depth of flavor to fermented beverages.

2.2 Chemistry of Oak Wood

Molecular Structure of Wood

The major structural components of wood cells are cellulose, hemicellulose, and lignin. Cellulose is the main constituent of wood, accounting for nearly 50% of its dry weight. Cellulose, only slightly soluble in water, is a polysaccharide consisting of tens of thousands of β-linked D-glucose moieties in a linear chain. These chains are linked by hydrogen bonds to create microfibrils. Microfibrils can either be present in a highly ordered crystalline structure of paralleled D-glucose chains, or in a less ordered amorphous type structure of antiparallel D-glucose chains. Hemicellulose, which is soluble in water, is a hetero-polysaccharide with branched chains typically much shorter than cellulose. The main hemicelluloses in wood are galactoglucomannan, arabinoglucuronoxylan, arabinogalactan, glucuronoxylan and glucomannan (Sjöström 1993). Hemicelluloses are used in the food industry as functional ingredients due to their solubility in water. Gum arabic is an example of a hemicellulose that is commonly used as a stabilizer in emulsifications. Lignin is the most complex structural component of wood and accounts for about 15-30% of the wood plant tissue. It is a heterogeneous

organic polymer responsible for the thickening of cell walls to make them rigid and impermeable. Its three main building blocks are monolignols, namely coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Vanholme et al. 2010). When incorporated into the lignin polymer these monlignols make up the p-hydroxyphenol (H), syringyl (S), and guaiacyl (G) units, respectively. Lignin composition is very diverse depending not only on the species of tree, but also on the type of cells within the tree. Lignin is a precursor of many aroma-active compounds responsible for the flavor oak casks impart to the aged spirits. These are formed by pyrolysis of the wood lignin during the toasting or charring step when manufacturing the barrel.

Sapwood

Sapwood is essentially the "living" part of the tree structure. Its function is the conduction of sap that caries water, mineral salts and nutrients from the roots to the leaves and other living tissues in the tree. It is also responsible for the synthesis and storage of photosynthates, the chemical products of photosynthesis, usually in the form of sugars or starch which are the main energy sources for the tree to maintain life and support further growth.

Heartwood and Extractives

The heartwood, in contrast to sapwood, is composed of essentially dead wood cells which no longer transport water, sap or nutrients. The heartwood goes through several changes during the process of cell death. A substantial loss of starch makes the heartwood less prone to microbial or insect attack because starch and free sugars, readily used by these invaders as energy sources, are no longer available. The loss of starch also results in a harder texture, enabling the heartwood to serve as part of the tree's structural support.

Wood extractives are also produced during the cell dying process. Extractives are natural, low molecular weight compounds products present in the wood cells apart from the cell wall components. They are extractable by either neutral organic solvents or water, hence the name extractives. Properties of extractives include protecting the wood from decay, increasing the strength of its texture, and enhancing color and odor (Rowe 1979). Odor is particularly important in repelling damaging insects. Extractives can be either a primary or secondary metabolite. Primary metabolites are biochemical compounds that are present in all living things, such as, simple sugars, amino acids, free fatty acids, etc. Secondary metabolites are more complex compounds, often species specific and not required for plant survival. Extractives are secondary metabolites common to all hardwood trees are phytosterols, simple terpenoids, phenylpropanoids, common flavonoids, simple tannins, and some coumarin type compounds. In the heartwood, these aromatic compounds are synthesized by biochemical condensation reactions from either acetate via malonyl CoA or directly from glucose via shikimic acid pathway as seen in Figure 2.1 (Higuchi 1976).

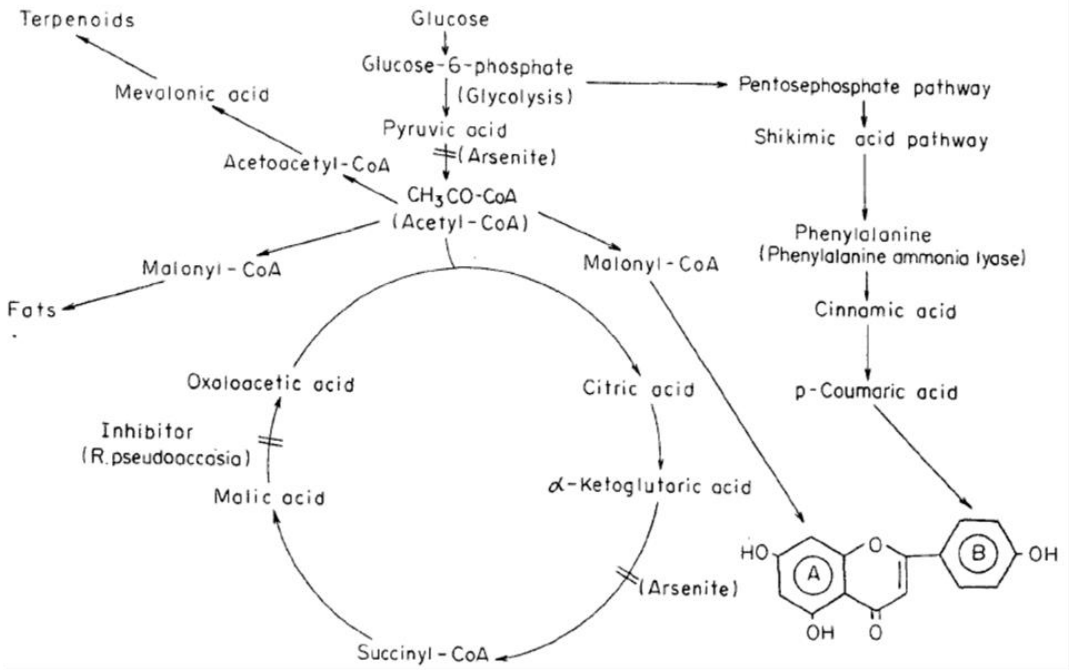

Figure 2.1: Biosynthetic pathway for heartwood extractive (Higuchi 1976).

These aromatics can include coumarins, ellagic acid, derivatives of cinnamic acid and p-coumaryl alcohol. Biosynthesis of the terpenoids, monoterpenes, diterpenes, sesquiterpenes and steroids, start with acetyl CoA and are formed from mevalonic acid as shown in Figure 2.2 (Umezawa 2001).

Artifacts can also be found in the extractives of wood. These are neither primary nor secondary metabolites, but are formed from autoxidation and non-enzymatic free-radical or acid-catalyzed condensations (Rowe 1979). Examples of artifacts include colored compounds, such as tannins. The darker color of the heartwood is a visible way of differentiating it from the sapwood (Taylor et al. 2002). Both the volatile extractives and the tannins are essential in flavoring distilled spirits during the barrel aging process.

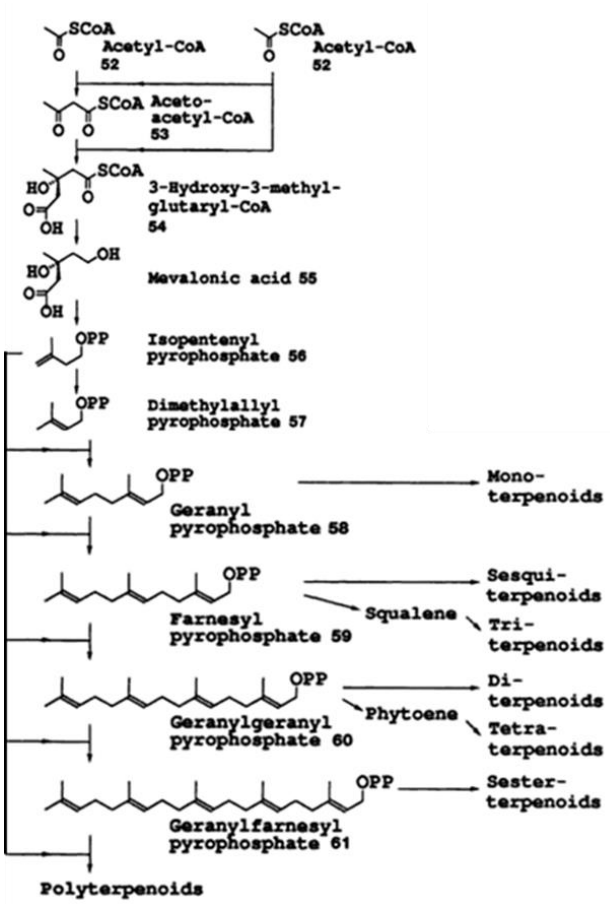

Figure 2.2: General scheme of terpenoid biosynthesis (with permission Umezawa 2001)

Oak Volatiles

Unlike other woods, oak is not used for its extractable oil. It does, however, contain a highly desired aroma, particularly for flavoring fermented and distilled beverages through the aging process. Since not all volatiles are aroma-active, only known odorants will be discussed in this review. A large majority of oak volatiles are formed during two essential steps of oak cask manufacturing, post seasoning and toasting (Figure 2.3).

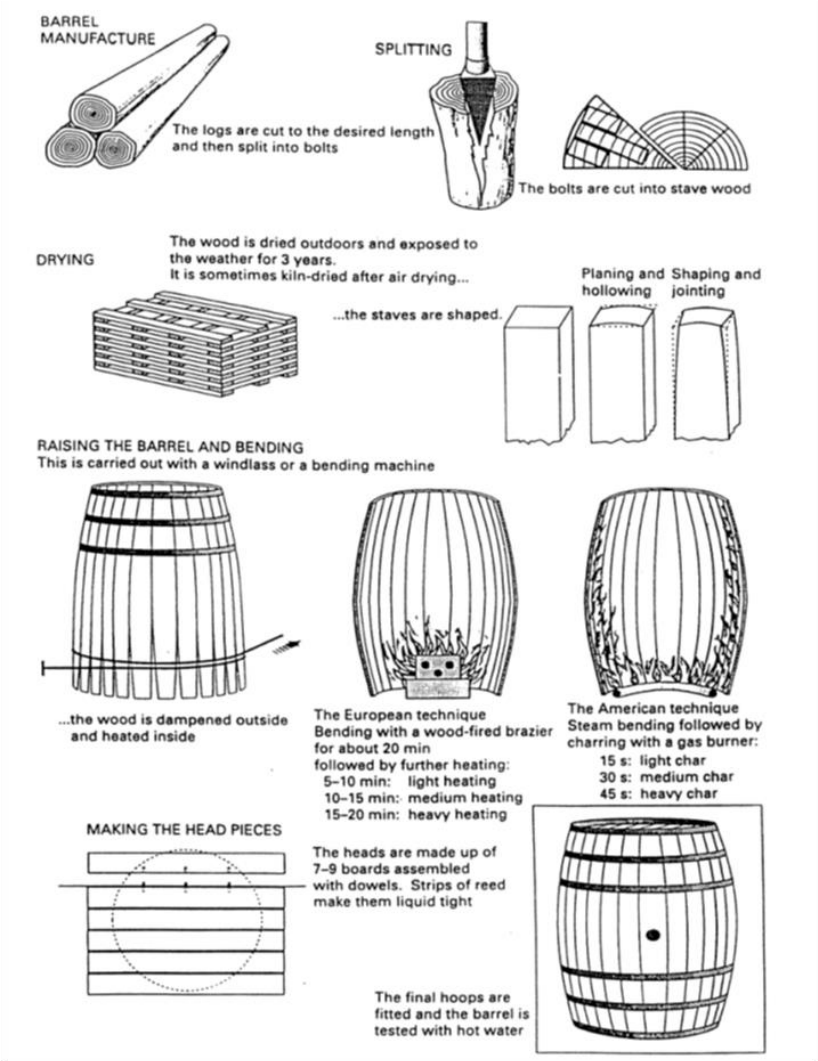

Figure 2.3: Manufacturing of oak casks (with permission Mosedale et al. 1998)

Seasoning is done by drying the cut wood, thereby equilibrating the moisture content to prevent further shrinkage or swelling. This process prepares the wood for toasting or burning and creates a higher concentration of volatiles due to loss of water from the wood. The toasting or charring step dramatically affects the volatile composition of oak through hydrothermolysis and pyrolysis reactions. Some of the more familiar aroma compounds derived from pyrolysis of the lignin during heating are shown in Figure 2.4. These include guaiacol, 4-ethylguaiacol, 4-vinylguaiacol (smoky compounds), eugenol and isoeugenol (spice or clove-like), syringol and syringaldehyde (sweet smoke), *p*-cresol (bandage), and vanillin (vanilla).

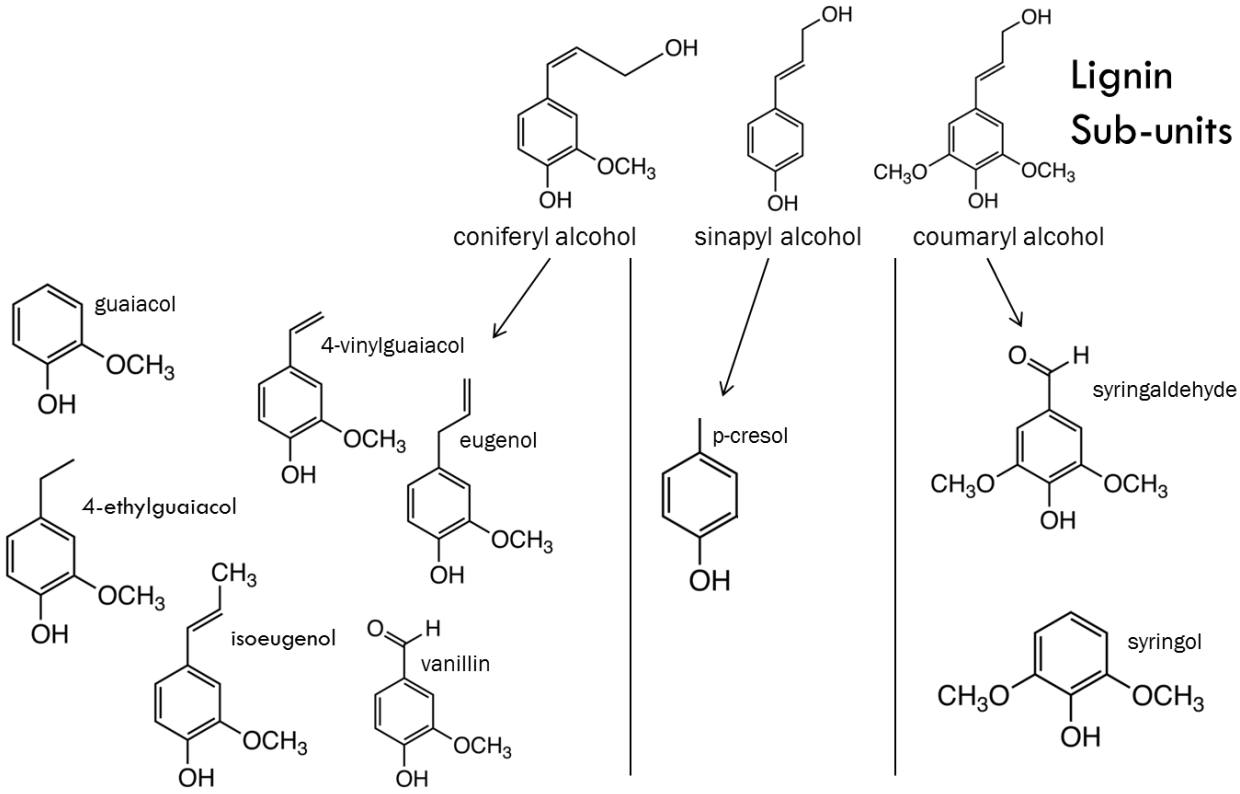

Figure 2.4: Aroma-actives formed from lignin

Other aroma compounds are present. Terpenoids such as linalool and thymol contribute by giving floral or fresh wood attributes. A unique characteristic of oak wood is the presence of carotenoids. Both β-carotene and lutein are precursors to

aroma compounds such as β-damascenone (cooked apple) and β-ionone (floral) (Chatonnet et al. 1998 and Alañón et al. 2009). Mailliard reaction type compounds arise from carbohydrates and proteins in the wood resulting in formation of furans: furfural (caramel), maltol (cotton candy), and furaneol (burnt sugar). Additional aldehydes, alcohols, and lactones are derived from lipids either by oxidation or rearrangement and include hexanal and nonenal (green), crotonolactone (buttery), butyrolactone (creamy), nonalactones and decalactones (fruity or peach-like). Most importantly to oak volatiles is *cis* and *trans*-β-methyl-γ-octalactone (coconut) shown in Figure 2.5 (Cutzach et al. 1997 and 1999, Doussot et al. 2002, Cadahia et al. 2003, Fernandez de Simon et al. 2009 and 2010).

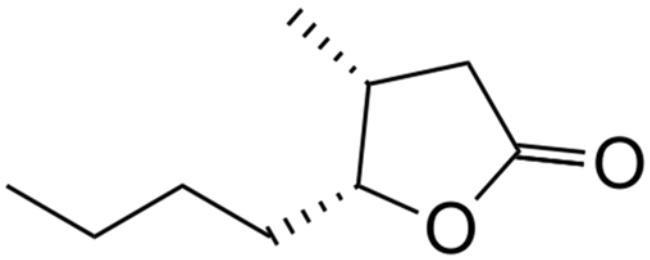

Figure 2.5: *cis*/*trans*-oak lactone

The lactones, c*is* and *trans*-β-methyl-γ-octalactone, were first discovered by Masuda and Nishimura (1971) in volatile extracts from oak wood, and were later discovered in distilled liquor (Otsuka et al. 1973). They are most famously known as "oak lactones", or "whiskey lactones", due to their importance in the aroma of oak wood. They lend a unique aroma to oak wood, and subsequently to whiskeys aged in oak barrels, confirming that the aroma-active compounds from oak wood can be directly transferred to the distilled spirit and have a noticeable impact on the flavor.

2.3 Distilled Spirits – Whiskeys

History

The history of barrel-aged distilled spirits naturally started with the invention of the wooden barrel. Wine can be traced back to as early as 6000 B.C. in Persia, modern day Iran, long before the invention of the wooden barrel. Originally wine resulted from the accidental spoilage of grapes. Over thousands of years, improvements in wine making were made by noting colors, varietals, and the effect of ripening trends, as well as many other cultivation techniques that influenced the characteristics of the final product. As the popularity of wine grew, the demand for exportation did as well. Clay pots (amphora) were the storage and transport vehicles for wines, despite their obvious flaws of cracking, breaking, and leaking. A less fragile option was needed, but it was neither the Romans nor the Greeks, both famous for manufacturing wine, that found a solution to this problem. Credit for that is given to the Celts (Preet 2012). Around 900 B.C. the Celts, then inhabiting the Danube River valley in central Europe, combined their woodworking and metallurgy skills to create the first wooden barrel, or cask. Not only was the barrel more efficient in storage and travel, but it was discovered that the wine underwent an unexpected change while in the barrel, with the barrel imparting a highly desirable flavor to the wine. It was found to be so desirable that even today barrel aging is used throughout the world though the need for the barrel to provide physical strength is obsolete. Thus the history of aged distilled spirits, in particular whiskey, stems from the invention of the wooden cask. When the Celts migrated to the Irish Isles, they found that the climate was unsuitable for the cultivation of grapes thus, an alternative starting material was found for the production of fermented beverages. Honey, which was readily available and also easily fermentable, replaced grapes and led to the production of mead (Preet 2012). It wasn't until the 12th century A.D. that ethanol distilled from fermented grains appeared in Irish monasteries. This beverage

was called *uisge beatha*, meaning "water of life" in Gaelic. Distillation first made an appearance in Spain and Portugal in the forms of Port, Sherry, and Madeira, which are wines to which distilled spirits are added. From there the practice of fermenting, distilling, and aging spread throughout the world (Fig 2.6). The Spanish settlers in the Americas brought the process of distillation to the indigenous people, who then distilled their fermented agave "beer" to create tequila and mescal. Rum, the product of sugarcane fermentation, made its first appearance after the British made their way to the Caribbean. Lastly, Tennessee whiskey and bourbon originated during the US colonial period when native corn was used as the starting material for these fermented beverages.

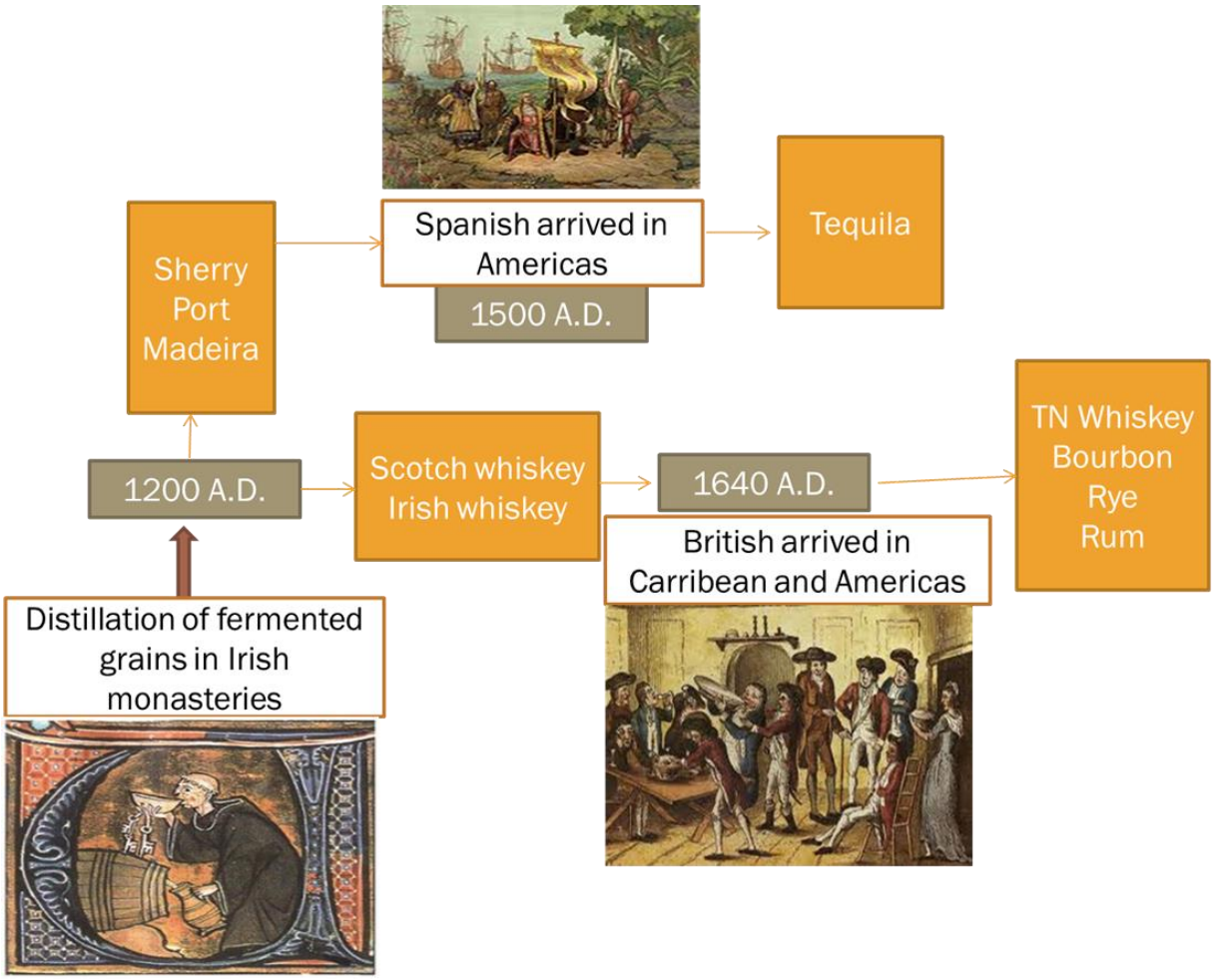

Figure 2.6: Iconographic timeline of aged distilled spirits

Manufacturing

There are three major steps in the production of distilled spirits. The first is fermentation. The starting or base material contains starch or sugars from which the fermented beverage is made. Whiskeys are generally made with grains, i.e. barley, rye, wheat, and/or corn, depending on the variety of whiskey being produced. Certain whiskeys, like bourbon, require a specific grain for production. By law, for a spirit to be called bourbon, the grain mixture must contain at least 51% corn by law (Code of Federal Regulations 2010). Tequila is made using syrup from the agave plant, while rum is made from molasses or sugar cane juice. Complex starches present in most of base materials require an extra step for yeast metabolism to occur. Yeast are only able to ferment simple sugars thus starch, a carbohydrate polymer, must be broken down into its component simple sugars. Malting is commonly used for this purpose. During the malting process grain is allowed to germinate during which enzymes (e.g. amylases) break down starch into its simple sugar sub-units. With the addition of yeast, commonly *Saccharomyces cerevisiae*, and sufficient water these free sugars are then fermented into ethanol (Figure 2.7). Each type of spirit is fermented for a specific period of time. For example, whiskeys are fermented for only 40 to 48 hours (Piggot at al. 2003).

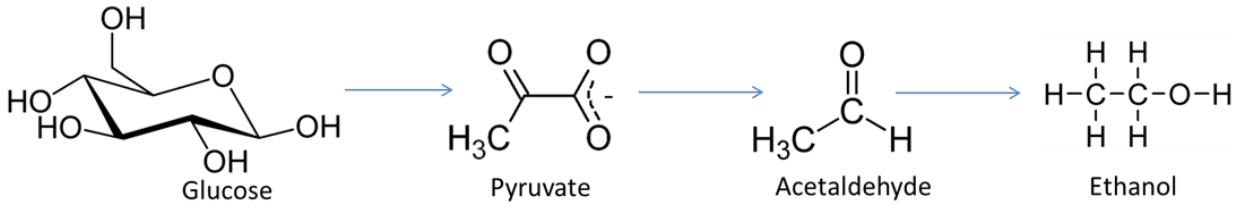

Figure 2.7: Yeast metabolism; Monosaccharide to ethanol

The second step in spirit manufacturing is distillation. Two methods exist, batch and continuous distillation, each with their own advantages. Batch or pot distillation typically results in a highly flavored spirit, whereas continuous distillation results in

lighter spirits. Batch distillation uses the pot, a swan neck vessel with a lyne arm, and a condenser, as shown in Figure 2.8. The fermented product or wash (5-7% ethanol) is transferred to the pot which is then heated, either directly by flame or indirectly by steam jacket. The pot is usually made of copper due to its good heat conduction and ability to remove unwanted odorous sulfur compounds (Whitby 1992). The lyne arm and condenser can be altered in length and orientation to obtain the desired % ethanol in the reflux which may affect flavor (Nicol 1989). This process is repeated in batches until a final ethanol content of 70-80% is obtained. Continuous distillation was invented by Aeneas Coffey in 1827 for the production of scotch whiskey (Piggot et al. 2003). In this process, shown in Figure 2.9, the wash is preheated by sending it through the second column (rectifier) and then fed into the top of the first column (analyzer), while steam enters at the base. In contrast to batch distillation, the fermentation liquid is continuously fed into the process. The volatiles are stripped from the wash and taken out from the top of the rectifier while the vapor returns to the bottom where water and alcohol are separated (Piggot et al. 2003).

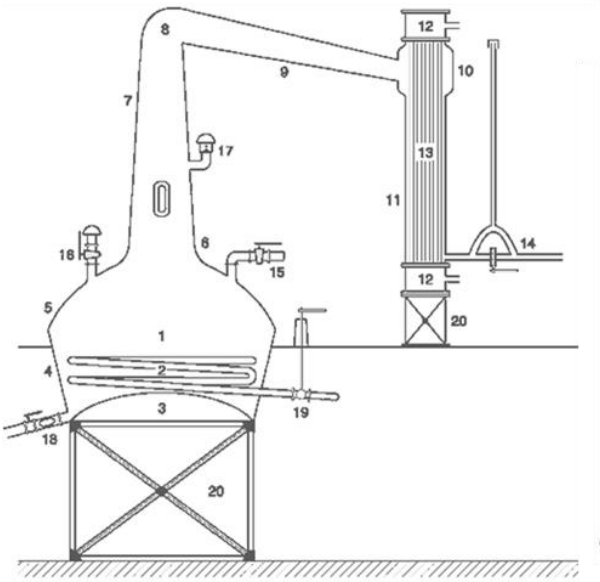

Figure 2.8: Batch distillation diagram (with permission Nicol 1989)

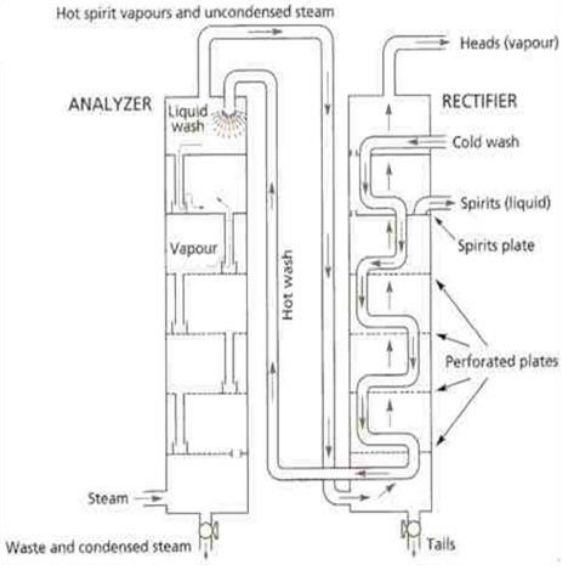

Figure 2.9: Continuous distillation diagram

Maturation, the final step, is generally considered to be the most important step for the overall flavor of aged spirits. Raw distilled whiskey has a very harsh, undesirable flavor. However, aging in white oak casks creates a product with desirable flavor characteristics. Prior to aging the distillate is cut with water to achieve an alcohol by volume (ABV) content of 60.0-62.5 percent. Standards of identity for Tennessee whiskey and bourbon require the distillate to be aged in new, charred, white American oak barrels, which is thought to result in the finest of whiskeys. Aging time is also controlled by law for Tennessee whiskey and bourbon, with a minimum time in barrel being at least two years (Waymack et al. 1995). Corn whiskey is not required to be aged and is better known as "moonshine". If it is aged, it is done briefly for six-months, either in un-charred or used oak casks. Whiskeys produced outside of the USA have their own standards. Canadian, scotch, and Irish whiskeys all require a minimum of three years aging in an oak barrel, however, the barrels are not required to be new. Both scotch and Irish whiskeys don't rely solely on oak aging for their flavor, as the malted grain is treated with peat smoke before fermentation, giving them their recognizably intense smoky aroma.

Flavor of Whiskeys

There are two major contributors to the flavor of whiskey, the starting grain and any subsequent treatment post distillation, i.e. oak aging. The starting grain, which is barley, corn, wheat or rye, should result in a fairly aroma neutral fermentation product. During the malting process Maillard reaction type volatiles are formed, resulting in a fermentation product that is very similar to an un-hopped beer (Cole et al. 2003). For Irish and scotch whiskey, the peating process on the malted grains adds a "smoky" or "peaty" flavor to the final product. Many of these "smoky" compounds are the same as the ones that come from the post distillation treatment of barrels, specifically from the charring of the oak. These include phenols, cresols, and guaiacols; such as syringol and

syringaldehyde, guaiacol, 4-ethyl guaiacol, 4-vinyl guaiacol, p-cresol, o-cresol, and m-cresol (Piggot et al. 2003, Poisson et al. 2008a, Fernanadez de Simon et al. 2010). This is likely the reason why Irish and scotch whiskeys don't require aging in new charred oak barrels, as their distillate already has a higher amount of these compounds that contribute to the "smoky" or "peaty" flavor. The aroma impacting compounds resulting from grain fermentation are fusel alcohols, acetates, and esters. These compounds, which impart a fruity or solvent-like characteristic, include: 2-methylbutan-1-ol, 3-methylbutan-1-ol, 2-methylbutyl acetate, acetaldehyde, isoamyl acetate and, 2-methyl-1-propanol.

Post-distillation oak aging is considered to be the most important step in developing the flavor of whiskey. The volatiles can come from three different sources: 1) ethanolysis of wood components, 2) lignin pyrolysis from charring the barrel and 3) direct extraction of wood volatiles. These have a wide range of odor descriptions, creating the complex flavor of whiskeys. The oak lactones, *cis*- and *trans*-β-methyl-γ-octalactone, are of particular importance, even being nicknamed "whiskey lactones". Other lactones present, including γ-nonalactone, δ-nonalactone, γ-decalactone, γ-dodecalactone and *cis*-6-dodeceno-γ-lactone contribute peachy and creamy flavors. Additional wood extractives known to contribute to the flavor include: eugenol, isoeugenol, β-ionone, β-damascenone and vanillin. Both eugenol and isoeugenol are described as clove-like, while β-ionone and β-damascenone impart floral and apple attributes. Vanillin is most commonly known to contribute most to the flavor of the vanilla bean. Lastly, ethanolysis of the wood acids results in fruity ethyl esters like ethyl propanoate, ethyl butanoate, ethyl hexanoate, and ethyl octanoate, along with several other branch chain ethyl esters (Conner et al. 1993, Conner et al. 2001, Piggot et al. 2003, Poisson et al. 2008a, Poisson et al. 2008b, Lahne 2010).

The importance of non-volatile, taste-active compounds extracted from the wood during maturation was recently studied with respect to both in-mouth flavor and

aroma-by-nose. These were identified as the ellagitannins: vescalagin, castalagin, grandinin, roburin A, B, C, D, and E, and 33-deoxy-33-carboxyvescalagin. These compounds contribute to astringency and bitterness along with matrix type effects. As a result of these findings, a procedure to deodorize whiskey became widely practiced. This procedure forms a non-biased base for recreating aroma models that include the taste-active ellagitannins which could be a major influence on the sensory attributes (Glabasnia et al. 2006).

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Chapter 3: Identification of Rotundone, a "Woody/Incense" Aroma Component of Oak Wood Casks

3.1 Abstract

Experiments were conducted in order to unambiguously identify a compound responsible for a "woody/incense" odor note in oak aged spirits. The target compound was isolated by rigorous simultaneous distillation extraction (SDE) of oak wood chips followed by several purification steps, as well as use of a custom built GC-MS/olfactometry system equipped with a heart-cutting system/internal CryoTrap (Deans' switch system), which enabled the acquisition of an interpretable electron-impact mass spectrum (EI-MS) for the compound. The EI-MS revealed the unknown compound possessed a molecular weight of 218; however, there was no match for the compound in the NIST database. A thorough investigation of naturally occurring organic compounds having a molecular weight of 218, plus additional experiments to determine the nature of any functional group(s) on the molecule, indicated numerous compounds as possible candidates. Most were found to occur naturally in a number of roots, spices, oils, and herbs, which were subsequently analyzed. Results of the analyses revealed that the compound was most likely the sesquiterpene compound 5-isopropenyl-3,8-dimethyl-3,4,5,6,7,8-hexahydro-1(*2H*)-azulenone, or rotundone. This presumptive identification was confirmed by comparison of the compound's EI-mass spectrum and GC retention indices on three different polarity phases against those of authentic rotundone obtained by chemical synthesis.

3.2 Introduction

Oak wood has been selected as the wood of choice for barrel making not only for its physical characteristics that lend itself to manufacturing a barrel, but also its unique

chemical properties that impart key flavors to aged spirits that are desired by the consumer.

Volatiles from oak wood can either be naturally present in the wood, or formed during post-harvest treatment. A common practice is to toast or char the wood, which is done to seal the wood, but this practice also creates hundreds of volatiles. These volatiles come from several sources. Lignin pyrolysis results in many phenolic compounds contributing to the aroma of toasted oak including: guaiacol, 4-methyl guaiacol, 4-vinyl guaiacol, eugenol, isoeugenol, syringol, vanillin, and syringaldehyde (Fernandez de Simon et al. 2009 and 2010, Cutzach et al. 1997 and 1999, Cadahia et al. 2003, Doussot et al. 2002.) Reactions with the lipids and carbohydrates present in oak result in the formation of a number of aldehydes, alcohols, esters, furans, lactones and most importantly *cis* and *trans*-β-methyl-γ-octalactone, endearingly called the "oak lactone" or "whiskey lactone" (Masuda et al. 1971 and Otsuka et al. 1973). Degradation of carotenoids to form terpenoids is unique to oak wood as oak contains both β-carotene and lutein which break down to form volatiles such as, β-ionone, β-damascenone, dihydroactinolide, and megastigmatrienones (Nonier et al. 2004 and Sefton et al. 1990). As thoroughly researched as oak wood and oak aged spirits are, the identity of the component responsible for the "woodiness" flavor attribute of age spirits is unknown. Previous research cited the presence of an unknown compound with a "woody/incense" aroma character, and mentioned that additional research was needed to identify it. Thus, the main objective of this study was to find and identify this unknown "woody/incense" compound, herein referred to as the target compound. It was hypothesized that this compound exists in both oak wood and in oak aged spirits potentially influencing the overall flavor of the spirit.

3.3 Materials and Methods

Materials

Toasted oak was purchased from Oak Chips Inc. (Waverly, OH). These included white American oak in light and medium toast levels and at two different sizes (chips and powder), and French oak (medium toast, chips). Commercially available oak wood extracts were purchased from commercial sources: Sinatin 17 (Crosby & Baker Ltd. (Westport, MA) and liquid oak extract (RJ Spagnols, Delta, BC, Canada.). *Cyrpus rotundus* "whole herb" (dried root) was purchased from Chinese Herbs Direct (Torrance, CA). Ground white peppercorn, *Piper nigrum,*(Spice Islands Trading Co., San Francisco, CA) was purchased from a local grocery store (Champaign, IL). Two samples of agarwood oil were provided by Orchidia Fragrances® (Downers Grove, IL).

Chemicals

The following chemicals used for volatile extraction, isolation, and chemical synthesis were purchased from Fisher Scientific Co. (Fair Lawn, NJ): dichloromethane, n-pentane, diethyl ether, sodium hydroxide, sodium bisulfite, sulfuric acid (conc), ethanol (99%)(Arcos Organics, Morris Plains, NJ), acetone, hydrochloric acid (concentrated), and sodium sulfite.

The following chemicals used for isolation and chemical synthesis were purchased from Sigma-Aldrich Co. (St. Louis, MO): 2,4-dinitrophenylhydrazine, Dess-Martin periodinane, guaiac wood oil, pyridine, thionyl chloride, acetonitrile, cobalt acetate tetrahydrate, *tert*-butyl hydroxide (5.0-6.0 M in decane), and silica gel (high-purity grade, 60A, 230-400 mesh particle).

Methods

Isolations of volatiles for GC-O analysis

Oak wood was isolated by simultaneous distillation-solvent extraction (SDE, Chrompack, Middelburg, Netherlands) as described by De Frutos (et al. 1988). Light and medium toasted American white oak chips (100 g) were added to a 1-L round bottom flask containing 500 mL of odor-free distilled-deionized water. Dichloromethane (50-75 mL) was used as a non-polar extraction solvent. Extraction was conducted for 3 h (reflux time). The extract was dried over anhydrous sodium sulfate and concentrated to 1 mL using a Vigreux column (45 °C) followed by further condensation of the extract using a gentle steam of N_2 gas.

The extract obtained from SDE of the oak wood was loaded onto a cooled (7 °C) jacketed glass column (45 cm x 1.5 cm) filled with silica 60A (pre-baked and then equilibrated with 5% w/w water post bake) in n-pentane. Under pressure (using N_2 gas at 1 psi), the extract was fractionated by polarity using five pentane : diethyl ether mixtures (v/v) (150 mL each; 100:0 (A), 95:5 (B), 90:10 (C), 80:20 (D), and 50:50 (E) as described by Poisson and Schieberle (2008a). Each fraction was dried over anhydrous sodium sulfate and concentrated to 0.5 mL using a Vigreux column followed by a gentle stream of N_2 gas.

Identification of aroma active volatiles

SDE extracts were subjected to evaluation by both gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS). The retention index (RI) was calculated for each aroma compound by comparing its retention time (RT) to those of standard n-alkanes (Van der Dool and Kratz 1963). Aroma-active compounds were identified based on three criteria 1) comparison of RIs on three different stationary columns (RTX-5, wax, and 1701) to that of literature values, 2) comparison of a compound's odor properties to published values, and 3) comparison of the electron ionization (EI) mass spectrum obtained by GC-MS analysis to those in the National Institute of Standards and Technology (NIST) database.

Gas Chromatography – Mass Spectrometry (GC-MS)

A 6890 GC-HP 5973N mass selective detector (Agilent Technologies Inc., Palo Alto, CA) was used for GC-MS analysis. Two μL of extract was injected into a cold splitless inlet CIS-4 inlet (Gerstel, Germany) held at -50°C for 0.10 min, then increased to 260°C at a rate of 12°C/sec. Separations were performed using a Supelco® SAC-5 column (30.0 m length x 0.25 mm i.d. x 0.25 μm film thickness; Sigma, St. Louis, MO) or Stabilwax® (30.0 m length x 0.25 mm i.d. x 0.25 μm film thickness; Restek. Helium was used as the carrier gas at a constant flow of 1.0 mL/minute. MS transfer line temperature was 280°C. Oven temperature was programmed as follows: initial temperature, 40°C (5 min hold), ramp rate, 4 °C/min, final temperature, 225 °C (45.0 min hold). The MSD conditions were as follows: capillary direct interface temperature, 280 °C; ionization energy, 70 eV; mass range, 35 to 300 amu; electron multiplier voltage (Autotune + 200 V); scan rate, 5.27 scans/s.

Gas Chromatography – Olfactometry (GC-O)

The GC-O system used for analysis of extracts consisted of a 6890 GC (Agilent Technologies Inc.) equipped with an flame ionization detector (FID) and sniff port (OD2, Gerstel, Germany). Separations were performed using a RTX®-Wax column (15 m length x 0.53 mm i.d. x 1 μm film thickness; Restek), RTX®-5 column (15 m length x 0.53 mm i.d. x 1μm film thickness; Restek) and RTX®-1701 (15 m length x 0.53 mm i.d. x 1 μm film thickness; Restek). Helium was used as the carrier gas at 5 mL/minute. FID temperature was 250°C. Oven temperature was programmed as follows: initial temperature, 40°C (5 min hold), ramp rate 8^0C/min, final temperature, 225°C (30 min hold).

Isolation of volatiles for unknown identification

Oak

Volatiles in oak wood were isolated by SDE as previous described with some modifications to the procedure. Oak samples, received pre-ground, were finely ground

using a Thomas Wiley® Mini Mill (Thomas Scientifict, Swedesborro, NJ) before addition of 500 g into a 5000 mL round bottom flask contaqining 2000 mL of odor-free DI water. Dicholoromethane (200 mL) was used as the extraction solvent. Extraction was conducted for 6 h (reflux time). The extract was dried over anhydrous sodium sulfate and concentrated to 1 mL using a Vigreux column (45 °C) followed by further concentration using a gentle stream of N_2 gas.

The extract obtained from oak wood was washed with 1M NaOH (3x 50 mL) to remove acids and phenolics before it was loaded onto a water-cooled glass column (45 cm x 1.5 cm) filled with silica 60A (pre-baked at 180° C, with 5% w/w water added post bake) in n-pentane to a height of 23 cm in the column. Under pressure, using N_2 gas (1 psi) in the flash column, the extract was fractionated by polarity using a succession of five 50 mL pentane:diethyl ether mixtures; 100:0, 90:10, 85:15, 80:20, 75:25, 50:50) Fractions possessing a woody/incense-like aroma detected by GC-O were collected and pooled. This flash column procedure was repeated four times to obtain an extract from 2 kg of oak wood.

Cyperus rotundus

The volatiles present in *C. rotundus* were extracted by SDE in the same manner as described above using a 25 g ground sample and 10 mL of dichloromethane. Fractionation was performed using the flash column method described above. Fractions having a woody/incense-like aroma were pooled and reduced to 1 mL for analysis.

White pepper

White peppercorn (pre-ground) was subjected to simple solvent extraction by placing 5g in a 50 mL test tube with 25 mL of diethyl ether and sealed with a PTFE cap. The prepared test tube was shaken for 1hr (DS-500 Orbital Shaker, VWR Scientific Products). After centrifugation, the solvent layer was drawn off, 25 mL of diethyl ether was added and a second extraction was performed. The extracts were pooled, washed

with 1 M NaOH (20 mL x 3) to remove acids and phenolics and then condensed to 1 mL for analysis.

Wood oils and extracts were analyzed directly (without extraction) since they were already in a suitable form.

Identification of target compound

GC-MS/GC-O Deans' switch system

A custom built gas chromatograph equipped with a Deans' switch heart-cutting system containing a CryoTrap on the cut section and a switching valve to direct flow to either the mass spectrometer or the olfactometer was used to selectively analyze for the target compound. The entire system consisted of an 6890 GC (Agilent Technologies Inc.) equipped with an FID and sniff port (OD2, Gersel), a 5973N mass selective detector (MSD, Agilent Technologies Inc.), a Deans' switch (Agilent Technologies Inc.), a JAS CryoTrap (Joint Analytical Systems; Newark, DE), and an Air and Electrically Actuated 2 Position Valve (Valco Instruments Co. Inc., Houston, TX.).

A schematic of this system is shown in Figure 3.1.

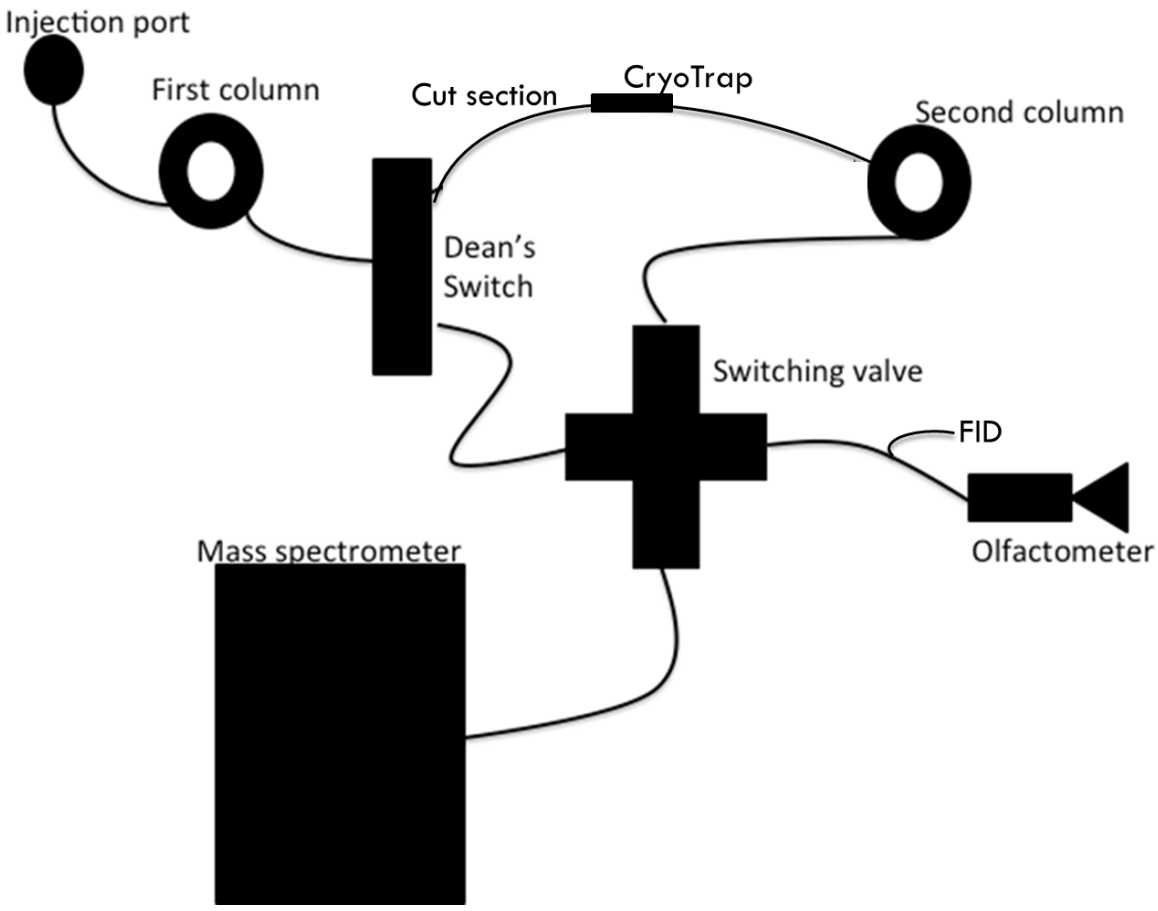


Figure 3.1: Schematic of GC-MS/O/FID system with Deans' switch and CryoTrap

In initial analyses, the volatiles were allowed to bypass the cut valve and flow directly to the olfactory port in order to determine the retention time and retention index (RI) of the target odorant with respect to the standard alkanes. This allowed the "cut" command to be programmed into the run at the correct time interval. For the target odorant the cut was made between an RI of 2200 – 2300 (Wax column), RI of 1700 – 1800 (5 column) and RI of 1800 -1900 (1701 column). Cuts were made onto columns of different polarities from the first column in order to obtain orthogonal chromatographic resolution. After samples were cut from the proper RI section to the MS, the valve was then switched for a second run to send the cut section to the olfactory port. The cut section sent to the olfactory port was sniffed and the retention time for the target odorant was marked on the chromatogram using a hand-held input device. The total

ion chromatogram (TIC) of the cut section from the MSD and the FID outputs were overlaid and the mass spectral data were evaluated for the target peak. The entire experiment was repeated several times using three different types of column and six different column configurations in order to unambiguously match the correct mass spectrum with the target compound. The Deans' switch requires specific pressure settings to ensure that no leaking occurs to the cut section and no back leaking occurs during the cut to the first column. This was adjusted through the front inlet pressure and a second pressure gauge installed which controls flow to the Deans' switch. Pressures were also adjusted depending on whether the valve was directed towards the olfactory port, or to the MSD. The pressures used are shown in Table 3.1 where valve position A directs the first column to the FID/O; cut section to the MS and position B directs the first column to the MSD; cut section to the FID/O.

Table 3.1: Pressure Settings for Deans' switch/CryoTrap system

Configuration	Wax (1^{st}) → 5 (cut)		5 (1^{st}) → Wax (cut)		5 (1^{st}) → 1701 (cut)	
Valve position	A	B	A	B	A	B
Front inlet pressure (psi)	60.0	37.0	60.0	37.0	60.0	57.0
Deans' switch pressure (psi)	57.7	27.0	57.7	28.0	61.2	50.0

Oven temperatures were programed with three ramps; the first functioned to control the oven during chromatography through the 1^{st} column, the second as an oven cool-down time while the cut section was trapped in the cryotrap, and the third functioned as to control the oven temperature during chromatography through 2^{nd} column. The first ramp consisted of an initial temperature of 50 °C (1 min hold), ramp rate 10 °C /min, final temperature 225 °C [1.95 min hold [5→wax (A)], 0.70 min hold [5 → wax (B)], 0.00 min hold [5 → 1701 (A)], 1.16 min hold [5 → 1701 (B)], 5.30 min hold [wax → 5 (A)],

2.50 min hold [wax → 5 (B)]. The second, cool-down ramp was 25 °C/min to a final temperature of 50 °C (2.00 min hold time). The third ramp consisted of an initial temperature of 50 °C (0.00 min hold), ramp rate 4 °C/min, final temperature 225 °C (20.00 min hold). The cryotrap was kept at 0 °C until the oven cooled down, both the third oven ramp and the cryotrap ramp started at the same time with the cryo ramp rate of 500 °C/min, final temperature of 260 °C (10.00 min hold).

Compound chemical characteristics

The presence and nature of functional groups were determined through a series of experiments designed to detect the presence of an aldehyde, ketone, or alcohol group on the target compound.

Reaction with sodium bisulfite

A 2 mL portion of the target compound fraction in dichloromethane solvent was washed with a 25% aqueous solution of sodium bisulfite to generate a water-soluble, addition product of carbonyls and bisulfite (Benn 1998). The solvent layer was then subjected to GC-O analysis to determine whether the target aroma was still present in the solvent phase. This indicated whether a carbonyl functional group was a moiety on the unknown compound.

Reaction with 2,4-dinitrophenylhydrazine

Another method to detect the presence of aldehydes or ketones made use of 2,4-dinitrophenylhydrazine (Allen 1930), where the a carbonyl group will react with 2,4-DNPH to form a solid. The 2,4-dinitrophenylhydrazine solution was prepared by first dissolving 4 g of 2,4-DNPH in 15 mL of concentration sulfuric acid then, with stirring, 20 mL of water was added, followed by 70 mL of 95% ethanol. A solution of 0.5 mL of 2,4-DNPH was then added to 2 mL of the target compound fraction and allowed to react for 10 min. This was quenched with water and the solvent layer was drawn off and subjected to GC-O analysis.

Reaction with Dess-Martin Periodinane

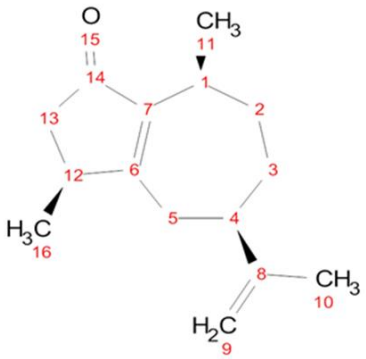
The Dess-Martin reaction is an effective method of oxidizing alcohols into aldehydes as described by Meyer et al. (1994). Briefly, water saturated dichloromethane was added, while stirring, to a mixture of 2 mL of the target compound fraction and 4 mL of DMP (0.3M in dicholormethane) (1.2 mmol). This was left to react overnight until all alcohols were oxidized to the aldehydes. The reaction was worked up and the solvent layer was subjected to GC-O analysis.

Compound Synthesis

Rotundone

The synthesis of rotundone was performed following the method of Mattivi et al. (2010) starting with the sesquiterpenoid alcohol, guaiol. Guaiol was isolated from guaic wood oil following a series of crystallization steps; first using acetone as the solvent followed by a 3:1 ethanol:water mixture (Minnaard et al. 1994). This yielded 99% pure guaiol as a crystaline material. The guaiol (10.0 g; 81 mmol) in pyridine (15 mL) was kept at -30°C under N_2 gas while thionyl chloride (6.18 g; 3.8 mL; 52 mmol) was added drop-wise. After 2 h, additional thionyl chloride (1.55 g; 0.95 mL; 13 mmol) was added. The resulting solution was kept overnight at 30°C. The product, a brown solution with white crystals, was worked up by adding a 25% HCl solution (50 ml) and extracting with diethyl ether. This resulted in a crude mixture of guaiene (87% yield). The crude mixture was passed through a bed of silica gel to remove any insoluble compounds. It was then dissolved in acetonitrile and the catalyst cobalt acetate tetrahydrate (0.60 g) was added along with an oxidizing agent, *tert*-butyl hydroxide (5.0-6.0 M in decane). The solution was left to react at room temperature. The reaction progress was monitored hourly by GC-MS until the guaiene peak was no longer detected. The reaction was quenched with and then washed with aqueous sodium sulfite (1 M) solution and extracted with ether. The ethereal extract contained the final product, rotundone, with a final yield of 8%. A detailed description of the synthesis can be found in the Appendix A.

Purification of rotundone was carried out by first subjecting the extract to a high vacuum transfer apparatus to remove any highly volatile compounds and then applying the extract it to a silica (60A) flash column (200 mL volume). Fractions fromvarying pentane:diethyl ether (v/v) mobile phase compositions (100:0, 94:6, 90:10, 80:20) were collected. Using this purification procedure, a 94% purity was achieved. EIMS, m/z (rel intensity) 219 (14), 218 ([M]$^+$, 100), 204 (8), 203 (82), 189 (9), 175 (25), 163 (46), 162 (35), 161 (55), 147 (53), 137 (59), 135 (25), 133 (42), 121 (32), 120 (40), 119 (61), 107 (38), 105 (67), 95 (34), 93 (43), 91 (87), 79 (53), 77 (53), 67 (48), 55 (29), 41 (33). Retention indices (RI) 1670 (RTX-1), 1715 (RTX-5), 1885 (DB-1701), 2260 (RTX-wax).



^{1}H NMR (500 MHz, $CDCl_3$), δ 4.75 – 4.60 (2H, overlapping m, H_{10}), 2.98 (1H, ddq, J = 11.2, 3.4, and 7.3, H_1), 2.64 – 2.42 (3H, overlapping m, $H_{12, 13a, 5a}$), 2.34 (1H, m, H_{6b}), 2.05 – 1.9 (2H, overlapping m, $H_{4, 13b}$), 1.81 – 1.75 (3H, overlapping m, $H_{3, 2a}$), 1.76 (3H, s, H_9), 1.53 (1H, m, H_{2b}), 1.10 (3H, d, J = 7.5, H_{16}), 0.99 (3H, d, J = 6.4, H_{11})

^{13}C NMR (500 MHZ, $CDCl_3$), δ 208.2, 177.2, 151.2, 145.6, 109.3, 46.4, 43.2, 38.1, 36.9, 32.8, 30.9, 27.0, 20.4, 19.4, 17.7.

^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (500 MHZ, $CDCl_3$) was obtained from the solutions in deuterochloroform with a Varian Unity (500 MHz, Quad Probe; Varian, Palo Alto, USA). Chemical shifts were referenced relative to the corresponding residual solvent signal. ^{13}C NMR spectra were obtained from the solutions in a deuterochloroform with a Varian VRX (500 MHz, Quad Probe).

The EI-MS and NMR spectra can be found in the Appendix B.

3.4 Results and Discussion

Volatile Identification

Extracts were prepared from both toasted French and American oak products via SDE. Based on the results of GC-O American oak had the stronger intensity of most characteristic oak odorants (Appendix E, Table A1). This finding was consistent with previous studies which indicated that toasted American oak has higher concentration of characteristic oak volatiles than toasted French oak (Cadahía et al. 2003). A comparison was also made between light and medium toasted American oak chips, revealing volatiles to be in higher abundance the medium toasted oak. Continuing with medium toasted American oak, GC-O analysis consistently allowed for the detection of 40 odor-active compounds (Table 3.2). All of the identified aroma compounds were in agreement with previous studies on volatiles of oak wood (Alañon et al. 2009, Fernández de Simón et al. 2009, Cadahía et al. 2003, Chatonnet and Dubourdieu 1998, Cutzach et al. 1997).

Of the 40 compounds detected, 7 were unknown including 2 (bolded, Table 3.2), which were described as "woody/incense". Other unknown compounds that may be of some interest imparted a "clove/pine" odor at RI 2099 (Wax column) and a"fresh wood/apple" odor at RI 2324 and 1463 (Wax and 5 columns, respectively).

Not all of these compounds will necessarily impact the flavor of the aged spirits. This may be due to how susceptible a compound is to ethanolic or aqueous extraction, or it might be present at a lower concentration that its detection threshold. Based on previous studies on aroma active compounds in aged spirits (Lahne et al 2012, Benn at al 1996, and Netto et al 2003), as well as the findings of this study, it was confirmed that

only the unknown compound [occurring at RIs of 2250 (Wax column), 1885 (1701 column), and 1722 (5 column)] was present as odor-active in oak aged spirits and thus qualified to proceed with identification. The GC-O analysis of the wood served to confirme that the target compound originates directly from the oak wood and that the target compound when extracted from the oak wood would be in a high enough concentration to be detectable by GC-MS since the oak wood extract will contain more volatiles than aged distilled spirits.

Table 3.2: Odor Active Compounds Extracted from Jim Beam Bourbon Aged 8 years

No.	Compound	Odor Description	RI		Identification[a]
			Wax	Rtx5	
1	ethyl propanoate	fruity/green		726	RI, O, MS
2	ethyl butanoate	fruity		808	RI, O, MS
3	ethyl isovalterate	ester fruity	1055	857	RI, O, MS
4	hexanal	green	1083	803	RI, O, MS
5	ethyl valerate	fruity	1129	890	RI, O, MS
6	ethyl 3-hydroxybutanoate	sweet	1522	928	RI, O, MS
7	ethyl hexanoate	fruity		1001	RI, O, MS
8	3-methyl-1-butanol	liquor	1211	736	RI, O, MS
9	heptanal	stale green	1243	965	RI, O
10	4-ethyl phenol	bandaid		1172	RI, O, MS
11	unknown	cooked/nutty	1307		O
12	ethyl octanoate	fruity	1420	1197	RI, O, MS
13	unknown	sour/mayonaise	1438		O
14	*trans*-2-nonenal	green hay	1530	1161	RI, O, MS
15	unknown	sweet citrus	1549	1199	O
16	*trans,cis*-2,6-nonadienal	green	1581	1150	RI, O
17	butyric acid	sweaty/cheesey	1615		RI, O
18	isovaleric acid	cheesy	1658		RI, O
19	*trans,trans*-2,4-nonadienal	hay	1694	1217	RI, O, MS
20	2-decenal	sawdust		1255	RI, O, MS
21	2-phenylethyl acetate	floral tea	1805	1260	RI, O, MS
22	β-damacenone	apple/lemon	1811	1391	RI, O, MS
23	guaicol	smokey/sweet	1840	1087	RI, O, MS
24	*trans*-whiskey lactone	coconut	1871	1295	RI, O, MS
25	phenylethyl alcohol	rose	1901	1120	RI, O, MS
26	β-ionone	floral/earthy	1904	1418	RI, O, MS
27	*cis*-whiskey lactone	coconut/sunscreen	1935	1329	RI, O, MS
28	γ-nonalactone	peach	2009	1368	RI, O, MS
29	*p*-cresol	burnt	2094	1111	RI, O, MS
30	eugenol	brown spice	2163	1361	RI, O, MS
31	*p*-vinylguaiacol	spice/curry	2171	1319	RI, O, MS
32	syringol	smokey bbq	2236	1355	RI, O, MS
33	ethyl cinnamate	spice		1466	RI, O
34	***unknown***	**woody/incense**	**2253**		**O**
	unknown	**woody/incense**		**1724**	**O**
35	unknown	fresh wood/apple	2322		O
36	*cis*-6-dodeceno-γ-lactone	cilantro	2377	1665	RI, O, MS
37	unknown	vanilla	2488		O
38	vanillin	vanilla/marshmellow	2519	1413	RI, O, MS
39	ethyl vanillate	brown spice/cinnamon	2600		RI, O, MS

[a] Identifcation criteria: rentention index (RI), odor quality (O), mass spectra (MS)

GC-MS/O/FID using Deans' switch system

To obtain an interpretable electron-impact mass spectrum (EI-MS) of the target compound, the RI range of the unknown compound was cut both to a Olfactory port/FID and a mass spectrometer. Figure 3.2 shows an example, in the RTX-wax (1st column) → RTX-5 (2nd cut section column) configuration, of comparing the two sections to find a peak corresponding to the odor. During the GC-O analysis of the cut section the time that the correct target compound aroma was detected was marked using an Olfactory Intensity Device (Gerstel USA Inc., Linthicum, MD).

This was repeated several more time using the RTX-5 → RTX-wax configuration (Figure 3.3) and the RTX-5 → RTX-1701 configuration (Figure 3.4).

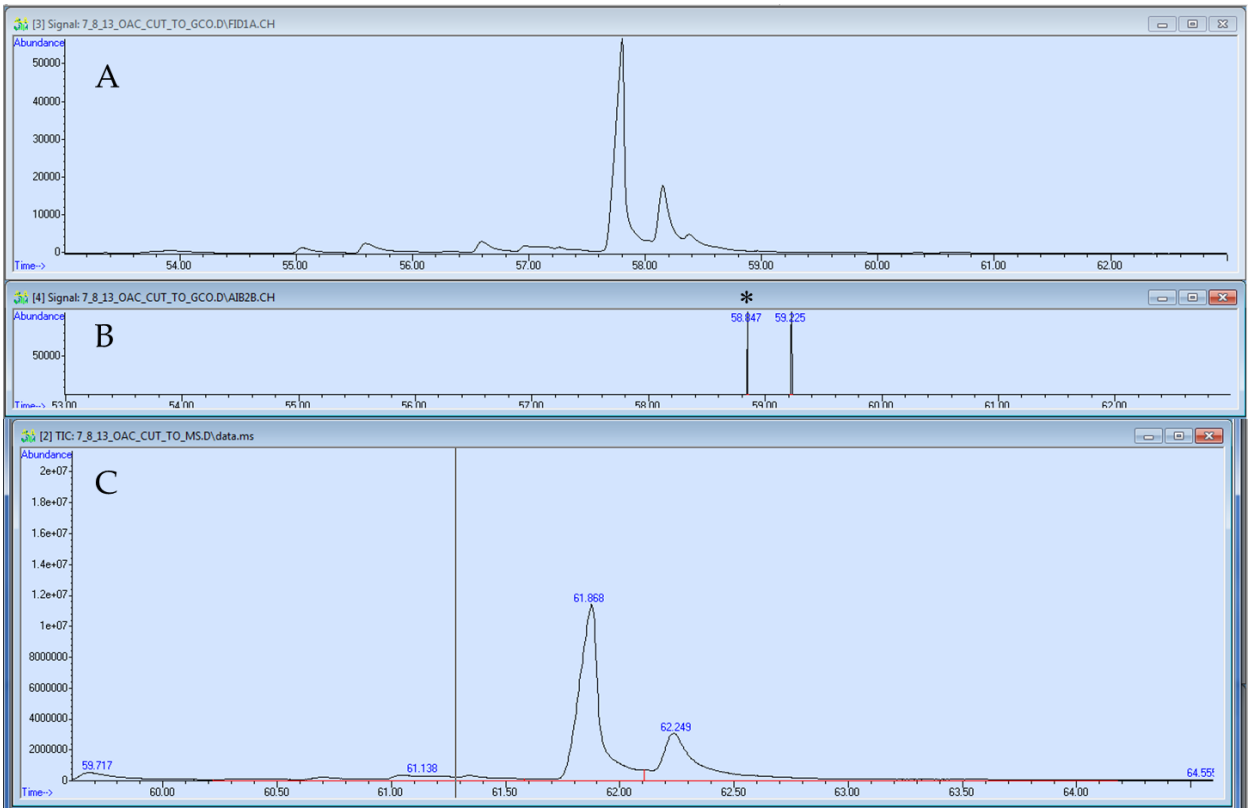


Figure 3.2: Screen shot example (wax → 5) of comparing FID/O cut section to the MS cut section run. Box A is the FID, box B is the odor marks, box C is the TIC from the MS. * indicated the target compound

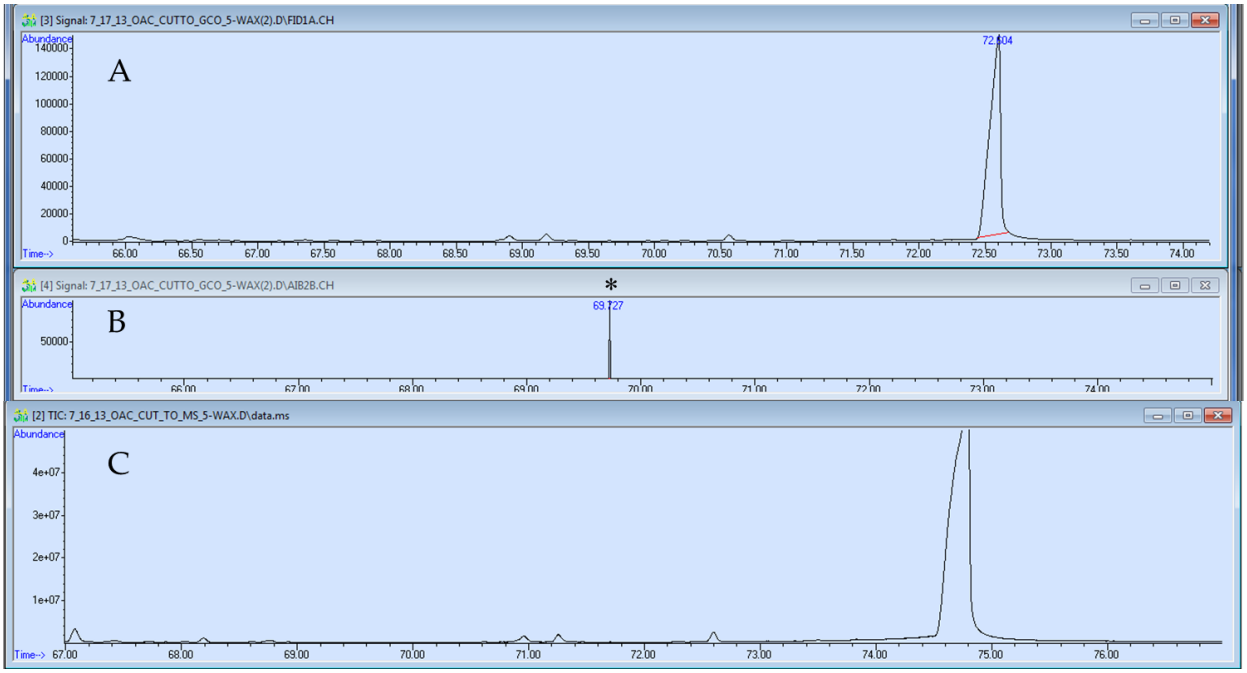


Figure 3.3: Screen shot example (5 → wax) of comparing FID/O cut section to the MS cut section run. Box A is the FID, box B is the odor marks, box C is the TIC from the MS. * indicated the target compound

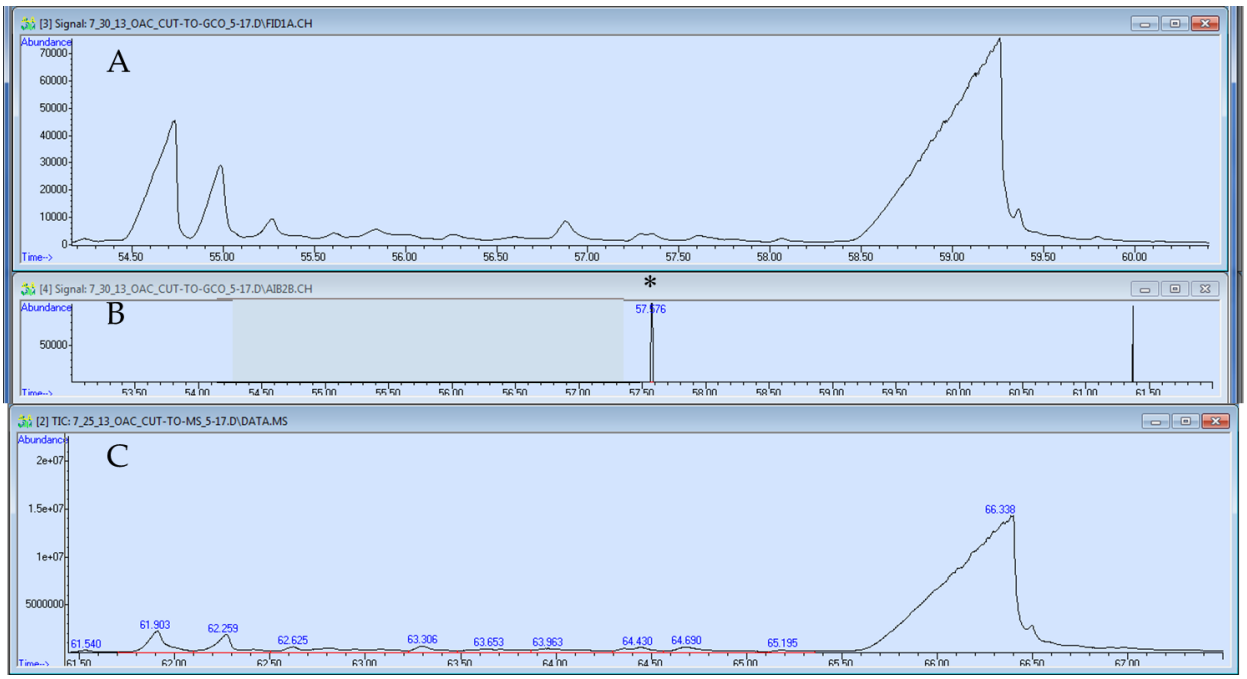


Figure 3.4: Screen shot example (5 → 1701) of comparing FID/O cut section to the MS cut section run. Box A is the FID, box B is the odor marks, box C is the TIC from the MS. * indicated the target compound

Only a certain mass ions were consistently present on all GC-MS/O/FID system configurations, occurring near the same area of the odor marks made during the GC-O run. The best estimate EIMS for the target compound is shown in Figure 3.5.

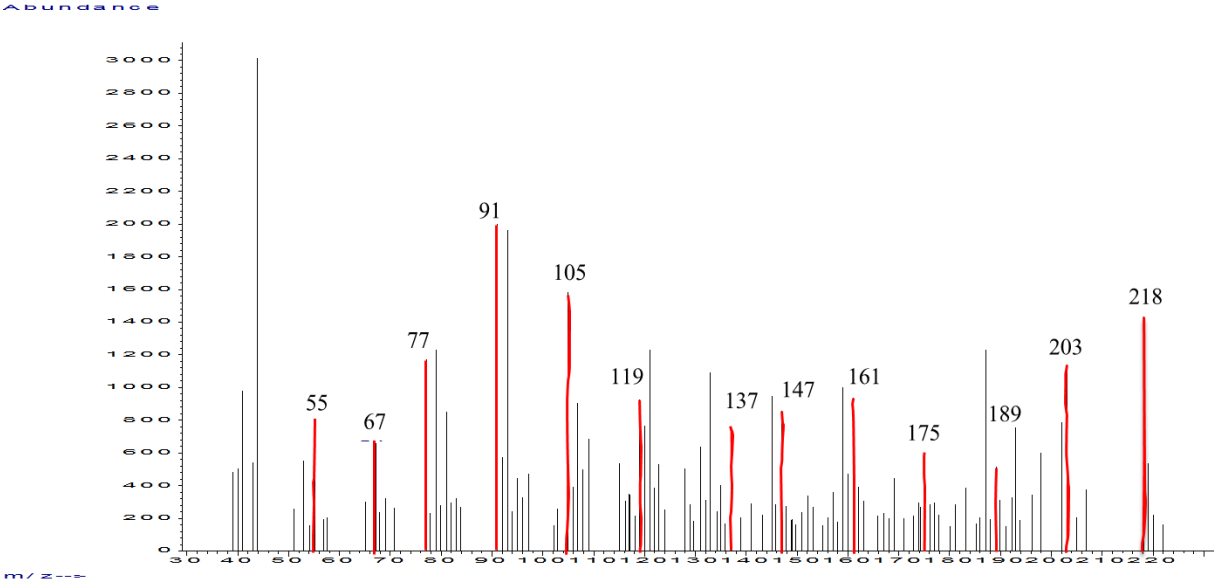


Figure 3.5: EI-MS of unknown target compound

The results suggest that the target compound has molecular weight of 218. However, a search of the NIST database did not contain any confirmed matches. Additional experiments were required to narrow down possible compounds based on type of compound and present functional groups.

Chemical characterization experiments

Each experiment used to detect functional groups was run on a GC-O and revealed whether the target compound was present or absent in the solvent phase of the experiment. The tabulated results are shown in Table 3.3.

Table 3.3: Results from the Characterization Tests

Test	Positive	Negative
Dess-Martin periodinane (DMP)	+	
sodium bisulfite		+
2,4-dinitrophenylhydrazine (DNPH)		+

The presence of the target compound by GC-O during the DMP test indicated that the compound did not contain an alcohol group. The DMP would have oxidized the alcohol and the unknown would not be detected. Both the sodium bisulfite and the DNPH test for the presence of an aldehyde or a ketone. The sodium bisulfite test complexes and solubilized the target into the aqueous phase resulting in a negative result. Meanwhile, the DNPH test forms a solid precipitate, thus also giving a negative result. Since both tests yielded a negative result for detection of the target compound in the solvent phase (by GC-O), it was therefore assumed that the unknown was indeed converted or consumed and, thus, was either an aldehyde or ketone. Based on the fact that aldehydes are unstable and are readily oxidized into acids, the unknown compound was assumed to be a ketone. This is based on the fact that oak casks are used for years and considering the small amount of unknown present, an aldehyde would most likely decrease over time and eventually be undetectable. Instead, this unknown remained detectable in spirits after many years of aging in an oak barrel.

Discovery of rotundone by investigation of other natural extracts

Spices, oil, and roots are used for their volatile content in many different applications ranging from cooking, medicine, insecticides and antimicrobials. There are a vast number of aroma-active compounds that have the potential to be the unknown target compound present in oak wood. A literature search revealed several possible 218 weight compounds. As a result an intense investigation of different wood oils, tobacco, hops, dried herbs and roots was undertaken using GC-O to confirm the identity of the target compound by using as the criteria odor property, retention index, potency, and chemical characteristics. Both the SDE extraction from *Cyperus rotundus* and the Agarwood oil contained an intense woody/incense-like peak at the same retention time as the unknown compound in the oak wood extracts. Investigations of these two samples resulted in the identifying the unknown in oak wood as 5-Isopropenyl-3,8-

dimethyl-3,4,5,6,7,8-hexahydro-1(2H)-azulenone, or rotundone. Its structure can be seen in Figure 3.6.

As just stated, rotundone was also identified in previous studies in the following products: Agarwood oil (Naef 2010, Ishihara et al. 1991), *Cyperus rotundus* (Kapadia 1967), and white peppercorn (Wood et al. 2008).

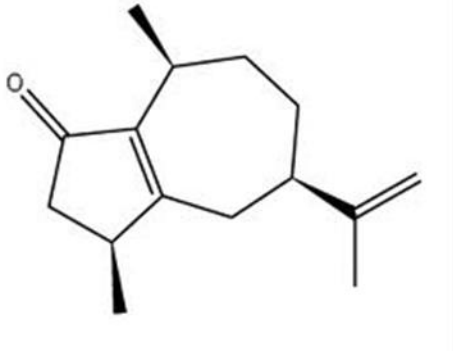


Figure 3.6: Rotundone

The successful synthesis of rotundone further confirmed the identity of the target compound in oak wood by providing an authentic standard for comparison. The rotundone standard and the unknown were analyzed on three different column phases and were found to have the same RI values on all phases. In addition, the proposed mass spectrum of the unknown was found to match that of the rotundone standard. Rotundone was first identified in the *Cyperus rotundus* root in 1967 (Kapadia et al. 1967). It went fairly unnoticed as a potent odorant except for being used in perfumery. It is found in Agarwood oil, a rare and expensive oil extracted from the heartwood of a mold infected tree, and used in perfume formulations (Naef 2010 and Ishihara et al. 1991). Most recently it was noted as a potent odorant in grapes (Wood et al. 2008), and was also identified in a number of products including white pepper, black pepper, wine, marjoram, geranium, rosemary, saltbush, basil, thyme, and oregano (Wood et al. 2009). Its potency was confirmed by its calculated threshold of 8 ng/L (parts per trillion) in water, and 16 ng/L in a wine matrix (Wood et al. 2008).

The discovery of rotundone in oak wood opens doors to its subsequent analysis in oak aged products. Importantly, it's extremely low threshold would make it very potent in any material in which it is found.

3.5 References

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Chapter 4: Quantification of Rotundone in Oak Aged Distilled Spirits

4.1 Abstract

The intense, "woody/incense" smelling aroma compound rotundone (5-isopropenyl-3,8-dimethyl-3,4,5,6,7,8-hexahydro-1(2*H*)-azulenone) was investigated as a potential aroma impact compound in oak aged distilled spirits. Accurate quantification of this trace level compound was done by stable isotope dilution analysis (SIDA) combined with gas chromatography-mass spectrometry. The analysis was conducted on a variety of distilled products including seven bourbons, which ranged in age from 4 to 12 years, rye whiskey, Tennessee whiskey, Scotch whiskey, aged rum, and añejo (aged) tequila. Interestingly, rotundone was also found in un-aged (silver) tequila which suggests that the compound may also be present in the agave plant. Some concentration trends were noted in terms of degree of aging; however, brand-to-brand variation was evident.

4.2 Introduction

Rotundone has been a fairly unassuming compound, first noted in 1967 (Kapadia et al. 1967) and not mentioned again in the flavor chemistry field until 2008, where it was identified as a potent odorant in Syrah grapes and wines (Wood et al. 2008). Rotundone was first isolated from the rhizome (or root) of the *Cyperus rotundus* plant, rotundone's namesake. *Cyperus rotundus*, more commonly known as nut grass, is used in traditional medicine acting mainly on the digestive system as a cure for spasms and pain. Claims were made that nut grass could also be used as an analgesic, antibacterial, antispasmodic, antitussive, aromatic, astringent, carminative, diaphoretic, diuretic, emmenagogue, litholytic, sedative, dermatological treatment, stimulant, stomachic, tonic and vermifuge (Anon. 2014a) . A brief assessment of the dried root reveals that it has a very potent aroma, being reminiscent of incense or black pepper. It was also

noted with interest that nut grass is quite common and is classified as an invasive noxious weed in at least 46 states in the US (Anon. 2014b).

Rotundone has also been identified in agarwood, a dark fragrant resinous material that develops primarily in the heartwood of trees in the genus Aquilaria. Agarwood is far more precious than nut grass; it is prized in Buddhist, Hindu, and Islamic regions for use as incense during religious ceremonies. It is also much harder to obtain, because the trees in which it develops grow in a fairly inaccessible region of India. In addition the trees must be infected with a fungus which then produces the oleoresin in the hardwood, and subsequently the fragrance associated with the tree. As a result of its rarity, agarwood oil has been priced as high at 100,000 (USD) per kilogram. Rotundone was first identified in agarwood in 1991 (Ishihara et al. 1991) and later confirmed as a volatile sesquiterpene constituent of the heartwood of the infected tree in 2010 (Naef 2010). The aroma of agarwood oil has been described as warm, sandalwood-like, rich, woody, ambergris, with balsamic notes, and generally regarded as having elegant characteristics.

The most recent report of rotundone being recognized as a potent odorant was its discovery in Syrah grape skin at a concentration of 0.62 μg/kg (Wood et al. 2008). This study also reported the compound in peppercorns where it is far more abundant (e.g. 1200 μg/kg in black peppercorns and 2025 μg/kg in white peppercorns. The discovery of rotundone in grapes prompted an interest in identifying and quantifying the compound in wines. This was successfully accomplished by Siebert et al. (2008), Mattivi et al. (2010) and Wood et al. (2008). Concentrations were reported in a range from 0.15 μg/L in Syrah wine (Wood et al. 2008) to 0.561 μg/L in vespolina wine (Mattivi et al. 2010). Although these low concentrations of rotundone don't at first sound impressive, one must consider that the compound has an odor threshold of about 0.008 μg/L (or 8 parts-per-trillion) in water (Wood et al. 2008). Therefore, even at extremely low concentrations rotundone may still be an extremely potent odorant.

In recent years, flavor chemists have relied on stable isotope dilution analysis (SIDA) to quantify compounds present at extremely low concentrations. This sensitive and accurate technique makes use of an isotopically labeled internal standard, which is synthesized by labeling the target compound with either deuterium or carbon-13. Samples are then spiked with a known concentration of the isotope before extraction, thus accounting for any possible losses of volatiles during the isolation step. The isotope varies slightly in mass from the target compound, so it is relatively easy to monitor both compounds by mass spectrometry. The mass ion peak area of the internal standard (of known mass) is related to the mass ion of the unlabeled target analyte by use of a mass ion response factor, which enables the calculation of the initial concentration of the target compound in the sample.

4.3 Materials and Methods

Materials

The seven bourbon whiskeys used in this study were: Jim Beam Bourbon (4 year), Jim Beam Black Bourbon (8 year), Jim Beam Signature Craft (12 year), Bulleit Bourbon (at least 6 years), Bulleit Bourbon 10 year (10 years), Elijah Craig Bourbon (12 years), W.L Weller Bourbon (12 years). Other aged spirits used in this study included: Jack Daniels Tennessee Whiskey (at least 4 years), Johnnie Walker Black Label Scotch Whiskey (at least 12 years), Bulleit Rye Whiskey (at least 4 years), Appleton Estates Extra Rum (12 years), Don Julio Añejo Tequila (18 months), and Milagro Tequila (un-aged). All of the above were commercially available and purchased at a local liquor store (Binny's Beverage Depot, Champaign, IL). The value in parentheses indicates the reported age for each product.

Chemicals

The following chemicals, used for volatile extraction, isolation, and chemical synthesis, were purchased from Fisher Scientific Co. (Fair Lawn, NJ): dicholormethane, diethyl ether, guaiac wood oil, hydrochloric acid (conc), n-pentane, sodium sulfite, sodium chloride, sodium sulfate.

The following chemicals, used for isolation and chemical synthesis, were purchased from Sigma-Aldrich Co. (St. Louis, MO): thionyl chloride, pyridine, acetonitrile, cobalt acetate tetrahydrate, *tert*-butyl hydroperoxide (5.0 – 6.0 M in decane), silica gel (high-purity grade, 60A, 230-400 mesh particle), deuterium oxide (99.9% atom D), and sodium deuteride.

Methods

Rotundone Synthesis

Rotundone was synthesized as previously described (Chapter 3). The synthesis involved extraction of guaiaol from guaiac wood oil, followed by dehydration of guaiaol to form guiaene, and finally random allylic oxidation of guiaene to obtain a crude mixture, yielding less that 10% rotundone. Purification of this crude mixture by flash column chromatography resulted in a final purity of 74% rotundone.

***d*$_4$-Rotundone Synthesis**

Isotopically labeled rotundone, *d*$_4$-dotundone, was synthesized by a simple exchange reaction using the method of Kotseridis et al. (1998) as described below. Sodium deuteride (3 drops) was added to a stirred solution (contained in a 40-mL amber vial and purged with N_2 gas) of rotundone (46 μmol, 10 mg) in pyridine (5 mL) and deuterium oxide (>100 molar excess, 1 mL). The vial was then purged with N_2 and sealed, and the solution allowed to stir at room temperature overnight. Exchange progress was monitored every few hours. Once complete, the reaction mixture was quenched with ice cold water. The pH of the solution was adjusted to 2 with aqueous HCl (4 N), and extracted with ether (3 x 10 mL). The ether extract was concentrated (1

mL) and passed through a bed (20 g) of silica using a mobile phase consisting of 80:20 pentane:ether to purify the target compound. Final purity was 84.5%.

Based on ^{1}H NMR analysis, the signals associated with the protons on C_{13} ($H_{13\alpha}$ and H_{13b}) and C_5 ($H_{5\alpha}$ and $H_{5\beta}$) were absent; confirming deuterium exchange at these two carbon atoms (Figure 4.1).

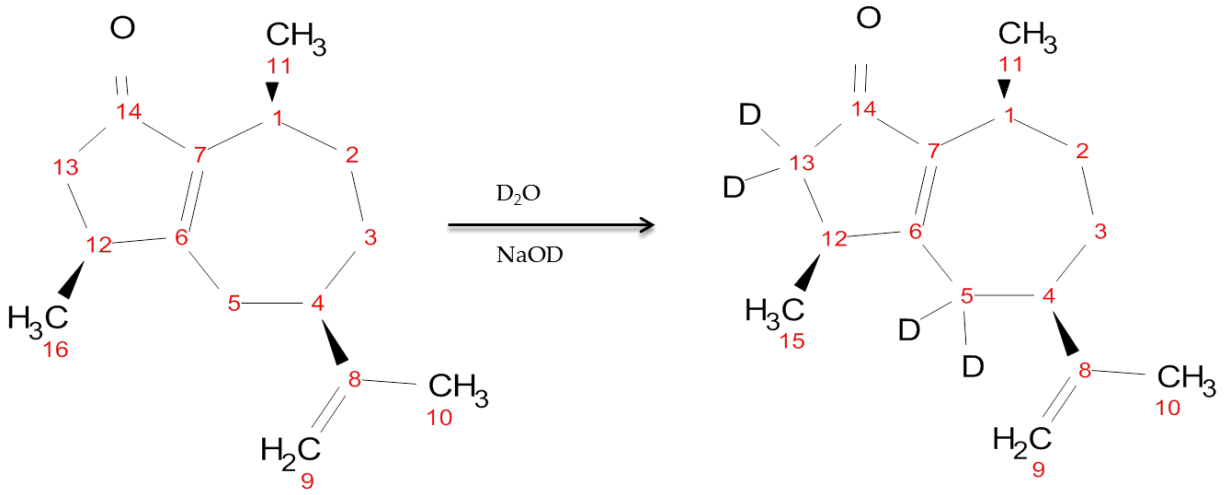

Figure 4.1: Synthesis of deutrium labeled (d_4)-rotundone from unlabeled rotundone.

d_4-Rotundone: EIMS *m/z* (rel intensity) 222([M]$^+$, 100), 221 (84), 207 (60), 206 (87), 166 (61), 165 (56), 164 (70), 163 (56), 150(48), 141 (51), 140 (56), 122 (56), 121 (73), 120 (59), 107 (56), 106 (48), 95 (58), 94 (44), 93 (60), 92 (48), 82 (50), 81 (47), 79 (52), 67 (44), 44 (40), 41 (52)

d_4-Rotundone: ^{1}H NMR (500 MHz, $CDCl_3$), δ 4.76-4.68 (2H, m, H_{10}), 2.99 (1H, m, H_1), 2.71 (1H, p, J=7.5, H_{12}), 2.01 (1H, m, H_4), 1.92-1.85 (3H, overlapping m, $H_{3,2b}$), 1.74 (3H, s, H_9), 1.53 (1H, m, H_{2b}), 1.14 (3H, d, J=4.2, H_{15}), 1.01 (3H, d, 7.0, J=7.0, H_{11})

^{1}H NMR (500 MHz, $CDCl_3$) was obtained from the solutions in deuterochloroform with a Varian Unity (500 MHz, Quad Probe; Varian, Palo Alto, USA). Chemical shifts were referenced relative to the corresponding residual solvent signal.

The EIMS and NMR chromatograms can be found in the Appendix B.

Volatile Extraction for Quantification

Each spirit sample (10 mL) was transferred to a 50-mL glass centrifuge tube to which 10 ng of d_4-rotundone was added as the internal standard. Ethanol in the sample was reduced to about 10% (alcohol by volume; ABV) by the addition of 30 mL of deodorized deionized-distilled water. The tube was sealed with a PTFE-lined cap and vigorously shaken (5 min) by hand. Dichloromethane (2 mL) was then added to the tube which was recapped, and again vigorously shaken (5 min) by hand. The tube was then centrifuged at 7500 rpm for 15 min (IEC HN-SI; Damon/IEC Division, Needham Heights, Massachusetts) to separate the solvent from the aqueous phase. The solvent (lower) phase (dichloromethane) was transferred to a 20 mL vial containing sodium sulfate (2 g) to remove any residual water. The extraction, with dichloromethane (2 mL), was repeated two more times. The final dried extract was condensed to 0.25 mL using a gentle stream of N_2 gas and stored at -20°C prior to analysis. This dichloromethane extraction was performed in duplicate (or triplicate) on all samples.

GC-MS analysis

A 6890 GC-HP 5973N mass selective detector (Agilent Technologies Inc.) was used for GC-MS analysis. Two μL of spiked extract was injected into a CIS-4 inlet (Gerstel, Germany) in the cold splitless mode (-50°C for 0.10 min, then increased at 12°C/sec to 260°C and held for 20 min). Separations were performed using a Stabilwax® column (30.0 m length x 0.25 mm i.d. x 0.25 μm film thickness; Restek, Bellefonte, PA). Oven temperature was programmed as follows: initial temperature 50°C (5 min hold), ramp rate 4°C/min to a final temperature of 225°C (45 min hold time). Helium was used as the carrier gas at a constant flow of 1.0 mL/minute. The MSD conditions were as follows: capillary direct interface temperature, 280 °C; ionization energy, 70 eV; mass range, 35 to 300 amu; electron multiplier voltage (Autotune + 200 V); scan rate, 5.27 scans/s. Data acquisition was performed using the simultaneous full scan (35-300 m/z) and selected ion monitoring (SIM) (ions 218, 203, 222, 221, 206 m/z) modes.

Stable Isotope Dilution Analysis (SIDA)

A calibration curve was generated using solutions of rotundone and d_4-rotundone in varying mass ratios (unlabeled:labeled) of approximately 10:1, 5:1, 1:1:, 1:5, and 1:10. Each solution was analyzed by GC-MS using the aforementioned conditions. Areas for both rotundone and d_4-rotundone were taken by extraction of selected mass ions from the resulting SIM chromatograms, and peaks were integrated with the assistance of Enhanced Data Analysis Software (Agilent Technologies, USA). The actual mass ratios (unlabeled:labeled) were plotted against the selected mass ion area ratios (unlabeled:labeled) and a best fit line equation was determined. The slope of the best fit line was used to determine the response factor (R_f) for the compound. Alternatively, the R_f can also be calculated using the following equation:

$$Rf = \frac{[area\ of\ ion_{target}/area\ of\ ion_{isotope}]}{[mass_{target}/mass_{isotope}]}$$

Rotundone was quantified in seven different bourbons (Jim Beam Bourbon (4 year), Jim Beam Black Bourbon (8 year), Jim Beam Signature Craft (12 year), Bulleit Bourbon (at least 6 years), Bulleit Bourbon 10 year (10 years), Elijah Craig Bourbon (12 years), W.L Weller Bourbon (12 years)), a Tennessee whiskey (Jack Daniels Tennessee Whiskey (at least 4 years)), a Scotch whiskey (Johnnie Walker Black Label Scotch Whiskey (at least 12 years)), rye whiskey (Bulleit Rye Whiskey (at least 4 years)), rum (Appleton Estates Extra Rum (12 years)), aged tequila (Don Julio Añejo Tequila (18 months)), and un-aged tequila (Melagro Silver). These sprits were selected to provide a full range of aged spirits but were biased on bourbons because they have strict requirements as to the type and treatment of the oak cask using in aging. The Jim Beam whiskeys were analyzed in triplicate to more strictly monitor the effect of aging time. All other spirits were analyzed in duplicate.

For rotundone quantification GC-MS data acquisition was conducted in the selected ion mode (SIM), which is more sensitive relative to a full scan mode, as rotundone could be

present in the low parts per trillion range. The ions selected for SIM were: 218 and 203 for rotundone and 222, 221, and 206 for d_4-rotundone. Due to small peak sizes, manual integration was necessary using the Enhanced Data Analysis Software. Microsoft Excel was used to relate the actual mass ratios (unlabeled:labeled) with respect to the calculated selected mass ion area ratios (unlabeled:labeled) using 218 for rotundone and 206 for d_4-rotundone. Ions were selected based on interference of other compounds in the extracts. Concentrations of the compound were then calculated using the following equation:

$$concentration_{target} = concentration_{isotope} \times Rf \times \frac{area_{target\ ion}}{area_{isotope\ ion}}$$

Statistical Analysis

Data were analyzed by one-way Analysis of Variance (ANOVA) for each compound concentration using the Minitab 16 program (Minitab Inc, State College, PA). For attributes with significant differences across products, Fisher's LSD was used for means separation, reporting differences at alpha=0.05.

4.4 Results and Discussion

This is the first report of the synthesis of deuterium-labeled rotundone using the methods described in this study. A previously reported synthesis method involved the exchange of hydrogens under extreme alkaline conditions using sodium ethoxide in ethan(ol-d) (Siebert et al. 2008). This method was attempted but it was found that the isotope exchange varied with time and that a consistent isotope exchange was difficult to achieve leading to a mixture of rotundone isotopologues with d_4 to d_6 exchanges. Considering the mild conditions and fairly high yield obtained in our current study, it is recommended that in future studies d_4-rotundone be synthesized following the method described herein, using D_2O and sodium deuteride in pyridine.

A SIM mode GC-MS scan of the spiked extract, where mass ions of 218 and 203 were monitored for rotundone and 222, 221, and 206 for d_4-rotundone, revealed that ions 218 and 206 were the best ions to monitor for the respective compounds on the basis of the *m*/*z* relative intensity and the ability to obtain resolved peaks with no interference from neighboring compounds. An example taken from the analysis of W.L. Weller 12yr Bourbon is shown in Figure 4.2.

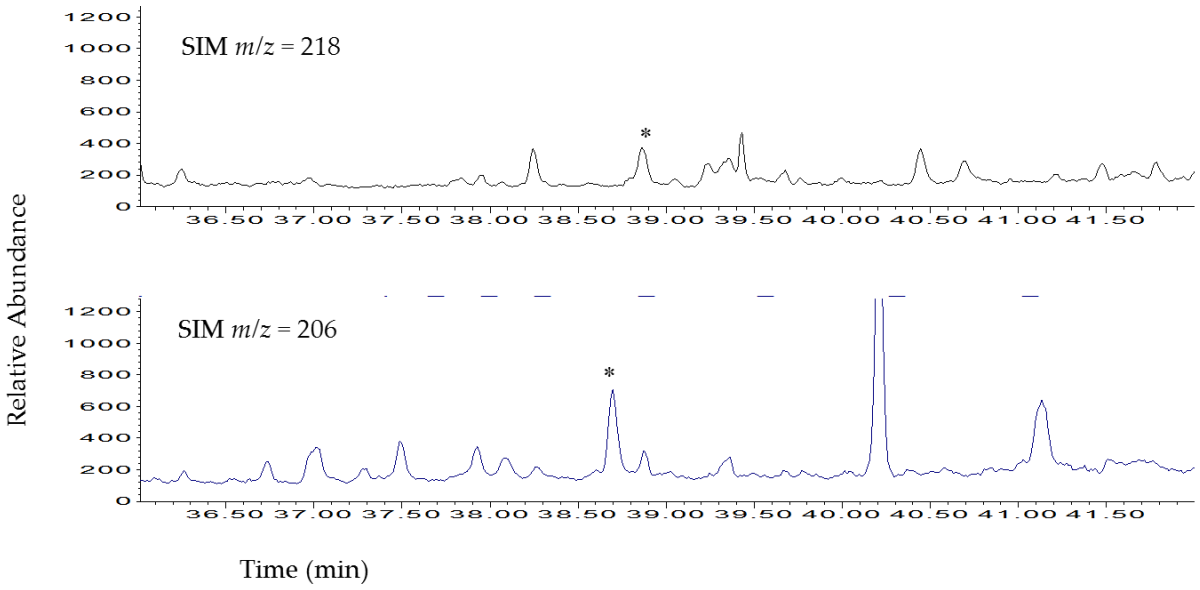

Figure 4.2: GC profiles obtained from SIM of 218 (rotundone) and 206 (d_4-rotundone). * indicates the targeted compounds

The results from rotundone quantification in bourbons and other aged spirits are shown in Table 4.1. These results indicate a unique success, as quantifying rotundone has been accomplished in only a few previous studies and never before in oak-aged spirits. They also confirm that rotundone is, indeed, transferred from the oak wood into the distilled spirit, potentially having a significant impact on its flavor. Of the oak-aged spirits analyzed, Johnnie Walker Black Label Scotch Whiskey and Appleton Estates Extra Rum had the lowest concentration of rotundone at 0.150 µg/L and 0.152 µg/L, respectively. This is not surprising as both of these employ used barrels in their manufacturing

procedure. Scotch maturation is done in casks that are used, repaired and then reused for as long as they remain intact, or until they no longer have an effect on flavoring of the distilled spirit. Even then, manufacturers may simply re-char the interior of the barrel to regenerate some of the aroma compounds (Piggott and Conner 2003). However, only certain compounds come from charring of the barrel, while others will be completely lost over time, so this technique is not an ideal solution for regenerating the oak cask. Rum has no legal requirements as to the cask used in aging, but it is required that the casks be coded according to their origin or previous history, marking them F1 or F2 for fresh first or second fill, respectively, while an unclassified refill must be marked "UR" (Nicol 2003). The only statement made on Appleton Estates Extra Rum was the guarantee that aging is done in "select American oak barrels." Jack Daniels Tennessee Whiskey was found to contain only a slightly higher concentration of rotundone (0.166 μg/L) than the rum and Scotch whiskeys analyzed. It is difficult to make any assumptions about this finding as Jack Daniels does not put an age statement on their bottle. Although Jack Daniels uses new charred white oak barrels, the company ages their whiskey to taste, not based on time elapsed, so the whiskey is not aged for a specific amount of time (Arnett 2014). Thus, a correlation between aging time and rotundone concentration is not possible for Jack Daniels whiskies.

In contrast, the bourbons and rye may be validly compared to one another, because they have the same legal aging requirements. An interesting observation is that age is apparently not the only factor contributing to the rotundone concentration considering Jim Beam Signature Craft (12 year) had a lower concentration (0.342 μg/L) than the Bulleit Bourbon (at least 6 years) (0.694 μg/L). Bulleit Bourbon 10 year contained the highest concentration of 1.345 μg/L. This is almost 10 fold higher than samples that were aged the least (~4 yrs).

Importantly, the two Bulleit bourbons and the Bulleit Rye were among those samples found to contain the highest rotundone concentrations, suggesting that factors in their

manufacturing process, other than age, affected their rotundone concentrations. Such factors may include the climate under which aging occurs, the characteristics of the tree or wood used for the barrel, and variations in the coopering process.

Humidity and temperature may influence volatile extraction. Generally, in lower humidity climates, water evaporates from casks resulting in spirits with higher ethanol content as well as a higher concentration of extracted volatiles (Nose 2004). Aging at higher temperatures has also been reported to result in an increase in the extraction of oak flavoring volatiles (Nicol 2003). However, it is unlikely that environmental conditions alone account for different rotundone concentrations in these bourbons as they are produced in the same region of Kentucky, and are therefore, aged under similar environmental conditions. It is important to consider that 10 different species of American Oak may be used for cooperage. Distilleries may use different oak species for their barrels which will likely affect the concentrations of rotundone in a particular spirit. Additionally, barrel size may affect extraction of volatiles. It has been shown that the volume to wood surface area has a marked effect on volatile concentration, i.e. the smaller the barrel volume, the more concentrated the volatiles from oak (Pérez-Prieto et al. 2002 and 2003).

This is supported by a study which showed that the geographical origin of the trees used for the barrels, the seasoning of the wood, and the coopering method all have an effect on the volatile compounds in wine. For example, cultivation at higher altitudes appeared to be an important parameter affecting volatile composition (Alañón et al. 2011). It is also notable that seasoning in Australia differs from seasoning in the USA or France (Spillman et al. 2004).

Conclusive statements concerning the concentration of rotundone and aging time can be drawn from the data obtained in the current study as demonstrated both in the Jim Beam bourbons and the Bulleit bourbons. The Bulleit bourbons almost doubled in rotundone concentration due to barrel aging, from 0.694 μg/L after ~6 years to 1.346

μg/L after 10 years. The Jim Beam bourbons also showed an increase, although not as great, from 0.342 μg/L (4 year) to 0.403 μg/L (8 year), and finally to 0.453 μg/L (12 year). The linear (R^2 = 0.9848) increase of rotundone was confirmed over time. An ANOVA determined that these values were statistically significant (p-value <0.05) showing that rotundone concentrations increased with barrel aging time.

One of our more interesting observations was that rotundone was present not only in aged tequila, but also in un-aged, silver tequila. This was confirmed by direct injection GC-O analysis of the un-aged tequila in which an odorant peak was detected with the same retention index and had the same odor property as rotundone. Quite possibly rotundone in the un-aged tequila originated from the agave leaves used its manufacture. Agave leaves, the starting material for manufacturing tequila, are known to contain a wide variety of monoterpenes and sequiterpenes. A study profiling the terpene content of a variety of agave leaves, by GC-MS, reported 9 in *Agave salmiana*, 8 in *Agave angustifolia*, and 32 in *Agave tequilana* (Peña-Alvarez et al. 2004). The research group followed up this report demonstrating that these terpenes are also in the final distilled spirit, tequila, by identifying 28 different monoterpenes and sequiterpenes by GC-MS (Peña-Alvarez 2006). Rotundone was not among those identified, however, at low concentrations rotundone is difficult to detect unless it is specifically targeted. It would be interesting to determine if Agave leaves were the source of the rotundone found in the silver tequila analyzed in the current study.

It has only been in the past few years that rotundone has gone from an obscure sesquiterpene, known to exist in a single root, to being identified in an array of herbs, spices, fruits, and now oak trees and possibly agave leaves. We can speculate that it probably exists in far more many natural materials, and the more well-known it becomes, the more likely it is to be viewed as a common odorant. With an extremely low threshold of 8 ng/L, rotundone is likely to be potent in anything in which it is present.

Table 4.1: Concentrations of Rotundone in Oak-Aged Distilled Spirits

Product	Concentration (μg/L)(ppb)[a]	RSD (%)[b]
Jim Beam Bourbon (4 year)	0.342[c]	0.54
Jim Beam Black Bourbon (8 year)	0.403[c]	1.4
Jim Beam Signature Craft (12 year)	0.453[c]	1.9
Bulleit Bourbon (~ 6 year)	0.694	0.12
Bulleit Bourbon 10 (10 year)	1.35	0.85
W.L Weller Bourbon (12 year)	0.393	1.8
Elijah Craig Bourbon (12 year)	0.694	10
Bulleit Rye Whiskey (~ 4 year)	0.434	0.58
Jack Daniels Tenneesee Whiskey	0.166	0
Johnnie Walker Black Label Scotch Whiskey (~ 12 year)	0.150	2.2
Appleton Estates Extra Rum (12 year)	0.152	1.8
Don Julio Añejo Tequila (18 month)	0.307	0.85
Milagro Silver Tequila	0.100	1.1

Age statements are indicative of years aged in an oak cask. [a] The mean value obtained by analyzing each product in duplicate samples taken from the same bottle. [b] The relative standard deviation in %. [c] The mean value was obtained in triplicate samples for use in further studies.

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Chapter 5: Potency of Rotundone in Bourbon Whiskey in Comparison to Other Aroma Actives Observed Over Barrel Aging Time

5.1 Abstract

A potent sesquiterepene ketone, rotundone, was recently identified and quantified in various oak aged spirits. To demonstrate its potential importance to the overall flavor of aged spirits, in particular bourbon, aroma extract dilution analysis (AEDA) was performed followed by quantification of all potent odorants and subsequent calculation of their odor activity values (OAV). Analyses were performed on bourbons of three different ages (4, 8, and 12 years) to determine the relationship between rotundone concentration and potency with increased aging. Monitoring of all potent odorants at various ages revealed some interesting trends. It was found that ethyl esters tended to increase over time due to continuous ethanolysis of wood acids. Some typical charred oak compounds also increased over time. These included vanillin, guaiacol, syringaldehyde and the *cis*- and *trans*-whiskey lactones. Isoeugenol and eugenol showed an interesting inverse relationship over time in which eugenol decreased as isoeugenol increased. The compound of interest, rotundone, also showed a linear increase over time. The OAV of rotundone was calculated to be 42 (4 year) to 56 (12 year) ranking it in the top 10 of 25 potent odorants in the bourbons. It is generally regarded that any compound with an OAV over 1 is detectable, thus, the relatively high OAV of rotundone confirms that it is a potent odorant in bourbon.

5.2 Introduction

Until recently, rotundone was an ambiguous sesquiterpene discovered in tubers of the grass, *Cyperus rotundus* (Kapadia et al. 1967). The first report of rotundone as a potent aroma compound demonstrated that it had a threshold of about 8 parts-per-trillion

(Wood et al. 2008), indicating that it had the potential to strongly impact the flavor of any material in which it was identified. To date, its presence was confirmed in white pepper, black pepper, wine, marjoram, geranium, rosemary, saltbush, basil, thyme and oregano (Wood et al. 2009). In the current study, its presence was confirmed in oak wood, and subsequently quantified in a range of oak aged spirits (Chapters 3 and 4).

It is widely regarded that oak aging is the most important factor influencing the quality and flavor of distilled spirits, contributing to the complexity of the finished product. The volatiles can originate from three main sources: 1) ethanolysis of wood components, 2) charring of the barrel and 3) direct extraction of wood volatiles. During the charring step, lignin pyrolysis creates "smoky" compounds, these include phenols, cresols, and guaiacols; such as syringol and syringaldehyde, guaiacol, 4-ethyl guaiacol, 4-vinyl guaiacol, *p*-cresol, eugenol, isoeugenol, and vanillin (Poisson 2008a, Fernanadez de Simon 2010, Piggot et al. 2003). Both eugenol and isoeugenol are described as clove-like, while vanillin is most commonly associated with the flavor of the vanilla bean. Other flavor compounds resulting from charring come from the rearrangement of lipids to form lactones. The oak lactones, *cis*- and *trans*-β-methyl-γ-octalactone, are of particular importance, even being nicknamed "whiskey lactones". Other lactones present, including γ-nonalactone, δ-nonalactone, γ-decalactone, γ-dodecalactone and *cis*-6-dodeceno-γ-lactone, contribute a peachy and creamy notes. Additional wood extractives known to contribute to the flavor include β-ionone and β-damascenone, which impart floral and cooked apple-like attributes, respectively. Lastly, ethanolysis of the wood acids results in formation of fruity ethyl esters like ethyl propanoate, ethyl butanoate, ethyl hexanoate, and ethyl octanoate, along with several other branched chain ethyl esters (Poisson 2008a, 2008b, Conner 1993, Conner 2001, Piggot 2003, Lahne 2010).

To demonstrate a compound's potency within a sample, it is a common practice to calculate its odor activity value (OAV), which is the ratio between the compound's

concentration and its aroma detection threshold. A compound must have an OAV of at least 1 to be considered detectable and, thus, important to the overall flavor. Although rotundone was identified in oak wood and quantified in oak aged spirits, this is not exactly representative of its potency. The goal of this current study is to prove that rotundone is important to the flavor of aged spirits, in particular whiskey, by calculation of its OAV along with all potent volatiles.

5.3 Materials and Methods

Materials

Three different commercially available bourbon whiskeys were purchased at a local liquor store (Binny's Beverage Depot, Champaign, IL). Their reported age statements were: Jim Beam Bourbon (4 year), Jim Beam Black Bourbon (8 year), Jim Beam Signature Craft (12 year).

Chemical

The following chemicals used for volatile extraction, isolation, and chemical synthesis were purchased from Fisher Scientific Co. (Fair Lawn, NJ): dicholoromethane, ethanol, sodium sulfate, diethyl ether, and sulfuric acid, n-pentane.

Octanoic acid, used for chemical synthesis, was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Standard Compounds

The standard compounds used for quantification and listed in Table 5.2 were purchased or supplied from the following companies: Sigma-Aldrich Co. (St. Louis, MO): phenthyl alcohol (**1**), ethyl octanoate (**2**), syringaldehyde (**3**), 3-methyl-1-butanol (**4**), isoamyl acetate (**5**), 2-methyl-1-butanol (**6**), ethyl hexanoate (**7**), phenethyl acetate (**9**), whiskey lactone mixture of *cis*/*trans* (**10** and **18**), ethyl butyrate (**11**), ethyl isobutyrate (**13**), ethyl vanillate (**14**), syringol (**16**), ethyl isovalerate (**17**), γ-nonalactone (**19**), eugenol

(**20**), 4-ethyl phenol (**21**), guaiacol (**22**), p-cresol (**24**); Fluka (Switzerland), vanillin (**12**); Alpha Aesar (Lancaster, UK), isoeugenol (**15**); Firmenich (Switzerland), β-damascenone (**23**); Fischer Scientific (Fair Lawn, NJ), and 2-methyl propanol (**8**).

Isotope Standard Compounds

Isotopically labeled standards obtained commercially (C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada) included d_7-ethyl butyrate (**I-11**), d_3-guaiacol (**I-22**), d_{11}-ethyl hexanoate (**I-7**) and d_3-p-cresol (**I-24**).

Methods

Aroma Extract Dilution Analysis

Each of the three bourbon samples (10 mL) was added to individual 50-mL glass centrifuge tubes. The alcohol by volume ratio was lowered to about 10% ethanol by the addition of 30 mL of deodorized DI water. Tubes were sealed with a PTFE-lined cap and vigorously shaken for 5 min by hand to obtain a fully mixed solution. This was followed by the addition of dichloromethane (2 mL) after which the tube was recapped, and vigorously shaken for another 5 min. The tube was then centrifuged at 7500 RMP for 15 min (IEC HN-SI; Damon/IEC Division, Needham Heights, Massachusetts) to separate the solvent from the aqueous phase. The lower phase (dichloromethane) was transferred to a 20 mL vial with sodium sulfate (2 g) to remove any water. Extraction with dichloromethane (2 mL) was repeated two more times. The final dried extract was condensed to 1.0 mL using a gentle stream of N_2 gas and stored at -20°C prior to analysis.

Starting with 1.0 mL extract, AEDA was performed using a 1:3 dilution series. For this, 0.5 mL was diluted into 1.5 mL of dichloromethane serially to obtain 1:3 ($Log_3FD = 1$), 1:9 ($Log_3 FD = 2$), 1:27 ($Log_3FD = 3$), 1:81 ($Log_3FD=4$), 1:243 ($Log_3FD=5$), 1:729 ($Log_3FD=6$), 1:2187 ($Log_3FD=7$), and 1:6561 ($Log_3FD=8$) dilution ratios. Each dilution was stored in a 1.5 mL septum-capped Target DP vial (National Scientific, Rockwood, TN) at -20°C

prior to analysis. The GCO system used for analysis of SAFE extracts consisted of a 6890 GC (Agilent Technologies Inc.) equipped with an FID and Olfactory port (OD2, Gerstel, Germany). Two μL of spiked extract was injected into a CIS-4 inlet (Gerstel, Germany) in the cold splitless mode (-50°C for 0.10 min, then increased at 12°C/sec to 260°C and held for 20 min). Separations were performed using a RTX®-Wax column (15 m length x 0.53 mm i.d. x 1.0 μm film thickness; Restek; Bellefonte, PA). Helium was used as the carrier gas at 5.0 mL/minute. FID temperature was 250°C. Oven temperature was programmed as follows: initial temperature, 40 °C (5 min hold), ramp rate 8 °C/min, final temperature, 225 °C (30 min hold). To aid in identification, analysis was also conducted using a RTX®-5MS column (15 m length x 0.53 mm i.d. x 1.0 μm film thickness; Restek). Evaluations were performed by three panelists. Results are based on consensus scores on 2 out of 3 panelists. Compound identifications were confirmed by comparison of retention indices (RI), odor properties, and EIMS spectra of unknowns with those of authentic reference standards.

GC-MS Analysis

A 6890 GC-HP 5973N mass selective detector (Agilent Technologies Inc.) was used for GC-MS analysis. One μL of extract was injected into a cold splitless inlet CIS-4 inlet (Gerstel, Germany) held at -50°C for 0.10 min, then increased to 260°C at a rate of 12°C/sec. Separations were performed using a Stabilwax® column (30.0 m length x 0.25 mm i.d. x 0.25 μm film thickness; Restek). Oven temperature was programmed as follows: initial temperature 50°C (5 min hold), ramp rate 8°C/min to a final temperature of 225°C (45 min hold time). Helium was used as the carrier gas a constant flow of 1.0 mL/minute. MS transfer line temperature was 280°C.

Stable Isotope Dilution Analysis (SIDA)

Compounds chosen for quantification, shown in Table 5.2, were based on results from AEDA, shown in Table 5.1, and also abundance observed during GC-MS analysis. Compounds were included that may not have been deemed potent by results of AEDA, but were in high abundance and may have an influence on the overall flavor.

Chemical synthesis

d_4-ethyl octanoate (**I-2**) was synthesized in a one-step acid-catalyzed esterification using ethanol, sulfuric acid and the corresponding C8 carboxylic acid, d_4-octanoic acid, as described by Vogel (1989). A large molar excess (1:100) of ethanol was added to d_4-octanoic acid in a vial followed by the addition of a few drops of sulfuric acid. The vial was capped and incubated at 40°C overnight. The reaction was quenched with water (50 mL) followed by extracting with pentane (3 x 10 mL). The detailed synthesis is given in the Appendix A and EIM is given in Appendix B.

Rotundone (**25**) was synthesized as previously described in Chapter 3 following the method of Mattivi et al. (2010) which involved the extraction of guaiaol from guaiac wood oil, dehydration of guaiaol to guiaene, and finally random allylic oxidation to obtain a crude mixture yielding less that 10% rotundone. The rotundone yield of this mixture was increased to 74% using flash column chromatography purification. The detailed synthesis is given in the Appendix A.

Synthesis of d_4-rotundone (**I-25**) was done as previously described in Chapter 4 following the method of Kotseridis et al. (1998), involving a simple exchange reaction of Rotundone in D_2O and sodium deuteride. The detailed synthesis is given in the Appendix A.

The following isotopes were prepared as previously described by Lahne (2010): 2-[d_3]-methoxy-6-methoxyphenol (**I-16**); 2-[1,2-$^{13}C_2$]-phenethyl alcohol (**I-1**) using the method of (Schuh et al. 2006); 4-[d_5]-ethylphenol (**I-21**); (*E*)-1-(2,6,6-trimethylcyclohexa-1,3-dien-

1-yl)-[1-$^{2}H_3$,3-$^{2}H_1$]-but-2-en-1-one (d_4-β-damascenone)(**I-23**) following the method of (Kotseridis et al. 1998); *cis*-/*trans*-β-methyl-γ-[3,4-d_2]-octanolide (d_2-(*cis*/*trans*)-whiskey lactone)(**I-10** and **I-18**); [d_5]-ethyl isobutyrate (**I-13**); ethyl 3-methyl-[3,4-d_2]-butyrate (d_2-ethyl isovalerate) (**I-17**); [d_5]-ethyl vanillate (**I-14**); 2-[d_3]-methoxy-4-(2-propenyl)-phenol (d_3-eugenol) (**I-20**); [3,4-d_2]-γ-nonalactone (**I-19**); 3-methyl-[3,4-d_2]-butyl acetate (d_2-isoamyl acetate) (**I-5**); [1,2-$^{13}C_2$]-phenylethyl acetate (**I-9**) using the method of (Furniss et al., 1989); 4-hydroxy-3-[d_3]-methoxy-5-methoxybenzaldehyde (d_3-syringaldehyde) (**I-3**); 4-hydroxy-3-[d_3]-methoxybenzaldehyde (d_3-vanillin) (**I-12**) using the method of (Schneider and Rolando 1992).

[d_3]-(E)-isoeugenol (**I-15**) were prepared as previously described by Lorjaroenphon (2012).

The structures for all above labeled isotopes are shown in Figure 5.1.

Calibration Curves

Standards (Table 5.2) were divided into three groups to avoid of co-elution with other analytes, especially those containing similar mass ions. Group 1 contained vanillin/d_3-vanillin (**12 and I-12**), p-cresol/d_3-p-cresol (**24** and **I-24**), 4-ethyl phenol/4-[d_5]-ethylphenol (**21 and I-21**), ethyl isovalerate/d_2-ethyl isovalerate) (**17** and **I-17**), guaiacol/d_3-guaiacol (**22** and **I-22**), isoeugenol/d_3-isoeugenol (**15 and I-15**), and ethyl octanoate/d_4-ethyl octanoate (**2** and **I-2**). Group 2 contained syringol/d_3-syringol (**16 and I-16**), ethyl vanillate/d_5-ethyl vanillate (**14 and I-14**), (cis/trans)-whiskey lactone/d_2-(*cis*/*trans*)-whiskey lactone)(**10** and **18** and **I-10** and **I-18**), β-damascenone/d_4-β-damascenone (**I-23**), eugenol/d_3-eugenol (**20** and **I-20**), and syringaldehyde/d_3-syringaldehyde (**3** and **I-3).** Group 3 contained ethyl butyrate/d_7-ethyl butyrate (**11** and **I-11**), γ-nonalactone/d_2-γ-nonalactone (**19** and **I-19**), isoamyl acetate/d_2-isoamyl acetate) (**5** and **I-5**), phenethyl acetate/$^{13}C_2$-phenethyl acetate (**9** and **I-9**), phenethyl alcohol/$^{13}C_2$-phenethyl alcohol (**1** and **I-1**), ethyl hexanoate/d_{11}-ethyl hexanoate (**7** and **I-7**), and ethyl isobutyrate/d_5-ethyl isobutyrate (**13** and **I-13**).

A calibration curve was created for each target analyte using solutions of unlabeled and labeled standard in varying mass ratios (unlabeled:labeled) of approximately 10:1, 5:1, 1:1:, 1:5, and 1:10. Each solution was analyzed by GC-MS using cold splitless injection and an RTX-wax column as previous described. Areas for each compound were determined using selected mass ions in the chromatogram. Peaks were integrated with the assistance of Enhanced Data Analysis Software (Agilent Technologies, USA). The mass ratios (unlabeled:labeled) were plotted against the selected mass ion area ratios (unlabeled:labeled), and a best fit line equation was determined. The slope of the best fit line was used to determine the Response Factor (R_f) for the specific compound, or the Rf can be calculated using the following equation:

$$Rf = \frac{[area\ of\ ion_{target}/area\ of\ ion_{isotope}]}{[mass_{target}/mass_{isotope}]}$$

Calibration curves for each target compound are found in the Appendix (pgs. ***-***).

Quantification

Compounds were quantified in three different bourbons (Jim Beam Bourbon (4 year), Jim Beam Black Bourbon (8 year), Jim Beam Signature Craft (12 year)). These were selected as representing bourbons aged for three different sequentially spaced aging times that originated from the same distillery. All samples were analyzed in triplicate for statistical validity of calculated data.

Target compounds (Table 5.2) were placed into three different groups based on predicted concentration. The high abundance target group consisted of phenethyl alcohol (**1**), ethyl octanoate (**2**), syringaldehyde (**3**), isoamyl acetate (**5**), and vanillin (**12**). The medium abundance group included ethyl hexanoate (**7**), phenethyl acetate (**9**), *cis*/*trans*-whiskey lactone (**10** and **18**), ethyl butyrate (**11**), ethyl vanillate (**14**), isoeugenol (**15**), syringol (**16**), γ-nonalactone (**19**), eugenol (**20**), and guaiacol (**22**). The low abundance target group contained ethyl isovalerate (**17**), 4-ethyl phenol (**21**), β-

damascenone (**23**), p-cresol (**24**), and rotundone (**25**). The high and medium target compounds were monitored on the Mass Spectrometer (MS) by Scan Mode, whereas, the low target compounds required the Selected Ion Monitoring (SIM) mode to be used during MS analysis. For SIM analysis, retention times (RTs) were obtained using authentic standards and used time-windows around each expected RT to fully capture both target compound and labeled compound peaks. To confirm the target peak, two ions for the target compound and two ions for the labeled analogue were monitored during SIM analysis.

Table 5.1: Compounds, Selected Ions and Response Factors used for SIDA

Compound		Selected Ion (*m*/z)		
Target	Isotope	Unlabeled	Labeled	R_f
phenethyl alcohol (**1**)	2-[1,2-$^{13}C_2$]-phenethyl alcohol (**I-1**)	122	124	0.145
ethyl octanoate (**2**)	d_4-ethyl octanoate (**I-2**)	127	131	1.18
syringaldehyde (**3**)	d_3-syringaldehyde (**I-3**)	182	185	1.79
3-methyl-1-butanol (**4**)		--[a]	--	0.467
isoamy acetate (**5**)	3-methyl-[3,4-d_2]-butyl acetate (**I-5**)	70	72	0.651
2-methyl-1-butanol (**6**)		--	--	0.529
ethyl hexanoate (**7**)	d_{11}-ethyl hexanoate (**I-7**)	99	110	0.944
2-methyl propanol (**8**)		--	--	0.615
phenethyl acetate (**9**)	[1,2-$^{13}C_2$]-phenylethyl acetate (**I-9**)	104	106	3.40
cis-oak lactone (**10**)	*cis*-β-methyl-γ-[3,4-d_2]-octanolide(**I-10**)	99	101	0.452
ethyl butyrate (**11**)	d_7-ethyl butyrate (**I-11**)	71	78	0.802
vanillin (**12**)	4-hydroxy-3-[d_3]-methoxybenzaldehyde (**I-12**)	152	155	0.243
ethyl isobutyrate (**13**)	[d_5]-ethyl isobutyrate (**I-13**)	116	121	1.20
ethyl vanillate (**14**)	[d_5]-ethyl vanillate (**I-14**)	196	201	1.56
isoeugenol (**15**)	[d_3]-(E)-isoeugenol (**I-15**)	164	167	0.066
syringol (**16**)	2-[d_3]-methoxy-6-methoxyphenol (**I-16**)	154	157	0.933
ethyl isovalerate (**17**)	ethyl 3-methyl-[3,4-d_2]-butyrate (**I-17**)	115	117	0.759
trans-oak lactone (**18**)	*trans*-β-methyl-γ-[3,4-d_2]-octanolide (**I-18**)	99	101	0.375
γ-nonalactone (**19**)	[3,4-d_2]-γ-nonalactone (**I-19**)	85	87	0.950
eugenol (**20**)	2-[d_3]-methoxy-4-(2-propenyl)-phenol (**I-20**)	164	167	1.39
4-ethyl phenol (**21**)	4-[d_5]-ethylphenol (**I-21**)	122	127	1.02
guaiacol (**22**)	d_3-guaiacol (**I-22**)	124	127	0.603
β-damascenone (**23**)	d_4-β-damascenone (**I-23**)	64	73	0.759
p-cresol (**24**)	d_3-*p*-cresol (**I-24**)	108	111	0.953
rotundone (**25**)	d_4-rotundone (**I-25**)	218	206	0.902

[a]Quantifcation performed by internal standard method

Areas for target compounds and isotopically labeled compounds were determined by integrating only selected mass ions in the chromatogram shown in Table 5.1. Peaks were integrated with the assistance of Enhanced Data Analysis Software (Agilent Technologies, USA). Ratios of the integrated area for labeled and unlabeled selected

mass ions were calculated and used to determine the actual mass of the target compound as follows:

$$concentration_{target} = concentration_{isotope} \times Rf \times \frac{area_{target\ ion}}{area_{isotope\ ion}}$$

Volatile extraction

Spirit samples (10 mL) in the high abundance group were placed in individual 20 mL vials along with the internal standards. The vial was sealed with a PTFE-lined cap and vigorously shaken for 5 min by hand. Samples were then analyzed by direct injection. Spirit samples (10 mL) from the medium abundance group were added to individual 50-mL glass centrifuge tubes containing the internal standards. The alcohol content by volume was reduced to about 10% ethanol with the addition of 30 mL deodorized DI water. The tube was resealed and shaken as described above to obtain a fully mixed solution. After, dichloromethane (1 mL) was added and the tube was recapped and vigorously shaken by hand for another 5 min. The tube was then centrifuged at 7500 RMP for 15 min (IEC HN-SII, Damon/IEC Division, Needham Heights, Massachusetts) to separate the solvent from the aqueous phase. The lower phase (dichloromethane) was transferred to a 1.5 mL vial with sodium sulfate (100 mg) to remove any water and was stored at -20°C prior to analysis.

Low abundance - spirit samples (10 mL) and the internal standards were added to individual 50-mL glass centrifuge tubes. The alcohol content by volume was reduced to about 10% ethanol with addition of 30 mL of deodorized DI water. The tube was sealed with a PTFE-lined cap and vigorously shaken 5 min by hand for to obtain fully mixed solution. This was followed by the addition of dichloromethane (2 mL) after which the tube was recapped and vigorously shaken by hand for another 5 min. The tube was then centrifuged at 7500 RMP for 15 min to separate the solvent from the aqueous phase. The lower phase (dichloromethane) was transferred to a 20 mL vial with sodium

sulfate (2 g) to remove any water. The extraction with dichloromethane (2 mL) was repeated two more times. The final dried extract was condensed to 0.25 mL using a gentle stream of N_2 gas and stored at -20°C prior to analysis.

Quantification of 2-methyl-propanol, 2-methyl-1-butanol, and 3-methyl butanol

The compounds 2-methyl-propanol (**8**), 2-methyl-1-butanol (**6**), and 3-methyl-1-butanol (**4**) were quantified by internal standard methodology and calibration curves were based on the target compound as compared to 2-pentanol. Bourbon samples (10 mL) were spiked with 5 µL of 2-pentanol as the internal standard. The vial was sealed with a PTFE-lined cap and vigorously shaken for 5 min by hand.

Calibration solutions were prepared by spiking a whiskey mimic matrix [40% ABV, prepared by mixing 95% grain alcohol (Everclear®) by volume with 100% natural spring water (ICE mountain, Nestlé Waters North America Inc., Stamford, CT)] with the internal standard, 2-pentanol (5 µL), while varying the amount of target compound added (2, 5, 10, or 15 µL). Each solution was analyzed by gas chromatography using an Agilent 6890 GC equipped with an FID detector. Separations were performed on a DB-5 column (50m length x 0.32 mm i.d. x 1 µm film thickness, J&W Scientific, Folsom, CA). Samples were introduced directly using hot split injection (30:1 split ratio; 260 °C). GC oven temperature was programmed as follows: 40 °C (5 min hold), ramp rate 10°C/min to a final temperature of 225°C (40 min hold time). Helium was used as the carrier gas at a constant flow of 1.6 mL/minute. Calibrations were created as previously described using the area under the compound peak. Samples for quantification were performed in triplicate.

Statistical Analysis

Data were analyzed by one-way Analysis of Variance (ANOVA) for each compound concentration using the Minitab 16 program (Minitab Inc., State College, PA). For

attributes with significant differences across products, Fisher's LSD was used for means separation, with reporting differences at alpha=0.05.

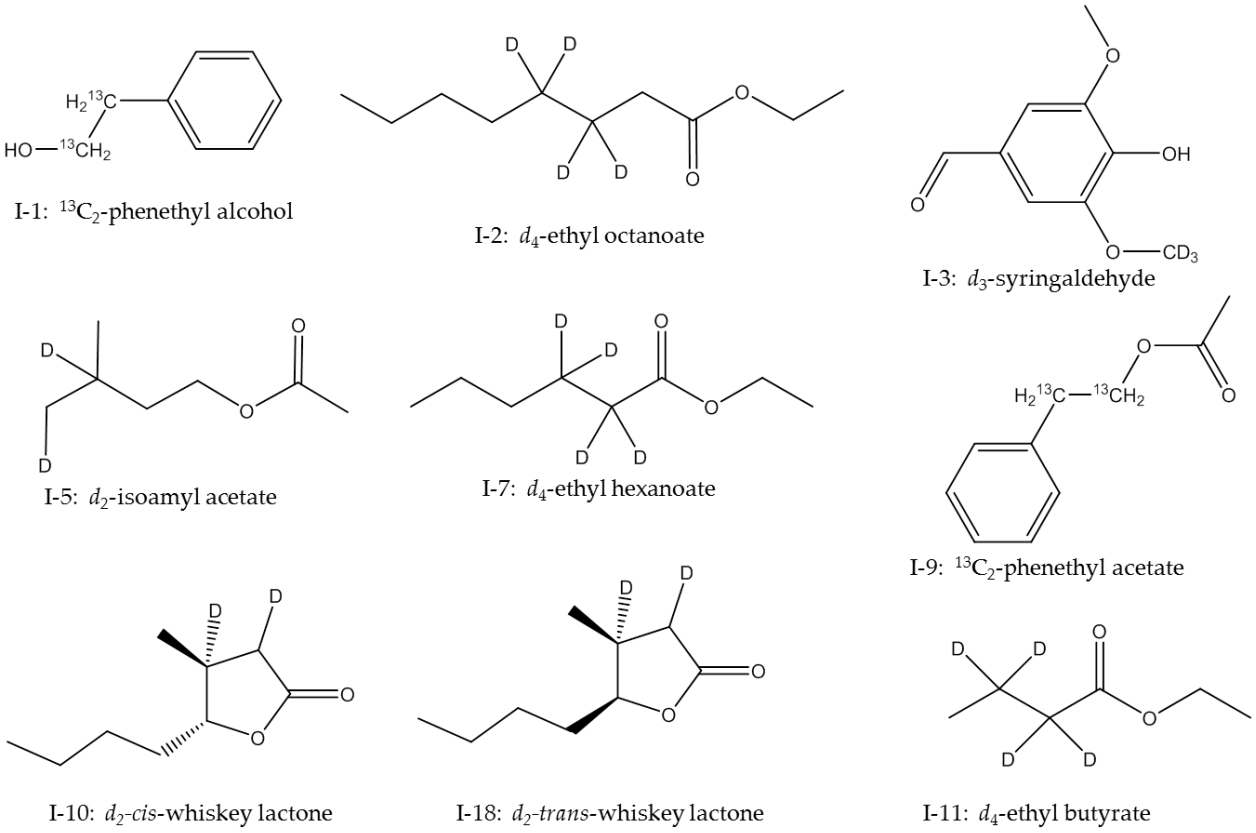
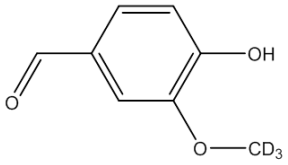
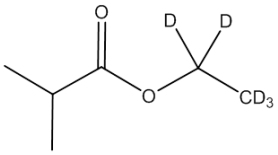


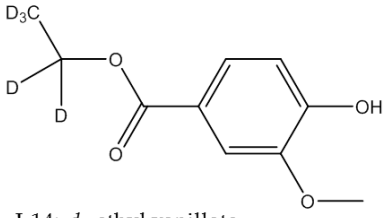
Figure 5.1: Isotopically labeled standards used for quantification



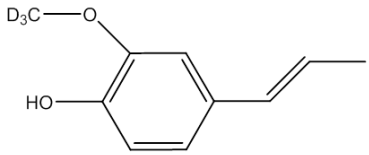
I-12: d_3-vanillin



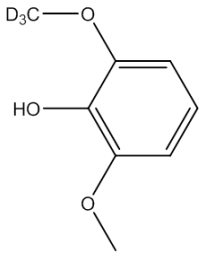
I-13: d_5-ethyl isobutyrate



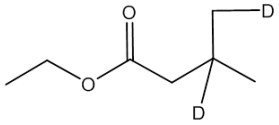
I-14: d_5-ethyl vanillate



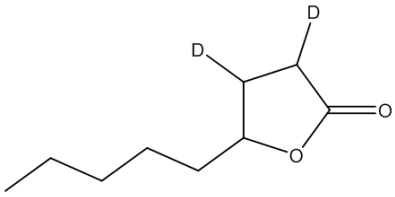
I-15: d_3-isoeugenol



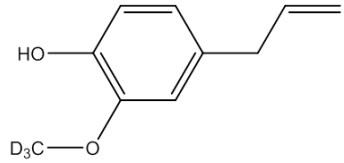
I-16: d_3-syringol



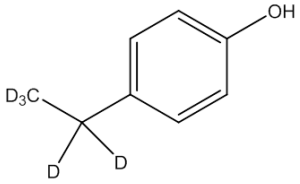
I-17: d_2-ethyl isovalerate



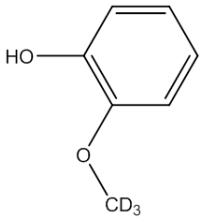
I-19: d_2-γ-nonalactone



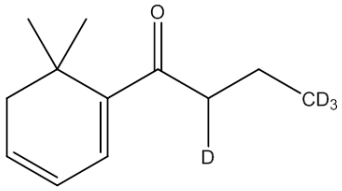
I-20: d_3-eugenol



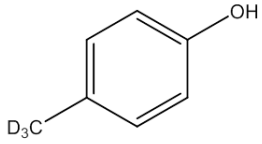
I-14: d_5-4-ethyl phenol



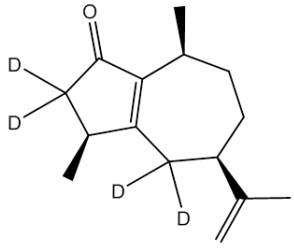
I-22: d_3-guaiacol



I-23: d_3-β-damascenone



I-24: d_3-p-cresol



I-25: d_4-rotundone

Figure 5.1 cont: Isotopically labeled standards used for quantification

5.4 Results and Discussion

Identification of Potent Odorants in Bourbon Whiskey by aroma extract dilution analysis (AEDA)

A total of 38 odorants were identified in the extracts of three bourbon whiskies by AEDA (Table 5.2). Results show that the three bourbons analyzed were consistent with respect to one another's potency rankings of the majority of compounds detected. The present identification results are in agreement with previous studies on the volatile analysis of whiskey (Câmara et al. 2007, Conner et al. 2001, Demyttenaere et al. 2003, Lahne 2010, MacNamara et al. 2000, Poisson et. al. 2008). Interestingly, only two earlier studies reported using AEDA to investigate spirits. One of these studies examined bourbon whiskey (Poisson et al. 2008) and the other rye whiskey (Lahne 2010). Almost all of the potent odorants identified in our study were derived from the oak barrel during aging. The only exceptions were the branched short-chained alcohols 2/3-methyl-1-butanol (**4,6**), and phenethyl alcohol (**1**), which are products of fermentation. The studies on bourbon and rye, noted above, also ranked 2/3-methyl-1-butanol as a high potency odorant.

There were two exceptions in our study with respect to consistency in compound potency rankings among the three bourbons analyzed. These were for *p*-cresol (**24**), described as "band aid", and an unknown compound described as "cereal/burnt". The compound *p*-cresol was detected on the Rtx5 column in two of the bourbons (JB 12 and JB 8) at higher $\log_3$FD factors than on the wax column. Previously, *p*-cresol was undetected in bourbon (Poisson et al. 2008), but was identified as an odorant in rye whiskey (Lahne 2010). The study on rye compared dilutions prepared using two different extraction methods: 1) continuous liquid-liquid extraction dilution and 2) dilution of the neat sample. It was found that *p*-cresol was actually more potent when

using the dilution of a neat sample method. This indicated that *p*-cresol may be ineffectively isolated by solvent extraction, which might explain the inconsistent results of that compound in the present study.

In our study, the most potent odorants in bourbon whiskey, detected consistently in the last or second to last dilution on both columns and all samples, were vanillin (**12**), *cis*-whiskey lactone (**10**), syringol (**16**), phenethyl alcohol (**1**), and guaiacol (**22**). The medium potency compounds were mostly ethyl esters (**7**, **17**, **11**, **13**, **14** and **2**). These are sometimes reversed between samples; which is to be expected. Ethyl esters are formed during the aging process in which ethanol reacts with the wood acids to produce ethyl esters. As these samples were of different ages, the degree at which ethyl esters are formed would be expected to differ, and thus, would not be consistent among the samples. Our study was the first to identify the presence of rotundone (**25**), the target compound of interest, in bourbon whiskey. It ranked among the medium potency odorants, being detected at $\log_3$FD factors from 2 to 4, thus indicating its potential impact on the overall flavor of bourbon. The remaining compounds identified in the medium potency range are well known in oak aged spirits. These included: eugenol (**20**), exhibiting a clove-like aroma, and its isomer isoeugenol (**15**), which has a more fresh/sweet clove aroma; β-damascenone (**23**), typically described as "cooked apple"; syringaldehyde (**3**), a relatively volatile smoky vanilla compound; 4-ethyl phenol (**21**), similar to *p*-cresol with a "bandage" aroma; γ-nonalactone (**19**), described as peach-like and reported to be the second most potent in the previous bourbon study (Poisson et al. 2008) ; phenethyl acetate (**9**), having a floral aroma and resulting from fermentation; and 2-methylpropanol (**8**), described as chocolate or malty.

A ranking of compounds, shown in Table 5.3, was made to determine which compounds would be subjected to quantification and determination of odor activity values (OAV). These calculations were based on AEDA results, and included compounds detected at log3FD of 3 or above. Also included were potentially important

compounds noted in previous studies (e.g. *p*-cresol (**24**)) (Lahne 2010) and compounds that were expected to be in high abundance [e.g., isoamyl acetate (**5**), and *trans*-whiskey lactone (**18**)]. Quantification and subsequent OAV calculation provided the necessary evidence for proof of rotundone's potency among all bourbon odorants.

Table 5.2: Odor Active Compounds Extracted From Bourbon Whiskey

No.	Compound	Odor Description	RI		Flavor Dilution Factor (Log_3FD)[a]						Identification[b]
					JB 4		JB 8		JB 12		
			Wax	Rtx5	Wax	Rtx5	Wax	Rtx5	Wax	Rxt5	
12	vanillin	vanilla	2529	1413	6	5	7	5	8	5	RI, O, MS
10	*cis*-whiskey lactone	coconut	1949	1329	5	5	8	6	8	6	RI, O, MS
16	syringol	smokey/bbq	2241	1355	7	4	8	5	7	5	RI, O, MS
1	phenethyl alcohol	rose	1900	1120	6	4	6	5	7	4	RI, O, MS
22	guaiacol	smoke	1854	1087	7	4	7	5	7	4	RI, O, MS
4,6	2/3-methyl-1-butanol	chocolate/malt	1212	836	8	5	7	5	7	5	RI, O, MS
15	isoeugenol	sweet/fresh/spice	2335	1459	6	3	6	3	6	4	RI, O, MS
20	eugenol	clove	2154	1361	5	4	5	4	6	3	RI, O, MS
23	β-damascenone	cooked apple	1823	1391	4	4	5	4	4	4	RI, O, MS
7	ethyl hexanoate	fruit/grape	1245	1001	4	3	4	3	4	5	RI, O, MS
17	ethyl isovalerate	fruit/berry	1070	857	3	3	4	3	4	4	RI, O, MS
11	ethyl butyrate	fruit	1046	808	3	2	3	3	4	4	RI, O, MS
13	ethyl isobutyrate	fruit	<1000	<800	4	4	4	4	4	4	RI, O, MS
	cis-2-nonenal*	green	1505	1149	2	2	3	2	4	3	RI, O
3	syringaldehyde	smokey vanilla	2884	1671	4	3	4	3	3	3	RI, O, MS
14	ethyl vanillate	sour spice	2642	1580	3	1	3	2	3	1	RI, O, MS
25	rotundone	woody incense	2262	1720	2	2	4	4	3	3	RI, O, MS
21	4-ethyl phenol	smoky bandaid	2167	1180	1	1	1	3	3	1	RI, O, MS
19	γ-nonalactone	peach	2020	1370	3	2	3	3	3	2	RI, O, MS
9	phenethyl acetate	floral	1812	1258	3	1	3	1	3	1	RI, O, MS
2	ethyl octanoate	fruit/tropical	1428	1193	3	1	3	1	3	2	RI, O, MS
8	2-methyl-propanol	chocolate	1099		1	ND	1	ND	3	ND[c]	RI, O, MS
	isovaleric acid	feet	1662		3	ND	3	ND	3	ND	RI, O
5	isoamyl acetate	banana	1147	875	--	1	--[d]	1	2	2	RI, O, MS
	unknown	fruit	1198		1	--	1	ND	2	ND	
	2-octenal*	cereal	1314	1052	2	1	1	1	2	2	RI, O
	acetic acid	sour/vinegar	1444	832	1	ND	--	ND	2	1	RI, O, MS
	trans-2-nonenal*	green	1537	1164	1	--	2	1	2	1	RI, O
	butyric acid	cheese	1619	853	2	1	2	--	2	2	RI, O, MS
	trans,trans-2,4-nonadienal*	cucumber	1700	1157	2	--	1	1	2	2	RI, O
	unknown	cereal/burnt	1801	1050	2	1	4	--	4	2	
18	*trans*-whiskey lactone	coconut	1882	1291	2	1	2	1	2	2	RI, O, MS
	p-vinyl guaiacol	curry	2174	1320	2	ND	1	1	2	2	RI, O
	unknown	soapy	2456	1555	1	ND	1	--	2	--	
24	*p*-cresol	bandaid	2074	1109	1	1	--	3	1	3	RI, O
	cis-6-dodeceno-γ-lactone	cilantro	2384	1666	1	--	--	ND	1	1	RI, O
	1-octen-3-one*	metallic	1306	982	1	ND	--	1	--	2	RI, O
	γ-decalactone	peach	2139	1465	ND	ND	ND	ND	--	--	RI, O
	β-ionone	floral		1427	ND	ND	ND	--	ND	--	RI, O
	4-ethyl guaiacol	spice	1994	1283	--	ND	--	ND	--	2	RI, O

[a] Log3FD assigments, * denotes tentative identification, [b] Identifcation criteria: rentention index (RI), odor quality (O), mass spectra (MS)

1 = 1:3, 2 = 1:9, 3 = 1:27, 4 = 1:81, 5 = 1:243, 6 = 1:729, 7 = 1:2173, 8 = 1:6561, [c] ND, not deteced, [d] -- detected in original extract.

Concentrations and odor activity values (OAV) of potent odorants in bourbons aged in oak (4, 8, and 12 years).

A total of 25 odorants identified in bourbon whiskeys were subject to quantification by SIDA (Table 5.3). Before a discussion of rotundone's ranking among the potent odorants, some interesting observations as to its concentration with respect to aging times can be made. An ANOVA, performed considering each compound's concentration as compared to the time aged in an oak barrel, revealed that all 25 compounds significantly differed over time. Furthermore, 16 of the 25 compounds showed a defined linear trend. Although concentrations changed over time, the order from most to least concentrated within each sample was fairly consistent with phenethyl alcohol (**1**) being the most abundant while compounds **22**-**25**, although the least abundant across all ages, were consistent with respect to order. Our target compound, rotundone, was consistently present at the lowest concentration among all odorants across all samples.

Several reports were published about the effect of aging on the concentration of volatiles in wine (Pérez-Prieto et al. 2003 and Arapitsas et al. 2003), cider (Fan et al. 2006 and Madrera et al. 2003), and whiskey (MacNamara et al. 2000). They report that the ethyl esters (ethyl ocatanoate, ethyl hexanoate, ethyl butyrate, ethyl isobutyrate, ethyl vanillate, and ethyl isovalerate) increased over time. These findings illustrates that ethanolysis was not selective as to the acid backbone structure with which it reacts. This was supported in the present study as aliphatic, branched chain, and aromatic acids showed similar trends, i.e. increasing concentrations over an 8 year aging period (4 years to 12 years). These observations also agree with the past studies done on cider which monitored changes in ethyl acetate, octanoic acid, and ethyl octanoate (Madrera et al. 2003) and which demonstrated that ethyl acetate steadily increased showing a change in as little as 30 months aging time.

Table 5.3: Concentrations of Selected Odorants in Bourbons Aged at Different Times

No.	Compound	Concentration (μg/L)(ppb) Jim Beam Bourbon (4 year)	Jim Beam Black Bourbon (8 year)	Jim Beam Signature Craft (12 year)	ANOVA[a] R^2 value
1	phenethyl alcohol	45300 (2.5)[b]	40800 (0.90)[b]	39100 (3.6)[b]	0.845
2	ethyl octanoate	9380 (0.74)	9930 (1.7)	15500 (2.4)	0.810
3	syringaldehyde	7530 (2.8)	10400 (0.74)	14400 (2.4)	0.985
4	3-methyl-1-butanol	5797 (10)	8660 (10)	9330 (6.8)	c
5	isoamy acetate	5960 (1.3)	4250 (3.4)	6670 (10)	c
6	2-methyl-1-butanol	1871 (5.6)	3040 (10)	4240 (6.0)	0.951
7	ethyl hexanoate	1890 (4.4)	2270 (2.9)	3310 (1.1)	0.922
8	2-methyl propanol	1450 (10)	1930 (7.7)	2780 (10)	0.862
9	phenethyl acetate	1980 (8.7)	1400 (1.7)	2030 (4.0)	c
10	*cis*-whiskey lactone	1080 (3.1)	1460 (3.6)	1670 (3.4)	0.947
11	ethyl butyrate	421(10)	861 (5.0)	1200 (1.7)	0.977
12	vanillin	619 (1.9)	950 (2.3)	1410 (1.5)	0.988
13	ethyl isobutyrate	179 (9.2)	394 (1.7)	600 (7.0)	0.977
14	ethyl vanillate	115 (9.4)	274 (5.1)	422 (3.8)	0.987
15	isoeugenol	306 (1.9)	368 (2.8)	416 (6.3)	0.874
16	syringol	205 (10)	265 (10)	219 (3.9)	c
17	ethyl isovalerate	74 (8.6)	157 (8.6)	208 (1.0)	0.977
18	*trans*-whiskey lactone	119 (1.1)	169 (3.4)	205 (3.9)	0.966
19	γ-nonalactone	159 (4.9)	190 (5.8)	164 (3.2)	c
20	eugenol	207 (3.2)	197 (1.6)	131 (4.3)	0.825
21	4-ethyl phenol	727 (0.58)	58.4 (0.67)	91.2 (7.4)	c
22	guaiacol	39.1 (6.2)	56.7 (3.9)	63.5 (3.5)	0.894
23	β-damascenone	2.63 (6.8)	4.14 (9.8)	3.02 (6.1)	c
24	*p*-cresol	1.80 (3.5)	2.32 (3.7)	2.36 (6.4)	c
25	rotundone	0.342 (0.54)	0.403 (1.4)	0.453 (1.9)	0.985

[a]one-way Analysis of Variance (ANOVA) for each compound concentration. For attributes with significant differences across products, Fisher's LSD was used for means separation, reporting differences at alpha=0.05. [b]the standard deviation of the mean (%). c = a significant difference was found but could not be linearly defined.

More interestingly, both octanoic acid and its corresponding ethyl ester, ethyl octanoate, were monitored in the same study and, as expected, octanoic acid decreased over time while ethyl octanoate gradually increased. It is noteworthy that in the current study an increase in ethyl octanoate was observed even in bourbons aged for 12 years. MacNamara et al. (2000) also observed this trend occurring with ethyl vanillate in

whiskeys aged 10 years. One might expect that a plateau would eventually be observed, as the starting material for the ethanolysis becomes depleted. However, the concentrations could also increase as a result of ethanol evaporation. As the ethanol decreases reducing the total volume, the resulting product would be more concentrated. One way of confirming if there is a steady increase in the ethyl ester over time would be to monitor the acids to see if, in fact, they are still available after many years, or if the increase is due to other factors.

A linear increase with time was also observed for the important oak wood extractives vanillin, guaiacol, syringaldehyde, *cis*-whiskey lactone and the *trans*-whiskey lactone. These compounds are derived directly from the oak wood, so a valid assumption may be that the longer the spirit is in contact with the wood, the greater the amount that would be extracted. The whiskey lactones, in particular, are considered to be among the most important components of the oak influence on whiskey and correlate with a positive assessment of whiskey flavor (Otsuka et al. 1974). An increase in the concentration of whiskey lactones over time was consistently observed in previous studies. Their high concentration in spirits is attributed to the layer of active carbon on the cask, produced during the charring step, which increases the extraction of the whiskey lactones (Madrera et al. 2003). In our study, vanillin had the strongest linear relationship to increased aging time with an R^2 value of 0.988. This is in agreement with previous reports (Spillman, et al. 1998). The compound vanillin was observed to form not only during the charring step, and subsequently extracted into the spirit, but it also was formed while aging by a hydrolytic mechanism, in which whiskey being slightly acidic causes the acid hydrolysis of the lignin during the aging process resulting in the formation of vanillin (Spillman, et al. 1998). Guaiacol and syringaldehyde are also formed during the charring step as a result of lignin pyrolysis. However, the concentration of guaiacol, with an odor threshold of about 10 ppb, was found at considerably lower concentrations than vanillin, only being found in the 30 ppb range

in the 4 year bourbon. Its increase over time has the potential to impact the flavor of an older whiskey resulting in a more pronounced smoky note, whereas in a young whiskey it may be undetected.

The relationship between eugenol and isoeugenol is also of great interest. Both have a linear trend during aging. However, the isoeugenol concentration increased over time; whereas, the concentration of eugenol decreased over time. This was also observed in model studies involving the artificially aging of apple cider using oak chips (Fan et al. 2006). Commonly, isoeugenol is synthesized from eugenol by migration of the double bond (Kishore et al. 2004), but it has also been observed in nature in some plants which contain a high level of isoeugenol. These plants produce an enzyme which catalyzes the formation of isoeugenol from eugenol (Koeduka et al. 2006). It can, therefore, be speculated that oak wood might also contain such an enzyme. Both phenethyl alcohol, 2-methyl-propanol and 2-methyl-1-butanol, products of yeast fermentation, increased as a function of aging time. Although they increased linearly with time, it is unlikely that their increasing concentrations are related to extraction from the oak cask. The concentrations would naturally be influenced by fermentation time, yeast species present, and distillation technique. Their increasing during barrel aging, however, may be a result of concentration effects due to evaporation of water over time. The last compound to increase linearly over time was rotundone, our target compound of interest. Although it was found at the lowest concentration among all compounds quantified, this was not a direct reflection of its potency.

It is common to calculate the odor activity value (OAV), the ratio of the concentration of a compound to its odor detection threshold, for each compound. For this purpose, aroma thresholds, previously measured using a ~10% ABV wine matrix, were used as we diluted our bourbon sample to 10% to obtain maximum aroma extraction. Results from the OAV calculation are shown in Table 5.4. Generally the odor of a compound with an OAV above 1 is considered to be detectable in a product. Of the compounds

quantified, 18 out of the 25 have OAVs above 1. Most of the ethyl esters fall into this category with the exception of ethyl vanillate, which was much lower than 1. Of the oak derived odorants, 4 of the 13 did not qualify as potential flavor contributors. The OAV of syringol, described as smoky/sweet, is close to or just at about 1. At this value it could be manipulated by increasing its concentration during wood production to potentially create a whiskey with a more pronounced smoky/sweet note of syringol. Those compounds with OAVs well above 1 are: isoeugenol, β-damascenone, rotundone, eugenol, *cis*-whiskey lactone, vanillin, guaiacol, and γ-nonalactone. The compound 4-ethyl phenol was well above 1 in the 4 year bourbon, but well below that in the 8 and 12 year bourbons. This was noted elsewhere (Lahne 2010) with 4-ethyl phenol differing by 10 fold between 2 similar rye whiskeys, suggesting that several factors contribute to its concentration in aged spirits. The *trans*-whiskey lactone along with *p*-cresol and syringaldehyde were all below 1. Both syringaldehyde and *trans*-whiskey lactone have fairly high thresholds, meaning their concentrations would need to relatively high to be detectable.

This was the first study in which rotundone was identified and quantified in spirits. Although rotundone was measured in the parts-per-trillion range, it's extremely low threshold yielded an OAV well above 1, i.e. 42.8 in 4 year to 50.4 in 8 year to 56.6 in 12 year aged bourbons. Thus, it can be concluded that rotundone was distinctly detectable and impacted the flavor of these bourbon whiskeys.

Table 5.4: Odor Activity Values of Potent Aroma Compounds in Bourbon

No.	Compound	Threshold (μg/L)(ppb)	Odor Activity Value[a] Jim Beam Bourbon (4 year)	Jim Bean Black Bourbon (8 year)	Jim Beam Signature Craft (12 year)
2	ethyl octanoate	2[b]	4690	4960	7750
7	ethyl hexanoate	5[b]	379	455	662
5	isoamy acetate	30[b]	199	142	222
17	ethyl isovalerate	2[b]	37.2	78.6	104
15	isoeugenol	6[g]	51.0	61.4	69.4
23	β-damascenone	0.05[b]	52.5	82.7	60.4
11	ethyl butyrate	20[b]	21.1	43.1	60.2
25	rotundone	0.008[h]	42.8	50.4	56.6
13	ethyl isobutyrate	15[b]	11.9	26.3	40.0
20	eugenol	5[b]	41.4	39.4	26.2
10	*cis*-whiskey lactone	67[e]	16.1	21.7	25.3
9	phenethyl acetate	250[b]	7.94	5.59	8.11
12	vanillin	200[b]	3.10	4.75	7.03
22	guaiacol	9.5[d]	4.11	5.97	6.69
19	γ-nonalactone	30[d]	5.29	6.32	5.48
1	phenethyl alcohol	10000[b]	4.53	4.08	3.91
16	syringol	250[c]	0.818	1.06	0.876
14	ethyl vanillate	900[c]	0.128	0.305	0.468
4	3-methyl-1-butanol	30000[b]	0.193	0.289	0.311
3	syringaldehyde	50000[b]	0.151	0.209	0.289
18	*trans*-whiskey lactone	790[e]	0.151	0.214	0.260
21	4-ethyl phenol	440[f]	1.653	0.133	0.207
24	*p*-cresol	12[f]	0.150	0.193	0.197
6	2-methyl-1-butanol	30000[b]	0.062	0.101	0.141
8	2-methyl propanol	40000[b]	0.036	0.048	0.070

[a]OAVs were calculated using the thresholds reported from the following: [b]Guth 1997 (10% v/v ethanol/water matrix, [c]Lopez et al. 2002 (10% v/v ethanol/water matrix), [d]Ferreira et al. 2000 (11% v/v ethanol, 7 g/L glycerine, 5 gof tartaric acid, pH adjusted to 3.4), [e]Otsuka et al. 1974 (30% v/v ethanol/water), [f]Boidron et al. 1988 (12% v/v ethanol/water, 8g/L glycerin, and different salts), [g]Cullere et al. 2004 (10% water/ethanol mixture, 5 g/L tartaric acid pH adjusted to 3.2), [h]Wood et al (water)

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Chapter 6: Summary, Conclusions, and Future Research

The practice of barrel aging of spirits has been used for centuries. It may have begun as an alternative storage and transportation method, but aging in an oak cask is now as so used as a means to impart flavor to the spirits. Oak wood has been selected as the wood of choice for barrel making not only for its physical characteristics that lend themselves to manufacturing a barrel, but also due to its unique chemical properties that impart to aged spirits the key flavors that are desired by the consumer. All woody and plant material contain lignin. When heat treated, as occurs during the charring step of barrel making, lignin degrades to produce volatile compounds which impart smoky, spiced, vanilla, etc. notes to the aged spirits. Oak, however, has additional components that when charred bring out unique flavor characteristics. One example is the formation of lactones formed by rearrangement of free fatty acids. The most infamous of these in oak are *cis*/*trans*-β-methyl-γ-octalactone isomers, also known as the "oak" lactone, or "whiskey" lactone. These lactones and in particular the *cis* isomer is considered one of the most important flavor compounds in whiskey, contributing a coconut-like aroma. Not only is it relatively abundant, but it is also very potent. Another example is the presence of two distinct carotenoids, β-carotene and lutein, in oak wood. Flavor compounds derived from these carotenoids include β-damascenone and β-ionone. Both of these are also found in aged spirits, and attribute a cooked apple and floral note, respectively. As thoroughly researched as oak wood and oak aged spirits are, the identity of the component responsible for the "woodiness" flavor attribute of age spirits was, prior to this report, unknown. Previous research cited the presence of an unknown compound with such an aroma character, and mentioned that additional research was needed to discover its identity. Thus, the main objective of this study was to find and identify this unknown woody compound. It was hypothesized

that this compound exists in both oak wood and in oak aged spirits. The goal was to isolate the compound; quantify it; and calculate its relative potency within whiskey to establish if it contributes to the overall flavor of aged distilled spirits.

Aroma actives in oak wood were first identified via gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS) analysis. The presence of an unknown compound was confirmed, and described as "woody/incense". Due to its extremely low abundance within an extremely complex extract, i.e. oak wood contains thousands of compounds, a custom built GC-MS/O/FID instrument equipped with a heart-cutting Dean's switch and a CyroTrap was implemented to acquire interpretable and searchable electron-impact mass spectrum (EI-MS) for the compound. While positive EI-MS match was not initially found for the compound, the spectrum did indicate that it had a molecular mass of 218. A widespread search for compounds with this mass previously identified in other aromatic woods, spices, herbs, and roots was performed in hopes of finding a source containing a higher abundance of the compound. With clues of its molecular weight from the EIMS and its functional groups from reactionary experiments, a highly potent sesquiterpene ketone from an obscure root, *Cyperus rotundus*, was a suspect and identified to be 5-isopropenyl-3,8-dimethyl-3,4,5,6,7,8-hexahydro-1(*2H*)-azulenone, or rotundone. The compound was then positively identified by comparison of its chromatographic (retention indices) and spectral data (EI-MS) to those of the authentic reference standard (obtained by synthesis).

The concentration of rotundone in various oak aged spirits was measured though application of stable isotope dilution analysis (SIDA) and the synthesis of a labeled isotope of rotundone. A representative sampling set of spirits, including seven bourbon whiskeys, Tennessee whiskey, Scotch whiskey, rye whiskey, aged rum, and añejo tequila were selected and analyzed. Results confirmed that rotundone is indeed transferred from the oak wood into the aged spirits and that it was present in an

extremely low concentrations ranging from 0.150 μg/L to 1.345 μg/L in this particular set of samples. It became clear that the amount of rotundone was influenced by several factors including aging time and potentially species of oak wood, location of tree cultivation, post-harvest wood handling, humidity and temperature of the storage conditions while aging, and barrel size to spirit volume ratio.

It is suggested that future studies determine rotundone's origin and concentration within the oak wood to potentially control its migration into the spirits. It is also strongly suggested that the kinetics of rotundone's extraction from oak and into the ethanol/water matrix be determined as this current study does show evidence of aging time being a direct influence of rotundone within aged spirits.

During this study, interestingly rotundone was also found to be present in un-aged, silver tequila. It is hypothesized that agave leaves, which are already known to contain monoterpenes and sesquiterpenes, might also contain rotundone. It would be important to any future studies on tequila to confirm whether or not rotundone also comes from agave, and to show how much in the final aged product comes from the raw distillate versus the oak barrel.

Although rotundone was identified in oak wood and quantified in oak aged spirits, it was important to determine its relative potency or influence to the flavor of aged spirits. This study performed an aroma extract dilution analysis (AEDA) on three bourbons of different oak aging times to determine which compounds were the most potent. Compounds were ranked more accurately in order of importance through the calculation of their odor-activity values (OAVs). To calculate OAVs, each compound was first quantified in the three different bourbons aged 4, 8, and 12 years. From the quantification data some interesting aging trends were established that demonstrate some effects of barrel aging. In general, potent ethyl esters, as a result of ethanolysis of the wood acids increased with time as well as some other compounds derived from the oak such as guaiacol, vanillin, syringaldehyde, β-damascenone and the *cis*/*trans*-

whiskey lactone. The compounds eugenol and isoeugneol both showed a linear trend over time, not surprisingly as isoeugenol increased, eugenol decreased. It is generally thought that isoeugenol is formed from eugenol via a double bond migration. The compounds originating from fermentation did not show clear trends during oak aging, and are best monitored during that step in spirit manufacturing. The target compound, rotundone, also increased with aging time; showing its potential to be more impactful in longer aged spirits. It would be important for future studies to monitor the concentration of these important compounds starting from time zero. Although we showed the trends from 4 to 12 years, it is unknown what happened during the first four years of aging. It would be useful to know whether compounds rapidly increased in concentration early in the aging process and slowly level out, or whether there was a steady increase in concentration over time.

Calculation of OAVs was the final step in demonstrating the importance of rotundone to whiskey flavor. It is generally understood that compounds with an OAV above 1 (i.e., when a compound's concentration is higher than its aroma threshold), are considered detectable in the product. With an OAV of 42.8 - 56.6, rotundone lies well above this requirement and is among the top 10 odorants quantified in these samples. Thus we conclude that rotundone is an important contributor to the flavor of bourbon whiskey. Future studies are suggested, possibly employing aroma models and omission studies, to demonstrate our conclusion of rotundone as an important contributor. Lastly, it would be interesting to see how the concentration of rotundone affects the overall flavor attributes of whiskey, and whether it conclusively increases the woody descriptor in aged spirits as its concentration increases.

Appendix A: Chemical Synthesis

Rotundone

1) In a 100 mL round bottom flask, with a N_2 gas purge, guaiaol (10 g; 45 mmol) in pyridine (15 mL) was kept at -30°C while thionyl chloride (6.18 g; 3.8 mL; 52 mmol) was added drop-wise.
2) After 2 hours, additional thionyl chloride (1.55 g; 0.95 mL; 13 mmol) was added and keep under N_2 gas overnight at -30°C.
3) Quench with 25% HCl (50 mL) and extract with diethyl ether (3 x 30 mL).
4) After removal of the solvent, add the crude mixture containing guaiene to acetonitrile (125 mL) in a 250 mL round bottom flask kept 0°C. While stirring the solution, using a magnetic stir bar, add cobalt acetate tetrahydrate catalyst (0.600 mg) and tert-butyl hydroperoxide (60 mL of 5.0-6.0 M in decane).
5) Leave stirring at 0°C and check hourly by GC-MS until the guaiene peak is gone.
6) Quench with saturated sodium sulfite (150 mL) to remove acetonitrile and extract with diethy ether (3 x 30 mL).
7) Wash diethyl ether extract with saturated sodium bicarbonate (3 x 50 mL), and subject crude mixture, containing rotundone to purification by silica flash column.

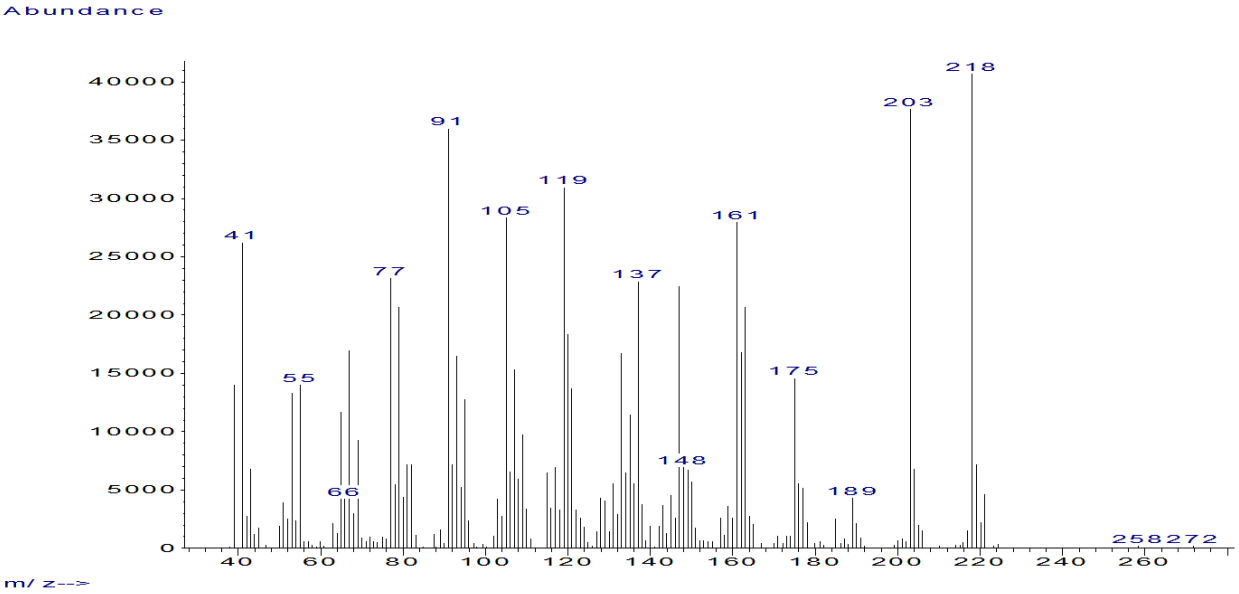
d$_4$-Rotundone

1) In an amber vial equipped with a stir bar, rotundone (10 mg, 0.0459 mmol) in pyridine (5 mL) was placed under a gentle stream of N_2 gas.
2) While stirring, add ^2H_2O (1.0 mL; molar xs) and 3 drops of NaOD, purge the vial and leave stirring at room temperature for 24 hrs. Check for completion periodically by extracting 50 µL of reaction mixture and transfer to a vial containing 1 mL of aqueous HCl (1 N) and 0.5 mL diethyl ether. Mix the contents and check the solvent layer by GC-MS analysis.
3) Quench with ice cold water (25 mL) and slowly adjust the pH to ~2 with HCl and extract with diethyl ether (3 x 10 mL).
4) Wash ether extract with aqueous saturated sodium chloride (3 x 5 mL) and dry over 2 g anhydrous sodium sulfate.

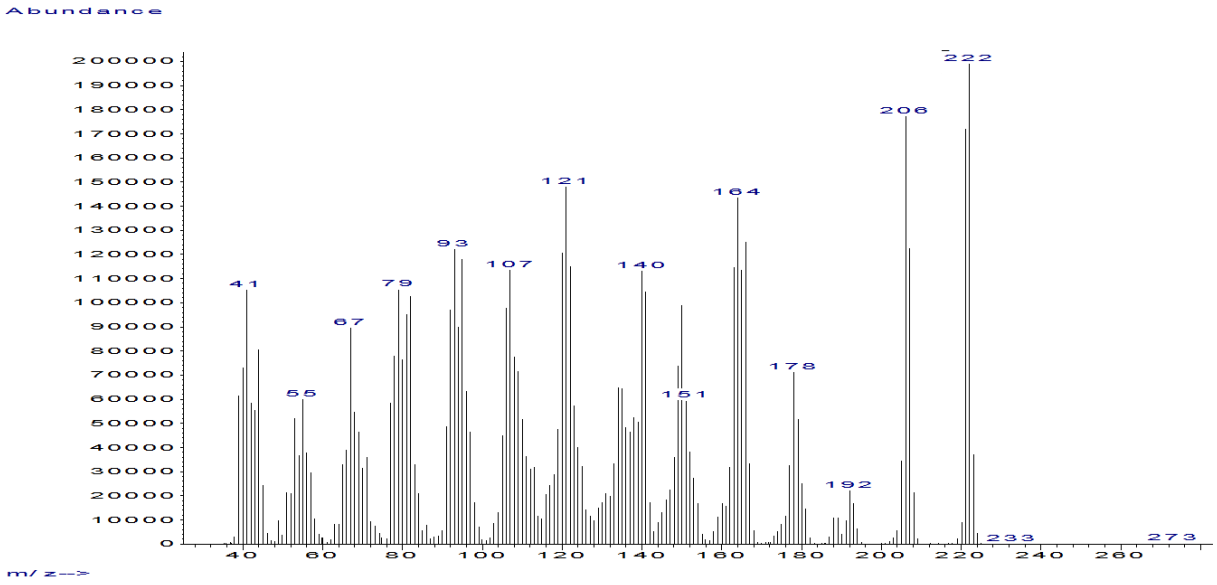
d_4-Ethyl octanoate

1) In a vial (25 mL) add d_4-octanoic acid (0.2 g; 1.35 mmol), ethanol (5 mL) and 3 drops of sulfuric acid.
2) Bake at 60°C for 3 hours.
3) Quench reaction with water (50 mL) and extract with diethyl ether (3 x 30 mL).
4) Dry over sodium sulfate.

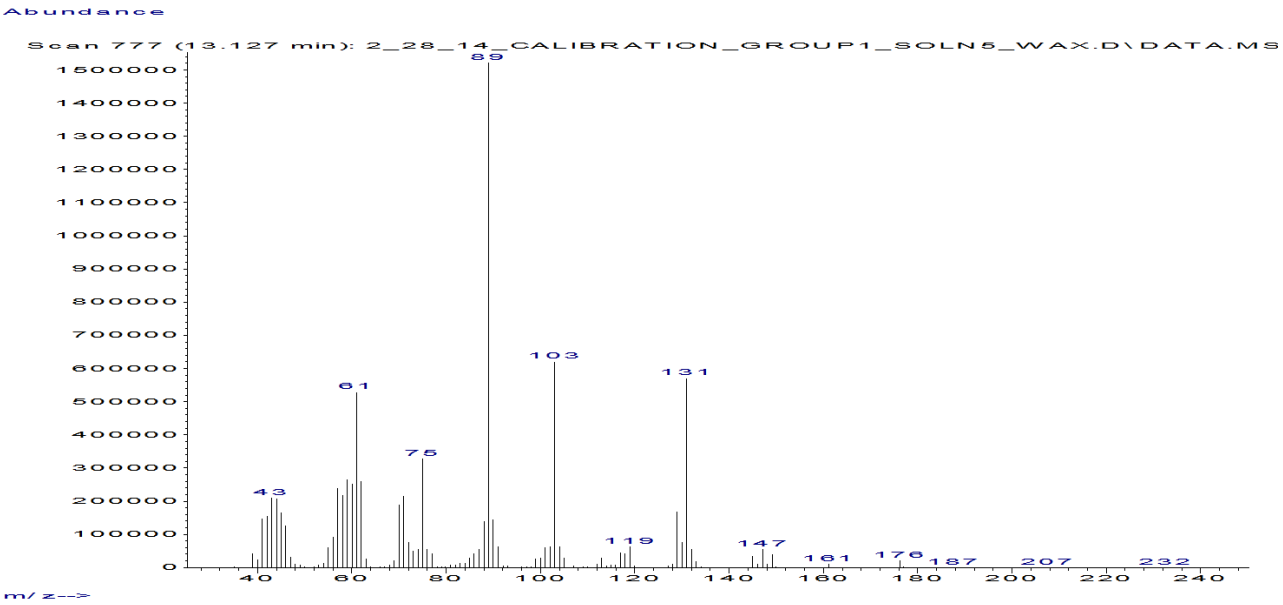
Appendix B: Chemical Spectrum



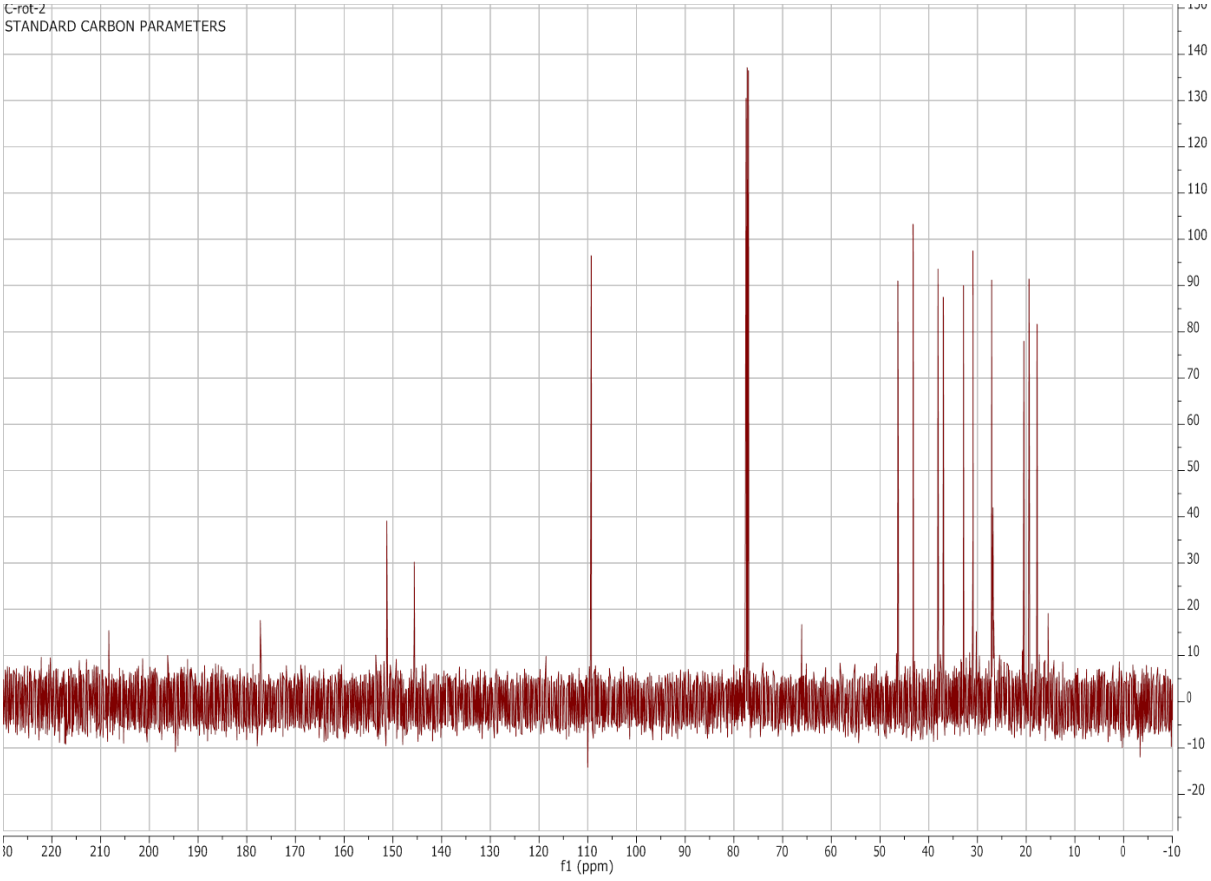
Mass Spectra Graph - rotundone



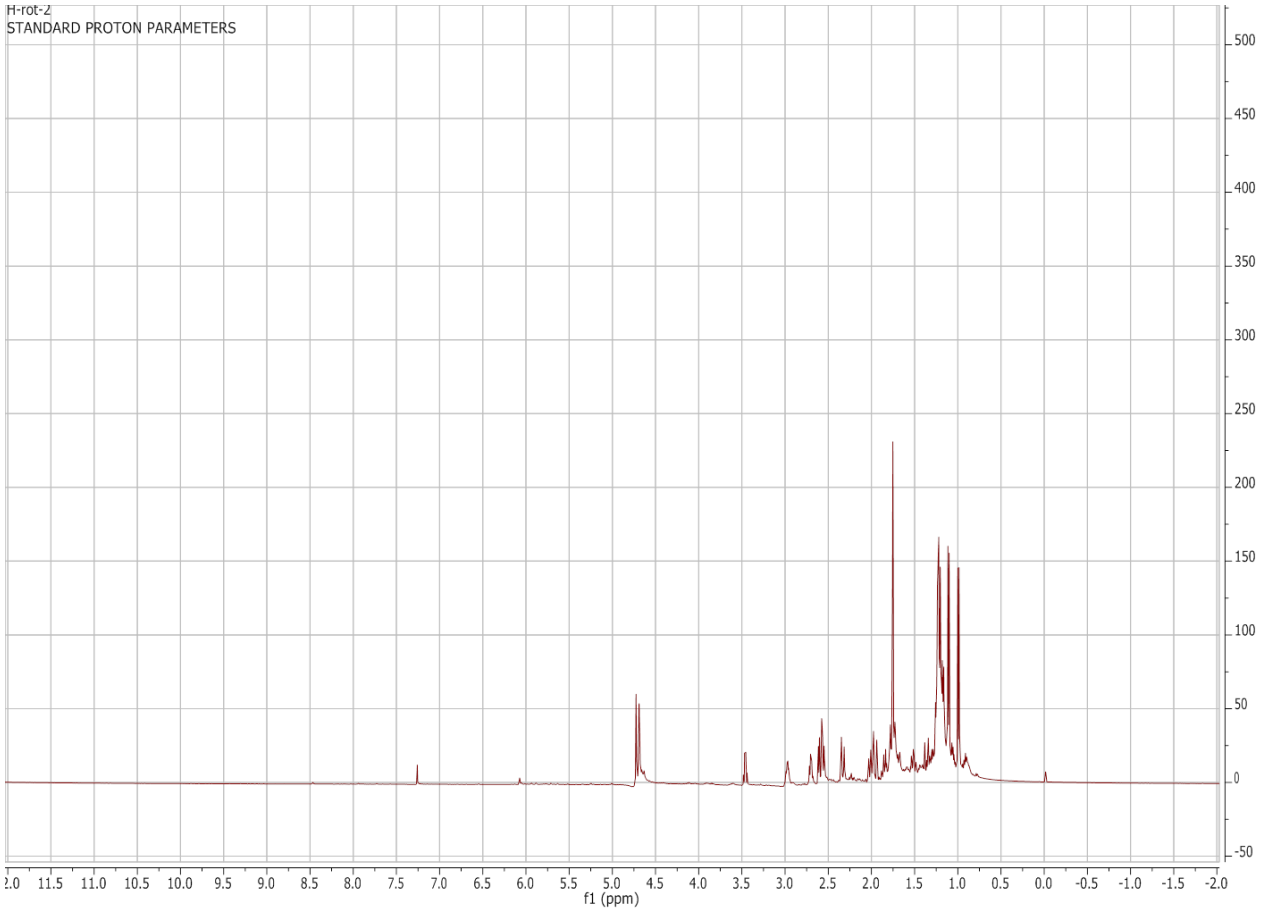
Mass Spectra Graph – d_4-rotundone



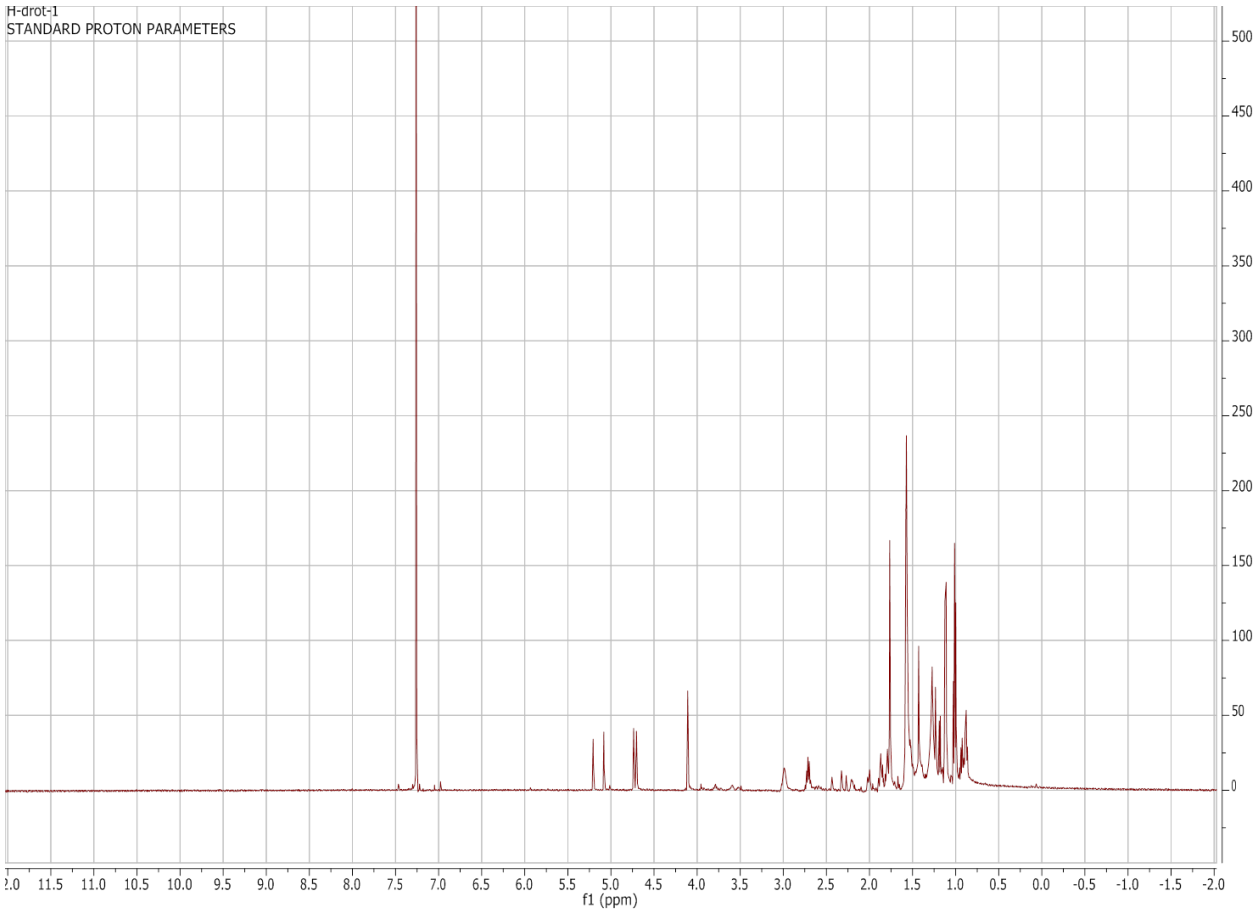
Mass Spectra Graph – d_4-ethyl octanoate



C-NMR Spectra Graph –rotundone



H-NMR Spectra Graph –rotundone

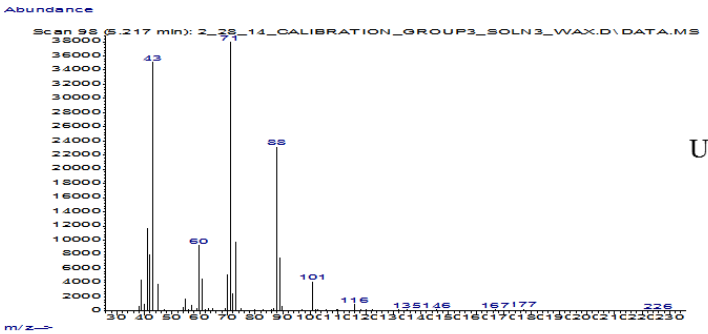


H-NMR Spectra Graph – d_4-rotundone

Appendix C: Calibration Curves

Response Factor of d_7-ethyl butyrate

	Isotope	Unlabeled
Standard:	d_7-ethylbutyrate	ethylbutyrate
CAS:	N/A	105-54-4
Mfg/Reference:	CDN	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-17	283; E1,570-1; 077721kQ
% Purity (by GC-FID)	N/A	99.90%



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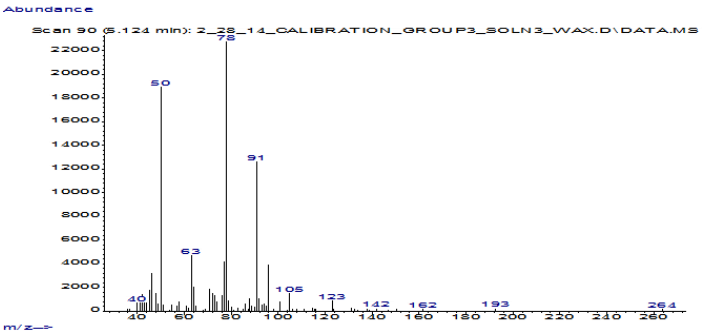
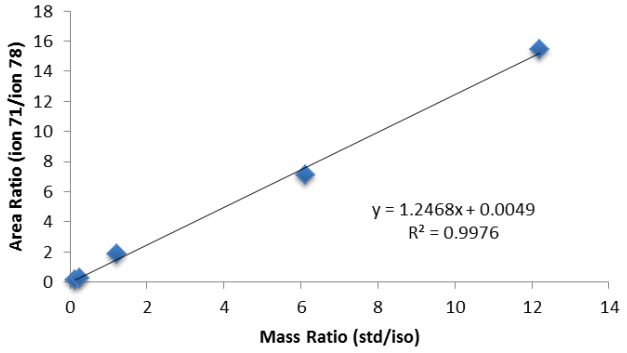


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		78	71	
mass ratio	0.12	17363764	2373168	0.136673592
	0.24	8489608	2104865	0.247934298
	1.22	816028	1532438	1.877923307
	6.11	465710	3322575	7.134429151
	12.21	1017154	15703404	15.43857076

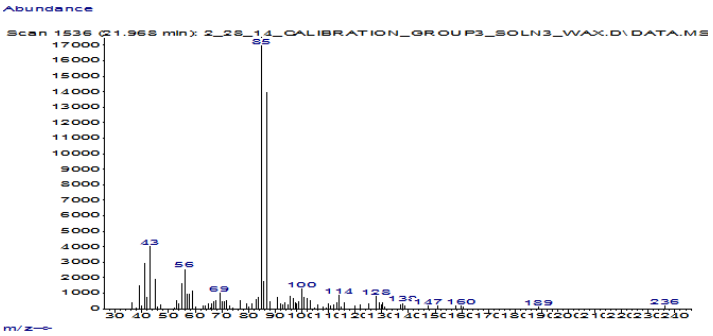
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
1.2468	0.802

Response Factor of d_2-γ-nonalactone

	Isotope	Unlabeled
Standard:	d_2-γ-nonalactone	γ-nonalactone
CAS:	N/A	104-61-0
Mfg/Reference:	synthesized	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-58	438; W27810-6; 07001HD
% Purity (by GC-FID)	N/A	99.80%



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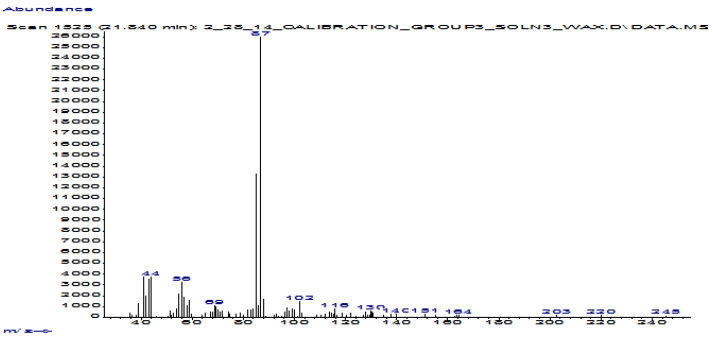
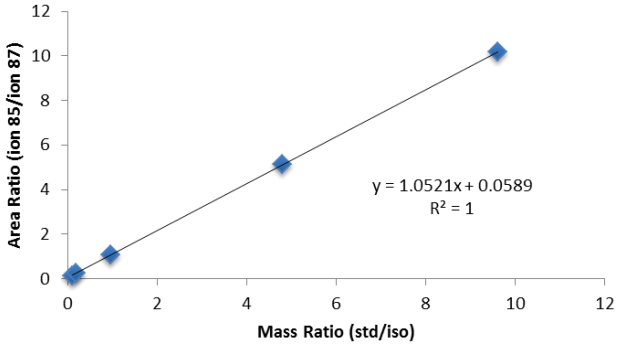


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		87	85	
mass ratio	0.1	90296597	12545463	0.138936166
	0.19	43712493	11117498	0.25433228
	0.96	3539143	3856227	1.089593441
	4.81	2382121	12246382	5.140957155
	9.62	7726804	78566978	10.16810806

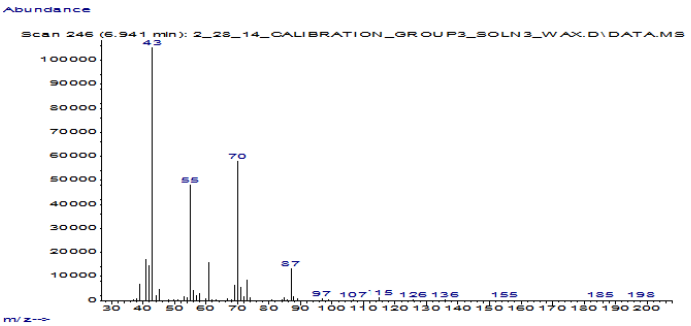
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
1.0521	0.950

Response Factor of d_2-isoamyl acetate

	Isotope	Unlabeled
Standard:	d_2-isoamyl acetate	isoamyl acetate
CAS:	N/A	123-92-2
Mfg/Reference:	synthesized	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-84	74; 11267-4; N/A
% Purity (by GC-FID)	N/A	97.57%



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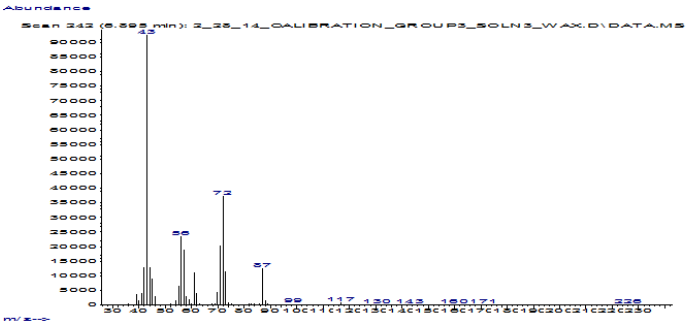
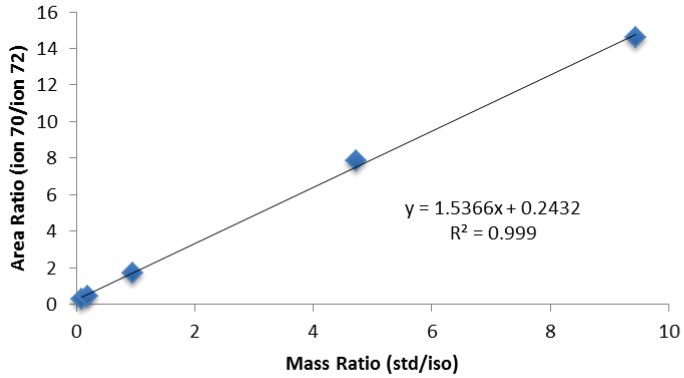


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		72	70	
mass ratio	0.09	15391803	4696250	0.305113702
	0.19	7935627	3585716	0.45185037
	0.95	970882	1637343	1.686449023
	4.73	604233	4746904	7.856082008
	9.45	1306127	19064353	14.59609441

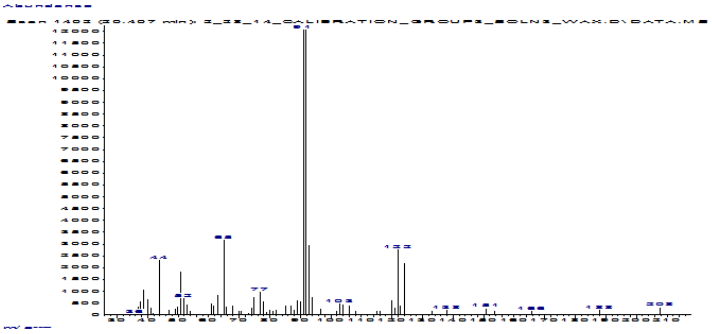
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
1.5366	0.651

Response Factor of $^{13}C_2$-phenethyl alcohol

	Isotope	Unlabeled
Standard:	$^{13}C_2$-phenethyl alcohol	phenethyl alcohol
CAS:	N/A	60-12-8
Mfg/Reference:	synthesized	SAFC, St. Louis, MO
No.; Catalog#; Batch#/Lot#:	ISO-75	377; W285803; MKBG5642V
% Purity (by GC-FID)	N/A	99.80%



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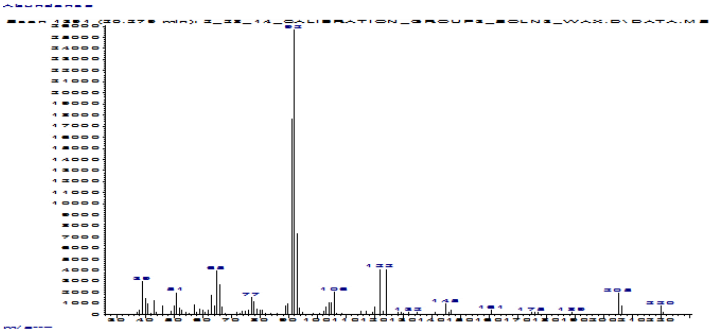


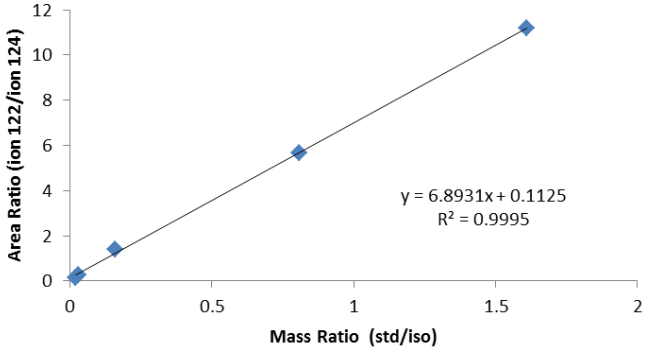
labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		124	122	
mass ratio	0.02	17421825	2799409	0.160684027
	0.03	10660232	2659521	0.249480593
	0.16	796569	1116958	1.402211233
	0.81	438442	2487343	5.673140347
	1.61	1481237	16598153	11.20560248

Methods

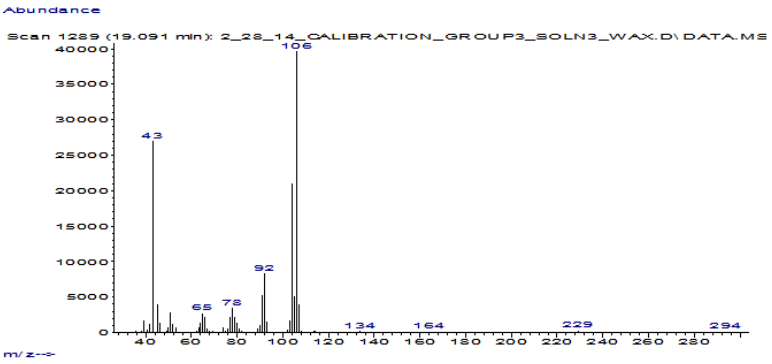
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
6.8931	0.145

Response Factor of $^{13}C_2$-phenethyl acetate

	Isotope	Unlabeled
Standard:	$^{13}C_2$-phenethyl acetate	phenethyl acetate
CAS:	N/A	103-45-7
Mfg/Reference:	synthesized	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-427	427; 29058-0; N/A
% Purity (by GC-FID)	N/A	98.97%



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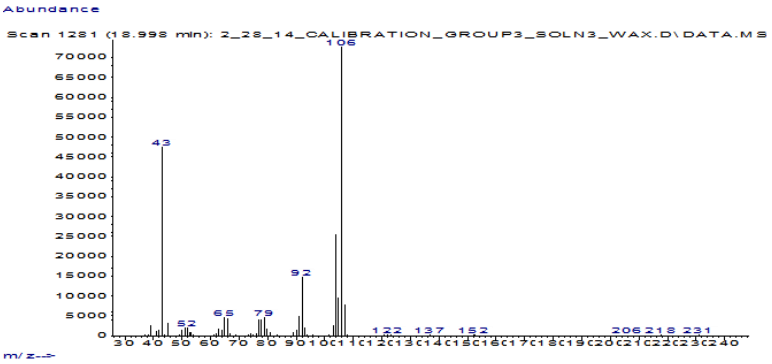
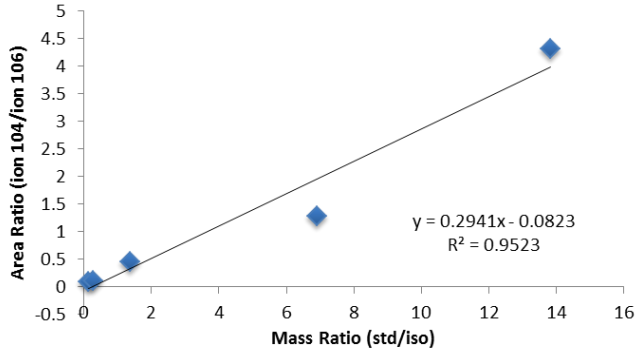


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		106	104	
mass ratio	0.14	130056240	10821066	0.083202974
	0.28	89495583	9151019	0.102251069
	1.38	8049941	3635804	0.451655981
	6.91	4642210	5912175	1.273569054
	13.83	15973731	68809650	4.307675521

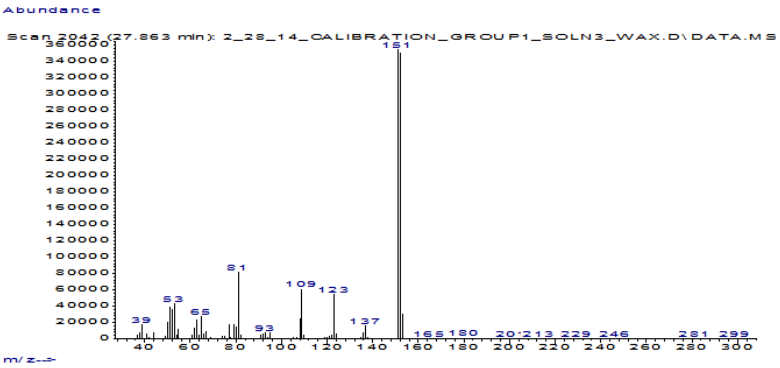
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



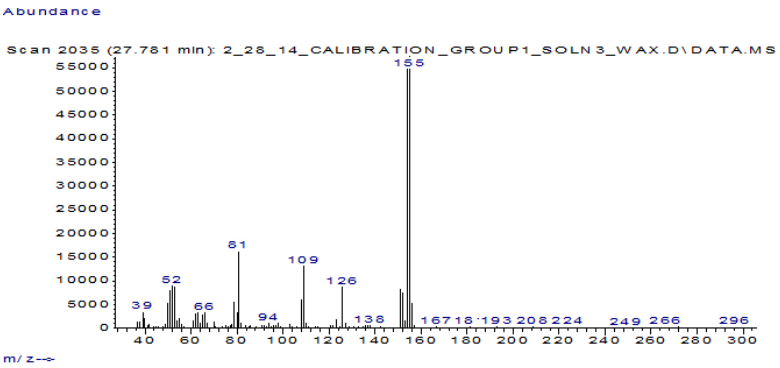
slope	Rf
0.2941	3.400

Response Factor for d_3-vanillin

	Isotope	Unlabeled
Standard:	d_3-vanillin	vanillin
CAS:	N/A	121-33-5
Mfg/Reference:	synthesized	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-475	70; V11-4; N/A
% Purity (by GC-FID)	N/A	99.90%



Unlabeled

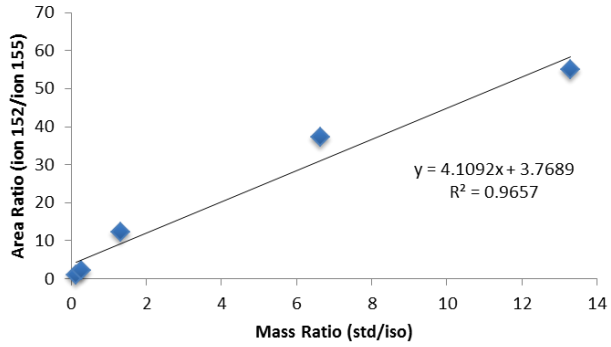


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		155	152	
mass ratio	0.13	30703250	33346068	1.086076165
	0.27	16337861	35679582	2.18385883
	1.33	3019985	36893343	12.21639942
	6.64	4154911	155111454	37.33207619
	13.29	3668568	201885577	55.03116666

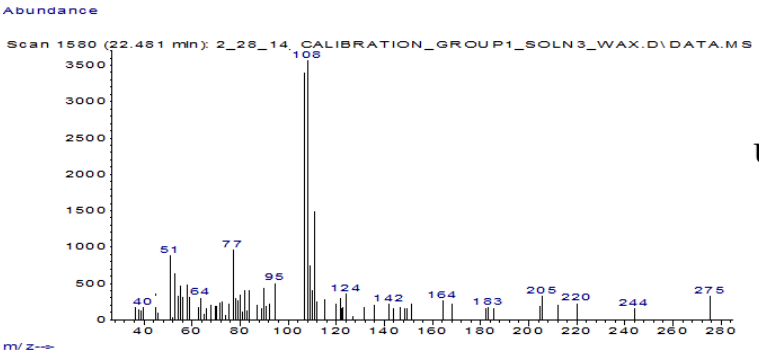
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
4.1092	0.243

Response Factor for d_3-p-cresol

	Isotope	Unlabeled
Standard:	d_3-p-cresol	p-cresol
CAS:	108561-00-8	106-44-5
Mfg/Reference:	CDN, Quebec, Canada	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-425; D-5628; R653P1	425; C8,575-1; 09410PI
% Purity (by GC-FID)	N/A	99.90%



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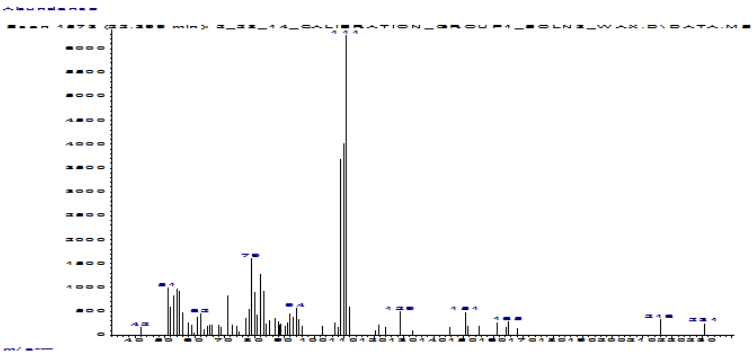
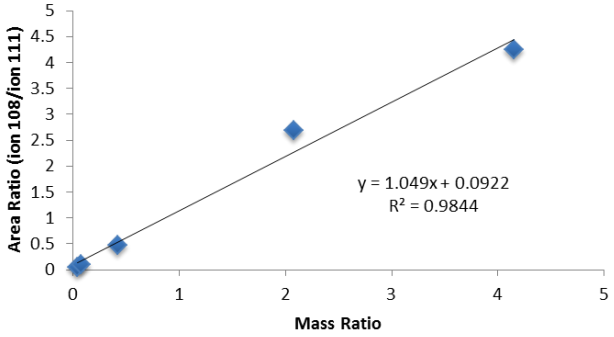


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		111	108	
mass ratio	0.04	3442276	178422	0.051832567
	0.08	1725142	185328	0.107427678
	0.42	297609	140698	0.47276124
	2.08	376128	1008327	2.680808129
	4.15	342265	1454646	4.250057704

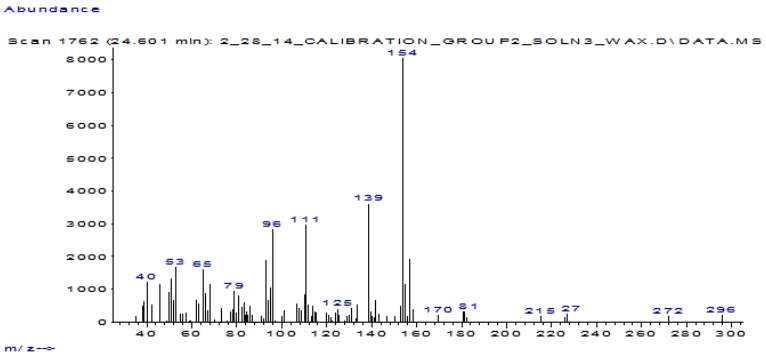
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
1.049	0.953

Response Factor of d_3-syringol

	Isotope	Unlabeled
Standard:	d_3-syringol	syringol
CAS:	N/A	91-10-1
Mfg/Reference:	synthesized	Sigma-Aldrich, St. Louis, MO
No.; Catalog#; Batch#/Lot#:	ISO-61	611; D135550; 06013TD
% Purity (by GC-FID)	N/A	99.90%



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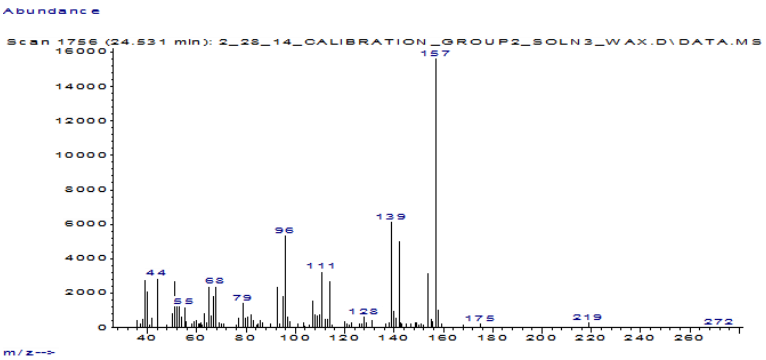
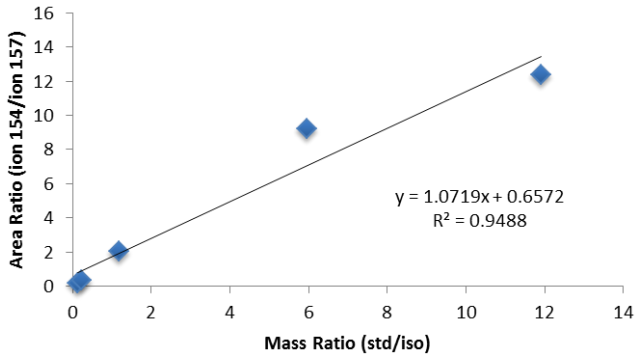


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		157	154	
mass ratio	0.12	20432631	3360370	0.164460955
	0.24	5610044	1955762	0.348617943
	1.19	645051	1319537	2.045632051
	5.96	1823006	16740605	9.182967582
	11.91	2422507	29942019	12.35993085

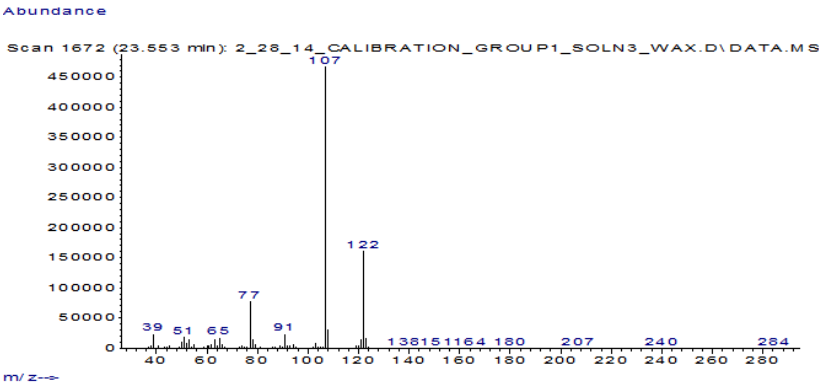
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
1.0719	0.933

Response Factor of 4-[d_5]-ethylphenol

	Isotope	Unlabeled
Standard:	4-[d_5]-ethylphenol	4-ethyl phenol
CAS:	N/A	123-07-9
Mfg/Reference:	synthesized	Aldrich F&F, St. Louis, MO
No.; Catalog#; Batch#/Lot#:	ISO-77	403; W315605; N/A
% Purity (by GC-FID)	98.70%	98.50%



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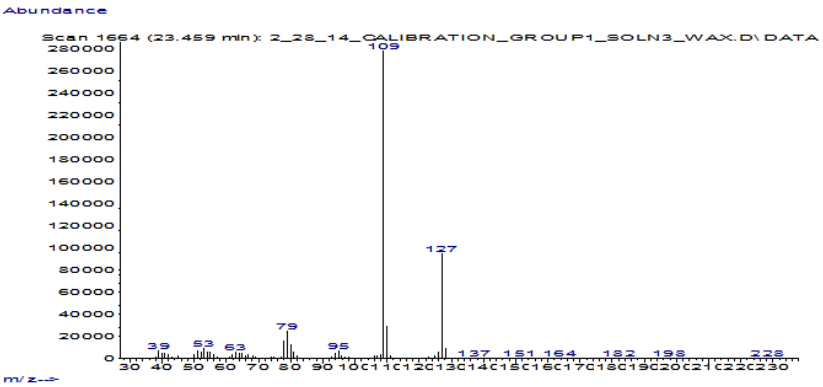
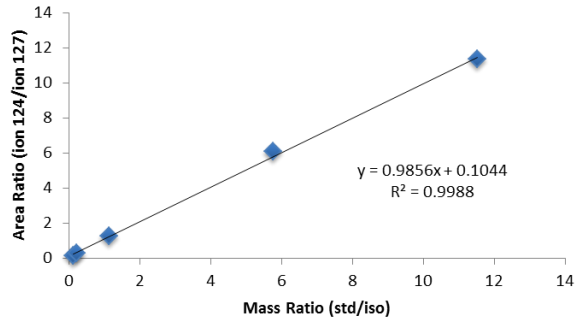


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		127	122	
mass ratio	0.12	35366814	4413549	0.124793514
	0.23	17906517	4735508	0.264457236
	1.15	3614359	4554737	1.260178361
	5.76	4172684	25309225	6.065454513
	11.53	3718981	42123598	11.3266505

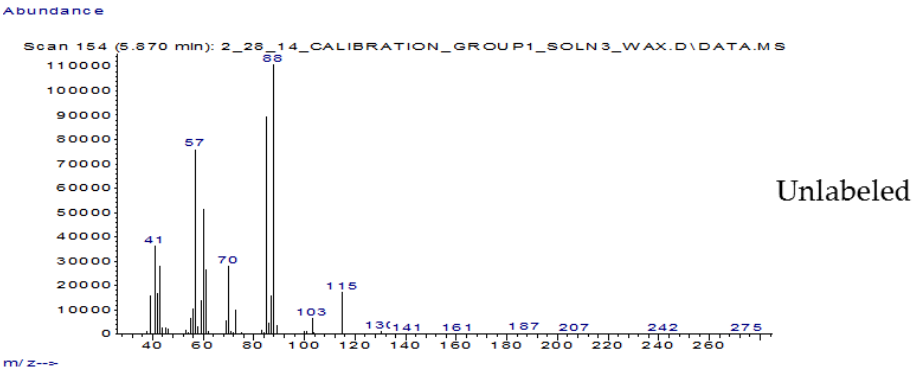
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
0.9856	1.015

Response Factor of d_2-ethyl isovalerate

	Isotope	Unlabeled
Standard:	d_2-ethyl isovalerate	ethyl isovalerate
CAS:	N/A	108-64-5
Mfg/Reference:	synthesized	Sigma-Aldrich, St. Louis, MO
No.; Catalog#; Batch#/Lot#:	ISO-74	324, 112283; 07131AEV
% Purity (by GC-FID)	N/A	99.88%



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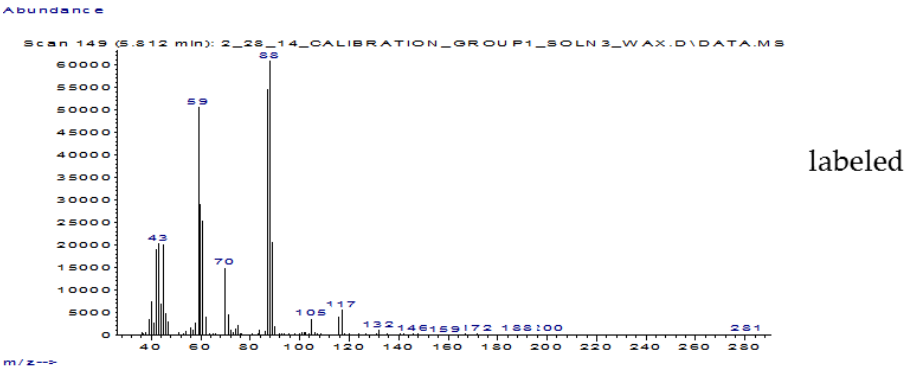
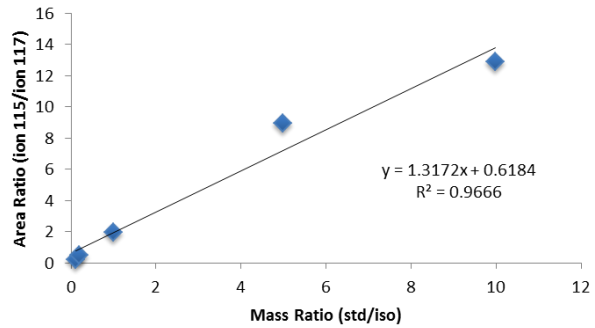


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		117	115	
mass ratio	0.1	1963257	404675	0.206124313
	0.2	1049199	520040	0.495654304
	1	211409	415862	1.967096954
	4.99	196336	1760509	8.966817089
	9.99	216313	2790553	12.90053302

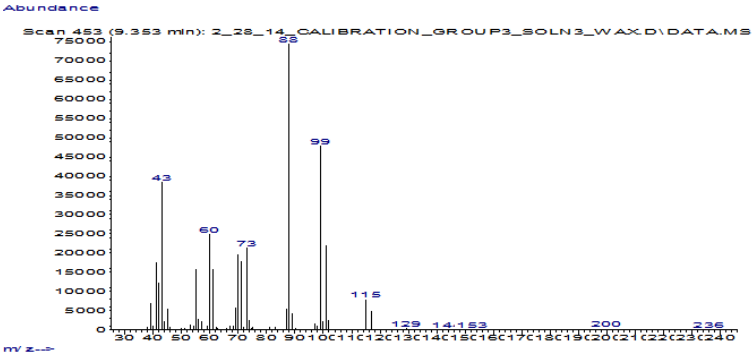
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
1.3172	0.759

Response Factor of d_{11}-ethyl hexanoate

	Isotope	Unlabeled
Standard:	d_{11}-ethyl hexanoate	ethyl hexanoate
CAS:	N/A	123-66-0
Mfg/Reference:	CDN, Quebec, Canada	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-21	287; 14,896-2; 15201MR
% Purity (by GC-FID)	N/A	99.90%



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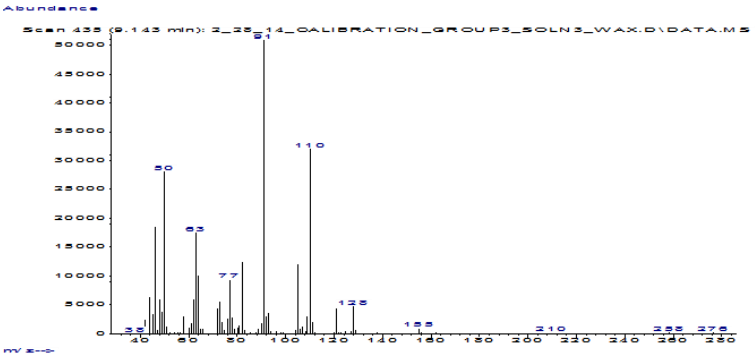
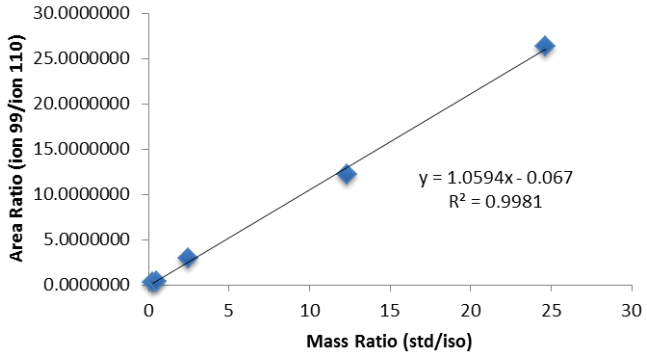


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		110	99	
mass ratio	0.25	13832772	4134563	0.2988962
	0.49	9619785	3534085	0.3673767
	2.46	742216	2196238	2.9590281
	12.31	478011	5824305	12.1844581
	24.62	1038750	27390915	26.3691119

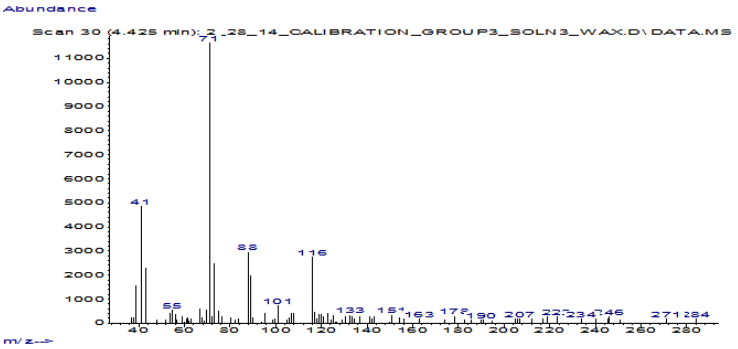
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
1.0594	0.944

Response Factor of d_5-ethyl isobutyrate

	Isotope	Unlabeled
Standard:	d_5-ethyl isobutyrate	ethyl isobutyrate
CAS:	N/A	97-62-1
Mfg/Reference:	synthesized	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-81	341; 246085; 01319ME
% Purity (by GC-FID)	N/A	99.90%



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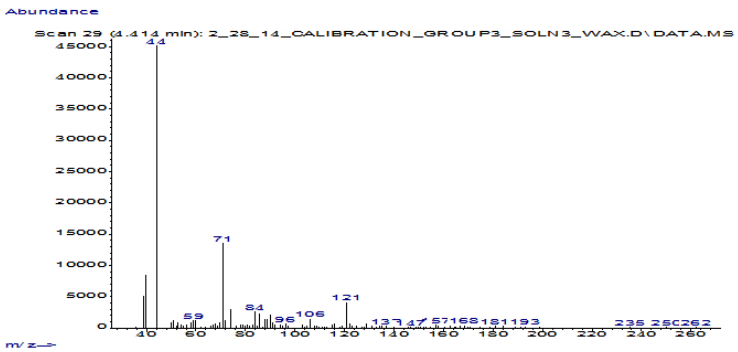
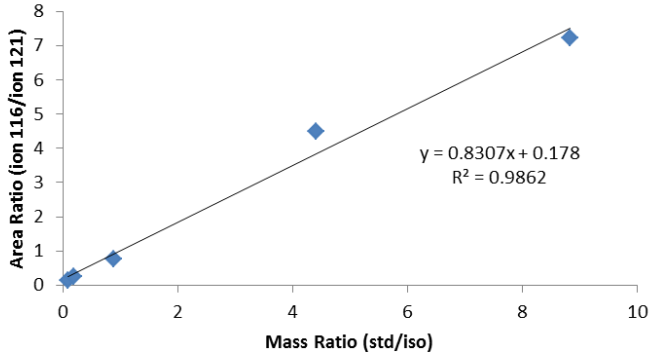


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		121	116	
mass ratio	0.09	572120	82360	0.143955813
	0.18	339238	85389	0.25170824
	0.88	80801	60876	0.753406517
	4.41	81130	363874	4.485073339
	8.83	96864	698388	7.209985134

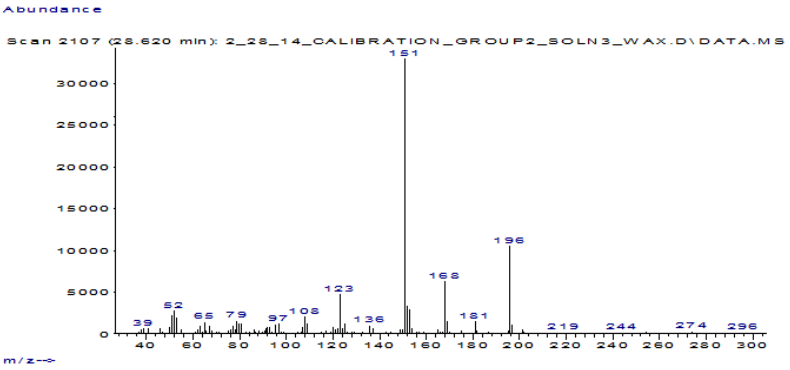
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax (chromatograms: "liz-wood")



slope	Rf
0.8307	1.204

Response Factor of d_5-ethyl vanillate

	Isotope	Unlabeled
Standard:	d_5-ethyl vanillate	ethyl vanillate
CAS:	N/A	617-05-0
Mfg/Reference:	synthesized	Alfa Aeasar, Lancaster, UK
No.; Catalog#; Batch#/Lot#:	ISO-73	1123; L05798; 10108450
% Purity (by GC-FID)	N/A	99.90%



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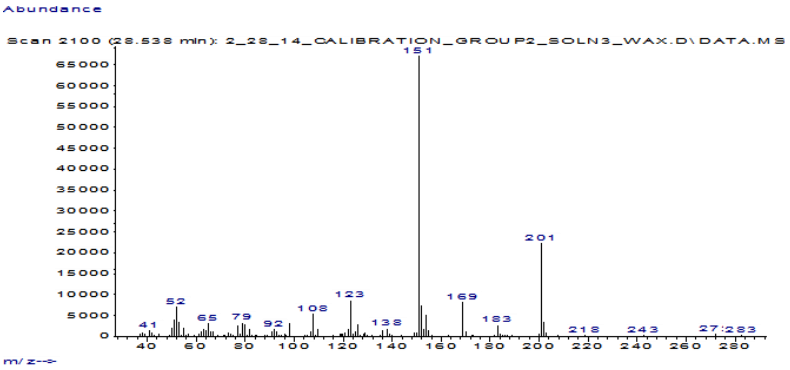
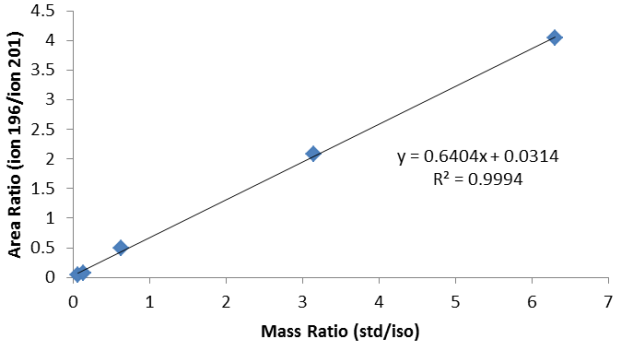


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		201	196	
mass ratio	0.06	25433181	948425	0.037290852
	0.13	7823075	596575	0.076258377
	0.63	776061	381049	0.491003929
	3.15	2420990	5052510	2.086960293
	6.3	2436029	9847099	4.042274948

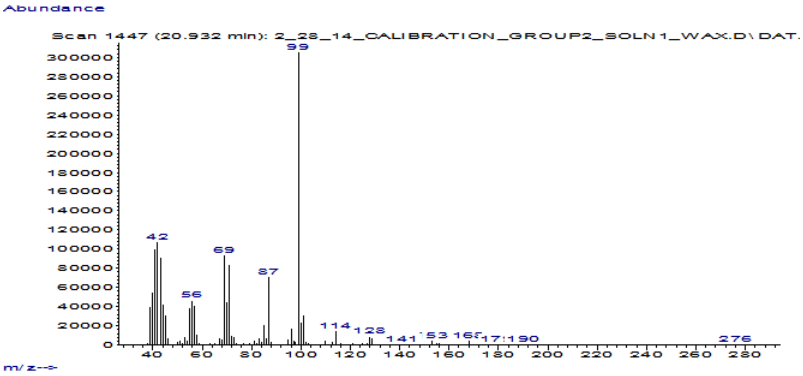
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax (chromatograms: "liz-wood")



slope	Rf
0.6404	1.562

Response Factor of d_2-*cis*-whiskey lactone

	Isotope	Unlabeled
Standard:	d_2-(*cis*)-whiskey lactone	*cis*-whiskey lactone
CAS:	N/A	39212-23-2
Mfg/Reference:	synthesized	SAFC, St. Louis, MO
No.; Catalog#; Batch#/Lot#:	ISO-70	666; W380318; S44624
% Purity (by GC-FID)	N/A	47.10%



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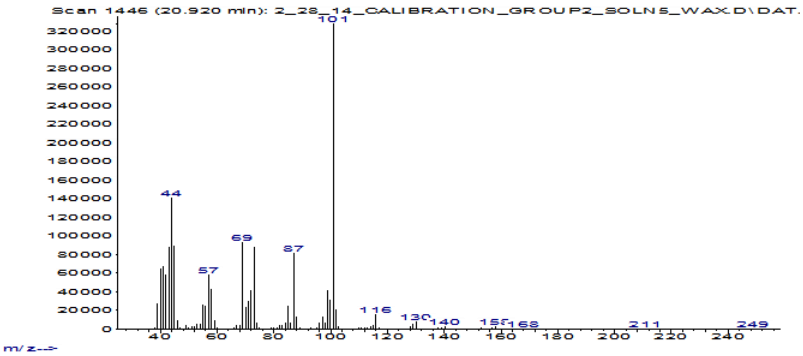
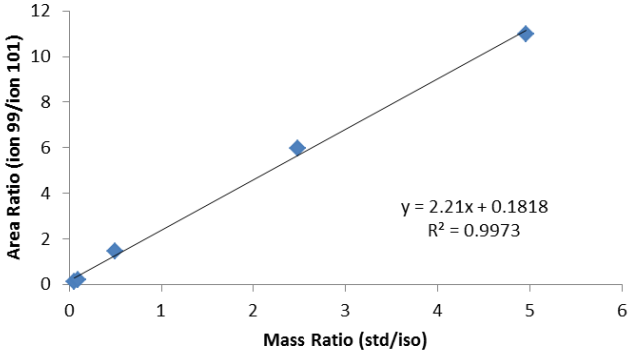


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		101	99	
mass ratio	0.05	13583504	1713042	0.126111937
	0.1	3742196	838042	0.223943909
	0.5	375284	557620	1.485861374
	2.48	1253677	7502283	5.984223209
	4.96	1411896	15485619	10.9679601

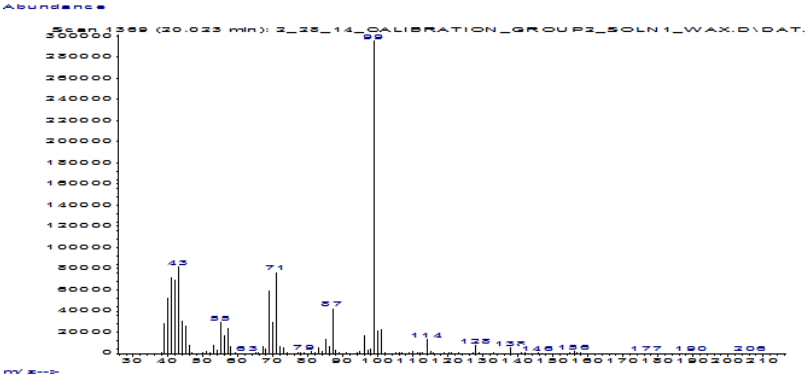
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
2.21	0.452

Response Factor of d_2-*trans*-whiskey lactone

	Isotope	Unlabeled
Standard:	d_2-(*trans*)-whiskey lactone	*trans*-whiskey lactone
CAS:	N/A	39212-23-2
Mfg/Reference:	synthesized	SAFC, St. Louis, MO
No.; Catalog#; Batch#/Lot#:	ISO-70	666; W380318; S44624
% Purity (by GC-FID)	N/A	52.90%



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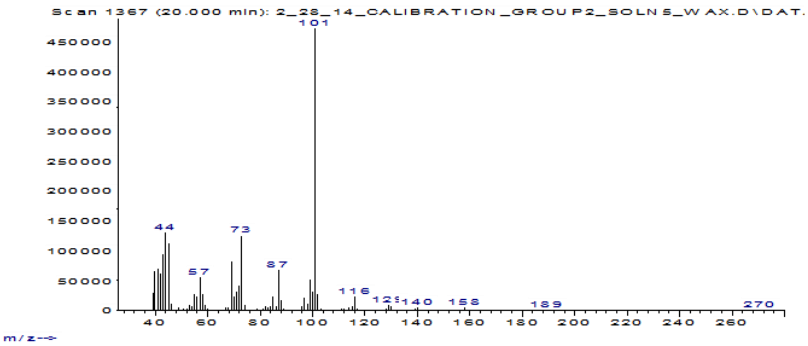
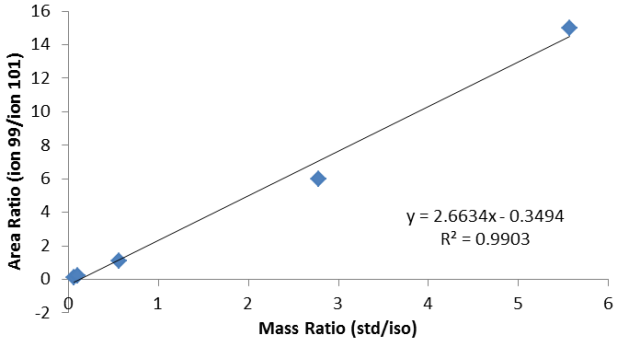


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		101	99	
mass ratio	0.06	17713610	2086307	0.117779888
	0.11	4334241	903425	0.208439032
	0.56	380162	420533	1.106194201
	2.78	1203795	7215872	5.994269788
	5.57	1291360	19383816	15.01038905

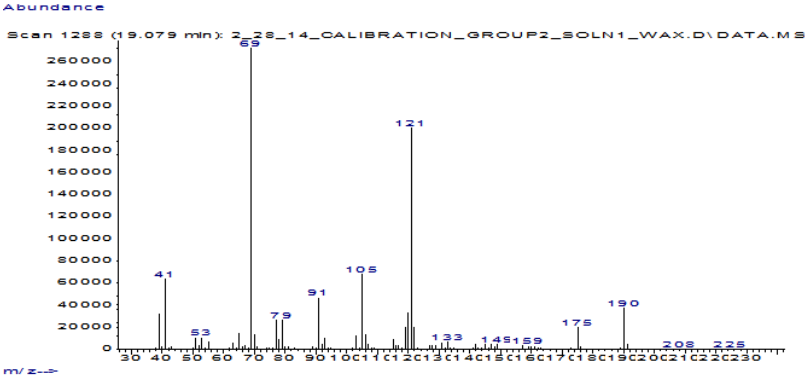
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
2.6634	0.375

Response Factor of d_4-β-damascenone

	Isotope	Unlabeled
Standard:	d_4-β-damascenone	β-damascenone
CAS:	N/A	23696-85-7
Mfg/Reference:	synthesized	Firmenich, Switzerland
No.; Catalog#; Batch#/Lot#:	ISO-1085	1075
% Purity (by GC-FID)	N/A	95.01%



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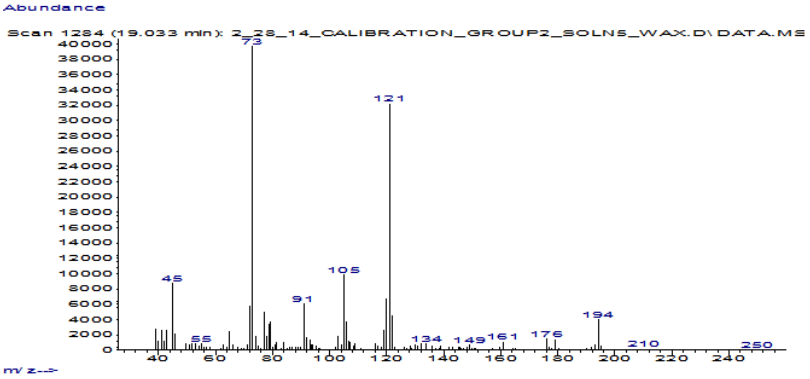
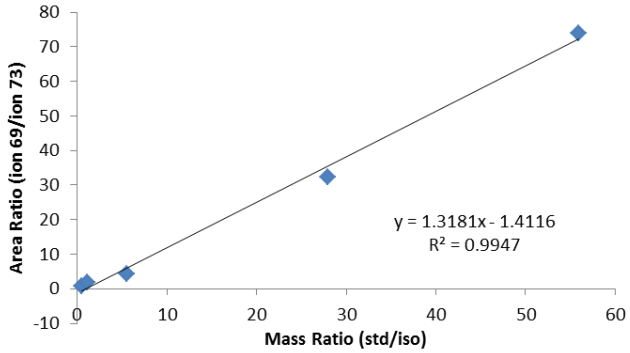


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		73	69	
mass ratio	0.56	930592	764489	0.821508244
	1.12	186493	336649	1.805156226
	5.59	30319	126140	4.160427455
	27.94	64490	2081610	32.2780276
	55.89	84966	6283604	73.95433468

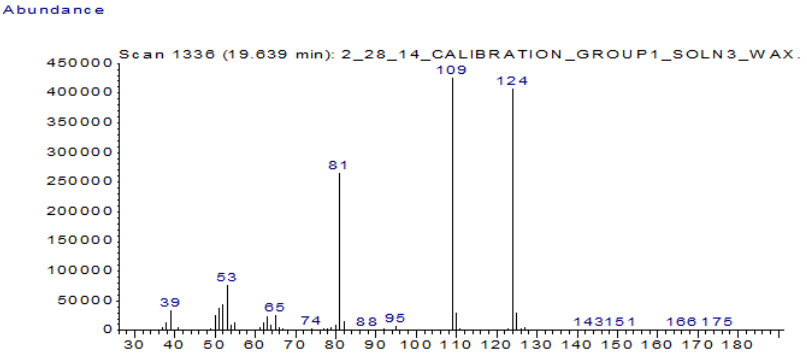
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



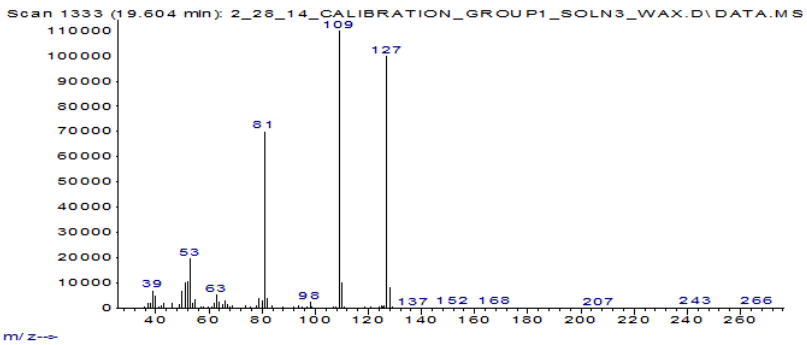
slope	Rf
1.3181	0.759

Response Factor of d_3-guaiacol

	Isotope	Unlabeled
Standard:	d_3-guaiacol	guaiacol
CAS:	74495-69-5	90-05-1
Mfg/Reference:	CDN, Quebec, Canada	Sigma, St. Louis, MO
No.; Catalog#; Batch#/Lot#:	ISO-9; D-5968; W321P3	617; G5502-119F3505
% Purity (by GC-FID)	N/A	99.34%



Unlabeled

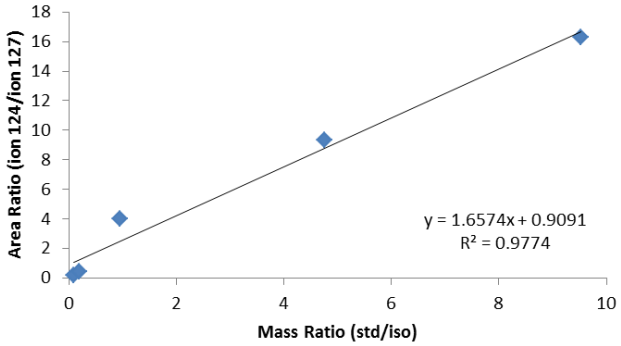


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		127	124	
mass ratio	0.1	29928806	5759954	0.192455188
	0.19	15086724	6524579	0.432471556
	0.95	2485240	9988935	4.019303971
	4.76	2924687	27289295	9.330671966
	9.52	2901737	47277281	16.29275189

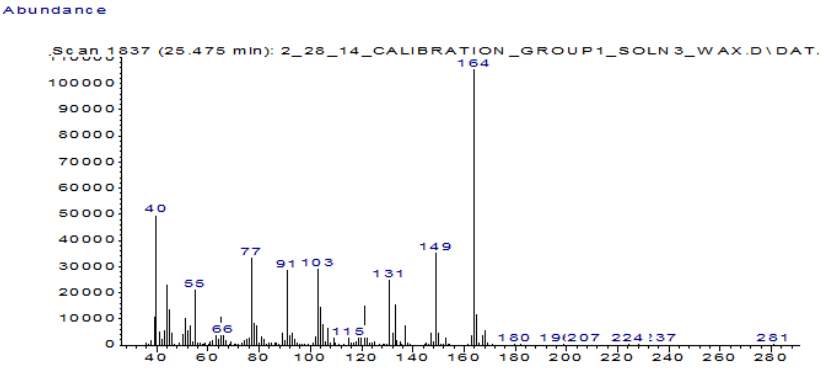
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



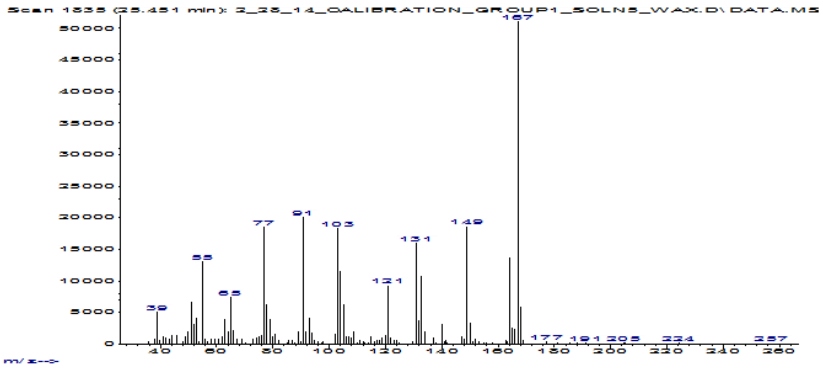
slope	Rf
1.6574	0.603

Response Factor of d_3-isoeugenol

	Isotope	Unlabeled
Standard:	d_3-isoeugenol	isoeugenol
CAS:	N/A	97-54-1
Mfg/Reference:	synthesized	Alfa Aesar, Lancaster, UK
No.; Catalog#; Batch#/Lot#:	ISO-43	183; B24541; G7410A
% Purity (by GC-FID)	N/A	92.16%



Unlabeled

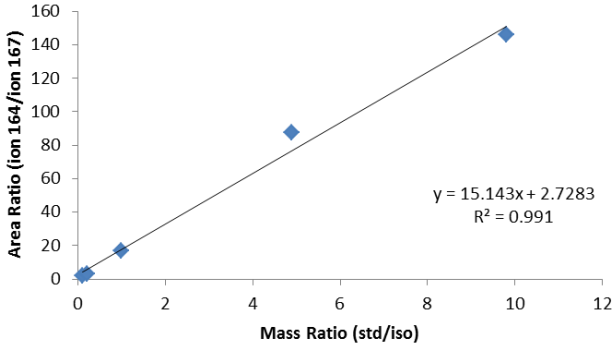


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		167	164	
mass ratio	0.1	1491825	3149289	2.11103112
	0.2	869516	2831523	3.256435764
	0.98	176776	2974798	16.8280649
	4.9	256933	22451268	87.38179992
	9.8	230470	33660890	146.053239

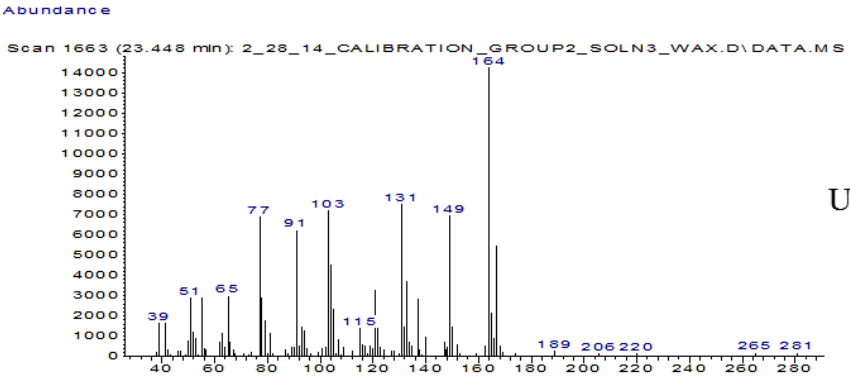
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



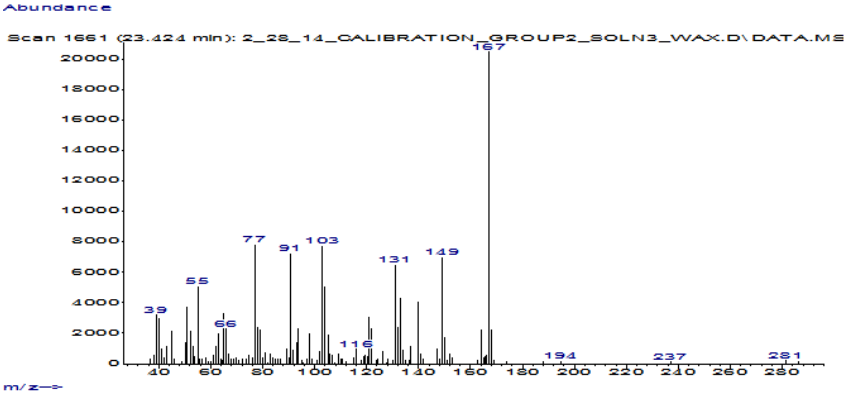
slope	Rf
15.143	0.066

Response Factor of d_3-eugenol

	Isotope	Unlabeled
Standard:	d_3-eugenol	eugenol
CAS:	N/A	97-53-0
Mfg/Reference:	synthesized	Aldrich, Milwaukee, WI
No.; Catalog#; Batch#/Lot#:	ISO-640	640; E5,179-1; 01114CV
% Purity (by GC-FID)	N/A	98.80%



Unlabeled

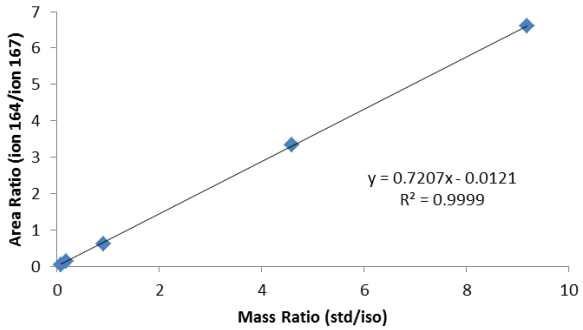


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		167	164	
mass ratio	0.09	39785298	2024895	0.050895559
	0.18	7319183	1023381	0.139821753
	0.92	837172	510091	0.609302509
	4.6	2422205	8086825	3.338621215
	9.19	2664946	17581552	6.597338933

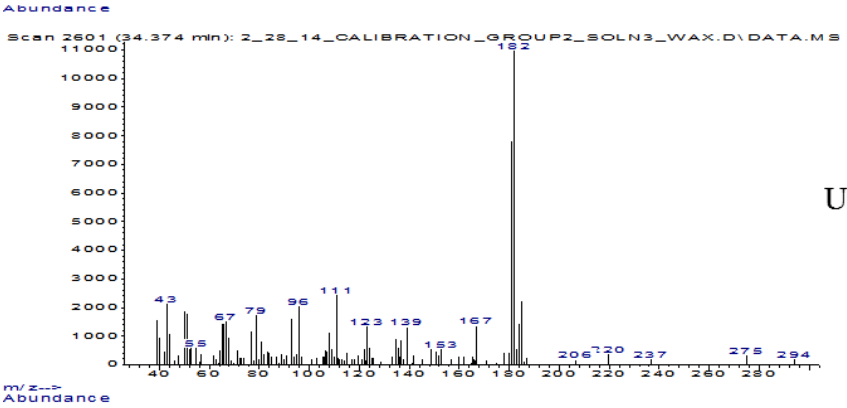
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



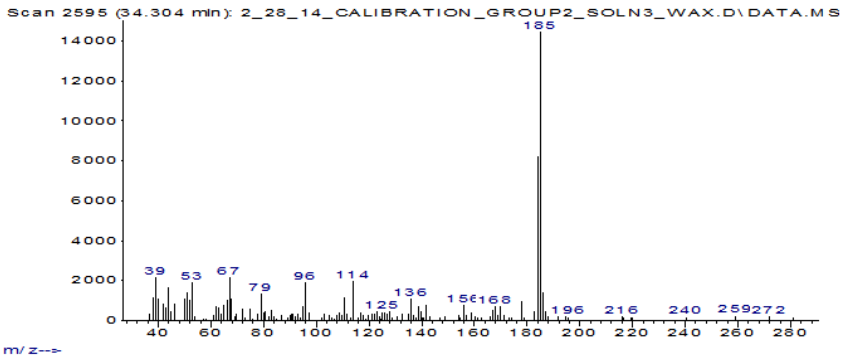
slope	Rf
0.7207	1.388

Response Factor of d_3-syringaldehyde

	Isotope	Unlabeled
Standard:	d_3-syringaldehyde	syringaldehyde
CAS:	N/A	134-96-3
Mfg/Reference:	synthesized	SAFC, St. Louis, MO
No.; Catalog#; Batch#/Lot#:	ISO-78	1065; W404926; MKBN1483V
% Purity (by GC-FID)	N/A	98.90%



Unlabeled

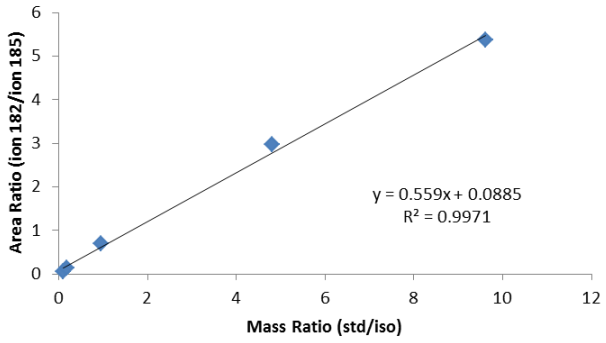


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		185	182	
mass ratio	0.1	32310506	2003531	0.062008654
	0.19	9613267	1213144	0.126194768
	0.96	1227146	841102	0.685413146
	4.81	2986413	8859961	2.966756775
	9.62	3120046	16748771	5.368116688

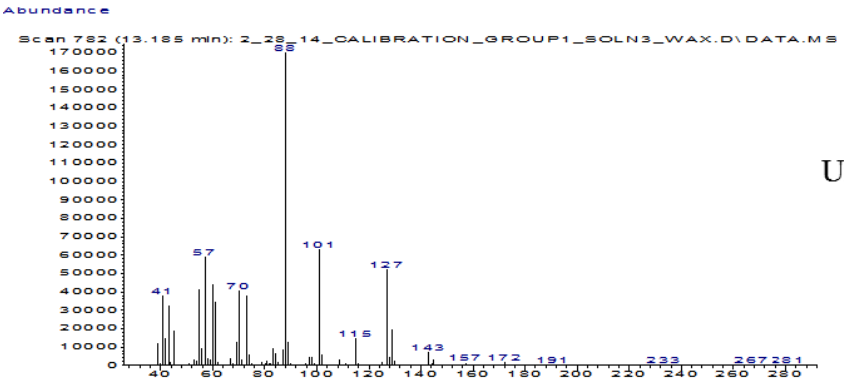
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



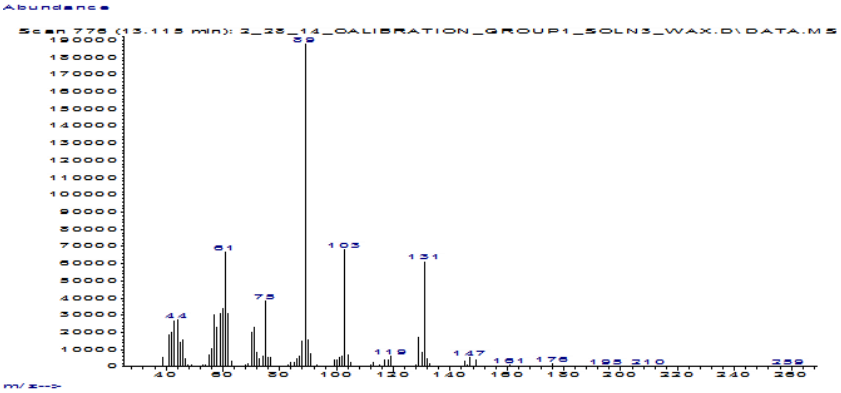
slope	Rf
0.559	1.789

Response Factor of d_4-ethyl octanoate

	Isotope	Unlabeled
Standard:	d_4-ethyl octanoate	ethyl octanoate
CAS:	N/A	106-32-1
Mfg/Reference:	synthesized	synthesized
No.; Catalog#; Batch#/Lot#:	N/A	N/A
% Purity (by GC-FID)	N/A	99.90%



Unlabeled

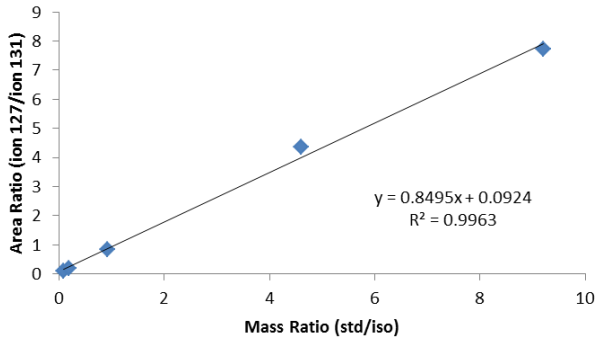


labeled

Standard Curve

		isotope	unlabeled	area ratio
selected ion		131	127	
mass ratio	0.09	11072852	1102536	0.099571095
	0.18	6486502	1124100	0.173298336
	0.92	1194823	994816	0.832605332
	4.6	1276023	5554982	4.3533557
	9.2	1225953	9486187	7.737806425

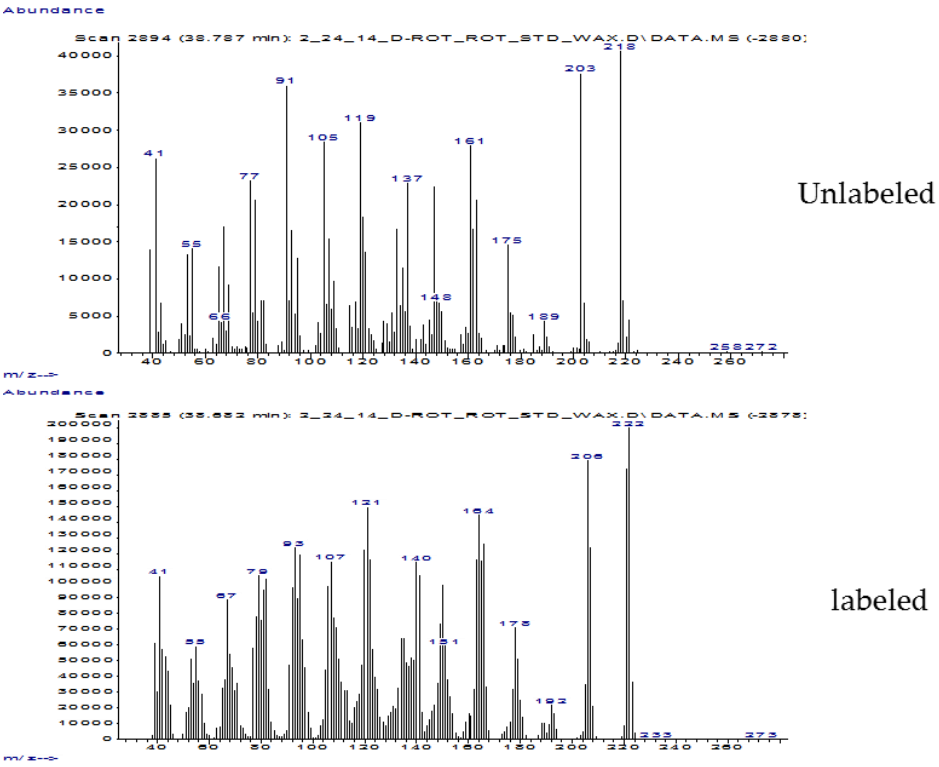
Methods
Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



slope	Rf
0.8495	1.177

Response Factor of d_4-rotundone

	Isotope	Unlabeled
Standard:	d_4-rotundone	rotundone
CAS:	N/A	18374-76-0
Mfg/Reference:	synthesized	synthesized
No.; Catalog#; Batch#/Lot#:	N/A	N/A
% Purity (by GC-FID)	N/A	74.5%



Unlabeled

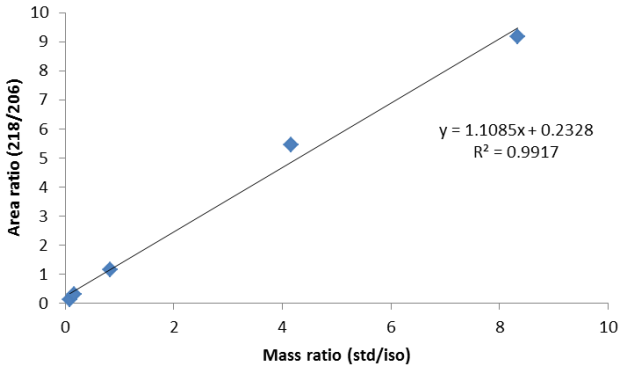
labeled

Standard Curve

	isotope	unlabeled	area ratio
selected ion	206	218	
mass ratio			
0.08	23391	167220	0.1398816
0.17	26581	91052	0.2919321
0.83	32289	27920	1.1564828
4.17	109934	20116	5.4650030
8.33	183112	19973	9.1679768

Methods

Matrix:ethanol
Injection: cold split-less
Column: RTX-wax
(chromatograms: "liz-wood")



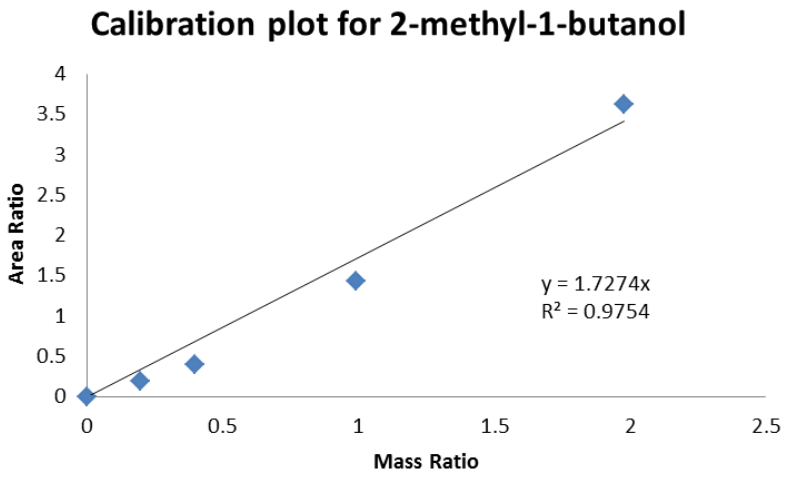
slope	Rf
1.1085	0.90212

2-Methyl-1-butanol Calibration and Quantification

		R. time	Purity
2-pentanol (density g/mL)	0.812	10.87	
2-MB (density)	0.815	12.12	98.608

	Vol std (uL)	Mass std (mg)	Vol IS (uL)	mass IS (mg)	mass ration	area ratio	area (2-MB)	Area (IS)
Solution 1	0	0	5	4.06	0	0	0	92.0543
Solution 2	1	0.804	5	4.06	0.19794463	0.197643	11.63718	58.8798
Solution 3	2	1.607	5	4.06	0.39588926	0.398312	37.39955	93.8951
Solution 4	5	4.018	5	4.06	0.98972315	1.434808	93.57462	65.2175
Solution 5	10	8.037	5	4.06	1.97944631	3.628223	172.16507	47.4516

Response Factor 0.528657569

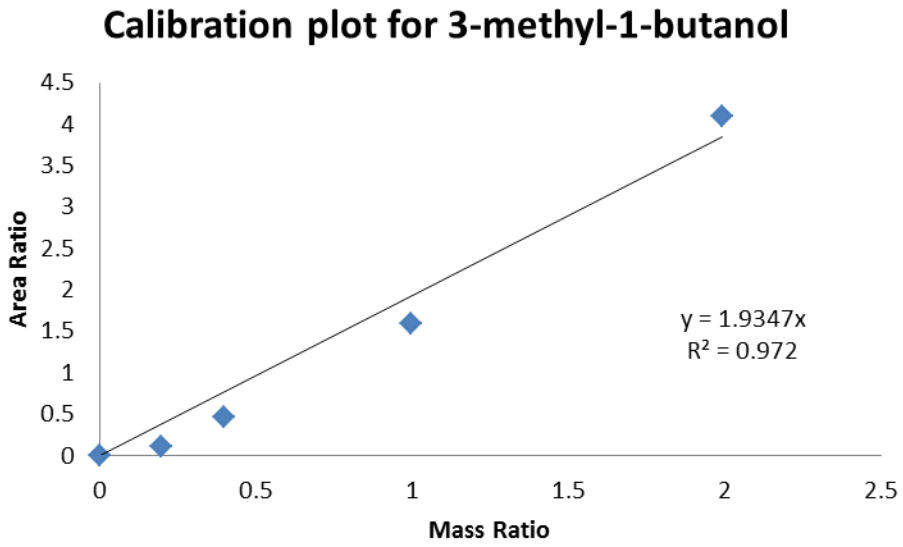


3-Methyl-1-butanol Calibration and Quantification

		R. time	Purity
2-pentanol (density g/mL)	0.812	10.87	
3-MB (density)	0.809	11.98	99.801

	Vol std (uL)	Mass std (mg)	Vol IS (uL)	mass IS (mg)	mass ration	area ratio	area (2-MB)	Area (IS)
Solution 1	0	0	5	4.06	0	0	0	92.05428
Solution 2	1	0.807	5	4.06	0.198865	0.11172	6.57803	58.87981
Solution 3	2	1.615	5	4.06	0.397729	0.475055	44.60534	93.89508
Solution 4	5	4.037	5	4.06	0.994323	1.60085	104.4035	65.21753
Solution 5	10	8.074	5	4.06	1.988646	4.094948	194.3119	47.45162

Response Factor 0.466672

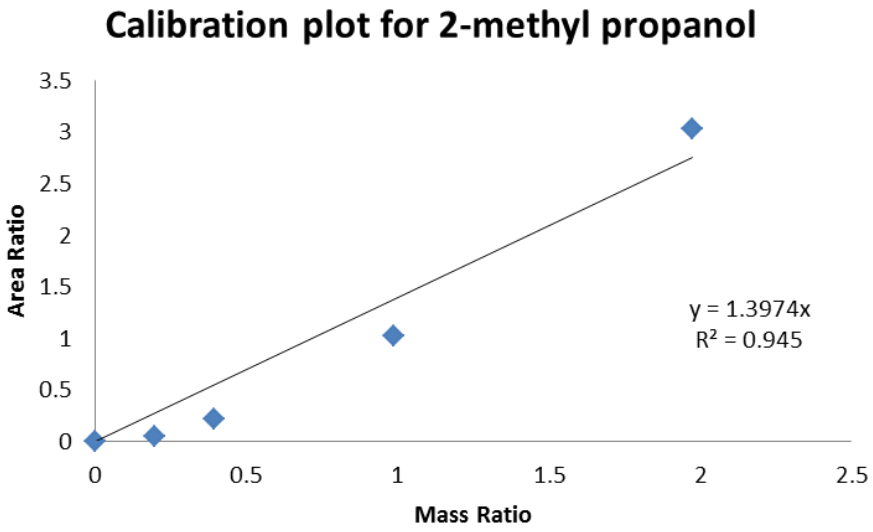


2-Methyl-1-propanol Calibration and Quantification

		R. time	Purity
2-pentanol (density g/mL)	0.812	10.87	
2-Methylpropanol (density)	0.802	8.65	99.976

	Vol std (uL)	Mass std (mg)	Vol IS (uL)	mass IS (mg)	mass ration	area ratio	area (2-MPropanol)	Area (IS)
Solution 1	0	0	5	4.06	0	0	0	92.05428
Solution 2	1	0.8018	5	4.06	0.19749	0.057768	3.40139	58.87981
Solution 3	2	1.6036	5	4.06	0.394979	0.218102	20.47868	93.89508
Solution 4	5	4.0090	5	4.06	0.987448	1.0223	66.67187	65.21753
Solution 5	10	8.0181	5	4.06	1.974895	3.0271	143.64082	47.45162

Response Factor 0.615262



Appendix D: Statistics

One-way ANOVA: phenethyl alcohol versus product

```
Source   DF        SS        MS      F      P
product   2  42148541  21074270  12.55  0.007
Error     6  10073637   1678940
Total     8  52222178

S = 1296   R-Sq = 80.71%   R-Sq(adj) = 74.28%

                         Individual 95% CIs For Mean Based on Pooled StDev
Level  N   Mean  StDev     -+---------+---------+---------+--------
JB 12  3  39058   1738     (------*-------)
JB 4   3  44260   1347                          (------*------)
JB 8   3  40780    451             (------*------)
                           -+---------+---------+---------+--------
                         37500     40000     42500     45000

Pooled StDev = 1296

Grouping Information Using Fisher Method

product  N   Mean  Grouping
JB 4     3  44260  A
JB 8     3  40780    B
JB 12    3  39058    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product  Lower  Center  Upper  -------+---------+---------+---------+--
JB 4      2614    5203   7792                           (--------*------)
JB 8      -866    1723   4312                  (------*------)
                          -------+---------+---------+---------+--
                             -3500         0      3500      7000

product = JB 4 subtracted from:

product  Lower  Center  Upper  -------+---------+---------+---------+--
JB 8     -6069   -3480   -891  (------*------)
                          -------+---------+---------+---------+--
                             -3500         0      3500      7000
```

One-way ANOVA: syringaldehyde versus product

```
Source   DF         SS        MS     F      P
product   2   76097567  38048783  9.32  0.014
Error     6   24488396   4081399
Total     8  100585963

S = 2020   R-Sq = 75.65%   R-Sq(adj) = 67.54%

                         Individual 95% CIs For Mean Based on
```

```
                        Pooled StDev
Level  N   Mean  StDev  -------+---------+---------+---------+--
JB 12  3  14432    429                     (-------*-------)
JB 4   3   7526    264  (--------*-------)
JB 8   3  12488   3463                (-------*-------)
                        -------+---------+---------+---------+--
                            7000     10500     14000     17500

Pooled StDev = 2020

Grouping Information Using Fisher Method

product  N   Mean  Grouping
JB 12    3  14432  A
JB 8     3  12488  A
JB 4     3   7526    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product   Lower  Center  Upper     --+---------+---------+---------+-------
JB 4     -10943   -6906  -2870     (-------*-------)
JB 8      -5981   -1944   2092               (-------*-------)
                                 --+---------+---------+---------+-------
                               -10000     -5000         0      5000

product = JB 4 subtracted from:

product  Lower  Center  Upper     --+---------+---------+---------+-------
JB 8       926    4962   8998                          (-------*-------)
                                 --+---------+---------+---------+-------
                               -10000     -5000         0      5000
```

One-way ANOVA: ethyl octanoate versus product

```
Source   DF        SS        MS       F      P
product   2  68963240  34481620  393.30  0.000
Error     6    526040     87673
Total     8  69489279

S = 296.1   R-Sq = 99.24%   R-Sq(adj) = 98.99%

                        Individual 95% CIs For Mean Based on
                        Pooled StDev
Level  N   Mean  StDev  -----+---------+---------+---------+----
JB 12  3  15507    459                                 (--*-)
JB 4   3   9379     85  (-*-)
JB 8   3   9928    212     (-*-)
                        -----+---------+---------+---------+----
                         10000     12000     14000     16000

Pooled StDev = 296

Grouping Information Using Fisher Method

product  N     Mean  Grouping
JB 12    3  15506.6  A
```

```
JB 8     3   9928.2    B
JB 4     3   9379.3    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  ----+---------+---------+---------+-----
JB 4     -6718.9  -6127.3  -5535.7  (--*--)
JB 8     -6169.9  -5578.3  -4986.8     (--*--)
                                   ----+---------+---------+---------+-----
                                   -6000     -4000     -2000         0

product = JB 4 subtracted from:

product  Lower  Center   Upper  ----+---------+---------+---------+-----
JB 8     -42.6   549.0  1140.6                                  (--*--)
                              ----+---------+---------+---------+-----
                              -6000     -4000     -2000         0
```

One-way ANOVA: vanillin versus product

```
Source   DF      SS      MS       F      P
product   2  935901  467950  851.43  0.000
Error     6    3298     550
Total     8  939198

S = 23.44   R-Sq = 99.65%   R-Sq(adj) = 99.53%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  -------+---------+---------+---------+--
JB 12  3  1406.0   26.4                                   (*-)
JB 4   3   619.5   14.3  (-*)
JB 8   3   949.6   27.3               (*)
                         -------+---------+---------+---------+--
                              750      1000      1250      1500

Pooled StDev = 23.4

Grouping Information Using Fisher Method

product  N     Mean  Grouping
JB 12    3  1405.98  A
JB 8     3   949.59    B
JB 4     3   619.46      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  ----+---------+---------+---------+-----
JB 4     -833.36  -786.52  -739.69  (-*)
```

```
JB 8     -503.23  -456.39  -409.55            (*)
                                    ----+---------+---------+---------+-----
                                     -700      -350         0       350

product = JB 4 subtracted from:

product   Lower  Center   Upper  ----+---------+---------+---------+-----
JB 8     283.29  330.13  376.97                                  (*-)
                                ----+---------+---------+---------+-----
                                 -700      -350         0       350
```

One-way ANOVA: isoamyl acetate versus product

```
Source   DF        SS       MS     F      P
product   2   7834480  3917240  7.44  0.024
Error     6   3159207   526534
Total     8  10993687

S = 725.6   R-Sq = 71.26%   R-Sq(adj) = 61.68%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean   StDev  ---+---------+---------+---------+------
JB 12  3  6415.0  1240.6                    (-------*--------)
JB 4   3  5964.5    97.3                (--------*-------)
JB 8   3  4249.3   176.0  (-------*--------)
                         ---+---------+---------+---------+------
                         3600      4800      6000      7200

Pooled StDev = 725.6

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  6415.0  A
JB 4     3  5964.5  A
JB 8     3  4249.3    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center   Upper  --------+---------+---------+---------+-
JB 4     -1900.2   -450.5   999.2          (-------*------)
JB 8     -3615.3  -2165.6  -715.9  (------*------)
                                  --------+---------+---------+---------+-
                                      -2000         0      2000      4000

product = JB 4 subtracted from:

product    Lower   Center   Upper  --------+---------+---------+---------+-
JB 8     -3164.8  -1715.1  -265.4    (------*-------)
                                  --------+---------+---------+---------+-
                                      -2000         0      2000      4000
```

One-way ANOVA: ethyl butyrate versus product

```
Source   DF      SS      MS       F      P
product   2  924611  462305  164.47  0.000
Error     6   16865    2811
Total     8  941476

S = 53.02   R-Sq = 98.21%   R-Sq(adj) = 97.61%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ------+---------+---------+---------+---
JB 12  3  1204.2   25.2                                 (--*--)
JB 4   3   421.1   70.7  (--*--)
JB 8   3   861.4   52.9                   (--*--)
                         ------+---------+---------+---------+---
                             500       750      1000      1250
Pooled StDev = 53.0

Grouping Information Using Fisher Method

product  N     Mean  Grouping
JB 12    3  1204.23  A
JB 8     3   861.44    B
JB 4     3   421.13      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%


product = JB 12 subtracted from:

product    Lower   Center    Upper   --+---------+---------+---------+-------
JB 4     -889.02  -783.09  -677.17   (-*--)
JB 8     -448.71  -342.79  -236.87             (-*--)
                                   --+---------+---------+---------+-------
                                  -800      -400         0       400

product = JB 4 subtracted from:

product   Lower  Center   Upper   --+---------+---------+---------+-------
JB 8     334.38  440.30  546.23                                 (--*--)
                                 --+---------+---------+---------+-------
                                -800      -400         0       400
```

One-way ANOVA: y-nonalactone versus product

```
Source   DF    SS   MS     F      P
product   2  1617  809  7.65  0.022
Error     6   634  106
Total     8  2251

S = 10.28   R-Sq = 71.84%   R-Sq(adj) = 62.45%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ----+---------+---------+---------+-----
JB 12  3  164.36   6.53      (---------*--------)
```

```
JB 4   3  158.84   9.69  (---------*---------)
JB 8   3  189.64  13.44                       (--------*---------)
                        ----+---------+---------+---------+-----
                          150       165       180       195

Pooled StDev = 10.28

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 8     3  189.64  A
JB 12    3  164.36    B
JB 4     3  158.84    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product   Lower  Center  Upper  -------+---------+---------+---------+--
JB 4     -26.06   -5.52  15.02          (------*------)
JB 8       4.74   25.27  45.81                      (-----*------)
                          -------+---------+---------+---------+--
                               -30         0        30        60

product = JB 4 subtracted from:

product  Lower  Center  Upper  -------+---------+---------+---------+--
JB 8     10.26   30.79  51.33                     (------*------)
                        -------+---------+---------+---------+--
                             -30         0        30        60
```

One-way ANOVA: phenethyl acetate versus product

```
Source   DF      SS      MS      F      P
product   2  815397  407698  20.80  0.002
Error     6  117617   19603
Total     8  933013

S = 140.0   R-Sq = 87.39%   R-Sq(adj) = 83.19%

                        Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean  StDev     +---------+---------+---------+---------
JB 12  3  2026.5   99.8                         (------*-----)
JB 4   3  2046.5  219.1                          (-----*------)
JB 8   3  1398.2   29.4     (------*-----)
                            +---------+---------+---------+---------
                         1200      1500      1800      2100

Pooled StDev = 140.0

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 4     3  2046.5  A
JB 12    3  2026.5  A
JB 8     3  1398.2    B
```

```
Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product   Lower  Center   Upper  ---------+---------+---------+---------+
JB 4     -259.7    20.0   299.7               (----*-----)
JB 8     -908.0  -628.3  -348.6   (----*-----)
                                  ---------+---------+---------+---------+
                                        -500         0       500      1000

product = JB 4 subtracted from:

product   Lower  Center   Upper  ---------+---------+---------+---------+
JB 8     -928.0  -648.3  -368.5  (-----*-----)
                                  ---------+---------+---------+---------+
                                        -500         0       500      1000
```

One-way ANOVA: syringol versus product

```
Source   DF     SS    MS     F      P
product   2   6006  3003  2.50  0.162
Error     6   7195  1199
Total     8  13202

S = 34.63   R-Sq = 45.50%   R-Sq(adj) = 27.33%

                        Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean  StDev   -+---------+---------+---------+--------
JB 12  3  219.04  10.44       (-----------*-----------)
JB 4   3  204.53  27.78   (-----------*-----------)
JB 8   3  265.12  52.12                  (-----------*------------)
                         -+---------+---------+---------+--------
                        160       200       240       280

Pooled StDev = 34.63

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 8     3  265.12  A
JB 12    3  219.04  A
JB 4     3  204.53  A

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product   Lower  Center   Upper  ---------+---------+---------+---------+
JB 4     -83.70  -14.51   54.67         (---------*---------)
JB 8     -23.10   46.08  115.27                   (---------*--------)
                                  ---------+---------+---------+---------+
                                        -70         0        70       140
```

```
product = JB 4 subtracted from:

product  Lower  Center   Upper  ---------+---------+---------+---------+
JB 8     -8.59   60.60  129.78                    (---------*---------)
                                ---------+---------+---------+---------+
                                       -70         0        70       140
```

One-way ANOVA: ethyl hexanoate versus product

```
Source   DF       SS       MS       F      P
product   2  3217687  1608843  252.94  0.000
Error     6    38163     6360
Total     8  3255850

S = 79.75   R-Sq = 98.83%   R-Sq(adj) = 98.44%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ----+---------+---------+---------+-----
JB 12  3  3308.6   45.3                              (-*-)
JB 4   3  1893.7  101.6  (-*-)
JB 8   3  2273.3   81.9         (-*--)
                         ----+---------+---------+---------+-----
                          2000      2500      3000      3500

Pooled StDev = 79.8

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  3308.6  A
JB 8     3  2273.3    B
JB 4     3  1893.7      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  ------+---------+---------+---------+---
JB 4     -1574.2  -1414.8  -1255.5  (-*--)
JB 8     -1194.6  -1035.3   -876.0        (--*-)
                                   ------+---------+---------+---------+---
                                     -1200      -600         0       600

product = JB 4 subtracted from:

product  Lower  Center  Upper  ------+---------+---------+---------+---
JB 8     220.2   379.5  538.9                              (-*--)
                                ------+---------+---------+---------+---
                                  -1200      -600         0       600
```

One-way ANOVA: ethyl vanillate versus product

```
Source   DF      SS     MS       F      P
product   2  140571  70286  244.26  0.000
```

```
Error     6    1726    288
Total     8  142298

S = 16.96   R-Sq = 98.79%   R-Sq(adj) = 98.38%

                        Individual 95% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev   -+---------+---------+---------+--------
JB 12  3  421.54  19.85                                  (-*--)
JB 4   3  115.49  13.24   (--*-)
JB 8   3  274.35  17.14                 (-*--)
                         -+---------+---------+---------+--------
                        100       200       300       400

Pooled StDev = 16.96

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  421.54  A
JB 8     3  274.35    B
JB 4     3  115.49      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  ---+---------+---------+---------+------
JB 4     -339.94  -306.05  -272.16  (--*-)
JB 8     -181.08  -147.19  -113.30            (-*-)
                                   ---+---------+---------+---------+------
                                   -300      -150         0       150

product = JB 4 subtracted from:

product   Lower  Center   Upper  ---+---------+---------+---------+------
JB 8     124.97  158.86  192.75                                (--*-)
                                ---+---------+---------+---------+------
                                -300      -150         0       150
```

One-way ANOVA: cis-oak lactone versus product

```
Source   DF      SS      MS      F      P
product   2  579360  289680  80.02  0.000
Error     6   21721    3620
Total     8  601081

S = 60.17   R-Sq = 96.39%   R-Sq(adj) = 95.18%

                        Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean  StDev     +---------+---------+---------+---------
JB 12  3  1694.1   70.3                                (----*---)
JB 4   3  1077.9   41.3     (---*---)
JB 8   3  1456.4   64.9                       (---*---)
                           +---------+---------+---------+---------
                        1000      1200      1400      1600
```

```
Pooled StDev = 60.2

Grouping Information Using Fisher Method

product  N     Mean  Grouping
JB 12    3  1694.05  A
JB 8     3  1456.41    B
JB 4     3  1077.91      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper    -+---------+---------+---------+--------
JB 4     -736.35  -616.14  -495.93    (--*---)
JB 8     -357.85  -237.64  -117.44               (--*---)
                                     -+---------+---------+---------+--------
                                   -700      -350         0       350

product = JB 4 subtracted from:

product   Lower  Center   Upper    -+---------+---------+---------+--------
JB 8     258.29  378.49  498.70                                (---*--)
                                  -+---------+---------+---------+--------
                                -700      -350         0       350
```

One-way ANOVA: trans-oak lactone versus product

```
Source   DF       SS      MS       F      P
product   2  11257.1  5628.6  116.03  0.000
Error     6    291.1    48.5
Total     8  11548.2

S = 6.965   R-Sq = 97.48%   R-Sq(adj) = 96.64%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  ----+---------+---------+---------+-----
JB 12  3  205.31   9.71                              (--*---)
JB 4   3  119.08   1.61  (---*--)
JB 8   3  169.39   6.97                  (--*---)
                         ----+---------+---------+---------+-----
                           120       150       180       210

Pooled StDev = 6.96

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  205.31  A
JB 8     3  169.39    B
JB 4     3  119.08      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product
Simultaneous confidence level = 89.08%
```

```
product = JB 12 subtracted from:

product    Lower  Center   Upper     +---------+---------+---------+---------
JB 4     -100.15  -86.23  -72.32     (--*--)
JB 8      -49.83  -35.92  -22.00               (--*--)
                                    +---------+---------+---------+---------
                                  -100       -50         0        50

product = JB 4 subtracted from:

product  Lower  Center  Upper     +---------+---------+---------+---------
JB 8     36.40   50.31  64.23                              (--*--)
                                 +---------+---------+---------+---------
                               -100       -50         0        50
```

One-way ANOVA: guaiacol versus product

```
Source   DF       SS      MS      F      P
product   2   956.01  478.01  60.36  0.000
Error     6    47.51    7.92
Total     8  1003.53

S = 2.814   R-Sq = 95.27%   R-Sq(adj) = 93.69%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  -----+---------+---------+---------+----
JB 12  3  63.510  2.753                           (---*--)
JB 4   3  39.055  2.963  (---*---)
JB 8   3  56.710  2.720                   (---*---)
                         -----+---------+---------+---------+----
                             40        50        60        70
Pooled StDev = 2.814

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  63.510  A
JB 8     3  56.710    B
JB 4     3  39.055      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper    +---------+---------+---------+---------
JB 4     -30.078  -24.455  -18.833    (---*--)
JB 8     -12.422   -6.800   -1.178               (--*---)
                                    +---------+---------+---------+---------
                                  -30       -15         0        15

product = JB 4 subtracted from:

product   Lower  Center   Upper    +---------+---------+---------+---------
JB 8     12.033  17.655  23.277                                (---*---)
                                  +---------+---------+---------+---------
                                -30       -15         0        15
```

One-way ANOVA: iso eugenol versus product

```
Source   DF     SS    MS      F      P
product   2  18236  9118  21.82  0.002
Error     6   2507   418
Total     8  20743

S = 20.44   R-Sq = 87.91%   R-Sq(adj) = 83.89%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  -----+---------+---------+---------+----
JB 12  3  416.18  32.22                        (-----*-----)
JB 4   3  306.24   7.22  (-----*-----)
JB 8   3  368.48  12.77              (-----*----)
                          -----+---------+---------+---------+----
                             300       350       400       450

Pooled StDev = 20.44

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  416.18  A
JB 8     3  368.48    B
JB 4     3  306.24      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center   Upper   --+---------+---------+---------+-------
JB 4     -150.78  -109.94  -69.10   (-----*-----)
JB 8      -88.54   -47.70   -6.86            (-----*-----)
                                   --+---------+---------+---------+-------
                                  -140       -70         0        70

product = JB 4 subtracted from:

product  Lower  Center   Upper   --+---------+---------+---------+-------
JB 8     21.40   62.24  103.08                          (-----*-----)
                                --+---------+---------+---------+-------
                               -140       -70         0        70
```

One-way ANOVA: eugenol versus product

```
Source   DF       SS      MS       F      P
product   2  10118.6  5059.3  119.17  0.000
Error     6    254.7    42.5
Total     8  10373.3

S = 6.516   R-Sq = 97.54%   R-Sq(adj) = 96.73%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
```

```
Level  N    Mean  StDev   -+---------+---------+---------+--------
JB 12  3  131.22   6.91   (--*---)
JB 4   3  206.77   8.02                              (---*--)
JB 8   3  196.89   3.90                          (---*--)
                          -+---------+---------+---------+--------
                         125       150       175       200

Pooled StDev = 6.52

Grouping Information Using Fisher Method

product  N     Mean  Grouping
JB 4     3  206.768  A
JB 8     3  196.889  A
JB 12    3  131.216    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product   Lower  Center   Upper  --------+---------+---------+---------+-
JB 4     62.534  75.552  88.569                                (---*----)
JB 8     52.656  65.673  78.691                             (---*---)
                                 --------+---------+---------+---------+-
                                         0        30        60        90
product = JB 4 subtracted from:

product    Lower  Center  Upper  --------+---------+---------+---------+-
JB 8     -22.896  -9.879  3.139  (----*---)
                                 --------+---------+---------+---------+-
                                         0        30        60        90
```

One-way ANOVA: ethyl isobutyrate versus product

```
Source   DF      SS      MS       F      P
product   2  266520  133260  127.57  0.000
Error     6    6268    1045
Total     8  272788

S = 32.32   R-Sq = 97.70%   R-Sq(adj) = 96.94%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev   -+---------+---------+---------+--------
JB 12  3  600.37  51.48                               (--*--)
JB 4   3  178.87  20.31   (--*--)
JB 8   3  393.97   8.43               (--*--)
                          -+---------+---------+---------+--------
                         150       300       450       600
Pooled StDev = 32.32

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  600.37  A
JB 8     3  393.97    B
JB 4     3  178.87      C
```

```
Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  ----+---------+---------+---------+-----
JB 4     -486.07  -421.49  -356.92  (--*--)
JB 8     -270.97  -206.40  -141.82            (---*--)
                                   ----+---------+---------+---------+-----
                                    -400      -200         0       200

product = JB 4 subtracted from:

product   Lower  Center   Upper  ----+---------+---------+---------+-----
JB 8     150.52  215.10  279.67                                 (--*--)
                                 ----+---------+---------+---------+-----
                                  -400      -200         0       200
```

One-way ANOVA: 4-ethyl phenol versus product

```
Source   DF        SS        MS         F      P
product   2  853230.0  426615.0  13364.19  0.000
Error     6     191.5      31.9
Total     8  853421.6

S = 5.650   R-Sq = 99.98%   R-Sq(adj) = 99.97%

                        Individual 95% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev  -------+---------+---------+---------+--
JB 12  3   91.21   8.31   (*
JB 4   3  727.32   5.14                                 *)
JB 8   3   58.35   0.48  *
                        -------+---------+---------+---------+--
                             200       400       600       800
Pooled StDev = 5.65

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 4     3  727.32  A
JB 12    3   91.21    B
JB 8     3   58.35      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product   Lower  Center   Upper  ---------+---------+---------+---------+
JB 4     624.82  636.11  647.40                                       *
JB 8     -44.15  -32.86  -21.57                   *
                                 ---------+---------+---------+---------+
                                       -350         0       350       700
```

```
product = JB 4 subtracted from:

product    Lower   Center    Upper  ---------+---------+---------+---------+
JB 8     -680.26  -668.97  -657.68  *
                                   ---------+---------+---------+---------+
                                         -350         0       350       700
```

One-way ANOVA: ethyl isovalerate versus product

```
Source   DF      SS      MS       F      P
product   2  266520  133260  127.57  0.000
Error     6    6268    1045
Total     8  272788

S = 32.32   R-Sq = 97.70%   R-Sq(adj) = 96.94%

                        Individual 95% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev   -+---------+---------+---------+--------
JB 12  3  600.37  51.48                              (--*--)
JB 4   3  178.87  20.31   (--*--)
JB 8   3  393.97   8.43                (--*--)
                         -+---------+---------+---------+--------
                        150       300       450       600

Pooled StDev = 32.32

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  600.37  A
JB 8     3  393.97    B
JB 4     3  178.87      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  ----+---------+---------+---------+-----
JB 4     -486.07  -421.49  -356.92  (--*--)
JB 8     -270.97  -206.40  -141.82            (---*--)
                                   ----+---------+---------+---------+-----
                                    -400      -200         0       200

product = JB 4 subtracted from:

product   Lower  Center   Upper  ----+---------+---------+---------+-----
JB 8     150.52  215.10  279.67                                   (--*--)
                                ----+---------+---------+---------+-----
                                 -400      -200         0       200
```

One-way ANOVA: p-cresol versus product

```
Source   DF      SS      MS      F      P
product   2  0.5969  0.2985  17.51  0.003
Error     6  0.1023  0.0170
```

```
Total     8  0.6992

S = 0.1305   R-Sq = 85.37%   R-Sq(adj) = 80.50%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ------+---------+---------+---------+---
JB 12  3  2.3594  0.1843                        (------*-------)
JB 4   3  1.7950  0.0779  (-------*------)
JB 8   3  2.3213  0.1054                     (-------*------)
                          ------+---------+---------+---------+---
                              1.75      2.00      2.25      2.50

Pooled StDev = 0.1305

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  2.3594  A
JB 8     3  2.3213  A
JB 4     3  1.7950    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  -------+---------+---------+---------+--
JB 4     -0.8252  -0.5643  -0.3035  (-----*----)
JB 8     -0.2988  -0.0380   0.2228             (----*----)
                                  -------+---------+---------+---------+--
                                      -0.50      0.00      0.50      1.00

product = JB 4 subtracted from:

product   Lower  Center   Upper  -------+---------+---------+---------+--
JB 8     0.2655  0.5263  0.7871                       (-----*----)
                                -------+---------+---------+---------+--
                                   -0.50      0.00      0.50      1.00
```

One-way ANOVA: ethyl isovalerate versus product

```
Source   DF      SS      MS       F      P
product   2  266520  133260  127.57  0.000
Error     6    6268    1045
Total     8  272788

S = 32.32   R-Sq = 97.70%   R-Sq(adj) = 96.94%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev   -+---------+---------+---------+--------
JB 12  3  600.37  51.48                              (--*--)
JB 4   3  178.87  20.31   (--*--)
JB 8   3  393.97   8.43                (--*--)
                          -+---------+---------+---------+--------
                          150       300       450       600
Pooled StDev = 32.32
```

```
Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  600.37  A
JB 8     3  393.97    B
JB 4     3  178.87      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  ----+---------+---------+---------+-----
JB 4     -486.07  -421.49  -356.92  (--*--)
JB 8     -270.97  -206.40  -141.82            (---*--)
                                   ----+---------+---------+---------+-----
                                    -400      -200         0       200

product = JB 4 subtracted from:

product   Lower  Center   Upper  ----+---------+---------+---------+-----
JB 8     150.52  215.10  279.67                                  (--*--)
                                ----+---------+---------+---------+-----
                                 -400      -200         0       200
```

One-way ANOVA: B-damascenone versus product

```
Source   DF     SS     MS      F      P
product   2  3.684  1.842  15.88  0.004
Error     6  0.696  0.116
Total     8  4.380

S = 0.3406   R-Sq = 84.11%   R-Sq(adj) = 78.81%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean   StDev  ---------+---------+---------+---------+
JB 12  3  3.0179  0.2261       (------*------)
JB 4   3  2.6264  0.2194  (------*------)
JB 8   3  4.1364  0.4988                      (------*------)
                         ---------+---------+---------+---------+
                                2.80      3.50      4.20      4.90

Pooled StDev = 0.3406

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 8     3  4.1364  A
JB 12    3  3.0179    B
JB 4     3  2.6264    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%
```

```
product = JB 12 subtracted from:

product    Lower   Center   Upper  --------+---------+---------+---------+-
JB 4     -1.0720  -0.3915  0.2890           (-----*----)
JB 8      0.4380   1.1185  1.7990                      (----*-----)
                                  --------+---------+---------+---------+-
                                       -1.2       0.0       1.2       2.4

product = JB 4 subtracted from:

product   Lower  Center   Upper  --------+---------+---------+---------+-
JB 8     0.8295  1.5100  2.1905                       (-----*----)
                                 --------+---------+---------+---------+-
                                     -1.2       0.0       1.2       2.4
```

One-way ANOVA: 2-methyl-propanol versus product

```
Source   DF       SS       MS      F      P
product   2  2728485  1364243  22.90  0.002
Error     6   357521    59587
Total     8  3086007

S = 244.1   R-Sq = 88.41%   R-Sq(adj) = 84.55%

                        Individual 95% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev   --+---------+---------+---------+-------
JB 12  3  2780.9  323.7                        (----*-----)
JB 4   3  1449.0  227.4   (-----*-----)
JB 8   3  1931.6  149.2           (-----*-----)
                         --+---------+---------+---------+-------
                        1200      1800      2400      3000
Pooled StDev = 244.1

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  2780.9  A
JB 8     3  1931.6    B
JB 4     3  1449.0    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center   Upper  ------+---------+---------+---------+---
JB 4     -1819.7  -1332.0  -844.3  (------*------)
JB 8     -1337.0   -849.4  -361.7         (------*------)
                                  ------+---------+---------+---------+---
                                    -1400      -700         0       700

product = JB 4 subtracted from:

product  Lower  Center  Upper  ------+---------+---------+---------+---
JB 8      -5.1   482.6  970.3                           (------*------)
                            ------+---------+---------+---------+---
                              -1400      -700         0       700
```

One-way ANOVA: 2-methyl-butanol versus product

```
Source   DF       SS       MS      F      P
product   2  8437405  4218702  58.81  0.000
Error     6   430423    71737
Total     8  8867828

S = 267.8   R-Sq = 95.15%   R-Sq(adj) = 93.53%

                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean  StDev    -+---------+---------+---------+--------
JB 12  3  4242.9  253.6                                (----*----)
JB 4   3  1871.4  104.0    (---*----)
JB 8   3  3037.0  374.3                (----*----)
                          -+---------+---------+---------+--------
                         1600      2400      3200      4000
Pooled StDev = 267.8

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  4242.9  A
JB 8     3  3037.0    B
JB 4     3  1871.4      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  ----+---------+---------+---------+-----
JB 4     -2906.7  -2371.6  -1836.5  (---*----)
JB 8     -1741.1  -1205.9   -670.8           (----*---)
                                   ----+---------+---------+---------+-----
                                   -2400     -1200         0      1200

product = JB 4 subtracted from:

product  Lower  Center   Upper  ----+---------+---------+---------+-----
JB 8     630.5  1165.6  1700.7                              (----*---)
                                ----+---------+---------+---------+-----
                                -2400     -1200         0      1200
```

One-way ANOVA: 3-methyl butanol versus product

```
Source   DF        SS        MS      F      P
product   2  21178891  10589445  13.01  0.007
Error     6   4882495    813749
Total     8  26061385


S = 902.1   R-Sq = 81.27%   R-Sq(adj) = 75.02%
```

```
                      Individual 95% CIs For Mean Based on
                      Pooled StDev
Level  N  Mean  StDev   --+---------+---------+---------+-------
JB 12  3  9334    634                        (-------*-------)
JB 4   3  5797    801   (-------*-------)
JB 8   3  8663   1182                    (-------*-------)
                       --+---------+---------+---------+-------
                      4800      6400      8000      9600

Pooled StDev = 902

Grouping Information Using Fisher Method

product  N    Mean  Grouping
JB 12    3  9334.5  A
JB 8     3  8663.1  A
JB 4     3  5797.0    B

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%

product = JB 12 subtracted from:

product    Lower   Center    Upper  --------+---------+---------+---------+-
JB 4     -5339.7  -3537.5  -1735.2  (-----*-----)
JB 8     -2473.6   -671.4   1130.9            (-----*-----)
                                   --------+---------+---------+---------+-
                                       -3000         0      3000      6000

product = JB 4 subtracted from:

product   Lower  Center   Upper  --------+---------+---------+---------+-
JB 8     1063.8  2866.1  4668.4                       (-----*-----)
                                --------+---------+---------+---------+-
                                    -3000         0      3000      6000
```

One-way ANOVA: Rotundone versus product

```
Source   DF       SS       MS       F      P
product   2  1.86470  0.93235  242.00  0.000
Error     6  0.02312  0.00385
Total     8  1.88781

S = 0.06207   R-Sq = 98.78%   R-Sq(adj) = 98.37%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean   StDev  -----+---------+---------+---------+----
JB 12  3  4.5304  0.0887                                   (-*--)
JB 4   3  3.4171  0.0185  (--*-)
JB 8   3  4.0264  0.0579                    (-*--)
                         -----+---------+---------+---------+----
                            3.50      3.85      4.20      4.55

Pooled StDev = 0.0621

Grouping Information Using Fisher Method
```

```
product  N    Mean  Grouping
JB 12    3  4.5304  A
JB 8     3  4.0264    B
JB 4     3  3.4171      C

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of product

Simultaneous confidence level = 89.08%


product = JB 12 subtracted from:

product    Lower   Center    Upper  -----+---------+---------+---------+----
JB 4     -1.2373  -1.1133  -0.9893  (--*-)
JB 8     -0.6280  -0.5040  -0.3800              (--*-)
                                   -----+---------+---------+---------+----
                                     -1.00     -0.50      0.00      0.50


product = JB 4 subtracted from:

product   Lower  Center   Upper  -----+---------+---------+---------+----
JB 8     0.4853  0.6093  0.7333                                    (-*--)
                                -----+---------+---------+---------+----
                                  -1.00     -0.50      0.00      0.50
```

Appendix E: Additional Results

Table A1. Odor active compounds extracted from toasted American and French Oak

No.	Compound	Odor Description	Rtx-5		Identification[a]
			French	American	
1	hexenal	green	811	811	RI, O, MS
2	heptanal	fatty/stale	904	904	RI, O, MS
3	3-octanol	mushroom	982	982	RI, O, MS
4	*cis*-2-nonanal	green	1149	1149	RI, O, MS
5	*trans,cis*-2,6-nonadienal	cucumber	1156	1156	RI, O, MS
6	*trans*-2-nonenal	hay/stale	1161	1161	RI, O, MS
7	*trans,trans*-2,4-nonadienal	oaty/stale/bandaid		1217	RI, O, MS
9	unknown	mushroom/chemical		912	RI, O, MS
11	*trans*-2-undecenal	mushroom/chemical/cilantro		1366	RI, O
13	dihydromaltol*	sweet/spice/good		1101	RI, O
15	guiacol	pleasant/smokey/vanilla	1085	1085	RI, O, MS
18	*trans*-whiskey lactone	spicey/coconut/herbaceous	1294	1294	RI, O, MS
19	maltol	sweet smokey		1110	RI, O
20	*cis*-whiskey lactone	spicey/wood/celery	1331	1331	RI, O, MS
21	o-cresol	plastic/unripe		1062	RI, O
22	4-ethylguaiacol	sweet	1274	1274	RI, O
23	furaneol	fruity/creamy	1094	1096	RI, O
24	γ-nonalactone	spicey wood/coconut		1368	RI, O, MS
26	*p*-cresol	barn		1083	RI, O
28	γ-decalactone	peach/apple		1463	RI, O, MS
29	eugenol	cloves	1368	1368	RI, O, MS
30	sotolon	resionous/maple		1110	RI, O
31	δ-decalactone	spicey wood		1501	RI, O, MS
32	p-vinyl guicol	soapy/wet/resinous	1321	1321	RI, O, MS
33	thymol	pencil shavings		1293	RI, O, MS
34	syringol	lactone/wood/bbq	1356	1356	RI, O, MS
35	***unknown***	**woody/incense**	**1710**	**1715**	**O**
38	unknown	fresh wood/apple	1463	1463	O
39	*cis*-6-dodecen-γ-lactone	cheesy/cilantro	1665	1665	RI, O, MS
40	vanillin	vanilla	1392	1392	RI, O, MS

[a] Identification criteria: retention index (RI), odor quality (O), and mass spectra (MS)

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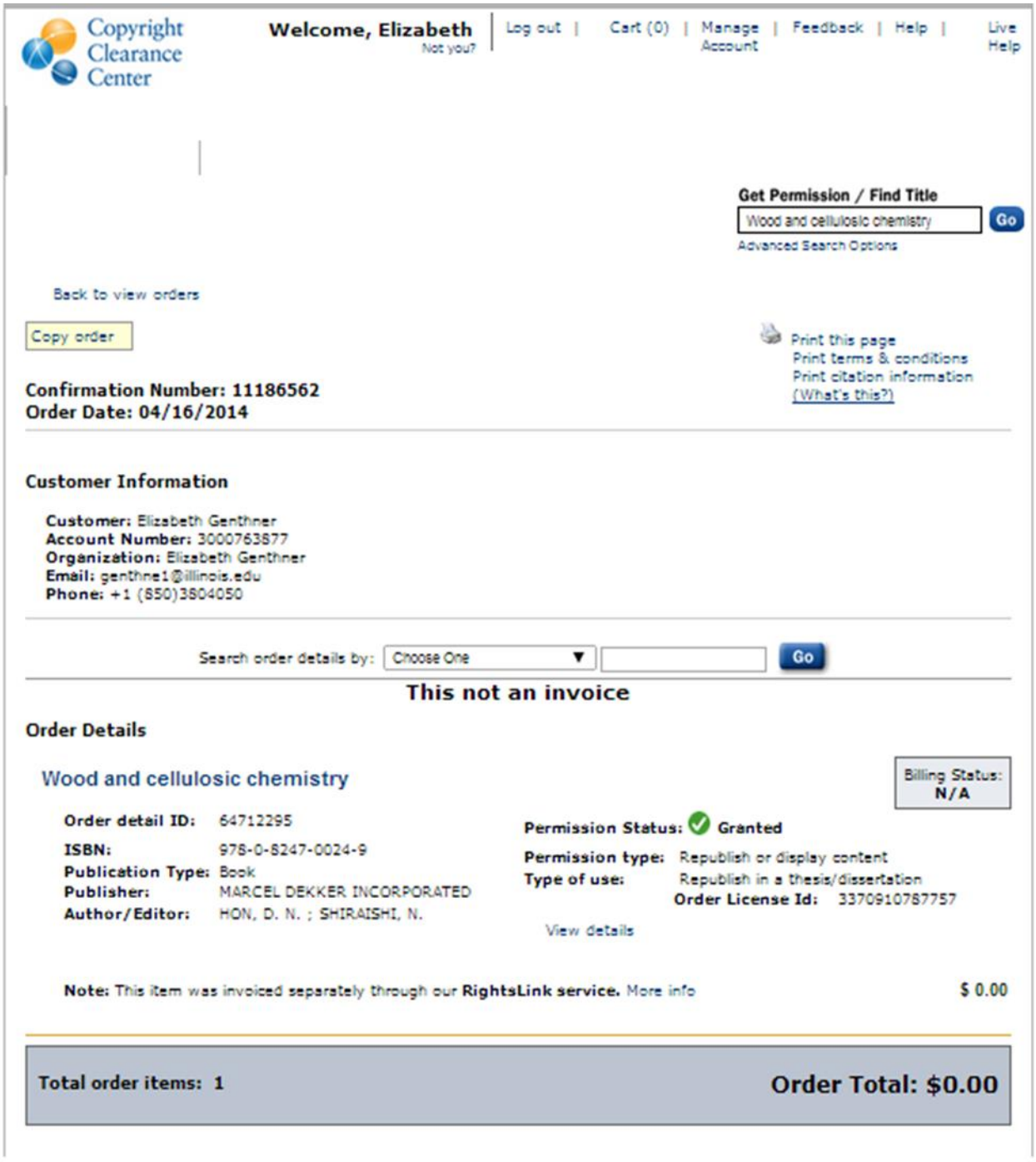

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