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CHANGES IN THE PHENOLIC ACID CONTENT DURING WORT BOILING AND WHIRLPOOL

Dominik Szwajgier, Monika Bancarzewska
University of Life Sciences in Lublin

Background. Phenolic acids were repeatedly pointed out as powerful antioxidants. The studies in the past prove the differences in the phenolic acids content in malts and worts. In this work, the influence of wort boiling and whirlpool separation on the phenolic acid content was studied.
Material and methods. Worts were produced in the local brewery by the infusion method using pale pilsner-type barley malt. Samples were analysed at the beginning of the boil, after the boil and after whirlpool separation (5 and 30 min). Free and total alkali extractable phenolic acids contents were analysed using HPLC-DAD.
Results. The main phenolic acid in all worts was ferulic acid in the free (35.47 ±3.28--117.51 ±4.40 mg·dm^{-3}) as well as total alkali extractable form (193.49 ±4.84-294.72 ±2.65 mg·dm^{-3}). With both forms no decrease was seen after boiling of wort (80 min at 100-100.5°C) followed by wort separation in the whirlpool. Similarly, no significant changes in the free and total form of p-coumaric acid content were seen.
Conclusions. It can be concluded that an elevated temperature during wort boiling and separation in whirlpool had no significant influence on the content of phenolic acids (at least in the case of the specific mashing program applied in this brewery: equipment, enzyme preparations, mashing, time-temperature parameters etc.). The differences in the phenolic acids levels could be rather attributed to different supplies of malt used for the production.

Key words: ferulic acid, phenolic acid, wort, wort boiling, antioxidant

INTRODUCTION

Barley, a cereal commonly used in the brewing industry, contains considerable amounts of phenolic antioxidants among which phenolic acids, flavan-3-ols derivatives and flavon glycosides are the most prominent [Gorinstein et al. 2000, Liu and Yao 2007, Pascoe and Ames 2003]. Phenolic acids were previously pointed out as powerful anti-

Corresponding author – Adres do korespondencji: Dr inż. Dominik Szwajgier, Department of Biotechnology, Human Nutrition and Science of Food Commodities of University of Life Sciences in Lublin, Skromna 8, 20-704 Lublin, Poland, e-mail: dominik.szwajgier@up.lublin.pl

oxidants *in vitro* [Cuvelier et al. 1992, Gerhäuser 2005, Maillard and Berset 1995, Maillard et al. 1996, Nardini et al. 1995] and *in vivo* [Itagaki et al. 2009, Liu and Yao 2007, Saija et al. 2000, Tanaka et al. 1993, Young et al. 2008] with ferulic acid as a very active biomolecule. During the brewing, the content of phenolic acids can be decreased after each production stage [Olthof et al. 2003]. Especially, it can be diminished in the presence of oxygen (at elevated temperatures) or some microorganisms due to the formation of volatile phenolic flavour compounds [Hughes 1997, Walters et al. 1997, Vanbeneden et al. 2008 a, b]. Ferulic acid is the main phenolic acid present in barley, malt [Maillard and Berset 1995, Maillard et al. 1996] and in beer [Nardini et al. 2002 a, Nardini and Ghiselli 2004, Vanbeneden et al. 2006] in the ester form with arabinoxylans. Ester-bound phenolic acids can be released in the gastrointestinal tract by bacterial esterases [Couteau et al. 2001] and can be directly accessible via intestinal absorption [Nardini et al. 2006]. It was proved that ferulic acid played a crucial role in the prevention of Alzheimer's disease and this acid was far more potent than vanillic, coumaric or cinnamic acid [Kanski et al. 2002, Joshi et al. 2006]. Moreover, it was active against β-amyloid-42 induced toxicity in mice brains in long-term experiments [Yan et al. 2001]. The concept that phenolic acids can be the most important group of food phenolic antioxidants is supported by the considerable number of papers proving that these compounds are not degraded in the human gastrointestinal tract prior the absorption [Ghiselli et al. 2000, Hollman and Katan 1998, Nardini et al. 2002 b, 1997, 2006, Olthof et al. 2003, Plumb et al. 1999, Rechner et al. 2002, Rondini et al. 2002, Scalbert and Williamson 2000]. Indeed, a considerable content of phenolic acids was detected in blood [Choudhury et al. 1999] and in urine [Bourne and Rice-Evans 1998]. The more complex phenolics are prone to microbial degradation in the intestines (e.g. proanthocyanidins [Deprez et al. 2000]) so their real *in vivo* antioxidant activities must be revised. Also, the excretion of phenolic acids after the consumption of more complex phenolic compounds was pointed out in the past [Clifford et al. 2000, Deprez et al. 2000, Gross et al. 1996, Rechner et al. 2001 a, b]. Taking under consideration the important role of phenolic acids, especially ferulic acid as a strong, natural antioxidant, the changes in phenolic acids concentrations at different points of wort boiling with hops and whirlpool separation were studied.

MATERIAL AND METHODS

Chemical reagents

Phenolic acids standards (purity 99% or higher) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade reagents were used (P.O.Ch. Gliwice, Poland) for HPLC-DAD. For other analyses, reagents of analytical grade were used (P.O.Ch. Gliwice, Poland).

Worts

The worts (provided by Perła Browary Lubelskie S.A., Lublin, Poland) were produced using a lager brewery malt (for lager pale bottom-fermented pilsner beer). Two production lines (later reffered as L1, L2) were parallelly used. The two lines were simi-

lar to each other except the minor difference in the diameter of the filtration vessels. Therefore, two paralell sets of wort samples originating from two mashing vessels (Perła "Strong" 14.0% (w/w), later referred as 14L1 and 14L2) were collected and directly analysed. Samples of worts were produced using the standard infusion method including the following production stages: heating up to approx. 52°C (protein pause), 63°C (β-amylase pause), 72°C (α-amylase pause) and 76-78°C (including pumping into filtration tank), wort filtration, wort boiling with hops, whirlpool separation. The enzyme preparations Viscozyme and Celluclast (both at 0.5 g/kg of malt) were added to the mash at the mashing-in at 52°C. After one month, other two sets of worts (Perła "Full", 12.2% (w/w), later referred as 12.2L1 and 12.2L2) were produced and analysed in the same way but with the use of the another supply of malt of the same kind. The selected parameters of malts as declared by the producer were as follows: total protein content 10.7%, Kolbach index (38-42% depending on malt supply), malt colour ≤ 3.5°EBC, colour after boiling ≤ 4.8 EBC, extractability 78-80%, Hartong 45°C in the range of 38-40, congress wort viscosity < 1.45 mPas. All worts were boiled with hops granulates for 80 min at approx. 100-100.5°C (slight overpressure). Non-isomerized, type 90 granulates were used (bittering hops, alpha-acids content 6.7% at the boiling-start, aromatic hops at the end of the boiling, alpha-acids content 4.0%). Directly after the boiling, worts were pumped into whirlpool. In this work, the following stages of beer production were analysed: boiling-start, boiling-end, wort separated for 5 and 30 min in whirlpool.

Extraction of free phenolic acids from worts

Worts were centrifuged (6°C, 20 min, 12 000 x g) and phenolic acids were extracted according to Nardini and Ghiselli [2004] with some modifications. After the adjustment of pH to 1.0 using HCl solution (2 $M \cdot dm^{-3}$), 0.5 g of KCl was added to each wort. Samples were then extracted three times by vortexing (BioVortex V1 Plus, Biosan, Latvia), each time using a new portion (10 cm^3) of ethyl acetate. After each extraction, wort sample was centrifuged (6°C, 20 min, 12 000 x g), ethyl acetate phase was transferred into a separate tube and another portion of ethyl acetate was added. The whole ethyl acetate phase was then evaporated to dryness (35°C, 0.01 MPa). The sample was directly dissolved in methanol (10 cm^3), filtered (Millipore, 0.45 μm) and directly analysed in HPLC-DAD system. Extraction was in triplicate and mean values were calculated.

Mild alkaline hydrolysis and extraction of phenolic acids from worts

The hydrolysis was performed according to Nardini et al. [2002 a] with slight modifications. 30 cm^3 of wort was mixed with 15 cm^3 of NaOH solution (2 $M \cdot dm^{-3}$) containing ethylenediamine-tetraacetic acid (10 $mM \cdot dm^{-3}$) and 1% (w/w) of ascorbic acid and hydrolyzed in darkness for 30 min, followed by the adjustment of pH to 1.0 using HCl solution (2 $M \cdot dm^{-3}$) and addition of KCl (0.5 g). The sample was then extracted using ethyl acetate as described above and phenolic acids content was analysed using HPLC--DAD as described below. The recovery of all phenolic acid standards (1 $mM \cdot dm^{-3}$) was in the range of 93% to 101% (detailed data not shown) was used during the recalculation of HPLC-DAD results.

HPLC-DAD

The HPLC system consisted of two Gilson 306 Separation Module piston pumps, Gilson PhotoDiode Array Detector 170, Gilson loop (0.02 cm^3), manometric module Gilson 805, dynamic mixer 811C and Gilson 234 Autoinjector. Waters Symmetry C18 column (USA, 250 mm, 4.6 mm i.d., 5 μm), and Waters Symmetry® C_{18} pre-column (5 μm, 8 × 20 mm) were used for the separations. Eluents used were: A-1% (v/v) acetic acid solution in deionized water. Eluent B – 50% (v/v) HPLC-grade acetonitrile in deionized water. Signals were monitored at 320 nm, 280 nm, 260 nm and 360 nm. The program applied was as follows: START 92% A, 8% B 0-10 min; 70% A, 30% B 10-40 min; 60% A, 40% B 40-55 min; 92% A, 8% B 55-70 min. The eluent flow was 0.8 $cm^3 \cdot min^{-1}$ (2050 p.s.i.). The coefficients of variation for ferulic acid and p-coumaric acid HPLC standards in within-one-day repeatability tests were 3.5% and 5.0%, respectively. In the analysis of reproducibility of HPLC method, the coefficients of variation were 4.7% and 6.1%, for ferulic and p-coumaric acid, respectively. The statistical analysis of the results was performed using STATISTICA 8.0 (StatSoft, Poland). The routine statistical calculations were applied (mean values calculation with standard deviation, one-dimenstional and multidimensional analysis of variations). The $p \leq 0.05$ value was taken as the measure of the statistically significant results.

RESULTS

Ferulic acid was the most abundant phenolic acid, both in the free (Fig. 1) as well as in the total alkali extractable form in all worts (Fig. 2). The differences in the free ferulic acid content (ranging from 35.47 ±3.28 $mg \cdot dm^{-3}$ to 117.51 ±4.40 $mg \cdot dm^{-3}$) in four worts (Fig. 1) can be attributed to the differences in the malt:water ratio and the use of different malt supplies. The total alkali extractable ferulic acid content (in the range of 193.49 ±4.84 to 294.72 ±2.65 $mg \cdot dm^{-3}$) was significantly ($p \leq 0.05$) higher than corresponding free ferulic acid content in the case of all worts. The free ferulic acid per-cent share in the total alkali extractable ferulic acid content in worts 14L1, 14L2, 12.2L1 and 12.2L2 in whirlpool was 50.2%, 37.5%, 24.8% and 35.1%, respectively. It must be underlined that no significant ($p > 0.05$) changes in the free ferulic acid content during wort boiling and whirlpool separations were seen in the case of all studied worts. In the case of 14L1, 14L2 worts (Fig. 2) the significant ($p \leq 0.05$) decrease of the total alkali extractable ferulic acid content was seen when the boiling-start and the end of the separation in whirlpool are compared. In the case of 12.2L1 wort, a significant ($p \leq 0.05$) increase of the total alkali extractable ferulic acid content was seen after the separation in whirlpool (30 min) in comparison to the corresponding content at boiling-start. No statistically significant ($p > 0.05$) change of the total alkali extractable ferulic acid was seen in the case of 12.2L2 wort during the whole process. Taking under consideration the action of the temperature during the boiling of wort and the decrease of the wort volume during cooling, the increase or stabilization of the total alkali extractable ferulic acid levels can be assumed. It can be supposed that the high temperature had no or restricted influence, at least in this study, on the change of ferulic acid contents in worts, in free as well as in the bound form.

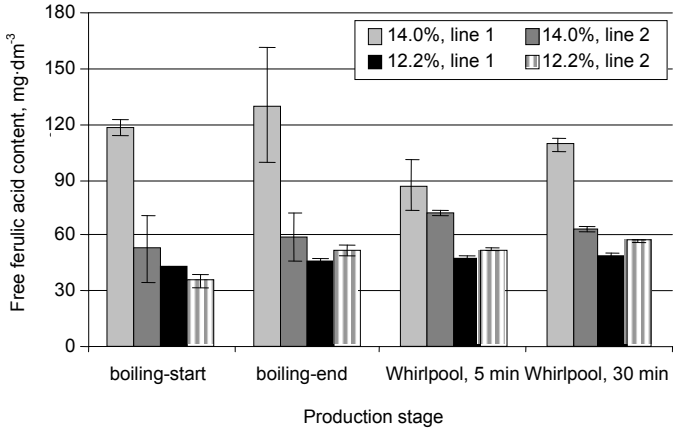

Fig. 1. Free ferulic acid concentrations in worts (n = 3)

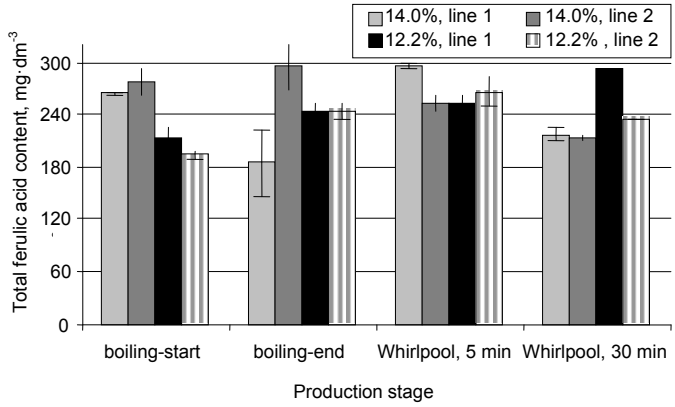

Fig. 2. Total (free + alkali extractable) ferulic acid concentrations in worts (n = 3)

Other phenolic acids separated and successfully identified in HPLC-DAD system were: p-coumaric, syringic, protocatechuic, 4-OH-benzoic, chlorogenic, caffeic and sinapic acid. In the case of worts 14L1 and 14L2, no significant ($p > 0.05$) changes in the free p-coumaric acid content (Table 1) during the whole production process were seen except the significant ($p \leq 0.05$) decrease of the free p-coumaric acid content in 14L1 wort after 30 min in whirlpool. In the case of 12.2L1 wort, the significant ($p \leq 0.05$) increase of the free p-coumaric acid content was seen in whirlpool after 30 min of separation in comparison to the boiling-start. In the case of 12.2L2 wort the significant ($p \leq 0.05$) increase of the free p-coumaric content was observed during the whole process. The total alkali extractable p-coumaric acid content in 14L2, 12.2L1 and 12.2L2 worts (Table 2) was significantly ($p \leq 0.05$) higher than the corresponding free forms. In the case 14L1 wort, no statistically significant increase of the p-coumaric acid content after the hydrolysis was seen except the end of the separation in whirlpool

Table 1. Free p-coumaric acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**16.96 ±0.13[a]**	18.27 ±3.91	19.09 ±0.70	**19.48 ±0.79[b]**
12.2 (L2)	**14.39 ±2.40[a]**	20.78 ±0.94	22.19 ±0.04	**24.22 ±0.10[b]**
14.0 (L1)	**37.07 ±0.20[a]**	31.80 ±4.92	28.31 ±0.16	**24.51 ±0.98[b]**
14.0 (L2)	22.80 ±2.82	19.76 ±4.50	21.99 ±0.96	21.05 ±0.29

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

Table 2. Total alkali extractable p-coumaric acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**24.54 ±0.98[a]**	28.56 ±1.11	29.14 ±0.12	**32.28 ±0.49[b]**
12.2 (L2)	**23.59 ±0.97[a]**	31.39 ±2.26	36.89 ±3.70	**31.68 ±0.09[b]**
14.0 (L1)	**39.54 ±1.43[a]**	44.98 ±11.75	29.63 ±4.52	**28.08 ±1.43[b]**
14.0 (L2)	27.50 ±1.37	31.78 ±4.12	27.83 ±0.61	25.76 ±1.22

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

($p \leq 0.05$). The changes in the free caffeic acid content in 14L1 and 14L2 worts were not significant ($p > 0.05$) during the process except the significant ($p \leq 0.05$) elevation of the caffeic acid content in 14L1 wort cooled for 5 min (Table 3), but this result must be revised in the future. Also, it is worth to mention that the free caffeic acid content in 14L1 wort was significantly ($p \leq 0.05$) lower than the corresponding content in other worts at the same production stages. On the other hand, the results obtained in the case of less concentrated worts (12.2L1, 12.2L2) pointed out the significant ($p \leq 0.05$) increase of free caffeic acid content after each step of the wort production. The free caffeic acid content after the alkaline hydrolysis (Table 4) at least partly originated from the hydrolysis of chlorogenic acid so the discussion of these results can be omitted. No significant ($p > 0.05$) changes in the free chlorogenic acid content were seen in the case of three wort series during the wort boiling and separation (14L1, 12.2L1 and 12.2L2; Table 5). After the alkaline hydrolysis, chlorogenic acid content in wort sample was decreased due to the alkaline hydrolysis (detailed data not shown, personal communication dominik.szwajgier@up.lublin.pl). The changes of the free sinapic acid content were not significant ($p > 0.05$) in 14L1, 12.2L1 and 12.2L2 worts but the significant ($p \leq 0.05$) decrease of this acid content was seen in 14L2 wort after wort--boiling. Free sinapic acid content was lower in both more concentrated worts (14L1, 14L2; Table 6) than in the more diluted ones (12.2L1 and 12.2L2) and this can be explained probably by the use of different malt supplies during the wort production.

Table 3. Free caffeic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**3.91 ±0.01**[a]	4.32 ±0.09	4.94 ±0.17	**5.20 ±0.52**[b]
12.2 (L2)	**2.86 ±0.24**[a]	4.23 ±0.21	4.39 ±0.82	**5.43 ±0.04**[b]
14.0 (L1)	0.12 ±0.02	0.11 ±0.03	2.41 ±1.67	0.13 ±0.02
14.0 (L2)	8.30 ±4.39	4.29 ±0.93	4.95 ±0.57	4.90 ±0.01

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

Table 4. Total alkali extractable caffeic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**3.13 ±0.06**[a]	3.23 ±0.40	4.05 ±0.01	**4.79 ±0.24**[b]
12.2 (L2)	**3.15 ±0.05**[a]	4.62 ±0.38	6.30 ±0.24	**5.13 ±0.24**[b]
14.0 (L1)	**19.23 ±0.10**[a]	14.11 ±2.96	10.91 ±5.87	**16.86 ±0.82**[b]
14.0 (L2)	**21.15 ±1.07**[a]	24.27 ±1.23	23.33 ±0.30	**16.61 ±0.46**[b]

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

Table 5. Free chlorogenic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**7.58 ±0.01**[a]	8.47 ±0.26	9.06 ±0.35	**9.28 ±0.43**[b]
12.2 (L2)	2.99 ±0.07	3.89 ±0.32	5.19 ±0.30	3.19 ±0.06
14.0 (L1)	6.32 ±0.20	6.52 ±0.91	5.27 ±0.81	6.88 ±0.46
14.0 (L2)	**1.71 ±0.91**[a]	2.51 ±0.87[b]	0.42 ±0.01[c]	**0.46 ±0.01**[b]

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

After the alkaline hydrolysis the significant ($p \leq 0.05$) increase of the total (free + released) sinapic acid contents was seen in all samples of the worts (Table 7). The assumption of the significant differences in the contents of phenolic acids in both of supplies of malts was strongly supported because free protocatechuic acid was detected in both less concentrated worts 12.2L1, 12.2L2 (Table 8) and not in the more concentrated ones. The content of free as well as total alkali extractable protocatechuic acid

Table 6. Free sinapic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**4.14 ±0.24**[a]	3.81 ±0.55	4.77 ±0.05	**5.31 ±0.02**[b]
12.2 (L2)	**3.93 ±0.16**[a]	4.88 ±0.32	4.36 ±0.28	**4.71 ±0.06**[b]
14.0 (L1)	**2.74 ±0.02**[a]	1.54 ±0.03	2.77 ±0.02	**2.43 ±0.03**
14.0 (L2)	**1.76 ±1.61**[a]	3.12 ±1.04	not detected	**0.10 ±0.02**[b]

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

Table 7. Total alkali extractable sinapic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	6.42 ±0.09	6.66 ±0.13	6.63 ±0.22	6.77 ±0.22
12.2 (L2)	**4.64 ±0.37**[a]	6.58 ±0.20	7.19 ±0.04	**7.84 ±0.06**[b]
14.0 (L1)	**8.36 ±0.39**[a]	8.89 ±3.48	5.92 ±0.94	**7.35 ±0.20**[b]
14.0 (L2)	**8.75 ±0.60**[a]	6.44 ±0.01	6.30 ±0.15	**4.08 ±0.15**[b]

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

Table 8. Free protocatechuic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**0.32 ±0.01**[a]	0.61 ±0.14	1.02 ±0.05	**1.19 ±0.17**[b]
12.2 (L2)	**0.37 ±0.20**[a]	0.60 ±0.10	1.15 ±0.06	**1.37 ±0.04**[b]

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

in 12.2L1, 12.2L2 worts increased significantly ($p \leq 0.05$) after wort-boiling and also after separation in whirlpool for 5 min. Then, the statistically insignificant changes of this phenolic acid content were seen after the last step of separation (Tables 8, 9) except the free protocatechuic acid content in wort 12.2L2 (Table 8) which increased until the end of the separation in whirlpool. Free 4-OH-benzoic acid content in more concentrated worts was lower than corresponding content in the more diluted ones (Table 10). Moreover, 4-OH-benzoic acid content in 14L1, 14L2 worts was constant during the whole production process. The free 4-OH-benzoic acid content significantly ($p \leq 0.05$) increased in whirlpool after 5 min of separation or at the end of wort boiling (12.2L2).

Table 9. Total alkali extractable protocatechuic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**0.52 ±0.02**[a]	0.64 ±0.05	0.98 ±0.06	**1.13 ±0.14**[b]
12.2 (L2)	**0.42 ±0.06**[a]	0.68 ±0.07	1.19 ±0.06	**1.00 ±0.05**[b]
14.0 (L2)	not detected	0.38 ±0.01	0.23 ±0.02	0.43 ±0.02

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

Table 10. Free 4-OH-benzoic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	0.84 ±0.34	0.93 ±0.07	1.31 ±0.06	1.29 ±0.01
12.2 (L2)	**0.30 ±0.20**[a]	1.12 ±0.02	2.02 ±0.06	**1.98 ±0.07**[b]
14.0 (L1)	0.30 ±0.02	0,28 ±0.01	0.16 ±0.02	0.32 ±0.01
14.0 (L2)	0.64 ±0.01	0.47 ±0.06	0.61 ±0.02	0.61 ±0.01

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

This phenolic acid was present in all worts predominantly in the free form (as seen in Tables 10 and 11). Last but not least, syringic acid was simultaneously present in both free and total alkali extractable form only in one, less concentrated wort (12.2L1, Tables 12 and 13), but the free form of the acid was detected in this wort only in the boiling kettle and not in the whirlpool. At each step of beer production, total alkali extractable syringic acid content was higher ($p \leq 0.05$) than corresponding free acid content. In 12.2L2 and 14L2 worts, the significant ($p \leq 0.05$) increase of the total alkali extractable syringic acid was seen from the boiling-start until the separation in whirlpool for 5 min followed by the decrease of the acid content after 30 min of the separation (Tables 12 and 13). The presented results confirm that the most distributed phenolic acids in worts were ferulic acid and p-coumaric (in free as well as total alkali extractable forms), followed by chlorogenic and caffeic acid. It was shown that most of the major phenolic acids were present in all studied wort samples in ester-bound, water soluble forms. It is interesting that the content of some minor phenolic acids (e.g. syringic, protocatechuic, 4-OH-benzoic acid) was higher in the more diluted worts (12.2% (w/w)) what was probably caused by the use of different malt supplies. The total alkali extractable ferulic acid or p-coumaric contents were similar in all four worts and this was especially clearly seen in the case of both major phenolic acids in samples taken from whirlpool. The worts were produced using parallel production lines and the main difference was the diameter of the filtration vessels. In that case, it can be assumed that the obtained results were affected by the malt supplies used rather than the minor differences in the construction of the equipment in both lines.

Table 11. Total alkali extractable 4-OH-benzoic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	0.73 ±0.02	1.02 ±0.32	1.26 ±0.13	1.90 ±0.07
12.2 (L2)	**0.77 ±0.20**[a]	1.79 ±0.05	2.50 ±0.06	**1.68 ±0.30**[b]
14.0 (L1)	0.41 ±0.03	0.5 ±0.02	0.37 ±0.02	0.33 ±0.02
14.0 (L2)	0.47 ±0.01	1.75 ±0.05	0.23 ±1.76	not detected

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

Table 12. Free syringic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	0.81 ±0.03	0.60 ±0.15	not detected	not detected
14.0 (L2)	2.83 ±0.22	3.59 ±0.89	5.66 ±3.82	2.44 ±0.35

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

Table 13. Total alkali extractable syringic acid content in worts, n = 3

Wort, %	Phenolic acid content, mg/L			
	wort boiling-start	wort boiling-end	wort cooling, 5 min	wort cooling, 30 min
12.2 (L1)	**1.47 ±0.06**[a]	1.56 ±0.12	2.24 ±0.07	**2.57 ±0.05**[b]
12.2 (L2)	**1.52 ±0.08**[a]	2.39 ±0.37	3.46 ±0.09	**2.36 ±0.37**[b]
14.0 (L2)	2.83 ±0.22	3.59 ±0.89	5.66 ±3.82	2.44 ±0.35

Statistically significant ($p \leq 0.05$) changes within one wort at the beginning and the end of the process are marked by superscripts (a, b).

DISCUSSION

This work focused on free phenolic acids changes during wort boiling and whirlpool. The concentration of free forms of these compounds in worts can be indirectly connected to the extent of off-flavors formation (via decarboxylation) [Vanbeneden et al. 2008 a] during wort boiling, fermentation or beer maturation and storage. Therefore, the changes in free phenolic acids content in worts were more thoroughly analysed whereas the total alkali extractable phenolic acid content seem to be less important from this point of view.

The source of phenolic acids in beer is malt (in Europe usually barley malt) exclusively, especially when the *Rheinheitsgebot* is obeyed, as in the case of the herein presented study. Due to the inconsiderable addition of hops portion into wort in comparison to malt, the role of phenolic acids from hops can be omitted. Many previous studies undoubtely prove that the main phenolic acid in malts and worts was ferulic acid. For example, Floridi et al. [2003] identified a wide range of free phenolic acids in worts (ferulic, p-coumaric, chlorogenic, gallic, protocatechuic, p-OH-benzoic, 2,6-di-OH--benzoic, vanillic, caffeic, m-OH-benzoic, syringic, sinapic, o-m-coumaric, gallic acid). Vanbeneden et al. [2006] detected ferulic acid (2.63 $mg \cdot kg^{-1}$), followed by p-coumaric and sinapic acid (1.48 and 0.13 $mg \cdot kg^{-1}$, respectively) as the most abundant phenolic acids in the experimental beer. Vanbeneden et al. [2007] detected that the main free phenolic acids in pale malts were ferulic (42.4-70.2 $\mu g \cdot g^{-1}$ of dry malt) and p-coumaric acid (6.1-13.0 $\mu g \cdot g^{-1}$ of dry malt). Both phenolic acids were also present in significant concentrations in the bound form in all corresponding Congress worts (3.95-5.73 $mg \cdot kg^{-1}$ and 0.17-0.50 $mg \cdot kg^{-1}$ for ferulic acid and p-coumaric acid, respectively). Another subject of the studies is the evolution of phenolic acids content in worts and beers. Pascoe and Ames [2003] determined the phenolic acids content in worts at the early stages of the mashing and in beers up to 10 weeks of beer storage. These authors proved that ferulic acid was the most abundant phenolic acid during the whole brewing process and the addition of hops had no effect on the content of phenolic acids. The boiling of wort caused a significant increase of the p-coumaric acid content whereas no change in the concentration of other phenolic acids was seen. Whirlpool separation caused no change in the phenolic acids levels in wort. These results are in agreement with herein presented work. Other authors studied the content of hydroxycinnamic acids and corresponding volatile phenols in Congress worts and different types of beers (bottom-fermented pilsner, top fermented ale beers, Belgian white beers, German Weizenbeers, blond and dark specialty beers). The considerable loss of ferulic acid was caused by the decarboxylation leading to 4-vinylguaiacol formation [Vanbeneden et al. 2008 a]. No 4-vinylguaiacol was detected in the wort heated up to 80°C and the significant decrease of the free ferulic acid content during wort heating at 90°C--100°C yielding 4-vinylguaiacol was seen. The linear relationship between the loss of ferulic acid and the time of wort heating at both temperatures was found. Vanbeneden et al. [2008 b] studied the effect of mashing-in temperature, time and pH of mashing, grist coarseness and composition and stirring regime on ferulic acid release into wort. The authors detected the elevation of the free ferulic acid content at mashing-in until the end of the holding time at 62°C. From this point of time, the free ferulic acid content in wort was constant until the mashing-off and filtration in the lauter tun. At this stage of wort production, the decrease of free ferulic acid content was attributed to the dilution of wort after sparging with water. The authors concluded that the increase of the free ferulic acid content by 10% after the boiling of wort for 90 min could be attributed to the evaporation of water. After the whole period of whirlpool hold, the free ferulic acid content decreased by 9% partially due to the transformation to 4-vinylguaiacol. This result is in agreement with our observations because no decrease of the ferulic acid content was seen in our worts after the boiling. Vanbeneden et al. [2008 b] suggested that the increase of the free ferulic acid content in the boiled wort could be caused by hop addition but these authors did not study this problem in their work. As it was previously stressed, the work of Pascoe and Ames [2003] as well as our HPLC-DAD analyses (results not shown) suggest that hop addition had no influence on the phenolic acids

content in worts. Another interesting aspect is the form of phenolic acid presence in wort, because bound (predominantly esterfied) forms of phenolic acids are less prone to decarboxylation. After the consumption, phenolic acids can be deesterified by the bacterial esterases and absorbed from the gastrointestinal tract. Phenolic acids in beer were found mainly in the bound form and the mild alkaline hydrolysis resulted in a considerable increase of the free ferulic, p-coumaric, caffeic, sinapic and vanillic acids content [Nardini and Ghiselli 2004]. Other phenolic acids were present in wort only in the free form. This observation is in agreement with our results. On the other hand, in other worts [Vanbeneden et al. 2007] p-coumaric acid was present mainly in the free and ferulic acid mainly in the bound form.

Phenolic acids can play an important role as wort and beer phenolic antioxidants because of their considerable contribution to the total phenolics content in beer. Fantozzi et al. [1998] determined that as much as 82% of the total phenolics in worts were non-tannin, non-flavonoid compounds, mainly phenolic acids. Moreover, phenolic acids percent participation in the total phenolics content in wort increased until the filtered and non-pasteurised beer. This phenolic acids to total phenolics ratio was not changed after the beer pasteurisation and bottling. It can be supposed, that phenolic acids-rich beers can be produced from worts containing elevated concentrations of phenolic acids, mainly in bound forms. The possibility of preventing of phenolic acids from the release leading to the risk of their decarboxylation should be studied in the nearest future.

SUMMARY

1. In all studied worts, ferulic acid was the most abundant phenolic acid, both in the free and in the total alkali extractable form. Other most distributed free acids were p-coumaric followed by chlorogenic and caffeic acid.

2. In the case of free p-coumaric, caffeic, protocatechuic or 4-OH-benzoic, either no significant changes in the acid content during the wort boiling or whirlpool was seen or the increment of the acid content was observed at different stages of wort production.

3. No significant changes in the free chlorogenic acid content during the wort boiling and whirlpool were seen in the case of three worts.

4. The free sinapic acid content changes were not significant in three studied worts whereas the decrease of the acid level was seen in the case of one wort after wort boiling.

5. The levels of free sinapic, protocatechuic, 4-OH-benzoic and syringic acids were higher in the more diluted worts (12.2%) than in the more concentrated (14.0%).

6. The differences in the free phenolic acids between the individual worts can be attributed to the use of different malt supplies rather than to the influence of the production parameters.

7. Taking into consideration the above presented conclusions, it can be stated that the free phenolic acids content in worts was not influenced by the wort boiling and whirlpool separation.

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ZMIANY ZAWARTOŚCI KWASÓW FENOLOWYCH PODCZAS GOTOWANIA BRZECZKI PIWNEJ I KLAROWANIA W WHIRLPOOLU

Wstęp. Kwasy fenolowe są wskazywane jako bardzo efektywne związki o charakterze przeciwutleniaczy. Badania wykonane w przeszłości wskazują na różnice w zawartościach kwasów fenolowych w słodach i brzeczkach. Celem pracy było sprawdzenie wpływu gotowania brzeczki oraz jej klarowania w separatorze Whirlpool na zawartości kwasów fenolowych.

Materiał i metodyka. Brzeczki były produkowane w miejscowym browarze metodą infuzyjną z użyciem jasnego jęczmiennego słodu pilzneńskiego typu lager. Próbki brzeczek analizowano na początku gotowania, po gotowaniu oraz po klarowaniu w separatorze Whirlpool (po 5 and 30 min). Zawartości kwasów fenolowych wolnych oraz po alkalicznej hydrolizie (całkowite zawartości kwasów fenolowych) analizowano za pomocą HPLC-DAD.

Wyniki. Kwas ferulowy był głównym kwasem fenolowym we wszystkich próbach brzeczek piwnych, zarówno w formie wolnej (35,47 ±3,28-117,51 ±4,40 $mg \cdot dm^{-3}$), jak i po hydrolizie alkalicznej (193,49 ±4,84-294,72 ±2,65 $mg \cdot dm^{-3}$). Ponadto, we wszystkich badanych brzeczkach wykryto duże stężenie kwasu p-kumarowego w formie zarówno wolnej, jak i związanej. Nie stwierdzono obniżenia stężenia obu wymienionych, najważniejszych ilościowo, kwasów fenolowych po gotowaniu brzeczek piwnych (80 min, 100,5°C) i klarowaniu w separatorze typu Whirlpool.

Wnioski. Dowiedziono, że podwyższona temperatura w czasie gotowania brzeczki nie miała statystycznie istotnego wpływu na zawartości wolnych form głównych kwasów fenolowych (po zastosowaniu opisanego w pracy programu produkcji brzeczki – określone wyposażenie warzelni, preparaty enzymatyczne, program czasowo-temperaturowy etc.). Różnice w stężeniach kwasów fenolowych przypisano raczej użyciu słodów jęczmiennych pochodzących z różnych dostaw.

Słowa kluczowe: kwas ferulowy, kwas fenolowy, brzeczka piwna, gotowanie brzeczki, przeciwutleniacz

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