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Analytical Methods

Authentication of "Cereza del Jerte" sweet cherry varieties by free zone capillary electrophoresis (FZCE)

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ABSTRACT

The purpose of this work was to develop a procedure based on protein analysis by free zone capillary electrophoresis (FZCE) that can be used as an alternative to other methods in the determination of sweet cherry varieties for the authentication of "Cereza del Jerte". Two autochthonous varieties of sweet cherry type "Picota", 'Ambrunés' and 'Pico Negro', and the foreign variety 'Sweetheart' were used in the study. Two protocols for extracting the methanol-soluble proteins were tested. On the basis of the results, direct evaporation with nitrogen of a methanol extract was included in the extraction protocol for routine analysis. This method was found to give excellent repeatability of the corrected migration time (CMT), and showed greater effectiveness in discriminating sweet cherry varieties than the SDS–PAGE technique. Three peaks found in the FZCE electropherograms were investigated as a basis for discriminating between varieties. In addition, the FZCE analysis of methanol-soluble proteins provides information about the physico-chemical parameters relevant to the sensorial quality of the sweet cherries.

1. Introduction

The sweet cherry "Cereza del Jerte" is a high quality fruit of *Prunus avium* L. varieties from the "Jerte Valley" region, in the centre-west of Spain. Autochthonous varieties, mainly 'Ambrunés', 'Pico Negro', are hand-picked without stems (type "Picota") and marketed under the Registry of the Protected Designation of Origin "Cereza del Jerte" (Valdastilla, Cáceres, Spain). The environmental conditions give the "Cereza del Jerte" a more highly valued flavour than other commercial sweet cherry varieties. The falsification of this product with foreign sweet cherry varieties of an inferior quality, primarily to increase profit margins, has been a concern for many years within the "Cereza del Jerte" industry. 'Sweetheart' in particular is the foreign variety most frequently used in the falsification of "Cereza del Jerte". Traditionally, product falsification has been recognized using morphological and sensory parameters, such as shape, colour and flavour. However, these subjective methods depend on the ripening stage of the fruit (Serrano, Guillén, Martínez-Romero, Castillo, & Valero, 2005). 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 falsification and adulteration (Barnwell, McCarthy, Lumley, & Griffin, 1994; Skarpeid, Moe, & Indahl, 2001; Toorop, Murch, & Ball, 1997). Analyses of proteins in these complex matrices are presently performed mainly by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and reversed-phase high-performance liquid chromatography (RP-HPLC). Capillary electrophoresis (CE) may be used as an alternative method since it offers several advantages over SDS–PAGE, such as more rapid analysis, detection, and on-column quantification, as well as increased efficiency and resolution (Cancalon, 1995; Manabe, 1999). In addition, CE methods involve the simple extraction of proteins using small quantities of organic solvents in comparison with RP-HPLC. A subset of CE called capillary free zone electrophoresis (FZCE) has been applied to analyze complex protein systems. Numerous papers have reported the potential of FZCE to discriminate, or fingerprint, such foodstuffs as cereal grain cultivars, *Vicia* species, and pepper varieties (Bean & Lookhart, 1998; Bean, Lookhart, & Bietz, 2000; Lookhart & Bean, 1995a, 1995b; Piergiovanni & Taranto, 2005a, 2005b). Proteins of different fractions in pepper, for example, can be separated into 20–30 peaks and shoulders by FZCE in phosphate buffers, differentiating paprikas elaborated with different pepper varieties (Hernández, Martín, Aranda, Bartolomé, & Córdoba, 2006, 2007). Thus, FZCE might be useful to detect falsification in sweet cherries.

The aim of the present work was to develop a procedure based on protein analysis by FZCE that can be used as an alternative to

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other methods, such as SDS–PAGE, in the identification of "Picota" varieties with respect to other kinds of sweet cherry. In addition, the profile proteins were correlated with several quality parameters such as °Brix, titratable acidity, pH, and maturation index.

2. Materials and methods

2.1. Sample collection

Samples of sweet cherry (*Prunus avium* L.) used in this study were obtained from an experimental farm in the "Jerte Valley" (Cabrero, Cáceres, Spain). Two autochthonous varieties of "Picota" cherry, 'Ambrunés' and 'Pico Negro', and the foreign variety 'Sweetheart', were harvested and grouped into three different ripening stages in accordance with homogeneity in size and skin colour (Serrano et al., 2005). Colour parameters of the sweet cherry varieties at the different ripening stages were as specified in Table 1. For each ripening stage, 25 fruits were homogenized and assayed for soluble solids content, titratable acidity, and pH. The rest of the sweet cherry samples were frozen in liquid N_2 and stored in a freezer.

2.2. Soluble solids content, titratable acidity, pH, and maturation index

The soluble solids content was determined twice per homogenate with an automatic temperature-compensated DR101 digital refractometer (Optic Ivymen System, Barcelona, Spain), and the results were expressed as °Brix.

Titratable acidity and pH were determined twice per homogenate in a 5 g aliquot diluted to 50 ml with de-ionized water obtained with a Milli-Q water purification system (Millipore, Bedford, MA, USA), using a 716 DMS automatic titrator (Metrohm, Herisau, Swiss). Samples were titrated with 0.1 N NaOH until the pH reached 8.1. The results were expressed as malic acid equivalents, gram of malic acid per litre of solution.

The maturation index was calculated as the ratio between soluble solids content (°Brix) and titratable acidity (grams of malic acid per litre of solution).

2.3. Extraction of proteins for CE

Direct extraction of sweet cherry samples with water may lead to components other than proteins being removed, and the consequent poor resolution of the FZCE. In particular, these extracts may also contain compounds such as polysaccharides, polyphenols, anthocyanins, tannins, DNA, free aminoacids, and sugars that could potentially bind to the inner walls of the silica capillaries (Bean & Lookhart, 2001). Hence, the homogenized samples were filtered with glass wool, centrifuged at 5800*g* for 2 min, and then the following pre-extraction step was performed. To a volume of supernatant it was added a volume of methanol and filtered through anhydrous sodium sulfate. After cooling to −80 °C for 5 min, the methanol extracts were centrifuged at 5800*g* for 1 min. In order to achieve fast and efficient protein extraction, two protocols were tested. In the first, an aliquot of 0.5 ml of methanol-soluble protein extract was partially precipitated with 1 ml of chloroform and centrifuged at 24,000*g* for 5 min. The pellets were cleansed twice with chloroform to free them of pigment, and then resuspended in 100 μl of 30% (v/v) acetonitrile. In the second protocol, a 0.5 ml aliquot of the methanol-soluble protein extract was directly evaporated with nitrogen and then resuspended in 100 μl of 30% (v/v) acetonitrile (Hernández et al., 2006).

2.4. FCZE analysis

The protein extracts, after filtration through a 0.2 μm filter, were analyzed by FZCE. The separations were done on an automated PACE 5500 device (Beckman Instrument Inc., Palo Alto, CA, USA). Run buffer was prepared with HPLC-grade water obtained from a Milli-Q water purification system, and consisted of 8.75 mM phosphate 20.6 mM tetraborate at a nominal pH of 9. This buffer has been successfully used for the determination of vegetable proteins by FZCE (Flurer, Crowe, & Wolnik, 2000; Hernández et al., 2006, 2007). 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 de-ionized water for 5 min. They were rinsed between separations for 2 min with 100 mM NaOH, for 2 min with de-ionized 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 214 nm. 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 visualized at 254 nm 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.

2.5. SDS–PAGE analysis

The proteins for SDS–PAGE were pre-extracted with methanol as described above for FZCE. The pellets, obtained from 1.5 ml of the methanol-soluble protein extracts directly evaporated with nitrogen, were previously cleansed with chloroform and mixed with 20 μl of PAGE loading buffer (62.5 mM Tris–HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue), and incubated at 99 °C for 5 min for protein denaturing. The electrophoresis conditions were those described by Laemmli (1970), and the concentrations of acrylamide (29:1 acrylamide/bisacrylamide) in the gels were 4% (w/v) for stacking gels and 15% (w/v) 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

Table 1
Colour parameters of the sweet cherry varieties at the different ripening stages studied

Variety	Stage	N[a]	Colour parameters[b]		
			L^*	Chroma	Hue angle
Ambrunés	1	100	36.93^3	38.41^2	25.09^3
	2	100	33.79^4	34.15^3	22.06^4
	3	100	31.20^5	28.70^4	15.15^5
Pico Negro	1	100	33.18^4	34.76^3	21.09^4
	2	100	26.68^6	19.43^5	12.50^6
	3	100	25.72^6	17.08^6	10.04^7
Sweetheart	1	100	46.52^1	47.58^1	32.33^1
	2	100	38.14^2	46.46^1	27.42^2
	3	100	26.12^6	28.67^4	15.90^5

[a] Number of samples.
[b] For a given column, values with different numbers are significantly different ($P < 0.05$).