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Comprehensive Sensomics Analysis of Hop-Derived Bitter Compounds during Storage of Beer

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Supporting Information

ABSTRACT: For the first time, quantitative LC−MS/MS profiling of 56 hop-derived sensometabolites contributing to the bitter taste of beer revealed a comprehensive insight into the transformation of individual bitter compounds during storage of beer. The proton-catalyzed cyclization of *trans*-iso-α-acids was identified to be the quantitatively predominant reaction leading to lingering, harsh bitter tasting tri- and tetracyclic compounds such as, e.g. the cocongeners tricyclocohumol, tricyclocohumene, isotricyclocohumene, tetracyclocohumol, and epitetracyclocohumol, accumulating in beer during storage with increasing time and temperature. The key role of these transformation products in storage-induced *trans*-iso-α-acid degradation was verified for the first time by multivariate statistics and hierarchical cluster analysis of the sensomics data obtained for a series of commercial beer samples stored under controlled conditions. The present study offers the scientific basis for a knowledge-based extension of the shelf life of the desirable beer's bitter taste and the delay of the onset of the less preferred harsh bitter aftertaste by controlling the initial pH value of the beer and by keeping the temperature as low as possible during storage of the final beverage.

KEYWORDS: sensomics, taste, iso-α-acids, bitter taste, hop, beer, aging, storage, shelf life

INTRODUCTION

Besides its sedative activity, beer has been attracting consumers over centuries due to its refreshing character, its typical aroma, and its desirable bitter taste profile. Whereas microbiological spoilage and haze formation determined the quality of beer in the past, the deterioration of their pleasant flavor is nowadays the shelf life limiting factor of beer products.[1−7] Besides changes in the composition and aroma impact of the fraction of odor-active volatiles,[1−4] in particular, a slight increase of sweetness and a decrease of the bitterness intensity accompanied by a shift of the taste profile toward a harsh and long-lasting bitter aftertaste are well-known phenomena observed during aging of beer.[5−7]

As the typical beer bitterness is mainly attributed to the addition of cones, pellets, or extracts of hop (*Humulus lupulus* L.) during wort boiling, a huge number of studies performed during the last century focused on the chemistry of hop-derived polyketides, namely α-acids and *β*-acids, prenylated chalcones such as xanthohumol, as well as their specific transformation products formed upon wort boiling. The group of α-acids, **1** (Figure 1), is the quantitatively predominating class of polyketides accounting for up to 10% of hops dry matter and comprising at least six congeners differing in the alkanoyl side chain. Whereas the cohumulone (**1a**), *n*-humulone (**1b**), and adhumulone (**1c**) account for more than 98% of the total amount of α-acids, the minor congeners, namely, postdhumulone, predhumulone, and adprehumulone, hardly reach a relative quantity of 2%.[8] Upon beer manufacturing, these α-acids (**1**) were found to undergo complex chemical transformations and, in addition, to be heavily adsorbed to yeast cells, trub, and filter materials, thus inducing a drastic reduction of their concentrations to reach levels of 1−5 *μ*mol/L in the final beverage accounting to less than 15% of amount initially added during wort boiling.[9] Although these α-acids are well-known to elicit a distinct bitter taste,[10] they are considered to have only a marginal impact on the beer taste.[11]

Upon wort boiling, the α-acids (**1**) were shown to be transformed via various pathways involving rearrangements, cyclizations, and oxidation reactions.[12−14] After protonation and α-ketol-rearrangement the α-acids (**1**) are known to be converted into the so-called iso-α-acids, which exhibit two chiral carbon atoms at the five-membered ring and exist as a mixture of trans- and cis-isomers, **2** and **3** (Figure 1). During beer manufacturing, the trans- (**2**) and cis-iso-α-acids (**3**) are formed in a ratio of about 0.4, thus indicating the *cis*-isomers as the dominating stereoisomer accounting for approximately 70% of the total amount of iso-α-acids.[15] Quantitative sensomics profiling throughout a full-scale beer manufacturing process recently confirmed the iso-α-acids (**2**, **3**) as the main transformation products accounting for up to 70% of the amount of α-acids initially added during wort boiling.[9,16] The comparatively high concentration in the final beverage and their low bitter taste thresholds strengthen their role as major bitter contributors in beer.[10,12,17,18] Accounting for less than 10% of the initial amount of α-acids, the

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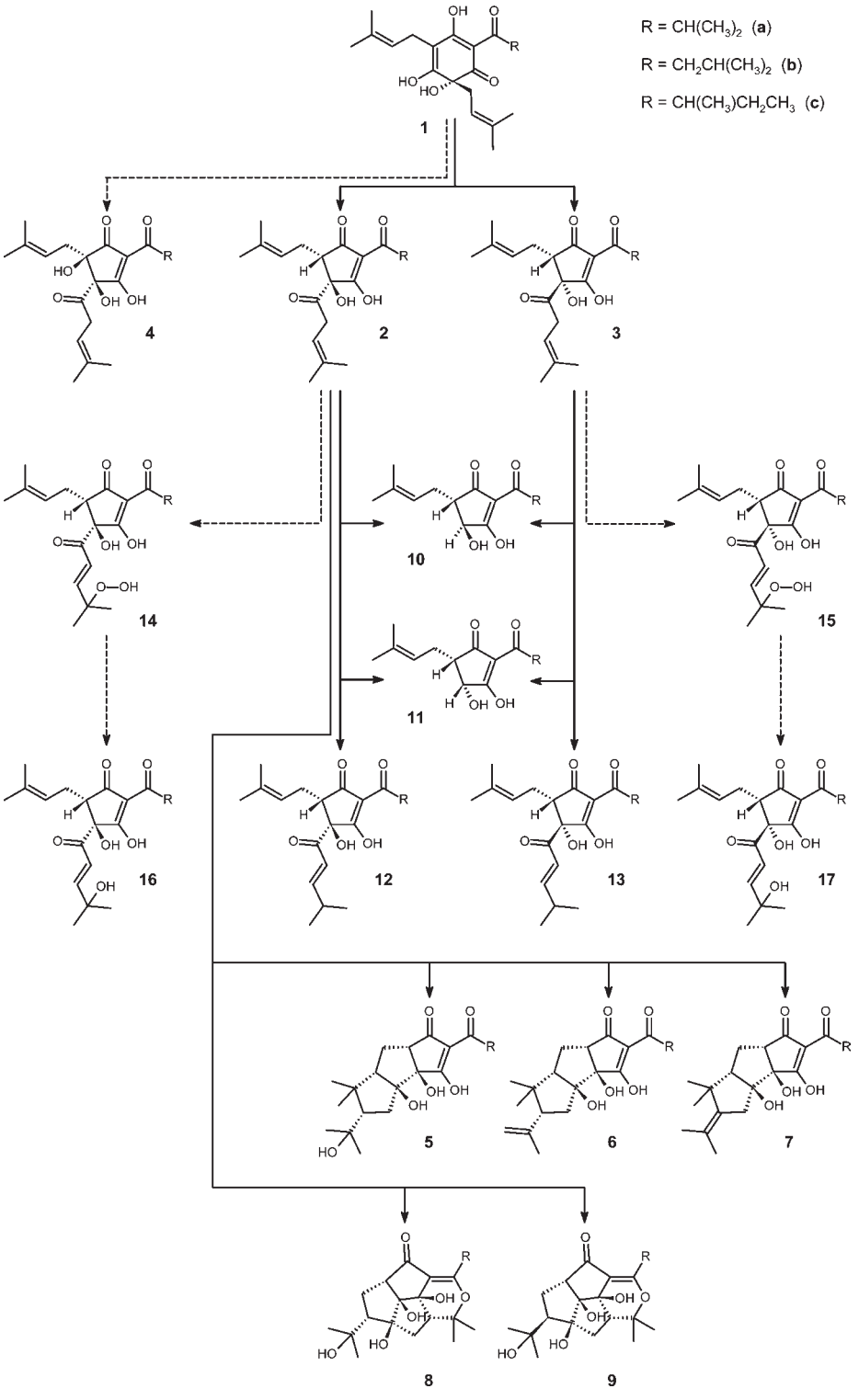

Figure 1. Structures of the α-acids cohumulone (**1a**), humulone (**1b**), adhumulone (**1c**) and their respective transformation products *trans*-isocohumulone (**2a**), *trans*-isohumulone (**2b**), *trans*-isoadhumulone (**2c**), *cis*-isocohumulone (**3a**), *cis*-isohumulone (**3b**), *cis*-isoadhumulone (**3c**), cohumulinone (**4a**), humulinone (**4b**), adhumulinone (**4c**), tricyclocohumol (**5a**), tricyclohumol (**5b**), tricycloadhumol (**5c**), tricyclocohumene (**6a**), tricyclohumene (**6b**), tricycloadhumene (**6c**), isotricyclocohumene (**7a**), isotricyclohumene (**7b**), isotricycloadhumene (**7c**), tetracyclocohumol (**8a**), tetracyclohumol (**8b**), tetracycloadhumol (**8c**), epitetracyclocohumol (**9a**), epitetracyclohumol (**9b**), epitetracycloadhumol (**9c**), *trans*-cohumulinic acid (**10a**), *trans*-humulinic acid (**10b**), *trans*-adhumulinic acid (**10c**), *cis*-cohumulinic acid (**11a**), *cis*-humulinic acid (**11b**), *cis*-adhumulinic acid (**11c**), *trans*-alloisocohumulone (**12a**), *trans*-alloisohumulone (**12b**), *trans*-alloisoadhumulone (**12c**), *cis*-alloisocohumulone (**13a**), *cis*-alloisohumulone (**13b**), *cis*-alloisoadhumulone (**13c**), hydroperoxy-*trans*-alloisocohumulone (**14a**), hydroperoxy-*trans*-alloisohumulone (**14b**), hydroperoxy-*trans*-alloisoadhumulone (**14c**), hydroperoxy-*cis*-alloisocohumulone (**15a**), hydroperoxy-*cis*-alloisohumulone (**15b**), hydroperoxy-*cis*-alloisoadhumulone (**15c**), hydroxy-*trans*-alloisocohumulone (**16a**), hydroxy-*trans*-alloisohumulone (**16b**), hydroxy-*trans*-alloisoadhumulone (**16c**), hydroxy-*cis*-alloisocohumulone (**17a**), hydroxy-*cis*-alloisohumulone (**17b**), and hydroxy-*cis*-alloisoadhumulone (**17c**).

humulinones, **4** (Figure 1), exhibiting an additional hydroxyl function at the five-membered ring system when compared to **2** and **3**, respectively, are supposed to be generated during wort boiling by means of an oxidative mechanism.[9,14,19] In contrast to the iso-α-acids, the humulinones emerge solely as the trans-configured isomer, thus strengthening the assumption that **4** is formed via a different reaction pathway when compared to **2**/**3**.

Besides the complex processes occurring during beer brewing, the aging of beer has been found to induce additional molecular transformations of the hop-derived bitter molecules. Most important, the degradation of the iso-α-acids has been found to strongly influence the overall bitter taste of beer.[20] Quantitative studies and model experiments revealed a huge influence of the stereochemistry on the reactivity of the iso-α-acids and demonstrated a rapid degradation of *trans*-iso-α-acids (**2**), whereas the corresponding cis-isomers (**3**) were found to be comparatively stable.[8,15,21] Recent molecular studies led to the discovery of harsh and lingering bitter tasting tricyclohumols (**5**), tricyclohumenes (**6**), isotricyclohumenes (**7**), tetracyclohumols (**8**), and epitetracyclohumols (**9**) as the major transformation products of *trans*-iso-α-acids (**2**) in model experiments as well as in authentic beer samples (Figure 1).[22] However, the structure of some additional, minor transformation products remained unknown. Stable-isotope ^{18}O-labeling studies with LC−MS/MS detection and computational studies demonstrated that the tri- and tetracyclic molecules **5**−**9** are formed via an oxygen-independent, proton-catalyzed trans-specific cyclization of iso-α-acids.[22,23] As outlined in Figure 1, additional degradation products were found to be formed independent of the iso-α-acid stereochemistry such as, e.g., *trans*-/*cis*-humulinic acids (**10**/**11**) and *trans*-/*cis*-alloisohumulone (**12**/**13**) formed by proton-catalyzed transformations[24,25] and hydroperoxy-*trans*/*cis*-alloisohumulones (**14**/**15**) and hydroxy-*trans*/*cis*-alloisohumulones (**16**/**17**) formed upon autoxidation involving air oxygen.[26]

Following the iso-α-acids, the β-acids (**18**) are the second major class of hop phytochemicals and, depending on the structure of the alkanoyl side chain, are named colupulone (**18a**), lupulone (**18b**), and adlupulone (**18c**) (Figure 2). Since β-acids have one additional prenyl group in contrast to **1**, these polyketides are even less water-soluble and were found to adsorb rather quantitatively to yeast, trub, and filter material during beer manufacturing.[9] In consequence, the content of β-acids (**18**) in beer is rather low and reaches levels of only 0.05−0.2 μmol/L, corresponding to a utilization rate of less than 4% of the initial amount of β-acids added with the hop dosage.[9,27] Recent model studies confirmed that, besides the iso-α-acid, also the β-acids are transformed upon wort boiling to give the so-called hulupones, **19** (Figure 2), which account for about 10% of the initially added amount of β-acids.[9,28,29] Upon cleavage of the variable alkanoyl side chain, the various hulupone congeners (**19a**, **19b**, **19c**) are truncated to give hulupinic acid, **20** (Figure 2).[30] Although the formation mechanisms of **19** and **20** are yet not completely understood, there is some evidence for oxidative reactions.[30,31] Very recently, a family of previously not reported tricyclic β-acid transformation products, **21**−**29** (Figure 2), could be identified and a radical reaction pathway was proposed for their formation.[28,29] With the exception of the hulupones (**19**), the concentrations of these β-acid transformation products in beer samples were found to be rather low and, therefore, the latter molecules are expected to show only a marginal impact on the bitter taste of beer.[28,29,32]

In addition to the polyketides, another major group of hop phytochemicals is the prenylated flavonoids such as, e.g., the chalcons xanthohumol and desmethylxanthohumol, **30** and **31** (Figure 3). Chalcone **30** shows only a limited water solubility and reaches rather low concentrations of 0.01−0.2 μmol/L in the final beer,[8] while **31** is considered as highly reactive and could yet not be detected in authentic beer samples.[33] As shown in Figure 3, wort boiling induces the cyclization of the chalcones to give the flavanones isoxanthohumol (**32**) from xanthohumol (**30**) and 6-prenylnaringenin (**33**) as well as 8-prenylnaringenin (**34**) from desmethylxanthohumol (**31**), respectively.[34,35] Pilsner-type beer was found to contain isoxanthohumol in levels of up to 3 μmol/L, whereas **33** and **34** are present only at trace levels.[8,36]

Although a vast number of bitter tasting transformation products of hop-derived phytochemicals have been identified in beer and monitored quantitatively throughout a full-scale beer manufacturing process, the information on the development of the individual sensometabolites during storage of beer is rather fragmentary. Although recent studies demonstrated the formation of harsh and lingering bitter tasting tri- and tetracyclic transformation products **5**−**9** upon storage of *trans*-iso-α-acid solutions, the structures of some additional, minor transformation products still remain unknown.[22] Therefore, the objective of the present investigation was to determine the chemical structure of these unknown degradation products, to verify the occurrence of these molecules as well as the entire set of previously identified bitter compounds in beer and, then, to perform quantitative HPLC−MS/MS studies on these sensometabolites in authentic beer samples stored under controlled conditions.

MATERIALS AND METHODS

Chemicals and Materials. The following chemicals were obtained commercially: formic acid, hydrochloric acid (Grüssing, Filsum, Germany); acetonitrile (Merck, Darmstadt, Germany). Deuterated solvents and ^{18}O-labeled water (isotopic abundance >98%) were from Euriso-top (Saarbrücken, Germany). Deionized water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, MA, USA). Freshly manufactured, authentic Pilsner-type beer samples (A−J), bottled in brown glass, were obtained from the German brewing industry. These samples were analyzed before (A_0−J_0) and after storage under forced aging conditions for 8 months at 28 °C (A_8−J_8). In another set of experiments, samples of Pilsner-type beer D were kept in brown glass bottles in the dark for 8 months at 28 °C, for 4 years at 20 °C, and for 10 years at about 20 °C, respectively. Another beer sample was stored for 4 years at 20 °C in a polyethylene terephthalate (PET) bottle. Samples of beer D, bottled in brown glass, were kept under forced aging conditions for up to 582 days at 28 °C in the dark prior to opening. For the storage experiments performed with pH-adjusted beer samples, brewing trials were carried out in a 20 hL pilot plant at Bitburger brewery. A standard 2-mash decoction procedure was used to produce Pilsner-type beers from 300 kg of barley malt and 325 g of hop. Wort was boiled for 70 min at 100 °C and, then, fermented at 10.5 °C. Filled storage containers were 50 L barrels. The final beer samples were adjusted to pH 4.4, 4.3, and 4.2, respectively, by adding lactic acid and, then, stored 0, 30, 90, and 300 d at 20 °C in the dark. Purified reference material of **1a**, **2a**, **2b**, **3a**, **5a**−**11a**, **14a**−**19a**, **20**, **30**, and **32** was prepared following literature protocols.[8,22,24,26,29] Purified samples of **4a**, **33**, and **34** were provided by the Hallertauer Hopfenveredlungsgesellschaft mbH (Mainburg, Germany).

Transformation of *trans*-Isohumulone (2b) in a Model Solution. A sample of *trans*-isohumulone (**2b**; 0.9 g) was dissolved in methanol (10 mL), a water/methanol mixture (70/30, v/v; 490 mL)

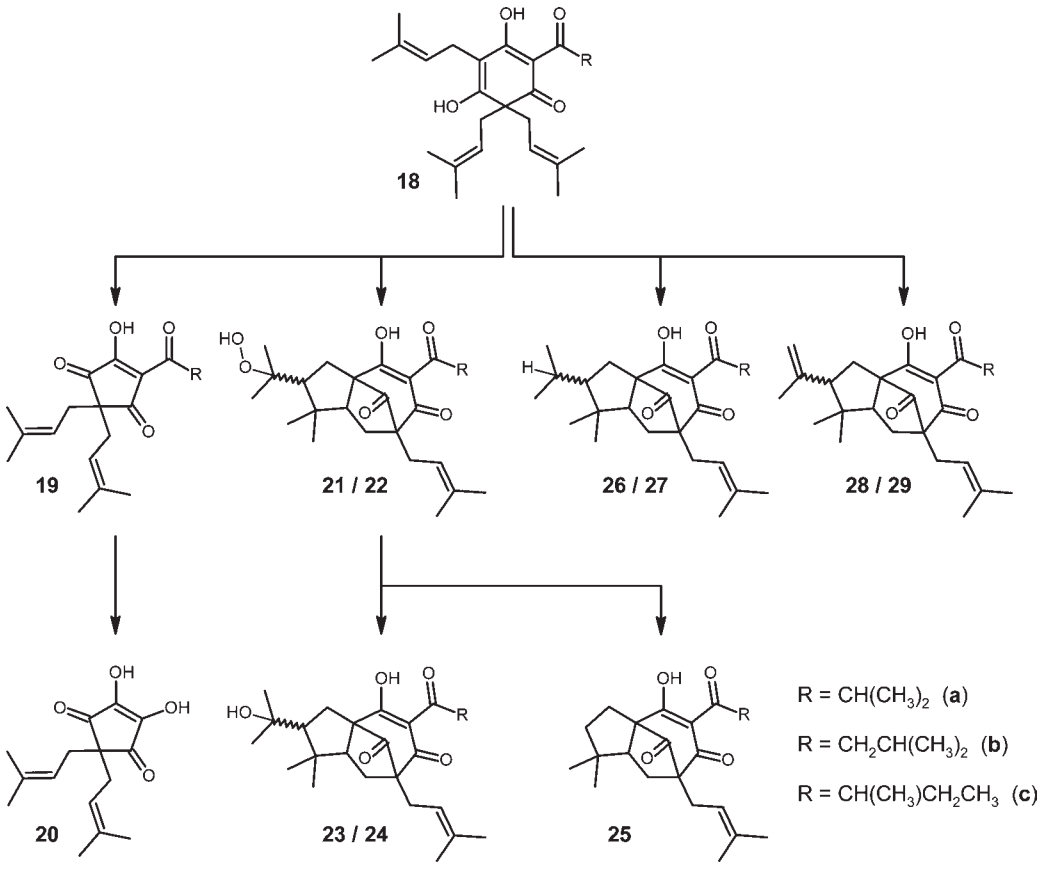

Figure 2. Structures of the β-acids colupulone (**18a**), lupulone (**18b**), adlupulone (**18c**) and their respective transformation products cohulupone (**19a**), hulupone (**19b**), adhulupone (**19c**), hulupinic acid (**20**), hydroperoxytricyclocolupone epimers (**21a**, **22a**), hydroperoxytricyclolupone epimers (**21b**, **22b**), hydroperoxytricycloadlupone epimers (**21c**, **22c**), hydroxytricyclocolupone epimers (**23a**, **24a**), hydroxytricyclolupone epimers (**23b**, **24b**), hydroxytricycloadlupone epimers (**23c**, **24c**), nortricyclocolupone (**25a**), nortricyclolupone (**25b**), nortricycloadlupone (**25c**), tricyclocolupone epimers (**26a**, **27a**), tricyclolupone epimers (**26b**, **27b**), tricycloadlupone epimers (**26c**, **27c**), dehydrotricyclocolupone epimers (**28a**, **29a**), dehydrotricyclolupone epimers (**28b**, **29b**), and dehydrotricycloadlupone epimers (**28c**, **29c**).

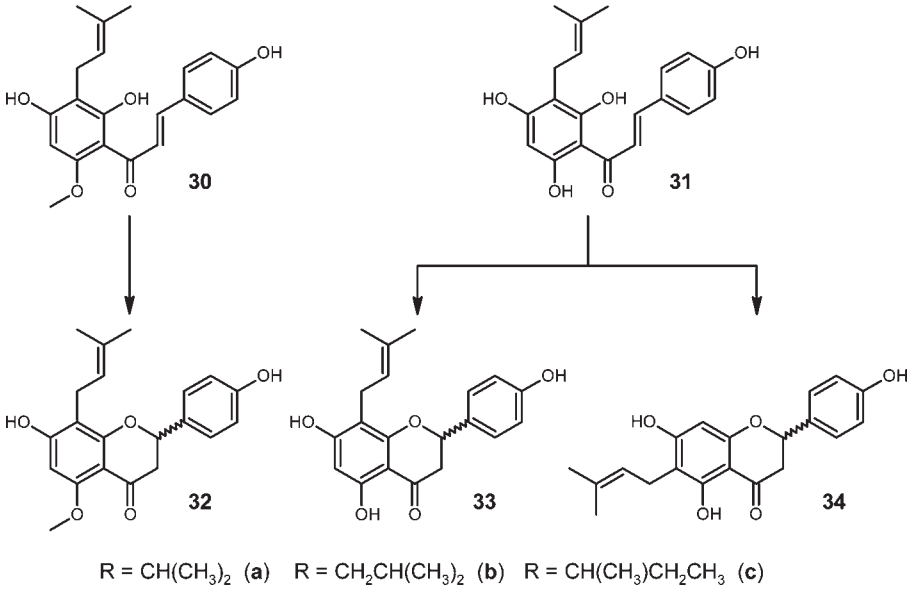

Figure 3. Structures of the prenylated flavanoids xanthohumol (**30**), desmethylxanthohumol (**31**), isoxanthohumol (**32**), 8-prenylnaringenin (**33**) and 6-prenylnaringenin (**34**).

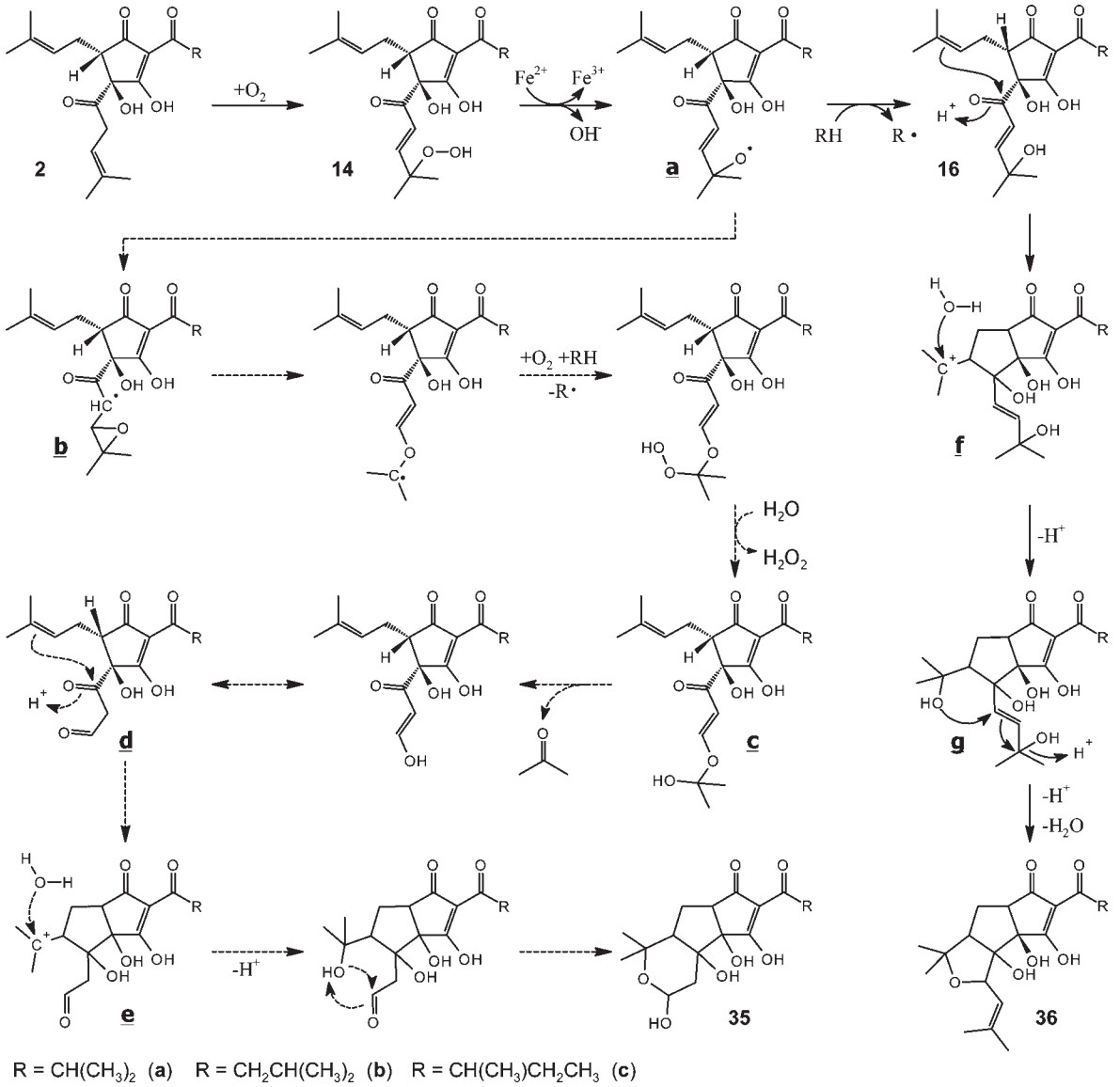

Figure 4. Chemical structures and hypothesized reaction route leading to tricyclocohumulactol (**35a**), tricyclohumulactol (**35b**), tricyclocadhumulactol (**35c**), scorpiocohumol (**36a**), scorpiohumol (**36b**) and scorpioadhumol (**36c**) from *trans*-iso-α-acids (**2**).

was added, and, after adjusting the pH to 4.0 by the addition of aqueous hydrochloric acid (1.0 mmol/L), the solution was kept for 6 h at 60 °C in a closed vessel. After cooling to room temperature, the reaction products were separated from solvent in vacuum, freeze-dried, taken up in acetonitrile, and then isolated by means of preparative RP-HPLC/UV (λ = 272 nm). A recently reported[22] analysis of the reaction products by means of HPLC−MS/MS-MRM using the compound-specific mass transitions experimentally determined for **5a**−**9a** and calculated for the corresponding homologues **5b**−**9b** revealed tricyclohumol (**5b**), tricyclohumene (**6b**), isotricyclohumene (**7b**), tetracyclohumol (**8b**), and epitetracyclohumol (**9b**) as major transformation products formed from *trans*-isohumulone, **2b** (Figure 1). In addition, two previously not reported reaction products, coined tricyclohumolactol, **35b** (Figure 4), and scorpiohumol (**36b**), were isolated and their chemical structures determined by means of UV/vis, NMR, and LC−MS experiments.

Tricyclohumolactol, ***35b****, Figure 4.* HRMS (ESI-): *m/z* 353.1665 (found), *m/z* 353.1605 (calcd for $[C_{18}H_{25}O_7 - H^+]^-$). LC/MS (ESI-): *m/z* (%) 353 (100, $[M - H^+]^-$). MS/MS (−30 V): *m/z* (%) 353 (20), 307 (40), 289 (75), 251 (100), 207 (70), 197 (75), 179 (35). ^{1}H NMR (400 MHz, CD_3OD): δ [ppm] 0.93 [d, 3H, *J* = 6.4 Hz], 0.94 [d, 3H, *J* = 6.4 Hz], 1.15 [s, 3H], 1.25 [m, 1H], 1.46 [s, 3H], 1.59 [m, 1H], 1.70 [m, 1H], 2.06 [m, 1H], 2.07 [m, 1H], 2.11 [m, 1H], 2.66 [dd, 1H, *J* = 6.9, 13.9 Hz], 2.72 [m, 1H], 2.76 [m, 1H], 5.14 [d, 1H, *J* = 9.2 Hz]. ^{13}C NMR (100 MHz, CD_3OD): δ [ppm] 22.8 [CH_3], 22.9 [CH_3], 25.4 [CH_2], 25.5 [CH_3], 26.6 [CH], 29.3 [CH_3], 37.2 [CH_2], 49.5 [CH_2], 51.5 [CH], 53.2 [CH], 75.1 [C], 80.1 [C], 86.5 [C], 89.6 [CH], 112.2 [C], 199.8 [C], 204.1 [2 × C].

Scorpiohumol, ***36b****, Figure 4.* HRMS (ESI-): *m/z* 377.1981 (found), *m/z* 377.1969 (calcd for $[C_{21}H_{29}O_6 - H^+]^-$). LC−MS (ESI-): *m/z* (%) 377 (100, $[M - H^+]^-$). MS/MS (−30 V): *m/z* (%) 377 (90); 359 (70); 233 (20); 197 (60); 179 (100). ^{1}H NMR (400 MHz, CD_3OD): δ [ppm] 0.91 [d, 3H, *J* = 6.9 Hz], 0.92 [d, 3H, *J* = 6.9 Hz], 1.21 [s, 3H], 1.39 [s, 3H], 1.49 [s, 3H], 1.71 [s, 3H], 1.87 [m, 1H], 2.11 [m, 1H], 2.13 [m, 1H], 2.51 [m, 1H], 2.66 [dd, 1H, *J* = 9.9, 9.5 Hz], 2.75 [m, 1H], 2.85 [m, 1H], 4.38 [d, 1H, *J* = 8.9 Hz], 5.41 [d, 1H, *J* = 8.9 Hz]. ^{13}C NMR (100 MHz, CD_3OD): δ [ppm] 18.9 [CH_3], 23.4 [CH_3], 23.5 [CH_3], 26.1 [CH_3], 26.2 [CH], 26.4 [CH_3], 26.8 [CH_2], 29.9

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Table 1. Optimized Mass Spectrometric Parameters for the Quantitative Analysis of Hop-Derived Sensometabolites by Means of LC−MS/MS Using Negative Electrospray Ionization (ESI^-)

compd no.[a]	mass transition Q1 → Q3	DP[b] [V]	CE[c] [V]	CXP[d] [V]
1a	*m/z* 347.1 → 277.9	−65	−28	−17
1b/c	*m/z* 361.2 → 292.0	−65	−28	−17
2a, 3a	*m/z* 347.0 → 251.0	−90	−22	−11
2b/c, 3b/c	*m/z* 361.0 → 265.0	−90	−22	−11
4a	*m/z* 363.1 → 209.2	−50	−22	−7
4b/c	*m/z* 377.1 → 223.2	−50	−22	−7
5a	*m/z* 365.3 → 165.0	−105	−48	−9
5b/c	*m/z* 379.3 → 179.0	−105	−48	−9
6a, 7a	*m/z* 347.3 → 165.0	−60	−52	−7
6b/c, 7b/c	*m/z* 361.2 → 179.0	−60	−52	−7
8a, 9a	*m/z* 365.3 → 193.1	−120	−46	−11
8b/c, 9b/c	*m/z* 379.3 → 207.1	−120	−46	−11
10a, 11a	*m/z* 251.0 → 140.8	−75	−28	−7
10b/c, 11b/c	*m/z* 265.0 → 154.8	−75	−28	−7
12a, 13a	*m/z* 346.9 → 181.1	−70	−45	−6
12b/c, 13b/c	*m/z* 361.0 → 195.0	−70	−45	−6
14a, 15a	*m/z* 379.0 → 347.0	−85	−26	−9
14b/c, 15b/c	*m/z* 393.0 → 361.0	−85	−26	−9
16a, 17a	*m/z* 363.0 → 261.0	−85	−28	−15
16b/c, 17b/c	*m/z* 377.0 → 275.0	−85	−28	−15
18a	*m/z* 399.2 → 286.9	−80	−38	−15
18b/c	*m/z* 413.2 → 301.2	−80	−38	−15
19a	*m/z* 317.0 → 204.9	−55	−40	−13
19b/c	*m/z* 331.1 → 218.9	−55	−40	−13
20	*m/z* 263.0 → 193.9	−50	−22	−11
30, 32	*m/z* 352.9 → 118.8	−95	−42	−7
31, 33, 34	*m/z* 339.1 → 219.0	−70	−30	−13
35a	*m/z* 339.2 → 193.0	−90	−38	−15
35b/c	*m/z* 353.2 → 207.0	−90	−38	−15
36a	*m/z* 363.3 → 164.9	−90	−46	−13
36b/c	*m/z* 377.3 → 178.9	−90	−46	−13

[a] Compound numbering refers to the chemical structures given in Figures 1−4. [b] Declustering potential. [c] Collision energy. [d] Cell exit potential.

[CH_3], 50.2 [CH_2], 56.9 [CH], 62.9 [CH], 78.7 [CH], 82.8 [C], 85.4 [C], 91.9 [C], 113.5 [C], 121.0 [CH], 139.6 [C], 201.0 [C], 201,1 [C], 205.3 [C].

^{18}O-Stable Isotope Labeling Experiment. An ^{18}O-stable isotope labeling experiment was performed in order to locate stably incorporated oxygen atoms in tricyclohumolactol (**35b**) and scorpiohumol (**36b**) generated during degradation of *trans*-isohumulone (**2b**). To achieve this, a solution of **2b** (30 μmol/L) in $H_2{}^{18}O$ (250 μL) was adjusted to pH 2.0 by adding trace amounts of concentrated hydrochloric acid and, then, kept for 4 h at 60 °C in the dark. After cooling to room temperature, the solvent was removed in vacuum and the residue was taken up in H_2O (250 μL) to achieve a re-exchange of instable bound ^{18}O atoms. In order to study the presence of stable-bound ^{18}O atoms in the degradation products formed, this solution was used for an LC−MS scan experiment. Isotopic distribution of the pseudo molecular ion $[M - H^+]^-$ found for tricyclohumolactol, **35b**: *m/z* 353 (0%), 355 (20%), 357 (60%), and 359 (20%). Isotopic distribution of the pseudo molecular ion $[M - H^+]^-$ found for scorpiohumol, **36b**: *m/z* 377 (0%), 379 (70%), 381 (30%).

Liquid Chromatography/Time-of-Flight Mass Spectrometry (LC/TOF-MS). High resolution mass spectra of the compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer and referenced to sodium formate.

High Performance Liquid Chromatography/Mass Spectrometry (HPLC−MS/MS). An Agilent 1100 Series HPLC-system consisting of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) or, alternatively, a PHD 4400 Hpsi-type syringe pump (Harvard Apparatus, Massachusetts, USA) was connected to an API 4000 Q-TRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany) which was equipped with an electrospray ionization (ESI) source operating in the negative ion mode. For HPLC−MS experiments, nitrogen was used as turbo gas at 400 °C. The compound-specific declustering potential (DP), cell exit potential (CXP), and collision energy (CE) were optimized for each substance prior to the analysis by infusion of pure reference solutions and are summarized in Table 1. The dwell time for each mass transition was 44 ms. For direct infusion experiments using the syringe pump, no additional turbo gas was used. The ion spray voltage was set to −4500 V. Data acquisition and processing was performed by using the Analyst software version 1.4.2 (AB Sciex Instruments, Darmstadt, Germany).

For screening and quantitative analysis of hop-derived compounds and their degradation products in beer samples by means of HPLC−MS/MS, a 150 × 2 mm, 4 μm, Synergi polar RP column (Phenomenex, Aschaffenburg, Germany) was used as the stationary phase, and a gradient of acetonitrile containing 0.1% formic acid (solvent A) and aqueous formic acid (0.1% in water; solvent B) was used as the mobile phase. Using a flow rate of 250 μL/min, chromatography was performed by increasing solvent A from 20 to 35% within 20 min, then to 70% within 20 min, to 100% within 10 min, holding 100% for 5 min, and, finally, decreasing within 2 min to 20%, and holding it for 3 min. Prior to the injection of the sample (5 μL), the system was equilibrated for 5 min. Each run started with the injection of the respective sample solution and was followed by a time-delayed injection of the internal ECHO standard as detailed recently.[8] As preliminary studies demonstrated that the use of isoxanthohumol (**32**) as the internal ECHO standard reveals data comparable to that found recently for a series of additional ECHO standards,[8] isoxanthohumol (**32**) was injected 15 min after starting of the HPLC−MS/MS analysis of the beer samples.

For the analysis of the ratio of individual isotopologues in the ^{18}O-labeling experiment, MS scan experiments were performed by means of HPLC−MS in the $Q1^-$ scan mode using the chromatographic conditions detailed above. The compound-specific declustering potential (DP) was set to an intermediate value of −70 V, and the ion spray voltage was set to −4500 V. The mass scan was performed from *m/z* 100 to 500 within 1 s per scan cycle.

Quantitative Analysis of Bitter Sensometabolites. Beer samples (5 μL) were degassed by ultrasonification and, after membrane filtration (0.45 μm, Sartorius, Göttingen, Germany), directly injected into the HPLC−MS/MS system. To enable the quantitation of compounds **1**−**11**, **14**−**20**, **30**, and **32**−**34**, 6-point external calibration curves were recorded for cohumulone (**1a**), *trans*-isocohumulone (**2a**), *cis*-isocohumulone (**3a**), cohumulinone (**4a**), tricyclocohumol (**5a**), tricyclocohumene (**6a**), isotricyclocohumene (**7a**), tetracyclocohumol (**8a**), epitetracyclocohumol (**9a**), *cis*-cohumulinic acid (**11a**), hydroperoxy-*cis*-alloisohumulone (**15b**), hydroxy-*cis*-alloisohumulone (**17b**), colupulone (**18a**), cohulupone (**19a**), hulupinic acid (**20**), xanthohumol (**30**), isoxanthohumol (**32**), and 8-prenylnaringenin (**33**), respectively. In order to obtain the best fit for all calibration curves, second-order polynomial equations were calculated. To avoid negative or exaggerated estimates at the low end of the concentration ranges, the functions were forced through zero, thus leading to correlation coefficients of >0.99 for all the reference compounds. The *n*- and ad-congeners of α-acids (**1**), iso-α-acids (**2**/**3**), humulinones (**4**), tricyclohumols (**5**), tricyclohumenes (**6**),

isotricyclohumenes (**7**), tetracyclohumols (**8**), epitetracyclohumenes (**9**), β-acids (**18**), and hulupones (**19**) were analyzed on the basis of the calibration curve of the corresponding cocongener (**1a**, **2a**, **3a**, **4a**, **5a**, **6a**, **7a**, **8a**, **9a**, **18a**, **19a**). Quantitative analysis of *cis*- and *trans*-humulinic acids (**10**/**11**) was performed by using the calibration function of *cis*-cohumulinic acid (**11a**). The *trans*- and *cis*-isomers of hydroperoxy- (**14**/**15**) and hydroxy-alloisohumulones (**16**/**17**) and were analyzed on the basis of the calibration curve of their *cis*-configured *n*-congeners **15b** and **17b**, respectively.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C and 2-D NMR data were acquired on a Bruker DMX-400 (Bruker BioSpin, Rheinstetten, Germany). CD_3OD was used as solvent, and chemical shifts were referenced to the solvent signal (δ_H = 3.31 Hz, δ_C = 49.05 Hz). For accurate NMR signal assignment, COSY-, HMQC-, and HMBC-experiments were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed by using XWin-NMR software (version 3.5; Bruker, Rheinstetten, Germany) as well as Mestre-C (Mestrelab Research, LA CORUÑA, Spain).

Multivariate Analysis. Principal component analysis (PCA) was performed using the open source software R (version 2.4.0, www.r-project.org). Therefore 12 variables (concentrations of **1a**−**3a**, **5a**−**11a**, **18a** and **19a**) were selected and a sample set of 144 beers was used for statistical analysis. For rotation of the variables, the function "prcomp" was applied with centered values under usage of the covariance matrix. By iteration linear combinations are calculated, leading to uncorrelated variables with maximized variance. The so-called principal components (PC) with the following relative variances were obtained (PC1−PC12 [%]; 82.36; 15.49; 2.11; 1.27; 0.41; 0.15; 0.029; 0.022; 0.013; 0.008; 0.003; 0.003). By multiplication of the linear transformed variables, the so-called loadings, with the individual concentration values of the sample coefficient matrices for all principal components, were obtained. Plotting the coefficients of two principal components leads to a scatter plot enabling a better distinction of samples of a multivariate data set.

For sensomics heatmapping, data analysis was performed within the programming and visualization environment R (version 2.10.0).[37] The sensomics heatmap was calculated using the heatmap.2 function of R based on the concentration data (see Supporting Information) after scaling the sum of all sensometabolites to an equal value for each beer sample. The dendrograms were constructed by means of an agglomerative linkage algorithm proposed by Ward[38] specifying the distance between two clusters as the increase in the error sum of squares after fusing two clusters into a single cluster and seeking for a minimum distance at each clustering step.

RESULTS AND DISCUSSION

Although recent studies demonstrated the formation of harsh and lingering bitter tasting tri- and tetracyclic transformation products **5**−**9** upon storage of *trans*-iso-α-acid solutions, the structure of two additional, minor transformation products still remained unknown.[22] In order to identify these unknown transformation products, *trans*-isohumulone (**2b**) was purified and degraded under controlled conditions.

Formation of Transformation Products of *trans*-Iso-α-acids (2). An aqueous solution (pH 2.0) of *trans*-isohumulone (**2b**) was kept for 6 h at 60 °C and, after cooling, the reaction products formed were isolated by means of RP-HPLC. As reported recently,[22] analysis of the reaction products was performed by means of HPLC−MS/MS-MRM using the compound-specific mass transitions experimentally determined for **5a**−**9a** and calculated for the corresponding homologues **5b**−**9b** (Table 2). Being well in line with our previous studies,[22] tricyclohumol (**5b**), tricyclohumene (**6b**), isotricyclohumene (**7b**), tetracyclohumol (**8b**), and epitetracyclohumol (**9b**) were found as the major *trans*-isohumulone transformation products (Figure 1). In addition, two unknown reaction products (**35b**, **36b**) were isolated, the chemical structures of which were determined in the following by means of UV/vis, NMR, and LC−MS experiments.

LC−MS analysis of compound **35b** revealed the pseudo molecular ion *m*/*z* 353 in the negative electrospray (ESI-) mode and demonstrated a difference in masses of 8 amu when compared to the pseudo molecular ion *m*/*z* 361 found for the parent *trans*-isohumulone (**2b**). LC−TOF-MS analysis showed the empirical formula of **35b** to be $C_{18}H_{26}O_7$, thus indicating the loss of three carbon atoms and four hydrogen atoms as well as the addition of two oxygen atoms when compared to **2b**. Comparison of the spectroscopic data obtained for **35b** from ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC experiments with those of the parent *trans*-isohumulone (**2b**) as well as tricyclohumol (**5b**) demonstrated that a C_3-fragment was split off from the *iso*-hexeneoyl side chain of **2b**. Careful assignment of all the 1D/2D-NMR signals led to the identification of **35b** as the previously not reported tricyclohumolactol (Figure 4).

LC−MS (ESI-) analysis of compound **36b** revealed *m*/*z* 377 as the pseudo molecular ion, differing by 16 amu from that of the parent *trans*-isohumulone (*m*/*z* 361) and suggesting the incorporation of an additional oxygen atom. This was further strengthened by means of LC−TOF-MS demonstrating an empirical formula of $C_{21}H_{30}O_6$. Comparison of the spectroscopic data obtained from one- (^{1}H, ^{13}C NMR) and two-dimensional NMR experiments (COSY, HMQC, HMBC) with those found for the precursor molecule **2b** and the transformation product tricyclohumol (**5b**) demonstrated a new carbon−carbon bond between the former carbonyl carbon of the isohexeneoyl side chain and the olefinic methylene carbon of the isoprenyl moiety as already observed in the structure of **5b**. In contrast to **5b**, the NMR data of **36b** did not show the presence of another new carbon−carbon bond, but gave strong evidence for a cyclic ether structure, fitting well with the chemical shifts of 78.7 and 82.8 ppm found for the adjacent carbon atoms. Taking all spectroscopic data into account, the structure of previously not reported transformation product was proposed to be tricyclic ether **36b**, coined scorpiohumol (Figure 4).

In order to gain a more detailed insight into the mechanism of the transformation of **2b** into tricyclohumolactol (**35b**) and scorpiohumol (**36b**), ^{18}O-labeling experiments with MS detection were performed to visualize the incorporation of oxygen atoms from water into the target molecules by means of LC−MS/MS. The transformation of compounds **35b** and **36b** was performed in a solution of $H_2{}^{18}O$ in order to visualize the stable insertion of ^{18}O atoms from water into the *trans*-iso-α-acid degradation products. To achieve this, a solution (pH 2.0) of *trans*-isohumulone (**2b**) in $H_2{}^{18}O$ was incubated for 4 h at 60 °C and, after separating the solvent in vacuum, the residue was taken up in H_2O to re-exchange labile bound ^{18}O atoms and, then, used for LC−MS/MS scan and fragmentation experiments. This experiment revealed an isotopic distribution of *m*/*z* 353 (0%), 355 (20%), 357 (60%), and 359 (20%) for tricyclohumolactol (**35b**) and *m*/*z* 377 (0%), 379 (70%), 381 (30%) for scorpiohumol (**36b**), thus demonstrating the stable incorporation of up to two and three ^{18}O atoms into **36b** and **35b**, respectively, from the solvent water into the target molecule.

On the basis of their chemical structure and the data of the ^{18}O-labeling experiment, a reaction pathway is proposed in Figure 4 for the formation of tricyclohumolactol (**35b**) and

Table 2. Concentrations (Standard Deviation) of Hop-Derived Bitter Compounds in Fresh and Stored Beer Samples

	concn [μmol/L] in beer sample[b]				
compd (no.)[a]	I	II	III	IV	V
cohumulone (**1a**)	0.41 (±0.03)	0.07 (±0.01)	0.02 (±0.01)	<0.01	<0.01
humulone (**1b**)	0.64 (±0.01)	0.19 (±0.01)	0.03 (±0.01)	0.01 (±0.01)	<0.01
adhumulone (**1c**)	0.07 (±0.01)	0.02 (±0.01)	<0.01	<0.01	<0.01
trans-isocohumulone (**2a**)	11.50 (±0.90)	3.50 (±0.10)	0.60 (±0.10)	0.20 (±0.03)	<0.01
cis-isocohumulone (**3a**)	29.60 (±0.50)	26.20 (±1.20)	26.20 (±1.70)	25.20 (±1.80)	0.20 (±0.01)
trans-isohumulone (**2b**)	12.60 (±0.30)	3.80 (±0.10)	0.80 (±0.10)	0.30 (±0.02)	<0.01
cis-isohumulone (**3b**)	35.70 (±1.40)	27.90 (±2.30)	21.80 (±1.70)	15.60 (±1.00)	0.21 (±0.01)
trans-isoadhumulone (**2c**)	2.90 (±0.10)	1.10 (±0.01)	0.30 (±0.10)	0.10 (±0.10)	<0.01
cis-isoadhumulone (**3c**)	10.00 (±0.40)	7.60 (±0.40)	6.80 (±0.40)	6.80 (±0.30)	0.04 (±0.01)
cohumulinone (**4a**)	0.84 (±0.05)	0.27 (±0.04)	0.15 (±0.03)	<0.01	nd
humulinone (**4b**)	1.07 (±0.01)	0.45 (±0.04)	0.25 (±0.04)	<0.01	nd
adhumulinone (**4c**)	0.34 (±0.01)	0.12 (±0.01)	0.06 (±0.01)	<0.01	nd
tricyclocohumol (**5a**)	1.00 (±0.00)	4.50 (±0.10)	7.20 (±0.50)	9.30 (±0.20)	6.10 (±0.81)
tricyclohumol (**5b**)	1.60 (±0.10)	7.60 (±0.50)	9.20 (±0.50)	7.40 (±0.60)	8.30 (±0.90)
tricycloadhumol (**5c**)	nq	nq	nq	nq	nq
tricyclocohumene (**6a**)	0.30 (±0.03)	1.03 (±0.09)	1.81 (±0.21)	3.10 (±0.26)	1.95 (±0.11)
tricyclohumene (**6b**)	0.36 (±0.03)	1.14 (±0.04)	1.70 (±0.16)	2.02 (±0.20)	2.43 (±0.11)
tricycloadhumene (**6c**)	nq	nq	nq	nq	nq
isotricyclocohumene (**7a**)	0.28 (±0.04)	1.25 (±0.09)	2.40 (±0.25)	5.08 (±0.44)	3.28 (±0.09)
isotricyclohumene (**7b**)	0.44 (±0.03)	1.66 (±0.10)	2.77 (±0.18)	4.17 (±0.38)	4.47 (±0.29)
isotricycloadhumene (**7c**)	0.12 (±0.03)	0.47 (±0.07)	0.94 (±0.08)	1.63 (±0.17)	1.14 (±0.15)
tetracyclocohumol (**8a**)	0.42 (±0.02)	1.95 (±0.13)	3.23 (±0.15)	4.31 (±0.36)	3.04 (±0.29)
tetracyclohumol (**8b**)	0.65 (±0.03)	3.05 (±0.30)	4.29 (±0.18)	3.98 (±0.32)	4.16 (±0.35)
tetracycloadhumol (**8c**)	0.14 (±0.01)	0.65 (±0.01)	1.05 (±0.04)	1.17 (±0.01)	0.95 (±0.01)
epitetracyclocohumol (**9a**)	0.08 (±0.02)	0.57 (±0.04)	1.17 (±0.04)	1.58 (±0.12)	1.18 (±0.11)
epitetracyclohumol (**9b**)	0.36 (±0.04)	1.42 (±0.11)	2.38 (±0.22)	2.36 (±0.07)	2.11 (±0.12)
epitetracycloadhumol (**9c**)	nq	nq	nq	nq	nq
trans-cohumulinic acid (**10a**)	0.10 (±0.01)	0.14 (±0.01)	0.24 (±0.02)	0.51 (±0.07)	0.60 (±0.06)
cis-cohumulinic acid (**11a**)	0.25 (±0.00)	0.54 (±0.04)	1.14 (±0.04)	2.36 (±0.16)	5.15 (±0.40)
trans-humulinic acid (**10b**)	0.12 (±0.01)	0.17 (±0.01)	0.22 (±0.03)	0.30 (±0.03)	0.54 (±0.01)
cis-humulinic acid (**11b**)	0.52 (±0.02)	1.05 (±0.06)	1.45 (±0.08)	1.91 (±0.15)	6.79 (±0.47)
trans-adhumulinic acid (**10c**)	0.09 (±0.01)	0.11 (±0.01)	0.16 (±0.02)	0.35 (±0.04)	0.47 (±0.01)
cis-adhumulinic acid (**11c**)	0.14 (±0.01)	0.26 (±0.02)	0.48 (±0.05)	0.83 (±0.11)	1.58 (±0.12)
hydroperoxy-*trans*-alloisocohumulone (**14a**)	0.04 (±0.01)	0.03 (±0.01)	<0.01	<0.01	<0.01
hydroperoxy-*cis*-alloisocohumulone (**15a**)	0.14 (±0.01)	0.17 (±0.01)	0.21 (±0.06)	0.16 (±0.03)	0.01 (±0.01)
hydroperoxy-*trans*-alloisohumulone (**14b**)	0.09 (±0.02)	0.05 (±0.01)	0.01 (±0.01)	<0.01	<0.01
hydroperoxy-*cis*-alloisohumulone(**15b**)	0.21 (±0.02)	0.22 (±0.04)	0.22 (±0.05)	0.12 (±0.02)	0.01 (±0.01)
hydroperoxy-*trans*-alloisoadhumulone (**14c**)	<0.01	<0.01	<0.01	<0.01	<0.01
hydroperoxy-*cis*-alloisoadhumulone (**15c**)	0.05 (±0.01)	0.05 (±0.01)	0.06 (±0.01)	0.04 (±0.01)	<0.01
hydroxy-*trans*-alloisocohumulone (**16a**)	0.02 (±0.01)	0.04 (±0.01)	<0.01	<0.01	<0.01
hydroxy-*cis*-alloisocohumulone (**17a**)	0.11 (±0.01)	0.43 (±0.03)	0.54 (±0.02)	1.00 (±0.07)	0.02 (±0.01)
hydroxy-*trans*-alloisohumulone (**16b**)	0.04 (±0.01)	0.06 (±0.05)	0.02 (±0.02)	0.02 (±0.01)	0.03 (±0.02)
hydroxy-*cis*-alloisohumulone (**17b**)	0.18 (±.02)	0.64 (±0.04)	0.61 (±0.02)	0.83 (±0.05)	0.02 (±0.01)
hydroxy-*trans*-alloisoadhumulone (**16c**)	nq	nq	nq	nq	nq
hydroxy-*cis*-alloisoadhumulone (**17c**)	0.04 (±0.01)	0.12 (±0.01)	0.15 (±0.01)	0.26 (±0.02)	0.01 (±0.01)
colupulone (**18a**)	0.07 (±0.01)	0.06 (±0.01)	0.03 (±0.01)	<0.01	<0.01
lupulone (**18b**)	0.05 (±0.01)	0.06 (±0.01)	0.03 (±0.01)	<0.01	<0.01
adlupulone (**18c**)	0.02 (±0.01)	0.02 (±0.01)	0.02 (±0.01)	<0.01	<0.01
cohulupone (**19a**)	1.60 (±0.09)	2.34 (±0.15)	2.34 (±0.25)	5.50 (±0.50)	2.48 (±0.16)
hulupone (**19b**)	0.94 (±0.04)	1.22 (±0.03)	1.00 (±0.07)	1.52 (±0.04)	1.57 (±0.05)
adhulupone (**19c**)	0.41 (±0.19)	0.52 (±0.01)	0.49 (±0.20)	1.19 (±0.78)	0.56 (±0.16)
hulupinic acid (**20**)	0.07 (±0.01)	0.05 (±0.01)	0.06 (±0.01)	0.43 (±0.15)	nd

Table 2. Continued

compd (no.)[a]	concn [μmol/L] in beer sample[b]				
	I	II	III	IV	V
xanthohumol (**30**)	0.03 (±0.01)	0.07 (±0.01)	0.08 (±0.01)	0.06 (±0.01)	0.05 (±0.01)
isoxanthohumol (**32**)	3.17 (±0.13)	4.23 (±0.04)	6.47 (±0.44)	5.34 (±0.11)	2.18 (±0.08)
8-prenylnaringenin (**33**)	0.03 (±0.01)	0.04 (±0.01)	0.06 (±0.01)	0.02 (±0.01)	nd
6-prenylnaringenin (**34**)	0.03 (±0.01)	0.06 (±0.01)	0.08 (±0.02)	0.03 (±0.01)	nd

[a] Chemical structures are given in Figures 1−3. [b] Concentrations (± standard deviation) are given as the mean of triplicates in fresh Pilsner-type beer D before (I) and after storage for 8 months at 28° C in a glass bottle (II), Pilsner-type beer stored for 4 years at 20° C in a glass bottle (III), Pilsner-type beer stored for 10 years at room temperature in a can (IV), Pilsner-type beer stored for 4 years at room temperature in a PET-bottle (V); nd, no signal detected; nq, not quantified due to coelution with other compounds.

scorpiohumol (**36b**) from *trans*-isohumulone (**2b**). Autoxidation of **2b** reveals the hydroperoxy-*trans*-alloisohumulone (**14b**), which is further degraded by a Fenton-type reaction to give its alkoxy radical (a) and, further on, the hydroxy-*trans*-alloisohumulone (**16b**) as reported recently.[26] Cyclization of the alkoxy radical (a) gives an epoxy intermediate (b) which, after rearrangement as well-known in fatty acid autoxidation, followed by an additional peroxidation, reveals the hemi ketal c. Release of one molecule of acetone from c, followed by enolization, gives the β-oxo-aldehyde d, which undergoes a proton-catalyzed cyclization in analogy to the formation of the tricycles **5b**−**7b**[23] to give the carbocation e. After addition of a molecule of water, followed by intramolecular ring closure, the hemiacetal tricyclohumolactol (**35b**) is formed. The isotopic distribution found for tricyclohumolactol (**35b**) in the ^{18}O-labeling experiment indicating the stable incorporation of up to three ^{18}O atoms is well in line with the proposed formation pathway as both the keto and the aldehyde function in intermediate d will undergo partial ^{16}O/^{18}O exchange and carbocation e reacts selectively with $H_2{}^{18}O$ when the reaction is performed in $H_2{}^{18}O$.

For the formation of scorpiohumol (**36b**), hydroxy-*trans*-alloisohumulone (**16b**) undergoes a proton-catalyzed carbonyl-ene reaction as found for the transformation of *trans*-iso-α-acids (**2**) into the tri- and tetracyclic compounds **5**−**9**.[22,23] This initial cyclization transforms the keto function of the *iso*-hexenoyl chain to a hydroxyl moiety in which the oxygen atom is not able to undergo ^{16}O/^{18}O-exchange with the solvent water anymore. As previously reported for the tricyclic molecules **5**−**7**, this cyclization leads to an incorporation rate of 30% of ^{18}O in the hydroxyl function formed.[23] The resulting carbocation (f) adds one molecule of water to give the tertiary alcohol (g), thus leading to the incorporation of one ^{18}O atom (100%) in the ^{18}O-labeling experiment. Proton-catalyzed elimination of water from the allylic alcohol, followed by the nucleophilic attack of the tertiary hydroxyl group, gives rise to scorpiohumol (**36b**).

LC−MS/MS Screening of Hop-Derived Sensometabolites in Aged Beer Samples. Aimed at detecting the individual hop-derived sensometabolites with high selectivity using the multiple reaction monitoring (MRM) mode, sample solutions of the individual analytes were repeatedly injected into the tandem mass spectrometer to determine the pseudomolecular ion ($[M - H]^-$) as well as the daughter ions in full scan mode in the range from 100 to 500 amu. Upon flow injection of the reference substances, the instrument settings were optimized between −120 and −50 for the declustering potential, between −52 and −22 for the collision energy, and between −17 and −7 for the cell exit potential (Table 1), thus enabling the maximization of the product ion intensity.

By direct injection of the degassed beer sample it was possible to detect both the major and the minor bitter compounds in beer within a single HPLC−MS/MS-MRM run. The traces recording the mass transitions of 60 sensometabolites in a Pilsner-type beer stored for eight month at 28 °C are displayed in Figure 5. The compounds *cis*-alloisohumulone (**13**), desmethylxanthohumol (**31**), and the β-acid degradation products **21**−**29** were present in beer just in trace levels and were, therefore, not considered anymore in the following studies. In addition, **6c**, **9c**, **12c**, **16c**, **35**, and **36** could not be unequivocally detected due to their low concentration and/or coelution with isobaric molecules. Based on this MS-profiling method, a total of 52 sensometabolites could be successfully detected in the stored beer sample.

Quantitative Analysis of Hop-Derived Sensometabolites in Fresh and Stored Beer Samples. For quantitative analysis of the bitter compounds, the ECHO technique was used to overcome ionization caused error effects during MS analysis.[8] As preliminary studies demonstrated that the use of isoxanthohumol (**32**) as the internal ECHO standard reveals data comparable data to that found recently for a series of additional ECHO standards,[8] a sample of isoxanthohumol was injected as pseudo internal standard (**e32**) 15 min after starting of the HPLC−MS/MS analysis of the beer samples and all peak areas were referenced to its signal (Figure 5). To enable the quantitation of the sensometabolites in fresh and aged beer samples, 6-point external calibration curves were determined by means of HPLC−MS/MS revealing correlation coefficients of >0.99 for all reference compounds.

Using this methodology, the sensometabolites were quantitatively determined in brown-glass bottled fresh beer (beer D) and beer samples stored in brown-glass bottles for 8 months at 28 °C, for 4 years at 20 °C, and for 10 years at 20 °C, respectively. In order to visualize the influence of the packaging material, another beer sample was stored for 4 years at 20 °C in a PET bottle. As shown in Table 2, all five beer samples contained significant amounts of the prenylated flavonoids **30** and **32**−**34**, among which isoxanthohumol (**32**) was the quantitatively predominant representative with concentrations between 2.18 and 6.47 μmol/L. Most interestingly, even after 10 years in a brown-glass bottle or after 4 years in a PET bottle, the concentrations of the prenylated flavonoids were not drastically different from those found in the fresh beer, thus demonstrating the enormous stability of this class of compounds.

Quantitation of the β-acids (**18a**−**c**) and their degradation products, namely, hulupones (**19a**−**c**) and hulupinic acid (**20**), revealed rather low amounts of 0.02−0.07 μmol/L ($\Sigma_{\mathbf{18a-c}}$ = 0.14 μmol/L) for the nonpolar lupulone congeners and of 0.07 μmol/L for the hulupinic acid (**20**), respectively, in fresh

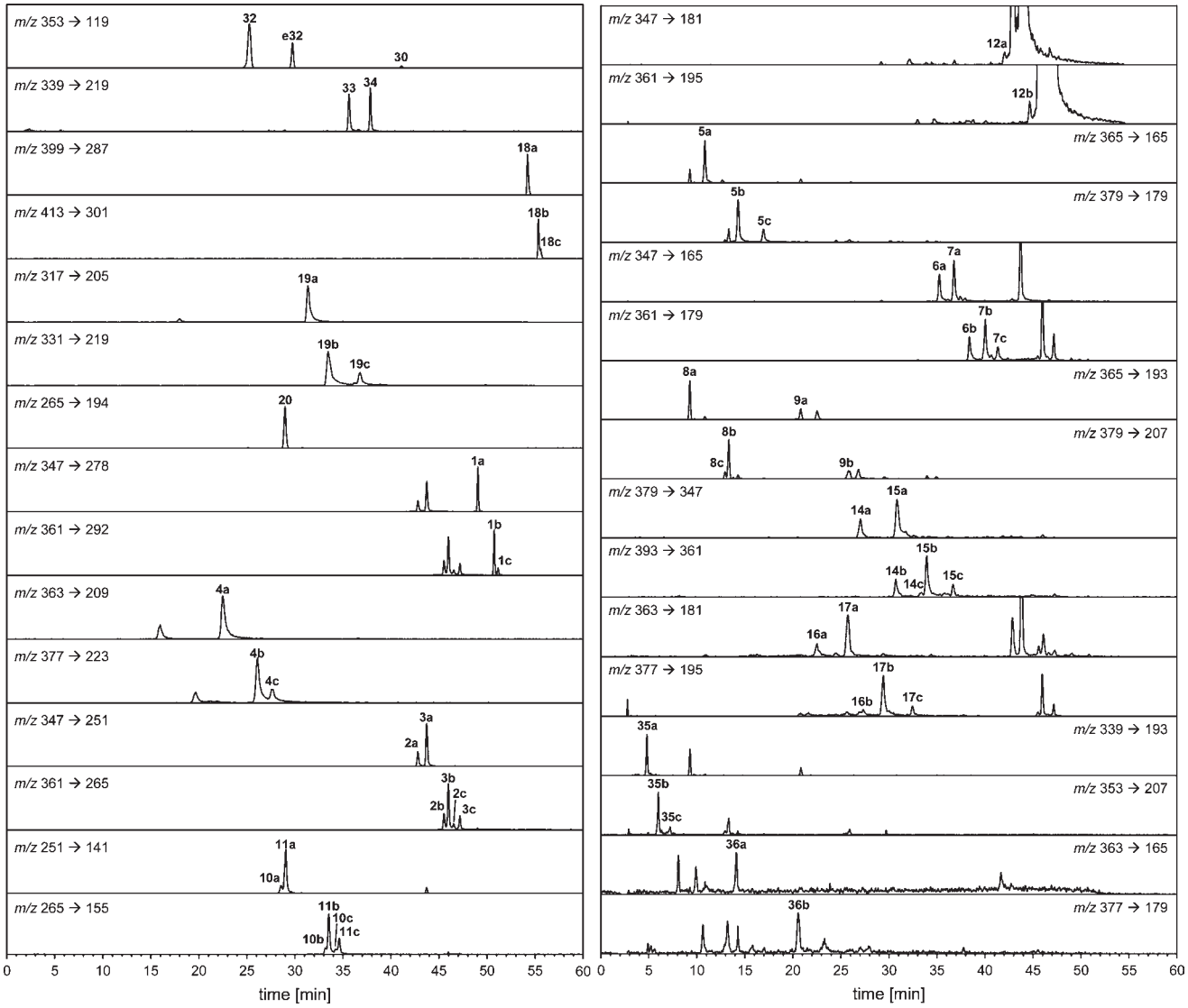

Figure 5. HPLC−MS/MS-MRM (ESI-) chromatograms of a Pilsner-type beer sample stored for 8 months at 28 °C. Signal intensity of each mass transition is normalized. Peak numbering refers to the chemical structures given in Figures 1−4.

beer sample A, while the hulupone congeners (**19a**/**b**/**c**) occur in significantly high concentrations of up to 1.6 μmol/L ($\Sigma_{\mathbf{19a-c}}$ = 2.95 μmol/L). This finding is well in line with previous studies demonstrating the high affinity of β-acids to bind to trub and filter materials during the brewing process.[9]

Compared to the β-acids (**18a**−**c**), the α-acids (**1a**−**c**) were present in fresh beer in ten times higher concentrations reaching levels of up to 0.64 μmol/L ($\Sigma_{\mathbf{1a-c}}$ = 1.12 μmol/L), being well in line with the increased water solubility of this class of polyketides (Table 2). Also the more polar α-acid oxidation products, the humulinones (**4a**−**c**), were found in levels of about 1 μmol/L ($\Sigma_{\mathbf{4a-c}}$ = 2.25 μmol/L) in the fresh beer sample. Although the concentrations of humulinones (**4a**−**c**) are two times above those of the parent α-acids, the humulinones account for only about 2% of the levels of the iso-α-acids (**2**, **3**), the major transformation products of α-acids. This result confirms that α-acids are more prone to isomerization than to oxidation during the brewing process.

With concentrations of 27 and 75 μmol/L, respectively, the *trans*- (**2a**−**c**) and *cis*-iso-α-acids (**3a**−**c**) were confirmed as the quantitatively dominating group of hop-derived phytochemicals ($\Sigma_{2,3}$ = 102 μmol/L) among all hop-derived bitter compounds in beer (Table 2). The trans/cis-ratio of the iso-α-acids in the fresh beer was 0.36 matching the value typically found for fresh beer samples. Being well in line with literature data,[15] this ratio is strongly reduced in the stored beer sample. After storing the beer for 8 months at 28 °C, the trans/cis-ratio decreased to 0.14. The beers stored for 4 and 10 years contained only trace amounts of *trans*-iso-α-acids, whereas the corresponding cis-isomers were found to be only slightly reduced. Most interesting, the beer sample stored for 4 years within a PET-bottle contained only trace amounts of *trans*- as well as *cis*-iso-α-acids. These data indicate a nonstereospecific oxidative degradation of the iso-α-acids when the beer is maintained in oxygen permeable PET bottles.[26]

Interestingly, noticeable amounts of the *trans*-specific iso-α-acid degradation products **5**−**11** were already detectable in this beer, e.g. a total amount of 7.9 μmol/L was found for compounds **5**−**11**, among which tricyclohumol (**5b**) and tricyclocohumol (**5a**) were the major representatives with concentrations of 1.6 and 1.0 μmol/L (Table 2). In comparison, a

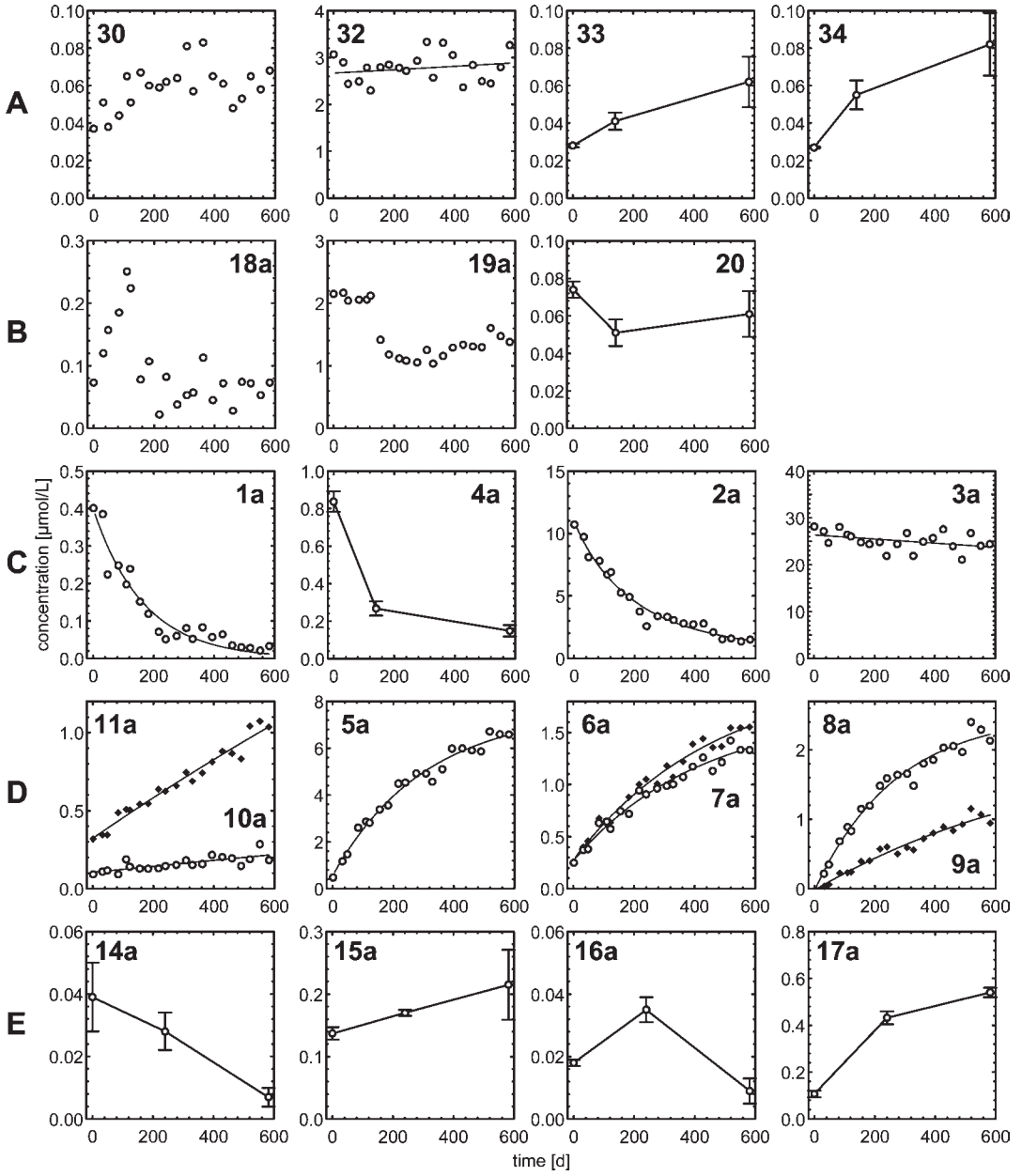

Figure 6. Influence of time on the concentrations of hop-derived bitter sensometabolites during storage of beer at 28 °C. (A) prenylated flavonoids, (B) β-acids and their transformation products, (C) α-acids and their transformation products, (D) iso-α-acids and their proton-catalyzed transformation products, (E) iso-α-acid autoxidation products; compound numbering refers to the chemical structures given in Figures 1−3.

rather low amount of 0.52 µmol/L was found for the sum of the hydroperoxy-alloisohumulones (**14a−c**, **15a−c**) formed upon iso-α-acid oxidation. Also the hydroxy-alloisohumulones (**16a−c**, **17a−c**) were present in this fresh beer in only rather low concentrations, e.g. a total amount of 0.39 µmol/L was determined (Table 2). Upon storage, the most drastic increase was found for the concentrations of the *trans*-specific iso-α-acid degradation products **5−11** (Table 2). Upon storage for 8 months at 28 °C, the total amount of **5−11** raised from 7.9 µmol/L in beer sample A to 29.3 µmol/L, that is more than 40% of the iso-α-acids present in that beer sample. Among these transformation products, tricyclocohumol (**5b**) was found as the quantitatively predominating compound with concentrations of 7.6 µmol/L in the beer stored for 8 month at 28 °C, for 4 years at 20 °C, and for 10 years at 20 °C, respectively. Interestingly, the beer sample stored in the oxygen-permeable PET bottle contained similar levels of **5b**, thus demonstrating the oxidative stability of this transformation product.

On the basis of these data, it can be concluded that the *trans*-specific iso-α-acid degradation products **5−9** are the most abundant aging induced transformation products (Σ_{5-9} = 25.3 µmol/L) in the beer sample stored for 8 months at 28 °C, while the oxidation products **14−17** reached only a total amount of 1.8 µmol/L and the humulinic acids **10** and **11** accounted for only 2.3 µmol/L.

Influence of Storage Time on the Sensometabolites in Pilsner-Type Beer. In order to gain a more detailed insight into the kinetics of the storage-induced changes of hop-derived sensometabolites, a series of beer samples, collected every two weeks from a full-scale beer manufacturing line, were collected

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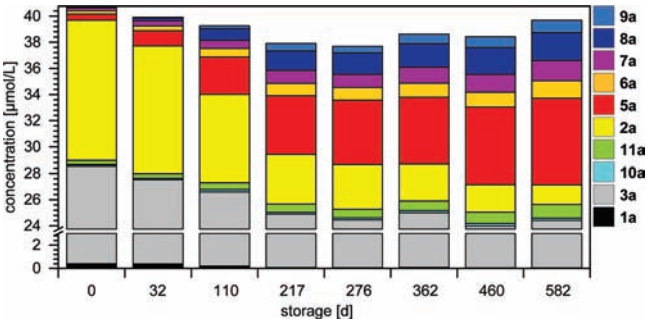

Figure 7. Staggered plot showing the quantitative balance of the hop-derived sensometabolites cohumulone (**1a**), *trans*-isocohumulone (**2a**), and *cis*-isocohumulone (**3a**), and their transformation products tricyclocohumol (**5a**), tricyclocohumene (**6a**), isotricyclocohumene (**7a**), tetracyclocohumol (**8a**), epitetracyclocohumol (**9a**), *trans*-cohumulinic acid (**10a**), and *cis*-cohumulinic acid (**11a**) during storage at 28 °C.

during a time period of 582 days and maintained under forced-aging conditions at 28 °C in the dark until analysis. Using the HPLC−MS/MS method reported above, the entire set of sensometabolites was quantitatively determined in each individual sample. Since preliminary quantitative studies did not show any significant influence of storage on the individual congeners forms of a compound class, only the cocongeners are presented in Figure 6. The amount of the prenylated flavonoids **30** and **32**−**34** were found to increase slightly over the 582 days of storage (Figure 6A), thus providing evidence for a enormous stability of these molecules during storage of beer. Also β-acids such as **18a** and the corresponding transformation products **19a** and **20** did not show any significant trend upon storage (Figure 6B). Since even the beer sample stored for 4 years (Table 2) still contained these molecular species (**18**−**20**) in concentrations matching those of the fresh beverage, these compounds seem to be rather stable during storage of beer. In comparison, the levels of the α-acids such as cohumulone (**1a**) were found to decrease strongly during beer aging (Figure 6C). From the experimental data obtained for **1a**, a reaction order of $n = 1.3$ could be deduced and thus implying a half-life of **1a** of $t_{1/2} = 100$ days. Also the transformation products cohumulinone (**4a**), formed upon oxidative degradation of **1a**, and *trans*-isocohumulone (**2a**), formed upon isomerization of **1a**, depleted upon storage to a rather similar extend. With a half-life of $t_{1/2} = 140$ days (reaction order $n = 1.3$), compound **2a** turned out to be only slightly more stable than its precursor **1a**, e.g. only small amounts were left after the 582 days (Figure 6C). In contrast, the concentration of the epimeric *cis*-isocohumulone (**3a**) was hardly reduced upon the storage time of 582 days. By extrapolation, a half-life of approximately 10 years could be estimated for **3a**.

Besides the pronounced decay of the *trans*-iso-α-acids, the transformation products, exemplified for the cocongeners **5a**−**9a**, were observed to be generated to a comparable extent (Figure 6D). Following a logarithmic function, the concentration of these compounds was increasing and approximated a maximum level after 582 days of storage, thus demonstrating that the tri- (**5**−**7**) and tetracyclic degradation products (**8**, **9**) are solely formed from the *trans*-iso-α-acids (**2**). Since these compounds did not show any decay in concentration with increasing storage time and even the 10 years aged beer sample contained high amounts of these substances (Table 2), the transformation products **5**−**11** seem to be rather stable end products of the reaction pathway. In contrast to the latter compounds, the *trans*- and *cis*-humulinic acids such as the cocongeners **10** and **11** still showed a strong increase even after the *trans*-iso-α-acid was almost fully consumed after 582 days of storage. This observation is well in line with the fact that **10** and **11** are formed from both *trans*- (**2**) and *cis*-iso-α-acids (**3**). Even after *trans*-isocohumulone is almost completely consumed after 582 days, the cis-isomer could still function as precursor of **10** and **11**.

Quantitative analysis of iso-α-acid autoxidation products such as **14a**−**17a** revealed that the freshly brewed beer contained about 0.04 μmol/L hydroperoxy-*trans*-alloisocohumulone (**14a**) and 0.14 μmol/L of the corresponding cis-isomer (**15a**) (Figure 6E). Upon storage, a decrease of **14a** was found to go along with a slight increase of **15a**. Since the initial trans/cis-ratio (**14a**/**15a**) of 0.29 was only slightly smaller than the trans/cis-ratio of 0.39 found for the iso-α-acids, rather similar relative formation rate might be assumed. However, compound **15a** accumulated during storage, while the isomer **14a** degraded faster as it formed, thus indicating a favored deterioration of the trans-isomer (**14a**). By comparison of the corresponding hydroxy-alloisocohumulones **16a** and **17a**, it became evident that again the cis-configumer **17a** accumulated during storage, while the trans-isomer **16a** decreased. Although **17a** reached the highest concentration within this group of compounds and, in consequence, was seemingly more stable than its precursor **15a**, the corresponding trans-isomers (**14a**, **16a**) occurred in almost equal concentrations, respectively.

In order to evaluate whether the considered transformation products account for the observed decay of *trans*-iso-α-acids, the total amount of the individual molecular cospecimens **1a**, **2a**, **3a**, and **5a**−**11a** were calculated for selected time points of the storage experiment (Figure 7). The data clearly show for the first time that the total amount of ∼40 μmol/L found for the sum of the individual molecules **1a**, **2a**, **3a**, and **5a**−**11a** remained rather constant during the 582 days of beer storage. In consequence, it can be concluded that **1a** and **5a**−**11a** are the quantitatively predominant key transformation products formed from iso-α-acid during storage of beer.

Hierarchical Cluster Analysis and Multivariate Statistics of Fresh and Stored Pilsner-Type Beers. In order to show that the observed behavior is not a unique phenomenon of a certain beer brand, ten commercially available Pilsner-type beer samples (A−J) were quantitatively analyzed in triplicate for the bitter sensometabolites (see Table S1 in the Supporting Information). To examine the multivariate distances between the individual sensometabolites throughout beer storage, the concentrations determined for each compound in ten beer samples before (samples A_0−J_0) and after storage for 8 months at 28 °C (samples A_8−J_8) (Figure 8) were scaled and a hierarchical cluster analysis was performed on the basis of these data. The results were visualized in a sensomics heatmap that was combined with hierarchical agglomerative clustering of the selected sensometabolites **1a**−**3a**, **5a**−**11a**, **18a**, **19a**, **30**, and **32** (Figure 8). The cluster analysis quantifies the degree of similarity between the sensometabolites as well as the 20 beer samples, respectively, by calculating the distance between all possible pairs of molecules and samples. As clustering was performed based on Ward's method,[38] the distance between two clusters was calculated as the increase of the sum of squares after merging the clusters, followed by the formation of clusters while keeping the sum of squares as small as possible. For example, the two most similar sensometabolites were then grouped together and the distance measure recalculated. This iterative process was continued until

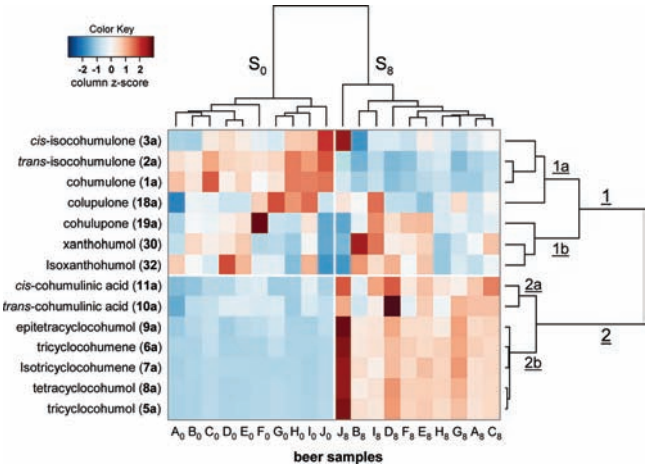

Figure 8. Sensomics heatmap calculated from quantitative data of ten commercially available Pilsener-type beer samples before (samples A_0−J_0) and after storage for 8 months at 28 °C (samples A_8−J_8) after scaling of the sensometabolite concentrations of all beer samples (see Supporting Information). The dendrograms are based on Ward's agglomerative linkage algorithm.[38].

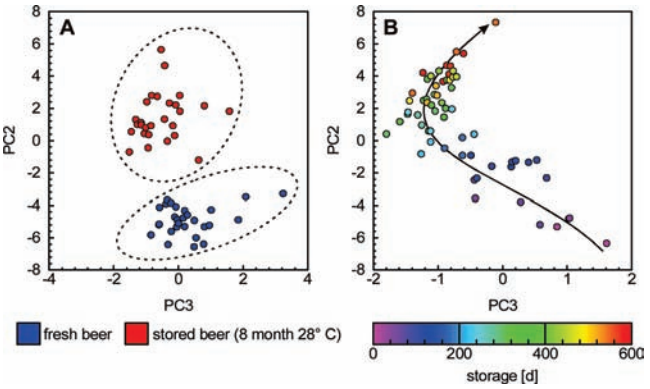

Figure 9. Principal component analysis (PCA) of fresh and stored beer samples using the quantitative data determined for compounds **1a**−**3a**, **5a**−**11a**, **18a**, and **19a**; scatterplots of PC2 versus PC3 are depicted for the comparison of beer samples before and after storage for 8 months at 28 °C (A) and for beer samples stored for up to 582 days (B).

all sensometabolites were members of a single cluster. This resulting hierarchical clustering is visually displayed as a dendrogram (Figure 8). The closer the sensometabolites are to each other in the dendrogram, the smaller the differences in their concentration patterns throughout the storage.

The hierarchical analysis arranged the 20 beer samples into two large clusters and clearly separated the fresh beer samples (S_0) and the 8 months aged samples (S_8). Also the individual sensometabolites were arranged into the two large clusters, labeled 1 and 2 (Figure 8). Cluster 1 consisted of the *cis*- and *trans*-isocohumulone (**3a**, **2a**), cohumulone (**1a**), and the colupulone (**18a**), all of which showed an aging induced decrease in concentration and, therefore, were grouped in the subcluster 1a. Cohulupone (**19a**), xanthohumol (**30**), and isoxanthohumol (**32**) did not followed a clear trend upon storage and were grouped in subcluster 1b.

Cluster 2 comprised all the transformation products generated upon beer aging. The cleavage products *cis*- and *trans*-cohumulinic acid (**11a**, **10a**) were separated in the subcluster 2a, whereas the series of tri- (**5a**−**7a**) and tetracyclic degradation products (**8a**, **9a**), formed upon proton-catalyzed cyclization of **2a**, clustered in 2b (Figure 8). The latter compounds (**5a**−**9a**) were present in the cluster of the fresh beer samples (S_0) only in small amounts and strongly increased in concentration upon storage (S_8). In particular, the beer sample J showing the highest level of *trans*-isocohumulone (**2a**) prior to storage (S_0) contained by far the highest concentrations of tri- (**5a**−**7a**) and tetracyclic degradation products (**8a**, **9a**) after storage (S_8). In contrast, aging of sample D induced the formation of rather high levels of the cleavage products *cis*- and *trans*-cohumulinic acid (**11a**, **10a**).

In addition to the sensomics heatmapping, further multivariate statistics were carried out using a principal component analysis (PCA) to study the complex relationship of the selected components **1a**−**3a**, **5a**−**11a**, **18a**, **19a**, **30**, and **32** analyzed in duplicate in 27 beer samples before and after forced aging for 8 months at 28 °C (Figure 9). By means of PCA the variables of this highly correlated data set are transformed by rotation to new uncorrelated variables with a maximized total variance. Herein, each principal component (PC) is based on a linear combination, whereas the respective factors are called loadings. For detailed comparison of the aging behavior of different beer samples, the coefficients matrices of the samples were calculated. By plotting the coefficients of different principal components a distinction could visually been drawn. While PC1 (81% of the total variance) was determined mainly by variation of the initial composition of the hop compounds, the aging induced changes were better represented by PC2 (15%) and PC3 (2%) (see Figure S1 in the Supporting Information). Although all these 54 beer samples differed significantly in their composition, the fresh beer samples were clearly separated from the stored samples when PC2 was plotted versus PC3 (Figure 9A), thus demonstrating this procedure as a suitable tool for characterizing the aging state of Pilsner-type beer. Figure 9B displays the PC2/PC3 plot for one individual Pilsner beer sample throughout 582 days of storage, thus visualizing how the principal components are changing during storage.

Influence of the pH Value on the Sensometablites in Fresh and Stored Beer. As recent molecular studies demonstrated a clear pH influence on the degradation kinetics of *trans*-iso-α-acids,[23] the question arose as to whether the pH value is decreasing during aging of beer. Therefore, the pH value was measured in the beer samples throughout 582 days of forced aging at 28 °C directly after opening the bottles and degassing. Within a natural variation of less than 0.1 unit, the pH value in these beer samples was extraordinarily constant over 582 days of aging (Figure 10A), thus suggesting, besides storage temperature and time, the initial pH value of a beer to be crucial for *trans*-iso-α-acid stability.

In order to study the range of initial pH values found in fresh commercial Pilsner-type beers, fresh samples (A_0−J_0) and 8 month (28 °C) aged samples (A_8−J_8) from ten different Pilsner-beer brands were selected, and the pH values of A_0−J_0 were determined as the means of triplicates and correlated against the decrease of *trans*-isocohumulone (**3a**) determined in percent from the concentrations of **3a** in the fresh (A_0−J_0) and aged samples (A_8−J_8). The linear correlation, shown in Figure 10B, gives evidence that already the small differences found for the initial pH value of commercial beers (pH 4.30−4.55) influence their bitter taste stability and that the stability of *trans*-isocohumulone is increasing with increasing pH value.

As proof of principle, brewing trials with subsequent adjustment of the pH value to 4.2, 4.3, and 4.4 were conducted and,

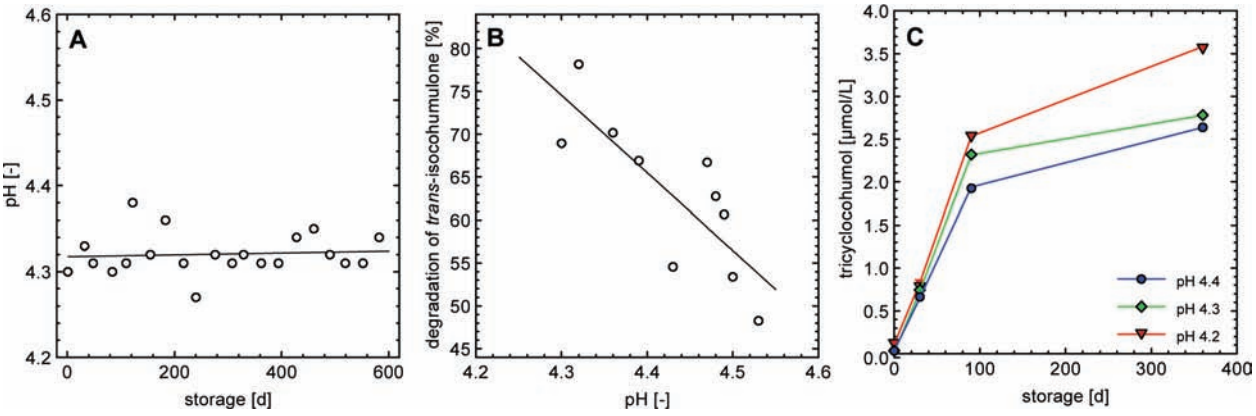

Figure 10. (A) Influence of storage at 28 °C on the pH value of a Pilsner-type beer (sample D); (B) correlation of storage-induced *trans*-isocohumulone degradation and pH values measured for ten commercial Pilsner-type beer samples (A-J); (C) increase of tricyclocohumol (**5a**) during storage of beer samples slightly differing in the pH value (20 °C).

after storage for up to 360 days at 20 °C, samples were withdrawn and analyzed for the bitter transformation products. As an example, the influence of pH value and storage time on the concentration of tricyclocohumol (**5a**) is depicted in Figure 10C. Although the pH difference between the beer samples is only 0.1, the three beer samples showed distinct differences in the tricyclocohumol concentration already after 90 days of storage. After 360 days the concentrations of **5a** in the samples, which were adjusted to pH 4.4 and pH 4.2 prior to storage, differed by 0.8 μmol/L and, therefore, confirmed that a more acidic pH values triggers the degradation of trans-iso-α-acids during beer storage.

In conclusion, quantitative profiling of the sensometabolites contributing to the bitter taste of beer revealed a comprehensive insight into the transformation of hop-derived bitter compounds during storage of beer. The time and temperature dependent, proton-catalyzed cyclization of *trans*-iso-α-acids (**2**) was identified to be the quantitatively predominant reaction leading to lingering, harsh bitter tasting tri- and tetracyclic compounds (**5**–**9**) upon storage of beer. Due to their stability, these transformation products, such as, e.g., tricyclocohumol (**5a**), are proposed as suitable indicator molecules to analytically monitor the aging status of beer. The sensomics data obtained during this study offers the scientific basis for a knowledge-based optimization of the beer bitter taste by controlling the initial pH value of the beer by technological means and by keeping the temperature as low as possible during storage of the final beverage.

■ ASSOCIATED CONTENT

Supporting Information. Principal component analysis of fresh and stored beer samples and concentrations of selected sensometabolites in beer samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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