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Aroma of Alcohol-Free Beer and the Influence of Dry-Hopping as a Possibility to Enhance Consumers' Acceptance

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“Ganz besonders wollen wir, daß forthin allenthalben in unseren Städten, Märkten und auf dem Lande zu keinem Bier mehr Stücke als allein Gersten, Hopfen und Wasser verwendet und gebraucht werden sollen.“

Bayrisches Reinheitsgebot

Wilhelm IV. – 1516

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Contents

Abbreviations

AEDA	Aroma extract dilution analysis
AF	Acidic fraction
AW	Aromawert
CI	Chemical ionization
EI	Electronical ionization
FD	Flavor dilution
FID	Flame ionization detector
GC	Gas chromatography
GC-O	Gas chromatography-olfactometry
HCA	Hallertauer Cascade
HHA	Hallertauer Mittelfrüh
HMB	Hallertauer Mandarina Bavaria
HRGC	High-resolution gas chromatography
KFO	Key food odorants
KFT	Key food tastants
LOD	Limit of detection
LOQ	Limit of quantitation
MS	Mass spectrometry
OAV	Odor activity value
OR	Odor receptor
SAFE	Solvent assisted flavor evaporation
SIDA	Stable isotope dilution analysis
SPME	Solid phase microextraction
TOF	Time-of-Flight

1 Summary

While the total consumption of beer decreased, alcohol-free beer was getting more and more popular during the past years. Furthermore, the number of micro-breweries is increasing, due to the craft beer trend. As every dealcoholization method produces beers with some typical aroma deficits, dry-hopping and special flavor hop varieties could be an opportunity to mask or compensate these off-notes in alcohol-free beer.

As, up to now, there have not been a lot of studies on dry-hopping of alcohol-free beers, the behavior of different hop varieties in this matrix, and the influence of different dealcoholization methods on the aroma composition of the final product, this study aimed to clarify the behavior of key odorants in alcohol-free beer regarding their stability, formation, degradation, or loss during dealcoholization using a thermal approach and a stopped fermentation method. Furthermore, the transfer of important hop odorants was analyzed during dry-hopping.

To get an overview over different hop varieties, a traditional Hallertauer Mittelfrüh, an aroma variety Hallertauer Cascade, and a special flavor hop Hallertauer Mandarina Bavaria were analyzed. As a basis for the following investigations, the key odorants of these three hop varieties were characterized using the molecular sensory science concept and revealed 41 aroma-active compounds of which 31 were successfully identified. The highest odor activity values (OAVs) were determined for linalool and myrcene, followed by geraniol and 3-methylbutanoic acid.

To get an insight into the changes of the aroma composition of beers during the dealcoholization, key odorants were quantitated before and after stopped fermentation or thermal dealcoholization. During the heat treatment, high losses of most of the key odorants like 2-phenylethanol or 3-methylbutyl acetate, resulting in OAVs <1, were observed, whereas during stopped fermentation odorants like ethyl butanoate and 2-phenylethanol reached OAVs >1, but clearly lower as in the corresponding alcoholic samples.

After quantitation of the most important hop odorants in different alcohol-free beer samples, it became clear that the initial idea of enhancing the hoppy aroma of alcohol-free beer by dry-hopping can be confirmed, as linalool, geraniol, and myrcene showed high transfer rates from hops into alcohol-free beer between 20% and 90%, comparable to the transfer into alcoholic beer. Furthermore, a formation of fruity

smelling esters was observed. This formation could be reproduced in model system experiments, showing other hop-derived esters like 2-methylbutyl methylpropanoate or methylpropyl methylpropanoate as possible precursors for a transesterification to the respective ethyl esters. By evaluating the transfer and formation of odorants during dry-hopping over time, this ester formation was shown as an exponential process, starting at day four of dry-hopping. Other odorants, namely, linalool, geraniol, myrcene, and propyl 2-methylbutanoate reached their maximal concentrations already in the first three days.

Further sensory investigations showed, that for an increase of the fruity aroma note, the contact time during dry-hopping can be extended, whereas for a stronger hoppy, citrus-like odor, the hop dosage must be increased. In case of linalool, too high concentrations showed a negative influence on the aroma profile, as the odor quality changed to an unpleasant soapy impression. Nevertheless, the contact time of seven days used in this study already showed the potential of dry-hopping for alcohol-free beers, as clear sensorial and analytical differences were observed in samples before and after dry-hopping, and thus, the initial idea of enhancing the consumers' acceptance by dry-hopping of alcohol-free beers can be supported.

2 Zusammenfassung

Während der Gesamtbierkonsum in Deutschland weiter abnimmt, wurde alkoholfreies Bier in den letzten Jahren immer beliebter. Außerdem steigt die Zahl sogenannter Mikrobrauereien aufgrund des Craft Beer Trends auch hierzulande an. Da die verschiedenen Herstellungsmöglichkeiten alkoholfreier Bier immer auch zu typischen Aromafehlern führen, könnten die Kalthopfung und spezielle Flavor Hopfensorten eine Möglichkeit darstellen, diese Aromadefizite in alkoholfreiem Bier zu maskieren oder kompensieren.

Bisher wurden nur wenige Studien zu alkoholfreiem Bier, dem Einfluss von Hopfenaromastoffen auf diese Produktklasse und dem Einfluss verschiedener Entalkoholisierungsmethoden publiziert. Diese Arbeit soll daher Aufschluss über das Verhalten der Schlüsselaromastoffe in alkoholfreiem Bier bezüglich Stabilität, Bildung, Abbau und Verlust während der Entalkoholisierung geben. Dazu wurden ein thermischer Prozess und eine Methode mit gestoppter Gärung betrachtet. Außerdem wurde der Übergang wichtiger Hopfenaromastoffe während der Kalthopfung analysiert.

Um einen Überblick über verschiedene Hopfensorten zu gewinnen, wurden die traditionelle Sorte Hallertauer Mittelfrüh, die Aromasorte Hallertauer Cascade und die Flavorsorte Hallertauer Mandarina Bavaria untersucht. Als Grundlage für die folgenden Untersuchungen wurden die Schlüsselaromastoffe dieser drei Hopfensorten charakterisiert. Mithilfe des Konzepts der Molekularen Sensorik konnten 41 aromaaktive Substanzen detektiert und 31 von ihnen erfolgreich identifiziert werden. Die höchsten Aromawerte (AWs) wurde dabei für Linalool und Myrcen ermittelt, gefolgt von Geraniol und 3-Methylbuttersäure.

Um einen Einblick in die Veränderungen der Aromazusammensetzung der Biere während der Entalkoholisierung zu bekommen, wurden Schlüsselaromastoffe vor und nach der gestoppten Gärung beziehungsweise der thermischen Entalkoholisierung quantifiziert. Während der Hitzebehandlung traten hohe Verluste fast aller untersuchten Aromastoffe wie 2-Phenylethanol oder 3-Methylbutylacetat auf. Diese Aromastoffe zeigten nach der Entalkoholisierung AWs <1. Während der gestoppten Gärung hingegen wurden Aromastoffe wie Ethylbutanoat und 2-Phenylethanol in

Konzentrationen gebildet, die AWs >1 ergaben, jedoch deutlich niedriger waren als in vergleichbaren alkoholhaltigen Bieren.

Die Ergebnisse der Analysen der kaltgehopften Biere machen deutlich, dass die Eingangsfrage, ob das Hopfenaroma in alkoholfreien Bieren mittels Kalthopfung intensiviert werden kann, mit Ja beantwortet werden muss. Linalool, Geraniol und Myrcen zeigten Tranferraten von Hopfen in alkoholfreies Bier zwischen 20 und 90 %, vergleichbar mit den Übergängen in alkoholhaltigem Bier. Des Weiteren wurde eine Bildung verschiedener fruchtig riechender Ester beobachtet. Diese Esterbildung konnte in Modellexperimenten erfolgreich nachgestellt werden. Dabei konnten andere Ester im Hopfen wie 2-Methylbutyl-methylpropanoat oder Methylpropyl-methylpropanoat als potenzielle Vorläufer identifiziert werden. Indem die Transfer- und Bildungsraten der untersuchten Aromastoffe über die gesamte Kontaktzeit von sieben Tagen verfolgt wurden, konnte außerdem gezeigt werden, dass die Bildung der Ester ab dem vierten Kalthopfungstag exponentiell verläuft. Linalool, Myrcen, Geraniol und Propyl-2-methylbutanoat hingegen erreichten ihre maximale Konzentration im Bier bereits in den ersten drei Tagen.

Weitere sensorische Untersuchungen zeigten, dass durch eine Verlängerung der Kontaktzeit während der Kalthopfung das fruchtige Aroma verstärkt werden kann, wohingegen eine intensivere hopfige, zitronige Note nur durch die Erhöhung der Hopfendosage erreicht werden kann. Im Fall von Linalool zeigten zu hohe Konzentration einen negativen Einfluss auf das Aomaprofil, da sich die Geruchsqualität zu einem unangenehm seifigen Eindruck änderte. Nichtsdestotrotz zeigt die hier verwendete Methode mit einer Kontaktzeit von sieben Tagen bereits das Potential der Kalthopfung für alkoholfreies Bier. Da analytisch und sensorisch deutliche Unterschiede im Aromaprofil vor und nach der Kalthopfung nachgewiesen werden konnten, kann die Eingangshypothese unterstützt werden, ob die Verbraucherakzeptanz durch Kalthopfung von alkoholfreiem Bier erhöht werden kann.

3 Introduction

During evolution, the ability to detect smell had different functions, like the identification of danger,[1] the ability to locate and evaluate food,[2,3] or the interaction between insects.[4,5] For humans, smell is important to specify foods, evaluate their quality, or detect spoilage. Therefore, aroma is one of the most important quality attributes of food, deciding about the consumers' acceptance.

3.1 The Olfactory System

Humans can perceive odorants via different classes of odor receptors (ORs). The volatile substances can reach the olfactory epithelium either directly via an orthonasal inhalation or indirectly via a retronasal airflow from the mouth across the throat to the nose during food intake.[6] In 1991, G protein-coupled 7 transmembrane helix receptors were reported as the dominant receptor family expressed in the olfactory epithelium, that is responsible for the detection of odorants.[7] By activating the G protein, odorants initiate a secondary signal cascade including a calcium influx into the olfactory cell that leads to a neuronal signal (Figure 1).[8]

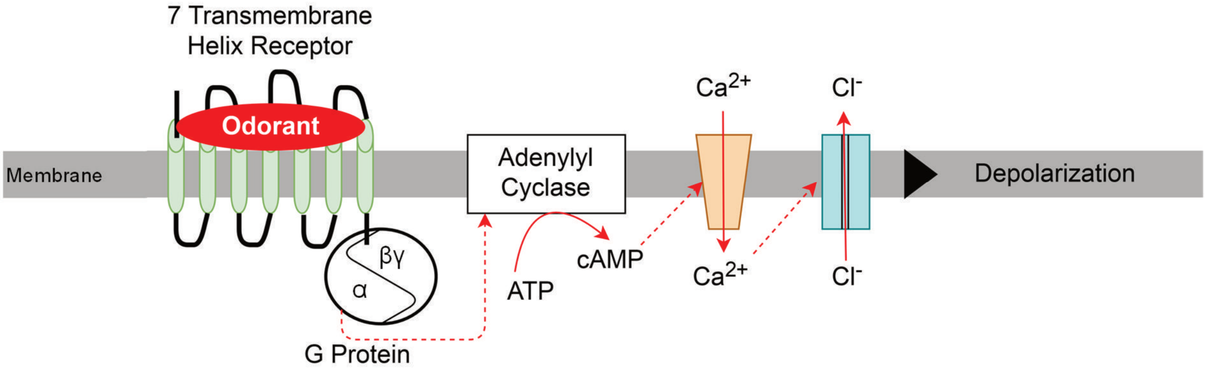

Figure 1: Signal Cascade of Odor Receptors (according to Krautwurst 2008[8])

Up to now, over 400 of these G protein-coupled receptors are known. One odorant can activate different ORs and one OR can be activated by different odorants. As one olfaction neuron expresses only one receptor type, all similar neurons merge in one glomerulus (bulbus olfactorius). The activation of receptors leads to a certain pattern of active globuli, that is processed to an odor quality in the brain.[6,8]

3.2 Aroma and Taste in Food

In the beginnings of flavor research, all volatiles that could be detected by gas chromatography were identified in different foods. Thus, over 8000 volatiles have been characterized so far.[9,10] But, by developing the molecular sensory science concept, the focus was shifted to the relevance of single volatiles to the overall aroma. The huge number of volatiles was reduced to only 3-40 key food odorants (KFOs) for one certain food aroma.[9,10] Within the non-volatile food constituents, 10-40 key food tastants (KFT) are needed for an authentic taste recombinate.[10] Of all known food volatiles, only 226 of them function as KFOs (based on the analysis of 227 food samples) (Figure 2).[9,10] Some of these KFOs are ubiquitous in almost all analyzed foods and called 'generalists', whereas the so-called 'specialists' are exclusive for one food or only a small number of foods.

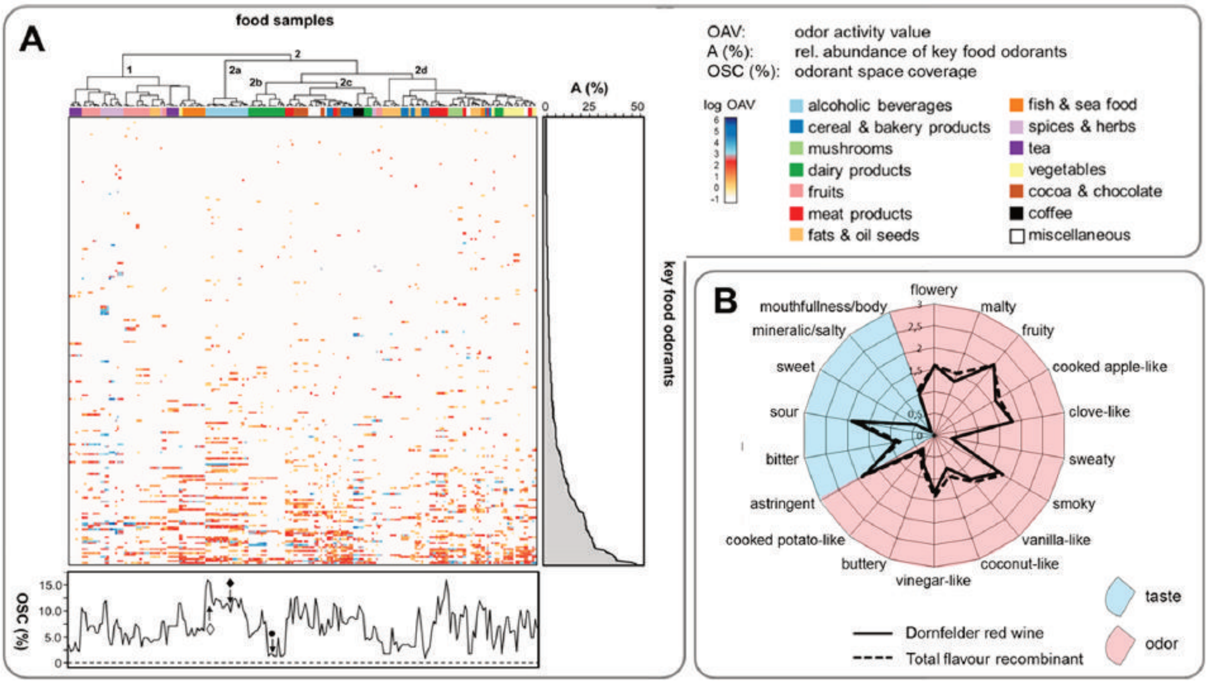

Figure 2: Key Food Odorants and Key Food Tastants in Flavor Research (reprinted (adapted) with permission from Hofmann et al.[10] © 2018 American Chemical Society) A: Odor activity values of 226 KFOs in 227 food samples, their abundance A (%) in foods, and their proportion OSC (%) of the total odor-active volatiles in the sample, highlighted: ◊ cognac (36KFOs), ♦ beer (18KFOs), and ● butter (3KFOs); B: Sensory profile of red wine and its recombinate (28 KFOs and 35 KFTs).

Beside the basic research on KFOs and KFTs of different foods, flavor research is also responsible to elucidate the reasons for an off-flavor formation or flavor deficits, but also to explore possibilities to increase the nutritional value of food without negative effects on its aroma and taste. Production processes can be optimized by tracing changes in the aroma composition and the formation of aroma- or taste-active substances released or generated from respective precursor molecules. Knowledge about the formation processes of undesired off-flavors can help to prevent their occurrence in food due to environmental factors, production processes, or other influences before, during, and after food production. In different juices, thermal treatment causes the formation of off-flavor compounds,[11,12] a steam treatment of rapeseed oil leads to a fishy aroma note caused by trimethylamine,[13] and a smoky odor of cocoa can influence the aroma of the final chocolate.[14] Other earlier studies identified odorants contributing to the off-flavors of boiled beef,[15] soybean oil,[15] milkfat,[15] and spinach.[16] For optimizing the development of a protein isolate from peas, different fatty acid derivates were identified as bitter off-tastants.[17]

Furthermore, analyzing the typical pattern of odorants and other volatile metabolites can also provide information on the authenticity of a food, for example on the botanical origin of honey[18] or the geographical origin of olive oils.[19]

3.3 Molecular Sensory Science Concept

The first step for the characterization of odorants in food and other samples is to extract them from their matrix without changing the composition of the volatile fraction. The development of the solvent assisted flavor evaporation (SAFE) technique in 1999[20] provided a gentle aroma extraction via high vacuum distillation with less degradation on the one side, and less artefact formation on the other side, compared to previous methods. This technique replaced a high-vacuum transfer method applied in flavor research in 1970 by Weurman et al.[21]

With the invention of gas chromatography-olfactometry (GC-O) the basis for the modern sensomics research was set. Fuller et al. published the first GC coupled with a human perfumer detector in 1964.[22] In the following years, the system was refined and also used for other applications like the research on insect pheromones.[23] Since 1986, GC-O is used to determine the so-called D-value (dilution value, today known as FD (flavor dilution) factor) of odorants in extracts of volatiles by sniffing dilution series of the sample (aroma extract dilution analysis, AEDA), to rank the aroma compounds according to their sensory importance.[24,25]

In 1966, Sweeley et al. used a stable isotopically labeled internal standard for the first time for the quantitation of glucose.[26] Nowadays, this method is known as stable isotope dilution assay (SIDA) and is applied to odorants, tastants, mycotoxins, and other food constituents.[27,28] First attempts to link the quantitation results with sensory data led to the definition of the odor activity value (OAV; ratio of odorant concentration to the respective odor threshold), to allow a direct comparison of odorants and their contribution of a single aroma-active compound to the overall aroma.[29]

On the basis of these milestones, the molecular sensory science concept could be developed as it is known today (Figure 3).[30,31]

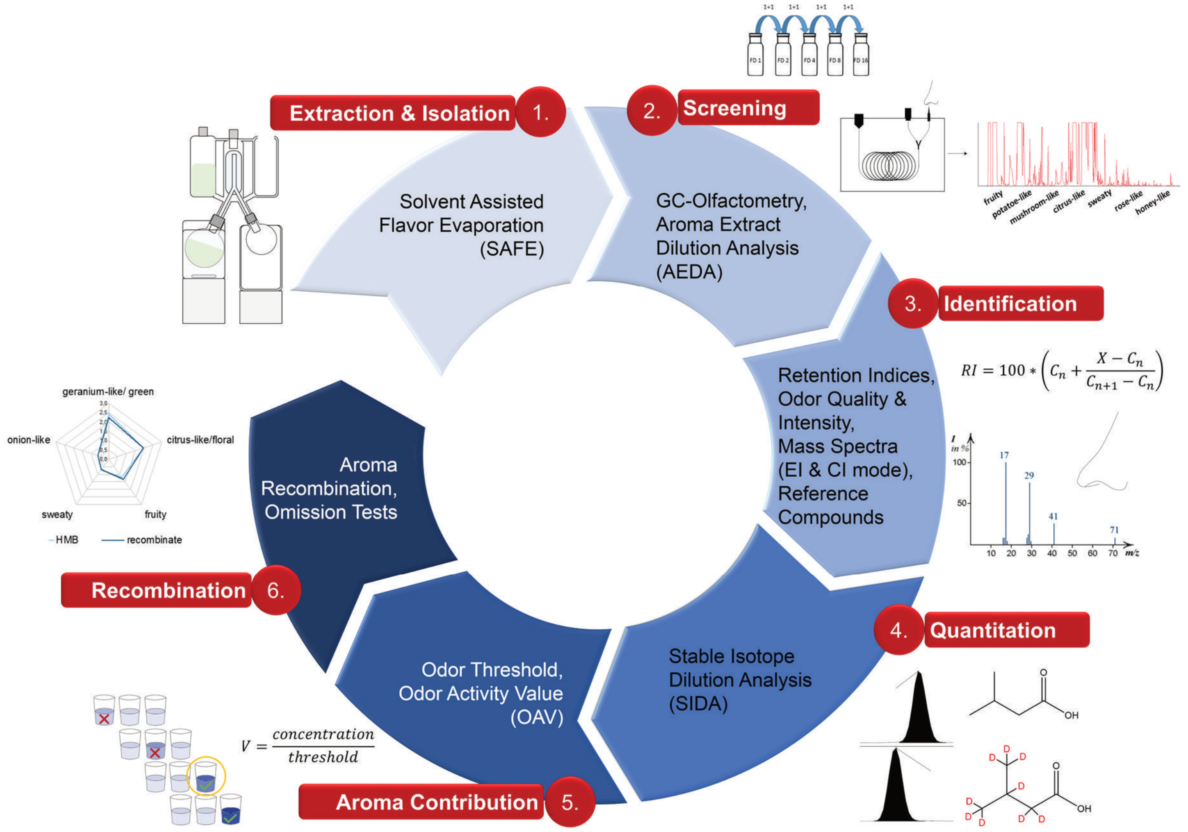

Figure 3: The Molecular Sensory Science Concept

This methodology allows the analysis of food odorants regarding its actual contribution to the aroma of the respective food. Whereas early aroma research focused on all volatiles in a sample, using GC coupled with mass spectrometry (MS), the additional usage of the human sense of smell as a detector during GC-O and the consideration of odor thresholds for the calculation of OAVs in the sensomics approach enables the identification of key food odorants.

For the isolation of volatiles, the SAFE technique provides a possibility of an extraction at low temperatures, and thus, with less thermal load and thermally induced artefact formation or degradation of thermo-labile odorants.[20] Under high vacuum, the aqueous sample or a solvent extract of a sample is distilled at app. 40 °C and the distillate is collected in a flask cooled with liquid nitrogen.

After extraction and isolation, GC-O (Figure 4) and AEDA can be used to screen the distillate for odor-active compounds and to locate them in the chromatogram.[32,33] Therefore, the sample is simultaneously detected by a flame ionization detector (FID) and the human nose at the sniffing port during GC-O. To estimate the importance of the single odorants for the overall aroma of a certain food, AEDA can be used. By a stepwise dilution of the sample (1+1, v+v), the FD factors are determined, defined as the last dilution, in which the odor is still perceivable at the sniffing port.

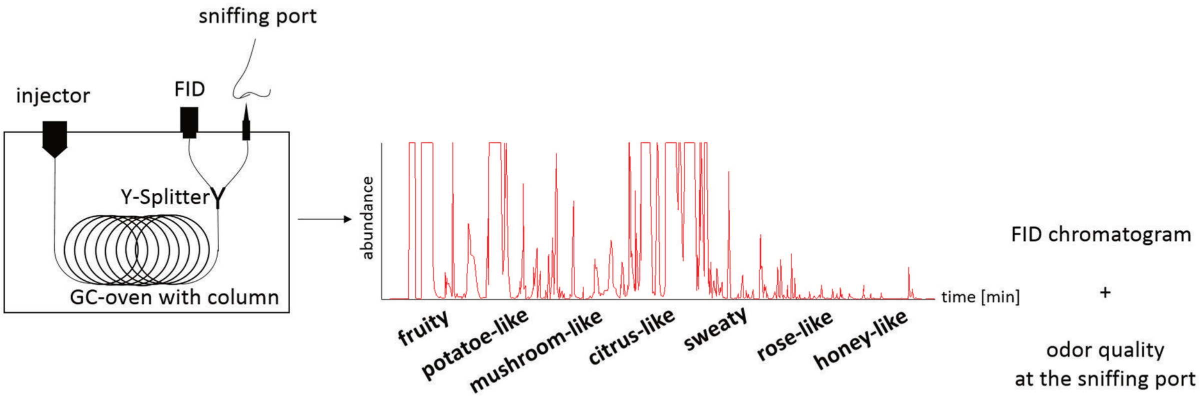

Figure 4: Principle of Gas Chromatography-Olfactometry (GC-O)

To identify the odorants in the sample, different criteria are used. Beside the retention indices on two columns of different polarities and mass spectra in electronical ionization (EI) and chemical ionization (CI) mode, also the odor quality and intensity perceived at the sniffing port are compared to the properties of authentic reference compounds.

The application of SIDAs to different foods was shown to produce quantitative results for odorants with the highest accuracy.[27,34,35] A stable isotopically labeled internal standard (**I**, Figure 5) is added to the sample at the beginning of the workup procedure. Thereby, losses during the sample workup are compensated due to the nearly identical physical and chemical properties of analyte and internal standard, whereas a structurally different internal standard (**II**, Figure 5) would not suffer the same changes and losses during workup.

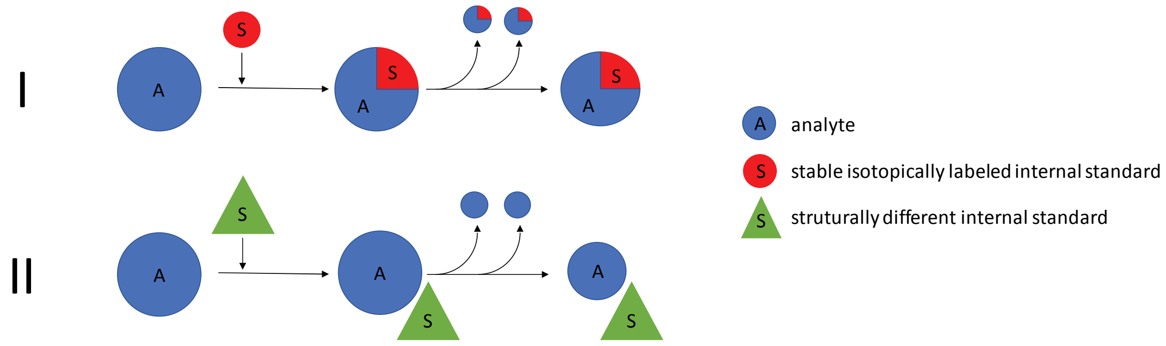

Figure 5: Stable Isotope Dilution Assay – Comparison of the Behavior of a Stable Isotopically Labeled and a Structurally Different Internal Standard During Sample Workup

Without taking sensory properties like orthonasal odor thresholds into consideration, the importance of an odorant to the overall aroma cannot be finally evaluated. Therefore, OAVs can be calculated as already explained. An OAV ≥ 1 means a direct contribution to the overall aroma of the food, as the concentration is above the respective odor threshold, while odorants with an OAV < 1 can only contribute due to additive or synergistic effects.

Afterwards, the results are verified by recombination experiments. The odorants, that have been identified as key food odorants (OAV ≥ 1), are mixed in their naturally occurring concentrations in a suitable odorless matrix. If the sensory comparison of the aroma profiles of the sample and the respective recombinate shows a high similarity, all key aroma compounds have been correctly identified and quantitated. If there is no similarity between the two aroma profiles, also odorants with OAVs < 1 should be taken into consideration to check possible additive and synergistic effects of these compounds.

In a last step, omission tests can be performed. By omitting single odorants from the recombination model (recombinate) and by comparing the aroma profiles (full recombinate vs. partial recombinate), the necessity of a single odorants to mimic the overall aroma can be verified.

3.4 Beer Brewing

Since centuries, fermented beverages based on different grains have been brewed, representing the ancestors of what we know as “beer” today. Nowadays, in Germany the production of beer is regulated by the German beer law (VorlBierG), including the purity law in § 9, allowing only water, malt, hops, and yeast as beer ingredients.

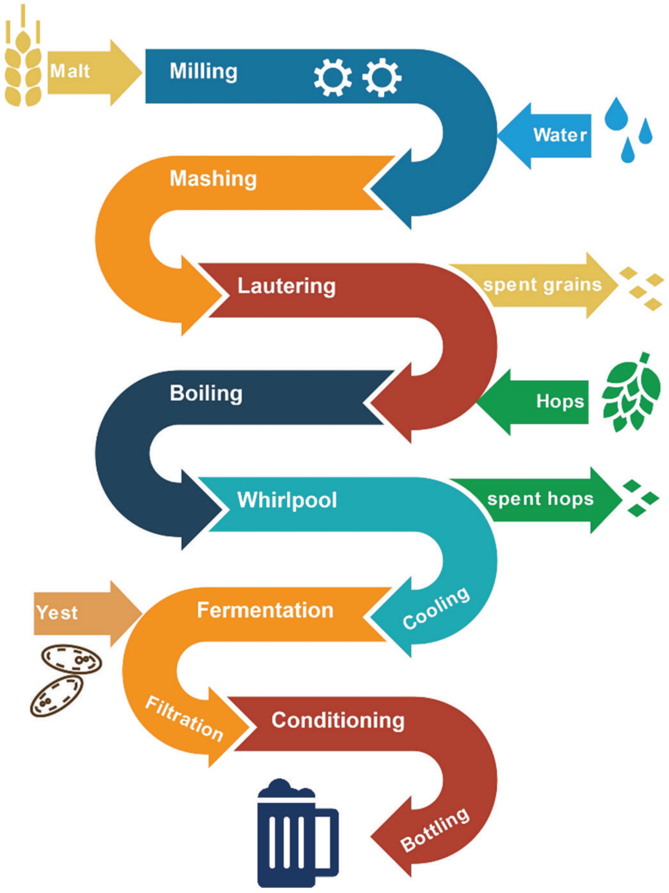

Figure 6: The Brewing Process

The brewing process (Figure 6) starts with the milling step. Afterwards, water is added to the malt. In this mashing step, insoluble malt ingredients are solubilized via physical, enzymatic, and chemical reactions. Thereby, starch is converted into fermentable sugars, mainly maltose, by amylases.[36] In the lauter tun, the remaining insoluble residue is separated from the liquid wort. During the boiling step, hops are added to the wort. By isomerization of hop α-acids into iso-α-acids, bitterness is added to the product. Also a formation of aroma and color substances due to Maillard reactions takes place during wort boiling, as well as an elimination of undesired odorants (e.g. diacetyl) via evaporation.[36] In the whirlpool, insoluble hop residues are removed, before applying the cooled wort to fermentation. By the addition of yeast, the wort is transferred into beer by the production of ethanol, CO_2, and other byproducts of the yeast metabolism such as odor-active compounds.[37] Therefore, either a top-fermenting or a bottom-fermenting yeast can be used. After fermentation, a cold storage at 0 °C is performed for approximately one month called conditioning or maturation. Meanwhile, trub substances can set down before filtration. After filtration, the product can be bottled or filled into barrels and is ready for consumption.

3.4.1 Hops

Already in 736, hops have been reported to be grown in the Hallertau in Germany.[39] In the beginning, they were mainly used because of their medical properties. Later, hops have been used in beer brewing for their bitterness, aroma compounds, and antioxidative activity. When brewing started to grow to a commercial scale, also hop breeding expanded in the 13th century.[39] The large German hop growing regions of today, Spalt, Hersbruck, and Hallertau, developed between the 16th and 19th century.[39]

Table 1: Chemical Composition of Hops (Dried Cones).[38]

component	amount [%]
cellulose	43
waxes, steroids	25
total resin	15-30
proteins	15
water	10
ash	8
polyphenols	4
pectin	2
sugars	2
essential oil	0.5-3
amino acids	0.1

Only unfertilized female plants of *Humulus Lupulus* L. develop hop cones. Therefore, they must be grown separately from male plants. Male plants, that carry quality-related genes, are used for breeding programs to develop new varieties. Thereby, the main goals of hop breeding are, beside an increased level of bitter acids or essential oil, the increase of yield, storage stability, or disease resistance.

All target compounds like aroma-active molecules and bitter acids are accumulated in the hop essential oil, that is located in the lupulin glands. To increase the stability of hop cones after harvesting, they are dried from a moisture content of 75-80% to 10%.[38] The chemical composition of these dried cones is shown in Table 1.

Hop resin consists of two main fractions: α- and β-acids (Figure 7). The bitterness of hops in beer is mainly based on the isomerization of α-acids into iso-α-acids during wort boiling.

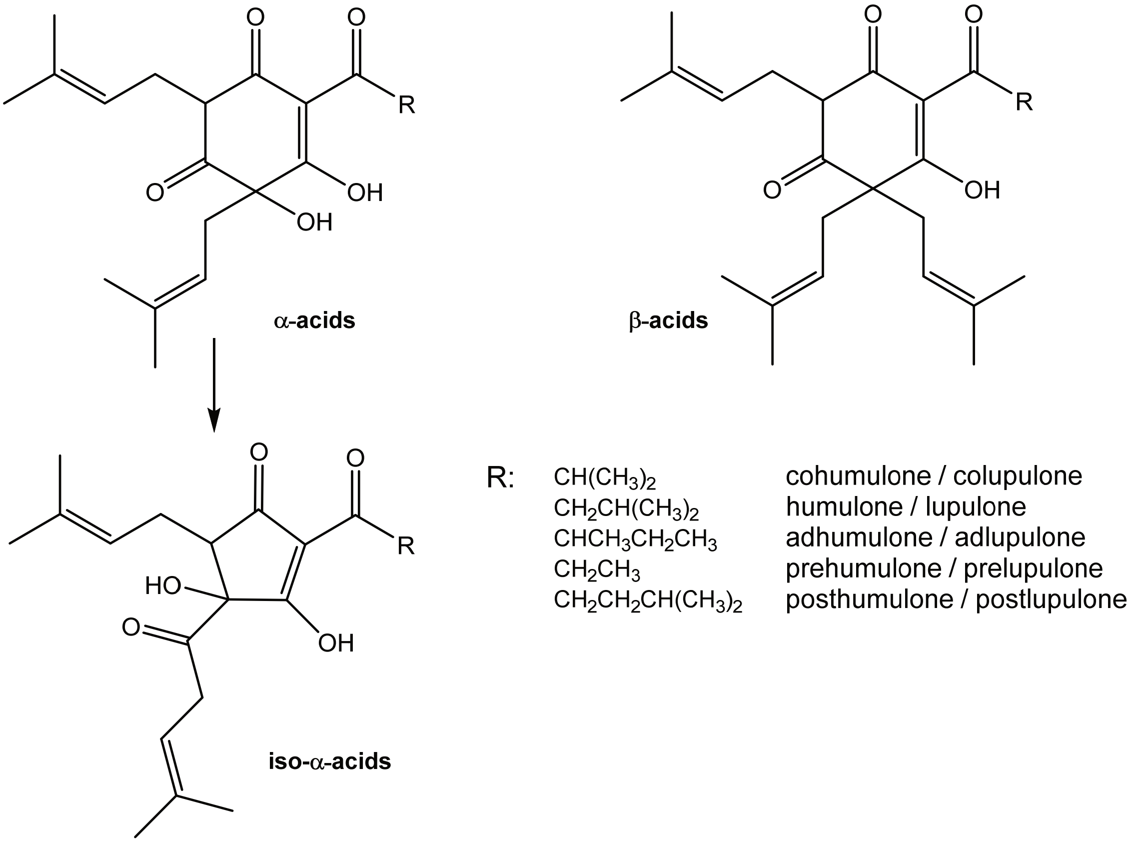

Figure 7: Structure of α-, iso-α-, and β-Acids (according to Ting and Ryder 2018[40])

The most important hop growing countries are Germany and the United States.[38] Most of the time, the main goal of hop cultivation was the increase of the α-acid content or a pronounced classical noble hoppy aroma. But in the past time, as dry-hopping was getting more and more important, new aroma and flavor hop varieties were bred. In 2006, the Hop Research Center Huell started new breeding programs with the aim of special fruity aroma varieties.[41] In the US and Australia, special aroma varieties are already available since the 1980s, like Amarillo, Citra, Simcoe (US), or Topaz, Stella, and Galaxy (Australia).

Until now, several hundred volatiles have been identified in hops in different studies using GC-FID, GC-O, or GC-MS.[42–47] In 1956, Howard applied GC to the identification of hop oil constituents for the first time and detected 18 compounds, e.g., myrcene and humulene.[48] GC is still the state-of-the-art analysis of hop essential oils with modern techniques like GC-time-of-flight-mass spectrometry (GC-ToF-MS). Roberts and Lewis (2002) identified 40 volatiles simultaneously in a fast approach with a run time of only 5 minutes.[45] By applying a comprehensive multidimensional GCxGC-ToF-MS > 100 hop volatiles were identified in one run, beside 1000 unidentified and not fully separated peaks.[49]

The composition of the essential oil of hops is dominated by terpenoid structures, such as monoterpenoids (e.g., myrcene, linalool, geraniol) and sesquiterpenoids (e.g., humulene, caryophyllene, farnesene) with up to 85%.[46] They are formed via the mevalonate (MVA; in the cytoplasm of eukaryotes) or methylerythritol phosphate (MEP; in plastids of plants) pathway. In all these studies about the volatile composition of hops, myrcene and humulene were the quantitatively most important components, but there has been no data about their sensory properties, and thus, about their actual contribution to the aroma of hops.

For the evaluation of the importance of each component for the overall aroma, odor thresholds and odor activity must be taken into consideration. Guadagni et al. (1966) ranked the identified molecules by calculating so-called odor units, referring to the odor threshold, resulting in myrcene as the most important odorant.[42] By applying AEDA to hop samples, important aroma components can be located due to their odor-activity (odor-intensity and odor-quality). Thereby, all odor-active compounds can be distinguished from the non-aroma-active volatiles (see chapter 3.3). This concept was applied to several hop products and varieties, determining FD factors or Charm values to estimate the importance of the odorants.[43,50–54] In all studies, myrcene and linalool were one of the most important odorants in hops followed by geraniol and 3-methylbutanoic acid.

Also, some varietal particularities have been identified. While high concentrations of ethyl 2-methylbutanoate, ethyl methylpropanoate, and propyl 2-methylbutanoate are specific for the variety Huell Melon, 3-methylbutyl acetate and 1,8-cineol are responsible for the typical aroma of Polaris hops.[52] 4-Mercapto-4-methylpentan-2-one is typical for different US varieties like Citra, Eureka, and Simcoe.[55,56] 4-Mercapto-4-methylpentan-2-one and other thiols were also detected in high concentrations in Nelson Sauvin hops.[57,58]

3.4.1.1 Hop Products

Beside the usage of whole hop cones, different hop products can be used during brewing. For a better extraction and storage stability, dried hops are ground and pressed into a pellet form. Today, over 40% of the annual hop harvest is processed to hop pellets.[39] Type 90 (90 kg pellets out of 100 kg raw hops) pellets show the same composition as raw hops, whereas type 45 pellets (45 kg pellets out of 100 kg raw hops) are enriched and standardized in their lupulin content.

Hop extracts can be gained via an extraction of hops with supercritical CO_2 or ethanol, which are both naturally occurring by-products during fermentation in the brewing process. Isomerized hop extracts or isomerized hop pellets are used for a better control of the bitterness in the final product, as iso-α-acids mainly contribute to the beer bitterness. By an isomerization under controlled conditions, the yield of iso-α-aids can be increased.[39] So-called downstream products are used after wort boiling.

To increase the hoppy aroma in beer, hop oil products can be used. They can be produced using supercritical CO_2 extraction or vacuum steam distillation.[39] Other special extracts are xanthohumol or tannin extracts, containing just specific fractions of the hop cones.

In Germany, the usage of hop pellets or hop extract, that are just concentrated but not modified in its content of hop ingredients, are in line with the German beer legislation. After wort boiling, only hop cones, pellets, or powder can be added to the beer (§ 9 VorlBierG).

3.4.2 Malt

Grains like barley, but also wheat, are used for beer brewing. Beside hops and yeast (fermentation), they are one of the most important factors influencing the aroma profile of the product. During malting and mashing, a lot of different aroma-active substances are formed. Thereby, one of the most important aroma formation reaction is the Maillard reaction, producing, among others, aroma-active pyrazines or furanones like furaneol® from sugars and amino acids during heat treatment. During the Strecker degradation different aldehydes are formed and represent an important group for the aroma of barley malt.[59] Also coloring substances are formed during this reaction, influencing the color of the finished product. Kilning temperatures vary from 65-105 °C, for specialty malts up to 220 °C depending on the desired color of the beer.[60] But also non-volatile compounds derived from malt are important for the beer quality such as astringent hordatine-derivates affecting the beer taste.

By the variation of different factors during malting, the formed amount of odorants can be influenced. During a longer time of germination, more sugars and amino acids are released from the grains as precursors leading to higher concentrations of specific aroma compounds.[61] Also by elevated temperatures during kilning, the concentrations of furanones can be increased. However, high temperatures at the beginning, and thus, a fast decrease of humidity in the kiln can reduce the aroma formation.[61] Under dry conditions the formation of pyrazines is more likely, whereas with a higher humidity higher amounts of Strecker aldehydes are formed.[60]

3.4.3 Brewing Yeast

Mostly, yeasts of the genus *Saccharomyces* are used for brewing. These yeast species must fulfill different requirements, for example, an ethanol tolerance. Thereby, it can be differentiated between top- and bottom-fermenting yeasts, depending on the behavior at the end of the fermentation to accumulate on the top or bottom of the fermentation tank.

Beside the production of ethanol and CO_2 from carbohydrates the formation of odorants is an important aspect of beer fermentation. Beside higher alcohols and fruity esters also thiols, lactones, furanones, and mono- and sesquiterpenoids are formed during the yeast metabolism.[62] With the selection of a suitable yeast strain the aroma profile of the finished product can be affected due to different enzyme spectra.

Through the Ehrlich pathway different (acetate) esters and (fusel) alcohols like 3-methylbutyl acetate and 3-methyl-1-butanol (isoamyl alcohol), 2-methyl-1-butanol, or methyl-1-propanol are formed from the amino acids leucine, isoleucine, and valine (Figure 8).[62–64] Also 2-phenylethanol, one of the most important odorants of beer, is derived from an amino acid, namely phenylalanine (Figure 8).[62]

Recently, other yeast strains than *Saccharomyces* were tested for brewing, providing a different aroma spectrum and alternative flavor characteristics. Using wild yeast species, a wider variability of beer types can be produced. But also some problems like a poor filterability or the formation of different off-flavors were reported.[65]

Also, to produce alcohol-free beer, an alternative yeast strain can be an opportunity. Special yeasts that only have a limited ability to use carbohydrates for their metabolism can lead to lower alcohol contents, while typical beer odorants are formed.[65] Some yeast strains are able to overproduce special odorants, and thus, to compensate the reduced fermentation time.[66]

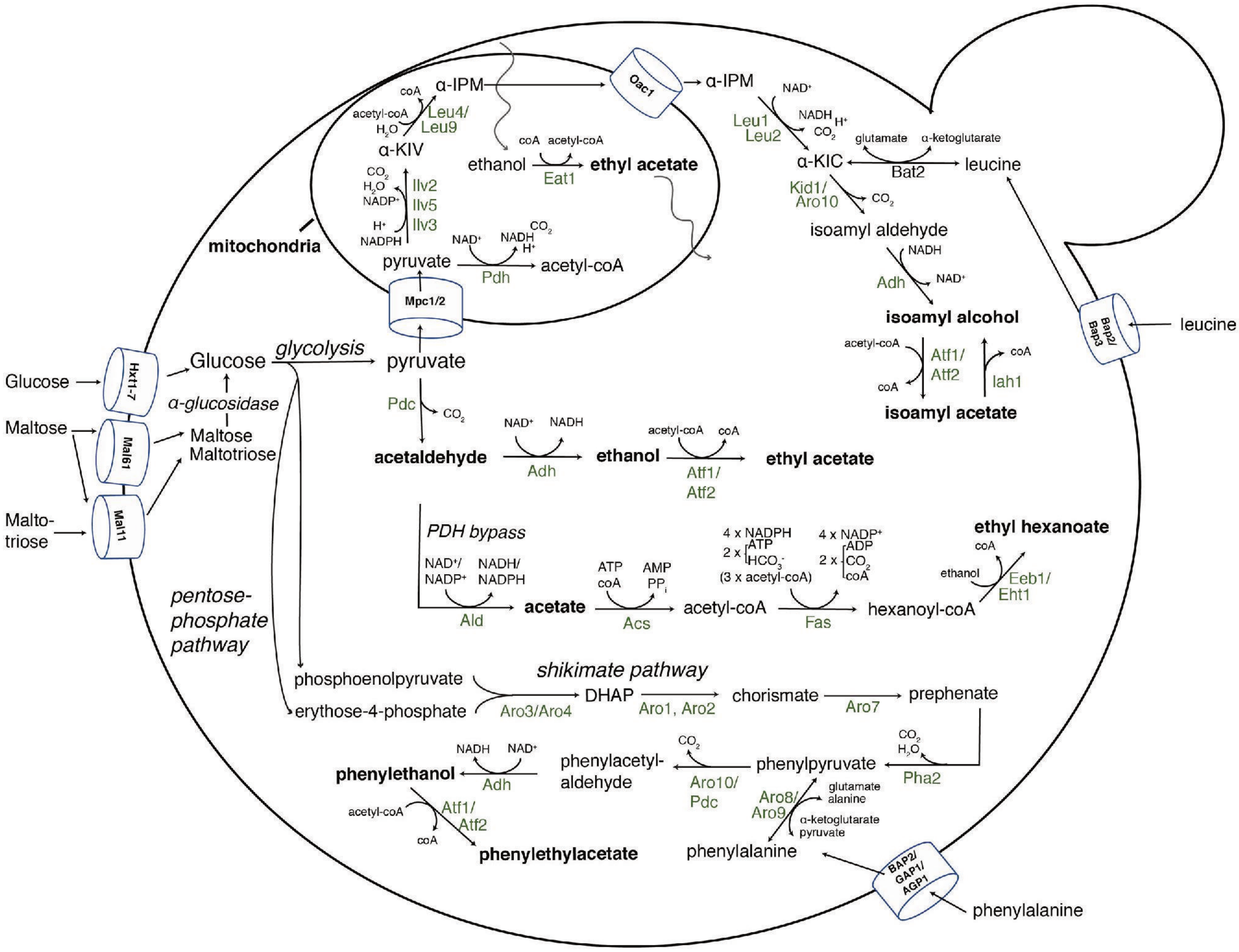

Figure 8: Formation of Different Alcohols and Esters in Yeast Biosynthesis Pathways. (Holt et al.[62]; © FEMS 2018.This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium.)

Another important consequence of the enzyme activities in yeast is the biotransformation of different hop-derived terpenoids. One possible pathway is the transformation of geraniol to citronellol, nerol, linalool, and α-terpineol.[67–71] Also from linalool, α-terpineol is formed during fermentation.[68] As the odor threshold of α-terpineol is much higher than those of geraniol or linalool, the biotransformation of these monoterpenes leads to a decrease in the citrus-like aroma-note of beer.[69]

The aroma profile of beer can also be influenced by the release of glucosidically bound odorants, e.g., citronellol by yeast *β*-glucosidases.[70–73] Also a *de novo* formation of monoterpene alcohols by yeast during fermentation was reported.[74] Thus, especially for the citrus-like aroma of beer, the choice of the right yeast strain is important.

3.5 Alcohol-Free Beer

In the past years, the market of regular beer declined, while a growing demand on alcohol-free alternatives emerged.[75] Thereby, alcohol-free beer is in line with the latest trends, being isotonic and containing less calories. In the first half year of 2018 Germany had domestic sales of alcohol-free beer of 3.3 million hL and exports of 500,000 hL.[76] In 2019, the total sales numbers of beer in Germany declined by about 1.9%, while the market of alcohol-free alternatives (alcohol-free beer, malt beer, and “Fassbrause”) only had a minus of 0.2% compared to 2018.[77] Most alcohol-free beer producing breweries are located in Bavaria while the Krombacher brewery in North Rhine-Westphalia shows the highest sales.[76]

There is no clear legal basis for alcohol-free beer and how much the ethanol content must be reduced to label it as alcohol-free. Following the wine regulations (WeinV § 47), a maximum of 0.5 vol % of ethanol is allowed in Germany. A declaration of “0.0%” is only possible if the ethanol content is < 0.05 vol % of ethanol.[78] Also other European countries, China, Great Britain, and the US apply the threshold of 0.5 vol % to label their product as “alcohol-free” or “non-alcoholic”.[78] In Japan, mainly “0.00” beer is produced with an alcohol-content of ≤ 0.005 vol %.

One of the main reasons of the increasing consumers’ interest in alcohol-free beer is an increased awareness of the own health and the functionality of food products.[79] Beside the lower alcohol content, alcohol-free beer or low-alcohol beer also contain less calories. Several medical studies verified the physiological harmlessness of the ethanol traces ≤ 0.5 vol % in alcohol-free beer.[80] Also other food items like mature fruit, fruit juice, bread, or kefir contain naturally occurring ethanol in the same concentration range because of fermentation processes. Thus, this amount of alcohol should not have a measurable influence on the blood alcohol content and should not have any negative physiological effects on the human body that are associated with the consumption of alcoholic beverages.[80]

Despite the dealcoholization process, alcohol-free beer still contains substances with positive physiological effects on human health. Among others, vitamins, polyphenols, flavonoids, and amino acids, partially derived from hops, show positive effects in regard to diabetes, cardiovascular diseases, or neurodegenerative diseases.[38,78]

3.5.1 Dealcoholization of Beer

In the production of alcohol-free beer, physical and biological methods can be distinguished. Biological methods like stopped fermentation or the usage of special brewing yeasts lead to beers with a higher content of unfermented sugars and, consequently, a sweeter taste.[81] The advantage of these methods is that they can be applied without the need of any special equipment.[82] If an immobilized yeast is used for stopped fermentation, the process is highly controllable and efficient.[83] Using physical methods, the alcohol is removed subsequently from a traditionally fermented beer. The removal of ethanol via physical methods can be divided into different groups, depending on the underlying physical properties of alcohol.[78] Boiling point (thermal dealcoholization: distillation, steam stripping, evaporation), concentration differences (dialysis or reverse osmosis), or particle size (nanofiltration) are used to separate ethanol from beer.[84,85] Also via adsorption or desorption principals ethanol can be removed from beer using supercritical CO_2 or silica gel.[78] Physical methods are the only possibility to produce a beer with an alcohol-content of 0.0 vol %.[82]

Obviously, all different methods to produce alcohol-free beer result in products that have clear sensory differences in comparison to an alcoholic beer. Beside the already mentioned sweeter taste of incompletely fermented beers, they also lack odor-active byproducts of the fermentation, while showing higher concentrations of remaining wort odorants.[66,85–87] Beers after vacuum distillation are reported as less full-bodied and with a weaker overall aroma due to the evaporation of most of the typical odorants.[88,89] And, last but not least, ethanol is one of the most important key odorants for the aroma of beer showing the highest OAV, making it impossible to produce alcohol-free beer with exactly the same aroma profile as an alcoholic beer.[90] To preserve some of the typical beer aroma properties, different methods were developed to blend the alcohol-free beer with an aroma extract of the alcoholic starting product, obtained during distillation.[91]

3.6 Dry-Hopping

In the 1980s, the craft brewers' movement started. Creative brewers wanted to produce new beer types with more alcohol, more bitterness, or a more intense hoppy or special aroma.[41] Due to the German purity law, no other ingredients than water, malt, hops, and yeast are allowed to use for beer-brewing. Thus, dry-hopping, the addition of hops into the cold area, is one of the few possibilities to influence the beer aroma after the finished fermentation. The main advantage of dry-hopping compared to the hop addition during wort boiling is a clearly lower loss of aroma-active compounds caused by high temperatures in the brew kettle.

For the dosage of hops, different methods are used. Dry-hopping with hop cones can be performed either by close-meshed nets or the so-called 'Torpedo' system. In the latter, hop cones are placed in a sieve and the beer circulates in the tank to extract the cones.[92] The easiest way to perform dry-hopping with hop pellets is the simple addition of the pellets to the storage tank or via a 'Hop Cannon' and circulation of the beer. However, the hop particles must be removed afterwards. Therefore, different technologies have been invented. The DryHopnik® allows dry-hopping under exclusion of oxygen. In the HopGun® green beer circulates between the lager

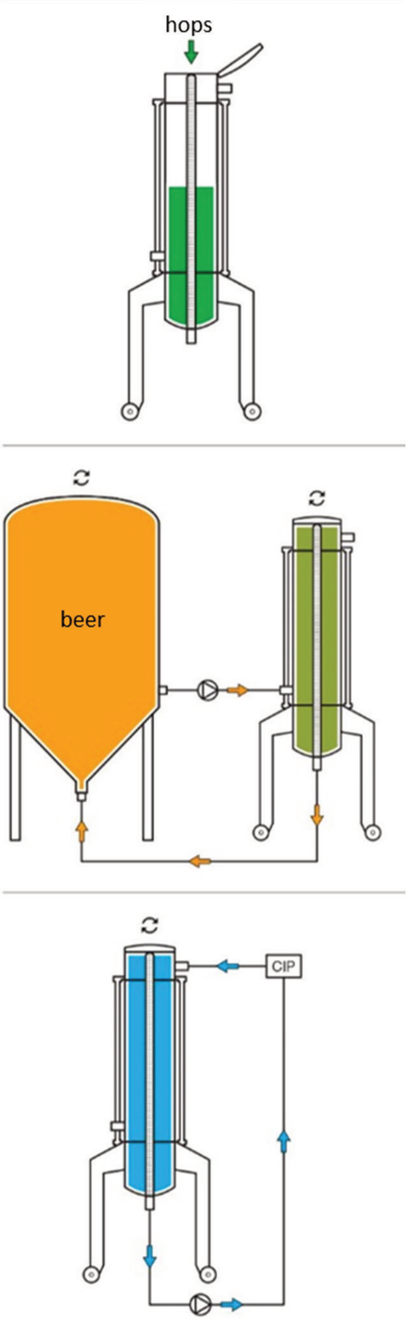

Figure 9: HopGun® Dry-Hopping Process (with kind permission of BrauKon GmbH, Seeon, Germany)

tank and the cylinder that is filled with either hop cones or hop pellets (Figure 9).[93] Thereby, particles remain in the cylinder so that no filtration is needed afterwards.

Alcohol-free beer can benefit from dry-hopping in different ways. Since during difference production processes high losses of aroma-active compounds can negatively affect the aroma profile of alcohol-free beer (see chapter 3.5), dry-hopping can be an opportunity to mask and compensate aroma deficits as well as to introduce new odor impressions into the product. Beside this possibility to influence the aroma profile of the product, hop constituents, e.g. bitter acids, can also have a positive impact on the microbiological stability. Due to its lower ethanol content and higher content of unfermented sugars, alcohol-free beer has to be classified as a microbiologically sensitive product.[94]

The transfer of odorants from hops into alcohol-free beer during dry-hopping has not been well studied up to now. Forster and Gahr analyzed the possibility to compensate aroma deficits of low alcohol beers (< 1.2 vol % ethanol), describing a transfer of linalool of 45% and an increase of palatefulness of low-alcohol and light beers by dry-hopping.[95] To the best of our knowledge, data about alcohol-free beer (< 0.5 vol %) has not been published.

More data is available on dry-hopping of alcoholic beer. Linalool has already been identified as one of the most important odorants in dry-hopped beer, characteristic for the hoppy aroma with higher transfer rates the later the hop dosage is performed.[96] Also for geraniol, high transfer rates of > 50%, accompanied by an additional formation of geraniol from its precursor geranyl acetate, present at elevated amounts in some varieties, have been reported.[97,98] Different esters reached high transfer rates as well and possibly showed some additional formation in dry-hopped beer.[97,99] In contrast, myrcene showed only very low transfer rates (max. 0.3%) as it adsorbs to yeast cells or crown caps.[100–102] Also the transfer of different sesquiterpenes like β-caryophyllene (max. 0.6%) and α-humulene (max. 1.9%) and sesquiterpenoids like caryophyllene oxide (max. 21%) and humulene epoxides I+II (max. 8.7%) has been observed, without any correlation to their odor activity.[97] In a previous study, it has been shown that sesquiterpenes and sesquiterpenoids do not contribute to the aroma of (dry-hopped) beer.[103]

Recently, the research focused on different sulfur compounds in hops as they show very low odor thresholds, and thus, are important odorants for dry-hopped beers. Not only free sulfur compounds but also bound precursors were identified in hops and hopped beer.[104] The concentrations of these compounds highly depend on the hop variety (see chapter 3.4.1). One well-studied representative of this compound group in hops and beer is 4-mercapto-4-methylpentan-2-one, also known in wine[105], showing a characteristic black currant-like odor. In 45 different hop varieties from different countries the highest concentration of 4-mercapto-4-methylpentan-2-one was found in the US variety Citra with 114 µg/kg, and also Eureka, Simcoe, and Apollo (all US) showed amounts >20 µg/kg.[55] According to that, Kishimoto et al. reported the highest intensity of a black currant-like odor in beers dry-hopped with the US varieties Simcoe, Summit, Apollo, and Cascade, with concentrations of 4-mercapto-4-methylpentan-2-one between 17 and 116 ng/L.[56] But also other sulfur compounds like methylthiohexanoate and methyl-2-methylthiopropanoate were identified in hops.[106] The formation of these thioesters was proposed as a reaction of methanethiol (from methionine) with acetyl-CoA by a thiolysis.

4 Objective

The main goal of this project was to identify the possibilities of dry-hopping to modify the aroma of alcohol-free beer regarding the aroma deficits that are typical for the respective dealcoholization method. Therefore, thermal dealcoholization and stopped fermentation should be investigated in both top- and bottom-fermented beers.

The first step was the evaluation of key odorants in three hop varieties, that should be used for dry-hopping in the later brewing experiments. Thereby, the three hop varieties Hallertauer Mandarina Bavaria (HMB), Hallertauer Cascade (HCA), and Hallertauer Mittelfrüh (HHA) were chosen because of their different flavor characteristics.

To evaluate the molecular level of the sensorially detected aroma deficits and differences of alcohol-free beers, the changes during the production of alcohol-free beers should be traced analytically. By comparing the content of key odorants in unfermented wort (for stopped fermentation) or the alcoholic reference beer (for thermal dealcoholization) to the respective alcohol-free beer, loss and formation rates should be calculated. To analyze the transfer of hop-derived odorants from hop pellets into alcohol-free beer during dry-hopping, the quantitative results of the odorants in alcohol-free beer before and after dry-hopping should be compared. As a formation of ethyl esters of different hop-derived monocarboxylic acids was observed during dry-hopping, the formation reaction should be examined regarding its chemical and enzymatic nature, investigating the influence of several parameters, e.g., temperature, pH value, or salt concentration.

To get an insight into the behavior of the analyzed odorants in the alcohol-free beer matrix, sensory evaluations on odor thresholds and dose-response-relationships should be conducted. Furthermore, using lab-scaled dry-hopping experiments, the transfer of odorants should be traced over 7 days in total. This knowledge can be linked to the sensory data to optimize the dry-hopping process regarding hop dosage and contact time.

In scale-up experiments, the transferability of the results to a semi-industrial scale should be evaluated.

5 Published Work

This publication-based thesis comprises several original-work articles. In the following, these publications are summarized in a thematic logical order, also indicating the individual contributions of all authors to the work. The full publications are shown in the Appendix.

First, the key aroma compounds in all hop varieties that have been used for dry-hopping trials of alcohol-free beer were characterized. Subsequently, the influence of dealcoholization or stopped fermentation on the volatile profile and aroma of alcohol-free beer, always in comparison to alcoholic reference beers or reference worts, was clarified, followed by the investigation of the aroma transfer from hops into alcohol-free beer. As a formation of ethyl and methyl esters has been observed, some model system studies were performed to elucidate the circumstances of this reaction. Finally, data on the sensory properties (odor thresholds, dose-response-relationships) of important odorants in dry-hopped alcohol-free beer and the time-dependence of the aroma transfer were generated.

5.1 Characterization of Key Aroma Compounds in Pellets of Different Hop Varieties (*Humulus lupulus* L.) by Means of the Sensomics Approach

To evaluate the contribution of dry-hopping to the aroma of alcohol-free beer, the key aroma compounds in the used hop varieties must be known. Therefore, they were characterized in pellets of the varieties Hallertauer Mandarina Bavaria (HMB), Hallertauer Cascade (HCA), and Hallertauer Mittelfrüh (HHA). During AEDA, 41 aroma-active areas were detected during GC-O, of which 39 could be identified. The highest FD factors were determined for myrcene, linalool, and 2-/3-methylbutanoic acid in all samples. Differences between the samples appeared in the FD factors of, for instance, 4-mercapto-4-methylpentan-2-one and different esters.

In the next step, 16 odorants with the highest FD factors were quantitated via stable isotope dilution analysis. By calculating OAVs, their contribution to the overall aroma was evaluated. The highest OAV resulted for linalool (15,000 – 33,000), followed by myrcene (1,800 – 5,100) and 3-methylbutanoic acid (1,500 – 3,600) in all hop varieties. All quantitated odorants revealed OAVs ≥ 1, and thus, a direct contribution to the aroma, except for ethyl 3-methylbutanoate and ethyl methylpropanoate in HHA. Further significant differences in the aroma composition of the different varieties were observed for ethyl 2-methylbutanoate with an OAV of 94 in HMB and only 7 in HHA. The other esters showed similar tendencies. While 4-mercapto-4-methylpentan-2-one showed OAVs > 350 in HMB and HCA, it was not detected in HHA.

For aroma recombination, cellulose was chosen as a matrix as the lupulin glands are destroyed in hop pellets and the hop oil is distributed in the ground material. All 14 – 16 (depending on the hop variety) odorants with OAVs ≥ 1 were added to the matrix in their original concentrations. For all three varieties, the aroma profiles of the sample and of the respective recombinate showed a high similarity. Thus, all key odorants were successfully identified and quantitated.

S. Brendel carried out the experiments of identification and quantitation of odorants in hop pellets, as well as the evaluations of aroma profiles, odor thresholds, and recombination experiments. S. Brendel evaluated and processed the analytical results and drafted the manuscript. T. Hofmann coordinated the research project and revised the publication draft. M. Granvogl supervised the research project and the work of S. Brendel, discussed the results, and revised the manuscript.

5.2 Dry-Hopping to Modify the Aroma of Alcohol-Free Beer on a Molecular Level – Loss and Transfer of Odor-Active Compounds

In this study, the molecular sensory science concept was applied to characterize the aroma-active compounds in different alcohol-free beers dry-hopped with three different hop varieties that have been analyzed previously in regard to their key odorants (Chapter 5.1). Therefore, thermal dealcoholization and stopped fermentation was used to produce the alcohol-free samples.

First, the changes in the aroma composition during the production processes were investigated, analyzing both alcoholic beer and alcohol-free beer (thermal dealcoholization) or unfermented wort and alcohol-free beer (stopped fermentation). The quantitative data of the thermally dealcoholized samples showed high losses up to 100% for key aroma compounds like 3-methyl-1-butanol, 2-phenylethyl acetate, ethyl hexanoate, or 3-methlbutyl acetate. During stopped fermentation, odorants like 3-methylbutyl acetate, ethyl butanoate, or 2-phenylethanol were formed in relevant concentrations with OAVs ≥ 1, and thus, contributed to the overall aroma. However, these concentrations were clearly lower as in the corresponding alcoholic beer.

Second, the transfer of hop-derived odorants into beer during dry-hopping was analyzed, supporting the initial idea of the study to influence the sensory profile of alcohol-free beers. Linalool, geraniol, and different fruity smelling esters reached OAVs ≥ 1, with transfer rates of 20-90%. Also, myrcene exceeded its odor threshold, even if it showed only very low transfer rates due to its bad water solubility and adsorption to yeast cells or crown caps. Additionally, the hop addition seemed to induce the formation of ethyl esters of hop derived monocarboxylic acids, such as 2-methylbutanoic acid, 3-methylbutanoic acid, and methylpropanoic acid.

S. Brendel carried out the experiments on the identification and quantitation of odorants in wort and beer, as well as the evaluations of aroma profiles and odor thresholds. S. Brendel evaluated and processed the analytical results and drafted the manuscript. T. Hofmann coordinated the research project and revised the publication draft. M. Granvogl supervised the research project and the work of S. Brendel, discussed the results, and revised the manuscript.

5.3 Hop-Induced Formation of Ethyl Esters in Dry-Hopped Beer

Different ethyl esters, namely, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, and ethyl methylpropanoate, showed a formation during dry-hopping in earlier experiments (chapter 5.2), most likely being induced by the addition of hops. Thus, this formation was investigated in this study using different model system experiments to clarify the role of hops in this reaction.

Different dry-hopping model systems were performed, using water and ethanol as a matrix, to exclude the influence of both the complex beer matrix and yeast enzymes on the formation of odorants. To get hints on the reaction type, the influences of light, oxygen, heat, and different additives were investigated. Also, the influence of various hop extracts was examined.

The model systems using hop pellets showed that not the concentration of monocarboxylic acids but other hop-derived esters may influence the formation of ethyl esters. Precursors like 2-methylbutyl methylpropanoate, 3-methylbutyl methylpropanoate, and methylpropyl methylpropanoate can be transesterified to the respective ethyl esters. Via a semiquantitative quantitation it was shown that the potential precursor esters mentioned above were present in the model systems in much higher concentrations than the ethyl esters. As ethyl esters have significantly lower odor thresholds than the precursor esters, the transesterification is an important factor for the aroma of dry-hopped beer.

The variation of different reaction conditions like oxygen concentration, temperature, light, salt concentration, or the presence of antioxidants revealed unclear results regarding a radical or enzymatic reaction type.

In the experiments using hop extracts an influence of monocarboxylic acids on the concentrations of ethyl esters was shown. Thus, beside the transesterification of hop esters, also the esterification of acids with ethanol can be an influencing factor for the formation of ethyl esters.

S. Brendel came up with the research question, carried out the experiments, and evaluated and processed the analytical data. She also drafted the manuscript. T. Hofmann coordinated the research project and revised the manuscript. M. Granvogl supervised the research project and the work of S. Brendel, discussed the research question, the experimental setup, and the results, and revised the manuscript.

5.4 Studies on the Odorant Concentrations and their Time Dependencies During Dry-Hopping of Alcohol-Free Beer

To complete the picture of the aroma improvement of alcohol-free beer by means of dry-hopping, investigations on the time course of the transfer of odorants are needed. Furthermore, only by taking odor thresholds and dose-response relationships into consideration, the achievements of the dry-hopping technique to improve the sensorial quality of alcohol-free beers can be evaluated.

Following the transfer of aroma compounds over the period of one week, a maximum in the transfer of geraniol, linalool, and myrcene was shown after 2-3 days. Especially for myrcene, a decline in the concentration was observed with a longer dry-hopping time, e.g., due to the adsorption to yeast cells.[68,100,101]

Like it has already been proved in earlier publications (chapters 5.2 and 6.3), different ethyl esters showed concentrations > 100% compared to the content in hop pellets and an 100% transfer. This formation started after 3-4 days and was also observed for methyl 2-methylbutanoate. In contrary, for propyl 2-methylbutanoate a maximal transfer was observed in the first 24 hours, followed by a constant decline of the concentration.

As enantiomers can show quite different aroma properties, the enantiomeric ratios of the odorants were determined in dry-hopped beer and, if appropriate, enantiopure reference substances were used for all sensory experiments. The sensory evaluations showed that odor thresholds in alcohol-free beer were 4-65 times higher than in water, providing the importance of the knowledge of these matrix-related threshold values to successfully modify the overall aroma of alcohol-free beers by dry-hopping. Sensorial investigations on the dose-response relationship of the concentration and the perceived odor intensity of various key odorants in dry-hopped alcohol-free beers showed different multi-step courses for geraniol, linalool, and myrcene. In contrast, with an increasing concentration, all investigated esters showed a constant and almost linear increase in the odor intensity.

Especially for linalool and all analyzed esters, the odor quality changed negatively with a higher concentration, leading to a synthetic sweet odor (esters) or a soapy aroma impression (linalool), whereas geraniol and myrcene only showed an intensification of a pleasant hoppy and floral odor.

The obtained data can be used for a prediction of the aroma intensity of different odor impressions in the final product, depending on the used hop variety and amount of pellets. Combining the knowledge about transfer time of odorants during dry-hopping and their sensorial behavior in different concentrations, defined aroma profiles can be reached in dry-hopped beer by optimizing the hop dosage and contact time and minimizing off-flavors due to too high concentrations.

S. Brendel prepared the lab-scaled experiments on the time dependence of aroma transfer during dry-hopping and carried out all experiments on the enantiomeric ratios of odorants in beer and the quantitation of odorants in potential matrices for sensorial experiments. S. Brendel prepared all sensorial investigations, evaluated and processed all analytical results, and drafted the manuscript. T. Hofmann coordinated the research project and revised the publication draft. M. Granvogl supervised the research project and the work of S. Brendel, discussed the results, and revised the manuscript.

6 Further Experiments

6.1 Scale-Up Experiments of Dry-Hopping of Alcohol-Free Beer

6.1.1 Objective

In lab-scaled experiments, the suitability of several hop varieties and optimization of the used amount was determined, depending on the beer type and the desired odor impression of the final product. It has already been described that the influence of dry-hopping on the aroma of beer and the transfer of odor-active compounds clearly depends on the batch scale.[107] Especially for the transfer of myrcene, linalool, and geraniol, differences in a lab-scaled, semi-industrial scaled, and industrial scaled dry-hopping trials were detected, while the influence on the transfer of esters like ethyl 2- and 3-methylbutanoate was less pronounced.[107] Important parameters affecting the transfer during dry-hopping were the headspace volume in the tank and the pellet distribution, while particle size and pellet density did not change the aroma concentration.[107,108]

Therefore, the results of the previous studies in this project should be confirmed by scale-up experiments to a semi-industrial production scale using a HopGun® system. The influences of the changed contact time and different hopping methods were analyzed by quantitating the transfer of important odorants and comparing the results to the transfer rates obtained during the lab-scaled trials (chapter 5.2).

6.1.2 Materials and Methods

Hop Pellets. Type 90 hop pellets of the variety Hallertauer Mittelfrüh, harvested in 2016, were obtained from HVG (Wolnzach, Germany). Pellets were stored under vacuum at -22 °C in non-transparent bags prior to analysis.

Beer Samples. Beer samples were provided by the Chair of Brewing and Beverage Technology (Technical University of Munich, Freising, Germany). Top-fermented (yeast WB06) and bottom-fermented beer (yeast 34/70) (700 hL each) were dealcoholized via thermal dealcoholization at a Dealcotec® system (Centec, Maintal, Germany) as previously described (Chapter 5.2).[109,110] After dealcoholization, the samples were dry-hopped with HHA according to 1.5 mL oil content/hL, using a

HopGun® (BrauKon, Seeon, Germany) apparatus. The contact time was set to 3 h hours. The samples were analyzed before and after dry-hopping.

Chemicals. All chemicals were obtained commercially: liquid nitrogen (Linde, Munich, Germany); diethyl ether, ethanol, sodium carbonate, sodium chloride, and anhydrous sodium sulfate (Merck, Darmstadt, Germany). Argon, helium, hydrogen, and nitrogen (Westfalen, Münster, Germany). Diethyl ether was distilled freshly before used.

The following reference compounds were also obtained commercially: ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, geraniol, linalool, (*R*)-linalool, 2-methylbutanoic acid, 3-methylbutanoic acid, methyl 2-methylbutanoate, (*S*)-methyl 2-methylbutanoate, methylpropanoic acid, myrcene, and propyl 2-methylbutanoate (Aldrich; Sigma-Aldrich, Taufkirchen, Germany).

Stable Isotopically Labeled Internal Standards. [$^2H_2$]-Methylpropanoic acid (C/D/N Isotopes, Quebec, Canada) and [$^2H_6$]-myrcene (Santa Cruz Biotechnology, Dallas, TX) were commercially obtained. Further internal standards were synthesized as described in literature: [$^2H_5$]-ethyl 2-methylbutanoate, [$^2H_5$]-ethyl 3-methylbutanoate, [$^2H_5$]-ethyl methylpropanoate, [$^2H_3$]-methyl 2-methylbutanoate, and [$^2H_3$]-propyl 2-methylbutanoate were synthesized via esterification of the respective carboxylic acid with the respective alcohol;[111] [$^2H_2$]-geraniol;[112] [$^2H_{2-3}$]-linalool;[113] and [$^2H_9$]-3-methylbutanoic acid.[114]

Sample Workup. Stable isotopically labeled internal standards were added to the beer samples (0.03-2 µg dissolved in diethyl ether; depending on the analyte concentration in the respective sample determined in a preliminary experiment). After stirring for 15 minutes at room temperature for equilibration, the samples (1 mL for acids, 40 mL for geraniol, linalool, and myrcene) were extracted via liquid-liquid extraction with diethyl ether (2 x 20 mL / 2 x 50 mL). The organic phases were combined and applied to high vacuum distillation using the SAFE[20] technique. For the quantitation of geraniol, the acidic fraction (AF) was removed by a treatment with aqueous sodium carbonate solution (2 x 100 mL, 0.5 mol/L). After drying the extract over anhydrous sodium sulfate and filtration, it was concentrated to ~100 µL, using a Vigreux column (50 cm x 1 cm id) and a microdistillation apparatus.

High-Resolution Gas Chromatography-Mass Spectrometry (HRGC-MS). For the quantitation of methylpropanoic acid, 2-methylbutanoic acid, and 3- methylbutanoic acid, a HRGC-MS system was used.[115] For both 2- and 3-methylbutanoic acid, [^{2}H$_{9}$]-3-methylbutanoic acid was used as internal standard. The ratio of both isomers was determined via typical mass fragments in EI mode as previously described.[115]

Two-Dimensional High-Resolution Heart-Cut Gas Chromatography-Mass Spectrometry (HRGC/HRGC-MS). For quantitating geraniol, linalool, and myrcene, a two-dimensional HRGC/HRGC-MS system with heartcut-coupling was used.[115]

Solid Phase Microextraction-Comprehensive High-Resolution Gas Chromatography-Time-of-Flight-Mass Spectrometry (SPME-HRGCxHRGC-ToF-MS). Esters were quantitated via SPME-HRGCxHRGC-ToF-MS, following the same procedure as described before (Chapter 5.2).[110]

6.1.3 Results and Discussion

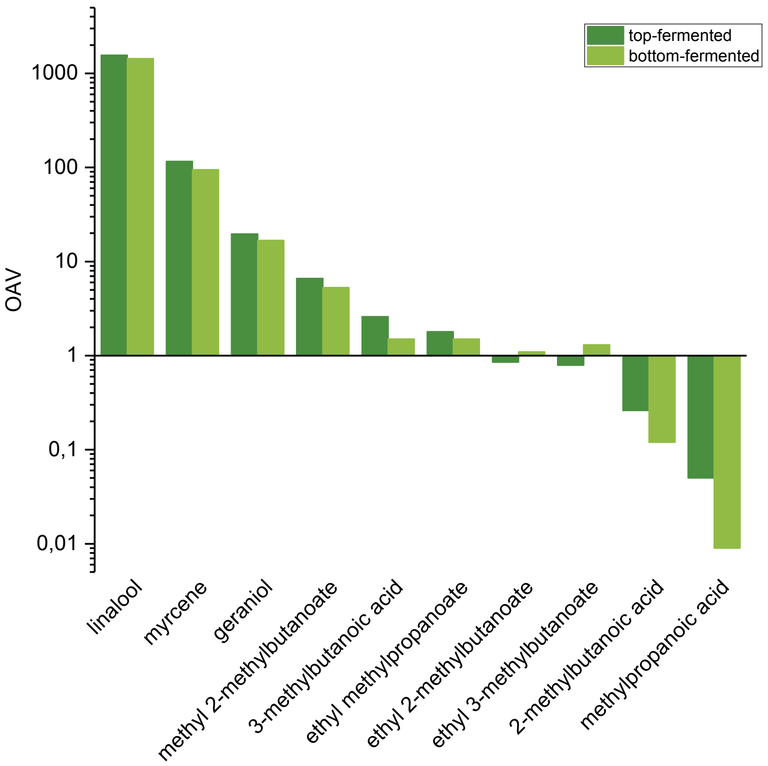

Figure 10: Odor Activity Values of Top- and Bottom-Fermented Alcohol-Free Beers after Dry-Hopping with Hallertauer Mittelfrüh Using a HopGun®.

The scale-up experiments revealed quite different results compared to the samples obtained in a research scale experiments (Table 2). Only for geraniol, linalool, and myrcene, in contrast to literature,[107] similar transfer rates were found. Regarding the formation of different esters, that has been investigated earlier,[116] higher formation rates were observed for methyl 2-methylbutanoate in the scale-up samples, whereas the formation of ethyl esters was significantly lower. In the top-fermented samples, there was even no formation of ethyl esters noticed after dry-hopping with the HopGun® system, but losses up to 0.445 µg/L (Table 2). As it has already been described that monocarboxylic acids can derive from hop bitter acid side chains, the increase in the concentrations of methylpropanoic acid, 2-methylbutanoic acid, and 3-methylbutanoic acid can be explained. Differences in the amounts of formed acids in top-fermented and bottom-fermented beers can be linked to the formation of ethyl esters in this study. In the bottom-fermented sample with a higher formation of ethyl esters, the amount of formed acids was lower, whereas in the top-fermented sample, not showing any formation of ethyl esters, the increase of the acid concentrations was higher. But this effect cannot be explained only by the concentrations of the analyzed esters and, as reported earlier, a direct formation of ethyl esters from theses acids has not been verified in model system experiments.[116]

While the amounts of methyl 2-methylbutanoate were app. six times higher than the odor threshold (see OAVs in Figure 10), the OAVs of the three ethyl esters showed values around 1, and thus, they had no or only a low influence on the overall aroma in these scale-up trials.

The results showed a transferability of the knowledge from the research-scaled dry-hopping experiments to the semi-industrial scale for those odorants, that just undergo a simple transfer from hop pellets into alcohol-free beer. The dimensions of hop-induced ester formation cannot be predicted, as the contact time with hops in the HopGun® (3 hours) was much shorter than in the experiments on the vibration table (7 days). The postulated enzymatic reaction of ethyl ester formation[116] seems to be more likely after a longer contact time, as experiments have shown the start of this formation after 3 days with an exponential growth (chapter 5.4). Furthermore, the formation of methyl esters in the HopGun® was the higher the lower the formation of ethyl esters was, indicating a possible transesterification between these two ester groups.

In summary, the transfer of geraniol, linalool, and myrcene during dry-hopping is comparable between research scale and semi-industrial scale, and also between dry-hopping on a vibration table and with a HopGun®. But for all analyzed ethyl and methyl esters, undergoing a formation during the dry-hopping process, research scale and semi-industrial scale cannot be compared. As long as the mechanisms (radically or enzymatically driven) that lead to the formation of these esters are not fully understood, the influence of the dry-hopping scale and method on this reaction cannot be finally evaluated.

Table 2: Concentrations and Transfer/Formation Rates (TFR) of Selected Odorants in Thermally Dealcoholized Beers Before and After Dry-Hopping with Hallertauer Mittelfrüh (HHA) Using a HopGun®.

compound	maximal concentration[a] [µg/L]	top-fermented				bottom-fermented			
		concentration [µg/L]		TFR[b] scale-up [%]	TFR[b] research-scale[110] [%]	concentration [µg/L]		TFR[b] scale-up [%]	TFR[b] research-scale[110] [%]
		before	after			before	after		
linalool	169	0.32	136	80	91	0.52	125	74	80
myrcene	7 430	0.59	139	1.9	1.3	2.49	114	1.5	0.2
geraniol	22.3	0.79	21.7	93	61	0.38	18.4	81	89
methyl 2-methylbutanoate	0.06	0.048	0.320	450	167	0.017	0.255	390	148
ethyl methylpropanoate	0.01	0.604	0.159	nt[c]	908	0.068	0.133	460	1219
ethyl 2-methylbutanoate	0.004	0.047	0.013	nt[c]	208	0.012	0.015	84	445
ethyl 3-methylbutanoate	< LOD[d]	0.067	0.018	nc[e]	nc[e]	0.021	0.030	nc[e]	nc[e]
propyl 2-methylbutanoate	0.03	< LOQ[f]	< LOQ[f]	nc[e]	nc[e]	< LOQ[f]	< LOQ[f]	nc[e]	nc[e]
methylpropanoic acid	12.5	2230	2 920	5 500	994	509	525	124	298
2-methylbutanoic acid	5.82	616	796	3 100	nt[c]	355	377	370	453
3-methylbutanoic acid	24.3	963	1 270	1 300	nt[c]	616	755	570	97

[a] Calculated as a theoretical transfer of 100%, based on the content in the used amount of hop pellets.[115] [b] Calculated on the basis of the concentrations in the used hop amount as 100%. [c] No transfer. [d] Limit of detection (LOD) in hops = 0.5 µg/kg, based on a signal-to-noise ratio of 3. [e] Not calculable due to concentration < LOD/LOQ. [f] Limit of quantitation (LOQ) = 0.051 µg/L, based on a signal-to-noise ratio of 10.

7 General Discussion

After investigations on the key aroma compounds of hops and beer and the transfer of them during dry-hopping, the initial question of this study, if dry-hopping is an opportunity to influence the aroma profile of alcohol-free beer which shows clear deficits in its sensorial properties, can be affirmed.

7.1 Characterization of Key Odorants in Hops

The analysis of pellets of the hop varieties Hallertauer Mandarina Bavaria, Hallertauer Cascade, and Hallertauer Mittelfrüh, following the molecular sensory science concept, revealed 14-16 KFOs (Table 3). During AEDAs using GC-O and after identification experiments, myrcene, linalool, and 3-methylbutanoic acid were detected with FD factors ≥ 512. After SIDAs, highest OAVs (1 200-33 000) were calculated for linalool, 3-methylbutanoic acid, myrcene, and dimethyl trisulfide in all analyzed varieties. 4-Mercapto-4-methylpentan-2-one was important for HMB and HCA. Methyl 2-methylbutanoate, geraniol, and butanoic acid also had a great impact on the overall aroma with OAVs between 50 and 450.

For the validation of the characterized KFOs, recombination experiments were performed in cellulose (Figure 11). Therefore, all odorants that were quantitated via stable isotopically labeled internal standards and contributed to the aroma of the three hop varieties (OAVs ≥ 1) were used for the recombinate, (Table 3).

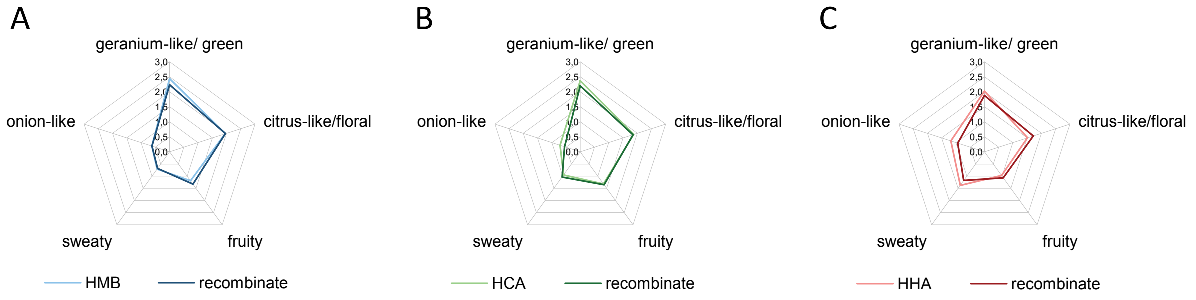

Figure 11: Aroma Recombinates of Hallertauer Mandarina Bavaria (A), Hallertauer Cascade (B), and Hallertauer Mittelfrüh (C).[115]

Varietal differences were mainly seen in the quantitative results for the odorants, less in the qualitative pattern, in contrast to some other German special flavor hop varieties.[52] While HHA showed the highest amounts of linalool and dimethyl trisulfide, HMB contained higher concentrations of the esters methyl 2-methylbutanoate, ethyl 2-methylbutanoate, propyl 2-methylbutanoate, ethyl 3-methylbutanoate, and ethyl methylpropanoate. One odorant typical for HCA and HMB (bred from HCA) was 4-mercapto-4-methylpentan-2-one, that could not be detected in HHA pellets. The highest myrcene concentration was determined in HCA pellets.

Table 3: Key Food Odorants and their Odor Activity Values in the Hop Varieties Hallertauer Mandarina Bavaria (HMB), Hallertauer Cascade (HCA), and Hallertauer Mittelfrüh (HHA).[115]

odorant	Odor Activity Values		
	HMB	HCA	HHA
linalool	15 000	19 000	33 000
3-methylbutanoic acid	3 600	2 100	1 500
myrcene	1 800	5 100	2 800
dimethyl trisulfide	1 200	2 400	3 000
4-mercapto-4-methylpentan-2-one	660	380	<19[a]
methyl 2-methylbutanoate	450	410	140
geraniol	250	110	55
butanoic acid	160	67	58
ethyl 2-methylbutanoate	94	41	7
2-methylbutanoic acid	80	50	35
methylpropanoic acid	63	44	15
propyl 2-methylbutanoate	12	4	2
ethyl 3-methylbutanoate	7	5	<1
ethyl methylpropanoate	4	3	<1
nonanal	3	4	6
vanillin	3	2	2

[a] Calculated with limit of detection (LOD) = 0.5 g/kg.

7.2 Influence of the Dealcoholization Method on the Aroma Profile of Alcohol-Free Beer

For the studies on alcohol-free beer, the first interest was the change in the aroma composition during the dealcoholization or stopped fermentation process. Therefore, previously reported key odorants of beer like 2-phenylethanol, 3-methyl-1-butanol, (*E*)-*β*-damascenone, and 2-methoxy-4-vinylphenol were quantitated in wort, beer, and alcohol-free beers.[90,117,118] For the studies on the aroma transfer from hops into beer during dry-hopping, 11 odorants have been selected. Linalool, myrcene, and geraniol already have been reported to be relevant for the hoppy aroma in dry-hopped beers.[99,119,120] Five different esters (ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, methyl 2-methylbutanoate, and propyl 2-methylbutanoate) were analyzed, because there were differences detected in their concentrations in the three hop varieties (chapter 7.1). Three monocarboxylic acids (2-methylbutanoic acid, 3-methylbutanoic acid, and methylpropanoic acid) were also analyzed because of their high OAVs in hop pellets.

First, the investigations showed the losses of odorants during the thermal dealcoholization. Therefore, the GC-O profiles of important odorants of the reference beers (top- and bottom-fermented) before dealcoholization were investigated. As expected, 2-phenylethanol, 3-methyl-1-butanol, and 2- and 3-methylbutanoic acid reached high FD factors ≥ 128 in the alcoholic reference beers, as they are already known as key aroma compounds of beer from literature.[90,117] Also the differences of top-fermented and bottom-fermented beers in the importance of 2-methoxy-4-vinylphenol have been shown, as it reached an FD factor of 4096 in the top-fermented sample, but only 512 in the bottom-fermented beer. As it is already known that 2-methoxy-4-vinylphenol is mainly formed from top-fermenting yeasts, the concentrations were expected to be higher in the top-fermented sample.[118,121]

The comparison of the samples before and after thermal dealcoholization using a cAEDA already showed big differences in the chromatograms and the detected FD factors. By quantitating the most important odorants, this first impression could be confirmed, as high losses of nearly 100% were observed for 2-phenylethyl acetate, 3-methyl-1-butanol, 3-methylbutyl acetate, and other key odorants. The losses of carboxylic acids like 2- and 3-methylbutanoic acid were less with 40% at most. To evaluate the impact of the heat treatment during dealcoholization on the aroma profile of the samples, OAVs were calculated. Considering all quantitated odorants, only ethyl 3-methylbutanoate, linalool, 3-methylbutanoic acid, nonanal, and phenylacetic acid still showed OAVs ≥ 1, and thus, a remaining contribution to the overall aroma. But the OAVs were quite low with values ≤ 7.

Furthermore, the impact of the stopped fermentation and the changes in the aroma profile from wort to alcohol-free beer were studied. Prior to fermentation, important malt odorants were predominant in the unfermented wort. Therefore, high FD factors of 512-4096 were evaluated for vanillin, furaneol®, and sotolone. During fermentation, the aroma profile changed due to degradation of odorants like vanillin or myrcene, and formation of 3-methyl-1-butanol, 2-phenylethanol, ethyl butanoate, and other products of the yeast metabolism. Calculation of OAVs proved the importance of yeast-induced odorant formation for the typical overall beer aroma. However, key odorants like 3-methyl-1-butanol reached significantly higher OAVs in an alcoholic reference beer (OAV 300) compared to the alcohol-free beer after stopped fermentation (OAV 34), as they are formed by the Ehrlich pathway in the yeast metabolism.[63,122] Thus, in the aroma of this type of alcohol-free beer the typical beer odorants are less pronounced, while malt odorants are more dominant than in fully fermented beer.

7.3 Investigation of the Aroma Transfer from Hops into Beer during Dry-Hopping

To clarify the influence of the three analyzed hop varieties on the overall aroma of dry-hopped alcohol-free beer, the transfer rates of aroma-active odorants from hops into beer were calculated, based on the concentrations in the unhopped alcohol-free beer and the concentrations in the dry-hopped alcohol-free beer. High transfer rates were found for linalool (35-91%) and geraniol (53-98%). For both compounds, an additional formation during dry-hopping by hydrolysis of different precursors was already described in literature.[97–99] In this study, this could only be confirmed for the formation of geraniol from geranyl acetate in the samples dry-hopped with HCA. In HCA, 17 900 µg/kg geranyl acetate were quantitated, leading to transfer/formation rates of 400%. In the other two hop varieties, only 487 µg/kg (HMB) and 1 980 µg/kg (HHA) geranyl acetate were found.

In all samples, myrcene showed only low transfer rates of 1.3% in maximum, due to its nonpolar structure, bad water solubility, and adsorption to crown caps, yeast cells, or during filtration.[68,100,101]

Furthermore, the transfer of different hop-derived esters was investigated. While for propyl 2-methylbutanoate transfer rates of up to 57% were calculated, all other analyzed esters reached higher transfer/formation rates from 280% (methyl 2-methylbutanoate) up to 23 000% (ethyl esters) in maximum. In literature, different possible formation pathways were postulated. Beside a formation from monocarboxylic acids with ethanol,[123,124] also a transesterification from different hop-derived precursor esters was discussed.[97,125]

In the end, the actual contribution of the odorants to the aroma of the dry-hopped alcohol-free samples can be assessed using OAVs. As it has already been described for alcoholic beer, linalool was also the most important odorant in the alcohol-free samples with the highest OAVs of 480-1 800.[96,126] But also geraniol highly contributed to the overall aroma impression with OAVs of 13-144, obviously showing higher OAVs in samples hopped with HCA, according to the additionally formed geraniol from its precursor geranyl acetate. The aroma input of myrcene was inconsistent with OAVs of 2-90, as the concentration strongly depended on the storage time of the beer, and thus, on the probability of losses due to adsorption.

In most of the samples, the hop-derived esters showed no or only little contribution to the overall aroma with OAVs of 0.64-25. But in the samples with very high ester formation rates, they reached OAVs up to 107. Thus, the ester formation can increase the importance of especially ethyl esters for the dry-hopped aroma of alcohol-free beer and enhance its fruity odor note.

7.4 Formation of Ethyl Esters in Dry-Hopped Beer

During the analysis of different dry-hopped beer samples, transfer rates of >100% were determined for ethyl esters, indicating an additional formation of ethyl esters induced by the addition of hops. Also in alcoholic beer, this phenomenon has already been described, but without any proven explanation.[99] During fermentation, the formation of esters has already been studied. Different acetyl-CoA:ethanol *O*-acetyltransferases (AETAs) were identified in yeast.[124,127,128] In apples, different amino acids like isoleucine were identified as the precursors for this enzymatic formation of a wide spectrum of ethyl and acetate esters.[129] To verify the hypothesis of the involvement of hops in an ester formation reaction, model system experiments were performed in a water-ethanol mixture to simulate dry-hopping under exclusion of the activity of yeast enzymes. Different modifications of the initial model system were performed to gain more information about this reaction.

Beside the participation of different precursor (hop-derived esters and acids) in the formation reaction, also the question, whether a radical or enzymatic mechanism is responsible for the formation of ethyl esters, should be investigated. A formation pathway that has already been suggested in other studies is the esterification of monocarboxylic acids, that can be released of the side chains of hop bitter acids, with ethanol.[124,130]

By comparing the model systems with and without added monocarboxylic acids, no significant changes in the ester concentrations were observed. Thus, the formed esters did not seem to originate from an esterification of hop-derived acids released from hop bitter acids and ethanol. In contrast to the hop pellets approaches, the model system studies performed with different hop extracts showed an influence of the addition of monocarboxylic acids on the ester concentrations.

Another possible formation pathway is the transesterification of other hop-derived esters like methylpropyl methylpropanoate, 3-methylbutyl methylpropanoate, or 2-methylbutyl methylpropanoate, that were already identified and quantitated in different hop varieties and hopped beers.[97,131] But as the concentrations of these possible precursors were much higher in hops, beer, and the model systems (e.g., 2-methylbutyl methylpropanoate > 100 µg/L) compared to the formed ethyl esters (e.g., ethyl methylpropanoate < 3 µg/L), no significant decrease could be observed to confirm this hypothesis.

Different influencing factors on the formation mechanism were also analyzed. While the presence of oxygen did not show any influence on the ester concentrations, the formation clearly increased under the influence of light. Furthermore, the use of an antioxidant inhibited the ester formation, as well as the addition of calcium chloride and a heat treatment of the hop pellets. While the influence of light and antioxidants indicate radical reaction mechanism, an involvement of hop enzymes can be postulated based on the inhibition by a higher salt concentration and temperature.

Also, different hop extracts were analyzed, enriched in α-acids, β-acids, xanthohumol, or tannins. In contrast to the hop pellet approaches, an influence of additionally added monocarboxylic acids was now observed. Overall, higher ester formation rates were observed in the hop bitter acid rich extracts (α- and β-acids), while the xanthohumol and tannin extracts did not show that much influence.

In general, the formation of ethyl esters is an important factor for the fruity aroma of dry-hopped beer, as they show relatively low odor thresholds.[125] As the concentrations of the possible precursors mentioned above (hop-derived esters and acids) are much higher compared to the concentrations of ethyl esters in hop pellets, their formation is more important than their transfer for the fruity odor note of dry-hopped beers.

7.5 Time Dependency of Hop Transfer during Dry-Hopping

In lab-scaled experiments, the transfer of the aroma compounds during dry-hopping of alcohol-free beer was displayed over 7 days of contact time. Therefore, the samples were analyzed every 24 hours. As matrix a commercially available alcohol-free beer with very low amounts of the target odorants was chosen to ensure OAVs < 1 for these compounds in the matrix.[132]

The experiments showed maximal transfer rates of all analyzed odorants already after 2-3 days. Afterwards, linalool and geraniol reached stable concentration levels (linalool: 35 µg/L; geraniol: 100 µg/L), as it has already been shown by Schnaitter et al. for alcohol-free beers with a maximal transfer within the first day of dry-hopping.[107] The myrcene concentration showed a steady decrease in the following days. Again, this could be explained by the already described loss of myrcene due to different adsorption processes and a bad water solubility. Also, propyl 2-methylbutanoate showed a constant decrease, after the maximal transfer was reached already after 24 hours. Therefore, to increase the concentration of theses odorants, and thus, the intensity of a mainly hoppy, citrus-like odor impression of the dry-hopped beer, an increased hop dosage would be more suitable than a longer contact time.

For the different ethyl esters and methyl 2-methylbutanoate, after the transfer during the first 2-3 days, an additional formation started on day 4, indicated by calculated ‘transfer’ rates > 100% as it has already been described above (chapter 7.3 and 7.4). Towards day 7 the concentrations of these esters showed an exponential increase. Thus, even higher ester concentrations, and therefore, a more intense fruity aroma might be reached by an extended contact time.

In summary, hop dosage and contact time during dry-hopping must be well selected depending on the desired odor impressions in the final product. A more hoppy, citrus-, and geranium-like aroma can be achieved via a higher hop dosage, however, changing to an unpleasant soapy odor in too high concentrations, while the fruity odor note can be intensified with a longer dry-hopping process.

7.6 Sensory Investigations on the Hoppy Aroma in Dry-Hopped Beer

To predict the behavior of the hop odorants over a wider concentration range, odor thresholds in alcohol-free beer and sensory dose-response curves were measured.

It is well-known that enantiomers can clearly differ in their odor qualities and thresholds.[133] Therefore, the enantiomeric ratios of all chiral odorants were investigated, as different enantiomers can show quite different odor thresholds. While for linalool the (*R*)-enantiomer was predominant in the beer samples and has already been identified as the common enantiomer in hops,[96] the (*S*)-enantiomer predominated for all analyzed esters in hops. For methyl 2-methylbutanoate the (*S*)-enantiomer was present at 100% also in wort and beer before and after dry-hopping. Propyl 2-methylbutanoate showed a shift towards a racemic distribution during fermentation and a higher amount of the (*S*)-enantiomer after dry-hopping. Ethyl 2-methylbutanoate ended up with a racemic distribution in the dry-hopped samples. Thus, for the sensory experiments, the respective dominating enantiomers in dry-hopped beers were used for linalool and methyl 2-methylbutanoate and a racemic reference for ethyl 2-methylbutanoate. As there was no enantiopure reference available, for propyl 2-methylbutanoate, also a racemic mixture was used. Because the odor threshold in air was significantly higher for the (*R*)-enantiomer (< 1215 ng/L air) than for the (*S*)-enantiomer (6.08 ng/L air), this should have no influence on the sensory experiments.

In general, odor thresholds were 4-65 times higher in alcohol-free beer compared to the thresholds in water. The biggest differences were seen for myrcene (water: 1.2 µg/L; beer: 76 µg/L) and propyl 2-methylbutanoate (water: 0.02 µg/L; beer: 1.3 g/L), whereas linalool (water 0.58 µg/L; beer: 4.7 µg/L) and ethyl methylpropanoate (water 0.089 µg/L; beer: 0.34 µg/L) only showed a 4-8 fold increase.

Furthermore, the behavior of the sensory properties of the odorants in alcohol-free beer in different concentrations was investigated. Therefore, different concentrations of the single odorants in alcohol-free beer were evaluated in sensory tests, using a row of increasing concentrations. A sensory panel rated the odor intensities and described the odor impressions in comparison to reference solutions of the beer without the odorant and beer with the maximal odorant concentration.

While all analyzed esters showed linear relations between concentration and odor intensity, linalool, myrcene, and geraniol showed different multistep relations. These three odorants had all individual concentration ranges, in which the odor intensity increased particularly steep, while in other concentration ranges a plateau was observed.

Myrcene showed a plateau of the odor intensity around its odor threshold up to 120 µg/L before a linear intensity increase was observed. As the concentrations observed in the dry-hopped samples reached 108 µg/L at maximum, a higher hop dosage could be a possibility to significantly increase the intensity of a typical hoppy aroma impression.

For linalool the concentrations in dry-hopped beer ranged in the same area as the intensity-plateau in the sensory tests (41-140 µg/L). Thus, only with a high increase in the hop dosage concentrations above the plateau can be reached. In much higher concentrations (> 500 µg/L) the panelists described the odor as “soapy” and unpleasant. Thus, an overdosing of hops could lead to an undesired aroma note.

The dose-response curve of geraniol was comparatively flat, only between 60 and 170 µg/L it showed a steeper ascent. As the geraniol concentration ranged between 14 and 160 µg/L in dry-hopped alcohol-free beer samples, an increase in the hop dosage may only be an opportunity for an increase in the floral note for the beers with lower geraniol concentrations. As already shown, another possibility could also be the usage of hop varieties that are rich in geraniol precursors like geranyl acetate in HCA.

These findings allow the optimization of the dry-hopping process towards the desired sensory properties by changing the hop dosage, and thus, the concentration of selected aroma-active substances. Both, hop amount and contact time, can influence the odor note and also the odor intensity that is achieved in the dry-hopped alcohol-free beer.

7.7 Scale-Up Experiments

To examine the transferability of the research scale process to a semi-technical scale, the experiments were repeated using a HopGun® for dry-hopping that allows a higher throughput in a much shorter time. The experiments showed a comparability of the transfer of linalool, geraniol, and myrcene into alcohol-free beer, as the transfer rates were in the same dimensions as in the research scale. But for all odorants that additionally undergo some formation processes beside the simple transfer, the results of the dry-hopping trials in the smaller scale on the vibration table could not be transferred to the HopGun® process. As contact time seems to be an important factor for the formation of ethyl and methyl esters, like it was shown in the monitoring of transfer over time in the lab-scale experiments, the drastically reduced contact time of 3 h (HopGun®) in contrast to 7 days (research scale), may explain, why a lower or even no formation of ethyl esters was observed in the scale-up experiments.

7.8 Conclusion

All in all, dry-hopping has been shown as an opportunity to modify the aroma of alcohol-free beers. The individual aroma deficits for the different dealcoholization methods were clearly shown. Thermally dealcoholized beers were characterized by the loss of many key odorants that normally contribute to the typical beer aroma. Alcohol-free beers produced via stopped fermentation contained higher amounts of typical wort odorants, while the beer odorants were present in clearly lower amounts as in the corresponding alcoholic beers.

After dry-hopping, important odorants like linalool, geraniol, and myrcene, that were already shown as key odorants for the hoppy aroma of beer,[96,99,119] reached concentrations above their odor thresholds, and therefore, contributed to the aroma of the dry-hopped products. Also, different fruity esters can play a role for the aroma profile of dry-hopped beers, but as the influencing factors for their formation are not clearly identified, their contribution to a fruity aroma profile cannot be clearly predicted and must be evaluated depending on the dry-hopping method. Since it has been shown before that consumers prefer a gentle fruity odor note in alcohol-free beers, while a too strong hoppy odor along with an enhanced bitterness was less favored, an increase of the ester formation might be desirable for the aroma profile of alcohol-free beers.[134]

In conclusion, the project was successful in producing alcohol-free beers with a modified aroma profile by using dry-hopping methods. The overall aroma clearly showed a shift to more citrus-like and floral notes, but also to a fruitier aroma impression. This changed aroma character of alcohol-free beers could be an opportunity to enhance the consumers' acceptance of alcohol-free beers. The results of the study also indicated possible strategies to enhance desired aroma notes in alcohol-free beers by modifying the hop dosage, hop variety, contact time, or dry-hopping method.

As the aroma transfer in the HopGun® system has not been well studied yet, more research must be done regarding the transfer mechanisms and the possibilities to influence this aroma transfer. Also, the formation of fruity (ethyl) esters after the hop addition is not fully understood up to now, and thus, cannot completely be predicted and controlled.

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9 Appendix

9.1 Characterization of Key Aroma Compounds in Pellets of Different Hop Varieties (*Humulus lupulus* L.) by Means of the Sensomics Approach

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Characterization of Key Aroma Compounds in Pellets of Different Hop Varieties (*Humulus lupulus* L.) by Means of the Sensomics Approach

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ABSTRACT: The use of hops in beer brewing is mainly based on its content of bitter acids and aroma compounds. Due to the loss of volatile odorants during wort boiling, the so-called dry hopping is a possibility to intensify the hoppy aroma in the final beer. To clarify the potential of different hop varieties for aroma modulation of beer via dry hopping, key aroma compounds of three different hop varieties were characterized using the sensomics approach. Aroma extract dilution analysis revealed 41 aroma-active compounds, of which 39 were identified via gas chromatography-olfactometry and gas chromatography-mass spectrometry. The highest flavor dilution factor was determined for myrcene with a geranium-like odor. Fourteen substances were quantitated by stable isotope dilution analysis and further two odorants via the internal standard method; all of them revealed odor activity values (OAVs; ratio of concentration to odor threshold) ≥ 1. Linalool, 3-methylbutanoic acid, myrcene, and dimethyl trisulfide showed the highest OAVs (>1000) in all analyzed hop varieties. For validation of the analytical data, reconstitution models were prepared by adding all quantitated aroma compounds with OAVs ≥ 1 in their naturally occurring concentrations to cellulose as matrix. All three recombinates showed a very high similarity to the aroma profile of the respective hop sample, confirming the correct identification and quantitation of all key aroma compounds.

KEYWORDS: *hops, molecular sensory science concept, aroma extract dilution analysis, stable isotope dilution analysis, odor activity values, aroma recombination*

INTRODUCTION

Hops are used in the beer-brewing process due to the content of bitter compounds and, therefore, are added during wort boiling. However, hops also influence the final aroma of beer. Thus, in the past few years, a later hop dosage has been applied to reduce thermal-induced losses of aroma compounds during brewing.[1] To achieve optimal transfer rates of aroma-active and bitter compounds from hops into beer, hop cones, pellets, or hop extracts are added at different times during the brewing process.[2] Addition of hops after the whirlpool into cold beer is called dry hopping and leads to pronounced hoppy aroma notes in beer.[3]

Germany and the United States, as the biggest hop producers with 19 500 and 22 900 ha of acreages,[4] increased the cultivation of the so-called aroma hops or special flavor hops since craft beer brewing gained greater importance in the past few years. In 2012, the first four flavor hop varieties—Mandarina Bavaria, Hüll Melon, Hallertau Blanc, and Polaris—were placed on the market in Germany.[5] With 310 ha, Mandarina Bavaria is the most cultivated variety of flavor hops in Germany.[6] Hallertauer Mittelfrüh with 540 ha is one of the five most important aroma hop varieties, and the US variety Cascade is also grown in Germany (86 ha) today.[6]

To date, several studies on the volatile fraction of different hop varieties have been performed. Thereby, >1000 volatile compounds were detected in hop essential oil using time-of-flight-mass spectrometry (TOF-MS).[7] In a direct thermal desorption-gas chromatography assay, nearly 300 volatiles were found in dried hop cones.[8] Using a simultaneous distillation-extraction apparatus, about 100 volatiles were extracted from hop cones of different American aroma hop varieties.[9]

Due to the fact that for each food only a small set of volatiles contributes to the specific smell, an activity-guided method such as aroma extract dilution analysis (AEDA) based on gas chromatography-olfactometry (GC-O), in combination with gas chromatography-mass spectrometry (GC-MS), is used as state-of-the-art methodology to identify the respective key odorants.[10]

A number of studies have been performed that only focused on volatile substances in hops without paying attention to the odor qualities and orthonasal odor thresholds of the identified molecules. In 1966, a first correlation between quantitative amounts and odor thresholds of hop oil components was reported by calculating odor activity values (OAVs),[11] identifying myrcene as an important aroma compound in hops. In the past two decades, more studies applied GC-O and odor thresholds to evaluate the importance of odor-active compounds. Using AEDA, myrcene, linalool, and 3-methylbutanoic acid were found as the most important aroma

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compounds in hop cones and pellets of different varieties, followed by *trans*-4,5-epoxy-(*E*)-2-decenal, geraniol, and (*E*,*Z*)-1,3,5-undecatriene.[12,13] First studies on the flavor hops Hüll Melon and Polaris confirmed these results also for some of these new cultivars, but they also pointed out the potential importance of different esters (Hüll Melon) and 1,8-cineol (Polaris) on their special aroma notes.[14] Further, 4-mercapto-4-methylpentan-2-one was reported as a potent hop odorant mainly in various American and some modern German varieties.[14−16]

Although several studies were related to key aroma compounds in hops, aroma recombination was only performed once for one variety but never in comparison between different ones.[17] Thus, the aim of this study was to identify and quantitate the most important aroma-active compounds in the hop varieties Hallertauer Mandarina Bavaria, Hallertauer Cascade, and Hallertauer Mittelfrüh to analyze the differences in the aroma of these hop samples on a molecular level using the sensomics concept, including AEDA, identification experiments, quantitation by stable isotope dilution analysis (SIDA), calculation of OAVs, and a final validation of the data by aroma recombination experiments.[18]

■ MATERIALS AND METHODS

Hop Pellets. Hop pellets type 90 of the varieties Hallertauer Mandarina Bavaria (HMB), Hallertauer Cascade (HCA), and Hallertauer Mittelfrüh (HHA), harvest years 2015 (HMB) and 2016 (HCA and HHA), were provided by Hopsteiner (Mainburg, Germany; variety HMB), HVG (Wolnzach, Germany; HCA), and Joh. Barth & Sohn (Nuremberg, Germany; HHA) and stored under vacuum at −22 °C prior to analysis.

Chemicals. The following reference compounds were commercially available: dimethyl trisulfide, 4-methyl-4-mercaptopentan-2-one, nonanal, and 1-octen-3-one (Alfa Aesar, Karlsruhe, Germany); *p*-cresol, γ-decalactone, δ-decalactone, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, β-ionone, 2-methylbutanoic acid, 3-methylbutanoic acid, methyl 2-methylbutanoate, methyl phenylacetate, methylpropanoic acid, myrcene, phenylacetaldehyde, phenylacetic acid, 2-phenylethanol, and tetrahydrolinalool (Aldrich; Sigma-Aldrich, Taufkirchen, Germany); hexanoic acid, linalool, and α-phellandrene (Fluka, Neu-Ulm, Germany); butanoic acid and vanillin (Merck, Darmstadt, Germany); acetic acid, 2,3-butanedione, geraniol, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one, 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one, methional, (*E*,*Z*)-2,6-nonadienal, and (*E*,*Z*)-2,6-nonadienol (Sigma-Aldrich); and propyl 2-methylbutanoate (TCI, Eschborn, Germany).

Trans-4,5-epoxy-(*E*)-2-decenal,[19] (*E*,*E*,*Z*)-2,4,6-nonatrienal,[20] (*Z*)-1,5-octadien-3-one,[21] and (*E*,*Z*)-1,3,5-undecatrien[12] were synthesized as described in the literature.

The following chemicals were commercially obtained: liquid nitrogen (Linde, Munich, Germany); dichloromethane, diethyl ether, pentane, silica gel 60, and anhydrous sodium sulfate (Merck); argon, helium, hydrogen, and nitrogen (Westfalen, Münster, Germany); and *n*-alkanes (C5−C26) and dithiothreitol (Sigma-Aldrich). Dichloromethane, diethyl ether, and pentane were freshly distilled prior to usage. Mercurated agarose gel was prepared from Affigel 10 (Bio-Rad Laboratories, Munich).[22]

Stable Isotopically Labeled Internal Standards. [$^{2}H_{2}$]-Methylpropanoic acid (CDN Isotopes, Quebec, Canada) and [$^{13}C_{6}$]-vanillin (Cambridge Isotope Laboratories, Tewksbury, MA) were commercially available.

The following stable isotopically labeled internal standards were synthesized as previously described: [$^{2}H_{2}$]-butanoic acid;[23] [$^{2}H_{6}$]-dimethyl trisulfide;[24] [$^{2}H_{5}$]-ethyl 2-methylbutanoate, [$^{2}H_{5}$]-ethyl 3-methylbutanoate, [$^{2}H_{5}$]-ethyl methylpropanoate, [$^{2}H_{3}$]-methyl 2-methylbutanoate, and [$^{2}H_{3}$]-propyl 2-methylbutanoate were synthesized by esterification of the respective alcohol with the respective carboxylic acid;[25] [$^{2}H_{2}$]-geraniol;[26] [$^{2}H_{9}$]-3-methylbutanoic acid;[27] and [$^{13}C_{4}$]-4-mercapto-4-methylpentan-2-one.[28] [$^{2}H_{4}$]-Nonanal was synthesized starting with non-3-yn-1-ol following the procedure described for [$^{2}H_{4}$]-hexanal.[29]

The concentrations of the labeled standards were determined as recently described.[30]

Isolation of the Volatiles. Hop pellets were ground with mortar and pestle, and an aliquot of the sample (7.5 g) was extracted with diethyl ether (2 × 125 mL) by stirring at room temperature (2 × 45 min). To separate the volatile and nonvolatile compounds, the combined filtered organic extracts were subjected to high vacuum distillation using the solvent assisted flavor evaporation (SAFE) technique.[31] The distillate obtained was dried over anhydrous sodium sulfate and, after filtration, concentrated to 1 mL using a Vigreux column (50 cm × 1 cm). The concentrated extract was used for AEDA.

For additional identification experiments, the distillate obtained after SAFE was separated into two fractions, containing neutral/basic compounds (NBF) and acidic compounds (AF). The distillate was extracted with aqueous sodium carbonate solution (0.5 mol/L; 2 × 100 mL), whereby the NBF remain in the organic phase. The aqueous phases, containing the AF, were combined, adjusted to pH 3 with hydrochloric acid, extracted with diethyl ether (2 × 100 mL), and, finally, the organic phases, now containing the AF, were combined. Both fractions (NBF and AF) were dried over anhydrous sodium sulfate and concentrated to ∼1 mL as described above. During concentration of the NBF, diethyl ether was stepwise replaced with pentane by adding pentane to the concentrated sample several times and concentrating it again to a volume of ∼1 mL. For further separation, the NBF was fractionated by column chromatography. Therefore, a water-cooled glass column (230 mm × 5 mm; 12 °C) was closed with defatted cotton wool and filled with a slurry of silica gel (7% of water content; prepared according to Esterbauer[32]) in pentane. The fraction of hydrocarbons (HCF) was eluted with pentane (10 mL) and the fraction of oxygenated compounds (OF) with diethyl ether (10 mL). Both fractions were concentrated to ∼1 mL as described above and were used for identification experiments.

Aroma Extract Dilution Analysis (AEDA) and Identification Experiments. AEDA was performed as a screening method for aroma-active compounds in the different hop pellets. Therefore, the aroma extract was diluted stepwise 1 + 1 (v + v) with diethyl ether, and the original extract as well as all dilutions were analyzed via high-resolution gas chromatography-olfactometry (HRGC-O) by at least three trained panelists to avoid potential overlooking of aroma-active compounds. At the end, a so-called flavor dilution (FD) factor (highest dilution in which the odorant was perceived for the last time) is assigned to each aroma-active compound. Identification of the odorants was based on their retention indices on two capillary columns of different polarities (DB-FFAP and DB-5; both 30 m × 0.32 mm i.d., 0.25 μm film thickness; J&W Scientific; Agilent Technologies, Waldbronn, Germany), their odor qualities and intensities perceived at the sniffing port during HRGC-O, and their mass spectra obtained in both electron ionization (EI) and chemical ionization (CI) mode. These data were compared to data of reference compounds from an in-house database containing >1000 volatile compounds.

High-Resolution Gas Chromatography-Olfactometry (HRGC-O). For HRGC-O, a Trace GC Ultra (Thermo Scientific, Dreieich, Germany) was used with manual cold on-column injection. For separation, either a DB-FFAP or a DB-5 column (both 30 m × 0.32 mm i.d., 0.25 μm film thickness; J&W Scientific) was applied. Aliquots (1 μL) of the samples were analyzed with helium as the carrier gas. The oven was held at 40 °C for 2 min and then heated at 6 °C/min to 230 °C (held for 5 min). To split the effluent into two equal parts at the end of the column, a Y-type quick-seal glass splitter (Chrompack, Frankfurt, Germany) was used. One part was led to a flame ionization detector (FID; 250 °C) and the other part to a sniffing port (230 °C). Thus, the odor quality and the FID signal could be detected simultaneously using an analogue writer (Servogor 124; BBC Goerz Metrawatt, Nuremberg, Germany).

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Table 1. Stable Isotopically Labeled Standards, Selected Ions for Quantitation, Response Factors, and Systems Used for Stable Isotope Dilution Analysis

		ion (m/z)[a]			
compound	isotope label	analyte	standard	response factor	system[b]
butanoic acid	[$^2H_2$]	103	105	0.89	I
dimethyl trisulfide	[$^2H_6$]	127	133	0.97	II
ethyl 2-methylbutanoate	[$^2H_5$]	131	136	0.99	III
ethyl 3-methylbutanoate	[$^2H_5$]	131	136	0.98	III
ethyl methylpropanoate	[$^2H_5$]	117	122	1.00	IV
geraniol	[$^2H_2$]	137	139	1.00	I
4-mercapto-4-methylpentan-2-one	[$^{13}C_4$]	99	103	0.96	III
methyl 2-methylbutanoate	[$^2H_3$]	117	120	0.95	III
2/3-methylbutanoic acid[c]	[$^2H_9$]	117	126	1.04	I
methylpropanoic acid	[$^2H_2$]	89	91	0.65	I
nonanal	[$^2H_4$]	143	147	0.97	V
propyl 2-methylbutanoate	[$^2H_3$]	145	148	0.99	III
vanillin	[$^{13}C_6$]	152	158	0.99	VI

[a] Ions used for quantitation. [b] System used for quantitation. I: GC-MS (DB-FFAP); II: SPME-GC-MS (DB-FFAP); III: GC/GC-MS (DB-FFAP/OV-1701); IV: GC/GC-MS (DB-WAX/OV-1701); V: GC/GC-MS (DB-FFAP/DB-5), all in CI mode; VI: GCxGC-ToF-MS (DB-FFAP/DB-1701) in EI mode. [c] Quantitated as a sum of both isomers with [$^2H_9$]-3-methylbutanoic acid as the internal standard.

Using a series of *n*-alkanes (C6−C26 for DB-FFAP and C6−C18 for DB-5, respectively), linear retention indices (RIs) were determined.[33]

High-Resolution Gas Chromatography-Sector Field Mass Spectrometry (HRGC-MS) for Identification. HRGC-MS for identification was performed with a gas chromatograph type 5890 series II (Hewlett-Packard, Waldbronn) connected to a sector field mass spectrometer type MAT 95 S (Finnigan, Bremen, Germany). Columns and temperature program were used as described above. Mass spectra were obtained in EI (70 eV) and CI (115 eV) mode using isobutane as the reactant gas.

Two-Dimensional High-Resolution Gas Chromatography-Mass Spectrometry for Identification (HRGC/HRGC-MS). For identification of overlapping volatiles or minor components, a two-dimensional HRGC/HRGC-MS system was used. A mega 2 series gas chromatograph (Fisons Instruments, Mainz, Germany) was coupled to a gas chromatograph type 3800 (Carlo Erba, Hofheim, Germany), which was connected to an ion trap mass spectrometer type 800 (Finnigan). Mass spectra were recorded in EI and CI mode with methanol as the reactant gas (both 70 eV). For separation, a DB-FFAP column in the first oven and a DB-5 column (both 30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific) in the second oven were used. The sample was injected cold on-column onto the first column, using a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland), and transferred onto the second column by means of a moving capillary stream switching (MCSS) system (Fisons Instruments) using a liquid nitrogen trap.

Quantitation by Stable Isotope Dilution Analysis (SIDA). For quantitation, diethyl ether (20−300 mL, depending on the amount of hop sample) and the stable isotopically labeled standards (0.1−10 μg, dissolved in dichloromethane or diethyl ether; amounts depending on the concentration of each analyte determined in preliminary experiments) were added to ground hop pellets (0.2−15 g). After equilibration, the workup procedure followed the description above for the isolation of the volatiles. For the quantitation of all acids, AF was used.

To determine the respective response factors (R_f), mixtures of each analyte and its corresponding stable isotopically labeled standard (5 + 1, 3 + 1, 1 + 1, 1 + 3, 5 + 1; *m* + *m*) of known concentrations were analyzed.

Quantitation of 4-Mercapto-4-methylpentan-2-one. 4-Mercapto-4-methylpentan-2-one was isolated using a mercurated agarose gel. To ground hop pellets (15 g), dichloromethane (3 × 100 mL) and [$^{13}C_4$]-4-mercapto-4-methylpentan-2-one (0.05 μg, dissolved in diethyl ether) were added, and extraction was performed by stirring at room temperature (3 × 60 min). After filtration, the combined extracts were distilled under high vacuum via SAFE,[31] dried over anhydrous sodium sulfate, and concentrated to ∼1 mL as described above. The concentrated extract was applied onto a glass column (100 mm × 5 mm) filled with mercurated agarose gel (1 g). After the column was rinsed with a mixture of pentane and dichloromethane (2 + 1, v + v; 15 mL), the thiols were eluted with dithiothreitol (7.7 g in 50 mL of dichloromethane). The eluate was again subjected to SAFE, dried over anhydrous sodium sulfate, and concentrated to ∼50 μL using a Vigreux column (50 cm × 1 cm) and microdistillation prior to mass spectrometry.

Quantitation of 2- and 3-Methylbutanoic Acid. As the two isomers of methylbutanoic acid could not chromatographically be separated by the used columns, they were quantitated in sum by SIDA in the CI mode. Subsequently, the ratio was determined in EI mode via the intensities of the typical mass fragments *m*/*z* 60 (3-methylbutanoic acid) and *m*/*z* 74 (2-methylbutanoic acid).[34] The concentrations in the samples were calculated based on a calibration curve using mixtures containing the two isomers in different defined ratios (0:100; 15:85; 30:70; 50:50; 70:30; *m*:*m*). The ratio of area counts of *m*/*z* 60 and the sum of area counts of *m*/*z* 60 and *m*/*z* 74 was plotted against the concentration percentage of 3-methylbutanoic acid in the calibration solution.[35]

High-Resolution Gas Chromatography-Mass Spectrometry (HRGC-MS) for Quantitation. For quantitation, a Varian 431 gas chromatograph (Darmstadt) connected to a Varian 220 ion trap mass spectrometer was used, recording the mass spectra in CI mode with methanol as the reactant gas (70 eV). The gas chromatograph was equipped with a DB-FFAP capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific). Cold on-column injection of an aliquot (2 μL) of the sample was performed by means of a Combi PAL autosampler (CTC Analytics). In general, the oven was held at 40 °C for 2 min and then heated at 6 °C/min to 230 °C (held for 5 min), with some specific modifications for selected compounds. The peak areas of the analyte and the respective labeled standard were determined via the protonated molecular masses or selected fragments (Table 1).

Two-Dimensional High-Resolution Gas Chromatography-Mass Spectrometry (HRGC/HRGC-MS) for Quantitation. In the case of overlapping volatiles, two-dimensional gas chromatography was performed. A Trace 2000 Series gas chromatograph (ThermoQuest, Egelsbach, Germany) was coupled to a CP-3800 gas chromatograph (Varian), which was connected to a Saturn 2000 ion trap mass spectrometer (Varian) recording the mass spectra in CI mode with methanol as the reactant gas (70 eV). The first GC was equipped with a DB-FFAP column (30 m × 0.25 mm i.d., 0.25 μm film thickness) or a DB-WAX column (30 m × 0.32 mm i.d., 1 μm film thickness; both J&W Scientific) and the second GC with an OV-1701 or a DB-5 column (both 30 m × 0.25 mm i.d., 0.25 μm film thickness; both J&W Scientific). Cold on-column injection of an aliquot (2 μL) of the sample was performed by means of a Combi PAL autosampler (CTC Analytics). The part of the eluate containing the substance that should be quantitated was transferred onto the second GC column by means of an MCSS system using a liquid nitrogen trap. Individual temperature programs were applied.

Comprehensive High-Resolution Gas Chromatography-Time-of-Flight-Mass Spectrometry (HRGCxHRGC-ToF-MS) for Quantitation of Vanillin. Vanillin was quantitated using a 6890 N gas chromatograph (Agilent Technologies) equipped with a DB-FFAP column (30 m × 0.25 mm i.d., 0.25 μm film thickness) in the first dimension and a DB-1701 column (2 m × 0.15 mm i.d., 0.3 μm film thickness; both J&W Scientific) in the second dimension and coupled to a Pegasus III time-of-flight mass spectrometer (LECO Instrumente, Moenchengladbach, Germany) recording mass spectra in EI mode (70 eV). Injection of an aliquot (1 μL) of the sample via a split−splitless-injector was performed by means of a GC PAL autosampler (CTC Analytics).

Headspace Solid Phase Microextraction High-Resolution Gas Chromatography-Mass Spectrometry (HS-SPME-HRGC-MS) for Quantitation of Dimethyl Trisulfide. Dimethyl trisulfide was quantitated via HS-SPME-HRGC-MS using a divinylbenzene/polydimethylsiloxane fiber (Supelco; Sigma-Aldrich). [$^2H_6$]-Dimethyl trisulfide (0.1 μg, dissolved in ethanol) was added to ground hop pellets (1 g) in a headspace vial (20 mL). Absorption was performed for 5 min at 30 °C; thermodesorption was performed for 5 min at 250 °C by an autosampler (GC sampler 80; Agilent). The 7890 B gas chromatograph (Agilent) was equipped with a DB-FFAP column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific) and coupled to an Agilent 240 ion trap mass spectrometer (CI mode, methanol, 70 eV).

High-Resolution Gas Chromatography-Flame Ionization Detection (HRGC-FID) for Quantitation of Myrcene and Linalool. For quantitation of substances present in amounts >50 mg/kg in hops and not showing overlapped peaks in the chromatogram, quantitation was performed by the internal standard method using HRGC-FID. Thereby, standards not naturally occurring in the samples and showing structural similarity to the analytes (α-phellandrene for myrcene, 5 mg; tetrahydrolinalool for linalool, 0.1 mg, both dissolved in diethyl ether) were added to ground hop pellets (2.5 g). The extraction with diethyl ether (2 × 75 mL) and the following workup procedure were performed as described above for the isolation of the volatiles. The extract obtained was concentrated to ∼5 mL. Quantitation was performed with a Trace GC Ultra (Thermo Scientific) equipped with a DB-FFAP column (30 m × 0.32 mm i.d., 0.25 μm film thickness; J&W Scientific) and an FID. Cold on-column injection of an aliquot (2 μL) of the sample was performed by means of a TriPlus autosampler (Thermo Scientific). The concentrations of the odorants were calculated via relative response factors (α-phellandrene: 0.91; tetrahydrolinalool: 1.08).

Determination of Orthonasal Odor Thresholds (OTs). OTs were determined in odorless refined sunflower oil as previously described.[36]

Aroma Profile Analysis (APA). For APA, the sensory panel evaluated five odor attributes on a seven-point linear scale from 0 (not perceivable) to 3 (strongly perceivable) in steps of 0.5 using aqueous solutions of reference compounds in concentrations 100 times above their respective odor thresholds: geranium-like/green (myrcene), citrus-like/flowery (linalool and geraniol), fruity (ethyl 3-methylbutanoate and methyl 2-methylbutanoate), sweaty (butanoic acid and 3-methylbutanoic acid), and onion-like (dipropyl trisulfide). The panel consisted of 20 experienced assessors, trained their abilities to recognize and describe different odor qualities in weekly sensory sessions. All sensory analyses were performed in a sensory room divided into single booths at 21 ± 1 °C. The samples (2 g) were presented in covered glass vessels (40 mm i.d., total volume = 45 mL).

Aroma Recombination Experiments. Aroma recombination was performed with cellulose as the matrix. All quantitated aroma compounds with an OAV ≥ 1 were added to cellulose in their naturally occurring concentrations determined in the respective hop sample using either the pure reference compound (only for myrcene) or odorant solutions in ethanol or water (for all acids to avoid esterification of ethanol), not exceeding the odor threshold of ethanol in cellulose. APA of the recombinates was performed as described above.

■ RESULTS AND DISCUSSION

To determine the differences in the overall aroma of the selected hop varieties, first, aroma profile analyses were performed (Figure 1). Thereby, HHA revealed differences to the other hop varieties, whereas HMB and HCA showed quite similar aroma profiles because of the descent of HMB from HCA and a male culture stock from Hüll. Nevertheless, HMB and HCA could significantly be differentiated in a three-alternative forced-choice test, following DIN EN ISO 4120, with a significance level of $\alpha = 0.001$. In comparison to HHA, HMB and HCA showed a more citrus-like/flowery and geranium-like/green odor impression and lower intensities for the sweaty and onion-like odor qualities. To characterize these differences on a molecular level, the sensomics approach[18] was used.

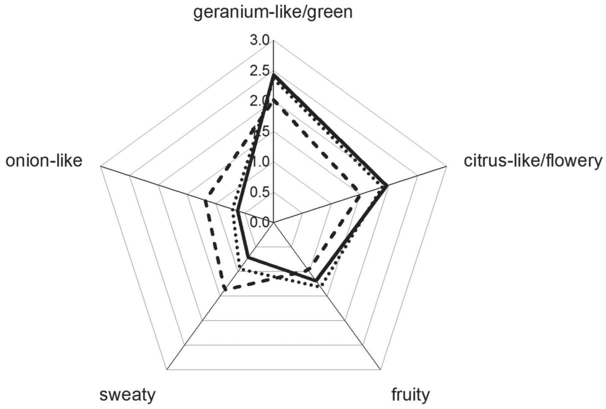
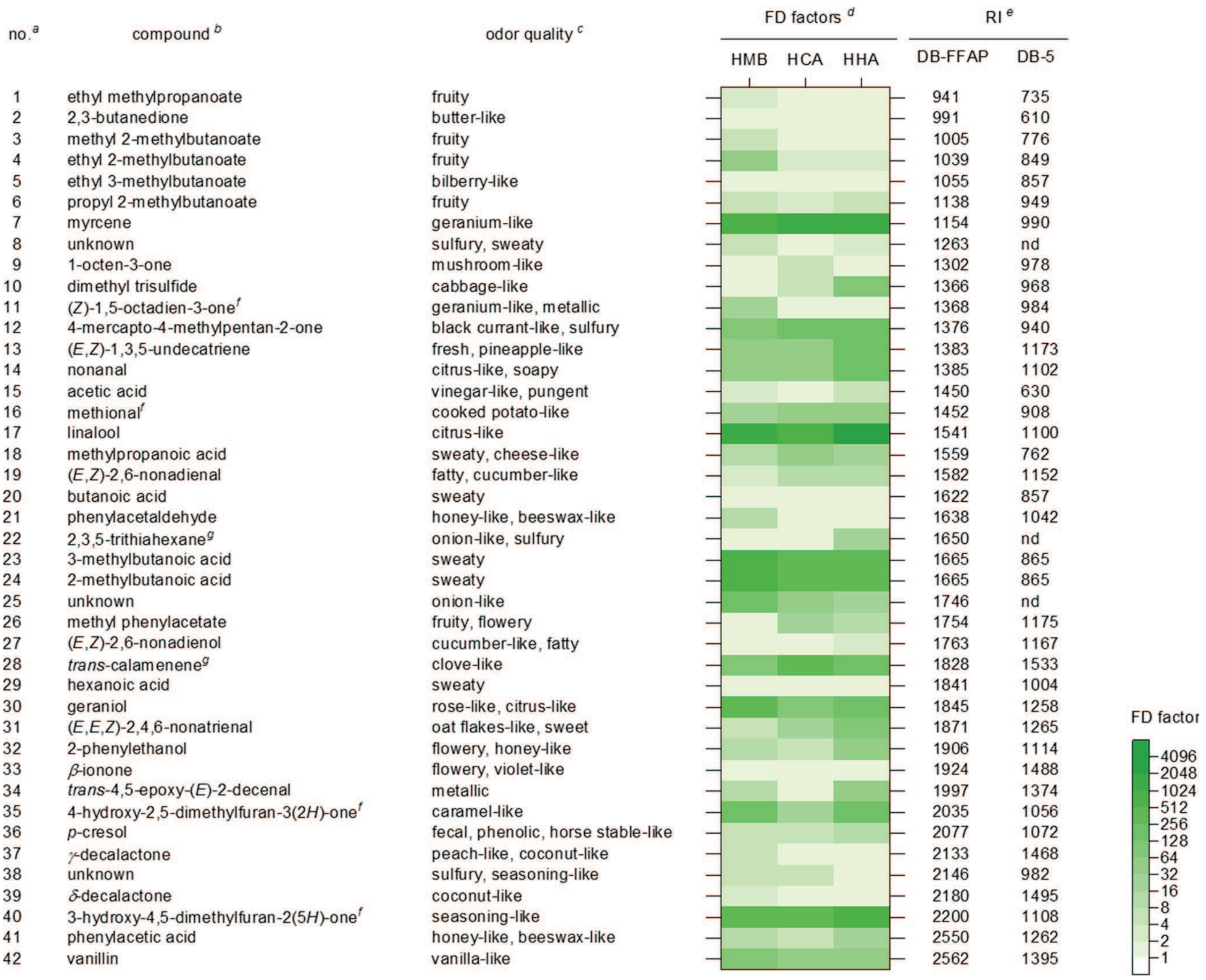


Figure 1. Aroma profile analyses of Hallertauer Mandarina Bavaria (HMB; solid line), Hallertauer Cascade (HCA; dotted line), and Hallertauer Mittelfrüh (HHA; broken line).

Identification of Aroma-Active Compounds in Hop Pellets. A comparative AEDA (cAEDA) was applied to the concentrated distillates of the pellets of the varieties HMB, HCA, and HHA obtained after solvent extraction and high vacuum distillation by the SAFE[29] technique. Thereby, 42 odor-active areas were detected in a flavor dilution (FD) factor range from 1 to 4096 (Figure 2). The highest FD factors between 256 and 4096, depending on the hop variety, were found for compounds **7** (geranium-like), **17** (citrus-like), **23** (sweaty), **24** (sweaty), and **40** (seasoning-like). Other important substances with FD factors from 64 to 256 were detected for compounds **12** (black current-like, sulfury), **13** (fresh, pineapple-like), **14** (citrus-like, soapy), **28** (clove-like), **30** (rose-like, citrus-like), and **35** (caramel-like).

For identification of the detected odor-active compounds, retention indices on two columns of different polarities were determined. After comparison to an in-house database containing >1000 volatiles, the respective reference compounds were applied to GC-O. Comparison of retention indices, aroma quality and intensity, and mass spectra in EI and



no.[a]	compound[b]	odor quality[c]	RI[e] DB-FFAP	RI[e] DB-5
1	ethyl methylpropanoate	fruity	941	735
2	2,3-butanedione	butter-like	991	610
3	methyl 2-methylbutanoate	fruity	1005	776
4	ethyl 2-methylbutanoate	fruity	1039	849
5	ethyl 3-methylbutanoate	bilberry-like	1055	857
6	propyl 2-methylbutanoate	fruity	1138	949
7	myrcene	geranium-like	1154	990
8	unknown	sulfury, sweaty	1263	nd
9	1-octen-3-one	mushroom-like	1302	978
10	dimethyl trisulfide	cabbage-like	1366	968
11	(*Z*)-1,5-octadien-3-one[f]	geranium-like, metallic	1368	984
12	4-mercapto-4-methylpentan-2-one	black currant-like, sulfury	1376	940
13	(*E*,*Z*)-1,3,5-undecatriene	fresh, pineapple-like	1383	1173
14	nonanal	citrus-like, soapy	1385	1102
15	acetic acid	vinegar-like, pungent	1450	630
16	methional[f]	cooked potato-like	1452	908
17	linalool	citrus-like	1541	1100
18	methylpropanoic acid	sweaty, cheese-like	1559	762
19	(*E*,*Z*)-2,6-nonadienal	fatty, cucumber-like	1582	1152
20	butanoic acid	sweaty	1622	857
21	phenylacetaldehyde	honey-like, beeswax-like	1638	1042
22	2,3,5-trithiahexane[g]	onion-like, sulfury	1650	nd
23	3-methylbutanoic acid	sweaty	1665	865
24	2-methylbutanoic acid	sweaty	1665	865
25	unknown	onion-like	1746	nd
26	methyl phenylacetate	fruity, flowery	1754	1175
27	(*E*,*Z*)-2,6-nonadienol	cucumber-like, fatty	1763	1167
28	*trans*-calamenene[g]	clove-like	1828	1533
29	hexanoic acid	sweaty	1841	1004
30	geraniol	rose-like, citrus-like	1845	1258
31	(*E*,*E*,*Z*)-2,4,6-nonatrienal	oat flakes-like, sweet	1871	1265
32	2-phenylethanol	flowery, honey-like	1906	1114
33	*β*-ionone	flowery, violet-like	1924	1488
34	*trans*-4,5-epoxy-(*E*)-2-decenal	metallic	1997	1374
35	4-hydroxy-2,5-dimethylfuran-3(2*H*)-one[f]	caramel-like	2035	1056
36	*p*-cresol	fecal, phenolic, horse stable-like	2077	1072
37	*γ*-decalactone	peach-like, coconut-like	2133	1468
38	unknown	sulfury, seasoning-like	2146	982
39	*δ*-decalactone	coconut-like	2180	1495
40	3-hydroxy-4,5-dimethylfuran-2(5*H*)-one[f]	seasoning-like	2200	1108
41	phenylacetic acid	honey-like, beeswax-like	2550	1262
42	vanillin	vanilla-like	2562	1395

Figure 2. Aroma-active compounds identified in Hallertauer Mandarina Bavaria (HMB), Hallertauer Cascade (HCA), and Hallertauer Mittelfrüh (HHA). [a]Odorants were consecutively numbered according to their retention indices on capillary DB-FFAP. [b]Odorant identified by comparison of its odor quality and intensity, retention indices on capillaries DB-FFAP and DB-5 as well as mass spectra (EI and CI mode) with data of reference compounds. [c]Odor quality detected at the sniffing port. [d]Flavor dilution factors determined during AEDA on capillary DB-FFAP. Color intensity from white to black is related to FD factors (1-4096). [e]Retention indices determined using a homologous series of *n*-alkanes. [f]No unequivocal mass spectrum was obtained; identification based on remaining criteria in footnote b. [g]No reference material available; identification based on mass spectra obtained from databases and ref 14. nd: not detected.

CI mode led to an unequivocal identification of most of the odorants. For identification of overlapping volatiles, the hop distillates were fractionated in acidic and neutral/basic compounds using silica gel column chromatography.

With this methodology, the structures of 39 volatiles were elucidated (Figure 2). The compounds showing the highest FD factors during AEDA were identified as myrcene (**7**, geranium-like), linalool (**17**, citrus-like), 3- and 2-methylbutanoic acid (**23** and **24**, sweaty), and 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one (**40**, seasoning-like). Furthermore, 4-mercapto-4-methylpentan-2-one (**12**, black currant-like, sulfury), (*E*,*Z*)-1,3,5-undecatrien (**13**, fresh, pineapple-like), nonanal (**14**, citrus-like, soapy), *trans*-calamenene (**28**, clove-like), geraniol (**30**, rose-like, citrus-like), and 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (**35**, caramel-like) were identified as important odorants, showing high FD factors.

Quantitation of Key Aroma Compounds in Hop Pellets via Stable Isotope Dilution Analysis (SIDA) as well as Internal Standard Method and Determination of Odor Activity Values (OAVs). By applying cAEDA to food samples, the number of volatiles can be reduced to a smaller set of key odorants, which play an important role in the overall aroma. However, the screening by AEDA does not consider the influence of the food matrix on the odor release. Therefore, in a next step, a precise quantitation method has to be used to obtain reliable results. Consequently, 14 aroma compounds, showing high FD factors or differences in their FD factors between the three hop varieties during AEDA, were quantitated by stable isotope dilution analysis (Tables 1 and 2). For quantitation of myrcene and linalool, the internal standard method using GC-FID was applied due to their high concentrations and good separation of other peaks in the chromatograms.

Odorant Concentrations. The highest concentration (>3 g/kg) was found for myrcene in all three samples. Linalool (up to 113 mg/kg in HHA) and geraniol (up to 67 mg/kg in

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Table 2. Concentration of Selected Aroma-Active Compounds in Hallertauer Mandarina Bavaria (HMB), Hallertauer Cascade (HCA), and Hallertauer Mittelfrüh (HHA)

compound	concentrations[a] [μg/kg]		
	HMB	HCA	HHA
myrcene	3 320 000	9 090 000	4 950 000
geraniol	66 600	28 400	14 900
linalool	50 500	63 100	113 000
3-methylbutanoic acid[b]	39 100	22 800	16 200
methylpropanoic acid	33 900	23 700	8340
2-methylbutanoic acid[b]	8850	5500	3880
butanoic acid	5450	2290	1970
nonanal	1990	2630	3450
vanillin	360	232	212
propyl 2-methylbutanoate	145	47.9	22.5
methyl 2-methylbutanoate	136	122	40.7
ethyl methylpropanoate	95.6	68.6	9.42
ethyl 2-methylbutanoate	34.8	15.0	2.53
dimethyl trisulfide	34.6	72.8	90.1
ethyl 3-methylbutanoate	6.89	5.01	<LoD[c]
4-mercapto-4-methylpentan-2-one	1.05	0.613	<LoD[d]

[a] Mean values of at least triplicates, standard deviations ≤ 15%. [b] Concentrations were calculated from the sum of 2- and 3-methylbutanoic acid (via GC-MS in the CI mode) and the ratio determined by GC-MS in the EI mode. For further details, see Materials and Methods. [c] LoD = 0.5 μg/kg. [d] LoD = 0.03 μg/kg.

Table 3. Orthonasal Odor Thresholds and Odor Activity Values (OAVs) of Selected Aroma-Active Compounds in Hallertauer Mandarina Bavaria (HMB), Hallertauer Cascade (HCA), and Hallertauer Mittelfrüh (HHA)

compound	odor threshold in oil[a] [μg/kg]	OAVs[b]		
		HMB	HCA	HHA
linalool	3.4	15 000	19 000	33 000
3-methylbutanoic acid	11	3600	2100	1500
myrcene	1800	1800	5100	2800
dimethyl trisulfide	0.03	1200	2400	3000
4-mercapto-4-methylpentan-2-one	0.0016[c]	660	380	<19[d]
methyl 2-methylbutanoate	0.3	450	410	140
geraniol	270[c]	250	110	55
butanoic acid	34	160	67	58
ethyl 2-methylbutanoate	0.37	94	41	7
2-methylbutanoic acid	110	80	50	35
methylpropanoic acid	540	63	44	15
propyl 2-methylbutanoate	12[c]	12	4	2
ethyl 3-methylbutanoate	0.98	7	5	<1[d]
ethyl methylpropanoate	22	4	3	<1
nonanal	610	3	4	6
vanillin	140	3	2	2

[a] Orthonasal odor threshold was determined in odorless refined sunflower oil. Thresholds from an in-house database. [b] OAVs were calculated as the ratio of the concentration to the respective odor threshold. [c] Newly determined in this study. [d] Calculated with LoD (cf. Table 2).

HMB) were also present at high amounts in all analyzed varieties. High concentrations were also determined for the monocarboxylic acids 3-methylbutanoic acid, methylpropanoic acid, 2-methylbutanoic acid, and butanoic acid (all 2 - 39 mg/kg), as well as for nonanal (>1.99 mg/kg) (Table 2). Various esters revealed specific differences in their concentrations depending on the respective hop varieties. For example, ethyl 3-methylbutanoate was not detectable in HHA (<LoD); ethyl methylpropanoate, ethyl 2-methylbutanoate, and propyl 2-methylbutanoate were present in amounts 6−10 times higher in HMB and HCA compared with HHA. Differences in the concentrations of methyl 2-methylbutanoate were not so much pronounced but followed the same pattern. 4-Mercapto-4-methylpentan-2-one was also not detectable in HHA but in both other samples. Concentrations of dimethyl trisulfide ranged between 35 (HMB) and 90 μg/kg (HHA).

Overall, HMB showed the highest concentrations in most of the quantitated aroma-active compounds, although the highest values for linalool, nonanal, and dimethyl trisulfide were determined in HHA and for myrcene in HCA (up to 9 g/kg; Table 2).

Odor Activity Values (OAVs) of Key Aroma Compounds. Concentrations do not finally determine if a certain odorant contributes to the overall aroma. Thus, quantitative data has to be combined with the respective odor thresholds leading to the so-called OAVs (ratio of the concentration divided by the respective odor threshold). Therefore, odor thresholds in oil were used because of the compartmentalization of the hop oil

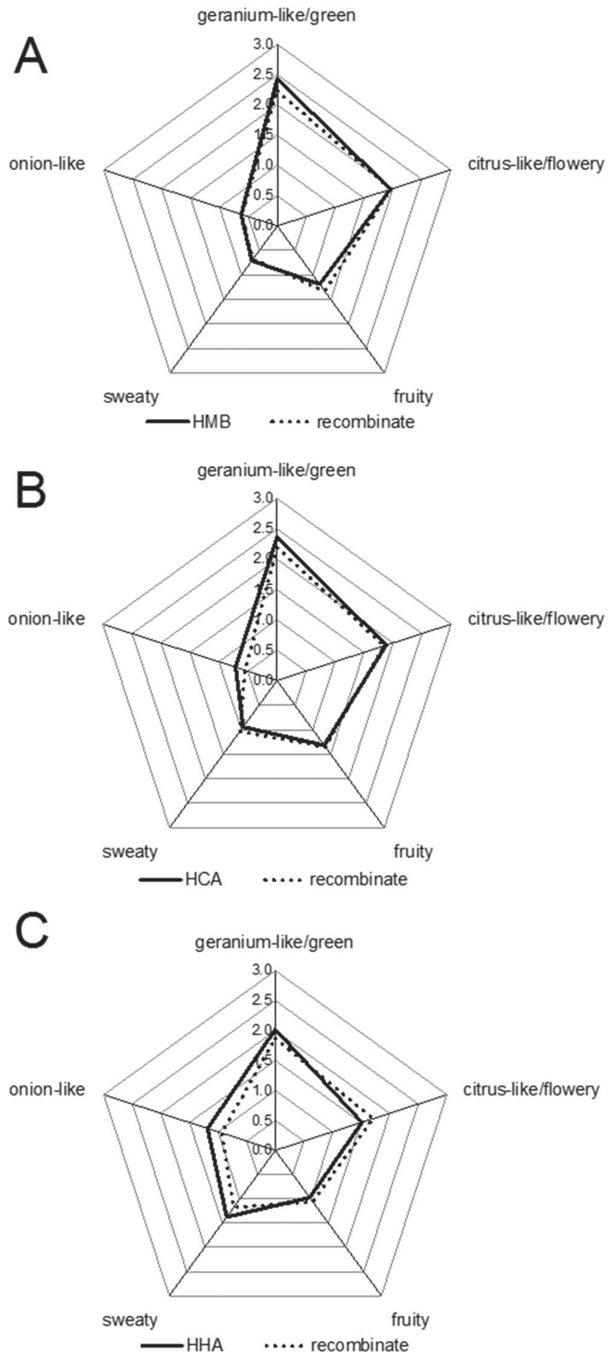

Figure 3. Aroma profile analyses of HMB (A), HCA (B), and HHA (C) (all solid lines) and the corresponding recombinates (all dotted lines).

(also containing the aroma compounds) in the lupulin glands in a lipophilic environment (Table 3).

Linalool showed the highest OAV > 10 000 in all three varieties, followed by OAVs > 1000 for 3-methylbutanoic acid, myrcene, and dimethyl trisulfide. The quantitated esters contributed to the overall aroma with OAVs between 2 (propyl 2-methylbutanoate in HHA) and 450 (methyl 2-methylbutanoate in HMB). Only in hops of HHA, ethyl methylpropanoate and ethyl 3-methylbutanoate showed OAVs < 1.

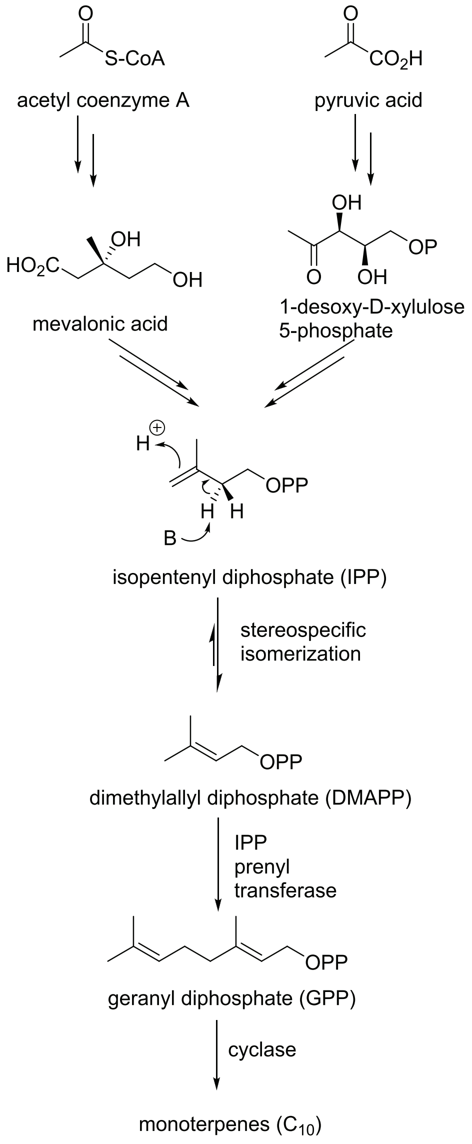

Figure 4. Biosynthesis of terpenes (according to ref 38).

Aroma Simulation. To verify the analytical data, all odorants with an OAV ≥ 1 were added to an odorless matrix in their naturally occurring concentrations. Cellulose was used as a suitable matrix to simulate hop pellets, in which the lupulin glands are no longer intact and the hop oil components are dispersed in the plant material (cellulose content of 50%).[37] Solutions of the odorants were prepared in ethanol or in water (for the acids to avoid possible esterification) and were added in the respective concentrations to cellulose without exceeding the odor threshold of ethanol in cellulose. Only myrcene was added directly to cellulose due to its very high concentration. For sensory evaluation, the original hop samples were presented in parallel with the respective recombinates to a trained sensory panel consisting of 20 panelists. For all recombinates (HMB, HCA, and HHA), very good similarities to the original hop pellets were achieved (Figure 3), proving a successful identification and quantitation of all key odorants.

Sources of Key Odorants. Terpenes and terpenoids (e.g., myrcene, linalool, and geraniol) are some of the largest groups

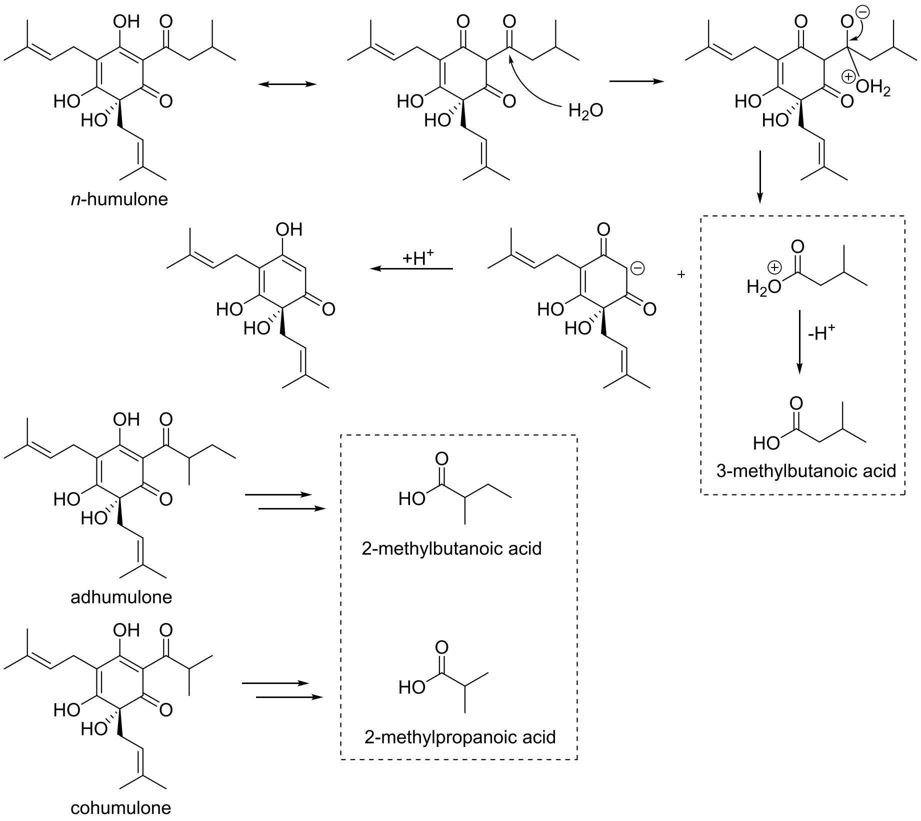

Figure 5. Proposed formation pathway of 3-methylbutanoic acid from *n*-humulone (modified to ref 39).

of plant metabolites and the major aroma-active compounds in hop oils. Analogous to the isoprenyl side chains of hop bitter acids, terpenoid aroma compounds are biosynthesized via the mevalonate or the pyruvate pathway (Figure 4).[38]

The bitter acid part of hops consists of α- and β-acids, humulones, and lupulones. During hop storage, short-chain carboxylic acids, namely, methylpropanoic acid, 2-methylbutanoic acid, and 3-methylbutanoic acid, can be released from the side chains of hop bitter acids (Figure 5).[39] Another possible pathway is the formation of medium-chain carboxylic acids, for example, pentanoic acid, during lipid synthesis.[39]

4-Mercapto-4-methylpentan-2-one (4-MMP) is known as an important odorant for the aroma of wine,[40] grapefruit,[41] or tea.[42] In wine, 4-MMP is released from nonvolatile precursors like glutathione or cysteine conjugates during fermentation.[43,44] Also in hops, especially in American hop varieties, 4-MMP has already been identified.[15,16] The absence of 4-MMP in European hop varieties was correlated with the use of copper sulfate as a fungicide and the corresponding high concentrations of copper ions in hop plants.[16] Also in this study, 4-MMP was not present (<LoD) in Hallertauer Mittelfrüh. In contrast, in Hallertauer Cascade, originating from the corresponding US variety, and Hallertauer Mandarina Bavaria, again originating from the Cascade species, 4-MMP was present (Table 2).

For dimethyl trisulfide, different precursors have been described in the literature, for example, methional originating from methionine during Strecker degradation and its reduced form methionol in aged beer.[45] In hops, *S*-methylcysteine sulfoxide was suggested as a precursor of dimethyl trisulfide formed during storage.[46]

Branched-chain esters of different monocarboxylic acids in plants and fruits can originate from amino acids like leucine, isoleucine, and valine. A biosynthesis pathway from isoleucine to ethyl 2-methylbutanoate via 2-methylbutanoyl-CoA has been proposed in the literature, analogous to methylpropyl esters from valine and 3-methylbutyl esters from leucine.[47,48]

In summary, this study characterized the key odorants in pellets of different hop varieties and verified the data by recombination experiments for the first time. Some aroma compounds of US Cascade hops have already been described in previous studies, but their contribution to the overall aroma was only estimated based on FD factors or Charm values.[13,49] In the past, myrcene, linalool, and 3-methylbutanoic acid were shown as important odorants; however, in contrast to our study, dimethyl trisulfide was identified but evaluated as not important with an FD factor <1.[13] Katsiotis et al. analyzed mainly terpenes and sesquiterpenes in Hallertauer Mittelfrüh with respect to their relative amounts without any relation to their sensory properties.[50] In the present study, only one harvest year for each variety was evaluated, not considering annual variations. However, studies with German flavor hop varieties on the influence of the harvest year on the concentration of odorants showed that there were only slight differences in concentrations.[14] The obtained results of this study will be used for further experiments based on the transfer of hop odorants into the final beer by dry hopping.

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Notes

The authors declare no competing financial interest.

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9.2 Dry-Hopping to Modify the Aroma of Alcohol-Free Beer on a Molecular Level – Loss and Transfer of Odor-Active Compounds

Title	Dry-Hopping to Modify the Aroma of Alcohol-Free Beer on a Molecular Level – Loss and Transfer of Odor-Active Compounds
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Dry-Hopping to Modify the Aroma of Alcohol-Free Beer on a Molecular Level—Loss and Transfer of Odor-Active Compounds

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ABSTRACT: There are mainly two options for the dealcoholization of beer: evaporation of ethanol by heat treatment, whereby desired aroma-active compounds are also removed, and stopped fermentation that leads to beers still containing high amounts of unfermented sugar in parallel with lower amounts of aroma-active fermentation products. Thus, dry-hopping could be an opportunity to compensate for these aroma deficiencies. Therefore, following the sensomics approach, odorants were characterized in dry-hopped (Hallertauer Mandarina Bavaria, Hallertauer Cascade, or Hallertauer Mittelfrüh) top- and bottom-fermented alcohol-free beers either after thermal dealcoholization or stopped fermentation. Twenty-three odorants were quantitated via stable isotope dilution analysis, and odor activity values (OAVs; ratio of concentration to odor threshold) were calculated. Thermally dealcoholized samples showed high losses (up to 100%) of key odorants like 3-methyl-1-butanol or 3-methylbutyl acetate. During stopped fermentation, aroma compounds like ethyl butanoate or 2-phenylethanol were formed in relevant concentrations, leading to OAVs ≥ 1, but the amounts were significantly lower compared to beers with normal alcohol contents. For hop-derived odorants (linalool, geraniol, myrcene, and esters), transfer rates between 20 and 90% were found, leading to OAVs ≥ 1 in beer. Furthermore, hop addition apparently induced the formation of ethyl esters of hop-derived monocarboxylic acids.

KEYWORDS: *hops, Humulus lupulus L., alcohol-free beer, dry-hopping, sensomics concept, aroma extract dilution analysis, stable isotope dilution analysis, odor activity values, thermal dealcoholization, stopped fermentation*

INTRODUCTION

During the past years, the sale rates of alcohol-free beer [alcohol content ≤ 0.5% vol in most EU countries, China, and Great Britain (labeled "alcohol-free") and the US (labeled "non-alcoholic")] strongly increased.[1] Different alcohol contents are needed for further specific labeling, for example, ≤0.05% vol to label the product as "0.0%" (Germany) or as "non-alcoholic" (Great Britain) and ≤0.005% vol to label the product as "0.00%" (Japan). In comparison to their alcohol-containing counterparts, clear differences in their aroma profiles and their matrices occur.[2,3] In general, two main dealcoholization methods can be differentiated: physical methods to remove selectively the alcohol, for example, by temperature during thermal dealcoholization (TD), but also via nanofiltration, dialysis, or reverse osmosis, and biological methods, based on a stopped fermentation (SF) principle by removing (filtration, centrifugation) or inactivating (cooling, pasteurization) the yeast before an alcohol content of 0.5% vol is exceeded. Therefrom, TD and SF are the most often applied dealcoholization methods in small and medium-sized breweries. However, thermally dealcoholized beer has often been described with an unpleasant odor impression and a lack of the typical aroma,[4] based on the fact that desired volatile odorants are also removed during the heat treatment.[5] In contrast, beer after SF has a sweeter and more wort-like aroma, whereas fruity fermentation notes are less pronounced.[4] Important odorants for the characteristic aroma of a regular beer, such as 2-phenylethanol, 3-methyl-1-butanol, 3-methylbutyl acetate, and ethyl hexanoate,[6,7] are formed during fermentation and therefore are less present after a shorter fermentation time. Additionally, maltose is not completely fermented, leading to a sweet taste impression.

Because of the German purity law (§ 9 VorlBierG), the use of additives in the brewing process is not allowed. Permitted ingredients are only water, malt, yeast, and hops. Hops are mainly used for their contents of bitter acids and odorants, as well as for their natural preservative properties. Hops added before the boiling step normally have no or only a marginal contribution to the aroma of the final beer because of the occurring losses of aroma-active compounds during the heat-processing steps, leading to concentrations of hop-derived odorants below their respective odor thresholds (OTs).[8] However, with a later hop dosage into the whirlpool or in the cold area, volatile hop compounds are transferred into beer without being evaporated during wort boiling.[9] This so-called dry-hopping can be a possibility to influence the aroma of beer along the purity law by the use of special hop cultivars with an intense aroma. Thus, dry-hopping might also be an opportunity to mask and compensate the aroma deficiencies

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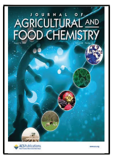

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Table 1. Selected Ions (m/z) of Analytes and Stable Isotopically Labeled Standards and Response Factors (R_f) Used in the Stable Isotope Dilution Assays

		ion (m/z)[a]			
Compound	isotope label	analyte	standard	calibration line equation[b]	R^2
ethyl 2-methylbutanoate	$[^2H_5]$	102/131[c]	107/136[c]	$1.0596x + 0.0005/0.8343x + 0.1072$	0.999/1.000
ethyl 3-methylbutanoate	$[^2H_9]$	88/131[c]	91/140[c]	$1.0864x - 0.0241/0.8343x - 0.1072$	1.000/1.000
ethyl methylpropanoate	$[^2H_5]$	116/117[c]	121/122[c]	$0.9160x - 0.0263/0.8855x - 0.0154$	1.000/0.999
methyl 2-methylbutanoate	$[^2H_3]$	101/117[c]	104/120[c]	$1.2309x - 0.0929/0.9461x + 0.0108$	0.997/1.000
propyl 2-methylbutanoate	$[^2H_3]$	116/145[c]	119/148[c]	$0.9914x - 0.0134/0.8936x + 0.1253$	0.999/0.996
butanoic acid	$[^2H_2]$	103	105	$0.8332x + 0.0507$	0.999
(*E*)-*β*-damascenone	$[^2H_{2-8}]$	191	193−199[d]	$1.0305x - 0.0610$	0.999
ethyl butanoate	$[^2H_3]$	117	120	$0.9745x + 0.0709$	0.999
ethyl hexanoate	$[^2H_3]$	145	148	$0.8541x + 0.0349$	1.000
geraniol	$[^2H_2]$	137	139	$1.1358x + 0.0109$	0.997
linalool	$[^2H_{2-3}]$	137	139 + 140[d]	$0.9434x + 0.0303$	0.999
2-methoxy-4-vinylphenol	$[^2H_3]$	151	154	$1.2176x + 0.0024$	1.000
2-/3-methylbutanoic acid[e]	$[^2H_9]$[e]	117	126	$1.0365x + 0.0161$	1.000
3-methyl-1-butanol	$[^2H_{11}]$	71	82	$1.0641x - 0.0191$	0.999
3-methylbutyl acetate	$[^2H_2]$	131	133	$0.9544x + 0.5958$	0.933
methylpropanoic acid	$[^2H_2]$	89	91	$0.8686x - 0.0486$	1.000
myrcene	$[^2H_6]$	137	143	$0.8826x + 0.0174$	1.000
nonanal	$[^2H_4]$	143	147	$1.0988x - 0.0129$	1.000
phenylacetic acid	$[^{13}C_2]$	137	139	$1.0159x + 0.1357$	0.998
2-phenylethanol	$[^2H_5]$	105	110	$0.9021x + 0.0723$	0.993
2-phenylethyl acetate	$[^2H_5]$	105	110	$0.8272x - 0.0182$	1.000
vanillin	$[^{13}C_6]$	153	159	$0.9984x - 0.0001$	0.999

[a] Ions used for quantitation via HRGC−MS or HRGC/HRGC−MS in CI mode. [b] Determined via a 5-point-calibration using mixtures of known amounts of unlabeled analyte and corresponding labeled internal standard. [c] Ions used either for quantitation via HRGCxHRGC-TOF-MS in EI mode or via HRGC/HRGC−MS in CI mode. Quantifier ions in EI mode may not be fully labeled because of fragmentation. [d] Internal standard was used as a mixture of isotopologues. [e] Quantitated as sum of both isomers with $[^2H_9]$-3-methylbutanoic acid as the internal standard.

of alcohol-free beers. In a recent study, late- or dry-hopping was shown as a possibility to produce low-alcohol beers (0.5−1.2% vol) with equivalent sensory properties to light beers (2.5−4.5% vol).[10]

Beside different aroma hop varieties like Cascade or Hallertauer Mittelfrüh (HHA), new special flavor hops like Mandarina Bavaria with exotic aroma profiles have been developed, eliciting a specific pattern of key odorants. Applying the sensomics concept, linalool, 3-methylbutanoic acid, myrcene, and dimethyl trisulfide were shown as the most important odorants in Hallertauer Mandarina Bavaria (HMB), Hallertauer Cascade (HCA), and HHA.[11] Differences between the overall aroma impressions of these hop varieties were mainly based on the contents of different esters. In addition, 4-mercapto-4-methylpentan-2-one was identified as a characteristic odorant for the varieties Cascade and Mandarina Bavaria.[11]

Therefore, the aim of this study was to evaluate the possibility of dry-hopping of alcohol-free beers to improve their aroma properties. Different beer types [top-fermented (TF) and bottom-fermented (BF)] were thermally dealcoholized under vacuum or shortly fermented to an alcohol content ≤0.5% vol. After TD or SF, the samples were dry-hopped with either HMB, HCA, or HHA. To determine the influences of the dealcoholization method and dry-hopping step on the final aroma, the aroma-active compounds were analyzed by stable isotope dilution analysis (SIDA) in combination with gas chromatography−mass spectrometry (GC−MS) in the samples with and without dry-hopping.

■ MATERIALS AND METHODS

Hop Pellets. Hop pellets type 90 of HMB, HCA, and HHA, harvest years 2015 (HMB) and 2016 (HCA and HHA), were provided by Hopsteiner (HMB; Mainburg, Germany), Joh. Barth & Sohn (now BarthHaas) (HCA; Nuremberg, Germany), and HVG Hopfenveredelungsgenossenschaft (HHA; Wolnzach, Germany). The same batches were recently used for the analysis of the respective hop key odorants.[11] *α*-Acids were analyzed by Hopsteiner: 6.2% (HMB), 4.2% (HCA), and 6.8% (HHA).

Beer Samples. All beer samples were provided by the Chair of Brewing and Beverage Technology (Technical University of Munich, Freising, Germany) in three technological replicates. For all of them, either the top-fermenting yeast strain WB06 or the bottom-fermenting yeast strain 34/70 was used.

For TD, an alcohol-containing reference beer (TF: 4.7% vol; BF: 4.9% vol) was dealcoholized to an ethanol content <0.1% vol by a DeAlcoTec system (Centec, Maintal, Germany) under vacuum at 36 °C.[12] Using this alcohol-free beer, three dry-hopped beers (either with HMB, HCA, or HHA) were produced, in each case one TF and one BF beer.

For SF, a reference wort (RW) was used to produce a partially fermented beer. Fermentation was started at 7 °C and stopped after 48 h by filtration, equivalent to an alcohol content of 0.4% vol. Using this alcohol-free beer, three dry-hopped beers (either with HMB, HCA, or HHA) were produced, again in each case one TF and one BF beer.

Dry-hopping was performed according to Schnaitter et al.[13] with the following modifications: hop dosage was performed by pellets representing 1.5 mL of hop oil/hL, and storage was performed on a vibration table (vibrating for 30 min twice a day) for 1 week. The unfiltered samples were bottled in brown glass bottles (500 mL) and stored at 4 °C prior to analysis.

Chemicals. The following reference compounds were commercially available: 2-aminoacetophenone, *p*-cresol, *γ*-decalactone, decanoic acid, 1,1-diethoxyethane, ethyl butanoate, ethyl hexanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, ethyl 3-phenylpropanoate, furaneol, geraniol, *β*-ionone, maltol, methional, methionol, 3-methylbutanal, 2-methylbutanoic acid, 3-methylbutanoic acid, 3-methyl-1-butanol, 3-methylbutyl acetate, methyl 2-methylbutanoate, methylpropanoic acid, myrcene, *γ*-nonalactone, octanoic acid, phenylacetic acid, 2-phenylethanol, 2-phenylethyl acetate, and sotolone (Sigma-Aldrich, Taufkirchen, Germany); 2-methoxy-4-vinylphenol, nonanal, and pentanoic acid (Alfa Aesar, Karlsruhe, Germany); butanoic acid and vanillin (Merck, Darmstadt, Germany); linalool (Fluka, Neu-Ulm, Germany); 4-vinylphenol (Lancaster, Mühlheim/Main, Germany); and propyl 2-methylbutanoate (TCI, Eschborn, Germany). (*E*)-*β*-Damascenone was kindly provided by Symrise (Holzminden, Germany). 2-Acetyl-1-pyrroline was synthesized as previously described.[14]

The following chemicals were commercially obtained: liquid nitrogen (Linde, Munich, Germany); dichloromethane, diethyl ether, sodium chloride, and anhydrous sodium sulfate (Merck); argon, helium, hydrogen, and nitrogen (Westfalen, Münster, Germany); and *n*-alkanes (C5−C26) (Sigma-Aldrich). Dichloromethane and diethyl ether were freshly distilled prior to use.

Stable Isotopically Labeled Internal Standards. The following stable isotopically labeled internal standards were commercially obtained: [$^2H_{11}$]-3-methyl-1-butanol and [$^2H_2$]-methylpropanoic acid (C/D/N Isotopes, Quebec, Canada); [$^2H_6$]-myrcene (Santa Cruz Biotechnology, Dallas, TX); [$^{13}C_2$]-phenylacetic acid (Sigma-Aldrich); and [$^{13}C_6$]-vanillin (Cambridge Isotope Laboratories, Tewksbury, MA).

The following stable isotopically labeled internal standards were synthesized as previously described: [$^2H_2$]-butanoic acid;[15] [$^2H_{2-8}$]-(*E*)-*β*-damascenone;[16] [$^2H_3$]-ethyl butanoate;[16] [$^2H_3$]-ethyl hexanoate;[16] [$^2H_5$]-ethyl 2-methylbutanoate, [$^2H_9$]-ethyl 3-methylbutanoate, [$^2H_5$]-ethyl methylpropanoate, [$^2H_3$]-methyl 2-methylbutanoate, and [$^2H_3$]-propyl 2-methylbutanoate were synthesized by esterification of the labeled carboxylic acid with the respective alcohol;[17] [$^2H_2$]-geraniol;[18] [$^2H_{2-3}$]-linalool;[19] [$^2H_3$]-2-methoxy-4-vinylphenol;[20] [$^2H_9$]-3-methylbutanoic acid;[21] [$^2H_2$]-3-methylbutyl acetate;[16] [$^2H_4$]-nonanal was synthesized starting with 3-nonyn-1-ol following the procedure described for [$^2H_4$]-hexanal;[22] [$^2H_5$]-2-phenylethanol;[23] and [$^2H_5$]-2-phenylethyl acetate.[24]

The concentrations of the stable isotopically labeled internal standards were determined as recently described.[25]

Isolation of the Volatiles. An aliquot of the beer samples (250 mL) was extracted with diethyl ether (2 × 200 mL) via liquid−liquid extraction. In case of alcoholic beer, ethanol was removed by extracting the combined organic phases with saturated aqueous sodium chloride solution (2 × 200 mL). After high vacuum distillation using the solvent assisted flavor evaporation (SAFE) technique,[26] the distillate obtained was dried over anhydrous sodium sulfate, filtered, and concentrated to 1 mL using a Vigreux column (50 cm × 1 cm id).

Aroma Extract Dilution Analysis (AEDA) and Identification Experiments. AEDA and identification of aroma compounds were performed as recently described.[11] Identification was based on the comparison of odor qualities and intensities detected at the sniffing port during AEDA, retention indices (RIs) on two capillaries of different polarities (DB-FFAP and DB-5), and mass spectra in electron ionization (EI) and chemical ionization (CI) mode to data of reference compounds available in an in-house database containing >1000 odorants.

High-Resolution Gas Chromatography−Olfactometry (HRGC−O). HRGC−O and the determination of RIs for all aroma compounds were performed as recently described.[11]

High-Resolution Gas Chromatography−Sector Field Mass Spectrometry (HRGC−MS) for Identification. The identification of aroma compounds in EI and CI mode with HRGC−MS was performed as recently described.[11]

Quantitation by Stable Isotope Dilution Analysis (SIDA). For quantitation experiments, stable isotopically labeled internal standards (0.1−10 μg, dissolved in dichloromethane or diethyl ether; amounts depending on the analyte concentrations determined in preliminary experiments) were added to beer (1−200 mL) or wort (1−40 mL). After equilibration for 15 min under continuous magnetic stirring at room temperature, the samples were extracted with diethyl ether (2 × 20−200 mL, depending on the sample amount), followed by the workup procedure as described above for the isolation of the volatiles. Determination of response factors for all analytes was performed as recently described.[11]

Quantitation of 2- and 3-Methylbutanoic Acid. The two isomers of methylbutanoic acid were quantitated in sum using [$^2H_9$]-3-methylbutanoic acid as the internal standard based on GC−MS in CI mode. The ratio was determined in EI mode based on the intensities of their typical mass fragments as recently described.[11,27,28]

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC−MS) for Quantitation. HRGC−MS was performed as recently reported.[11] Peak areas of analytes and stable isotopically labeled internal standards were determined by using the protonated molecular masses or selected mass fragments obtained during GC−MS in CI mode with methanol as reagent gas (Table 1).

Two-Dimensional Heart-Cut High-Resolution Gas Chromatography−Mass Spectrometry (HRGC/HRGC−MS) for Quantitation. For minor analytes overlapped by major compounds, HRGC/HRGC−MS was applied using a J&W DB-FFAP capillary column in the first dimension and a J&W OV-1701 capillary column in the second dimension (both 30 m × 0.25 mm id, 0.25 μm film thickness; Agilent Technologies, Waldbronn, Germany).[11]

Headspace Solid Phase Microextraction Comprehensive High-Resolution Gas Chromatography−Time-of-Flight Mass Spectrometry (HS-SPME-HRGC×HRGC-TOF-MS) for Quantitation of Hop-Derived Esters. The stable isotopically labeled internal standards (1−30 ng, dissolved in ethanol; amounts depending on the analyte concentrations determined in preliminary experiments) were added to beer or wort (50 mL each), and the mixture was equilibrated (stirring for 15 min at room temperature). An aliquot of the sample (5 mL) and sodium chloride (2 g) were filled into a headspace vial (20 mL). HS-SPME was performed by a divinylbenzene/polydimethylsiloxane fiber (Supelco, Bellefonte, PA) using an MPS autosampler (Gerstel, Mühlheim/Ruhr, Germany). Adsorption onto the fiber was performed for 10 min at 30 °C and desorption for 5 min with an inlet temperature of 250 °C. The 7890A gas chromatograph (Agilent Technologies) was equipped with a J&W DB-FFAP capillary column (30 m × 0.25 mm id, 0.25 μm film thickness; Agilent Technologies) in the first dimension and a J&W VF-5-MS capillary column (2 m × 0.15 mm id, 0.30 μm film thickness; Agilent Technologies) in the second dimension. The GC was coupled to a Pegasus 4D time-of-flight mass spectrometer (LECO Instrumente, Moenchengladbach, Germany), recording mass spectra in EI mode (70 eV).

Headspace Solid Phase Microextraction−High-Resolution Gas Chromatography−Mass Spectrometry (HS-SPME-HRGC−MS) for Quantitation of Ethyl Butanoate, Ethyl Hexanoate, and 3-Methylbutyl Acetate. The respective stable isotopically labeled internal standards (0.5−5 ng, dissolved in ethanol; amounts depending on the analyte concentration determined in preliminary experiments) were added to beer or wort (1 mL each; diluted 1:10, if necessary) placed in a headspace vial (20 mL). After equilibration (stirring for 15 min at room temperature), quantitation was performed as previously described.[11]

Determination of Orthonasal Odor Thresholds (OTs) in Water and Calculation of Odor Activity Values (OAVs). Orthonasal OTs in water were determined as recently described[29] to calculate OAVs (ratio of the concentration of the odorant to its OT).

Aroma Profile Analysis (APA). For APA, six odor descriptors with the respective reference compounds were chosen: banana-like/fruity (3-methylbutyl acetate), caramel-like (furaneol), clove-like/smoky (2-methoxy-4-vinylphenol), honey-like/floral (2-phenylethanol), malty (3-methyl-1-butanol), and pungent/sour (acetic acid). Sensory analyses were performed as recently reported.[11]

RESULTS AND DISCUSSION

Aroma profile analyses showed clear differences between different dealcoholized beers and the respective regular beer. In comparison to its alcoholic counterpart, thermally dealcoholized beer clearly lacked in nearly all evaluated odor qualities, except for the malty note. In contrast, beer after SF showed a clearly increased caramel-like odor but also a stronger malty note (as shown in Figure 1 for TF beer).

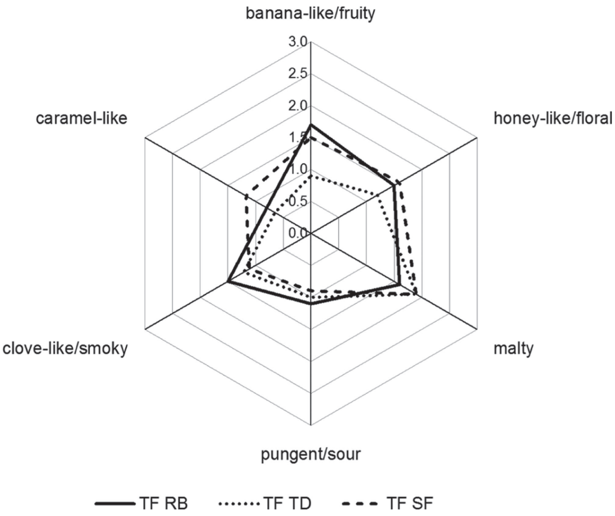

Figure 1. Aroma profiles of TF reference beer (RB; solid line), TF thermally dealcoholized beer (TD; dotted line), and TF beer after stopped fermentation (SF; broken line).

Changes in the Aroma Profile of Beer During TD. Comparative aroma extract dilution analysis (cAEDA) revealed 34 odor-active regions in BF beer and 37 in TF beer. Odor qualities and intensities, RIs on two columns of different polarities, and mass spectra in EI and CI mode, in comparison to data of reference compounds, led to the identification of 35 odorants (BF TD and TF TD; Figure 2).

Before dealcoholization, the highest FD factors (1024−4096) were determined for 2-phenylethanol, phenylacetic acid, and furaneol in the TF and BF beers. Other byproducts from the yeast metabolism, such as 3-methyl-1-butanol and 2- and 3-methylbutanoic acid, all formed via the Ehrlich pathway,[30,31] also reached high FD factors (≥128). Other Ehrlich products and yeast metabolites like ethyl butanoate, ethyl hexanoate, 3-methylbutyl acetate, 2-phenylethyl acetate, and methylpropanoic acid were present at lower FD factors. 2-Methoxy-4-vinylphenol showed a high FD factor (4096) in TF beer as a typical odorant formed by top-fermenting yeasts.[32]

Already the comparison of the flame ionization detector (FID) chromatograms of the samples before and after dealcoholization illustrates the loss of highly volatile substances (in the first 10 min) because of the heat treatment (Figure 3). The quantitative data of the regular beer and the dealcoholized beers (BF TD and TF TD; Table 2) showed high losses up to 100% for most of the quantitated odorants, among them typical beer aroma compounds such as ethyl butanoate, ethyl hexanoate, 3-methylbutyl acetate, 3-methyl-1-butanol, and 2-phenylethyl acetate but also of hop-derived odorants like linalool and geraniol. Carboxylic acids showed moderate losses between 34 and 40% in BF beer and <25% in TF beer. Only butanoic acid was nearly completely evaporated during the dealcoholization process because of its higher volatility.

Calculated OAVs clearly showed the influence of the TD on the perceived final beer aroma. Nearly all odorants were present at concentrations < their respective OTs in water after dealcoholization (cf. Tables 3 and 4), and thus, did not contribute to the overall beer aroma anymore with OAVs < 1. These results clearly explained the lack of aroma in alcohol-free beers produced by heat treatment (Figure 4).

Changes in the Aroma Profile of Wort During SF. Applying cAEDA to the unfermented wort samples and the respective SF beers (TF and BF) revealed 38 (BF) and 36 (TF) odor-active regions. Subsequent identification experiments enabled the elucidation of 32 odorants (BF SF and TF SF; Figure 2).

In wort, typical malt aroma compounds[33] like vanillin, sotolone, and furaneol showed the highest FD factors (512−4096), followed by (*E*)-*β*-damascenone and maltol with lower FD factors.

The comparison of the concentrations of selected aroma compounds (Table 2) in wort before and alcohol-free beer after SF showed a degradation of some aroma compounds like vanillin and myrcene. The loss of myrcene is in accordance with literature data and can occur during fermentation, filtering, and storage because of adsorption to yeast cells or to the sealing of crown caps caused by its apolarity.[34−37] Also, geraniol showed a slightly reduced concentration because of its biotransformation into citronellol, its acetic acid ester, or other terpenoids by yeast.[35,38−40]

In contrast, byproducts of the yeast metabolism like 3-methyl-1-butanol, 2-phenylethanol, 2-phenylethyl acetate, ethyl butanoate, ethyl hexanoate, 3-methylbutyl acetate, and acids like butanoic acid and methylpropanoic acid were formed. Again, the different activities of top- and bottom-fermenting yeasts were clearly visible in the concentrations of 2-methoxy-4-vinylphenol, which increased during the fermentation in the TF but not in the BF sample. Also, (*E*)-*β*-damascenone showed a different behavior in the samples, degraded in the BF sample but formed during fermentation in the TF sample. No significant changes during fermentation were found for 2- and 3-methylbutanoic acid, phenylacetic acid, nonanal, linalool, geraniol, and different fruity smelling esters derived from 2- and 3-methylbutanoic acid (Table 2).

OAVs showed that the limited fermentation time is still long enough for the formation of most of the relevant odorants in concentrations > their respective OTs, and thus, for a contribution to the overall aroma. However, in comparison to the corresponding alcoholic beers (samples before TD), OAVs were clearly lower, for example, an OAV of 3-methyl-1-butanol of 300 in alcoholic beer versus an OAV of 34 in alcohol-free beer obtained after SF (Figure 4). Thus, the beer-like aroma expression of these samples was significantly lower than in fully fermented beers.

Transfer Rates of Hop-Derived Odorants During Dry-Hopping. For evaluating the transfer rates of hop-derived aroma compounds into beer during dry-hopping, selected odorants were quantitated in the beer samples, dry-hopped either with HMB, HCA, or HHA. Odorants were chosen based on their importance for the hop aroma,[11] their potential to elicit a hoppy aroma in beer because of low OTs in water, and their changes found during cAEDA with the non-hopped samples.

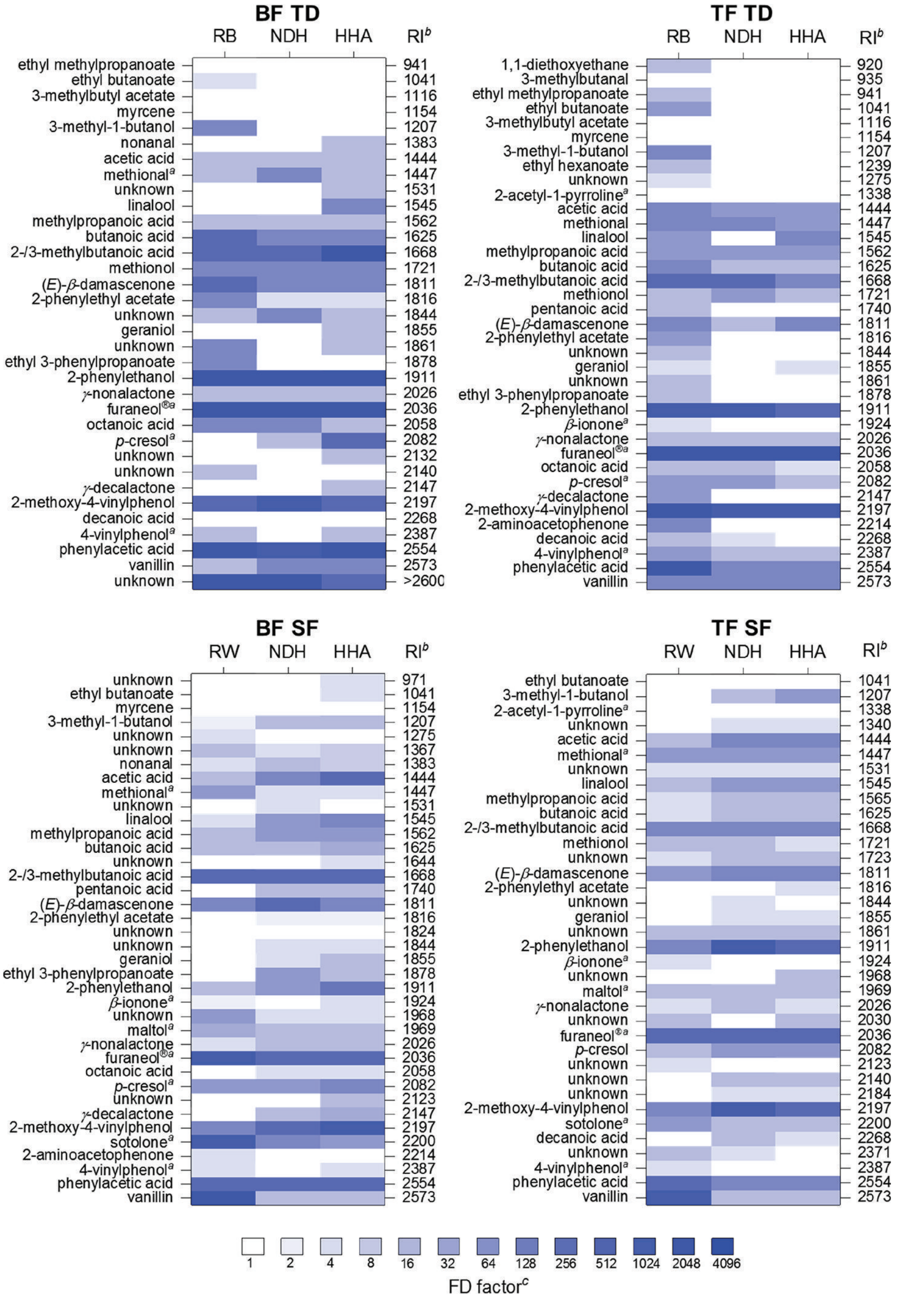

Figure 2. AEDA of BF and TF beers produced either via thermal dealcoholization (TD) or stopped fermentation (SF). Reference beer (RB), reference wort (RW), not dry-hopped alcohol-free beer (NDH), and dry-hopped alcohol-free beer with HHA (HHA). [a]No unequivocal mass spectrum obtained; identification based on remaining criteria (see Materials and Methods). [b]Retention indices (RIs), calculated from the retention time of the compound and the retention times of adjacent *n*-alkanes by linear interpolation. [c]Flavor dilution factors determined by AEDA on a DB-FFAP capillary column. FD factors are illustrated using a color code from 1 (white) to 8192 (blue).

Using the concentrations in beer (Tables 2 and 3), the concentrations in hop pellets determined in a previous study,[11] and the hop dosage (1.5 mL of hop oil/hL, which is equivalent to 1.5 g of hops/L for HMB and HHA and 1.25 g of hops/L for HCA, respectively), the transfer rates were calculated (Table 5).

As already known, linalool is one of the most important hop-derived odorants in dry-hopped beers.[41,42] Also, in the present study, linalool reached concentrations up to 154 μg/L in dry-hopped beers and transfer rates of 91% in maximum. An additional formation of linalool, leading to a theoretical transfer rate ≥100%, as reported in literature,[43] could not be confirmed. In contrast, myrcene showed very low transfer rates of <1.3% because of its nonpolar structure, and consequently low water solubility, as well as its adsorption capability to crown caps or losses during filtering.[34−37]

For geraniol, transfer rates of 53−98% were determined in samples dry-hopped with HMB or HHA. For the hop variety HCA, theoretical transfer rates up to 400% were calculated, indicating a formation of geraniol during dry-hopping,

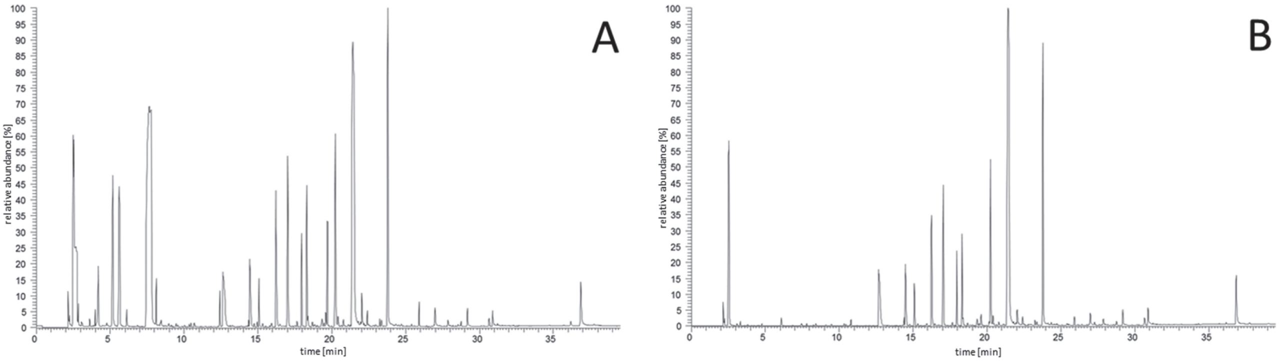

Figure 3. GC−FID chromatograms of BF reference beer (A) and BF thermally dealcoholized beer (B).

Table 2. Concentrations of Selected Odorants in BF and TF Beer before and after Thermal Dealcoholization (TD) and Stopped Fermentation (SF)

	concentration [μg/L][a]							
	BF TD		BF SF		TF TD		TF SF	
compound	before	after	before	after	before	after	before	after
ethyl 2-methylbutanoate[b]	0.54	<0.01[c]	<0.011[c]	0.011	0.548	<0.011[c]	<0.011[c]	0.021
ethyl 3-methylbutanoate[b]	2.55	0.07	0.013	0.030	1.120	<0.007[c]	0.013	0.018
ethyl methylpropanoate[b]	nq[d]	<0.01[c]	0.012	0.087	13.8	<0.012[c]	0.034	0.209
methyl 2-methylbutanoate[b]	0.05	<0.01[c]	0.076	0.045	0.053	<0.029[c]	0.168	0.073
propyl 2-methylbutanoate[b]	<0.01[c]	<0.01[c]	<0.051[c]	<0.051[c]	<0.051[c]	<0.051[c]	0.102	<0.051[c]
butanoic acid	15,000	487	3350	4160	15,900	850	1860	2790
(*E*)-*β*-damascenone	nq[d]	nq[d]	4.23	1.12	nq[d]	nq[d]	0.67	1.60
ethyl butanoate	64.7	0.10	0.12	2.01	105	0.12	0.16	2.95
ethyl hexanoate	98.8	0.11	0.04	2.05	156	0.70	2.09	4.12
geraniol	3.07	0.01	13.0	8.97	7.05	0.57	9.11	8.94
linalool	5.19	0.56	28.6	26.3	58.6	0.35	13.3	18.8
2-methoxy-4-vinylphenol	nq[d]	nq[d]	155	90.8	nq[d]	nq[d]	62.0	88.3
2-methylbutanoic acid	968	642	136	165	779	594	145	220
3-methylbutanoic acid	864	573	566	598	966	951	313	377
3-methyl-1-butanol	51,200	5.89	247	5850	65,200	126	157	7470
3-methylbutyl acetate	915	1.16	0.86	15.7	2230	9.04	1.60	31.2
methylpropanoic acid	690	416	485	579	3670	3790	251	1280
myrcene	0.27	0.14	11.4	0.08	1.00	0.33	79.7	1.84
nonanal	9.04	6.72	3.05	3.41	2.97	3.39	2.80	2.57
phenylacetic acid	306	336	205	215	362	375	131	137
2-phenylethanol	nq[d]	nq[d]	144	1410	nq[d]	nq[d]	155	1600
2-phenylethyl acetate	695	13.6	1.24	7.67	789	11.8	3.17	56.8
vanillin	4.50	5.00	205	15.8	nq[d]	nq[d]	249	11.5

[a] Mean values of triplicates, not differing more than ±15%. [b] Quantitated either via HRGC/HRGC−MS in CI mode (for BF TD) or via HRGCxHRGC-TOF-MS in EI mode (for BF SF, TF TD, and TF SF). [c] Limit of quantitation (LOQ) was determined on the basis of a signal-to-noise ratio of 10. [d] Not quantitated.

additional to the transfer from hops. The same phenomenon was already described earlier,[9] and a hydrolysis of geranyl acetate to geraniol was proposed later.[44] This hypothesis was supported by quantitative data obtained for geranyl acetate in the hop varieties used in this study, showing clearly higher concentrations (17,900 μg/kg in HCA, 1,980 μg/kg in HMB, and 487 μg/kg in HHA) only in Cascade hops.

The theoretically calculated transfer rates of ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, and ethyl methylpropanoate from 63 to >8000% cannot be explained by a simple transfer from hops into beer during dry-hopping. Rather they indicate a formation of these esters, induced by the hop dosage. Also, methyl 2-methylbutanoate showed transfer rates >100% but not as high as the ethyl esters. For propyl 2-methylbutanoate, transfer rates up to 57% were determined. In contrast to other studies, which reported the absence of ethyl 3-methylbutanoate and methyl 3-methylbutanoate in dry-hopped beers,[43,45] ethyl 3-methylbutanoate was quantitated in amounts between 0.03 and 2.46 μg/L in this study (Table 3), and methyl 3-methylbutanoate was identified by its mass spectrum, although it was not perceived during AEDA and also not quantitated.

In literature, the formation of ethyl esters, namely, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, and ethyl methylpropanoate, is described as an esterification of the hop-derived monocarboxylic acids with ethanol, and that it can be increased by the usage of aged hops.[46,47] Thereby, the monocarboxylic acids in hops derive from the degradation of bitter acids.[47,48]

Table 3. Concentrations of Selected Odorants in BF and TF Beer after Thermal Dealcoholization (TD) and Stopped Fermentation (SF), Dry-Hopped with Either HMB, HCA, or HHA

	concentration [μg/L][a]											
	BF TD			BF SF			TF TD			TF SF		
compound	HMB	HCA	HHA	HMB	HCA	HHA	HMB	HCA	HHA	HMB	HCA	HHA
ethyl 2-methylbutanoate[b]	0.21	0.03	0.03	0.082	0.047	0.053	0.062	0.035	0.018	0.356	0.172	0.064
ethyl 3-methylbutanoate[b]	2.46	0.56	0.34	0.104	0.070	0.078	0.034	0.044	0.036	0.290	0.210	0.087
ethyl methylpropanoate[b]	1.22	0.26	0.18	0.835	1.14	0.632	0.191	0.316	0.139	1.53	1.80	0.763
methyl 2-methylbutanoate[b]	0.13	0.12	0.10	0.261	0.157	0.214	0.293	0.168	0.129	0.415	0.332	0.240
propyl 2-methylbutanoate[b]	0.13	0.02	0.02	<0.051[c]	<0.051[c]	<0.051[c]	0.123	<0.051[c]	<0.051[c]	0.169	0.051	<0.051[c]
butanoic acid	472	457	428	4090	3960	3850	779	900	779	2700	2550	2530
geraniol	74.3	38.9	19.8	81.3	105	30.9	53.1	108	14.3	91.2	158	24.1
linalool	47.2	49.7	137	61.1	54.0	97.1	41.6	55.8	154	70.0	70.1	142
2-methylbutanoic acid	786	709	668	197	179	163	592	572	548	242	227	217
3-methylbutanoic acid	701	633	596	708	651	625	1010	948	807	462	398	390
methylpropanoic acid	648	569	453	714	736	697	4200	4200	3840	1410	1390	1390
myrcene	4.80	21.9	14.4	19.8	2.05	2.13	64.9	108	93.2	44.6	86.5	67.2
nonanal	4.62	4.06	5.76	3.37	3.10	3.58	2.98	3.00	3.52	2.46	3.62	3.71

[a] Mean values of triplicates, not differing more than ±15%. [b] Quantitated either via HRGC/HRGC−MS in CI mode (for BF TD) or via HRGCxHRGC-TOF-MS in EI mode (for BF SF, TF TD, and TF SF). [c] LOQ was determined on the basis of a signal-to-noise ratio of 10.

Table 4. Odor Thresholds (OTs) in Water of the Quantitated Aroma Compounds

compound	OT [μg/kg][a]
ethyl 2-methylbutanoate	0.014[b]
ethyl 3-methylbutanoate	0.023
ethyl methylpropanoate	0.089
methyl 2-methylbutanoate	0.14
propyl 2-methylbutanoate	0.15
butanoic acid	2400
(*E*)-*β*-damascenone	0.006
ethyl butanoate	0.75
ethyl hexanoate	1.2
geraniol	1.1
linalool	0.58
2-methoxy-4-vinylphenol	21
2-methylbutanoic acid	3100
3-methylbutanoic acid	490
3-methyl-1-butanol	220
3-methylbutyl acetate	7.2
methylpropanoic acid	60,000
myrcene	1.2
nonanal	2.8
phenylacetic acid	68
2-phenylethanol	140
2-phenylethyl acetate	360
vanillin	53

[a] Data from an in-house database. [b] OT newly determined in the present study.

Also, in this study, an increase of the acid concentrations was found for most of the analyzed samples. The non-enzymatic formation of acids and esters is postulated to be an aging reaction during beer storage, which is not caused by yeast.[46,48,49] However, this formation would be a rather slow reaction. An enzymatic esterification caused by yeast was postulated as a second possible formation pathway of these esters, catalyzed by acetyl-CoA:ethanol *O*-acetyltransferases (AEATs).[47,50,51] Also, the effect of an increase in the concentrations of these esters with a later hop addition was described.[47]

However, all explanations for elevated ethyl ester concentrations cannot explain the high concentrations in the analyzed dry-hopped samples in the present study (Table 3). On the one hand, already in the non-hopped samples, the concentrations of the respective monocarboxylic acids would have been high enough (in the same ranges as some of the dry-hopped beers) for a storage-caused ester formation. Due to the fact that the samples before and after dry-hopping were stored under the same conditions and for the same time, ester formations should take place in both, and only the transfers from acids into the beer cannot explain this phenomenon. On the other hand, yeast metabolism can also not be the origin of these ethyl esters because then no difference between the dry-hopped and non-hopped samples should be obtained.

Another possible biosynthetic pathway of these esters could be the formation from different amino acids acting as precursors, catalyzed by AEATs. Yeast-originating AEATs, producing acetic acid esters and fatty acid ethyl esters during beer fermentation, are well-known.[52−55] The two different AEATs Eht1 and Eeb1 have been identified in yeast, catalyzing the formation of ethyl esters with medium chain fatty acids by a condensation reaction.[56,57] In this way, isoleucine will be the biosynthetic precursor of 2-methylbutyl and 2-methylbutanoate esters, leucine of 3-methylbutyl and 3-methylbutanoate esters, and valine of methylpropyl and methylpropanoate esters.[58,59] Thus, the presence of AEATs in hops could corroborate the assumption of a hop-induced enzymatic ester formation.

Contribution of Hop-Derived Aroma Compounds to the Aroma of Dry-Hopped Beers. In a next step, the contribution of the quantitated odorants to the hoppy aroma of dry-hopped beer was evaluated by calculating OAVs on the basis of the analytical quantitative data combined with the sensorial data of the respective OTs in water (Table 5). Thereby, several hop-derived odorants revealed OAVs ≥ 1, and thus, a clear influence of dry-hopping on the overall aroma of alcohol-free beer was proven (Figure 4).

Highest OAVs of 480−1800 were calculated for linalool in all samples. Linalool is already known for its importance for the hoppy aroma in beer.[41,42] For myrcene, the OAVs in dry-hopped beers strongly differed from 2 to 90 because of losses

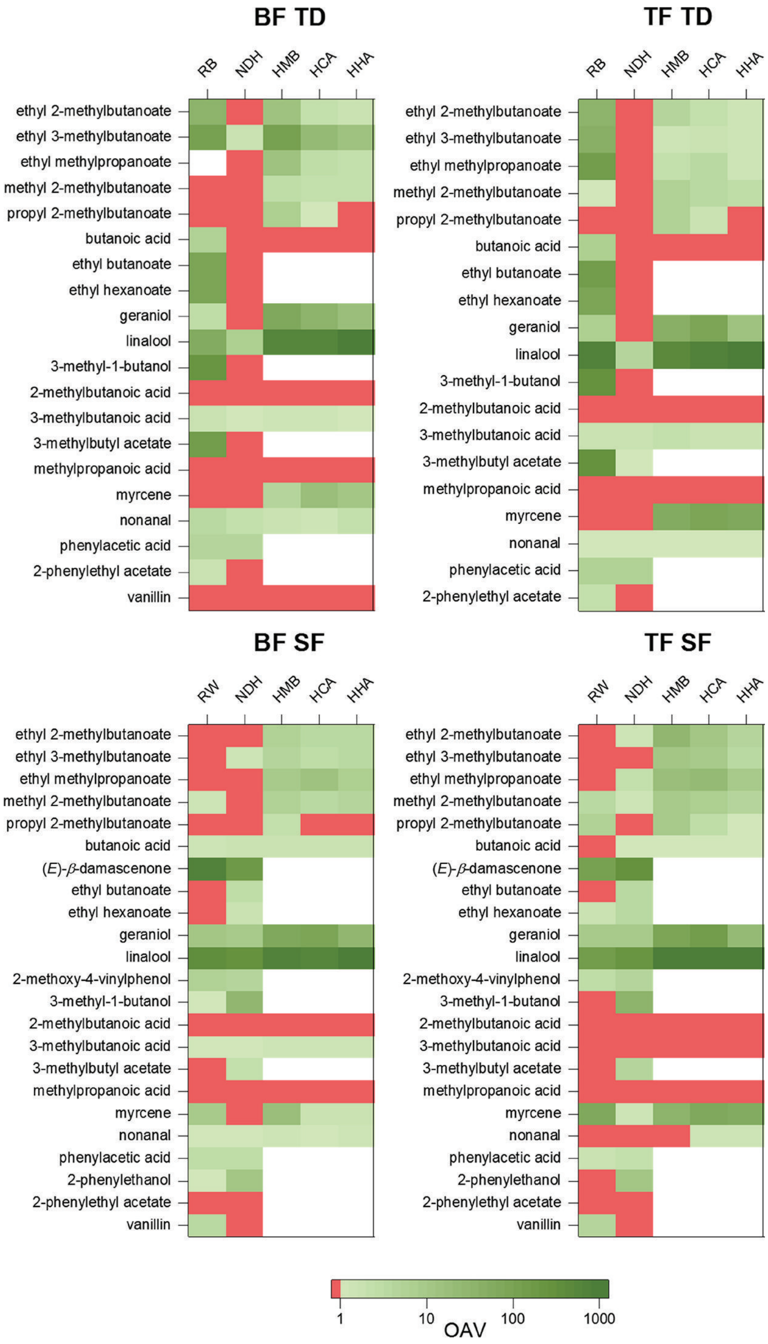

Figure 4. OAVs of BF and TF beers produced either via thermal dealcoholization (TD) or stopped fermentation (SF). Reference beer (RB), reference wort (RW), not dry-hopped alcohol-free beer (NDH), dry-hopped alcohol-free beer with HMB (HMB), HCA (HCA), or HHA (HHA). OAVs are illustrated using a color code from <1 (red) to >1000 (green). White areas: compounds not quantitated.

during beer staling as mentioned above. Thereby, its contribution to the overall aroma was more pronounced for TF beers (OAVs = 37−90) compared to BF beers (OAVs = 2−18). Also, the quantitated esters contributed to the aroma of dry-hopped beers with OAVs up to 10. The additionally formed ethyl esters reached OAVs of 15−110 in some of the samples.

In summary, this study proved that dry-hopping is a possibility to modify the aroma of alcohol-free beers and mask aroma deficiencies caused by TD or SF. Consequently, based on a market with a high demand for alcohol-free or alcohol-reduced beers, dry-hopping can help to increase the popularity of alcohol-free beer for consumers, especially for those who refused it, up to now, because of its aroma differences to regular alcoholic beer.

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Table 5. Transfer and Formation Rates of Selected Hop-Derived Odorants in BF and TF Beer after Thermal Dealcoholization (TD) and Stopped Fermentation (SF), Dry-Hopped with Either HMB, HCA, or HHA

	transfer/formation rate [%][a]											
	BF TD			BF SF			TF TD			TF SF		
Compound	HMB	HCA	HHA	HMB	HCA	HHA	HMB	HCA	HHA	HMB	HCA	HHA
ethyl 2-methylbutanoate	390	110	450	140	190	1100	99	130	210	640	800	1100
ethyl 3-methylbutanoate	24,000	8300	nc[b]	720	640	nc[b]	280	600	nc[b]	520	3100	nc[b]
ethyl methylpropanoate	840	290	1200	520	1200	3900	130	360	910	2600	1900	3900
methyl 2-methylbutanoate	61	71	150	110	74	280	130	93	170	170	170	270
propyl 2-methylbutanoate	57	22	16	0[c]	0[c]	0[c]	34	0[c]	0[c]	55	2.9	0[c]
geraniol	74	110	89	72	270	98	53	300	61	82	420	68
linalool	61	62	80	46	35	42	54	70	91	59	58	66
myrcene	0.09	0.19	0.19	0.40	0.02	0.03	1.3	0.94	1.3	0.83	0.74	0.88

[a] If the amount in the sample was <LOQ, LOQ was taken for the calculation of transfer/formation rates. [b] Not calculable because odorant was not quantitated in hops. [c] Concentrations in dry-hopped beer <LOD, resulting in a transfer rate of "0".

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NOMENCLATURE

2-acetyl-1-pyrroline, 1-(3,4-dihydro-2*H*-pyrrol-5-yl)ethanone; 2-aminoacetophenone, 1-(2-aminophenyl)ethanone; *p*-cresol, 4-methylphenol; (*E*)-*β*-damascenone, (2*E*)-1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one; *γ*-decalactone, 5-hexyldihydro-2(3*H*)-furanone; furaneol, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone; geraniol, (2*E*)-3,7-dimethyl-2,6-octadien-1-ol; geranyl acetate, (2*E*)-3,7-dimethyl-2,6-octadien-1-yl acetate; *β*-ionone, (3*E*)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one; linalool, 3,7-dimethyl-1,6-octadien-3-ol; maltol, 3-hydroxy-2-methyl-4*H*-pyran-4-one; methional, 3-(methylsulfanyl)propanal; methionol, 3-(methylsulfanyl)-1-propanol; myrcene, 7-methyl-3-methylene-1,6-octadiene; *γ*-nonalactone, 5-pentyldihydro-2(3*H*)-furanone; *γ*-octalactone, 5-butyldihydro-2(3*H*)-furanone; sotolone, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone; vanillin, 4-hydroxy-3-methoxybenzaldehyde

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9.3 Hop-Induced Formation of Ethyl Esters in Dry-Hopped Beer

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Hop-induced formation of ethyl esters in dry-hopped beer

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Abstract

Fruity smelling esters play an important role for the aroma of hops and beer and they have been characterized as key aroma compounds in different hop varieties. Studies on the transfer of hop-derived compounds into beer during dry-hopping showed calculated transfer rates of different ethyl esters far above 100%, leading to the assumption that these esters must be newly formed. To investigate this formation, dry-hopping was imitated in water to eliminate the influence of the beer matrix on the formation of these odorants. Thereby, the formation of ethyl esters of 2-methylbutanoic acid, 3-methylbutanoic acid, and methylpropanoic acid, induced by the addition of hops, was shown. Different approaches inhibiting enzyme activities and experiments with different hop extracts might lead to the assumption that enzymes are involved in the formation of these esters, beside possible transesterification.

Keywords: Hops, Dry-hopping, Beer, Ethyl esters, Aroma formation, Radicals, Enzymes

Introduction

Previous studies on dry-hopping have evaluated the transfer of, among other odorants, different ethyl esters from hops into beer. Thereby, calculated transfer rates far above 100% were observed (Brendel et al. 2020; Neiens and Steinhaus 2019). It is already known that hops can be the source of monocarboxylic acids, that are needed for the formation of these esters, namely, 2- and 3-methylbutanoic acid and methylpropanoic acid, formed by the degradation of humulones (Rettberg et al. 2014; Williams and Wagner 1979). Thus, a subsequent esterification of the respective monocarboxylic acids with ethanol was assumed, but has not been verified by further investigations (Schnaitter et al. 2016a, b). In beer, different formation pathways of ethyl esters have already been suggested, e.g., a non-enzymatic reaction during beer storage (Vanderhaegen et al. 2003, 2007; Williams and Wagner 1978, 1979). Other studies have postulated the formation of ethyl esters via transesterification of other hop-derived esters like methylpropyl methylpropanoate, 3-methylbutyl methylpropanoate, and 2-methylbutyl methylpropanoate during fermentation (Takoi et al. 2018; Forster and Gahr 2013). A further possibility is the enzymatic esterification during yeast fermentation by acetyl-CoA:ethanol *O*-acetyltransferases (AEATs) (Dank et al. 2018; Rettberg et al. 2014; Saerens et al. 2008). Two AEATs in yeast have already been identified as Eht1 and Eeb1, catalyzing a condensation of medium chain fatty acids with ethanol (Saerens et al. 2006; Mason and Dufour 2000). Isoleucine, leucine, and valine are known as precursors for 2-methylbutyl and 2-methylbutanoate esters, 3-methylbutyl and 3-methylbutanoate esters, and methylpropyl and methylpropanoate esters (Matich and Rowan 2007; Rowan et al. 1996).

However, in a very recent study (Brendel et al. 2020), ethyl ester formation has mainly been observed in dry-hopped samples, even if the concentrations of the respective monocarboxylic acids as precursors would have been high enough (in the same ranges as some of the dry-hopped beers) in the non-hopped reference samples. Due to the fact that the samples before and after dry-hopping were stored under the same conditions and for the same time, ester formations should have taken place in both. Thus, only the transfers from acids into the beer, followed by their esterification cannot explain this

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phenomenon. On the other hand, yeast metabolism can also not be the origin of these ethyl esters, because then, no difference between the dry-hopped and non-hopped samples should have been obtained. If hops contain unknown AEATs, an enzymatic ester formation might be induced by hop addition into beer (Brendel et al. 2020).

Thus, this study should clarify the role of hops in the formation of ethyl esters and give hints on monocarboxylic acids or methylpropanoic acid esters as precursors within a possible transesterification. Therefore, model studies in water were performed to confirm the formation of the target esters and to exclude the influence of yeast enzymes on the reaction. To arrive at a more accurate picture, different hop extracts were used in the model system studies and different methods of enzyme inhibition should give deeper insights into the reaction type.

Materials and methods

Hop samples

Hallertauer Mandarina Bavaria hop pellets type 90, harvest year 2015, were provided by Hopsteiner (Mainburg, Germany). Identification and quantitation of odorants in this batch have already been performed in a previous study (Brendel et al. 2019). Hop extracts were also provided by Hopsteiner: Alpha extract contained 20.0 ± 1.0% α-acids. Beta rich hop extract was specified with 40 ± 0.2% β-acid content. XanthoFlav™ extract was coated on diatomaceous earth with a specification of 7–12% xanthohumol (without carrier material). Tannin extract contained 2–6% low molecular weight polyphenols.

Chemicals

The following reference compounds were purchased from commercial sources: ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, 2-methylbutanoic acid, 3-methylbutanoic acid, and methylpropanoic acid (Sigma-Aldrich, Taufkirchen, Germany).

The following chemicals were obtained commercially: calcium chloride dihydrate, diethyl ether, ethanol, anhydrous sodium sulfate (Merck, Darmstadt, Germany); L-ascorbic acid (Sigma-Aldrich); and argon, helium, hydrogen, and nitrogen (Westfalen, Münster, Germany).

Stable isotopically labeled internal standards

The stable isotopically labeled internal standards were synthesized as previously described (Guth and Grosch 1993): [$^2H_5$]-ethyl 2-methylbutanoate, [$^2H_9$]-ethyl 3-methylbutanoate, and [$^2H_5$]-ethyl methylpropanoate via esterification of the respective labeled carboxylic acid with ethanol.

Isolation of the esters from hop extracts

To hop extract (0.1–1 g), diethyl ether (2 × 100 mL) and the stable isotopically labeled internal standards (0.2–0.8 μg, dissolved in ethanol; amounts depending on the concentration of the respective analyte, determined in preliminary experiments) were added. After equilibration, the sample was extracted by stirring for 2 × 1.5 h at room temperature. The extract obtained was subjected to high vacuum distillation using the solvent assisted flavor evaporation (SAFE) technique (Engel et al. 1999). After drying over anhydrous sodium sulfate, the filtered sample was concentrated to ~ 100 μL using a Vigreux column (50 cm × 1 cm id), followed by microdistillation. The concentrated extract was used for two-dimensional high-resolution heart-cut gas chromatography-mass spectrometry (HRGC/HRGC-MS).

Model system studies with hops

For the dry-hopping model, water (500 mL), ethanol (250 μL), hops (0.8 g), 2-methylbutanoic acid, 3-methylbutanoic acid, and methylpropanoic acid (250 μg each) were used to prepare six different models: model A containing all compounds mentioned above; model B without hops; model C without ethanol; model D without carboxylic acids; model E without argon atmosphere; and model F in a clear glass bottle. The samples were stirred at room temperature for 10 days in amber glass bottles (except for model F) under argon (except for model E). The concentrations of the esters were measured via headspace solid phase microextraction comprehensive two-dimensional high-resolution gas chromatography-time-of-flight mass spectrometry (HS-SPME-HRGCxHRGC-TOF-MS).

Model system studies to evaluate the reaction type

A further model in a clear glass bottle with the addition of ascorbic acid (5 g) as antioxidant was prepared (model G). Next, model A (as described above) was modified by the addition of calcium chloride (5 g) to evaluate the influence of enzyme activities (model H) on the ester formation. For the next experiment, hops were heat-processed in water under reflux for 20 min prior to the addition of ethanol and the acids (model I) to evaluate the influence of a heat treatment on a possibly enzymatic activity. The concentrations of the esters were analyzed again via HS-SPME-HRGCxHRGC-TOF-MS.

Model system studies with hop extracts

For each hop extract, two further models were prepared: water (100 mL), ethanol (50 μL), and the respective hop extract (0.15 g) were stirred under argon in amber glass bottles for 10 days at room temperature, once without the addition of carboxylic acids (model J) and once with carboxylic acids (250 μg each) (model K). Again, the concentrations of the esters were measured via HS-SPME-HRGCxHRGC-TOF-MS.

Two-dimensional high-resolution heart-cut gas chromatography-mass spectrometry (HRGC/HRGC-MS)

For quantitation of the esters in the hop extracts, HRGC/HRGC-MS was performed as recently described (Brendel et al. 2019).

Headspace solid phase microextraction-comprehensive two-dimensional high-resolution gas chromatography-time-of-flight mass spectrometry (HS-SPME-HRGCxHRGC-TOF-MS)

To an aliquot of the aqueous samples (50 mL), the stable isotopically labeled internal standards (10–35 ng, dissolved in ethanol; amounts depending on the concentration of the respective analyte, determined in preliminary experiments) were added and the mixture was stirred for 15 min at room temperature for equilibration. An aliquot (5 mL) and sodium chloride (2 g) were filled into a headspace vial (20 mL) and the measurements were performed as previously described (Brendel et al. 2020).

Results and discussion

Model system studies with hops

The study setup using water, ethanol, hops, and monocarboxylic acids was chosen to simulate the dry-hopping process and to exclude the possible influence of the beer matrix with yeast enzymes on the formation of the odorants. By performing the experiments with an alcohol content of 0.05% vol, also alcohol-free beers can be included into the resulting considerations, for which an ester formation has also been observed (Brendel et al. 2020). Therefore, the study should also answer the question whether this low alcohol content, that for instance can be reached via thermal dealcoholization, is sufficient for esterification.

Figure 1 shows the results of all models, for which hop pellets were used, in comparison to the concentrations of the respective esters in the used amount of hops. All models are compared to model A (consisting of water, ethanol, hops, and acids in an amber glass bottles under argon), which functions as a dry-hopping model without any additives or special treatments. Already the comparison of the results obtained for model A (0.37–2.3 μg/L) to the concentrations of ethyl esters in hop pellets

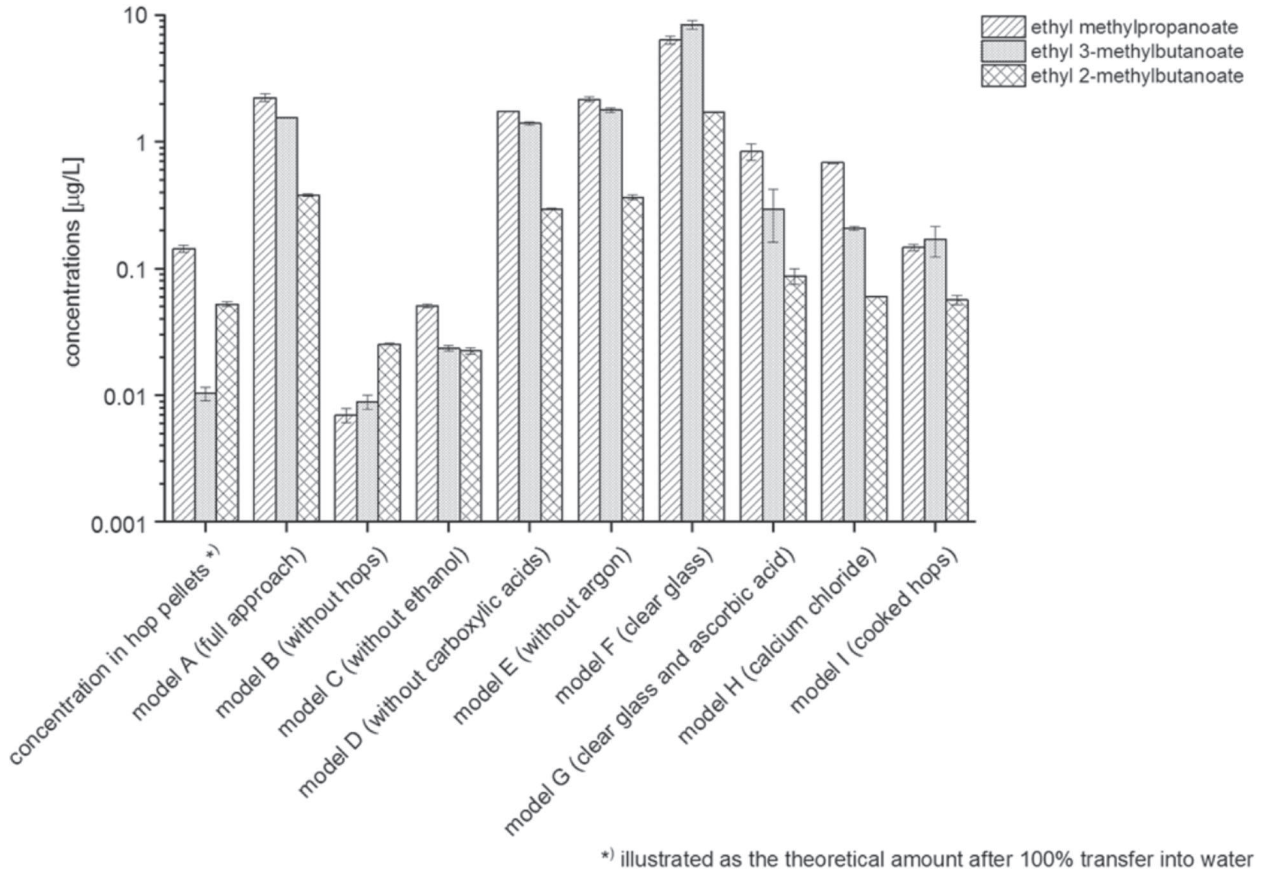


Fig. 1 Concentrations of ethyl esters on a logarithmic scale in the model system studies using hop pellets

(6.9–96 μg/kg, which is equivalent to 0.011–0.15 μg/L, based on a theoretical 100% transfer from hops into water) underlines the hypothesis, that a formation of these esters is caused by the addition of hops due to their clearly increased concentrations. In contrast, the esterification of the added carboxylic acids with ethanol without hop addition (model B) was clearly lower (0.007–0.025 μg/L) than in the dry-hopped approach (model A). This experiment also showed that the fermentation process and the activity of yeast enzymes is not relevant for the observed formation of ethyl esters in real beer samples. As a control, the model without ethanol (model C) showed ester concentrations in the same range as the concentrations in the used hop pellets, showing an ester transfer during dry-hopping.

Interestingly, the added monocarboxylic acids in model A did not influence the ester formation since their concentrations (0.29–1.8 μg/L) did not change significantly without the addition of the acids (model D). These results indicated that monocarboxylic acids derived from hop humulones and lupulones may not be the precursors for the esterification. Also Takoi et al. concluded that the concentrations of the formed esters should be much higher, if they are formed from the respective acids, as there is a high excess of hop bitter acids in beer (Takoi et al. 2018). Thus, a transesterification of other hop-derived esters seems to be more likely than the esterification of ethanol with hop-derived acids.

It has already been shown that also methyl 2-methylbutanoate is formed during dry-hopping of (alcohol-free) beer (Brendel et al. 2020; Neiens and Steinhaus 2019). This increase interferes with a possible decrease of methyl ester concentrations by transesterification to ethyl esters, and thus, cannot be used as indicator for the ethyl ester formation. The same behavior could be shown by a semi-quantitative estimation of the contents of methyl 3-methylbutanoate and methyl methylpropanoate in hops and dry-hopped beer (data not published). However, also 2-methylbutyl methylpropanoate, 3-methylbutyl methylpropanoate, and methylpropyl methylpropanoate have been postulated as precursors for the formation of ethyl esters in beer (Forster and Gahr 2013). These esters have been identified in different hop varieties and dry-hopped beer (Forster and Gahr 2013, 2014; Schmidt and Biendl 2016). Thereby, transfer rates of 40–72% were reported during dry-hopping with Hallertauer Cascade and Hallertauer Mandarina Bavaria and, therefore, the remaining 28–60% might be transesterified to ethyl esters (Forster and Gahr 2013, 2014). On the other hand, after dry-hopping with Hallertauer Huell Melon, Hallertauer Blanc, and Hallertauer Polaris, transfer/formation rates up to 161% (3-methylbutyl methylpropanoate) and 222% (2-methylbutyl methylpropanoate) were observed (Forster and Gahr 2013), and thus, these esters did not seem to be the precursors of ethyl esters, or these esters did show even higher transfer/formation rates and were partially transesterified to ethyl esters.

In previous studies, the concentrations of 2-methylbutyl methylpropanoate (40–300 mg/kg), 3-methylbutyl methylpropanoate (10–40 mg/kg), and methylpropyl methylpropanoate (10–120 mg/kg) were quantitated in Hallertauer Mandarina Bavaria, Hallertauer Cascade, and Hallertauer Mittelfrüh (Forster and Gahr 2013, 2014). The concentrations were much higher compared to those of the investigated ethyl esters in the hop varieties of the present study, with a maximum concentration of 96 μg/kg (Brendel et al. 2019). In beer, concentrations of 22–26 μg/L (3-methylbutyl methylpropanoate), 32–63 μg/L (methylpropyl methylpropanoate), and 200–340 μg/L (2-methylbutyl methylpropanoate) were reported (Forster and Gahr 2013, 2014).

In the model experiments, the concentrations of possible “precursor esters”, e.g., 2-methylbutyl methylpropanoate and methylpropyl methylpropanoate, were semiquantitated (due to the missing corresponding labeled standard) in model A and model C using the concentration of ethyl methylpropanoate as a reference value (Table 1).

While the concentration of ethyl methylpropanaote was much higher in model A compared to model C (without ethanol), the related esters 2-methylbutyl methylpropanoate and methylpropyl methylpropanoate only showed a slight and not significant increase in their concentrations from model C to model A (Table 1). Furthermore, based on literature data, the estimated concentrations correspond to a transfer from hops into the solution of < 100%, so the differences can also be caused by a variability of transfer (Forster and Gahr 2013). In the case of transesterification of the precursor esters to the corresponding ethyl esters, the precursor concentrations should be higher in model C. But as the formed amount of ethyl methylpropanoate is quite low related to the concentrations of the possible precursor esters, the possibility of transesterification cannot be excluded based on this semiquantitative data.

The presence of oxygen (model E without argon atmosphere) did not show any effects on the amount of

Table 1 Quantitative data of ethyl methylpropanoate and semi-quantitative data of the possible precursor esters 2-methylbutyl methylpropanoate and methylpropyl methylpropanoate in model A and model C

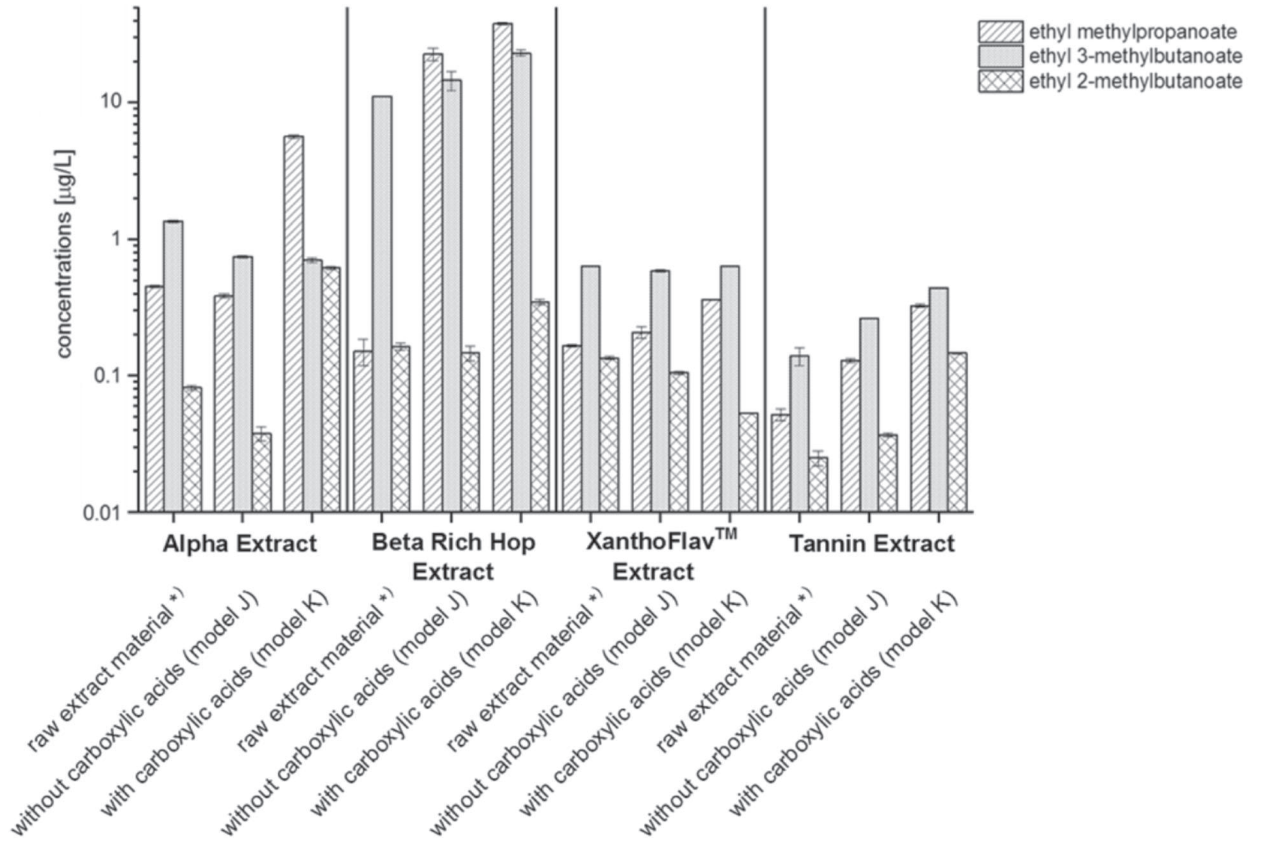
Sample	Concentrations [μg/L]		
	ethyl methylpropanoate	2-methylbutyl methylpropanoate	methylpropyl methylpropanoate
Model A	2.2	140	46
Model C	0.05	110	43

the formed esters (0.36–2.2 μg/L). Next, the influence of light on the formed amounts of esters was analyzed using a clear glass bottle (model F). Thereby, the ester formation increased by a factor of 3–5 to 1.7–8.3 μg/L, which might be a hint to a radical mechanism in ester formation. To reduce radical, but also enzymatic reactions, the clear glass sample was additionally treated with ascorbic acid (model G), which has antioxidative properties and influences the pH value as well. With this approach, the ester concentrations decreased by 62–81% to 0.087–0.84 μg/L. Normally, a lower pH value should lead to a faster formation of esters of monocarboxylic acids and ethanol and also to a faster transesterification. If the reaction occurs due to an enzymatic activity, the respective enzymes can be inhibited by a suboptimal pH value. Possibly, beside the change of the pH value of the solution, the influence of the antioxidant properties of ascorbic acid on the reaction might be secondary, as hops already contain antioxidants like xanthohumol and other polyphenols.

To further test the involvement of enzymatic reactions, heat treatment and salt addition was used to decrease the enzymatic activity. Using calcium chloride (model H), the ester concentrations decreased by 69% for ethyl methylpropanoate (0.68 μg/L), by 84% for ethyl 2-methylbutanoate (0.06 μg/L), and by 87% for ethyl 3-methylbutanoate (0.21 μg/L). Also a heat treatment of the hops (model I) for a thermal inactivation of enzymes prior to the addition of ethanol decreased the ester concentrations by 85% for ethyl 2-methylbutanoate (0.06 μg/L), by 89% for ethyl 3-methylbutanoate (0.17 μg/L), and by 93% for ethyl methylpropanoate (0.15 μg/L). Thus, hops that is added during wort boiling may not have this ester formation activity. Since the addition of calcium chloride and an elevated temperature showed a clear impact on the ester formation, it can be postulated that the formation is induced by enzymes that are present in hops.

Model system studies with hop extracts

While the addition of monocarboxylic acids did not show an effect on the ester formation in the model studies above, performing the same model approach with different hop extracts did show an influence of acids on the formation of the ethyl esters (Fig. 2). A clear increase in the ester concentrations was found for alpha and beta



*) illustrated as the theoretical amount after 100% transfer into water

Fig. 2 Concentrations of ethyl esters on a logarithmic scale in the model system studies on different hop extracts

Table 2 Concentrations of newly formed ethyl esters in the model studies with different hop extracts

Extract	Acid addition	Concentrations [μg/L][a]		
		ethyl methylpropanoate	ethyl 3-methylbutanoate	ethyl 2-methylbutanoate
Alpha Extract	no	0.084	<LOD[b]	<LOD[b]
	yes	5.7	0.70	0.62
Beta Extract	no	22	7.1	0.037
	yes	38	23	0.35
XanthoFlav™ Extract	no	0.097	0.17	0.016
	yes	0.36	0.63	0.053
Tannin Extract	no	0.094	0.17	0.020
	yes	0.32	0.44	0.15

[a] Calculated as the difference between total ester content of the model samples and content in the used amount of hop extract

[b] Limit of detection (LOD) was determined based on a signal-to-noise ratio of 3: ethyl methylpropanoate 12 ng/L, ethyl 3-methylbutanoate 21 ng/L, and ethyl 2-methylbutanoate 61 ng/L

extracts (both containing bitter acids), whereas the models treated with xanthohumol or tannin extracts did only show low rates of ester formation. However, the alpha and beta extracts cannot be directly compared, as they did not have an equal bitter acid concentration. Furthermore, the beta extract also had a residual content of α-acids of 0.5%. Therefore, they differ in their potential to release the respective monocarboxylic acids. It is also unknown, how effective the ethyl esters, that are already present in the hop extracts, are transferred into the aqueous system, as the extracts showed different textures and solubility in water.

Overall, the xanthohumol and tannin extracts showed the lowest ester formation activity, also if carboxylic acids were added. These models with xanthohumol and tannin extracts did not exceed the concentrations of formed esters (total concentration minus the potential transfer from the extract) of 0.63 μg/L (ethyl 3-methylbutanoate in the xanthohumol extract with the addition of acids). Without the addition of acids, the xanthohumol and tannin extracts only formed 0.016–0.17 μg/L of the different esters (Table 2, Fig. 2). Also by estimating the concentrations of the possible “precursor esters” semi-quantitatively, the lowest amounts were observed in the xanthohumol extract.

The alpha extract showed nearly no ester formation activity, yielding not more than 0.08 μg/L (sum of all three ethyl esters), if no carboxylic acids were added. However, with the addition of carboxylic acids, especially the concentration of newly formed ethyl methylpropanoate increased to 5.7 μg/L. The beta extract showed the highest activity already in the samples without acid addition (22 μg/L ethyl methylpropanoate, 7.1 μg/L ethyl 3-methylbutanoate). After adding the respective carboxylic acids, ester formation increased up to 38 μg/L (ethyl methylpropanoate) (Table 2, Fig. 2).

Generally, the highest formation rates of ethyl methylpropanoate and ethyl 3-methylbutanoate were observed in the beta extract, of ethyl 2-methylbutanoate in the alpha extract. The xanthohumol and tannin extracts only showed concentrations < 0.2 μg/L for each ester without the addition of carboxylic acids, but up to 0.63 μg/L, if acids were added (Table 2, Fig. 2). In contrast to the models using hop pellets (A-I), an increase of esters was observed by adding monocarboxylic acids to the models with hop extracts (J and K).

Conclusions

In conclusion, the present study demonstrated that hops induce the formation of ethyl esters of 2- and 3-methylbutanoic acid and methylpropanoic acid during dry-hopping. Thereby, also the residual alcohol content of alcohol-free beer is sufficient for this esterification. The reaction type cannot be clearly specified based on the performed model studies, but according to the inhibition of the reaction via pH value, heat treatment, and salt concentration, an enzymatic pathway can be postulated. Therefore, the transfer of these esters from hops into beer during dry-hopping is secondary, as the amounts of newly formed ethyl esters is much higher than their original contents in hops.

The question about the precursors of the ethyl esters could not fully be answered within this study, as by simulating dry-hopping with hop pellets, humulone- and lupulone-derived acids did not show a great influence on the concentration of ethyl esters, whereas in dry-hopping models with hop extracts, the addition of these acids clearly led to higher concentrations of ethyl esters. Furthermore, other hop-derived esters as 2-methylbutyl methylpropanoate and methylpropyl methylpropanoate can also act as possible precursor structures for an ester formation via transesterification. As already reported earlier, this would be an important reaction regarding the sensory properties of the final product, as the odor thresholds of the ethyl esters are much lower compared to those of the “precursor esters” (Takoi et al. 2018).

Abbreviations

AEAT: Acetyl-CoA:ethanol *O*-acetyltransferase; HRGC/HRGC-MS: Two-dimensional high-resolution heart-cut gas chromatography-mass spectrometry; HS-SPME-HRGCxHRGC-TOF-MS: Headspace solid phase microextraction-comprehensive two-dimensional high-resolution gas chromatography-time-of-flight mass spectrometry

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Authors' contributions

Miss S. Brendel and Prof. Dr. M. Granvogl came up with the research question. S. Brendel investigated the analytical method, performed the measurements, evaluated the received data, and wrote the draft manuscript. M. Granvogl contributed in acquiring the research money, in supervising the full project, in discussing and interpreting the results, and in revising the manuscript. Prof. Dr. T. Hofmann supervised the project as owner of the chair at the Technical University of Munich, where the project was performed. The author(s) read and approved the final manuscript.

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Availability of data and materials

All needed data is presented in this manuscript. Other necessary data, that leaded to the underlying theory, has already been published and is cited at the corresponding position within this publication.

Competing interests

The authors declare that there is no competing interests.

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9.4 Studies on the Odorant Concentrations and their Time Dependencies During Dry-Hopping of Alcohol-Free Beer

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RESEARCH ARTICLE

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Studies on the odorant concentrations and their time dependencies during dry-hopping of alcohol-free beer

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Abstract

Both the market for alcohol-free beer and the number of craft beer breweries increased in the past years. Thus, dry-hopping as a possibility to compensate/mask aroma deficiencies of alcohol-free beers is gaining more and more interest. To better understand the transfer of odorants from hops into alcohol-free beer during dry-hopping, their concentrations were monitored over a period of 7 days in a laboratory-scale experiment simulating the dry-hopping process. Thereby, a main transfer occurred during the first 2-3 days. However, the relevance of the transferred odorants to the overall aroma can only be evaluated by taking their sensory properties into consideration. Therefore, orthonasal odour thresholds and sensorial dose-response relationships were investigated in an alcohol-free beer matrix. Sensory tests showed a nearly linear increase in the odour intensities of different esters, whereas further typical hop odorants, such as linalool, geraniol, and myrcene, showed a multistage increase. Overall, the present study clearly corroborated that dry-hopping of alcohol-free beers can be optimised regarding the hop dosage and the contact time, depending on the desired flavour of the final product.

KEYWORDS
alcohol-free beer, aroma transfer, contact time, dose-response relationship, dry-hopping

1 | INTRODUCTION

Hops as beer ingredient offer different possibilities to modify the aroma of the final beer. Therefore, many studies focused on the key odorants of different hop varieties, to evaluate their potential for the brewing industry. By applying the molecular sensory science concept, linalool, myrcene, 3-methylbutanoic acid, and geraniol were identified as important aroma-active compounds in hop varieties.[1-3] For some hops, variety-specific odorants were identified like ethyl 2-methylbutanaote in Huell Melon or 4-mercapto-4-methylpentan-2-one in US varieties like Cascade, Citra, and Eureka.[4] Especially by late- or dry-hopping, higher concentrations of these hop odorants can be achieved in the final product. So that the transfer of hop odorants is not affected by evaporation during wort boiling, a more intense hoppy aroma can be achieved by hop addition into the whirlpool or in the cold area. While, among others, 3-methyl-1-butanol, 2-phenylethanol, 2-methoxy-4-vinylphenol, and 2- and 3-methylbutanoic acid were identified as important key odorants in different beer types,[5,6] hop key odorants like linalool, geraniol, or 4-mercapto-4-methylpentan-2-one were shown as highly important for the typical aroma of dry-hopped beers.[4,7-9] Also, different esters like ethyl 3-methylbutanoate and ethyl methylpropanoate were demonstrated to be present in concentrations above their odour thresholds in regular and alcohol-free dry-hopped beers.[7,10,11]

In the past years, the market for alcohol-free beers strongly increased. But the industry is still facing some challenges in regard to alcohol-free beers. On the one side, thermally dealcoholised beers are lacking in the typical aroma and in the full body elicited by regular beers. On the other side, the sweeter taste along a wort-like odour is an issue of alcohol-free beers produced by stopped fermentation.[12] With a growing number of craft beer breweries, dry-hopping also gained more importance for low alcohol and alcohol-free beers as an opportunity to increase the popularity and acceptance of these beer types. While studies on dry-hopping of alcoholic beers[7,13-15] and of low alcohol beers[16] have already shown a clear improvement of beer attributes like full body and bitter quality, in parallel with the compensation of aroma deficiencies due to the lower alcohol content, limited data are available for the transfer of odorants into alcohol-free beer. In a very recent study, it has been shown that dry-hopping can be a possibility to mask aroma deficits of alcohol-free beers, and therefore, increase its consumer's acceptance. Thereby, especially linalool, geraniol, myrcene, and ethyl esters of hop-derived monocarboxylic acids were identified as important contributors to the aroma of alcohol-free beer after dry-hopping.[10]

Recent studies on aroma-active compounds in beer established odour thresholds only in an alcoholic beer matrix or in model solutions.[17-19] For alcohol-free beer, Piornos et al. published orthonasal and retronasal odour thresholds of typical beer odorants like 3-methyl-1-butanol, 2-methoxy-4-vinylphenol, or 2-phenylethanol in a model alcohol-free beer, also considering different calculation methods. Thereby, a comparison of the retronasal thresholds in alcohol-free beer to data in alcoholic beer showed lower thresholds in alcohol-free beer on the one side, but most of the time higher thresholds compared to those in water on the other side.[20]

Perpète and Collin studied the retention of odorants in dependency of the alcohol and sugar contents, with a higher retention of aldehydes in solutions with a higher ethanol content. In addition, a ‘salting out’ effect in solutions with higher sugar content, as it is likely in alcohol-free beers after stopped fermentation, was shown.[21]

Beside the effects of alcohol or sugar contents on the release of odorants, and thus, on the respective odour thresholds, also chirality can have an influence on the sensory perception of odorants. It is well established that enantiomers can clearly vary in their sensory properties. Beside differences in their odour qualities, like the well-known example of (*S*)-carvon (‘caraway-like’) and (*R*)-carvon (‘spearmint-like’), also big differences in their odour thresholds are well-known.[22] For hops, the enantiomeric distribution of linalool is well examined,[8,23] whereas enantiomeric ratios of other chiral hop odorants have not been published yet.

Thus, the aims of the present study were first the evaluation of the time dependency of the transfer of odorants during dry-hopping into alcohol-free beer. Second, sensory experiments like the determination of odour thresholds and dose-response relationships were performed in alcohol-free beer to get an insight into changes of sensory properties of different odorants depending on their respective concentrations. In addition, the enantiomeric ratios were determined in hops and alcohol-free beer for all sensorial investigated odorants, as their odour characteristics can be very different.

2 | EXPERIMENTAL

2.1 | Beer samples

Wort and beer samples for the determination of the enantiomeric ratios of chiral odorants were provided by the Chair of Brewing and Beverage Technology (Technical University of Munich, Freising, Germany). Samples were produced via stopped fermentation and dry-hopped on a vibration table for one week with hop pellets, equivalent to 1.5 mL of hop oil/hL, as described very recently.[10] For all other experiments, different alcohol-free beers were purchased at local supermarkets.

2.2 | Hop pellets

Hop pellets type 90 of Hallertauer Mandarina Bavaria (HMB), harvest year 2015, were provided by Hopsteiner (Mainburg, Germany). The pellets were stored under vacuum at −20 °C prior to use, ensuring the aroma quality. Identification and quantitation of the odorants have been performed in a previous study.[1]

2.3 | Chemicals

The following odorants were obtained commercially: linalool and (*R*)-linalool (Fluka, Neu-Ulm, Germany); ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, geraniol, methyl 2-methylbutanoate, (*S*)-methyl 2-methylbutanoate, and myrcene (Sigma-Aldrich, Taufkirchen, Germany); and propyl 2-methylbutanoate (TCI, Eschborn, Germany).

The following chemicals were purchased from commercial sources: liquid nitrogen (Linde, Munich, Germany) and diethyl ether, ethanol, and anhydrous sodium sulphate (Merck, Darmstadt, Germany).

2.4 | Stable isotopically labelled internal standards

$[^2H_6]$-Myrcene (Santa Cruz Biotechnology, Dallas, TX) was obtained commercially.

$[^2H_5]$-Ethyl 2-methylbutanoate, $[^2H_9]$-ethyl 3-methylbutanoate, $[^2H_5]$-ethyl methylpropanoate, $[^2H_3]$-methyl 2-methylbutanoate, and $[^2H_3]$-propyl 2-methylbutanoate were synthesized by esterification of the respective labelled carboxylic acid with the respective alcohol.[24] $[^2H_2]$-Geraniol[25] and $[^2H_{2\text{-}3}]$-linalool[23] were synthesized as recently described.

2.5 | Laboratory-scale dry-hopping of alcohol-free beer

Seven portions of hops (150 mg each) were filled into tea bags and hung into an amber glass bottle (1 L). Alcohol-free beer (700 mL) was added and stirred at room temperature for 7 days. Each 24 hours, hops (1 bag) and beer (100 mL) were removed from the bottle and the odorant concentrations were determined. The experiment was conducted in two technical duplicates.

2.6 | Quantitation by stable isotope dilution analysis (SIDA)

The isolation and quantitation of the volatiles in alcohol-free beer were performed as recently reported.[10]

2.7 | Two-dimensional high-resolution heart-cut gas chromatography-mass spectrometry (HRGC/HRGC-MS)

For quantitation of geraniol, linalool, and myrcene, HRGC/HRGC-MS was performed as recently described.[10] For the determination of the enantiomeric ratios of ethyl 2-methylbutanoate, linalool, methyl 2-methylbutanoate, and propyl 2-methylbutanoate, a chiral capillary column (BGB-175 or BGB-176; both 30 m × 0.25 mm i.d., 0.25 μm film thickness; BGB Analytik, Boeckten, Switzerland) was used in the second GC.

2.8 | Solid phase microextraction-comprehensive high-resolution gas chromatography-time-of-flight mass spectrometry (SPME-HRGCxHRGC-TOF-MS)

For quantitation of several esters, the SPME-HRGCxHRGC-TOF-MS approach was applied following the recently published procedure.[10]

2.9 | High-resolution gas chromatography-olfactometry (HRGC-O) and odour thresholds in air

Determination of odour thresholds in air was performed following a formerly published protocol.[26] For this, a recently described HRGC-O system[1] equipped with a chiral BGB-176 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; BGB Analytik) and (*E*)-2-decenal as the internal standard with an odour threshold of 2.7 ng/L air[27] were used.

2.10 | Odour thresholds in water and in alcohol-free beer

Orthonasal odour thresholds were determined via three-alternative forced-choice tests as recently described,[28] using either water or alcohol-free beer as the matrices.

2.11 | Sensorial dose-response relationships of odorants in alcohol-free beer

A solution of each odorant was prepared in ethanol and added separately to alcohol-free beer. Thereby, concentrations in beer were approximately 100 μg/L for all esters, 4800 μg/L for geraniol and linalool, and 3200 μg/L for myrcene, leading to concentrations about 50-100 times higher than previously determined in dry-hopped alcohol-free beer.[10] This stock solution was diluted with alcohol-free beer stepwise 1 + 2 (v + v). Seven to 10 of these dilution steps were prepared, depending on the applied concentration range starting always with the lowest concentration below the known odour threshold in alcohol-free beer and ending with an odorant-specific chosen maximum concentration. These solutions were presented in increasing concentrations to a trained sensory panel consisting of at least 10 weekly trained persons. Assessors were asked to rate the intensity of the odorant in the solutions on a line scale (15 cm), as described by the German Agricultural Society (DLG, Deutsche Landwirtschafts-Gesellschaft).[29] Starting and end point of the scale were represented by reference solutions. For the starting point, alcohol-free beer without the addition of an odorant was used; for the end point, a solution of the maximal concentration of the odorant, that was used in the sensory test, was presented. For data processing, the ratings of each assessor were standardised to values between 0 and 10.

3 | RESULTS AND DISCUSSION

3.1 | Evaluation of a suitable matrix for analytical and sensory experiments

For the laboratory-scale dry-hopping experiments and sensory tests, first, the concentrations of the target odorants were determined in 6 different commercially purchased alcohol-free beers. The beer with the lowest concentrations of these analytes was chosen as the matrix for the following experiments. Thereby, beer **F** showed the lowest concentrations for all analytes, which were also below the respective odour thresholds in water (except for linalool, with only a slightly higher concentration and ethyl 2-methylbutanoate with < LOQ that was slightly above the respective odour threshold; Table 1).

3.2 | Time dependency of the transfer of odorants during dry-hopping

A laboratory-scale experiment was chosen to evaluate the time dependency of the odorant transfer from hops into alcohol-free beer during dry-hopping. Every 24 hours, the concentrations of eight different odorants (ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, geraniol, linalool, methyl 2-methylbutanoate, myrcene, and propyl 2-methylbutanoate) were determined, which have already been identified as important odorants in hops and dry-hopped alcohol-free beer.[1,10]

For geraniol, linalool, and myrcene, the maximal transfer rates were reached after 2-3 days (Figure 1). Afterwards, a decline in concentrations was observed. But while the concentrations of geraniol and linalool reached a stable level after one week (geraniol around 100 μg/L; linalool around 35 μg/L), for myrcene a constant decrease was noticeable. The latter might be explained

TABLE 1 Concentrations of selected odorants in different commercially purchased alcohol-free beers. Odour thresholds in water are given for comparison

Odorant	Concentrations (μg/L)						Odour threshold in water[a] (μg/L)
	Beer A	Beer B	Beer C	Beer D	Beer E	Beer F	
linalool	72.8	7.34	3.13	2.11	1.77	1.20	0.58
myrcene	21.1	0.80	0.14	0.13	0.17	0.10	1.2
geraniol	6.90	0.89	0.90	0.89	0.20	0.03	1.1
ethyl methylpropanoate	0.790	0.393	0.023	0.098	0.753	< 0.012	0.089
propyl 2-methylbutanoate	0.297	<0.051[b]	<0.051[b]	<0.051[b]	<0.051[b]	<0.051[b]	0.15
ethyl 3-methylbutanoate	0.149	0.173	0.013	0.066	0.166	< 0.007	0.023
methyl 2-methylbutanoate	0.149	0.038	<0.029	<0.029	<0.029	< 0.029	0.048
ethyl 2-methylbutanoate	0.068	0.071	<0.011[b]	0.023	0.085	<0.011[b]	0.008

[a] Data from an in-house database obtained via three-alternative forced-choice tests.

[b] Limit of quantitation (LOQ) was determined based on a signal-to-noise ratio of 10.

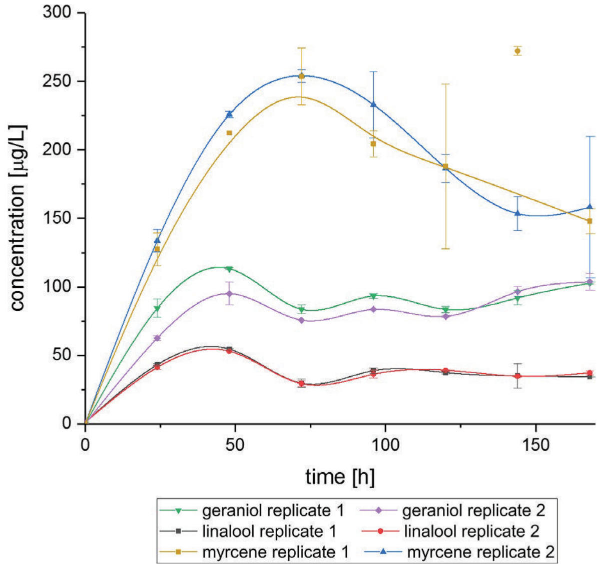

FIGURE 1 Time-dependent transfer of geraniol, linalool, and myrcene during dry-hopping of alcohol-free beer. [Colour figure can be viewed at wileyonlinelibrary.com]

by adsorption processes to yeast cells or crown caps during storage.[30,31] A quite similar course of the curve for linalool and geraniol was already shown for dry-hopped alcoholic beer in different production scales.[32] In a semi-industrial scale, a maximal transfer of linalool and geraniol in the first 24 hours and a slight decline of the concentrations in the following days were reported.[32] An increase of geraniol concentration at the end of the dry-hopping period can be attributed to the use of the hop variety Mandarina Bavaria, that has been shown to have high contents of geranyl acetate, acting as geraniol precursor.[10] In a laboratory-scale experiment, also a decline in the myrcene concentration was observed between days 1 and 7.[32] In contrast, Wolfe et al. showed almost constant concentrations of linalool and myrcene during a dry-hopping period of 7 days.[33]

For all analysed esters, transfer rates up to 91%, referred to the concentrations in hops, were obtained during the first 2-3 days (Figure 2). After days 3-4, an additional formation of ethyl and methyl esters started (Figure 2A-D). This phenomenon of a hop-induced ester formation has been recently described.[11] As the reaction type has not yet been completely clarified, it cannot finally be explained, which external factors caused the differences between the two replicates. Nevertheless, it was observed that a higher formation rate of methyl esters seems to correlate with a lower rate of ethyl esters (except for propyl 2-methylbutanoate with a maximal transfer rate of 60% within the first 24 hours, followed by a constant decrease of its concentration; Figure 2E). In a very recent study, it has been reported that the propyl ester is not affected by the hop-induced ester formation.[11]

As it has already been shown, that the aroma transfer during dry-hopping, especially of myrcene and linalool, is highly dependent on the batch size,[32] it is noteworthy, that in the actual study, the concentrations of all odorants were at the same level as it was the case for dry-hopped alcohol-free beers, that have been analysed in a previous study using the same hop pellets.[10] Thus, the results of the laboratory-scale experiment should be comparable to a bigger research scale approach. Nevertheless, parameters like the headspace volume in the dry-hopping tank or the way of assuring a distribution of the hops (by circulation, vibration table, manual turn-over, HopGun®, etc) had an influence on the odorant transfer,[32] while pellet properties like density and particle size did not have a significant influence on the mass transfer.[33]

3.3 | Enantiomeric ratio of chiral aroma compounds in different alcohol-free beers

Since decades, it is well-known that enantiomers can clearly differ in their odour qualities and odour thresholds.[22] Therefore, the ratio of (*R*)- and (*S*)-enantiomers of ethyl 2-methylbutanoate, linalool, methyl 2-methylbutanoate, and propyl 2-methylbutanoate

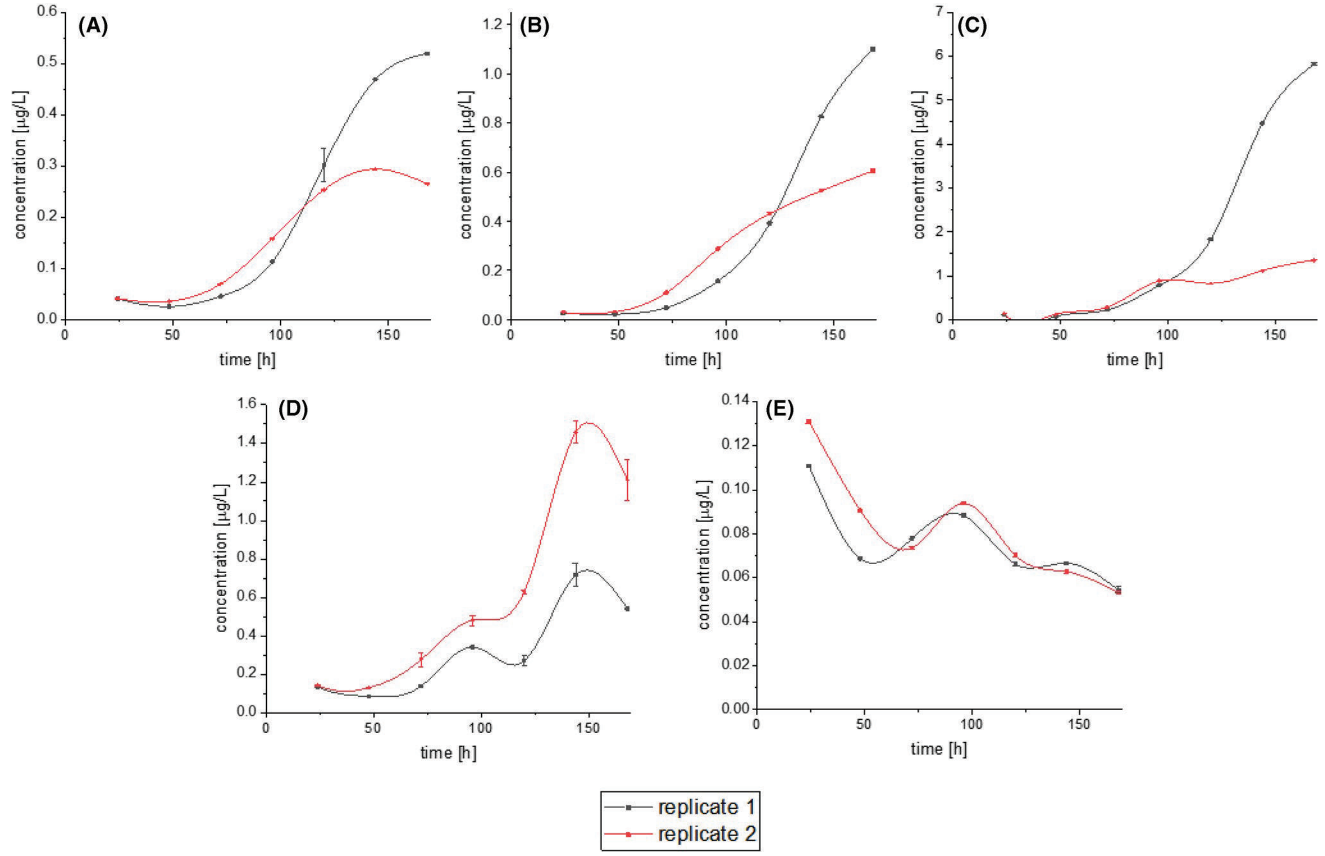

FIGURE 2 Time-dependent transfer of ethyl 2-methylbutanoate (A), ethyl 3-methylbutanoate (B), ethyl methylpropanoate (C), methyl 2-methylbutanoate (D), and propyl 2-methylbutanoate (E) during dry-hopping of alcohol-free beer. [Colour figure can be viewed at wileyonlinelibrary.com]

were determined in different hop varieties, wort, and beer samples (Figure 3; Table 2). For the following sensory experiments, either the quantitatively predominant enantiomer in the dry-hopped samples (for linalool and methyl 2-methylbutanoate) or the racemate (for ethyl 2-methylbutanoate, which was nearly present in a racemic distribution in dry-hopped beers (beers 3 and 4; Table 2), and for propyl 2-methylbutanoate, due to the lack of enantiopure (*S*)-propyl 2-methylbutanoate) were used.

By comparing the enantiomeric ratios in the analysed wort and beer samples after stopped fermentation, the influence of the fermentation can be evaluated. While for methyl 2-methylbutanoate, no changes were observed, propyl 2-methylbutanoate changed from 100% of the (*S*)-enantiomer in wort to a racemic mixture after fermentation (beers 1 and 2; Table 2). Also, ethyl 2-methylbutanoate showed mainly the (*S*)-enantiomer in wort, while the (*R*)-enantiomer was predominant after fermentation. These data were of interest due to the fact that in a recent study, nearly 100% of (*S*)-ethyl 2-methylbutanoate was reported in different types of beer.[34] For linalool, only minor changes occurred during fermentation (Table 2). For fully fermented beers that can be dealcoholised by different physical methods (e.g. heat treatment, nanofiltration, or dialysis), the changes in the enantiomeric ratios of odorants could also be influenced, but the performed experiments clearly showed the influence of fermentation on the enantiomeric distribution.

In all hop samples, only or mainly the (*S*)-enantiomer of the different esters were present. Consequently, the dry-hopping process (beers 3 and 4; Table 2) led to a clear shift in the enantiomeric ratios in favour of the (*S*)-enantiomer of ethyl 2-methylbutanoate and propyl 2-methylbutanoate in the beer samples. In contrast, (*R*)-linalool (ratio not determined in the actual study) was already known as the predominating enantiomer in hops.[8] Accordingly, 92% and 90% of (*R*)-linalool were determined in dry-hopped beers (beers 3 and 4; Table 2) compared to only 75% and 70% in the corresponding non-hopped beers (beers 1 and 2; Table 2), which was in accordance with earlier studies on hopped beer.[8,23]

3.4 | Sensory experiments I: Odour thresholds in air and in alcohol-free beer

To evaluate the orthonasal odour perception of important aroma-active compounds in dry-hopped alcohol-free beer, odour thresholds in an 'analyte-free' matrix (beer F) were determined. Based on the enantiomeric ratios in different beer samples, for linalool ((*R*)/(*S*) = 92/8 and 90/10) the (*R*)-enantiomer, for methyl 2-methylbutanoate ((*R*)/(*S*) = 100/0) the (*S*)-enantiomer, and for ethyl 2-methylbutanoate ((*R*)/(*S*) = 57/43) the racemate were used (beers 3 and 4, Table 2). Both enantiomers of ethyl 2-methylbutanote showed an

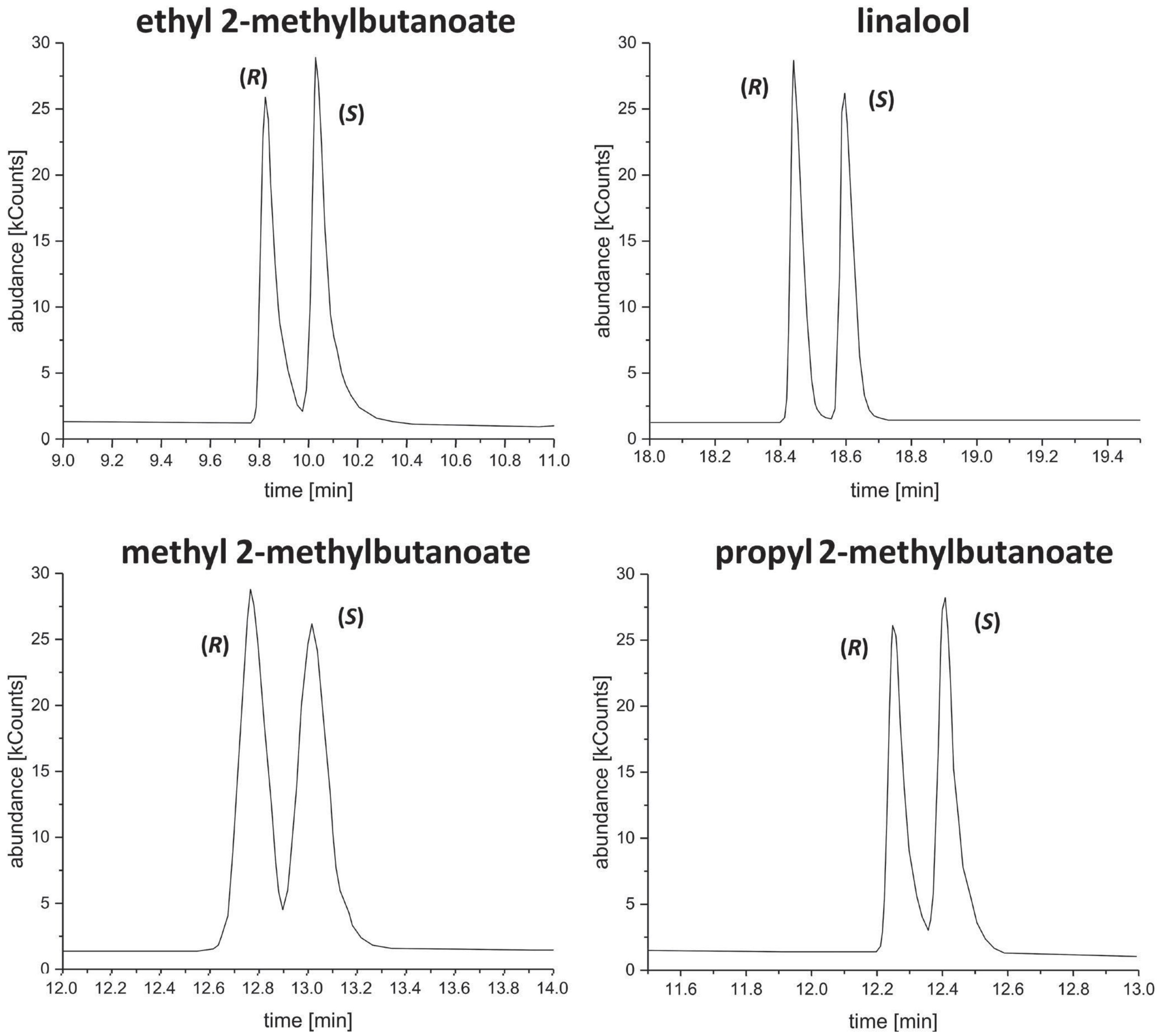

FIGURE 3 Separation of reference racemates (ethyl 2-methylbutanoate, linalool, methyl 2-methylbutanoate, and propyl 2-methylbutanoate) on a chiral GC capillary column

odour threshold in air of 1.27 ng/L air and the same odour quality. Thus, it can be assumed that they are likely showing the same sensory properties also in other matrices, which has also been shown in a previous study.[34]

For propyl 2-methylbutanoate, no enantiopure reference was available. To assess the differences of the two enantiomers, the odour thresholds in air of each enantiomer were determined after GC separation. Thereby, (*S*)-propyl 2-methylbutanoate showed an odour threshold of 6.08 ng/L air, whereas the threshold of (*R*)-propyl 2-methylbutanoate was >1215 ng/L air. Due to this high discrepancy, the sensory experiments were performed with the racemate (consequently, for threshold determination, only the half of the applied amount was considered), as there should be no sensory influence of the (*R*)-enantiomer.

As shown in Table 3, odour thresholds in alcohol-free beer were 4 (ethyl methylpropanoate: 0.34 μg/L in alcohol-free beer vs 0.089 μg/L in water) to 65 ((*S*)-propyl 2-methylbutanoate: 1.3 μg/L in alcohol-free beer vs 0.02 μg/L in water) times higher compared to the respective odour thresholds in water. Also in a recent study, Piornos et al. reported that orthonasal odour thresholds of aroma compounds in an alcohol-free model beer were most of the time higher than in water.[20] The present data clearly corroborated the influence of the beer matrix, and of the sensory complexity in general, on the perception of different odorants.

3.5 | Sensory experiments II: Dose-response relationships

It is also well accepted that the odour quality of aroma-active compounds can change in dependency of the respective concentrations.

TABLE 2 Enantiomeric ratios of chiral odorants in hop varieties, wort, and beers after stopped fermentation

	Enantiomeric ratios (%)							
	ethyl 2-methylbutanoate		linalool		methyl 2-methylbutanoate		propyl 2-methylbutanoate	
Sample	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)
HMB[a]	7	93	nq	nq	0	100	0	100
HCA[b]	3	97	nq	nq	0	100	0	100
HHA[c]	1	99	nq	nq	2	98	0	100
Wort 1	0	100	89	11	0	100	0	100
Wort 2	13	87	64	36	0	100	0	100
Beer 1[d]	70	30	75	25	0	100	49	51
Beer 2[e]	72	28	70	30	0	100	50	50
Beer 3[f]	43	57	92	8	0	100	0	100
Beer 4[g]	43	57	90	10	0	100	23	77
Sensory tests[h]	racemate		(*R*)		(*S*)		racemate	

[a] Hallertauer Mandarina Bavaria.

[b] Hallertauer Cascade.

[c] Hallertauer Mittelfrüh.

[d] Beer 1 = top-fermented beer after stopped fermentation.

[e] Beer 2 = bottom-fermented beer after stopped fermentation.

[f] Beer 3 = beer 1, dry-hopped with Hallertauer Mandarina Bavaria.

[g] Beer 4 = beer 2, dry-hopped with Hallertauer Mandarina Bavaria.

[h] Selected odorant(s) for the sensory experiments.

nq = not quantitated.

TABLE 3 Comparison of odour thresholds in water and in alcohol-free beer

	Odour thresholds (μg/L)	
Odorant	In water[a]	In alcohol-free beer
myrcene	1.2	76
geraniol	1.1	19
(*R*)-linalool	0.58	4.7
ethyl methylpropanoate	0.089	0.34
(*S*)-methyl 2-methylbutanoate	0.048	2.5
ethyl 3-methylbutanoate	0.023	0.24
(*S*)-propyl 2-methylbutanoate	0.020	1.3
ethyl 2-methylbutanoate	0.014	0.24

[a] Data from an in-house database obtained via three-alternative forced-choice tests.

Thus, sensorial dose-response experiments were performed to evaluate (a) such possible changes in the alcohol-free beer matrix and (b) the possible sensorial impact of these odorants to the overall aroma of dry-hopped alcohol-free beers in dependency on their concentrations, which might affect not only the odour intensity but also the odour quality.

Myrcene, present at maximal concentrations of approximately 250 μg/L in the transfer experiments before declining to about 150 μg/L, showed a plateau in the sensory curve between 40 and 120 μg/L (Figure 4A). Intensities > 0 below the respective odour threshold can be explained by single panellists, whose personal threshold was lower than the average threshold. A relevant increase in odour intensity started at about 120 μg/L, and thus, a higher hop dosage can definitively lead to a strengthened odour perception. The panellists described the odour quality in alcohol-free beer in this concentration range as 'floral, green', higher concentrations led to a typical 'hoppy, geranium-like' odour, enabling not only an intensified aroma, but also the possibility to get a clearly hoppy odour note by a higher hop dosage during dry-hopping.

Also for (*R*)-linalool, a plateau in the sensory dose-response relationship was determined for concentrations at around 100 μg/L of alcohol-free beer (Figure 4A). The concentrations in the actual transfer experiments were between 40 and 50 μg/L, which led to a 'citrus-like, fruity' aroma. In higher concentrations (>500 μg/L), the odour quality changed to an unpleasant 'soapy' impression. Thus, the content of linalool (known as a key odorant in alcoholic dry-hopped beer[8]) of the used hop variety must be considered when choosing the hop dosage for dry-hopping, as a maximal transfer is already reached after 2-3 days and should not exceed a certain concentration to avoid an undesired aroma note (Figure 4A).

Geraniol showed a quite flat increase in its odour intensity, except for the concentration range between 60 and 170 μg/L. As the dry-hopped beer samples showed amounts of about 100 μg/L, a relevant increase in its odour intensity can be reached by the use of hop varieties either high in geraniol or in its precursor geranyl acetate or by the increase of the hop dosage. The odour was described as 'floral, rose-like' at concentrations around 100 μg/L (Figure 4A).

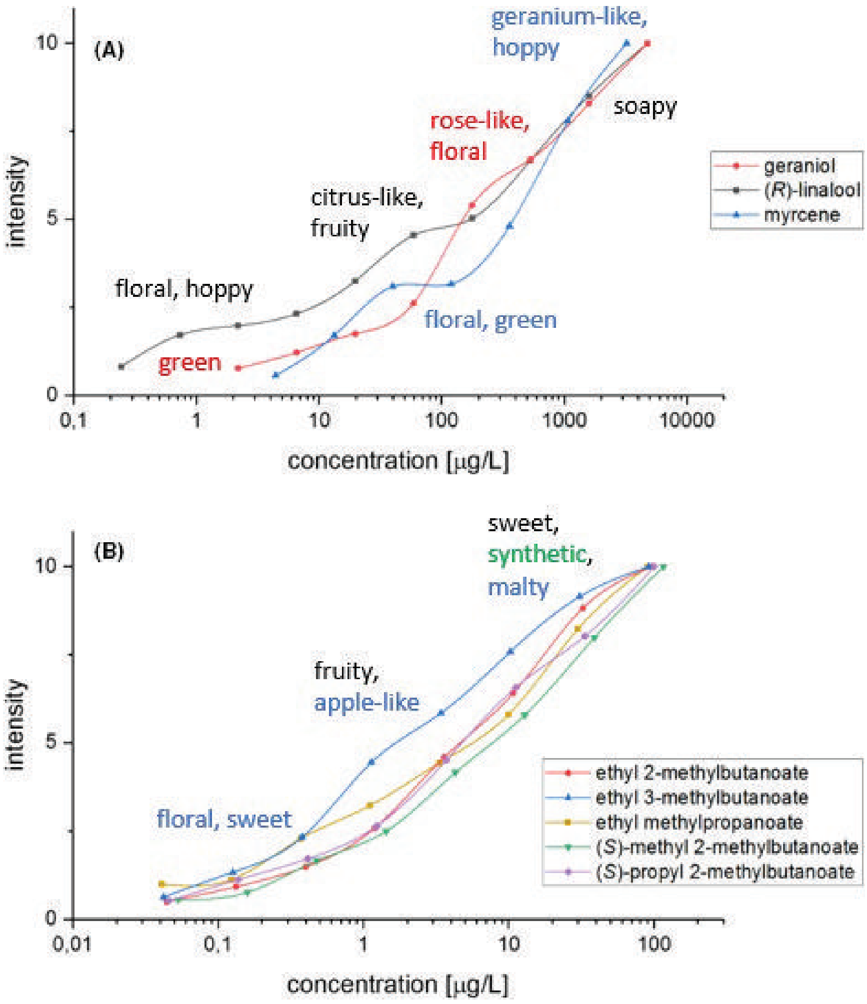

FIGURE 4 Sensorial dose-response relationship of geraniol, (*R*)-linalool, and myrcene (A) and of ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, (*S*)-methyl 2-methylbutanoate, and (*S*)-propyl 2-methylbutanoate (B; black odour descriptors related to all esters, coloured odour descriptors are related to the specific esters) in alcohol-free beer. [Colour figure can be viewed at wileyonlinelibrary.com]

All analysed esters showed a nearly linear increase in regard to odour perception with increasing concentrations, without any plateau formation (Figure 4B). The odour quality was described as ‘fruity’ at lower concentrations and changed to ‘sweet’ notes at higher concentrations (100 µg/L). Thus, also for the esters, a too high amount must be avoided, but is normally not reached by common practices. Since the major amount of these esters in alcohol-free beer is caused by the hop-induced ester formation[11] and not by the transfer during dry-hopping, a higher hop dosage does not necessarily lead to an increase in the odour intensity.

On the one side, these dose-response relationships clearly proved the opportunities of dry-hopping to influence and vary both the odour quality and intensity of alcohol-free beers, which can help to increase their acceptance by consumers. On the other side, detailed knowledge is necessary to avoid too high concentrations of certain odorants, which can finally lead to an aroma disliked by the consumers, as shown for linalool and the esters.

4 | CONCLUSION

In summary, this study gives a deeper insight into the sensorial impact of hop odorants on the aroma of alcohol-free beer. It is known that transfer rates during dry-hopping of alcohol-free beer do not differ too much from those of alcoholic beer.[10] But since the aroma profiles of alcohol-free beers strongly differ from those of regular beers,[10] depending on various production and dealcoholisation processes, the sensory influence of the transferred hop odorants can be totally different in alcohol-free beers. By knowing the sensory properties of important key odorants of hops in the alcohol-free beer matrix, the dry-hopping process can be optimised to reach and modify desired aroma characteristics in the final product, and to specifically mask undesired odour properties of alcohol-free beers. Sensory data like odour thresholds and dose-response relationships in alcohol-free beer can help to decide about the amount of hop dosage used for dry-hopping, depending on the desired intensities of aroma qualities like ‘citrus-like’ or ‘fruity’. The results confirmed that a higher hop dosage is not always the method of choice to obtain, for example a typical citrus-like odour note in alcohol-free beers, as the odour quality of (*R*)-linalool can change to an undesired ‘soapy’ odor attribute in higher concentrations. The present study was the first on the time dependency of the aroma transfer during dry-hopping of alcohol-free beer, while this issue has already been examined in alcoholic beer.[32,33] It was shown that a longer contact time does not necessarily increase the transfer of linalool, myrcene, or geraniol into beer, but can be used if a higher concentration of ‘fruity’ esters is desired. Using this knowledge, the dry-hopping process can also be optimised in regard to the contact time to receive, for example, a more pronounced hoppy aroma in alcohol-free beers, which can lead to a higher consumer's acceptance of this beer type.

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CONFLICTS OF INTERESTS

The authors declare no competing interests.

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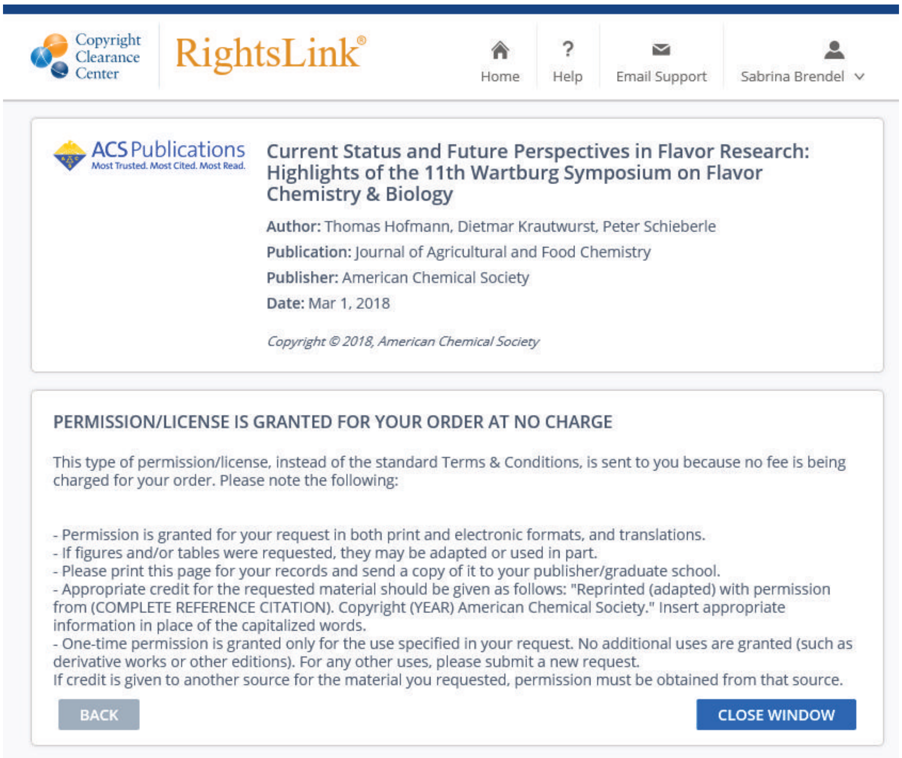

Current Status and Future Perspectives in Flavor Research: Highlights of the 11th Wartburg Symposium on Flavor Chemistry & Biology

Author: Thomas Hofmann, Dietmar Krautwurst, Peter Schieberle
Publication: Journal of Agricultural and Food Chemistry
Publisher: American Chemical Society
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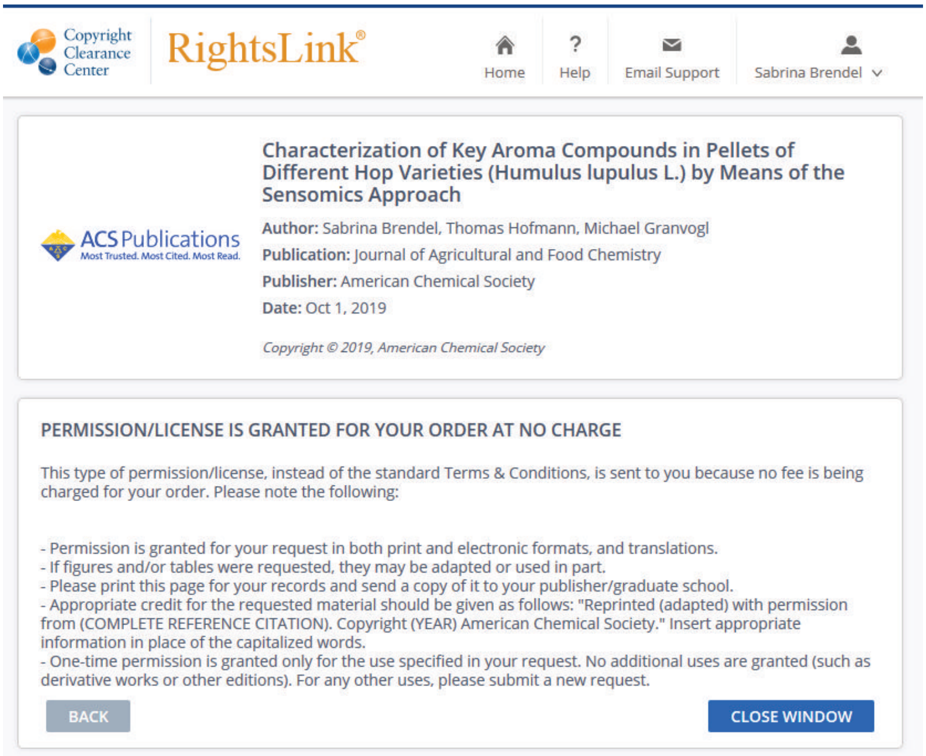

Characterization of Key Aroma Compounds in Pellets of Different Hop Varieties (Humulus lupulus L.) by Means of the Sensomics Approach

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Publication: Journal of Agricultural and Food Chemistry
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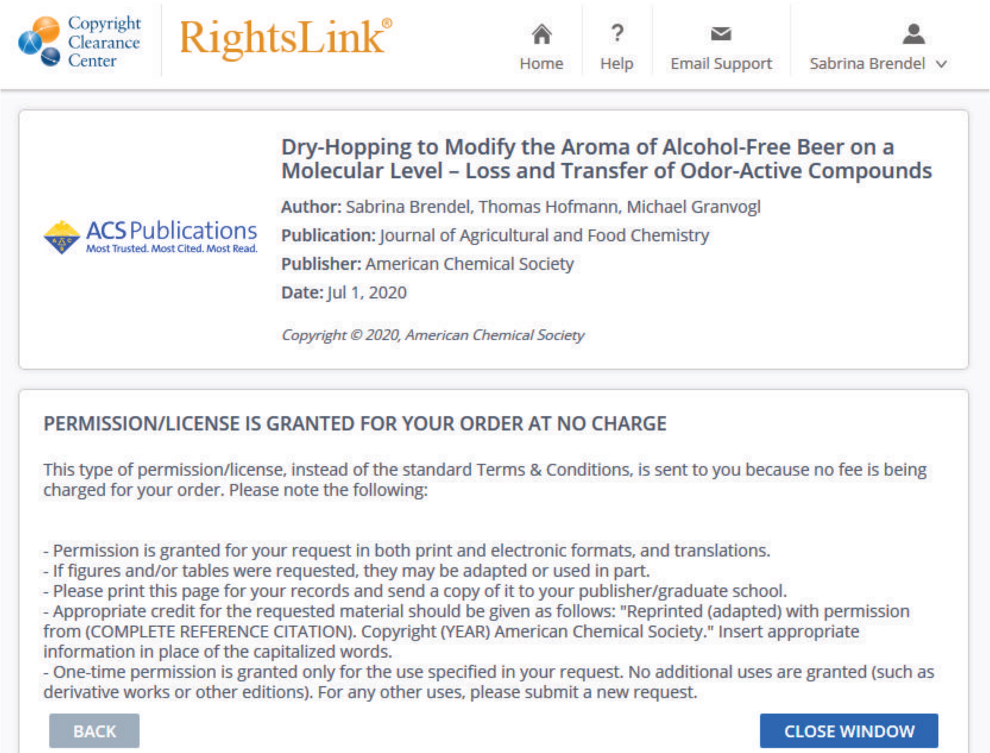

Dry-Hopping to Modify the Aroma of Alcohol-Free Beer on a Molecular Level – Loss and Transfer of Odor-Active Compounds

Author: Sabrina Brendel, Thomas Hofmann, Michael Granvogl
Publication: Journal of Agricultural and Food Chemistry
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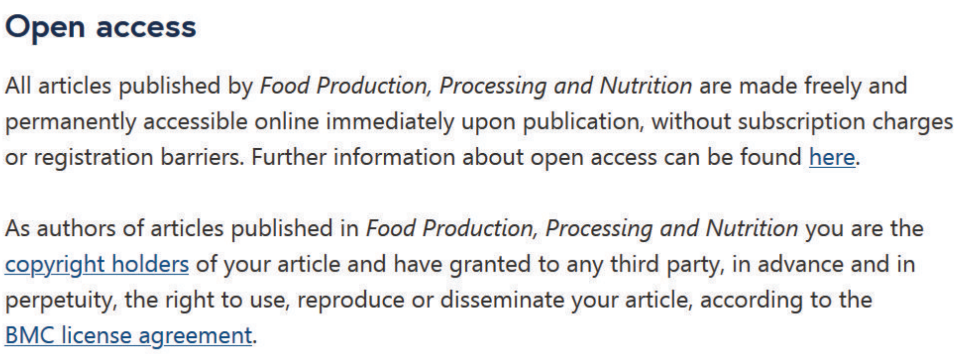

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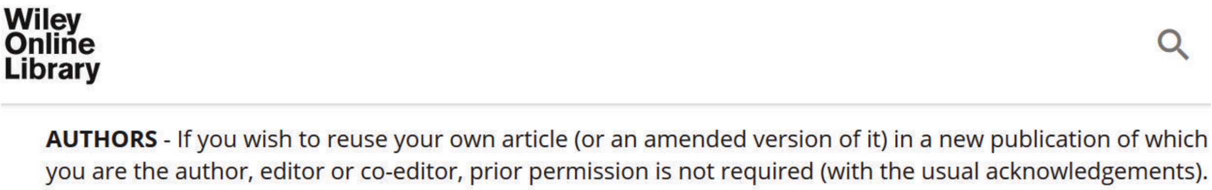

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Reprint Permission 5: Studies on the Odorant Concentrations and their Time Dependencies During Dry-Hopping of Alcohol-Free Beer (Chapter 10.4)

9.6 List of Publications

Peer-Reviewed Publications

Brendel, S.; Hofmann, T.; Granvogl, M. Characterization of key aroma compounds in pellets of different hop varieties (*Humulus Lupulus* L.) by means of the sensomics approach. *J. Agric. Food Chem.* **2019**, *67*, 12044-12053, DOI 10.1021/acs.jafc.9b05174.

Brendel, S.; Hofmann, T.; Granvogl, M. Dry-hopping to modify the aroma of alcohol-free beer on a molecular level – loss and transfer of odor-active compounds. *J. Agric. Food Chem.* **2020**, DOI 10.1021/acs.jafc.0c01907.

Brendel, S.; Hofmann, T.; Granvogl, M. Studies on the odorant concentrations and their time dependencies during dry-hopping of alcohol-free beer. *Flavour Frag. J.* **2020**, accepted.

Brendel, S.; Hofmann, T.; Granvogl, M. Hop-induced formation of ethyl esters in dry-hopped beer. *Food Prod. Process. And Nutr.* **2020**, accepted.

Non-Peer-Reviewed Publications

Brendel, S.; Hofmann, T.; Granvogl, M. Charakterisierung von Schlüsselaromastoffen in verschiedenen Hopfensorten anhand des Konzepts der Molekularen Sensorik. *Lebensmittelchemie* **2018**, *72*, 96, ISSN 0937-1478.

Brendel, S.; Hofmann, T.; Granvogl, M. Charakterisierung von Schlüsselaromastoffen in verschiedenen Aroma- und Flavor-Hopfensorten. *Lebensmittelchemie* **2018**, *72*, 158, ISSN 0937-1478.

Oral Presentations

Brendel, S.; Hofmann, T.; Granvogl, M. Charakterisierung von Schlüsselaromastoffen in verschiedenen Hopfensorten anhand des Konzepts der molekularen Sensorik. *Arbeitstagung des Regionalverbandes Bayern der Lebensmittelchemischen Gesellschaft,* **March 21, 2018**.

Brendel, S.; Hofmann, T.; Granvogl, M. Odor-active compounds in different hop varieties – the sensomics approach. *EBC Symposium: Recent Advances in Hop Science,* **September 11, 2018**.

Brendel, S.; Hofmann, T.; Granvogl, M. Application of dry-hopping to modify the aroma of alcohol-free beer. *2019 ASBC Meeting*, *American Society of Brewing Chemists*, **June 24, 2019**.

Poster Presentations

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