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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).

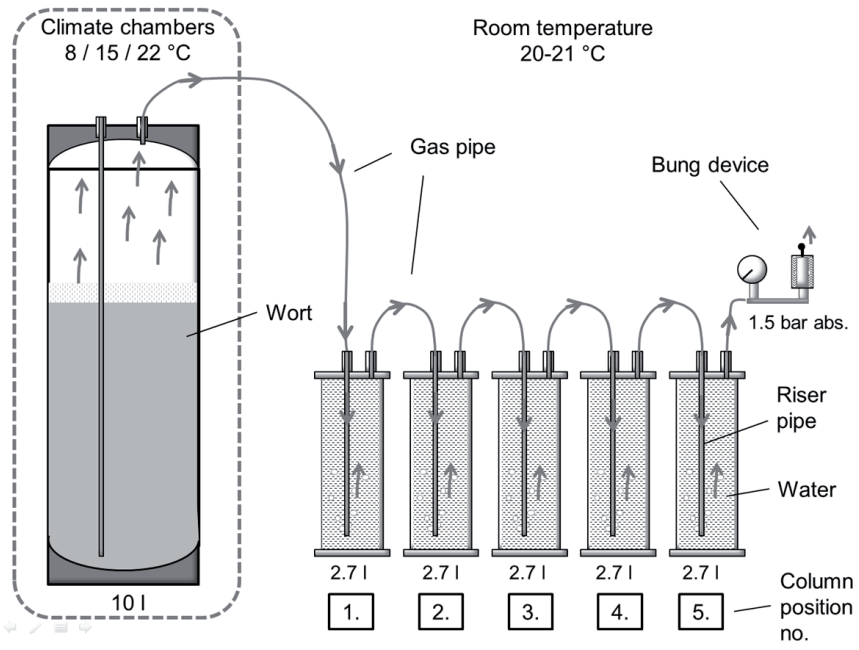

Fig. 1 Experimental set-up for the dissolution of volatiles in beer wort headspace by five bubbling water columns connected in series

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

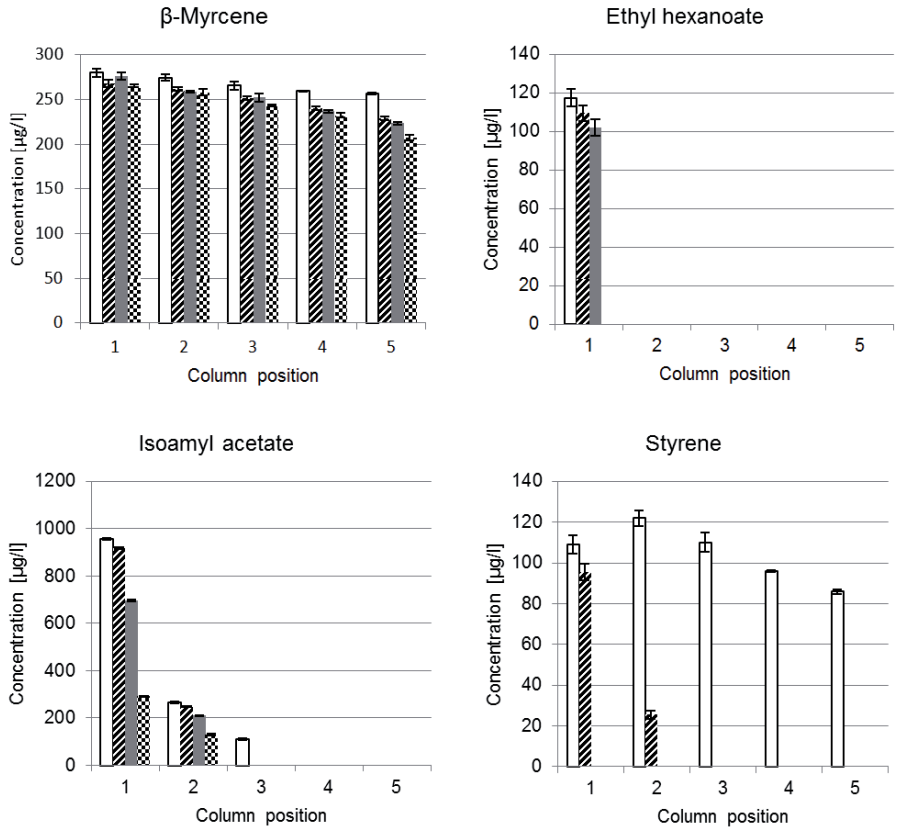

Fig. 2 Contents of four compounds in bubbling water columns originating from the headspace of wort during fermentation in µg/l; TUM 68, 22 °C; TUM 68, 15 °C; TUM 34/70, 15 °C; TUM 34/70, 8 °C

(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

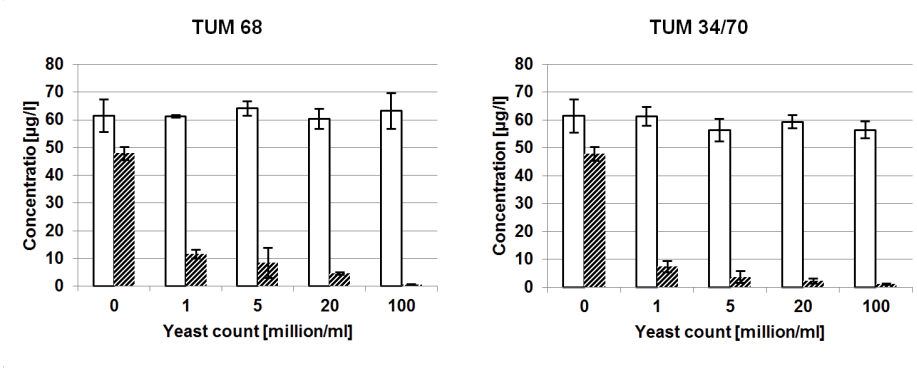

Fig. 3 **Contents of ☐ linalool and ▨ β-myrcene [µg/l] in lager beer after contact with different yeast counts TUM 68 (left) and TUM 34/70 (right)**

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.

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