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Further insights into the role of melanoidins on the antioxidant potential of barley malt

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ABSTRACT

The role of Maillard reaction products isolated from barley malt by gel permeation chromatography and ultrafiltration on the antioxidant potential of *pale*, *melano80* and *black* malts was evaluated. The roasting process is responsible for the polymerisation of early formed lower molecular weight compounds (<10 kDa) into high molecular weight melanoidins (>300 kDa). Melanoidins showed 3-fold higher capacity to scavenge radicals than the lower molecular weight colorants by the metmyoglobin assay. However, a significant decrease of the capacity of *black* malt and high molecular weight melanoidins to inhibit Fenton induced hydroxyl degradation of deoxyribose was observed. As the high molecular weight fraction, isolated from the *black* malt extract, exhibited 4-fold higher reducing power than the lower molecular weight fraction, our results support a pro-oxidant effect due to the catalytic formation of hydroxyl radicals in the presence of ferric ions.

1. Introduction

Malt is one of the main ingredients used in beer production, mainly as a source of starch but also contributing to the colour and organoleptic characteristics of beer. Malted grains are produced in a process called malting involving 3 steps: steeping (early growth of the embryo), germination (formation of green malt) and drying or roasting (final heat treatment) of green malt. Dark malts are prepared using high temperatures, resulting in a non-enzymatic browning also known as Maillard reaction (Coghe, Adriaenssens, Leonard, & Delvaux, 2004; Coghe, D'Hollander, Verachtert, & Delvaux, 2005; Coghe, Gheeraert, Michiels, & Delvaux, 2006; Yahya, Linforth, & Cook, 2014). During this step a group of compounds denominated Maillard reaction products (MRP) are formed by the reaction between reducing sugars with amino acids and amino groups of peptides or proteins. During the final stages of the Maillard reaction the formation of melanoidins (MLD) occurs by polymerisation (Coghe et al., 2006; Wang, Qian, & Yao, 2011). MLD are described as nitrogenous compounds with high molecular weight, high reducing potential and an intense brown colour (Echavarría, Pagán, & Ibarz, 2012).

During malt kilning (up to 80 °C) and roasting (110–250 °C) the formation of antioxidants may occur through the Maillard reaction, such as reductones and MLD (Vanderhaegen, Neven, Verachtert, & Derdelinckx, 2006). In fact, some authors stated that the most important properties of dark malts are their colour and flavour, but also their antioxidant capacity resulting from the Maillard reaction (Coghe, Adriaenssens, Leonard, & Delvaux, 2003; Samaras, Camburn, Chandra, Gordon, & Ames, 2005). The study of the compounds that can affect the antioxidant capacity of malt is of extremely importance not only due to their contribution to the oxidative stability of beer (Vanderhaegen et al., 2006), but also to the benefits that they could bring to consumers' health, preventing and neutralising reactive oxygen species (ROS) associated with numerous diseases: cancer, cardiovascular and neuronal diseases (Halliwell, 1987; Landete, 2013). For that reason, health benefits of MLD are the focus of many researchers in food science.

MLD are reported to have important beneficial effects such as antiradical, antimutagenic, antimicrobial, antihypertensive, antiallergenic, antioxidant and cytotoxic properties (Echavarría et al., 2012; Wang et al., 2011). Studies about the antioxidant properties of MRP suggest that MLD are the main contributors to the

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FC, Folin–Ciocalteu; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; GPC, gel permeation chromatography; HMW, high molecular weight; IMW, intermediate molecular weight; LMW, lower molecular weight; MLD, melanoidins; MRP, Maillard reaction products; MW, molecular weight; MWCO, molecular weight cut-off; ROS, reactive oxygen species; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; TE, Trolox equivalents; TPC, total phenolic content; TPTZ, 2,4,6-tris(2-pyridyl)-*s*-triazine; UF, ultrafiltration.

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antioxidant capacity of malt (Coghe et al., 2003), honey (Brudzynski & Miotto, 2011) and coffee (Gómez-Ruiz, Ames, & Leake, 2008). This capacity is often ascribed to the metal-chelating properties, reducing power and radical-scavenging capacity (Echavarría et al., 2012; Sovrano, Buiatti, & Anese, 2006; Wang et al., 2011). However, recent studies showed that MLD can exert a pro-oxidant activity as well (Cämmerer et al., 2012; Hoff, Lund, Petersen, Jespersen, & Andersen, 2012). The pro-oxidant properties of MLD can be related with the formation of radicals by a Fenton mechanism, due to the presence of iron or copper cations (Hoff et al., 2012), suggesting that the reducing groups of MLD may be responsible for the pro-oxidative properties of roasted malts (Wunderlich, Wurzbacher, & Back, 2013).

Considering the antioxidant properties of malt, beer oxidative stability and quality, the present work aims at evaluating the effect of MRP on the overall antioxidant potential of malts with different degrees of roasting. Isolation of MRP was carried out according to their molecular weight, by ultrafiltration (UF) and gel permeation chromatography (GPC). The antioxidant potential was evaluated by different methods, based on the radical-scavenging activity and reducing power. The metmyoglobin assay and the deoxyribose assay were used to assess the antiradical capacity, whereas the ferric reducing antioxidant power (FRAP) was applied to evaluate the reducing properties.

2. Material and methods

2.1. Reagents and standards

All chemicals used, unless stated otherwise, were of analytical grade or highest purity available and were purchased from Sigma–Aldrich (St. Louis, MO). Stock standard solutions (1000 mg L^{-1}) of phenolic compounds were prepared by rigorous dissolution of the commercial reagent in methanol. Standard solutions were stored at −20 °C and used for further dilutions. High-purity water from a Direct-Q 3 UV water purification system (Millipore Iberian, Spain) was used for all analyses and glassware washing.

2.2. Malt samples

Pale, *melano80* and *black* malts used throughout this work were kindly supplied by *Os Três Cervejeiros, Lda* (Porto, Portugal) and *Unicer – Bebidas de Portugal, S.A.* (S. Mamede de Infesta, Portugal). Characteristics of each malt type, namely the colour range and kilning temperature are listed in Table 1. Malt samples were milled in a laboratory EBC mill (Casela, London, UK) and extracted as detailed below.

2.3. Preparation of malt extracts

Malt extracts were prepared by mixing 50 g (±1 g) of ground sample and 200 mL water at 45 °C. The mash was continuously stirred and incubated for 1 h at 45 °C. After extraction the mixtures were cooled to room temperature and more water (at room temperature) was added to a final volume of 300 mL. The extracts were then filtered with a Whatman (Maidstone, UK) no. 1 filter paper and freeze-dried (Unicryo MC4L system; Progen Scientific, Merton, UK).

Table 1
Colour range and kilning temperatures of the different malt types used in this work.

Malt	Color range/EBC units	Kilning temperature/°C
Pale	3.5–5.7	80–85
Melano80	75–85	130
Black	1300–1500	230

2.4. Isolation of MRP from malt extracts

2.4.1. Ultrafiltration (UF)

Malt extracts were fractionated by UF in order to isolate MRP within different MW ranges. Approximately 100 mg of each freeze-dried extract were resuspended in 5 mL of water and subjected to stepwise ultrafiltration process using Microsep™ Advance centrifugal devices (Pall Corporation, Port Washington, NY) with molecular weight cut-offs (MWCOs) of 100, 30 and 10 kDa. The total sample volume (5 mL) was added to the sample reservoir of the device of 100 kDa, followed by centrifugation at 13,500 rpm, for 30 min. More water was added and centrifuged again to ensure that all the lowest molecular weight compounds were separated from the highest molecular weight compounds. The remaining residue in the sample reservoir (HMW fraction) was recovered. The solution in the filtrate receiver was applied to the 30 kDa MWCO device and from this to the 10 kDa MWCO. Four fractions were collected at the end: one HMW fraction (UF1 > 100 kDa), two intermediate-molecular-weight fractions (IMW, 30 < UF2 < 100 kDa and 10 < UF3 < 30 kDa) and one LMW fraction (UF4 < 10 kDa). All fractions were freeze-dried and stored at −20 °C until further use. The quantitatively dominant fractions recovered from each sample, according to Table 2, were subjected to further characterisation.

2.4.2. Gel permeation chromatography (GPC)

The distribution of the molecular weight of MRP present in the malt extracts was estimated in more detail by means of GPC. For this purpose, 500 μL of each malt extract were injected into a Superdex 200 column (1.6 × 40.9 cm; Amersham Biosciences, Uppsala, Sweden). Coloured compounds were eluted with ammonium acetate buffer (0.2 M, pH 6.0) at 0.5 mL/min and on-line detected at 420 nm. At the end of the chromatographic run, 30 fractions of 2.0 mL were collected (F1–F30). The void volume was determined with blue dextran 2000 and the total volume with glycine. Ribonuclease A (13.7 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) were used as molecular weight calibration standards (GE Healthcare, Fairfield, IL). All the fractions obtained were subjected to further characterisation.

2.5. MLD standard preparation and quantification

The MLD standard was obtained from a glucose and glycine model system and used for determination of MLD content in the samples. The standard was prepared by dissolving 0.05 mol of glucose and glycine in 100 mL water. The solution was freeze-dried to constant weight. The white mixture of carbonyl compound and amino acid was placed in a fan oven, preheated at 125 °C. The mixture was heated for 2 h without covering. After cooling to room temperature in a desiccator, the brown solid was transferred to a mortar and carefully ground to a fine powder. A 5-g aliquot of

Table 2
Fractions obtained by ultrafiltration of the different malt extracts. Each fraction was lyophilised after extraction and the yield of compounds within the different MW ranges was determined in each sample.

Fraction (kDa)	Yield (%, w/w)		
	Pale	*Melano80*	*Black*
UF_1 > 100 kDa	1.7	2.3	39.2
100 < UF_2 < 30 kDa	3.4	2.3	0.8
30 < UF_3 < 10 kDa	2.5	6.2	2.4
UF_4 < 10 kDa	92.4	89.1	57.6