

Characterization of Novel Single-Variety Oxygenated Sesquiterpenoid Hop Oil Fractions via Headspace Solid-Phase Microextraction and Gas Chromatography–Mass Spectrometry/Olfactometry

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ABSTRACT: The volatile composition of novel varietal oxygenated sesquiterpenoid hop oil fractions (“spicy essences”) was characterized by headspace solid-phase microextraction in combination with gas chromatography–mass spectrometry. Oxygenated sesquiterpenes represent the major chemical compound class, accounting for at least 65% of the total volatile fraction. In addition to oxygenated sesquiterpenes, spicy hop essences consist of several ketones, sesquiterpene and monoterpene hydrocarbons, and a relatively high number of unidentified compounds. On the basis of their relative composition, spicy hop essences can be fully differentiated according to their varietal origin. Multidimensional gas chromatography in combination with time-of-flight mass spectrometry on spicy hop essence cv. Spalter Select further demonstrated the enormous complexity of this particular hop oil fraction. The aromagram obtained via gas chromatography–olfactometry comprised nine odor-active regions described in terms of “citrus”, “green”, “haylike”, “earthy”, “woody”, and “spicy”. 2-Undecanone, 2-tridecanone, γ -cadinene, α -calacorene, calarene, humuladienone, caryolan-1-ol, caryophyllene oxide enantiomers, and humulene epoxide II are tentatively identified in the odor-active zones.

KEYWORDS: *spicy/herbal/noble hop aroma, spicy hop essence, oxygenated sesquiterpenoids, headspace solid-phase microextraction, gas chromatography–mass spectrometry/olfactometry, two-dimensional gas chromatography*

■ INTRODUCTION

Hops (*Humulus lupulus* L.) are at the origin of the unique, characteristic, and desirable hoppy aroma of beer. The typical and pleasant aroma characteristics of fresh hops are assigned to the composition of hop essential oil present in the lupulin glands of the female hop flowers. Hop essential oil composition is enormously complex: at present, more than 450 volatiles have already been identified,¹ and it has even been suggested that hop oil comprises over 1000 different volatile compounds.² Hop oil constituents are generally classified into three chemical groups, namely hydrocarbons, oxygenated compounds, and organosulfur compounds accounting for 50–80%, 20–50%, and less than 1% of total hop oil,³ respectively.

The typical aroma that hops impart to beer depends on the hopping technology used during the brewing process, i.e., selection of particular hop varieties or hop products, points of addition, and levels added.^{4–8} Sensory impressions in beer originating from hop essential oils are usually described in terms of floral, citrus, fruity, and spicy or herbal flavor notes. During the last decades, extensive scientific research has been carried out by several research groups to identify flavor-active constituents imparting hop-derived scents in beer. As a result, a relatively high number of character impact compounds have been proposed in literature. Mainly monoterpene alcohols including linalool, geraniol, and citronellol appear to be key elements of the floral and citrus bouquet of hoppy aroma in beer.^{5,7,9–17} In addition, typical esters (e.g., ethyl 3-methylbutanoate, ethyl 2-methylpropanoate), hop-derived sulfur

compounds (e.g., 4-mercapto-4-methylpentan-2-one, 3-sulfanyl-4-methylpentan-1-ol, 3-sulfanyl-4-methylpentan-2-one), esters of linalool and geraniol, and some uncommon cyclic ethers, such as Karahana ether and hop ether, are often mentioned as key contributors to the floral–fruity hop-derived aroma in beer.^{7,9,17–21}

While there is general agreement on the nature of particular compounds associated with floral, fruity, and citrus scents, the spicy or herbal aspects of hop character are ill defined. The spicy aspect of hoppy aroma, which is believed to be associated with the highly desirable “noble” or “kettle hop aroma”, is much more complex, and the nature of compounds responsible for spicy hop character is still far from clear. In several previous studies, it has been concluded that humulene epoxides and related compounds are collectively involved in this very specific and unique flavor impression.^{7,10,22–27} However, the relevance of humulene and caryophyllene oxidation products with regard to particular hop-derived spicy/herbal flavor impressions in beer remains to be proven.²⁸ Two major problems arise when considering humulene and caryophyllene oxidation products. First of all, the concentrations of these compounds in beer remain in most cases below their flavor threshold value and,

Received: June 6, 2013

Revised: August 6, 2013

Accepted: August 7, 2013

Published: August 7, 2013

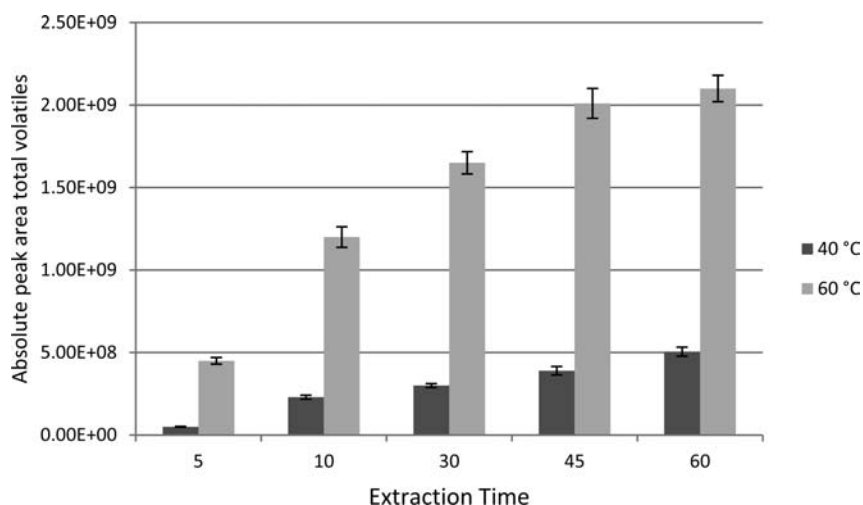


Figure 1. Extraction time–temperature profile for SPME extraction of spicy hop essence cv. Spalter Select (extraction fiber: PDMS (100 μm); all experiments were repeated three times; error bars represent standard deviation from the mean value).

second, their aroma attributes do not really correspond with spicy sensory impressions.²⁹

More recently, further arguments for the potential impact of hop sesquiterpenoids on the spicy aspect of hoppy aroma were reported. GC-O analyses on beers strongly hopped with different hop varieties allowed detecting 30 hop-derived odorants, among which three had spicy characteristics.⁷ Although the precise identity of these compounds could not be revealed, it was suggested that they are of sesquiterpenoid nature because elution occurred in a region abundant in sesquiterpenoid constituents. Eyres et al.²⁸ were able to tentatively identify 14-hydroxy- β -caryophyllene as a highly odor-active compound in commercially available spicy hop oil fractions derived from four different hop varieties. This tentative identification was possible by olfactometric analyses combined with highly advanced heart-cut multidimensional gas chromatography coupled to time-of-flight mass spectrometry. Using comparable analytical techniques, a caryophyllene alcohol, tentatively identified as caryophylla-3,8-(13)-dien-5- β -ol, was detected as being responsible for the spicy/noble/cedarwood aroma in hops and corresponding ale beers.³⁰ Nevertheless, it appears that other key flavor constituents of spicy and herbal hop character still remain to be identified.

In our previous studies we reported on the sensory characteristics of novel varietal oxygenated hop oil fractions (“spicy essences”) and demonstrated their impact on beer flavor via aromatization experiments. When added in appropriate amounts to a nonaromatized pilot lager, such single-variety “spicy” essences prepared from various European aroma hops impart revealing sensory impressions, i.e., pleasant herbal/hoppy aroma reminiscent of typical “noble” hop aroma, and in particular, increased bitterness intensity, mouthfeel, and fullness.^{26,31}

The present study aims at detailed characterization of the volatile composition of our novel spicy hop essences prepared from four German aroma varieties and at determination of the odor-active constituents present in the essences. For that purpose, an analytical procedure based on headspace solid-phase microextraction (HS-SPME) is developed and olfactometric analysis is performed on the GC effluent of SPME extracts from the spicy essence cv. Spalter Select. Results from GC-O are combined with GC-MS analysis in an attempt to

identify and allocate “spicy” hop aroma impact compounds. For more detailed investigation of the volatile composition of spicy hop essence (cv. Spalter Select), comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GCxGC-TOFMS) is carried out.

MATERIALS AND METHODS

Chemicals. All reference compounds were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade: α -humulene ($\geq 98.0\%$), α -pinene (98.0%), β -caryophyllene (98.5%), β -myrcene ($\geq 95.0\%$), β -pinene (99.0%), (–)-caryophyllene oxide (sum of enantiomers $\geq 99.0\%$), 2-dodecanone (97.0%), linalool (98.5%), methyl octanoate (99.8%), methyl decanoate (99.5%), nonanal (95.0%), 2-nonanone (99.5%), 2-tridecanone (97.0%), 2-undecanone (99.0%).

Plant Material. Single-variety spicy hop essences were prepared from hop pellets T90 (crop year 2007) from the varieties Hallertau Tradition, Saphir, Spalter Select, and Tettang Tettanger (HVG, Wolnzach, Germany). Pellets (250 g) were stored under recommended conditions (cold storage at 0 °C, packaged under vacuum in metalized polyethylene laminates)³² to prevent oxidative transformations of the brewing principles. Prior to extraction, the hop material (50 g) was disrupted using a mortar and pestle to facilitate subsequent extraction. Stainless steel extraction cells (10 mL) were filled with ground hop pellets (6 g), and duplicate extractions using supercritical carbon dioxide were performed as described below.

Preparation of Single-Variety Spicy Hop Oil Essences. Single-variety spicy essences were prepared according to our hop aroma extraction technology, based on density programmed supercritical fluid extraction (SFE) using carbon dioxide and subsequent solid-phase extraction (SPE) using ethanol/water mixtures for further fractionation of SFE extracts.

Ground hop pellets T90 were extracted via two-step SFE using a Dionex SFE-703 supercritical fluid extractor (Dionex, Sunnyvale, CA). The first extraction was performed using a carbon dioxide density of 0.29 g/mL and was finished when 25.0 L of gaseous carbon dioxide was collected. The remaining hop solids were further extracted by applying a carbon dioxide density of 0.50 g/mL until a volume of 25.0 L of gaseous carbon dioxide was collected, yielding the “crude” spicy SFE extract. Further fractionation of the SFE extracts was performed via solid-phase extraction. Varian Bond Elut C18 cartridges (500 mg) (Varian, Palo Alto, CA) were employed for this purpose. For more details on the extraction/fractionation procedure, reference is made to our paper on the production of novel varietal hop aromas by supercritical fluid extraction of hop pellets.³¹

Headspace Solid-Phase Microextraction (HS-SPME) for Isolation of Hop Oil Volatiles. Under the applied experimental conditions for SFE, less than 10% of nonvolatile hop acids present in the hop pellets were extracted, and consequently the spicy hop essences were not suitable for liquid injection. HS-SPME allows analysis of the volatiles of this particular hop oil fraction by GC without the need for additional cleanup steps. Together with the many advantages that SPME has (e.g., automation of the extraction and especially high enrichment of the volatiles) and because spicy hop oil fractions have not been investigated in detail using SPME in previous studies, we decided to investigate our novel spicy hop essences via SPME.

Headspace solid-phase microextractions of hop oil preparations were automated using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland). Prior to extraction of the headspace volatiles by SPME, spicy essences were diluted by pipetting a volume of 50 μL into 5 mL of milli-Q water in a carbon dioxide purged extraction vial (20 mL). Next, the extraction vial was immediately closed with a magnetic bimetal crimp cap containing a silicone/Teflon septum (Thermo Fisher Scientific, Austin, TX). For SPME isolation of the volatiles, an extraction fiber with PDMS coating (100 μm) was selected. The extraction fiber was exposed into the headspace of the vial (25 mm). The effects of the extraction time (5, 15, 30, 45, and 60 min, respectively) and temperature (40 $^{\circ}\text{C}$ or 60 $^{\circ}\text{C}$) on the extraction performance are shown in Figure 1. Clearly, the applied extraction temperature shows a large impact on the total amount of volatiles extracted from spicy essence. Performing the extraction at 60 $^{\circ}\text{C}$ is highly favorable in terms of an increased detector response for all volatiles when compared to that of 40 $^{\circ}\text{C}$. Furthermore, the absolute peak area is affected by the extraction time, i.e., longer extraction times result in higher peak areas. For SPME extraction at 60 $^{\circ}\text{C}$, the overall detector response is practically at maximum after 45 min, whereas at 40 $^{\circ}\text{C}$, the maximum and thus equilibrium is not reached after 45 min. In conclusion, for a maximum detector response, an extraction temperature of 60 $^{\circ}\text{C}$ and duration of 45 min were selected for further GC-MS characterization of spicy hop essences.

GC-MS Conditions for Separation and Detection of the Extracted Volatiles. Gas chromatographic operating conditions were as follows. SPME fibers with extracted volatiles were thermally desorbed in the heated inlet (split/splitless injector, 250 $^{\circ}\text{C}$) of the Ultra Trace gas chromatograph (Thermo Fisher Scientific, Austin, TX) for 3 min. Helium (Alphagaz 2, Air Liquide, Luik, Belgium) was used as a carrier gas at a constant flow of 1.0 mL/min. Injection was performed in the split mode (split ratio: 1/10) for 3 min at 250 $^{\circ}\text{C}$. Separation of the injected compounds was performed on a 40 m \times 0.18 mm i.d. \times 0.20 μm film thickness RTX-1 capillary column (Restek Corporation, Bellefonte, PA). The oven temperature program for separation of the volatiles was as follows: 3 min at 35 $^{\circ}\text{C}$, followed by a temperature increase at 5 $^{\circ}\text{C}/\text{min}$ up to 250 $^{\circ}\text{C}$ (1 min isotherm).

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). The ion source temperature was set at 240 $^{\circ}\text{C}$, and the electron multiplier voltage was 1445 V. Analyses were performed in the full scan operating mode ($m/z = 40\text{--}400$). 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 "Flavor MS library for Xcalibur 2003" spectral libraries (Interscience, Louvain-la-Neuve, Belgium), retention times of authentic reference compounds, and calculation of retention indices of the volatiles. Retention indices were determined by using a homologous series of normal alkanes (C8–C18; Sigma-Aldrich, St. Louis, MO). When no reference compounds were available constituents were "tentatively identified" using following criteria: (1) MS match factor >650 and calculated RI = literature RI \pm 5 or (2) MS match factor >750 when no literature RI available. Compounds having MS match factor <750 and literature RI significantly different from the calculated RI, were considered as "unknown".

Determination of Coefficients of Variation and Regression Coefficients. Coefficients of variation for selected markers of spicy

hop essence (caryophyllene oxide, humuladienone, humulene epoxide I and II, humulenol II), isolated, and analyzed under the above selected experimental conditions for SPME extraction (60 $^{\circ}\text{C}$, 45 min) and GC-MS, range from 1.4% (humulene epoxide II) to 4.1% (caryophyllene oxide) (five SPME extractions; CV calculations on basis of absolute peak areas). The regression coefficient for (–)-caryophyllene oxide was determined at 0.9972 via SPME of standard solutions (concentration range: 0.5–200 μg (–)-caryophyllene oxide/L water).

Two-Dimensional Gas Chromatography–Mass Spectrometry (GCxGC-TOFMS). HS-SPME combined with two-dimensional gas chromatography–time-of-flight mass spectrometry (GCxGC-TOFMS) was performed on spicy hop essence cv. Spalter Select. HS-SPME isolation of volatiles was performed as described above.

Gas chromatographic operating conditions were as follows. SPME fibers with extracted volatiles were thermally desorbed for 3 min in the heated injector (split/splitless, 250 $^{\circ}\text{C}$) of the Ultra Trace GCxGC equipped with a dual jet modulator. A dual jet modulator consists of two jets to supply localized cryogenic cooling (CO_2 , modulation time: 4 s). The principle is as follows: compounds eluting from the first capillary column are trapped via the first cooled jet. Next, the cooling switches to the second jet and as a result the trapped fraction moves on to the second jet and refocuses. After the cooling to the first jet is switched back, the compounds are injected on the second column. (Thermo Fisher Scientific, Austin, TX). Injection was performed in the splitless mode. Helium (Alphagaz 2, Air Liquide, Luik, Belgium) was used as a carrier gas at a constant flow of 1.2 mL/min. Volatiles were separated on a RTX-PONA (100% polydimethylsiloxane, nonpolar) capillary column (50 m \times 0.25 mm i.d. \times 0.5 μm film thickness; Restek Corporation, Bellefonte, PA) in the first dimension. The second dimension column was a BPX-50 (50% phenyl polysilphenylene–siloxane, midpolarity) capillary column (2 m \times 0.15 mm i.d. \times 0.15 μm film thickness; SGE Europe Ltd., UK). Both columns were placed in a single oven. Oven start temperature was set at 45 $^{\circ}\text{C}$ and was increased at 2 $^{\circ}\text{C}/\text{min}$ up to a final temperature of 320 $^{\circ}\text{C}$ where it was held for 20 min.

Mass spectrometric analysis was performed by time-of-flight mass spectrometry (Almsco BenchTOF-dx, Llantrisant, Wales, UK). The TOFMS was operated in the full scan monitoring mode, scanning ions at $m/z = 40\text{--}400$ (detection frequency: 100 scans/s). Data processing was performed by the Chrom-Card software v.2.5.0 (Thermo Fisher Scientific, Austin, TX). For compound identification, mass spectra were automatically compared with mass spectral data from the "NIST98" library and the "Flavor MS library for Xcalibur 2003".

Olfactometry. For olfactory assessment, a Sniffer 9000 system (Brechtbüchler Inc., Schlieren, Switzerland) was coupled to the GC-MS. The effluent was split to the mass spectrometer (50%) and the sniffing port (50%) using a passive split. The transfer line connecting the GC to the olfactory port, as well as the heated block of the sniffing device, was maintained at 280 $^{\circ}\text{C}$. Volatiles eluting at the sniffing port were presented to the assessors in a stream of humidified air. Assessors were asked to describe the odor of eluting compounds and to record the duration of odor perception by using a hand-held control unit with a cursor wheel for signal generation. Assessors were thoroughly trained for odor detection and description using reference compounds (linalool, β -myrcene, 2-nonanone, 2-undecanone, methyl octanoate, nonanal, α -humulene, β -caryophyllene, (–)-caryophyllene oxide) and total hop essential oil prepared by SFE as described by Van Opstaele et al.³³

Preliminary GC-O sniffing of volatiles from undiluted spicy hop essence resulted in a constant green-woody-haylike background odor at the sniffing port during the whole time of analysis. Further sniffing experiments showed that a 10-fold dilution of the spicy essence was appropriate for subsequent GC-O analysis. Both a detection frequency method (olfactory global analysis, OGA) and aroma extract dilution analysis (AEDA) were applied in different sessions. GC-O AEDA was performed via splitless injection (dilution: 1) and split injection (split ratio: 1/10, dilution: 10; split ratio: 1/20, dilution: 20) of the extracted sample. OGA was carried out by sniffing the effluent of splitless injected SPME extract of 10-fold diluted spicy hop essences. Sniffing

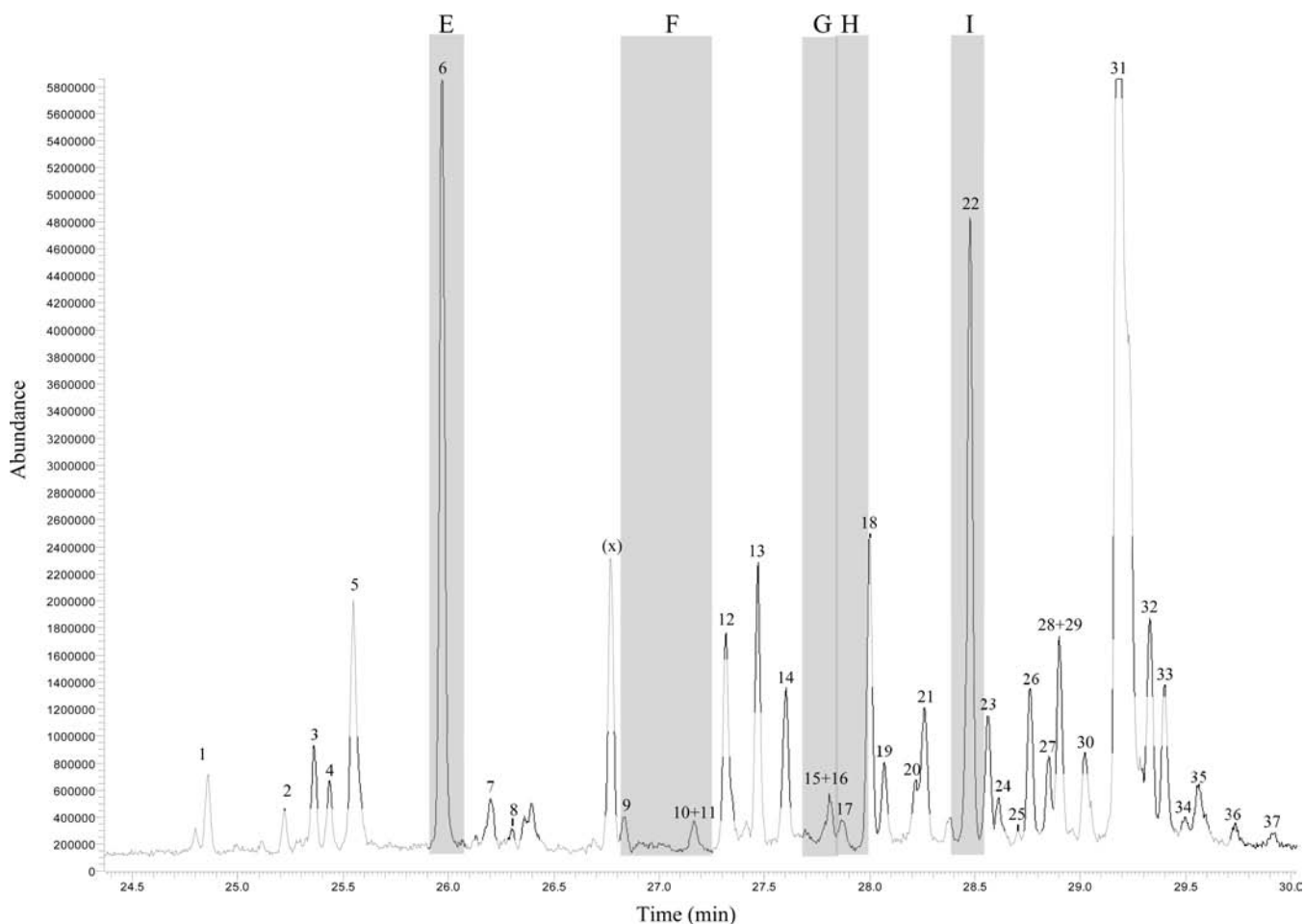


Figure 2. Partial HS-SPME-GC-MS pattern (TIC) of spicy SFE/SPE hop essence cv. Spalter Select (peak numbering in accordance with numbering in Table 1; (x): column or septum bleed). Odor-active regions (E–I) are indicated by gray areas.

sessions were carried out by three trained assessors, each assessor performing five analyses. Assessors were asked to describe the odor characteristics of eluting components and to generate a signal by the cursor wheel of the olfactometric unit when odorants were perceived (on signal) until the odor changed in character or vanished (off signal).

RESULTS AND DISCUSSION

Analytical Characterization of Single-Variety Spicy Hop Oil Essences. *Analysis of Spicy Hop Essence via Monodimensional Gas Chromatography in Combination with Quadrupole MS.* Single-variety spicy hop essences were prepared from pellets T90 cv. Hallertau Tradition, cv. Saphir, cv. Spalter Select, and cv. Tettanng Tettannger via supercritical fluid extraction, followed by solid-phase fractionation as described by Van Opstaele et al.³¹ Figure 2 shows the partial (sesquiterpenoid) volatile pattern of spicy essence cv. Spalter Select, as obtained by extracting and detecting the volatiles via the optimized HS-SPME-GC-MS procedure. In total, 45 components were assigned upon analysis of all spicy essences (see Table 1). Spicy hop oil constituents were further classified into 22 (tentatively) identified and unidentified oxygenated sesquiterpenes, 5 nonoxygenated sesquiterpenes, and a group comprising miscellaneous compounds, i.e., 2 monoterpene hydrocarbons, 7 ketones, 4 esters, and 5 “unknowns”. Spicy hop essences are especially enriched in oxygenated sesquiterpenes, accounting for 65–69% of total peak area. Among the 22

constituents grouped in this chemical compound class, 8 components were tentatively identified as α -humulene or β -caryophyllene oxidation products. In all spicy essences, humulene epoxide II appears to be the predominant constituent in the group of tentatively identified oxygenated sesquiterpenes, followed by (–)-caryophyllene oxide (see Table 1). Furthermore, spicy hop essence cv. Saphir contains the highest levels of all marker oxygenated sesquiterpenes. According to Deinzer and Yang,²⁹ oxygenated hop sesquiterpenes are not likely products of the plant biosynthetic apparatus. However, because oxidation reactions take place rapidly, oxygenated sesquiterpenes such as humulene epoxides, humulenol II and caryophyllene oxide, are present in the oils of fresh hops. With respect to their sensory attributes, many different descriptors (floral, fruity, lemon, woody, cedar, spicy, citrus, herbal, lime, sagebrush, spicy, haylike, fatty) have been used to characterize the odor of oxygenated hop sesquiterpenes (see Table 1).

Though we were able to classify a relatively large number of hop oil constituents into the group of oxygenated sesquiterpenes on the basis of mass spectral information (e.g., occurrence of fragment ions at $m/z = 79, 93, 161, 189$, and detection of the molecular ion at $m/z = 220, 222$, or 236), the precise identity of many of these constituents remains unknown. This is due to high similarity between sesquiterpenoid mass spectra, the lack of literature retention indices of compounds of interest, and, above all, the lack of reference MS

Table 1. Composition of Single-Variety Spicy Hop Essences (cv. Hallertau Tradition (HAL), cv. Saphir (SAP), cv. Spalter Select (SPA), and cv. Tett nang Tett nanger (TET)) via HS-SPME-GC-MS

compound	peak no. ^a	RI ^b	relative composition (%) ^c				literature odor description ^c	identification ^d
			HAL	SAP	SPA	TET		
OXYGENATED SESQUITERPENES								
(tentative) identification								
humuladienone ^f	15	1564	1.05	0.685	0.634	0.344	kettle hop aroma (B), floral (D)	MS, RI
caryolan-1-ol ^f	16						gasoline, fruity, lemon (A)	MS, RI
caryophyllene oxide enantiomer	17	1568	0.624	0.457	0.461	0.457		MS, RI, RC
(-)-caryophyllene oxide	18	1571	8.94	7.27	3.91	6.37	cedar, lime, floral (D, E, F)	MS, RI, RC
globulol	19	1574	n.d.	0.694	0.859	n.d.	floral, rose (C)	MS, RI
viridiflorol	20	1589	n.d.	0.516	0.528	n.d.	herbal, green (C)	MS, RI
humulene epoxide I	21	1595	2.81	1.66	2.05	2.87	haylike (D, F)	MS, RI
humulene epoxide II	22	1605	19.7	8.45	8.13	17.0	moldy, cedar, lime (E, F)	MS, RI
10- <i>epi</i> - α -cadinol	24	1611	2.55	0.325	0.421	0.887	herbal, wood (E)	MS, RI
humulene epoxide III ^f	28	1627	6.20	2.22	2.32	3.93	cedar (D), sagebrushlike (F)	MS, RI
humulenol II ^f	29						floral, spicy, citrus, fatty (D)	MS, RI
τ -cadinol	30	1634	6.26	0.905	1.42	1.88	herbal (F)	MS, RI
unidentified								
<i>m/z</i> = 93, 109, 161, 189, 204, 222 (MW)	14	1556	n.d. ^h	1.54	2.14	n.d.		
<i>m/z</i> = 93, 109, 161, 189, 204, 220, 222 ^g	23	1608	n.d.	0.890	1.62	n.d.		
<i>m/z</i> = 93, 109, 161, 189, 204, 220, 222 ^g	26	1619	0.550	1.16	2.02	0.179		
<i>m/z</i> = 105, 119, 161, 189, 204, 220 ^g	27	1624	2.82	0.644	0.913	0.890		
<i>m/z</i> = 67, 79, 93, 164, 179, 204, 220, 222 ^g	31	1646	15.1	35.1	37.1	30.4		
<i>m/z</i> = 59, 93, 105, 149, 161, 189, 204, 222 (MW)	32	1658	0.555	1.65	2.68	0.530		
<i>m/z</i> = 59, 93, 105, 149, 161, 189, 204, 222 (MW)	34	1670	n.d.	0.155	0.280	n.d.		
<i>m/z</i> = 93, 133, 161, 189, 204, 222 (MW)	35	1675	0.534	0.338	1.01	n.d.		
<i>m/z</i> = 67, 79, 87, 91, 109, 121, 220, 236 (MW)	36	1690	n.d.	0.252	0.357	0.452		
<i>m/z</i> = 81, 97, 111, 126, 222, 236 (MW)	37	1706	n.d.	0.134	0.203	n.d.		
compound class %			67.7	65.0	69.1	66.2		
NONOXYGENATED SESQUITERPENES								
β -caryophyllene	1	1426	0.727	0.528	1.03	0.218	green, spicy, woody (A)	MS, RI, RC
β -farnesene	3	1451	n.d.	n.d.	1.31	n.d.	oily, fruity, citrus, woody (A)	MS, RI
α -humulene ^g	5	1459	6.72	2.32	3.95	2.74	oily, green, woody (A)	MS, RI, RC
γ -cadinene	9	1521	0.819	0.347	0.465	0.279	herbal, thyme, woody (A)	MS, RI
calarene (coelution with α -calacorene)	10 (11)	1532	n.d.	0.391	0.485	n.d.	dry-woody (G)	MS, RI
compound class %			8.27	3.59	7.24	3.24		
MONOTERPENES								
β -pinene		972	0.190	0.381	0.636	0.258		MS, RI, RC
β -myrcene		988	0.851	1.40	0.340	0.911		MS, RI, RC
compound class %			1.04	1.78	0.976	1.17		
KETONES								
5-undecen-2-one		1257	0.017	0.012	n.d.	0.028		MS
2-undecanone		1276	0.432	1.17	1.76	1.08	fruity, citrus (A)	MS, RI, RC
2-dodecanone		1377	0.123	0.170	0.137	n.d.	fruity, citrus, orange (C)	MS, RI, RC
methyl ketone	2	1443	0.881	0.158	0.436	0.122		
<i>cis</i> -5-tridecen-2-one	4	1453	2.08	2.67	0.794	4.95		MS
2-tridecanone	6	1479	8.16	5.92	9.95	2.41	fatty, herbal (C)	MS, RI, RC
6- <i>cis</i> -pentadecen-2-one		1661	0.437	1.29	1.79	1.09		MS, RI
compound class %			12.1	11.4	14.9	9.68		
ESTERS								
methyl <i>trans</i> -4-decenoate		1292	0.142	0.215	0.259	0.182		MS, RI
methyl geranate		1304	0.283	0.059	n.d.	0.053		MS, RI
methyl decanoate		1308	0.052	0.021	n.d.	0.079	oily, fruity (A)	MS, RI, RC
geranyl isobutyrate	8	1495	0.623	1.50	0.263	0.311	rosy, fruity (C)	MS, RI
compound class %			1.10	1.80	0.522	0.625		

Table 1. continued

compound	peak no. ^a	RI ^b	relative composition (%) ^e				literature odor description ^c	identification ^d
			HAL	SAP	SPA	TET		
UNKNOWNNS								
unknown(s) 1 ^g	7	1485	4.89	4.22	0.745	9.32		
unknown 2 (<i>m/z</i> = 67, 79, 93, 150, 165)	12	1541	2.73	5.80	3.18	4.94		
unknown 3 (<i>m/z</i> = 67, 82, 95, 109, 124)	13	1548	2.15	5.13	3.31	4.26		
unknown 4 (<i>m/z</i> = 85, 100, 113, 139)		1559	n.d.	0.954	n.d.	0.489		
unknown 5 (<i>m/z</i> = 71, 113, 150, 168)	25	1617	n.d.	0.274	0.119	0.068		
compound class %			9.77	16.4	7.35	19.1		

^aPeak number in accordance with peak numbering in Figure 2. ^bCalculated retention index (RTX-1 capillary column, 40 m × 0.18 mm i.d. × 0.20 μm film thickness). ^cOdor/aroma descriptors found in literature: (A) El Sayed;³⁶ (B) Peacock and Deinzer;⁹ (C) <http://www.thegoodscentscompany.com> (accessed Aug 2, 2010); (D) Lermusieau et al.;⁴² (E) Deinzer and Yang;²⁹ (F) Fukuoka and Kowaka;⁴⁶ (G) <http://www.flavornet.org/flavornet.html> (accessed Aug 2, 2010). ^dCompounds identified on the basis of (i) mass spectral comparison with the reference databases (MS), (ii) comparison with literature retention index (RI), and (iii) comparison of mass spectrum and RI with authentic reference compounds (RC). ^eRelative peak areas represent the mean of triplicate HS-SPME-GC-MS analyses. ^fCoeluting compounds. ^gCoelution with unknown compound(s). ^hn.d.: not detected.

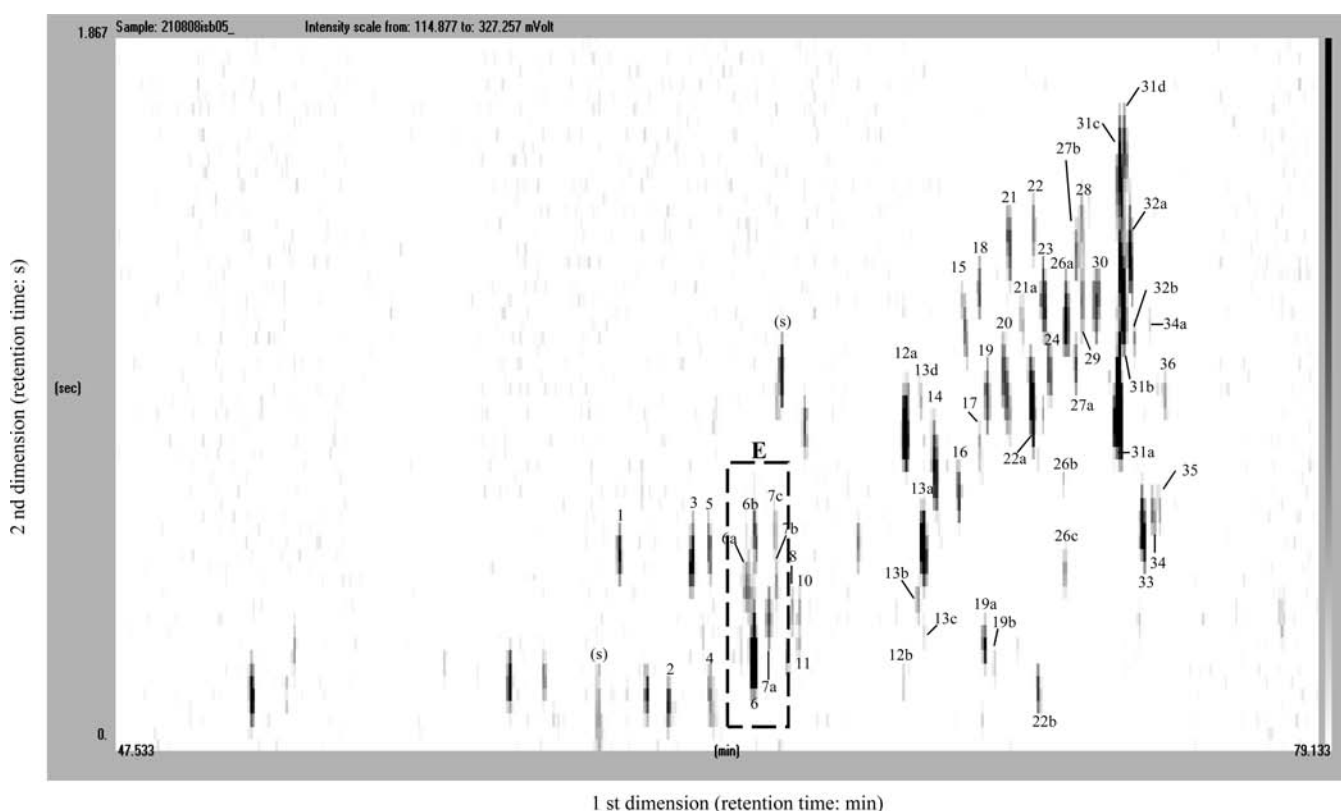


Figure 3. Two-dimensional TIC plot obtained by HS-SPME GCxGC-TOFMS on spicy hop essence cv. Spalter Select (peak numbering in accordance with numbering in Tables 1 and 2; (s): chromatographic system peak). Odor-active zone E: area defined by dotted line.

spectra in the reference spectral libraries. Furthermore, coelution with components tentatively classified in the group of oxygenated sesquiterpenes (see, for example, compounds at RI = 1608, 1619, 1624, 1646) hampers identification because of mixed spectral information.

Table 1 further displays the relative proportions of the minor compound classes in the spicy hop essences. The ketone group accounts for 9.7% (cv. Tettanager) up to 14.9% (cv. Spalter Select) of total peak area in the essences. Unknown compounds represent 7.4% (cv. Spalter Select) up to 19.1% (cv. Tettanager), and nonoxygenated sesquiterpenes 3.2% (cv. Tettanager) up to 8.3% (cv. Hallertau Tradition) of total peak area. Monoterpene hydrocarbons and esters represent only

minor fractions in spicy hop essences (relative peak areas in all essences below 2%).

Analysis of Spicy Hop Essence via Two-Dimensional Gas Chromatography (GCxGC) in Combination with Time-of-Flight Mass Spectrometry (TOFMS). The above results suggested considerable coelution of volatiles during mono-dimensional GC-MS analysis of HS-SPME extracts from single-variety spicy hop essences. To further unravel the volatile composition of this complex hop oil fraction, GCxGC combined with TOFMS was performed on HS-SPME extract from spicy essence cv. Spalter Select. The result of this analysis, as shown in Figure 3, illustrates the greater complexity of the volatile pattern of spicy essence when compared to single

column analysis (Figure 2) and thus demonstrates the better separation performance of GCxGC. This is further apparent from Table 2, displaying the resolution of 30 components via

Table 2. Separation of Spicy Hop Oil Compounds, Coeluting in Monodimensional GC, via GCxGC-TOFMS and Tentative Identification (identifications based on mass spectral comparison with the mass spectral library “Flavor MS library for Xcalibur 2003” and literature data (Tressl et al.;⁴⁴ Szczepaniak et al.,⁴⁵ Adams,⁴⁷ NIST online database 2011: <http://webbook.nist.gov> (accessed June 4, 2013))

RI ^a	peak no. ^b	GC-MS	peak no. ^c	GCxGC-TOFMS
1479	6	2-tridecanone	6	2-tridecanone
			6a	43(100)*, 57(82), 85(72), 127(64), 142(50), 153(42), 210(50)
			6b	cyclotridecanone
1485	7	unknown(s) 1	7a	δ -guaiene
			7b	α -selinene
			7c	unidentified oxygenated sesquiterpene
1541	12	unknown 2	12a	43(55), 55(50), 67(70), 79(100), 93(25), 109(10), 126(10), 150(15), 165(5)
			12b	43(100), 58(98), 71(76), 82(15), 96(10)
1548	13	unknown 3	13a	54(100), 67(50), 82(40), 96(25), 111(20), 124(15), 142(5), 152(10), 210(5)
			13b	<i>trans</i> -nerolidol
			13c	44(55), 55(100), 61(40), 70(25), 81(30), 88(55), 96(35), 138(25), 180(5)
			13d	elemol
1574	19	globulol	19	globulol
			19a	2-tetradecanone
			19b	43(100), 56(34), 71(92), 95(6), 111(8)
1605	22	humulene epoxide II	22	humulene epoxide II
			22a	69(100), 81(12), 95(22), 109(40), 183(18)
			22b	59(100), 87(85), 93(55), 133(30), 161(18), 189(18), 204(20), 206(35), 235(50)
1619	26	oxygenated sesquiterpene	26a	γ -eudesmol
			26b	43(100), 55(46), 67(26), 81(40), 99(24), 127(52)
			26c	67(100), 81(84), 95(42), 110(50), 150(10)
1624	27	oxygenated sesquiterpene	27a	1- <i>epi</i> -cubenol
			27b	τ -muurolol
1646	31	oxygenated sesquiterpene	31a	43(65), 55(70), 67(68), 79(100), 93(20), 164(10), 222(5)
			31b	43(70), 55(90), 67(60), 79(100), 91(45), 107(35), 165(5)
			31c	β -eudesmol
			31d	α -eudesmol
1658	32	oxygenated sesquiterpene	32a	α -cadinol
			32b	14-hydroxy- β -caryophyllene; caryophylla-3,8(13)-dien-5 β -ol

^aCalculated retention index (RTX-1 capillary column, 40 m \times 0.18 mm i.d. \times 0.20 μ m film thickness). ^bPeak number in accordance with peak numbering in Table 1 and Figure 2. ^cPeak number in accordance with peak numbering in Figure 3. * Mass spectral data (EI, TOFMS) show the most intense fragment ions and their relative intensity: m/z (relative intensity).

GCxGC compared to the 10 components resolved in the single column application. Using the GCxGC approach we were able to confirm the presence of 2-tridecanone, globulol, and humulene epoxide II in coeluting zones of the spicy essence. Moreover, we tentatively identified 10 sesquiterpene alcohols (*trans*-nerolidol, elemol, γ -eudesmol, 1-*epi*-cubenol, τ -muurolol, β -eudesmol, α -eudesmol, α -cadinol, 14-hydroxy- β -caryophyllene, and caryophylla-3,8(13)-dien-5 β -ol), two sesquiterpene hydrocarbons (δ -guaiene, α -selinene), and two ketones (cyclotridecanone, 2-tetradecanone) on the basis of mass spectral comparison. These compounds have been mentioned previously as constituents of hop essential oil.^{1,34}

A relatively large number of compounds remains unknown because reference spectra are not included in the mass spectral libraries used for identification. The lack of availability of reference compounds represents a crucial problem in view of further in-depth characterization of the sesquiterpenoid hop oil fraction. Thus, further research focusing on (1) preparation of reference compounds by chemical transformation of hop oil sesquiterpenes into their respective epoxides and alcohols, and (2) structure elucidation of these oxidized hop oil components by state-of-the-art spectroscopic techniques, is required.

Determination of Odor-Active Constituents in Spicy Hop Essence cv. Spalter Select by HS-SPME-GC-O/MS.

The results from HS-SPME-GC-O/MS analysis of the spicy hop essence cv. Spalter Select are summarized in Table 3. In total, nine odor-active regions were detected and, where possible, compounds present in the odorous zones were identified. In contrast to earlier GC-O analysis on floral hop essence,³⁵ only weak odor impressions were noticed for the eluting compounds originating from the spicy essence. This is apparent from the fact that the odors were only detected when performing splitless injection of the SPME extract of a 10-fold diluted sample of spicy essence (see Table 3). GC injection of SPME extract in the split mode (split ratio 1/10, 1/20) failed to show any odor-activity at the sniffing port.

The odor-active compounds of spicy hop essence cv. Spalter Select were determined by a detection frequency method. The ability of the assessors, when applying splitless injection, to detect and describe odor-active regions of spicy hop essence in terms of “citrus”, “green”, “haylike”, “earthy”, “woody”, and “spicy” is clearly shown in Table 3. Five narrow odor intervals are registered at RI = 1276, 1319, 1410, 1415, and 1565. A citrus odor is perceived at RI = 1276, corresponding to the volatile identified as 2-undecanone. A “green” odor is ascribed to the region at RI = 1565 corresponding to coeluting volatiles identified as humuladienone and caryolan-1-ol. “Green” and “haylike” odor characteristics, and an unpleasant “earthy” smell are perceived in odorous regions at RI = 1319, 1410, and 1415, respectively. However, the identity of the components present in these regions of the aromagram remains unknown because MS signals were too weak to give any relevant structural information.

Other regions showing odor-activity are less clearly defined (see also Table 3: RI = 1479–1485; 1521–1535; 1568–1573; 1605–1608) and comprise more than one chromatographic peak. Possible reasons for longer-lasting odor perception during GC-O analysis have been reported by Eyres et al.²⁸ During analysis of the less volatile spicy essence containing mainly sesquiterpenoids, partial condensation of eluting semivolatiles at the outlet surface of the sniffing port and perception of coeluting odorants could be the main reasons for these broad intervals of olfactory sensation.

Table 3. Odor-Active Compounds Detected by GC–Olfactometry in Spicy Hop Essence cv. Spalter Select

odor-active zones	RI ^a	odorant ^b	odor descriptor ^c	dilution ^d	detection frequency ^e	identification ^f
A	1276	2-undecanone	citrus	1	12	MS, RI, RC
B	1319	unknown ^g	green	1	9	–
C	1410	unknown ^g	haylike	1	8	–
D	1415	unknown ^g	earthy	1	9	–
E	1479–1485	2-tridecanone, coeluting peaks	green, woody	1	14	MS, RI, RC
F	1521–1535	γ -cadinene, α -calacorene, calarene	spicy, woody	1	13	MS, RI
G	1565	humuladienone/caryolan-1-ol	green	1	12	MS, RI
H	1568–1573	caryophyllene oxide enantiomer (–)-caryophyllene oxide	green, spicy	1	13	MS, RI, RC
I	1605–1608	humulene epoxide II + 2 unidentified coeluting compounds	green, haylike	1	15	MS, RI

^aCalculated retention index (RTX-1 capillary column; 40 m \times 0.18 mm i.d. \times 0.20 μ m film thickness) of odor active regions in the aromagram.

^bCompounds identified in odor-active regions. ^cOdor descriptors given by three trained assessors in 15 separate sessions. ^dHighest dilution at which odor-activity is perceived (serial dilution of SPME extract was obtained by adjusting split ratio (1; 1/10, 1/20) at the GC-inlet). ^eIn total, 15 sniffing sessions were carried out (3 trained assessors, each assessor performing 5 analyses). ^fCompounds were identified on the basis of (i) mass spectral comparison using reference libraries (MS), (ii) comparison of retention index (RI), and (iii) comparison of retention time, RI, and mass spectrum with those of authentic reference compounds (RC). ^gUnknown compounds (MS signal too weak for interpretation).

The intricate composition of sesquiterpenoid spicy hop essences has been demonstrated above. As a result of this complexity, considerable coelution of hop oil constituents occurs when applying monodimensional GC. Therefore, allocation of a perceived odor to a specific chemical compound should be done with care. For example, from monodimensional GC analysis of spicy essence, it seems that the “green/woody” odor-active region (RI = 1479–1485) comprises two to three different volatiles that could be involved in the perceived odor. However, GCxGC analysis revealed that at least six different compounds coelute in this region (see Figure 3, odorant zone E). Which component(s) eluting in the green/woody region (RI = 1479–1485) causes the ascribed odor impressions cannot be unequivocally determined at the moment.

γ -Cadinene, α -calacorene, and calarene represent volatiles detected in the spicy/woody region with RI = 1521–1535 (see Table 3). These compounds are known for their woody odor characteristics³⁶ and have been reported previously as possible character impact compounds in plant essential oils and exotic fruits.^{37–39} Thus, these volatiles are potential contributors to the odor of the spicy/woody zone.

Two compounds with a highly similar mass spectrum were found in the green/spicy odor region at RI = 1568–1573. This region can be allocated to (–)-caryophyllene oxide and a caryophyllene oxide enantiomer via analysis of the (–)-caryophyllene oxide reference compound and comparison of RI and mass spectra.

Finally, from monodimensional GC-O/MS analysis, humulene epoxide II is identified in the green/haylike odor region (RI = 1605–1608), on the basis of mass spectral comparison and retention index (RI = 1605) (see Table 3). However, the odor description given by the assessors (green, haylike) is clearly different from previously reported odor attributes of humulene epoxide II, such as “moldy”, “lime”, or “cedar”.²⁹ This suggests, together with our GCxGC data revealing several unidentified compounds (for mass spectral information see Table 2: compounds 22a and 22b) coeluting with humulene epoxide II, that the haylike odor impression perceived in this particular region of the aromagram is possibly due to compounds different from humulene epoxide II.

Humulene and caryophyllene oxidation products, such as humuladienone, humulene epoxide I, humulene epoxide II, humulene epoxide III, humulenol II, humulol, and caryophyl-

lene oxide, have been associated with herbal/spicy hop-derived flavor impressions in beer.^{9,15,26,29,40,41} However, in present study, most of these compounds did not show odor-activity at the sniffing port, although they are clearly present in varietal spicy hop essence. Nevertheless, our findings are in agreement with literature data because, with the exception of humuladienone,⁴² none of the above-mentioned humulene and caryophyllene oxidation products were reported as character impact compounds in hop cones, hop pellets, commercial spicy hop essences, or beer on the basis of GC-olfactometric assessment.^{6,28} Surprisingly, although present in the spicy essence investigated in this study (see Table 2, 32b), the high character-impact compounds 14-hydroxy- β -caryophyllene and caryophylla-3,8-(13)-dien-5- β -ol imparting spicy, noble, and cedarwood aromas in hops and beer as proposed by Eyres et al.²⁸ and Nielsen,³⁰ respectively, did not show odor-activity on the basis of our GC-O analyses.

In conclusion, spicy hop essence cv. Spalter Select clearly contains constituents that are odor-active. However, because of the very complex chemical composition of the essence, it is not possible to identify those compounds causing the perceived odors. Chromatographic coelution and the lack of authentic reference compounds to verify analytical data (mass spectra, retention indices) and sensory descriptions hamper identification of key character impact compounds responsible for odors such as green/woody or green/haylike present in the spicy hop oil fraction. Therefore, further in-depth studies of the spicy fraction are proposed by applying highly sophisticated techniques, such as heart-cut multidimensional GC combined with olfactometry.^{28,30} The use of such advanced separation and detection techniques together with the synthesis of pure sesquiterpenoid reference compounds is indispensable for further unraveling the nature of spicy hop character.

Finally, the question as to which compounds present in spicy hop essence impact mouthfeel and bitterness of beer, as observed in our previous studies^{26,31} remains unanswered. To tackle these issues comprising nonvolatile beer sensory attributes such as mouthfeel and bitterness perception, requires a different approach than GC-O⁴³ (e.g., addition of authentic reference sesquiterpenoid compounds to beer or model solutions and subsequent real tasting experiments). Taken all together, the preparation of pure reference components, in particular of oxygenated sesquiterpenoids naturally present in

hop oil, appears to be needed for a detailed, comprehensive flavor evaluation of this intriguing fraction of hop essential oil.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank members of the sensory panel of our institute for performing GC-O experiments. We are grateful to Jo Vervenne and Dr. Joeri Vercammen (Interscience, Louvain-la-Neuve, Belgium) for the opportunity to perform GCxGC-TOFMS on spicy hop essence cv. Spalter Select. We thank HVG – Hopfenverwertungsgenossenschaft e.G. (Wolnzach, Germany) for financial support of this study.

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