C. Schmidt, A. Lagemann, A. Stephan and G. Stettner

Impact of the Use of Inline Pre-isomerized Hop Products on Analytical and Sensory Markers for Beer Ageing

The main quality challenge of beer is the change of its chemical composition during storage. It is known that the typical bitterness of fresh beer declines in the intensity and changes in the quality with an increasing age of the beverage. The bitter tasting compounds *trans*-iso-α-acids converse into lingering and harsh bitter tasting tri- and tetracyclic degradation products. In order to investigate the evaluation of beer flavour stability, the behaviour of *trans*-iso-α-acids, the *trans/cis* ratio of bitter acids, and the formation of tricyclocohumol and tricyclohumol as representatives of acidic-catalyzed degradation products of *trans*-iso-α-acids were chosen as analytical markers in wort, fresh beer as well as in 2 and 4 months aged beer samples with and without inline pre-isomerization. The characteristics of these analytical parameters were determined using HPLC-DAD and HPLC-MS/MS analysis. Supplementary to quantitative data, fresh beer as well as 2 and 4 months stored beer samples with or rather without the use of pre-isomerization were evaluated for the attributes aroma, taste, and beer ageing by a trained sensory panel.
The performed experiments with a hop yield enhancer in comparison to conventional hopping showed no effect on the flavour stability of Pilsener beer. Nevertheless, the tricyclic degradation products tricyclocohumol and tricyclohumol were suitable as solid analytical parameters to estimate the flavour stability during storage.

Descriptors: iso-α-acids, pre-isomerization, beer ageing, tricyclic degradation products, HPLC-MS/MS analysis, sensory evaluation

1 Introduction

Non-volatile bitter compounds of beer have been investigated in the last few decades, and it is agreed upon that the typical beer bitterness is caused by adding hop products such as pellets or extracts during wort boiling. A number of isomerization processes during the wort boiling procedure have been reported to be of major importance for bitter taste development in the final beer product. Moreover, the iso-α-acids have been identified as the major bitter contributors in beer [1] and were demonstrated to be generated upon a re-arrangement reaction of their hop-derived precursors, namely the α-acids cohumulone (1a), humulone (1b), and adhumulone (1c) (e.g.Fig.1) [2, 3]. The iso-α-acids occur as *cis* (2a-c) and *trans* isomers (3a-c) with three different alkanoyl side chains (e.g.Fig.1). Already *De Cooman* et al. [4] pointed out that particularly the *trans*-iso-α-acids (3a-c) are prone to degradation. In contradiction to previous findings, *Intelmann et al*. [5, 6] revealed an acid-catalytic decomposition pathway for *trans*-iso-α-acids (3a-c) to tri- and tetracyclic degradation products. An overview over the formation pathway of the tricyclic degradation products tricyclocohumol (4a), tricyclohumol (4b), and tricycloadhumol (4c) demonstrates figure 1. The degradation compound tricyclocohumol (4a) was generated in this connection by carbonyl-ene-reaction of *trans*-iso-cohumulone (3a) [6].

The main quality challenge of beer products is the change of its chemical composition during storage for which reason the evaluation of beer flavour stability is usually based on the determination of one or few analytical parameters like e.g. the change in concentration of *trans*-2-nonenal, strecker aldehydes, β-damascenone or the *trans*/*cis* ratio of the bitter acids [7-10]. The consequence of beer ageing during the storage is a significant decrease in the intensity of the bitterness as well as a change of the bitter taste quality accompanied by the descent of the *trans*-iso-α-acids [11].

Corresponding to literature bitter compounds are detected by a specific subset of taste receptor cells of the tongue and palate epithelia and characterized by the expression of members of the TASTE 2 G protein-coupled Receptors (TAS2R or T2R) gene family encoding bitter taste receptor candidates [12-14]. Elucidating the candidate receptors which mediate the bitter taste of hop-containing beverages such as beer *Intelmann et al*. [15] identified three hTAS2R bitter taste receptors hTAS2R1, hTAS2R14, and hTAS2R40 responding to the bitter compounds from hop and beer including α-acids, β-acids, *cis*- and *trans*-iso-α-acids, isoxanthohumol, xanthohumol, and 8-prenylnaringenin.

To study the possibilities to enhance the hop yield during the brewing process, *Hertel and Dillenburger* examined the significant parameters regarding the behaviour of bitter-acids among the different manufacturing steps [16-18] and presented the benefits of a hop yield enhancer which can influence the degree

Authors

Christina Schmidt, Dr. Annika Lagemann, Dr. Andreas Stephan, Dr. Georg Stettner, Bitburger Braugruppe GmbH, Bitburg, Germany; corresponding author: christina.schmidt@bitburger-braugruppe.de

Tables and figures see Appendix

of the isomerization of α-acids in all hop products in a way that maximum possible isomerization ratio can be achieved in a very short period by increasing the solubility of the bitter-acids [19]. In comparison to the conventional hopping where high amounts of unisomerized bitter components precipitate during the fermentation step, these substances keep solved up to the resulting beer. A significant increase of the yield of bitter-acids was described as the result of pre-isomerization.

To investigate the influence of pre-isomerization the present study was conducted to monitor the content of iso-α-acids and the tricyclic degradation products of *trans*-iso-α-acids, in particular tricyclocohumol and tricyclohumol, by means of liquid chromatography coupled with mass spectrometry in comparison to sensory behaviour of fresh beer as well as 2 and 4 months aged beer samples with or rather without pre-isomerized hop.

2 Materials and methods

Chemicals and materials

Following chemicals were obtained commercially: methanol, acetonitrile (BDH Prolabo, VWR International, Darmstadt, Germany), methanol (LiChrosolv), ammonium acetate, ortho-phosphoric acid (85 %), citric acid monohydrate, disodium hydrogen phosphate dihydrate (Merck KGaA, Darmstadt, Germany). Deionized water was prepared by Synergy UV and Elix UV water purification systems (Millipore, Billerica, MA).

Quantification of iso-α-acids was done by external calibration using dicyclohexylamine salts of *trans*-iso-α-acids for HPLC analysis of isomerized and reduced isomerized α-acids (DCHA-Iso, ICS-I3, Labor Veritas AG, Zürich, Switzerland).

The standards of tricyclic degradation products were achieved from Chair of Food Chemistry and Molecular Sensory Science (Technische Universität München, Freising, Germany).

The trials were carried out in a 20 hL pilot plant at Bitburger brewery. A standard 2-mash decoction procedure was used to produce Pilsener type beer from 300 kg barley malt and 670 g hops. For pre-isomerization trial hop extract (50 % HHM/25 % HTU/25 % HHS; amount of α-acids: 45.6 %) and hop pellets (50 % HPE/50 % HHT; amount of α-acids: 6.5 %) were heated for 20 minutes at constant pressure (1.2 bar) and temperature (120 °C) in a 10 L pressure vessel in water before adding to the wort. Figure 2 demonstrates the schematic diagram of the used pressure vessel for hop yield enhancing. Wort was boiled for 75 minutes at 100 °C and fermented at 10.5 °C. 50 L kegs were used as filled storage containers. The dosed amount of α-acids was 8.1 g/hL cast wort.

The wort and beer samples were examined fresh, as well as after 2 and 4 months of storage at 28 °C.

Sensory analysis

Beer samples were analyzed by 12 trained assessors in separate sensory cabins using triangle test with followed profile analysis where the panelists had to judge aroma, taste, and beer ageing on a scale from 0 (not detectable) up to 9 (strong perception) in comparison to a fresh Pilsener type beer. Beer samples were always presented in brown glasses. Data evaluation was executed with help of FIZZ software (Biosystemes, Couternon, France).

Solid Phase Extraction

For sample preparation of iso-α-acids, wort and beer samples were cleaned-up using reversed phase solid phase extraction (SPE) columns (Strata C18-E, Phenomenex, Aschaffenburg, Germany). Prior to analysis fresh and stored beer samples were degassed and filtered, while wort samples were filtered after centrifugation. A defined volume of the wort and beer samples (200 mL) was treated with ortho-phosphoric acid (85 %; 400 µL). After conditioning of the SPE columns with water/methanol mixture (50/50; v/v, acidified with 200 µL ortho-phosphoric acid (85 %)), 50 mL of the beer and wort sample, respectively, were given to the SPE columns. The treated columns were washed with acidified water (200 µL ortho-phosphoric acid (85 %)/100 mL water) and eluted with methanol/water (90/10; v/v with 200 µL ortho-phosphoric acid (85 %)). The achieved samples were analyzed by means of HPLC-DAD with following conditions.

High-Performance Liquid Chromatography (HPLC)

BIO-TEK Kontron Instruments HPLC-system consisted of a pump, a degasser, an autosampler, a thermostated column oven and a diode array detector (BIO-TEK Kontron Instruments, Neufahrn, Germany) was used for the quantitative analysis of iso-α-acids (2a-c, 3a-c). For chromatography, an analytical 250 x 4.6 mm, 3 µm, Gemini RP18 column (Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same type was used as the stationary phase at 40 °C and citric acid/phosphate buffer (42.02 g citric acid monohydrate was dissolved in 2 L deionized water and mixed with 17.8 g Na_2HPO_4 x 2 H_2O/500 mL water) as solvent A and acetonitrile/water (90/10; v/v) as solvent B. Monitoring the effluent flow (0.7 mL/min) at 270 nm, chromatography was executed by increasing the amount of solvent B from 50 to 60 % within 18 min, and from 60 to 100 % within 5 min, thereafter, maintaining for 100 % solvent B for additional 17 min.

High-Performance Liquid Chromatography – Mass spectrometry (HPLC-MS/MS)

Shimadzu Corporation HPLC-system consisted of a binary pump, a degasser, an autosampler, and a thermostated column oven (Shimadzu Corporation, Kyoto, Japan) was coupled with API 4000 Q-TRAP mass spectrometer (AB SCIEX, Darmstadt, Germany) equipped with the electrospray ionization (ESI) source running in the negative ion mode.

Samples were introduced by HPLC with a solvent flow of 200 µL/min requiring the use of the turbo gas at a temperature of 450 °C. The ion spray voltage was set to – 4500 V, the declustering potential and the MS/MS parameters were optimized for each substance to induce fragmentation of the pseudo molecular ion $[M-H]^-$ to the corresponding target product ions after collision-induced dissociation. The dwell time for each mass transition was 80 msec. The

collision energy (CE), the declustering potential (DP) as well as the cell exit potential (CXP) were set as given in table 1. Nitrogen was used as the collision gas. The quantification was done using the multiple reaction monitoring (MRM) mode of the instrument with the fragmentation parameters optimized prior to analysis. Data processing and integration was performed by using Analyst software version 1.5 (AB SCIEX, Darmstadt, Germany). Liquid chromatography separation was performed using a 50 x 2.0 mm internal diameter Chromolith® Fast Gradient RP-18 encapped column (Merck KGaA, Darmstadt, Germany) at 40 °C. For elution of the compounds 10 mM ammonium acetate as solvent A and methanol as solvent B was applied. Chromatography was performed by increasing solvent B from 30 to 50 % within 20 min, then to 60 % within 20 min, to 90 % within 5 min, and kept constant for another 5 min. The tricyclic degradation products 4a-b were examined in degassed beer samples without the need of any clean-up procedures.

3 Results and discussion

To study the evaluation of beer flavour stability, the behaviour of *trans*-iso-α-acids (**3a-c**), the *trans*/*cis* ratio of bitter acids (3a-c/2a-c), and the formation of tricyclocohumol (4a) and tricyclohumol (4b) as representatives of acidic-catalyzed degradation products of *trans*-iso-α-acids (3a-c) were chosen as analytical markers in wort, fresh as well as in 2 and 4 months aged beer samples with and without pre-isomerization.

Therefore the amounts of *cis*- and *trans*-iso-α-acids (2a-c, 3a-c) were determined using high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD), while the quantification of the tricyclic degradation products (4a-b) was executed by means of LC-MS/MS analysis. To enhance the selectivity for the degradation products, the experiments were carried out using the multiple reaction monitoring (MRM) mode. To exemplify the received LC-MS/MS results, figure 3 shows a chromatogram of a beer sample. The investigation of the selected samples of wort, fresh and stored beer was feasible without the need of any clean-up procedures. Prior to quantification, the optimization of the MS/MS parameters for the tricyclic degradation products tricyclocohumol (4a) and tricyclohumol (4b) was executed. The achieved total ion chromatogram (TIC) of a beer sample is illustrated on the right side of figure 3. The specific mass transitions of the compounds 4a (m/z 365 → 165) and 4b (m/z 379 → 179) are given on the left side of the same figure. The additional optimized LC-MS/MS parameters are presented in table 1.

Table 2 presents the quantitation results of *cis*- (2a-c) and *trans*-iso-α-acids (3a-c), *trans/cis* ratio of the bitter acids (3a-c/2a-c) and the tricyclic degradation products (4a-b) in wort, fresh, 2 and 4 months aged beer samples without as well as with inline pre-isomerization. The total amount of the analyzed degradation products tricyclocohumol (4a) and tricyclohumol (4b) varied between 0.22 mg/L in wort samples without pre-isomerization and 0.24 mg/L in samples with an inline pre-isomerization, respectively, and 5.0 mg/L in 4 months aged beer samples with and without pre-isomerized hop products (e.g.Table 2). The opposite development of 4a-b in comparison to the *trans*-iso-α-acids (3a-c) demonstrates figure 4. Conversely to the described results of the acidic-catalyzed degradation products (4a-b), the total amount of *trans*-iso-α-acids (3a-c) decreased with an increasing period of storage between 13.54 mg/L in wort samples and 5.87 mg/L in 4 months aged beer samples without pre-isomerization of the used hop products and between 14.14 mg/L (wort) and 6.61 mg/L (4 months) in samples using the handling of the hop yield enhancer (e.g.Fig.4). In accord with literature [4, 9-11], the concentrations of *cis*-iso-α-acids (2a-c) showed only a slight decrease in fresh beer samples compared to the concentrations of 2a-c in wort samples with and without pre-isomerization, respectively. Within the storage the concentrations of 2a-c remained totally unchanged in both types of sample preparation (e.g.Table 2). A*raki et al*. found 2002 a new parameter to estimate the flavour of stored beer without the need of sensory evaluation. Therefore the *trans*/*cis* ratio of the bitter acids must be calculated. This ratio was similar for fresh beer regardless of the beer specification, but it decreased in stored beer with differences between variable beers [10]. In agreement with [10], the *trans/cis* ratio of the investigated samples decreased in a range from 0.46 in wort samples with and without pre-isomerized hop products to 0.24 in beer products without pre-isomerized hop and 0.28 in beer samples with inline pre-isomerization (e.g.Table 2). *Araki et al.* also described a good correlation between the *trans*/*cis* ratio and the stale flavour intensity of beer [10].

Concerning the quantitation results of different analytical markers such as *trans*/*cis* ratio (3a-c/2a-c), the concentrations of *cis*- (2a-c) and *trans*-iso-α-acids (3a-c) as well as the tricyclic degradation products (4a-b), the storage behaviour of diverse beer samples with and without the use of inline pre-isomerization showed no significant difference.

Supplementary to the evaluations by means of high-performance liquid chromatography, the beer samples were analyzed by a trained sensory panel by comparing the attributes aroma, taste, and beer ageing of fresh beer prepared with pre-isomerized hop as well as 2 and 4 months aged beer samples with and without the use of pre-isomerization with those of a fresh Pilsener type beer. Demonstrating in figure 5 the panelists ranged the impressions aroma and taste in a fresh beer sample with 8.7 for aroma in both types of beer and between 8.7 and 8.9 for taste in beer samples without and with pre-isomerized hop products, respectively. Ageing flavour does not play a role in a fresh beer with or rather without pre-isomerization. The results for 2 and 4 months aged beer were ranged, on a scale from 0 (not detectable) to 9 (strong perception), between 5.4 (2 months) and 4.8 (4 months) for the aroma in beer without pre-isomerization and between 6.7 (2 months) and 5.9 (4 months) in beer with pre-isomerized hop. The achieved findings for the taste impression showed values between 4.6 (2 months) and 4.3 (4 months) in samples prepared without pre-isomerization as well as between 6.2 (2 months) and 6.1 (4 months) in beer with pre-isomerized hop products. The ageing flavour was evaluated by the panelists between 4.5 (2 months) and 4.8 (4 months) in beer with conventional hopping and between 2.7 (2 months) and 3.5 (4 months) in beer with a hop yield enhancer.

Similarly to the described quantitation results, the sensory analysis of the fresh and aged beer samples did not yield significant taste differences between the samples with and without pre-isomerized

hop products. Nevertheless, half of the assessors described differences in bitter aftertaste, and ageing flavour in beer samples with pre-isomerized hop products.

4 Conclusion

In this study the influence of the inline pre-isomerization of hop products like extracts and hop pellets on selected analytical and sensory markers for beer ageing were observed. The performed investigations with a hop yield enhancer showed no effect on the flavour stability of beer samples. However, the acidic-catalyzed degradation products tricyclocohumol and tricyclohumol offered solid analytical markers to estimate the flavour stability during storage.

5 References

1. Govaert, F.; Verzele, M.; Anteunis, M.; Fontyn, F. and Stockx, J.: Xanthohumol, Experientia, **13** (1957), pp. 105-160.
2. De Keukeleire, D. and Verzele, M.: The absolute configuration of the isohumulone and the humulinic acids, Tetrahedron, **27** (1971), pp. 4939-4945.
3. Jaskula, B.; Kafarski, P.; Aerts, G. and De Cooman, L.: Kinetic study on the isomerization of hop α-acids, Journal of Agriculture and Food Chemistry, **56** (2008), pp. 6408-6415.
4. De Cooman, L.; Aerts, G.; Overmeire, H. and De Keukeleire, D.: Alterations of the profiles of iso-α-acids during beer ageing, marked instability of trans-iso-α-acids and implications for beer bitterness consistency in relation to tetrahydroiso-α-acids, Journal of the Institute of Brewing, **106** (2000), pp. 169-178.
5. Intelmann, D.; Kummerlöwe, G.; Haseleu, G.; Desmer, N.; Schulze, K.; Fröhlich, R.; Frank, O.; Luy, B. and Hofmann, T.: Structures of storage-induced transformation products of the beer´s bitter principles, revealed by sophisticated NMR spectroscopic and LC-MS techniques, Chemical European Journal, **15** (2009), pp. 13047-13058.
6. Intelmann, D.; Demmer, O.; Desmer, N. and Hofmann, T.: ^{18}O stable isotope labeling, quantitative model experiments, and molecular dynamics simulation studies on the trans-specific degradation of the bitter tasting iso-α-acids of beer, Journal of Agriculture and Food Chemistry, **57** (2009), pp. 11014-11023.
7. Lermusieau, G.; Noël, S.; Liegéois, C. and Collin, S.: Nonoxidative mechanism for development of *trans*-2-nonenal in beer, Journal of the American Society of Brewing Chemists, **57** (1999), pp. 29-33.
8. Bravo, A.; Herrera, J. C.; Scherer, E.; Ju-Nam, Y.; Rübsam, H.; Madrid, J.; Zufall, C. and Rangel-Aldao, R.: Formation of α-dicarbonyl compounds in beer during storage of pilsner, Journal of Agriculture and Food Chemistry, **56** (2008), pp. 4134-4144.
9. Vanderhaegen, B.; Neven, H.; Verachtert, H. and Derdelinckx, G.: The chemistry of beer aging – a critical review, Food Chemistry, **95** (2006), pp. 357-381.
10. Araki, S.; Takashio, M. and Shinotsuka, K.: A new parameter for determination of the extent of staling in beer, Journal of the American Society of Brewing Chemists, **60** (2002), pp. 26-30.
11. Intelmann, D.; Haseleu, G. and Hofmann, T.: LC-MS/MS Quantitation of hop-derived bitter compounds in beer using the ECHO technique, Journal of Agriculture and Food Chemistry, **57** (2009), pp. 1172-1182.
12. Adler, E.; Hoon, M. A.; Mueller, K. L.; Chandrashekar, J.; Ryba, N. J. P. and Zuker, C. S.: A novel family of mammalian taste receptors, Cell, **100** (2000), pp. 693-702.
13. Chadrashekar, J.; Mueller, K. L.; Hoon, M. A.; Adler, E.; Feng, L.; Guo, W.; Zuker, C. S. and Ryba N. J. P.: T2Rs function as bitter taste receptors, Cell, **100** (2000), pp. 703-711.
14. Meyerhof, W.; Bartram, C.; Kuhn, C.; Brockhoff, A.; Chudoba, E.; Bufe, B.; Appendino, G. and Behrens, M.: The molecular receptive ranges of human TAS2R bitter taste receptors, Chemical Senses, **35** (2010), pp. 157-170.
15. Intelmann, D.; Bartram, C.; Kuhn, C.; Haseleu, G.; Meyerhof, W. and Hofmann, T.: Three TAS2R bitter taste receptors mediate the psychophysical responses to bitter compounds of hops (*Humulus lupulus L.*) and beer, Chemosensory Perception, **2** (2009), pp. 118-132.
16. Hertel, M. and Dillenburger, M.: Möglichkeiten zur Erhöhung der Bitterstoffausbeute bei der Bierbereitung (Teil 1), BRAUWELT, **10** (2009), pp. 254-257.
17. Hertel, M. and Dillenburger, M.: Möglichkeiten zur Erhöhung der Bitterstoffausbeute bei der Bierbereitung (Teil 2), BRAUWELT, **14** (2009), pp. 394-398.
18. Hertel, M. and Dillenburger, M.: Möglichkeiten zur Erhöhung der Bitterstoffausbeute bei der Bierbereitung (Teil 3), BRAUWELT, **19** (2009), pp. 532-534.
19. Hertel, M. and Dillenburger, M.: Möglichkeiten zur Erhöhung der Bitterstoffausbeute bei der Bierbereitung (Teil 4), BRAUWELT, **23** (2009), pp. 640-644.

Received 31 August 2011, accepted 14 October, 2011

Appendix

Table 1 **Specific mass transitions and optimized parameters for the LC-MS/MS analysis of tricyclocohumol (4a) and tricyclo-humol (4b) using electrospray ionization in the negative mode (ESI^-)**

compound no.[a]	mass transition *m/z* Q1 → Q3	DP[b] [V]	CE[c] [V]	CEP[d] [V]
4a	365 → 165	- 81	- 52	- 10
4b	379 → 179	- 105	- 47	- 10

[a] Numbering of compounds and chemical structures refer to figure 1. [b] Declustering potential. [c] Collision energy. [d] Cell exit potential.

Table 2 **Quantitation results of *cis*- and *trans*-iso-α-acids (2a-c, 3a-c), *trans/cis* ratio of the bitter acids (3a-c/2a-c) and degradation products (4a-b) in wort, fresh beer, 2 and 4 months aged (28 °C) beer samples without (A) and with pre-isomerization (B)**

compound no.[a]	wort[b]		fresh beer[b]		2 months[b]		4 months[b]	
	A	B	A	B	A	B	A	B
2a-c	29.69	30.60	23.71	24.23	23.86	24.26	24.18	23.49
3a-c	13.54	14.14	10.42	10.85	8.29	8.65	5.87	6.61
3a-c/2a-c	0.46	0.46	0.44	0.45	0.35	0.36	0.24	0.28
4a	0.08	0.10	0.16	0.15	0.81	0.79	1.86	1.73
4b	0.14	0.14	0.45	0.42	1.30	1.25	3.13	3.03

[a] Numbering of compounds and chemical structures refer to figure 1.
[b] Results are given in mg/L

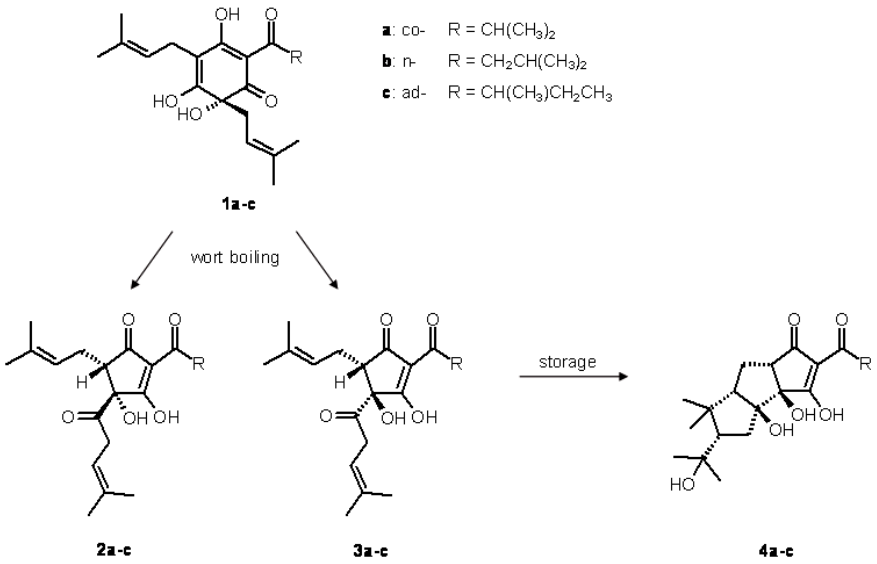

Fig. 1 **Formation of *cis*- (2a-c) and *trans*-iso-α-acids (3a-c) by isomerization reaction of α-acids (1a-c) during wort boiling, and the formation of tricyclic degradation products (4a-c) during storage of beer**

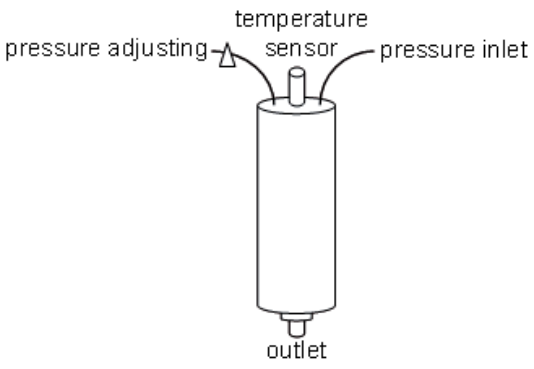

Fig. 2 **Schematic diagram of used pressure vessel for hop yield enhancing**

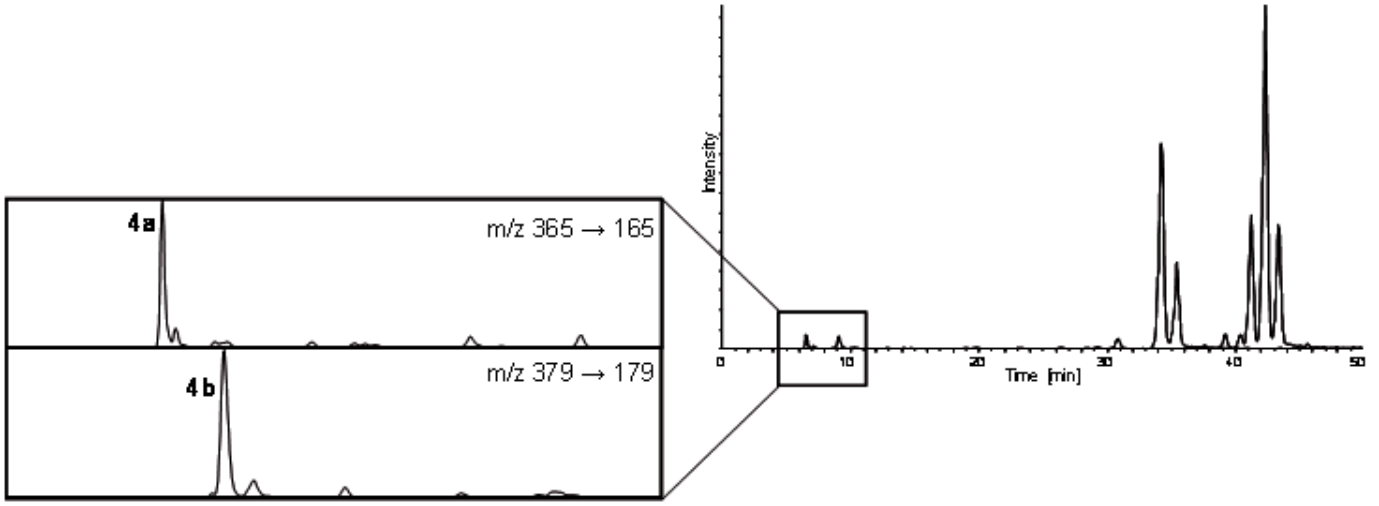

Fig. 3 HPLC-MS/MS chromatogram of a beer sample: Total ion chromatogram (right side) and mass transitions of tricyclocohumol (4a) and tricyclohumol (4b) with normalized signal intensity (left side)

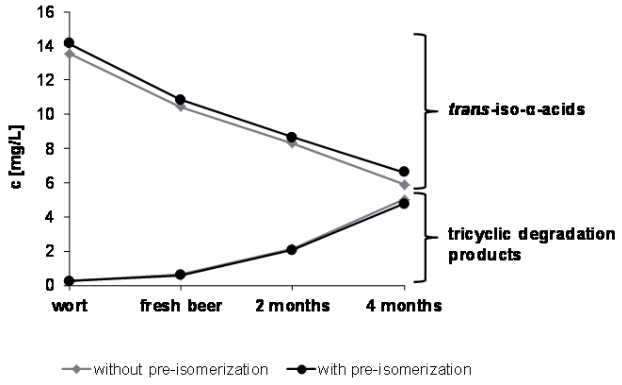

Fig. 4 Comparison of analytical markers 3a-c and 4a-b analyzed in wort, fresh, 2 and 4 months aged (28 °C) beer samples

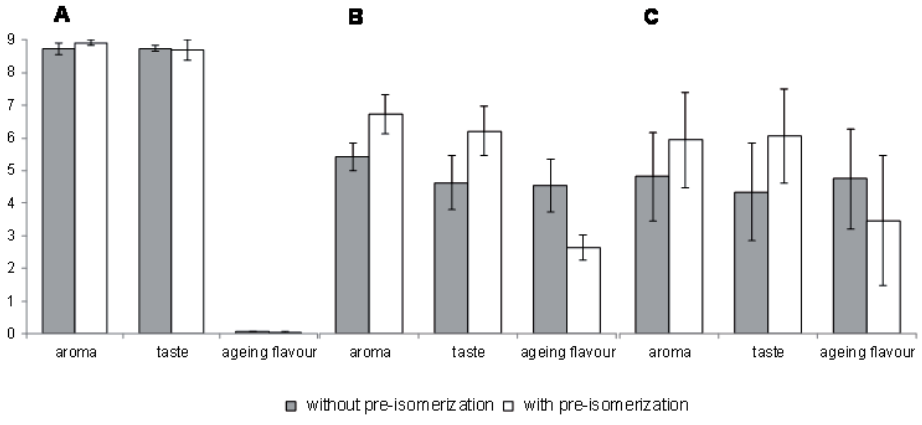

Fig. 5 Results of the sensory evaluation, given with standard deviation, of fresh (A), 2 months aged (B), and 4 months aged (C) (28 °C) beer samples