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Aroma Active Monocarboxylic Acids – Origin and Analytical Characterization in Fresh and Aged Hops

During hop storage secondary metabolites, primarily bitter acids, terpene hydrocarbons and terpenoids, undergo qualitative and quantitative changes. One reaction that influences both – bitter properties and aroma – is the release of extremely flavour active short chain monocarboxylic acids by cleavage of the acyl-side chain of bitter substances. Short chain acids exhibit very intense unpleasant cheesy, sweaty, and rancid smells, however they are key precursors of highly positive flavourings of late hopped beer.
In the current paper a stable isotope dilution assay for precise quantification of short chain carboxylic acids was integrated into conventional hop oil analysis. Short chain acids, terpene hydrocarbons, terpenoids, and hop bitter substances where analyzed in fresh as well as in moderately and excessively aged hop samples.

Descriptors: hop aroma, bitter acid degradation, ester formation, mass spectrometry

1 Introduction

Hop has remarkable impact on the final beer flavour and hop pronounced beers are enjoying rising popularity. During the past decades extensive research has been performed to unravel the secrets of hop aroma by identification of the high impact odorants of hop essential oil [e.g. 1, 2, 3, 4]. Recent publications concentrate on identifying key odorants of hoppy beer flavour, as well as measures for controlled and reproducible enrichment of hop derived volatiles within beer production [e.g. 5, 6, 7, 8].

Today, the major challenge remains that both – hop aroma and hoppy beer flavour – differ strongly. Discrepancy is caused by the fact, that the constituents of the hop essential oil are not quantitatively transferred into beer, and that most of them undergo chemical modification and/or biochemical conversion during beer processing. The majority of hop pronounced beers is produced by adding at least two hop dosages: The first hop addition is performed for bittering purposes at the beginning of the kettle boil. Hop bitter acids dissolve in the hot wort, α-acids isomerise into water soluble and bitter tasting iso-α-acids, which are transferred into bright beer. For most hop aroma substances are evaporated during kettle boil, so called late and dry hopping techniques are applied. Late hopping is the addition of (a second portion) of hops at the end of the boil or during whirlpool rest, in dry hopping aroma hops is added in the cold phase of beer production.

Both hopping procedures aim an effective enrichment of hop flavourings, whereas the aroma profiles of late and dry hopped beers differ considerably [9, 10]. In late hopping several hop essential oil constituents are oxidized and create spicy, herbal, or woody flavours [11]. In dry hopping hop aroma compounds are basically extracted, some are converted by action of yeast enzymes [12, 13, 14]. Yeast action on volatiles or their non-volatile precursors (i.e. glycosides) produces a variety of compounds accounting for fruity and floral characteristics of dry hopped beers.

The biotransformation of principal hop derived aroma compounds in fermentations with *Saccharomyces cerevisiae* are as follows:

- carbonyl compounds are reduced to alcohols [e.g. 15, 16]
- ethers undergo reduction into e.g. diols [e.g. 17]
- monoterpene alcohols isomerize [e.g. 12, 13, 14]
- glycosidically bound aroma precursors are released [e.g. 18, 19, 20]
- esters are *trans*-esterified or hydrolysed [e.g. 21, 15]
- acids are converted into (ethyl) esters [e.g. 22, 23, 24]

1.1 Formation of hop derived ethyl esters

Fermentation of hopped wort yields numerous esters absent in beers derived from fermentation of unhopped wort. The five principal esters arising from fermentation of *Saccharomyces cerevisiae* upon hopped wort are ethyl 3-methylbutanoate, ethyl 2-methylbutanoate, ethyl 2-methylpropionate, ethyl 4-methylpentanoate, and 2-phenethyl 3-methylbutanoate [22–24]. *Kishimoto* [23] and *Nielsen* [24] proposed, simultaneously but self- contained, that these esters are produced by esterification of hop derived short chain monocarbo-

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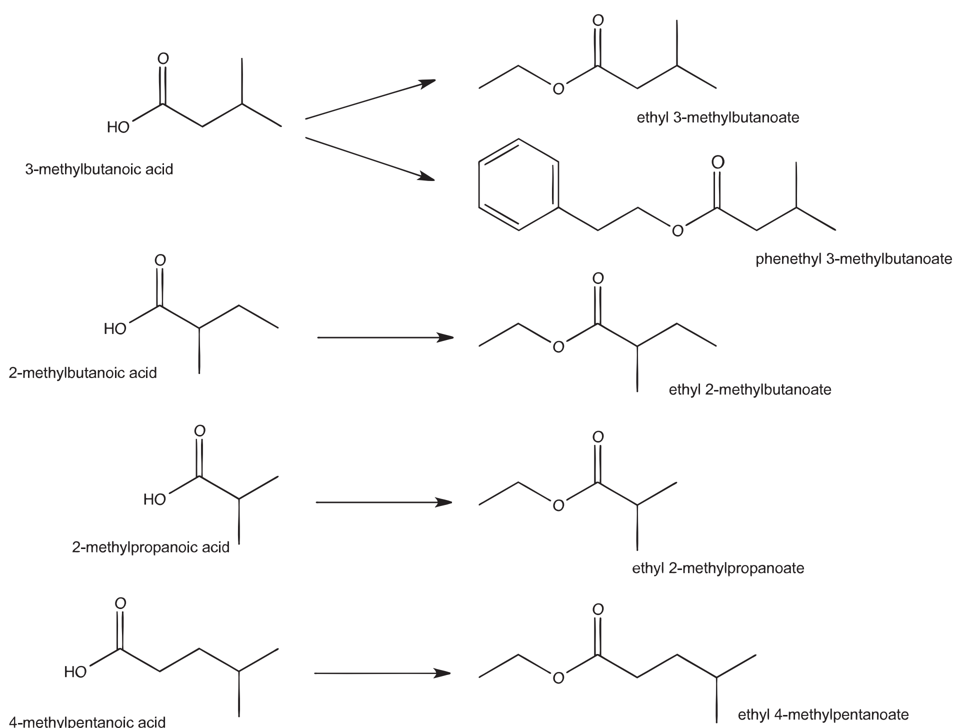

Fig. 1 Esterfication of short chain carboxylic acids into their corresponding ethyl esters as proposed by Kishimoto [23] and Nielsen [24]

xylic acids (cf. Fig. 1), and that those esters strongly contribute to pleasant fruity aromas of (late) hopped beers.

Both researchers agreed, that ester concentration in beer can be increased by using aged hops rich in short chain acids (cf. 1.3). In their studies short chain acids in hops were not quantified, however Nielsen found that the concentration of ethyl esters correlates with the concentration of short chain acids in beer.

Furthermore, ester concentration in beer is influenced by several technological measures: Increased ester concentration was observed when hop was added after wort boiling. It was hypothesised, that during kettle boil short chain acids are mostly evaporated and/or removed by adsorption onto trub. During fermentation the concentration of hop derived ethyl esters increases, whereas their esterification advances after the ester synthesis of lipid derived straight chain esters has finished. The accumulation of hop derived ethyl esters was shown to proceed after diacetyl is attenuated. Own, up to date unpublished investigations show, that elevated maturation temperatures and circulation of (late hopped) end fermented beer is very effective in increasing the formation of hop derived ethyl esters. Biochemically, there are two primary reasons for yeast catalyzed esterification. Firstly, short and medium chain monocarboxylic acids exhibit strong antimicrobial activity and are toxic to the yeast [25]. Consequently, the esterification of acids into ethyl esters is a detoxification process. Secondly, the ester formation in late fermentation and maturation reduces the acetyl charge, namely the balance between acetyl-coenzyme A and coenzyme A, which is essential for the yeast cell [26, 27].

During beer storage *Williams* and *Wagner* [28, 29] observed ester accumulation due to condensation of ethanol and organic acids. Non-yeast catalysed ester formation is rather slow, elevated ester concentrations were found when beer was subject to forced aging [30].

Ethyl 3-methylbutanoate, ethyl 2-methylbutanoate, ethyl 2-methylpropionate, ethyl 4-methylpentanoate have very intensive sweet, estery, fruity, pineapple-like odor qualities. The aliphatic ethyl esters have very low flavour thresholds in beer [31, 32, 33], also strong additive effects among these compounds were observed [34]. 2-Phenylethyl 3-methylbutanoate exhibits a floral and minty impression [22]. There is no information concerning its flavour threshold available in literature.

1.2 Compositional changes of hop essential oil upon storage

During storage of hops secondary metabolites, primarily bitter acids and volatiles (hop essential oil), undergo changes that strongly affect hop quality [35, 36, 37, 38]. Hop essential oil is an extremely complex mixture of several hundred volatiles [39]. The major volatiles of fresh hops are terpene hydrocarbons (myrcene, α-humulene, and β-caryophyllene), which account for up to 80 % of the total oil. Remaining volatiles are grouped as the so called “oxygenated fraction”, which predominately includes terpenoids, but also various esters, ketones, aldehydes, as well as traces of sulphur compounds. Basically, these volatiles are found in much lower concentrations compared to the abundant terpene hydrocarbons (factor 100–1000). Still, they have remarkable aroma characteristics (fruity, floral but also spicy, herbal, and woody notes) and are distinguished by extremely low flavour thresholds. It is agreed, that they are significant contributors to hop aroma and hoppy beer flavour [3]. The concentration of oxygenated compounds rises during ripening of the hop cone [40, 41], but also during hop processing and in the early stage of hop storage [42, 43, 44, 45, 46, 47]. The concentration of oxygenated compounds increases when hop is subject to short term storage under pro-oxidative conditions [42, 43], hence a moderate hop storage prior to brewing has been proposed to positively affect the aroma properties of the final beer [42]. Long term hop storage (several months) leads to a decrease of total volatiles (terpene hydrocarbons and oxygenated fraction) accompanied by loss of favourable aroma characteristics [15].

1.3 Oxidation of bitter acids and release of short chain monocarboxylic acids

Hop bitter substances primarily consist of two related series of structures, the α-acids (n-humulone, co-humulone, and ad-humulone) and the β-acids (lupulone, co-lupulone, and ad-lupulone). In addition, there are some minor hop acids called post-humulone and pre-humulone, or post-lupulone and pre-lupulone, respectively [48].

Prolonged hop storage at elevated temperatures (> 0 °C) and exposed to atmospheric oxygen decreases the concentration of those valuable bitter acids. Their oxidation results in multiple and aggregate structures, prominent non-volatile derivates are humu-

linic acids, humulinone, or the so called hulupones [49]. Here, the prior ranking fact is, that oxidized α-acids can no longer isomerize into iso-α-acids, whereby the bittering potential of hop decreases [50]. It was found that the oxidation of α- and β-acids is impaired by storage conditions (oxygen, temperature, duration), but also by hop variety. Differences among varieties are attributed to the antiradical activity of hops, as well as the polyphenol content. Generally, β-acids are more stable towards oxidation than α-acids [35].

Whereas a comprehensive discussion on the multiple degradation reactions of hop bitter acids is beyond the scope of this paper, one reaction has been postulated that simultaneously reduces bittering potential and fresh hop aroma: the cleavage of the acyl-side chain of bitter acids is proposed to release flavour active short chain monocarboxylic acids [51]. Degradation of n-humulone releases 3-methylbutanoic acid, 2-methylpropanoic acid results from degradation of co-humulone, 2-methylbutanoic acid from degradation of ad-humulone, 4-methylpentanoic acid from post-humulone, and hexanoic acid from pre-humulone. These bitter substance derived acids exhibit intense sour, sweaty, rancid, and cheesy odors.

In literature there is no comprehensive study focussing on the metabolism of short chain acids in aging hops, the only available data were published by *Tressl* et al. [51] more than 30 years ago: Tressl and coworkers semiquantified more than twenty monocarboxylic acids in several fresh hop varieties (namely Bullion, Hüller Bitter, Fuggles, Hersbrucker Spät, Cluster, Yakima, Northern Brewer, and Brewers Gold), as well as in fresh and aged Spalter hops. Their analysis resulted, that the concentration of methyl branched short chain monocarboxylic acids in fresh hops differed throughout the varieties. They postulated that the ratio of 2-methylpropionic and 2-/3-methylbutyric to be a varietal characteristic, that correlates with the concentration of n-humulone and co-humulone. Storage trials (3 years, 0 °C) evidenced a remarkable increase in the concentrations of 2-methylpropionic acid, 2- and 3-methylbutyric acid. Also several straight chain (saturated and unsaturated) acids such as pentanoic acid, hexanoic acid, octanoic acid, (2E)-hexenoic acid, and (3E)-hexenoic acid were traced in the aged samples. Increasing concentrations of those straight chain acids was attributed to the oxidative degradation of linoleic and linolenic acid, as well as to the cleavage of corresponding methyl esters.

1.4 Aim of the current paper

Hop derived short chain carboxylic acids are potent odorants of the hop essential oil and key precursors of high impact odorants of hopped beers. It has been shown, that hop varieties differ in their initial concentration of short chain acids, whereas increasing concentrations were observed when hop was subjected to long time storage. However, no comprehensive data set, including bitter acids, short chain acids, and other key volatiles from hop is described in literature. Data for short chain acid concentrations in moderately aged hops are missing. Also straightforward analytical tools for the identification and precise quantification of short chain acids from hops have not been described before. These facts initiated the study described herein, which followed three principal aims:

- Establish a selective analytical route to isolate short chain monocarboxylic acids from hops, provided compatibility with the analysis of terpene hydrocarbons and oxygenated flavourings of hop essential oil.
- Establish a sensitive GC-MS route and a stable isotope dilution assay for unambiguous identification and precise quantification of monocarboxylic acids from hops.
- Perform storage experiments under aerobic conditions in order to evaluate concentration changes in regard to short chain monocarboxylic acids and hop bitter substances.

Table 1 List of analytical targets and stable isotope standards including registry numbers of the Chemical Abstracts Services (CAS), formula in Hill notation, boiling point in °C, as well as the m/z (EI-MS) of quantifier and qualifier ions of the corresponding methyl esters

analyte	CAS No.	formula (Hill notation)	Bp [°C]	m/z[a]
2-methylpropanoic acid	79-31-2	$C_4H_8O_2$	155	**43**, 59, 71, 87
2-methylbutanoic acid	1730-91-2	$C_5H_{10}O_2$	176	41, **88**, 101
3-methylbutanoic acid	503-74-2	$C_5H_{10}O_2$	176	41, **74**, 101
pentanoic acid	109-52-4	$C_5H_{10}O_2$	187	43, 57, **74**, 85
4-methylpentanoic acid	646-07-1	$C_6H_{12}O_2$	199	43, **74**, 59,87
hexanoic acid	142-62-1	$C_6H_{12}O_2$	206	43, **74**, 59, 87
(2E)-hexenoic acid	13419-69-7	$C_6H_{10}O_2$	217	**55**, 87, 97, 113
heptanoic acid	111-14-8	$C_7H_{14}O_2$	222	43, 55, 59, **74**, 87
6-methylheptanoic acid	929-10-2	$C_8H_{16}O_2$	232	43, 55, 57, **74**, 87
octanoic acid	124-07-2	$C_8H_{16}O_2$	237	43, 55, **74**, 87, 158
nonanoic acid	112-05-0	$C_9H_{18}O_2$	255	43, 55, 59, **74**, 87
decanoic acid	334-48-5	$C_{10}H_{20}O_2$	268	**74**, 87, 143
(4Z)-decenoic acid	505-90-8	$C_{10}H_{18}O_2$	278	67, 69, **74**, 110, 152
undecanoic acid	112-37-8	$C_{11}H_{22}O_2$	280	43, **74**, 87, 143
dodecanoic acid	143-07-7	$C_{12}H_{24}O_2$	299	55, **74**, 87, 143
10-methylundecanoic acid	2724-56-3	$C_{12}H_{24}O_2$	305	**74**, 87, 143, 171
standards		**linear formula**	**Bp [°C]**	**m/z[a]**
^{13}C-butanoic acid	/	$CH_3(CH_2)_2{}^{13}CO_2H$	162	43, 72, **75**, 88
^{13}C-octanoic acid	/	$CH_3(CH_2)_6{}^{13}CO_2H$	237	**75**, 88, 159

[a] acids were analyzed as fatty acid methyl esters. Mass to charge (m/z) ratios of prominent ions were selected for qualification and quantification. Bold letters indicate quantifier ions

Discuss changes in brewing value and aroma profile with respect to other key odorants of hop essential oil.

2 Materials and Methods

2.1 Hop samples and storage conditions

Commercial hop pellets (type 90) of five US grown varieties from the 2012 harvest were available: Columbus was selected as a so called super high alpha variety, Chinook and Nugget as high-alpha varieties, Cascade and Mount Hood (MT Hood) as aroma hop varieties. Prior to the storage trials pellets were stored vacuum packed at –23 °C in undamaged wrappings (each 50 g, metalized polyethylene laminates, vacuum packed). For the storage trials wrappings were cut open, one set of samples was analyzed directly (fresh), one was stored at room temperature (RT, 21 °C in average), another set at 0 °C. After four and twenty for weeks α- and β-acids (cf. 2.3) were analyzed in repeat determination, terpene hydrocarbons, terpenoids, and short chain acids were analyzed in triplicate determination (cf. 2.4 and 2.5) for each variety.

2.2 Reference materials and chemicals

Short chain monocarboxylic acids examined within this study are compiled in table 1. Corresponding analytical standards were purchased in purity of ≥ 96 % from Sigma-Aldrich Chemie GmbH (D-89555 Steinheim), except for 6-methylheptanoic acid which was purchased from Alfa Aesar GmbH & Co KG A (D-76057 Karlsruhe). (4*Z*)-decenoic acid was only available in form of its corresponding methyl ester. Reference materials for the analysis of terpenes and terpenoids are listed below. If not stated elsewise they were also purchased from Sigma-Aldrich Chemie GmbH in purity ≥ 96 %: Myrcene, limonene ((+)-Carvene, ≥ 90 %), ocimene (≥ 90 %), *cis*- and *trans*-linalooloxid (2-(5-Methyl-5-vinyltetrahydro-1-furyl)-2-propanol), linalool, α-terpineol, nerol, neral, geraniol, geranial, methylgeranate, geranylacetate, ß-caryophyllene, α-humulene, geranylpropionate, methyl 6-methylheptanoate, methyl octanoate, methyl 6-methyloctanoate, and methyl decenoate.

Methyl tert-butyl ether (MTBE, analytical grade), methanol (HPLC grade), *ortho*-phosphoric acid (85 %), sodium sulfate (Na_2SO_4), and sodium-hydrogen-carbonate ($NaHCO_3$) were purchased from Carl Roth GmbH + Co. KG (D-76185 Karlsruhe). Ethylacetate (analytical grade) and diethyl ether (DEE, analytical grade) from Merck KGaA (D-64293 Darmstadt). MTBE and DEE were further purified by distillation.

2.3 Reversed-Phase- High Performance Liquid Chromatography (RP-HPLC) analysis of bitter acids

The analysis of α- and β-acids from fresh and aged hop pellets was based on Analytica-EBC 7.7 [52] using a Shimadzu LC-2010CHT Liquid Chromatograph equipped with a Bluespher C18 column (endcapped, 100 mm length, 2 mm inner diameter, pore size 100 Å, particle size 2.0 µm) from Herbert Knauer GmbH (D-14163 Berlin). Mobile phase was HPLC grade methanol, ultrapure water drawn from a Merck Millipore Synergy® UV water purification system (D-64293 Darmstadt), and *ortho*-phosphoric acid (85 %) in a ratio of 775/210/9 by volume. Injection volume was 10 µL, column oven temperature 40 °C. Isocratic elution was performed at a flow rate of 0.25 mL/min. UV-Detector was operated at a wavelength of 314 nm. Identification and quantification of α-acids (co-humulone, n- and ad-humulone) and β-acids (co-lupulone, n- and ad-lupulone) was based on comparison of retention times and peak areas with those obtained by analysis of the International Calibration Extract (ICE-3) purchased from Labor Veritas AG (CH-8002 Zürich).

2.4 Sample preparation

Isolation of volatiles from hops

Volatiles were isolated by simultaneous distillation extraction (SDE) according to a method previously described [53, 54]. The internal standards (IS) for both, essential oil and monocarboxylic acid quantification were added at the very beginning of sample preparation. Standards for the quantification terpenoids were 6-Methyl-5-[1,1,1,3,3-$^2H_5$]hepten-2-one (D_5-6-Methyl-5-hepten-2-one), 3,7-[10,10,10-$^2H_3$]Dimethyl-1,6-octadien[4,4-$^2H_2$]-3-ol (D_5-linalool), terpene hydrocarbons were quantified using 2-octanol. D_5-6-Methyl-5-hepten-2-one and D_5-linalool were synthesized according to the method of *Kreck* et al. [55], 2-octanol (purity ≥ 97 %) was purchased from Sigma-Aldrich Chemie GmbH. For quantification of the short chain carboxylic acids 1-^{13}C-butanoic acid (99 atom % ^{13}C) and 1-^{13}C-octanoic acid (99 atom % ^{13}C) were used. Both standards were purchased from Sigma-Aldrich Chemie GmbH. SDE extracts (100 mL) were concentrated to a volume of approx. 15 mL by Vigreux-distillation. An aliquot of 10 mL was extracted to enrich the short chain acids, another aliquot (100µL) was directly analyzed by GC-MS.

Isolation and derivatization of monocarboxylic acids

The extraction procedure was based on the prescript of Tressl et al. [51], modifications and especially miniaturizing have been applied: 10 mL extract from SDE were extracted thrice with each 25 mL of a saturated solution of sodium-hydrogen-carbonate ($NaHCO_3$; 7 mg/ml; pH 10). Sodium-hydrogen-carbonate phase was combined, acidified to pH 2 by addition of phosphoric acid (H_3PO_4), thereafter extracted twice with each 10 mL DEE. The DEE phase was dried over sodium sulfate and concentrated to 1 mL by Vigreux-distillation. For the GC-MS analysis an aliquot of 80 µL was transferred into a GC-vial, in order to revise separation perfomance on DB 5 ms column (cf. 2.5) monocarboxylic acids where converted into fatty acid methyl esters (FAME) by addtion of 20 µl of trimethylsulfonium hydroxide (TMSH) in methanol. Hop contains traces of fatty acid (methyl) esters which were basically removed by the LLE. However undervatized samples (blind samples) were analyzed to detect any carryover into the polar extracts. The blind samples were prepared by addition of 20 µl methanol (without derviatisation agent).

2.5 Gas chromatography – mass spectrometry (GC-MS)

GC-MS analysis was performed on Shimadzu GCMS-QP2010 Plus applying a DB 5ms column (inner Ø = 0.25 mm, 0.25 µm film thickness, length = 30 m) from Agilent Technologies (D-71034

Böblingen). GC was operated in linear flow mode (linear velocity = 40.0 cm/s), helium was used as carrier gas.

For analysis of the monocarboxylic acids (as their corresponding FAME) the injector temperature was set to 250 °C. A sample volume of 1 µL was injected in split injection mode (split 10). Column oven temperature program was set at an initial temperature of 40 °C, after 3.5 minutes of sampling time temperature was raised by a rate of 10 °C/minute until reaching 200 °C, than 70 °C/minute up to 300 °C (hold for 60 s). DB 5 ms phase showed very suitable performance to separate all target compounds, except for the methyl esters of 2- and 3-methylbutanoic acid which could be distinguished by MS (cf. Table 1). Ion source of quadrupole mass spectrometer (QP-MS) was adjusted to 200 °C, the interface temperature to 250 °C. Analyte ionization was reached by electron impact ionization (EI). For data acquisition QP-MS was operated in scan mode (m/z = 29–400, scan rate = 2500). Target identification was based on comparison of retention time and EI mass spectra with those of reference standards (cf. 2.2), mass to charge ratios (m/z) of qualifier and quantifier ions are given in table 1.

For direct analysis of the SDE extracts GC temperature program was as follows: Oven temperature program was set at an initial temperature of 60 °C, then raised by a rate of 6 °C/minute until reaching 220 °C, than by a rate of 70 °C/minute up to 300 °C (hold for 60 s). Ion source of QP-MS was adjusted to 200 °C, the interface temperature to 220 °C. Ions ranging from m/z = 29 to m/z = 400 were detected in scan mode (scan rate = 2500). Target identification was based on comparison of retention time and EI mass spectra with those of reference standards. Data evaluation focussed on terpene hydrocarbons, principal terpenoids, as well as fatty acid methyl esters. The compounds that were not available as analytical standards were identified by means of their relative retention index (R_I) and their mass spectrum in comparison with NIST 2008 database. R_I was determined according to *Kovats* [56], reference R_I were taken from the compilation published by *Adams* [57]: α-limonene-diepoxid (R_I=1132), α-bisabolene (R_I=1506), geranyl isobutyrate (R_I=1606), caryophylenne oxide (R_I=1582), humulene monoepoxid II (R_I=1608).

In sum 17 terpenoids, five terpene hydrocabons, and five methyl esters, were dircetly quantified from the SDE extract. In addtion 16 monocarboxylic acids were quantifiied from the methylated LLE extract.

2.6 Calibration and validation

Calibration

Calibration curves were established for all monocarboxylic acids (IS = 1-^{13}C- butanoic acid and 1-^{13}C-octanoic acid), terpene hydrocarbons (IS = 2-octanol), terpenoids and methyl esters (IS= D_5-6-Methyl-5-hepten-2-one and D_5-linalool), except for those compounds that were not commercially available (cf. 2.5). Quantification of those compounds was performed using the calibration function ß-caryophyllene. For calibration analytes were mixed with the respective IS in molar ratios ranging from 0.1 to 10, than diluted to match the dynamic range of MS detection. In case of the short chain acids derivatization with TMSH was performed. The coefficient of determination (R^2) was at least 0.98 for all compounds.

Recovery rate (RR)

The monocarboxylic acids focused in this study have boiling points ranging from 155 °C (2-methylpropanoic acid) to approx. 300 °C (10-methylundecanoic acid or dodecanoic acid), also the polarity of short chain (methyl branched) and medium chain (unsaturated) acids differ strongly. The water solubility of 2-methylpropanoic acid is comparably high (21 g/100mL, at 20 °C), whereas those of dodecanoic acid is very limited (0.006 g/100 mL at 20 °C). The sample preparation includes three extraction steps, thus the application of proper (stable isotope labeled) internal standards, but also detailed knowledge in regard to the extraction properties of the analytes is needed. To assure proper quantification recovery experiments where carried out: 5 g spent hops was spiked with an analyte mixture and the internal stable isotope labeled standards (1-^{13}C-butanoic acid and 1-^{13}C-octanoic acid) in ratios of 2:1, 1:1, and 1:2. SDE, LLE, derivatization and analysis were carried out as described above. Spent hops was prepared by extraction (2 * 8 hours) of commercial hop pellets with MTBE in a Soxhlet apparatus. The recovery rate was defined as: recovery rate = concentration analyte/concentration standard * 100.

Coefficient of variation (CV)

CV was determined by sixfold repeat of the whole analytical procedure for terpene hydrocarbons, terpenoids, and methyl ester (weighted sample, SDE, GC-MS analysis), as well as for the short and medium chain acids (weighted sample, SDE, LLE, derivatization, GC-MS analysis).

Limit of quantification (LOQ)

LOQ was determined by the signal-to-noise (S/N) method. The LOQ was defined as the analyte concentration by which the peak area of the quantifier ion (S) is at least by factor ten higher compared to the noise (N). For calculation of N, the signal of the quantifier ion was integrated over a distance equal to approx. 20 times of the width at half-height of the respective peak. For a detailed directive concerning the determination of RR and CV refer to *Kromidas* [58].

3 Results and discussion

3.1 Method development and validation

The enrichment of low concentrated short chain carboxylic acids from hops by SDE and LLE is an effective and reproducible multi-step sample preparation procedure: The coefficient of variation was below 14 % for all short chain monocarboxylic acids (cf. Table 3). The isolation of the polar acids by LLE of the SDE extract was very efficient, abundant terpene hydrocarbons, terpene alcohols, but also traces of fatty acid methyl esters were removed. Blind samples did not contain signals for terpenoids and methyl esters, there was only a minor carryover of myrcene, ß-caryophyllene, and α-humulene. The latter seems inevitable due

Table 2 **Mean concentrations of α- and β-acids in fresh hop pellets**

	Columbus	Nugget	Chinook	MT Hood	Cascade
α-acids [g/100g]	13.9	13.0	11.8	6.1	5.5
co-humulone [g/100g]	4.8	3.0	3.5	1.3	1.8
n- + ad-humulone [g/100g]	9.1	10.0	8.3	4.8	3.7
β-acids	4.3	4.2	2.8	5.1	5.5
co-lupulone [g/100g]	2.3	2.1	1.5	2.4	2.2
n- + ad-lupulone [g/100g]	2.0	2.1	1.3	2.7	2.2
[a] total resins[g/100g]	18.2	17.2	14.6	11.2	11.0
ratio (co-/ n-+ad-humu-lone)	0.53	0.30	0.42	0.27	0.49

[a] the total resins [g/100g] equal the sum of α- and β-acids

to the high abundance of those compounds in the SDE extracts of fresh hop samples. However, signals did not interfere with any of the qualifier and quantifier ions of the FAME, thus they did not disturb identification and quantification. The recovery experiments resulted in very acceptable RR ranging from 91–116 % for either 1-^{13}C-butanoic acid or 1-^{13}C-octanoic acid. Based on the results of the recovery experiments monocarboxylic acids with 4-6 carbons atoms where quantified using 1-^{13}C-butanoic acids, whereas acids ranging from 7–12 carbon atoms were quantified using 1-^{13}C-octanoic acid. RR reveal, that the stable isotope standards with four respectively eight carbon atoms were very suitable to cover the range of C4–C12 acids in the hop samples. The LOQ of the method was 0.1 µg analyte/g hop pellet. Similar LOQ for all analytes results from the fact that matching quantifier ions were used and that RR was similar (cf. Table 1).

3.2 Concentration of α- and β-acids in fresh and aged hop samples

The results of RP-HPLC analysis of the five pellet varieties prior to aerobic storage are shown in table 2. The total concentration of α-acids was calculated from the peak areas for co-humulone, n-humulone and ad-humulone, whereas the latter were not separated by LC. Accordingly β-acids were calculated from co-lupulone, n-lupulone and ad-lupulone signals. The total resins given in table 2 equal the sum of α-acids and β-acids.

In the fresh hop samples highest amount of total resins (18.2 g/100 g) and α-acids (13.9 g/100 g) was found in Columbus hops. Cascade contained the lowest portion of both: Total resins were 11.0 g/100 g, whereas an α-acid content of 5.5 g/100 g was found. Cascade and Columbus have similar ratios of co-humulone (approx. 0.5), whereas MT Hood and Nugget contained less co-humulone (0.27).The concentration of β-acids was in the range of 5.5 g/100 g (Cascade) to 4.2 g/100 g (Nugget).

In the storage experiments a decrease of α- and β-acids was observed across all hop varieties. The concentration (decrease) of α- and β-acids was dependent on storage temperature (0 °C vs. RT) and storage time (4 weeks vs. twenty four weeks). The comprehensive data set for α- and β-acids throughout the storage trials is visualized in figure 2. The relative changes, namely a loss of α- and β-acids compared to the initial concentration, are indicated by bar charts for all five hop varieties. The total amount of α- and β-acids is split into the respective parts for co-humulone and n- + ad-humulone (Fig. 2 a), or co-lupulone and n- + ad-lupulone (Fig. 2 b).

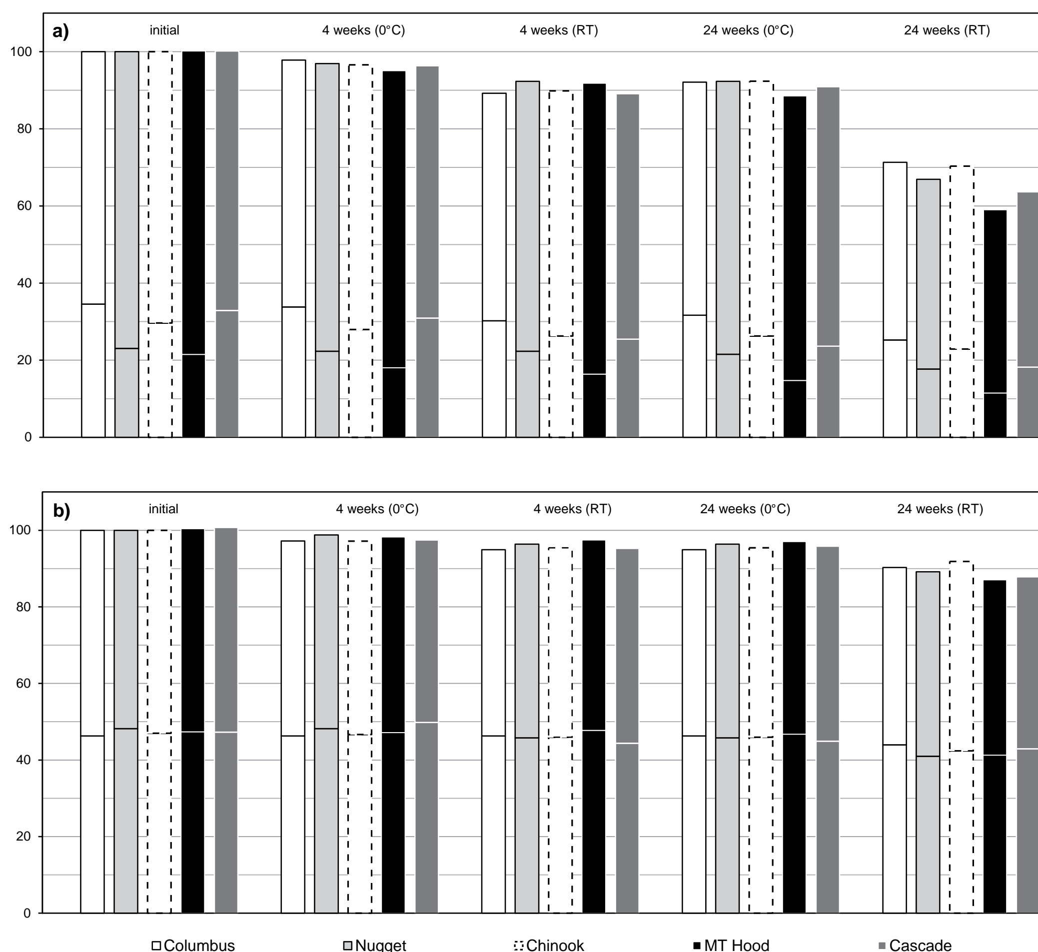

Fig. 2 **Relative decrease [%] of α-acid (a) and β-acid (b) concentration during aerobic hop storage at 0 °C and RT. Relative decrease [%] of α-acid (a) and β-acid (b) concentration during aerobic hop storage at 0 °C and RT. Bar height indicates the relative amount [%] of α- and β-acids drawn to the initial concentration. Bars are split into the respective parts for co-humulone (bottom) and n- + ad-humulone (top a) or co-lupulone (bottom) and n- + ad-lupulone (top b)**

During four week storage at 0 °C the concentration of α-acids decreased at a minimum of –2.2 % in Columbus, and at a maximum of -4.9 % in Cascade. After twenty four weeks at 0 °C losses between –7.6 % (Chinook) and –9.8 % (MT Hood) were observed. At RT the decrease of α-acids was much higher: After four weeks a reduction of –10.9 % was observed in Cascade hops, –9.2 % were found in the MT Hood samples. After twenty four weeks maximal losses of –41.0 % for MT Hood, and minimal losses of –29.7 %in the Chinook pellets were observed. Data does not indicate a causal correlation between the initial content of α-acids and their %-decrease upon storage. The ratio of co-humulone/ n- + ad-humulone remained almost constant throughout storage. A tendency towards slightly higher losses for n- + ad-humulone was observed in Columbus, Nugget, and Chinook. In the aroma hops (MT Hood and Cascade) a tendency towards higher losses for co-humulone was found. The ratios (concentration co-humulone/ concentration n- + ad-humulone) after twenty four weeks storage at RT were: Columbus = 0.57, Nugget = 0.36, Chinook = 0.48, MT Hood = 0.24, Cascade = 0.40.

The data is in accordance to literature [e.g. 59] and to the assessment published by the Hops Growers of America as the distributor of the hop samples analyzed within this study [60]. *Canbas* et al. [59] found losses ranging from 30.8 % in hop pellets variety Brewers Gold to 36.4 % in pellets type 90 of Northern Brewer. *Mikyska* and *Krofta* [35] found losses of up to 30 % (24 weeks, RT) for several Czech aroma hop varieties. The Hops Growers of America estimated losses of approx. 50 % for Cascade, Columbus, and MT Hood, 25–30 % for Nugget and Chinook. Differences in the stability of bitter acids from different hop varieties have been attributed to polyphenol content and anti-radical properties of hop varieties [35]. These parameters were not determined within this study.

Storage trials positively showed, that β-acids are more stable towards oxidation reactions. The decrease of β-acids was considerably lower than those of the α-acids: When hop was stored at RT for twenty four weeks the highest loss were found in Cascade (–12.2 %), whereas in Chinook only losses of –8.1 % were found. Cold storage (0 °C) was very effective in preventing β-acid degradation. Maximal losses of –5.1 % were found in Columbus hops (twenty four weeks of storage). Very similar β-acid concentrations were found when hop was stored at RT for four weeks. These results are in accordance to previously published data [35, 59].

3.3 Short chain and medium chain monocarboxylic acids in fresh and stored hop samples

Hop essential oil is a complex mixture of hundreds of volatiles, thus GC chromatograms usually contain several hundred signals as well. Abundant terpene hydrocarbons, but also some alcohols, esters, sesquiterpene epoxydes, and ketones dominate chromatograms, signal overlaps exclude the identification of trace concentrated

Table 3 **Mean concentrations short chain monocarboxylic acids [µg/g] in commercial hop pellets. The short chain acids are divided into bitter substance derived acids and other acids. CV [%] is given for each of the analytes**

	CV	Columbus	Nugget	Chinook	MT Hood	Cascade
bitter substance derived acids	[%]			[µg/g]		
2-methylpropanoic acid	12	14.65 ± 1.76[a]	3.98 ± 0.48	14.56 ± 1.75	8.45 ± 1.01	10.07± 1.21
2-methylbutanoic acid	11	14.24 ± 1.57	4.25 ± 0.47	8.36 ± 0.92	6.39 ± 0.70	6.62 ± 0.73
3-methylbutanoic acid	8	21.50 ± 1.72	8.18 ± 0.65	24.65 ± 1.97	16.44 ± 1.32	16.82 ± 1.35
hexanoic acid	6	9.77 ± 0.59	5.43 ± 0.33	4.92 ± 0.30	5.14 ± 0.31	3.90 ± 0.23
4-methylpentanoic acid	11	3.12 ± 0.34	0.61 ± 0.07	1.90 ± 0.21	0.10 ± 0.01	0.89 ± 0.10
sum		63.3	22.4	54.4	36.5	38.3
(2-methylpropanoic acid) / 2 + 3-methylbutanoic acid)		0.41	0.32	0.44	0.37	0.43
(co-/ n- + ad-humulone)		0.53	0.30	0.42	0.27	0.49
other short and medium chain acids				[µg/g]		
pentanoic acid	13	0.33 ± 0.4	0.21 ± 0.03	0.18 ± 0.02	< 0.1	0.24 ± 0.03
(2E)-hexenoic acid	15	0.12 ± 0.02	0.51 ± 0.08	< 0.1	0.75 ± 0.11	0.43 ± 0.06
heptanoic acid	9	12.43 ± 1.12	44.90 ± 4.04	8.81 ± 0.79	15.97 ± 1.44	18.85 ± 1.70
6-methylheptanoic acid	10	63.49 ± 6.35	11.15 ± 1.12	19.68 ± 1.97	8.38 ± 0.84	15.23 ± 1.52
octanoic acid	4	25.12 ± 1.00	68.37 ± 2.73	41.61 ± 1.66	27.95 ± 1.12	24.97 ± 1.00
nonanoic acid	5	43.11 ± 2.16	25.86 ± 1.29	27.53 ± 1.38	9.55 ± 0.48	25.10 ± 1.25
(4Z)-decenoic acid	11	68.51 ± 7.54	25.00 ± 2.75	44.12 ± 4.85	20.48 ± 2.25	18.34 ± 2.02
decanoic acid	13	35.51 ± 4.62	47.78 ± 6.21	23.41 ± 3.04	4.51 ± 0.59	17.09 ± 2.22
undecanoic acid	13	1.60 ± 0.19	1.36 ± 0.16	0.32 ± 0.04	0.20 ± 0.02	1.53 ± 0.18
10-methylundecanoic acid	12	2.79 ± 0.34	2.84 ± 0.34	1.52 ± 0.18	1.42 ± 0.17	7.61 ± 0.91
dodecanoic acid	11	13.86 ± 1.52	10.64 ± 1.17	12.20 ± 1.34	9.38 ± 1.03	13.58 ± 1.49
sum		266.9	238.6	179.4	98.7	143.0

[a] deviations (e.g. ± 0.2 µg/g) of the mean concentrations were calculated using the CV (cf. 2.6)

odorants such as monocarboxylic acids. For this reason SDE extracts were purified by LLE, GC-MS analysis of the LLE extracts enabled the analysis of trace concentrated polar volatiles from hops. GC-MS analysis verified the occurrence of 16 short and medium chain monocarboxylic acids with 4 to 12 carbon atoms (cf. Table 3). These 16 acids were quantified in fresh and aged hop samples. In order to simplify presentation and discussion of the experimental data, the short chain acids are divided into two groups (cf. 1.3):

- Bitter substance derived (short chain) monocarboxylic acids, namely 2-methylpropanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, 4-methylpentanoic acid, and hexanoic acid.
- Medium chain monocarboxylic acids with 7 to 12 carbon atoms, including pentanoic acid and (2E)-hexenoic acid. These acids are either derived from lipid synthesis and breakdown, or from cleavage of their corresponding methyl esters (cf. 1.3).

The analytical data presented and discussed in the following reveals three central declarations:

1. Hop varieties (commercial pellets type 90) differ in their content of short chain and medium chain monocarboxylic acids.
2. During aerobic storage the concentration of the bitter substance derived short chain acids increased significantly. The highest concentration of these short chain acids was observed in samples stored at RT for twenty four weeks.
3. During aerobic hop storage the concentration of the other short and medium chain monocarboxylic acids decreased. Generally, the highest concentration of these acids was observed in fresh hops, the lowest after twenty four weeks of storage at RT.

3.3.1 Concentration of short and medium chain acids in fresh hop samples

The quantification of monocarboxylic acids showed, that commercially available hop pellets differ in their content of short and medium chain monocarboxylic acids. Table 3 summarizes the results of quantification for all 16 acids, whereas deviations from the mean (e.g. ± 0.2 µg/g) were calculated by means of the CV for each analyte. Additionally, table 3 includes the sums for both groups of acids, namely for the bitter substance derived short chain acids and other short and medium chain acids.

The principal volatile acids of fresh pellets are decanoic acid, nonanoic acid, octanoic acid, and 6-methylheptanoic acid. Their individual concentration ranges from approx. 30 to 80 µg/g hop pellets (cf. Table 3). As sum of the other medium and short chain acids concentrations of 267 µg/g (Columbus) to 98.7 µg/g (MT Hood) were found. These acids were reported to result from lipid synthesis or breakdown [51]. Their concentration correlates with the total concentration of volatiles in the fresh hop pellets: Pellets with high concentration of volatiles (e.g. Columbus) contain more medium chain fatty acids than varieties with low concentrations of volatiles (e.g. Cascade, cf. Table 4).

The concentration of bitter substance derived short chain monocarboxylic acids in fresh pellets is considerably lower than those of the medium chain acids: 2-methylpropanoic acid was found in concentrations ranging from 3.98 µg/g (Nugget) to 14.65 µg/g (Columbus), 2-methylbutanoic acid ranged from 4.25 µg/g (Nugget) to 14.24 µg/g (Columbus), 3-methylbutanoic acid ranged from 8.18 µg/g (Nugget) to 24.65 µg/g (MT Hood). Maximal concentrations of hexanoic acid (9.77 µg/g) and 4-methylpentanoic acid (3.12 µg/g) were traced in Columbus.

In sum the bitter substance derived acids ranged from 63.3 µg/g in Columbus to 22.4 µg/g in Nugget. The concentration in Chinook pellets was 54.4 µg/g, 36.5 µg/g in MT Hood, and 37.5 µg/g in Cascade.The results of quantification are in accordance to the data of Tressl et al. [51], who also reported acid concentrations in the low µg/g (ppm) range.

The concentrations of bitter substance derived short chain acids correlates with the concentrations of the corresponding bitter substances: Co- + n- + ad-humulone (lupulone) are the principal

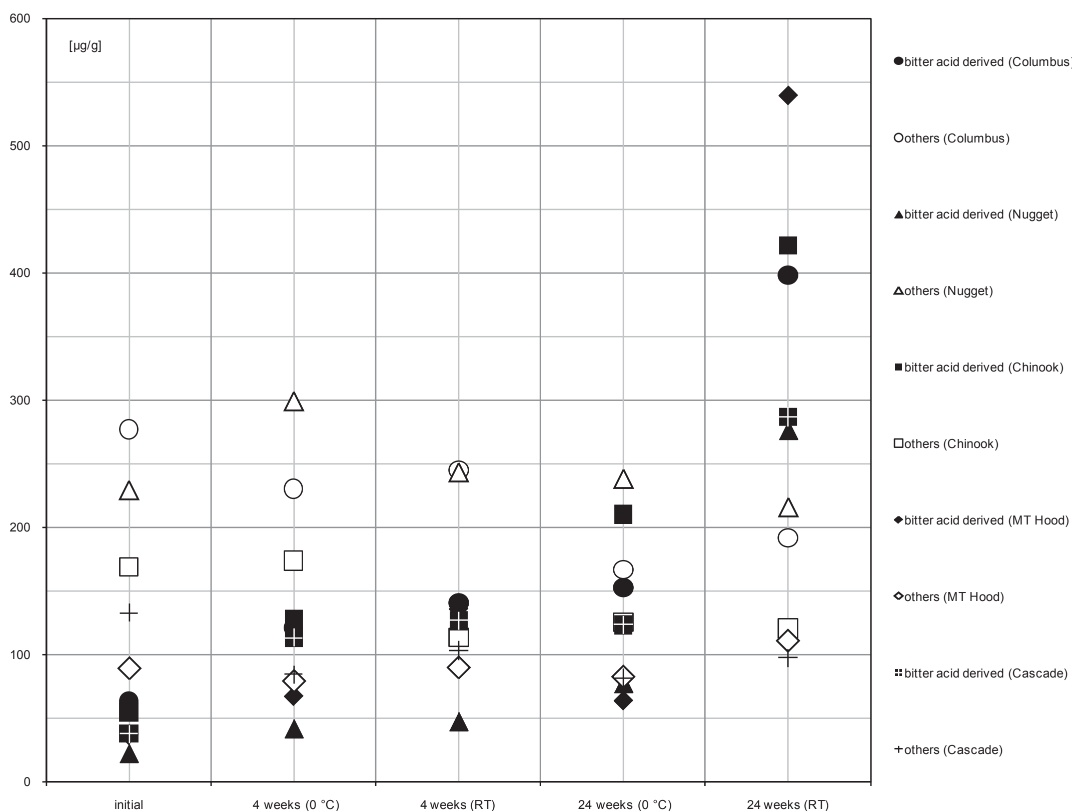

Fig. 3 Mean concentrations of short chain and medium chain acids during aerobic hop storage of Columbus, Nugget, Chinook, MT Hood, and Cascade pellets. Labels indicate the concentration of either bitter substance derived acids or other medium and short chain acids

bitter acids of hops, 2-methylpropanoic acid and 2- + 3-methylbutanoic acid are the principal methyl branched short chain acids. Minor bitter substances are pre- and posthumulone (lupulone), 4-methylpentanoic acid and hexanoic acid are also present in lower ratios. However, data does not indicate a direct correlation between the total α-acid concentration of hops (cf. Table 2) and the total concentration of bitter substance derived short chain acids (cf. Table 3): MT Hood (6.1 g/100 g α-acid) and Cascade (5.5 g/100g α-acid) show higher concentrations of short chain acids compared to Nugget, whereas the α-acid concentration in Nugget (13 g/100 g) is considerably higher. Also, the concentration of bitter substance derived acids did not correlate with concentration of total volatiles (cf. Table 4).

Tressl et al. [51] proposed a correlation between the concentrations of co-humulone and 2-methylpropanoic acid, as well as of n- + ad-humulone and 2/3-methylbutanoic acid. The concentration ratios of co-humulone/n+ad-humulone and 2-methylpropanoic acid/2- + 3-methylbutanoic acid are listed in table 3. Those ratios were very similar for Nugget and Chinook, they differed in Columbus, MT Hood and Cascade. Based on these results we can not confirm a direct correlation between co-humulone and 2-methylpropanoic acid, as well as of n- + ad-humulone and 2/3-methylbutanoic acid in fresh hop pellets. Differences might be attributed to different drying procedures.

3.3.2 Concentrations of short and medium chain acids during aerobic hop storage

During aerobic storage of hops the concentration of the bitter substance derived monocarboxylic acids, namely of 2-methylpropanoic, 2 + 3-methylbutanoic acid, 4-methylpentanoic acid, and hexanoic acid increased strongly, whereas the concentration of the other short and medium chain acids with 7 to 12 carbon atoms (+ pentanoic acid and (2E)-hexenoic) decreased. Figure 3 illustrates the concentration changes of both acid groups upon storage all five pellets varieties. Black labels of the scatter plot indicate the concentrations the bitter acid derived acids, white labels represent the concentrations of other short and medium chain acids. In fresh hops the sum of bitter substance derived acids was lower than the sum of the other short and medium chain acids in all varieties, whereas after 24 weeks of storage at RT maximal concentrations of bitter substance derived acids were found. At this sampling time concentrations of bitter acid derived acids were higher compared to the other short and medium chain acids in all samples. A detailed discussion of the individual results for both acids is given below.

Bitter substance derived monocarboxylic acids

The initial concentration of bitter acid derived acids ranges from 63.0 µg/g in Columbus to 21.90 µg/g in Nugget. Concentration increased by factor 1.8 (Nugget) to 3.2 (Cascade) within four weeks of cold storage, and by factor 2.1 (Nugget) to 3.7 (Cascade) within four week storage at RT. After twenty four weeks of cold storage acid concentrations were by factor 2.4 (Columbus) to 3.8 (Chinook) higher compared to their initial value. Concentrations raised by factor 6.2 (Columbus) to 18.3 (MT Hood) within twenty four weeks of storage at RT. The increase correlates with the decreasing concentrations of bitter acids (cf. Fig. 2): Cold aerobic storage (0 °C, 4 weeks and 24 weeks) resulted in reduced bitter acid degradation and shortened release of monocarboxylic acids across all samples. Aerobic storage at RT (4 weeks and 24 weeks) resulted in losses of bitter acids of up to 40 %, simultaneously remarkable concentrations (up to 550 µg/g) of bitter substance derived monocarboxylic acids were found. The short chain acids are extremely flavour active, they exhibit very unpleasant cheesy, sweaty, rancid, and goat like smells, that dominate the aroma of excessive aged hops.

Figure 4 shows the individual concentration of 2-methylpropanoic, 2+3-methylbutanoic, 4-methylpentanoic, and hexanoic acid in Columbus hops, figure 5 shows the corresponding data for fresh and aged Chinook hops.

Among these acids 3-methylbutanoic acid was found in the highest concentration in fresh and aged hop samples. It was followed by 2-methylpropanoic acid, 2-methylbutanoic acid, and hexanoic acid. The concentration of 4-methylpentanoic acid is considerably lower.

Data shown in figures 2–5 indicates a connection between bitter substance concentration and changes in concentration of short

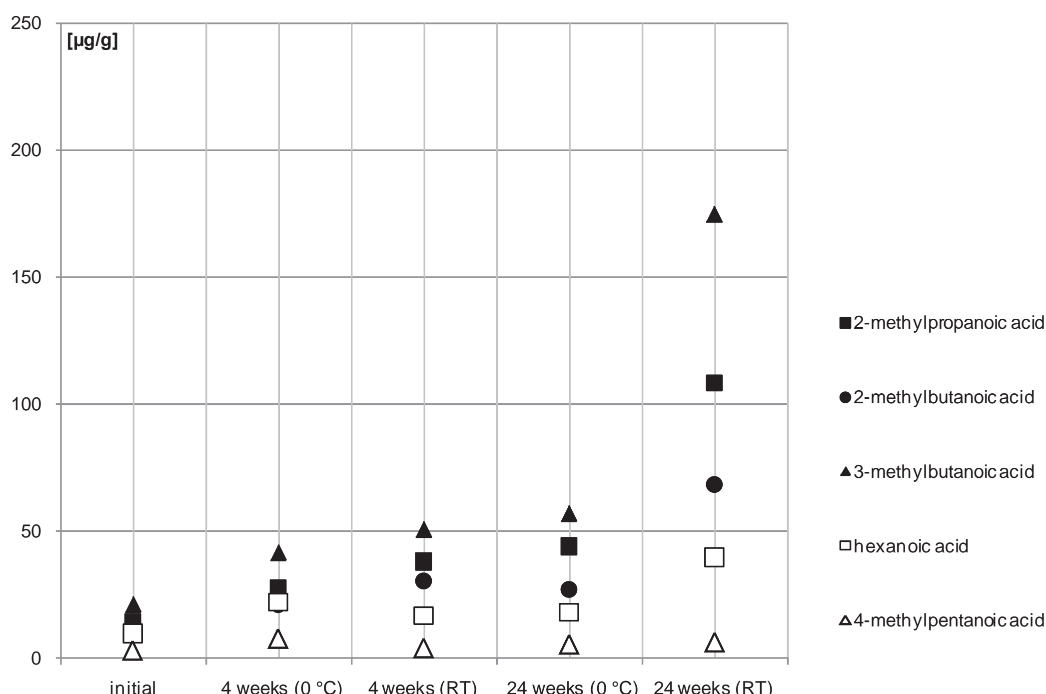

Fig. 4 Concentration of bitter substance derived monocarboxylic acids [µg/g] in fresh and stored Columbus hops

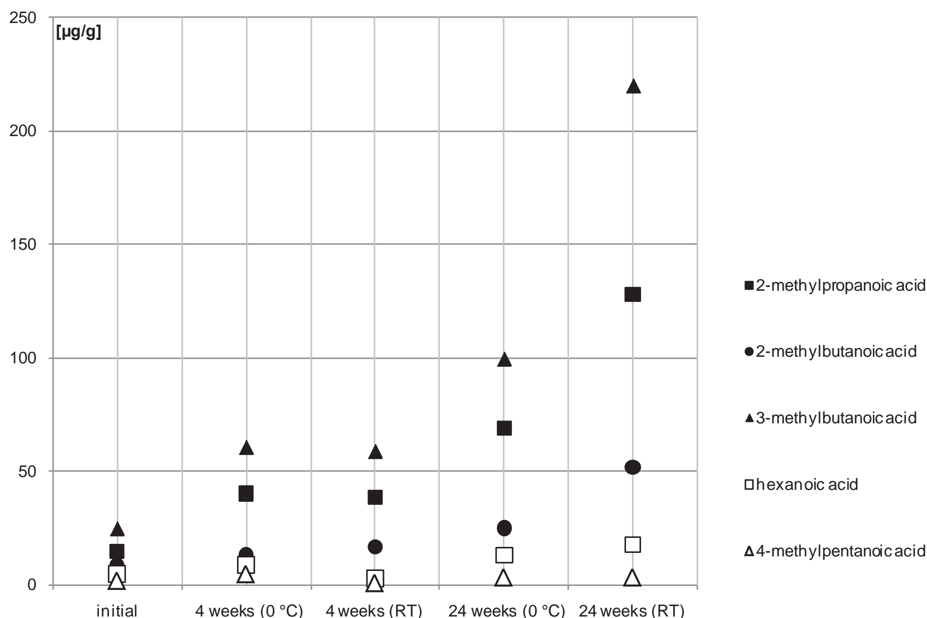

Fig. 5 Mean concentrations of bitter substance derived monocarboxylic acids [µg/g] in fresh and stored Chinook hops

Based on the experimental data of this study it seems very likely, that short chain acids are in fact released by cleavage of the acyl-side chains of bitter acids. In figure 6 a reaction scheme for the release of monocarboxylic acids from hop bitter acids by so called "acid cleavage" is proposed: The central six-ring of hop bitter acids represents a 2-acyl-1,3-diektone moiety, the charge distribution of this structure triggers a nucleophilic attack of water onto the carbonyl function of the acyl side chain. Analogue to the degradation of n-humulone and the release of 3-methylbutanoic acid shown in figure 6, the acyl side chains of the remaining bitter acids can release 2-methylpropanoic acid (co-humulone/-lupulone), 2-methylbutanoic acid (ad-humulone/-lupulone), 4-methylpentanoic acid (post-humulone/-lupulone), and hexanoic acid (pre-humulone/-lupulone). The non-volatile reaction product shown in figure 6 has not been analyzed, further degradation or rearrangement reactions might occur.

chain acids. The concentration of co- + n- + ad-humulone decreases when hops is stored, whereas 2-methylpropanoic and 2- + 3-methylbutanoic acid are released as the principal short chain acids. The concentrations of short chain acids in aged hops correlate with ratios of the corresponding bitter substances, however there seems to be no causal correlation between bitter substance losses and release of short chain acids amongst varieties. The results presented herein confirm the conclusion of Tressl et al., who originally proposed a connection between bitter acid degradation and release of flavour active short chain acids during hop storage. It has to be remarked, that their proposal was not based on the presentation of comprehensive data for both substance groups.

Other short and medium chain monocarboxylic acids

Short chain acids as well as straight chain, methyl branched, and unsaturated medium chain acids (C > 7) are either intermediates of plant lipid synthesis [61], the result of lipid breakdown, or hydrolysis products of corresponding methyl esters [51]. The concentration of short and medium chain acids in fresh hop is displayed in table 3, concentration changes during aerobic storage did not show a consistent trend. Changes in the total concentration displayed in

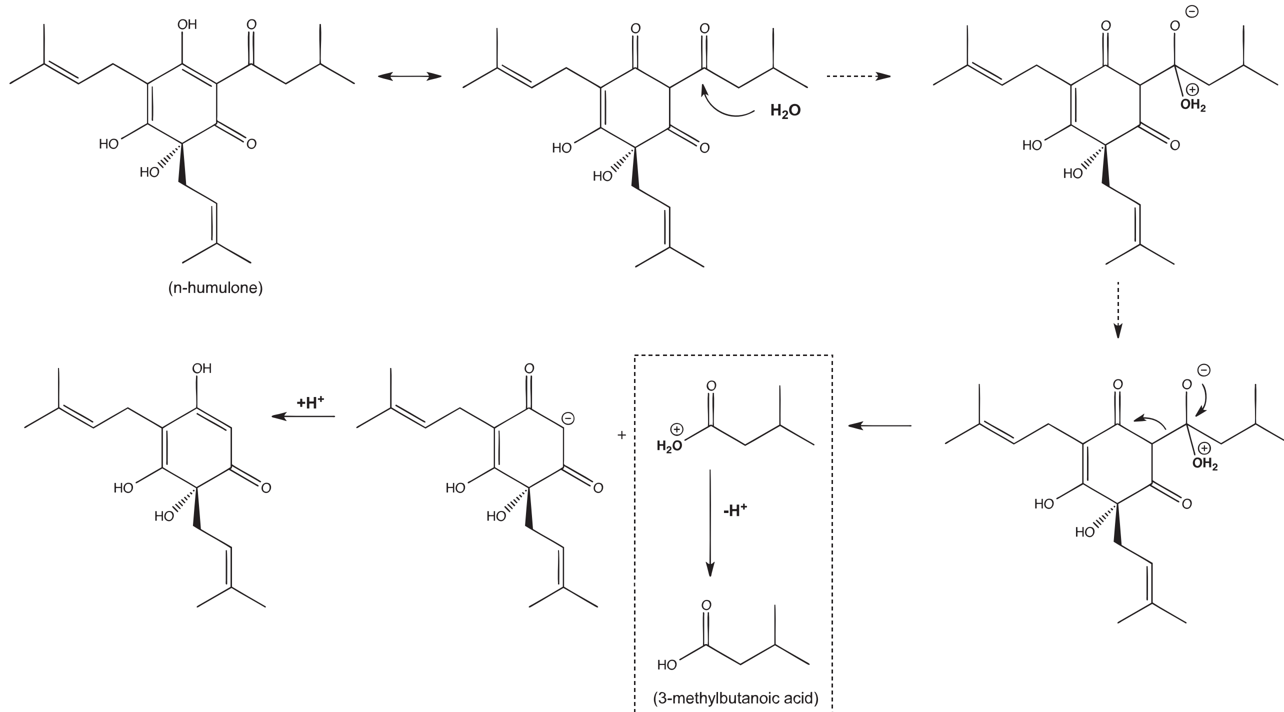

Fig. 6 Proposed reaction scheme of n-humulone into 3-methylbutanoic acid, namely acid cleavage of a 2-acyl-1,3-diektone moiety

Table 4 **Mean concentrations of volatiles in fresh and aged hop pellets. The sum of volatiles is derived by addition of the concentration of terpene hydrocarbons and terpenoids. The Concentration of myrcene is given individually**

	Columbus	Nugget	Chinook	MT Hood	Cascade
fresh samples					
[a] sum [µg/g]	18075	22269	12140	15818	6670
myrcene [%]	68.8	53.0	43.7	37.7	61.5
ß-caryophyllene [%]	6.9	10.8	12.2	13.1	8.2
α-humulene [%]	16.4	28.3	27.4	43.4	17.7
terpene hydrocarbons [%]	93.2	95.9	83.4	94.5	87.9
terpenoids [%]	6.8	4.1	16.6	5.5	12.1
4 weeks (0 °C)					
sum volatiles [µg/g]	14692	17619	7389	10914	7042
[b] loss of volatiles [%]	–18.7	–20.9	–39.1	–31.0	–5.3
myrcene [%]	66.7	55.1	46.6	50.4	46.6
terpene hydrocarbons [%]	88.3	93.4	83.9	93.7	85.6
terpenoids [%]	11.7	6.6	16.1	6.3	14.4
4 weeks (RT)					
sum volatiles [µg/g]	9975	11685	6588	7640	4011
loss of volatiles [%]	–44.8	–47.5	–45.7	–51.7	–39.9
myrcene [%]	66.0	49.5	57.4	35.6	57.4
terpene hydrocarbons [%]	90.8	92.5	86.4	92.9	67.1
terpenoids [%]	9.2	7.5	13.6	7.1	32.9
24 weeks (0 °C)					
sum volatiles [µg/g]	3202	5083	3174	4001	2176
loss of volatiles [%]	–82.3	–77.2	–73.9	–74.7	–67.4
myrcene [%]	3.7	3.1	1.9	2.1	0.9
terpene hydrocarbons [%]	58.1	73.3	53.4	74.6	46.8
terpenoids [%]	41.9	26.7	46.6	25.4	53.2
24 weeks (RT)					
sum volatiles [µg/g]	1611	1115	412	561	281
loss of volatiles [%]	–91.1	–95.0	–96.6	–96.5	–95.8
myrcene [%]	5.6	5.6	9.2	7.8	2.1
terpene hydrocarbons [%]	62.8	65.7	58.5	31.7	36.9
terpenoids [%]	37.2	34.3	41.5	68.3	63.1

[a] the sum of volatiles does not include the concentrations of short and medium chain acids
[b] loss is referred to the concentration of volatiles in the fresh samples

figure 3 were basically determined by changes in the concentration of abundant medium chain acids, namely octanoic acid, 6-methylheptanoic acid, (4Z)-decenoic acid, and decanoic acid (cf. Table 3). Within the first four weeks of storage concentrations increased in Nugget and Chinook, decreasing or stable concentrations were observed for Cascade, Columbus, and MT Hood. Comparison of the initial concentrations with those after 24 weeks at RT, showed a concentration decrease for all varieties except MT Hood (cf. Fig. 3). Comparison of the concentrations after four respectively 24 weeks (at either 0 °C or RT) indicates a tendency towards lower acid concentrations in case hops was stored cold (cf. Fig. 3). The overall concentration decrease indicates, that losses (e.g. by evaporation) proceed at a higher rate than the formation of short and medium chain acids.

The occurrence of relevant concentrations of short and medium chain monocarboxylic acids in fresh hops indicates lipid degradation and cleavage of methyl esters during hop processing and drying, however there is also indication for both processes during hop storage: In the SDE extracts of hop pellets five methyl ester were traced in concentrations > 5 µg/g, the average concentrations in the fresh pellets of all five varieties are given in brackets: methyl 6-methylheptanoate (Ø = 38 µg/g), methyl octanoate (Ø = 51 µg/g), methyl 6-methyloctanoate (Ø = 32 µg/g), methyl (4Z)-decenoate (Ø = 129 µg/g), and methyl decenoate (Ø = 89 µg/g). The concentration of these esters decreased upon aerobic hop storage (24 weeks at RT): Methyl 6-methylheptanoate –95 %, methyl octanoate –90 %, methyl 6-methyloctanoate –91 %, methyl (4Z)-decenoate –90 %, and methyl decenoate –95 %.

Pentanoic acid, (2*E*)-hexenoic, and heptanoic acid, are products of lipid oxidation [62]. Hops contain considerable amounts of lipids [63], the relevance of lipid oxidation during hop processing/drying has been shown by characterization of aroma active lipid degradation products such as (2*E*)-hexenal or *trans*-4,5-epoxy-(2*E*)-decenal [3]. The concentration of lipid derived acids in fresh pellets was in the low µg/g range (cf. Table 3), their concentration decreased during storage. Obviously, lipid oxidation during aerobic storage seems of minor importance, however the analysis of lipid derived acids might be a suitable measure to quantify lipid oxidation during hop drying and processing.

3.4 Changes in terpene hydrocarbons and terpenoids

The results of hop essential oil analysis (GC-MS of the SDE extracts) are summarized in table 4. For clarity the volatiles were grouped into terpene hydrocarbons (myrcene, limonene, ocimene, ß-caryophyllene, α-humulene, α-bisabolene) and terpenoids (linalool, *cis-/trans*-linalool oxide, α-terpineol, nerol, neral, geraniol, geranial, methyl geranate, geranyl acetate/isobutyrate/propionate, caryophyllene oxide, humulene monoepoxid, α-limonene-diepoxid). Added concentrations of terpene hydrocarbons and terpenoids equal the sum of total volatiles presented and discussed in the following. Short and medium chain acids as well as their corresponding methyl ester are not included.

Data given in table 4 shows an overall concentration decrease of volatiles during aerobic hop storage. Also a shift in the ratio between the terpene hydrocarbons and the terpenoids can be observed. In fresh hops terpene hydrocarbons account for approx. 85–95 % of total volatiles, whereas in aged hops (24 weeks, RT) only 30–60 % were found.

After 24 weeks of storage at RT approx. 90 % of the volatiles were lost, cold storage for twenty four weeks still resulted in losses of 50–70 %. In fresh hops terpenoids accounted for maximal 17 % (Chinook), whereas 68 % were found in aged MT Hood hops. This data is in accordance to previously published data [cf. 15, 16, 42]. The decrease of total volatiles was primarily caused by a strong decrease in myrcene concentration: In fresh pellets myrcene accounted for 38 % up to 69 % of the total volatiles, which equals at maximum 12 435 µg/g hops (Columbus). Upon storage a reduction to < 10 % was observed, this equals a concentration of 90 µg/g (Columbus). The %-share of myrcene (drawn to the total volatiles) is displayed separately in table 4. Basically, myrcene concentration decreases with incremental storage time and temperature.

For the terpenoids an increase in absolute concentration after four weeks was observed, after 24 weeks of storage concentrations fell below the initial concentration (data not shown).The shift in the %-share of terpene hydrocarbons vs. terpenoids primarily results from losses of the terpene hydrocarbons by evaporation and partial transfer into oxygenated derivates, in addition polymerization might occur.

Except for myrcene, no individual concentration changes of other volatiles are displayed. In brief the quantitative and qualitative changes were as follows:

Monoterpenes (myrcene/limonene/ocimene)

In accordance to the changes observed for myrcene limonene and ocimene decreased upon storage. Compared to the other volatiles of the hop essential oil myrcene, limonene, and ocimene are characterized by low boiling points (167° to 177°C), thus their evaporation during aerobic hop storage seems very likely. However, it has to be remarked, that the initial concentration of limonene and ocimene can not be compared to those of myrcene, both hops were found in concentrations < 40 µg/g in all varieties.

Sesquiterpenes (ß-caryophyllene and α-humulene)

The concentration of both primary sesquiterpenes decreased during hop storage. Basically, losses increased with time and temperature: After four weeks at 0°C approx. 30 % ß-caryophyllene were lost as an average across all five varieties, 60 % were lost after four weeks at RT. During 24 weeks of storage losses accumulated to 65 % (0 °C) and 90 % (RT), respectively. Similar results were obtained for α-humulene.

Humulene oxide (humulene monoepoxide II) and caryophyllene oxide

The sesquiterpene oxides are formed by oxidation of either ß-caryophyllene and α-humulene. In fresh hops caryophyllene oxide concentration ranged from 12 µg/g (Chinook) to 24 µg/g (Cascade), humulene monoepoxide II was found in the range of 27 µg/g (Nugget) – 65 µg/g (Chinook). Increasing concentrations during aerobic storage were observed in all hop varieties, whereas the maximal concentration of caryophyllene oxide was 131 µg/g in aged Chinook hops (24 weeks, RT), and 332 µg/g humulene monoepoxide II in MT Hood (24 weeks, RT).

Linalool and linalool oxides

The concentration of linalool slightly increased (+ max. 5 %) when hops was stored at 0 °C for four weeks. The increase of linalool might be due to the oxidation of myrcene (cf. 46) or by hydrolysis of non-volatile precursors. Storage for 24 weeks at 0 °C, or at room temperature (four and 24 weeks) led to a concentration decrease. Reduced linalool concentrations correlated with increasing concentrations of the linalool oxides, however there is no quantitative transfer of linalool into linalool oxides. In MT Hood hops linalool concentration decreased from 98 µg/g to 16 µg/g, whereas *cis*- and *trans*-linalool oxides increased from 9 to 27 µg/g. In Chinook hops a decrease of linalool from 74 µg/g to 14 µg/g was observed, linalool oxides increased from 9 to 22.5 µg/g.

Geranyl esters (methyl geranate, geranyl acetate, geranyl propionate, geranyl isobutyrate) and geraniol

The concentration of the geranyl esters and geraniol differed strongly throughout the hop varieties. Geranyl isobutyrate, methyl geranate, and geranyl acetate are the principal geranyl esters, their concentration was in the range of 60–290 µg/g fresh hops. Their concentration decreased during storage, whereas the lowest concentrations were found after 24 weeks at RT. The decrease of the geranyl ester might be due to hydrolysis, losses of geraniol might be primarily caused by evaporation.

The changes described above are in accordance to literature [15, 16, 21], a detailed and comprehensive survey is beyond the scope of this paper.

4 Summary and conclusion

In the current paper an analytical route for isolation, identification, and quantification of short and medium chain monocarboxylic acids from hops is described. Since the concentration of these substances in (fresh) hops is comparably low a SDE / LLE route was introduced to effectively enrich the target compounds. In order to cover analyte loss during sample preparation two stable isotope standards where used for quantification. The analytical setup was very suitable to monitor changes in the concentration of short and medium chain acids during hop storage. Method validation proved the analytical setup to be highly reproducible, therefore the current method is proposed as a meaningful supplement of conventional hop essential oil analysis.

Data presented herein showed the decrease of important secondary metabolites upon aerobic hop storage: The total concentration of terpene hydrocarbons, terpenoids and valuable bitter acids decreased dependent on storage time and temperature. The

concentration of terpene hydrocarbons was strongly reduced. Besides an oxidation into terpenoids, polymerization reactions, but primarily evaporation seems likely. The oxidation of linalool, α-humulene and ß-caryophyllene led to the formation of water soluble aroma active derivates (linalool oxides, humulene monoepoxide II, and caryophyllene oxide), which are key odorants of noble hop aroma in beer.

Upon aerobic storage the concentration of bitter substances decreased, whereas α-acids were more susceptible to oxidation compared to β-acids. The degradation of α- and β-acids was accompanied by remarkable concentration increases (up to 500 %) of five short chain acids, namely 2-methylpropanoic, 2- + 3-methylbutanoic acid, 4-methylpentanoic acid, and hexanoic acid. Those acids presumably result from acid-cleavage of the acyl-side chain of the bitter acids, however their concentration in excessive aged hops is comparable to the concentration of terpenes and terpenoids. 2-Methylpropanoic, 2- + 3-methylbutanoic acid, 4-methylpentanoic acid, and hexanoic acid, exhibit very unpleasant cheesy, sweaty, rancid, and goat like smells, that brewers broadly attribute to "old hops smell". Bitter substance derived short chain acids are essential contributors to the taste of lager beers [33, 64, 65], but first and foremost they are key precursors of highly acceptable esters in (late and dry hopped) beer. In contrast to the unpleasant odors of the short chain acids, corresponding ethyl esters exhibit very intensive sweet, estery, fruity and pineapple odor qualities, their flavour thresholds in beer are by factor 100 below those of the corresponding acids.

Storage experiments, accompanied by comprehensive analytical characterization of important hop metabolites confirmed, that prolonged storage at RT reduces the brewing value of hops, whereas a moderate short term storage is very promising in refining the aroma properties of hops: After 4 weeks of aerobic storage (0 °C) a slight increase in the concentration of terpenoids was found. Most notably, short chain acid concentrations were increased by approx 200 %, whereas the decrease of bitter acids was in the range of 5 %. During storage the concentration of medium chain acids decreased, obviously their formation by either lipid oxidation or cleavage of corresponding methyl esters proceeds at lower rate than their evaporation. A comprehensive data set, including quantitative analysis of aroma active short and medium chain acids from fresh and aged hops has not been published before.

As hop market increasingly lacks in aroma varieties, measures to enhance the aroma properties of bitter hop varieties might become relevant to some practical brewers. Especially, in terms late or dry hopping methods are applied, the concentration of water/beer soluble volatiles such as terpenoids and short chain acids is by far more important than bitter acid content or the concentration of terpene hydrocarbons.

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