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Application of temperature-induced phase partition of proteins for the detection of smoked paprika adulteration by free zone capillary electrophoresis (FZCE)

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Abstract

A procedure for protein extraction was developed for use in the determination, by free zone capillary electrophoresis (FZCE), of smoked paprika "Pimentón de La Vera" adulteration with paprika elaborated from varieties of pepper foreign to the "La Vera" region, in the centre-west of Spain. Two autochthonous varieties of pepper, Jaranda and Bola, and the varieties Papri Queen, Papri King and Sonora, foreign to the "La Vera" region, were used in the study. Several Tris–HCl buffer concentrations and pH values were tested for the extraction of the hydrophilic and hydrophobic protein fractions obtained by temperature-induced phase partition with Triton X-114. On the basis of the results, 0.5 mM Tris–HCl buffer, pH 7.4, with 150 mM sodium chloride, was adopted as the optimal extraction buffer. Five peaks found in the FZCE electropherograms of the hydrophilic protein fraction were investigated as a basis for detecting and estimating the adulteration of smoked paprika. The adulteration detection limits varied from 10% to 40% of paprika elaborated from foreign varieties within a satisfactory working range of admixture (5–80%) sufficiently large to cover the adulteration levels of interest. In addition, a peak of this fraction was identified as a marker for the smoke-drying process. With respect to the hydrophobic proteins, the use of the peak denominated M and the ratio of peaks M and K as markers for determining adulteration gave the best results, with an adulteration detection limit of 5% (w/w), and correlation coefficients greater than 0.965.
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Keywords: Paprika; Adulteration; Temperature-induced phase partition; FZCE

1. Introduction

Smoked paprika "Pimentón de La Vera" is a high quality product obtained by drying the fruit of autochthonous varieties of pepper (*Capsicum annuum* L.) from the region of "La Vera" in the centre-west of Spain (Lozano & Montero de Espinosa, 1999). Oak logs are burnt 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 flavour 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 is the variety foreign to the "La Vera" region most frequently used in the adulteration of smoked paprika, although other emerging pepper varieties, such as Sonora or Papri King, are also used.

Free zone capillary electrophoresis (FZCE), to analyze protein patterns, is recognized as a fast, economic, and efficient method for the detection of adulteration in several

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foodstuffs, including smoked paprika (Bonetti et al., 2004; Flurer, Crowe, & Wolnik, 2000; Hernández, Martín, Aranda, Bartolomé, & Córdoba, 2006). By means of FZCE analysis of the methanol-soluble proteins, the adulteration detection limit of smoked paprika with the Papri Queen variety is 5% (w/w) for the Bola variety (Hernández et al., 2006). However, for the Jaranda variety and the mixture of Bola and Jaranda varieties, the detection limits are 10% (w/w). It would be interesting to lower this latter detection limit, since most marketed smoked paprika "Pimentón de La Vera" is a mixture of these two autochthonous varieties. Adulteration with other foreign varieties, such as Papri King and Sonora, also needs to be tested.

To overcome these problems, there is a need to develop a new protein extraction protocol aimed at obtaining adequate FZCE profiles for the detection of the lower limits of commercial smoked paprika adulteration. General procedures for the extraction of plant proteins for analysis by electrophoresis involve the evaluation and modification of existing methods. In this sense, the Tris–HCl extraction protocols, and the classical trichloroacetic acid/acetone precipitation of total proteins, are among the methods most successfully used for plant materials, including peppers (Anu & Peter, 2003; Carpentier et al., 2005; Granier, 1988; Kannamkumarath, Wrobel, & Wuilloud, 2005; Odeigah, Oboh, & Aghalokpe, 1999; Song, Braun, Bevis, & Doncaster, 2006; Zukas & Breksa, 2005).

Polyoxyethylene-type non-ionic detergents can be used in the solubilization and purification of plant proteins, owing to their ability to produce aqueous two-phase system (ATPS) in the biocompatible temperature range (Balasubramaniam, Wilkinson, Van Cott, & Zhang, 2003; Platis & Labrou, 2006; Vaidya, Suthar, Kasture, & Nene, 2006). The use of micelle/polymer systems offers unique possibilities for the extraction of hydrophobic proteins, due to the formation of two-phase systems at low temperatures with many commonly used non-ionic detergents. Phase separation in detergent/polymer/water mixtures has been studied for a number of systems. Triton X-114 is probably the most widely used and certainly one of the best characterized of commercially available polydisperse compounds (Bordier, 1981; Sánchez-Ferrer, Pérez-Gilabert, Núñez, Bru, & García-Carmona, 1994; Sivars & Tjerneld, 2000; Wissing, Heim, Flohé, Bilitewski, & Frank, 2000).

The aim of the present work was to develop a simple procedure for protein extraction from paprika, based on temperature-induced phase partition with Triton X-114, that allows high sensitivity in the determination of smoked paprika adulteration with foreign paprika by FZCE analysis.

2. Materials and methods

2.1. Sample collection

Pepper varieties of *Capsicum 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 non-autochthonous varieties Papri Queen, Papri King and Sonora, were air-dried in an industrial dryer. Additionally, a batch of Bola peppers was also air-dried to study the effect of the drying process on the protein fractions. The varieties were separated into batches, and taken to the processing plant to be milled into paprika. Paprika samples were collected from five different processing lines. A total of fifteen samples of each batch (three samples from each processing line) were collected. The samples (50 g approximately) were put into plastic bags, and kept under dry conditions prior to assay in the laboratory within 1–2 days after collection.

2.2. Extraction of proteins by temperature-induced phase partitioning in Triton X-114

The two-phase system containing detergent/polymer/water for in situ solubilization was based on the protocol described by Wissing et al. (2000). The paprika samples were dissolved (1:10 w/v) in a Tris–HCl buffer with 150 mM sodium chloride. In order to obtain high-protein extraction yields, different Tris–HCl concentrations (100 mM, 50 mM, and 10 mM) and pH's (4.5, 6.5, 7.4, 9, and 11) were tested. The samples were mixed for 3 min and the unsolubilized material was removed by ultracentrifugation at 5800*g* for 2 min. A longer extraction time did not improve the effectiveness of the extraction. The supernatant was filtered before the addition of Triton X-114 to a final concentration of 5% (w/v) under refrigerated conditions. The two-phase system was briefly vortexed and incubated for 10 min at 30 °C by gentle inversion. The two phases were separated by centrifuging at 24,000*g* for 1 min. Both the detergent-depleted (hydrophilic fraction) and detergent-enriched (hydrophobic fraction) phases were cleansed and then analyzed by FZCE.

2.3. FZCE analysis

Both the hydrophilic and hydrophobic protein fractions were filtered through a 0.2 μm filter after addition of acetonitrile to a final concentration of 30% (v/v), and assayed by FZCE. The separations were done on an automated PACE 5500 device (Beckman Instrument, Inc., Palo Alto, CA, USA). Buffers were prepared with HPLC-grade water obtained with a Milli-Q water purification system, and consisted of 8.75 mM phosphate 20.6 mM tetraborate at a nominal pH of 9 (Hernández et al., 2006). Uncoated fused silica capillaries of 75 μm i.d. and 57 cm total length (50 cm to window detector) were used (Supelco, Tecknocroma, Barcelona, Spain). The capillaries were initially conditioned with 100 mM NaOH for 10 min, and then with deionized water for 5 min. They were rinsed between separations for 2 min with 100 mM NaOH, for 2 min with

deionized water, and then with separation buffer for 2 min. When not in use, the capillaries were rinsed with 100 mM NaOH for 10 min, followed by water for 10 min, and finally dried by nitrogen gas for 10 min. The separation voltage was 263 V/cm (15 kV) and the separation temperature was 23 °C. The wavelength used to monitor the assays was 256 nm. This wavelength has been successfully used for the determination of methanol-soluble proteins of paprika by FZCE (Hernández et al., 2006). Samples were injected under pressure (0.5 psi) for 5 s and the protein spectra were monitored from 190 to 300 nm with a PACE diode array detector (Beckman Instrument Inc., Palo Alto, CA, USA). 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. Protein peaks were identified using corrected migration times and UV absorbance spectra. The Beckman P/ACE Station (Version 1.21) software package was used to store, manipulate, and compare the electropherograms.

2.4. Statistical analysis

The batches of paprika were distinguished on the basis of differences in the peak areas of the FZCE electropherograms. Peak areas were studied by one-way analysis of variance (ANOVA). The means were separated by Tukey's honest significant difference test using the SPSS software package vers. 10.0.6 from SPSS Inc. (Chicago, IL, USA).

With respect to the analysis of artificially adulterated samples, six adulteration levels (5%, 10%, 20%, 40%, 60%, and 80%) were used. Five analyses were made 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.

3. Results and discussion

3.1. Optimization of protein extraction for FZCE analysis

Smoked paprika samples from the Jaranda autochthonous pepper variety were used to optimize the protein extraction protocol. The initial extractions of the hydrophilic proteins were carried out with the buffers adjusted to pH 7.4. Similar pH values are used in general procedures for the extraction of plant proteins for analysis by electrophoresis, including seeds of peppers (Anu & Peter, 2003; Odeigah et al., 1999). We first tested the effect of the different buffer concentrations used for the hydrophilic protein extraction. The results did not show any relevant differences in the hydrophilic protein profiles obtained by FZCE analysis for the same sample extracted with the different concentration buffers (Fig. 1). The resolution and migration times for these proteins were appropriate, being resolved in 20 min and separated into 22 peaks and shoulders. These results were somewhat surprising, since additional steps are often used to remove such compounds as

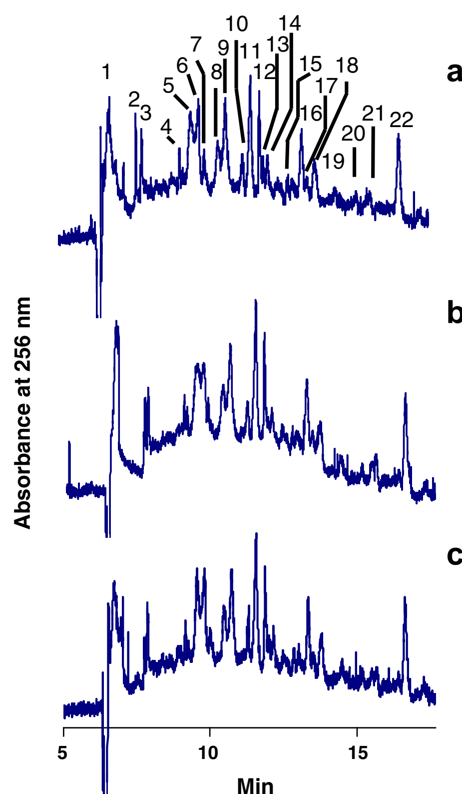

Fig. 1. Effect of buffer concentration on FZCE patterns of the hydrophilic protein fraction of paprika: (a) 10 mM Tris–HCl buffer with 150 mM sodium chloride, (b) 50 mM Tris–HCl buffer with 150 mM sodium chloride, and (c) 100 mM Tris–HCl buffer with 150 mM sodium chloride.

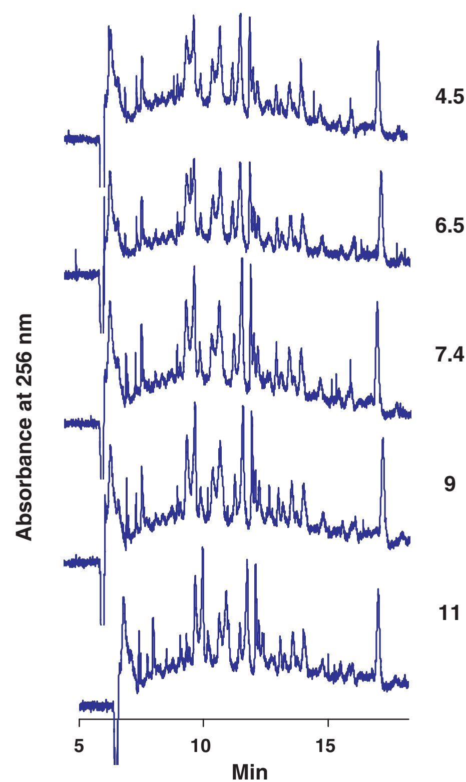

Fig. 2. Effect of varying the extraction buffer pH on the paprika protein concentration: pH's 4.5, 6.5, 7.4, 9, and 11.

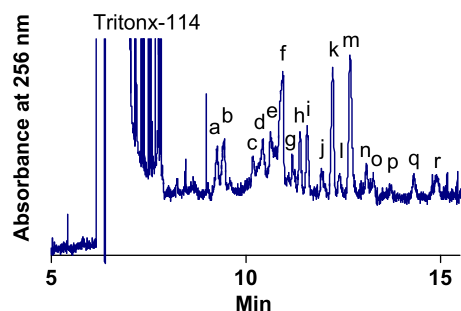

Fig. 3. Free capillary electrophoresis pattern of the hydrophobic protein fraction of smoked paprika elaborated from the Jaranda pepper variety.

polysaccharides, polyphenols, tannins, DNA, free amino acids and sugars, that could lead to a poor FZCE resolution of plant proteins (Bean & Lookhart, 1998; Cifuentes, 2006; Frazier & Papadopoulou, 2003; Manabe, 1999). On the basis of these results, the middle buffer concentration, 50 mM Tris–HCl buffer with 150 mM sodium chloride, was chosen as optimal for the extraction of the hydrophilic proteins of the paprika samples.

The effect of pH was next studied using the selected buffer concentration for sample extraction (Fig. 2). The amount of the hydrophilic proteins extracted seemed to increase as the buffer pH was increased from 4.5 to 7.4. At buffer pHs of 9 and 11, the results were similar to pH 7.4, so that this value was chosen as optimal for the extraction of the hydrophilic proteins of the paprika samples. This is in accordance with the results of Hernández et al. (2006) for the suspension of the methanol-soluble proteins of paprika.

The effect of buffer concentration and pH was also examined for the extraction of the hydrophobic protein fraction. It was found that the profiles of this protein fraction were similar for all the extraction conditions tested (data not shown). The FZCE profiles of the Jaranda samples had 18 peaks, with a migration time range of 8–16 min (Fig. 3). The peak corresponding to Triton X-114 was resolved between 6 and 8 min. Thus, 0.5 mM Tris–HCl pH 7.4 with 150 mM sodium chloride was used as extraction buffer for the rest of the study.

3.2. FZCE analysis of paprika elaborated with different drying processes

The effect of the drying process on the hydrophilic protein fraction was first studied using the smoke-dried and air-dried samples of paprika elaborated from peppers of the Bola variety. Peaks 2, 3, 4, 8, 9, 11, 13, 16, and 22 showed relevant differences between batches of smoked and non-smoked paprika (Fig. 4). In particular, peak 22 showed the greatest differences as it was absent from the profiles of the air-dried samples. This peak showed the maximum absorbance at 250 nm and could correspond to compounds related to the smoke, such as phenol derivatives, carbonyls, organic acids and their esters, lactones, pyrazines, pyroles, and furan derivatives (Simon, de la Calle, Palme, Meier, & Anklam, 2005). Hence, this peak may be used as a marker for the smoke-dried process.

With respect to the FZCE analysis of the hydrophobic proteins, minor differences were found in peaks K and M between smoke-dried and air-dried samples (Fig. 4).

3.3. FZCE analysis of paprika elaborated from pure pepper varieties

In order to explore the potential of the FZCE profiles of the two protein fractions applied to paprika variety dis-

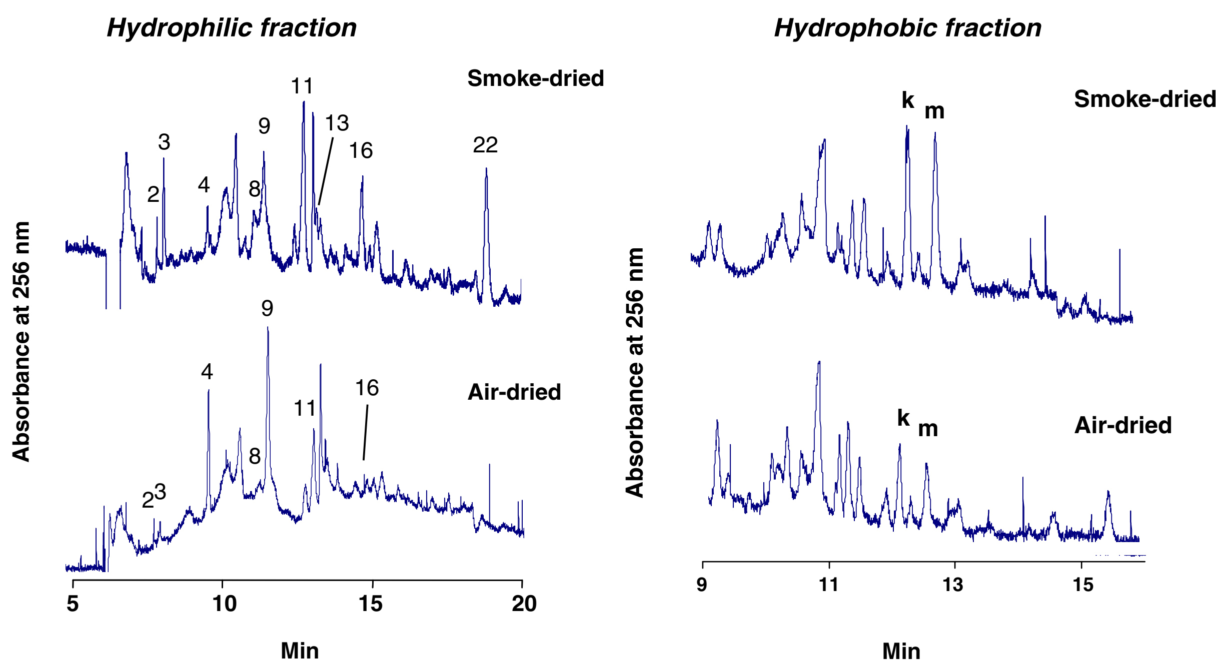

Fig. 4. Effect of drying method on the profile of the hydrophilic and hydrophobic protein fractions of paprikas elaborated from the Bola pepper varieties.

Table 1
Analytical parameters and peak areas of hydrophobic proteins with significant differences in the paprika batches studied[a]

Peaks	Migration time		Autochthonous varieties		Foreign varieties			P
	CMT[b]	RDS %[c]	Bola	Jaranda	Papri Queen	Papri King	Sonora	
Differences by drying								
P2	7.74	0.57	4430[1d]	4473[1]	2876[2]	2547[2]	2688[2]	0.045
P3	7.96	0.84	6269[2]	9154[1]	3458[3]	3587[3]	3541[3]	0.000
P4	9.11	0.38	2948[2]	2861[2]	5661[1]	3851[1,2]	5427[1]	0.001
P8	10.44	1.31	9609[1]	11073[1]	4219[2]	2568[2]	3841[2]	0.000
P9	10.92	1.01	20464[2]	23783[1,2]	27937[1]	30540[1]	30254[1]	0.042
P13	12.27	0.87	3046[2]	4215[1]	n.d.[3e]	n.d.[3]	n.d.[3]	0.000
P22	17.08	1.01	27613[1]	31319[1]	n.d.[2]	n.d.[2]	n.d.[2]	0.000
Differences by variety								
P10	11.46	0.81	7114[1]	8534[1]	2875[2]	2542[2]	2457[2]	0.000
P11	11.75	1.02	22856[2,3]	20694[2,3]	31483[1]	24547[2]	15800[3]	0.000
P12	12.13	0.75	11368[1]	13333[1]	8255[2]	5243[3]	6995[2,3]	0.000
P16	13.66	0.84	12868[3]	12647[3]	29932[1]	30504[1]	22147[2]	0.000
P18	14.14	0.89	12372[1]	14672[1]	5215[2]	5032[2]	6542[2]	0.000

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

crimination, intra-variety differences were evaluated and compared.

In the case of the hydrophilic proteins, qualitative and quantitative differences were detected by comparing the electropherograms of the paprika batches, especially between autochthonous and foreign varieties (Table 1). Part of these differences can be explained by the drying process, as was described above. The analysis of the hydrophilic proteins from the autochthonous varieties, Bola and Jaranda, showed all the peaks, while peaks 13 and 22 were not detected in the Papri Queen, Papri King or Sonora samples (Fig. 5). Likewise, peaks 2, 3, 8, 10, 12, 16, and 18 showed significant differences between batches of autochthonous and foreign varieties. Between the

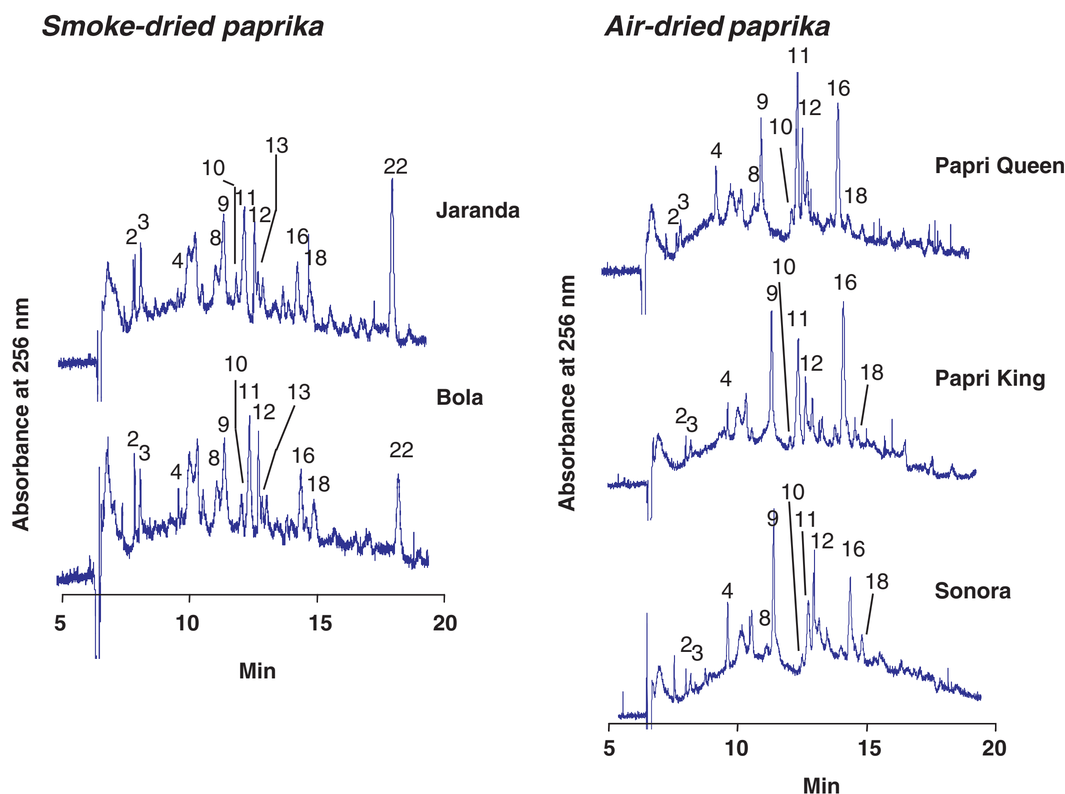

Fig. 5. Electropherograms of the hydrophilic protein fraction of the paprika batches studied.

Table 2
Analytical parameters and peak areas of hydrophobic proteins with significant differences in the paprika batches studied[a]

Peaks	Migration time		Autochthonous varieties		Foreign varieties			P
	CMT[b]	RDS %[c]	Bola	Jaranda	Papri Queen	Papri King	Sonora	
Differences by variety								
D	10.80	0.90	3818[2d]	4428[2]	6502[1]	6674[1]	7481[1]	0.027
G	10.97	0.92	3547[1]	3254[1,2]	1974[1,2]	985[2]	1854[1,2]	0.045
H	11.67	1.07	7700[1]	7634[1]	1847[2e]	1254[2]	1521[2]	0.000
J	12.47	0.89	3632[2]	2855[2]	8054[1]	6547[1]	3687[2]	0.000
K	12.64	0.78	19880[1]	22718[1]	1522[2]	2217[2]	2104[2]	0.000
L	12.80	1.16	2980[2]	3165[2]	5951[1]	4581[1,2]	4268[1,2]	0.041
M	13.09	1.14	32659[2]	32075[2]	89526[1]	81004[1]	82798[1]	0.000

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

Jaranda and Bola varieties, the areas of peaks 3 and 13 were greater in the Jaranda variety samples. Only peaks 11, 12, and 16 showed relevant differences between paprika samples of foreign varieties (Table 1).

Likewise, the overall profiles of the hydrophobic proteins of the two autochthonous varieties were very similar. In fact, no significant difference was found between Bola and Jaranda (Table 2; Fig. 6). Also, these profiles showed relevant differences with respect to the foreign samples. The Jaranda and Bola varieties could be distinguished from Papri Queen, Papri King, and Sonora varieties by the significant differences in peaks D, H, K, and M.

These results showed that the FZCE analysis of the hydrophilic and hydrophobic protein fractions is able to discriminate the paprika batches studied. The differences found in the FZCE profiles of the two fractions may be appropriate for studying low adulteration levels of autochthonous smoked paprika.

3.4. Detection of smoked paprika adulteration

In view of the relative concentrations of the different hydrophilic proteinaceous compounds found in individual batches, as given in Table 1, and of the resolution of peaks shown in Fig. 4, peaks 16 and 22 were selected as potential diagnostic peaks to determine the level of smoked paprika adulteration. The ratio of peaks 9/8 and 16/18 were also used as markers to calculate the level of adulteration.

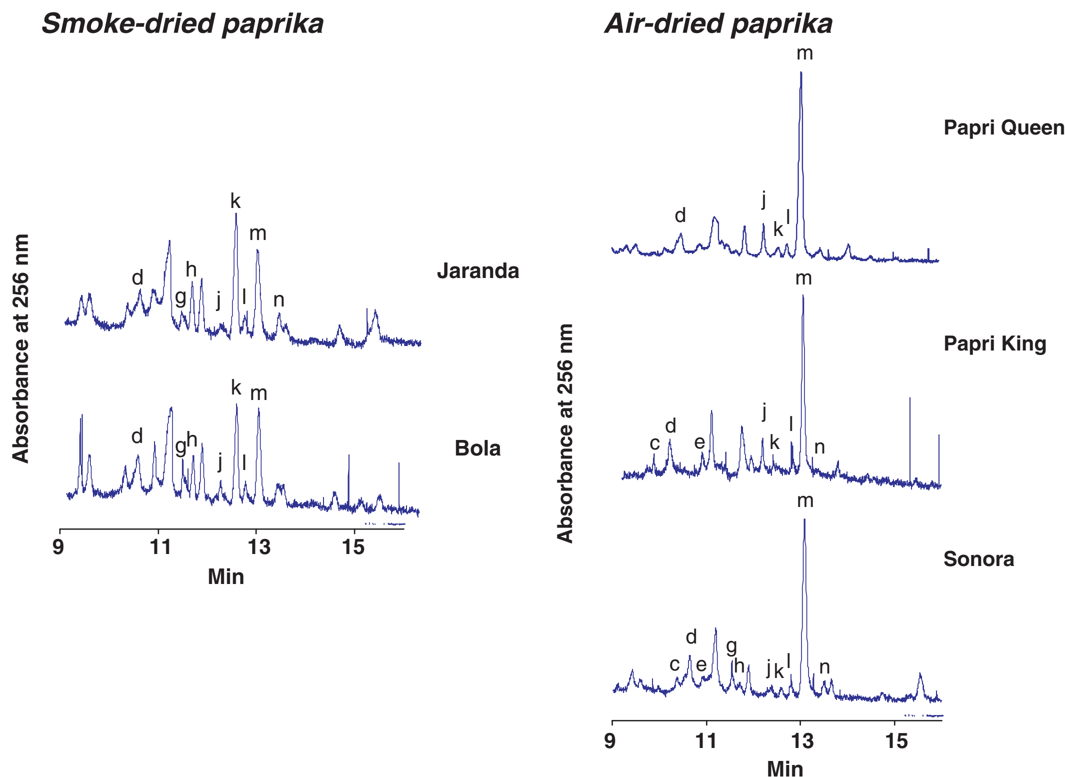

Fig. 6. Electropherograms of the hydrophobic protein fraction of the paprika batches studied.

The effect of smoked paprika adulteration with a mixture of paprikas, obtained from Papri Queen, Papri King, and Sonora varieties, on the electropherogram of hydrophilic proteins is illustrated in Fig. 7. The area of peak 16 and the ratios of peaks 9/8 and 16/18 increased, whereas the area of peak 22 decreased. The linear relationship between the areas of the aforementioned peaks and the adulteration level of autochthonous smoked paprika with paprika from the foreign varieties can be seen in Table 3. The FZCE analysis of this protein fraction was found to give adulteration detection limits varying from 10% to 40% of foreign paprika with correlation coefficients greater than 0.90,

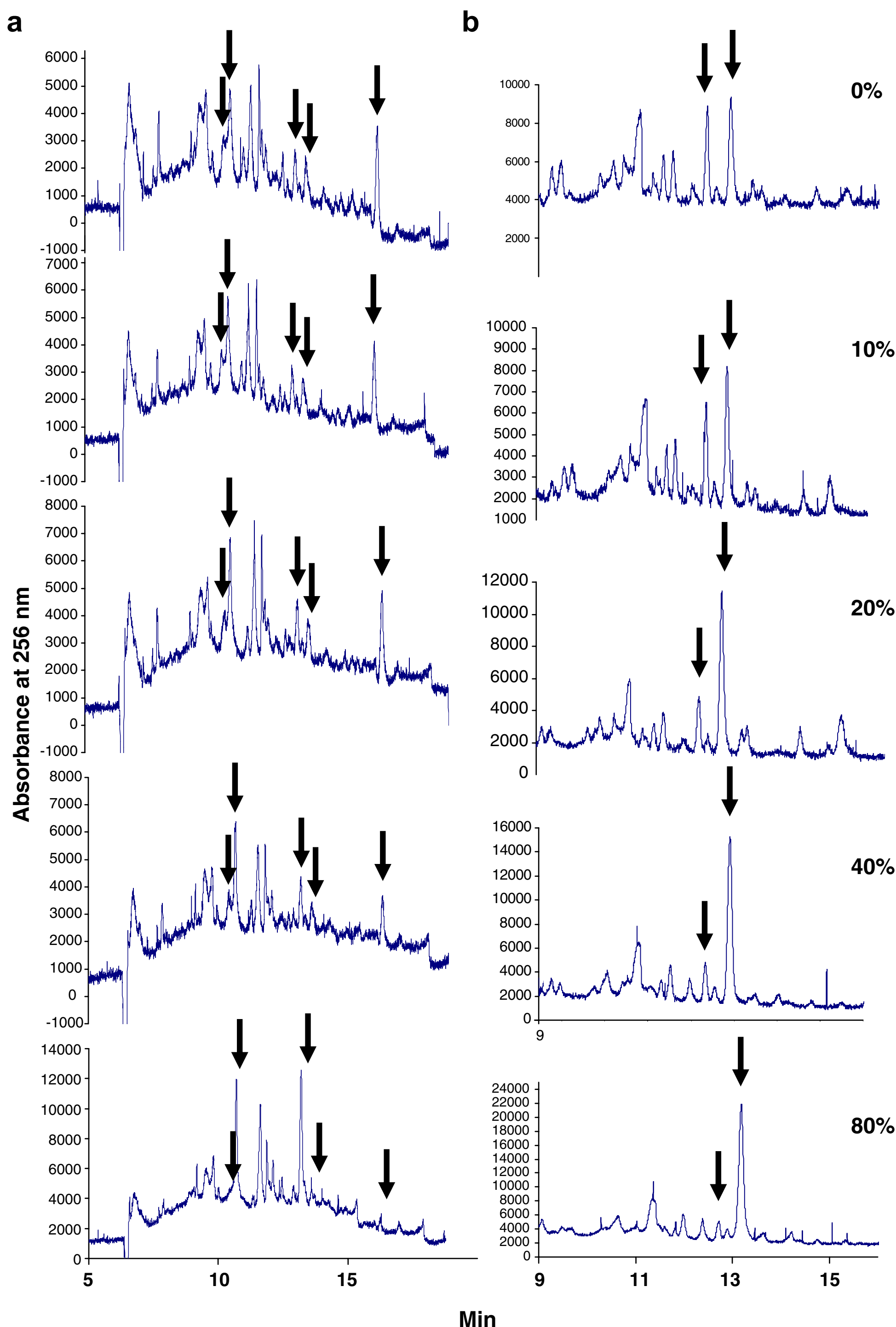

Fig. 7. FZCE profiles illustrating the effect of adulteration of smoked paprika elaborated with Bola and Jaranda varieties (1:1) with paprika elaborated from a mixture of Papri Queen, Papri King and Sonora varieties (1:1:1). (a) Electropherograms of the hydrophilic protein fraction. (b) Electropherograms of the hydrophobic protein fraction: no adulteration, 10%, 20%, 40%, and 80% of adulteration (w/w). The arrows indicate the diagnostic peaks for the determination of smoked paprika adulteration.

Table 3
Analytical parameters of diagnostic peaks for the determination of smoked paprika adulteration with paprika elaborated from the foreign varieties

Varieties	Peaks	Regression equation parameters[a]				Detection limit (%)
		Working range (%)	a	b	R^2	
Hydrophilic fraction						
Jaranda-foreign mixture[b]	Ratio 9/8	5–80	1.444	0.730	0.959	20
	16	5–80	3258	8199	0.945	10
	Ratio 16/18	5–80	0.585	0.069	0.900	20
	22	5–80	−4625	31461	0.991	10
Bola-foreign mixture	Ratio 9/8	5–80	1.636	1.141	0.957	20
	16	5–80	3718	11358	0.924	10
	Ratio 16/18	5–80	0.832	0.613	0.927	20
	22	5–80	−6535	42877	0.963	10
Jaranda/Bola (1:1)-foreign mixture	Ratio 9/8	5–80	1.526	0.906	0.962	40
	16	5–80	3647	10714	0.934	10
	Ratio 16/18	5–80	0.690	0.302	0.905	40
	22	5–80	−5444	36354	0.987	10
Hydrophobic fraction						
Jaranda-foreign mixture	K	5–80	−4594	32295	0.971	5
	M	5–80	20594	−5267	0.979	5
	Ratio M/K	5–80	2.170	−2.538	0.985	5
Bola-foreign mixture	K	5–80	−1779	15967	0.940	10
	M	5–80	22682	−1969	0.975	5
	Ratio M/K	5–80	4.353	−5.003	0.978	5
Jaranda/Bola (1:1)-foreign mixture	K	5–80	−3278	24716	0.961	10
	M	5–80	21160	−3158	0.966	5
	Ratio M/K	5–80	3.714	−5.141	0.974	5

[a] Regression equation: $A = ax + b$, where A is the peak area or ratio, a is the slope, x is the percentage of adulteration, and b is the intercept.
[b] Foreign mixture: Papri Queen, Papri King, and Sonora (1:1:1).

within a satisfactory working range of admixture (5–80%). In particular, the area of peak 22 was found to be the best marker in the hydrophilic proteins for determining the adulteration of smoked paprika, with an adulteration detection limit of 10% (w/w) for the Bola variety, the Jaranda variety, and the mixture the two, with correlation coefficients greater than 0.963.

With respect to the hydrophobic proteins, the results for the peaks and ratios selected as markers (K, M, and M/K) are also listed in Table 3. With these diagnostic peaks, we were able to detect adulteration levels of 5% (w/w) in most of the cases studied, the correlation coefficients being greater than 0.940. In particular, for peak K, the adulteration detection limit was 5–10% (w/w), with correlation coefficients of 0.940–0.971. However, peak M and the ratio of peaks M and K were found to be the best markers for determining the adulteration of smoked paprika with the foreign varieties, showing an adulteration detection limit of 5% (w/w) for the Bola and the Jaranda varieties and the mixture of the two, and correlation coefficients greater than 0.965.

4. Conclusion

The present protocol for the extraction of proteins from paprika by temperature-induced phase partition with Triton X-114 allows the detection of smoked paprika adulteration with less than 10% of paprika elaborated from peppers of foreign varieties by the FZCE analysis of the hydrophobic protein fraction. In addition, the FZCE analysis of the hydrophilic fraction provides a method for the determination of which drying process was used for autochthonous varieties of peppers. We therefore propose this extraction protocol, to be used routinely for the quality control of "Pimentón de La Vera" smoked paprika.

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