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Structural Identification and Distribution of Proanthocyanidins in 13 Different Hops

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Ten newly isolated hop proanthocyanidin oligomers and flavan-3-ol monomers from 13 different hops have been identified as gallocatechin, gallocatechin-(4α→8)-catechin, gallocatechin-(4α→6)-catechin, catechin-(4α→8)-gallocatechin, catechin-(4α→6)-gallocatechin, afzelechin-(4α→8)-catechin, catechin-(4α→8)-catechin-(4α→8)-catechin, epicatechin-(4β→8)-epicatechin-(4β→8)-catechin, catechin-(4α→8)-gallocatechin-(4α→8)-catechin, and gallocatechin-(4α→8)-gallocatechin-(4α→8)-catechin, together with seven previously isolated oligomers, namely, catechin, epicatechin, epicatechin-(4β→8)-catechin, epicatechin-(4β→8)-epicatechin, catechin-(4α→8)-catechin, catechin-(4α→8)-epicatechin, and epicatechin-(4β→8)-catechin-(4α→8)-catechin. These compounds were subjected to acid-catalyzed degradation in the presence of phloroglucinol or by partial or complete acid-catalyzed degradation and reaction with benzyl mercaptan followed by desulfurization. The resultant adducts when compared to authentic samples by high-performance liquid chromatography−atmospheric pressure chemical ionization tandem mass spectrometry and high-performance liquid chromatography−electrospray ionization tandem mass spectrometry served to identify the precursors. The composition of proanthocyanidins from 13 different hops was similar, but the concentration of individual compounds showed some differences, which indicated that hop proanthocyanidin profiles are affected by geographic origin and are variable depending on the cultivars.

KEYWORDS: Hops; proanthocyanidins; *Humulus lupulus*; Cannabinaceae; HPLC/APCI−MS/MS; HPLC/ESI−MS/MS

INTRODUCTION

Of all the herbs that have been used in beer brewing, only the hop (*Humulus lupulus* L., Cannabinaceae) plant is regarded as an essential raw material in the beer brewing industry (*1*). Hops are perennial plants grown on trellises, and different varieties are derived via breeding programs. The hop plant is dioecious and cultivated in most temperate zones of the world for its female inflorescences, commonly referred to as hop cones or hops. The female flower clusters are partly covered with lupulin glands, while male flowers have only a few glands in the crease of their anthers and on their sepals. The resin secreted by these glands contains bitter acids, essential oils, and flavonoids (flavonol glycosides, prenylflavonoids, and tannins) (*2*). Since hops are used exclusively to give beer its characteristic aroma, bitterness, foam and light stability, the investigations of hop oil constituents have continued (*2*−*8*). In comparison with some other constituents, the brewing value of hop proanthocyanidins is not well-understood. Proanthocyanidins, also known as condensed tannins, are flavan-3-ol oligomers and polymers that give anthocyanidins upon acid depolymerization reactions. Because of the difficulty of extracting and purifying proanthocyanidins, together with their instability and structural complexity, understanding the chemistry of this class of polyphenols has been challenging (*9*).

Proanthocyanidins are widely distributed throughout the plant kingdom. There is a growing body of evidence linking these compounds with plant defense mechanisms, organoleptic characteristics, and potential health benefits (*6*−*11*). Hop proanthocyanidins have received special attention in the brewing industry because they contribute to haze formation (*12*). They also stabilize the organoleptic properties and color and contribute to the astringency and bitterness. The estimated amount of total hop proanthocyanidins ranges from 0.5 to 5% on a dry weight basis, depending on the variety, geographic origin, freshness, and harvesting procedure (*6*, *7*). Previous studies have also shown that up to 30% of the proanthocyanidins present in beer is derived from hops. Surprisingly, only a few oligomeric proanthocyanidins and monomeric flavan-3-ols have been reported in hops. These include catechin (*6*), epicatechin (*6*), epicatechin-(4β→8)-catechin (procyanidin B1) (*13*), epicatechin-(4β→8)-epicatechin (procyanidin B2) (*12*, *13*), catechin-(4β→8)-catechin (procyanidin B3) (*12*, *13*), and catechin-(4β→8)-epicatechin (procyanidin B4) (*12*, *13*). Trimeric procyanidins have also been reported to be present, but their structures were not elucidated (*6*). All of the effects of hop proanthocyanidins on beer values seem to depend on their affinity for proteins. Since little is known about their structures and composition,

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their nutritional significance or complexation ability with proteins is unclear. The possible sensory properties in beer also have not been studied.

The aim of this work was to isolate and elucidate the structures of unknown hop proanthocyanidins and to study their composition and distribution in 13 different hops. Ten proanthocyanidin oligomers and flavan-3-ol monomers isolated from hops are reported here for the first time in addition to seven that were previously isolated and identified.

MATERIALS AND METHODS

Materials. The 13 different hops chosen for study include Willamette hop cones (Oregon, Idaho, and Washington, USA), Vanguard pellet (USA), Palisade pellet (USA), Tettnang−Hallertauer pellet (Germany), Hallertauer−Hallertauer pellet (Germany), North American Hallertauer pellet (USA), Zeus pellet (USA), Cascade pellet (USA), Saaz 36 pellet (USA), Saaz 72 pellet (USA), and Glacier pellet (USA). Hop pellets are made from dried hop cones by milling in a hammer mill and then compressing the hop powder through a 6 mm die to form pellets of about 10−25 mm in length. The chemistry, content, and brewing value of these pellets were not changed in any way other than a slight loss of moisture content. The hops were all commercial samples, and all commercial hops are female. They were harvested at maturity in 2004 except for the Washington−Willamette hops, which were harvested in 2003.

(+)-Catechin, (−)-epicatechin, (+)-gallocatechin, and (−)-epigallocatechin were purchased from Sigma-Aldrich (Milwaukee, WI), and (+)-afzelechin and (−)-epiafzelechin were gifts kindly provided by Prof. Tak H. Chan of The Hong Kong Polytechnic University; (+)-taxifolin for the synthesis of catechin-(4α→2)-phloroglucinol was also purchased from Sigma-Aldrich (Milwaukee, WI; grape seeds were kindly provided by Dr. James A. Kennedy of Oregon State University that were extracted to prepare epicatechin-(4β→2)-phloroglucinol and epigallocatechin-(4β→2)-phloroglucinol; and black currant leaves were kindly provided by Mrs. Kim Hummer of the USDA ARS National Clonal Germplasm Repository for tannin extracts that were used to prepare gallocatechin-(4α→2)-phloroglucinol.

Hexane, dichloromethane, acetone, ethanol, and methanol were of HPLC grade and purchased from Fisher Scientific (Santa Clara, CA). Glacial acetic acid, formic acid, benzyl mercaptan, phloroglucinol, and Raney nickel were purchased from Sigma (St. Louis, MO). Sephadex LH-20 and Toyopearl TSK HW-40S were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The Synergi C_{18} column and Luna phenyl-hexyl C_{18} column were purchased from Phenomenex (Torrance, CA). Water was purified to HPLC grade with a Millipore Milli-Q apparatus (Bedford, MA). All solvent−water mixtures used for column chromatography contained 0.1% (v/v) formic acid (Fluka brand, Sigma-Aldrich, Milwaukee, WI) and were degassed by helium sparging prior to use. Hydrogen (H_2), nitrogen (N_2), argon (Ar), helium (He), and sulfur dioxide (SO_2) were all high-purity grade.

Preparation of Proanthocyanidins from Hops. Air-dried hop cones (100.0 g) were briefly immersed in dichloromethane (CH_2Cl_2) and stirred for 1 h, and the extract was decanted. The hop cones were further washed with CH_2Cl_2 3 times (1.5 L × 3) to extract the resins, pigments, and lipids, dried in a stream of air in a fume hood, and then ground with a Wiley mill (sieve no. 20) to obtain hop granules (72.4 g). The hop granules (72.4 g) were extracted with 1 L of acetone/water (7:3, v/v). This extraction step was repeated 3 times with 1 L of acetone/water (7:3, v/v). The combined acetone/water extracts were separated from the hop granules by filtration and then concentrated on a rotary evaporator under vacuum at less than 35 °C to remove the acetone. The resulting extract was washed with hexane (0.5 L × 2) 2 times and subsequently with CH_2Cl_2 (0.5 L × 2) 2 times to remove more pigments and nonpolar material, rotary evaporated to remove the residual organic solvents, and then passed through a 30 cm × 4 cm column of Sephadex LH-20 preequilibrated with water. The LH-20 column was successively eluted with water (500 mL), methanol/water (500 mL, 1:3, v/v), methanol/water (500 mL, 1:1, v/v), methanol/water (500 mL, 3:1, v/v), methanol (500 mL), and finally with acetone/water (500 mL, 7:3, v/v) at a flow rate of 100 mL/h. Each fraction (500 mL) was collected and monitored by HPLC−UV and two-dimensional (2-D) TLC on cellulose plates developed first with *t*-butyl alcohol/water/acetic acid (3:1:1, v/v/v), dried, then developed in the second dimension with 6% aqueous acetic acid, and visualized with a vanillin-HCl reagent. Fractions that contained mainly oligomeric proanthocyanidins and monomeric flavan-3-ols were combined and concentrated by rotary evaporation and lyophilization to yield 4.2 g of crude proanthocyanidins. The crude proanthocyanidin mixture (4.2 g) was passed through a 45 cm × 4 cm column of Sephadex LH-20 preequilibrated with water. The LH-20 column was successively eluted with water (1 L), methanol/water (1 L, 1:1, v/v), methanol (1 L), and finally with acetone/water (1 L, 7:3, v/v) at a flow rate of 100 mL/h. Each fraction (1 L) was collected and monitored by 2-D TLC and ESI−MS and then concentrated by rotary evaporation and lyophilized to give fraction 1 (0.5 g) consisting of glycosides and other materials, fraction 2 (0.4 g) consisting of monomeric flavan-3-ols and proanthocyanidin dimers, fraction 3 (0.3 g) consisting of proanthocyanidin oligomers, and fraction 4 (3.0 g) consisting of proanthocyanidin polymers.

Fractions 2 and 3 were further chromatographed on a 30 cm × 1.5 cm column of Toyopearl TSK HW-40 S using methanol as the eluent at a flow rate of 1 mL/min. Fractions of 10 mL each were collected and examined by HPLC−UV at 280 nm. The comparatively pure constituents of the hop oligomeric proanthocyanidins and monomeric flavan-3-ols were isolated by semipreparative HPLC using a linear solvent gradient from 5% B (MeOH) to 40% B in A (1% aqueous formic acid) over 40 min at a flow rate of 4 mL/min. The UV trace was recorded at 280 nm. Peak fractions identified by mass spectrometry were collected manually, the solvents were removed by rotary evaporation, and the remainder was lyophilized to dryness and stored at −15 °C.

The chemical structures of hop proanthocyanidins (**Figure 1**) consist of **1** (catechin), **2** (epicatechin), **3** (gallocatechin), **4** (epicatechin-(4β→8)-catechin, procyanidin B1), **5** (epicatechin-(4β→8)-epicatechin, procyanidin B2), **6** (catechin-(4α→8)-catechin, procyanidin B3), **7** (catechin-(4α→8)-epicatechin, procyanidin B4), **8** (gallocatechin-(4α→8)-catechin), **9** (gallocatechin-(4α→6)-catechin), **10** (catechin-(4α→8)-gallocatechin), **11** (catechin-(4α→6)-gallocatechin), **12** (afzelechin-(4α→8)-catechin), **13** (catechin-(4α→8)-catechin-(4α→8)-catechin, C2), **14** (epicatechin-(4β→8)-catechin-(4α→8)-catechin), **15** (epicatechin-(4β→8)-epicatechin-(4β→8)-catechin), **16** (catechin-(4α→8)-gallocatechin-(4α→8)-catechin), and **17** (gallocatechin-(4α→8)-gallocatechin-(4α→8)-catechin).

Electrospray ionization tandem mass spectrometry (ESI/MS−MS) was usually performed on a PE Sciex API III triple-quadrupole mass spectrometer in the positive ion MS mode. Samples diluted to 10 μg/mL were loop-injected into methanol/0.5% aqueous formic acid (2:1, v/v) flowing at 8 μL/min into the electrospray source. Ionization voltage was 5 kV, and the orifice was set at 60 V.

Atmospheric pressure chemical ionization tandem mass spectrometry (APCI/MS−MS) was also performed on a PE Sciex API III triple-quadrupole mass spectrometer in the positive ion MS mode, and the source was equipped with a heated nebulizer interface kept at 480 °C. Samples were introduced into the mass spectrometer by high-performance liquid chromatography (HPLC). APCI/MS−MS experiments were carried out with argon−10% nitrogen as the target gas at a thickness of ca. 1.9×10^{14} atoms per cm^2 using a collision energy of 20 V. Analytical HPLC separations were performed on a 250 mm × 4.6 mm Synergi 4 μm Hydro-RP-80A column with a linear gradient from 5 to 50% methanol in 1% aqueous formic acid over 50 min at 0.8 mL/min (procedure 1), and semipreparative HPLC was run on a 250 mm × 10 mm, 10 μm Econosil C_{18} column.

Acid-Catalyzed Degradation of Proanthocyanidins in the Presence of Phloroglucinol. According to the reported procedure (**Figure 2**) (*14*), a solution of proanthocyanidin dimer **1** (0.1 mg), phloroglucinol **18** (2 mg), and acetic acid (2 μL) in the solvent mixture of ethanol/water (100 μL, 1:3, v/v) was sparged with nitrogen, sealed, and heated to 100 °C for 20 min. The aliquot of the mixture was then diluted exactly with ethanol/water (1:3, v/v) to reduce the concentration of the main phloroglucinol adduct **19** below 0.5 mg/L. This sample was then analyzed directly by analytical HPLC/APCI−MS/MS on a 250

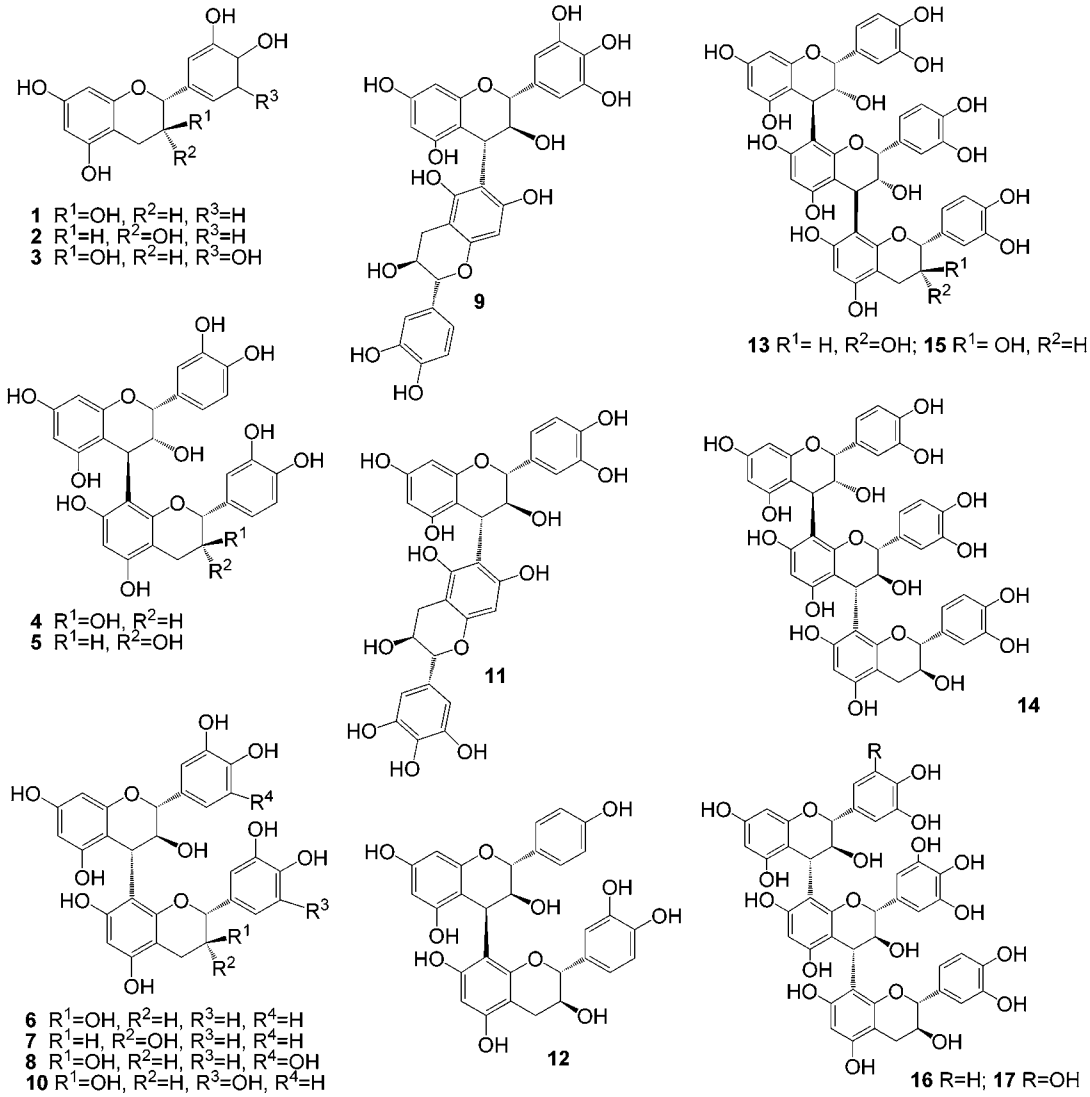

Figure 1. Chemical structures of hop proanthocyanidins.

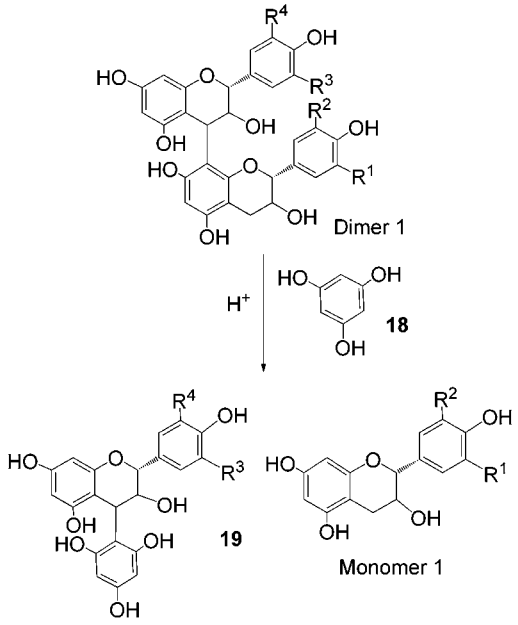

Figure 2. Degradation of proanthocyanidins in the presence of phloroglucinol.

mm × 4.6 mm i.d, 4 μm Synergi C_{18} column protected by a guard column containing the same material, using a binary gradient of mobile phases containing 1% v/v aqueous acetic acid (mobile phase A) and methanol (mobile phase B) at a flow rate of 0.8 mL/min. Eluting peaks were monitored at 280 nm; the gradient was maintained at 5% B for 10 min, then from 5 to 20% B over 20 min, and finally from 20 to 40% B over 25 min. The column was then washed with 90% B for 10 min and reequilibrated with 5% B for 5 min before the next injection (procedure 2).

The authentic sample of catechin-(4α→2)-phloroglucinol was prepared from commercial (+)-taxifolin according to a previous report (*14*). The solution of (+)-taxifolin (20 mg) and sodium borohydride (10 mg) in absolute ethanol (4 mL) was sparged with nitrogen and stirred at room temperature for 1 h, and then phloroglucinol (70 mg) in 4 mL of hydrochloric acid (0.1 N) was added and further stirred for 30 min. The solution was diluted with 4 mL of water, extracted with ethyl acetate (4 mL × 3), dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and evaporated under reduced pressure at less than 35 °C. The crude product was purified by a semipreparative HPLC (procedure 2) and lyophilized to obtain 2.2 mg (62% yield) of dry white powder, which was characterized by ^{1}H NMR (400 MHz, Bruker) and LC/APCI−MS (API 300, PE Sciex) operated in the positive mode and was confirmed as catechin-(4α→2)-phloroglucinol: APCI-MS, *m/z* 415 $[M + H]^+$; ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 4.40 (H-2, d, J = 8.8 Hz), 4.45 (H-4, d, J = 7.6 Hz), 4.61 (H-3, dd, J = 8.8, 7.6 Hz), 5.84 (phloroglucinol-2H, d, J = 2.1 Hz), 6.05 (A-ring, 2H, d, J = 2.4 Hz), 6.68 (B-ring, 1H, dd, J = 1.5, 7.8 Hz), 6.72 (B-ring, 1H, d, J = 7.8 Hz), 6.79 (B-ring, 1H, d, J = 1.5 Hz).

The authentic samples of epicatechin-(4β→2)-phloroglucinol and epigallocatechin-(4β→2)-phloroglucinol were prepared by acid-

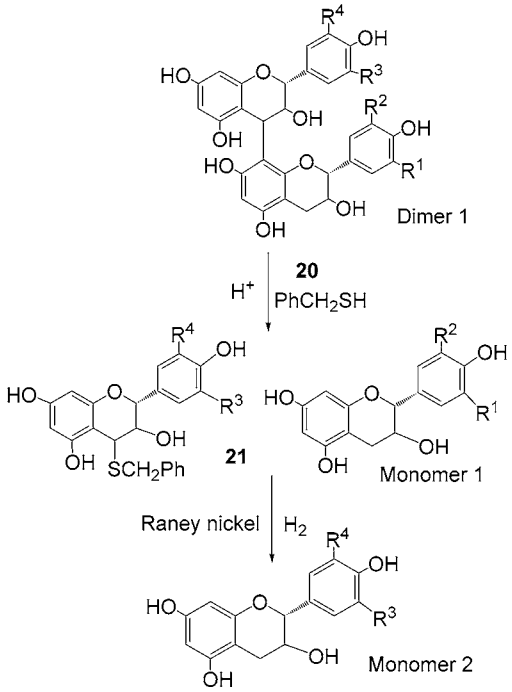

Figure 3. Degradation of proanthocyanidins in the presence of benzyl mercaptan.

catalyzed degradation of grape seeds. The solution of phloroglucinol (1 g), ascorbic acid (200 mg), and hydrochloric acid (0.1 N) in methanol (4 mL) was sparged with nitrogen, and the proanthocyanidin mixture (50 mg) extracted from grape seed was allowed to react in this solution at 50 °C for 20 min and then was combined with 5 vol of aqueous sodium acetate (0.04 M) to stop the reaction. The crude product was purified by semipreparative HPLC (procedure 2) to give two major products as follows: epicatechin-(4β→2)-phloroglucinol, white amorphous solid (3.5 mg); APCI−MS, *m*/*z* 415 $[M + H]^+$; ^{1}H NMR (CD_3-OD, 400 MHz) δ (ppm): 3.93 (H-3, dd, J = 3.3, 0.9 Hz), 4.54 (H-4, d, J = 3.3 Hz), 5.07 (H-2, d, J = 0.9 Hz), 5.94 (phloroglucinol-2H, d, J = 2.0 Hz), 6.07(A-ring, 2H, d, J = 2.3 Hz), 6.70 (B-ring, 1H, dd, J = 1.8, 8.2 Hz), 6.78 (B-ring, 1H, d, J = 8.2 Hz), 7.0 (B-ring, 1H, d, J = 1.8 Hz). Epigallocatechin-(4β→2)-phloroglucinol, white amorphous solid (1.2 mg); APCI−MS, *m*/*z* 431 $[M + H]^+$; ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 4.03 (H-3, dd, J = 2.3, 0.9 Hz), 4.62 (H-4, d, J = 2.3 Hz), 5.03 (H-2, d, J = 0.9 Hz), 5.94 (phloroglucinol-2H, d, J = 2.0 Hz), 6.04 (A-ring, 2H, d, J = 2.3 Hz), 6.50 (B-ring, 2H, s). By the same method, an authentic sample of gallocatechin-(4α→2)-phloroglucinol was prepared by acid-catalyzed degradation of black currant leaves (*Ribes nigrum* Raven) to give a white amorphous solid (2.1 mg), APCI−MS, *m*/*z* 431 $[M + H]^+$; ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 4.24 (H-2, d, J = 9.1 Hz), 4.43 (H-4, d, J = 8.0 Hz), 4.61 (H-3, dd, J = 9.1, 8.0 Hz), 5.87 (phloroglucinol-2H, d, J = 2.4 Hz), 6.01(A-ring, 2H, d, J = 2.4 Hz), 6.59 (B-ring, 2H, s).

Acid-Catalyzed Degradation of Hop Proanthocyanidins in the Presence of Benzyl Mercaptan (*15*). A solution of proanthocyanidin dimer 1 (0.1 mg), benzyl mercaptan **20** (1 μL), and acetic acid (1 μL) in a glass capillary tube loaded with ethanol (100 μL) was sparged with nitrogen, sealed, and heated to 100 °C for 13 h (**Figure 3**). The aliquot of the mixture was directly analyzed by analytical HPLC/APCI−MS/MS and then injected into the semipreparative HPLC column consisting of 250 mm × 10 mm i.d., 10 μm Econosil C_{18} column to isolate the benzyl mercaptan adduct **21** and monomer 1, both of which were collected and lyophilized to dryness. The mixture of benzyl mercaptan adduct **21**, monomer 1, and aqueous Raney nickel (50 μL) was added in a glass tube, which was sparged with nitrogen, shaken several times at regular intervals over a period of 1 h, directly analyzed by analytical HPLC/APCI−MS/MS, and then injected into the semipreparative HPLC to isolate monomer 1 and the desulfurized product, monomer 2. Monomers 1 and 2 were identified by HPLC/APCI−MS/MS.

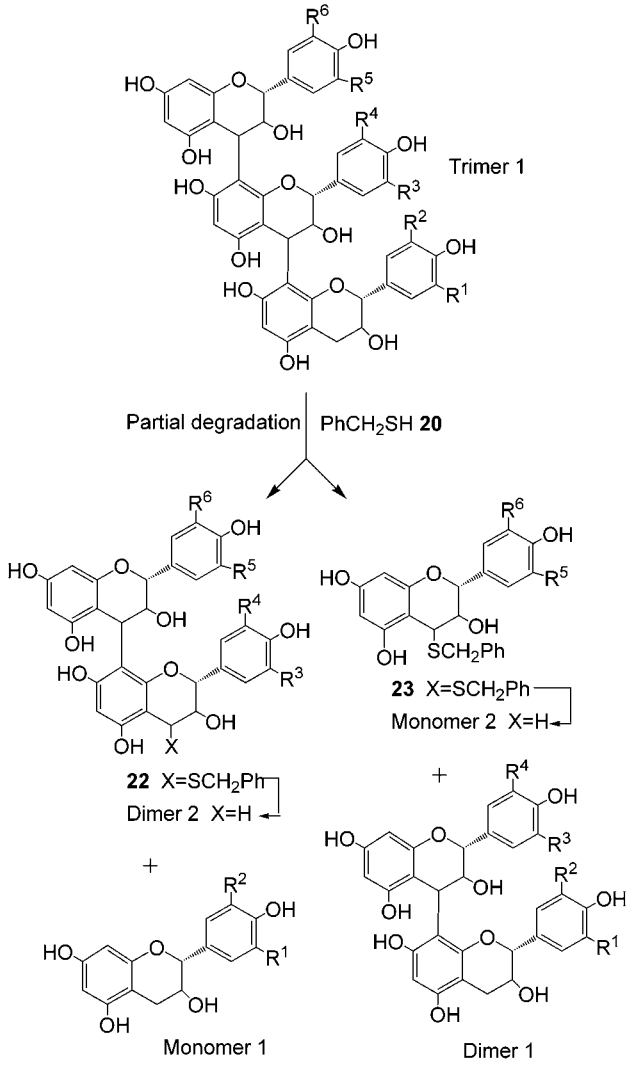

Figure 4. Partial degradation of proanthocyanidins in the presence of benzyl mercaptan.

Partial Acid-Catalyzed Degradation of Hop Proanthocyanidins in the Presence of Benzyl Mercaptan (*17*−*19*). As shown **(Figure 4)**, a solution of hop proanthocyanidin trimer (0.1 mg), benzyl mercaptan (0.5 μL), sulfur dioxide (SO_2, 0.2 μL), and acetic acid (0.3 μL) in a glass capillary tube loaded with ethanol/water (100 μL, 1:1, v/v) was sparged with nitrogen, sealed, and heated to 60 °C for 0.5−6 h depending on the rate of degradation as detected by analytical HPLC/APCI−MS/MS. Monomer 1 and dimer 1 were detected by cochromatography with authentic samples. The benzyl mercaptan adducts **22** and **23** were isolated by analytical HPLC, collected, lyophilized to dryness, and then mixed with aqueous Raney nickel (100 μL) in a glass tube, which was sparged with nitrogen, sealed, shaken several times at regular intervals over a period of 1 h, and directly analyzed by reverse-phase HPLC/APCI−MS. The identities of desulfurized products were established by cochromatography with authentic samples.

RESULTS AND DISCUSSION

Isolation and Purification of Hop Proanthocyanidins. A number of chromatographic procedures using Sephadex G25, Sephahex LH-20, and Toyopearl TSK HW 40 (*6*, *19*, *20*) have been developed for fractionating and isolating proanthocyanidins on a preparative scale. Various hops were extracted with aqueous acetone several times, and then the acetone was removed by evaporation under reduced pressure. The resulting extracts were washed with hexane to remove nonpolar material and then with dichloromethane to remove pigment, flavonoids, and lipids.

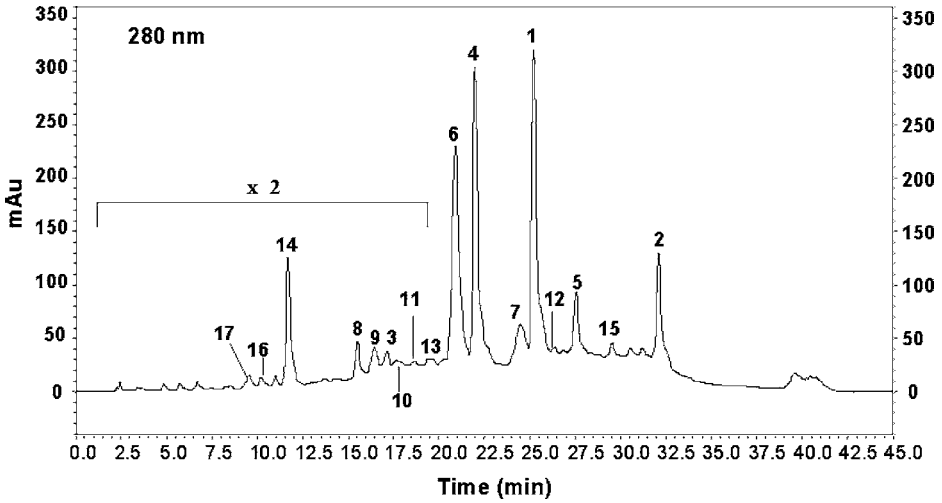

Figure 5. HPLC chromatogram of the Oregon–Willamette hop proanthocyanidins.

Table 1. HPLC (Procedure 1), Retention Times (R_t), Molecular Ions [M + H]$^+$ (APCI), and Mass Spectrometric Fragments of the Oregon–Willamette Hop Proanthocyanidins[a]

compd	R_t (min)	[M + H]$^+$	daughter ions of [M + H]$^+$
1	25.31	291	275 (25), 123 (100)
2	32.22	291	275 (20), 123 (100)
3	17.19	307	181 (11), 139 (100)
4	22.06	579	561 (11), 427 (100), 409 (95), 291 (34)
5	27.66	579	561 (12), 427 (100), 409 (87), 291 (25)
6	20.99	579	561 (6), 427 (100), 409 (92), 291 (28)
7	24.58	579	561 (8), 427 (100), 409 (92), 291 (33)
8	15.55	595	443 (37), 425 (55), 305 (54), 291(75), 287 (100)
9	16.50	595	443 (24), 425 (35), 305 (41), 291 (60), 287 (100)
10	17.74	595	427 (51), 409 (51), 307 (78), 289 (75), 247 (100)
11	18.74	595	427 (43), 409 (41), 307 (65), 289 (70), 247 (100)
12	26.48	563	427 (100), 411 (68), 291 (32), 273 (45)
13	19.68	867	715 (45), 579 (100), 427 (20), 409 (41)
14	11.72	867	715 (55), 579 (100), 427 (30), 409 (47)
15	28.50	867	715 (40), 579 (100), 427 (31), 291 (10)
16	10.25	883	731 (46), 605 (14), 595 (18), 593 (35), 579 (100)
17	9.59	899	747 (22), 731 (46), 609 (24), 595 (100)

[a] See Materials and Methods for further details.

Various oligomeric proanthocyanidins and monomeric flavan-3-ols were obtained by column chromatography on Sephadex LH-20 (2 times) and then on Toyopearl TSK HW-40S. The hop proanthocyanidin oligomers and flavan-3-ol monomers (**Figure 1**) consist of catechin, epicatechin, procyanidin B1, procyanidin B2, procyanidin B3, procyanidin B4, and epicatechin-(4β→8)-catechin-(4α→8)-catechin, all of which have been reported previously as being present in hops. The other 10 hop constituents are reported here for the first time. They are gallocatechin, gallocatechin-(4α→8)-catechin, catechin-(4α→8)-gallocatechin, afzelechin-(4α→8)-catechin, gallocatechin-(4α→6)-catechin, catechin-(4α→6)-gallocatechin, epicatechin-(4β→8)-epicatechin-(4β→8)-catechin, C2, catechin-(4α→8)-gallocatechin-(4α→8)-catechin, and gallocatechin-(4α→8)-gallocatechin-(4α→8)-catechin, which were clearly resolved by HPLC using procedure 1 (**Figure 5**) and characterized by HPLC retention times, mass spectrometric molecular ions, and molecular fragments (**Table 1**). Moreover, to obtain as pure a compound as possible, compounds **3**, **8**−**11**, and **13** were further chromatographed on a 250 mm × 4.6 mm Luna 5 μm phenyl-Hexyl C_{18} column.

Identification of Hop Proanthocyanidins. Hop flavan-3-ol monomers were identified by HPLC/APCI−MS with authentic samples for comparison using procedure 1. Compounds **1** and **2** (**Table 1**) showed molecular ions with *m*/*z* 291 [M + H]$^+$ and were confirmed as catechin and epicatechin, respectively, by cochromatography with authentic samples (HPLC/APCI−MS). Compound **3** ([M + H]$^+$, *m*/*z* 307) was identified as gallocatechin by cochromatography with authentic samples (HPLC/APCI−MS).

Most of the hop proanthocyanidin dimers were identified by acid-catalyzed degradation in the presence of phloroglucinol. Since their interflavonoid C−C linkage bonds are easy to cleave relative to other C−C bonds, it was relatively straightforward to determine their subunit composition by acid-catalyzed degradation with phloroglucinol present, which reacts to form the adduct (*14*). Hop proanthocyanidin dimers (dimer 1) can be degraded (**Figure 2**) to release terminal subunits (i.e., the flavan-3-ol monomers (monomer 1)) and extension subunits as intermediate C-4 carbocations and trapped with phloroglucinol (**18**) to generate the analyzable phloroglucinol adducts **19** by HPLC/APCI−MS.

The precursors for epicatechin-(4β→2)-phloroglucinol ([M + H]$^+$, *m*/*z* 415, R_t 27.5 min (procedure 2)) and epigallocatechin-(4β→2)-phloroglucinol ([M + H]$^+$, *m*/*z* 431, R_t 17.0 min (procedure 2)) were prepared by acid-catalyzed degradation of grape seeds in the presence of phloroglucinol (*14*). Similarly, the gallocatechin-(4α→2)-phloroglucinol precursor ([M + H]$^+$, *m*/*z* 431, R_t 15.2 min (procedure 2)) was prepared by acid-catalyzed degradation of black currant leaves (*Ribes nigrum* Raven) (*21*). The authentic samples of these three phloroglucinol adducts were confirmed by comparing their spectroscopic and mass spectrometric data with those reported previously (*14*, *22*). The authentic sample of catechin-(4α→2)-phloroglucinol ([M + H]$^+$, *m*/*z* 415, R_t 26.9 min (procedure 2)) was prepared from commercial (+)-taxifolin according to a previous report (*14*).

Compounds **4**−**7** in **Table 1** all showed molecular ions with *m*/*z* 579 [M + H]$^+$, indicating that they were proanthocyanidin dimers. After acid-catalyzed degradation in the presence of phloroglucinol, compound **4** yielded epicatechin-(4β→2)-phloroglucinol and catechin ([M + H]$^+$, *m*/*z* 291, R_t 37.1 min (procedure 2)), compound **5** yielded epicatechin-(4β→2)-phloroglucinol and epicatechin ([M + H]$^+$, *m*/*z* 291, R_t 46.6 min (procedure 2)), compound **6** yielded catechin-(4α→2)-phloro-

glucinol and catechin, and compound **7** yielded catechin-(4α→2)-phloroglucinol and epicatechin. Therefore, the original compounds **4**−**7** were, respectively, epicatechin-catechin, epicatechin-epicatechin, catechin-catechin, and catechin-epicatechin, and these were finally identified as epicatechin-(4*β*→8)-catechin (procyanidin B1), epicatechin-(4*β*→8)-epicatechin (procyanidin B2), catechin-(4α→8)-catechin (procyanidin B3), and catechin-(4α→8)-epicatechin (procyanidin B4), respectively, on the basis of our previously reported spectral characteristics (*6*).

Compounds **8** and **10** (**Table 1**) both showed molecular ions with *m*/*z* 595 $[M + H]^+$, indicating that they were also proanthocyanidin dimers. After acid-catalyzed degradation and reaction with phloroglucinol, compound **8** gave gallocatechin-(4α→2)-phloroglucinol and catechin, and compound **10** gave catechin-(4α→2)-phloroglucinol and gallocatechin ($[M + H]^+$, *m*/*z* 307, R_t 17.4 min (procedure 2)). Compounds **8** and **10** were, thus, gallocatechin-catechin and catechin-gallocatechin, respectively, and were further confirmed as gallocatechin-(4α→8)-catechin and catechin-(4α→8)-gallocatechin according to previous reports (*23*, *24*). The method used here would also be helpful for the identification of 4 → 6 linked hop proanthocyanidins. Compounds **9** and **11** (**Table 1)** both showed molecular ions with *m*/*z* 595 $[M + H]^+$, again indicating that they were proanthocyanidin dimers. After acid-catalyzed degradation and reaction with phloroglucinol, compound **9** gave gallocatechin-(4α→2)-phloroglucinol and catechin, and compound **11** gave catechin-(4α→2)-phloroglucinol and gallocatechin. Thus, the original compounds **9** and **11** were identified as gallocatechin-catechin and catechin-gallocatechin, respectively, and were tentatively deduced as gallocatechin-(4α→6)-catechin and catechin-(4α→6)-gallocatechin as they eluted later on the HPLC column and required a higher collision energy for fragmentation than did the corresponding 4 → 8 linked analogues.

One hop proanthocyanidin dimer (compound **12**, **Table 1**) was identified by acid-catalyzed degradation in the presence of benzyl mercaptan followed by desulfurization with hydrogen and Raney nickel. The acid-catalyzed degradation of hop proanthocyanidin dimers (dimer 1) in the presence of benzyl mercaptan (**20**, **Figure 3**) yielded the terminal subunits as flavan-3-ol monomers (monomer 1) and the extended subunits as intermediate C-4 carbocations that could be trapped by benzyl mercaptan to give adducts **21**, which were reductively desulfurized to generate the analyzable monomer 2. Compound **12** showed a molecular ion with *m*/ *z* 563 $[M + H]^+$, indicating that it was a proanthocyanidin dimer. After acid-catalyzed degradation and reaction with benzyl mercaptan, compound **12** released catechin and the benzyl mercaptan adduct of (epi)-afzelechin that was desulfurized by hydrogen with Raney nickel to give afzelechin ($[M + H]^+$, *m*/*z* 275, R_t 44.5 min (procedure 2)). Compound **12** was characterized as afzelechin-catechin and was tentatively identified as afzelechin-(4α→8)-catechin by comparison of the specific rotation ($[\alpha]^{26}_D$ −186.2° (*c* = 0.2, acetone)) with that from the literature (ref *17*: $[\alpha]^{28}_D$ −189.6° (*c* = 0.5, acetone)).

The hop proanthocyanidin trimers were identified by partial acid-catalyzed degradation and reaction with benzyl mercaptan and desulfurization by hydrogen with Raney nickel. As shown (**Figure 4**), partial acid-catalyzed degradation in the presence of benzyl mercaptan (**21**) gave the terminal subunits (monomer 1), the benzyl mercaptan adducts of the upper central subunits **22**, the central-terminal subunits (dimer 1), and the benzyl mercaptan adducts of the upper subunits **23**. Compounds **23** and **22** were desulfurized by hydrogen with Raney nickel catalyst to give the corresponding upper subunits (monomer 2) and upper central subunits (dimer 2). The structures of hop proanthocyanidin trimers (trimer 1) could be deduced from the corresponding upper central subunits (dimer 2) and central-terminal subunits (dimer 1).

Compound **13** (**Table 1**) showed a molecular ion with *m*/*z* 867 $[M + H]^+$, indicating that it was a proanthocyanidin trimer. After partial acid-catalyzed degradation with benzyl mercaptan, compound **13** gave its central-terminal subunit ($[M + H]^+$, *m*/*z* 579) as catechin-(4α→8)-catechin (**6**) and the benzyl mercaptan adduct of the upper central subunit ($[M + H]^+$, *m*/*z* 701) that was further desulfurized by hydrogen/Raney nickel to give the corresponding upper central subunit as catechin-(4α→8)-catechin. Catechin-(4α→8)-catechin was confirmed by cochromatography with authentic samples, so that compound **13** was identified catechin-(4α→8)-catechin-(4α→8)-catechin. Compound **14** (**Table 1**) showed a molecular ion with *m*/*z* 867 $[M + H]^+$, indicating that it was a proanthocyanidin trimer. After partial acid-catalyzed degradation in the presence of benzyl mercaptan, compound **14** gave its central-terminal subunit as catechin-(4α→8)-catechin and the benzyl mercaptan adduct of the upper central subunit ($[M + H]^+$, *m*/*z* 701) that was further desulfurized by hydrogen and Raney nickel to give the corresponding upper central subunit ($[M + H]^+$, *m*/*z* 579) as epicatechin-(4*β*→8)-catechin (**4**). Epicatechin-(4*β*→8)-catechin and catechin-(4α→8)-catechin were confirmed by cochromatography with authentic samples, so that compound **14** was identified as epicatechin-(4*β*→8)-catechin-(4α→8)-catechin.

Compound **15** (**Table 1**) showed a molecular ion with *m*/*z* 867 $[M + H]^+$, indicating that it was a proanthocyanidin trimer. After partial acid-catalyzed degradation in the presence of benzyl mercaptan, compound **15** gave its central-terminal subunit as epicatechin-(4*β*→8)-catechin and the benzyl mercaptan adduct of the upper central subunit ($[M + H]^+$, *m*/*z* 701) that was further desulfurized by hydrogen and Raney nickel to give the corresponding upper central subunit ($[M + H]^+$, *m*/*z* 579) as epicatechin-(4*β*→8)-epicatechin (**5**). Epicatechin-(4*β*→8)-epicatechin and epicatechin-(4*β*→8)-catechin were confirmed by cochromatography with authentic samples, so that the original compound **15** was epicatechin-(4*β*→8)-epicatechin-(4*β*→8)-catechin. Compound **16** (**Table 1**) showed a molecular ion with *m*/*z* 883 $[M + H]^+$, indicating that it was also a proanthocyanidin trimer. After partial acid-catalyzed degradation and reaction with benzyl mercaptan, compound **16** gave its central-terminal subunit ($[M + H]^+$, *m*/*z* 595) as gallocatechin-(4α→8)-catechin (**8**) and the benzyl mercaptan adduct of the upper central subunit ($[M + H]^+$, *m*/*z* 717) that was further desulfurized by hydrogen with Raney nickel to give the corresponding upper central subunit ($[M + H]^+$, *m*/*z* 595) as catechin-(4α→8)-gallocatechin (**10**). Catechin-(4α→8)-gallocatechin and gallocatechin-(4α→8)-catechin were confirmed by cochromatography with authentic samples, so that compound **16** was catechin-(4α→8)-gallocatechin-(4α→8)-catechin.

Compound **17** (**Table 1**) showed a molecular ion with *m*/*z* 899 $[M + H]^+$, indicating that it was a proanthocyanidin trimer. After partial acid-catalyzed degradation and reaction with benzyl mercaptan, compound **17** gave gallocatechin-(4α→8)-catechin (**8**, central-terminal subunit) and catechin (**1**, terminal subunit), both of which were confirmed by cochromatography with authentic samples and the benzyl mercaptan adducts that were further desulfurized by hydrogen/Raney nickel to give gallocatechin (**3**, upper subunit) and gallocatechin-(4α→8)-gallocatechin (upper central subunit, $[M + H]^+$, *m*/*z* 611) that were identified according to the previous report (*25*, *26*). Compound

Table 2. Proanthocyanidin Oligomer Profiles of the 13 Different Hops[a]

	1#													
compd	1a#	1b#	1c#	2#	3#	4#	5#	6#	7#	8#	9#	10#	11#	av
1	21.7	16.5	7.7	12.8	14.7	29.5	11.4	17.4	13.2	23.6	32.1	17.7	9.8	17.6
2	20.8	22.7	8.7	12.0	18.3	19.0	17.5	13.8	14.5	12.8	5.5	10.4	15.3	14.7
3	2.5	1.7	3.2	1.6	1.2	1.9	1.8	2.2	2.9	2.2	2.0	4.2	2.2	2.3
4	12.2	13.5	20.7	18.3	14.2	12.0	11.5	10.7	20.0	12.0	19.5	11.3	16.1	14.8
5	8.2	12.0	13.1	5.4	8.7	6.3	4.0	2.2	5.3	7.7	6.6	5.7	8.0	7.2
6	12.8	11.3	18.9	24.7	14.8	6.5	22.7	19.8	15.2	14.9	11.7	4.7	20.2	15.2
7	5.4	6.1	10.0	12.1	18.0	6.0	15.2	20.7	6.8	11.3	4.8	23.4	20.3	12.3
8	1.7	1.2	0.9	1.0	1.3	4.4	1.8	2.1	2.6	1.7	4.5	2.2	1.1	2.0
9	2.2	1.9	3.0	1.0	0.9	2.4	2.7	2.0	4.0	1.7	1.4	2.8	1.0	2.1
10	2.1	1.8	1.0	0.8	0.4	0.7	1.3	1.7	2.3	0.9	0.8	1.9	0.5	1.2
11	2.1	0.9	2.5	1.5	0.2	1.0	1.7	3.0	2.1	0.9	1.2	7.5	0.3	1.9
12	2.1	4.4	4.3	3.7	0.6	3.1	2.5	0.8	0.7	1.0	2.3	0.7	0.6	2.1
13	1.2	0.9	1.6	0.8	0.7	1.1	1.4	1.7	1.9	1.5	1.8	2.8	1.0	1.4
14	2.3	2.6	1.2	0.9	2.2	2.5	0.5	0.4	2.1	2.7	1.4	1.3	0.6	1.6
15	2.2	1.9	2.6	3.1	3.7	3.2	3.7	1.0	5.9	4.8	3.8	3.0	3.0	3.2
16	0.3	0.2	0.3	0.1	0.1	0.2	0.1	0.1	0.3	0.3	0.2	0.1	0.04	0.2
17	0.4	0.2	0.4	0.02	0.1	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.04	0.2

[a] Abundance (mol %) of the total hop proanthocyanidins determined by HPLC (Procedure 1) at 280 nm. 1# (Willamette) [1a# (Oregon–Willamette), 1b# (Idaho–Willamette), and 1c# (Washington–Willamette)]; 2# (Vanguard); 3# (Palisade); 4# (Tettnang–Hallertauer); 5# (Hallertauer–Hallertauer); 6# (Zeus); 7# (Idaho–Hallertauer); 8# (Cascade); 9# (Saaz 36); 10# (Saaz 72); and 11# (Glacier).

17, therefore, was deduced as gallocatechin-(4α→8)-gallocatechin-(4α→8)-catechin.

Relative Amounts of Individual Proanthocyanidins in 13 Different Hops. Thirteen different hops were examined to determine whether the composition of their proanthocyanidin oligomers had any value in hop variety identification. The compositions of the 13 different hop proanthocyanidin oligomers (**Table 2**) were similar, consisting mostly of three flavan-3-ol monomers, nine proanthocyanidin dimers, and five proanthocyanidin trimers, but the concentrations of these individual compounds showed some differences.

The percent (mol %) compositions of proanthocyanidins were based on a comparison of peak integrations at 280 nm. The detector response of proanthocyanidin dimers and trimers was estimated using molar absorption coefficients relative to the monomers. Molar absorptivities of three representative compounds were measured: monomer (catechin, $\epsilon_{280:}$ 3975); dimer (procyanidin B1, ϵ_{280}: 6725); and trimer (epicatechin-(4β→8)-epicatechin-(4α→8)-catechin, ϵ_{280}: 11360), so the relative molar response ratios of monomers, dimers, and trimers were 1:1.69:2.86. Although other factors such as environment and harvesting procedure were not taken into account, the results (**Table 2**) provide some useful information for the understanding of the relative composition of the samples and the profiles of different hop varieties.

On the whole, proanthocyanidins (88.0%) were dominant in all samples, and small quantities of prodelphinidins (9.9%) and propelargonidin (2.1%) were also present (**Table 2**). The reported hop catechin (**1**, 17.6%), epicatechin (**2**, 14.7%), procyanidin B1 (**4**, 14.8%), procyanidin B2 (**5**, 7.2%), procyanidin B3 (**6**, 15.2%), and procyanidin B4 (**7**, 12.3%) together with the newly identified procyanidin trimer epicatechin-(4β→8)-epicatechin-(4β→8)-catechin (**15**, 3.2%) were the major hop proanthocyanidin oligomers, and the other 10 hop proanthocyanidin oligomers amounted to 15.0% on the total (**Table 2**).

Catechin (**1**, 17.6%) was the dominant flavan-3-ol monomer, followed by epicatechin (**2**, 14.7%) and gallocatechin (**3**, 2.3%). Saaz 36 and Tettnang−Hallertauer hop proanthocyanidin oligomers had the highest content of catechin (i.e., 32.1 and 29.5%,

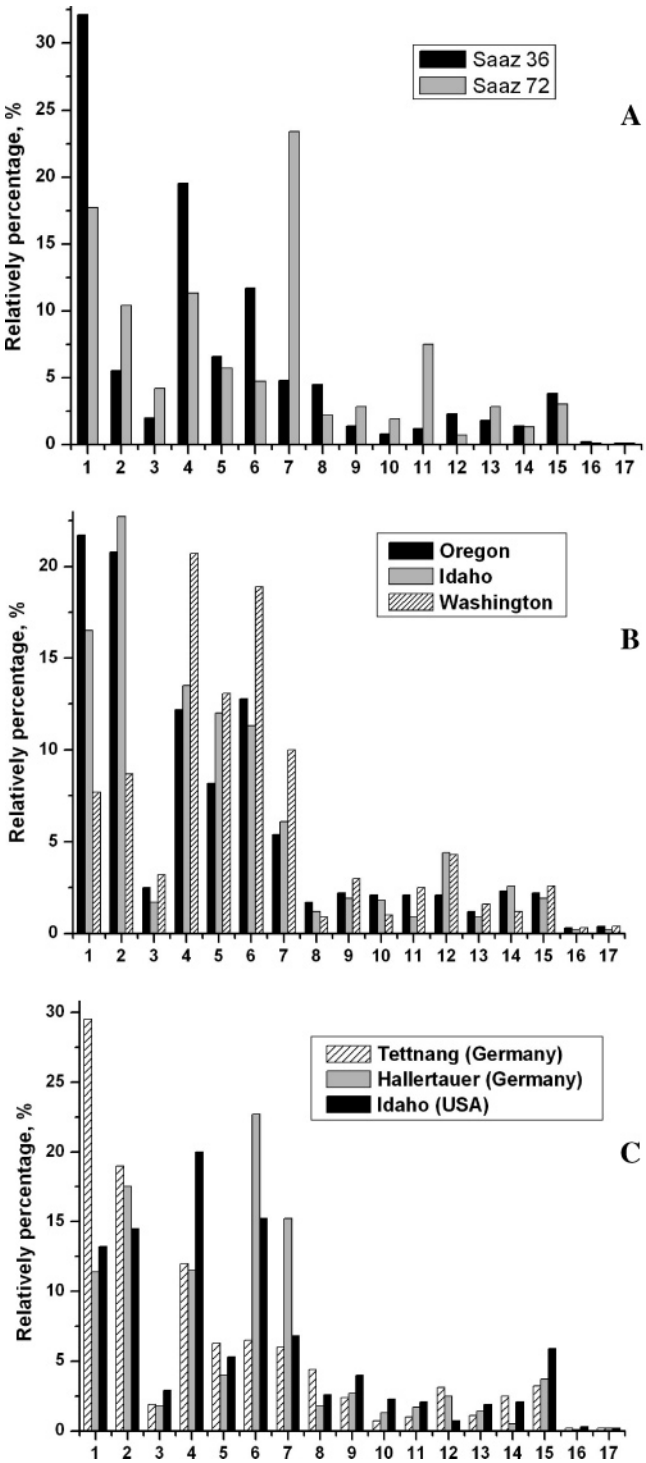

Figure 6. (**A**) Proanthocyanidin oligomer profiles of Saaz 36 and Saaz 72 Hops. (**B**) Proanthocyanidin oligomer profiles of Willamette hops grown in Oregon (2004), Idaho (2004), and Washington (2003). (**C**) Proanthocyanidin oligomer profiles of Hallertauer hops grown in Tettnang (Germany, 2004), Hallertauer (Germany, 2004), and Idaho (2004).

respectively), but the Washington−Willamette and Glacier hop proanthocyanidin oligomers were exactly the opposite, 7.7 and 9.8%, respectively (**Table 2**). With regard to proanthocyanidin dimers, procyanidin B3 (**6**, 15.2%) and procyanidin B1 (**4**, 14.8%) were dominant, followed by procyanidin B4 (**7**, 12.3%) and procyanidin B2 (**5**, 7.2%). The procyanidin B3 content in Vanguard, Hallertauer−Hallertauer, and Glacier hop proanthocyanidin oligomers was 24.7, 22.7, and 20.2%, respectively, but Saaz 72 hop proanthocyanidin oligomers contained only

4.7% procyanidin B3 (**Table 2**). Prodelphinidin dimers (compounds **8**−**11**) and the propelargonidin dimer (afzelechin-(4α→8)-catechin, **12**) were generally present in hop proanthocyanidin oligomers. Tettnanger−Hallertauer and Saaz 36 hop proanthocyanidin oligomers contained gallocatechin-(4α→8)-catechin (**8**) at 4.4 and 4.5%, respectively. Saaz 72 hop proanthocyanidin oligomers contained 7.5% catechin-(4α→6)-gallocatechin (**11**), and the compound **12** content in Idaho−Willamette and Washington−Willamette hop proanthocyanidin oligomers was 4.4 and 4.3%, respectively (**Table 2**). Proanthocyanidin trimers included epicatechin-(4*β*→8)-epicatechin-(4α→8)-catechin (**15**, 3.2%), C2 (**13**, 1.4%), and epicatechin-(4*β*→8)-catechin-(4α→8)-catechin (**14**, 1.6%). Idaho−Hallertauer and Cascade hop proanthocyanidin oligomers contained compound **15** at 5.9 and 4.8% levels, respectively, but Zeus hop proanthocyanidin oligomers contained only 1.0%. Catechin-(4α→8)-gallocatechin-(4α→8)-catechin (**16**, 0.2%) and gallocatechin-(4α→8)-gallocatechin-(4α→8)-catechin (**17**, 0.2%) were very limited in hop proanthocyanidin oligomers (**Table 2**).

Clearly, there were differences in relative amounts of compounds **1**, **5**, and **7** in Saaz 36 and Saaz 72 hop proanthocyanidin oligomers (**Figure 6A**). This was surprising because these two hops were supposedly genetically identical, and they were grown in the same location in Idaho. These clones were established many years apart, and it has often been observed that clonal selections from varieties established long ago in a given locality might be different. Anecdotally, they were reported to have different brewing characteristics as well.

Willamette hops (Oregon, Idaho, and Washington) and Hallertauer hops (Germany Tettnang, Germany Hallertauer, and Idaho) were selected to study the effect of geographic origin on their proanthocyanidin profiles (**Table 2** and **Figure 6B**,**C**). It is evident that most hop constituents are affected by geographic origin. For example, the relative percentage of compound **1** in Willamette hop proanthocyanidin oligomers, from Oregon, Idaho, and Washington, was 21.7, 16.5, and 7.7%, respectively (**Table 2** and **Figure 6B**). The relative percentages of compound **6** in Hallertauer hop proanthocyanidin oligomers, from Tettang (Germany, 2004), Hallertauer (Germany, 2004), and Idaho (2004) were 6.5, 22.7, and 15.2%, respectively (**Table 2** and **Figure 6C**). These results suggest clearly that the proanthocyanidin profiles of the 13 different hops are affected by geographic origin.

In summary, this study on the composition and distribution of hop proanthocyanidins in 13 different hops gives a clear picture of proanthocyanidin profiles and further showed that hop proanthocyanidin profiles were affected by geographic origin and were variable depending on the cultivars. Seventeen hop proanthocyanidin oligomers and flavan-3-ol monomers were identified by chemical degradation, HPLC/APCI−MS/MS and HPLC/ESI−MS/MS. As far as we know, the prodelphinidins containing gallocatechin units and propelargonidin containing afzelechin units have now been identified in hops for the first time. This study will provide useful information for determining the impact of hop proanthocyanidins on beer flavor and stability and may also pave the way to the discovery of the real interaction between proanthocyanidins and proteins in beer.

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