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Comprehensive Characterisation of the hop-derived Sesquiterpenoid Fingerprint of American Kettle hopped Lager Beers

In common brewing practice the typical so-called kettle hoppy aroma of lager beer results from the addition of aroma hop varieties to the boiling kettle. Years of extensive research has elucidated that hop-derived volatile flavour-active oxygenated compounds are most likely to impart this kettle hoppy aroma. In particular the oxygenated sesquiterpenoids are considered to play a key role in the spicy and herbal impression of 'kettle hoppy' aroma of traditional kettle hopped lager beers. However, accurate analytical detection and identification of these compounds in lager beers is everything but straightforward since these compounds are mostly present at the low ppb level. Therefore, in this study, three American kettle hopped lager beers expressing clear hoppy aroma characteristics were analysed via HS-SPME-GC-MS to obtain hop-derived volatile profiles. A relatively simple methodology using Solid Phase Extraction (SPE) was developed to enrich the hop oil volatiles, in particular the oxygenated sesquiterpenoids, present in beer and to distribute them over different fractions to overcome the analytical problem of co-elution. After analysis of these fractions, a remarkable number of 63 compounds was detected in the chromatographic region of interest. Amongst the 41 tentatively identified compounds, 33 proved to be oxygenated sesquiterpenoids. We also report for the first time the presence of iso-korajol and 3 caryophyllene derivatives, i. e. 4S-dihydrocaryophyllene-5-one, 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al and 6(5→4)-abeo-caryophyll-8(13)-en-5-al in lager beer. In addition, olfactory assessment via GC-O sniffing analyses was performed on the SPE-derived fractions of a lager beer, exclusively hopped with noble aroma hop varieties. Flavour-active zones were frequently allocated to α-humulene and β-caryophyllene derived epoxides and alcohols, sustaining the general view that these compounds might indeed play a key role in kettle hoppy aroma.

Descriptors: lager beer, hops, flavour-activity, hoppy aroma, oxygenated sesquiterpenoids, HS-SPME-GC-MS

1 Introduction

Lager beers are relatively new on the global beer market when comparing to the production of ales, which has already been going on for centuries. Nowadays, the craft brewing scene, in particular that of the USA, is stimulating the rise of specialty and ale beers, frequently delivering beers with unique hop-derived aromas and flavours. Despite this fact, lager beers still make up the most popular beer type and are consumed worldwide. The consumer's preference for lager beer types could partly be explained by their drinkability, refreshing power, low calorie content but most of all by a refined and well-balanced hoppy aroma.

This desirable flavour characteristic is derived from the volatiles present in hop essential oil and obtained by addition of aroma hops to the boiling kettle [1–3]. During the boiling of hops, many hop essential oil constituents are stripped out of the wort, polymerised, adsorbed to haze or oxidised into more water-soluble compounds [2, 4–12]. Next, the fermentation process has a clear impact on the spectrum of hop-derived volatiles in the final beer since hop oil constituents (e. g. particular monoterpenoids) could undergo biotransformation by the yeast [13–16]. In addition, nonpolar terpenes that survived the boiling process adsorb to trub and yeast cells or migrate to the foam layer, and are consequently almost completely lost [2, 4, 6, 8, 14, 17]. Summarised, the various brewing process steps that the hop oil compounds pass through alter the original hop volatile profile. As a result, the hop-derived constituents, ending up in the final beer, may differ significantly from the original hop oil volatiles [11] and consequently the aroma of raw hops as such and hoppy aroma of beer are clearly different [1, 18–21]. When the final aroma displays a herbal or spicy note, this is generally referred to as 'noble' kettle hoppy aroma [3, 19]. The general agreement is that the spicy hop impression is obtained by adding aroma hops early to the kettle [22] and that oxygenated sesquiterpenoids, in particular β-caryophyllene and α-humulene oxidation products, are at least correlated to the so-called kettle hoppy aroma [4, 5, 20, 23–26]. Traditional European hop varieties are used to impart this particular sensorial impression [1, 3, 19]. However, several non-European hop varieties such as Helga and Sylva originating from Australia, Motueka from New Zealand, Southern Star bred in South Africa and the American varieties Santiam, Ultra and Vanguard also express 'noble' aroma characteristics [27]. In contrast to kettle hopping, the practice of dry hopping directly transfers the original hop oil constituents to beer [11].

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Since decades, research has focused on the α-humulene and β-caryophyllene oxidation and hydrolysis products as key impact flavour compounds for hoppy aroma [4, 5, 25, 28 –31] and although their relevance was questioned, recent research again points in their direction [23, 32–35]. τ-Cadinol [36, 37], α-eudesmol [37] and the humulene derivatives humuladienone, humulene epoxide I, II, III, humulenol II, humulol, and humulene diepoxides A–D [1, 2, 4, 5, 7, 9, 37, 38] were already proposed to be important in respect of hoppy aroma in beer several decades ago. Recently, several oxygenated caryophyllene derivatives were proven to be flavour-active [33–35]. Despite the advances in chromatography, researchers claim that there are still minor flavour-active sesquiterpenoids that have yet to be identified [23, 32–36]. In addition, *Van Opstaele* and coworkers [39] reported a flavour threshold value of oxygenated sesquiterpenoid fractions in beer of 10–20 ppb, pointing to the high flavouring potential of these particular compounds.

Summarised, it can be stated that years of extensive scientific research piled up enough data supporting the impact of oxygenated sesquiterpenoids on hoppy aroma. However, despite this fact, a lot of difficulties remain to actually pinpoint the compounds responsible. The reasons therefore are quite fundamental and range from the occurrence of synergetic effects [2, 40–43] and lack of reference compounds and high quality mass spectra (hampering adequate identification) [33, 35, 44] to high degrees of co-elution, hampering allocation of an apperceived aroma to a particular compound when performing GC-O analyses [35, 44, 45]. Another obstacle that should not be underestimated is the low level of oxygenated sesquiterpenoids that survives the brewing process and is found in the final beer (typically in the range of 10 to 100 ppb). Basically, determining the impact of the oxygenated sesquiterpenoids on hoppy aroma is rather challenging since the actual detection limits of the chromatographic systems don't always permit a clear detection of some particular compounds in beer. This was certainly a bottle-neck in the early years of hop essential oil research and indeed, various researchers reported about extraction of large volumes of beer and concentration methods prior to GC-MS analysis for detection and identification of hop oil volatiles in beer [4, 5, 7, 19, 25, 26, 29, 36, 46– 48]. Nowadays, methods such as SPME are available for extraction of volatiles. In particular headspace SPME has frequently been employed to extract and concentrate volatiles from hops, hop essences and beer samples [34, 49–52]. This particular method is based on an equilibrium between volatiles in the solid/aqueous phase and in the gaseous phase. A fibre, coated with a polymer, is introduced into the headspace. Subsequently, an equilibrium between volatiles in the gaseous phase and volatiles adsorbed to the fibre is reached, whereupon the volatiles are desorbed in the gas chromatograph. Despite some drawbacks such as equilibrium drift with increasing fibre age, influence of temperature and time on the equilibrium and influence of major constituents on the adsorption of the target analyte [50], SPME has proven to be a rapid and solvent-free extraction method. Nevertheless, at present, the number of papers in which an attempt is made to characterize the detailed spectrum of oxygenated sesquiterpenoids, is extremely limited. However, the detection and identification of these hop-derived constituents in beer is essential in order to further unravel the complex issue of kettle hoppy aroma of beer. Consequently, we worked in this paper towards comprehensive analytical characterisation of the sesquiterpenoid fingerprint of three American kettle hopped lager beers. To overcome the problem of co-elution when performing monodimensional GC, we developed a new methodology based on solid phase extraction (SPE) for fractionation of our compounds of interest, which are subsequently detected and tentatively identified with state-of-the-art GC-MS. Finally, we aimed at pinpointing of odour-active compounds in the hop-derived sesquiterpenoid hop oil fraction of the investigated beers via gas chromatography-olfactometry.

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-heptanol (98 %); hexane (≥ 95 %); nonadecane (≥ 99.8 %); nonane (≥ 99.8 %); octane (≥ 99 %); tetradecane (99 %); tridecane (≥ 99 %); (E)-nerolidol (≥ 98 %); trans-β-farnesene (≥ 90 %).

The following reference compounds were purchased from Merck (Darmstadt, Germany) and were of analytical grade:
decane (> 99 %); undecane (> 99 %); dodecane (> 97 %); heptadecane (> 98 %); heptane (97 %); hexadecane (> 99 %); octadecane (> 98 %); pentadecane (> 99 %).

Ethanol absolute (≥ 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 American lager beers

Three commercial kettle hopped American lager beers (A, B and C) expressing a distinct kettle hoppy aroma, were transferred from the USA and stored at 4 °C until analysis. The alcohol percentage of beer A, B and C is resp. 5.20 %, 4.90 % and 4.90 %, the bitterness 30 IBU, 30 IBU and 30–35 IBU, whereas the original gravity is resp. 13 °P, 13 °P and 11–12.5 °P. For beer A, amongst other varieties, the following noble aroma hops were used: Hallertau Mittelfrüh and Vanguard. Beer B was exclusively brewed with the German noble aroma hop varieties Hallertau Mittelfrüh and Tettnang Tettnanger. Beer C was exclusively brewed with Northern Brewer.

2.3 Fractionation of the hop-derived compounds in lager beer via Solid Phase Extraction (SPE)

For detailed analysis of the oxygenated sesquiterpenoid fraction of three American kettle hopped lager beers, we used a Solid Phase Extraction (SPE) based methodology. This method allows for enrichment and fractionation of hop-derived compounds in beer in order to facilitate separation, detection and identification via HS-SPME-GC-MS analysis. Prior to SPE, 200 mL of each beer (A, B and C) was degassed using an ultrasonic bath (Julabo USR 3, Belgolabo, Overijse, Belgium). For each beer, a Bond Elut C18 cartridge (Mega Bond Elut Flash) (1 g, 60 mL, 40 µm, Agilent Technologies, Lake Forest, USA) was placed on a stopcock, inserted in

the cover of a glass SPE manifold. Vacuum was obtained by connecting the vacuum port with a water jet pump. The 3 columns were pre-conditioned by eluting respectively 3 volumes of HPLC-grade ethanol, three volumes of MQ-water and 3 volumes of a 5 v/v % ethanol/MQ-water solution through the column. Degassed beer was pipetted onto each column for enrichment of the hop-derived compounds and the effluent was collected in a waste container. The hop-derived compounds, adsorbed to the column, were subsequently eluted with 5mL HPLC-grade ethanol and the effluent was collected in a vial (10 mL, clear glass, Chromacol, Welwyn Garden City, UK). The effluent of each beer was diluted by addition of 5 mL MQ-water. These dilutions were further fractionated using 3 Bond Elut C18 cartridges (500 mg, 6 mL, Agilent Technologies, Lake Forest, USA). The columns were pre-conditioned with three volumes ethanol, MQ-water and 50/50 v/v % ethanol/MQ-water and each dilution was pipetted onto a column. The sample effluent was collected in a vial (20 mL, amber glass, Chromacol, Welwyn Garden City, UK) to check the absence of hop-derived compounds. Next, the adsorbed compounds were fractionated by desorbing the compounds using an ethanol gradient. Respectively 3 mL of a 50, 60, 70, 80, 90 and 100 v/v % HPLC-grade ethanol/MQ-water solution was pipetted onto the column and each fraction was collected in a separate vial (20 mL, amber glass, Chromacol, Welwyn Garden City, UK). All fractions were stored in the freezer (–18 °C) until further analysis.

All fractions were analysed via HS-SPME-GC-MS. For determination of the level of oxygenated sesquiterpenoids in the different fractions, the peak area of the oxygenated sesquiterpenoids was standardised by taking the internal standard (2-heptanol) into account. In figure 1, this standardised peak area is plotted as a function of the analysed fraction. For all the beers, practically no oxygenated sesquiterpenoids were detected in the sample effluent, implying that the hop-derived compounds were adsorbed onto the column as intended. The fractions eluting with 70 % and 80 % ethanol contain the highest levels of oxygenated sesquiterpenoids (see also Fig. 1). The oxygenated sesquiterpenoid fingerprint of these fractions will be comprehensively characterised in this study.

2.4 HS-SPME-GC-MS analysis of the SPE-derived fractions

2.4.1 HS-SPME extraction of the volatiles in the SPE-derived fractions

The fractions, derived from SPE fractionation of beers, were analysed by adding 4.400 mL MQ, 500 µL of the fraction and 1.5 g sodium chloride in a HS-SPME vial (20 mL, clear glass, Chromacol). Before the vials were closed with bimetal magnetic caps with silicon/Teflon septum (Supelco, Bellefonte, USA), 100 µL 2-heptanol (253 ppm stock solution in ethanol) was pipetted into the vial to a final concentration of 5 ppm to serve as an internal standard. Automated extraction of the hop-derived compounds was achieved via headspace solid-phase microextraction (HS-SPME) using a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland), controlled via Pal Real Time software. A polydimethylsiloxane (PDMS) coated extraction fibre (100 µm, Supelco Inc., Bellefonte, PA, USA) was introduced through the septum into the headspace

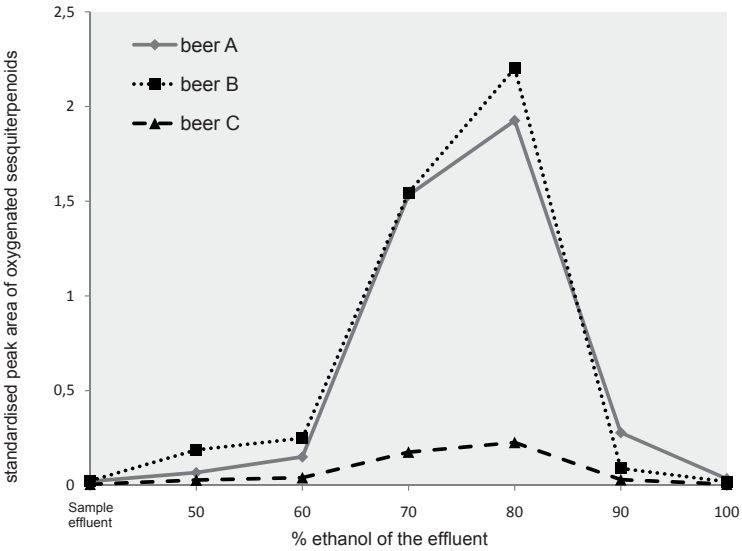

Fig. 1 Standardised peak area (area sesquiterpenoid fraction/area 2-heptanol (internal standard)) as a measurement for the level of oxygenated sesquiterpenoids, found in the effluent of the sample and fractions with increasing ethanol concentration after Solid Phase Extraction (SPE) treatment of three American lager beers (A, B, C)

of the vial (vial penetration: 22 mm). After extraction at 60 °C during 45 minutes (250 rpm, agitation: 5 s on – 2 s off) [35], the fibre was automatically placed into the heated inlet of the Ultra Trace gas chromatograph (Thermo Fisher Scientific, Austin, TX) for thermal desorption of the extracted volatiles (splitless injection, 3 minutes, 220 °C), after which the fibre was post-conditioned in the needle heater (5 min at 250 °C).

2.4.2 Gas chromatographic separation of the volatiles, extracted from the SPE-derived fractions

For separation of the volatiles, a nonpolar PDMS coated RTX-1 capillary column (40 m x 0.18 mm i.d. x 0.2 µm film thickness, Restek Corporation, Bellefonte, PA) was employed. Helium (Alphagaz 2, Air Liquide, Liège, Belgium) was used as a carrier gas at a constant flow of 1.2 mL/min. The oven program 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, 20 °C/min up to 250 °C, hold 3 min.

2.4.3 Mass spectrometric detection of volatiles

Mass spectrometric detection of volatiles was performed by a Dual Stage Quadrupole MS (DSQ I, Thermo Fisher Scientific, Austin, TX) operating in the electron ionisation mode (EI, 70 eV). The ion source was set at 240 °C and the detection gain was 2 x 10^5 (electron multiplier voltage: 1446 V). Analyses were performed in the full scan operating mode (m/z = 40–265). The MS was programmed to detect positive ions and the total scan time was 0.25 s (4.0323 scans/s, scan rate: 995.8 amu/s). Specific screening of the full scan chromatograms for oxygenated sesquiterpenoids was achieved by post analysis selected ion monitoring (extracted ion chromatograms were obtained by plotting specific mass fragments: m/z 79, 91, 93, 105, 121, 131, 133, 149, 202, 205, 218, 220, 222). The detected compounds were identified by mass spectral comparison via the Xcalibur software (v.1.4 SR1, Thermo Fisher Scientific, Austin, TX), using the "NIST98" and "Flavour MS library for Xcalibur 2003" spectral libraries (Interscience, Louvain-la-Neuve, Belgium), via

mass spectra that are found on the NIST website (http://webbook.nist.gov/chemistry/) and in books, containing mass spectral information [53, 54]. Kovàts retention indices (RI) [55] from literature data were compared with the calculated retention indices of the volatiles, determined by analysis of a homologous series of normal alkanes (C6–C19; Sigma-Aldrich, St. Louis, MO). Compounds were only (tentatively) identified if there was a match for both mass spectrum (MS match factor > 650) and RI (calculated RI = RI literature ± 5). If the mass spectrum and/or RI was not comparable with literature data, the compound was indicated as 'unknown'.

2.5 Determination of flavour-active compounds in beer B via GC-O

Volatiles of beer B, eluting in the oxygenated sesquiterpenoids chromatographic region, were extracted via HS-SPME and flavour-active constituents were determined by GC-olfactometry with a Sniffer 9000 system (Brechbüchler Inc., Schlieren, Switzerland), coupled to the GC-MS device. For this purpose, the method described in 2.4 was employed. The effluent from the GC-column was split to the mass spectrometer and the sniffing port (50/50 %). The temperature of the transfer line (connection GC and sniffing port) was set at 250 °C. The effluent was mixed with a stream of humidified air before leaving the sniffing port. Three trained assessors were asked to sniff the sample and to indicate flavour-active zones as well as to record the duration of the odour perception, which was achieved by using a hand-held control unit with cursor wheel for signal generation. Assessors were highly trained for detection of oxygenated sesquiterpenoids by sniffing the oxygenated sesquiterpenoid fraction of hop (oil) and beer samples on a regular basis. Aromagrams were automatically generated via the Xcalibur software. The fractions of beer B that elute with 70 % and 80 % ethanol contain the highest level of oxygenated sesquiterpenoids (see Fig. 1) and were therefore selected for GC-O analysis. For analysis, 500 µL of the fraction was pipetted to 4500 µL MQ-water in a HS-SPME vial. Both the 70 % and 80 % ethanol fraction were analysed in triplicate and the detection frequency (DF) is calculated as the number of times a particular odour-active zone was detected out of three analyses.

3 Results and discussion

3.1 GC-MS fingerprinting of the hop-derived oxygenated sesquiterpenoid spectrum of American kettle hopped lager beers

Investigation of the composition of the hop-derived oxygenated sesquiterpenoid fraction in beer is not straightforward and a challenging task for analysts on account of the high complexity of this particular fraction, the high degree of co-elution (hampering accurate identification) when performing standard monodimensional GC-MS and the extremely low levels at which these constituents are present in beer. To tackle this problem, an SPE-based methodology was developed to extract hop oil constituents, in particular oxygenated sesquiterpenoids, from beer and to enrich and distribute our compounds of interest over different fractions. As discussed in 2.3, the fractions eluting with 70 % and 80 % ethanol contain the highest level of oxygenated sesquiterpenoids and therefore, these two fractions were analysed via HS-SPME-GC-MS in order to characterise the oxygenated sesquiterpenoid profile of three American kettle hopped lager beers.

On the basis of the calculated Kovàts indices in combination with mass spectra, compounds that elute in the oxygenated sesquiterpenoids chromatographic region were tentatively identified. The results are summarised in table 1. In total, 63 different compounds were detected in the chromatographic region where oxygenated sesquiterpenoids elute and moreover, 41 compounds were (tentatively) identified. Clearly, 33 identified volatiles belong to the chemical class of oxygenated sesquiterpenoids. In addition, 11 more compounds, although their precise identity could not be revealed, are proposed to be oxygenated sesquiterpenoids on account of their mass spectra (i. e. typical fragmentation pattern and recognition of the molecular ion at m/z 218, 220 or 222). Thus, the results show that 70 % of the detected compounds are oxygenated sesquiterpenoids, proving that the proposed SPE based methodology allows accurate determination of the oxygenated sesquiterpenoid hop oil fraction in beer. Apparently, this class of compounds survives the brewing process and therefore they are very important in lager beer from an analytical point of view (analytical fingerprinting). Moreover, according to literature data, they are also considered to be important regarding sensory characteristics as they are suggested to contribute to kettle hoppy aroma of beer [4, 5, 20, 23–26]. Next to the oxygenated sesquiterpenoids, we also detected 5 sesquiterpene hydrocarbons (n° 3, 9, 12, 13, 53) and 3 compounds that probably didn't originate from hops since they don't show a terpene-derived chemical structure (n° 7, 8, 58).

The major part of the hop-derived oxygenated sesquiterpenoid fraction consists of β-caryophyllene and α-humulene derivatives. These compounds are oxidation products from their parent sesquiterpene hydrocarbon molecule, formed during ageing and possibly during the boiling of hops [2, 4, 7, 8, 23]. Nine compounds are clearly derived from β-caryophyllene (Table 1, n° 14, 15, 19, 21, 22, 38, 45, 50, 54), whereas 10 compounds are α-humulene derivatives (n° 4, 20, 25, 26, 27, 29, 34, 36, 56, 62). The chemical structure of compound n° 11, i. e. iso-korajol (IUPAC name: (1R,2R,5S,6R,9R)-2,6,10,10-tetramethyltricyclo[7.2.0.$0^{2,5}$]undecan-6-ol), suggests that this compound is derived from β-caryophyllene or α-humulene. This compound has not been reported in literature data in the context of hops and beer but its mass spectrum, Kovàts index and chemical structure were reported by *Tkachev* [54].

Two β-farnesene derivatives (n° 61, 63) were detected in beer B and C. Trace levels of β-farnesene (n° 3) were also found in beer B and C, suggesting that these beers were brewed with a β-farnesene rich hop variety (e. g. cv. Saaz, Lublin, Styrie, Backa, Spalt, Tettnang and Hallertau [56–58]). This observation is in accordance with the information which was given on beer B (see paragraph 2.2), i. e. hop aromatisation with hops cv. Hallertau Mittelfrüh and Tettnang Tettnanger.

Remarkably, all the other identified oxygenated sesquiterpenoids not discussed above, except for gleenol (n° 24) and nerolidol (n° 18), depict a highly similar chemical structure ($C_{15}H_{26}O$) with a molecular mass of 222 and contain two 6-ring structures (having 2 adjacent carbon atoms in common), one hydroxyl group and one unsaturated bound. Particular compounds of this group, such as

Table 1 **Compounds detected in the chromatographic region where the oxygenated sesquiterpenoids elute (full scan detection (m/z 40-265) and subsequent extracted ion chromatograms (m/z 79, 91, 93, 105, 121, 131, 133, 149, 202, 205, 218, 220, 222)). All identifications are tentative and based on a match for both RI and mass spectrum, except no. 4 (only match for mass spectrum). [R] = verification of identity by pure reference compound. no. = peak number, RI = calculated Kovàts index, t_R = retention time (min), * = oxygenated sesquiterpenoid (if the compound is unknown, the mass spectrum suggests an oxygenated sesquiterpenoid structure), x = detected in beer A, B and/or C. Compounds in bold are detected in all investigated beers**

no.	RI	t_R	compound	A	B	C
1	1436	42.77	**unknown (m/z 69, 81, 95, 109, 123, 205, 220)***	**x**	**x**	**x**
2	1441	43.12	unknown (m/z 135, 149, 163, 177, 205, 207, 220, 222)*	x		x
3	1443	43.28	β-farnesene[R]		x	x
4	1464	44.86	**1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.0^{6,9}]dodecene***	**x**	**x**	**x**
5	1470	45.31	unknown (m/z 69, 81, 95, 109, 123, 205, 220)*			x
6	1481	46.07	unknown (m/z 69, 83, 121, 139)	x	x	
7	1488	46.6	**ionol**	**x**	**x**	**x**
8	1490	46.73	**3,5-di-tertylbutyl-phenol**	**x**	**x**	**x**
9	1493	46.97	δ-cadinene	x	x	
10	1493	46.95	unknown (m/z 177, 220)*	x		
11	1505	47.88	iso-korajol*	x	x	
12	1516	48.82	**trans-cadina-1,4-diene**	**x**	**x**	**x**
13	1521	49.17	**α-calacorene**	**x**	**x**	**x**
14	1527	49.66	4S-dihydrocaryophyllene-5-one*	x	x	
15	1527	49.73	6(5→4)abeo-8,12-cyclo-caryophyllan-5-al*	x	x	
16	1538	50.58	unknown (79, 80, 81, 150, 157, 220)	x		
17	1538	50.58	unknown (m/z 93, 205, 220)*		x	
18	1543	50.97	**(E)-nerolidol*[R]**	**x**	**x**	**x**
19	1548	51.39	**caryolan-1-ol***	**x**	**x**	**x**
20	1548	51.44	**humuladienone***	**x**	**x**	**x**
21	1553	51.85	6(5→4)-abeo-caryophyll-8(13)-en-5-al*		x	
22	1560	52.42	**clovenol***	**x**	**x**	**x**
23	1564	52.76	unknown (m/z 107,135,161, 218)*	x	x	
24	1565	52.77	gleenol*	x	x	
25	1572	53.42	**humulene epoxide I***	**x**	**x**	**x**
26	1577	53.79	**humulol***	**x**	**x**	**x**
27	1582	54.23	**humulene epoxide II***	**x**	**x**	**x**
28	1589	54.76	**unknown (m/z 81,123,135,161, 179, 189, 204, 207)***	**x**	**x**	**x**
29	1591	54.92	humulene allylic alcohol*		x	
30	1593	55.1	**1,10-di-epi-cubenol***	**x**	**x**	**x**
31	1596	55.32	junenol*	x	x	
32	1600	55.65	unknown (m/z 70)	x		x
33	1601	55.76	unknown (m/z 59, 81, 135, 149, 161, 164, 179, 189, 204)*	x		
34	1604	56.00	**humulene epoxide III***	**x**	**x**	**x**
35	1606	56.16	**1-epi-cubenol***	**x**	**x**	**x**
36	1607	56.23	**humulenol II***	**x**	**x**	**x**
37	1608	56.32	**unknown (m/z 119, 161, 179, 189, 204)***	**x**	**x**	**x**
38	1611	56.6	caryophylla-4(12),8(13)-diene-5-ol*		x	
39	1617	57.09	**τ-cadinol***	**x**	**x**	**x**
40	1618	57.16	τ-muurolol*			x
41	1621	57.45	**cubenol***	**x**	**x**	**x**
42	1624	57.70	β-eudesmol*	x	x	
43	1627	57.91	**selin-11-en-4-α-ol***	**x**	**x**	**x**
44	1629	58.10	**α-cadinol***	**x**	**x**	**x**
45	1632	58.39	(3Z)-caryophylla-3,8(13)-diene-5α-ol*	x	x	
46	1634	58.54	unknown (m/z 79, 80, 81,164, 222)	x	x	
47	1636	58.73	unknown (m/z 79, 80, 81,162, 220)	x		
48	1638	58.90	**unknown (m/z 119, 121, 191)**	**x**	**x**	**x**
49	1641	59.09	intermedeol*	x		
50	1641	59.09	14-hydroxy-β-caryophyllene*	x	x	
51	1644	59.41	unknown (m/z 93,137)	x	x	
52	1644	59.41	unknown (m/z 191)	x	x	
53	1646	59.50	cadalene	x	x	
54	1647	59.60	(3Z)-caryophylla-3,8(13)-diene-5β-ol*	x	x	
55	1649	59.77	unknown (m/z 55, 69, 82, 93, 120, 138, 222)	x	x	
56	1654	60.19	humulene allylic alcohol*	x	x	
57	1654	60.23	unknown (m/z 79, 80, 81)		x	
58	1661	60.82	tetradecanol	x	x	
59	1665	61.15	unknown (m/z 69, 82)	x	x	
60	1667	61.34	eudesm-7(11)-en-4α-ol*	x	x	
61	1671	61.66	farnesal*		x	x
62	1686	62.90	humulene diepoxide A*	x	x	
63	1698	63.93	farnesol*		x	x

the cadinols (derived from the cadinane skeleton), tend to have a higher concentration in noble aroma hops and are probably not formed by chemical oxidation of sesquiterpene hydrocarbons but instead related to the hop plant biosynthesis [25, 59]. Although this class of compounds is not correlated to the β-caryophyllene and α-humulene oxidation products, these compounds (cadinols but also muurolols, eudesmols, cubenols etc.) might be important with respect to hoppy aroma, since they clearly survive the brewing process and are detected in the beers investigated in our study. Furthermore, τ-cadinol has been proposed in literature as a contributor to hoppy aroma [36, 37] .

There should be emphasised that despite interference from fermentation fusel alcohols and esters, the relatively low level of hop-derived volatiles in beer and co-elution of various compounds, a large series of oxygenated sesquiterpenoids has been detected and identified in the volatile profile of lager beer using SPE-enrichment and fractionation. Amongst them, some compounds have already been detected, investigated and identified in hops and beer decades ago (Table 1, e. g. n° 18, 19, 20, 25, 26, 27, 34, 35, 36, 39, 41, 42, 43, 44, 62, 63 [1, 2, 4, 5, 7, 20, 25, 36, 46, 47, 58, 60, 61], whereas the presence of other compounds was only recently confirmed in hops (e. g. n° 24, 50 [33, 35, 62]) and/or beer (e. g. n° 24, 30, 38, 49, 50, 54 [34, 51]).

The main caryophyllene oxidation product, i.e. caryophyllene oxide, was not detected in the beers in our study, which is in agreement with previous findings [1, 4] and can be attributed to the fact that this compound is prone to hydrolysis and isomerisation reactions [1, 29, 31]. Interestingly, some caryophyllene oxide derived compounds were already found in hop essential oil in the 90's, but were at that time not found in beer [29]. It concerns 2 allylic alcohols (caryophylladienol = caryophylla-4(12),8(13)-diene-5-ol and caryophyllenol = caryophylla-3,8(13)-diene-5-ol) a caryophyllene

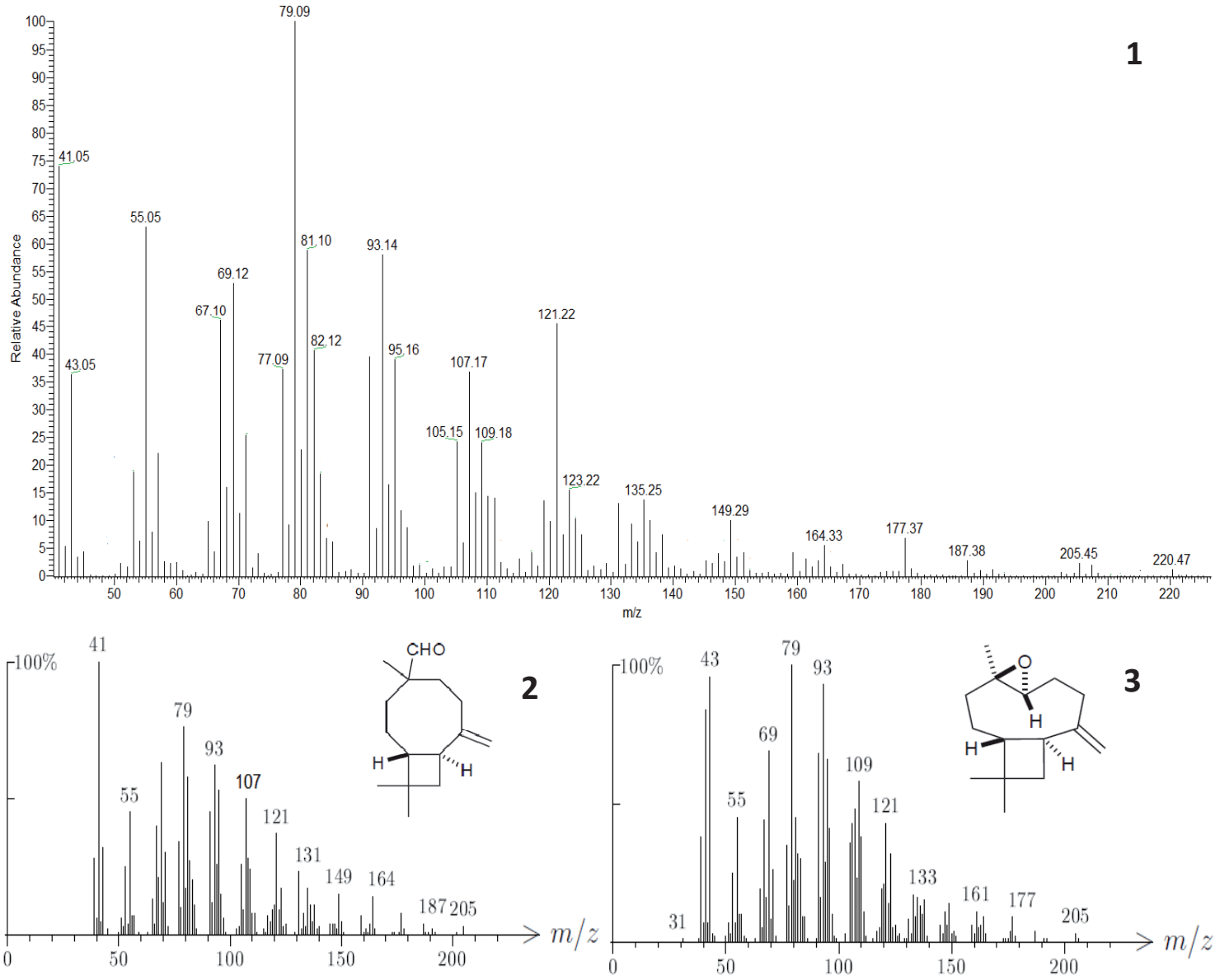

Fig. 2 **1. Mass spectrum of compound n° 21 (in accordance with Table 1), detected in beer B.**

2. Mass spectrum of 6(5→4)-abeo-caryophyll-8(13)-en-5-al [54].

3. Mass spectrum of caryophyllene oxide [54]

derived aldehyde (6(5→4)-abeo-caryophyll-8(13)-en-5-al) and a ketone (dihydrocaryophyllene-5-one). The ketone as well as the 2 allylic alcohols each have 2 isomers [54]. The ketone that was detected in hop oil by *Yang* and coworkers [29] is the S-isomer whereas the detected caryophylladienol and caryophyllenol structure are the α-isomer.

We tentatively identified caryophylla-4(12),8(13)-diene-5-ol in beer B. However, under our GC-MS conditions, no distinction between caryophylla-4(12),8(13)-diene-5α-ol and caryophylla-4(12),8(13)-diene-5β-ol can be made, due to the identical Kovàts index and mass spectrum. Therefore, the isomeric form of compound n° 38 is not further specified and also *Dresel* and coworkers assigned a general name, i.e. 'caryophylladienol', to this volatile after detecting it in wort samples, hopped with cv. Amarillo, and in both a kettle and dry hopped beer, brewed with cv. Wilamette [51]. Although this caryophyllene derivative is only a minor compound, it can easily be recognised by the distinct mass fragment at m/z 136 in the mass spectrum.

Caryophylla-3,8(13)-diene-5α-ol and caryophylla-3,8(13)-diene-5β-ol could, despite their identical mass spectrum, be distinguished on the basis of their Kovàts index [54]. The β-isomer was detected quite recently in hop oil and in ale beer [34], whereas the α-isomer was detected in hop oil several years ago [29]. Our research group recently detected the latter compound in both a kettle and dry hopped beer, brewed with a single hop variety cv. Wilamette [51]. Also *Tressl* and coworkers reported about the presence of 'caryophyllenol' in beer, although the isomeric form was not further specified [61]. In our current work, we detected both isomers in beer A and B (n° 45 and 54, Table 1).

In contrast to the caryophyllene allylic alcohols discussed above, the caryophyllene derived aldehyde has hitherto never been detected in beer. This could possibly be attributed to confusion with caryophyllene oxide, which has a similar Kovàts index and depicts a highly similar mass spectrum (Fig. 2). However, these two compounds can be distinguished since the mass spectrum of 6(5→4)-abeo-caryophyll-8(13)-en-5-al shows high relative intensities for the fragment ions at m/z 41, 107 and 164, whilst the caryophyllene oxide mass spectrum is characterised by higher intensities for m/z 43, 109 and 161. Based on the mass spectrum and Kovàts index, we tentatively identified compound n° 21, detected in beer B, as 6(5→4)-abeo-caryophyll-8(13)-en-5-al.

The caryophyllene derived ketone (n° 14) is also detected in this study for the first time in beers A and B. Both the S and R-isomer depict highly similar mass spectra [54]. However, on the basis of the calculated Kovàts index the compound was tentatively identified as 4S-dihydrocaryohpyllene-5-one, which was already reported in hop essential oil [29]. The experimental mass spectrum of this compound is depicted in figure 3.

In conclusion, both the abovementioned caryophyllene derived aldehyde and ketone were previously reported in hop oil but were so far not detected in beer. Next to these compounds, we would like to report 2

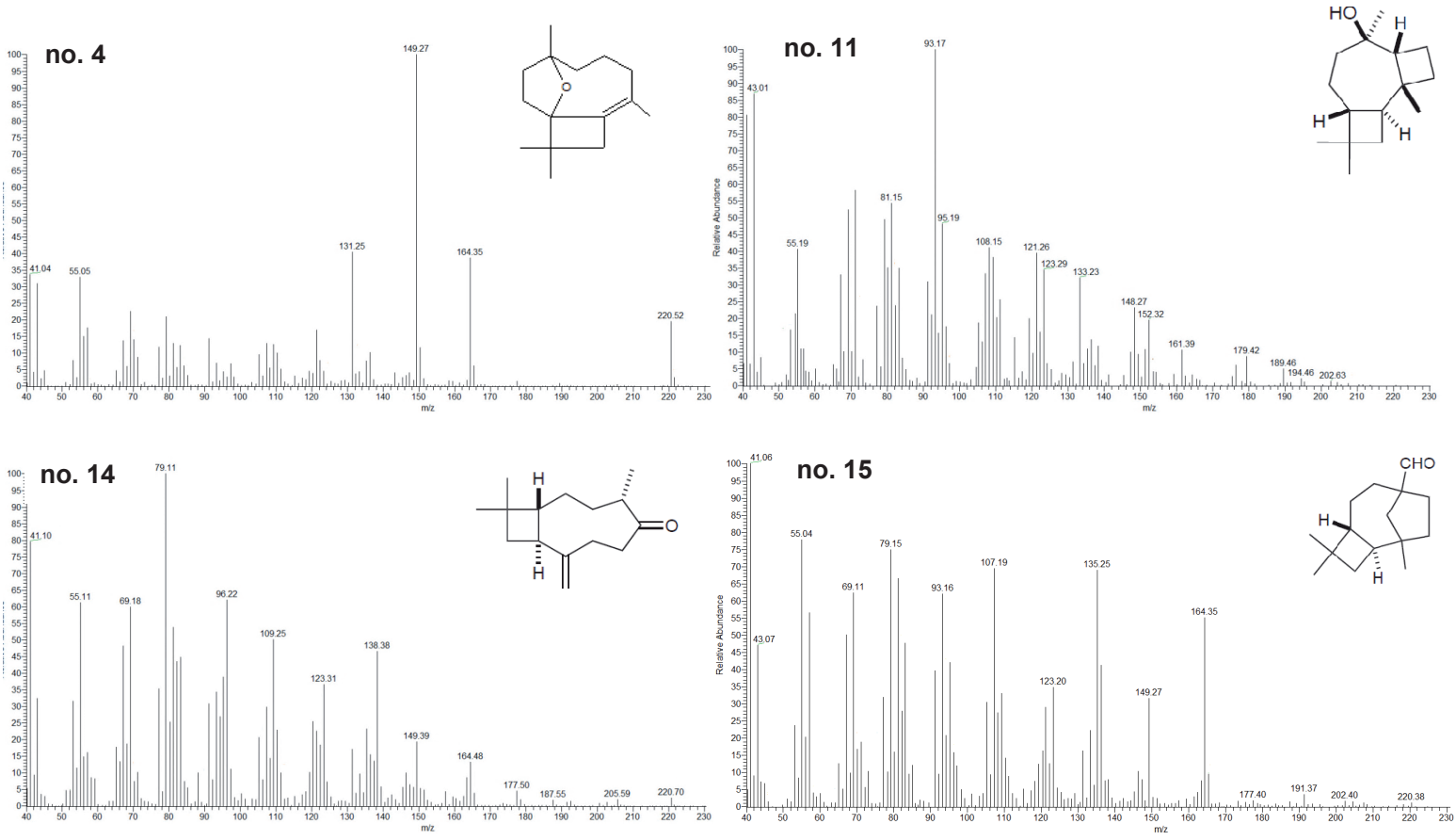

Fig. 3 Experimental mass spectra of compound no. 4 (1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.0^{6,9}]dodecene), no. 11 (iso-korajol = (1R,2R,5S,6R,9R)-2,6,10,10-tetramethyltricyclo[7.2.0.0^{2,5}]undecan-6-ol), no. 14 (4S-dihydrocaryophyllene-5-one) and no. 15 (6(5→4) abeo-8,12-cyclo-caryophyllan-5-al). Chemical structures are reproduced from literature data [30, 54]

other compounds, which have hitherto neither been detected in hops, nor in beer. It concerns compound n° 15, which was tentatively identified as 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al, and compound n° 11, tentatively identified as iso-korajol on the basis of both Kovàts index and mass spectrum [54]. The experimental mass spectra and chemical structures of these compounds are depicted in figure 3.

We also underline the presence of a series of compounds which have not frequently been reported in literature data. One of them is compound no. 4, which was already identified in a mixture of reaction products, obtained via reflux boiling of humulene epoxide II and III by Yang and *Deinzer* [30] and was later detected in beer [28]. We were able to tentatively identify compound n° 4 as 1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene on the basis of its highly characteristic mass spectrum. According to Yang and Deinzer, the mass spectrum is characterised by high fragment ion intensities at m/z 55, 131, 149, 164 (relative intensities of 23, 31, 100 and 71 % respectively) and shows an intense molecular ion at m/z 220 (relative intensity of 45 %) [30], which is exactly identical to our experimental mass spectrum for compound no. 4 (see Fig. 3).

Clovenol (n° 22) and junenol (n° 31) were detected in beer in this study. The latter compound was mentioned in the context of beer by *Tressl* and coworkers [61], although they did not find this compound in hops earlier [59]. Finally, 2 humulene derived oxygenated

Table 2 **Compounds detected in flavour-active chromatographic regions in the aromagrams, obtained via GC-O analysis of the SPE-derived 70% and 80% ethanol fractions of beer B. All identifications are tentative and are based on a match for both RI and mass spectrum. RI = calculated Kovàts index, t_R = retention time (min), start = start flavour-active region, end = end flavour-active region. DF = detection frequency. Compounds in bold are detected 3 times or more out of the 6 analyses**

RI start	RI end	Compound	70 % DF out of 3	80 % DF out of 3	total DF out of 6
1463	1468	1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene	2	0	2
1468	1472	unclear mass spectrum	2	0	2
1489	1494	3,5-di-tertylbutyl-phenol/δ-cadinene	2	0	2
1502	1504	iso-korajol	1	1	2
1517	1518	unclear mass spectrum	1	1	2
1520	1521	α-calacorene	2	0	2
1522	**1524**	**unclear mass spectrum**	**3**	**1**	**4**
1536	1541	unknown (m/z 93, 205, 220)	2	0	2
1537	**1539**	**β-calacorene**	**2**	**1**	**3**
1539	1542	unclear mass spectrum	0	2	2
1548	1549	caryolan-1-ol/humuladienone	1	1	2
1557	1559	clovenol	2	0	2
1569	1574	unclear mass spectrum	0	2	2
1569	**1572**	**humulene epoxide I**	**2**	**3**	**5**
1573	**1576**	**humulol**	**2**	**2**	**4**
1584	**1586**	**unclear mass spectrum**	**2**	**2**	**4**
1587	1590	unknown (m/z 81, 123, 135, 161, 179, 189, 204, 207)/ humulene allylic alcohol	1	1	2
1594	1600	junenol	1	1	2
1596	1598	unclear mass spectrum	2	0	2
1601	1604	humulene epoxide III	1	1	2
1605	**1608**	**humulenol II**	**2**	**2**	**4**
1609	**1613**	**caryophylla-4(12),8(13)-diene-5-ol/ phenyl ethyl hexanoate**	**2**	**3**	**5**
1616	**1619**	**τ-muurolol**	**2**	**2**	**4**
1616	1619	cubenol	1	1	2
1622	1625	β-eudesmol	1	1	2
1628	1629	α-cadinol	2	0	2
1627	1630	3Z-caryophylla-3,8(13)diene-5α-ol	1	1	2
1635	**1639**	**14-hydroxy-caryophyllene**	**1**	**2**	**3**
1636	1641	unknown (m/z 93, 137)	0	2	2
1643	**1645**	**cadalene/3Z-caryophylla-3,8(13)diene-5β-ol**	**2**	**3**	**5**
1668	**1671**	**unclear mass spectrum**	**1**	**2**	**3**

sesquiterpenoids, containing an allylic alcohol in their chemical structure (n° 29, 56), were detected. Humulenol II also belongs to the chemical group of humulene-derived allylic alcohols. Next to humulenol II, Yang and Deinzer also detected another humulene allylic alcohol, i.e. 2,6,6,9-tetramethyl-2,4,8-cycloundecatrien-1-ol, in the mixture of hydrolysates of humulene epoxide II and III and both in hop oil and beer [28, 30]. This compound might match our compound n° 29 or 56, although this can't be verified since the authors didn't provide spectral information.

From table 2, it is clear that the hop-derived oxygenated sesquiterpenoid spectrum strongly varies between the beers. Some compounds are found in all the investigated beers and show a relatively high peak area (e.g. humuladienone, humulene epoxide I, II, III, humulol, humulenol II, τ-cadinol and humulene diepoxide A). Strikingly, these compounds were also thoroughly investigated in literature. Other compounds are not detected in all beers or only show minor peaks, which can explain why they were frequently looked over in earlier studies. Indeed, several years ago *Siebert* stated: "It is highly likely that, despite the advances in analytical methodology, we still can't measure compounds at low enough concentrations to know all those responsible for hoppy flavour" [2]. This statement seems valid up to date, since new minor constituents are still found flavour-active [33–35].

3.2 GC-O analysis for the determination of flavour-active oxygenated sesquiterpenoids in SPE-derived fractions of American kettle hopped lager beer

To obtain insights in which particular sesquiterpenoids actually contribute to the (kettle) hoppy aroma of beer, sensory assessment was performed via GC-O analysis on the SPE fractions derived from beer B. Beer B was chosen for profound olfactometric evaluation on the basis of the high level of oxygenated sesquiterpenoids in the SPE fractions as reported earlier (see Fig. 1).

Table 2 displays odour-active regions (31 in total) that were detected at least twice upon GC-O analysis and, where possible, the identity of the compounds detected in the respective odour-active regions are reported.

A relatively high number of chromatographic regions showed weak odour-activity based on the detection frequency (DF=2), and in many cases it was impossible to reveal the identity of the compound responsible for the perceived odour (low quality mass spectra or level of the compound below detection limit). However, we were able to (tentatively) identify 1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$] dodecene (literature odour descriptors: 'cedar', 'camphor' [1, 28]), δ-cadinene (literature odour descriptor: 'herbal'*)*, iso-korajol, clovenol, junenol, and α-calacorene (literature odour descriptor: 'wood' [35, 48]) in weak odorous zones. The latter compound was already detected in an odour-active region of a hop oil-derived spicy fraction [35] and was related to the citrusy and spicy character of beer [48]. In this study, its isomer β-calacorene was also tentatively identified in the odorous zone ranging from RI 1537–1539.

In this study, caryolan-1-ol and humuladienone were detected in the odour-active region from RI 1548–1549. *Shimazu* and coworkers [7] already proposed humuladienone as a flavour-impact compound for hoppy aroma, since its content in beer (30–70 ppb) is near to the flavour threshold (100 ppb). Several years ago, humuladienone was detected in beer via GC-O [38] and only recently, Van Opstaele and coworkers [35] reported a highly flavour-active zone in a spicy hop essence that consists of humuladienone and caryolan-1-ol. The presence of caryolan-1-ol in beer has amply been demonstrated [20, 25, 51, 61] but the question whether caryolan-1-ol is odour-active remains unanswered.

Humulene epoxide III, described in literature data as 'cedar' [28], was present in the odour-active zone ranging from RI 1601–1604. Although this compound is a humulene oxidation product, its chromatographic peak was yet relatively small, probably due to the conversion of this compound to various hydrolysis and isomerization products [30].

Bicyclic sesquiterpenoid alcohols (e. g. cubenol, β-eudesmol, α-cadinol) were identified in specific odour-active regions. In literature, β-eudesmol (RI 1622–1625) was proposed as a key aroma compound related to hoppy characteristics in beer using PCA by *Inui* and coworkers [48].

The odorous zone comprising humulene epoxide I showed high flavour-activity (DF = 5). It was suggested in literature that humulene epoxide I contributes to hop aroma in beer since its concentration in beer (125 ppb [61]) is far above its reported threshold value (10 ppb in water [25]). A relatively high peak area of humulene epoxide I in the registered chromatogram in this study, in particular in comparison to the peak areas of humulene epoxide II and III, points to high levels in beer. In general, the concentration of humulene epoxide I in hops is lower than the level of humulene epoxide II [59]. However, the reverse is observed in beer [61], which can be assigned to the fact that humulene epoxide I is fairly resistant to hydrolysis whereas humulene epoxide II and III can extensively be converted into a series of alcohols [1, 30]. Humulene epoxide II was not found to be odour-active on the basis of our sniffing analyses and although this compound was previously detected in an odour-active region of an oxygenated sesquiterpenoid hop oil fraction, the odour was proposed to be imparted by minor co-eluting constituents [35].

In sharp contrast with humulene epoxide II, we observed high levels of humulenol II in a highly flavour-active region (RI 1605–1608, DF = 4) in the registered aromagrams. The higher level of humulenol II compared to the level of humulene epoxide II is possibly due to the chemical conversion of humulene epoxide II to humulenol II as was reported in literature [4, 5, 25, 30]. Our findings on the odour-activity of humulenol II are in agreement with literature data. *Peacock* and coworkers [25] found that humulenol II should at least be partly responsible for hoppy aroma of beer, in particular, for beers brewed with traditional aroma hop varieties (noble kettle hop aroma) [5, 25] while *Lam* and coworkers reported the contribution of humulenol II to the herbal/spicy note of beer [4].

Finally, our GC-O results clearly show the presence of particular sesquiterpene alcohols, i.e. humulol, τ-muurolol, caryophylla-4(12),8(13)-diene-5-ol, 14-hydroxy-caryophyllene and 3Z-caryophylla-3,8(13)diene-5β-ol, in several highly odour-active

regions. Most of these constituents have already been reported in earlier studies as potential flavour-impact compounds for hop(py) aroma. For example, 14-hydroxy-caryophyllene was found to be flavour-active on the basis of GCxGC-TOFMS and GC-O analysis of the spicy fraction of hops by *Eyres* and coworkers [33], while 3Z-caryophylla-3,8(13)diene-5β-ol has been indicated as a character-impact compound for the hop aromatic character of ale beer [34]. Although the contribution of humulol to hoppy aroma of beer was questioned on the basis of its high threshold value [47], we were able to find evidence for the odour-activity of humulol in the investigated beer. To our knowledge, caryophylla-4(12),8(13)-diene-5-ol is reported for the first time as potential odour-impact compound in beer.

In conclusion, GC-O clearly demonstrated the odour-activity of oxygenated sesquiterpenoid fractions isolated from an American kettle hopped lager beer. Although we were able to allocate and tentatively identify particular constituents in odour-active regions we would like to emphasise that odour-activity of the reported compounds has not been proven unambiguously since phenomena such as co-elution and minor constituents, present below the actual detection limits, hamper allocation of a perceived odour to a particular compound. Therefore, our findings call for continued research aiming at further development and application of highly sophisticated analytical methodologies/techniques, and preparation of authentic sesquiterpenoid reference compounds for further unravelling the true nature of the (kettle) hoppy aroma of beer, associated with the oxygenated sesquiterpenoid fraction derived from hops.

4 Conclusion

A relatively simple Solid Phase Extraction (SPE) based methodology was developed and combined with HS-SPME-GC-MS in order to facilitate separation, detection and tentatively identification of oxygenated sesquiterpenoids in three American kettle hopped lager beers, expressing clear hoppy aroma characteristics. The oxygenated sesquiterpenoid fractions eluting with 70 % and 80 % ethanol were thoroughly characterised. As a result, 63 compounds were detected in the chromatographic region where oxygenated sesquiterpenoids elute, of which 33 were identified as oxygenated sesquiterpenoids. In addition, the presence of iso-korajol, 4S-dihydrocaryophyllene-5-one, 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al and 6(5→4)-abeo-caryophyll-8(13)-en-5-al in lager beer is reported for the first time.

GC-O sniffing analysis on the SPE fractions (70 % and 80 % ethanol) derived from the beer exclusively hopped with noble aroma hop varieties, revealed a high number of flavour-active regions. α-Humulene-derived epoxides and both α-humulene and β-caryophyllene derived alcohols were frequently detected in these flavour-active zones, implying that these compounds might, in agreement with the general view, be important regarding the hop aroma characteristics of kettle hopped lager beers.

Exactly two decades ago, in the context of flavour-active compounds and the deficiency of analytical tools to detect them at that time, *Siebert* stated [2]: "Two approaches appear appropriate in this situation. The more tedious is to fractionate a hoppy beer, at each step adding each fraction back to beer for tasting… An alternative approach is to use the separating power of gas chromatography combined with the sensitivity of the human nose as detector". Summarized, two research approaches are suggested, i. e. reconstitution/omission experiments and GC-olfactometry. Although in this study we did not add each fraction back to beer, we basically combined the two proposed approaches by fractionating beer and perform GC-O on these fractions. This approach allowed us to point out flavour-active oxygenated sesquiterpenoids. Most of them were already related to hoppy aroma in beer decades ago, although GC-olfactometry was not available at that moment. Later on, the role of the α-humulene and β-caryophyllene oxidation and hydrolysis products in particular was seriously questioned since at first they were not detected via GC-O. However, more recently, several of these products were found to express green, woody, cedar and spicy odours [33–35] and also our current work suggests flavour-activity of oxygenated sesquiterpenoids. With respect to the still ongoing debate about flavour-active volatiles imparting the illusive kettle hoppy aroma, (non-identified) oxygenated sesquiterpenoids once again arise as potential aroma impact compounds.

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