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Influences on the concentration of hop flavor components during dry hopping

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Preface

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Peer-reviewed publications

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1. **Haslbeck, K.**; Jerebic, S. and Zarnkow, M.: Characterization of the unfertilized and fertilized hop varieties Progress and Hallertauer Tradition – analysis of free and glycosidic-bound flavor compounds and β-glucosidase activity, Brewing Science, 70 (2017), no. 11/12, pp. 148-158, DOI: 10.23763/BrSc17-15haslbeck

2. **Haslbeck, K.**; Bub, S.; von Kamp, K.; Michel, M.; Zarnkow, M.; Hutzler, M. and Coelhan, M.: The influence of brewing yeast strains on monoterpene alcohols and esters contributing to the citrus flavour of beer, Journal of the Institute of Brewing, 124 (2018), no. 4, pp. 403-415. DOI: 10.1002/jib.523

3. **Haslbeck, K.**; Bub, S.; Schönberger, C.; Zarnkow, M.; Jacob, F. and Coelhan, M.: On the fate of β-myrcene during fermentation – the role of stripping and uptake of hop oil components by brewer´s yeast in dry-hopped wort and beer, Brewing Science, 70 (2017), no. 11/12, pp. 159-169, DOI: 10.23763/BrSc17-16haslbeck

4. **Haslbeck, K.**; Minkenberg, D. and Coelhan, M.: Investigations into the transfer rate of volatile compounds in dry hopping using an octanol-water partition coefficient model, Journal of the American Society of Brewing Chemists, 76 (2018), no. 3, pp. 169-177, DOI: 10.1080/03610470. 2018.1483701

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Abbreviations

Abbreviation	Meaning
α	Level of significance
abs.	Absolute
amu	Atomic mass unit
approx.	Approximately
ANOVA	Analysis of variance
ASBC	American Society of Brewing Chemists
ATF	Acetyltransferase
B	Blind (without enzyme treatment)
β-Glc	Glycoside
C	Control
°C	Degree Celsius
CA	Cascade
cf.	confer
CIC	Character impact compounds
cm	Centimeter
CO_2	Carbon dioxide
d	Diameter
DLG	Deutsche Landwirtschafts-Gesellschaft (German Agricultural Society)
EBC	European Brewery Convention
e.g.	exempli gratia (lat.: for example)
et al.	et alia (and others)
EtOH	Ethanol
EU	Eureka!
eV	Electron volt
F	Fertilized
FD	Flavor dilution
Ferm.	Fermentation
FID	Flame-ionization detector
FU	Flavor unit
g	Gram
GB	Great Britain
GC	Gas chromatography
GC-MS	Gas chromatography coupled with mass spectrometry
GC-TOF-MS	Gas chromatography coupled with time-of-flight mass spectrometry
GC-O	GC-olfactometry
h	Hour
HB	Hallertau Blanc
HE	Hersbrucker
HHT	Hallertauer Tradition
HHV	Hallertauer Hopfenveredelungsgesellschaft mbH
hl	Hectoliter
HM	Hallertauer Magnum
HSI	Hop storage index
HVG	Hopfenverwertungsgenossenschaft e.G.
IBU	International bittering units

IPA	India pale ale
i.d.	Inner diameter
i.e.	Id est (lat.: that is to say)
IL	Illinois
Inc	Incorporated
K_{OW}	Octanol-water partition coefficient
kg	Kilogram
kPa	Kilopascal
L	Liter
L.	Linnaeus
LC	Liquid chromatography
ld	Low dosage
log	Logarithm
m	Meter
M	Molar
MA	Massachusetts
MB	Mandarina Bavaria
MCC pathway	Leucine catabolism pathway
MEBAK	Mitteleuropäische Brautechnische Analysenkommission (Central European Commission for Brewing Analysis)
2MIB	2-methylbutyl isobutyrate
min	Minute
µl	Microliter
ml	Milliliter
µm	Micrometer
mm	Millimeter
4MMP	4-methyl-4-mercaptopentan-2-one
4MSP	4-methyl-4-sulfanyl pentan-2-one
MN	Minnesota
MS	Mass spectrometer
MS/MS	Mass spectrometry/mass spectrometry (Tandem mass spectrometry)
m/z	Ratio mass-to-charge
n	Number
NA	Non-alcoholic
Na_2SO_4	Sodium sulfate
NC	North Carolina
n. d.	Not detected
NEIPA	New England IPA
ng	Nanogram
n. i.	Not identified
nm	Nanometer
No.	Number
n/a	Not available
OAV	Odor activity value
OG	Original gravity
OR	Oregon
OYE	Old yellow enzyme
°P	Degrees plato
PA	Pennsylvania
PAD	Phenolic acid decarboxylase

PDMS	Polydimethylsiloxane
PG	Progress
pNP	*p*-nitrophenol
pNP-β-Glc	*p*-nitrophenyl-β-glucopyranoside
POF	Phenolic off-flavor
ppt	Parts per trillion
PTR	Proton-transfer reaction
QP	Quadrupole
Quant.	Quantification
r	Correlation coefficient
R	Samples treated with Rapidase F64
R^2	Coefficient of determination
Rap	Rapidase F64
RC	Reference compound
Ref.	Reference
RI	Retention index
rpm	Revolutions per minute
RT	Retention time
s	Second
S.	*Saccharomyces*
SD	Standard deviation
SAFE	Solvent-assisted flavor evaporation
3SH	3-sulfanylhexan-1-ol
SIM	Select ion monitoring
3S4MP	3-sulfanyl-4-methyl pentan-1-ol
3S4MPA	3-sulfanyl-4-methyl pentyl acetic acid
SPE	Solid phase extraction
SPME	Solid phase microextraction
TE	Tettnanger
TOF	Time of flight
Tr	Trace
TUM	Technical University of Munich
U	Unit
U	Unfertilized
U.K.	United Kingdom
U.S.	United States
U.S.A.	United States of America
UV–vis	Ultraviolet–visible
vol	Volume
v/v	Volume/volume
w/w	Weight/weight

Summary

Hops are an indispensable und valuable raw material in beer production. The cultivation and breeding of the hop plant (*Humulus lupulus* L.) from the Middle Ages have resulted in a multitude of varieties with different aroma properties for brewers to choose from. In recent years, there has been an increase in the production of intensely flavored specialty beers using new hop varieties. These “creative beers”, referred to as craft beers, are often brewed with the flavoring method of dry hopping, which achieves a recognizable hoppy flavor. However, knowledge regarding the formation of dry hoppy beer flavor is limited. The lack of process control leads to high losses of valuable flavor components in dry hopping, as well as unwanted variations in the beer flavor.

In this thesis, potential factors that significantly influence the concentrations of hop flavor components during dry hopping were studied with a focus on the investigation of conversion reactions, loss- and extraction processes. Consequently, glycosides and glucosidases of the hop raw material were analyzed, and biotransformations, adsorption- and volatilization processes and the extraction properties of the base beer were investigated. The experiments confirmed that essential oil and glycosides of hop contain multiple flavor-active components. Furthermore, evidence was provided that hop patterns are suitable for use in dry hopping regardless of the seed percentage 1.3 ± 0.3 % or 18.9 ± 2.3 %. In the scope of the present studies, dried hop cones and brewing yeast strains showed glycoside-hydrolyzing activity, 0.11 ± 0.01 U/g and 0.07–0.15 U/l, respectively. During dry hopping these enzymes lead to a catalytic release of flavor components, which are present in a glycosidically bound form (flavor-precursor) in hops. The investigated brewing yeast strains influenced to varying degrees the concentration of monoterpene alcohols in dry-hopped wort by *de novo* synthesis of, e.g. linalool, geraniol, β-citronellol, nerol and α-terpineol. Linalool, geraniol, and β-citronellol are proven to be important components for the citrus hoppy beer flavor. There was a difference in geraniol concentration of 5.1 µg/l between a wheat beer yeast strain (TUM 68) and a lager beer yeast strain (TUM 69). The decrease in geraniol concentration by geraniol metabolism depended on the brewing yeast strain and increased with the level of wort´s original gravity; the highest and lowest relative decrease in concentration in the test series were 83 % (TUM 193; 18 °P) and 36 % (TUM 506; 7 °P).

In addition to the monoterpene alcohols, the brewing yeast also influenced volatile and hydrophobic terpenes from the hop, e.g. β-myrcene. The volatilization into the gas phase and adsorption on the yeast cell significantly reduced the concentration of β-myrcene in dry-hopped young beer. At cell counts that are common during the main fermentation, approx. 100 million cells/ml, 98-99 % of the dissolved terpene was irreversibly bound by the yeast

strain, regardless of whether it was a bottom- or top-fermenting type. The rise in dry hopping temperature and basic beer ethanol content to levels present during main fermentation (20 °C) or strong beers (8.1 % v/v ethanol) influenced the transfer rates of flavor-active volatiles from hop pellets into test beers. The increase varied depending on the component. For β-myrcene the increase was higher (factor 3.2 or 3.4) compared to linalool (factor 1.2 or 1.6). In addition, a lower yield of essential oil constituents was confirmed at higher hop dosage amounts. An increase in the dosage by a factor of 5 led to an increase in β-myrcene and linalool by a factor of 1.7 and 3.0, respectively. A theory explaining different yields between the essential oil constituents in the series was found. A solubility model was established based on the octanol-water partition coefficient (log K_{OW}) of essential oil constituents. The transfer rates of the volatile components into the base beer correlated with their log K_{OW} values, the higher the hydrophilicity, the higher the transfer rate. The differences in solubility properties explained low rates of β-myrcene (0.3–2.7 %) compared to linalool (71–146 %). In connection with the extraction of aroma components, indications were confirmed that other groups of substances, such as the α-acids, influence the yield of the essential oil components during dry hopping. The tendency towards lower transfer rates with increasing α-acid content was recorded.

Overall, the results of this thesis support the approach that the flavor of dry-hopped beer is created by selecting the hop variety, brewing yeast strain, basic beer starter material, and by setting process parameters.

Zusammenfassung

Hopfen ist ein unverzichtbarer und wertvoller Rohstoff bei der Bierbereitung. Die Kultivierung und Züchtung der Hopfenpflanze (*Humulus lupulus* L.) ab dem Mittelalter haben eine Vielzahl an Sorten mit unterschiedlichen Aromaeigenschaften hervorgebracht, auf die Brauer zurückgreifen können. In den letzten Jahren werden vermehrt Spezialbiere eines aromaintensiven Typs unter der Verwendung neuer Hopfenzüchtungen hergestellt. Diese „Kreativ-Biere“, sogenannte Craftbiere, werden oftmals mit der Aromatisierungsmethode „Kalthopfung“ gebraut, wodurch ein deutliches Hopfenaroma erzielt werden kann. Trotz des Erfolges dieser Biere, ist das Wissen zu der Ausbildung des Kalthopfungsaromas begrenzt. Die fehlende Prozesskontrolle bei der Kalthopfung führt zu hohen Verlusten wertgebender Aromakomponenten, sowie ungewollten Variationen im Bieraroma.

In dieser Thesis wurden potenzielle Faktoren untersucht, die während der Kalthopfung signifikante Konzentrationsänderungen flüchtiger Hopfenaromakomponenten bewirken können mit dem Fokus auf der Untersuchung von Umwandlungsreaktionen, Verlustprozessen und Extraktionsvorgängen. Folglich wurde die Analyse der Glycosid- und Glucosidase-ausstattung des Hopfenrohmaterials sowie die Untersuchung von Biotransformationen, Adsorptions- und Verflüchtigungsvorgängen und der Extraktionseigenschaften des Grund-bieres durchgeführt.

Die Experimente bestätigten, dass neben dem ätherischen Öl in den Glycosiden des Hopfens eine Vielzahl an aromaaktiven Komponenten enthalten sind. Es wurde der Nachweis erbracht, dass Hopfenmuster unabhängig der Samenanteile 1,3 ± 0,3 % oder 18,9 ± 2,3 % für den Einsatz bei der Kalthopfung geeignet sind. Des Weiteren zeigten getrocknete Hopfendolden und Brauhefestämme 0,11 ± 0,01 U/g bzw. 0,07–0,15 U/l glycosidhydrolysierende Aktivität. Die Anwesenheit dieser Enzyme bei der Kalthopfung von Bier führt zur katalytischen Freisetzung von Aromakomponenten, die in glycosidisch gebundener Form (Aroma-Precursor) in Hopfen vorliegen. Die untersuchten Brauhefestämme beeinflussten in unterschiedlichem Maß die Konzentrationen monoterpener Alkohole durch die *De-novo*-Synthese von beispielsweise Linalool, Geraniol, β-Citronellol, Nerol und α-Terpineol. Linalool, Geraniol, und β-Citronellol sind nachweislich wichtige Komponenten für das Citrus-Hopfenaroma von Bier. Zwischen einem Weizenbierhefestamm (TUM 68) und einem Lagerbierhefestamm (TUM 69) lag etwa eine Differenz der Geraniolkonzentration von 5,1 µg/l vor. Die Abnahmerate der Geraniolkonzentrationen durch den Geraniolmetabolismus hing ebenfalls von dem Brauhefestamm ab und stieg mit der Höhe des Stammwürzegehaltes; die höchste bzw. niedrigste Konzentrationsverringerung in der Versuchsreihe betrug 83 % (TUM 193; 18°P) bzw. 36 % (TUM 506; 7 °P).

Neben den monoterpenen Alkoholen wurden Konzentrationen flüchtiger hydrophober Terpene des Hopfens, z.B. β-Myrcen, durch die Brauhefe beeinflusst. Die Ausgasung von β-Myrcen in die Gasphase und die Adsorption an den Hefezellen reduzierten signifikant dessen Konzentrationen in Jungbier. Bei Zellzahlen wie sie während der Hauptgärung üblich sind, ca. 100 Mio. Zellen/ml, wurden 98–99 % des gelösten Terpens von den Hefestämmen irreversibel gebunden, unabhängig des Typs ober- oder untergärig.

Die Erhöhung der Temperatur und des Alkoholgehaltes des Grundbiers, auf Werte die bei der Hauptgärung (20 °C) bzw. der Fermentation von Starkbieren vorkommen (8,1 % v/v Ethanol), steigerten die Transferraten flüchtiger Hopfenkomponenten aus dem Hopfenpellet in das Bier. Die Zunahme schwankte je nach Komponente, für β-Myrcen war die Steigerung höher (Faktor 3,2 bzw. 3,4) im Vergleich zu Linalool (Faktor 1,2 bzw. 1,6). Darüber hinaus wurde bestätigt, dass mit höheren Hopfendosagemengen die Ausbeute sinkt. Eine Erhöhung der Dosage um den Faktor 5 führte zu Zunahme an β-Myrcen und Linalool um die Faktoren 1,7 bzw. 3,0. Für die erfassten Unterschiede zwischen den ätherischen Ölkomponenten in den Ausbeuten in den Versuchsreihen wurde eine Erklärung gefunden. Ein Löslichkeitsmodel basierend auf den Octanol-Wasser Verteilungskoeffizienten (log K_{OW}) wurde aufgestellt. Die Transferraten der ätherischen Ölkomponenten in das Grundbier korrelierten mit ihren log K_{OW}-Werten, je höher die Hydrophilie, desto höher die Transferrate. Die Unterschiede in den Löslichkeitseigenschaften erklärten die niedrigen Raten von β-Myrcen (0,3–2,7 %) im Vergleich zu Linalool (71–146 %). Im Zusammenhang der Extraktion von Aromakomponenten wurden Hinweise bestätigt, dass weitere Stoffgruppen, etwa die α-Säuren, die Ausbeute der ätherischen Ölkomponenten bei der Kalthopfung beeinflussen. Die Tendenz zu geringeren Transferraten mit steigendem α-Säure-Gehalt wurde erfasst.

Die Ergebnisse dieser Dissertation zeigen, dass das Aroma kaltgehopfter Biere über die Auswahl der Hopfensorte, den Brauhefestamm, das Grundbier und die Einstellung der Prozessparameter gestaltet werden kann.

1 Introduction and motivation

For centuries, hops have been a crucial raw material to impart microbiological stability to beer. Hop-derived components in beer are primarily associated with many aromatic impressions and taste experiences. In beer brewing, hops are generally added between the start of wort boiling and final beer filtration. The hop addition timing influences different process-related reactions that impact the utilization and extraction of volatile and non-volatile hop components and their subsequent contribution to the beer aroma [1, 2]. Beers with hoppy flavor are usually brewed by adding multiple and late dosages. For this, hops are added to the wort kettle towards the end of boiling, during the whirlpool rest (late hopping) and to green and lager beer, when it is called dry hopping. Dry hopping refers to the addition of the hop product, e.g. cones or pellets to green or bright beer during primary and secondary fermentation and beer lagering. This “rediscovered” old traditional flavoring technique [3] can produce intensely hoppy beers, whose flavor profiles differ considerably from kettle- or late-hopped beers [4–6]. The odor of dry-hopped beer is often described as being reminiscent of the raw hop aroma. The flavor of dry-hopped beers is the result of multiple interactions of compounds that are disproportionately extracted from complex raw material. Lots of technical and analytical issues are often not completely understood, although much research has been conducted in the field of hopping beer [7]. Explaining key factors that influence the transfer of volatile hop constituents into wort or beer could be beneficial for creating hoppy beer flavors. Controlling the method of dry hopping in a more targeted manner would be a great way of creating extraordinary beers with various flavors.

Currently, there is interest in using the aroma potential of the hop raw material for beer odor. In the last two decades, not only in the U.S. but also in many countries with a beer tradition, such as Belgium, Germany and the Czech Republic, more and more breweries have successfully introduced a special type of beer, termed craft beer, into a highly competitive market [1]. Many of these craft brewers use hops primarily as a spice within dry hopping that contributes significantly to the beer character. Although craft beers represent 2 % of the world beer market share (2019), 20 % of the world´s hop production is used for these beers [8]. The breeding and cultivation of hop varieties with intense exotic fruit-like or citrus fruit-like aromas are closely related to craft beers brewed with these hops, which have proved popular with beer consumers. Thus, hops play a key role in this movement.

1.1 The hop plant

The genus *Humulus* is a dioecious, perennial, climbing vine capable of heights ranging from 2–6 meters on trellis structures. This genus belongs to the Cannabaceae family of the Urticales suborder that belongs to the natural order of Rosales [9]. The only other genus in the family is *Cannabis* (Figure 1), which is solely represented by *C. sativa* (i.e. Indian hemp, marijuana, or hashish) [10]. The genus *Humulus* is represented by three species, the "common hop" *Humulus lupulus* L., the "Japanese hop" *H. japonicus* Zieb. et Zucc [11] and *Humulus yunnanensis* Hu [12].

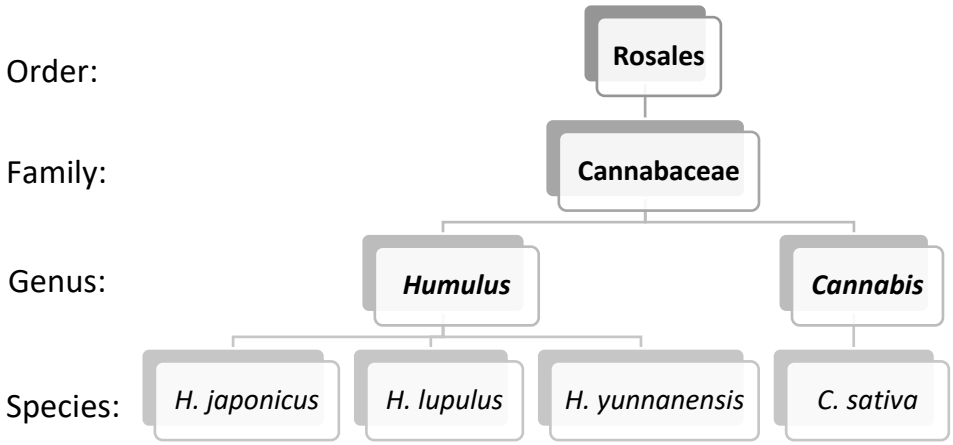


Figure 1 Classification of the hop plant [9, 13]

1.1.1 Cultivation of unfertilized and fertilized hops

The female plant of *Humulus lupulus* L. (Figure 2) is the only hop species that contains components relevant to beer brewing [1]. Apart from the minimal use of *Humulus lupulus* L. in pharmacology or as ornamentals, about 97 % of cultivated hops worldwide are used for brewing purposes. The hop plant is cultivated in temperate climate regions that are located between latitudes 35° and 55° of the northern and southern hemispheres. Consequently, a considerable amount of the growing season has more than 13 hours of daylight, which is a prerequisite for flowering. Additionally, the crop also has a steady supply of water.

The hop flower, which is called a cone, consists of a spindle and leaves with glands that secrete lupulin, a fine yellow resinous powder (Figure 2) that contains valuable compounds for brewing (cf. chapter 1.1.3–4). Hops are harvested in late summer or early autumn when the hop cones have ripened, and the content of bitter-tasting substances is highest [1]. Freshly picked, green hop cones (wet hops) have a water content of about 75–80 %. Since hops cannot be stored in this state, they are dried immediately after harvesting at a maximum temperature

of 65 °C to a residual water content of 9–11 %. The plant, which is native to Central Europe, has been used for beer brewing since the 9th century [14]. It has been cultivated since the 13th century in Central Europe and since the 18th century in North America. Today, the world´s hop production is dominated by Germany and the U.S., representing about 76.8 % (2018) of the total hop (weight) output [15]. The largest hop-growing areas include the Hallertau region in Germany and the Yakima Valley in the U.S., which make up 54 % of the hop area worldwide under cultivation. Other hop-growing countries are the Czech Republic, Poland, Slovenia, U.K., Ukraine, China, South Africa, Australia, and New Zealand [1].

Figure 2 Hop cones (left); lupulin glands on bracts (middle); electron micrograph of lupulin glands (right) [1]

Currently, there is a farming method to produce unfertilized or fertilized hops for brewing. In many commercial hop-growing regions such as in Germany, male plants are prohibited to prevent the fertilization of the female plants, and, therefore, the production of seeds [1]. Hop seeds have a high lipid content of up to 32 % [16]. Since several decades it is believed that oxidation of the seed fatty acids might produce off-flavors in beer, which is considered to have a damaging impact on lager beer quality [17]. Furthermore, a loss of lupulin content or reduction in essential oil (hereinafter referred to as “oil” or “hop oil”) content was determined for some varieties [18]. The reasons to cultivate fertilized hops may be agronomic, e.g. earlier closing of flowering, reduced disease susceptibility, and economic, e.g. seed content is heavier and larger clusters [19]. Another feature of fertilized hops might be interesting for brewers; hops produce several enzymes that can influence the result of the brewing process, and it was shown that specific enzyme activities such as maltase in dry hops could be higher in fertilized plants than in unfertilized plants [20].

1.1.2 Hop types and products

There are more than 200 hop varieties that can be used for brewing [1]. The shape of the hop cone is a significant botanical distinguishing criterion for a hop variety. However, hop varieties are classified as “aroma hop” and “bitter hop” varieties based on their α-acid content. The α-acids within the lupulin glands are converted into iso-α-acids during wort boiling. The iso-α-acids are the main bittering substances in beer. Brewers select a hop variety according to its classification to add pleasant bitterness or flavors to beers [21]. Thus, the grouping of hop varieties is indicative of the intended use in the brewery. The official designation of a new variety is carried out by the regulatory authorities according to the breeder´s instructions. Aroma hops are usually characterized by a mild, pleasant aroma, higher polyphenol content, and α-acid levels well below 10 %. For bitter varieties, the α-acid content is usually over 10 %. The effort of hop breeders led to the development of “high-α hop” with the α-acid content over 12 % and the “super-high-α hop” containing over 15 % α-acids. Due to vintage-related fluctuations some varieties can have higher or lower α-acid levels than defined by their classification. Major bittering hop varieties traded on the world market are Galena, Hallertauer Magnum, Hallertauer Taurus, Herkules, Nugget, and Millennium. Popular aroma hop varieties are Hallertauer Mittelfrüh, Hallertauer Perle, Hallertauer Tradition, Spalter Select, Hersbruck Hersbrucker, Tettnang Tettnanger, Saaz, and Cascade. The aroma hops, sometimes called “flavor hop”, impart floral, fruity, citrus, and exotic fruit-like flavors to a beer. The aroma hop varieties Mandarina Bavaria, Hallertau Blanc, Huell Melon, Callista and Ariana, were bred at the Hop Research Center Hüll, Germany, and introduced in 2012 [22]. It should be mentioned that “dual-purpose hops”, such as Nelson Sauvin, Chinook, and Simcoe®, offer diverse applications in beer brewing because they combine the properties of typical aromas and bitter varieties.

Until the 20th century, only whole hop cones were used for beer brewing [1]. Since the 1970s, conventional hop products include packaged hop cones, regular (type-90) and enriched (type-45) pellets, CO_2 extract, and ethanol extract. No chemical additives or substance separation processes are used to manufacture these products. Outside the German purity law, hop suppliers offer hop products (e.g. light-stable and pre-isomerized extracts) for precisely defined purposes (e.g. flavoring, bittering, foam-improving) and time of additions, e.g. at whirlpool rest, beer filtration, and final beer.

1.1.3 Valuable hop components

In beer brewing, only the inflorescences of the female plants from the hop cones (strobile) are used [14]. The hop oil, the bitter acids, and the polyphenols (Figure 3) are the valuable ingredients for brewing. These three fractions are secondary metabolites of the hop plant.

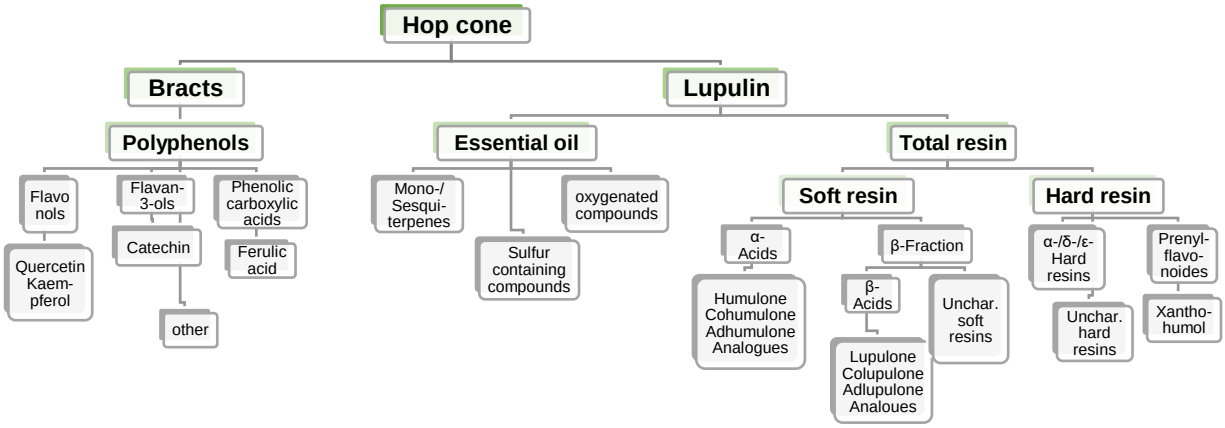


Figure 3 Schematic classification of the most important secondary metabolites of hop [13, 21]

The overall average chemical composition of fresh, dried hop cones is shown in Table 1. Most polyphenolic compounds are present in the bracts [1]. Hops (dry matter) contain about 2–5 % polyphenols [23] that are considered to have various positive effects on human health through antioxidative effects, for example. The oil and the bitter acids are produced in the lupulin glands. Only the lupulin glands contain enzymes that can introduce so-called prenyl groups as side chains in terpenophenolic or polyphenolic basic structures. The hop oil represents 0.5–5.0 % of the dried hop cone (Table 1). The composition and role in beer flavor of hop oil are discussed in chapters 1.1.4 and 1.2.1. Hops contain a high percentage of bitter-tasting substances, known as resins. The hop resin is differentiated as soft resins (soluble in hexane) and hard resins (soluble in methanol, not in hexane), which account for 10–30 % and 2–3 % of a dried hop cone, respectively [1]. Both fractions combined are called total resin. The main components of the soft resin are α- and β-acids. These are mixtures of homologs with chemically comparable structures and part of the soft resin is isomerized during wort boiling, as mentioned before. Consequently, part of them increases solublility in wort or beer and affects the bitter beer taste, predominantly the isomerized α-acids that are called iso-α-acids. Detailed information regarding the bitter hop compounds is given in literature references cited in this chapter. It should be mentioned that xanthohumol from the hard resin fraction might have an anticarcinogenic effect. However, regulatory approval of xanthohumol as medicine is still pending.

Table 1 Average chemical composition of dried hop cones [1][1], [2][23]

Constituent	Amount (%)	Constituent	Amount (%)
• α-Acids	2–20 [1]	• Proteins	1 [2]
• β-Acids	1–10 [2]	• Waxes and steroids	- [2]
• Essential oil	0.5–5 [2]	• Pectins	2.0 [2]
• Polyphenols	2–5 [2]	• Ash	10 [2]
• Monosaccharides	2.0 [2]	• Moisture	8–12 [1]
• Amino acids	0.1 [2]	• Cellulose etc.	40–50 [2]

Apart from the oil, the β-glycosides are flavor precursors and another potential aroma source extracted during beer production [24, 25]. Glycosides are present not only in the lupulin glands but also in the cones and surrounding vegetative tissue. The process of glycoconjugation allows the hop plant to produce and store volatile molecules in a soluble and inactive state until they are needed, e.g. as defense against an insect predator or attractant for pollination [26]. The composition of the hop cone depends primarily on the hop variety. However, the vintage, provenance, and harvest time ("ripeness") also influence the contents of (valuable) compounds [1, 27] and consequently the flavor that is achieved through dry hopping of beer [28, 29]. Later harvest dates can lead to increased oil contents and in case of Cascade hops used for dry hopping to higher intensity of citrussy in beer [30].

1.1.4 Free and bound oil components

The hop oil is characterized by a pleasant aromatic odor. It is a complex mixture of substances, which are generally obtained from cones by steam distillation, pressing or extraction. The boiling point of the oil constituents is in the range from 50 to 320 °C [31]. The composition and amount of the hop oil is the result of terpene biosynthesis and secondary reactions (e.g. oxidation) that depend mainly on genetics, cultivation (e.g. cultivar, geography, as mentioned before), kilning, processing, and lastly, hop storage [32–34].

1.1.4.1 Biosynthesis of terpenes and terpenoids

In the physiology of hop plants, the hop oil constituents have various tasks. The most important reasons for their synthesis and emission are to attract pollinators and seed disseminators, and to defend the hop plant against insect predators and pathogens [35]. A detailed discussion of the synthesis and composition of relevant hop aroma compounds within the plant is provided in a review article in 2018 [7]. In short, the biosynthesis of terpenes primarily takes place in the lupulin glands. Within a strictly organized reaction cascade, the dimethylallyl diphosphate and isopentenyl diphosphate serve as synthesis components. These

compounds result from either pyruvate and glyceraldehyde-3-phosphate or acetyl-CoA, which are formed by the plastidial 2-C-methylerythritol 4-phosphate and the cytosolic mevalonic-acid pathway, respectively [36]. Several enzymes are involved in pathways such as the monoterpene biosynthesis. The prenyltransferases, for example, catalyze condensations, the reaction of dimethylallyl diphosphate and isopentenyl diphosphate into neryl diphosphate or geranyl pyrophosphate. Cyclic terpenes such as limonene are produced from the neryl diphosphate that is an essential precursor. Many non-cyclic monoterpenes, such as β-myrcene, originate from geranyl pyrophosphate [37]. Another condensation reaction, which represents a crucial step in sesquiterpene synthesis, is the connecting of two dimethylallyl diphosphate molecules and isopentenyl diphosphate to produce farnesyl pyrophosphate. Farnesyl pyrophosphate is a precursor of β-farnesene, which is produced following the elimination of pyrophosphoric acid – alternatively, further cyclization of pyrophosphoric acid results in the isomers α-humulene and β-caryophyllene. A large family of enzymes, the terpene synthases/cyclases, is involved in the generation of some mono- and sesquiterpenes [38]. These enzymes also catalyze reactions resulting in compounds of the terpenoid group (e.g. linalool and geraniol) [39], which are very important qualitatively, though not quantitatively.

1.1.4.2 Composition of terpenes and terpenoids

Hop oil consists of many different volatile compounds, about 440 have been identified and chemically characterized to date [40]. Roberts et al. supposed there are potentially over 1000 hop volatiles [41]. These can be divided into three main classes, illustrated in Figure 4; hydrocarbons, which account for 40–80 % of the total oil [42], compounds that contain oxygen, and compounds that contain sulfur.

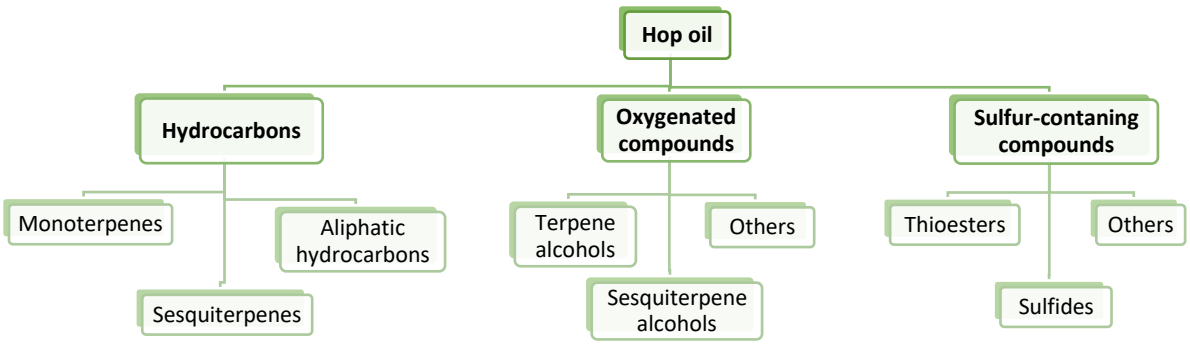


Figure 4 Classification of hop oil according to Sharpe and Laws [18]

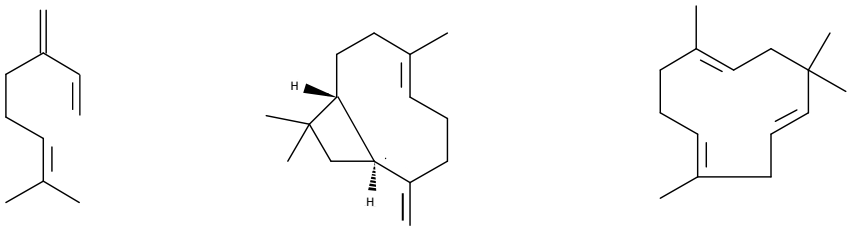
The hydrocarbon fraction consists of the group of monoterpenes with β-myrcene (10–73 %) as the main component, the group of sesquiterpenes, with the major components being α-

humulene (15–42 %) and β-caryophyllene (3–15 %) and residual aliphatic hydrocarbons [40]. In the following, Table 2 lists important hop oil constituents.

Table 2 Average concentration (%) of selected compounds in hop oil [40]

Constituent	Amount (%)	Constituent	Amount (%)
Monoterpenes		**Aldehydes**	
• β-myrcene	10–73	• hexanal	< 0.01
• γ-terpinene	< 0.38	• (E)-2-hexenal	< 0.01
• α-pinene	< 0.01–0.4	• geranial	< 0.01
• β-pinene	< 0.01–1.8	**Ketones**	
• limonene	< 0.02–0.5	• 2-decanone	< 0.01–0.3
Sesquiterpenes		• 2-undecanone	< 0.01–1.5
• α-humulene	15–42	• (E)-β-damascenone	not available
• β-caryophyllene	3–15	**Alcohols**	
• β-farnesene	< 0.01–16	• linalool	< 0.1–1.1
• δ-cadinene	0.1–3.7	• geraniol	< 0.1–1.5
• α-selinene	< 0.01–7.0	• α-terpineol	< 0.02
Epoxides		• nerol	< 0.01–0.1
• humulenepoxid I	< 0.01–0.42	• 1-octen-3-ol	< 0.01
• humulenepoxid II	< 0.01–1.86	• β-citronellol	< 0.02
• β-caryophyllenoxid	< 0.01–0.64	**Esters**	
Sulfurous compounds		• geranyl acetate	< 0.01–1.4
• dimethyl disulfide	< 0.001	• neryl acetate	< 0.01–0.15
• thiols (4MSP, 3SH)	not available	• isobutyl isobutyrate	< 0.01–0.92
• myrcene disulfide	< 0.001	• methyl-4-decenoate	< 0.01–5.6

The β-myrcene appeared to be the most abundand and dominant sensory flavor component in various varieties, e.g. Spalter Select, Cascade and Northern Brewer hop cones [32, 43]. It´s impact on the flavor is described as being “herbaceous”, “balsamic” and geranium-like. By applying the sensomics concept and aroma recombination experiments using a cellulose matrix, a recent study confirmed key role of β-myrcene for the flavor of hop pellets of varieties Hallertauer Mandarina Bavaria, Hallertauer Cascade, and Hallertauer Mittelfrüh [44]. Figure 5 illustrates the structural formulas of major hydrocarbons in hop oil.



β-Myrcene β-Caryophyllene α-Humulene

Figure 5 Structural formulas of major hydrocarbons of the hop oil [42]

The β-farnesene is a non-cyclic sesquiterpene that is present at relevant concentrations in some aroma hop varieties such as Saaz, Lublin and Styrie [45]. Hops such as Hersbrucker Spaet can also contain several bicyclic and tricyclic terpene hydrocarbons, e.g. bergamotene, aromadendrene, α- and β-selinene, germacrene, and amorphene [46]. In addition to terpene hydrocarbons, some terpenoids consist of a terpene carbon skeleton and primarily carboxyl, ester, and ether functional groups. These functional groups can add a hydrophilic effect to these compounds. The terpenoid fraction, which accounts for 10–30 % of the hop oil [1], consists of esters, alcohols, and ketones. Interestingly, the concentration of terpenoids increases not only during the ripening of the hop cone [28, 29], but also during hop processing, and (aerobic) storage [33]. The carboxylic acid esters are the largest group of oxygenated compounds and the third largest group of substances in total. The hop oil contains methyl esters of various straight-chain primarily or branched carboxylic acids, but also their ethyl, propyl, or (iso-)butyl esters [47, 48]. The primary esters in hop oil are geranyl acetate, geranyl propionate, and geranyl isobutyrate. Free carboxylic acids or fatty acids, e.g. butanoic acid and isovaleric acid, which can be formed during the hop storage, have a rancid butter-like or cheesy odor [1]. The monoterpene alcohols linalool, geraniol, and their isomer β-citronellol are vital compounds in terms of citrus hoppy beer flavor [49], although the maximum concentration is usually lower than 1.5 % in hop oil (Table 2). Linalool is classified being a key indicator substance for a hoppy flavor [50]. A correlation was found between the linalool content and a floral, fruity hop aroma in beer [51]. This monoterpene alcohol can significantly exceed the odor threshold of around 10 µg/l (10 ppb, (R)-linalool) in beer, even within a conventional hopping regime during the wort boil. Linalool is chiral and is mainly present in hops (90 %) as an odor-active R-enantiomer (Figure 7) [52]. In terms of beer flavor, the threshold of (R)-linalool is lower than the (S)-isomer and their (racemic) mixture. The composition of enantiomers is retained even during hop processing, but changes during beer production and storage.

Hop oil contains various sulfurous compounds (cf. Table 2) that can contribute to desired flavors such as “exotic fruit” and grapefruit-like notes to beer [53]. Thiophenes, methyl- or polysulfides, methyl thioesters and polyfunctional thiols have been determined [7]. Sulfur adducts of several hop oil components such as myrcene disulfide, α-humulene, or β-caryophyllene were shown to exist [54]. Some of the compounds known as mercaptans are extremely odor-active, such as specific thiols that contribute to the hop varietal impact on beer flavor, for instance by Nelson Sauvin and Tomahawk hops [55]. A popular thiol is 4-methyl-4-sulfanyl pentan-2-one (4MSP, also known as 4MMP) which is a potent hop flavor component, contributing to the muscat-like flavor to beer and is considered reminiscent of the smell of the hop variety U.S.-Cascade [56]. In addition to the cultivar, the growing location and conditions of a hop plant influence the levels of thioesters and sulfur compounds [57]. In

the case of 4MSP, the growing region plays a crucial role, as especially the usage of copper-containing fungicides used in European hop gardens leads to decrease of its contents [58]. Other odoriferous sulfurous compounds of hop oil are 3-sulfanyl-4-methyl pentan-1-ol (3S4MP) and 3-sulfanyl-4-methyl pentyl acetic acid (3S4MPA) found in Nelson Sauvin hops [59]. Interestingly, synergystic effect by 3S4MP enhanced the flavors of 3S4MPA and 2-methylbutyl isobutyrate (2MIB) in model solution [60].

1.1.4.3 Composition of glycosides

In hops, compounds such as terpene alcohols can be present as free volatiles but are also esterified and bound to monosaccharides, referred to as glycosides, as mentioned before. Glycosides are non-volatile components that consist of a carbohydrate (usually β-D-glucose) group that is bound to the hydroxyl group of a non-sugar component (aglycone) [61]. Figure 6 shows linalyl-β-D-glycopyranoside, which is the glycoside of linalool and β-D-glucose.

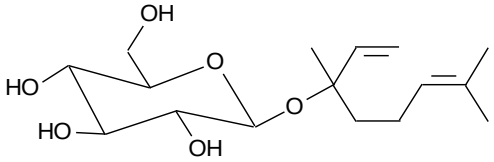
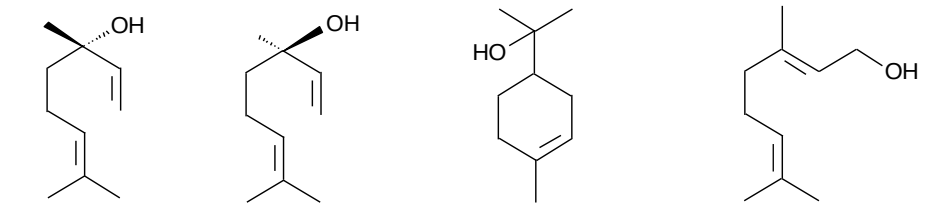
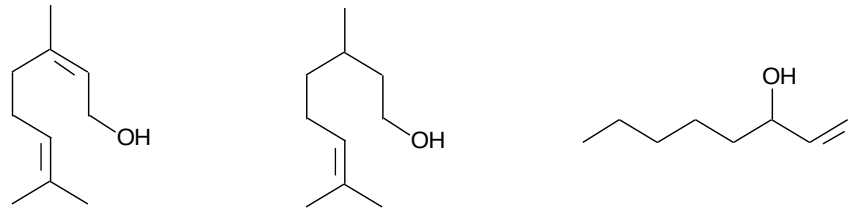


Figure 6 Linalyl-β-D-glucopyranoside [62]

Goldstein et al. determined 60 different aglycones in hops with the major sugar moiety glucose (92 %) in the C1 position (55 %) in the molecular structure [24]. Besides monoterpene alcohols, e.g. linalool, α-terpineol, and hop glycosides can contain aliphatic alcohols, e.g. (Z)-3-hexen-1-ol, 1-octen-3-ol, aromatic structures such as benzyl alcohol, vanillin, and norisoprenoid compounds such as 3-hydroxy-7,8-dihydro-β-ionol and β-damascenone. Figure 7 shows the chemical structures of some aglycones.



(*S*)-Linalool (*R*)-Linalool α-Terpineol Geraniol



Nerol β-Citronellol 1-Octen-3-ol

Figure 7 Compounds that can occur in a glycosidically bound state [63]

Wilhelm determined through analyzing five different hop varieties (Perle, Smaragd, Hersbrucker, Golding, Cascade) that the quantity of glycosidically bound linalool and geraniol in hops depends significantly on the cultivar [64]. Furthermore, a correlation was shown between the concentrations of free and bound linalool in hops; the bound linalool corresponded to 21–36 % of the total linalool amount detected (sum of free and bound linalool). With regard to varietal specific contents of terpenyl glycosides, a recent study showed significant differences in contents of bound terpene alcohols, especially between Columbus and Centennial hops [65]. However, there is no comprehensive data on the type of aglycones or exact glycoside ratios in the hops, for more than 200 varieties. The aglycones from glycosides in hops can be released by enzymatic, thermal, and acid-catalyzed cleavage reactions during beer production, which can lead to increased concentrations of hop volatiles in beer [62]. A similar effect was documented for esterified terpene alcohols such as geranyl acetate, which leads to increased levels of geraniol in beer [66]. This theory is supported by the fact that compounds such as geranyl acetate and geranyl isobutyrate occur in several hop varieties, e.g. Polaris and Cascade that is also rich in geraniol, however, these esters are hardly found in final beer [67].

1.2 Dry hopping and beer flavor

The main objective of dry hopping is to transfer flavor components from the hops into beer, thereby exerting the lowest impact on colloidal stability and oxygen content. Apart from the beer flavor, dry hopping also influences the taste and mouthfeel of a beer, but this will not be considered in any further detail for the purpose of the present dissertation. However, it should be noted that dry hopping respectively the unintended extraction of nonvolatile hop constituents can affect bitterness and stability of dry-hopped beer, which is concluded by a recent review article [68]. The (dry) hoppy flavor of beers that is formed by volatile flavor-active compounds derived from the hop oil and their conversion products being addressed in the following chapter 1.2.1.

1.2.1 Hop volatiles in beer

Assessing hop aroma and hoppy beer flavor has been a challenging target for numerous research groups. Despite decades of research in this field, the hoppy flavor of a beer is still far from fully understood. Today, there is a consensus that the odor of beer is the result of a vast number of flavor-active compounds and compositions of these, as well as combinatory (synergy, masking) effects between beer constituents [4, 7]. Hop-derived components and their conversion products significantly influence the flavor profile of a beer. As well as

glycosides, the hop oil is another source of volatile compounds in the hop plant. Most of them originate either directly from plant metabolism, or they arise from secondary reactions, e.g. oxidation or hydrolysis of volatile and non-volatile precursor molecules. The secondary reactions proceed throughout the brewing process. Consequently, not the entire quantity of hop-derived flavorings is found in their initial state in beer. Despite degradation reactions of oil constituents, similar flavors can be detected in hop cones or dry-hopped beers, although dry hopping usually flattens hop varietal characteristics [69]. The chemical composition of the hop oil is critical to the overall hop aroma intensity, particularly since the specific volume of oil in hop bears difficulties to indicate hoppiness potential in dry-hopped beer when comparing different lots of a cultivar, e.g. Cascade [70]. A recent study showed that concentrations of oil constituents such as geraniol or β-pinene can be better marker than total oil content for hoppyness intensity of dry-hopped beer using Cascade or Centennial hops [71]. With regard to flavor potential of dry-hopped beers, partial least squares (PLS) regression is a prommissing approach to estimate flavor intensities based on concentrations of particular oil constituents, e.g. β-myrcene, 2-methyl butyl-2-methyl propanoate, linalool and α-humulene [72].

The complex composition of hop, the contribution of individual constituents to the flavor of hop, and hoppy beers have been subject to several reviews [7, 21, 73]. The following gives a brief selection of compounds that are currently considered might be used to comprehensively characterize hoppy beer flavor. A very volatile and flavor-active hop oil constituent, whose contribution to beer flavor has been proven by many research groups, is linalool, as mentioned before. The monoterpene alcohol was found to be generally present above its flavor threshold in conventionally hopped beer, as only a part is lost during boiling and fermentation [74]. The comparably good solubility (1.667 g/l in water [75]) in wort and beer is attributed to its relative hydrophilic character. It has been proposed as an analytical marker for both the intensity and quality of the hoppy flavor of beer [76–78]. Furthermore, the effect of the hopping regime, boiling time, boiling system, and beer staling on the linalool concentration has been thoroughly investigated [51, 79].

In addition to linalool, monoterpene alcohols such as geraniol and β-citronellol are reported to contribute to the floral-/citrus-like flavor of beer and to induce synergies that increase this flavor [49]. With regard to mentioned alcohols, dry hopping leads to higher concentrations compared to dosages during wort boiling [80]. Interestingly, in a recent study highest concentrations were found in beers hopped during whirlpool rest compared to kettle or dry hopping using Simcoe® hops [6]. It should be noted that other important alcohols such as α-terpineol and nerol might have comparable effects on the beer flavor but to a lower extent [49]. The group of esters is another representative from the oxygenated hop compounds, also known for its contribution to fruity and citrus-like beer flavors [53, 72]. Some esters such as

isobutyl isobutyrate, isoamyl propanoate, 2-, and 3-methyl butyl-2-methyl propanoate could exceed their threshold levels in beer, especially in dry-hopped beers [81]. In the case of isobutyl isobutyrate, it was found to decrease during wort boiling and fermentation [82, 83]. The mentioned esters were suggested being important with regard to variety specific flavors derived during (late or) dry hopping [84]. Neiens and Steinhaus reported that fruity-smelling esters ethyl 2-methyl propanoate, methyl 2-methyl butanoate, ethyl 2-methyl butanoate, and propyl 2-methyl butanoate are variety-specific aroma components of Huell Melon hops in beer [85]. In another recent study it was shown that red fruit-like flavors, which are related to the presence of esters such as ethyl isobutanoate and ethyl butanoate [86] can be increased by late or dry hopping using Barbe Rouge hop variety [87]. However, the authors did not analyze the source of mentioned esters.

Several other compounds derived from hop oil were evidenced in the final beer and have been proposed as being contributors to hoppy flavors. These include oxidation products of compounds such as α-humulene, β-farnesene, β-citronellol, geraniol, and α-terpineol [56, 79, 88]. The “noble-” or “kettle hop aroma” is generally characterized as being “herbal”, “spicy”, and “woody”. It was suggested that sesquiterpene oxidation products might be formed during wort boiling and could give rise to subtle spicy flavors [89]. The oxidation of hop-derived oil constituents may occur not only during wort boiling but also during hop storage [33]. Interestingly, in a recent study using oxidized hop for dry hopping, no adverse effect was determined on the overall taste of the final beer [90]. The test beers that were dry hopped with the oxidized hops had significantly higher sensory ratings for woody and herbal attributes. Thus, those hops may also serve to enhance the noble hop aroma in dry-hopped lager beer. Hop-derived aldehydes, e.g. (Z)-3-hexenal, and hexanal that were determined in hop oil were attributed with green and grassy flavors [32]. In case of hexenal, the oil constituent has been identified as a source of the green flavor of wet hops (freshly picked hop, 75–80 % water content). It was suggested that this is an essential flavor component in beers brewed using wet hops [1]. A recent study showed the importance of oxygen containing compounds by identifying commonalities in single varietal dry-hopped beers according to the type of character impact compounds (CIC) for hoppy flavor as being 2-furan methanol, linalool, geraniol, cis-geranic acid methyl ester, and n-decanoic acid [91]. An evident variation between the varieties of Chinook or Centennial and Cascade was shown with regard to 2-phenyl ethanal. Sulfurous compounds of the hop oil can emit an intense aroma even at negligible concentrations of a few nanograms per liter (ppt) in beer [55, 92]. 4-Sulfanyl-4-methyl pentan-2-one (4MSP) is known as a source of blackcurrant- or muscat-like odor in beer having a low threshold value of 1.5 ppt (i.e. 1.5 ng/l) [56]. The volatile thiols 3-sulfanyl-4-methyl pentan-1-ol (3S4MP) and 3-sulfanyl-4-methyl pentyl acetate (3S4MPA) were detected for the first time in the New Zealand hop variety Nelson Sauvin and the beer produced from them [59, 60]. In

beer, they give rise to an odor that is comparable to Sauvignon Blanc wine flavor. Other thiols such as 3-sulfanyl hexane-1-ol (3SH) contribute to a grapefruit-/rhubarb-like flavor in beer.
The contribution of hydrocarbons, e.g. β-myrcene, and α-humulene to hoppy beer flavor depends significantly on the hopping technology. Apart from the volatile character, hydrocarbons are relatively hydrophobic [93]. Consequently, most of these compounds are lost by evaporation during the wort boiling process, which leads to trace amounts in beers that are hopped conventionally [94, 95]. In addition, terpenes can be vaporized during fermentation [96]. Furthermore, terpene oxidation and polymerization reactions were shown to occur readily in hot wort [89].

1.2.2 Technology of dry hopping

Dry hopping can be done using different hop products. A traditional material still in frequent use is dried whole cone hops [1]. In the U.K., whole-cone hop is usually compressed into cylindrical cakes (plugs) called grafts that can be transferred directly into barrels. Pelleted hops, mostly pellet type-90, are commonly used in the U.S. and Europe for dry hopping. In practice, blends of different hop varieties are often used to react on shortages of a particular variety. Similar flavor profiles can be achieved by using varying blends of Centennial, Cascade and Chinook hops [97]. Hop oil products are used increasingly by breweries to simplify the dry hopping process and maximize efficiency. The oil is extracted from hop material using liquid or supercritical CO_2 [1].
Hop cones or pellets are usually added using a single-stage or two-stage dry hopping regime to increase extraction efficiency [98]. The popular form of dry hopping is the direct injection of hop into fermentation and storage tank before the tank is filled with wort or green beer. The hops are usually placed in a mesh bag before being added to a vessel to facilitate their separation from the beer after the dry-hopping treatment, which is shown in Figure 8. Although using a mesh bag could reduce the transfer of hop flavor components [99], it is crucial to minimize the oxygen input, which is more problematic when using cones instead of pellets, for example, because of the larger surface area. Another way to approach this problem is to dry hop the green beer. It is assumed that most of the oxygen is consumed by the yeast that is present before it can significantly oxidize the beer. The distribution of hop material can be impaired especially when dry hopping on an industrial scale [100]. Brewers often consider the methods of stirring or “rousing” hops to react and yet improve the aroma extraction when dry hopping [1]. A common method is to inject CO_2 from the bottom port of the cylindroconical vessel to promote the distribution of hop material. It should be noted that if the supplied CO_2 gas can leave the vessel, very volatile flavor compounds with poor solubility might also be lost from the beer.

Typically, when static dry hopping, the contact time ranges from a period of one to three weeks, although recent studies conclude best flavor results can be achieved when dry hopping two days up to four days maximum [101, 102]. In the context of effectiveness of short dry hopping durations, another recent study showed highest intensities of fruity and especially black currant-like flavors after two days of dry hopping with Eureka! hops [103]. The treatment described seems to be the usual scenario for small and medium-sized breweries. However, there is no standard approach to dry hopping. Actually, a survey of nine U.S. breweries revealed that each brewery dry hopped their beers in an individual way [104]. A recent questionnaire of 50 breweries showed that the extraction temperature ranges between 0 and 25 °C, with the highest proportion of dry hopping at 0–5 °C [105]. The dosage based on hop weight is sometimes very high, with 40 % of the surveyed brewers adding over 5 g/l. In breweries using large tank units, which often have neither a utility hole nor top opening, this form of static dry hopping is problematic. In the last decade, specialized devices called dynamic systems have been invented to automate the dry-hopping treatment [1]. Figure 8 shows the application of static and dynamic systems in a brewery.

Figure 8 (1) Static dry hopping at Orval Brewery; (2, 3) dynamic dry hopping equipment at Sierra Nevada Brewery [1]

The use of automated (dynamic) approaches usually involves pumping beer through a separate vessel that contains hop material that is retained on a sieve. The contact time is set by the duration of beer pumping through a circulatory system. Several different systems have been developed so far, although dry hopping in this way can result in the introduction of large quantities of hop plant material [68].

1.3 Influence on dry-hopping outcome

Dry hopping aims to dissolve volatile hop components out of the hops. The extracted amount depends on the hop material and the dry-hopping parameters. The dry-hopping parameters are usually the result of the actual brewing process step. Thus, the properties of the extraction medium (chilled wort, green, and bright beer) can differ significantly with regard to the primary and secondary fermentation processes.

1.3.1 Pellet composition and dry-hopping parameters

Pellet processing influences pellet density and particle size [1]. Various pelletizing processes could lead to differences among pellet patterns and, therefore, during static dry hopping, to the formation of a layer of the medium near the surface and another at the base of the vessel. The degree of dispersion could also influence the flavor component extraction, although it is not thought to affect the outcome during longer intervals [106]. With respect to the goal to impart hoppy flavors to beer, the dosing quantity should be based on the oil content of the hop and not on the weight or α-acid content, especially when using different types of pellets, e.g. type-45 and -90, respectively [107]. It is accepted practice to use the amount of oil added to beer within pellet dosage as an indicator for hoppiness potential, although this method might not be sufficiently specific regarding oils of different varieties, as mentioned before (cf. chapter 1.2.1).

The relative amount of the mass transfer (transfer rate) of a particular compound can be calculated based on the concentrations in the hop material, in the base beer and in the dry-hopped beer. Wide differences between volatile hop constituents were determined, e.g. β-myrcen (< 1 %) and linalool (> 80 %) [81, 108]. The transfer rates in oil constituents and, therefore, flavor intensities in dry-hopped beer are influenced by several factors, including hop variety, product (e.g. cone or pellet), dosing amount and method, contact period and temperature. It was proposed that higher α-acid contents could decrease the swelling volume of hop pellets and further influence the flavor component extraction during static dry hopping [109]. In the same year, another study showed different hop pellet swelling volume as a consequence of hop variety [110]. In the context of varietal specific effects the transfer rates of oil constituents such as geraniol can vary from 38 to 269 % between different hop varieties Hallertauer Mittelfrüh and Polaris [66]. Varietal effects on transfer rate of oil constituents, such as linalool, during kettle hopping were also previously determined [111]. The process management can impact the yield and dry hopping outcome. With regard to dosage amount, higher hopping rates lead to decrease of extraction efficiency resulting in increased amounts of components with brewing value in spent material [112]. The transfer of oil constituents, e.g. linalool and, geraniol and consequently the intensity of hoppy flavor was shown to

increase at a higher dosage of hop pellets [113], although the transfer rate could decrease as determined for several components, e.g. β-myrcene, β-caryophyllene and α-humulene [114]. In the aforementioned study, maximum β-myrcene transfer rates decreased about 20 % from 2.0 to 1.6 % when doubling the hop pellet dosage (from 2 to 4 g/l). The extraction temperature should be above 0 °C, as this low temperature was reported to inhibit the swelling of plant material [107]. Furthermore, higher temperatures such as 20 °C can accelerate the extraction [99], although the actual impact of the dry-hopping temperature on the extraction rate varies with the individual compound [105]. The ethanol content of the base beer was considered to have the potential to influence the extraction of hop components [114]. The period of dry hopping and application of dynamic systems are addressed in chapter 1.2.2. The studies cited in this chapter indicate that hops can potentially be used more efficiently by controlling process parameters and as such have inspired this research project.

1.3.2 Brewing yeast and fermentation

In beer brewing, the yeast is mainly used to ferment wort (70–85 % final attenuation) to produce regular (12 °P original gravity) and high-gravity beer (13–22 °P original gravity) [115]. The most commonly used brewing yeast strains for this purpose include the top-fermenting *Saccharomyces cerevisiae* and bottom-fermenting *Saccharomyces pastorianus* species, as well as a few "environment-associated" fermented beers [116]. Brewing yeasts such as the lager beer strain TUM 34/70 (*S. pastorianus*, Figure 9) and the wheat beer strain TUM 68 (*S. cerevisiae*) are widespread in beer production and especially popular in Germany [117].

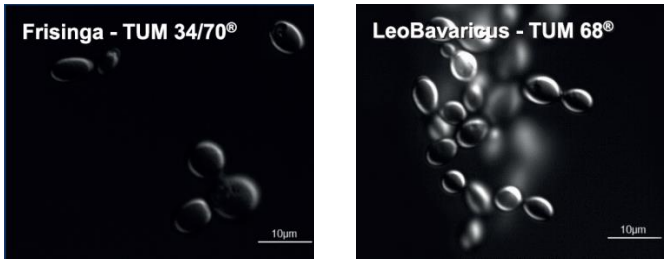


Figure 9 TUM 34/70 (*S. pastorianus*) and TUM 68 (*S. cerevisiae*) [118]

Yeast is single-celled, with a round or elliptical-oval shape at 5–12 µm in length and 5–10 µm in width. The surface of the yeast cell is, on average, 150 μm^2 [119]. Yeast propagation through sprouting (rarely spore formation) results in cell counts of about hundred million cells per milliliter during beer fermentation. Thus, a considerable yeast biomass surface of 15 m^2 per liter wort is expected. The yeast cell surface is considered to impact hydrophobic compounds present in fermenting wort, as the surface of a yeast cell is also hydrophobic. The level of hydrophobicity increases with cell age, which is caused by scars [120], and positively correlates

with the flocculation of yeasts [121]. It was reported that yeast hydrophobicity increases when various cell wall-associated proteins (encoded by the FLO gene family) are built [122]. Due to the ionization of carboxyl and phosphodiester groups of cell wall proteins and phosphomannans, respectively, the yeast cell wall has a net negative charge. In addition, the repulsion of like charges prevents cells from approaching sufficiently close and acts as an effective barrier to cell aggregation [123]. High quantities of the significantly hydrophobic components of the hop oil, such as mono- and sesquiterpenes, disappear during fermentation. Several research groups suggest that these are adsorbed on the hydrophobic surface of the yeast cell [124–126]. A study showed that hop terpenes, e.g. β-myrcene and α-humulene, can be vaporized into the headspace of fermenting wort [96], as mentioned before. Recently it was shown that the choice of yeast strain could influence their concentrations in dry-hopped beer with regard to concentration of terpenes and terpenoids [127, 128]. Outside of a beer-like environment, Bishop et al. illustrated the encapsulation of peppermint oil (40 % w/w) by *S. cerevisiae* cells [129].

The yeast produces about 0.035 kg carbon dioxide during the fermentation of a liter of wort at 12 °P original gravity, of which approx. 0.002 kg is bound in beer. The excess fermenting carbon dioxide must be exhausted, which is approx. 16.8 liter of gas per liter of wort [119]. The rise of carbon dioxide bubbles within a fermenting medium, e.g. wort and grape must, supports the migration of volatile components into the headspace [130–132]. Fermentation vessels are designed to release undissolved carbon dioxide to avoid a critical overpressure that would impact the yeast´s fermenting capacity. It is this fermentation that promotes the volatilization of flavour-active components.

The yeast produces various odor-active compounds during fermentation, e.g. isoamyl acetate (Table 3), which has a decisive influence on the beer flavor. The fermentation intensity and the speed of yeast propagation are crucial to the formation and excretion of fermentation by-products into beer. Additionally, their contents are influenced through process control, wort composition, and the choice of yeast strain [119]. The odor-active yeast products are classified into two groups: the green beer bouquets and aromatic (bouquet) components. The green beer bouquet substances are intermediates, e.g. acetaldehyde and diacetyl, which are usually removed from the beer by the yeast through biochemical pathways during maturation. In contrast, the bouquet substances are not degraded by yeast. The bouquet components are characteristic of a specific beer type, e.g. 4-vinyl guaiacol for German wheat beer. Other vital compound categories of bouquets are esters and higher alcohols. The odor-active (fruit-like) esters produced within yeast metabolism, e.g. ethyl hexanoate, are considered to have an intensifying effect on the hoppy flavor of beer [53].

Synergies among odor-active alcohols were discussed in chapter 1.2.1. Sulfur metabolism should also be mentioned, as the content of sulfur dioxide can be influenced by process control and, thus, the antioxidant characteristics of a beer.

Table 3 Selected fermentation by-products with flavor description and threshold in beer [115]

Compound	Compound category	Flavor description	Flavor threshold
Acetaldehyde	Aldehyde	Green-apple-like	5–25 mg/l
Diacetyl	Diketone	Buttery, sweet	0.08 mg/l
4-Vinylguaiacol	Phenol	Clove-like	0.3 mg/l
Ethyl hexanoate	Ester	Fruity	0.2 mg/l
Isoamyl acetate	Ester	Banana-like	0.6 mg/l
Isobutanol	Alcohol	Alcoholic	10–200 mg/l
n-Propanol	Alcohol	Alcoholic	2–50 mg/l
Styrene	Aromatic hydrocarbon	Synthetic-material-like	20 µg/l
2-Methyl butanol-1	Alcohol	Malt-like	15–65 mg/l

Brewing yeast produces various enzymes, which are classified into hydrolases, transferases, oxidoreductases, lyases, isomerases, and ligases [119]. The enzymatic conversion of hop-derived compounds (biotransformation) within yeast metabolism is discussed in chapter 1.3.3.

1.3.3 Biotransformation of hop-derived compounds

Brewing yeast can enable the biotransformation of several hop-derived compounds, which is considered to impact the hoppy flavor of beer significantly. However, there is a limited number of studies discussing this topic. An in-depth discussion of yeast-initiated hop component biotransformation is provided in a review article in 2011 [133]. Figure 10 gives a brief overview of the latest knowledge on biotransformation pathways of hop-derived compounds by *S. cerevisae* strains during wort fermentation.

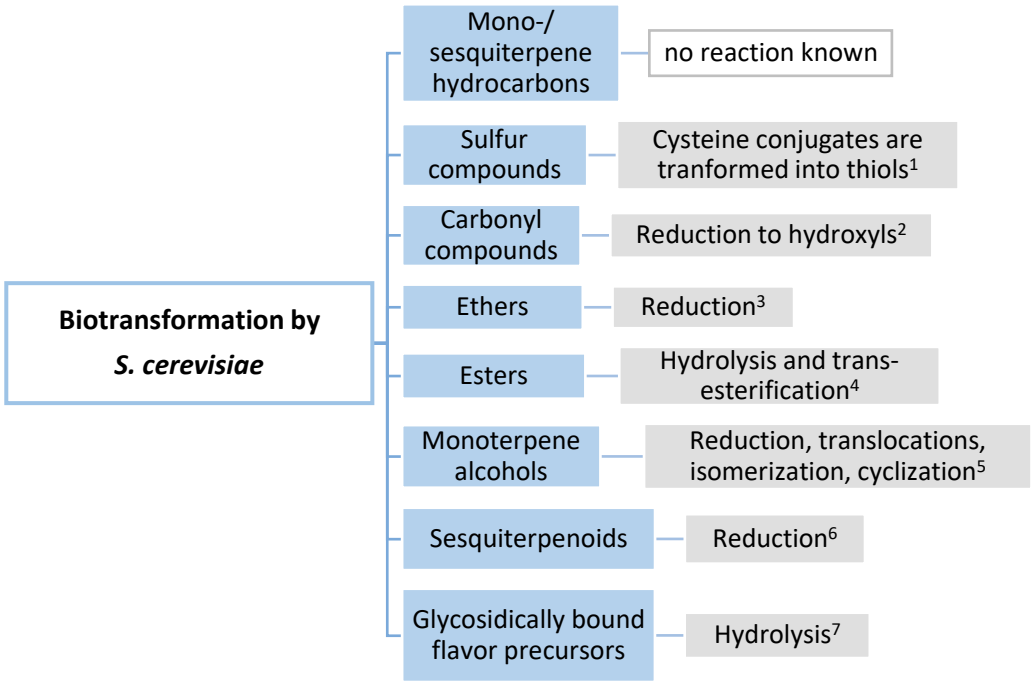


Figure 10 Overview of biotransformations of hop-derived compounds by *S. cerevisiae*; [1][134]; [2][126]; [3][135]; [4][82, 88]; [5][136, 137]; [6][138]; [7][24]

The biotransformation of monoterpenes by *S. cerevisiae* is not known, but from studying particular sesquiterpenes [139]; from model studies and fermentations, it was deduced that humulol II could be a yeast reduction product of humulene epoxide II [138]. Due to the reducing activity of yeast, terpenes containing heterocyclic sulfur atoms (e.g. myrcene disulfide) can undergo ring opening, which results in the formation of thiols [140].

In investigations on the bottle refermentation process, Nizet et al. detected increased levels of many thiols such as sulfanyl alkyl alcohols, sulfanyl alkyl acetates, and sulfanyl alkyl carbonyls, creating a powerful sensorial impact [134]. They suggested that hop cysteine adducts might be hydrolyzed by yeast-derived lyases and that the Ehrlich pathway also remained efficient during refermentation. Yeast can induce reduction of carbonyl compounds found in hop oil to alcohols [126]. Enzymes such as dehydrogenases and reductases are involved in these reactions, e.g. the reduction of methyl ketones to the corresponding secondary alcohols. Cyclic ethers such as the cis- and trans-linalool oxide, hop ether, and rose oxide were determined in hop oil and suggested that they contribute to the hoppy beer flavor [88]. Studies on a wine-fermentation process indicated that the yeast might reduce the precursor 3,7-dimethyl octa-2,5-diene-1,7-diol (geranyl diol I) yielding 3,7-dimethyl-5-octene-1,7-diol (citronellyl diol I) that gives rise to rose oxide after acid-catalyzed cyclization [135]. Although this reaction was determined in must fermentation, the presence of rose oxide in hop oil and beer has been reported [48].

Several studies have shown that esters of the hop oil are influenced by hydrolysis and transesterification through yeast fermentation [5, 88, 125]. Methyl esters, which exist in a homologous series from hexanoate to dodecanoate in hop oil, for example, can undergo both hydrolysis and transesterification into acids or ethyl esters [48, 126]. Yeast-derived esterase activity could lead to degradation of various esters or to transesterification of isobutyric esters, e.g. isobutyl isobutyrate, isoamyl isobutyrate, and 2-methyl butyl isobutyrate into ethyl esters [83]. In this context, a study by King and Dickinson showed that different brewing yeast strains or species could have different effects on hop oil-derived monoterpene alcohols [124]. Acetate esters of geraniol and β-citronellol have been formed in model solutions by a specific lager yeast strain *S. bayanus* NCYC 1324, but not by the ale yeast strain *S. cerevisiae* NCYC 1681. The same authors reported that monoterpene alcohols might be the subject of further biotransformation during fermentation [136]. They proposed that the transformations of geraniol and nerol into linalool, cis-trans isomerization of nerol to geraniol, reduction of geraniol to β-citronellol, and the cyclization of nerol and linalool to α-terpineol may be catalyzed by *S. cerevisiae* (strain IWD72). In these model fermentations, traces of cis-terpin hydrate were detected that were probably formed by hydroxylation of α-terpineol to terpin, which may be further hydrated. Seaton et al. have proposed the transformation of geraniol to β-citronellol by yeast metabolism previously [82]. With regard to geraniol or β-citronellol in beer dry-hopped during fermentation, a recent study confirmed that different brewing yeast strains can have different impact on their concentrations [141]. Takoi et al. performed several studies on the fate of monoterpene alcohols during beer fermentation using different hop varieties [67, 137, 142]. In two studies in 2010, they showed that a decrease in geraniol and an increase in β-citronellol could be particularly significant during beer wort fermentation [49, 137], confirming earlier findings by Lam et al. [143]. In these studies, Takoi et al. determined a fast decline of geraniol concentration a continuous increase in β-citronellol concentration. Subsequently, in finished beers, the concentrations of geraniol and β-citronellol increased, depending on the initial level of geraniol in the wort. They supposed that biotransformation (in addition to geraniol metabolism) might have occurred, such as the release of glycosidically bound compounds. Hanke et al. reported a minor increase or a significant decrease in the geraniol content in fermentation trials [111]. They suggested that different biotransformations of geraniol took place such as formation of cintronellol or cleavage of precursors as a consequence of different initial geraniol concentrations.

Slight changes in the amounts of the specified monoterpene alcohols during fermentation even below threshold concentrations could have a noticeable effect on citrus beer flavor, as synergies among these compounds were found even below their threshold levels [49]. The enzymatic release of aglycones, among them highly odor-active aroma compounds, e.g. linalool and geraniol as mentioned before, might be significant for hoppy beer flavor. The

aglycone and the sugar moiety can be cleaved by β-1,4-glucosidase enzyme activity. Yeast can induce hydrolase activity towards glycosidically bound compounds extracted from hops [144, 145]. The optimal functionality of these enzymes was determined at pH 4.5–5.2. Various *Saccharomyces* brewing yeast strains, e.g. *S. cerevisiae,* are reported to show glucoside hydrolase activity based on exo-β-glucanase activity [137, 146]. Thus, *S. cerevisiae* can be essential to release flavor-precursor of the hop. Variable impact of different brewing yeasts was shown by a recent study on a lager-strain and an ale-strain stressing out the ability to release terpene alcohols [65]. The yeast exo-β-glucanase activity is generated independently of the carbon source [147] and first secreted to the periplasmic space and then released into the culture medium [148]. However, recent studies indicate that activities of yeast-derived glucoside hydrolases in beer media seem to be low [149, 150]. Enzymatic hydrolysis of glycosides depends on the specificity of a given enzyme for the substrate. It was shown that β-glucosidase activity, which is most efficient in releasing flavor-active compounds from hop glycosides, could be found in some non-*Saccharomyces* yeast cells, e.g. *Brettanomyces custersii*, isolated from fermenting Lambic [146]. The acid hydrolysis of glycosides has been shown to occur starting at around pH 4.4, and the reaction rate increases as the pH drops [25, 151], which might occur during brewing. As stated in this chapter the influence of dry hop media and yeast fermentation activities during dry hopping on the formation of a hoppy beer flavor are manifold, although the impact of the brewing yeast on specific hop-derived flavors is still insufficiently explored.

1.4 Purpose of the study and research hypotheses

Brewers aim to produce extraordinary beer flavors to meet the needs of consumers. Fine hoppy flavors contribute significantly to the quality of a beer and are an important characteristic of beer types such as “Pilsner” or “IPA”. An effective method of creating intense hoppy flavors is dry hopping, which is often used to brew craft beers. There is currently great interest in these flavor-intense beers. Due to the diverse settings of dry-hopping parameters, several beers with different aroma characteristics can be brewed by using just one hop variety. In recent years, the opportunities to differentiate hoppy flavor impressions in beer have also been effectively enriched by new breeds of flavor hops, increasing the number of hop varieties available for brewing to over 200. The high potential of dry hopping beer for flavoring is well described in the research literature. However, it is not yet possible to assess the full range of hoppy flavors in beers that can be created by dry hopping. Furthermore, the efficient usage of hop in dry hopping must be investigated in more detail, especially as hops are a valuable raw material. There is therefore a great need to better understand and control flavor-relevant processes in dry hopping.

The previous chapters pointed out that the factors that influence the concentration of flavor-active hop constituents during dry hopping are diverse. Due to the findings on flavor potential in the raw hop material, (bioactive) metabolic products of brewing yeasts, and further dry-hopping-process parameters that might impact beer flavor, the following working hypotheses were investigated in this dissertation:

- There is a lack of information in the literature on the role of flavor-precursor compounds in hops with regard to dry hoppy beer flavor. Dry hopping beer may result in the enzymatic release of flavor components from hop-derived precursors.
- The presence of (active) brewing yeast or its metabolic products influences the concentration of flavor-active hop components generated during dry hopping.
- The chemical nature of the extract, basic beer composition, and dry hopping parameters impact the extraction rate of hop oil constituents during dry hopping.

These hypotheses need to be investigated to guarantee the strict brewery quality guidelines on beer flavor. A consistently high-quality beer aroma is also an essential requirement for conventionally hopped and dry-hopped beers. As hops are an agricultural product with crop- and storage-based fluctuations, in-depth knowledge of the flavor-relevant reactions involved in dry hopping is fundamental to produce a brand with consistent flavor.

A reproducible result in terms of beer flavor is an essential factor for brand recognition, which is especially crucial for industrial breweries that produce globally available products. Indeed,

dry hopping is no longer limited to small craft breweries – larger breweries have also been using this technique for a long time. Due to ever-increasing cost pressures and the goal of responsible raw material usage, the excessive use of hops should be evaluated from the perspective of high yield of flavor components. Especially at the high production volume of industrial brewing groups, even minor interventions in the dry hopping process could have a significant impact on resource consumption.

Apart from the raw material, different dry hopping methods could be used that are associated with varying degrees of technical or economic effort. Indeed, long storage phases during dry hopping, for example, could lead to decreased flexibility or high energy consumption for cooling requirements. Dynamic systems could reduce the storage period; however, these dry hopping applications involve additional investment and operating effort. As dry hopping significantly impacts both the quality of beer and the raw materials used in beer production, it is important to explore factors influencing the yield of hop oil components relevant for beer hoppy flavor.

2 Results (thesis publications)

2.1 Summary of results

The thesis publications are each summed up in the following paragraphs with a description of authorship contribution followed by full copies of the publications. Table 4 shows an overview of the publications.

Table 4 Short overview of the four publications with title of the publication, major objective, applied method and main findings

Publication			
Publication 1 **Pages 30 - 41** Characterization of the Unfertilized and Fertilized Hop Varieties Progress and Hallertauer Tradition – Analysis of Free and Glycosidic-Bound Flavor Compounds and β-Glucosidase Activity	**Publication 2** **Pages 42 - 55** The Influence of Brewing Yeast Strains on Monoterpene Alcohols and Esters Contributing to the Citrus Flavour of Beer	**Publication 3** **Pages 56 - 67** On the Fate of β-Myrcene during Fermentation – The Role of Stripping and Uptake of Hop Oil Components by Brewer´s Yeast in Dry-Hopped Wort and Beer	**Publication 4** **Pages 68 - 77** Investigations into the Transfer Rate of Volatile Compounds in Dry Hopping using an Octanol-Water Partition Coefficient Model
Major objective			
To characterize free and glycosidically bound flavor components, study β-glucosidase activity in fertilized and unfertilized brewer´s hop and use for dry hopping beer.	To analyze biotransformation of hop oil constituents by brewing yeast strains during dry hopping and its impact on beer flavor.	To investigate the influences on concentrations of hop oil constituents when dry hopping during main fermentation besides biotransformation.	To identify important factors influencing the transfer of hop oil constituents during dry hopping and verify if flavor transfer could be explained by an abstracted model.
Applied methods			
Cultivation of fertilized and unfertilized hops, glycoside extraction, β-glucosidase activity test and GC-O and GC-MS, trial dry hopping and trained panelists.	Glucoside hydrolase activity enzyme assay, trial fermentation, detection of hop oil constituents using HS-GC-MS, sensory evaluation by trained panelists.	Trial fermentations, fermentation gas trapping by bubbling water column method. Extractions and determination of flavorings using ethanol or SPE and GC-FID, GC-MS and HS-GC-MS, respectively.	Dry hopping on a laboratory scale, applying different standardized process conditions using ethanol and climate chambers. HS-GC-MS of beer and hop oil analysis by GC-FID.
Main findings/Conclusion			
Free and glycosidically bound flavor-active components and β-glucosidase activity were found in hop samples. No adverse impact by fertilized hop on dry hoppy beer flavor was detected.	Brewing yeasts can produce terpenoids, show different geraniol metabolism activities that further depend on wort original gravity and influence citrus flavor of test beers	Significant decreasing impact of brewing yeasts on amounts of β-myrcene was shown. Uptake by the yeast and evaporation during fermentation were identified as main factors.	Introduction of an explanatory model of the transfer rates of volatiles during dry hopping using log K_{OW}. Impact of ethanol content, temperature, dosage and hop variety on dry hopping result.

Publication 1 Page 30 - 41	**2.2 Characterization of the Unfertilized and Fertilized Hop Varieties Progress and Hallertauer Tradition – Analysis of Free and Glycosidic-Bound Flavor Compounds and β-Glucosidase Activity**

In recent years there has been a high demand for beers with intense hoppy flavors. Aroma hop varieties and the technique of dry hopping are often used for this purpose. The glycosidically bound terpene alcohols and nor-carotenoids in hops (flavor precursors) are believed to contribute to the hoppy flavor in beer. There might be further potential to intensify the hoppy beer flavor by controlling the cleavage of these compounds during brewing. Despite the great interest in these flavor precursors, many aspects such as agronomical and varietal factors influencing their amounts in hops, the relationship of free and bound flavor compounds, and their role for hoppy beer flavor, are far from being fully understood.

In this study, samples of fertilized and unfertilized hop varieties Progress and Hallertauer Tradition were prepared and analyzed in terms of the range of their free and glycosidically bound flavor components. Several compounds, for example, aliphatic alcohols, terpene alcohols, and C_{13}-norisoprenoid compounds were identified as being released by the Rapidase F64 enzyme that is related to the hydrolysis of glycosides. The flavor potential of the released compounds was confirmed by gas chromatography olfactometry (GC-O). The β-glucosidase enzyme activity, which is shown by specific yeast strains, is reported to release the flavor-active components from glycosides. A glycoside-hydrolytic activity in hops was identified and verified at approx. 0.11 U/g (dry matter hops) on average in all examined samples. The inherent enzyme activity of hops might play a significant role in the cleavage of glycosides in the brewery´s cold process area. Fertilized and unfertilized hop patterns were used for the dry hopping of a lager beer. In the sensory evaluation, minor differences were observed regarding flavor attributes. However, the flavor quality and stability were not significantly influenced by the insemination. In the cultivation of brewer´s hops in Germany, male plants are prohibited and only used for controlled pollination. Prejudices have been established regarding the qualification of fertilized hop plants for brewing purposes; nevertheless, in several growing regions such as English hop gardens, pollinated cones are accepted. The proclaimed disadvantages of fertilized hops regarding decreased oil and α-acid contents of hops were not confirmed.

Authors/Authorship contribution:

Haslbeck, K.: Literature search, conception and statistical analysis of tastings, discussion of data, writing, conception and design of manuscript; **Jerebic, S.**: Data creation and analysis; **Zarnkow, M.**: Study conception and supervision, critical review of draft.

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K. Haslbeck, S. Jerebic, and M. Zarnkow

Characterization of the Unfertilized and Fertilized Hop Varieties Progress and Hallertauer Tradition – Analysis of Free and Glycosidic-Bound Flavor Compounds and β-Glucosidase Activity

Fertilized or unfertilized, hops have an impact on the characteristics of beer that determine quality. Unique, comparable samples of unfertilized and fertilized plants of the Progress (Goudhurst, United Kingdom) and Hallertauer Tradition (Hüll, Germany) varieties were cultivated. Different cultivation methods were applied depending on the growing region. The success of the methods was verified by high seed contents of fertilized plants and only minimal formation of semen by plants that were prevented from pollinating. The comparability of the samples was targeted by similar growth and harvest conditions, managed at the same location. No significant differences occurred in the composition of the essential oils of fertilized or unfertilized samples. The disadvantages of fertilized hops as a result of decreased α-acid content or a lower essential oil quantity as described in the literature could not be confirmed. The glycosidically bound flavorings of fertilized or unfertilized hop samples were released by preparation of the Rapidase F64 enzyme. Approximately 2 µg of glycosidically bound linalool could be released from one gram of hops (dry matter). In the hops, β-glucosidase enzyme activity could be verified at approximately 0.11 U/g (dry matter hops) on average in all examined samples.

Descriptors: *Humulus lupus* L., unfertilized and fertilized hops, glycosides, essential oil, gas chromatography, β-glucosidase activity

1 Introduction

Today there is a similar wide distribution of both farming methods for unfertilized and fertilized (brewers) hops [1, 2, 3]. In countries such as the United Kingdom, USA and Australia wind pollination of female hop plants is accepted or even desired. The reasons may be agronomic (earlier closing of flowering, reduced disease susceptibility) and economic (seed content is heavier, larger clusters). Nevertheless, in many countries there are prejudices against hops with an increased seed share [4]. Male plants are actually forbidden in growing regions in Germany [5]. Along with other reasons (some hop varieties: a loss of lupulin content or reduction of essential oil [3, 5]) this is due to the high lipid content of hop seeds (up to 32 %), which is claimed to have a damaging impact on the quality of lager beers [6]. In this context it was demonstrated that only a minimal proportion of the fatty acids of seeds merges into the finished product when hops are added at wort boiling [7, 8]. Various working groups performed comparative brewing experiments with unfertilized and fertilized hops. It is not possible to derive a tendentious adverse influence on the quality of beer from that. In most cases no significant differences between the beers were detected [1, 4, 9, 10]. However, these results were not confirmed by all studies [11, 12, 13]. From an agronomic point of view the fertilization and prevention of fertilization of female hop plants have both advantages and disadvantages.

Hop plants have certain enzymes to synthesize glycosides. These enzymes could be used in reverse to release glycosidically bound flavor substances. Glycosidically bound flavor substances are odorless, non-volatile molecules that consist of an aglycone and a sugar residue. The aglycone represents the flavor-active substance [14]. Examples of flavor-active aglycones that have been identified in various plants and fruits are medium-chain alkanols and alkenols, derivatives of shikimic acid, C_{13}-norisoprinoides, and monoterpene and sesquiterpene alcohols [15]. The diastatic activity of hops, which was first described by *Janicki and Kotasthane* is an interesting feature for brewers [16]. Hops added to beer during storage can cause a secondary fermentation via the hydrolysis of dextrines to fermentable sugars. They showed that the diastatic activity is greater in fertilized plants than in unfertilized plants [16]. With regard to the release of flavor-active aglycones from hop glycosides, these results have yet another meaning. *Kollmannsberger* and *Nitz* showed that a commercially available

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amylase preparation splits glycosides very poorly; nonetheless the aglycones were still released [17].

Based on these considerations, samples of fertilized and unfertilized hops Progress and Hallertauer Tradition were prepared and analyzed in terms of the range of their free and glycosidically bound flavor substances. Furthermore, the glycoside-hydrolytic activity of both patterns was verified in the present work.

2 Materials and methods

The hop varieties Hallertauer Tradition HHT (Hüll, D-85253 Wolnzach) and Progress PG (Cranbrook, GB-TN17 Kent) were grown in a field test in season 2013. Each of the fertilized and unfertilized plants were cultivated in the same field at similar agronomic conditions. In Germany, six HHT vines (HHT-F) were artificially fertilized with 2 g pollen of different male plants and covered with a plastic film to prevent further fertilization (plastic film was removed after three days). Twelve plants were grown conventionally (HHT-U). In UK, wind pollination was (virtually) prevented by packing the female PG hop bines (before bloom) into pollination bags, containers for controlling pollination. Three different types of hops were prepared and nine vines of each were grown. Among these unfertilized hops (packed in pollination bags, PG-U), fertilized hops (ripened without pollination bags, PG-F) and a control sample (comparatively seeded hops packed after bloom in pollination bags, PG-C). All bines were manually plucked and dried at 60 °C. Hop cones were crushed using a knife mill (Retsch GM 300, NATECO$_2$, Wolnzach, Germany) in reverse rotation (10 s at 2000 rpm) to prevent damaging the semen. The hops were then vacuum-packed in 30 g bags and stored at 0 °C.

The water content of the hops was analyzed according to MEBAK methods [18]. The seed, α-acid and essential oil content of the hops were analyzed according to the Analytica-EBC methods [19, 20]. The essential oil was used for further gas chromatographic analysis.

Glycoside extracts of hops were produced and then cleaved using the technical glycosidase Rapidase F64 (DSM Food Specialties, Düsseldorf) into free aglycones and equivalent amounts of sugar according to Kollmannsberger and Nitz [17]. Briefly, the solid residue of an ether extract of hops (5 g) was suspended in methanol and 2 µmol of phenyl-β-D-glucopyranoside (Sigma-Aldrich, Taufkirchen, Germany) added. After a total of 72 hours for the exposure phase and two filtration steps, the methanolic phase was concentrated to dryness in a round-bottom flask. The residue was incorporated in 50 ml of McIlvaine buffer solution (pH 5.00) and two 25-g-samples of each were transferred into 50-ml flasks with a ground glass stopper. One batch was used as a blank sample, 25 mg Rapidase F64 was added to the second one for cleavage. Both samples were sealed and incubated for 67 h at 40 °C. The free aglycones were isolated in diethyl ether, dried over Na_2SO_4 and concentrated in a Vigreux column (40 °C) to approx. 1 ml.

The β-glucosidase activity of hops was determined by the ability to cleave the synthetic glucoside p-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich Chemie, Taufkirchen, Germany) into the aglycone p-nitrophenol and glucose. The method is based on spectrophotometric analysis of the β-glucosidase activity of different yeasts by *Rosi* and *Vinella* [21]. Aqueous extracts of hops at two different degrees of grinding were prepared for this purpose; a fine type by ceramic mortar (crushed seeds) and a coarse type by knife mill (intact seeds). The samples were then concentrated on a rotary evaporator. The liberated aglycones were strongly photoactive in the alkaline range and could be recorded at a wavelength of 400 nm by a spectrophotometer.

GC-TOF-MS analysis of essential oil and aglycone extracts: an Agilent 6890N gas chromatograph was directly coupled to a SensiTOF mass spectrometer (Five Technologies, Munich, Germany). Separation was achieved using a DB 5 (J & W Scientific, CA, USA) 30 m × 0.25 mm capillary column (0.25 µm film thickness). The oven was programmed at a rate of 5 °C/min from 60 °C (5 min isotherm) to 240 °C. Carrier gas used was helium (1.5 ml/min); split 1:10; injection volume: 0.5–1.5 µl; injector: 250 °C; transfer line: 220°C; ion source temperature, 200 °C; ionization: –70 eV; mass range: 35–600 amu. Data analysis by MASPEC data system 2.11, version 14.0f (1998).

GC-FID analysis of essential oil and aglycone extracts: a Siemens SiChromat 3 gas chromatograph directly coupled to a Merck-Hitachi D2500 Integrator FID. The capillary column was a DB 5 (J & W Scientific, CA, USA) 30 m × 0.25 mm (0.25 µm film thickness); carrier gas: helium (1.0 ml/min, 60 °C); split 1:20 and splitless, respectively (aglycone extracts); injector/detector: 250 °C; fuel gases: hydrogen and air (each 2 bar). The temperature program of the oven was at a rate of 5 °C/min from 60 °C (5 min isotherm) to 250 °C. The injection volume was 1.0 µl and 4.0 µl, respectively (aglycone extracts).

GC-O analysis of hop extract: a Siemens Sichromat II gas chromatograph was directly coupled to Finnigan MAT 8222 magnetic sector field mass spectrometer (EI mode, –70 eV, 35–350 amu), capillary column: SPB5 (Supelco) 30 m × 0.53 mm (film thickness = 1.5 µm); carrier gas: helium (3 ml/min); split: 1:10. The oven was programmed at 5 °C/min to 250 °C starting at 100 °C; injector: 250 °C; transfer line: 200 °C. The GC eluent was divided by a live-T switching device to allow simultaneous sniffing analysis and mass spectrometric identification. Data analysis by MASPEC data system 2.11, version 14.0f (1998). Volatile compounds of each hop extract (5 g) were adsorbed in a 20 ml vial at 35 °C by the SPME fiber (Stable Flex Divinylbenzol/Carboxen/PDMS 50/30 µm, Supelco, Bellafonte, PA/USA) for 30 min. The enriched substances were desorbed in the injector of the gas chromatograph for 30 s at 250 °C.

Effects on beer quality of fertilized hops were tested by sensory evaluation of a lager beer single dry-hopped with equally gained fertilized and unfertilized hops. After a longer storage period, only samples of varieties Pilgrim and Challenger – fertilized and unfertilized – had adequate Hop Storage Index (HSI), and only these varieties have been used for sensory evaluation. So samples of both varieties were used for dry hopping trials. A pale filtered lager beer in 50-l-Kegs (industrially produced, 4.8 % ethanol vol/vol) was static dry-hopped at 1.5 ml essential oil per hectoliter for seven days at 1 °C. Beers were tasted and evaluated by a sensory panel of 7 DLG-certified tasters (Deutsche Landwirtschafts-Gesellschaft) subsequently of dry-hopping and after storage for

Table 1 HHT harvest data; fertilized plants n = 6, analysis of 12 samples; unfertilized plants n = 6, analysis of 13 samples; oil content fourfold determination; harvest date 03.-06. 09. 2013

		HHT-U		HHT-F	
		Sum		Sum	
green hops	[g]	24961		22087	
dried hops	[g]	5809		5752	
		Mean	SD	Mean	SD
H_2O after drying	[%]	4.82	0.48	4.75	0.34
α-acid (water free)	[%]	6.75	0.64	6.72	1.05
Oil content	[ml/100 g]	1.0		1.1	
		Seed share	SD	Seed share	SD
dried hops	[%]	1.3	0.3	18.9	2.3
		Oil content	SD	Oil content	SD
dried hops	[mL/100 g]	0.97	0.08	1.05	0.06

Table 2 PG harvest data, triple determination; 9 plants cultivated each case; start point treatment pollination bags: unfertilized: 08. 07. 2013, fertilized (control): 29. 07. 2013; harvest date 09.-13. 09. 2013

		PG-F		PG-C		PG-U	
		Sum		Sum		Sum	
dried hops	[g]	835		425		220	
		Mean	SD	Mean	SD	Mean	SD
H_2O after drying	[%]	5.42	0.39	6.15	0.43	5.91	0.41
		Seed share	SD	Seed share	SD	Seed share	SD
dried hops	[%]	21.6	1.9	22.1	1.0	0.2	0.1

3 month at 8 °C. The examination of the beer samples was done accordingly to the DLG-scheme for beer (attributes: smell, taste, body, rezenz, bitterness). Secondly, a descriptive tasting was conducted; intensities of eleven typical descriptors of hoppy flavor in beers (e.g. fruity, hoppy, green, spicy, herbal, resinous, citrussy, floral, tea and white wine) were rated by panelists. Every attribute was evaluated from 0, meaning not noticeable, to 5, extremely noticeable. Significant differences among flavor attributes were assessed by one-way analysis of variance (ANOVA) using the SPSS Version 24.0 statistical package for Windows (SPSS Inc, Chicago, IL, USA). Statistical differences between means were evaluated using Games-Howell's test at 0.05 % level in order to evaluate the significance of the analysis.

3 Results and discussion

3.1 Characterization of fertilized and unfertilized hop samples

The fertilized and unfertilized hop plants (harvest data, analytical characteristic, Tables 1 and 2) were cultivated in the United Kingdom (PG) and Germany (HHT) respectively in the 2013 season. Two different insemination methods were applied, depending on the country. In general, the growth and harvest of the hop plants proceeded normally. The visual inspection revealed nothing out of the ordinary, a variety-specific pure and lasting odor was recognized for all samples. Irrespective of the variety, the cones of fertilized hops were bigger and had greatly enlarged bracts; the seeds were smaller than those of unfertilized plants. The plants treated using pollination bags showed evidence in some cases of mildew infestation and slightly higher water content. This is due to the reduced air exchange and condensation formation. The maturation of cones and thus harvest was thereby negatively influenced. For HHT, the water, α-acid and oil contents were very similar; the fertilized hop samples were less homogeneous than their unfertilized patterns. The average essential oil content was determined of both fertilized and unfertilized HHT cones. No significant difference was noted. The disadvantages of fertilized hops due to a decreased α-acid content or a lower essential oil quantity described by two different research groups could not be confirmed [3, 5], although general conclusions are not to be derived due to limited data material. Significant differences were observed between unfertilized hop patterns PG and HHT when determining seed shares. The pollination bag method prevented wind pollination effectively. It was recognized that even in hop-growing areas in Germany it is not possible to entirely exclude wind pollination. In this context minimum formation of semen in the HHT pattern was accepted (1.3 ± 0.3 %). The artificial fertilization of HHT plants in Hüll, Germany, gave similar results to the wind-fertilized PG plants in Kent, UK. Derogations may be based on varietal differences or on deviating cone ripening conditions.

3.2 Quantification of flavor compounds in essential oils

Essential oil fractions of HHT were examined qualitatively and quantitatively by GC-TOF-MS and GC-FID, respectively (Table 3). In both extracts of fertilized and unfertilized hops 90 compounds of each were identified and 52 compounds quantified. Contents of important compounds of hop essential oil such as β-myrcene, α-humulene and β-caryophyllene form over 80 % of the essential oil fraction that is comparable to reference [22] regardless of fertilized or unfertilized samples. Linalool, which is key compound for hoppy flavor of beer [23] formed about 0.8 %, which is relatively low (1.0–1.5 %). In case of esters 3-methylbutyl-2-methyl propanoate and methyl-6-methyl heptanoate contents significantly (student t, n = 4, α = 0.05) varied between the samples. Sum of analysed compounds was 6.6 % higher in unfertilized than fertilized samples, 1042 µg/g and 973 µg/g, respectively. We conclude instrumental results of both HHT samples are well comparable with each other and references.

3.3 Analysis of β-glycosidically bound flavor compounds in hops

The existence of glycosidically bound flavor substances in hops was

Table 3 Concentration of hop constituents in µg/g hops dry matter; unfertilized (-U) and fertilized (-F).
[1] significant difference between the concentrations in the unfertilized and fertilized samples; [2] eluted together

					HHT-U		HHT-F
	compound	RI TOF	RI GC	mean	SD	mean	SD
1	2-Methylpropyl-2-methylpropanoate	911	911	10.7	2.62	17.2	4.59
2	α-Pinene	929	929	3.3	0.49	1.6	0.57
3	2-Methylbutyl-propanoate	967	968	11.5	2.87	12.3	2.95
4	β-Pinene	972	972	37.7	6.23	32.8	5.17
5	Myrcene	991	993	3447.3	342.68	3130.8	530.29
6	3-Methylbutyl-2-methylpropanoate[1]	1011	1012	6.6	0.90	14.8	3.20
7	2-Methylbutyl-2-methylpropanoate	1014	1015	46.7	7.46	68.1	12.38
8	Methylheptanoate	1022	1023	50.8	11.23	36.9	9.84
9	Methyl-4-methyl-2-hexanoate	1024	1025	63.1	8.12	49.2	7.54
10	Methyl-6-methylheptanoate[1]	1085	1087	33.6	4.76	19.7	2.95
11	Nonan-2-one	1090	1092	10.7	3.20	7.4	3.77
12	Linalool	1099	1101	69.7	18.29	64.8	15.91
13	2-Methylbutyl-2-methylbutanoate	1103	1104	17.2	1.31	14.8	1.48
14	Methyl octanoate	1124	1125	60.7	14.19	57.4	12.22
15	Decan-2-one	1191	1191	5.7	1.48	4.1	0.66
16	Methyl-3-nonenoate	1211	1212	7.4	2.13	6.6	0.08
17	Methylnonanoate	1224	1225	17.2	3.77	14.8	2.87
18	*iso*-Undecan-2-one	1255	1256	9.0	1.89	7.4	3.36
19	*iso*-Undecen-2-one	1274	1277	9.8	3.61	5.7	2.46
20	Methyl-8-methylnonanoate	1288	1289	8.2	1.23	4.9	1.07
21	Undecan-2-one	1292	1294	82.0	11.64	58.2	8.36
22	Methyl-4-decenoate	1308	1309	136.1	18.53	105.0	14.68
23	Methyl-4,8-decadienoate	1314	1314	94.3	3.94	90.2	16.07
24	Methyldecanoate	1325	1325	10.7	3.69	18.9	3.61
25	α-Cubebene[2]	1344	1347	11.5	1.39	11.5	3.28
26	Octyl-2-methylpropanoate[2]	1345					
27	α-Ylangene	1364	1367	6.6	2.54	7.4	2.13
28	α-Copaene	1369	1372	24.6	2.95	23.8	4.10
29	Dodecan-2-one	1393	1395	10.7	3.20	7.4	2.95
30	*E*-β-Caryophyllene	1408	1416	803.6	37.72	761.0	35.34
31	β-Copaene	1421	1425	33.6	2.87	36.9	1.48
32	α-Humulene	1446	1453	2776.5	244.61	2740.4	59.78
33	Selina-4,11-diene	1467	1471	18.9	3.94	19.7	4.51
34	γ-Muurolene	1469	1474	64.0	5.58	60.7	2.30
35	α-Amorphene	1473	1477	9.8	0.98	8.2	0.90
36	β-Selene	1476	1482	22.1	2.71	20.5	0.74
37	α-Selinene	1486	1491	41.8	6.89	41.8	5.33
39	Tridecan-2-one	1494	1496	70.5	17.88	71.3	7.71
40	γ-Cadinene	1506	1511	94.3	13.37	82.0	2.95
41	δ-Cadinene	1517	1522	129.6	16.73	99.2	33.62
42	Cadina-1,4-diene	1524	1530	12.3	3.77	19.7	12.55
43	α-Cadinene	1530	1535	14.8	3.85	13.9	0.82
44	*iso*-Tetradecen-2-one	1567	1571	5.7	1.48	8.2	3.03
45	Caryophyllene oxide	1571	1578	3.3	1.39	1.6	1.31
46	Humulene oxide A	1587	1594	4.9	0.33	4.9	1.97
47	Tetradecan-2-one	1596	1598	9.0	2.05	6.6	2.05
48	Humulene oxide B	1597	1604	31.2	6.56	22.1	9.02
49	Pentadecadien-2-one	1657	1660	52.5	15.09	35.3	1.48
50	Pentadecatrien-2-one	1662	1665	13.9	4.10	9.0	0.49
51	Pentadecen-2-one	1668	1670	21.3	6.31	17.2	5.99
52	Pentadecan-2-one	1697	1699	8.2	2.46	5.7	0.41
	Sum			1042.1		973.1	

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Table 4 Identified compounds of glycoside extracts in the fertilized and unfertilized Hallertauer Tradition (HHT) hop variety with the retention indices (RI) and main mass fragments (m/e)

	Standard	RI TOF	RI GC	m/e	Identification
	Phenol	982	979	94, 66, 65, 39	MS; RT
	Methyl heptanoate	1024	1025	74, 43, 87, 113	MS; RT
	Aliphatic alcohols	**RI TOF**	**RI GC**	**m/e**	
1	3-Methylbutan-1-ol	722	722	55, 42, 70, 43, 41	MS; RT
2	2-Methylbutan-1-ol	726	726	57, 41, 56, 70, 44	MS; RT
3	Pentan-1-ol	757	756	55, 42, 70, 42, 41	MS; RT
4	3-Methyl-2-buten-1-ol	765	766	71, 53, 41, 67, 68, 86	MS; RT
5	3-Methylpentan-2-ol	780	780	45–56, 41,69,84,87	MS; RT
6	4-Methylpentan-2-ol	784		45–43, 96, 84, 87, 57	MS
7	3-Z-Hexenol	845	852	67, 41, 82–55, 69	MS; RT
8	Hexanol	858	862	56–43, 41, 69, 55, 84	MS; RT
9	1,5-Octadien-3-ol	978		57, 72, 99, 110	MS
10	1-Octanol	1071		41, 56, 55, 70, 84, 112	MS
	Aromatic compounds	**RI TOF**	**RI GC**	**m/e**	
11	Benzaldehyde	956		79, 108, 109, 77	MS
12	Benzylalcohol	1032	1036	108, 107, 79, 77	MS; RT
13	Phenylacetaldehyde	1039	1046	91, 69, 79, 108	MS; RT
14	Guajacol	1085	1090	55, 109, 81, 124, 69	MS; RT
15	2-Phenylethanol	1109	1118	91, 92, 122, 65	MS; RT
16	Methyl salicylate	1191	1198	120, 152, 92	MS; RT
17	4-Vinylphenol	1220	1220	119, 91, 65, 39	[28]
18	4-Vinylguajacol	1311	1322	135, 107, 77, 151	[28]
19	4-Hydroxy-benzaldehyde	1368	1379	121, 122, 65, 103	MS; RT
20	Vanillin	1395	1408	151, 152, 81, 109	MS; RT
21	Tyrosol	1424	1430	107, 138, 77	MS; RT
22	4-Vinylcatechol	1444	1479	136, 89, 90, 110, 77, 63	[28]
23	Coniferylaldehyde	1731	1748	178, 135, 147, 107, 77, 51	[28]
24	*p*-Coumarin	1778	1794	43, 123, 163, 209, 224	MS; RT
25	Ferulic acid	1845		194, 179, 133, 77, 105	MS
	Terpene compounds	**RI TOF**	**RI GC**	**m/e**	
26	α-Pinene	931	938	93, 91, 92, 55, 77, 79, 67	MS; RT
27	β-Pinene	982		94, 66, 65, 55	MS
28	Linalool	1098	1102	71, 93, 41, 55, 80, 121, 136	MS; RT
29	α-Terpineol	1189	1196	59, 93, 121, 136, 68	MS; RT
30	*Z*-8-Hydroxy-linalool	1343	1348	43, 67, 71, 55, 68	[29]
31	*E*-8-Hydroxy-linalool	1362	1368	43, 67, 71, 55, 68	[29]
32	*p*-Menth-1-en-7,8-diol	1468	1479	59, 79, 93, 94	[30]
	C_{13}-norcarotinoid compounds	**RI TOF**	**RI GC**	**m/e**	
33	Theaspirane 1	1298		138, 82, 96, 109, 123	MS
34	Theaspirane 2	1315		138, 82, 96, 123	MS
35	3-OH-β-damascone	1614	1627	69, 43, 121, 175, 193, 208	[31]
36	3-OH-7,8-dihydro-β-ionol	1659	1671	121, 43, 119, 93, 105, 136, 212	[32, 33]
37	3-OH-5,6-epoxy-β-Ionol	1667	1674	43, 125, 109, 82, 208, 107, 166	[33]
38	Vomifoliol	1790	1804	124–43, 79, 135,150, 168	[32, 34]
39	7,8-Dihydro-vomifoliol	1853	1869	43,110, 111,152 ,96 ,68 ,170	[32]
	Fatty acids	**RI TOF**	**RI GC**	**m/e**	
40	Palmitin acid	1965	1964	43, 73, 60, 256	MS;RT
41	Linoleic acid	2136	2147	67, 81, 55, 41, 95, 280, 109	MS;RT
42	Linolenic acid	2142	2152	79, 67, 55, 95, 108, 222, 278	MS;RT
	Other compounds	**RI TOF**	**RI GC**	**m/e**	
43	n. i.	1371		107, 121, 136, 192	MS
44	n. i.	1676	1682	126, 85, 69, 168, 111	MS; RT

Table 5 Quantification of compounds in the glycoside extracts (Δ = deviation dual approach) of HHT hops in µg/g hops (dry matter); unfertilized (-U) and fertilized (-F); n. i. = not identified; Tr = trace (< 1 µg/g)

		HHT-U		HHT-F	
Standard	**RI**				
Methyl heptanoate	1025	40		40	
Aliphatic alcohols	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
3-Methylbutan-1-ol	722	2	Tr	3	Tr
2-Methylbutan-1-ol	726	1	Tr	2	Tr
Pentan-1-ol	756	Tr	Tr	Tr	Tr
3-Methyl-2-buten-1-ol	766	1	Tr	1	Tr
3-Methylpentan-2-ol	780	2	Tr	4	Tr
3-*Z*-Hexenol	852	2	Tr	3	Tr
Hexanol	862	Tr	Tr	Tr	Tr
Aromatic compounds	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
Benzylalcohol	1036	15	Tr	13	Tr
Phenylacetaldehyde	1046	Tr	Tr	1	Tr
Guajacol	1090	Tr	Tr	Tr	Tr
2-Phenylethanol	1118	7	Tr	6	Tr
Methyl salicylate	1198	4	Tr	4	Tr
4-Vinylphenol	1220	349	41	279	5
4-Vinylguajacol	1322	64	7	59	1
4-Hydroxy-benzaldehyde	1379	2	Tr	2	Tr
Vanillin	1408	1	Tr	2	Tr
Tyrosol	1430	3	1	4	Tr
4-Vinylcatechol	1479	160	38	154	Tr
Coniferyl aldehyde	1748	34	2	43	3
p-Coumarin	1794	10	Tr	10	Tr
Terpene compounds	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
α-Pinene	938	TR	Tr	1	Tr
Linalool	1102	2	Tr	2	Tr
α-Terpineol	1196	3	3	3	Tr
Z-8-Hydroxy-linalool	1348	4	Tr	3	Tr
E-8-Hydroxy-linalool	1368	14	2	12	Tr
p-Menth-1-en-7,8-diol	1479	5	1	6	5
C_{13}-Norcarotinoid compounds	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
3-OH-β-Damascone	1627	2	Tr	2	Tr
3-OH-7,8-Dihydro-β-ionol	1671	9	2	10	Tr
3-OH-5,6-Epoxy-β-ionol	1674	2	Tr	3	1
Vomifoliol	1804	2	Tr	2	Tr
7,8-Dihydro-vomifoliol	1869	3	Tr	3	Tr
Fatty acids	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
Palmitic acid	1964	143	3	142	35
Linoleic acid	2147	116	2	119	28
Linolenic acid	2152	124	3	120	18
Other compounds	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
n. i.	1682	4	1	5	< 1

clearly demonstrated by several studies [17, 24, 25, 26, 27]. In this project, the glycosidically bound fraction of hops was isolated and cleaved into its aglycones and sugar residues by Rapidase F64 enzyme preparation. The quantitative yield of the enzyme splitting was determined based on the cleavage of synthetic glycoside phenyl-β-D-glucopyranoside. The average yield of the unfertilized samples was 93 %. The average yield of the fertilized samples was 89 % [17]. In the extracts prepared from both unfertilized and fertilized hops HHT, the same variety of alcohols and diols could be released that were not included in the control. In total, 44 compounds (Table 4) were determined by GC-TOF-MS; 35 compounds were quantified by GC-FID (Table 5). A total of seven aliphatic alcohols, 13 aromatic compounds, six terpene compounds, five C_{13}-norisoprenoid compounds, three fatty acids and an unidentified one were determined. Figures 1–3 (see page 154) shows important correlations. The samples of the extract PG-U were lost

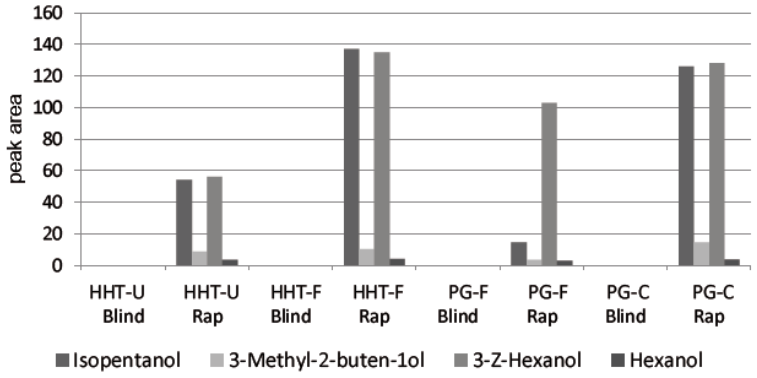

Fig. 1 Peak areas of selected aliphatic alcohols of glycoside extracts; unfertilized (-U), fertilized (-F) and control (-C, fertilized with pollination bag)

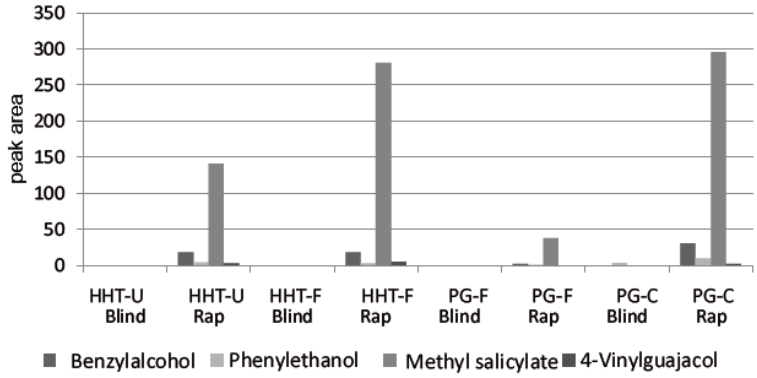

Fig. 2 Peak areas of selected aromatic compounds of glycoside extracts; unfertilized (-U), fertilized (-F) and control (-C, fertilized with pollination bag)

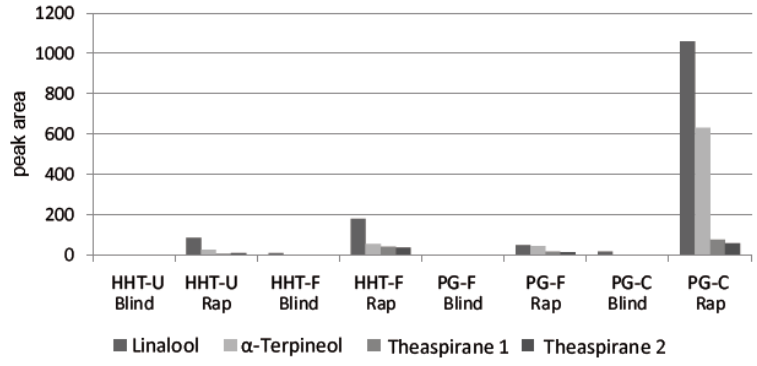

Fig. 3 Peak areas of selected terpene alcohols and C_{13}-norisoprenoid compounds of glycoside extracts; unfertilized (-U), fertilized (-F) and control (-C, fertilized with pollination bag)

due to technical problems during the measurement process. The quantities of liberated aglycones are rather low compared with the concentrations of the individual hop oil components. For example, it was possible to recover a quantity of approx. 2 µg linalool per gram of hops in the glycoside extracts. In the same amount of hops approx. 70 µg free linalool is included as a proportion of essential oil. In comparison, *Wilhelm* proved a quantity of up to 41 µg glycosidically bound linalool in his studies [27]. Concentrations of aliphatic alcohols were generally higher than in the unfertilized samples with the exception of hexanol. In other substance classes no clear differences were recognized between unfertilized and fertilized samples. The aromatic compounds formed the biggest substance class despite a tannin-side stabilization of the methanol extracts by Polyclar. Components such as 4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol were measured at high concentrations. These compounds are attributed to the phenol carboxylic acid esters that are present in hops [35, 36] and esterase activity of the hemicellulase preparation Rapidase F64 [30, 37]. The esterase activity is probably responsible for the high concentrations of fatty acids such as palmitic acid, linoleic acid, and linolenic acid in the extracts, leading to shares 10–13 % of extracts. There were no significant differences in the concentrations of each individual fatty acid between the unfertilized and fertilized samples, representing in total a share of 33.7 % and 35.6 % of extracted glycosides. This is interesting in terms of the high fat content of seeds in general, especially the unsaturated fatty acids which are held responsible for the deterioration in beer flavor stability [6, 7, 8]. Released substances that were identified using HS-SPME-GC-O (Table 6) included aliphatic alcohols (3-methylbutan-1-ol, 3-methyl-2-buten-1-ol, 3-Z-hexenol and hexanol), aromatic compounds (benzyl alcohol, phenylethanol, methyl salicylate and 4-vinylguaiacol), terpene alcohols (α-terpineol), and two theaspiranes (related to C_{13} norisoprenoid derivatives). A fungus-like smell was assigned to the compound 1-octen-3-ol, but this compound could only be detected in the sample "PGB-C" treated with Rapidase F64. The compounds furaneol (sweetish, flowery) and phenylacetaldehyde (gummy) were eluted to similar RT. The furaneol was detected only in the samples treated with Rapidase F64. Linalool (citrus-like, flowery) could be detected in all samples, except for "PGB-F" blind. The odor impression of the samples treated with Rapidase F64 was generally stronger than that of the untreated blind samples.

3.4 Analysis of β-glucosidase activity in hops

Figure 4 shows the specific β-glucosidase activity for PG and HHT unfertilized and fertilized hop samples. The differences for each

Table 6 Odor-relevant compounds of glycoside extracts, with their associated olfactory impressions; [1] compound was identified by their retention time and their typical odor; n. i. = not identified; B = "blind" without enzyme; R = samples treated with Rapidase F64; data of unfertilized PG samples got lost

				HHT-U		HHT-F		PG-F		PG-C	
compound	RT	RI	odor	B	R	B	R	B	R	B	R
Hexanal	2.4	802	green, grassy	x	x	x	x	x	x	x	x
Ethyl-3-methylbutanoate	3.1	842	sweet, fruit jelly			x	x	x	x	x	x
3-*Z*-Hexanol	3.2	851	green, banana								
1-Octen-3-ol	5.5	976	mushrooms	x	x	x	x	x	x	x	x
1,5-Octadien-3-one[1]	5.6	983	metallic	x	x	x	x	x	x	x	x
Octanal	6.2	1006	orange peel, floral			x	x			x	
Furaneol[1]	7.4	1055	sweet, floral		x		x		x		x
Phenylacetaldehyde	7.5	1059	rubbery			x				x	
1-Nonen-3-one	8.2	1083	mushrooms, chanterelles							x	x
n. i.	8.6	1099	green, cucumber, sweet				x	x		x	x
Linalool	8.7	1103	sweet, citrussy, floral	x	x	x	x		x	x	x
2-*Z*-Nonenal[1]	10.1	1152	burnt rubber		x	x	x	x	x	x	x
2,6-Nonadienal	10.3	1159	sweet, cucumber, floral	x	x	x	x		x	x	x
2-*E*-Nonenal	10.5	1165	roasted almonds	x	x	x	x	x	x	x	x
n. i.	11.2	1190	green, acrid floral smell	x	x		x	x		x	x
n. i.	12.3	1225	sweet, rose							x	x
n. i.	13.5	1265	rubber, tar		x		x		x		
4-Vinylguajacol	15.5	1331	clove				x				
β-Damascenone[1]	17.7	1403	fruity, apple juice			x	x			x	x
β-Damascone[1]	18.6	1432	cherry, fruit preserve				x	x	x		x

of the hop samples were very low. Nevertheless result of HHT hints towards tendency that specific β-glucosidase activity could be stronger in fertilized hops than in unfertilized hops. However, this should be investigated using a suitable sample material of further varieties. The analysis revealed an overall average of 114 mU/g (hop dry matter), maximum specific activity 139 mU/g and minimum 100 mU/g. Despite the small result range, differences between the samples could be recognized. Janicki and Kotasthane showed that diastatic activity originating from hops depends on the seed content and crushing ratio [16]. In this study, coarse samples (intact seeds) of the HHT hop variety showed no significant differences. By breaking the seeds (fine grinding), the fertilized samples showed a significantly higher specific β-glucosidase activity than the unfertilized samples.

The mold contamination of clusters ripened in the pollination bags led to increased enzyme activity that hampered any further interpretation. However, in the PG hop solutions prepared with coarse hop pellets the fertilized hops showed a highly significant (student t: α = 0.01) higher specific β-glucosidase activity than the unfertilized hops. Breaking up the seeds of the PG variety revealed a significant increase of specific β-glucosidase activity in the fertilized hop samples whereas it decreased slightly for the unfertilized samples. The control (fertilized with a pollination bag) showed a significantly higher activity in the coarse variant than the fertilized samples and a significantly lower activity than the unfertilized samples. It decreased for the fine variant to approximately the same level of the fertilized samples (without pollination bags). The increased β-glucosidase activity of the fine control could possibly be explained by the mold growth in the pollination bags [38]. This

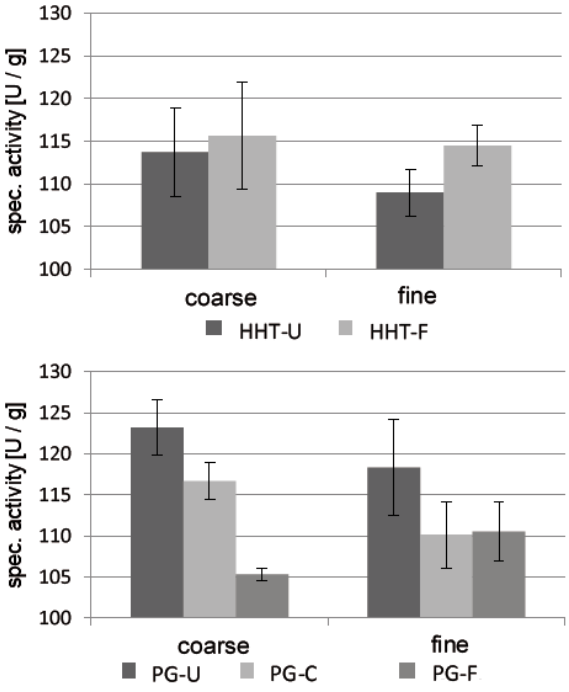

Fig. 4 Average specific β-glucosidase activity of the Progress PG and Hallertauer Tradition HHT hops; unfertilized (-U), fertilized (-F) and control (-C, fertilized with pollination bag); confidence intervals (Student t; α = 0.05)

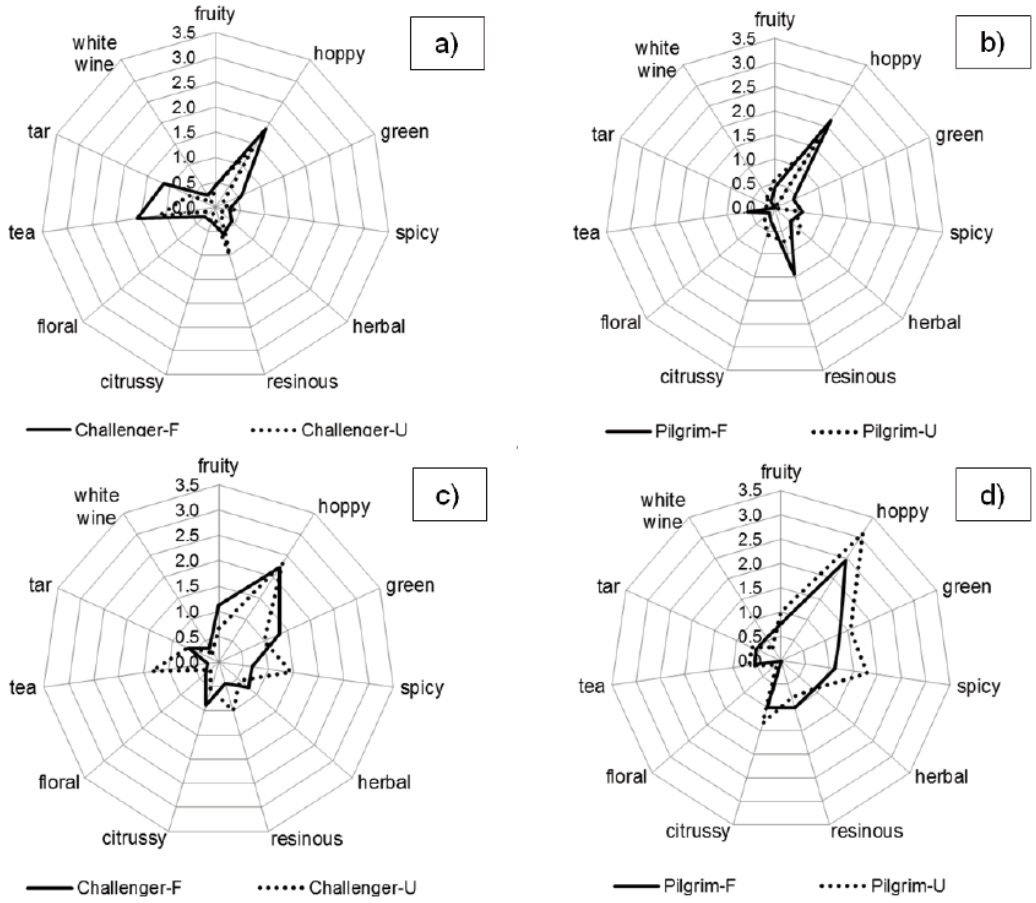

Fig. 5 **Average intensities of flavor descriptors of beers dry-hopped with Pilgrim or Challenger; sensory evaluation of fresh (a, b) and stored samples for 3 months (c, d); six-point intensity range (0–5); unfertilized (-U), fertilized (-F)**

might be reason for the high level of specific β-glucosidase activity of PG-U compared to the low level of HHT-U.

3.5 Sensory evaluation of lager beer single dry-hopped with fertilized and unfertilized hop samples

A lager beer of one batch was single dry-hopped (1.5 ml/hl) in 50-l- Kegs with Pilgrim unfertilized (97 g/hl), Pilgrim fertilized (126 g/hl), Challenger unfertilized (150 g/hl) and Challenger fertilized (273 g/hl). All samples of both hops were produced according to the method described above. Unfortunately, the samples of HHT and PG, which were subject of several analyses above showed no adequate HSI for test-brews after a longer storage period. The examination of the beer samples accordingly to the DLG-scheme approved pureness of all produced beers. In figure 5 the average intensities of eleven flavor descriptors of dry-hopped lager beers with Pilgrim and Challenger, fertilized and unfertilized, respectively, are shown. In descriptive tasting of beers produced by fertilized or unfertilized Challenger hops, slight differences in the intensities of the attributes “tea”, “tar” and “resinous”, +0.4, +0.5, –0.5, respectively, were found. In stored samples (three months), slight differences were observed by tasters with regard to the characteristics “tea” and “spicy” between fertilized unfertilized Challenger hops, –0.9 and –0.7, respectively. In the case of (fresh) beers dry-hopped by fertilized Pilgrim hops “resinous” was perceived more intensively (+0.7) than in dry-hopped beers with unfertilized Pilgrim ops. After storage of dry-hopped beers with fertilized hops, attributes such as “hoppy” and “spicy” were slightly weaker described by tasters than in beers with and unfertilized patterns, –0.7 and –0.7, respectively. In fresh and stored samples, in none of the tested characteristics, a significant difference was observed between beers dry-hopped with fertilized and unfertilized hop samples (ANOVA, $\alpha = 0.05$, $n = 7$). This confirms results of various working groups that performed comparative brewing experiments with unfertilized and fertilized hops [1, 4, 9, 10].

4 Conclusion/Summary

In this study it was shown that it is possible to produce comparable fertilized and unfertilized hop samples. The study was conducted

in field trials in the presence or absence of male plants. This was achieved by artificially fertilized hop plants grown in Hallertau (Germany), accomplished by preventing the wind fertilization of hop plants in Kent (UK). An especially interesting finding is that proclaimed disadvantages such as reduced α-acid and oil contents of fertilized hops were not observed [3, 5]. Glycosidically bound flavor-active substances, for example aliphatic alcohols, terpene alcohols and C_{13}-norisoprenoid compounds were identified confirming previous studies [17, 25, 26, 39, 40]. The substances mentioned above are related to a kettle hoppy flavor in beer [26, 41, 42]. Confirmation is also provided for glycoside hydrolysis in hops in the form of β-glucosidase activity. The observation that hops with a high seed share such as the fertilized HHT samples can have an increased β-glucosidase activity was already shown in previous studies [16]. In this context the mold contamination of clusters ripened in pollination bags hampered further interpretation. No adverse effects on the sensory between dry-hopped beers with unfertilized and fertilized hops in fresh and stored samples were noticed.

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Publication 2 Page 42 - 55	**2.3 The Influence of Brewing Yeast Strains on Monoterpene Alcohols and Esters Contributing to the Citrus Flavour of Beer**

In Publication 1, several highly flavor-active alcohols and esters in free and bound form (flavor precursors) were determined in hop raw material that could be biotransformed by the brewing yeast. The investigation into the interaction of hop volatiles and the brewing yeast might gather relevant information for brewers concerning the implementation of dry hopping during the primary or secondary fermentation. One problem in evaluating the impact of yeast on monoterpene alcohols and esters is that different effects could coincide that might increase or decrease levels of compounds.

In this study, several test fermentation set-ups were designed to analyze the biotransformation potential of popular brewing strains often used in beer production in Germany. A clear impact of different hopping timings on the final levels of hop volatiles in beer was confirmed. Despite lower concentrations of oil constituents, dry hopping green beer led to the highest citrus flavor intensity. It was shown that different outcomes might be attributed to brewing yeast activity by dry hopping green or lager beer. Hydrolase activity was identified in both green and bright beer yeast.

The yeast strains showed different potential to decrease geraniol concentrations during primary fermentation. In statistical analysis (ANOVA) and sensory evaluation using a geraniol reference compound, the importance of geraniol for citrus flavor in beer was confirmed. The high potential of the brewing yeast strain TUM 506 to influence citrus flavor in beer was attributed to the production of flavor-active compounds and a slight geraniol decreasing effect during main fermentation. The analysis of isobutyl isobutyrate showed the synergistic and antagonistic effect of the ester for citrus flavor in beer depending on the compound concentration. The different impact of the flavor-active compound on flavor descriptors indicated that the complex issue of combinatory effects is involved in shaping beer flavor.

Authors/Authorship contribution:

Haslbeck, K.: Literature search, writing, data creation, study conception and design; **Bub, S.**: Data analysis and interpretation (Enzyme assay, HS-GC-MS system); **Von Kamp, K.**: Creation of the research plan (fermentation); **Michel, M.**: Critical review (fermentation), supported statistical analysis tasting; **Zarnkow, M.**: Support at statistical analysis of data; **Hutzler, M.**: Supported creation of the research plan (yeast strain selection); **Coelhan, M.**: Supervised the project, critical content review.

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The influence of brewing yeast strains on monoterpene alcohols and esters contributing to the citrus flavour of beer

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'Aroma hops' and especially newly bred 'flavour hops' are used throughout the world to impart citrus-like and/or exotic fruit-like flavours to craft beers. Citrus-like flavours in beer are known to be influenced by yeast fermentation when transforming certain secondary metabolites of the hops such as monoterpene alcohols. In this study, the influence of different *Saccharomyces cerevisiae/pastorianus* brewing yeast strains on the citrus flavour of beers hopped at different times during beer production (30 min boiling, added during fermentation, added during maturation) with Hersbrucker, Mandarina Bavaria or Hallertauer Magnum was investigated. Yeast strains TUM 68, TUM 506, TUM 511, TUM 34/70, TUM 69 and TUM 193 that are widespread in worldwide beer production were used for standardised laboratory scale fermentations. The yeast strains showed similarly low glucoside hydrolase activity in fermenting beer. The *de novo* synthesis of monoterpene alcohols was identified, and the geraniol decrease during fermentation was confirmed using HS-GC–MS. This research indicates that the impact of monoterpene alcohols on the citrus flavour of beer could be significantly influenced by flavour active esters owing to the combinatory effects of the flavouring substances. The results of instrumental analysis and sensory evaluation suggest that the choice of yeast strain is significant for the intensity of citrus flavour in beer. © 2018 The Institute of Brewing & Distilling

Keywords: *Saccharomyces cerevisiae/pastorianus*; *Humulus lupulus* L; citrus beer flavour; β-glycosidic activity; geraniol

Introduction

It has been known for centuries that hops (*Humulus lupulus* L.) have properties that are relevant from a nutritional and physiological point of view and that they have a positive effect on beer *(1)*. They are used in the production of beer not only to impart bitterness, but also for flavour, foam enhancement, antioxidant and anti-microbiol properties *(1)*. Hop varieties are generally categorised as 'aroma hops' or 'high-alpha hops' (bitter hops). 'Aroma hops' contain relatively small amounts of alpha acids and are mainly used as a flavouring ingredient for beer. Usually, 'aroma hops' are added to wort kettle at the end of boiling, in the whirlpool or during cold processing by dry hopping of beer *(2)*. 'High-alpha hops' contain large quantities of alpha acids and are mainly used as a bittering agent. They are usually added to the wort kettle at the beginning of boiling. The so-called 'dual-purpose hops' combine characteristics of both 'aroma' and 'high-alpha hops'. The trend in consumption over the last decade, which is linked to the growth in global craft brewing, is towards specialty beers with distinctive citrus-like and exotic fruit-like flavours. This has led to the establishment of a new category of hops – the 'flavour hops'. These days, a wide variety of flavours can be introduced into beers with up to 250 available hop varieties *(2)*.

Along with other secondary metabolites, the odour source of hops – the essential oil – is located in the lupulin of hop cones. These volatile compounds form 0.2–3.0% of the dry hop material *(2)*. There are presumed to be >1000 different components in the hop oil fraction *(3)*, which are classified into three main groups: hydrocarbons, sulphur- and oxygen containing compounds *(1)*. The group of oxygen containing compounds (~30%) consists of a complex mixture of alcohols, esters and ketones that can impart floral, fruity and citrus flavours to beer *(2,4)*. In 1986, Lam *et al. (5)* assumed that the monoterpene alcohols linalool, geraniol and β-citronellol, which are highly flavour active, contribute to the floral/citrus flavour of a late hopped beer. In 2010, Takoi *et al. (6)* identified synergy among the monoterpene alcohols noted above that contribute to the citrus flavour of beer. They observed additive effects of geraniol and β-citronellol even below threshold levels. α-Terpineol and nerol, other monoterpene alcohols, may have comparable effects for beer flavour but at lower levels *(4,6)*. The group of esters that is particularly well represented in German flavour hops – Mandarina Bavaria, Hüll Melon, Hallertauer Blanc and Polaris – is generally attributed with citrus beer flavours *(4,7)*. It was shown that certain highly volatile thiols that have a grapefruit like flavour contributed to the citrus character of beer made with the Nelson Sauvin hop *(8)*. However, no underlying

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theory for the formation of specific citrus-like or other hoppy flavours could be established.

Recently, several studies showed that neglected traditional and newly identified brewer's yeast strains have a high potential to decisively and positively influence the flavour of beer *(9,10)*. Flavours known from exotic fruits (banana, pineapple, passion fruit) and citrus fruits (tangerine, grapefruit, lemon) were generated by ale yeasts during beer wort fermentation *(10)*. Besides flavour active secondary metabolites, brewing yeasts influence beer flavour by the biotransformation of hop derived flavour compounds *(11,12)*. In 2000, King and Dickinson *(13)* reported the transformation of monoterpene alcohols such as linalool, geraniol, β-citronellol, α-terpineol and nerol via geraniol metabolism of brewer's yeast. It was shown that the decrease in geraniol and an increase in β-citronellol can be significant during fermentation *(6)*. The levels of the above compounds can furthermore be influenced by the glucosidase enzyme of brewer's yeast *(11)*. 1,4-β-Glucosidase can lead to increased amounts of essential oil constituents by the hydrolytic cleavage of β-glycosides (aroma precursors) *(14,15)*. For geraniol, the content in beer can be increased by hydrolysing geranyl acetate and geranyl isobutyrate *(16)*. The influence of fermentation on the formation of hoppy beer flavour are manifold *(12)*. However, Steyer et al. *(17)* in a study using kettle hopped worts, four French hop varieties and three yeasts (ale and two lager), suggested an interaction between hops compounds and yeast metabolism which requires further investigation. Furthermore the impact of brewer's yeast on specific hop derived flavours under conditions comparable with industrial scale beer fermentation is still insufficiently explored.

In this study, the effect of several brewing yeasts on differently hopped worts and beers and resultant beer flavour were investigated focusing on the citrus flavour of the test beers. Test beers were produced using a pilot scale brewery and a laboratory scale fermentation unit that creates conditions comparable to a large scale fermentation. 1,4-β-Glucosidase activity and the impact on monoterpene alcohol contents during fermentation were investigated in separate tests. Data from instrumental measurement and sensory evaluation were analysed to determine any correlation. Test beers were reproduced with selected flavourings using beer and water matrices.

Material and methods

Chemicals

All reference compounds were of analytical grade: β-citronellol (97.3%), geraniol (99%), nerol (99.1%), methyl hexanoate (>99.9%), methyl octanoate (99.5%), internal standard pulegone (98%) and *p*-nitrophenol were purchased from Sigma-Aldrich (Steinheim, Germany); methyl heptanoate (99%) was obtained from Merck (Darmstadt, Germany), isobutyl isobutyrate (98%) was purchased from Safe Chemicals Co. (Seoul, Korea) and (*R*)-linalool (>95%) was obtained from Honeywell Fluka (Schwerte, Germany). β-Glucosidase (activity 40 U/mL) was purchased from Megazyme (USA) and *p*-nitrophenol-β-glycoside was obtained from Calbiochem (San Diego, CA, USA).

Yeast strains

Pure cultures of top fermenting brewing yeasts TUM 68, TUM 506, TUM 511 and bottom fermenting TUM 34/70, TUM 69 and TUM 193 were obtained from the Yeast Center, Research Center Weihenstephan for Brewing and Food Quality (TU München, Germany). Table 1 shows information about the yeasts and fermentation conditions.

Hop raw material

Hop pellets type-90 of Hersbrucker (HE), Mandarina Bavaria (MB) and Hallertauer Magnum (HM) were provided by HVG (Hopfenverwertungsgenossenschaft e.G., Wolnzach, Germany). All hops were grown and pelletised in Germany in 2013. Analytical data are given in Table 2. The total essential oil in hops was determined according to standard ASBC method *(18)*.

Brewing

The wort used for yeast propagation and brewing was based on (unhopped) pilsner (all-)malt wort extract (Weyermann GmbH & Co. KG, Bamberg, Germany). It was diluted with deionised boiling water to an original gravity of 12.0°P.

Table 1. Yeast strains and fermentation conditions

Yeast strain	Original habitat/typical production of beer type	Pitching cell count (million cells/mL)	Fermentation	Maturation	Counter pressure (bar) fermentation/maturation
TUM 68	Bavarian/German wheat beer	15	≥10 days, 20°C	21 days, 0°C	0.5
TUM 506	British ale	15	≥10 days, 20°C	21 days, 0°C	0.5
TUM 511	US ale	15	≥10 days, 20°C	21 days, 0°C	0.5
TUM 34/70	Lager	30	≥10 days, 15°C	21 days, 0°C	0.5
TUM 69	Lager	30	≥10 days, 15°C	21 days, 0°C	0.5
TUM 193	Lager	30	≥10 days, 15°C	21 days, 0°C	0.5

Table 2. Hop varieties (harvest 2013, Germany) used in this work

Hop variety	Abbreviations	Type of hops	α-Acid content (%)	Hop oil content (µL/g)
Hersbrucker	HE	'Aroma hop', 'land race variety'	3.3	4.80
Mandarina Bavaria	MB	'Flavour hop'	7.7	6.07
Hallertauer Magnum	HM	'Bittering hop'	12.7	17.3

Propagation Yeast was inoculated from agar slants into 70 mL of sterile wort (described above) in a 100 mL Erlenmeyer flask. Incubation in this and the following steps took 96 h at ambient temperature (20°C) and atmospheric pressure. The incubation in glass vessels of wort (1–4 L) was repeated until the required amount of yeast was obtained. Yeast cell concentration (cells/mL) was determined using a cell counter (Nexcelom Bioscience, Lawrence, MA, USA) that was calibrated for the corresponding yeast strain.

Influence of hopping procedure on hop flavour compounds The test beers were produced using a special hopping procedure (Fig. 1, Table 3). The wort or beers were hopped exclusively at a single time point to obtain kettle hopped and dry hopped beers at similar total hop dose based on essential oil concentration in beer (1.5 mL/hL). The protocol for one fermentation was as follows: the wort was boiled for 30 min in a pilot scale wort kettle; a batch of 40 L wort was boiled without hops and 20 L in the presence of hops (hopping timing 1, Table 3). In order to prepare different worts afterwards, samples of hopped and unhopped worts were placed in 10 and 20 L vessels (Cornelius, NC, USA). After the vessels were cooled to pitching temperature, propagated yeast was pitched aseptically at 30×10^6 cells/mL (bottom fermenting) and 15×10^6 cells/mL (top fermenting). The worts were not oxygenated. After the vessels were agitated to mix the contents, the worts were divided into 2 kg portions in stainless steel fermenters. Some of the fermenters containing unhopped wort were prepared with hops (the hop bag was fixed on the riser pipe of each fermenter, hopping timing 2, Table 3).

Influence of original gravity on geraniol and ß-citronellol during fermentation A seperate test series were prepared of unhopped worts at 7, 12 and 18°P. They were prepared as the control samples (brewed without hops) except that, after yeast pitching, 70 µg/L geraniol was added to the worts using the reference compound (200 µL of 0.7 g/L geraniol–ethanol solution).

Fermentation Fermenters ($n = 27$) with dimensions of 10 cm diameter × 36 cm height (2.7 L capacity) were used according to Müller-Auffermann *et al. (19)*. The caps of each container were

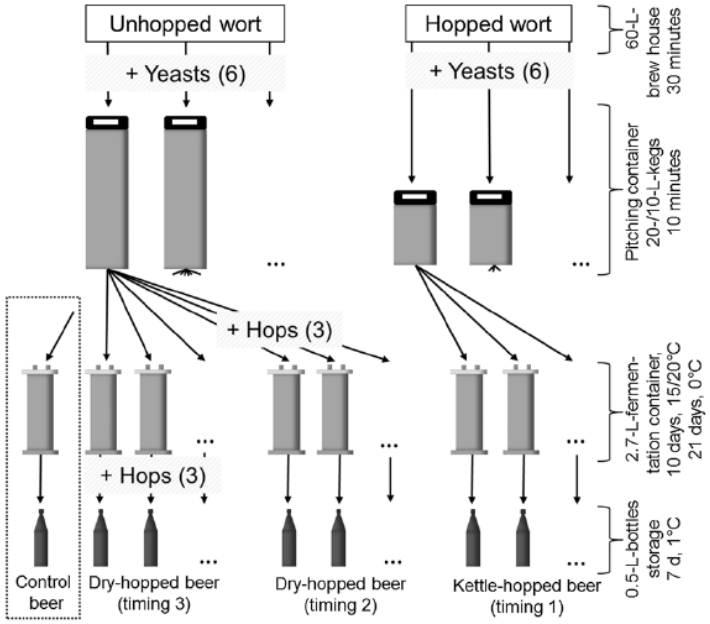

Figure 1. Set-up of the dry hopping experiment. In total six batches for testing three different hop varieties and six different yeast strains. Brewing of control beers in separate test series without any hop addition. Fermentations were in triplicate.

Table 3. Timing of (single) hop addition and dosage in brewing trials with 1.5 mL hop oil added per hL

Timing	Details	Volume (L)	HE (g)	MB (g)	HM (g)
—	'Control', unhopped wort and beer	—	—	—	—
1	Hopping of kettle-wort, 30 min boiling	20	69.2	49.6	16.0
2	Unhopped wort, dry hopping beginning when pitching yeast to wort in fermenters, duration and temperature see conditions at (primary) fermentation (Table 1)	2	6.92	4.96	1.60
3	Unhopped wort, dry hopping bottled beer at storage (after maturation), 7 days, 1°C	0.5	1.73	1.24	0.40

equipped with two gas ports, one of which was connected to a riser pipe. Sealed containers were hermetically connected by gas lines. To imitate fermentation in vessels on a large scale, a head pressure of 0.5 bar was applied simulating liquid heights of 10 m (median hydrostatic pressure) during fermentation and maturation *(19)*. Temperatures of 15 °C (bottom fermentation) and 20°C (top fermentation) were maintained for at least 10 days of primary fermentation. Primary fermentation was considered complete after the specific gravity had remained constant for two consecutive days. Maturation was performed by keeping the green beer in fermenting vessels for 21 days at 0°C. Test fermentations were performed in triplicate.

Bottling After maturation, beers were packaged into pre-evacuated 0.5 L brown glass NRW bottles by applying a positive pressure with carbon dioxide (in fermenter) to minimis oxygen pick-up. Aluminium foil was inserted between the mouth of the filled bottle and crown cork to inhibit the migration of volatile compounds into crown cork liner polymers during storage *(20)*. Some of the bottles containing unhopped beer were prepared with hops just before placing the aluminium foil on the bottle mouth prior to sealing according to hopping timing 3, Table 3. Subsequently the bottles were inverted five times and stored. The oxygen uptake was determined as being 0.051 ± 0.018 mg/L when dosing 3 g of hop pellets per litre (n = 20, α = 0.05) using an Orbisphere 3650/3655 (Hach Company, Loveland, CO, USA). The bottled test beers were stored for 7 days at 1°C until sensory evaluation and GC analysis of volatile compounds.

Glucoside hydrolase activity during fermentation The glucoside hydrolase activity in control samples was determined on the third and tenth day of fermentation according to Takoi *et al. (11)*. The reaction is based on the enzymatic cleavage of the model glycoside *p*-nitrophenyl-β-glucopyranoside (pNP-β-Glc) to *p*-nitrophenol (pNP) and glycoside (β-Glc). One unit (U) of glucoside hydrolase activity is defined as the quantity of enzyme that incorporates pNP-β-Glc into 1 µmoL of pNP and β-Glc per minute under the conditions of the assay. The pNP content is proportional to the enzyme activity and was detected using Evolution 300 UV–vis spectrophotometer (Thermo Fisher Scientific, MA, USA). The wavelength was 405 nm *(11)* and the pH of the sample was controlled at >11 for all measurements *(21)*. The activity was measured as follows: pNP-β-Glc substrate solution (172 mg pNP-β-Glc dissolved in 100 mL 0.05 M acetate buffer at pH 4.0) and 8 mL test tubes were pre-heated for 5 min at 40°C in a drying chamber; 0.7 mL of 4-NPG solution and 0.3 mL sample was added to a tube. Blank samples with 2 mL of a 1 M sodium carbonate solution and then pNP-β-Glc was added to prevent enzymatic reactions. The tubes were then incubated at 40°C for 180 min. The enzymatic reaction was stopped by adding 2 mL of 1 M sodium carbonate. Subsequently, samples were analysed spectrophotometrically against the corresponding blank using quartz glass cuvettes (d = 1 cm). Calibration was performed by measuring the absorbance of pNP standard solutions (0.02, 0.04, 0.06 and 0.08 µmol/mL). Glucoside hydrolase activity (U/L) of each sample solution was calculated from an absorbance of the reaction mixture and the absorbance of pNP standard solutions.

HS-GC–MS of test beer

A Shimadzu GC-2010 gas chromatograph was directly coupled to an MS-QP2010 Ultra (Nakagyo-ku, Japan). Separation was performed using a ZB-WAX (Phenomenex Inc., Torrance, CA, US) 30 m × 0.25 mm capillary column (0.25 µm film thickness). The system was equipped with a headspace sampler HS-20 (Shimadzu, Nakagyo-ku, Japan). Measurement was as follows: 5 mL samples in nitrogen purged 20 mL vials containing 3 g sodium chloride were equilibrated for 30 min at 80°C immediately before injection of the 1 mL headspace gas sample; split 1:5 (loop system). The carrier gas helium was adjusted to 200 kPa. The temperature programme of the GC oven increased at a rate of 4°C/min from 50 to 130°C, increased at 8°C/min to 180°C and then at 15°C/min to 240°C. Ion source temperature was set to 200°C; interface temperature was set to 250°C. Measurements were taken using the SIM mode (70 eV ionisation). Data analysis was by LabSolutions GCSolutionAnalysis and LabSolutions GCMSsolution version 2.72. The monoterpene alcohols (linalool, α-terpineol, β-citronellol, nerol and geraniol) and esters (isobutyl isobutyrate, methyl-4-decenoate, methyl hexanoate, methyl heptanoate and methyl octanoate) were quantified in the SIM mode, with selection of the following ions: m/z 74 (for methyl-4-decenoate), 89 (isobutyl isobutyrate), 93 (nerol, β-citronellol and geraniol), 121 (linalool), 113 (methyl hexanoate, methyl heptanoate), 117 (methyl octanoate) and 136 (α-terpineol). Standard addition calibration curves were determined using lager beer (5% ethanol, targeted compounds <5 µg/L) containing the monoterpene alcohols and esters at additions of 0, 5, 10, 20, 40, 80 and 160 µg/L. All calibrations produced a linear response with an R^2 value >0.98 over the concentration range analysed. Calibration curves for samples of 7, 12 or 18°P were determined using water including 3, 5 or 10% ethanol containing the monoterpene alcohols at final concentrations ranging from 0 to 40 µg/L. All calibrations produced a linear response with an R^2 value >0.98 over the concentration range.

Sensory evaluation

The DLG (Deutsche Landwirtschafts-Gesellschaft) scheme for beer and descriptive tasting were performed by a panel of seven DLG certified and trained tasters in triplicate. In each session a maximum of 10 beers was tested. Tasting of samples at 12°C took place in individual walled tasting cabins under controlled environmental conditions. Intensity of 'citrus' and, if implemented, additional flavour attributes of 'fruity', 'green', 'artificial', 'floral' and 'sweet' were tested in a six-point range from 0, meaning 'not noticeable', to 5, 'extremely noticeable'. Tastings using reference compounds at 0.7 µg/L ethanol solution, were performed on lager beer produced using TUM 34/70 from a commercial brewery and model solutions (composition details in Table 8), which were prepared according to the method described in Takoi *et al. (11)*. The tasting procedure was as follows: a 50 mL aliquot of each test beer (geraniol or nerol reference compounds was added to bottled beers that were subsequently sealed with crown corks at minimised oxygen pick-up as described previously, four hours prior to sensory testing) or sample solution was presented in a brown glass and the five flavour characters (floral, fruity, citrus, green and artificial or sweet) scored from 0 (no flavour) to 5 (strong flavour).

Data analysis

Correlation analyses were carried out using Excel 2013 (Microsoft, CA, USA), measuring the Pearson correlation coefficient. Multivariate analysis and one-way analysis of variance (ANOVA) were used with the SPSS Version 24.0 statistical package for Windows (SPSS Inc., Chicago, IL, USA). Statistical differences between means were

evaluated using Games–Howell's test at 0.05% level in to evaluate the significance of the analysis.

Results and discussion

Brewing yeast strains

The yeast strains used here (Table 1) are currently popular for beer production in Germany *(23)*. They are a wide range of domestication trajectories to consider to gain a comprehensive overview of the brewing yeast strains. In 2016, Gonçalves *et al. (22)* showed that, at a genome wide level, *S. cerevisiae* strains used to produce top-fermented (ale type) beer and wine are clearly discernible. Beer strains are more diverse and show clustering according to beer/genome type. The Bavarian/German wheat beer strain TUM 68 clusters in the sake clade, British ale strain TUM 506 clusters in the baker's yeast clade and US ale strain TUM 511 clusters in the wine yeast clade *(22)*. TUM 34/70, which belongs to the Frohberg group, is popular commercially used yeast strain for lager production *(22,24)*. To the best of our knowledge no information is available regarding the origin of TUM 69 and TUM 193.

Unhopped wort fermentations

Glucoside hydrolase activity Recently, several authors have reported the occurrence of glycosidically bound flavour compounds in hops and hopped beers *(11,15,25)*. Flavour potential of several monoterpene alcohols in beer were determined *(11)*. Various brewing yeasts with glucoside hydrolase activity depending on *exo-β*-1,3-glucanase were identified *(14,26)*. Table 4 shows glucoside hydrolase activity in fermenting control beers, which is attributed to the *exo-β*-1,3-glucanase activity of the yeasts *(11,14)*. Activities of 0.067–0.168 U/L were determined, which is below the levels (0.4–0.8 U/L) determined in fermenting beers by Takoi *et al. (11)* using a similar enzyme assay. In this study, glycosidic cleavage activity by brewer's yeasts was determined to be relatively equal, suggesting a similar potential to influence the flavour of beers as described above, which is consistent with Sharp *et al. (26)*. However, the *Saccharomyces* yeasts tested here showed relatively weak glucoside hydrolase activity compared with other genera such as *Brettanomyces/Dekkera* yeasts *(14)*, which can also play a role in the production of beer *(27)*.

Table 4. Comparison of glucoside hydrolase activity (U/L) in fermenting control (unhopped wort) samples

Yeast	Day 3 of fermentation	Day 10 of fermentation
TUM 68	0.15 ± 0.03	0.01 ± 0.01
TUM 506	0.11 ± 0.01	0.17 ± 0.00
TUM 511	0.08 ± 0.02	0.09 ± 0.00
TUM 34/70	0.14 ± 0.00	0.12 ± 0.01
TUM 69	0.09 ± 0.02	0.14 ± 0.03
TUM 193	0.07 ± 0.01	0.07 ± 0.00

Mean values ± standard deviation, $n = 3$.

***De novo* synthesis of monoterpene alcohols** Table 5 shows the amount of monoterpene alcohols in unhopped beers. All malt wort used for fermentations did not contain any of the monoterpene alcohols. The results show that, for the beer produced with TUM 68 (12.1 μg/L), higher levels of monoterpene alcohols were detected than in beers produced with ale strains TUM 506 (5.2 μg/L) and TUM 511 (6.7 μg/L) and lagers using TUM 34/70 (5.6 μg/L), TUM 69 (4.6 μg/L) and TUM 193 (5.7 μg/L). Geraniol was the monoterpene alcohol produced in the greatest abundance. The highest content of geraniol at 6.8 ± 1.7 μg/L was found in beer produced with TUM 68, which is within the range of threshold concentration. Linalool was produced by TUM 68 at higher levels than by lager yeasts, 2.6 ± 1.2 and 0.9–1.3 μg/L, respectively; however, linalool was not detected to be produced by ale yeasts TUM 506 and TUM 511. Traces of *β*-citronellol, *α*-terpineol and nerol were measured in beers, 0.4–0.9, 0.4–1.0 and 0.2–1.4 μg/L, respectively. In 2006, Kishimoto *et al. (28)* determined linalool in unhopped beer using GC-O. Takoi *et al. (11)* and Hanke *et al. (29)* determined a slight increase in geraniol during fermentation at lower levels of this compound in hopped worts. To the best of our knowledge this is the first quantification of monoterpene alcohols identified as being produced by *S. cerevisiae* and *S. pastorianus* brewing yeast strains during unhopped all malt wort fermentation. It can be assumed that the same reaction takes place in hopped all malt wort. Carrau *et al. (30)* detected the *de novo* biosynthesis of terpene alcohols by (environmental) *S. cerevisiae* wine yeasts up to 4 μg/L linalool or *α*-terpineol using fermentation media mimicking grape juice. They discussed the leucine catabolism pathway (MCC pathway) as a hypothetical model for the formation of monoterpenes in *S. cerevisiae* in relation to the sterol biosynthetic pathway.

Decrease in geraniol and increase in *β*-citronellol Figure 2 shows the contents of geraniol (a) and *β*-citronellol (b) in unhopped beers produced using worts at different original gravities supplemented with 70 μg/L geraniol. The levels of geraniol decreased during fermentation, which might be caused by biotransformation reactions via yeast metabolism *(6,13)*. The *de novo* production of geraniol as analysed above should be considered in the discussion of the results. Generally, lower contents of

Table 5. Concentrations (μg/L) of monoterpene alcohols in unhopped wort and resultant beer

Compounds	Wort	TUM 68	TUM 506	TUM 511	TUM 34/70	TUM 69	TUM 193
Linalool	n.d.	2.6 ± 1.2		n.d.	1.2 ± 0.2	1.3 ± 0.4	0.9 ± 0.2
α-Terpineol	n.d.	1.0 ± 0.5	0.9 ± 0.2	0.8 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.1
β-Citronellol	n.d.	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.7 ± 0.1	0.8 ± 0.0	0.9 ± 0.2
Nerol	n.d.	1.3 ± 0.3	1.4 ± 0.3	1.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
Geraniol	n.d.	6.8 ± 1.7	4.0 ± 0.7	2.7 ± 0.4	3.0 ± 1.0	1.7 ± 0.4	3.2 ± 0.3
Sum	—	12.1	6.7	5.2	5.6	4.6	5.7

n.d., Not detected.
Mean values ± standard deviation, $n = 3$.

geraniol were determined in samples produced from worts at higher original gravities. The decrease in geraniol are considered significant for the respective beer flavours. The exceptions are beers produced with TUM 506 in which the geraniol concentration was high (40.9–44.8 µg/L) regardless of the wort original gravity. It is assumed that this is due to TUM 506 having the lowest geraniol metabolic activity; it is also very probable that there was no decrease as a result of yeast metabolism. The evaporation or partitioning of certain terpenoids into the cell membranes of yeasts, which has been reported by several authors *(13,31,32)*, may have led to results below the set values of geraniol. A previous study determined that geraniol could be retained during fermentation by choosing yeast with low *OYE2* gene activity (for NADPH oxidoreductase) and acetyltransferase (*ATF1*) enzyme activity which were both strain dependent *(33)*. Levels of β-citronellol increased at higher original gravities except for TUM 69; however, it is assumed that the decrease in geraniol as well as the increase in β-citronellol may depend on the fermentable extract. Contents of β-citronellol in samples were similar to control samples produced without geraniol. In addition, levels of other potential metabolites *(13)*, such as linalool and α-terpineol, have also been detected in the range of *de novo* synthesis (data not shown). Accordingly, it is proposed that the hypothesis that the increase in β-citronellol is primarily due to the *de novo* production by yeasts rather than by the reduction of geraniol.

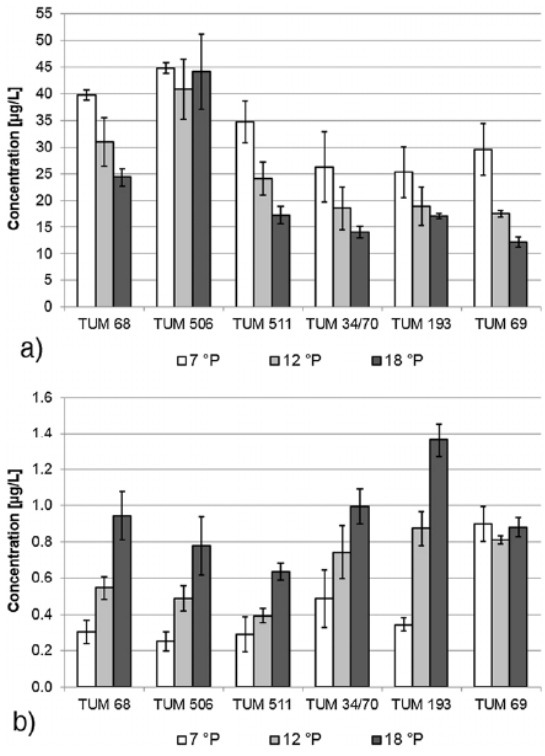

Figure 2. Contents of geraniol (a) and β-citronellol (b) in beers produced at different original gravities using unhopped wort containing geraniol (70 µg/L).

Hop derived monoterpene alcohols and selected esters

Influence of hop variety Brewing was conducted using type-90 pellets of hop cultivars HE, MB or HM that contribute a citrus odour (as well as other notes). Single variety hopping clearly caused differences in the profile of monoterpene alcohols and esters in the beers (Figs 3 and 4), which are related to the citrus flavour of beer *(6,34)*. HE led to comparably higher amounts of linalool, up to 136 µg/L (hopping timing 3), which is regarded as a key compound for a 'hoppy' beer flavour *(35)*. In MB beers, comparably high amounts of geraniol were detected. In 2010, Takoi *et al*. *(6)* determined that geraniol rich hops such as Citra can impart a 'citrus' flavour to beers; they showed that high contents of geraniol in the initial wort could lead to high contents of geraniol and β-citronellol in the finished beer. β-Citronellol was determined at low levels ($\leq$6.7 µg/L), which is comparable with previous studies *(11)*. In another study *(36)*, the same authors suggest that the comprehensive behavior of β-citronellol is commonly observed during fermentation using the geraniol rich hop varieties. The concentration of esters (4.9–35.4 µg/L) and the level of especially isobutyl isobutyrate 23.8 µg/L were comparably in MB beers, which is consistent with previous studies *(7)*. Isobutyl isobutyrate was reported in samples of several hops such as Hallertauer Tradition, Hallertauer Magnum, Czech Saaz and Nelson Sauvin *(8)*. In this study, in HM beers, the concentration of esters was between those of the other varieties MB and HE and the lowest contents of monoterpene alcohols were determined, 6.4–29.7 and 8.0–63.6 µg/L, respectively. With regard to the compounds analysed, HM – which is generally used for bittering – contributed comparable amounts of flavouring.

Influence of hop addition timing Single hopping timings were applied, dosing the same amount of essential oil (1.5 mL/hL) for each sample. Generally, the contents of analysed compounds in test beers were higher when hops were added at a later stage in the brewing process (Figs 3 and 4, timing 2 and especially timing 3). This confirms that the timing of hop addition has an influence on the content of monoterpene alcohols and esters, despite good solubility in wort kettle and fermenting beer *(27,37)*. The lowest amounts found in beers brewed with hop addition timing 1 are partly attributed to evaporation during wort boiling *(38)*. Seaton *et al*. *(39)* proposed that isobutyric acid esters such as isobutyl isobutyrate are possibly unstable as they decrease during boiling and fermentation. The flavour transfer during static dry hopping at timing 2 or 3 is influenced by several factors such as the disintegration of pellets, which depends on the alpha acid content of hop variety, duration and temperature *(40,41)*. Using hop bags exclusively in timing 2, which is expected to decrease the aroma extraction *(42)*, should be considered when comparing the two dry hopping timings. With regard to the flavour potential being assumed in hop monoterpene glycosides, Sharp *et al*. *(26)* determined similar amounts of monoterpene glycosides extracted from hops regardless of hopping regime for the hop varieties Columbus, Hallertau Mittelfrüh and Simcoe.

Influence of yeast strains Differing levels of monoterpene alcohols were determined in test beers that used the same hop variety but were produced with different yeast strains. In MB- or HM-hopped beers (timing 1) produced with top-fermenting strains (TUM 68, TUM 506 or TUM 511) the total monoterpene alcohol content was higher than for lager beers (TUM 34/70, TUM 69, TUM 193), 31.9–45.2 and 10.1–28.1 µg/L, respectively. The *de novo* synthesis of monoterpene alcohols (see discussion above; Table 5)

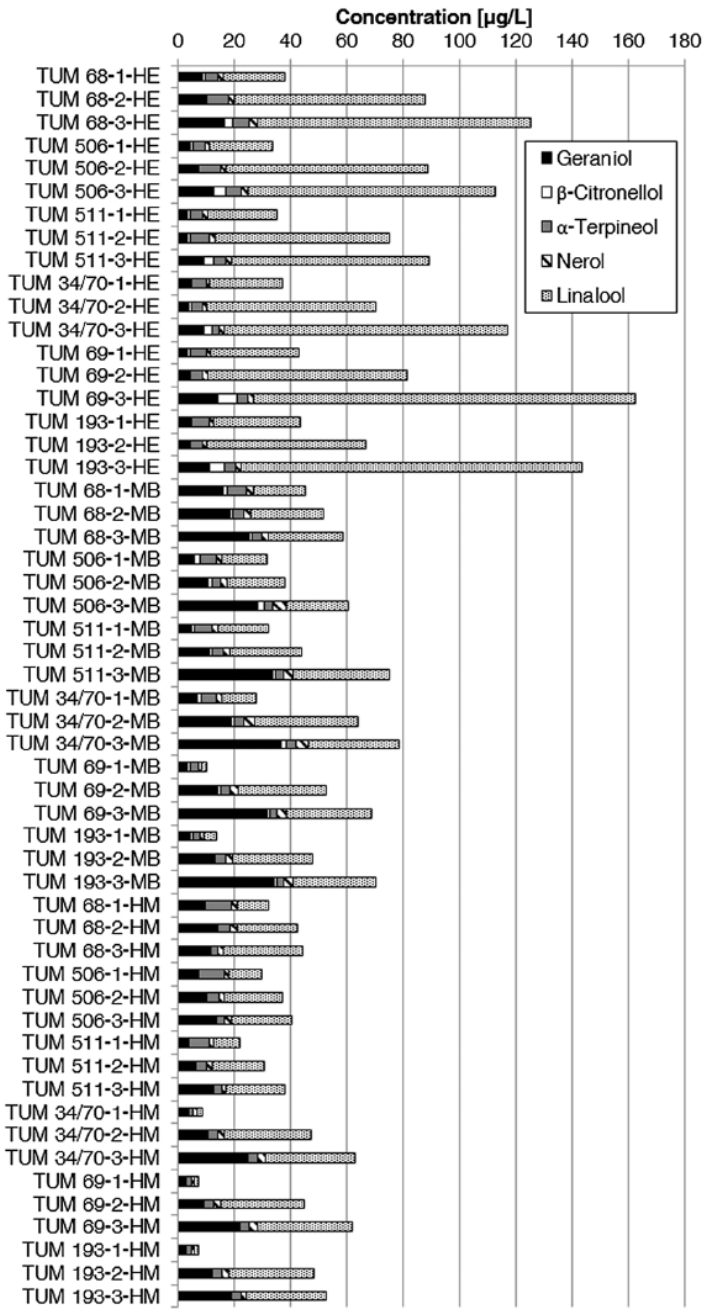

Figure 3. Contents of monoterpene alcohols in test beers ($n = 3$). Beers produced with yeasts TUM 68, TUM 506, TUM 511, TUM 34/70, TUM 69 or TUM 193, hopped at timing (1) '30 min wort boiling', (2) 'beginning dry hopping when pitching the yeast into wort' or (3) 'dry hopping at maturation' using Hersbrucker (HE), Mandarina Bavaria (MB) or Hallertauer Magnum (HM) hops.

could only partly explain the higher content in top-fermented beers. In lager beers hopped with MB or HM (timing 1), geraniol was determined at comparably low levels, which might be attributed to higher biotransformation of geraniol (see discussion above; Fig. 2). Different flavour potential in test beers produced with different yeast strains regarding monoterpene glycosides are unlikely because relatively equal activity of glucoside hydrolase activities from yeast were found (see discussion above; Table 4).

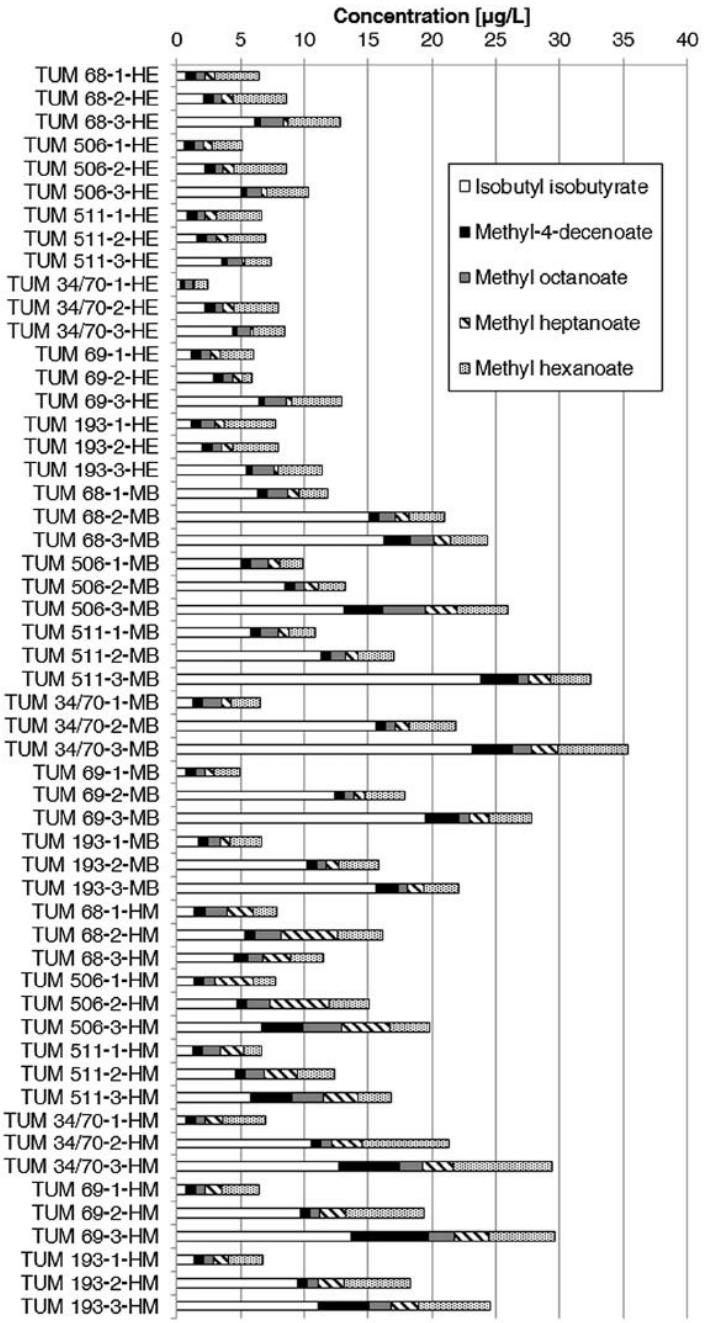

Figure 4. Contents of esters in test beers ($n = 3$). Beers produced with yeasts TUM 68, TUM 506, TUM 511, TUM 34/70, TUM 69 or TUM 193, hopped at timing (1) '30 min wort boiling', (2) 'beginning dry hopping when pitching the yeast into wort' or (3) 'dry hopping at maturation' using HE, MB or HM hops.

However, in HE-hopped beers (timing 1) similar levels of total monoterpene alcohols were determined regardless of yeast strain. Therefore it is assumed that the impact of yeast on the content of hop-derived monoterpene alcohols may depend on the hop variety used. In a previous study *(17)*, esters derived from yeast metabolism were found to be influenced by the hop variety, although neither of the esters nor their precursors are known to be present in hops. A model that could fully explain compound

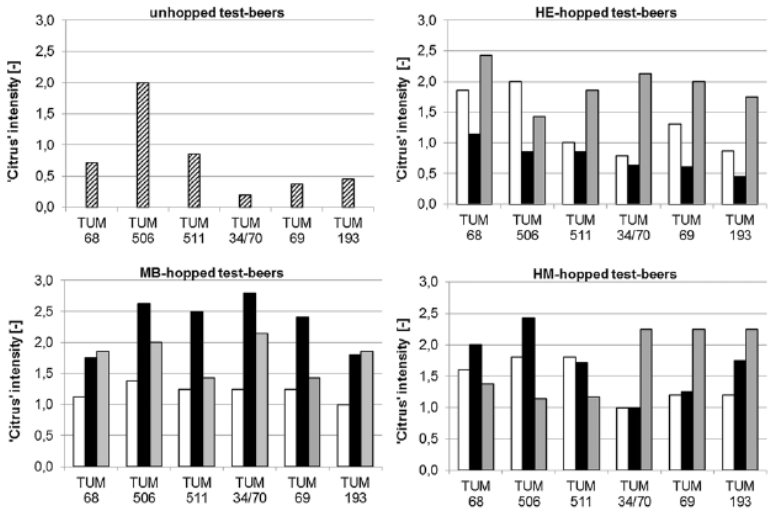

Figure 5. 'Citrus' intensity of beers ($n = 3$; seven tasters) unhopped and hopped at different timings with Hersbrucker (HE), Mandarina Bavaria (MB) or Hallertauer Magnum (HM) hops. 0, 'Not noticeable', to 5, 'extremely noticeable'. Black hatched, unhopped; pale grey, hopped at timing 1 ('30 min wort boiling'); black, hopped at timing 2 ('beginning dry hopping when pitching the yeast into wort'); dark grey, hopped at timing 3 ('dry hopping at maturation').

biotransformation during fermentation has yet to be established. Lower contents of monoterpene alcohols at timing 2 compared with timing 3 might be due to yeast metabolism during the fermentation *(37)*. For linalool, decrease during fermentation by yeast uptake or purging by carbon dioxide is unlikely, *(33)*. Previous studies have proposed transformation of esters during fermentation *(12,39)*. It is well known that hop derived methyl esters are hydrolysed or converted into ethyl esters by yeasts *(34)*. The study of King and Dickinson *(43)* revealed that yeasts may also contribute to the presence of terpenoid esters in beer. They interpreted the formation of acetate esters of geraniol and β-citronellol with a lager strain but not a ale strain as a reflection of the genetic (and consequent biochemical) differences between both species. In this study, there was a maximum difference of 7 µg/L between the esters of beers identically hopped but fermented by different strains. These small differences lead us to conclude that the yeast strains had similar effects on the analysed esters under the set of experimental conditions.

Citrus flavour of test beers

Sensory evaluation revealed no sensory errors in any of the test beers. In the unhopped beers produced with TUM 506, 'citrus' was rated highest followed by TUM 68/TUM 511, 2.0 and 0.7/0.9, respectively, whereas in lager beers the intensities were comparably low (<0.5, Fig. 5). It is well known that top fermented beers can contain significant levels of flavour active esters affecting a variety of flavours including exotic and citrus fruits *(10,44)*. Different varieties were used when producing single hopped test beers; Hersbrucker hops have flavour descriptors such as 'spicy', 'hay', 'orange' and 'tobacco' *(45)*. Mandarina Bavaria is the daughter of Cascade and a Huell wild hop-derived male *(43)*. This family tree explains its relatively intense citrus flavours such as 'tangerine', 'grapefruit' and 'lemon'. The flavours of Hallertauer Magnum hops are defined as 'citrus', 'fruity', 'green pepper' and 'apple' *(45)*. The hopping of worts or dry hopping of beers generally led to an increase in the citrus note of the resulting beers (Fig. 5). This corresponds to the calculated odour activity values (OAV: ratio of concentration to odour threshold) of hop derived compounds that are attributed as 'citrus', shown in Table 6. The table also lists olfactory descriptions, perception thresholds and concentration ranges in test beers. Tasters attributed the highest 'citrus' intensitie (≥2.5) to beers produced with TUM 506, TUM 511 or TUM 34/70 that were hopped (timing 2) with Mandarina Bavaria. Intense citrus odours in MB dry hopped beers were also shown in previous studies *(7)*. Both ale strains were found to be suitable for creating beers with specific hoppy flavours such as 'citrus'. The 'wheat beer yeast' TUM 68 is well known for its phenolic flavours *(44)*. These were also noted in the sensory evaluation of the test beers and have an adverse effect on hoppy beer flavour *(27)*. In addition, 'US ale yeast' strain TUM 511 was determined as *POF+* (Phenolic Off-Flavour) in a previous study *(10)*. In some cases, hopping resulted in lower citrus intensities compared with unhopped beers, e.g. TUM 506 beers hopped at timing 3 with HE or HM. This may be attributed to antagonistic effects between flavour compounds that can occur in heterogeneous mixtures *(46,47)*. However, no definite trend could be identified regarding a particular hopping timing for the highest intensities for that specific flavour. Despite the high popularity of dry hopping for use as an aroma hopping method, a recent study showed that whirlpool-hopped beers could produce even more intensely aromatic beers than dry hopping *(27)*. In this study the intensity of citrus flavour depended on hop variety, hopping timing and yeast strain.

Correlation analysis of compound concentration with citrus flavour intensity of test beers

The assumption that individual compounds in test beers might contribute to the citrus flavour in beers, was tested using the standard Pearson correlation analysis *(48)*. This approach gave a numerical value for how well the intensity of citrus flavour correlates with the levels of specific compounds in beer. In addition, using multivariate analysis, no further results were achieved

Table 6. Olfactory description, perception threshold, concentration and odour activity value (OAVs) of analysed compounds in test beers

Reference compound	Olfactory description[a]	Olfactory perception threshold[b]	Concentration (this study)	OAV[c] (this study)
Linalool (µg/L)	Lavender	5	1.1–136	0.22–27.2
Geraniol (µg/L)	Rose	6	3.1–36.7	0.52–6.17
β-Citronellol (µg/L)	Lemon, lime	8	nd; 6.7	nd; 0.84
α-Terpineol (µg/L)	Lilac	2000	1.6–6.2	<0.01
Nerol (µg/L)	Floral, citrus	500	0.7–4.5	<0.01
Isobutyl isobutyrate (µg/L)	Fruity, pineapple	30[d]	0.3–23.1	0.01–0.77
Methyl octanoate (µg/L)	Fruity	200[d]	0.6–2.1	<0.01
Methyl-4-decenoate (µg/L)	Fruity	n/a	0.4–6.1	n/a
Methyl heptanoate (µg/L)	Cheesy, fermented	4[d]	0.2–2.7	0.05–0.68
Methyl hexanoate (µg/L)	Fruity	84[d]	0.8–7.8	<0.09

[a]Odour descriptors from the literature *(6,52–55)*. [b] Odour thresholds from the literature *(6,52–55)*. [c] OAVs were calculated by dividing the concentrations by the respective thresholds in beer. [d] Olfactory perception threshold in water. n.d., Not detected. n/a, Not available.

Table 7. Significant correlations ($r \geq 0.67$; *: $\alpha \leq 0.05$; **: $\alpha \leq 0.01$; $n = 9$) of concentration of volatile compounds (determined in test series: 'influence of hopping procedure on hop flavour compounds', Fig. 3 and 4) *versus* intensity of 'citrus' (Fig. 5)

	TUM 34/70		TUM 193	
	r	Trend line function	r	Trend line function
Geraniol	0.71*	$y = 0.0490x + 0.8971$	0.70*	$y = 0.0412x + 0.9573$
Nerol	0.73*	$y = 0.4893x + 0.4628$	0.78*	$y = 0.7276x - 0.0123$
Isobutyl isobutyrate	0.70*	$y = 0.0682x + 1.0188$	0.82**	$y = 0.0899x + 0.8571$

* $\alpha = 0.05$; ** $\alpha = 0.01$.

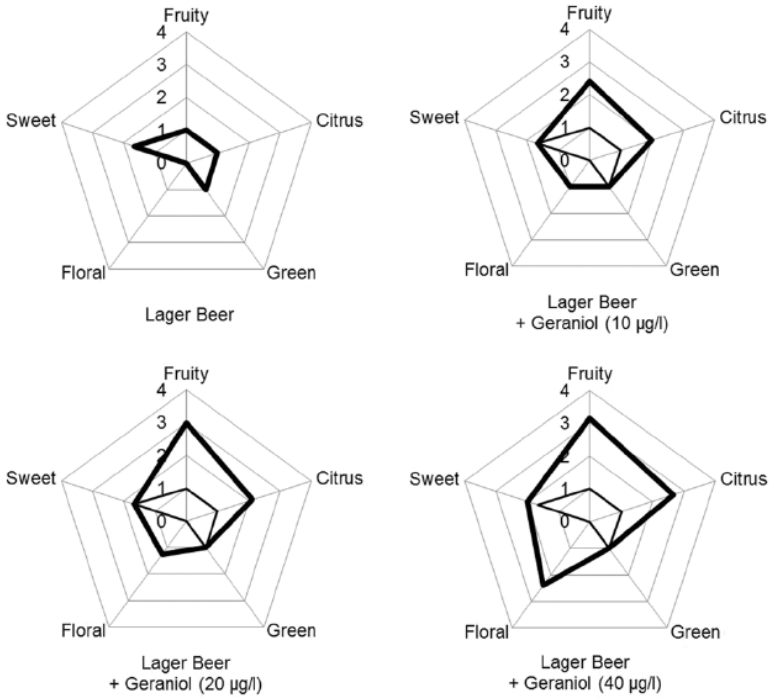

Figure 6. Flavour profile of lager beer ($n = 3$; seven tasters) containing 2.1 µg/L geraniol and geraniol reference compound; 0, 'not noticeable', to 5, 'extremely noticeable'.

owing to multicollinearity and the multivariate regression model may not give valid results about any individual predictor, or about which predictors are redundant with respect to others. Table 7 shows data for significant correlation. In lager beers produced with TUM 34/70 and TUM 193 the intensity of citrus flavour correlated with the corresponding content of geraniol, nerol and isobutyl isobutyrate ($r \geq 0.67$; $\alpha \leq 0.05$; $n = 9$). No further significant correlation of other substances or single strain test beers with citrus notes were found. However, it can be assumed that a variety of other flavour compounds and different combinations thereof can result in citrus flavours in beer *(4)*. In addition, the intensity of the citrus flavour in beers of TUM 34/70 and TUM 193 was not significantly higher than other test beers (ANOVA, $\alpha = 0.05$; $n = 54$). This indicates that the contribution of individual substances to 'citrus' is influenced by further flavour compounds present in test beers. It is well known that beer flavour is very complex and created by the interactions of several hundred flavour substances *(1,49)*.

Table 8. Comparison of the composition of monoterpene alcohols in test beer[a] with the highest intensity of citrus flavour (2.8, Fig. 5) and model solution

	Test beer[a]	Model solution[b]
Linalool (µg/L)	36.7	20[c]
β-Citronellol (µg/L)	1.0	2
Geraniol (µg/L)	18.9	20
Nerol (µg/L)	3.5	—
α-Terpineol (µg/L)	3.8	—
Isobutyl isobutyrate (µg/L)	15.6	0, 10, 30 or 80

[a]Test beer was produced using TUM 34/70 and dry-hopped at yeast pitching with 2.48 g/L Mandarina Bavaria hop.
[b]Ethanol–water solution (1:100, *v*/v).
[c]Content of linalool were set to 20 µg/L owing to the strong layering effect at higher concentrations in model solution. Mean value, $n = 3$.

The impact of geraniol on beer flavour and the role of nerol

The impact of geraniol on the flavour of test beers suggested by the correlation analysis (above) was further evaluated. Different geraniol contents in test beers (Table 6) were set by adding the compound to samples of a German style lager beer containing small amounts of geraniol (2.1 µg/L). The resulting sensory profiles are shown in Fig. 6. It is remarkable that adding geraniol at contents slightly above the threshold level of geraniol in beer significantly increased the flavour attributes of 'flowery', 'citrus' and 'fruity'. These flavour descriptors intensified with higher geraniol additions. The 'citrus' levels rose with the addition of 10, 20 or 40 µg/L, to +1.0, +1.1 and +1.7, respectively. This confirms the important role of geraniol in beer flavour as noted previously *(6)*. The impact of different geraniol levels on flavour profile is also meaningful in terms of a decrease in geraniol at comparable amounts of 30–50 µg/L in 12°P wort (Fig. 2) by yeast fermentation as discussed above. The contribution of nerol to the citrus note as indicated by correlation analysis was not confirmed in sensory analysis using the reference compound (data not shown). We assume

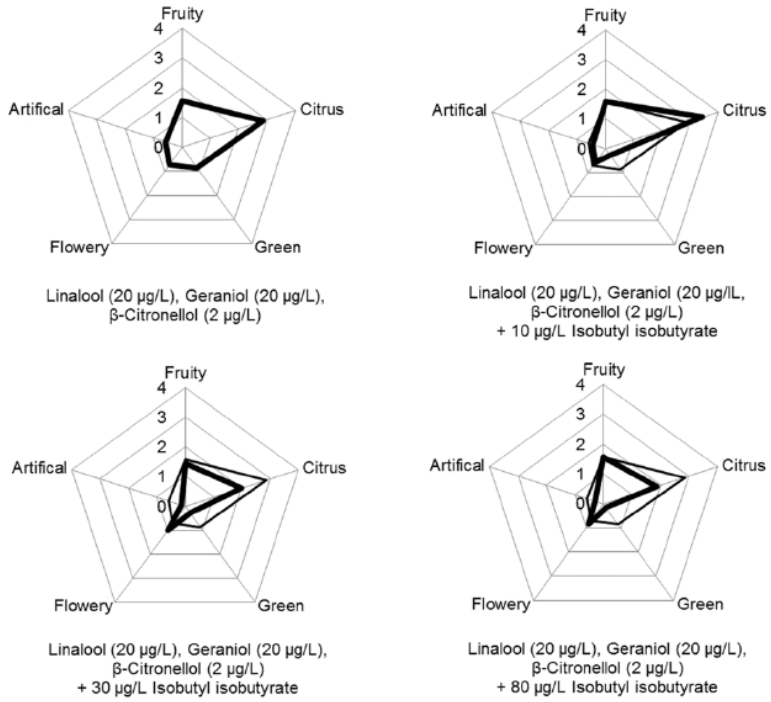

Figure 7. Flavour profiles of model solutions ($n = 3$; seven tasters) simulating the composition of three monoterpene alcohols and isobutyl isobutyrate in test beer TUM 34/70 hopped with MB at timing 2; 0, 'not noticeable', to 5, 'extremely noticeable'.

the correlation of content in the beers of TUM 34/70 and TUM 193 with the citrus flavour intensity is due to its highly significant correlation with geraniol (Pearson correlation, $\alpha = 0.001$, $n = 9$), $r = 0.92$ and $r = 0.93$, respectively, whose contribution to 'citrus' is highly probable as discussed above. The minor sensory impact of nerol to hoppy beer flavour has been suggested by several authors *(6)*.

Combinatory effects of isobutyl isobutyrate and monoterpene alcohols

Isobutyl isobutyrate was determined in test beers at OAVs 0.01–0.77 (Table 6). The reference compound has fruity flavours equivalent to green apples and apricot, which is typical for isobutyric acid esters *(8,50)*. However, significant correlation with citrus flavours of TUM 34/70 and TUM 193 beers was suggested by correlation analysis. The role of isobutyl isobutyrate to the citrus flavour of test beer was evaluated according to the procedure in Takoi *et al. (11)*. This used model solutions (1% ethanol–water solution) containing geraniol, linalool and β-citronellol at levels representative of test beer with highest citrus intensity (Table 8). At levels below the threshold concentration, a minor increase in the intensity of citrus flavour was shown (Fig. 7). At levels equal to the threshold and 2.5 times above the threshold, tasters noted lower intensity. These results point to combinatory effects of the monoterpene alcohols with isobutyl isobutyrate. Synergistic and antagonistic effects of flavour compounds are well known in heterogeneous mixtures *(46,47)*. In terms of beer flavour, in 2010, Hanke *et al. (51)* identified an increase or decrease in the perceived intensity of particular flavours in beer depending on the concentration of individual compounds such as linalool, ethyl acetate and isoamyl acetate.

Conclusions

The *S. cerevisiae* and *S. pastorianus* yeast strains showed a variable impact on the monoterpene alcohol content during fermentation, although similarly low levels were determined for glycosidic hydrolase activity. The *de novo* synthesis of monoterpene alcohols was identified and the highest production was shown by yeast TUM 68 at levels that could be of sensory importance to beer flavour. The decrease in geraniol that was previously reported by several authors appeared to depend on the level of wort original gravity. The rise in β-citronellol in test beers was not confirmed as corresponding to geraniol reduction. A direct impact on the citrus flavour of test beers as result of monoterpene alcohol modification by yeast was found to be likely. Indeed, it was established that the choice of yeast can be significant for the citrus intensity of resultant beers. Generally, higher amounts of monoterpene alcohols and esters were determined when the hops were added at a later stage in the brewing process, although the citrus flavour did not relate to the timing of hop addition. The impact of geraniol and isobutyl isobutyrate on the citrus flavour of TUM 34/70 and TUM 193 test beers was suggested by statistical significant correlations, and was verified by further sensory evaluation. The important role of geraniol for citrus flavour of beer was confirmed. An interesting finding is that isobutyl isobutyrate showed synergistic and antagonistic effects dependent upon concentration with monoterpene alcohols, contributing to the citrus flavour of test beers. These investigations indicate that combinatory effects among flavouring substances are important in the formation of hoppy beer flavours and should be the subject of further research.

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2.4 On the Fate of β-Myrcene during Fermentation – The Role of Stripping and Uptake of Hop Oil Components by Brewer´s Yeast in Dry-Hopped Wort and Beer

In several studies, the brewing yeast was identified as having an impact on the terpenoid oil constituents during fermentation. However, the change in concentration of terpene oil constituents such as mono- and sesquiterpenes during the main fermentation is sometimes more significant. Despite their importance for dry-hopped beer flavor, there is limited knowledge of the factors leading to losses, which can be highly significant.

In Publication 3, the evaporation of volatile compounds during fermentation of dry-hopped all-malt wort was investigated. The method of bubbling water columns was used to trap the fermentation gases. A laboratory-scale fermentation unit was used that imitated conditions comparable with a large-scale fermentation vessel. Considerable amounts of β-myrcene were determined in the water of bubbling columns that were evaporated during fermentation, which is related to its hydrophobicity. The fermentation temperature level had a (minor) influence on the amounts evaporated, which is due to the temperature dependency of the compound volatility. The top-fermenting or bottom-fermenting yeast strain used in trials influenced the amounts of flavor-active yeast products evaporated, such as ethyl hexanoate, isoamyl acetate, and styrene. Usually, green beer has approx. hundred million yeast cells per milliliter, which combined, have a high surface area with hydrophobic effect. Due to the presence of yeast in a test medium (non-alcoholic beer), β-myrcene concentration was significantly decreased. At the highest cell count (100 million cells/ml), 98–99 % of the dosed reference compound was removed. In a separate test, a strong binding effect of yeast was determined that led to the conclusion that in a beer-like environment, bound β-myrcene content will not contribute to the beer flavor. Thus, there is an expected impact of the brewing yeast on dry-hopped beer flavor (when dry hopping green beer), especially for flavors such as “resinous” and “green”, which are associated with monoterpene and sesquiterpene hydrocarbons. In addition, the hydrophilic compound linalool was not affected by evaporation or adsorption effects at the test conditions used, confirming its relatively good solubility in wort and beer.

Authors/Authorship contribution:

Haslbeck, K.: Literature search, writing, data creation, study conception and design; **Bub, S.**: Data analysis and interpretation; **Schönberger, C.**: Critical review of trial design and manuscript; **Zarnkow, M.**: Supported study conception and design; **Jacob F.**: Critical content review; **Coelhan, M.**: Supported the creation of research plan, critical content review, supervised the project.

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On the Fate of β-Myrcene during Fermentation – The Role of Stripping and Uptake of Hop Oil Components by Brewer's Yeast in Dry-Hopped Wort and Beer

Hops play a significant role in determining the aroma of beer. The essential oil of hops contains a large number of flavor-active components. Concentrations of essential oil constituents in beer depend on factors such as the time of hop addition in the brewing process and hop amount added. Generally, compound classes such as mono- and sesquiterpenes do not reach the threshold concentrations in the final product, but in dry-hopped beers after main fermentation they often do. Two factors that potentially cause decreased amounts of terpenoids in beer were investigated. In case of the non-polar compound β-myrcene, losses due to releases into the gas phase during standardized laboratory-scale fermentations were studied. Samples of industrially produced all malt wort (11.5 °P) were dry-hopped at pitching with Mosaic hops. Two yeast strains that are widespread in German beer production were used in trials, TUM 68 (*S. cerevisiae*) and TUM 34/70 (*S. pastorianus*). A method for dissolving fermentation gases in bubbling water columns was used. The hops, SPE-water extracts and beer samples were analyzed by several chromatographic systems using two different GC-FID, nanoLC-MS/MS, GC-MS and HS-GC-MS, respectively. Tendency was shown that higher temperatures at primary fermentation cause increased releases of aroma compounds into the gas phase, which was observed on model fermentations in previous studies. The reversible uptake of β-myrcene by yeast cells, identified in separate test series, was determined as being a highly effective factor decreasing amounts in beer systems. In bottled beers 100 million cells/ml led to decreased amounts of about 98–99 %. It was shown that solvent systems with similar properties to beers (5 % and 10 % ethanolic solution) are inadequate for re-dissolving compounds attached to yeasts. The absorbed amount in yeast therefore cannot contribute to the flavor of beer. Incomplete recovered amounts of β-myrcene even in pure ethanol suspensions indicate that there are strong bonds between yeast cells and the odor compound. Linalool, on the other hand, was not affected by the test conditions used.

Descriptors: *S. cerevisiae* and *S. pastorianus*, *Humulus lupus* L., dry hopping, fermentation, beer flavor, β-myrcene and linalool

1 Introduction

In recent years the interest in beers with special and diverse flavors has grown. Many brewers use newly developed raw materials such as flavor hops, more variety in aroma intense yeast strains and apply rediscovered traditional techniques such as dry hopping [1, 2]. Aroma compounds in beer originate from malt, hops (that are partially transformed in process steps such as wort boiling) and arise from the metabolic activity of brewing yeast [3, 4]. Hops play a significant role in determining the aroma of beer and there are a large number of popular beer types with a pleasantly enhanced hop bouquet. Research in the field of hoppy flavor of beer focuses on essential oil as the primary source of hop flavoring. More than 1000 different constituents are assumed in the essential oils [5]. β-Myrcene and linalool in hop essential oil were identified as some of the most potent odorants by applying AEDA to the volatile fraction isolated from a hop cultivar (Spalter Select) [6, 7]. In beer, the concentrations as well as the combinations of key compounds such as linalool ("floral", "fruity") [8] determine the final particular hoppy flavor in beer [3, 9]. Roughly summarized, the type of hop flavor can be distinguished as kettle hop or dry hop flavor. Differences occur due to the time of addition in the brewing process. The kettle hop flavor is formed when boiling wort in the presence of hops. Essential oil constituents such as sesquiterpenes are partly oxygenated and can evoke spicy flavors in beer [10]. Other volatile compounds like monoterpenes are usually reduced to traces [1, 11, 12]. It is assumed that their generally non-polar and very volatile character might lead to adsorption to the trub and evaporation

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with wort steam [12]. Hopping beer after the main fermentation can lead to monoterpene concentrations above threshold values contributing to a particular dry hop beer flavor [1, 13]. β-Myrcene in particular is an important component of the essential oil of hops that is described as "herbaceous", "resinous", "green", "balsamic", "fresh hops" and often found in dry-hopped beers [12, 14]. Losses of monoterpenes such as β-myrcene were noted not only at wort boiling, but also during yeast fermentation, which can be significant [15]. Decreasing contents of linalool were also documented during fermentation, but in much smaller amounts [16]. With regard to their importance for beer aroma there is a high level of interest in obtaining information on factors that may lead to the loss of these pleasantly aromatic essential oil constituents.

In several studies on fermentations of beer worts or wine musts it was shown that volatile compounds are partly transported to the surface of the media by fermentative carbon dioxide and subsequently released into the gas phase [17–19]. Besides, little is known about the fate of compounds produced by yeast or pre-existing odorant compounds and losses through stripping during fermentation which is why the final flavor of the beer is not always uniform [20]. In recent years, methods for the real-time monitoring of stripped aroma compounds during beer fermentation were developed [17, 21]. In 2013 *Haefliger* and *Jeckelmann* determined mass flows with 5 minutes resolution of released gases from yeast metabolism and compounds derived from hops, including monoterpenes, sesquiterpenes and some esters in the headspace of wort during fermentation. The mass flows were determined by gas chromatography mass spectrometry (GC-MS) equipped with an automatic cryotrapping sampling system [18]. In 2014, *Keupp* and *Zardin* observed dynamic changes in the release of acetic acid, ethyl acetate, isobutyl acetate and isoamyl acetate by proton-transfer-reaction mass spectrometry (PTR-MS) directly in the headspace of fermenting wheat beer wort [19]. Real-time monitoring of fermentation gases can provide extensive information on the dynamics of aroma compound release that could contribute to controlling various processing parameters with the objective of creating the final aroma of beer.

However, real-time applications could only be achieved to date in a laboratory-like environment at limited scales of fermentations. It is well known when upscaling brewing batches that differences in aroma profiles will occur and so there is a need to further develop existing systems or to use other methods [22, 23]. In the field of chemical engineering, different kinds of gas sampling methods are used, which allow the subsequent analysis of gas constituents [24, 25]. The bubbling water column is an example of when gases become specifically dissolved in solvents. In this very flexible and robust method, a gas stream is passed through a water column. Gaseous substances present in small bubbles are absorbed by the water [24, 26]. The absorption rate of a dissolved gas in bubbling columns is determined by the density of the water, the gas mass fraction and gas diffusivity [24]. The water of bubbling columns containing compounds that are transferred from fermenting worts can be used for gas chromatographic analysis.

When addressing the issue of loss of hop essential oil constituents during fermentation, many authors believe that adsorption at the surface of hydrophobic yeast cells [4, 27–33] and migration to the foam layer might occur [34]. It is worth mentioning that enzymatic cleavage of glycosidically-bound constituents and biotransformations of monoterpene alcohols such as linalool, geraniol, α-terpineol, citronellol and nerol can affect the amounts of essential oils during fermentation [9, 31, 33, 35]. Other hop constituents such as bitter acids were determined in spent brewer's yeast at reasonable amounts depending on the hopping regime [36]. So far, the effect of brewer's yeast regarding the large hydrophobic surface of yeast cells in fermenting wort and beer [4] on concentrations of odor compounds has been little studied. These considerations are directly connected with a pronounced hydrophobic character of a part of the essential oil constituents [37]. There are large differences between the solubility of a relatively polar component such as linalool and a relatively non-polar component such as β-myrcene in water: 10.1 ± 0.61 mmol/l and 0.22 ± 0.02 mmol/l (measured at 25 °C by *Fichan* and *Larroche*), respectively [38]. It is assumed that this is the primary reason of the differences in the varying levels of different aroma compounds in wort and beer, which is an essential part of the following investigations.

In this study, a brewing trial at standardized fermentations at a 10-l laboratory-scale was conducted. Mosaic hop was added at the pitching stage of all malt wort that was produced on an industrial scale. Fermentations were achieved using the brewing yeast strains TUM 68 (*S. cerevisiae*) and TUM 34/70 (*S. pastorianus*) at low and high temperatures for each strain. Hop samples were analyzed by GC-FID and nanoLC-MS/MS. Volatile compounds in beer samples were analyzed using GC-FID, HS-GC-MS and nanoLC-MS/MS. In this approach bubbling water columns were used between each fermentation vessel and bung apparatus in order to dissolve the fermentation gases in water, then extracted by SPE and analyzed by GC-MS. In separate experiments, the affinity of brewer's yeast for β-myrcene and linalool, respectively, was investigated.

2 Materials and methods

2.1 Hop raw material

Mosaic hop pellets type-90 of crop 2015 (USA) were provided by Barth Haas (84048 Mainburg, Germany). The total essential oil in hops was determined according to standard ASBC methods [39]. The essential oil was used for further gas chromatographic analysis.

2.2 Brewing trial

2.2.1 Dry-hopped pitching wort

Lager beer wort used for the brewing trial (Table 4, see page 164) was produced on an industrial scale (300 hl batch). The wort was moderately kettle-hopped with Perle 16 (65 g/hl). Samples of a batch were taken after whirlpool rest and directly inserted in 10-kg-portions into four fermenting vessels (Cornelius NC) and subsequently cooled down in water baths until they reached pitching temperatures. Wort and yeast samples were prepared for pitching using climate chambers at 8 °C, 15 °C and 22 °C. Immediately prior to pitching, a sterile nylon fiber bag containing 9.6 g Mosaic pellets (Table 1) was added to each of the four fermentation vessels. Hop

Table 1 Dry hopping doses for 1.5 ml hop oil/hl

Variety	α-Acids (% w/w)	Oil Content (ml/100 g)	Dosage (g/kg)
Mosaic	12.3	1.55	0.96

bags attached to stainless steel weights using 15 cm long nylon cords were positioned on the vessel bottom in order to prevent floating to the surface.

2.2.2 Propagation

Yeast was propagated from pure culture provided by Yeast Center of the Research Center Weihenstephan for Brewing and Food Quality (Freising, TU München, Germany). Isolates were inoculated from agar slants into 70 ml of sterile wort medium in a 100-ml-Erlenmeyer flask. The wort was made using an unhopped pilsner barley malt extract (Weyermann GmbH & Co. KG, Bamberg, Germany). The extract was diluted with distilled boiling water to an original gravity of 12.0 °P to guarantee sterile conditions. Incubation in this and the following steps took 96 hours at ambient temperature (20 °C) and pressure. After the first incubation period yeast was transferred to 1 l of sterile wort in a 2.5-l-glass vessel and further incubated. Then the supernatant was decanted and yeasts were transferred to 3.5 l of sterile wort in a 5.0-l-glass vessel. After incubation, yeasts were added to two 5.0-l-glass vessels each containing 3.5 l of sterile wort and incubated. The incubation in two 5-l-vessels was repeated until the desired amount of yeast for trials was reached. Before fermentation, yeasts were softly tempered within 24 hours until they reached pitching temperatures (8 °C, 15 °C, and 22 °C). Yeast cell concentrations (cells/ml) were determined using a cell counter (Nexcelom Bioscience, Lawrence, MA, USA) that was calibrated for the corresponding yeast strains.

2.2.3 Fermentation

Laboratory-scale fermentations were performed using Cornelius NC stainless steel vessels with dimensions of 21.6 cm diameter x 62.9 cm height (18.9 l) and sealed by caps that were equipped with gas ports (Cornelius, Inc., Osseo, MN, USA). Pure cultures of *S. cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70 (Research Center Weihenstephan for Brewing and Food Quality, Freising, TU München, Germany) were used as representative brewer's top- and bottom fermenting strains, respectively. The wort was not oxygenated. Fermentations at different test set-ups were achieved in single-issue approaches. The fermentation was started by adding 30 million cells/ml of propagated yeast TUM 34/70 to both vessels in cooling chambers at 8 °C and 15 °C, respectively and 15 million cells/ml of propagated yeast TUM 68 to both vessels at 15 °C or 22 °C chambers. In order to imitate fermentation in vessels on an industrial scale, a head pressure of 0.5 bar was applied by a bung apparatus simulating liquid heights of 10 m (median hydrostatic pressure) [22]. The temperatures were maintained for at least 10 days of primary fermentation. Primary fermentation was considered complete after the specific gravity remained constant for two consecutive days. Maturation was carried out for three weeks at 0 °C. The beer samples were then filled in 0.5-l-portions with pilot scale bottle filler (Esau-Hueber, Schrobenhausen, Germany) into 0.5-l-brown glass (NRW-) beer bottles under anti-oxidizing conditions. The alcohol content, residual extract and fermentation degree of the beers were determined from filtered (Whatman folded filter paper, diameter: 320 mm, GE Healthcare Europe GmbH, Freiburg, Germany) samples using a DMA 35N (Anton-Paar GmbH, Graz, Austria). In beers, the hop essential oil constituents were analyzed by HS-GC-MS and nanoLC-MS/MS, fermentation by-products were measured by GC-FID.

2.2.4 Bubbling water column

Five bubbling water columns bound in series were connected to the gas line between each fermentation vessel and a bung apparatus. Thus fermentation gases were forced to pass five water columns before escaping via the bung apparatus. Therefore stainless steel containers with dimensions of 10 cm diameter × 36 cm height (2.7 l) were filled completely with (non-carbonated) mineral water of a single batch (ja!, REWE Group) ensuring standardized conditions, slight pH-buffering capacities and non-hazardous handling. The caps of each container were equipped with two gas ports, one of which was connected with a riser pipe. Sealed containers were hermetically connected by gas lines plugged into the ports so that fermentation gases could escape the riser pipe at the bottom of each container and leave the container via the gas port in the cap (Fig. 1). The containers were placed outside of climate chambers at room temperature (20–21 °C) during fermentation in order to ensure equal conditions for the dissolution of the released gases [24]. After fermentation, 1-l-samples of each column were extracted by SPE and subsequently analyzed by GC-MS (see 2.4.3 for details of preparation of SPE extracts and 2.4.4 for GC-MS of SPE extracts).

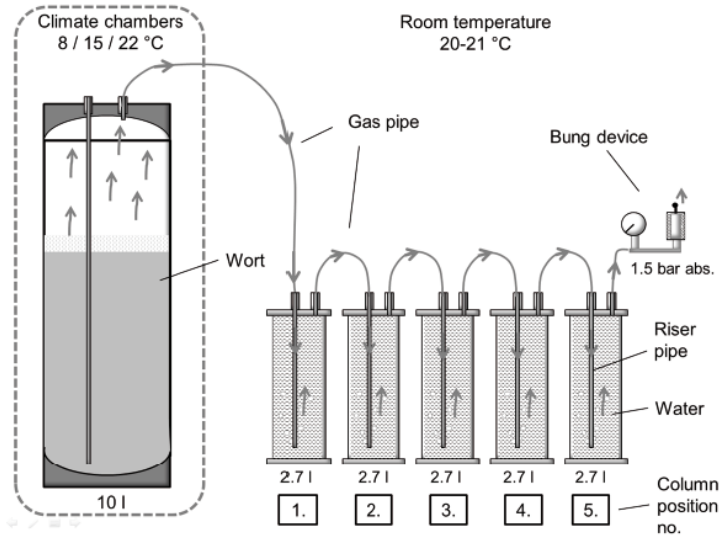

Fig. 1 Experimental set-up for the dissolution of volatiles in beer wort headspace by five bubbling water columns connected in series

2.3 Concentration of aroma compounds in yeast

2.3.1 Recovery of β-myrcene and linalool in beer

A pale filtered non-alcoholic lager beer filled in 0.5-l-brown glass NRW-bottles was used in these test series. The beer was industrially produced from a comparable batch of wort and yeast strain (TUM 34/70) as that utilized in the brewing trial. The experimental set-up, which included contact duration, temperature, slight agitation, cell count and medium, was selected to reflect the main fermentation. At the same time losses by outgassing were avoided. Four different yeast counts of 1, 5, 20 and 100 million cells/g were prepared in bottled beers by adding propagated and washed yeast samples to opened bottles. The method of yeast washing was based on references [36] and [40]. Briefly, 500 g of propagated yeast suspensions adjusted to 100 million cells/g was centrifuged in 600-ml centrifuge tubes at 4000 rpm for 10 minutes using a Megafuge 40R (Thermo Scientific, Waltham, MA, USA). The supernatant was replaced by deionized water and the (centrifuge) tube content subsequently treated using an ARE magnetic stir bar (VELP Scientifica, Usmate Velate, Italy) at medium stirring speed for 10 min in order to suspend the sedimented yeast. The washing procedure for each portion of yeast was repeated four times. Yeast quantities for setting the desired cell concentrations (cells/ml) were determined using a cell counter calibrated for the corresponding yeast strain (Nexcelom Bioscience, Lawrence, MA, USA). 50 µl of β-myrcene (0.7 g/l, tetrahydrofuran solution) or linalool (0.7 g/l ethanol solution) was added to beers to set the concentrations to 70 µg/l, which is within the characteristic range for moderately dry-hopped beers [41]. Aluminum foil was inserted between the bottle mouth and crown cap and subsequently sealed to inhibit migration of β-myrcene into crown cork liner polymers [42]. The prepared beer bottles were then agitated for one week at 75 rpm (20 °C) using VKS-75 Control (Edmund Bühler GmbH, Hechingen, Germany). A reference sample was treated the same way but without yeast addition. The trial was conducted in triplicate. Before gas chromatographic analysis, yeast cells were removed from beer samples by centrifuging the entire bottle contents in 600-ml tubes at 4000 rpm for 10 min using a Megafuge 40R (Thermo Scientific, Waltham, MA, USA).

2.3.2 Recovery of β-myrcene and linalool from yeast

In consecutive steps, propagated amounts of yeasts TUM 68 and TUM 34/70 were washed, then brought into contact with β-myrcene or linalool, washed again and subsequently treated with solvents; finally solvent extracts (SPE) were analyzed by GC-MS.

For contact with aromatics and performing the test in duplicate, 600 ml deionized water in a 1-l-SCHOTT bottle was set to 100 million cells/g using washed yeast and split equally between six 250-ml-Erlenmeyer flasks. Concentrations (cells/ml) of yeast cells were determined using a cell counter calibrated for the corresponding yeast strain (Nexcelom Bioscience, Lawrence, MA, USA). Into three 250-ml-Erlenmeyer flasks containing 100 ml yeast-water suspensions (100 million cells/ml) 70 µg of β-myrcene were added, which is within a characteristic range of that particular compound for strongly dry-hopped beers [1]. This was also done for linalool. For the amount of 70 µg, 100 µl of the β-myrcene pure substance solution (0.7 g/l, tetrahydrofuran-ethanol (1:1 [v/v]) solution) or 100 µl of the linalool pure substance solution (0.7 g/l, ethanol solution) was used. Erlenmeyer flasks were sealed with glass stoppers and agitated at 75 rpm for 16 hours at 20 °C using VKS-75 Control (Edmund Bühler GmbH, Hechingen, Germany). Control samples were treated equally until this step without yeast contents and subsequently extracted (SPE) and analyzed (GC-MS). After agitation step, entire quantities of yeast-water suspensions in Erlenmeyer flasks were washed four times as described before to remove any

Table 2 Chromatography system applications and settings

Sample type (targeted comp.)	Hop essential oil	Beer (hop essential oil constituents)	Beer (fermentation by-products)	Water SPE-extracts (full scan volatile comp.)
System	GC-FID	HS-GC-MS	GC-FID	GC-MS
Manufacturer	Perkin Elmer	Shimadzu	Perkin Elmer	Thermo Scientific
Sampler	(integrated)	HS-20 10-ml vial, 5 ml sample vol.	Turbo Matrix 40 (HS) 20-ml vial, 2 ml sample vol.	AS 3000
GC	Clarus 580	GC-2010 Plus	Clarus 580	Trace GC Ultra
MS transfer line temp., ion source temp.	–	GCMS-QP2010 Ultra 250°C, 200°C	–	DSQ II 230 °C, 230 °C
Column	ZB-WAX	ZB-WAX	INNOWAX	TR-5MS
Film thickness [µm]	0.5	0.25	0.5	0.25
Length [m]	60	30	60	30
[mm]	0.25	0.25	0.32	0,25
Injection volume	2.0 µl	1 ml	pressure controlled	1.0 µl
Carrier gas	helium 5.0 ECD-quality	helium 5.0 ECD-quality	helium 5.0 ECD-quality	helium 5.0 ECD-quality
Split	20 ml/min	1:5	20 ml/min	1:10
Internal standard	*p*-cymene	pulegone	*p*-cymene	pulegone
Software	TotalChrom	LabSolutions	TotalChrom	Thermo Electron

residual amounts of flavor compounds. Each of the sedimented yeast portions was subsequently suspended with 100 ml ethanol-water solutions in 250-ml-Erlenmeyer flasks containing 5 %, 10 % and 100 % [v/v] ethanol, respectively. Erlenmeyer flasks were sealed with glass stoppers and agitated at 75 rpm for 3 days at 20 °C. Then, suspensions were centrifuged at 4000 rpm for 10 min at 20 °C. Supernatants were adjusted to solutions at 5 % [v/v] ethanol contents with distilled water in 2-l-SCHOTT bottles setting samples at similar properties to calibration medium of SPE method. The entire sample quantity was subsequently extracted by SPE. The extracts were used for gas chromatographic analysis.

2.4 Analytical methods

In this study five different chromatography systems were used to analyze volatiles in essential oil, beer, and water samples. Table 2 shows the system applications. NanoLC-MS/MS of thiols in hop and beer samples was performed by laboratory Nyseos, sample processing and system application according to *Roland* and *Viel* in 2016 [43].

2.4.1 Chromatographic analysis of essential oil

The essential oil was analyzed using a gas chromatograph connected with a FID. Separation was achieved using in a ZB-WAX. The oven was programmed at a rate of 5 °C/min from 45 °C (11 min isotherm) to 210 °C, increased at 20 °C/min to 240 °C (8 min hold) and at 10 °C/min to 260 °C (5 min hold).

2.4.2 Chromatographic analysis of beer

Essential oil constituents in beer samples were quantified using a gas chromatograph that was directly connected to a mass spectrometer (Table 2). The system was equipped with a headspace sampler loop system. Samples were equilibrated for 30 min at 80 °C. The temperature program of the oven was at a rate of 4 °C/min from 50 °C to 130 °C, increased at 8 °C/min to 180 °C and at 15 °C/min to 240 °C. Samples were assessed in SIM mode. Fermentation by-products were analyzed by GC-FID equipped with a headspace sampler. Vials containing beer samples were equilibrated at 60 °C for 25 min. 1 min after injection at 50 °C the temperature was increased at 7 °C/min to 85 °C. After 1 min hold 190 °C was reached at 25 °C/min (4 min hold).

2.4.3 Preparation of SPE extracts

SPE was performed using 6-ml HR-P-cartridges filled with 500 mg polystyrene-divinylbenzene (Chromabond, Macherey Nagel, Düren, Germany). A vacuum port with gauge was used to control the vacuum applied to the chamber at 0.8 bar abs. using a vacuum pump to accelerate flow rates. Cartridges were pretreated successively with 5 ml dichloromethane, 5 ml methanol and 5 ml deionized water. Then samples of bubbling water columns (1 l) or yeast extracts (0.1–2 l) were increased by 50 µl of internal standard pulegone (400 mg/l, ethanol solution) in 2-l-SCHOTT bottles and subsequently loaded on pretreated cartridges at flow rates of approx. 15 ml/min. Loaded cartridges were washed with 5 ml 2-% [v/v] methanol solution and eluted twice with 4 ml dichloromethane. The eluent was collected in 10-ml glass tubes equipped with a length gauge. The eluent was reduced down to a volume of about 200 µl using a fine nitrogen stream and stored at –20 °C until the GC-MS analysis.

Table 3 **Contents of 35 selected aroma compounds in Mosaic essential oil in µg/g pellet. 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA), 4-methyl-4-mercaptopentan-2-one (4MMP)**

	Mosaic
Linalool	85 ± 2.3
Geraniol	61 ± 0.1
β-Citronellol	129 ± 0.4
Menthol	5 ± 1.7
1-Octen-3-ol	16 ± 1.1
α-Pinene	14 ± 0.4
β-Pinene	56 ± 3.9
β-Myrcene	4,288 ± 67.3
α-Humulene	97 ± 7.2
Trans-β-Farnesene	5 ± 1.8
Trans-Caryophyllene	188 ± 1.4
Limonene	101 ± 4.6
γ-Terpinene	28 ± 1.2
Heptanol	3 ± 0.2
2-Octanol	23 ± 0.8
Isobutyl isobutyrate	41 ± 0.9
Geranyl acetate	12 ± 0.6
Cis-4-methyl-decenoate	1,120 ± 8.0
Methyl decanoate	3 ± 0.1
C11-Methyl ester	85 ± 22.1
Methyl hexanoate	225 ± 6.0
Neryl acetate	9 ± 1.2
β-Selinene	36 ± 10.0
Methyl nonanoate	5 ± 0.2
Methyl octanoate	11 ± 0.5
Citronellal	15 ± 0.8
β-Damascenone	14 ± 2.5
2-Decanone	61 ± 7.8
2-Nonanone	68 ± 6.0
2-Undecanone	43 ± 19.3
Carvone	67 ± 1.0
Dimethyl disulfide	3 ± 0.8
4MMP (ng/g)	22
3MH (ng/g)	54
3MHA (ng/g)	6
Sum	**6,917**

2.4.4 GC-MS of SPE extracts

A gas chromatograph/mass spectrometer system was equipped with an automatic liquid injection system. The temperature of the GC oven was increased at a rate of 8 °C/min from 50 °C (7 min isotherm) to 150 °C, 20 °C/min to 280 °C (5 min isotherm) and 10 °C/min to 330 °C. The samples were measured in full scan

mode at the mass range 50–250 amu.

3 Results and discussion

3.1 Hop analysis

The aroma hop cultivar Mosaic is the daughter of YCR 14 Simcoe (multi-purpose hop variety) and a Nugget (high-alpha variety) derived male. This family tree explains the relatively high contents of α-acids for a flavor variety. Table 3 lists the analysis results of 35 substances in Mosaic pellets. Mosaic, a cultivar released in 2012, shows some specific characteristics such as having polyfunctional thiols 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA) and 4-methyl-4-mercaptopentan-2-one (4MMP). These three thiols have been linked to several hop cultivars such as Nelson Sauvin and Cascade by exhibiting typical blackcurrant bud and grapefruit notes detected by GC-olfactometry [44]. β-Myrcene was determined in hop oil at a level of 62 %. That is slightly above a common value with regard to variety data sheet (47-53 %) [45]. The linalool content, which is often used as one of the primary markers for hop aroma in beer [8], was measured at a typical share of essential oil such as 1.2 % [1]. Esters such as cis-4-methyl-decenoate and methyl hexanoate were determined at relatively high contents [41], 16.2 and 3.3 %, respectively, possibly contributing to the fruity character of the pelletized hop samples [1].

3.2 Brewing trial

3.2.1 Wort and beer analysis

The wort and the brewed beers were analyzed comprehensively (Table 4). Similar levels of residual extracts (real), alcohol contents and final fermentation degrees (real) indicate to good comparability of four brews.

Table 5 shows the values of 40 analyzed aroma components in the pitching wort before dry hopping and brewed beers. These include 30 hop-derived aroma compounds. Increased amounts in beers are due to the dry hopping of the pitching wort that was moderately kettle hopped. The threshold value of linalool was exceeded in all beers (33.4 ± 0.8 – 36.4 ± 0.9 µg/l). In the case of geraniol, threshold value was also achieved, though there was a great difference between yeast strains. Contents in TUM 68-beers were recorded at 72.8 ± 0.2 µg/l (22 °C) and 69.2 ± 3.3 µg/l (15 °C) whereas levels in TUM 34/70-beers were determined at 54.3 ± 0.8 µg/l (15 °C) and 30.9 ± 0.9 µg/l (8 °C). Deviations might be caused by the cleavage of geranyl glycoside and the release of the corresponding geraniol [9, 35]. Furthermore, differences in degradations of geraniol by biotransformations might have occurred [9, 31, 33]. Mono- and sesquiterpenes such as α- and β-pinene, β-caryophyllene, α-humulene, β-famesene were generally determined at trace amounts and below threshold levels, among these the highest contents of β-myrcene were determined at 15.9 ± 2.6 µg/l in TUM 68-beer (22 °C) and 6.9 ± 0.8 µg/l in TUM 34/70-beer (8 °C). Regarding thiols, in case of 4MMP (blackcurrant, muscat-like, fruity) and 3MH (fruity, catty, thiol-like) threshold levels at 10–50 and 55 ng/l [46] were achieved in all beers (30–40 ng, 350–450 ng).

Ten important flavor compounds produced by yeast metabolism were analyzed by GC-FID (Table 5). A variation in the production of fermentation by-products for both yeast strains was determined. Top-fermenting TUM 68 showed higher amounts of alcohols such as i-butanol and amyl alcohols and esters such as isoamyl acetate ("fruity", "banana";), a key-compound for top-fermented wheat beers [20], compared with bottom-fermented beers, +42 mg/l, +10 mg/l, +0.9 mg/l (higher temperature attempts), respectively.

3.2.2 Fermentation gas analysis

Figure 2 shows the levels of hop-derived compound β-myrcene and the three products of yeast metabolism, isoamyl acetate, ethyl hexanoate and styrene dissolved in the water of bubbling columns after the main fermentation. This points to stripping of the compounds mentioned above during standardized conditions which were inspired by large-scale beer fermentations [22]. Releases of aroma compounds have been determined before by several authors using real-time monitoring methods [17–19]. β-Myrcene was measured in column position number 1 (Fig. 1) at levels of about 256–280 µg/l. In the subsequent column positions 2 to 5, 228–268 µg/l, 223–276 µg/l, 207–265 µg/l, respectively, decreasing quantities were detected. This was attributed to the depletion of β-myrcene from the fermentation gases. The highest dissolved amounts in columns were determined at 22 °C fermentation temperature and the lowest at 8 °C regardless of column position. In this study, tendencies towards higher released amounts at primary fermentation are probably attributed to the increased volatility of aroma compounds as proposed by *Schneiderbanger* and *Hutzler* [20]. They determined increased releases of aroma compounds into the gas phase at higher temperatures from water systems when simulating beer fermentations [20]. Considering the fact that ethyl hexanoate was only detected in column position 1 and isoamylacetate only in positions 1–3, it becomes clear that the test set-up in its present form is highly suitable for dissolving hydrophilic aroma compounds [38, 52] in bubbling water columns. Nonetheless, no linalool, which is equally highly water soluble, could be detected

Table 4 General analysis data of the wort and four beers

	Wort	Beer			
		TUM 68		TUM 34/70	
		Ferm. 22 °C	Ferm. 15 °C	Ferm. 15 °C	Ferm. 8 °C
Extract [°P] (residual)	11.47	3.49	3.55	3.61	3.52
Alcohol [% vol/vol]	0.00	5.66	5.66	5.75	5.77
Final fermentation degree [%]	–	72.2	71.8	71.8	72.3

Table 5 Contents of 40 selected aroma compounds in pitching wort (before dry hopping) and four beers

		Wort	TUM 68		TUM 34/70		Threshold*
			Ferm. 22 °C	Ferm. 15 °C	Ferm. 15 °C	Ferm. 8 °C	
Linalool	[µg/l]	3.6 ± 0.33	33.4 ± 0.80	36.1 ± 1.15	36.4 ± 0.87	35.1 ± 1.01	5, 27, 80
Geraniol	[µg/l]	2.7 ± 0.41	72.8 ± 0.17	69.2 ± 3.32	54.3 ± 0.82	30.9 ± 0.92	36
Citronellol	[µg/l]	nd	1.2 ± 0.10	1.8 ± 0.29	1.2 ± 0.05	1.7 ± 0.03	5
α-Terpineol	[µg/l]	nd	0.6 ± 0.23	0.2 ± 0.07	2.8 ± 0.15	0.2 ± 0.07	300, 2000
Nerol	[µg/l]	nd	7.7 ± 0.25	7.6 ± 0.44	6.9 ± 0.23	5.2 ± 0.10	50, 1200
1-Octen-3-ol	[µg/l]	nd	nd	nd	2.4 ± 0.04	1.9 ± 0.04	10**
α-Pinene	[µg/l]	nd	nd	nd	nd	nd	2.5-62
β-Pinene	[µg/l]	nd	nd	nd	nd	nd	140
β-Myrcene	[µg/l]	0.3 ± 0.12	12.1 ± 1.12	15.9 ± 2.58	7.4 ± 0.81	6.9 ± 0.85	30, 1000
α-Humulene	[µg/l]	nd	0.6 ± 0.10	1.2 ± 0.41	0.4 ± 0.04	0.4 ± 0.05	800
β-Famesene	[µg/l]	nd	0.5 ± 0.18	nd	nd	nd	2000
β-Caryophyllene	[µg/l]	nd	0.2 ± 0.06	0.3 ± 0.12	nd	nd	450
1-Heptanol	[µg/l]	5.0 ± 0.82	6.2 ± 0.24	5.7 ± 0.28	4.9 ± 0.28	4.5 ± 0.22	1000
Isobutyl isobutyrate	[µg/l]	0.5 ± 0.11	6.6 ± 0.37	8.0 ± 0.25	8.3 ± 0.27	8.5 ± 0.30	n/a
Methyl hexanoate	[µg/l]	0.5 ± 0.20	24.7 ± 0.92	23.7 ± 15.82	33.0 ± 1.50	37.1 ± 1.25	n/a
Methyl heptanoate	[µg/l]	nd	nd	nd	nd	nd	n/a
Methyl octanoate	[µg/l]	nd	0.7 ± 0.11	0.7 ± 0.10	0.8 ± 0.09	0.7 ± 0.05	n/a
Methyl nonatoate	[µg/l]	nd	0.2 ± 0.15	nd	nd	nd	n/a
Methyl decanoate	[µg/l]	nd	0.4 ± 0.15	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	n/a
4-Methyl-decenoate	[µg/l]	5.5 ± 0.90	0.4 ± 0.01	0.4 ± 0.04	0.4 ± 0.05	0.4 ± 0.02	n/a
Geranyl acetate	[µg/l]	nd	2.3 ± 0.07	2.1 ± 0.21	2.2 ± 0.04	1.8 ± 0.03	9, 460
Citronellal	[µg/l]	nd	0.4 ± 0.02	0.4 ± 0.01	0.4 ± 0.02	0.4 ± 0.01	n/a
2-Undecanone	[µg/l]	nd	2.1 ± 0.17	1.4 ± 0.47	2.6 ± 0.11	2.1 ± 0.03	400
2-Dodecanone	[µg/l]	nd	0.6 ± 0.05	0.4 ± 0.01	0.5 ± 0.01	0.4 ± 0.03	n/a
Neryl acetate	[µg/l]	nd	1.1 ± 0.22	0.9 ± 0.11	0.9 ± 0.10	0.9 ± 0.06	n/a
2-Nonanone	[µg/l]	nd	2.4 ± 0.09	2.5 ± 0.09	3.1 ± 0.09	2.7 ± 0.08	200
2-Tridecanone	[µg/l]	nd	0.8 ± 0.05	0.7 ± 0.02	0.7 ± 0.01	0.7 ± 0.01	100
4-methyl-4-mercaptopentan-2-one (4MMP)	[ng/l]	nd	31	37	40	40	10, 50
3-mercaptohexan-1-ol (3MH)	[ng/l]	29	399	398	475	359	55
3-mercaptohexyl acetate (3MHA)	[ng/l]	nd	4	8	10	4	9
Acetaldehyde	[mg/l]	0.88 ± 0.078	4.51 ± 0.003	2.09 ± 0.007	2.90 ± 0.170	3.21 ± 0.127	5, 25
Ethyl formiate	[mg/l]	0.79 ± 0.021	0.77 ± 0.057	0.75 ± 0.049	0.59 ± 0.014	0.81 ± 0.007	150
Ethyl acetate	[mg/l]	nd	32.84 ± 0.09	31.56 ± 0.849	34.14 ± 1.450	27.27 ± 0.092	30
Ethyl propionate	[mg/l]	nd	0.23 ± 0.004	0.21 ± 0.007	0.22 ± 0.003	0.24 ± 0.002	150
n-Propanol	[mg/l]	0.14 ± 0.004	19.65 ± 0.580	17.30 ± 0.417	13.16 ± 0.049	12.34 ± 0.191	2, 50
Ethyl butanoate	[mg/l]	nd	0.10 ± 0.007	0.12 ± 0.007	0.09 ± 0.001	0.12 ± 0.007	0.3
i-Butanol	[mg/l]	0.23 ± 0.003	51.89 ± 1.280	52.59 ± 1.365	10.93 ± 6.859	11.29 ± 0.156	200
Isoamyl acetate	[mg/l]	nd	1.77 ± 0.021	2.23 ± 0.035	1.40 ± 0.078	1.30 ± 0.021	1.6
Amyl alcohol	[mg/l]	0.48 ± 0.032	75.24 ± 0.792	78.71 ± 0.877	67.64 ± 0.940	59.92 ± 0.361	70
Ethyl hexanoate	[mg/l]	0.04 ± 0.007	0.10 ± 0.003	0.13 ± 0.004	0.13 ± 0.007	0.12 ± 0.004	0.2

* Odor thresholds in beer found in the literature [3, 16, 46-51]; n/a if not available; ** determined in ethanolic solution

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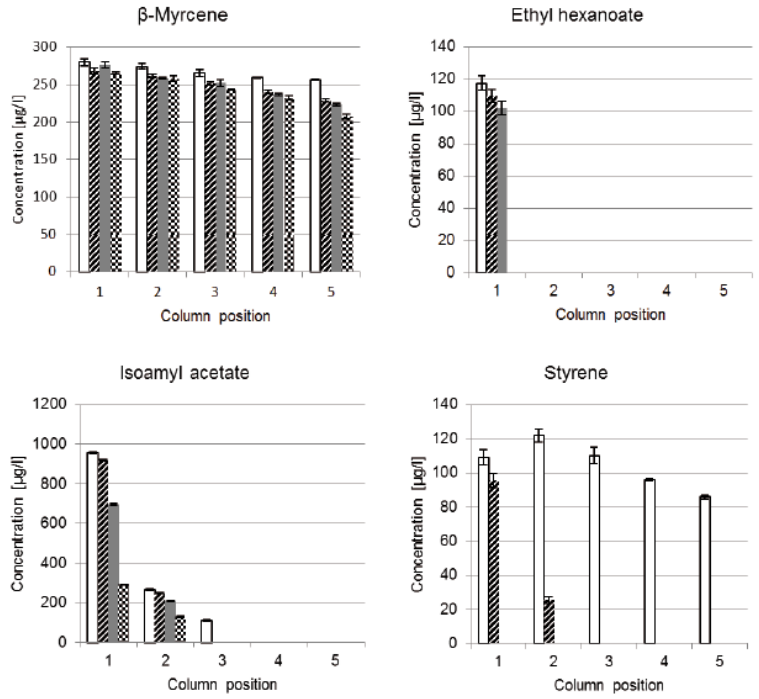

Fig. 2 Contents of four compounds in bubbling water columns originating from the headspace of wort during fermentation in µg/l; ☐ TUM 68, 22 °C; ▨ TUM 68, 15 °C; ■ TUM 34/70, 15 °C; ▩ TUM 34/70, 8 °C

(Table 6), which is attributed to the excellent dissolution of this substance in young beer and was not released.

The experimental set-up can be modified for further investigation into non-polar compounds using a higher number of bubbling columns or other solvents with higher capacities to dissolve compounds like β-myrcene since columns at position 5 contained β-myrcene in the range 207–265 µg/l. It is most likely that the compound was still present in outgoing gases although no solubility limits were reached in the water of bubbling columns for any of the analyzed compounds (Table 6) [38, 52]. However, using the method of five bubbling water columns in its present form, tendencies towards differences in releases of β-myrcene between fermentation temperatures were observed. Furthermore, this was achieved in simultaneous fermentation approaches with different yeast strains at uniform yeast vitalities, yeast viabilities and wort characteristics, which is the basis of acknowledged methods for characterization of fermentations in brewing research [2, 22].

3.3 Uptake of aroma compounds by yeast

3.3.1 Recovery of β-myrcene and linalool in beer

Figure 3 shows concentrations of β-myrcene and linalool in defined media after contact with yeast strains TUM 68 and TUM 34/70 under particular storage conditions to imitate conditions during main fermentations. In 2003, *King* and *Dickinson* assumed that rising alcohol contents had an effect on the concentrations of terpenoids during fermentation, enabling more of the terpenoids to dissolve [31]. With this background, a non-alcoholic beer was used in this trial as contact media and the impact of different alcohol contents was tested in separate test series (3.3.2). Amounts of β-myrcene in the beer were decreased depending on cell concentrations. At the highest counts (100 million cells/g) only traces like 0.5 ± 0.2 µg/l (TUM 68) and 1.0 ± 0.2 µg/l (TUM 34/70) remained in beers, corresponding to decreases of 99.0 % (TUM 68) and 98.0 % (TUM 34/70) compared with contents in control tests that were not increased by yeasts (47.9 ± 2.5 µg/l). It is assumed that the non-polar substance β-myrcene was attached to the non-polar surface of the yeast cells [4, 31], which were separated from the samples by centrifugation. Linalool amounts in beers were not noticeably affected by the presence of yeast and were determined at comparable concentrations to the control samples. Linalool is relatively soluble in hydrophilic solutions like beer [38] and therefore not effectively influenced by non-polar particles such as yeast cells".

3.3.2 Recovery of β-myrcene and linalool from yeast cells

Table 7 shows amounts of β-myrcene recovered from yeasts cells that were previously in contact with a defined quantity of β-myrcene at yeast counts of about 100 million cells/ml. Recovery (%) was calculated using determined amounts in solvents such as 8.2 µg (TUM 68) and 8.1 µg (TUM 34/70) and quantified amounts in control samples at 46.0 µg.

Using the relatively hydrophobic solvent in this test series pure ethanol compared to aqueous solutions, 17.2 % from yeasts TUM 68 and 16.8 % from TUM 34/70 were recovered of the spent β-myrcene. At ethanol contents of 5 % and 10 %, β-myrcene was not recovered from yeasts. This is consistent with the results of a previous study, in which 50 % ethanol solution failed to dissolve a measurable amount of terpenoids possibly concentrated in yeast pellets [31].

Table 6 Solubility limits [38, 52] and maximum recovery of released aroma compounds in water of bubbling columns in mg/l; nd = not detected

	Solubility limit in water	Maximum contents determined
Linalool	1556	nd
β-Myrcene	4.0–30.0	0.280
Ethyl hexanoate	630–650	0.117
Isoamyl acetate	2000	0.956
Styrene	160-300	0.122

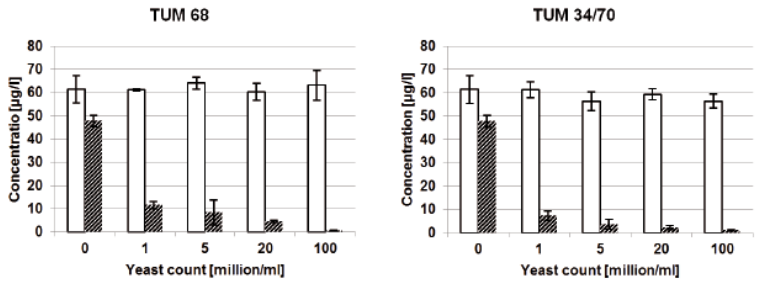

Fig. 3 Contents of □ linalool and ▨ β-myrcene [µg/l] in lager beer after contact with different yeast counts TUM 68 (left) and TUM 34/70 (right)

Table 7 Recovery of β-myrcene from yeast cells in µg and %

		Solvent		
		5 % ethanol (aqueous solution)	10 % ethanol (aqueous solution)	100 % ethanol
TUM 68	β-myrcene [µg]	nd	nd	8.2
	Recovery [%]	nd	nd	17.2
TUM 34/70	β-myrcene [µg]	nd	nd	8.1
	Recovery [%]	nd	nd	16.8

It is probable that pure ethanol was still unsuitable to completely recover β-myrcene from yeast when comparing clearly higher losses of β-myrcene about 99.0 % (TUM 68) respectively 98.0 % (TUM 34/70) in beer-yeast suspensions (3.3.1). We assume that unrecovered amounts of β-myrcene (about approx. 80 %) are still concentrated in yeast. Considering both trials regarding concentrations of aroma compounds in brewer's yeast it is concluded that solvents with similar polarity to beer systems (5–10 % [v/v] ethanol) are insufficient to re-dissolve compounds attached to yeasts such as β-myrcene. Therefore, the uptaken amounts are unable to contribute to the aroma of beer. Linalool was used at the same contents as β-myrcene, but was not recovered. This confirms good solubility of linalool in hydrophilic solvents such as beer and no indication that it is uptaken by yeast cells. The present method could be adapted especially for the analysis of non-polar flavorings by using solvents such as hexane or dichloromethane.

4 Conclusion/Summary

Standardized fermentations of dry-hopped worts and two separate test series showed very large losses of β-myrcene during beer fermentation and two principal causes were identified. With the help of the bubbling water column used for these brewing tests, releases of aroma compounds into the gas phase were confirmed [17–19]. In addition, higher fermentation temperatures resulted in a tendency to increase the release of flavor compounds as identified in studies on model fermentations [20]. It was shown that the method used is very suitable for determining released volatile compounds from the headspace during fermentations; nonetheless no linalool could be detected, which is attributed to the excellent dissolution of this substance in beer. Using water as a solvent in bubbling columns proved to be unsuitable to determine absolute stripped-off amounts of non-polar compounds such as β-myrcene. However, there were great advantages in the flexibility and robustness of the method, although the experimental series was carried out in a single-issue experiment. In a separate test series, the uptake of β-myrcene by yeast cells was determined as it was assumed by several authors [4, 27-33]. In a test set-up that prevented evaporation, 99.0 % (TUM 68) and 98.0 % (TUM 34/70) of β-myrcene was absorbed by yeast at cell counts (100 million cells/ml) occurring during fermentations. Furthermore, it is highly probable that beer is an inappropriate solvent for dissolving β-myrcene uptaken by yeast with the consequence that these quantities do not contribute to the flavor of beer. The level of linalool, on the other hand, could not be detected as being affected by yeast in these experiments. These results can help to shape the flavor of strongly kettle- or dry-hopped beers in a more targeted way, especially for hydrophobic flavor compounds such as monoterpenes.

5 Literature

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Publication 4 Page 68 - 77	**2.5 Investigations into the Transfer Rate of Volatile Compounds in Dry Hopping using an Octanol-Water Partition Coefficient Model**

The knowledge about theoretical principles of dry hopping is minimal. Due to its increasing popularity, there is demand for controlling the extraction process in a more targeted way. Therefore, a better understanding of the basic reactions during dry hopping is needed.

In Publication 4, factors influencing the volatile profile of beer during the dry hopping process were examined. Non-alcoholic beer (0.1 % v/v EtOH) that was previously set to 5.0 and 8.1 % v/v EtOH was used for dry hopping with the hop variety Tettnanger. Higher basic beer ethanol contents led to higher amounts of volatile hop compounds extracted, especially of terpenes. Higher extraction temperatures, 20 or 4 °C compared with 1 °C, had a similar effect on dry-hopped beer. The increase in the dosage of pellets, 7.3 g/l compared with 1.5 g/l, led to a higher proportion of terpenoids in beer, although not all analyzed compounds were uniformly increased. The varietal impact on the transfer rate of volatiles was confirmed, although only three varieties (Cascade, Hallertau Blanc, Eureka!) were compared. Despite improved extraction conditions in this study, the analyzed terpenes and C_{11}-ester were transferred at relatively low rates during dry hopping; a maximum 13 % (α-pinene) was reached in trials. The alcohols and C_8-esters (hydrophilic) were transferred at rates above 23 %. Levels above 100 % (max.: 1-octen-3-ol at 411 %) are attributed to β-glycosidic bound compounds released during dry hopping.

The octanol-water partition coefficient (K_{OW}) of a hop oil constituent was determined as the key parameter for the transfer rate and the resulting concentration in dry-hopped beer. Process parameters were only a secondary influence on the volatile profile of dry-hopped test beers. The log K_{OW} is the ratio of the level of a compound in a mixture of the immiscible phases water and octanol at equilibrium. It is, therefore, a measure of the hydrophobicity of a compound and is used to comprehensively assess the thermodynamics of partitioning and the environmental behavior of chemicals.

Authors/Authorship contribution:

Haslbeck, K.: Literature search, writing, data creation, study conception and design; **Minkenberg, D.**: Data acquisition, processing and interpretation; **Coelhan, M.**: Project idea, critical review and revision of manuscript, supervised the project.

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Investigations into the Transfer Rate of Volatile Compounds in Dry Hopping Using an Octanol-Water Partition Coefficient Model

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ABSTRACT

Dry hopping is a powerful practice for imparting a multitude of flavors into beer. In this study, the influence of ethanol content, temperature, dosage, and hop variety on the transfer of essential oil during dry hopping was examined on a laboratory scale. The dry hopping was performed with nonalcoholic beer and beer containing 5.0 and 8.1% ethanol at 1 and 20°C using the hop varieties Tettnanger, Cascade, Hallertau Blanc, and Eureka. The results showed that the basic beer, hop variety, and dry hopping regime influence the composition of hop essential oil constituents in dry-hopped beer. The increase of the basic beer ethanol content, and especially the rise in temperature, led to a significant increase in the proportion of monoterpenes such as β-myrcene among hop volatiles in dry-hopped beers. Increasing hop dosage led to higher proportions of alcoholic compounds (linalool). Furthermore, the transfer rates of particular volatile hop-derived substances correlated with their octanol-water partition coefficients (log K_{OW}), which is a measure of the hydrophobicity of a compound, regardless of tested factors in dry hopping. Therefore, it is proposed that the log K_{OW} could be a useful model for the prediction of transfer rates of hop oil flavor components in dry hopping. However, the transfer rates of the alcohols linalool, geraniol, α-terpineol, and 1-octen-3-ol were higher than the expected levels from the log K_{OW} values. These compounds are reported present in bound form in hops and released during dry hopping.

KEYWORDS

Beer; dry hopping; gas chromatography; *Humulus lupus* L.; octanol-water partition coefficient; transfer rate

Introduction

The contribution of essential oil components to a hoppy flavor and the mechanism of varietal aroma formation has been the subject of several studies.[1–3] From a chemical perspective, components of hop essential oils can be classified into three main groups: hydrocarbons, oxygenated compounds, and sulfurous compounds.[4] The compound β-myrcene, which belongs to the group of monoterpene hydrocarbons, usually forms the largest proportion in hop essential oils regardless of variety.[5] In addition, α-humulene, β-caryophyllene, and, in some hop varieties, β-farnesene are other common main constituents of the hydrocarbon group. The class of oxygenated compounds of the hop oil (approx. 30%) consists of a complex mixture of alcohols, esters, and ketones.[6] Further oxygenated terpenoids include acids, aldehydes, and epoxides.[6] Generally, sulfur-containing substances form a small proportion of the essential oil of hops; however, they can influence the flavor significantly.[7] The dry-hopping technique is associated with a pleasant and generally distinctive raw hop flavor in beer. For this purpose, hop is added in the cold process area of the brewery.[5] A common practice when dry hopping beer is to add hops to lagering tanks for several days.[8] The late addition in the brewing process minimizes the evaporation of flavorings.[9,10] Several researchers have identified factors that influence the levels of essential oils in dry-hopped beers such as hop variety, hop product (cones, type-90 and type-45 pellets), dosing technique, and essential oil composition.[11–15] It was determined that the transfer rate was reduced at increased dosages, especially for β-myrcene.[8,16] The temperature of dry hopping seems to have an accelerating role on the flavor extraction, but has minimal effect on the final concentrations of compounds such as linalool and β-myrcene.[16,17] Pellets dosed in loose form can increase the extraction efficiency of linalool by almost 50% compared with pellets packed in a finely woven net.[17] It was shown that the rate of essential oil extraction is not significantly influenced by the properties of hop pellets and appears to be finished rapidly within a few hours.[18]

There are indications that the basic beer can influence the essential oil amount extracted in dry-hopped beers.[19,20] Furthermore, in addition to extracting compounds in beer systems, hop oil constituents can biotransform. The hydrolytic cleavage of β-glycosides (aroma precursor substances) by 1,4-β-glucosidase activity can increase the concentrations of specific terpenoids.[21,22] Different studies [12,14] have shown similar levels for transfer rates of amounts of essential oil constituents depending on compounds such as linalool (transfer rate: 63–111%) and β-myrcene (transfer rate: 0.2–2.0%) during dry hopping. This point of view was the focus of this study, that is, to highlight the extract-solvent relationship based on a better understanding of the mechanisms involved in dry hopping. In this study, several factors that were selected based on literature data[8,14,16,23] such as ethanol content, extraction temperature,

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dosage, and hop variety were examined in terms of their effect on the transfer rates of essential oil components during dry hopping. To the best of our knowledge, there is no report interpreting different transfer rates as a result of differing log octanol-water partition coefficient (log K_{OW}) values. The K_{OW} is a dimensionless distribution coefficient that indicates the ratio of the concentrations of a chemical in a two-phase system of n-octanol and water.[24] Therefore, K_{OW} serves as a measure of the relationship between lipophilicity (lipid solubility) and hydrophilicity (water solubility) of a substance. Thus, the study is concerned with introducing the K_{OW} as a model for the extraction of hop essential oil constituents in dry hopping.

Experimental

Hop samples

Type-90 hop pellets of the varieties Tettnanger TE (Germany), Cascade CA (U.S.A.), Hallertau Blanc HB (Germany), and Eureka EU (U.S.A.) of crop 2015 kindly provided by HHV (Hallertauer Hopfenveredelungsgesellschaft mbH, Mainburg, Germany) were used for the trials. The essential oil and α acid contents of hops were determined according to standard ASBC methods.[25] A mean hop oil density of 0.82 g/mL was used for calculations.[26]

Beer samples for dry hopping

A nonalcoholic beer (bottom-fermented, 11.4°P lager beer dealcoholized to 0.1% (v/v), packaged in 0.5 L bottles) was used for the dry hopping trials. In addition, to test the effect of ethanol, 24.5 and 40.0 mL of 0.5 L bottled nonalcoholic beer was exchanged for pure ethanol (Analytical Standard, Sigma-Aldrich, Steinheim, Germany) to achieve a 5.0 and 8.1% (v/v) ethanol content, respectively. A pale "doppelbock" beer [8.1% (v/v) ethanol, bottom-fermented, kettle- and late-hopped with Hallertau Perle and Opal] of a single batch was used for further dry-hopping trials. The beer was brewed, filtered, and casked on an industrial scale. Part of the "doppelbock" was diluted to 5.0% (v/v) ethanol content in casks with carbonized (5.0 g/L CO_2) pure water (mini-UP Plus, Berrytec GmbH, Munich, Germany). Both beer samples were bottled in 0.5-L-portions using a pilot scale bottle filler (Esau-Hueber, Schrobenhausen, Germany) into 0.5-L-brown glass beer bottles type NRW (bottle height: 26.0 cm; diameter at widest point: 6.8 cm) prior to dry hopping under oxygen-free conditions.

Single-variety dry hopping on a laboratory scale

The dosage for dry hopping was based on the essential oil content of hops. All trials were performed in triplicate. Calculated quantities of hop pellets were added directly into beer bottles without a hop bag to enable distribution and expansion of the pellets in the media. Subsequently, ingressed air in the bottle headspace gas was mitigated by bringing the beer to foaming. This was accomplished by slightly vibrating the bottle. A piece of aluminum foil was inserted between the bottle mouth and crown cap before resealing, to avoid migration of hop volatiles into the plastic liner. Bottles were turned over five times to complete the distribution of pellet particles in the beer. After seven days of dry hopping, samples were filtered using a fine-meshed polyamide fabric and centrifuged at 4000 g (m/s^2) for 10 min at 20°C. Subsequently, 5-mL-portions of the samples were transferred into gas chromatograph (GC) vials (20 mL) using 5-mL-glass pipettes and further prepared for GC analysis in duplicate.

Gas chromatograph-flame ionization detector (GC-FID) analysis of essential oils

Hop oil (50 μL) was dissolved in a 20-mL-volumetric flask using tetrahydrofuran that contained p-cymene at 250 μg/mL as an internal standard. Analyses were conducted using a Perkin Elmer 580 GC equipped with FID (Waltham, Mam U.S.A.). For chromatographic separation, a ZB-WAX capillary column film thickness 60 m × 0.25 mm i.d. × 0.25 μm, was used (Phenomenex Inc., Aschaffenburg, Germany). The GC temperature program was as follows: 40°C for 10.5 min, then ramp at 5°C/min until 245°C (5 min hold). The flow of helium carrier gas was maintained at 1.2 mL/min. The injection volume in split mode was 1.0 μL and the split ratio was 20. The injector temperature was set at 250°C. Data acquisition and processing were performed using Perkin Elmer TotalChrom Version 6.3.2.

Head space-gas chromatography-mass spectrometry (HS-GC-MS) analysis of beer

A Shimadzu GC-2010 was used to analyze the beer volatiles and was equipped with a headspace sampler (HS-20, Shimadzu) coupled to a Shimadzu MS-QP2010 Ultra quadrupole mass spectrometer (Nakagyo-ku, Japan). The beer samples (5 mL) were equilibrated for 30 min at 80°C. The injection volume (loop system) was 1.0 mL using a split ratio of 1:5. The capillary column used was a ZB-WAX 30 m × 0.25 mm i.d. × 0.25 μm film thickness (Phenomenex Inc., Aschaffenburg, Germany). Column head pressure for carrier gas helium was set at 200 kPa. The GC temperature program was: ramp at 4°C/min from 50 to 130°C, then ramp at 8°C/min to 180°C, and, subsequently, at 15°C/min to 240°C. The ion source temperature and interface temperature were 200 and 250°C, respectively. Mass spectra were obtained in electron ionization mode (70 eV) with selected ion monitoring (SIM). Calibrations were prepared according to the alcohol content of the beer samples (0.1, 5.0, and 8.1% ethanol). Data acquisition and processing were performed using LabSolutions GCSolutionAnalysis and LabSolutions GCMSsolution Version 2.72.

Statistical analyses

Significant differences among transferred amounts from single-variety dry hopping were assessed by one-way analysis of variance (ANOVA) using the SPSS Version 24.0 statistical package for Windows (SPSS Inc, Chicago, IL, U.S.A.). Statistical differences between means were evaluated using Games-Howell's test at the 0.05% level to evaluate the analytical significance.

Results and discussion

The results from the hop and hop oil analyses are shown in Table 1 and Table 2. As expected from the high α acid content, the dual-purpose cultivar EU contained the highest amount of essential oil (27.4 μL/g), followed by HB, CA, and TE with 13.7,

Table 1. Chemical technical analysis of hop pellets type-90 of crop 2015 and dry hopping dosage.

	Tettnanger	Cascade	Hallertau Blanc	Eureka
Oil content (μL/g)	3.47	9.12	13.70	27.40
α Acids content (%)	3.0	5.8	10.1	17.6
Quotient oil-/α acids content (μL/g/% α acids)	1.2	1.6	1.4	1.6
Dosing quantity[a] (g/L)	1.50; 7.30	1.64	1.09	0.55
Dosed α acids in tests (mg/L)	45; 219	95	110	97

[a]Dosing related to oil concentration in beer; 0.5, 2.5 mL/HL (TE) and 1.5 mL/HL (CA, HB, EU).

9.12, and 3.47 μL/g, respectively. Twenty-two compounds were quantified in pellets of each variety. The β-myrcene made up 36.1 (TE), 46.6 (HB), 48.0 (CA), and 56.9% (EU) of total essential oil. The TE and CA β-myrcene values are consistent with those from the literature,[27] showing 20–35 and 40–60%, respectively. One of the most potent hop flavor compounds, linalool,[28] was contained in the highest amounts in EU at 170 μg/g, which was almost four times higher than in HB (47 μg/g).

Transfer of volatile compounds from single-variety dry hopping

The outcome of dry hopping depends on factors such as the vessel size.[29] On a small scale, influences such as failure of distribution of the pellets in the vessel can be reduced and therefore a standard volume of 0.5 L was chosen. The hop dosage in the experiments was based on essential oil content. Using hop oil allowed for high, medium, and low dry-hopped dosage rates at 2.5, 1.5, and 0.5 mL/HL, respectively, based on the recent literature.[5,11,29]

Dry hopping of nonalcoholic beer

Tettnanger was used for dry hopping in these experimental trials because of its low α acid content (3.0%), as higher levels of α acid could adversely influence flavor extraction rates.[23] The nonalcoholic (NA) beer contained only β-myrcene and linalool at measurable levels of 1.6 and 2.1 μg/L, respectively, before dry hopping (Table 3).

Table 2. Gas chromatographic analysis of hop essential oils in hop pellets.

Compound	Tettnanger μg/g	Cascade μg/g	Hallertau Blanc μg/g	Eureka μg/g
α-pinene	2.29 ± 0.07	7.30 ± 0.05	14.5 ± 0.3	22.9 ± 0.4
isobutyl isobutyrate	0.82 ± 0.01	11.3 ± 0.1	45.5 ± 0.5	9.27 ± 0.55
β-pinene	12.0 ± 0.07	32.5 ± 0.4	31.0 ± 0.3	92.7 ± 1.1
β-myrcene	1,028 ± 12	3,595 ± 28	5,243 ± 7	12,792 ± 33
D-limonene	4.30 ± 0.02	17.0 ± 0.2	26.3 ± 0.1	40.9 ± 0.5
γ-terpinene	11.5 ± 0.0	30.3 ± 0.1	46.1 ± 0.5	90.6 ± 1.1
methyl heptanoate	nd[a]	1.09 ± 0.03	1.92 ± 0.27	3.82 ± 0.55
1-octen-3-ol	1.3 ± 0.05	2.74 ± 0.18	3.84 ± 0.02	6.55 ± 1.09
citronellal	1.67 ± 0.03	5.11 ± 0.07	7.95 ± 0.26	14.2 ± 0.2
methyl nonanoate	1.60 ± 0.21	4.01 ± 0.04	10.7 ± 0.8	16.4 ± 0.1
linalool	13.1 ± 0.2	35.6 ± 0.2	47.2 ± 1.1	170 ± 2
β-caryophyllene	307 ± 7	663 ± 3	225 ± 4	1,652 ± 13
methyl decanoate	0.97 ± 0.14	3.10 ± 0.55	4.95 ± 0.03	16.9 ± 10.4
methyl-4-decenoate	14.4 ± 0.2	22.3 ± 0.1	108 ± 1	468 ± 3
α-humulene	517 ± 3	1,166 ± 22	1,420 ± 13	4,275 ± 6
β-farnesene	169 ± 1	295 ± 1	412 ± 0	570 ± 1
β-damascenone	1.39 ± 0.09	3.28 ± 0.09	3.02 ± 0.82	5.45 ± 2.18
α-terpineol	1.60 ± 0.07	4.93 ± 0.18	12.0 ± 0.3	18.6 ± 0.1
geranyl acetate	1.37 ± 0.14	105 ± 1	19.2 ± 2.6	37.1 ± 0.1
neryl acetate	1.60 ± 0.07	6.20 ± 0.02	9.05 ± 0.81	13.1 ± 0.4
caryophyllene oxide	6.80 ± 0.14	8.76 ± 1.06	1.37 ± 0.24	1.09 ± 0.03
geraniol	2.61 ± 0.08	35.5 ± 4.1	35.0 ± 3.1	17.9 ± 0.1

[a]nd = not detected.

Effect of ethanol content on extraction

In the first test setup, the dependency of transfer on the ethanol concentration was investigated (Table 3). Treating NA beer and alcohol fortified NA beers with Tettnanger hop pellets led to a different composition of hop oil components in the resulting beers compared with the hops (Figure 1). At 1028 μg/g, β-myrcene dominated the hop oil composition of pellets, whereas linalool was the sixth major component at only 13 μg/g (Table 2). However, after dry hopping (under "control" conditions), linalool was present at a concentration of 86.8 μg/L and was the predominant component in NA beer, followed by β-myrcene at 26.3 μg/L (Table 3). Increasing the ethanol content from 0.1 to 5.0% resulted in higher levels of 18 hop volatiles in beer except for α-pinene, D-limonene, γ-terpinene, and neryl acetate, which were not detected in any of the samples. Interestingly, β-farnesene and geranyl acetate could not be detected in samples with added ethanol. This suggests that other chemical reactions were at work, such as aldol condensation. With 5.0% ethanol, linalool showed the highest concentration at 120 μg/L, followed by β-myrcene at 76.7 μg/L, and geraniol at 19.5 μg/L. A further increase in ethanol content from 5.0 to 8.1% resulted in significantly increased levels of many of the analyzed hop volatiles. Generally, increased ethanol levels improve the solubility of hydrophobic compounds in aqueous solutions.[30] Ethanol is a molecular dipol, which combines hydrophilic and lipophilic properties. In order to enable increases in transfer rates due to higher alcohol contents, the extraction temperature must be above 0°C, which has been reported to inhibit the swelling of plant material.[13] A previous study found no significant effects on the transfer of essential oil components from Hallertauer into aqueous systems at 0°C with increasing ethanol contents from 5 to 10%.[14]

Effect of temperature on extraction

The results of studying the effect of tempersture on extraction of hop oil components into beer are shown in Table 3. Temperatures selected in test dry hoppings were set to reflect levels in the cold process area of the brewery, such as the fermentation and lagering cellar, and in the literature.[13,17] By increasing the temperature from 1 to 4°C with dry hopping (of nonalcoholic beer), the concentrations of the following compounds changed significantly; β-pinene, β-myrcene, β-caryophyllene, α-humulene, β-farnesene, 2-undecanone, and 2-tridecanone. With other compounds, the changes, if any, were below 10%. The level of linalool changed slightly from 86.9 to 90.8 μg/L, which is consistent with previous reports.[16,17] The concentration of the second most abundant compound β-myrcene in beer doubled from 26.3 to 50.0 μg/L. At a significantly higher temperature of 20°C, the highest increase once again was in the concentration of β-myrcene at 24.9 μg/L. The level of α-humulene in the essential oil of TE was half of that of β-myrcene. However, when dry hopping beer, the level of α-humulene was significantly lower than half of the β-myrcene concentration at any extraction temperature

Table 3. Gas chromatographic analysis of Tettnanger dry-hopped beers.

				Low dosage	High dosage							
Compound	Quant. ion[a] m/z	Ref. ions[b] m/z	Basic beer µg/L	1°C[c] 0.1% ETOH µg/L	1°C 0.1% ETOH µg/L	4°C 0.1% ETOH µg/L	20°C 0.1% ETOH µg/L	1°C 5.0% ETOH µg/L	1°C 8.1% ETOH µg/L	Odor description[d]	Od. thres. beer[e] µg/L	Ident.
α-pinene	121	93-136	nd[f]	nd	nd	nd	nd	nd	nd	orange peel, pine	2.5, 62	MS, RC
isobutyl isobutyrate	89	71-101-91	nd	0.9 ± 0.1[g]	2.3 ± 0.2	2.4 ± 0.2	3.1 ± 0.3	3.7 ± 0.5	4.2 ± 0.4	fruity, pineapple	30	MS, RC
β-pinene	93	136-121	nd	0.1 ± 0.1	0.4 ± 0.0	0.9 ± 0.2	1.5 ± 0.0	1.5 ± 0.2	1.5 ± 0.4	resinous, woody	140	MS, RC
β-myrcene	93	136-121	1.6 ± 0.1	15.4 ± 3.3	26.3 ± 1.5	50.0 ± 8.3	84.9 ± 1.9	76.7 ± 8.8	89.4 ± 19.7	resinous	30, 1000	MS, RC
D-limonene	136	121-107	nd	nd	nd	nd	nd	nd	nd	citrus, orange-like	n/a	MS, RC
γ-terpinene	93	136-121	nd	nd	nd	nd	nd	nd	nd	herbal, citrus, lemon	n/a	MS, RC
methyl heptanoate	113	101-87	nd	0.1 ± 0.0	0.1 ± 0.3	0.1 ± 0.2	0.1 ± 0.0	0.7 ± 0.4	1.2 ± 0.3	cheesy, fermented	4	MS, RC
methyl octanoate	127	58-87-158	nd	0.7 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.9 ± 0.1	2.5 ± 0.4	3.1 ± 0.2	fruity	n/a	MS, RC
2-nonanone	58	142-98	nd	3.8 ± 0.2	7.3 ± 0.6	7.4 ± 0.6	9.2 ± 0.3	11.9 ± 1.8	14.9 ± 0.8	varnish	200	MS, RC
1-octen-3-ol	57	85-99-56	nd	7.4 ± 0.1	3.3 ± 0.2	3.6 ± 0.3	4.6 ± 0.2	20.0 ± 1.3	19.8 ± 0.4	mushroom, earthy	10[h]	MS, RC
linalool	121	136-121	2.1 ± 0.2	28.7 ± 0.4	86.9 ± 5.4	90.8 ± 5.1	104 ± 4.2	120 ± 11.5	138 ± 4.3	floral, citrus	5, 27, 80	MS, RC
β-caryophyllene	133	161-95-189	nd	0.4 ± 0.1	1.7 ± 0.6	3.3 ± 1.1	4.5 ± 0.6	3.0 ± 0.9	3.9 ± 1.5	spices, woody	450	MS, RC
2-undecanone	71	112-110-170	nd	2.7 ± 0.5	3.6 ± 0.3	4.3 ± 0.4	6.1 ± 0.3	7.7 ± 1.1	11.5 ± 0.9	varnish, green	400	MS, RC
methyl-4-decenoate	74	152-184	nd	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.2 ± 0.0	1.8 ± 0.2	2.8 ± 2.4	fruity	n/a	MS, RC
α-humulene	121	147-93	nd	2.4 ± 0.4	7.3 ± 1.7	13.3 ± 3.9	18.5 ± 2.2	13.1 ± 3.0	16.8 ± 3.4	balsamic	800	MS, RC
β-farnesene	133	204-93	nd	0.9 ± 0.3	1.6 ± 1.0	5.0 ± 1.6	6.5 ± 0.8	nd	nd	woody, citrus, sweet	2000	MS, RC
α-terpineol	136	139-121-93	nd	1.5 ± 0.1	3.5 ± 0.2	3.8 ± 0.2	10.6 ± 0.2	5.0 ± 0.5	5.2 ± 0.3	floral, citrus, woody	300, 2000	MS, RC
geranyl acetate	69	93-121-136	nd	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	nd	nd	floral, roses	9, 460	MS, RC
neryl acetate	121	154-196-136	nd	nd	nd	nd	nd	0.4 ± 0.0	0.3 ± 0.1	floral, citrus	n/a	MS, RC
β-citronellol	93	138-93	nd	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.2 ± 0.4	citrus	5	MS, RC
nerol	93	121-153-139	nd	1.1 ± 0.1	2.3 ± 0.2	2.3 ± 0.1	2.8 ± 0.1	3.2 ± 0.4	3.6 ± 0.2	floral, citrus	50, 1200	MS, RC
2-tridecanone	58	198-140	nd	0.1 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	1.1 ± 0.1	varnish	100	MS, RC
geraniol	93	58-154-139	nd	6.0 ± 0.2	13.8 ± 1.0	14.4 ± 1.0	16.7 ± 0.5	19.5 ± 2.2	26.3 ± 0.5	floral, roses	36	MS, RC
Sum			3.7	73.6	164	205	277	292	344			

[a]Quantified ion measured in SIM mode.
[b]Reference ions measured in SIM mode for identification.
[c]Static dry hopping temperature, basic beer ethanol (ETOH) content and dosage related to oil concentration in beer; 0.5 mL/HL at low dosage and 2.5 mL/HL at high dosage.
[d]Odor descriptors from the literature.[7,28,37,38]
[e]Odor thresholds from the literature;[7,13,28,37–42] n/a = not available.
[f]nd = not detected.
[g]Confidence interval ($\alpha = 0.05$, $n = 6$).
[h]Threshold determined in ethanol solution.

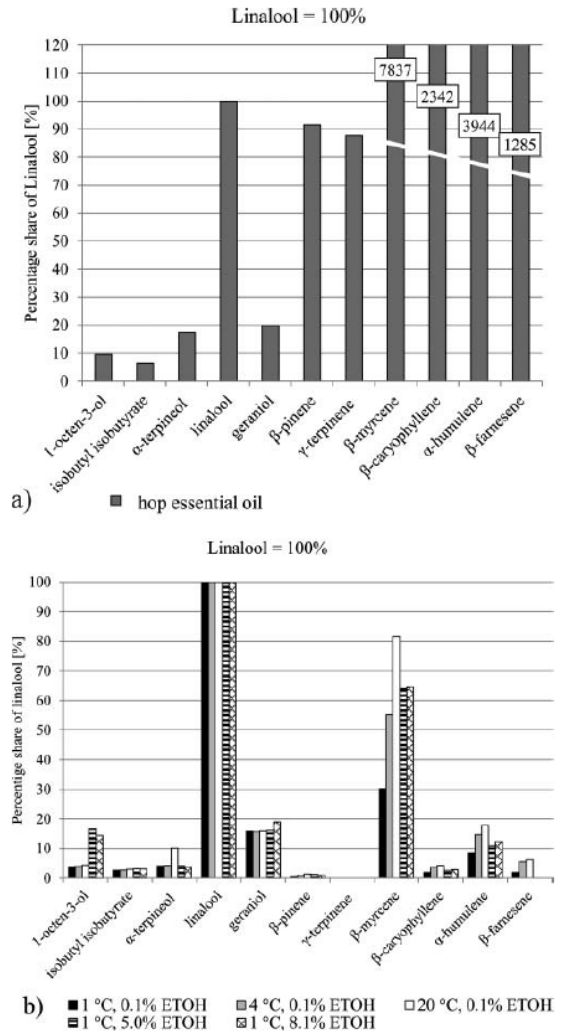

Figure 1. Essential oil composition (%) compared with linalool (= 100%) in (a) Tettnanger TE pellets and (b) TE nonalcoholic beers set to different ethanol (ETOH) contents dry-hopped at different temperatures. Dosage related to oil concentration in beer; 2.5 mL/HL.

tested. This finding also suggests other important parameter(s) are influencing the extraction. In general, higher temperatures increase the diffusion and reduce the viscosity of solvents and therefore improve the extraction;[24] however, the increase with higher temperature led to different compositions of hop oil components (Figure 1 b).

Effect of dosage on extraction

Although increasing the TE dosage from 1.5 to 7.3 g/L, which resulted in a factor of 4.9, led to higher levels of hop volatiles in beer with 0.1 % ethanol at 1°C, the increase in the total levels went from 73.4 to 164 μg/L (Table 3), a factor of 2.2. At the low dosage, only β-myrcene and linalool were present at concentrations above 10 μg/L, while geraniol was the third most abundant compound in the row with high dosage. The increase in the linalool concentration was highest from 28.7 to 86.9 μg/L, whereas the level of β-myrcene only changed from 15.4 to 26.3 μg/L. It can be concluded from the measurements that increasing the dosage leads to higher levels of the particularly alcoholic components of hop essential oil, changing the composition of hop volatiles to a greater degree than the terpene β-myrcene. Consequently, the hop-derived flavor in beer may be expected to change at various dry-hopping rates.

Effects of the hop varieties Cascade, Hallertau Blanc, and Eureka on the concentrations of volatile compounds in a high-gravity beer

The Cascade CA, Hallertau Blanc HB, and Eureka EU hops were selected to represent low, medium, and high α hops. For these hop varieties, the ratio of essential oil and α acid contents could be compared more easily (1.6, 1.4, and 1.6 μL/g/% α acid) than by using TE (1.2 μL/g/% α acid) in this test series (Table 1). In general, hop varieties with higher α acid contents are also rich in essential oil.[25] The sum of transferred amounts of hop compounds after dry hopping using beer of 5.0% ethanol at 1°C (8.1% ethanol at 20°C) was 357 (704), 269 (469), and 311 (509) μg/L for CA, HB, and EU (Table 4), respectively. A relationship between extraction and the α acid content is observed when comparing the hop oil/α acid contents ratios of the varieties. HB showed the lowest level (1.4 μL/g/% α acid) and also lowest transferred amounts. If the quantities of α acids per liter (Table 1) are considered, which are 95, 110, and 97 mg for CA, HB, and EU, respectively, the highest dosed quantity of α acid (HB) corresponds to the lowest level transfers, while the lowest dosed level of α acid (CA) shows the highest transfers. Despite the fact that only three hop varieties were compared, the results indicate the influence of hop variety α acid content in addition to temperature and ethanol content as previously shown.

Dependency of transfer rates on octanol-water partition coefficients with dry hopping

The physical-chemical properties of a compound are determinant in terms of transfer rates. Typically, in extraction systems and environmental chemistry, the log octanol-water partition

Table 4. Total amount of transferred essential oil constituents into dry-hopped[a] beers.

	Cascade		Hallertau Blanc		Eureka	
	5.0% ETOH 1°C[b] (μg/L)	8.1% ETOH 20°C (μg/L)	5.0% ETOH 1°C (μg/L)	8.1% ETOH 20°C (μg/L)	5.0% ETOH 1°C (μg/L)	8.1% ETOH 20°C (μg/L)
Amount of transferred essential oil constituents	357	704	269	469	311	509

[a]Dosing related to oil concentration in beer; 1.5 mL/HL.
[b]Dry hopping of diluted high gravity beer at 1°C and high gravity beer at 20°C

Table 5. Transfer rates of Tettnanger hop volatiles into nonalcoholic beer and log K_{OW} when dry hopping.

Compound	Log K_{OW}[a] [log (mol mol^{-1})]	Low dosage 0.1% ETOH 1°C[b] (%)	Low dosage 0.1% ETOH 1°C (%)	High dosage 5.0% ETOH 1°C (%)	High dosage 8.1% ETOH 1°C (%)	High dosage 0.1% ETOH 4°C (%)	High dosage 0.1% ETOH 20°C (%)
1-octen-3-ol	2.64	179	397	220	218	200	266
isobutyl isobutyrate	2.68	37	70	62	69	39	51
α-terpineol	3.28	21	42	30	31	23	64
linalool	3.50	89	146	123	142	93	107
geraniol	3.56	73	155	102	138	75	88
methyl-4-decenoate	3.91	nd[c]	nd	1.7	2.6	nd	nd
β-pinene	4.16	0.5	nd	1.8	1.8	1.1	1.7
β-myrcene	4.34	0.3	1.0	1.0	1.2	0.6	1.1
β-caryophyllene	6.30	0.1	0.1	0.1	0.2	0.1	0.2
α-humulene	6.95	0.2	0.3	0.3	0.4	0.4	0.5
β-farnesene	7.10	0.1	0.4	nd	nd	0.4	0.5

[a]Log K_{OW} values given in the literature.[43–46]
[b]Static dry hopping, basic beer ethanol (ETOH) content (0.1, 5.0, and 8.1%) and temperature (1°C and 20°C); dosage related to oil concentration in beer; 0.5 mL/HL at low dosage or 2.5 mL/HL at high dosage.
[c]nd = not detected.

coefficient (log K_{OW}) is used to comprehensively assess the thermodynamics of partitioning and environmental behavior of chemicals such as persistent organic pollutants (POPs).[24] The log K_{OW} is a criterion for the hydrophobicity of a compound as its distribution is measured between the two phases, octanol (hydrophobic) and water (hydrophilic).[31] Log K_{OW} is often correlated with water solubility.[30] When comparing two compounds, a lower log K_{OW} indicates better water solubility.[30] For investigations into the transfer rate of volatile compounds in dry hopping using the log K_{OW} model, hop pellets due to their hydrophobic character correspond with the octanol-phase.

Transfer rates were obtained by calculating the percentage ratio of absolute quantities of an essential oil compound in dry-hopped beer and the hops used in dosing (Table 5 and Table 6). Different groups of hop volatiles were produced from all the dry-hopping trials investigating transfer rates. The C_{11}-ester, monosesquiterpenes, and sesquiterpenes showed transfer rates between 0.1 and 13%, while alcohols and C_8-esters showed transfer rates above 23%. In Figure 2 and Figure 3, the correlation of transfer rates of particular volatile hop-derived substances in dry hopping trials with their log K_{OW} is shown, except for alcohols that are prone to transformation reactions, which are discussed in a further section of this article. Therefore, the log K_{OW} may be a useful model for the prediction of transfer rates of hop oil flavor components in dry hopping, regardless of tested process parameters. None of the compounds studied had a transfer rate between 13 and 23%, which is due to the lack of an appropriate compound with a log K_{OW} value between 3.5 and 4.0. Generally, 12°P beers consist of 90–92% water, 3.8–4.2% ethanol [4.7–5.2% (v/v)], 0.42–0.55% carbonic acid, and 4.0–4.5% extract;[4] thus, the polarity is closer to that of pure water than to that of hydrophobic solvents. Hop oil components analyzed herein such as alcohols (log K_{OW} 2.46–3.56) and C_8-esters (log K_{OW} 2.68 and 2.83) are more readily soluble in aqueous solutions than C_{11}-ester (log K_{OW} 3.91), monoterpenes (log K_{OW} 4.16–4.44), and sesquiterpenes (log K_{OW} 6.3–7.1). Of all the hop oil components, 1-octen-3-ol

Table 6. Transfer rates of hop volatiles into high-gravity beer and log K_{OW} when dry hopping.

Compound	Log K_{OW}[a] log (mol^*mol^{-1})	High gravity beer[b] (μg/L)	Cascade 5.0% ETOH[c] 1°C (%)	Cascade 8.1% ETOH 20°C (%)	Hallertau Blanc 5.0% ETOH 1°C (%)	Hallertau Blanc 8.1% ETOH 20°C (%)	Eureka 5.0% ETOH 1°C (%)	Eureka 8.1% ETOH 20°C (%)
1-octen-3-ol	2.64	1.9 ± 0.4[d]	143	411	109	341	76	229
isobutyl isobutyrate	2.68	1.4 ± 0.2	54	68	56	58	81	87
methyl heptanoate	2.83	0.6 ± 0.1	58	94	89	113	84	120
α-terpineol	3.28	7.5 ± 1.1	47	116	31	83	38	70
linalool	3.50	89.2 ± 4.9	97	113	71	78	101	83
geraniol	3.56	8.5 ± 1.3	168	350	103	181	100	135
methyl-4-decenoate	3.91	nd[e]	nd	nd	3.5	5.0	2.5	3.2
β-pinene	4.16	nd	4.1	6.5	4	6.2	2.3	4.2
γ-terpinene	4.25	nd	1.9	2.2	nd	nd	nd	nd
β-myrcene	4.34	nd	1.0	2.7	0.7	2.0	0.8	2.2
D-limonene	4.38	nd	4.6	10	3.4	6.4	nd	nd
α-pinene	4.44	nd	10	13	7.2	8.7	nd	nd
β-caryophyllene	6.30	1.3 ± 0.2	1.4	4.0	0.7	1.8	5.2	9.0
α-humulene	6.95	nd	0.9	2.6	0.5	1.3	1.3	2.9
β-farnesene	7.10	nd	1.1	3.1	nd	nd	nd	nd

[a]Log K_{OW} values given in the literature.[43–46]
[b]Not dry hopped.
[c]Static dry hopping, basic beer ethanol (ETOH) content and temperature; dosage related to oil concentration in beer; 1.5 mL/HL.
[d]Confidence interval ($\alpha = 0.05$, $n = 3$).
[e]nd = not detected.

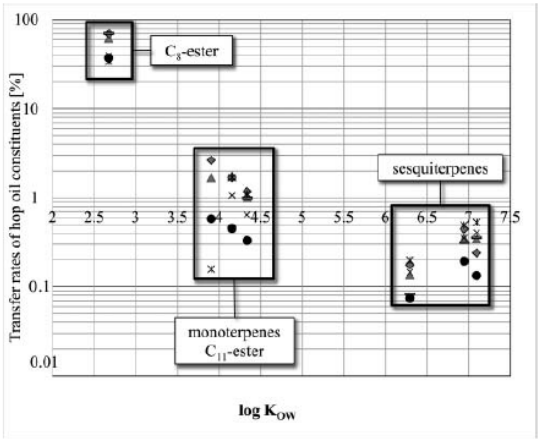

Figure 2. Classification of transfer rates of hop essential oil constituents from static dry hopping (at 1, 4, and 20°C) of nonalcoholic basic beer (0.1% or set to 5.0 and 8.1% ethanol) with Tettnanger TE by log K_{OW} (levels in Table 5). Dosage related to oil concentration in beer; 2.5 mL/HL and 0.5 mL/HL at low dosage (ld). C_8-ester: isobutyl isobutyrate; C_{11}-ester: methyl-4-decenoate; monoterpenes: β-pinene, β-myrcene; sesquiterpenes: β-caryophyllene, α-humulene, β-farnesene. Diagram point labels include dry hopping parameters, basic beer ethanol, (ETOH) content, and temperature:
●0.1% ETOH, 1°C; 0.1% ETOH, ▬1°C (ld); 5.0% ETOH, △1°C; 8.1% ETOH, ◆1°C; X0.1% ETOH, 4°C; ✱0.1% ETOH, 20°C.

showed the highest transfer rates for all three hop varieties. Particularly at 1°C, transfer rates of 1-octen-3-ol using beer with 5.0% ethanol were 143, 109, and 76% for CA, HB, and EU, respectively. With high gravity beer at 20°C, the rates were even higher at 411, 341, and 229% for CA, EU, and HB, respectively. Because the amounts transferred into beer are much

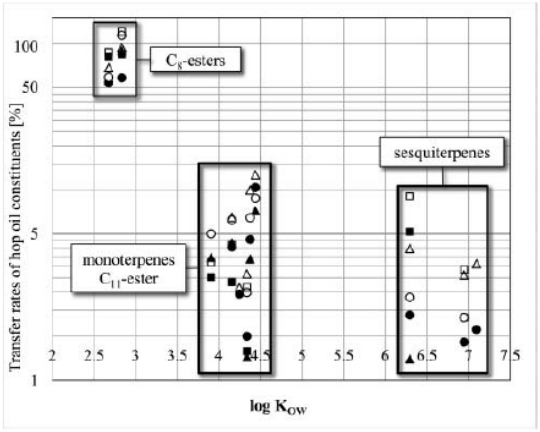

Figure 3. Classification of transfer rates of hop essential oil constituents (CA: $n = 15$, HB: $n = 14$ and EU: $n = 12$) from dry hopping (1°C or 20°C) of high-gravity basic beer (5.0 or 8.1% ethanol) with Cascade CA, Hallertau Blanc HB or Eureka EU by log K_{OW} (levels in Table 6). Dosage related to oil concentration in beer; 1.5 mL/HL. C_8-esters: isobutyl isobutyrate, methyl heptanoate; C_{11}-ester: methyl-4-decenoate; monoterpenes: β-pinene, γ-terpinene, β-myrcene, D-limonene, α-pinene; sesquiterpenes: β-caryophyllene, α-humulene, β-farnesene. Diagram point labels include dry hopping parameters hop variety, basic beer ethanol (ETOH) content, and temperature:
●CA, 5.0% ETOH, 1°C; ○CA, 8.1% ETOH, 20°C; ▲HB, 5.0% ETOH, 1°C; △HB, 8.1% ETOH, 20°C; EU, 5.0% ETOH, 1°C; ■EU, 8.1% ETOH, 20°C.

higher than they are present in free form in the pellets, the majority of 1-octene-3-ol may not be present in free form in the studied hops. Other compounds with high transfer rates over 100% are geraniol and linalool. For transfer rates above 100%, it is speculated that the levels of analyzed alcohols were influenced by transformation reactions, such as the release of free alcoholic compounds from their glycosidically bound form. The glucosidase (1,4-β-glucosidase) enzyme activity of brewer's yeast can lead to increased amounts of several essential oil constituents by the hydrolytic cleavage of certain β-glycosides.[21,22] For example, glycosidically bound compounds are reported to be present in wine,[32] as well as in hops and beer; although, recent studies suggest that the amounts of glucosides can be significantly lower for some hop varieties, especially in dual purpose hop varieties, compared with the free fractions.[21,33–36] In hop oil, only the free forms of volatile flavor compounds are present, which need to be considered when calculating the transfer rates. In regard to alcoholic compounds, it is not known which part of the transferred amount comes from the free form in hop pellets. As for 1-octen-3-ol, the proportion coming from the free form may be roughly estimated by comparing the level of isobutyl isobutyrate, since both compounds have similar log K_{OW} values and biotransformation reactions are not known for isobutyl isobutyrate. For example, the levels in beer with 8.1% ethanol using TE at 20°C are 19.8 and 4.2 μg/L for 1-octen-3-ol and isobutyl isobutyrate, respectively. The resulting transfer rates are 218 and 69% for 1-octen-3-ol and isobutyl isobutyrate, respectively. If only the free form 1-octen-3-ol was present in the hops, then the maximum level in beer is 9.1 μg/L (19.8 μg/L × 100% / 218%) providing a transfer rate of 100%. Thus, the portion 6.3 μg/L (19.8 μg/L × 69% / 218%) is derived from the free form. However, conflicting results were determined for EU hops. The transfer rates were significantly different at 0.8 and 2.2% for β-myrcene in the beers with 5.0 and 8.1% ethanol, respectively, compared with 5.2 and 9.0% for β-caryophyllene. The log K_{OW} value of β-myrcene at 4.34 is much lower than that of β-caryophyllene at 6.3. Consequently, the expected transfer rate for β-caryophyllene should be significantly lower than for β-myrcene. Deviations to the expected values can be due to the complexity of beer and hop phases. Nevertheless, the log K_{OW} value is a useful tool in estimating the relative concentrations of hop volatiles in any beer, at any temperature, with dry hopping.

Conclusions

Dry-hopping conditions significantly influence the concentrations and compositions of hop oil components in beer. Although higher dosages increased the concentrations of hop oil components, the increase was not equal for all compounds. In particular, the proportion of monoterpenes such as β-myrcene and sesquiterpenes was lower with dry hopping at 7.3 g/L compared with 1.5 g/L, despite a subsequent higher concentration in beer. In other words, an increase in dosage led to a higher proportion of monoterpene alcohols and C_8-esters, in particular linalool and geraniol, in dry-hopped beer under the experimental conditions. The temperature and alcohol content showed similar effects, resulting in significantly higher levels of hop oil components than for increased dosage. Although the

proportion of β-myrcene increased markedly with increasing temperature and/or alcohol content, which presumably impacts the flavor, the proportions of 1-octen-3-ol in beers of 5.0 and 8.1% alcohol was much higher than with any other condition. Due to presence of the glycosidically bound form of some compounds (a recognized theory), in particular linalool, geraniol, and 1-octen-3-ol, much higher levels in dry-hopped beers were detected than expected from their levels in hop oil. Hop variety apparently plays an important role with regard to transfer rates. It appears that a high dosage of α acids (when dosing is based on essential oil content in beer) could adversely impact the transfer rate of essential oil constituents, although only three varieties were compared in this study. Depending on the dry-hopping conditions, not only do the levels of hop oil components change but also their compositions. Consequently, these two factors should lead to different flavors in dry-hopped beers, even when using just one hop variety. Since the overall flavor is a result of all flavorings, different beers dry hopped under identical conditions should provide differing aromas. The results revealed that the log K_{OW} value of a hop oil component is the key parameter for transfer rate and the resulting concentration in dry-hopped beer; although, known K_{OW} values may be expected to be quite different from those for beer-hop pellet systems. Thus, the log K_{OW} has been introduced as a consistent model for the extraction of hop essential oil constituents in dry hopping.

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3 Discussion

The use of hops in the cold area of a brewery opens a wide range of opportunities for the creation of beers with exceptional flavors. One of the main obstacles is to efficiently extract the hop oil. However, doing this and to further achieve consistent quality presents brewers and researchers with diverse challenges due to the complexity of the hop material, the extraction medium including green and bright beer, and the dry hopping process itself with numerous potential factors influencing final result. When comparing hop oils, there are substantial differences in their composition given the existence of more than 200 different hop varieties available for brewing. Moreover, the green and bright beer matrices contain biologically active components (e.g. enzymes [134, 146]) and microparticles with specific surface properties (e.g. yeast cells [121]). It is expected that interactions of the derived hop, malt, and yeast compounds impact the formation of beer flavors. To date, interactions of dry hopping-derived flavorings and matrix compounds have received scant attention in the research literature. Little is known about controlling the extraction to influence hoppy flavor of beer, and nor is it clear if there are any other factors that impact the concentrations of extracted components during the dry hopping period. The major factors influencing concentrations of volatile components during dry hopping and investigated in the present dissertation are as follows:

- Hop raw material:
  The transfer rates of volatile hop components during dry hopping and consequently the composition in beer depend on the substance properties. The flavor potential of precursor substances in hops is released through dry hopping.

- Brewing yeast fermentation:
  Interactions of volatile hop components with yeast cells, yeast metabolism-derived enzymes or undissolved carbon dioxide have an impact on the concentration of hop flavor components in the beer matrices.

- Dry hopping process:
  The basic beer composition and dry hopping parameters, in turn, influence the extraction of flavor components.

3.1 Utilization of hop flavor components and precursors in dry hopping

Hops are characterized by a high proportion of secondary metabolites. Thus, hop is a raw material versatile for beer production. Today it is well established that the oil of hops is a source of the hoppy flavor of beer [4, 56]. Analyzes of the flavor profile of beers showed that individual substances such as linalool and geraniol can play a key role in the formation of hoppy beer flavors [49]. The evaluation of additional components, e.g. β-myrcene, 2-methyl butyl-2-methyl propanoate, and α-humulene can provide a reliable indication of the sensory perception of the total hoppy content of dry-hopped beers, as shown in a current study by Machado et al. [72]. Although researcher analyzed glycosides as possible beer flavor precursors [62], the impact of precursors especially on dry-hoppy beer flavor is not clear yet. New insights presented herein will feed into the discussion regarding flavor potential and hydrolysis pathways of glycosides. Part of the aim of this dissertation is to assess the contribution of different hop material components to the flavor of dry-hopped beers. This chapter discusses new findings about transfer rates of hop aroma components in dry hopping, using a solubility model (log K_{OW}) introduced within a study that is part of the present dissertation. There is also a focus on the qualitative evaluation of seeded compared to unseeded hops for brewing purposes. The application of current and newly introduced methods allows a reassessment of hop samples with different seed contents, especially regarding their suitability for usage in dry hopping. It should also be considered that initial field trials were carried out to produce high-quality, standardized fertilized and un-fertilized hop patterns.

3.1.1 Hop oil constituents and their octanol-water partition coefficients

The concentration change in oil constituents during dry hopping has been studied for several years, however, these studies failed to establish a model theory explaining the different yields or transfer rates of oil constituents from hops. Previously, it was shown that the transfer rates vary during dry hopping depending on the oil constituent [81, 114]. In particular, there were significant differences in the transfer ranges between selected terpenoids (> 49 %; < 178 %; Table 5) and terpenes (> 0.1 %; < 2.6 %). In the course of this dissertation, higher transfer rates of terpenoids (> 71 %; < 350 %) and terpenes (> 0.1 %; < 9.0 %) were determined [152] compared to the aforementioned study results. In addition to the influence of the hop variety (discussed below), this is primarily due to the dry hopping parameters that can increase the yield of oil components from hops (cf. chapters 3.3.2/3). In Table 5, the transfer rates obtained in a study within present dissertation are divided according to base beer alcohol contents. A

direct comparison of the transfer rates indicates the impact of the base beer properties, such as the extraction-increasing influence of increased base beer ethanol contents. It should be noted that hop cones were used in the study by Forster et al. [81], which usually results in comparably lower extraction rates than for pellets [106]. However, comparing study results in table 5, in case of linalool and geraniol, Forster et al. determined comparably higher transfer rates than Krottenthaler et al. Thus, further factors besides degree of dispersion of the hop material had impact on transfer rates. The role of different dry hopping parameters on transfer rates of oil constituents is discussed in chapters 3.3.2/3.

Table 5 Log K_{OW}-levels [log (mol mol^{-1})] and transfer rates (%) of oil constituents during dry hopping from hop into (bright) beer

Compound	Log K_{OW} [153]	Forster et al. [81]	Krottenthaler et al. [114]	Haslbeck et al. [152] [1]	Haslbeck et al. [152] [2]
Linalool	3.50	100–111	63–96	71–146[3]	78–142
Geraniol	3.56	49–178	49–86	73–168	135–350
β-Myrcene	4.34	0.1–0.3	0.3–2.0	0.3–1.0	1.2–2.7
β-Caryophyllene	6.30	0.02–0.6	0.1–2.6	0.1–5.2	0.2–9.0
α-Humulene	6.95	0.1–1.9	0.1–2.6	0.2–1.3	0.4–2.9

[1]beer EtOH content ≤ 5.0 v/v, hop dosage based on oil in beer 1.5–2.5 ml/hl;
[2]beer EtOH content 8.1 v/v; hop dosage based on oil in beer 2.5 ml/hl;
[3]low hop dosage amount led to increased transfer rates, not considered in test setup using beer at 8.1 v/v EtOH;

Despite the differences described, there is a convergence in the results of the previous studies and those in the context of the dissertation, namely the difference in the transfer rates of terpenes (≤ 9.0 %, Table 5) and terpenoids (≥ 49 %). The yield of terpenes and terpenoids differs significantly and is independent of the use of different hop varieties, products, and dry hopping methods. Other recently published studies on dry hopping extraction efficiency using different scales confirm relatively low transfer rates of terpenes compared to terpenoids [108, 112]. As a result of compound-specific transfer rates, the compositions of the oil differ between hop and beer that were dry hopped with the same batch, which can be demonstrated using linalool and β-myrcene as representatives of the specified compound groups. In the previously mentioned study conducted as part of dissertation, the transfer rates during dry hopping with Tettnanger (TE) hops were 1.0 % for β-myrcene and 123 % for linalool (Table 6) [152]. Linalool made up a low proportion of 0.5 % in the oil of pellets (type-90), whereas it had the highest proportion in dry-hopped beer at 41.1 %. The β-myrcene formed the highest proportion in the oil of the same pellets with 36.1 % and had a minor proportion of 26.3 % in the dry-hopped beer.

Table 6 Concentrations of oil constituents in hop and beer and transfer rates during dry hopping from hop into beer [152]

Compound	Tettnanger (pellet) µg/g	Tettnanger (pellet) %	Dry-hopped beer[2] µg/l	Dry-hopped beer[2] %	Transfer rate %
Linalool	13.1 ± 0.2	0.5	120 ± 11.5	41.1	123
β-Myrcene	1,028 ± 12	36.1	76.7 ± 8.8	26.3	1.0
Sum oil	2,845 [1]	100	292	100	-

[1] oil content: 3.47 µl/g;
[2] lager beer at 5 % v/v EtOH; dry hopping at 1 °C;

The most important finding was that transfer rates of oil constituents depended primarily on their solubility properties. The octanol-water partition coefficients (log K_{OW}; Table 5) of the hop oil constituents, which are used as a measure of the solubility of a substance in aqueous solutions [93, 154], correlated with their transfer rates during dry hopping [152]. Higher log K_{OW}-levels of terpenes (≥ 4.34), for example, indicate the comparatively higher solubility in the hop matrix having rather non-polar and hydrophilic characteristics. Consequently, the transfer rates of terpenes into beer, which have more polar and hydrophilic features, will be rather low (≤ 9.0 %). A certain drawback associated with the use of the log K_{OW} solubility model is that this model uses n-octanol rather than the hop matrix that was used in experiments. The hop matrix is understood to correspond to the non-polar phase represented by n-octanol, and the beer phase corresponds to the (deionized) water phase. However, the model in its present form is reliable and a consistent explanatory approach for the limited concentrations of terpenes, and also larger ester (C_{11}) in dry-hopped beers. This is contrary to concentrations of alcohols and shorter esters (C_8), which were extracted up to quantitative amounts. Although the levels of the transfer rates were identified being substance-specific, they can be influenced within certain ranges via the dry-hopping process control as mentioned before.

It should be noted that the transfer rates listed in Tables 5 and 6 are not based exclusively on the extraction of the oil components from the lupulin glands. In addition, biotransformations of hop flavor components can take place during dry hopping. In a study within the course of the present dissertation, the maximum transfer rate of an oil constituent (isobutyl isobutyrate), of which no concentration-increasing reaction is known, was 87 % [152]. Higher transfer rates, especially those above 100 %, are due to conversion reactions, such as the release of aglycones from the hop material, e.g. linalyl- or geranyl glycosides (cf. chapter 3.1.3). In the case of geraniol, the transfer rates were particularly high (max. 350 %), which indicates the presence of further precursors in the hop material, e.g. geranyl acetate as previously suggested [81].

3.1.2 Seed content of hops

Hops for brewing come in similar proportions worldwide from growing areas with fertilized and un-fertilized hops. In Germany, great importance is attributed to the fact that all harvested hops are preferably unfertilized (seed content < 2 %), whereas in other countries, e.g. U.S.A., U.K., Australia, the seed content is not an evaluation criterion and varies between 10–30 % [1, 18]. Since contradicting statements were made in previous studies regarding the effect of seeded hops on beer quality, in a study within the present dissertation it was investigated whether the proportion of seeds influences the aroma quality in the specific case of dry-hopped beers. What stands out in this research is that the hop samples investigated were produced in standardized field trials. The method of growing fertilized or unfertilized plants at the same location was introduced to minimize external influences [155]. Hop patterns that had a high proportion of seeds (18.9 ± 2.3 %) or a minimal proportion of seeds (1.3 ± 0.3 %) were used, although the hop plants were cultivated under the same environmental influences. Hallertauer Tradition (HHT) was the main test object, as it is traditionally one of the most important aroma varieties in Germany and employed in 2019 with 13.5 % of the total cultivation area [15]. Visual inspection confirmed that the fertilized cones are larger than the unfertilized patterns and have greatly enlarged bracts [19]. The relatively large cones combined with the increased proportion of seeds lead to a relatively high cone weight, which can be seen as an economic advantage. In the analysis of α- and oil-contents of HHT patterns with different seed proportions (Table 7), no significant differences were found. The α-contents around 6.7 % were slightly above the 5- (6.4 %) or 10-year mean (6.3 %) of this variety cultivated in the Hallertau [22]. There is little published information on the effect of seeds on hop quality parameters. In 1981, a review was published that offers contradictory findings about the oil and α-acid content of hops with different seed proportions [18]. Several studies were summarized which compared the α- and oil contents of various hop varieties (Fuggles, Northern Brewer, Hallertauer, Wye Target, Wye Challenger, Wye Northdow, Bullion, Wye Saxon) cultivated in different growing areas in the U.K. and Germany. There was a noticeable tendency towards a higher oil content and, depending on variety, also a higher α-content with a lower proportion of seeds. However, these studies have been unable to demonstrate that differences between patterns were not affected by external influences, e.g. environmental conditions.

The analysis of the oil fraction of the HHT patterns from the aforementioned field trials showed high similarity [155]. The same 90 substances were identified in unfertilized and fertilized hops, respectively. Differences were found in a few cases within the 52 quantified substances, such as some esters. The concentrations of 3-methyl butyl-2-methyl propanoate were significantly higher in the fertilized samples than in the unfertilized samples, whereas in

the unfertilized samples, the concentrations of 6-methyl heptanoate were higher (student t, n = 4; α = 0.05; Table 7). In the context of terpenoids and flavor contributions, several esters proved to be important for the flavor of hops [44]. Isobutyl isobutanoate and methyl-4-decenoate can be essential for the flavor of dry-hopped beers using Styrian Golding [5]. In a study as part of this dissertation it was shown that isobutyl isobutyrate can be a flavor component of dry-hopped beers using Hersbrucker, Mandarina Bavaria or Hallertauer Magnum [156].

Table 7 Selected hop analysis results [155]

Hop sample	α-Content %	Oil content ml/100 g	3-Methyl butyl-2-methyl propanoate µg/l	6-Methyl heptanoate µg/l
$HHT_{unfertilized}$	6.75 ± 0.64	0.97 ± 0.8	0.8 ± 0.11	4.1 ± 0.58
$HHT_{fertilized}$	6.72 ± 1.05	1.05 ± 0.6	1.8 ± 0.39	2.4 ± 0.36

Another critical test method assessing the quality of hop material is the sensory evaluation of their impact on beer flavor. In a previous study it was reported that hop seeds could impair the taste stability of beers due to their high lipid content of up to 32 % [16, 17]. In the context of hop samples from the above-mentioned field trials, dry-hopped beers using these hops and with different proportions of seeds showed a comparably high-quality aroma profile immediately after production and after three months of storage [155]. In the descriptive tasting by trained tasters, no significant difference was found in eleven examined aroma attributes (ANOVA, α = 0.05; n = 7). Thus, important indications were provided that hop patterns are equally suitable for dry hopping regardless of their seed content.

3.1.3 Hop glycosides

Over the past two decades, only a few studies have directly addressed if hop glycosides could contribute to hoppy beer flavors. Goldstein et al. postulated that hops contain glycosides or other flavor precursors from which flavorings may be released during fermentation [24]. They identified 60 different aglycones in the varieties Galena, Tettnanger, and Cascade [25]. An early quantification of 14 different aglycones using GC-MS within a study by Kollmannsberger et al. indicated dependency of the amount of glycosidically bound flavoring on variety [62]; samples of Hallertauer Hersbrucker (HHE) and Hallertauer Tradition (HHT) had comparatively higher contents of released linalool than Hallertauer Magnum (HHM) (peak areas; HHE: 27; HHT: 25; HHM: 12). In the context of the present dissertation, 44 different aglycones were identified in HHT samples (from field trial mentioned previously; crop 2013) by using GC-TOF-MS, of which 35 substances were quantified by GC-FID [155], which is a higher number than

in the previous study. It is unclear if the number of aglycones varies between studies due to the analysis methods used or if another factor such as crop has an impact. Among the quantified compounds in glycoside extracts from HHT field trial samples, e.g. 2.0 µg/g linalool was detected, which corresponds to only 3 % of total linalool. This is an even lower share compared to the 21–36 % bound linalool in total linalool determined by Wilhelm [64]. This researcher group detected 6.6–41.1 µg bound linalool per gram of hops in the varieties Perle, Smaragd, Hersbrucker, Golding and Cascade. Interestingly, for linalool, the content differed by a factor of 6 between the examined varieties. In the context of hop glycosides as flavor precursors, a recent study by Cibaka et al. underlined that their contribution to beer flavor is supposed to be low [149]. They determined a relatively low proportion of glycosidically bound terpinols at 0.6–28.6 µg/g compared to the corresponding free form of aglycons at 7.8–109.2 µg/g in the varieties Amarillo, Citra, Hallertau Blanc, Mosaic, and Sorachi Ace. Data from several studies suggest that the concentrations of glycoside flavor precursors in hops are relatively low, however, it should be noted that the extraction rates of the glycosides from the hop material into the wort or beer can be considered to be high due to their relatively hydrophilic character. In a study on hopping regimes with the Simcoe®, Hallertau Mittelfrüh and Columbus hop varieties, Sharp et al. determined that comparable amounts of glycosides were extracted regardless of the use of the hops in kettle, late or dry hopping [150].

The present study in the course of the dissertation provides the first comprehensive assessment of glycosides in fertilized or unfertilized hop patterns [155]. Different seed contents did not significantly influence aglycon composition in samples of the varieties HHT and Progress (PG) from the previously mentioned field tests. The analysis of the aglycones of HHT and PG, which included GC-O, contributed additional evidence that hops involve precursors of potent flavor components for beer. Identified aglycones of the compound groups aliphatic alcohols, terpene alcohols and C_{13}-norisoprenoids are to be emphasized, since they include some components that have been attributed as contributing to hoppy flavors of pellets and beer, e.g. 1-octen-3-ol, phenyl acetaldehyde, linalool, α-terpineol and β-damascenone [4, 24, 44, 56, 74]. Interestingly, the concentrations of the released fatty acids of the glycoside extracts of the HHT and PG samples showed no significant differences between the unfertilized and fertilized patterns [155]. This is a striking result as in previous reports the high fat content of the seeds and their unsaturated fatty acids in particular were considered to be the trigger for a deteriorating taste stability of beers brewed with hops at increased seed contents [16, 17].

In the investigation of aglycones, different enzyme preparations can be used to break down the glycosides. For example, glucosidase from *Aspergillus niger* was used in a previous study to release the bound linalool [64], while another group used Hemicellulase Rapidase F64 [61]. Since the high effectiveness of the Hemicellulase Rapidase F64 was also confirmed by another group [65], after a review, this enzyme preparation was used in the context of the present dissertation. Phenyl-β-D-glucopyranoside was used as a glycoside for this test [155]. An average yield of 93 % was determined in the matrix of the unfertilized hop pattern. In the matrix of the fertilized hop pattern, the yield was 89 %, thus confirming the high effectiveness of Rapidase F64.

3.1.4 Glycoside hydrolysis pathways

In the past two decades, there has been a surge of interest in using the aromatic potential of the hop glycosides by releasing the aglycones. A few years after their identification, Kaltner et al. suggested that the slight increase in linalool concentrations during fermentation might be due to the hydrolyzing effect of brewer´s yeast on glycosides [77]. In the following, Daenen et al. identified the glycoside hydrolase activities by using *Saccharomyces* and *Brettanomyces* yeasts [146]. In a direct comparison, brewing yeasts (*S. cerevisiae*; *S. pastorianus*) showed a lower activity than non-*Saccharomyces* yeasts. Significant differences in activity levels occur even within brewing yeasts. Takoi et al. detected 0.4–0.8 U/l glycoside hydrolase activities in young and matured beer based on exo-β-1,3-glucanases from an *S. pastorianus* strain (lager yeast, strain not named) [137]. Based on the enzyme assay published by the group, glycoside hydrolase activities of 0.07–0.15 U/l were determined in the context of the present dissertation, originating from various *S. cerevisiae* (TUM 68, TUM 511, TUM 506) and *S. pastorianus* (TUM 34/70, TUM 193, TUM 69) brewing yeast strains [156]. The comparison of both studies using a similar enzyme assay shows that the glycoside hydrolase activities of brewing yeast strains can vary significantly (factor of 5) and, regardless of the absolute level, the activities during the main fermentation and maturation remain at relatively constant. Previous studies did not respond satisfactorily to the question of the effectiveness of the glycoside-hydrolyses at those low activity levels. In the context of low activity levels, a study by Kanauchi and Bamforth provided further facts that the influence of brewer´s yeast on the hydrolysis of glycosides in the wort is to be classified as relatively low, since only a small part (2.5 %) of the β-glucosidase activities of ale and lager yeast was extracellular [145]. It is also unclear whether parallels can be drawn between brewing and other research disciplines. However, in enology it is common practice to dissolve glycosides as flavor precursor compounds through enzymes formed by certain yeast strains, including *Saccharomyces* yeasts [157, 158].

In light of the above-mentioned results, alternative glycoside-hydrolysis pathways are needed. Interestingly, hop cones from field trials mentioned before (cf. chapter 3.1.2/3), showed little β-glucosidase activity (0.11 U/g) [155]. This is the first description of an alternative source for glycoside-hydrolyzing enzymes (besides technical preparations), which is an important finding within a study of the present dissertation. Assuming a moderate dry hopping dose of 1 g/l, the expected level of activity is comparable to the exo-β-1,3-glucanase activity that was detected from six different brewing yeast strains previously mentioned in this chapter (TUM 68, TUM 511, etc.) [156]. The samples of the hop variety PG (0.11 ± 0.01 U/g), grown in the field trial in Kent, U.K., showed the same β-glucosidase activity as the variety HHT grown in Hüll (0.11 ± 0.01 U/g). Due to the small sample size it was not clarified whether the enzymatic potential of cone hops depends on the variety. In the context of enzyme activities originating from hop samples, a previous study evaluating diastases found increased activities in fertilized hop patterns [20]. However, the findings of the current study as part of this dissertation did not support the theses that fertilization generally leads to higher activities of hydrolases. Fertilized and unfertilized hop samples of the HHT variety did not show any significantly different β-glucosidase activities (t-test; $\alpha = 0.05$) [155]. It should be mentioned that the cone hops were dried for approx. five hours at 60 °C before analyzing the enzyme activity. In the case of glucosidases, denaturation rises significantly at temperatures around 50 °C [145]. Considering the heat exposure, the activity levels determined are even more noticeable. With regard to the use of hop pellets for dry hopping, only slightly lower enzyme activities are to be expected in comparison to cone hops, since temperatures up to 60 °C and 50–55 °C are only reached temporarily when the hops are homogenized (drying step) and pelletized [1].

3.2 Influence of brewing yeast fermentation on dry hopping-derived flavorings

The brewing yeast metabolizes the (fermentable) extract of the beer wort and uses the energy obtained for cell propagation, which creates a significant biomass. The composition of the beer matrix is also changed by products of alcoholic fermentation such as ethanol and carbonic acid. During the fermentation and maturation of beer, significant changes sometimes occur in the concentration of hop aroma components. Proof was provided that several hop-derived components undergo transformation during the brewing process such as oxidation in case of sesquiterpenes during wort boiling [89]. However, a deeper understanding of the influence of brewing yeast fermentation on hoppy beer flavor is needed. Thus, in the following, potential influences on the flavor of dry-hopped beers that are induced by brewing yeast activities are discussed.

3.2.1 Vaporization of β-myrcene

The drop in concentrations of mono- and sesquiterpenes during wort fermentation is significant. Depending on the substance content in the pitching wort and the fermentation conditions, the concentrations can be reduced below the detection limit in final beer [95]. In the case of dry hopping during the main fermentation, study results in the course of this dissertation showed that only small amounts of terpenes, e.g. β-myrcene, get dissolved [159]. The transfer rate of β-myrcene was determined at 0.3 %, leading to concentrations in the low microgram range in beer. This level is low compared to transfer rates of 0.3–2.7 % during the dry hopping of matured beer in a closed pressure tank using hop pellets (Table 5) [114, 152].
A source of losses of volatile compounds is based on a characteristic function of fermentation vessels, the discharge of excess fermentation carbon dioxide. Up to now very little has been published about the volatilization of flavorings during the fermentation of beverages, especially for beer fermentation. Furthermore, previous studies failed to provide data on the losses of flavorings within experiments representative of beer production. In a study as part of this dissertation, the evaporation of flavorings during dry hopping of fermenting wort in standardized small-scale experiments (10 l) was investigated for the first time. Losses have been identified via the trapped gas from the headspace of the fermenting wort. In the case of β-myrcene, an average of about 250 µg/l of vaporized β-myrcene for each test batch was dissolved in trap containers (bubbling water columns) [159]. Consequently, an average of 3.38 mg of vaporized β-myrcene was dissolved per test batch, which corresponded to 8.2 % of the β-myrcene (41.16 mg) contained in the dry hopping dose (Mosaic, pellet type-90). However, these data must be interpreted with caution because the test setup was not designed to quantitatively trap vaporized β-myrcene, but other (less nonpolar) compounds as well. Besides hop oil constituents, the same test setup also trapped fermentation products such as ethyl hexanoate, isoamyl acetate, and styrene in case of top-fermentation. Previously, Haefliger et al. used a cryotrapping sampling system to investigate fermentation gases in lab-scale fermentations (0.12 l) [96]. In the gas phase of fermenting wort they determined mono- and sesquiterpenes, e.g. β-myrcene, α-pinene, β-pinene, α-humulene, β-caroyphyllene, and some esters derived from alcohols 2-methyl propyl alcohol, 2- and 3-methyl butyl alcohol. No terpene alcohols were detected among the volatilized hop components, which is in line with the results of the study in this dissertation [159]. An important finding in the specified study is that indications were given that higher temperatures during the main fermentation could lead to the increased release of hop flavor components into the gas phase. An increased volatility at higher temperatures of flavorings typically produced by yeasts was previously described in model fermentations [132].

The volatilization of hop components is closely related to yeast activities. The rise of carbon dioxide bubbles in the fermenting wort particularly promotes the expulsion of relatively hydrophobic components (cf. log K_{OW}; chapter 3.1.1). In addition to the polarity of a substance, its volatility is a criterion for its evaporation. A terpene like β-myrcene has a comparably lower boiling point of 176 °C and therefore higher volatility than the terpene alcohol linalool, which has a boiling point of 198 °C [160]. This is thought to be part of the explanation for different behaviors in green beer.

3.2.2 Adsorption of β-myrcene

In green beer, yeast propagation results in a high concentration of microparticles. The surface of the yeast cell is non-polar, which plays an important role during flocculation, for example. In aqueous solutions, hydrophobic effects can lead to the aggregation of molecules with a non-polar surface [160], such as yeast cells and hop bitter acids [161]. In a study within this present dissertation, it was demonstrated for the first time that significant amounts of volatile hop components can be bound at the yeast biomass [159]. In laboratory tests using model solutions, the content of β-myrcene (initial concentration 70 µg/l) was reduced depending on the concentration of yeast cells. At cell counts typical of the range at the end of the main fermentation (approx. 100 million cells/ml), the terpene was almost completely adsorbed by the brewing yeast strains TUM 68 or TUM 34/70, 98 % and 99 %, respectively. With regard to the reduction in β-myrcene concentration, no difference was determined between the top- (TUM 68) and bottom-fermenting (TUM 34/70) yeast strain. In contrast to bottom-fermenting yeasts, top-fermenting yeasts form budding chains, which is thought to reduce the cell surface and lead to decreased adsorption effects towards yeast cells. However, taking almost equal decrease rates into account, no significant difference was observed.

From the point of view of imparting flavorings to beer within the method of dry hopping, it is of great interest if these binding processes are reversible. Thus, in further test series within the scope of this dissertation study, the solvent efficiency of ethanol, which is a natural ingredient of beer, was tested at different concentrations. Even in pure ethanol, which can be used as a solvent for relatively hydrophobic substances, only 17.2 % (TUM 68) or 16.8 % (TUM 34/70) of the originally dosed amount of β-myrcene was solubilized. A weaker solvent (5 or 10 % v/v EtOH solution), on the other hand, could not release any bound β-myrcene. Thus, this case study showed that intermolecular attractions between a comparably hydrophobic flavor component and the cell surface are relatively strong. Consequently, adsorbed β-myrcene and substances of similar characteristics will not contribute to beer flavor. In contrast to the monoterpene, linalool was not influenced by the presence of yeast cells, which in turn is due to its comparatively higher hydrophilicity.

3.2.3 Production of monoterpene alcohols

It is well known that brewing yeasts produce various flavor-active components, however, similarities to hop flavourings have rarely been studied. Kishimoto et al. provided the first indication of the ability of brewer´s yeast to form monoterpene alcohols [56]. They found evidence that linalool was present in unhopped beer using GC-O. Takoi et al. detected a slight increase in geraniol during the fermentation of a wort hopped with HHT, which was preceded by a rapid decrease in the first three days of fermentation [137]. A slight increase in geraniol concentration during the fermentation of hopped wort was also determined by Hanke et al. [111]. Within a study in the scope of this dissertation, *de novo* synthesized amounts of linalool, geraniol, β-citronellol, nerol and α-terpineol by yeast were quantified for the first time in the brewing sector [156]. What is remarkable about this finding is that it is evidence of the high conformity among important flavorings in beer regardless of the source. Furthermore, this insight partly explains the harmony in beer flavor due to synergies among both hops and yeasts. The results were obtained using (unhopped) worts based on malt extract in order to exclude the possibility that precursors from the hops could be source of the increase in terpenoids during the test fermentation (cf. chapter 3.1.3).

Table 8 Selected brewing yeast metabolism activities that influence the dry hopping outcome [156]

Yeast strain		Glycoside hydrolase activity (main ferm.)	*De novo* production			Geraniol metabolism		
			Linalool	β-Citronellol	Geraniol	Geraniol conc. decrease[1] at different original gravities: 7 °P	12 °P	18 °P
		U/l	µg/l			% (µg l^{-1})	% (µg l^{-1})	% (µg l^{-1})
Top-fermenting	**TUM 68** "wheat beer"	0.15 ± 0.03	2.6 ± 2.2	0.7 ± 0.2	6.8 ± 1.7	43 (30.1)	56 (39.2)	65 (45.5)
	TUM 506 "ale"	0.11 ± 0.01	n. d.	0.8 ± 0.0	4.0 ± 0.7	36 (25.2)	42 (29.4)	37 (25.9)
	TUM 511 "ale"	0.08 ± 0.02	n. d.	0.7 ± 0.0	2.7 ± 0.4	50 (30.5)	66 (46.2)	75 (52.5)
Bottom-fermenting	**TUM 34/70** "lager"	0.14 ± 0.00	1.2 ± 0.2	n. d.	3.0 ± 1.0	63 (44.1)	73 (51.1)	80 (56.0)
	TUM 69 "lager"	0.09 ± 0.02	1.3 ± 0.4	n. d.	1.7 ± 0.4	64 (44.8)	73 (51.1)	76 (53.2)
	TUM 193 "lager"	0.07 ± 0.01	0.9 ± 0.1	n. d.	3.2 ± 0.1	58 (40.6)	75 (52.5)	83 (58.1)
	Average	0.11	1.5	0.7	3.5	52 (36.4)	64 (44.8)	69 (48.3)

[1] initial geraniol concentration set to 70 µg/l using reference substance

The Table 8 shows the concentrations of monoterpene alcohols by *de novo* production in beers using different *S. cerevisiae* and *S. pastorianus* brewing yeast strains commonly used in beer production in Germany [117]. Interestingly, there were differences in the ability to synthesize monoterpene alcohols. The TUM 68 showed the highest production of an individual component in the case of geraniol at 6.8 ± 1.7 µg/l. TUM 68 and the three bottom-fermenting yeast strains examined synthesized small amounts of linalool (0.9–2.6 µg/l), but the ale strains TUM 506 and TUM 511 did not. Traces of β-citronellol (0.7–0.8 µg/l) were produced exclusively by the top-fermenting yeast strains. Although the synthesis of the specified alcohols is in the low microgram range, it is still important for the beer flavor. Linalool (flowery, citrus-like), geraniol (flowery, rose-like) and β-citronellol (citrus-like) have very low odor thresholds (5–10 µg/l) and even below these concentrations they positively influence the beer flavor through synergistic effects [49, 51]. The concentration decreases of geraniol in the course of geraniol metabolism listed in Table 8 are discussed in chapter 3.2.4., glycoside hydrolase activities are addressed in chapters 3.1.4 and 3.2.5.

3.2.4 Behavior of geraniol and β-citronellol

Several studies in the past three decades have documented interesting behavior of geraniol and β-citronellol during fermentation and maturation. However, there is still no systematic understanding of how yeast influences the concentrations of both compounds. Study results within the present dissertation provide important insights into the concentration changes that were also observed in previous investigations on beer fermentations. Lam et al. and Takoi et al. determined that the decrease in the concentration of geraniol or the increase in the β-citronellol concentration can be significant [67, 143]. What is remarkable about β-citronellol is that it is usually absent in worts or hop oils, but often present in beer. For trial fermentations using Citra hops, traces of β-citronellol in pitching wort increased to 10–20 µg/l during fermentation, whereas geraniol concentration decreased from 60–120 to 10–20 µg/l [49]. King et al. suggested that the reduction of geraniol to β-citronellol is part of the yeast´s geraniol metabolism [136]. They detected biotransformations of monoterpene alcohols in model fermentations including reductions, hydrations, and isomerization reactions. Based on the experimental data, they proposed a reaction cascade. In the context of transformation reactions, Sharp et al. also previously described a slight increase in β-citronellol concentration in dry-hopped fermenting wort [6]. However, indications were found that the changes in concentration are not necessarily based on the direct reduction of geraniol to β-citronellol [137]. In a study conducted as part of this dissertation, evidence was provided that the decrease in the concentration of geraniol does not necessarily lead to an increase in the β-citronellol concentration [156]. In model fermentations using malt extract wort, significant geraniol concentration decreases of up to 83 % (58.1 µg/l; Table 8) occurred, while a minor

increase in β-citronellol concentration was determined at maximum of 1.5 µg/l. It was concluded that the traces of β-citronellol have most likely originated from *de novo* synthesis (cf. chapter 3.2.3). Consequently, the geraniol could most likely be excluded from being a source of β-citronellol in the present test series. An explanation might be found in yeast genetics. In wine fermentation using *Saccharomyces cerevisiae* yeasts role of OYE2 gene on reduction of geraniol to β-citronellol was studied [162]. Wild type yeast strain not deleted from OYE2 gene showed comparably highest production of β-citronellol.

The most surprising aspect discovered in this particular study is the opposite effect of yeast metabolism on geraniol concentration in hopped wort [156]. In the absence of geraniol, the *de novo* production of the yeast was detected (cf. chapter 3.2.3). When adding a geraniol reference compound to the pitching wort, however, its concentration was reduced by the yeast geraniol metabolism, which is in line with earlier studies mentioned before. Since the concentration-reducing effect of the geraniol metabolism is comparatively stronger, the *de novo* production in beer fermentation is only noticeable by slightly decreasing the geraniol concentration. Another interesting finding in the present study was that the reduction was influenced by wort properties, which is an important insight in the field of geraniol metabolism. In model fermentations using worts of different original gravities 7, 12, and 18 °P, the reduction of geraniol concentration increased with the level of the original wort (Table 8). The highest decrease in geraniol concentration at 83 % was detected when fermenting wort of original gravity at 18 °P, the lowest was 36 % at 7 °P. In addition, the degradation rates varied between tested yeast strains; in fermentation of 18 °P wort, the maximum difference in degradation between the yeast strains TUM 193 and TUM 506 was 46 % (32.2 µg/l). Within the yeast selection tested, the strain TUM 506 provided indications that no decrease of geraniol concentration took place due to metabolic activity. In the worts fermented by the strain, a consistently low decrease in the geraniol concentration at 36–42 % was detected regardless of original gravity. Thus, in case of TUM 506, it was concluded that losses must have occurred besides those from biotransformations, e.g. minor volatilization. The impact of brewing yeast metabolism and the role of geraniol concentrations on hoppy beer flavors is discussed as part of a case study in the following chapter.

3.2.5 Case study of contribution of brewing yeast to citrussy dry hop flavor

It is now well established that brewer´s yeast can have a decisive positive impact on beer flavors, however, its influence on dry hoppy flavors has remained unclear. In the aforementioned study on brewing yeasts in the context of the present dissertation, the significant impact of an ale yeast strain (TUM 506) on the citrussy flavor of dry-hopped beers was the subject of further investigations [156]. Interestingly, even in unhopped TUM 506-beer

panelists determined decidedly citrus notes with an intensity of 2.0 on a 6-point scale (0–5; descriptive tasting). A clearer comparison is provided by intensities of 0.5 ± 0.2 in beers prepared using other yeast strains. A plausible explanation for this might be that TUM 506 formed fermentation products that led to a citrus-like beer flavor. Candidates would be flavor-active esters, e.g. ethyl-2-methyl butyrate, ethyl butyrate, ethyl hexanoate, and ethyl-3-hydroxy hexanoate [115], however, unfortunately there was no analysis of these compounds in beers.

In the context of citrussy in hopped beers, strain TUM 506 minimized the negative impact on geraniol concentration resulting from biotransformations. As previously mentioned, the yeast strain showed a relatively high *de novo* production at 4.0 ± 0.7 µg/l whilst showing comparably lower geraniol metabolism and resulting in a concentration decrease of hop-derived geraniol at only 42 % (at 12 °P; Table 8); decrease for the other tested strains was 68 % on average. In previous studies, geraniol has been identified as being a contributor to citrussy flavors of beers, even when present at minimal levels in the low microgram range (per liter) [49]. However, the influence of concentration changes in the context of geraniol metabolism on beer flavor has not yet been evaluated. A series of tasting-tests within a study for this dissertation showed that even small changes in concentration can have a strong impact on the beer flavor [156]. In a moderately hopped lager beer at low initial geraniol concentration (2.1 µg/l), a geraniol substance was added to achieve the target concentrations of 10, 20 or 40 µg/l in beers. As a result of the tastings, it was found that a slight increase in geraniol concentration to 10 µg/l already resulted in an increase in the aroma attribute “citrus-like” from 1.0 to 2.0 on a six-point scale (0–5). The increase to 20 or 40 µg/l resulted in 2.1 or 2.7 intensity points, respectively. Taking significant differences in geraniol reduction between the yeast strains at 4.9–32.2 µg/l into account (Table 8), the selection of yeast strains appears relevant in preventing losses of hopped-derived geraniol, and consequently, citrussy intensity in beer. In the context of other important monoterpene alcohols that contribute to citrussy flavor, concentrations of linalool and β-citronellol appeared to be equally influenced by the tested yeast strains. On the basis of the listed data and considering the hydrolytic enzyme activity with regard to geraniol precursors such as glycosides (cf. chapter 3.1.4), it is thought to be a consistent conclusion that the strain TUM 506 is particularly suitable to support the citrus aroma of dry-hopped beers. A recently published study on dry-hopped beers (NEIPA) produced by nine different yeast strains confirmed variable contribution of different yeasts to fruity (and juicy) flavors and in some cases even suppression of mentioned attributes [128]. Variable impact on concentrations of flavor-active hop components and/or synergistic effects of fermentation products and hop oil constituents were derived to be major influencing parameters for resultant beer flavors.

A possible explanation for differences in flavor formation during brewing yeast fermentation might have been provided by researcher groups using population genomics to study the domestication of beer and wine yeasts [163, 164]. They found that yeast strains that are used today for brewing beer were developed from a variety of different trajectories and led from a wild form to a contemporary brewer´s yeast (= domestication). The result of the different family trees is that the flavor impression that a certain yeast strain creates in the fermentation of a beer wort is characteristic, and consequently, differs from other yeast strains. Within the previously mentioned study the yeast strains tested were selected based their high diversity, which is one explanation for the different influences on flavor formation in the fermentation of dry-hopped wort. The present study has provided a deeper insight into the interactions of brewing yeast and hop-derived components that influence citrus flavor of dry-hopped beers. However, brewing yeasts could influence concentrations of hop-derived compounds in several further ways. Steyer et al. analyzed the influence of brewing yeast and hop variety on 39 volatile compounds in beer [127]. Nine out of 39 compounds (including monoterpenes), were influenced only by hop variety, two by yeast only (isoamyl alcohol, styrene), and the remaining compounds by both hop and yeast. The brewing yeast metabolism activities include the production of various different enzymes and consequently, they can acetylate (ATF1/ATF2), decarboxylate (PAD), or reduce (OYE2) several of the volatiles or precursors of the volatiles found in beer [165, 166]. Another possible explanation for the variability in the impact of yeasts on volatile compounds is the mutation in the enzyme gene(s) involved in these pathways [127]. This approach could also be used to interpret the results of this study conducted in the course of the dissertation.

3.3 Effect of dry-hopping process parameters on the extraction of volatiles

The method of dry-hopping offers a highly effective measure to create intense hoppy beer flavors that can even be used as a style-defining element. However, controlling the extraction of flavor components via process management, which is an important goal from a quality assurance perspective, is a major challenge for brewers. Indeed, in the context of the fate of hop-derived components in the cold area, several key questions about the extraction and solubilization of oil constituents remain unresolved. The following discussion refers to the potential to influence the extraction of volatile hop components during static dry hopping via process control and the basic beer.

3.3.1 Hop addition timing and impact on flavor

Hops can be added during several process steps in the cold area, basically, to the pitching wort, the green beer and the maturated beer. Obviously, major differences in extraction properties occur in potential matrices regarding the basic beer temperature, ethanol or carbonic acid content. However, there is no comprehensive analysis of the impact of dry hopping addition timing on volatiles. Briefly summarizing the insights of previously mentioned studies conducted in the course of the present dissertation: processes of loss of hop terpenes do clearly depend on the timing of hop-addition, this applies in particular to additions during ("early" timing) or after the main fermentation. Firstly, this is due to the process vessels varying between "open", which enables volatilization as previously mentioned (cf. chapter 3.2.1), and "closed" for lagering ("late" timing). Secondly, a negative impact of yeast biomass concerns hop additions before yeast separation (cf. chapter 3.2.2).

For monoterpene alcohols concentrations, the dry-hop addition timing does not have a comparably uniform and significant impact. As previously mentioned, during fermentation, maturation, and lagering, the concentration of several monoterpene alcohols can change, and not always in the same direction (cf. chapters 3.1/2). To gain greater clarity, a study in the context of this dissertation focused on the fate of selected terpenoids derived by different dry hopping regimes [156]. Three hop varieties, three hop addition timings and six yeast strains (Table 8) were used in a series of single-hop and -addition experiments. It was found that the average concentration of the sum of linalool, geraniol, β-citronellol, nerol and α-terpineol in dry hopping during the main fermentation (15–20 °C; addition during pitching) was 33 % lower than dry hopping during cold maturation (1 °C; closed system) using the same dosing amount of hops. For esters, the sum of isobutyl isobutyrate, methyl hexanoate, methyl heptanoate, methyl octanoate, and methyl-4-decenoate was 30 % lower at the "earlier" dry hopping timing. Other alcohols and esters and additional terpenoid groups such as ketones, are thought to undergo similar concentration losses. As a result, despite concentration-increasing reactions in the case of the monoterpene alcohols (e.g. precursor hydrolysis, *de novo* synthesis; cf. chapters 3.1.4, 3.2.3), the final concentrations in beers that are dry hopped during the main fermentation are significantly lower than beers dry hopped in the subsequent process step. The present study showed that losses of terpenoids vary with dry hop addition timing, although at lower levels than terpenes. In a comparison of both compound groups, it was found that minor losses of terpenoids are based on their relatively higher polarity. The findings herein are in line with previous study results. Takoi et al. determined a significant decrease in the concentrations of α-terpineol and nerol during the main fermentation [137]. The same group detected a halving of the linalool concentration during the main fermentation [49]. Previously, a decrease in monoterpene alcohols during fermentation was attributed to

yeast metabolism and adsorption on the yeast cells [94], although investigations conducted as part of this dissertation showed that adsorption processes of these substances can be assumed as being unlikely (cf. chapter 3.2.2). Concentration decrease in case of esters might be attributed to degradations induced by yeast-derived esterase activities [83].
It is difficult to associate these findings with a specific impact on beer flavor, as a perceived flavor is basically the result of the amount and composition of flavor-active compounds, and both the amount and composition of flavorings differ between various dry hopping timings. A cautious approach to interpret the flavor impact would be that “early” dry hopping helps to emphasize fruity, citrus-like or floral contributions of a hop variety. Taking into account the clear influence of the addition timing on the relationship between two groups of substances, terpenoids and terpenes, early addition enhances the contribution of terpenoids to beer flavor. The representatives of the terpenoids, e.g. alcohols, and esters of hops can be roughly categorized as fruity, citrus-like or floral as mentioned before, in contrast to other categories green-grassy, spicy and vegetal. For citrussy this statement is supported by tasting results within the aforementioned study that include the varieties Hersbrucker (HE), Mandarina Bavaria (MB), and Hallertauer Magnum (HM) in the context of this dissertation [156]. Citrus intensity in test beers dry-hopped at the “early” timing using MB were rated highest (≥ 2.5; 6-point scale) when they were produced using ale-strains (TUM 506, TUM 511) or a lager beer strain (TUM 34/70). A conclusion of study results regarding flavor impressions that are typical of terpenes, such as “herbal”, and “balsamic” (e.g. β-myrcene, β-pinene [32]) would be that those exclusively occur in “late” dry-hopped beers [159]. Previous studies consistently determined significant amounts of terpenes exclusively solubilized during the dry hopping of bright beer [5, 95]. Thus, based on the presented findings and earlier reports adding hops after the yeast cells harvest appears to be an effective measure to impart green and resinous flavors to beer.

3.3.2 Hop variety and dosage amount

Hop varieties can be distinguished based on their chemical composition, which is a result of genetically controlled factors. However, there is no systematic investigation into varietal influences on extraction efficiency during dry hopping. In the context of varietal impacts, dry-hopping experiments conducted in the course of this dissertation showed that the transfer rates fluctuated to some extent between hop varieties. The hop variety with the highest α-acid oil-content ratio (Hallertau Blanc: 0.74 % α-acid $\mu l^{-1} g$) caused the lowest increase in oil (269 µg/l) and the varieties with lower α-acid oil-content ratio (Cascade or Eureka!: 0.64 % α-acid $\mu l^{-1} g$) led to higher increases in oil concentration (Cascade: 357 µg/l; Eureka!: 311 µg/l). These findings suggest that a higher content of α-acids could negatively affect the extraction of the volatile hop components. Based on the previously discussed log K_{OW} model, an

explanatory approach would be based on the solubility of the oil in solutions that contain both a polar and a non-polar phase; accordingly, the relatively hydrophobic oil could be “retained” by higher contents of the relatively non-polar α-acids in the hop matrix and be solubilized by the polar beer phase in comparatively smaller amounts [152]. However, with a small sample size, caution must be applied, as these findings might not be applicable to other hop varieties. Previously, Engstle et al. detected a comparatively low swelling volume of pellets of resin-rich hop varieties and concluded that this can result in a lower yield of volatile substances during dry hopping [109]. A varietal impact on the hop pellet swelling volume, and therefore extraction, was reported in other recent study [110].

Obviously, the dosage amount of hops as the major source of oil is an important factor for the content of oil in beer. In the context of absolute oil concentrations, studies confirm expectations that the increase in the amount of hop dosage causes an increase in the concentration of hop oil components, and consequently an intensification of hop flavor attributes in beer [112, 113]. Furthermore, indications were provided regarding a decrease in the oil yield within hop dosage increase [114]. However, as the previous study has suffered from considerable design limitations, transfer rates in the context of hop dosages were also the subject of investigations conducted as part of the present dissertation. What was surprising in these investigations is that dosage amount and concentration changes were inconsistent in terms of the oil constituents [152]. An increase in the pellet dosage (Tettnanger “TE”) by a factor of 5.0 (dosages based on oil in beer: 0.5–2.5 ml/hl) only led to an increase in the concentration of β-myrcene by a factor of 1.7. Conversely, the linalool concentration increased by a factor of 3.0. As previously mentioned, the two substances can be used as representatives of the relatively hydrophobic or hydrophilic flavorings of hops. Firstly, this example clearly illustrates that higher amounts of hop material lead to lower transfer rates of oil constituents. For terpenes in particular, an important insight is that considerably higher hop dosages will not significantly increase their contents in beer. Secondly and most interestingly, there is a shift in the oil composition in beer with an increase in dosage. Thus, increasing the dosage will lead to a higher proportion of esters and monoterpene alcohols, e.g. isobutyl isobutyrate, linalool and geraniol, compared to mono- and sesquiterpenes. Consequently, the hop-derived flavor in beer is expected to change towards fruity, floral and citrus-like flavors when increasing dry hopping rates. The differences in dosage effects between compound groups are again based on the different solubility properties in beer (cf. chapter 3.1.1).

In short, besides variety and amount, the type of hops is crucial for the flavor impact due to dry hopping. Pelletized hops are primarily used for dry hopping in comparison to the studies mentioned in this chapter. When using pellets, higher transfer rates are to be expected compared to cone hops, as already mentioned in chapter 3.1.1. In the pelletizing process the

hop lupulin glands are squeezed, i.e. the cell membranes are broken [1, 106]. In chemical engineering, the extraction of squeezed membranes, which could be considered leaching, is described as more efficient compared to extracting intact cell membranes [167].

3.3.3 Basic beer temperature and ethanol content

In previous studies, several basic beer parameters were suggested as being potential influencing factors on the result of dry hopping. However, most of these investigations have suffered from methodological limitations or depth of analysis. In the context of the present dissertation, the basic beer temperature and ethanol content appeared to be most effective in influencing the (static) dry hopping result [152]. Various test media temperatures (1, 4, 20 °C) and ethanol contents (0.1, 5.0, 8.1 % w/w) were selected in dry hopping experiments using TE pellets type-90, as those parameters can occur when dry hopping during fermentation, maturation or lagering. In case of β-myrcene, the concentrations were increased significantly by a factor of 3.2 or 3.4 as a result of the increase in temperature from 1 to 20 °C (basic beer: 0.1 % EtOH w/w) or ethanol content from 0.1 to 8.1 % EtOH w/w (basic beer: 1 °C), respectively. This is a remarkable finding considering the minor levels of this kind of compound usually found in beer (cf. chapters 3.1.1, 3.2.1/2). Under the same experimental conditions, the linalool concentrations only increased by a factor of 1.2 or a factor of 1.6. The results confirm a minor increase in the linalool concentration when comparing different base beer temperatures. Previously, base beer temperatures in a range between 0 °C and 20 °C during dry hopping were tested [99, 105, 107]. The results show that different temperatures or ethanol contents during dry hopping similarly lead to significant concentration changes of hop oil constituents in beer. Interestingly, apart from differences in the levels of a compound, individual impacts on the transfer rates of hop volatiles will lead to shifts in the composition of hop oil in beer. Due to different compound properties, an increase in each of the parameters will shift the ratio independently in favor of mono- and sesquiterpenes. The explanation for this would be that the rise in the dissolving capacity of the base beer via the increase in temperature or the improved solvent properties due to the higher ethanol content, will have a comparably stronger effect on relatively hydrophobic substances [168]. An important insight derived from the obtained results is that the same dry hopping regime used with different basic beers, e.g. non-alcoholic or strong beer, will result in different hop oil compositions, and consequently in different flavors. In a nutshell, dry hopping strong beers will emphasize certain flavor categories, e.g. “herbal”, and “green”.

In a case study part of this dissertation comparing the dry-hopping results of lager beer (5.0 % EtOH w/w) or high-gravity beer (8.1 % EtOH w/w) at low (1 °C) or high (20 °C) base beer temperatures, higher ethanol content and temperatures resulted in an increase of the β-myrcene transfer rates by a factor of about 2.9, 2.7, and 2.8, using Hallertau Blanc, Cascade, and Eureka!, respectively [152]. With regard to the impact of hop variety on transfer rates discussed in chapter 3.3.2, adjustments had slightly different influences on transfer rate increases. This chapter presented opportunities to influence the extraction of the hop oil components via the base beer. However, in the context of minimal transfer levels below 3.0 % in the case of β-myrcene, despite adjustments to process parameters, there are obvious limitations to extraction during dry hopping (cf. chapter 3.1.1).

4 Conclusions

The present dissertation disclosed that the concentrations of volatile hop components in dry-hopped wort or beer are essentially determined by the composition of the hop material, the brewer´s yeast including fermentation products and the process of dry hopping itself. The effect of dry hopping was examined using volatile aroma components in hops and beer. The categorization of the transfer rates of oil constituents based on the octanol-water partition coefficient model served as an explanatory approach for their disproportional extraction from hops. The high-quality hop samples from the standardized field tests provided evidence that no qualitative losses in the beer are to be expected using hops for dry hopping having an increased proportion of seeds. It was confirmed that hop contain glycosidic flavor precursors and different ways were highlighted out of releasing aglycones during dry hopping. In the in-depth analysis of the obtained hop samples, β-glucosidase activity was identified. Thus, an alternative source of enzymes was described, which have a hydrolyzing effect on glycosidically bound flavor precursors. The activity levels of the glycoside-hydrolyzing enzymes from hops and the brewing yeasts of the studies in the context of the present dissertation were comparatively minor. Therefore, the impact of hop- or yeast-derived enzymes on the hydrolysis of glycosides is regarded as being low. In the context of glycosides, the present research project failed to assign the proportions of flavorings present in beer, e.g. linalool and geraniol, to the potential sources of hop oil or flavor precursors. This kind of information would be helpful to more precisely define flavor-relevant processes during dry hopping.

Brewer´s yeast was identified as being an important factor that influences the concentrations of volatile hop components. Different metabolic reactions and interactions caused changes in concentrations, especially of monoterpene alcohols. Concentration-increasing effects were firstly determined in case of *de novo* synthesis, and new insights into the geraniol metabolism were presented. Geraniol concentration decrease occurred was found to be yeast strain-specific and influenced by the wort original gravity. As a result of the specified yeast activities, possible impacts on dry hoppy beer flavor were consistently demonstrated, especially in the case of citrussy notes. Further yeast activities were proven to influence terpenes. It was confirmed that adsorption on the yeast cell surface and volatilization are major factors for significant losses of monoterpenes such as β-myrcene during the main fermentation. With regard to the dry hopping regime, hop additions during main fermentation led to losses of terpenoids by one third compared to additions to matured beer. Terpenes were only solubilized in significant amounts when adding hops to matured beer. It was concluded that in general, early dry-hopping additions will emphasize floral, fruity and citrus-like flavors, whereas only late additions will lead to herbal, and resinous beer flavors. Investigating

different hop varieties showed that aroma-, bitter- and dual-purpose hops are equally suitable for dry hopping, and only a slight decrease in the transfer rates of samples with high α-contents together with low oil contents was identified. The possibilities and limits of increasing the extraction of the hop flavor components process parameters within a static dry-hopping regime were clearly demonstrated. Besides the hop dosage amount, the temperature and the alcohol content of the base beer were determined as effective parameters to influence dry hopping result. Adjustments to the dosing and the two basic beer properties mentioned previously not only influenced the concentrations of the oil components in beer, but also their composition. Consequently, various options were provided to influence the dry hoppy flavor of beer. This research paper sheds light on the complex relationship between the hop raw material, yeast metabolism activities, dry-hopping regime and the contents of hop flavorings in final beer. The presented insights into flavor-relevant factors of the dry hopping process can help to shape beer flavor.

5 Outlook

The investigation into the factors that influence the concentration of volatile hop aroma components in the cold area is a challenging task. Reactions with opposite effects can take place simultaneously, particularly in the case of dry hopping during fermentation. Concentrations of a compound such as geraniol can decrease in the course of yeast metabolism, and effects that increase this concentration can also occur due to transformations of geranyl acetate or glycosides. In order to unequivocally determine the relevant processes for the dry hop aroma, model tests were carried out in the context of this dissertation in which, for example, unhopped malt extract wort was used to study *de novo* synthesis (cf. chapter 3.2.3) or reference substances to study geraniol metabolism (cf. chapter 3.2.4) instead of wort or hop material. In all model and authentic dry-hopping approaches, standardized laboratory-scale test setups were used. When up-scaling dry-hopping attempts, it must be taken into account that there may be changes in the beer aroma. The impaired homogenization of hop pellets in storage vessels on an industrial scale has been described as causing reduced extraction rates [100]. Nevertheless, studies should also be carried out on an industrial scale in order to test the effects of the identified factors on the beer flavor outside of a laboratory environment. In the context of industrial brewing, the beer production steps of stabilization, filtration, bottling, and also storage may further influence the concentrations of hop volatiles in beer [169, 170], and should therefore be considered in future studies in the field of dry hoppy beer flavor.

The fact that several flavor-relevant processes take place at the same time in dry hopping, e.g. adsorptions, vaporizations, glycoside hydrolysis, and previously mentioned reactions must also be considered when investigating process parameters. Changing a parameter can influence both, the extraction properties and biochemical reactions in the beer matrix. In the case of an increase in the temperature of the base beer, this may firstly lead to an increased capacity to dissolve hop components, and, secondly, to increased enzyme activity, e.g. β-glucosidases are to be expected [145]. Accordingly, it would be interesting to investigate whether an increased β-glucosidase activity in dry hopping leads to an increase in the yield of aglycones. In addition to the base beer temperature, the effect of the enzymes introduced via yeast metabolism or hop material could be influenced by further adjustments, e.g. the base beer pH, and agitation. It is advisable to expand the investigations of enzyme activities to other enzymes that may potentially influence concentrations of flavor components. β-lyases are of particular interest, as they can release sulfur-containing volatiles by breaking cysteine bonds [134]. Among them, polyfunctional thiols such as 4MSP or 3SH were identified, which are known to be flavor-active in beer even in the low ppt range [55]. Recently, in a research project conducted in the broader scope of the present dissertation, it was demonstrated that many

brewer´s yeasts have the genetic prerequisite to form β-lyases, and actually showed these enzyme activities in model fermentations [171]. Thus, by using a particular yeast strain in beer production, there appears to be plausible impact on the thiol profile of dry-hopped beers. In another recent study it was discussed that certain yeast strains could release comparatively higher levels of thiols, e.g. 4MSP and 3SH, although proof was not provided [141].

The octanol-water partition coefficient model for transfer rates of volatile substances in dry hopping proved to be a useful explanatory tool. Therefore, it should be expanded to include other substances that can also influence beer flavor. Substances at log K_{OW} levels between 4.5–6.3 (e.g.: β-eudesmol: 4.88; α-copaene: 5.71 [172]) would be particularly suitable candidates, since there is no test data at those levels in the present model. In the context of the present dissertation, the volatilization of β-myrcene during the main fermentation was detected and interpreted as a result of its relatively poor solubility in aqueous solvents. However, quantitative data on the losses of hydrophobic substances would be a further step towards a better understanding of processes during dry hopping. Due to the differences in polarity of hop oil constituents, the solvent used in bubbling columns should be adapted for non-polar volatiles in further studies. For example, hexane would be suitable to study β-myrcene. In future studies on fermentation gases, different geometries and scales of the fermentation vessels should be tested. Both factors influence the convection stream of the fermenting wort, which in turn influences the volatilization of flavor components [119]. High circulation rates of fermenting wort can lead to increased outgassing, which is known in the case of isoamyl acetate, for example, during wheat beer production [173]. Furthermore, the future use of innovative analytical methods and the establishment of previously unused methods, such as the electronic nose [174], could help to gain new insights into the flavor of dry-hopped beers.

6 References

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7 Appendix

7.1 Non-reviewed papers

- Haslbeck, K.: Geschmacksstabilität des Bieres – Mögliche Optimierungsmaßnahmen in der Brauerei, Der Weihenstephaner 3 (2014), no. 82, pp. 118-121.
- Haslbeck, K.; Englmann, J. and Jacob, F.: Bedeutender Kostenfaktor Würzekochung – Beurteilungskriterien für Würzekochsysteme Teil 1, Brauindustrie 5 (2016), pp. 20-24.
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- Haslbeck, K.; Minkenberg, D.; Zarnkow M.; Jacob F. and Coelhan, M.: Möglichkeiten und Grenzen der Kalthopfung – Was verrät der Octanol-Wasser Verteilungskoeffizient über das Hopfenaroma?, Der Weihenstephaner 4 (2018), no. 86, pp. 159-163.
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- Haslbeck, K.; Bub, S., Schönberger, C., Zarnkow, M., Jacob, F., Coelhan, M.: Volatile hop aroma substances in the brewing process – Loss processes, Brauwelt International 3/19, June, Volume 37 (2019), pp. 197-200.

7.2 Oral presentations with first authorship

- Haslbeck, K.; Riedl, R.; Koob, J. and Hutzler, M.: Dry Hopping – eine Bewertung aus mikrobiologischer Sicht, 2. Seminar Hefe und Mikrobiologie, Freising Weihenstephan, 2014.
- Haslbeck, K.: Beurteilungskriterien für Würzekochsysteme, 10. Praxisseminar, Rosenheim, 2015.
- Haslbeck, K.: Einfluss von Hefestämmen und Hopfungstechnologien auf den Biercharakter, 11. Praxisseminar, Ingolstadt, 2016.
- Haslbeck, K.: Hopfen- und Hefeinteraktion – Auswirkungen auf das Aroma, 5. Seminar Hefe und Mikrobiologie, Freising Weihenstephan, 2017.
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- Haslbeck, K.; Bub, S.; Zarnkow, M.; Jacob, F. and Coelhan, M.: On the fate of β-myrcene and monoterpene alcohols during fermentation, 39th Brewing and Malting Seminar, Pilsen, Czech Republic, 2018.
- Haslbeck, K.; Zarnkow, M. and Coelhan, M.: Transfer flüchtiger Aromakomponenten bei der Kalthopfung, Fa. Hopsteiner, Mainburg, 2019.
- Haslbeck, K.: Rules and challenges of dry hopping, Czech Beer Forum, Prag, Czech Republic, 2020.
- Haslbeck, K.: Challenges of dry hopping, Czech Beer Forum, Brno, Czech Republic, 2020.

7.3 Presenting author of joint project

- Michel, M.; Haslbeck, K.; Cheng, A.; Zarnkow, M.; Coelhan, M. and Jacob, F.: Yeast strain specific release of cystein-conjugated thiols like 4-MSP by β-lyase activity 37th EBC Congress, Antwerps, Belgium, 2019.

7.4 Poster presentation with first authorship

- Haslbeck K.; Minkenberg D.; Zarnkow M. and Coelhan M.: Dependency of transfer of flavor-active compounds during dry-hopping on the octanol-water partition coefficient, Trends in Brewing, Ghent, Belgium, 2018.

7.5 Permission of publishers for imprints of publications

Haslbeck, K.; Jerebic, S. and Zarnkow, M.: **Characterization of the unfertilized and fertilized hop varieties Progress and Hallertauer Tradition – analysis of free and glycosidic-bound flavor compounds and β-glucosidase activity**, Brewing Science, 70 (2017), no. 11/12, pp. 148-158, DOI: 10.23763/BrSc17-15haslbeck

Haslbeck, K.; Bub, S.; Schönberger, C.; Zarnkow, M.; Jacob, F. and Coelhan, M.: **On the fate of β-myrcene during fermentation – the role of stripping and uptake of hop oil components by brewer´s yeast in dry-hopped wort and beer**, Brewing Science, 70 (2017), no. 11/12, pp. 159-169, DOI: 10.23763/BrSc17-16haslbeck

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Sehr geehrter Herr Haslbeck,

hiermit erteilen wir Ihnen die Genehmigung zur Zweitverwertung folgender Artikel für Ihre Promotion:

Haslbeck, K, Jerebic, S., Zarnkow, M.: Characterization of the Unfertilized and Fertilized Hop Varieties Progress and Hallertauer Tradition _ Analysis of Free and Glycosidic-Bound Flavor Compounds and β-Glucosidase Activity, in: BrewingScience Vol. 70, (November/December 2017), S. 148-158

Haslbeck, K, Bub, S, Schönberger, C., Zarnkow, M., Jacobs, F., Coelhan, M.: On the Fate of β-Myrcene during Fermentation – The Role of Stripping and Uptake of Hop Oil Components by Brewer's Yeast in Dry-Hopped Wort and Beer, in BrewingScience Vol. 70, (November/December 2017, S. 159-169.

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