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Control of antioxidant beer activity by the mashing process

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Beer is considered to be a good source of antioxidants. The composition and the quantity of the antioxidant compounds depend not only on the qualities of the raw materials, but also on the technology processes. Barley and malt represent the main source of antioxidant compounds in beer and the contribution of the hop antioxidants is lower. The influence of the mashing process on the antioxidant activity and polyphenol concentration is crucial. The antioxidant state of the sweet wort and the hopped wort are dependent on the technology processes and the raw materials used. The spontaneous sorption of polyphenols onto wort dregs and the polymerization of catechin and epicatechin lead to decreasing concentrations of individual polyphenols in the final beer. Two methods, based on electron spin resonance were used to determine antioxidant activity. These were the DPPH (2,2-diphenyl-1-picryl-hydrazyl) assay and the 'lag time' assay using free radical spin-trapping agent PBN (N-tert-butyl-α-phenylnitrone). HPLC with CoulArray detection was used to measure the concentration of the individual polyphenols. This study focused on the antioxidant compounds and on the correlation of their concentrations with the values of total antioxidant activity depending on the mashing process. A good correlation was found between the decline in the concentrations of DPPH (expressed as ARA2) and concentrations of catechin and epicatechin (in sweet wort samples $R^2 = 0.970$, $R^2 = 0.961$, respectively, and in hopped wort samples $R^2 = 0.949$, $R^2 = 0.956$ respectively). Copyright © 2012 The Institute of Brewing & Distilling

Keywords: HPLC; polyphenols; CoulArray detection; DPPH; ESR lag time; antioxidant activity; beer

Introduction

Many scientists are interested in the antioxidant characteristics of foodstuffs. Antioxidants are compounds that have a positive effect on human health. They are able to remove reactive oxygen and radicals from the organism and in this way prevent oxidative damage, such as ageing and degenerative diseases, cancer and cardiovascular disease.

Phenolic compounds, as natural antioxidants, arise from the raw materials, malt and hops during brewing process. The literature indicates that approximately 70–80% of beer polyphenols originate from malt and about 20% from hops *(1)*. These compounds play a key role in antioxidant activity *(2)* and they can also influence the sensory stability of the beer.

Antioxidant activity can also be influenced by the mashing system used. During mashing, starch from malt is converted into fermentable sugars by the malt enzymes. Generally, mashing systems are divided into two methods, infusion mashing and decoction mashing.

Infusion mashing is the simplest process. The principle of infusion mashing is the gradual heating of the whole mash in the same vessel. A decoction mashing system is based on removing a portion of the mash (the decoction) for heating to the conversion temperature in the mash kettle. After decoction, this part of the mash is brought to a boil to inactivate the enzymes and then is returned to the main mash to increase the temperature in the entire volume. There are three-step, two-step and single-step mash decoction systems, according to the number of recycling stages and rests. The rests are kept at the specified temperatures needed for the cleavage of saccharides and proteins *(3)*.

This study focused on the comparison of the antioxidant activity of hopped wort and beer as influenced by the technological process. Many modern analytical methods for the determination of antioxidant activity of brewing raw materials, sweet wort, hopped wort and beer, as well as for the measurement of concentrations of polyphenols in beer, have been published in recent years. A summary of analytical methods for the determination of the antioxidant activity of brewing materials, intermediate products and beers can be found in the paper by Moll *(4)*. The analytical methods for the assessment of antioxidant activity include both chemical and physical methods. In the chemical methods based on oxidation–reduction (redox) reactions, what is measured spectrophotometrically is the intensity of colouration or decolouration of the studied environment as a result of concentration changes of the added stable radicals. The reducing activity of the beer is measured by the method described by Kaneda *(5,6)*, in which the reaction is based on the added stable-colour free radical, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), with reducing substances.

The electron spin resonance (ESR) method is the most common physical method used in breweries over recent years. For assessment of the endogenous antioxidant activity of beer, the 'lag time' is used. During the oxidative forcing test (i.e. incubation of beer at 60°C with a spin-trapping agent, *N*-Tert-butyl-α-phenylnitrone, PBN) OH radicals start the generation and initiation of oxidative reactions. The beer itself has endogenous antioxidant activity, i.e. the capability to prevent OH radical generation. The duration of this resistance against

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oxidation (period before OH radical generation, lag time) is determined *(7,8)*. The polyphenol composition of malt and barley has been studied by many authors using HPLC with various means of detection of individual polyphenols. All polyphenolic compounds can be analysed by UV detection *(9)* or mass spectrometry detection *(10)*. The –OH groups of polyphenols can be electrochemically oxidized. The oxidative potential reflects the difficulties of oxidation molecules, which are given by –OH and $–OCH_3$ substituents and their location on the aromatic scaffold *(11,12)*. The first studies of free phenolic acids in beer and wort based on electrochemical oxidation of polyphenols using HPLC with CoulArray detection were published in papers by Floridi *et al. (13)* and Škeříková *et al. (14)*. There is also a new method of direct current polarography based on the measurement of the decreasing hydrogen peroxide anodic current in the presence of antioxidants *(15)*. The influence of polyphenols on colloidal stabilization and the composition of beer polyphenols and other features of beer (colour, haze, taste, foam stability, acidity) using stabilization technology were recently described in a detailed study *(16)*. The real content of polyphenols in barley and malt was shown by pressure solvent extraction. These results were compared with the results of the congress mash technique *(17)*. The wide spectrum of antioxidants consisted of phenolic acids and more complex flavonoids. The major part of phenolic acids was reported to be in the bound form and the simple phenolic acids were present in lower amounts in both the barley and the malt. The main groups of free flavonoids were the flavanols in monomeric (flavan-3-ols) and in oligomer (proanthocyanidins) forms *(18)*.

Materials and methods

Samples

The sweet worts, hopped worts and beers were prepared in the pilot plant using infusion mashing and a mash decoction process. Brews of 12 and 15°P all-malt pale lager beers were prepared using the same malt and hops. The five brews (A–E) were prepared as follows: (A) 12°P infusion mashing; (B) 12 and (C) 15°P, both single-step mash decoction processes; and (D) 12°P and (E) 15°P, both two-step mash decoction processes. Samples for analysis were taken after mashing (sweet wort), after wort boiling (hopped wort) and after bottling with pasteurization.

Measurement of antioxidant activity

Reagents and solutions. *N*-Tert-butyl-α-phenylnitrone (98%), DPPH, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) and ethanol for assays were obtained from Sigma-Aldrich Co (St Louis, MO, USA). A solution of DPPH was freshly prepared by dissolving 16.3 mg/L of DPPH in a mixture of ethanol and acetate buffer (2:1). The acetate buffer (pH 4.3) was prepared by mixing a solution of acetic acid ($c = 0.1$ mol/L) and sodium acetate ($c = 0.1$ mol/L) and adjusted to the exact pH value of 4.3.

Electron spin resonance for the determination of antioxidant activity of the brewing intermediate products and finished beers was used. The measurement was performed on the Spectrometer MiniScope MS 200, Magnettech GmbH, Germany. For both assays, the preparation of samples was the same. A wort sample was cooled to 0°C immediately after sampling and then was centrifuged at 9000 ***g*** for 20 min to obtain the clear supernatant for the ESR measurement. A beer sample was degassed by centrifugation, twice, at 13,000 ***g*** for 15 min at 5°C.

DPPH assay. The stable free radical compound DPPH was used in our modified method *(2)*. The ESR analytical conditions were as follows: magnetic field, 3 377 G; field sweep, 48 G; modulation amplitude, 1500 mG; attenuation, 10 dB; and sweep time, 34 s. The procedure was started by adding 1 mL of the sample to 14 mL DPPH solution in the test tube and this was immediately transferred into an autosampler and the measurement started. The signal value of the free radical DPPH in 1 min intervals, for 10 min, was measured. The temperature was maintained at 30°C. A DPPH solution was used as a blank. Data processing was carried out with an ESR mathematic apparatus. The results were expressed in percentage of decline in the DPPH value 10 min after beginning of the reaction (ARA2).

Lag time assay. The ESR analytical conditions were as follows: magnetic field, 3372 G; field sweep, 12 G; modulation amplitude, 1000 mG; attenuation, 10 dB; and sweep time, 46 s. A solution of 2.55 mol/L PBN in 50% ethanol and of 10 μmol/L TEMPOL, both in ultrapure water from a Millipore system, were used. A 0.26 mL aliquot of 2.55 M PBN was added to 13 mL of sample (final concentration 0.05 M PBN) and mixed vigorously. The sample tube was placed into the autosampler and incubated at 60 °C for 180 min. Automatic sampling was into a flat quartz ESR cuvette. The ESR spectrum was measured with a spectrometer MiniScope MS 200. The compound TEMPOL was used as a standard for determination of the concentration of free radicals.

Determination of total polyphenols

Total polyphenols were determined by the EBC Analytica method 9.11 *(19)*.

Measurement of individual polyphenols by HPLC

The free polyphenols analysed were the following: gallic acid, protocatechuic acid, gentisic acid, esculin, *p*-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, (+)-catechin, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, (−)-epicatechin, vanillin, *p*-coumaric acid, umbelliferone, scopoletin, ferulic acid, sinapinic acid, rutin, naringin, myricetin, 4-hydroxycoumarin, daidzein, quercetin, genistein and apigenin. Standards were obtained from Fluka (Buchs SG, Switzerland).

Calibration standards. Mixed stock solutions were prepared at 10 mg weighed with accuracy to 0.1 mg of each compound and dissolved in 100 mL methanol (Sigma Aldrich, Seelze, Germany). The stock solutions were stored at −4 °C for a maximum of 3 months. The calibration standard solutions were prepared at four concentration levels (1.0, 0.5, 0.1 and 0.01 mg/L) by diluting the stock solution in 100 mL measuring flasks by phase A.

The analyses of individual polyphenols were realized by HPLC with electrochemical detection. The HPLC system consists of two ESA pumps, model 542 Autosampler and 5600 A CoulArray detector (ESA, Chelmsford, MA, USA). The detector consisted of eight electrochemical cells formed from porous graphite electrodes, palladium reference electrode and platinum counter electrode. The polyphenols were separated on a Synergi 4 μm Hydro-RP 80A 250 × 4.6 mm column (Phenomenex, Torrance, CA, USA) under binary gradient elution. The mobile phases consisted of 0.005 M ammonium acetate (Sigma Aldrich) in ultrapure water (Millipore) of ensured conductivity, and acetonitrile (Sigma Aldrich) that was used as an organic modifier (phase A contained 5% acetonitrile and phase B contained 50%

acetonitrile). The gradient for separation was: 0–5 min, 0% B; 5–18 min, 0–13% B; 18–77 min, 13–21% B; 77–120 min, 21–95% B; 120–125 min; and 95–100% B. The gradient returned to 0% B after cell cleaning in 126–127 min and equilibrium took 18 min.

The analytes eluted from the column went through the array of electrodes with potentials ordering uplink 250, 300, 400, 500, 600, 700, 800 and 900 mV and were oxidized and emerging currents were measured as peaks (the area of peak corresponds to the passed charge) on all potentials. The highest response (dominant peak) was used for calibration and for the determination of each analyte's retention time.

Results and discussion

The experimental comparison of the influence of the mashing technology on antioxidant features and content of polyphenols was performed three times. Figures 1 and 2 show the dependence of the total content of polyphenols and antioxidant activities (ARA2) in sweet wort and hopped wort, respectively, on the type of technology. The dependence of the antioxidant activity of beers is expressed in term of lag time on total polyphenols (Fig. 3).

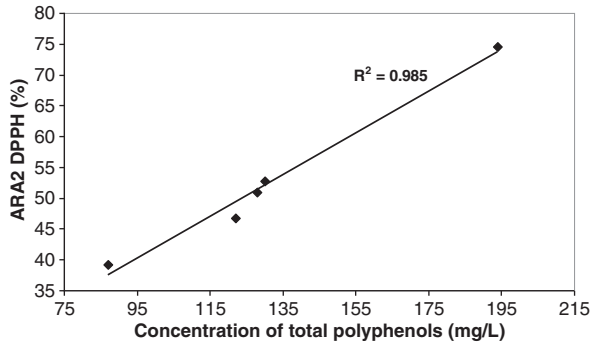

Figure 1. Dependence of antioxidant activity on polyphenols concentration in sweet wort from different mashing procedures.

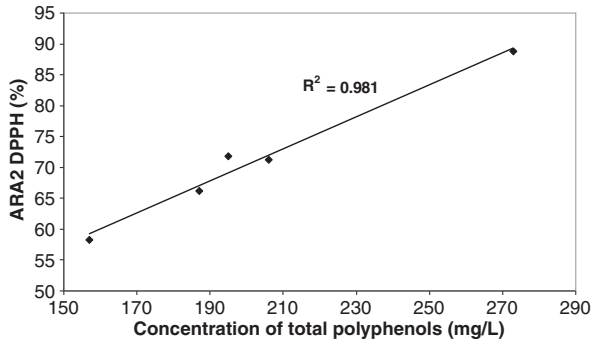

Figure 2. Dependence of antioxidant activity on polyphenols concentration in hopped wort from different mashing procedures.

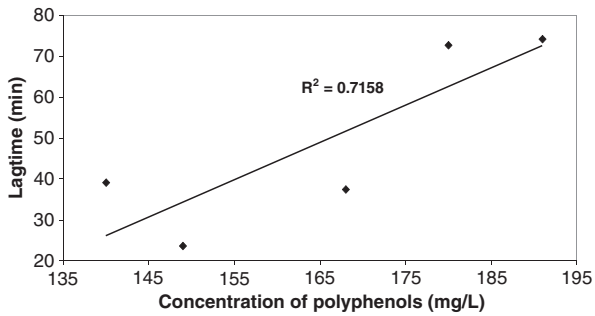

Figure 3. Dependence of antioxidant activity on polyphenols concentration in beers from different mashing procedures.

It has been shown that the technology process significantly affects the antioxidant activity of the beer and the semi-finished products (sweet wort and hopped wort) using the same raw materials (hops and malt). The antioxidant activity of the beer, sweet worts and hopped worts depends strongly on the concentrations of the polyphenols. This can be controlled by choosing different mashing processes.

Several ways of achieving the required polyphenol concentrations and corresponding antioxidant activity were tested. The first was simply an infusion mash process (samples A). Second were decoction processes, where the efficiency of polyphenol extraction depended on the number of decoction cycles (samples B and C used a single-step mash decoction system and samples D and E used a two-step mash decoction system). The base concentrations of polyphenols were due to malt variety and also, as expected, the amount of raw materials used in the brew. Thus, the levels in samples C and E (15°P) were higher than in samples B and D (12°P).

The influence of the reducing abilities of the polyphenols and their contribution to antioxidant activity was evaluated by studying the individual polyphenols by CoulArray detection. The compounds with a lower oxidative potential play a more active

Table 1. Oxidative potential of polyphenols in the mobile phase

Polyphenol substance	Oxidative potential (mV)	Channel
Gallic acid	400	3
Protocatechuic acid	600	5
Gentisic acid	400	3
Esculin	900	8
4-Hydroxybenzoic acid	900	8
4-Hydroxyphenylacetic acid	900	8
Catechin	500	4
Chlorogenic acid	500	4
Vanillic acid	800	7
Caffeic acid	400	3
Syringic acid	400	3
Epicatechin	400	3
Vanillin	900	8
4-Coumaric acid	800	7
Umbelliferone	900	8
Scopoletin	800	7
Ferulic acid	700	6
Sinapinic acid	600	5
Rutin	400	3
Naringin	900	8
Myricetin	300	2
4-Hydroxycoumarin	900	8
Daidzein	800	7
Quercetin	400	3
Genistein	800	7
Apigenin	900	8
Formononetin	900	8
Biochanin A	900	8

Table 2. Polyphenols found in sweet worts as determined by CoulArray detection (mg/L) and total polyphenols according to Analytica EBC

Polyphenol substance	Sample A	Sample B	Sample C	Sample D	Sample E
Gallic acid	0.02	0.02	0.03	0.03	0.06
Protocatechuic acid	0.08	0.06	0.10	0.12	0.15
Gentisic acid	0.02	0.03	0.04	0.02	0.03
Catechin	1.54	1.67	2.30	2.34	4.44
Chlorogenic acid	0.11	0.19	0.25	0.53	0.91
Vanillic acid	0.31	0.15	0.31	0.04	0.87
Caffeic acid	0.10	0.08	0.13	0.19	0.24
Syringic acid	0.23	0.21	0.28	0.28	0.42
Epicatechin	0.10	0.16	0.23	0.18	0.49
4-Coumaric acid	0.15	0.12	0.07	0.13	0.05
Scopoletin	0.05	0.10	0.03	0.04	0.07
Ferulic acid	4.28	2.92	5.80	5.22	6.22
Sinapinic acid	0.38	0.27	0.37	1.05	1.30
Rutin	0.37	1.12	1.55	1.41	0.10
Myricetin	0.00	0.01	0.00	0.00	1.42
Daidzein	0.00	0.00	0.08	0.03	0.03
Quercetin	0.10	0.03	0.15	0.07	0.02
Genistein	0.01	0.01	0.02	0.02	0.07
Sum	7.87	7.15	11.72	11.67	16.77
Sum polyphenols oxidized on 400–500 mV	2.59	3.51	4.95	5.04	6.71
Total polyphenols	87	122	128	130	194

Table 3. Polyphenols found in hopped worts determined by CoulArray detection (mg/L) and total polyphenols according to Analytica EBC

Polyphenol substance	Sample A	Sample B	Sample C	Sample D	Sample E
Gallic acid	0.10	0.07	0.08	0.10	0.10
Protocatechuic acid	0.12	0.15	0.18	0.20	0.21
Gentisic acid	0.00	0.00	0.04	0.01	0.00
Catechin	3.10	3.72	3.86	4.40	6.30
Chlorogenic acid	0.25	0.33	0.36	0.36	0.09
Vanillic acid	0.36	0.39	0.50	0.50	1.14
Caffeic acid	0.10	0.10	0.15	0.22	0.24
Syringic acid	0.14	0.15	0.21	0.19	0.28
Epicatechin	0.81	1.02	1.07	1.15	1.85
4-Coumaric acid	0.87	0.86	1.31	1.24	1.76
Scopoletin	0.03	0.03	0.03	0.50	0.08
Ferulic acid	3.62	3.30	5.46	5.08	5.36
Sinapinic acid	0.56	0.62	0.85	0.96	1.23
Rutin	0.87	1.42	1.54	1.72	0.59
Myricetin	0.06	0.00	0.00	0.00	0.00
Daidzein	0.01	0.01	0.01	0.01	0.04
Quercetin	0.00	0.00	0.01	0.00	0.04
Genistein	0.04	0.05	0.11	0.07	0.24
Sum	11.06	12.22	15.76	17.42	16.71
Sum polyphenols oxidized on 400–500 mV	5.38	6.81	7.32	8.14	9.48
Total polyphenols	157	187	195	206	273

role than the compounds with higher oxidative potential (Table 1). The concentrations of analysed polyphenols in sweet worts, hopped worts and beers (average of three determinations) are summarized in Tables 2–4. The grey-scale colour in Tables 1–4 label indicates the groups of antioxidant compounds with the same electrochemical behaviour. Very low detection selectivity at the highest potential, 900 mV, can cause overvaluation of the measured concentrations of polyphenols oxidized at this potential. When diluting samples in the mobile phase and some matrix compounds with the same retention time as polyphenols, these can be oxidized together with them. Indeed,

Table 4. Polyphenols found in beers determined by CoulArray detection (mg/L) and total polyphenols according to Analytica EBC

Polyphenol substance	Sample A	Sample B	Sample C	Sample D	Sample E
Gallic acid	0.05	0.03	0.05	0.04	0.06
Protocatechuic acid	0.23	0.25	0.30	0.29	0.21
Gentisic acid	0.00	0.00	0.04	0.01	0.02
Catechin	1.20	1.31	1.25	1.58	4.08
Chlorogenic acid	0.21	0.65	0.69	0.22	0.12
Vanillic acid	0.46	0.49	0.69	0.27	0.83
Caffeic acid	0.12	0.12	0.16	0.22	0.16
Syringic acid	0.23	0.24	0.32	0.25	0.25
Epicatechin	0.79	0.91	0.82	1.02	1.09
4-Coumaric acid	0.72	0.70	1.09	1.29	1.11
Scopoletin	0.18	0.04	0.00	0.01	0.09
Ferulic acid	3.76	3.24	5.20	5.14	3.58
Sinapinic acid	0.55	0.59	0.77	0.88	0.74
Rutin	1.46	1.77	1.37	1.51	0.34
Myricetin	0.00	0.35	0.00	0.01	0.12
Daidzein	0.05	0.02	0.03	0.02	0.01
Quercetin	0.16	0.01	0.00	0.02	0.18
Genistein	0.00	0.25	0.19	0.10	0.01
Sum	10.17	10.94	12.97	13.58	12.89
Sum polyphenols oxidized on 400–500 mV	4.22	5.03	4.70	4.87	6.29
Total polyphenols	140	191	149	180	168

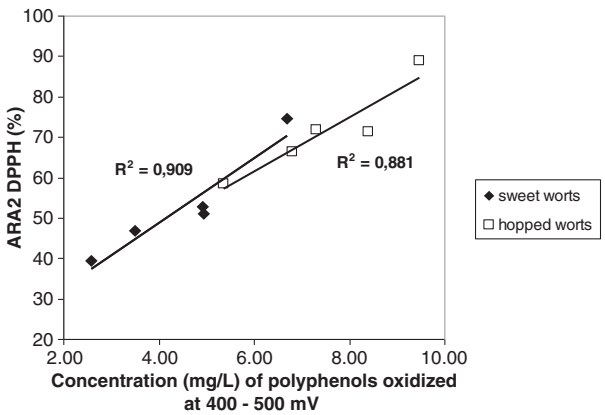

Figure 4. Correlation between antioxidant activities ARA2 DPPH (%) and concentrations of sum of simple polyphenols oxidized at 400–500 mV in sweet worts and hopped worts.

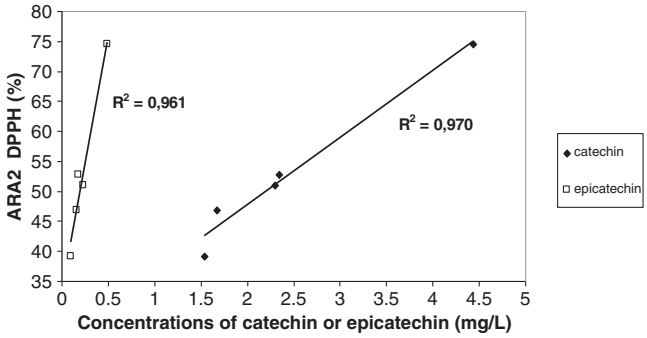

Figure 5. Relationship between concentrations of catechin or epicatechin and antioxidant activities in sweet worts.

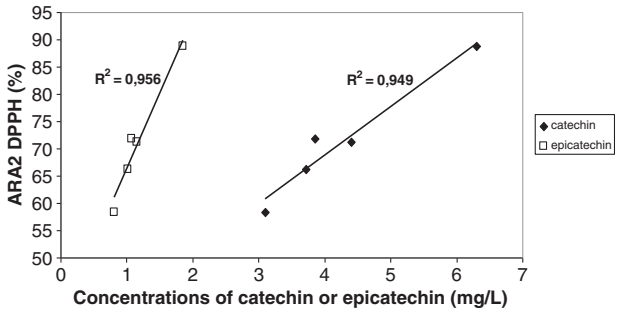

Figure 6. Relationship between concentrations of catechin or epicatechin and antioxidant activities in hopped worts.

we found overvalued concentration of polyphenols oxidized at potential 900 mV (e.g. 4-hydroxycoumarin or 4-hydroxybenzoic acid) in this chromatography system, and so these are not discussed further.

The correlation between the sum of compounds with oxidative potentials 400–500 mV and ARA2 was around 0.9 (Fig. 4) in all samples of sweet worts and hopped worts. Stronger correlations were found between ARA2 and concentrations of catechin and epicatechin in sweet wort samples ($R^2 = 0.970$, $R^2 = 0.961$, respectively) and in hopped wort samples ($R^2 = 0.946$, $R^2 = 0.956$, respectively; Figs 5 and 6). These data-correlations of catechins and ARA2 were not found for the beer samples owing to the sorption of polyphenols on the dregs, which led to a decreasing concentration in beer, and also to a change in the oxidation–reduction conditions (e.g. the rise of sulphur dioxide during fermentation).

The sorption and polymerization processes cause the unpredictable concentration changes in the polyphenol substances in beer. The sum of the polyphenols oxidized on 400–500 mV and total polyphenols in sweet and hopped wort increased with wort concentration and the number of mashing cycles (Tables 2 and 3). The beer samples behaved in an irregular manner. Beer sample A had the lowest concentration of polyphenols and this was in compliance with the infusion technology. Increasing concentrations in the other samples were only seen with beers C and E (from 15°P wort), and polyphenol concentration in samples B and D (from 12°P wort) did not follow this trend. There was a much better correlation with ESR lag time used for measuring antioxidant activity in the beer (Fig. 3). A chromatogram of the polyphenols analysed can be seen in Fig. 7.

Conclusions

The type of mashing had a strong impact on the antioxidant features of semi-finished products of sweet wort and hopped wort, using the same raw materials. The main group responsible for antioxidant activity is the group of flavan-3-ols.

The antioxidant activity properties of the sweet wort and hopped wort were well characterized by the ARA2 DPPH method, whose values correlated well with the content of some simple polyphenols (catechin and epicatechin) and total polyphenols. However, for beer the situation is different, as during cooling the wort, polyphenols are adsorbed onto the dregs and during fermentation sulphur dioxide is produced. This results in a change in the redox properties of beer. The ESR lag time method was found to be better for measuring the antioxidant activity of the beer.

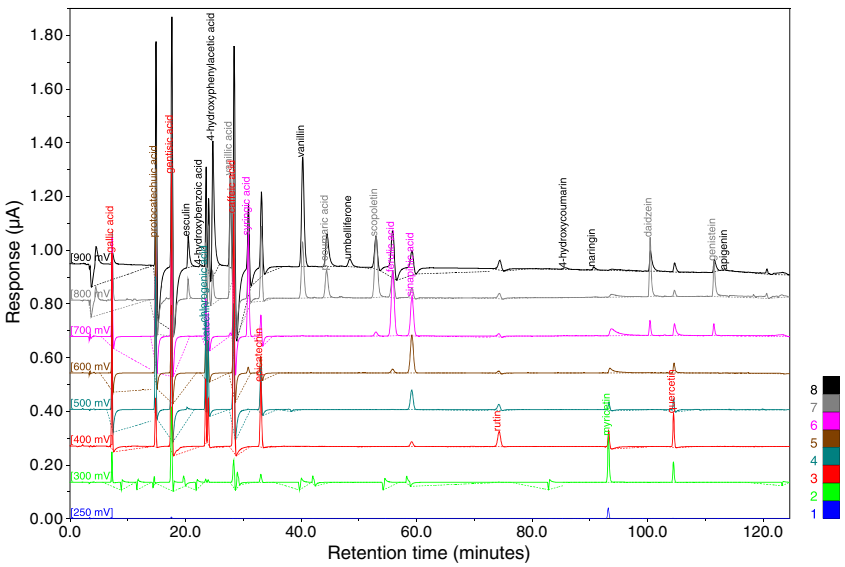

Figure 7. The chromatogram of standards of the analysed polyphenols (1 mg/L of each compound) by Coul Array detection.

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