Faculty of Pharmaceutical Sciences

Bioavailability of Hop-derived Bitter Acids: Key Factors for the Health-Beneficial Properties of Beer

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Table of Contents

List of abbreviations 9
I. Literature Review 19
  1.1 History of hop and beer 19
  1.2 Phytogeography of hop 22
  1.3 Botanical description of hop 23
  1.4 Chemistry of hop 26
    1.4.1 Hop essential oil 28
    1.4.2 Hop polyphenols 29
    1.4.3. Hop bitter acids 29
  1.5 Purification and Analysis of Hop Acids 40
    1.5.1 Considerations regarding complexity of hop acids mixtures 42
    1.5.2 HPLC analysis of hop-derived bitter acids mixtures 45
    1.5.3 LC-MS analysis of hop-derived bitter acids mixtures 46
  1.6 Biological activity of hop-derived bitter acids 48
    1.6.1 Anticancer Potential of Hop Bitter Acids 49
    1.6.2 Hop Bitter Acids in Inflammatory Disorders 57
    1.6.3 Hop Bitter Acids Improve Markers for Metabolic Syndrome 60
    1.6.4 Role of Hop Bitter Acids in Osteoporosis 63
    1.6.5 Effects of Hop Bitter Acids on the Central Nervous System 64
    1.6.6 Bactericidal Activity of Hop Bitter Acids 66
    1.6.7 Hop Bitter Acids as Potent Antioxidants 69
    1.6.8 Effects of Hop Bitter Acids on the Gastrointestinal Tract 71
    1.6.9 Conclusions 72
  1.7 Toxicology of Hop Bitter Acids 72
II. State-of-the-art and Objectives 75
  2.1 Background on bioavailability of hop-derived bitter acids 75
  2.2. Objectives 78
III. Transport of hop-derived bitter acids across Caco-2 cell monolayers 81
  3.1 Introduction 82
  3.2 The Caco-2 cell monolayer system 83

3.2.1 Expression of phase-I enzymes 84
3.2.2 Expression of phase-II enzymes 85
3.2.3 Expression of Efflux transporters.. 85
3.2.4 Transport mechanisms across Caco-2 monolayers...................................... 87
3.3 Materials... 89
3.4 Methods 90
3.4 Results & Discussion.. 100
3.4.1 Caco-2 studies with hop α-acids and β-acids 104
3.4.2 Caco-2 cell experiments of iso-α-acids and reduced derivatives 119
IV. Bioavailability of hop-derived bitter acids using the rabbits as an *in vivo* model 127
4.1 *In vivo* studies on the bioavailability 127
4.1.1. Introduction 128
4.1.2 Materials and methods... 129
4.1.3 Results... 137
4.1.4 Discussion.. 147
4.2 Metabolism of hop-derived bitter acids 151
4.2.1 Introduction 151
4.2.2 Materials and methods... 152
4.2.3 Results... 157
4.2.4 Discussion.. 166
V. General Conclusion and Future Perspectives 171
Summary 175
Samenvatting... 177
Curriculum Vitae 179
Bibliography 183

List of abbreviations

HBA	hop bitter acids
AA	α-acids
BA	β-acids
IAA	iso-α-acids
DHIAA	dihydro-iso-α-acids
THIAA	tetrahydro-iso-α-acids
HHIAA	hexahydro-iso-α-acids
RF	riboflavin
MBT	3-methylbut-2-ene-1-thiol
$NaBH_4$	sodium boron hydride
H_2/Pd-C	hydrogenation/ catalyzed by Pd on carbon substrate
e.g.	exempli gratia ("for example").
UV	ultraviolet
^{1}H-NMR	proton nuclear magnetic resonance
TLC	thin layer chromatography
GC	gas chromatography
HPLC	high performance liquid chromatography
Nm	nanometer
EBC	European Brewing Convention
ASBC	American Society of Brewing Chemists
EDTA	ethylenediamine tetraacetate
MeOH	methanol
MeCN	acetonitril
ESI	electrospray ionisation

LC-MS	liquid chromatography-mass spectrometry
ESCOP	European Scientific Cooperative on Phytotherapy
ADME	Absorption – distribution – metabolism - elimination
HL-60 cells	Human promyelocytic leukemia cells
U937 cells	Human leukemic monocyte lymphoma cell line; macrophage-like cell line
IC_{50}	concentration of an inhibitor where the response (or binding) is reduced by half
Bcl-2	B-cell lymphoma 2; family of proteins regulate apoptosis by controlling mitochondrial permeability
Bax, Bad, Bid	pro-apoptotic Bcl-2-family proteins
Fas	the death receptor on the surface of cells that leads to apoptosis
FasL	ligand of Fas; transmembrane protein that belongs to the tumor necrosis factor (TNF) family.
MDAMB- 231	type of human breast carcinoma cell line
SK-MES	type of human lung carcinoma cell line
DNA	deoxyribonucleic acid
cytochrome c	small heme protein associated with inner membrane of the mitochondrion; part of the electron transport chain in mitochondria
GADD153	growth arrest of DNA damage-inducible gene 153
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor related apoptosis inducing ligand
SW480 cells	type of human colon adenocarcinoma cell line
SW620	type of human colon adenocarcinoma cell line
p53	type of tumor suppressor protein which regulates the cell cycle and is involved in preventing cancer.
Mcl-1	induced myeloid leukemia cell differentiation protein. Bcl-2 family protein, regulating cell apoptosis.

MAPKs	mitogen-activated protein kinases
JNK	Jun *N*-terminal kinase
ERK	extracellular signal-regulated kinase
p38	class of mitogen-activated protein kinases that are responsive to stress stimuli and are involved in cell differentiation, apoptosis and autophagy.
PFT-α	pifithrin-α. p53 inhibitor
RNA	ribonucleic acid
G0	resting phase of cell-division cycle
G1	cell increase growth phase of cell-division cycle
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TNF-α	tumor necrosis factor alpha
K562	human immortalised myelogenous leukemia line
HEL cells	human erythroleukemia cell line
KU812 cells	human leukemic cell line
THP-1 cells	human acute monocytic leukemia cell line
ML-1 cells	human myeloblastic leukemia cell line
CAMs	chick embryo chorioallantoic membranes
ED_{50}	the median dose of a drug that produces a quantal effect (all or nothing) in 50% of the population that takes it.
KOP2.16 cells	murine endothelial cell line
bFGF	basic fibroblast growth factor
VEGF	vascular endothelial growth factor
Co26 cells	murine transplantable colon cancer model
HUVEC	Human umbilical vein endothelial cells
NO	nitric oxide
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
IFN-γ	interferon-γ cytokine, critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor

	control. important activator of macrophages.
mRNA	messenger ribonucleic acid
COX-2	cyclooxygenase-2 or prostaglandin-endoperoxide synthase 2 enzyme that converts arachidonic acid to prostaglandin endoperoxide H2.
PGE_2	prostaglandin E2
RAW264.7	type of mouse macrophage cell line
IKKβ	subunit of the IκB kinase (IKK) enzyme complex that is involved in propagation of the cellular response to inflammation
NF-κB	the transcription factor nuclear factor-kappaB (NF-κB). Plays critical roles in inflammation, control of cell death pathways and cell proliferation.
ERK1/2	extracellular signal-regulated protein kinases 1 and 2. Members of the mitogen-activated protein kinase super family that can mediate cell proliferation and apoptosis
CRE	cAMP response element for CREB
cAMP	Cyclic adenosine monophosphate. Is a second messenger used for intracellular signal transduction
CREB	cAMP response element-binding protein. Is a cellular transcription factor. It binds to certain DNA sequences called CRE, thereby increasing or decreasing the transcription of the downstream genes
GSK3	glycogen synthase kinase 3 (GSK-3). Is a serine/threonine protein kinase that mediates the addition of phosphate molecules onto serine and threonine amino acid residues.
SB216763	synonym: 3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione. Is a potent and selective ATP-competitive inhibitor of the GSK-3 α and β isozymes.
RANKL	receptor activator of nuclear factor kappa-B ligand
TRAP	tartrate-resistant acid phosphatase

IL-1β	interleukin-1 beta (IL-1β). Member of the interleukin 1 cytokine family. Is a cytokine protein. Important mediator of the inflammatory response, and involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.
MMP-13	matrix metallopeptidase 13. Member of the matrix metalloproteinase (MMP) family which are involved in the breakdown of extracellular matrix in normal physiological processes.
SW1353 cells	human bone chondrosarcoma, fibroblast-like cell line
IκB	inhibitory kappaB binding proteins of mammalian NF-κB transcription factor
p65	nuclear factor NF-kappa-B p65 subunit. Part of the p50/p65 heterodimer, which is the most abundant form of NF-κB
PI3K	phosphatidylinositide 3-kinase. Member of a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking, which in turn are involved in cancer.
Akt	protein kinase B. Is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration.
CYP-450	cytochrome P450 superfamily. Are the major enzymes involved in drug metabolism and bioactivation.
ACF	aberrant cryptic foci. Clusters of abnormal tube-like glands in the lining of the colon and rectum.
DMBA	7,12-dimethylbenz[α]anthracene
ID_{50}	the median dose that causes 50% inhibition
TxA_2	thromboxane A2
COX-1	cyclooxygenase-1. Constitutive isozyme of the cyclooxygenase

	enzyme in the biosynthetic pathway to prostaglandins from arachidonic acid
MC3T3-E1 cells	osteoblast precursor cell line
PBMC	human peripheral blood mononuclear cells
FCS	fecal calf serum
ip	intraperitoneal
PBS	phosphate-buffered saline
DMSO	dimethylsulfoxide
WOMAC	Western Ontario and McMaster Universities Arthritis Index. Proprietary set of standardized questionnaires used by health professionals to evaluate the condition of patients with osteoarthritis of the knee and hip, including pain, stiffness, and physical functioning of the joints. The WOMAC has also been used to assess back pain, rheumatoid arthritis and fibromyalgia.
VAS	visual analog ue scale. Psychometric response scale which can be used in questionnaires.
PPARs	peroxisome proliferator-activated receptors. Group of nuclear receptor proteins that function as transcription factors regulating the expression of genes which play essential roles in the regulation of cellular differentiation, development, and metabolism, and tumorigenesis of higher organisms.
PPAR-α	α-subtype of PPAR
PPAR-γ	γ-subtype of PPAR
HepG2 cells	human liver carcinoma cell line. *In vitro* model for polarized human hepatocytes
CV-1 cells	normal African green monkey kidney fibroblast cells
GAL-4	yeast transcription factor. Archetypal transcription factor isolated as an activator of the genes for galactose metabolism

	in *Saccharomyces cerevisiae*
PPRE	PPAR response element
COS1L2A cells	transformed (Simian Virus 40) African green monkey kidney fibroblast cells; derived from CV-1 cells
KK-A^y mice	mice with non-insulin dependent diabetes. Widely used as an experimental model for type 2 diabetes mellitus.
ACO	acyl-coenzyme A oxidase. Enzyme participating in fatty acid metabolism, polyunsaturated fatty acid biosynthesis, and PPAR signaling pathway.
FAT	fatty acid translocase. Long chain fatty acid transporter present at the plasma membrane
ADRP	adipose differentiation related protein
LPL	lipoprotein lipase
C57BL/6N mice	diet-induced obese diabetic mice
HDL	high density lipoprotein. Enables lipids like cholesterol and triglycerides to be transported within the water-based bloodstream
ACS	acyl-Coenzyme A synthetase. Involved in metabolism of carbon sugars, catalyzing the formation of acetyl-coenzyme A
FATP	fatty acid transport protein
hemoglobin A1c	glycosylated hemoglobin
BMI	Body Mass Index
CNS	central nervous system
GABA	γ-aminobutyric acid
EPM test	Elevated Plus Maze test
E. coli.	Escherichia coli
L. monocytogenes	*Listeria monocytogenes*
MIC	minimum inhibitory concentration
ROS	reactive oxygen species
DPPH	2,2-diphenyl-1-picrylhydrazyl

RSA	radical scavenging activities
LIA	lipid peroxidation inhibitory activity
FRAP	ferric-reducing ability of plasma
TRAP	total radical trapping by anti-oxidants
GRAS	generally recognized as safe
LD_{50}	median lethal dose for 50% of subjects
NOAEL	no-observed-adverse-effect level
8-PN	8-prenylnaringenin
IsoX	isoxanthohumol
C_{max}	maximum concentration
T_{max}	time to reach the maximum concentration
AUC	area-under-the-curve
MDR1	multidrug resistance protein 1 also known as P-glycoprotein 1
PXR	human nuclear xenobiotics pregnane X receptor
Caco-2	human epithelial colorectal adenocarcinoma cells
P-gp	phospho-glycoprotein
SLC	solute-carrier superfamily
ABC	ATP-binding cassette
MRP	multidrug resistance associated protein
BCRP	breast cancer resistance protein
ATP	Adenosine-5'-triphosphate
P_{app}	apparent permeability coefficient
L-DOPA	L-3,4-dihydroxyphenylalanine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBSS	Hank’s balanced salt solution
TEER	transepithelial electrical resistance
FBS	fetal bovine serum
DMEM	Dulbecco’s Modified Eagle Medium
HCOOH	formic acid
AP	apical

BL	basolateral
EtOAc	ethyl acetate
EtOH	ethanol
AP-to-BL	apical-to-basolateral transport
BL-to-AP	basolateral-to-apical transport
CMFDA	5-chloromethylfluorescein diacetate
H_3PO_4	phosphoric acid
NaOAc	sodium acetate
H. pomatia	*Helix pomatia*
ER	efflux ratio
ANOVA	one-way analysis of variance
APCI	atmospheric pressure chemical ionization
UV/VIS	ultraviolet/visible
SIM	selected-ion monitoring
LOD	limit of detection
LOQ	limit of quantification
RSD	relative standard deviation
S/N	signal-to-noise-ratio
%RE	percentage relative error
logD	octanol/water distribution coefficient
clog P	calculated log octanol/water partition coefficient
PK	pharmacokinetic
ILVO	Institute for Agricultural and Fisheries Research
$t_{1/2}$	half life
HED	human equivalent dose
K_m factor	based on the ratio of the body weight (kg) and body surface area (m^2) of a species.
BSA	body surface area
S9 fraction	defined by the U.S. National Library of Medicine's "IUPAC Glossary of Terms Used in Toxicology" as the "Supernatant

	fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000 g for 20 minutes in a suitable medium; this fraction contains cytosol and microsomes.
Tris-HCl buffer	buffer based on hydrogen chloride salt of tris(hydroxymethyl)aminomethane
NADPH	nicotinamide adenine dinucleotide phosphate
NRS	NADPH-regenerating system
G6P	glucose-6-phosphate
β-$NADP^+$	nicotinamide adenine dinucleotide phosphate ion
G6PDH	glucose-6-phosphate dehydrogenase
MgCl2	magnesium chloride
MRM	multimode reaction monitoring
TIC	total ion chromatogram
EIC	extracted ion chromatogram
FDA	US Food and Drug Administration

I. Literature Review

1.1 History of hop and beer

The history of farming and cultivation of the hop plant (*Humulus lupulus* L.) is highly connected to beer brewing and goes back to the ancient times. Historical artifacts of beer brewing date even back to 7000 B.C. in Babylon,[1] the remains of the city present day known as Al-Hillah in Iraq. Even the ancient Egyptians prepared beer from malted barley and a primitive type of wheat.[2] The first written proof on the use of hops dates back to the first century as depicted by Plinius the Elder (23-79 A.D.). In his encyclopedic collection "Naturalis Historia", hop was mentioned as a garden plant that was used for the consumption of the young shoots. He described hop as "lupus salictarius" (or "the wolf of the willow"), because of the comparison of the strangling growth of wild hops among willows and the destructive behavior of wolfs towards sheep.[3, 4] From this Latin term, the latter botanical name *Humulus lupulus* was derived.

Although expertise on the brewery techniques passed on from the ancient civilizations to the Greeks and Romans, their preferred drink however was wine. In fact, beer and the development of brewing was more linked to the culture of the Celtic and Germanic agrarian population of Northern and Central Europe in the medieval and early modern period. Fortunately, following tradition, the medieval monks (8th - 9th century) kept a comprehensive collection of written proofs of their activities. As a result we now know that the cultivation of hops started in Germany (Freising, Bavaria), where monasteries and cloisters were often surrounded by large hop gardens and hops were principally used for medicinal purposes.[5] In particular, hop preparations were mainly recommended for the treatment of sleeping disorders, as a mild sedative, liver/bile disorders, and for the activation of gastric function as bitter stomachic.[6, 7]

Since education and scientific research was only reserved for the monks, it was there that experimental use of hops in beer brewing started, as an antiseptic and flavoring agent. One of the first documented evidences was recovered in the statutes of the abbot from the monastery of Corvey on the river Weser (North Rhine-Westphalia, Germany).[8] In fact, at the beginning, hop was utilized as an alternative to *Myrica gale* (bog myrtle or sweet gale), which at that time was the established beer additive in Europe, where it was native. The common name for this beer agent was called '*gruit*' or '*gruut*' in the Netherlands and Northern Germany, where the trade of '*gruut*' was a royal business in the medieval time. Remarkably, Hildegard von Bingen (1098-1179) was against the use of hops for making beer and recommended the use of '*gruut*' instead (in combination with other aromatic herbs) to flavor beer. During the medieval period, there was a strong competition between both types of beer (based on hops and on sweet gale), but due to its lower shelf life resulting from microbiological instability, beer made from sweet gale was not really suited for long-distance transportation. This important feature boosted the hop flavored beer and the use of *H. lupulus* overcame that of *M. gale* due to its better preserving property.[9] As a consequence, hop beer export became more important but also hop cultivation followed this export through the whole of Europe for use in local brewing. From that moment, the decline of *gruut* beer was inevitable, while in the 18th century, it was even thought to be poisonous and production of sweet gale beer was forbidden in Germany.

As a protectionism measure, William IV, Duke of Bavaria, adopted in 1516, the *Reinheitsgebot* (purity law), in which it was stipulated that the only allowed ingredients of beer were water, hops, and barley-malt.[10] Later, when the knowledge on fermentation was established by Pasteur, yeast was added to this list. Although, this food-quality regulation was abandoned in 1987 following a European Commission decree, it is still in use until today among German brewers to maintain their position in the German market.

In the time of the medieval monks, the fermentation of sugars to ethanol and carbon dioxide in the process of brewing took place spontaneously. The first observations of yeast

cells were reported in the 17th century by Antonie van Leeuwenhoek (1632-1723). Later, in the 19th century, a real breakthrough in this field was achieved by the publication of L. Pasteur's "*Etudes sur la bière*". Essential in this work was the discovery of yeast as a microorganism which applied fermentation of sugars as an energy source for growing (instead of sunlight).[11] In addition to this, the progress of the brewing scientist Emil Christian Hansen from the Carlsberg brewery (Denmark) was crucial for modern brewing industry. In 1883, he obtained several pure yeast cultures and selected especially the strain "*Saccharomyces carlsbergensis*" for the production of pilsner beers, which is still in use today.[12] This concept was soon applied in practical brewing, proving of major importance in standardizing yeasts for reliable fermentations. By selection of the most appropriate yeast strains for beer brewing, reproducible beers could be produced.

During the 19th century, the industrial revolution, and the related population growth, income rise and increased consumption provoked the transition of artisanal domestic brewing to industrial brewing. The development of hydrometers and thermometers introduced process control during brewing.[13] During the 20th century, the scientific knowledge on malting and brewing flourished and, as a result, the modern brewing industry applies a whole spectrum of novel technical, biochemical, microbiological, and genetic inventions.[14]

Today, the brewing industry is a global business, consisting of several dominant multinationals and many thousands of smaller regional and local breweries and brewpubs. The fusion between Interbrew (Belgium) and AmBev (Brazil) (2004) forming InBev and, more recently, the fusion with Anheuser-Bush to form AB InBev (2009), makes this company now the biggest brewer in the world. On the opposite hand, nowadays, there is an upcoming trend in the establishment of local breweries in many countries producing specific beers that increase consumer's options. Despite the global success of InBev, beer consumption is decreasing in Belgium, as well as in most European countries.

This modern time, the hop plant (*Humulus lupulus L.*) is an essential ingredient in beer and about 95% of world-wide cultivated hops is destined for brewing purposes, while the remaining fraction is nowadays used in fully developing new markets, such as the domain of phytopharmaceuticals and dietary supplements. Also, particularly in Belgium*,* shoots of hop plants are served as a delicacy, though prices are expensive.[5]

1.2 Phytogeography of hop

The genus *Humulus* belongs to the Cannabinaceae family, which consists of three species: H. *lupulus* Linnaeus, H. *japonicus* Siebold & Zucc. And H. *yunnanensis* Hu.[15, 16] The origin of the genus has been suggested to be in China, since all of the *Humulus* species were found in this area. From China, an advancing eastward migration to Japan and America and a migration to the west to Europe could be responsible for the actual distribution of the Humulus species.[16-18] Successful cultivation of hops requires optimal growth conditions, especially with respect to the length of daylight, the summer temperature, the amount of rain, and the fertility of the soil. Therefore, hops are found in the moderate climatic zones of the Northern and Southern hemispheres, with Germany and the United States by far the largest producing countries.[19-22] The farming of hops for long times has caused the existence of hundreds genetically close cultivars.[16] The major reason was the need for hop plants delivering specific organoleptic properties to improve the flavor and the aroma of beer. As a result, cultivars with an increased content in volatile oil and bitter acids have been selected throughout the years.[23]

1.3 Botanical description of hop

Figure 1.1 *Humulus lupulus* L.

Figure 1.2 Hop field.

The hop plant (*Humulus lupulus L.*) is a dioecious perennial climbing plant characterized by a very fast growth each spring from the rhizomes of an underground rootstock. Each autumn and winter, the annual parts from aboveground of the plant die, though the plant has a fair resistance to frost thanks to an extended root system which burrows deep into the ground. Every year in spring, numerous shoots sprout from the remaining expanding rootstock, from which new plants can develop. Hop can grow amazingly fast, sometimes growing up to 30 cm per day and can reach heights from 6 up to 8 m. The climbing plant is grown in a hop field, trained up wires which support the plants and allow them significantly greater growth with the same sunlight profile. The stem is rough, hexagonal squared, and swings clockwise around trees and other supports, attached by fine two-pointed hairs. The leaves of the hop plant are rough, dark green, heart-shaped with a long stalk and finely toothed edges. The leaves can grow up to 15 cm long, are singular, cross-opposed, with a hand-shaped nervure and they possess little supportive leaves that are single-lobed.

Figure 1.3 Inflorescences on the male plant of *Humulus lupulus* L.

Figure 1.4 Hop cones on the female plant of *Humulus lupulus* L.

During the blooming period, in July and August, the flowers spring from the axils of the leaves. Although individual monoecious plants are frequently found in some wild North-American hop populations, hop is known as a dioecious plant. Accordingly, unisexual male and female flowers grow on separate plants. The male plants carry small inflorescence, while the flowers of the female plants develop into greenish-yellow, hop cones called strobiles that are formed of membranous partially overlapping scales (bracts and bracteoles). This morphological difference between male and female flowers is the only morphological characteristic to distinguish and identify the sex of the plant.[22]

At the end of the summer or the beginning of autumn, the female inflorescence has reached ripening and can be harvested as ovoid, greenish hop cones. The inside of the bract and the bracteoles contains oleoresin glands that appear as small yellow-orange granules and constitute lupulin,[22, 24, 25] a resinous, yellow-orange powder. Lupulin contains numerous compounds that are of economic interest especially in the beer brewing industry and, therefore, only the female hop plants are being cultivated.

In some hop growing areas, particularly in England, male plants are cultivated together with the females in order to produce seeds in the hop cones following pollination and to improve crop yields. However, in other global parts, it is forbidden to cultivate a male

plant in vicinity of females. In Belgium, wild males must be removed within 5 km from a female plant, because fertilized hop cones contain hard greasy seeds which adversely affect beer quality.[21, 26, 27] Especially the fats and fatty acids have a bad influence on the beer foam. At harvest, the hop cones have a moisture content of 75-80 %. In order to prevent deterioration during storage or processing, drying of the hop cones is necessary to reduce the water content below 12%. This step, achieved by hot air dryers, is very critical with respect to the hop quality, since higher temperatures, especially above 65°C, may accelerate oxidative decomposition of major constituents. Dried hops are recommended to be stored at low temperature prior to processing or further use.[16, 22]

The bitter and aroma components in the hop cones are very sensitive to oxidation. For this reason, the hop plant is frequently processed into more stable products, such as non-isomerized supercritical or liquid carbon dioxide extracts, distilled hop oil fractions, and potassium solutions of pre-isomerized hop acids that can be directly added to the brewing kettle (non-isomerized and pre-isomerized non-purified products) or post-fermentation (hop oils and pre-isomerized purified products).[1, 28, 29] Hop extracts and hop oil fractions are also used as flavoring products in non-alcoholic beverages and foods.[24]

1.4 Chemistry of hop

Female hop cones contain glandular structures in which lupulin powder is secreted (a yellow and sticky powder), in which a variety of secondary metabolites can be identified. Nagel *et al.* used microscopic techniques to visualize the morphology of hop cones and lupulin glands (Figure 1.5).[30] The typical composition of air dried hops is presented in Table 1.1.[21, 22]

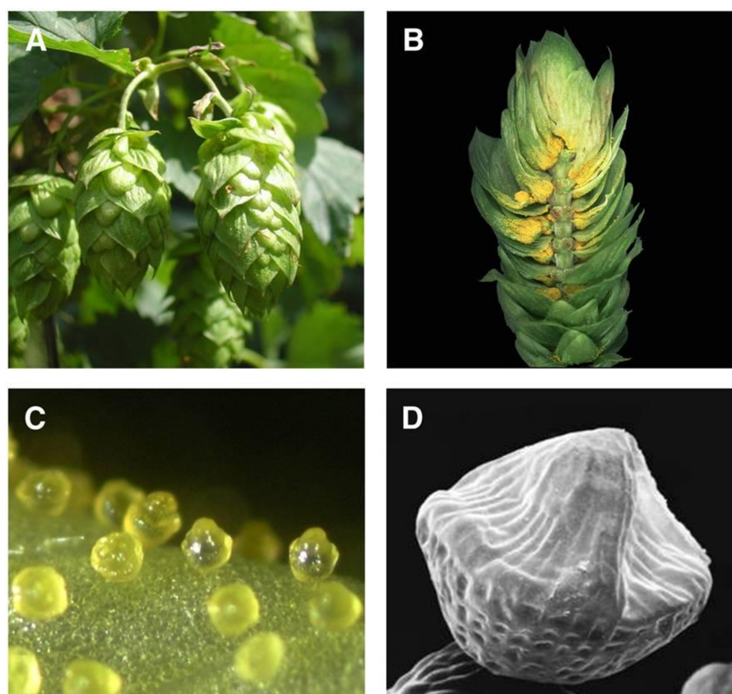


Figure 1.5 Morphology of hop cones and lupulin glands. A: Cones of hop cultivar Taurus (cones are ~5 cm in length). B: Longitudinal cross-section of a hop cone showing lupulin glands at the base of bracteoles. C: A light-microscopy image of ripe lupulin glands. D: Scanning electron micrograph of a ripe lupulin gland showing the peaked appearance of the filled subcuticular sac. [30]

Table 1.1 Composition of air-dried hops.

Compounds	% (m/m)
α-acids	2-19
β-acids	2-10
amino acids	0.1
ash/salts	10
cellulose-lignin	40-50
monosaccharides	2
oils and fatty acids	1-5
pectins	2
polyphenols and tannins	3-6
proteins	15
volatile oil	0.4-3.4 (v/m)
water	8-12

Among the hundreds of compounds, the bitter acids, the volatile essential oil, and the polyphenols can be classified as the most important constituents[31] both as chemical markers to support type differences in hop varieties and for the commercial use of hops. A schematic description of the composition of hops is presented in Figure 1.6.

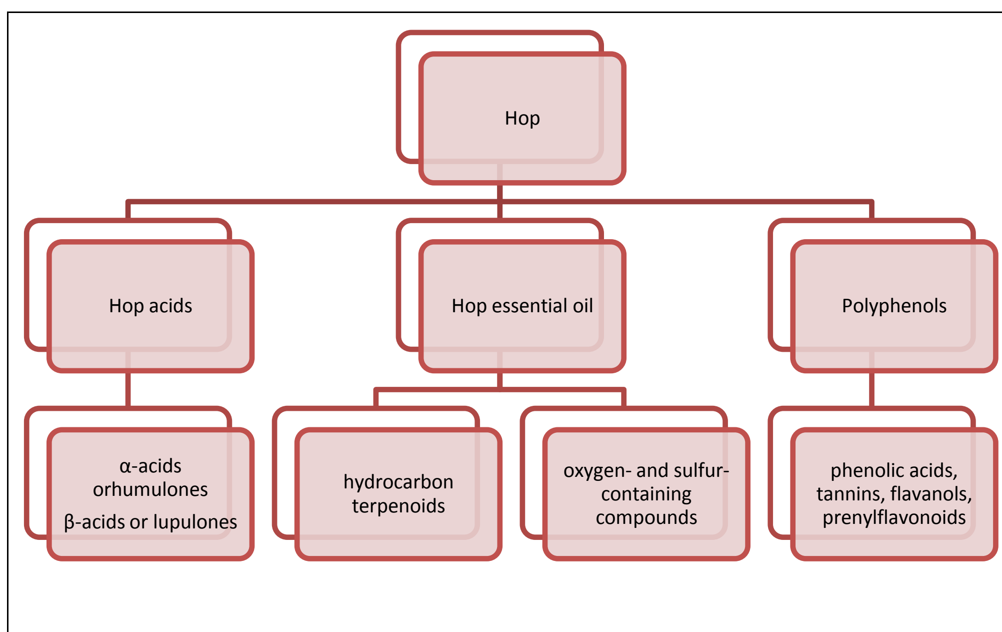


Figure 1.6 Composition of hops.

1.4.1 Hop essential oil

The essential or volatile hop oil consists mainly of a hydrocarbon terpenoid fraction such as the monoterpene β-myrcene (30-50%), and the sesquiterpenes humulene, β-caryophyllene, and farnesene (40-80%).[16, 21, 32, 33] Some of the major compounds are depicted in Figure 1.7. Due to the susceptibility of terpenoids to oxidation, significant fractions of oxygenated products can be found as a result of post-harvest handling and storage.[33] These include alcohols (linalool, geraniol), acids (2-methylbutyric acid, 3-methylbutyric acid), and esters (2-methylpropylisobutyrate, 2-methylbutylisobutyrate) in combination with aldehydes, ketones, and epoxides.[21]

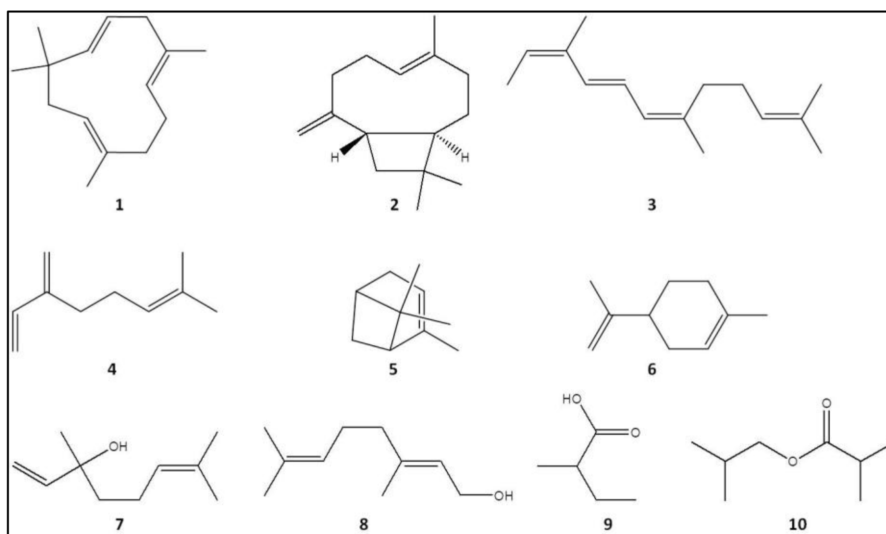


Figure 1.7 Terpenoids and oxygenated derivatives in hop essential oil. 1. humulene, 2. β-caryophyllene, 3. farnesene, 4. β-myrcene, 5. α-pinene, 6. limonene, 7. linalool, 8. geraniol, 9. 2-methylbutyric acid, 10. 2-methylpropylisobutyrate.

Among all constituents in hop essential oil, less than 1% accounts for organosulfur compounds including thiols, sulfides, polysulfides, thioesters, thiophenes, and episulfides. Either their origin is from the plant itself (for tissue repair purposes) or they can be formed as a result of the spraying of sulfur-containing fungicides during farming. Moreover, it is unclear how many of such organosulfur compounds are formed after harvest as a result of drying and storage.

1.4.2 Hop polyphenols

Polyphenols (Figure 1.7) in hop include phenolic acids, flavonol glycosides, tannins, together with a number of prenylated chalcones, such as xanthohumol and desmethylxanthohumol. These latter ones are precursors of the isomeric flavanones isoxanthohumol (main prenylflavonoid present in beer) and 8-prenylnaringenin, along with 6-prenylnarigenin, respectively.

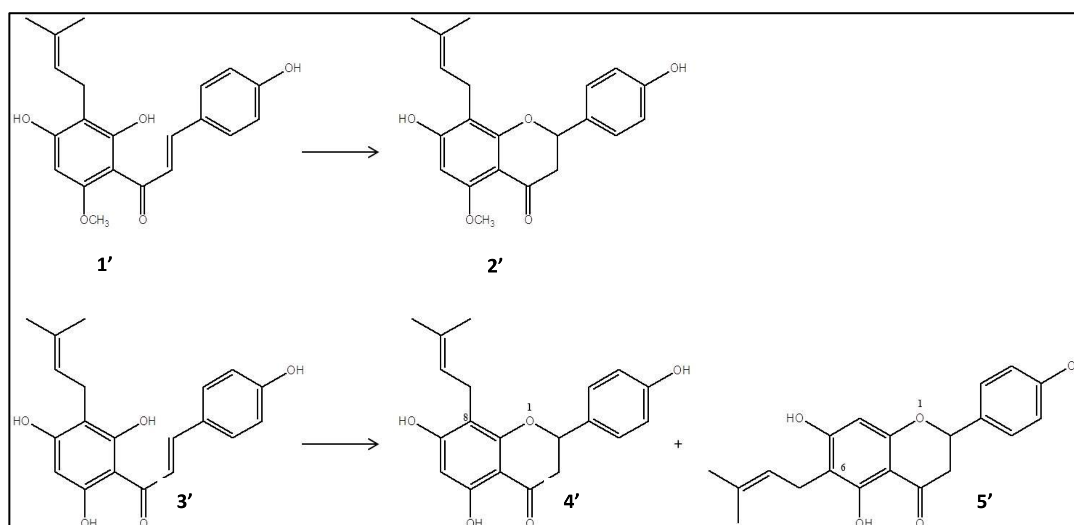
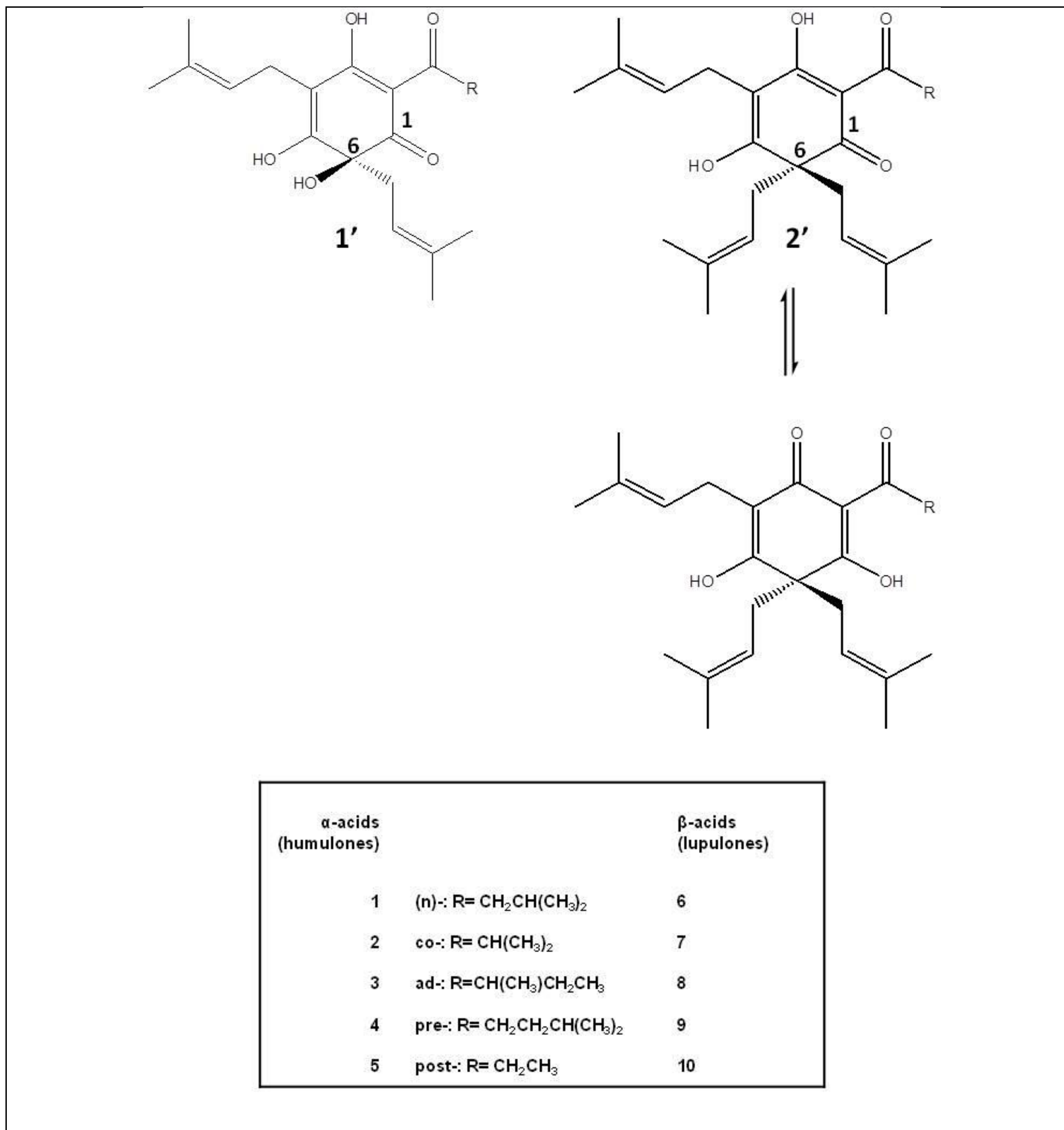


Figure 1.8 Hop-derived prenylflavonoids. 1': xanthohumol; 2': isoxanthohumol; 3': desmethylxanthohumol; 4': 8-prenylnaringenin; 5': 6-prenylnaringenin.

1.4.3. Hop bitter acids

Hop bitter acids consist of two related series, the α-acids (or humulones) and the β-acids (or lupulones), which are both characterized as prenylated phloroglucinol derivatives (Figure 1.8). These compounds occur as pale yellowish oils (or so-called soft resins) that are soluble in hydrocarbon solvents, such as hexane. Depending on the nature of the acyl side chain, five analogues can be identified: isovaleroyl in n-, isobutyroyl in co-, 2-methylbutyroyl in ad-, isohexanoyl in pre-, and propanoyl in post-bitter acids.



α-acids (humulones)		β-acids (lupulones)
1	(n)-: R= $CH_2CH(CH_3)_2$	6
2	co-: R= $CH(CH_3)_2$	7
3	ad-: R=$CH(CH_3)CH_2CH_3$	8
4	pre-: R= $CH_2CH_2CH(CH_3)_2$	9
5	post-: R= CH_2CH_3	10

Figure 1.9 Chemical structures of hop α-acids (1') and β-acids (2').

The relative amounts of α-acids and β-acids, as well as the concentrations of individual representatives, depend strongly on the hop variety and the conditions of growing. Hop bitter acids are very sensitive to oxidation, thus forming a mixture of ill-defined products that are soluble in diethyl ether, but no longer in hexane (this fraction of compounds is referred to as hard resins). Deterioration of hops as a function of time is accompanied by the development of a strong odor that is generally not welcomed by brewers.[26] To prevent this,

hops are rapidly dried after harvesting, pelleted, and stored in airtight bags, preferably at low temperatures. Today, next to the use of pellets, many beers are rendered bitter-tasting with hop extracts, which leads to the benefits of an increased utilization of brewing principles, increased stability, improved uniformity, and easier handling.[28]

The α-acids are the most important constituents of hops, that make up to 20% of the dry weight of the hop cones (Table 1.1). The weak-acidic, salt-forming, and chelating properties of α-acids reside in their β-triketo system. It appears that only the R-configuration at C-6 within a fully enolized β-triketo system is formed stereoselectively in nature (Figure 1.9).[22] Only recently, the absolute structure of humulone was fully established by Urban *et al.* by X-ray crystallography: the chiral center at C-6 in humulone was unequivocally assigned to be 6S,[34] contradicting the reports of De Keukeleire and Verzele in the 70's, who determined that the configuration at C-6 in humulone was the R-configuration using a combination of chemical, spectrometric, and chiroptical techniques.[35]

Determination of the composition of the α-acids mixture is important, as high levels of cohumulone are generally associated with a lower hop quality, although several authors reported that the differential contribution of the individual α-acids to the bitterness seems negligible.[36-38] In general, the analogues n-humulone, cohumulone, and adhumulone are the main constituents of the hop α-acids, representing 35-70%, 20-65%, and 10-15% of the total levels, respectively. The pre- and post-humulones represent only a minor part of the α-acids. The relative amounts of n-humulone and cohumulone are depending on hop variety, while the amount of adhumulone is fairly constant.[21] For research purposes, α-acids can be isolated from a hop acids mixture (e.g., a liquid carbon dioxide extract) by pH-selective liquid-liquid extraction (isooctane, diethyl ether)[31] or by precipitation upon addition of lead(II) acetate, thus forming yellow-colored highly stable lead salts that can be stored for years without deterioration.[22] The exact structure of the salts is not known, but the tertiary alcohol function must be involved, as the β-acids cannot form such salts. From the mixture of α-acids, n-humulone can be isolated by complexation with 1,2-diaminocyclohexane followed

by repeated crystallization,[39] while isolation of cohumulone and adhumulone requires further preparative HPLC.

During brewing, the concentration of α-acids remaining in the final product is drastically reduced to reach levels of few mg/L due to several reasons including isomerization or other chemical reactions during wort boiling, their limited solubility in aqueous solutions, and adsorption on yeast cells, trub, and filter materials.[40] In dry-hopped beers, residual concentrations of α-acids up to 14 mg/L can be present.[36] The α-acids improve foam stability, suppress gushing, and contribute to the preservation of beer. However, their main contribution to beer is the isomerization during the boiling of wort with hops, thereby forming the iso-α-acids, the largest contributor to beer bitterness (see below).[41]

The β-acids are less acidic than the α-acids, because the tertiary alcohol function at C-6 is replaced by an extra prenyl side chain. This difference enables separation of β-acids and α-acids from a hop acids mixture by pH-selective liquid-liquid extraction from a solution in hexane. The α-acids (pKa ~ 5.4)[42] can be extracted with sodium bicarbonate, while the less acidic β-acids (pKa ~ 6.1)[22] require a stronger base, like sodium hydroxide. In contrast to α-acids, two different enolization patterns prevail for each β-acid (Figure 1.8). However, β-acids are mostly represented in the predominant conjugated dienolic form, which corresponds to that of the α-acids. Most hop varieties contain approximately equal levels of n-lupulone and colupulone (ca. 20-55%), next to less variable levels of adlupulone (10-15%). Prelupulone and postlupulone are only present in trace quantities.

Hop β-acids are extremely sensitive to oxidation, which is initiated by air (auto-oxidation), but also during wort boiling giving rise to a number of oxidized compounds and derivatives.[40, 43, 44] One particular and most important oxidative reaction leads to formation of the highly stable hulupones (Figure 1.10). In contrast to the non-bitter β-acids, hulupones have a very bitter taste (threshold concentration of 7.9 µmol/L) and can be present in beer in quantities of few mg/L. Other oxidation products reported in the literature include

tricycloderivatives (Figure 1.10). In comparative sensory tests, these showed bitterness threshold concentrations in line with their precursors or higher (37.9 – 90.3 μmol/L).[44]

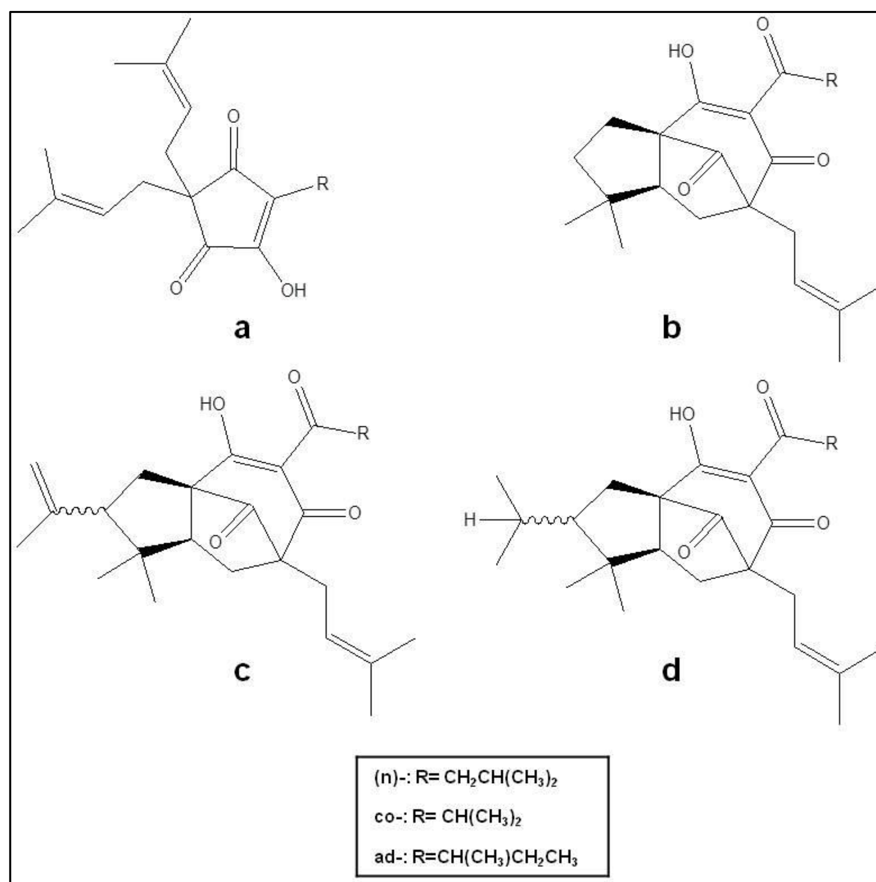


Figure 1.10 Chemical structures of hop β-acids oxidation products. a: hulupones; b: nortricyclolupulones; c: dehydrotricyclolupulones; d: tricyclolupulones.

During the brewing process, α-acids are converted via a thermal isomerization to the more water-soluble iso-α-acids via an acyloin-type ring contraction, thereby yielding concentrations ranging from 10 up to 100 mg/L of iso-α-acids in beers. The higher solubility in aqueous medium is associated to their higher acidity (pKa 3.0-4.0).[22] Conversion of each iso-α-acid analogue results in an epimeric pair of *cis-* and *trans*-isomers, where the stereochemical notation refers to the relative orientation of the hydroxyl at C-4 and the prenyl group at C-5 (Figure 1.11). Consequently, six major iso-α-acids are present in beers, the *cis-* and *trans-* epimers of isocohumulone, isohumulone, and isoadhumulone. According to the

new discoveries by Urban and co-workers, the isomerization of α-acids into iso-α-acids proceeds by configuration-retention of the tertiary alcohol in (6S)-humulone to the α-hydroxy ketone on C-4 in isohumulones.[34] This is in contrast with the proposed isomerization mechanism found in most reports, which assume that the conserved stereocenter is on C-5, while cis and trans differ stereochemically at C-4. Considering that the oxygen atoms possess negative charges during the isomerization, one might imagine the chelation of two vicinal oxygen atoms to a divalent cation, a process that is known to accelerate the rate of isomerization, while limiting decomposition.

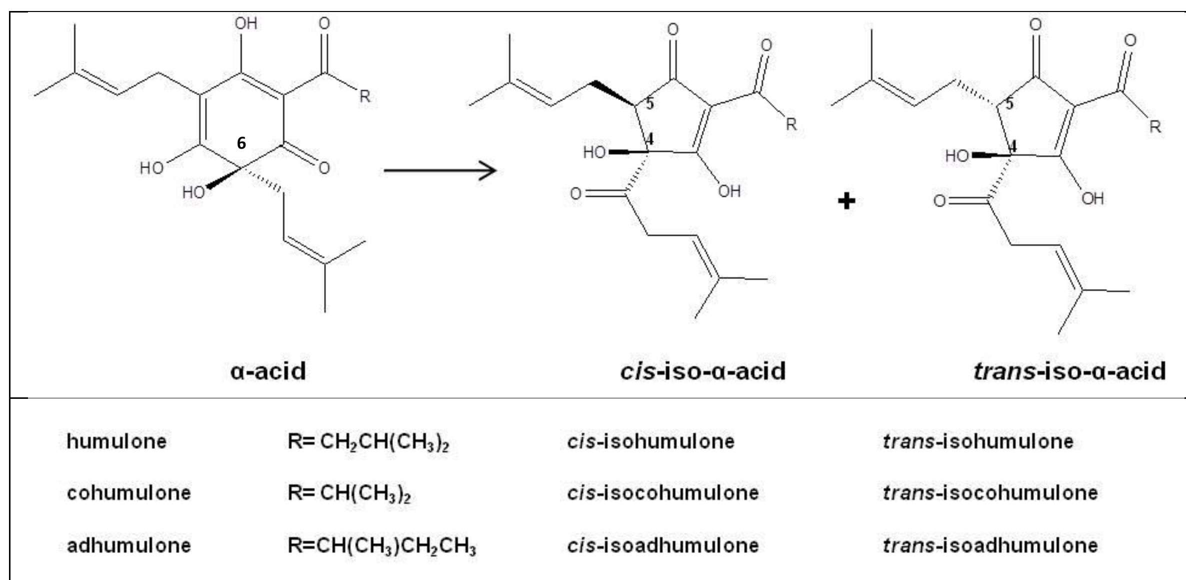


Figure 1.11 Chemical structures of hop-derived *cis-* and *trans*-iso-α-acids.

The ratio of *cis*/*trans* depends on the reaction conditions, but is typically 68:32 under normal brewing settings. The cis-isomer is thermodynamically more stable in view of the least steric hindrance between the two large vicinal side chains.[41] Iso-α-acids can be formed from α-acids under a variety of conditions. During the brewing process, α-acids are isomerized by boiling hops or hop extracts in the aqueous wort medium at a pH of 5.0-5.5. In

practice, a final α-acid utilization yield of only 25-35% is reached in the beer. This is also subject to variations, even from brew to brew. Many kinetic studies have been carried out on the transformation of the hop α-acids into the corresponding *cis*- and *trans*-iso-α-acids during the wort boiling process. [22, 45-50] The wort-boiling of α-acids follows first-order kinetics and the rate is influenced by the temperature, the pH, and the concentrations of divalent cations. The reaction can be favored by boiling α-acids in alkaline media in the presence of divalent cations as catalysts. Alternatively, isomerization of α-acids can be achieved by irradiation of a solution in methanol of α-acids with UV-light. This photo-isomerization proceeds in a fully regio- and stereoselective way and produces exclusively *trans*-isomers.[51] Inter-conversion of *cis*- into *trans*-epimers, as well as conversion of iso-α-acids into their parent compounds, is feasible.[22] Iso-α-acids represent well above 80% of all hop-derived components in beer. The high concentrations of these five-membered ring compounds in the final beverage and their low bitter taste thresholds support their function as major bitter agents in beer.[52-55] Besides, they possess tensioactive properties, thereby stabilizing the beer foam, and protect beer against micro-organisms (preservative role).[56, 57] On the other hand, iso-α-acids are key ingredients in the formation of the so called “lightstruck flavor” in beer. When exposed to light, iso-α-acids decompose via a series of excited states and radical-type intermediates to unpleasant smelling “skunky” thiols, mainly 3-methylbut-2-ene-1-thiol (next to dehydrohumulinic acid) (Figure 1.12).[58, 59] This reaction is initiated by riboflavin (vitamin B2) as a photosensitizer, since iso-α-acids cannot absorb visible light directly.

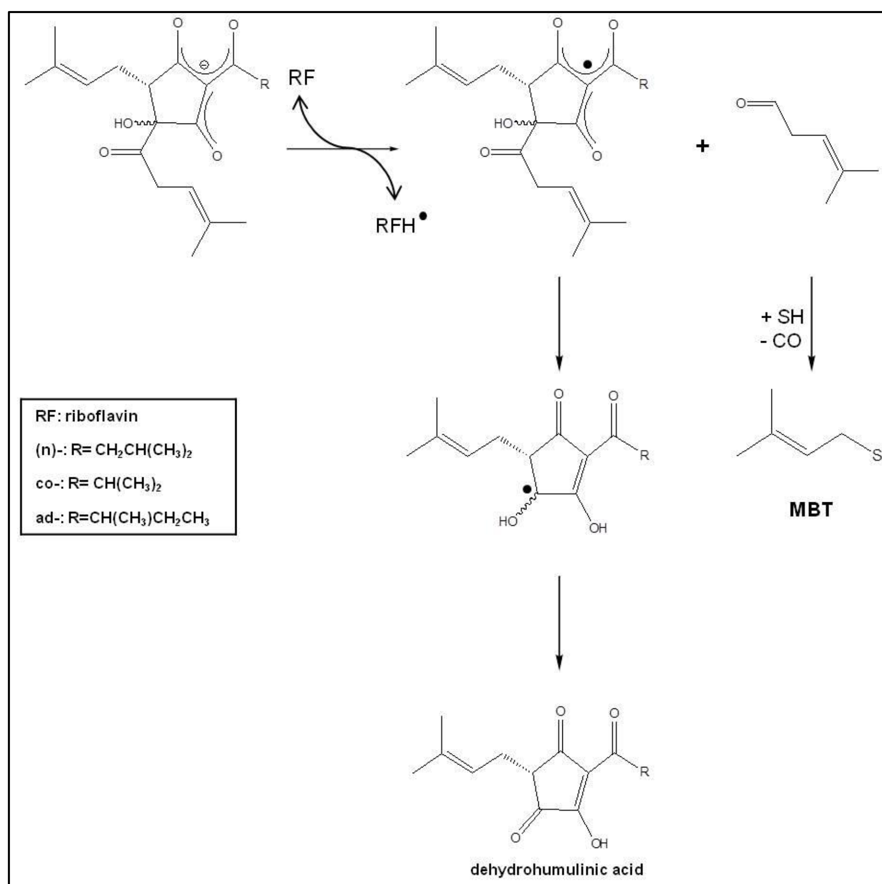


Figure 1.12 Formation of the "lightstruck flavor" in beer. RF: riboflavin. MBT: 3-methylbut-2-ene-1-thiol.

Enhanced resistance to the "lightstruck flavor" is provided by the use of reduced derivatives of iso-α-acids. Three major types can be considered depending on the number of added hydrogen atoms: dihydro-, tetrahydro-, and hexahydro-iso-α-acids (Figure 1.13). They are formed either by hydrogenation (H_2/Pd-C) or by reaction with sodium boron hydride ($NaBH_4$), or by a combination of both.[60] The reduced derivatives are light-proof, and also more stable with respect to oxidation, thus allowing brewers to bottle beers with an increased shelf life. The tetrahydro-iso-α-acids (THIAA) and hexahydro-iso-α-acids (HHIAA) have the extra-advantage of enhancing beer foam stability.[29, 41, 61]

During formation of dihydroiso-α-acids (DHIAA) and HHIAA, an additional chiral centre on the acyl side chain is introduced, leading to two epimeric reaction products for each iso-α-analogue. As a result, theoretically, the group of DHIAA and HHIAA can consist

of twelve stereoisomeric products.[1] THIAA are formed by hydrogenation of the double bonds present in the side chains of IAA, thus also consisting of *cis*- and *trans*- isomeric pairs, leading to six stereoisomers.[62, 63] Iso-α-acids and reduced derivatives are commercially available as aqueous solutions of their corresponding potassium salts. They can be applied post-wort boiling, e.g., during lagering or even just prior to bottling, since isomerization is no longer required.[28]

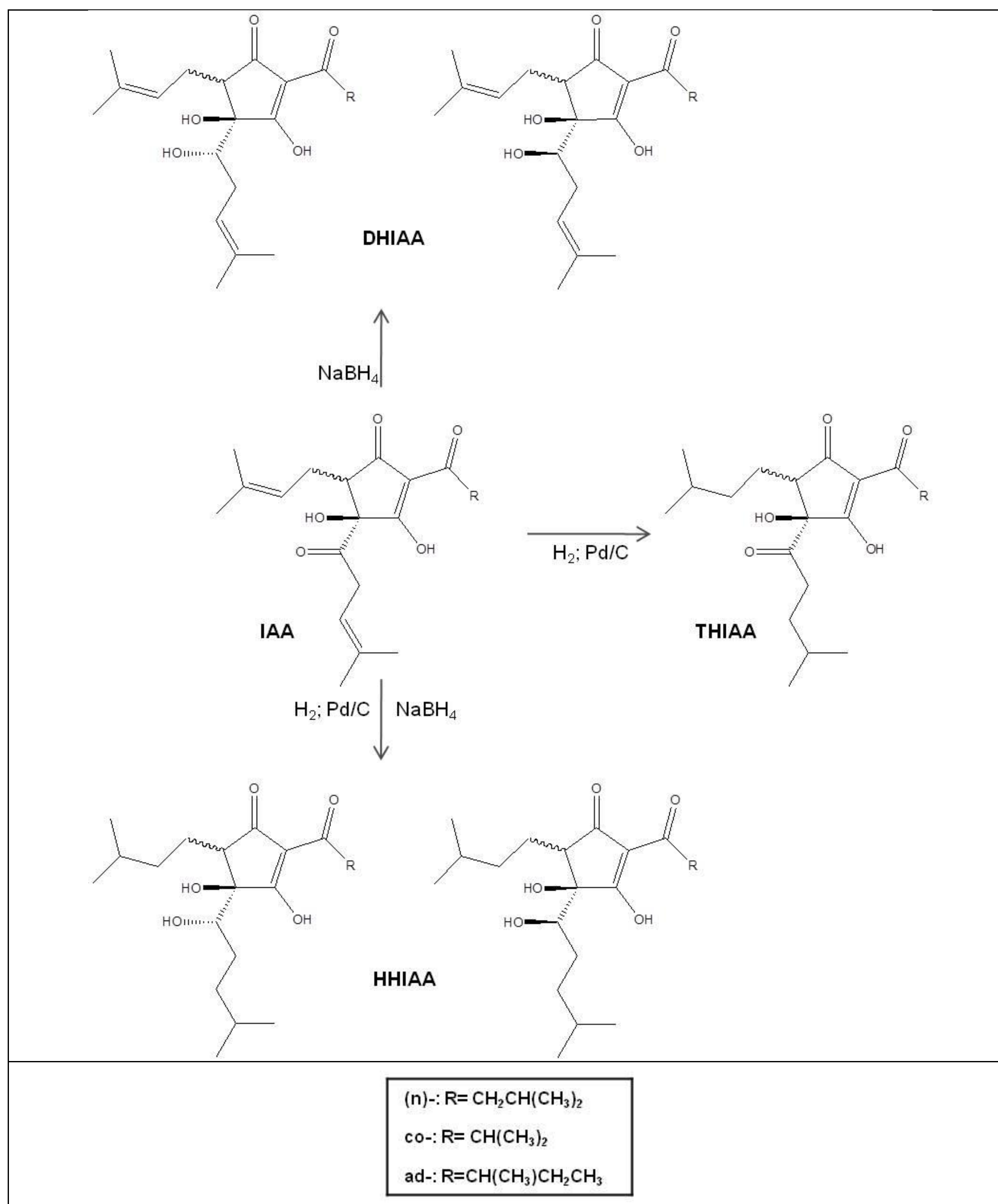


Figure 1.13 Chemical structures of reduced derivatives of iso-α-acids. IAA: iso-α-acids; DHIAA: dihydro-iso-α-acids; THIAA: tetrahydro-iso-α-acids; HHIAA: hexahydro-iso-α-acids.

Besides the formation of off-flavors occurring during light-exposure of beer, molecular transformations of the hop-derived iso-α-acids during beer ageing have an essential impact on the overall bitter taste of beer.[40, 64, 65]

A rapid degradation of *trans*-iso-α-acid is observed, whereas the corresponding *cis*-stereomers were found to be relatively stable.[66-68] Detailed investigations discovered tri- and tetracyclic molecules (Figure 1.14) that are formed via acid-induced cyclization of *trans*-iso-α-acids.[64, 69, 70] Also, additional degradation products independent of the *cis*-/*trans*-stereochemistry were found, including hydroxy- and hydroperoxy-*trans*-/*cis*-alloisohumulones produced upon auto-oxidation involving air oxygen,[71] plus formerly described *trans*-/*cis*-humulinic acids formed by proton-catalyzed transformations (Figure 1.14).[72, 73]

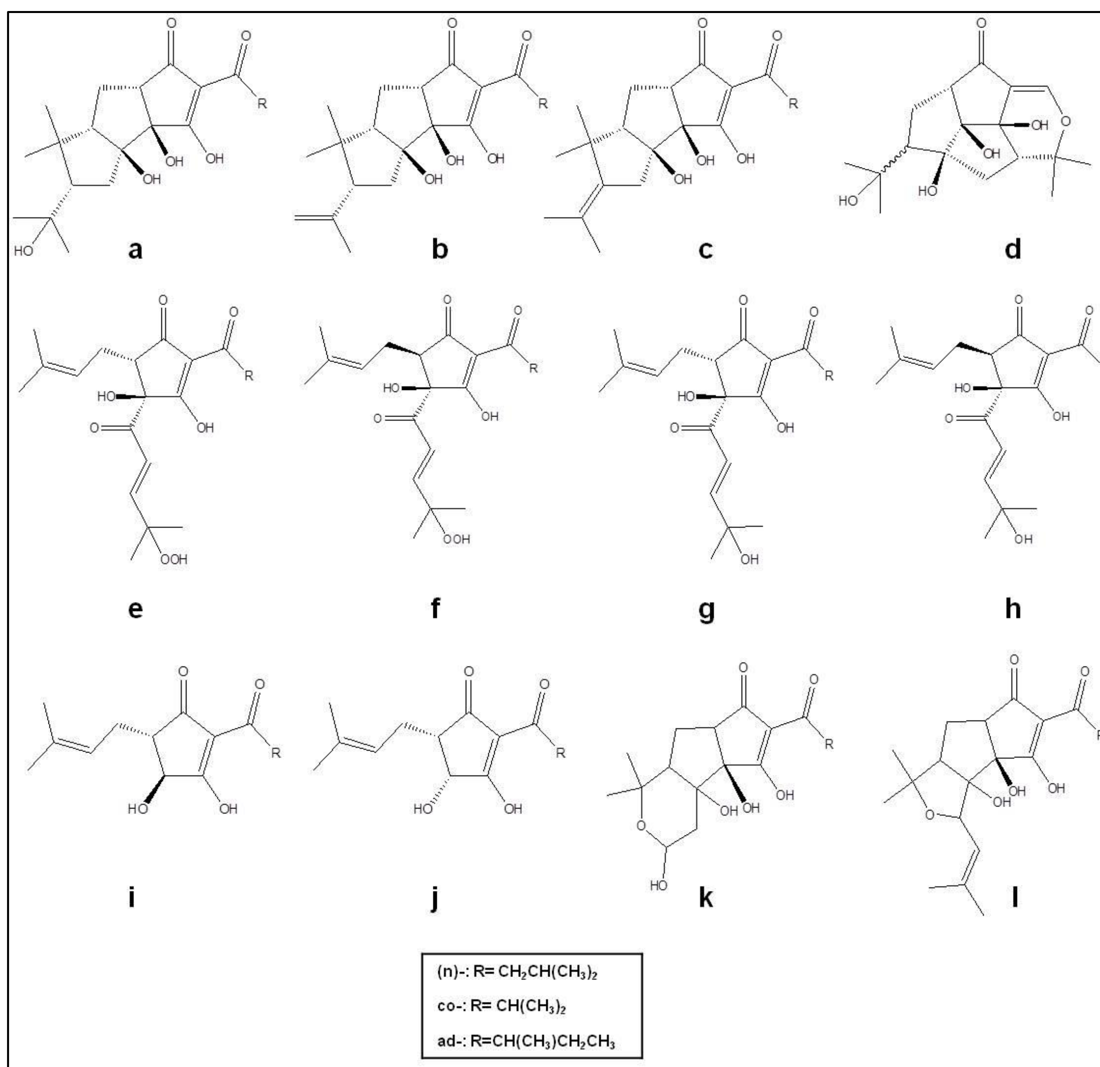


Figure 1.14 Chemical structures of oxidized derivatives of iso-α-acids in beer: tricyclohumols (a), tricyclohumenes (b), isotricyclohumenes (c), tetracyclohumols (d), hydroperoxy-*trans*/*cis*-alloisohumulones (e, f), hydroxy-*trans*-/*cis*-alloisohumulones (g, h), *trans*-/*cis*-humulinic acids (i, j), tricyclohumolactol, (k), and scorpiohumol (l)

1.5 Purification and Analysis of Hop Acids

Hop acids can be separated from the vegetative material by extraction with solvents of different polarity. Extraction with supercritical or liquid carbon dioxide yields a dark-green or a yellow-golden paste containing high levels of α-acids and β-acids without the more polar tannins, hard resins, and salts.[29] This type of extract serves as a suitable material for further manipulations. From this, α-acids can be purified by precipitation of their lead(II) salts or by

liquid-liquid extraction with aqueous sodium bicarbonate, whereas β-acids need a more alkaline environment to be extracted (for example sodium hydroxide).[22]

Iso-α-acids and reduced derivatives are commonly purified from their commercially available potassium salt solutions. An efficient separation of *trans*- and *cis*-iso-α-acids by complexation with β-cyclodextrin has been described using a molar ratio of β-cyclodextrin to iso-α-acids from 1:1 to 1:4.[74, 75] Individual hop bitter acids are obtained from extracts by (semi)-preparative HPLC coupled to detection by UV, mass spectrometry, ^{1}H-NMR spectroscopy, or a combination of techniques.[76-82]

A number of variables determine the final composition of hop bitter acid preparations. There are substantial variations in the starting material, depending on the hop variety, the harvest time, the growing conditions, the processing, and the storage. Furthermore, hop bitter acids are very sensitive to oxidation and degradation. For research purposes, it is therefore essential that purification and storage of these compounds is well controlled and the relative composition of the extracts must be frequently monitored. However, researchers tend to underestimate this issue, making it hard to compare results from different studies with the “same” compounds. The specification of the exact composition of the hop extracts used should be included in the reports or individual compounds could be used. Further information on the chemistry, preparation, and purification of hop bitter acids can be found in reviews by Verzele and De Keukeleire, [22] and Ting and Goldstein.[62]

Due to their importance for beer bitterness, various analytical approaches were undertaken in the past to measure iso-α-acids. Spectrometric analysis according to a method of the European Brewing Convention is the most common procedure.[83] Bitter units are calculated from the total UV-absorption at 275 nm, but detailed information on the exact composition of the hop-derived bitter compounds in beer samples is lacking, since this method is incapable of distinguishing between the individual bitter acids. To overcome this limitation, specific analytical methods were developed based on separation techniques such as countercurrent distribution.[84] It gives a good separation of bitter substances, but requires large amounts of organic solvents and is time-consuming. Others developed thin-layer

chromatography (TLC) separation techniques for detection of *trans*- and *cis*-isohumulone, together with hulupones.[85] However, the sensitivity and the resolution are insufficient. This problem can be solved by the use of high-performance liquid chromatography (HPLC) and gas chromatography (GC). HPLC with UV-detection has been intensively used for the analysis of hops and beer[76, 86-90] next to other techniques such as capillary electrophoresis[91] and micellar electrokinetic chromatography.[92] All hop-derived bitter substances are UV-active and demonstrate maximum molar absorption coefficients of the same order of magnitude as simple aromatic compounds at appropriate wavelengths (i.e., of the order of 10^4). The α- and β-acids show UV-maxima at wavelengths of around 310-340 nm, while the iso-α-acids and reduced derivatives show UV-maxima around 250-270 nm. Nowadays, chromatographic separation can also be combined with sophisticated detection techniques based on NMR analysis [80, 93] or mass spectrometry. [78, 94-96] The latter, being more and more the state-of-the-art in analytical detection, in different types and configurations (single quad, time-of-flight, tandem…)

1.5.1 Considerations regarding complexity of hop acids mixtures

The hop acids (α- and β-acids) occur as a mixture of homologues and structural isomers, with the three major components labeled as co-, n-, and ad-homologues. The co-species have one methylene (CH_2) group less than the n- and ad-compounds, being less polar and readily resolved from the n- and ad-counterparts. The n- and ad-variants are isomers which differ only in the location of the side chain branching point, which renders the separation more challenging. For quantification purposes, it is assumed that these compounds (including co-, n-, and ad-) show the same response factor under the chromatographic conditions applied.

This issue is even more complex in the group of iso-α-acids and derivatives due the fact that each α-acid gives rise to two stereoisomeric *cis*- and *trans*-iso-α-acids, varying in the stereochemistry of one carbon. The resolution of the 3 *cis*-iso-α-acids from their 3 *trans*-epimers requires strictly pH-controlled mobile phases. The same applies to the corresponding tetrahydro-iso-α-acids (with the double bonds hydrogenated), which also consist of three *cis*- and *trans*-pairs, totaling six congeners. The dihydro-iso-α-acids are derived from the borohydride reduction of the iso-α-acids. From each iso-α-acid, two epimers are formed upon reduction of the carbonyl moiety to the corresponding secondary alcohol, so there are theoretically 12 related compounds possible. However, the *trans*-dihydro-iso-α-acids are mostly absent from a dihydro-iso-α-acid preparation, probably due to the steric hindrance experienced by the borohydride moiety when approaching the carbonyl along the isopentenoyl-side chain of the *trans*-iso-α-acids.[62] An overview of the chemical structures of the major hop-derived bitter acids studied in this work are shown in Fig. 1.15. Pure calibration standards of individual hop acids for use as external standards are not commercially available. One of the reasons is the inherent instability of these compounds. Although isolation of pure analogues of α- and β-acids should be feasible, the calibration standard available (Labor Veritas, Switzerland) contains only a mixture of co-, n-, and ad-analogues of α- and β-acids and are calibrated for these compounds on a percentage (w/w) basis. In the case of iso-α-acids, pure *trans*-isohumulones can readily be prepared by photoisomerization of humulone,[97] but decomposition occurs even when stored under nitrogen atmosphere at low temperature. Instead, the mixture of dicyclohexyl-ammonium (DCHA) salts of *trans*-iso-α-acids has been widely used as a reference for the quantification of iso-α-acids and is commercially available. Until today, a standard including the *cis*-isomers is not commercially available. For the tetrahydro-iso-α-acids, a mixture of *cis*-and *trans*-forms of co-, n-, and ad-homologues is commercially available, while, for the dihydro-iso-α-acids, a purified preparation of the dicyclohexyl-ammonium salts of *cis*-dihydro-iso-α-acids is commercialized.

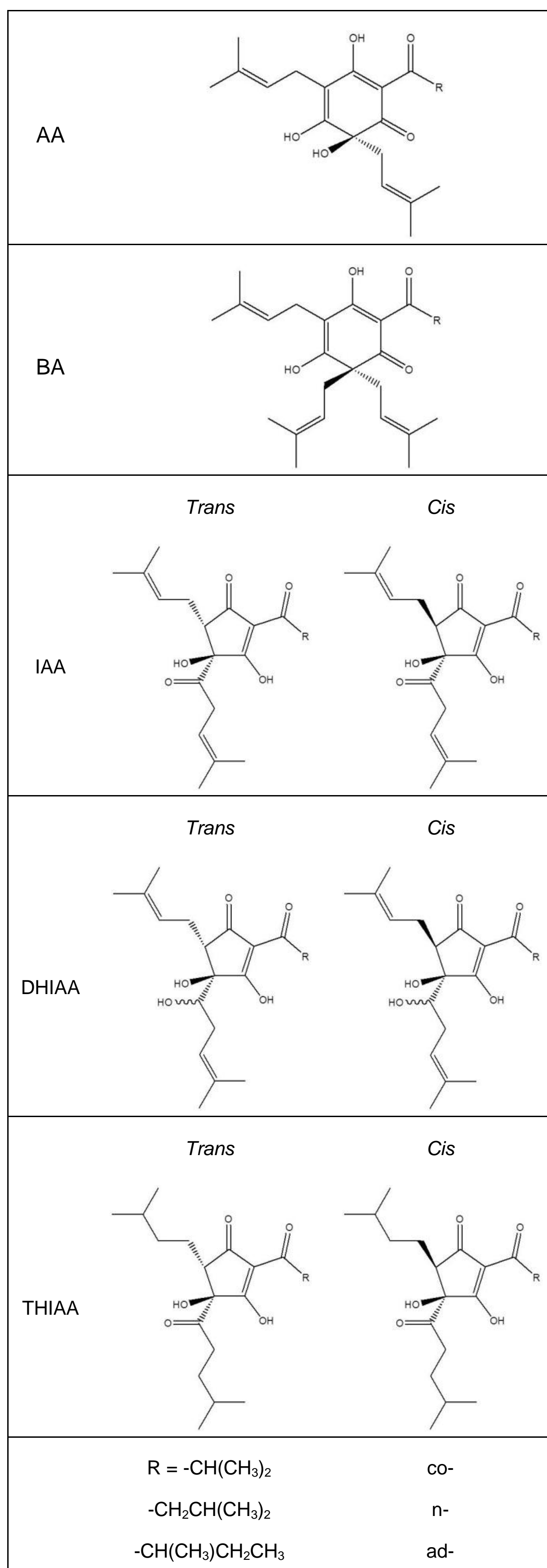


Fig.1.15 Molecular structures of hop-derived bitter acids. AA: α-acids, BA: β-acids, IAA: iso-α-acids, DHIAA: dihydro-iso-α-acids, THIAA: tetrahydro-iso-α-acids.

1.5.2 HPLC analysis of hop-derived bitter acids mixtures

An overview of the methods described in literature for the determination of α- and β-acids by HPLC for is given Table 1.2.

Table 1.2 Recommended HPLC methods for the HPLC analysis of hop α- and β-acids.

Mobile phase	Stationary phase	T (°C)	Flow rate (ml/min)	Ref.
Gradient: MeCN/ MeOH/ tris buffer pH 7.5	Nucleosil C_{18} 250 x 3 mm, 5 µm	40	0.55	[76]
MeCN/ phosphoric acid buffer pH 2.8	Alltima C_{18} 150 x 4.6 mm, 5 µm	ambient	1.8	[90]
Gradient: MeCN/ MeOH/ 5%HCOOH	Nucleosil C_{18} 250 x 4.6 mm, 5 µm	35	0.9	[31]
MeOH/ H_2O/ phosphoric acid	C_{18} 250 x 4.6 mm, 5 µm	ambient	0.8	ASBC, EBC

The methods recommended by the European Brewing Convention (EBC) and the American Society of Brewing Chemists (ASBC) rely on the use of phosphoric acid to suppress the ionization of the acidic components and to enhance retention on the column. Others tried other acidic additives, such as formic acid, but needed higher concentrations for the same performance. Also, problems related with interaction of the solutes with trace metals in the chromatographic system resulting in poor resolution have been reported. Quenching of these metal ions may be achieved by the addition of both phosphoric acid and ethylenediamine tetraacetate (EDTA).[88]

As for the α- and β-acids, methods are recommended for the HPLC analysis of iso-α-acids (Table 1.3). Separation of the *cis*-isohumulone from *trans*-iso-adhumulone proved most challenging. Ionization suppression is usually achieved by phosphoric acid. The chromatography of iso-α-acids is considered more sensitive to trace metal ions.[22] When

simultaneous analysis of iso-α-acids and its reduced derivatives is needed, citrate buffers were proposed to attain maximum resolution.

Table 1.3 Recommended HPLC methods for the HPLC analysis of hop-derived iso-α-acids and reduced derivatives.

Mobile phase	Scope	Stationary phase	T (°C)	Flow rate (ml/min)	Ref.
Gradient: MeCN/MeOH/citric acid buffer pH 7	Iso-α-acids (IAA), dihydro-iso-α-acids (DHIAA), Tetrahydro-iso-α-acids (THIAA), Hexahydro-iso-α-acids (HHIAA)	Zorbax Eclipse XDB-C_8 250 x 4.6 mm, 5 µm	25	1.0	[98]
Gradient: MeCN/MeOH/ citric acid buffer pH 7	IAA, DHIAA, THIAA	Nucleosil C_{18} 250 x 3.0 mm, 5 µm	40	0.55	[76]
MeCN/ phosphoric acid buffer pH 2.8	IAA	Alltima C_{18} 150 x 4.6 mm, 5 µm	ambient	1.8	[90]
MeCN / water/ phosphoric acid	IAA	Hipersil C_{18} 250 x 4.6 mm, 5 µm	ambient	1.5	[99]

1.5.3 LC-MS analysis of hop-derived bitter acids mixtures

During the last two decades, the optimization of the chromatographic separation parameters as well as the advantage of mass spectrometric detection significantly improved selectivity and sensitivity of the analysis of hop-derived bitter acids. Most HPLC-methods presented above use non-volatile buffer additives, such as phosphate and citrate, for the separation of hop acids and its derivatives, but they are not compatible with mass spectrometry. Some reports have been published on the LC–MS analysis of hop acids. Using ammonium acetate or acetic acid as mobile phase additives (Table 1.4), hop acids

were analyzed in beers by electrospray ionization (ESI) in the negative mode, after direct injection.[95] Vanhoenacker *et al.* described a LC-MS method for the simultaneous analysis of iso-α-acids and reduced derivatives using an ammonium acetate buffer at high pH (pH = 9.95) in combination with a solid phase that is stable at such elevated pH levels.[78]

Table 1.4 Recommended LC-MS methods for the HPLC analysis of hop-derived mixtures of α-acids, β-acids, iso-α-acids, and reduced derivatives (dihydro-iso-α-acids, tetrahydro-iso-α-acids, hexahydro-iso-α-acids).

Mobile phase	Scope	Stationary phase	T (°C)	Flow rate (ml/min)	Ref.
MeOH / 0.01M sodium acetate buffer in 20% MeOH without pH adjustment.	IAA, DHIAA, THIAA, HHIAA	Hypersil C_{18} 100 x 4.6 mm, 5 µm	ambient	1.0	100
MeCN / 8% HCOOH	AA, BA	Inersil ODS3 25 x 0.46 cm, 5 µm	ambient	0.8	94
MeCN / MeOH / ammonium acetate pH 8	IAA	XTerra C_{18} 15 x 0.21 cm, 3.5 µm	40	0.23	96
Gradient: MeCN/ EtOH/ Ammoniumacetate pH 9.95	AA, BA, IAA, DHIAA, THIAA, HHIAA	Two Zorbax Extend C_{18}	35	1.0	78

1.6 Biological activity of hop-derived bitter acids

Since ancient times, hops have been used in folkloric medicine for their claimed anti-inflammatory, antiseptic, antidiuretic, (an)aphrodisiac, hypnotic, sedative, and stomachic properties.[5, 25, 101-103] Indian tribes drank hop tea to alleviate nervousness and heated a small bag of leaves to apply in cases of ear- or toothache.[104] King George III slept on a pillow stuffed with hop cones to alleviate symptoms of porphyria.[5, 19, 105] The German Commission E approved a monograph on hops for use in mood and sleep disturbances. Similar indications are described in an ESCOP (European Scientific Cooperative on Phytotherapy) monograph.[19, 24, 106, 107]

Today, a wide range of over-the-counter preparations containing hop extracts or hop-derived products is available on the market, in particular for use in the phytotherapy of sleep disorders or pain relief and in alleviation of menopausal symptoms.[20, 101, 108, 109]

In line with a growing interest in the health benefits of plants used in traditional medicine, researchers have been trying to identify the bioactive ingredients in hops and to elucidate the underlying molecular mechanisms by which they exert their activities.

During the past decade, many pharmacological investigations *in vitro* and *in vivo* tried to produce scientific evidence of the reported traditional uses.

In recent years the estrogenic properties as well as the potential chemopreventive activities of hops have been investigated, in which much attention has gone to the bioactivity of the polyphenolic content of hops. Among this group, especially 8-prenylnaringenin has been identified as one of the most potent phytoestrogens currently known, while xanthohumol showed to have an important function in several cancer-inhibiting mechanisms.[110-112] Recently, increasing evidence reveals that the hop bitter acids, which represent up to 30% of the total lupulin content of hops, exhibit interesting effects on human health. A rising number of studies show bioactive concentrations in the lower micromolar range.

In the next section, a comprehensive overview of the current evidence for the bioactivities and pharmacological properties of hop-derived bitter acids is outlined, as a framework for the study on the aspects of the bioavailability (ADME; absorption – distribution – metabolism - elimination) of hop-derived bitter acids. The following segment is based on the review of Van Cleemput *et al.*, and updated with recent data on this subject.

1.6.1 Anticancer Potential of Hop Bitter Acids

Several natural compounds, including hop bitter acids, have been identified as promising molecules for the use in cancer chemotherapy or cancer chemoprevention. Plant-derived substances may lower the risk of developing cancer by preventing metabolic activation of pro-carcinogens, or alternatively, they can inhibit cancer development by arresting or reversing the processes of tumor initiation, promotion, and progression.[113]

1.6.1.1 *In vitro* activity: induction of apoptosis

Hop bitter acids target cancer via the induction of controlled cell death (or apoptosis) in fast-growing tumor cells. The first report dates from 1997, when Tobe and co-workers reported the apoptosis-inducing properties of humulone in promyeloid leukemia HL-60 cells. Humulone (1-100 μg/mL) induced DNA fragmentation into (oligo)-nucleosomal units, a characteristic for apoptosis, in a time- and dose-dependent manner. Interestingly, treatment with iso-α-acids (100 μg/mL) did not induce DNA breakdown, not even after overnight incubation.[114] In later studies, scientists attempted to unravel the molecular targets of hop bitter acid-initiated apoptosis. Chen and Lin used a standardized hop extract, consisting of 49.39% α-acids and 24.94% β-acids, which dose-dependently induced apoptosis in human leukemia HL-60 cells (IC_{50} 8.67 μg/mL) and, albeit to a much lesser extent, in human histocytic lymphoma U937 cells (IC_{50} 58.87 μg/mL). In this study, hop bitter acids activated the intrinsic mitochondrial apoptotic pathway by disrupting the mitochondrial membrane

potential and enhancing membrane permeability by altering the expression of the Bcl-2 family of proteins, consisting among others of the anti-apoptotic Bcl-2 and the pro-apoptotic Bax. This eventually resulted in activation of a cascade of caspases, which function as cysteine proteases thereby causing proteolytic breakdown of structural cell proteins. Furthermore, hop bitter acids stimulated the extrinsic pathway, which involved increased expression of the death receptor Fas and its ligand, FasL.[115] Recently, inhibition of proliferation by humulone and lupulone was confirmed by Tyrrell *et al.* in MDAMB- 231 (breast cancer) and SK-MES (lung cancer) cell lines in a dose- and time-dependent manner. Exposure of SK-MES cells to humulone or lupulone induced significant DNA fragmentation suggesting cell death by caspase-dependent apoptosis. Also, treatment of SK-MES cells with hop bitter acids led to a significant inhibition of adhesion to both mineralized and non-mineralized matrices, which has important implications for processes that control the development of metastases. [116]

Consistent with the above, Liu *et al.* examined the antiproliferative effects of β-acids (BA) and the structurally related compounds, hexahydro-β-acids on HL-60 cells. Hexahydro-β-acids (IC_{50} 0.71 μg/ml) and BA (IC_{50} 2.31 μg/ml) displayed strong growth-inhibitory effects against HL-60 cells and were able to induce apoptosis in a concentration- and time-dependent manner. Treatment with hexahydro-β-acids caused a rapid loss of mitochondrial trans-membrane potential, released mitochondrial cytochrome c into cytosol, increased levels of Bad and Bax, and promoted the up-regulation of Fas prior to activation of pro-caspase-8 and cleavage of Bid, suggesting the involvement of a Fas-mediated pathway in HBA-induced cells. Moreover, these changes occurred upon HBA-induced irreparable DNA damage, and enhanced expression of (GADD153) protein (growth arrest of DNA damage-inducible gene 153) in a concentration- and time-dependent manner, triggering apoptosis in HL-60 cells. [117]

In a next study of the same authors, lupulones (40 μg/mL) were reported to up-regulate Fas and FasL expression in a human metastatic colon carcinoma-derived cell line (SW620 cells). Again, mitochondrial membrane permeability was augmented in association

with an altered expression of Bcl-2 and Bax proteins.[118] More detailed investigation revealed that a crucial role was determined for the TNF (tumor necrosis factor)-related apoptosis-inducing ligand (TRAIL)-R1 and -R2 receptors, which were up-regulated and activated by lupulones (40 µg/mL) in both TRAIL-sensitive (SW480) and TRAIL-resistant colon cancer cells (SW620).[119] Because p53 plays a central role in the response to cellular stresses by up-regulating the transcription of several genes controlling apoptosis, the involvement of p53 on lupulone-triggered apoptosis was studied in a successive investigation. Interestingly, both cell lines SW480 and SW620, which exhibit the same p53 mutations, showed opposing responses of p53 upon lupulone treatment (40 µg/ml). In SW620 cells, lupulone up-regulated p53 gene expression and caused a cloistering of p53 in the nucleus, allowing p53 to play a pro-apoptotic role by activating the TRAIL-death receptor pathway. In contrast, in SW480 cells, p53 was translocated to the cytoplasm where it initiated a survival response with the up-regulation of anti-apoptotic Bcl-2 and Mcl-1 proteins in an attempt to preserve mitochondrial integrity. These pro-survival effects of p53 in lupulone-treated SW480 cells were inverted by pifithrin-α (PFT-α), p53 function inhibitor, which caused a blocking of p53 in the nucleus leading to the down-regulation of Bcl-2 and Mcl-1, the up-regulation of pro-apoptotic Bax protein and TRAIL-death receptors leading to enhanced cell death.[120] Since the mitogen-activated protein kinases (MAPKs) control fundamental cellular processes such as apoptosis, the role of Jun *N*-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 inhibitors on lupulone-triggered apoptosis was determined. Data showed that only p38 played a major role in by activation of p53 and the TRAIL-death receptor apoptotic pathway in SW 620 cells.[121]

1.6.1.2 Antiproliferative activity of hop bitter acids

Hop bitter acids show antiproliferative activity, thus arresting cell growth of invasive cancer cells.[118, 122] The β-acids, lupulone and colupulone, and a semi-synthetic derivative, hexahydrocolupulone, inhibited cell growth of several human cell lines: hexahydrocolupulone

was the most potent variant with a wide spectrum of activity against solid tumors and leukemia's, as well as against drug-resistant cell lines (IC_{50} values of 0.85 and 2.19 μM, respectively). It caused cell cycle arrest (G0/G1 phases) and affected the incorporation of precursors into their macromolecules, resulting in disrupted DNA, RNA, and protein synthesis.[122]

Humulone inhibited the proliferation of human leukemia U937 cells (IC_{50} 3.4 μM) and slightly induced their differentiation, as concluded from higher nitroblue tetrazolium reducing and lysozyme activities, both typical differentiation markers. Agents that inhibit proliferation and enhance the conversion of premalignant cells to differentiated cells are expected to reduce cancer development. Humulone enhanced the differentiation of U937 monocytes induced by vitamin D3, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), all-*trans*-retinoic acid, and tumor necrosis factor alpha (TNF-α). These effects were similar in other myelogenous leukemia cells, such as K562, HEL, KU812 erythroleukemia cells, promyelocytic leukemia HL-60 cells, monoblastic THP-1 cells, and myeloblastic ML-1 leukemia cells.[123]

1.6.1.3 Inhibition of Angiogenesis

The formation of new capillary blood vessels for the supply of oxygen and nutrients, also named angiogenesis, plays a key role in the development of malignant tumors. Shimamura and co-workers reported that humulone dose-dependently prevented angiogenesis in chick embryo chorioallantoic membranes (CAMs), with an ED_{50} (the "effective dose" at which 50% of angiogenesis is inhibited) of 1.5 μg/CAM. Humulone (10 μM) inhibited tube formation by vascular lung endothelial cells from rats and reduced cell growth of endothelial mouse KOP2.16 cells, stimulated by basic fibroblast growth factor (bFGF), by 20%. Furthermore, 100 μM humulone suppressed the expression of vascular endothelial growth factor (VEGF), which contributes to angiogenesis, more significantly in tumor cells (Co26s) than in endothelial cells (KOP2.16).[124]

Lupulone (2.5-50 μg/mL) induced a concentration-dependent inhibition of HUVEC endothelial cell proliferation and chemotaxis toward fibronectin. Furthermore, the formation of closed capillary-like structures was reduced in a Matrigel morphogenesis assay, indicating a strong inhibitory effect on neovascularization.[125] Nitric oxide (NO) is a gaseous free radical involved in the production of VEGF, the overexpression of which induces angiogenesis and vascular hyperpermeability, and accelerates tumor development. The ethyl acetate-soluble fraction of hop cones, containing hop bitter acids, inhibited both NO-production and expression of inducible nitric oxide synthase (iNOS) in RAW 264.7 mouse macrophages, stimulated by a combination of lipopolysaccharide (LPS) and interferon-γ cytokine (IFN-γ). Within this fraction, the strongest effect was observed for xanthohumol, whereas lupulone and some of its oxidative degradation products inhibited NO-production, but without reducing iNOS-expression. The oxidation products, exhibited either much weaker inhibitory activities on NO-production than lupulone or false inhibitions with strong cytotoxicity, indicating that oxidation may reduce the inhibitory activity of lupulone on NO-production.[126]

Hexahydro-β-acids, reduced derivatives of β-acids (BA), significantly inhibited protein and mRNA expression of iNOS and cyclooxygenase-2 (COX-2) in murine RAW 264.7 macrophages activated with lipopolysaccharide (LPS). Both BA and hexahydro-β-acids inhibited concentration-dependently nitrite-production (indicator for NO), but the inhibitory effect of hexahydro-β-acids (IC_{50} 3.3 μg/mL) was more significant, compared to the BA (IC_{50} 8.9 μg/ml). For PGE_2-production, hexahydro-β-acids inhibited PGE_2- increase in LPS-stimulated RAW264.7 cells in a dose-dependent manner, while the inhibitory effect for BA was only observed under higher concentration.[127]

Several authors provided experimental support for the tumor promoting function of IKKβ and the classical NF-κB pathway in several distinct models of cancer [32, 45,47, 48, 57], supporting the hypothesis that transcription factor NF-κB (nuclear factor kappa B) and the signaling pathways that control its activity provide a molecular link between inflammation and cancer. Inflammatory signaling is highly regulated by a network of transcription factors that modulate gene transcription in response to pro-inflammatory stimuli, such as cytokines,

pathogens, and oxidative stress. For example, when TNF-α triggers its cognate membrane receptor, an intracellular cascade of kinases is activated, which leads to the release of NF-κB from its inhibitor in the cytoplasm. Released NF-κB can then translocate to the nucleus, where it initiates the formation of a functional transcriptome, leading to increased expression of cytokines, enzymes, and adhesion molecules. Furthermore, constitutive NF-κB-activation is often detected in cancer.

In a mechanistic study in LPS-stimulated RAW 264.7 cells, DHIAA selectively inhibited the NF-κB pathway, while having no effect on ERK1/2, p38 and JNK phosphorylation, nor on the transactivation of CRE (a known transcriptional factor regulated by MAPK), demonstrating specifically inhibiting the NF-κB signaling pathway but not MAPK pathways. GSK3 (α and β) were inhibited by DHIAA and phosphorylation of a known GSK-3 substrate, β-catenin, was inhibited by DHIAA in, showing similar mode of action to a known GSK-3 inhibitor, SB216763, which also inhibited β-catenin. In addition, DHIAA inhibited NF-κB-mediated inflammatory markers in various cell models, including NO in LPS-stimulated RAW 264.7 cells, RANKL-mediated tartrate-resistant acid phosphatase (TRAP) activity in transformed osteoclasts, and TNF-α/IL-1β-mediated MMP-13 expression in SW1353 human chondrosarcoma cells.[128]

Hexahydro-β-acids inhibited the transcriptional activity of NF-κB in LPS-stimulated murine macrophages by blocking phosphorylation of inhibitor κB (IκB) α and p65. Also, hexahydro-β-acids inhibited LPS-induced activation of PI3K/Akt, extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK. Independently, our research group established a dose-dependent reduction of NF-κB-dependent gene transcription by α-acids, β-acids (0.5-10 μM), and iso-α-acids (25-200 μM).[129]

It was observed that dihydro-iso-α-acids (DHIAA) and tetrahydro-iso-α-acids (THIAA) dose-dependently reduced NF-κB nuclear translocation and abundance in LPS-stimulated RAW 264.7 macrophages. Also, a commercially available extract, consisting of DHIAA

(META060) dose-dependently inhibited prostaglandin E_2-and NO-formation, inducible but not constitutive COX-2 abundance, and NF-κB activation in LPS-stimulated RAW 264.7 macrophages.

1.6.1.4 Induction of CYP-450 Enzymes

Several compounds induce the expression of detoxification enzymes of the cytochrome P450 system, which are very important in the metabolism and subsequent activation and/or inactivation of many xenobiotics including pro-carcinogens. As a part of a mouse diet, colupulone (0.18%) increased the P-450 content of the liver microsomes and stimulated various phase I enzyme activities, such as those responsible for demethylation of ethylmorphine and aminopyrine and the hydroxylation of aniline and benzo[*a*]pyrene.[130] An up-regulation of multiple CYP-450 enzymes, in particular of CYP3A and CYP2B, was detected independently by western and northern blotting.[130, 131] Similar results were obtained after administration of a hop hexane extract (0.33%) and crude hops (1%).[130]

If hops and colupulone, in particular, are able to induce CYP-450 enzymes in species other than the mouse, then ingestion might have a significant impact on the bioactivation and/or detoxification of food-borne pro-mutagens. However, short-term administration of colupulone to the rat (0.36%) did not alter the *ex-vivo* CYP450-mediated conversion of the pro-mutagens alfatoxin B1 and benzo[*a*]pyrene to their mutagenic forms, as measured in *Salmonella typhimurium* and mammalian microsomal assays.[131] In two comparative studies, published by Foster and co-workers, several brands of beer were examined for their potential to affect human cytochrome P450-mediated biotransformation. Initial findings were confirmed that some beers had a potential to affect the safety and efficacy of medications and supplements metabolized by CYP2D6, CYP3A4, or CYP2C9 isozymes. Moreover, dose-dependent inhibition of CYP3A4, CYP3A5, CYP3A7, and CYP19 (aromatase) by β-acid content was significant. Further studies are required to determine the clinical significance of these findings.[132, 133] Up to the present, no further studies addressing the

effects of long-term administration of hops, or individual hop constituents, on *in vivo* CYP-450 enzyme activity have been carried out.

Hall and co-workers presented a study probing the potential inhibition of cytochrome P450 (CYP) catalytic activity of six isozymes CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, collectively involved in the metabolism of approximately 85% of all phase I-metabolized drugs and other xenobiotics,[134] by a commercial mixture of dried magnesium salts of dihydro-iso-α-acids (DHIAA). Results showed CYP2C9 was the most strongly inhibited CYP isozyme (IC_{50} of 0.30 μg/mL DHIAA), while CYP2C19 was moderately inhibited (IC_{50} 6.3 μg/mL). CYP3A4 was weakly inhibited by DHIAA. No inhibition was noted at the highest concentrations of DHIAA tested for 1A2 (>100 μg/ml), CYP2D6 (>100μg/ml) and CYP2E1 (>50 μg/ml).[135]

1.6.1.5 *In vivo* activity

In rats, adding lupulone (0.001% and 0.005%) to the diet reduced the development of colon carcinogenesis, initiated by azoxymethane, in a dose-dependent way. Both the number of pre-neoplastic lesions (aberrant cryptic foci, ACF) and the total number of tumors in the colon were dramatically reduced.[118] In a similar experimental protocol, oral administration of an isomerized hop extract containing 30% iso-α-acids (0.01% or 0.05%) proved to reduce the number of ACF in the colon, as well as the prostaglandin E2 (PGE_2) levels in the mucosa.[136] Topical application of humulone (1 mg/mouse) protected against tumor formation in mouse skin, initiated by 7,12-dimethylbenz[α]anthracene (DMBA) and promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA).[137]

Humulone significantly inhibited TPA-induced epidermal cyclooxygenase-2 (COX-2) expression, for which the levels are up-regulated during carcinogenesis and inflammation.[138] Additionally, orally administered lupulone (0.01% in drinking water for 21 days) inhibited new vessel formation in mice by 50%. Neovascularization was determined by measuring the

hemoglobin content of Matrigel plugs, implanted under the mouse skin, and was standardized for control plugs of mice receiving tap water containing excipient.[125]

1.6.2 Hop Bitter Acids in Inflammatory Disorders

Since long ago, the hop plant has been known for its anti-inflammatory properties, as American Indians (the Delaware) used hops traditionally to relieve toothache and earache.[104] Yasukawa and co-workers screened 100 edible plant extracts against TPA-induced inflammation in mice in an attempt to find new herbal anti-inflammatory compounds. They identified a hop methanolic extract as a potent inhibitor of TPA-induced ear edema and identified humulone as the active compound. In comparison with standard drugs, humulone (ID_{50} 0.2 mg/ear) was a less effective inhibitor than the steroid hydrocortisone (ID_{50} 0.03 mg/ear), but compared well with the inhibition potency of the nonsteroidal indomethacin (ID_{50} 0.3 mg/ear).[139] Similarly, humulone was found to inhibit ear edema in mice, induced by arachidonic acid.[137]

1.6.2.1 *In vitro* activity

Cyclooxygenases (COX) are key enzymes required for the transformation of arachidonic acid to a wide range of prostanoids, including PGE_2 and thromboxane A2 (TxA_2). The COX-2 isoform is highly up-regulated by cytokines at sites of inflammation, whereas, in contrast, COX-1 is constitutively expressed in many cell types, where it has homeostatic functions in gastric cytoprotection and platelet activation.[140] The so-called "COX-2 hypothesis" assumes that the gastroduodenal toxicity of traditional nonsteroidal anti-inflammatory drugs (NSAIDs), which block both COX isoforms, is mainly related to their inhibition of COX-1-dependent PGE_2- and TxA_2-formation in the gastric epithelium and platelets, while COX-2 has a major role in pain mediation, inflammation, and pyresis.[141] Specific inhibitors for the COX-2 isoform, including celecoxib (Celebrex), are used for the

treatment of osteoarthritis and rheumatoid arthritis, particularly in patients at high risk of developing gastrointestinal complications.[142]

From several studies, individual hop acids demonstrated interesting anti-inflammatory therapeutic properties, by selectively inhibiting COX-2 up-regulation by pro-inflammatory mediators. Humulone suppressed the TNF-α-dependent release of PGE_2 in murine osteoblastic MC3T3-E1 cells (IC_{50} 0.030 μM) and reduced COX-2 enzyme activity, mRNA-expression, and promoter activity. These effects were similar for the glucocorticoid dexamethasone, but, for humulone, the glucocorticoid receptor was not involved. Results from *in vitro* enzymatic assays showed inhibition of the catalytic activity of COX-2 by humulone with an IC_{50} value of 1.6 μM, whereas COX-1-activity was not inhibited below 10 μM.[143] On screening hop bitter acid-containing formulations for COX-2-inhibition in LPS-induced mouse macrophages RAW 264.7, a number of these emerged as strong anti-inflammatory agents with no effects on PGE_2 originating from the constitutive form of the enzyme. Furthermore, the hop acids studied left constitutively secreted COX-1 in human gastric mucosa cells unaffected, which is predictive of a low gastrointestinal toxicity.[144] Interestingly, in a study presented by Liu and co-workers, hexahydro-β-acids significantly blocked protein and mRNA expression of COX-2 (but not COX-1) and iNOS in LPS-induced RAW264.7 macrophages in a dose-dependent manner.

Also, reduced iso-α-acids (dihydro-iso-α-acids and tetrahydro-iso-α-acids) (1-20 μg/mL) inhibited PGE_2-release from LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner by inhibiting inducible COX-2 protein expression.[135, 145]

Furthermore, after an interferon-γ-combined stimulation with LPS, iso-α-acids inhibited PGE_2-production in a dose-dependent way.[136] Independently, a standardized carbon dioxide extract from hops dose-dependently inhibited PGE_2-production in LPS-stimulated human peripheral blood mononuclear cells (PBMC) without compromising metabolic activity (IC_{50} 3.6 μg/mL). Using human blood (whole blood assay), PGE_2 production was not decreased after selective activation of COX-1 by calcimicin.

In contrast, the hop extract inhibited PGE_2-concentrations in blood, pretreated with aspirin to inactivate COX-1 and supplemented with LPS to induce COX-2. Thus, the hop extract was concluded to inhibit COX-2 selectively with a calculated IC_{50} of 20.4 µg/mL. The large differences in IC_{50} (PBMC vs. WBA) was explained by different experimental conditions: in whole blood assays, using 50% blood, a larger concentration of plasma proteins is present, which might interfere with the hop extract when compared to the PBMC, cultured in 10% FCS.[146]

1.6.2.2 *In vivo* activity

Our research group studied the effects of hop bitter acids in mice, in which acute inflammation was induced by subcutaneous injection of zymosan in the paw. Intraperitoneal (ip) administration of 250 µg of iso-α-acids or α-acids effectively inhibited paw edema, a characteristic symptom of inflammation, and this effect was similar for administration as potassium salts in phosphate-buffered saline (PBS) or as neutral acids in dimethylsulfoxide (DMSO), suggesting that both formulas are equally well absorbed from the peritoneal cavity.[129]

In contrast, Hougee and co-workers administered a carbon dioxide hop extract orally to mice (1.25 mg by oral gavage for 10 days) in which acute arthritis was induced by injection of zymosan into the knee, but failed to detect a reduction of inflammation-related symptoms. The orally administered hop carbon dioxide extract neither inhibited joint swelling, nor restored the inhibited proteoglycan synthesis in the arthritic cartilage.

On the other hand, upon stimulating the blood of mice with LPS *ex vivo*, PGE_2-production was 24% lower in samples from mice treated with the hop extract, compared to vehicle-treated mice, thus suggesting that the extract does become bioavailable. These contradictory findings can be explained by the low bioavailability of hop bitter acids after oral

intake. It was suggested, therefore, by the authors that the dose of 1.25 mg of hops extract be increased in order to lead to a detectable reduction of parameters for inflammation.[146]

Mice with collagen-induced arthritis were orally dosed with 10, 50, or 250 mg/kg of DHIAA, and symptoms of joint swelling and the arthritic index were evaluated. It was found that DHIAA and THIAA dose-dependently reduced the arthritis index and, at 250 mg/kg, their efficacy was similar to that of 20 mg/kg of celecoxib, the positive control.[147, 148] Analyses of individual markers revealed that, compared to the diseased controls, DHIAA (250 mg/kg)-treatment significant reduced joint destruction, cartilage degradation, and bone erosion.[128] Similar results were obtained by oral administration of META060, a commercially available extract consisting of dihydro-iso-α-acids, in mice with carrageenan-induced acute inflammation.[149]

Next to these results in animal studies, proof of effectiveness of anti-inflammatory activity of hop-derived bitter acids in humans is limited and suggestive: in an 6-week, open-label trial of human subjects exhibiting knee osteoarthritis, the anti-inflammatory activity of DHIAA (1000 mg/day for 6 weeks) was assessed using the validated WOMAC and visual analog scale (VAS) questionnaires. In all questionnaires, a reduction in score was due to a reduction in symptoms, such as pain and stiffness. After 6 weeks, RIAA administration led to a 54% reduction in the WOMAC global score.[135]

1.6.3 Hop Bitter Acids Improve Markers for Metabolic Syndrome

In modern western society, the prevalence of metabolic diseases is taking on epidemic proportions and implicates a high risk of mortality due to cardiovascular complications. The so-called “metabolic syndrome” is defined as a cluster of abnormalities covering insulin resistance, central obesity, impaired glucose tolerance or type 2 diabetes, dyslipidemia, hypertension, hypercoagulability, atherosclerosis, and elevated rates of inflammatory blood markers.[150] Current treatment is based on diet, exercise, and specific

lipid-altering drug therapy, supplemented with anti-diabetic agents that improve some of the associated complex atherogenic parameters. Plants, as extracts or isolated pure compounds, have already been shown to play a valuable role in the prevention or treatment of lifestyle-related disorders.[151] Iso-α-acids have been shown to improve health by positively influencing lipid metabolism, glucose tolerance, and body weight.

1.6.3.1 *In vitro* activity

Peroxisome proliferator-activated receptors (PPARs) are a class of nuclear receptors that are essentially involved in the regulation of fatty acid and carbohydrate metabolism. Fibrates, agonists of the PPAR-α subtype, are clinically used for the treatment of dyslipidemia, whereas the glitazones, PPAR-γ agonists, improve insulin sensitivity in type II diabetes. Positive results of hop iso-α-acids on the lipid profile in rodents suggested a direct agonistic effect on PPARs. Indeed, these compounds dose-dependently (1-30 μM) activated PPAR-α and PPAR-γ in HepG2 and CV-1 cells, respectively. Using chimeric expression plasmids in which the ligand-binding domain of PPAR-α or PPAR-γ was fused to the DNA-binding domain of the yeast transcription factor GAL-4, iso-α-acids bound to PPAR-α as well as PPAR-γ, thereby increasing the transcription of a co-transfected reporter gene, containing five GAL-4 binding sites coupled to the luciferase-encoding sequence.[152] However, this result could not be substantiated in our research group when transfecting full-length receptors together with a PPRE (PPAR response element) containing luciferase reporter in COS1L2A cells.[129]

1.6.3.2 *In vivo* activity

In a mouse model of non-insulin-dependent diabetes (KK-A^y mice), co-administration of iso-α-acids improved hyperglycemia and hyperlipidemia, similar to the PPAR-γ agonist pioglitazone, but, without a concomitant increase in body weight. In the liver, genes for acyl-

CoA oxidase (ACO) and fatty acid translocase (FAT) were highly up-regulated, resulting in an enhanced lipid metabolism. Unexpectedly, iso-α-acids increased only moderately the expression of the adipose differentiation related protein (ADRP) and lipoprotein lipase (LPL) genes, involved in lipid uptake and storage in white adipose tissue. Similar effects were observed in diet-induced obese diabetic C57BL/6N mice. Co-administration to C57BL/6N mice of a high-fat diet and an isomerized hop extract, containing high amounts of iso-α-acids, dose-dependently reduced body weight gain, improved glucose tolerance, and slightly reduced insulin resistance, compared to the control group. In white adipose tissue, apoptosis of hypertrophic adipocytes was induced, next to an increased number of small adipocytes, thus improving insulin sensitivity.[152]

When feeding the mice an iso-α-acid-rich diet, supplemented with high amounts of cholesterol, a drastic improvement of atherosclerotic clinical parameters was observed. Indeed, an increase in plasma HDL-cholesterol and a reduction in the liver content of cholesterol and triacylglycerol were observed. Similar results were obtained feeding the animals a standard diet containing only hop iso-α-acids.[153] In general, lipid metabolism in the liver was enhanced by up-regulated levels of acyl-CoA oxidase (ACO), acyl-CoA synthetase (ACS), and fatty acid transport protein (FATP) mRNA, which control cellular fatty acid uptake and peroxisomal *β*-oxidation, next to elevation of apoprotein CIII and lipoprotein lipase (LPL) content, crucial for the metabolism of triacylglycerol.[153-155] The changes in lipid metabolism correspond to those of the PPAR-α agonist, fenofibrate, and were not found in PPAR-α-deficient mice, thus suggesting that iso-α-acids operate via an analogous mechanism.[154, 155] Iso-α-acids also inhibited absorption of dietary fat in rats, which further supports a negative effect on body weight gain. As a possible target, isomerized hop extract decreased the pancreatic triacylglycerol lipase activity in a dose-dependent manner, thus elevating the undigested lipid content in the feces.[153]

Concerning cardiovascular parameters, rats on a high-salt regimen did not develop a higher mean blood pressure when iso-α-acids were incorporated into the diet (0.3%). It was

proposed that these compounds reduce oxidative stress and restore the lower levels of bioavailable nitric oxide (NO) caused by the high-salt diet. As NO is a critical messenger molecule for the kidney to maintain salt and water homeostasis, increased bioavailable NO could protect against developing hypertension.[156]

One pilot study in humans has been carried out in which oral iso-α-acids ameliorated insulin sensitivity in mild type 2 diabetic patients by decreased blood glucose and hemoglobin A1c levels.[152] Above results were confirmed in a 12-week double-blind human study in which pre-diabetic subjects ingested capsules with iso-α-acids (16 mg, 32 mg or 48 mg p/day). Next to a decrease in blood glucose and hemoglobin A1c, treatment resulted in a decreased body mass index (BMI) and total fat area.[157]

1.6.4 Role of Hop Bitter Acids in Osteoporosis

Osteoporosis develops when the balance between bone formation and bone resorption is disturbed, and, consequently, it is considered feasible to prevent osteoporosis by promoting bone formation or by inhibiting bone resorption. Humulone inhibited the formation of osteoporotic lesions in dentine slices (pit formation assay) with an IC_{50} value of 5.9 nM. The ad-homologue was equally active, while cohumulone showed no inhibitory activity. Also, lupulone was reported to be a strong inhibitor of bone resorption.[158, 159]

However, the question remains as to what extent these *in vitro* data are valuable in the *in vivo* setting. It was reported that long-term administration to rats of a hop powder-enriched diet (further undefined), either alone or combined with isometric strength training, did not improve bone parameters. However, the authors concluded that the body weights were significantly lower in those rats fed with the hop diet than in the control group. Therefore, it might have been difficult to detect positive effects of hops on bone, because lower body mass is associated with lower bone mass.[160] It should also be mentioned that recent studies have established an unequivocal relationship between osteoporosis and inflammation.[161] For example, elevated serum levels of systemic inflammation markers such

as interleukin-6, TNF-α, and high-sensitivity C-reactive protein are correlated significantly with a lower bone mineral density.[162, 163] Therefore, the direct anti-inflammatory effects of hop acids could also contribute in the prevention and treatment of osteoporosis.

1.6.5 Effects of Hop Bitter Acids on the Central Nervous System

For quite a long time, from when it was observed that hop pickers tired easily, the hop plant has been reputed to possess sedative properties.[20, 164] At present, hop-based preparations, mostly in combination with valerian, are marketed widely as a natural remedy for sleeping disorders, nervousness, and insomnia.[165-167]

Already by the beginning of the 20th century, the sedative activity of various hop extracts and components was shown using frogs. In general, hop extracts reduced the excitability of the striated muscles and motor nerve endings, diminished the irritability of the nervous system, and induced narcosis.[168, 169] However, the effect seemed to be highly species-dependent, since in rabbits, hops caused opposite effects such as increases in body temperature and dyspnea.[169]

As both hop extract, freed from hop acids, and hop essential oil were devoid of activity, as early as 1938, Sikorski and Rusiecki pointed to the hop bitter acids humulone and lupulone as active compounds for the observed sedative activity upon administration of hops to pigeons and small birds.[170] Later on, Hänsel and co-workers suggested that it was not the hop bitter acids but rather a degradation product produced during storage that is responsible for the tranquilizing properties of hops.[171-173] They reported that degradation of humulones and lupulones can result in the formation 2-methylbut-1-en-3-ol, a C_5-alcohol, by a radical-type auto-oxidation in the presence of atmospheric oxygen.[174] Both in rats[175] and mice,[176] ip administration of this decomposition product showed sedative activity at low doses and induced transient deep narcosis at higher doses.

However, only hop teas and balneotherapeutic preparations were found to contain effective amounts of 2-methylbut-1-en-3-ol, whereas its content in "sedative dragees" was negligible. Therefore, their effectiveness could be questionable. Still, these dragees contain high amounts of hop acids, which, according to Hänsel *et al.*, could act as precursors of the active compound via a similar radical-type degradation reaction *in vivo*.[173]

Over the past decade, the neuropharmacological activity of hops has been reinvestigated in more detail using recent techniques including receptor binding assays. Both a carbon dioxide hop extract and an isolated α-acid fraction proved to modulate CNS activity in rats.[177] It was found that both products dose-dependently enhanced pentobarbital-induced sleeping time without influencing locomotor activity, a marker for motor behavior. In addition, anti-depressant activity similar to the reference drug imipramine was observed for both hop preparations (forced swimming test), whereas no anxiolytic effects could be observed (Elevated Plus Maze test).

In contrast, oral administration of a β-acid fraction increased locomotor activity and caused a reduced percentage of animals falling asleep on pentobarbital administration. It was shown that β-acids can interfere with the GABAergic system (GABA; γ-aminobutyric acid), leading to a general reduced neurotransmission in CNS.[178] Negri *et al.* investigated the anxiolytic properties [Elevated Plus Maze (EPM) test and Neophobia test] of a hydroethanolic hop extract in male Wistar rats, which received orally doses of 250, 500, 750 and 1000 mg/kg of a hop extract. Results showed that the extract of significantly increased the time of permanence on the open arms of the EPM with all doses tested. The extract also increased significantly the amount of food intake during the 60 min of evaluation in the Neophobia test.[179]

In contrast to the work by Zanoli *et al.*, reduced locomotor activities on administration of either carbon dioxide or ethanolic hop extracts to mice were observed by Schiller *et al.*, albeit using much higher dosages. Interestingly, they did observe an increased sleeping time on treatment with a narcotic drug and, moreover, they observed a reduced body temperature, another parameter indicating sedative activity. Furthermore, by using a range

of different enriched fractions, it was indicated that various components including α-acids, β-acids, and hop oil all contribute to the overall sedative activity of hops.[180]

1.6.6 Bactericidal Activity of Hop Bitter Acids

Initially, hops were used for prolonged storage of beer. Adding hops reduces the growth of *Lactobacillus*, the main beer contaminant, which otherwise would affect yeast performance, cause losses in ethanol yield, and form undesirable off-flavors.[181] The preservative properties of hops have been investigated for many years and, despite some reports on the anti-bacterial activity of hop oil,[182] the bitter acids seem to be the main active compounds. The target bacteria are Gram-positive species, such as *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Micrococcus*, and *Bacillus*.[25, 183-189] In contrast, Gram-negative bacteria, such as *Escherichia coli*, are either resistant or only affected at very high concentrations of hop acids. However, the combination of hop resins with sodium hexametaphosphate showed strong antimicrobial activity against *E. coli*.[190] Yeast is not inhibited, which is very important for the use of hops in beer production.[187, 191] Some inhibitory activity has also been reported for certain fungi, such as *Penicillium* and *Aspergillus* species.[185, 192, 193]

In general, lupulone has greater antimicrobial activity than humulone, which is, in turn, more active than isohumulone.[194] It appears that the more prenyl groups (three in the β-acids) are present, the stronger the bacteriostatic action is. However, the role of iso-α-acids in beer preservation is of great value, since they represent quantitatively the main contribution of hops to beer. In all studies, hop acids may behave as either bacteriostatic substances or bactericides, depending on the conditions employed.

Growth of *Listeria monocytogenes* was inhibited in culture media and in certain foods by hop extracts containing varying concentrations of α- and β-acids. The lowest inhibitory concentrations were observed for extracts containing the highest concentrations of β-acids.

In food matrix, these hop extracts showed varying magnitudes of inhibition, but, overall, the anti-microbial activity in food appeared to increase with acidity and lower fat content.[194]

In bacteriological broth, the addition of β-acids (1.0-5.0 µg/mL) inhibited growth of *L. monocytogenes*, with inhibition being more pronounced at higher concentrations and at lower storage temperature (4°C). Moreover, the anti-listerial activity of β-acids (0.5 to 3.0 µg/mL) was enhanced when combined with sodium diacetate, acetic acid, or potassium lactate. [195] In a succeeding study on the storage of frankfurters, dipping of *L. monocytogenes* inoculated-frankfurters in solution of β-acids (0.03-0.10 w%), before low-temperature storage for up to 90 and 48 days, caused *L. monocytogenes* reductions and complete suppression of pathogen growth for 30 to 50 days (4°C) or 20 to 28 days (10°C), with anti-listerial effects increasing with higher concentrations. Further analysis revealed that the presence of β-acids resulted in an extension of the lag-phase duration of the pathogen, and decreased growth rate. [196] It was also found that a low level of iso-α-acids in beer wort is sufficient to inhibit growth of *L. monocytogenes*.[197] It has to be mentioned that food preservation with hop acids requires quite high levels, which may impart undesirable flavors and aroma characteristics.

Shimwell noted that the anti-septic potency of hops increased at low pH, which was attributed to changes in permeability of the bacterial cell wall.[186] This hypothesis was confirmed in *Bacillus subtilis*, in which lupulone, humulone, and isohumulone caused cell wall lesions by incorporation into the cytoplasmic membrane. This activity resulted in inhibition of active transport of sugars and amino acids and, subsequently, led to inhibition of cellular respiration and synthesis of proteins, RNA, and DNA.[191]

Later on, Simpson identified the mechanism by which *trans*-isohumulone inhibits the growth of the beer-spoilage bacterium *Lactobacillus brevis*. Apparently, the iso-α-acids act as mobile carrier ionophores, catalyzing electroneutral influx of undissociated molecules, as well as their internal dissociation and efflux of their complexes with divalent cations such as Mn^{2+}. Consequent loss of the proton gradient inhibits the uptake of sugars and causes starvation in bacterial cells. The properties of other hop acids are similar to those of *trans*-

isohumulone, confirming a similar mechanism.[57] Since hop acids are weak acids and only undissociated forms are active, the antibacterial properties fall with higher pH-values. Furthermore, the potency is enhanced by increasing the hydrophobicity of the molecules, as determined by the acyl side chain length and the number of prenyl groups.[57, 187] In a comparative study on the antibacterial activity of iso-α-acids (IAA), dihydroiso-α-acids (DHIAA), and tetrahydroiso-α-acids (THIAA) against some Gram-positive bacteria (*Lactobacillus* and *Pediococcus*), it was observed that the minimum inhibitory concentration (MIC) decreased when the degree of hydrogenation of the analyzed compounds increased, $[MIC]_{THIAA} < [MIC]_{DHIAA} < [MIC]_{IAA}$. This confirms the former observation that increased hydrophobicity (lipophilicity) leads to a greater anti-microbial activity. The authors stated that the increased hydrophobicity renders a compound more prone to interaction with the cell membrane, thus explaining the observed effects.[198]

Varying applications have been examined to exploit the bacteriostatic activity of the β-acids, for example, to control and reduce bacterial activity in the sugar industry. Pollach and co-workers presented a study on the application of lupulones as bacteriostatic in the sugar industry in order to potentially replace formaldehyde as disinfectant. It was found that addition of hop β-acids could help reducing the formation of lactic acid. [199] Also, addition of a commercially available alkaline solution of hop β-acids, named BetaStab® 10A, (0 – 160 mg/L), to a thick juice (a concentrated intermediate product in the production of beet sugar) delayed acidification which is related to bacterial growth and the development of fastidious bacteria in a concentration-dependent manner. The addition of β-acids extended the lag time of colony formation, thus leaving the number of colonies unaffected. In this way, β-acids do not prevent the thick juice from deteriorating, but they could significantly delay its degradation.[200]

In a study on the evaluation of the anti-bacterial properties of hop bitter acids (α-acids, β-acids, iso-α-acids, and reduced derivatives) against different strains of bacteria involved in primary or secondary skin and soft tissue infections (*Propionibacterium acnes,*

Staphlylococcus epidermidis, Staphylococcus aureus, Kocuria rhizophila and, *Staphylococcus pyogenes*), all hop acids tested showed inhibitory effects, though lowest MIC-values were observed for lupulones; 0.1 mg/ml against *Propionibacterium acnes* and *Staphylococcus pyogens* and 1 mg/ml against *S. epidermidis*, *K. rhizophila*, and *S. aureus*, respectively. These low MIC values are comparable to the most commonly prescribed antibiotics for topical acne treatment (e.g. clindamycin and erythromycin).[201]

Also, there are a few reports on the antibiotic properties of hops in relation to tuberculosis infection. Chin *et al.* demonstrated that lupulone inhibits the growth of a virulent strain of *Mycobacterium tuberculosis in vitro* and considerably suppressed the development of tuberculotic lung lesions in mice when administrated either intramuscularly or intragastrically.[184, 202] Humulone also proved to be effective, although to a lesser extent, while the iso-α-acids were negative.

A detailed study of influencing parameters showed that lupulone remains active, regardless of experimental variations in pH, NaCl-concentration, and serum content.[203] Indeed, in a small-scale study in tuberculosis patients, daily oral administration of 5 g of lupulone for 3 months was considered therapeutically active, without toxicity.[204] However, the most suitable method of administration and the possible development of drug resistance have not been investigated.

Limited evidence is available on the anti-viral activity of hop acids. The iso-α-acids were shown to have a low to moderate anti-viral activity against several DNA and RNA viruses (IC_{50} in low µg/ml range), whereas no anti-viral activity was detected for the hop β-acids.[205]

1.6.7 Hop Bitter Acids as Potent Antioxidants

Various health-promoting effects of plant compounds can be attributed to their intrinsic anti-oxidant activities: they neutralize cell damage caused by reactive oxygen species (ROS) and reactive nitrogen species such as free radicals, singlet oxygen, and hydroperoxides. Cell damage caused by free radicals appears to be a major contributor to ageing and degenerative diseases of ageing such as cancer, cardiovascular disease, immune system decline, diabetes mellitus, inflammation, brain dysfunction, and stress, among others. Phytochemicals may assist the body's own defense enzymes, such as superoxide dismutase and glutathione peroxidase, to scavenge or quench free radicals to protect the body against deleterious effects. There are many *in vitro* assays available for determining anti-oxidative activities, including measuring 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, superoxide, or peroxynitrite radical scavenging activities (RSA), lipid peroxidation inhibitory activity (LIA), the ferric-reducing ability of plasma (FRAP), total radical trapping by anti-oxidants (TRAP), xanthine oxidase activity, and determination of hydrogen peroxide hemolysis. Often, a combination of methods is applied to characterize a compound as an anti-oxidant.

Humulone inhibited hydrogen peroxide-induced hemolysis (IC_{50} 28 μM).[114] Furthermore, humulone and lupulone were shown to be radical scavengers in the DPPH-RSA assay with IC_{50} values of 32 and 25 μM, respectively. Both compounds also inhibited lipid peroxidation (IC_{50} 7.9 μM for humulone and 39 μM for lupulone). Interestingly, hop acids were more potent than the natural anti-oxidants α-tocopherol and ascorbic acid in this assay. The β-triketone moiety seems pivotal in view of its radical stabilizing property.[206] Ting *et al.* suggested that the β-triketo group and the fully conjugated cyclic dione of α-acids can lead to stable phenoxyl radicals, which can act anti-oxidatively.[207] In this investigation on the radical scavenging capacity of pure hop compounds and their derivatives, components having a 6-membered ring configuration (semi-quinone or quinoid) had the strongest radical-suppression activity.[207]

This is consistent with Mikyska *et al.*, who reported that hop α- and β-acids showed significant radical-quenching abilities, while iso-α-acids displayed a negligible effect.

Moreover, iso-α-acids may be slightly pro-oxidative by acting as electron donors and thereby leading to the formation hydrogen peroxide.[208] Another report gave the following order of decrease in OH-RSA: α-acids > β-acids > dihydroiso-α-acids > hexahydroiso-α-acids > tetrahydroiso-α-acids (IC_{50}: 0.21, 0.96, 1.36, 1.40, and 1.78 mg/mL, respectively). α-Acids and β-acids are potent scavengers of free radicals, whereas iso-α-acids and reduced derivatives show decreased activities in this regard. However, in terms of lipid peroxidation, the order of potency was as follows: α-acids > β-acids > iso-α-acids > tetrahydroiso-α-acids > dihydroiso-α-acids > hexahydroiso-α-acids.[209]

In vivo anti-oxidative effects can be determined by the *in situ* fluorescent detection of ROS and NO in tissues or by indirect assays, such as measuring urinary NOx excretion and quantifying ROS-production in the blood. Iso-α-acids have been reported to inhibit oxidative damage in rats fed a high-salt diet, thus preventing renal tissue damage. They decreased the production of ROS in renal tissues and increased bioavailable NO to basal levels. Increased ROS inactivates NO, critical for maintaining salt and water homeostasis in the kidney, thereby generating peroxynitrites, which, in turn, modify tyrosine residues of proteins to produce nitrotyrosine. Iso-α-acids in a high-salt diet, indeed, reduced the levels of renal nitrotyrosine, as detected by western blotting.[156]

1.6.8 Effects of Hop Bitter Acids on the Gastrointestinal Tract

Hops are used as over-the-counter products to improve gastric function. The bitterness of the hop acids is supposed to stimulate gastric secretion, similar to other bitter plant substances such as quinine. Only one study has addressed the effect of hops on gastric function, which uses the rat pylorus-ligated model. Oral administered hops clearly increased gastric juice volume without affecting acidity, which was not the case for intra-gastric administration. The effects after oral administration were similar to those obtained with carbachol, a cholinergic agonist, whereas they were completely blocked by the cholinergic antagonist atropine. It was concluded that the increase in gastric juice volume by

hops could be mediated by the cholinergic nervous system. However, since this study was carried out with an undefined hop extract, suspended in physiological saline at pH 4.5, it is not clear as to what extent these effects were caused by the bitter acid content of hops.[210]

1.6.9 Conclusions

Through their wide range of biological and pharmacological effects, hop acids have proved being interesting candidates for the treatment and/or prevention of several human disorders, including cancer, diabetes mellitus, osteoarthritis, osteoporosis, and cardiovascular disease. Hop acids may accounts at least partially for some of the health-beneficial effects of moderate beer consumption, as reported by a battery of epidemiological studies.[211-221]

In conclusion, hop bitter acid research has led to a better understanding of the effects of these compounds on health and this knowledge has already been translated into the production of hop-derived phytomedicines and dietary supplements.

1.7 Toxicology of Hop Bitter Acids

In general, skin contact with hops is well tolerated. Occasional hop allergy has been reported, most frequently after long-term exposure by direct contact or inhalation, for example by hop-pickers. Allergic reactions, mostly mild, include skin symptoms, such as urticaria, dermatitis, erythema, and pruritus of the uncovered skin, as well as respiratory disorders, including rhinitis, conjunctivitis, and asthma.[5, 26, 101, 222-224] Due to long-term uses in brewing and herbal medicine, hops are generally recognized as safe (GRAS) for oral intake.[225]

In vivo toxicity studies showed that only very large doses of hop intake are toxic, causing respiratory irregularities and central respiratory depression terminating in paralysis, in frogs, pigeons, small birds, rabbits, and mice.[168, 169, 171] Regarding individual hop acids, intravenous injection of small doses of lupulone stimulated respiration in rabbits and cats.[184] In mice, oral doses of 10 to 100 mg/kg body weight of lupulone did not cause any adverse effects.[171] Repeated intravenously injected humulone, at doses of 1-10 mg/kg, caused hyperventilation and hyperthermia in cats. In rabbits, the effects were much weaker and also of shorter duration. Lethal doses of humulone caused an abnormally severe rigor mortis, which appeared rapidly after death, suggesting that humulone affects muscular metabolism.[226]

A safety study of pre-isomerized hop acids revealed that the LD_{50}-values of iso-α-acids and dihydroiso-α-acids are approximately 1000 mg/kg body weight in the rat, when administered as single doses in a 50% corn oil solution. Long-term addition of iso-α-acids, dihydroiso-α-acids, tetrahydroiso-α-acids, or hexahydroiso-α-acids to the diet (1% for 90 days) caused a reduction in body weight gain in rats, without behavioral and histopathological changes. A dose of 150 mg/kg body weight was considered to be the no-observed-adverse-effect level (NOAEL). Furthermore, tetrahydroiso-α-acids, hexahydroiso-α-acids, and dihydroiso-α-acids did not cause mutagenic or genotoxic effects. In the dog, subchronic oral administration of tetrahydroiso-α-acids and hexahydroiso-α-acids was well tolerated with NOAEL values of 50 and 100 mg/kg body weight, respectively. Undigested material could be retrieved in the feces, suggesting a poor gastrointestinal absorption. In

general, toxic effects of high doses of pre-isomerized hop acids were limited to the gastrointestinal tract, most probably due to irritation by these bitter compounds.[227]

There are only a small number of reports addressing the safety of hop bitter acids in humans. A daily oral administration of 5000 mg of lupulone for three months was not toxic for the liver, kidney, bone marrow, or myocardium. However, each patient experienced some degree of gastrointestinal irritation, ranging from epigastric burning pain, abdominal cramping, diarrhea, nausea, and vomiting.[204]

A formula containing dihydroiso-α-acids (Meta050) (440 mg daily for eight weeks) did not result in clinically relevant changes in blood pressure, complete blood counts, or liver and kidney function. Furthermore, there was no negative impact on gastrointestinal markers normally affected by selective COX-2 enzyme inhibitors, as concluded from normal fecal calprotectin excretion.[228, 229] Similar data were obtained after administration of pure dihydroiso-α-acids (450 mg daily for 2 weeks).[228, 229]

II. State-of-the-art and Objectives

2.1 Background on bioavailability of hop-derived bitter acids

There is increasing scientific evidence that the health-beneficial properties of moderate beer consumption not only relate to the presence of alcohol (ethanol),[230-232] but, in particular, to unique biological activities of hop-derived constituents[111, 124, 233] - in analogy to the health-protective activity of red wine-that is associated with the presence of anthocyanins and stillbenoids-.

During the last 50 years, first the department of organic chemistry and later the lab for pharmacognosy and phytochemistry at Ghent University has gained a lot of experience on the chemistry, purification, stability, and analysis of substances present in hops and beers, including the photo-stability of beer-bittering substances, and the bioactivity and bioavailability of hop-derived prenylflavonoids and derivatives.

A number of studies report on the phytoestrogenic and cancer-chemoprotective activities of hop-derived prenylflavonoids. Some beers contain quite a lot of isoxanthohumol (IsoX), the pro-phytoestrogen for 8-prenylnaringenin (8-PN); in the range of 3-4 mg/L. This could in principle provide estrogenic activities in specific persons (low natural estrogen level and high conversion of IsoX to 8-PN by microbiota).

On the other hand, current investigations focus on highly interesting health-beneficial effects by hop-derived bitter acids (α-acids, β-acids) and derivatives (iso-α-acids, dihydro-iso-α-acids, tetrahydro-iso-α-acids). As presented in the previous chapter, a steadily growing number of publications have been reporting bioactive concentrations with IC_{50}-values in the lower micromolar range (anti-inflammatory and anti-angiogenic properties, improving lipid profiles, and counteracting diabetes type 2).[6, 128, 157, 229, 234, 235]

Surprisingly, until recently, only little was known on absorption, distribution, metabolism, and excretion (ADME) of iso-α-acids and derivatives, a topic that is highly relevant when assessing the usefulness of these compounds for either preventive or therapeutic uses. Insights into the bioavailability and the effective bioactivity of these compounds are essential to support, on a sound scientific basis, any health-related claim associated to nutraceuticals based on hop-derived bitter acids. In both the US and Europe, Metagenics Inc commercializes hop-based nutraceuticals and medical foods that contain hop-derived bitter acids varying from a few 100 mg up to 1000mg.[236] In beer, typical concentrations of α-acids are low, but techniques such as "dry hopping" can introduce levels of α-acids up to 14 mg/l.[36] The β-acids are generally not present in beer. On the other hand, iso-α-acids and reduced derivatives can be present in beer in much larger quantities, varying from 10 up to 100 mg/l, depending on the bitterness.[67, 68, 237] The presence of such high concentrations of hop-derived bitter acids could possibly explain the positive health effects associated with ingestion of preparations containing these products. Below, an overview of the limited knowledge on the ADME of hop bitter acids is presented.

In the evaluation for anti-inflammatory efficacy of a defined mixture of dihydroiso-alpha-acids (DHIAA), support for its bioavailability was determined by administration of a single oral dose of 1000 mg DHIAA to 2 normal healthy subjects. The bioavailable DHIAA was determined by the measurement of 2 separate diastereomers of the n-analog of DHIAA: *trans*-(6*R*)-n- and *cis*-(6*S*)-n-dihydroisohumulones. Both diastereomers were present in the plasma following oral administration and a reached maximum concentration (C_{max} was 1-3 μg/ml) at 4 h after dosing. The $AUC_{0–8h}$ for the *trans*-(6*R*)-n- and *cis*-(6*S*)-n-diastereomers was 18.8 and 7.15 μg*h/ml, respectively.[135]

In another study, presented by Desai *et al.,* reporting on the anti-inflammatory properties of a tetrahydro-iso-α-acids mixture, META060, its bioavailability was determined in a small human trial, as part of a study to address its therapeutic efficacy to treat chronic inflammation. META060 is a mixture of n- (45%), co-(39%), and ad-(8%) analogues of

tetrahydroiso-α-acids. Four healthy volunteers consumed 5 softgel capsules delivering 940 mg of META060 as the free acid. META060 was detected in the plasma of 4 human subjects within 1 h following a single oral dose of 940 mg; peak levels were observed in 3 of the 4 subjects at 4 h. While inter-subject variability was evident, C_{max} ranged from 4–15 μg/ml and T_{max} from 2–4 h. In the absence of data from systemic administration of META060, it was not possible to determine the absolute bioavailability, but the area-under-the-curve ($AUC_{0–8\ h}$) ranged from 15–98 μg*h/ml when normalized to a dose of 10 mg/kg. [238]

Recently, induction of the quinone reductase activity by α-acids and iso-α-acids and activation of CYP3A4, CYP2B6, and some MDR1 levels in human hepatocytes have been reported.[233, 239] It appears that hop acids stimulate both phase I and phase II detoxification processes. Activation of CYP3A4 is noteworthy because this gene product is the most abundant of all the cytochromes P450, clearing more than half of all prescription drugs.[240] This may be relevant with respect to the bioavailabilities as enhanced activities stimulate metabolism and excretion, resulting in lower overall bioavailabilities on repeated ingestion.

In one of the studies cited above, conducted by Teotico and co-workers, data indicated that hop bitter acids induced CYP3A4 and other drug-metabolizing genes by activating the human nuclear xenobiotics pregnane X receptor (PXR). PXR has a key role in the transcriptional regulation of genes that encode multidrug resistance efflux pumps and xenobiotic metabolism enzymes including cytochromes P450, glutathione transferases, UDP glucuronosyltransferases, sulfotransferases. The β-bitter acid colupulone was demonstrated to be a direct activator of (PXR).[241] The crystal structure of the ligand binding domain of human PXR in complex with colupulone was elucidated, and colupulone was observed to bind in a single orientation stabilized by both van der Waals and hydrogen bonding contacts. The crystal structure also indicated that related hop-derived α- and β- acids have the capacity to serve as PXR agonists as well.[239]

2.2. Objectives

The goal of this PhD project is the characterization of the factors governing the absolute bioavailability of hop-derived bittering substances using both *in vitro* and *in vivo* approaches, in combination with state-of-the-art analytical techniques. Furthermore, given the subtle differences in molecular structure between the compounds under investigation in this work, comparison of the results allows identification of possible structure-activity relationships.

To achieve this objective, the following topics were addressed in this research project:

1) As a first approach to study the bioavailability, we have investigated the in vitro intestinal permeabilities of hop α-acids, β-acids, iso-α-acids, dihydro-iso-α-acids and tetrahydro-iso-α-acids using Caco-2 cell monolayers (a commonly used screening tool for the prediction of intestinal absorption). Because of the wide use of hops as main ingredient for beer brewing and the increasing amount of hop-based food supplements, the *in vitro* absorption of both hop-derived α-, β-, iso-α-acids, dihydroiso-α-acids, and tetrahydroiso-α-acids using Caco-2 cell monolayers was investigated.

2) In addition, *in vivo* experiments have been carried out in which various hop acids and derivatives (α- and β-acids, iso-α-acids, dihydro- and tetrahydro-iso-α-acids) were administered to rabbits (oral vs. intravenous administration). The pharmacokinetic profiles of the compounds were used to calculate the absolute bioavailability of these substances in rabbits. In addition, in a second type of experiments, the different types of hop acids were administered orally to rabbits and urine and feces were collected over a period of 24h to elucidate elimination pathways for these compounds.

3) As a final part of the investigations, hop bitter acids were incubated with liver microsomes as an *in vitro* model to investigate phase-I and phase-II metabolism. In addition, the urine samples of the *in vivo* experiments were evaluated for the presence of possible metabolites and/or degradation products.

III. Transport of hop-derived bitter acids across Caco-2 cell monolayers

<ins>This chapter is based on:</ins>

Cattoor, K.; Bracke, M.; Deforce, D.; De Keukeleire, D.; Heyerick, A., *In vitro transport of hop bitter acids across Caco-2 monolayers, Journal of Agricultural and Food Chemistry* **2010,** *58* (7), 4132-4140

3.1 Introduction

In drug development, oral ingestion is the most common -or most desired- way of intake. Knowledge on the absorption and metabolism of xenobiotics at the intestinal mucosal level is of high importance, since the oral bioavailability is defined as the fraction of an oral dose reaching the systemic circulation in an unchanged form. Typically *in vivo* trials of potent bioactive molecules are preceded by *in vitro* screening methods and *in silico* computer simulations, based on ethical considerations and/or because of cost control issues. The most important advantage of *in vitro* work is that it permits an enormous level of simplification of the system under study, so that scientists can focus on a small number of components. Other possible advantages include options for high throughput research, mechanistic studies, etc. The primary disadvantage of *in vitro* experimental studies lies in the translation from the *in vitro* results back to the biology of the intact organism and/or humans. Care must be taken to avoid over-interpretation of the *in vitro* results.

In this chapter, the intestinal absorption of hop-derived bitter acids was studied using an *in vitro* approach. This study focuses on hop α- and β-acids as pure cohumulone and colupulone, respectively, and a mixture of n-humulone + adhumulone and n-lupulone + adlupulone, respectively. Also, the intestinal absorption of iso-α-acids (IAA), dihydroiso-α-acids (DHIAA), and tetrahydroiso-α-acids (THIAA) was studied. For the exact molecular structure of all the investigated compounds, see Figures 1.9; 1.15. The concentrations of hop acid chosen (30-120 µM) in the presented study can be reasoned by taking into account that an intestinal exposure to 30-120 µM hop bitter acid is in line with moderate consumption of a dry-hopped beer or a single oral dose of a food supplement containing about 10-20 mg of hop acids, while commercially available hop-based food supplements may contain up to 1000 mg of hop-derived bitter acids.[236]The results provide first insights into the intestinal absorption of hop acids.

During the past decade, numerous *in vitro* screening techniques have been developed to predict human intestinal absorption. One of the most intensively applied assays for permeability assessment has been the Caco-2 monolayer system.[242, 243] Less laborious methods with assays utilizing artificial membranes[244, 245] have also gained popularity, but the Caco-2 monolayers are regarded as the best model in terms of reliability.[246-250]

3.2 The Caco-2 cell monolayer system

The human epithelial Caco-2 cell monolayer model using differentiated Caco-2 cells is commonly applied as a screening tool for the prediction of intestinal absorption of compounds and for mechanistic studies of drug transport across epithelial layers.[250] Notably, *in vitro* permeability coefficients measured for reference compounds obtained in the Caco-2 cell model have shown good correlation with results based on *in vivo* studies.[251, 252]

Although derived from a colon carcinoma, Caco-2 cells can differentiate spontaneously, when grown on a suitable substrate, into a monolayer to exhibit the morphological characteristics of small intestinal cells including the formation of intercellular tight junctions and apical microvilli. The adjacent cells adhere through tight junctions formed at the apical side of the monolayer and form a clear separation of the apical compartment from the basolateral compartment. These compartments correspond to the intestinal lumen side and serosal side (bloodstream), respectively. A schematic picture of the Caco-2 cell monolayer set-up is shown in Figure 3.1.

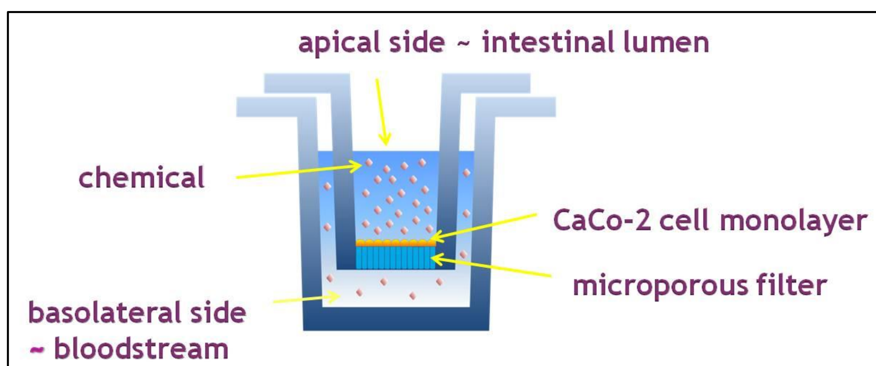


Fig 3.1 Schematic view of a Caco-2 cell monolayer set-up using a Transwell insert with a microporous filter.

3.2.1 Expression of phase-I enzymes

The first studies on the Caco-2 cell line demonstrated that these cells, upon differentiation, express small intestine hydrolase enzyme activities (i.e. sucrose-isomaltase, lactase, aminopeptidase N, dipeptidylpeptidase IV) on the apical membrane.[253] Certain cytochrome P450 enzymes are abundant in the human small intestine, such as CYP3A4, which accounts for ≈50% of all CYP isozymes in this tissue.[254] The specific expression of CYP1A1 in Caco-2 cells was proven by Boulenc *et al.*[255], while expression of CYP3A4 has been reported in Caco-2 cell monolayers by Gan and co-workers,[256] albeit at low levels. However, others have reported neither immunological nor functional evidence of phase-I enzymes in Caco-2 cells.[257] The under-expression of CYP enzymes compared to human small intestine, limits the use of Caco-2 cells as a model for intestinal phase-I metabolism of orally administered compounds. Investigators have tried to overcome this by treating Caco-2 cell monolayers at confluence with 1α,25-dihydroxyvitamin D_3, resulting in an increase of the CYP3A4 mRNA expression, next to increased levels of NADP cytochrome P450 reductase.[258] Confirmation was obtained by studying metabolic kinetics of midazolam by CYP3A4 enzymes, giving results that were similar to those observed *in vivo*.[259]

3.2.2 Expression of phase-II enzymes

Although CYP enzymes are poorly expressed in Caco-2 cells, many other drug metabolizing enzymes are expressed at levels, useful for intestinal metabolism studies.[260, 261] For example, hydrolases, carboxylesterases, glucuronyltransferases, glutathione-*S*-transferases, sulfotransferases, and catechol-*O*-methyltransferase are present and functional in Caco-2 cells. Among these, the phase-II sulfotransferases and glucuronyltransferases are particularly significant in the determination of bioavailability of orally administered compounds, since conjugation usually results in reduction or elimination of their biological activity. Caco-2 cells have been shown to produce sulfate and glucuronide conjugates of resveratrol,[262] flavonoids, like chrysin[263] and epicatechin,[264] and also of hop-derived prenylated flavonoids, such as 8-prenylnaringenin.[265]

3.2.3 Expression of Efflux transporters

Efflux transporters play an important role in disposition and elimination of many substances including metabolites, pharmacological drugs, dietary compounds, and other xenobiotics. The known intestinal efflux transporters responsible for carrier-mediated transport are divided in two categories: the solute-carrier superfamily (SLC) and the ATP-binding cassette (ABC) transporter superfamily. ABC transporters involve P-glycoprotein (P-gp, multidrug resistance protein 1 (MDR1)), multidrug resistance-associated proteins (MRPs), and the breast cancer resistance protein (BCRP). These ABC transporters function to suppress the intracellular accumulation of their substrates by preventing the influx and facilitating the efflux out of cells. In intestinal epithelia, P-gp, MRP2, and BCRP are expressed on the apical membrane and MRP3 is expressed on the basolateral membrane.

P-glycoprotein

The apical efflux transporter P-gp is responsible for the trans-membrane transport of different compounds by an ATP–depending process. P-gp mainly recognizes a variety of structural and pharmacologically unrelated neutral and positively charged hydrophobic compounds.[266] Caco-2 cells possess an elevated activity of P-gp, compared to human intestine, which is important to consider when using these cells as a model for intestinal bioavailability. Also, P-gp expression in Caco-2 cells was observed to be influenced by culture conditions and drug exposure.[253] This implies that the purpose of Caco-2 cell assays must be focused mainly to screen whether a compound interacts with P-gp, but not to quantify the interaction.[267]

MRP

The MRP's belong to the same ABC-superfamily as P-gp and transport relatively hydrophilic substrates, including glucuronide, glutathione, and sulfate conjugates, endogenous and exogenous compounds.[268] Thus, the substrates are predominantly anionic substances. MRP1 to MRP6 are known to be expressed both in humans and Caco-2 cells at medium to low levels. Also, MRP-2 and MRP-3 may have greater roles than other MRP's due to their higher expression levels.[269] It was shown that MRP-2 was only present at the apical membrane of Caco-2 cells[270] and similarly it is localized at the luminal membrane of the small intestine. In contrast, MRP-3 is located at the basolateral membrane in Caco-2 cells and various epithelia.[271]

BCRP

BCRP is a third member of the ABC-superfamily with wide substrate specificity and is expressed in many normal human tissues.[272] BCRP can transport a structurally and functionally diverse range of organic substrates including both hydrophobic and hydrophilic agents.[266] In Caco-2 cells, BCRP was found to be located on the apical membrane.[273, 274]

3.2.4 Transport mechanisms across Caco-2 monolayers

The intestinal absorption (see Figure 3.2) of various compounds across the intestinal epithelia into the blood circulation is mediated primarily by two routes: the paracellular (route A) and the transcellular route. Transcellular transport is carried out by either of the following three mechanisms: simple passive diffusion across the lipid bilayer (route B), carrier-mediated uptake for compounds which can act as substrates for intestinal transporters (route C), and endocytosis (transcytosis) (route D). Efflux pumps localized at the apical side, such as P-gp and MRP-2, serve as a protective barrier to intestinal transport by exporting xenobiotics back into the intestinal lumen (E). Absorption via the paracellular route is typically restricted by the relatively small pore size of the paracellular canal and the presence of tight junctions acting as a barrier to absorption,[275] reducing the pore radius to a few Ångstrom (1 Å = 0.1 nm). Moreover, the relative total surface area of the intercellular space is negligible compared to the total area of the epithelium, hence this paracellular pathway is only significant for chemicals showing slow transport across the cell membrane. In general, the more lipophillic compounds are rapidly transported across cells, while the most hydrophilic compounds have lower permeability coefficients.[276] For hydrophilic compounds that exhibit poor membrane permeability, and which do not serve as substrates for membrane uptake carriers,[242] the paracellular route may be an important absorption mechanism.

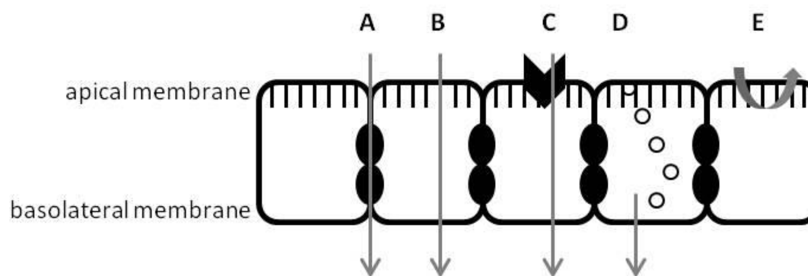


Fig 3.2 Schematic representation of routes and mechanisms for intestinal transport of molecules. Following oral intake, a compound can be absorbed into the systemic circulation by passive diffusion via paracellular transport between cells (A), transcellular transport across the cell membrane (B), or via active transport by carrier-mediated uptake (C) and transcytosis (D). Compounds can also be pumped back to the intestinal lumen by apical localized efflux pumps like P-gp, MRP-2, or BCRP-types (E).

Carrier-mediated uptake is possible for compounds having a molecular structure resembling those of actively transported nutrients. Often, transport is mediated partly by the carrier and partly by passive routes. Since, carrier-mediated transport is saturable, the contribution of the passive route will increase with increasing dose. If the compound has a low passive permeability, saturation of the carrier will result in a decreased absorbed fraction. Endocytosis is mainly considered for compounds which are excluded from other transport ways due to their large size.

Permeability data from former research with Caco-2 monolayers revealed that compounds known for complete human absorption were found to have a high apparent permeability coefficient ($P_{app} \geq 1 \times 10^{-6}$ cm/s), while poorly absorbed substances had a low permeability coefficient ($P_{app} \leq 0.1 \times 10^{-6}$ cm/s). It has been shown, from *in vivo* absorption data, that the usefulness of Caco-2 systems is appropriate for relative ranking of permeability of compounds (for example, classification in low and high absorption), but not for exact quantitative prediction of human absorption.[277] Lennernäs *et al.* investigated the effective permeability of different classes of drugs in Caco-2 monolayers and in human jejunum *in situ.*

The permeabilities of the rapidly and completely absorbed compounds (transported by a passive transcellular route) differed only 2- to 4- fold between the two models, whereas the correlation of the permeabilities of the slowly and incomplete absorbed drugs (transported by a passive paracellular route) were transported at a 30- to 80-fold slower rate in the Caco-2 monolayers than in the human jejunum. The permeability for the drug L-DOPA (L-3,4-dihydroxyphenylalanine), which is normally completely and rapidly absorbed, mainly via the carrier for large neutral amino acids in the human jejunum, was transported at a >100-fold slower rate in Caco-2 monolayers than in the jejunum in the investigated concentration interval.[278] Thus, it seems possible that the strength of the Caco-2 monolayers vs. *in vivo* correlations may vary for different groups of compounds.

3.3 Materials

Atenolol, propranolol, verapamil hydrochloride, indomethacin, sulfatase type H1 (from *Helix pomatia*), sodium fluorescein, 4–(2-hydroxyethyl)–1–piperazine-ethane sulphonic acid (HEPES) sodium salt 99%, D-(+)-glucose, and all HBSS buffer constituents were purchased from Sigma-Aldrich (Bornem, Belgium). Glutamine, non-essential amino acid solution, penicillin G, fungizone, amphotericin B, and fetal bovine serum (FBS) were purchased from Gibco (Invitrogen, Merelbeke, Belgium). The liquid/supercritical CO_2 extract was obtained from Hopsteiner (Mainburg, Germany). Isohop®, Redihop®, and Tetrahop Gold® were obtained from the Barth-Haas Group (Botanix, Kent, UK). International calibration standards for α- and β-acids (ICE-2), iso-α-acids (DCHA-iso, ICS-I3), dihydroiso-α-acids (all *cis*-dihydro-iso, ICS-R2), and tetrahydroiso-α-acids (ICS-T3) were all obtained from Labor Veritas (Zürich, Switzerland). HPLC/LC-MS solvents (analytical grade) were purchased from Biosolve (Valkenswaard, The Netherlands).

3.4 Methods

Caco-2 cell culture

Caco-2 cells (American Type Culture Collection (ATCC), Rockville, Maryland, USA) originating from a human colorectal carcinoma were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing GLUTAMAX™, supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, 100 U/ml penicillin, and 100 µg/mL streptomycin. Cells were grown in 25 cm^2 culture flasks (Corning Costar, New York, USA) in an atmosphere of 10% CO_2 and 90% relative humidity at 37°C (Forma Scientific, Marietta, Ohio, USA). Cells were passaged every 7 days (90-95% confluence) at a split ratio of 1:10. For transport studies, Caco-2 cells were seeded at a density of 1 $x10^5$ cells/insert on Transwell® membrane inserts (0.4 µm pore diameter, 6.5 mm diameter, Corning Costar, Corning, New York, USA) and cultured until late confluence. In the experiments with iso-α-acids, dihydro-iso-α-acids, and tetrahydro-iso-α-acids, membrane inserts with 0.4 µm pore diameter and 24 mm insert diameter were used. The cell culture medium was changed every other day. Monolayers were investigated, 18 to 24 days post-seeding. Cells with passage numbers 25-50 were used. The integrity of each monolayer of differentiated cells was monitored by measuring the transepithelial electrical resistance (TEER) with a Millicel-ERS volt-ohmmeter (Millipore, Bedford, Massachusetts, USA). TEER is a measure for the presence of tight junctions between adjacent cells. Volumes amounted to 200 µl at the apical side and 750 µl at the basolateral side of the monolayer (or 1.5 ml and 2.6 ml at the apical and basolateral side in case of 6 well plates).

Purification of α-acids and β-Acids

Individual hop α- and β-acids were separated on the basis of a pH-dependent liquid-liquid extraction, followed by semi-preparative HPLC. The hop CO_2 extract (10 g) was dissolved in diethyl ether (50 mL) in a separation funnel. α-Acids (pKa 4.7-5.6) were extracted with Na_2CO_3 (0.15M, 3 x 50 mL) and β-acids (pKa 6.7-7.6) with NaOH (0.1 mol/l, 3 x 50 mL).[279] The aqueous phases containing either the deprotonated α-acids or β-acids were acidified with HCl (12 M, pH 2) and extracted with diethyl ether (3 x 200 mL). The fractions enriched in either α-acids or β-acids were further purified by preparative HPLC (Gilson, Villiers-le-bel, France). The injection volume of a concentrated solution (300-400 mg/mL in MeOH) was 200 µL. A Varian C-18 column (250 x 21.4 mm, 10 µm) was used. Isocratic elution with a flow rate of 15 ml/min was applied with a mobile phase consisting of 30:70 (v/v) H_2O/CH_3CN + acidified with 0.025% HCOOH. The residues of the respective fractions after evaporation (cohumulone, n-humulone + adhumulone, colupulone, n-lupulone + adlupulone) were extracted with diethyl ether after acidification with HCl (12 M, pH < 2). Purities (> 98%) were confirmed by LC-MS and were stored at -20°C.

Caco-2 control measurements

The low-permeability standard atenolol (50 µM) and the high-permeability standard propranolol (20 µM) were added to the monolayers simultaneously (in the same well) with the test compounds. TEER was measured before and after the experiments. Monolayers with low TEER-values assumed to exhibit extensive leakage through imperfect occluding junctions or holes in the monolayer were discarded. After the transport studies, sodium fluorescein was used as a paracellular leakage marker. HBBS (200µl) containing 1 mg/mL sodium fluorescein was added to the AP chamber of each monolayer. After 1 h of

incubation, the amount of fluorescein transported to the BL chamber was measured by fluorescence spectrophotometry (λ_{exc} = 480 nm, λ_{em} = 530 nm).

Bidirectional Transport Studies of α-acids and β-acids

The transport medium was Hanks' buffered saline solution (HBSS) containing 10 mM HEPES + 25 mM D-(+)-glucose, adjusted to pH 7.4. The osmolarity was ± 0.35 Osm/l, verified with an osmometer (Knauer, Berlin, Germany). Prior to the experiments, the cell culture was removed from both AP and BL chambers of the Transwell plate. The cells were washed three times and preincubated (37°C, 10% CO_2) with transport medium for 30 min. TEER was measured before the experiments. Only cells with initial TEER-values >300 Ω x cm^2 were used. Stock solutions (20 mM in EtOH) of cohumulone, n-humulone + adhumulone, colupulone, and n-lupulone + adlupulone were diluted in the transport medium to a final concentration of 50 µM. Due to co-elution of ad- and n-analogues in the preparative HPLC method, these compounds were applied as a mixture (n-humulone + adhumulone and n-lupulone + adlupulone, respectively). Final EtOH contents were <0.5%.

The transport experiment was initiated by adding 50 µM hop sample to either the AP chamber (for absorptive transport study AP-to-BL) or the BL chamber (for secretive transport study BL-to-AP). Blank transport medium was added to the other (receiving) chamber. Each experiment was performed in triplicate (three sequential wells with Caco-2 monolayers were tested, in correspondence with methodologies described elsewhere).[275, 280] Samples from the receiving compartment were collected after 10, 20, and 30 min, respectively. During the experiments, each sampling volume was replaced by an equal volume of blank transport medium. Samples (apical (200 µl) and basolateral (750 µl) taken from the Caco-2 assay at different incubation times were spiked with the internal standard (IS) (1.0 µg). 4-hydroxybenzophenone was applied as internal standard in case of samples containing α-acids and β-acids. Next, samples were acidified (pH 2) with H_3PO_4 (0.1 mol/l; 1.5 volumes)

followed by extraction with ethyl acetate (EtOAc; 4 volumes; extraction was repeated once). Collected organic phases were evaporated (N_2) and residues were reconstituted in 100 µl methanol (MeOH). Samples were stored at -20°C until LC-MS analysis.

Cellular Uptake Experiments of α-acids and β-acids

The same pre-incubation protocol was followed as described under 'Bidirectional Transport Studies of α-acids and β-acids'. In cellular uptake studies, the hop acids (final concentration of 50 µM) were added to the AP chambers. Blank transport medium was added to the BL chamber. Different monolayers were incubated with hop acids for 15, 30, and 60 min, respectively. Each experiment was performed in triplicate. At the end of the experiment, samples were withdrawn from the AP (200µl) and the BL (750µl) chambers, followed by EtOH extraction of the cell monolayers. For this, excessive transport medium was removed and monolayers were extracted with EtOH (200 µl AP; 750 µl BL) during 30 minutes. Afterwards, EtOH samples from both the AP and BL chambers were combined and stored at -20°C until LC-MS analysis.

Inhibitor Studies of transport of α-acids and β-acids

In transporter inhibition studies, either verapamil (100 µM) or indomethacin (100 µM) was added into both AP and BL chambers. After a pre-incubation period in the presence of specific inhibitors, the same experimental protocol was followed as described under 'Bidirectional Transport Studies of α-acids and β-acids'. Furthermore, the transport of probe substrates rhodamine 123 (as substrate for P-gp) and 5-chloromethylfluorescein diacetate (CMFDA) (as substrate for MRP-2) was monitored as positive controls.

Bidirectional Transport Studies of iso-α-acids and reduced derivatives

The same pre-incubation protocol was followed as described under 'Bidirectional Transport Studies of α-acids and β-acids'. Different donor concentrations (30, 60, and 120 µM) were applied by adding a solution of IAA, DHIAA, or THIAA to either the AP compartment (for absorptive transport study; AP-to-BL) or to the BL compartment (for secretive transport study; BL-to-AP). Donor solutions were diluted from commercially available solutions of potassium salts of hop-derived bitter acids at pH 8-10 (Isohop®, Redihop®, and Tetrahop Gold® containing 200, 300, and 90 mg/ml of IAA, DHIAA, and THIAA, respectively) in HBSS (dilution factor > 4000). Blank transport medium was added to the other (receiving) compartment. After 1, 2, and 4 hours of incubation, samples were taken out from the basolateral (2600 µl) (for AP-to-BL transport) or apical (1500µl) (for BL-to-AP transport) side and the volume was replaced with blank transport medium. At the last sampling point (4 h), an aliquot of the donor compartment was included as a sample (respectively 1500 µl from the AP chamber or 2600 µl from the BL chamber).

In order to quantify the absorbed intracellular amounts, excessive transport medium was removed and monolayers were extracted with EtOH (1500 µl AP; 2600 µl BL) during 30 minutes. Afterwards, both EtOH fractions (1500µl; 2600 µl) of both sides were combined. Each experiment was performed in triplicate (three sequential wells with Caco-2 monolayers were tested). Samples (apical (1500µl) and basolateral (2600 µl)) taken from the Caco-2 assay at different incubation times were spiked with the internal standard (IS) (1.0 µg). THIAA were applied as internal standard in case of samples containing IAA and DHIAA. In samples following dosing of THIAA, DHIAA were used as internal standard. Next, samples were acidified (to pH < 2) with H_3PO_4 (0.1 mol/l; 1.5 volumes) followed by extraction with ethyl acetate (EtOAc; 4 volumes; extraction was repeated once). The collected organic phases were evaporated (N_2) and residues were reconstituted in 100 µl methanol (MeOH). Samples were stored at -20°C until LC-MS analysis.

Enzymatic Hydrolysis of Caco-2 monolayer extracts

Conjugated levels of hop-derived acids were quantified by enzymatic hydrolysis based on a method validated by Wyns *et al.*[281] Cell fraction extract aliquots (EtOH; 375 µl) were evaporated until dryness. Afterwards, residues were re-dissolved in 5 volumes NaOAc buffer (0.1 mol/l, pH 5) and 30 µl of a solution in NaOAc buffer (0.1 mol/l, pH 5) containing both β-glucuronidase and sulfatase (activities of 10,000 units/ml and 330 units/ml, respectively) *H. pomatia* from was added. Samples were incubated for 2 h at 37 °C. Subsequently, samples were acidified with H_3PO_4 (200 µl; 1.0 mol/L, pH 2) and extracted with EtOAc (4 volumes, extraction was repeated once). The collected organic phases were evaporated (N_2) and residues were reconstituted in 100 µl methanol (MeOH). Samples were stored at -20°C until LC-MS analysis. Replicate control samples were included in absence of enzyme treatment to determine the extent of glucuronidation and/or sulfation. Cellular levels of conjugated hop-derived acids were calculated by subtracting the amount of free hop acid (no enzyme treatment) from the amount of total hop-derived acids (+ β-glucuronidase/ sulfatase). Similarly, aliquots of the medium from the basolateral (300 µL) and the apical (100 µL) compartments were combined with the mixture of β-glucuronidase/sulfatase and further processed as described above.

Data Presentation of Caco-2 Experiments

The results of the transport experiments are expressed as an apparent permeability coefficient P_{app} (cm/s), calculated as described by Artursson *et al.*[252]

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with $\delta Q/\delta t$ (nmol/s): transport of the compound in the receiving chamber over time, A (cm^2): the membrane surface area of the cell monolayer, C_0 (nmol/cm^3): the initial compound concentration in the donor compartment. Other important parameters are the percent amount transported to the receiving chamber compared with the amount added to the donor compartment (%T) and the efflux ratio (ER), which is used to determine the extent of efflux, calculated according the following equation:

with P_{appBA} and P_{appAB}, being the apparent permeability coefficients for transport from the basolateral to apical compartment and from the apical to basolateral compartment, respectively.

Statistical Analysis

SPSS release 17.0 for Windows (SPSS, Chicago, Illinois, USA) was used for all statistical analyses. All experiments had a minimum of 3 independent observations for each test group. Each experiment was replicated at least once such that N=6, unless indicated otherwise. Data were expressed as means ± SEM when applicable. Normality of distribution was investigated using the Shapiro-Wilk test and the homogeneity of variances was evaluated using the Levene's test. Comparison of means between more than 2 groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance. Linear regression was performed when correlation analysis was warranted. Results were considered to be statistically significant when $P < 0.05$.

Purity of donor solutions of IAA, DHIAA, and THIAA

The purity of the donor solutions from commercially available Isohop®, Redihop® and Tetrahop Gold® was evaluated by dilution with methanol at a concentration of 0.1 and 1.0 µg/ml and analyzed by LC-MS to determine quantities of IAA, DHIAA, and THIAA in each solution. A purity > 98% was confirmed for each of the class of hop acids.

Stability of Hop Bitter Acids

The stability of hop-derived bitter acids in the experimental conditions was evaluated. Stock solutions of 20 mM (EtOH) of α- and β-acids and commercial available solutions of IAA, DHIAA, THIAA were diluted to a final concentration of 50 µM for each class of hop acids, and incubated at 37°C for 4 h. Afterwards, samples (500 µL) were extracted as described in the sections 'Bidirectional Transport Studies of α-acids and β-acids' and 'Bidirectional Transport Studies of iso-α-acids and reduced derivatives'.

LC-Analysis of Samples

Extracted samples from the Caco-2 experiments were analyzed using LC/MS analysis (Agilent 1200 LC-MS, Agilent, Waldborn, Germany). MS analysis was performed using an AT multimode ionization source coupled to a single quadrupole detector (MSD), SL version. The Agilent Chemstation software package (Rev.B.02.01) was used to control the analytical system as well as for data acquisition as for processing. For the analysis of atenolol and propranolol, a Zorbax SB C_{-18} column, was used. The mobile phase consisted of 10 mM ammonium acetate pH 5.0 + 2% CH_3CN (A) and methanol (B). The initial mobile phase, 10% B, was increased linearly to 90% B over 8 min, maintained for 4 min, and further increased to 100% B in 1 min and maintained during 4 min. Finally, the mobile phase was

adjusted to 10% B in 1 min and re-equilibrated at 10% B for 4 min prior to the next injection. In case of samples containing α- and β-acids, iso-α-acids, dihydroiso-α-acids, and tetrahydroiso-α-acids, an Xbridge C_{18} column (150 x 30 mm; 3.5 μm) (Waters, Zellik, Belgium) was used. The mobile phase consisted of 10 mM ammonium acetate pH 9.75 + 20% MeOH (A) and 100% MeOH (B). The flow rate was 0.5 ml/min and the column temperature 40°C. The initial mobile phase, 27% B, was increased linearly to 60% B over 24 min, maintained for 5 min, and further increased to 95% B in 5 min and maintained during 4 min. Finally, the mobile phase was re-adjusted to 27% B in 1 min and re-equilibrated at 27% B for 6 min prior to the next injection. The MS-parameters in the negative atmospheric pressure chemical ionization (APCI) mode were tuned to maximize formation of the deprotonated analyte. 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 0.1 MPa, capillary voltage 1000 V, corona current 6 μA, and charging voltage 1000 V. In each analysis, qualitative identification was performed in the negative ion scan mode (m/z 150–700) and quantitative data were obtained by construction of the extracted-ion chromatogram following measurement in the selected-ion monitoring (SIM) mode. Quantitative analysis of atenolol and propranolol was operated in the SIM positive-ion mode using target ions at [M-H](+) m/z 260.3 and 267.3 for propranolol and atenolol, respectively. The values of the target ions [M-H](-) m/z, used for quantification of the different hop acid compounds, are presented in Table 3.1. Calibration curves were established by linear least squares regression analysis using the ratio of the peak area of total hop acid versus IS against the concentration of hop-derived acids in 6 standards (blank HBSS medium) covering the range of 0.5 to 200 μM. Daily prepared calibration curves were constructed by injecting calibration standards at the beginning and at the end of each batch of samples.

To evaluate the accuracy and precision of the method, recovery experiments were carried out on three consecutive days by adding 2 different concentrations of AA, IAA, DHIAA, and THIAA (10 and 50 μM) to blank transport medium. The accuracy was expressed in terms of relative error, and the precision was evaluated in terms of the relative standard

deviation (RSD). The limit of detection (LOD) and the limit of quantification (LOQ) of the method were defined as the lowest concentration with a signal-to-noise ratio of 3 and 10, respectively, in spiked samples, where the noise data were taken from the analysis of blank matrices.

Table 3.1 Molecular ions used for selected-ion monitoring (SIM) mode for the various compounds in LC-MS analysis.[a]

Compound	m/z (negative ionization)
AA	
cohumulone	347
n-humulone +adhumulone	361
BA	
colupulone	399
n-lupulone +adlupulone	413
IAA	
cis-isoco *trans*-isoco	347
cis-isoad *cis*-iso-n+ *trans*-isoad *trans*-iso-n	361
DHIAA	
cis-DHisoco *cis-DHisoco*	349
cis-DHisoad *cis*-DHiso-n *cis*-DHisoad *cis-DHiso-n*	363
THIAA	
cis-THisoco *trans*-THisoco	351
cis-THisoad *cis*-THiso-n *trans*-THisoad *trans*-THiso-n	365

[a] AA: α-acids; BA: β-acids; IAA: iso-a-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids.

3.4 Results & Discussion

LC-MS analysis of hop-derived bitter acids in Caco-2 samples

In this study, an LC-MS method is described that enables simultaneous analysis of, in total, 24 hop-derived bitter acids. A mobile phase with a high pH is used based on the work of Vanhoenacker and co-workers.[78] This study showed that an alkaline mobile phase demonstrated improved peak shape and efficiency, implying better resolution. As buffer additive, the volatile ammonium acetate was chosen at pH 9.75, thus requiring selection of a stationary phase, stable at high pH. The column, an Xbridge C_{18} is stable in the pH range 1-12. In accordance with the study by Vanhoenacker *et al.*, APCI was used in this work, instead of ESI. Figure 3.3 shows an LC-MS analysis of a mixure of all hop-derived hop acids in MeOH. The chromatograms of each group of compounds are reconstructed by the extracted-ion function. Peak identification was based on the retention time and elution order of detected MS-ions, UV-spectra, and literature data.[76, 98]

For the α-acids, full separation can be observed for all 3 homologues; while for the β-acids, n- and ad-compounds are co-eluting. Generally, in routine analysis of iso-α-acids not all individual iso-α-acids are resolved. Mostly 2 peaks appear, the first peak representing the *cis-* and *trans*-isocohumulones and the second peak the *cis-* and *trans*-isomers of isohumulone and isoadhumulone. In the method presented in this study, full separation of the 6 major iso-α-acids can be observed (Fig 4.1). Also for the tetrahydroiso-α-acids, all 6 constituents were separated with this method. In case of the *cis*-dihydroiso-α-acids, separation of five of the 6 constituents could be obtained. The small unassigned peaks in the chromatogram could be assigned to minor presence of *trans*-dihydroiso-α-acids.

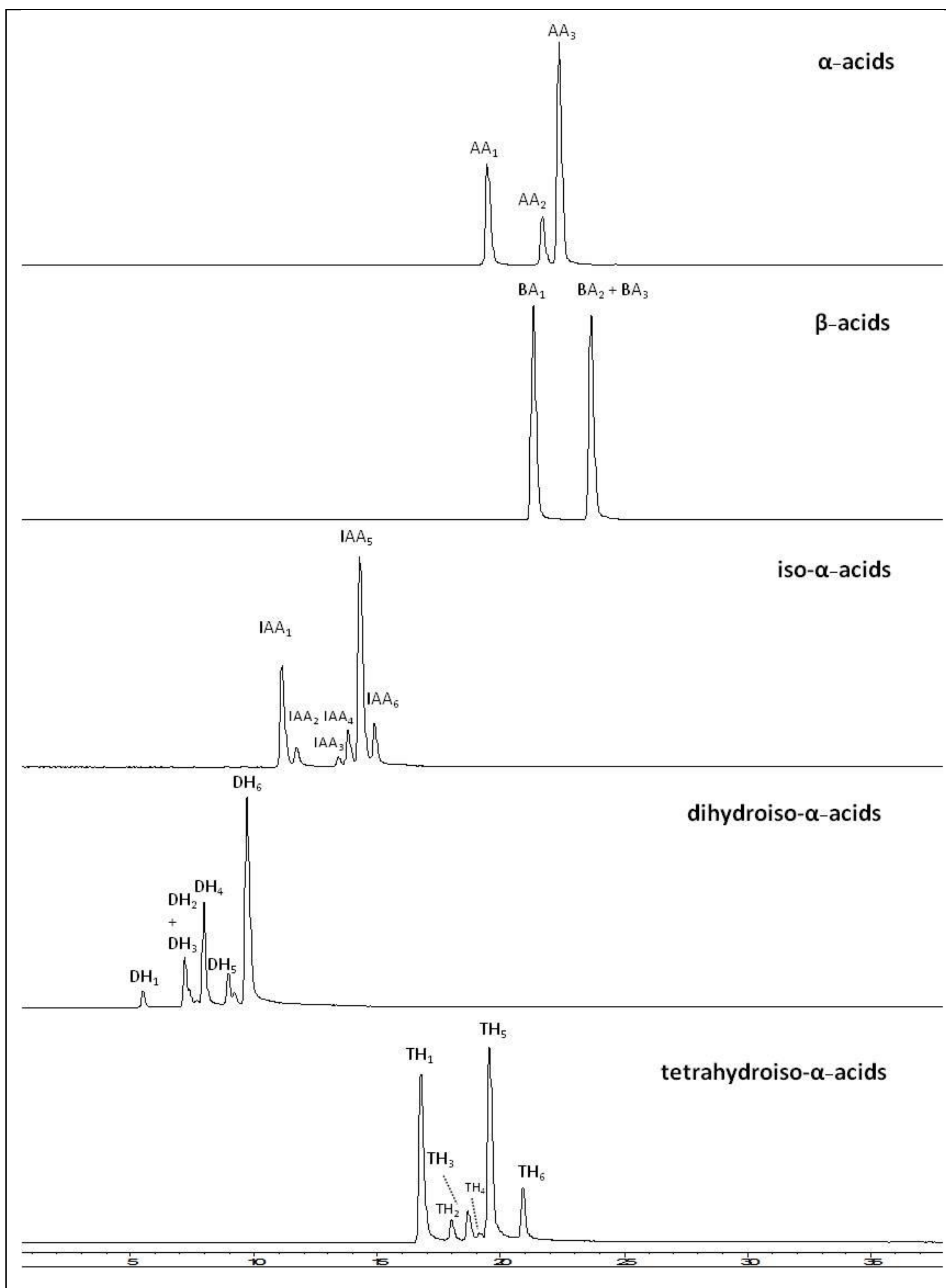


Figure 3.3 Representative LC-MS chromatograms of various hop-derived bitter acids in MeOH.

The standard curves for the determination of α-acids, β-acids, iso-α-acids, dihydro-iso-α-acids, and tetrahydro-iso-α-acids in blank transport medium were all linear over the concentration range of 0.5-200 μM. The correlation coefficients were always higher than 0.995. The limits of detection (S/N = 3) for all compounds are listed in Table 3.2. The LODs ranged from 0.010 μM to 0.015 μM.

Table 3.2 Limit of detection (LOD) in Caco-2 transport medium (HBSS)

	HBSS LOD (μM)
AA	0.014
BA	0.010
IAA	0.012
DHIAA	0.015
THIAA	0.012

The accuracy and precision, expressed as the percentage relative error (%RE) and relative standard deviation (RSD), was within the acceptable ranges of 15% for all compounds (Table 3.3). The accuracy ranged from 6.7% to 12.5%. The intra-assay precision ranged from 2.6% to 5.7%. The inter-assay precision ranged from 6.9% to 10.7%.

Table 3.3 Accuracy (%RE) and precision (RSD) (intra- and inter-assay) in Caco-2 transport medium (HBSS)

compound	accuracy	precision	
		intra-assay	*inter-assay*
AA	12.0	3.1	8.7
BA	6.7	4.3	6.9
IAA	7.1	5.4	10.7
DHIAA	10.7	5.7	8.5
THIAA	12.5	2.6	7.4

Bidirectional Transport of Marker Compounds and Monolayer Integrity

The Caco-2 cell monolayers were assessed with respect to their barrier properties using the model compounds atenolol (paracellular transport) and propranolol (transcellular transport), known for passive diffusion. Propranolol and atenolol showed P_{app} values of 53.1 ± 5.0 x 10^{-6} and 0.45 ± 0.18 x 10^{-6} cm/s, respectively, which is in correspondence with reported data.[249, 251, 252, 282, 283] Reference values for the atenolol flux are usually lower than 1.0 x 10^{-6} cm/s while the propranolol flux is typically higher than 10 x 10^{-6} cm/s.[275] The results indicate that the monolayers can be used to discriminate between compounds with low and high permeabilities. Final concentrations did not affect TEER or transepithelial transport, hence the transport of compounds did not influence TEER, which was confirmed by preservation of the integrity of the monolayers, also shown by fluorescein transport values < 1%/h for Transwell membrane inserts with 6.5 mm diameter and < 0.3%/h for inserts with 24 mm diameter.[284]

Stability of Hop-derived Bitter Acids in HBSS

To investigate the stability of hop-derived bitter acids, control experiments with various classes of compounds were carried out. After 4 h of incubation in blank transport medium, recoveries were all >98%, indicating that no significant degradation was observed for all hop-derived bitter acids during the experimental conditions applied.

3.4.1 Caco-2 studies with hop α-acids and β-acids

3.4.1.1 Bidirectional Transport of α-acids and β-Acids across Caco-2 Cell Monolayers

The present study was undertaken to investigate the *in vitro* transport of hop bitter acids as cohumulone and n-humulone + adhumulone (α-acids) and colupulone and n-lupulone + adlupulone (β-acids) using Caco-2 cell monolayers. The transport characteristics were determined for two directions, apical-to-basolateral (AP-to-BL) and basolateral-to apical (BL-to-AP). The cumulative amounts transported into the receiving chamber as a function of time are shown in Figures 3.4 and 3.5.

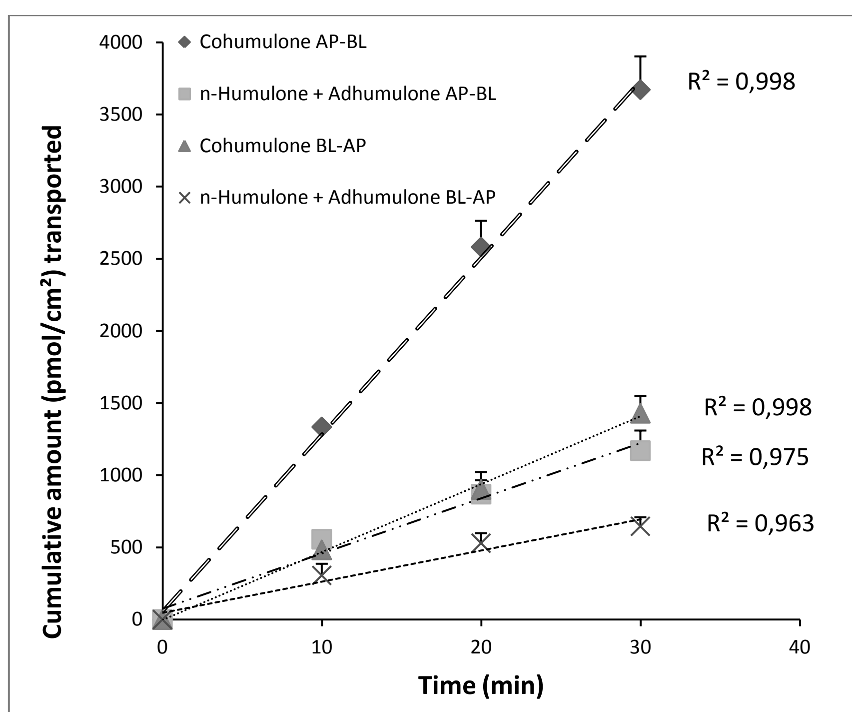


Fig. 3.4 Apical (AP)-to-basolateral (BL) and basolateral (BL)-to-apical (AP) transport of α-acids (cohumulone and n-humulone + adhumulone) across Caco-2 monolayers. Hop α-acids (50 µM) were added to either the apical (AP-to-BL) or the basolateral (BL-to-AP) compartment of monolayers at time zero. Samples from the receiving compartment were collected at 10, 20, and 30 min, and analyzed by LC-MS as described under Materials and Methods. Data are means ± SEM for three replicates. Trendlines from linear regression analysis fitted through zero show good linearity (R^2 > 0.95).

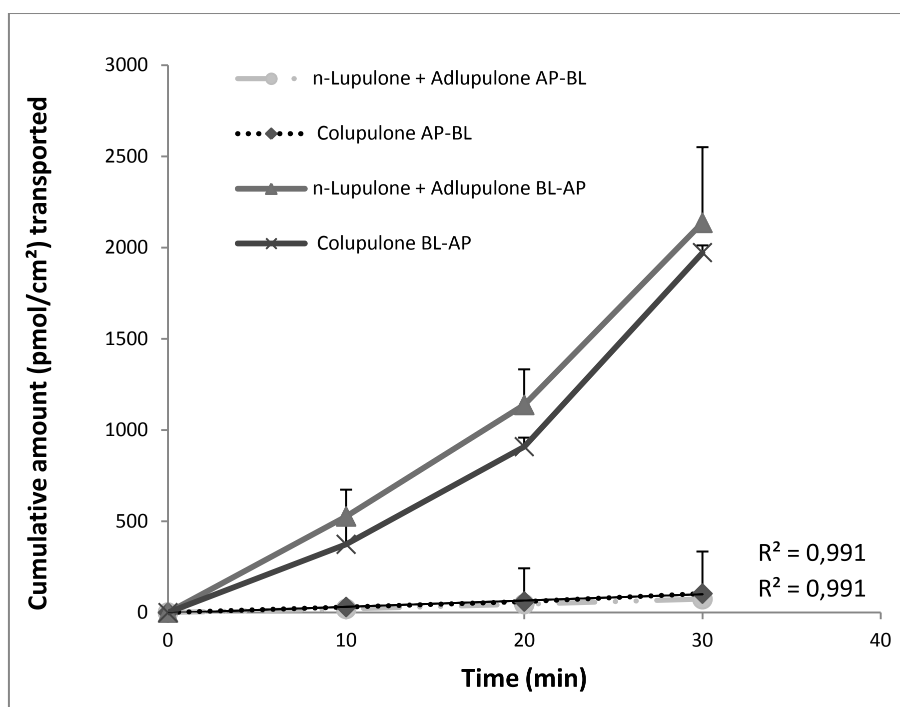


Fig. 3.5 Apical (AP)-to-basolateral (BL) and basolateral (BL)-to-apical (AP) transport of β-acids (colupulone and n-lupulone + adlupulone) across Caco-2 monolayers. Hop β-acids (50 µM) were added to either the apical (AP-to-BL) or the basolateral (BL-to-AP) compartment of monolayers at time zero. Samples from the receiving compartment were collected at 10, 20, and 30 min, and analyzed by LC-MS as described under Materials and Methods. Data are means ± SEM for three replicates. Trendlines from linear regression analysis fitted through zero show good linearity for AP-to-BL transport (R^2 > 0.95). BL-to-AP transport showed enhanced transport upon longer treatment periods.

Significantly higher amounts of α-acids were transported to the basolateral chamber compared with the β-acids. For the α-acids, the fractions transported into the receiver chambers increased linearly within 30 min in both absorptive and secretive directions. For the β-acids, the transport in the absorptive direction also showed a linear relationship, but, in efflux transport, a different relationship was observed. Table 3.4 presents the transport parameters P_{app} and efflux ratios (ratio of efflux versus influx) for the hop acids in the AP-to-BL as well as in the BL-to-AP direction.

Table 3.4 Apparent permeability coefficients (Papp) and efflux ratios in the absence and presence of the specific inhibitors verapamil and indomethacin.[a]

	COH (50 μM)	N-HUM + ADHUM (50 μM)	COL (50 μM)	N-LUP + ADLUP (50 μM)
P_{appAB} (x 10^{-6} cm/s)	41 ± 2	14 ± 2	2.1 ± 0.9	0.9 ± 0.1
P_{appBA} (x 10^{-6} cm/s)	16 ± 2	7.2 ± 0.6	21 ± 3	8.9 ± 1.0
efflux Ratio	0.4 ± 0.1	0.5 ± 0.1	10 ± 4	10 ± 4
P_{appAB} (x 10^{-6} cm/s) +verapamil	37 ± 3	18 ± 3	2.5 ± 0.6	1.4 ± 0.3
P_{appBA} (x 10^{-6} cm/s) + verapamil	16 ± 1	10 ± 2	10 ± 2	10 ± 2
P_{appAB} (x 10^{-6} cm/s) +indomethacin	41 ± 3	17 ± 2	27 ± 1	14 ± 5
P_{appBA} (x 10^{-6} cm/s) +indomethacin	15 ± 1	10 ± 1	5.6 ± 0.4	2.7 ± 0.5
Efflux ratio + verapamil	0.4 ± 0.1	0.5 ± 0.2	4.2 ± 2.0*	7.0 ± 1.7
Efflux Ratio + indomethacin	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.1**	0.2 ± 0.1**

[a]Hop α-acids (50 μM) (cohumulone = COH and (n-humulone + adhumulone) = N-HUM + ADHUM) and β-acids (colupulone = COL and (n-lupulone + adlupulone) = N-LUP + ADLUP) were added to the apical (AP-BL) or basolateral (BL-AP) compartment of monolayers at time zero. After 10, 20, and 30 min, samples were withdrawn from the receiving compartment and analyzed by LC-MS as described under Materials and Methods. Data are means ± SEM for three replicates. Comparison of efflux ratios (in the absence and presence of inhibitors) between more than two groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance. Statistically significant differences versus the efflux ratios in the absence of inhibitors are indicated: *$p < 0.05$; **$p < 0.005$.

The P_{appAB} values ranged from 0.9 ± 10^{-6} to 40.9 ± 10^{-6} cm/s. The highest membrane permeability was determined for cohumulone (40.9 ± 10^{-6} cm/s), being about 3-fold higher than the P_{appAB} values of (n-humulone + adhumulone) and 20-40 times higher than the transport rates of the β-acids.

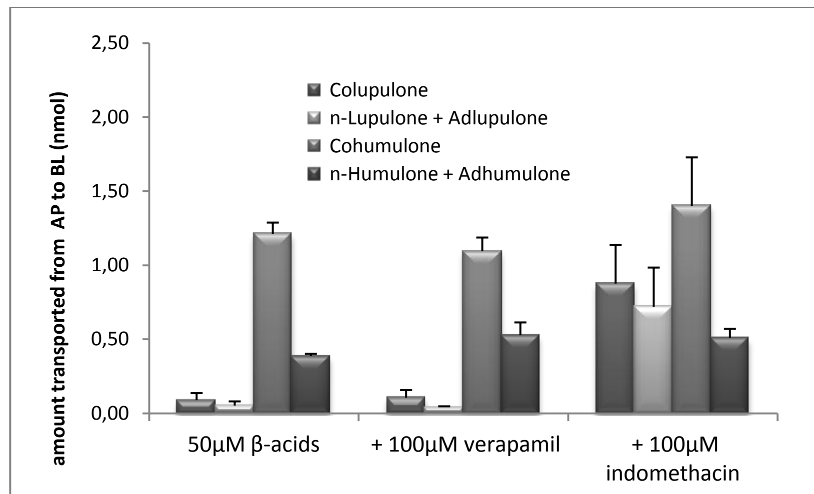
In secretive transport, the P_{appBA} of the α-acids in the BL-to-AP direction was 15.7 ± 10^{-6} cm/s for cohumulone and 7.2 ± 10^{-6} cm/s for (n-humulone + adhumulone). Cohumulone and (n-humulone + adhumulone) showed higher absorption than secretion with efflux ratios of 0.4 for cohumulone and 0.5 for (n-humulone + adhumulone). Notably, colupulone and (n-lupulone + adlupulone) exhibited substantially different bidirectional P_{app} values with efflux ratios of 10.3 and 9.7, respectively.

3.4.1.2 Effect of Inhibitors on the Transport of α-acids and β-Acids

To confirm the expression of efflux pumps in the Caco-2 cells, the transport of the probe substrates rhodamine 123 (as substrate for P-gp) and CMFDA (as substrate for MRP-2) was monitored as positive control. The transport of rhodamine 123 showed a substantial efflux with an efflux ratio of 3.0, which was significantly reduced to 0.98 in the presence of verapamil as a P-gp inhibitor. For CMFDA, the presence of indomethacin as a MRP-inhibitor caused reduction of the efflux ratio from 6.4 to 0.91. The results confirmed that P-gp and MRP-2 were both present in the Caco-2 monolayers. To identify involvement of efflux transporters in the bidirectional transport of colupulone and (n-lupulone + adlupulone) across Caco-2 monolayers, their transport properties were examined in the presence and the absence of specific transport inhibitors. As a control experiment, bidirectional transport of α-acids in the presence of verapamil and indomethacin was also examined. As expected, the transport of cohumulone and (n-humulone + adhumulone) was not influenced.

The amounts transported in bidirectional transport of α-acids and β-acids in the presence of selective inhibitors (P-gp influence) and indomethacin (MRP-2 influence) are

shown in Figure 3.6 and the corresponding efflux ratios are listed in Table 3.2. For β-acids in the presence of verapamil (100 μM), a significant decrease of the efflux ratio was observed compared to the data in the absence of the P-gp-inhibitor (from 10.3 and 9.7 to 4.2 and 7.0, respectively). The presence of indomethacin (MRP-2-inhibitor) (50 μM) caused almost complete inhibition of the efflux of colupulone and (n-lupulone + adlupulone) (Table 3.2, Figure 3.6). A significant decrease in the efflux ratio from 10.3 and 9.7 to 0.2 for colupulone and (n-lupulone + adlupulone), respectively, was observed. The results suggest that both MRP and P-gp are involved in the efflux of hop β-acids.



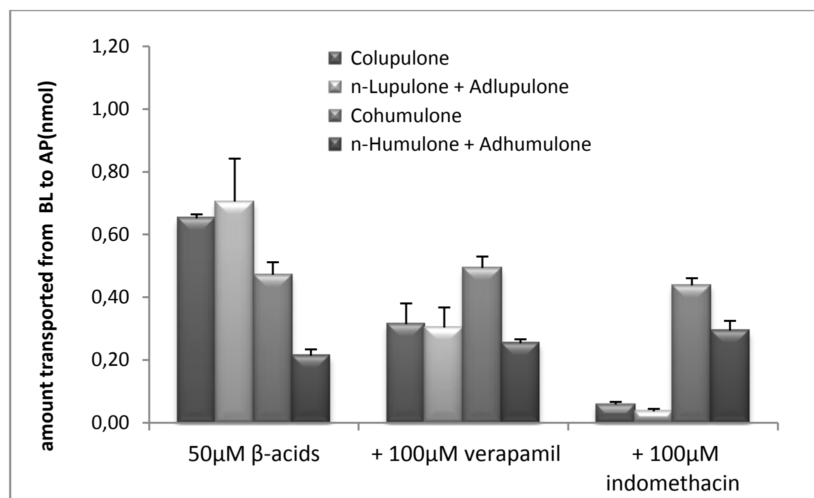


Figure 3.6 Apical (AP)-to-basolateral (BL) transport of hop bitter acids across Caco-2 monolayers in the presence/ absence of verapamil and indomethacin. Hop α- acids and β-acids (50 µM) were added to either the apical (AP-to-BL) or the basolateral (BL-to-AP) compartment of monolayers at time zero. Samples from the receiving compartment were collected at 10, 20, and 30 min, and analyzed by LC-MS as described under Materials and Methods. Data are means ± SEM for three replicates.

3.4.1.3 Cellular Uptake of α- and β-Acids

The amounts of α- and β-acids recovered in the AP chamber, BL chamber, and cell monolayer are shown in Figure 3.7.

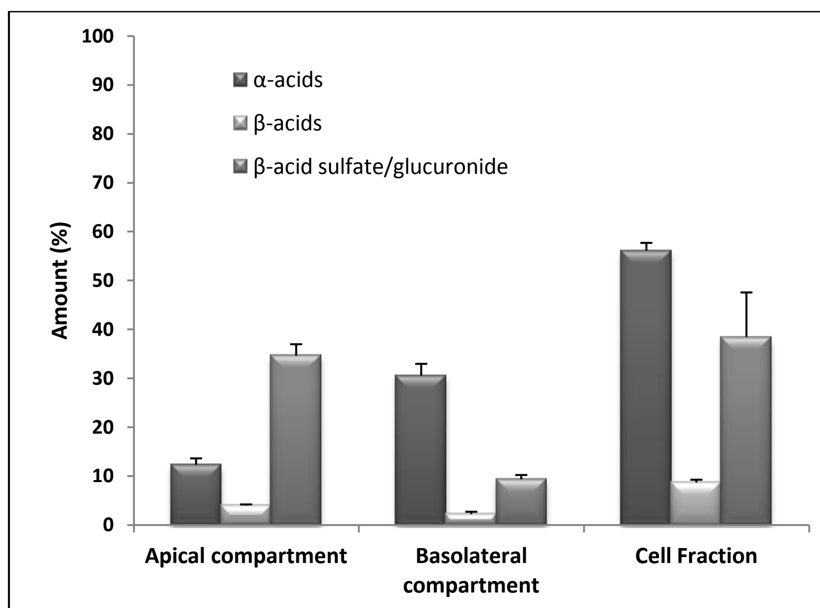


Fig. 3.7 Distribution of α-acids and β-acids in the apical compartment, the basolateral compartment, and the cell fraction. Hop α- acids and β-acids (50 μM) were added to the apical compartment of monolayers at time zero. After 60 min, samples were withdrawn from the apical compartment, basolateral compartment and cell fraction and analyzed by LC-MS as described under Materials and Methods. Data are means ±SEM for three replicates.

After 60 min of incubation of the α-acids with the Caco-2 cells, the amount absorbed in the cell fraction was 60%, whereas 30% was transported to the BL-chamber. A residual amount of 10% was still present at the AP-chamber. After 60 min of incubation of the β-acids with the Caco-2 cells, the amount recovered in the cell fraction was only 8%, whereas 2.1% was present unchanged at the BL-chamber. A residual 4.0% was recovered in the AP-chamber. More than 95% of the administered amount of α-acids was recovered unchanged. The formation of phase-II metabolites of α-acids seems unlikely within the duration of the

experiments. The recovery of the β-acids was <15%. These observations suggest formation of phase II metabolites and/or degradation products of β-acids in the Caco-2 cells within the duration of the experiment (60 min).

3.4.1.4 Enzymatic Hydrolysis of Cellular Uptake Samples of β-acids

As a consequence of the low recovery of the β-acids (<15%), enzymatic hydrolysis of fractions from apical and basolateral compartments, and cell monolayers was carried out with a mixture of sulfatase and glucuronidase to determine the amount of sulfate and/or glucuronide conjugates (Fig 3.7, Table 3.5).

The total amount(%), quantified after deconjugation, rendered a recovery of 97.5 ± 13.6%. Therefore, about 81-90% of the total amount of β-acids present in the apical compartment, the basolateral compartment, and cell fraction was present as conjugated sulfate or glucuronidated products. Following apical supplementation, 38.7 ± 2.5% of the total amount of the β-acid + conjugates were present in the apical compartment and 11.6 ± 1.4% in the basolateral compartment, whereas 47 ± 10% remained in the cell fraction.

Table 3.5 Amounts (%) of free and conjugated β-acids (as sulfates and glucuronides) in the apical compartment, basolateral compartment, and cell fraction[a]

	% β-acid conjugated	% β-acid non-conjugated	SUM % β-acid conjugated + non-conjugated	Relative amount (%) Conjugated β-acids / Total β-acids
AP	34.7 ± 2.3	4.0 ± 0.2	38.7 ± 2.5	90
BL	9.4 ± 0.9	2.2 ± 0.5	11.6 ± 1.4	81
CF	38.5 ± 9.1	8.7 ± 0.6	47.2 ± 9.7	82
SUM β-acids (%)	82.6 ± 12.3	14.9 ± 1.3	97.5 ± 13.6	85

[a]Hop β-acids (50 µM) were added to the apical (AP) compartment of monolayers at time zero. After 60 min, samples were withdrawn from the apical compartment, the basolateral (BL) compartment and cell fraction (CF). Enzymatic hydrolysis with a mixture of sulfatase and β-glucuronidase was carried out in order to determine the amount of conjugated β-acids. Control samples without addition of the enzyme mixture were included. Samples were analyzed by LC-MS as described under Materials and Methods. Data are means ±SEM for three replicates.

3.4.1.5 Discussion

Oral bioavailability is one of the most important pharmacokinetic properties of drugs. Various intestinal processes such as permeation, efflux, and intestinal metabolism may affect the bioavailabilities of hop acids. As a first aspect of the study of the bioavailability of hop acids, permeability experiments of hop acids in both absorptive (AP-to-BL) and secretive (BL-to-AP) modes were carried out to determine whether a carrier-mediated transport or a carrier-mediated efflux mechanism is involved. The P_{app}-values determined for the α-acids approximate the P_{app}-value of propranolol. Propranolol is a lipophillic compound, which is known for its high permeability across epithelial membranes. Rapidly and completely absorbed compounds are generally lipophillic and distribute readily into the cell membranes. Because the surface area of the brush border membranes is >1000-fold larger than the paracellular surface area,[285] it is suggested that the hop α-acids are quickly and efficiently transported exclusively by the transcellular route[286] in the absorptive direction.

The absorptive transport of both α-acids and β-acids shows a linear increase during 30 min, indicating the absence of saturation characteristics under the experimental conditions applied,[280] which may suggest passive transport, although AP-to-BL and BL-to-AP permeabilities with a wider concentration range (for example, 5-200 µM) on the initial side are necessary to confirm this observation. The vast majority of well-absorbed drugs are transported passively across cell membranes.[250] Active transport of dissociated forms with anionic transporters or facilitated diffusion would rather lead to a sigmoid-like relationship. Even though, this would be observed only at sufficiently high concentrations, due by saturation of the carrier-mediated transport. If a compound has a low passive permeability, saturation of the carrier will result in a decreased absorbed fraction.[242, 262]

There was no significant difference between transport in the absorptive direction and transport in the secretive direction of the α-acids, since P_{appAB} and P_{appBA} are comparable. Influx exceeded efflux, albeit with small differences. It may be derived that α-acids show a bidirectional transport by passive diffusion (i.e., not transporter-mediated). This observation

suggests that passive diffusion of α-acids could play a role in permeation across intestinal cells, not only in the Caco-2 monolayers, but possibly also *in vivo*. The P_{app}-values of the β-acids (≤1 x 10^{-6} cm/s) were comparable to the P_{app} of atenolol, which is a marker for low permeability. This observation suggests that intestinal absorption of β-acids is predicted to be poor, which might be associated with a low bioavailability. Factors influencing diffusion include physico-chemical properties of molecules including molecular weight, lipophilicity, molecular surface polarity, hydrogen bonds, and charge.[242] In a study reported by Hou *et al.*, lipophilicity is recognized as one of the key descriptors governing permeability across Caco-2 cells, reporting that measured logD explains ~50% (i.e. r^2 ~0.5) of the total variance in a dataset of 77 permeability measurements.[287] The extra isoprenyl moiety present in the β-acids compared to the α-acids contributes to a higher lipophilicity and increased steric hindrance, resulting in lower absorption. Differences in lipophilicity are also demonstrated by the corresponding clog P-values (calculated using "Molinspiration MiTools", based on ZINC database)[288] of α-acids ranging from 3.2 to 3.9 for cohumulone and n-humulone, respectively in contrast with 6.2-6.9 for colupulone and n-lupulone, respectively. Furthermore, Gleeson *et al.* reported clogP values 3-5 for good permeability based on an analysis of > 50000 compounds with measured artificial membrane permeability data. Weak acids can indeed cross cytoplasmatic lipophilic membranes in undissociated form.[289, 290] Knowledge on the pH value of the transport medium and the pKa value of the hop acid under consideration allows to calculate the concentration of undissociated molecules at any pH value because [undissociated acid] = [total acid administered]/[$10^{(pH-pKa)}$ + 1] by rearrangement of the Henderson-Hasselbalch equation. Under the experimental conditions of pH 7.4, the ratio of dissociated/undissociated molecules was approximately 100. The presence of a high percentage of the dissociated forms seems contradictory with the high absorption of α-acids. Presumably, fast transport of the undissociated species from the apical compartment into the cell compartment, followed by a fast transport further to the basolateral compartment, is maintained by significant protonation of ionized α-acids due to rebalancing of the shifted acid/conjugated base equilibrium at the apical compartment. Therefore, it would be

interesting to study the effects of the pH of the transport medium on the epithelial transport of hop acids.

Often, transport of compounds across the intestinal epithelial is accompanied by phase-II conjugation reactions, which may affect drug absorption. Enzymatic hydrolysis of samples following a 60 min-incubation of β-acids with Caco-2 cells indicate that the largest fraction of the β-acids is present as sulfate and/or glucuronide conjugates, suggesting that intestinal absorption of the lipophillic β-acids is limited by substantial glucuronidation and/or sulfation by the enterocytes. For Caco-2 cells derived from human colon carcinoma, expression of human phase II metabolizing enzymes has been confirmed.[291] Therefore, the β-acid-derived phase-II metabolites found in the present study indicate that similar types of metabolites of β-acids might also be found in the human small intestine. It is also interesting to notice that the intracellular formed sulfated and glucuronidated β-acids were highly abundant in the apical compartment, whereas only low levels of those metabolites were found in the basolateral compartment. This suggests that a certain type of active transporter is involved in the selective efflux of the metabolites formed in the Caco-2 cells. Membrane transporters, especially the efflux transporters P-gp and MRP-2, are known to affect absorption and oral bioavailability of drugs. Previous studies demonstrated that P-gp- and MRP-type efflux pumps are capable of transporting phase-II metabolites such as glutathione, and glucuronide and sulfate conjugates.[292]

Verapamil and indomethacin are representative inhibitors for *in vitro* screening and appropriate standards for such experiments. In drug discovery, for the P-gp-substrate specificity, the so-called “Rule of Four”, can be applied as an aid in predicting whether or not a test compound is likely to be a candidate for P-gp-involvement. The rule can be summarized as follows: a compound is more likely to be a P-gp-substrate when it has $(N + O) \geq 8$ and a molecular weight (MW) > 400 with weak acidic character ($pKa > 4$). Most P-gp-substrates can be defined as lipophillic and neutral species, while MRP-2 is directly responsible for the intestinal elimination of organic anions, complementing the substrate specificity of P-gp.

The bidirectional transport of colupulone and (n-lupulone + adlupulone) exhibited efflux ratios of 10.3 and 9.7, respectively, suggesting the involvement of carrier-mediated transport for β-acids (active efflux pathway by P-gp-, BCRP-, and/or MRP-2-type efflux pumps). Co-administration of verapamil or indomethacin did not modify the transport of hop α-acids, as expected. In contrast, in the current study, both P-gp and, to a larger extent, MRP-2 decreased the efflux of β-acids to the apical compartment and increased the disposition of the β-acids to the basolateral compartment. Further study is needed to determine the possible interference of verapamil and indomethacin with phase-II conjugation, since several phase-II metabolites of verapamil and indomethacin have been reported.[293, 294]

For the α-acids as well as for the β-acids, the different nature of the side chain at C-1 (isopentanoyl in n- and ad-, isobutyroyl in co-analogues) resulted in different absorption efficiencies. The co-analogues seemed to be more efficiently absorbed than the (n- + ad)-analogues. The different nature of the side chain is reflected by differences in diffusion and transport rates. The above observations suggest that the dissimilarity in the molecular structure and corresponding lipophilicity between the investigated α-acids and β-acids is sufficiently high to result in substantial differentiated absorption efficiencies, substrate affinity for efflux transporters, and metabolizing enzymes.

Although no substantial degradation in the experimental setup was observed, experiments including the stability of these acids during gastrointestinal digestion by *ex vivo* studies in the stomach and intestinal fluid could be a great contribution to the discussion of the findings of this study in a broader context.

In conclusion, the present study demonstrates fast and efficient absorption of hop α-acids. Cohumulone showed higher absorption than (n-humulone + adhumulone). On the basis of the low apparent permeability coefficient of β-acids, the *in vivo* absorption of orally administered β-acids is predicted to be low. The limiting factors in the absorption of β-acids could be the involvement of P-gp- and MRP-2-type efflux transporters and/or substantial

phase-II metabolism reactions. The results from the Caco-2 cell culture model must be interpreted with caution because clearance and toxicity have not been measured, although they should have an impact *in vivo*.

3.4.2 Caco-2 cell experiments of iso-α-acids and reduced derivatives

3.4.2.1 Bidirectional transport of iso-α-acids and reduced derivatives across Caco-2 monolayers

In this study, the absorption of IAA, DHIAA, and THIAA was investigated using an *in vitro* model system based on Caco-2 cell monolayers. The transport characteristics were determined in both the apical-to-basolateral (AP-to-BL) and the basolateral-to-apical (BL-to-AP) direction. In Figure 3.8A (AP-to-BL) and 3.8B (BL-to-AP), the cumulative amounts (pmol/cm²) transported into the receiving chamber after 4h of incubation as a function of the donor concentration of IAA, DHIAA, and THIAA is presented.

Significantly higher amounts ($P < 0.0001$) of IAA and THIAA were transported to the receiving compartment compared with DHIAA, both in absorptive (15.1 ± 0.8 nmol/cm² and 16.4 ± 2.2 nmol/cm² for IAA and THIAA versus 7.8 ± 0.6 nmol/cm² for DHIAA for a concentration of 120 µM in the apical compartment) and secretive (28.9 ± 7.2 nmol/cm² and 32.4 ± 3.4 nmol/cm² for IAA and THIAA versus 9.5 ± 2.6 nmol/cm² for DHIAA for a concentration of 120 µM in the basolateral compartment) directions. All transport results showed a linear dose-dependent relationship. The cumulative amounts (nmol/cm²) transported into the receiving compartment for a concentration of 120 µM as a function of time are shown in Figures 3.9A (AP-to-BL) and 3.9B (BL-to-AP)

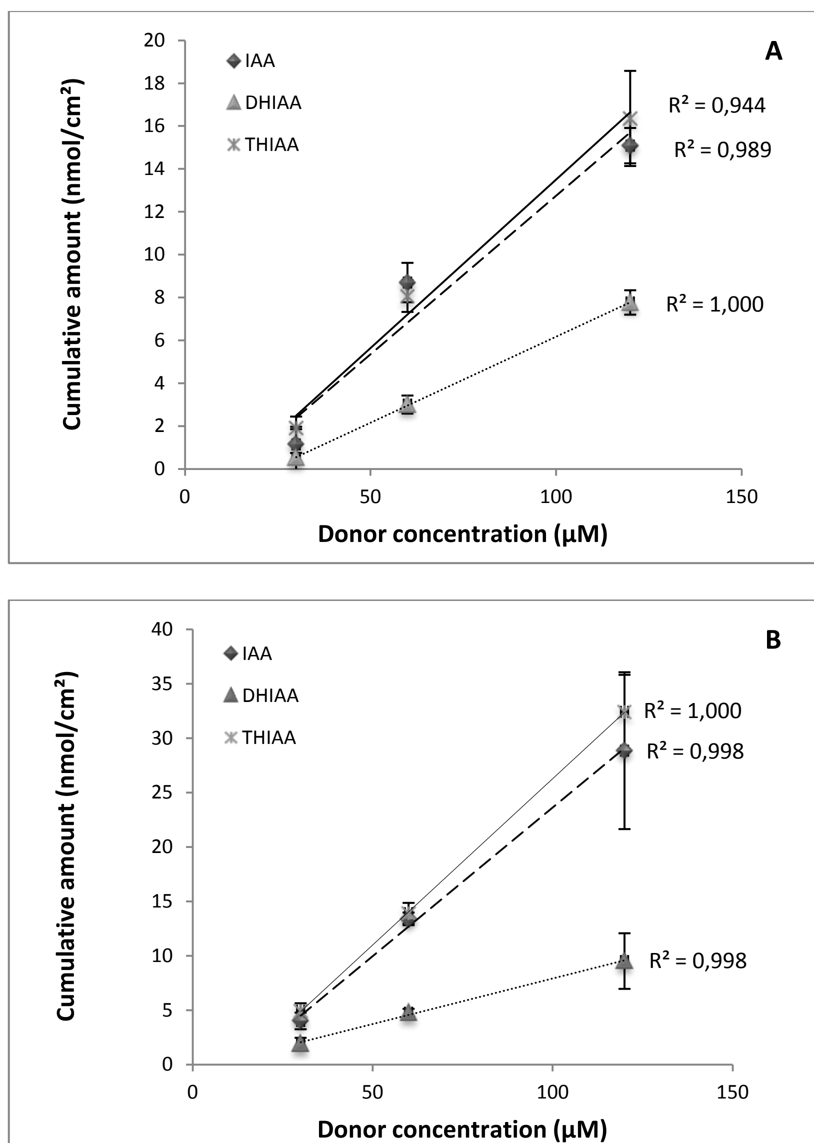


Fig. 3.8 Cumulative amounts (nmol/cm²) of IAA, DHIAA, and THIAA transported across Caco-2 monolayers in absorptive (A) (AP-to-BL) and secretive (B) (BL-to-AP) directions in function of different donor concentrations for 4 h of incubation. Each point was the mean ± standard deviation of three independent experiments. Trend lines fitted through zero show good linearity for AP-to-BL and BL-to-AP transport (r^2 > 0.95). IAA: iso-α-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids.

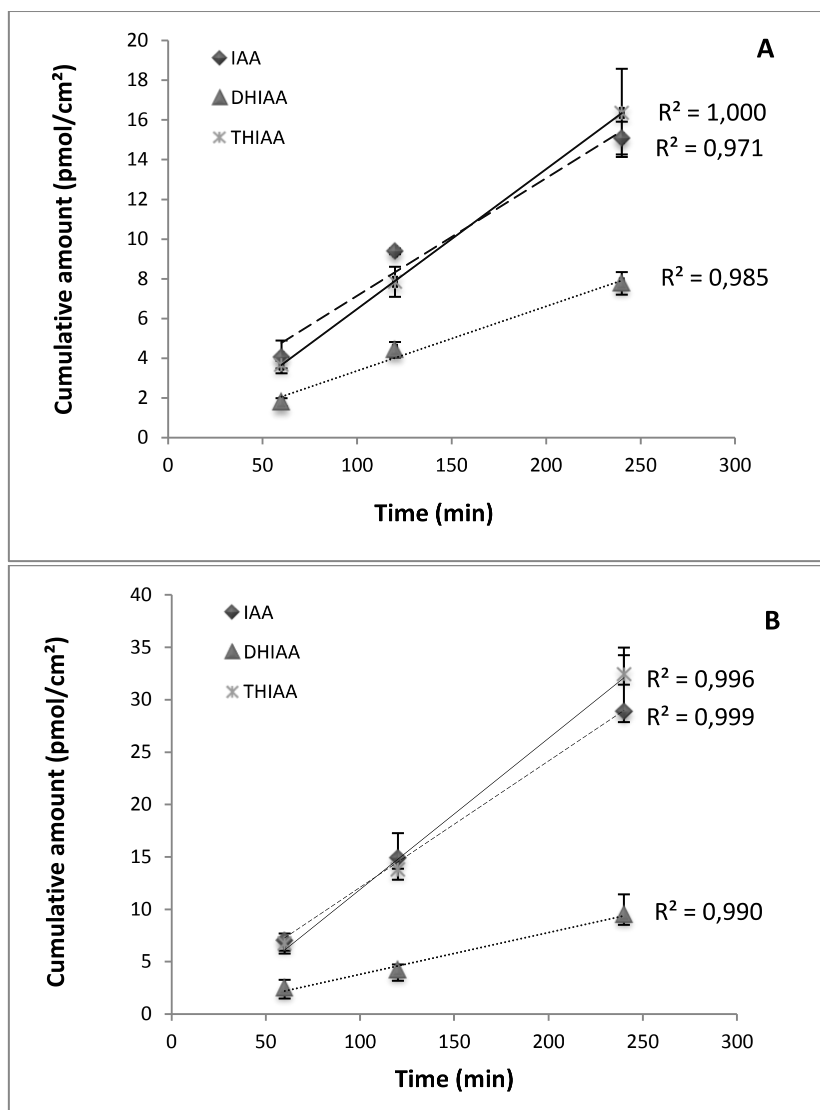


Fig. 3.9 Cumulative amounts (nmol/cm²) of IAA, DHIAA, and THIAA transported across Caco-2 monolayers in absorptive (A) (AP-to-BL) and secretive (B) (BL-to-AP) directions in function of time for a donor concentration of 120 µM. Each point was the mean ± standard deviation of three experiments. Trend lines fitted through zero show good linearity for AP-to-BL and BL-to-AP transport (r^2 > 0.97). IAA: iso-α-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids.

For all the hop-derived acids, the amount transported into the receiver chambers increased linearly with time, in both absorptive and secretive directions. The rates of transport are presented in Table 3.6, which shows the values for the apparent permeability coefficients P_{app} and efflux ratios for the hop-derived acids in the AP-to-BL, as well as in the BL-to-AP direction. The P_{appAB}-values ranged from 1.58 x 10^{-6} to 5.57 x 10^{-6} cm/s. Both IAA and THIAA showed similar absorption transport rates, since differences in their P_{appAB}-values were not statistically significant. In secretive transport, the P_{appBA} of the hop-derived acids varied from 5.68 x 10^{-6} to 16.28 x 10^{-6} cm/s. Efflux ratios of IAA and THIAA were similar around 3, slightly lower than the value for DHIAA which was around 3.5.

Table 3.6 Permeability in the AP-to-BL and BL-to-AP directions and efflux ratio of IAA, DHIAA, and THIAA across Caco-2 monolayers. Values are presented as mean values ± standard deviation. IAA: iso-α-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids[a]

	P_{app} (x 10^{-6} cm/s)		
Compound	**AP-to-BL**	**BL-to-AP**	**Efflux ratio**
IAA	4.62 ± 1.29	13.02 ± 3.13	2.8 ± 1.4
DHIAA	1.58 ± 0.22*	5.68 ± 0.87**	3.5 ± 1.0
THIAA	5.57 ± 1.22	16.28 ± 2.71	2.9 ± 1.1

***Significant difference in Papp (AP-to-BL) of DHIAA versus IAA and THIAA. P < 0.0001**
****Significant difference in Papp (BL-to-AP) of DHIAA versus IAA and THIAA. P < 0.0001**
[a] Comparison of P_{app} between IAA, DHIAA, and THIAA was performed using one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni. Statistically significant differences versus the P_{app} between IAA, DHIAA, and THIAA are indicated.

3.4.2.2 Intracellular accumulation of iso-α-acids and reduced derivatives in Caco-2 cells

The mass balance was determined from the sum of the cumulative amount transported, the amount remaining in the donor compartment, and the amount accumulated in the cells during the transport experiment in relation to the initial amount in the donor compartment. The total amount of IAA and THIAA recovered was 85-90 % in all experiments indicating that there was no significant breakdown, metabolism, nor sorption to the surface of the Transwell® microplates and inserts.

In contrast, recovery of DHIAA was lower than 45%. The percentages of hop-derived acids associated to the cells with respect to the initial dose were found to be 1.5% with IAA, 0.9% DHIAA, and 2.0% with THIAA. This showed that hop-derived acids were not significantly accumulated in the cells during the transport experiment. In contrast, a substantial amount was transported across the monolayer.

To probe possible phase-II metabolism of IAA, DHIAA, and THIAA, enzymatic hydrolysis of fractions from cell monolayers was carried out with a mixture of sulfatase and glucuronidase (sulfatase from *H. Pomatia* with secondary β-glucuronidase activity). The presence of conjugates could not be demonstrated in samples of IAA and THIAA. The amounts, quantified after deconjugation, were not significantly different from the non-hydrolyzed levels, indicating that formation of phase-II metabolites of IAA and THIAA seems unlikely. However, enzymatic hydrolysis of cellular fractions of DHIAA showed that up to 60 % of the intracellular amount was conjugated as glucuronides and/or sulfates.

3.4.2.3 Discussion

Hop-derived bitter acids, have been reported to show important bioactive properties, including anti-inflammatory, anti-angiogenic properties, lipid metabolism enhancement, and counteracting diabetes type 2. However, a review of the literature shows that data on bioavailability of these compounds are lacking. In this study, the *in vitro* transport characteristics, of IAA, DHIAA, and THIAA were assessed. The doses of hop acid applied in the present Caco-2 assay (30-120 μM) are relevant, as beer may contain up to 100 mg/l (or equal to 250 μM) hop-derived bitter acids (depending on beer type and brand).[90] The dose solutions used in the experiments are diluted from commercially available Isohop®, Redihop® and Tetrahop Gold®, which are stable solutions (pH 8-10) of potassium salts of IAA, DHIAA, and THIAA, respectively. For optimal stability and solubility, dose formulations were obtained by dilution with HBSS buffer at pH 7.4. Results from the Caco-2 experiments showed that permeability (AP-to-BL) mechanisms other than passive diffusion seem unlikely, as indicated by the linear dose-transport and time-transport relationships, and the lack of saturation effects. A passive diffusion transport mechanism occurs most probably transcellularly, since the paracellular pathway is restricted by the tight junctions of intestinal epithelium.[295] Also, the surface area of the luminal cell membrane of the intestinal epithelium is 1000-fold larger than that of the paracellular space.[285] For the concentration range used in this study, the linear dose-transport relationship indicated that P_{app} values were independent of the dose applied for all hop-derived bitter acids.

Based on the P_{appAB} of IAA, DHIAA, THIAA in combination with the results of the α-acids (AA) and β-acids (BA) of the previous subsection, a ranking in absorption is suggested as follows, AA > THIAA ≈ IAA > DHIAA > BA. In studies attempting to correlate passive drug permeability in Caco-2 experiments with drug absorption in 5 humans after oral administration, it was observed that moderately to well-absorbed compounds (20-80% fraction absorbed) had permeability coefficients of $1 \times 10^{-6} < P_{app} < 10 \times 10^{-6}$ cm/s, whereas poorly absorbed drugs had $P_{app} < 0.1 \times 10^{-6}$ cm/s (< 20 % fraction absorbed).[252, 296] Taking

this into account and based on the values obtained in this study, a fair to good intestinal absorption of this range of compounds could be expected.

For all hop-derived acids examined, secretion (BL-to-AP) showed a linear relationship between dose and amount transported suggesting that secretion is also expected to occur by passive diffusion. Efflux would only become important, when concentrations at the luminal side attain 20-50% of that at the blood side, independent of the involvement of active transporters (since diffusion is forced by a concentration gradient). Most likely, this is only reached just before complete absorption. However, efflux permeability coefficients (BL-to-AP) P_{appBA} ranged from 6 to 16 x 10^{-6} cm/s and efflux ratios (around 3-3.5) were substantial, hence additional experiments using specific inhibitors for efflux pumps (Pgp, MRP-2, and BCRP) providing proof of possible active efflux mechanism involvement would be important. In earlier work (results presented in subsection above),[297] the *in vitro* transport of hop α-acids and β-acids across Caco-2 monolayers has been investigated, showing efficient epithelial transport of hop α-acids (P_{app} > 10 x 10^{-6} cm/s), whereas the permeability of β-acids was limited by the involvement of Pgp and MRP-2 type efflux transporters and phase-II metabolism.

The lower P_{appAB}-value of DHIAA compared to the values for IAA and THIAA could be explained by a substantial conjugation of DHIAA, following absorption in the Caco-2 cells, from where the major fraction being conjugated can transfer to the BL-compartment, or back into the AP-compartment, whether or not with the involvement of active transport mechanisms, most often of the MRP-type family.[272] The absence of conjugation of IAA and THIAA provides an indication for conjugation via the accessible alcohol group in the molecular structure of DHIAA. The enolic group, present in the molecular structures of IAA and THIAA, may be inactive for conjugation in view of its acidity or due to intramolecular hydrogen bonding with the adjacent carbonyl group in the acyl side chain.

Although the Caco-2 cell model is recognized rather as a model for human intestinal absorption than for studying phase-II intestinal metabolism, there are examples of conjugation reactions of xenobiotics by Caco-2 cells in the literature,[263, 264, 298] including

flavonoids originating from hops, which are known to be extensively conjugated by Caco-2 cells.[265]

In conclusion, the present study demonstrates fast and efficient intestinal absorption of hop iso-α-acids and reduced derivatives. In the next chapter, the tissue culture data obtained in these Caco-2 studies will be compared with pharmacokinetic data generated *in vivo.*

IV. Bioavailability of hop-derived bitter acids using the rabbits as an *in vivo* model

4.1 *In vivo* studies on the bioavailability

<u>This subchapter is based on:</u>

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 and Function* **2011,** *2 (7)*, 412-422

4.1.1. Introduction

To express the efficacy of a compound to enter the systemic circulation, the term 'bioavailability' is used. Bioavailability can be described as a numerical parameter which reflects the unchanged fraction of a compound that reaches the systemic circulation following administration. Following intravenous dosing, bioavailability is 100%. For every other way of administration, the bioavailability varies between 0 and 100%. Factors affecting an incomplete bioavailability can be numerous, including incomplete dissolution, intestinal instability, or metabolism.

Oral dosage dominates contemporary drug therapy, as it is regarded to be safe, efficient and easily accessible with minimal discomfort compared to other routes of administration, such as sublingual, rectal, subcutaneous, intramuscular, transdermal, ocular, or intranasal dosing. However, despite the advantages of oral administration compared to alternatives, many of the mechanisms of drug uptake following oral administration remain to be fully characterized. Previously, for example, drug development projects were frequently terminated on grounds of poor gastrointestinal absorption and bioavailability.[299] Today, however, it is fully accepted that it is important to consider the total biopharmaceutical (e.g. solubility, permeability, first pass effect) and pharmacokinetic variables (clearance, half-life, volume of distribution) in pharmaceutical drug development.

The bioavailability is determined from the corresponding pharmacokinetic (PK) profiles obtained by *in vivo* research. Pharmacokinetics (PK) is the study of a drug and/or its metabolite kinetics in the body. More precisely, it refers to the temporary evolution of a compound and its metabolites in plasma, (or whole blood), and target tissues/ organs over time. The body is a very complex system and a compound undergoes many steps as it is being absorbed, distributed through the body, metabolized, and/ or excreted (ADME). In general, pharmacokinetics can be divided into two categories of study: absorption and disposition. Disposition is further subdivided into the study of distribution and elimination.

The term elimination includes both metabolism and excretion, since a compound is considered being eliminated when it is no longer in its original chemical structure, even if the resulting metabolites remain in the body. The dynamic relationship between ADME and PK-properties provide the basis for drug discovery. Usually, ADME studies by *in vitro*, *in situ (e.g.* perfusion of specific organ*)*, *in vivo*, or *in silico* techniques are focused solely in one specific pharmacokinetic aspect (Absorption, Distribution, Metabolism, Excretion) whereas the name 'pharmacokinetics' is normally reserved to *in vivo* studies where an approach of all the ADME processes together is taken.

In the present study, different aspects of the bioavailability of the hop-derived bitter acids were investigated. The pharmacokinetic parameters for AA, BA, IAA, DHIAA, and THIAA were investigated upon both intravenous and oral dosing in New Zealand white rabbits. From these values, the absolute bioavailability of the different HBA was determined. Knowledge on the bioavailabilities and the pharmacokinetic parameters of hop-derived AA, BA, IAA, DHIAA, and THIAA is essential to understand possible health benefits associated to preparations containing hop-derived compounds such as beer and hop-based food supplements.

4.1.2 Materials and methods

4.1.2.1 Materials

Isohop® (20% w/v iso-α-acids), Redihop® (35% w/v dihydroiso-α-acids), and Tetrahop Gold® (10% w/v tetrahydroiso-α-acids) were obtained from Barth-Haas Group (Botanix, Kent, UK). LC-MS solvents (analytical grade) were from Biosolve (Valkenswaard, the Netherlands).

International calibration standards for α-acids and β-acids (ICE-2; 49.39% α-acids (w/w), 24.94% β-acids (w/w)), iso-α-acids (DCHA-Iso, ICS-I3; 62.3% *trans*-iso-α-acids), dihydroiso-

α-acids (all *cis*-dihydroiso, ICS-R2; 65.3 % w/w *cis*-dihydroiso-α-acids), and tetrahydroiso-α-acids (ICS-T3; 99.4% w/w tetrahydroiso-α-acids) were all obtained from Labor Veritas (Zurich, Switzerland). Sulfatase type H1 (from Helix pomatia), was purchased from Sigma-Aldrich (Bornem, Belgium).

4.1.2.2 Methods

In vivo evaluation

The protocol of the animal experiments was approved by the Ethics Committee of the Institute for Agricultural and Fisheries Research (ILVO) (Merelbeke, Belgium). New Zealand white rabbits (3.0 ± 0.5 kg) were fasted 16 h prior to the experiment. Water was available *ad libitum*. In accordance with the regulations that apply to animals in laboratories in the 'Guide for the Care and Use of Laboratory Animals',[300] the rabbits were sedated with an intramuscular injection of 0.05 ml kg^{-1} Placivet® (Codifar, FL, USA) immediately after either intravenous (i.v.) or oral administration.

Collection of plasma samples

A first group of 12 rabbits was divided in 4 groups of 3 animals. Each group (n = 3) received an i.v. dose in the marginal ear vein of 25 mg kg^{-1} (dose volume 1 ml) of AA + BA, IAA, DHIAA, or THIAA, respectively. Donor solutions containing 75 mg ml^{-1} of (AA + BA) (containing 75 mg ml^{-1} of a mixture of AA and BA) were prepared in ammonium acetate buffer (0.1 mol/l, pH 10) by dilution of a methanolic solution made up by dissolving a define amount of calibration standard.

Donor solutions containing 75 mg ml^{-1} of IAA, DHIAA, or THIAA were prepared in ammonium acetate buffer (0.1 mol/l, pH 10) by dilution of commercially available aqueous

solutions (potassium salts) of hop-derived bitter acids at pH 8–10 (Isohop® 30%, Redihop® 35%, Tetrahop Gold® 9%). The pH of the solution was increased to allow solubility of the compounds of interest. It is not expected that this significantly influences the pharmacokinetics upon both intravenous and oral intake as the total dosed volume was low (1 ml) and the recipient compartments (blood or stomach) have sufficient buffering capacity. Blood samples were collected from the ear vein at 0.5 min before and at 0.5, 2, 5, 10, 15, 30, 45, 60, and 120 min after i.v. dosing; (in case of α-acids + β-acids, samples up to 60 min. were collected). A second group of 24 rabbits was randomly divided in 4 groups of 6 animals. Each group (n = 6) was administered, via oral gavage using a syringe, an oral dose of 25 mg kg^{-1} (dose volume of 1 ml) of AA + BA, IAA, DHIAA, or THIAA, respectively. Similar to the i.v. administration, dosing solutions were diluted in ammonium acetate buffer (0.1 mol/l, pH 10) to a final concentration of 75 mg ml^{-1} from commercially available Isohop® 30%, Redihop® 35%, and Tetrahop Gold® 9%. Blood samples were collected at 0.5 min before and at 0.5, 1, 2, 4, 6, 8, 12, 16 and 24 h after oral dosing. After addition of heparin (LEO Pharma, Wilrijk, Belgium) to the blood samples, plasma was separated by centrifugation (700 x g, 5 min) and samples were stored at -20 °C until further processing.

Collection of faeces and urine

In a separate experiment, 12 rabbits were divided in 4 groups of 3 animals. Each group was either administered a single oral dose or single i.v. dose in the marginal ear vein of 25 mg kg^{-1} solution of AA + BA, IAA, DHIAA, or THIAA, respectively. Donor solutions were diluted in ammonium acetate buffer (0.1M, pH 10) from commercially available solutions of potassium salts of HBA at pH 8–10 (Isohop® 30%, Redihop® 35%, and Tetrahop Gold® 9%, respectively).

Urine and faeces were collected over a period of 24 h after oral and i.v. dosing. All samples were immediately frozen at -20°C and analyzed within 2 months.

Sample extraction

The total volume of urine was determined and a sample of the total urine (500 µl) was diluted with 2.5 ml sodium acetate (NaOAc) buffer (0.1 mol/l; pH 5.0). Faecal material was lyophilized, weighted and grinded. Samples of total faeces (1.0 g) were diluted with 3 ml H_2O and homogenized. To 1.0 g of this homogenate, 5 ml NaOAc buffer (0.1 mol/l; pH 5.0) was added. Subsequently, the urine, faecal, and plasma samples (300 µl) were spiked with internal standard (IS) (1.0 mg). THIAA were applied as internal standard in case of samples containing AA + BA, IAA, and DHIAA. In plasma samples following dosing of THIAA, DHIAA were used as internal standard. After addition of IS, samples were acidified (pH 2) with H_3PO_4 (0.1 mol/l; 1.5 volumes) followed by extraction with EtOAc (4 volumes). The EtOAc phase was evaporated (N_2) and residues were reconstituted in 100 µl MeOH. Samples were stored at -20 °C prior to LC-MS analysis.

Stability

The short-term stability of (AA + BA), IAA, DHIAA, and THIAA in rabbit plasma and urine was evaluated. Blank plasma (500 µl) and urine (300 µl) were spiked with (AA+BA), IAA, DHIAA, or THIAA at concentrations of 0.1 and 1.0 mg ml^{-1} and left at room temperature for 12 h. Next, the samples were extracted following the procedure described in the section ‘‘Sample extraction’’ and analyzed using LC-MS. The concentration of each compound was compared to that of fresh samples and expressed in terms of degradation.

Enzymatic hydrolysis of samples

The extent of phase-II conjugation of the HBA as sulfate or glucuronide in the different samples (plasma, urine, faeces) was determined by enzymatic hydrolysis, based on a method validated including by Wyns *et al.*[281] In this study, a bioanalytical method for the

detection of 13 phytoestrogens and their conjugates (including xanthohumol and 8-prenylnaringenin) has been fully validated. Plasma samples (300 µl) and a sample of total urine (500 µl) were diluted with 5 volumes NaOAc buffer (0.1 mol/l; pH 5.0). A sample (1.0 g) of total faeces was diluted with 3 ml H_2O and homogenized. To 1.0 g of this homogenate, 5 ml NaOAc buffer (0.1 mol/l; pH 5.0) was added. To each of these mixtures, a preparation containing both β-glucuronidase (10000 units ml^{-1}) and sulfatase (330 units/ml) from *H. pomatia* (30 µl) from a solution in NaOAc buffer (0.1 mol/l, pH 5) was added. Samples were incubated for 2 h at 37 °C. Afterwards, samples were extracted as described in the section 'Sample extraction'. Replicate control samples of urine, faeces, and plasma were included with no enzyme treatment to determine the extent of glucuronidation and/or sulfation. Urine, plasma and faecal levels of conjugated hop-derived bitter acids were calculated by subtracting the amount of free hop acid (no enzyme treatment) from the amount of total hop-derived bitter acids (+ β-glucuronidase/sulfatase).

LC-MS analysis of samples

The extracted samples from the different biological media (urine, faeces, plasma) were analyzed using LC/MS analysis (Agilent 1200 LC-MS, Agilent, AT, Santa Clara, California, USA). The Agilent Chemstation software package (Rev.B.02.01) was used to control the analytical system as well as for data acquisition and processing. As stationary phase, an Xbridge C_{18} column (150 x 30 mm, 3.5 µm) (Waters, Zellik, Belgium) connected to a C_{18} guard column (20 x 3.0 mm; 3.5 µm) (Waters, Zellik, Belgium) was used. The mobile phase consisted of 10 mM ammonium acetate pH 9.75 + 20% MeOH (A) and 100% MeOH (B). The mobile phase was degassed by the integrated AT 1200 series vacuum degasser. The flow rate was 0.5 ml min^{-1} and the column temperature was 40 °C. The initial mobile phase, 27% B, was increased linearly to 60% B over 24 min, maintained for 5 min, and

further increased to 95% B in 5 min and maintained during 4 min. Finally, the mobile phase was re-adjusted to 27% B in 1 min and re-equilibrated at 27% B for 6 min prior to the next injection. UV-detection was performed at 314 nm for α-acids and β-acids and at 270 nm for iso-α-acids, dihydroiso-α-acids, and tetrahydroiso-α-acids. MS analysis was performed using an AT multimode ionization source coupled to a single quadrupole detector (MSD), SL version. A standard APPI/APCI calibration mix was used for daily tuning of the MSD-source. The MS-parameters in the negative atmospheric pressure chemical ionization (APCI) mode were tuned to maximize formation of the deprotonated analyte. Interface settings were as following: N_2 drying gas temperature 250 °C, N_2 drying gas flow 5 l min^{-1}, APCI vaporizer temperature 150 °C, nebulizer pressure 10^5 Pa, capillary voltage 1000 V, corona current 6 µA, and charging voltage 1000 V. In each analysis, qualitative identification was performed in the negative ion scan mode (*m/z* 150–700) and quantitative data were obtained by construction of the extracted ion chromatogram following measurement in the selected ion monitoring (SIM) mode. The $[M - H]^{(-)}$ m/z-values, used for quantification of AA, BA, IAA, DHIAA, and THIAA, and the retention times of the different compounds are presented in Table 4.1. The quantitative data of the analysis of THIAA were assessed as an example to determine a possible difference in bioavailability between the *cis*- and *trans*-stereoisomers or between the different co- versus n-homologues. Calibration curves were established by linear-least-squares regression analysis using the ratio of the peak area of total AA, BA, IAA, DHIAA, or THIAA versus IS against the concentration of hop-derived acids in 6 standards (blank plasma medium) covering the range of 0.1 to 4.0 µg ml^{-1} (or 0.30 to 12 µM) in three replicates.

Daily prepared calibration curves were constructed by injecting calibration standards at the beginning and at the end of each batch of samples.

To evaluate the accuracy and precision of the method, recovery experiments were carried out on three consecutive days by adding different concentrations of AA, IAA, DHIAA, and THIAA (0.1 and 1.0 µg ml^{-1}) to blank urine and plasma. The accuracy was expressed in terms of relative error, and the precision was evaluated in terms of the relative standard

deviation (RSD). The limit of detection (LOD) and the limit of quantification (LOQ) of the method were defined as the lowest concentration with a signal-to-noise ratio of 3 and 10, respectively, in spiked samples, where the noise data were taken from the analysis of blank matrices.

Table 4.1 Molecular ions used for the selected ion monitoring (SIM) mode for the various hop acids in LC-MS analysis.

	Compound		MW (g/mol)	m/z (-)
α-acids	cohumulone	AA_1	**348**	**347**
	adhumulone	AA_2	**362**	**361**
	humulone	AA_3		
β-acids	colupulone	BA_1	**400**	**399**
	adlupulone	BA_2	**414**	**413**
	lupulone	BA_3		
iso-α-acids	*cis*-isocohumulone	IAA_1	**348**	**347**
	trans-isocohumulone	IAA_2		
	cis-isoadhumulone	IAA_3	**362**	**361**
	trans-isoadhumulone	IAA_4		
	cis-isohumulone	IAA_5		
	trans-isohumulone	IAA_6		
dihydroiso-α-acids	*cis*-dihydroisocohumulone	DH_1	**350**	**349**
	cis-dihydroisocohumulone	DH_2		
	cis-dihydro-isoadhumulone	DH_3	**364**	**363**
	cis-dihydro-isoadhumulone	DH_4		
	cis-dihydro-isohumulone	DH_5		
	cis-dihydro-isohumulone	DH_6		
tetrahydroiso-α-acids	*cis*-tetrahydro-isocohumulone	TH_1	**352**	**351**
	trans-tetrahydro-isocohumulone	TH_2		
	cis-tetrahydro-isoadhumulone	TH_3	**366**	**365**
	trans-tetrahydro-isoadhumulone	TH_4		
	cis-tetrahydro-isohumulone	TH_5		
	trans-tetrahydro-isohumulone	TH_6		

Pharmacokinetic calculations

The plasma concentration-time profiles were analyzed by Win-NonLin® (version 5.2.1, Pharsight Corporation, Mountain View, California, USA). The pharmacokinetic parameters were determined from the individual plasma concentration-time profiles by non-compartmental analysis. According to the plasma concentration-time curves, the area-under-curve ($AUC_{0\text{-}t}$) was calculated by the linear trapezoidal rule from zero to the last time point showing a measurable concentration of the analyte. The terminal half-life ($t_{1/2}$) was calculated as $[\ln(2)]\ \lambda_z^{-1}$ and λ_z was the terminal elimination rate constant and was estimated from the slope of the terminal regression line. The $AUC_{0\text{-}\infty}$ was calculated as $AUC_{0\text{-}t} + [C_t\ \lambda_z^{-1}]$, where C_t is the last detectable plasma concentration and t is the time at which this concentration occurred. In all of the cases, the degree of extrapolation of $AUC_{0\text{-}\infty}$ was lower than 20%. The peak concentration (C_{max}) and the time at which this occurred (t_{max}) were obtained from the observed data. The oral bioavailability (F) was determined by the ratio of the $AUC_{0\text{-}\infty}$ following oral and i.v. dosing.

Statistical analysis

SPSS release 17.0 for Windows (SPSS, Chicago, Illinois, USA) was used for all statistical analyses. The *in vivo* experiments had 3 independent observations for each test group (an identical dose of (AA + BA), IAA, DHIAA, and THIAA, respectively, was applied to 3 rabbits), except for the oral plasma study in which 6 individual rabbits received an identical oral dose. Data were expressed as means ± standard deviation. The normality of distribution was investigated using the Shapiro-Wilk test and the homogeneity of variances was evaluated using Levene's test. Comparison of means between more than 2 groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance. Results were considered to be statistically significant when $P < 0.05$.

4.1.3 Results

Stability

To investigate the short-term stability of the studied compounds in rabbit plasma and urine, control experiments with (AA + BA), IAA, DHIAA, and THIAA were carried out. Almost all (>95%) spiked hop-derived bitter acids were recovered unchanged from rabbit urine and plasma, indicating no significant degradation for (AA + BA), IAA, DHIAA, or THIAA. In addition, no inter-conversion of AA to IAA, or from the *trans*- to *cis*-form of IAA, DHIAA, and THIAA was observed.

LC-MS Method for quantification of hop-derived bitter acids in plasma and urine

For more details on the chromatographic LC-MS method used for separation of α-acids, β-acids, iso-α-acids, dihydro-iso-α-acids, and tetrahydro-iso-α-acids, please refer to the previous chapter. The limits of detection (S/N = 3) for all compounds, both in urine and plasma, are listed in Table 4.2. In urine, the LODs ranged from 3.0 ng/ml to 40.1 ng/ml, and in plasma, from 4.0 ng/ml to 46.9 ng/ml. The limits of quantification were set as the lowest point of the calibration curves with a signal-to-noise ratio of at least 10. With regard to specificity, only a few small peaks were observed in the mass chromatograms of different blank urine and plasma samples. Since the present method also makes use of extracted ions for quantification, the background is further reduced and the sensitivity as well as specificity are increased. Moreover, simultaneous recording of characteristic retention times and UV spectra favored accurate identification and quantification of the target compounds. The linearity was tested in urine for the range of concentrations 0.1–4 μg/ml for all compounds. Calibration curves showed linear responses for all analytes over the dynamic ranges and the corresponding regression correlation coefficients (r^2) were all >0.995.

Table 4.2 Limit of detection (LOD) in urine and plasma

	Plasma LOD (ng/ml)	Urine LOD (ng/ml)
AA	46.9	9.3
BA	2.3	3.0
IAA	4.0	31.0
DHIAA	4.1	14.2
THIAA	10.0	40.1

The accuracy and precision, expressed as the percentage relative error (%RE) and relative standard deviation (RSD), was within the acceptable ranges of 15% for all compounds at all concentrations (Table 4.3), except for the α-acids in plasma (18.4% RSD). In urine, the accuracy ranged from 2.9% to 11.0% and, in plasma, from 5.3% to 10.0%. The intra-assay precision ranged from 3.4% to 5.3% and from 1.3% to 8.9% in urine and plasma, respectively. The inter-assay precision ranged from 4.9% to 13.2% and from 5.4% to 18.4% in urine and plasma, respectively. Based on the data above, this method was considered suitable for our purpose.

Table 4.3 Accuracy (%RE) and precision (RSD) (intra- and inter-assay) in urine and plasma

	Plasma			**Urine**		
	accuracy	precision		accuracy	precision	
		intra-assay	*inter-assay*		*intra-assay*	*inter-assay*
AA	5.3	8.9	18.4	11.0	3.5	13.2
BA	7.3	4.7	6.2	10.4	5.3	9.9
IAA	4.9	1.3	8.9	6.5	4.5	4.9
DHIAA	5.4	2.4	5.4	12.0	3.4	8.9
THIAA	10.0	4.5	10.1	2.9	3.6	12.1

Pharmacokinetics of AA, BA, IAA and reduced derivatives

In neither of the fractions (urine, plasma, fecal), no detectable amount of unchanged β-acids could be observed upon IV and oral dosing of β-acids. Therefore, results on bioavailability of β-acids have been left out in this work.

Plasma concentration-time curves following i.v. and oral administration of AA, IAA, DHIAA, and THIAA are presented in Fig. 4.2 and Fig. 4.3, respectivelyThe corresponding pharmacokinetic parameters calculated from the non-compartmental analysis are shown in Table 4.4 and Table 4.5. After i.v. injection of 25 mg kg^{-1} of AA, IAA, DHIAA, and THIAA, the plasma levels of AA and IAA declined faster (clearance of AA and IAA was 707 ± 17, and 931 ± 91 ml h^{-1}, respectively) than those of DHIAA and THIAA, which showed both a similar elimination slope (Fig. 4.2). This is also illustrated by a half-life ($t_{1/2}$) of 0.34 ± 0.02 h for AA and 0.32 ± 0.03 h for IAA versus 0.72 ± 0.10 h and 0.69 ± 0.07 h for DHIAA and THIAA, respectively.

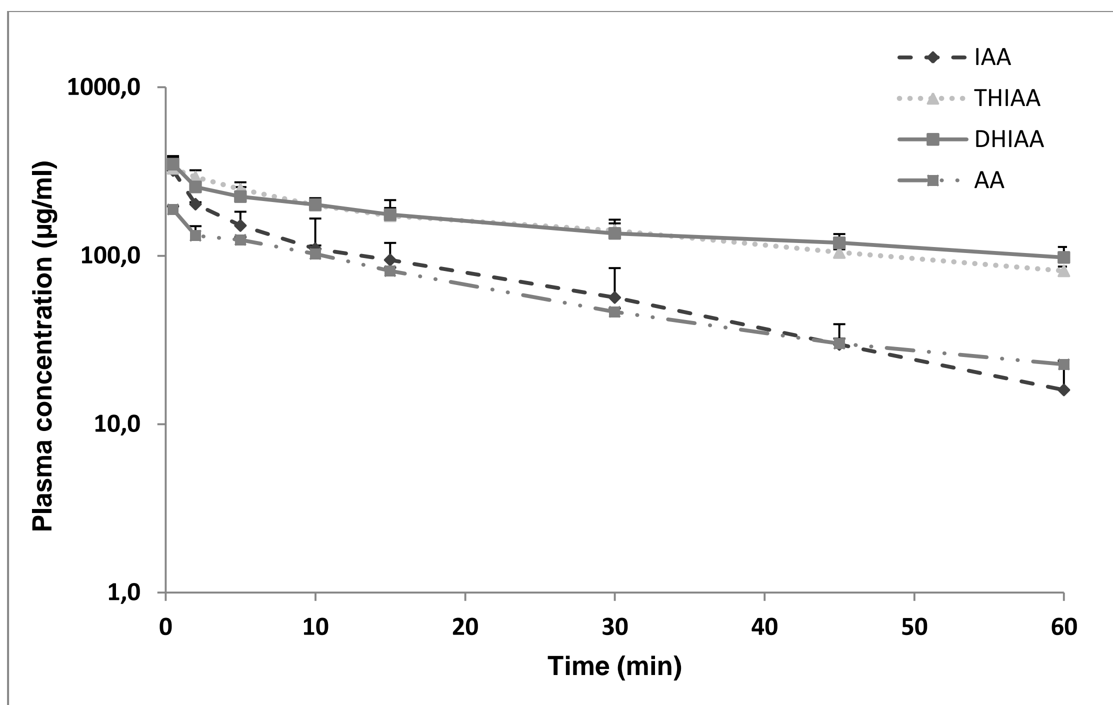


Figure 4.2 Plasma concentration-time curves in rabbits following intravenous (0.5 – 60 min; n = 3) administration of 17 mg/kg AA or 25 mg/kg IAA, DHIAA, and THIAA. Values represent the mean plasma concentration and error bars represent the standard deviation. AA: α-acids IAA: iso-α-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids

Table 4.4 Non-compartmental analysis of pharmacokinetics of hop-derived bitter acids after a single intravenous (n=3) dose of 25 mg kg^{-1}.(17 mg/kg for AA). Values are presented as mean values ± standard deviation. AA: α-acids; IAA: iso-α-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids. Comparison of parameters following i.v. dosing between AA, IAA, DHIAA, and THIAA was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance. Statistically significant differences versus parameters between AA, IAA, DHIAA, and THIAA are indicated.

	i.v. administration			
Parameter	**AA**	**IAA**	**DHIAA**	**THIAA**
Cl (ml/h)	707 ± 17[a]	931 ± 91[b]	258 ± 67	300 ± 7
$t_{1/2}$ (h)	0.34 ± 0.02[c]	0.32 ± 0.03[d]	0.88 ± 0.29	0.69 ± 0.07
$AUC_{0-\infty}$ (h*µg/ml)	71 ± 2[e]	81 ± 8[f]	252 ± 7	250 ± 6

[a] Significant difference in Cl of AA versus DHIAA and THIAA upon i.v. dosing. P < 0.05

[b] Significant difference in Cl of IAA versus DHIAA and THIAA upon i.v. dosing. P < 0.001

[c] Significant difference in $t_{1/2}$ of AA versus DHIAA and THIAA upon i.v. dosing. P < 0.05

[d] Significant difference in $t_{1/2}$ of IAA versus DHIAA and THIAA upon i.v. dosing. P < 0.05

[e] Significant difference in $AUC_{0-\infty}$ of AA versus DHIAA and THIAA upon i.v. dosing. P < 0.001

[f] Significant difference in $AUC_{0-\infty}$ of IAA versus DHIAA and THIAA upon i.v. dosing. P < 0.001

When orally dosed, the plasma concentration-time profile of the individual rabbits (n = 6) was obtained, as shown in Fig. 4.3. Since large inter-individual variations were observed for the time to reach the absorption maximum, t_{max} was described as a range of values (Table 5.3). For DHIAA, t_{max} ranged 4.0–12 h post-dosing, while t_{max} of IAA and THIAA varied 0.5–6 h after ingestion. The time to reach the maximum concentration for AA ranged 2.0-8.0 h post-dosing. For AA and IAA, a C_{max} of 2.5 ± 1.6 µg ml^{-1} (or equal to 6.9 µM), and 3.7 ± 3.0 µg ml^{-1} (or equal to 10.3 µM), respectively, was determined, while for DHIAA, a maximum concentration was found, which was 2-3-fold higher than for AA or IAA (6.6 ± 3.8 µg ml^{-1} equaling 18.2 µM). Following oral dosing of THIAA, a C_{max} of 7.7 ± 4.3 µg ml^{-1} (or equal to 21.2 µM) could be measured. The area-under-curve $AUC_{0-\infty}$ upon oral gavages for DHIAA and THIAA were 70.7 ± 48.4 µg h ml^{-1} and 57.4 ± 9.0 mg h ml^{-1}, respectively. This was almost 6-7-fold higher than the value calculated for IAA, for which

$AUC_{0-\infty}$ was 10.6 ± 5.3 µg h ml^{-1}. Also, for AA, a higher $AUC_{0-\infty}$ was found compared to IAA (18.2 ± 9.0 for AA versus 10.6 ± 5.3 µg h ml^{-1} for IAA), despite the lower dose (17 versus 25 mg/kg).

The lowest absolute bioavailability was determined for IAA, being 13.0 ± 6.5, while the AA and the reduced derivatives, however, reached higher absolute bioavailabilities of 25.6 ± 13.2% for AA, 28.0 ± 19.4% for DHIAA and 23.0 ± 3.6% for THIAA.

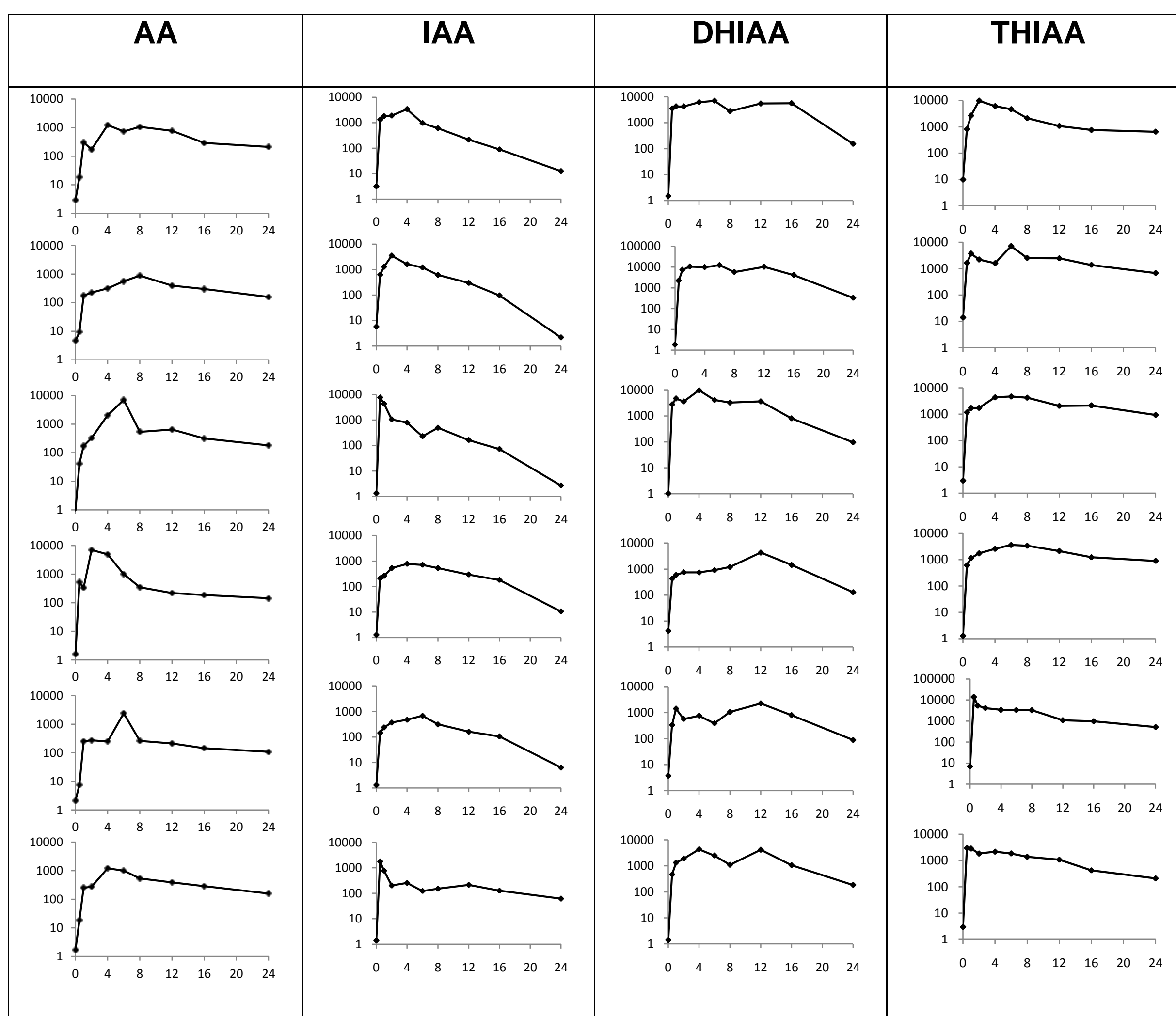


Figure 4.3 Individual plasma concentration-time profiles (log concentration (µg/ml) versus time (h) in rabbits following oral administration (0.5 – 24 h; n = 6) of 25 mg/kg AA, IAA, DHIAA, and THIAA. AA: α-acids; IAA: iso-α-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids

Table 4.5 Non-compartmental analysis of pharmacokinetics of hop-derived acids after a single oral (n=6) dose of 25 mg/kg. Values are presented as mean values ± standard deviation. Values for t_{max} are presented as ranging values of the individual rabbits. AA: α-acids; IAA: iso-α-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids. Comparison of parameters (following i.v. dosing and oral dosing) between IAA, DHIAA, and THIAA was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance.

	Oral administration			
Parameter	**AA**	**IAA**	**DHIAA**	**THIAA**
$AUC_{0-\infty}$ (h*µg/ml)	18.2 ± 9.0	10.6 ± 5.3	71 ± 48	57 ± 9
C_{max} (µg/ml)	3.7 ± 3.0	2.5 ± 1.6	6.6 ± 3.8	7.7 ± 4.3
t_{max} (h)	[2.0 – 8.0]	[0.5 – 6.0]	[4.0 – 12.0]	[0.5 – 6.0]
F (%)	25.6 ± 13.2	13.0 ± 6.5	28.0 ± 19.4	23.0 ± 3.6

To investigate possible phase-II metabolism of AA, IAA, DHIAA, and THIAA, enzymatic hydrolysis of plasma samples following oral treatment was carried out with a mixture of sulfatase and glucuronidase. The amounts of AA, IAA and THIAA, quantified after the enzymatic reactions, were not significantly different from the non-hydrolyzed levels; hence, the presence of conjugates could not be demonstrated in samples of AA, IAA and THIAA. However, enzymatic hydrolysis of plasma samples of DHIAA resulted in a value for $AUC_{0-\infty}$ of 140 ± 43 mg h ml^{-1}. From these results, the percentage of DHIAA being conjugated as a sulfate or a glucuronide could be estimated to be around 50%.

The pharmacokinetic data of THIAA were assessed as a model to determine a possible difference in bioavailability between the *cis*- and *trans*-stereoisomers or between the different co- versus n-homologues (Table 4.6 and Table 4.7). The different side chain at C_1 (isopentanoyl in n- and ad-, isobutyryl in co-analogues) resulted in a slightly higher bioavailability of the n-analogues compared to the co-analogues (25% versus 18%). On the other hand, in case of *cis*- and *trans*-tetrahydroisocohumulone there was no difference in bioavailability observed in favor of *cis*- (17%) compared to *trans*-analogue (19%).

Apparently, a higher grade of lipophilicity (of the n- versus co-analogue), and the presence of the side chains on different faces of the five-membered ring of the *cis*- and *trans*-analogues did not have substantial effect on the bioavailabilities.

Table 4.6 Pharmacokinetic parameters of *cis*- versus *trans*-stereoisomers of THIAA after a single oral (n=6) dose of 25 mg/kg. Values are presented as mean values ± standard deviation. Comparison of the bioavailability (F) between cis- (cis-THIAA: cis-tetrahydroisocohumulone) versus trans-stereoisomers (trans-THIAA: trans-tetrahydroisocohumulone) homologues of THIAA was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance.

	***cis*-THIAA**	***trans*-THIAA**
F (%)	17.0 ± 3.0	19.0 ± 4.0

Table 4.7 Pharmacokinetic parameters of co- versus n-homologues of THIAA after a single oral (n=6) dose of 25 mg/kg. Values are presented as mean values ± standard deviation. Comparison of the bioavailability (F) between co- (co-THIAA: sum of *cis*-tetrahydroisocohumulone and *trans*-tetrahydroisocohumulone) versus n- homologues (n-THIAA: sum of *cis*-tetrahydroisohumulone and *trans*-tetrahydroisohumulone) of THIAA was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance. Statistically significant differences versus parameters between co- versus n-homologues are indicated.

	co-THIAA	**n-THIAA**
F (%)	17.6 ± 2.6	25.1 ± 2.7

Fractions excreted in urine and faeces

The percentages of dose (%dose) of the compounds excreted unchanged in urine and faeces following oral and i.v. administration of AA, IAA, DHIAA, and THIAA are shown in Table 4.8. After oral gavage, the urinary %doses of intact IAA and AA were 0.13 ± 0.05% and 0.4 ± 0.2%, respectively.

This fraction was very small when compared with the levels of THIAA and DHIAA, for which the %dose was 1.0 ± 0.1% and 12.1 ± 4.8%, respectively. In the faeces, the %dose of intact THIAA following oral application was 25.6 ± 7.6%, while the %doses of DHIAA and IAA were 13.1 ± 1.1% and 6.0 ± 1.8%, respectively.

Table 4.8 Percentages (% of dose) of IAA, DHIAA, and THIAA 24 h after dosing in urine and faeces upon a single oral and i.v. dose of 25 mg/kg; n=3. The unabsorbed fraction is calculated as the difference in the amounts in faecal samples following oral and i.v. dosing. Values are presented as mean values ± standard deviation. IAA: iso-α-acids; DHIAA: dihydroiso-α-acids; THIAA: tetrahydroiso-α-acids. Comparison of amounts (following i.v. dosing and oral dosing) between IAA, DHIAA, and THIAA was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance. Statistically significant differences versus amounts between IAA, DHIAA, and THIAA are indicated.

Compound	Intravenous		Oral		Fraction unabsorbed (% of dose)
	Urine	Faeces	Urine	Faeces	
AA	0.3 ± 0.1	< LOD	0.4 ± 0.2	0.18 ± 0.05	-
IAA	0.5 ± 0.2	0.9 ± 0.2[b]	0.13 ± 0.05	6.0 ± 1.8	5.1 ± 2.0
DHIAA	15.4 ± 1.4[a]	8.4 ± 1.8	12.1 ± 4.8[c]	13.1 ± 1.1	4.7 ± 2.9
THIAA	0.8 ± 0.2	10.1 ± 2.1	1.0 ± 0.1	25.6 ± 3.6	15.5 ± 4.9

[a]Significant difference in urinary amount of DHIAA versus AA, IAA and THIAA upon i.v. dosing. $P < 0.005$
[b]Significant difference in faecal amount of IAA versus DHIAA and THIAA upon i.v. dosing. $P < 0.05$
[c]Significant difference in urinary amount of DHIAA versus AA, IAA and THIAA upon oral dosing. $P < 0.001$

Following i.v. administration, the urinary %dose of intact DHIAA was determined to be 15.4 ± 1.4%. The levels of AA, IAA, and THIAA were comparable: 0.3 ± 0.1 %, 0.50 ± 0.2% and 0.8 ± 0.2%, respectively. The faecal %dose of intact IAA after i.v. dosing was again very low compared to the doses of the reduced derivatives: 0.9 ± 0.2%. No detectable amount could be determined for AA. For DHIAA and THIAA, comparable percentages were found: 8.4 ± 1.8% and 10.1 ± 2.1% for DHIAA and THIAA, respectively.

From these data, it was possible to calculate the non-absorbed fraction as the difference in the faecal %dose following oral and i.v. administration. This was around 5% of the ingested dose for IAA and DHIAA, in contrast with THIAA, for which a non-absorbed fraction of 15.5 ± 4.9% was calculated. Large differences between the different classes of hop-derived acids could be observed: the urinary excretion of DHIAA upon both oral and i.v. dosing exceeded the values of AA, IAA and THIAA, which were significantly lower. The differences in urinary excretion between IAA and THIAA (and AA versus THIAA) were not statistically significant. Samples of faeces and urine were also subjected to enzymatic hydrolysis with a mixture of sulfatase and glucuronidase to screen for the presence of possible phase-II conjugates of IAA, DHIAA, and THIAA. Subtracting the amounts upon enzymatic hydrolysis from the control samples (in the absence of enzyme treatment) showed no significant conjugation of AA, IAA or THIAA in urine or faeces. However, enzymatic hydrolysis of urine samples of DHIAA following oral ingestion showed that up to 22% of the excreted amount of DHIAA was conjugated as a sulfate or a glucuronide.

4.1.4 Discussion

Hop-derived bitter acids, the main flavoring agents in beer, have been reported to show important bioactive properties, including anti-inflammatory, anti-angiogenic properties, lipid metabolism enhancement, and counteracting diabetes type 2. However, a review of the literature shows that data on the pharmacokinetics of these compounds are lacking. In this study, the pharmacokinetic parameters and oral bioavailability of hop-derived bitter acids (α-acids, iso-α-acids and reduced iso-α-acids) were assessed. Quantities of hop-derived bitter acids up to 100 mg l^{-1} may be present in beer (depending on beer type and brand) equaling 250 µM HBA.[90]

The dose solutions used in the rabbit trials are diluted from commercially available Isohop®, Redihop® and Tetrahop Gold®, which are stable solutions of potassium salts of respectively IAA, DHIAA, and THIAA, respectively, at pH 8–10. For stability and solubility issues, the dose formulations were obtained by dilution with ammonium acetate buffer (pH 10). Nevertheless, a potential influence on bioavailability by the use of non-physiological pH should be considered for further investigation. An animal dose of 25 mg kg^{-1} in the pharmacokinetic study could be translated to a human equivalent dose (HED) using the following formula: HED = 25 mg kg^{-1} x [K_m factor (rabbit)] / [K_m factor (human)], in which the K_m factor (kg m^{-2}) is calculated from the ratio of the body weight (kg) and body surface area (m^2) of a species. The FDA-draft guidelines report values of K_m = 12 for rabbits and 37 for humans, based on the ratio of the body weight and the body surface area (BSA).[301, 302] In this way, a HED of 8 mg kg^{-1} could be calculated, which would correspond with a dose of ± 500 mg for a human weight of 60–70 kg. This is in the line with the amounts of hop-derived acids present in commercially available dietary supplements (usually 400 mg or more) and the doses (single or frequent dosing) used in clinical trials with animals [128, 152, 156] or humans.[135, 229, 234] However, this dose is not conform to moderate beer consumption, since an amount of 400–500 mg hop-derived acids would require a beer intake of at least 5 liters. In all publications cited, no adverse effects were reported upon ingestion of these types of

compounds. In one report, Chappel *et al.* conducted a study to determine the effect associated with subchronic oral administration of THIAA (as well as hexahydroiso-α-acids) in the dog. Most materials were excreted in the faeces and the no-observed-adverse-effect level (NOAEL) of the compounds was 100 and 50 mg kg^{-1} body weight for THIAA and hexahydro-iso-α-acids, respectively. As for dogs, the observations showed that these compounds were generally well tolerated.[227]

Also, an observational human trial to investigate the efficacy of a formula containing DHIAA (Meta050) (440 mg daily for eight weeks) on pain in patients with rheumatic disease did not result in clinically relevant changes in blood pressure, complete blood counts, or liver and kidney function. Furthermore, there was no negative impact on gastrointestinal markers normally affected by selective COX-2 enzyme inhibitors, as concluded from normal fecal calprotectin excretion. Similar data were obtained after administration of pure DHIAA (450 mg daily for 2 weeks).[228, 229]

We found that the absolute oral bioavailability of iso-α-acids was less than 15% in NZ white rabbits, while the bioavailabilities of the α-acids and reduced derivatives were higher (25.6% for AA, 23% for THIAA and 28% for DHIAA). However, differences in bioavailabilities were determined to be not statistically significant, because of large inter-individual variations in the animals. Factors limiting a high bioavailability can be diverse, but typically include inefficient absorption and rapid metabolism (i.e., the first-pass effect).

In previous results in Caco-2 experiments studying intestinal absorption, the apparent permeability coefficients were determined for all compounds under investigation as $> 1 \times 10^{-6}$ cm/s indicating efficient permeability of these compounds, which would result in efficient absorption. In correspondence, the calculated non-absorbed fractions (5% for IAA and DHIAA and 15% for THIAA) obtained from the amounts determined in the faeces upon oral and i.v. application suggest efficient absorption of IAA, DHIAA, and THIAA. However, this calculation of the non-absorbed fraction is likely an underestimated value, since a significant fraction of the administered compounds can be metabolized and/or degraded by the

microbiota into diverse metabolites which were not detected in the analysis. Nevertheless, absorption of hop-derived acids is suggested to be efficient in rabbits, indicated by substantial plasma concentrations in the µg ml^{-1} (or lower µM) scale. From the pharmacokinetic data of DHIAA obtained following oral application, an $AUC_{0\text{-}8h}$ of 30.6 h mg ml^{-1} could be determined, which is in accordance with the results published by Hall *et al.*, taken into account an HED of 560 mg. Following oral application of a dose of 700 mg DHIAA administered to 2 healthy human subjects, an $AUC_{0\text{-}8h}$ of 26 mg h ml^{-1} was calculated, which is in line with the results of our study.[135]

Prior to absorption and introduction of a compound in the systemic circulation and exposure to liver enzymes, intestinal epithelial cells (enterocytes) provide the first site for CYP-catalyzed and phase-II metabolism,[303] since the highest catalytic activity resides in the proximal region of the small intestine.[254, 304, 305] Following oral dosing, only minor amounts of intact AA and IAA were determined in urine and faeces. In our study, the mass balance (a summed total of %dose in urine and faeces of intact and conjugated forms over 24 h) was lower than 6% for IAA and lower than 1% for AA, indicating that metabolism/degradation of AA and IAA are the most important paths of elimination. This seems consistent with the low levels recovered in urine and faeces after i.v. administration of IAA and AA. Previous 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.[306] This could suggest the involvement of P450-enzymes in the metabolism of α-acids and β-acids, which could explain the low levels of the unchanged α-acids found in urine and faeces. Also, the absence of detectable amounts of unchanged β-acids in plasma, urine, and faecal fractions could be caused by a fast biotransformation of the β-acids. Furthermore, two reports describe induction of quinine-reductase activity by AA and IAA and activation of CYP3A4, CYP2B6, and some multi-drug resistance (MDR1) levels in human hepatocytes indicating that these hop-derived acids can stimulate both elimination and metabolism detoxification processes.[233, 239] In addition, recent reports of Intelmann and Hofmann on the identification of degradation products of IAA formed upon beer ageing confirm the proneness of IAA to

undergo oxidative degradation.[71] Similar findings have been reported previously.[72, 73, 307-311] Next to phase-I metabolism of IAA, phase-II conjugation of IAA cannot be completely ruled out, but, in none of the plasma, urine, and faecal samples, proof for sulfation or glucuronidation could be demonstrated. The same situation was observed for THIAA. This also corresponds with previous results for enzymatic hydrolysis of Caco-2 monolayer samples. However, other possible phase-II metabolism reactions (conjugation of glutathione or amino acids) should be further investigated.

Compared to IAA, the mass balance of the reduced derivatives following oral dosing was substantially higher, totaling 47% for DHIAA and 25% for THIAA of the dose administered, showing that a substantial part of orally administered DHIAA and THIAA escapes metabolism/degradation *in vivo* in rabbits. On the contrary, in the plasma and urine samples following DHIAA-ingestion, substantial sulfation or glucuronidation was evident. The presence of an accessible alcohol group in DHIAA explains the absence of conjugation for IAA and THIAA. The enolic group, present in the molecular structures of AA, IAA and THIAA, may be inactive for conjugation in view of its acidity or due to intramolecular hydrogen bonding with the adjacent carbonyl group in the acyl side chain.

Conclusions

In the study presented above, the bioavailabilities in rabbits of hop-derived bitter acids, which have been reported to show important bioactive properties, have been investigated. At least in rabbits, the maximum exposure levels reached for AA, IAA, DHIAA and THIAA are in the range of 2–8 μg ml^{-1} (or equal to 7–20 μM), which is in line with bioactive concentrations previously suggested elsewhere.[135, 136, 152, 156] The bioavailabilities of AA and IAA were lower compared to those of the reduced derivatives (DHIAA, THIAA) and they are largely affected by phase-I metabolic interference. The bioavailabilities of DHIAA were influenced by substantial phase-II metabolism.

4.2 Metabolism of hop-derived bitter acids

This subchapter is based on:

Cattoor K.; Dresel M.; De Bock L.;, Boussery K.; Van Bocxlaer J.; Remon J.P.; De Keukeleire D.; Deforce D.; Hofmann T.; Heyerick A., Metabolism of Hop-Derived Bitter Acids*, Journal of Agricultural and Food Science,* Manuscript in revision

4.2.1 Introduction

In a previous study, it has been shown that pre-systemic breakdown or metabolism (either gastrointestinal or in hepatic tissues) was the most important factor influencing bioavailabilities of α-acids, iso-α-acids and reduced derivatives.[312] Recently, induction of the quinone reductase activity by α-acids and iso-α-acids and activation of CYP3A4, CYP2B6, and some MDR1 levels in human hepatocytes have been reported.[233, 239] It appears that hop acids stimulate both phase I and phase II detoxification processes.

The present study focuses on the CYP-metabolism of the hop-derived bitter acids (α-acids, β-acids, 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 bioavailabilities, 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-acids, β-acids, and iso-α-acids with liver microsomes, isolated from New Zealand white rabbits.

The most common reaction catalyzed by cytochrome P450-enzymes is a mono-oxygenase reaction; therefore, especially the formation of oxidative breakdown products was

investigated by specific HPLC-MS and LC-MS/MS methods in order to identify metabolites of iso-α-acids, α-acids, and β-acids following microsomal incubations.

4.2.2 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-acids and β-acids (ICE-2), and iso-α-acids (DCHA-Iso, ICS-I3), were obtained from Labor Veritas (Zurich, Switzerland). Glucose 6-phosphate sodium salt, glucose 6-phosphate dehydrogenase from baker's yeast (*S. cerevisiae*), β-nicotinamide adenine dinucleotide phosphate hydrate (β-$NADP^+$) were purchased from Sigma-Aldrich (Bornem, Belgium). All other reagents were of analytical grade and 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). De-ionized water used for chromatography was purified by means of a Mill-Q Gradient A10 system (Millipore, Billerica, Massachusetts, USA).

Pure reference compounds (Figure 5.2) (**1-17**) used for identification by means of HPLC-MS/MS Qtrap analysis, were obtained following the recently published protocols of Intelmann and Haseleu.[40, 69-71]

Animals

The study protocol (approval number 2009/98) of the animal experiments was approved by the Ethics Committee of the Institute for Agricultural and Fisheries Research (ILVO) (Merelbeke, Belgium). New Zealand white rabbits (breeding approval number LA2400369) (3.0 ± 0.5 kg) were fasted 16 h prior to the experiment. Water was available *ad libitum*.

Preparation of Rabbit Liver Microsomes

Six rabbits (n=6) were anesthetized using CO_2 and killed by asphyxiation. The livers were quickly removed, cut-up into smaller pieces (1 cm^3), and frozen at -80°C before use. Aliquots of the liver of 6 different rabbits were washed with phosphate buffer containing 1.15% KCl (0.25 mol/l, pH=7.25) to remove blood and the weight 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 liver) containing 1.15% KCl (0.25M, pH=7.25) and homogenized using an IKA Ultra-Turrax® equipped with a Potter-Elvehjem tissue grinder (VWR International, West Chester, Pennsylvania, USA). The homogenate was centrifuged at 10.000 *g* for 25 min at 4°C and the pellet (nuclei, mitochondria and debris) was discarded. The supernatant (S9 fraction) was further centrifuged at 100.000 *g* for 80 min at 4°C in order 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 liver) containing 1.15% KCl (0.25 mol/l, pH=7.25), homogenized, and centrifuged again at 100.000 *g* for 80 min at 4°C. The final microsomal pellet was resuspended in phosphate buffer (1.5 ml/g liver) containing 1.15% KCl and 30 % glycerol (0.25 mol/l, pH=7.4) and microsomes were stored at -80°C until use. The protein concentration of liver microsomes was determined according to the method described by Bradford.[313]

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 mol/l, pH=7.4) consisted of 1 mg/ml of pooled liver microsomal protein (n= 6), a nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system (NRS) consisting of 1mM 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 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 pre-incubation for 5 min, the reaction was initiated by the addition of NRS. Incubations were carried out at 37°C for 120 min with continuous shaking in an Eppendorf Thermomixer (VWR International, West Chester, Pennsylvania, USA). Control incubations were performed without the addition of NRS or microsomes to ensure that metabolite formation was dependent of the presence of microsomes and NADPH. Reactions were terminated by acidification (pH 2) with H_3PO_4 (0.1 mol/l, 2 volumes) to the incubation mixture (500 µl), followed by extraction with ethyl acetate (EtOAc; 4 volumes). The EtOAc-phase was evaporated to dryness under a nitrogen flow. The residues were reconstituted in 100 µl MeOH and stored at -20°C until analysis. Comparative studies with human liver microsomes (purchased from Celsis IVT, Chicago, Illinois, USA) were also included using the incubation protocol described above.

LC-MS Analysis

LC-MS analysis of samples from the different incubations and of urine was performed using an Agilent 1200 LC-MS system (SL) equipped with a dual ionization source (ESI/ APCI) and DAD detector (Agilent, Waldbronn, Germany). The Agilent Chemstation software package (Rev.B.02.01) was used to control the analytical system as well as for data

acquisition and processing. As stationary phase, an Xbridge 3.5 µm C-18 column (150 x 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 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, and further increased to 95% B in 5 min and maintained for 4 min. Finally, the mobile phase was re-adjusted 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-acids, dihydroiso-α-acids, and tetrahydroiso-α-acids) and 314 nm (for α-acids and β-acids). The MS parameters in the negative atomic pressure chemical ionization (APCI) mode were tuned to maximize formation of the deprotonated analyte. 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, and charging voltage 1000 V. In each analysis, qualitative 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 are presented in Table 4.8.[78, 95, 96, 100]

Table 4.8 Molecular ions used for the selected-ion monitoring (SIM) mode for the various hop acids in LC-MS analysis.

	Compound		MW (g/mol)	m/z (-)
α-acids	cohumulone	AA_1	**348**	**347**
	adhumulone humulone	AA_2 AA_3	**362**	**361**
β-acids	colupulone	BA_1	**400**	**399**
	adlupulone lupulone	BA_2 BA_3	**414**	**413**
iso-α-acids	*cis*-isocohumulone *trans*-isocohumulone	IAA_1 IAA_2	**348**	**347**
	cis-isoadhumulone *trans*-isoadhumulone *cis*-isohumulone *trans*-isohumulone	IAA_3 IAA_4 IAA_5 IAA_6	**362**	**361**

HPLC-MS/MS Q-Trap Analysis

In order to identify hop acid metabolites, samples from microsomal incubations and urine were analyzed using an Agilent 1200 Series HPLC-system, consisting of 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 electrospray ionization (ESI) source and operated in the negative ionization mode. Data processing and integration was performed by use of Analyst software version 1.5 (AB Sciex Instruments, Darmstadt, Germany). As stationary phase, a Synergi 4 µm Hydro-RP column (150 x 2.0 mm) (Phenomenex, Aschaffenburg, Germany) was used. The mobile phase consisted of acetonitrile (MeCN) + 0.1 % formic acid (HCOOH) as solvent A and H_2O + 0.1 % HCOOH as solvent B. Using a flow rate of 0.25 ml/min, chromatographic separation was achieved by gradient elution increasing solvent A from 20 to 60% within 20

min and further increased to 70% in 15 min, to 92% during 28 min, and, finally, to 100% within 2 min. This was maintained at 100% for 5 min, following by re-adjustment to 20% within 1 min, and re-equilibrated for 5 min prior to the next injection. MS/MS parameters settings were based on the methods described by Haseleu and co-workers.[16] Qualitative analysis was performed by mean of multiple reaction monitoring (MRM) mode using the fragmentation parameters and retention times by mean of co-chromatography with authentic reference compounds, obtained using protocols reported by Intelmann and co-workers.[16-18, 25]

4.2.3 Results

In vitro Metabolism of Hop-derived Acids

In Figure 4.4, the percentage of compounds metabolized following incubation of 20 μM of α-acids, β-acids, and iso-α-acids with liver microsomes isolated from rabbit liver and human microsomes are shown. Results show that, after 120 min, 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 biotransformations, still a substantial amount of 48 ± 3% was metabolized. In studies with human liver microsomes (Fig. 5.1), highly comparable values were obtained. The percentages of α-acids, β-acids, and iso-α-acids metabolized amounted to, 73 ± 3%, 95 ± 1%, and 48 ± 2%, respectively.

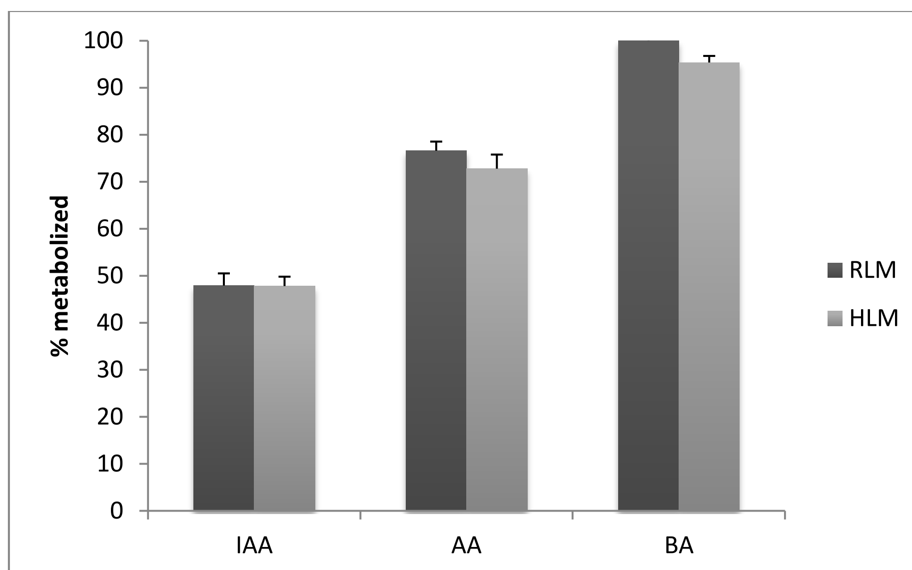


Figure 4.4 Phase-I metabolism of 20 μM hop α-acids, β-acids, and iso-α-acids following 2 h incubation with rabbit liver microsomes (RLM) and human liver microsomes (HLM). Experiments were applied in triplicate. Amounts are presented as the percentage metabolized of the administered amount ± standard deviation. IAA: iso-α-acids; AA: α-acids; BA: β-acids

Initially, all incubation mixtures were analyzed by LC-MS (APCI; negative mode) both quantitative (see results above), as well as qualitative. Figures 4.5-4.7 illustrate a set of total ion chromatograms (TIC) and extracted ion chromatograms (EIC) of parent compounds and potential metabolites of respectively, α-acids (Figure 4.5), β-acids (Figure 4.6), and iso-α-acids (Figure 4.7) following 120 min incubation with rabbit liver microsomes. Microsomal incubation of α-acids (Figure 4.5) resulted in a quite complex TIC (Figure 4.5A). In the EIC of the parent compounds (Figure 4.5B) with pseudo molecular ion [M-H]- m/z 347 and 361, some proof of conversion of α-acids into iso-α-acids could be observed. Study of the full scan spectrum of potential metabolites of the α-acids revealed that the main biotransformation products had a pseudo molecular ion [M-H]- m/z 363 and 377, in which the molecular mass of the parent compound was increased by 16 Da, suggesting mono-oxygenation reaction (epoxidation, hydroxylation…) as the most important metabolism product (Figure 4.5C).

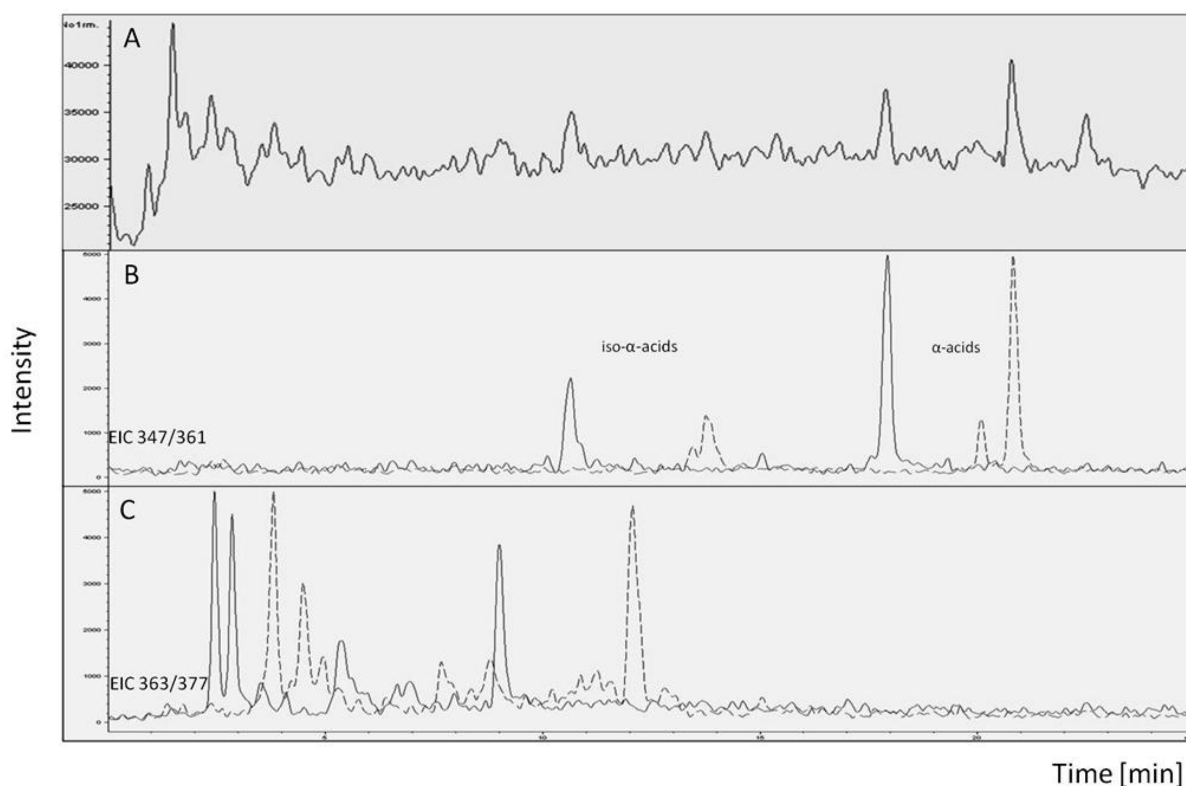


Figure 4.5 Representative HPLC-MS chromatogram following 2h incubation of α-acids (20 µM) in microsomes. In each chromatogram, the co-analogue and n+ad-analogues are respectively represented by a solid line, and a dotted line. A: TIC. B: EIC of parent compounds with m/z 347 and 361. C: EIC of mono-oxygenated metabolites with m/z 363 and 377.

Figure 4.6 represents an LC-MS chromatogram following microsomal incubation of iso-α-acids. The most important metabolites formed (Figure 4.6C and Figure 4.6D), showed a molecular mass increase of the parent compound by 16 or 32 Da, showing the incorporation of one or two oxygen atoms as major microsomal biotransformation products of iso-α-acids.

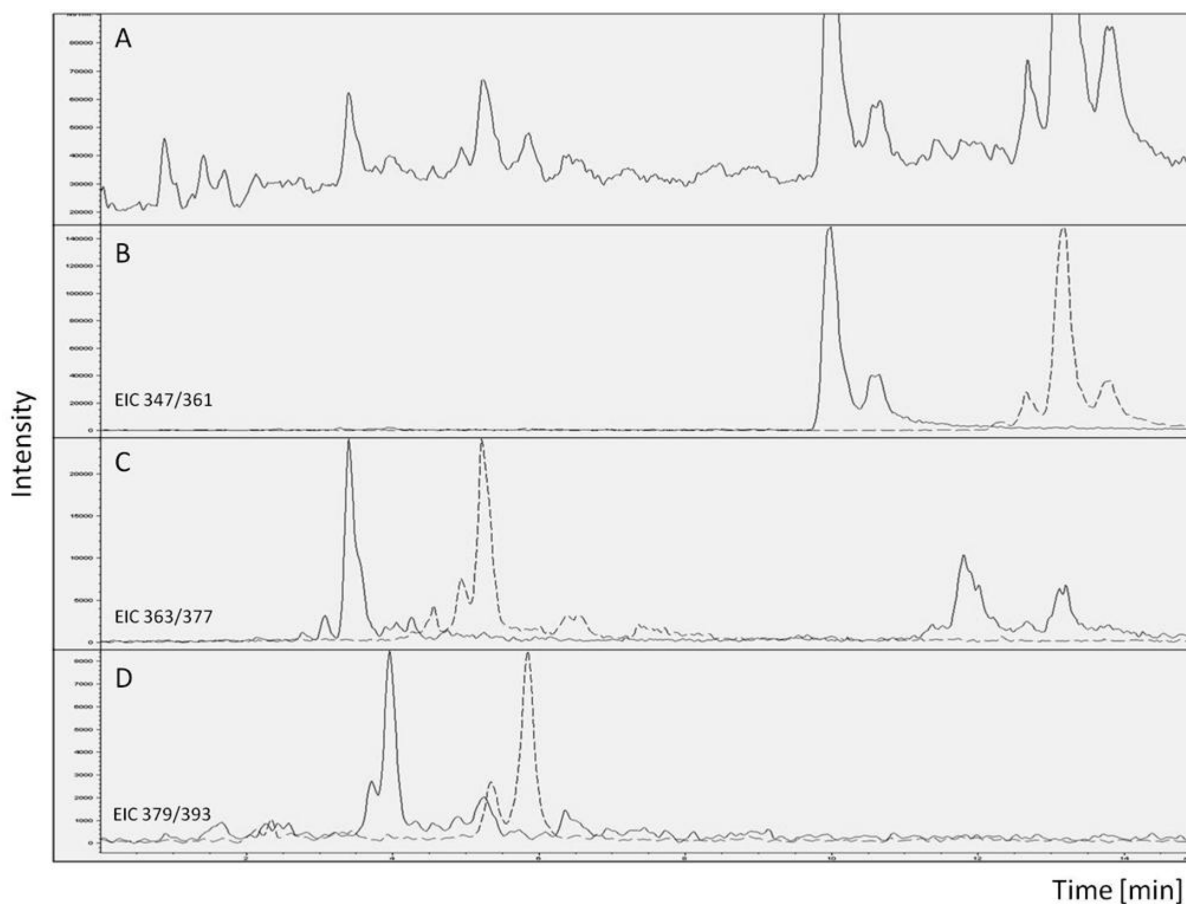


Figure 4.6 Representative HPLC-MS chromatogram following 2h incubation of iso-α-acids (20µM) in microsomes. In each chromatogram, the co-analogue and n+ad-analogues are respectively represented by a solid line, and a dotted line. A: TIC. B: EIC of parent compounds with m/z 347 and 361. C: EIC of mono-oxygenated metabolites with m/z 363 and 377. D. EIC of di-oxygenated metabolites with m/z 379 and 393.

The metabolism of β-acids by microsomal enzymes resulted in several distinct products (Figure 4.7). In the β-acids mixture used in this experiment, only trace amounts of n+adlupulone were present compared to colupulone. Therefore only signals related to colupulone (parent compound with m/z 399) were used as a model for 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 4.7C), which corresponded with 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 di-oxygenated biotransformation products were determined, with detection of pseudo molecular ions $[M-H]^-$

m/z 415 and 431 (Figure 4.7D and Figure 4.7E). Furthermore, a metabolite with pseudo molecular ion $[M-H]^-$ m/z 397 was determined.

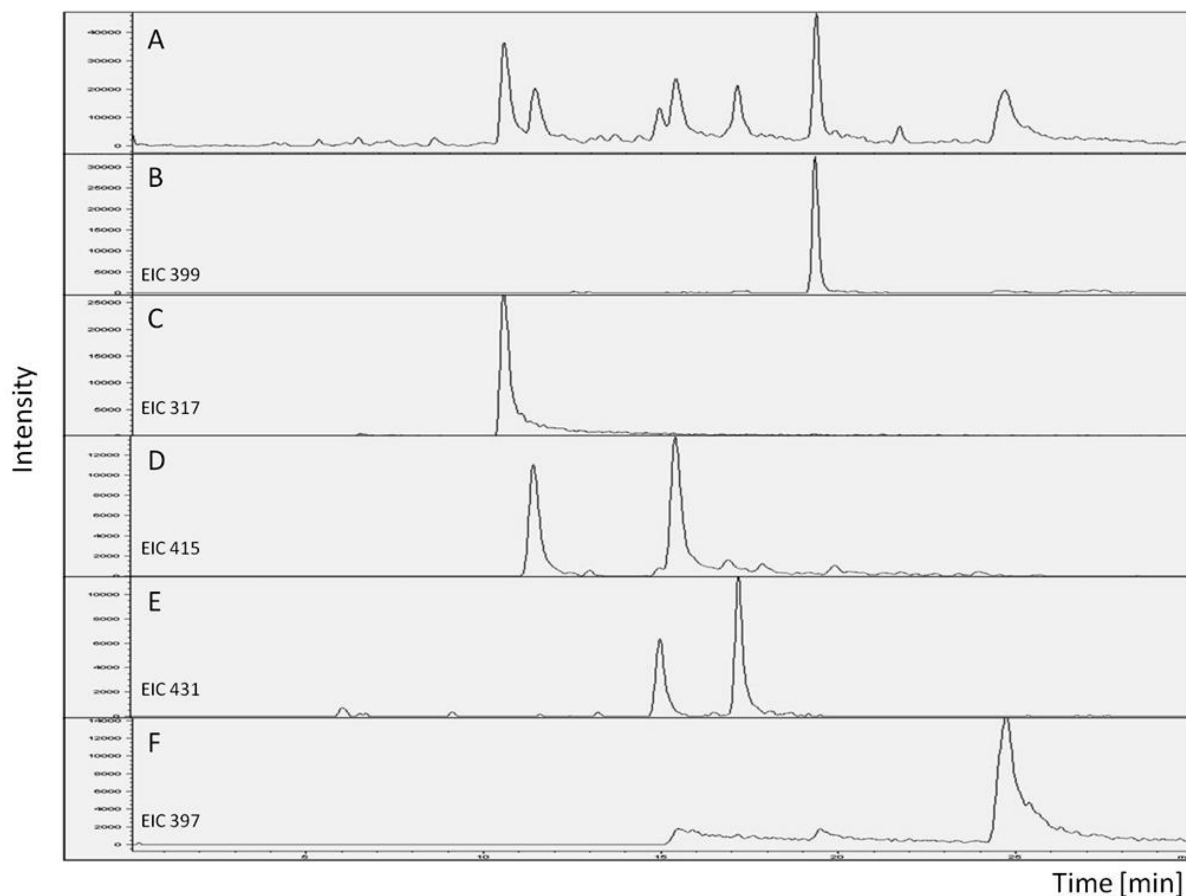


Figure 4.7 Representative HPLC-MS chromatogram following 2h incubation of β-acids (20 µM) in microsomes. A: TIC. B: EIC of parent compound with m/z 399. C: EIC of metabolite with m/z 317. D. EIC of mono-oxygenated metabolites with m/z 415. E. EIC of di-oxygenated metabolites with m/z 431. F. EIC of metabolite with m/z 397.

Though, by the use of LC-MS analysis, little structural information could be obtained for the biotransformation products formed. Based on data of the metabolism pathway of other prenylated hop-derived constituents such as xanthohumol and 8-prenylnarigenin (8-PN), retention times, 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, correlate to a great extend with the oxidative degradation products of hop bitter acids in aged beer & wort mixtures, recently described by Intellmann *et al.*[16-18, 25, 26] Based on 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 multiple reaction monitoring (MRM) methods for the analysis of hop bitter acids and these degradation products observed in oxidatively aged beer. Confirmation of the identified products was possible by co-chromatography 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 ageing. The corresponding representative chromatograms of the identified products (for the co-congener) analyzed by LC-MS/MS operating in the MRM mode of products following microsomal incubation of α-acids and β-acids, and iso-α-acids are presented in Figure 4.8. 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 4.9.

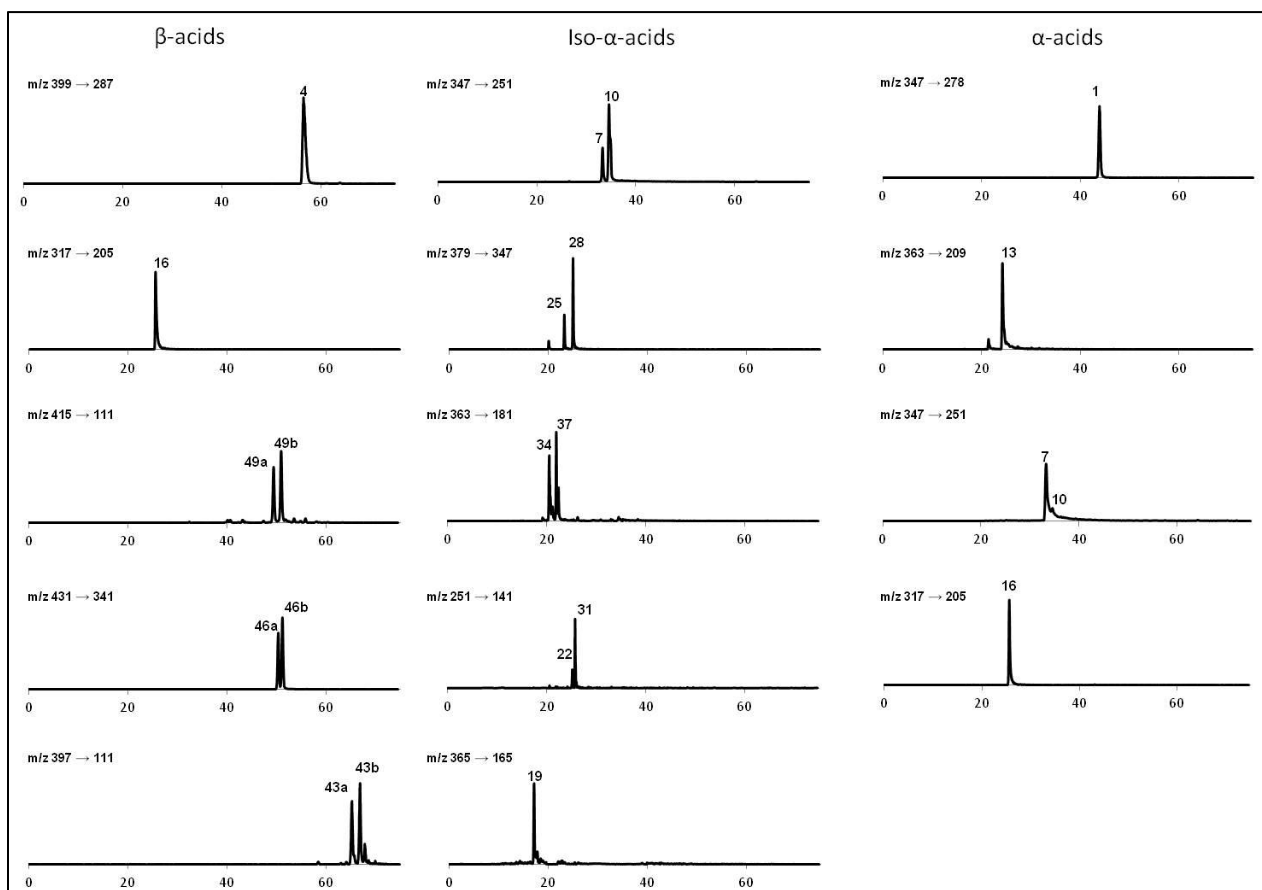


Figure 4.8 Representative LC-MS/MS chromatograms (MRM transitions) of parent compounds and identified microsomal metabolites of hop-derived α-acids, β-acids, and iso-α-acids. In each of the cases, the co-congener has been shown as matter of example.

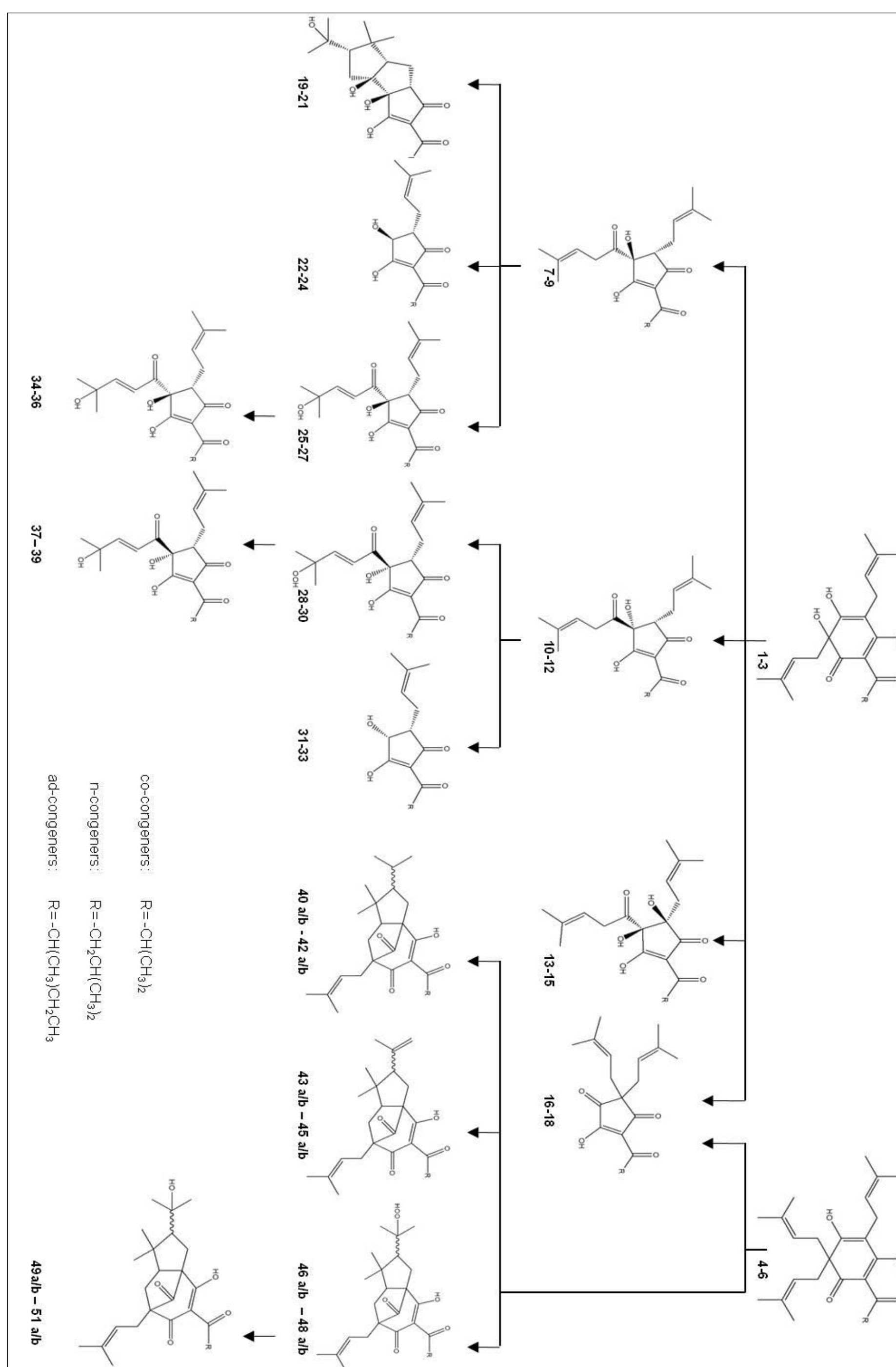


Figure 4.9 Reaction routes for the biotransformation of hop-derived α-acids (1-3), β-acids (4-6), trans- (7-9) and cis-iso-α-acids (10-12) leading to the formation of humulinones (13-15), hulupones (16-18), trans- (22-24), and cis-humulinic acids (31-33), epimeric pairs of tricyclic β-acid cyclization products (40a/b-42a/b, 43a/b-45a/b, 46a/b-48a/b), trans- (25-27) and cis-alloisohumulonehydroperoxides (28-30), trans- (34-36) and cis-alloisohumulonehydroxides (37-39), and tricyclohumols (19-21).

Upon incubation with rabbit liver microsomes, α-acids (**1-3**) were mainly conversed into *trans*- (**7-9**) and *cis*-iso-α-acids (**10-12**), together with also humulinones (**13-15**), and surprisingly, also 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**), in resemblance with the products recently identified by Haseleu and co-workers.[25, 26] Microsomal metabolism of iso-α-acids (**7-9**, **10-12**) resulted mainly in the formation of *trans*- (**22-24**) and *cis*-humulinic acids (**31-33**), in addition to *trans*- and *cis*- pairs of hydroperoxy- (**25-27**, **28-30**) and hydroxylated-allo-iso-α-acids (**34-36**, **37-39**). Furthermore, the conversion of iso-α-acids into cyclic degradation substances, including tricyclohumols (**19-21**) was observed.

4.2.4 Discussion

Studies on ADME of hop-derived bitter acids, showing promising multipotent bioactive activities, such as anti-inflammatory, anti-osteoporotic or anti-carcinogenic,[235] are of great importance. In the study above, a first insight is gained into the possible metabolic pathways of hop-derived bitter acids. Several biotransformation products of α-acids, β-acids, and iso-α-acids were identified in microsomal mixtures. The discovery of an array of breakdown products of these compounds was in line with the preceding findings on the bioavailabilities of hop-derived bitter acids in rabbits. Results showed a fast elimination for α-acids and iso-α-acids, as well as low amounts of unchanged products in urine indicating breakdown by gut related microorganisms or phase I/II metabolism in gastrointestinal or hepatic tissues.[312]

Several previous investigations have been reported on the structures of (oxidative) degradation of hop-derived bitter acids as well as their underlying formation mechanisms.[40, 54, 59, 69-73, 84, 307-311, 314-330] 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 can be oxidized further or undergo further (de-) hydration and cyclization reactions. However, the focus in the cited studies was mainly on their decomposition in beer. Although, physiological biotransformation conditions (including metabolism) are nothing like beer deterioration, these studies provided preliminary suggestion to possible structures of metabolites of these compounds.

Upon microsomal incubation, the metabolism of α-acids showed biotransformation into humulinones, besides *cis*- and *trans*-iso-α-acids, and surprisingly, hulupones, which is a well-known oxidation product of β-acids. Isomerization of humulone is the most important reaction in the brewing process.[35, 49, 53, 97, 331, 332] Interestingly, this conversion could also be

catalyzed by microsomal enzymes. As the results showed, among the microsomal bioconversion of β-acids, the formation of 2 main types of reaction products could be identified, namely hulupones and tricyclic degradation products. The identified metabolites of iso-α-acids comprised predominantly humulinic acids, and hydroxy-/peroxy-alloisohumulones. These findings indicate that liver oxidation catalyzed by cytochrome P450-enzymes could be an important metabolic pathway for α-acids, β-acids, and iso-α-acids. Moreover, this suitability to microsomal breakdown could suggest a significant first-pass effect upon oral ingestion of such compounds, resulting in a fast clearance following absorption.

Recently, some oxidation products of iso-α-acids and β-acids were identified upon oxidative breakdown in beer by Intelmann[40, 71] and Haseleu.[43, 44] In these studies, the transformation of iso-α-acids in hydroxyl- and hydroperoxy-alloisohumulones and β-acids into tricyclic compounds, including nortricyclo-, dehydrotricyclo-, and tricyclolupulones is described. Despite the different reaction conditions (beer matrix versus microsomal enzymes), some interesting similarities could be drawn. In the beer ageing studies, the authors proposed a lipid peroxidation mechanism (oxidation of unsaturated fatty acids), which is based on the same principles used 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 and leading to the corresponding hydroperoxy- and hydroxy-alloisohumulones. For the β-acids, hydrogen abstraction is followed by cyclization steps and oxygenation, leading to nortricyclo-, dehydrotricyclo-, and tricyclolupulones by different reaction routes. CYP metabolism pathways include iron-oxo species which abstracts 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 NADPH.[60]

Apart from transformation of iso-α-acids into the hydroperoxy- and hydroxy-alloisohumulones, formation of *cis-*/*trans*-humulinic acids was observed. These compounds result from cleavage of the (un)modified hexenoyl side chain, directly from iso-α-acids, or from another intermediate (alloiso-α-acids, hydroxy-/hydroperoxy-alloiso-α-acids). Furthermore, also cyclic metabolites of iso-α-acids were identified such as tricyclohumols. These products have been previously identified by Intelmann *et al.* upon acid-catalyzed intramolecular cyclization of *trans*-iso-α-acids in aged beer.[69, 71] Although, it is unclear whether these compounds were the result of microsomal conversion or were formed during the acidic extraction conditions throughout the processing of the samples. Intelmann and co-workers showed that 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).[70] Besides, this kind of acid-catalyzed degradation might also occur in the stomach and/or intestinal zones with lower pH upon oral ingestion of iso-α-acids. Moreover, only *trans*-iso-α-acids were affected, since only for these stereomers the interacting carbon atoms possessed the correct 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 for substances with hydroxylated positions in the prenyl side chains of α-acids and β-acids, although exact assignment of the molecular structures requires further experiments including NMR spectroscopy confirmation of isolated compounds. However, the occurrence of metabolites with hydroxylated prenyl (or 3-methyl-2-butenyl) side chains of α-acids and β-acids is in line with CYP-metabolites of another hop constituent, 8-prenylnaringenin, (8-PN). In a study conducted by Nikolic *et al.*, the most abundant pathway of hepatic metabolism of 8-PN was hydroxylation of one of the terminal methyl's 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.[333] Moreover, in two recent studies Negri *et al.* and Lupinacci and coworkers have reported an oxidized α-acids product, formed upon oxidation of the prenyl side chains in humulinone (oxidation product of α-acids), followed by cyclization, resulting in a compound wherein 2 oxygen atoms were incorporation.[179, 334] These examples suggest 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.

The absence of a hexenoyl side chain in the molecular structure of dihydro-iso-α-acids, and the absence of double bounds in the molecular structure of tetrahydroiso-α-acids, could suggest that these reduced derivatives resist to a great extent the types of biotransformation described above. To support this, additional investigations with reduced iso-α-acids are necessary to support this. In the study on the bioavailabilities of hop-derived iso-α-acids and reduced derivatives in rabbits (see section 4.1), a slower clearance and a higher unmodified amount excreted in urine and faeces was observed for the dihydro-iso-α-acids and the tetrahydro-iso-α-acids.[312]

In conclusion, HPLC-MS/MS operating in the MRM-mode allowed for the first time detection of biotransformation products of hop-derived α-acids, β-acids, and iso-α-acids in microsomal incubation mixtures. Interestingly, the identified metabolites showed similarity to products formed upon oxidative decomposition of these hop constituents during beer ageing. The findings show a first insight into the metabolites of hop-derived bitter acids.

V. General Conclusion and Future Perspectives

The objective of this PhD research project was to gain insight in the different aspects influencing the bioavailability of hop-derived bitter acids.

In Caco-2 cell culture experiments, fast and efficient absorption of hop α-acids, iso-α-acids, and reduced derivatives could be demonstrated. On the basis of the low apparent permeability coefficient of β-acids, the *in vivo* absorption of orally administered β-acids is predicted to be low. The limiting factors in the epithelial absorption of β-acids across Caco-2 monolayers could be the involvement of P-gp- and MRP-2-type efflux transporters and/or substantial phase-II metabolic reactions. It was also shown that the transport properties of dihydroiso-α-acids are influenced by phase-II metabolism.

In rabbits, used as an *in vivo* animal model, the maximum exposure levels reached for AA, IAA, DHIAA and THIAA are in the range of 2–8 μg ml^{-1} (or equal to 7–20 μM), which is in line with bioactive concentrations previously reported.[135, 136, 152, 156] and confirms the values found in the Caco-2 assays. Amounts of β-acids could not be detected, which should be due to gastrointestinal pre-systemic instability. In addition, the bioavailability of IAA was lower compared to those of their precursor AA and the reduced derivatives (DHIAA, THIAA) and was largely affected by microbial and/or metabolic breakdown. The bioavailability of DHIAA was influenced by substantial phase-II metabolism. Fast biotransformation was evident for α-acids, β-acids, and iso-α-acids in microsomal incubations (isolated from rabbits and humans). Interestingly, metabolites show structural similarities to the compounds formed upon oxidative decomposition during beer ageing. As such, our findings should have significant practical implications.

Because of the limited predictive power of animal studies for humans, extrapolations from the pharmacokinetic rabbit data obtained in this PhD should be used with caution. To reach the maximum exposure levels found in this study (2-8 µg ml^{-1}), a corresponding human intake of 400-500 mg of hop bitter acids can be estimated based on the calculated HED following the FDA-draft guidelines (for a human weight of 60-70 kg).[301, 302] This is in the line with the amounts of hop-derived bitter acids present in commercially available dietary supplements (usually 400 mg or more) on the market. Metagenics Inc. produces food supplements and nutraceuticals containing hop bitter acids and pre-formulates (400-500 mg) predominantly reduced derivatives (DHIAA, THIAA) in their products, which showed higher bioavailabilities according to our results. In a study presented by Hall et al., following an oral dose of 700 mg DHIAA administered to 2 healthy human subjects, maximum exposure levels of 2-4 µg ml^{-1} were found, which is in line with the results of our study.[135] Additional human trials are necessary to draw conclusions about human bioavailability.

With regard to the topic 'health & beer', a dose of 400-500 mg is not conform to moderate beer consumption, since such amount of hop-derived bitter acids would require a beer intake of at least 5 liters. In the beer brewing industry, mostly iso-α-acids are used as main bitter flavoring compounds. The ADME properties of the iso-α-acids determined in this study suggest a significant absorption, followed by a fast biotransformation and elimination, indicating that the possibility of reaching bioactive tissue concentrations of unchanged hop-derived bitter acids is more likely for beers flavored with reduced iso-α-acids. In a non-regulated trial we conducted, urine (12h post intake) was collected and analyzed from a series of healthy subjects following oral intake of 660 ml of beer. Results showed that, in none of samples, iso-α-acids could be recovered unchanged whereas for the reduced derivatives (DHIAA, THIAA), unchanged amounts could be determined in urine (data not shown).

Also, it might be important to assess the biological activity of the main metabolites of iso-α-acids, such as hydroxylated allo-iso-α-acids, humulinic acids, and unidentified forms. For example, microbial transformation in the colon may increase the biological activity of ingested compounds, a process that has been described for different phytoestrogens, such as prenylflavonoids from hops, for which the pseudo estrogenic activity is determined by intestinal bacterial activation followed by absorption of the microbial metabolites.[335]

A number of aspects have been investigated in this research project on the bioavailabilities of hop-derived bitter acids. Nevertheless, further research is essential to enable full understanding of the real health-beneficial properties of iso-α-acids and derivatives as important beer constituents and as part of nutraceuticals.

- A study on the intestinal microbial metabolism, since intestinal microbiota are capable to induce a multitude of transformations in the diet and may play an important role in bioavailabilities and biological effects of specific compounds. First, the *in vitro* stability of iso-α-acids and reduced derivatives in the stomach and the small intestine can be studied. In a next step, the target compounds can be incubated with human faecal samples to investigate inter-individual differences of intestinal metabolism. In a last phase, evaluation of microbial transformations over a longer period of time and under steady-state conditions of feeding can be envisaged. Besides the influence of microbiota on the target compounds, the effects (on composition & activity) of exposure of these microbiota to iso-α-acids and reduced derivatives should be evaluated.
- Animal studies, in which administration of multiple doses (long term) of iso-α-acids or reduced derivatives is applied by oral gavage to determine steady-state concentrations in faeces, urine and blood.
- Human studies, where healthy human volunteers will be asked to ingest a single dose of iso-α-acids and/or reduced derivatives. Fractionated urine samples and

blood samples could be used to establish the most important pharmacokinetic parameters. Next, multiple doses of iso-α-acids and/or reduced derivatives could be applied in order to establish steady-state concentrations in both urine and blood. On the practical side, the matrix effect, such as prevailing in beer, should be integrated in comprehensive future studies.

Summary

In comparison to the continuously growing number of publications targeting the health-beneficial effects of hop bitter acids, very little is known on their ADME-characteristics (absorption, distribution, metabolism, excretion), which is highly relevant when assessing the usefulness of these compounds for either preventive of therapeutic use. The background on hop-derived bitter acids and the current situation on bioactivity of these target compounds are addressed in a literature review in Chapter 1. Following a description of the history, chemistry, and analytical methods of hop constituents and beer, current data on bioactivity and toxicity of the target compounds is described.

In Chapter 2, the current state-of-the-art and -until now known- data on the bioavailability aspects (ADME) of hop-derived bitter acids is outlined, followed by the description of the different objectives of this work.

As a first approach to study the bioavailability, in Chapter 3, the *in vitro* intestinal permeabilities of hop bitter acids using Caco-2 cell monolayers (a commonly used screening tool for the prediction of intestinal absorption) was studied, preceded by an introduction on Caco-2 cells, their properties and uses for studying intestinal transport. It appears that α-acids are efficiently absorbed, while the permeability of β-acids is low. The limiting factors in the absorption of β-acids involve P-gp- and MRP-2-type efflux transporters. In addition, phase-II metabolic reactions may also contribute to the reduced permeability of β-acids versus α-acids. In the comparative Caco-2 study with iso-α-acids and its reduced derivatives (dihydroiso-α-acids, tetrahydroiso-α-acids), efficient absorption was evident for all compounds, but lower transport rates were determined for dihydroiso-α-acids, due to influences by phase-II metabolism.

Chapter 4 describes the *in vivo* evaluation of various hop acids and derivatives (α-acids (AA) and β-acids (BA), iso-α-acids (IAA), dihydroiso-α-acids (DHIAA), tetrahydroiso-α-

acids (THIAA)) in rabbits (oral vs intravenous administration). The most important pharmacokinetic parameters (C_{max}, t_{max}, half life, clearance, $AUC_{0-\infty}$) and the absolute bioavailabilities were determined for each class of hop acid. After oral and i.v. dosing to New Zealand white rabbits, the absolute bioavailability for IAA was determined to be 13.0%. The AA and reduced derivatives reached higher bioavailabilities with 25.6% for AA, 28.0% for DHIAA and 23.0% for THIAA. The area-under-curve $AUC_{0-\infty}$ upon oral gavage for DHIAA and THIAA was 70.7 ± 48.4 µg h ml^{-1} and 57.4 ± 9.0 mg h ml^{-1}, respectively, while that for AA and IAA was 18.2 ± 9.0 and 10.6 ± 5.3 mg h ml^{-1}, respectively. Gastrointestinal instability and/or metabolism were indicated as the main factors limiting the bioavailabilities of IAA. The bioavailability of DHIAA is mostly influenced by phase-II metabolism as shown by enzymatic hydrolysis of plasma samples upon administration of DHIAA. As a final part of the investigations, a second part of Chapter 4 highlights metabolism of α- acids, β-acids, and iso-α-acids. Iso-α-acids and α- and β-acids were incubated with microsomes, isolated from New Zealand (NZ) white rabbits, and fractions were subjected to LC-MS/MS analysis for identification of oxidative biotransformation products of α-acids, β-acids, and iso-α-acids. The metabolism of β-acids was characterized by conversion into hulupones, as well as by formation of a series of tricyclic degradation products, named dihydrotricyclolupulones, hydroxytricyclolupulones, and hydroperoxytricyclolupulones. The most important metabolites of α-acids were identified as humulinones and hulupones. Iso-α-acids, were found to be mainly metabolized into *cis*- and *trans*-humulinic acids, in addition to hydroperoxy and hydroxylated alloiso-α-acids. Interestingly, phase-I metabolites were highly analogous to oxidative degradation products in beer.

Samenvatting

In vergelijking met het groeiend aantal publicaties over de gunstige effecten van hopzuren op de gezondheid is zeer weinig bekend over hun absorptie, distributie, metabolisme en eliminatie (ADME), die belangrijke aspecten bij de beoordeling van de effecten van deze verbindingen voor zowel preventief als therapeutisch gebruik.

Een inleiding over hopbitterzuren en hun situering wordt beschreven in Hoofdstuk 1. Na een overzicht van de geschiedenis, de chemie en de analysemethoden van hop-afgeleide verbindingen en bier, volgt een weergave van de bekende gegevens over de bio-activiteiten, en toxiciteit van deze verbindingen. In Hoofdstuk 2 wordt een overzicht gegeven van de huidige - tot nu toe beperkte - kennis op het vlak van biologische beschikbaarheid van hopbitterzuren, gevolgd door de beschrijving van de verschillende doelstellingen van dit onderzoek.

In een eerste benadering van de biologische beschikbaarheid werd de *in vitro* intestinale absorptie van hopbitterzuren bestudeerd aan de hand van Caco-2 celculturen (een veelgebruikt screeningmiddel voor de voorspelling van intestinale absorptie). In Hoofdstuk 3 wordt een inleiding gegeven over Caco-2 cellen, hun eigenschappen en hun gebruik voor het bestuderen van intestinaal transport, gevolgd door de studie die werd uitgevoerd om de absorptie van α-zuren, β-zuren, iso-α-zuren en derivaten te bepalen. De resultaten toonden aan dat de α-zuren efficiënt worden geabsorbeerd, terwijl de permeabiliteit van β-zuren laag is. De beperkende factoren in de absorptie van β-zuren houden verband met actief transport door P-gp- en MRP-2-type efflux pompen. Ook kunnen fase-II metabolische reacties bijdragen aan de verminderde permeabiliteit van β-zuren in vergelijking met α-zuren. In de vergelijkende Caco-2 studie met iso-α-zuren en derivaten (dihydroiso-α-zuren, tetrahydroiso-α-zuren) bleek efficiënte absorptie op te treden voor alle verbindingen, maar lagere transportsnelheden werden gevonden voor dihydro-iso-α-zuren, gezien hun transport beïnvloed weerd door fase-II metabolisme.

Hoofdstuk 4 beschrijft de *in vivo* evaluatie van verschillende hopzuren en derivaten (α-zuren (AA) en β-zuren (BA), iso-α-zuren (IAA), dihydro-iso-α-zuren (DHIAA), tetrahydro-iso-α-zuren (THIAA)) bij konijnen (orale versus intraveneuze toediening). De belangrijkste farmacokinetische parameters (C_{max}, t_{max}, half life, klaring, $AUC_{0-\infty}$) en de absolute biologische beschikbaarheden werden bepaald voor elke klasse hopzuur. Na orale en intraveneuze toediening aan konijnen werd een absolute biologische beschikbaarheid voor iso-α-zuren (IAA) van 13.0% bepaald. De α-zuren, dihydro- en tetrahydro-derivaten bereikten een hogere biologische beschikbaarheid van 25.6% voor AA, 28.0% voor DHIAA en 23.0% voor THIAA. Het oppervlak-onder-de-curve $AUC_{0-\infty}$ na orale toediening voor DHIAA en THIAA was 70.7 ± 48.4 µg h ml-1 en 57.4 ± 9.0 mg h ml-1, terwijl voor AA en IAA waarden van, respectievelijk, 18.2 ± 9.0 en 10.6 ± 5.3 mg h ml-1 bepaald werden. Gastrolintestinale instabiliteit en/of metabolisme werden aangegeven als de belangrijkste factoren die de biologische beschikbaarheid van IAA beperkt. De biologische beschikbaarheid van DHIAA wordt vooral beïnvloed door fase-II metabolisme zoals aangetoond door enzymatische hydrolyse van plasmastalen bij toediening van DHIAA. Het tweede deel van Hoofdstuk 4 stelt een laatste deel van het onderzoek voor, waarin metabolisme van α-zuren, β-zuren en iso-α-zuren werd bestudeerd. Iso-α-zuren en α-zuren en β-zuren werden geïncubeerd met microsomen, geïsoleerd uit witte Nieuw-Zeeland (NZ) konijnen en stalen werden onderworpen aan LC-MS/MS analyse voor de identificatie van oxidatieve biotransformatieproducten van α-zuren, β-zuren en iso-α-zuren. Het metabolisme van β-zuren werd gekenmerkt door omzetting tot huluponen, evenals door vorming van een reeks van tricyclische afbraakproducten, genaamd dihydrotricyclolupulonen, hydroxytricyclolupulonen en hydroperoxytricyclolupulonen. De belangrijkste metabolieten van α-zuren werden geïdentificeerd als humulinonen en huluponen. Iso-α-zuren bleken voornamelijk te worden gemetaboliseerd tot *cis*- en *trans*-humulinezuren, naast hydroperoxy en gehydroxyleerde allo-iso-α-zuren. Het is interessant te vermelden dat de fase-I metabolieten overeenkomsten vertonen met oxidatieve afbraakproducten in bier.

Curriculum Vitae

Personalia

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1996-2002: High School: Science-Mathematics (8h)

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Working Experience

01/2013- now: **Plant Technical Engineer**

Amcor Flexibles

- Projects on flexible food packaging films
- Complaint handling of Food packaging products
- Qualification of new raw materials (films, adhesives, inks)
- Quality control of flexible films
- Collaborations with suppliers, end-users, partner enterprises and research institutes
- Skills:
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    - Analytical: DMA, DSC, IR, viscosity, O2/gas barrier experiments, mechanical characterization

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05/2011- 09/2012: **R&D Project Chemist**

Basaltex

- Managing of R&D projects on basalt fibers & its applications
- Technical support
- Quality control (tensile strength, loss on ignition) of basalt fibers
- Dissemination of results on conferences & fairs
- Collaborations with suppliers, end-users, partner enterprises and research institutes
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    - Composites (mechanical analysis, manufacturing)
    - Coatings, polymers
    - Analytical: TGA, DMA, DSC, IR, viscosity

10/2006 – 12/2010 **PhD student,** Ghent University**,** Faculty of Pharmaceutical Sciences**,** Laboratory of Phytochemistry and Pharmacognosy

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- Collaborative research project at the Technical University of Munich – Department of food Science and Molecular Sensory
- Skills:
    - Drug development, nutraceuticals, food chemistry
    - Natural product isolation and structure determination

- Cell culture experiments
- Absorption, Pharmacokinetics, Metabolism
- Analytical: Mass spectroscopy (LC-MS, LC-MS/MS), HPLC, NMR)

Languages

Dutch:	mother tongue
French:	good
English:	very good
Mandarin:	basic (Survival Chinese A; UCT UGent)

Computer skills

MS Office:	very good
Statististical software (SPSS):	good

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Publications

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, *Journal of Natural Products.,**2009**, Vol.72, pp. 1220 – 1230*

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