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Fate of polyphenols and antioxidant activity of barley throughout malting and brewing

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ABSTRACT

Phenolic contents of barley and malt extracts and their corresponding antioxidant activities were investigated using a chromatographic online antioxidant detection system. Ethyl acetate extracts of barley and malt were separated using reverse phase HPLC and compounds eluting from the column were submitted to two UV–visible detections: one for the phenolic compounds; and the other for the reduced form of the radical cation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) after the compounds were allowed to react online with it. Prodelphinidin B3 and procyanidin B3 were identified as two major contributors in the antioxidant activity of barley, in addition to catechin. Malting had a dramatic impact on these three compounds by resulting in a sharp decrease in their detected amounts and the associated antioxidant activities. Two other antioxidants, ferulic and sinapic acids, showed a better ability to withstand not only malting but also brewing steps. As for the overall phenolic content and antioxidant capacity, the study showed that malting allowed a better release and/or extraction of phenolic compounds, while the first brewing step caused the most significant damage by drastically decreasing the total polyphenols and their activity. Hopping however did not significantly affect neither the phenolic content nor the antioxidant activity.

1. Introduction

The beneficial effects of beverages containing polyphenols such as teas, coffees, fruit juices, wines and beers are today well recognized (Gorinstein et al., 2000; Kaur and Kapoor, 2002). As far as beer is concerned, a number of studies have outlined its phenolic content (Fantozzi et al., 1998), and reported correlations between the level of beer polyphenols and the antioxidant activity (Gorinstein et al., 2007; Gorjanovic et al., 2010), between its alcohol content and the absorption of polyphenols (Bourne et al., 2000), and between beer consumption and human health (Preedy, 2009). In addition to their physiological properties, beer polyphenols are technologically crucial: they are involved in foam maintenance, physico-chemical stability and shelf-life of beer (Mikyska et al., 2002).

Beer polyphenols are derived from cereal grains (e.g. barley, rice, wheat, sorghum, oat, etc.) and hop; and their levels depend on malt and hop varieties (Agu, 2002; Derdelinckx, 2008; Liu and Yao, 2007). Barley and its derived malt are the focus of attention due to their high phenolic content (benzoic and cinnamic acids, proanthocyanidins, tannins, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds) and the associated antioxidant activity (Goupy et al., 1999; Hernanz et al., 2001; Liu and Yao, 2007; Qingming et al., 2010).

Yet, the levels of polyphenols in beer also depend on the malting and brewing processes. Data showed that the brewing process had a considerable impact on the phenolic content and the antioxidant activity of beer, mainly due to reactions undergone by polyphenols (Derdelinckx, 2008; Fantozzi et al., 1998; Gorjanovic et al., 2010; Pascoe et al., 2003). In most cases however, the antioxidant activity is studied without separation of beer's individual compounds, which takes into account the matrix complexity. Yet, from an analytical

Abbreviations: $ABTS^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); ANOVA, analysis of variance; BBME, brewed and boiled malt extract; BE, barley extract; BME, brewed malt extract; COADS, chromatographic online antioxidant detection system; ESI, electrospray ionization; HBBME, hopped, brewed and boiled malt extract; HE, hop extract; HPLC, high performance liquid chromatography; ME, malt extract; SD, standard deviation.

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point of view, this hampers the identification of antioxidant compounds and the changes they individually undergo during the processing steps. In a recent study, we used a Chromatographic Online Antioxidant Detection System (COADS) to assess the effects of brewing, boiling and fermentation on beer polyphenols (Leitao et al., 2011). It has been shown that with global determinations, the interactions occurring, while more or less representative of the food system, affect the assessment of the actual phenolic activities.

As far as malting is concerned, several studies investigated its effect on total polyphenols and antioxidant activity (Friedrich and Galensa, 2002; Lu et al., 2007; Qingming et al., 2010; Samaras et al., 2005). It has been found that malt had a higher antioxidant activity and phenolic content than the corresponding unmalted barley, which suggested that the malting process was of significance for this increase, especially the later stages of germination and subsequent kilning. Yet, the effect of malting on the fate of individual polyphenols and their corresponding antioxidant activities remains to be determined. The novelty of the present study is that it set out to determine the fate of individual phenolic compounds during the malting process. A further monitoring during the subsequent brewing is also presented.

2. Experimental

2.1. Chemicals, products and plant materials

2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), p-hydroxybenzoic acid, chlorogenic acid, 4-hydroxycinnamic acid (p-coumaric), sinapic acid, catechin and epicatechin were HPLC-grade and were purchased from Sigma–Aldrich (Seelze, Germany). Barley (*Hordeum vulgare*) was from the Sunshine variety and was malted by Brasseries Kronenbourg (Strasbourg, France). Hop was purchased from Yakima Chief, Inc (Sunnyside WA, USA) in the form of a resinous phase of alpha acids, beta acids, oils and uncharacterized resins produced by CO_2 supercritical extraction.

2.2. Extraction of phenolic compounds

The phenolic compounds extraction procedure used was adapted from Samaras et al. (2005) with slight modifications. Barley/malt samples were finely ground in liquid nitrogen (Air Liquide, Paris, France) during 9 min with a cryogenic ground (6870 Freezer/Mill, Spex CertiPrep, Stanmore, U.K.) at a rate of 20 impacts/sec. Powdered samples (1.63 g) were homogenized in 10.72 mL of acetate buffer (50 mM ammonium acetate, pH 5.4) for 2 min using a pestle and mortar (kept on ice). This corresponded to 10 mL of wort at 13 °Plato (g of sugar/100 g of wort). After centrifugation of the extracts (3000 rpm, 10 min), supernatants were filtered through a Whatman No.1 filter paper, to remove particulate material. Samples were adjusted to pH 2.0 by the addition of HCl (37%), and 0.5 g of sodium chloride was added. As previously described (Leitao et al., 2011), extraction was carried out in 50 mL Corning centrifuge tubes with 10 mL of ethyl acetate (three times, for periods of 15 min) on an orbital shaker at 200 rpm. The extracts were centrifuged (5000 rpm, 10 min), and the supernatants were collected and evaporated to dryness under vacuum (30 °C, 80 mbar). Samples were then redissolved in 1 mL methanol/water (50/50, v/v), membrane-filtered (0.45 μm, Macherey–Nagel, Hoerdt, France) and injected (20 μL) in the chromatographic system.

2.3. Brewing process

The brewing heating steps were applied to malt's ethyl acetate extract (ME). Brewed malt extract (BME) was obtained by mixing ME with water (to be equivalent to 10 mL of wort at 13 °Plato) and heating it as follows: 0–20 min, 37 °C; 20–34 min, 37–50 °C; 34–44 min, 50 °C; 44–59 min, 50–65 °C; 59–69 min, 65 °C; 69–82 min, 65–76 °C; 82–92 min, 76 °C. Brewed and boiled malt extract (BBME) was obtained by boiling BME (1 h, 100 °C). Hopped, brewed and boiled malt extract (HBBME) was obtained by boiling BME with hop extract (HE) (1 h, 100 °C) (Fig. 1).

2.4. HPLC analysis

The conditions and equipment used for COADS method were as previously described (Leitao et al., 2011). Briefly, separation was carried out using a Waters HPLC system (Waters, Saint-Quentin-Fallavier, France), a hypersil BDS C18 HPLC column (5 μm, 250 × 4.6 mm i.d., ThermoScientific, Gometz-le-Châtel, France), and a mobile phase consisting of a water (0.1% formic acid, v/v)/methanol gradient delivered at 1 mL/min. Detection was done at 254 nm for phenolic compounds and at 412 nm for their respective antioxidant activities after post-column reaction with $ABTS^{\bullet+}$ prepared as previously described (Leitao et al., 2011).

2.5. Identification of phenolic compounds with LC-ESI-MS

Identification of phenolic compounds was performed with an electrospray ionization (ESI) mass spectrometer after online HPLC separation. High-resolution mass spectra were obtained with

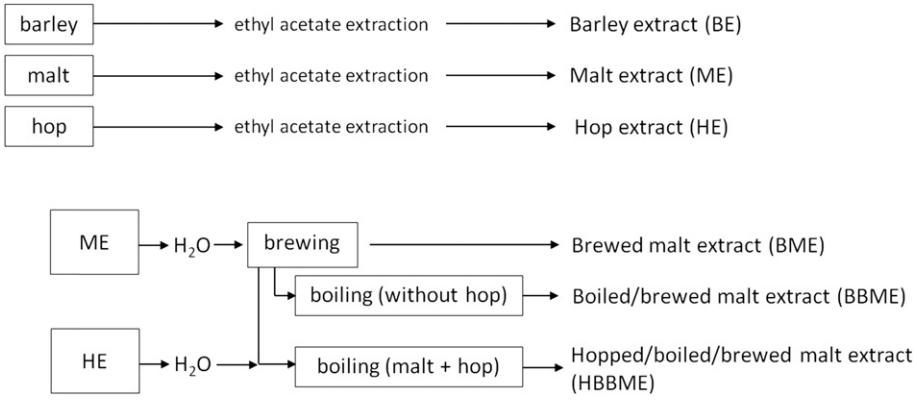

Fig. 1. Sampling and extraction at different stages of malting and brewing.

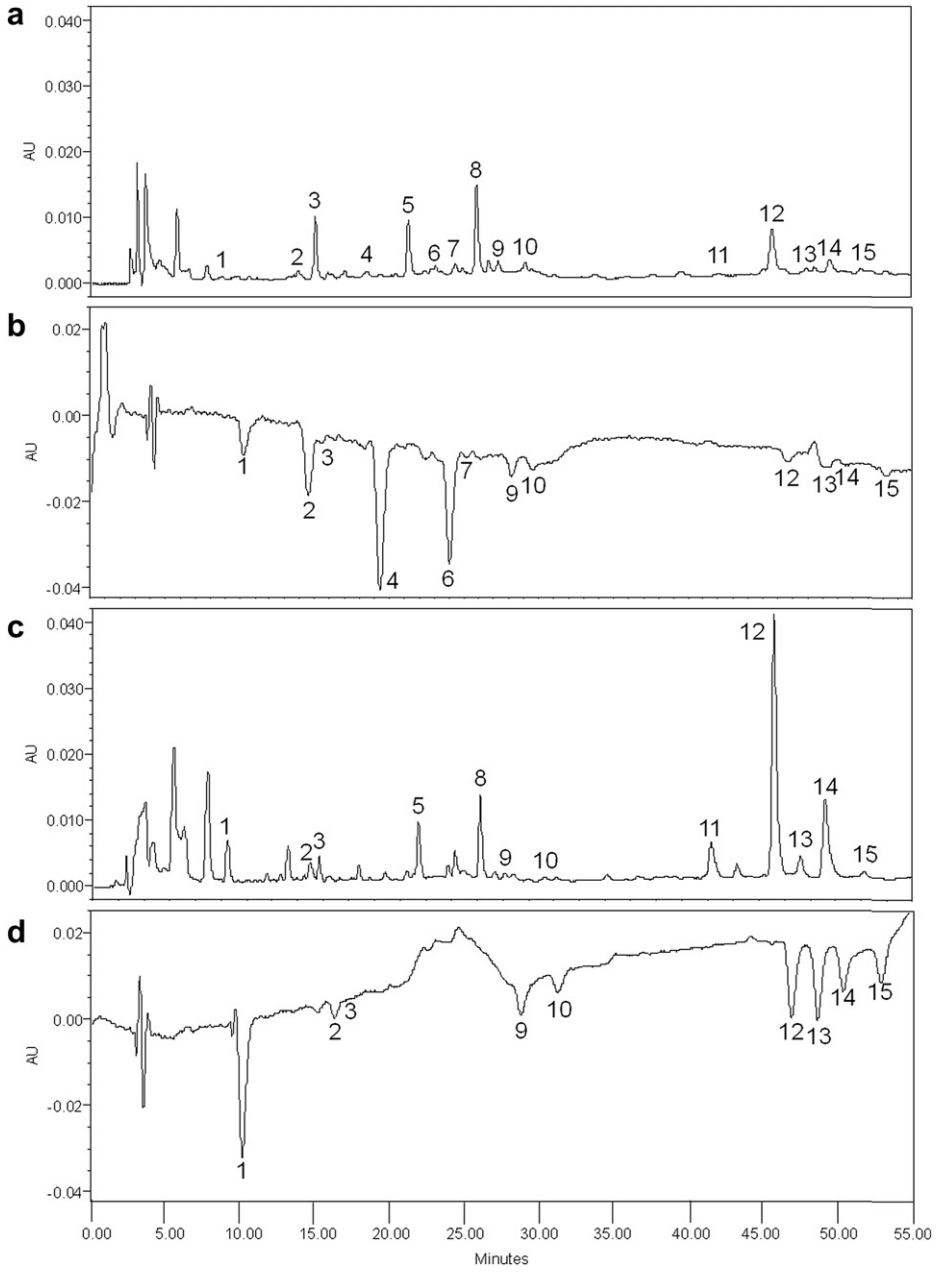

Fig. 2. Chromatographic determination of phenolic compounds (a, c) and their corresponding antioxidant activities (b, d) in extracts of barley (a, b) and malt (c, d). Peaks are as follows: 1, unidentified compound; 2, prodelphinidin B3; 3, protocatechuic acid; 4, procyanidin B3; 5, p-hydroxybenzoic acid; 6, catechin; 7, chlorogenic acid; 8, vanillic acid; 9, caffeic acid; 10, epicatechin; 11, p-coumaric acid; 12, ferulic acid; 13, sinapic acid; 14, unidentified compound; 15, unidentified compound.

a 6520 Accurate Mass Q-TOF spectrometer (Agilent, Massy, France), with separation on a C18 HPLC column (1.9 μm, 100 × 1 mm i.d.) and an isocratic water/acetonitrile mobile phase containing 0.01% (v/v) formic acid and delivered with a 1200 solvent delivery module (Agilent).

2.6. Procyanidin B3 synthesis

Multi protected Procyanidin B3 was prepared according to the method developed by Tarascou et al. (2006) by stoichiometric coupling reaction between 3′, 4′, 5, 7-tetra-*O*-benzylcatechin and 3′, 4′, 5, 7-tetra-*O*-benzyl-8-bromo-4β-(2-hydroxyethoxy) catechin in the presence of $TiCl_4$ (1M in dichloromethane) in tetrahydrofuran and dichloromethane. Procyanidin B3 was obtained by debenzylation and debromination using Pearlman's catalyst in a 1:1 mixture of methanol/ethyl acetate in the presence of triethylamine.

2.7. Calibration graphs

Calibration graphs for each phenolic compound were drawn from data of three replicate injections of 20 μL of standard mixtures obtained by dilution (methanol/water (50/50, v/v)) at various levels of the stock standard solutions. The curves (six data points, $n = 3$) were linear with R^2 values higher than 0.99. Each known phenolic compound was quantified by reference to its appropriate authentic standard by UV absorption detection, while the antioxidant potential was calculated as the concentration of Trolox required to produce an equivalent antioxidant activity and expressed as Trolox equivalent (μM).

2.8. Statistical analysis

Data were analyzed by ANOVA (at significance level of 95%) using Statgraphics Plus software. All samples were analyzed in triplicate.

3. Results and discussion

Fig. 2 shows COADS chromatograms obtained with phenolic compounds in ethyl acetate extracts of barley (BE) and malt (ME) (Fig. 2a, c) and their corresponding antioxidant activities (Fig. 2b, d). Ten phenolic compounds could be identified using chromatographic standards: protocatechuic acid (peak 3); p-hydroxybenzoic acid (peak 5); catechin (peak 6); chlorogenic acid (peak 7); vanillic acid (peak 8); caffeic acid (peak 9); epicatechin (peak 10); p-coumaric acid (peak 11); ferulic acid (peak 12) and sinapic acid (peak 13). Compounds 2 and 4 were identified by LC-ESI-MS analysis in positive ion mode as prodelphinidin and procyanidin dimers, respectively (McMurrough et al., 1996). Compound 2 showed an $[M + H]^+$ of 595 m/z and an exact mass of 594.14534 and was identified as prodelphinidin B3, a gallocatechin-catechin dimer. Compound 4 showed an $[M + H]^+$ at 579 m/z and an exact mass of 578.15039 and was identified as procyanidin B3, a catechin–catechin dimer (Goupy et al., 1999; McMurrough et al., 1996). Procyanidin B3 was synthesized and fully characterized using IR, NMR (^1H, ^{13}C) (400 MHz, H_2O/10% D_2O, 22 °C) and MS experiments (LSIMS: $[M-H]^+$ at 577 m/z) as previously reported (Tarascou et al., 2006). Chromatographic and mass data confirmed the identity of the isolated compound. Compounds corresponding to peaks 1, 14 and 15 could not be identified.

As far as the antioxidant activity is concerned, with the exception of p-hydroxybenzoic, vanillic and p-coumaric acids, all identified compounds reacted with ABTS radical. The three unidentified compounds 1, 14 and 15 also showed antioxidant activity (Fig. 2). Using PBS buffer at pH 7.4 as dilution solvent for $ABTS^{\bullet+}$, instead of ethanol, as recommended by Shi et al. (2009), resulted in an increase of the antioxidant response only with ferulic acid, while it had insignificant or no effect on the other compounds.

In barley, the largest antioxidant contribution came from catechin, procyanidin B3 and prodelphinidin B3, which together corresponded to 53% of the total antioxidant activity (Table 1). This is consistent with previous data showing that catechin and flavonoids are generally abundant in barley (Leitao et al., 2011). Malting apparently resulted in a decrease in the amounts of these compounds and the antioxidant activity that could be associated with them. Such a decrease has already been reported for proanthocyanidins and catechins, and has been attributed to glycosylation reactions during malting (Friedrich and Galensa, 2002). The malting process would therefore not result in a degradation of these compounds, which is in agreement with our previous findings showing that prodelphinidin B3, procyanidin B3 and catechin were indeed found in wort (Leitao et al., 2011).

On the other hand, the largest antioxidant contribution in malt came from ferulic and sinapic acids, as well as compound 1, which together represented 52% of the total antioxidant activity (Table 1). The activity due to ferulic and sinapic acids was three-fold higher, and that of compound 1 was five-fold higher in malt than in barley, which is obviously due to higher contents of these compounds (Fig. 2a, c). This increase could be attributed to a better extraction following the release of bound molecules during kilning (Woffenden et al., 2002). Ferulic acid and p-coumaric acid (the amount of which also increases after malting) have already been identified as the most important bound phenolic compounds in barley grains (Nordkvist et al., 1984). In addition, the friable nature of malt as compared to barley would probably allow a better extraction of phenolic compounds.

After malting and after each step of the subsequent brewing process, COADS chromatograms were determined and peak areas were summed and used to represent the total phenolic content and the total antioxidant activity, which were expressed as average ± SD from triplicates of three determinations (Fig. 3). It appeared that total phenolic content was four-fold higher in malt than in barley (Fig. 3a). This difference was mainly due to higher concentrations of p-coumaric, ferulic and sinapic acids, as well as compound 14 in malt (Fig. 2a, c). They corresponded to 22 and 77% of the total phenolic content of barley and malt, respectively.

The total antioxidant activities, expressed as Trolox equivalent (μM), did not follow a similar trend, since there was no significant ($p > 0.05$) difference between barley and malt (Fig. 3b). Yet, the two antioxidant activity profiles were dissimilar, as the decrease in the antioxidant contribution of compounds like catechin, procyanidin B3, protocatechuic acid, and prodelphinidin B3 was balanced by the increase in that of ferulic and sinapic acids, as well as compounds 1, 14 and 15 whose levels have increased (Fig. 2b, d).

As far as the subsequent processing steps (brewing, boiling and hopping) are concerned, relevant heating steps were applied to ethyl acetate extract of malt, in order to investigate their influence

Table 1
Amounts of antioxidant compounds and their antioxidant activities in ethyl acetate extracts ($n = 9$) of barley (BE), malt (ME), brewed malt (BME), boiled and brewed malt (BBME) and hopped, boiled and brewed malt (HBBME).

Phenolic compounds		BE		ME		BME		BBME		HBBME	
Peak	Identity	CC[a]	AA[b]	CC	AA	CC	AA	CC	AA	CC	AA
1	Unidentified	nq	2 ± 1	nq	10 ± 3	nq	6 ± 2	nd	nd	nd	nd
2	Prodelphinidin B3	nq	5 ± 2	nq	2 ± 1	nd	nd	nd	nd	nd	nd
3	Protocatechuic acid	12 ± 1	3 ± 1	3 ± 1	2 ± 1	nd	nd	nd	nd	nd	nd
4	Procyanidin B3	nq	10 ± 6	nd	nd	nd	nd	nd	nd	nd	nd
5	p-Hydroxybenzoic acid	7 ± 0	–	5 ± 1	–	5 ± 1	–	4 ± 1	–	4 ± 0	–
6	Catechin	15 ± 8	7 ± 3	nd	nd	nd	3 ± 0	nd	nd	nd	nd
7	Chlorogenic acid	5 ± 0	2 ± 0	nd	nd	nd	nd	nd	nd	nd	nd
8	Vanillic acid	17 ± 1	–	10 ± 1	–	5 ± 0	–	5 ± 1	–	5 ± 1	–
9	Caffeic acid	3 ± 0	2 ± 1	1 ± 0	3 ± 1	nd	1 ± 0	nd	nd	nd	nd
10	Epicatechin	24 ± 7	2 ± 0	15 ± 3	3 ± 1	nd	nd	nd	nd	nd	nd
11	p-Coumaric acid	3 ± 1	–	30 ± 8	–	21 ± 2	–	19 ± 2	–	22 ± 4	–
12	Ferulic acid	20 ± 2	2 ± 1	73 ± 7	6 ± 2	37 ± 3	1 ± 1	40 ± 2	2 ± 1	40 ± 5	2 ± 0
13	Sinapic acid	1 ± 0	2 ± 1	8 ± 2	6 ± 1	31 ± 5	6 ± 1	20 ± 4	5 ± 1	24 ± 5	4 ± 2
14	Unidentified	nq	2 ± 0	nq	5 ± 1	nq	2 ± 1	nq	1 ± 0	nq	3 ± 1
15	Unidentified	nq	2 ± 0	nq	5 ± 2	nd	nd	nd	nd	nd	nd

[a] concentration in μM.
[b] antioxidant activity in μM of trolox equivalent; -no antioxidant activity; nq, not quantified (no standards); nd, not detected.

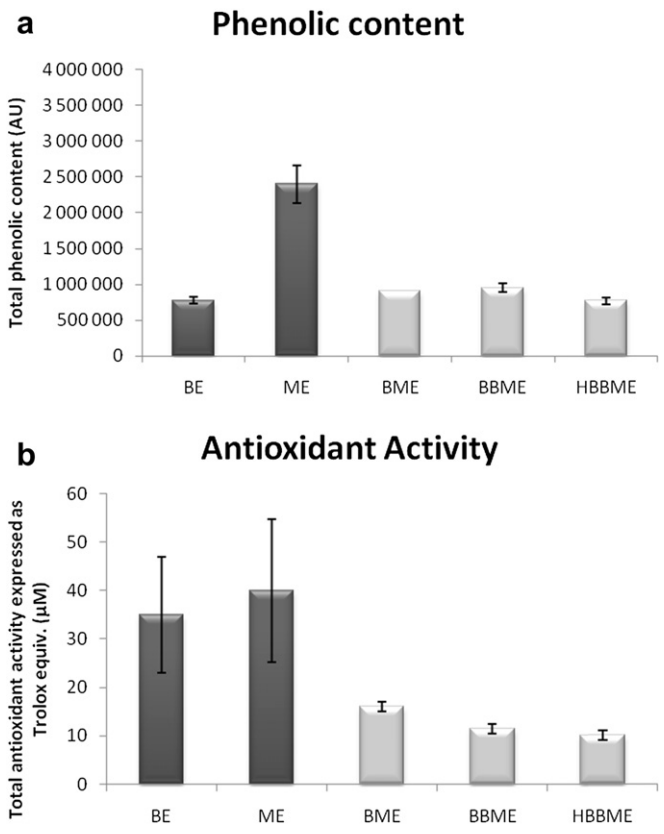

Fig. 3. Total phenolic content (a) and antioxidant activity (b) of barley extract (BE) and malt extract (ME) at different steps of brewing: BME, brewed malt extract; BBME, boiled and brewed malt extract; and HBBME, hopped, boiled and brewed malt extract.

on its total phenolic content and antioxidant activity. Extracts (BME, BBME and HBBME) representing the brewing process applied to ME were analyzed using the COADS method (Fig. 1).

During brewing, the total phenolic content of ME decreased by 62% (Fig. 3a), parallel to a similar decrease in antioxidant activity (Fig. 3b). This reflected a decrease of the concentrations of all identified phenolic compounds, except p-hydroxybenzoic acid, whose concentration remained unchanged, and sinapic acid, whose concentration showed a four-fold increase, while its antioxidant activity remained unchanged (Table 1). During the boiling step however, the phenolic content remained unchanged, while the total antioxidant activity showed a further decrease by 30%. Both were unaffected by the hopping step (Fig 3b). It is however noteworthy that, if as suggested, prodelphinidin B3, procyanidin B3 and catechin are glycosylated under malting conditions, their extraction by ethyl acetate would be hampered.

The present study allowed the identification of prodelphinidin B3 and the procyanidin B3 as two major contributors in the antioxidant activity of barley, in addition to catechin. It also showed that the malting and the brewing processes had a dramatic impact on these compounds by resulting in a sharp decrease in their detected amounts and the associated activities. On the other hand, two other antioxidants, ferulic and sinapic acids, showed a better ability to withstand the heat treatments of processing steps. As for the overall phenolic content and antioxidant capacity, the study showed that malting allowed a better release and/or extraction of phenolic compounds, while the first brewing step caused the most significant decrease in the total polyphenols extracted and the antioxidant activity.

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