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Starting hop lipidomics – Isolation and characterization of non-polar, neutral and polar hop lipids

Lipids are very diverse in chemical structure and properties. They are spread over a wide polarity range, thus comprehensive lipid analysis (lipidomics) from biological samples such as hops is challenging. The current study presents a straightforward route for extraction, classification and quantification of hop lipids. Offline LC-GC-MS enables separation of polar, neutral and non-polar lipids, as well as the individual analysis of fatty acid type and content. Unambiguous identification and precise quantification of C14-C22 fatty acids was performed by use of four appropriate internal standards and GC-EI-MS analysis of fatty acid methyl esters.

Descriptors: fatty acid, lipid, gushing, hops, flavor, beer staling

1 Introduction

For decades fatty acids (FA) have attracted the attention of brewing researchers and practical brewers. Free fatty acids (FFA) with 14 to 18 carbon atoms influence beer foam [1–3] and fermentation [4, 5]. Enzymatic and autoxidative degradation of polyunsaturated fatty acids (PUFA) are closely linked to beer flavour (in)stability [6, 7]. Also the contribution of FFA to the beer gushing phenomenon is constantly discussed [8–10]. For their impact on beer quality, FFA from barley, malt, adjuncts, yeast, mash, wort and beer have been extensively analyzed [11–15]. Also factors that promote oxidative degradation of free and esterified PUFA throughout the malting and brewing process have been surveyed in detail [16–19]. In contrast to this, hop derived lipids are poorly studied. This fact might be caused by the challenges going along with lipid analysis from hops, and not by their insignificance: Hop lipids were already suggested to be involved in beer staling and gushing in the nineteen seventies [9, 10, 20]. It has been shown that post-fermentation bittering increases FA levels and reduces the flavour stability of lager beer [21]. In addition, lipid oxidation products such as *trans*-4,5-epoxy-(*E*)-2-decenal are amongst the most potent odorants of fresh and dried hop cones [22].

1.1 Hop lipids in brewing research

There are comparably limited studies focusing on hop lipids and none of them is giving a comprehensive overview. Several early works describe the occurrence of hop waxes, such as the long chain ester triacontyl hexadecanoate or the long chain alcohol 1-hexacosanol [23, 24]. Triacontyl hexadecanoate, 1-hexacosanol and other waxes are insoluble in cold methanol, thus they precipitate during the analysis of hop bitter substances. FFA with chain lengths of C12–C20 in hops were reported by *Clarke* et al. [25] *Roberts and Stevens* [26] and *Delanghe* et al. [27]. Delanghe et al. [27] also traced aliphatic alcohols with chain lengths of 18 to 30 carbon atoms, whereas *Jarolimek* and coworkers [28] found several long chain alkanes and methyl paraffines. *Carrington* et al. [9] and *Sandra* et al. [10] quantified FFA in several hop varieties and hop products. Both research groups agreed that palmitic acid, linoleic and α-linolenic acid are the major FFA in hops. Carrington et al. reported FFA concentrations in hops from 0.04 % up to 0.4 % dry matter. In pre-isomerized hop extracts up to 1.5 % FFA have been found. From these results they [9] concluded that processing techniques strongly influence the FFA concentration of hop products. Recently *Bravi* et al. [29] analyzed the lipid content of Saazer hops. They reported a total lipid content of 5.7 %. Palmitic acid, linoleic acid and α-linolenic acid were reported as the major long chain FA in Saazer hops.

1.2 Lipids are diverse

Data on hop lipids is rare and particularly focused on FFA and non-polar substances such as alkanes, (very) long chain alcohols and waxes. These approaches ignore the complexity of lipids usually found in plants. The compounds that make up the lipid family are grouped due to their relative insolubility in water [30]. Nevertheless, lipids in biological samples are spread over wide polarity range. They are very diverse in their respective structures and cellular functions [31]. Figure 1 shows the chemical structures of L-α-phosphatidylcholine (1-hexadecanoyl-2-(9*Z*,12*Z*-octadecadienoyl)-*sn*-glycero-3-phosphocholine), linoleic acid, cholesteryl oleate (3β-hydroxy-5-cholestene 3-oleate), triacontyl hexadecanoate, and a triacylglyceride (1-hexadecanoyl-2-(9*Z*-octadecenoyl)-3-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-*sn*-glycerol). Biologically lipids

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have four functions in nature: They are energy stores, membrane constituents, serve as secondary messengers, and are precursors of important bioactive molecules such as leucotriens. The most abundant plant lipids are triacylglycerides, phosphoglycerides, glycosylglycerides and waxes. Less abundant but also relevant plant lipids are sterol derivates, sphingolipids, polyketides and prenol lipids.

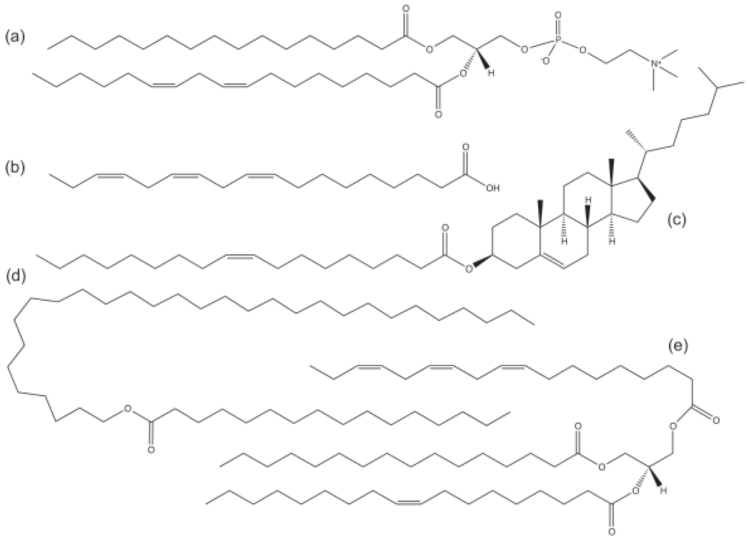

Fig. 1 **Lipid structures can be found over a wide polarity range. L-α-phosphatidylcholine (a) is a polar lipid, linoleic acid (b) a neutral, whereas cholesteryl oleate (c), triacontyl hexadecanoate (d) and triacylglycerides (e) are non-polar lipids**

Triacylglycerides (TAG) are the primary form of energy storage. In TAG three FA are esterified to a glycerol backbone (cf. Fig. 1). Depending on the degree of FA saturation, TAG can be either solid (called fats) or liquid (called oils) at room temperature. TAG hydrolysis by lipase enzymes yields diacylglycerides (DAG), monoacylglycerides (MAG) and FFA. FFA are readily oxidized in the β-oxidation cycle to gain energy. Phosphoglycerides (PG) and glycosylglycerides (GG) are the major polar plant lipids. Their structure is similar to those of TAG: In PG and GG the glycerol is esterified with two FA, a phosphate group or carbohydrate respectively. PG and GG are amphiphilic, their polar or ionic head group (carbohydrate and phosphate group) is water soluble, their non-polar aliphatic ending (FA) is soluble in fat. PG and GG are able to form a double layer with the polar ends pointing outwards and the nonpolar ends pointing inwards. This arrangement is called lipid bilayer, it keeps the watery interior of the cell separate from the exterior. Cell membranes have to be fluid under environmental conditions. Plant membrane lipids contain high amounts of PUFA, which have considerable lower melting points than saturated FA. Plant surfaces, especially leaves, are covered by plant cuticle impregnated with waxes. Waxes are mixtures of hydrophobic compounds, typically esters of saturated FA and long chain aliphatic alcohols. They form a protective surface layer that prevents water loss, entry of microbial spoilers and pollutants. Waxes are insoluble in water and solid at room temperature [32].

1.3 Aim of the present work

Aim of the present survey was to establish an analytical route for isolation, separation, purification, and analysis of major hop lipids. Basically, this includes analytical techniques for initial extraction of lipids from plant material, separation of lipids from bitter substances and polyphenols, as well as classification of lipids according to their polarity. Transesterification of polar lipids (PG, GG, and MAG), neutral lipids (FFA, FAME, and DAG) and non-polar lipids (TAG, cholesterol derivates, and waxes) into volatile fatty acid methyl esters (FAME) is performed to enable unambiguous identification by GC-MS. The use of 1,2,3-tritridecanoylglycerol (glyceryl tritridecanoate), methyl heptadecanoate, pentadecanoic acid and lab-made 2,2-$^2H_2$-heptadecanoic acid (D_2-heptadecanoic acid) as internal standards enabled precise quantification of FAME throughout all lipid fractions. The current study aims to give detailed insights into the occurrence and concentration of individual fatty acids esterified either in simple (TAG, DAG, MAG, wax) or complex lipid structures (PG and GG). By FAME quantification the total lipid content of hops shall be calculated. Lipid analysis is supplemented by quantification of hop essential oil and bitter substances in order to detect correlations between these the groups of important hop constituents.

2 Experimental

2.1 Reference materials and chemicals

Analytical standards for GC-MS analysis and thin layer chromatography (TLC) were purchased from Sigma-Aldrich Chemie GmbH (D-89555 Steinheim). The following substances were available in purity ≥ 97 %, if not stated elsewise: Myristic acid, methyl myristate, palmitic acid, methyl palmitate, stearic acid, methyl stearate, oleic acid, methyl oleate, linoleic acid, methyl linoleate, α-linolenic acid, methyl linolenate, arachidic acid, behenic acid, α-humulene, β-caryophyllene, myrcene, linalool, nerol, geraniol, and α-terpineol. Internal standards for lipid analysis were glyceryl tritridecanoate, methyl heptadecanoate, pentadecanoic acid and lab-made D_2-heptadecanoic acid. Additional reference materials for thin layer chromatography (TLC) were: Cholesteryloleate, triolein (> 65 % (Assay: GC)), dioleoylglycerol (mixture of glycerol-1,2- and -1,3-dioleate isomers), L-α-phosphatidylcholine (> 60 % (Assay: TLC)) and 1-oleoyl-glycerol. Triacontanyl hexadecanoate was isolated from bee wax by TLC separation on silica gel 60 [33]. Glucose oleate was synthesized by esterification of glucose with oleic acid chloride. Reaction conditions and isolation procedure were according to the protocol of *Reinefelder and Korn* [34].

Labmade 2,2-$^2H_2$-heptanoic acid (D_2-heptadecanoic acid) was synthesized by base catalyzed exchange of ^1H atoms of the alpha-carbon: 250 mg heptadecanoic acid were solved in 12 g D_2O and 0.4 g NaOD. Reaction mixture was heated to 150 °C in a high-pressure lab-autoclave. After 72 h at 150 °C, the reaction mixture was cooled to room temperature, acidified to pH 2 (2N hydrochloric acid), D_2-heptadecanoic acid was extracted with pentane.

The solvents, except methanol, were of analytical grade. Methyl tert-butyl ether (MTBE) and methanol (HPLC grade) were purchased from Carl Roth GmbH + Co. KG (D-76185 Karlsruhe), n-pentane, light petroleum and ethylacetate from Merck KGaA (D-64293 Darmstadt), n-hexane from Sigma-Aldrich Chemie GmbH (D-89555 Steinheim). MTBE, pentane, and light petroleum were further purified by distillation. Ortho-phosphoric acid (85 %), hydrochloric acid, and acetic acid were purchased from Carl Roth GmbH + Co. KG (D-76185 Karlsruhe).

2.2 Hop samples

Pellets type 90 of five US hop varieties Nugget, Columbus, Chinook, Cascade and Mount Hood (MT Hood) all from the 2011 harvest were available. Pellets type 90 were chosen, because they undergo minimal processing and therefore have tight relation to bale hops. Pellets were stored at –23 °C, prior to any further analysis the water content was determined according to Analytica-EBC (7.2.), α- and β-acids were quantified in triplicate determination by RP-HPLC (*cf. 2.4*).

2.3 Isolation and classification of hop lipids

To isolate hop lipids, bitter acids, and hop essential oil from insoluble and solid hop constituents, to separate and enrich polar, neutral and non-polar lipids, as well as to analyze the concentration of fatty acids in each fraction (polar, neutral, non-polar) the following work up procedure was carried out triplicate for each hop variety:

- *Soxhlet extraction*
  5 g pellets were finely ground by pestle and mortar, than mixed with 15 g sea sand. Extraction was carried out for 10 hours in a Soxhlet apparatus using MTBE as solvent. MTBE has been shown as a very suitable solvent for quantitative lipid recovery of all major lipid classes [35]. Extraction was followed by solvent removal (rotary evaporation), the remaining “Soxhlet extract” was quantified gravimetrically. The concentration of α- and β-acids in the Soxhlet extract was analyzed by RP-HPLC (*cf. 2.4*).
- *LC separation of polar, neutral and non-polar lipids*
  500 mg Soxhlet extract were resolved in MTBE. Internal standards (glyceryl tritridecanoate, methyl heptadecanoate, and pentadecanoic acid) were added. The extract was separated into 30–40 eluate fractions (5–10 mL each) by liquid chromatography. The stationary phase for LC separation was silica gel 60 (20 g), the mobile phase was a quasi-gradient: 100 mL light petroleum + ethylacetate + acetic acid (700 + 300 + 1 by volume), 150 mL light petroleum + ethylacetate + acetic acid (500 + 500 + 1 by volume). For final elution of polar lipids the column was flushed with 100 mL ethylacetate.
- *Assignment of lipid classes by thin layer chromatography (TLC)*
  Aliquots of all eluate fractions were spotted onto silica gel 60 TLC plates from Merck KGaA (D-64293 Darmstadt). For TLC separation a mobile phase of hexane + ethylacetate + acetic acid (600 + 400 + 1 by volume) was used. Spots were highlighted by spraying with phosphomolybdic acid. The polarity range of the substances in each fraction was judged by reference plates, that contained lipids of different polarities, terpene hydrocarbons, terpene alcohols, and bitter acids. The retention factors (R_F) of the reference materials are given in table 1.
- *Preparation of fatty acid methyl esters*
  Fractions with RF = 1.0–0.69 (non-polar), RF = 0.68–0.39 (neutral), RF = 0.38–0.15 (polar I), and those with RF < 0.15 (polar II) were combined and solvent was removed. Each fraction was analyzed gravimetrically. The fraction polar I, which contains MAG, PG and GG was additionally spiked with lab-made D2-heptadecanoic acid. To enable gas chromatographic separation esterified fatty acids and FFA where converted into fatty acid methyl esters (FAME). Briefly, transesterification was carried out as follows: Extracts (non-polar, neutral, polar I) were re-dissolved in MTBE. Aliquots of 100 µL were transferred in screw-cap vials with micro insert (200 µL), than 50 µL of 2.0 M Trimethylsulfoniumhydroxid (TMSH) in methanol from Macherey und Nagel GmbH & CO. KG (D-52355 Düren) were added. The solution was Vortex-mixed for 30 seconds, allowed to react for 20 minutes at room temperature. FAME were separated and analyzed as described in *2.6*.

Table 1 **Retentions factors (R_F) of 13 reference materials on silica gel 60 TLC plates using hexane + ethylacetate + acetic acid (700 + 300 + 1 by volume) as mobile phase**

reference material	Retention factor (Rf)	classification
triacontanyl palmitate	0.92	non-polar
cholesteryl oleate	0.86	
glyceryl trioleate	0.82	
terpenes (e.g. myrcene)	0.80–0.90	
oleate	0.69	
terpene alcohols (e.g. linalool)	0.50	neutral
glycerol-1,2- and -1,3-dioleate	0.35–0.45	
oleic acid	0.39	
L-α-phosphatidylcholine	0.25	polar I
glucose oleate	0.15	
1-oleoyl-glycerol	0.15	
bitter substances	0.1–0.15	polar II

2.4 Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) analysis

The analysis of α- and β-acids from hop pellets and Soxhlet extracts was based on method Analytica-EBC 7.7 [36]. The analysis was performed on a Shimadzu LC-2010CHT Liquid Chromatograph using 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, bidest. water drawn from a Merck Millipore Synergy® UV ultrapure 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- and β-acids 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).

2.5 Analysis of hop essential oils

Hop essential oils were isolated by simultaneous distillation extraction (SDE) according to a method previously described [37, 38]. Internal standards were 6-methyl-5-[1,1,1,3,3-$^{2}H_{5}$]hepten-2-one (D_5-6-methyl-5-hepten-2-one), 3,7-[10,10,10-$^{2}H_{3}$]dimethyl-1,6-octadien[4,4-$^{2}H_{2}$]-3-ol (D_5-linalool), 2-octanol and 1(^{13}C)-methyl-octanoate. D$_5$-6-methyl-5-hepten-2-one and D$_5$-linalool were synthesized according to the method of *Kreck* et al. [39]. 1(^{13}C)-methyl-octanoate was obtained by methylation of 1(^{13}C)-octanoic acid (99 atom % ^{13}C, Sigma-Aldrich) using an ethereal solution of diazomethane (CH_2N_2). Diazomethane was prepared by hydrolysis of Diazald™ (N-methyl-N-(*p*-tolylsulfonyl)nitrosamide, Sigma–Aldrich) in diethyl ether with 10 % sodium hydroxide in methanol. 2-Octanol (purity ≥ 97 %) was purchased from Sigma-Aldrich Chemie GmbH (D-89555 Steinheim).

2.6 Gas chromatography – mass spectrometry (GC-MS)

GC-MS analysis was performed on Shimadzu GCMS-QP2010 Plus applying a DB 5UI ms column (inner Ø = 0.25 mm, 0.25 µm film thickness, length = 30 m) from Agilent Technologies (D-71034 Böblingen). This non-polar phase showed suitable performance to separate target compounds, except for oleic and linoleic acid which were distinguished by MS only. Helium was used as carrier gas. For FAME analysis the injector temperature was set to 250 °C, head pressure to 69,4 kPa. 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 120 °C, raised by a rate of 6 °C/minute until reaching 250 °C, than 70 °C/minute up to 300 °C (hold for 60 sec). 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=29–600). For the analysis of hop essential oils column oven temperature program was set at an initial temperature of 80 °C, raised by a rate of 6 °C/minute until reaching 220 °C, than 70 °C/minute up to 300 °C (hold for 60 sec). Ion source of quadrupole mass spectrometer (QP-MS) was adjusted to 200 °C, the interface temperature to 220 °C. Compound identity was confirmed by comparison of retention times and EI mass spectra with analytical standards. Identification of γ-linoleic acid (6*Z*,9*Z*,12*Z*)-6,9,12-octadecatrienoic acid), stearidonic acid (6*Z*,9*Z*,12*Z*,15*Z*)-6,9,12,15-octadecatetraenoic acid, eicosadienoic acid ((11*Z*,14*Z*)-11,14-eicosadienoic acid), henicosanoic acid, erucic acid ((13*Z*)-docosenoic acid), n-hexacosane, 1-hexacosanol, n-heptacosane, 1-heptacosanol, 2-methyloctadecane, n-octacosane, 1-eicosanol, and 1-docosanol was reached comparison of EI mass spectra of the respective peaks with NIST 2008 database.

2.7 Quantification, calibration and validation

Linear calibration curves were established for all major fatty acids, arachidic acid, and behenic acid. Analytes were mixed with all four internal standards in molar ratios from 0.1 to 5.0 and diluted to match the dynamic range of MS detection. The coefficient of determination (R^2) was at least 0.98. The quantification of γ-linoleic acid and stearidonic acid was carried out using the calibration curve of α-linolenic acid, for eicosadienoic acid the calibration curve of linoleic acid was applied. Henicosanoic acid, erucic acid, hexacosane and 1-hexacosanol were quantified using the calibration curve of stearic acid. All analytical procedures (extraction, LC separation, gravimetrical, GC-MS and RP-HPLC analysis) were carried out in triplicate. For the results obtained by GC-MS analysis of FAME the coefficient of variation was below 12 % for all varieties and fractions within the triplicate determination. The only exception was myristic acid with a coefficient of variation below 15 %.

3 Results and discussion

3.1 Extraction of hop pellets using MTBE

The extraction of hop pellets using MTBE as solvent yielded 35.5–50.7 % extractable substances drawn to pellet dry matter. By LC the total Soxhlet extract was separated into non-polar, neutral and polar fractions. Drawn to the Soxhlet extract the polar compounds were the most abundant fraction (47–67 %). 25–43 % of the polar substances in the Soxhlet extract were identified as α- and β-acids by RP-HPLC. Hop varieties high in total resins (α- and β-acids) had the highest yield in MTBE extractable compounds. The concentration of α- and β-acids in the Soxhlet extract correlates with the original α- and β-acid concentration except for Cascade and MT Hood (cf. Table 2, see page 180).The neutral fraction accounts for only approx. 4–6 % of the Soxhlet extract, 3 % drawn to pellet dry matter respectively. Non-polar substances were in the range of 8.6–14.0 % of pellet dry matter. Varieties with high total Soxhlet extract yield, such as Columbus or Nugget, contained more non-polar substances that e.g. Cascade or MT Hood pellets. Table 2 gives a detailed overview on the amount and composition of the MTBE extractable hop constituents and the concentration of α- and β-acids in the original pellets.

Table 2 **Concentration of MTBE extractable fractions from hop pellets given as g/100g pellet or as % of the Soxhlet extract. Data from RP-HPLC analysis is indicated by"**", data derived from gravimetrical analysis by "*"**

		Columbus	Nugget	Chinook	Cascade	MT Hood
Total Soxhlet extract*	(g/100 g pellet)	50.7 ± 0.5	41.6 ± 0.3	38.6 ± 0.3	39.5 ± 0.4	35.5 ± 0.3
α- and β-acids**	(g/100 g pellet)	18.21 ± 0.3	17.15 ± 0.5	14.63 ± 0.3	10.97 ± 0.3	11.2 ± 0.2
non-polar fraction*	(g/100 g pellet)	14.01 ± 0.3	10.78 ± 0.2	9.72 ± 0.3	8.63 ± 0.2	8.72 ± 0.2
	% of Soxhlet extract	27.6	21.3	19.2	17.0	17.2
neutral fraction*	(g/100 g pellet)	2.84 ± 0.05	2.34 ± 0.05	2.95 ± 0.05	3.19 ± 0.05	2.59 ± 0.05
	% of Soxhlet extract	5.6	4.6	5.8	6.3	5.1
polar fraction*	(g/100 g pellet)	33.84 ± 0.3	28.49 ± 0.3	25.91± 0.4	27.67 ± 0.4	24.23 ± 0.3
	% of Soxhlet extract	66.8	56.2	51.1	54.6	47.8
α- and β-acids in polar fraction**	(g/100 g pellet)	22.21 ± 0.5	15.62 ± 0.3	14.96 ± 0.4	13.07 ± 0.2	12.78 ± 0.3
	% of Soxhlet extract	43.8	30.8	29.5	25.8	25.2
polar compounds (excl. α-/β-acids)	(g/100g pellet)	11.63	12.87	10.95	14.60	11.45
	% of Soxhlet extract	23.0	25.4	21.6	28.8	22.6

3.2 Identification and quantification of polar, neutral and non-polar esterified fatty acids

Individual transesterification of non-polar, neutral and polar fractions (polar I) followed by GC-MS analysis enabled identification and quantification of 13 long chain FA, four medium chain FA, several long chain alcohols and alkanes. The major long chain FA were myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3), arachidic acid (C20:0) and behenic acid (C22:0). Minor long chain fatty acids were γ-linolenic acid, stearidonic acid, eicosadienoic acid, henicosanoic acid and erucic acid. Medium chain fatty acids such as octanoic acid (caprylic acid), decanoic acid (capric acid), docosanoic acid (lauric acid) and (4*Z*)-decenoic acid were identified in the neutral lipid fraction. Aside from major and minor FA the following (minor) alkanes and long chain alcohols were identified in the non-polar fractions: n-hexacosane, 1-hexacosanol, n-heptacosane, 1-heptacosanol, 2-methyloctadecane, n-octacosane, 1-eicosanol, and 1-docosanol. The concentration of the minor FA, n-hexacosane and 1-hexacosanol is given as sum (“others”) in the following. The minor alkanes and long chain alcohols were not quantified. By means of their respective GC-MS peak areas their concentration is estimated below 0.3 mg/g pellet dry matter.

The individual analysis of polar, neutral and non-polar fractions reveals differences in the concentration of individual FA. From this data the relative distribution of FA of each fraction was calculated. Figure 2 shows the relative FA distribution of the polar fraction, whereas figure 3 shows the relative FA distribution of the non-polar fraction.

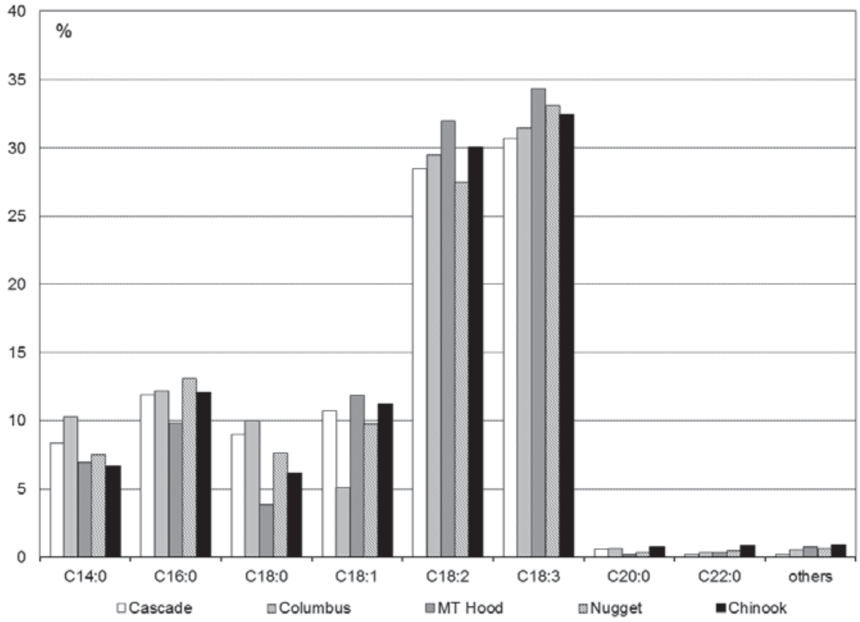

Fig. 2 Relative FA distribution (%) of the polar fraction (PG, GG and MAG) of five pellets varieties

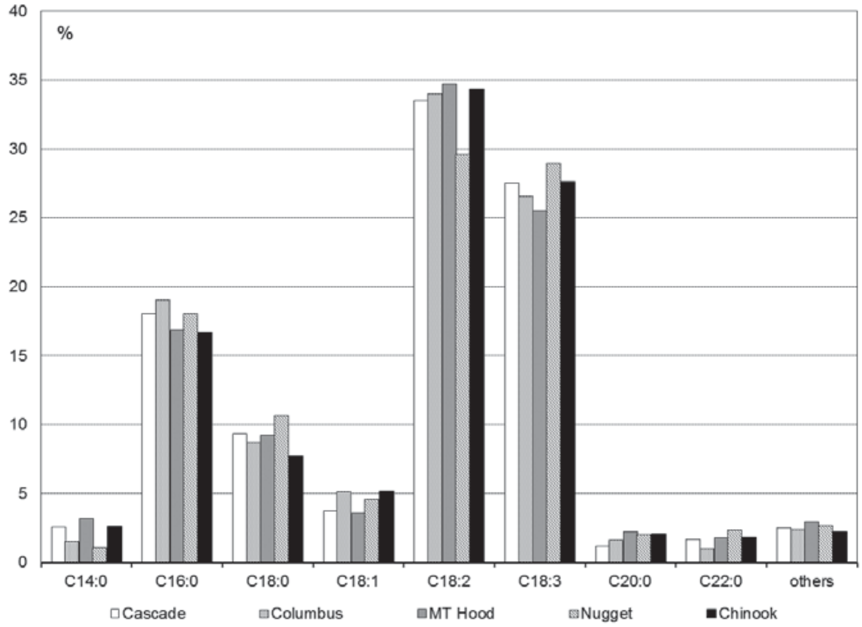

Fig. 3 Relative FA distribution (%) of the non-polar fraction (TAG and wax) of five pellets varieties

In the polar fraction of all five hop varieties α-linolenic acid is the most abundant FA (31.6–34.3 %). Linoleic acid (27.4–31.9 %) is also highly abundant, the concentrations of palmitic acid and oleic acid are in the range of approx. 10 %. In Nugget pellets the concentration of oleic acid is low compared to the other varieties (5.5 %), but elevated concentrations of myristic acid (10.2 %), palmitic acid (12.9 %), and stearic acid (9.9 %) were found.

In the non-polar fraction the concentration of linoleic acid (29.5–34.7 %) is higher than those of α-linolenic acid (25.5–28.9 %) for all pellet varieties. Palmitic acid is present in a share of 16.6–19.1 % of the non-polar hop lipids. Numerical this means: The non-polar fraction contains 9.6–14.5 mg linoleic acid per g pellet, 8.6–11.3 mg α-linolenic acid per g pellet, and 4.7–8.1 mg palmitic acid per g pellet. Palmitic acid concentration in the non-polar fraction is considerably higher than those found in the polar fraction. As described introductory, palmitic acid is usually esterified in waxes, for elevated concentrations in the non-polar fractions could be expected. The non-polar fraction also contains comparable amounts of C20:0 and C22:0 FA, the minor long chain FA were traced in higher concentrations compared to the polar-fraction.

Major alkanes (n-hexacosane, 1-hexacosanol), minor alkanes and long chain alcohols were absent in the neutral and polar fractions. GC-MS chromatograms of the non-polar fractions were also characterized by the presence of terpene hydrocarbons (myrcene, α-humulene, β-caryophyllene), whereas in the neutral fraction linalool could be easily identified. Terpenes were not quantified after LC separation, nevertheless relation of the peak areas (e.g. of myrcene, α-humulene, and β-caryophyllene) was very similar to those obtained after SDE. The FA distribution of the neutral fraction is not illustrated. FA distribution of the neutral fraction is as follows: Linoleic acid (32.5–37.7 %), α-linolenic acid (28.1–32.2 %), palmitic acid (10–12.5 %). In addition to the long chain FA octanoic acid, decanoic acid and (4*Z*)-

decenoic acid were identified. The concentration of these medium chain fatty acids was below 0.01 mg/g pellets which equals 0.5–1 % of the overall FA concentration of the neutral fraction.

3.3 Calculation of the total lipid content

The concentration of total lipids was calculated as sum of FA esterified in polar, neutral and non-polar lipids. Figure 4 shows the relative total FA distribution of all five pellets varieties.

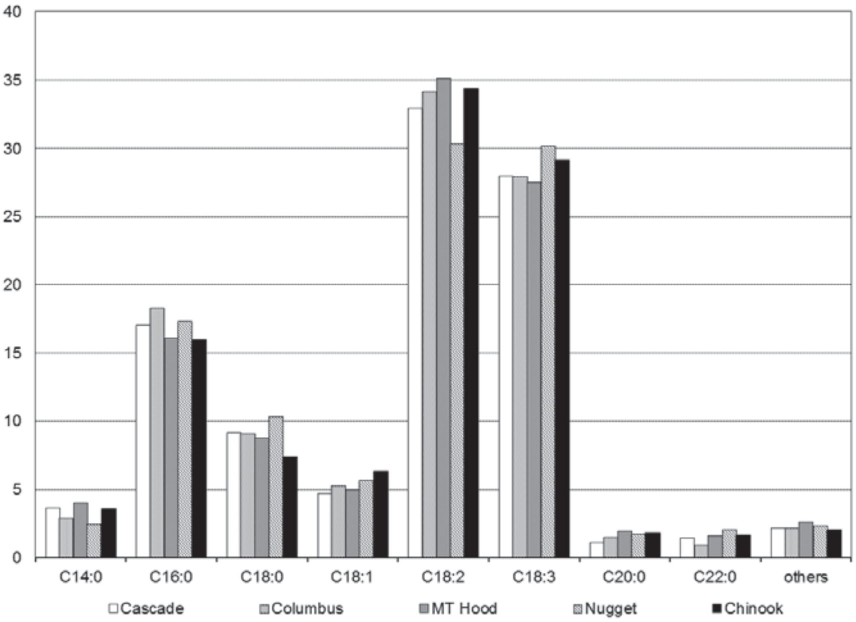

Fig. 4 **Total relative FA distribution (%) of five pellets varieties**

The major FA in hops are linoleic acid (30–36 %), α-linolenic acid (26–31 %) and palmitic acid (16–18 %). In accordance to Carrington et al. [9] and Bravi et al. [29] PUFA represent approx. 70 % of FA in hops. Palmitic acid is the most abundant saturated FA. C20, C22 and the minor FA were in the range of 1–2 %. The total FA distribution (cf. Fig. 4) is very similar across all five varieties, except the Nugget pellets, where α-linolenic acid was found in concentrations equal to linoleic acid. In all other varieties linoleic acid was the most abundant FA. Again, the total lipid content of hop pellets reflects the calculated sum of the individual long chain FA concentrations in the polar, neutral and non-polar fraction. The lipid distribution grouped by polarity is given in table 3. The lipid content is given in mg/g pellets and as a relative distribution in percent (%).

The total lipid content deviates from 35.4 mg/g (3.5 %) in the Chinook pellets to 50.7 mg/g (5.1 %) in the Columbus pellets. Cascade, MT Hood, and Nugget pellets contained approx. 41 mg lipids per g pellet dry matter. The total lipid concentrations are lower than those published by Bravi et al. [29]. For no detailed information on the quantification procedure was given in their work, a detailed discussion is complicated. In their calculation medium chain fatty acids such as caprylic, capric, lauric acid were included. These FA represented up to 12 % of total FA in Saazer hops. We cannot confirm these results. By GC-MS co-elution of lipid and non-lipid material was observed. In case GC-FID equipment in combination with external calibration is used, imprecise quantification – namely over estimation of FAME signals – seems very likely.

The concentration (mg lipids/g pellet dry matter) of non-polar, neutral and polar fractions differ throughout the five hop varieties. Columbus hop pellets contain 42.6 mg/g non-polar lipids, whereas Cascade pellets only contain 33.7 mg/g. However, the relative distribution (given as % of total lipids) of polar, neutral and non-polar lipids is constant throughout all five varieties (cf. Table 3):

- The non-polar lipids (TAG, wax) are always the most abundant fraction and account for approx. 80 % of total lipids (TL).
- The neutral lipids (DAG and FFA) represent the smallest fraction in all pellet varieties. DAG and FFA are either a result of TAG, GG and PG synthesis or their breakdown respectively. Drawn to hop dry matter DAG and FFA concentration is 2 mg/g or 0.2 %. This data is in accordance to the findings of Sandra et al. [10] who found up to 0.4 % FFA in hop cones.
- The occurrence of polar hop lipids has not been described before. The polar lipids range from 11–16 % of total lipids in all five pellet varieties. For brewers polar lipids might be special interest due to two aspects: They are rich in PUFA (cf. Fig. 3), which are susceptible to oxidative degradation. Polar lipids i.e. PG and GG are soluble in wort and beer. They might be easily transferred from hops into product (green or bright beer). Esterified FA are subject to oxidation throughout the brewing process [40] and possibly during beer storage. In accordance to glycosidically bound flavour compounds a liberation of FA during the brewing process and beer storage is likely [41, 42]. In this study a closer examination of polar lipids was not conducted. Targeted analysis of polar lipids is possible using ESI-MS-MS instrumentation, experiments to gain detailed information need to be performed.

3.4 Combined data for lipids, bitter acids and hop essential oil

In addition to lipid analysis bitter substances and steam volatile hop constituents have been analyzed. In table 4 the results of hop essential oil analysis are summarized, also the concentration of bitter substances (α- acids, sum of α- and β-acids) is given for each variety. Twelve principal hop volatiles have been identified

Table 3 **Distribution of non-polar, neutral and polar lipids in five pellet varieties. Results for each fraction are given as mg lipids/g pellet dry matter and as share of total lipids in %**

		non-polar	neutral	polar	sum
Cascade	lipids (mg/g)	33.7	2.0	5.0	40.9
	share in %	82.6	5.0	12.4	
Columbus	lipids (mg/g)	42.6	2.6	5.6	50.8
	share in %	83.8	5.1	11.1	
MT Hood	lipids (mg/g)	33.7	2.5	5.5	41.6
	share in %	80.9	5.9	13.2	
Nugget	lipids (mg/g)	32.9	2.6	5.5	41.0
	share in %	80.3	6.4	13.3	
Chinook	lipids (mg/g)	28.2	1.8	5.4	35.4
	share in %	79.6	5.1	15.2	

and quantified. Their concentration is given as sum of the terpene hydrocarbons (myrcene, α-humulene, β-caryophyllene, farnesene), as sum of the oxygenated terpenes and as sum of both fractions. Terpene hydrocarbons range from 0.55 g/100 g pellets (Cascade) to 1.88 g/100 g pellets (Columbus). The composition of the oxygenated fraction is very complex. The major oxygenated terpenes were: Linalool, nerol, geraniol, α-terpineol, citral, caryophyllene oxide, farnesol, *cis*- and *trans*-linalool oxides. In sum the concentration of oxygenated terpenes ranged from 0.012 g/100 g (MT Hood) to 0.049 g/100 g (Columbus).

Table 4 Concentrations of terpene hydrocarbons, oxygenated terpenes, as well as the α- and β-acids

analytes (g/100g pellets)	Columbus	Nugget	Chinook	Cascade	MT Hood
terpene hydrocarbons	1.88± 0.2	1.86± 0.04	0.82± 0.03	0.55± 0.04	1.45± 0.02
oxygenated terpenes	0.049 ± 0.002	0.028 ± 0.003	0.020 ± 0.003	0.019 ± 0.002	0.012 ± 0.003
∑ volatiles	1.93	1.89	0.84	0.57	1.46
α-acids	13.9 ± 0.2	13 ± 0.2	11.8 ± 0.2	5.5 ± 0.1	6.1 ± 0.1
∑ α- and β-acids	18.21 ± 0.3	17.15 ± 0.5	14.63 ± 0.3	10.97 ± 0.3	11.2 ± 0.2

Columbus and Nugget pellets are especially rich in bitter acids and volatiles, Cascade and MT Hood are very similar in regard to their bitter acid content, whereas MT Hood contains much higher concentrations of volatile substances. The bitter hop varieties Columbus and Nugget contain higher amounts of bitter acids and oils compared to the aroma varieties Cascade and MT Hood. The Chinook variety is something in between, bitter substance concentration is close to those of Columbus and Nugget, the concentration of volatiles is between Cascade and MT Hood. The amount of bitter acids, the composition and concentration of hop essential oil is basically a varietal characteristic. A correlation between bitter acid content and oil content has been described before [43]. Commonly bitter varieties contain many more lupulin glands than aroma varieties, thus they are high in oil and bitter acids [44]. Data for α-acids, the sum of volatiles hop constituents and the lipid content of all five varieties is combined in figure 5.

Columbus and Nugget, both rich in bitter substances and volatiles, are also characterized by high lipid concentrations. In contrast MT Hood and Cascade contain low concentrations of all three substance groups. Chinook hops has relative high concentrations of α-acids, volatiles and lipids are comparably low.

4 Summary and outlook

Lipid analysis has historically been a challenging task. Lipid structures can be found over a wide polarity range, thus comprehensive lipid analysis (lipidomics) from complex biological samples usually requires several analytical techniques [e.g. 45–47]. The isolation and analysis of hop lipids is challenging due to the high concentration of bitter acids. Bitter acids strongly influence lipid extraction and analysis. Their non-volatile nature requires complete removal within sample preparation for GC. In the current study we established an indeed labor intensive, but straightforward route for lipid analysis from hops. LC separation and fractionation of lipid extracts enables the individual analysis of polar, neutral and non-polar lipids. Offline 2D-LC has been shown as powerful technique in lipid analysis [48]. The current paper presents offline LC-GC-MS coupling for hop lipidomics. The transesterification of FA from LC separated polar, neutral, non-polar into FAME, followed by GC-MS analysis, enabled identification and quantification of long chain fatty acids, alkanes and alcohols. GC separation of FAME is an established technique, it enables separation according to carbon number and saturation. GC in combination with EI-MS ensures definite structure assignment and resolves co-elution problems with e.g. terpene hydrocarbons. The separation of positional isomers, e.g. α- and γ-linolenic acid, is an example for a GC-MS feature that is not readily offered by HPLC [45]. Analysis of five hop varieties (pellets type 90) showed to following results: Pellets type 90 contain 30–50 mg lipids/g dry matter. This equals 3–5 % total lipids. Non-polar lipids, such as TAG and waxes, account for approx. 80 % of total lipids. The polar lipids account for approx. 15 %, the neutral lipids for approx. 5 %. Linoleic and α-linolenic acid are the major unsaturated FA, palmitic acid is the principal saturated FA in hops. Differences between the fatty acid patterns of polar and non-polar lipids were observed: The polar fraction was rich in α-linolenic and linoleic acid (> 70 % in sum), the non-polar fraction also contained considerable amounts of palmitic acid (≤ 19 %), also long chain FA with carbon chains of C20–C22,

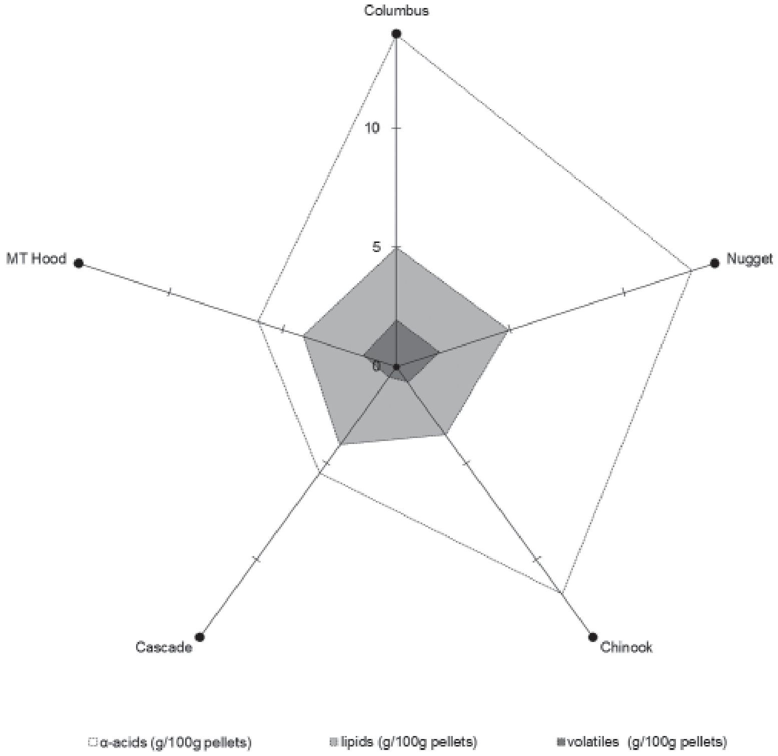

Fig. 5 Spider web diagram including the concentration of volatiles (sum terpene hydrocarbons and terpenes, lipids and α-acids given in g/100 g pellets

long chain alkanes and alcohols were traced. High concentrations of PUFA in the polar fraction result from PG and GG, which are integral membrane lipids. High concentrations of palmitic acid in the non-polar fraction originate from storage fats, and waxes. A correlation of α-acids, hop essential oil concentration and lipids was observed: Hops that are rich in α-acids and essential oil appear to have higher lipid content as well. Analytical techniques to analyze hop lipids and detailed knowledge of hop lipids are important for several aspects: Free and bound unsaturated FA are precursors of potent odorants (such as *trans*-4,5-epoxy-*(E)*-2-decenal) during hop processing. Polar and neutral lipids are soluble in beer, meaning that late hop additions increase the lipid content of beer and might impact flavor stability. At this the hop lipid content might be an additional parameter of hop (product) quality. It has been described that hop processing has strong influence on the lipids in hop products [10], here the current analytical route enables detailed analysis. For hop produces polar lipids might of special interest: PG and GG are very valuable (by-) products of hop extraction. Polar lipids might be used as foam stoppers [49] in the brewing process, but also as detergents in nature cosmetics.

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