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Phytochemical and Morphological Characterization of Hop (*Humulus lupulus* L.) Cones over Five Developmental Stages Using High Performance Liquid Chromatography Coupled to Time-of-Flight Mass Spectrometry, Ultrahigh Performance Liquid Chromatography Photodiode Array Detection, and Light Microscopy Techniques

Adam R. Kavalier,[†] Amy Litt,[‡] Chunhui Ma,[†] Nicholi J. Pitra,[§] Mark C. Coles,[§] Edward J. Kennelly,*[,†] and Paul D. Matthews[§]

[†]Department of Biological Sciences, Lehman College and The Graduate Center, The City University of New York, 250 Bedford Park Boulevard West, Bronx, New York 10468, United States

[‡]New York Botanical Garden, Pfizer Laboratory, 200th Street and Kazimiroff Boulevard, Bronx, New York 10458, United States

[§]Hopsteiner, S.S. Steiner Inc., 655 Madison Avenue, New York, New York 10065, United States

Supporting Information

ABSTRACT: Hop (*Humulus lupulus* L.) inflorescences, commonly known as "hop cones", are prized for their terpenophenolic contents, used in beer production and, more recently, in biomedical applications. In this study we investigated morphological and phytochemical characteristics of hop cones over five developmental stages, using liquid chromatography coupled to time-of-flight mass spectrometry (LC−TOF-MS), and ultrahigh performance liquid chromatography photodiode array detection (UHPLC−PDA) methods to quantitate 21 polyphenolics and seven terpenophenolics. Additionally, we used light microscopy to correlate phytochemical quantities with changes in the morphology of the cones. Significant increases in terpenophenolics, concomitant with glandular trichome development and associated gross morphological changes, were mapped over development to fluctuations in contents of polyphenolic constituents and their metabolic precursor compounds. The methods reported here can be used for targeted metabolic profiling of flavonoids, phenolic acids, and terpenophenolics in hops, and are applicable to quantitation in other crops.

KEYWORDS: hops, *Humulus lupulus* L., α-acids, β-acids, terpenophenolics, polyphenolics, cone development, gross morphology, phytochemical accumulation

INTRODUCTION

Humulus lupulus (L.), commonly known as hops, is an agricultural crop valued for its rich terpenophenolic, polyphenolic, and essential oil contents, used primarily in the brewing industry but also in biomedical research.[1−3] Hops contain two main classes of terpenophenolics, namely, prenylflavonoids and prenylated acylphloroglucinols, the latter of which are typically known as α- and β-acids (Figure 1). Recently, prenylflavonoids xanthohumol and 8-prenylnaringenin have been the focus of biomedical[1,4,5] and molecular genetics[6,7] research, because of their antiprolific and proestrogenic activities. Furthermore, in addition to a traditional focus on terpene essential oils as flavor components of beer, major brewers have become interested in hop polyphenolic contributions to foam stability, reduction−oxidation effects, and bitterness intensity as well as in marketing of the functionality of beverages, i.e., the health and nutritive effects of ingredients.[8,9] Nevertheless, hops are produced primarily for α-acids, or humulones, which impart a bitter flavor to beer.[10] Finally, new-use interest in the antimicrobial activity of β-acids, or lupulones, in fodder and fermentation of biofuels, merits careful analytical attention to terpenophenolics.[11,12] Since only the female inflorescence is used in beverage production and unfertilized cones are preferred in brewing because seed fatty acids can impart undesired flavors to beers, we describe the development of female hop cones that have not been exposed to pollen.

Terpenophenolics and essential oils accumulate in sessile glandular trichomes which are highly metabolically active structures most abundant in the inflorescence, but also present on vegetative leaves.[13] Two types of glandular trichomes have been described in hops: peltate glands, which are large and contain 100−200 cells; and bulbous glands, which are much smaller, containing eight cells at maturity (Figure 2).[14] Trichome development can be classified into two main stages, the growth stage and the biosynthetic-secretory stage.[14,15] The growth stage includes the growth and development of gland stalk cells which support a layer of gland head cells, the main site of biosecretory production. In peltate trichomes, the cuticle thickens at the apical side of head cells and the number of plastids significantly increases just prior to secretion.[15,16] During the secretory stage the apical cell wall of the head cells splits to form an intrawall cavity; terpenophenolics secreted by the head cells accumulate in this secretory cavity. The resulting mature gland is a biconal

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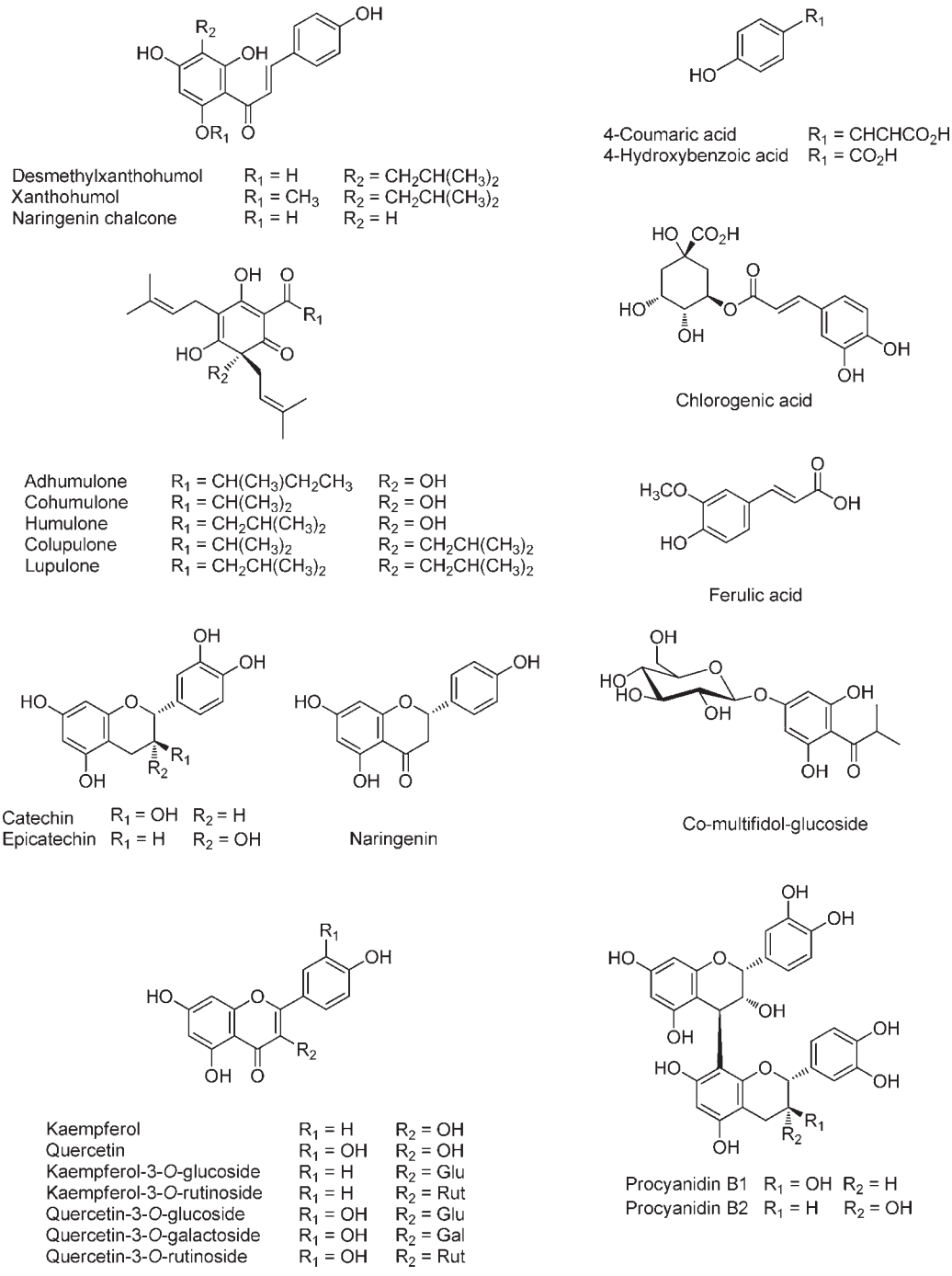

Figure 1. Chemical structures of terpenophenolics, flavonoids, and phenolic acids quantitated in this study.

structure, filled with secretions contained by the cuticle and subcuticular wall.[15,16] Developmental stages of bulbous glands are difficult to identify due to their small size and rapid development, and have not been as well studied.[14]

Coinciding with the development of the glandular trichomes, terpenophenolics accumulate over the progression of cone development.[17,18] Terpenophenolic accumulation has been studied over three developmental stages which were distinguished on the basis of cone length.[18] It was shown that terpenophenolics accumulate as cones increase in length, which corresponds to the elongation of bracts and bracteoles. In contrast to terpenophenolic-rich glandular trichomes, the green tissues of the bracts and bracteoles contain a diverse set of polyphenolic constituents.

The most common polyphenols found in hops are catechins, phenolic acids, flavonol glycosides, namely glycosides of quercetin and kaempferol, and procyanidins (Figure 1).[19] In addition to their ability to protect the plant from pests, polyphenols are important to brewers as they impart unique and complex flavors to beer.[8,20,21] Some of these polyphenols have been shown to decrease in content at late stages of hop cone development.[20]

Hop cone development is a rapid and dynamic process, and in-depth phytochemical profiles mapped to morphological assessment of developmental stages are useful to experimental,

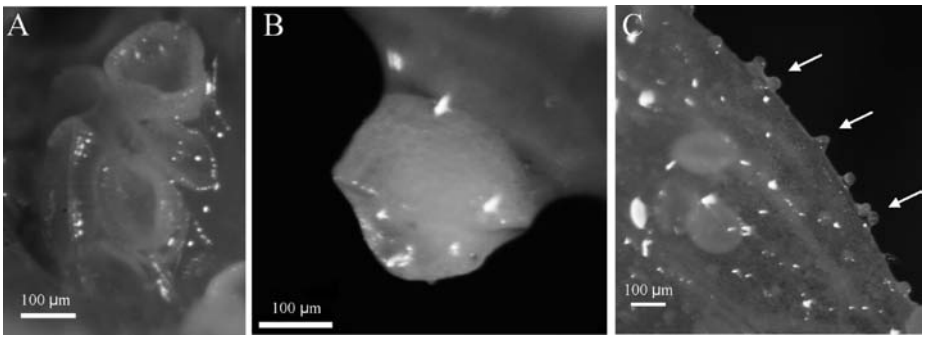

Figure 2. Hop cone trichomes. (A) Fully grown peltate trichomes prior to secretion. (B) Fully developed peltate trichomes containing biosecretions. (C) Fully developed bulbous trichomes (shown by arrows) are much smaller than peltate trichomes, but also contain biosecretions.

agronomic, and industrial applications. In this context, here we report a targeted metabolic approach to characterize changes in agronomically relevant compounds from hops over five developmental stages in two cultivars. We accomplished morphological and phytochemical characterization for two commercially prevalent hop cultivars, Willamette and Zeus, over five morphologically defined developmental stages. Willamette and Zeus cultivars were selected to represent the two classes of commercially grown hops: low-α-acid-content (2−7% w/w), "aroma" hops; and high-α-acid-content (10−20% w/w), "alpha" hops, respectively. Aroma hops, like Willamette, are added to the brewing process as whole hops for complex flavoring arising from infusion of polyphenols, terpenes, and terpenophenolics into the wort; while alpha hops are mainly used for the production of α-acid-enriched hop extracts. Polyphenols, terpenes, and lupulones are byproducts of the production of α-acid extracts, used for bittering of large production-scale beers, but these byproducts are nevertheless botanicals used in cosmetics, functional beverages, and medicines. Over the last three years in the U.S.A., Willamette and Zeus have occupied the most acreage within their respective aroma and alpha classes.[22]

Using LC−TOF-MS and UHPLC−PDA we accomplished the simultaneous quantitation of 21 polyphenolic and seven terpenophenolic constituents, and correlated the phytochemical accumulation with detailed morphology of each cone stage using light microscopy. Seasonal, climatic, edaphic and biotic stress-induced variation in both terpenophenolic and polyphenolic content of hop cones can be large; therefore, an index relating morphological and phytochemical development can be useful in providing developmental reference points for experimental research, for harvest-ripeness determination, and for product quality.[17,20]

MATERIALS AND METHODS

Plant Material. Two hop (*H. lupulus*) cultivars, Willamette and Zeus, were grown under standard agronomic conditions at Golden Gate Ranches, Hopsteiner, S.S. Steiner, Inc., near Prosser, WA. A total of 150 samples were collected from the upper third section of plants at five developmental stages on the following dates in 2008: Willamette (28 July, 2 August, 7 August, 14 August, and 21 August); and Zeus (30 July, 5 August, 12 August, 17 August, and 21 August). Cones collected on these five dates were characterized as stages I−V, each collection date corresponds to a single developmental stage and each stage was sequentially collected in order of developmental stage (I−V). Willamette cones ripen earlier than Zeus cones, so we started the collection of Willamette cones earlier than that of Zeus. Cones were collected from each of fifteen hop vines for each sample group.

Digital Morphometrics. A high-throughput method was developed to assess the approximate volumes of cones in each sample. Silhouettes of 10−20 cones for each cultivar from each sampling time point were produced in multiplex by transillumination and photography with a C-type 1.3 megapixel video camera driven by iREZ-iNSPECTX capture software (Global Media LLC, Canada). Silhouettes were analyzed using SigmaScan Pro 5.0 margin recognition software (Systat Software Inc., San Jose, CA). Cone perimeters were measured, and the average radius was used to calculate approximate cone volumes based on radial symmetry. Cones were also weighed, and cone density (in mg/cm^3) was calculated.

Microscopy. Hop cones were preserved in formalin−propionic acid−ethanol 50% (FPA, 1:1:18 v/v) under vacuum. Five cone stages were studied using a Nikon SMZ1500 stereoscopic dissecting microscope. Images were recorded using a Nikon DXM1200F digital camera and Nikon ACT-1 software. Flowers develop acropetally, so basal flowers were characterized for each cone developmental stage. While detailed observations of basal flowers were used to characterize cone stages, information from apical flowers was included when informative.

Chemicals. Authentic phytochemical standards catechin, epicatechin, 4-coumaric acid, 4-hydroxybenzoic acid, chlorogenic acid, ferulic acid, naringenin, phenylalanine, procyanidins B-1 and B-2, quercetin, kaempferol, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-rutinoside were purchased from Extrasynthese (Genay, France), and leucine, naringenin chalcone, and valine were purchased from Chromadex (Irvine, CA). Prenylflavonoids xanthohumol and desmethylxanthohumol, and prenylated acylphloroglucinols, adhumulone, cohumulone, humulone, colupulone, lupulone, and comultifidol glucoside were provided by Hopsteiner, S.S. Steiner, Inc. (Mainberg, Germany).

Extraction and UHPLC Analysis of Terpenophenolics. Hop cones were lyophilized for 24 h (2−4% moisture) and extracted in 100% MeOH by stir plate agitation for 20 min on a DPC Micromix 5 using program 40 (15 Hz at 900 rpm). The resulting extract was then subjected to UHPLC−PDA analysis. Seven terpenophenolics, xanthohumol, desmethylxanthohumol, adhumulone, cohumulone, humulone, colupulone, and lupulone, were quantitated by UHPLC−PDA using a previously developed method.[23]

Extraction and Sample Preparation of Polyphenols. After removing 0.25 g of sample for terpenophenolic analysis, the remaining lyophilized tissue was extracted by supercritical carbon dioxide. Samples were extracted by CO_2 in a custom-built industrial extractor at the following parameters: 2,400 psi and 30 °C. The resulting tissue was used to produce a polyphenol-rich extract. Aliquots of CO_2 extracted tissue (0.25 g) were weighed out and extracted by sonication for 1 h in 10 mL of 80% MeOH. The resulting extract was centrifuged at 3,500 rpm for

15 min, and the supernatant was decanted. Liquid extract was dried under nitrogen gas, lyophilized, and weighed. Dried extracts were reconstituted at 10 mg/mL, filtered using a 0.45 μm nylon filter disk and syringe, and then subjected to HPLC−TOF-MS analysis. Serial dilutions (1:20) were also subjected to HPLC−TOF-MS analysis in order to quantitate major constituents within the linear range.

LC−TOF-MS Analysis. Samples were analyzed by LC−MS using a Waters LCT Premiere XE time-of-flight (TOF) mass spectrometer (Waters Corp., Milford, MA). Ionization was achieved using a multimode source in electrospray (ESI) mode at the following conditions: +ESI capillary 3000 V, −ESI capillary 2800 V, 20 V, aperture 1:0 V, ion guide 1:0 V, and multichannel plate (MCP) 2500 V. Nitrogen was used for both cone and desolvation gases, with a cone gas flow of 20 L/h, and desolvation gas flow of 600 L/h at 400 °C. The source temperature was 120 °C. Leucine-enkephalin (m/z 556.2771) was used as a reference mass and infused by a secondary reference probe. The reference mass was scanned once every five scans for each positive and negative data collection. Both positive and negative ESI data were collected using a scan time of 0.2 s, with an interscan time of 0.01 s, and a polarity switch time of 0.3 s. MS data were collected in centroid mode using MassLynx V4.1 Scn 727 software (Waters Corp., Milford, MA).

LC separation was conducted using a Waters Alliance 2695 HPLC coupled to a Waters 2998 PDA detector. Separation was achieved on a 150 × 2.0 mm, 2.6 μm, Kinetex C-18 column (Phenomenex, Torrance, CA), held at a constant temperature of 45 °C and using a gradient system composed of A, 0.1% formic acid in water, and B, 0.1% formic acid in MeCN, at a flow of 0.2 mL/min (except where noted): 0−2.7 min B, 7−9%; 2.7−13.8 min B, 9%; 13.8−14.1 min B, 9−13%; 14.1−54.0 min B, 13−30%; 54.0−67.0 min B, 30−80.5%; 67.0−67.5 min B, 80.5−100%; 67.5−67.6 min B, 100%; flow increased to 0.3 mL/min, 67.6−78.0 min B, 100%. The LC eluent was diverted to waste at two points in the run (0−0.9 min and 67.0−78.0 min) to avoid salt and lipophilic compounds from entering the ESI source.

Quantitation. All MS data were processed using QuanLynx software (Waters). Quantitated components were identified by comparison of retention time and exact mass of both $[M + H]^+$ and $[M - H]^-$ ions with those of authentic standards. Phenolic acids were quantitated using the −ESI mode as they produced a greater signal in negative mode; all other compounds reported here were quantitated using the +ESI mode. QuantLynx mass tolerance was set to ±5 mDa with a retention time window of ±0.3 min.

UHPLC−PDA data were processed using Empower2 software (Waters). Prenylflavonoids were quantitated at 370 nm, and desmethylxanthohumol quantitation was based on the calibration curve of xanthohumol; α- and β-acids were quantitated at a wavelength of 325 nm. This method was developed and validated in our previous work.[23]

Validation. HPLC−TOF-MS analytical methods were validated for linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision. For each standard, LOD and LOQ were determined as 3:1 and 10:1 S/N, respectively. Calibration curves were obtained using six concentrations of each analyte above the LOQ and within the linear range.

Statistical Analysis. Statistical tests were conducted using JMP 8.01 (SAS, Cary, NC) software. Statistical significance was determined using Tukey−Kramer HSD analysis within a one-way ANOVA where significance was ascribed to differences among means where $p < 0.05$, and are described here as being significant. Values present in all tables include letter notations ascribed to values using Tukey−Kramer HSD analysis.

RESULTS AND DISCUSSION

Morphological Characterization of Hop Cones for Correlation with Accumulation of Secondary Metabolites. We observed distinct morphological traits to aid in the identification of each stage (I−V) of cone development (Figure 3). The most informative morphological changes included stigma senescence, bract and bracteole elongation, and development of peltate glandular trichomes. Stigma senesce in stage I is followed by the elongation of bracts and bracteoles which occurs in stages II through V. Glandular trichomes are apparent at stage II, and fill with secretions from stages III−V. The ovary senesces in stages III and IV, but the perianth remains persistent and contains high densities of developing glandular trichomes. At stage V bracts and bracteoles are fully elongated and glandular trichomes are fully expanded, filled with secretions. Using these morphological characters we are able to distinguish five morphologically distinct stages; we have combined the morphological and phytochemical constituents for each stage of development for use as a comprehensive developmental stage index (Table 1).

Cone Volume, Mass, and Density. In the Zeus cultivar, cones increased in average volume and mass over the five developmental cone stages (Figures 4A−4C). Zeus cone volume and mass increased between each stage from 1.5 (stage I) to 12.1 cm^3 (stage V), and 119.6 (stage I) to 302.6 mg (stage V), respectively. Average cone volume significantly increased between each sequential stage. Density followed an opposite trend, decreasing from stages I through V, with no significant changes occurring between stages III and V.

Willamette cones, as in Zeus, showed significant increases in volume and mass over the five developmental stages, while cone density significantly decreased overall (Figures 4D−4F). Willamette cones increased in volume from 0.6 (stage I) to 6.9 cm^3 (stage V), and this increment was statistically significant between all stages, except for between stages I and II. Cone mass increased over the five developmental stages, from 45.2 (stage I) to 145.2 mg (stage V), but the only significant increases occurred between stages II and III. Willamette cone density decreased from 79.5 (stage I) to 21.2 mg/cm^3 (stage V), with the only significant decreases occurring between stages II and III, and between stages III and IV.

In both cultivars least-squared regression of average cone mass increases (Willamette, $R^2 = 0.99$; Zeus, $R^2 = 0.98$) and volume increases (Willamette, $R^2 = 0.94$; Zeus, $R^2 = 0.97$) was essentially linear over the five stages; and, thus, mass and volume are not useful in stage demarcation, but do allow tracking of relative growth progression among cones. Cone density decreases can be used to demarcate the early stages of bract and bracteole formation from the latter stages of elongation and expansion.

Validation of Analytical Methods. A sensitive and suitable HPLC−TOF-MS method to quantitate a large panel of hop constituents was achieved (Figure 5). The calibration curves indicated good linearity for all standards analyzed between the peak area and concentration ($R^2 \geq 0.99$), which occurred between 0.016 and 5 mg/mL for phenolic acids and flavonol aglycones, 0.26−100 mg/mL for flavonol glycosides, 0.26−100 mg/mL for flavan-3-ols and procyanidins, and 0.26−25 mg/mL for amino acids. The limit of detection (LOD) values for phenolic acids, flavonols and flavonol glycosides, flavan-3-ols and procyanidins and amino acids ranged from 3.9 to 4.9, 3.3 to 7.6, 22.9 to 33.2, and 21.6 to 24.2 ng/mL, respectively, and the limit of quantitation (LOQ) values, for the same metabolites, from 11.9 to 17.4, 9.9 to 22.6, 69.1 to 100.5, and 56.7 to 73.5 ng/mL, respectively.

Accuracy was determined using recovery experiments in which each analyte was spiked into the plant material prior to extraction. Recovery ranged from 97.2 to 101.3% for phenolic acids, 96.0

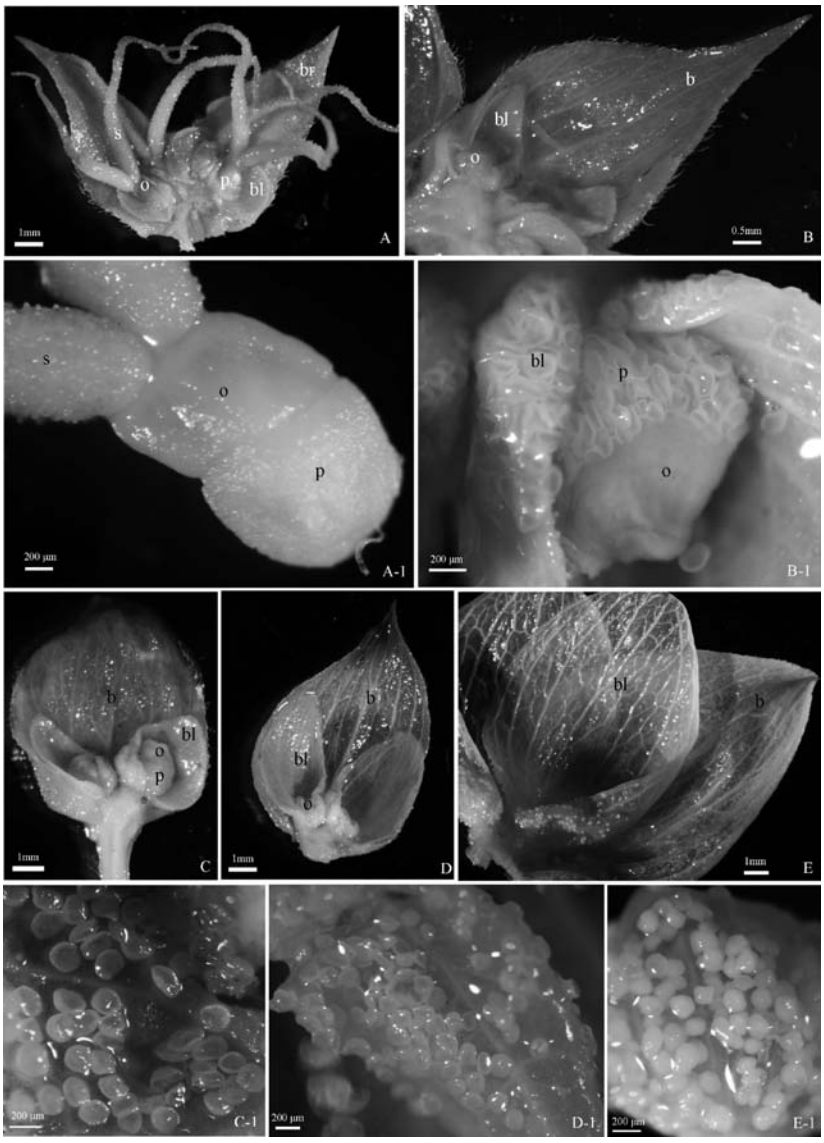

Figure 3. Basal flowers were photographed from (A) stage I, (B) stage II, (C) stage III, (D) stage IV, and (E) stage V. Letters represent (b) bract, (bl) bracteole, (p) perianth, (o) ovary, and (s) stigma.

to103.2% for flavonols and flavonol glycosides, 97.7 to 99.3% for flavan-3-ols and procyanidins, and 95.8 to 102.4% for amino acids.

The repeatability of the analytical method was evaluated in terms of the intermediate precision by analyzing the inter- and intraday variation, which were calculated as the relative standard deviation (RSD) of each analyte quantitated from a mixture of the 21 polyphenols. Polyphenolic standards were quantitated at three concentrations that spanned the concentrations of analytes found in all samples. Intraday analysis occurred three times within the same day, and interday analysis occurred over three separate days. In general, all intraday and interday variations were below 4.3% and 7.1%, respectively. More specifically, intraday variation for phenolic acids, flavonols and flavonol glycosides, flavan-3-ols and procyanidins, and amino acids ranged from 2.2 to 3.4%, 1.6 to 4.3%, 0.8 to 3.6%, and 2.0 to 4.1%, respectively. Interday variation of the same compounds ranged from 2.0 to 5.0%, 2.2 to 7.1%, 1.5 to 3.4%, and 2.2 to 4.3%, respectively.

Quantitation of Terpenophenolics and Polyphenols. We quantitated seven terpenophenolics and 21 polyphenols by UHPLC−PDA and LC−TOF-MS, respectively, for Zeus and Willamette cones over five developmental stages.

Terpenophenolics. The accumulation of terpenophenolics over cone maturation directly corresponded to the morphological development of both bulbous and peltate glandular trichomes as observed over the five developmental stages of cone growth (Figures 2 and 3). All seven terpenophenolic constituents significantly increased in Zeus and Willamette cones between developmental stages I and V, and were coincident with morphological changes in trichomatous glands. The development of peltate glandular trichomes most closely corresponded with terpenophenolic accumulation. Prenylflavonoids desmethylxanthohumol and xanthohumol from Zeus significantly increased from 0.75 and 7.25 mg/g (stage I) to 2.73 and 14.86 mg/g (stage V), respectively (Figure 6A). In Willamette cones, a significant increase in xanthohumol occurred

Table 1. Summary of Morphological and Terpenophenolic Data[a] for Each Developmental Stage, I−V, for Zeus and Willamette Hop Cultivars

stage	morphology	cultivar	mass (mg)	volume (cm^3)	density (mg/cm^3)	prenylflavonoids (mg/g)	α-acids (mg/g)	β-acids (mg/g)
I	stigma, tips browning; bracteoles, slightly longer than ovary and flat; peltate glands, not apparent	Willamette	45.2 ± 10.3	0.57 ± 0.04	79.5 ± 5.94	DMX 1.34 ± 0.04 XN 3.53 ± 0.20	AdHum 0.83 ± 0.13 CoHum 1.11 ± 0.19 Hum 2.06 ± 0.46	CoLup 4.80 ± 0.61 Lup 7.34 ± 0.77
		Zeus	119.6 ± 41.2	1.45 ± 0.18	82.36 ± 3.49	DMX 0.75 ± 0.04 XN 7.25 ± 0.43	AdHum 8.53 ± 0.67 CoHum 18.31 ± 1.46 Hum 47.41 ± 3.30	CoLup 26.85 ± 1.10 Lup 29.56 ± 0.85
II	stigma, not present on basal flowers, present at mid and apical flowers; bracteoles, enclose ovary; peltate glands, cup shaped, no secretions	Willamette	66.6 ± 13.0	0.77 ± 0.07	85.97 ± 8.86	DMX 1.59 ± 0.07 XN 6.54 ± 0.34	AdHum 12.66 ± 0.52 CoHum 23.42 ± 1.03 Hum 52.65 ± 2.33	CoLup 30.68 ± 1.31 Lup 33.10 ± 1.30
		Zeus	120.1 ± 24.0	2.31 ± 0.32	52.07 ± 4.68	DMX 1.01 ± 0.06 XN 10.31 ± 0.56	AdHum 23.67 ± 1.34 CoHum 51.48 ± 3.19 Hum 121.02 ± 6.90	CoLup 43.28 ± 1.71 Lup 41.47 ± 1.46
III	stigma, not present on any flowers; bracteoles, half length of bract; ovary, senescing; peltate glands, filling with secretions	Willamette	112.9 ± 14.0	2.69 ± 0.20	41.91 ± 2.26	DMX 1.63 ± 0.09 XN 7.03 ± 0.20	AdHum 15.84 ± 2.02 CoHum 26.29 ± 1.34 Hum 59.57 ± 6.59	CoLup 35.81 ± 0.96 Lup 38.18 ± 2.22
		Zeus	157.4 ± 21.8	5.33 ± 0.55	29.53 ± 1.14	DMX 1.25 ± 0.07 XN 10.68 ± 0.33	AdHum 33.85 ± 1.74 CoHum 56.67 ± 3.82 Hum 155.12 ± 13.88	CoLup 44.45 ± 1.79 Lup 45.02 ± 1.67
IV	bracteoles, elongated, over half length of bract; ovary, senesced and brown; peltate glands, filling with secretions	Willamette	131.1 ± 14.7	5.55 ± 0.53	23.61 ± 1.61	DMX 1.70 ± 0.11 XN 7.53 ± 0.33	AdHum 17.15 ± 1.04 CoHum 27.57 ± 1.52 Hum 66.51 ± 4.26	CoLup 36.68 ± 1.84 Lup 36.33 ± 2.01
		Zeus	214.7 ± 19.8	9.49 ± 0.50	22.62 ± 1.92	DMX 2.03 ± 0.11 XN 13.77 ± 0.63	AdHum 38.50 ± 1.55 CoHum 72.79 ± 3.67 Hum 186.98 ± 9.97	CoLup 51.83 ± 2.20 Lup 49.25 ± 2.06
V	bracteoles, fully elongated, almost bract length; ovary, inconspicuous; peltate glands, biconal shape, filled with secretions	Willamette	145.2 ± 8.1	6.85 ± 0.38	21.2 ± 1.20	DMX 1.85 ± 0.07 XN 7.72 ± 0.24	AdHum 39.93 ± 0.79 CoHum 30.80 ± 1.30 Hum 99.12 ± 3.95	CoLup 38.21 ± 1.23 Lup 37.55 ± 1.47
		Zeus	302.6 ± 23.8	12.09 ± 0.57	25.02 ± 1.31	DMX 2.73 ± 0.10 XN 14.86 ± 0.52	AdHum 71.23 ± 1.75 CoHum 94.96 ± 3.31 Hum 213.65 ± 6.81	CoLup 58.04 ± 1.31 Lup 55.28 ± 1.31

[a] Compound abbreviations: DMX, desmethylxanthohumol; XN, xanthohumol; CoHum, cohumulone; Hum, humulone; AdHum, adhumulone; CoLup, colupulone; Lup, lupulone.

from 3.53 (stage I) to 7.72 mg/g (stage V) (Figure 7A). In addition, levels of xanthohumol metabolic precursor desmethylxanthohumol followed the same trend in Willamette cones, significantly increasing from 1.34 (stage I) to 1.85 mg/g (stage V). In both cultivars significant increases also occurred in desmethylxanthohumol and xanthohumol between most sequential stages of development.

Like the prenylflavonoids, α- and β-acids also significantly increased over the five developmental stages in both Zeus and Willamette cones, which also corresponded with glandular trichome development (Figures 6B and 7B). In general, the Zeus cultivar showed significant increases in α-acids adhumulone, cohumulone, and humulone, and β-acids colupulone, and lupulone over the five stages of cone development; levels of α-acids adhumulone, cohumulone, and humulone significantly increased from 8.53, 18.31, and 47.41 mg/g (stage I) to 71.23, 94.96, and 213.65 mg/g (stage V), respectively; levels of β-acids, colupulone and lupulone, followed the same trend, as colupulone significantly increased from 26.85 (stage I) to 58.04 mg/g (stage V), and lupulone from 29.56 (stage I) to 55.28 mg/g (stage V). In Willamette cones, α- and β-acids measured also significantly increased over the five developmental stages; levels of α-acids adhumulone, humulone, and cohumulone significantly increased from 0.83, 2.06, and 1.11 mg/g (stage I) to 39.93, 99.12, and 30.80 mg/g (stage V), respectively. In the case of β-acid accumulation, the majority occurred between stages I and II when colupulone increased from 4.80 to 30.68 mg/g, and lupulone increased from 7.34 to 33.10 mg/g, respectively. Colupulone and lupulone levels continued to increase over developmental stages II−V, but increases were not as large as those between stages I and II.

As expected, accumulation of terpenophenolics in both Zeus and Willamette hop cones directly correlated with the development and maturation of peltate glandular trichomes at most

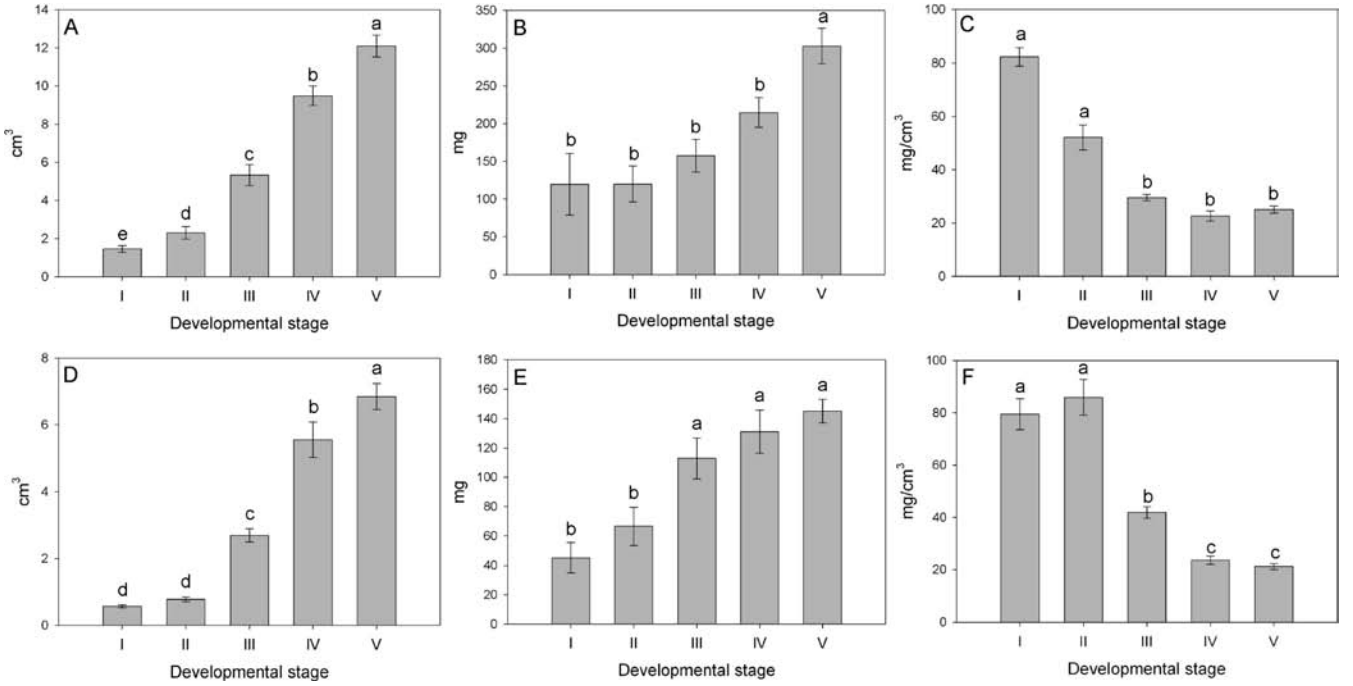

Figure 4. Zeus (A) cone volume, (B) cone mass, and (C) cone density over the five developmental stages. Willamette (D) cone volume, (E) cone mass, and (F) cone density over the five developmental stages. Values that share the same letters are not significantly different where $p > 0.05$ as calculated by Tukey−Kramer HSD analysis within a one-way ANOVA.

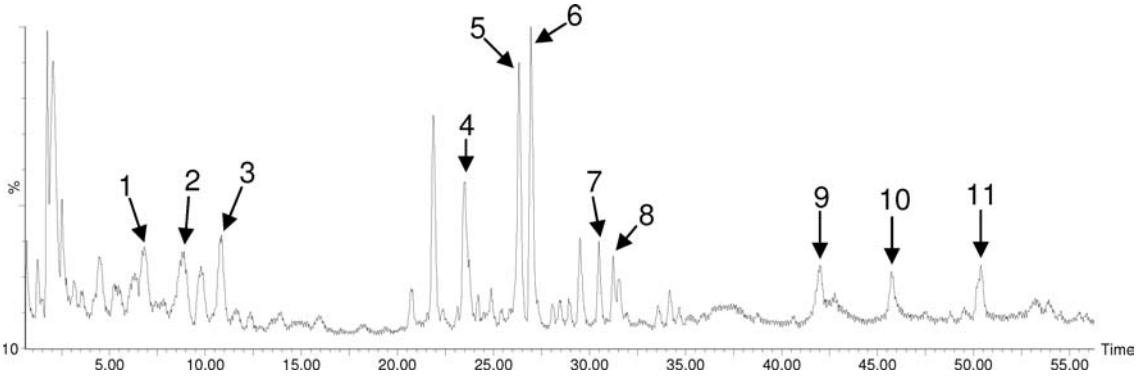

Figure 5. Total ion chromatogram (TIC) from +ESI-LC−TOF-MS analysis. Compounds showing peaks in TIC are labeled as (1) catechin, (2) procyanidin b1, (3) epicatechin, (4) comultifidol glycoside, (5) quercetin-3-*O*-rutinoside, (6) quercetin-3-*O*-glucoside, (7) kaempferol-3-*O*-rutinoside, (8) kaempferol-3-*O*-glucoside, (9) quercetin, (10) chalcone naringenin, and (11) kaempferol.

developmental stages. Numerous fully developed bulbous glandular trichomes, filled with biosecretions, were present on the abaxial and adaxial surface of bracts and bracteoles at stage II. Some terpenophenolic accumulation at stage II may be associated with developed bulbous glands. Further work is required to isolate the contribution of bulbous glands to metabolite accumulation. A coincidence between gland development and terpenophenolic accumulation of hop cones was incremental over developmental time, and these occurrences are key identifiers of developmental stage. In addition to terpenophenolic accumulations, trends in some classes of polyphenolics were similar over the developmental stages of Zeus and Willamette cones.

Amino Acids. Phenylalanine is the precursor to several phenolic acids, and leucine and valine degradation products are the precursors to α- and β-acid synthesis; therefore the levels of these substrates were of interest to this study. In addition, they are also used in multiple and diverse cellular processes from signaling to protein production. Nevertheless, the decrease in levels of these compounds with the increased production of flavonoids and terpenophenolics might be related.

In Zeus cones, leucine and phenylalanine content showed a decrease over the five developmental stages while valine levels did not change (Figure 6C). Leucine content decreased, but was not significantly different at stage V compared to stage I. Phenylalanine levels significantly decreased from 0.26 (stage I) to 0.10 mg/g (stage V), and decreases were observed between each sequential stage. In contrast to Zeus, leucine and valine contents of Willamette cones decreased between almost all sequential developmental stages (Figure 7C). Leucine and valine

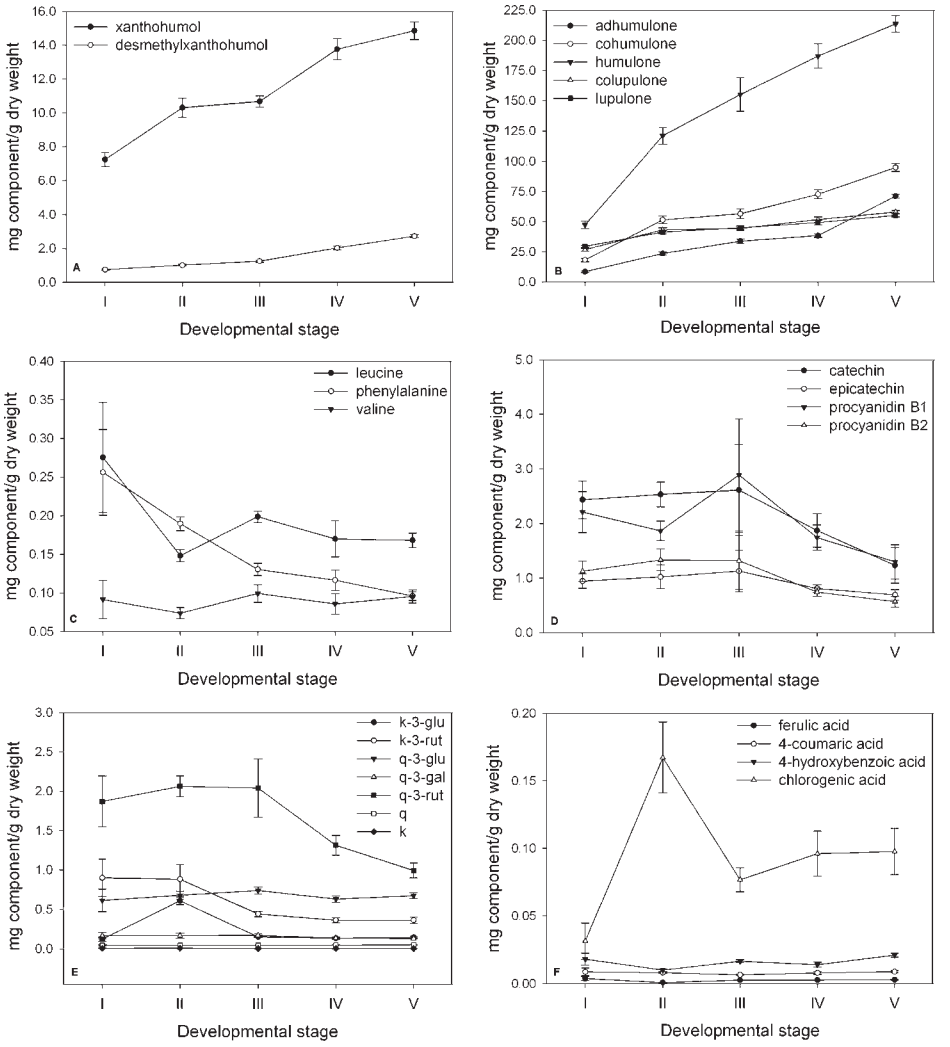

Figure 6. Contents of (A) prenylflavonoids, (B) α- and *β*-acids, (C) amino acids, (D) flavan-3-ols and procyanidins, (E) flavonols and flavonol glycosides, and (F) phenolic acids of Zeus hop cones over five developmental stages, labeled I—V. For each measurement $n = 15$, and error bars represent standard error.

content significantly decreased from 86.4 and 27.9 mg/g (stage I) to 38.1 and 14.1 mg/g (stage V), respectively.

Overall, decreases, many of which were statistically significant between stages, were observed among select amino acids over the five stages in both Zeus and Willamette cones. However, due to the chloroplastic origins and multiple cellular fates of amino acids leucine, valine, and phenylalanine, changes in contents are a result of more than just utilization for terpenophenolic synthesis. Nonetheless, it is important to note that terpenophenolic production is a large metabolic sink late in development for the activated degradation products, namely isovaleryl-, isobutyl-, and coumaryl-coenzyme As, and may therefore coincide with decreased phenylalanine and reduction in branched amino acid production in late seasonal cone development. While only steady-state amino acid levels are reported here, we expect different pools of branch-chain amino acids to be synthesized in glandular secretory cell elioplasts and in bract and bracteole chloroplasts and for them to have different fates specific to organ types. It is not known whether amino acids produced in hop leaf chloroplasts are transported to secretory gland cells for the production of gland secondary metabolites.

Flavan-3-ols and Procyanidins. Flavan-3-ols and their dimers, procyanidins, are highly antioxidant and of interest to several agricultural products such as tea and wine, but also for beer. These compounds are also well-known for their biomedical implications. Polyphenol levels are also of concern to brewers as antioxidants and haze-reducing agents in beer production and storage. Catechin, epicatechin, and procyanidins B-1 and B-2 levels were highly variable in Zeus cones; the only significant decreases occurred at stage V, but a decreasing trend was apparent in all four compounds between stages III and V (Figure 6D). In Willamette cones, flavan-3-ols, catechin and epicatechin, significantly decreased from 0.69 and 0.06 mg/g (stage I) to 0.30 and 0.03 mg/g (stage V), respectively (Figure 7D). Procyanidin B-2 levels fluctuated in Willamette cones over the season, significantly increasing between stages I and II, and significantly decreasing between stages IV and V, but no overall trend was observed. Procyanidin B-1 was present below the LOQ in Willamette MeOH cone extracts, and was therefore not quantitated.

Flavonols and Flavonol Glycosides. Flavonol glycosides make up a large percentage of hop polyphenols. These compounds,

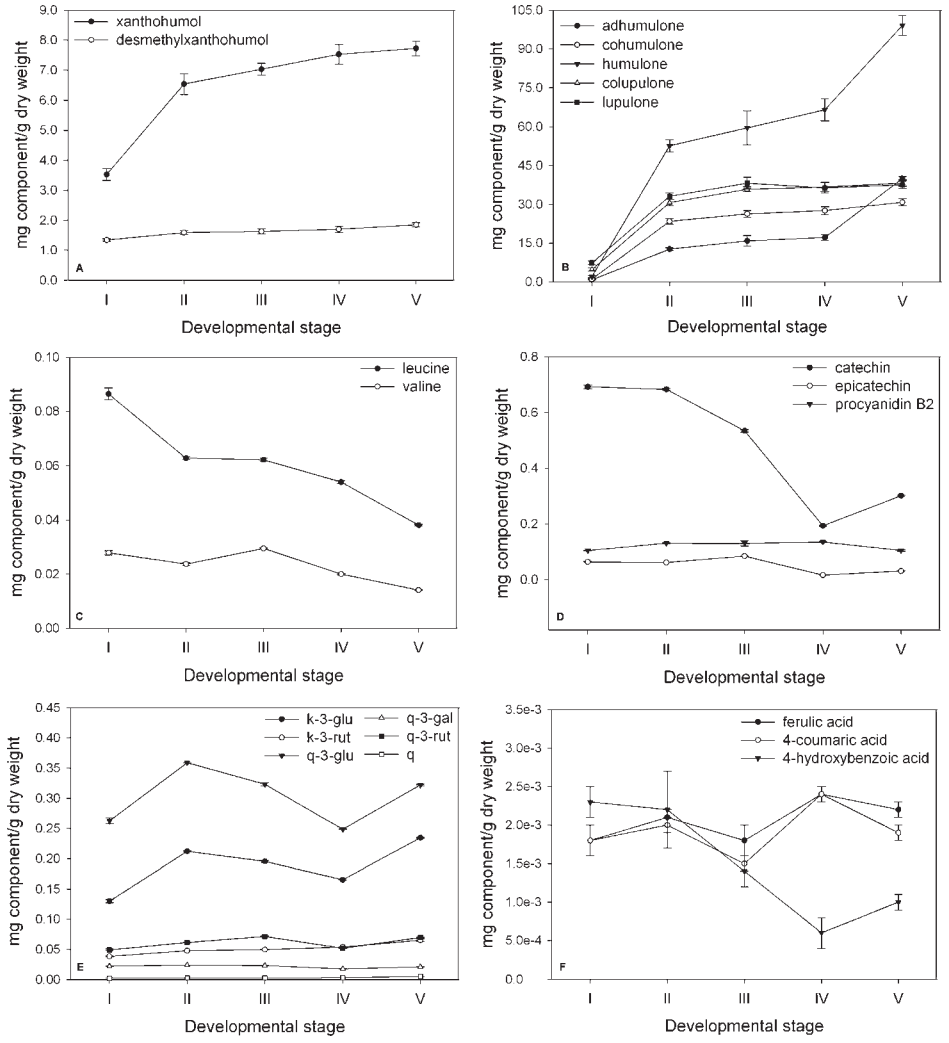

Figure 7. Contents of (A) prenylflavonoids, (B) α- and *β*-acids, (C) amino acids, (D) flavan-3-ols and procyanidins, (E) flavonols and flavonol glycosides, and (F) phenolic acids of Willamette hops over five developmental stages, labeled I—V. For each measurement $n = 15$, and error bars represent standard error.

especially the glycosides, are stable and soluble throughout the brewing process and control the haze and stability of beer. In Zeus cones, the content of the flavonols, quercetin and kaempferol, did not significantly change over the five developmental stages. Flavonol glycoside levels had mixed trends, where the levels of glucosides slightly increased, and contents of the galactosides and rutinosides slightly decreased over developmental time; only one of the five flavonol glycosides measured significantly changed from stage I to stage V (Figure 6E). In Zeus hops, kaempferol-3-*O*-rutinoside levels significantly decreased from 0.90 (stage I) to 0.36 mg/g (stage V). In Willamette hops, quercetin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-rutinoside all significantly increased in content over the five developmental stages (Figure 7E); all flavonol glycosides, with the exception of quercetin-3-*O*-galactoside, significantly increased over the five developmental stages, although changes between each stage were variable.

Overall, with the exception of quercetin-3-*O*-galactoside, flavonol and flavonol glycoside levels significantly increased over Willamette cone development. These trends were generally not observed in Zeus cone flavonols and flavonol glycosides over cone development. Varietal and environmental variation may be large in hop flavonol accumulation, which may also specifically respond to abiotic and biotic stress, and additional developmental experiments may be necessary in order to elucidate repeatable trends among cultivars.

Phenolic Acids. Phenolic acids are the precursors to flavonoids and were of interest in this study as they are precursors to prenylflavonoid production. Some phenolic acids changed over the five developmental stages, but no correlation among phenolic acids or among cultivars was apparent. In Zeus cones, chlorogenic acid levels significantly increased between stages I and II, and ferulic acid significantly decreased between stages I and II; no other major changes were noted in phenolic acids in Zeus cones (Figure 6F). In Willamette cones, 4-hydroxybenzoic acid was the only phenolic acid which significantly changed over developmental time; 4-hydroxybenzoic acid decreased from 2.3 (stage I) to 1.0 mg/g (stage V), and decreases occurred between most developmental stages (Figure 7F). Overall, phenolic acids were highly variable in both hops cultivars over the five developmental stages, and no overall trend was apparent.

The entire hop industry, from growers to end users, holds great interest in phytochemical accumulation during hop cone development, mainly because of the impact of time-of-harvest on product quality. Therefore we have provided a detailed staging index for hop cones spanning five stages of terpenophenol and polypenol accumulation. At stage V, terpenophenolics have accumulated to their maximum levels in each variety, which correlate to spectrophotometric measurements of commercially harvested cones over several seasons for these varieties (results not shown). Additionally, flavonoid and phenolic acid contents are sensitive to biotic and abiotic factors,[24] which can also influence the time-of-harvest. However, phytochemical accumulation alone does not indicate complete maturation of hop cones for use in the brewing industry; additional characteristics, such as cone moisture content, cone size, cone density, and the onset of decline in α-acid contents, are also used to determine commercial harvest time.

Our cone staging index can be used to inform future investigations into developmentally sensitive hop research. For example, transcriptomics has delivered a plethora of putative gene functions,[6,13,25,26] often with multiple gene family members indicated, whose functional characterization depends on spatial and temporal placement within the compartmentalized biochemistry of cone development. Similarly, application of growth regulators during cone maturation, such as prohexidione-calcium, has produced agronomically positive yet developmentally sensitive effects on terpenophenolic and cone biomass yields.[27] Successful time-of-application of growth regulators to increase yield and subsequent assessment of the chemical substantial equivalence of the agricultural food product can be based on developmental chemical profiling and analytical advances reported here.

In conclusion, here we reported the application of a new LC−TOF-MS method and a previously reported UHPLC−PDA method to quantitate 28 industrially pertinent compounds in hop cones. These methods provide a rapid and most detailed analysis to date of the polyphenolic and terpenophenolic makeup of hop cones with sensitive, accurate, and repeatable results. The analytical methods also have wider applicability beyond hops and can be used for targeted metabolite quantitation in other crops. Application of these methods to hops has generated a novel, useful picture of morphological and phytochemical development of hop cone maturation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Table of validation data for LC−TOF-MS method and detailed botanical description of five stages of hop cone development with photomicrographs for use in keying cones. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 718-960-1105. Fax: 718-960-8236. E-mail: edward.kennelly@lehman.cuny.edu.

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