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Free-Zone Capillary Electrophoresis Analysis of Hordein Patterns at Different Stages of Barley Malting

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We have carried out a comparison of hordein patterns at different stages of the malting process using free-zone capillary electrophoresis (FZCE). FZCE has proved to be a suitable technique for the separation and characterization of hordeins in barley seeds. Assays of protein extraction and electrophoretic procedures led us to conclude that hordeins were best extracted with 40% ethanol and analyzed using 50 mM phosphate−glycine, pH 2.5, containing 20% ACN and 0.05% HPMC, at 12.5 kV and 45 °C, with 10 s hydrodynamic injection at 0.5 psi and 50 μm i.d. × 31 cm uncoated fused-silica capillary. Our results afford useful information about changes in the composition of these proteins in barley during malting.

KEYWORDS: Proteins; hordeins; barley; malt; free-zone capillary electrophoresis

INTRODUCTION

Barley is an important cereal crop, used as both animal and human food, as well as the preferred grain for malt production in the brewing industry. Barley grain is converted into malt by soaking in water until it sprouts under controlled conditions to form enzymes and modify its nutrient reserves (*1*). Malt quality is greatly influenced by the barley protein content.

Grain protein comprises a complex mixture of polypeptides that are classified by their extractability and solubility characteristics: albumins (soluble in water), globulins (insoluble in water but soluble in dilute salt solutions), glutelins (insoluble in the above solutions but soluble in weak acid or basic solutions), and prolamins (insoluble in the above solutions but soluble in alcohol/water mixtures) (*2*−*4*).

The alcohol-extractable fraction of barley grain contains the main endosperm storage proteins, the hordeins. Hordeins accumulate in protein bodies of the starchy endosperm during seed development and comprise 30−50% of the total grain protein. They are extremely heterogeneous in composition in different barley cultivars. In accordance with electrophoretic mobility, hordeins are classified into four groups of polypeptides, known as B, C, D, and γ. The B hordein (sulfur-rich) and C hordein (sulfur-poor) are the two main fractions, while D hordein (high molecular weight) and γ hordein (sulfur-rich) are minor components. B and C hordeins account for 70−80% and 10−20% of the hordein fraction, respectively, while γ and D hordeins make up less than 5% of the total hordein fraction. The proportions of the different hordein fractions present in mature grain depend on cultivar and growth conditions (*5*).

Hordeins are a dominant component of the protein matrix that surrounds the starch granules within the cells of the endosperm. One of the most important physical−chemical changes that occurs during malting is the degradation of the hordeins in this matrix and their conversion into soluble peptides and amino acids to provide substrates for the synthesis of proteins in the growing embryo. The degradation of hordeins during malting is also necessary to allow enzymes access to the starch, thus facilitating its complete hydrolysis (*5*, *6*).

Albumin and globulin proteins are relatively resistant to proteolysis, whereas our results show that hordeins undergo a dramatic breakdown during malting, with D hordein being degraded more rapidly than B hordein and C hordein being the most resistant (*7*−*9*). Protein content is a key characteristic used for accepting or rejecting barley for malting, and therefore it is important to have methods that can separate, characterize, and identify barley proteins. The two most commonly used identification techniques, polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC), have recently been reviewed (*10*−*13*). Matrix-assisted laser desorption ionization (MALDI)−time of flight mass spectrometry (TOF-MS) also seems to be a useful alternative technique for the identification of these barley prolamins, with a detection sensitivity of about 50−100 ng total protein loaded (*14*). Another approach to identify barley varieties is isoelectric focusing. Isoelectric focusing performed with immobilized pH gradients was found to be superior to other commonly used electrophoretic methods for the identification of barley cultivars, but evaluation was more complicated because of the larger number of protein bands detected (*15*).

In recent years a new, more powerful technique for the analysis of storage proteins in cereals, capillary electrophoresis (CE), has been developed. Using only a small sample, it is

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capable of rapid, high-resolution, quantitative digital analysis of storage proteins in such cereal crops as wheat, oats, rice, and maize (*16*−*23*). In particular, free-zone capillary electrophoresis (FZCE) produces little hazardous waste and is easily automated, which makes it an attractive method for separating hordeins (*24*, *25*). Lookhart et al. and Yan et al. have described the optimum conditions for the extraction, separation, and characterization of hordein subclasses and applied their method to differentiate barley cultivars.

We describe here a simpler and faster FZCE method to characterize hordein patterns during different stages of malting.

EXPERIMENTAL PROCEDURES

Chemicals and Samples. Hordeins were extracted either with 1-propanol or with ethanol (Panreac, Barcelona, Spain) using DL-dithiothreitol from Sigma Chemical Co. (St. Louis, MO) as reducing agent.

Sodium dihydrogen phosphate 1-hydrate, glicine, and acetonitrile, used for the CE running buffers, were from Panreac (Barcelona, Spain), hydroxypropylmethyl-cellulose (hypromellose, HPMC) was from Sigma Chemical Co. (St. Louis, MO), and phosphoric acid was from Merck (Darmstadt, Germany). The buffers were prepared by weighing the concentrations indicated in doubly distilled water and adjusting the pH to 2.5. All solutions were filtered through a 0.5 μm Millipore (Bedford, MA) membrane filter before being injected into the capillary. Distilled water was deionized using a Milli-Q system (Millipore, Bedford, MA).

Ten barley samples of the variety Scarlett taken at different stages of the malting process were provided by the company Intermalta S. A. (Spain): sample M1, initial barley of variety Scarlett; sample M2, green malt at the end of steeping; samples M3, M4, M5, M6, M7, and M8, green malt obtained from six different substages of germination. Each sample is soaked and kept in forced ventilation for 17 h at a humidity of around 40% and temperature between 17 and 22 °C. When the barley passes from one stage to another it is watered abundantly, except for samples M7 and M8. Samples M9 and M10 were malt obtained from the two substages of the roasting. The first substage (sample 9) is carried out with a hot air flow of 130.000 m^3/h per 50 tons for 17 h at a maximum temperature of 65 °C. The malt is dried until reaching 14−16% humidity. In the second substage, the air flow is less (80.000 m^3/h per 50 tons), and the process is carried out for 12 h with an increase in temperature from 60 to 80 °C.

Equipment. CE experiments were made with a Beckman P/ACE System MDQ capillary electrophoresis instrument equipped with a built-in 0−30 kV high-voltage power supply and a diode array detector and GOLD software for system control and data handling. All capillaries (fused silica) had an internal diameter of 50 μm and were 31.2 cm in total length (Beckman Instrument Inc., Fullerton, CA). Temperature was controlled using a fluorocarbon-based cooling fluid.

Electrophoretic Procedure. CE separation was carried out on a fused silica capillary (50 μm i.d., 375 μm o.d., total length 31.2 cm; a detection window was created at 21 cm from the capillary inlet). New capillaries were preconditioned by rinsing with 1 M phosphoric acid for 10 min, followed by a 5 min rinse with Milli-Q water and 15 min with buffer (50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC). To ensure good repeatability, after each separation the capillary was rinsed with 1 M phosphoric acid for 5 min, followed by a 5 min flush with Milli-Q water. The capillary was equilibrated with the running buffer for 15 min before each sample injection.

Samples were injected hydrodynamically under low-pressure (0.5 psi) for 10 s into the anodic end. Electrophoretic separation was performed at 12.5 kV, and the temperature was kept at 45 °C. All solutions and buffers were filtered through a 0.20 μm syringe filter. UV detection was performed at 200 and 280 nm simultaneously.

Hordein Extraction Procedure. The barley grains taken at different stages of the malting process were dried in an oven for 2.5 h at 50 °C to remove humidity. The dried grains were milled to powder in a commercial grinder, and 300 mg of the milled samples was extracted with 1 mL of 40% ethanol for 5 min using a vortex. The extracts were then centrifuged at 4800 rpm for 10 min. The supernatants containing the hordeins were filtered through 0.20 μm filters and transferred to a microvial before being analyzed by capillary electrophoresis.

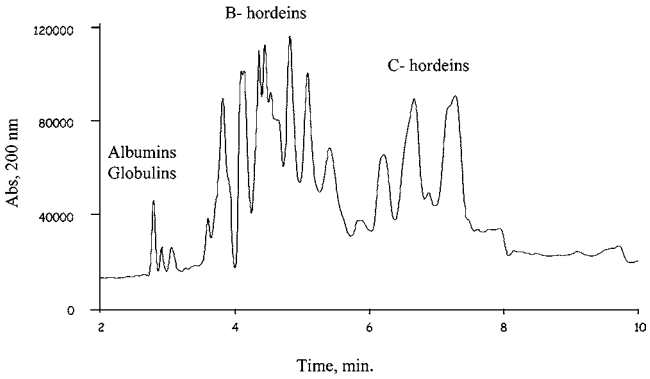

Figure 1. Identification of the different groups of peaks in the optimum electropherogram obtained with a malt sample provided by the company Grupo Cervezas Alhambra. Extraction conditions: ethanol 40%. Experimental conditions: buffer 50 mM phosphate–glycine containing 20% ACN and 0.05% HPMC; 50 μm i.d. fused silica capillary, 31.2 cm detector and total length, 12.5 kV, 10 s hydrodynamic injection at 0.5 psi.

RESULTS AND DISCUSSION

Optimization of the CE Method. We studied the effects of different separation parameters to obtain the best conditions of selectivity, sensitivity, and resolution.

The CE method was optimized using the extract obtained with 40% ethanol from a malt sample provided by Grupo Cervezas Alhambra S.L. The main obstacle to the separation of proteins was the tenacious affinity of ionized silanols toward a proteinaceous surface due to the multiple ionizations occurring on the silica surface at any pH above 3 and to the multiple charges typically present on a protein surface. One simple strategy consists of the use of electrolyte solutions at acidic pH values for suppressing silanol dissociation (*26*). A final pH of 2.5 was chosen for the running buffer because of the basic properties of the hordeins and their positive electrophoretic mobility at acidic pH.

First, we studied different running buffers at this pH, such as 100 mM phosphoric acid, 50 mM phosphate containing 20% ACN, 50 mM phosphate containing 20%ACN and 0.05% HPMC, and 50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC. The best resolution and fastest separation was achieved using 50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC as running buffer. These results are in accordance with the bibliography because in general organic solvents in low-pH phosphate buffers, particularly ACN, tend to improve the resolution of CE separations of storage proteins in wheat (*17*, *21*), barley (*24*), maize, and sorghum (*18*).

To obtain better resolution between the hordein peaks, we also tested the effect of the ionic strengths of the running buffer: 25 mM phosphate−glycine containing 20% ACN and 0.05% HPMC, 50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC, 75 mM phosphate−glycine containing 20% ACN and 0.05% HPMC, and 100 mM phosphate−glycine containing 20% ACN and 0.05% HPMC. The resolution did not improve when the buffer concentration was increased, and for lower concentrations, the ionic strength was not enough to obtain separation.

We also tested the effects of different voltages, temperatures, and injection times. The applied voltage was varied between 10 and 30 kV, and a voltage of 12.5 kV was found to produce

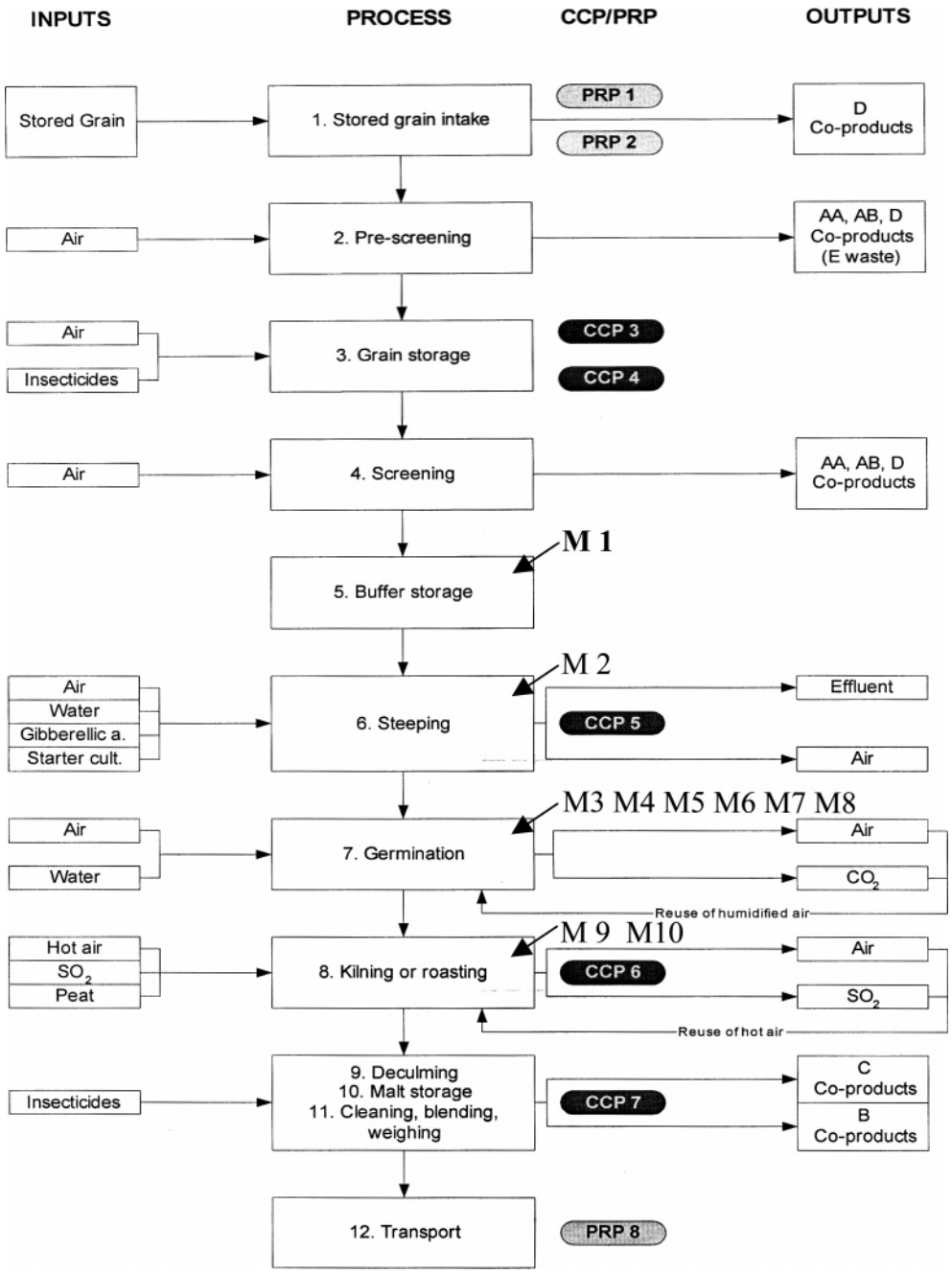

Figure 2. Diagram of the different stages of the malting process indicating where the ten samples have been taken: sample M1, initial barley of variety Scarlett; sample M2, green malt at the end of steeping; samples M3, M4, M5, M6, M7, and M8, green malt obtained during the six successive substages of germination; samples M9 and M10, malt obtained during the two substages of roasting.

fast separation and good resolution. Two temperatures (30 and 45 °C) were also tested, and the best resolution was obtained at 45 °C. Assays of different injection systems, hydrodynamic injection (between 5 and 10 s) and electrocinetic injection (8 s, 10 kV) proved that hydrodynamic injection for 10 s produced the best results.

Consequently, we chose the following CE conditions: a running buffer of 50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC, pH 2.5, voltage 12.5 kV, 45 °C, 10 s hydrodynamic injection at 0.5 psi, and 50 μm i.d. × 31 cm uncoated fused-silica capillary. The main differences between our method and those of Lookhart et al. and Yan et al. are the running buffer, the temperature, and the dimension of the capillary, which have allowed us to obtain shorter analysis times (<10 min).

Repeatability was tested by analyzing seven consecutive injections under the optimum conditions described above, and the retention time was 1.5%.

Comparative Studies of Extraction Procedure. To extract the hordeins in the samples, we studied three types of possible mixtures: 1-propanol 50% with 1% DTT, ethanol 40%, and ethanol 40% with 1% DTT. All the solvents were compared under the same optimum CE conditions described above.

The time needed to extract the hordeins was investigated previously by extracting samples for 5 to 60 min with a wide variety of solvents and temperatures. These assays proved that 5 min was enough to extract hordeins (*13, 24*). Therefore we extracted for 5 min 300 mg of milled samples of grain dried in an oven for 2.5 h at 50 °C with 1 mL of the different solvents studied using a vortex. The extracts were then centrifuged at

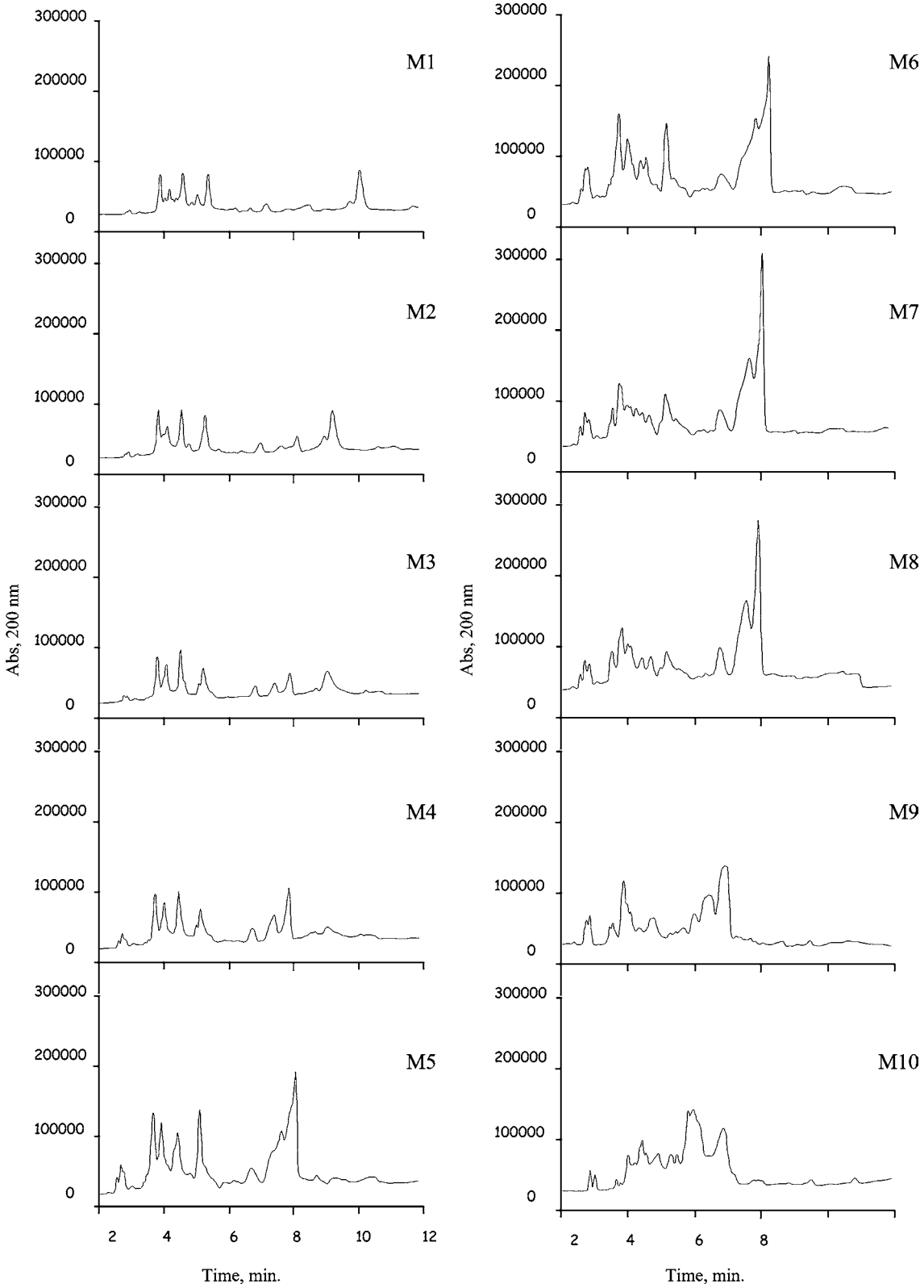

Figure 3. Comparison of hordein patterns of ten samples taken at different stages of the malting process. The separation conditions were as described in Figure 1.

4800 rpm for 10 min. The supernatants containing the hordeins were filtered through 0.20 μm filters and transferred to a microvial before being analyzed by capillary electrophoresis.

The results obtained with 40% ethanol extracts showed better resolution than those with 50% 1-propanol and 1% DTT. Apart from this, the addition of a reducing agent did nothing to improve the resolution of the hordein peaks. Therefore, the samples were successfully separated by FZCE after extraction with 40% ethanol under nonreducing conditions, and the resolution was better than that with the reduced samples.

Identification of Hordeins. The optimum electropherogram of the protein pattern can be seen in **Figure 1**. The proteins were resolved into three groups of peaks. The first group was identified as albumins and globulins since previous work on

the separation of wheat proteins by FZCE showed that the albumin and globulin proteins had the highest mobilities of the proteins tested (*27*). The other two well-separated groups apparently represented the B and C hordeins. The hordein CE patterns were similar to those reported by Lookhart et al. (*24*) and by Yan et al. (*25*).

Application. The aim of this study was to follow the behavior of hordein samples taken at different stages of the malting process with FZCE.

The different stages of the malting process can be seen in **Figure 2**. The barley used for the malting process is stored under suitable conditions with enough oxygen to keep the embryo alive after it is screened to remove other grains or foreign objects. The clean grains are then steeped in oxygenated water until reaching a moisture content of 45%. Then the grains are allowed to germinate, and at the correct moment, germination is interrupted to roast the grains with a hot air current. The malted grain is stored and should remain stable for months or even years. The barley proteins before and after germination comprise a complex mixture difficult to characterize. This is because during the germination carbohydrates are consumed in a respiratory process, so the protein content seems to increase and nitrogenous substances are used to synthesize proteins of the embryo (*1*).

A set of ten samples comprising different phases of the malting process (M1−M10) were analyzed to study the capacity of FZCE to differentiate the protein profiles of malting and to test any changes that might take place during this process.

The comparison of hordein patterns at different stages is shown in **Figure 3**. This comparison shows important changes among the ten samples during the main phases of the process. During the first stages of the malting process, reception and steeping (samples M1 and M2), the patterns of B and C hordeins were approximately the same, indicating that significant changes do not occur in these proteins during steeping. The samples taken at these stages also exhibited fewer peaks in the C hordein range. During the germination stages more differences could be seen. Although the B hordeins did not show any significant changes during the first two stages, a slight increase in the height of the peaks could be observed in the last sample. But the main qualitative and quantitative differences among samples were found in the area of the C hordeins. C hordeins increased in quantity throughout the six stages of the germination process, exhibiting the highest peaks in the samples taken during the last two stages (M7, M8). In the roasting process, the extreme conditions of temperature and humidity brought about an important change in the protein patterns of both B and C hordeins.

Capillary electrophoresis allows a fast determination of the hordein fractions in barley, and our results provide useful information to help evaluate the changes in proteins during malting to differentiate and monitor the stages of this process. Since C hordeins show the most significant changes throughout the stages, they could be used as reliable indicators to follow the malting process.

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