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Electrophoretic and HPLC methods for comparative study of the protein fractions of malts, worts and beers produced from Scarlett and Prestige barley (*Hordeum vulgare* L.) varieties

Filipe Silva [a,b], Luciana C. Nogueira [c], Cristina Gonçalves [d], António A. Ferreira [d], Isabel M.P.L.V.O. Ferreira [a,*], Natércia Teixeira [b,e]

[a] *REQUIMTE – Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[b] *Serviço de Bioquímica, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[c] *Laboratório de Bioquímica Nutricional e de Alimentos, Instituto de Química, Universidade Federal do Rio de Janeiro, Brazil*
[d] *UNICER – Bebidas de Portugal SGPS, SA, Leça do Balio, 4466-955, S. Mamede de Infesta, Portugal*
[e] *IBMC, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal*

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Abstract

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high performance liquid chromatography (RP-HPLC) methods were used for studying the protein fractions (hordeins; albumins and other soluble proteins) of Scarlett and Prestige barley malts and to follow changes of the protein profile of worts and beers from these two malt varieties. Similar industrial brewing conditions were applied for both varieties.

Statistical analyses of RP-HPLC data showed that hordeins were exposed to a proteolytic process during germination, which reduced its content and originated less hydrophobic peptides. In contrast, albumins and other soluble proteins increased during the germination process. Some malt water-soluble proteins result from the hordein proteolysis. Quantitative differences were observed between the protein fractions of the two malt varieties.

SDS-PAGE patterns indicate that most of the components present in the worts were also detectable in final beers. However, chemometric analysis of the HPLC data showed quantitative differences between Scarlett and Prestige worts quantitative protein profiles. Scarlett wort contained more protein than Prestige worts. However, final beer samples presented a quantitative protein profile more similar than the respective worts.

The optimized methodologies can be successfully used to compare the protein fractions of malts produced from two barley varieties, to follow the evolution of protein fraction during germination and the evolution of protein fraction content of worts and beers.

Keywords: *Hordeum vulgare* L.; Malt hordeins; Malt albumins; RP-HPLC; SDS-PAGE; Wort; Beer

1. Introduction

Barley is the most important raw material for beer production. It is a widely grown cereal crop, used for human and animal feed and for brewing, due to the high enzymatic content, that is conversion of starch into fermentable sugars and being a cereal with a husk that protects the embryo during the handling of the grain and is an important aid during the wort filtration. In fact, the aim of the malting process is the production and activation of enzymes. These molecules also contribute to the hydrolysis of β-glucans and hordeins (water insoluble proteins), which would otherwise restrict access of enzymes to the starch granules (Hughes & Baxter, 2001).

* Corresponding author. Tel.: +351 222078929; fax: +351 222003977.
E-mail address: isabel.ferreira@ff.up.pt (I.M.P.L.V.O. Ferreira).

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Barley grain germination is initiated by the uptake of water. The grain imbibes water during controlled cycles of water spraying, or water immersion, followed by aeration, until the water content of the grain reaches 42–48%. Water enters the grain via the embryo, and after approximately 24 h, the first visible sign of germination is the appearance of the root, as a white 'chit'. Germination is typically allowed to proceed over a period of around 5 days to obtain green malt, which is then stopped during the Dewatering and Kilning phase by forced flow of hot air. Hydrolases produced during malting are partially inactivated during this process. The malt is stable for storage and has a friable texture suitable for the milling process, which precedes brewing.

Breeders are involved in the development of high-quality value barley cultivars for the malting and brewing industries. Barley selected for use in the brewing industry must meet special quality requirements and be must be approved for malting and beer production. The malt quality of a given barley variety is determined by its genetic background and the physical conditions during growth, harvest and storage. The validation process for brewing includes trials in micro and pilot malting and brewing plants before introduction at production scale (Østergaard, Melchior, Roepstorff, & Svensson, 2002).

Proteins are among the barley components that are essential for the quality of malt and beer. Thus, the total protein quantity in the barley grain is a crucial factor for final beer quality. High protein contents decrease available carbohydrates, with a negative influence on the brewing process (Fox, Onley-Watson, & Osman, 2002; Peltonen, Rita, Aikasalo, & Home, 1994). The protein content in barley grains represents, approximately, 8–15% of its total mass. Hordeins are the most abundant proteins (40–50%) found in a barley grain (Osman et al., 2002). In addition to hordeins, other proteins have been identified, including albumins, glutelins (globulins), friabilin, enzymes, serpins and other inhibitors, chaperones and other proteins with unknown functions (Borén, Larsson, Falk, & Jansson, 2004; Finnie, Melchior, Roepstorff, & Svensson, 2002; Fox et al., 2002; Osman, Coverdale, Onley-Watson, Bell, & Healy, 2003; Østergaard, Finnie, Laugesen, & Roepstorff, 2004; Østergaard et al., 2002).

Hordeins, as the main storage protein fraction in barley seeds, accounts for up to half of the total protein in the mature grains, and may be classified into four groups named B, C, D and γ hordeins based on their electrophoretic mobilities. The B (30–45 kDa) and C (45–75 kDa) fractions account for ~70–80% and ~10–12%, respectively, of the total hordeins, while the D and γ fractions are minor components. Hordeins exist both in monomeric and aggregated forms. (Brennan, Smith, Harris, & Shewry, 1998; Fox et al., 2002; Lookhart, Bean, & Jones, 1999; Molina-Cano et al., 2001; Peltonen et al., 1994; Schmitt, Gille, Gaucher, & Montembault, 1989; Shewry, Kreis, Parmar, Lew, & Kasarda, 1985).

Information about albumins, found in the literature, is mainly related to protein Z and lipid transfer protein 1 (LTP1). Protein Z is a 40 kDa hydrophobic glycoprotein, with an isoelectric point of 5.5–5.8 (barley form) or 5.1–5.4 (beer form) (Curioni, Pressi, Furegon, & Peruffo, 1995; Hejgaard & Kaersgaard, 1983; Hejgaard, 1982; Leiper, Stewart, & McKeown, 2003; Lusk, Cronan, Chicoye, & Goldstein, 1987; Sørensen, Bech, Muldbjerg, Beenfeldt, & Breddam, 1993; Yokoi, Maeda, Xiao, Kamada, & Kamimura, 1989). In barley and malt, protein Z can be found in two isoforms: Protein Z4 (80%) and Z7 (20%) (Evans, Nischwitz, Stewart, Cole, & MacLeod, 1999). On the other hand, LTP1, which was initially named probable amylase/protease inhibitor (PAPI) (Leiper et al., 2003), is a 9.7 kDa glycoprotein, with an isoelectric point of 9 (Jégou, Douliez, Mollé, Boivin, & Marion, 2000; Jones & Marinac, 1997; Vaag, Bech, Cameron-Mills, & Svendsen, 1999). LTP1 contains 91 amino acid residues (Lindorff-Larsen & Winter, 2001), organized into four α-helix segments, which are stabilized by four disulphide bonds (Bech, Vaag, Heinemann, & Breddam, 1995).

The majority of beer protein lies in the 10–40 kDa size range (Leiper et al., 2003). Mostly, the origin of this protein is malted barley (Hughes & Baxter, 2001). Some beer proteins appear to have no function in beer except their contribution to mouthfeel, flavor, texture, body, color, and nutritional value (Leiper et al., 2003; Osman et al., 2003). Protein Z, LTP1, and other proteins present in beer have been associated to foam formation and/or stabilization (Evans & Sheehan, 2002; Ferreira, Jorge, Nogueira, Silva, & Trugo, 2005; Lusk, Goldstein, & Ryder, 1995; Nierop, Evans, Axcell, Cantrell, & Rautenbach, 2004; Perrocheau, Rogniaux, Boivin, & Marion, 2005). Protein Z has also been related to beer haze (Curioni et al., 1995).

Scarlett and Prestige barleys were two rowed spring varieties. Scarlett has very good brewing quality and is considered a standard variety, by the European Brewery Convention (EBC). Presently, Prestige is also proposed as standard variety for some regions, it presents good agronomic characteristics, including high plague resistance. A comparative study of the protein fractions (hordeins; albumins and other soluble proteins) of Scarlett and Prestige malts is important to contribute to the characterization of these barley varieties. Chromatographic (Osman et al., 2003; Schmitt et al., 1989) and electrophoretic (Echart-Almeida & Cavalli-Molina, 2001; Leisegang & Stahl, 2005; Molina-Cano et al., 2001; Villiers & Laubscher, 1989) techniques are frequently used to study malt proteins, thus, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high performance liquid chromatography (RP-HPLC) techniques were chosen for comparison of malt proteins from Scarlett and Prestige varieties. Studies were performed for two different germination times (60 and 120 h).

In a posterior phase, both malts were used to produce beer. Similar industrial brewing conditions were separately applied to Scarlett and Prestige malts. Sodium dodecylsul-

fate-polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high performance liquid chromatography (RP-HPLC) were used to examine the protein profiles of the worts and beers produced from these two malt varieties.

2. Materials and methods

2.1. Reagents and standards

A mixture of molecular weight standards from 6.5 to 205 kDa (SigmaMarker – Wide molecular weight range, Sigma, St. Louis, MO, USA) was used in the electrophoretic separations. A 98% purity Bovine Serum Albumin (BSA) solution (Sigma, St. Louis, MO, USA) was also employed as standard, to confirm the relative position of the molecular weight standards.

Eluents used for the chromatographic separations were ultra-pure water (obtained from a Seral – Seralpur Pro 90 CN water purifying system) and LiChrosolv acetonitrile (Merck, Darmstradt, Germany). Trifluoroacetic acid (TFA) (Fluka, Seelze, Germany) was added to both eluents to make them 0.1% solutions of the former.

2.2. Sample preparation

Malt samples were produced from Portuguese barleys, Scarlett and Prestige varieties, (harvested in Alentejo, (2003)), using the same malting conditions. Samples of 60 and 120 h of germination were collected, kilned, milled to a mixture of flour and husk, and subsequently submitted to two different protein extraction methods. Hordeins were extracted using the Schmitt et al. (1989) procedure. Briefly, 1 g of ground malt was submitted to extraction with 8 mL of 62 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 8% (v/v) 2-mercaptoethanol. After vigorous stirring, the mixture was kept at room temperature for 3 h. The extract was centrifuged at 5000 rpm, for 15 min at room temperature, and the supernatant was collected. Albumins and other soluble proteins were extracted by procedure described by Howard, Gayler, Eagles, and Halloran (1996). Briefly, proteins were extracted twice, each time for 1 h, from 1 g of ground malt with 10 mL of 0.15 M potassium phosphate pH 8.0, 5 mM dithiothreitol (DTT) at room temperature. The extracts were combined and centrifuged at 5000 rpm, for 8 min at room temperature, and the supernatant was collected. All supernatants were filtered, using 0.45 μm Teknokroma (TR-200106 – PVDF, 25 mm ∅, PK/100) syringe filters, divided into aliquots, and stored at −20 °C.

Beers were produced in industrial scale from Scarlett and Prestige malts. 100% of the malt used in each case was from a single barley cultivar. Similar mashing, fermentation, and filtration procedures were applied. Wort and filtered beer were collected. Analyses were performed in quadruplicate. All the samples were filtered, using 0.45 μm Teknokroma (TR-200106 – PVDF, 25 mm ∅, PK/100) syringe filters, divided into aliquots, and stored at −20 °C.

2.3. Malts physico-chemical characterization

The malt samples were characterized according to the analytical methods of Analytica-EBC, 1998 concerning extract, total protein, soluble protein, diastatic power, free α-amino nitrogen, and β-glucans.

Extract was determined by method 4.5.1 (“Extract of malt: Congress mash”), Issue 2004; total protein was evaluated by method 4.3.1 (“Total nitrogen of malt: Kjeldahl method”), Issue 1998, and soluble protein by method 4.9.1 (“Soluble nitrogen of malt: Kjeldahl method”) Issue 1998, Diastatic Power was determined by method 4.1.2 (“Diastatic power of malt”, Issue 1998, free α-amino nitrogen was determined by method 4.10 (“Free aminonitrogen of malt by spectrophotometry”), Issue 1997.

2.4. Sodium dodecylsulfate-polyacrylamide gel electrophoresis

The protein content of samples analyzed by the electrophoretic procedure was determined by the Bradford (1976) method. BSA was used as standard.

The electrophoretic separations were achieved in a Hoefer Scientific Instruments SE 280 vertical electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA), coupled with a UniEquip Unipack 2000 electric source (UniEquip, Munich, Germany). All gels were photographed and digitalized using a UV/VIS Vilber Lourmat digital camera (Vilber Lourmat, Marne-la-Vallée, France) and the software BioCaptMW for Windows, version 95.05 s.

The electrophoretic analyses were carried out according to the Laemmli (1970) discontinuous buffer system. Separation gels (1.5 mm thick, 12 × 16 cm) consisted of a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel. Samples were mixed 1:1 (v/v) with the sample buffer, in order to achieve a final protein quantity of 2 μg. The wide molecular weight standards and BSA were used as standards. Separations were carried out at 25 mA in the stacking gel, and at 35 mA in the resolving gel. During separation, the electrophoresis system was kept at 4 °C. All samples were analyzed, at least, in triplicate. Gel fixation and staining was accomplished using the Morrissey (1981) silver staining procedure.

2.5. Reversed-phase high performance liquid chromatography

The chromatographic analyses were carried out using a HPLC unit (Jasco, Tokyo, Japan) composed of a low-pressure quaternary pump (Jasco PU-1580 intelligent HPLC pump), a degasification unit (Jasco DG-1580-54 4-line degasser), a type 7981 Jones Chromatography column heater (Jones Chromatography, Hesperia, CA,

USA), a type 7725i Rheodyne injector (Rheodyne, Rohnert Park, CA, USA), and a UV/VIS detector (Jasco UV-970 intelligent UV/VIS detector). The column was a Chrompack P 300 RP (polystyrenedivinylbenzene copolymer, 8 μm, 300 Å, 150 × 4.6 mm i.d.) (Chrompack, Middleburg, The Netherlands). Data acquisition was accomplished using the Borwin Controller Software, version 1.50 (JMBS Developments, Le Fontanil, France). Eluents were degassed using an ultrasonic bath (Fungilab, Barcelona, Spain).

Gradient elutions were carried out with a mixture of two eluents. Eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile. The flow-rate was 1 mL/min, the column was kept at a temperature of 65 °C, and the detection was achieved at 214 nm. An injection volume of 500 μL was used. All samples were analyzed, at least, in triplicate. Hordeins were eluted with a serie of linear gradients, starting with 20% of solvent B during 3 min, from 20 to 25% of B over 3 min, keeping this conditions during 3 min, from 25 to 30% B over 3 min, keeping this conditions during 3 min, from 30 to 35% of B over 3 min, keeping this conditions during 3 min, from 35 to 40% of B over 3 min, keeping this conditions during 3 min, from 40 to 45% of B over 3 min, keeping this conditions during 3 min, from 45 to 50% of B over 3 min, keeping this conditions during 3 min, from 50 to 60% of B over 3 min, finishing with 60% of B in 3 min and 5 min for column re-equilibration. Albumins and other soluble proteins were eluted with 5% of solvent B during 5 min, from 5% to 10% of B over 5 min, from 10% to 55% B over 20 min, and 5 min for column re-equilibration.

Daily, before the chromatographic separation procedures, a 1 mg/mL BSA solution was injected, to detect possible modifications in the HPLC system.

2.6. Statistical analysis

All statistical analyses involving RP-HPLC data were performed using the software SPSS for Windows, version 13.0 (SPSS, Chicago, IL, USA). Data was studied as chromatographic peak areas. One-Way Analyses of Variance (One-Way ANOVA) at the 5% significance level, with Tukey HSD *post-hoc* test, were carried out so as to ascertain significant differences between varieties and/or germination times. Principal components analysis (PCA) was performed in an attempt to simplify the results and discriminate between the malt samples, according to their protein content. In the specific case of the hordeins PCA, an orthogonal variables rotation method (Varimax with Kaiser normalization) was applied. Coefficients of variation (CV%) were calculated for each variable in order to evaluate the variation of results.

Student's *t*-tests were carried out to test differences between varieties for significance at the 5% confidence level. In addition, Principal components analysis (PCA) was performed, in order to compare the results obtained from wort and beer chromatograms. Variables used in this PCA were the areas of major proteins from worts and beers.

3. Results and discussion

3.1. Malts physico-chemical characterization

The physico-chemical characterization of final malts revealed similar characteristics concerning pH, diastatic power and free α-amino nitrogen. However, Scarlett samples presented slightly higher quantities of total and soluble proteins than Prestige samples, (respectively, 9.80% and 4.30% for Scarlett and 9.50% and 4.20% for Prestige). On the other hand, the Prestige extract value was higher than that found in the Scarlett variety (respectively, 83.6% dm and 81.3% dm). It should be pointed out that the total protein quantity of the malt grains influences the malt extract value and is critical for the brewing process, (Fox et al., 2002; Peltonen et al., 1994).

3.2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis

3.2.1. Malts

3.2.1.1. Hordeins. The gel obtained from the hordein electrophoretic separation is presented in Fig. 1a. Samples applied in lanes 3, 4, and 5 were Scarlett malt extracts, while samples applied in lanes 6, 7, and 8 were extracted from Prestige malts. Hordeins were extracted simultaneously from all samples, at three different days. The electrophoretic patterns were identical for the three extractions and, therefore, no significant differences were observed due to the extraction process.

The patterns were also very similar between the two germination times of each variety. As to the varieties, some differences were observed between the B and C hordein fractions (29–55 kDa hordeins). Bands a and b, in the B hordein zone, are only visible in the Scarlett malt samples. Besides this qualitative differentiation, some quantitative differences were also observed between varieties. Bands c and d, in the B hordein zone, and bands e and f, in the C hordein zone, were detected in both varieties, however appearing more intense in the Scarlett samples. On the other hand, band g, in the B hordein zone, was more intense in the Prestige variety. No bands were observed in the D hordein zone and no differences were obtained between varieties in the A hordein zone.

Scarlett malts exhibit not only a higher number of bands, but also more intense ones, in the B and C hordein zones (29–55 kDa). Howard et al. (1996) referred that high quantities of B and/or C hordeins can be correlated to poor malt extract values.

SDS-PAGE analytical methods have been applied in the study of barley hordeins, with the aim of identifying barley varieties (Villiers & Laubscher, 1989) or determining the malting quality of different barleys (Brennan et al., 1998; Molina-Cano et al., 2001; Peltonen et al., 1994).

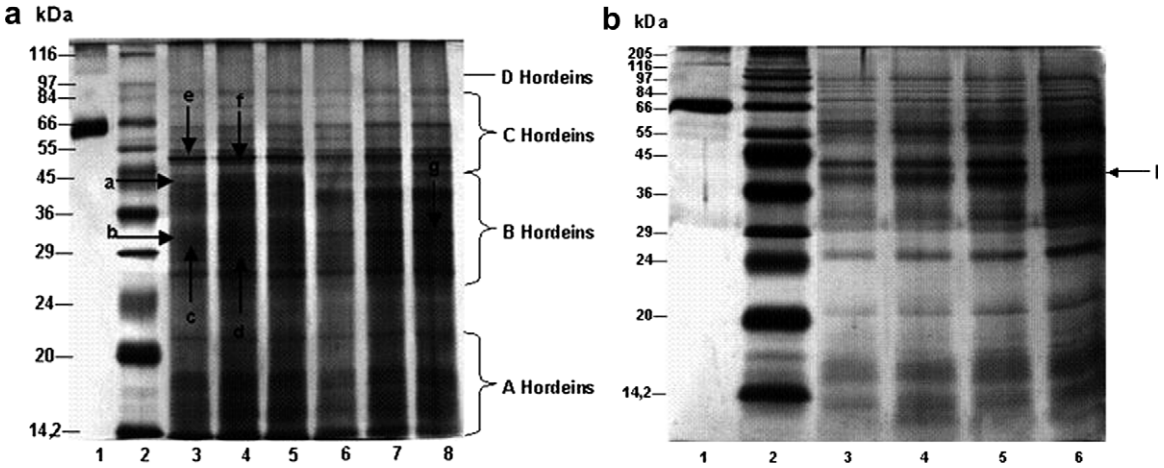

Fig. 1. SDS-PAGE of malt hordeins. 1: BSA; 2: Molecular weight standards; 3: Scarlett (extraction 1); 4: Scarlett (extraction 2); 5: Scarlett (extraction 3); 6: Prestige (extraction 1); 7: Prestige (extraction 2); 8: Prestige (extraction 3). Samples after 60 h of germination. (b) SDS-PAGE of malt albumins and other soluble proteins. 1: BSA; 2: Molecular weight standards; 3: Scarlett, 60 h of germination; 4: Scarlett, 120 h of germination; 5: Prestige, 60 h of germination; 6: Prestige, 120 h of germination.

3.2.2. Albumins and other soluble proteins

Concerning the electrophoretic separation of malt albumins and other soluble proteins, no differences were obtained between varieties or germination times (Fig. 1b). Nevertheless, it was possible to detect an intense band (I), of approximately 40 kDa, correspondent to the protein Z, commonly mentioned in the literature (Curioni et al., 1995; Hejgaard & Kaersgaard, 1983; Villiers & Laubscher, 1989).

3.2.3. Worts and beers

The electrophoretic patterns of the worts produced with the two varieties were very similar (results not shown). An intense band, of approximately 40 kDa (I) was observed in both worts. Other bands were detected between 97 and 116 kDa and between 24 and 36 kDa. In a similar study of wort samples, Kordialik-Bogacka & Ambroziak (2004) described the existence of a 66 kDa band, which was not detected using our electrophoretic conditions.

Most of the components present initially in wort were also detectable in final beer. No differences were observed between samples produced from different beer varieties concerning protein profile (not shown). An intense band (I) of proteins with molecular mass around 40 kDa was detected in all beer samples. In addition to this 40 kDa band, other fainter bands were observed, including one between 116 and 205 kDa, other between 97 and 116 kDa, and some between 24 and 36 kDa. A faint 66 kDa band, previously described in beer (and malt samples) by Mohan, Smith, Kemp, & Lyddiatt (1992), was also detected. This band is likely to be the same as that found in wort samples by Kordialik-Bogacka & Ambroziak (2004).

The most abundant protein constituents in beer have been described as the 40 kDa proteins and a group of proteins with a molecular mass ranging from 8 to 17 kDa in a SDS-PAGE pattern, which implied that the 40 kDa protein was protein Z4/Z7. During the brewing process about 16% of the lysine content of protein Z is glycated through Maillard reactions (Hejgaard & Kaersgaard, 1983). Partial modification of lysine residues with sugars may explain the migration of protein Z as a diffuse zone (Perrocheau et al., 2005). Acilation and glycation through Mailard reactions lead to a complex pattern for LTP1s.

Recently, Hao et al., 2006 used electrospray ionization-ion trap mass spectrometry to identify 7 and 15 proteins corresponding, respectively, to 40 and 7–17 kDa bands in SDS-PAGE pattern of beer. The majority originated from the barley albumins which were resistant to enzymes during brewing. In the 7–17 kDa area there were also two hordein-related proteins and fragments from protein Z4. It was clarified that at least a trace of protein Z4 was degraded by protease during brewing. These previously unknown beer proteins of barley origin have similar molecular weights as proteins Z4 or LTP1 and are overlapped in the two major SDS-PAGE bands. Thus, it is not surprising that no differences were noted between beer samples from the two malt varieties.

3.3. Reversed-phase high performance liquid chromatography

The peak areas obtained from the daily injection of the external standard (1 mg/mL BSA) were not significantly different and, consequently, the normalization of the samples peaks areas was not utilized.

3.3.1. Malt

3.3.1.1. Hordeins. A gradient previously used by other authors for studying barley hordeins (Schmitt et al., 1989) was optimized to separate malt peptides and hordeins. Nine chromatographic peaks were obtained for all malt extracts (Fig. 2). Those peaks were numbered according to their relative hydrophobicity, being peak 1 the least hydrophobic, while peak 9 was the most hydro-

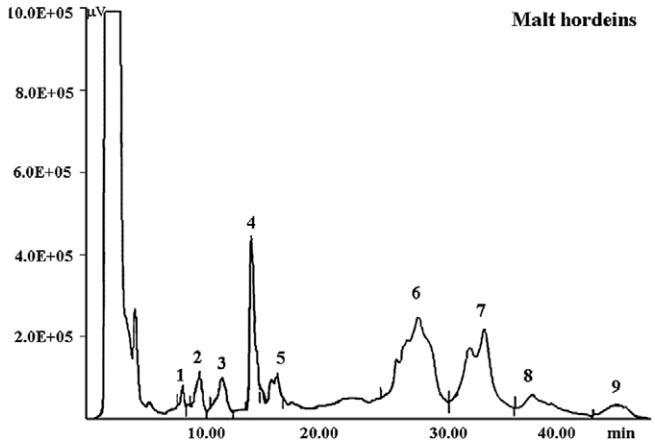

Fig. 2. Typical chromatogram obtained for the malt hordein RP-HPLC separations. Scarlett sample, 60 h of germination.

phobic. This procedure was followed in all peak identifications.

Similar qualitative profiles were observed in all samples. However, quantitative differences were obtained between varieties and germination times. In some cases, those differences were statistically significant. The variation of results was evaluated by calculating the coefficients of variation. The precison of the method was good, since the mean coefficient of variation was 5.8%.

Principal components analysis was applied in the study of data obtained from the hordein chromatographic separations. Two new variables were obtained component 1, which explains 53.0% of the data variance, and Component 2, which explains approximately 36.2% of the data variance. The sum of the data variance explained by these two components is approximately 89.2%. Thus, the dimensionality of the results was reduced from nine variables to two Principal Components, with approximately 10.8% loss of information. Component 1 was high in peaks 6, 7, 8 and 9, while Component 2 was high in peaks 3, 4 and 5. The scores of all malt samples, as a function of the first two Principal Components, are plotted in Fig. 3. In this figure, the most important peaks needed for the definition of these Components are shown on the axis edges, indicating the direction in which their values increase.

Samples are grouped into four clusters, according to their higher or lower content in the different peaks. It is noticeable the clear separation between varieties and germination times. Scarlett samples demonstrated higher protein contents in all peaks. Samples with 60 h of germination had the highest content of more hydrophobic peaks (peaks 6, 7, 8, and 9), while those with 120 h of germination had higher proportion of less hydrophobic peaks, (peaks 3, 4, and 5). In both varieties, from 60 to 120 h of germination, it was observed a decrease of peaks 6, 7, 8, and 9, corresponding to hordeins, and an increase of peaks 3, 4, and 5, corresponding to peptide fragments. Our results are in agreement with other authors, who propose that hordeins are exposed to proteolysis during germination (Chandra, Proudlove, & Baxter, 1999; Osman et al., 2002; Kihara et al., 2002), which originates less hydrophobic peptides. The results of the present study suggest that peaks 6, 7, 8, and 9 are hydrophobic unaltered hordeins, and peaks 3, 4, and 5 are less hydrophobic hordein-derived peptides. The chromatographic profiles obtained by Schmitt et al. (1989) and Lookhart et al. (1999) for barley extracts, using similar chromatographic conditions, are correlated with our results. C hordeins eluted with a proportion of approximately 40% (Schmitt et al., 1989) or 45% acetonitrile (Lookhart et al., 1999). In the present work, the peak that eluted with identical proportions of acetonitrile was the one identified as 6. Consequently, this peak corresponds to C hordeins. On the other side, B hordeins eluted with a proportion of approximately 45% (Schmitt et al., 1989) or 50% acetonitrile (Lookhart et al., 1999). In the same way, peak 7, which eluted with a similar proportion of acetonitrile, corresponds to B hordeins. On the contrary, Schmitt et al. (1989) and Lookhart et al. (1999) did not obtain any peaks that corresponded to peaks 3, 4, and 5. However, these authors have analyzed barley samples, instead of malts that underwent germination.

3.3.1.2. Albumins and other soluble proteins. The chromatogram of albumins and other soluble proteins extracted from malt samples presented seven major peaks. Peaks 4 and 5 seemed to be composed by different proteins and were therefore identified as group 4 (G4) and group 5 (G5).

Once more, no qualitative differences were found between varieties or germination times, given that the RT of the peaks obtained from these separations was also very similar. However, quantitative differences were obtained. The mean coefficient of variation was 7.9%, which speaks for good precision.

A PCA was performed to simplify the results obtained from the chromatographic separations of albumins and other soluble proteins. Two new variables were introduced: component 1, which explains approximately 52.3% of the data variance, and component 2, which explains approximately 32.1% of the data variance. The sum of the data variance explained by the two Components is approximately 84.4%. Peaks 1, 2, 3, G5 and 7 were positively associated to Component 1, whereas peaks 2 (negative influence), G4 and 6 (positive influence) were correlated to Component 2. The results are depicted on a two-dimensional plot (Fig. 4).

As observed in Fig. 5, it is possible to distinguish four clearly separated groups, each one representing a different sample. This separation is based on the individual content of albumins and other soluble proteins. Comparing samples with 60 h of germination, the Scarlett variety presented higher protein content in peaks 1, 2, 3, G5, and 7, whereas the Prestige variety displayed higher protein content in the peaks identified as G4 and 6. In both varieties, an increase in the protein content from 60 to 120 h of germination was observed, although the protein pattern was similar. The increase of the protein content suggests that during the germination process occurs protein synthesis. However, some

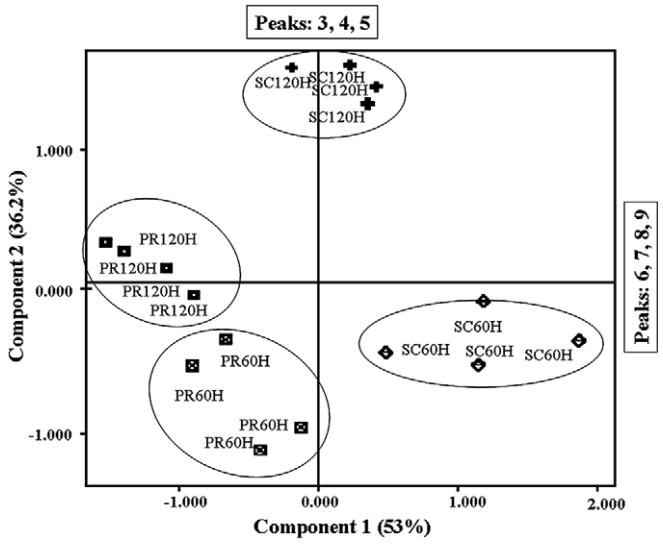

Fig. 3. Two-dimensional plot representing the PCA of data obtained from the malt hordeins RP-HPLC separations. SC60H:Scarlett, 60 h of germination; SC120H: Scarlett, 120 h of germination; PR60H: Prestige, 60 h of germination; PR120H: Prestige, 120 h of germination.

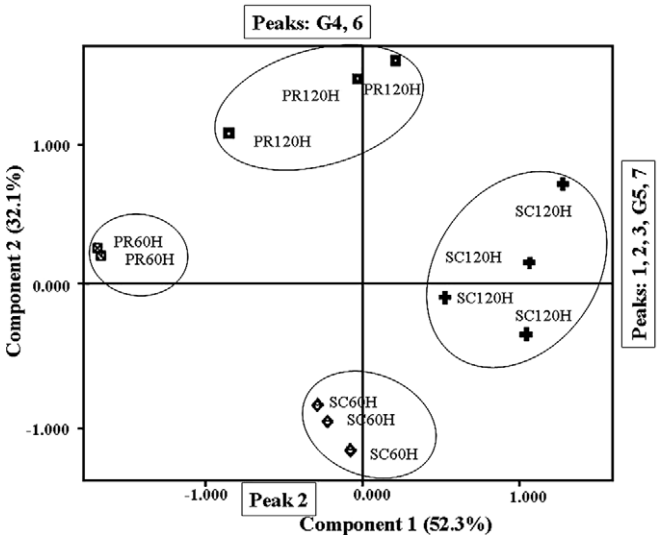

Fig. 4. Two-dimensional plot representing the PCA of data obtained from the malt albumins and other soluble proteins RP-HPLC separations. SC60H: Scarlett, 60 h of germination; SC120H: Scarlett, 120 h of germination; PR60H: Prestige, 60 h of germination; PR120H: Prestige, 120 h of germination.

authors propose that the quantity of water-soluble proteins and peptides increases, during the malting process, as a result of the release of some latent proteins and/or the hydrolysis of insoluble hordeins (Osman et al., 2002, 2003). Peak G5, which increases in both varieties from 60 to 120 h of germination, is most certainly composed by those soluble hordein-derived peptides. This peak eluted with a proportion of approximately 30% acetonitrile. In the hordein analysis, the peaks that eluted with an equivalent acetonitrile quantity were those identified as 3, 4, and 5, which were composed by the less hydrophobic peptides. The method used to extract albumins and other soluble proteins has, probably, also extracted the hordein-derived less hydrophobic peptides.

Differences between varieties observed by the electrophoretic method were in agreement with the chromatographic results. One-Way ANOVA, with Tukey HSD *post-hoc* test, revealed that the hordein content of peak 6 (C hordeins) was higher in the Scarlett malts than in the Prestige malts ($p = 0.003$ for 60 h of germination; $p = 0.024$ for 120 h of germination). In relation to peak 7 (B hordeins), Scarlett samples also presented higher hordein contents than Prestige samples ($p = 0.000$ for both germination times).

The higher content of B and C hordeins in the Scarlett variety can also be pointed out as responsible for its lower extract value (Howard et al., 1996).

3.3.2. Worts and beers

The chromatographic separations of wort proteins presented analogous qualitative profiles in Scarlett and Prestige samples, with four major peaks showing similar retention times (results not shown). Those peaks were designed according to their relative hydrophobicity, i.e. peak 1 was the least hydrophobic, and peak 4 was the most hydrophobic. The identification of peaks obtained from the beer proteins separations has followed the same criterion. The daily injection of the external standard (1 mg/mL BSA solution) showed that the system response was basically unchanged, thus, the samples peak areas were not normalized.

Quantitative differences were observed between worts. Student's *t*-test revealed that Scarlett samples presented higher protein contents than Prestige samples ($p = 0.000$). The respective coefficients of variation were evaluated. The mean coefficient of variation was 7.9%, indicating that good precision was obtained.

The chromatographic conditions used for wort proteins were the same used in the separation of malt albumins and other soluble proteins. It is important to stress that no peaks eluted in the more hydrophobic zone of the chromatogram (with a retention time longer than 25 min). Therefore, only the less hydrophobic proteins were extracted from malt to wort, during the mashing process.

The analysis of filtered beer samples, produced from Scarlett and Prestige barley varieties, presented similar chromatographic profiles. Five major peaks were obtained for all samples (Fig. 5). The peaks designation followed their correspondence to wort samples. Three of those peaks were clearly separated (2, 3, and 4), one was not observed in the wort samples (2b), and peak 1 was not always well resolved from the injection peak. For that reason, the areas of this peak were not utilized in the statistical analyses.

No qualitative differences were detected among samples, since comparable retention times were obtained. In general, Scarlett samples presented higher protein contents than Prestige samples. Using Student's *t*-tests, it was observed that, concerning filtered beers, significant differences were

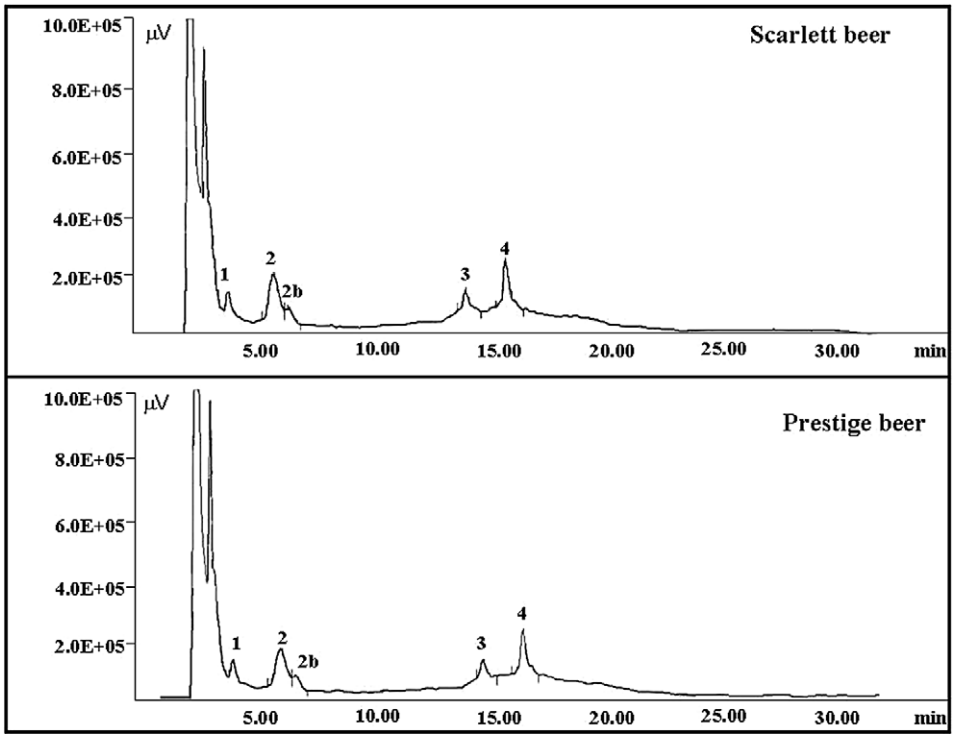

Fig. 5. Typical chromatogram obtained from the beer proteins RP-HPLC separations.

obtained in peaks 2 ($p = 0.029$), 3 ($p = 0.027$), and 4 ($p = 0.000$). The mean coefficients of variation were 5.5%.

After analyzing the results of wort and beer samples individually, data was combined and studied by the PCA statistical method, in order to compare the protein fraction of all samples. The less hydrophobic peak (1) was not used, due to its poor resolution in beer samples. With this method, two new variables were found: component 1 and component 2 which explains, 66.3% and 32.6% of the data variance, respectively. Together, these two components explain approximately 98.9% of the variance in data set. Peaks 2, 3, and 4 had high loadings on Component 1 while Peaks 2b and 4 had high loadings on Component 2. A two-dimensional plot representing this PCA is depicted in Fig. 6.

It is possible to distinguish three well-defined groups. Two of these contain the wort samples produced from the two malt cultivars. Scarlett worts contained higher amounts of protein than Prestige worts. The third group is comprises the filtered beer samples from both malt cultivars. The beers have more similar protein profile than the respective worts. However, the protein content was higher in Scarlett beer when compared to Prestige beer.

In conclusion, the results showed that both HPLC and SDS-PAGE methods were successfully applied in the characterization of protein fraction, including hordeins, albumins and other soluble proteins, of Scarlett and Prestige malts, to follow the proteolytic process during malt germination and the evolution of protein fraction content of worts and beers. Both methodologies can play a key role in the analysis and quality control of malt. Knowledge of barley protein changes during malting is of great technological relevance as it can improve beer quality. Multivariate statistical analysis of results helps to follow the evolution of protein profile during beer production and to find similarities and differences between protein profiles of samples from different varieties. More studies should be performed to correlate malt and wort composition with physico-chemical characteristics of foam.

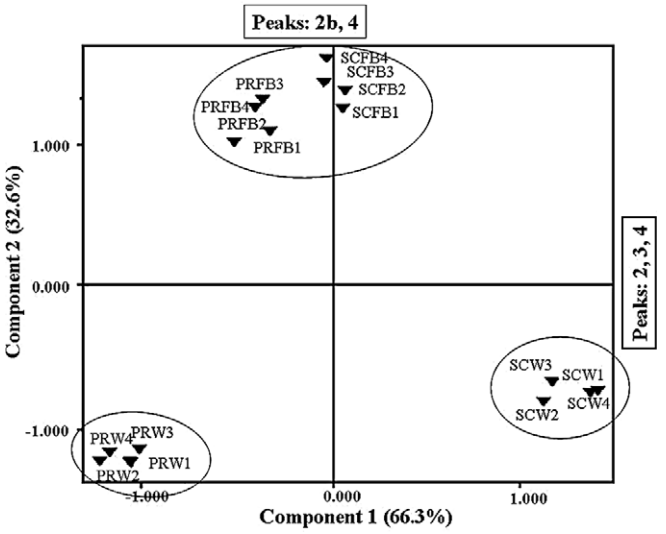

Fig. 6. PCA score plot obtained from the RP-HPLC protein results for wort and beer proteins. SCW: Scarlett wort; PRW: Prestige wort; SCFB: Scarlett filtered beer; PRFB: Prestige filtered beer.

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