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LC-MS/MS Analysis of Hop Flavonoids in Dry-Hopped Beers

As a major ingredient during beer brewing, hops give a characteristic bitter taste to the final product. The quantitative analysis as well as the knowledge of the contribution of hop bitter compounds to the overall bitter taste of beer is therefore essential. HPLC-UV analysis of selected bitter substances like alpha-acids or humulinones (oxidized alpha-acids) in dry-hopped beers is feasible. Reliable structure identification and quantification of hop flavonoids like multifidol, kaempferol and quercetin glycosides require the use of HPLC-MS/MS technique. New findings about key bitter compounds from hops (e.g. co-multifidol glucoside) and their contribution to the bitter profile of beer were introduced and discussed in literature recently. To monitor selected hop flavonoids in dry-hopped beers, an in-house HPLC-MS/MS method was developed. Dry-hopped beers produced with different hop varieties showed significant differences in their hop flavonoids pattern.

Descriptors: dry hopping, hop flavonoids, beer analysis, bitter taste, co-multifidol glucoside

1 Introduction

The use of hops for brewing beer is extremely important, not only due to the characteristic bitterness originating from hop ingredients, but also because hop ingredients influence aroma, microbiological stability, foam and haze formation.

The spectrum of bitter substances in hops includes α- and β-acids which undergo isomerization, transformation or degradation during wort boiling [1–4] and are known to be present in the soft resin fraction of the hop cone. The more polar hard resins consist primarily of prenylflavonoids and glucopyranosides. The total polyphenol content of dried hop cones varies between 3 % and 8 %, depending on the hop variety [5].

Multifidol glucosides (Fig. 1, see page198) were isolated from hops in 2005, for the first time [6]. These compounds are intermediate products of the biosynthesis of α- and β-acids and have the same (acyl) side chains as these bitter compounds. Anti-inflammatory activities were described for multifidol glucosides [6]. The amount in hops is about 0.5 % [5]. Both multifidol glucosides and prenylflavonoids (e.g. xanthohumol) are typical for hops. Xanthohumol (Fig. 1) was discovered in hops more than 100 years ago. The structure determination of this compound was in 1961. The amount in dried hop cones accounts for up to 1.2 %, depending on the hop variety [5]. Xanthohumol is converted to the prenylated flavonoid isoxanthohumol (Fig. 1) during wort boiling [7]. This compound is the major prenylated flavonoid in beer. The hop plant produces 8-prenylnaringenin (8-PN, Fig. 1) by cyclization of desmethylxanthohumol. This reaction also forms 6-prenylnaringenin (6-PN, Fig. 1). The ratio of these two isomers is approximately 1:3 (8-PN:6-PN). The amount of 8-prenylnaringenin in hops is extremely low with less than 0.01 %. The described ratio of 1:3 can also be observed in the beer. In recent years, several positive physiological and pharmacological properties have been reported for the xanthohumol and other prenylflavonoids from hops [8–11].

In recently published work, *Dresel* and coworkers [12] took a closer look to investigate the occurrence of known and unknown bitter compounds in the hard resin fraction of hops and to evaluate their contribution to the overall bitter profile of beer by means of human threshold concentrations. Furthermore, they developed an HPLC-MS/MS method for the quantification of these bitter compounds in hop products, hop extracts and beer samples. Besides a series of literature known xanthohumol derivatives, multifidol glucosides, flavon-3-on glycosides, and p-coumaric acid esters, a total of 11 bitter compounds were reported in this study for the first time [12]. In addition, brewing trials were carried out with hard and soft resin and revealed that the bitter quality of the final beer is positively influenced by hard resin [12]. In another study, Dresel and coworkers [13] quantitatively monitored over 40 bitter compounds in 75 hop varieties and observed their behavior in hops during storage [13].

Flavonol glycosides are a further group of chemical compounds found in hops and many other plants. The amount varies within the different hop varieties but it doesn't exceed 1 % [5]. The composition of quercetin and kaempferol glucosides in the world hop collection (121 different varieties from 17 countries) was investigated by Kammhuber in 2012 [14] and was described to be suitable

https://doi.org/10.23763/BrSc17-20schmidt

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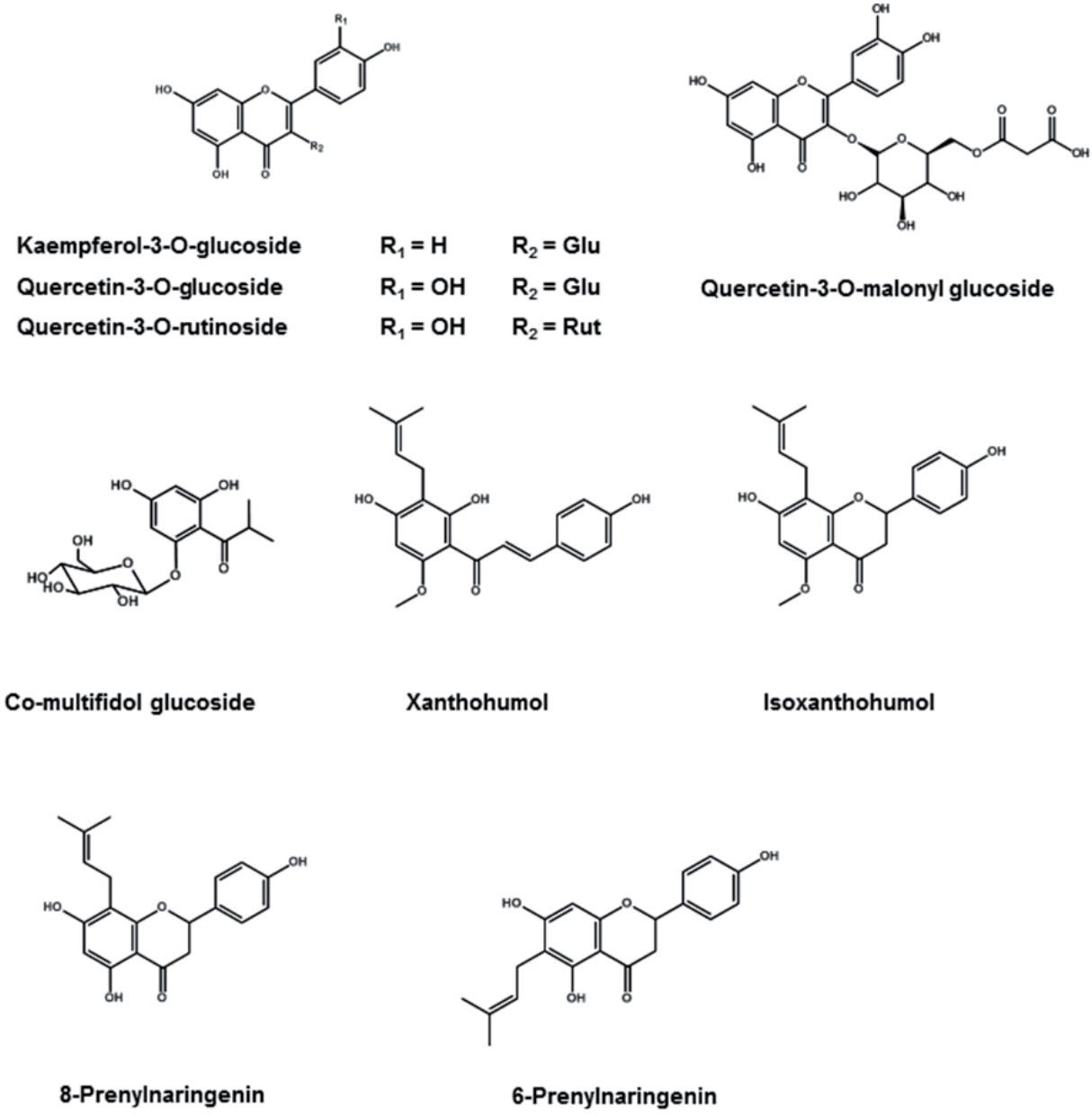

Fig. 1 Chemical structures of hop flavonoids

to differentiate hop varieties. In addition, co-multifidol glucoside, quercetin-3-O-galactoside, quercetin-3-(malonyl)hexoside as well as kaempferol-3-(malonyl)hexoside were identified in different hop varieties in this study.

The human perception of bitterness is imparted by the hTAS2R receptor family with approximately 25 G-protein coupled receptors (GPCRs) [15–19]. Molecular biological studies show that the iso-α-acids, found in beer, as well as the prenylflavonoids xanthohumol, isoxanthohumol and 8-prenylnaringenin activate the three bitter taste receptors hTAS2R1, hTAS2R14 and hTAS2R40 [20].

HPLC-UV analysis of bitter substances like α-acids or humulinones (oxidized α-acids) in dry-hopped beers is feasible. Reliable structure identification and quantification of hop flavonoids like multifidol, kaempferol and quercetin glycosides require the use of an HPLC-MS/MS technique. For this reason, the present study was executed to investigate a suitable HPLC-MS/MS method for monitoring hop flavonoids in dry-hopped beers.

2 Materials and methods

2.1 Reagents

Following chemicals were obtained from commercial sources: water and methanol for LC-MS: (Chemsolute®, Th. Geyer GmbH & Co. KG, Renningen, Germany); formic acid and ammonium formate (Merck, Darmstadt, Germany). The substances quercetin-3-O-glucoside, quercetin-3-O-rutinoside, quercetin-3-O-malonylglucoside, kaempferol-3-O-glucoside were obtained from Merck (Darmstadt, Germany). The internal standards bentazon, dicamba, and nicarbazin were obtained from LGC Standards GmbH (Wesel, Germany). The purified standard for the compound co-multifidol glucoside was

provided by Technical University of Berlin. The calibration standards xanthohumol, isoxanthohumol and 8-prenylnaringenin were purchased from Orgentis Chemicals (Gatersleben, Germany).

2.2 Sample preparation

For the analysis of beer samples, the beverage was decarbonated by manual shaking. After dilution steps (1:100, 1:50, 1:20 (v/v)) with a mixture of HPLC solvent A/solvent B (50/50, v/v) and addition of internal standards (final concentration: c(dicamba) = 10 ng/ml, c(bentazon) = 10 ng/ml, c(nicarbazin) = 1 ng/ml), the samples were analyzed by LC-MS/MS.

2.3 Sample analysis with liquid chromatography – tandem mass spectrometry (LC-MS/MS)

The HPLC system, consisting of a binary pump, a degasser, an auto-sampler and a thermostatted column oven (Shimadzu Corporation, Kyoto, Japan), was coupled with API 3000 mass spectrometer (SCIEX, Darmstadt, Germany) running in the negative ion mode. Samples were introduced by HPLC at a solvent flow of 200 µl/min, which required the use of turbo gas at a temperature of 490 °C. The ion spray voltage was set to −4200 V, the declustering potential and the MS/MS parameters were optimized for each substance to induce fragmentation of the pseudo molecular ion $[M-H]^-$ to the corresponding target product ions after collision-induced dissociation. The collision energy (CE), the declustering potential (DP) as well as the cell exit potential (CXP) were set as given in table 1. Nitrogen was used as the collision gas. The quantitation was done using the scheduled multiple reaction monitoring (MRM) mode of the instrument with the fragmentation parameters optimized prior to analysis and the retention times of the corresponding reference compounds. Data processing was performed by using Analyst software version 1.5.1 and data integration was done by MultiQuant software version 3.0.2 (SCIEX, Darmstadt, Germany). For chromatography, an analytical 50 x 2.0 mm Synergi 4µ Fusion-RP 80A column (Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same type (Phenomenex, Aschaffenburg, Germany) was used. 5 mM ammonium formate containing 0.1 % formic acid in water was used as solvent A and methanol with 5 mM ammonium formate and 0.1 % formic acid as solvent B. The temperature of the column oven was set at 40 °C. The injection volume was 20 µl. Chromatography was performed by increasing solvent B from 20 to 100 % within 8 min and holding for 2 min. Quantitation was done by external calibration in a range between 5 and 500 ng/ml. 6-Prenylnaringenin was quantified using 8-prenylnaringenin because of the lack of a reference compound for 6-prenylnaringenin.

Table 1 Specific mass transitions and optimized parameters for the LC-MS/MS analysis of hop flavonoids

Compound	mass transitions *m/z* Q1→Q3	DP[a] [V]	CE[b] [V]	CXP[c] [V]
6-Prenylnaringenin	339.0 → 218.8[d]	–81	–30	–15
	339.0 → 119.0	–81	–42	–7
8-Prenylnaringenin	339.0 → 218.8[d]	–81	–30	–15
	339.0 → 119.0	–81	–42	–7
Bentazon (IntStd)	239.0 → 197.0[d]	–66	–26	–15
	239.0 → 131.9	–66	–38	–9
Co-multifidol glc	357.0 → 194.7[d]	–76	–18	–13
	357.0 → 150.9	–76	–50	–9
Dicamba (IntStd)	218.9 → 174.8[d]	–36	–8	–11
	218.9 → 35.0	–36	–30	–3
Isoxanthohumol	353.1 → 118.9[d]	–76	–38	–7
	353.1 → 232.9	–76	–24	–21
Kaempferol-3-O-glc	446.9 → 283.8[d]	–76	–38	–15
	446.9 → 254.8	–76	–58	–13
Nicarbazin (IntStd)	301.0 → 136.5[d]	–31	–44	–19
	301.0 → 137.0	–31	–16	–9
Quercetin-3-O-glc	463.0 → 300.1[d]	–86	–38	–17
	463.0 → 271.0	–86	–62	–21
Quercetin-3-O-mal-glc	549.1 → 505.1[d]	–56	–14	–15
	549.1 → 299.9	–56	–42	–21
Quercetin-3-O-rut	609.0 → 300.0[d]	–76	–50	–19
	609.0 → 271.1	–76	–82	–15
Xanthohumol	353.1 → 118.9[d]	–86	–40	–1
	353.1 → 232.8	–86	–26	–19

[a] Declustering potential. [b] Collision energy. [c] Cell exit potential. [d] Quantifier ion. IntStd: Internal Standard

Table 2 Limit of detection (LOD) and limit of quantification (LOQ) in mg/L for hop flavonoids

Compound	LOD [ppm]	LOQ [ppm]
8-Prenylnaringenin	0.01	0.05
Co-multifidol glucoside	0.05	0.1
Isoxanthohumol	0.05	0.1
Kaempferol-3-O-glucoside	0.01	0.05
Quercetin-3-O-glucoside	0.01	0.05
Quercetin-3-O-rutinoside	0.01	0.05
Quercetin-3-O-mal-glucoside	0.01	0.05
Xanthohumol	0.05	0.1

The limits of detection as well as the limits of quantification for all substances tested are given in table 2.

2.4 Beer samples

Beers (Pale Ales) from two different brewing trials (with two different base beers) were investigated by the LC-MS/MS method described above.

Table 3 Dry hopping – Recipe

	Variety	Product	Quantity
Trial 1			
Beer No. 1	Apollo	P90	500 g/hl
Beer No. 2	Bravo	P90	500 g/hl
Beer No. 3	Calypso	P90	500 g/hl
Beer No. 4	Denali	P90	500 g/hl
Beer No. 5	Lemondrop	P90	500 g/hl
Trial 2			
Pale Ale No. 1	Lemondrop	P90	500 g/hl
Pale Ale No. 2	Bravo	P90	500 g/hl

P90 = pellets type 90

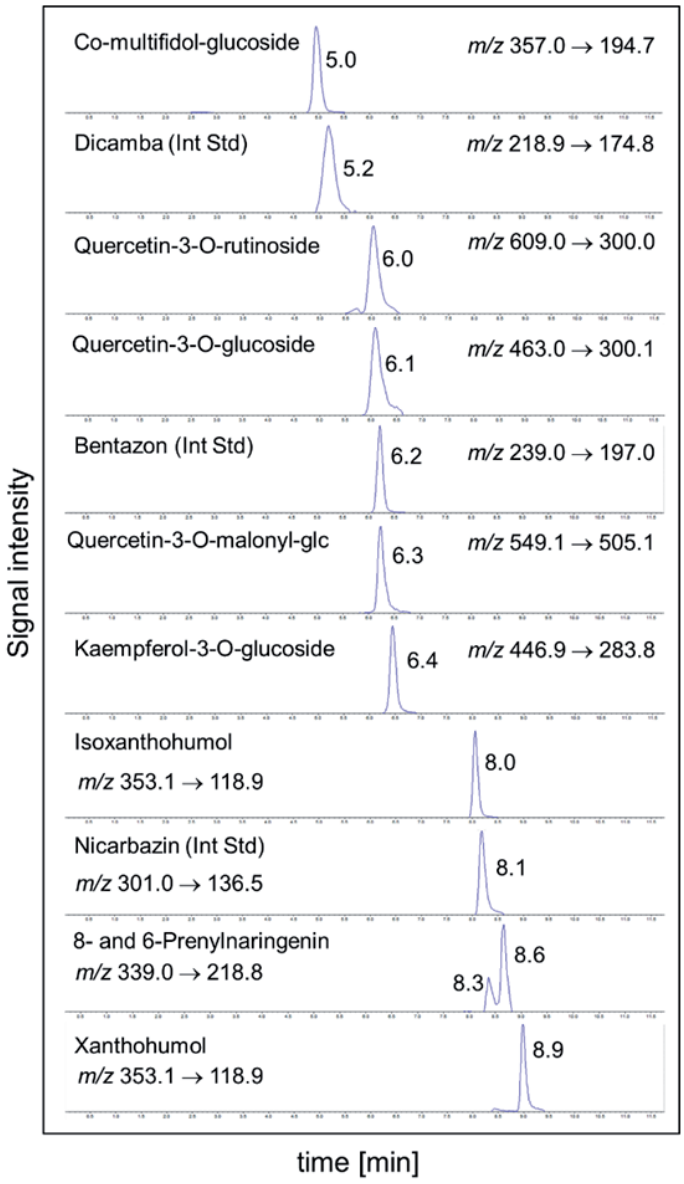

Fig. 2 LC-MS/MS chromatograms of selected hop flavonoids in a dry-hopped beer

2.4.1 Trial 1

For the first trial, pure alpha extract [21] as the only hop product was dosed at the begin of boiling to achieve approximately 20 IBUs. This base beer (4.6 vol.-% alcohol, 21 IBUs, pH 4.0) was then split into different parts and each was dry-hopped using one of the five varieties shown in table 3. Dry-hopping was done in a static way in Cornelius kegs for 7 days at 3 °C. The beers were not filtered.

2.4.2 Trial 2

For the second trial, another Pale Ale base beer was brewed (6.0 vol.-% alcohol, 24 IBUs, pH 4.3). In the brewhouse Hallertau Tradition (pellets type P90 with an α-acid content of 3.8 %) has been used for the Pale Ale base beer. Therefore, 70 % of it were added at the start of boiling and 30 % of it 20 minutes prior to knock out. The base beer was split and dry-hopping was done in a dynamic way using the HopGun (BrauKon GmbH, Seeon, Germany) with two different hop varieties Lemondrop and Bravo (Table 3), followed by conditioning for 14 days at 2 °C. The beers were not filtered.

3 Results and discussion

The developed HPLC-MS/MS method allows the identification and quantitation of 9 hop flavonoids in dry-hopped beers using a single LC-MS/MS run with selective mass transitions as given in figure 2. As internal standards dicamba, bentazon and nicarbazin were applied to the method. These compounds cover different retention time areas and are absent in beer. Dicamba was used as internal standard for co-multifidol glucoside, bentazon was taken for kaempferol and quercetin glycosides and finally nicarbazin was in use for the remaining compounds.

The base beer of trial 1 was prepared using alpha extract only for bittering. None of the substances tested was detected by this method in the base beer. The results for beers No.1-5 show the influence of dry hopping on the hop flavonoid composition. The quantitative data are summarized in table 4.

The highest amount for co-multifidol glucoside with 3.32 mg/L was observed in beer No. 2 with the hop variety Bravo. The lowest amount (0.38 mg/L) was detected in beer No. 4, dry-hopped with the hop variety Denali. Significant differences based on different hop varieties were observed for the bitter compound co-multifidol glucoside with exception of beer No. 1 and beer No. 5.

The highest amounts for all substances were determined in beer No. 2 (with Bravo) and the lowest amounts showed beer No. 4, with the hop variety Denali. 6- and 8-prenylnaringenin were not detected in the dry-hopped beers because of the extremely low amounts of these substances in hops. The concentration of isoxanthohumol was not higher than 0.11 mg/L. The amount of this compound is higher if the hop dosage takes place before wort boiling due to the conversion of xanthohumol to isoxanthohumol [7]. Xanthohumol was determined with a concentration range between 0.10 and 0.18 mg/L. These results are conformable to literature data [22]. *Gahr* et al. described amounts of 0.10, 0.13 and 0.17 mg/L of xanthohumol in dry-hopped beers [22].

The analysis of Pale Ale base beer (trial 2) gives quantitative data for 8 of 9 hop flavonoids tested. The results, given in figure 3, show the transfer of these substances into the beer during wort boiling

already. The impact of dry hopping on the hop flavonoids pattern in beer is obvious after subtraction of the results from the Pale Ale base beer. 8-Prenylnaringenin is absent in this figure because the concentrations in beers were below the quantitation limit of 0.05 mg/L.

Both dry-hopped beers, Pale Ale No. 1 and No. 2, showed a significant increase in comparison with the base beer for co-multifidol glucoside, quercetin-3-O-glucoside, kaempferol-3-O-glucoside and xanthohumol. No changes were observed after dry-hopping for the two remaining quercetin derivatives quercetin-3-O-rutinoside and quercetin-3-O-malonyl-glucoside in Pale Ale No. 1 but there was a significant increase for these compounds in Pale Ale No. 2 compared with the base beer. No impact of dry-hopping on the amounts of 6-prenylnaringenin was determined. Isoxanthohumol decreased in both Pale Ales in comparison with the base beer.

Table 4 Quantitation results in mg/L (± standard deviation, n = 3) for hop flavonoids in beers of Trial 1

Compound	Base beer	Beer No. 1	Beer No.2	Beer No.3	Beer No.4	Beer No.5
Co-multifidol glc	n.d.	1.48	3.32	1.15	0.38	1.59
		(± 0.23)	(± 0.01)	(± 0.02)	(± 0.03)	(± 0.05)
Quercetin-3-O-glc	n.d.	0.72	1.72	0.63	0.48	0.38
		(± 0.03)	(± 0.05)	(± 0.01)	(± 0.01)	(± 0.01)
Quercetin-3-O-rut	n.d.	0.46	0.54	0.33	0.22	0.34
		(± 0.02)	(± 0.01)	(± 0)	(± 0)	(± 0.02)
Quercetin-3-O-mal-glc	n.d.	0.30	1.24	1.30	0.43	0.55
		(± 0)	(± 0.09)	(± 0.07)	(± 0)	(± 0.08)
Kaempferol-3-O-glc	n.d.	0.21	0.38	0.19	0.11	0.52
		(± 0.01)	(± 0)	(± 0)	(± 0)	(± 0.02)
Isoxanthohumol	n.d.	0.10	0.11	0.10	0.10	
		(± 0)	(± 0)	(± 0)	(± 0)	n.d.
Xanthohumol	n.d.	0.10	0.16	0.18	0.10	n.d.
		(± 0)	(± 0.01)	(± 0.02)	(± 0)	
6-Prenylnaringenin#	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-Prenylnaringenin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

6-Prenylnaringenin quantified with 8-prenylnaringenin. n.d. = not detected.

For the ratio of quercetin-3-O-glucoside and kaempferol-3-O-glucoside in beer No. 5 (trial 1) and Pale Ale No. 1 (trial 2), both dry-hopped with the hop variety Lemondrop, a different behavior was detected in comparison with the ratio of these 2 compounds to all other beers tested. It could be observed that the amounts of these 2 compounds were equal in these 2 beers whereas the concentration of quercetin-3-O-glucoside was higher in comparison with the kaempferol-3-O-glucoside amount in other beers. The composition of quercetin and kaempferol glucosides is genetically determined and therefore depends on the variety [14].

To evaluate the influence of bitter compounds on the overall bitter profile of a dry-hopped beer, the knowledge of so called flavour thresholds is necessary. A direct contribution can be assumed if the concentration found in the beer is higher than the flavour threshold for this compound. Apart from that, additive effects play a role. Dresel et al. published in 2015 [12] human recognition threshold concentrations of taste compounds found in hops. The published threshold concentrations were determined in aqueous ethanolic solution with a pH of 4.4. Table 5 (see page 202) gives the flavour thresholds from this article. Co-multifidol glucoside and all the prenylflavonoids were perceived exclusively bitter whereas the glycosides of quercetin and kaempferol evoke also an astringent taste. The flavour threshold for co-multifidol glucoside is 1.8 mg/L [12]. The concentrations of this bitter compound in the 3 beers from trial 2 exceed the bitter flavour threshold. Therefore the contribution to the overall bitter profiles of these dry-hopped beers can be expected. Also the amount of co-multifidol glucoside in beer No. 2 from trial 1 exceeds the flavour thresholds for bitterness. The concentrations of quercetin-3-O-glucoside and kaempferol-3-O-glucoside were lower than the bitter flavour thresholds for these compounds. However the detected amounts exceeded the astringent flavour thresholds in the 3 beers of trial 2 as well as in beer No.2 of trial 1. A contribution to the overall astringent profiles of these dry-hopped beers can be expected. The

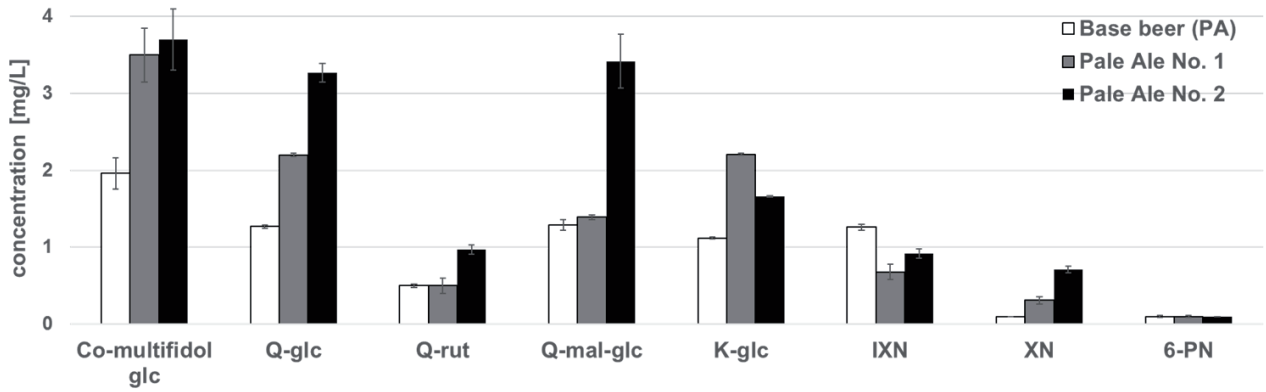

Fig. 3 Amounts [mg/L] and standard deviation (n = 3) of hop flavonoids in beers of trial 2. (Q-glc = quercetin-3-O-glucoside; Q-rut = quercetin-3-O-rutinoside; Q-mal-glc = quercetin-3-O-malonyl-glucoside; K-glc = kaempferol-3-O-glucoside; IXN = isoxanthohumol; XN = xanthohumol; 6-PN = 6-prenylnaringenin, quantified with 8-prenylnaringenin)

Table 5 Sensory quality and threshold concentrations in mg/L of selected hop flavonoids taken from [12]

Compound	sensory quality	threshold concentration [mg/L]
Co-multifidol glucoside	bitter	1.8
Kaempferol-3-O-glucoside	bitter/astringent	13/0.5
Quercetin-3-O-glucoside	bitter/astringent	13/0.9
Xanthohumol	bitter	3.5
Isoxanthohumol	bitter	5.6
6-Prenylnaringenin	bitter	3.4
8-Prenylnaringenin	bitter	2.7

determined concentrations of xanthohumol, isoxanthohumol and 6-prenylnaringenin were below the flavour thresholds in all beers tested. Dry-hopped beers in combination with roasted malt, like for example a dry-hopped stout, can lead to xanthohumol amount of e.g. 6 mg/L, which is over its flavour threshold [23].

4 Conclusion

The developed HPLC-MS/MS method allows the identification and quantitation of selected hop flavonoids in dry-hopped beers using a single LC-MS/MS run. Dry-hopped beers produced with different hop varieties showed significant differences in their hop flavonoids pattern. The contribution of these bitter substances to the overall bitter profile of beer could be evaluated using the quantitative data and the flavour thresholds known from literature.

Acknowledgements

We thank Dr. Philip Wietstock from Technical University of Berlin for providing the purified standard of co-multifidol glucoside.

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Received 17 August 2017, accepted 21 November 2017