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De novo Formation of Sesquiterpene Oxidation Products during Wort Boiling and Impact of the Kettle Hopping Regime on Sensory Characteristics of Pilot-Scale Lager Beers

Many brewers aim at a balanced 'kettle hop' aroma in their lager beers and therefore add aroma hops to the boiling kettle. Whereas the application of 'late' hop additions to acquire an intense 'kettle hop' aroma with a 'floral/citrusy' bouquet is scientifically quite understood, brewers have also been adding rather expensive (European/noble) aroma hops at the onset of boiling in an empirical way to impart 'noble kettle hop' aroma, typically described by delicate 'spicy/herbal' notes, to their beers. Although many researchers suggested generation of hop oil-derived terpene oxidation products during wort boiling and associated oxygenated sesquiterpenoids with these refined 'spicy/herbal' notes, actual *de novo* formation of such compounds during wort boiling has up to date not been proven unambiguously in real brewing practice and consequently, there remain many questions with regard to this subject. This study tackles this problem by investigation of 4 conventionally hopped lagers, thereby varying the time point of hop addition (pellets cv. Saaz). HS-SPME-GC-MS analysis of samples taken along the wort boiling process of an 'early' hopped beer revealed *de novo* formation of oxygenated sesquiterpenoids. The impact of the hopping regime on the hop-derived flavour of the beers was demonstrated via sensory analysis by our taste panel. The 'early' hopped beer clearly expressed 'spicy/herbal' aroma. These notes were also clearly detected in the beer hopped with a combination of 'early' and 'late' hopping, and, moreover, this beer expressed 'floral/citrusy' notes and was scored highest for both 'kettle hop' flavour and general appreciation. Our observations suggest that expression of 'noble kettle hop' aroma characteristics in lager beer might not simply be dependent on the absolute level of (flavour-active) oxygenated sesquiterpenoids present, but also on the ratio of volatiles imparting 'floral' aroma and 'spicy' aroma.

Descriptors**:** Kettle hop aroma, kettle hopping, wort boiling, whirlpool, oxygenated sesquiterpenoids, HS-SPME-GC-MS

1 Introduction

Many researchers and brewers agree that a fine and balanced 'noble kettle hop' aroma is an essential quality characteristic of lager beer. Especially for traditional Pilsner-type beers, usually produced by higher amounts of hop compared to lager beer [1], a fine hop aroma can be regarded as 'the soul' of the beer [2]. 'Kettle hop' flavour has been defined as the hop-derived flavour of beer, obtained by boiling of hop cones or pellets and subsequent fermentation [3]. Especially 'noble' kettle hop aroma which is obtained after vigorous boiling of 'noble/European' aroma hops, has been associated with 'spicy/herbal' and 'fragrant' notes [4], whereas late-hopping increases these notes and adds 'floral', 'citrus' and 'resinous' notes [5].

Many parameters, such as hop variety, growing region, hop product and hopping regime, influence hop flavour in beer and the time point of hop addition is decisive in this regard [6–9]. The impact of 'late' and 'whirlpool' hopping technologies on 'hoppy' flavour is scientifically quite understood and linalool has been proven to be an important contributor to the resulting 'floral' notes [10–13]. On the other hand, insights into 'early' hopping and the resulting 'spicy/herbal' aspect of 'kettle hop' flavour appear to be more elusive. Humulene and caryophyllene oxidation and hydrolysis products have been linked to the 'spicy/herbal' notes typical for 'kettle hop' aroma [10, 14–17] and recent studies by our research group demonstrated a cause-effect relationship between the presence of these compounds and expression of 'spicy/herbal' and 'woody' notes in beer [18–20]. These oxidation products were proven to be formed upon lab scale boiling of total hop essential oil and hop oil-derived sesquiterpene

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hydrocarbons. Although many researchers suggested that they might also be formed during wort boiling [10, 21–23], *de novo* formation of sesquiterpene oxidation products has, up to date, not been unambiguously demonstrated during brewing practice. The impact of addition of hops at the onset of wort boiling on 'kettle hop' flavour has even been questioned. *Meilgaard* and *Peppard* stated that beers resulting from this hopping practice would rarely exhibit any appreciable degree of hop character [24] and research results from Kaltner and coworkers would point to the fact that oxidation products are not involved in contributing to hop aroma in beer [6, 25, 26]. *Fritsch* and *Schieberle* did not detect additionally formed compounds as an effect of 'early' kettle hopping and stated that this result is contradictory to the often discussed formation of new odour-active compounds when hops are boiled [27]. Summarised, the impact of 'early kettle' hopping with regard to generation of new odorants and the 'hoppy' flavour in the final beer remains a matter of debate.

To shed light on this complex issue we have been conducting lab scale boiling experiments with total hop essential oil (cv. Saaz) in simplified model solutions [19]. We demonstrated a general increase in the level of spicy compounds which was attributed to oxidation of sesquiterpene hydrocarbons, and, also pinpointed differences between the hop oil-derived fingerprint of volatiles in unboiled and boiled hop essential oil dilutions. Boiled hop essential oil was spiked to non-aromatised iso-α-acid-bittered beer, and, remarkably, this beer expressed 'spicy' and 'hoppy' notes. Moreover, many of the α-humulene and β-caryophyllene oxidation products were previously detected in flavour-active zones upon GC-O analysis of a spicy fraction derived from a commercial kettle hopped beer, suggesting relevance for real brewing practice [28]. Our observation indicated that increases in levels of sesquiterpene oxidation products as a consequence of boiling might play a role into development of 'kettle hop' aroma. In our following study [20], we further focused on these sesquiterpene oxidation products by isolation of a sesquiterpene hydrocarbon fraction from total hop essential oil cv. Saaz, lab scale boiling of this fraction and subsequent isolation of the newly formed sesquiterpene oxidation products. The resulting fraction, which consisted of various α-humulene and β-caryophyllene oxidation and hydrolysis products, was added to non-aromatised iso-α-acid bittered lager beer and clearly resulted in a shift of the flavour profile towards 'woody', 'spicy' and 'hoppy' notes. This sesquiterpene oxidation product fraction, which expressed interesting sensory characteristics, was further investigated via GC-O analysis, revealing two highly flavour-active intervals in which humulene epoxide III/humulenol II/caryophylla-4(12),8(13)-diene-5-ol and (3Z)-caryophylla-3,8(13)-diene-5-ol (α and β)/14-hydroxy-β-caryophyllene eluted. In our current study, we aim at verifying our results, obtained on a lab scale, in real brewing practice. Four different conventionally aromatised lager beers are prepared at our pilot-scale brewery and exclusively hopped with a noble hop variety (cv Saaz), varying the time point of hop addition. Samples are taken along the wort boiling and whirlpool process and analysed via HS-SPME-GC-MS, aiming at obtaining insights into the behaviour of hop oil-derived volatiles during these processes. To investigate the impact of the hopping regime on the 'hoppy' flavour in those beers, sensory evaluation by our trained taste panel is performed.

2 Materials and methods

2.1 Chemicals

The following reference compounds were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade: 2-decanone (99.5 %); 2-dodecanone (97.0 %); 2-heptanol (98 %); 2-nonanone (99.5 %); 2-tridecanone (97.0 %); 2-undecanone (99.0 %); caryophyllene oxide (≥ 99.0 %); decanal (≥ 98.0 %); geraniol (≥ 99.0 %); limonene (97.0%); linalool (98.5 %); methyl 3-nonenoate (99.8 %); methyl decanoate (99.5 %); methyl geranate; methyl nonanoate (99.8 %); methyl octanoate (99.8 %); nerol (≥97.0 %); ocimene (≥ 90.0 %, mixture of isomers); p-cymene (≥ 99.0 %); terpinen-4-ol (≥ 95.0 %); terpinolene (≥ 90.0 %); trans-β-farnesene (≥ 90 %); α-copaene (≥ 90 %); α-humulene (≥ 98.0 %); α-pinene (98.0 %); β-caryophyllene (≥ 98.5 %); β-damascenone (≥ 98.0 %); β-ionone (≥ 97.0 %); β-myrcene (≥ 95.0 %); β-pinene (99.0 %); γ-terpinene (≥ 97.0 %).

For additional confirmation of tentative identification of oxygenated sesquiterpenoids, reference mixtures of α-humulene, isocaryophyllene and β-caryophyllene epoxidation products were prepared (resp. code HEP, IEP, CEP). α-humulene and β-caryophyllene epoxide rearrangement products were obtained via acid-catalysed rearrangement (resp. code HHP and CHP) and allylic alcohols were prepared by photosensitised oxidation of α-humulene and β-caryophyllene (resp. code HAA and CAA). We refer to our previous papers for these procedures [19, 20].

Ethanol absolute (EtOH) (≥ 99.8 %) was purchased from VWR International (Zaventem, Belgium); Milli-Q water was obtained from a Milli-Q purification system (Synergy 185, Millipore S.A., Molsheim, France); Sodium chloride was purchased from Merck (for analysis, 1 kg, Darmstadt, Germany).

2.2 Plant material

Hop pellets T90 cv. Saaz (crop year 2014) were kindly provided by the Barth-Haas Group (Joh. Barth & Sohn GmbH & Co. KG, Nürnberg, Germany). Pellets (5 kg) were vacuum packed in laminated foils with an aluminium layer as a barrier to prevent oxygen diffusion and, stored in the freezer (–18 °C) to avoid oxidative degradation of hop oil compounds.

2.3 Hop oil content determination via steam distillation

The hop oil content of T90 pellets cv. Saaz was determined on the basis of the IOB method 6.3 using steam distillation. There proved to be 0.50 mL hop oil per 100 g pellets (n = 8, CV = 0.3%).

2.4 Preparation of pilot-scale lager beers

Five pilsner beers were prepared at the pilot brewery (4-hL scale) of KU Leuven (lab EFBT, Technology Campus Ghent, Belgium). The brewing installation is a prototype for innovative wort production as described by *De Rouck* et al. [29]. Four beers were hopped by addition of hop pellets (noble hop variety cv. Saaz) to the boiling kettle, whereas one beer was exclusively bittered with iso-α-acids

(beer ISO) and used as reference. In order to understand the impact of the hopping procedure on the hop oil-derived spectrum of volatiles and flavour characteristics of the resulting beer, the time point of hop addition of the 4 lagers was varied (hop additions standardised by weight) whereas all other parameters were kept constant. Beer E was hopped with 300 g/hL Saaz pellets at the onset of boiling ('early kettle hopping'), aiming at a final iso-α-acid concentration in the beer of 25 mg/L (taking into account an initial α-acid content of 2.37 % (on the basis of HPLC analysis) and an utilisation of 35 %). For the late hopped beer (beer L), an equal amount of hop pellets was added 10 minutes before the end of wort boiling and iso-α-acid extract (Botanix, Paddock Wood, England) was added to compensate for the bitterness (7.1 mg iso-α-acids /L on the basis of an utilisation rate of 10 %, addition of 17.9 mg/L isomerised extract). A combination of these two hopping regimes was obtained by addition of 150 g/hL pellets at the onset and 150 g/hL pellets towards the end of boiling (beer EL: 'early' and 'late' hopping). For compensation of the bitterness, 8.9 mg/L isomerised extract was added (16.1 mg/L iso-α-acids derived from pellets). Finally, a beer (beer W) was bittered exclusively by addition of 25 mg/L isomerised hop extract to the kettle and then aromatised by 'whirlpool' hop addition (300 g/hL pellets). Since there is a limitation in the number of brews (no replication), results only apply on the current brews.

For brewing, the following conditions were used: 87 kg fine milled Pilsner malt (wet disc mill, Meura, Péruwelz, Belgium) is mixed with 2.5 hL reversed osmosis brewing water with addition of $CaCl_2$ (80 ppm Ca^{2+}) and lactic acid (2 mL/L); mashing-in: temperature: 64 °C; pH 5.2; brewing scheme: 64 °C (30 min), 72 °C (20 min), 78 °C (1 min) (temperature increase: 1 °C/min); wort filtration: membrane assisted thin bed filter; sparging up to 11.5 °P sweet wort; wort boiling: 60 min atmospheric boiling using a double jacket for heating (evaporation: 5 %); at the end of boiling, 0.2 ppm Zn^{2+} ions were added, as well as iso-α-acids extract aiming at 25 ppm iso-α-acids in the finished beer; wort clarification: whirlpool; after cooling and aeration, the wort (original gravity: 12 °P) was pitched with 10^7 yeast cells/mL (inoculum: dry yeast, strain KO5 (Fermentis), hydrated for 1 hour in sterile water with a volume of 10 times the weight of the dry yeast); primary fermentation: 9–13 days at 12 °C in cilindroconical tanks (diacetyl management by addition of Maturex); maturation: 14 days at 0 °C in 50 L casks; beer filtration: kieselguhr/cellulose sheets (pore size 1 µm); CO_2 saturation up to 5.6 g/L; packaging: 6 head rotating counter pressure filler (monobloc, CIMEC, Italy) using double pre-evacuation with intermediate CO_2 rinsing and overfoaming with hot water injection before capping (final oxygen levels: below 50 ppb).

2.5 Sampling along the brewing process

Samples (500 mL) were taken along the boiling process of beer E and during the whirlpool stage of beer W for analysis of hop-derived volatiles. For all the hopped beers (E, EL, L and W), samples were taken at the end of wort boiling and at the end of the whirlpool process. Chemical reactions were immediately stopped by cooling the samples in liquid nitrogen (–196 °C), and samples were kept frozen (–18 °C) until further HS-SPME-GC-MS analysis. For a detailed oversight of all samples taken for the different beers, see table 1.

2.6 HS-SPME-GC-MS analysis of wort and beer samples

Wort and beer samples were analysed by adding 5 mL beer and 20 µL internal standard (2-heptanol, 253 ppm stock solution) in a HS-SPME vial (20 mL, clear glass, Chromacol) containing 1 g of NaCl. Vials were closed with bimetal magnetic caps with silicon/Teflon septum (Supelco, Bellefonte, USA). Hop-derived volatiles were extracted via headspace solid-phase microextraction (HS-SPME) (fibre coating: polydimethylsiloxane (PDMS), extraction time: 45 min, extraction temperature: 60°C) as previously described by our research group [30]. All samples were analysed by splitless injections. Except for the temperature program, gas chromatographic conditions for separation of the volatiles were similar to our previous work [30]. In this study two different oven programs were used for separation of the volatiles via the RTX-1 capillary column (nonpolar fused silica column, dimensions: 40 m x 0.18 mm x 0.25 µm): The oven program for analysis of the full hop-derived volatile profile was as follows: hold 1 min at 40 °C, 10 °C/min up to 72 °C, hold 1 min, 2 °C/min up to 137 °C, hold 1 min, 1 °C/min up to 160 °C, hold 1 min, 10 °C/min up to 250 °C, hold 3 min (total acquisition time of 74.7 min). For determination of the level of oxygenated sesquiterpenoids in the hopped lager beers, the following oven program was employed: hold 3 min at 35 °C, temperature ramp of 6 °C/min up to 250 °C, hold 5 min (total acquisition time of 45 min). Mass spectrometric detection of volatiles was performed by a Dual Stage Quadrupole MS (DSQ I, Thermo Fisher Scientific, Austin, TX) operating in the electron ionization mode (EI, 70 eV). For instrumental parameters

Table 1 **Overview of samples taken along brewing process of beer E, beer EL, beer L and beer W. = hop addition (T90 pellets cv. Saaz)**

Samples	Beer E	Beer EL	Beer L	Beer W
0 min, before hopping	x			
Early hop addition				
5 min of boiling	x			
10 min of boiling	x			
20 min of boiling	x			
30 min of boiling	x			
40 min of boiling	x			
50 min of boiling	x			
Late hop addition				
60 min of boiling (end boiling)	x	x	x	x
Transfer to whirlpool				
Whirlpool hop addition				
0 min whirlpool (start whirlpool)				x
5 min whirlpool				x
10 min whirlpool				x
15 min whirlpool				x
20 min whirlpool (end whirlpool)	x	x	x	x

and further information on mass spectral libraries used, we refer to our previously published work [19]. If no reference compound is available, tentative identifications are based on a match for both mass spectra (MS) and retention indices (RI) and, in the case of various sesquiterpene oxidation products and derivatives, on comparison with mass spectra and retention indices of volatiles in mixtures of reference compounds (see section 2.1).

2.7 Determination of caryophyllene oxide equivalents in beers

Levels of oxygenated sesquiterpenoids in beer E, EL, L and W were determined by external calibration using the reference compound caryophyllene oxide. The 8-point calibration curve ranged from 0 to 50 µg/L (1 g NaCl, 5 % EtOH, 20 µL internal standard stock solution (253 mg/L), 0 to 50 µL caryophyllene oxide stock solution (5 mg/L)). Using this calibration curve, levels of oxygenated sesquiterpenoids can be expressed in caryophyllene oxide equivalents (lack of other oxygenated sesquiterpenoid reference compounds).

2.8 Sensory evaluation of lager beers by taste panel

In first instance, the significance of sensory differences between the reference beer (beer ISO) and hopped lager beers (beer E, EL, L and W), and, between beer E and beers EL, L and W, were investigated by the trained taste panel of our institute (8 panellists) via triangular tests (α-level: 0.05). During each (separate) triangular test (7 in total), 3 samples were served (randomised order) and panellists were asked to indicate the different sample. Subsequently, odour and aroma characteristics of the lager beers were evaluated via descriptive sensory analysis by our trained taste panel. The panel was trained using reference compounds, total hop essential oils and hop-derived essences (total oils, polar, floral, citrus and spicy essences prepared as described by *Van Opstaele* et al. [31, 32], PHA® Spicy, Citrusy, Floral, Herbal and Sylvan, Botanix, U.K.). In separate sessions, the non-aromatised reference lager (beer ISO) was compared to a hopped lager. Panel members were instructed to score the intensity of pre-selected odour/aroma descriptors (malt/worty, fruity, floral, citrusy, spicy/herbal, woody, hay/straw, resinous, grass/green, earthy, general intensity of 'kettle hop aroma', general appreciation, bitterness, quality bitterness, mouthfeel and astringency) on a scale ranging from 0 to 8 (0 = not detectable, 8 = very high intensity).

3 Results and discussion

3.1 Progress of hop oil-derived volatiles during wort boiling

In order to gain insight into the impact of the 'kettle' hopping regime on the analytical composition of the hop-oil derived spectrum of volatiles in the wort, the evolution of hop-derived compounds throughout the brewing process of an 'early kettle' hopped beer (beer E) was investigated. Samples were taken at different time points during the boiling process (Table 1) and the volatile composition was determined using HS-SPME-GC-MS analysis. Peak areas of chemical compound classes (monoterpene hydrocarbons, floral fraction (i.e. ketones, esters, alcohols, oxygenated monoterpenoids) [33], sesquiterpene hydrocarbons and spicy fraction (i.e. ketones, esters, alcohols, oxygenated sesquiterpenoids) [30]) were normalised (internal standard taken into account for compensation of variation due to SPME extraction) and the average normalised peak area (duplicate analysis) is plotted in figure 1A. Obviously, 'early' kettle hopping introduces both mono- and sesquiterpene hydrocarbons as well as floral and spicy compounds to the wort. Levels of monoterpene and sesquiterpene hydrocarbons clearly decrease with increasing boiling time, due to known processes such as stripping and probably polymerisation. Compounds within the floral fraction also show a decrease. Although these compounds are better soluble into the wort compared to terpene hydrocarbons, these molecules are still relatively volatile which could explain their loss. *De novo* formation of oxygenated monoterpenoids by oxidation of monoterpene hydrocarbons is however not excluded, since losses due to volatilisation could (over)compensate for increases, resulting in a nett decrease. Remarkably, spicy compounds show a rather low but however significant increase in their level with increasing boiling time after 20 minutes of boiling. This is a most

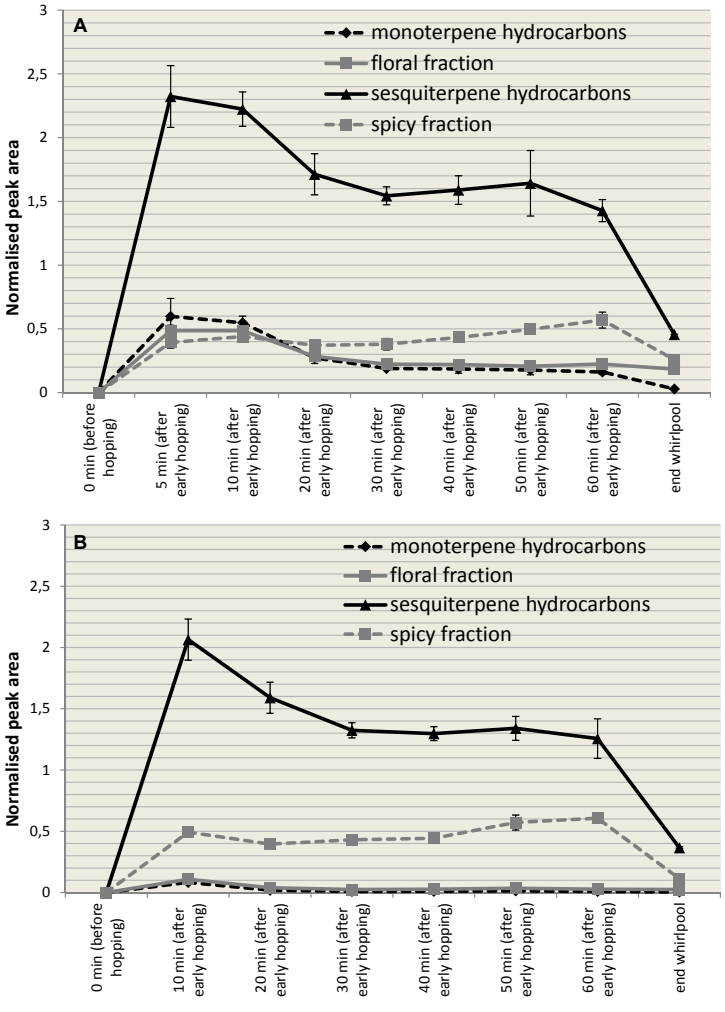

Fig. 1 **Average standardised peak area for different chemical compound classes of hop oil (-derived) volatiles, detected via HS-SPME-GC-MS analysis, as a function of samples taken along the wort boiling process and at the end of the whirlpool process of beer E ('early' kettle hopping with Saaz). A = results of beer E. B = results of replicate of brew (parameters as for beer E, this wort was however not fermented and didn't result in a beer)**

interesting observation that might be explained by long extraction times (i.e. slow transfer of these volatiles from hop pellets into the wort), or, by oxidation of sesquiterpene hydrocarbons into oxygenated sesquiterpenoids. *De novo* formation of oxygenated sesquiterpenoids during wort boiling has amply been suggested in literature [10, 21, 22, 23, 24, 34–36]. Also by our own research group [19, 20], *de novo* formation has been proven to occur during lab scale boiling experiments. However, up to date, this has not been demonstrated during real brewing practice. In an attempt to confirm the observed results, wort was brewed in an identical way (same malt, parameters and hopping regime as beer E, hopped wort was in this case not fermented). Figure 1B confirms the results discussed above, i.e. an increase in the level of spicy compounds (incl. oxygenated sesquiterpenoids) with increasing wort boiling time in real brewing practice.

To verify to which extent this increase concerns *de novo* formation and to exclude the possibility that this observation is due to slow extraction of oxygenated sesquiterpenoids from hops to wort, we looked for differences in the behaviour of sesquiterpene oxidation products (e.g. epoxides and their hydrolysis products) and oxygenated sesquiterpenoids that are related to the hop plant metabolism (e.g. cadinols [15]). As observed in our previous work [19, 20], the latter group did not increase in their level upon lab scale boiling of total hop essential oil (cv. Saaz) or a hop oil-derived sesquiterpene hydrocarbon fraction. On the other hand, a significant increase in the levels of α-humulene and β-caryophyllene oxidation products was demonstrated. Therefore, T-cadinol, α-cadinol and several α-humulene and β-caryophyllene oxidation products were selected amongst the spicy compounds as marker compounds. For each volatile, the normalised peak areas in the different samples was expressed as a percentage of the normalised peak area found after 5 minutes of boiling. These recoveries (%) upon boiling are displayed in figure 2 and depict the progress of the marker compounds with increasing boiling time. In graph A, one can see the progress of the cadinols. Their level reaches a maximum after 10 minutes, which might be the extraction time required for these compounds. Although, from there on, their recovery varies around 100 % (recovery compared to the level detected in the samples taken after 5 min of boiling), a clear increase with increasing boiling times is not observed. On the contrary, the oxidation products in graph B, also showing a local maximum at 10 minutes of boiling, show a remarkable and significant increase in their level with increasing boiling times. From these compounds, caryophyllene oxide, followed by humulene epoxide, show less pronounced increases in their level. These observations confirm our previous lab scale results, during which these two volatiles showed slightly different behaviour compared to other β-caryophyllene and α-humulene-derived oxidation products. This observation was explained by the fact that these epoxides are relatively prone to hydrolysis and rearrangement reactions [10, 14–17, 37, 38].

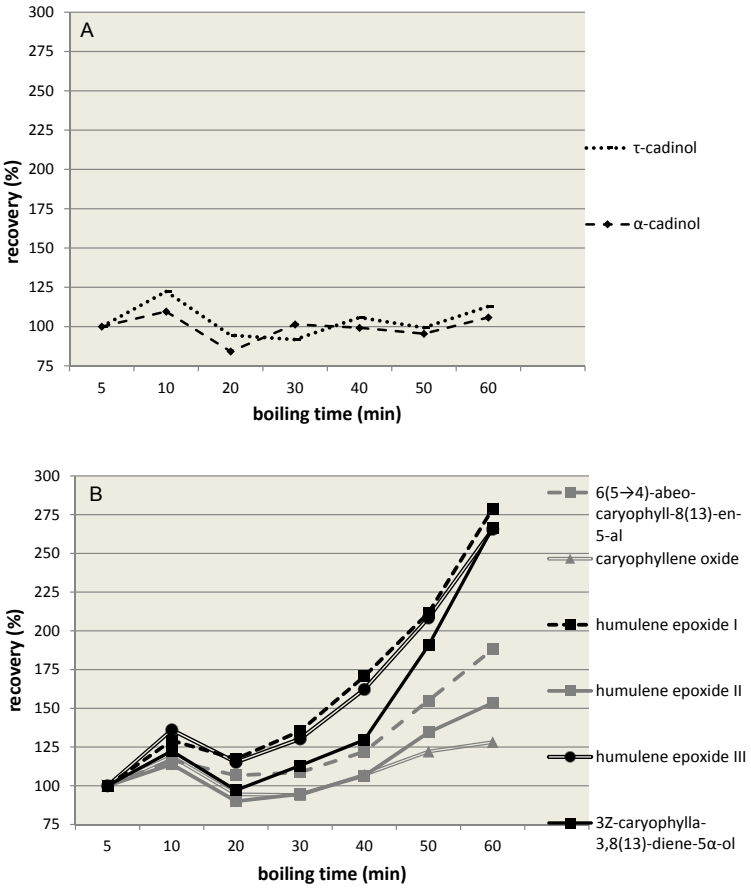

Fig. 2 Recovery (on basis of average standardised areas, determined in SIM mode for increased accuracy) of selected cadinols (A) and α-humulene and β-caryophyllene oxidation and hydrolysis products(B) upon wort boiling of beer E (in %, compared to sample taken after 5 minutes of wort boiling).

Since there is a clear indication for *de novo* formation of several compounds upon wort boiling, a comprehensive profiling of hop-derived volatiles was performed. The recovery of each (detected) volatile upon boiling was estimated via normalised peak areas for the samples taken after 5 min and 50 min of wort boiling. Because of the high degree of co-elution of the volatiles in the HS-SPME-GC-MS-derived chromatograms, peak areas were determined in the SIM (selected ion monitoring) mode. This mode allows for selection of specific and unique mass fragments of the relevant compound for accurate determination of increases. The (tentatively) identified volatiles characterised by an increase in their level upon wort boiling (*i.e.* recovery higher than 100 %) are summarised in table 2. These results do not unambiguously exclude *de novo* formation of other compounds upon boiling, since potential increases in levels of these volatiles might not be detected due to losses by other phenomena such as adsorption to trub and stripping effects. However, a high number of volatiles proves to increase in their level upon boiling. P-cymene, a disproportionation product of limonene [39], was detected amongst such volatiles. In addition, the β-carotene oxidative degradation products β-damascenone and β-ionone were also found to increase in their level upon wort boiling. An increase in the β-damascenone level during wort boiling was previously observed by *Kishimoto* and coworkers [9]. With respect to sensory properties, the odour of β-damascenone (flavour threshold: 0.009 µg/L [40]) was described as 'apple, peach' and 'honey-like' [1, 11]. This volatile was perceived during GC-O sniffing analyses of Pilsner beer by Fritsch and Schieberle [1] and GC-O analysis of both unhoped beer and beers hopped with Challenger and Saaz by *Lermusiau* and coworkers [11]. The dilution factor at which this compound could be detected was clearly higher in the hopped beers. On

the other hand, it was suggested that β-ionone does probably not influence the beer hoppy character since this compound was not perceived upon GC-O analysis of beer [11]. However, this compound (flavour threshold: 0.008 µg/L [40]) was described as 'floral' and 'violet-like' [12, 41], and, both carotenoids are present in beer at levels at which they may be important contributors to hoppy aroma of beer [42]. To this respect, increases in the levels of these carotenoids degradation products during wort boiling may play a role into development of hoppy aroma. Most remarkably, all (detected) α-humulene and β-caryophyllene oxidation products are characterised by a recovery higher than 100 %, proving *de novo* formation of these compounds upon wort boiling by oxidation of their parent sesquiterpene hydrocarbon molecule. On the contrary, cadinols and cubenols did not depict a recovery higher than 100 %. Amongst the sesquiterpene hydrocarbon oxidation products, isocaryophyllene epoxide was not detected in the samples taken after 5 min of boiling, whereas it was detected in the samples taken after 50 minutes of boiling. This observation indicates that also qualitative changes in the hop oil-derived volatile profile occur as a result of boiling hops. Literature data proving increases in the level of sesquiterpene oxidation products as a result of 'early' addition of hops to the boiling kettle is scarce. Possibly, such an increase was not detected previously due to more significant losses of these compounds by stripping effects, which would result in a nett decrease, whereas during our current experiment, evaporation losses were limited.

Table 2 Tentative identification and recoveries (%) of volatiles characterised by an increase in their level upon wort boiling (detected in samples taken after 50 minutes of wort boiling compared to samples taken after 5 minutes) of beer E. RI = retention index (calculated on RTX-1 column). SIM = selected ion monitoring (selection of specific characteristic mass fragments for accurate determination of normalised peak areas and recovery upon boiling). R (%) = recovery (sample after 50 min of boiling compared to samples after 5 min of boiling), based on normalised SIM peak areas. Identification on basis of MS (mass spectrum), RI (retention index) and/or RC (reference compound) or comparison with mixtures of reference compounds (HEP, IEP, CEP, HHP, CHP, HAA, CAA, see section 2.1). N = detected after 50 min of boiling but not detected after 5 min of boiling.

Compound	RI	SIM mass fragments	R (%)	Identification
p-Cymene	1002	119, 134	156	MS/RI/RC
β-Damascenone	1361	69, 121, 190	272	MS/RI/RC
Cis-α-bergamotene	1408	93, 119	104	MS/RI
Unknown oxygenated sesquiterpenoid				
(m/z 69, 81, 95, 109, 123, 138, 149, 191, 205, 220)	1438	Full scan	145	MS
β-Ionone	1462	177	137	MS/RI/RC
Unknown oxygenated sesquiterpenoid				
(m/z 69, 81, 95, 109, 123, 138, 149, 191, 205, 220)	1473	191, 205 220	137	MS
4S-Dihydrocaryophyllene-5-one	1530	79, 96, 109, 138, 164, 220	211	MS/RI
Isocaryophyllene epoxide A	1531	106	N	MS/RI/IEP
4R-Dihydrocaryophyllene-5-one	1534	79, 96, 109, 138, 164, 220	275	MS/RI
Unknown oxygenated sesquiterpenoid				
(m/z 93, 107, 121, 205, 220)	1544	93, 205, 220	219	MS
Humuladienone	1550	67, 96, 109, 138	135	MS/RI
Caryolan-1-ol	1550	111	130	MS/RI
6(5→4)-Abeo-caryophyll-8(13)-en-5-al	1556	79, 93, 107, 121, 164, 205, 220	162	MS/RI
E-Dendrolasin	1556	69, 81	215	MS/RI
Caryophyllene oxide	1560	Full scan	119	MS/RI/CEP
Clovenol	1563	161, 205, 220	117	MS/RI/CHP
Humulene epoxide I	1574	93	206	MS/RI/HEP
Humulol	1579	82, 83	163	MS/RI/HHP
Humulene epoxide II	1585	96, 109, 138	133	MS/RI/HEP
Humulene allylic alochol	1593	105, 107, 109, 159, 177, 205, 220	158	MS/RI/HAA
Humulene epoxide III	1606	81	307	MS/RI/HEP
Humulenol II	1608	119	115	MS/RI/HAA
Caryophylla-4(12),8(13)-diene-5-ol	1613	136	154	MS/RI/CAA
3Z-Caryophylla-3,8(13)-diene-5α-ol	1634	Full scan	187	MS/RI/CAA
3Z-Caryophylla-3,8(13)-diene-5α-ol	1649	Full scan	152	MS/RI/CAA
Humulene allylic alcohol	1655	Full scan	136	MS/RI/HAA

In summary, typical 'noble kettle hop aroma', achieved by 'early' addition of aroma hop varieties which are usually rich in α-humulene [4, 37, 43–45], is described by 'spicy' and 'herbal' notes [4, 46–48]. Moreover, a cause-effect relationship between sesquiterpene oxidation products and these odour characteristics has been proven by addition of a sesquiterpene oxidation product fraction (obtained by lab scale boiling of an enriched sesquiterpene hydrocarbon fraction cv. Saaz) to non-aromatised iso-α-acid-bittered lager beer [20]. In addition, many of the sesquiterpene hydrocarbon oxidation products have been found to elute in flavour-active intervals, detected upon GC-O analysis of spicy fractions obtained by SPE-fractionation of a commercial kettle hopped lager beer [28]. Increases of such α-humulene and β-caryphyllene oxidation products, previously demonstrated to occur upon lab scale boiling [19, 20], has now also been proven during the wort boiling process in real brewing practice by monitoring hop oil-derived volatiles of an 'early' kettle hopped lager beer. Basically, there can be concluded that boiling of aroma hops definitely alters the hop oil composition and that *de novo* formation of sesquiterpene oxidation products plays a key role into development of 'kettle hop' aroma.

3.2 Progress of hop oil-derived volatiles during whirlpool process

The impact of 'whirlpool hopping' was further investigated by HS-SPME-GC-MS analysis of wort samples taken along the whirlpool process of beer W (see Table 1). Normalised peak areas of chemical compound classes are depicted in figure 3, showing that terpene hydrocarbons as well as oxygenated compounds are introduced to the wort via the whirlpool process. However, terpene hydrocarbons are lost to a great extent, which could be attributed to volatilisation and adsorption to hot break. Losses of oxygenated compounds appear to be less pronounced, due to their higher solubility in wort. Nevertheless, a general increase in the level of spicy compounds, as was detected during wort boiling, was not detected during the whirlpool stage.

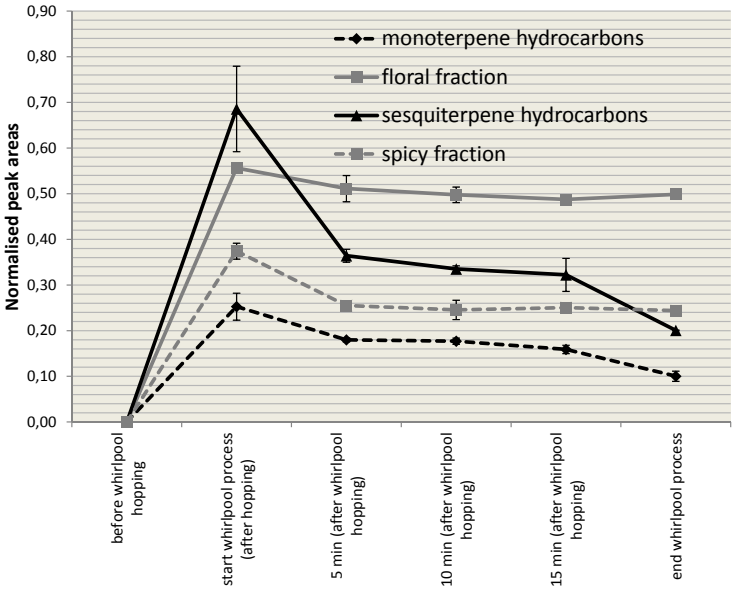

Fig. 3 Average standardised peak area for different chemical compound classes of hop oil (-derived) volatiles, detected via HS-SPME-GC-MS analysis, as a function of samples taken along the whirlpool process of beer W ('whirlpool' hopping with Saaz)

Since the temperature of the wort during the whirlpool stage is still relatively high (100 °C at the start of whirlpooling, 90 °C at the end of the process), oxidation of terpene hydrocarbons and *de novo* formation of several oxygenated compounds might still occur during this process step. Moreover, glycosidically bound volatiles, present in the hop vegetative matter, are extracted into the hot wort. Hydrolysis reactions might release such volatiles from their sugar moiety, causing an increase in their level during the whirlpool process. Therefore, the full spectrum of volatiles was obtained via HS-SPME-GC-MS analysis of samples taken at the start and end of the whirlpool process of beer W (see Table 3). Although some of the detected volatiles are (at least partly) wort-derived (they also appeared in samples taken before hopping, e.g. phenylacetaldehyde, borneol, vinylguaiacol, β-damascenone), the largest part is clearly derived from the hop essential oil. Whirlpool hopping introduces a broader spectrum of volatiles to the wort compared to 'early hopping' since many monoterpenoid compounds, not detected in the wort samples of beer E, are now detected in the wort samples of beer W. Some examples of such compounds are dihydro-ocimene, myrcenol, terpinen-4-ol, nerol and several unidentified monoterpenoids. The absence of these compounds in the wort samples of beer E can be rationalised by stripping effects since temperatures in the boiling kettle are higher than in the whirlpool. A series of compounds was characterised by an increase in their level during the whirlpool stage, although the recoveries of most of these compounds are only slightly higher than 100 %. To investigate whether these particular recoveries are due to slight increases in levels as a function of the whirlpool time or are rather due to variation, the progress of these volatiles along the whirlpool process was investigated into more detail by determination of the normalised peak areas in each sample (start, 5 min, 10 min, 15 min and end whirlpool) via the SIM-mode and plotting as a function of the whirlpool time. As a result, a distinction between the progress of several volatiles could be made. The unknown monoterpenoid at RI 1060, borneol (RI 1146), an unknown at RI 1156, an unknown at RI 1183 and geraniol (RI 1235) showed recoveries between 106 and 123 % (see Table 3). From their progress in figure 4A it remains dubious whether these volatiles are actually formed *de novo* during the whirlpool stage or not, since a clear, significant and consistent increase in their level as a function of the whirlpool time is not observed. The behaviour of α-terpineol, (RI 1171), nerol (RI 1211), 3 unknowns (at RI 1257, 1264 and 1381) and humulol (RI 1574) is depicted in figure 4B, indicating that these volatiles slightly increase in their level upon the whirlpool stage. Finally, the progress of volatiles characterised by a clear increase in their level as a function of the whirlpool time are depicted in figure 4C. β-Damascenone, which was also found to be formed *de novo* during the wort boiling process and is found in both wort and hop oil, appears to further increase in its level during the whirlpool stage. Also 4-vinylguaiacol is characterised by an increase in its level, although this volatile is wort-derived [49]. The norisoprenoid dihydroedulan showed a clear increase and reached a recovery of 265 % after 20 minutes in the whirlpool. This rather atypical compound was identified for the first time in a glycosidic extract form Saaz spent hops and hopped beer by *Daenen* [50]. The increase in the level of dihydroedulan, as well

Table 3 **Tentative identification and recoveries (%) of the full spectrum of volatiles detected in samples taken at the end of the whirlpool process compared to samples taken at the start of the whirlpool process of beer W.** RI = retention index (calculated on RTX-1 column). Area% = relative composition of the sample, based on peak areas. W start = sample taken right after hopping (duplicate analysis; a and b). W end = sample taken after 20 minutes, at the end of the whirlpool process (duplicate analysis; a and b). R (%) = recovery (sample end whirlpool compared to samples start whirlpool), based on full scan normalised peak areas. Identification on basis of MS (mass spectrum), RI (retention index) and/or RC (reference compound) or mixtures of reference compounds (HEP, IEP, CEP, HHP, CHP, HAA, CAA, see section 2.1). Bold = increase in level of the particular volatile upon the whirlpool process

Compound	RI	W start a Area%	W start b Area%	W end a Area%	W end b Area%	R (%)			Identification
α-Pinene	<1000	0.03	0.03	0.03	0.02	38	±	5	MS/RI/RC
6-Methyl 5-hepten-2-one	<1000	0.12	0.14	0.21	0.22	91	±	2	MS/RI/RC
β-Pinene	<1000	0.55	0.53	0.40	0.36	39	±	0	MS/RI/RC
β-Myrcene	<1000	13.01	12.08	8.99	7.79	37	±	0	MS/RI
α-Phellandrene	<1000	0.06	0.06	0.07	0.07	62	±	5	MS/RI
Unknown (m/z 55, 82, 110, 111, 127, 142)	1007	1.17	1.25	2.06	1.96	92	±	3	
Phenyl acetaldehyde	1015	0.10	0.12	0.19	0.20	98	±	0	MS/RI
Limonene	1020	0.33	0.31	0.41	0.38	68	±	1	MS/RI/RC
Cis-β-ocimene	1026	0.08	0.08	0.14	0.13	92	±	5	MS/RI/RC
Cis-dihydro-ocimene	1034	0.08	0.08	0.10	0.08	64	±	3	MS/RI
Trans-β-ocimene	1037	0.19	0.19	0.28	0.26	77	±	0	MS/RI/RC
Methyl 2-methylheptanoate	1047	0.13	0.13	0.21	0.19	86	±	6	MS/RI
2-Nonanol	1051	0.00	0.00	0.00	0.00	105	±	26	MS/RI
Unknown monoterpenoid (67, 71, 79, 81, 93, 107, 122)	**1060**	0.03	0.03	0.07	0.06	**123**	±	**8**	
2-Nonanone	1070	1.12	1.16	2.01	1.88	94	±	2	MS/RI/RC
Terpinolene	1078	0.05	0.05	0.08	0.08	86	±	2	MS/RI/RC
Linalool	1084	3.02	3.34	5.96	5.55	100	±	7	MS/RI/RC
Perillene	1086	0.64	0.69	1.06	1.04	87	±	1	MS/RI
Unknown monoterpenoid (m/z 67, 71, 79, 81, 109, 123, 137, 152)	1097	0.07	0.08	0.10	0.09	74	±	7	
Myrcenol	**1102**	0.02	0.02	0.07	0.08	**188**	±	**6**	MS/RI
Methyl octanoate	1108	0.10	0.11	0.16	0.15	85	±	2	MS/RI/RC
Unknown monoterpenoid (m/z 69, 79, 91, 107, 121, 152)	1118	0.09	0.09	0.12	0.11	70	±	3	
Unknown (m/z 67, 69, 71, 79, 91, 137, 156)	1137	0.07	0.09	0.11	0.12	81	±	0	
Unknown (m/z 69, 79, 91, 107, 121, 150)	**1142**	0.02	0.02	0.03	0.04	**112**	±	**1**	
Borneol	**1146**	0.00	0.01	0.01	0.01	**106**	±	**1**	MS/RI
Unknown (m/z 43, 54, 67, 81, 96, 111, 125, 136, 154)	**1156**	0.14	0.16	0.32	0.32	**114**	±	**4**	
Terpinen-4-ol	1159	0.07	0.08	0.13	0.14	100	±	8	MS/RI/RC
α-Terpineol	**1171**	0.14	0.15	0.33	0.34	**126**	±	**2**	MS/RI
2-Decanone	1171	1.37	1.45	2.55	2.56	100	±	2	MS/RI/RC
Ethyl octanoate	1181	0.00	0.00	0.00	0.00	80	±	6	MS/RI
Unknown (m/z 85)	**1183**	0.00	0.00	0.01	0.01	**109**	±	**3**	
Decanal	1184	0.10	0.14	0.20	0.20	90	±	14	MS/RI/RC
2-Decanol	1187	0.30	0.32	0.58	0.56	103	±	4	MS/RI
Methyl 3-nonenoate	1193	0.43	0.44	0.76	0.78	98	±	6	MS/RI/RC
Dodecane	1199	0.02	0.02	0.03	0.04	88	±	26	MS/RI
Unknown (m/z 69, 100)	1202	0.11	0.10	0.16	0.18	88	±	17	
Methyl nonanoate	1207	0.21	0.20	0.28	0.28	73	±	6	MS/RI/RC
Nerol	**1211**	0.01	0.01	0.02	0.02	**135**	±	**1**	MS/RI/RC
Geraniol	**1235**	0.54	0.62	1.19	1.30	**118**	±	**2**	MS/RI/RC
Unidentified methyl ketone	1237	0.83	0.88	1.32	1.29	84	±	0	

Ethyl ester	1245	0.19	0.18	0.19	0.17	53	±	1	
Unknown (unclear mass spectrum)	1252	0.05	0.05	0.08	0.06	75	±	8	
5-Undecen-2-one	1253	1.82	1.94	3.40	3.47	100	±	2	MS/RI
Unknown (m/z 43, 55, 93, 111, 123)	**1257**	0.14	0.15	0.34	0.32	**128**	±	**2**	
Unknown (m/z 69, 114)	1259	0.11	0.12	0.22	0.20	100	±	2	
Methyl ester	1263	0.08	0.07	0.11	0.11	79	±	5	
Unknown (m/z 67, 81, 95, 110)	**1264**	0.19	0.21	0.46	0.42	**121**	±	**13**	
2-Undecanone	1273	4.05	4.07	5.83	5.72	78	±	3	MS/RI/RC
Dihydroedulan	**1278**	0.06	0.06	0.12	0.13	**114**	±	**14**	MS/RI
Vinyl guaiacol	**1285**	0.01	0.02	0.04	0.05	**146**	±	**15**	MS/RI
Methyl trans-4-decenoate	1289	4.17	4.18	6.19	6.25	82	±	5	MS/RI
Unknown (m/z 85, 150)	1292	1.69	1.81	3.17	3.16	99	±	0	
Unknown (m/z 137)	1295	0.08	0.09	0.15	0.14	98	±	4	
Methyl cis-4-decenoate	1299	0.06	0.06	0.08	0.08	72	±	3	MS/RI
Methyl geranate	1301	2.02	2.09	3.45	3.53	93	±	4	MS/RI/RC
Methyl decanoate	1307	0.07	0.07	0.08	0.07	57	±	3	MS/RI/RC
Unknown (m/z 69, 93, 105, 121, 148)	1310	0.06	0.06	0.09	0.10	88	±	12	
α-Cubebene	1342	0.02	0.02	0.01	0.01	28	±	3	MS/RI
Unknown (m/z 43, 54, 68, 82, 96, 124, 161, 189)	1349	0.05	0.05	0.09	0.08	86	±	4	
β-Damascenone	**1358**	0.14	0.15	0.34	0.34	**130**	±	**0**	MS/RI/RC
α-Ylangene	1363	0.08	0.08	0.10	0.09	60	±	3	MS/RI
α-Copaene	1368	0.15	0.13	0.09	0.08	32	±	2	MS/RI/RC
2-Dodecanone	1374	0.44	0.44	0.45	0.45	56	±	2	MS/RI/RC
Unknown (m/z 58, 69, 111, 126) / sesquiterpene hydrocarbon	1378	0.05	0.06	0.07	0.07	70	±	4	
Unknown (m/z 69, 152, 196)	**1381**	0.22	0.24	0.51	0.50	**122**	±	**4**	
Tetradecene	1387	0.17	0.19	0.23	0.22	68	±	5	MS/RI
Unknown (m/z 79, 80, 81, 83, 122, 136, 164)	1390	0.31	0.33	0.45	0.45	77	±	3	
Isocaryophyllene	1395	0.05	0.04	0.03	0.04	44	±	15	MS/RI/RC
Sesquiterpene hydrocarbon									
(m/z 91, 105, 119, 147, 161, 175, 204)	1402	0.03	0.03	0.03	0.04	55	±	8	
β-Caryophyllene	1407	4.82	4.34	2.46	2.43	29	±	3	MS/RI/RC
Caryophylla-4(12),8(13)-diene	1414	0.02	0.02	0.01	0.01	23	±	1	MS/RI
β-Copaene	1416	0.17	0.15	0.07	0.06	23	±	2	MS/RI
Unknown (m/z 69, 111, 126)	**1418**	0.06	0.06	0.13	0.13	**120**	±	**7**	
Trans-α-bergamotene	1425	0.78	0.70	0.45	0.41	32	±	2	MS/RI
Sesquiterpene hydrocarbon (m/z 69, 91, 105, 119)	1430	0.04	0.06	0.06	0.06	65	±	9	
Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 177, 191, 205, 220)	1433	0.74	0.92	0.70	0.69	46	±	5	
α-Humulene	1439	21.39	19.76	11.60	12.00	31	±	4	MS/RI/RC
β-Farnesene	1442	8.69	7.44	2.82	2.96	20	±	4	MS/RI/RC
Unknown (m/z 43, 67, 81, 96, 110, 138)	1444	0.31	0.33	0.36	0.37	62	±	2	
Oxygenated sesquiterpenoid (m/z 91, 191, 187, 202)	1450	0.12	0.16	0.14	0.14	53	±	7	
Unknown (m/z 123)	1452	0.07	0.10	0.14	0.14	88	±	16	
β-Ionone	1456	0.33	0.39	0.43	0.50	72	±	3	MS/RI/RC
γ-Muurolene	1460	0.52	0.52	0.34	0.34	36	±	2	MS/RI
α-Amorphene	1463	0.15	0.18	0.16	0.15	50	±	4	MS/RI

Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 177, 191, 205, 220)	1468	0.85	1.01	1.21	1.20	71	±	5	
2-Tridecanone	1472	0.93	0.93	0.73	0.79	45	±	4	MS/RI/RC
Cis-cadina-1,4-diene	1477	0.20	0.21	0.19	0.21	52	±	5	MS/RI
α-Selinene	1479	0.29	0.31	0.23	0.22	42	±	1	MS/RI
Epi-zonarene	1482	0.10	0.10	0.08	0.09	46	±	7	MS/RI
Unknown (m/z 79, 80, 81,136) / α-muurolene	1484	0.89	0.97	1.07	1.14	65	±	2	MS/RI
δ-Amorphene	1491	0.04	0.06	0.07	0.08	79	±	6	MS/RI
(E,E)-α-Farnesene	1492	0.06	0.06	0.03	0.04	34	±	6	MS/RI
β-Bisabolene/γ-cadinene	1496	0.80	0.79	0.51	0.51	35	±	3	MS/RI
Trans-calamenene	1500	0.30	0.30	0.26	0.26	47	±	3	MS/RI
δ-Cadinene	1506	0.91	0.86	0.49	0.55	32	±	6	MS/RI
Trans-cadina-1,4-diene	1514	0.15	0.16	0.16	0.17	59	±	5	MS/RI
α-Calacorene	1517	0.11	0.12	0.12	0.13	58	±	5	MS/RI
4S-Dihydrocaryophyllene-5-one/6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al	1523	0.12	0.15	0.19	0.19	77	±	8	MS/RI
6(5-4)-Abeo-caryophyll-7-en-5-al	1532	0.04	0.05	0.07	0.07	81	±	12	MS/RI
Unknown oxygenated sesquiterpenoid									
(m/z 93, 107, 121, 205, 220)	1534	0.03	0.05	0.07	0.07	94	±	20	
Unknown (m/z 79, 80, 81, 150, 157)	1536	0.23	0.25	0.30	0.31	69	±	0	
E-Nerolidol / caryophylla-4(12),8(13)-dien-5-one	1541	0.10	0.12	0.16	0.15	79	±	9	MS/RI
Caryolan-1-ol	1543	0.01	0.01	0.02	0.02	93	±	1	MS/RI
Humuladienone	1544	0.68	0.79	0.73	0.70	53	±	5	MS/RI
6(5-4)-Abeo-caryophyll-8(13)-en-5-al	1550	0.59	0.73	0.81	0.79	67	±	7	MS/RI
Caryophyllene oxide	1554	0.64	0.70	0.75	0.85	66	±	5	MS/RI/CEP
Clovenol	1555	0.14	0.18	0.24	0.26	85	±	8	MS/RI/CHP
Unknown oxygenated sesquiterpenoid (m/z 107, 135, 218)	1561	0.13	0.15	0.29	0.25	105	±	17	
Humulene epoxide I	1568	1.07	1.28	1.69	1.86	83	±	0	MS/RI/HEP
Humulol	**1574**	0.02	0.02	0.06	0.07	**179**	±	**4**	MS/RI/HHP
Humulene epoxide II	1579	2.79	3.20	2.54	2.85	49	±	2	MS/RI/HEP
Humulene allylic alcohol	1586	0.24	0.30	0.39	0.40	80	±	8	MS/RI/HAA
1,10-Di-epi-cubenol	1589	0.18	0.22	0.31	0.33	88	±	3	MS/RI
Junenol/α-corocalene	1591	0.05	0.06	0.08	0.08	86	±	4	MS/RI
Humulene epoxide III	1600	0.59	0.70	0.81	0.86	71	±	1	MS/RI/HEP
Humulenol II	1603	2.57	3.28	3.60	3.66	69	±	7	MS/RI/HAA
Caryohylla-4(12),8(13)-diene-5-ol	1606	0.41	0.50	0.62	0.66	77	±	4	MS/RI/CAA
τ-Cadinol	1612	0.46	0.56	0.87	0.99	101	±	1	MS/RI
Cubenol	1616	0.13	0.16	0.23	0.27	97	±	2	MS/RI
Selin-11-en-4-ol	1621	0.06	0.07	0.10	0.13	100	±	6	MS/RI
α-Cadinol	1624	0.03	0.04	0.06	0.07	101	±	10	MS/RI
3Z-Caryophylla-3,8(13)-diene-5α-ol	1627	0.55	0.67	0.73	0.77	68	±	4	MS/RI/CAA
Unknown (m/z 79, 80, 81, 164, 222)	1631	1.06	1.03	0.81	0.87	44	±	6	
Unknown (m/z 79, 91, 93, 95)	1633	0.37	0.42	0.42	0.50	64	±	5	
Unknkown (m/z 93, 137)	1637	0.08	0.10	0.10	0.11	63	±	0	
3Z-Caryophylla-3,8(13)-diene-5α-ol	1639	0.18	0.23	0.23	0.25	64	±	3	MS/RI/CAA
Unknown (m/z 82)	1644	0.20	0.21	0.16	0.20	48	±	8	
Humulene allylic alcohol	1647	0.40	0.50	0.41	0.44	52	±	4	MS/RI/HAA

as terpineol, geraniol and nerol, might originate from glycosidically bound volatiles in hops. Also β-damascenone can be derived from glycoconjugated precursors after acid catalysed conversion [50]. Myrcenol, a β-myrcene-derived monoterpene alcohol detected in the oil of hops by *Gildemeister* and *Hoffman* [51], also clearly depicts *de novo* formation upon the whirlpool process.

Although the identity of many of the volatiles discussed above remains unknown, it is clear that monoterpenoid alcohols (such as myrcenol, borneol, α-terpineol, nerol, geraniol) and noriso-

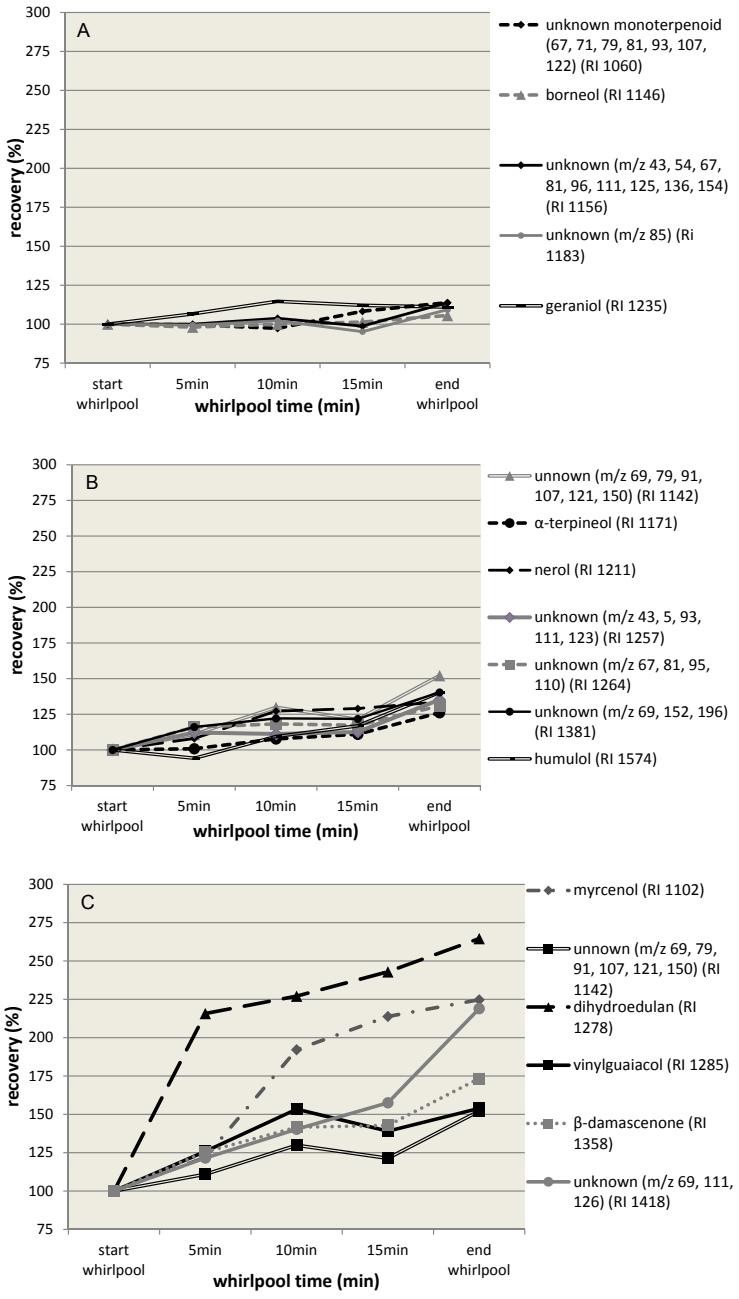

Fig. 4 Recovery (on basis of average standardised areas, determined in SIM mode for increased accuracy) of volatiles upon the whirlpool process of beer W (in %, compared to sample taken at the start of the whirlpool process). For volatiles in graph A, increases are too low to state *de novo* formation. Volatiles in graph B show a slight increase in their level, whereas volatiles in graph C show a clear increase in their level, probably due to *de novo* formation

prenoids (β-damascenone, dihydroedulan) are present amongst the volatiles characterised by an increase in their level upon the whirlpool process. These compounds might be (indirectly) formed by thermal oxidation of monoterpene hydrocarbons and degradation of carotenoids, due to the relatively high remaining temperature of the wort during the whirlpool stage. Also release of glycosidically bound volatiles might explain the observed increases of particular volatiles during the whirlpool process. However, an increase in the levels of these volatiles (except for β-damascenone) was not found during wort boiling. It is tempting to assume that these chemical reactions also occur during wort boiling but that the reactions products are, due to their high volatility, quickly stripped out of the wort before any detection is possible. The more gentle temperature conditions in the whirlpool, combined with limited adsorption to trub since these compounds are better soluble into the wort compared to terpenes and oxygenated sesquiterpenoids, might allow these products to survive the whirlpool process. On the other hand, an increase in the level of sesquiterpene oxidation products, which was clearly detected during wort boiling, is not found during the whirlpool process. Temperatures in the whirlpool are possibly not high enough for significant oxidation of sesquiterpene hydrocarbons or, in case oxidation would occur, formation of these volatiles is quickly compensated by losses due to adsorption to trub.

In summary, it can be concluded that the whirlpool process also induces some changes in the volatile hop oil-derived fingerprint. Yet, the analytical profiles of 'early kettle' hopped wort and 'whirlpool hopped' wort are clearly different from both a quantitative and qualitative point of view. As a result, it can be expected that beer E and beer W will express completely different flavour characteristics.

3.3 Oxygenated sesquiterpenoid levels in pilot-scale hopped lager beers

Levels of oxygenated sesquiterpenoids in beers were quantified using a caryophyllene oxide calibration line (see section 2.7) (correlation coefficient R: 0.9981). The levels in the 'early' kettle hopped beer (beer E), the 'early' and 'late' kettle hopped beer (beer EL), the 'late' hopped beer (beer L) and the 'whirlpool' hopped beer (beer W) were determined at 15.37, 23.59, 19.25 and 19.19 µg/L respectively. These levels may appear relatively low taken into account the hopping rate of the beers (300 g pellets/hL wort for each beer, hop pellets addition according to the EBC manual hops and hop products: 25–300 g/hL for early hopping and 10–20 g/hL for 'mid', 'late' or 'whirlpool' additions [52]). However, at most 12.81 mg/L hop oil was introduced to the wort for each beer, due to low hop oil contents in Saaz hops. These results would implicate that less than 1 % of the hop oil-derived volatiles survived the brewing process. Indeed, a large relative proportion of total hop oil is made up by sesquiterpene hydrocarbons and ketones (up to 90 % [53–55]) that do not survive the brewing process (caused by processes such as volatilisation, polymerisation, adsorption to yeast/trub and migration to the foam layer [10, 23, 34, 56–59]). Moreover, levels of oxygenated compounds in lager beer have been reported at 15–50 ppb [35, 59] and also our research group estimated levels of oxygenated sesquiterpenoids in commercial lagers (exhibiting relatively distinct kettle hop flavour) at 33 to 109 ppb (87 ppb on average) [20]. Taken into account these observations, the oxygenated sesquiterpenoid levels in our current beers

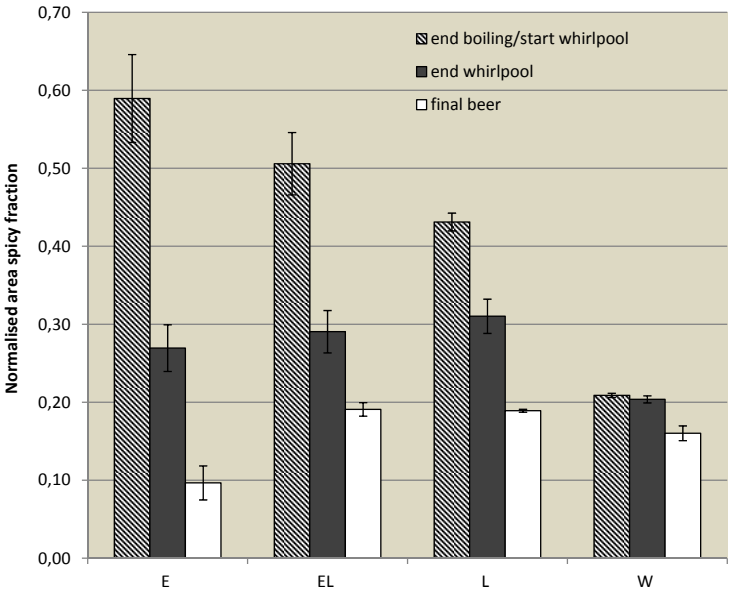

Fig. 5 Normalised peak area (n = 2) of spicy fraction in samples taken at the end of the boiling process, at the end of the whirlpool process and in the final beer for beer E, EL, L and W

lie within the normal range. Despite the low level at which these oxygenated sesquiterpenoids are detected, these compounds might have a high impact on the hop-derived flavour of the beers. Indeed, these water-soluble hop oil-derived compounds have been reported to be detectable up to levels as low as 5.8 ppb upon addition to beer [35] and also Goiris and coworkers determined the flavour threshold of an oxygenated sesquiterpenoid hop essence at 5 ppb. However, levels of 20 ppb where preferred and introduced a pleasant spicy hop flavour and enhanced mouthfeel, fullness and bitterness perception, whereas higher addition rates were described as overwhelming for pilsner beer types [18]. Later on, addition of a hop-derived spicy essence to beer confirmed these results [32]. In this respect, it appears that our applied hopping rates result in an oxygenated sesquiterpenoid level which might find itself within the ideal concentration range to impart subtle yet balanced 'kettle hop' flavour.

From the 4 hopped beers, beer E proved to contain the lowest oxygenated sesquiterpenoid level. Nevertheless, from section 3.1, it became clear that 'early' addition of hop pellets leads to an increase in the spicy fraction due to *de novo* formation of sesquiterpene oxidation products and subsequently, one would expect this beer to have increased oxygenated sesquiterpenoid levels compared to the other beers. Therefore, normalised peak areas of spicy compounds in hopped samples at the end of the boiling process (start whirlpool process for beer W), at the end of the whirlpool process and in the final beer were plotted in figure 5. From this graph, it can be concluded that levels of spicy compounds in beer E are indeed elevated at the end of the boiling process and clearly, the later hop pellets were added, the lower spicy compound levels. However, spicy compounds are lost to a great extent during the whirlpool process and also during subsequent process steps such as fermentation, lagering and filtration. Higher percentages of losses

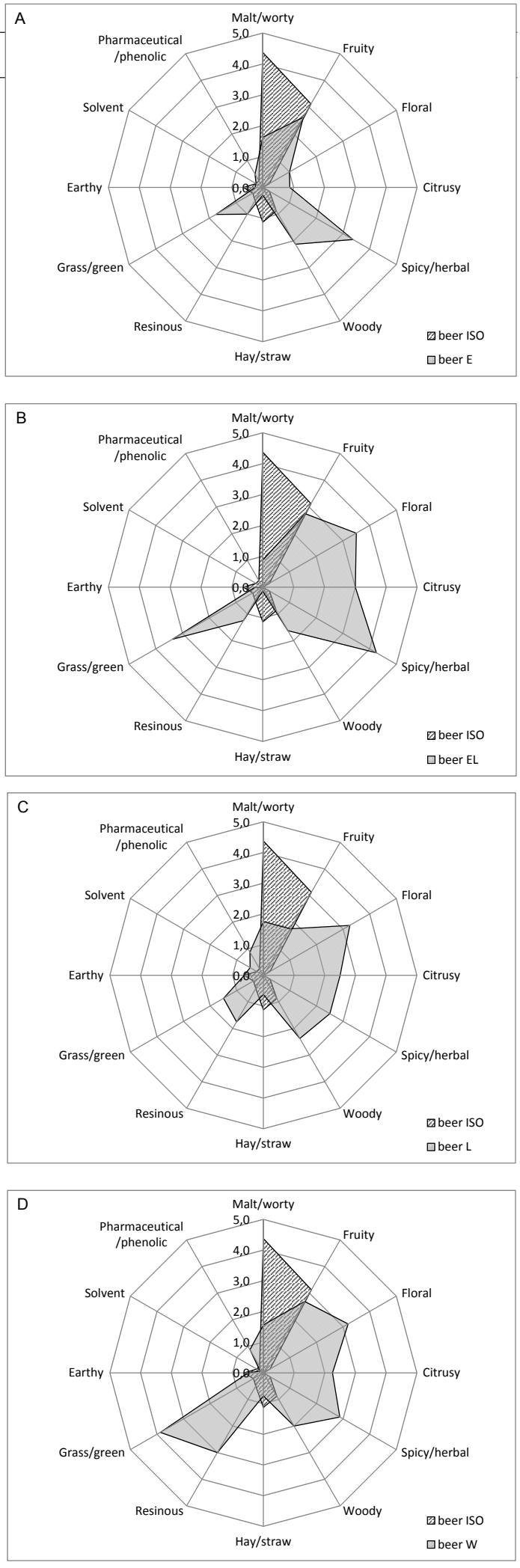

Fig. 6 (right) Spider plots depicting flavour profile of beer E, beer EL, beer L and beer W (resp. graph A, B, C and D) compared to an unhopped beer, bittered with iso-α-acids (ISO), based on average score (8 panellists) for pre-selected odour/flavour descriptors

of these compounds in beer E (54 % losses during the whirlpool process of beer E versus 43 %, 28 % and 2 % losses for resp. beer EL, beer L and beer W), due to adsorption to hop vegetative matter, hot and cold break and yeast cells, might clarify why beer E is characterised by the lowest oxygenated sesquiterpenoid level. Unfortunately, losses of hop oil compounds during such process steps are highly variable, since they depend on various parameters such as for example yeast cell growth during fermentation, and are therefore difficult to maintain constant.

3.4 Sensory evaluation of hopped lager beers

During a first series of triangular tests, sensory differences between the non-aromatised iso-α-acid-bittered beer (ISO) and the hopped beers E, L, EL and W were investigated. The results indicate that all investigated hop technologies impart significant (at the presumed α-level of 5 %) sensory differences compared to an unhopped beer. Also, although some brewers believe 'early kettle' hopping does not induce hop-derived aroma because all hop volatiles are stripped out of the wort, addition of aroma hops at the onset of boiling clearly imparts hop-derived aroma since this beer could be distinguished from the ISO beer. Moreover, during a second series of triangular tests, sensory differences between beer E and beer EL, L and W were demonstrated. This observation confirms that addition of hops at the onset of wort boiling or later in the process (or even during the whirlpool stage) clearly results in beers with different flavour attributes and that the time point of hop addition definitely has an impact on 'hoppy' aroma. Moreover, although levels of hop oil volatiles remaining in the final beer are relatively low (ppb range, see also section 3.3), these quantities are clearly sufficient to impart distinct hop-derived flavour characteristics to lager beer.

During descriptive sensory evaluation, panellists were asked to score their general appreciation for the different beers from 0 to 8 (score 0 = not appreciated). The ISO beer, beer E, beer EL, beer L and beer W were assigned an average score of 4.1, 5.6, 7.5, 5.4 and 5.4 respectively. Clearly, hopping with Saaz pellets, regardless of the applied hopping regime, consistently resulted in higher appreciation compared to the unhopped ISO beer. Beer EL, which was hopped by addition of Saaz pellets both at the onset and towards the end of boiling received the highest appreciation. Such a hopping regime is frequently applied in brewing practice and is characteristic for a classic Pilsner type beer. Interestingly, also beer E received a (slightly) higher score compared to beer L and beer W, indicating that some degree of 'early kettle' hopping has value towards improved hop-derived flavour characteristics.

The average of the scores assigned for the various descriptors for each hopped beer is compared to the scores given for the ISO beer in separate spider plots. Figure 6A represents the flavour profile of beer E, on which one can see that 'early kettle' hopping impacts the flavour of lager beer by masking typical 'malty' and 'worty' flavours. Also 'fruity' flavours, which are in general imparted by fermentation esters, slightly decreased as a consequence of 'early kettle' hopping. Some 'floral', 'citrusy', 'grass/green' and 'resinous' notes are detected and flavour attributes described by 'spicy/herbal' and 'woody' clearly come to expression as a consequence of 'early kettle hopping'. The remarkable increase in 'spicy/herbal' and 'woody' aromas compared to the ISO beer can be clarified by the presence of oxygenated sesquiterpenoids, which confirms our previous study, in which unhopped iso-α-acid-bittered lager beer demonstrated 'spicy/herbal' and 'woody' aroma upon addition of a sesquiterpene oxidation product fraction [20]. Beer EL (see Fig. 6B), for which a portion of the hop pellets was added 'early' whereas another portion was added 'late', also expresses these 'spicy/herbal' and 'woody' flavours. In addition, as can be expected from the late hop addition, scores for the descriptors 'floral', 'citrusy' and 'grass/green' were significantly elevated compared to beer E. Apparently, beer EL expresses both the 'spicy/herbal' aromas typical for 'noble kettle hop' aroma and 'floral/citrus' notes, which might explain why this beer was so highly appreciated by our panellists. The flavour profile of beer L, depicted in figure 6C, shows that addition of all the hop pellets towards the end of wort boiling did not lead to elevated scores for 'floral' and 'citrus' compared to beer EL. Moreover, 'grass/green' and 'fruity' notes were scored much lower and also 'spicy/herbal' did not appear to be as distinct as in beer EL. On the other hand, our panellists detected increased 'woody' aroma characteristics in beer L. The flavour profile of beer W, exclusively hopped during the whirlpool stage and depicted in figure 6D, showed a comparable profile to beer EL, although 'spicy/herbal' notes were less pronounced and the beer clearly expressed a strong 'resinous' aroma. Compared to beer L, beer W expressed much stronger 'grass/green' and 'resinous' aroma. Panellists specifically mentioned that beer W was most comparable to beer EL, but, also agreed that the distinct 'resinous' aroma had a rather negative impact on their general appreciation for beer W. In general, beer EL was described as having the most intense 'kettle hop aroma'. Although beer E also clearly expressed the 'spicy/herbal' notes typical for 'noble kettle hop' aroma, panellists agreed that the hop-derived aroma of beer EL was more complex, which can most likely be brought into relation to the highest general appreciation for this beer.

Although beer E contains relatively low oxygenated sesquiterpenoid levels compared to beer EL, L and W (despite *de novo* formation of sesquiterpene oxidation products during the boiling process of beer E, see section 3.1), beer E was scored relatively high for 'spicy/herbal' notes. This observation might be clarified by less masking by 'late hop' flavours (floral, citrusy) which are typically less subtle than the delicate 'spicy' aroma. Linalool, for example, has been proven to be a contributor to the floral aroma of beer [1, 11–13, 60, 61]. On the basis of the normalised peak area of linalool in beer E, EL, L and W, it could be concluded that beer EL, L and W contain resp. 2.7, 3.7 and 4 times more linalool than beer E and also the descriptor 'floral' was scored significantly higher in these beers. Accordingly, the expression of 'spicy/herbal' notes, characteristic for 'noble kettle hop' aroma in lager beer, might not simply be dependent on the absolute level of flavour-active oxygenated sesquiterpenoids present, but rather on the ratio of volatiles imparting 'floral' aroma and 'spicy' aroma. The ratio of spicy compounds versus linalool was calculated on the basis of standardised peak areas, resulting in a ratio of 29, 25, 17 and 14 for beer E, EL, L and W respectively. Clearly, for beer E and EL this ratio is much higher than for beer L and W, and also the descriptor 'spicy/herbal' was scored significantly higher for beer E and EL. Apparently, the relatively high oxygenated sesquiterpenoid levels, combined with an ideal balance with 'floral/citrus' odorants, might

explain the high general appreciation score attributed to beer EL and its 'noble kettle hop 'aroma characteristics.

Also scores for other flavour attributes such as bitterness intensity and quality, mouthfeel and astringency were assigned to all the beers. It could be concluded that hopping leads to a significant increase of all these flavour attributes compared to the unhopped ISO-beer. Beer L and W were characterised by the highest scores for 'astringency', which was, according to the panellists, one of the reasons why beer L and W received the lowest scores for general appreciation. In addition, the high appreciations of beer EL could also be assigned to an increased bitterness quality and a positive effect on mouthfeel.

Basically, the hopping regime affects different flavour aspects of the final beer and, despite the low levels of hop oil-derived volatiles in the final beer, differences amongst the hop-derived aroma of the differently hopped beers are clearly detectable. Addition of a portion of rather expensive aroma hops at the onset of boiling seems indeed to make sense since the 'spicy/herbal' notes in both beer E and EL were highly appreciated by our panellists. Beer EL contained the highest level of oxygenated sesquiterpenoids and showed pronounced 'spicy/herbal' notes. In addition, our panellists agreed that beer EL expressed the most intense 'kettle hop' flavour. Clearly, such flavour characteristics, in combination with some 'floral/citrus' notes, are highly valued in lager beer.

4 Conclusion

In conclusion, we brewed one unhopped and four hopped lager beers and varied the time point of hop addition ('early', 'late', 'early and late', 'whirlpool' hopping). By analysis of wort samples, we proved for the first time that *de novo* formation of sesquiterpene oxidation products by oxidation of sesquiterpene hydrocarbons, which has already extensively been demonstrated on a lab scale by our research group [19, 20], also occurs during wort boiling when hop pellets are added 'early' to the process under our applied brewing conditions. During the whirlpool process of a beer for which 'whirlpool hopping' was applied, nett increases in the level of such sesquiterpene oxidation products are not observed. On the other hand, several oxygenated monoterpenoids increased in their level as a consequence of the whirlpool process and amongst the floral compounds, many volatiles were not detected in samples taken during the wort boiling process of the 'early kettle hopped' beer. Since there is no replication of the different beers, it is not possible to generalise the findings to other brews. However, the hopping regime clearly has a major impact on the composition of hop oil volatiles detected in wort. Although many compounds are lost during subsequent brewing process steps and hop oil levels that survived up to the final beer are in the low ppb level (impeding detection of individual hop oil-derived volatiles), the 4 differently hopped lager beers clearly expressed different flavour characteristics. During our experiment, the increase in the level of sesquiterpene oxidation products upon 'early kettle' hopping was lost during the whirlpool process, fermentation, lagering and filtration. Nevertheless, 'early kettle' hopping has a positive impact on 'spicy/herbal' aroma characteristics of lager beer. These flavours, characteristic for 'noble kettle hop' aroma, are better detectable when 'floral/citrus' notes, typically imparted by 'late' and 'whirlpool' hop additions, are not too pronounced. During our brew trials, addition of a portion of the hops at the onset of boiling and a portion at the end resulted in a highly appreciated and well-balanced beer with intense 'kettle hop' aroma. Brewing beers that express such a refined and highly desired flavour characteristic seem to require a delicate balance between the 'spicy/herbal' and 'floral/citrus' bouquet and remains therefore the ultimate challenge for brewers of traditional Pilsner beer types.

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