K. Haslbeck, S. Jerebic, and M. Zarnkow

Characterization of the Unfertilized and Fertilized Hop Varieties Progress and Hallertauer Tradition – Analysis of Free and Glycosidic-Bound Flavor Compounds and β-Glucosidase Activity

Fertilized or unfertilized, hops have an impact on the characteristics of beer that determine quality. Unique, comparable samples of unfertilized and fertilized plants of the Progress (Goudhurst, United Kingdom) and Hallertauer Tradition (Hüll, Germany) varieties were cultivated. Different cultivation methods were applied depending on the growing region. The success of the methods was verified by high seed contents of fertilized plants and only minimal formation of semen by plants that were prevented from pollinating. The comparability of the samples was targeted by similar growth and harvest conditions, managed at the same location. No significant differences occurred in the composition of the essential oils of fertilized or unfertilized samples. The disadvantages of fertilized hops as a result of decreased α-acid content or a lower essential oil quantity as described in the literature could not be confirmed. The glycosidically bound flavorings of fertilized or unfertilized hop samples were released by preparation of the Rapidase F64 enzyme. Approximately 2 µg of glycosidically bound linalool could be released from one gram of hops (dry matter). In the hops, β-glucosidase enzyme activity could be verified at approximately 0.11 U/g (dry matter hops) on average in all examined samples.

Descriptors: *Humulus lupus* L., unfertilized and fertilized hops, glycosides, essential oil, gas chromatography, β-glucosidase activity

1 Introduction

Today there is a similar wide distribution of both farming methods for unfertilized and fertilized (brewers) hops [1, 2, 3]. In countries such as the United Kingdom, USA and Australia wind pollination of female hop plants is accepted or even desired. The reasons may be agronomic (earlier closing of flowering, reduced disease susceptibility) and economic (seed content is heavier, larger clusters). Nevertheless, in many countries there are prejudices against hops with an increased seed share [4]. Male plants are actually forbidden in growing regions in Germany [5]. Along with other reasons (some hop varieties: a loss of lupulin content or reduction of essential oil [3, 5]) this is due to the high lipid content of hop seeds (up to 32 %), which is claimed to have a damaging impact on the quality of lager beers [6]. In this context it was demonstrated that only a minimal proportion of the fatty acids of seeds merges into the finished product when hops are added at wort boiling [7, 8]. Various working groups performed comparative brewing experiments with unfertilized and fertilized hops. It is not possible to derive a tendentious adverse influence on the quality of beer from that. In most cases no significant differences between the beers were detected [1, 4, 9, 10]. However, these results were not confirmed by all studies [11, 12, 13]. From an agronomic point of view the fertilization and prevention of fertilization of female hop plants have both advantages and disadvantages.

Hop plants have certain enzymes to synthesize glycosides. These enzymes could be used in reverse to release glycosidically bound flavor substances. Glycosidically bound flavor substances are odorless, non-volatile molecules that consist of an aglycone and a sugar residue. The aglycone represents the flavor-active substance [14]. Examples of flavor-active aglycones that have been identified in various plants and fruits are medium-chain alkanols and alkenols, derivatives of shikimic acid, C_{13}-norisoprinoides, and monoterpene and sesquiterpene alcohols [15]. The diastatic activity of hops, which was first described by *Janicki and Kotasthane* is an interesting feature for brewers [16]. Hops added to beer during storage can cause a secondary fermentation via the hydrolysis of dextrines to fermentable sugars. They showed that the diastatic activity is greater in fertilized plants than in unfertilized plants [16]. With regard to the release of flavor-active aglycones from hop glycosides, these results have yet another meaning. *Kollmannsberger* and *Nitz* showed that a commercially available

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Authors

Korbinian Haslbeck, Martin Zarnkow, Technical University of Munich, Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany; Sebastian Jerebic, corosys Prozeßtechnik und Sensoren GmbH, Hofheim, Germany; corresponding author: Martin.Zarnkow@tum.de

amylase preparation splits glycosides very poorly; nonetheless the aglycones were still released [17].

Based on these considerations, samples of fertilized and unfertilized hops Progress and Hallertauer Tradition were prepared and analyzed in terms of the range of their free and glycosidically bound flavor substances. Furthermore, the glycoside-hydrolytic activity of both patterns was verified in the present work.

2 Materials and methods

The hop varieties Hallertauer Tradition HHT (Hüll, D-85253 Wolnzach) and Progress PG (Cranbrook, GB-TN17 Kent) were grown in a field test in season 2013. Each of the fertilized and unfertilized plants were cultivated in the same field at similar agronomic conditions. In Germany, six HHT vines (HHT-F) were artificially fertilized with 2 g pollen of different male plants and covered with a plastic film to prevent further fertilization (plastic film was removed after three days). Twelve plants were grown conventionally (HHT-U). In UK, wind pollination was (virtually) prevented by packing the female PG hop bines (before bloom) into pollination bags, containers for controlling pollination. Three different types of hops were prepared and nine vines of each were grown. Among these unfertilized hops (packed in pollination bags, PG-U), fertilized hops (ripened without pollination bags, PG-F) and a control sample (comparatively seeded hops packed after bloom in pollination bags, PG-C). All bines were manually plucked and dried at 60 °C. Hop cones were crushed using a knife mill (Retsch GM 300, NATECO$_2$, Wolnzach, Germany) in reverse rotation (10 s at 2000 rpm) to prevent damaging the semen. The hops were then vacuum-packed in 30 g bags and stored at 0 °C.

The water content of the hops was analyzed according to MEBAK methods [18]. The seed, α-acid and essential oil content of the hops were analyzed according to the Analytica-EBC methods [19, 20]. The essential oil was used for further gas chromatographic analysis.

Glycoside extracts of hops were produced and then cleaved using the technical glycosidase Rapidase F64 (DSM Food Specialties, Düsseldorf) into free aglycones and equivalent amounts of sugar according to Kollmannsberger and Nitz [17]. Briefly, the solid residue of an ether extract of hops (5 g) was suspended in methanol and 2 µmol of phenyl-β-D-glucopyranoside (Sigma-Aldrich, Taufkirchen, Germany) added. After a total of 72 hours for the exposure phase and two filtration steps, the methanolic phase was concentrated to dryness in a round-bottom flask. The residue was incorporated in 50 ml of McIlvaine buffer solution (pH 5.00) and two 25-g-samples of each were transferred into 50-ml flasks with a ground glass stopper. One batch was used as a blank sample, 25 mg Rapidase F64 was added to the second one for cleavage. Both samples were sealed and incubated for 67 h at 40 °C. The free aglycones were isolated in diethyl ether, dried over Na_2SO_4 and concentrated in a Vigreux column (40 °C) to approx. 1 ml.

The β-glucosidase activity of hops was determined by the ability to cleave the synthetic glucoside p-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich Chemie, Taufkirchen, Germany) into the aglycone p-nitrophenol and glucose. The method is based on spectrophotometric analysis of the β-glucosidase activity of different yeasts by *Rosi* and *Vinella* [21]. Aqueous extracts of hops at two different degrees of grinding were prepared for this purpose; a fine type by ceramic mortar (crushed seeds) and a coarse type by knife mill (intact seeds). The samples were then concentrated on a rotary evaporator. The liberated aglycones were strongly photoactive in the alkaline range and could be recorded at a wavelength of 400 nm by a spectrophotometer.

GC-TOF-MS analysis of essential oil and aglycone extracts: an Agilent 6890N gas chromatograph was directly coupled to a SensiTOF mass spectrometer (Five Technologies, Munich, Germany). Separation was achieved using a DB 5 (J & W Scientific, CA, USA) 30 m × 0.25 mm capillary column (0.25 µm film thickness). The oven was programmed at a rate of 5 °C/min from 60 °C (5 min isotherm) to 240 °C. Carrier gas used was helium (1.5 ml/min); split 1:10; injection volume: 0.5–1.5 µl; injector: 250 °C; transfer line: 220°C; ion source temperature, 200 °C; ionization: –70 eV; mass range: 35–600 amu. Data analysis by MASPEC data system 2.11, version 14.0f (1998).

GC-FID analysis of essential oil and aglycone extracts: a Siemens SiChromat 3 gas chromatograph directly coupled to a Merck-Hitachi D2500 Integrator FID. The capillary column was a DB 5 (J & W Scientific, CA, USA) 30 m × 0.25 mm (0.25 µm film thickness); carrier gas: helium (1.0 ml/min, 60 °C); split 1:20 and splitless, respectively (aglycone extracts); injector/detector: 250 °C; fuel gases: hydrogen and air (each 2 bar). The temperature program of the oven was at a rate of 5 °C/min from 60 °C (5 min isotherm) to 250 °C. The injection volume was 1.0 µl and 4.0 µl, respectively (aglycone extracts).

GC-O analysis of hop extract: a Siemens Sichromat II gas chromatograph was directly coupled to Finnigan MAT 8222 magnetic sector field mass spectrometer (EI mode, –70 eV, 35–350 amu), capillary column: SPB5 (Supelco) 30 m × 0.53 mm (film thickness = 1.5 µm); carrier gas: helium (3 ml/min); split: 1:10. The oven was programmed at 5 °C/min to 250 °C starting at 100 °C; injector: 250 °C; transfer line: 200 °C. The GC eluent was divided by a live-T switching device to allow simultaneous sniffing analysis and mass spectrometric identification. Data analysis by MASPEC data system 2.11, version 14.0f (1998). Volatile compounds of each hop extract (5 g) were adsorbed in a 20 ml vial at 35 °C by the SPME fiber (Stable Flex Divinylbenzol/Carboxen/PDMS 50/30 µm, Supelco, Bellafonte, PA/USA) for 30 min. The enriched substances were desorbed in the injector of the gas chromatograph for 30 s at 250 °C.

Effects on beer quality of fertilized hops were tested by sensory evaluation of a lager beer single dry-hopped with equally gained fertilized and unfertilized hops. After a longer storage period, only samples of varieties Pilgrim and Challenger – fertilized and unfertilized – had adequate Hop Storage Index (HSI), and only these varieties have been used for sensory evaluation. So samples of both varieties were used for dry hopping trials. A pale filtered lager beer in 50-l-Kegs (industrially produced, 4.8 % ethanol vol/vol) was static dry-hopped at 1.5 ml essential oil per hectoliter for seven days at 1 °C. Beers were tasted and evaluated by a sensory panel of 7 DLG-certified tasters (Deutsche Landwirtschafts-Gesellschaft) subsequently of dry-hopping and after storage for

Table 1 **HHT harvest data; fertilized plants n = 6, analysis of 12 samples; unfertilized plants n = 6, analysis of 13 samples; oil content fourfold determination; harvest date 03.-06. 09. 2013**

		HHT-U		HHT-F	
		Sum		Sum	
green hops	[g]	24961		22087	
dried hops	[g]	5809		5752	
		Mean	SD	Mean	SD
H_2O after drying	[%]	4.82	0.48	4.75	0.34
α-acid (water free)	[%]	6.75	0.64	6.72	1.05
Oil content	[ml/100 g]	1.0		1.1	
		Seed share	SD	Seed share	SD
dried hops	[%]	1.3	0.3	18.9	2.3
		Oil content	SD	Oil content	SD
dried hops	[mL/100 g]	0.97	0.08	1.05	0.06

Table 2 **PG harvest data, triple determination; 9 plants cultivated each case; start point treatment pollination bags: unfertilized: 08. 07. 2013, fertilized (control): 29. 07. 2013; harvest date 09.-13. 09. 2013**

		PG-F		PG-C		PG-U	
		Sum		Sum		Sum	
dried hops	[g]	835		425		220	
		Mean	SD	Mean	SD	Mean	SD
H_2O after drying	[%]	5.42	0.39	6.15	0.43	5.91	0.41
		Seed share	SD	Seed share	SD	Seed share	SD
dried hops	[%]	21.6	1.9	22.1	1.0	0.2	0.1

3 month at 8 °C. The examination of the beer samples was done accordingly to the DLG-scheme for beer (attributes: smell, taste, body, rezenz, bitterness). Secondly, a descriptive tasting was conducted; intensities of eleven typical descriptors of hoppy flavor in beers (e.g. fruity, hoppy, green, spicy, herbal, resinous, citrussy, floral, tea and white wine) were rated by panelists. Every attribute was evaluated from 0, meaning not noticeable, to 5, extremely noticeable. Significant differences among flavor attributes were assessed by one-way analysis of variance (ANOVA) using the SPSS Version 24.0 statistical package for Windows (SPSS Inc, Chicago, IL, USA). Statistical differences between means were evaluated using Games-Howell's test at 0.05 % level in order to evaluate the significance of the analysis.

3 Results and discussion

3.1 Characterization of fertilized and unfertilized hop samples

The fertilized and unfertilized hop plants (harvest data, analytical characteristic, Tables 1 and 2) were cultivated in the United Kingdom (PG) and Germany (HHT) respectively in the 2013 season. Two different insemination methods were applied, depending on the country. In general, the growth and harvest of the hop plants proceeded normally. The visual inspection revealed nothing out of the ordinary, a variety-specific pure and lasting odor was recognized for all samples. Irrespective of the variety, the cones of fertilized hops were bigger and had greatly enlarged bracts; the seeds were smaller than those of unfertilized plants. The plants treated using pollination bags showed evidence in some cases of mildew infestation and slightly higher water content. This is due to the reduced air exchange and condensation formation. The maturation of cones and thus harvest was thereby negatively influenced. For HHT, the water, α-acid and oil contents were very similar; the fertilized hop samples were less homogeneous than their unfertilized patterns. The average essential oil content was determined of both fertilized and unfertilized HHT cones. No significant difference was noted. The disadvantages of fertilized hops due to a decreased α-acid content or a lower essential oil quantity described by two different research groups could not be confirmed [3, 5], although general conclusions are not to be derived due to limited data material. Significant differences were observed between unfertilized hop patterns PG and HHT when determining seed shares. The pollination bag method prevented wind pollination effectively. It was recognized that even in hop-growing areas in Germany it is not possible to entirely exclude wind pollination. In this context minimum formation of semen in the HHT pattern was accepted (1.3 ± 0.3 %). The artificial fertilization of HHT plants in Hüll, Germany, gave similar results to the wind-fertilized PG plants in Kent, UK. Derogations may be based on varietal differences or on deviating cone ripening conditions.

3.2 Quantification of flavor compounds in essential oils

Essential oil fractions of HHT were examined qualitatively and quantitatively by GC-TOF-MS and GC-FID, respectively (Table 3). In both extracts of fertilized and unfertilized hops 90 compounds of each were identified and 52 compounds quantified. Contents of important compounds of hop essential oil such as β-myrcene, α-humulene and β-caryophyllene form over 80 % of the essential oil fraction that is comparable to reference [22] regardless of fertilized or unfertilized samples. Linalool, which is key compound for hoppy flavor of beer [23] formed about 0.8 %, which is relatively low (1.0–1.5 %). In case of esters 3-methylbutyl-2-methyl propanoate and methyl-6-methyl heptanoate contents significantly (student t, n = 4, α = 0.05) varied between the samples. Sum of analysed compounds was 6.6 % higher in unfertilized than fertilized samples, 1042 µg/g and 973 µg/g, respectively. We conclude instrumental results of both HHT samples are well comparable with each other and references.

3.3 Analysis of β-glycosidically bound flavor compounds in hops

The existence of glycosidically bound flavor substances in hops was

Table 3 Concentration of hop constituents in µg/g hops dry matter; unfertilized (-U) and fertilized (-F).
[1] significant difference between the concentrations in the unfertilized and fertilized samples; [2] eluted together

					HHT-U		HHT-F
	compound	**RI TOF**	**RI GC**	**mean**	**SD**	**mean**	**SD**
1	2-Methylpropyl-2-methylpropanoate	911	911	10.7	2.62	17.2	4.59
2	α-Pinene	929	929	3.3	0.49	1.6	0.57
3	2-Methylbutyl-propanoate	967	968	11.5	2.87	12.3	2.95
4	β-Pinene	972	972	37.7	6.23	32.8	5.17
5	Myrcene	991	993	3447.3	342.68	3130.8	530.29
6	3-Methylbutyl-2-methylpropanoate[1]	1011	1012	6.6	0.90	14.8	3.20
7	2-Methylbutyl-2-methylpropanoate	1014	1015	46.7	7.46	68.1	12.38
8	Methylheptanoate	1022	1023	50.8	11.23	36.9	9.84
9	Methyl-4-methyl-2-hexanoate	1024	1025	63.1	8.12	49.2	7.54
10	Methyl-6-methylheptanoate[1]	1085	1087	33.6	4.76	19.7	2.95
11	Nonan-2-one	1090	1092	10.7	3.20	7.4	3.77
12	Linalool	1099	1101	69.7	18.29	64.8	15.91
13	2-Methylbutyl-2-methylbutanoate	1103	1104	17.2	1.31	14.8	1.48
14	Methyl octanoate	1124	1125	60.7	14.19	57.4	12.22
15	Decan-2-one	1191	1191	5.7	1.48	4.1	0.66
16	Methyl-3-nonenoate	1211	1212	7.4	2.13	6.6	0.08
17	Methylnonanoate	1224	1225	17.2	3.77	14.8	2.87
18	*iso*-Undecan-2-one	1255	1256	9.0	1.89	7.4	3.36
19	*iso*-Undecen-2-one	1274	1277	9.8	3.61	5.7	2.46
20	Methyl-8-methylnonanoate	1288	1289	8.2	1.23	4.9	1.07
21	Undecan-2-one	1292	1294	82.0	11.64	58.2	8.36
22	Methyl-4-decenoate	1308	1309	136.1	18.53	105.0	14.68
23	Methyl-4,8-decadienoate	1314	1314	94.3	3.94	90.2	16.07
24	Methyldecanoate	1325	1325	10.7	3.69	18.9	3.61
25	α-Cubebene[2]	1344	1347	11.5	1.39	11.5	3.28
26	Octyl-2-methylpropanoate[2]	1345					
27	α-Ylangene	1364	1367	6.6	2.54	7.4	2.13
28	α-Copaene	1369	1372	24.6	2.95	23.8	4.10
29	Dodecan-2-one	1393	1395	10.7	3.20	7.4	2.95
30	*E*-β-Caryophyllene	1408	1416	803.6	37.72	761.0	35.34
31	β-Copaene	1421	1425	33.6	2.87	36.9	1.48
32	α-Humulene	1446	1453	2776.5	244.61	2740.4	59.78
33	Selina-4,11-diene	1467	1471	18.9	3.94	19.7	4.51
34	γ-Muurolene	1469	1474	64.0	5.58	60.7	2.30
35	α-Amorphene	1473	1477	9.8	0.98	8.2	0.90
36	β-Selene	1476	1482	22.1	2.71	20.5	0.74
37	α-Selinene	1486	1491	41.8	6.89	41.8	5.33
39	Tridecan-2-one	1494	1496	70.5	17.88	71.3	7.71
40	γ-Cadinene	1506	1511	94.3	13.37	82.0	2.95
41	δ-Cadinene	1517	1522	129.6	16.73	99.2	33.62
42	Cadina-1,4-diene	1524	1530	12.3	3.77	19.7	12.55
43	α-Cadinene	1530	1535	14.8	3.85	13.9	0.82
44	*iso*-Tetradecen-2-one	1567	1571	5.7	1.48	8.2	3.03
45	Caryophyllene oxide	1571	1578	3.3	1.39	1.6	1.31
46	Humulene oxide A	1587	1594	4.9	0.33	4.9	1.97
47	Tetradecan-2-one	1596	1598	9.0	2.05	6.6	2.05
48	Humulene oxide B	1597	1604	31.2	6.56	22.1	9.02
49	Pentadecadien-2-one	1657	1660	52.5	15.09	35.3	1.48
50	Pentadecatrien-2-one	1662	1665	13.9	4.10	9.0	0.49
51	Pentadecen-2-one	1668	1670	21.3	6.31	17.2	5.99
52	Pentadecan-2-one	1697	1699	8.2	2.46	5.7	0.41
	Sum			1042.1		973.1	

Table 4 Identified compounds of glycoside extracts in the fertilized and unfertilized Hallertauer Tradition (HHT) hop variety with the retention indices (RI) and main mass fragments (m/e)

	Standard	RI TOF	RI GC	m/e	Identification
	Phenol	982	979	94, 66, 65, 39	MS; RT
	Methyl heptanoate	1024	1025	74, 43, 87, 113	MS; RT
	Aliphatic alcohols	**RI TOF**	**RI GC**	**m/e**	
1	3-Methylbutan-1-ol	722	722	55, 42, 70, 43, 41	MS; RT
2	2-Methylbutan-1-ol	726	726	57, 41, 56, 70, 44	MS; RT
3	Pentan-1-ol	757	756	55, 42, 70, 42, 41	MS; RT
4	3-Methyl-2-buten-1-ol	765	766	71, 53, 41, 67, 68, 86	MS; RT
5	3-Methylpentan-2-ol	780	780	45–56, 41,69,84,87	MS; RT
6	4-Methylpentan-2-ol	784		45–43, 96, 84, 87, 57	MS
7	3-Z-Hexenol	845	852	67, 41, 82–55, 69	MS; RT
8	Hexanol	858	862	56–43, 41, 69, 55, 84	MS; RT
9	1,5-Octadien-3-ol	978		57, 72, 99, 110	MS
10	1-Octanol	1071		41, 56, 55, 70, 84, 112	MS
	Aromatic compounds	**RI TOF**	**RI GC**	**m/e**	
11	Benzaldehyde	956		79, 108, 109, 77	MS
12	Benzylalcohol	1032	1036	108, 107, 79, 77	MS; RT
13	Phenylacetaldehyde	1039	1046	91, 69, 79, 108	MS; RT
14	Guajacol	1085	1090	55, 109, 81, 124, 69	MS; RT
15	2-Phenylethanol	1109	1118	91, 92, 122, 65	MS; RT
16	Methyl salicylate	1191	1198	120, 152, 92	MS; RT
17	4-Vinylphenol	1220	1220	119, 91, 65, 39	[28]
18	4-Vinylguajacol	1311	1322	135, 107, 77, 151	[28]
19	4-Hydroxy-benzaldehyde	1368	1379	121, 122, 65, 103	MS; RT
20	Vanillin	1395	1408	151, 152, 81, 109	MS; RT
21	Tyrosol	1424	1430	107, 138, 77	MS; RT
22	4-Vinylcatechol	1444	1479	136, 89, 90, 110, 77, 63	[28]
23	Coniferylaldehyde	1731	1748	178, 135, 147, 107, 77, 51	[28]
24	*p*-Coumarin	1778	1794	43, 123, 163, 209, 224	MS; RT
25	Ferulic acid	1845		194, 179, 133, 77, 105	MS
	Terpene compounds	**RI TOF**	**RI GC**	**m/e**	
26	α-Pinene	931	938	93, 91, 92, 55, 77, 79, 67	MS; RT
27	β-Pinene	982		94, 66, 65, 55	MS
28	Linalool	1098	1102	71, 93, 41, 55, 80, 121, 136	MS; RT
29	α-Terpineol	1189	1196	59, 93, 121, 136, 68	MS; RT
30	*Z*-8-Hydroxy-linalool	1343	1348	43, 67, 71, 55, 68	[29]
31	*E*-8-Hydroxy-linalool	1362	1368	43, 67, 71, 55, 68	[29]
32	*p*-Menth-1-en-7,8-diol	1468	1479	59, 79, 93, 94	[30]
	C_{13}-norcarotinoid compounds	**RI TOF**	**RI GC**	**m/e**	
33	Theaspirane 1	1298		138, 82, 96, 109, 123	MS
34	Theaspirane 2	1315		138, 82, 96, 123	MS
35	3-OH-β-damascone	1614	1627	69, 43, 121, 175, 193, 208	[31]
36	3-OH-7,8-dihydro-β-ionol	1659	1671	121, 43, 119, 93, 105, 136, 212	[32, 33]
37	3-OH-5,6-epoxy-β-Ionol	1667	1674	43, 125, 109, 82, 208, 107, 166	[33]
38	Vomifoliol	1790	1804	124–43, 79, 135,150, 168	[32, 34]
39	7,8-Dihydro-vomifoliol	1853	1869	43,110, 111,152 ,96 ,68 ,170	[32]
	Fatty acids	**RI TOF**	**RI GC**	**m/e**	
40	Palmitin acid	1965	1964	43, 73, 60, 256	MS;RT
41	Linoleic acid	2136	2147	67, 81, 55, 41, 95, 280, 109	MS;RT
42	Linolenic acid	2142	2152	79, 67, 55, 95, 108, 222, 278	MS;RT
	Other compounds	**RI TOF**	**RI GC**	**m/e**	
43	n. i.	1371		107, 121, 136, 192	MS
44	n. i.	1676	1682	126, 85, 69, 168, 111	MS; RT

Table 5 **Quantification of compounds in the glycoside extracts (Δ = deviation dual approach) of HHT hops in µg/g hops (dry matter); unfertilized (-U) and fertilized (-F); n. i. = not identified; Tr = trace (< 1 µg/g)**

		HHT-U		HHT-F	
Standard	**RI**				
Methyl heptanoate	1025	40		40	
Aliphatic alcohols	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
3-Methylbutan-1-ol	722	2	Tr	3	Tr
2-Methylbutan-1-ol	726	1	Tr	2	Tr
Pentan-1-ol	756	Tr	Tr	Tr	Tr
3-Methyl-2-buten-1-ol	766	1	Tr	1	Tr
3-Methylpentan-2-ol	780	2	Tr	4	Tr
3-*Z*-Hexenol	852	2	Tr	3	Tr
Hexanol	862	Tr	Tr	Tr	Tr
Aromatic compounds	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
Benzylalcohol	1036	15	Tr	13	Tr
Phenylacetaldehyde	1046	Tr	Tr	1	Tr
Guajacol	1090	Tr	Tr	Tr	Tr
2-Phenylethanol	1118	7	Tr	6	Tr
Methyl salicylate	1198	4	Tr	4	Tr
4-Vinylphenol	1220	349	41	279	5
4-Vinylguajacol	1322	64	7	59	1
4-Hydroxy-benzaldehyde	1379	2	Tr	2	Tr
Vanillin	1408	1	Tr	2	Tr
Tyrosol	1430	3	1	4	Tr
4-Vinylcatechol	1479	160	38	154	Tr
Coniferyl aldehyde	1748	34	2	43	3
p-Coumarin	1794	10	Tr	10	Tr
Terpene compounds	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
α-Pinene	938	TR	Tr	1	Tr
Linalool	1102	2	Tr	2	Tr
α-Terpineol	1196	3	3	3	Tr
Z-8-Hydroxy-linalool	1348	4	Tr	3	Tr
E-8-Hydroxy-linalool	1368	14	2	12	Tr
p-Menth-1-en-7,8-diol	1479	5	1	6	5
C_{13}-Norcarotinoid compounds	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
3-OH-β-Damascone	1627	2	Tr	2	Tr
3-OH-7,8-Dihydro-β-ionol	1671	9	2	10	Tr
3-OH-5,6-Epoxy-β-ionol	1674	2	Tr	3	1
Vomifoliol	1804	2	Tr	2	Tr
7,8-Dihydro-vomifoliol	1869	3	Tr	3	Tr
Fatty acids	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
Palmitic acid	1964	143	3	142	35
Linoleic acid	2147	116	2	119	28
Linolenic acid	2152	124	3	120	18
Other compounds	**RI**	**Mean**	**Δ**	**Mean**	**Δ**
n. i.	1682	4	1	5	< 1

clearly demonstrated by several studies [17, 24, 25, 26, 27]. In this project, the glycosidically bound fraction of hops was isolated and cleaved into its aglycones and sugar residues by Rapidase F64 enzyme preparation. The quantitative yield of the enzyme splitting was determined based on the cleavage of synthetic glycoside phenyl-β-D-glucopyranoside. The average yield of the unfertilized samples was 93 %. The average yield of the fertilized samples was 89 % [17]. In the extracts prepared from both unfertilized and fertilized hops HHT, the same variety of alcohols and diols could be released that were not included in the control. In total, 44 compounds (Table 4) were determined by GC-TOF-MS; 35 compounds were quantified by GC-FID (Table 5). A total of seven aliphatic alcohols, 13 aromatic compounds, six terpene compounds, five C_{13}-norisoprenoid compounds, three fatty acids and an unidentified one were determined. Figures 1–3 (see page 154) shows important correlations. The samples of the extract PG-U were lost

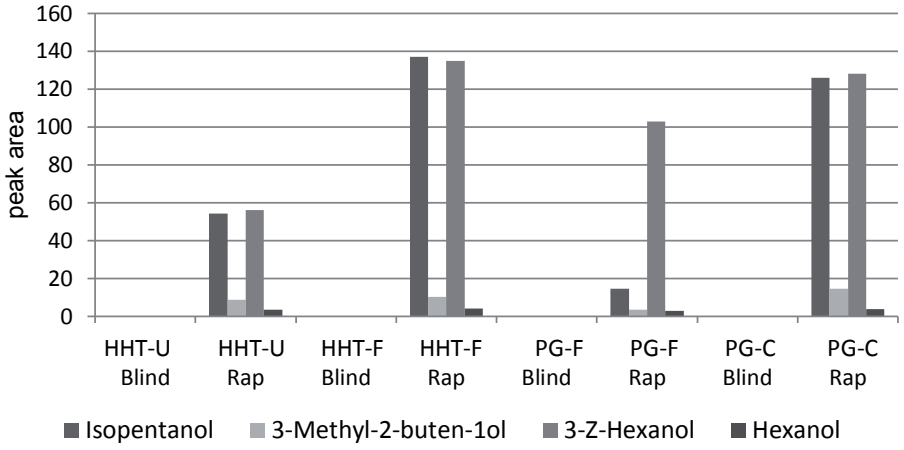

Fig. 1 **Peak areas of selected aliphatic alcohols of glycoside extracts; unfertilized (-U), fertilized (-F) and control (-C, fertilized with pollination bag)**

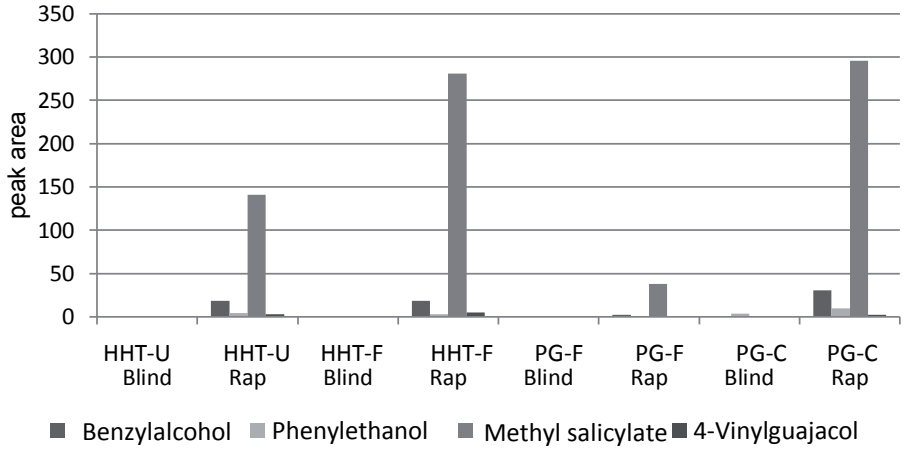

Fig. 2 **Peak areas of selected aromatic compounds of glycoside extracts; unfertilized (-U), fertilized (-F) and control (-C, fertilized with pollination bag)**

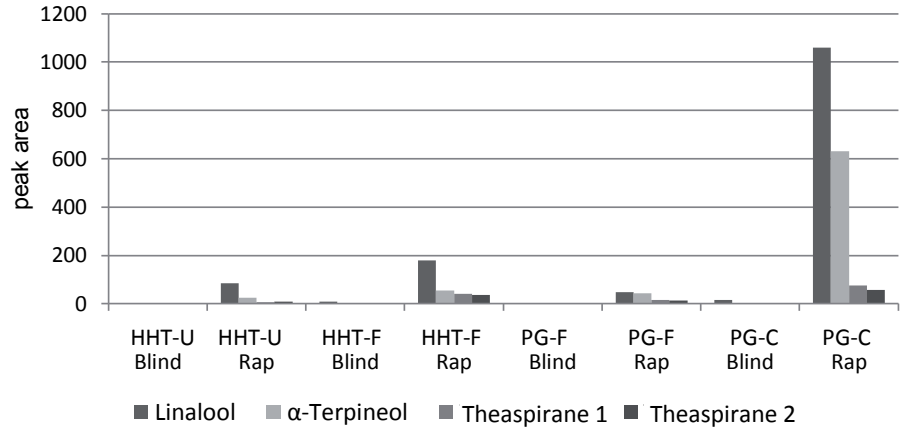

Fig. 3 **Peak areas of selected terpene alcohols and C_{13}-norisoprenoid compounds of glycoside extracts; unfertilized (-U), fertilized (-F) and control (-C, fertilized with pollination bag)**

due to technical problems during the measurement process. The quantities of liberated aglycones are rather low compared with the concentrations of the individual hop oil components. For example, it was possible to recover a quantity of approx. 2 µg linalool per gram of hops in the glycoside extracts. In the same amount of hops approx. 70 µg free linalool is included as a proportion of essential oil. In comparison, *Wilhelm* proved a quantity of up to 41 µg glycosidically bound linalool in his studies [27]. Concentrations of aliphatic alcohols were generally higher than in the unfertilized samples with the exception of hexanol. In other substance classes no clear differences were recognized between unfertilized and fertilized samples. The aromatic compounds formed the biggest substance class despite a tannin-side stabilization of the methanol extracts by Polyclar. Components such as 4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol were measured at high concentrations. These compounds are attributed to the phenol carboxylic acid esters that are present in hops [35, 36] and esterase activity of the hemicellulase preparation Rapidase F64 [30, 37]. The esterase activity is probably responsible for the high concentrations of fatty acids such as palmitic acid, linoleic acid, and linolenic acid in the extracts, leading to shares 10–13 % of extracts. There were no significant differences in the concentrations of each individual fatty acid between the unfertilized and fertilized samples, representing in total a share of 33.7 % and 35.6 % of extracted glycosides. This is interesting in terms of the high fat content of seeds in general, especially the unsaturated fatty acids which are held responsible for the deterioration in beer flavor stability [6, 7, 8]. Released substances that were identified using HS-SPME-GC-O (Table 6) included aliphatic alcohols (3-methylbutan-1-ol, 3-methyl-2-buten-1-ol, 3-Z-hexenol and hexanol), aromatic compounds (benzyl alcohol, phenylethanol, methyl salicylate and 4-vinylguaiacol), terpene alcohols (α-terpineol), and two theaspiranes (related to C_{13} norisoprenoid derivatives). A fungus-like smell was assigned to the compound 1-octen-3-ol, but this compound could only be detected in the sample "PGB-C" treated with Rapidase F64. The compounds furaneol (sweetish, flowery) and phenylacetaldehyde (gummy) were eluted to similar RT. The furaneol was detected only in the samples treated with Rapidase F64. Linalool (citrus-like, flowery) could be detected in all samples, except for "PGB-F" blind. The odor impression of the samples treated with Rapidase F64 was generally stronger than that of the untreated blind samples.

3.4 Analysis of β-glucosidase activity in hops

Figure 4 shows the specific β-glucosidase activity for PG and HHT unfertilized and fertilized hop samples. The differences for each

Table 6 **Odor-relevant compounds of glycoside extracts, with their associated olfactory impressions; [1] compound was identified by their retention time and their typical odor; n. i. = not identified; B = "blind" without enzyme; R = samples treated with Rapidase F64; data of unfertilized PG samples got lost**

				HHT-U		HHT-F		PG-F		PG-C	
compound	**RT**	**RI**	**odor**	**B**	**R**	**B**	**R**	**B**	**R**	**B**	**R**
Hexanal	2.4	802	green, grassy	x	x	x	x	x	x	x	x
Ethyl-3-methylbutanoate	3.1	842	sweet, fruit jelly			x	x	x	x	x	x
3-*Z*-Hexanol	3.2	851	green, banana								
1-Octen-3-ol	5.5	976	mushrooms	x	x	x	x	x	x	x	x
1,5-Octadien-3-one[1]	5.6	983	metallic	x	x	x	x	x	x	x	x
Octanal	6.2	1006	orange peel, floral			x	x			x	
Furaneol[1]	7.4	1055	sweet, floral		x		x		x		x
Phenylacetaldehyde	7.5	1059	rubbery			x				x	
1-Nonen-3-one	8.2	1083	mushrooms, chanterelles							x	x
n. i.	8.6	1099	green, cucumber, sweet				x	x		x	x
Linalool	8.7	1103	sweet, citrussy, floral	x	x	x	x		x	x	x
2-*Z*-Nonenal[1]	10.1	1152	burnt rubber		x	x	x	x	x	x	x
2,6-Nonadienal	10.3	1159	sweet, cucumber, floral	x	x	x	x		x	x	x
2-*E*-Nonenal	10.5	1165	roasted almonds	x	x	x	x	x	x	x	x
n. i.	11.2	1190	green, acrid floral smell	x	x		x	x		x	x
n. i.	12.3	1225	sweet, rose							x	x
n. i.	13.5	1265	rubber, tar		x		x		x		
4-Vinylguajacol	15.5	1331	clove				x				
β-Damascenone[1]	17.7	1403	fruity, apple juice			x	x			x	x
β-Damascone[1]	18.6	1432	cherry, fruit preserve				x	x	x		x

of the hop samples were very low. Nevertheless result of HHT hints towards tendency that specific β-glucosidase activity could be stronger in fertilized hops than in unfertilized hops. However, this should be investigated using a suitable sample material of further varieties. The analysis revealed an overall average of 114 mU/g (hop dry matter), maximum specific activity 139 mU/g and minimum 100 mU/g. Despite the small result range, differences between the samples could be recognized. Janicki and Kotasthane showed that diastatic activity originating from hops depends on the seed content and crushing ratio [16]. In this study, coarse samples (intact seeds) of the HHT hop variety showed no significant differences. By breaking the seeds (fine grinding), the fertilized samples showed a significantly higher specific β-glucosidase activity than the unfertilized samples.

The mold contamination of clusters ripened in the pollination bags led to increased enzyme activity that hampered any further interpretation. However, in the PG hop solutions prepared with coarse hop pellets the fertilized hops showed a highly significant (student t: α = 0.01) higher specific β-glucosidase activity than the unfertilized hops. Breaking up the seeds of the PG variety revealed a significant increase of specific β-glucosidase activity in the fertilized hop samples whereas it decreased slightly for the unfertilized samples. The control (fertilized with a pollination bag) showed a significantly higher activity in the coarse variant than the fertilized samples and a significantly lower activity than the unfertilized samples. It decreased for the fine variant to approximately the same level of the fertilized samples (without pollination bags). The increased β-glucosidase activity of the fine control could possibly be explained by the mold growth in the pollination bags [38]. This

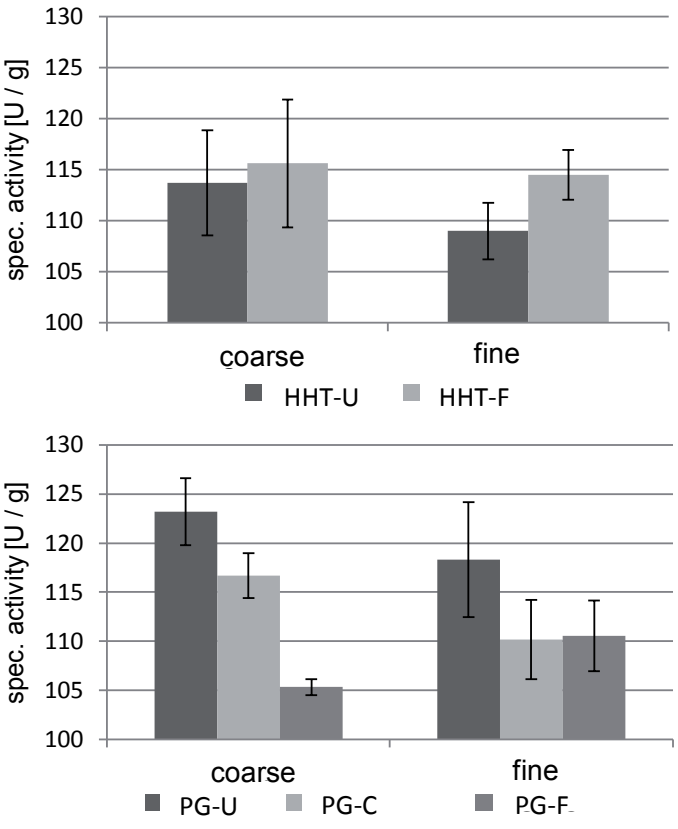

Fig. 4 **Average specific β-glucosidase activity of the Progress PG and Hallertauer Tradition HHT hops; unfertilized (-U), fertilized (-F) and control (-C, fertilized with pollination bag); confidence intervals (Student t; α = 0.05)**

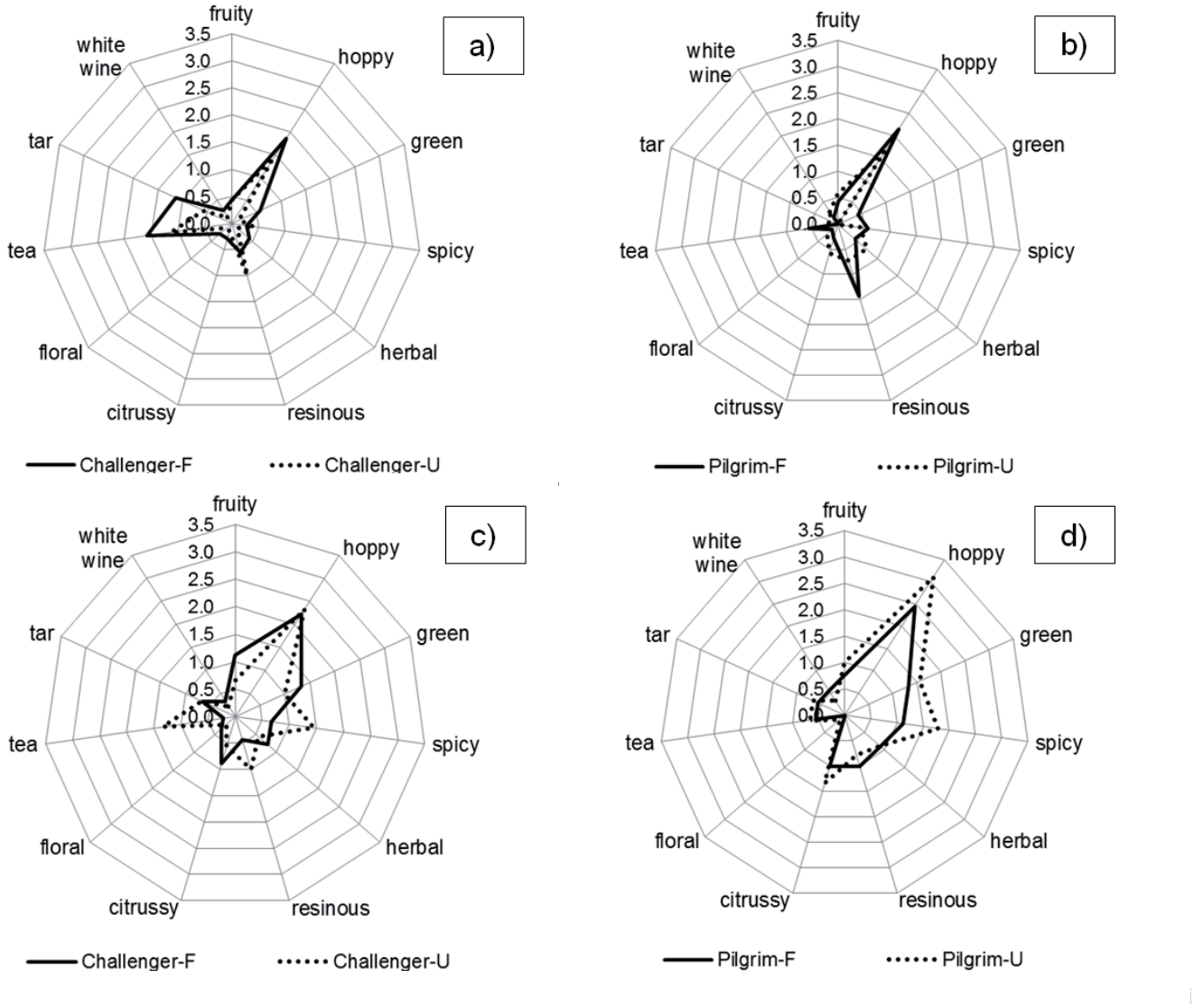

Fig. 5 **Average intensities of flavor descriptors of beers dry-hopped with Pilgrim or Challenger; sensory evaluation of fresh (a, b) and stored samples for 3 months (c, d); six-point intensity range (0–5); unfertilized (-U), fertilized (-F)**

might be reason for the high level of specific β-glucosidase activity of PG-U compared to the low level of HHT-U.

3.5 Sensory evaluation of lager beer single dry-hopped with fertilized and unfertilized hop samples

A lager beer of one batch was single dry-hopped (1.5 ml/hl) in 50-l- Kegs with Pilgrim unfertilized (97 g/hl), Pilgrim fertilized (126 g/hl), Challenger unfertilized (150 g/hl) and Challenger fertilized (273 g/hl). All samples of both hops were produced according to the method described above. Unfortunately, the samples of HHT and PG, which were subject of several analyses above showed no adequate HSI for test-brews after a longer storage period. The examination of the beer samples accordingly to the DLG-scheme approved pureness of all produced beers. In figure 5 the average intensities of eleven flavor descriptors of dry-hopped lager beers with Pilgrim and Challenger, fertilized and unfertilized, respectively, are shown. In descriptive tasting of beers produced by fertilized or unfertilized Challenger hops, slight differences in the intensities of the attributes “tea”, “tar” and “resinous”, +0.4, +0.5, –0.5, respectively, were found. In stored samples (three months), slight differences were observed by tasters with regard to the characteristics “tea” and “spicy” between fertilized unfertilized Challenger hops, –0.9 and –0.7, respectively. In the case of (fresh) beers dry-hopped by fertilized Pilgrim hops “resinous” was perceived more intensively (+0.7) than in dry-hopped beers with unfertilized Pilgrim ops. After storage of dry-hopped beers with fertilized hops, attributes such as “hoppy” and “spicy” were slightly weaker described by tasters than in beers with and unfertilized patterns, –0.7 and –0.7, respectively. In fresh and stored samples, in none of the tested characteristics, a significant difference was observed between beers dry-hopped with fertilized and unfertilized hop samples (ANOVA, $\alpha = 0.05$, $n = 7$). This confirms results of various working groups that performed comparative brewing experiments with unfertilized and fertilized hops [1, 4, 9, 10].

4 Conclusion/Summary

In this study it was shown that it is possible to produce comparable fertilized and unfertilized hop samples. The study was conducted

in field trials in the presence or absence of male plants. This was achieved by artificially fertilized hop plants grown in Hallertau (Germany), accomplished by preventing the wind fertilization of hop plants in Kent (UK). An especially interesting finding is that proclaimed disadvantages such as reduced α-acid and oil contents of fertilized hops were not observed [3, 5]. Glycosidically bound flavor-active substances, for example aliphatic alcohols, terpene alcohols and C_{13}-norisoprenoid compounds were identified confirming previous studies [17, 25, 26, 39, 40]. The substances mentioned above are related to a kettle hoppy flavor in beer [26, 41, 42]. Confirmation is also provided for glycoside hydrolysis in hops in the form of β-glucosidase activity. The observation that hops with a high seed share such as the fertilized HHT samples can have an increased β-glucosidase activity was already shown in previous studies [16]. In this context the mold contamination of clusters ripened in pollination bags hampered further interpretation. No adverse effects on the sensory between dry-hopped beers with unfertilized and fertilized hops in fresh and stored samples were noticed.

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