S. Wunderlich, M. Biendl, A. Zürcher and W. Back

# Reduced Xanthohumol Recovery after Solid Phase Extraction of Dark Beers

**In wort and beer the hop polyphenol xanthohumol (XN) is often determined after solid phase extraction (SPE). Recently researchers described substances in roasted malts that help to enrich XN in dark beers. The carrier effect that reduces losses during beer production may influence the SPE as well. We investigated the XN recovery in dark beer samples with and without SPE sample preparation. In SPE eluates the XN recovery significantly decreased with increasing XN content compared to directly injected samples. Experiments on XN recovery in SPE passes repeatedly treated by SPE showed that this is not due to an overload of the sorbent. The use of SPE prior to XN determination in samples containing roasted substances proved to be inefficient compared to direct injection.**

Descriptors: hops, polyphenol, roasted malt, SPE, HPLC-DAD

## 1 Introduction

*Power et al.* isolated xanthohumol from hops in 1913 for the first time. They named the yellowish substance as xanthohumol (XN) after “xanthos“ (greek: yellow) and „humulus“ (latin: hops) [17]. XN has been investigated by several medical scientists [6, 11, 18, 22, 23]. It showed widespread health benefits such as antioxidative or antimicrobial properties that have been summed up by *Malaghäes et al.* [15].

Beer is the only beverage that is traditionally produced with hops. But XN (Table 1) is extensively eliminated during the brewing process due to its non-polar properties or it is transformed into its isomeric flavanone isoxanthohumol [10, 19]. So, because of the potential benefits of XN, various researchers work on technologies for its enrichment in beer [1, 2, 3, 9, 13]. Special interest is on XN enrichment by roasted substance in dark beers. Several authors described the “carrier-effect“ of roasted substances that results in significantly increased XN recoveries in beer [4, 5, 24]. Investigations by *Wunderlich et al.* confirmed this result and the authors pointed out that these relevant roasted substances occur in chocolate roasted malt [26]. Formation of these substances parallels colour development. They also appear to have a molecular size between 300 and 600 kDa. Due to these “carrier”-substances, XN contents above 10 mg/l can be attained in beer. Higher grist load ratios of chocolate roasted malt may cause a burnt taste, but the resulting beers can be filtered or stabilised by PVPP without major losses [25, 26].

Authors:
Dipl.-Ing. Sascha Wunderlich*, TU München-Weihenstephan, Lehrstuhl für Technologie der Brauerei I, Germany; Dr. Martin Biendl, Hopsteiner – Hallertauer Hopfenveredlungsgesellschaft, Germany; Dr. Achim Zürcher, Feldschlösschen Getränke AG, Switzerland; Prof. Dr. Werner Back, TU München-Weihenstephan, Lehrstuhl für Technologie der Brauerei I, Germany; *corresponding author: sascha.wunderlich@wzw.tum.de

Tables and figures see Appendix

Up to now neither MEBAK [16] nor Analytica-EBC [7] describe a specific XN analysis method in hops and beer. Other publications on XN use liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) or high performance liquid chromatography coupled with diode array detector (HPLC-DAD) applications [3, 8, 12, 14, 20, 24, 25]. The latter is more likely to be available in the modern brewing laboratory. It records spectra at each time point by the simultaneous detection of multiple wavelengths. Often EBC methods 7.7 and 7.8 [7] are used to determine iso-alpha-, alpha-, and beta-acids in hops and isomerised hop extracts. XN is co-extracted during sample preparation of these methods. It appears in the chromatogram and can be easily determined. Detection of XN in beer is more difficult due to the complex matrix. Some authors suggest a sample clean up and analyte concentration by solid phase extraction (SPE). *Walker et al.* carried out investigations on XN levels in stout and porter beers [24]. They observed higher XN contents in samples measured by LC-MS without SPE clean up compared to measurements by HPLC and SPE sample preparation [24]. The efficiency of SPE procedures, which are based on adsorption and desorption under different chemical conditions, may be influenced by roasted substances that proved to be strong “carriers”. The aim of this study was to investigate suitability of sample clean-up by SPE for dark beer prepared with roasted malt.

## 2 Materials and methods

### Experimental beers

An XN enriched beer and a conventional beer were produced. Initially both were prepared with pale wort using exclusively Pilsner malt. At the start of wort boiling liquid malt colour solely produced from chocolate roasted malt (SINAMAR®, Weyermann®, Germany) was added to achieve a wort colour of 80 EBC. Bitterness hopping was achieved by carbon dioxide extract to generate 75 mg alpha-acid/l. Additionally, an 80 % (w/w) XN hop product (XANTHOFLAV®, Hopsteiner®, Germany) was used for the XN enriched beer. This was added 5 min before end of boiling to achieve 40 mg XN/l. Yeast (15,000,000 cells/ml) was pitched into both 12° Plato cast worts. Fermentation proceeded for 7 days at 12°C and was followed by 2 days of maturation and one week of storage at 0°C. Both beers were filtered by KS 200 sheet filters (Pall Corporation, Germany). Finally the XN enriched and the conventional beers were blended to different XN contents (0 to 10 mg XN/l) at similar colour of 80 EBC.

**Sample preparation SPE**

Chemicals for SPE clean up were all of HPLC grade and purchased from J.T. Baker, Holland. Solutions for conditioning, washing, and elution were acidified by phosphoric acid (20 $\mu$l of 85 % per 10 ml). Preparing the SPE, C18ec columns (Strata 18E, 1000 mg, 6 ml, Phenomenex, Germany) were washed with 10 ml methanol and conditioned by 10 ml of an acidified methanol-water solution (1:2, v:v). Beer samples (100 ml) were sonicated to remove carbon dioxide. Foam residues that clung to the vessel were resolved by gently agitating the sample. Solids were separated by centrifugation at 3640 g for 15 min. Prior to SPE, the samples were carefully decanted and 10 ml supernatant was applied per column (Fig. 1). Slowly (approx. 3 ml/min) the samples were pulled through the column by vacuum (“pass”). The sorbent was cleaned up with 10 ml demineralised, acidified water (“washing water”). After 45 sec of drying by applying a vacuum, XN was eluted with 7 ml acidified methanol into a 10 ml volumetric flask, which was finally filled up to 10 ml with acidified methanol (“eluate”). The cleaned up sample was filtered through a syringe filter (Macherey-Nagel chromafill PET-45/25, Germany) into a vial for HPLC detection, discarding the first 2 ml as forerun.

**Sample preparation direct injection**

Analogous to SPE sample preparation, beer samples were treated in an ultrasonic bath and centrifuged. The supernatant was decanted and then directly transferred into a vial for HPLC detection.

**Further investigations**

Besides measurement of samples without SPE clean up and complete SPE clean up, respectively, the “pass” (Fig. 1) was directly injected to the HPLC system to identify XN that is not retained by the column material. In further investigations, the “pass” of one beer sample (10 mg XN /l) was repeatedly cleaned up (three times) by SPE. In doing so, 20 ml sample was applied to the SPE column and the resulting “passes” were analysed by direct injection.

**HPLC parameters**

XN contents were determined by a Hewlett Packard 1090 Series II HPLC system coupled with an internal DAD. The HPLC conditions are described in table 2. Chromatograms of XN were manually integrated. Pure XN (Phytochem, Germany) was used as external standard for quantification.

## 3 Results and discussion

In comparison to direct injection the XN recovery in SPE cleaned up samples decreases with increasing XN content (Fig. 2). Additionally, the XN recovery in the SPE pass increases, so that the sum of eluate and pass XN contents is approximately the same as the XN content determined by direct injection. Therefore further losses during the washing step can be neglected. Reasons for the reduced performance of the SPE may be due to an overload of the C18 sorbent. The pass was tested again by repeated SPE (Fig. 3). Although the XN content decreases from SPE to SPE, there is always a similar portion that is not retained by the sorbent. This indicates that the predominant equilibrium between the mobile and solid phases influences XN retention. It seems likely that this is due to the reaction between XN and chocolate roasted substances, which change chemical properties of XN or at least result in the formation of complexes that have different chemical properties. These chemical properties allow inhibition of XN isomerisation observed by Walker et al. [24] and may explain the finding by Wunderlich et al. that PVPP treatment is less efficient for XN removal in dark beers than in pale beers [26]. The high XN concentrations in dark beers (e.g. 10 mg XN/l), that significantly exceed the ethanol:water (5:95; v:v) solubility of XN (3.5 mg/l, Table 1) confirm that chemical properties have changed.

## 4 Summary

The aim of this study was to identify influences of SPE on XN recovery in dark beers. Measurements of directly injected samples were compared to samples measured after SPE clean up. With increasing XN content the XN recovery in SPE eluats decreased. Repeated SPE clean up of SPE passes revealed that reduced efficiency of SPE is not due to an overload of the sorbent but due to changed chemical conditions. We conclude that the SPE clean-up methodology applied in this study is not a suitable approach for the quantification of XN in liquids with roasted substances.

**Acknowledgements**

*The investigations were supported by the Weihenstephaner Jubiläumsstiftung 1905 e.V. and the Wissenschaftsförderung der Deutschen Brauwirtschaft e.V. (B81 I). We thank the Malzfabrik Weyermann for providing us with raw materials.*

## 5 References

1. Back, W.: Ausgewählte Kapitel der Brauereitechnologie, Fachverlag Hans Carl GmbH, Nürnberg, Germany, 2005.

2. Back, W.; Zürcher, A. and Wunderlich, S.: Natural extract containing xanthohumol, and method for the production thereof and products produced therefrom, Patent Publication number WO/2006/066941, 2006.

3. Biendl, M.; Mitter, W.; Peters, U. and Methner, F. J.: Einsatz eines xanthohumolreichen Hopfenproduktes bei der Bierherstellung, BRAUWELT, **140** (2000), pp. 2006-2011.

4. Biendl, M. and Walker, C.: The effects of Stout and Porter brewing technology on levels of hop compound Xanthohumol, Scandiniavan Brewer‘s Review, **61** (2004), pp. 20-22.
5. Biendl, M.; Methner, F.-J.; Stettner, G. and Walker, C.: Brauversuche mit einem xanthohumolreichen Hopfenprodukt, BRAUWELT, **144** (2004), pp. 236-241.
6. Buckwold, V. E.; Wilson, R. J.; Nalca, A.; Beer, B. B.; Voss, T. G.; Turpin, J. A.; Buckheit, R. W.; Wei, J.; Wenzel-Mathers, M.; Walton, E. M.; Smith, R. J.; Pallansch, M.; Ward, P.; Wells, J.; Chuvala, L.; Sloane, S.; Paulman, R.; Russell, J.; Hartman, T. and Ptak, R.: Antiviral activity of hop constituents against a series of DNA and RNA viruses, Antiviral research, **61** (2004), pp. 57-62.
7. EBC-Analysis-Committee (ed.): Analytica-EBC, Fachverlag Hans Carl GmbH, Nürnberg, 2008.
8. Forster, A.; Gahr, A.; Ketterer, M.; Beck, B. and Massinger, S.: Xanthohumol in Bier – Möglichkeiten und Grenzen, Monatsschrift für Brauwissenschaft, **55** (2002), pp. 184-194.
9. Forster, A.; Ketterer, M. and Gahr, A.: Ein neuartiger xanthohumolangereicherter Hopfenextrakt und sein Einsatz für unfiltrierte Biere, Hopfen Rundschau International, 2003, pp. 65-71.
10. Forster, A. and Koeberlein, A.: Der Verbleib von Xanthohumol aus dem Hopfen während der Bierbereitung, BRAUWELT, **138** (1998), pp. 1677-1679.
11. Gerhauser, C.; Alt, A.; Heiss, E.; Gamal-Eldeen, A.; Klimo, K.; Knauft, J.; Neumann, I.; Scherf, H. R.; Frank, N.; Bartsch, H. and Becker, H.: Cancer chemopreventive activity of Xanthohumol, a natural product derived from hop, Molecular cancer therapeutics, **1** (2002), pp. 959-969.
12. De Keukeleire J.; Janssens, I.; Heyerick, A.; Ghekiere, G.; Cambie, J.; Roldan-Ruiz, I.; van Bockstaele E. and de Keukeleire D.: Relevance of organic farming and effect of climatological conditions on the formation of alpha-acids, beta-acids, desmethylxanthohumol, and xanthohumol in hop (*Humulus lupulus L.),* Journal of Agricultural and Food Chemistry, **55** (2007), pp. 61-66.
13. Magalhães, P. J.; Dostalek, P.; Cruz, J. M.; Guido, L. F. and Barros, A. A.: The Impact of a Xanthohumol-Enriched Hop Product on the Behavior of Xanthohumol and Isoxanthohumol in Pale and Dark Beers: A Pilot Scale Approach, Journal of the Institute of Brewing, **114** (2008), pp. 246-256.
14. Magalhães, P. J.; Guido, L. F.; Cruz, J. M. and Barros, A. A.: Analysis of xanthohumol and isoxanthohumol in different hop products by liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry, Journal of Chromatography A, **1150** (2007), pp. 295-301.
15. Magalhães, P. J.; Carvalho, D. O.; Cruz, J. M.; Guido, L. F. and Barros, A. A.: Fundamentals and health benefits of xanthohumol, a natural product derived from hops and beer, Natural Product Communications, **4** (2009), pp. 591-610.
16. Pfenninger, H. (ed.): Brautechnische Analysenmethoden. Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK) Band III, Freising-Weihenstephan, 1996.
17. Power, F.B.; Tutin, F. and Rogerson, H.: The Constituents of Hops, Journal of the Chemical Society, Transactions, **103** (1913), pp. 1267-1292.
18. Stevens, J. F.; Miranda, C. L.; Frei, B. and Buhler, D. R.: Inhibition of peroxynitrite-mediated LDL oxidation by prenylated flavonoids. The alpha,beta-unsaturated keto functionality of 2’- hydroxychalcones as a novel antioxidant pharmacophore, Chemical research in toxicology, **16** (2003), pp. 1277-1286.
19. Stevens, J. F.; Taylor, A. W.; Clawson, J. E. and Deinzer, M. L.: Fate of xanthohumol and related prenylflavonoids from hops to beer, Journal of Agricultural and Food Chemistry, **47** (1999), pp. 2421-2428.
20. Stevens, J. F.; Taylor, A. W. and Deinzer, M. L.: Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography tandem mass spectrometry, Journal of Chromatography A, **832** (1999), pp. 97-107.
21. Thieme Chemistry (ed.): RÖMPP Online – Version 3.1, Stuttgart, 2008.
22. Tobe, H.; Muraki, Y.; Kitamura, K.; Komiyama, O.; Sato, Y.; Sugioka, T.; Maruyama, H. B.; Matsuda, E. and Nagai, M.: Bone resorption inhibitors from hop extract, Bioscience, biotechnology, and biochemistry, **61** (1997), pp. 158-159.
23. Vogel, S. and Heilmann, J.: Synthesis, cytotoxicity, and antioxidative activity of minor prenylated chalcones from Humulus lupulus, Journal of Natural Products, **71** (2008), pp. 1237-1241.
24. Walker, C.; Lence, C. F. and Biendl, M.: Untersuchungen zum hohen Xanthohumol-Gehalt in Bieren vom Typ Stout/Porter, BRAUWELT, **143** (2003), pp. 1709-1712.
25. Wunderlich, S.; Back, W.: Brewing technological limits of xanthohumol enrichment in beer, Proceedings of the 31th EBC Congress, Fachverlag Hans Carl , Nürnberg, Germany, 2007, pp. 997-1003
26. Wunderlich, S.; Zürcher, A. and Back, W.: Enrichment of xanthohumol in the brewing process, Molecular Nutrition & Food Research, **49** (2005), pp. 874-881.

*Received 09 June, 2009, accepted 06 July, 2009*

# Appendix

**Table 1** **Chemical properties of XN [21]**

| | |
|---|---|
| CAS registry number | 569-83-5, 6754-58-1 |
| Synonym | 1-[2,4-dihydroxy-6-methoxy-3-(3-methyl-but-2-enyl)-phenyl]-3-(4-hydroxy-phenyl)-propenone |
| Molecular formula | $C_{21}H_{22}O_5$ |
| Molecular weight | 354.40 |
| Melting point | 172°C |
| solubility | |
| in $H_2O$ | 0.0013 g/l (23°C), 0.0011 g/l (8°C) |
| in ethanol: $H_2O$ (5:95; v:v) | 0.0035 g/l (23°C), 0.0018 g/l (8°C) |

**Table 2** **HPLC-DAD conditions for XN detection**

| | |
|---|---|
| Column | Macherey-Nagel EC-125/4<br>Nucleodur 100-5 C18ec<br>Ser.No.: 7111-087 |
| Flow rate | 0.8 ml/min |
| Temperature | 38°C |
| Injection volume | 25 $\mu$l |
| Eluate A | Distilled $H_2O$ with 1 % phosphoric acid |
| Eluate B | Acetonitrile |
| Eluate gradient | 0–3 min: A = 60 %, B = 40 %<br>3–11,5 min: A = 25 %, B = 75 %<br>11,5–16 min: A = 0 %, B = 100 %<br>16–18 min: A = 60 %, B = 40 % |
| Wavelength | 370 nm |

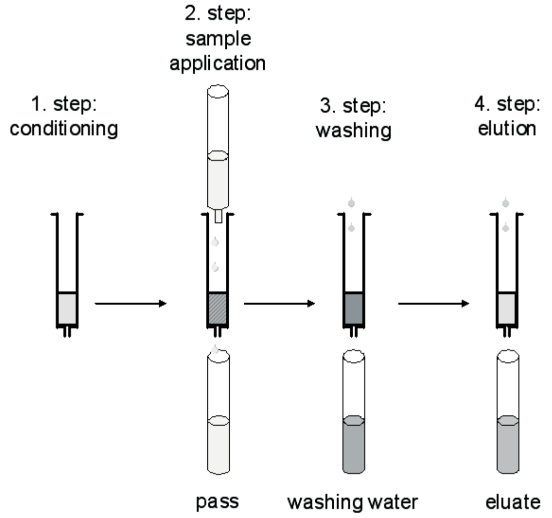
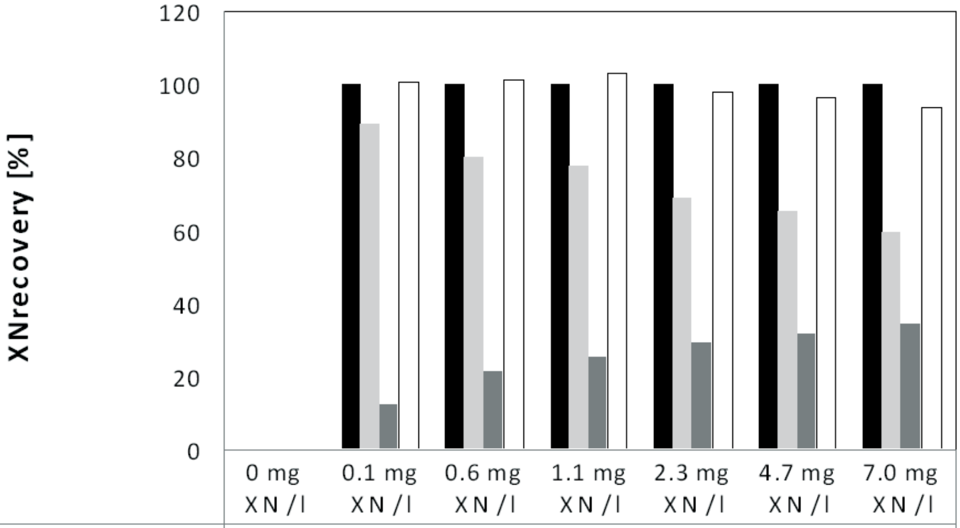
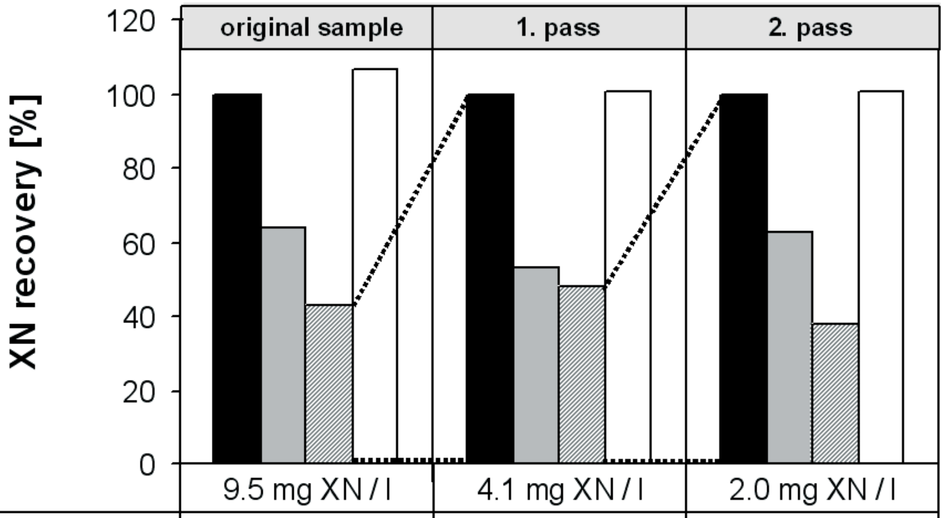


**Fig. 1** **SPE clean up**



| | 0 mg XN / l | 0.1 mg XN / l | 0.6 mg XN / l | 1.1 mg XN / l | 2.3 mg XN / l | 4.7 mg XN / l | 7.0 mg XN / l |
|---|---|---|---|---|---|---|---|
| ■ direct injection % | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| ■ complete SPE % | 0 | 89 | 80 | 78 | 69 | 65 | 60 |
| ■ pass % | 0 | 12 | 21 | 25 | 29 | 32 | 34 |
| □ Σ complete SPE and pass % | 0 | 101 | 101 | 103 | 98 | 97 | 94 |

**Fig. 2** XN recovery with and without SPE sample preparation



| | 9.5 mg XN / l | 4.1 mg XN / l | 2.0 mg XN / l |
|---|---|---|---|
| ■ direct injection % | 100 | 100 | 100 |
| ■ complete SPE % | 64 | 53 | 63 |
| ▨ pass % | 43 (4.1 mg XN / l) | 48 (2.0 mg XN / l) | 38 |
| □ Σ complete SPE and pass % | 107 | 101 | 101 |

**Fig. 3** XN recovery in passes repeatedly treated by SPE