Fate of Xanthohumol and Related Prenylflavonoids from Hops to Beer

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The fate of three prenylated flavonoids of the chalcone type, xanthohumol, desmethylxanthohumol, and 3′-geranylchalconaringenin, was monitored with LC/MS-MS from hops (*Humulus lupulus* L.) to beer in two brewing trials. The three prenylchalcones were largely converted into their isomeric flavanones, isoxanthohumol, prenylnaringenins, and geranylnaringenins, respectively, in the boiling wort. Losses of prenylflavonoids were due to incomplete extraction from the hops into the wort (13−25%), adsorption to insoluble malt proteins (18−26%), and adsorption to yeast cells (11−32%) during fermentation. The overall yield of xanthohumol, after lagering of the beer and largely in the form of isoxanthohumol, amounted to 22−30% of the hops' xanthohumol. About 10% of the hops' desmethylxanthohumol, completely converted into prenylnaringenins, remained in the beers. 3′-Geranylchalconaringenin behaved similarly to desmethylxanthohumol. Solubility experiments indicated that (1) malt carbohydrates form soluble complexes with xanthohumol and isoxanthohumol and (2) solubility does not dictate the isoxanthohumol levels of finished beers.

Keywords: *Beer; hops; Humulus lupulus; prenylated flavonoids; xanthohumol*

INTRODUCTION

Hops, the female inflorescences of the hop plant (*Humulus lupulus* L.), are used in the brewing industry to add flavor and bitterness to beer. The bitter principles of beer, the iso-α-acids, are formed from the hops' α-acids by thermal isomerization in the brew kettle. Because the bitter acids occur together with prenylated flavonoids of the chalcone type in the resin fraction of hops (Hänsel and Schulz, 1988; Stevens et al., 1997), conversion of prenylchalcones into their isomeric flavanones (Bohm, 1989) is expected to take place in the brewing process. In fact, beer contains higher levels of prenylflavanones than prenylchalcones (Stevens et al., 1999). Beer prenylflavanones are largely derived from the hop chalcones because hops are very poor in prenylflavanones, and other beer ingredients such as barley, wheat, and rice lack prenylated flavonoids (see Figure 1 for structures and nomenclature).

Earlier studies on the fate of phenolic compounds during the brewing process have been summarized by Charalambous (1981). Most of these studies, however, were focused on total phenolics or on proanthocyanidins as a group of polyphenols. More recently, Moll et al. (1984) investigated changes in the levels of individual catechins from barley to finished beer by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. They attributed the loss of procyanidin dimer B3 (∼75%) to incomplete extraction from barley and to chemical degradation (depolymerization) during the later brew stages. Because proanthocyanidins have been associated with the formation of beer haze, many investigators have studied the removal of these phenolics from beer by adsorption to nylon and polyvinylpolypyrrolidone [see, e.g., McMurrough et al. (1995)]. The prenylflavonoids of hops have not received nearly as much attention for two main reasons. First, the value of these polyphenols to the brewer is not well understood. However, recent studies revealing various biological effects of hop prenylflavonoids in vitro (Miranda et al., 1999; Tabata et al., 1997; Tobe et al., 1997) have fueled a renewed interest in these phenolics as dietary flavonoids. Second, the levels of prenylflavonoids in typical American beers (0.6 ppm; Stevens et al., 1999) are low in comparison with the levels of iso-α-acids (13−20 ppm; Peacock, 1998) and proanthocyanidins [1−40 ppm, depending on the method used; see, e.g., McGuinness et al. (1975) and Delcour (1988)]. This makes HPLC with UV detection less suitable for analysis of beer prenylflavonoids without sample pretreatment. We have recently developed a sensitive and selective method for direct quantitation of prenylflavonoids in hops and beer by HPLC/tandem mass spectrometry (MS) (Stevens et al., 1999). In the present study, we used this method in two brewing trials to investigate the fate of xanthohumol and related prenylflavonoids from hops to beer.

EXPERIMENTAL PROCEDURES

Chemicals. Xanthohumol, isoxanthohumol, desmethylxanthohumol, and 3′-geranylchalconaringenin were isolated from hops as before (Stevens et al., 1997). Prenylnaringenins were prepared by prenylation of naringenin (Indofine, Somerville, NJ) according to the method of Jain et al. (1978). A similar procedure was used for the preparation of 6-geranylnaringenin and is described below. The internal standard, 2′,4-dihydroxychalcone, was purchased from Indofine.

Preparation of Geranylnaringenins. 6-Geranylnaringenin was prepared by treatment of naringenin with (±)-linalool in the presence of BF_3−etherate as follows. Boron trifluoride−etherate (0.54 mL) was added dropwise to a stirred

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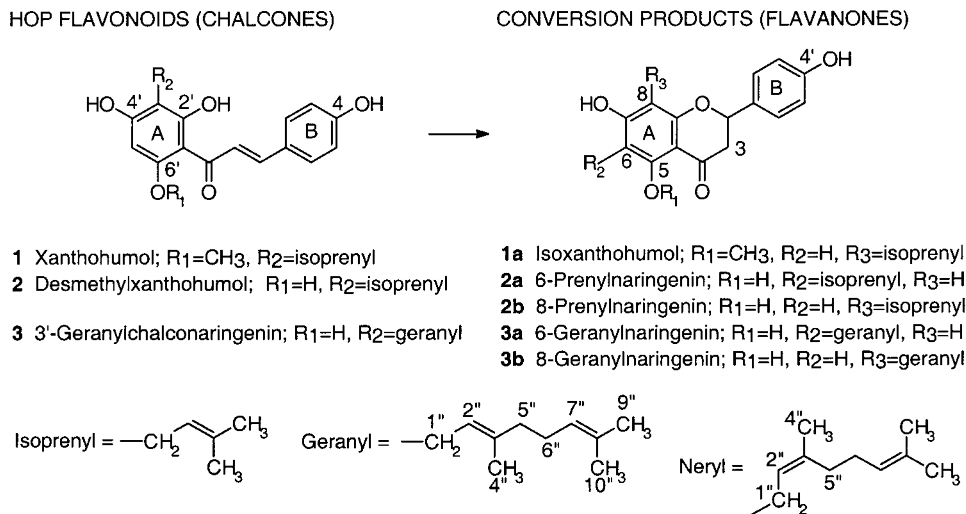

Figure 1. Major prenylated flavonoids of hops (left) and their conversion products formed during the brewing process (right).

solution of naringenin (0.55 g) in dioxane (10 mL) under nitrogen. Linalool (0.8 mL) was then added dropwise to the reaction mixture. The solution was stirred for 2.5 h at room temperature and then diluted with ethyl ether (150 mL). The ether layer was washed with water (3 × 100 mL), dried over anhydrous Na_2SO_4, and evaporated to dryness on a rotary evaporator. The residue was taken up in MeOH and submitted to preparative HPLC. The reaction products were separated on a 10 μm Econosil RP-18 column (22 × 250 mm) using a linear gradient starting from 40 to 100% acetonitrile in 1% aqueous formic acid over 45 min at a flow rate of 11.2 mL/min. The UV trace was recorded at 290 nm. Peak fractions were collected manually and dried by rotary evaporation and lyophilization. 6-Geranylnaringenin was obtained as a white powder (15 mg): UV (MeOH) λ_{max} (log ϵ) 294 (4.24) and 336 (3.55) nm; APCI-MS, m/z (rel intensity) 409 $[MH]^+$ (100); MS-MS (collision energy, 11 V) m/z 409 (71), 285 $[MH - C_9H_{16}]^+$ (100), 165 $[^{1,3}AH-C_9H_{16}]^+$ (25); ^1H NMR (DMSO-d_6, 600 MHz) δ 12.43 (s, OH-5), 10.72 (s, OH-7), 9.57 (s, OH-4′), 7.30 (2H, d, J = 8.5 Hz, H-2′/H-6′), 6.78 (2H, d, J = 8.5 Hz, H-3′/H-5′), 5.96 (1H, s, H-8), 5.40 (1H, dd, J = 2.8 and 12.8 Hz, H-2), 5.12 (1H, t, J = 7.1 Hz, H-2″), 5.04 (1H, t, J = 7.1 Hz, H-7″), 3.23 (1H, dd, J = 12.8 and 17.0 Hz, H-3_{ax}), 3.12 (2H, d, J = 7.0 Hz, H-1″), 2.67 (1H, dd, J = 2.9 and 17.0 Hz, H-3_{eq}), 1.99 (2H, t, J = 7.4 Hz, H-6″), 1.90 (2H, t, J = 7.6 Hz, H-5″), 1.69 (3H, s, H-4″), 1.60 (3H, s, H-10″), 1.53 (3H, s, H-9″); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 196.5 (C-4), 164.2 (C-7), 160.6 (C-5), 160.5 (C-9), 157.7 (C-4′), 133.8 (C-3″), 130.6 (C-8″), 129.0 (C-1′), 128.3 (C-2′/C-6′), 124.1 (C-7″), 122.4 (C-2″), 115.1 (C-3′/C-5′), 107.5 (C-6), 101.6 (C-10), 94.3 (C-8), 78.3 (C-2), 42.1 (C-3), 39.0 (C-5″), 26.2 (C-6″), 25.5 (C-10″), 20.6 (C-1″), 17.5 (C-9″), 15.9 (C-4″).

8-Geranylnaringenin was prepared by isomerization of 6-geranylnaringenin. The latter compound (12 mg) was dissolved in 20 mL of 5% NaOH in MeOH. The mixture was refluxed under nitrogen for 1 h and then poured into 200 mL of 2 N HCl. The aqueous mixture was extracted with EtOAc (1 × 150 mL, 2 × 75 mL). The combined EtOAc layers were washed with water (3 × 100 mL), dried over Na_2SO_4, and evaporated. The major product, 8-geranylnaringenin, was isolated from the residue by preparative HPLC along with 3′-geranylchalconaringenin and 6-geranylnaringenin. 8-Geranylnaringenin was obtained as a white powder (3 mg): UV (MeOH) λ_{max} (log ϵ) 294 (4.23) and 338 (3.58) nm; APCI-MS, m/z (rel intensity) 409 $[MH]^+$ (100); MS-MS (collision energy, 11 V) m/z 409 (100), 285 $[MH - C_9H_{16}]^+$ (58), 165 $[^{1,3}AH-C_9H_{16}]^+$ (39); ^1H NMR (DMSO-d_6, 600 MHz) δ 12.10 (s, OH-5), 10.74 (s, OH-7), 9.55 (s, OH-4′), 7.30 (2H, d, J = 8.5 Hz, H-2′/H-6′), 6.78 (2H, d, J = 8.5 Hz, H-3′/H-5′), 5.97 (1H, s, H-6), 5.41 (1H, dd, J = 2.9 and 12.5 Hz, H-2), 5.09 (1H, t, J = 7.1 Hz, H-2″), 5.03 (1H, t, J = 7.1 Hz, H-7″), 3.19 (1H, dd, J = 12.6 and 17.1 Hz, H-3_{ax}), 3.08 (2H, d, J = 7.1 Hz, H-1″), 2.72 (1H, dd, J = 3.1 and 17.1 Hz, H-3_{eq}), 1.97 (2H, t, J = 7.4 Hz, H-6″), 1.87 (2H, t, J = 7.6 Hz, H-5″), 1.59 (3H, s, H-10″), 1.53 (3H, s, H-4″), 1.52 (3H, s, H-9″); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 196.7 (C-4), 164.3 (C-7), 161.1 (C-5), 159.7 (C-9), 157.5 (C-4′), 133.7 (C-3″), 130.6 (C-8″), 129.2 (C-1′), 128.0 (C-2′/C-6′), 124.1 (C-7″), 122.4 (C-2″), 115.1 (C-3′/C-5′), 106.9 (C-8), 101.8 (C-10), 95.2 (C-6), 78.2 (C-2), 41.9 (C-3), 38.2 (C-5″), 26.1 (C-6″), 25.5 (C-10″), 21.1 (C-1″), 17.5 (C-9″), 15.8 (C-4″).

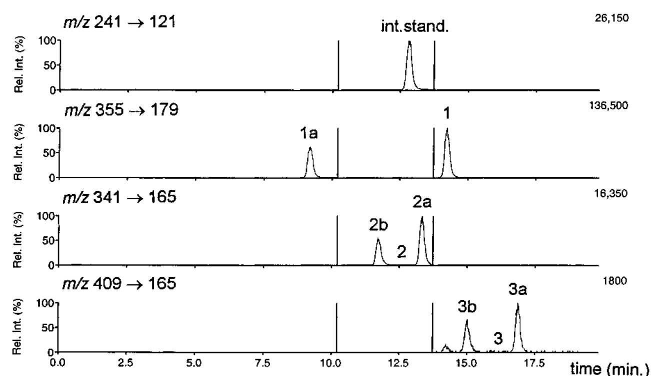

Figure 2. LC/MS chromatogram of a methanolic extract of the cold trub, recovered from the single-hopped wort. For key to peaks, compare Figure 1. Peaks 2 and 3 are included to indicate their relative positions in the chromatogram.

Instrumentation and Analysis. Prenylflavonoids were analyzed in hops, wort, and beer by LC/MS as described previously (Stevens et al., 1999). In short, hop extracts and brew products were separated on a 5 μm C_{18} column (250 × 4 mm) using a linear solvent gradient from 40 to 100% B (acetonitrile) in solvent A (1% aqueous formic acid) over 15 min, followed by 100% solvent B for 5 min. The flow rate was 0.8 mL/min. The HPLC instrument was connected to a PE Sciex API III Plus triple-quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. The APCI source was operated in the positive ion mode. The HPLC effluent was introduced into the mass spectrometer via a heated nebulizer interface at 500 °C. Ionization of the analyte vapor mixture was initiated by a corona discharge needle at ~8 kV and a discharge current of ~6 μA. Six prenylflavonoids were selectively detected by multiple reaction monitoring (MRM) in the tandem MS mode. Argon−nitrogen (9:1) was used as target gas (~1.8 × 10^{14} atoms/cm^2). The collision energy was 30 V. For MRM, specific daughter ions produced by collision-induced dissociation of $[MH]^+$ ions of selected prenylflavonoids were detected (for a typical chromatogram, see Figure 2). Quantitation was carried out using 4,2′-dihydroxychalcone as the internal standard. Calibration curves were recorded for each of the six prenylfla-

vonoids on the days the samples were analyzed; samples and standards were run at least twice.

Nuclear magnetic resonance spectra were recorded on a multinuclear Bruker DRX 600 instrument at 600 MHz for ^{1}H and at 150.9 MHz for ^{13}C. Samples were dissolved in DMSO-d_6 and analyzed at room temperature. DMSO resonances (δ_H 2.50 and δ_C 39.51) were used as internal chemical shift references. $^{1}H-^{13}C$ HMQC and HMBC experiments were performed using standard pulse sequences. One-dimensional NOE experiments were recorded using the double-pulsed field gradient spin−echo (DPFGSE) sequence as described by Stott et al. (1995).

Ultraviolet spectra were recorded in MeOH on a Cecil 3021 spectrophotometer.

Beer Production. Two beers were produced in 265 L batches according to normal brewing procedures. Both beers were of the lager type, and they were brewed using 68% two-rowed malted barley (Great Western Malting) and 32% rice syrup solids. Before boiling, the wort volume was 303 L (pH 5.6). The volume after wort boiling was 265 L. Lactic acid was added to the wort during the boil, resulting in pH 5.2 at the end of the boil.

The hopping rate for each beer was intended to result in the same number of bittering units (BU), that is, 12 BU. For the first brew, whole hops (172 g, cv. Willamette harvested in 1997) were added in one portion 15 min after the wort was brought to boiling. Boiling was continued for 75 min. For the second brew, whole hops from the same bale were added in two portions. The first portion (128 g, accounting for 75% of the BU) was added 15 min after the wort was brought to boiling; the second portion (137 g, accounting for 25% of the BU) was added 5 min before the end of the 90 min-boiling period.

After wort boiling, a whirlpool was formed in the kettle using a paddle, and the solids were allowed to settle for 15 min. The wort was then pumped to the fermentation tank via a heat exchanger, which cooled the wort to 13 °C. During the transfer, the wort [specific gravity = 8.5 °Plato (°P)] was oxygenated with sterile oxygen. The finished wort was cooled to 4 °C and held overnight to allow further settling. The wort was then warmed to 13 °C and inoculated with a lager yeast (Wyeast Laboratories, strain 2035). Primary fermentation was carried out at 13 °C for 7 days. The temperature was raised to 15.5 °C for 48 h (diacetyl rest) and then dropped to 3 °C for secondary fermentation, which lasted 15 days. The raw beer was transferred into 19 L kegs and stored at 2 °C for 7 days; it was then aged at 1 °C for another 2−3 weeks (lagering period).

Brew Samples and Sample Preparation. Samples were collected at various stages during the brewing process and treated as follows prior to LC/MS analysis:

(1) Hops. A 5 g sample of hop cones was ground with a Wiley mill to pass a 20 mesh sieve. An aliquot (0.400 g) was extracted in 100.0 mL of MeOH by sonication for 10 min. A portion of the extract was centrifuged (1300*g*, 15 min). An aliquot of the supernatant (2.5 mL) and 50 μL of internal standard solution (2′,4-dihydroxychalcone, 1 mg/mL DMSO) were placed in a 100 mL volumetric flask, and the contents were made up to volume with MeOH/HCOOH (99:1 v/v). The rest of the ground hops was lyophilized to determine the moisture content (9.3 and 9.7% for hop cones used in the first and second brews, respectively).

(2) Unhopped Wort. A 0.5 L sample of the wort was taken before the hops were added. A portion of the liquid was centrifuged (1300*g*, 15 min), and 100.0 mL of supernatant was spiked with 50 μL of internal standard solution.

(3) Wort Sampled 15 min after Addition of Hops. This was treated in the same manner as the unhopped wort.

(4) Wort Sampled 30 min after Addition of Hops. This was treated the same as the unhopped wort.

(5) Wort Sampled 70 min after Addition of Hops. This was treated the same as the unhopped wort.

(6) Wort Sampled after Whirlpool. This was treated the same as the unhopped wort.

(7) Spent Hops. After whirlpool and transfer of the wort to the fermenter, solids remaining in the kettle were allowed to settle for ~5 min, and the spent hops were quantitatively recovered using a coarse sieve. The weight of the wet spent hops was determined. A weighed portion was lyophilized. The lyophilized material was weighed, ground, and further treated as hops.

(8) Hot Trub. Fine solids were allowed to resettle after recovery of the spent hops. The deposit, consisting mainly of coagulated protein (trub) and finer hop particles, was drained as a slurry from the bottom of the brew kettle. The slurry was weighed, and an aliquot was filtered through Whatman No. 1 paper. The weight of the hot trub was determined after lyophilization. A 0.400 g sample of the lyophilized material was extracted with 100 mL of MeOH by sonication for 10 min. The extract was centrifuged (1300*g*, 15 min), and 25.0 mL of the supernatant was spiked with 50 μL of internal standard solution and made up to 100 mL with MeOH/HCOOH (99:1, v/v).

(9) Preyeast Pitch Wort. After the cold trub was removed from the fermenter, a sample of the supernatant was taken prior to inoculation with the yeast and further treated as unhopped wort.

(10) Cold Trub. During the overnight chilling of the wort in the fermenter, another portion of coagulated protein and other solids settled in the coned-shaped bottom of the fermenter, which was drained as a slurry and further treated the same as hot trub.

(11) End of Primary Fermentation. A sample was drawn from the fermenter and treated the same as the unhopped wort.

(12) Seven Days of Secondary Fermentation. A sample was drawn after 7 days of secondary fermentation and treated as the unhopped wort.

(13) Yeast Slug. The yeast settled out during the secondary fermentation and was drawn off as a slurry from the cone-shaped bottom of the fermenter. The yeast slurry was further treated the same as hot trub.

(14) Raw Beer. Raw beer was treated the same as the unhopped wort.

(15) Beer after Lagering. This was treated the same as the unhopped wort.

Solubility of Xanthohumol and Isoxanthohumol in Different Media. The following media were studied: water, 5% ethanol in water (v/v), beer (a major U.S. brand containing no detectable amounts of prenylflavonoids), low-gravity wort (8.5 °P ≅ 8.4 g of extract solids/100 g of wort), and high-gravity wort (13 °P ≅ 12.9% w/w). Before the start of the experiments, the worts were refrigerated and then centrifuged (1300*g*, 15 min) to remove any cloudiness. To 1.0 mg portions of xanthohumol and isoxanthohumol was added 25.0 mL of the above media. The mixtures were shaken from time to time and kept at 80 °C for 30 min, resulting in complete dissolving of the solids. The mixtures were cooled and kept at room temperature for at least 4 h and then centrifuged (1300*g*, 15 min). Aliquots of the supernatants (100 μL) and 50 μL of the internal standard solution (0.10 mg/mL DMSO) were diluted to 10.0 mL with MeOH/H_2O/HCOOH (50:49:1 by vol). The centrifuged mixtures were then resuspended by shaking and kept at 8 °C for at least 4 h. After centrifugation (1300*g*, 15 min), 100 μL aliquots were diluted as described for the room temperature supernatants. Additional 1.0 mg portions of isoxanthohumol were mixed with 5.0 mL of the beer and worts and further treated and analyzed as described above.

Isomerization of Xanthohumol in a Model System. The reaction medium was prepared by dissolving 10 g of sucrose in a mixture of 10 mL of ammonium acetate buffer (100 mM, adjusted with acetic acid to pH 5.5) and 50 mL of water, finally diluted with water to 100 mL. This solution was brought to a boil, and xanthohumol (200 μg, dissolved in 100 μL of DMSO) was added. The reaction mixture was left boiling (101 °C) under reflux conditions, and 4 mL samples were drawn from the reaction mixture at 5, 15, 30, and 60 min after addition of

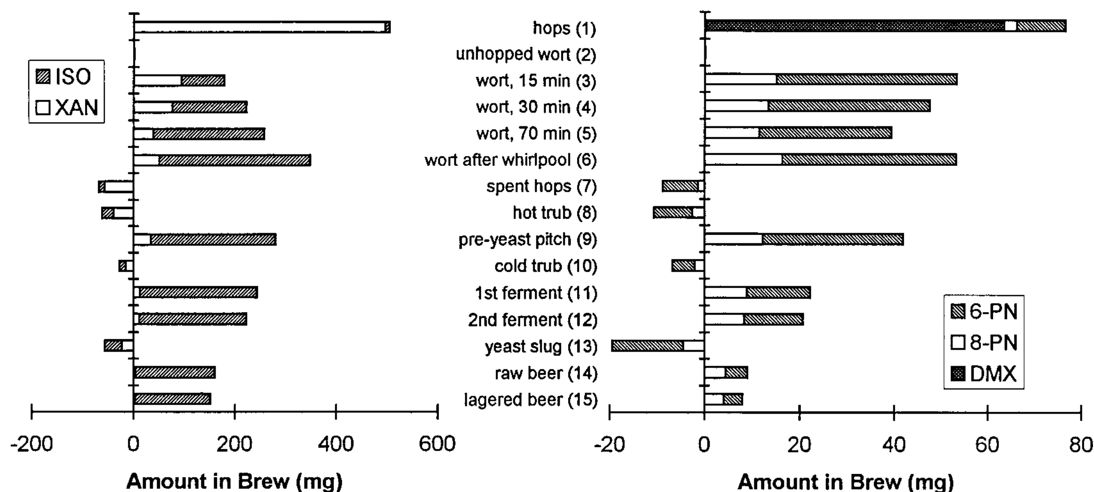

Figure 3. Prenylflavonoids measured in single-hopped brew. Flavonoids: ISO, isoxanthohumol; XAN, xanthohumol; 6-PN, 6-prenylnaringenin; 8-PN, 8-prenylnaringenin; DMX, desmethylxanthohumol. Brewing stages: for more details, see Brew Samples and Sample Preparation under Experimental Procedures.

xanthohumol. After the samples had cooled to room temperature, 2.5 mL aliquots were spiked with 5 μL of internal standard solution (2′,4-dihydroxychalcone, 1.0 mg/mL DMSO) and made up to 10 mL with 10 mM ammonium acetate buffer. The time = 0 min sample (pH 5.4) was prepared by adding xanthohumol (200 μg, dissolved in 100 μL of DMSO) to 100 mL of reaction medium at room temperature; a 2.5 mL aliquot was diluted to 10 mL with 10 mM ammonium actetate buffer after the addition of 5 μL of internal standard solution. These solutions were analyzed by LC/MS as described above. At the end of the experiment and after cooling to room temperature, the reaction mixture was at pH 5.4.

RESULTS AND DISCUSSION

The prenylated flavonoids of hops are predominantly of the chalcone type (Figure 1). No less than 95% of the prenylflavonoid fraction of hops consists of xanthohumol, desmethylxanthohumol, and 3′-geranylchalconaringenin (3′-geranyl-2′,4′,6′,4-tetrahydroxychalcone). Hops contain minor quantities of prenylated flavanones, which are most likely formed during drying and storage of commercial hops (Stevens et al., 1997). By contrast, beer contains much higher levels of prenylflavanones than prenylchalcones (Stevens et al., 1999). Obviously, beer prenylflavanones are formed from their isomeric chalcones during the brewing process. The conversion of chalcones into flavanones requires the presence of a 2′- or 6′-hydroxyl group. Because xanthohumol has one hydroxyl available for ring closure, isoxanthohumol is the only possible cyclization product. Desmethylxanthohumol and 3′-geranylchalconaringenin, on the other hand, have two hydroxyl functions that can participate in cyclization, yielding two flavanones for each chalcone (Figure 1). Other ingredients of beer, barley (*Hordeum vulgare*), rice (*Oryza sativa*), and corn (*Zea mays*), do not contain prenylflavonoids. The flavonoids of barley are of the proanthocyanidin type (McMurrough, 1981) which do not interfere with the prenylflavonoids of hops in our LC/MS method.

Effect of Early Hopping on the Overall Yield. In the first brewing experiment, a lager-type beer was brewed with a single dose of hops (172 g), calculated to give a beer with ~12 BU. The hops were added 15 min after the wort was brought to a boil and remained in contact with the boiling wort for 75 min. Flavonoid amounts present in the brew at various stages or in the waste materials were determined and expressed as percent of the prenylflavonoid amounts in the hops (Figure 3). Because 13% of the hops' xanthohumol was found in the spent hops, at least 87% must have been extracted. However, the amount of xanthohumol measured in the post-whirlpool wort was equal to 69%. The difference (18%) could largely be accounted for by adsorption to the coagulated proteins (hot trub, 12%), which settled out of the hot wort at the end of the boil. Later in the brew, an additional 17% was lost due to adsorption to the cold-precipated proteins (cold trub, 6%) and yeast (11%). The overall yield of xanthohumol dropped to 30% in the beer after lagering; at this stage, 98% of the amount of xanthohumol was present as isoxanthohumol. A substantial amount of xanthohumol (27%) could not be accounted for.

Similar results were obtained with desmethylxanthohumol (Figure 3). This prenylchalcone went more quickly into solution than xanthohumol, most likely due to its higher hydrophilicity, and showed a much faster conversion into its isomeric prenylflavanones. In fact, desmethylxanthohumol had completely isomerized by the time the first wort sample was drawn from the brew kettle, 15 min after addition of the hops. The overall yield of desmethylxanthohumol (in the form of 6- and 8-prenylnaringenin) amounted to 11% in the beer after lagering. The combined losses (spent hops, trub, and yeast) totaled 60%, leaving 29% of the amount of desmethylxanthohumol initially present in the hops unaccounted for.

Effect of Late Hopping on the Overall Yield. It is common practice in the brewing industry to add a second portion of hops late in the wort boil to retain a greater portion of the hops' essential oils in the beer, resulting in a stronger aroma, usually referred to as "late hop aroma". The effect of late hopping on the fate of xanthohumol and desmethylxanthohumol was also investigated (Figure 4). To obtain a beer with ~12 BU, the total amount of hops added was increased to 265 g, of which 128 g was added as "kettle" hops and 137 g as "finish hops". The kettle hops remained in the boiling wort for 75 min, and the finish hops were added during the last 5 min of the wort boil. Shortly after removal of the (combined) spent hops, the xanthohumol amount in the wort was 53% of the amount in the total of hops added (as compared to 69% in the first brew), reflecting

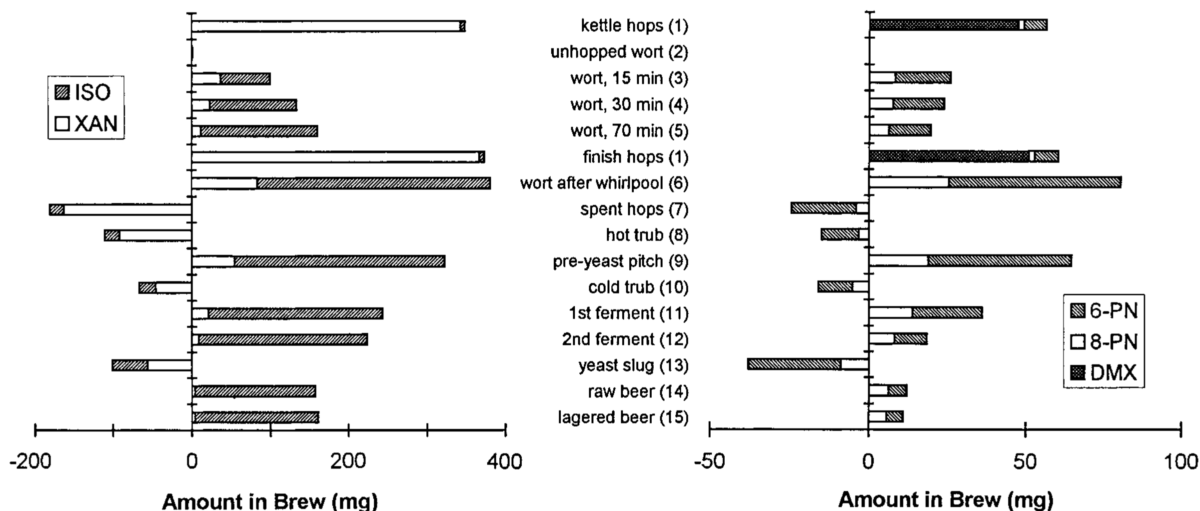

Figure 4. Prenylflavonoids measured in double-hopped brew. See Figure 3 for keys to flavonoids and brewing stages.

the brief contact between the finish hops and the boiling wort. At this stage, 40% of total added xanthohumol was found in the spent hops and the hot trub. Further losses were due to adsorption to the cold-precipitated proteins (cold trub, 9%) and to the yeast slug (14%). About 22% of the hops' xanthohumol was found in the beer after lagering, largely in the form of isoxanthohumol. The losses totaled 64%, leaving 14% unaccounted for.

With desmethylxanthohumol, a significant increase in the levels of 6- and 8-prenylnaringenin was seen 20 min after addition of the finish hops (this period included 5 min of wort boiling and 15 min of whirlpool settling) (Figure 4). Moreover, this increase almost equals the amount of desmethylxanthohumol present in the finish hops, indicating a virtually quantitative extraction of desmethylxanthohumol. By contrast, a mere 45% was found in the wort 15 min after addition of the kettle hops, and the total amount of prenylnaringenins dropped to 36% of the hops' desmethylxanthohumol just before addition of the finish hops. Apparently, extraction and isomerization of desmethylxanthohumol are complete within a few minutes, after which time a gradual loss of prenylnaringenins from solution sets in as a result of adsorption to the spent hops (21%) and insoluble proteins (hot trub, 13%). Although a further 14% precipitated with the cold trub, most of the prenylnaringenins were lost during fermentation as a result of adsorption to the yeast cells (32%). About 10% of the hops' desmethylxanthomumol remained, in flavanone form, in the lager beer, leaving 11% unaccounted for.

Adsorption of Prenylflavonoids to Trub Proteins and Yeast. Several processes determine the fate of xanthohumol from hops to beer: extraction, isomerization, and adsorption. Whereas extraction and isomerization have already been dealt with in some detail, the process of adsorption is more complex and needs further discussion. Many studies on the interaction between polyphenols and proteins [see, e.g., McMurrough et al. (1995) and Siebert and Lynn (1998)] have led to the general belief that hydrophobic forces and hydrogen bonding play important roles in the interaction between phenolic groups and proteins (Outtrup, 1992; Haslam, 1996). Most of these studies were focused on catechin, epicatechin, and their oligomers (condensed tannins), which are much more soluble in water than are the hop prenylflavonoids.

Table 1. Apparent Solubility of Xanthohumol and Isoxanthohumol

medium	temp (°C)	concn[a] (mg/L) xanthohumol	concn[a] (mg/L) isoxanthohumol
water	23	1.3	5.0
	8	1.1	3.9
5% ethanol	23	3.5	5.5
	8	1.8	4.4
beer	23	5.3	27.2
	8	4.0	23.8
low SG wort[b]	23	8.7	39.6
	8	7.0	37.6
high SG wort[c]	23	9.5	43.3
	8	8.9	37.3

[a] Mean of two measurements by LC/tandem MS or LC/UV. [b] Specific gravity = 1.034 (8.5 °P). [c] Specific gravity = 1.052 (13 °P).

As with other polyphenols, adsorption of prenylflavonoids is assumed to be an equilibrium process in which the distribution of bound and unbound molecules depends on the nature of the complexing materials and the solubility of the prenylflavonoid in the medium. The solubilities of xanthohumol and isoxanthohumol were determined in five different aqueous media (Table 1). Isoxanthohumol is more soluble in the true solvents, water and 5% ethanol, than xanthohumol. It was also more soluble in the beer and in the worts. The beer and the worts contain carbohydrates (1, 8.4, and 13% w/w, respectively), which keep a higher amount of both prenylflavonoids in solution, presumably as soluble complexes (referred to as "apparent solubility" in Table 1). In support of the complexation hypothesis, an increase in the apparent solubility was observed with the wort having a higher specific gravity, that is, a higher concentration of carbohydrates.

Taking the results of the brew trials and the solubility experiments together, it seems reasonable to assume that the amounts of freely dissolved xanthohumol and isoxanthohumol are small compared to the amounts bound to trub proteins and wort carbohydrates. After whirlpool settling, there is much more isoxanthohumol than xanthohumol in the brew as a result of isomerization, yet more xanthohumol was recovered from the hot trubs in both brews. Isoxanthohumol shows a higher affinity for the wort carbohydrates than xanthohumol (Table 1), and this interaction may reduce the pool of free isoxanthohumol available for binding to trub pro-

teins. Differences in the relative affinity of xanthohumol and isoxanthohumol for the trub and yeast surfaces could also play a role; however, this was not investigated in this study. It is hypothesized that these differences in prenylflavonoid–macromolecule affinities result in the greater proportion of xanthohumol measured in the protein residues of the trub materials and the yeast. Yeast proteins accessible to prenylflavonoids are located in the outer layer of the cell walls (Stewart and Russell, 1981). In the solubility experiments, the contribution of trub proteins to prenylflavonoid–macromolecule complexation was assumed to be minimal because any cloudiness formed on refrigeration of the worts was removed by centrifugation prior to addition of the flavonoids.

There is little doubt that adsorption caused a gradual decrease of the prenylnaringenin levels during wort boiling in both brewing trials. Wort boiling induces denaturation of proteins, thereby enhancing the surface available for interaction with prenylflavonoids (Haslam, 1996). Denaturated proteins may form aggregates, which become visible as a trub-like precipitate on cooling of the wort samples taken during the boil. These precipitates were removed by centrifugation prior to LC/MS analysis, hence the apparent decrease of the prenylnaringenin levels in the supernatants. Whirlpool settling resulted in precipitation of trub particles while the wort was still hot. Under these circumstances, it is more favorable for the prenylnaringenins to remain in solution, even after cooling. This may explain why lower prenylnaringenin levels were measured in the prewhirlpool worts. Although less prominent, the same probably underlies the sudden jump in the xanthohumol and isoxanthohumol levels after whirlpool settling in the first brew. Unlike the prenylnaringenins, the combined xanthohumol/isoxanthohumol concentration shows a net increase, caused by a positive balance between continuing release of xanthohumol from the hops and loss due to adsorption.

The solubility experiments also demonstrate that solubility hardly dictates the isoxanthohumol levels of commercial beers, which vary greatly, from absence in beers bittered with hop extract preparations to 3.44 mg/L in highly hopped beers (Stevens et al., 1999). From Table 1 it can be seen that a saturated solution of isoxanthohumol in beer contains ~8 times more isoxanthohumol than a beer richest in prenylflavonoids (3.44 mg/L). The brew trials suggest rather that adsorption of prenylflavonoids is more important than the hop dosage for the final prenylflavonoid concentration in the beer. Despite the extra xanthohumol added in the second brew, both brews yielded beers with about the same isoxanthohumol levels (0.62 and 0.65 mg/L, respectively).

Fate of the "Missing" Flavonoid Amounts. Substantial amounts of the hops' xanthohumol (27%) and desmethylxanthohumol (29%) were missing from the total of amounts measured in the final products (i.e., beer, yeast, trubs, and spent hops). Part of these amounts could be attributed to less than full recovery of "lost" fractions and measurement errors. Although best efforts were made to recover the spent hops, trubs, and yeast slug, some losses did occur; on the basis of visual inspection, these are thought to be <5% in all cases. Regarding measurement error, it can be argued that the LC/MS method underestimates xanthohumol in wort and beer, as shown in a previous study (Stevens et al., 1999). However, the prenylflavonoids in beer are largely in the flavanone form, which can be quantified by the method with sufficient accuracy (±5% for isoxanthohumol and 6-prenylnaringenin, ±8% for 8-prenylnaringenin). Furthermore, the accuracy with regard to xanthohumol was shown to vary within ±5% when dissolved in methanol, also used in this study for extraction of hops, spent hops, trubs, and yeast. In addition, the extraction of the trubs and yeast with methanol proved to be satisfactory: a second extraction of the remaining solids yielded only 0–3.7% of the flavonoids found in the initial extracts. Therefore, some cause(s) other than experimental error must account for the bulk of the missing 27% of the initial xanthohumol.

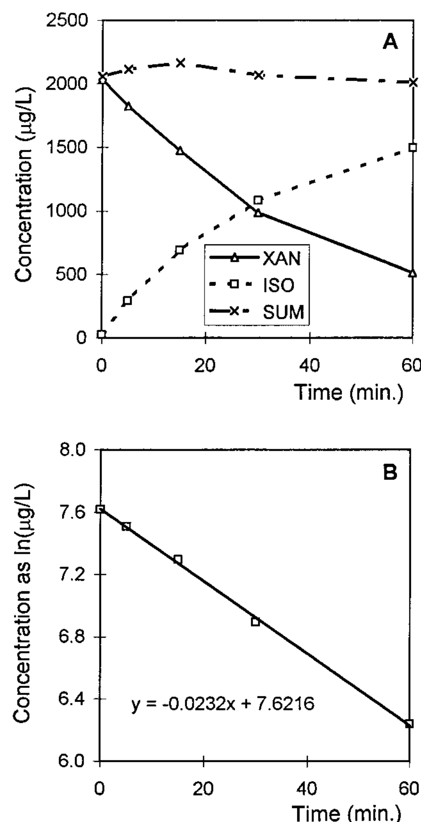

Figure 5. (A) Isomerization of xanthohumol in a model system (boiling water, 10% sucrose, pH 5.4). (B) Plot of ln[xanthohumol] vs boiling time (half-life = 30 min).

Another explanation for the missing amounts could be the participation of xanthohumol and desmethylxanthohumol in chemical reactions besides isomerization. Therefore, the conversion of xanthohumol into isoxanthohumol was carried out in a model system to eliminate loss of xanthohumol as a result of adsorption. The decrease of xanthohumol was in very good balance with the formation of isoxanthohumol in boiling water (Figure 5). Assuming first-order kinetics, the half-life of xanthohumol was calculated to be 30 min. These results led to the conclusion that the contribution of possible side reactions to the degradative loss of xanthohumol is negligible in boiling water. It is assumed the same is true for boiling wort.

Fungal Metabolism. It is known that fungi such as *Botrytis cinerea* and *Saccharomyces cerevisiae* (brewers' yeast) contain monooxygenases that are capable of epoxidizing double bonds of isoprenoid moieties (Satoh et al., 1993; Tanaka and Tahara, 1997). Xanthohumol and related prenylflavonoids could be epoxidized in a

similar fashion and then hydrolyzed or reduced to alcohols. To test this hypothesis, a concentrated methanolic extract of yeast, recovered from the raw beer, was analyzed by LC/MS using single ion monitoring and daughter ion scanning. Several compounds were detected that had masses consistent with that of oxygenated (iso)xanthohumol (M_r 370); daughter ion mass spectra allowed these products to be characterized as prenylated chalcones or flavanones. These compounds were present only in trace amounts, and some occur naturally in hops, which left doubt as to whether these trace constituents are produced by the hops or by the yeast. At any rate, these xanthohumol derivatives do not nearly make up for the amount of missing xanthohumol in the brew trials (14–27%).

Geranylflavonoids. The geranyl analogue of desmethylxanthohumol, 3′-geranylchalconaringenin, is the only known geranylchalcone from hops (Stevens et al., 1997). The fate of this geranylflavonoid could not be quantified because it and 8-geranylnaringenin were not available in sufficient amounts at the time the brewing trials were conducted. However, the chromatographic profiles indicated that 3′-geranylchalconaringenin behaved very similarly to desmethylxanthohumol in both brewing experiments. After addition of the hops to the boiling wort, 3′-geranylchalconaringenin was rapidly converted into two products, which were identified as 8- and 6-geranylnaringenin by LC/MS comparison with authentic markers (Figure 2).

A small amount of 6-geranylnaringenin was obtained by alkali treatment of 3′-geranylchalconaringenin isolated from hops. The position of its geranyl substituent was determined by 2-D NMR: in the $^{1}H-^{13}C$ HMBC spectrum, cross-peaks showing interaction between the chelated 5-hydroxyl and C-6 and between H-1″ of the geranyl group and C-6 left no doubt that the geranyl group was located ortho to the hydroxyl at C-5. Furthermore, the only aromatic A-ring proton showed cross-peaks with all A-ring carbons but C-5 and was therefore assigned to H-8.

A larger amount of 6-geranylnaringenin was obtained by geranylation of naringenin with (±)-linalool. The semisynthetic product was identical to the above 6-geranylnaringenin by UV, LC/MS, and 2D-NMR. It was treated with alkali to give 3′-geranylchalconaringenin and an earlier eluting product, which was identified as 8-geranylnaringenin by MS and 2-D NMR spectroscopy. In the HMBC spectrum of the latter compound, the aromatic A-ring proton was assigned to H-6 because it showed cross-peaks with all A-ring carbons but C-9. Carbon-9 interacted with H-1″ of the geranyl substituent at C-8. The identity of carbons 5, 6, and 10 was confirmed by interactions between these carbons and the hydrogen-bonded 5-hydroxyl proton. Full assignments of proton and carbon resonances for both geranylnaringenins are listed under Experimental Procedures.

The condensation of naringenin with linalool results in the formation of a double bond between C-2″ and C-3″ of the monoterpenyl substituent (Figure 1). Double-bond formation may give rise to cis and trans isomers, that is, neryl- and geranylnaringenins, and this problem was investigated by means of 1-D NOE experiments. Irradiation of the H-2″ resonance of 8-geranylnaringenin resulted in enhancement of the H-1″ and H-5″ signals, whereas the intensity of the CH_3-4″ singlet remained unaltered. This was taken as proof for H-2″ being located trans to the CH_3-4″ group, thus confirming the presence of a geranyl, not neryl, substituent.

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LITERATURE CITED

Bohm, B. A. Chalcones and aurones. In *Methods in Plant Biochemistry, Vol. 1. Plant Phenolics*; Harborne, J. B., Ed.; Academic Press: London, U.K., 1989; pp 237–281.

Charalambous, G. Involatile constituents of beer. In *Brewing Science*; Pollock, J. R. A., Ed.; Academic Press: London, U.K., 1981; Vol. 2, pp 167–254.

Delcour, J. A. Malt and Hop Flavanoids in Pilsner Beer. In *Modern Methods of Plant Analysis, Vol. 7, Beer Analysis*; Linskens, H. F., Jackson, J. F., Eds.; Springer-Verlag: Berlin, Germany, 1988; pp 225–240.

Hänsel, R.; Schulz, J. Desmethylxanthohumol: Isolierung aus Hopfen und Cyclisierung zu Flavanonen. *Arch. Pharm. (Weinheim)* **1988**, *321*, 37–40.

Haslam, E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J. Nat. Prod.* **1996**, *59*, 205–215.

Jain, A. C.; Gupta, R. C.; Sarpal, P. D. Synthesis of lupinifolin, di-O-methylxanthohumol and isoxanthohumol and related compounds. *Tetrahedron* **1978**, *34*, 3563–3567.

McGuinness, J. D.; Laws, D. R. J.; Eastmond, R.; Gardner, R. J. The estimation and significance of catechin and dimeric catechin in beer. *J. Inst. Brew.* **1975**, *81*, 237–241.

McMurrough, I. High-performance liquid chromatography of flavonoids in barley and hops. *J. Chromatogr.* **1981**, *218*, 683–693.

McMurrough, I.; Madigan, D.; Smyth, M. R. Adsorption by polyvinylpolypyrrolidone of catechins and proanthocyanidins from beer. *J. Agric. Food Chem.* **1995**, *43*, 2687–2691.

Miranda, C. L.; Stevens, J. F.; Helmrich, A.; Henderson, M. C.; Rodriguez, R. J.; Deinzer, M. L.; Barnes, D. W.; Buhler, D. R. Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*Humulus lupulus*) in human cancer cell lines. *Food Chem. Toxicol.* **1999**, in press.

Moll, M.; Fonknechten, G.; Carnielo, M.; Flayeux, R. Changes in polyphenols from raw materials to finished beer. *Tech. Q.—Master Brew. Assoc. Am.* **1984**, *21* (2), 79–87.

Outtrup, H. Proanthocyanidins, the brewing process, and the quality of beer. In *Plant Polyphenols*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: New York, 1992; pp 849–858.

Peacock, V. Fundamentals of hop chemistry. *Tech. Q.—Master Brew. Assoc. Am.* **1998**, *35* (1), 4–8.

Satoh, T.; Horie, M.; Watanabe, H.; Tsuchiya, Y.; Kamei, T. Enzymatic properties of squalene epoxidase from *Saccharomyces cerevisiae*. *Biol. Pharm. Bull.* **1993**, *16*, 349–352.

Siebert, K. J.; Lynn, P. Y. Comparison of polyphenol interactions with polyvinylpolypyrrolidone and haze-active protein. *J. Am. Soc. Brew. Chem.* **1998**, *56*, 24–31.

Stevens, J. F.; Ivancic, M.; Hsu, V. L.; Deinzer, M. L. Prenylflavonoids from *Humulus lupulus*. *Phytochemistry* **1997**, *44*, 1575–1585.

Stevens, J. F.; Taylor, A. W.; Deinzer, M. L. Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **1999**, *832*, 97–107.

Stewart, G. G.; Russell, I. Yeast Flocculation. In *Brewing Science*; Pollock, J. R. A., Ed.; Academic Press: London, U.K., 1981; Vol. 2, pp 61–92.

Stott, K.; Stonehouse, J.; Keeler, J.; Hwang, T.-L.; Shaka, A. J. Excitation sculpting in high-resolution nuclear magnetic resonance spectroscopy: application to selective NOE experiments. *J. Am. Chem. Soc.* **1995**, *117*, 4199–4200.

Tabata, N.; Ito, M.; Tomoda, H.; Omura, S. Xanthohumols, diacylglycerol acyltransferase inhibitors from *Humulus lupulus*. *Phytochemistry* **1997**, *46*, 683–687.

Tanaka, M.; Tahara, S. FAD-Dependent epoxidase as a key enzyme in fungal metabolism of prenylated flavonoids. *Phytochemistry* **1997**, *46*, 433–439.

Tobe, H.; Muraki, Y.; Kitamura, K.; Komiyama, O.; Sato, Y.; Sugioka, T.; Maruyama, H. B.; Matsuda, E.; Nagai, M. Bone resorption inhibitors from hop extract. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 158–159.

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