AN ABSTRACT OF THE THESIS OF

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Title: The Potential Role of Aliphatic γ- and δ-Lactones in Beer Fruit Aroma

Abstract approved:

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Thomas H. Shellhammer

This work set out to examine the potential contribution of five aliphatic lactones, γ-nonalactone, γ-decalactone, γ-dodecalactone, δ-decalactone and δ-dodecalactone to stone fruit aroma in beer. This work consists of three related studies; lactone olfaction thresholds, additive/synergistic aroma effects and a gas-chromatography-mass-spectrophometry method of analysis. First was the determination of these lactone's thresholds in an unhopped pale ale beer base. This same base was used for the second investigation, which assessed the influence of these lactones on overall fruit aroma in combination with hop-derived esters, a terpene alcohol and a norisoprenoid in descriptive analysis. Lastly, the third body of work consisted of developing a simple and accessible instrumental method for the detection of lactones in a beer matrix.

The olfaction detection threshold of five aliphatic lactones were determined in an unhopped pale ale using the ASTM E679 "best estimate threshold" standard methodology. Twenty-five to twenty-nine panelists assessed the sets of 3-alternative forced choice tests and the group's geometric mean calculated from each panelist's individual threshold served as the olfaction detection threshold in this base. The calculated thresholds in unhopped pale ale were above published thresholds previously reported in water and ranged from 238 – 750 μg/L.

To assess the additive or synergistic effects of lactones (γ-nona and deca lactone, and δ-decalactone) in combination with hop-derived esters (ethyl 2- and ethyl 3-methylbutanoate) and oxygenated terpenes (linalool and β-damascenone) descriptive analysis was performed with a trained panel with a final ballot possessing *stone fruit/peach*, *coconut/oily*, *red berry* and *melon* plus *overall fruity intensity* as descriptors. All compounds were spiked into unhopped pale ale at published and commercially realistic levels detected in beers. Lactones individually yielded low average scores in all categories and only significantly differed from the base on coconut/oily and overall intensity. However, when mixed with ethyl esters and oxygenated terpenes, overall fruity intensity significantly increased, as did the stone fruit/peach descriptor.

The development of the Headspace Solid Phase Microextraction (HS-SPME) coupled with gas chromatography and mass spectrometery (GC-MS) method for lactones in a beer matrix was adapted from multiple methods developed for the detection of lactones in wine. A model system (5% ABV ethanol, pH 4.5) was utilized to determine GC-MS identification parameters for Selected Ion Monitoring (SIM) mode. All subsequent calibration curves were performed in an American light lager base. The developed method was adequate at detecting spiked lactones in beers at trace levels (<5 µg/L). Analysis of all lactones in OSU research dry-hopped beers and a sampling of commercially produced beers were below the calculated olfaction thresholds and the limits of quantitation for the GC method thus providing evidence that lactones are found at trace levels in beer. Further studies into optimal extraction techniques of lactones from beer are required.

The Potential Role Aliphatic γ- and δ-lactones in Beer Fruit Aroma

By

Rachel Ann Hotchko

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APPROVED:

_____

Major Professor, representing Food Science and Technology

_____

Head of the Department of Food Science and Technology

_____

Dean of the Graduate School

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Rachel Ann Hotchko, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

Page

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

Page

LIST OF FIGURES

LIST OF TABLES

Table Page

LIST OF APPENDICES

LIST OF APPENDIX FIGURES

Figure Page

LIST OF APPENDIX TABLES

Table Page

CHAPTER 1. A REVIEW OF HOP-DERIVED BEER AROMA COMPOUNDS AND THE POTENTIAL ROLE OF ALIPHATIC LACTONES IN BEER FRUIT AROMA

1.1 Introduction

Fermented grains have been a source of enjoyment since antiquity, and yet, the majority of beer production world-wide today still relies on four main ingredients: water, malted barley, hops and yeast (106). The final aroma and flavor of beer are shaped by each ingredient, the brewing process and product storage. Today's beer consumers experience the marriage of flavor and aroma provided by each beer's raw materials and the brewing techniques. There is arguably more variety now for the beer consumer than there ever was before.

The first step in beer production requires the extraction of sugars from milled malted barley via enzyme attack on starch contained in the malted barley's endosperm (74). This is achieved by adding the barley to hot water in the mash/lauter tun, allowing the enzymatic process to be carried out, and separating the sugary solution (called wort) from the spent grain. After this extraction, the wort is transferred to the kettle where it is boiled to both sterilize and enhance flavor. Next, hops are added. After a desired boiling time, the hopped wort is processed to remove the spent hops, chilled, and aerated before yeast is added thereby transforming the liquid into beer (Figure 1.1). As mentioned, each ingredient and process contributes to the overall flavor and aroma. Malt and hops synergistically provide the flavor, mouthfeel, and aroma of the brew. The yeast also contributes to aroma, but most importantly, it produces carbon dioxide and ethanol via biochemical pathways that occur within the yeast cells.

Hop aroma and flavor (mostly derived from the hops' essential oil) have become highly desired qualities by brewers and consumers alike and as such has become a popular area of research (111, 113). Hops, the cones from the female *Humulus lupulus* plant, have been dosed into beer for centuries (74). This age old practice results in increased

microbiological stability of the beers (140). Hops provide the desired bitterness and aroma to the beer from the thermally isomerized alpha acids and their essential oil compounds, respectively (74). Inarguably, the alpha acids are very important to the brewer for they provide the bitterness that balances the malt flavors. Brewers' goal to highlight hop aroma qualities in their products drives the breeding and selection of hops based on essential oil composition and perceived aroma (116).

While hops are traditionally added at specific times, there are numerous points throughout the entire brewing process in which hops, and consequently their aroma compounds, can be imparted to the beer. Techniques such as adding hops to the beer after primary fermentation, deliver potent hop aroma to the product. This technique, referred to as dry-hopping, has become especially popular among craft brewers. However, dry-hopping techniques are wildly variable and can lead to the presence of a plethora of aromas ranging from grassy to cheesy to fruity (84). As such, a better understanding of aroma compounds' origins and of the potential additive, synergistic or masking effects of hop-derived compounds is required.

1.2 Exploration of Beer Fruity Aroma

Brewers aim to adjust raw material usage and techniques in such a way that showcases one or more aromas, flavors, or tastes. With the development of new hops and varying hopping techniques, a sweet/stone fruit descriptor (peach, apricot, plum) has emerged on the flavor wheel. These descriptors expand upon the "fruity" label which encompasses the commonly used citrus/tropical and green fruit (apple/pear) characteristics.

In order to better understand the complex factors that comprise "stone fruit aroma" in beer, lactones were identified as potential sources of this aroma. Aliphatic lactones have been shown to possess potent stone fruit and coconut aromas (79). Trace amounts of aliphatic lactones have been identified in alcoholic beverages, including beer, and could potentially play a role in the perceived fruity aroma (18, 32, 68, 130, 141). These

lactones are typically derived from fatty acid precursors and both hops and malt contribute lipids and fatty acids to the wort (30). Little attention has been given to the perceived intensities and organoleptic properties of combined compounds in a beer matrix. These lactones combined with known levels of hop-derived esters and terpenes could synergistically enhance beer's overall fruity profile as they have in wine (77).

This thesis proposes that these lactones, when in mixtures with other known hop-derived compounds, can enhance the final fruity profile of beers. Also, the optimization of an easily automated, simple, and solvent free extraction technique like Headspace Solid Phase Microextraction (HS-SPME) to detect these compounds is desirable. But most importantly, the exploration of these compounds can contribute to the overall knowledge of beer fruity aroma and the initial perception of a product that can influence consumers. This exploration could also lead to product consistency, improvement and diversification in an increasingly competitive market. There is also a recent trend of pairing food with beer which has allowed for the exposure of new flavors to a wider population and as such, differences between consumers' perception based on gender and familiarity with beers and food have arisen (22, 23)

Preliminary instrumental data suggests that aliphatic lactones are below 5ppb in a beer matrix. Fortunately, we can assess these compounds' influence through sensory evaluation. First, the thresholds of individual compounds were determined. A trained panel then assessed the influence of these lactones at threshold and sub-threshold levels in combination with other compounds commonly found in hops and beer. A terpene alcohol, linalool and a norisoprenoid, β-damascenone (further referred to as oxygenated terpenes), were combined with esters, ethyl 2-methylbutanoate and ethyl 3-methylbutanoate. These were chosen because they are either extracted into the fermenting medium, released from glucose units in the presence of β-glucosidase or produced during fermentation and therefore would be likely to be present in most beers.

1.3 Beer Quality and Perception

Aroma is the product of a multitude of complex interactions with perceived odor (ortho- and retro-nasal) heavily influencing the overall flavor perception. Minor contributions arise from basic tastes and tactile sensations (121). Furthermore, flavor consistency is expected by consumers thus making it paramount for brewers to produce consistent as well as creative brews. Therefore, a better understanding of the biotransformation and additive effects of hop-derived compounds is required.

Different beer styles typically possess unique flavors; these can be attributed to the use of specific hop cultivars that exhibit the desired aroma. For example, “noble hops” such as Hallertau mittelfüh and Saaz, which were originally cultivated in Europe, are considered to possess distinctive spicy and herbal characteristics that are transferred to the beer especially when used during kettle boil (70). This attribute is thought to be derived from higher levels oxygenated hop terpenes (31, 42, 66, 87). On the other hand, citrus and tropical fruit flavors which are characteristic of many New World hops such as Cascade and Centennial, are readily accepted as being derived from terpenes, esters and thiols (45, 59, 89, 124). Many of the craft breweries today use large amounts of these New World hops during dry-hopping of their pale ales and India pale ales (IPA) to produce very fruity, floral and sweet smelling beers (Table 1.2).

1.4 Fruity Aroma Compounds

More often than not, the hops commonly used for dry-hopping are called “aroma” hops and possess citrus, fruit and floral notes. Many researchers over the decades have aimed to identify and quantify specific hop-derived aroma compounds, primarily found in the hop essential oils (118). Hop oil compounds, such as terpenes, terpenoids and their epoxides, can be above sensory detection threshold levels in beer products, thereby impacting consumers’ beer experience (31, 56, 60). There are other potential contributors to fruity aroma in addition to esters and hop oil constituents. Additionally, compounds

found at low levels may interact with these compounds to change the perception of fruity aromas (46, 54, 73).

1.5 Lactones

In order to understand beer fruity aroma, each component must first be explored. Lactone is a generic term for a cyclic ester (11). It possesses a "lactone ring" consisting of two or more carbons bonded to an oxygen with an assortment of side chains and rings. Five and six membered rings, labeled gamma (γ) and delta (δ), respectively are the most sterically stable rings (11, 103). The classification of lactone encompasses many structures ranging from simple three membered aliphatic ring structures to complex macrocyclic units (Figure 1.5). The structure defines the sensorial and chemical properties of lactones (27,79,97).

Γ- and δ-lactones possess potent organoleptic properties (low sensory thresholds) and because of their stability they are influential aroma compounds in many foods (27,31,79,119). The γ and δ-C_9-C_{12} aliphatic lactones have aromas reminiscent of fatty/oily, coconut, peach, and apricot (Table 1.1) (27,47,105). Due to the high demand and production of these aromas by the dairy, flavor and fragrance industries, yeast and bacteria biochemical pathways have been extensively studied and discussed in subsequent sections (105). These lactones are also naturally found in all food classes and beverages, but typically found in levels above thresholds in fruits and sub threshold levels in wines and other alcoholic beverages (47).

Aliphatic γ and δ-$C_{9\text{-}12}$ lactones have low odor thresholds ranging from 7-100 µg/L in water and have been found in low concentrations in wines (Table 1.1). They are often below sensory threshold in grape wine (19,71), but can be found above detection threshold in fruit wines such as mango wine (95). Despite the low levels of these compounds detected in alcoholic beverages, lactones have been shown to exhibit a synergistic effect when combined with cinnamates, vanillins, and terpenes to significantly influence the final wine aroma profile (77).

1.5.1 γ-lactones in Beer

One of the first reports of aliphatic lactones' presence in a beer matrix was by Spence et. al (1973) (122). They tentatively identified γ-butyrolactone (γ-C_4), γ/δ-valerolactone (γ/δ-C_5), and γ-dodecalactone (γ-C_{12}) via GC-MS. They also mentioned that γ-C_4 and γ-C_5 lactones' flavor threshold were high (>10ppm) (122). In 1975, Tressl and Renner identified many lactones in fermented beverages ranging from 0.005 to 1.75 mg/L (Table 1.5) including aliphatic γ-$C_{6\text{-}10}$ lactones, A few years later published a more extensive list of 25 unsaturated and aliphatic lactones analyzed in beer (137). Despite the acknowledgment of lactones' contribution to the flavor of fermented beverages, there was no further mention of the magnitude of their sensory impact. In 1994, Dufosse mentioned that beer contained 10 total lactones: 9 γ-lactones and 1 δ-lactone. It was not until after the advancement of flavor (tandem GC's and Time of Flight GC-MS) and sensory analysis that lactones, especially aliphatic lactones, were indisputably identified and extensively studied for their flavor influence in alcoholic beverages.

γ-lactones may be found in malt (γ-C_9) and beer (γ-$C_{9,\ 10,\ 12}$) (13). γ-C_9, also referred to as coconut aldehyde, has been detected in wines and beers at low levels in multiple studies (41,73,110,138). In the case of beer, this lactone can contribute to a beer's sweet flavor (111).

γ-C_9 is believed to be primarily derived during mashing from 4-hydroxynonanoic acid or from lipoxygenase activity on malt linoleic acid, which produces hydroperoxides, 9- and 13-hydroperoxyoctadecadienoic acids (9- and 13-HODE) (40,41,66). It is important to note, however, that lipoxygenase activity is dependent on barley variety and heavily influenced by mashing temperatures (65). Lipases can release free linoleic and linolenic acids from malt lipids during mashing and make them available for hydroperoxide formation (87). Lipoxygenases, hydroxylases and similar enzymes are also utilized in the peroxidation of polyunsaturated fatty acids for large scale bioproduction of lactones

(105). These malt derived precursors result in lactones that the brewing yeast can utilize in subsequent metabolism (40).

Brewing adjuncts like rice, corn or soybean protein can also provide precursors to γ-C_9 formation. According to research performed by Tsuji and others (2010), beers brewed with 0% to 100% malt contained roughly an equal amount of γ-C_9 as detected by Stir Bar Sorptive Extraction on a GC-MS (138). This lactone concentration drastically increased in malt based beers and only slightly increased in non-malt beers over 4 weeks of storage (138).

γ-C_9, having a low reported odor threshold in water (30 µg/l) (47), has also been implicated in staling flavors yet the threshold determined in aged beer was considerably higher at 607 ppb (110). However, this lactone can alter the aroma of the beer after hopping even if γ-C_9 is absent in the hop products themselves (73). Langos and colleagues detected up to 84 µg/L of γ-C_9 in addition to trace amounts γ-C_{10}, and δ-C_{10} in wheat beers (72).

Γ-C_{10}, the main essence of peaches, is the most extensively studied aliphatic lactone as it is in high demand by the flavor and fragrance industry. The biosynthesis of γ-C_{10} in nature (fruits and foodstuffs) and in biotechnological reactors from ricinoleic acid by many microorganisms is well documented (1, 27). Studies performed by Haffner and Tressl (1976) using *Sporobolomyces odorus*, a commonly used yeast model for aliphatic lactone synthesis, determined that γ-C_{10} can be formed from oleic acid (50).

There is little published evidence of γ-$C_{10\ \&\ 12}$ presence in beer, but a recent study in 2013 determined the concentrations of γ-C_{10} and δ-C_{10} detected in wheat beers were 1.30 µg/L to 2.71µg/L, respectively (72). γ-C_{10} has been detected in wines and other fermented beverages like whisky (18, 141). γ-C_9, γ-C_{10} and γ-C_{12} lactones were reported at similar levels in immature and aged whisky which leads the authors to suggest that these lactones were formed from the brewer's yeast lipids after the alcoholic environment becomes inhospitable to the brewing yeast (146). The oleic and palmitoleic fatty acids released upon yeast cell death are converted to hydroxyl fatty acids, 10-

hydroxystearic and 10-hydroxypalmitic acids respectively. Conversion occurs in the presence of lactic acid bacteria which ultimately results in γ-C_{10} and γ-C_{12} lactones by distiller's yeast (145). Lactic acid bacteria are also employed during the production of sour beers, which are increasing in the North American market, and could in turn increase the levels of hydroxyl fatty acids in the medium, potentiating lactone production. It is known that Baker's yeast is able to produce γ-C_{12} from the respective hydroxyl acid, which arises from the bacteria hydroxylating oleic acid (44).

1.5.2 δ-lactones in Beer

δ-lactones have only been recently reported in yeast biomass and beer (37, 68, 133). In a study performed by W. Albrecht et al. (1992), they determined that the δ-lactones biosynthesized are reabsorbed by the organism (*S. odorus* yeast) whereas the γ-lactones represent metabolic end products (1). Another study conducted by Garbe et. al (2008), demonstrated that *S. cerevisiae* could further metabolize δ-decalactone and its hydrolysis product, (R)-5-hydroxydecanoic acid, into (S)-4-hydroxynonanoic acid and γ-C_9 (40). The re-adsorption of δ-lactones by multiple yeast strains could explain why δ-lactone's presence may be below detectable ranges in fermented beverages. Two recent studies detected low levels of δ-C_{10} (in wheat beer) and δ-C_{12} (in low to high malt beers), but these compounds were not considered to be highly influential aromatically (68, 133).

1.5.3 Potential Raw Ingredient Lactone Precursors

Aliphatic γ- and δ-lactones are deemed desirable, and as such it has led to in-depth research of the required precursors and the general peroxisome lactonization pathway in yeast. Malt and hop additions, provide abundant nutrient for the yeast, including lipids and thus free fatty acids, albeit at very low levels when utilizing traditional mashing techniques (0.1-3.0% lipid extraction) (3, 15). Yeast biotransformation is extensively researched for its aroma contributions, but the yeast itself also contributes free fatty acids and lipids to the medium. Individually, bound free fatty acids are typically odorless, yet

when in the presence of active yeast, can result in highly aromatic compounds via glucosidase liberation followed by esterification or lactonization (77). Monocarboxylic acids can possess cheesy, rancid aromas, however, they are also transformed during fermentation to produce esters (69). All ingredients used in beer possess lipids and therefore potentially provide free fatty acid precursors for lactone formation.

Malt and Wort Lipids

The malt basically contains all the lipids, free saturated and unsaturated fatty acids, necessary for yeast growth under anaerobic conditions (133). Yeast utilization of fatty acids in cell wall and organelle development prevents the fatty acids from completely transferring into the final product (133). But malt-derived fatty acids do impact the odor profile. Numerous studies have documented that the oxidation of malt-derived unsaturated fatty acids can lead to the stale flavor aldehyde, (*E)*-2-nonenal, which has been linked to aging and staling flavors especially under improper storage conditions (8, 9, 23, 63, 134). It is an imbalance of fatty acids in the wort and beer that leads to low yeast biomass, reduced fermenting ability and off-flavor development.

Analysis of wort lipids and fatty acids reveals that 0.1-3.0% of malt lipid materials are extracted into wort with long chain fatty acids (LCFA C_{14-20}) comprising 85-90% of the total free fatty acids (FFA) in the wort (9, 15, 128) while medium chain fatty acids (MCFA C_{8-12}) comprise 75-80% in beer (16). DeVries (1990) demonstrated that MCFA levels found in wort were significantly lower than those found in the fermented samples. Conversely, LCFA levels in the wort were higher than those detected in the fermented samples suggesting that the MCFA were produced and excreted by the yeast during fermentation while LCFA were utilized by the yeast for growth and health maintenance (21). MCFA and LCFA have been found to be mostly associated with residual malt and hop matter, called trub, and can therefore be removed during wort separation prior to fermentation (21).

The fatty acids that are detected in the final beer product are not wholly due to the degradation of wort LCFA. Fatty acids arise from yeast excretions and hop lipid

extractions as well (132). Hop-derived fatty acids that are introduced into the boiling wort and fermenting beer (via dry-hopping) can be utilized by the yeast for energy and production of potential aromatic compounds.

Hop Lipids

Hops are composed of mostly cellulose and lignin (40-43%) (52, 121) yet they do possess 1-5% by weight of lipid and fatty acids. This portion is comprised of mostly LCFA and a few MCFA (9, 14, 29, 96). Anness and Reed reported that whole seeded hops contributed 4.2% w/w lipids to the wort which is similar to the amount provided from the malt (3). The lipid content of hop seeds has also been investigated and it was noted that the seeds contain high levels of unsaturated fatty acids, but this acid is highly unstable and becomes rancid within a few weeks (98, 121). The FFA instability in addition to the low quantity of seeds found in hop cones and products (<2% w/w), suggests that seed lipids could only slightly increase the overall fatty acid content of the hop product. They are not the major contributor of FFA to beer.

Saturated and unsaturated MC and LCFA from hops and hop products, and especially hop extracts, can have a deleterious effect on overall product and flavor stability. Pre-isomerized hop extract can contain higher percentages of LCFA when compared to whole or pellet hops. LCFA percentage is highly dependent on the purification cycle during manufacturing (33, 108). If hop extracts are used properly, they provide superior bittering, foam and microbiological stability benefits (36). On the other hand, if used excessively, the extracts can potentially cause gushing, which is uncontrollable rush of contents out of the bottle, and reduce flavor stability from the production of stale aldehydes (14, 33).

In studies conducted by Carrington et. al (1972), promoters of gushing were determined to be attributed to high saturated LCFA levels while unsaturated LCFA served as suppressants (15). Therefore an imbalance of the FFA levels, favoring saturated LCFA, could result in gushing in the final product (15). If hop and hop products are used later in

the process, the polar and neutral lipids solubilize in the beer thus increasing final concentration (100). Typically when using whole or pellet hops, the ratio of unsaturated to saturated fatty acids are such that they will not promote gushing (112). Gushing is a minor threat to modern brewers due to improved malt and hop practices, but it is imperative to keep all ions, nutrients and raw material constituents in balance when brewing.

Yeast and Beer Lipids

The health of the yeast and therefore the overall quality of the product is determined by the uptake and utilization of nutrients present in the aerated (oxygenated) wort. The practice of wort oxygenation at low levels (5-8mg/l) after pitching the yeast ensures satisfactory fermentation. It also has been shown to have no effect on flavor stability (20).

The free unsaturated MCFA and LCFA in the presence of oxygen are required for growth; they are synthesized, metabolized and incorporated into essential organelle and structural elements (Figure 1.6) (5, 57, 95, 110). Unsaturated fatty acids (UFA $C_{16\ \&\ 18}$) are one of the major components synthesized and utilized during the growth phase and only second to sterol production (57) thus making UFA more prevalent than saturated fatty acids within the yeast and the fermenting environment (99). LCFA $C_{16\ \&\ 18}$ are in the highest concentration in the fermenting medium due to yeast biosynthesis and malt and hop additions (57). Oxygen deficiency at the beginning of fermentation leads to reduced yeast cell growth thus resulting in “stuck” or incomplete fermentations (61). Conversely, at high concentrations and extended exposure, oxygen can cause deleterious effects on odor profile (20).

Upon yeast cell death, the synthesized lipids and fatty acids, especially MCFA, are released into the medium resulting in an increase in the overall fatty acid concentration (132). While the protein content and quality in beer dictates the foam stability of the final product, the presence of foam negative materials like FFA (greater than 1 ppm linoleic

acid and 5 ppm palmitic acid), phospholipids and triglycerides in the beer may lead to foam instability and decreased quality (17, 21, 99). Because lipids are present at every stage of the brewing process, it is of interest to explore the possibilities of fatty acid derived aroma compounds.

1.5.4 Lactone Biotransformation

Lactone production is most commonly a result of fatty acid oxidation and degradation within the yeast peroxisome (27, 75). There are three main multi-enzyme catalyzed reactions that occur to form lactones from fatty acids: hydroxylation, α/β-oxidation, and cyclization or lactonization (100, 101) (Figure 1.7). These steps are both energy expending and producing, and yeast cells require high enough fatty acid levels to induce the production of peroxisome enzymes required for their metabolism (100, 136).

Hydroxylation and Transportation to Peroxisome

The presence of fatty acids in the medium induces growth, metabolism and transcription of enzymes. If the FFA is not already in the hydroxyl or epoxy form, it must first undergo the addition of a hydroxyl (OH) group to the carbon chain. The position of the hydroxyl group dictates the number of carbons and configuration of the lactone. When there is a hydroxyl group in an even carbon position (i.e. 10, 12, 14) from the carboxy end prior to beta-oxidation the resulting lactone is classified as a γ-lactone (5 membered ring) (105). If the hydroxyl group is on an odd carbon position (i.e. 11 or 13), a δ-lactone is produced (6 membered ring) (105).

Hydroxylated FFA enter through the yeast cell membrane into the cytoplasm either via diffusion or with the aid of transport proteins (7). The enzymes and specific pathways of fatty acid transportation of any length through the peroxisome membrane is still under investigation, but it has been postulated that SC and MCFA and those in high concentrations are transported via diffusion, while LCFA and those in low concentrations require the help of fatty acid transport or acyl-CoA binding proteins (6, 27). The pH inside

the peroxisome is lower than that of the surrounding cytosol and as such, it has also been suggested that the pH gradient plays a role in peroxisomal membrane transportation (30). Once imported into the cell, exogenous fatty acids are then activated by an acyl-CoA synthase either in the cytosol or peroxisome and can enter into the α or β-oxidation cycle (7,30) (Figure 1.8).

Peroxisomal α and β-oxidation

During metabolism, the activated hydroxylated CoA ester undergoes a specified number of oxidation cycles that results in the release of either one or two carbons via α or β-oxidation, respectively (30). The yeast metabolizes lipids and subsequent fatty acids for metabolites used in other areas of the cell and energy in the form of acetyl CoA (Figure 1.6). There are three major enzymes associated with β-oxidation: Acyl-CoA oxidase (Aox), followed by hydratase/dehydrogenase complex, and a thiolase (49, 74, 139). Acyl-CoA oxidase introduces a double bond between the α and β carbons. It has been suggested that the expression levels of Aox controls the flux through this fatty acid degradation pathway and is thought to be the limiting factor in lipid metabolism (4). Interestingly there is a positive correlation between lactone production and Aox activity in peaches (149).

A multifunctional hydratase/dehydrogenase enzyme complex is involved in peroxisomal β-oxidation. The hydratase adds a hydroxyl group on the γ carbon (3^{rd} carbon from carboxyl end) of the chain then the dehydrogenase transfers a hydride (H^-) to an available NAD^+ to result in a 3-ketoacyl-CoA (52). The final enzyme is a thiolase, which is also induced by fatty acid presence, and catalyzes the release of an acetyl-CoA compound from the 3-ketoacyl CoA ester (52). Depending on substrate levels and length of FFA, most FFA can be completely degraded. It was shown by Bloomfield and Bloch that *S. cerevisiae* is unable to completely degrade LCFA into many acetyl CoA units however new genomics studies demonstrate that the oxidase enzyme can accept LC, MC and SCFA (7, 100).

Lactonization

Lactonization is promoted in a heated and acidic environment when a hydroxyl group and a carboxyl acid group are in close proximity to each other on the same compound. Cyclocondensation forms the lactone ring in the presence of active yeast (105). It is to be noted that the hydroxyl fatty acid and the corresponding lactone are both excreted and present in the medium, but depending on the organism, the equilibrium shifts towards the lactone when pH is less than 2 (30). Lactonization is able to occur intra or intercellularly, although the location of this process is still not clearly defined. This pathway is dependent on the environment, transportation enzymes, and membrane permeability (30). The equilibrium between the accumulation and degradation of these compounds must favor the production of lactones before detectable quantities exist. While there is a potential for the lactones to be further degraded (δ-lactones reabsorbed by yeasts, oxidized, or hydroxylated on chain or ring) (85), it has not been empirically demonstrated that brewing yeast degrades aliphatic lactones or that it happens in an alcoholic environment. Those lactones explored by flavor chemists appear to be fairly stable in acidic conditions and remain in the medium (30).

1.6 Hop Essential Oil

A major contributor to fruity aroma in beers are the essential oils of hops. Constituents of the essential oil of hops are biosynthesized in trichomes of the inflorescences on the female plant during flowering (97, 114). Hundreds of compounds derived from the essential oil have been identified in recent decades and they greatly impact, usually positively, the odor and flavor of the beverage. Even though the hop cones only possess 0.5-3% oil by mass, the composition and ratio of compounds differ enough among varieties that oil composition can be used for classification of families of cultivars

(29, 71). The most abundant essential oil compounds are present in all cultivars and many possess fruity, floral and citrus- like aromas (Table 1.1).

The most abundant essential oil compounds are hydrocarbon chains called terpenes and the percentage found in hops differs between cultivars, time and year of harvest (112, 114). Terpenes are comprised of different quantities of 5 carbon units called isoprene units and therefore they vary greatly in structure from monoterpene (2 isoprene units) to sesquiterpene (3 isoprene units) (Figure 1.2). A-humulene, β-caryophyllene and myrcene are the terpenes typically found in the highest concentrations (97, 129). However concentrated the terpenes may be, their low boiling points typically result in the loss of this hydrocarbon fraction during wort boiling and are not thought to highly influence the aroma of beer when hops are introduced in the kettle (101).

Oxygenated terpenes, or terpenoids, are also highly aroma active. Terpenoids comprise a large percentage of the total oil composition due to their biosynthesis during cone production or formation during hop storage especially after exposure to oxygen (30, 116). Due to the presence of oxygen, these compounds are more water soluble and therefore remain in the final product after boiling and fermentation (120). This oil fraction can also undergo further modification; biotransformation from one terpenoid to another or liberated from a glucose unit, in the presence of yeast (60).

Terpenoid aromas range from floral to citrus to piney (88). Two of these floral and fruity terpenoids, linalool and β-damascenone, are able to survive the boiling and fermentation processes (73). Both linalool and β-damascenone drive the aroma of pilsner-type beers due to their low threshold values (37). Linalool is a major constituent of hop essential oil (up to 85% of terpenoid fraction) and is reported to possess a pronounced floral, rose-like aroma in hops and beer (59, 84, 89, 126). It is difficult to confidently state the detection thresholds of hop aroma compounds for they drastically differ among bases (water versus beer, for instance) and individual panelists. Table 1.4 presents the aroma descriptions and thresholds of many hop-derived aroma compounds. While thresholds for linalool range anywhere from 2.2 µg/L to 1 mg/L in beer (123) it is

well accepted that linalool concentrations in beer are often above sensory threshold (60, 88). B-damascenone is less prominent than linalool in hop oil, but increases in concentration during forced beer aging thus altering the organoleptic properties of the beer (17). B-damascenone is noted to produce apple, peach, fruity, and woody aromas (63) with an exceptionally low threshold in water (0.02-0.09 μg/L (13, 16) and widely variable threshold range of 1.6-157 μg/L in beer (Table 1.4) (115).

1.7 Biotransformed Compounds

Hop essential oil compounds have been the chief focus of hop aroma research, but the biotransformation of aroma compounds by brewer's yeast during primary fermentation has shown significant influence on beer aroma profile as well (56, 59, 125, 126). The biotransformation pathways and required enzymes are not clearly defined for every compound, but esterification and liberation of monoterpene alcohols from sugar units are known to contribute to hop aroma (113).

One area of focus within the field of beer flavor analysis is the biosynthesis and biotransformation of aroma compounds during fermentation; these may be produced pre or post primary fermentation from precursors provided by the raw materials and have major impact on final aroma profile and product stability (56, 126). Biosynthesis refers to the production of chemical compounds by metabolizing cells such as the biogenesis of humulene, whereas biotransformation refers to the use of microbial cells to perform modifications or interconversions of chemical structures such as the formation of esters or the hydrolysis of aromatic glycosides (122, 143). Yeast contains the required enzymes that are involved in aromatic compound transformation and thus have been the subject of study for decades.

The presence and biotransformation of monoterpene alcohols such as geraniol and linalool, into different terpenoids such as β-citronellol, nerol, and α-terpineol by both ale and lager yeast strains during fermentation results in fruity, citrus (β-citronellol, geraniol), and floral (geraniol, linalool, nerol) notes in the medium (55, 56). King and

Dickinson, later supported by Takoi et al. demonstrated that a portion of the geraniol present in the wort is converted to β-citronellol and nerol during fermentation (56, 125). Takoi suggests that the final concentration of β-citronellol can be controlled by the initial concentration of geraniol in the wort (56, 126).

1.7.1 Glycosides

In addition to the free forms of terpene alcohols, it has also been shown that terpene alcohols can be bound to a sugar unit via a glycosidic bond, like linalyl and geranyl glycosides, making them odorless and non-volatile. These bound compounds, which are present in hops and hop products, can potentially be liberated by the yeast and extracted into the beer medium (64, 129). Studies have shown that there is an increase in linalool during fermentation due to the presence and activity of yeast-derived β-glucosidase (51). β-damascenone may also be present in hops and beer as a glycoside (68), but more importantly, precursors to β-damascenone are also bound to a sugar unit via glycosidic bond (57). These precursors may be liberated in the presence of β-glucosidase thus increasing the levels of β-damascenone formed during forced beer aging (16, 40). This is in addition to the terpene alcohol's extraction from the hops during boiling and dry-hopping thereby imparting more linalool in the finished beer than would be expected by considering just the oil-derived sources in the hop.

1.7.2 Esters

Esterification of hop-derived acids occurs during fermentation in the presence of alcohols and enzymes. These compounds are actively studied due to their high ortho and retronasal impact. Esters are known to contribute intense fruity and floral notes with acetate esters and medium chain fatty acid ethyl esters forming the highest percentage of these compounds (95, 105). Dresel et al demonstrated that the concentration of esters before fermentation is variety dependent, however after primary fermentation, the levels are similar across all cultivars (25). Potent acetate esters are formed when an alcohol,

usually a higher alcohol (two or more carbons) are combined with acyl-CoA (66, 87). Acyl-CoA is obtained via lipid metabolism and availability of fatty acids. Higher alcohols are produced as yeast secondary metabolites (69). Van Laere and colleagues' stress the importance of controlling higher alcohol formation (by monitoring wort composition and temperature) because they can negatively impact the beer aroma with an alcoholic/solvent-like quality if higher alcohols are at concentrations above sensory threshold (50, 66). The number of carbons in the ester (four or more) dictate how easily it is excreted into the beer medium – acetate esters freely diffuse while ethyl ester transfer decreases as chain length increases (142). An imbalance of the ester profile due to changes in fermentation conditions can result in a decrease in beer quality (142).

Other highly aromatic compounds produced by esterification in yeast are fatty acid esters, specifically MCFA ethyl esters. Very high levels of unsaturated fatty acids can, however, decrease esters levels by repressing enzyme activity required for the production of esters (35, 129). Ethyl esters like ethyl 3-methylbutanoate (E3MB) and ethyl 2-methylbutanoate (E2MB) are known to provide citrus, fruity, estery, and apple-like aromas to beer in addition to possessing very low sensory thresholds (32, 61, 76, 86). Esters have received the most attention for their contributions to beer fruity aroma, however modification of free hydroxyl fatty acids may also occur during primary fermentation to produce unsaturated and saturated lactones (40). The influence of these precursors have on aroma development is far from completely understood with very few studies have exploring the formation, presence and influence of aliphatic lactones in a beer medium.

1.8 Sensory evaluation

Aroma of beverages can be measured through sensory evaluation, commonly with category intensity scaling and descriptive analysis (51, 80). The use of category or line scales allow the panelists to denote their perception of the intensity of aroma with

numbers (83). Descriptive analysis seeks to define the perceived sensory parameters of the product by use of descriptive and unique terms (51, 80).

1.8.1 Additive and Synergistic Sensory Effects

Sensory perception of beer aroma is complex; multiple factors influence the odor and consumer's experience. The synergistic, additive and masking effects of combined aroma compounds has been known for decades (49). Compounds, such as esters, are individually found near threshold values so an increase in concentration due to altered brewing practices could significantly alter the final aroma because of additive and synergistic effects (80). Takoi and colleagues demonstrated additive effects among linalool, geraniol, and β-citronellol in beers (130).

Recently, hop-derived ethyl esters, E3MB and E2MB have shown high aroma impact on hopped beers (64,89,124). Aliphatic lactones have also demonstrated synergistic effects in wine model systems. Mixtures of less than 10 ppb concentrations of terpenes, cinnamates, vanillins and lactones significantly enhanced the floral, sweet fruit and peach aromas (77). Jarauta, Ferreiria and Cacho determined that when 5 aliphatic γ-lactones (C_{8-12}) were mixed together at average concentrations found in Spanish red wines, the Odor Activity Value (OAV) increased 3.5 times (58). This indicates that compounds of similar properties or "families of odorants" can greatly impact the beverage aroma (58).

List of figures

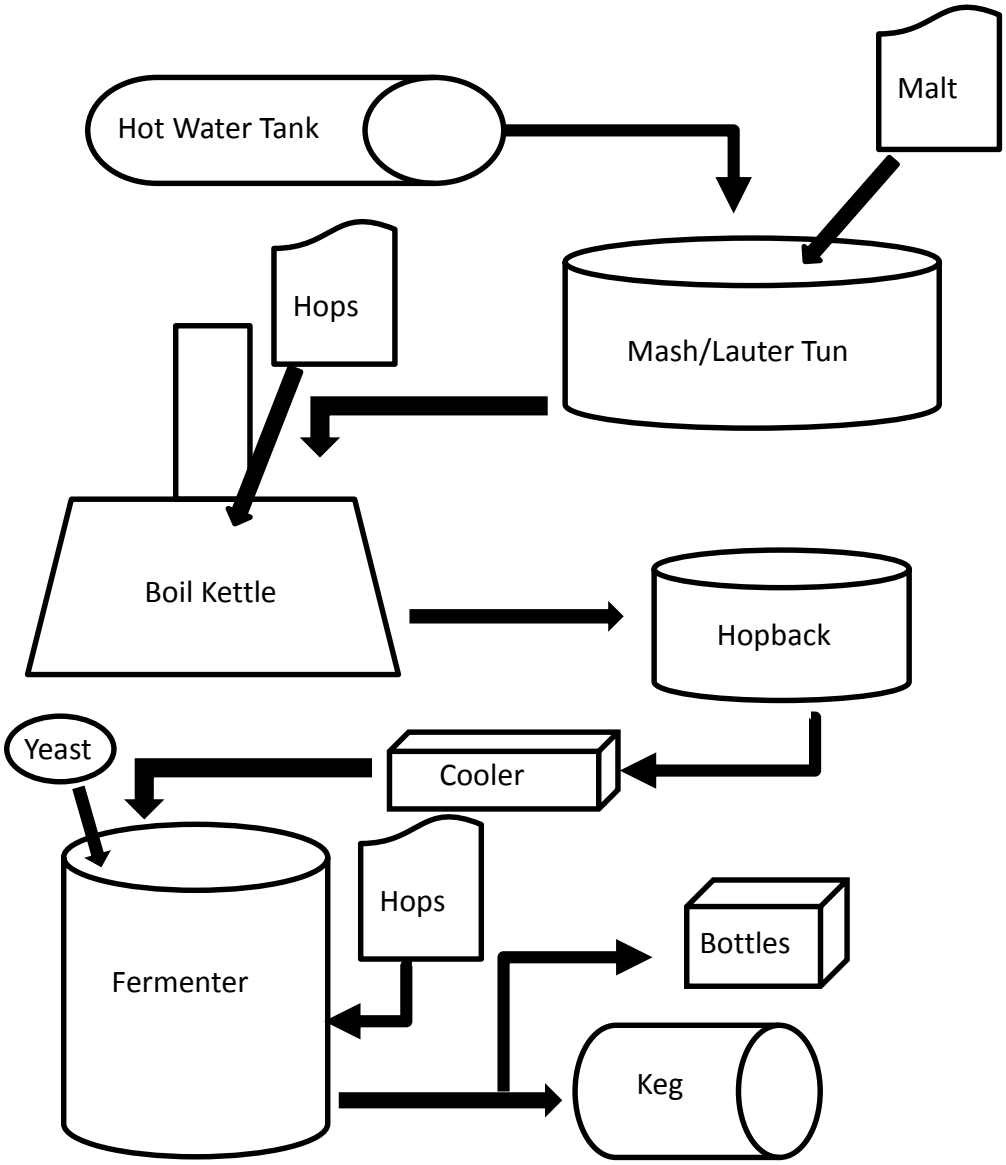

Figure 1.1. Flow diagram of the basic modern brewing process.

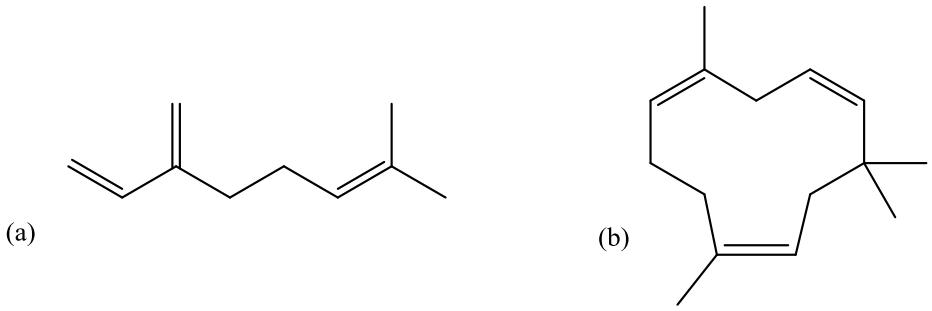

Figure 1.2. Monoterpene Myrcene (a) and sesquiterpene Humulene (b)

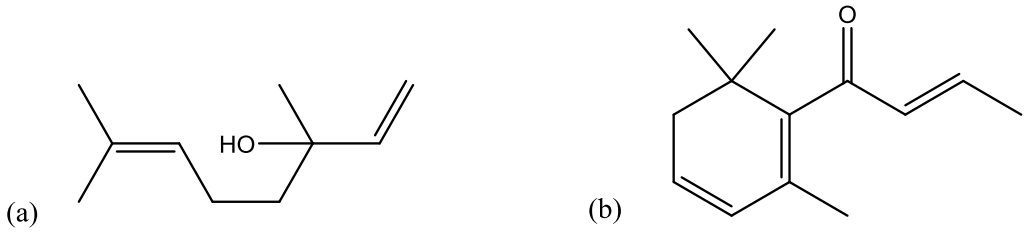

Figure 1.3. Terpene alcohol Linalool (a) and norisoprenoid β-damascenone (b)

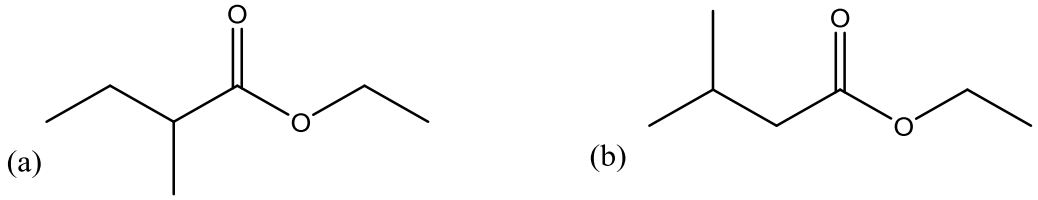

Figure 1.4. Ethyl esters ethyl 3-methylbutanoate (ethyl isovalerate) (a) and ethyl 2-methylbutanoate (b)

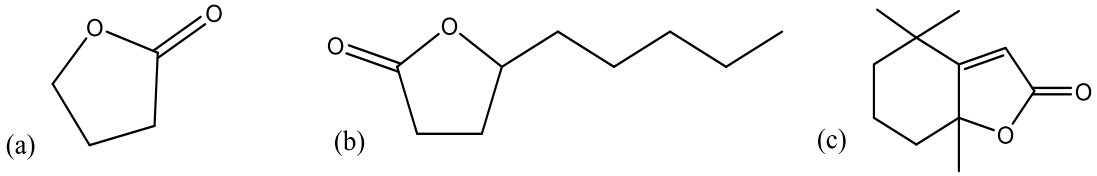

Figure 1.5. Examples of saturated (a) γ-butyrolactone, (b) γ-nonalactone and unsaturated lactone (c) Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone (derived from malting and wort boiling from β-carotene) found in beer from Tressl et. al (135)

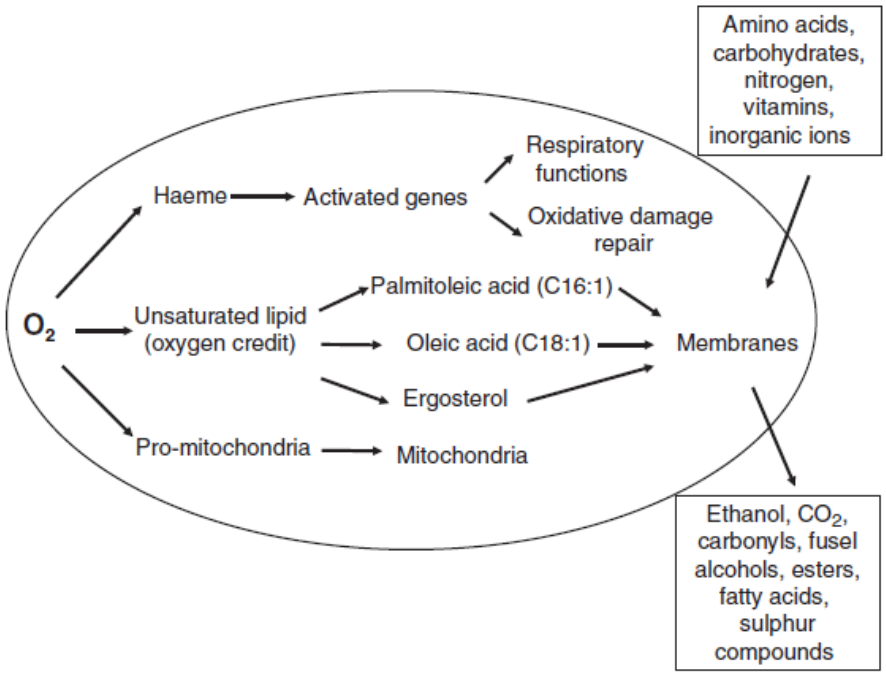

Figure 1.6. Use of oxygen for metabolism in the yeast cell (76)

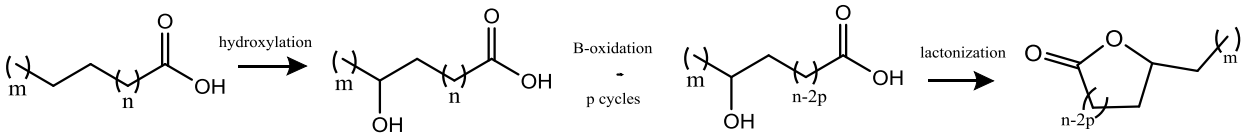

Figure 1.7. Generic scheme of lactonization (105)

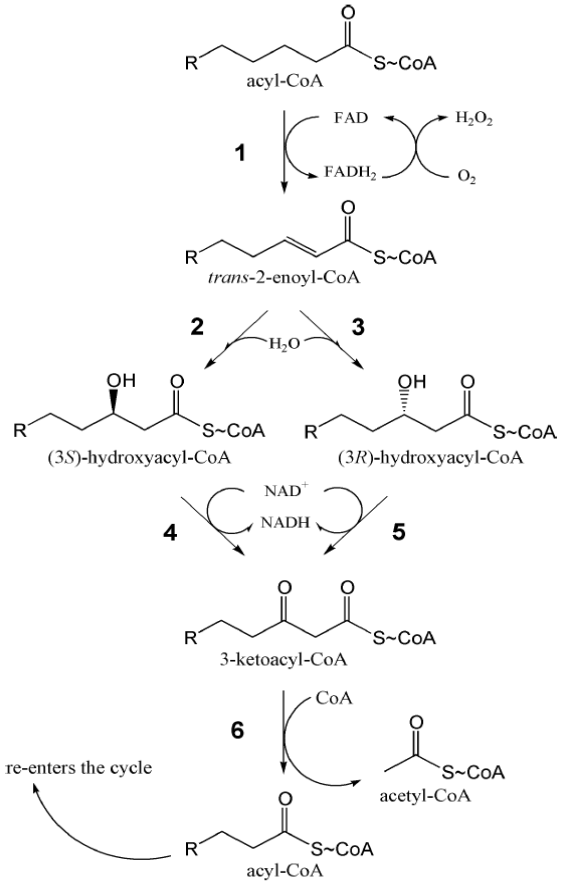

Figure 1.8. Generic scheme of B-oxidation.

List of tables

Table 1.1. The aroma quality, systematic names, and aroma detection thresholds in water of five aliphatic lactones reported in beer (47). Bolded descriptor is main aroma.

Compound	Aroma Quality	Systematic Name	Aroma threshold (ug/L, water)(31)
γ-nonalactone	**Coconut**, fatty	Dihydro-5-pentyl-2(3H)-furanone	30-65
γ-decalactone	**Peaches**, coconut, waxy	Dihydro-5-hexyl -2(3H)-furanone	11
γ-dodecalactone	**Fruity**, perfume, earthy	Dihydro-5-octyl-2(3H)-furanone	7
δ-decalactone	**Coconut,** oily	Tetrahydro-6-pentyl-2H-pyran-2-one	100
δ-dodecalactone	**Fruity**, tropical, fatty	Tetrahydro-6-heptyl-2H-pyran-2-one	50

Table 1.2. New World hops and their perceived aromas.

Hop Cultivar	Aroma
Citra ®	Strong citrus, tropical fruits, grapefruit, melon, lime, gooseberry, passion fruit and lychee
Mosaic ™	Tropical fruit, citrus, blueberry, floral, and herbal
Simcoe ®	Citrus, tropical and pine
Equinox ™	Citrus, lemon, lime, tropical, papaya, apple, herbal, floral and green pepper
Chinook	Spice, pine, and grapefruit
Centennial	Floral and citrus, lemon
Cascade	Floral, citrus and grapefruit

All descriptors found at hopunion.com

Table 1.3. Most abundant hop oil compounds (88)

Spicy/Herbal	Floral/Fruity	Citrus/Pine
Caryophyllene oxide	Geraniol	Limonene
Humulene Epoxide I	Geraniol Acetate	Farnesene
Humulene Epoxide II	Linalool	Citral
Humulene Epoxide III	Citronellol	Citranellal
Humulol	Nerol	Linalool
Humulenol II	B-damascenone	α-pinene
Myrcene	Citronellal	Ethyl-4-methylpentanoate
Eudesmol	Geranyl Isobutyrate	ethyl butyrate
Farnesol	B-ionone	
Ethyl Cinnamate		
Humulene		

Table 1.4. Aroma descriptors and thresholds of common hop-derived beer aroma compounds.

Compound	**Aroma descriptor**	**Olfaction Thresholds (μg/L)**
ESTERS		
Ethyl 2-methylpropanoate	Citrus, pineapple, sweet (a), fruity (c)	6.3 (a)
Ethyl butyrate	Citrus (a), papaya-like, apple-like (h)	1.0 (i), 400 (h)
Ethyl 2-methylbutanoate*	Citrus, apple-like (a), fruity, estery (b,c), sweet, artificial flavored candy (h)	0.1 - 1.1 (a) , 18 (g), 7-200 (h)
Ethyl 3-methylbutanoate*	Citrus, sweet, apple-like (a), fruity, estery (b), grape-like (h)	2.0 (a), 18-200 (h)
Isoamyl acetate	Banana, clove, solvent (a)	19 (i)
Ethyl 4-methylpentanoate	Citrus, pineapple (a), fruity (c)	1.0 (a)
Ethyl hexanoate	Citrus, estery (a), fruity (c), apple-like, aniseed-like (h)	170-210 (h)
LACTONES		
γ-valerolactone	Hay, tobacco (j)	10,000 (k)
γ-heptalactone	Sweet, caramel, nut-like (j) coconut, sweet, coumarin (k)	400 (l)
γ-nonalactone*	Coconut (c), peach, fruity (d), sweet (a,e)	30 (f), 30 - 65 (j,m)
γ-decalactone*	Peach, fruity, coconut, fatty (n)	2.6 (i), 11 (m)
δ-decalactone*	Peach-, apricot-like (j), coconut, oily	51 (i), 100 (m), 400 (o)
γ-dodecalactone*	Fatty, fruity, peach (l)	7 (m)
δ-dodecalactone*	Fresh-fruit, oily, peach-, plum-, pear-like (j)	50, 160 (j), 100 (p)

Table 3. continued

TERPENES/TERPENE ALCOHOLS		
Geraniol	Floral, rose-like (a)	4.0 (a), 3.2 (i)
Linalool*	Floral, citrus, terpenic (a, c)	0.14 (i), 1 (a), 27 - 100 (o, q)
β-ionone	Floral, violet-like, berry (a)	0.6 (a)
β-damascenone*	Citrus (a), grape, tobacco, rose (b), fruity (r), red fruits, strawberry (s), apple, peach (t), honey-like (c)	0.004 (i), 0.002-0.009 (u), 4 – 7 (w)
Myrcene	Spicy, resinous (v)	200 (v)

(a) in beer (64), (b) in beer (89), (c) in beer (37), (d) in beer (43), (e) in beer (73), (f) in wine (86), (g) in wine (35), (h) flavor threshold in beer (82), (i) in water (72), (j) in water (79), (k) in beer (39), (l) in water (39), (m) in water (47), (n) (71), (o) in beer (81), (p) in water (119), (q) in beer (93), (r) in beer (17), (s) in beer (43), (t) in beer (73), (u) in water (63), (v) in beer (117), (w) in wine (94). *Compounds that were used in sensory evaluation in this work.

Table 1.5. List of unsaturated lactones present in beer according to Tressl et. al. (126,136)

Lactone	Concentration (µg/L)
5,5-dimethyl-2(5H)-furanone	50-1750
Dihydro-5,5-dimethyl-2(3H)-furanone	20-1600
5-ethyldihydro-2(3H)-furanone	20
Dihydro-5-propyl-2(3H)-furanone	15
5-butyldihydro-2(3H)-furanone	20-50
Hydroxynonanoic acid lactone	320
Dihydro-5-pentyl-2(3H)-furanone*	100-500
5-hexyldihydro-2(3H)-furanone**	5-20
Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	30

* γ-nonalactone, **γ-decalactone

CHAPTER 2. HS-SPME GC-MS LACTONE DETECTION METHOD

2.1 Introduction

Headspace Solid Phase Microextraction (HS-SPME) is a commonly used extraction and concentration technique for aromatic compounds in the food and beverage industries. This technique is coupled with a gas chromatograph and often a mass spectrometer (GC-MS) or olfaction port (GC-O), which aid in the detection and identification of aromatic compounds. In order to evaluate the levels of lactones in a beer matrix, a straightforward and accessible HS-SPME method was adapted from published methods for the detection of five aliphatic lactones, γ-nonalactone (γ-C_9), γ-decalactone (γ-C_{10}), γ-dodecalactone (γ-C_{12}), δ-decalactone (δ-C_{10}) and δ-dodecalactone (δ-C_{12}) in beer.

2.2 Headspace Solid Phase Microextraction (HS-SPME) Technique

Extraction techniques generally fall into two categories: solvent assisted or solvent-less. HS-SPME is very appealing for it does not require the use of a solvent for extraction, is relatively inexpensive, concentrates volatile compounds quickly and can be used for solid in addition to liquid samples (150). This technique consists of a fiber with various coatings and thicknesses ranging from polar polyacrylate (PA) to nonpolar polydimethylsiloxane (PDMS) to mixed coatings of Carboxen/PDMS/Divinylbenzene (Car/PDMS/DVB). The adsorption of volatiles onto the fiber is dependent on the matrix composition, analyte concentration and fiber coating, length and thickness (46). Headspace sampling of the gaseous phase is not only representative of the compounds responsible for the perceived aroma of the beverages, but extraction is also faster than sampling the liquid phase directly (150). Equilibrium between the gaseous and liquid phases of most analytes is quickly reached when the salt saturated sample is stirred and extraction temperature is increased (150). A limiting factor of this technique is the fiber space available for compounds – competition between analytes, especially ethanol, can result in a misrepresentation of headspace composition and must be taken into

consideration when working with alcoholic beverages. The SPME fiber with absorbed headspace analytes is easily introduced and thermally desorbed in a GC injector thus allowing for quick identification of headspace compounds.

2.3 Instrumental Methodology

2.3.1 Sample Preparation

Theoretically, increasing the temperature of the sample will increase the concentration of analytes in the headspace which reduces extraction time (46). As such, elevated temperatures (30 - 70°C) are typically used depending on the molecular weight and boiling point of the compounds of interest. To optimize for lactone detection, two temperatures were chosen: 40 and 60 °C using a Car/PDMS/DVB fiber (Sigma-Aldrich) with three extraction times of 40, 50 and 60 minutes. A final temperature method of 50 min at 60°C was chosen for all sample analyses. In an effort to reduce ethanol competition, samples were diluted to 2% ABV. A volume of 9mL of diluted sample was dispensed to a 20 mL vial (Sigma-Aldrich) and saturated with 3.0 g NaCl (J.T. Baker).

A Gerstel MPS 2 was used for automation of vial penetration, extraction and injection program was as follows: sample was incubated for 5 min at 60 °C with agitation at 250rmp. Compounds were extracted at 60 °C for 50 min with agitation at 250 rpm. Immediately after extraction, fiber was desorbed in the GC inlet at 250 °C held constant for 10 min in splitless mode.

2.3.2 Operating GC-MS Instrumental Conditions

Carrier gas was UHP Helium at a flow rate of 1 mL/min with column head pressure of 7.2 psi. Oven temperature started at 40°C and held for 5 minutes, followed by 15°C/min ramp to 170°C, held for 0.5 minutes then 3°C/min ramp to 240°C and held for 10 minutes. SPME fiber was automatically baked out after each sample desorption for 10 minutes at 250°C. GC-MS transfer line was 240°C. MS operated in electron impact mode at 70 eV and

collected data at a rate of 3.2 scans/s over a mass range of m/z 50-350. Ion source was at 160 °C, detector voltage was set to 2165V and detector temp was 250 °C.

2.3.3 Validation of method

Calibration curves (1.56 – 12.5 µg/L) created by the method of standard addition of known compounds in light American lager beer, were run in duplicate. All analytical grade lactones were purchased from Sigma-Aldrich. Blank injections showed slight memory effect for γ-C_{12} thus a correction was applied by subtracting the peak areas of known carry over. The calibration curves were plotted as the relative peak areas of analyte to internal standard of γ-undecalactone as a function of compound concentration (Figure 2.1). The plots appeared to be fairly linear (r^2 >0.89) (Table 2.1).

The limit of detection (LOD, corresponding to 3 times signal to noise) and limit of quantification (LOQ, corresponding to 10 times signal to noise) were calculated from the ratio of peak areas to the average noise before and after each peak. LOQ values range from 1.2 µg/L to 14.2 µg/L and are lower than respective olfaction detection thresholds in unhopped pale ale. This method was repeated using five replicates of 12.5 µg/L in 2% ABV American light lager (Table 2.1). Reproducibility was determined by duplicates of 12.5 µg/L on three different days. Complete explanation of method is presented in Appendix A.

2.4 Conclusions

HS-SPME is an efficient and precise technique for most compounds present in beer, especially ones of higher concentration. However, the low pbb levels of these aliphatic lactones have proven difficult to reliably detect and quantify with this method at this time. The calibration curve is fairly linear, but further optimization is required to reach a mean correlation coefficient of >0.98 for each compound of interest. The repeatability was conducted with a new fiber, while the reproducibility was performed with two fibers of different quality, which is reflected in the high reproducibility RSD%. It

is also recommended to perform recovery studies in multiple styles of beer to observe any matrix effects. For future studies, solvent-assisted (Solvent-assisted Flavor Evaporation) or direct extraction (Stir Bar Soprtive Extraction) techniques should be explored and combined with HS-SPME.

List of figures and tables

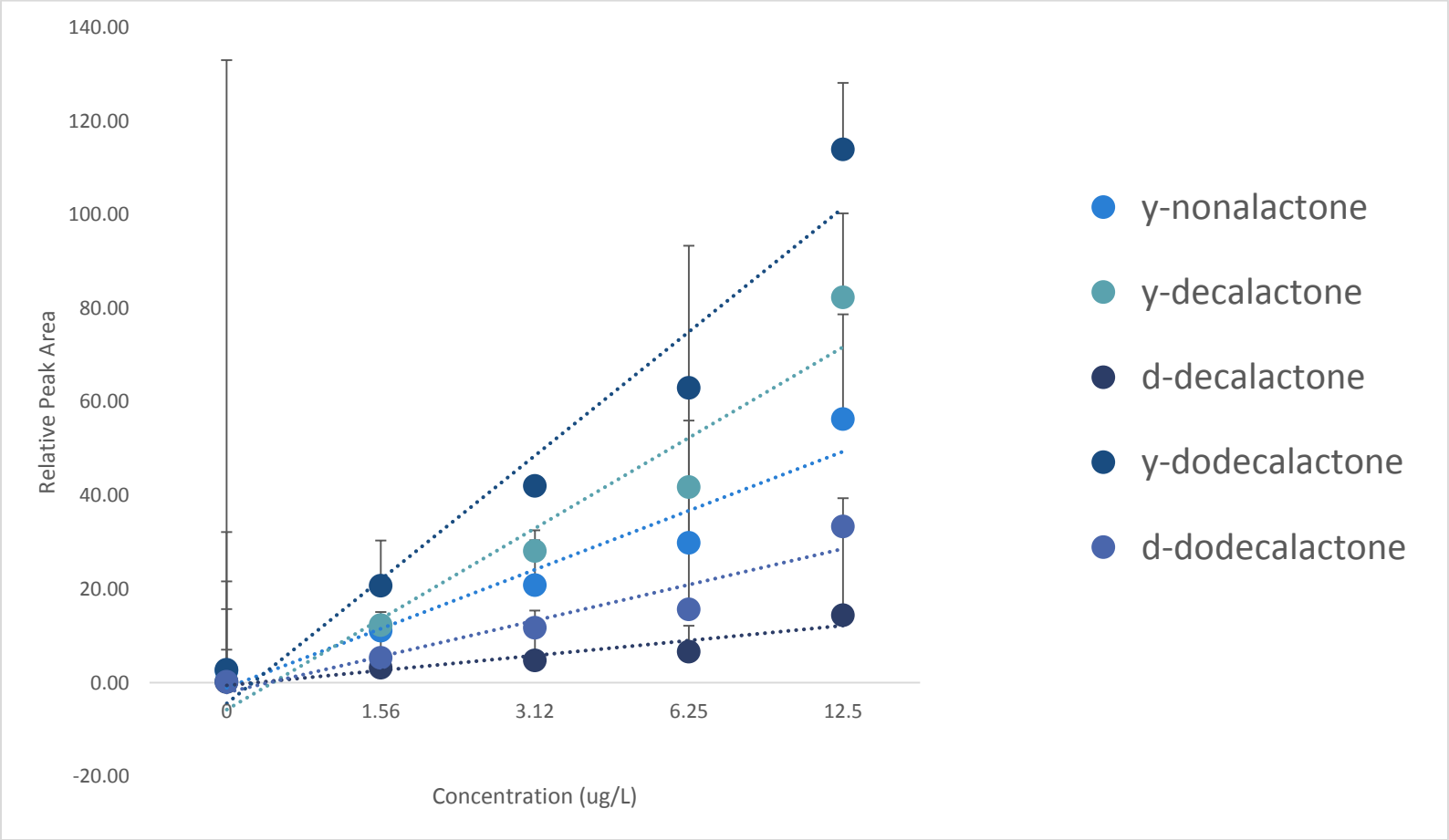

Figure 2.1. Calibration curve (1.56 – 12.5 µg/L) for five lactones in 2% ABV American light lager.

Table 2.1. Linearity, LOD, LOQ, repeatability, and reproducibility of proposed method.

Compound	Internal standard	Mean r^2	LOD (µg/L)	LOQ (µg/L)	Repeatability [a] RSD %	Reproducibility [b] RSD %*
γ-C_9	γ-C_{11}	0.9296	0.9	3.0	7.9	40.4
γ-C_{10}	γ-C_{11}	0.929	0.6	1.9	5.8	41.6
γ-C_{12}	γ-C_{11}	0.9462	0.4	1.2	4.9	46.8
δ-C_{10}	γ-C_{11}	0.8901	4.3	14.2	8.6	37.1
δ-C_{12}	γ-C_{11}	0.9106	2.0	6.6	5.7	32.7

[a] Repeatability was calculated from five replicates of 2 % ABV American light lager spiked with 12.5 µg/L on the same day.

[b] Reproducibility was calculated from duplicates of 2 % ABV American light lager spiked with 12.5 µg/L over three different days.

*Different quality of fibers were used over the course of reproducibility tests

CHAPTER 3. INFLUENCE OF ETHYL ESTERS, OXYGENATED TERPENES, AND ALIPHATIC Γ AND Δ-LACTONES (C_{9-12}) ON BEER FRUIT AROMA

(To be submitted to the Journal of the ASBC)

Abstract

Aliphatic γ- and δ- lactones with nine to twelve carbons (γ-C_{9-12} and δ-C_{10-12}) possess rich peach and coconut odors and have been identified as a potential source of fruity, especially stone fruit (i.e. peach, apricot), aroma in beers. In order to assess the influence of these lactones in a complex beer matrix, low levels (µg/L) of these lactones in combination with oxygenated terpenes (linalool and β-damascenone) and ethyl esters (ethyl 2- and 3-methylbutanoate) were evaluated by a descriptive analysis panel in addition to group olfaction detection Best Estimate Thresholds (BET) determination. Descriptive analysis demonstrated that lactones enhanced the final overall fruity aroma profile and contributed stone fruit, peach aromas when combined with the terpenes and esters. Group BET were calculated to be 376 µg/L (γ-C_9), 239 µg/L (γ-C_{10}), 238 µg/L (γ-C_{12}), 750 µg/L (δ-C_{10}) and 256 µg/L (δ-C_{12}) in an unhopped ale base according to ASTM E679 methodology. Headspace solid phase microextraction (HS-SPME) methodology was applied to commercial and controlled dry-hopped beers where levels of γ-lactones ranged from 1.2 to 2.3 µg/L, while δ-lactones ranged from 1.8 to 3.9 µg/L. Concentrations of detected lactones are well below group BET and appear to play a minor role in overall beer fruit aroma.

3.1 Introduction

Throughout modern history, many brewing scientists have aimed to identify and quantify aroma compounds in beer. Beer is composed of a multitude of chemical groups including but not limited to, esters, acids, higher alcohols, and terpenoids derived from all raw materials (2, 55, 63, 92, 95, 107). Stone fruit aroma (peach, apricot, plum) has emerged as a sensory descriptor of the aroma of some heavily hopped beers. Due to their

intense peach and coconut aromas, lactones are hypothesized to contribute to stone fruit aroma alone and/or in combination with other hop- or fermentation-derived compounds. The organoleptic significance of mixtures has been explored in many systems (48, 76, 79), and this paper aims to assess the impact of lactones individually and in combination with oxygenated terpenes and ethyl esters on beer fruity aroma. Individual compounds below or near their calculated detection thresholds may have an enhancing or masking effect on the perceived aroma when combined with other aroma compounds.

Lactones, with their potent oily, stone fruit, and coconut aroma qualities, are present in all food classes ranging from fruit to dairy to alcoholic beverages (144, 146, 147). Lactones are formed in fermented beverages when *Saccharomyces cerevisiae* transforms the fatty acids into cyclic esters (30). Malt and hops contribute the lipid and fatty acid precursors that can then potentially be transformed by yeast into potent lactones (40). Most commonly reported lactones in alcoholic beverages are γ and δ-lactones (5 and 6 membered rings, respectively) (18, 70, 72, 94, 144). However, there is very little data available on the levels of aliphatic lactones with 9 or more carbons present in beer, especially γ-deca and dodeca lactones (γ-C_{10} and γ-C_{12}), and δ-deca and dodeca lactones (δ-C_{10} and δ-C_{12}).

Γ-nonalactone (γ-C_9) was the first aliphatic lactone to be indisputably identified in multiple beer studies (40,73,110). In recent years, γ/δ- $C_{10\ \&\ 12}$ have also been detected in beers (71, 121, 136). Previous studies have reported the odor detection thresholds of γ-$C_{9,\ 10,\ \&\ 12}$ and δ-$C_{10\ \&\ 12}$ in water and wine where they range anywhere from 7 to 793 µg/L (30, 34, 41, 46, 57, 70, 85). Γ-C_9 odor detection threshold ranges from 11.2 to 607 µg/L in beer (109, 126) while γ- and δ-C_{10} have reported flavor thresholds of 400 µg/L (81). However, the thresholds of these five aliphatic lactones have never been published in the same unhopped pale ale matrix. Interestingly, the published concentrations of these lactones in beer are considerably lower than the ranges of thresholds in other alcoholic beverages and published literature does not demonstrate that these five aliphatic lactones are the main drivers of stone fruit aroma in beers. Hop essential oil compounds,

for example linalool and β-damascenone, and fermentation-dervied esters, such as ethyl 2- and ethyl 3-methylbutanoate (E2MB and E3MB), are commonly detected above sensory threshold in beers and are therefore considered to be major contributors to fruit aroma in beer. .These four hop- and fermentation-derived compounds are known to possess fruity aromas so in combination with lactones, may potentiate stone fruit aroma.

In order to assess the contribution of lactones, olfaction thresholds and aroma qualities of individual lactones and mixtures of oxygenated terpenes and ethyl esters were determined. A Headspace Solid Phase Microextraction (HS-SPME) technique was optimized to provide an instrumental analysis of the volatiles in the headspace above the beer samples. The levels detected by this method were compared to sensory findings to evaluate whether or not these lactones' contribute to stone fruit aroma. It is important to note that quantification by HS-SPME is highly dependent on original analyte concentration so data obtained with this methodology should take into consideration these limitations.

3.2 Materials and Methods

3.2.1 Reagents

Analytical standards of >98% purity of γ-$C_{9, 10, 11 \& 12}$, and δ-$C_{10 \& 12}$ were obtained (Sigma-Aldrich Corp, St. Louis, MO). Analytical grade sodium chloride was obtained from JT Baker (Center Valley, PA). Milli-Q water was obtained from a Milli-Q Plus water system (Darmstadt, Germany). The solid phase microextraction (SPME) fiber was 1 cm 23 gauge Divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) Stable Flex with 30/50 µm coating thickness (Sigma-Aldrich Corp, St. Louis, MO)(109). 20mL clear glass SPME vials with screw tops with silicone septa were used for sample extraction (Sigma-Aldrich Corp, St. Louis, MO).

3.2.2 Beer Production

An unhopped pale ale was brewed for all sensory evaluations and dry-hop trials. The base malt consisted of 98.5% 2-Row pale malt (Great Western Malting, Vancouver, WA) and 1.5% acidulated malt (Weyermann, Bamberg, Germany) with an original gravity of 13.0° Plato. The wort was boiled for 60 minutes without the addition of yeast or hops, then cooled, aerated and fermented with American Ale™ yeast (1056 Wyeast, Odell, OR) at 20 °C for 7 days followed by lagering at 2°C for 4 days. The beer with 5.6% ABV and real extract of 2.5° Plato was filtered bright and kegged with 0.5-1 volumes of CO_2. The beer was left uncarbonated for all sensory evaluations to decrease variability in carbonation levels and perception during testing.

3.2.3 Dry-hopping and Yeast Addition Protocol

The unhopped pale ale was allowed to reach 68°F (20°C) in 3 gallon (11.3 L) Cornelius kegs prior to dry-hopping treatments. Citra, Centennial, Chinook, Mosaic and Cascade hops were weighed according to a dosing rate of 1 lb/bbl (3.8 g/L) and contained in a sterilized synthetic cheese cloth bag. To ensure the bag did not float during treatment, a clean tri-clamp fitting was added to the bag to provide additional mass. The bag containing hops and clamp were then placed in mylar bags that were flushed with N_2, sealed and stored in at -10°F (-23°C) until use.

To dry-hop, the kegs were filled with 8.00 kg of beer and headspace was flushed with CO_2. An equivalent of 10^6 cells/mL were added to each keg to mimic the amount of yeast still in suspension in a commercial facility. The synthetic cheesecloth bag containing the hops was added to the vessel and top closed all while under low flow of CO_2.Kegs were mixed and stored at 68°F (20°C) for the duration of the experiment.

3.2.4 Olfaction Threshold Determination

For all sensory evaluations, the desired volume of uncarbonated beer was pushed from keg with CO_2 into a sealable holding vessel and allowed to warm to room

temperature for more than 1 hour prior to sample preparation for sensory evaluation. Samples were served at room temperature to decrease sample temperature variability that potentially arises when distributing to multiple panelists.

A preliminary bench test was performed to gather an appropriate range of lactone concentrations required for the execution of the threshold tests. Analytical standard lactones were diluted to 10,000, 1,000, 100, and 10 mg/L stock solutions into 10% ethanol solutions using 95% ABV food grade ethanol (EMB Millipore, Darmstadt, Germany) with a micro pipette (Rainin L300, Mettler-Toledo, Columbus, OH) and analytical balance. Lactone stock solutions were dosed into 125 mL serving glasses (Libbey Glass, Toldeo, OH) according to the range of concentrations listed in Table 3.1 with micropipette (20-300 µg/L of corresponding stock solutions). The mass of beer, 50g for thresholds and 30g for descriptive analysis, was subsequently covered with plastic lids (Sysco Corp. Houston, TX) to contain the aroma in the headspace.

The prepared samples were blind coded with a 3-digit number and presented in a randomized order for each panelist using a paper ballot (Appendix C). All threshold olfaction tests were conducted at room temperature over the course of three sequential days at the same time in the afternoon each day in the same room. The detection threshold for the five aliphatic lactones was executed and reported according to the standard “best estimate threshold” methodology (ASTM E679-04) for the determination of odor thresholds by ascending concentration (29). A minimum of 25 assessors were recruited and pooled for each testing session (5). Participants were trained on how to complete the 3-alternative forced choice (3-AFC) tests, which consists of three stimuli – one spiked sample and two blank bases. The samples were open and visible for all panelists to observe. Panelists were instructed to circle the three-digit code that corresponded to what they believed contained the dosed lactone. Prior to commencing the assessments, panelists were provided a sensory description, sample of the chemical standard dosed above threshold in the base and a blank reference and were instructed to only smell the samples.

Panelists were presented with seven sets of 3-AFC tests and evaluated two to three threshold compounds each session. The concentrations of the test sample doubled in each succeeding set and ranged from 12.5 – 25600 µg/L. Re-evaluation was administered if the assessor correctly identified the spiked sample in the first set (lowest concentration) of the primary session. If retesting for a specific panelist was not possible, the panelist's data were removed from final group calculations (Appendix D).

3.2.5 Olfaction Threshold Calculations (Best Estimate Threshold)

Using the protocol outlined in the ASBC Standard Method for detection thresholds and the ASTM E679 (29), the individual assessor threshold values were calculated as the geometric mean between the last incorrect and the first correct response (Table 3.1). The individual threshold values and frequency were plotted to determine population normalcy and the standard deviation of the logarithmic transformed individual values was calculated for each compound. The group "best estimate threshold" (BET) for each lactone was calculated from the logarithmic mean of the individuals' threshold values (Appendix F).

3.2.6 Olfaction Descriptive Analysis

Eight panelists, five of whom had previous experience with beer descriptive analyses, were trained and participated in a descriptive analysis of selected beer treatments. Panelists participated in three training sessions to introduce themselves the 0-15 point scale (0 = none and 15 = very strong), the aroma types and intensities expected during testing, and to establish the descriptive lexicon. Anchoring the scale was achieved by using the un-dosed base beer as a low signal and all compounds combined together as the high signal in addition to 10 real fruit standards to promote lexicon development. The finalized ballot contained the following sensory descriptors: Overall Aroma Intensity, Stone Fruit/Peach, Coconut/Oily, Red Berry, and Melon.

Five samples containing various mixtures of lactones (γ-C_9, γ-C_{10} and δ-C_{10}), terpenes (linalool and β-damascenone), and esters (ethyl 2- and ethyl 3-methylbutanoate) were evaluated. Concentrations of compounds used were selected based on previously published values found in beer (42, 63, 71, 72) (Table 3.2). The panel was conducted and reported using an approach adapted from a descriptive analysis methodology established by Hootman and Stone for the description of perceptions (54). The presentation order of the beer samples was blocked by replication such that all five treatments were presented to each panelist within each replication. Presentation order was randomly assigned within each panelist. Panelists were instructed to scale all sensory attributes using the 16-point scale for a particular beer treatment before moving onto the next sample. Testing was carried out over three sessions with which two individual sensory replications were evaluated, thereby achieving a total of six replications in a complete randomized block design Panelists were instructed to take a 5 minute break between each replicate set of five samples. Analysis of Variance (ANOVA), Tukey's HSD multiple comparisons test, and Principle Component Analysis (PCA) were performed using XLStat to evaluate panelists' performance and differences among samples (Addisoft, Coppell, TX).

3.2.7 HS-SPME Sample Preparation for Instrumental Analyses

Dry-hopped samples (~200 mL) were collected into a sampling vessel after 24 hours by dispensing them from the dry-hopping kegs with CO_2. The beers were filtered through 0.45 µm Whatman filter paper, 9 mL aliquots transferred into 20 mL glass SPME vials, purged with N_2, capped and stored in a freezer until HS-SPME-GC-MS analysis was performed. Commercial beers samples were prepared similarly.

To decrease ethanol competition on the SPME fiber, all samples were standardized to 2% ABV with Milli-Q water using original and final gravities if brewed at Oregon State University or according to the published ABV on commercial bottles. Samples were then volumetrically transferred to 20 mL SPME vials. Headspace SPME (HS-

SPME) was performed using 9 mL sample and saturated with 3.0 g NaCl (35.7% w/v) with the addition of an internal standard of γ-C_{11} added at a final concentration of 50 µg/L. All samples were purged with N_2 prior to extraction. Sample vials were placed in Gerstel MPS2 sample tray at room temperature (Gerstel, Germany). Gerstel MPS2 was used for automation of extraction and injection of the headspace sample and method was as follows: samples were incubated at 60°C for 5 min with 250 rpm agitation, volatiles were extracted onto the SPME fiber at 60°C for 50 minutes with 250 rpm agitation, finally the compounds were desorbed in the GC using a septumless injector for 10 min at 250 °C. This method was based off of previous studies of γ/δ-lactones in wine (19,71) with adjustment of extraction time and temperature.

3.2.8 Instrumental GC-MS analysis

Preliminary analyses were performed on an HP5890 with a FID, but was deemed too insensitive for the low concentrations of lactones in a beer matrix. Therefore, a method was developed for an HP6890 GC coupled with a HP 5972 single quadrupole MS in scan and selected ion Monitoring (SIM) modes. The GC conditions were as follows: Injections were performed in splitless mode for 2 min with temperature held at 250°C for the duration of fiber desorption. The column used was a Supelcowax 10 (30m x 0.25mm x 0.5µm film thickness, Supelco, St. Louis, MO) with ultra-high purity Helium carrier gas at a flow rate of 1 mL/min with column head pressure of 7.2 psi. Oven temperature started at 40°C and held for 5 minutes, followed by 15°C/min ramp to 170°C, held for 0.5 minutes then 3°C/min ramp to 230°C, then 10°C/min ramp to 240 °C and held for 10 minutes. SPME fiber was automatically baked out after each sample desorption for 5 minutes at 250°C. GC-MS transfer line was 240 °C. The mass selective detector operated in electron impact mode at 70 eV and collected data over a mass range of m/z 50-350 in scan mode. Ion source was set at 160 °C, detector voltage was set to 2165V and detector temp was 250 °C.

Lactones were identified and quantified using scan and SIM modes of quantifier and qualifier ions. Previous studies demonstrate that the major ions for γ-lactones and δ-lactones are m/z 85 and m/z 99, respectively, and were thus used as the quantifier ions. Scan mode was utilized to select unique qualifier ions (m/z ions) to help eliminate the interference of co-eluting compounds (Table 3.3). To identify lactones, sample results were compared with pure lactone standard retention times and mass spectra, and confirmed with NIST mass spectra library. External calibration curves (1.56 – 12.5 µg/L) were determined by the method of standard addition of the six analytical grade lactone compounds in light American lager beer adjusted to 2% ABV (Table 3.4). Blank injections showed a slight memory effect for γ-C_{12} thus a correction was applied by subtracting the peak areas of known carry over.

3.3 Results

3.3.1 Olfaction Group Threshold Determination

A total number of 58 panelists (14 female and 44 male) ranging from 21-52 years old with a median age of 27 participated in threshold testing over the course of the study. Population distributions of two of the five aliphatic lactones appeared to have normality for γ-C_{10} and δ-C_{10} (Appendix F) while the other three displayed non-normality. For instance, γ-C_{12} appeared to be approaching a bi-modal distribution where a population of highly sensitive panelists (<28 µg/L geometric mean concentration) was present alongside a considerably less sensitive population (Figure 3.1). Figure 3.2 represents a normally distributed population for δ-C_{10}. A few panelists' data were removed from the final data set due to suspicious results or they were unable to be retested on a lower concentration range for the specific compounds, but at least 25 assessors were used to generate group detection thresholds for each compound (Appendix D).

γ-lactones (γ-C_9, $C_{10,}$ and C_{12}) group BET concentrations were calculated to be 376, 239, and 238 µg/L, respectively, while the δ-lactones (δ-C_{10} and C_{12}) were 750 and 256

µg/L, respectively (Table 3.1). γ-C_{10} had the smallest range of concentrations detected – 71 to 566 µg/L whereas γ-C_9 and δ- C_{10} had the most variable ranges of detection.

3.3.2 Descriptive Analysis

Eight panelists (3 female and 5 male) ranging from 26-50 years old with a median age of 30 evaluated five samples by scaling descriptive attributes using a 16-point scale. Panelist performance was evaluated and seven out of the eight panelists could discriminate on one or more attributes, so all scores from all panelists were included in subsequent statistical analysis (Appendix G). The unhopped pale ale base was also included among the sample set to determine if panelists could distinguish the base from any spiked sample. In all cases, the base received the lowest average scores while the beer treatment with all aromatic compounds combined received the highest average score (Table 3.6).

The addition of compounds did not result in significant differences across treatments for the Coconut/Oily descriptor other than being significantly higher than the base. In addition, no significant differences were found among any of the treatments including the base for the Melon descriptor. Conversely, Overall Intensity, Stone Fruit/Peach and Red Berry averaged scores increased with the addition of compounds resulting in significant differences among treatments. Interestingly, when sub-detection threshold concentrations of lactones were dosed, there were no significant differences between lactones and the base with respect to Stone Fruit/Peach and Red Berry aromas. Yet when esters and terpenes were added Stone Fruit/Peach and Red Berry qualities increased significantly.

Principal Components Analysis (PCA) was performed on descriptive data and 98.1% of the variance was explained by the first two components with the beer samples separated primarily on the first component while the aroma descriptors are separated on the second component (Figure 3.3). Overall Intensity was highly correlated with Stone Fruit/Peach whereas Stone Fruit/Peach and Coconut/Oily were not. The dosed samples

linearly trended towards the Stone Fruit/Peach and Overall Intensity cluster as more compounds were dosed into samples; when all compounds were present, the sample had the highest Stone Fruit/Peach while solely lactones produced a beer with the lowest intensity. The PCA also showed the base being the farthest removed from all descriptors and other dosed samples in a completely separate quadrant.

3.3.3 Instrumental Results of Beers

A sampling of commercially produced and experimental dry hopped (DH) beers were analyzed in duplicate and the concentrations of the five aliphatic lactones were measured (Table 3.5). All detectable lactones had concentrations less than 4 µg/L which was below calculated LOQ for four of the lactones and significantly lower than all five of the olfaction detection thresholds. γ-C_{10} and δ-C_{10} levels were in agreement with previously published concentrations in beer (72).

3.4 Discussion

The olfaction detection thresholds for five aliphatic lactones were determined in an ale matrix using ASTM E679 methodology. Testing these compounds in unhopped ale as opposed to water or an ethanol:water model system allowed for the most accurate and relevant representation of the lactone thresholds in beer. Despite the panelists' exposure to the compounds prior to testing, the group BET values were considerably higher than published values in water, wine and model solution matrices, which was expected due to differences in matrix composition and variability in assessor population (Table 3.1).

Threshold tests only involve and represent a small portion of the population, which results in a wide range of individual thresholds. Figures 3.1 and 3.2 demonstrate two very different individual threshold concentration distributions; one is nearly bi-modal while the other is normal, thus the group BET number does not fully explain the distributions. The group threshold value for γ-C_{12} poorly represented the sensitivities and

detection levels of the assessors involved; nearly half of the assessors (12 out of 28) were considerably more sensitive than would be expected from the calculated group threshold value. On the other hand, the individual threshold values for δ-C_{10} were normally distributed and the calculated group threshold value more accurately represents the sensitivities of a majority of the assessors. While the group threshold concentration is an important index for comparing aromatic compounds and assessing their sensory impact in food systems, some caution must be exercised. It is important to be keep in mind the total range of threshold concentrations in a population and the nature of the population distribution when claiming a singular threshold concentration. For instance, bimodality of the threshold concentration distribution implies that two separate distributions may exist, one for a group of assessors that are particularly sensitive to the compound in question and another group that is relatively less sensitive. The non-normal distributions for four out of the five compound suggested that these thresholds are not good representatives of these compounds' thresholds in unhopped pale ale beer. There are limitations with Best Estimate Thresholds, as there are with any method, so perhaps the use of a different technique, such as signal detection method and calculating d' would be more appropriate for threshold determinations.

Interestingly, δ-C_{10} had the highest calculated group threshold concentration (750 µg/L) when compared to the other four aliphatic lactones, which in and of themselves were relatively similar to each other (238 – 376 µg/L). This suggested that higher levels of this compound were required for the average assessor to detect a difference from the base beer. δ-C_{10} is typically found in low levels in fruits and other alcoholic beverages potentially due to the fact that it can be reabsorbed and metabolized by the yeasts resulting in γ-C_9 or other end products (1). It has also been reported that γ- lactones have lower thresholds than their corresponding δ-lactones in water and oil mediums (119), which in this study was the case for γ-C_{10} but not for γ-C_{12}.

The instrumental method developed in this study demonstrated that lactones can be detected via HS-SPME-GC-MS. The sampling of commercially produced beers

encompassed different styles, from beers that are not perceived as having stone fruit aromas (ESB) to ones that were brewed with fruit including peaches and pluots. The tested pale ale had been collectively described by a sensory panel as having pronounced stone fruit aroma during a separate experiment. The hops chosen for the dry-hopping trials of experimental beers are commonly used for late and/or dry-hopping by American craft brewers to produce fruity aromas in beer, which in some cases present stone fruit/peach aroma qualities.

Data collected by this HS-SPME method demonstrates that all aliphatic lactones of interest, regardless of style and hop cultivar used, were at very low levels (4 µg/L) with δ-C_{10} tentatively present in the matrix at the highest levels. However, δ-C_{10} levels are still below its calculated LOD and measured levels corresponding to γ-C_9 and γ-C_{10} are also below their respective LOQ levels so further studies are required (Table 3.5). The trace levels of lactones in beer are difficult to detect via headspace methods due to their low concentration and higher molecular weight and therefore more sensitive instruments, such as tandem MS or Time of Flight MS, and extraction techniques, such as stir-bar sorption and solvent assisted methods, should be explored and used alone or in combination with HS-SPME.

It is interesting to note that all instrumentally detectable levels of lactones were considerably lower than the group detection thresholds and as such, individually these lactones most likely do not have a large impact on the final aroma profile in beer. However, there exists the possibility that these lactones could potentiate stone fruit aromas at sub threshold levels. According to studies performed by Loscos et al., when less than 10 ppb concentrations of aliphatic lactones were dosed into a model wine matrix individually, there was no significant difference from the un-dosed sample. However, when combined with low ppb levels of terpenes, cinnamates, and vanillins, the aroma of a model wine solution was described as peach and sweet fruit (72). Similar observations were made in our study.

To test the hypothesis that low levels of lactones could potentiate stone fruit aroma in beer, a trained descriptive panel was conducted using an unhopped beer that was dosed with a range of compounds at previously published and realistic concentrations (Table 3.2). Results indicated a statistically significant aroma effect among the five treatments for four out of the five sensory descriptors. The increase in average Overall Intensity scores as more compounds were dosed indicated that there is little to minimal masking effect among the three groups of compounds (Table 3.6). In fact, the opposite was observed. It is interesting to note that the sample dosed with all compounds combined was significantly different in Red Berry aroma than any other treatment, which could be attributed to additive effects between the lactones, esters and linalool, which are known to be major contributors to raspberry and blackberry aromas (28).

The sample dosed solely with lactones was not significantly different from the base beer in Stone Fruit/Peach or Red Berry aromas, which confirmed that the lactone concentrations used were below or near panelists' detection thresholds. As esters and terpenes were added to the mixture, the Stone Fruit/Peach aroma increased (Table 3.6 and Figure 3.3). Individually at super threshold concentrations, these lactones have potent oily coconut and peach aromas, yet in the present study our panelists did not find any correlation between these qualities. While the Coconut/Oily descriptor increased significantly above that of the base after the addition of any lactone or lactone-ester/terpene combination, the increase was minimal and independent of increases in Stone Fruit/Peach aroma. This result can be observed in the PCA biplot (Figure 3.3) along with the distinct separation of the base beer from any of the dosed samples. However, it is important to note that the dosed samples are separated along the first component that describes 86.5% of the variance while the aroma descriptors are separated along the second component which only describes 11.6% of the variance. This suggests that while lactones may influence the perceived the fruit aroma, they are not a major driver of stone fruit aroma in beers.

3.5. Conclusions

The five aliphatic lactones examined in this study individually have group detection thresholds ranging from 238 – 750 µg/L, consistently higher than published values in water and wine. However the levels of these lactones tentatively detected in dry-hopped and commercially produced beers were considerably lower, by at least two orders of magnitude, than the calculated group detection thresholds. When these lactones were combined at low ppb or subthreshold levels with other hop- and fermentation-derived compounds, Stone Fruit/Peach aroma quality increased as did the Overall Intensity. These sensory findings coupled with instrumental data suggested that the levels of lactones are not significantly influencing beer aroma individually, but could still potentiate stone fruit and peach aromas when in the presence of other fruity volatiles. Further exploration of other fruity aromatic compounds that were not tested in this work and the unambiguous detection of lactones and other lipid-derived flavor molecules will lead to a better understanding of their contributions to the aroma profile of beer.

List of figures

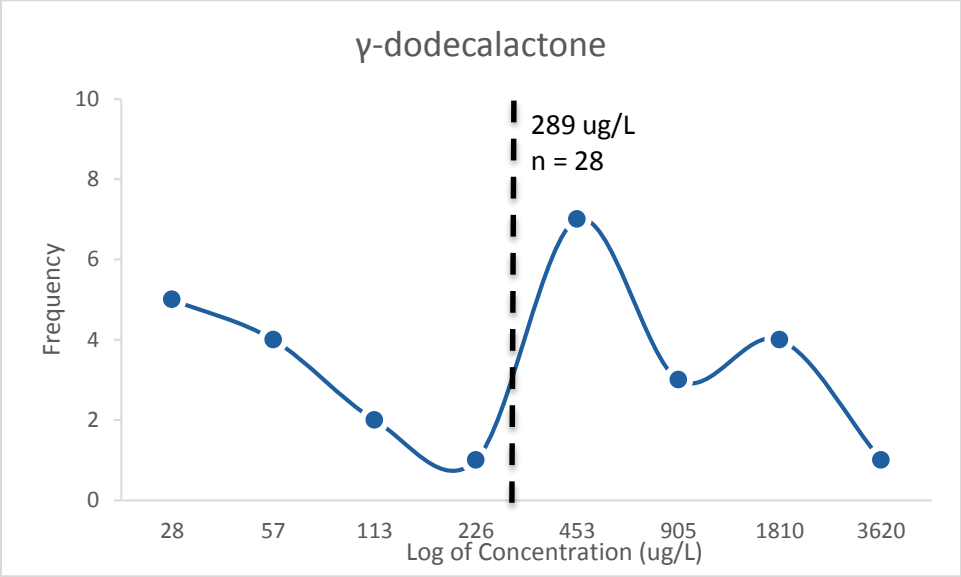

Figure 3.1. An example of a population approaching bi-modal distribution for γ-dodecalactone threshold concentrations.

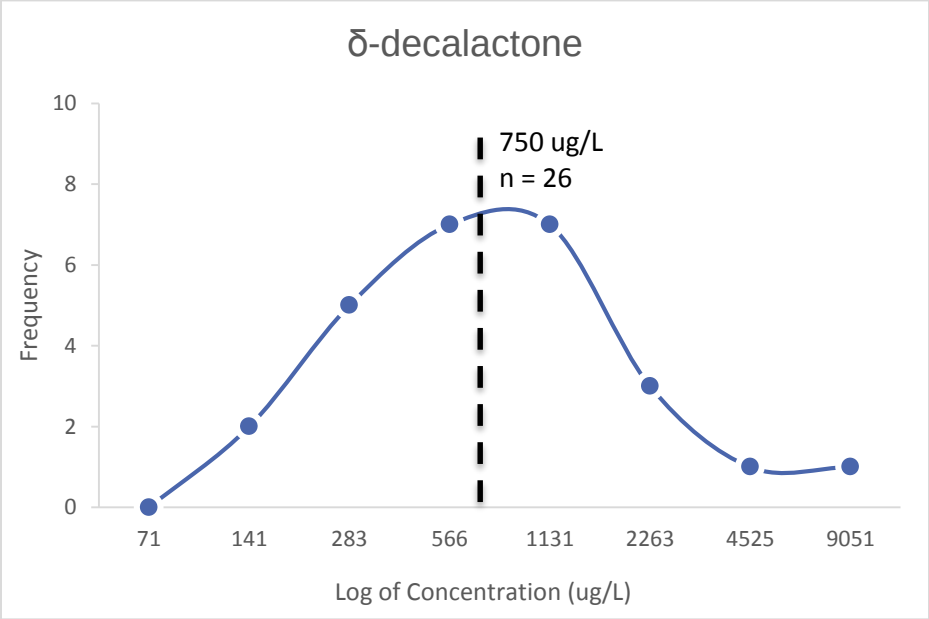

Figure 3.2. An example of a normally distributed population for δ-decalactone threshold concentrations.

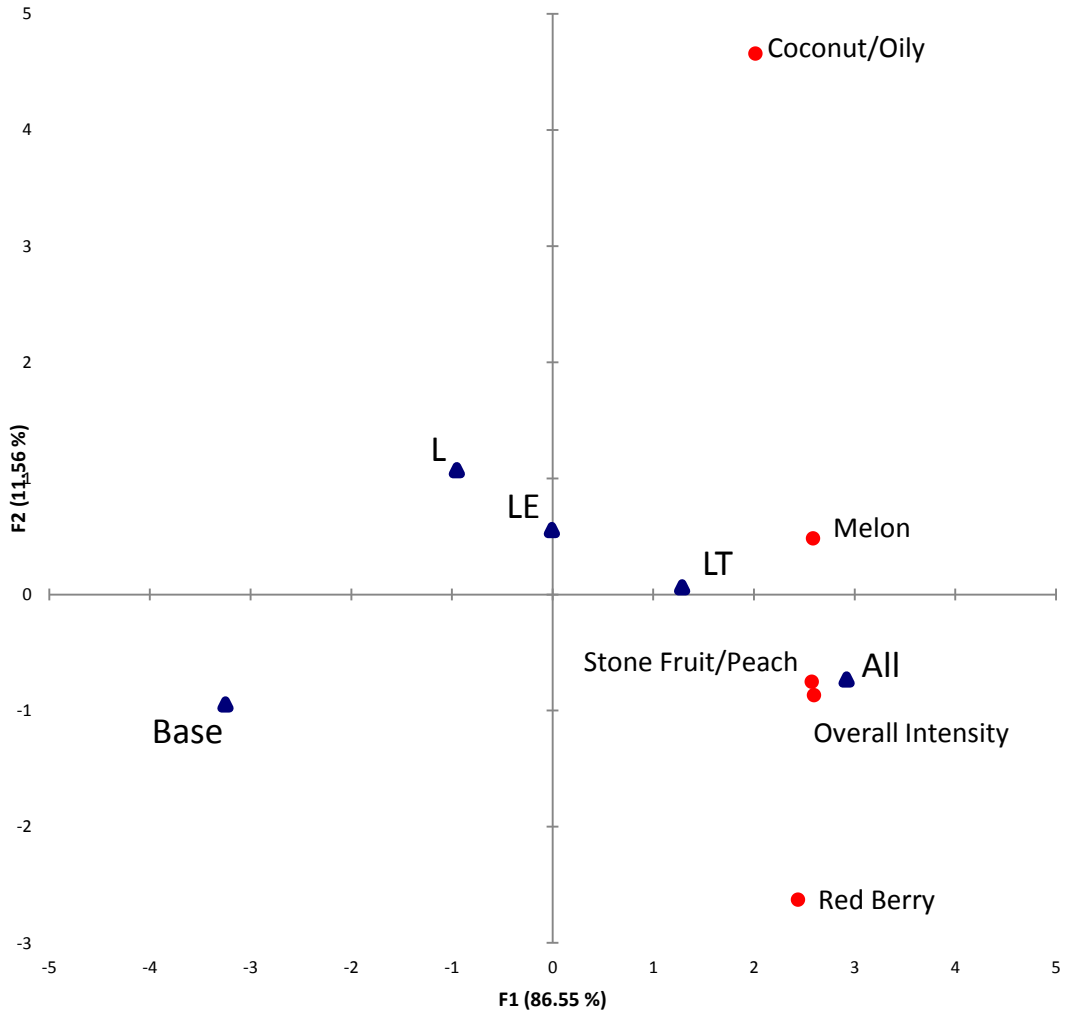

Figure 3.3. Principal Component Analysis of descriptive analysis scores averaged over all panelist.

Base – unhopped pale ale
L – Lactones
LE – Lactones + Esters
LT – Lactones + Terpenes
All – Lactones + Esters + Terpenes.

List of tables

Table 3.1. Individual geometric mean concentration ranges, number of assessors and group Best Estimate Thresholds of five aliphatic γ- and δ-lactones.

Compound	Individual concentration ranges (µg/L)	No. of Assessors	Group BET (µg/L)	Reported Thresholds (µg/L)	Source
γ-C_9	26 – 6788	29	376	30 in wine 30 - 65 in water	(47,79,86)
γ-C_{10}	71 – 566	25	239	2.6 – 11 in water	(47,72)
γ-C_{12}	28 – 3620	28	238	7 in water	(47)
δ-C_{10}	141 – 9051	26	750	51 - 100 in water 400 in beer*	(47,72,81)
δ-C_{12}	35 – 2263	28	256	50 – 160 in water	(79,119)

*Flavor threshold in beer

Table 3.2. Concentrations of lactones, oxygenated terpenes and ethyl esters dosed into samples used for descriptive analysis and published levels found in beer.

Compound	Aroma	Concentrations (µg/L)	Reported levels (µg/L)	Source
γ-C_9	Coconut	30	20-84.1	(43,72,73)
γ-C_{10}	Peach	2	1.3	(72)
δ-C_{10}	Fruity	3	2.7	(72)
Linalool	Floral, citrus	100	1.31-167.0	(62,63,64)
β-damascenone	Citrus, red fruits	3	0.6-9.0	(43,63)
Ethyl 2-methylbutanoate	Fruity, estery	6	0.02-9.78	(62,64)
Ethyl 3-methylbutanoate	Fruity, citrus	6	0.01-5.32	(64)

Table 3.3. Quantifier (bold numbers) and qualifier ions for five saturated lactones evaluated in this study.

Compound	m/z
γ-C_9	**85**, 86, 100, 128
γ-C_{10}	**85**, 86, 100, 128
γ-C_{12}	**85**, 86, 100, 128, 136
δ-C_{10}	55, 71, **99**, 114
δ-C_{12}	71, **99**, 114, 134
γ-C_{11} (IS)	**85**, 100, 114, 128

Table 3.4. Limit of Detection and Limit of Quantification for five aliphatic lactones.

Compound	LOD (µg/L)	LOQ (µg/L)
γ-C_9	0.9	3.0
γ-C_{10}	0.6	1.9
γ-C_{12}	0.4	1.2
δ-C_{10}	4.3	14.2
δ-C_{12}	2.0	6.6

Table 3.5. Lactones levels detected (μg /L) in a sample of commercial and 24 hour dry-hopped (DH) beers

	Concentration (μg/L)				
Beer	γ-C_9*	γ-C_{10}*	δ-C_{10}**	γ-C_{12}	δ-C_{12}
IPA	1.6	-	-	1.5	-
ESB	1.2	-	-	-	-
Wheat	2.2	-	-	1.3	-
Fruit brewed	-	-	-	2.2	3.1
Pale Ale	-	-	3.9	-	-
Peche	2.3	1.6	3.8	-	-
Light Lager	1.3	-	-	1.3	-
Unhopped Pale Ale	2.0	-	-	-	-
Experimental DH	1.6	-	2.1	-	1.8
Citra DH	1.4	-	-	-	-
Mosaic DH	1.6	-	2.6	1.3	-
Centennial DH	1.5	-	2.7	-	-
Chinook DH	1.5	1.5	3.0	-	-

*Levels below calculated LOQ
** Levels below calculated LOD

Table 3.6 Mean sensory descriptive scores for all attributes averaged over all panelists and all replications.

Treatment	Mean Score				
	Overall Intensity	Stone Fruit / Peach	Coconut / Oily	Red Berry	Melon
Base	4.5^{C}	1.9^{C}	1.5^{B}	0.8^{C}	1.4^{A}
Lactones	5.9^{B}	2.8^{C}	3.2^{A}	1.1^{C}	1.9^{A}
Lactones + Esters	6.5^{B}	4.2^{B}	3.1^{A}	1.8^{C}	1.5^{A}
Lactones + Oxy Terpenes	8.4^{A}	$5.1^{A,B}$	3.1^{A}	3.0^{B}	2.1^{A}
All	9.1^{A}	6.1^{A}	3.0^{A}	4.8^{A}	2.3^{A}

[A] Treatments with different superscripts are significantly different within each attribute using Tukey's Honestly Significant Difference (HSD) at $\alpha < 0.05$.

CHAPTER 4. FUTURE WORK

This work provides information about the levels of four aliphatic lactones that have not been individually extensively studied in beers, namely γ-$C_{10\ \&\ 12}$, and δ-$C_{10\ \&\ 12}$. Lipid-derived flavors in beer is a wide area of research and lactones are only very small portion of that area. As sensory and flavor analysis methodology advances, it will become easier to accurately explore unsaturated/saturated lactones with techniques and tools such as SAFE and SBSE coupled with TOF-MS or GC-MS-MS. It has already been shown that beers possess unsaturated and hydroxyl lactones (3-hydroxy-γ-decalactone) (143) so further exploration into their magnitude of influence on final beer products through sensitive analytical and sensory methods is an open avenue. For a complete aroma profile evaluation of trace compounds, it is best to have multiple sample extraction and concentration techniques such as Stir Bar Sorptive Extraction. The optimization of SPME methodology and fiber coating for beers would greatly improve analysis of aroma impact compounds.

Lactones are in low ppb levels in beers, partially due to the already low concentration of lipids present, and may have minimal aroma impact when compared to esters, terpenes, terpene alcohols and thiols. The use of more sensitive techniques would aid in the exploration of oxygen containing compounds such as terpenoids and esters, which are considered to be the major drivers of fruit and floral aromas in beer. It would also be of interest to explore the beer consumers' acceptance and preference for fruity/floral beers and even explore pairings with various styles of beers and common foods.

Sulfur containing aroma compounds are of great interest to brewing scientists and many possess potent tropical fruit and citrus aromas. These sulfur containing compounds are also at low levels but have shown to be impactful (48, 62, 128) thus any optimization of flavor analysis methodology for thiols would greatly enhance the knowledge of beer aroma.

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APPENDICES

Appendix A. HS-SPME and GC-MS analysis methodology

This assay was developed to examine aliphatic lactones in beer via sampling the headspace volatiles with solid-phase microextraction. The SPME takes approximately 60 min including samples preparation, followed by a 42.7 min GC-MS program.

Reagents and Materials

(a) Analytical standards of >98% purity of γ-$C_{9, 10, 11 \& 12}$, δ-$C_{10 \& 12}$, 4- Octanol.
(b) Analytical grade NaCl.
(c) Analytical grade Internal Standard, such as γ-C_{11} or 4-octanol.
(d) 20 mL glass vials with silicone septa.
(e) SPME fiber assembly. Tri-phase Stable Flex SPME fiber 23 gauge 30/50 µm DVB/CAR/PDMS.
(f) GC-MS with polar Carbowax column 30m x 0.25mm x 0.5 µm film thickness. Any column that can be used for oxygenated compounds will suffice.
(g) Gerstel MPS 2 for automation of SPME extraction and injection into GC.

Sample Preparation

Degas packaged beer via beaker transfer and filter samples from fermentation or conditioning vessels. Weigh 3.0g NaCl into 20 mL vial and add 9 mL of sample volumetrically. Add desired concentration of internal standard that is lower than the largest aroma compound and higher than the smallest aroma compound.

Autosampler Method

Program Gerstel MPS 2 to incubate sample vial for 5 min at 60 °C with agitation at 250rmp. After incubation, set appropriate depths for vial penetration and extract at 60 °C for 50 min. Immediately after extraction, desorb the fiber in the GC inlet at 250 °C for 10 min.

Operating GC-MS Instrumental Conditions

Inject in splitless mode for 2 min with temperature held constant at 250°C and desorp from SPME fiber for 10 minutes in the injector. Carrier gas is UHP Helium at a flow rate of 1 mL/min with column head pressure of 7.2 psi. Oven temperature starts at 40°C and holds for 5 minutes, followed by 15°C/min ramp to 170°C, hold for 0.5 minutes then 3°C/min ramp to 240°C and hold for 10 minutes. SPME fiber is automatically baked out after each sample desorption for 10 minutes at 250°C. GC-MS transfer line is 240C. MS operaes in electron impact mode at 70 eV and collects data at a rate of 3.2 scans/s over a mass range of m/z 50-350. Ion source is at 160 °C, detector voltage set to 2165V and detector temp is held at 250 °C.

Quantification Protocol

For quantification, create calibration curves that span the expected concentrations in beer. Lactones are fairly low in concentration so a 5 point curve was built ranging from 1.56 to 12.5 µg/L. Calculation of analyte concentration is done by finding the relative peak area to that of the known concentration and area of internal standard. Limit of Detection (LOD) and Limit of Quantification (LOQ) are calculated as three and ten times the ratio of background noise to signal height, respectively, as a function of concentration (Table 5.1). Repeatability and reproducibility tests are recommended to validate method (Table 5.1).

Table 5.1. Limit of Detection, Limit of Quantification, repeatability and reproducibility percent relative standard deviation for five aliphatic lactones in 2% ABV American light lager beer.

Compound	Concentration added (µg/L)	LOD (µg /L)	LOQ (µg /L)	Repeatability % RSD	Reproducibility % RSD*
γ-C_9	12.5	0.9	3.0	7.9	40.4
γ-C_{10}	12.5	0.6	1.9	5.8	41.6
δ-C_{10}	12.5	4.3	14.2	8.6	46.8
γ-C_{12}	12.5	0.4	1.2	4.9	37.1
δ-C_{12}	12.5	2.0	6.8	5.7	32.7

*****The high reproducibility % RSD is due to different qualities of fibers that occurred over the course of 5 days.

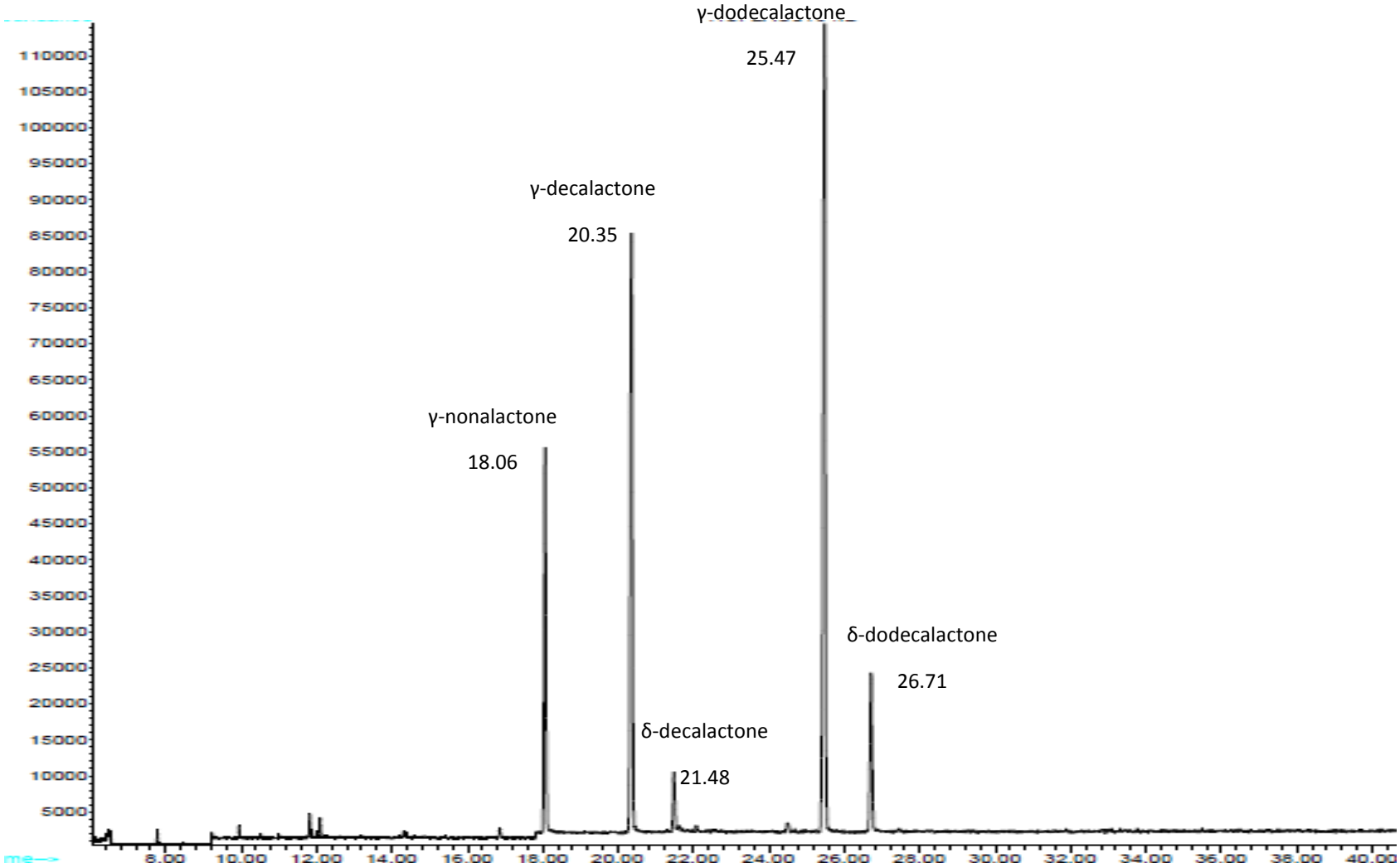

Figure 5.1. Representative GC-MS chromatogram of six aliphatic lactones spiked into 2% ABV American light lager. Retention times are approximate.

1

Appendix B. Olfaction Threshold Dilution Calculations

Initially, stock solutions of 10, 100, 1,000 and 10,000 mg/L were made for each of the five aliphatic lactones based on density of each compound and the specific gravity of 95% food grade Ethanol (Table 5.2). The density served to convert mass to volume and vice versa to get a final volume of compound into 50 grams of unhopped base beer.

Table 5.2. Specific gravities of five aliphatic lactones used in the creation of stock samples for olfactory threshold tests.

Compound	Density (g/cm^3) at 25°C
γ-C_9	0.976*
γ-C_{10}	0.952*
γ-C_{12}	0.936*
δ-C_{10}	0.954*
δ-C_{12}	0.942*
95% EtOH	0.985
Unhopped Pale Ale	1.0152-1.0104

*All values acquired from Sigma-Aldrich MSDS sheet accompanying pure analytical standards.

Specific volumes of stock solutions were added to 50 grams of base beer solution to create the spiked sample in each threshold test. Appropriate conversions between weight and volume were utilized. Below is a calculation example of the generic first dilution calculation and an example that produced a final concentration of 0.15 mg/L γ-nonalactone in 50 g of base beer with SG ov 1.0105 g/ml from 100 mg/L γ-nonalactone stock solution.

$$First\ dilution = \left(\frac{\left(first\ dil.(\frac{mg}{L}) \times \left(\frac{50g\ base}{SG\ of\ base\ (\frac{g}{mL})} \right) \right)}{compound\ stock\ solution \frac{mg}{L}} \right) \times 1000uL = x\ \frac{ul\ compound}{50g\ base\ beer}$$

$$\gamma - nonalactone\ first\ dilution = \left(\frac{\left(\frac{0.15mg}{l} \times \left(\frac{50g}{\frac{1.0105g}{ml}} \right) \right)}{100 \frac{mg}{L}} \right) \times 1000uL = 74.2\ \frac{ul\ compound}{50g\ base\ beer}$$

In order to decrease glassware usage, volume of compound was added to weight of base beer and the different concentrations of stock solutions were used so that the same pipette was utilized throughout the entire process.

Appendix C. Threshold Testing Ballot

Triangle Test - Hop Compound A

5/6/2014

Name_____

You will be given multiple sets of three samples. Smell the samples in the order listed below. Two of the samples are the same, one has an added substance. Circle the number that corresponds to the sample that is different. You may revisit samples but only left to right. Only smell the samples. DO NOT CONSUME SAMPLES.

set 1	**361**	647	215
set 2	730	413	**526**
set 3	946	**870**	104
set 4	574	631	**218**
set 5	**145**	471	739
set 6	999	486	**742**
set 7	116	**564**	932

Figure 5.2. An example of detection threshold ballots used in this study.

Appendix D. Explanation of panelists removed

A few assessors' data were removed from the BET calculations due to suspicious or unavailability for retesting on compounds or that they identified correctly in the first set. No assessors were removed from the γ-nonalactone data set. Three assessors were removed from the γ-decalactone data, one for suspicious data (incorrectly identified spiked sample at the highest level) and two that correctly identified all spiked samples in every set, but were unavailable to be retested at lower concentrations. One assessor was removed for suspicious data (incorrectly identified the final three level spikes, but correctly identified lower concentration spikes) in the γ-dodecalactone set. One assessor was removed from the δ-decalactone data set due to unavailability for retesting at lower concentrations and one assessor was also removed from the δ-dodecalactone set for suspicious data (correctly identified lower concentrations, but missed second to last spike sample).

Appendix E. Best Estimate Threshold values for five aliphatic lactones all panelists

Panelists	γ-nonalactone in unhopped pale ale										Best-Estimate Threshold (BET)	
	Concentration (ug/l)											
	18.7	37.5	75	150	300	600	1200	2400	4800	9600	Value	log of Value
1				0	+	+	+	+	+	+	212	2.33
2				0	+	+	0	0	+	+	3394	3.53
3				0	+	+	+	+	+	+	212	2.33
4				0	0	0	0	+	0	+	6788	3.83
5				0	0	0	+	0	+	+	3394	3.53
6				0	+	0	+	+	+	+	849	2.93
7				0	0	+	+	+	+	+	424	2.63
8				0	+	+	+	+	+	+	212	2.33
9	0	0	0	+	+	+	+	+	+	+	106	2.03
10				0	0	0	+	+	+	+	849	2.93
11				0	+	+	+	+	+	+	212	2.33
12				0	0	0	0	+	+	+	1697	3.23
13				0	+	+	+	+	+	+	212	2.33
14				+	0	0	+	+	+	+	849	2.93
15				+	0	+	+	+	+	+	424	2.63
16				0	+	0	0	0	+	+	3394	3.53
17				+	+	0	+	+	+	+	849	2.93
18				0	+	+	+	+	+	+	212	2.33
19	0	+	+	+	+	+	+	+	+	+	26	1.42
20	0	+	0	+	+	+	+	+	+	+	106	2.03
21				0	+	+	+	+	+	+	212	2.33
22				+	0	0	+	+	+	+	849	2.93
23	0	+	0	+	+	+	+	+	+	+	106	2.03
24				0	+	+	+	+	+	+	212	2.33
25	0	+	+	+	+	+	+	+	+	+	26	1.42
26	0	0	0	+	+	+	+	+	+	+	106	2.03
27	0	+	0	+	+	+	+	+	+	+	106	2.03
28				0	+	+	+	+	+	+	212	2.33
29				0	0	0	0	+	+	+	1697	3.23
							Group BET Geometric Mean				$10^{2.58}$ →	**376**
							Standard deviation					0.613

Figure 5.3. γ-nonalactone odor threshold panelist data. No assessors were removed from data set.

Panelist	γ-decalactone in unhopped pale ale										Best-Estimate Threshold (BET)	
	Concentration (ug/l)											
	25	50	100	200	400	800	1600	3200	6400	12800	Value	log of Value
1				+	0	+	+	+	+	+	566	2.75
2				+	0	+	+	+	+	+	566	2.75
3				0	0	+	+	+	+	+	566	2.75
4				0	+	+	+	+	+	+	283	2.45
5	+	0	+	0	+	+	+	+	+	+	141	2.15
6	0	0	+	+	+	+	+	+	+	+	71	1.85
7	+	0	+	+	+	+	+	+	+	+	71	1.85
8				0	+	+	+	+	+	+	283	2.45
9	+	+	0	+	+	+	+	+	+	+	141	2.15
10	0	0	0	+	+	+	+	+	+	+	141	2.15
11				0	0	+	+	+	+	+	566	2.75
12				0	+	+	+	+	+	+	283	2.45
13				0	0	+	+	+	+	+	566	2.75
14				0	+	+	+	+	+	+	283	2.45
15				+	0	+	+	+	+	+	566	2.75
16				0	+	+	+	+	+	+	283	2.45
17	0	0	+	+	+	+	+	+	+	+	71	1.85
18				0	+	+	+	+	+	+	283	2.45
19				0	0	+	+	+	+	+	566	2.75
20				0	+	+	+	+	+	+	283	2.45
21	0	0	+	+	+	+	+	+	+	+	71	1.85
22				0	0	+	+	+	+	+	566	2.75
23	0	0	0	+	+	+	+	+	+	+	141	2.15
24	+	0	+	+	+	+	+	+	+	+	71	1.85
25				0	+	+	+	+	+	+	283	2.45
							Group BET geometic mean				$10^{2.38}$ →	**239**
							Standard deviation					0.340

Figure 5.4. γ-decalactone individual olfaction threshold. Three assessors were removed from data set.

Panelist	γ-dodecalactone in unhopped pale ale										Best-Estimate Threshold (BET)	
	Concentration (ug/l)											
	40	80	160	320	640	1280	2560	5120	10240	20480	Value	log of Value
1	+	+	+	+	+	+	+	+	+	+	28	1.45
2				+	+	0	+	+	+	+	1810	3.26
3				0	0	+	+	+	+	+	905	2.96
4				+	+	+	+	+	+	+	226	2.35
5	0	0	+	+	+	+	+	+	+	+	113	2.05
6				0	+	+	+	+	+	+	453	2.66
7				0	0	+	+	+	+	+	905	2.96
8				0	+	+	0	+	+	+	3620	3.56
9	0	+	+	+	+	+	+	+	+	+	57	1.75
10				0	+	+	+	+	+	+	453	2.66
11	0	+	+	+	+	+	+	+	+	+	57	1.75
12				0	0	+	+	+	+	+	905	2.96
13	+	+	+	+	+	+	+	+	+	+	28	1.45
14				0	+	+	+	+	+	+	453	2.66
15				0	+	+	+	+	+	+	453	2.66
16	0	0	+	+	+	+	+	+	+	+	113	2.05
17	0	+	+	+	+	+	+	+	+	+	57	1.75
18				0	+	+	+	+	+	+	453	2.66
19	+	+	+	+	+	+	+	+	+	+	28	1.45
20				0	+	0	+	+	+	+	1810	3.26
21				0	+	+	+	+	+	+	453	2.66
22				0	+	0	+	+	+	+	1810	3.26
23				0	+	+	+	+	+	+	453	2.66
24				+	0	0	+	+	+	+	1810	3.26
25	0	+	+	+	+	+	+	+	+	+	57	1.75
26	0	+	+	+	+	+	+	+	+	+	57	1.75
27	+	+	+	+	+	+	+	+	+	+	28	1.45
28	+	+	+	+	+	+	+	+	+	+	28	1.45
							Group BET geometic mean				$10^{2.38}$ →	**238**
							Standard deviation					0.680

Figure 5.5. γ-dodecalactone individual olfaction threshold. One assessor was removed from data set.

Panelist	δ-decalactone in unhopped pale ale										Best-Estimate Threshold (BET)	
	Concentration (ug/l)											
	50	100	200	400	800	1600	3200	6400	12800	25600	Value	log of Value
1	0	0	0	+	+	+	+	+	+	+	566	2.75
2				+	0	+	+	+	+	+	1131	3.05
3	0	0	0	+	+	+	+	+	+	+	283	2.45
4				+	0	0	0	0	+	+	9051	3.96
5	0	0	0	+	+	+	+	+	+	+	283	2.45
6				0	0	0	+	+	+	+	2263	3.35
7				0	0	+	+	+	+	+	1131	3.05
8	0	0	0	+	+	+	+	+	+	+	283	2.45
9	+	+	0	+	+	+	+	+	+	+	283	2.45
10	0	0	0	+	+	+	+	+	+	+	283	2.45
11				+	0	+	+	+	+	+	1131	3.05
12				0	0	+	+	+	+	+	1131	3.05
13				0	0	+	+	+	+	+	1131	3.05
14				0	+	+	+	+	+	+	566	2.75
15				+	0	0	+	+	+	+	2263	3.35
16				0	0	+	+	+	+	+	1131	3.05
17				0	+	+	+	+	+	+	566	2.75
18				0	+	+	+	+	+	+	566	2.75
19				0	0	+	+	+	+	+	1131	3.05
20				0	+	+	+	+	+	+	566	2.75
21	+	0	+	+	+	+	+	+	+	+	141	2.15
22				+	+	0	+	+	+	+	2263	3.35
23				0	+	+	+	+	+	+	566	2.75
24				0	+	+	+	+	+	+	566	2.75
25	+	0	+	+	+	+	+	+	+	+	141	2.15
26				0	+	+	+	+	+	+	566	2.75
27				0	0	0	0	+	+	+	4525	3.66
											Group BET geometic mean	**750**
											Standard deviation	0.428

Figure 5.6. δ-decalactone individual olfaction thresholds. One assessor was removed from data set.

Panelists	δ-dodecalactone in unhopped pale ale										Best-Estimate Threshold (BET)	
	Concentration (ug/l)										Value	Log of Value
	12.5	25	50	100	200	400	800	1600	3200	6400		
1				0	0	0	+	+	+	+	566	2.75
2				0	+	+	+	+	+	+	141	2.15
3				0	0	0	0	+	+	+	1131	3.05
4				0	+	+	0	+	+	+	1131	3.05
5				0	0	+	+	+	+	+	283	2.45
6				0	+	+	+	+	+	+	141	2.15
7				+	+	0	+	+	+	+	566	2.75
8	0	0	0	+	+	+	+	+	+	+	71	1.85
9				0	0	+	+	+	+	+	283	2.45
10				0	+	0	+	+	+	+	566	2.75
11	0	0	+	+	+	+	+	+	+	+	35	1.55
12				0	0	0	0	+	+	+	1131	3.05
13				0	+	+	+	+	+	+	141	2.15
14	+	+	0	+	+	+	+	+	+	+	71	1.85
15				0	0	0	+	+	+	+	566	2.75
16	+	0	+	+	+	+	+	+	+	+	35	1.55
17				+	0	+	+	+	+	+	283	2.45
18				+	0	+	+	+	+	+	283	2.45
19				0	+	+	+	+	+	+	141	2.15
20				+	+	0	+	+	+	+	566	2.75
21	0	0	0	+	+	+	+	+	+	+	71	1.85
22				0	0	0	0	+	+	+	1131	3.05
23				+	0	+	+	+	+	+	283	2.45
24				0	+	+	+	+	+	+	141	2.15
25				0	0	+	0	0	+	+	2263	3.35
26				0	+	+	+	+	+	+	141	2.15
27				0	+	+	+	+	+	+	141	2.15
28				0	+	+	+	+	+	+	141	2.15
											$10^{2.41}$ →	**256**
											Group BET geometric mean	
											Standard Deviation	0.476

Figure 5.7. δ-dodecalactone individual olfaction threshold. One assessor was removed from data set.

Appendix F. Olfaction Threshold Geometric Mean Calculations and Histograms

To calculate the individual thresholds, the geometric mean was calculated from the last incorrect response and the first consecutive correct response. The Best Estimate Threshold for the group was then calculated from the logarithmic mean of each individual's values. A sample calculation for γ-nonalactone with an assessor whose last incorrect concentration was 150 ug/L and first correct concentration was 300 ug/L is as follows.

$$Individual\ geometric\ mean\ = \sqrt{150\ \times 300} = 212\ ug/L$$

$$Individual\ logarithmic\ mean\ = \log_{10} 212 = \ 2.33$$

$$Group\ logarithmic\ mean = \ 2.58$$

$$Group\ BET\ geometric\ mean\ (ug/L) = \ 10^{2.58} = 376\ mg\ /L$$

Olfaction threshold histograms

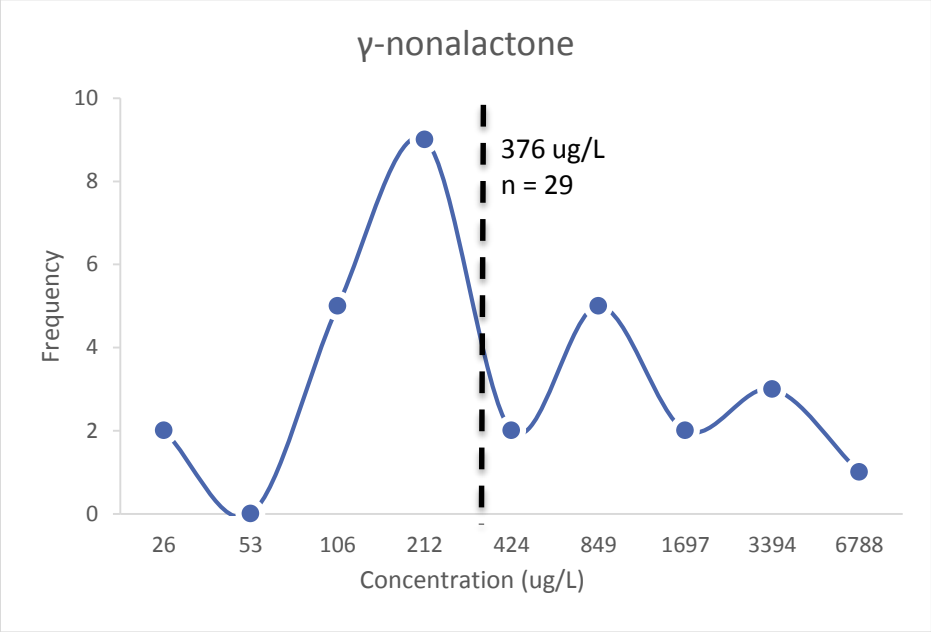

Figure 5.8. γ-nonalactone geometric mean threshold concentration frequencies

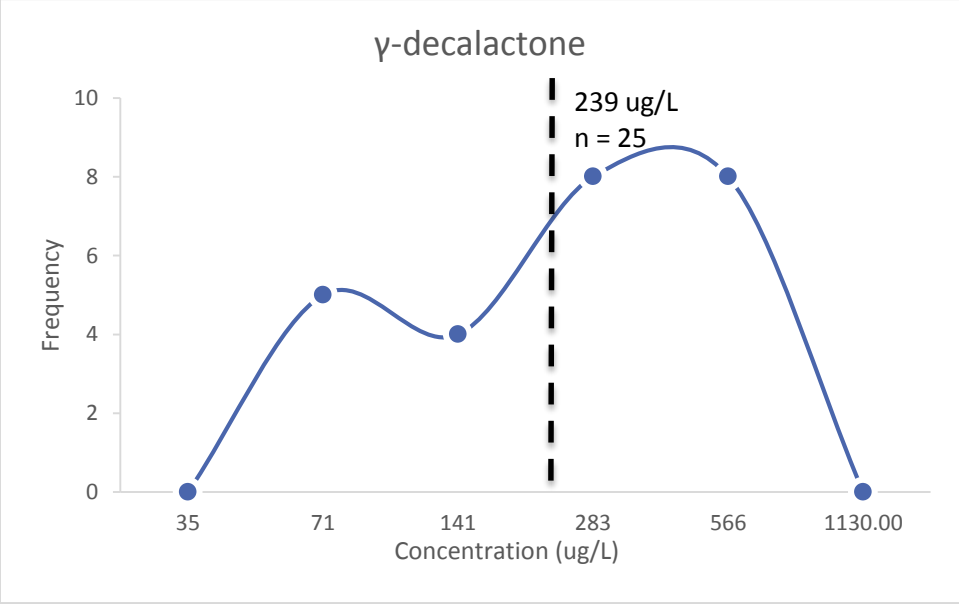

Figure 5.9. γ-decalactone geometric mean threshold concentration frequencies

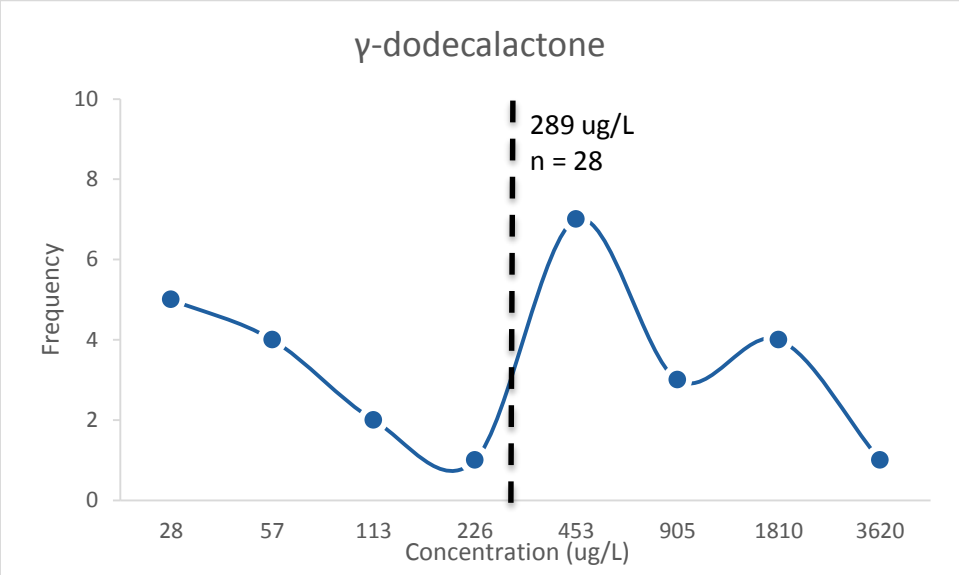

Figure 5.10. γ-dodecalactone geometric mean threshold concentration frequencies

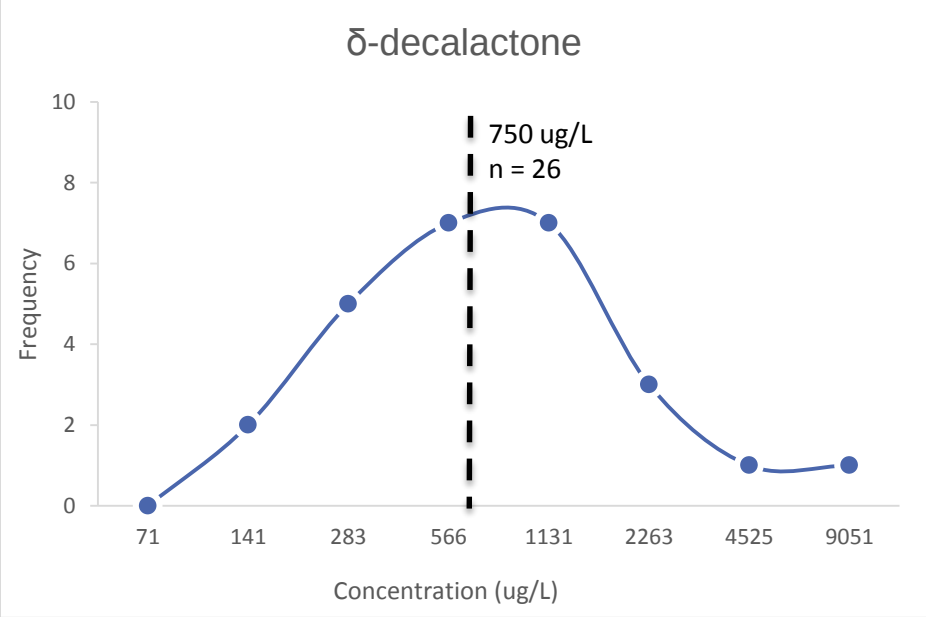

Figure 5.11. δ-decalactone geometric mean threshold concentration frequencies

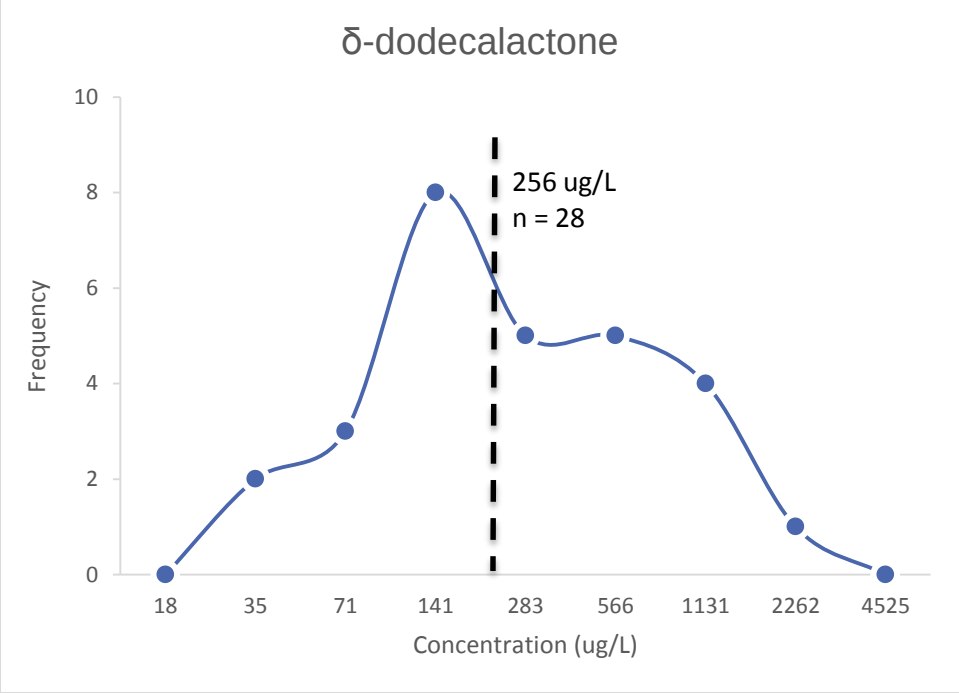

Figure 5.12. δ-dodecalactone geometric mean threshold concentration frequencies

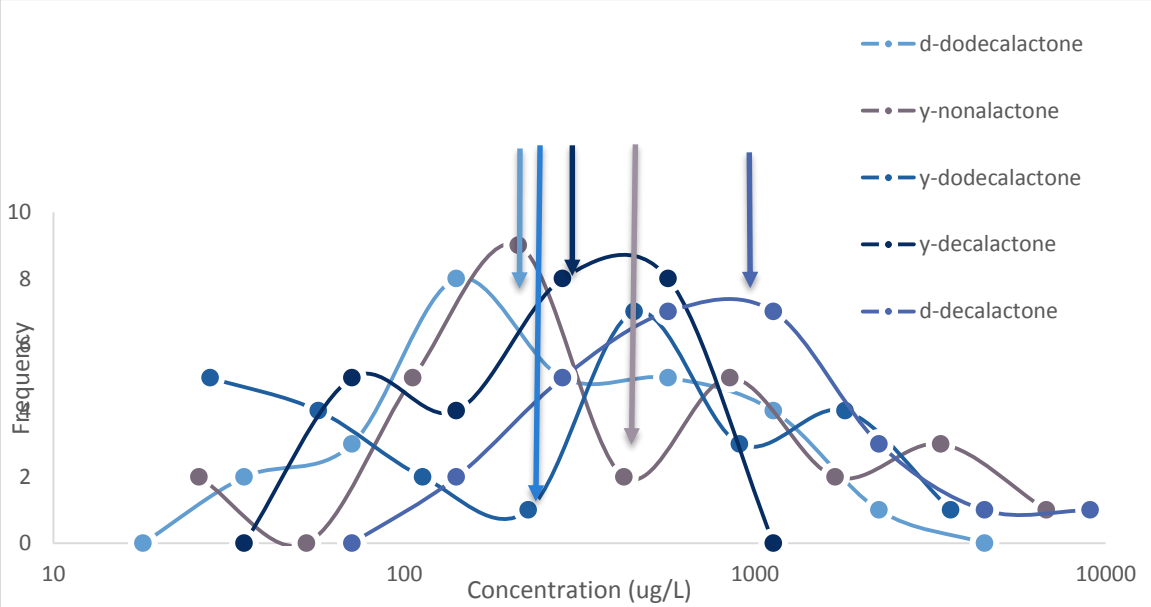

Figure 5.13. Group detection BET for five aliphatic lactones assessed in this study.

Appendix G. Panelist Replication for Descriptive Analysis

Panelist performance was evaluated by performing one-way Analyses of Variances (ANOVA) on each panelist and on every attribute. The purpose of evaluating panelist is to determine if each assessor can discriminate on any of the attributes given and if they are able to replicate their data. This ensures that the data was appropriately collected and can be confidently analyzed. If the p-value is significant (<0.05) for the attributes, then the panelist is able to discriminate among samples on that attribute. This is highly desirable. On the other hand, when testing for panelist replication, it is desirable that the p-value is not significant (>0.05). If the p-value is significant for replication, then the panelist is inconsistently using the scale to describe the same samples and cannot replicate their own data. From the table below, seven out of eight panelists are able to discriminate on one or more attribute and one panelist was not able to replicate data (p-value = 0.49) on one attribute albeit only very minimally.

Table 5.3. Panelist replication and discrimination one-way ANOVA p-value results for descriptive analysis.

Blue highlighted cells indicate significant p-value for treatments while pink highlighted cells indicate not significant p-value for replicate.

Panelist	ANOVA Factor	Overall Intensity	Stone Fruit/Peach	Coconut/Oily	Red Berry	Melon	*Count*
1	Rep	0.467	0.916	0.765	0.308	0.927	**0**
	Treatment	< 0.0001	0.000	0.445	< 0.0001	0.000	**4**
2	Rep	0.987	0.997	0.965	0.998	0.993	**0**
	Treatment	0.000	< 0.0001	0.335	< 0.0001	0.264	**3**
3	Rep	0.999	0.989	0.997	0.752	0.379	**0**
	Treatment	0.587	0.795	0.976	0.086	0.754	**0**
4	Rep	0.537	0.079	0.449	0.987	0.419	**0**
	Treatment	0.124	0.009	0.132	0.013	0.195	**2**
5	Rep	0.158	0.049	0.097	0.631	0.679	**1**
	Treatment	< 0.0001	< 0.0001	0.039	0.961	0.746	**3**
6	Rep	0.287	0.129	0.827	0.193	0.776	**0**
	Treatment	0.164	0.140	0.904	0.576	0.050	**1**
7	Rep	0.999	0.995	0.879	0.989	0.872	**0**
	Treatment	0.022	0.009	0.005	0.031	0.431	**4**
8	Rep	0.979	0.863	0.409	0.626	0.978	**0**
	Treatment	0.019	0.000	0.020	0.001	0.260	**4**
		5	**6**	**3**	**5**	**2**	
		0	**1**	**0**	**0**	**0**	

Appendix H. Category Scale Sensory Evaluation

One approach explored for determining the influence of lactones on beer fruit aroma was through category scaling sensory evaluations. Levels of two lactones (γ-C_9 & C_{10}), two ethyl esters (E2MB and E3MB), and two oxygenated terpenes (linalool and β-damascenone) were chosen to be near detection threshold concentrations that were determined in two previous sensory studies. Lactone detection concentrations are described in Chapter 3. The elevated levels were chosen as such to ensure panelists could easily detect and rate aromas. However, these levels were not commercially relevant to beer levels thus this study removed from the manuscript. The results showed a similar trend to the descriptive analysis results described in chapter 3 – the addition of more compounds resulted in significantly increased fruity aroma.

Eight panelists, five panelists with previous sensory panel experience and training, (3 female and 5 Male) aging 26-50 with a median age of 30 were trained and tested for the category scaling of Overall Fruity Aroma Intensity only. The panelists were trained over two sessions to introduce the scale, and the aroma types and intensities expected during testing. This was achieved by using low, moderate and high concentrations of γ-C_{10}, ethyl 2-methylbutanoate (E2MB), and linalool dosed into the unhopped pale ale base to anchor the scale (Table 5.4). Eight samples containing the base beer and mixtures of esters, oxygenated terpenes and lactones near panelists' thresholds were used for Category Scale evaluation. Three testing sessions took place where every sample was presented twice to each panelists in a randomized order and they rated the overall fruity intensity using a sixteen-point scale (0-15) (Table 5.4).

Tukey's HSD ($\alpha<0.05$) results indicate that when all compounds are combined, the sample did not significantly differ from lactones plus terpenes or esters plus terpenes (Table 5.6). Linalool and β-damascenone were at very high concentrations (Table 5.4) and could have potentially been driving the intensity of the aroma, overpowering other compounds present. The unhopped pale ale base was also included in each set of samples

to determine if the panelists could distinguish the base from dosed samples and results demonstrate that it was significantly different than all dosed samples (Table 5.6). An ANOVA was performed on all panelists' averaged Overall Intensity scores, over all replications, and for all treatments and indicated differences could be detected among data was replicated (Table 5.5). However, when panelists performance was determined, the ANOVA p-value results revealed that all panelist could discriminate samples on Overall Intensity (p-value <0.05), but two out of the eight panelists had difficulty replicating their data or were using the scale very differently between repetitions (p-value >0.05) (Table 5.7). The observations from this study suggested that the addition of esters and terpenes increased the overall fruity intensity so we decided to test if the trend could be replicated with commercially relevant concentrations.

Table 5.4. Concentrations of lactones, terpenes and esters dosed into unhopped pale ale base for anchors for category scaling sensory evaluation.

Compound	Concentration (µg/L)
Low standard	γ-C_{10}: 100 E2MB: 10
Moderate standard	γ-C_{10}: 300 E2MB: 10
High standard	γ-C_{10}: 700 E2MB: 20 Linalool: 1000
γ-C_9	300
γ-C_{10}	200
Linalool	1000
β-damascenone	3000
Ethyl 2-methylbutanoate (E2MB)	10
Ethyl 3-methylbutanoate (E3MB)	10

Table 5.5. Category Scale ANOVA results for Overall Fruit Aroma for all panelists and attributes.

Source	DF	Sum of squares	Mean squares	F	Pr > F
Panelist	7	169.7	24.2	3.534	0.001
Treatment	7	1979.8	282.8	41.227	< 0.0001
Replicate	5	42.6	8.5	1.241	0.290
Panelist*Treatment	49	896.5	18.3	2.667	< 0.0001
Panelist*Replicate	35	173.0	4.9	0.721	0.878
Treatment*Replicate	35	284.9	8.1	1.187	0.228

Table 5.6. Sensory intensity score of all beer treatments averaged over all panelists

Category	Mean	Groups*				
All	10.771	A				
TE	9.708	A	B			
TL	9.688	A	B			
T	8.562		B	C		
LE	7.437			C	D	
L	6.312				D	E
E	5.542					E
Base	3.646					F

*Treatments with different letters are significantly different using Tukey's HSD at $\alpha < 0.05$, compared across all panelists and within each treatment. N = 8

All – all compounds combined
TE – Terpenes + Esters
TL – Terpenes + Lactones
T – Terpenes
LE – Lactones + Esters
L – Lactones
E – Esters
Base – Unhopped pale ale

Table 5.7. Panelist replication for Category Scale evaluations

Panelist	ANOVA Factor	Overall Intensity	Count
1	Rep	0.944	**0**
	Treatment	<0.0001	**1**
2	Rep	0.905	**0**
	Treatment	0.004	**1**
3	Rep	0.988	**0**
	Treatment	0.029	**1**
4	Rep	0.013	**1**
	Treatment	<0.0001	**1**
5	Rep	0.022	**1**
	Treatment	<0.0001	**1**
6	Rep	0.651	**0**
	Treatment	0.014	**1**
7	Rep	0.955	**0**
	Treatment	<0.0001	**1**
8	Rep	0.954	**0**
	Treatment	0.0003	**1**
		8	
		2	