JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Metabolism of Hop-Derived Bitter Acids

Ko Cattoor,*[,†] Michael Dresel,[⊥] Lies De Bock,[‡] Koen Boussery,[‡] Jan Van Bocxlaer,[‡] Jean-Paul Remon,[§] Denis De Keukeleire,[†] Dieter Deforce,[†,∥] Thomas Hofmann,[⊥] and Arne Heyerick[†]

[†]Laboratory of Pharmacognosy and Phytochemistry, [‡]Laboratory of Medical Biochemistry and Clinical Analysis, [§]Laboratory of Pharmaceutical Technology, and [∥]Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Ghent University (UGent), Harelbekestraat 72, B-9000 Ghent, Belgium

[⊥]Chair of Food Chemistry and Molecular Sensory Science, Technische Universität Munchen, Lise-Meitner-Strasse 34, D-85354 Freising, Germany

ABSTRACT: In this study, in vitro metabolism of hop-derived bitter acids was investigated. Besides their well-known use as bitter compounds in beer, in several studies, bioactive properties have been related to these types of molecules. However, scientific data on the absorption, distribution, metabolism, and excretion aspects of these compounds are limited. More specific, in this study, α-acids, β-acids, and iso-α-acids were incubated with rabbit microsomes, and fractions were subjected to LC−MS/MS analysis for identification of oxidative biotransformation products. Metabolism of β-acids was mainly characterized by conversion into hulupones and the formation of a series of tricyclic oxygenated products. The most important metabolites of α-acids were identified as humulinones and hulupones. Iso-α-acids were found to be primarily metabolized into *cis*- and *trans*-humulinic acids, next to oxidized alloiso-α-acids. Interestingly, the phase I metabolites were highly similar to the oxidative degradation products in beer. These findings show a first insight into the metabolites of hop-derived bitter acids and could have important practical implications in the bioavailability aspects of these compounds, following ingestion of hop-based food products and nutraceuticals.

KEYWORDS: *hop bitter acids, α-acids, β-acids, iso-α-acids, metabolism, liver microsomes*

INTRODUCTION

The worldwide use of hop (*Humulus lupulus* L) in the brewing industry is mainly based on the contribution of hop-derived bitter acids, contained in the bright yellow lupulin glands of female hop flowers, to the overall microbial stability (as natural preservatives), a pleasant bitter flavor, and an enhanced foam stability. The main hop bitter acids in hops are α- and β-acids, also called humulones and lupulones, which are present as a mixture of three congeners, co, *n*, and ad, depending on the side chain in the chemical structure of the molecules (Figure 1). During brewing, the relatively insoluble α-acids are transformed into their corresponding mixture of beer-soluble diastereomeric pairs of iso-α-acids (*cis*- and *trans*-isomers), thus resulting in six iso-α-acids originating from the three main α-acids (Figure 1). Next to their application in beers, an increase in global research interest in the health-beneficial aspects of hop-derived bitter acids has been observed[1,2] as evidenced by the increasing number of reports on various promising bioactivities with IC_{50} values in the lower micromolar range (anti-inflammatory[3,4] and antiangiogenic properties,[5,6] improving lipid profiles,[7,8] and counteracting diabetes type 2).[9]

In comparison to the growing knowledge on their health-beneficial effects, little is known about the absorption, distribution, metabolism, and excretion (ADME) of these compounds. In previous papers, we reported on the in vitro intestinal permeabilities of α-acids and β-acids and the pharmacokinetics and bioavailability in rabbits of iso-α-acids, dihydroiso-α-acids, and tetrahydroiso-α-acids.[10,11] This latter study showed that presystemic breakdown or metabolism (either gastrointestinal or in hepatic tissues) was the most important factor influencing the bioavailability of iso-α-acids and reduced derivatives.

Recent experiments reported by Aniol et al. showed that hop α-acids and β-acids are totally degraded when incubated with peroxidase enzymes from plant extracts,[12] suggesting a potential proneness of hop acids to oxidative enzyme breakdown.

The present study focuses on the CYP metabolism of the natural hop-derived bitter acids (α-, β-, and iso-α-acids) present in beer and hop-based food supplements. Insights into the metabolism and/or degradation following ingestion of these substances are essential to understand their overall bioavailability and related health effects following intake of such preparations. Since CYPs are the major enzymes involved in drug metabolism and bioactivation, accounting for ∼75% of the total metabolism, phase I metabolism catalyzed by cytochrome P450 enzymes was investigated by incubation of α-, β-, and iso-α-acids with liver microsomes, isolated from rabbits. The most common reaction catalyzed by cytochrome P450 enzymes is a mono-oxygenase reaction; therefore, the formation of oxidative breakdown products was investigated by specific HPLC−MS and LC−MS/MS methods to identify metabolites of iso-α-acids, α-acids, and β-acids in microsomal incubations.

MATERIALS AND METHODS

Chemicals. Hop α-acids and β-acids were obtained as pure solutions in methanol and iso-α-acids as an isomerized extract from Hopsteiner (Mainburg, Germany). International calibration standards for α- and β-acids (ICE-2) and iso-α-acids (DCHA-Iso, ICS-I3) were all obtained from Labor Veritas (Zurich, Switzerland). Glucose 6-phosphate

Received: January 3, 2012
Revised: June 23, 2013
Accepted: July 30, 2013
Published: July 30, 2013

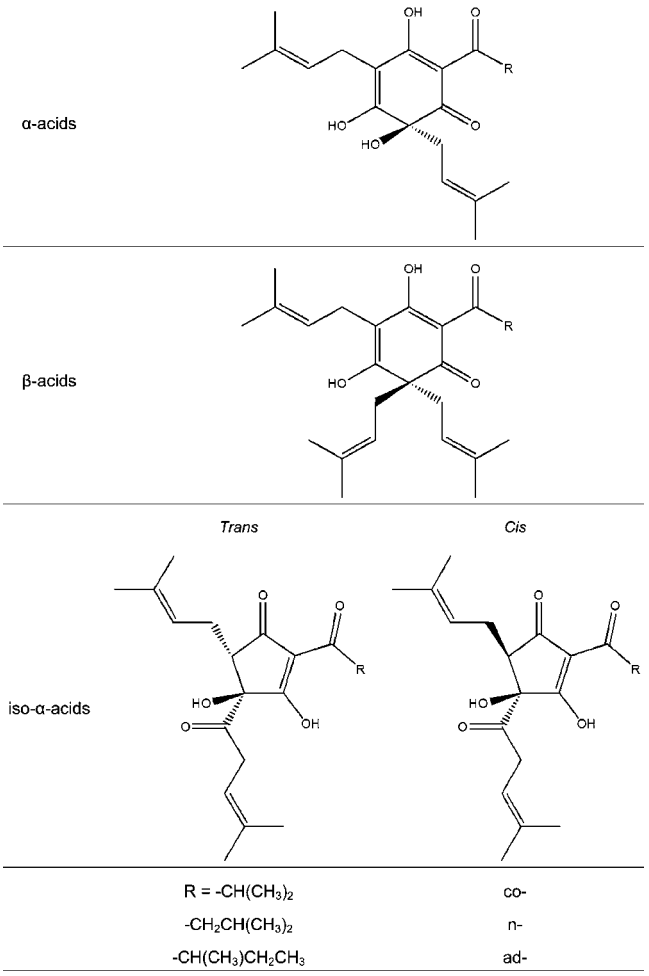

Figure 1. Molecular structures of hop-derived α-acids, β-acids, and iso-α-acids.

sodium salt, glucose 6-phosphate dehydrogenase from baker's yeast (*Saccharomyces cerevisiae*), and β-nicotinamide adenine dinucleotide phosphate hydrate (β-NADP$^+$) were purchased from Sigma-Aldrich (Bornem, Belgium). All other reagents were of analytical grade and were used without further purification. Water (ULC−MS grade) and all other LC−MS solvents (LC−MS quality) were obtained from Biosolve (Valkenswaard, The Netherlands) and Merck (Darmstadt, Germany). Formic acid was obtained from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) was purchased from Riedel-de-Haen (Seelze, Germany). Deionized water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, MA). Pure reference compounds for identification by means of HPLC−MS/MS Qtrap analysis were obtained following the recently published protocols of Intelmann and Haseleu.[13−15]

Preparation of Rabbit Liver Microsomes. Six rabbits (n = 6) were anesthetized using CO_2 and killed by asphyxiation. The livers were quickly removed, cut into smaller pieces (1 cm^3), and frozen at −80 °C before use. Aliquots of the liver of six different rabbits were washed with phosphate buffer containing 1.15% KCl (0.25 M, pH 7.25) to remove blood, and the mass of one sample was adjusted to 3.1 ± 0.1 g. Then, all liver pieces in one sample were mixed with phosphate buffer (10 mL/g of liver) containing 1.15% KCl (0.25 M, pH 7.25) and homogenized using an IKA Ultra-Turrax equipped with a Potter-Elvehjem tissue grinder (VWR International LLC, West Chester, PA). The homogenate was centrifuged at 10000g for 25 min at 4 °C, and the pellet (nuclei, mitochondria, and debris) was discarded. The supernatant (S9 fraction) was further centrifuged at 100000g for 80 min at 4 °C to separate the S9 fraction into a cytosolic fraction (supernatant) and a microsomal pellet. The microsomal pellet was resuspended in phosphate buffer (10 mL/g of liver) containing 1.15% KCl (0.25 M, pH 7.25), homogenized, and centrifuged again at 100000g for 80 min at 4 °C. The final microsomal pellet was resuspended in phosphate buffer (1.5 mL/g of liver) containing 1.15% KCl and 30% glycerol (0.25 M, pH 7.4), and the microsomes were stored at −80 °C until use. The protein concentration of the liver microsomes was determined by the method described by Bradford.[16]

Table 1. Molecular Ions Used for Selected Ion Monitoring (SIM; Negative) Mode for α-Acids, β-Acids, and Iso-α-acids in LC−MS Analysis

	compd	m/z		compd	m/z
α-acids	cohumulone	347	iso-α-acids	*cis*-isocohumulone	347
	adhumulone	361		*trans*-isocohumulone	347
	humulone	361		*cis*-isoadhumulone	361
β-acids	colupulone	399		*trans*-isoadhumulone	361
	adlupulone	413		*cis*-isohumulone	361
	lupulone	413		*trans*-isohumulone	361

In Vitro Metabolism of Hop-Derived Acids. The microsomal fractions were thawed on ice on the day of the assay and diluted to the required protein concentration using ice-cold Tris−HCl buffer. A typical 0.5 mL biotransformation incubation mixture in Tris−HCl (0.1 M, pH 7.4) consisted of 1 mg/mL pooled liver microsomal protein (n = 6), a nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system (NRS) consisting of 1 mM β-NADP$^+$, 10 mM glucose-6-phosphate (G6P), and 1 unit/mL glucose-6-phosphate dehydrogenase (G6PDH), 5 mM $MgCl_2$, and 20 μM α-acids, β-acids, or iso-α-acids as the substrate (this was a serial dilution in Tris−HCl of a 20 mM solution in methanol (MeOH) to the required concentrations). The final MeOH concentration did not exceed 1% (v/v) in the mixture. After preincubation of 5 min, the reaction was initiated by the addition of the NRS. Incubations were carried out at 37 °C for 120 min with continuous shaking in an Eppendorf Thermomixer (VWR International LLC, West Chester, PA). Control incubations were performed without the addition of NRS or microsomes to ensure that metabolite formation was dependent on the presence of microsomes and NADPH. Reactions were terminated by acidification (pH 2) with H_3PO_4 (0.1 M, 2 volumes) added to the incubation mixture (500 μL), followed by extraction with ethyl acetate (EtOAc; 4 volumes). The EtOAc phase was evaporated to dryness under nitrogen flow. The residues were reconstituted in 100 μL of MeOH and stored at −20 °C until analysis. Comparative studies with human liver microsomes (purchased from Celsis IVT, Chicago, IL) were also included, using the incubation protocol described above.

LC−MS Analysis. LC−MS analysis of samples from the different incubations was performed using an Agilent 1200 LC−MS system (SL) equipped with a dual ionization source (electrospray ionization/atomic pressure chemical ionization, ESI/APCI) and diode array detector (DAD) (Agilent, Waldbronnn, Germany). The Agilent Chemstation software package (revision B.02.01) was used to control the analytical system as well as for data acquisition and processing. As the stationary phase, a 3.5 μm Xbridge C-18 column (150 × 30 mm) (Waters, Zellik, Belgium) was used. The mobile phase consisted of 10 mM ammonium acetate, pH 9.75, + 20% MeOH (A) and MeOH (B). The flow rate was 0.5 mL/min, and the column temperature was maintained at 40 °C. The initial mobile phase, 27% B, was increased linearly to 60% B over 24 min, maintained for 5 min, further increased to 95% B in 5 min, and maintained for 4 min. Finally, the mobile phase was readjusted to 27% B in 1 min and re-equilibrated for 6 min prior to the next injection. UV/vis detection was performed at 270 nm for iso-α-acid and 314 nm for α- and β-acids. The MS parameters in the negative APCI mode were tuned to maximize formation of the deprotonated analyte. The interface settings were as follows: N_2 drying gas temperature, 250 °C; N_2 drying gas flow, 5 L/min; APCI vaporizer temperature, 150 °C; nebulizer pressure, 10^5 Pa; capillary voltage, 1000 V; corona current, 6 μA; charging voltage, 1000 V. In each analysis, qualitative

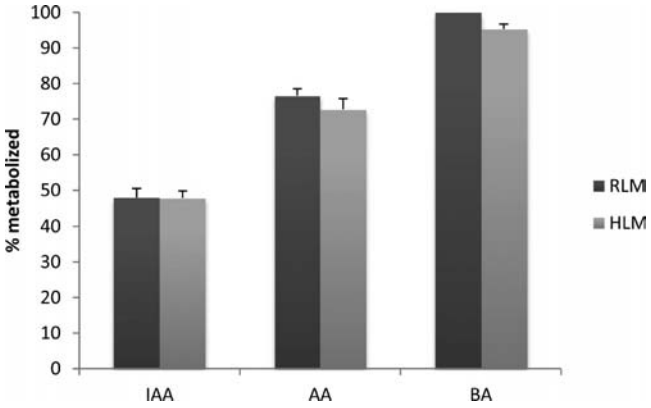

Figure 2. Percentage of metabolism of 20 μM hop α-acids, β-acids, and iso-α-acids following 2 h of incubation with rabbit liver microsomes (RLMs) and human liver microsomes (HLMs). Experiments were applied in triplicate. Amounts are presented as the percentage metabolized of the administered amount ± standard deviation. IAA = iso-α-acids, AA = α-acids, and BA = β-acids.

identification was performed in the negative ion scan mode (m/z 150–700) and quantitative data were obtained by reconstruction of the extracted ion chromatogram following measurement in the selected ion monitoring (SIM) mode. The $[M - H]^-$ m/z values, used for quantification of α-acids, β-acids, and iso-α-acids of the different compounds are presented in Table 1.[17−19]

HPLC−MS/MS Q-Trap Analysis. To identify hop acid metabolites, samples from microsomal incubations were analyzed using an Agilent 1200 series HPLC system consisting of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) connected to an API 4000 Q-TRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany) which was equipped with an ESI source and operated in negative ionization mode. Data processing and integration was performed by use of Analyst software, version 1.5 (AB Sciex Instruments, Darmstadt, Germany).

Chromatographic separation and determination of MS/MS parameter settings for qualitative analysis was performed by means of the multiple reaction monitoring (MRM) mode using the fragmentation parameters and retention times by means of cochromatography with authentic reference compounds, obtained using protocols reported by Intelmann and co-workers.[13,20,21]

RESULTS

In Vitro Metabolism of Hop-Derived Acids. In Figure 2, the percentages of compounds metabolized following incubation of 20 μM α-acids, β-acids, and iso-α-acids with liver microsomes isolated from rabbit liver and human microsomes are shown. The results show that after 120 min of incubation in rabbit liver microsomes, the total amount of the applied β-acids was metabolized. The percentage of α-acids metabolized was 77 ± 0.8%. Although the iso-α-acids proved to be more resistant to biotransformation, still a substantial amount (48 ± 3%) was metabolized. In comparative studies with human liver microsomes (Figure 2), similar values were obtained. The percentages

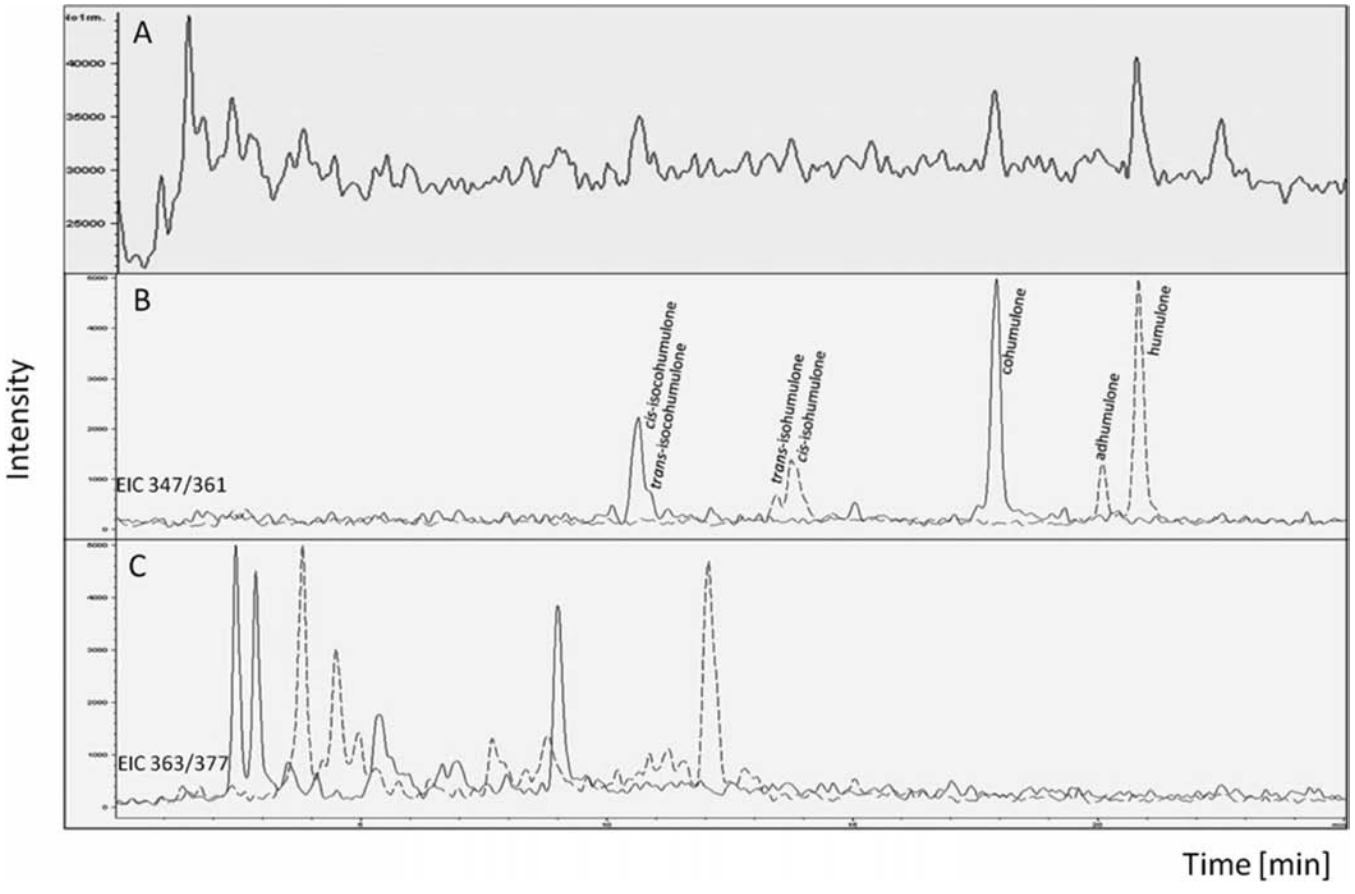

Figure 3. Representative HPLC−MS chromatogram following 2 h of incubation of α-acids (20 μM) in microsomes. In each chromatogram, the co-analogue and n- plus ad-analogues are respectively represented by a solid line and a dotted line. (A) TIC. (B) EIC of parent compounds with m/z 347 and 361. (C) EIC of mono-oxygenated metabolites with m/z 363 and 377. On the basis of the LC−MS/MS analysis, relevant peaks were assigned.

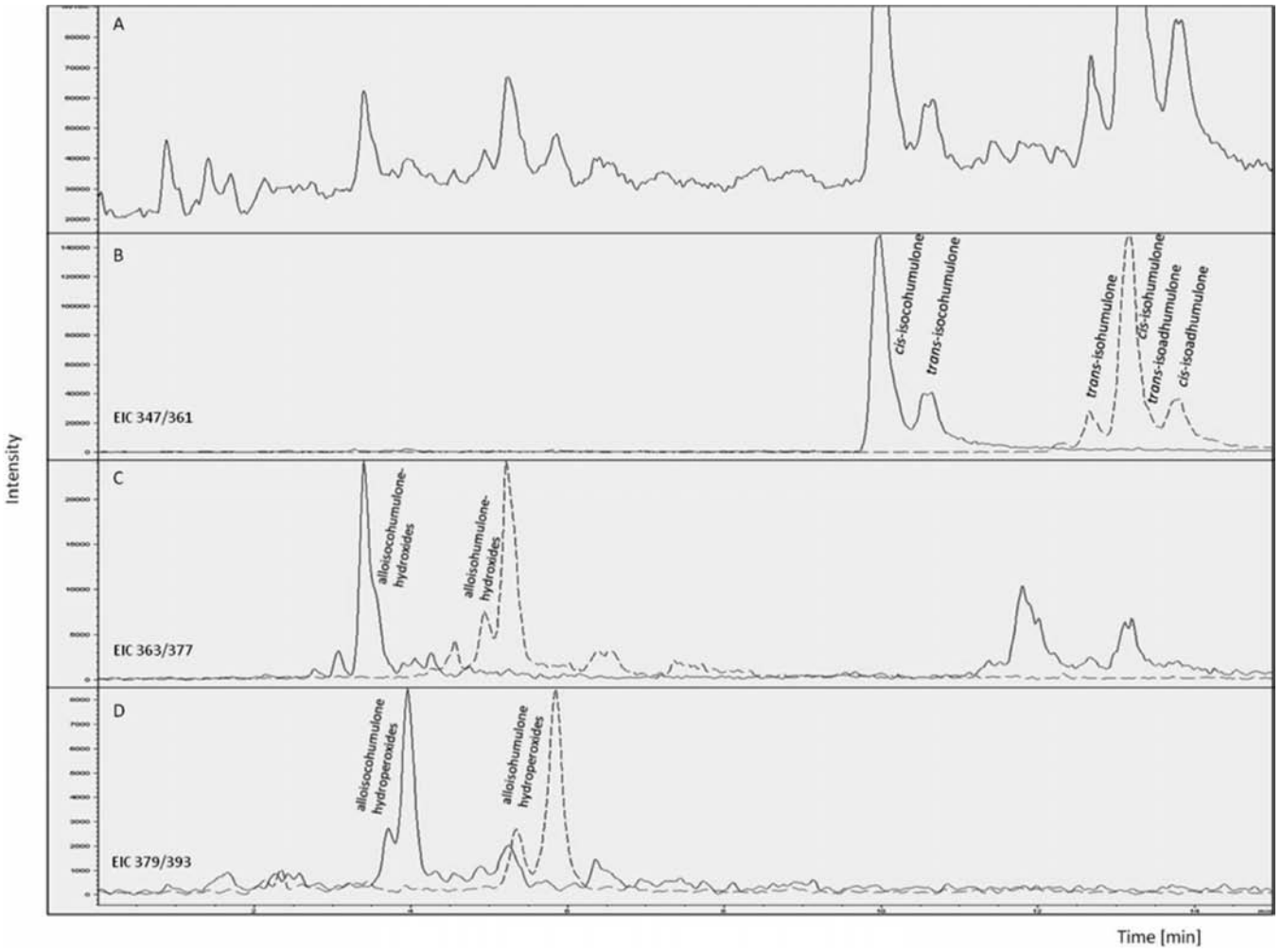

Figure 4. Representative HPLC−MS chromatogram following 2 h of incubation of iso-α-acids (20 μM) in microsomes. In each chromatogram, the co-analogue and *n*- plus ad-analogues are respectively represented by a solid line and a dotted line. (A) TIC. (B) EIC of parent compounds with m/z 347 and 361. (C) EIC of mono-oxygenated metabolites with m/z 363 and 377. (D) EIC of dioxygenated metabolites with m/z 379 and 393. On the basis of the LC−MS/MS analysis, relevant peaks were assigned.

of α-acids, β-acids, and iso-α-acids metabolized amounted to 73 ± 3%, 95 ± 1%, and 48 ± 2%, respectively.

Initially, all incubation mixtures were analyzed by LC−MS (APCI, negative mode), both quantitatively (see the results above) and qualitatively. Figures 3−5 illustrate a set of total ion chromatograms (TICs) and extracted ion chromatograms (EICs) of parent compounds and potential metabolites of respectively α-acids (Figure 3), β-acids (Figure 4), and iso-α-acids (Figure 5) following 120 min of incubation with rabbit liver microsomes.

As shown in the LC−MS analysis of the incubation mixture of α-acids (Figure 3), a quite complex TIC is obtained (Figure 3A). In the EIC of the parent compounds (Figure 3B) with pseudo molecular ions $[M - H]^-$ m/z 347 and 361, some proof of conversion of α-acids into iso-α-acids is observed. Study of the full-scan spectrum of potential metabolites of the α-acids revealed the main biotransformation products were pseudo molecular ions $[M - H]^-$ m/z 363 and 377, in which the molecular mass of the parent compound was increased by 16 Da, suggesting mono-oxygenation (epoxidation, hydroxylation, ...) as the most important metabolism product (Figure 3C).

Figure 4 represents an LC−MS chromatogram following microsomal incubation of iso-α-acids. In Figure 4C,D, the most important metabolites formed show an increase of the molecular mass by 16 or 32 Da compared to that of the parent compound, demonstrating the incorporation of one or two oxygen atoms to give the major microsomal biotransformation products of iso-α-acids.

The metabolism of β-acids by microsomal enzymes results in several distinct products (Figure 5). In the β-acid mixture used in this experiment, only a minor amount of *n*-lupulone plus adlupulone was present compared to colupulone. Therefore, only signals related to colupulone (parent compound with m/z 399) were used in Figure 5 to demonstrate the metabolism of all the β-acids. Among the several metabolites detected, formation of a product with pseudo molecular ion $[M - H]^-$ m/z 317 was observed (Figure 5C), which could correspond to the molar mass of hulupones (318 Da), a well-known oxidation product of β-acids. Also, in correspondence with the metabolism of α-acids and iso-α-acids, mono- and dioxygenated biotransformation products were determined, with detection of pseudo molecular ions $[M - H]^-$ m/z 415 and 431 (Figure 5D,E). Furthermore, a metabolite with pseudo molecular ion $[M - H]^-$ m/z 397 was determined.

Nevertheless, by use of LC−MS analysis, little structural information could be obtained for the biotransformation products. On the basis of the data of the metabolism pathway

dx.doi.org/10.1021/jf300018s | J. Agric. Food Chem. 2013, 61, 7916−7924

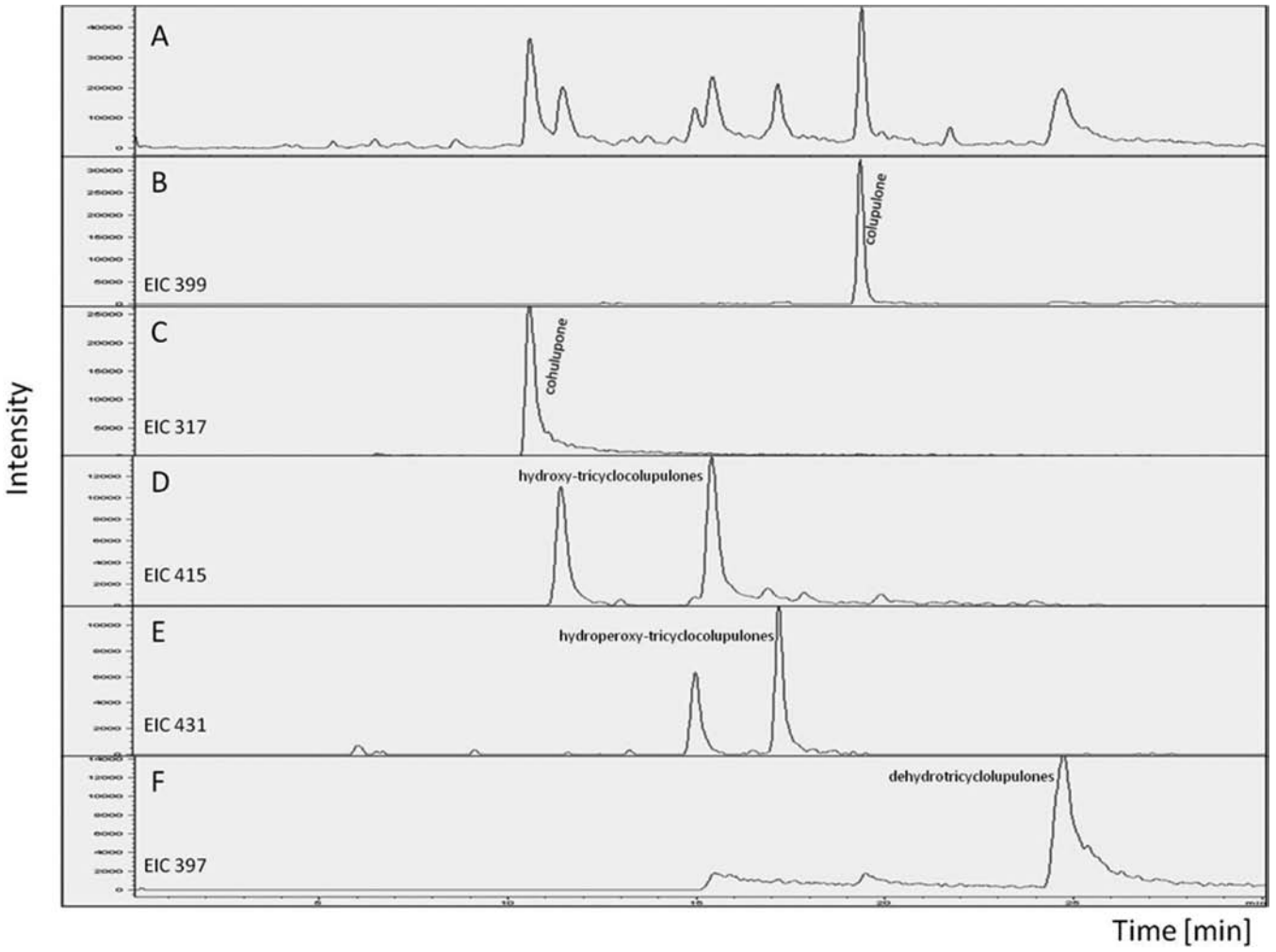

Figure 5. Representative HPLC−MS chromatogram following 2 h of incubation of β-acids (20 μM colupulone) in microsomes. (A) TIC. (B) EIC of parent compound with m/z 399. (C) EIC of the metabolite with m/z 317. (D) EIC of the mono-oxygenated metabolite with m/z 415. (E) EIC of the dioxygenated metabolite with m/z 431. (F) EIC of the metabolite with m/z 397. On the basis of the LC−MS/MS analysis, relevant peaks were assigned.

of other prenylated hop-derived constituents such as xanthohumol and 8-prenylnarigenin (8-PN), retention time, UV spectra, and molecular mass of the detected metabolites, it was only possible to make some suggestions on the molecular structure.

However, for the products described above, the molecular mass determined in the LC−MS analysis correlates to a great extent with the oxidative degradation products of hop bitter acids in aged beer and wort mixtures, recently described by Intellmann et al.[13−15,20] On the basis of this assumption, more structural and (semi)quantitative information on possible degradation products and/or metabolites was obtained by detailed LC−MS/MS analyses using recently optimized MRM methods for the analysis of hop bitter acids and these degradation products, which were recently isolated from oxidized beer and unequivocally assigned by NMR by Haseleu and co-workers.[20] Confirmation of the identified products was possible by cochromatography with authentic reference compounds. In this way, it was possible to demonstrate similarity in phase I metabolites of hop-derived bitter acids (α-acids, β-acids, and iso-α-acids) and products formed upon oxidative decomposition of these hop constituents during beer aging. The corresponding representative chromatograms of the metabolites identified by LC−MS/MS analysis operating in the MRM mode of products (co-congener) following microsomal incubation of α-acids, β-acids, and iso-α-acids are presented in Figure 6. Analogous results were also obtained for the ad- and *n*-congeners.

An overview of possible reaction routes for the biotransformation of hop-derived α-acids, β-acids, and iso-α-acids is shown in Figure 7.

Upon incubation with rabbit liver microsomes, α-acids (**1**−**3**) were mainly converted into *trans*-iso-α-acids (**7**−**9**) and *cis*-iso-α-acids (**10**−**12**), together with humulinones (**13**−**15**), and surprisingly hulupones (**16**−**18**). To the best of our knowledge, hulupones are a well-known oxidation product exclusively formed from β-acids. Besides the conversion of β-acids (**4**−**6**) into hulupones (**16**−**18**), biotransformation products of β-acids also comprised a series of epimeric pairs of tricyclic cyclization products, named dehydrotricyclolupulones (**43a**/**b**-**45a**/**b**), hydroxytricyclolupulones (**49a**/**b**-**51a**/**b**), and hydroperoxytricyclolupulones (**46a**/**b**-**48a**/**b**), resembling the products recently identified by Haseleu and co-workers.[13,22]

Microsomal metabolism of iso-α-acids (**7**−**9**, **10**−**12**) resulted mainly in the formation of *trans*-humulinic acids (**22**−**24**) and *cis*-humulinic acids (**31**−**33**), in addition to *trans* and *cis* pairs of hydroperoxy-alloiso-α-acids (**25**−**27**, **28**−**30**) and hydroxylated

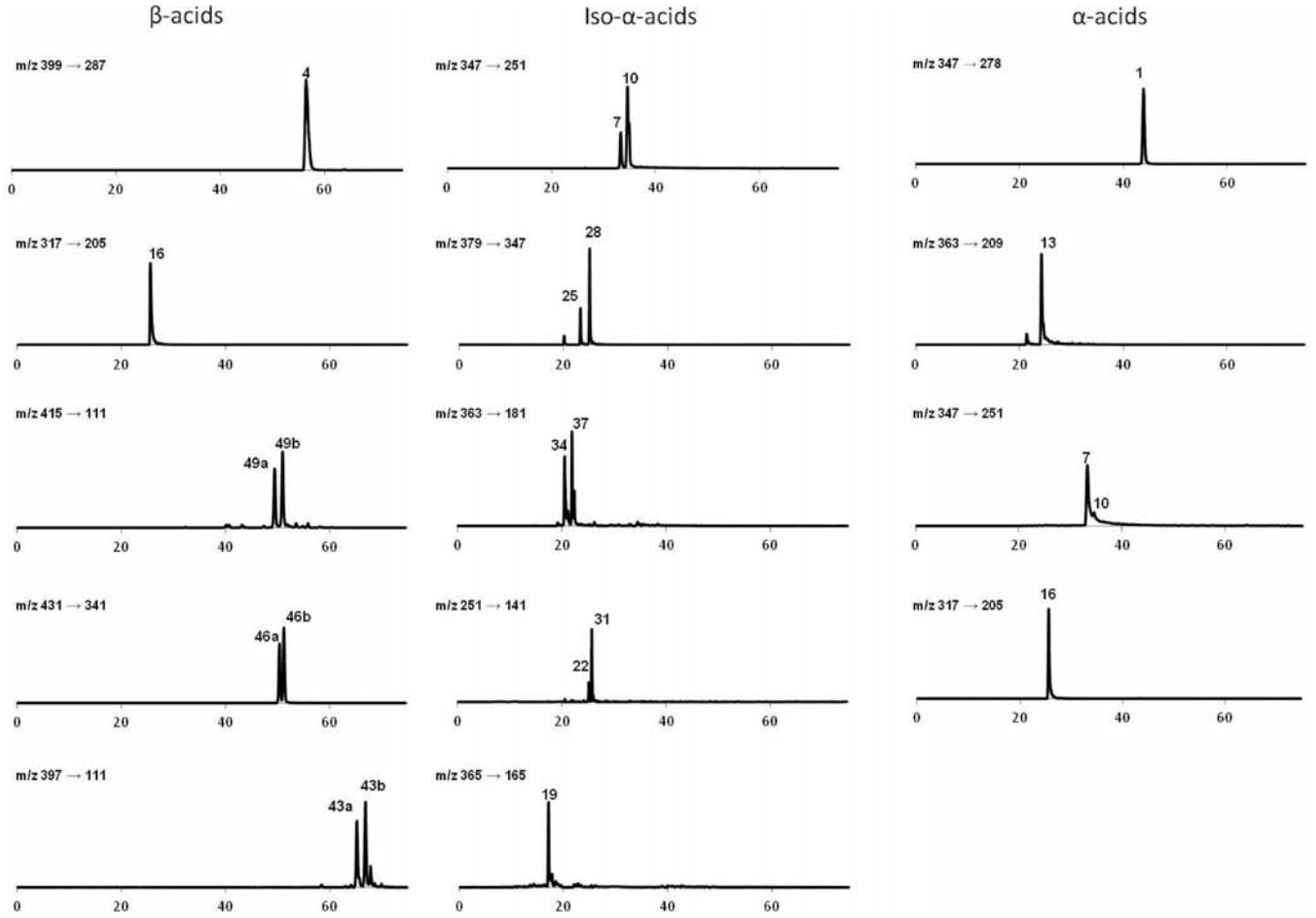

Figure 6. Representative LC−MS/MS chromatograms (MRM transitions) of parent compounds and identified microsomal metabolites of hop-derived α-acids, β-acids, and iso-α-acids. In each of the cases, the co-congener is shown as a matter of example.

alloiso-α-acids (**34**−**36**, **37**−**39**). Furthermore, the conversion of iso-α-acids into cyclic degradation substances, including tricyclohumols (**19**−**21**), was observed.

DISCUSSION

Studies on the ADME of hop-derived bitter acids, showing promising multipotent bioactive activities as, for example, anti-inflammatory agents against osteoporosis[23] or as anticarcinogenic medicines,[5] are of great importance. In this study, a first insight is gained into the possible metabolism pathway of hop-derived bitter acids. Several biotransformation products of α-acids, β-acids, and iso-α-acids were detected, both in microsomal mixtures and in urine samples. The discovery of an array of breakdown products of iso-α-acids confirmed previous findings of the authors: according to a preceding paper on the bioavailability of hop-derived iso-α-acids and reduced derivatives in rabbits, a lower bioavailability of iso-α-acids was obtained, since it was greatly influenced by breakdown by gut-related microorganisms or phase I/II metabolism in gastrointestinal or hepatic tissues.[11]

In earlier presented work, the in vitro transport of hop α-acids and β-acids across Caco-2 monolayers has been studied, showing that the permeability of β-acids was limited by phase II metabolism.[10] Currently, comparable studies on the bioavailability of α- and β-acids are being carried out by the authors.

Several previous investigations[13−15,22,24−38] have been reported on the structures of (oxidative and light-induced) degradation of hop-derived bitter acids as well as their underlying formation mechanisms. In particular, the 3-methyl-2-butenyl side chains of α-acids, β-acids, and iso-α-acids and the isohexenoyl side chain of iso-α-acids are very sensitive to oxidation, either at the double bounds or in the allylic positions. Most often, the native oxidized compounds are oxidized further or transformed by hydration and/or elimination. However, this was always focused on their decomposition in beer. Although physiologic biotransformation conditions (including metabolism) are unlike the beer deterioration situation, these studies provided preliminary indication of structures possible to expect following biotransformation. In these reports, most common products included humulinones, hulupones, alloisohumulones and *cis*/*trans*-humulinic acids.

Upon microsomal incubation, metabolism of iso-α-acids comprised predominantly conversion into humulinic acids and hydroxy/peroxyalloisohumulones, while α-acids showed biotransformation into humulinones, besides *cis*- and *trans*-iso-α-acids, and surprisingly hulupones, which are well-known β-acid oxidation products. Isomerization of humulone is the most important reaction in the brewing process.[39] Interestingly, this conversion could also be catalyzed by microsomal enzymes.

As the results showed, among the microsomal bioconversion of β-acids, the formation of two main types of dominating products could be identified, hulupones and tricyclo degradation products. This could indicate that liver oxidation is an important metabolism reaction of α-, β-, and iso-α-acids, catalyzed by cytochrome P450 enzymes. Moreover, this proneness to oxidative

dx.doi.org/10.1021/jf300018s | J. Agric. Food Chem. 2013, 61, 7916−7924

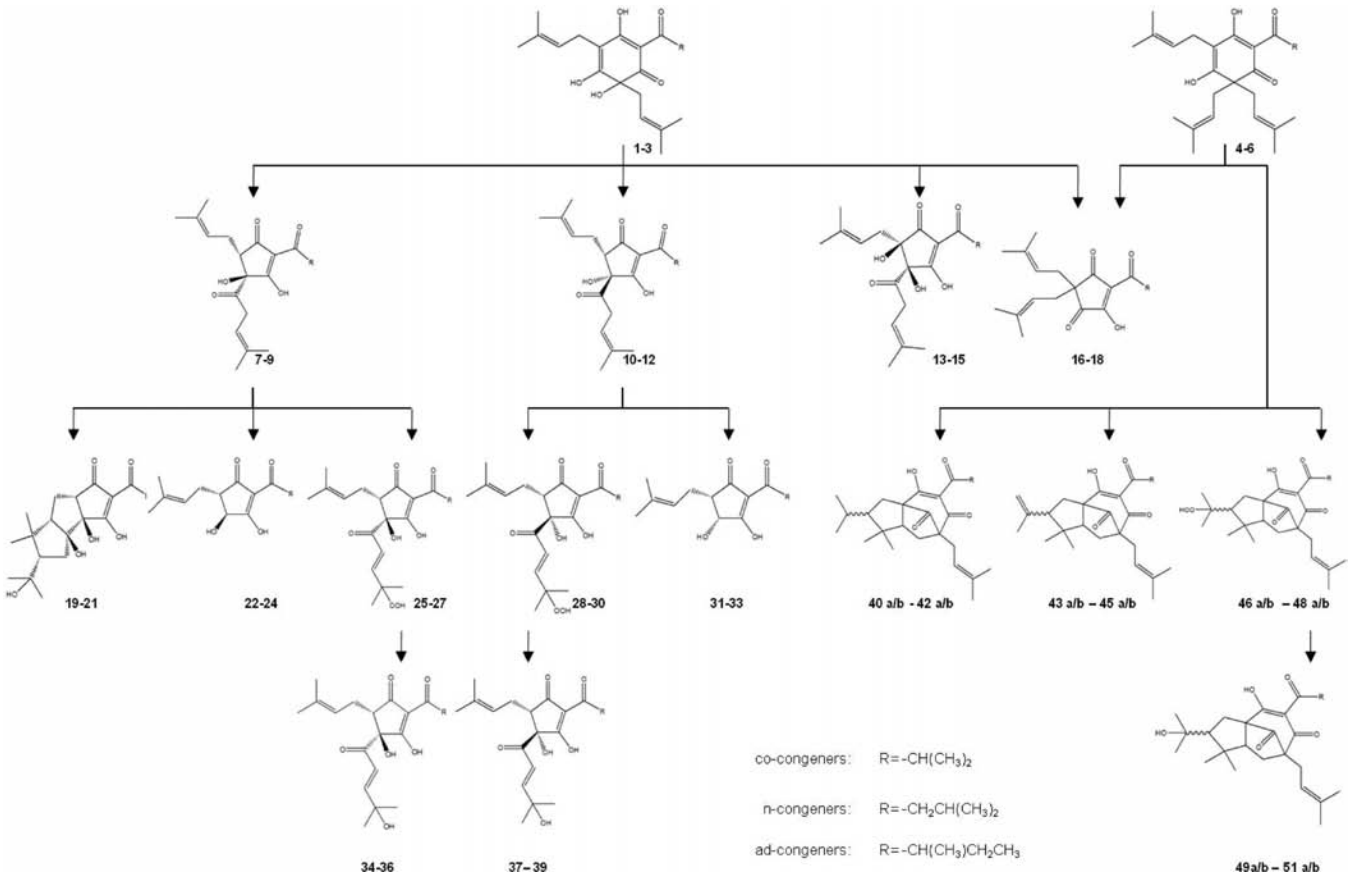

Figure 7. Reaction routes for the biotransformation of hop-derived α-acids (**1**−**3**), β-acids (**4**−**6**), *trans*-iso-α-acids (**7**−**9**), and *cis*-iso-α-acids (**10**−**12**) leading to the formation of humulinones (**13**−**15**), hulupones (**16**−**18**), *trans*-humulinic acids (**22**−**24**), *cis*-humulinic acids (**31**−**33**), epimeric pairs of tricyclic β-acid cyclization products (**40a,b**−**42a,b**, **43a,b**−**45a,b**, **46a,b**−**48a,b**, **49a,b**−**51a,b**), *trans*-alloisohumulone hydroperoxides (**25**−**27**), *cis*-alloisohumulone hydroperoxides (**28**−**30**), *trans*-alloisohumulone hydroxides (**34**−**36**), *cis*-alloisohumulone hydroxides (**37**−**39**), and tricyclohumols (**19**−**21**).

microsomal breakdown could suggest a significant first-pass effect upon oral ingestion of such compounds, resulting in fast clearance following absorption. Generally, CYP metabolism pathways include iron−oxo species which abstract a hydrogen atom from the alkyl group of a substrate to give a radical that subsequently displaces the hydroxyl group from iron in a homolytic substitution reaction (hydrogen abstraction−oxygen rebound), utilizing two electrons that are provided by NAD(P)H via a reductase protein.[40]

The transformation of iso-α-acids and β-acids in respectively hydroxyl- and hydroperoxyalloisohumulones and tricyclo products, including nortricyclo-, dehydrotricyclo-, and tricyclolupulones, could also include a similar mechanism. Recently, these same oxidation products of iso-α-acids and β-acids were identified upon radical-assisted oxidative breakdown in beer by Intelmann and Haseleu.[13−15,22] Despite the different reaction conditions (beer matrix versus microsomal enzymes), some interesting similarities could be drawn. In the beer aging studies, the authors proposed a lipid peroxidation mechanism (like oxidation of unsaturated fatty acids) which is based on the same principles occurring in cytochrome P450 enzymatic pathways: first a hydrogen atom is abstracted, leading to a resonance-stabilized radical in the isohexenoyl side chain (iso-α-acids), followed by addition of oxygen, leading to the corresponding hydroperoxy- and hydroxylalloisohumulones. In the case of the β-acids, hydrogen abstraction is followed by cyclization steps and oxygenation, leading by different reaction routes to products such as nortricyclo-, dehydrotricyclo-, and tricyclolupulones.

Apart from the hydroperoxy- and hydroxyalloisohumulones, formation of *cis*/*trans*-humulinic acids was observed. These products are a result of cleavage of the (un)modified hexenoyl side chain, directly from iso-α-acids, or from another intermediate (alloiso-α-acids, hydroxy/hydroperoxyalloiso-α-acids). Their detailed formation mechanism is still unclear, but might also be catalyzed by CYP enzymes.

Furthermore, exclusively in urine, the presence of tri- and tetracyclic metabolites, including tricyclohumols, tetracyclohumols, and tricyclohumenes, was observed, like the decomposition products identified by Intelmann et al. upon acidic degradation of *trans*-iso-α-acids in aged beer.[41] These products are formed by proton-catalyzed intramolecular nucleophilic cyclization reaction and were absent in microsomal incubation mixtures. This suggests a nonliver enzymatic mechanism, but presumably an acid-catalyzed degradation in the stomach and/or intestinal zones with lower pH, since Intelmann and co-workers showed this reaction was strongly pH-dependent. At pH 1.0, the most rapid degradation was observed, whereas none of these compounds were formed at pH 6.0.[41] Moreover, just *trans*-iso-α-acids were affected, since only for these stereomers the interacting carbon atoms possessed the right steric geometry that enabled an overlapping of the corresponding π-orbitals for bonding.

Using the MRM method in the LC−MS/MS analysis, it was also possible to detect transitions representative of substances with hydroxylated positions in the prenyl side chain of α- and β-acids, though exact assignment of the molecular structure requires further experiments, including NMR spectroscopy confirmation of isolated compounds. Nevertheless, the occurrence of products with hydroxylated 3-methyl-2-butyenyl (or prenyl) side chains of α-acids and β-acids is in line with the products formed following CYP metabolism of another hop constituent, 8-prenylnaringenin (8-PN). In a study conducted by Nikolic et al., the most abundant pathway of liver microsomal metabolites of 8-PN was hydroxylation of one of the terminal methyls of the prenyl group. Hydrogen abstraction at a terminal methyl group is favorable due to the formation of a stable allyl radical, and these groups are readily accessible for enzymatic attack.[42] Moreover, in two recent studies, Negri et al. and Lupinacci and co-workers have reported an oxidized α-acid derivate, formed upon the oxidation of the 3-methyl-2-butenyl side chains in humulinone (oxidation product of α-acids), followed by cyclization, resulting in an oxidized α-acid derivative in which two oxygen atoms were incorporated.[43,44] These examples indicate the susceptibility of the 3-methyl-2-butenyl side chains of hop-derived bitter acids for oxidation. The evaluation of formation of similar products among metabolites of α- and β-acids should be further investigated.

In conclusion, HPLC−MS/MS operating in the MRM mode allowed for the first time detection and quantification of hop-derived biotransformation products α-acids, β-acids, and iso-α-acids in microsomal incubation samples. Interestingly, the results of this study demonstrated similarity in metabolites and products formed upon oxidative decomposition of these hop constituents during beer aging. The results from this study show first insight into the metabolites of hop-derived bitter acids.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ko.cattoor@UGent.be.

Funding

K.C. is a recipient of an academic scholarship, "malting and brewing", provided by the InBev-Baillet Latour Fund, Leuven, Belgium.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefuly acknowledge the technical assitance of Daniel Tensy.

REFERENCES

(1) Zanoli, P.; Zavatti, M. Pharmacognostic and pharmacological profile of *Humulus lupulus* L. *J. Ethnopharmacol.* **2008**, *116* (3), 383−396.

(2) Van Cleemput, M.; Cattoor, K.; De Bosscher, K.; Haegeman, G.; De Keukeleire, D.; Heyerick, A. Hop (*Humulus lupulus*)-derived bitter acids as multipotent bioactive compounds. *J. Nat. Prod.* **2009**, *72* (6), 1220−1230.

(3) Konda, V. R.; Desai, A.; Darland, G.; Bland, J. S.; Tripp, M. L. *Rho* iso-alpha acids from hops inhibit the GSK-3/NF-κB pathway and reduce inflammatory markers associated with bone and cartilage degradation. *J. Inflammation* **2009**, *6*, 26.

(4) Hall, A. J.; Babish, J. G.; Darland, G. K.; Carroll, B. J.; Konda, V. R.; Lerman, R. H.; Bland, J. S.; Tripp, M. L. Safety, efficacy and anti-inflammatory activity of rho *iso*-alpha-acids from hops. *Phytochemistry* **2008**, *69* (7), 1534−1547.

(5) Bohr, G.; Klimo, K.; Zapp, J.; Becker, H.; Gerhauser, C. Cancer chemopreventive potential of humulones and isohumulones (hops α- and iso-α-acids): induction of NAD(P)H:quinone reductase as a novel mechanism. *Nat. Prod. Commun.* **2008**, *3* (12), 1971−1976.

(6) Shimamura, M.; Hazato, T.; Ashino, H.; Yamamoto, Y.; Iwasaki, E.; Tobe, H.; Yamamoto, K.; Yamamoto, S. Inhibition of angiogenesis by humulone, a bitter acid from beer hop. *Biochem. Biophys. Res. Commun.* **2001**, *289* (1), 220−224.

(7) Obara, K.; Mizutani, M.; Hitomi, Y.; Yajima, H.; Kondo, K. Isohumulones, the bitter component of beer, improve hyperglycemia and decrease body fat in Japanese subjects with prediabetes. *Clin. Nutr.* **2009**, *28* (3), 278−284.

(8) Miura, Y.; Hosono, M.; Oyamada, C.; Odai, H.; Oikawa, S.; Kondo, K. Dietary isohumulones, the bitter components of beer, raise plasma HDL-cholesterol levels and reduce liver cholesterol and triacylglycerol contents similar to PPARalpha activations in C57BL/6 mice. *Br. J. Nutr.* **2005**, *93* (4), 559−567.

(9) Yajima, H.; Ikeshima, E.; Shiraki, M.; Kanaya, T.; Fujiwara, D.; Odai, H.; Tsuboyama-Kasaoka, N.; Ezaki, O.; Oikawa, S.; Kondo, K. Isohumulones, bitter acids derived from hops, activate both peroxisome proliferator-activated receptor alpha and gamma and reduce insulin resistance. *J. Biol. Chem.* **2004**, *279* (32), 33456−33462.

(10) Cattoor, K.; Bracke, M.; Deforce, D.; De Keukeleire, D.; Heyerick, A. In vitro transport of hop bitter acids across Caco-2 monolayers. *J. Agric. Food Chem.* **2010**, *58* (7), 4132−4140.

(11) Cattoor, K.; Remon, J. P.; Boussery, K.; Van Bocxlaer, J.; Bracke, M.; De Keukeleire, D.; Deforce, D.; Heyerick, A. Bioavailability of hop-derived iso-α-acids and reduced derivatives. *Food Funct.* **2011**, *2* (7), 412−422.

(12) Aniol, M.; Bartmanska, A.; Huszcza, E.; Maczka, W.; Zolnierczyk, A. K. Degradation of hop bitter acids by plant enzymes. *Przem. Chem.* **2009**, *88* (5), 392−395.

(13) Haseleu, G.; Intelmann, D.; Hofmann, T. Identification and RP-HPLC−ESI-MS/MS quantitation of bitter-tasting β-acid transformation products in beer. *J. Agric. Food Chem.* **2009**, *57* (16), 7480−7489.

(14) Intelmann, D.; Kummerlowe, G.; Haseleu, G.; Desmer, N.; Schulze, K.; Frohlich, R.; Frank, O.; Luy, B.; Hofmann, T. Structures of storage-induced transformation products of the beer's bitter principles, revealed by sophisticated NMR spectroscopic and LC-MS techniques. *Chem.—Eur. J.* **2009**, *15* (47), 13047−13058.

(15) Intelmann, D.; Hofmann, T. On the autoxidation of bitter-tasting iso-α-acids in beer. *J. Agric. Food Chem.* **2010**, *58* (8), 5059−5067.

(16) Bradford, M. M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* **1976**, *72* (1−2), 248−254.

(17) Vanhoenacker, G.; De Keukeleire, D.; Sandra, P. Analysis of iso-alpha-acids and reduced iso-alpha-acids in beer by direct injection and liquid chromatography with ultraviolet absorbance detection or with mass spectrometry. *J. Chromatogr.* **2004**, *1035* (1), 53−61.

(18) Ting, P. L.; Kay, S.; Ryder, D. Separation and identification of stereoisomers of isomerized alpha-acid derivatives by HPLC/DAD and electrospray HPLC/MS. *J. Am. Soc. Brew. Chem.* **2007**, *65* (1), 9−14.

(19) Hofta, P.; Dostalek, P.; Sykora, D. Liquid chromatography-diode array and electrospray high-accuracy mass spectrometry of iso-alpha-acids in DCHA-Iso standard and beer. *J. Inst. Brew.* **2007**, *113* (1), 48−54.

(20) Haseleu, G.; Lagemann, A.; Stephan, A.; Intelmann, D.; Dunkel, A.; Hofmann, T. Quantitative Sensomics Profiling of hop-derived bitter compounds throughout a full-scale beer manufacturing process. *J. Agric. Food Chem.* **2010**, *58* (13), 7930−7939.

(21) Intelmann, D.; Haseleu, G.; Hofmann, T. LC−MS/MS quantitation of hop-derived bitter compounds in beer using the ECHO technique. *J. Agric. Food Chem.* **2009**, *57* (4), 1172−1182.

(22) Haseleu, G.; Intelmann, D.; Hofmann, T. Structure determination and sensory evaluation of novel bitter compounds formed from

beta-acids of hop (*Humulus lupulus* L.) upon wort boiling. *Food Chem.* **2009**, *116* (1), 71−81.
(23) Holick, M. F.; Lamb, J. J.; Lerman, R. H.; Konda, V. R.; Darland, G.; Minich, D. M.; Desai, A.; Chen, T. C.; Austin, M.; Kornberg, J.; Chang, J. L.; Hsi, A.; Bland, J. S.; Tripp, M. L. Hop *rho* iso-alpha acids, berberine, vitamin D_3 and vitamin K_1 favorably impact biomarkers of bone turnover in postmenopausal women in a 14-week trial. *J. Bone Miner. Metab.* **2010**, *28* (3), 342−350.
(24) De Cooman, L.; Aerts, G.; Witters, A.; De Ridder, M.; Boeykens, A.; Goiris, K.; De Keukeleire, D. Comparative study of the stability of iso-α-acids, dihydroiso-α-acids and tetrahydroiso-α-acids during beer ageing. *Cerevisia* **2001**, *26*, 3.
(25) Kokubo, E.; Kowaka, M.; Kuroiwa, Y. New bitter substances—lupoxes a, lupoxes b and lupdoxes a. *J. Am. Soc. Brew. Chem.* **1971**, 265−279.
(26) Kowaka, M.; Kokubo, E.; Kuroiwa, Y. New bitter substances of beer. Lupoxes c and lupdoxes b. *J. Am. Soc. Brew. Chem.* **1972**, 42−46.
(27) Kowaka, M.; Kokubo, E.; Kuroiwa, Y. New bitter substances of beer. Lupdeps and lupdols. *J. Am. Soc. Brew. Chem.* **1973**, *7*, 66−71.
(28) Verzele, M.; Van de Velde, N.; Dewaele, C. High performance liquid chromatography analysis of colupulone oxidation products in hops and in beer. *J. Am. Soc. Brew. Chem.* **1983**, *41*, 57−60.
(29) Masuda, S.; Kikuchi, K.; Harayama, K.; Sakai, K.; Ikeda, M. Determination of lightstruck character in beer by gas chromatography-mass spectroscopy. *J. Am. Soc. Brew. Chem.* **2000**, *58* (4), 152−154.
(30) Burns, C. S.; Heyerick, A.; De Keukeleire, D.; Forbes, M. D. E. Mechanism for formation of the lightstruck flavor in beer revealed by time-resolved electron paramagnetic resonance. *Chem.—Eur. J.* **2001**, *7* (21), 4553−4561.
(31) Vanderhaegen, B.; Neven, H.; Verachtert, H.; Derdelinckx, G. The chemistry of beer aging—a critical review. *Food Chem.* **2006**, *95* (3), 357−381.
(32) Huvaere, K.; Andersen, M. L.; Olsen, K.; Skibsted, L. H.; Heyerick, A.; De Keukeleire, D. Radicaloid-type oxidative decomposition of beer bittering agents revealed. *Chem.—Eur. J.* **2003**, *9* (19), 4693−4699.
(33) Clarke, B. J.; Hilldebrand, R. P. The isomerization of humulone III. The degradation of isohumulone. *J. Inst. Brew.* **1967**, *73* (3), 282−293.
(34) Cann, M. R.; Davis, A. M.; Shannon, P. V. R. New rearrangement and oxidation products from humulone. *J. Chem. Soc., Perkin Trans. 1* **1982**, No. 2, 375−383.
(35) De Keukeleire, D. Oxidation products of the hop α-acids and the iso-α-acids. *Cerevisia* **1981**, 73−80.
(36) Vanhoey, M.; Vandewal., M.; Verzele, M. Oxidation products of isohumulones. *Bull. Soc. Chim. Belg.* **1970**, *79* (9−10), 499−509.
(37) Hashimoto, N.; Eshima, T. Oxidative degradation of isohumulones in relation to flavor stability of beer. *J. Inst. Brew.* **1979**, *85* (3), 136−140.
(38) Huvaere, K.; Andersen, M. L.; Skibsted, L. H.; Heyerick, A.; De Keukeleire, D. Photooxidative degradation of beer bittering principles: a key step on the route to lightstruck flavor formation in beer. *J. Agric. Food Chem.* **2005**, *53* (5), 1489−1494.
(39) Verzele, M.; Vanboven, M. Isomerization mechanism of humulone. *Bull. Soc. Chim. Belg.* **1971**, *80* (11−1), 677.
(40) Meunier, B.; de Visser, S. P.; Shaik, S. Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes. *Chem. Rev.* **2004**, *104* (9), 3947−3980.
(41) Intelmann, D.; Demmer, O.; Desmer, N.; Hofmann, T. O-18 stable isotope labeling, quantitative model experiments, and molecular dynamics simulation studies on the trans-specific degradation of the bitter tasting iso-α-acids of beer. *J. Agric. Food Chem.* **2009**, *57* (22), 11014−11023.
(42) Nikolic, D.; Li, Y. M.; Chadwick, L. R.; Grubjesic, S.; Schwab, P.; Metz, P.; van Breemen, R. B. Metabolism of 8-prenylnaringenin, a potent phytoestrogen from hops (*Humulus lupulus*), by human liver microsomes. *Drug Metab. Dispos.* **2004**, *32* (2), 272−279.
(43) Negri, G.; di Santi, D.; Tabach, R. Bitter acids from hydroethanolic extracts of *Humulus lupulus* L., Cannabaceae, used as anxiolytic. *Braz. J. Pharmacogn.* **2010**, *20* (6), 850−859.
(44) Lupinacci, E.; Meijerink, J.; Vincken, J. P.; Gabriele, B.; Gruppen, H.; Witkamp, R. F. Xanthohumol from hop (*Humulus lupulus* L.) is an efficient inhibitor of monocyte chemoattractant protein-1 and tumor necrosis factor-α release in LPS-stimulated RAW 264.7 mouse macrophages and U937 muman monocytes. *J. Agric. Food Chem.* **2009**, *57* (16), 7274−7281.