ELSEVIER

Journal of Chromatography A, 803 (1998) 321–327

JOURNAL OF CHROMATOGRAPHY A

Short communication

Separation of alcohol-soluble proteins from tobacco seeds by capillary zone electrophoresis

Bin Deng*, Jianpin Lu, Jinhui Yan

Yunnan Tobacco Research Institute, Yunnan 653100, China

Received 15 April 1997; received in revised form 9 December 1997; accepted 9 December 1997

Abstract

The separation of alcohol-soluble proteins of tobacco seeds by free-solution capillary zone electrophoresis (CZE) has been described. The entire separation was achieved in less than 12 min using on-column UV detection at 214 nm. As buffer modifier, 20% acetonitrile was tested for use with 100 m*M* phosphate buffer (pH 2.5) containing 0.05% hydroxypropylmethylcellulose. This buffer provided high resolution and reproducibility of alcohol-soluble proteins of tobacco seed. Among four tested tobacco cultivars (Virginia tobaccos speight G-28, Yunyan-317, Hongda and Yunyan-85), each cultivar was found to possess a unique protein CZE peak pattern and this pattern could be regarded as a genetic marker to identify tobacco cultivar. © 1998 Elsevier Science B.V.

Keywords: Tobacco; Proteins

1. Introduction

Tobacco is made of *Solanaceae nicotiana* economic crops. There are many cultivars and the number of new cultivars of tobacco in the world steadily increases.

Their seeds are so small that 1 g seed contains 10 000–18 000 grains. The protein content of tobacco seed is 23–28% and most of them are globulins. The seed also has a high lipid content of 37–45%. So there are some difficulties in identifying and classifying them by morphological characteristics and general chemical methods. As a rapid, simple and efficient method, electrophoresis has been widely applied for the past ten years to separate seed proteins and identify the variety [1]. It is well known that the proteins of tobacco seeds are expressing products of tobacco nuclear genomic DNA, so the peak or band pattern of proteins in an electropherogram could be an expression of genetic trait and a genetic marker of tobacco seeds themselves.

Polyacrylamide gel electrophoresis (PAGE), acid–PAGE and sodium dodecyl sulfate–PAGE (SDS–PAGE) are the established methods for protein separation and are used as reliable tools in cultivar identification of several important crops [2–5] and grass cultivars [6]. A total of 28 cultivars of perennial ryegrass were characterized by their unique band patterns and band intensity ratios of total seed proteins [7]. However this technique did not differentiate between very closely related ryegrass cultivars [7].

Capillary zone electrophoresis (CZE) in free solution has been widely used as a powerful separation tool which combines gel electrophoretic with chromatographic techniques. It offers a higher resolution

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01256-9

and a shorter analysis time than high-performance liquid chromatography (HPLC) and gel electrophoresis [8]. The sample amount injected is very small (nl or pl) and the detection limits are low (amol levels) [9]. So the CZE approach is very suitable to separate tobacco seed proteins at trace levels for cultivar identification and genetic purity assessment. Some cereal proteins such as wheat, oats and rice have been analyzed by CZE [10,11]. The electrophoretic method, resolution and reproducibility were optimized by Lookhard and Bean [12]. However, no prior research has been reported on the separation of alcohol-soluble proteins from tobacco seeds by CZE.

Experimental samples G-28, Y-317, Hongda and Y-85 are four closely related tobacco cultivars. They are all classified as Virginia tobaccos. This paper describes the separation of proteins from four different Virginia tobacco seeds and shows their own characteristic peaks in CZE for tobacco cultivar identification.

2. Experimental

2.1. Chemicals and reagents

Sodium phosphate and hydroxypropylmethylcellulose (HPMC) were purchased from Sigma (St. Louis, MO, USA) and acetonitrile (ACN) from BDH (Poole, UK). All other chemicals were analytical-reagent grade from China, and the water used was deionized using the Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Experimental tobacco samples G-28, Yunyan-317 (Y-317), Hongda and Yunyan-85 (Y-85) were a gift from China Tobacco Breeding Center (South). G-28 was introduced from the USA. The others were bred by Yunnan Tobacco Co., Yunnan, China.

Tobacco seeds (100 mg) were ground to a fine powder with a mortar and pestle. The powder was extracted at room temperature (25±1°C) with 1 ml 0.1 *M* sodium chloride solution in a 1.5-ml plastic centrifuge tube for 1 h with brief vortexing every 15 min. The tube was centrifuged at 14 000 *g* for 10 min. After extraction of salt-soluble globulin, pellet was resuspended with 1 ml of 70% aqueous ethanol [5] and left on a shaker for 4 h at room temperature (25±1°C). The tube was centrifuged at 14 000 *g* for 10 min. The supernatants were filtered through a 0.45-μm filter into one 0.5-ml P/ACE vial for immediate CZE analysis or stored at −20°C.

2.3. Instrument and procedure

CZE experiments were performed on a Beckman P/ACE system (Beckman Instruments, Fullerton, CA, USA) Model 5510 instrument which consisted of uncoated fused-silica capillary, a power supply, a detection and a data collection system. The fused-silica capillary (Beckman Instruments) was 37 cm (30 cm to the detector)×50 μm I.D.×375 μm O.D.

CZE separations were performed in 100 m*M* sodium phosphate buffer, pH 2.5, containing 20% ACN and 0.05% HPMC, using an applied voltage of 15 kV [12]. Protein samples were pressure injected for 5 s, and separated proteins were detected at 214 nm. Operating temperature was maintained at 45±0.1°C.

The capillary was rinsed with run buffer for 2 min (containing no ACN) and then for 4 min with the phosphate buffer containing 20% ACN before injections and with the 1 *M* phosphoric acid for 2 min between injections [12].

The program is controlled by an AST personal computer with Gold System Personal chromatography software (version 8.10) and detected by a Beckman UV absorbance P/ACE detector.

3. Results and discussion

Tobacco seeds contain large amounts of globulins, so 0.1 *M* NaCl for the first extraction is useful to decrease this factor. CE separations of 70% EtOH, 55% isoamylalcohol and 25% 2-ethylenechlorohydrin which extract salt-soluble globulins were compared: 70% EtOH extract was found optimal to extract tobacco seed alcohol-soluble protein (data not shown). In order to avoid the influence of EtOH UV absorbance, a wavelength of 214 nm was chosen to detect the separation of proteins. The reproducibility of this isolation procedure was ver-

ified by extracting seed globulins and alcohol-soluble proteins from three batches of seed source for each cultivar. In the CZE system, sample preparation is very easy and rapid, especially in the salt-protein, because CZE methods do not require desalting and special treatment required for HPLC that needs a high sample purity and special solvent.

The HPCE pattern of Hongda alcohol-soluble proteins in the pH 2.5 phosphate containing 0.05% HPMC buffer is shown in Fig. 1A and with the addition of 20% ACN in Fig. 1B. The separation resolution of protein in the run buffer with ACN is more effective than without ACN buffer. The buffer modified with ACN produced almost the same migration time patterns as the buffer without ACN. However, the buffer with ACN provided nearly baseline resolution and provided more peaks.

ACN is a relatively hydrophilic solvent, the resolution may be enhanced by altering the selectivity of the separation. The presence of ACN in the buffer also decreases the current flow during a separation [12]. In addition, an important operating parameter, the electroosmotic flow (EOF), is very low at this low pH value [13]. The migration times of proteins in the CZE analysis are mainly affected by two factors: the EOF and the ionic state of the solute. Both factors can be controlled by altering the pH of the run buffer from 2.0 to 11.5 to optimize the separation [14].

The separation is based on the differences in the charge-to-mass ratio of the different analytes. A properly high temperature during CZE separation can decrease the association degrees of protein; as a result, it will increase the charge-to-mass ratio of the analytes and the migration velocity of each protein. Using this buffer with ACN, the best resolution was found at 40–50°C; 45°C and 15 kV were previously found as optimum to separate wheat gliadin [12]. These results have also been tested in our experiments of separating tobacco seed protein under different temperatures. Fig. 2A,B show the separation of alcohol-soluble protein of Hongda at 25°C and 35°C. Compared with the separation at 45°C, the migration times of the same peak are higher and the resolution of protein separation decreases with the drop in temperature.

Based on the high resolution and sensitivity of the CZE protocol described in this paper, tobacco seed

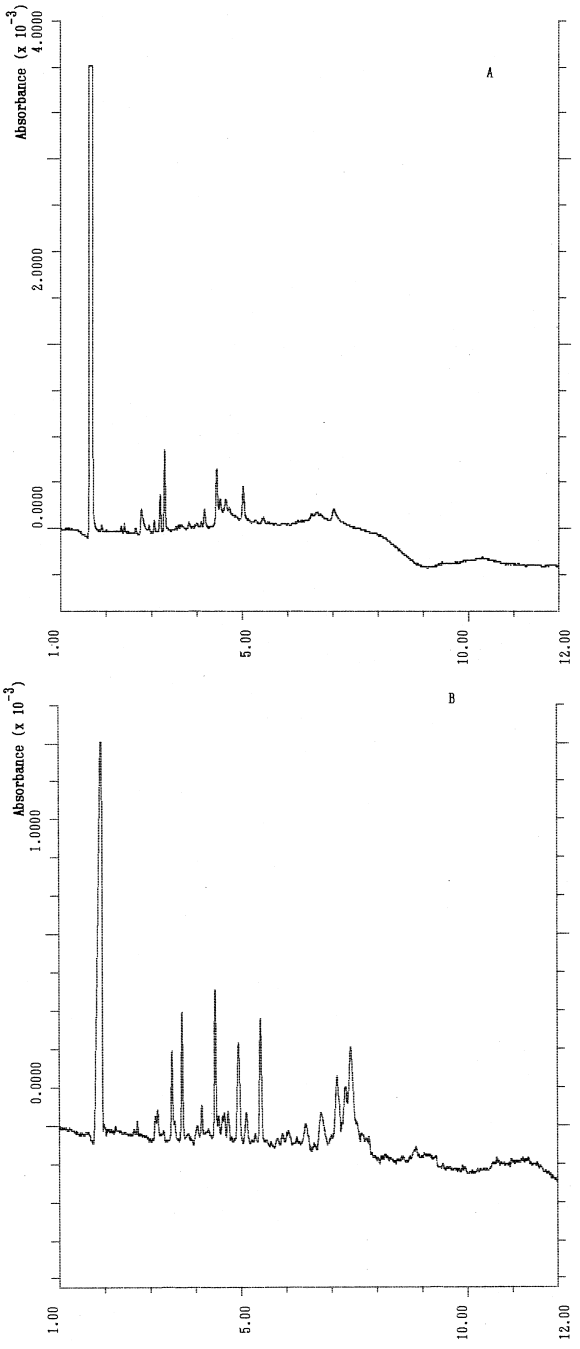

Fig. 1. Separation of alcohol-soluble proteins of Hongda by CZE with 100 m*M* phosphate buffer, pH 2.5, containing 0.05% HPMC (A) or buffer A plus 20% ACN (B). Separations were carried out using 37 cm (30 cm separation length)×50 μm I.D. capillaries at 15 kV (+ to −) and 45°C. Samples were pressure injected (5 s). UV detection at 214 nm.

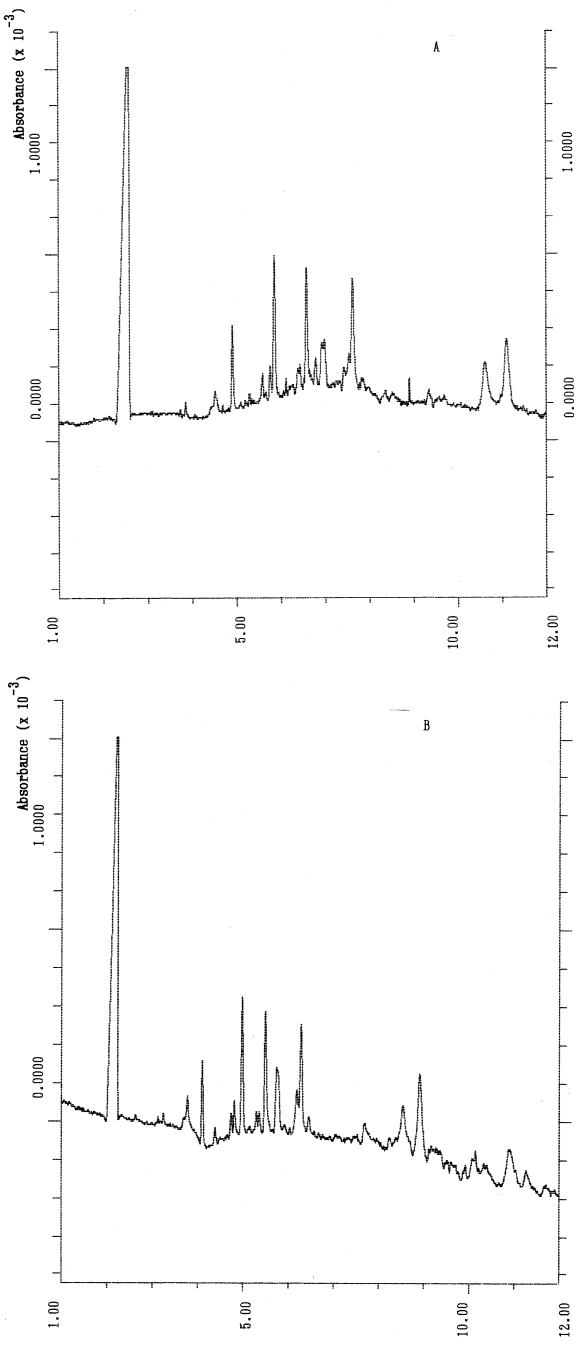

Fig. 2. Separation of alcohol-soluble proteins of Hongda by CZE with 100 m*M* phosphate buffer (pH 2.5) containing 0.05% HPMC and 20% ACN at different temperatures. (A) 25°C, (B) 35°C. Other experimental conditions as in Fig. 1.

proteins have been separated into numerous components (peaks). Among the four Virginia tobacco cultivars, diversities of the protein peaks were distinguished except for the first peak at 1.8 min as shown in Fig. 3A–D. These differences can be easily observed and compared with computer assisted magnification. Each cultivar has its own unique peak pattern distinguishable from the others, which can be regarded as a genetic marker. In the 2–6 min range, there are five to ten major peaks and a few minor peaks.

Comparison of the HPCE pattern of different cultivars revealed several unique and reproducible CE characteristics of each cultivar. Both qualitative and quantitative differences were observed. The intensity and the number of peaks of Y-317 appear to be much higher than that of the other cultivars. In the 6–8 min range, Y-85 shows four peaks with high intensity. Hongda and G-28 have five peaks with low intensity in the protein HPCE patterns. However, there is a larger 1:2 doublet at about 7.2 min for Hongda and a larger 1:1.5:1.7 triplet for G-28 at about 7 min. Y-317 has six peaks with high intensity and a larger 3:2:1 triplet at 7 min. In addition, for Y-317 there are five little peaks in the 8–10 min range compared with no peaks for the other cultivars in this range. Y-317 was selected from Hongda cross maize, so there was a little difference compared with others.

Reproducibility of the separation with phosphate–ACN buffer under optimal conditions was excellent [12]. Using this protocol, relative standard deviation (R.S.D.) for migration times of 17 major peaks of G-28 ranged from 0.1 to 0.3% over 20 runs (Table 1). R.S.D.s for peak heights on the same peaks averaged 4–11% R.S.D. (Table 2). Consistent results of other samples was found in the protein HPCE patterns (data not shown). To ensure good reproducibility, fresh buffer was used every 10 runs (20 min per run, using 4.5-ml vials). The volatility of ACN also made it difficult to keep the buffer composition constant over a period of 10 runs.

Tobacco proteins extracted by 70% EtOH were separated by PAGE at a pH of 3.1 and by SDS–PAGE in 1996 [15]. The experiments showed that the protein band patterns had some distinct differences in the interspecific varieties but not in the very

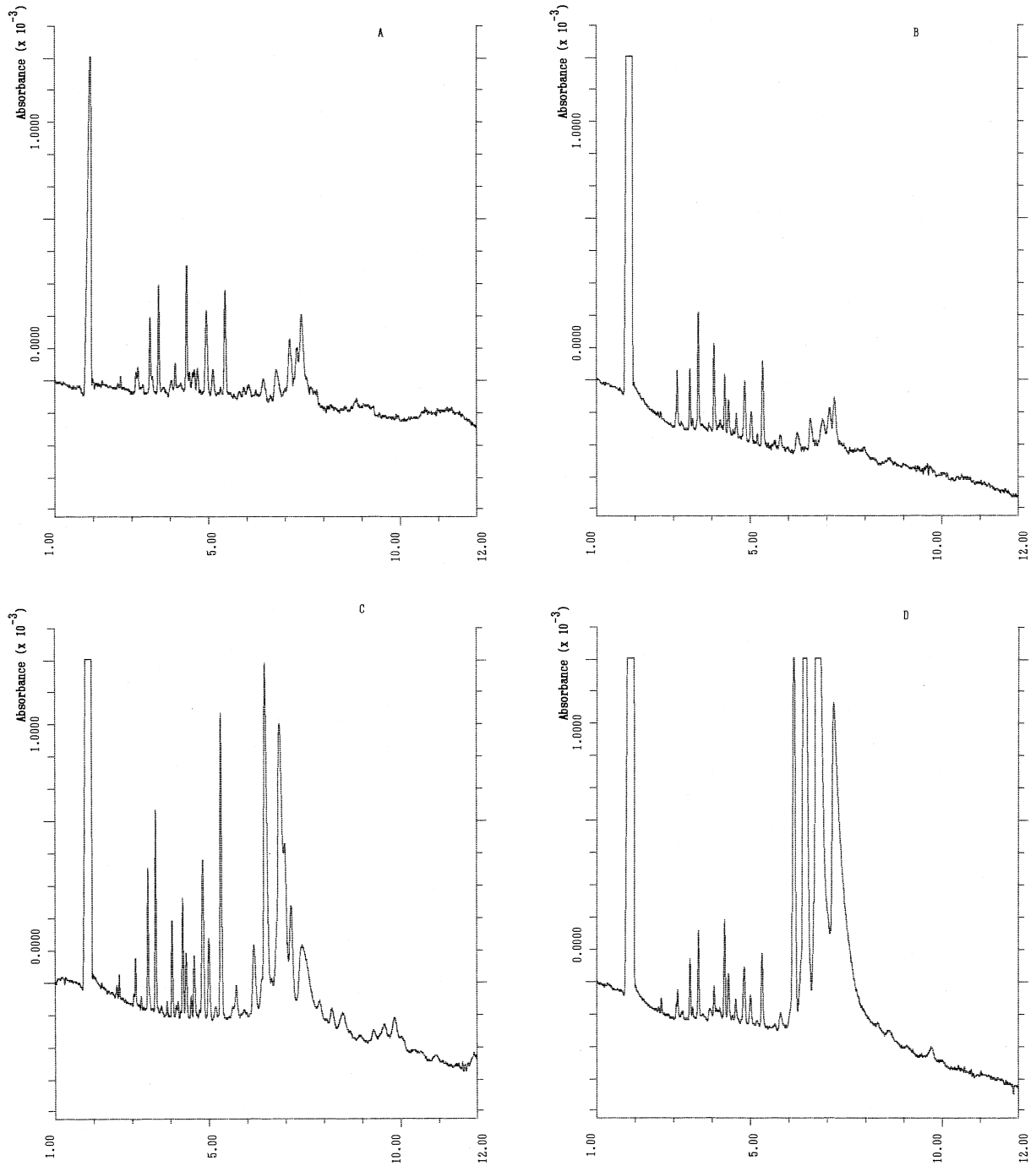

Fig. 3. CE patterns of alcohol-soluble proteins of four different tobacco cultivars by CZE (A) Hongda, (B) G-28, (C) Yunyan-317, (D) Yunyan-85. Run buffer was 100 m*M* phosphate buffer (pH 2.5) containing 0.05% HPMC and 20% ACN. Other experimental conditions as in Fig. 1.

Table 1
Reproducibility of migration times of 17 G-28 alcohol-soluble protein peaks using the 100 m*M* phosphate buffer (+0.05% HPMC) containing 20% ACN over 20 runs

Peak No.	Migration time	
	Mean±S.D. (min)	R.S.D. (%)
1	1.930±0.0026	0.13
2	3.072±0.0052	0.17
3	3.439±0.0072	0.21
4	3.663±0.0080	0.22
5	4.070±0.0090	0.22
6	4.307±0.0092	0.21
7	4.440±0.0096	0.22
8	4.607±0.0102	0.22
9	4.871±0.0113	0.23
10	5.008±0.0115	0.23
11	5.336±0.0128	0.24
12	5.776±0.0134	0.23
13	6.243±0.0150	0.24
14	6.544±0.0166	0.25
15	6.878±0.0182	0.27
16	7.045±0.0180	0.26
17	7.178±0.0196	0.27

closely related cultivars such as the same Virginia tobacco G-28, Y-317, Hongda, Y-85 and so on (data not shown) because of its low resolution and sensitivity. From the other reports of identifying crop cultivars by slab gel electrophoresis, the minor quantitative and qualitative differences among closely related cultivars in the relative intensities in protein patterns often cannot be used as the main criteria for identifying cultivars, because these changes in protein levels can be influenced by environmental conditions as well as by fertilizer factors [16,17]. Compared with CZE, the disadvantages of slab gel electrophoresis are lack of on-line sample detection, difficultly of quantitation and automation and the separation medium cannot be reused for a number of runs. In CZE of proteins, run times are typically not more than 20 min. No additional time is needed for detection and data reduction. Considering the gel preparation, casting, gel transferring and film development of slab gel electrophoresis, run times of CZE per sample have to be determined.

Table 2
Reproducibility of peak heights of 17 G-28 alcohol-soluble protein peaks using the 100 m*M* phosphate buffer (+0.05% HPMC) containing 20% ACN over 20 runs

Peak No.	Peak heights	
	Mean±S.D. (mAU)[a]	R.S.D. (%)
1	0.86580±0.06200	7.16
2	0.01187±0.00097	8.17
3	0.01179±0.00112	9.50
4	0.02782±0.00285	10.24
5	0.02223±0.00145	6.52
6	0.01190±0.00051	4.29
7	0.00892±0.00065	7.29
8	0.00674±0.00044	6.53
9	0.02049±0.00087	4.25
10	0.00723±0.00041	5.67
11	0.02871±0.00190	6.61
12	0.00432±0.00035	8.10
13	0.00720±0.00059	8.20
14	0.01002±0.00069	6.89
15	0.00922±0.00070	7.60
16	0.00996±0.00054	5.42
17	0.01101±0.00081	8.09

[a] Millabsorbance units.

Other techniques such as two-dimensional electrophoresis and electrofocussing have helped to identify closely related cultivars, but they are relatively difficult and slow to perform [18,19]. In addition, reversed-phase HPLC has also been used to differentiate cereal cultivars for some cereal crops [5,20,21]. A recent review of electrophoretic methods to identify cereals has been published [22].

The quantity of CE exhausted solvent is so small that one 5-ml run buffer can be used 10 to 20 times. As a result, the generation of laboratory waste is considerably lessened. In addition, the CZE capillary can be reused 100–1000 times and its cost is far less than that of a HPLC column. All of these advantages overcome the problems associated with HPLC while the high resolution and sensitivity are more accurate and rapid than those of protein gel electrophoresis.

4. Conclusions

Alcohol-soluble proteins of tobacco seed are a complex and heterogeneous group of proteins. They can be separated rapidly and reproducibly by HPCE with 100 m*M* phosphate buffer containing 0.05% HPMC and 20% ACN at 45°C. The CZE patterns were used successfully for tobacco cultivar identification.

Acknowledgements

Funds and research support were provided by Yunnan Tobacco Co.

References

[1] R.J. Cooke, Electrophoresis, VCH, Weinheim, 1986, p. 203–207.
[2] M.B. McDonald Jr., J. Seed Technol. 5 (1980) 88–101.
[3] P.R. Shewry, A.J. Faulks, H.M. Pratt, B.J. Miflin, J. Sci. Food Agric. 29 (1978) 847–849.
[4] W. Bushuk, R.R. Zillman, J. Plant Sci. 58 (1978) 505–515.
[5] J.A. Bietz, T. Burnouf, L.A. Cobb, J.S. Wall, Cereal Chem. 61 (1984) 129–135.
[6] H.B. Krishnan, D.A. Sleper, Crop Sci. 37 (1997) 215–219.
[7] J.M. Ferguson, D.F. Grabe, Crop Sci. 26 (1986) 170–176.
[8] T. Tsuji, J. Chromatogr. 550 (1991) 823–830.
[9] Y. Ma, Z. Wu, H.C. Furr, C. Lammi-Keefe, N.E. Craft, J. Chromatogr. 616 (1993) 31–37.
[10] G.L. Lookhart, S.R. Bean, Cereal Chem. 72a (1995) 42.
[11] G.L. Lookhart, S.R. Bean, Cereal Chem. 72b (1995) 312.
[12] G.L. Lookhart, S.R. Bean, Cereal Chem. 73 (1996) 81–87.
[13] R.M. McCormick, Anal. Chem. 60 (1988) 2322.
[14] S.S. Yang, I. Smetena, A.I. Goldsmith, J. Chromatogr. A 756 (1996) 131–136.
[15] J. Lu, B. Deng and W. Ma, 50th Tobacco Chemists Research Conference, Richmond, VA, 20–24 October 1996, p. 34.
[16] F.R. Huebner, J.A. Bietz, Cereal Chem. 65 (1988) 362–366.
[17] F.R. Huebner, J.A. Bietz, B.D. Webb, B.O. Julian, Cereal Chem. 67 (1990) 129–135.
[18] Y.J. Guo, R. Bishop, H. Ferhnstrom, G.Z. Yu, Y.N. Lian, S.D. Huang, Cereal Chem. 63 (1986) 1–3.
[19] L.S. Robert, C. Nozzolillo, I. Altosaar, Cereal Chem. 60 (1983) 438–442.
[20] G.L. Lookhart, B.O. Juliano, B.D. Webb, Cereal Chem. 66 (1991) 396–400.
[21] G.L. Lookhart, Cereal Chem. 62 (1985) 345–350.
[22] G.L. Lookhart and C.W. Wrigley, in C.W. Wrigley (Editor), Identification of Food Grain Varieties, American Association of Cereal Chemists, St. Paul, MN, 1995, Ch. 4, pp. 55–71.