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LIQUID CHROMATOGRAPHIC DETERMINATION OF POLYPHENENOLS IN CZECH BEERS DURING BREWING PROCES

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ABSTRACT

High performance liquid chromatographic (HPLC/UV) method was adapted for simultaneous determination of seven polyphenols, including derivatives of benzoic (gallic and vanillic acids) and cinnamic acids (p-coumaric, ferulic and sinapic acids), flavan-3-ols (catechin) and flavonols (rutin) in worts and beers at the various stages of the brewing process. Based on the semi-quantitative HPLC analysis, total polyphenols chromatographic index (TPCI) was in the ranges of 5.18 – 19.4 mg/L and 7.37 – 20.7 mg/L for all worts and beers, respectively. The HPLC analyses showed that relatively high levels of (+)-catechin and gallic acid were in all the worts and the beers, while the values were much lower for ferulic acid, rutin, vanillic acid, sinapic acid and p-coumaric acid. Polyphenols with relatively high concentrations, that were detected in all tested worts and beers, were gallic acid (1.29 – 4.75 mg/L resp. 2.59 – 4.97 mg/L), (+)–catechin (1.66 – 7.95 mg/L resp. 4.70 – 10.0 mg/L) and ferulic acid (0.41 – 4.53 mg/L resp. 1.05 – 2.87 mg/L). On the other side, the sinapic acid (0.72 – 1.59 mg/L resp. 0.72 – 2.5 mg/L), rutin (1.17 – 2.03 mg/L resp. 1.16 – 2.85 mg/L), p–coumaric acid (ND – 4.73 mg/L resp. ND – 1.44 mg/L) and vanillic acid (ND – 1.52 mg/L resp. 0.75 – 1.81 mg/L) were detected in lowest concentrations. In both, worts and beers investigated in this study, the changes in the contents of individual polyphenols were not uniform. In the case of some polyphenols, a decrease in the content was observed after boiling the worts with hops or after the main fermentation until maturation and filtration, but with some polyphenols, the concentrations were constant until the end of the process or even increased.

Keywords: **beers**; **worts; brewing technology; polyphenols; HPLC; UV–VIS diode array detection**

INTRODUCTION

Beer polyphenols have been mostly investigated in the light of their potential antioxidant activity claimed to enhance beer flavor and stability or even human health **(Cortacero–Ramírez et al., 2003; Kondo, 2004; Nardini and Natella, 2006)**. The majority of polyphenols of beer are derived from malt (70–80%), whereas about 20–30% are derived from hops **(Gerhauser, 2005)**. Further, polymerization of phenolics and formation of polyphenols, and their chemical changes can occur during wort boiling and possibly during fermentation and storage of beer. Polyphenolic constituents of beer represent a large structural variety and belong to the classes of simple phenols, benzoic and cinnamic acids derivatives, coumarins, catechins, di–, tri– and oligomeric proanthocyanidins, (prenylated) chalcones and flavonoids **(Gerhauser and Becker, 2009)**.

In recent years, significant efforts have been made to avoid the oxygen pick–up during brewing process, the level of total packaged oxygen might be as low as 0.1 mg/L, but oxidative staling of beer is still noticeable. Minimizing the formation and reducing activity of reactive oxygen species (O_{2}^{-}, $HOO^{\bullet}$, H_2O_2 and $HO^{\bullet}$) in beer and wort, must be the first step for improving beer flavor stability. Antioxidants reduce the rate of oxidation reactions. Therefore, attention is now increasingly shifting towards increasing the antioxidant activity of beer itself **(Lu et al., 2007)**. There are many endogenous antioxidants such as polyphenols, Maillard reaction products, and sulfite present in beer. Among these antioxidants, polyphenols are of particular interest to brewers because they play a key role in the brewing process by delaying, retarding, or preventing oxidation processes **(Lugasi and Hovari, 2003; Lu et al., 2007; Zhao et al., 2010)**.

Rapid analytical methods are necessary for the quality control department of beer producers to evaluate polyphenols that can adversely affect beer flavor and stability, what is of practical interest. Analytical methods for determining polyphenols in wort and beer are limited **(Madiga et al., 1994; Montanari et al., 1999; Andersen and Skibsted, 2001; Floridi et al., 2003)**. Several authors determined polyphenols in beer matrices by RP–HPLC followed by ultraviolet **(Hayes and Smyth, 1987)**, photodiode–array **(PDA, Sanchez–Moreno et al., 1998; Montanari et al., 1999)**, fluorimetric detection **(Dvorakova et al., 2008)**. Electrochemical detection (HPLC–ECD) has become a widely accepted and valuable technique **(Rehova et al., 2004; Skeriková et al., 2004)** because of its high sensitivity as well as its superior selectivity to UV absorption for analytes that are

electrochemically active, such as polyphenols **(Roston and Kissinger, 1981; Wang et al., 2002)**. Mass (MS) and nuclear magnetic resonance (NMR) spectrometric detection can provide additional structural information and solve co–eluting compounds in complex mixtures **(Whittle and Eldridge, 1999)**. Electrospray ionization (ESI) mass spectrometry provides the molecular masses as a soft ionization technique after chromatographic separation, while tandem mass spectrometry (MS/MS) provides extra information on the distribution of the substituents on the phenolic rings, useful for tentative identification but only rarely providing sufficient data for full structural analysis **(Careri et al., 1998)**. An overview of recent development in HPLC determination of phenolics in beers is presented in **(Chunsriimyatav et al., 2010a–c)**.

The general aim of this study was to detect, in a full scale industrial process, the polyphenols in all worts and beers, their fate during the main brewing steps and to compare the six kinds of "Czech brews" and their corresponding 28 worts and 17 beers from Janáček Brewery, Uherský Brod, Czech Republic from the point of view of identification and quantification of individual polyphenols by using HPLC method.

MATERIAL AND METHODOLOGY

Chemicals

Gallic acid, (+)–catechin, vanillic acid, p–coumaric acid, ferulic acid, sinapic acid, rutin, acetonitril (ACN), trifluoroacetic anhydride (TFAA) and methanol (all from Sigma–Aldrich), ferulic acid (Merck, Darmstadt, Germany), Na_2CO_3 and all other chemicals of p.a. purity were from Penta (Chrudim, Czech Republic). The stock standard solutions (ca. 1000 μg/mL) of each polyphenols were prepared in methanol by weighing approximately 0.001 g of the analyte into a 10 mL volumetric flask and diluting to volume. An intermediary mixed standard solution was prepared by dilution of the stock standard solutions in mobile phase A to give a concentration of ca. 10, 20, 30 and 50 μg/mL for each polyphenols. All standard solutions were stored in the dark at 4 °C and were stable for at least three months.

Instrumentation

A UV–VIS spectrophotometer Libra S6 (Biochrom Ltd, Cambridge, UK) and an ultrasonic bath (PSO 4000 A, Kraintek, Slovakia) were used for sample preparation. A HPLC system UltiMate 3000 system (Dionex Corporation, California, USA) consisted of a pump, an autosampler, a column compartment and a diode array detector. Chromatographic separation was carried out on a Supelcosil LC–18–DB column (250 x 4.6 mm, 5 μm, Supelco, USA) at 30°C using a gradient elution with a mobile phase consisting of solvent A (95% (v/v)) acetonitrile acidified with 0.35 mL TFAA) and solvent B (50% (v/v)) aqueous acetonitrile acidified with 0.25 mL TFAA). An injection volume 10 μL, flow rate of 1 mL/min, runtime 30 min were used. Phenolic compounds were identified on the basis of retention times (see Table 1) and UV spectra as compared to standard solutions of phenolic compounds. The concentrations of individual polyphenols in wort and beer samples were calculated using calibration curves constructed for all the phenolic compounds. The analytical parameters of the calibration curves were calculated with the Excel program.

Brew samples.

Six kinds of "Czech brews" (labeled as A – F) processed by different technologies from Janáček Brewery, Uherský Brod, Czech Republic and their corresponding 28 worts and 17 beers were collected at various stages during the brewing process as follows:

1) Malt wort – front part (fresh mash) "front part" – it is the intermediate product in the process of brewing beer. It is a sweet solution without hops, containing saccharides and proteins substance that appears during the percolate. The clear fresh mash is the first part running out of a percolate bowl. It contains the highest amount of polyphenolic substances.

2) The second malt wort – after skimming of the first malt wort (extract content 16–20%), the residual spent grist flushed with hot water for last running. The goal is to get saccharides out of spent grains as much as possible. The decrease of the amount of polyphenols, which is obvious from the graph, is caused by withholding of polyphenols in the spent grains (in the filtrating layer). Temperature is important during lautern, because increasing temperature decreases viscosity and lautern is accelerated. However, temperatures above 80 ºC are unfavorable. Then α–amylase is destroyed and un–dissolved starch cannot be saccharified. Wort will not be iodine normal and starch haze will result in beer.

3) Third malt wort – is the last running (extract content 0.5–1%). The main quantities of most substances have been already filtrated by previous out flowing with the previous aberration/excess. The volume of last running depends on aimed extract concentration. Extract content in spent grist fixes the end of lautern. Final extract content in spent grist has to be below 0.8%.

4) Unhoped malt wort – after lautering, brewer's wort mixed from fronts and all low wines (usually from three of them) malt wort (front, first and third) is combined and transferred to the brewing kettle, where it is boiled during at least one hour with the addition of hops. Aims of wort boiling are wort sterilization, predication of coagulated proteins and isomerization of hop bitter substances. Next to this during hop boiling coagulate proteins with polyphenols during complex compound inception and than they come out from the solution. Coagulation has to be perfect; otherwise the rests of proteins can disturb fermentation and create later fogs.

5) Hopped wort – hop (Červeňák, Žatec hop) is added during the wort boiling. The amount of hops needed is only a fraction of the substantial quantities of malt used in the brewery. Usually, a few grams of hops are sufficient as a quantitatively minor, but qualitatively major ingredient with crucial impact on well–defined beer features. Hop dosage at the beginning of wort boiling serves for bittering and is generally carried out with bitter hop. A second dosage at the end of boiling or into the whirlpool gives a favorable hop dose.

6) Young beer – after cooling and removal of spent hops, the hopped wort is being pumped to the fermentation vessels and yeast is being added under aeration for growth. The fermentation takes about one week thereby delivering a so–called 'young beer' or 'green beer', which not

drinkable, as a number of offending (bad taste and smell) compounds are formed during fermentation. During the anaerobic phase yeast cells convert sugars to ethanol and carbon dioxide.

7) Unfiltered beer – after fermentation, beers need a maturation or lagering period of several weeks at about 0 °C, during which the unwanted components are slowly decomposed. High concentrations of diacetyl and pentane–2,3–dione are particularly obnoxious for the quality of lager beers ('pilsner–type') and scrutinous monitoring is required. Only after the content has decreased below critical values (ppb–ranges), beer can be bottled.

8) Filtered beer – solid and hazy particles still present in the beer (yeast, protein–tannin particles, and hop resins) are removed by filtration. Filtration also improves biological and physico–chemical stability. Filtration is carried out at low temperature (possibly at 0 to -2°C) under a counter–pressure of carbon dioxide above its saturation level, and with minimum uptake of oxygen. Beer samples were degassed in ultrasonic bath PSO 4000 A before analysis (waiver of carbon dioxide). Degassed beers and worts were filtered through 0.45 µm Nylon membrane filter (13 mm, Gronus filter, part No FFNN1345–100, SMI–LabHut Ltd., Gloucester, UK).

RESULTS AND DISCUSSION

Analysis of individual polyphenols by analytical HPLC

To remedy the limitation of spectrophotometric methods **(Chunsriimyatav et al., 2010a–c)** for total polyphenols polyphenols including derivatives of benzoic and cinnamic, flavan–3–ols and flavonols were identified and quantified by HPLC analysis in six kinds of "Czech brews" and their corresponding worts and beers from various stages during the brewing process.

The seven polyphenols standard solutions prepared by dilution of the individual stock standard solutions in mobile phase A to obtain the desired concentrations of ca. 10, 20, 30 and 50 µg/mL^{-1} for each polyphenols. The working standard mixture was diluted 1:4, 3:7, and 1:1 (v/v) to obtain the calibration solutions. Table 2 lists the parameters of calibration curves and their calibration equations (with c = 0 as fixed point and omitting c = 0 point). The diode array detection was conducted by scanning between 205 nm, 210 nm and 275 nm (except of rutin). Comparing the absorbances at the three wavelengths, the absorbance at 210 nm showed considerable improvement in signal–to–noise ratio (better precision, sensitivity). The concentrations of seven polyphenols in worts and beers were determined using the calibration curves (with c = 0 as fixed point) listed in Table 2. The identification of the peaks was carried out by their retention times in comparison with standards, but also comparing the UV spectra in samples and standards by using a diode array detector. The standard polyphenols were used to examine phenol concentration in different kinds of worts and beers.

HPLC separation of standards of polyphenols

The retention times (RT) of seven standard compounds are reported in Table 1. The elution of polyphenols follows the decreasing polarity in reversed–phase HPLC, thus benzoic acid derivatives are eluted earlier than cinnamic acid derivatives. **Guo et al., (1997)** reported that the retention time of polyphenols increases with the number of $–OCH_3$ substituents. The elution order for benzoic acid is as follows: gallic acid > vanilic acid. Gallic acid is the first acid eluted (three –OH groups), whereas vanilic acid, the first $–OCH_3$ substituted among benzoic acids, has an RT of 11.77 min. Under the same condition, the elution order for cinnamic acids is p–coumaric acid > ferulic acid > sinapic acid. Ferulic acid eluted after p–coumaric, which indicates that the methoxy ($–OCH_3$) substituent is less polar, for it increases in retention.

Individual polyphenols

Table 3 reports the concentration of the seven polyphenols and the total phenolic chromatographic index (TPCI) as sum of all the polyphenolics classes calculated from the chromatogram in 28 worts and 17 beers. The standard deviation (SD) value ranges from 0.002 to 0.91 mg/L for worts and from 0.007 to 3.7 mg/L for beers. Polyphenols with relatively high concentrations, that were detected in all tested worts and beers, are gallic acid (1.29 – 4.75 mg/L resp. 2.59 – 4.97 mg/L), (+) catechin (1.66 – 7.95 mg/L resp. 4.70 – 10.0 mg/L) and ferulic acid (0.41 – 4.53 mg/L resp. 1.05 – 2.87 mg/L). On the other side, sinapic acid (0.72 – 1.59 mg/L resp. 0.72 – 2.5 mg/L), rutin (1.17 – 2.03 mg/L resp. 1.16 – 2.85 mg/L), p–coumaric acid (ND – 4.73 mg/L resp. ND – 1.44 mg/L) and vanillic acid (ND – 1.52 mg/L resp. 0.75 – 1.81 mg/L) were detected in low concentrations. Due to their low content, some individual polyphenols like p–coumaric acid could not be detected in a number of beer and wort samples.

Moreover, all the worts and beers tested in the current study exhibited relatively high levels of (+)–catechin and gallic acid, while the values were much lower for rutin, ferulic acid, sinapic acid, vanillic acid and p–coumaric acid. In both, worts and beers, the changes in the individual polyphenols were not uniform. In the case of some polyphenols, a decrease in the content was observed after boiling the worts with hops or after the main fermentation until maturation and filtration, but with some polyphenols, the concentrations were constant until the end of the technological processes or even increased (e.g., gallic acid and catechin in brew C, brew D and brew E). The concentrations of (+)–catechin and gallic acid were approximately constant or slightly decreased in most cases or increased during the brewing process (sweet wort → hopped wort→ fresh beer). The results also indicated a remarkable increase of (+)–catechin contents in all beers in comparison to the corresponding worts after maturation process.

Table 1 Retention times (RT) of polyphenols.

№	IUPAC name	Current name	Abbreviation	Peak-RT [a] (min)
1	3,4,5-trihydroxybenzoic acid	Gallic acid	GA	4.16
2	trans-3,3'4',5,7-pentahydroxyflavane	Catechin	Cat	10.08
3	4-hydroxy-3-methoxybenzoic acid	Vanilic acid	VA	11.77
4	*trans*-4-hydroxycinnamic acid	*p*-Coumaric acid	pCA	18.73
5	4-hydroxy-3-methoxycinnamic acid	Ferulic acid	FA	20.64
6	3,5-dihydroxy-4-hydroxycinnamic acid	Sinapic acid	SA	20.79
7	Quercetin-3-rutinoside	Rutin	Rut	21.46

[a] RT-Retention time in minutes

Table 2 Calibration curves and their calibration equations of polyphenols standards.

Compound	UV (nm)	Calibration equation [a]	R^2	Calibration equation [b]	R^2
Gallic acid	205	Y = 1552.5x - 1921	0.9907	Y = 1689.7x - 7135.2	0.9884
	210	Y = 1874.5x - 2306.8	0.9912	Y = 2039.3x - 8568.2	0.9912
	275	Y = 675.7x - 832.33	0.9925	Y = 735.15x - 3091.5	0.9925
Catechin	205	Y = 1390x - 1526.8	0.9959	Y = 1499.1x - 5671.1	0.9984
	210	Y = 946.61x - 985.34	0.9961	Y = 1017x - 3659.8	0.9982
	275	Y = 74.941x - 104.51	0.9957	Y = 82.406x - 388.18	0.9992
Vanilic acid	205	Y = 1336.1x - 385.26	0.9971	Y = 1363.6x - 1431	0.9977
	210	Y = 1337.6x - 251.56	0.9967	Y = 1355.6x - 934.37	0.9977
	275	Y = 412.52x – 33.041	0.9990	Y = 414.88x - 122.72	0.9977
p-Coumaric acid	205	Y = 654.17x + 627.88	0.9944	Y = 609.32x - 2332.1	0.9905
	210	Y = 678.88x + 644.97	0.9945	Y = 632.81x + 2395.6	0.9907
	275	Y = 823.09x + 709.47	0.9952	Y = 772.42x + 2635.2	0.9916
Ferulic acid	205	Y = 962.44 – 598.91	0.9936	Y = 1005.2x - 2224.5	0.9866
	210	Y = 1054.3x - 652.28	0.9936	Y = 1100.8x - 2422.8	0.9866
	275	Y = 746.46x - 437.55	0.9935	Y = 777.71x - 1625.2	0.9860
Sinapic acid	205	Y = 672.7x – 420.67	0.9970	Y = 702.74x - 1526.5	0.9951
	210	Y = 617.66x - 422.07	0.9969	Y = 647.81x - 1567.7	0.9955
	275	Y = 44.065x - 54.397	0.9938	Y = 254.76x - 647.51	0.9956
Rutin[c]	205	Y = 879.8x - 1396.8	0.9928	Y = 979.57x - 5188.3	1.0000
	210	Y = 734.11x - 677.09	0.9970	Y = 782.47x - 2514.9	0.9987
	275				

[a] with c=0 as fixed point, [b] omitting c= 0 point, [c] rutin not detected at 275 nm

The found values agree with phenolic concentrations determined by other authors in literature. **Floridi et al., (2003)** using HPLC with coulometric array detection, described a wide range of free phenolic acids in worts. **Nardini and Ghiselli (2004)** determined free and total alkali extractable phenolic acids in three beers of Italian, Austrian and German origin. Ferulic acid was the main phenolic acid in both forms, followed by other phenolic acids present in the three beers always in considerably lower levels than ferulic acid. Phenolic acids were present in these beers mainly in the bound form. **Vanbeneden et al., (2006)** using HPLC–ECD, determined the content of the three main phenolic acids: ferulic (main phenolic acid) followed by p–coumaric and sinapic acids, but their analytical technique was created primarily for the simultaneous detection of volatile phenols and not phenolic acids in worts or beers.

The sum of all the phenolic classes calculated from the chromatogram (total phenolics chromatographic index – TPCI) in different brews varied considerably,

Table 3 Individual polyphenols content (calculated using the corresponding calibration curves).

Brew	Comp	1	2	3	4	5	6	7	8
A	GA	4.16 ±0.08	2.89 ±0.04	4.25 ±0.26	4.75 ±0.06	3.51 ±0.05	3.85 ±0.37	3.72 ±0.48	4.16 ±0.08
B		4.15 ±0.09	2.82 ±0.004	2.95 ±0.07	3.26 ±0.09	2.87 ±0.13	3.45 ±0.48	4.15 ±0.09	2.82 ±0.004
C		3.72 ±0.05	3.79 ±0.14	2.02 ±0.01	3.22 ±0.2	1.85 ±0.07	3.64 ±0.08	2.59 ±0.01	4.85 ±0.04
D		2.31 ±0.16	1.91 ±0.06	3.17 ±0.02	2.74 ±0.32	3.76 ±0.05	4.69 ±0.01	4.97 ±0.01	2.31 ±0.16
E		3.06 ±0.04	1.95 ±0.01	1.40 ±0.003	3.28 ±0.09	3.54 ±0.91	3.36 ±0.02	4.71 ±0.02	3.06 ±0.04
F		3.27 ±0.25	3.20 ±0.01	1.29 ±0.01	3.51 ±0.02	3.37 ±0.02	4.19 ±0.02	3.70 ±0.02	4.16 ±0.01
A	Cat	4.77 ±0.07	4.06 ±0.06	5.92 ±0.02	7.53 ±0.006	7.09 ±0.007	8.44 ±0.10	4.70 ±0.55	4.77 ±0.07
B		6.98 ±0.05	5.29 ±0.06		4.05 ±0.04	4.27 ±0.01	6.56 ±0.56	5.43 ±1.2	
C		6.28 ±0.05	5.69 ±0.03	3.07 ±0.04	7.72 ±0.03	6.66 ±0.26	6.40 ±0.07	9.25 ±0.74	9.75 ±2.32
D		6.73 ±0.03	ND	4.51 ±0.46	5.46 ±0.35	8.12 ±0.02	6.27 ±0.01	5.78 ±0.03	6.73 ±0.03
E		4.94 ±0.06	2.59 ±0.29	1.66 ±0.01	5.68 ±0.06	7.95 ±0.09	10.0 ±0.16	7.45 ±0.09	8.07 ±0.06
F		3.66 ±0.3	4.02 ±0.02	ND	1.96 ±0.15	7.12 ±0.1	ND	7.91 ±1.05	6.94 ±0.59
A	pCA	-[a)]	-[a)]	0.66 ±0.06	-[a)]	-[a)]	-[a)]	-[a)]	-[a)]
B									
C		ND	ND	ND	ND	ND	ND	0.94 ±0.06	0.83 ±0.04
D		0.62 ±0.03	ND	ND	0.53±0.03	ND	1.29 ±0.09	1.23 ±0.03	0.62 ±0.03
E		ND	ND	ND	0.59 ±0.02	1.52 ±0.05	1.01 ±0.06	1.81 ±0.41	0.75 ±0.02
F		0.76 ±0.03	ND	ND	0.45 ±0.02	ND	0.89 ±0.02	1.14 ±0.05	0.55 ±0.03
A	FA	4.51 ±0.01	2.51 ±0.02	4.41 ±0.002	4.78 ±0.01	2.76 ±0.02	2.00 ±2.61	1.64 ±0.12	4.51 ±0.01
B		4.01 ±0.03	ND		4.23 ±0.007	4.45 ±0.01	2.87 ±0.15	1.60 ±0.05	
C		2.06 ±0.04	1.37±0.07	ND	-[a)]	-[a)]	-[a)]	-[a)]	0.31 ±0.03
D		4.73 ±0.02	ND	ND	2.40±0.03	-[a)]	-[a)]	-[a)]	4.73 ±0.02
E		4.24 ±0.005	1.42 ±0.02	1.01±0.004	0.97 ±0.01	0.95 ±0.28	1.12 ±0.009	1.22 ±1.21	1.12 ±0.05
F		4.26 ±0.1	0.94 ±0.09	-[a)]	-[a)]	-[a)]	-[a)]	1.44 ±0.09	1.02 ±0.05
A	SA	1.06 ±0.03	ND	0.94 ±0.06	ND	ND	ND	1.35 ±0.06	1.06 ±0.03
B		1.34 ±0.05	ND		1.23 ±0.02	1.58 ±0.03	1.18 ±0.04	1.29 ±0.07	
C		1.80 ±0.06	1.65 ±0.1	ND	1.85 ±0.06	1.59 ±0.04	1.79 ± 0.06	2.50 ±0.01	1.82 ±0.65
D		3.22 ±0.06	1.61 ±0.02	4.13 ±0.06	4.23 ±0.22	1.05 ±0.02	1.44 ±1.28	1.18 ±0.03	3.22 ±0.06
E		ND	ND	ND	1.01 ±0.02	0.88 ±0.04	0.87 ±0.07	1.71 ±0.09	1.52 ±0.01
F		1.05 ±0.09	0 .81 ±0.04	ND	0.94 ±0.02	0.90 ±0.02	1.07 ±0.02	1.34 ±0.05	1.10 ±0.03
A	Rut	1.19 ±0.04	ND	1.29 ±0.03	1.22 ±0.01	1.32 ±0.03	1.27	1.34 ±0.07	1.19 ±0.04
B		ND	ND		ND	1.55 ±0.05	1.33 ±0.06	1.22 ±0.05	
C		ND	ND	ND	ND	1.55 ±0.02	2.02 ±0.08	2.56 ±0.03	1.89 ±0.07
D		1.83 ±0.07	1.78±0.08	1.76 ±0.06	1.78±0.05	2.85±0.03	2.44 ±0.08	2.17 ±0.15	1.83 ±0.07
E		ND	ND	ND	1.65 ±0.07	2.03 ±0.05	2.13 ±0.09	2.38 ±0.04	1.24 ±0.01
F		0.87 ±0.09	1.21 ±0.02	ND	1.42 ±0.06	ND	ND	ND	1.05 ±0.02
A	TCPI[b]	15.7	9.5	17.5	18.3	14.7	15.6	12.8	
B		16.5	8.11		12.5	15.1	14.8	12.9	
C		18.4	14.8	5.18	13.2	14.4	14.6	19.2	20.7
D		19.4	6.02	13.6	18.1	16.7	17.4	16.6	19.4
E		12.2	5.96	4.07	13.2	16.9	18.5	19.3	16.8
F		15.1	11.3	1.29	9.5	12.6	7.37	16.7	17.1
		1.26 ±0.05	1.14 ±0.06	ND	1.17 ±0.02	1.19 ±0.05	1.22 ±0.06	1.16 ±0.05	2.31 ±0.41
		ND	0.72 ±0.01	ND	0.98 ±0.05	0.93 ±0.04	1.25 ±0.07	1.26 ±0.08	ND

Brew A – Patriot 11%, B – Olsavan 11%, C – in Comenius 14º, D– Extra 12º, E– Patriot PLTM 11º, F–Prima 10º, [a] each value is the mean ±standard deviation of triplicate determinations; ND – not determined, [a] – concentration below the detection limits, [b] Total Phenolic Chromatographic Index (TPCI) = sum of all the phenolic classes calculated from the chromatogram

ranging from 5.18 – 19.4 mg/L and 7.37 – 20.7 mg/L for all worts and beers, respectively. Moreover, significant differences in total polyphenols content determined by Folin–Ciocalteau and HPLC methods were found in the present study, which also verified the non–specificity of Folin–Ciocalteau method. Therefore, the measurement of phenolic profiles by HPLC method could give more information about their chemical characteristics and antioxidant activities.

CONCLUSION

HPLC analysis coupled with UV–VIS diode array detection allows separation of polyphenols in worts and beers during the brewing process. Based on the semi–quantitative HPLC analysis, total phenolics chromatographic index (TPCI) was in the ranges of 5.18 – 19.4 mg/L and 7.37 – 20.7 mg/L for worts and beers, respectively. All the beers from different technologies contained polyphenols at concentrations that generally were similar to those detected in their corresponding worts. The HPLC analysis showed that all worts and beers tested in the current study were relatively high levels of (+)–catechin and gallic acid, while the values were much lower for ferulic acid, rutin vanillic, sinapic and p–coumaric acids, most of which changed significantly during the brewing process. This HPLC–DAD analysis set up to routinely analyze up to seven polyphenols in order to control the brewing process

and the composition of the final product. The advantage of this procedure is that reproducible results are obtained by direct injection of worts and beers without sample preparation. The influence of the brewing process on the content of free phenolic acids and other polyphenols of worts and beers can be easily evaluated. Covalently bonded polyphenols in worts and beer will be investigated in future studies. A method will be developed for the hydrolysis and extraction for determining the total concentration (free or bound) of phenolic acids, including some other polyphenols resolved with this method but not determined in this work. On the results obtained from current study, further work on optimizing brewing processes will be the improvement of beer's flavor stability through raising selectively certain polyphenols.

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