

# Investigation into the high levels of xanthohumol found in Stout and Porter-style beers

Perhaps one of the most outstanding examples of these is the prenyl flavonoid xanthohumol, (Xn) which is unique to hops.

For example, last year the German Cancer Research Centre reported that Xn showed positive activity in a range of anti-cancer *in vitro* tests, and has been one of the most promising natural products that they have tested (Gerhauser *et al.*, 2002).

Since xanthohumol is unique to hops, beer would in principle be the only significant dietary source of this compound. Brewers have therefore been interested in assessing the levels of xanthohumol in beer, and also seeking ways to increase its levels. Unfortunately, the brewing process itself creates a problem. Xn, just like the alpha acids, isomerises during boiling and it forms the compound isoxanthohumol (Ix). Although Ix has also shown positive activity in *in vitro* tests, its activity does not look as promising as that of Xn. Therefore, if the brewer wishes to find a method to increase the levels of Xn in beer, this heat-induced isomerisation presents a problem.

For those brewing according to the German purity laws, the options for preventing the isomerisation of Xn are very limited, since hopping after the boil is not permitted. However, in recent work Back (2003) reported that high levels of Xn in beer could be achieved by a combination of high hopping rates with a Xn-enriched product, very late hopping and the use of repitched yeast. With these methods Xn levels of approx. 1 mg/litre were achieved in unfiltered beers. These compare to typical levels of 0.1 mg/litre or below found in most commercial beers and represent a significant improvement.

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**Many herbal remedies are now being tested in sophisticated *in vitro* assays by pharmaceutical researchers, who are keen to use this as a method for new drug discovery. Since hops have a history of being used to treat a range of disorders, hop compounds have come under such scrutiny. Over the last few years there have been many reports published in the medical literature of purified hop compounds showing positive activities in these tests.**

In this paper, high levels of Xn in commercial stout and porter style beers are reported. From laboratory model systems, we conclude that a component in dark malts, with a molecular weight below 3,000, inhibits the isomerisation of Xn. This component may well provide an alternative method for increasing the level of Xn in beer.

## ■ Methods

### Roasted barley extract preparation

Ground roasted barley (20 g) was stirred in 100 ml of distilled water for 5 min at 70 °C. The sample was cooled to room temperature, using an ice bath, centrifuged at 3000 rpm for 1 min, then quickly decanted to a beaker and filtered through fibreglass.

Microfiltration was performed using 6 and 2.7 mm Whatman filters, 0.45 and 0.2 Millipore filters and 10,000 and 3,000 molecular weight filters, successively.

### Boiling experiments

Roasted barley extract (44ml) and 2.5 ml of 400 mg/l Xn stock solution in methanol were made up to 500 ml with distilled water. The solution (2 mg Xn/l) was boiled for one hour and then cooled in an ice bath to room temperature. For the control, the barley extract was added after boiling and cooling.

For the isomerisation experiments pure Xn was supplied by Prof. Becker, University of Saarbrücken/Germany.

### Solid phase extraction and HPLC analysis

#### Materials

All solvents and materials according to method Analytica-EBC 7.8 (Grundwerk 1998):

- Solution A: 0,2 ml of o-phosphoric acid in 50 ml water and 50 ml methanol;
- solution B: 0,2 ml o-phosphoric acid in 100 ml water;
- solution C: 0,1 ml of o-phosphoric acid in 90 ml methanol and 10 ml water;
- solid-phase column (Bakerbond spe octadecyl/C18, 6 ml/1000 mg);
- "Vacubox" (Baker spe 12-G) with vacuum equipment.

#### Solid phase extraction

- Beer is degassed prior to analysis;
- mix 200 ml of (degassed) sample in a beaker with 1 ml of 85 % o-phosphoric acid;
- mount solid-phase column on "Vacubox" and set vacuum to 400 mbar;
- aspirate 20 ml of methanol through it;
- aspirate 10 ml of solution A through it;
- make sure that column does not dry out.
- Aspirate immediately 50 ml of acidified sample through.
- Aspirate 10 ml of solution B through.
- Dry column for 120 seconds by aspirating air through.

- Aspirate 10 ml of solution C through.
- Collect eluate in a 10 ml graduated flask.
- Add solution C to make up 10 ml.
- Mix intensively. This solution is used for HPLC measurement.

*HPLC measurement (modified method Analytica-EBC 7.8)*

Instead of the HPLC column "Nucleosil" currently recommended in the EBC method 7.8 the new column "Nucleodur" recently developed by Macherey-Nagel was used. For detection of Xn in the first part of the programme a wavelength of 370 nm was used. For detection of Ix a wavelength of 290 nm was used.

- Column: Nucleodur 5-100 C18ec, 125x4 (Macherey-Nagel);
- Flow rate: 1 ml/min;
- Temperature: 35 °C;
- Detection: 270 nm (370 nm, 290 nm);
- Eluent A: Methanol;
- Eluent B: 750 ml methanol / 240 ml water / 10 ml orthophosphoric acid;
- Programme:
 

0 – 9 min, 100 % B	270 nm (detection of iso-alpha acids)
	370 nm (detection of Xn)
	290 nm (detection of Ix)
9 – 17 min, 100 % B	314 nm

17 – 25 min, 65 % B	314 nm
25 – 30 min, 100 % B	314 nm

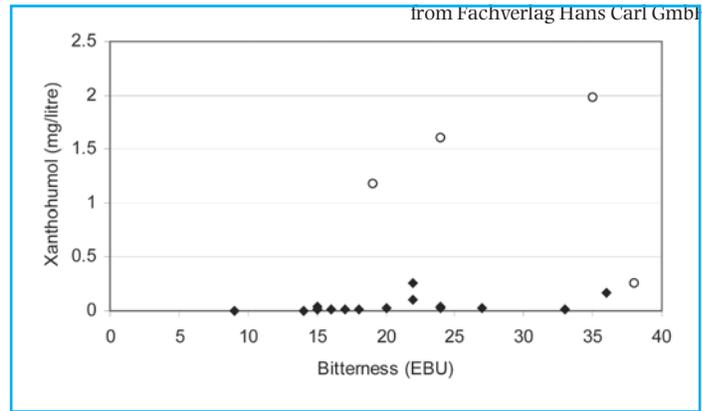
*Preparation of the standard solutions for external calibration*

Accurately weigh to 0,1 mg approximately 0,05 g of pure Xn (or Ix respectively) into a 100 ml graduated flask. Add 50 ml methanol and dissolve the standard using an ultrasonic bath. Make up to volume by meth-

anol at 20 °C. Pipette 1 ml of the solution at 20 °C into a 50 ml graduated flask and make up to volume with methanol at 20 °C. Pure Xn and Ix were purchased from *Phytochem Referenzsubstanzen GbRmbH*, 89335 Ichenhausen/Germany.

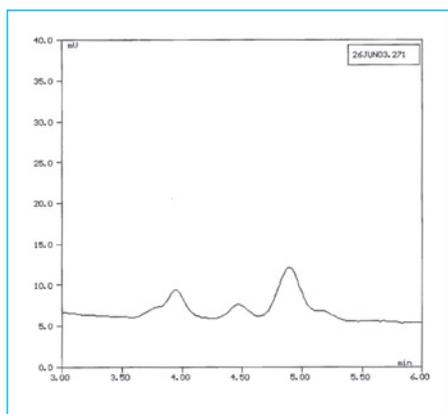
### LC-MS analysis

LC-MS analysis was carried out by the *Government Laboratories (CSL)* at York by

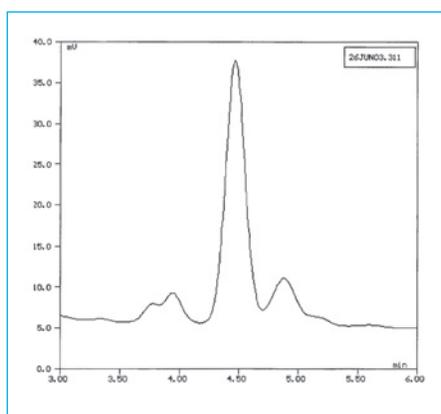


**Figure 1 Xanthohumol content in a variety of commercial beers in relation to their bitterness (open circles represent stout and porter samples; diamonds are lagers and ales)**

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**Fig. 2** HPLC-UV chromatogramme of a Porter with an Xn content < 0.1 mg/l



**Fig. 3** HPLC-UV chromatogramme of a Porter with an Xn content of 1.2 mg/l

**Table 1** Analysis of Xn in stout and porter beers by HPLC

Sample no.	Type	Xanthohumol (mg/litre)
1	Stout	0.5
2	Stout	0.1
3	Porter	0.6
4	Porter	0.2
5	Porter	< 0.1
6	Porter	1.2
7	Stout	0.4
8	Stout	0.2
9	Porter	0.4

Dr Don Clarke. The analysis method was that of Stevens *et al* (1999), and was by quantitative LC-MS/MS (Micromass Quattro Ultima).

## Results

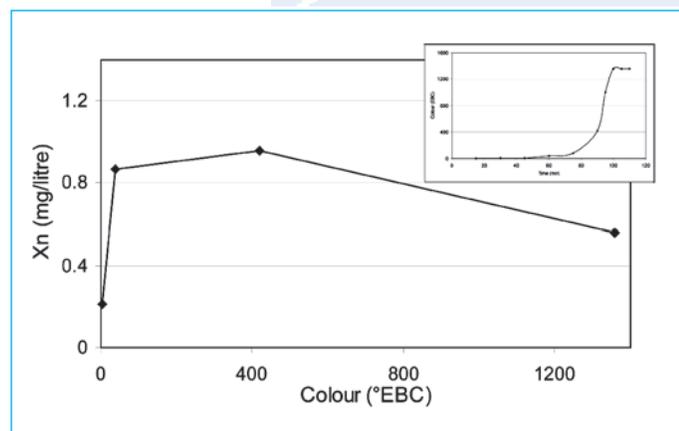
### LC-MS analysis of Xn

Another compound of medical interest is the phytoestrogen 8-prenylarigenin (8PN), which is present in low amounts in

hops. Like Xn, 8-prenylarigenin is a prenyl flavonoid. As a part of our work on surveying the levels of phytoestrogens in beer, we also included a measurement of Xn. Because of its low abundance, 8PN can only be reliably quantitated using a sophisticated LC-MS based analysis (Stevens *et al*, 1999). In this method, beer is injected without treatment onto the HPLC column, and several prenyl flavonoid compounds can be quantitated simultaneously.

**Table 2** The effect of roasting on the production of Xn isomerisation inhibitors

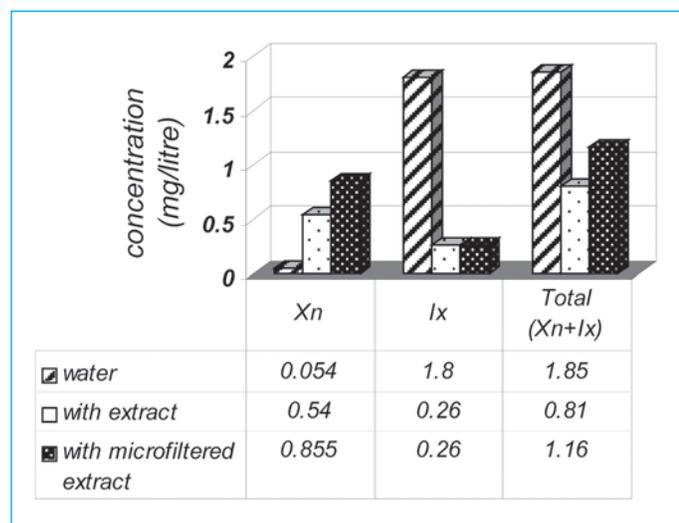
Time (min)	Colour (°EBC)	Xn mg/l	Ix mg/l	Total (Ix+Xn)
0	3	0.21	0.96	1.17
60	37	0.87	0.83	1.7
90	420	0.96	0.49	1.45
100	1360	0.56	0.27	0.83



**Fig. 4** Effect of roasted barely extract on the isomerisation of Xn during boiling

The survey set of beers for this study was designed to represent the range of commercially available beers in the UK, and included 8 lagers, 9 ales and 4 porters/stouts. The results of the phytoestrogen survey itself will be published separately, but the data for Xn are shown in Fig. 1.

The majority of beers had very low levels of Xn as would be expected due to isomerisation during the brewing process. However, three beers were clearly different, containing levels above 1 mg Xn/litre. All of these beers were either porters or stouts, although the Xn level in one stout sample was much lower.



**Fig. 5** Effect of malt extracts on Xn isomerisation according to colour (malt samples were taken at time points during the production of a chocolate malt)

This surprising result matched with a smaller survey made by Stevens and his group in 1999, where the same method of analysis was used. Levels of 0.69 and 0.34 mg Xn/litre were reported for a porter and stout respectively. The authors suggested that late hopping in the wort kettle caused these high levels of Xn. In order to check this theory, we contacted the brewers whose porters and stouts had been included in our survey. They kindly informed us that hopping was at the beginning of the boil, suggesting that there must be some other explanation for these high Xn levels.

The low level in one stout beer is caused by the use of CO<sub>2</sub> extract. This hop product

only contains traces of Xn whereas Xn is transferred almost quantitatively into ethanol extracts and pellets.

#### **Xn analysis by HPLC-UV**

Prior to the LC-MS measurement no purification of the sample was carried out. So it was possible that the presence of interfering substances in these dark beers might effect the quantitation of Xn. Several stout and porter samples were therefore analysed by HPLC-UV (using a modification of the EBC method for iso-alpha acid determination) after solid phase extraction of the beer. According HPLC chromatogrammes are shown in Figs. 2 and 3.

The results of the analysis in Table 1 show that several stouts and porters had very high levels of Xn. The beers came from Ireland (no. 1–3), Finland (no. 4), Denmark (no. 5, 6), the UK (no. 7, 8) and Germany (no. 9). For one beer, levels of 1.2 mg/litre were measured, but this beer also has a very high bitterness (> 50 BU).

Our data indicated that the LC-MS method could have over-estimated Xn levels in beer but the general observation that Xn levels can be unusually high in these dark beers seemed to be accurate.

#### **Laboratory investigations**

A possible explanation for high Xn levels in dark beers, was that a factor in these beers was able to inhibit the isomerisation of Xn. This theory was tested on the laboratory scale in a model system: Xn (2 mg/litre) was boiled in water. A concentrated extract of roasted barley (see methods) was added before or after boiling. Both the levels of Xn and Ix were quantitated after boiling. The results (Fig. 4) showed that when boiled in water, Xn almost quantitatively isomerised. Whereas when boiled with the roasted barley extract there were some losses but the ratio of Xn to Ix was > 2.

We suspected that the presence of particles in the roasted barley extract could be causing this effect by absorbing XN and thus preventing from chemical conversion. Therefore, the particles were removed from the roasted barley extract by microfiltration, such that only compounds with a molecular weight below 3,000 would be present. As shown in Figure 4, this particle-free extract also inhibited Xn isomerisation. The ratio of Xn to Ix was even higher than in case of adding the non filtered extract, suggesting that a chemical rather than a physical effect must be involved.

Using this lab-based system, several malt samples were tested for inhibition of

Xn isomeriation. Both chocolate malt and crystal malt proved to be inhibitory (data not shown). Therefore we tested malts taken at time intervals during the production of a chocolate malt, to see at what point these inhibitory components were forming (Fig. 5).

The data suggested that the components that were inhibiting isomerisation were produced very early during the roasting process, before the maximum phase of colour development takes place. Also, with the highly coloured extract we observed greater losses of Xn and Ix (Table 2).

#### **Conclusions**

Our studies suggest that components present in dark malts inhibit the isomerisation of Xn. These components seem to be soluble, and have a molecular weight below 3,000. Although we were not able to identify the active agents, it seems likely that they might be isolated in the future and used as a “special malt extract” for increasing Xn levels in beer. This method could represent an alternative or complementary technique to those already being proposed in the literature.

Already today commercial Stout and Porter-style beers are showing rather high concentrations of Xn as compared to other beer types. Further increases could be achieved by using a Xn-enriched hop product. A first pilot scale brewing trial already resulted in a Stout showing a Xn concentration of 3 mg/l. At the same time the bitterness was 37 bitter units. This will be reported on separately.

Whether the levels of Xn in beer can be sufficient to have any medical advantage must await results from the medical community on the effectiveness of Xn in animal and human trials.

#### **Acknowledgements**

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#### **References**

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