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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · AUGUST 2003

Impact Factor: 2.91 · DOI: 10.1021/jf034263z · Source: PubMed

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Formation and Accumulation of α-Acids, β-Acids, Desmethylxanthohumol, and Xanthohumol during Flowering of Hops (*Humulus lupulus* L.)

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Important secondary metabolites, present in hops (*Humulus lupulus* L.), include α-acids and β-acids, which are essential for the brewing of beer, as well as the prenylated chalcones, desmethylxanthohumol, and xanthohumol, which exhibit interesting bioactive properties. Their formation and accumulation in five selected hop varieties, Wye Challenger, Wye Target, Golding, Admiral, and Whitbread Golding Variety, were quantitatively monitored by high-performance liquid chromatography using UV detection. All target compounds were present from the onset of flowering, not only in female hop cones but also in male inflorescences, albeit in low concentrations. During development from female inflorescences to cones, levels of α-acids, β-acids, desmethylxanthohumol, and xanthohumol gradually increased, while each hop variety exhibited individual accumulation rates. Furthermore, these compounds were present in leaves of fully grown hops as well. The study demonstrated that key compounds for flavor and potential beneficial health effects associated with beer not only reside in the glandular lupulin structures but also are distributed over various parts of the hop plant.

KEYWORDS: α-Acids; β-acids; desmethylxanthohumol; xanthohumol; hops (*Humulus lupulus* L.); high-performance liquid chromatography

INTRODUCTION

The economic value of the hop plant (*Humulus lupulus* L.) is derived from its worldwide application as an essential flavoring ingredient for the brewing of beer. The impact of hops on beer quality is manifold, but by far, most important are specific features attributed to beer flavor including bitter taste and hoppy aroma. Pivotal constituents of hops are so-called α-acids and β-acids (**Figure 1**), also known as humulones and lupulones, respectively, because they are precursors of beer bittering agents; iso-α-acids or isohumulones are the most relevant (*1*). These hop acids are found in glandular structures (lupulin glands) that are abundantly present in cones of the female hop plant. Particular hop varieties contain up to 19% (w/w) α-acids (super-α-hops), which nowadays are, to a great extent, extracted using liquid or supercritical carbon dioxide. Further elaboration of these hop extracts gives rise to various advanced hop products that are increasingly being used in modern brewing practices (*2*). Flavonoids, more specifically prenylated flavonoids, are another class of secondary metabolites present in hops (*3*, *4*). Various bioactivities associated with these

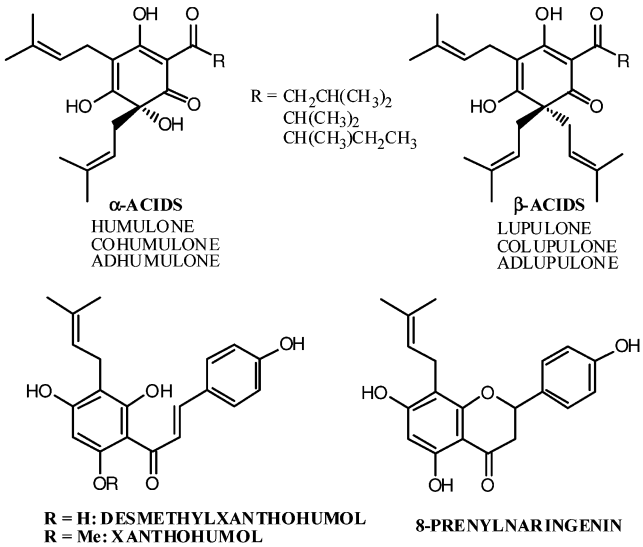

Figure 1. Structures of α-acids, β-acids, DMX, X, and 8-prenylnaringenin.

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10.1021/jf034263z CCC: $25.00
Published on Web 00/00/0000 **PAGE EST: 5.2**

compounds arouse currently high scientific and medical interest. Thus, 8-prenylnaringenin (**Figure 1**) reportedly is the most active phytoestrogen known in the plant kingdom (*5*), while xanthohumol (X) (**Figure 1**), the most abundant prenylated chalcone present in hops, exhibits potent cancer chemopreventive properties (*6*).

The biosynthesis of α-acids, *β*-acids, and prenylated flavonoids involves common building blocks including malonyl-coenzyme A ester and isoprenyl pyrophosphate (*7, 8*); hence, formation pathways may be competitive. It is, therefore, worthwhile to monitor their appearance and accumulation during development of hop cones and, possibly, their presence in other plant parts. In the course of our studies on the estrogenicity of hops, particular hop varieties were selected for their content of prenylated flavonoids, namely Wye Challenger (WC), Wye Target (WT), Golding (G), Admiral (A), and Whitbread Golding Variety (better known as WGV). It was intended to present here quantitative data on formation and accumulation of α-acids, *β*-acids, and two prenylated chalcones, desmethylxanthohumol (DMX) and X, during different stages of flowering of these selected hop varieties.

MATERIALS AND METHODS

Hop Sampling. Five hop varieties were investigated, WC, WT, G, A, and WGV. Duplicates of all samples were stored as voucher specimens in the facilities of the Botanical Garden of the Ghent University. The hop varieties were examined at three stages of flowering, from the appearance of inflorescences (stage 1; harvest date, 8 August 2002) to the formation of small (stage 2; harvest date, 19 August 2002) and full-grown hop cones (stage 3; harvest date, 3 September 2002). As for the hop varieties G and A, small (stage 2a) and medium-sized hop cones (stage 2b) could be distinguished, and both were harvested on the same day (19 August 2002). The length of small was between 8 and 12 mm, and that of medium-sized cones was between 12 and 16 mm. In addition, leaf material of each variety was collected at the moment of hop harvesting. All samples were available at the hop farm of Joris Cambie, Poperinge, Belgium. Furthermore, an opportunity was presented to collect male inflorescences in the wild. Care was taken to sample morphologically homogeneous inflorescences, cones, and leaves. Samples were transferred to round bottom flasks, lyophylized for 36 h (Heto Lyolab 3000 from Heto-Holten, Allerod, Denmark, in combination with a DUO 5 Pfeiffer Vacuum Pump from Pfeiffer Vacuum, Asslar, Germany), and stored at −20 °C.

Extraction. Samples of hop inflorescences (ca. 250 mg for stage 1), cones (500 mg to 1 g for stages 2 and 3), and leaves (1 g) were ground, suspended in methanol containing 0.01% formic acid (10 mL for stage 1 and 20 mL for both stages 2 and 3 and for leaves), vortex-mixed (1 min), and stirred for 1 h. A sample of the supernatant (ca. 1 mL) was withdrawn and filtered over a 0.2 *μ*m filter (regenerated cellulose, 13 *μ*m; Alltech, Lokeren, Belgium) prior to analyis by high-performance liquid chromatography (HPLC).

Analytical Procedure. Quantitative analyses were effected by HPLC using a Waters 2695 Alliance Separations Module equipped with a C18 reversed-phase column (Varian Omnisphere, 250 mm × 4.6 mm, 5 *μ*m). A gradient was used composed of solvent A (water acidified with 0.025% (v/v) formic acid) and solvent B (methanol acidified with 0.025% (v/v) formic acid). Gradient profile: 0−3 min, 45% B in A; 3−32 min, from 45% B in A to 95% B in A; 32−37 min, 95% B in A; 37−45 min, 95% B in A to 45% B in A; 45−47 min, 45% B in A. The sample size was 20 *μ*L, the flow rate was 1 mL/min, and the column temperature was 35 °C. Detection was done at 314 (for α-acids and *β*-acids) and at 370 nm (for DMX and X) using diode array detection. Peaks were identified by comparison of the retention times with those of authentic reference compounds, as well as by inspection of the respective UV spectra. A mixture of α-acids and *β*-acids (Versuchsstation Schweizerische Brauereien, Zurich, Switzerland) of well-known composition (ICE-1: International Calibration Extract-1; 17.69% cohumulone, 41.49% (humulone + adhumulone), 9.66% colupulone, 8.46% (lupulone + adlupulone)) served as external standard to quantify α-acids and *β*-acids. DMX and X were isolated according to published procedures (semipreparative HPLC) (*9, 10*) and identified by comparison of ^{1}H NMR and ^{13}C NMR data (Varian 300 MHz) with literature values (*10*). Injections were done in 2-fold for five preparations of each sample, and standard deviations were calculated based on peak area integration.

RESULTS AND DISCUSSION

Reliable quantification of target compounds in a single-run separation was achieved after suitable optimization of a gradient HPLC procedure (**Figure 2**) (*11*). The elution order was as follows: DMX, t_R = 23.9 min; X, t_R = 26.7 min; cohumulone, t_R = 32.5 min; (humulone + adhumulone), t_R = 33.4 min; colupulone, t_R = 34.9 min; (lupulone + adlupulone), t_R = 35.7 min. Humulone and adhumulone on one hand and lupulone and adlupulone on the other hand are structural isomers. While separation is feasible (*12, 13*), analyses in the present study were achieved using the respective mixtures, since adcompounds are very minor constituents (<15% of the mixture of α-acids) (*14*) and separate quantification of humulone/adhumulone and lupulone/adlupulone was not required for the purpose of the work. Moreover, standards of these individual constituents are not available. The prenylated chalcones DMX and X are naturally present in hops, and they are precursors of isomeric flavanones, the most important one being 8-prenylnaringenin (**Figure 2**) derived from isomerization of DMX. It was ascertained that during extraction and analysis and in the presence of 0.01% formic acid, isomerization of DMX to X was negligible.

For each of the hop varieties investigated, WC, WT, G, A, and WGV, samples were collected at three different stages, from the onset of flowering (stage 1) to small (stage 2) and full-grown cones (stage 3). During sampling of G and A, the opportunity was presented to collect cones of two different sizes and data of these small (stage 2a) and medium-sized (stage 2b) cones are included in the study.

Figure 3 highlights graphically the quantitative analyses of α-acids, *β*-acids, DMX, and X in the hop variety G, while results for all hop varieties are summarized in **Table 1**. Apart from specific features of each hop variety, some general observations are worth noting. All hop acids and the prenylated chalcones, DMX and X, are apparently present already during the first stages of flowering, although invariably below 1% for each parameter monitored, with the exception of hop variety A (1.66% *β*-acids). Evidently, their concentrations increase during cone development. However, (lupulone + adlupulone) constitute a striking exception, since the concentration reaches a maximum at the second stage (small cones) for all hop varieties except WC. It is well-known that *β*-acids are very prone to oxidation (*15*), and this sensitivity may be variety-dependent, although antioxidative mechanims are ill-defined. It is, furthermore, striking that the formation of *β*-acids precedes that of α-acids during the very beginning of flowering.

A remarkable observation pertains to the pronounced accumulation of α-acids during full flowering of WC (from 3.06 to 9.18%) coincident with a vigorous growth, i.e., rapid formation of hop cones (length of at least 16 mm) from inflorescences (length of less than 8 mm). For the other hop varieties, the greatest increase in concentrations of the target compounds occurred during early formation of cones. Clearly, the biochemical pathway leading to α-acids is preferred as soon as the initial inflorescences progress to develop cones. It is very likely that the pathways leading to hop acids and chalcones involve a common intermediate, such as phloroglucinol. Acylation by apolar amino acids (leucine, valine, isoleucine) gives

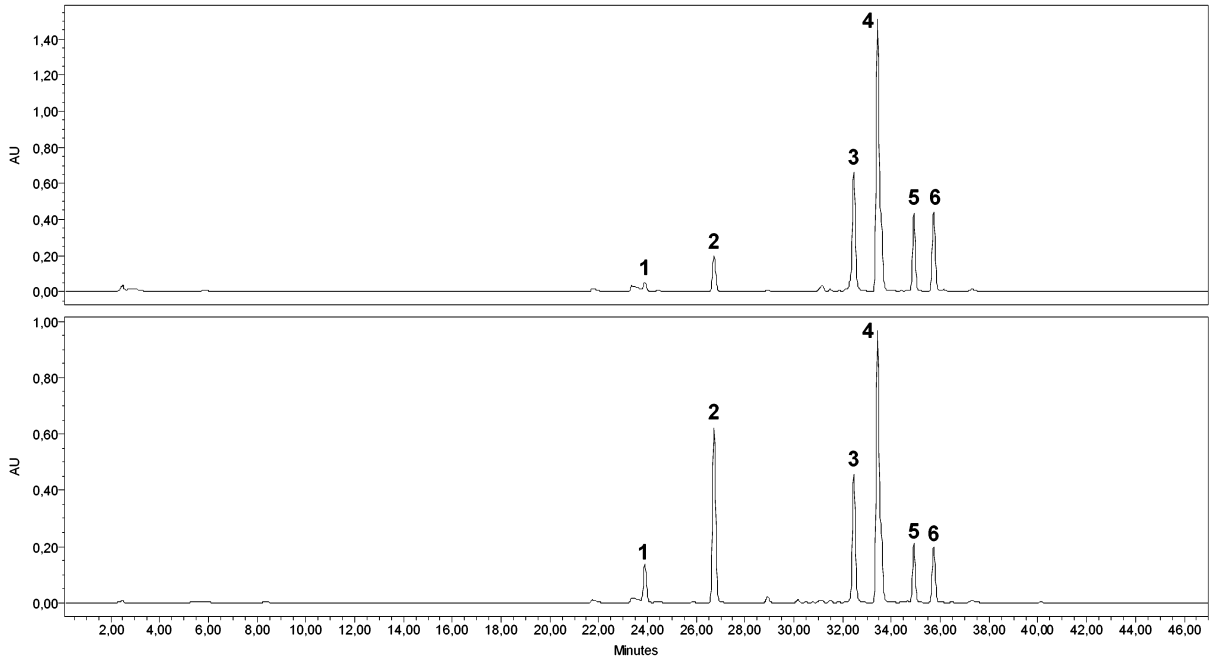

Figure 2. HPLC chromatograms with detection at 314 nm (upper chromatogram) and at 370 nm (lower chromatogram) of the separation of DMX (peak number 1; t_R = 23.9 min), X (peak number 2; t_R = 26.7 min), cohumulone (peak number 3; t_R = 32.5 min), (humulone + adhumulone) (peak number 4; t_R = 33.4 min), colupulone (peak number 5; t_R = 34.9 min), and (lupulone + adlupulone) (peak number 6; t_R = 35.7 min) in the hop variety G (for chromatographic conditions, see Materials and Methods).

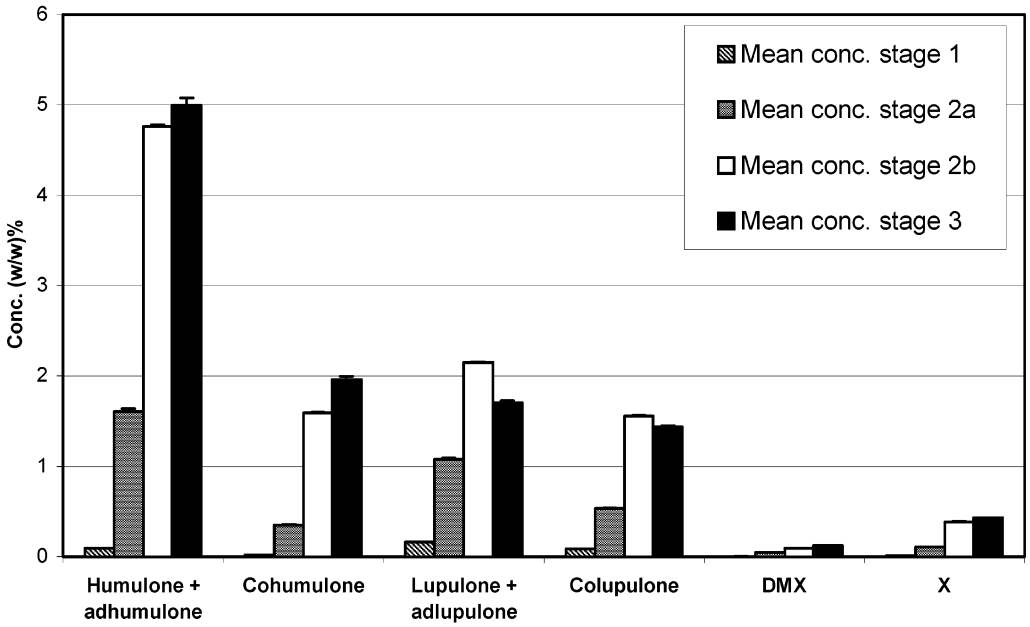

Figure 3. Quantification (%, w/w) of (humulone + adhumulone), cohumulone, (lupulone + adlupulone), colupulone, DMX, and X in the hop variety G during different stages of development of female inflorescences and cones.

rise ultimately to α- and β-acids. Similar acylation, e.g., by cinnamoylCoA, would lead to chalcones. It has been reported that valerophenone synthase (furnishing humulone/lupulone) has chalcone synthase activity (*16*); however, a true chalcone synthase is present in hops as well (*17*). The special features of WC are also illustrated by the high content of DMX as a proestrogen relative to X (ratio of DMX to X, 0.78) in contrast to WT (ratio, 0.13). This may bear relevance to the estrogenic potential of WC (*5*). It is, indeed, the concentration of DMX that is crucial in view of the estrogenic features. A plant with a high content of DMX would be a plant with a high estrogenic potential, since X is estrogenically inactive (*18*). Ratios have been reported in the present paper, because of the interest in X as a potential chemopreventive agent (*6*). Thus, the selection of a hop variety is aimed at having high contents of DMX and reasonable contents of X.

While it is commonly accepted that the secondary hop metabolites reside in the lupulin glands, this study showed the presence of the target compounds in the hop leaves on the day of harvest (**Table 2**). Indeed, the presence of lupulin-like glands on hop leaves is well-known. Large differences were noted among the concentrations of the constituents as well as among the hop varieties, although it must be noted that total concentrations were below 0.1%. The β-acids predominated largely over the α-acids. In particular, WT and A appeared to be rich both in cocompounds and in concentrations of (humulone +

Table 1. Quantification (%, w/w) of (Humulone + Adhumulone), Cohumulone, (Lupulone + Adlupulone), Colupulone, DMX, and X in Five Selected Hop Varieties during Different Stages of Development of Female Inflorescences and Cones (For Experimental Conditions, See Materials and Methods)

hop variety	(humulone + adhumulone)	cohumulone	(lupulone + adlupulone)	colupulone	DMX	X
			WC			
stage 1[a]	0.04 ± 0.01	0.02 ± <0.01	0.35 ± 0.03	0.22 ± 0.02	0.01 ± <0.01	0.02 ± <0.01
stage 2[b]	2.59 ± 0.12	0.47 ± 0.03	2.31 ± 0.11	0.99 ± 0.05	0.13 ± 0.01	0.13 ± 0.01
stage 3[c]	6.79 ± 0.07	2.39 ± 0.03	3.55 ± 0.04	2.72 ± 0.03	0.39 ± <0.01	0.50 ± <0.01
			WT			
stage 1	0.15 ± <0.01	0.07 ± 0.01	0.24 ± 0.01	0.20 ± 0.01	0.01 ± <0.01	0.05 ± >0.01
stage 2	8.51 ± 0.06	3.55 ± 0.03	3.62 ± 0.03	3.55 ± 0.06	0.06 ± <0.01	0.90 ± 0.01
stage 3	9.17 ± 0.05	5.03 ± 0.03	3.08 ± 0.01	3.69 ± 0.05	0.13 ± <0.01	1.02 ± 0.01
			G			
stage 1	0.09 ± <0.01	0.02 ± <0.01	0.16 ± <0.01	0.09 ± <0.01	0.01 ± <0.01	0.02 ± <0.01
stage 2a[b]	1.60 ± 0.03	0.35 ± <0.01	1.08 ± 0.01	0.54 ± 0.03	0.04 ± <0.01	0.11 ± <0.01
stage 2b[d]	4.76 ± 0.02	1.59 ± <0.01	2.15 ± <0.01	1.56 ± 0.02	0.09 ± <0.01	0.39 ± <0.01
stage 3	4.99 ± 0.08	1.96 ± <0.01	1.71 ± 0.02	1.43 ± 0.08	0.12 ± <0.01	0.43 ± <0.01
			A			
stage 1	0.59 ± 0.03	0.35 ± 0.01	0.81 ± 0.02	0.85 ± 0.02	0.03 ± <0.01	0.16 ± <0.01
stage 2a	2.13 ± 0.07	0.86 ± 0.03	1.07 ± 0.03	1.02 ± 0.07	0.03 ± <0.01	0.24 ± 0.01
stage 2b	8.63 ± 0.19	4.71 ± 0.11	3.06 ± 0.07	3.73 ± 0.19	0.09 ± <0.01	0.85 ± 0.02
stage 3	10.69 ± 0.07	7.26 ± 0.05	2.43 ± 0.01	3.83 ± 0.07	0.19 ± <0.01	1.33 ± 0.01
			WGV			
stage 1	0.03 ± <0.01	0.01 ± <0.01	0.17 ± <0.01	0.11 ± <0.01	0.01 ± <0.01	0.03 ± <0.01
stage 2	3.72 ± 0.05	1.34 ± 0.02	1.87 ± 0.03	1.36 ± 0.04	0.09 ± <0.01	0.34 ± <0.01
stage 3	4.79 ± 0.07	2.53 ± 0.04	1.63 ± 0.02	1.72 ± 0.05	0.13 ± <0.01	0.45 ± 0.01

[a] Inflorescences fully developed. [b] Small cones. [c] Full-grown cones. [d] Medium-sized cones.

Table 2. Quantification (%, w/w) of (Humulone + Adhumulone), Cohumulone, (Lupulone + Adlupulone), Colupulone, DMX, and X in Leaves of Five Selected Hop Varieties[a] and in Male Inflorescences (Belgian Wild Male) (For Experimental Conditions, See Materials and Methods)

hop variety	(humulone + adhumulone)	cohumulone	(lupulone + adlupulone)	colupulone	DMX	X
			leaves			
WC[a]	0.28 ± <0.01	0.07 ± 0.01	1.78 ± 0.07	1.49 ± 0.17	0.02 ± < 0.01	0.25 ± 0.01
WT[a]	0.84 ± 0.08	0.26 ± 0.02	9.62 ± 0.38	6.96 ± 0.30	0.16 ± 0.01	2.33 ± 0.07
G[a]	0.46 ± 0.20	0.08 ± < 0.01	1.10 ± 0.05	2.27 ± 0.07	0.02 ± < 0.01	0.51 ± 0.01
A[a]	0.66 ± 0.10	0.26 ± 0.07	7.72 ± 1.75	7.52 ± 1.69	0.16 ± 0.04	2.46 ± 0.54
WGV[a]	0.44 ± 0.04	0.12 ± 0.02	0.28 ± 0.03	0.25 ± 0.02	0.01 ± <0.01	0.12 ± 0.01
			male inflorescences			
Belgian wild male	0.10 ± 0.01	0.03 ± <0.01	0.09 ± 0.01	0.06 ± <0.01	<0.01 ± <0.01	0.02 ± <0.01

[a] Concentrations × 10^2.

adhumulone) and (lupulone + adlupulone). Remarkably, WGV was extremely poor in β-acids. The concentrations of α-acids were very low, and only WT contained 0.01% in the leaves. A most revealing observation pertained to the concentrations of X in WT and A, which largely outweighed the contents of α-acids, while concentrations of DMX were very low.

α-Acids, β-acids, and X were also detected in male inflorescences (Belgian wild male). The concentrations were 0.13, 0.15, and 0.02%, respectively; the concentration of (humulone + adhumulone) reached 0.1% (**Table 2**). The presence of hop acids in male inflorescences has been reported previously (*19*, *20*). Concentrations are comparable to those found during early female flowering. Even the concentrations of α-acids in male inflorescences were higher than those at stage 1 in the female inflorescences of WC, G, and WGV. In contrast, the concentrations of β-acids in the male inflorescences did not exceed those in the early (stage 1) female inflorescences in any of the hop varieties. Moreover, the presence of X in male inflorescences has also been confirmed, again in similar concentrations as in early female inflorescences.

Inspection of indvidual hop varieties leads to interesting conclusions, in particular when the respective ratios of α-acids to β-acids, cohumulone to (humulone + adhumulone), colupulone to (lupulone + adlupulone), and DMX to X are considered during the various stages of hop flowering (**Table 3**). WC showed a moderately high production of α-acids (9.18%) with an unusually low ratio of cohumulone to (humulone + adhumulone) (0.35), while remarkably, colupulone (2.72%) did not predominate over the other β-acids (3.55%). The β-acids were already present during the first stage of flowering (ca. 10-fold higher than α-acids) and the ratios of cohumulone to (humulone + adhumulone) as well as of colupulone to (lupulone + adlupulone) fluctuated significantly during hop flowering. Apparently, production of cocompounds proceeds more efficiently during later stages of flowering. Furthermore, the ratio of α-acids to β-acids is the lowest (1.46%) of all hop varieties investigated. WC is also an efficient producer of DMX with respect to X (ratio of DMX to X of 0.78).

The features of WT are entirely different from those of WC, as WT presents itself as a high-α hop variety (14.20% α-acids). Relatively high proportions of cocompounds are determined both in the α-acids and in the β-acids. Also, the concentrations of the hop acids changed only slightly during the last 2 weeks of flowering, thus characterizing this hop as a so-called early hop variety, which, in the present case, could have been harvested from mid-August on. Furthermore, the concentration of X slightly exceeded 1% but that of DMX was very low (ratio of DMX to X, 0.13).

Table 3. Weight Ratios of α-Acids to β-Acids, Cohumulone to (Humulone + Adhumulone), Colupulone to (Lupulone + Adlupulone), and DMX to X in Five Selected Hop Varieties during Different Stages of Development of Female Inflorescences and Cones (For Experimental Conditions, See Materials and Methods)

hop variety	α-acids/ β-acids	cohumulone/ (humulone + adhumulone)	colupulone/ (lupulone + adlupulone)	DMX/X
		WC		
stage 1[a]	0.11	0.50	0.63	0.50
stage 2[b]	0.93	0.18	0.43	1.00
stage 3[c]	1.46	0.35	0.77	0.78
		WT		
stage 1	0.50	0.47	0.83	0.20
stage 2	1.68	0.42	0.98	0.07
stage 3	2.10	0.55	1.20	0.13
		G		
stage 1	0.44	0.22	0.56	0.50
stage 2a[b]	1.20	0.22	0.50	0.36
stage 2b[d]	1.71	0.33	0.73	0.23
stage 3	2.21	0.39	0.83	0.28
		A		
stage 1	0.57	0.59	1.05	0.19
stage 2a	1.43	0.40	0.95	0.13
stage 2b	1.96	0.55	1.22	0.11
stage 3	2.87	0.68	1.58	0.14
		WGV		
stage 1	0.14	0.33	0.65	0.33
stage 2	1.57	0.36	0.73	0.26
stage 3	2.19	0.53	1.06	0.29

[a] Onset of flowering. [b] Small cones. [c] Full-grown cones. [d] Medium-sized cones.

G shows a pattern more or less similar to that of WT. Interestingly, small and medium-sized cones could be collected separately on the same day (19 August 2002). This allowed us to detect quite remarkable differences. The concentrations of α-acids and β-acids did not alter significantly, when these medium-sized cones were compared to the final cones harvested 2 weeks later. It may, therefore, be interesting for a grower to carefully observe the proportion of small cones, as these may significantly lower the yields of target constituents. It may also be noted that the concentrations of the β-acids decreased to some extent during the last 2 weeks of flowering.

Salient features of A are the exceptionally high content of α-acids (17.95%), which characterizes A as a remarkable super-α-hop (at least in the conditions of growing during the 2002 season in Poperinge), the nonnegligible formation of α-acids (0.94%) and β-acids (1.66%) at the onset of flowering, the elevated levels of cocompounds (by far the highest of all hop varieties) both for the α-acids (ratio of cohumulone to (humulone + adhumulone), 0.68) and for the β-acids (ratio of colupulone to (lupulone + adlupulone), 1.58), the high concentration of X (1.33%), and the very low concentration of DMX (0.19%).

Finally, the hop variety WGV showed a steady increase of all compounds of interest during flowering, except for (lupulone + adlupulone), the concentration of which decreased for more than 10% during the last 2 weeks before final harvest. Another feature worth noting is the very low concentrations of X and DMX, comparable to those observed for G.

Monitoring the production and accumulation of hop acids (α-acids and β-acids) and prenylated chalcones (DMX and X) in five selected hop varieties showed that these key constituents are present from the onset of flowering of hops (*H. lupulus* L.). Both female and male inflorescences contain the target compounds; however, the concentrations in the male inflorescences are very low. Levels increased significantly during development from female inflorescences to cones, but each hop variety exhibited specific features in this respect. Also, leaves of fully grown hops contain low but detectable levels of hop acids and even chalcones. Thus, it was demonstrated that key compounds for the beer taste and for interesting beneficial health effects associated to beer (however, not proven yet by epidemiological studies) are distributed over various parts of the hop plant. Careful control during growing of hops should result in determining the most suitable time of harvest, not only in view of optimized utilization for the brewing of beer but also with regard to exploiting the health potential of hops.

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Received for review March 19, 2003. Revised manuscript received May 14, 2003. Accepted May 18, 2003. We are indebted to the Special Research Fund of the Ghent University for financing our joint hop endeavors (Project No. 01100403) and for providing a predoctoral fellowship to J.D.K.

JF034263Z