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Influence of malt on the xanthohumol and isoxanthohumol behavior in pale and dark beers: A micro-scale approach

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ABSTRACT

The main objective of this work was to understand which compounds are responsible for the partial inhibition of xanthohumol (XN) thermal isomerization during wort boiling of dark malts. With such purpose, worts from different kinds of malt (pale, caramel and roasted) were chemically characterized (e.g. antioxidant activity, polyphenols and melanoidins contents) and studied by several chromatographic techniques. Molecular exclusion chromatography experiments showed that adsorption of XN to polyvinylpolypyrrolidone is lower for the high molecular weight fractions. It seems likely that this is due to the reaction between XN and coloured substances that changes chemical properties of XN or, at least, results in the formation of complexes that have different chemical properties. This leads to an inhibition of XN isomerization observed in this work. It was also verified that the melanoidins content was significantly higher in the higher molecular weight fractions, suggesting that probably these compounds are the main responsible for the XN isomerization inhibiting effect.

1. Introduction

The hop plant (*Humulus lupulus L.*) is a dioecious plant of the Cannabacea family, cultivated in most temperate zones of the world for its female inflorescences. Hops are used in the brewing industry to add flavour, aroma, bitterness and stability to beer (Magalhães, Carvalho, Cruz, Guido, & Barros, 2009; Magalhães, Carvalho, et al., 2009). Numerous flavonoid glycosides were found in the water-soluble part of hops, while the weakly polar contains prenylated flavonoids, such as prenyl dihydroflavonoid and prenylchalcones, among them 6-prenylnaringenin, 8-prenylnaringenin and XN which are potent phytoestrogens (Magalhães, Carvalho, et al., 2009). Recent research has examined the biological activities of single hop components, particularly XN. Anti-inflammatory, antioxidant, anti-lipoperoxidative activities as well as antiangiogenic, antiproliferative and apoptotic effects, mainly assessed in vitro studies, reasonably suggest a potential chemopreventive activity (Gerhauser et al., 2002; Magalhães, Carvalho, et al., 2009). In addition, XN displays broad-spectrum anti-infective activity against several micro-organisms (Magalhães, Carvalho, et al., 2009; Nowakowska, 2007). The reported beneficial health effects of XN have attracted attention in the brewing community concerning the production of XN enriched hop extracts and beers (Biendl, Methner, Stettner, & Walker, 2004; Magalhães, Dostalek, Cruz, Guido, & Barros, 2008; Wunderlich, Zürcher, & Back, 2005).

XN is largely converted into its isomeric flavanone, IXN, during the wort boiling. In general, this is the reason why commercial beers around the world are characterized by a very low content of XN (maximum of 0.15 mg/L in conventional pale beers) and a high content of IXN (ranging from 0.04 to 3.44 mg/L) (Stevens, Taylor, & Deinzer, 1999). However, there are other factors responsible for the low content of XN in beer. Large quantities of XN added with hops are removed during wort production together with the trub (Magalhães et al., 2008; Stevens, Taylor, Clawson & Deinzer, 1999). Losses can be explained by XN's hydrophobicity and the insufficient extraction of XN in wort. During fermentation and filtration, XN concentration decreases further (Magalhães et al., 2008; Stevens, Taylor, Clawson et al., 1999). Beer stabilization, especially with PVPP, is associated with a strong reduction of XN in beer (Magalhães et al., 2009). A study on special malts and cereals showed that the roasting process

Abbreviations: ARP, antiradical power; CE, catechin equivalent; DAD, diode array detection; EBC, European Brewing Convention; ESI-MS/MS, tandem mass spectrometry using electrospray ionization; FC, Folin–Ciocalteau; FRP, ferricyanide reducing power; GAE, gallic acid equivalent; GPC, gel permeation chromatography; HMW, high molecular weight; HPLC, high-performance liquid chromatography; IXN, isoxanthohumol; LMW, low molecular weight; MLD, melanoidins; PVPP, polyvinylpolypyrrolidone; QE, quercetin equivalent; SEC, size exclusion chromatography; TPC, total phenolic content; XN, xanthohumol.

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generates substances which can inhibit XN isomerization during wort boiling and increase the XN yield (Wunderlich et al., 2005). In this study, it was verified that 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. Recently, the influence of an XN enriched hop product on the contents of XN and IXN in pale and dark beers during the whole brewing process was studied (Magalhães et al., 2008). As expected, it was verified that XN was mainly converted into IXN during wort boiling in brewing trials with pale malt. However, and corroborating bibliographic data (Biendl & Walker, 2004; Wunderlich, Biendl, Zürcher, & Back, 2009), the use of dark malt appeared to inhibit isomerization of XN, resulting in notable high levels of XN in the stout production process. Presumably, XN is bound to the roasted substances present in roasted malt, that may act as a carrier for XN throughout wort boiling preventing its isomerization during the brewing process (Wunderlich et al., 2005, 2009).

Therefore, it was the logical step forward to investigate which compounds present in dark malts are responsible for the partial inhibition of the isomerization of XN during the production of stout/porter beers. Until nowadays these compounds have not been identified. With such purpose in mind, several kinds of malts (pale, caramel and roasted) were chemically characterized (antioxidant activity, polyphenols and MLD contents) with several chromatographic techniques such as HPLC with DAD and ESI-MS/MS and SEC in the form of GPC.

2. Materials and methods

2.1. Production of pale, caramel and roasted Congress worts: small scale mashing

Pale, caramel and roasted malts (colour: 2–3, 140 and 850 EBC units, respectively) were a kind gift from the Litovel brewery (Litovel, Czech Republic). 50 g (±1 g) of each malt sample were milled in a Buhler Miag disc mill, using a fine grind (0.2 mm) and coarse grind (0.7 mm). Mashes were carried out in stirred metal beakers, using EBC Congress mash. 200 mL of distilled water, at 45 °C, were added to the ground malt samples. The mash was continually stirred. After 30 min at 45 °C, the temperature was increased at the rate of 1 °C per minute up to 70 °C. More water (100 mL at 70 °C) was added. The temperature was maintained at 70 °C for 1 h. The mash was then cooled to 20 °C and filtered with a Whatman (Maidstone, UK) no. 1 filter paper.

2.2. Malt and wort analysis

Standard wort and malt analyses were carried out according to Analytica-EBC (EBC, 2003). Wort apparent extract (g/100 mL), colour (EBC units) and pH were monitored using a SCABA 5600 Automatic Beer Analyser (Tecator AB, Sweden).

2.3. Isomerization of XN in the different congress worts

500 mL of each wort were boiled at 100 °C for 110 min with a previous addition of 20.0 mg/L of XN at the beginning of boiling. During the boiling experiments, aliquots of 1.0 mL were collected for each 10 min in order to determine the contents of XN and IXN. The quantification of these phenolic compounds in the different aliquots was done by direct injection into the chromatographic system. XN (90% purity) was purchased from Hopsteiner (Mainburg, Germany), and IXN (≥98%) from Alexis Biochemicals (Lausen, Switzerland).

The HPLC system (Jasco Corporation, Tokyo, Japan) consisted of a low pressure quaternary gradient unit (model LG-1580-04) with an in-line DG-1580-54 degasser and a model AS-950 autosampler. The system is equipped with a photodiode array detector (model MD-1510 UV/Vis multiwavelength detector). Separations were achieved on a LiChroCart (Merck, Darmstadt, Germany) RP-C_{18} column (125 mm × 3.0 mm, 3 μm) with a linear solvent gradient, starting on injection, from 40% to 100% B, acetonitrile (HPLC grade, Sigma-Aldrich, St. Louis, USA), in A, 1% aq. formic acid (99%, Merck) over 15 min, followed by 100% B for 5 min. A guard column (LiChroCart RP-C_{18} 4.0 mm × 4.0 mm, 5 μm) was placed in front of the analytical column. The flow rate was 0.8 mL/min and the detection wavelengths of XN and IXN were about 368 nm and 286 nm, respectively. A total of 20 μL was injected into the column kept at room temperature. Analytes in each sample were identified by comparing their retention times and UV–Vis spectra with those of standard compounds. Peak purity was checked to exclude any contribution from interfering peaks.

2.4. TPC in worts

The TPC was determined by a modified FC method described in literature (Singleton & Rossi, 1965). The FC reagent was purchased from Merck, sodium carbonate and gallic acid were purchased from Sigma-Aldrich (St. Louis, USA). The TPC in the different samples was also measured as described in Analytica-EBC (EBC, 2003).

2.5. Determination of flavan-2-ols and proanthocyanidins in worts by the vanillin assay

The vanillin assay was done as previously described in literature (Sun, Ricardo-da-Silva, & Spranger, 1998). Vanillin reagent and (+) catechin were purchased from Sigma-Aldrich, sulphuric acid 98% and methanol, analytical grade, from Merck.

2.6. Determination of free, soluble ester and insoluble-bound phenolic in worts

The free, esterified, and bound phenolic compounds in wort extracts were isolated according to procedures described in literature (Naczk & Shahidi, 2004; Sosulski, Krygier, & Hogge, 1982) with slight modifications. Concentrated supernatant of crude extract (described above) was extracted with diethyl ether (3 × 10 mL). The ether extracts were collected and evaporated to dryness under vacuum at 35 °C. Phenolic acids extracted were labeled as free phenolics. The supernatants with esterified phenolic acids were then treated with 30 mL of 2 M NaOH for 2 h at room temperature (Dvořáková et al., 2008). The resultant hydrolyzate was acidified to pH 2 using 6 M HCl and extracted three times with diethyl ether. The ether extracts were then combined and evaporated to dryness at 35 °C under vacuum. The phenolic acids extracted were those liberated from their esters and labeled as esterified phenolic acids. The leftover meal after extractions was treated with 30 mL of 2 M NaOH for 2 h at room temperature. The samples were then acidified to pH 2 with 6 M HCl followed by centrifugation (6 kg for 10 min). The mixture was extracted three times with diethyl ether. The extracts were combined and evaporated to dryness under vacuum at 35 °C. The phenolic acids extracted were labeled as bound phenolics. Free, esterified and insoluble-bound phenolics were dissolved separately in 1 mL of mobile phase (for HPLC-MS/MS analysis) and 5 mL of methanol (HPLC grade, Labscan, Dublin, Ireland).

The quantitative analysis of the polyphenols in the different free, soluble ester and bound forms was performed by HPLC–DAD. The chromatographic conditions used were the following: flow rate 0.2 mL/min, sample injection volume of 20 μL and mobile phase A, 100% methanol, and B, 0.1% aq. formic acid. A gradient program was used as follows: 90% B in 0 min, from 90 to 60% B in 75 min, back to 90% B in 5 min and 15 min of reconditioning before the next injection. The photodiode array detection was conducted by scanning between 190 and 600 nm, and the quantification was conducted at 280 nm for monomeric flavan-3-ols ((+) catechin and (−) epicatechin) and