Detection of Smoked Paprika "Pimentón de La Vera" Adulteration by Free Zone Capillary Electrophoresis (FZCE)

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The purpose of this work was to develop a procedure based on protein analysis by free zone capillary electrophoresis (FZCE) that can be used in the determination of smoked paprika "Pimentón de La Vera" adulteration with paprika elaborated from varieties of pepper foreign to the "La Vera" region, in central western Spain. Two autochthonous varieties of pepper, Jaranda and Bola, and the variety Papri Queen, foreign to the "La Vera" region, were used in the study. Several aqueous solutions for solubilization of the methanol-soluble proteins were tested, and the FZCE conditions of capillary dimensions, FZCE buffer concentrations, and detection wavelengths were optimized. On the basis of the results, 30% (v/v) acetonitrile was adopted as the suspending solution for routine analysis, and the optimal FZCE parameters were 75 μm inner diameter and 57 cm total length capillaries, 8.75 mM phosphate/20.6 mM tetraborate as run buffer, and 256 nm as detection wavelength. This method was found to give excellent repeatability of the corrected migration time (CMT) with coefficients of variation (RSD %; $n = 5$) of <1% for most of the proteinaceous compounds analyzed and showed greater effectiveness in discriminating paprika varieties than the SDS-PAGE technique. Four peaks found in the FZCE electropherograms were investigated as a basis for detecting and estimating the adulteration of smoked paprika with paprika elaborated from the Papri Queen variety. The adulteration detection limits varied from 5 to 40% of the Papri Queen variety within a satisfactory working range of mixture (5−80%) sufficiently large to cover the adulteration levels of interest. The use of peak 6 as a marker for determining adulteration gave the best results, with an adulteration detection limit of 5−10% (w/w).

KEYWORDS: Paprika; adulteration; protein profile; FZCE

INTRODUCTION

Smoked paprika, "Pimentón de La Vera", is a high-quality product obtained by drying the fruits of autochthonous varieties of pepper (*Capsicum annuum* L.) from the region of La Vera, in central western Spain (*1*). Oak logs are burned as the heat source to dry this product. Only autochthonous varieties of pepper with a thin pericarp, such as Jaranda and Bola, are appropriate for this slow drying process. The smoke gives the product a more highly valued flavor as an ingredient in the processing of chorizo, a Spanish pork sausage, compared to paprikas obtained from sun-dried or hot-air-dried peppers. The adulteration of smoked paprika "Pimentón de La Vera" with foreign paprika of an inferior quality, primarily to increase profit margins, has been a concern for many years within the smoked paprika industry. Papri Queen in particular is the variety foreign to the La Vera region most frequently used in the adulteration

of smoked paprika. Traditionally, product adulteration has been investigated using the measure of color. However, this parameter depends on the degradation level of the different carotenoid fractions during drying (*2*) and can lead to variation in the test results. Thus, verification by another method would be desirable for legal purposes.

Proteins are suitable compounds for such a purpose. Various studies have described methods based on protein patterns to detect food adulteration (*3−5*). Analyses of proteins in these complex mixtures are presently performed mainly by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) and reversed-phase high-performance liquid chromatography (RP-HPLC). Indeed, several studies have characterized *Capsicum annuum* cultivars by SDS-PAGE analysis of seed proteins (*6−9*). However, this technique involves many manual steps, including gel preparation in gel molds, setting the gel molds in an electrophoresis apparatus, sample injection, removal of gels from the molds, staining and destaining of the gels, etc. (*10*). To quantify the stained proteins, further manual steps are required for the densitometry or image analysis of the stained gel. Capillary electrophoresis (CE) may be used as an alternative

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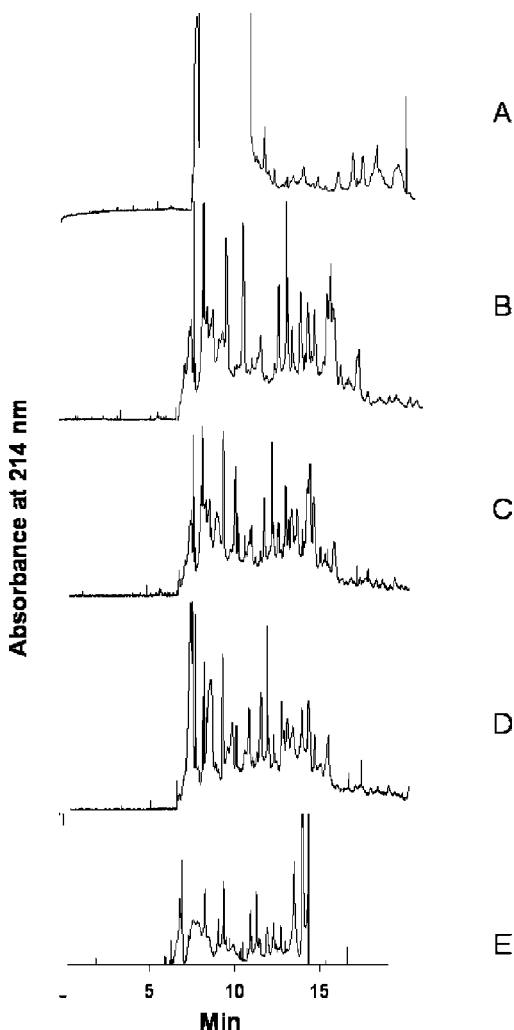[‡] Producción Vegetal.

Figure 1. Effect of aqueous solution on FZCE patterns of methanol-soluble proteins of paprika: (**A**) 70% (v/v) ethanol + 0.5% sodium acetate + 5% β-mercaptoethanol; (**B**) 30% (v/v) acetonitrile; (**C**) water; (**D**) 0.1 M sodium hydroxide; (**E**) 1.5% (v/v) orthophosphoric acid.

Figure 2. Effect of various run buffer concentrations on paprika protein separation.

method because it offers several advantages over PAGE, such as more rapid analysis, detection, and on-column quantification and increased efficiency and resolution (*11*, *12*). In addition, CE-based methods involve a simple extraction of proteins and small quantities of organic solvents in comparison with RP-HPLC. A mode of CE called free zone capillary electrophoresis (FZCE) has been applied to the analysis of complex protein systems such as foods (*13*−*16*). Numerous papers have reported the potential of FZCE to discriminate, or fingerprint, vegetable foodstuffs such as species of leguminous and cereal grain cultivars (*17*−*19*). Proteins of different fractions in cereals can be separated into 30−40 peaks and shoulders by FZCE in phosphate buffers, differentiating oat, rice, wheat, maize, and sorghum genotypes by analysis of the prolamin and gliadin fractions (*19*−*22*). In this way, the adulteration of such foodstuffs as durum wheat flour with flour from common wheat and locust bean gum with guar gum has been detected and estimated quantitatively by this technique (*23*, *24*). Thus, FZCE might be useful to detect adulteration in smoked paprika.

The aim of this work was to develop a procedure based on protein analysis by FZCE that can be used as an alternative to other methods, such as SDS-PAGE, in the determination of smoked paprika adulteration with other kinds of paprika.
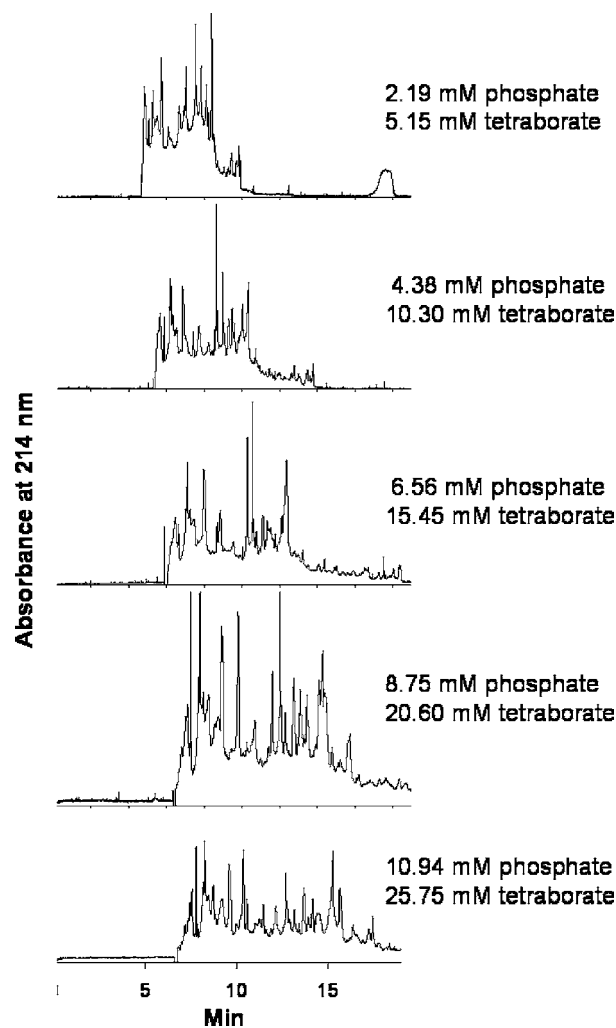
MATERIALS AND METHODS

Sample Collection. Pepper varieties of *C. annuum* L. used in this study were obtained from authenticated stocks held at the Registry of the Denomination of Origin "Pimentón de La Vera" (Jarandilla, Cáceres, Spain). Two autochthonous varieties of pepper, Jaranda and Bola, were smoke-dried for 15 days in a traditional dryer, and peppers of the nonautochthonous variety Papri Queen were air-dried in an industrial dryer. The varieties were separated into batches and taken to the processing plant to be ground into paprika. Paprika samples were collected on five different processing lines. A total of 15 samples of each batch (three samples from each processing line) were collected. The samples ($\approx$50 g) were put into plastic bags and kept under dry conditions until assay in the laboratory within 1−2 days after collection.

Extraction of Proteins for FZCE. Direct extraction of paprika samples with water may lead to components other than proteins being removed and, consequently, poor resolution of FZCE. In fact, these extracts may also contain compounds such as polysaccharides, polyphenols, tannins, DNA, free amino acids, and sugars that could all potentially bind to the inner walls of the silica capillaries (*25*). Hence, we performed a pre-extraction by mixing 1 g of paprika with methanol (3:10 w/v) for 10 min at room temperature. A longer extraction time did not improve the effectiveness of the extraction. The suspension was vortexed periodically and centrifuged at 5800*g* for 5 min, and the supernatant was collected. From an aliquot of 0.5 mL, methanol-soluble proteins were partially precipitated with chloroform (1:2 v/v) and centrifuged at 24000*g* for 5 min. The pellets were cleaned twice with chloroform, and the pigment-free pellets were collected. Finally, the pellets were suspended in 0.5 mL of a solution. The tested solutions were deionized water obtained with a Milli-Q water purification system
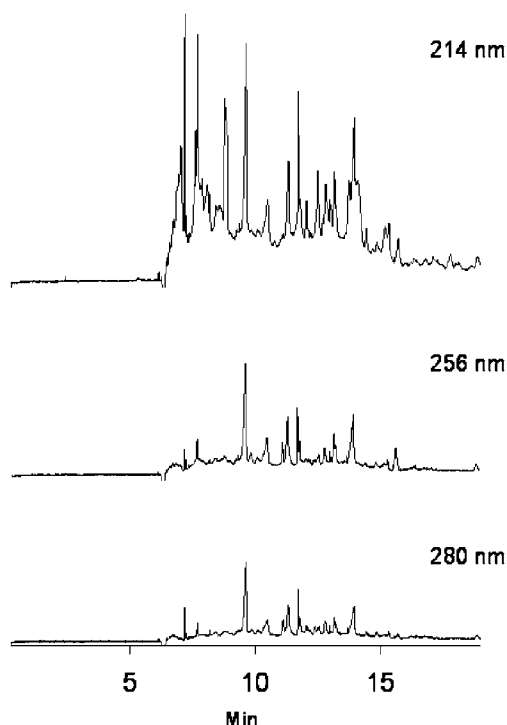
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Figure 3. Free capillary electrophoresis patterns at 214, 256, and 280 nm of paprika elaborated from the Jaranda pepper variety.

Table 1. Analytical Parameters and Peak Areas of Proteins Determined by FZCE under Optimized Conditions in the Paprika Batches Studied

| peak | migration time | | UAA[a] | | | P |
	CMT[b]	RDS %[c]	Bola	Jaranda	Papri Queen	
1	6.59	0.69	2475	2430	3229	0.535
2	7.05	0.71	2926	4992	1319	0.347
3	7.10	0.73	1002	1452	875	0.388
4	7.51	1.00	4085[1,2d]	7064[1]	2642[2]	0.015
5	8.25	0.68	591[2]	2316[1]	140[2]	0.001
6	8.63	0.59	5553[2]	5492[2]	32071[1]	0.000
7	9.10	0.53	5340	3598	4303	0.091
8	9.43	0.49	16656[2]	59959[1]	11703[2]	0.000
9	9.73	1.02	3695	4625	4500	0.752
10	10.10	0.97	5579	4647	6374	0.454
11	10.26	0.82	7885[2]	17787[1]	4900[2]	0.004
12	10.92	0.30	6801[1]	8831[1]	3158[2]	0.006
13	11.10	0.41	25068[2]	36199[2]	48046[1]	0.000
14	11.52	0.45	10276	15581	13666	0.134
15	11.62	0.54	2409[2]	5117[1]	5420[1]	0.006
16	11.72	0.71	1024	2426	2867	0.084
17	11.85	0.76	988[2]	167[2]	2498[1]	0.000
18	12.13	0.72	3039	3732	4445	0.769
19	12.22	0.73	893[2]	3873[1]	3526[1]	0.000
20	12.45	0.82	6895[2]	9669[2]	18825[1]	0.000
21	12.81	0.81	10816[1,2]	17829[1]	2762[2]	0.005
22	12.91	0.79	700[1,2]	1086[1]	nd[2e]	0.019
23	13.51	0.65	42644[1,2]	59173[1]	22298[2]	0.039
24	14.01	0.69	2407	2368	1986	0.164
25	14.33	0.89	2912	3017	2232	0.838
26	14.80	1.26	3111	3108	4451	0.317
27	15.16	1.02	3897[1,2]	7791[1]	1601[2]	0.013
28	16.29	0.82	1412[2]	987[2]	5608[1]	0.000

[a] UAA, arbitrary area units. [b] CMT, corrected migration time. [c] RDS %, relative standard deviation ($n = 5$). [d] For a given protein (row), values with different numbers are significantly different ($P < 0.05$). [e] nd, not detected.

(Millipore, Bedford, MA), an acidic solution [1.5% (v/v) orthophosphoric acid], a basic solution (0.1 M sodium hydroxide), 30% (v/v) acetonitrile as used to extract locust bean proteins (*23*), and 70% (v/v) ethanol + 0.5% sodium acetate + 5% β-mercaptoethanol as used to resuspend zein and kafirin fractions (*22*). For the last solution, protein denaturing was carried out at 99 °C for 5 min.

FZCE Analysis. The protein extracts were filtrated through a 0.2 μm filter and analyzed by FZCE. The separations were done on an automated PACE 5500 device (Beckman Instruments, Inc., Palo Alto, CA). To obtain fast, high-resolution, and reproducible separations, different FZCE buffer concentrations, capillary size, and detection wavelengths were tested. Buffers were prepared with HPLC-grade water obtained with a Milli-Q water purification system and consisted of phosphate/tetraborate at a nominal pH of 9. The buffer concentration was varied from 2.19 mM phosphate/5.15 mM tetraborate to 10.94 mM phosphate/25.75 mM tetraborate. Uncoated fused silica capillaries of 75 μm i.d. and 57 cm total length (50 cm to window detector) and 50 μm i.d. and 77 cm total length (70 cm to window detector) were tested (Supelco, Tecknocroma, Barcelona, Spain). The capillaries were initially conditioned with 100 mM NaOH for 10 min and then with deionized water for 5 min. The 50 μm i.d. capillaries were rinsed between separations for 3 min with 100 mM NaOH and for 3 min with deionized water and then with separation buffer for 2 min. For the 75 μm i.d. capillaries, rinse times were reduced to 2 min with 100 mM NaOH and 2 min with deionized water between runs and 2 min with separation buffer. When not in use, the capillaries were rinsed with 100 mM NaOH for 10 min, followed by water for 10 min, and finally by nitrogen gas for 10 min. The separation voltage was 263 V/cm (15 kV for the 75 μm i.d. capillaries and 20 kV for the 50 μm i.d. capillaries). The separation temperature was 23 °C because a low separation temperature (e.g., 20−30 °C) improves the FCZE protein separation (*22*, *26*). The six wavelengths tested for the monitoring of the analyses were 214, 256, and 280 nm. Samples were injected under pressure (0.5 psi) for 7 s with the 50 μm i.d. capillaries and for 5 s with the 75 μm i.d. capillaries. The protein spectra were monitored from 190 to 300 nm with a PACE diode array detector (Beckman Instruments, Inc.). Protein peaks were identified using corrected migration times and UV absorbance spectra. The Beckman P/ACE Station (version 1.21) software package was employed for storing, manipulating, and comparing the electropherograms.

SDS-PAGE Analysis. The proteins for SDS-PAGE were pre-extracted with methanol/chloroform as described above for FZCE. The pellets were mixed with 30 μL of PAGE loading buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.025% bromophenol blue) and incubated at 99 °C for 10 min for protein denaturing. The electrophoresis conditions were those described by Laemmli (*27*), and the concentrations of acrylamide (29:1 acrylamide/bisacrylamide) in the gels were 4% for stacking gels and 12% for separating gels. Gels were cast and run in a Miniprotean III device (Bio-Rad Laboratories, Richmond, CA). The molecular mass marker kits (Sigma Chemical Co., St. Louis, MO) contained proteins from 6.5 to 205 kDa. The gels were subsequently stained with 0.5% (w/v) Coomassie blue (G-250) dissolved in 45% water, 45% methanol, and 10% acetic acid for 30 min and destained with a solution consisting of 20% methanol, 10% acetic acid, and 70% distilled water for 4 h. A computer image analysis program (SynGene, Synoptics Ltd., Cambridge, U.K.) was used for the densitometric analysis of the gel electrophoreses.

Statistical Analysis. The cultivars were distinguished on the basis of differences in the peak areas of the FZCE electropherograms and SDS-PAGE densitograms. Peak areas were studied by one-way analysis of variance (ANOVA). The means were separated by Tukey's honestly significant difference test using the SPSS software package version 10.0.6 from SPSS Inc. (Chicago, IL).

With respect to the analysis of artificially adulterated samples, six adulteration levels (5, 10, 20, 40, 60, and 80%) were used. Five analyses were realized for each calibration point. The regression equations were calculated using the least-squares method. Confidence intervals ($P < 0.05$) were determined for each adulteration level.

RESULTS AND DISCUSSION

Optimization of FZCE Separation. Paprika samples from the Jaranda autochthonous pepper variety were used to optimize
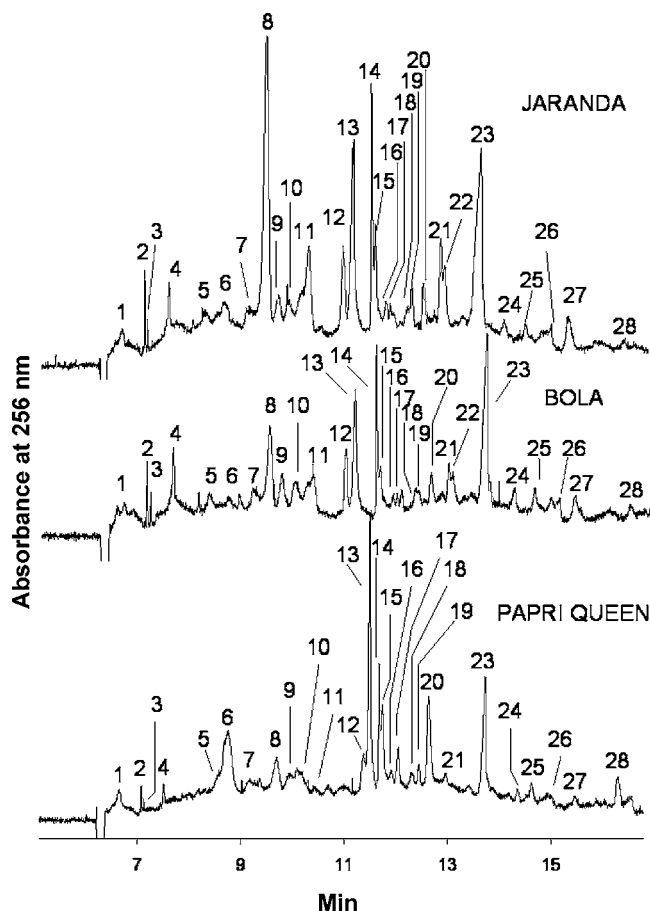
Figure 4. Electropherograms of methanol-soluble proteins of paprikas elaborated from the Jaranda, Bola, and Papri Queen pepper varieties.

Figure 5. Densitograms of methanol-soluble proteins of paprikas elaborated from the Jaranda, Bola, and Papri Queen pepper varieties.

the FZCE separation. The initial FZCE separations of the methanol-soluble proteins were carried out with a capillary of 75 μm i.d and 57 cm total length and 8.75 mM phosphate/20.6 mM tetraborate (pH 9) as separation buffer and monitored at 214 nm. This buffer concentration has been successfully used for the determination of locust bean and guar proteins by FZCE (*23*). We first tested the effect of the different solutions used to suspend the pellets of methanol-soluble proteins obtained after precipitation with chloroform. The results showed differences in the electropherograms obtained for the same sample suspended with the different solutions (**Figure 1**). The resolution and migration times for methanol-soluble proteins suspended in water, acidic and basic solutions, and 30% (v/v) acetonitrile were appropriate. The proteins suspended in these solutions were resolved in 20 min and separated into 30−40 peaks and shoulders. However, relatively small amounts of proteinaceous compounds were extracted from the pellets with water and extreme pH solutions. The analysis of this protein fraction suspended in the denaturing solution had poor resolution. On the basis of these results, 30% (v/v) acetonitrile was adopted as the suspending solution for routine analysis.

The effect of buffer concentration was next studied using the optimized sample extraction (**Figure 2**). Resolution of the proteins increased as the buffer concentration was increased from 2.19 mM phosphate/5.15 mM tetraborate to the initial concentration 8.75 mM phosphate/20.6 mM tetraborate. At a buffer concentration of 10.94 mM phosphate/25.75 mM tetraborate, the results were similar to the initial buffer concentration, but sensitivity was slightly lower. With respect to resolution, fast migration peaks increased slightly, particularly at 8−12 min. However, some of the most relevant slow
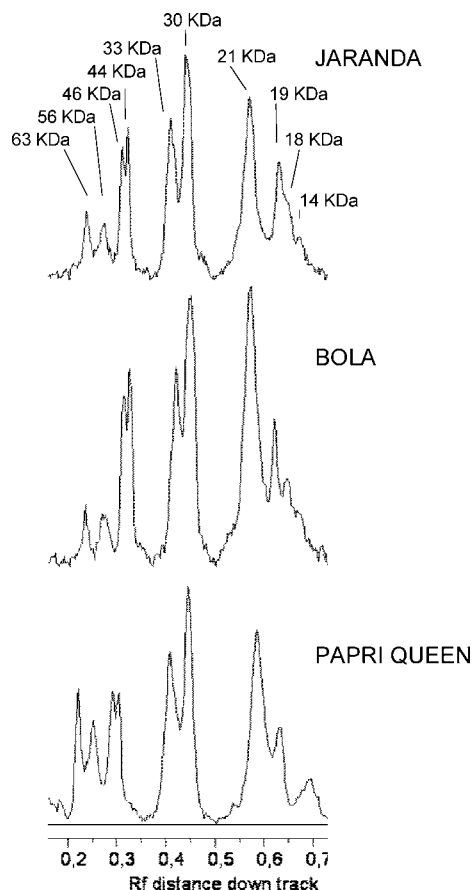
migration peaks had poorer resolution. Thus, the initial buffer concentration, 8.75 mM phosphate/20.6 mM tetraborate, was chosen as optimal for the FZCE separation of methanol-soluble proteins of the paprika samples.

Using these optimal separation conditions, the effect of increasing capillary diameter and length was examined. Separations in 50 μm i.d. and 77 cm total length capillaries were similar to those in 75 μm i.d. and 57 cm total length capillaries, but the larger inner diameter provided greater sensitivity. Bean et al. (*22*) obtained similar results for the FZCE separation of maize and sorghum prolamins when they compared capillaries of 25 and 50 μm i.d. Thus, 75 μm i.d. capillaries were used for the rest of the study.

Under the optimized conditions, attempts to improve the FZCE analysis of the methanol-soluble proteins were made by monitoring peaks at 256 and 280 nm (**Figure 3**). Although some peaks of proteinaceous compounds seen at 214 nm were not detected, the protein profiles were clearer at both of the tested wavelengths. The electropherograms obtained at 256 nm had a profile similar to that of the same samples monitored at 280 nm, but a greater sensitivity. Thus, the optimal wavelength was taken to be 256 nm for the analysis of methanol-soluble proteins of paprika.

For the determination of the analytical parameters, a negative acetonitrile peak was used to normalize peak areas and to calculate the corrected migration times (CMT) of the peaks. The optimized FZCE method was found to give excellent repeatability of the CMT with coefficients of variation (RSD %; $n = 5$) of <1% for most of the proteinaceous compounds analyzed (**Table 1**).

FZCE and SDS-PAGE Comparison. To explore the potential of the FZCE method applied to paprika variety discrimi-

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Table 2. Peak Areas of Proteins Determined by SDS-PAGE in the Paprika Batches Studied[a]

approx MW	Bola	Jaranda	Papri Queen	*P*
6300	1983	2305	2756	0.518
5600	1867	2210	2546	0.623
4600	2981	3745	3313	0.780
4400	2780	3856	3094	0.679
3300	4618	4313	4789	0.913
3000	6024	6308	6637	0.939
2100	4212	5423	4779	0.756
1900	2483	3226	2726	0.775
1800	938[1,2a]	1986[1]	nd[2b]	0.039
1400	520[2]	1248[1,2]	2380[1]	0.007

[a] For a given protein (row), values with different numbers are significantly different ($P < 0.05$). [b] nd, not detected.

nation, the intravariety differences were evaluated and compared with the SDS-PAGE technique.

The electropherograms obtained by FZCE under the optimized conditions had a total of 28 peaks for all of the paprika varieties analyzed (**Figure 4**). As seen in **Table 1**, qualitative and quantitative differences were detected by comparing the electropherograms of the paprika batches. The analysis of methanol-soluble proteins from the autochthonous varieties, Bola and Jaranda, showed all of the peaks, whereas peak 22 was not detected in the analysis of Papri Queen samples. Likewise, the areas of peaks 5, 8, and 11 were greater in the Jaranda variety samples, whereas peaks 15 and 19 were significantly lower in the Bola variety samples. Peaks 6, 12, 13, 17, 20, and 28 showed significant differences between batches of autochthonous varieties and Papri Queen variety (**Table 1**). These results showed that the optimized FZCE method is able to discriminate paprika elaborated with the varieties of pepper studied.

With respect to SDS-PAGE, the densitograms showed a total of 10 polypeptide bands with an approximate molecular mass range of 14–63 kDa (**Figure 5**). There were great similarities in the overall polypeptide profiles of the methanol-soluble proteins from the three varieties studied. However, the analytical system revealed moderate differences between batches (**Table 2**). The Jaranda and Bola varieties could be distinguished from Papri Queen by the presence of a polypeptide band with a molecular mass of 18 kDa. The 14 kDa band was significantly lower in the Bola variety than in the Papri Queen variety.
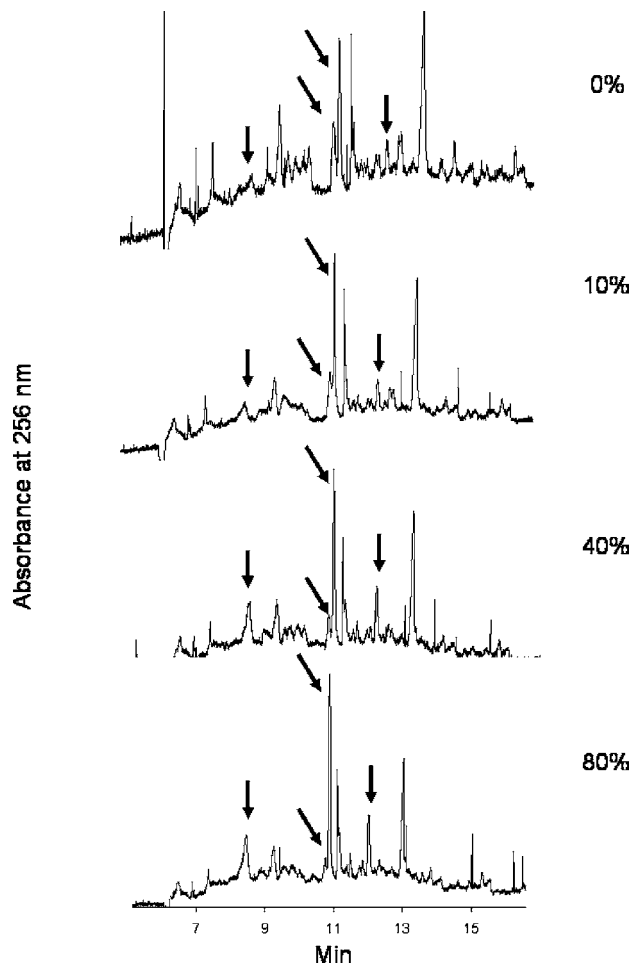

Figure 6. FZCE profiles illustrating the effect of smoked paprika adulteration (Bola variety) with paprika elaborated from the Papri Queen variety. Electropherograms: no adulteration; 10, 40, and 80% adulteration (w/w). The arrows indicate the diagnostic peaks for the determination of smoked paprika adulteration.

Therefore, SDS-PAGE might be a useful tool for identifying paprika obtained from a single pepper variety. However, the differences found with this technique permitted only the detection of severe adulteration of autochthonous smoked

Table 3. Analytical Parameters of Diagnostic Peaks for the Determination of Smoked Paprika Adulteration with the Papri Queen Variety

variety	peak	regression equation parameters[a]				detection limit (%)
		working range (%)	*a*	*b*	*R*[2]	
Jaranda–Papri Queen	6	5–80	384.68	2121	0.993	10
	12	10–80	−53.644	7262	0.920	40
	13	10–80	162.15	33550	0.950	20
	ratio 12/13	5–80	0.1306	4.047	0.963	10
	20	10–80	136.15	5907	0.973	20
Bola–Papri Queen	6	5–80	357.53	2115	0.999	5
	12	5–80	−54.416	5906	0.985	20
	13	10–80	224.8	23288	0.955	20
	ratio 12/13	5–80	0.2066	1.515	0.979	10
	20	5–80	55.51	13138	0.939	40
Jaranda/Bola (1:1)–Papri Queen	6	5–80	373.55	2062	0.996	10
	12	10–80	−49.938	6801	0.948	40
	13	10–80	208.36	28172	0.946	20
	ratio 12/13	5–80	0.1606	2.499	0.991	10
	20	10–80	89.23	9547	0.937	40

[a] Regression equation: $A = ax + b$, where A is the peak area, a is the slope, x is the percentage of adulteration, and b is the intercept.

paprika with paprika from the Papri Queen variety and was inappropriate for the purposes of the present study (data not shown).

Detection of Smoked Paprika Adulteration by FZCE. In view of the relative concentrations of the different proteinaceous compounds found in individual batches as given in **Table 1** and of the resolution of peaks shown in **Figure 4**, peaks 6, 12, 13, and 20 were selected as potential diagnostic peaks to determine the level of smoked paprika adulteration. The ratio of peaks 12 and 13 was also used as a marker to calculate the level of adulteration. The effect of smoked paprika adulteration with paprika obtained from Papri Queen variety on the electropherogram is illustrated in **Figure 6**. The areas of the diagnostic peaks 6, 13, and 20 increased, although the area of peak 12 decreased. The linear relationship between the areas of the aforementioned peaks and the adulteration level of autochthonous smoked paprika with paprika from the Papri Queen variety can be seen in **Table 3**. The FZCE method was found to give adulteration detection limits varying from 5 to 40% of the Papri Queen variety with correlation coefficients of >0.90, within a satisfactory working range of mixture (5−80 or 10−80%) sufficiently large to cover the adulteration levels of interest. In particular, for peaks 12, 13, and 20 the adulteration detection limit was 20−40% (w/w), with correlation coefficients of 0.920−0.985, 0.946−0.955, and 0.937−0.973, respectively. For the ratio of peaks 12 and 13, adulteration was detectable at 10% (w/w) with correlation coefficients from 0.963 to 0.991. However, peak 6 was found to be the best marker for determining the adulteration of smoked paprika with the Papri Queen variety, with adulteration detection limits of 5% (w/w) for the Bola variety and 10% (w/w) for the Jaranda variety and the mixture of both autochthonous varieties and excellent correlation coefficients of >0.99.

In conclusion, the present protocol of soluble-methanol protein extraction from paprika and the FZCE analysis procedure provides a fast, economic, and efficient method for the detection of smoked paprika adulteration with paprika elaborated from peppers of the Papri Queen variety. We have therefore proposed this technique to be used routinely for the quality control of "Pimentón de La Vera" smoked paprika. Further work is in progress to study the use of this FZCE method for the detection of smoked paprika adulteration with paprika elaborated from other emerging pepper varieties such as Sonora or Papri King.

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