AN ABSTRACT OF THE THESIS OF

Kathryn M. Kolpin for the degree of Master of Science in Food Science and Technology, presented on June 20, 2008.

Title: Human Bitterness Detection Thresholds of Hops Acids in Beer and Honey

Abstract approved:

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Thomas H. Shellhammer

Abstract

Alpha acids are found in the lupulin glands of hops cones. These acids have no bitterness of their own, but can be isomerized and subsequently hydrogenated to form bitter iso-α-acids and tetrahydroiso-α-acids respectively. Current literature values for the detection thresholds of iso-α-acid and tetrahydroiso-α-acid in lager beer have been reported using American Society of Testing and Materials (ASTM) method 679, a method commonly used to find detection thresholds of various compounds. ASTM method 679 is currently described as a "rapid method" and was a forerunner to ASTM method 1432, the current standard method for finding human detection thresholds. It has been argued that ASTM method 679 is statistically weaker than ASTM method 1432, as a group threshold found using ASTM method 679 is based on an individual "best estimate threshold" found using only one ascending series of 3-altrnative forced choice (3-AFC) presentations per panelist. The group threshold found using ASTM method 1432 is based on individual thresholds that are determined using data from 6 ascending series of 3-AFC presentations per panelist, the principle being that test repetition will

help nullify moment-to-moment panelist variation. However, test repetition takes time, and the lack of repetition required for ASTM method 679 makes the method quicker and simpler to implement than ASTM method 1432. Current literature detection threshold values for both iso-α-acids and tetrahydroiso-α-acids in lager beer have not been found using ASTM method 1432. The primary objective of the research presented in this paper was to use the current ASTM standard method to determine updated threshold values for both iso-α-acids and tetrahydroiso-α-acids in lager beer. The secondary objective of this research was to compare threshold results from the same data set using ASTM method 679 and ASTM method 1432 to evaluate whether these methods provide statistically similar results. Findings showed that implementation of ASTM method 679 to find individual thresholds did produce results that were not statistically different from individual results found using ASTM method 1432. All group thresholds except the Day 1 tetrahydroiso-α-acid threshold found using ASTM 679 were also statistically in agreement with group thresholds obtained using ASTM method 1432.

Beta acids, like alpha acids, are found in the lupulin glands of hops cones. Beta acids oxidize to form bitter compounds. Beta acids and tetrahydroiso-α-acids have been found to be miticidal against varroa mites, parasitic mites that feed off of and damage honeybees and bee larva. Though beta acids and tetrahydroiso-α-acids are FDA-declared GRAS substances, and will have no ill-effects on honey consumers, bees may transfer the bitter acids to their honey. Detectable levels of these bitter substances could lead to consumer rejection of honey from hives treated with beta acids or tetrahydroiso-α-acids.

In a second experiment discussed in this thesis, the detection thresholds of beta acids and tetrahydroiso-α-acids were found in light clover honey. A method for the extraction and quantification of beta acids and tetrahydroiso-α-acids in beer and wort was modified and verified for the extraction and quantification of hops acids in honey. This method combined with group threshold data obtained in this experiment can be used in an industrial setting to determine whether the concentrations of beta acids and/or tetrahydro-iso-α-acids that may have been transferred to honey are above or below the human detection threshold.

Human Bitterness Detection Thresholds of Hop Acids in Beer and Honey

by
Kathryn M. Kolpin

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Kathryn M. Kolpin, Author

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The Human Bitterness Detection Threshold of Hop Acids in Beer and Honey

Thesis Introduction

1.1 Hops

Hops, scientifically known as *humulus lupulus,* belong to the family Cannabaceae, and are native to temperate climates in Asia, Europe and North America (*1, 2*). This tall vine is a dioecious species (a species with separate male and female plants) that commonly grows to heights of 16 to 26 feet (*3*). Brewers utilize the fruiting body of the female hops plant, known as the cone. Male plants are not grown in hop yards because they do not produce cones, but they do fertilize female flowers, which leads to the development of seeds in the hops cones. These seeds impart undesirable flavors and astringency to beer. Hops cones consist of a series of petal-like bracteoles attached to an inner stem called a strig (figure 1.1). Small, yellow lupulin glands found at the base of these bracteoles contain acids, resins, and essential oils that provide bitterness and aroma for beer (figure 1.1) (*4, 5*). Alpha-acids are among the most important components present in the lupulin glands. The bitterness of beer comes primarily from the isomerization of these alpha-acids during wort boiling (*4*).

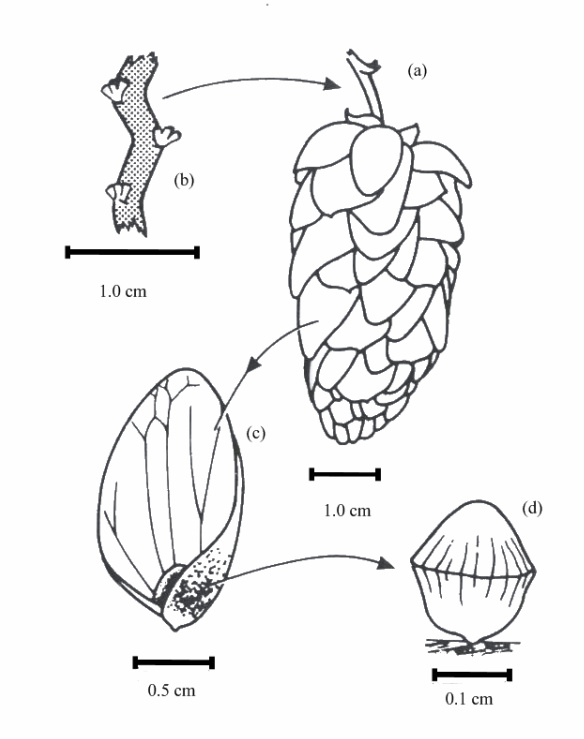


Figure 1.1: Diagram of the hop cone and its parts. The hops cone (a), consists of the central strig within the cone (b), bracteoles (c), and lupulin glands (d) (*6*)

1.2 Hops use in beer

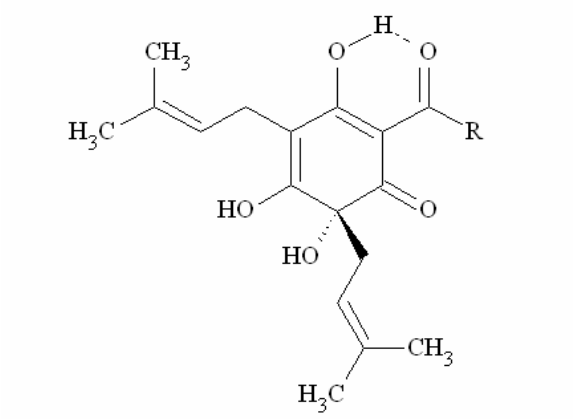
Hop use as a flavoring and preservative in beer has been heavily documented since about 1000 AD, and written references to hops appear as early as the 6th century BC (*7*). Hops provide bitterness to beer to balance the sweet and sour flavors of the malted grain. A point of equal importance; hops possess anti-microbial compounds that help protect the wort and beer against beer spoilage organisms like Gram-positive bacteria such as *pediococcus* and *lactobacillus* (*5, 8-10*). Beer is rarely brewed today without the use of hops or hop extracts, but hops were not always considered so necessary to

brewing. Hop cultivation began—most likely for medicinal purposes—in Eastern Europe around the 9th century AD (*1, 7*). The first documented practice of using hops in beer was by monks around the 11th century AD (*7*). Even though brewers in Germany became legally obligated to include hops in their beers through the adoption of the German purity law known as the Reinheitsgebot in 1516 (*11*), parts of England actually outlawed the use of hops—labeling them "a wicked and pernicious weed," as late as 1519 (*7*). Hops cultivation was eventually legalized in England by Parliament in 1554, and hops became a valuable crop in England (*7*). Brewing practices and hop cultivation techniques ultimately followed British, German, and Dutch emigrants to North America, Tasmania, South Africa, Japan and New Zealand by the mid 1800's, and the beverage we know today as "beer" made of hops, malted barley, water and yeast, was introduced to cultures and people around the world (*1, 7*). Currently, Germany, the United States and China are the world's top growers of hops, producing about 68% of the world's hops by weight (*1*).

1.3 Alpha acids

Humulones, also known as alpha-acids, are secondary metabolites found only in the soft resin fraction of the contents of the lupulin glands of hops cones (*12*) (figure 1.2). This makes alpha-acids chemotaxonomic, meaning if a plant contains these acids, it is classifiably a hops plant (*12*). The alpha acid portion of resin is comprised of about 15% adhumulone, 20-50% cohumulone, and 20-50% humulone with the amounts of cohumulone and adhumulone varying by hop variety (*12*). Alpha acids are arguably the

most important hop component for beer brewing. Though alpha acids are not bitter themselves, they are thermally isomerized during the wort-boiling phase of beer brewing to yield intensely bitter isomerized alpha acids that help balance the sweetness and sourness of malt. Alpha acids are also known to possess bacteriostatic qualities that are likely due to the prenyl side-chain interfering with cell plasma membrains of Gram-positive bacteria (*12*). Brewers utilize this quality to help protect wort and beer from spoilage organisms. Brewers add alpha-acids to wort by adding whole hops, ground hops, hops pellets, or resin extracts at the beginning of wort boil.



$R = CH_2CH(CH_3)_2$ = humulone

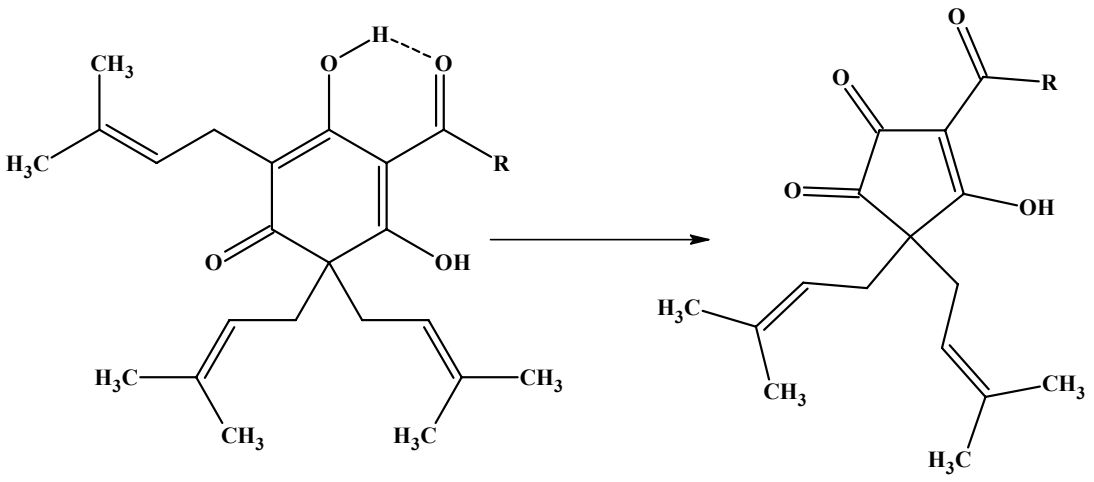
$R = CH(CH_3)_2$ = cohumulone

$R = CH(CH_3)CH_2CH_3$ = adhumulone

Figure 1.2: The chemical structure of alpha acid (*13*)

1.4 Beta acids

Beta acids, like alpha-acids are chemotaxonomic, secondary metabolites found in the soft resin fraction of the contents of the lupulin glands of hops cones (*12*) (figure 1.3). The presence of three prenyl side-chains make beta acids bacteriostatic as well (*12*). Beta acids are not bitter, and though they do not isomerize appreciably in during wort boil they are easily oxidized to hulupulones, which impart undesirable bitterness to beer (figure 1.3)(*3*). Though beta acids do not have much importance for brewing, these acids have been found to be miticidal to parasitic varroa mites that have been at least partially responsible for the demise of many United States bee hives (*14, 15*).



$R=CH_2CH(CH_3)_2$ = lupulone

$R=CH(CH_3)_2$ = colupulone

$R=CH(CH_3)CH_2CH_3$ = adlupulone

Figure 1.3: Beta-acid oxidation (*13*)

1.5 Alpha and beta acid extraction

Hop resins can be extracted using ethanol, hexane, liquid CO_2 or super-critical CO_2. As a highly non-polar solvent, hexane selectively extracts the non-polar soft resin fraction, leaving behind bitter hard resins and undesirable oxidation products that ethanol will dissolve (*16*). Both ethanol and hexane must be evaporated from the final extract by use of a vacuum evaporator. Vacuum evaporation also problematically facilitates a partial loss of desirable hop volatile compounds. Liquid and super-critical CO_2 extraction processes allow for significantly better retention of these aromatic compounds (*16*). Because temperatures can be carefully controlled in CO_2 extraction processes,

isomerization of alpha-acids and volatilization of aroma compounds is kept to a minimum. Liquid and super-critical CO_2 also dissolve the soft-resin portion of hops more selectively than hexane and ethanol without any risk of harmful solvent remaining in the final extract (*16*).

1.6 Isomerized-α-acids:

Desirable bitterness of traditionally hopped beer is due primarily to the presence of isomerized alpha acids (iso-α-acids) (*3, 10*). Due to the presence of both hydrophobic and hydrophilic molecular regions, iso-α-acids contribute to beer foam stability, and beer foam cling by acting essentially as emulsifiers between the gas and liquid matrices in beer foam (*10*). Iso-α-acids are produced when naturally present alpha acids undergo thermal isomerization when hops are boiled in the brewing process. This isomerization changes nearly tasteless humulone, cohumulone, and adhumulone (the alpha-acids) into the intensely bitter cis and trans isohumulone, isocohumulone, and isoadhumulone, the iso-α-acids (Figure 1.4) (*4, 10, 17*).

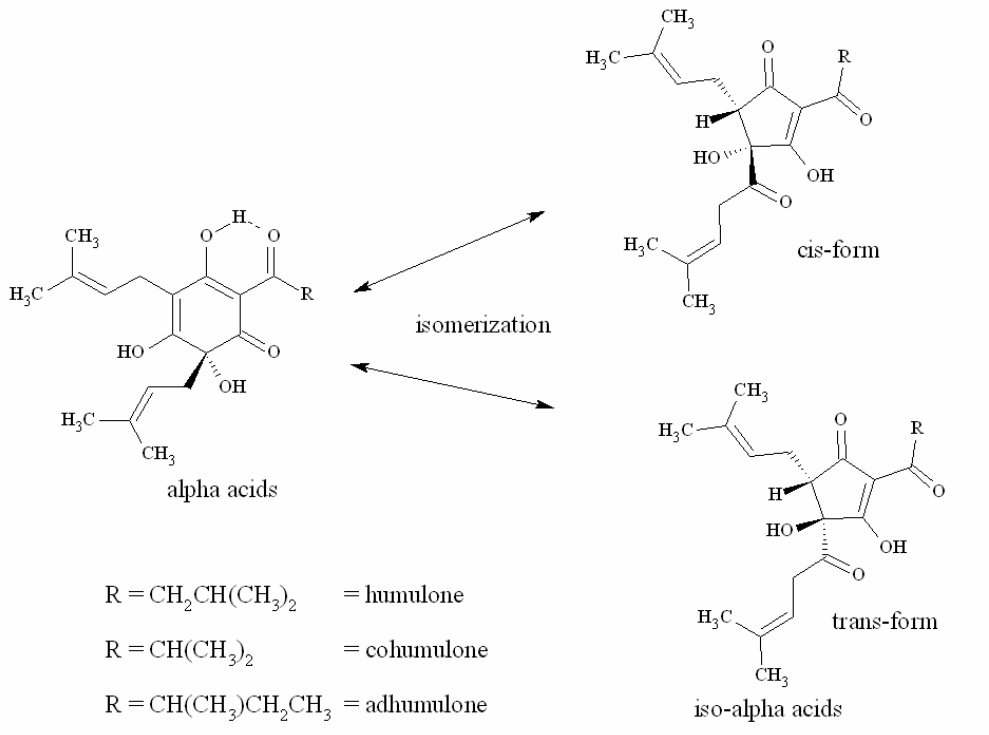


Figure 1.4: Isomerization of α-acids to iso-α-acids. (*13*)

Iso-α-acids are also available to brewers in the form of extracts. Iso-α-acid extracts are produced by heating α-acids in the presence of alkali metal salts like magnesium chloride (*16*). Brewers often use iso-α-acid extracts because they are easy to use and store, and they are standardized to allow for more predictable brewing results compared to conventional hops use (*16*).

1.7 Tetrahydro-iso-α-acids

Tetrahydro-iso-α acids (tetra acids) are a reduced form of iso-α-acids made by hydrogenating both carbon-carbon double bonds present in the prenyl side-chains of an

iso-α-acid molecule (figure 1.5) (*16, 18*). Tetra acids can also be made via hydration, oxidation and isomerization of beta acids, though this reaction is less efficient (figure 1.5) (*12*). Tetra acids impart bitterness to beer, while providing foam stability that nearly matches that of beer made with whole hops (*10, 18*). Like beta acids discussed earlier, tetra acids have been found to be miticidal to parasitic varroa mites (*14*).

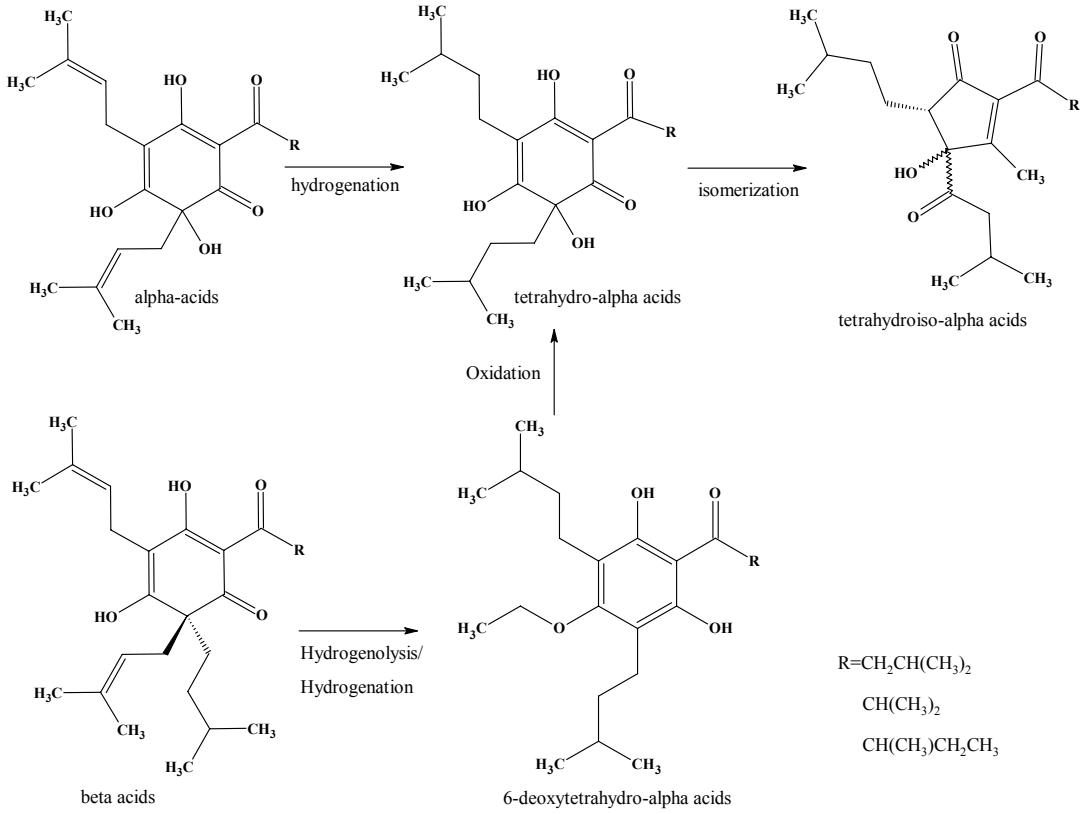


Figure 1.5: Formation of tetrahydroiso-α-acids from alpha acid and beta acid precursors (*12*)

Tetra acids have been deemed particularly valuable by brewers because they do not form mercaptans ("sun-struck" or "skunky" flavor) when exposed to UV light, unlike the non-reduced iso-α-acids (*18, 19*). Sun-struck aroma occurs when a 3-methyl-2-butenyl free radical, cleaved from an iso-α-acid through UV degradation reacts with a SH radical in the presence of riboflavin. These free radicals combine to form isopentenyl mercaptan (figure 1.6) (*3*). The saturated side-chains of tetra acid protect the hops acid from UV photo degradation, and thus, prevent the formation of the sun-struck aroma (*3, 18*).

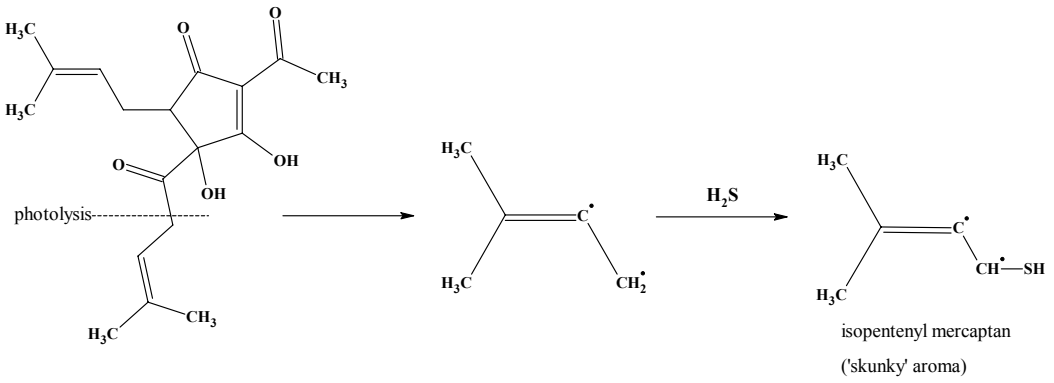


Figure 1.6: Photo-oxidation of hop acids forms the 3-methyl-2-butenyl free radical and the SH free radical, which combine to form isopentenyl mercaptan (*20*).

1.8 Dihydro- and Hexahydro-iso-alpha acid

Two other reduced isomerized alpha acids that are commonly used in brewing are known as hexahydro-iso-α-acids (hexa), and dihydro-iso-α-acids (rho). Rho is formed by reducing the carbonyl group in the side chain at carbon 4 with sodium borohydride (figure 1.7) (*12*). Hexa is formed via a simultaneous or successive reduction of the side-

chain carbonyl group and hydrogenation of the prenyl side-chain double bonds in the iso-α-acids (figure 1.7) (*12*). These bitter, reduced iso-α-acids are light-stable. Thus, like tetra, they are used in place of unreduced iso-α-acids to protect the beer from forming skunky isopentenyl mercaptan aromas. Neither of these acids was used for the research described in this thesis.

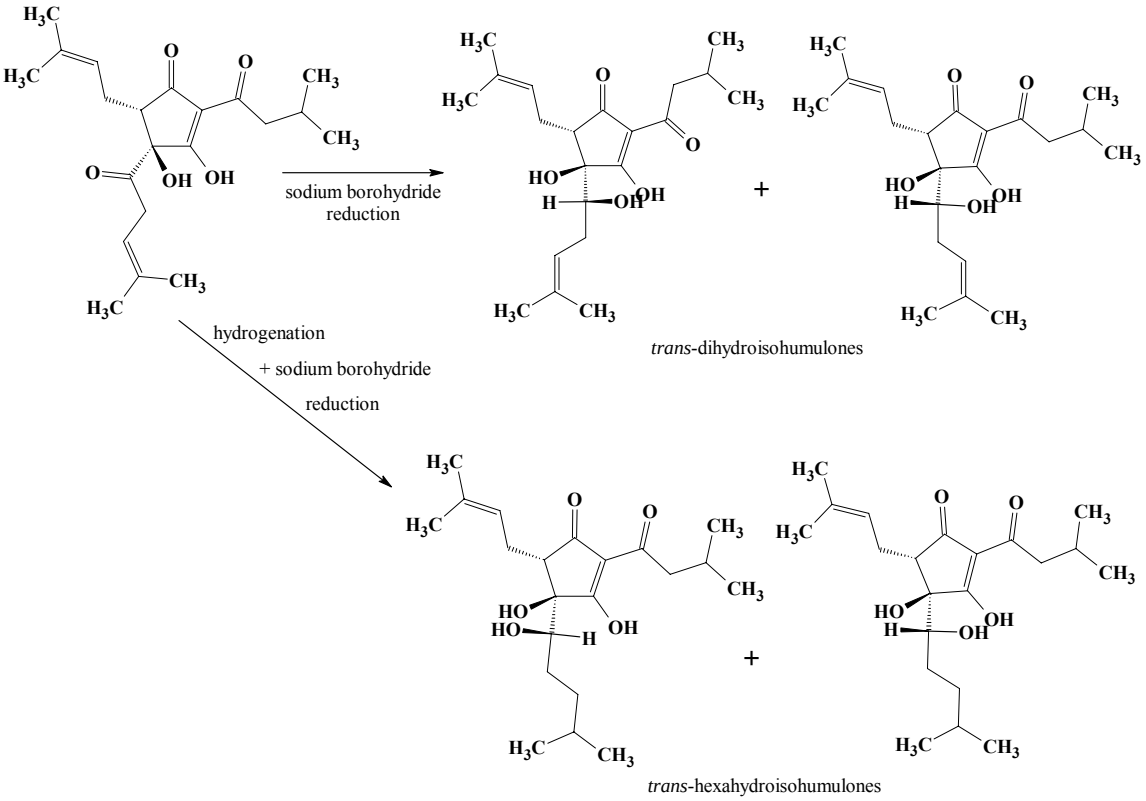


Figure 1.7: Formation of hexahydroisohumulone and dihydroisohumulone from an isohumulone precursor (*12*)

1.9 Sensory science:

Any question that concerns attributes that can be perceived by human senses relates to sensory science. Though these questions can seem straightforward, there is

often no answer that applies to all people. For instance, asking "what color is the sky to you?" will garner a reply, "blue," from the majority of the population, but a fully-colorblind individual would not answer "blue." He or she would instead answer "gray." Sensory science studies the reaction of the 5 senses to presented stimuli. In sensory science people can act as sensitive, analytical instruments or as general consumers in experiments that attempt to answer questions about human perception of the world around them.

Using humans as instruments can be troublesome. Individual responses vary constantly due to health state, level of distraction, and level of motivation (*4, 21*). This variability makes individual test results difficult to reproduce, and thus necessitates creation of statistically sound methods for testing subjects and the subsequent analysis of the data gained from those tests (*21*). There is also significant variation between panelists' sensitivities to different compounds (*4*). These differences are often attributable to genetics and age, and to a lesser extent, gender, and cultural background. The existence of these population-based differences requires that panelist groups be as representative as possible of the population at large in order to prevent bias.

Basic human psychology is also a factor that must be acknowledged by sensory scientists. Humans psychologically want to perform well at a task. This aspect of humanity can result in a person consciously or subconsciously giving a result they feel is expected of them, rather than the answer that best defines their perceptions in a poorly-designed sensory test. Samples must be coded and randomized to prevent this error of

expectation (*22*). Coding must be done carefully, because panelists tend to select familiar codes, for instance, a number associated with a local popular radio station. Panelists also tend to rank items subconsciously when they can. Using random, 3-digit codes to identify samples while avoiding numbers familiar to panelists prevents panelists from ranking, subconsciously selecting, or seeking to respond with an expected answer. Offering several repetitions of a test provides enough data for improved statistical power with regard to the validity of any individual results. Using large groups of panelists provides for a normal distribution of results.

Sensory scientists employ a myriad of techniques to evaluate sensorial aspects of substances based on qualitative factors like preferences, or quantitative factors like thresholds for example. As new sensory-related questions arise, new methods are developed to adequately answer those questions. Human flaws do not outweigh the importance of using human subjects in answering sensory questions. Analytical instruments can only tell us how much of a given substance is present. They cannot provide any information as to how that substance will be perceived and liked by the population at large (*23*). Human variability must simply be accounted for in the design of questions to be answered, and in the design of test methods to seek those answers. The experiment discussed in this thesis utilizes sensory science to find the human threshold of hops compounds in lager beer.

1.10 Matrix effect:

Perception of a substance varies greatly depending on the matrix in which the substance is placed. Lipids, proteins, and carbohydrates can interact with volatile compounds, causing them to be either less accessible or more accessible to sensory structures by masking or intensifying a compound's flavor respectively (*24, 25*). Compounds can also compete for the same physical receptors, causing one or both compounds to be perceived differently (*26*). Combinations of taste qualities, can also illicit a cognitive effect, meaning that people perceive combinations differently than lone taste qualities. This may be due to a neurological response for one compound inhibiting a or elevating neurological response to another compound (*26*).

There are 3 main types of matrix interactions that can occur in a food system: enhancement, synergy and suppression. Enhancement takes place when the presence of one compound increases the intensity of another (*25*). For example, the presence of ethanol appears to increase the intensity of bitter compounds, possibly by increasing their solubility and making them more available to taste buds (*27*). Synergy is said to occur when both compounds taste stronger in each other's presence (*25*). As an example, the sweetness of the low-calorie sweetener D-tagatose has been shown to perceptively increase in the presence of either aspartame or acesulfame K (*28*). Lastly, suppression is a decrease in the intensity of one compound due to the presence of another compound (*25*). Bitter taste is suppressed by sugar, by salt, and by increases in viscosity (*27, 29*). It is not entirely known how sugar and salt suppress bitterness, but current research points

to a peripheral (in-mouth) response instead of a neurological response (*30*). Higher matrix viscosity can prevent tastants from reaching sensory receptors, thus suppressing perception (*31*). Because these matrix effects can have a profound effect on the perception of flavor, a well-defined matrix is essential for discussing sensory perception of any particular aspect. An understanding of the matrix provides essential context of a taste threshold.

1.11 Bitterness

Rancid fats, secondary plant metabolites, and other potentially harmful compounds taste bitter to humans, so it is believed that bitter taste receptors evolved as a mechanism of warning (*32-34*). Though bitterness evolutionarily came to be equated with danger, humans still display a high degree of variability in their ability to taste bitterness (*33*). The evolutionary reasons for this high degree of person-to-person variability are not well understood, but current theories point to both the abundance of genes that encode for bitter receptors, and the presence of multiple pathways for sensing bitter taste (*33, 35*). A good illustration of genetic variation in the ability to taste bitter compounds can be found in humans' abilities to taste either 6-n-propylthiouracil (PROP) and phenylthiocarbamide (PTC). Both of these compounds taste intensely bitter to about 75% of people at low concentrations, but about 25% cannot taste them at all (*36*). A portion (55-85%) of the variation in ability to taste PROP and PTC is controlled by a difference in 3 amino acids on one gene called TSA2R38, with a proline-alanine-valine

substitution being associated with tasters, and alanine-valine-isoleucine substitution being associated with non-tasters (*37*). If a person possesses a double-recessive version of that gene, he or she often lacks the ability to taste either compound, though there have been some rare cases where a different gene appears to control the ability to taste these compounds (*32, 35*). Interestingly, the ability to taste the bitterness of PROP has not been conclusively shown to correlate with a person's ability to taste other bitter compounds, lending credence to the multiple pathways theory of bitterness perception (*27*). These multiple pathways could have evolved because bitter compounds do not all share a common chemical configuration, and thus will not chemically interact with taste structures in the same way. After all, the group of bitter-tasting chemicals includes many different types of compounds including hydrophobic compounds, amino acids, modified sugars, divalent cations, and sweetener analogs (*32*).

Age has also been cited as a factor in a person's ability to taste bitter compounds. With age a person's general ability to taste declines. This is especially the case with bitter compounds (*27*), (*38, 39*). In one study, elderly persons (65-83 years old) needed 74% more caffeine than young people in order to detect that substance in water (*40*). Quinine is also perceived as being less bitter by the elderly than by young people (*41*). Not all bitter compounds show this age-dependant decrease in sensitivity. Urea is perceived as having the same intensity regardless of age (*41*).

1.12 Thresholds

The term "threshold" has historically been arbitrarily used to represent many different aspects of a given food system in various experiments. The relevance of sensory thresholds as useful tools for determining the sensorial effect of substances in a matrix, however, is undeniable. Four main categories have evolved for the term "threshold." These include terminal thresholds, difference thresholds, recognition thresholds and detection thresholds. The term, "terminal threshold" describes a concentration of a substance that is high enough such that even if a higher concentration is presented, an individual will not be able to sense that difference (*21*). If a detectable concentration of a substance is already present in a given matrix, the term difference threshold describes how much more of a substance must be added before a change will be detected (*21*). This has been modeled using Fechner's law, which basically states that the response to a change in stimulus will be proportional to the relative increase in the stimulus (*21, 22*). Recognition thresholds describe how much of a substance must be added to a matrix before an individual will be able to correctly identify the substance (*21*). The threshold that describes the lowest detectable amount of a substance in a given matrix is called the detection threshold (*21*). The body of research presented in this thesis primarily includes this particular category of thresholds. Generally, thresholds are often the most direct way to learn something about the interaction and flavor influence of any particular compound present in any food system (*4*).

Numerous types of methods have been used to determine human thresholds. It has been quipped by G. Rolfe Morrison of MacMaster University, Ontario, Canada, that "there are just about as many methods for determining thresholds as there are papers written on the subject (*21*)". This multiplicity of methods may be due in part to our increasing understanding of human psychology and physiology over time. Whatever the reason for the existence of so many methods, numerous strategies for defining the same phenomenon can be troublesome. Different methods have defined the term "threshold" to mean different things. Older methods (pre-1981) captured only momentary thresholds (*42*). Even if the test was repeated with an individual panelist, there was no inclusion of replicate data in a statistical analysis. At best, experimenters arbitrarily looked at the replicates to see if the panelist appeared to be giving the same results over a period of time (*42, 43*). Thresholds have also been defined using a variety of numerical percentages of correct choices ranging from 50% to 75% correct choices (*42*). Thresholds have been defined as the lowest concentration that can be detected with a statistical significance of 0.01 while still not accounting for multiple replicates of data per panelist (*42*). A method that only requires one test per panelist doesn't accurately depict the detection threshold, because there is no single perfect value for a threshold where the panelist is always tasting the substance with statistical significance. An individual's threshold varies from moment to moment as previously discussed in the "sensory science" section of this thesis. Thresholds have also been defined historically as being the concentration at which the panelist correctly identifies the dosed sample 50% above

the chance of guessing (*42*). If left undefined by an equation, as it originally was, this can mean 50% correct choices, which is the chance of guessing the correct sample in a 2-sample test plus and additional 50%, or 100% correct answers (*44*). It can also mean that same 50% correct choices chance of guessing plus half of the probability of guessing incorrectly (50% of 50% in this case) or 75% correct choices (*44*). Similarly, in a 3-choice test, it can mean 50% in addition to the 33% chance of guessing the correct sample. This means that the threshold would be defined as the point where the panel or individual was correct 83% of the time (*44*). This can also mean the chance of guessing correctly (33%) plus half of the chance of guessing incorrectly (50% of 66%) which would define the threshold as the concentration where the panel or individual correctly identified the stimulus 66% of the time (*42*). All of this ambiguity arose from a lack of an equation-based definition for threshold, and outlines the need for well defined standard methods for defining flavor thresholds.

For research pertinent to this thesis, the term "threshold" is defined in 2 ways. In the case of ASTM method 679 (to be discussed later), the term "threshold" is defined as the geometric mean of the concentrations of the last incorrectly-identified sample, and the first correctly-identified sample. In the case of ASTM method 1432 (to be discussed later), the threshold is defined as the concentration where a panelist selects the dosed sample from a 3-alternative forced-choice test 66% of the time(*45*). This is the chance of guessing the correct sample (33%) plus 50% of the chance of guessing incorrectly(*45*). The threshold for iso-α-acids in a lager beer matrix with 1 to 30 mg/L iso-α-acids was

found to be between 6 and 15 mg/L using a method known as ASTM method 679, which will be discussed in detail later in this thesis (*46*). Because the research pertaining to the threshold of tetra acids in beer uses the ASTM method 679-related term "best estimate threshold," it is assumed that the threshold for tetra acids was also found using that method, though the sensory method is neither cited nor described in the tetra acid experiment (*47*). The best-estimate threshold of tetra acids in unhopped beer matrix was found to be 8.50 mg/L by Weiss and Schönberger in 2002 (*47*).

1.13 Detection Threshold Methods

There are three main categories of tests for determining human thresholds of substrates in media: staircase procedures, R-index measures, and alternative forced choice tests. These three methods will be discussed in this section.

1.13.1 Staircase procedure

The staircase procedure is a commonly-used method for finding both detection and difference thresholds of substances. First developed by T. N. Cornsweet in 1962, this test implements a series of reversing 2-alternative forced choice tests (2-AFC) to find a person's threshold (*48*). The 2-AFC presentation consists of a pair of samples—one that contains a stimulus, and one that does not. The panelist is asked to choose which sample contains the stimulus. If the panelist is incorrect the next stimulus presented will be of a

higher concentration. If the panelist correctly chooses the stimulus over the control, the test is repeated, at the same level. If the panelist correctly chooses the dosed sample a number of times specified by the experimenter (usually 2-3) the concentration of the next test is decreased (*49, 50*). A single wrong answer causes the concentration to increase again. As the name implies, if one thinks of the procedure in terms of a staircase, going down stairs corresponds with decreasing a panelist testing concentration and going upstairs is associated with increasing a panelists' concentration, and any time the panelist "changes direction" is known as a reversal. The testing procedure is continued until the panelist reaches a predetermined number of reversals—usually 6-8, though, no specific number is required (*49, 50*). The threshold can be defined as the signal intensity where the probability of a panelist being correct (p) is equal to the nth root of 0.5 where n is the number of correct responses required to cause a reversal (*49*). This number of responses is determined by the experimenter. The threshold is also described as the average of the stimulus levels at reversal points or the arithmetic mean of the concentrations between the last 4 and 5 reversal points (*49, 51*). This last discrepancy highlights one of the main problems with the staircase procedure: There is no specific definition of how many reversals are required, or how many samples must be right or wrong to cause a reversal (*50*). These aspects can change the results obtained with this procedure, and if they are left undefined, the data is not replicable, and cannot be compared to other literature values (*50*). The staircase procedure is also very resource intensive—requiring many samples per individual, and requiring individual attention for every panelist in every test

(*49*). Results from each test must be tabulated before another test can be delivered (*49*). Moreover, it is not specified that the staircase procedure be repeated with each individual panelist, and thus the procedure does not account for individual moment-to-moment variation, nor does it provide any way to check the reliability and repeatability of individual tests (*44, 45*). There is no ASTM standard method outlined for the staircase procedure.

Despite the lack of procedural definitions, this procedure has been used to find the threshold for haze in apple juice in an experiment that compared results found using this procedure with results found using ASTM method 679. The results indicated using the staircase procedure gave results were similar to results found using ASTM method 679 (*49*). The staircase procedure was also used to determine the thresholds of caffeine and alcohol in water by Richard Mattes in 1994 (*51*).

1.13.2 Signal Detection Theory and The R-index

When given a question to answer, humans will have four different potential answers: the person may be correct, and certain of his or her choice; the person may be correct, but uncertain of his or her choice; the person may be incorrect and unsure of his or her choice, or the person may be incorrect while still expressing certainty with his or her choice. This series of responses is the basis for signal detection theory (*52*). Signal detection theory is the basis for the use of a probability value known as the R-index. The

R-index is the predicted probability of giving a correct response when presented with a signal-noise pair, the principal being that if a difference between two stimuli is large, the probability of detecting the difference will be significantly greater than chance, and thus the R-index will be high (*53, 54*).

In this method, a panelist is presented with several randomly-organized "Signal" samples (S), and "noise" samples (N) which in the case of a threshold experiment would be the stimulus and blank samples respectively (*54*). The number of samples is determined by the experimenter. The panelist is asked to state whether the sample is S or N, and also must state whether or not he or she is sure of this choice (*54*). Panelist responses are organized in the form of a matrix (figure 1.8), which is used to calculate the number of correct paired comparisons. The R-index is calculated by dividing the number of correct paired comparisons by the maximum number of paired comparisons possible in the test. This R-index is compared to the R-index of chance (50%) of to see whether the panelist is actually differentiating between the samples (*54*). To calculate the R-index, one must calculate the total number paired comparisons between S and N samples presented in a test. This is simply the number of S samples multiplied by the number of N samples. Then one must calculate the number of times, if given either a signal of some concentration or a noise sample, the panelist would correctly identify the presented sample. As an example, in the case of figure 1.8 below, if a panelist were given a series of signals and noise samples, he or she would be said to have correctly detected the signal "x" times, and the noise "f," "g," and "h" times. Thus the number correct paired

comparisons for this hypothetical series of samples would be defined by the equation *x (f + g+ h)*. The total number of correct paired comparisons (Y) is summed and the probability of guessing correctly (50% of the paired comparison for any single N S response) is added to that sum.

$$Y = x(f+g+h) + b(g+h) + ch + 0.5(xe+bf + cg + dh)$$

Y divided by the total number of possible paired comparisons yields the R-index (*54*). In order to determine whether the calculated R-index is significantly greater than the chance level R-index, one must use a Wilcoxon-Mann-Whitney rank sums test and check for statistical significance (*54*).

		Judge's Response			
		Definitely S	Maybe S	Maybe N	Definitely N
Samples	S	x	b	c	d
Presented	N	e	f	g	h

Figure 1.8: Example matrix of responses in a R-index differentiation trial using one series of Signal samples and one series of Noise samples (*54*)

One possibly problematic aspect that becomes clear upon reading the description of the method is the relative complexity of the analysis of results. Numerous calculations and statistical procedures are required to simply answer the question of whether the panelist is distinguishing between one concentration of a sample and noise. As a

threshold test, this procedure would have to be repeated at higher or lower stimulus intensities until an R-index of differentiation of 50% above chance was reached. Proponents of the R-index still claim that this method advantageously provides accurate results with less sample preparation than other sensory threshold tests. However, accurate results obtained with an R-index require that each panelist can be given at least 20-40 presentations, which is the same number of presentations required by ASTM 1432 (*45, 53, 55*). Unlike other sensory methods, all replicates can be presented in one session, which significantly reduces the time required to perform a group threshold analysis (*55*). The lack of test repetition over a period of time does not address the effect of time on panelist sensitivity (*53*). It is known that panelists may learn the flavor of the substance over time, and panelists may also become less sensitive to a substance over time (*53*). The first 20-40 samples presented may not show the effect of learning or desensitization, but that sampling strategy might not provide an accurate representation of an individual panelist's threshold (*53*). It is also important to note that limited research has been done to compare the R-index method to threshold values found using standard methods for finding sensory thresholds of substances, and there is no current accepted standard method for using the R-index to find sensory thresholds (*55*).

The R-index was first used in 1974 by J. Brown when it was used to find the difference threshold for sodium chloride in water (*55, 56*). More recently, the R-index has been used to find flavor thresholds of caffeine, and bitter soy isoflavones (*55, 57*). As recently as 2005, the thresholds for caffeine in water obtained by K. M. Robinson

using both the R-index and ASTM method 679 were compared to each other, and to threshold values found in literature. Both methods yielded results that were within the range of literature threshold values (*55*).

1.13.3 ASTM Method 679

ASTM method 679 was originally approved in 1980 after Brown et al reviewed 13 different detection threshold determination methods and determined that 679 was the best method for finding relatively reproducible thresholds (*43, 56*). This method relies on the premise that for every person, there is a concentration of a substance above which the person can reliably detect that substance, and below which a person cannot (*43*).

ASTM 679 was originally known as the ascending method of limits (*43, 58*). In order to find an individual threshold, a panelist is presented with a series of 3-alternative forced choice (3-AFC) presentations. In each 3-AFC, one sample is dosed with a test compound, the other 2 samples are blanks, and the panelist is asked to identify the dosed sample. The concentration of the dosed sample is increased by a constant factor over the series of 3-AFC presentations. Theoretically, the panelist begins the test unable to reliably identify the dosed sample and completes the test having been able to reliably detect the dosed sample. As stated in ASTM method 679, the test is repeated "until there is no doubt that the threshold has been found (*43*)." Once a panelist has satisfactorily completed the test, his or her threshold is determined as the geometric mean of the

concentration of the highest missed detection and the next concentration on the scale (the first detected level) (*43*). If the panelist correctly identifies the lowest level, by procedure it is assumed that the panelist would have missed the next lowest concentration and the threshold is calculated as the geometric mean of the lowest concentration delivered and the next lowest scale concentration (*43*). Likewise, if the panelist misses the highest concentration presented to him or her, it is assumed that he or she would detect the next highest level, and his or her threshold is calculated as the geometric mean of the highest concentration presented and the next highest scale concentration. If replicate tests are given, the calculated threshold from each test are simply averaged to find the individual's threshold (*43, 53, 58*). The group threshold is calculated as the geometric mean of all the individual thresholds (*43, 58*).

The time and the number of samples required to properly carry out a sensory threshold method are very important factors to consider when planning a sensory test. ASTM method 679 minimally requires one series of 6, 3-AFC presentations compared to the 20-40 presentations required for both ASTM 1432 and the R-index method (*43, 45, 59*). Using fewer tests and samples reduces supply costs, cost of panelist compensation, and time required to administer the entire panel (*21, 58*). Fewer tests also reduce panelist fatigue and prevent issues relating to lack of panelist motivation (*58*). Both can be sources of bias that will affect experimental outcomes (*58*). The results gained from each test can be analyzed very easily, and have provided satisfactory results to the ASBC and ASTM for over 10 years (*21, 22*). With a large enough panel, data will be "normal" and

outliers will balance across the data set with reproducibility levels of 20-50% using a new panel (*4, 58*). These advantages are why ASTM method 679 is currently the most commonly used method for finding sensory thresholds (*42*).

At first glance, ASTM 679 appears to be a highly useful, rapid, easily applied sensory method for accurately finding sensory thresholds. When the ascending method of limits was accepted as an ASTM standard method in 1991, Morrison offered a dissenting opinion stating that "ASTM method 679 contains a number of logical and methodological errors of such seriousness that they completely invalidate the results (*21, 22*)." ASTM method 679 procedurally requires that the test be repeated with an individual until an experimenter is "certain" that the panelist's threshold has been captured (*43*). This requirement of repetition diminishes the economic superiority over other, more involved methods (*21, 43*). Moreover, ASTM 679 has no prescribed method of utilizing these test repetitions (*43*). ASTM 679 also neglects the possibility that data points outside of the highest missed concentration and the next detected concentration may be valuable (*21*). Essentially, this method works on the assumption that there exists a point above which a panelist will correctly identify a sample 100% of the time, and below which the panelist will miss 100% of the time (*21*). Based on current signal detection theory we know that this is simply not true. It has been found that the human ability to sense variation is more of a continuum, with a range of concentrations over which a panelist will be unable to sense a stimulus, a range on concentrations where a panelist will be able to sense a stimulus, and an intermediate range where the panelist

may or may not be able to sense a stimulus depending on a number of circumstances. The threshold lies in the last of these three ranges (*44*). ASTM method 679 neglects the reality of moment-to-moment variability of a panelist's ability to taste substances (*21, 58*).

ASTM method 679 also makes assumptions stated previously about panelists whose thresholds lie either above or below the presented ranges. The assumption that a panelist will miss the next lowest concentration if he or she has been correct through the entire test or that the panelist will identify the next highest concentrations when he or she missed the last presented concentration is nothing more than unproven speculation, and a way to artificially provide closure to a study.

Statistically, it must also be understood that if a panelist is not able to taste a presented concentration of substance, there is still a 33% chance in any given 3-AFC presentation that the panelist will correctly identify the dosed sample. As the panelist nears his or her threshold concentration, the probability of correctly choosing the dosed sample increases, because there will be a greater number of incidences where a panelist will actually detect the sample (though perhaps not yet reliably) in addition to the 33% chance of guessing correctly when the panelist does not actually detect the sample (*21*). These factors cannot be accounted for in single repetition of a threshold test using ASTM method 679. Chance guesses coupled with the choice of scale for concentration steps, and the actual concentration of a panelist's threshold can bias a group's threshold by a factor of 1.5 to 3 (and in some instances more) (*58*). Because panelist variability

contributes to a person's ability to taste, the only way to find an individual threshold while accounting for this variability through repeated testing and a method for analyzing that data inclusively (*21*).

The opinions stated in Morrison's papers cast serious doubt on the validity of ASTM method 679, and thus shed doubt on the validity of results obtained using that method. In recognition of the faults of ASTM method 679, the method was reviewed, and relegated to "rapid method" status for finding group thresholds in 1991. Individual thresholds found using this method were referred to as "best estimate thresholds," as it was decided that the one-test thresholds were no more than estimated thresholds. At the same time a new standard method for finding group and individual thresholds called ASTM method 1432 was adopted.

Despite its flaws, ASTM method 679 is still the most commonly used method for determining human thresholds in food sensory science. Its uses are also not limited exclusively to the sense of taste. It has recently been used to find the threshold for 2-isopropyl-3-methoxypyrazine (the aroma of ladybugs) in wine by G. J. Pickering (*60*). It has been used to find odor and flavor thresholds of citra-hexanal, beta-pinene, and limonene in orange juice (*24*). ASTM method 679 has also been used to find the visual detection threshold for haze in apple juice in a study that used this medium to compare ASTM method 679 to the staircase procedure (*49*). Method 679 was found to give similar results as the staircase procedure in that study (*49*).

1.13.4 ASTM Method 1432

ASTM method 1432 was the method for finding individual and group thresholds that replaced ASTM method 679 in 1991 when ASTM method 679 was relegated to "rapid method" status. Like the R-index method, ASTM 1432 is a method that attempts to find a statistical threshold for each panelist, which is defined as the concentration that the panelist has a probability of actual detection equal to p= 0.5 (*45, 58*).

Each panelist is presented with at least 20-40 3-AFC presentations spread over several tests—each test presented on a different day. Each test consists of 5-7, 3-AFC presentations given in ascending order by concentration including concentrations that the panelist cannot reliably taste at the lowest end and concentrations that the panelist can reliably taste at the highest end (*45*). As was the case in ASTM method 679, 2 of 3 samples presented in a 3-AFC test are blanks, and the panelist is asked to identify which sample is dosed with some test compound. Individual panelists' responses are tabulated, and this data is plotted as the percent of correct choices versus the log of each presented concentration the panelist tasted (*45*). A non-linear regression is used to model the data and provide an equation of a non-linear line of best fit. The equation for that line is used to calculate the concentration where a panelist will be able to detect the difference with a probability of p=0.66, or 50% above chance.

With regard to the economic factors of time and sample preparation, ASTM method 1432 is a very cumbersome method. It requires numerous repetitions per panelist. Specifically, determining a group threshold requires 500 total 3-AFC presentations or more to adequately reduce the degree of random error associated with the experimental design (*45*). The test for each panelist is repeated on 5 to 7 separate days that can be spread out over weeks. This lengthy period of time required to test panelists can become tedious to the panelists, resulting in a lack of motivation that can bias results (*21*). Because fewer panelists are needed for a group threshold using ASTM method 1432 compared to ASTM method 679 one must take special care to be certain that his or her group of panelists is representative of the population being studied (*45*). Due to its cumbersome nature, ASTM method 1432 is often overlooked for its seemingly easier counterpart—ASTM method 679.

ASTM method 1432 may be a long, tedious method to carry out, but it is the current ASTM standard method for determining group and individual thresholds (*45*). This status as the ASTM standard method is partly due to the fact that this method improved on older methods by incorporating many days of tests into one final threshold to account for day-to-day panelist variability (*45*, *58*). Because of the test repetition and statistical analysis of the data, individual thresholds found using ASTM methd 1432 are not referred to as "estimated thresholds" as are those found using ASTM method 679 (*43*, *45*). These thresholds are considered to be the true thresholds of the individuals on the panel. A group threshold calculated from actual individual thresholds is an actual

threshold of the group, rather than an estimate based on estimated thresholds that would be found using ASTM method 679. ASTM method 1432 also provides a very specific definition of the term "threshold," preventing ambiguity of results obtained through the method. When panelists are trained adequately, this method provides a valuable way to more precisely determine individual sensory thresholds (*45*). ASTM 1432 has been used to find the group threshold of carbon dioxide in yogurt by A. O. Wright et al in 2003 (*61*).

The Human Bitterness Detection Threshold of Iso-α-Acids and Tetrahydro-Iso-α-Acids in Lager Beer

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2.1 Abstract

Human taste thresholds of iso-α-acids (iso) and tetrahydro-iso-α-acids (tetra) were measured in an unhopped lager beer matrix using the ASTM method 1432 protocol. For each compound, a group of 14 volunteers participated in 3 training sessions and 6 to 13 tasting sessions where each panelist was presented with a series of 6, 3-alternative forced choice tests (3-AFC). Each series was presented in increasing concentration by a factor of 1.5 or 1.3 over the previous dosed sample for iso and tetra, respectively. The individuals' threshold concentrations were determined as the point on a sigmoidal curve fitted to their percent correct response data where the panelist could correctly choose the dosed sample 66% of the time. The group-wise detection threshold was determined as the point on a rank probability plot where 50% of the panelists could correctly identify the dosed sample. The group-wise detection thresholds and standard deviations of thresholds for iso and tetra were 7.1 mg/L (4.5-11.2 mg/L) and 2.7 mg/L (0.7-10.0 mg/L), respectively.

2.2 Introduction

Hops are used as the primary bittering agents in nearly all beer. Learning the human taste detection threshold—the lowest amount of these compounds humans can taste—helps scientists and brewers define and understand the complex mixture of chemicals that combine to create the flavor we recognize as beer. The current increase in use of hops extracts in beer and the trend toward reducing over-all bitterness of American

light lagers has made it relevant to find the lowest amount of iso and tetra the average person can taste in a similar beer matrix. The importance of this understanding was recognized in the mid 1970's and 1980's by M. C. Meilgaard, who set out to create a library of taste and aroma thresholds for a myriad of compounds that can be detected in beer (*46, 62*). He determined the threshold of iso-α-acids (iso) in beer with 0-30 mg/L of isohumulone to be between 7 and 15 mg/L using the ascending method of limits (ASTM method 679), which was the accepted method for determining group thresholds at the time (*43, 46, 58*). Around the time that Meilgaard was publishing results for thresholds of various compounds, G. Rolfe Morrison published two articles questioning the statistical validity of the ascending method of limits used in Meilgaard's threshold experiments (*21, 44, 46*). His main criticism was that the method of ascending limits did not require individual panelist repetition (*43*). Because individual thresholds vary moment to moment, concerns were raised that group thresholds were based on estimates of individual thresholds, and thus the group threshold was statistically only an estimate itself. Once these statistical shortcomings were noted, a modified version of the method of ascending limits, ASTM method 1432, was introduced (*45*). This method required each panelist to participate in several repetitions of an ascending series of triangle tests to get statistically defined individual thresholds thereby resulting in group thresholds with stronger statistical significance (*45*). Though the method used to gather the original data has been relegated to a "rapid method" known as ASTM 679, researchers still use the original resultant value of 7-15 mg/L when discussing the threshold value of iso (*63*).

The best-estimate threshold of tetra in unhopped beer was found to be 8.50 mg/L by Weiss and Schönberger in 2002 (*47*). The term "best-estimate threshold" suggests the implementation of ASTM method 679, but no sensory method was cited or described in the paper (*47*). To date, human threshold measurements of iso or tetra in beer using ASTM method 1432 have not been published. The primary objective of our research was to provide updated individual threshold values and group values for both iso and tetra in a well-defined beer matrix using current, standard methods. The secondary objective was to compare methods 1432 and 679 by using a common set of data.

2.3 Experimental

2.3.1 Sample Preparation

An unhopped lager beer was the medium for these sensory experiments. It was brewed in the Oregon State University pilot beer brewery with 90% of extract from pale malt (Great Western Malting Co.) and 10% of extract from rice syrup solids. The original gravity of the beer was 11.0 °P, and the final gravity was 2.2 °P. The final ethanol concentration was determined to be 3.47% by weight (*64*), and the real extract was calculated as 4.26 °P (*65*).

The iso-α-acid (iso) solution used in this experiment (John I. Haas Hops Company, Yakima, WA) was found to be 31.1% (wt/wt) iso using spectrophotmetry (*66*) and 29.7% (wt/wt) iso using HPLC under conditions outlined in the ASBC standard method for analysis of isomerized hop extracts by HPLC (*67*). This solution was dosed into the

unhopped beer medium and filtered to yield a stock solution that was 66 mg/L iso. This stock beer was further diluted with unhopped beer to 30 mg/L of iso. This highest concentration to be tested in this study was subsequently serially diluted by a factor of 1.5 to achieve all 11 levels used in this experiment ranging from 0.5 mg/L to 30 mg/L.

The tetrahydro-iso-α-acid (tetra) solution used in this experiment (John I. Haas Hops Company, Yakima, WA) was found to be 11.6% (wt/wt) tetra acids using spectrophotmetry (*66*) and 10 percent tetra acid using HPLC under conditions outlined in the ASBC standard method for analysis of isomerized hop extracts by HPLC (*67*). Tetra was dosed into the unhopped beer medium and filtered to yield a stock solution that was 36.1 mg/L tetra. This stock beer was further diluted with unhopped beer to 24.5 mg/L of tetra. This highest concentration to be tested in this study was subsequently serially diluted by a factor of 1.3 to achieve all 16 levels used in this experiment ranging from 0.3 mg/L to 24.5 mg/L. All concentrations of iso and tetra in the dosed beer samples were verified by HPLC using the ASBC standard method for quantification of isomerized alpha acids in wort and beer (*67*).

2.3.2 Sensory Panel

The panel for each threshold study was comprised of 14 volunteers from the Corvallis, Oregon community (7 females and 7 males for iso, or 6 females and 8 males for tetra) between the ages of 23 years and 57 years. In both cases, 8 of the 14 panelists

had 3 or more years of experience on taste panels for bitter compounds. In each experiment, all panelists participated in 3 one-hour training sessions that were held in order to introduce the panelists to the testing format, to determine proper testing levels for each panelist, and to familiarize the panelists with both the control beer and the beer containing iso or tetra.

2.3.3 Sensory Methodology

The group and individual iso and tetra thresholds of the panelists were determined using ASTM method 1432 (*45*). For the iso experiment, each panelist participated in 6 testing sessions. Each session consisted of 6, 3-alternative forced choice (3-AFC) presentations, presented in order of ascending concentration. The tetra experiment was carried out in the same manner as the iso experiment except panelists participated in 6 to 13 tasting sessions to capture individual thresholds. In several cases the concentrations required to include an individual panelist's detection threshold continued to shift after the 3-day training period. Extra sessions were offered to panelists as required until the concentration range given to each included at least two lower concentrations that the panelist was incorrectly identifying at least 66% of the time (chance), and two upper concentrations at which the panelist was correct more than 66% of the time.

Before each tasting session panelists were given a sample of the unhopped lager beer (control), and a "warm-up" sample dosed with either iso or tetra in the first and second studies respectively. This "warm-up" sample acted as a signal to re-familiarize

panelists with the flavor of the dosed sample. The dosed warm-up sample was at a concentration such that the individual panelists would be able to taste the concentration without being overwhelmed by the stimulus—usually the second-highest level the panelist would taste during his or her test. All beer samples were served on ice and were measured to be between 3 and 6 °C. During the tests, the panelists wore nose plugs and were instructed to circle the 3-digit code corresponding to the sample they believed to be the most bitter in each 3-AFC presentation. The odd sample in each 3-AFC was the dosed sample. After each 3-AFC presentation, panelists rinsed with a 1% pectin solution and with water, and waited 3 minutes to prevent adaptation and/or bitterness carryover from one sample to the next. Upon completion of testing, all panelists not on record as having already been tested for sensitivity to 6-n-propyl-2-thouracil (PROP) were asked to taste a 0.00032M solution of PROP and rate it on a 15 point scale. Panelists who rated the sample at or above 10 were considered "super tasters" and panelists who rated the sample below 10 were considered mild to moderate tasters. Panelists who could not taste the sample at all were considered non-tasters.

2.3.4 Statistical Analysis

In both experiments the group threshold was statistically analyzed with regard to demographic categories of individual panelists in order to see whether age (defined as above or below 35 years of age), gender, smoking status, experience on panels (defined

as more than 3 years experience) or ability to taste PROP (super taster or not a super taster) contributed to significant differences in taste thresholds. Each demographic category was divided into two subgroups (ie smokers or non-smokers). In each category, the thresholds of the subgroup populations were first analyzed using a 2-sample F-test to ensure equal variance. Upon finding the variance of subgroups' populations to be equal, the thresholds of those subgroups were compared using a pooled variance 2-sample t-test using the S-plus statistical analysis computer program.

In both the iso and tetra experiments Each individual's ASTM method 1432 threshold was compared to that individual's ASTM method 679 thresholds using a pooled variance 2-sample T-test. The lack of variance around an individual's ASTM method 1432 threshold, a single value, necessitated the assumption of equal variance for this analysis.

In the iso experiment and days 1 through 6 of the tetra experiment, the means of each ASTM method 679 daily threshold data were compared to the mean of the ASTM method 1432 threshold data using paired T-tests. A paired T-test was used in these cases, because each ASTM method 679 daily threshold population was exactly the same as the population used for the ASTM method 1432 threshold.

In the tetra experiment, the means of the ASTM method 679 daily threshold data for days 7 and 8 were compared to the ASTM method 1432 threshold data using a pooled variance 2-sample T-test. The populations for the ASTM method 679 threshold data for days 7 and 8 were not identical to the ASTM method 1432 threshold data population.

2.4 Results

2.4.1 Method 1432

Each panelist's data were plotted as the percent of correct choices vs. the log of the presented concentration (figure 2.1). Percent correct choices were calculated as the sum of correct responses at a stimulus concentration divided by the total number of responses at that concentration. All data points from all test sessions were used to calculate individual thresholds. Panelists 1, 6, 7, and 10 did not participate in both studies. With the exception of the iso data for panelist 3, a 4-parameter sigmoidal equation was fitted to each panelist's data.

$$y = y_0 + \frac{a}{1 + e^{\frac{(-x - x_0)}{b}}}$$

Panelist 3's iso data could not be fitted with a 4-parameter sigmoidal equation, but was fitted instead with a 4-parameter logistic equation.

$$y = y_0 + \frac{a}{1 + \left|\frac{x}{x_0}\right|^{|b|}}$$

For both equations, x represents the log of the concentration of iso or tetra, y represents the percent of correct choices for a panelist, y_o is the minimum value of y, a is the range of concentrations over which the response occurs ($y - y_o$), and x_o is the log concentration

of hops compound at one-half *a*, and *b* is the slope of the line at x_o. Each individual panelist's threshold was determined as the concentration at which the panelist correctly chose the sample spiked with iso or tetra 66% of the time (i.e., 50% above chance).

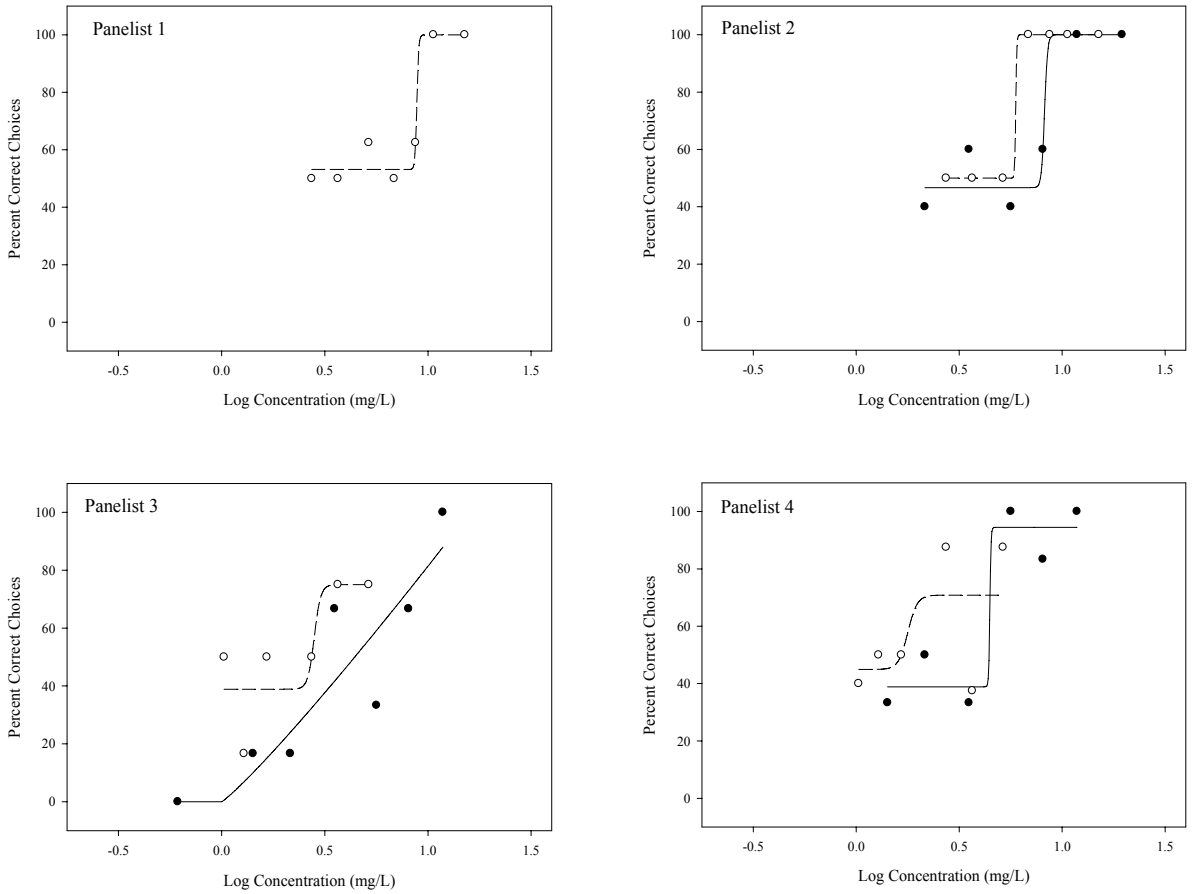
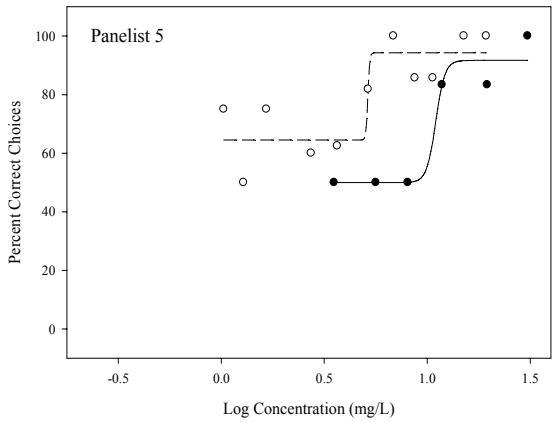
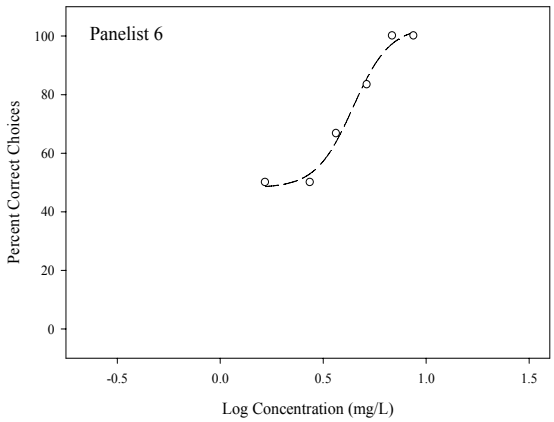
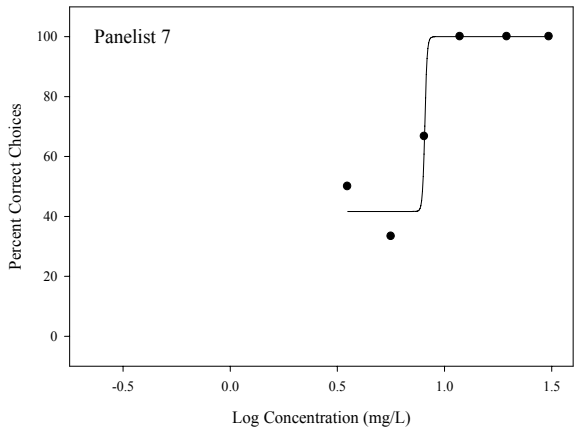


Figure 2.1 continued on pages 41-43

Figure 2.1 continued







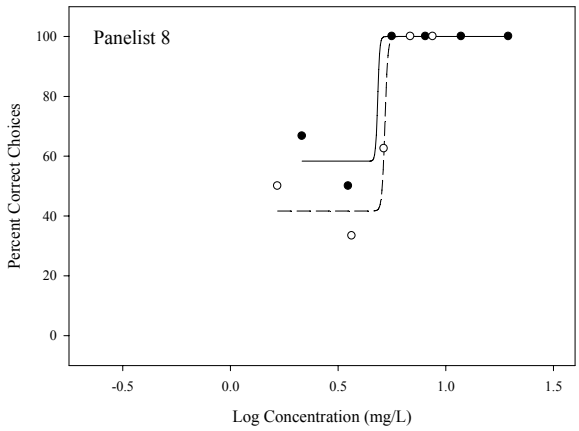
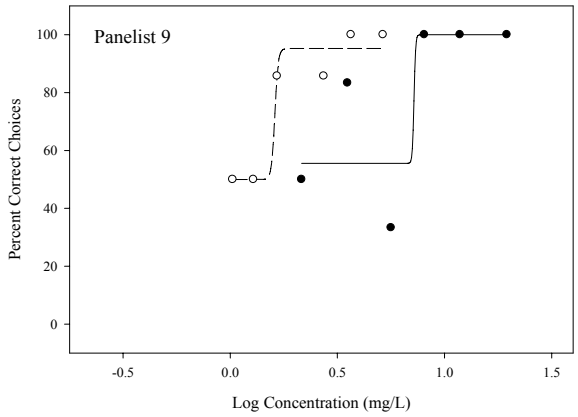
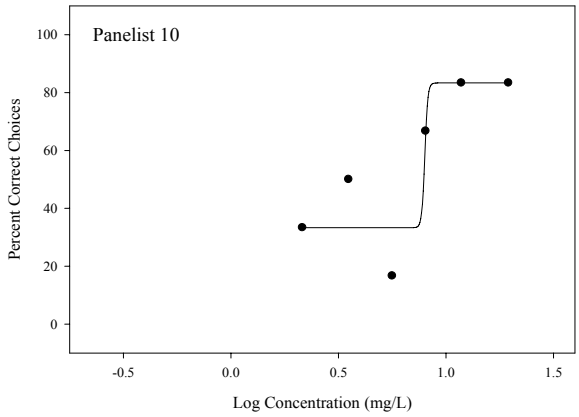
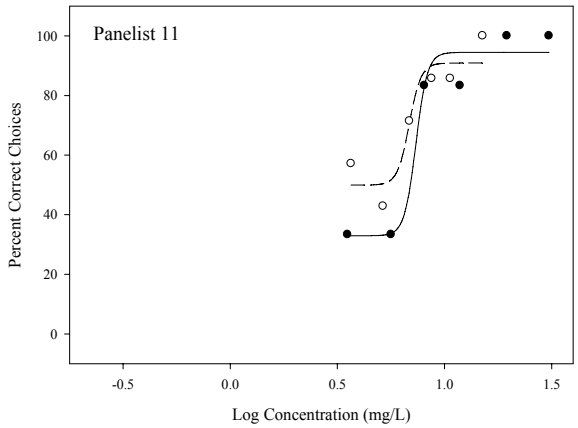


Figure 2.1 continued







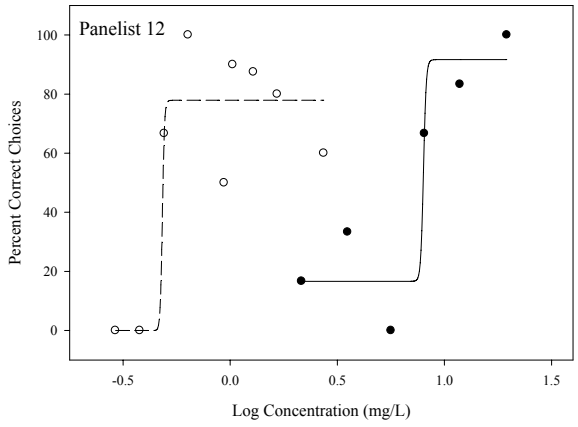


Figure 2.1 continued

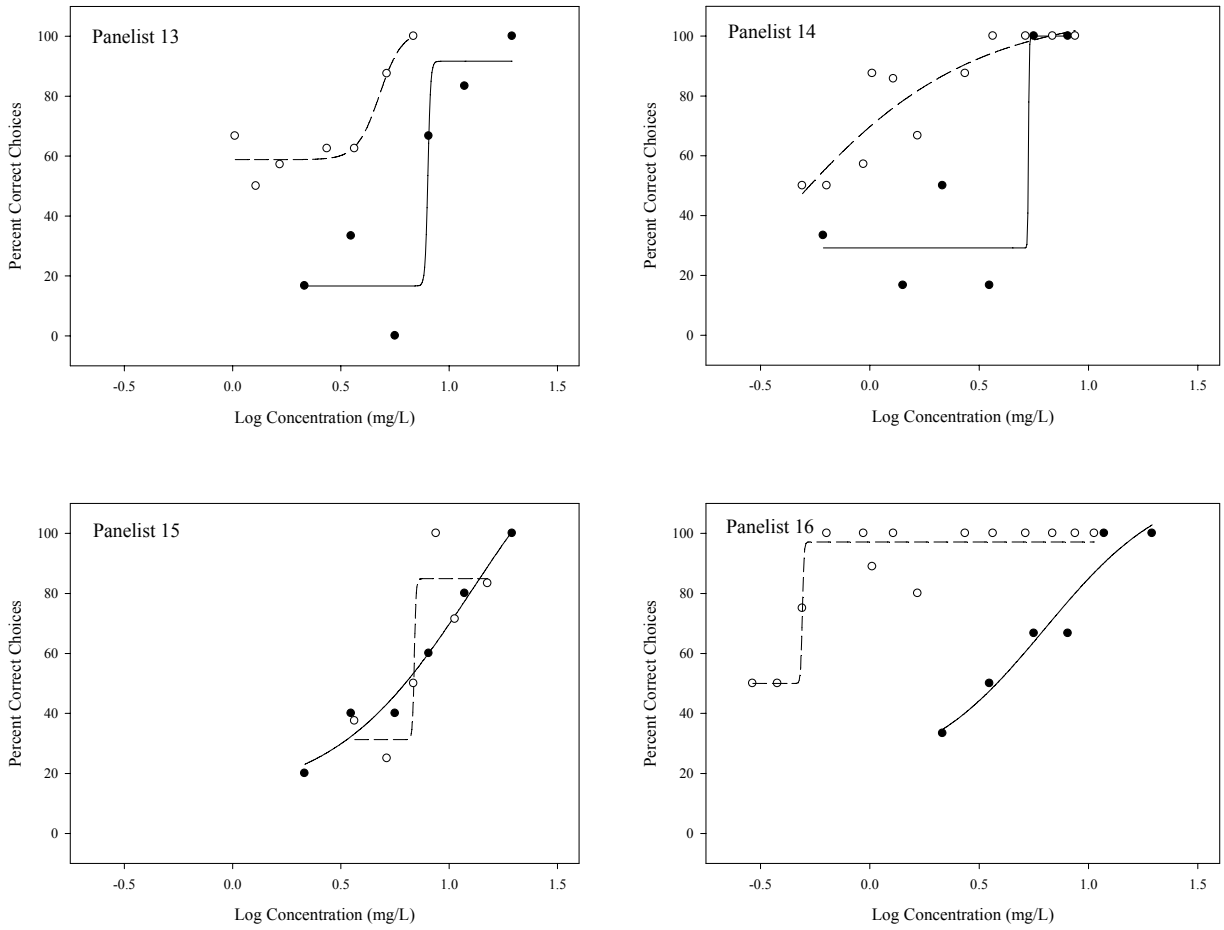


Figure 2.1: Individual panelists' dose-response data from a series of 3 AFC presentations for iso and tetra in lager beer where the plot with open circles (○) and a dashed line (---) refers to tetra data, and the plot with closed circles (●) and a solid line (──) refers to iso data.

In order to calculate the group thresholds for iso and tetra, the log values of the panelists' thresholds were ranked from lowest to highest, and were plotted against the panelists' rank position (figure 2.2). The rank position (%) was determined as being equal to $100\ i\ /\ n + 1$, where i represents the numerical rank of the panelist in relation to

the group, and *n* represents the number of panelists in the group (*45*). A linear regression was fitted to the data, and the group threshold was determined as the concentration where rank position is equal to 50% with +/- one standard deviation limits when rank position equals 84% and 16%, respectively (*45*).

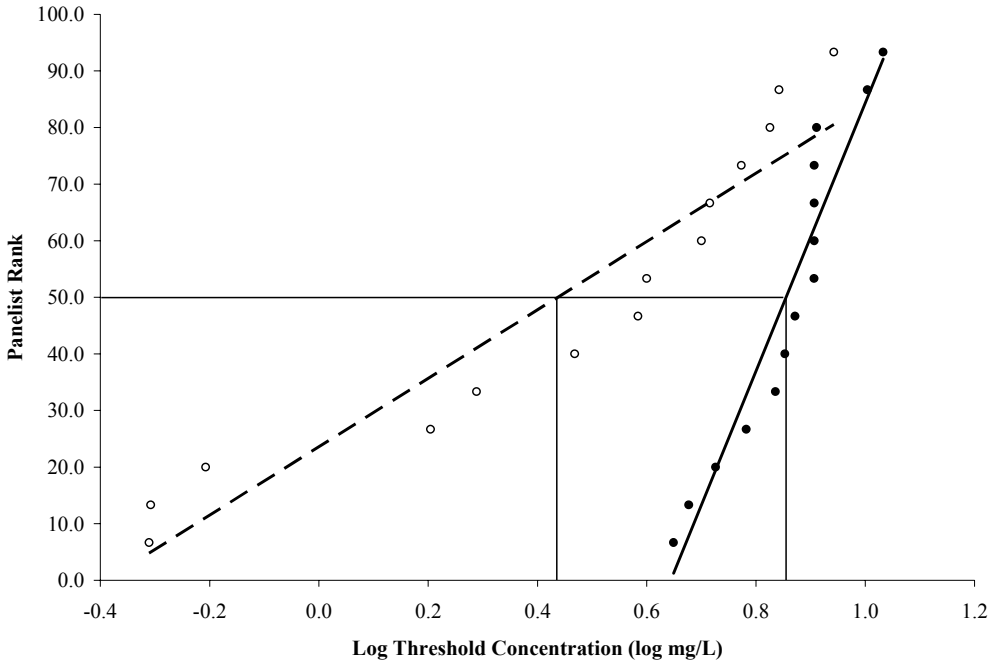


Figure 2.2: A group threshold rank-probability graph of the tetra threshold data (○) and the iso threshold data (●). A straight line fit to the iso data yielded an r^2 value of 0.911, while the tetra data yielded an r^2 value of 0.898. The vertical lines represent the concentration that corresponds to a panelist rank of 50 (horizontal line).

In the iso threshold study, the group threshold of iso in beer was calculated to be 7.1 mg/L with a +/- one standard deviation range of 4.5 to 11.2 mg/L based on 504 total 3-AFC presentations (6 testing sessions with 6 3-AFC presentations for 14 panelists). In

the tetra threshold study, the group threshold of tetra in beer was calculated to be 2.7 mg/L with a +/- one standard deviation range of 0.7 to 10.0 mg/L based on 738 total 3-AFC presentations (some panelists required more than 6 tests to adequately capture their thresholds). With the exception of day 1 for the tetra threshold measurements, there was no substantial evidence in either threshold study of group-wise or individual learning effects over the duration of the threshold testing procedure (Table 2.1, 2.2).

Table 2.1: ASTM method 679 best estimate iso thresholds (mg/L) for each panelist by day, and ASTM 1432 individual thresholds

	ASTM Method 679							**ASTM Method 1432**
Panelist	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Average 679 Threshold**	**1432 Threshold**
2	6.7	4.4	1.7	9.7	9.7		6.5	8.1
3	6.7	2.7	6.7	6.7	9.7	9.7	7.1	6.8
4	4.4	4.4	2.7	9.7	4.4	4.4	5.1	4.5
5	9.7	15.2	9.7	15.2	9.7	4.4	10.7	10.8
7	9.7	9.7	4.4	2.7	6.7	6.7	6.7	8.1
8	4.4	4.4	2.7	1.7	4.4	1.7	3.3	4.8
9	6.7	1.7	6.7	6.7	1.7	6.7	5.1	7.1
10	6.7	9.7	4.4	6.7	24.5	9.7	10.3	8.1
11	9.7	6.7	15.2	6.7	2.7	6.7	8.0	7.4
12	15.2	6.7	6.7	9.7	6.7	6.7	8.7	8.1
13	6.7	6.7	6.7	9.7	6.7	9.7	7.8	8.1
14	4.4	4.4	4.4	4.4	1.7	4.4	4.0	5.3
15	4.4	6.7	6.7	9.7	15.2	24.5	11.2	10.1
16	6.7	9.7	4.4	9.7	1.7	1.7	5.7	6.1
ASTM 679 group Thresholds by day:	6.8	5.8	5.2	6.9	5.6	5.9	**ASTM 679 average group threshold**	**ASTM 1432 group threshold**
							6.1	7.1

Table 2.2: ASTM method 679 best estimate tetra thresholds (mg/L) for each panelist by day

	ASTM Method 679														ASTM Method 1432
Panelist	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Average 679 Threshold**	**ASTM 1432 Threshold**
1	7.7	9.6	9.6	7.7	4.4	4.4	9.6	3.2						7.0	8.8
2	6.0	3.2	4.4	4.4	6.0	6.0								5.0	5.9
3	7.7	3.2	1.5	3.2	4.4	6.0	3.2	1.5						3.8	2.9
4	7.7	4.4	6.0	1.0	1.1	4.4	4.4	2.1						3.9	1.9
5	12.7	4.4	9.6	6.0	3.2	3.2	2.1	4.4	3.2	4.4	4.4			5.2	5.0
6	4.4	6.0	4.4	6.0	2.1	3.2	4.4	2.1						4.0	3.8
8	4.4	4.4	2.1	6.0	1.5	6.0	4.4	6.0						4.3	5.2
9	3.2	1.5	2.1	1.5	3.2	1.1	1.5	1.0						1.9	1.6
11	12.7	7.7	7.7	6.0	6.0	3.2	9.6							7.5	6.7
12	3.2	6.0	3.2	7.7	1.5	0.9	1.1	3.2	0.5	0.5	0.9	0.5	0.4	2.3	0.5
13	4.4	2.1	3.2	4.4	1.5	6.0	2.1	1.5						3.1	4.0
14	3.2	2.1	1.5	2.1	0.8	1.0	0.8	0.8	0.8	1.1	0.6			1.3	0.6
15	7.7	12.7	6.0	6.0	17.1	7.7	4.4	7.7						8.7	7.0
16	2.1	2.1	1.0	2.1	0.6	0.6	0.6	0.6	0.3	0.3	0.4			1.0	0.5
ASTM 679 group Thresholds by day:	5.4	4.1	3.5	3.9	2.5	2.9	2.7	2.2						**ASTM 679 average group threshold**	**ASTM 1432 group threshold**
														3.4	2.7

In both experiments the group threshold was statistically analyzed with regard to demographic categories of individual panelists using a pooled variance 2-sample t-test in order to see whether age (defined as above or below 35 years of age), gender, smoking status, experience on panels (defined as more than 3 years experience) or ability to taste PROP contributed to significant differences in taste thresholds. None of these factors was found to statistically contribute to deviations in the group threshold for iso or tetra (table 2.3). PROP was tested at a level of 0.00032M, which would be high enough to illicit a varying response among the 3 subgroups (non-tasters, tasters, and supertasters) (*69*). Non-tasters were defined as those who ranked the sample 0-1 on a 15-point scale. Tasters were defined as people who ranked the sample as less than 10, and super-tasters were defined as those who ranked the sample higher than 10. For the purpose of statistical analysis, tasters and non-tasters were grouped together, as there was only one non-taster.

Table 2.3: Analysis of panelist factors which might affect sensory thresholds using a 2-sample t-test:

Factor compared	Status A	Status B	Iso t-value	Iso p-value	Tetra t-value	Tetra p-value
Smoking status	Smoker I T 2 1	Non smoker I T 12 13	1.2301	0.242	NV	NV
Age	Over 35 I T 9 8	Under 35 I T 5 6	0.4242	0.678	0.1506	0.8815
Gender	Male I T 8 7	Female I T 6 7	-0.1139	0.911	0.6764	0.5048
PROP tasting status	Super-Taster I T 5 8	Other Taster I T 9 6	-0.3367	0.742	0.2054	0.8388
Experience on Panels	Over 3 years I T 8 8	Under 3 years I T 6 6	0.1885	0.853	0.1506	0.8815

Number of panelists in the category for the iso and tetra threshold experiments (I and T) respectively.
NV- "no value" as there was only one smoker in the tetra experiment

2.4.2 Method 679

According to ASTM method 679, one series of 6, 3-AFC tests is enough to estimate a panelist's individual threshold whereby the individual threshold is defined as the geometric mean between the sample concentrations of the last incorrect choice and the next highest concentration in a series of 3-AFC tests presented in order of ascending

concentration (*43*). In each experiment, panelists completed between 6 and 13 repetitions of ascending 3-AFC tests. For the purpose of comparison, each repetition was analyzed as one ASTM method 679 presentation resulting in 6 to 13 individual best-estimate thresholds for each panelist for iso (table 2.1) and tetra (table 2.2). The group threshold defined by ASTM method 679 is simply the geometric mean of the individual thresholds. The average of the per-panelist best-estimate thresholds for each compound (ASTM method 679) were compared to that panelist's ASTM method 1432 threshold using a pooled variance 2-sample t-test. For the sake of this comparison, it was necessary to assume equal variance, because there was no variance around the ASTM method 1432 individual threshold. There was no statistically significant difference between each individuals ASTM method 679 average threshold and the same individual's ASTM method 1432 threshold for either iso or tetra (significance at $p< 0.05$). Similarly, for the iso experiment and the tetra experiment days 1 through 6, the ASTM method 679 group thresholds were compared to the ASTM method 1432 group threshold using a pared t-test. ASTM method 679 group thresholds for days 7 and 8 of the tetra experiment were compared to the ASTM method 1432 group threshold using a pooled-variance 2-sample t-test. No statistically significant difference was found between any ASTM method 679 daily group threshold and the ASTM method 1432 group threshold in the iso experiment (significance at $p< 0.05$). The ASTM method 679 group tetra threshold for day 1 was found to be statistically greater than the ASTM method 1432 group threshold ($p=0.007$). This potentially displays a learning effect, but this trend does not persist in either

individual or group thresholds for the remainder of the duration of the experiment. No other ASTM method 679 group tetra threshold was found to have a statistically significant difference over the group tetra 1432 threshold.

2.5 Discussion

The ASTM method 1432 group threshold for iso in this experiment (7.1 mg/L) was found to be in agreement with threshold range for iso previously determined to be 6-15 mg/L by Meilgaard et al using ASTM method 679 (*46*). Though 4 of the 6 ASTM 679 group thresholds were less than 6 mg/L, none of these values were found to be statistically different from the ASTM method 1432 threshold obtained in this experiment. Therefore, these thresholds are statistically in agreement with the previously determined iso threshold range.

Group thresholds for tetra found in this experiment (2.7 mg/L using ASTM method 1432 or ranging from 2.2 to 5.4 mg/L using ASTM method 679) were much lower than 8.5 mg/L expressed in research by Weiss et al, with the possible exception of the ASTM method 679 threshold from day 1 (5.4 mg/L) (*47*). The panelists used for the present research appeared to have an initial, short learning period when they tasted tetra. This demonstrates that tetra is more difficult to recognize initially than iso, and panelists require even more training than was provided in this experiment to accurately identify tetra in beer. Implementation of ASTM method 679 for determining tetra thresholds could yield misleadingly high outcomes.

A significant amount of time is required to collect enough threshold data to determine a statistically valid result. This is one argument for using ASTM method 679 over ASTM method 1432 (*43*). Though fewer tests may reduce testing time, fewer tests may also compromise the statistical validity of the experimental outcome. The results of this experiment show that with a panel of 14 people, ASTM method 679 provides comparable group threshold results to ASTM method 1432 for both iso and tetra. In practice, ASTM method 1432 requires 6 times the amount of testing of ASTM method 679. In this research, ASTM method 679 repeatedly provided a group threshold that was not found to be statistically different than the threshold found using ASTM method 1432 for both tetra and iso. However, even though no individual was found to have an ASTM method 679 threshold that was significantly different than his or her ASTM 1432 threshold, it must be noted that ASTM method 679 can and did provide a wide range of individual thresholds. An example is panelist 15 in the iso experiment (table 2.1). Note that this individual's threshold ranged from 4.4 mg/L to 24.5 mg/L over the course of the test. Though this data was not particularly influential on group threshold values, it demonstrates that an individual threshold can and does vary greatly over a period of time. If time is of great importance in a threshold experiment and individual thresholds are not necessary, ASTM method 679 is an acceptable method of determining group thresholds. However, if individual threshold values are required, one should use the longer, more statistically strong ASTM method 1432.

The Human Detection Threshold of Beta Acids and Tetrahydroiso-α-Acids in Light Clover Honey

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3.1 Abstract:

The detection thresholds of two hop-derived compounds, beta acids and tetrahydroiso-α-acids, were determined in light clover honey. In order to determine the group threshold of beta acids and tetrahydroiso-α-acids in honey, we used a semi-trained panel of 17 tasters and followed the ASTM 1432 protocol for threshold calculations. The calculated concentration whereby 50% of the sample population could not taste beta acids and tetrahydroiso-α-acids in honey was 5.6 mg/L and 13.3 mg/L respectively. Because there is currently no standard method for the analysis and quantification of hops acids in honey, we modified procedures for the extraction of hop acids from beer. This modified method was verified over the range of beta and tetra acid concentrations used for this experiment and was implemented in analysis of our samples.

3.2 Introduction

It is estimated that about 90 crops, accounting for one-third of the United States diet, rely on pollination by honeybees (*15, 70*). So many bees are required to pollinate these crops that an entire industry has developed with the sole purpose of transporting millions of beehives around the country to pollinate these crops. Transporting beehives in this manner has lead to an increase in the transmission of pathogens and parasites among domestic honeybee species (*15*). One parasite that has been troublesome to beekeepers is the Varroa mite, which was first detected in the United States in the mid-

1980's and has since spread nation-wide (*15, 71*). It has been hypothesized that these mites play a part in colony collapse disorder—a phenomenon where entire hives of bees suddenly and inexplicably disappear, destroying hive infrastructure (*15, 70*). Varroa mites feed on and weaken developing bee larva, and bees, attaching themselves between the abdominal folds of a bee's thorax (*72*). Currently, EPA approved strips impregnated with fluvalinate, and strips impregnated with coumaphos, are used for Varroa mite control (*72*). However, mites are becoming resistant to these organophosphate pesticides, and there is some health concern about the possibility of pesticide transfer to honey (*72, 73*). It has been discovered that both beta acids (beta) and tetrahydroiso-α-acids (tetra) act as effective pesticides against the Varroa mite (*14*). Hops acids have been declared a GRAS substance by the FDA, and thus, any residual hops acids that may be transferred to honey by the bees will pose no threat to the health and safety of honey consumers. However, these hop acids taste bitter, and may pose a consumer acceptance problem if residual levels in honey are detectable. Determining the detection thresholds of beta and tetra was the primary objective of this research. The secondary objective of this research was to develop a procedure to extract beta and tetra from honey and quantify these acids using high performance liquid chromatography, both to verify the levels of these acids dosed into honey for this research, and to allow for quantification of these acids in honey that will be sold to consumers should these acids be used by beekeepers to control Varroa mites.

3.3 Experimental

3.3.1 Honey

Raw light clover honey used in this experiment was purchased from Queen Bee Apiarie (Corvallis, Oregon) in three to five gallon buckets. All buckets of honey were heated in a steam kettle and mixed to ensure homogeneity of honey samples. The final mixed honey was found to be 82.3 brix using a multiscale automatic refractometer (Bellingham and Stanley Inc.) at 25 °C. The honey was found to have a density of 1.46 g/ml using a digital densitometer (Mettler Toledo DE45).

3.3.2 Tetrahydroiso-α acid Sample Preparation

The tetrahydroiso-α-acid (tetra) solution used in this experiment (John I. Haas Hops Company, Yakima, WA) was found to be 11.6% (wt/wt) tetra using spectrophotmetry (*66*) and 10 % tetra using HPLC under conditions outlined in the ASBC standard method for analysis of isomerized hop extracts by HPLC (*67*). This solution was dosed into light clover honey to achieve a concentration of 101.2 mg/L by HPLC analysis, which was the highest concentration to be tested in this study. This initial concentration was serially diluted with raw clover honey by a factor of 1.5 to produce all 11 levels used in this experiment ranging from 1.8 mg/L to 101.2 mg/L.

3.3.3 Tetrahydroiso-α-acid Extraction and Quantification

A 2.91 g aliquot of honey dosed with tetra acids was added to 7 ml of acidified water (99.9: 0.1 milli-Q water:phosphoric acid (v/v)), and heated in a warm water bath (40 °C) to facilitate mixing. The fully-dissolved sample was drawn through an SPE LC-8 column (Supelco, Bellefonte, PA) that had been pre-conditioned with 2 ml of methanol followed by 2 ml of milli-Q water to remove any residues. The sample was then rinsed 3 times with 2 ml aliquots of acidified water, and a 2 ml rinse of an acidified 50:50 methanol and water solution (49.9:49.9:0.2 methanol:milli-Q water:phosphoric acid (v/v/v)) to elute polar and weakly non-polar compounds from the column while retaining non-polar tetra acids. These eluates were discarded. The tetra acids were eluted from the SPE column with 3, 0.6 ml aliquots of acidified methanol (99.9: 0.1 methanol: phosphoric acid (v/v)) into a 2 ml volumetric flask, brought to volume, and mixed well before quantification on an HPLC. Each preparation of tetra acids extracted from honey was verified by HPLC using the American Society of Brewing Chemists (ASBC) standard method (*67)*. ASBC International Calibration Extract ICS-T2, was used as the standard for all tetra measurements.

3.3.4 Beta Acid Sample Preparation

The solution of beta acids (beta) used in this experiment was 10% beta acid by weight, and was provided by John I. Haas Hops Company (Yakima, WA). This solution

was dosed into light clover honey to yield a solution that was 140 mg/L, which was the highest concentration to be tested in this study. This initial concentration was serially diluted with honey by a factor of 2 to produce all 11 levels used in this experiment ranging from 0.14 mg/L to 140 mg/L.

3.3.5 Beta-acid Extraction and quantification

A 2.91 g aliquot of honey dosed with beta acids was dissolved in 7 ml of a 50:50 water:methanol (v/v) solution using a warm water bath (40 °C) to facilitate mixing. Beta is non-polar and will not dissolve in water alone. The extraction of the fully-dissolved sample of beta acids on an SPE LC-8 column (Supelco, Bellefonte, PA) was carried out the in the same fashion as the tetra acid extraction except that this sample was not rinsed with acidified water. The lack of solubility of beta acids in water would cause the acids to salt out of solution, which would prevent them from associating with the matrix of the SPE LC-8 column (20μm polyethylene frit). If the concentration of beta acids in the sample was expected to be less than 20 mg/L, a greater mass of honey (up to 833 g) was extracted such that the concentration of beta acids in the final acidified methanol elution was at least 20 mg/L. This volume of honey was diluted 2:1 solvent:honey with 50:50 methanol and water, and extracted using the procedure that was outlined for the 2.91 g sample of honey. In instances where more than 58 g of honey were required to concentrate beta acids to 20 mg/L, the sample was diluted 2:1 solvent:honey with 50:50

methanol and water, and filtered with a 0.7 μm Whatman® Glass Microfibre Filter to remove particulates. The glass filter was rinsed with acidified methanol to elute any remaining beta acids. The entire filtered sample was run through an SPE column and quantified by HPLC (*67*).

The concentration of each preparation of beta acids extracted from honey was verified by HPLC using a Discovery C18 column. Samples were analyzed at 314 nm with an isocratic mobile phase consisting of an 85:17:1 ratio (v/v/v) of methanol, water and phosphoric acid respectively at a flow rate of 1.0 ml/min and a temperature of 40 ºC. ASBC International Calibration Extract ICE-2 (24.94% beta), was used as the standard for all measurements (*67*).

3.3.6 Threshold Measurement

The human sensory panel for this threshold study was comprised of 17 volunteers from the Corvallis, Oregon community (9 females and 8 males) between the ages of 22 years and 35 years. All panelists participated in 4, 1-hour training sessions in order to introduce the panelists to the 3-alternative forced choice test (3-AFC) format, to determine proper testing levels for each panelist, and to familiarize the panelists with both the control and the test compounds.

The group and individual beta and tetra thresholds of the panelists were determined using ASTM method 1432 (*45*). Each panelist took part in 6 tasting sessions

for both tetra and beta acids for a total of 12 tasting sessions. Each of the 6 sessions consisted of 7, 3 alternative forced choice (3-AFC) presentations of increasing intensity for a total of 42 forced-choice presentations per compound for each of the 17 panelists, and 714 total observations per compound. The odd sample in each 3-AFC presentation was always the sample of honey dosed with either tetra in the first experiment, or beta acids in the second experiment.

In both studies panelists were given a sample of plain honey that had not been dosed with tetra or beta (control), and a "warm-up" sample dosed with either tetra or beta acid in the first and second studies respectively to re-familiarize panelists with the flavor of the dosed sample before each tasting session. The dosed warm-up sample was the second-highest level the panelist would taste during his or her test. All samples of honey were served at room temperature. Panelists were instructed to circle the 3-digit code corresponding to the sample they believed to be the most bitter in each 3-AFC presentation. After each 3-AFC presentation, panelists rinsed with a 1% pectin solution and with water, and waited 3 minutes to prevent adaptation and/or bitterness carryover from one sample to the next.

3.4 Results

3.4.1 Threshold Analysis

Each panelist's data were plotted as the percent of correct choices at the log of the concentration the panelist tasted (Figure 3.1). A 4-parameter sigmoidal equation was fitted to the data (*45*).

$$y = y_0 + \frac{a}{1 + e^{\frac{(-x - x_0)}{b}}}$$

For this equation, x represents the log of the concentration of beta or tetra, y represents the percent of correct choices for a panelist, y_o is the minimum value of y, a is the range of concentrations over which the response occurs ($y - y_o$), x_o is the log concentration of hops compound at one-half a, and b is the slope of the line at x_o. Each individual panelist's threshold was determined as the concentration at which the panelist correctly chose the dosed sample 66% of the time (i.e., 50% above chance). Panelists 9 and 17 only participated in the beta study. All other panelists participated in both the beta and tetra studies.

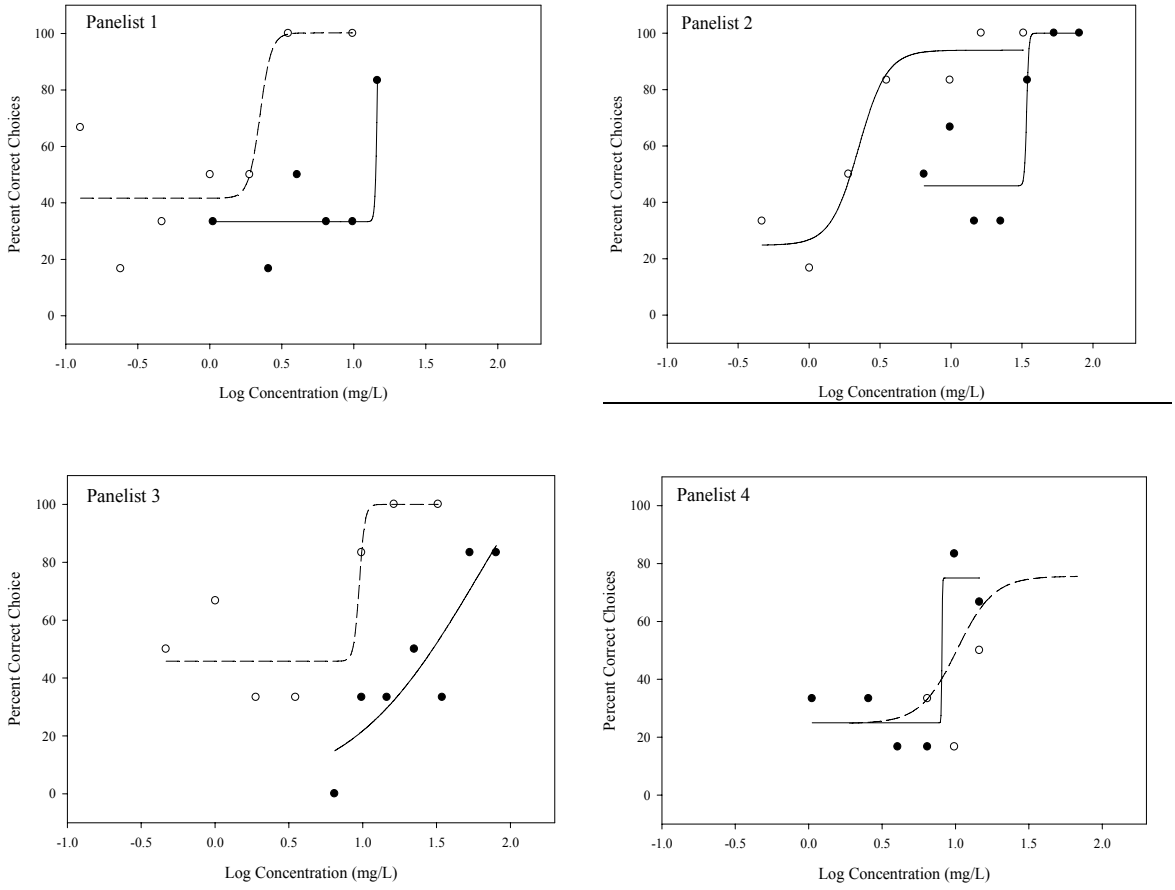


Figure 3.1 continued on pages 65-68

Figure 3.1 continued

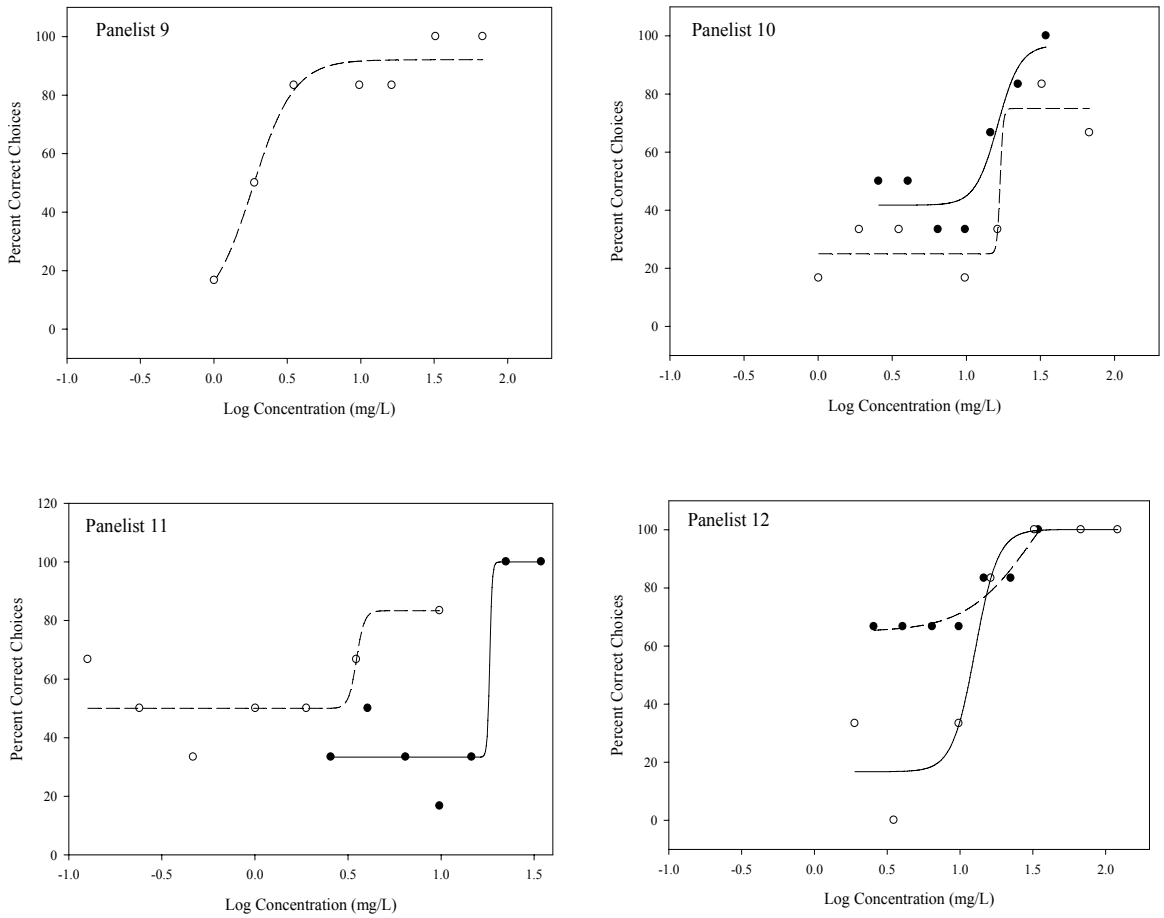
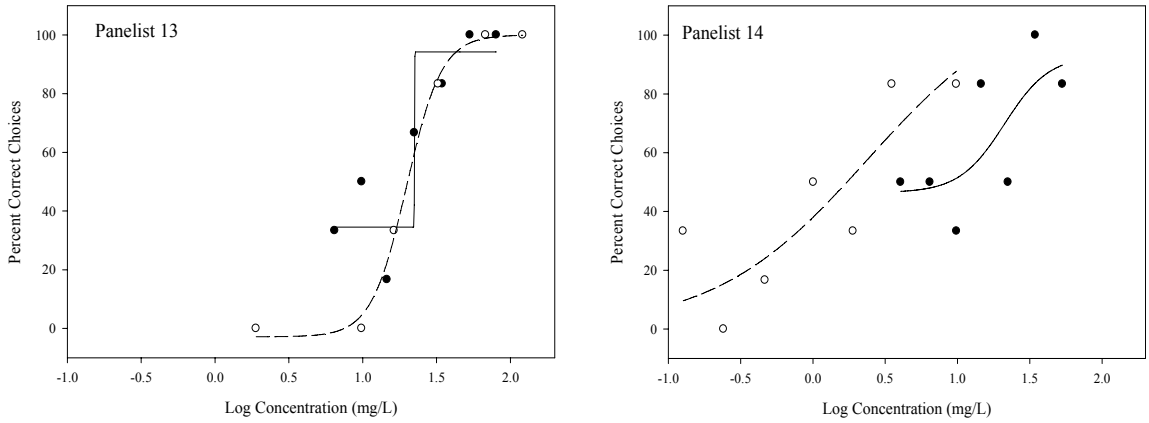


Figure 3.1 continued



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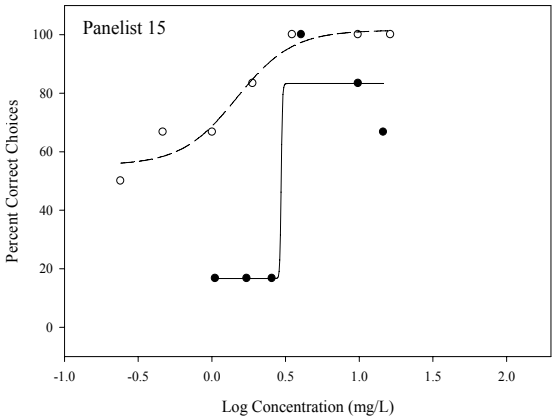

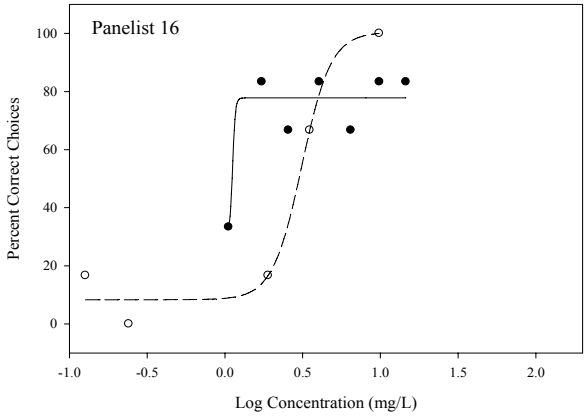

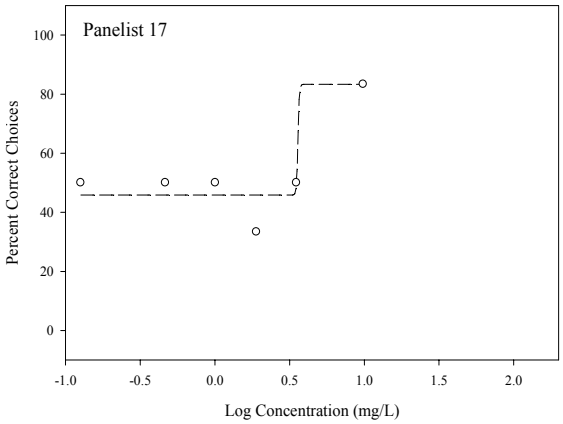

Figure 3.1: Individual panelists' dose-response data from a series of 3 AFC presentations for beta and tetra in honey where the plot with open circles (○) and a dashed line (---) refers to beta data, and the plot with closed circles (●) and a solid line (──) refers to tetra data.

Individual beta and tetra thresholds were calculated for each panelist who adequately completed the trial (table 3.1). In order to calculate the group thresholds for tetra and beta, the log values of the panelists' thresholds were ranked from lowest to highest, and were plotted against the panelists' rank position (figure 3.2). The rank position was determined as being equal to $100\ i\ /\ n + 1$, where *i* represents the numerical rank of the panelist in relation to the group, and *n* represents the number of panelists in the group (*45*). A linear regression was fitted to the data, and the group threshold was determined as the concentration where rank position is equal to 50% with one standard deviation limits occurring when panelist rank position equals 84% and 16% (+/-34%). (*45*). The group threshold of beta acids in honey was calculated to be 5.80 mg/L with a range of standard deviation from 1.89 mg/L to 17.8 mg/L. The group threshold of tetra acids in honey was calculated to be 16.3 mg/L with a range of standard deviation from 3.54 mg/L to 50.86 mg/L.

Table 3.1 Beta and tetra individual and group threshold results

Panelist	Threshold of Tetra (mg/L)	Threshold of Beta (mg/L)
1	14	2
2	34	3
3	48	9
4	8	16
5	20	3
6	26	4
7	6	6
8	53	8
9		3
10	16	18
11	18	4
12	5	14
13	22	24
14	19	4
15	4	3
16	1	4
17		4
Geometric mean	**13**	**5**

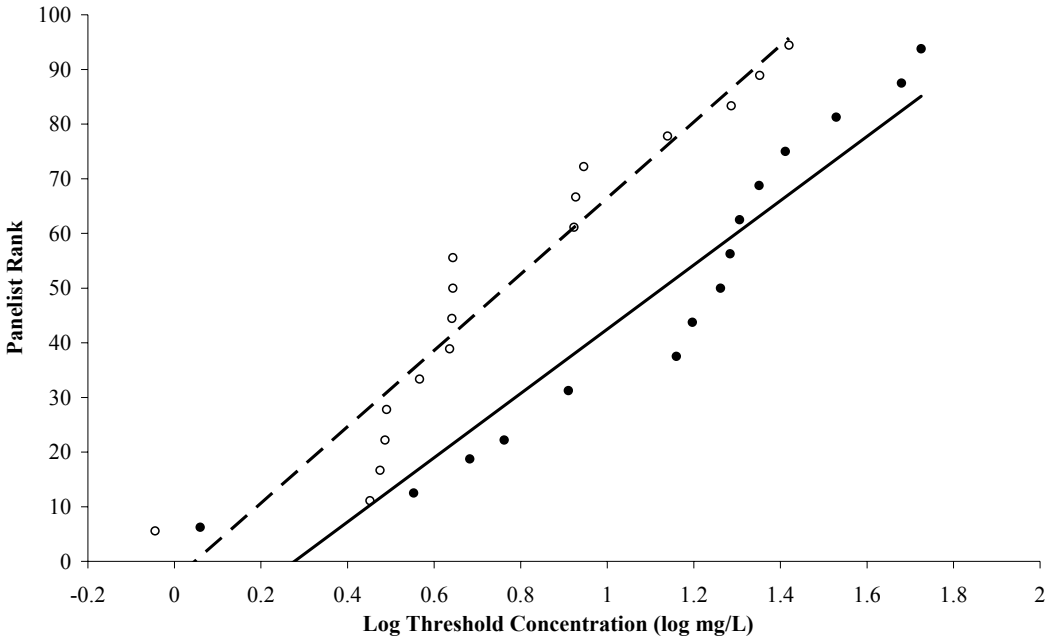

Figure 3.2: A group threshold rank-probability graph of tetra threshold data (●) and beta threshold data (○). A straight line fit to the beta data has an r^2 value of 0.912. A straight line fit to the tetra data has an r^2 value of 0.899.

3.4.2 HPLC Quantification of Hops Acids in Honey

The purpose of this research was to find the taste threshold of both beta and tetra acids in honey, and this required the extraction of beta acids and tetra from honey. There is currently no standard method available for extraction and quantification of these compounds from honey, so a suitable quantification method was developed based on existing methodology of analyzing these compounds in wort and beer.

Each sample of honey dosed with either tetra or beta acids was sampled, extracted, and analyzed three times in three separate experiments. During the beta acid analysis, concentrations of hop acids ranging from 0.13 to 121 mg/L were analyzed with an average recovery of 92% (95% confidence interval from 73% recovery to 108% recovery) calculated as the recovered concentration relative to the predicted or calculated concentration (Table 3.2).

Table 3.2: Comparison of expected and measured concentrations of beta acids dosed into light clover honey

Expected Conc. (mg/L)	Avg. Conc. (mg/L) (1)	Coefficient of Variation (%) (2)	Recovery (Actual / Expected)	Dilution Factor
0.14	0.13	4.02	93%	1.9
0.27	0.24	1.94	88%	1.9
0.55	0.47	2.59	85%	2.2
1.09	1.01	14.17	92%	1.9
2.19	1.90	2.16	87%	1.9
4.38	3.52	10.24	80%	2.8
8.75	9.84	3.85	112%	1.7
17.50	16.31	11.59	93%	2.0
35.00	32.43	10.19	93%	2.1
70.00	67.77	6.33	97%	1.8
140.00	121.16	5.04	87%	
		Average % CV	**Average Recovery**	**Average Dilution Factor**
		6.6	**91.6%**	**2.0**

(1) Average concentration was determined from three independent extractions at each concentration.
(2) The repeatability of the replicated extractions at each concentration is presented via the coefficient of variation.

During the tetra analysis, concentrations of hop acids ranging from 1.4 to 80 mg/L were analyzed with an average recovery of 91% (95% confidence interval from 74% recovery to 108% recovery) calculated as the recovered concentration relative to the predicted or calculated concentration (Table 3.3). For tetra, the continually decreasing compound recoveries can be explained as a slight initial dosing error that was amplified by the serial dilution process.

Table 3.3: Comparison of expected and measured concentrations of tetra acids dosed into light clover honey

Expected Conc. (mg/L)	Avg. Conc. (mg/L) (1)	Coefficient of Variation (%) (2)	Recovery (Actual / Expected)	Dilution Factor
1.39	1.06	7.32	76%	1.63
2.08	1.73	3.24	83%	1.48
3.12	2.56	5.83	82%	1.58
4.68	4.05	5.81	86%	1.60
7.02	6.46	5.73	92%	1.52
10.53	9.85	6.62	94%	1.48
15.80	14.61	2.63	92%	1.53
23.70	22.39	2.86	94%	1.54
35.56	34.59	2.04	97%	1.54
53.33	53.22	3.87	100%	1.51
80.00	80.17	2.87	100%	
		Average % CV	**Average % Recovery**	**Average Dilution Factor**
		4.4	**90.7 %**	**1.5**

(1) Average concentration was determined from three independent extractions at each concentration.

(2) The repeatability of the replicated extractions at each concentration is presented via the coefficient of variation.

3.5 Discussion

Modified methods were created and verified for the quantification of both beta and tetra in honey. Beta acids were dosed into honey at a level of 140 mg/L and serially diluted by a factor of 2.0 to achieve concentrations ranging from 0.14 mg/L to 140 mg/L. These samples were extracted, and quantified using HPLC. The average recovery for beta acids was 92%, the average coefficient of variation was 6.6%, and the average dilution factor was 2.0, which was the expected dilution factor. Similarly, tetra acids were dosed into honey at a level of 80 mg/L and serially diluted by a factor of 1.5 to achieve concentrations ranging from 1.4 mg/L to 80 mg/L. The average recovery for tetra acids was 91%, the average coefficient of variation was 4.4%, and the average dilution factor was 1.5, which was the expected dilution factor. The slight decrease in recoveries over the series of tetra acids is likely due to amplification of an early dosing error. Achieving the expected average dilution factors with high recoveries and small coefficients of variation demonstrates that the results found using the modified methods for beta and tetra acid extraction and quantification are valid. These modified methods were used to quantify the concentrations of beta and tetra that were used to find the group thresholds of these compounds in honey samples.

Group detection thresholds of beta and tetra acids in honey were found to be 5.6 mg/L and 13.3 mg/L respectively. A literature value for the threshold of beta acids has not been previously published. The threshold for tetra acid in beer has been found to be 8.5 mg/L by Weiss et al, and 2.7 mg/L in the research outlined in Chapter 2 of this thesis

(*47*). Both threshold values for tetra in beer are lower than the threshold for these acids in honey, however it is understood that sweetness suppresses the perception of bitterness (*27*). In this case, the sweetness of honey suppressing the bitterness of tetra acids would increase the threshold of tetra acids in honey over the thresholds of tetras in less sweet substances like beer.

Panelists described the tetra acids in honey as being distinctly and cleanly bitter while the beta acids in honey were described as "tasting like hops." Beta acids are not commonly extracted from hops for use in brewing. Beta acid extracts can contain aromatic oils and resins that smell distinctly like hops, but more highly refined, unscented versions are available. This experiment utilized an extract that contained some of these aromatic compounds, which may have lead to a lower threshold of beta acids in honey. Further research should focus on whether an unscented version of beta acids in honey would have a higher detection threshold. A semi-trained panel was used for this experiment, which may have also influenced the group threshold values for these compounds. The use of an untrained consumer panel instead of a trained panel could increase the detection threshold up to 1000-fold (*45*).

It is also important to note and understand the difference between a detection threshold (which we measured) and a consumer rejection threshold (i.e. that level of added substance that would render the base product unacceptable). More research would be required to determine the consumer rejection threshold of beta and tetra acids in honey.

General Conclusions

The first experiment outlined in this thesis was carried out in order to find the taste thresholds of iso-α-acids, tetrahydroiso-α-acids in beer. Current thresholds for iso and tetra have been found using ASTM method 679 (*43, 46, 58*). In the 1980's ASTM method 679 was found to have many statistical shortcomings, and an updated version, ASTM method 1432, was introduced. ASTM method 679 relegated to "rapid method" status (*45*). To date, human threshold measurements of iso or tetra in beer using ASTM method 1432 have not been published. In the research presented in this thesis, human taste thresholds of iso-α-acids (iso) and tetrahydro-iso-α-acids (tetra) were measured in an unhopped lager beer matrix using the ASTM method 1432 protocol. The group-wise detection thresholds and standard deviations of thresholds for iso and tetra were 7.1 mg/L (4.5-11.2 mg/L) and 2.7 mg/L (0.7-10.0 mg/L), respectively.

The ASTM method 1432 group threshold for iso in this experiment was found to be in agreement with threshold range for iso previously determined to be 6-15 mg/L by Meilgaard et al using ASTM method 679 (*46*). Group thresholds for tetra found in this experiment were lower than 8.5 mg/L expressed in research by Weiss et al, with the possible exception of the ASTM method 679 threshold from day 1 (5.4 mg/L) (*47*). The panelists used for the present research appeared to have an initial, short learning period when they tasted tetra that lasted longer than the learning period for the iso experiment. This demonstrates that tetra is more difficult to recognize initially, and panelists require

even more training than was provided in this experiment to accurately identify tetra in beer. Implementation of ASTM method 679 for determining tetra thresholds could yield misleadingly high outcomes.

In this research, ASTM method 679 repeatedly provided a group threshold that was not found to be statistically different than the threshold found using ASTM method 1432 for both tetra and iso. However, even though no individual was found to have an ASTM method 679 threshold that was significantly different than his or her ASTM 1432 threshold, it must be noted that ASTM method 679 can and did provide a wide range of individual thresholds. ASTM method 679 is an acceptable method of determining group thresholds. However, if individual threshold values are required, one should use the longer, more statistically strong ASTM method 1432.

The second experiment outlined in this thesis was carried out for the purpose of finding the thresholds of beta acids and tetrahydroiso-α-acids in honey. These acids have been found to be miticidal to Varroa mites, parasites that have been troublesome to beekeepers. It has been hypothesized that these mites play a part in colony collapse disorder—a phenomenon where entire hives of bees suddenly and inexplicably disappear, destroying hive infrastructure (*15, 70*). Hops acids have been declared a GRAS substance by the FDA, and thus, any residual hops acids that may be transferred to honey by the bees will pose no threat to the health and safety of honey consumers. However,

these hop acids taste bitter, and may pose a consumer acceptance problem if residual levels in honey are detectable.

The detection thresholds of beta acids and tetrahydroiso-α-acids, in light clover honey were determined using ASTM 1432 protocol for threshold calculations. The calculated concentrations whereby 50% of the sample population could not taste beta acids and tetrahydroiso-α-acids in honey were 5.6 mg/L and 13.3 mg/L respectively. A literature value for the threshold of beta acids has not been previously published. The threshold for tetra acid in beer has been found to be 8.5 mg/L by Weiss et al, and 2.7 mg/L in the research outlined in Chapter 2 of this thesis (*47*). Both threshold values for tetra in beer are lower than the threshold for these acids in honey, however it is understood that sweetness suppresses the perception of bitterness (*27*). In this case, the sweetness of honey suppressing the bitterness of tetra acids would increase the threshold of tetra acids in honey over the thresholds of tetras in less sweet substances like beer.

Because there is currently no standard method for the analysis and quantification of hops acids in honey, we modified procedures for the extraction of hop acids from beer and implemented these modifications in our analysis. These modified methods were verified and used for the quantification of both beta and tetra in honey.

Panelists described the tetra acids in honey as being distinctly and cleanly bitter while the beta acids in honey were described as "tasting like hops." This experiment utilized a beta acid extract that contained aromatic hop compounds, which may have lead to a lower threshold of beta acids in honey. Further research should focus on whether an

unscented version of beta acids in honey would have a higher detection threshold. It is also important to note and understand the difference between a detection threshold (which we measured) and a consumer rejection threshold (i.e. that level of added substance that would render the base product unacceptable). Finding a detection threshold only answers the question, "at what concentration is the substance detectable in a given medium?" One would expect that this detection threshold would be lower than a consumer rejection threshold, which is the concentration where a given product is no longer acceptable to the public. More research would be required to determine the consumer rejection threshold of beta and tetra acids in honey.

Future Research

The research performed for this thesis has generated various topics worthy of future and separate research. Further investigation into these areas would complement the subject matter included in this thesis.

- The threshold for an unscented version of beta acids should be found in honey. The aromatic compounds in the scented version of beta acids have likely depressed the threshold described in this research.

- Consumer rejection thresholds should be determined for both beta acids and tetrahydroiso-α-acids in honey to learn at what concentration the hops acids cause the honey to be unacceptable to consumers.

- Further research regarding the repeatability of both individual and group thresholds found using ASTM method 1432 should be performed. Understanding the repeatability of this method would help elucidate the strength and repeatability of ASTM method 679.

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