



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.

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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 “Jerre 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 N₂ 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 5800g 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 5800g 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 24,000g 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 Miniprotein 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 ($P < 0.05$).

(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 (Genetools, Synoptics Ltd., Cambridge, UK) was used for the densitometric analysis of the gel electrophoreses.

2.6. 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 honest significant difference test using the SPSS software package version 10.0.6 from SPSS Inc. (Chicago, IL, USA). The relationships between the physico-chemical parameters and peak area values were evaluated by Pearson correlation coefficients, and verified by linear regression analysis.

3. Results and discussion

3.1. Optimization of FZCE analysis

Cherry samples from the 'Ambrunés' autochthonous sweet cherry variety were used to evaluate the two protein extraction protocols tested. The results showed relevant differences in the electropherograms obtained for the same sample extracted with the different procedures (Fig. 1). The resolution and migration times for methanol-soluble proteins were appropriate with both extract protocols. The proteins were resolved in 25 min and separated into 15–26 peaks and shoulders. However, there were fewer peaks and relatively smaller amounts of proteinaceous compounds with the chloroform precipitation of the methanol-soluble proteins (Fig. 1, Trace A). This procedure was successfully used for extraction of methanol-soluble proteins from dried products, such as paprika (Hernández et al., 2006). For sweet cherry fruit, direct

evaporation with nitrogen was therefore adopted as the extraction protocol for routine analysis.

With respect to the analytical parameters, the 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 2).

3.2. FZCE and SDS–PAGE comparison

To explore the potential of the FZCE method to distinguish cherry varieties, the inter-variety differences were evaluated and compared with the SDS–PAGE technique.

The electropherograms obtained by FZCE under the optimized conditions had a total of 19 well-defined peaks for all of the varieties analyzed (Fig. 2A). As seen in Table 2, qualitative and quantitative differences were detected by comparing the electropherograms of the sweet cherry batches. The analysis of methanol-soluble proteins from the autochthonous variety 'Pico Negro' showed all of the peaks, whereas peaks 14 and 15 were not detected in the analysis of 'Ambrunés' and 'Sweetheart' samples. Also, peaks 12 and 17 were not detected in the foreign variety 'Sweetheart', and peaks 3, 11, and 19 showed significant differences between batches of autochthonous varieties and the 'Sweetheart' variety (Table 2). These results showed that the optimized FZCE method is able to discriminate the varieties of sweet cherry studied.

With respect to SDS–PAGE, the densitograms showed a total of only six polypeptide bands with an approximate molecular mass range of 20–92 kDa (Fig. 2B). The fewer effectiveness of this technique than the FZCE in discriminating of vegetable varieties have been previously described (Canalón, 1995; Hernández et al., 2006; Manabe, 1999). There were great similarities in the overall polypeptide profiles of the methanol-soluble proteins from the three varieties studied, and indeed this analytical system did not reveal any significant differences between batches (Table 3). Therefore, the analysis of methanol-soluble proteins by SDS–PAGE proved to be an ineffective tool for identifying sweet cherry varieties.

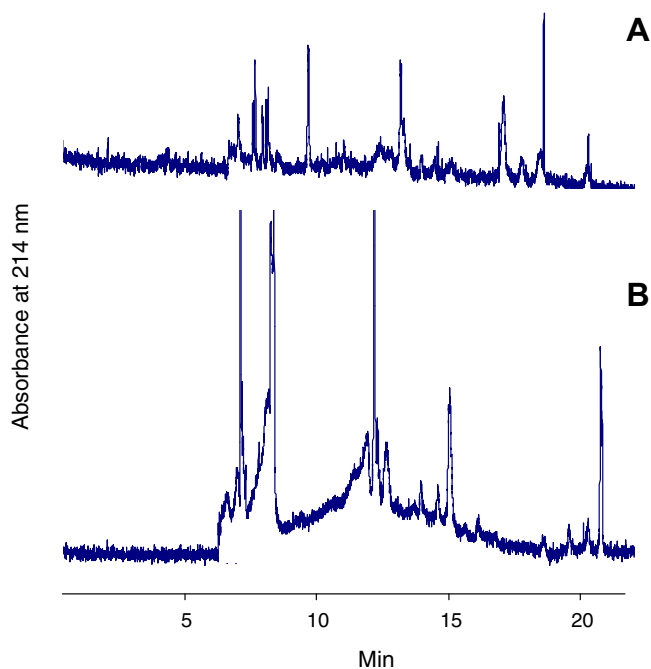


Fig. 1. Effect of the extraction protocol on FZCE patterns of methanol-soluble proteins of sweet cherry variety 'Ambrunés', ripening stage 3: (A) precipitated with chloroform (1:2 v/v); (B) directly evaporated with nitrogen.

Table 2

Analytical parameters and peak areas of proteins determined by FZCE under optimized conditions for the sweet cherry varieties studied^a

Peak	Migration time		UAA ^b			P
	CMT ^c	RSD% ^d	Ambrunes	Pico Negro	Sweetheart	
1	6.81	0.69	57,880 ²	148,349 ¹	17,408 ²	0.000
2	7.30	0.73	54,943 ¹	3755 ²	8273 ²	0.000
3	7.41	1.00	251,959 ¹	212,211 ¹	143,415 ²	0.000
4	8.21	0.68	19,721	31,044	41,484	0.069
5	8.53	0.59	375,739	349,750	340,637	0.702
6	12.64	0.53	183,360	151,485	225,354	0.062
7	12.9	0.56	310,085	365,083	253,612	0.400
8	13.51	0.41	76,580	123,871	131,638	0.730
9	14.86	0.45	53,717	41,768	39,842	0.465
10	15.51	0.71	26,656	25,076	38,190	0.203
11	16.00	0.76	143,456 ¹	151,481 ¹	107,499 ²	0.012
12	16.77	0.72	11,152 ¹	2034 ²	n.d. ^{2e}	0.001
13	17.16	0.73	14,652 ²	18,593 ^{1,2}	21,345 ¹	0.024
14	18.64	0.81	n.d. ²	19,391 ¹	n.d. ²	0.000
15	18.90	0.65	n.d. ²	7724 ¹	n.d. ²	0.000
16	19.81	0.69	17,037 ²	26,009 ¹	22,519 ^{1,2}	0.010
17	20.75	0.89	18,133 ¹	23,874 ¹	n.d. ²	0.000
18	21.23	0.82	22,375	29,151	17,934	0.057
19	21.57	1.24	119,922 ¹	127,666 ¹	59,469 ²	0.000

^a For a given protein (row), values with different numbers are significantly different ($P < 0.05$).

^b Arbitrary area units.

^c Corrected migration time.

^d Relative standard deviation ($n = 5$).

^e Not detected.

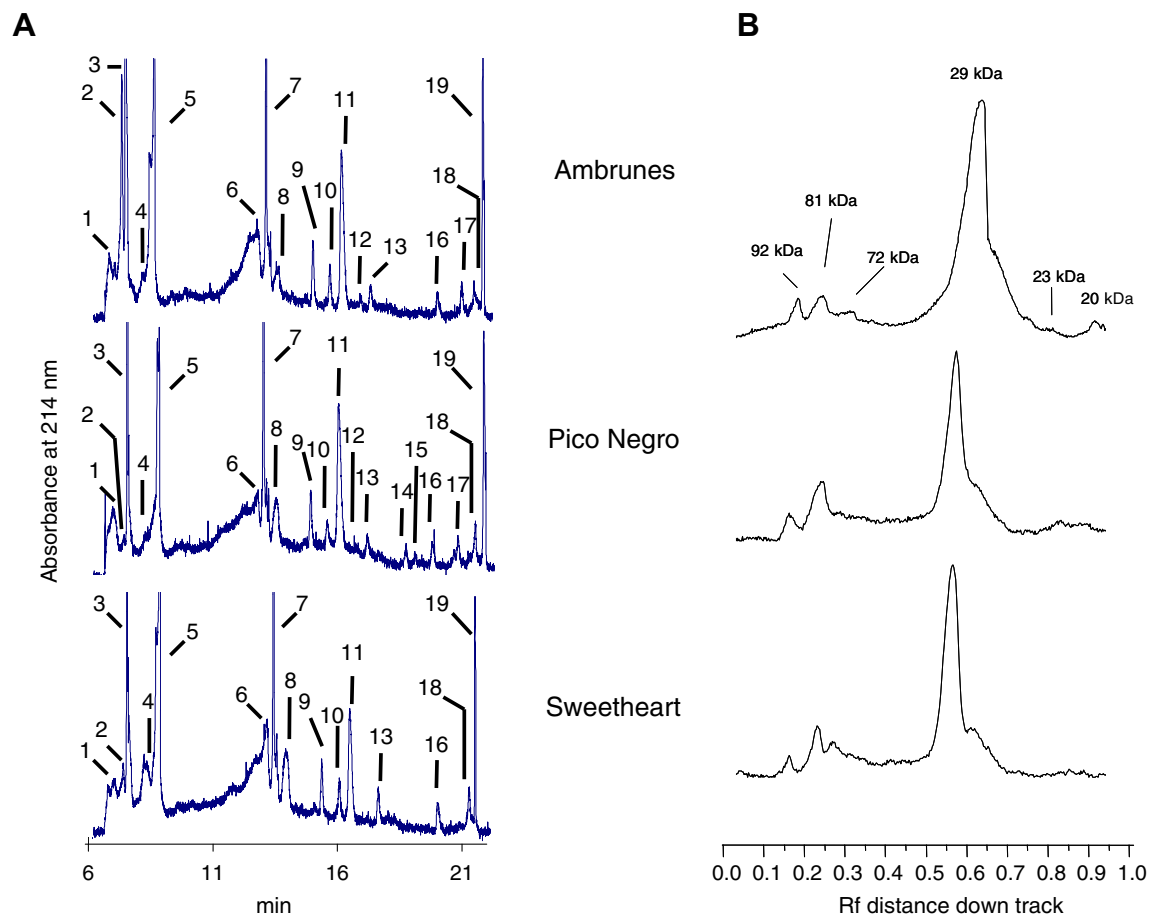


Fig. 2. Electropherograms (A) and densitograms (B) of methanol-soluble proteins of 'Ambrunes', 'Pico Negro', and 'Sweetheart' sweet cherry varieties in the ripening stage 3.

Table 3

Peak areas of proteins determined by SDS-PAGE in the sweet cherry batches studied^a

Approximate MW (kDa)	UAA ^b			P
	Ambrunes	Pico Negro	Sweetheart	
92	39,473	22,970	28,099	0.541
81	76,409	45,798	27,018	0.485
72	20,154	n.d. ^c	12,513	0.211
29	710,833	453,884	568,094	0.231
23	6864	3544	4380	0.514
20	7094	3892	3368	0.654

^a For a given protein (row), values with different numbers are significantly different ($P < 0.05$).

^b Arbitrary area units.

^c Not detected.

Table 4

Physico-chemical parameters of sweet cherry fruits at different ripening stage, and their correlation with peaks of FZCE electropherograms

Physico-chemical parameters	Stage				Correlations with the peaks of FCZE electropherograms							
	1	2	3	P ^a	Peak 1	Peak 3	Peak 6	Peak 7	Peak 8	Peak 9	Peak 13	Peak 14
°Brix	16.13 ¹	17.14 ¹	20.87 ²	0.000	0.225	0.207	0.441 [*]	0.588 ^{**}	0.464 [*]	0.585 ^{**}	0.013	0.024
Titratable acidity (mg g ⁻¹ f.w.)	7.93	8.28	7.78	0.859	-0.843 ^{**}	-0.524 [*]	-0.176	-0.379	-0.414	-0.122	-0.657 ^{**}	-0.662 ^{**}
pH	3.97	3.95	4.05	0.798	0.814 ^{**}	0.138	0.26	0.617 ^{**}	0.664 ^{**}	0.008	0.905 ^{**}	0.911 ^{**}
Maturation index (arbitrary units)	2.10 ¹	2.14 ¹	2.86 ²	0.042	0.663 ^{**}	0.329	0.379	0.693 ^{**}	0.630 ^{**}	0.404	0.506 [*]	0.520 [*]

^a P-value due to stage sampled.

^{*} $P < 0.05$.

^{**} $P < 0.01$.

3.3. Relationship between physico-chemical parameters and the FZCE profile in the sweet cherries

No relevant inter-variety differences were found in the study of the physico-chemical parameters of the sweet cherries (data not shown). Table 4 lists the physico-chemical characteristics of the three sweet cherry varieties studied at different stages and their correlations with the methanol-soluble proteins analyzed by FZCE. The °Brix and maturation index were greater in the samples of stage 3, whereas the titratable acidity and pH did not show significant differences between stages. Similar values of °Brix, titratable acidity, pH, and maturation index have been reported for different sweet cherry varieties, including the "Picota" type (Alique, Zamorano, Martínez, & Alonso, 2005; Bernalte, Hernández, Vidal-Aragón, & Sabio, 1999). There were correlations, however, between the

physico-chemical parameters and several peaks of the electropherograms. The areas of peaks 7 and 8 were positively correlated ($P < 0.05$) with high °Brix, pH, and maturation index values, and negatively ($P < 0.1$) with titratable acidity. Similar results were obtained for peaks 1, 3, 6, and 9 of the three varieties studied. Likewise, peaks 14 and 15 of the electropherograms of the 'Pico Negro' samples also showed strong correlations with high pH and maturation index values and low titratable acidity values.

4. Conclusion

The present protocol of methanol-soluble protein extraction from sweet cherry fruit and the FZCE analysis procedure provides a fast, economic, and efficient method authentication of the "Picota" type sweet cherry varieties. In addition, the FZCE analysis of methanol-soluble proteins provides information about the major physico-chemical parameters related to the sensorial quality of the fruit. We have therefore proposed that this technique be used routinely for the quality control of sweet cherries commercialized under the Registry of the Protected Designation of Origin "Cereza del Jerte".

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