Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 108 (2008) 287–296
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Multivariate optimisation of a capillary electrophoretic method for the separation of glutenins. Application to quantitative analysis of the endosperm storage proteins in wheat

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Received 6 February 2007; received in revised form 19 September 2007; accepted 4 October 2007

Abstract

A capillary electrophoretic method has been designed to allow separation of glutenins with high resolution. Several factors, such as buffer composition, running voltage and capillary temperature were optimised using factorial design and response surface methodology. On the other hand, quantification of content of glutenins and gliadins of different wheat varieties were achieved for the first time using a glutenin extract of wheat gluten and a gliadin extract as external standards, respectively, and using the lys-tyr-lys tripeptide as internal standard. The optimised method and an early reported method for the gliadin separation were validated by evaluating linearity, sensitivity, detection and quantitation limits, repeatability and precision.

Keywords: Gliadins; Glutenins; Endosperm storage proteins; Capillary electrophoresis; Wheat; Quantification; Validation; Multivariate optimization

1. Introduction

The endosperm storage proteins of cereals are constituted by two types of protein fractions (glutenins and gliadins) traditionally classified on the basis of their solubility in different solvents (Bean & Lookhart, 2000a). Glutenins are one of the components of the gluten and the endosperm storage proteins and it is well known that wheat breadmaking quality is mainly determined by glutenin proteins, especially high molecular weight glutenin subunits (Yan et al., 2003). On the other hand, gliadins are the other component of the gluten and the endosperm storage protein of wheat and they has been divided into four classes (α, β, γ and ω) based on electrophoretic migration (Lookhart & Bean, 1995). Because these endosperm storage proteins have important nutritional and functional roles, these cereal proteins have been intensively studied for many years (Bean & Lookhart, 2000b).

Today, capillary electrophoresis and high-performance liquid chromatography have become the most frequently used analytical techniques for the separation and characterization of cereal storage proteins (Bietz, 1985; Bonetti et al. 2004; Lookhart & Bietz, 1990; McCarthy, Cooke, Lumley, Scanlon, & Griffin, 1990). Capillary electrophoresis is increasingly recognized as an important separation technique because of its speed, efficiency, reproducibility, ultra-small samples volume and low consumption of solvents (Rodriguez-Nogales, Garcia, & Marina, 2006). Advancement in the analysis of cereal proteins by capillary electrophoresis has concentrated on the optimization of composition of running buffer to improve resolution and analysis time (Bean & Lookhart, 2001a; Hutterer & Dolnik, 2003; Righetti, Bossi, Olivieri, & Gelfi, 1999; Righetti et al., 2000). Different buffer systems have been used to separate cereal proteins by capillary electrophoresis (acidic,

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doi:10.1016/j.foodchem.2007.10.016

low-conductive, and isoelectric buffers) (Bean, Bietz, & Lookhart, 1998a, 1998b), however, the use of isoelectric buffer based on aspartic acid or IDA permitted rapid separations with high resolutions (Bean & Lookhart, 2001a). Moreover, for cereal proteins, a solubilisation agent of proteins (organic solvents, urea, and/or detergents) has been added to the buffer (Bean, Lookhart, & Bietz, 2000). In addition to solubilizers, some type of polymer modifier has also been added to the buffer to prevent protein-wall interactions (Bean & Lookhart, 2001b).

Separation of cereal proteins by capillary electrophoresis has been applied to the characterisation of normal and genetically modified cereals, cultivar differentiation, and prediction of quality, etc. (Bean et al., 1998a, 1998b; Bonetti et al. 2004; Rodriguez-Nogales et al., 2006). These studies were based on qualitative or semi-quantitative methods. Nevertheless, capillary zone electrophoretic methods for the reliable quantification of gliadins and glutenins in wheat flours have not been found in the literature.

The design of an efficient capillary electrophoretic method that permits the separation and quantification of cereal storage proteins is a multivariate procedure in which many factors can affect separation selectivity, mainly, characteristics of the running buffer, voltage and capillary temperature. In literature, it is very usual found researches applying a classic method for determining the optimal separation conditions varying one variable while keeping the other at a specified contact level. However, this one-dimensional approach is very laborious and often fails to guarantee the determination of optimal conditions (Box, Hunter, & Hunter, 1978; Wernimont, 1985). On the other hand, carrying out experiments with all possible factorial combinations of the test variables is impractical owing to the large number of experiments required (Haaland, 1989). In order to overcome these problems, optimisation studies have been performed using response surface methodology, a statistically designed experimental protocol in which several factors are varied simultaneously. This multivariate approach has advantages in terms of reductions in the number of experiments, improved statistical interpretation possibilities, and reduced requirements of overall analysis time. The response surface methodology has been successfully employed to improve the analytical results in capillary electrophoresis (Ben Hameda, Elosta, & Havel, 2005; Daali, Cherkaoui, Christen, & Veuthey, 1999; Frias-Garcia, Sanchez, & Rodriguez-Delgado, 2004; Martí, Aguilar, & Farran, 1999; Ortega, Albillos, & Busto, 2003; Servais et al., 2004; Spanila, Pazourek, Farkova, & Havel; 2005).

To our knowledge, it has not been found so far any work on the application of response surface methodology for the optimisation of conditions of separation of wheat glutenin by capillary electrophoresis. Furthermore, the quantification of glutenins and gliadin from different wheat cultivars by capillary electrophoresis has not been reported either. Thus, the goal of this work was to develop an analytical capillary electrophoretic method enabling the separation and quantification of glutenins. The validation of the optimised method for the glutenin separation and the validation of an early reported method for the gliadin separation were also performed.

2. Materials and methods

2.1. Sample preparation

The capillary electrophoresis (CE) optimization was carried out with whole wheat flour. Wheat samples from different varieties and wheat gluten where generously facilitated by Nickerson Sur (Navarra, Spain) and Cerestar (Barcelona, Spain), respectively. The sequential extraction of each class of proteins was the same Osborne method previously modified by other authors (Aja, Wang, & Rosell, 2003; Bean et al., 1998a, 1998b; Rosell, Wang, Aja, Bean, & Lookhart, 2002). Water and salt soluble proteins were preextracted from 50 mg of whole flour with 1.0 ml of tris-ClH buffer at pH 7.8 containing 50 mM KCl and 5 mM ethylenediaminetetraacetic acid (EDTA) (Bean & Tilley, 2003). After 5 min vortexing and centrifuging at 15,000 rpm for 5 min, the supernatant was discarded and the precipitate was washed twice with water. Gliadin extract was obtained by mixing the precipitate with 1.0 ml 50% 1-propanol, vortexing for 5 min and centrifuging at 15,000 rpm for 5 min. The remaining pellet was washed twice with 1.0 ml 50% 1-propanol and supernatants were discarded. Glutenins were obtained by mixing the gliadins-free pellet with 1.0 ml 50% 1-propanol containing 1% dithiothreitol (DTT) and then centrifuged at 15,000 rpm for 10 min. The study of glutenin extracts stability allowed us to store them until a maximum of 24 h under refrigeration, (4 ± 2 °C), prior to analysis without obtaining any significant difference in their proteins content. It was observed that at room temperature, samples were deteriorated in few hours. The use of 50 mg of flour, instead of 200 mg, as other authors have reported (Aja et al., 2003; Bean et al., 1998a, 1998b; Rosell et al., 2002), allowed us improving peak resolution in both gliadin and glutenin electropherograms (results not shown). Three replicates of each sample were prepared and each one was measured in duplicate.

All reagents were analytical grade and solutions were prepared with ultrapure water obtained from a Milli-Q water purification system (Millipore, Bedford, Massachusetts, USA). A Vortex MS2 Minisaker (IKA, Staufen, Germany) and a centrifuge Digiten 20 (Orto-Arlesa, Madrid, Spain) were alternately used to put into solution the different protein fractions and to separate them from the remaining pellets. Wheat samples were milled with a Perten 3100 mill (Perten Inst., Huddinge, Sweden).

2.2. Standard preparation

In order to reduce the lack of reproducibility usually obtained in electrophoretic analysis, internal standard cal-

ibration was employed. The lys-tyr-lys tripeptide (Sigma-Aldrich, Inc, USA) was used as internal standard. Two solutions of 1.5 mM tripeptide were prepared, one of them for gliadins analysis, where the solvent was 50% 1-propanol, and the other for glutenin analysis, where the solvent was 50% 1-propanol + 1% DTT. The wheat flour protein extracts, 800 μL, were mixed with the tripeptide, 200 μL, before the analysis in order to get in the injected solution a final internal standard concentration of 0.3 mM.

Gliadin isolated from wheat flour (Fluka, St. Gallen, Switzerland) was used as an external standard for quantification. Six gliadin standard solutions (10, 8, 6, 4, 3, 2 and 1 mg/ml in 50% 1-propanol) with 0.3 mM tripeptide concentration were used for the CE calibration of gliadins.

Due to the lack of any commercial glutenin, wheat gluten was used for extracting the glutenin fraction. This extract was used as external standard. The glutenin content of the wheat gluten was calculated through the determination of the gliadin and the insoluble residue (in 50% 1-propanol + 1% DTT) contents. The gliadin content obtained by CE from the mean of ten measurements was 34% ± 6% (dry basis). The mean of the gravimetric insoluble residue was 17% ± 5% (d.b.). The rest, 49% ± 11% (d.b.), was considered the glutenin content of the gluten. Taken into account this value, six glutenins standard solutions (10, 8, 6, 4, 2, 1 mg/ml in 50% 1-propanol + 1% DTT) with 0.3 mM tripeptide were prepared for the EC calibration of glutenin.

2.3. Capillary electrophoresis

CE was carried out with a Beckman P/AC System 2000 controlled by a System Gold Software data system version 810. The separation was performed using a silica uncoated capillary column (Sugelabor, Madrid, Spain) of 27 cm (20 cm to the detector window) and 0.50 μm I.D. The buffer used to run the samples was an isoelectric buffer containing iminodiacetic acid (IDA), hydroxy propyl methyl cellulose (HPMC) and acetonitrile (ACN). IDA concentrations were varied from 25.0 to 126.8 mM. HMPC and ACN concentrations were assayed from 0.05% to 0.12% and from 8.0% to 30.0%, respectively. Separation voltages were varied from 8.3 to 28.0 kV, and separation temperature was varied from 25.0 to 45.0 °C. All samples were injected into capillary with pressure of 0.5 psi for 4 s. During sample analysis, a constant voltage and temperature were applied. For all experiments, the protein components were detected by UV absorbance at 200 nm. Optimised conditions for the separation of wheat glutenin were: a buffer composition of 115.7 mM IDA, 20.7% ACN and 0.12% HPMC, and an electrophoretic separation of 10 kV at 40 °C. Between samples, the capillary was rinsed with water (2 min), 50% 1-propanol with 1% DTT (2 min), water (2 min), and running buffer (5 min). The conditions of separation of wheat gliadins were: a buffer composition of 50 mM IDA, 20% ACN, and 0.05% HMC, and an electrophoretic separation of 15 kV at 45 °C. Before each injection of gliadins, the capillary was washed with the running buffer for 2 min.

2.4. Experimental design and data treatment

The CE parameters were optimised using response surface methodology (Haaland, 1989). The design of the statistical experiments and the evaluation were preformed using the Statgraphics Plus for Windows 4.0 computer program (Statistical Graphics Corp., Rockville, MD, USA). The area percentage of every peak was calculated as the average of three replicates (injected in duplicate). The integration was performed by setting the baseline from valley to valley. Variance analysis (ANOVA) was done with Statgraphics Plus for Windows 4.0.

3. Results and discussion

3.1. Optimisation of the conditions of separation of glutenins

As starting point of our study, the running buffer composition used for the separation of gliadins was selected (Aja et al., 2003; Bean et al., 2000; Rosell et al., 2002). The buffer consisted of IDA in presence of HPMC and ACN. The electrophoretic separation was performed at 20 kV and 45 °C. Under these conditions, glutenin electropherogram had a first group of bad resolved peaks that ends in a pair of peaks (P1 and P2) usually of big size and only partially resolved (see Fig. 1A). At the end of the electropherogram some little peaks usually well resolved, can be seen.

The general aim of our optimization was to improve the separation of the first group of proteins without prolonging excessively the run time. A first step of the optimisation of the electrophoretic parameters is to establish the criteria that will define the quality of the analysis. In our case, five responses were selected in order to determine the optimal separation conditions: (i) the peak resolution (evaluated from the partially resolved peaks P1 and P2 at the end of the first peak group), (ii) the total peak number in the first group of peaks, (iii) the total area in the first group of peaks, (iv) the time of this group of peaks (evaluated from the time of the end of the group minus the time of the beginning), and (v) the total analysis time.

In order to obtain an electrophoretic method for the separation of wheat glutenin with the highest resolution possible in the shortest possible time, a preliminary study of the effect of the concentration of IDA, HMPC and ACN in the running buffer, the running voltage and the capillary temperature was studied. As a screening experiment, a fractional factorial design 2^{5-2} with three runs in the centerpoints (eleven runs) was chosen. Experiments at the centre were carried out to obtain an estimation of the experimental error (Montgomery, 2002). The concentration of IDA was assayed at 25.0, 62.5 and 100.0 mM; the concentration of HMPC at 0.050%, 0.075% and 0.100%; the concentration of ACN at 10.0%, 20.0% and 30.0%;

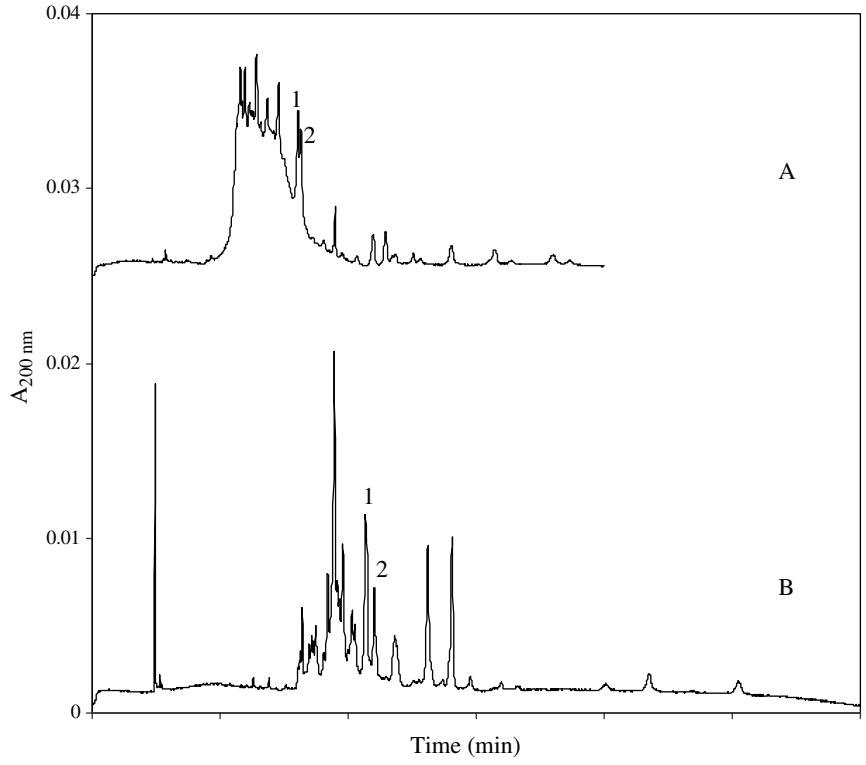

Fig. 1. Capillary electropherograms of glutenins of Berdum wheat separated with the initial electrophoretic method: 50 mM IDA, 20% ACN, 0.05% HMC, 20 kV, 45 °C (A), and with the optimised method: 115.7 mM IDA, 20.7% ACN, 0.12% HPMC, 10 kV, and 40 °C (B).

the voltage at 20.0, 24.0 and 28.0 kV; and the capillary temperature at 25.0, 35.0 and 45.0 °C. An extract of glutenin from whole wheat was injected by triplicate under these different conditions and the responses were studied.

Analysis of estimated effects of each of the parameters investigated that showed significant effect on the electrophoretic responses indicated that an increase of the concentration of IDA (with an estimated effect of +4.0) and HPMC (with an estimated effect of +3.5) provides a positive effect on the peak number, but buffers with high concentration of ACN (with an estimated effect of −4.5) reduced this response. The sign of the estimated effect shows the conditions necessary in order to obtain the maximum of minimum responses: When a factor has a positive effect, the response is higher at the high level; when a factor has a negative effect, the response is lower at the high level. On the other hand, the peak resolution was positively affected by high concentration of HPMC (estimated effect of +0.053). It should be noted that increases in the concentration of HPMC permitted an adequate reduction of the protein adsorption inside of the capillary wall (Capelli et al., 1998), which may account for a better glutenin separation. The presence of adequate levels of ACN is necessary to maintain the solubility of these hydrophobic storage proteins (Bean et al., 2000). On the other hand, the presence of high concentrations of ACN (with an estimated effect of −3.337) and HPMC (with an estimated effect of −0.637) permitted faster separations; however, increasing the level of IDA to 100 mM (estimated effect of +2.262) the separation was slower. By increasing running voltage from 20 to 28 kV, the peak number and area were reduced but faster separations were achieved (with estimated effect of −0.5 and −4.219, respectively). Due to the relatively low ionic strength of IDA, high voltages could be used to achieve rapid separations (Bean et al., 2000). However, a compromise between the resolution and analysis time must be determined for the separation of glutenins. The separation temperature presented positive influence on the peak number (estimated effect of +1.5) and higher temperatures provided faster separations (estimated effect of −3.463). The reason of this behaviour is that as the temperature increases, the viscosity of the buffer decreases and the electrophoretic mobility increases as well (Richard, Franzier, Ames, & Nursten, 2000).

In order to improve these results, all the factors were assayed in a second experimental design (fractional factorial design 2^{5-2}) under different factor levels. A new response was introduced in the experimental design in order to evaluate the baseline drift of the electropherograms. The concentration of IDA was assayed at 80.0, 100.0 and 120.0 mM; the concentration of HMPC at 0.080%, 0.100% and 0.120%; the concentration of ACN at 8.0%, 12.0% and 16.0%; the voltage at 10.0, 15.0 and 20.0 kV; and the capillary temperature at 40.0, 42.5 and 45.0 °C. Under these conditions, the best resolution and number of peaks were found at the highest concentrations of IDA, ACN and HMPC with estimated effects of

+0.072, +0.298 and +0.096, respectively. At the lowest values of running voltage and capillary temperature, the peak resolution improved (estimated effects of −0.171 and −0.154, respectively). The results showed that at 16% ACN and 10.0 kV the total area of the electropherograms was increased with estimated effects of +2.856 and −7.03, respectively. On the other hand, higher group and analysis times were obtained at the highest concentration of ACN (estimated effects of +0.227 and +5.0, respectively) and the lowest values of voltage (estimated effects of −2.025 and −8.5, respectively). In order to reduce the noise of the electrophoretic method, the percentage of IDA (estimated effect of −0.006) and ACN (estimated effect of −0.009) in the running buffer, and the running voltage (estimated effect of +0.021) have to be increased and reduced, respectively.

Regarding the values of estimated effects of this second experiment, the concentration of HPMC and the capillary temperature presented little influence on the responses; nevertheless the addition of HPMC at 0.12% and the decrease of temperature to 40 °C produced a little improvement in the separation of glutenin, so 0.12% and 40 °C was fixed for the remaining work. Therefore, the factors concentration of IDA and ACN, and running voltage were assessed in a third experiment. A central composite design (19 runs) was chosen as a 2^3 full design with star point and five supplementary trials at the centre. The concentration of IDA was assayed at five levels: 93.2, 100.0, 110.0, 120.0, and 126.8 mM; the concentration of ACN at 15.9%, 17.0%, 18.5%, 20.0%, and 21.0%; and the voltage at 8.3, 10.0, 12.5, 15.0, and 16.7 kV.

Mathematical models were built through regression based on the response results. The models were simplified by omitting non significant factors ($p > 0.05$) after variance analysis. The p-values of lack of fit for each one of the models were higher than 0.05, and the coefficients of determination (R^2) presented values between 89.74 and 98.41, indicating the adequate predictability of the models (Table 1). This means that these models can be applied in the subsequent optimisation stages. These mathematical equations were optimised by an iteration method using the statistical software package Statgraphics® Plus, v. 4.0 and they were used to determined the optimum local conditions by maximising resolution, peak number, area, and group time, and minimising the analysis time and the noise of the capillary electrophoretic method. The optimal values of variables obtained for the electrophoretic responses are shown in Table 1. Different optimal conditions were found for each response. A good resolution (1.24) was achieved at 126.8 mM IDA, 21% ACN and 11.3 kV. Maximum peak number was observed with a similar running voltage (11.7 kV) but at a lower level of IDA (110.5 mM) and ACN (15.9%). The optimum value of percentage of IDA for area (93.2 mM) was considerably lower that the other responses. Minimum values of analysis time and noise were achieved at a similar composition of running buffer and voltage.

In order to search the condition simultaneously satisfying the responses, multiple response optimisation was calculated by a desirability function. In this particular study, a desirability function was built on the premise that the resolution, peak number, area and group time were maximized, whereas the analysis times and baseline drift would be minimised. Different weights for each one of the response variables were introduced in the desirability function. Weights of 1.0 were applied for the resolution and the peak number, 0.5 for the total area of capillary electropherogram, and 0.2 for baseline drift, group and analysis time. The analysis of this combined equation resulted in the following optimum combination of the independent variables: 115.7 mM IDA, 20.7% ACN and 10 kV. Fig. 1B shows the capillary electropherograms obtained from glutenin under the optimised conditions. Under these conditions, an excellent separation of the glutenin proteins was achieved.

3.2. Method validation

The quantification of glutenins and gliadins in the samples presents the difficulty of the selection of a suitable standard. Indeed, there is a no certified reference material that could be used as standard for the determination of glutenins and gliadins. Protein isolated, concentrate of protein, and flour have been reported as external standard for the determination of vegetable proteins (Castro-Rubio, Garcia, Rodriguez, & Marina, 2005; Criado, Castro-Rubio, Garcia-Ruiz, Garcia, & Marina, 2005; Garcia-Ruiz, Garcia, Garcia, & Marina, 2006). For this study, the calibration of glutenins and gliadins were performed using as external standards a glutenin extract from wheat gluten and a gliadin extract (Fluka), respectively. In both methods, a calibration with internal standard was carried out using the lys-tyr-lys tripeptide.

For the validation of the capillary electrophoretic methods, the characteristics of the calibration plots and reproductively were obtained. The linear relationship between signal and wheat protein fraction was up to 10 mg/ml for determination of glutenin and gliadin. The calibration equations for both electrophoretic methods are shown in Table 2. The results demonstrated that the correlation between protein fraction concentrations and resulting peak areas in the electropherograms was linear, with high values for the correlation coefficient. The limits of detection and quantification were calculated using the internal and external standard methods. The values of these parameters for internal standard calibration were lower than those calculated using external standard calibration. The limits of detection for internal standard method (calculated as the concentration corresponding to a signal equal to the intercept plus three times the standard error of the internal standard calibration plot) were 13.4 mg/g of flour for glutenins and 17.8 mg/g of flour for gliadins. The limits of quantification for external standard method (calculated as the concentration corresponding to

Table 1
Second-order polynomial models for the central composite design 2^3 + star and optimal conditions for the electrophoretic parameters

Responses	Mathematical model	R^2	p-Value	Optimum value		
				C_{IDA} (mM)	C_{ACN} (%)	Voltage, V (kV)
Resolution	$19.46 - 0.16 \cdot C_{IDA} - 1.41 \cdot C_{ACN} + 0.41 \cdot V + 0.01 \cdot C_{IDA} \cdot C_{ACN} + 0.01 \cdot C_{ACN}^2 - 0.02 \cdot V^2$	94.53	0.4004	126.8	21.0	11.3
Peak number	$-198.83 + 3.42 \cdot C_{IDA} - 0.54 \cdot C_{ACN} + 7.29 \cdot V - 0.02 \cdot C_{IDA}^2 - 0.31 \cdot V^2$	89.74	0.1800	110.5	15.9	11.7
Area	$-56.05 + 0.11 \cdot C_{IDA} + 5.85 \cdot C_{ACN} - 0.61 \cdot V - 0.01 \cdot C_{IDA} \cdot C_{ACN} - 0.14 \cdot C_{ACN}^2 + 0.02 \cdot V^2$	97.23	0.1789	93.2	19.4	8.3
Group time (min)	$10.83 - 0.03 \cdot C_{IDA} - 0.49 \cdot C_{ACN} - 0.02 \cdot V + 0.01 \cdot C_{IDA} \cdot C_{ACN} - 0.0\ 1 \cdot C_{IDA} \cdot V - 0.02 \cdot C_{ACN} \cdot V + 0.03 \cdot V^2$	98.41	0.0730	126.7	17.6	8.3
Total time (min)	$71.79 - 0.71 \cdot C_{IDA} - 0.26 \cdot C_{ACN} - 0.22 \cdot V + 0.01 \cdot C_{IDA}^2 - 0.03 \cdot V^2$	90.78	0.2451	114.9	15.9	16.7
Baseline drift (ΔA_{210})	$-0.1928 + 0.0026 \cdot C_{IDA} + 0.0091 \cdot C_{ACN} - 0.0060 \cdot V + 0.0001 \cdot C_{IDA}^2 - 0.0002 \cdot C_{IDA} \cdot C_{ACN} - 0.0001 \cdot C_{IDA} \cdot V + 0.0002 \cdot C_{IDA}^2 + 0.0006 \cdot C_{ACN} \cdot V$	95.17	0.6227	111.5	16.7	16.5

Table 2
Validation of the electrophoretic methods for the analysis of glutenins and gliadins in wheat

	Glutenin method	Gliadin method
Linearity (mg/ml)	Up to 10	Up to 10
Internal standard Calibration curve ($n = 6$)[a]		
Slope	0.3034 ± 0.0104	0.4099 ± 0.0171
Intercept	0.1148 ± 0.1903	-0.4671 ± 0.2494
Correlation coefficient	0.9965	0.9948
Detection limit (mg/mL)[c]	0.67	0.89
Quantitation limit (mg/mL)[d]	2.26	2.98
External standard Calibration curve ($n = 6$)[b]		
Slope	0.8058 ± 0.0508	0.5143 ± 0.0472
Intercept	-1.1871 ± 0.4205	-0.8592 ± 0.5794
Correlation coefficient	0.9884	0.9753
Detection limit (mg/mL)[c]	1.24	1.98
Quantitation limit (mg/mL)[d]	4.14	6.59
Repeatability (RSD, %) ($n = 10$)[e]		
Internal standard method	6.70	3.62
External standard method	7.76	7.62
Intermediate precision (RSD, %) ($n = 10$)[f]		
Internal standard method	6.67	7.00
External standard method	11.94	9.28

[a] Equation of the straight line obtained when plotting the measured glutenin or gliadin concentration (mg/ml) versus the total electrophoretic area/internal standard area.
[b] Equation of the straight line obtained when plotting the measured glutenin or gliadin concentration (mg/ml) versus the total electrophoretic area.
[c] The detection limit was calculated as $3 \cdot \sigma/m$, where σ is the standard error and m is the slope of calibration curve of area versus concentration.
[d] The quantitation limit was calculated as $10 \cdot \sigma/m$.
[e] Calculated by injecting one wheat solution ten consecutive times in the same day.
[f] Determined by injecting ten independently extracted solutions obtained from the same wheat flour.

a signal equal to the intercept plus ten times the standard error of the external standard calibration plot) were 45.2 and 29.6 mg/g of flour for the quantification of glutenins and gliadins, respectively.

The repeatability was determined by performing replicated injections ($n = 10$) of a same protein extract and by measuring the protein concentration with internal and external standard methods. On the other hand, reproducibility was determined by injecting ten extracts of gliadins and glutenins obtained from 50 mg/mL of the same wheat flour. As can be seen in Table 2, both repeatability and intermediate precisions were improved with the internal standard method.

3.3. Application to wheat analysis

To test the ability of the optimised method to the successful differentiation of wheat cultivars, glutenins and gliadins from five different classes of Spanish wheat were separated and compared (Figs. 2 and 3).

The electropherogram of glutenins could be divided into three groups of peaks: one at the beginning of the electropherogram (migration time between 5.0 and 12.5 min), a second group at the middle of the electropherogram (migration time between 12.6 and 14.5 min), and the other at the end of the electropherogram (migration time between 14.6 and 30.0 min), corresponding with I-, II-

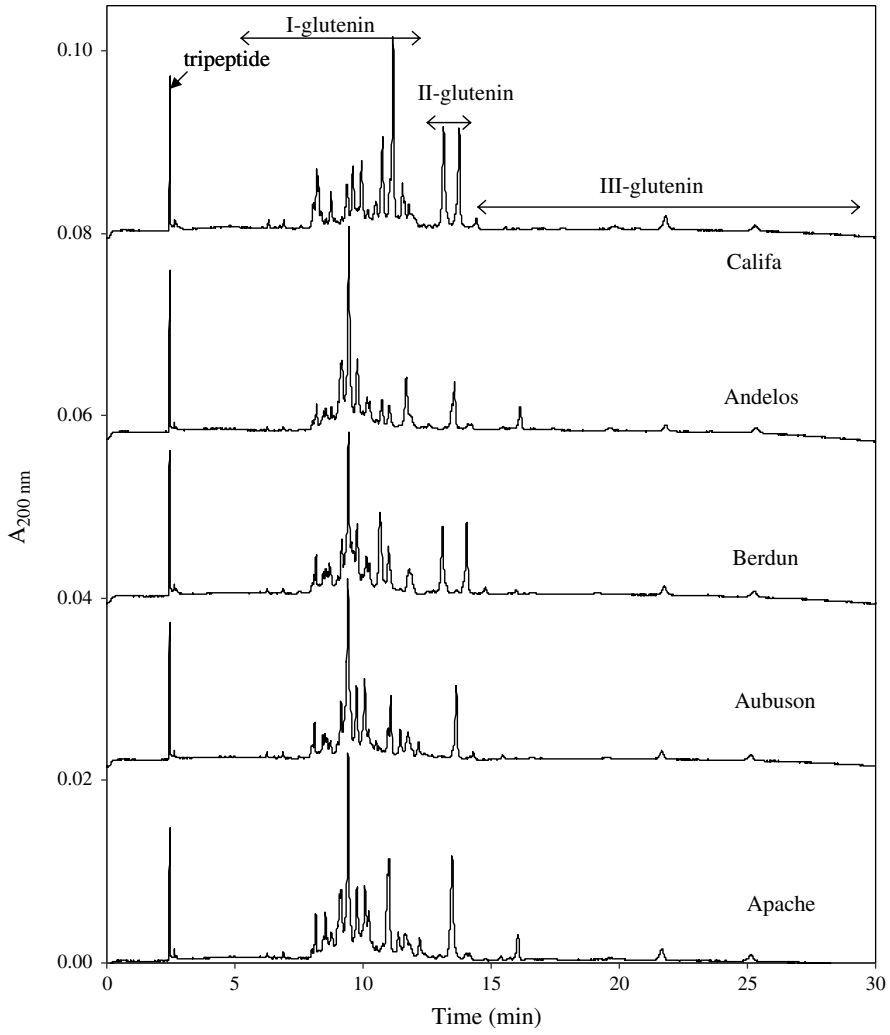

Fig. 2. Capillary electropherograms of glutenins from different wheat varieties. Separation conditions: 115.7 mM IDA, 20.7% ACN, 0.12% HPMC, 10 kV, and 40 °C.

and III-glutenin fractions, respectively. I-glutenin fraction consisted of several peaks with bigger areas, while II-glutenin fraction presented only one or two peaks according with the wheat variety. Finally, III-glutenin fraction corresponded with the proteins slower whose areas were very low.

A detailed analysis of the I-glutenin fraction revealed the existence of differences among wheat varieties. This fraction consisted of several peaks whose number and area varied from variety to variety. Apache, Aubuson, Berdun and Andelos presented a high peak at a retention time of 9.4 min, while for Califa the higher peak was observed at 11.7 min. The II-glutenin fraction appeared as two main peaks in Berdun and Califa varieties, however only a peak could be observed for the rest of samples.

For quantitative analysis, the concentrations of the glutenin fractions were calculated by interpolating the total peak area divided among the internal standard peak area in the calibration plot obtained by the internal standard method. This procedure was also applied for the determination of the content of gliadins in the wheat cultivars. Table 3 shows the contents of gliadin and glutenin fractions in these five varieties of wheat. The analysis of variance (ANOVA) performed disclosed significant differences for glutenin and gliadin fractions. A Tukey test ($\alpha = 0.05$) was carried out to test for statically differences between samples. Mean values with different superscripted letters differ significantly among the different wheat lines at 5% significance level. Apache and Califa varieties presented the highest values for total glutenins. The highest concentrations of I- and II-glutenin fractions were observed for Apache and Califa, respectively. These lines presented also higher amount of the III-glutenin fractions. Andelos and Apache showed the highest values for total gliadins. High values of $\alpha + \beta$- and γ-gliadin were also found for Andelos and Apache wheat varieties while the highest value of ω-gliadin was observed in Apache variety. Lower levels of the $\alpha + \beta$-, γ-, ω-gliadins were evaluated in Aubuson, Berdum and Califa.

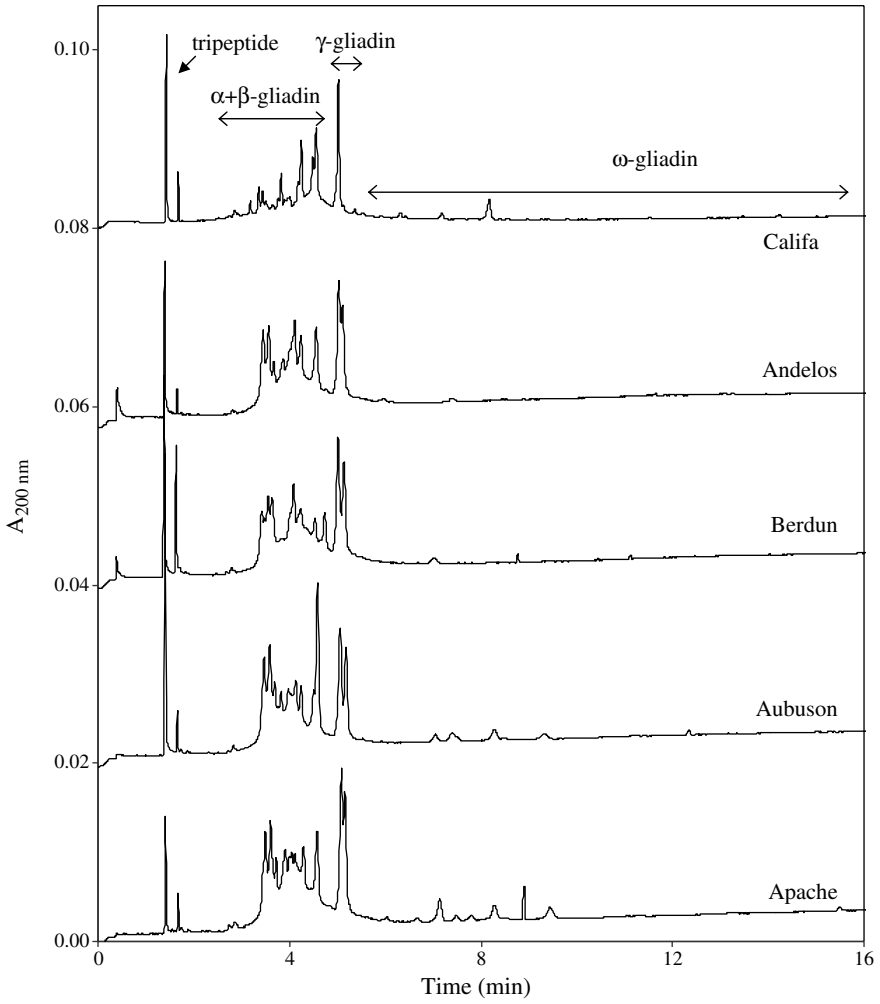

Fig. 3. Capillary electropherograms of gliadins from different wheat varieties. Separation conditions: 50 mM IDA, 20% ACN, 0.05% HMC, 15 kV, and 45 °C.

Table 3
Content of gliadin and glutenin fractions in different varieties of wheat flours

Wheat variety	Protein fraction concentration (g/100 g of flour)[A]							
	Gliadin	α + β-Gliadin	γ-Gliadin	ω-Gliadin	Glutenin	I-Glutenin	II-Glutenin	III-Glutenin
Andelos	6.66 ± 0.22^{b}	3.68 ± 0.13^{c}	2.62 ± 0.09^{b}	$0.36 \pm 0.05^{a,b}$	5.98 ± 0.88^{a}	4.82 ± 0.67^{a}	0.67 ± 0.05^{a}	0.49 ± 0.16^{a}
Apache	9.59 ± 0.71^{c}	4.66 ± 0.12^{d}	2.70 ± 0.69^{b}	2.22 ± 0.08^{c}	11.99 ± 0.95^{b}	8.72 ± 0.81^{c}	2.09 ± 0.14^{b}	1.20 ± 0.02^{b}
Aubuson	3.28 ± 0.18^{a}	2.04 ± 0.10^{b}	0.79 ± 0.05^{a}	0.44 ± 0.03^{b}	$9.04 \pm 0.39^{a,b}$	$7.24 \pm 0.35^{a,b,c}$	1.16 ± 0.03^{a}	0.64 ± 0.02^{a}
Berdun	2.29 ± 0.26^{a}	1.38 ± 0.16^{a}	0.74 ± 0.06^{a}	0.17 ± 0.03^{a}	7.54 ± 0.98^{a}	$5.36 \pm 0.55^{a,b}$	1.86 ± 0.25^{b}	0.31 ± 0.18^{a}
Califa	3.39 ± 0.18^{a}	2.01 ± 0.10^{b}	0.95 ± 0.05^{a}	0.42 ± 0.03^{b}	11.77 ± 0.07^{b}	$7.72 \pm 0.06^{b,c}$	3.29 ± 0.05^{c}	$0.77 \pm 0.03^{a,b}$

Mean within a column followed by a different letter are significantly different at 0.05 level as determined by the Tukey test.
[A] Expressed as mean ± standard ($n = 4$) deviation of content of different protein fractions.

4. Conclusions

This is the first time that a capillary electrophoretic method has been optimised and validated for the quantitative determination of glutenins. Factorial design and response surface methodology have been used successfully for the optimisation of the buffer composition, running voltage and temperature capillary affecting the separation of glutenins by capillary electrophoresis. These chemometric tools made it possible to model the optimisation of capillary electrophoretic method in fewer experiments than an univariate development process would have required. Excellent separation was achieved using a buffer composition of 115.7 mM IDA, 20.7% ACN and 0.12% HPMC, at 10 kV and 40 °C. The use of the lys-tyr-lys tripeptide as internal standard, and the glutenin extract from wheat

gluten and the gliadin extract as external standards for the quantification of glutenins and gliadins in wheat flours, respectively, enables a good estimation of their contents, being possible to detect up to 13.4 mg of glutenin/g of flour and 17.8 mg of gliadin/g of flour. Repeatability and precision of the electrophoretic methods using an internal standard can be considered adequate to achieve the quantification of glutenin and gliadin in wheat flours.

Acknowledgements

Authors thank the Laboratorio de Técnicas Instrumentales of Valladolid University and Dr. Alejandro Cifuentes (Fermentaciones Industriales, CSIC, Spain) for the use of their Capillary Electrophoresis equipments. We also thank Vanesa Sanz for capillary electrophoresis analysis.

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