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Enrichment of xanthohumol in the brewing process

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Xanthohumol (XN), a component of hops, is lost in significant quantities in the conventional brewing process. In commercial beers less than 0.2 mg XN/L are found. In order to increase the yield of XN in the brewing process, the parameters of XN recovery were studied. During wort boiling, XN is largely isomerised to isoxanthohumol. Further losses are owing to the precipitation and absorption of XN to yeast cells and haze particles and by filtration. The use of XN-enriched hop products combined with a late hop dosage during wort boiling proved to be effective in increasing the XN content in beer. The yield was further raised by a low-pitching rate and the abnegation of beer stabilisation. The use of dark malts had a positive effect on the XN recovery. Investigations of roasted malt extracts revealed several high-molecular substances that are able to form complexes with XN. These complexes proved to be stable in the brewing process. Depending on the addition of roasted malt or special XN-enriched roasted malt extracts, dark beers with more than 10 mg XN/L were achieved. Results obtained led to a brewing technology that produced on an industrial scale pale wheat beer with more than 1 mg XN/L.

Keywords: Brewing technology / Dark special malts / Hop products / 'XAN' technology

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1 Introduction

More than 90% of worldwide hop production is used in the brewing industry. Hops and hop products (pellets and extracts) are required to flavour and stabilise the beer. From the brewers point of view, the valuable constituents of hops are mainly the bitter acids (alpha acids), hop oils and polyphenols. Conventionally, hops are added to the boiling wort, where isoalpha acids are formed from the hops' alpha acids by thermal isomerisation. Isoalpha acids generate a basic bitter flavour, whereas a characteristic hoppy beer flavour is created by hop oils. As hop oils are evaporated or thermally degraded during wort boiling, 'late hopping' (hop dosage at the end of wort boiling) can be used to highlight the hoppy beer flavour. The value of hop polyphenols with regard to beer quality is not completely understood. On the one hand, phenolic compounds are associated with the formation of beer haze and many efforts are made to remove these substances from beer, *e. g.* by adsorption to stabilisers (polyvinylpolypyrrolidone (PVPP)). On the other hand, various biological effects of prenylated flavanoids, like the hop polyphenol xanthohumol (XN) are revealed. The chemopreventive properties of XN make this a potentially interesting flavanoid in beer [1, 2].

The first systematic studies about the fate of XN from hops to beer were published by Stevens *et al.* [3]. Large quantities of XN added with hops are removed during wort production together with the trub (Fig. 1). Losses can be explained by the hydrophobic character of XN and the insufficient extraction of XN in wort. Furthermore, similar to the alpha acids XN undergoes a thermal isomerisation reaction in the hot wort (Fig. 2). The isomerisation product isoxanthohumol (IX) has a conspicuously better solubility, even though its biological effects are less promising [1]. During fermentation and filtration, XN concentrations decrease further. Beer stabilisation, especially with PVPP, is associated with a strong reduction of XN in beer. In most commercial beers the concentration of XN is less than 0.1 mg/L. This is equivalent to a yield of 5% or even lower in the brewing process (Fig. 1).

Hop is the sole natural source for XN. Hop cones contain about 0.2–1.1% of XN. It is located in the lupulin glands together with the alpha acids, oils and other unspecified resins with bittering potential. Diverse extraction methods by ethanol and supercritical CO_2 at different pressures lead to residues that contain more than 30% XN. Other ingredients of XN-rich hop products are, *e. g.*, alpha acids, isoalpha

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Abbreviations: GPC, gel permeation chromatography; **IX**, isoxanthohumol; **PVPP**, polyvinylpolypyrrolidone; **XN**, xanthohumol

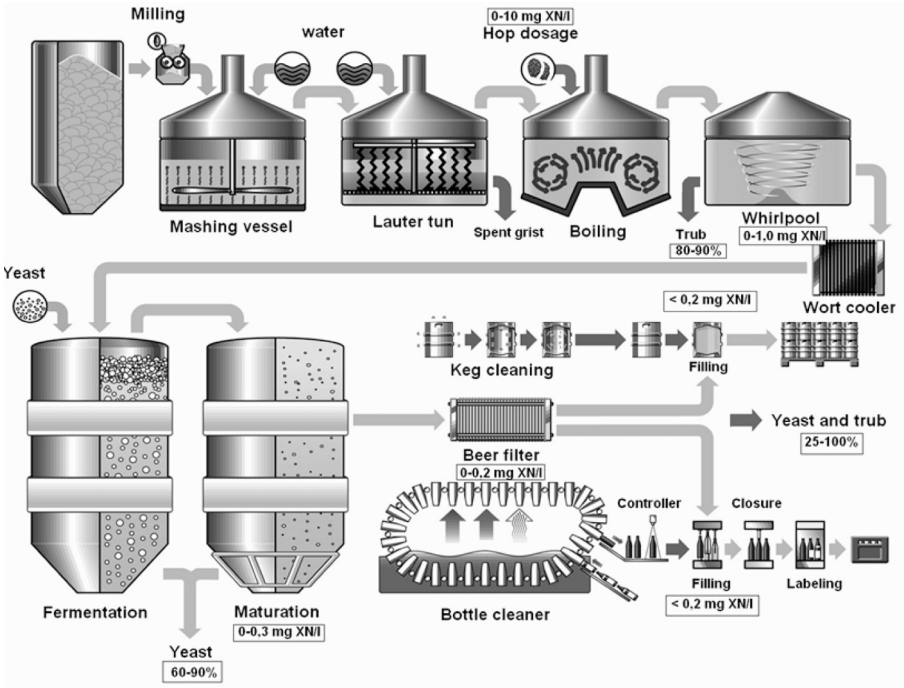
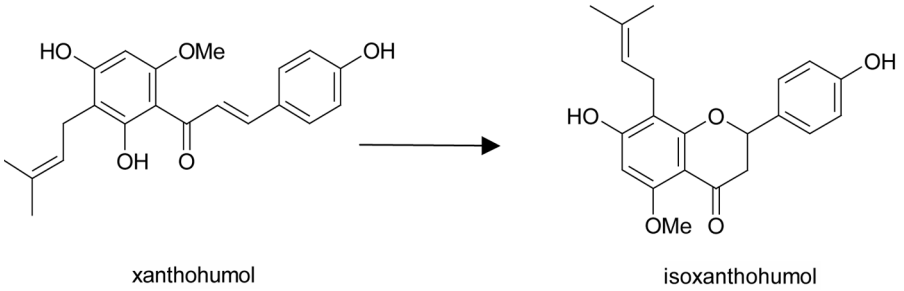


Figure 1. Brewing process (according to Gesellschaft für Öffentlichkeitsarbeit der Deutschen Brauwirtschaft e.V.) and XN losses.



xanthohumol isoxanthohumol

Figure 2. Structural formulas of XN and IX.

acids and IX. Kieselguhr is used to make pasty hop extracts free flowing [4–6].

Various brewing studies have been carried out using XN-enriched hop products. Stettner *et al.* [7] and Biendl *et al.* [8, 9] used a hop extract produced by ethanol extraction followed by supercritical CO_2 extraction. They reached an IX content greater than 8 mg/L, but observed only a small increase of the XN content. Tasting results of this beer showed a higher quality of beer bitterness compared to a conventionally hopped beer [7].

The use of higher XN-enriched hop products (30 – 90%) did not increase the XN recovery in conventionally produced filtrated beers [10]. Since an XN dosage prior to filtration also leads to high losses, Forster *et al.* [10] recommend the XN dosage after filtration to minimise costs. This procedure is limited to an XN solubility in beer of 3 mg/L [10] and does not conform to the German purity law for beer, which allows only the addition of hop extracts to hot wort [11].

Another method for XN enrichment in beer is the dosage of XN-enriched wort as feed for bottle fermentation [6]. The precipitation during secondary fermentation leads to no further losses, because it remains in the bottle.

In 2004, an XN-enriched beer was launched on the Bavarian beverage market for the first time. It was produced according to a method by Back *et al.* [12], named below as 'XAN' technology with patent applied, which implies a late hopping with an XN-enriched hop product and a fast cool down of the wort to inhibit the isomerisation. In commercial wheat beer, an XN content of about 1 mg/L was measured. Data on the development of this procedure according to the German purity law are not yet published.

Recently, Biendl *et al.* [13] and Walker *et al.* [14] reported a conspicuously high XN content in some stout and porter type beers. XN contents of 1.2 and 3.2 mg/L were measured in conventionally hopped beers. A study of special malts and cereals showed that the roasting process generated substances, which can inhibit the XN isomerisation process and increase the XN yield. In roasted barley extracts the XN content increased with higher colour up to 350 EBC [14].

Below, data on the development of the XAN technology and further investigations on the enrichment of XN in beer are

presented. In laboratory and small-scale brewing experiments, brewing technological parameters like wort temperature, time and method of XN dosage, wort pH value, pitching rate, filtration and stabilisation are studied. The idea of dark malts is further investigated. Different malts are compared according to their XN enrichment potential and the quality of the resulting beers is assessed. The aim is a maximum enrichment of XN in a sensorically immaculate beer produced according to the German purity law.

2 Materials and methods

2.1 Chromatographic methods

For determination of XN and IX, 200 mL of degassed sample was centrifuged and 400 µL phosphorous acid (85%) was added to the resulting supernatant. Samples were then injected directly into an HPLC system coupled with a diode array detector (DAD) Hewlett Packard 1090 Series II. The HPLC specifications are shown in Table 1. Quantification was done applying an external XN and IX standard (Phytochem®, Ichenhausen, Germany).

Table 1. HPLC-DAD specifications

Column	Macherey-Nagel EC-250/4 Nucleosil 100-5 C18 hop
Pump rate	0.8 mL/min
Column temperature	38°C
Injection volume	25 µL
Eluate A	$H_2O_{dem.}$ + 1% phosphorous acid
Eluate B	ACN
Eluate gradient	0–3 min: A = 60%, B = 40% 3–11.5 min: A = 25%, B = 75% 11.5–16 min: A = 0%, B = 100% 16–18 min: A = 60%, B = 40%
DAD wavelength	XN: 375 nm IX: 290 nm

Fractionation of roasted malt beer was carried out using size exclusion chromatography (SEC) in the form of gel permeation chromatography (GPC). It was performed by a Superdex 200 column (Amersham Bioscience) for molecule sizes from 100 to 600 kDa. For investigation of the XN enrichment potential of the resulting fractions, they were mixed with 35 mg XN/L. Samples were taken after 24 h resting at 4°C to reach equilibrium and one-half of each was additionally treated with PVPP. Roasted malt beer was investigated by ultrafiltration (PALL Macrosep 300 K omega) for further size classification.

2.2 Determination of beer characteristics

A UV-VIS photometer (CADAS 200, Dr. Lange, Düsseldorf, Germany) was used to determine wort and beer colour at a wavelength of 430 nm. Bitter units were measured at 275 nm (UV-VIS, CADAS 200, Dr. Lange) after extraction of alpha acids and isoalpha acids by isooctan. Foam stability was determined using the NIBEM foamtester. Tests were done according to MEBAK II [15]. For sensorical analysis, tastings according to Deutsche Landwirtschaftsgesellschaft (DLG, described in MEBAK II [15]) were conducted to find differences from type specific flavours using a panel of seven to ten tasters. Additionally, the tasters described their tasting impressions.

2.3 Brewing experiments

Studies on the influence of temperature, XN dosage and pH value were carried out on a laboratory scale (200 mL) with the first wort of pale malt brews. All other brewing experiments were performed in a pilot scale brewery (20 L) with a 30 L fermentation tank. XN was dosed in the form of a 2% XN hop product. The effects of different XN hop products on beer quality were studied with hop products containing 2, 30 and 80% XN, and a deoiled ethanolic extract containing 3% XN. Their properties are given in Table 2. In the temperature experiments 40 mg XN/L and in the dosage experiments 80–320 mg XN/L were added. In all other experiments 80 mg XN/L were dosed, if not otherwise stated.

We investigated different malts and their influence on XN enrichment (Table 3). If not otherwise stated, the malts were dosed according to the maximum manufacturers (Weyermann®, Bamberg, Germany) recommendation. Roasted malt beer was added to a pale malt wort until we reached the same colour of wort as in the wort with 5% roasted malt type II. The dark brews were produced according to 'XAN' technology [12]. All brews were fermented after addition of 10–15 × 10^6 yeast cell/mL at 12°C for 7 days and maturated at 16°C for 2 days, followed by storage of 1 wk at 0°C.

A KS 200 sheet filter (Seitz), PVPP, xerogel, activated carbon and kieselguhr were used for filtration and stabilisation experiments. In the experiments with dark brews, the stabilisation products were added at ten times the normal concentration.

3 Results

3.1 Characteristics of XN yield in the brewing process

After adding and dissolving XN isomerised in a boiling pale wort (Fig. 3), the XN content decreased while the IX content increased. Within 10 min of hopping, a maximum XN concentration of 4.5 mg XN/L was measured. The isomerisation was reduced after XN dosage to pale wort at 80°C. An XN concentration of 5.5 mg XN/L was reached

Table 2. Properties of (investigated) XN-rich hop products (investigated)

Hop product	XN content, %	IX content, %	Alpha acid content, %	Isoalpha acid content, %	Carrier	Establishing
2% XN product (Hopsteiner®)	2	0.3	0.8	1.4	Kieselguhr	Ethanol + CO_2 extraction
30% XN product (NATECO$_2$®)	30	1.8	3.76	1.84	–	Double CO_2 extraction
80% XN product (Hopsteiner®)	80	<0.1	0.3	0.29	–	Ethanol + CO_2 extraction + precipitation by ethanol/water
Ethanolic extract (deoiled)	3	0.25	38.5	1.4	–	Ethanol extraction

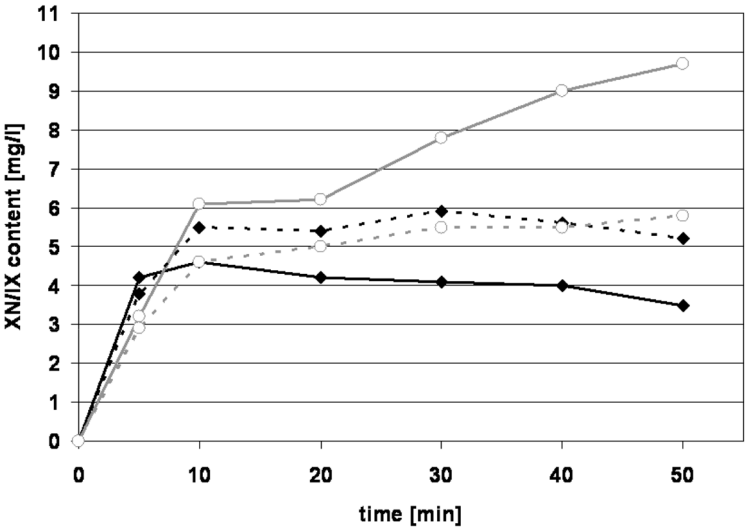


Figure 3. XN and IX content in pale wort during heating wort at 100 and 80°C (laboratory scale), dosage of 40 mg XN/L. (–♦–) XN 100°C, (–○–) IX 100°C, (– –♦– –) XN 80°C and (– –○– –) IX 80°C.

Table 3. Properties of the used malts (Malzfabrik Weyermann, Germany)

Malt	Colour, EBC	Required quantity, %
Brewing malt		
Pale malt	2 – 3	100
Münchner Malz type II	20 – 25	100
Caramel malt		
CARAHELL®	20–30	30
CARAAROMA®	300–400	15
CARAAMBER®	60–80	20
Roasted malt		
CARAFA® type II	1000–1100	5
Roasted rye malt	500–800	5
Roasted cereal		
Roasted barley	800–850	5
Roasted malt beer		
SINAMAR®	8000–9000	As necessary

after 10 min and was constant for the remainder of the experiment (Fig. 3).

XN recovery decreases with increasing XN dosages (Table 4). In pale wort 12.8 mg XN/L was measured as a maximum absolute XN concentration. pH has certain influences on the XN content in pale wort (Table 4). In wort 7.5 mg XN/L could be recovered at pH 4.5, while 12 mg XN/L were found at pH 6.5 under the same conditions.

After addition of fresh yeast to a filtrated pale malt brew, the XN content decreased from 1.1 to 0.4 mg XN/L (Tab. 4). In this experiment, the IX content remained at a level of 1.0 mg IX/L (data not shown). Another experiment with manifold reuse of yeast showed that XN losses during fermentation can be reduced by XN enrichment of the yeast, while the IX content was again kept constant (3.5 mg IX/L, data not shown). The addition of XN-enriched yeast (threefold reused) to filtrated beer confirmed this result. Fermentation temperature did not influence the XN content.

In pale malt brews, up to 46% of the XN is lost by filtration (sheet), xerogel and kieselguhr treatment (Table 4). In samples after stabilisation with PVPP or activated carbon, XN could be detected but could not be determined.

3.2 Special characteristics of dark malts

In investigations of 30 dark beers brewed according to the purity law, we found up to 0.27 mg XN/L at a colour of 73 EBC (data not shown). In order to determine the reasons for

Table 4. Results from small-scale and laboratory experiments with pale wort, pale and dark beer

Variable		XN content, mg/L		XN recovery, %	
XN dosage[a], mg/L	80	10.6		13	
	160	11.0		6	
	240	11.8		5	
	320	12.8		4	
pH value[b]	4.5	7.5		–	
	5.5	10.3		–	
	6.5	12.2		–	
Yeast[c]	Filtrated beer	1.1		100	
	Fresh yeast	0.4		36	
	XN-enriched yeast	1.0		91	
Yeast reuse[d]	Fresh yeast	2.3		–	
	1. Reuse	2.5		–	
	2. Reuse	3.1		–	
	3. Reuse	3.5		–	
		Pale	Dark	Pale	Dark
Filtration/stabilisation[e]	Untreated	1.1	7.7	100	100
	Xerogel	0.8	7.4	74	96
	Kieselguhr	0.7	7.2	65	94
	Sheet filtration	0.5	6.5	46	85
	Activated carbon	<0.05	4.2	<4	55
	PVPP	<0.05	4.0	<4	52

a) Content in pitching wort, dosage at 80°C to pale cast wort.
b) Content in pitching wort, dosage at 80°C to pale cast wort, pH adjustment by addition of H_3PO_4 or NaOH.
c) Pale malt brew, sheet filtration.
d) Unfiltered pale malt brew.
e) Use of filtration/stabilisation in conventional amounts in the pale malt brew, tenfold amount in the dark brew with 10% roasted malt type II.

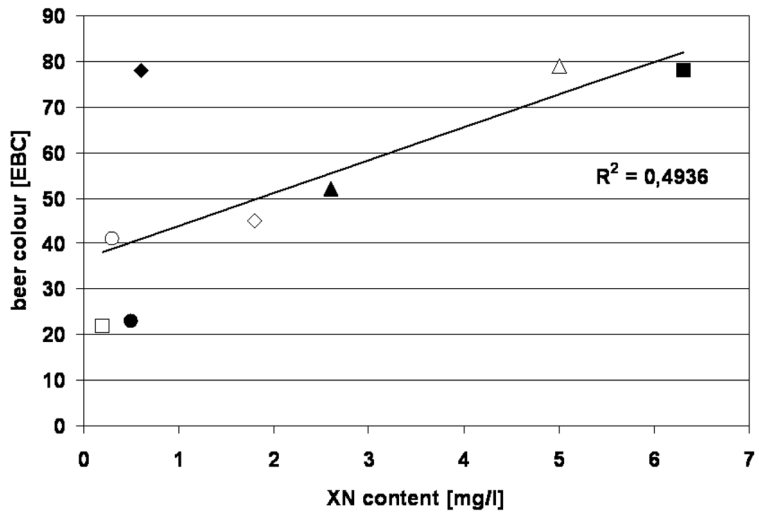


Figure 4. XN content and beer colour of different dark brews. (▫, 20% CARAAMBER®; ●, 30% CARAHELL®; ○, 100% Münchner Malz; ◆, 15% CARAAROMA®; ◇, 5% roasted rye malt; ▲, 5% roasted barley; △, 5% roasted malt type II and ■, 100% pale malt + roasted malt beer.

higher XN contents in international beers, we studied different malts, roasted barley and roasted malt beer (Tab. 3) on their XN enrichment potential in beer (Fig. 4). Brews with more intensive roasted malts, like roasted malt type II and roasted malt beer, had higher XN contents than those with less intensive roasted malts. However, no correlation between beer colour and XN enrichment of filtrated beer was found (Fig. 4). Figure 5 shows the results from experiments with varying proportions of roasted malt type II in pale malt brews with an XN dosage of 30 mg XN/L. The XN content increases in filtrated beer with increasing proportions of roasted malt. This linear correlation has a coefficient of determination of 0.98 (Fig. 5). The roasted malt brews are also characterised by high XN concentra-

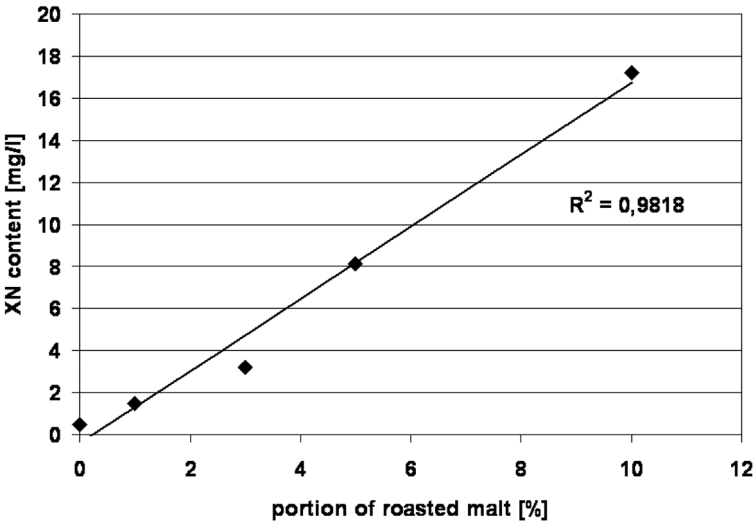


Figure 5. XN content in filtrated beers brewed with 80 mg XN/L and different proportions of roasted malt.

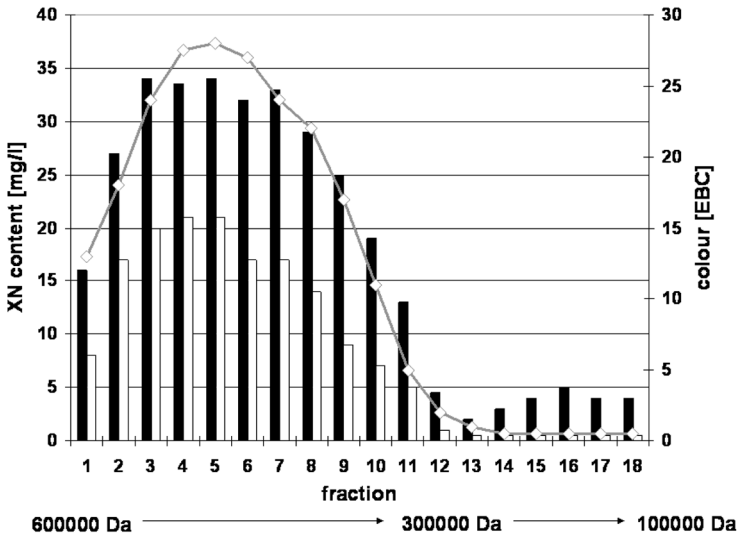


Figure 6. XN content and colour in GPC fractions of a roasted malt wort before and after PVPP treatment. ■, XN content (mg/L) before PVPP treatment; □, XN content (mg/L) after PVPP treatment and –◇–, colour (EBC).

tions in the pitching wort and low losses during the remaining brewing process. For example, 24 mg XN/L were found in a pitching wort with an XN dosage of 80 mg/L and a roasted malt portion of 10%. After fermentation, 19 mg XN/L were measured. An XN concentration of 17.2 mg/L was then found in the filtrated beer.

In this beer, XN was not removed as much as in pale brews by filtration and stabilisation products (Tab. 4). Ultrafiltration and GPC showed that wort fractions of a roasted malt beer in a size range of 300 – 600 kDa (Fig. 6, fraction 1 – 11) had the highest XN enrichment potential. In these fractions, PVPP treatment reduced the XN content up to 60%. In smaller fractions, the XN was almost completely eliminated by PVPP treatment.

3.3 Characteristics of XN-enriched beers

Among the XN content, we studied further properties of XN-enriched pale malt brews. Differences can be obtained by using different XN-rich hop products (Tab. 2). Beers produced according to XAN technology with the 2% XN product are characterised by a higher XN yield compared to the other products. In addition, in brews with ethanolic extracts higher EBC bitter units were measured. This is reflected in the results from the sensorical analysis. All brews were rated as typical with an average mark of 4.4 according to DLG tasting. The beers with the 2% XN product and the ethanolic extract were distinguished by a bouquet of hops with a pleasant hard resin bitterness. Additionally, they had a conspicuously stable foam. The brews with 30 and 80% XN product were neutral on sensoric and foam.

4 Discussion

4.1 XN enrichment in pale malt brews

The findings on the isomerisation of XN to IX are in accordance with the results of Stevens *et al.* [3], who observed high XN losses during wort boiling. The larger increase in the IX concentration compared to the decrease of XN in our experiment is probably due to a quicker loss by isomerisation than a release by extraction of XN. The experiments with different temperatures showed that the isomerisation can be reduced by dropping the temperature to 80°C. A high-gravity brewing and the addition of cold brewing water after XN dosage render the temperature and gravity adjustment in accordance to the German purity law. The precooling of wort to 80°C may lead to a deficient formation of the trub cone, resulting in an increased carryover of hot trub to the fermentation tank. Additionally, codosed alpha acids may affect the sensoric impression (bitterness) and the beer stabilities. The dosage of the XN-rich product, *e.g.* 5 min before the end of boiling followed by a precooling of the cast wort is proposed to solve these problems.

The dosage experiment showed no limiting by solubility although the XN yield decreased with increasing dosage. There may be substances in wort (*e.g.* kieselguhr), which act as mediators or carriers or just lead to a better distribution of XN in wort. With regard to the German purity law, the pH values can be ignored because during wort boiling they range from 5.2 to 5.6.

The impact of yeast is not yet clear. The decrease of the XN content during fermentation, as observed by Stevens *et al.* [3], may be owing to an adsorption of XN on the yeast. The lower XN concentration in a filtrated beer sample after addition of yeast and a 'saturation' of the yeast by reuse back this hypothesis. Further studies on the yeast have to be conducted to get more information about metabolism. Probably the saturation effect is caused by reduced yeast vitality and metabolism. Losses by decomposition of XN are also possible. The fact that IX is less influenced by yeast may be due to differences in structure and resulting differences in solubility. The 'XAN' technology recommends a reduced pitching rate with XN-enriched yeast to improve the XN recovery. The yeast vitality has to be considered, since it is decisive for beer quality, like foam, sensoric and physicochemical stabilities.

The almost complete elimination of XN in pale malt brews by PVPP and activated carbon is probably due to a high affinity of XN to substances like proteins and surface active substances (attached XN). Additionally, XN may react directly with the stabilisation products (dissolved XN) and may be removed by centrifugation during sample preparation by attachment to the stabilisation products.

4.2 XN enrichment in dark malt brews

Up to 1.2 mg XN/L are found in international dark beer brands [14]. Nevertheless, significantly higher XN contents were not measured in dark brews produced according to the German purity law compared to pale ones. The investigations on different dark malts and their XN enrichment potential showed that intensively roasted malts are most appropriate. Roasted malts and roasted malt beer are conventionally used in small amounts for dark beers produced according to the German purity law. Additionally, these beers are often brewed with low hops [16]. Walker *et al.* [14] also observed a correlation between the XN enrichment and the roasting intensity of the used malts. Our results of an increased XN content linear to the increased amount of used roasted malt confirm this finding (Fig. 5). In our small sampling, a correlation between XN enrichment and beer colour was not found (Fig. 4, $R^2 = 0.49$). It is conceivable that not all colouring substances are responsible for XN enrichment. In Fig. 4 the brews with roasted malts, roasted cereal and roasted malt beer showed a higher XN content than the other brews, although the CARAARO-MA® brew had a beer colour of 78 EBC. This may be due to different substances, *e.g.* Maillard products, which are synthesised during the kilning and roasting process depending on time and temperature [17]. The identification of these substances is difficult. Coghe and Andriaenssens [17] described colorants in black roasted malt of an average molecular weight of 320 000 Da. This finding may explain why we found especially roasted substances with a molecule size range of 600 000 – 300 000 Da important for XN enrichment. Differences in XN recovery of the GPC fractions 1–10 suggest that it is a matter of not only one but a variety of substances (Fig. 6). These substances are characterised by a high affinity to XN. Smaller molecules also associate XN but, in contrast, it can be removed almost completely by PVPP. Presumably, XN is bound to the roasted substances during wort boiling preventing isomerisation. The roasted substances act as a carrier and transport XN throughout the brewing process.

4.3 Characteristics of XN-enriched pale beers

It is not surprising that different hop products cause differences in the resulting beers. For example the codosage of alpha acids and the resulting isoalpha acids alter the sensorical impression of bitterness as observed by Stettner *et al.* [7]. Foam stability can also be explained by the isoalpha acid contents of the beers [18]. They probably are enriched in the foam and stabilise it. The better XN yield of the 2% XN product is due to an enlarged surface of this product by kieselguhr. A better distribution of the product in the wort is achieved and results in a more intensive contact between hop product and wort.

5 Conclusions

In pale malt brews produced according to 'XAN' technology about 3 mg XN/L is produced, and results of sensorical analysis showed that XN-enriched pale malt beers taste typical. Thus far it is not clear if this concentration is the most ideal. Higher concentrations are possible by the use of roasted malts or roasted malt beer. We assume that the use of an advanced 'XAN' technology, including the use of roasted malt or roasted malt beer, permits an XN enrichment in every beer type up to 20 mg XN/L. Different XN-rich hop products and technological changes should make it possible to meet the requirements of most brewers.

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