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Comparison of Odor-Active Compounds in the Spicy Fraction of Hop (*Humulus lupulus* L.) Essential Oil from Four Different Varieties§

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The "spicy" character of hops is considered to be a desirable attribute in beer, associated with "noble hop aroma". However, the compounds responsible have yet to be adequately identified. Odorants in four samples of the spicy fraction of hop essential oil were characterized using gas chromatography−olfactometry (GC-O) and CharmAnalysis. Four hop varieties were compared, namely, Target, Saaz, Hallertauer Hersbrucker, and Cascade. Odor-active compounds were tentatively identified using comprehensive two-dimensional gas chromatography (GC×GC) combined with time-of-flight mass spectrometry (TOFMS). An intense "woody, cedarwood" odor was determined to be the most potent odorant in three of the four spicy fraction samples. This odor coincided with a complex region where between 8 and 13 compounds were coeluting in each of the four spicy fractions. The peak responsible was determined by (i) correlating peak areas with Charm values in eight hop samples and (ii) heart-cut multidimensional gas chromatography−olfactometry (MDGC-O). The compound responsible was tentatively identified as 14-hydroxy-β-caryophyllene. Other important odorants identified were geraniol, linalool, β-ionone, and eugenol.

KEYWORDS: ***Humulus lupulus* L.; gas chromatography−olfactometry; comprehensive two-dimensional gas chromatography; time-of-flight mass spectrometry; multidimensional gas chromatography; hop aroma; character-impact odorants**

INTRODUCTION

Hops (*Humulus lupulus* L.) are an indispensable component of beer, with the essential oil responsible for imparting the distinctive hop aroma. Differences in aroma properties between hop varieties can be attributed to variations in the composition of their essential oils (*1*). However, not all character-impact odorants in hop essential oil have been identified, and hop aroma in beer is still not completely characterized or understood (*2*). The composition of hop essential oil is very complex, with 485 compounds currently identified in the literature (*3*, *4*), and recent research suggests that up to 1000 compounds may actually be present (*4*). In addition, many important odorants are present at only trace concentrations, having very low sensory thresholds.

"Noble hop aroma" is a particularly desirable character in beer and is a term commonly used in the literature. This character is usually associated with the use of traditional aroma varieties from Europe such as Hallertauer Hersbrucker and Saaz (*5*, *6*). The aroma description of this "noble" character is poorly defined, but is often described as herbal or spicy (*5*). Oxidation and hydrolysis products of sesquiterpenes have been associated with noble and spicy hop characters in beer (*5*−*7*). Good correlations between increasing concentrations of humulene epoxides and these hop characters have been demonstrated (*8*). However, a good correlation does not prove a cause-and-effect relationship (*5*, *8*), and the importance of these oxidation compounds for imparting hoppy aroma remains controversial (*7*, *9*, *10*). The compounds so far identified have exhibited concentrations below their detection thresholds, and their aroma characteristics do not correspond to the desired spicy or noble hop aroma (*6*).

Fukuoka and Kowaka (*10*) reported that two compounds predominantly responsible for the herbal odor of a fractionated hop sample had oxygenated sesquiterpenoid structures. Goiris et al. (*7*) found that adding 20 ppb of an oxygenated sesquiterpene fraction to a bland pilot beer produced a desirable spicy or herbal aroma reminiscent of noble hop aroma. Kishimoto et al. (*11*) detected three spicy odorants in beer, eluting in a region abundant in sesquiterpenoids, although the compounds responsible were not identified.

The complexity of hop aroma in beer has led to increasing trends for fractionated hop oils with specific odor characteristics

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to be added to beer postfermentation (*1*, *12*, *13*). Hop resins and essential oils are generally extracted using liquid CO_2 or supercritical CO_2, with the essential oil then isolated using molecular distillation under high vacuum (*1*, *14*). Fractionation is then achieved using a combination of distillation and chromatographic methods (*1*, *12*, *13*). One such commercial product, known as the spicy fraction [Pure Hop Aroma (PHA); Botanix Ltd., Paddock Wood, Kent, U.K.), is rich in monoterpene and sesquiterpene alcohols and has been previously described as having a sandalwood or oakmoss character (*13*, *15*). Spicy fractions obtained from different hop varieties retain distinct aroma profiles due to differences in their chemical composition (*1*, *13*, *15*).

The majority of past research on the character-impact odorants in hops and beer has used instrumental data only. However, it is well-established that the odor thresholds and odor intensities of volatile compounds may vary considerably between compounds (*16*, *17*). Because of the large variation in these two properties, the response of a physical GC detector (e.g., FID or MS) is not representative of odor activity, and the most abundant compound in a chromatogram may not be the most important odorant (*18*). Consequently, the impact a compound has on hoppy aroma must be evaluated using human assessors. A valuable tool for identifying character-impact odorants is gas chromatography−olfactometry (GC-O), where human assessors are used to detect and evaluate volatile compounds as they elute from a column following a GC separation (*17*).

Several different GC-O methodologies have been developed to evaluate the relative importance of odor-active compounds in a sample (*17*). In CharmAnalysis, a dilution series is prepared and each dilution is assessed by GC-O until no odors are perceived (*19*, *20*). The results quantify the odor potency of a compound, which is based upon the ratio of its concentration to its odor threshold in air (*19*). Because the spicy fraction products are designed to be added to beer postfermentation at typical dose rates of 50−100 ppb (*13*), only the most potent odorants will remain above threshold. Therefore, using CharmAnalysis to rate relative importance by odor potency (as opposed to odor intensity) is appropriate.

A limitation of GC-O is that peak coelution in complex samples makes identification of the compound(s) responsible for an odor difficult, particularly where trace odorants coelute with larger odor-inactive peaks. Coelution of odor-active compounds may also result in the perception of "odor clusters" during GC-O analysis (*18*). One possible solution for identifying character-impact odorants where coelution occurs is to use comprehensive two-dimensional gas chromatography (GC×GC). GC×GC consists of two columns with different stationary phases connected in series with a cryogenic modulator at the interface. Sequentially trapping and pulsing compounds from the first column to the second column (e.g., every 5 s) creates a two-dimensional separation based on two different column properties (*21*). For example, two compounds with similar boiling points that coelute on the first column may be resolved on the second column if they differ in their polarity. The greater peak capacity, resolution, and sensitivity of GC×GC provide superior analyses compared with single-column GC analysis (1DGC). Hyphenating GC×GC to time-of-flight mass spectrometry (TOFMS) presents researchers with a very powerful identification tool.

The primary objective of this research was to identify the character-impact odorants and compare the odorants in the spicy fraction of hop essential oil obtained from four different hop varieties.

MATERIALS AND METHODS

Samples. Commercial samples of the spicy fraction (Spicy PHA) of hop essential oil from four varieties were obtained from Botanix Ltd. The production process involves extraction of hop pellets using liquid CO_2 followed by isolation of the whole essential oil using molecular distillation under high vacuum. Fractionation is achieved using a propriety procedure involving a combination of distillation and chromatographic methods (*13*).

Odor-active compounds in the spicy fraction from four different hop varieties were investigated, namely, Cascade, Target, Hallertauer Hersbrucker (HHE), and Saaz. The Cascade and Target samples were supplied as pure oils, whereas the HHE and Saaz samples were supplied as 50% solutions in ethanol (v/v) due to their high viscosities.

Sample solutions were prepared using cyclohexane (pesticide analysis grade, 99.7%; BDH Laboratory Supplies, Poole, Dorset, U.K.) for GC-O analysis or *n*-hexane for GC×GC-TOFMS (Pestanal, ≥95%; Riedel-de Haën, Sigma-Aldrich Co., Seelze, Germany), GC×GC-FID, and MDGC-O (Nanograde, ≥95% *n*-hexane, ≥99.9% total hexanes; Mallinckrodt Baker, Inc., Phillipsburg, NJ) analyses.

Reference Compounds. 3-Methylbutanoic (isovaleric) acid (~99.0%) was purchased from Aldrich Chemicals (Sigma-Aldrich Co., St. Louis, MO); ethyl 3-methylbutanoate (ethyl isovalerate) (~99.7%) was purchased from Fluka Chemie GmbH (Sigma-Aldrich Co., Buchs, Switzerland); linalool (~97.0%) was purchased from ACROS Organics (Fair Lawn, NJ); eugenol, geraniol, and *E*-2-hexenal were gifts from McCormick Flavor Division (McCormick and Co., Inc., Hunt Valley, MD); β-damascenone was a gift from Firmenich SA (Corporate R&D Division, Geneva, Switzerland); and β-ionone (>95.0%) was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany).

Gas Chromatography−Olfactometry and CharmAnalysis. GC-O analyses were performed using a HP5890 series II plus gas chromatograph (Hewlett-Packard, Avondale, PA) connected to an olfactometry port (Datu Technology, Geneva, NY). The separation was carried out on a 25 m × 0.32 mm i.d. × 0.5 μm d_f BPX5 (5% phenyl polysilphenylene-siloxane stationary phase) capillary column (SGE International, Ringwood, Australia). Helium was used as the carrier gas with a constant flow of 2 mL min^{-1} with an initial column head pressure of 59 kPa at 60 °C. Sample volumes of 1 μL were manually injected using a 5 μL syringe (SGE International). The injector was operated with a split ratio of 50:1 at 220 °C. The GC oven was temperature programmed from 60 to 210 °C at a rate of 6 °C min^{-1}, then increased to 290 °C at 10 °C min^{-1}, and held for 20 min to elute higher boiling compounds and reduce the risk of carryover. Each GC-O sniff run had a maximum duration of 25 min to ensure the assessors did not suffer fatigue (*19*).

The olfactory port was maintained at 300 °C for GC-O analysis. As compounds eluted from the column they were presented to the assessor in a stream of humidified air at 50 °C to be evaluated. The assessor recorded the duration and the description of an odor, and the data were collected using Charmware software (version 1.08, Datu Technology). A series of alkanes (C8−C26) was run using flame ionization detection (FID) to establish retention indices (*I*) for olfactometry analysis.

GC-O analyses were performed by two experienced assessors on a series of dilutions starting with a 10% (v/v) sample of essential oil in cyclohexane. Serial dilutions (factor of 2) were sequentially assessed until no odors were detected. CharmAnalysis was performed on the results of each assessor using the Charmware software by integrating the GC-O results of each dilution using the Charm calculations described elsewhere (*19*, *20*). For each odor peak both assessors had in common, the Charm values were combined and then expressed as a percentage of the total Charm value (TCV), which was the sum of all odors detected by the two assessors.

Comprehensive Two-Dimensional Gas Chromatography. GC×GC separations were performed using two Agilent 6890A gas chromatographs, the first (Agilent Technologies, Little Falls, DE) equipped with a FID and the second (Agilent Technologies, Palo Alto, CA) coupled to a Pegasus III time-of-flight mass spectrometer (TOFMS; Leco Corp., St. Joseph, MI). Both instruments were retrofitted with an Everest model Longitudinally Modulated Cryogenic System (LMCS; Chromatography

Concepts, Doncaster, Australia). All columns for GC×GC were supplied by SGE International.

GC×GC-FID. GC×GC-FID was initially used to characterize the composition and determine separation conditions. The column set consisted of a 30 m × 0.25 mm i.d. × 0.25 μm d_f BPX5 primary column (^{1}D) coupled in series to a 1.1 m × 0.1 mm i.d. × 0.1 μm d_f BP20 (polyethylene glycol stationary phase) second-dimension column (^{2}D). A modulation period of 5 s was used with the cryogenic trap maintained at −15 °C for the duration of each analysis. The separation was performed with a linear temperature program of 60 to 225 °C at 3 °C min^{-1}. The oven temperature was subsequently increased to 240 °C at 10 °C min^{-1} and held for 20 min. One microliter samples of 1 and 10% (v/v) essential oil were injected using an Agilent 7683 series autosampler. The injector was operated at 220 °C with a split flow of 300 mL min^{-1}. Hydrogen was used as a carrier gas under constant flow mode with an initial inlet pressure of 178.4 kPa at 60 °C. Average linear velocity through both columns was measured with methane gas and was found to be 34.4 cm s^{-1} at 60 °C. The FID was operated at 260 °C at an acquisition rate of 100 Hz.

GC×GC-TOFMS. The column set consisted of a 30 m × 0.25 mm i.d. × 0.25 μm d_f BPX5 primary column (^{1}D) coupled in series to a 0.8 m × 0.1 mm i.d. × 0.1 μm d_f BP20 second-dimension column (^{2}D). A modulation period of 5 s was used with the cryogenic trap maintained at −20 °C. The separation was performed with a linear temperature program of 60 to 225 °C at 3 °C min^{-1} after an initial hold at 60 °C for 0.2 min. The oven temperature was subsequently increased to 250 °C at 10 °C min^{-1} and held for 20 min. One microliter samples of 1% (v/v) oil were injected using an Agilent 7683 series autosampler. The injector was operated at 250 °C with a split ratio of 50:1. Helium was used as a carrier gas at a constant flow of 2 mL min^{-1} with an initial inlet pressure of 264.1 kPa at 60 °C. Average linear velocity through both columns was measured with butane gas and was found to be 25.2 cm s^{-1} at 60 °C. This flow rate was chosen on the basis of recent literature that demonstrated that the velocity for optimum efficiency in both columns is lower than the value in conventional GC (*22*). This is due to the narrow diameter of the ^{2}D column, which creates a high velocity in the ^{2}D column and a high pressure in the ^{1}D column, reducing the mobile phase diffusion coefficients.

The column set was coupled to the TOFMS via a 30 cm × 0.11 mm fused silica transfer line maintained at 260 °C. Ions in the mass range of m/z 41−415 were acquired at a rate of 100 spectra s^{-1} after a solvent delay of 3 min. The TOFMS had a source temperature of 200 °C and a detector voltage of 1660 V. Data processing was performed automatically using the peak detection algorithm of the ChromaTOF software (Leco Corp.). Peaks were tentatively identified by comparing their mass spectra to the NIST library (1998), the Wiley library (7th edition, 2000), the Adams library of essential oil components (2001) (*23*) and the MassFinder 3.58 library of terpenoids and related constituents of essential oils (Hochmuth Scientific Consulting, Hamburg, Germany). A series of alkanes (C8−C22) were analyzed to establish first-dimension retention indices (^1I) for each peak using the van den Dool and Kratz equation (*24*). Experimental retention indices were compared to literature values of the TOFMS hits to confirm or disprove the identifications (*23*, *25*). When possible, compound identification was confirmed by analysis of pure reference compounds using GC×GC.

Identification of Compounds Responsible for Character-Impact Odorants. GC×GC-TOFMS was used to tentatively identify peaks eluting in the odor-active regions perceived during GC-O. Odor-active regions were located in GC×GC-TOFMS by comparison to the GC-FID chromatograms corresponding to the GC-O separation and by matching linear retention indices. The odor quality and thresholds of compounds identified in the odor-active regions were obtained from the literature. When available, pure reference compounds were analyzed using GC-O to evaluate whether the retention index and odor quality of the standard matched the odor perceived in the sample. It is generally accepted that this provides sufficient evidence to conclude that the identified compound is responsible for the odor perception (*19*).

Heart-Cut Multidimensional GC−Olfactometry (MDGC-O). MDGC separations were performed on two capillary columns with different stationary phases (^{1}D; ^{2}D) contained within a single Agilent 6890N GC oven (Agilent Technologies, Shanghai, China). An Agilent G2855B microfluidic Deans switch was used to selectively "heart-cut" discrete regions of eluate from the ^{1}D to the ^{2}D column to aid resolution of coeluting regions. Auxiliary electronic pressure control (EPC) supplied additional carrier gas to the Deans switch to effect flow switching, directing the eluate from the ^{1}D column either to FID 1 via a deactivated fused silica transfer line (^{1}D TL) or to the ^{2}D column. Deans switch calculator software (version A.01.01; Agilent Technologies) facilitated the selection of initial experimental conditions, which were then fine-tuned according to the method proposed by Deans (*26*). This ensures that 100% of the ^{1}D eluate is switched to either FID 1 or the ^{2}D column. The pneumatic resistance of the ^{1}D TL to FID 1 was matched to that of the ^{2}D column by calculating appropriate dimensions. Constant pressure mode was used to maintain the pressure balance between the two columns throughout the oven temperature program. Further details regarding the operation of the Deans switch interface are available elsewhere (*26*, *27*).

The first column (^{1}D) was a 30 m × 0.32 mm i.d. × 0.25 μm d_f HP5 (J&W Scientific, Agilent Technologies, Palo Alto, CA) and the second column (^{2}D) was a 30 m × 0.32 mm i.d. × 0.5 μm d_f Solgel Wax (SGE International). The ^{1}D TL to FID 1 was a 1.59 m × 0.15 mm i.d. section of deactivated fused silica. The oven was programmed from 60 to 240 °C at 6 °C min^{-1} followed by an increase to 280 °C at 10 °C min^{-1} and a 10 min hold. Sample injections of 1 μL, at 10% (v/v) essential oil, were made using an Agilent 7683 series autosampler. The injector was operated at 250 °C with a split ratio of 50:1. Hydrogen was used as a carrier gas with a constant inlet pressure of 107.6 kPa and a constant auxiliary pressure to the Deans switch of 73.1 kPa. These conditions gave a ^{1}D flow rate of 3.0 mL min^{-1} (39.0 cm s^{-1}) and a ^{2}D flow rate of 4.5 mL min^{-1} (57.0 cm s^{-1}) at 60 °C. The eluate from the ^{2}D column was split (1:1) between FID 2 and an odor port via two 0.7 m × 0.32 mm i.d. fused silica transfer lines. Auxiliary nitrogen at 206.9 kPa was supplied to the SGE column flow splitter (SGE International) by the EPC module to increase the velocity in the transfer lines.

The original design of the MDGC system included a commercial olfactory port attached to the side of the GC oven. However, problems were encountered with condensation of analytes in the transfer line to the nose cone (data not shown), so that late eluting odors could not be perceived. Therefore, the odor activity of compounds eluting from the ^{2}D column was assessed by sniffing the eluate issuing from the housing of FID 2 with the barrel and jet removed. The column was positioned in the detector at the same place as if the jet and barrel were installed. A glass nose cone was positioned in the FID housing to facilitate sniffing and to isolate the assessor's nose from the hot FID. The detector block was heated to 250 °C, but the nose cone remained cool enough to touch comfortably. Nitrogen was supplied at the base of the column at 80 mL min^{-1}. This rudimentary odor port was tested with 1 μL injections of *E*-2-hexenal, linalool, and geraniol standards at 0.1% (v/v) and a split ratio of 50:1. The odors perceived were clear with good intensity, and the onset and end of the odor perception closely matched those of the chromatographic peak width. Because this setup resulted in the loss of a detector, the MDGC analysis had to be performed in two stages. First, the ^{1}D TL was connected to the active FID to acquire the ^{1}D chromatogram to determine heart-cut times. The ^{1}D TL was then removed from the FID and replaced with the ^{2}D TL to obtain FID detection simultaneously with the olfactory evaluation. In either case, the outlet of the unconnected TL was fed outside the oven so that hydrogen was not purged into the oven. The active FID was operated at 300 °C with an acquisition rate of 20 Hz.

RESULTS AND DISCUSSION

GC×GC Separation. The GC×GC-FID contour plots of the spicy fractions from the four different hop varieties are compared in **Figure 1**. Considerable differences in the qualitative and quantitative composition between the four varieties can be easily distinguished by the GC×GC pattern. The outlined regions in

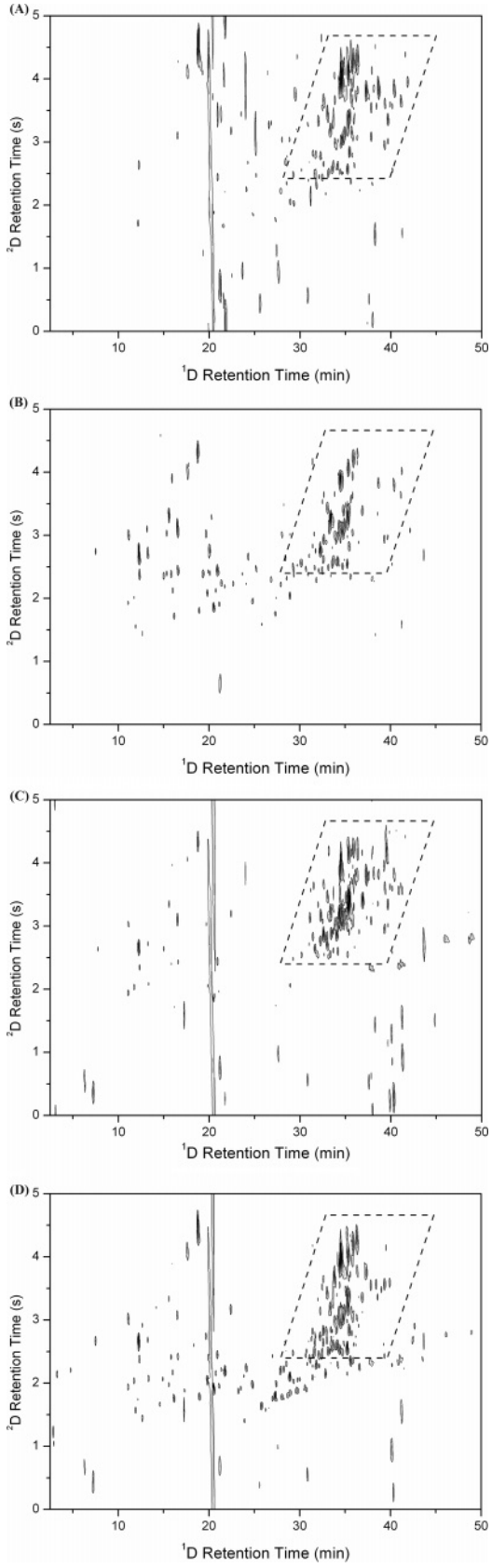

Figure 1. GC×GC-FID contour plots of the spicy fractions of (**A**) Cascade hops, (**B**) Target hops, (**C**) HHE hops, and (**D**) Saaz hops. Retention time on the ^{2}D column (*y*-axis) is plotted against retention time on the ^{1}D column (*x*-axis). Detector signal is plotted in the *z*-axis with peak height indicated by the contour levels and increasing shading. The outlined regions in each contour plot predominantly consist of oxygenated sesquiterpenes.

each contour plot predominantly consist of oxygenated sesquiterpenes, which demonstrate substantial levels of complexity. The vertical smears at 20 min in the Cascade, HHE, and Saaz samples are due to elution of 4-methyl-3-pentenoic acid, which has a poor peak shape on the BPX5 column and a long retention with broad peak widths (~4 s) on the polar ^{2}D column. This compound results from photo-oxidation of the ring structure of α- and β-acids in the hop resin during storage of the hop cones prior to extraction and distillation (*28*).

GC-O and CharmAnalysis. A total of 119 odor-active regions were detected by the two assessors during GC-O analysis for the four samples. The Cascade spicy fraction had the simplest aroma profile with a total of 38 odors detected by CharmAnalysis followed by Target spicy with 45 odor-active regions. Hersbrucker spicy and Saaz spicy were the most complex samples, with 70 and 71 odors, respectively. For simplicity, only the 25 most potent odors overall are presented, comprising 99.4, 90.6, 97.6, and 94.9% of the total Charm values (TCV) for Cascade, Target, HHE, and Saaz, respectively (**Table 1**). Compounds possibly responsible for the odor perception are listed along with the methods by which they were identified in GC×GC-TOFMS and whether their odor contribution was confirmed by assessing a reference standard with GC-O.

The most potent odorant in Cascade (66.1% TCV), HHE (77.5% TCV), and Saaz (41.8% TCV) and the second most potent odorant in Target (24.6% TCV) was an intense woody, cedarwood odor (peak 21a). Cascade had the greatest Charm value for this odor (96222), followed by HHE (65087), Saaz (26304), and Target (6296). This odorant will be dealt with in more detail later.

Overall, geraniol (peak 12; floral − rose, geranium) was the second most potent odorant, being ranked second for Cascade (29.6% TCV) and Saaz (24.4% TCV), third for Target (12.3% TCV), and fourth for HHE (2.1% TCV). Linalool (peak 5; floral − citrus) was the major character-impact odorant in the spicy fraction of Target hops, making up 33.9% of the odor-activity (TCV). This compound was the third most potent odorant in Saaz (7.0% TCV) and HHE (2.6% TCV), but of low importance in Cascade (0.2% TCV). Peacock et al. (*29*) concluded that geraniol and linalool were responsible for floral aroma in beer brewed with Cascade hops. Linalool in particular has been implicated as being important in overall hoppy aroma and the noble hop aroma (*30*, *31*). These compounds would be expected to produce a floral hop aroma in beer when added postfermentation but are also expected to survive fermentation (*9*).

β-Ionone (peak 15; floral − violet) was also a potent odorant in each sample, but particularly in the spicy fraction of Saaz hops (6.6% TCV). β-Ionone has previously been suggested to be an important odorant in hops and beer due to its low odor threshold (*32*), which ranges between 0.008 and 0.170 ppb in water (*33*). Kishimoto et al. (*11*) reported that β-ionone contributed to the floral odor of beer heavily hopped with Saaz, Hersbrucker, and Cascade. However, it is estimated that approximately a third of the population have a specific anosmia for β-ionone and, therefore, cannot detect it (*33*). In fact, one assessor used for CharmAnalysis was anosmic to this compound, whereas the other assessor was hypersensitive, being able to detect the odor in GC-O upon injection of 1 μL of a 1 ppt solution with a split ratio of 50:1. This demonstrates the danger of using too few assessors for GC-O and CharmAnalysis; therefore, when an inference is made about the importance of an odorant to a population, a panel of assessors is required (*17*).

A medicinal, clove odor (peak 13) was the second most potent odor in the HHE spicy fraction (3.2% TCV), and eugenol was

Table 1. Comparison of the Odor-Active Regions for the Spicy Fractions from Four Hop Varieties

			Cascade		Target		HHE		Saaz			
peak	peak *I*[a]	odor descriptors[b]	Charm	%	Charm	%	Charm	%	Charm	%	compd identified[c]	ID method[d]
1	777	fruity – SCFA ester			130	0.5	1118	1.3	353	0.6	*unknown ester*	MS
2	826	goaty, sweaty, cheesy	23	<0.1	59	0.2	227	0.3	248	0.4	3-methylbutanoic acid[e]	MS, RI, RC
3	843	fruity – SCFA ester			87	0.3	697	0.8	155	0.2	ethyl 3-methylbutanoate[e]	MS, RI, RC
4	847	fruity			23	<0.1	931	1.1	255	0.4	*SCFA ester*	MS
5	1107	floral – citrus	309	0.2	8654	33.9	2158	2.6	4416	7.0	linalool[e]	MS, RI, RC
6	1119	pungent, fruity, chemical					432	0.5	436	0.7	*pentyl 3-methylbutanoate*	MS
7	1130	pungent, winey, chemical	43	<0.1	4	<0.1	543	0.6	141	0.2	*unknown*	
8	1134	floral – rose, geranium	151	0.1			276	0.3	311	0.5	*unknown*	
9	1163	earthy, moldy, musty			127	0.5	20	<0.1	412	0.7	*decanone isomer*	MS
10	1175	aldehydic, cucumber, papery	159	0.1	92	0.4	290	0.3	373	0.6	*unknown*	
11	1181	aldehydic, papery, earthy	121	<0.1	229	0.9	271	0.3	807	1.3	*Z-linalool oxide (pyranoid)*	MS
12	1260	floral – rose, geranium	43106	29.6	3136	12.3	1755	2.1	15382	24.4	geraniol[e]	MS, RI, RC
13	1371	medicinal, cloves	47	<0.1			2700	3.2	260	0.4	eugenol[e]	MS, RI, RC
14	1396	cooked apple, apple sauce, sweet tobacco	109	<0.1	182	0.7	171	0.2	971	1.5	β-damascenone[e]	MS, RI, RC
15	1498	floral – violet	1530	1.1	655	2.6	828	1.0	4171	6.6	β-ionone[e]	MS, RI, RC
16	1561	fatty, green, aldehydic	240	0.2	13	<0.1	867	1.0	1147	1.8	*unknown*	
17	1568	fatty, green, aldehydic	463	0.3			368	0.4	172	0.3	*tetradecanone isomer*	MS
18	1582	fruity – artificial candy, floral	354	0.2			559	0.7	921	1.5	*unknown*	
19	1611	woody, cedarwood					1587	1.9	223	0.4	*unknown*	
20	1662	woody, cedarwood	123	<0.1					2021	3.2	*unknown*	
21a	1682	woody, cedarwood	96222	66.1	6296	24.6	65087	77.5	26304	41.8	*14-hydroxy-β-caryophyllene*	MS, RI
21b	1696	pungent, woody, spicy			2909	11.4					*unknown*	
22	1730	medicinal, pungent, woody	13	<0.1	424	1.7	129	0.2	236	0.4	*unknown*	
23	1744	pungent, woody, earthy					714	0.9			*unknown*	
24	1770	woody, cedarwood	1622	1.11	124	0.5	227	0.3			*unknown*	
		total Charm value (TCV)	144635	99.4	23144	90.6	81955	97.6	59715	94.9		

[a] Retention index of odor peak on a BPX-5 column in GC-O. [b] Odor descriptors generated by the two assessors during GC-O. Different terms are separated by a comma, and specific terms are separated by a dash. [c] Compounds identified in odor-active regions, possibly responsible for the odor perceived. Tentative identifications are in italics. [d] Compounds were identified on the basis of (i) comparison of their mass spectrum to reference databases (MS), (ii) comparison of retention index (RI), and (iii) comparison with reference compounds (RC). [e] Odor contribution confirmed by assessing a pure reference compound using GC-O.

suspected to be the compound responsible. The retention index and odor quality were confirmed by injection of a pure standard in GC-FID/O. Eugenol was not initially identified using GC×GC-TOFMS because it was present below the sensitivity level set for data processing. It was not until the odor was perceived during GC-O that it was identified by specifically searching in the corresponding region using its unique ion (*m*/*z* 164).

A number of aldehydic odors contributed to the odor character of the spicy fractions, with descriptors of fatty, green (peaks 16 and 17), papery, earthy (peak 11), and cucumber, papery (peak 10). A number of fruity odors were also perceived, the most potent of which contributed an odor reminiscent of an artificially flavored candy (peak 18). The other fruity odor regions all eluted early and were due to an unknown ester (peak 1), ethyl 3-methylbutanoate (peak 3), and an unidentified short-chain fatty acid (SCFA) ester (peak 4).

A distinctive cooked apple, applesauce, sweet tobacco odor (peak 14) was perceived in all samples but significantly contributed only to the odor of the Saaz spicy fraction (1.5% TCV). The compound responsible was suspected to be β-damascenone on the basis of its odor character and retention index, which were identical to that of a reference standard in GC-FID/O. A trace β-damascenone peak (S/N = 32) could only be located in the Saaz spicy fraction by specifically searching for its abundant ions (*m*/*z* 69 and 121) in the appropriate region of the GC×GC-TOFMS contour plot. β-Damascenone has previously been reported to be an important odorant in hops (*5*, *34*) and beer (*11*, *34*, *35*).

In addition to the major odorant (peak 21a), four other woody odors contribute to the odor character of the spicy fractions. In particular, peak 21b (I = 1696) is important in the Target sample, peak 20 (I = 1662) is important in the Saaz sample, peak 19 (I = 1611) is important in HHE, and peak 24 (I = 1770) is important in Cascade. These results suggest that "woody, cedarwood" characters contribute to the spicy nature of these PHA samples. However, the spicy character is a second-tier term in the beer flavor wheel grouped under "alcoholic", with synonyms of allspice, nutmeg, and peppery (*36*). Furthermore, the official reference standard of the European Brewery Convention (EBC) and the American Society of Brewing Chemists (ASBC) for spicy character is eugenol, which has an odor reminiscent of cloves (*37*). Sanchez et al. also used cinnamon, nutmeg, anise, and eugenol to train a panel for evaluating spicy hop aroma (*5*). **Table 1** shows that only the Hersbrucker spicy fraction exhibited an odor peak that matched this definition of spicy (peak 13; medicinal, cloves; eugenol). The woody, cedarwood odors found using CharmAnalysis appear to match the EBC and ASBC definitions of the term "resinous" (fresh sawdust, resin, cedarwood, pinewood, sprucy, and terpenoid) and the term "woody" (uncut seasoned wood) more closely than "spicy" (*36*). It is apparent that these descriptions require clarification when reported in the literature.

Varietal Differences. Although the four varieties had most odor peaks in common (**Table 1**), considerable quantitative differences in the Charm values were observed. For instance, Saaz spicy had the greatest concentration of β-ionone (peak 15; floral − violet) and β-damascenone (peak 14; apple, cooked apple), Hersbrucker spicy had a high level of eugenol (peak 13; medicinal, cloves), Cascade spicy was particularly rich in geraniol (peak 12; floral − rose, geranium), and Target spicy

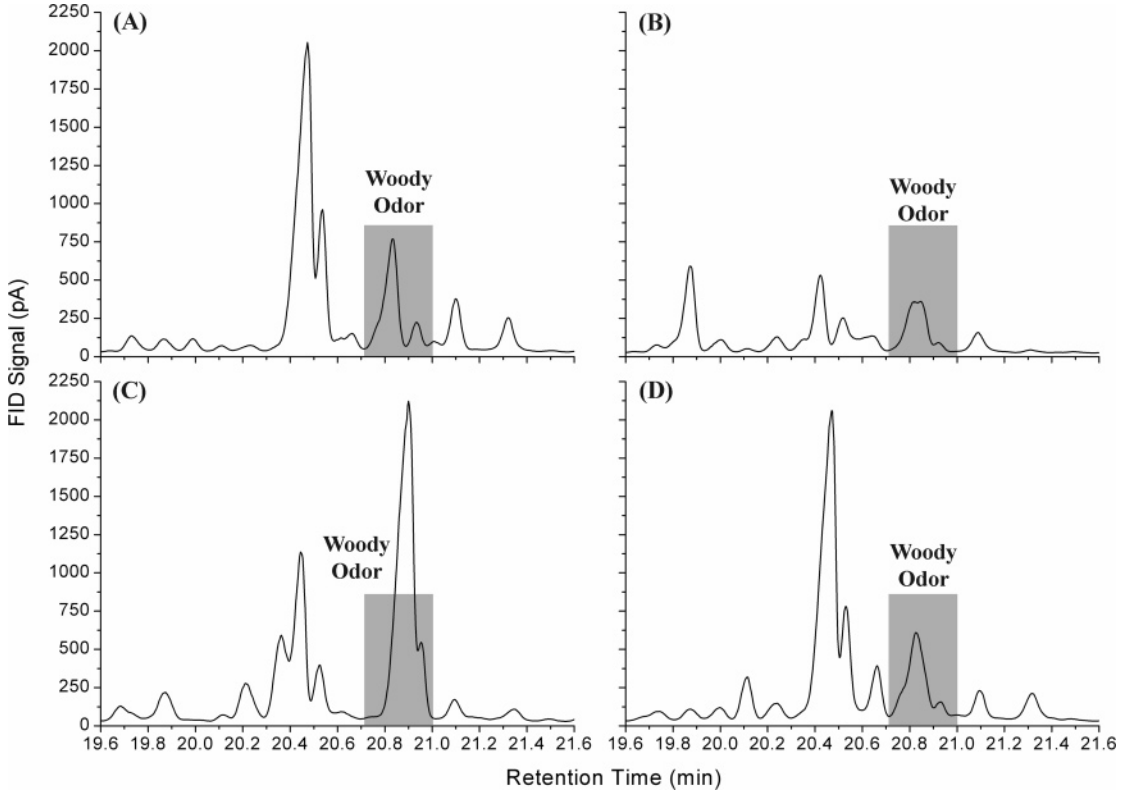

Figure 2. Selected regions of 1DGC-FID chromatograms of the spicy fractions of (**A**) Cascade hops, (**B**) Target hops, (**C**) HHE hops, and (**D**) Saaz hops collected under GC-O experimental conditions. The shaded region represents the duration of the woody, cedarwood odor (peak 21a) perceived during GC-O.

had the greatest amounts of linalool (peak 5; floral − citrus). This presumably accounts for varietal differences in odor characteristics between the four samples.

Identification of the Compound Responsible for Peak 21a. One reason for the large Charm value for the potent woody, cedarwood odor (peak 21a) was the long duration of the odor perception (maximum range $I = 1678-1736$). The duration of the perception is longer than expected and much greater than the chromatographic peak width. This could be due to a number of different factors including the high odor intensity, creating a long-lasting sensation; tailing of the chromatographic peak; perception of several coeluting odorants; slow release from the odor port; a physiological memory effect due to a continued receptor stimulus in the nose after elution of the peak; or a psychological memory effect of the assessor (*17*, *38*). Recording the start of an odor has also been shown to be much more reproducible than the recording of the end (*38*, *39*). However, it became apparent that more than one compound was contributing to the odor in this region. Only in the Target sample, where the odor potency was lowest, could the second odor region be distinctly resolved ($I = 1696$; peak 21b).

Peak 21a coincides with a complex region of the chromatogram where a large number of oxygenated sesquiterpenoids coelute during GC-O. **Figure 2** depicts where the woody odorant elutes during GC-O for each of the spicy fractions from the four hop varieties. It appears that at least three peaks may coelute from the single column during the perception of the odor. **Figure 3** compares the separation obtained in this region using GC×GC-FID for the four samples. The outlined region depicts where the woody odor is perceived, showing the number of coeluting compounds by GC×GC. Mass spectral deconvolution indicated that further coelution still occurred (data not shown), giving between 8 (Target and HHE) and 13 (Saaz) possibilities for the compound(s) responsible for the odor perceived.

The odor activity of each peak in this region could be determined by evaluating pure reference compounds. However, this cannot be achieved if standards cannot be obtained or synthesized or especially if the peaks themselves cannot be identified. The oxygenated sesquiterpenoid compounds eluting in this region exhibit very similar mass spectra, making unambiguous identification uncertain without reference standards. It is also difficult to source or to synthesize sesquiterpenoid reference compounds to test the odor activity of each individually. Thus, identifying each peak in this region would be time-consuming. A more effective approach would be to determine which peak is responsible for the odor perceived ***before*** identification.

Peaks eluting from a GC×GC separation are not ideal for olfactory detection by human assessors. The human nose is a slow detector dependent upon the assessor's breathing cycle (3−4 s), which is too slow to permit reliable assessment of the narrow, rapidly eluting peaks produced by GC×GC. The peaks eluting in the odor-active region of interest exhibit peak widths of 100−500 ms eluting within a 2.5 s time frame (**Figure 3**). In addition, each compound is modulated into multiple slices requiring several successive detections. These factors conspire to make GC×GC-O a difficult technique to implement for the direct determination of the odor activity of each peak.

In this study, four whole essential oils from the same hop varieties as the four spicy fractions were also assessed by CharmAnalysis and analyzed by GC×GC. This provided data on eight closely related samples, allowing peak areas to be correlated with the Charm values of the odor regions perceived. This is valid because odor potency and Charm values are

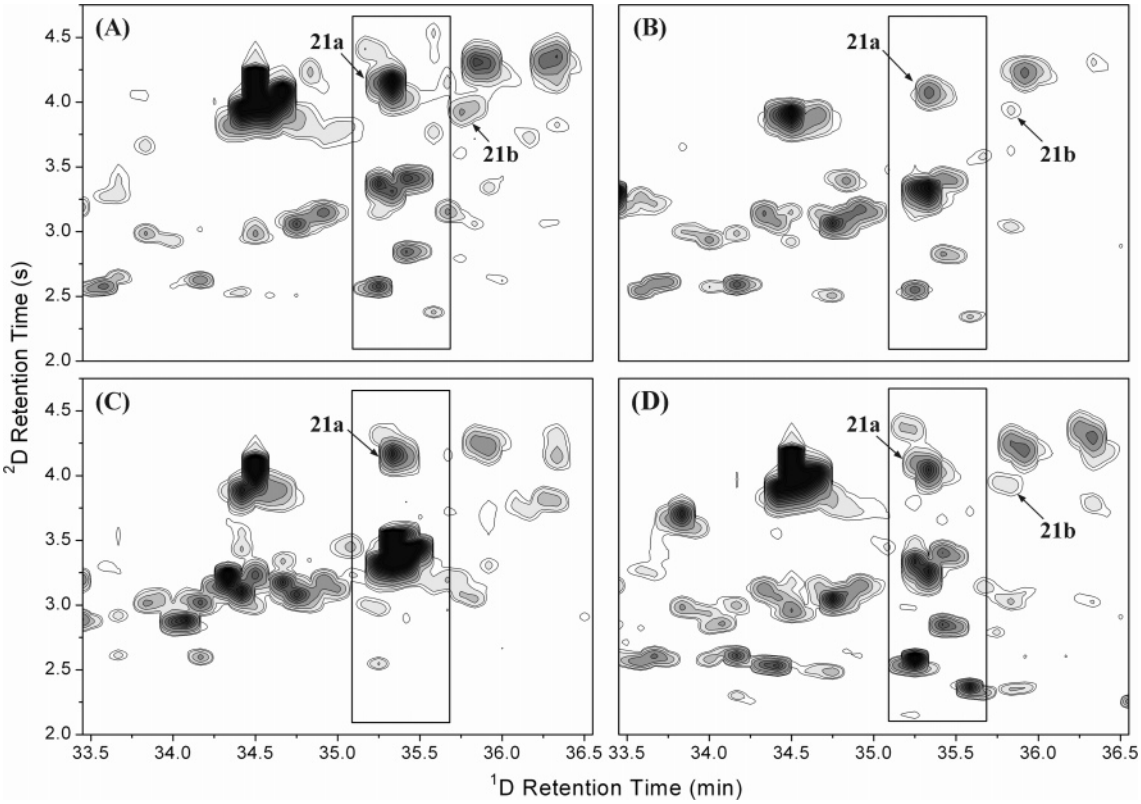

Figure 3. Selected regions GC×GC-FID contour plots of the spicy fractions of (**A**) Cascade hops, (**B**) Target hops, (**C**) HHE hops, and (**D**) Saaz hops. The outlined regions correspond to where the woody, cedarwood odor (peak 21a) was perceived during GC-O. The peaks determined to be responsible for the odors perceived in this region during GC-O (peaks 21a and 21b) are labeled.

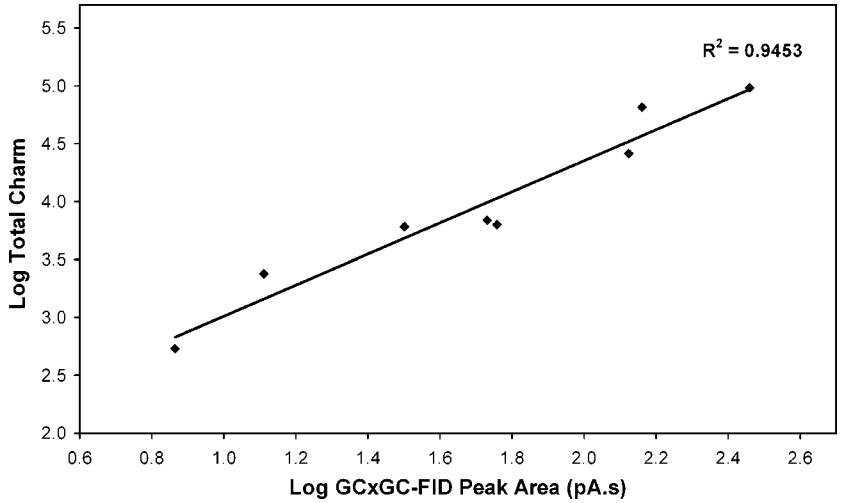

Figure 4. Correlation of log GC×GC-FID peak area with log Charm value for the woody odor perceived during CharmAnalysis of four spicy fraction and four whole oil hop samples.

proportional to concentration (*19*). Only one GC×GC peak was found to correlate with the Charm values of the woody odor in eight hop samples (R^2 = 0.9543; **Figure 4**) and is marked as peak 21a in **Figure 3**.

This peak was identified from its mass spectrum as 14-hydroxy-β-caryophyllene (bicyclo[7.2.0]undec-4-ene-4-methanol, 11,11-dimethyl-8-methylene-, [1*R*,4*Z*,9*S*]; CAS Registry No. 78683-81-5) using the MassFinder Terpenoid library. This compound was originally isolated from the wood of Cade juniper (*Juniperus oxycedrus*) and identified using IR, UV, and NMR spectroscopies and GC-MS (*40*−*42*). The stereochemistry was initially misreported as 14-hydroxy-9-*epi*-β-caryophyllene ([1*R*,4*Z*,9*R*]; CAS Registry No. 123355-03-3) but was later corrected to [1*R*,4*Z*,9*S*] and confirmed by synthesis (*43*, *44*). Demirci et al. (*45*) reported 14-hydroxy-β-caryophyllene to be the major component of steam-distilled birch bud oil (*Betula* species) (20.5−37.5%). The compound was isolated and then characterized by EI-MS, ^{1}H and ^{13}C NMR, and GC-FTIR and matched with the previous results obtained by Barrero et al. (*40*−*43*). However, nothing has been reported regarding its odor activity.

There are also two other possible isomers of 14-hydroxy-caryophyllene listed by CAS: 14-hydroxy-9-*epi*-(*E*)-caryophyllene ([1*R*,4*E*,9*R*]; CAS Registry No. 244226-09-3) and

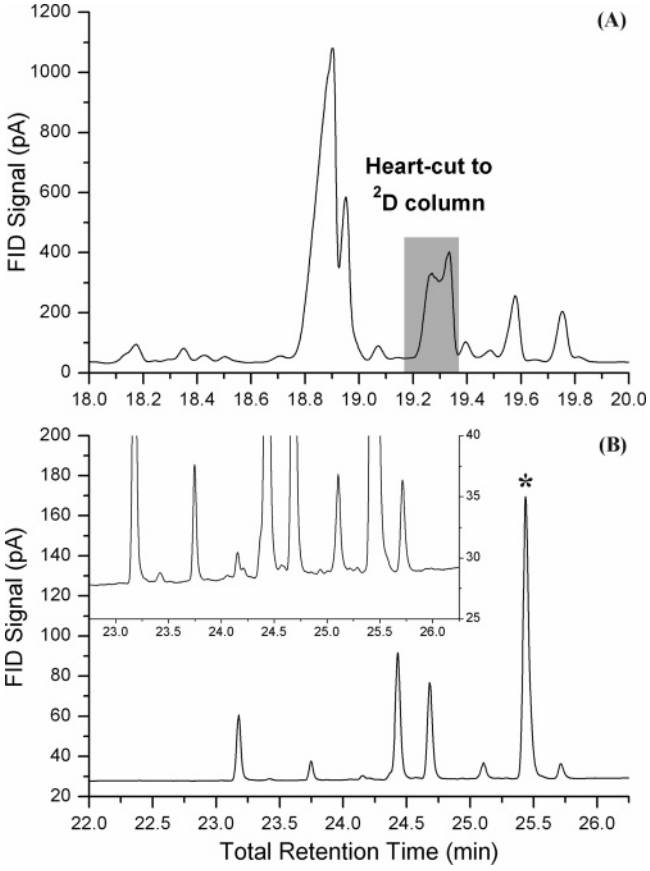

Figure 5. MDGC separation of the spicy fraction of Cascade hops. (**A**) 1D chromatogram corresponding to the GC-O separation in **Figure 2A**. The shaded area represents the 12 s region (19.17–19.37 min) heart-cut to the 2D column. (**B**) 2D chromatogram of the heart-cut region showing the resolution of seven significant peaks on the 2D column. Zooming in also reveals a number of minor peaks (inset). The peak responsible for the woody, cedarwood odor is marked with an asterisk.

14-hydroxyisocaryophyllene ([1*R*,4*E*,9*S*]; CAS Registry No. 79768-25-5). The experimental mass spectrum also matched the library spectrum of 14-hydroxy-9-*epi*-(*E*)-caryophyllene from the Adams library (*23*). The literature retention index of 1670 on a DB-5 type stationary phase closely matches the experimental value obtained ($I = 1682$), supporting (but not absolutely confirming) the identification of this compound. The larger experimental value is expected due to the use of a BPX5 stationary phase (5% phenyl polysilphenylene-siloxane), which is marginally more polar than a DB5-type phase (5% phenyl methylpolysiloxane). However, the stereochemistry and CAS Registry No. reported by Adams matches 14-hydroxyisocaryophyllene rather than 14-hydroxy-9-*epi*-(*E*)-caryophyllene. Furthermore, the origin of the reference spectrum and how the stereochemistry was determined are not known. Several papers in the literature have reported identifying 14-hydroxy-9-*epi*-(*E*)-caryophyllene using the Adams library, but it is apparent that there is considerable confusion regarding the identification of this compound, and it is possible that the stereochemistry has been misreported.

Pending confirmation of the stereochemistry, we have tentatively identified the compound responsible for peak 21a to be 14-hydroxy-β-caryophyllene on the basis of the robust identifications of Barrero et al. (*43*) and Demirci et al. (*45*). The retention index reported by Demirci et al. (*46*) on a polar stationary phase ($I = 2357$; Innowax) also matched where the woody odor was perceived ($I = 2376$) when GC-O was performed on a ZB-Wax column (Phenomenex, Torrance, CA). Although retention indices on polar columns may vary, the difference between these values is consistent with two other compounds identified by Demirci et al. (*46*) in common with the hop spicy fractions: geraniol (1857 vs 1866) and eugenol (2186 vs 2206). This further supports the proposed identification.

Multidimensional GC−Olfactometry (MDGC-O). The correlation approach used above cannot be applied to the analysis of a single sample to determine which peak is responsible for an odor perception in a coeluting region. An alternative technique that may be used to solve this problem is heart-cut MDGC-O. In MDGC, only specific zones (heart-cuts) of the 1D eluate are transferred to the second dimension, allowing the 2D column to be much longer than that used in GC×GC. Thereby, selected odor regions where coelution occurs in 1DGC may be heart-cut and resolved on the 2D column. MDGC generates one discrete peak per compound with a broader peak width, making the peaks more suitable for olfactory assessment than those generated by GC×GC.

Figure 5A presents the first column (1D) separation on the MDGC instrument of the same region where the woody odor was perceived in conventional GC-O of the spicy fraction of Cascade hops (**Figure 2A**). The shaded area shows the 12 s region that was selectively heart-cut to the 2D column. **Figure 5B** demonstrates the resolution achieved for the heart-cut region on the 2D column, showing that seven significant peaks and a number of minor peaks (inset) were coeluting on the 1D column. This corresponds well to the GC×GC contour plot in terms of total peak number (**Figure 3A**). The characteristic woody, cedarwood odor was detected as coinciding with the elution of the peak marked with an asterisk. This peak corresponds to the 14-hydroxy-β-caryophyllene peak identified using the correlation approach described above, confirming that result. The MDGC-O analysis was repeated for each spicy fraction sample (data not shown), with the same results. No other significant odors were detected for peaks eluting in this heart-cut for any of the spicy fraction samples.

The coeluting woody odor (peak 21b) was also assessed using MDGC-O and was determined to be the minor peak marked in **Figure 3**. The compound responsible, however, remains unidentified.

Final Remarks. The presented methodology presents a powerful approach to identify character-impact odorants in complex samples. Using heart-cut MDGC with simultaneous olfactory evaluation and mass spectral detection presents the natural progression of this methodology for identifying the compound responsible for coeluting odor clusters. However, analysis using GC×GC-TOFMS is still important to determine the overall composition of the sample in a single analysis.

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