Studies of iso-alpha-acids: analysis, purification, and stability.

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CHAPTER 1

General Introduction

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1.1. INTRODUCTION TO HOP

Hop is a member of the plant family Cannabaceae, which consists of two genera: *Humulus* and *Cannabis*. *Humulus* comprises two varieties: ordinary hop *(Humulus lupulus* L.) and the Japanese hop (*Humulus japonicus* Siebold & Zucc.). Japanese hop has no brewing value and is grown only as an ornamental plant. There is only one sort of *Cannabis*, the Indian hemp (*Cannabis sativa* L.) (Verhagen, 1988).

Hop is known since ancient times and has been used for several purposes, e.g. as ornamental plant, for bread-making, for stuffing of pillows, and as salad (Delyser and Kasper, 1994). It is still unclear when precisely hop was introduced in beer brewing. Cultivation of hop appears in the documents of the monastery Freising, Bavaria between year of 859 and 875. Monks of Picardy introduced the use of hop in brewing after they founded the Cobay cloister on the Weser in Northern Germany. Originally, hop was used not so much to flavour beer, but to improve its preservation time. Hop was generally accepted for bittering beer later in the 19th century (Behre, 1999; Moir, 2000; Verzele and De Keukeleire, 1991).

Today only cultivated hop is used. Hop is grown for commercial purposes in most of the moderate climate zones. Hop growing areas are situated between latitudes roughly 35^{o}-55^{o} in the Northern hemisphere. Hop plants were introduced to the Southern hemisphere in colonial times in Australia and New Zealand. The largest hop growing areas are situated in Europe and USA (Delyser and Kasper, 1994; Verzele and De Keukeleire, 1991).

Hop is a climber plant. The bracts of the cones have glands which contain the hop bitter agents (Rybáček, 1991). Hop gives beer the characteristic flavour, and it makes beer different from all other drinks. The female flowers are the source of beer bitter substances because they have a greater number of resin-containing lupulin glands than the male flowers (Verhagen, 1988).

Besides to provide bitterness and aroma in beer, hop is also used for culinary, medicinal, and cosmetic purposes. Hop is used for example for hair rinsing and as an ingredient in a massage cream. Basic extract, a by-product of isomerized extract that contains β-acids, is an alternative bacteriocide to reduce spoilage during the production of beet sugar (Delyser and Kasper, 1994; Moir, 2000).

1.2. CHEMICAL PROPERTIES OF HOP EXTRACT

Fresh hop cones contain about 80% moisture which is reduced to 8-12% by drying before storage. There are hundreds of components in hop cones, but the interesting compounds can be grouped in three classes: resin (containing α- and β- acids), hop oil and polyphenols. These three classes are important as biochemical markers to differentiate hop varieties (Benitez *et al*., 1997; De Cooman *et al*., 1998; Kovačevič and Kač, 2002; Verhagen, 1988; Verzele, 1986). The major components are shown in Table 1.1.

Table 1.1. The chemical composition of dried hop cones.

Components	Amount (%, w/w)
α-Acids	2-18
β-Acids	1-10
Essential oil	0.5-3.0
Polyphenols	2-5
Oil and fatty acids	Up to 25
Protein	15
Cellulose	40-50
Chlorophyll	2
Ash-salts	10
Water	8-12

Sources: Verzele (1986) and www.barthhaasgroup.com/pls/bhg/home

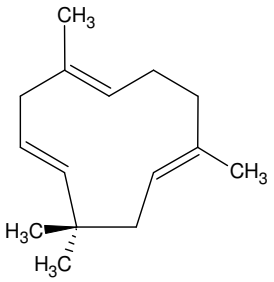
The dried hop cones contain polyphenols, mainly phenolic acids, prenylated chalcones, flavonoids, catechins and proanthocyanidins (Stevens *et al*., 1998; Taylor *et al*., 2003). Only 20–30% of beer polyphenols are derived from hop, whereas 70–80% originate from malt (Moir, 2000). Polyphenols derived from hop are likely to be different from those of malt (Benitez *et al*., 1997). Polyphenols are natural anti-oxidants which can protect beer against oxidation (Lugasi, 2003). Polyphenols also contribute to the colour of beer (Baxter and Hughes, 2001) and to haze formation (Papp *et al*., 2001). However, polyphenols may cause an unpleasant astringency to beer (Murray *et al*., 1994).

Phenolic acids can be precursors to specific beer flavours. Ferulic acid for example is a precursor of wheat aroma. The main prenylflavonoid of hop cones is xanthohumol. It is a simple prenylated chalcone only present in hop. It posseses an anticancer activity (Gerhäuser *et al*., 2002; Stevens *et al*., 1997; Stevens and Page, 2004). Other polyphenols, namely tannins play a role in clarifying beer by precipitating proteins during boiling (Delvaux *et al*., 2003).

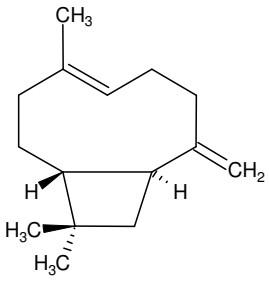
Hop oil contributes to the aroma of beer. There are more than 300 compounds in hop oil. It can be divided into a non polar (hydrocarbon) and a polar (oxygenated and sulphur-containing) fraction. Around 40-80% of hop oil is of hydrocarbon-nature and consists mainly of the monoterpene myrcene and the sesquiterpenes caryophyllene, humulene, and farnesene. Alcohols are also present in hop oil. Linalool is the major terpene alcohol found in hop, it is present up to 1% of total oil. Hop oils contain organic sulphur compounds which have a negative effect on beer flavour. Although sulphur compounds are present in very low quantities in hop, some have flavour thresholds of a few parts per billion or even lower (Baxter and Hughes, 2001; Benitez *et al*., 1997; Engan, 1981; Lermusieau and Collin, 2003).

While essential oils are responsible for the aroma, the resinous compounds, especially α-acids are responsible for the bitter taste of beer. Hop resin is species-specific compounds. There are two types of hop resins: α- and β-acids. They are called acids because they are proton-donators. Total resin is defined as the portion of a diethyl ether extract of hop that is soluble in cold methanol and consists of hard resin and soft resin. It is different from hop wax, a mixture of long-chain alcohols, acids, esters, and hydrocarbons, which are poorly soluble in cold methanol. Hard resin is defined as the resin that is insoluble in hexane, while soft resin is soluble in hexane. Hard resin consist of α-acids- and β-acids-oxidation products, xanthohumol, iso-xanthohumol, and some flavones. Soft resin is very important in brewing because it consists of deoxyhumulones, α-acids (humulone, cohumulone, adhumulone, prehumulone, and posthumulone) and β-acids (lupulone, colupulone, adlupulone, prelupulone, and postlupulone), known as bitter acids. Because soft resin is found in lupulin glands, it is also called lupulinic resin. For the brewery the most important compounds of hop are the α-acids. These are weak acids (pKa values of humulone = 5.1) and have a very poor solubility in aqueous beer medium (pH between 5.0 and 5.2 for pilsener beer). During wort boiling they are isomerized to the bitter-tasting iso-α-

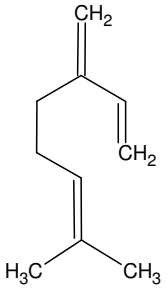
acids which are much more soluble (pKa values of *trans*-isohumulone = 3.1) (De Keukeleire *et al.*, 1992; Hughes and Simpson, 1994; Verhagen, 1988; Verzele, 1986).



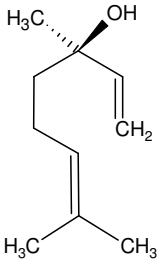
α-Humulene



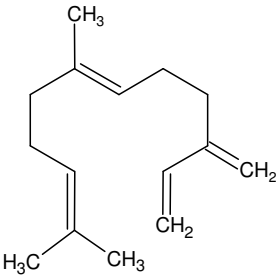
β-Caryophyllene



β-Myrcene



R-Linalool



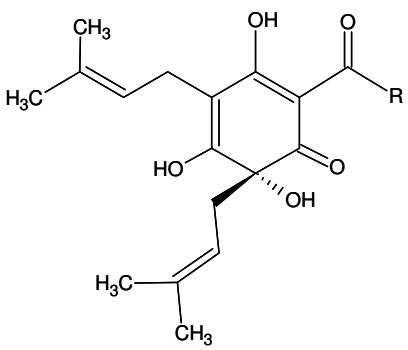
Farnesene

Fig. 1.1. Chemical structures of major hop oil compounds.

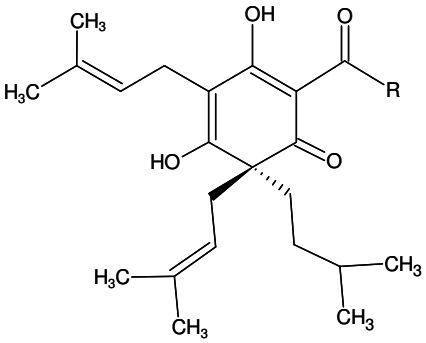
Compared to iso-α-acids, β-acids are much less bitter. Thus, these compounds do not play a role in the quality of beer. β-Acids tend to precipitate in wort and beer. They have pKa values around 6. They comprise 5 compounds, namely lupulone, colupulone, adlupulone, prelupulone, and postlupulone (Moir, 2000; Rybáček, 1991; Verzele, 1986).

Soft resin and hop oil are not stable products. They easily degrade at room temperature (Canbaş *et al.*, 2001). Oxidation of humulone produces the oxidation

products humulinone (γ-acids), tricyclodehydroisohumulone, and hydroxy-humulinic acids. None of these oxidation products is very bitter. Tricyclodehydrohumulone is about 70% as bitter as iso-α-acids. The oxidation of β-acids produces δ-acids (hulupones) (Verzele, 1986; Verzele and De Keukeleire, 1991).



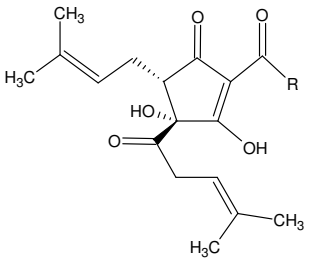
α-acids



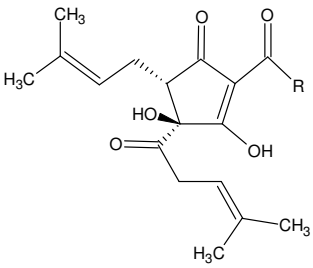
β-acids

R groups:	**α-acids**	**β-acids**
$-CH_2CH(CH_3)_2$	Humulone	Lupulone
$-CH(CH_3)_2$	Cohumulone	Colupulone
$-CH(CH_3)CH_2CH_3$	Adhumulone	Adlupulone
$-(CH_2)_2CH(CH_3)_2$	Prehumulone	Prelupulone
$-CH_2CH_3$	Posthumulone	Postlupulone

Fig. 1.2. Chemical structure of α- and β-acids.



cis-Iso-α-acids



trans-Iso-α-acids

R -$CH_2CH(CH_3)_2$: isohumulone

R -$CH(CH_3)CH_2CH_3$: isoadhumulone

R -$CH(CH_3)_2$: isocohumulone

Fig. 1.3. Chemical structures of iso-α-acids.

1.3. ISO-α-ACIDS

The isomerization of α-acids to iso-α-acids occurs during wort boiling by an acyloin-type ring contraction. Each α-acid converts to a pair of *trans*-/*cis*-iso-α-acids. These two epimers are different depending on the position of the tertiary alcohol function at C-4 and the prenyl side chain at C-5. Thus 3 major α-acids convert to 6 major iso-α-acids (*trans*-/*cis*-isocohumulone, *trans*-/*cis*-isohumulone, and *trans*-/*cis*-isoadhumulone) which are present in beer. The ratio of the iso-α-acids depends on the reaction conditions. In the wort medium it is normally 75:25 (*cis*-/*trans*-isomers). A major problem is the poor solubility of the α-acids in the wort medium which limits their conversion. α-Acids (pKa values around 5.5) are not well soluble in wort medium which has a pH between 5.0 and 5.2. On the other hand, iso-α-acids (pKa values around 3.0) are much more soluble in wort medium. Divalent cations, such as magnesium or calcium, strongly catalyze the isomerization reaction, producing a 1:1 of *cis*-/*trans*-isomers composition (De Keukeleire *et al.*, 1992; Koller, 1969; Moir,

2000). The isomerisation of the α-acids can also be performed by irradiation with UV light in the wavelength region of 365-366 nm. However, α-acids are converted exclusively to the *trans*-isomers by this method (Sharpe and Ormrod, 1991).

Ionisation of the acidic function of humulone **1** deconjugates the double bond system in **2**, so that ketonisation can occur yielding **3**, an intermediate with two isoprenyl groups in *trans*-position (Fig. 1.4). The ring contraction gives a mixture of *trans*- and *cis*-isohumulones. The relative amount of the two diastereoisomers formed, remains relatively constant (25% *trans*-form and 75% *cis*-form) over a wide range of pH when only monovalent cations are used. With bivalent or multivalent cations derived from e.g. magnesium, lead or iron other compositions or even equilibrium situations can occur (Verzele, 1986; Verzele and Van Boven, 1971).

The concentration of iso-α-acids in beer is quite low (15-80 ppm). However, the quality of beer is much influenced by these compounds since 80% of the bitter taste derives from iso-α-acids. The bitter taste threshold value of the iso-α-acids in water is around 6 mg/liter (Benitez *et al*., 1997; De Keukeleire *et al*., 1992).

Individual stereoisomers have different characteristics both in bitter taste and stability. Generally the *trans*-isomers are less bitter than the *cis*-counterparts (Hughes, 2000; Hughes *et al.,* 1997; Hughes and Simpson, 1994; Hughes and Simpson, 1996). The *trans*-isomers were also reported to be more prone to oxidation than their *cis*-counterparts, thus they are less stable (De Cooman *et al*., 2001). The quality of beer therefore may be improved by using only the *cis*-isomers mixture.

Iso-α-acids are very unstable compounds and their degradation products are thought to be partially responsible for the off-flavour characteristic of ageing beer including stale and cardboard flavours which are connected with their oxidative degradation. The compounds that are responsible for these off-flavours are unsaturated aldehydes, such as *trans*-nonen-2-al, formed by the oxidative degradation of isohumulones (Hashimoto and Eshima, 1979). Other compounds are the vicinal diketones which are formed by oxidative decarboxylation of 2-acetohydroxycarboxylic acids. The taste threshold values for these compounds are very low ($<10^{-2}$ mg/l), and even as low as to 5 x 10^{-4} mg/l for *trans* 2-nonenal. In higher concentration it will give beer a very unpleasant resinous taste. Beer is no longer drinkable if the concentration of these compounds is about 1 mg/l (Verzele and De Keukeleire, 1991).

Furthermore, iso-α-acids are sensitive to light, and their degradation products (3-methyl-2-butene-1-thiol and dehydrohumulinic acid) are responsible for the light struck flavour of beer. In order to reduce this, beer is usually bottled in dark-coloured glass. Alternately, light stable reduced-iso-α-acids are used (Hougen, 1963).

Iso-α-acids exhibit other interesting features: they stabilize the beer foam and inhibit the growth of gram-positive bacteria. However, lactic acid bacteria in beer are resistant to iso-α-acids. As isocohumulone is less foam-active compared to the other iso-α-acids, the cohumulone content of different hop varieties should be taken into consideration (Simpson and Smith, 1992; Smith *et al*., 1998).

1.4. REDUCED ISO-α-ACIDS

Reduced iso-α-acids have been used because these acids have a good stability against light and improve the foam stability. There are three types of reduced iso-α-acids depending on the number of hydrogen atoms incorporated during reduction, e.g. dihydroiso-α-acids (*rho*-iso-α-acids), tetrahydroiso-α-acids, and hexahydroiso-α-acids as shown in Fig. 1.5. Among them, *rho*- and tetrahydroiso-α-acids are now used worldwide. Dihydroiso-α-acids, are formed by reduction of the carbonyl group of the isohexenoic side chain at C-4 to an alcohol. Tetrahydroiso-α-acids (the most bitter) are obtained by hydrogenation of the double bonds in the side chains at C-4 of the iso-α-acids. Hexahydroiso-α-acids are formed in which the isohexenoic carbonyl group and the side chain carbon-carbon double bonds are reduced (Hughes and Simpson, 1993; Moir, 2000).

The chemical reduction impacts the properties of the compounds in which the greater degree of chemical reduction causes a more hydrophobic and foam positive characteristic of the hop bitter acids. United States Food and Drugs Administration permits the use of these products for human consumption with specified maximum levels of both solvent residues and boron, and they are commercially used in the USA (Hughes and Simpson, 1993).

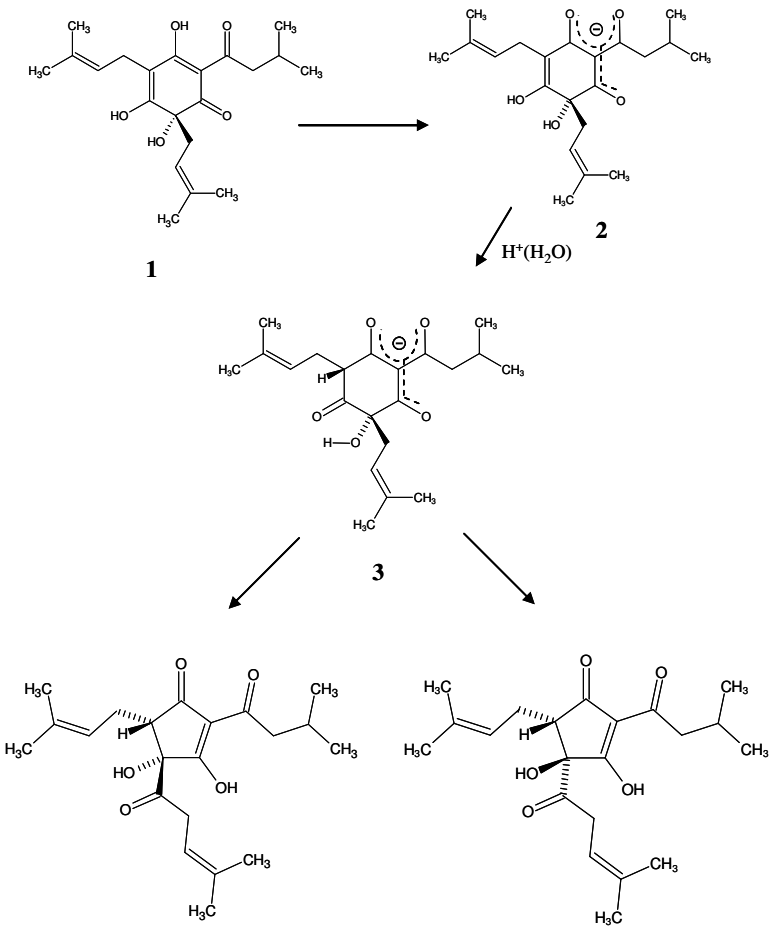
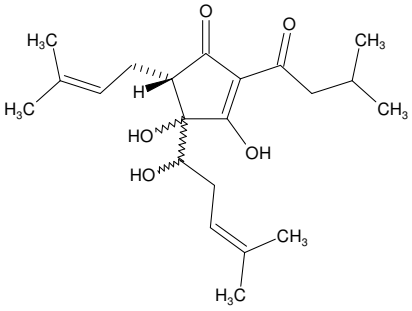
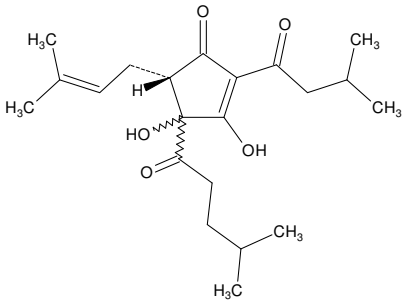


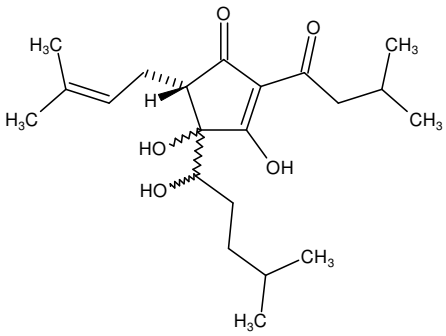
Fig. 1.4. Isomerisation of humulone to produce *trans*- and *cis*- isohumulone.



Dihydroisohumulone



Tetrahydroisohumulone



Hexahydroisohumulone

Fig. 1.5. Chemical structure of reduced isohumulone.

1.5. HOP PRODUCTS

Traditionally hop is used in their unprocessed form (whole hop) as compressed bales. However, there are several problems in using the unprocessed form in the brewery. The bales are bulky which causes a problem in storage, transport, and handling for automatic dosage into the kettle. The concentration of α-acids in bales is very low and not constant due to the variation between different batches. Furthermore, chemical residues from agrochemicals are always present in the bales. The conversion

of α-acids to iso-α-acids during wort boiling is low, usually in the range 30 to 40% due to the acidic pH of wort. In this acidic condition isomerization takes a long time (Benitez *et al*., 1997).

Because of those problems, the use of processed hop in the brewery is considered to be more efficient. The hop products can be classified into several types: hop powders/ pellets, enriched hop powders/pellets, specialty hop powders/pellets, hop extracts, specialty hop extracts, isomerized hop extracts, and hop oil (Clarke, 1986).

There are two type of hop pellets: type 90 and type 45. The value indicates the percentage of original bale in the pellet. Type 90 hop pellets are made by drying the hop cones and subsequently milling and packaging as a powder or converting into pellet form before packaging. The content of α-acids is standardized before pelleting. The pellets are packaged under an inert atmosphere, such as nitrogen or carbon dioxide. Type 90 pellets have several advantages compared to hop bales. The bulk density is four times higher compared to that of hop bales. The concentration of α-acids can be controlled by blending different hop varieties. Another advantage is an improvement in utilization, because the α-acids are easier to be extracted from the ruptured lupulin glands (Benitez *et al*., 1997; Hughes and Simpson, 1993).

Type 45 (enriched hop pellet) is made in order to increase the concentration of both α-acids and essential oils in the pellets. Both compounds are present only in lupulin glands. Therefore, the concentration of both compounds can be increased by isolation of lupulin glands from the bracts of hop cones. It can be performed by maceration at a temperature of -35 ^{o}C or less. Lupulin glands lose their stickiness at this temperature and can be separated from the bracts. In this way, the concentration of the α-acids and essential oils increase almost two-fold (Benitez *et al*., 1997; Hughes and Simpson, 1993).

Specialty hop products are produced among others by blending ascorbic acid with pelleted hop. Ascorbic acid can increase the stability of pelleted hop. Pelleted hop also can be mixed with magnesium or calcium salts. Making the α-acids salts improve the utilization and the speed of isomerization (Hughes and Simpson, 1993).

In order to increase the concentration of α-acids, hop extracts are made. Several solvents are used on the basis of selectivity, e.g. carbon dioxide, ethanol, and hexane. Water is not used since the bitter acids have a very low solubility in this solvent. The use of liquid and supercritical CO_2 hop extracts is very popular in breweries due to

safety reasons. Other advantages of CO_2 are that it is easily removed at room temperature and it is inert. The extractability of CO_2 can be changed by changing temperature and pressure. Both liquid and supercritical CO_2 extracts contain a high level of α-acids (27-60%). Liquid CO_2 extraction is selective, but it requires several hours extraction time. Supercritical CO_2 hop extraction is more rapid and overcomes this problem, although the costs are five times higher than that of liquid CO_2 extraction (Clarke, 1986; Hughes and Simpson, 1993; Kruger, 1980).

There are several type of specialty hop extracts. Hop extract can be blended with silicic acid in order to make a powdered form. This powder is easier to handle, and improves the colloidal stability of beer. Another type is a basic extract which contains β-acids and hop oils. It is the remaining fraction after separation of α-acids. Basic extracts are used to balance the hop flavour of beer bittered with isomerized hop products (Benitez *et al*., 1997; Hughes and Simpson, 1993).

Isomerization of hop extracts is catalyzed by potassium or magnesium salts. This process results in aqueous concentrates of iso-α-acids as their potassium salts or solid concentrates of iso-α-acids as their magnesium salts. These extracts are reasonably stable when stored at 5 ^{o}C. It can be added to beer post-filtration and thus improves the utilization (Benitez *et al*., 1997; Hughes and Simpson, 1993).

Hop oil-rich extract (non pure hop oil) can be obtained by extraction with liquid CO_2 or fractionation during production of pure resin extract with supercritical CO_2. Pure hop oil is produced by steam distillation of resin extract. Another method is molecular distillation by creating a thin film on a heated surface (120 ^{o}C). The oil is distilled off under a very high vacuum. Fractionated hop oils are also available commercially. Hop oil can be fractionated chromatographically to produce fractions of hop oil that give either floral, spicy, and herbal aromas to beer. Traditionally hop oils are added to the process, either towards the end of the wort boil (late hopping), or to the final container (dry hopping) (Benitez *et al*., 1997; Hughes and Simpson, 1993).

1.6. ANALYSIS OF HOP BITTER ACIDS

There are a number of analytical methods described for hop bitter acids. These can be divided into unspecific and specific methods. The traditional method for measuring

bitter acids, i.e. spectrometric analysis (Alderton *et al*., 1954; Maye *et al*., 2002) is incapable of distinguishing between the individual bitter acids.

Specific analytical methods for bitter acids were developed based on the separation techniques such as countercurrent distribution (Howard and Tatchell, 1957). It gives good separation of bitter substances, but is very time-consuming and requires large amounts of organic solvents.

A thin layer chromatography (TLC) method has been developed for analysing bitter acids (Aitken *et al*., 1968; Franiau and Mussche, 1974). Aitken *et al.* (1968) developed TLC system to detect *trans*- and *cis*-isohumulone, together with hulupone. However, the sensitivity and resolution of this method are low.

This problem can be overcome by high performance liquid chromatography (HPLC) and gas chromatography (GC) which have higher resolution and quantitative results for bitter acids (Harms and Nitzsche, 2001; Hermans-Lokkerbol and Verpoorte, 1994a; Vanhoenacker *et al.*, 2004). These can be coupled to UV, mass spectrometry (Vanhoenacker *et al*., 2004; Zhang *et al*., 2004), or NMR spectrometric detection (Pusecker *et al*., 1999) for the qualitative and quantitative detection of these components. Some examples of HPLC solvent systems can be seen in Table 1.2.

The iso-α-acids are extremely sensitive to oxidative degradation. This process is drastically enhanced by traces of metals present in the LC-column-packing material. Trace metals also can influence the retention time and alter the UV spectrum of hop bitter acids (Dewaele and Verzele, 1980). Therefore, the use of special demineralized stationary phases is recommended (ROSiL-C18, available from RSL-alltech-Europe, Eke, Belgium; Nucleosil-C18, available from Macherey-Nagel, Düren, Germany). The trace metal problem may be masked by the addition of both phosphoric acid and ethylenediamine tetraacetate to the eluent, giving correct results on commercial columns designed for hop analysis (De Keukeleire *et al.*, 1992).

Calibration of the quantitative analysis of iso-α-acids is a major problem. The efforts to obtain pure individual iso-α-acids for use as external standards seems not possible due to the inherent instability of those compounds. *trans*-Isohumulone, which can readily be prepared in pure state by photoisomerization of humulone decomposes even when stored under nitrogen at low temperature. It should be recrystallized prior to each series of analyses, which impedes practical application.

Table 1.2. Summary of representative reverse-phase HPLC-UV methods reported in the scientific literature for bitter acids analysis.

Mobile Phase	Scope	Column	T (°C)	Flow rate (ml/min)	Running time (min)	Reference
Methanol: water: phosphoric acid (85:17:0.25)	α- and β-acids , xanthohumol, xanthogalenol	Nucleosil RP C_{18}, 15 x 0.31 cm, 5 µm, Macherey Nagel, Düren, Germany	50	0.6	25	Hampton *et al.* (2002)
Methanol: water: phosphoric acid (85:17:0.5)	α- and β-acids	ROSiL-C18-D, 25 x 0.46 cm, 5 µm, RSL-Alltech-Europe, Eke, Belgium	ambient	1	25	Verzele and Van de Velde (1987)
0.05 M triethanolamine in methanol-water (65:35,v/v) pH 6.85	α- and β-acids	Hypersil 5 C_{18}, 25 x 0.46 cm, 5 µm, Phenomenex, Torrance, CA, USA	ambient	1.75	25	Hermans-Lokkerbol and Verpoorte (1994a)
Gradient: Acetonitrile/ methanol/ citric acid buffer pH 7 (17:25:57, to 55:0:45,v/v/v)	Iso-α-acids, *rho*-iso-α-acids, tetrahydro iso-α-acids	Nucleosil 100-5 C_{18} AB, ET 250/3, Macherey Nagel, Düren, Germany	40	0.55	25	Harms and Nitzche (2001)
Gradient: Acetonitrile/methanol / tris buffer pH 7 (17:25:57) to acetonitrile/ methanol/ tris buffer pH 7.5 (50:8:42)	α- and β-acids	Nucleosil 100-5 C_{18} AB, ET 250/3, Macherey Nagel, Düren, Germany	40	0.55	65	Harms and Nitzche (2001)
Gradient: 5mM ammonium acetate in 20% ethanol to acetonitrile/ ethanol (60:40,v/v)	Iso-α-acids, reduced iso-α-acids, α- and β-acids	Two Zorbax Extend C_{18} columns, 15 x 0.46 cm, 5 µm, Agilent Technologies, Germany	35	1	60	Vanhoenacker *et al*. (2004)

The lack of a suitable standard is evidenced by analyses of commercial isomerized extracts on the demineralised stationary phases, which show that the actual concentrations of iso-α-acids were 20 to 30% lower than the advertised values. Hydrogenated iso-α-acids or dicyclohexylammonium (DCHA) salts of iso-α-acids have been proposed as stable standards, but their utility still remains to be determined. The use of internal standards of different chemical nature has been advocated but the response factor of such substances still has to be related to that of the pure iso-α-acids (De Keukeleire *et al.*, 1992). This mixture of *trans*-iso-α-acids-DCHA salts has been used widely as a reference compound and is commercially available. However, the *cis*-isomers are not available to date. Therefore, the development of an efficient isolation method for iso-α-acids and a method to increase the stability still remain as a major issue.

Another analytical technique is micellar electro-kinetic chromatography (MEKC) which is becoming popular for bitter acids analysis. The absence of trace-metal containing packing material makes this technique particularly useful for the analysis of hop α-acids and iso-α-acids. The separation of 6 major iso-α-acids and distinguishing them from reduced iso-alpha-acids has been performed by this method (Cortacero-Ramírez *et al*., 2003; McLaughlin *et al*., 1996; Royle *et al*., 2001). MEKC was successfully used to separate the oxidation products of the α-acids and thus to monitor the stability of hop products (Royle *et al*., 2001).

NMR had been used for qualitative and quantitative analysis of bitter acids. NMR has several advantages for analytical purposes. It can detect a wide range of compounds in a single measurement. It is the only method in which the intensity of the signals is directly correlated to the molar concentration. That means that the amount of all compounds can be directly compared, eliminating the need for calibration curves for each individual compound. NMR has also the great advantage of high reproducibility as the spectra are based on the physical properties of a molecule. However, this technique needs a large amount of sample. Hoek *et al*. (2001) developed the method for α-acids. Some of the proton signals of cohumulone, humulone, and adhumulone can be clearly detected as a signals separated in the NMR spectrum. Up to date there is no report about the use of this method for other bitter acids including iso-α-acids.

1.7. AIM OF THE THESIS

The aim of this thesis project was the development of a separation of all individual iso-α-acids. To achieve this goal we first developed an HPLC system for the analysis of the iso-α-acids. Using this system the separation and stabilization of the iso-α-acids by means of cyclodextrins were studied. This method proved to give an excellent separation of the *cis*- and *trans*-isomers. Subsequently the mechanism of this separation was studied in more detail, as well as the effect on stability. Finally an analytical method to detect a wide range of components in beer including iso-α-acids using two dimensional J resolved NMR was developed.

1.8. OUTLINE OF THE THESIS

The hop bitter acids have been studied extensively and a large number of publications were produced as reviewed above. However, there still remain many questions regarding efficient techniques to separate and to isolate *trans*- and *cis*-iso-α-acids.

In **Chapter 2** the analysis of iso-α-acids using HPLC is discussed. This method was applied to analyze the purity of iso-α-acids fractions obtained during the development of the separation and isolation methods for iso-α-acids using β-cyclodextrin which is discussed in **Chapter 3** and **4**.

The separation of *trans*- and *cis*-iso-α-acids from an isomerized CO_2 hop extract using β-cyclodextrin is discussed in **Chapter 3**. This technique can also be applied to isolate the pure iso-α-acids (**Chapter 4**). The confirmation of the chemical structures of the isolated pure iso-α-acids was checked using NMR and mass spectrometry (**Chapter 5**).

The main problem of iso-α-acids is their low stability especially in the presence of light and oxygen. The study on the stability of these compounds in β-cyclodextrin complexes is described in **Chapter 6**. Also in this chapter the characterization of the binding of iso-α-acids to β-cyclodextrin is described.

Iso-α-acids are not the only compounds that influence the quality of beer. There are others components that also contribute. However, HPLC can not detect all the

compounds in beer. In order to be able to detect a wide range of components in beer including iso-α-acids, a method using two dimensional J resolved NMR has been developed as discussed in **Chapter 7**.