

# Rapid separation and characterization of grain water-soluble proteins in bread wheat cultivars (*Triticum aestivum* L.) by capillary electrophoresis

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Wang, A., Pei, Y., Li, X., Zhang, Y., Zhang, Q., He, Z., Xia, X., Appels, R., Ma, W., Huang, X.-Q. and Yan, Y. 2008. **Rapid separation and characterization of grain water-soluble proteins in bread wheat cultivars (*Triticum aestivum* L.) by capillary electrophoresis.** Can. J. Plant Sci. **88**: 843–848. Water-soluble (WS) proteins in wheat grain are considered to represent the suite of biologically active enzymes and enzyme inhibitors in the grain. In this study, a rapid capillary electrophoresis (CE) method for WS protein separations was developed using untreated fused-silica columns and an acidic phosphate-glycine buffer system. In order to optimize the resolution and reproducibility of CE separation, different protein extraction methods, organic modifiers in phosphate-glycine buffer and capillary electrophoresis conditions, including capillary length and inner diameter (ID), operating temperature, performance voltages, sample injection times, etc., were investigated. High resolution and reproducibility of WS proteins were achieved using 20% ethanol as the extracting buffer. The optimal condition to separate these proteins was 50  $\mu\text{m}$  ID  $\times$  31.5 cm (26.5 cm to the detector) capillary at 11.0 kV and 35°C. The optimum buffer was 0.1 M phosphate-glycine (pH 2.5) containing 20% acetonitrile (ACN) and 0.05% hydroxypropylmethylcellulose. Using this method, the WS proteins were well separated in less than 10 min. A total of 120 Chinese bread wheat cultivars were analyzed. The CE patterns of most bread wheat cultivars showed a higher level of polymorphisms compared with SDS-PAGE patterns. All cultivars analyzed could be readily differentiated based on their WS protein profiles. Results indicate that the WS proteins are useful biochemical markers for wheat genetics and breeding research and CE is expected to become a new and powerful tool for the separation and characterization of grain WS proteins in bread wheat.

**Key words:** *Triticum aestivum*, bread wheat, water-soluble proteins, capillary electrophoresis, biochemical markers

Wang, A., Pei, Y., Li, X., Zhang, Y., Zhang, Q., He, Z., Xia, X., Appels, R., Ma, W., Huang, X.-Q. et Yan, Y. 2008. **Séparation et caractérisation rapides des protéines hydrosolubles dans le grain des cultivars de blé tendre (*Triticum aestivum* L.) par électrophorèse capillaire.** Can. J. Plant Sci. **88**: 843–848. Les protéines hydrosolubles, estimes-t-on, correspondent à l'ensemble des enzymes biologiquement actives et des inhibiteurs des enzymes présents dans le grain du blé tendre. Les auteurs ont mis au point une technique rapide d'électrophorèse capillaire (EC) pour séparer les protéines hydrosolubles en recourant à des colonnes de silice fondue et à une solution de phosphate acide tamponnée à la glycine. Pour la meilleure résolution et la reproductibilité optimale de la séparation par EC, ils ont examiné diverses méthodes d'extraction des protéines, plusieurs modificateurs organiques du tampon phosphate-glycine et différents paramètres de l'électrophorèse capillaire, parmi lesquels la longueur et le diamètre intérieur (DI) des capillaires, la température et la tension durant l'opération et la durée d'injection de l'échantillon. On parvient à une haute résolution et à une bonne reproductibilité de la séparation des protéines hydrosolubles en utilisant de l'éthanol à 20% comme tampon pour l'extraction. La combinaison optimale de facteurs pour séparer les protéines est la suivante: capillaires de 50  $\mu\text{m}$  de DI  $\times$  31,5 cm de longueur (26,5 cm jusqu'au détecteur), à 11,0 kV et 35°C. Le tampon idéal est une solution de phosphate-glycine à 0,1 M (pH 2,5) contenant 20% d'acétonitrile et 0,05% d'hydroxypropylméthylcellulose. Avec cette méthode, on réussit à séparer les protéines hydrosolubles en moins de 10 min. Les auteurs ont analysé 120 cultivars chinois de blé tendre. Les profils obtenus par EC pour la majorité d'entre eux révèlent un plus grand polymorphisme que celui indiqué par électrophorèse en gel de polyacrylamide en présence de dodécyl sulfate de sodium (SDS-PAGE). On pourrait aisément différencier tous les cultivars examinés d'après le profil de leurs protéines hydrosolubles. Les résultats montrent que ces dernières constituent des

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**Abbreviations:** ACN, acetonitrile; CE, capillary electrophoresis; HMW, high molecular weight; HPMC, hydroxypropylmethylcellulose; DTT, dithiothreitol; WS, water-soluble

marqueurs biochimiques utiles pour la recherche sur la génétique du blé et pour l'amélioration de cette culture; l'EC devrait devenir un outil puissant pour la séparation et la caractérisation des protéines hydrosolubles du blé tendre.

**Mots clés:** *Triticum aestivum*, blé tendre, protéines hydrosolubles, électrophorèse capillaire, marqueurs biochimiques

Wheat grain proteins are classified into two major groups: the main storage proteins of the endosperm (gliadins and glutenins) called prolamins, and the non-prolamins consisting of water-soluble (WS) albumins and salt-soluble globulins (Majoul et al. 2004). The albumin and globulin fractions represent about 9 and 5% of wheat seed proteins, respectively, and they are mainly present in embryo and aleurone (Singh and Skerritt 2001). The molecular weights of albumins and globulins are mostly between 12 to 60 kD, with the high molecular weight (HMW) albumins in the range of 45–65 kD and triticins 22–58 kD (Singh and Skerritt 2001; Bean and Tilley 2003; Majoul et al. 2004). In some cases, HMW albumins and triticins are storage proteins, which have the characteristics of wheat storage proteins (Singh and Shepherd 1985, 1987; Gupta et al. 1991). Compared with the gluten proteins, albumins and globulins contain higher levels of essential amino acids, such as lysine and methionine (Singh and Skerritt 2001) that are important for balanced diets. Both albumins and globulins serve not only nutritional but also important functional roles in metabolic activities and structure. They contain important biologically active enzymes and protease inhibitors such as alpha-amylase, beta-amylase, lipid transfer proteins, serine carboxypeptidase, polyphenol oxidase, peroxidase (Weiss et al. 1997; Singh and Skerritt 2001; Dicko et al. 2002; Bean and Tilley 2003), which play an important role in the wheat grain development. Meanwhile, some of them are food allergens, which can cause anaphylactic reactions especially in young children, such as baker's asthma, urticaria, vomiting, etc. (Weiss et al. 1997).

Capillary electrophoresis (CE) is one of the fastest growing analytical techniques in separation science (Bean and Lookhart 2001). As a new technology, considerable methods have been reported for separating the storage proteins of cereal grains, especially gliadin and glutenin proteins (Lookhart and Bean 1995, Bean and Lookhart 1998, 2000; Yan et al. 1998, 1999, 2003a, b, c, 2004; Zhu and Khan 2001). However, methods for separating WS proteins of wheat grain have seldom been reported. Recently, Bean and Tilley (2003) and Piergianni (2007) reported a method that can produce high-resolution, reproducible separations of the water-soluble proteins of cereals by capillary electrophoresis, which could be helpful for characterizing the relationship between albumin and globulin fractions and wheat quality.

For the characterization of high molecular weight glutenin subunits of wheat, Yan et al. (2004) developed a rapid method of acidic capillary electrophoresis. In the present study, we focused on developing a rapid CE

method for the separation and characterization of seed WS proteins of bread wheats.

## MATERIALS AND METHODS

### Wheat Cultivars

Wheat samples used in this study were 120 bread wheat cultivars (*Triticum aestivum* L.,  $2n=6x=42$ , AABBDD) from major wheat growing areas of China, including some elite cultivars with good quality and high yield traits such as Zhongyou 9507, Shanyou 225, Gaocheng 8901, Jimai 20, Zhengzhou 9023, Jing 411, etc. All samples were from germplasm collections of the Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS).

### Sample Preparation

Both whole ground meal and flour (30 mg) were used for water-soluble protein extraction with different solutions (300  $\mu$ L), including distilled water, 10, 20, 30, 40, 50 and 70% aqueous ethanol, and ethanol plus 1% dithiothreitol (DTT). Different extraction times (5–30 min) at room temperature with periodic agitation were tested. Extracts were clarified by centrifugation (10 000 rpm for 10 min).

### Capillary Electrophoresis

The BioFocus 3000 instrument (Bio-Rad, Hercules, CA) was used to carry out CE separations (Yan et al. 2004). In order to obtain a rapid method for water-soluble protein analysis by acidic capillary electrophoresis in untreated fused-silica capillaries, different organic modifiers in phosphate-glycine buffer, CE performance conditions and different sample extraction times were tested and optimized. Four different buffers were investigated: 0.1M phosphate-glycine buffer (pH 2.5) containing 0.05% hydroxypropylmethylcellulose (HPMC) and 20% (vol/vol) methanol, 0.1 M phosphate-glycine buffer (pH 2.5) containing 0.05% HPMC and 20% 2-methoxyethanol, 0.1 M phosphate-glycine buffer (pH 2.5) containing 0.05% HPMC and 20% acetonitrile (ACN), and 0.1 M phosphate-glycine buffer (pH 2.5) containing 0.05% HPMC, 20% ACN and 50 mM CHAPS. The untreated, fused-silica columns used included 25.5-cm and 31.5-cm lengths (20.5 cm and 26.5 cm to the detector) with 25  $\mu$ m and 50  $\mu$ m inner diameters (ID), respectively. Several performance voltages (9.0, 10.0, 11.0 and 12.0 kV) were tested and separation temperature ranged from 25 to 40°C. Different pressure injection times of samples were also examined. All samples were detected by UV absorbance at 200 nm.

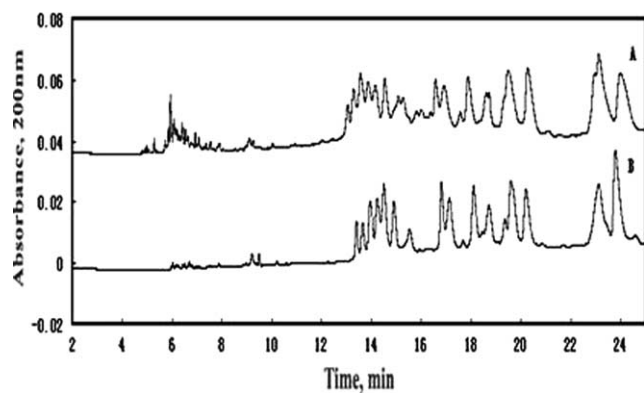
### Capillary Rinsing Protocol

Before use, new capillaries were rinsed with separation buffer for 1 h (old capillaries for 20 min). After each separation, capillaries were rinsed with 1 M phosphate solution followed by separation buffer for 3 min, respectively, unless otherwise stated.

## RESULTS AND DISCUSSION

### Optimization of Sample Extraction

In order to confirm that the aqueous ethanol solution was suitable for WS protein extraction and CE separation, different experimental procedures were carried out. As shown in Fig. 1, two CE patterns of proteins from cultivar Zhongyou 9507 were compared, one generated from proteins extracted directly by 20% ethanol and the other by first removing albumins and globulins prior to 20% ethanol extraction. According to the method reported by Marin et al. (1994), 30 mg crushed seeds were extracted twice with 400 mL 50 mM Tris-HCl buffer (pH 7.8, containing 100 mM KCl and 5 mM EDTA) for 10 min to remove albumins and globulins. The pellet was then extracted twice with 400 mL water for 5 min to remove residual albumins and salts, and finally extracted with 20% ethanol for 30 min. Both samples were analyzed by CE for 25 min with the same separation conditions. As shown in Fig. 1B, there were no peaks before 8 min with 20% ethanol extraction after albumins and globulins being removed. It was obvious that the protein peaks at 4–8 min belonged to WS proteins; although, some contamination could not be excluded. Furthermore, gliadin patterns at 8–24 min in both samples were nearly identical. This indicated that



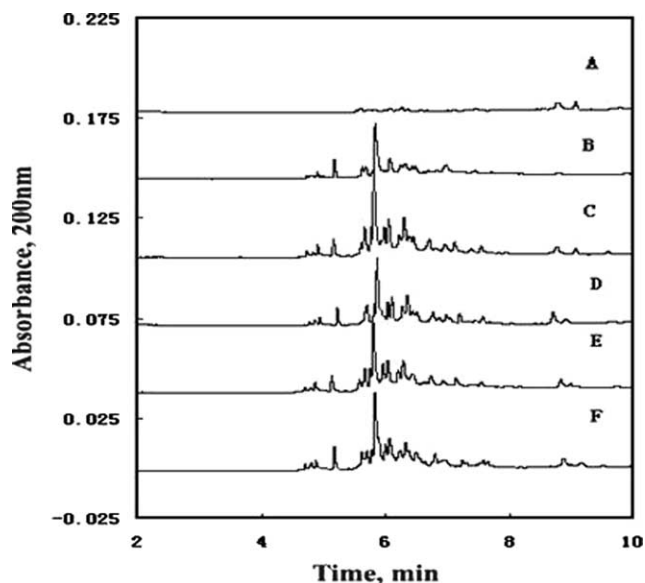
**Fig. 1.** Capillary electrophoresis (CE) separation of extractions from Chinese bread wheat cultivar Zhongyou 9507 and the WS proteins were confirmed to be present at 5–8 min. (A) 20% ethanol extraction (B) 20% ethanol extraction after removing albumins and globulins. Extraction pressure-injected time was 6 s in a 31.5-cm length and 50  $\mu$ m ID untreated fused-silica capillary at 35°C and 11.0 kV. Separation buffer was 0.1 M phosphate-glycine buffer (pH 2.5) containing acetonitrile (ACN) and 0.05% hydroxylpropyl-methylcellulose (HPMC). Detection wave length was 200 nm.

water-soluble proteins and gliadins were easily differentiated by CE.

Various extracts were tested to ascertain optimal conditions for solubilising WS proteins for CE separation. The major extractants tested were distilled water, aqueous ethanol and ethanol plus 1% DTT. It was found that resolution of proteins extracted with 20% ethanol appeared considerably better than those with distilled water and 10% ethanol, but similar to 30, 40, 50 and 70% aqueous ethanol. Resolution of proteins extracted with ethanol plus DTT was poorer than those extracted with 20% aqueous ethanol alone. Furthermore, the CE patterns from the whole ground meal and flour extractions showed only minor quantitative differences, which are similar to these reported by Bean and Tilley (2003). Therefore, whole ground meal was used for protein extraction in this study. Furthermore, results of different extraction times (5, 10, 20 and 30 min. Fig. 2) revealed that 5 min was adequate for WS protein extraction.

### Optimization of CE Separation Conditions

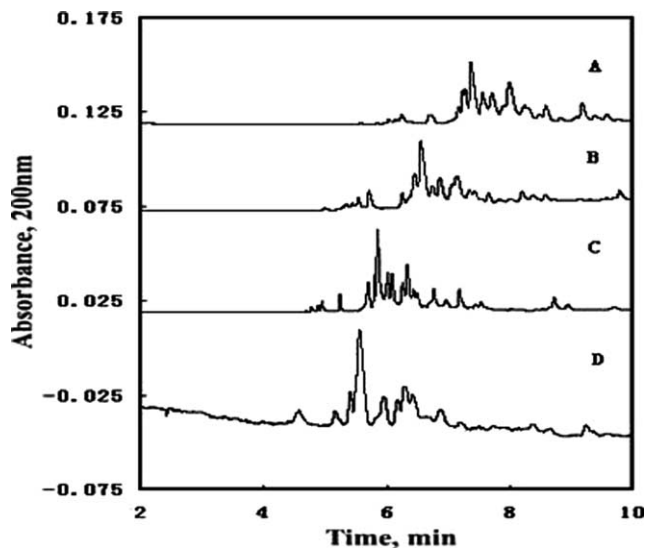
To develop an optimal CE method for WS protein separation, different separation conditions, including different organic modifiers in the CE buffer, capillary lengths and ID, operating temperatures, performance voltages and injection-times of samples were tested.



**Fig. 2.** CE patterns of WS proteins from Chinese cultivar Zhongyou 9507 by different extraction methods. (A) 20% ethanol extraction after removing albumins and globulins for 10 min. (B) 100% distilled water extraction for 10 min. (C) 20% ethanol extraction for 5 min. (D) 20% ethanol extraction for 10 min. (E) 20% ethanol extraction for 20 min. (F) 20% ethanol extraction for 30 min. Separation conditions as in Fig. 1.

Four organic modifiers (20% methanol, 20% 2-methoxyethanol, 20% ACN and 20% ACN with 50 mM CHAPS) were used to analyze their effects on the resolution of WS protein separations in the acidic buffer system (0.1 M phosphate-glycine buffer, pH 2.5, containing 0.05% HPMC). The results showed that the best resolution could be obtained by using 20% ACN as organic modifier (Fig. 3). Both methanol and 2-methoxyethanol resulted in poor resolution and delay of migration time. Although 20% ACN and 50 mM CHAPS resulted in slightly faster separation, the resolution was decreased. As shown in previous reports, the organic modifier ACN can improve the separation of water-soluble proteins (Bean and Tilley 2003) and cereal storage proteins (Bean and Lookhart 1998). In general, higher resolution of CE separations of wheat storage proteins and water-soluble proteins can be achieved with low pH phosphate-glycine buffer plus some additives such as ACN, HPMC or hexane sulfonic acid (Lookhart and Bean 1996; Bean and Lookhart 1998; Yan et al. 2003a, b, c; Bean and Tilley 2003).

According to the previous work (Bietz and Schmalzried 1995; Yan et al. 1999), higher temperature and voltage can generally result in proteins eluting earlier, but may produce poor resolution. Several parameters were tested to improve CE resolution: different temperatures (25, 30, 35 and 35°C), voltages (9, 10, 11, 12 and 13 kV), capillary lengths (25.5 and 31.5 cm) using 0.1 M phosphate-glycine buffer (pH 2.5) containing 20% ACN and 0.05% HPMC at the detection wavelength of 200 nm. The results showed that the optimal

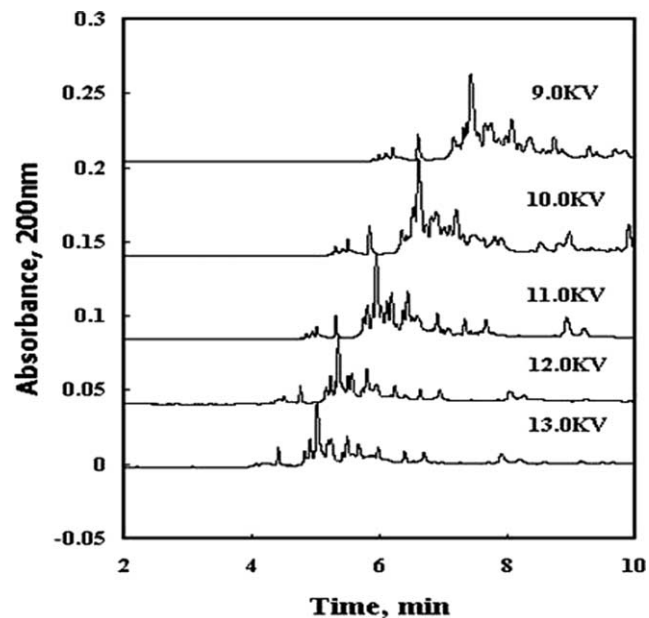


**Fig. 3.** Effects of different organic modifiers in 0.1 M phosphate-glycine buffer (pH 2.5) containing 0.05% HPMC on the separations of WS proteins from Chinese cultivar Zhongyou 9507. (A) 20% methanol. (B) 20% 2-methoxyethanol. (C) 20% ACN. (D) 20% ACN and 50 mM CHAPS. The other separation conditions are described as in Fig. 1.

temperature and voltage were 35°C and 11.0 kV (Fig. 4). The 31.5-cm length (26.5 cm to detector) and 50  $\mu$ m ID capillary could produce more sensitive and higher resolution CE separations (Fig. 5). The separation of one sample could be completed in less than 10 min.

Different experiments were performed to improve the reproducibility of CE separation. When proteins were extracted with distilled water, migration times drifted widely because of sample instability. Sample extractions with different ethanol concentrations showed that when samples were extracted with 20% ethanol, the reproducibility and sensitivity of CE patterns were good. Bean and Tilley (2003) demonstrated that the reproducibility could be greatly improved after adding 10% separation buffer to the sample buffer. In our work, however, the resolution was similar by this method. In addition, sample injection-time was an important factor to affect reproducible separations. Injection times of 2, 4, 6 and 8 sec were tested at 11.0 kV with consecutive injections, and the 6 s injection time was found to be optimal.

Reproducibility is essential for storage protein separations (Bietz and Schmalzried 1995; Bean and Lookhart 1998; Yan et al. 1999, 2003c). The capillary rinsing protocol is highly important to obtain reproducible separation by CE. Many combinations of capillary rinsing procedures were investigated and the optimal rinsing protocols were as follows: new capillaries were rinsed for 1 h (old capillaries for 20 min) with separation buffer. After each separation, capillaries were rinsed with 1 M phosphate and separation buffer for 3 min.



**Fig. 4.** Effects of CE performance with voltage on the separations of WS proteins from Chinese cultivar Zhongyou 9507. Separation conditions as in Fig. 1.

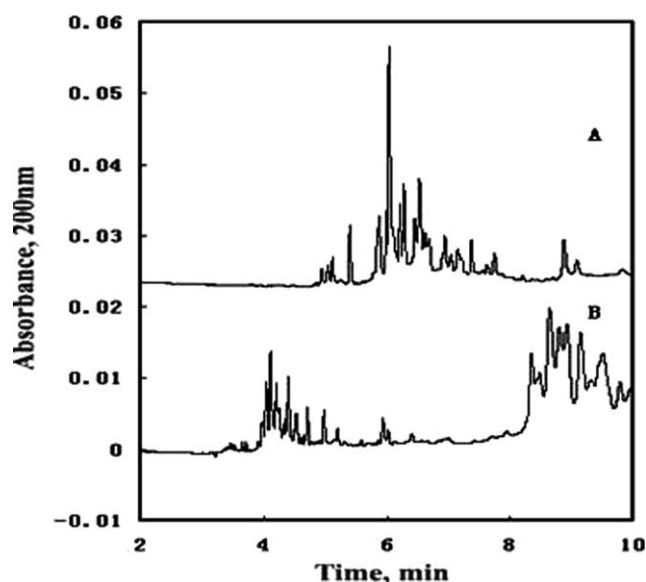


Fig. 5. Effects of capillary lengths on the separations of WS proteins from Chinese cultivar Zhongyou 9507. (A) 31.5 cm. (B) 25.5 cm. Separation conditions as in Fig. 1.

#### Potential Applications of the CE Method

The optimized CE method described above was used to separate WS proteins of 120 Chinese bread wheat cultivars that included the major elite bread wheat cultivars planted in different areas of China at present. The WS proteins of these cultivars were initially analyzed by one-dimensional SDS-PAGE and a low level of polymorphisms was detected. However, a higher level of polymorphisms in WS proteins was detected when separated by CE. The WS protein patterns of six typical Chinese bread wheat cultivars (Zhengzhou 9023, Jing 411, Gaocheng 8901, Shanyou 225, Zhongyou 9507 and Jimai 20) are shown in Fig. 6, and revealed that each cultivar displayed clear qualitative differences and high polymorphism in WS protein composition. The major WS protein components were present at 5–8 min elution. Generally, there were 3–6 major peaks at 5.5–6.5 min, which were highly polymorphic among cultivars. These components could be used as useful biochemical markers for discriminating between different cultivars. In particular, some good breadmaking quality cultivars, such as Jimai 20, displayed characteristic protein peaks. According to our study, CE is expected to become a powerful tool for wheat cultivar differentiation and germplasm screening and for the research on the structures and functions of WS proteins. Some other potential applications of CE procedures for WS protein separations include the relationships between WS protein patterns and their allergenic level, the effects of environmental factors on water-soluble protein expression, protein synthesis and accumulation at different development stages and their relations to quality performance.

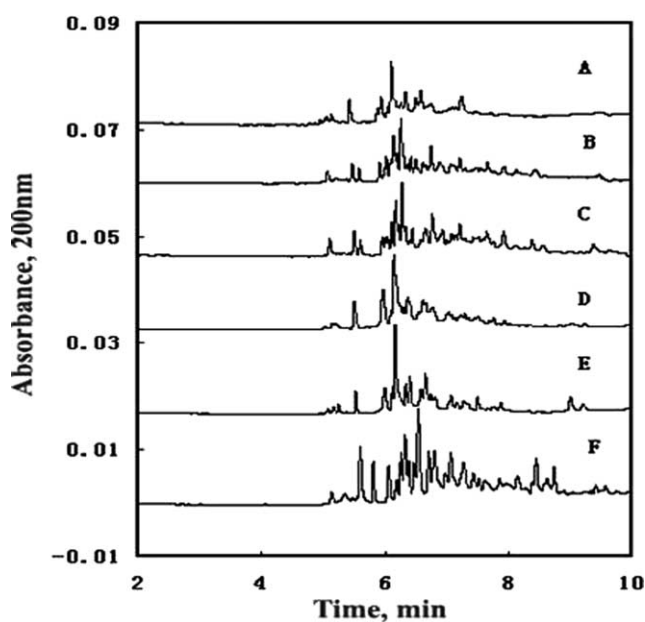


Fig. 6. Characterization of WS proteins from six Chinese bread wheat cultivars by CE. (A) Zhengzhou 9302. (B) Jing 411 (C) Gaocheng 8901. (D) Shanyou 225. (E) Zhongyou 9507. (F) Jimai 20. CE conditions were as in Fig. 1.

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