Sandra Katharina Tschurnig, BSc

Oxidation of polyphenols in hops

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Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. Michael Murkovic

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AFFIDAVIT

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Abstract

Beer is the most consumed alcoholic beverage and contains hops as an essential compound. The prenylated chalcone Xanthohumol (XH) occurs in lupulin glands of the hop plant and obtained much attention in the last decades due to its beneficial and biological effects in human health. During brewing the XH content decreases due to isomerisation reaction which converts the chalcone into its isomeric form called Isoxanthohumol (IXH). However the prenylated flavanone IXH has less positive and healthy effects compared to XH. Not only isomerisation reaction decreases the XH content but also fermentation, separation and filtration steps are responsible for this process. Since the advantages of XH are known, the brewing industry researches for suitable production technologies to produce XH-enriched beer. Investigators also look for an appropriate enrichment of XH with the addition of hops in other edibles like for example in herbal tea. Furthermore the hop plant itself is applied in extract capsules as dietary supplement and it is also utilised in the cosmetic industry for several beauty products.

The aim of this master thesis was to determine the ratio of hop polyphenols (XH and IXH) in different hop pellet species and beer samples. XH and IXH were also analysed during wort boiling to show the isomerisation reaction which occurs in the brewing process. Finally the maltose and glucose concentrations were determined in boiled wort samples.

Zusammenfassung

Bier ist das meist konsumierte alkoholische Getränk und enthält Hopfen als essentiellen Bestandteil. Das prenylierte Chalcone Xanthohumol (XH) kommt in den Lupulin Drüsen der Hopfenpflanze vor und bekam in den letzten Jahrzehnten große Aufmerksamkeit aufgrund von nützlichen und biologischen Eigenschaften für die menschliche Gesundheit. Während des Brauprozesses wird der XH Gehalt im Hopfen aufgrund von Isomerisationsreaktion verringert und dadurch wird das Chalcone in seine isomerische Form, genannt Isoxanthohumol, umgewandelt. Das prenylierte IXH hat hingegen weniger positive und gesundheitsfördernde Eigenschaften im Vergleich zum XH. Nicht nur die Isomerisationsreaktion verringert den XH Gehalt, sondern auch Fermentations-, Filtrations- und Abtrennungsschritte sind verantwortlich für diesen Vorgang. Seitdem die gesundheitsfördernden Eigenschaften von XH bekannt sind, untersucht die Brauindustrie geeignete Produktionsverfahren, um XH angereichertes Bier herzustellen. Forscher suchen auch nach einer Anreicherung von XH durch die Verwendung von Hopfen in anderen Nahrungsmitteln, wie zum Beispiel Kräutertee. Desweiteren wird die Hopfenpflanze in Extrakt-Kapseln als Nahrungsergänzungsmittel angewendet und auch in der Kosmetik-Industrie für verschiedene Schönheitsprodukte.

Das Ziel dieser Masterarbeit war es das Verhältnis von Hopfenpolyphenolen (XH und IXH) in verschiedenen Hopfenpellets-Arten und Bierproben zu bestimmen. XH und IXH wurden auch während der Würzekochung analysiert um die Isomerisationsreaktion, welche im Brauprozess auftritt, zu zeigen. Zum Schluss wurde auch noch die Maltose und Glucose Konzentration in gekochten Würzeproben untersucht.

Contents

1. Introduction

A polyphenol, named “Xanthohumol”, is the major prenylated chalcone in hop cones. Hop is an important ingredient of beer which is a symbol of our culture and a modern lifestyle beverage consumed all over the world [1]. This alcoholic beverage is the third most popular drink ranking behind water and tea [5].

1.1 History of Beer

The alcoholic drink started its history in Sumer, a civilization of city states in south-eastern Mesopotamia. The Sumerians produced beer from bread and malt and finally they flavoured it with honey [1]. Beer was also used as a currency at this time and it played a key role in ritual and religious practices [2]. In the prehistoric town Godin Tepe (now western Iran) some excavations were done. A 5500 year old pottery jar which contains calcium oxalate was found [1]. This chemical substance was the evidence of beer production, because it is a principal (insoluble) component of beer-stone. The inorganic and scale-like deposit accumulates in beer storage tanks and fermentation vessels [2].

It is assumed by historians that beer-like beverages were brewed in China as early as 7000 BC, but the first documents which show evidence of beer consumption is known from 2800 BC in Mesopotamia. At 3000 BC the beer culture spreads from Mesopotamia to Egypt and already at 30 BC beer was the most favourite beverage of Egyptian people. When Egypt fell under Roman domain, a wine culture was established in the region. So wine was a drink for the nobles because it was the conqueror´s beverage and beer was the drink of the “barbarians”.

Before the expansion of the Roman Empire occurred, all Celtic peoples from France, Spain, Portugal, Belgium, Germany and Britain consumed beer as their most favourite beverage. After expansion the development of the wine culture was imported. When Germany conquered Western Europe in the fifty century AD by losing control of Romans, beer was again the drink for sovereign.

The first evidence of commercial brewing derived from the old drawings of a brewery in the monastery of Saint Gall in Switzerland, which dates back to 820 AD [4]. Since that time it was demonstrated that every northern European monastery has a

brewery [1]. At this time only monasteries started to produce large amounts of beer [4]. The monks were allowed to sell beer in so called monastery pubs to make extra income [1]. So the basis of the brewing industry began to grow in the urban centres where large markets started to emerge [4]. In around 900, some monasteries obtained a beer monopoly for the production of gruit, which is a combination of herbs to flavour the beer. Many years later (15th century) hops replaced gruit. When beer was exported in other countries there was a problem with the stability of the unhopped product. The beer spoiled rapidly and was impossible to trade. So due to that, the time of hops started because of its antimicrobial effect. Addition of hops was a preservative and beer could stay fresh for weeks and months. Furthermore, hops provided a unique flavour and aroma to the beer.

The first historical document which describes the use of hops in beer was found from 822. It was a list of rules written by the abbot Adalhard (751-827) for the monastery of St. Peter and St. Stephen in Corbie, northern France. At this time there was no evidence that hops were cultivated so it was probably collected from the nature. The period from 859-875 indicates a cultivation of hops at the Abbey of Freisingen in Bavaria, southern Germany [1].

1.2 Ingredients

The usual ingredients of the fermented beverage, according to the Beer Purity Law from traditional German beer, are water, malt, hops and yeast [3]. This kind of food regulation, the *Reinheitsgebot* (1487), is the oldest act in the history. The “German Beer Purity Law” or also called as “Bavarian Purity Law” was originally designed to prevent the use of wheat or rye as starch source for the brewing process. Today’s Purity Law (*Vorläufiges Biergesetz*) is a slightly modified version of the *Reinheitsgebot*. It limits the ingredients for making bottom-fermented beers, compared to top-fermented beers, where different kinds of malt and sugar adjuncts are allowed [4].

Although finished brewed beer varies at the composition, the approximate average compounds are:

Table 1: Beer composition [1]

Water	92.9 %
Ethanol	3.9 %
Carbohydrates	2.5 %
Carbon dioxide	0.5 %
Protein	0.2 %

Beer also contains other nutritious components like vitamins, minerals and phenolic compounds. Approximately 70-80 % of the polyphenols occurring in beer originate from the malt and 20-30 % from hops [3]. At the beginnings, beer contained such an amount of yeast, starch and proteins that it was cloudy and a nearly complete food. Nowadays it is clear that beer is not nourishing but it contains desirable nutrients like niacin, silicon and folic acid. Furthermore the beverage has no fat and only 2.5 % sugar which is another advantage. High consumption of beer is absolute unhealthy.
On the other hand there is a perception that beer has a very fattening aspect. This assumption of beer tends to the food which is consumed with it. For example, 1 litre beer, a portion of chicken wings and a piece of pizza have a content of 1816 calories. But two beers, a couple of carrots and some celery sticks would come to less than 400 calories [1].

1.2.1 Water

High amounts of water are used for the beer production itself, but it is also utilized for some rinsing and cleaning. The “liquor” as it is called from the brewers, defines water which is used for the brewing process. It determines the quality of beer and continuous monitoring of the brewing liquor has to be executed which includes the chemical and microbial analyses. The water has to be potable and must be free of pathogens. The mineral ions from the water are another important aspect. Some ions could affect the brewing process and are essential for the final beer taste. For example, sulphates could increase the hardness and dryness of beer. Also the hop bouquet is favoured through it. High amounts of iron and manganese can modify the colour and taste of beer. Another important ion is calcium which protects the enzyme α-amylase from the early inactivation by lowering the pH-value toward the optimum for enzymatic activity. In the fermentation process calcium is also essential for yeast

flocculation and at the boiling process it prevents the over-extraction of components in hops [4]. Zinc ions act as a cofactor for the yeast and help with stress tolerance. They are important for the yeast enzymes and a lack of zinc could impede yeast from budding. Each yeast strain needs different amounts of zinc, but the average required level for optimal fermentation ranges from 0.48 to 1.07 ppm [6]. Nitrites are the antagonists which impede yeast growth and fermentation but are available in water only in low concentrations [4].

1.2.2 Malt

Malt from barley (*Hordeum vulgare*) is the most used grain for brewing process [1]. The reproductive parts (seeds) of the plant are the starch source and provide many enzymes to break down the polysaccharide starch into small molecules of sugars that can be fermented [1], [4]. There are two types of barley (fig. 1) which are used for the beer production. The six-row barley with three grains per node has a higher protein but a lower starch content. This barley would be the best choice if enzymatic strength is the aim at the brewing process. The two-row barley with one grain per node is used if increased extract content is desired [4].

Figure 1: Different types of barley.
Left: two-row barley, right: six-row barley [7]

Many breweries also use alternative starch sources (adjuncts) in addition to malted barley. They use adjuncts to enhance colour, flavour and aroma of the beer and also the final cost of the recipe could be reduced by this way. Some examples for most commonly used adjuncts are un-malted barley, wheat, rice, corn or other sugar

sources like starch, sucrose, glucose and corresponding syrup. The use of adjuncts is controlled from each country. For example in Germany the Bavarian Purity Law regulates the allowed amount of adjuncts. Whereas in other “outlaw” countries like USA and Brazil, an increased input of adjuncts can be noticed. In the USA, the commercial breweries are allowed to use 34 % (w/w) of un-malted cereals (adjuncts) of the total weight of grist. In Brazil, they are allowed to use un-malted grains (e.g. corn and rice) as high as 45 % of the total recipe content [4].

1.2.3 Yeast

The yeast *Saccharomyces* has always been involved in the fermentation process (e.g. of beer) but our ancestors did not know anything about it. Antonie van Leeuwenhoek was the first scientist who saw yeast cells through a microscope in 1680. Afterwards, Louis Pasteur studied on the conversion of wort into beer by living cells. He investigated the growth of brewing yeast cells and proved that they are necessary for the fermentation.

Yeasts which are used for the brewing process are eukaryotic, unicellular, heterotrophic and facultative anaerobic microorganisms [4].

Reproduction of the single-cell fungus occurs via budding [1]. The yeast cell has an ovoid shape, can bud approximately 10-30 times and measures a diameter of 5-10 µm [4].

There are two different types of yeasts for the brewing process (top fermenting and bottom fermenting) but all cells have the same organisation (fig. 2) whose components are cell wall, nucleus, cytoplasm and bud [9].

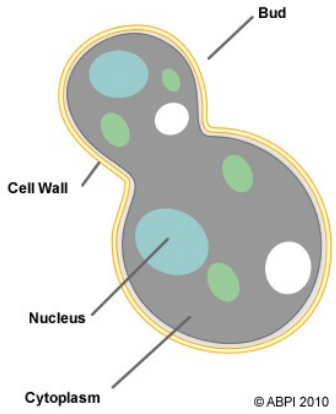

Figure 2: Structure of yeast cell [9]

The yeast cell is surrounded by a wall which exists for the shape of the cell [9]. It also offers strength to the cell and plays an important role for cell-cell interactions [10]. Inside the cell there is the nucleus which is responsible for the regulation of cell activation and reproduction and it also contains the genetic material. In the cytoplasm many chemical reactions occur and at the bud new yeast cells could be formed by asexual reproduction [9]. New daughter cells are built through cell division from the mother cell as a bud. These new bud leaves a “bud scar” on the surface of the mother cell before the new daughter cell is separated off [10].

As already mentioned there are two types of brewing yeasts. They have different properties but both could be used for the brewing process. The ale yeasts cannot ferment the sugar melibiose, which is a disaccharide of glucose and galactose. Lager yeasts can hydrolyze the indicator (5-bromo-4-chloro-3-indolyl-α-D-galactoside) and grow blue colonies in the media which is containing this substance, compared to ale yeasts which form uncoloured colonies.
The species *Saccharomyces cerevisiae* is a top-fermenting yeast because the cells accumulate in the foam during fermentation. It ferments at relative high temperatures (18-25°C), resulting in fast fermentations and is used for the production of ales [4]. Lager yeasts (formerly S. *carlsbergensis* and *S. uvarum*, current accepted nomenclature of *S. pastorianus*) are bottom-fermenting yeasts because they flocculate to the bottom of the tank after fermentation. Here the fermentation temperature is lower (8-12°C) and this kind of yeast needs much more time to ferment the sugar products compared to the top-fermenting yeasts [8].

1.2.4 Hops

Hops are the most important ingredient in this master thesis and therefore this chapter will be describing it in more detail.
The last of the four ingredients which is needed for brewing is hops. Only the female inflorescences (hop cones) of the plant of *Humulus lupulus* L. can be used [11]. These hop cones are able to secrete the fine yellow resinous powder. The main brewing principles of hops are fixed in these lupulin glands, where resins and essential oils are produced and accumulated [13]. The amount of added hops which belongs to the family of Cannabinaceae, is the lowest compared to water and malts, but contributes to the final character of beer [4].

Figure 3: Hop cones [15]

The genus name *Humulus* consists of three species: *Humulus lupulus* L., *Humulus japonicas* Siebold & Zucc. and *Humulus yunnanensis* Hu. There are five taxonomic varieties of the species *Humulus lupulus* which were classified by Small in 1978. The species were based on morphological characteristics and geographical locations. These are var. *lupulus* Small for European hops, the var. *cordifolius* Small for Japanese hops, the var. *neomexicanus* Nelson & Cockerell, the var. *pubescens* Small and the var. *lupuloides* Small for North American hops [11].

Hops are cultivated all over the world and are destined for brewing processes. Germany and USA are the main producing countries and produce 75-80 % of the total hop output. The Hallertau region in Germany and the states of Washington, Oregon and Idaho in the USA are the largest hop growing areas in the world.

The chemical composition of hops (tab. 2) comprises several components. For the brewing industry, the precursors of the flavour- and bitter-active compounds, which are found in resins (secreted by lupulin glands), are the important values. Also the hop essential oils are significant for the brewer because they provide flavour and aroma properties to the beer [13].

Table 2: Chemical composition of hops [13]

Constituent	Amount (%)
Total resins	15 - 30
Essential oils	0.5 - 3
Proteins	15
Monosaccharides	2
Polyphenols (tannins)	4
Pectins	2
Amino acids	0.1
Waxes and steroids	traces - 25
Ash	8
Moisture	10
Cellulose	43

Because of their important functions, the three components which were marked grey are subsequently described more in detail. Total resins and hop essential oils will be described right afterwards. Hop polyphenols which are the most important components of hops for this master thesis will be described in chapter 1.4.

1.2.4.1 Total resins

The total resin content depends on the hop variety and growing conditions. The resin content can range between 15-30 % of the total weight of a dried hop cone. Figure 3 shows an overview of the classification and nomenclature of hop resins [13].

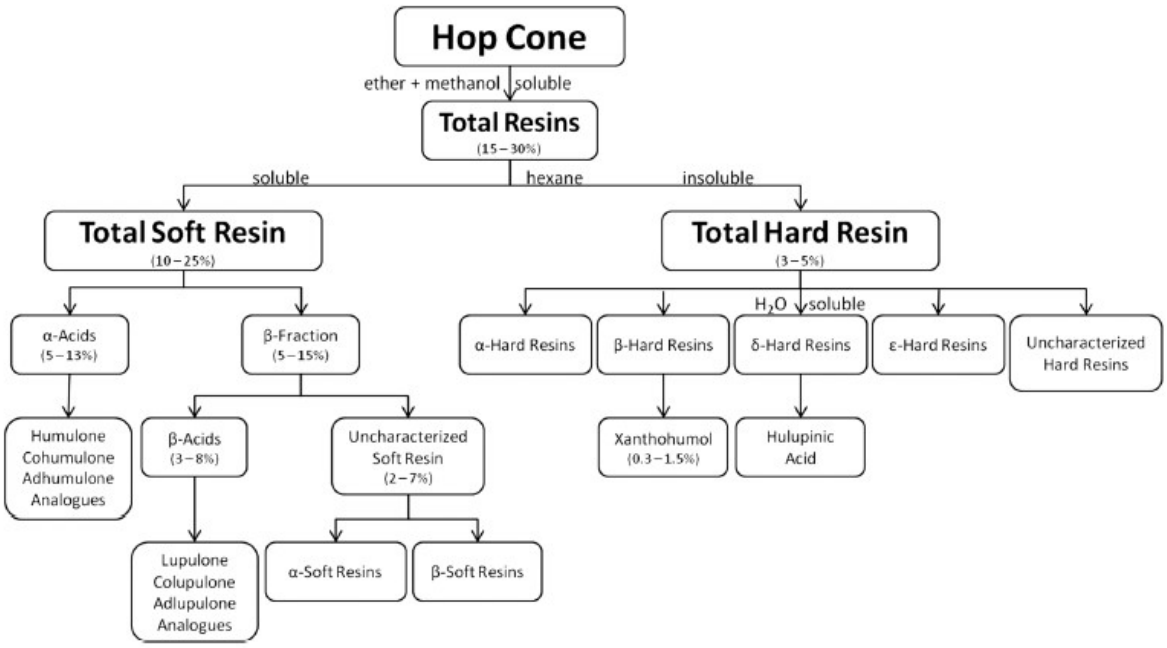

Figure 4: Overview about classification and nomenclature of hop resins [13]

The composition of the α-acid fraction depends on the hop varieties and further on the hop harvesting time. The most important α-acids in hops are humulone, cohumulone and adhumulone.

The β-fraction has two groups, the β-acids and the uncharacterized soft resins. The group of β-acids is less extensively studied compared to α-acids. The main β-acids are lupulone, colupulone and adlupulone [13]. All compounds of α- and β-acids differ only through the character of their acyl side chains [2].

It was evidenced by some scientists that β-acids are poorly soluble in water. During wort boiling, the α-acids undergo isomerisation while the β-acids do not. So it was

accepted (from Haseleu *et al.*) that these fraction is lost in the brewing process and does not contribute to beer bitterness.
The hexane insoluble total hard resins are fractioned in five groups. Xanthohumol, an important prenylated chalcone derived from the fraction of β-hard resin, is the major portion of the total hard resins [13].

1.2.4.2 Hop essential oils

The next constituents from hops which are described more in detail are the hop essential oils. Like the hop resins, these hop oils are also secreted in the lupulin glands [13]. Compared to α-acids which are responsible for beer bitterness, the hop oils contribute to the aroma or bouquet of a beer [2]. The essential oils are secondary metabolites and defined as the volatile fraction of hop cones [13]. The hop oil content ranges between 0.5-3 % of the total weight of a dried hop cone. In general approximately 200 hop oil compounds exist [2]. The composition and content of essential hop oils in hop cones depends on several factors. These are hop variety, growing and drying conditions, harvesting time, oxidation and finally age and storage conditions [13]. Fractionation of total hop oil provides two different kinds of oils, the "spicy" oil and the "floral" oil. In 1980, the hop oils were classified in three main groups, the hydrocarbons (50-80 %), the oxygenated compounds (20-50 %) and the sulphur-containing compounds (less than 1 %). Terpenes are the major group in the hop oil fraction of hydrocarbons. The monoterpene β-myrcene (fig. 5) and the two sesquiterpenes α-humulene (fig. 6) and β-caryophyllene (fig. 7) make 80-90 % of the total essential oils in hops [2].

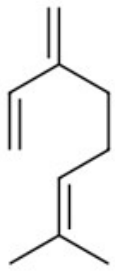

Figure 5: β-myrcene [14]

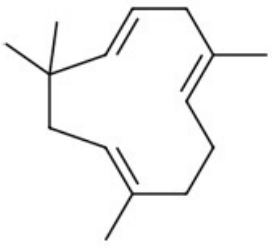

Figure 6: α-humulene [14]

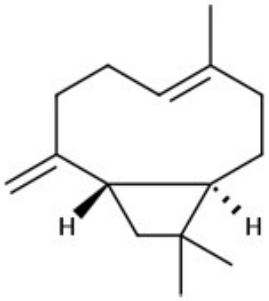

Figure 7: β-caryophyllene [14]

As already mentioned the hop polyphenols will be described in chapter 1.4.

1.2.4.3 Miscellaneous about hops

For the brewing process there are two different groups of hops which are known as “aroma/flavour hops” and “bitter hops”. All hops varieties contain α-acids for the bitterness. So aroma hops have less α-acids but more essential oils. Conversely, bitter hops contain higher amounts of α-acids but less essential oil [4].
At first the hop plant was used as preservative for its antimicrobial activity, afterwards it was used to add a bitter flavour to beer [11].

Bitterness:
At the boiling process, where the wort is heated with hops, many complex chemical reactions occur. During the transformation of wort the hop bitter principles are extracted. The isomerisation reaction of insoluble α-acids into soluble iso-α-acids is the most important conversion during wort boiling. From the reaction of one α-acid, two iso-α-acids are generated as diastereoisomers (*cis* and *trans*). The *cis* molecule is significantly more bitter than the *trans*. The bitterest compound of the six major iso-α-acids is *cis*-isohumulone and the least bitter compound is *trans*-isocohumulone. So the bitter taste is attributed to iso-α-acids which were formed from the hop resins during wort boiling. Approximately over 85 % of the pleasant bitterness is from iso-α-acids [13].

Foam:
Today it is known that hops not only contribute to beer bitterness, but also play an important role in beer foam formation and stabilisation. Foam is a high quality factor in the beer production process. In 1976 the scientists Fly and Chicoye observed the correlation of iso-α-acids and their potential of beer foam stability. It has been shown that the highly hydrophobic components of iso-α-acids were responsible for the stabilisation of beer foam. It is assumed that the hydrophobic compounds have a high tendency to leave the beer and concentrate in the foam, which increases the foam stability [13].

Microbial stability:
Antibacterial activity on Gram-positive bacteria is attributed to humulone and lupulone. The α-acids and β-acids are resistant against some species of *Micrococcus, Staphylococcus, Mycobacterium* and *Streptomycetes.* The reason for this function is the primary membrane leakage because of the interaction of

hydrophobic parts of molecules with the bacterial cell wall. The bitter acids also provide antifungal activity against *Candida albicans, Trichophyton, Fusarium*, and *Mucor* species. Some studies showed that also hop essential oils have an antimicrobial activity against Gram-positive bacteria like *Staphylococcus aureus*, but no effect on the Gram-negative bacterium *Escherichia coli* and fungus *Candida albicans.* Also the prenylated chalcone xanthohumol has desired properties. The broad spectrum of antiinfective agents is resistant against Gram-positive bacteria (*Staphylococcus aureus, Streptococcus mutans*), viruses (cytomegalovirus, herpes simplex virus type 1 and 2, human immunodeficiency virus 1), fungi (*Trichophyton spp.*) and malarial protozoa (*Plasmodium falciparum*). The wide spread functions of xanthohumol are still under investigation.

Furthermore, our ancestors have been using hops as medicinal resource against different pains. It was applied as a mild sedative for treatment of sleeping disorders and nervousness and as a bitter stomachic for the activation of gastric functions [11].

Hop products:

After harvesting, the hop cones from the plant, has to be processed for the modern brewing industry. At first the water content has to be reduced from 65-80 % to 8-10 % [11]. In a small amount of breweries the raw hop cones are used but pellets and hop extracts are used in the most breweries. The storage is easier and they have a longer shelf life [4]. Pellets are much more used than hop extracts [12]. Pellets are produced by drying, grinding, screening, mixing and pressing to pellets. Hop extracts are prepared by extraction with ethanol or supercritical carbon dioxide. The end product is a highly concentrated, resin-like and sticky substance [4]. Supercritical CO_2 extraction is the most commonly used method for the production of hop extracts [12]. Supercritical carbon dioxide is a solvent which is non-polar and rather selective. It dissolves soft resin and oils from hop but does not attack its polar components [11]. After extraction the hop extract contains nearly all important oils from hops, a high ratio of α-acids to β-acids, a low amount of hard resins and minor traces of triglycerides, waxes, chlorophylls and inorganic salts [12]. Different temperature and pressure profiles were tested because they influence the yield, the composition of bitter compounds, and the presence of volatile components. It has been observed

that parameters of 40°C and 200,000 hPa were the best conditions for the extraction of bitter compounds and volatiles [11].

Hop varieties:

First of all hop varieties have traditionally been classified in bittering hops and aroma hops. For the brewer it is an important choice which hop is needed for the different beer styles. Related to the content of α-acids and hop oils the classification was done. The bittering hop varieties are divided into three groups, depending on the α-acid content. The groups are bittering hops, high alpha and super high alpha. For the aroma hop varieties two groups were established. The fine aroma hops and aroma hops. Table 3 gives an overview of the several varieties of bittering and aroma hops [13].

Table 3: Overview of different hop varieties [13]

Bittering hop	Aroma hop
Hallertauer Magnum	Hallertauer Perle
Hallertauer Taurus	Hallertauer Tradition
Herkules	Spalter Select
Galena	Hallertauer Mittelfrüh
Nugget	Hersbruck Hersbrucker
Millennium	Tettnang Tettnanger
CTZ (Columbus, Tomahawk, Zeus)	Saaz
	Cascade

Another hop variety which became more popular in the hop and brewing industry are the “flavour hops”. These kinds of hops are used to give bold tastes as well as distinct aroma and flavour characteristics to beer. Introduction of new flavour profiles with tropical and fruity notes are ensured. The most important flavour hops from USA are Cascade, Simcoe, Centennial and Citra. In Australia flavour hops like Summer, Topaz, Galaxy, Ella and Vic Secret are used. Nelson Sauvin, Kazbek and Aramis are cultivated in New Zealand, Czech Republic and France [13].

1.3 Brewing process

After detailed description of all ingredients the whole brewing process will be explained in this chapter. In the brew house the raw materials are received and converted into cooled hopped wort and afterwards it is ready for fermentation process [2]. There are eight basic steps for the standard beer production. These are milling, mashing, wort separation, boiling, chilling, fermenting, conditioning, and packaging [1].

But as a very first step another operation has to be executed which is called malting. This step is not part of the brewing process since it is not done by the brewery but by a malt house. The aim is to prepare malt which enables the brewer to produce wort, containing a balance of amino acids, polypeptides, fermentable sugars and essential metabolites sufficient to support yeast growth. The three main phases of malting are steeping, germination and kilning [2].

1.3.1 Milling

Milling is the process where malt which consists of seeds of grain (usually barley) is crushed. Each seed involves a packet of starch and small amounts of sugar. The compartment is called endosperm which is surrounded by the aleurone layer (living cells), seed coat (made of waxy material) and a hull (woody material). With the aid of milling the endosperm is broken into small pieces. So the endosperm without hull and seed coat has plenty of contact with water during mashing. The procedure is done by a device called mill and crushed grain is called grist. Some breweries also use the grain bed as a filter medium to clarify the wort. So the wort flows through a bed of grist. Because of the production of fine dust during milling, the process has to be separated from the other brewing steps. This dust can burn or explode due to the large surface area. The dust also contains bacteria which have a bad effect on the beer. Furthermore it is unhealthy to breathe. Based on these problems the dust air has to be filtered before it can be released. Another way to eliminate the dust problems is to moisten the grain before milling [1].

1.3.2 Mashing

The next step in brewing is called mashing where the grist is treated with hot water. The aim of this process is to convert most of the starch into sugar that can be used

from yeast. A mash tun is the vessel where mashing occurs and the procedure takes about one hour. There are three important steps for mashing. These are gelatinization, liquefication and saccharification [1].

Gelatinization:

In the first phase the starch gelatinizes because it absorbs water and swells. The purpose of gelatinization is to move water into the starch granules and separate the sugar chains. Barley is prized for the brewing process because its starch gelatinizes at low temperatures (approximately 70°C) compared to other grains [1].

Liquefication:

In the second step liquefication occurs. Starch consists of long sugar chains, amylose with straight chains and amylopectin with branched chains. The starch molecule is not soluble in water. Liquefication breaks the long sugar chains into smaller pieces called dextrins, which are better soluble in water. With the aid of the enzyme α-amylase sugar units are spilt in the middle of a chain. Yeasts cannot use dextrins but this kind of smaller sugar chains provide other chain ends for the next enzyme to work on [1].

Saccharification:

The last step of mashing is called saccharification, where dextrins and starch are converted into sugar so that yeast can be used for fermentation. The enzyme β-amylase splits a two sugar molecule which is called maltose from the end of a starch chain or dextrin [1].

1.3.3 Wort Separation

After the process of mashing, the water which contains dextrins and fermentable sugars, have to be separated from spent grains (called draff). There are different methods for separation. In some breweries the solution of sweet wort including draff is pumped into another vessel which is called lauter tun. In this separation method the bed of grain is used as a filter itself. Wort runs through the grain bed until it is clear. Afterwards, hot water is sprayed on the grain bed to wash all the sugars out. This process is called sparging. Another method of separation is filtration, where the mash is pumped under control of pressure through porous polymer pads. The spent grains are kept back and are often used as cattle feed [1].

1.3.4 Wort Boiling

The wort boiling process is the least understood part in brewing due to the reactions that occur. Ancient brewers observed that boiled wort produced a robust and resistant beer. Due to that discovery the heating step was an essential phase in the brewing process [2]. The separated and clearly sweet wort is pumped to a vessel called kettle or copper. Hops are added to the vessel in different dosages during the boil. It contributes bitterness and flavour to the beer. Some breweries also add hops at the end of the boil or even after chilling, to get aroma compounds from the hops into the beer. For the boiling process heat in form of steam is used and the procedure takes about one hour at a temperature of 98°C. The outcome of boiling is the hopped wort [1].

The purposes of wort boiling are:

- Sterilisation of the wort (bacteria, wild yeasts and other microorganisms will be destroyed)
- Inactivation of any malt-derived enzymes (termination by coagulation)
- Concentration of the wort (removal of water via evaporation)
- Finishing of chemical reactions that started during mashing (as a result lowering of pH)
- Coagulation of proteins and tannins from grain (they clump together and can be removed)
- Elimination of unwanted volatile compounds (e.g. dimethyl sulphide, volatile substance that is formed from precursors in malt, give a cooked vegetable flavour to beer; they are removed with the steam)
- Improvement of wort colour (via oxidation of polyphenols and interaction of carbohydrates and nitrogen containing compounds)
- Extraction of essential oils and polyphenols from hops
- Bittering the wort (because of isomerisation reactions of hop resins) [1], [2].

After boiling the hot hopped wort has to be separated from hop debris and hot trub which is a mixture of proteins, polyphenols, hop acids and carbohydrates [24]. This process occurs in a device called whirlpool. Proteins coagulate by heat and are sedimenting as hot trub particles [2].

1.3.5 Chilling

Before hopped wort is ready for the fermentation process, the liquid must be cooled down. The cooling takes place in a device called heat exchanger and brings the temperature from boiling (100°C) to fermentation temperature (7-22°C). Rapid cooling should be done because it prevents flavour defects. Also proteins coagulate during wort cooling (cold break) and are removed before the cold hopped wort is used for fermentation [1].

1.3.6 Fermentation

After chilling and aeration, which results in cold hopped wort, the product is transferred into a vessel, called fermenter, where the fermentation process occurs. Then the selected yeast strain is added to the fermenter and the sugars from the wort are converted into alcohol and carbon dioxide. This process is called pitching [1].

The sugar uptake of yeast has a general pattern (sucrose-glucose-fructose-maltose-maltotriose) [2]. Sucrose which is a dissacharide of glucose and fructose is hydrolysed by the enzyme invertase outside the cell. The sugars are transported into the cytoplasma for further processing. However, the major wort sugars maltose and maltotriose are converted into glucose by α-glucosidase inside the cell [4]. The uptake of wort sugars is regulated by size of molecule, the concentration of sugar in wort and the availability of enzyme systems required for metabolism. Figure 8 shows an overview of the sugar uptake from wort by yeast [2].

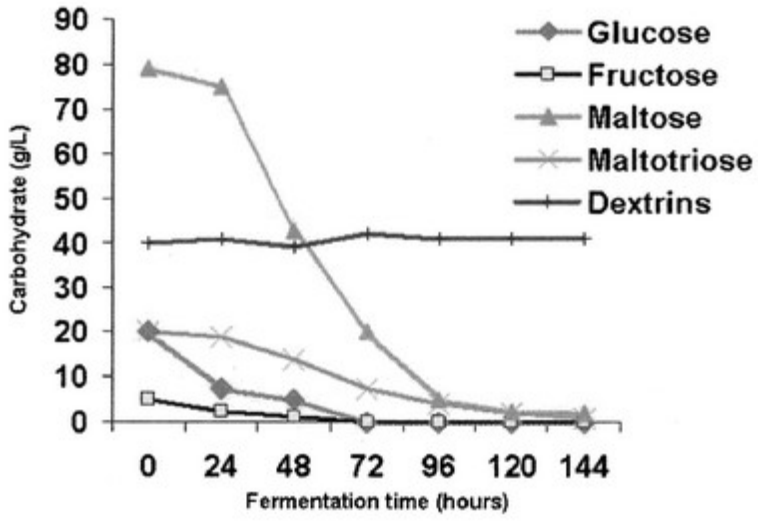

Figure 8: Diagram of sugar uptake by yeast [2]

In the first 24 hours yeast consumes major amounts of glucose and fructose which are present in low concentrations. Due to a constitutive glucose permease carrier in the cell membrane, the consumption of monosaccharides rapidly increases [2]. Glucose and fructose compete for the same permease in the cell wall, but glucose has a higher affinity to the permease and is transported first. Afterwards if glucose is mostly depleted, fructose can pass [4]. After 24 hours maltose – which is the major sugar component in wort and present in high concentrations (50-60 %) – is used by the yeast. In the lag phase (first 24 hours) an active transport system has to be synthesized to transfer maltose. The transport carrier is called maltose permease. Maltotriose is at least fermented if maltose concentration is low to permit the induction of maltotriose permease. Pentose sugars and dextrins which include maltotetraose and other starch breakdown products are not fermented from yeast [2].

The fermenter is equipped with a one-way valve which regulates the outflow of the produced gas. The fermentation of sugars to alcohol (fig. 9) is an anaerobic reaction [1].

$$C_6H_{12}O_6(glucose) \xrightarrow{yeast} 2\,CH_3CH_2OH\,(ethanol) + 2\,CO_2 + energy$$

Figure 9: Equation of anaerobic alcoholic fermentation [1]

The fermentable sugar glucose is converted by the yeast into ethanol and carbon dioxide. The aim of yeast is to get energy in a form which the cells can use for their life processes. Ethanol and carbon dioxide are waste products for yeast. The fermentation reaction itself does not consume oxygen. Oxygen is only needed by the yeast strains for growth and reproduction [1]. In an initial stage of the fermentation, sufficient oxygen supply which is dissolved in the wort is important for the yeast. Yeasts need oxygen for rapid cell growth but the oxygen regulation should be monitored because of aerobic respiration. It means, if there is enough oxygen the yeast converts the sugar into water, carbon dioxide and biomass [2].

1.3.7 Conditioning

Finished fermented beer from the fermenter is called green beer. The beer has to be in contact with yeast for a while to achieve a mature flavour and to get clarified. The yeast cells sediment to the bottom of the vessel slowly. For ale beer styles the conditioning lasts a few days. For lager beer types some weeks for conditioning should be given. The last step of conditioning is a filtration or centrifugation to eliminate the yeast and other particles that did not sediment. Filtration is executed with the aid of diatomaceous earth (Kieselguhr). The beer is pumped through the bed of powdered highly porous rock and becomes clear. Due to the spinning bowls in the centrifuge the particles in fermented green beer are separated. Some breweries skip the conditioning step and clarification takes place in the bottle or cask after packaging [1].

1.3.8 Packaging

The purpose in the packaging step is to introduce carbon dioxide for the characteristic beer foam. The most common method is called forced carbonation where carbon dioxide is added with pressure to the bottle, casks, cans or kegs. In general it is important to exclude oxygen because it makes the beer stale. Also the glass bottles should have a brown colour to exclude sunlight. Otherwise the beer gets a flavour defect which is called lightstruck. It results in a skunky smell based on the reactions of hop products. Other processes in packaging which affect the beer flavour eliminate or kill microbes to stabilize the beer. One method is the heat treatment of consumer-size packages which is called pasteurization. Also microbial filtration is a possibility for more beer stability [1].

After description of every single step in the brewing process, figure 10 shows an overview of it.

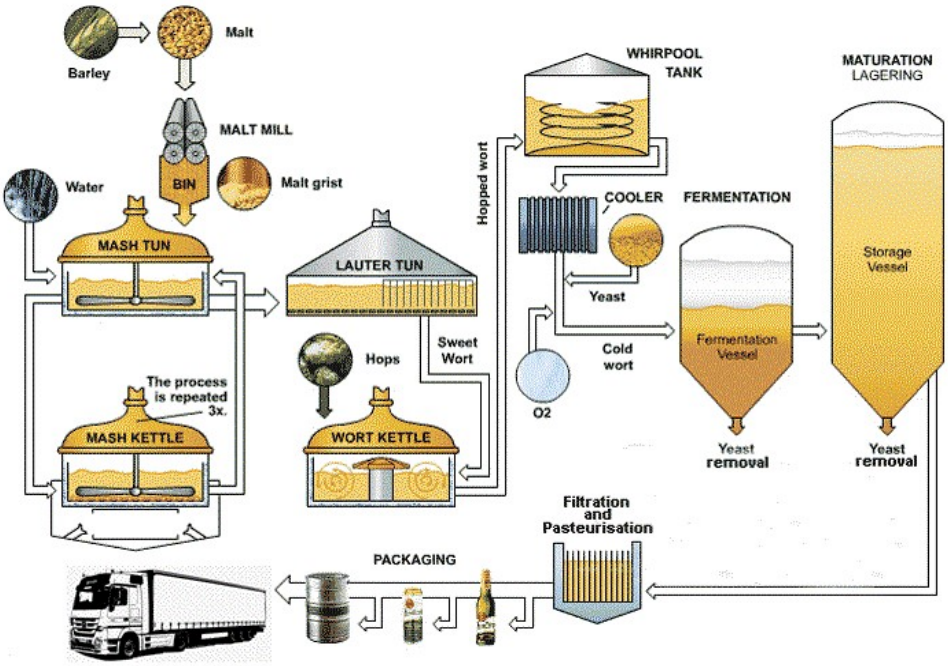

Figure 10: Overview of a brewing process [16]

1.4 Hop polyphenols

The most important chemical components of hops for this master thesis are the hop polyphenols. In this chapter they are described in very detail and were analysed in the practical part.

Above from table 2 it is apparent that the hop polyphenols comprise up to 4 % of the total weight of dried hop cones. The content of polyphenols in hops depends on variety. Some scientists have shown that certain polyphenols are unique to a specific hop variety. Several factors influence the composition of hop polyphenols which are variety, cultivation, harvesting time and technique and the degree of aging. It has been observed that aged hops contain higher amounts of polyphenols than fresh hops. Another study showed that aroma hops contain higher amounts of low molecular weight polyphenols than bitter hops.

The fraction of hop polyphenols is a wide spread class of compounds consisting of multiple phenol units and share a common structure. The whole heterogeneous fractions of hop polyphenols exist of an aromatic ring with at least two hydroxyl groups.
Over 100 compounds of hop polyphenols were identified. The hop polyphenols can be divided into four major groups (fig. 11) [13].

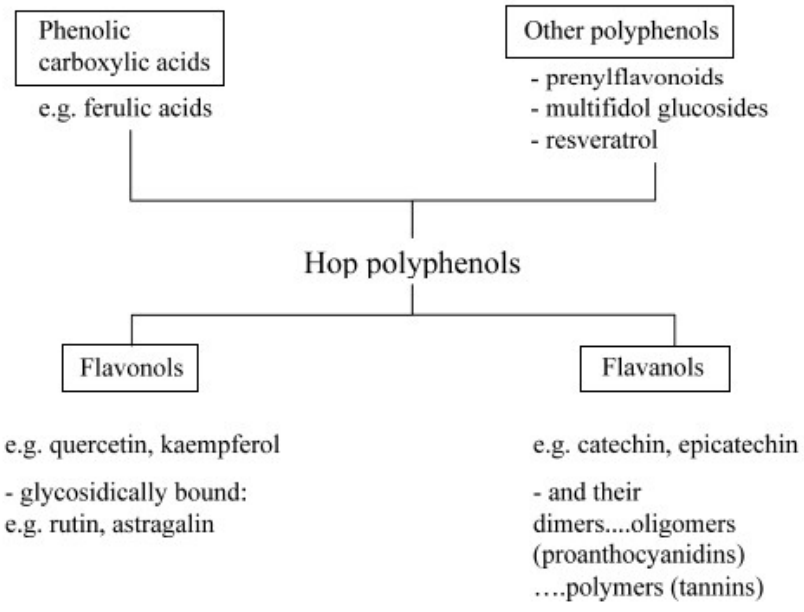

Figure 11: Classification of hop polyphenols [17]

These are flavonols [e.g. kaempferol (fig. 12) and quercetin (fig. 13)], flavanols [e.g. catechin (fig. 14) and epicatechin (fig. 15)], phenolic carboxylic acids (e.g. ferulic acid) and other polyphenols. Most of these polyphenols are widely spread in plant kingdom, compared to other polyphenols which are found only in a few plants [17].

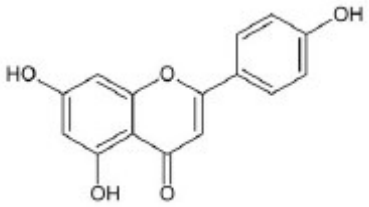

Figure 12: Kaempferol [19]

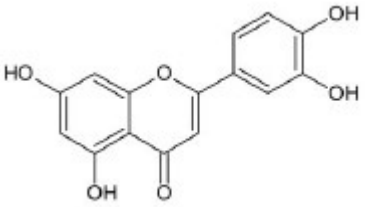

Figure 13: Quercetin [19]

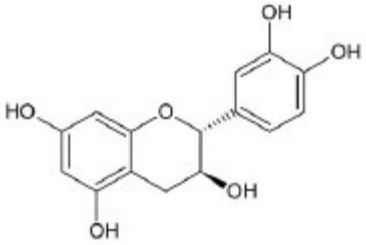

Figure 14: Catechin [19]

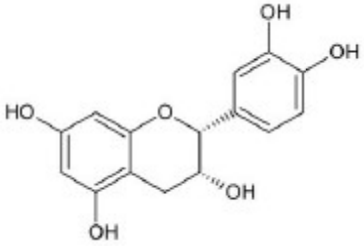

Figure 15: Epicatechin [19]

The two structures of flavonols (quercetin and kaempferol) do not occur free in hops but they are glycosylated. Quercetin is more hydrophilic than kaempferol due to the second hydroxyl group. It has a higher antioxidative potential compared to kaempferol which is more present in aroma hops. Hop polyphenols have different solubility. Catechin and epicatechin from the flavanol group are water soluble substances. These two chemical structures are the monomeric building blocks of dimers, trimers and higher polymeric molecules. Substances which consist of up to eight flavanol monomer units are called proanthocyanidins (oligomers) and are soluble in wort and beer. Molecules with a higher number of monomers are identified as tannins (polymers) which are also soluble.

The concentrations of the different hop polyphenols are various. Table 4 shows an overview about the average amounts of the four major groups of hop polyphenols. The content of individual kinds of polyphenols is also shown [13].

Table 4: Concentrations of hop polyphenols [17]

Component	Content (%)
Total	
Flavanols	7
Prenylflavanoids	1.3
Flavonols	0.5
Phenolic carboxylic acids	0.05
Individual	
Xanthohumol	1.1
Co-multifidol glucoside	0.3
Catechin	0.2
Quercetin	0.2
Kaempferol	0.1
Desmethylxanthohumol	0.1
6-Prenylnaringenin	0.03
8-Prenylnaringenin	0.01
Ferulic acid	0.01
Resveratrol	<0.01

Prenylflavonoids are categorized to the main group of “other polyphenolic compounds” from figure 11. Xanthohumol, a prenylated chalcone, is the major compound of this group. The phenolic compound and smaller amounts of desmethylxanthohumol occur in the lupulin glands. The prenylated chalcones are precursors of their isomeric flavanones and convert during the brewing process. Desmethylxanthohumol (fig. 16) is a mixture of 6-prenylnaringenin (fig. 17) and 8-prenylnaringenin (fig. 18) [13]. 8-prenylnaringenin was identified as the most important phytoestrogen [21]. Phytoestrogens are substances from plants with oestrogenic effects. It was observed that they resemble the effect of sex hormones and induce oestrus. The content of the phytoestrogen 8-prenylnaringenin in different beer types is various. It ranges from <1.6 µg/l in non-alcoholic beers to 138.5 µg/l in stouts [23].

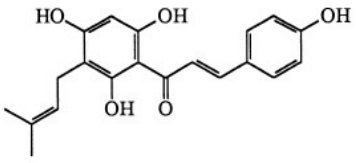

Figure 16: Desmethylxanthohumol [22]

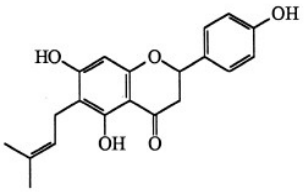

Figure 17: 6-Prenylnaringenin [22]

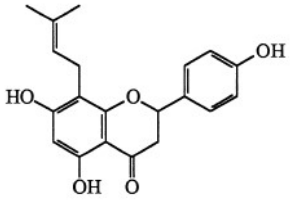

Figure 18: 8-Prenylnaringenin [22]

1.4.1 Polyphenols in beer

Phenolic constituents in wort originate mostly from malt (70-80 %). Only 20-30 % originates from the hop material. Polyphenols of malt undergo modifications during malting and brewing, so they are less characterised than hop polyphenols [3].

Hop polyphenols contribute not only to flavour and bitterness in the brewing process. They also interact with protein and precipitate, forming a non-biological haze, which limits the shelf-life of bottled beer [13]. Due to chemical reactions between proteins (which are rich in the amino acid prolin) from malt and haze active polyphenols originated from malt and hops, haze develops. Oxidation and polymerisation processes of polyphenols in beer are the reason for the chemical interactions between the two substances. Freshly filtered and bottled beer has no indication of haze because no oxidation and polymerisation occurs. The haze does not affect flavour, aroma or mouthfeel of the beer. It is only an aesthetic effect because ales and lager beer styles are characterised for brilliantly clearness [24]. Due to this problem some scientists have searched for a method to remove the phenols from beer. The haze active polyphenols were eliminated by adsorption on nylon and polyvinylpolypyrrolidone (PVPP) [27].

Another important function from polyphenols in beer is their action as strong antioxidants. The polyphenols scavenge free radicals by inhibiting the lipoxygenases or acting as metal chelators [13]. The reducing character of some polyphenols can react with oxygen to protect other beer constituents. Hop polyphenols in oxidised form, catalyse the reaction of alcohols and fatty acids to aldehydes and prevent beer from an aging taste [18]. The natural antioxidants consist of low-molecular-weight and higher-molecular-weight polyphenols. The last one affect the colour of beer and haze formation. The insoluble precipitates are products of proanthocyanidins or tannins with proteins. The low-molecular polyphenols protect beer against oxidation and improve the taste stability [13].

1.4.2 Xanthohumol

A yellow compound which is named “Xanthohumol” (XH) is the most popular prenylflavonoid in hops. It is also the major component which was measured in this study followed by the isomeric form called “Isoxanthohumol” (IXH).

Recently, XH has received much attention because of its benefit for health and the wide spectrum of positive properties. XH (fig. 19) is secreted with bitter acids, hop resins and essential oils in high quantities in the female inflorescence of the lupulin glands in comparison to the male hop plant which contains less lupulin glands and by this reason also less content of XH [20]. In the hop resins XH is the major prenylflavonoid and it occurs at 10-100 fold higher concentrations compared to the other 13 related chalcones [26].

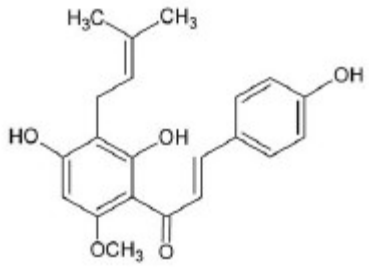

Figure 19: Xanthohumol [19]

The concentration of the prenylated chalcone XH is up to 1.5 % of the total weight in dried hop cones. Because of its positive effects on human health, brewers have searched for a method to produce XH-enriched beers. They bounced on two main problems which are the low solubility of XH and the isomerisation to its isomeric flavanone IXH. A further disadvantage is the loss of insoluble XH during wort production where it is removed together with the trub after boiling [25]. During fermentation and filtration steps in the brewing process, the XH content decreases again. Beer stabilization substances like PVPP (Polyvinylpolypyrrolidone) also reduce XH amounts in beer. Due to these problems there is a low general XH content in commercial beers. The concentration of XH in conventional pale beers is approximately 0.15 mg/l. The higher IXH content ranges from 0.04 to 3.44 mg/l [26].

The content of XH in different hop species is various. The highest XH content was found in the variety “Taurus” which is cultivated in the Hallertau region. The ratio from XH to α-acids is relatively stable and therefore the XH input could be derived from α-

acid dosage [28]. It means a high content of α-acids provide also a high content of XH [29]. Hops from other countries like England, USA, New Zealand and South Africa contain a low XH content. It has been observed that the ratio of XH to α-acids is also low. There is a consideration that the production of XH is promoted in Central Europe. Hop species like Perle, Nothern Brewer, and Magnum which are spread in Germany are also cultivated in USA. Comparison of the hops from the different countries indicates that the Hallertau region has an increased XH content and a better α-acid ratio. It is supposed that the growing area of Hallertau has a moderate climate which promotes the production of XH and low-molecular polyphenols [28].

The common substance XH has a broad-spectrum of biological effects which are beneficial for human health. The functions are wide spread and range from anti-carcinogenic functions to antioxidative effects [20]. The properties of the cancer chemopreventive agent are induced by inhibition of procarcinogens, activation of carcinogen-detoxyifying enzymes and deactivation of tumour growth at early stage [21]. Some tests which were executed in vitro represent that XH may have therapeutic utilization as cancer prevention agent for treatment of breast and prostate cancer. Other possible utilities of XH are the treatment of osteoporosis, alleviation of hot flashes, inhibition of HIV-1 and effect as antioxidant for treatment of atherosclerosis [26].

Cancer related studies show that XH and 8-Prenylnaringenin prevent the metabolic activation of IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) which is a procarinogen that occurs in cooked meat. The inhibition of IQ takes place due to the inhibition of enzymes from cytochrome P450 [21].

Increased production of hormone like mediator such as prostaglandin is associated with uncontrolled growth and spreading of tumour cells. Anti-inflammatory activity of XH was researched by inhibition of cyclooxygenase enzymes (COX-1 and COX-2) which are important for the synthesis of prostaglandin [21].

1.4.3 Isoxanthohumol

The isomeric molecule of XH is the flavanone IXH (fig. 20) which is formed during wort boiling due to isomerisation reactions. The content of formed IXH correlates with the amount of XH which is present in hops. Due to the chemical isomerisation, IXH is the most abundant flavonoid in hopped beers [22]. The solubility of IXH is higher compared to XH [25]. IXH has also beneficial effects on human health but less effective than XH. The disadvantage could eventually be compensated by increasing the concentrations of IXH in beer. It could also be possible that IXH has other new positive effects which will be discovered by pharmaceutical investigations compared to XH. Recent studies showed that the anti-inflammatory character of IXH and its "anti-aging" property were evaluated higher than that of XH [26].

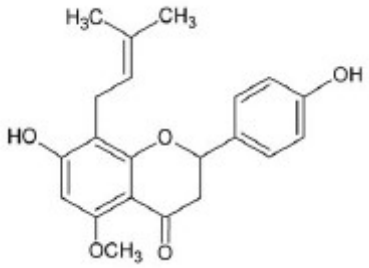

Figure 20: Isoxanthohumol [19]

The stability of IXH is affected in the same way as XH by PVPP which reduces the content by about 30 %. [30]. Other materials which decrease the XH and IXH content are diatomaceous earth, tannic acid, polyamide 6 and BEERPAP (diatomaceous earth which is covered with a layer of polyamide 6). Diatomaceous earth is used as a filtration material to remove yeast cells from beer and a significant portion of XH is linked to the yeast surface. Polyamide substances have a high affinity for XH and eliminate polyphenols from beer. The addition of tannic acid is used to remove haze-active proteins [25].

During the brewing process many chemical reactions take place, especially at wort boiling. An important step which influences the XH content intensively is the isomerisation reaction (fig. 21) [2]. The free hydroxyl group of XH and conditions of high temperatures during wort boiling convert the chalcone to its isomeric flavanone IXH. Thereby a low XH and a high IXH content results in final beer [29].

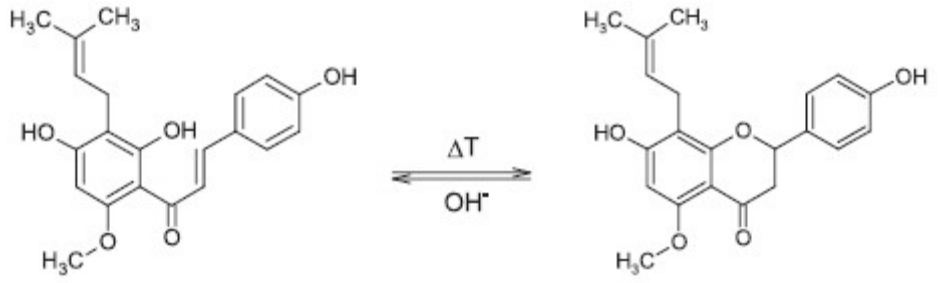

Figure 21: Isomerisation reaction [25]

The isomerisation reaction also occurs in hops but not yet in the lupulin glands. Chalcone isomerase (CI) is the enzyme which converts the chalcones to its corresponding flavanones through ring closure. Without the enzyme of chalcone isomerase the prenylated chalcones would isomerise chemically. XH has one free hydroxyl group for cyclisation to yield the prenylated flavanone IXH. Desmethylxanthohumol exists of two hydroxyl groups available for ring closure to yield the two flavanones 6-prenylnaringenin and 8-prenylnaringenin. Thereby the low content of flavanones could be explained compared to the chalcones XH and Desmethylxanthohumol. Isomerisation is promoted by alkali treatment during extraction and purification process, thereby yielding higher amounts of prenylflavanones. By this reason scientists consider that the substance 8-prenylnaringenin is an artefact and not a real compound in hops [31].

As already mentioned due to the positive effects of XH on human health, many brewers try to find a method for the production of beer with a high XH content. Some scientists tried to enrich XH in beer.

One method is to use dark roasted malts which have a positive effect on the isomerisation reaction of XH. The prenylated chalcone produce a stable complex with a substance from the roasted malt (probably melanoidins). This process inhibits the isomerisation reaction of XH to IXH and a higher XH content is gained [25].

Another option for XH enrichment in beer is the application of “XAN” technology. In this method the hop dosage and temperature of wort cooling are the important steps. The addition of hop is carried out by a XH enriched hop product, which is added 5 minutes before the end of boiling. Afterwards the wort must be cooled down to less

than 80°C to inhibit the isomerisation reaction [26]. This technology facilitates a XH concentration of 1 mg/l in pale beers (compared to the usually XH concentration of 0.1 mg/l). In general, the highest rate of isomerisation was observed at 100°C. The optimal temperature for hop addition to get a high XH amount is about 60°C or lower.

A unique process to enrich XH in beer is the addition of an enrichment solution which consists of XH extract, hot wort and yeast. This mixture is added to the beer before it is filled into bottles and kegs. This process enables for different types of beers a XH concentration of about 2.5 mg/l [25].

Although there are some methods to get XH-enriched beers, not every method is applicable for brewing according to the traditional German Beer Purity Law, which only permits the addition of hop to hot wort [26].

2. Materials and Methods

2.1 Materials

2.1.1 Samples

The analysis of XH and IXH was done at different compounds of beer which are explained below.

2.1.1.1 Hop pellets

For the analysis of XH and IXH in hop pellets, different hop samples (fig. 22) were provided from a brewery in Carinthia (Villacher Brauerei). The hop pellets Cascade, Citra and Topaz are classified into aroma hops. They have a fruity note and are used for the production of aroma beer styles. The Aurora hops has a bitter character and is used for commercial lager beer types like “Villacher”.

Figure 22: Hop pellets of different species

2.1.1.2 Beer samples

Beer samples were provided from a brewing course (brau campus, KFU). XH and IXH content were measured in different production steps (5 minutes after hop addition, hop boiling finish and filling).

2.1.1.3 Wort samples

Samples of wort boiling for determination of hop polyphenols (XH, IXH) and the sugars maltose and glucose were also provided from the Villacher brewery. The

samples were taken before boiling (wort without hops), after hop addition of 5, 15, 25, 35, 44, 50 and 60 minutes boiling time.

2.1.2 Chemicals

Several chemicals and reagents were used for the analysis of XH, IXH and the brewing sugar maltose and glucose. Table 5 shows an overview of the utilized standard solutions and table 6 lists the chemicals which were used as eluent for HPLC and TLC.

Table 5: Used chemicals for standard measurements

Chemicals	Characteristics	Producer
Xanthohumol ($C_{21}H_{22}O_5$)	MW: 354.40 g/mol	Carl Roth GmbH + Co. KG
Isoxanthohumol ($C_{21}H_{22}O_5$)	MW: 354.40 g/mol	Carl Roth GmbH + Co. KG
Maltose (Monohydrate) crystal.($C_{12}H_{22}O_{11} * H_2O$)	MW: 360.32 g/mol	E. Merck, Darmstadt
D (+) Glucose (Monohydrate) ($C_6H_{12}O_6 * H_2O$)	MW: 198.17 g/mol	E. Merck, Darmstadt

Table 6: Used eluent for HPLC and TLC

Chemicals	Characteristics	Producer
Methanol (CH_3OH)	MW: 32.04 g/mol	Chem-Lab NV
Acetic Acid (CH_3COOH)	MW: 60.05 g/mol	J.T. Baker
96 % Sulphuric Acid (H_2SO_4)	MW: 98.08 g/mol	E. Merck, Darmstadt
Toluene (C_7H_8)	MW: 92.14 g/mol	Carl Roth GmbH
1,4-Dioxane ($C_4H_8O_2$)	MW: 88.11 g/mol	Carl Roth GmbH
Distilled Water (H_2O)	MW: 18.00 g/mol	Millipore System

2.1.3 Equipment

The hop polyphenols (XH, IXH), maltose and glucose were analysed by High Pressure Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) (tab. 7). Each of the three HPLC´s has a different detector for analysing several parameters in hops, wort, and beer.

HPTLC plates (20 x 10 cm silica gel 60, from E. Merck) were used as stationary phase for determination of XH and IXH in different hops via TLC. A chromatographic chamber with a lid was also needed. The TLC was coupled with an automatic TLC sampler 4 from “Camag”, which sprayed the samples on the HPTLC plates and the detection was done with the aid of a TLC Scanner 3 which was also provided from “Camag”.

Table 7: Equipment for analysis

Equipment	Software	Producer
HPLC	HP-1100, UV-FLD	Hewlett Packard Series 1100
TLC	WinCATS Planar Chromatography Manager	Camag
HPLC	Chem Station Agilent Technologies, refractive Index Detector	Hewlett Packard Series 1100
HPLC	Chem Station Agilent Technologies, DAD	Hewlett Packard Series 1100

2.2 Methods

2.2.1 Sample preparation

Before analysis of XH, IXH, maltose and glucose in different hops, in wort and in beer samples, a sample preparation had to be performed. The sample must be prepared in a shape which is suitable for the used chromatographic method. The techniques for preparation range from easy dilutions till complex multi-step extractions. The aim of sample preparation is to create a preliminary chromatographic clean-up and pre-concentration of the sample extract [36].

2.2.1.1 Hops

Experiment a) Determination of XH and IXH in different hop pellets

Hop polyphenols were measured via HPLC and TLC in different hop species. Approximately 30 mg of hop pellets were pulverized with the aid of a mortar. Afterwards 2 ml MeOH were added and the Eppendorf reaction tube which includes those two components was given into an ultrasonic bath for 10 minutes. Subsequently the sample was centrifuged for 10 minutes at 14,000 rpm and 10°C. After centrifugation 1 ml from the supernatant was collected and measured via HPLC and TLC. For the measurement via TLC an eluent of toluene-dioxane-acetic acid in a ratio of 77+20+3 was used.

Experiment b) Behaviour of XH and IXH during isomerisation reaction

Due to isomerisation reaction in hops the XH and IXH content varies. For the analysis of hop polyphenols at different temperatures and time, a thermocycler was used.

- TLC:

30 mg of hop pellets were weighed in an Eppendorf reaction tube and 950 µl H_2O were added. In this way 7 samples were generated. Then all samples were given into an ultrasonic bath for 10 minutes. Afterwards the homogenous solutions were given onto a thermocycler with 95°C. At the starting point (0 min) and after every 10 minutes (up to 1 hour), a sample was taken from the thermocycler and 950 µl MeOH were added. In the end all 7 samples (0-60 min) were centrifuged for 10 minutes at 14,000 rpm and 10°C. 1 ml of the supernatant was collected and 1 µl was sprayed onto a silica gel plate. Afterwards the plate was put into a chromatographic chamber with a lid and an eluent of toluene-dioxane-acetic acid (49+49+1) was used for development. The development time was about 20 minutes. After development the plate was dried in the dark for 10 minutes. In the end the HPTLC plate was scanned for XH at 368 nm and for IXH at 290 nm.

- HPLC:

The procedure of hop sample treatment for HPLC was not exactly the same as for TLC. 30 mg of hop pellets were weighed in a reaction tube and 600 µl H_2O were added. In this way 10 samples were generated. Afterwards all samples were given

into an ultrasonic bath for 5 minutes and following onto the thermocycler with 95°C. After that every 10 minutes one sample was taken from the heat plate and 600 µl MeOH were added. Then the reaction tubes were placed on ice to stop the isomerisation reaction. In the end the samples were centrifuged (10 min, 14,000 rpm and 10°C) and 500 µl from the supernatant were taken and measured via HPLC at several wavelengths for XH (368 nm) and IXH (290 nm). Due to the detection of XH and IXH at different wavelengths, a method for HPLC measurement was established. IXH elutes at 3.8 minutes and XH at 9.1 minutes. Therefore the wavelength of the VWD-detector was changed at 6.5 minutes from 290 nm (for IXH) to 368 nm (for XH).

2.2.1.2 Beer and wort

Experiment c) Determination of XH and IXH in beer and wort

For the analysis of polyphenols in beer and wort, a sample preparation was executed. The substances of interest (XH and IXH) had to be isolated from complex biological matrices [32]. With the aid of solid phase extraction (SPE) the analytes could be collected through a C-18 column (Bond Elut, Varian). The cartridge in the column consists of a 500 mg frit, which is covered with hydrophobic bonded silica sorbents. Different solvents were used for the washing and elution steps. Figure 23 shows the general workflow for sample preparation of wort or beer samples to yield the desired polyphenols.

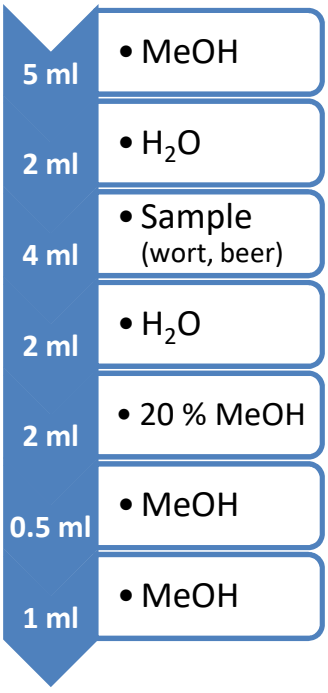

Figure 23: Scheme of sample preparation

The cartridge was conditioned with 5 ml MeOH and 2 ml H_2O. Afterwards 4 ml of the desired sample were filled and subsequently another washing step followed to remove salts and proteins from the column. Then 2 ml of 20 % MeOH and 0.5 ml MeOH were used for further washing steps. Previously it was analysed at which MeOH fraction XH and IXH elute. In the last step 1 ml MeOH was used to elute hop polyphenols from the column. Figure 24 shows SPE columns at different stages during preparation process. The resulting solution which contains the polyphenols had a yellow colour.

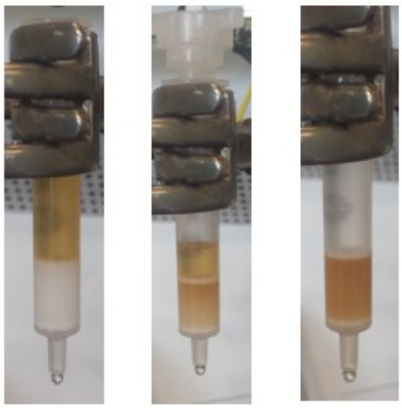

Figure 24: Solid phase extraction (SPE)

The following described two steps (which were not noted so far) are important for the whole procedure of sample preparation. First, before beer or wort is filled into the SPE-cartridge, the sample has to be prepared specially. The solid particles which precipitated from frozen beer or wort samples were homogenised in an ultrasonic bath for 5 minutes. The second important step is the centrifugation of the extracted samples (5 minutes at 14,000 rpm, 10°C) because also from the yellow solution, solid particles fell out.

2.2.1.3 Sugar

Experiment d) Determination of maltose and glucose during wort boiling

Sample preparation for the determination of maltose and glucose in wort samples were done by simple dilutions. 1 ml from each wort sample was homogenised by ultrasound for 5 minutes. Afterwards the samples were centrifuged at same conditions as before. In the end a 1:40 dilution (25 µl from supernatant + 975 µl distilled water) was made and measured via HPLC with the aid of a numeric

refractive index detector (nRID). Conditions and parameters of the HPLC measurement are shown in table 9.

2.2.2 Principle of HPLC

HPLC enables rapid separation of different substances which are non-volatile and thermal instable. The chromatographic method is a physical separation where desired substances are spread between two phases. The construction of a HPLC consists of a reservoir for eluent, an injector, a pump, a separating column and a detector. The separating column includes a solid stationary phase and the eluent represents the mobile phase. The soluble sample is pumped via pressure through the column. Separation occurs via gradient elution or isocratic elution. The composition of the eluent for isocratic elution is always the same and does not change during whole analysis compared to gradient elution which modifies during separation [33]. Gradient elution has the advantage that the analysis time could be reduced by different retention times of specific substances [34].

Reversed phase chromatography (RP-HPLC) was executed for the measurements of XH and IXH in different hop, beer and wort samples. The RP-HPLC consists of a non-polar stationary phase and a polar mobile phase [33]. The elution of RP-HPLC depends on hydrophobic interactions between the stationary column and the compounds from the sample. If there are strong interactions between these two phases, the substance stays in the column for a longer time. If there are weak interactions, the substances reach the detector earlier via mobile phase [34].

There are several detectors for the determination of unknown compounds by HPLC. The detector recognizes changes in the mobile phase and detects it. These alterations are converted into an electric signal and are analysed through a data system. Examples for UV-Vis detectors are diode array detector (registering detectors) and VWD-detector (variable wavelength detectors). Also fluorescence detectors are commonly used [35].

The differently separated substances could be identified in a chromatogram with the aid of retention times. Furthermore the concentration of a substance could be calculated by the area of the signal [34]. Table 8 gives an overview about the different conditions for the measurement with RP-HPLC and the aid of a VWD-

detector. The following graph (fig. 25) shows the gradient elution of methanol and 0.075 % acetic acid.

Table 8: Conditions of UV-HPLC for determination of XH and IXH in different hops, beer and wort samples

Approaches	Parameters
Injection volume	5 µl
Eluent	0-12 min: **60 %** Methanol and **40 %** 0.075 % Acetic Acid 12-13 min: **84 %** Methanol and **16 %** 0.075 % Acetic Acid 13-20 min: **100 %** Methanol
Flow rate	0.6 ml/min
Column	Kinetex® 5 µm EVO C18, 150 x 3.00 mm (Phenomenex)
Column temperature	25°C
Time	Stop time: 20 min, Post time: 4 min
Detection	VWD Detector: XH (368 nm), IXH (290 nm)

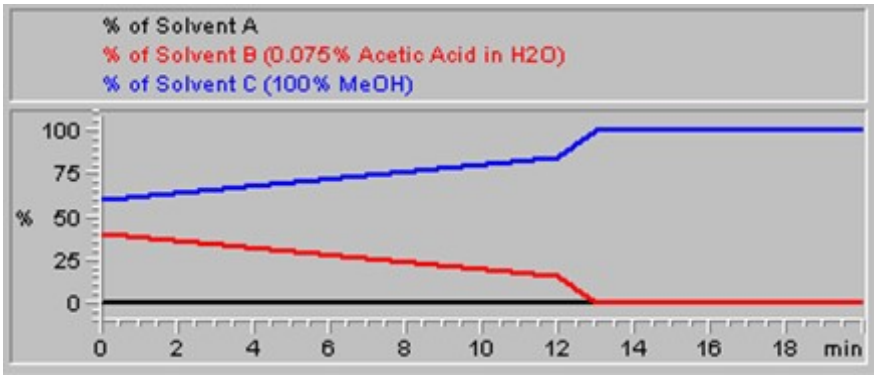

Figure 25: Gradient elution of UV-HPLC

Table 9: Conditions of nRID-HPLC for determination of maltose and glucose in wort

Approaches	Parameters
Injection volume	20 µl
Eluent	5 mM Sulphuric acid
Flow rate	0.6 ml/min
Column	HPLC Fast Acid Analysis, 100 x 7.80 mm (Bio Rad)
Optical unit temperature	35°C
Time	Stop time: 5 min,
Detection	numeric refractive index detector (nRID)

2.2.3 Principle of TLC

Thin layer chromatography was established for separation and semi-quantitative determination for individual components. It is a simple and rapid analytical technique. The most important advantages of TLC are the usage of a great variety from different stationary and mobile phases for the analytical procedure, as well as the ability to identify a wide variety of organic and inorganic substances [37]. The measurement performance of TLC is worse than from GC (gas chromatography) or HPLC because of problems which contribute to the poor reproducibility (including sample application, development, validation and undefined parameters). The precision and accuracy of this method have to be improved. Otherwise the chromatographic technique could not be sensitive enough for separation and determination processes [38].

3. Results

3.1 Thin Layer Chromatography

3.1.1 Standard calibration curve

At first a calibration curve of XH and IXH were established to determine the concentrations in different hop pellet species. A standard stock solution of XH (120 µg/ml) and IXH (320 µg/ml) was produced. Different concentrations were measured via TLC and the following calibration curves (fig. 26) were created. XH was measured in the concentration range from 6-24 µg/ml. For the last measurement (0.96 µg/ml) no signal was detected. IXH was measured in a concentration range from 4-16 µg/ml. Also the last measurement (0.64 µg/ml) produced no signal.

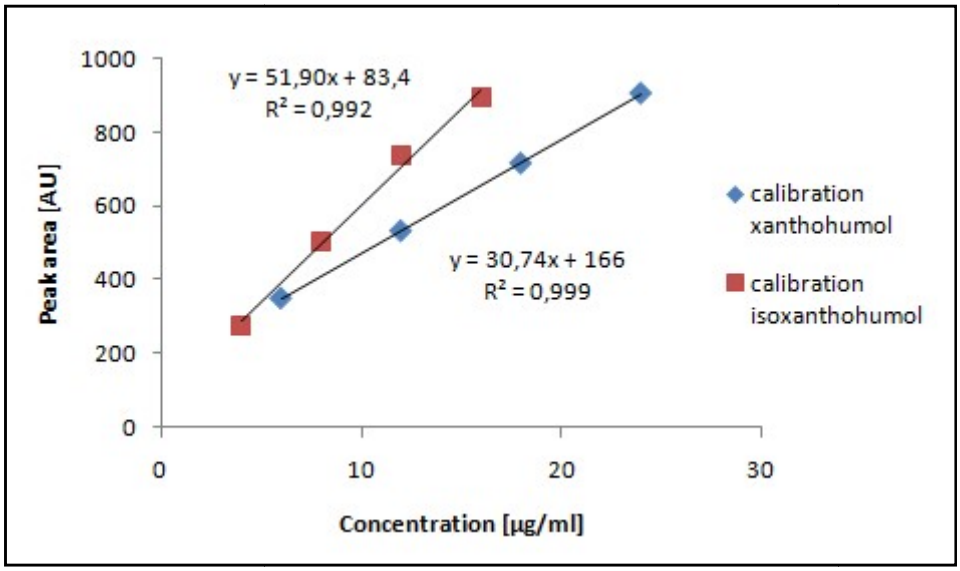

Figure 26: Calibration curves of XH and IXH. The concentration of XH and IXH are plotted over peak area, area units [AU]. The blue dots represent the XH concentrations and the red dots indicate the IXH concentrations. Next to the linear regressions the corresponding linear equations are indicated.

According to the measurements of a XH standard solution, which provides an R_f-value of 0.54, a signal could be detected. The IXH standard solution generated an R_f-value of 0.21.

Figure 27 shows a TLC chromatogram of a XH standard solution (24 µg/ml).

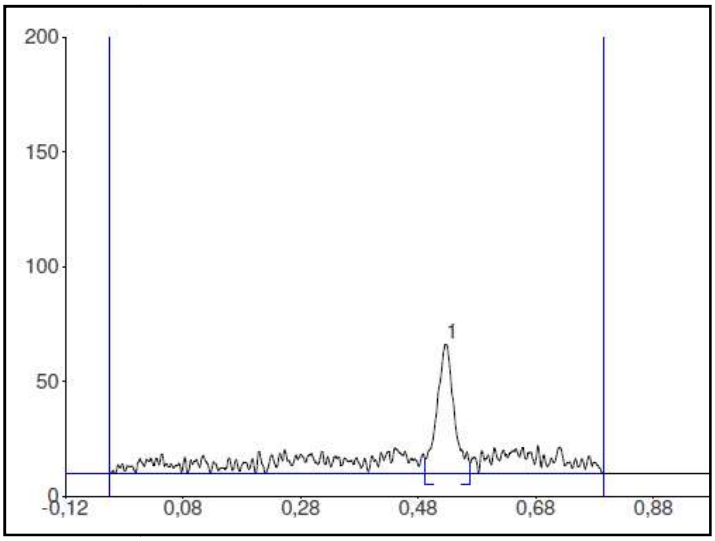

Figure 27: TLC chromatogram of XH. The peak shows the polyphenol XH. The diagram is plotted over the R_f-value. XH was scanned at 368 nm.

3.1.2 Experiment a) Determination of XH and IXH in different hop pellets

After development of calibration curves, the two hop polyphenols (XH and IXH) were measured in different hop pellet species via TLC. The following two chromatogram´s (fig. 28 and fig. 29) show the detected XH and IXH peak in different hop pellets.

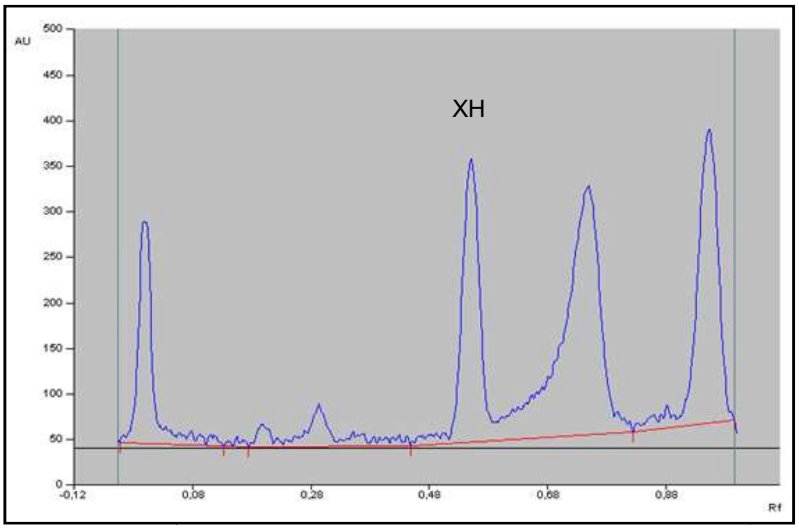

Figure 28: TLC chromatogram of XH in aurora hops. The measured peak has an R_f-value of 0.59 and a peak area of 6291.6 AU.

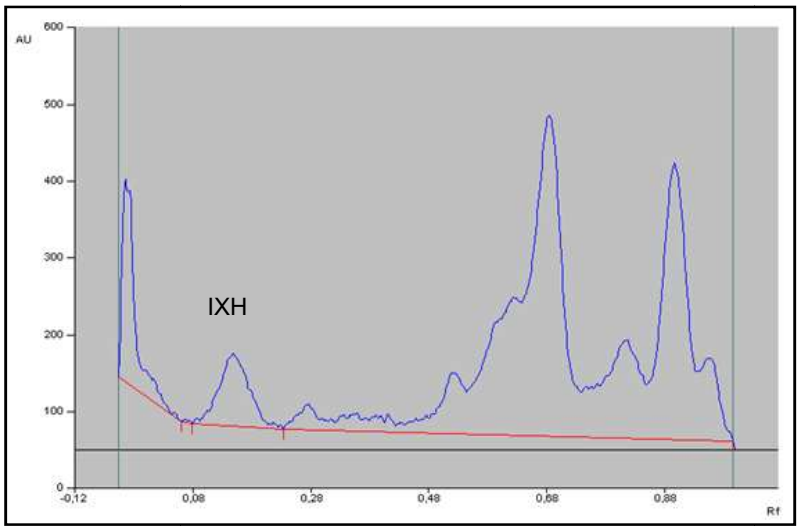

Figure 29: TLC chromatogram of IXH in topaz hops. The measured peak has an R_f-value of 0.19 and a peak area of 2417.2 AU.

With the aid of standard calibration curves the different concentrations of XH and IXH can be calculated (see table 10). The polyphenols were measured in four different hop samples. For the calculation of XH and IXH the conversion of the initial weight has to be included. The concentration units (µg/mg) result from the weighted sample of 30 mg which were dissolved with 2 ml MeOH. Therefore the measured peak areas were calculated with the aid of the linear equations from the calibration curve (in µg/ml) and afterwards the factor was multiplied by 2 ml and then divided by 30 mg.
It was determined that the hop pellet “Aurora” provides the highest XH and the lowest IXH content compared to the other hop species.

Table 10: Overview of the calculated concentrations of XH and IXH in four different hop pellet species

Hop sample	XH [µg/mg]	IXH [µg/mg]
Cascade	7.39	1.96
Citra	9.36	2.35
Topaz	10.3	2.86
Aurora	13.1	0.370

3.1.3 Experiment b) Behaviour of XH and IXH during isomerisation reaction

Due to the isomerisation reaction in hops during wort boiling, the conversion of XH to its isomeric form IXH was analysed. Therefore hop pellets (Aurora hops) were given on a thermocycler for different times. The thermocycler has a temperature of 95°C and every 10 minutes a sample was taken from the heating plate to determine the different concentrations of XH and IXH. After cooling at room temperature the samples were analysed via TLC. Figure 30 represents a chromatogram of XH which was measured under known conditions by TLC.

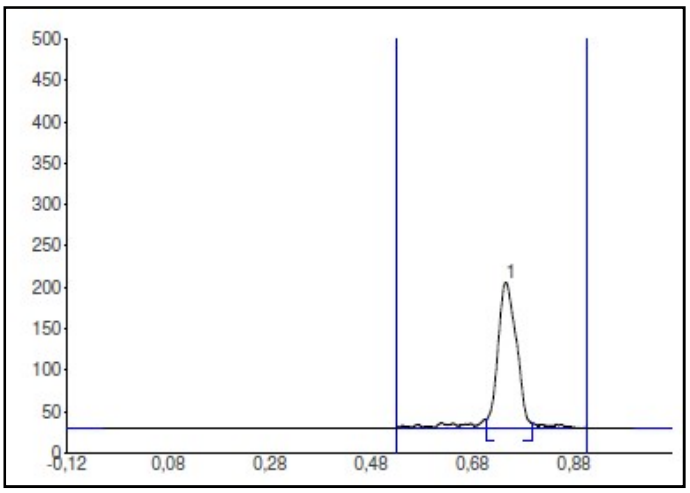

Figure 30: TLC chromatogram of XH at 50 minutes heating at 95°C. The x-axis represents the [R_f]-value and the y-axis exhibits the measured area units [AU].

During heating process (0-60 min) of hops it was observed that the XH content was increased in the first 10 minutes. After extraction of 10 minutes by heating at 95°C, the XH concentration was reduced. Table 11 represents the measured and calculated values for XH during heating. For the calculation of the concentration of XH in µg/mg, the conversion of the initial weight has to be included. It results from the weighed hop sample of 30 mg and the addition of 950 µl water and 950 µl MeOH.

Table 11: Summary of the measured units and calculated concentrations for XH during heating

Heating time [min]	R_f-value	Area Units [AU]	XH conc. [µg/mg]
0	0.80	7181.9	14.5
10	0.80	7724.3	15.6
20	0.80	7243.5	14.6
30	0.80	6762.9	13.6
40	0.79	5820.3	11.6
50	0.80	4999.9	9.96
60	0.81	3623.0	7.122

The values for IXH were analysed at the same conditions as for XH. The following chromatogram (fig. 31) represents the peak of IXH in hops during heating.

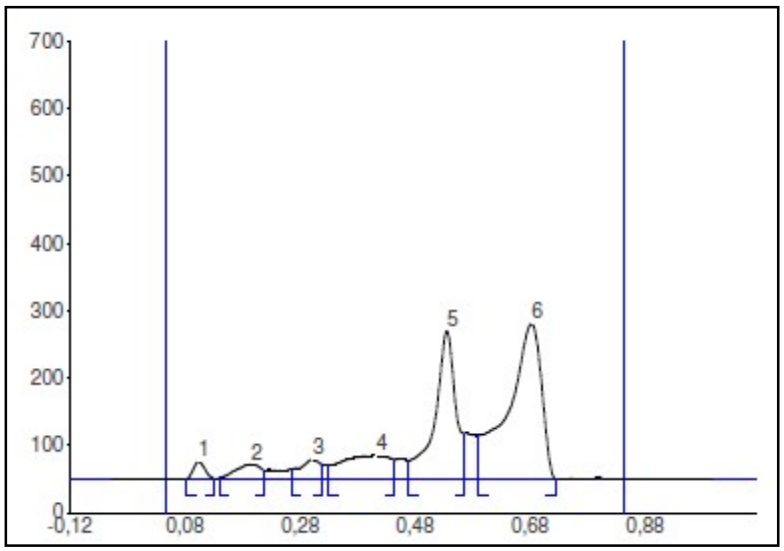

Figure 31: TLC chromatogram of IXH at 50 minutes heating at 95°C. The first peak represents IXH. The y-axis, which stands for the measured area units [AU], are plotted over the x-axis which stands for the [R_f]-value.

3.2 High Pressure Liquid Chromatography

3.2.1 Standard calibration curve

Same as for the TLC measurement a standard calibration curve of XH (fig. 32) and IXH (fig. 33) was created to determine the concentrations of the two hop polyphenols in different hop pellet species. The concentration range of XH reaches from 0.24-6

µg/ml. The LOD (limit of detection) for XH was analysed at a concentration of 0.047 µg/ml. IXH was measured in a concentration range from 0.64-16 µg/ml.

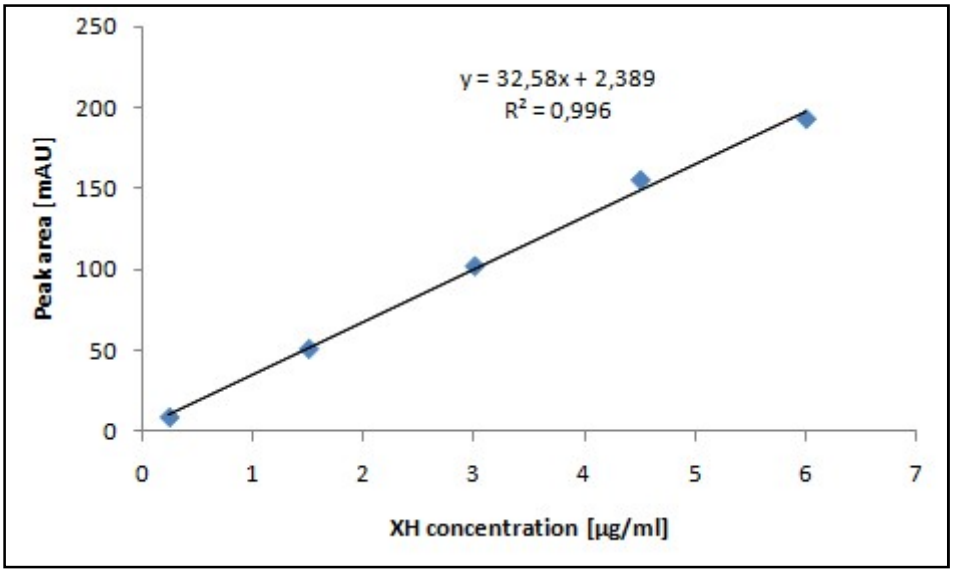

Figure 32: Calibration curve of XH. The concentration of XH is plotted over the measured peak area [mAU]. The blue dots represent the XH concentrations and next to the linear regression the corresponding linear equation is indicated.

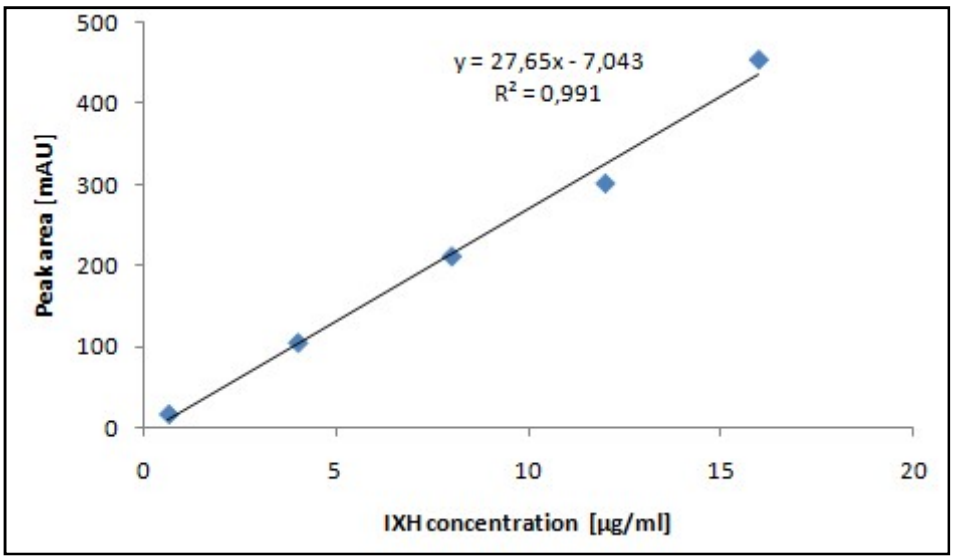

Figure 33: Calibration curve of IXH. The IXH concentration [µg/ml] is plotted over the measured peak area [mAU].

Figure 34 shows a chromatogram of XH and IXH standard solutions which were measured via HPLC. Both standard solutions have a concentration of 30 µg/ml and the drawing represents that IXH elutes first, followed by XH.

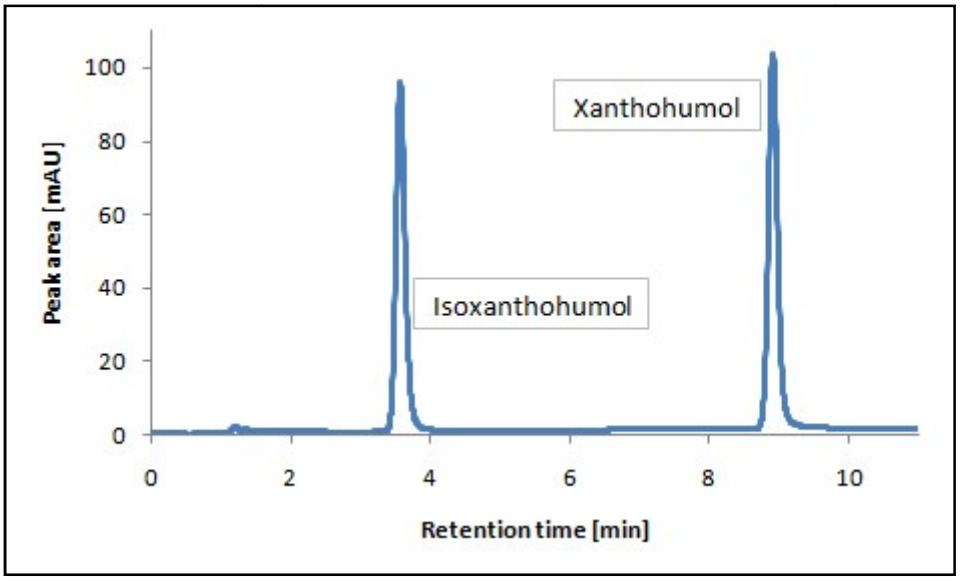

Figure 34: HPLC chromatogram of XH and IXH standard solutions. The retention time [min] is plotted over the measured peak area [mAU]. IXH has a retention time of 3.8 minutes and XH has a retention time of 9.1 minutes. The VWD-detector switches at 6.5 minutes from 290 nm to 368 nm.

3.2.2 Experiment a) Determination of XH and IXH in different hop pellets

Different hop pellet species (Cascade, Citra, Topaz and Aurora) were analysed via HPLC at the same conditions for sample preparation as TLC (30 mg hop + 2 ml MeOH). Table 12 shows the determined concentrations for XH and IXH in different hop pellets.

Table 12: Overview of the calculated values for XH and IXH in different hop pellets

Hop sample	XH [µg/mg]	IXH [µg/mg]
Cascade	4.48	0.215
Citra	5.45	0.122
Topaz	5.76	0.204
Aurora	7.44	0.211

The table with the calculated values for the two polyphenols exhibits that the Aurora hops has the highest XH content (7.44 µg/mg) in comparison to the Cascade hop pellet which has the lowest one (4.48 µg/mg). The highest IXH content could be determined in Cascade (0.215 µg/mg) compared to Citra which represents the lowest IXH content (0.122 µg/ml). Figure 35 shows a chromatogram where the high XH content in Aurora hop pellet was measured.

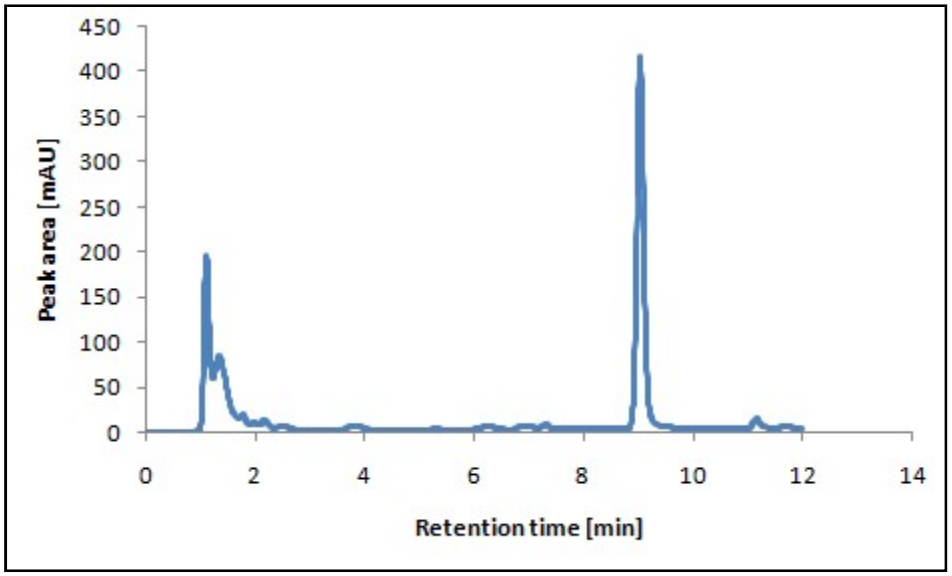

Figure 35: HPLC chromatogram of XH from hop pellet "Aurora". It represents the XH peak which has a retention time of 9.1 minutes.

3.2.3 Experiment b) Behaviour of XH and IXH during isomerisation reaction

Due to isomerisation reaction during wort boiling the XH and IXH content was analysed by HPLC. To analyse the altered concentrations of XH and IXH during brewing a model was created. Aurora hop pellets were put on a thermocycler (95°C) for different times to achieve the same conditions for hops during wort boiling at the brewing process. Due to heating, the prenylated chalcone XH is converted to its isomeric flavanone IXH [29]. The following diagrams represent the measured peak areas for XH and IXH at 0 minutes of isomerisation (fig. 36) and after 90 minutes of isomerisation (fig. 37).

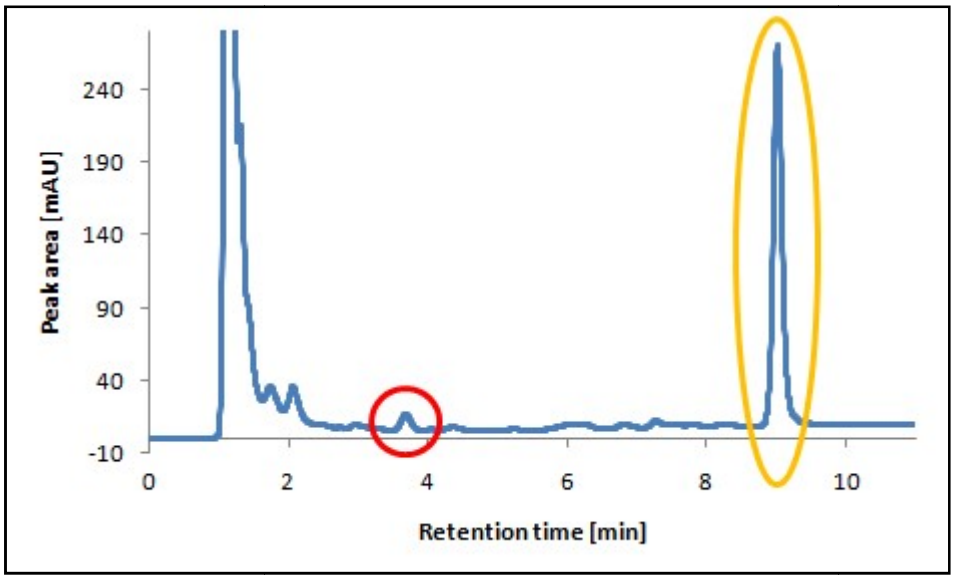

Figure 36: HPLC chromatogram of XH and IXH before isomerisation (0 min). The measured IXH peak is marked in red and XH is marked in yellow. The chromatogram indicates a reduced IXH peak and an increased XH peak.

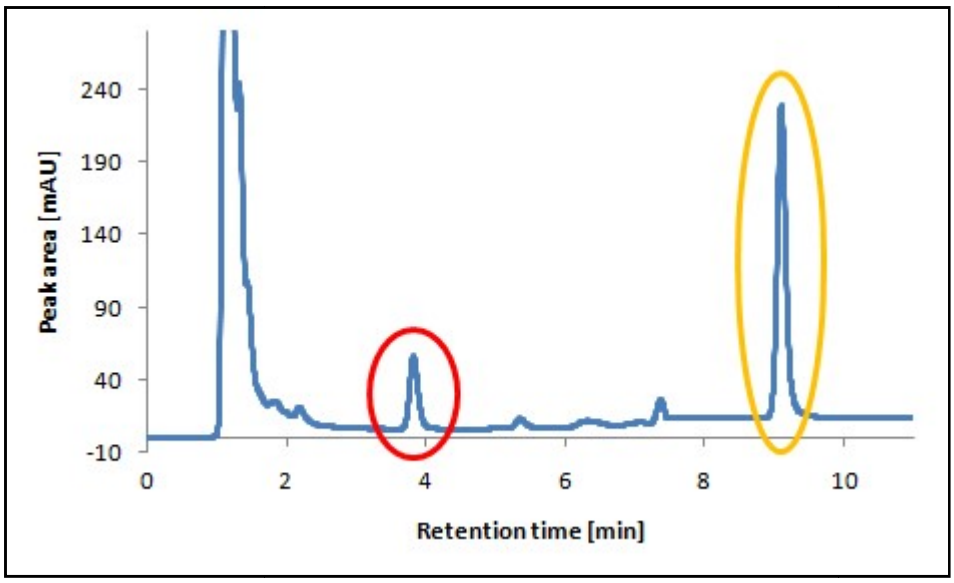

Figure 37: HPLC chromatogram of XH and IXH after isomerisation (90 min). IXH is marked in red and XH is marked in yellow. The chromatogram shows an increased IXH peak and a decreased XH peak.

The following result (tab. 13) shows the measured concentrations for XH and IXH during isomerisation reaction. The conversion of the initial weight was included due to 30 mg weighed hop sample and the addition of 600 µl water and 600 µl MeOH.

Table 13: Overview of the calculated concentrations of XH and IXH during isomerisation

Heating time [min]	XH conc. [µg/mg]	IXH conc. [µg/mg]
0	2.94	0.159
10	3.53	0.239
20	3.62	0.285
30	3.37	0.344
40	3.17	0.409
50	3.04	0.468
60	3.38	0.533
70	2.75	0.550
80	2.82	0.609
90	2.38	0.658

The whole conversion from XH to IXH due to isomerisation reaction was determined by HPLC. The next result (fig. 38) represents a model of hops boiling which occurs during wort boiling at the brewing process.

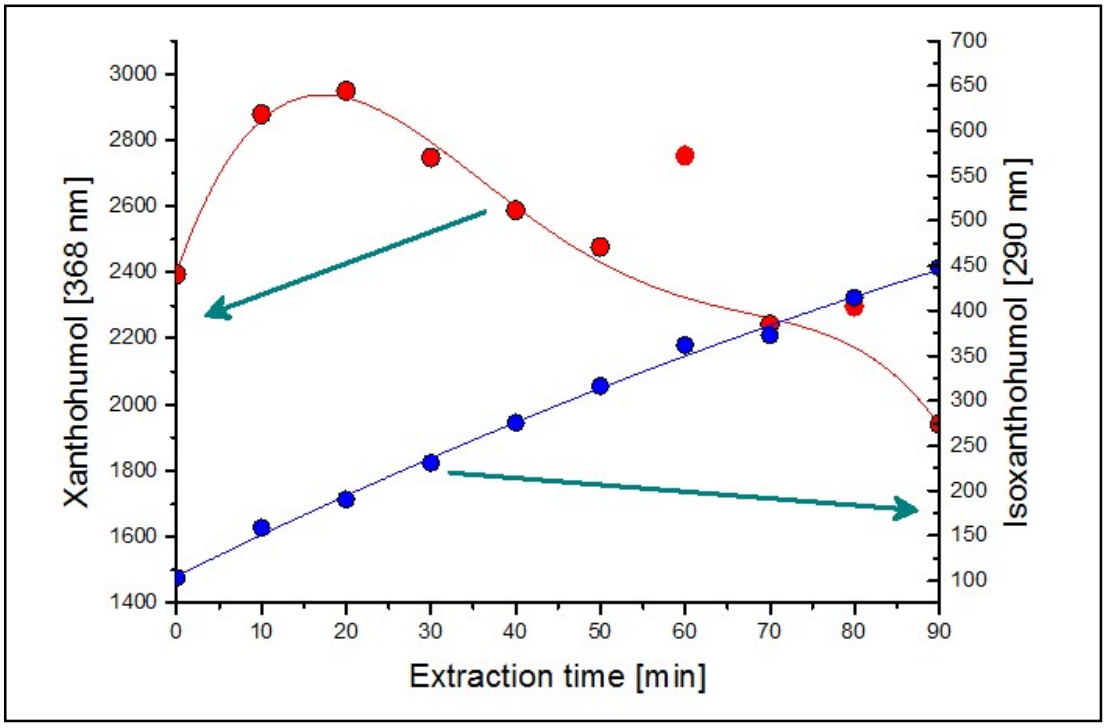

Figure 38: Isomerisation reaction of XH to IXH during heating. The red dots represent the measured peak areas for XH and the blue dots represent the measured peak areas for IXH. The x-axis denotes the extraction time of heating from 0-90 minutes at 95°C. XH was measured with UV-detector at 368 nm and IXH at 290 nm. The blue line (IXH) increases during the whole extraction time, compared to the red line (XH) which increases in the first 20 minutes and afterwards decreases to the end of the extraction time.

3.2.4 Experiment c) Determination of XH and IXH in beer

The concentrations of XH and IXH were analysed in beer from a brewing course. The sample preparation was done by SPE and afterwards the samples were measured via HPLC. The beer samples were obtained from following three production steps which are: 5 minutes after hop addition, hop boiling finish and filling. Figure 39 shows a chromatogram of XH in beer from the production step of filling and the diagram (fig. 40) below represents the measured peak areas for XH and IXH in beer.

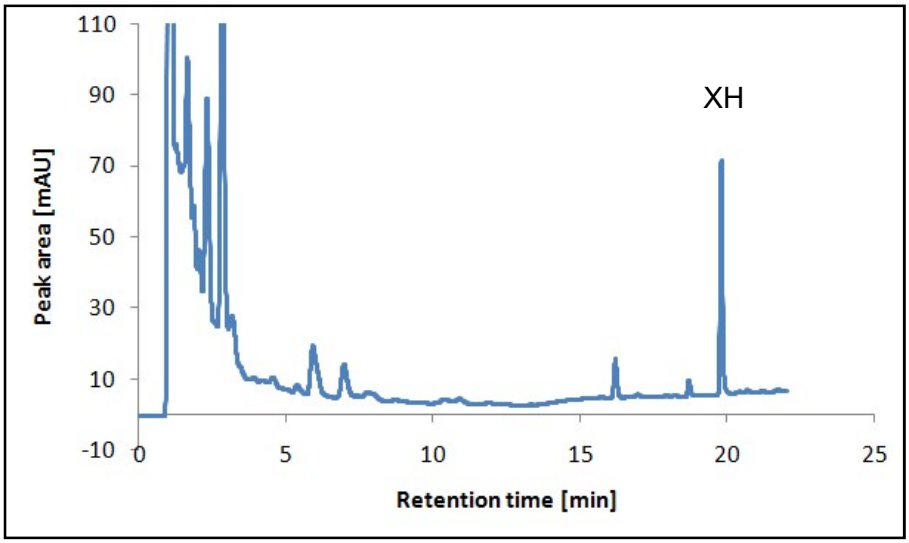

Figure 39: HPLC chromatogram of XH in beer. The XH peak has a retention time of 19.8 minutes.

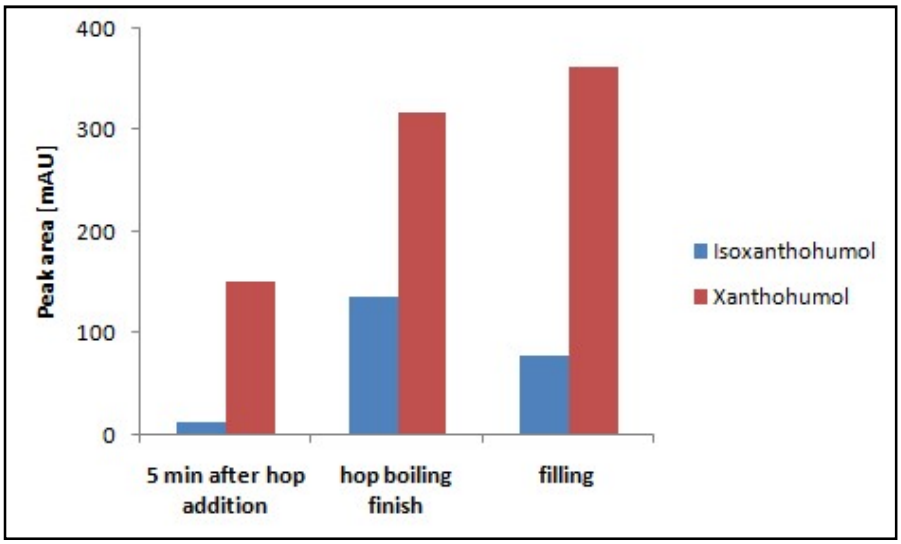

Figure 40: Diagram of XH and IXH measured beer samples. The red balk represents XH and the blue balk shows IXH. The blue balk (IXH) increases from the first to the second production step and decreases in the end. The red balk (XH) increases during the whole production.

3.2.5 Experiment d) Determination of XH and IXH in wort

After determination of XH and IXH concentrations in heated hops, another experiment which analyses the behaviour of these two polyphenols was executed. Due to occurrence of isomerisation reaction during wort boiling, the chalcone XH and the flavanone IXH were measured in wort samples, which were obtained from the brewery. The wort samples were taken at different boiling times. Eight wort samples were obtained which are: wort without hops and wort with the addition of hops after 5, 15, 25, 35, 44, 50 and 60 minutes of boiling. The frozen wort samples have to be defrosted and afterwards 1 ml was put into an ultrasonic bath for 5 minutes. Then the homogenous solution has to be centrifuged for 5 minutes to separate the solid particles. In the end a sample preparation via SPE was executed. Figure 41 shows a chromatogram of a wort sample with the measured IXH peak for identification.

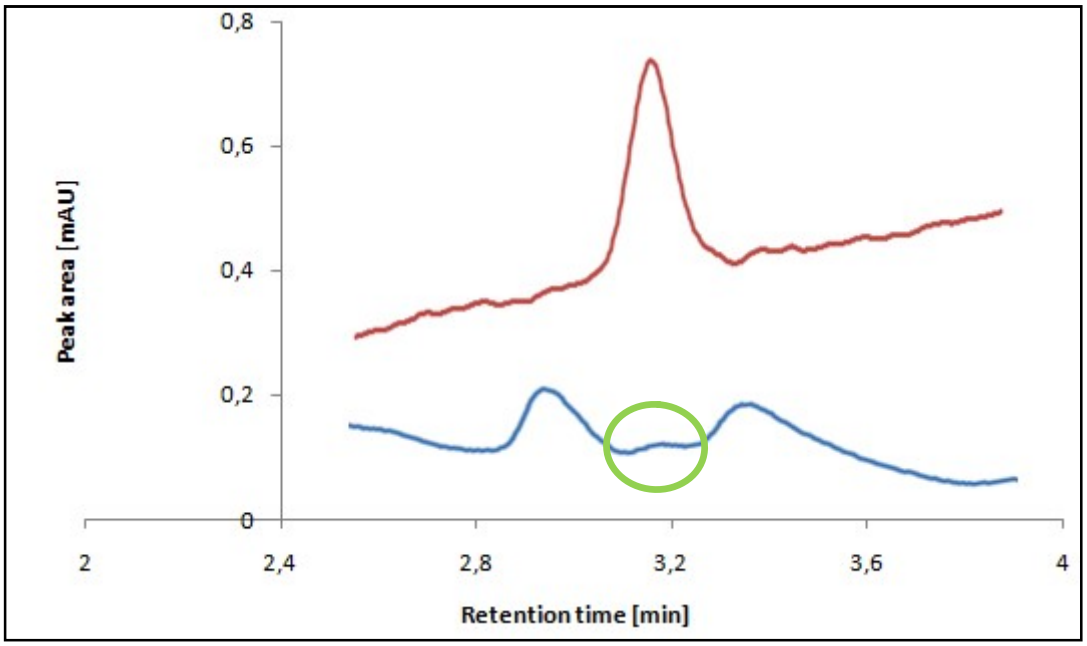

Figure 41: HPLC chromatogram of wort sample and IXH standard solution. The red line represents the IXH standard solution with a concentration of 30 µg/ml and the blue line indicates the wort sample at 44 minutes of boiling. The IXH peak of the wort sample is marked green and located between two higher peaks.

After measurement of the wort samples via HPLC, a diagram was constructed with six of the eight wort samples (fig. 42). In general it was observed a low detection of signals for both polyphenols (XH and IXH). A related behaviour of XH and IXH could be monitored compared to the experiment of isomerisation with heating hops.

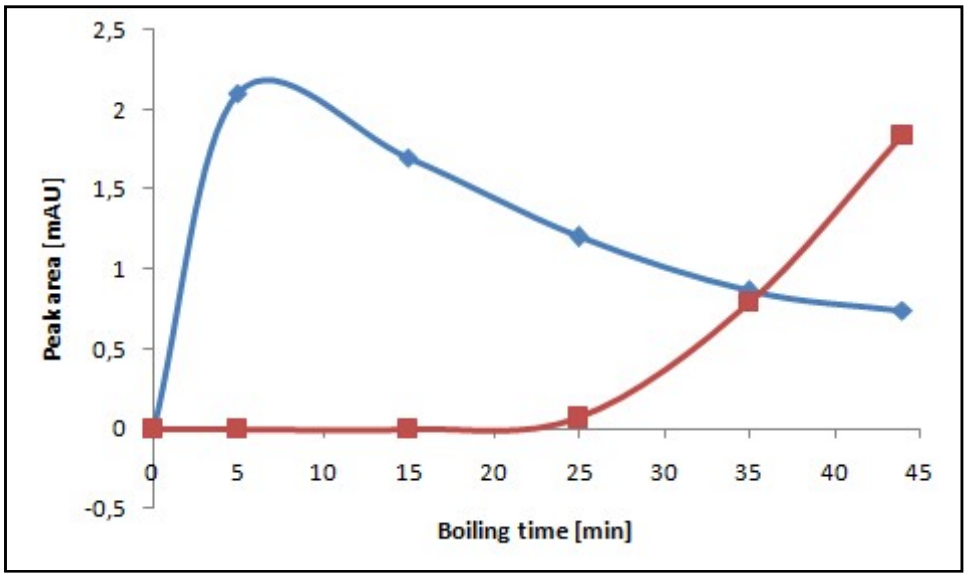

Figure 42: Diagram of wort boiling. The blue line indicates the XH content and the red line represents the IXH content during wort boiling. The XH content increases at the first 5 minutes and afterwards it decreases till to the end. The IXH content begins with a stationary phase followed by an increased phase.

3.2.6 Experiment e) Determination of sugars during wort boiling

For the determination of maltose and glucose in wort samples during boiling, two calibration curves (fig. 43 and fig. 44) were established. Therefore different maltose and glucose concentrations were measured via HPLC. The carbohydrate signal was detected with the aid of a numeric refractive index detector. The maltose and glucose calibration curves have a linear regression with a R^2 of 0.99. Different concentrations of maltose were manufactured with maltose monohydrate powder. The same was done for glucose with glucose monohydrate powder. The peak of maltose exhibits a retention time of 2.7 minutes and the peak of glucose shows a retention time of 3.1 minutes. Prior to the HPLC measurement the wort samples were diluted 1:40.

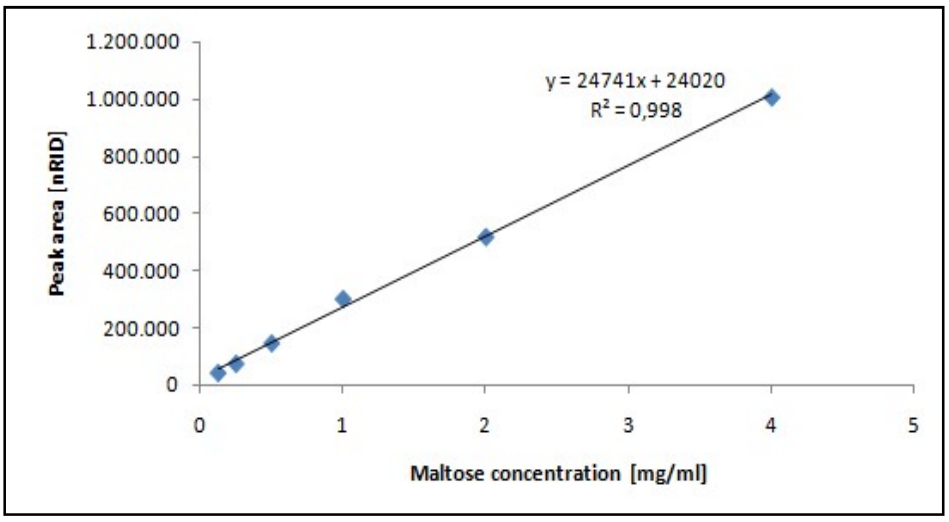

Figure 43: Calibration curve of maltose. The maltose concentration is plotted over the peak area [nRID]. The blue dots represent the measured maltose concentrations and next to the linear regression the corresponding linear equation is indicated.

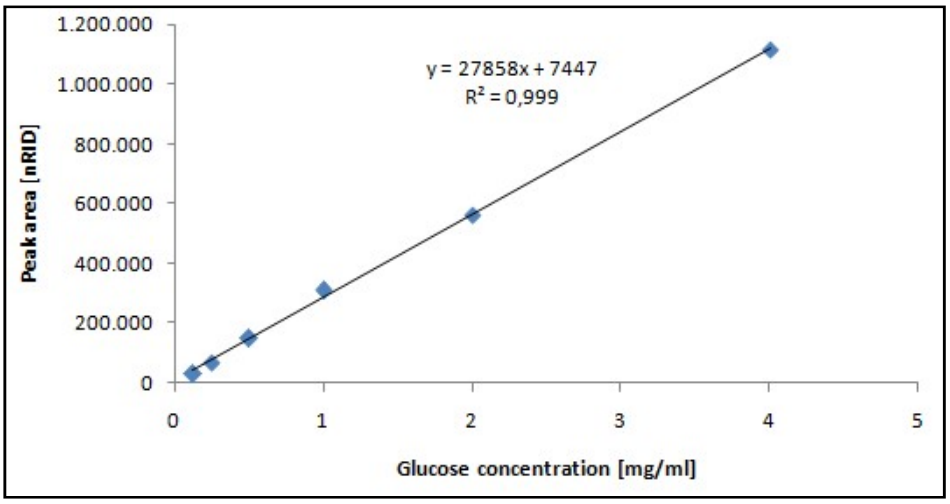

Figure 44: Calibration curve of glucose. The glucose concentration is plotted over the peak area [nRID] and the blue dots represent the measured glucose concentrations.

After measurement of several wort samples, a diagram of maltose and glucose concentrations were created (fig. 45).

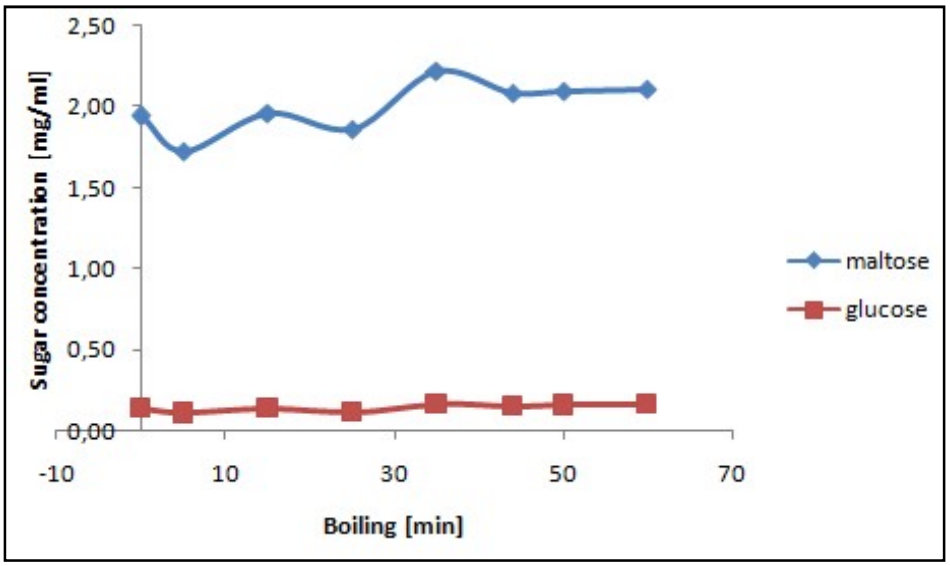

Figure 45: Diagram of maltose and glucose concentrations in wort. The blue line indicates the maltose concentration and the red line represents the glucose concentration. The red line (glucose) shows a slightly increased phase and the blue line (maltose) represents an increase by a followed stationary phase. The sugar concentrations were calculated in the units [mg/ml].

3.2.7 Experiment f) UV adsorption spectra´s of XH and IXH

XH and IXH were measured via UV light at two different wavelengths. From several publications it could be noticed that XH is measured at 368 nm and IXH at 290 nm. To estimate the correct wavelength itself, a DAD-HPLC was executed for the two hop polyphenols. The figures 46 and 47 show the UV absorption spectra of IXH and XH.

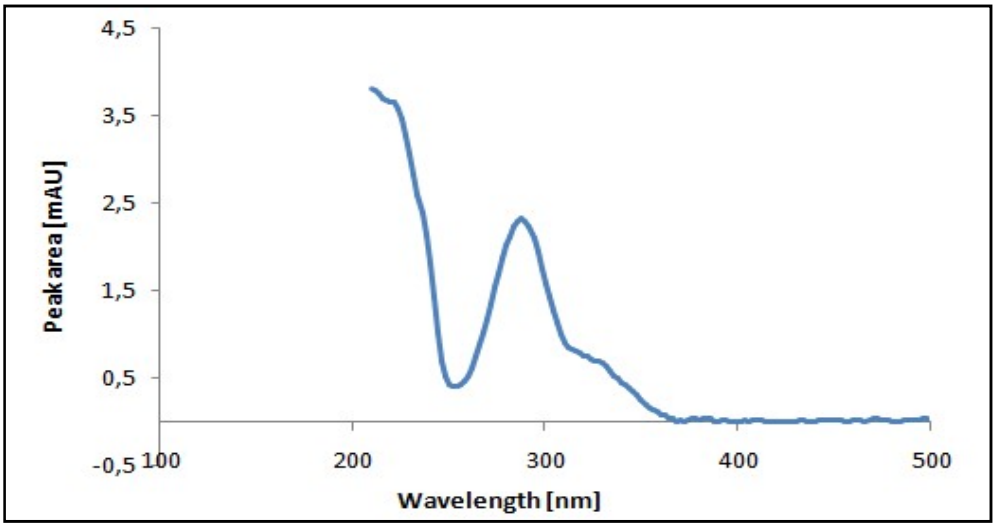

Figure 46: UV absorption spectra of IXH. The HPLC chromatogram represents IXH which has the maximum adsorption at λ = 290 nm.

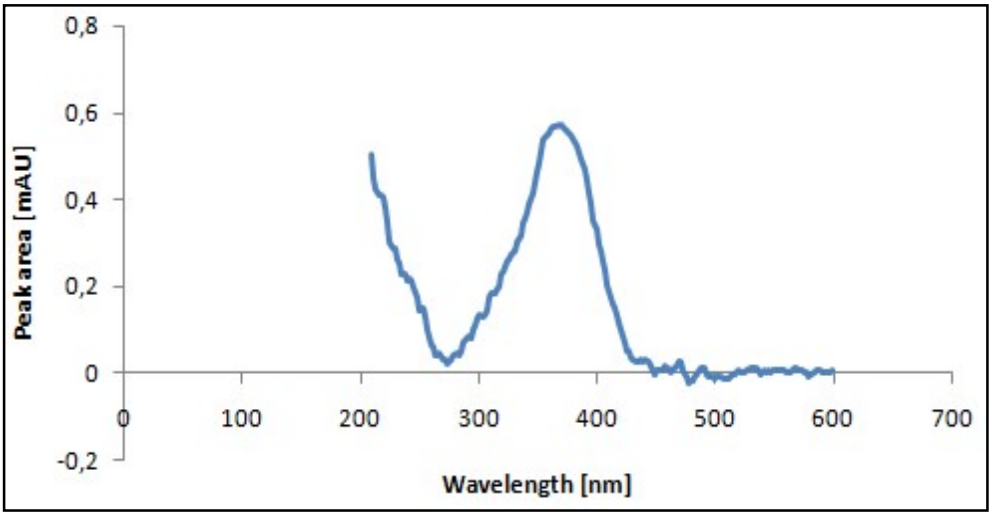

Figure 47: UV absorption spectra of XH. The HPLC chromatogram represents XH which has the maximum adsorption at λ = 368 nm.

4. Discussion

Standard calibration curves for the hop polyphenols XH and IXH were established. The linear regressions were determined with TLC and HPLC. Each calibration achieved a linear regression with a R^2 of 0.99 and could be used for the calculation of the different concentrations for XH and IXH. Also maltose and glucose calibration curves were developed for the measurement of maltose and glucose in several wort samples. The linear regressions have a R^2 of 0.99 and were used for the calculation of the different maltose and glucose concentrations in wort.

Experiment a) Determination of XH and IXH in different hop pellets

The concentrations of the hop polyphenols XH and IXH were analysed in different hop varieties via TLC and HPLC. For both sample preparations 30 mg of hop pellet were used and extracted with 2 ml MeOH. The results for the measurement via TLC are available in table 10. The hop pellet species Cascade and Citra originate from USA and are classified as aroma hops. The hop Cascade has a flowery, spicy aroma and at higher dosages it leaves a smooth bitter taste in beer. According to the TLC measurement the hop Cascade contains 7.39 µg/mg XH and 1.96 µg/mg IXH. The hop Citra (also called fruit salad of the hop species) shows a XH concentration of 9.36 µg/mg and an IXH concentration of 2.35 µg/mg. The Australian hop Topaz is a high-alpha hops and showed a measured XH concentration of 10.3 µg/mg and an IXH concentration of 2.86 µg/mg [39]. The highest analysed XH concentration was measured in Aurora hop which is classified as bitter hops. With the aid of TLC a XH concentration of 13.1 µg/mg and an IXH concentration of 0.370 µg/mg were determined in Aurora. In comparison with HPLC the measurements of XH and IXH concentrations in different hop pellet species show reduced but accurate values. The analysed concentrations of XH and IXH via HPLC are pictured in table 12. The hop pellet Aurora demonstrates the highest XH concentration (7.44 µg/mg) and the hop Cascade has the lowest XH content (4.48 µg/mg). Citra represents the lowest IXH concentration (0.122 µg/mg) and Cascade shows the highest IXH content (0.215 µg/mg). According to both chromatographic measurements (HPLC and TLC), the XH concentrations are increased in the same order which is also shown in table 10 and

12. However, the TLC measurement achieves higher values for XH than HPLC. For IXH concentrations no correlation could be seen between HPLC and TLC. For example the Topaz showed the highest IXH concentration (2.86 µg/mg) measured with TLC while the Cascade had the highest IXH concentration (0.215 µg/mg) analysed with HPLC. Since the HPLC is anyhow a more precise measurement methodology and also more sensitive compared to TLC, the result from the TLC analysis for IXH can be ignored. The reason why there was still a correlation at XH is, because XH occurs in a higher amount in hops and so also the TLC can measure it in a right way. IXH occurs in a lower amount in hops and so the TLC is not enough sensitive anymore to measure right results.

Experiment b) Behaviour of XH and IXH during isomerisation reaction

The determination of XH and IXH during isomerisation reaction were analysed with TLC and HPLC. The sample preparations for TLC and HPLC were different. For the TLC measurement 30 mg weighed hop sample were used and 2 ml MeOH were added. In the experiment with HPLC also 30 mg hops were used but the addition of the solution was different. For this chromatographic method 600 µl H_2O and 600 µl MeOH were added. The conditions were changed to yield a higher solubility of hops. XH was measured in the prior experiment with TLC and a signal was detected with an R_f-value of 0.54. The TLC measurements for the second experiment show an R_f-value of 0.80 for XH due to a changed eluent composition. The eluent for TLC measurement in the first experiment (XH concentration in different hop pellet species) was toluene-dioxane-acetic acid in relation of 77+20+3. For the second experiment (behaviour of XH and IXH during isomerisation reaction) the composition of the eluent was changed to 49+49+1 due to enhanced separation results.

In theory, the XH content is decreased and the IXH content increased during extraction time because of isomerisation. That means that the chalcone XH is converted to its isomeric form IXH due to heat which activates isomerisation reaction. In the hop plant the isomerisation reaction occurs with the aid of the enzyme chalcone isomerase [31].

In general a trend for the measured XH content could be observed. The TLC measurements show that XH is increased during the first 10 minutes of extraction and afterwards the content decreased. The XH content increased in the first 10 minutes because of the hops is getting more soluble with increasing temperature. The measurement results for XH with TLC were presented in table 11. The determination of IXH with TLC showed no significant results because of the reduced amounts of IXH in hops and therefore the TLC methodology cannot work in a right way. It could be the same problem as in the prior experiment with the different hop pellet species. Due to the low IXH content in hops, no relevant values were measured for IXH with TLC.

The calculated concentrations for XH and IXH with HPLC were pictured in table 13. A correlation of the yielded XH concentrations which were measured with HPLC and TLC could be observed. With HPLC the XH concentrations increased in the first 20 minutes and afterwards it decreased. The same behaviour of XH was monitored with HPLC and therefore the same consideration as by TLC could be noted. The results in figure 36 and 37 represent the measured peak areas for XH and IXH during heating. Figure 36 indicates that the XH is initially higher than IXH. The following result (fig. 37) exhibits the XH and IXH peak after 90 minutes of heating which yielded a reduced XH peak and an increased IXH peak. Figure 38 shows the isomerisation from XH to IXH. The blue (IXH) and red (XH) lines represent the behaviour of XH and IXH during heating. IXH increases the whole extraction time and XH increases in the first 20 minutes and then decreases until the end of boiling. In the first 20 minutes XH has a higher solubility due to heat and afterwards isomerisation takes place and converts XH to its isomeric form IXH.

A reason for the different times of solubility for XH could be that the samples which were prepared for the HPLC method were kept directly on ice to stop the reaction. During sample preparation of TLC the samples were put from the thermocycler and were cooled to room temperature without ice.

Experiment c) Determination of XH and IXH in beer

For the identification of XH and IXH in different beer samples, a SPE was executed for the sample preparation step. Afterwards the extracted samples were measured via UV-HPLC. The chromatogram (fig. 39) shows a XH peak in beer with a retention time of 19.8 minutes. The reason for the high retention time in that chromatogram is that the HPLC conditions were changed in the course of time. Figure 40 represents the measured peak areas for XH and IXH in different samples during several beer production steps. It was observed that XH is increased till to the end of the filling step due to additional hop dosages during production. The IXH showed an increased value after the boiling step where isomerisation occurs and therefore a higher IXH content could be measured. At the end, in the finished brewed and bottled beer, the IXH content is reduced due to following brewing steps of fermentation, storage and filling processes.

The hop polyphenols XH and IXH were also measured in commercial lager beer but no significant results were obtained. Due to a lot of industrial processes during brewing, the hop polyphenols were reduced till to the end by fermentation, separation and filtration steps. On these grounds, values for XH and IXH could not be determined by chromatographic method, because in beer the concentration of XH and IXH was below the LOD.

Experiment d) Determination of XH and IXH in wort

The occurrence of isomerisation reaction was determined during wort boiling. Wort samples were prepared with the aid of SPE and afterwards they were measured with HPLC. The XH and IXH were analysed in different wort samples. The diagram in figure 42 shows the measured peak areas for XH and IXH during wort boiling. The IXH signal was not measured in the first 25 minutes of boiling due to the low amount of IXH in hops and therefore also in wort. Afterwards slightly increased IXH peak areas were determined at 35 and 44 minutes of boiling because of the isomerisation reaction which occurs. The measured peak areas for XH were increased in the first 5 minutes due to addition of hops and the higher solubility of the hop polyphenol. Afterwards they were decreased through the whole boiling process due to

isomerisation reaction. The HPLC measurement was done for the first 44 minutes, subsequently a further (second) hop addition takes place at 45 minutes. The third and last hop addition occurs at 55 minutes. Then the wort was boiled for further 5 minutes and after it the liquid has to be cooled down for the following fermentation step.

In general a correlation of the measured values for XH and IXH could be determined in experiment b, where isomerisation occurs in heated hops and in experiment d, where isomerisation was analysed in boiled wort samples. The behaviour of XH increased in the first few minutes and afterwards the content decreased till to the end of the experiment. The line of IXH shows a slight increase during the whole experiment.

Experiment e) Determination of sugars during wort boiling

Since wort samples were obtained from the brewery, also maltose and glucose concentrations in several wort samples were analysed. At first standard calibration curves were established to calculate the final maltose and glucose concentrations. Figure 45 shows a diagram of the calculated maltose and glucose concentrations in wort boiling. The samples were measured during the heating process of 60 minutes. Heat is induced with the aid of a boiler which is anchored inside of the wort kettle. The boiler heats with steam and since the boiler is separated with the wort kettle, no mixture of the wort with the steam is possible. Consequently dilutions of the sugar concentrations cannot appear. The glucose concentrations (red line) represent a minimal increase during the whole boiling process and range from 0.154-0.205 mg/ml. The increase could be described due to the evaporation of water and therefore the extracts in wort were concentrated [40].

The maltose concentration (blue line) starts with a high value (1.94 mg/ml), which indicates the sample name “wort without hop”. It means the sample was taken from the finished filled wort kettle at approximately 92°C. This first wort sample is followed by a reduced maltose concentration of 1.72 mg/ml. It demonstrates the sample wort after hop addition which was boiled for 5 minutes. The first hop addition occurs at 95-96°C and the hop is added via a bypass-system to the wort kettle. Thereby no

dilutions occurred during this process which would describe the reduced maltose concentration of 1.72 mg/ml. There is the assumption that this value is a runaway. The next calculated maltose concentration has a value of 1.96 mg/ml and it demonstrates the wort sample which is boiled for 15 minutes. The maltose concentration (blue line) represents the first increase in the diagram, due to evaporation of water the wort extracts were concentrated [40]. Afterwards a maltose concentration of 1.86 mg/ml follows and indicates another decrease of the blue line, which is supposed to be a runaway as well. It stands for the wort sample which was boiled for 25 minutes. At 35 minutes of boiling the maltose concentration was determined at 2.22 mg/ml and represents the second increase in the diagram due to evaporation of water which concentrated the wort extracts. The last three measurements of wort boiling samples (at 44, 50 and 60 minutes) were calculated to values that range from 2.08-2.11 mg/ml. These concentrations show a stationary phase related to the blue line of maltose in the diagram.

It could be summarized that the maltose concentrations represent a linear increase (when ignoring two runaways) and a following stationary phase during boiling. The reason for the two mentioned runaways is an experimental failure of measurement. The glucose concentrations indicate a slight increase as well. These two enhancements could be explained by the evaporation of water due to heating during the wort boiling process.

5. List of figures

6. List of tables

7. Literature

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