Hard-To-Cook Beans (*Phaseolus vulgaris*): Involvement of Phenolic Compounds and Pectates

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Hard-to-cook (HTC) is a textural defect that affects legume seeds stored in adverse conditions. Phenolic acids were extracted from dehulled soft (control) and HTC beans. HPLC analyses of the phenolic fractions indicated the presence of caffeic, *p*-coumaric, sinapic, and ferulic acids. The highest content of phenolic acids was present as methanol-soluble esters in the control samples (approximately 45 times more than in HTC). Phenolic acids bound to the water-soluble pectin fraction were 2 times higher in HTC than in control beans, wheras the content of ferulic acid bound to the water-insoluble residue of the cell walls was 4 times higher in the control than in HTC samples. Through Fourier transform infrared spectroscopy accumulation of pectates and binding of phenolics to cell wall components were detected in HTC samples.

Keywords: *Beans; common beans; Phaseolus vulgaris; HTC; hard-to-cook; phenolics*

INTRODUCTION

Beans are some of the most nourishing vegetables in the human diet (Ensminger et al., 1994). However, their consumption can be affected by poor cooking quality. For example, prolonged storage of common beans at high temperature and humidity causes the textural defect known as hard-to-cook (HTC). HTC seeds have poor soaking imbibition and, despite prolonged cooking times, do not attain adequate texture, due to a failure of cotyledon cells to separate upon cooking. A survey on major consumer bean quality characteristics identified cooking time as one of the most important factors, followed by the HTC defect (Van Herpen, 1991). Moreover, texture is not the only quality attribute of beans that is affected by HTC; the nutritional value is also impaired by a loss of vitamins and decreased protein availability (Sgarbieri and Whitaker, 1982). Economic losses result either through the rejection of beans by consumers for its poor texture or due to the need for increased energy required for cooking. Prolonged cooking is a factor that prevents the increase of production and use of legume seeds in many communities (FAO, 1990).

Several causes have been suggested to explain the HTC phenomenon. (1) *Formation of insoluble pectates* at the cell wall−middle lamella would render the tissue more refractive to cell separation during cooking (Chang et al., 1977; Kon and Sanshuck, 1981; Jones and Boulter, 1983; Moscoso et al., 1984; Hentges et al., 1991). In fact, ultrastructural studies have demonstrated that in HTC beans cell separation is prevented (Shomer et al., 1990). (2) *Degradation of cell membranes* was also considered in the development of HTC. Varriano-Marston and Jackson (1981) suggested that increased peroxidation within the cytoplasm could lead to loss of membrane integrity. Richardson and Stanley (1991) related loss of membrane functionality to HTC. (3) *Involvement of phenolic compounds* was also implicated by several authors. Hincks and Stanley (1987) suggested that a lignification process may be involved in the development of HTC phenomenon. Srisuma et al. (1989) failed to detect significant changes in lignin content during hardening of beans but reported a large increase in free hydroxycinnamic acids, which they supposed was associated with increased hardening. Rozo (1982) described a decrease in the extraction of soluble tannins of the seed coat during hardening. It was inferred that this could be related to an increase in condensed tannins in the seed coat and a possible migration of soluble tannins to the cotyledon. (4) *A combination of mechanisms* was also suggested (Hincks and Stanley, 1986; Aguilera and Ballivian, 1987).

In this paper we describe changes in the profile of phenolic compounds during storage in accelerated conditions of hardening of Carioca beans (*Phaseolus vulgaris*). We also investigated the partitioning of some phenolic compounds in isolated cell wall fractions, and, for the first time, Fourier transform infrared spectroscopy was applied to the study of bean cell wall constituents.

MATERIALS AND METHODS

Materials. Common bean (*P. vulgaris* cv. Carioca) seeds were supplied by the Instituto Agronômico de Campinas (SP, Brazil). Carioca beans have a cream color with tan stripes. A control lot of seeds was stored at 5 °C/40% relative humidity (RH) for 6.5 months, and another lot of seeds was allowed to harden under storage at 35 °C/75% RH for the same duration. A third lot of seeds was kept at room temperature (20−25 °C/40% RH, on average) for 2 years and used only for determination of the crystallinity index (see below). A modified Mattson cooking device (Jackson and Varriano-Marston, 1981) was used to determine cooking time of Carioca beans: control sample (33 min), hard beans (HTC 272 min), and the lot kept at room temperature (101 min). After the storage period, the seeds were dehulled and the cotyledons were freeze-dried, ground, and sieved through a 0.5 mm mesh.

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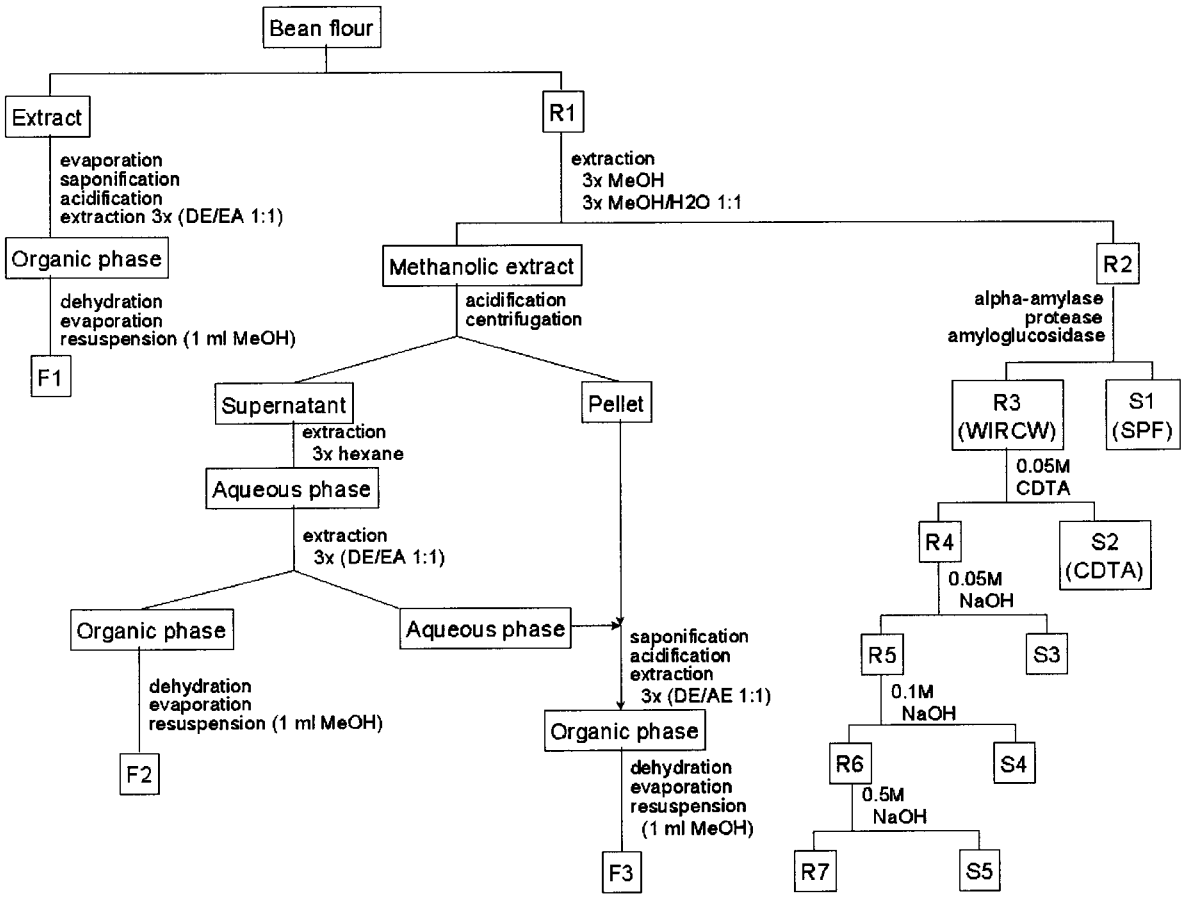

Figure 1. Outline of the isolation procedure of phenolic fractions from dehulled bean cotyledons: F, fraction; R, residue; S, supernatant; WIRCW, water-insoluble residue of the cell wall preparations; SPF, soluble pectin fraction; CDTA, cyclohexanediaminetetraacetic acid-soluble fraction.

Chemicals. Heat-stable α-amylase A-3306, protease P-3910, amyloglucosidase A-9913, and all phenolic standards were obtained from Sigma Chemical Co. (St. Louis, MO). KBr was purchased from Merck (Darmstadt, Germany).

Extraction of Phenolic Compounds. The extraction of phenolic compounds was conducted according to the procedure of Srisuma et al. (1989). All steps followed for the extraction are outlined in Figure 1.

Isolation of Phenolic Compounds Associated with the Water-Insoluble Residue of the Cell Wall (WIRCW) Preparations. The residue R_2 (Figure 1) was freed of starch and proteins by means of a sequence of enzymatic hydrolyses. Initially the residue was treated with a thermoresistant α-amylase (0.1 mL of enzyme to 500 mg of sample) at pH 6.0, for 30 min in a boiling water bath. After cooling, the pH was adjusted to 7.5, and a treatment with a protease (at a ratio of 1:100 w/w) for 1 h at 60 °C followed. The pH was readjusted to 3.0, and then an amyloglycosidase (0.3 mL of enzyme to 500 mg of sample) was added (1 h at 60 °C). The suspension was allowed to cool and centrifuged, and the supernatant was added to 4 volumes of 95% ethanol; after 12 h at 0 °C, a pectin fraction (SPF) had precipitated. SPF was rinsed three times with 80% ethanol, suspended in water, and freeze-dried for storage. The residue of the enzymatic treatments was rinsed twice with each of the following: 0.5 M sodium phosphate buffer, pH 7.2; deionized water; a 1:1 mixture of chloroform and methanol at 45 °C for 30 min; and water/methanol. The remaining residue was added to 90% DMSO and sonicated for 20 min, centrifuged, rinsed, centrifuged, and freeze-dried. This fraction was called the WIRCW.

Thirty milligrams of WIRCW was extracted for 16 h at 22 °C by stirring with 10 mL of 0.05 M cyclohexanediaminetetraacetic acid at pH 6.5 with a few drops of toluene. The suspension was centrifuged at 1500*g* for 5 min. The supernatant was filtered through a GF/F (Whatman) filter, and the filtrate was extracted with 1:1 (v/v) diethyl ether/ethyl acetate, resulting in the fraction called CDTA (or S_2; see Figure 1). The residue (R_4) was sequentially treated with 10 mL of 0.05, 0.10, and 0.50 N NaOH containing $NaBH_4$ (3 mg/mL) for 1 h under N_2. After each extraction, the suspension was centrifuged at 1500*g* for 15 min and the supernatant filtered and kept; the pH was adjusted to a value of 2.0 with HCl. Each fraction of bound phenolic was extracted with diethyl ether/ethyl acetate, resulting in fractions S_3, S_4, S_5, and the final residue R_7.

Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC). All of the fractions obtained were analyzed by TLC using silica gel 60 Chromatoplates (Merck) developed with toluene/acetic acid (9:1). Phenolic compounds were detected by UV fluorescence before and after exposure to ammonia vapor and also by spraying with the Folin−Ciocalteu reagent followed by NH_3 vapor.

Phenolic acids were determined by HPLC using a Shimadzu (Kyoto, Japan) chromatograph equipped with an LC-9A pump, a SIL-B6 autoinjector, and a model SPD-M6A photodiode array detector. A C_{18} column was used (3.9 × 150 mm; 60 Å, 4 μm; NovaPak, Waters). As mobile phase two solvents were used: 0.03 M KH_2PO_4, pH 2.5 (solution A); and 1:1 methanol/0.03 M KH_2PO_4 (solution B), adapted from Spanos and Wrolstad (1990). The elution program was 5% solvent B isocratic for 3 min, followed by a 28 min linear gradient to 100% B, and holding for 5 min isocratic with 100% B; flow rate was 1.2 mL/min, and elution was carried out at room temperature. The detection was performed at 280 and 320 nm (the later for hydroxycinnamates). The results are the average of four determinations (duplicate HPLC analysis of two phenolic extractions).

Fourier Transform Infrared Spectroscopy (FTIR) of the Cell Wall Material. Four fractions were analyzed by FTIR: the soluble pectic fraction, the water-insoluble residue

Table 1. Content of Phenolic Compounds Extracted from Cotyledons of Carioca Beans Stored at 4 °C (Control) and at 35 °C for 6.5 Months (HTC)[a]

phenolic acid (μg/g of cotyledon)	free phenolic acids		methanol soluble esters	
	control	HTC	control	HTC
caffeic acid	nd	0.61 ± 0.1	1.2 ± 0.1	nd
p-coumaric acid	0.50 ± 0.1	2.30 ± 0.2	13.2 ± 0.7	nd
ferulic acid	3.25 ± 0.1	14.78 ± 0.3	80.4 ± 0.7	2.04 ± 0.2
sinapic acid	1.14 ± 0.2	4.03 ± 0.1	32.3 ± 0.3	0.8 ± 0.1
total	4.89	21.72	127.1	2.84

[a] Results are expressed on a dry weight basis. nd, not detected.

of the cell wall after soluble pectin extraction, the cyclohexanediaminetetraacetic acid-soluble fraction, and the final residue R_7. Dried samples (1 ± 0.02 mg) were mixed with KBr (90 ± 1 mg) and made into pellets. Spectra were obtained on a Bomem MB-102 IR-spectrophotometer (Bomem, Inc., Quebec, Canada); all spectra were obtained at a resolution of 4 cm^{-1}.

Scanning Electron Microscopy of Isolated Cell Walls. The procedure described by Hincks and Stanley (1987) was followed. Fifty grams of dehulled beans was macerated in 250 mL of deionized water at 4 °C for 18 h. Using a Polytron homogenizer (Kinematica AG, Brinkman Instruments), the cotyledons were homogenized at 4 °C, 2 × 5 min, with a 1 min interval to avoid heating of the samples. The resulting homogenates were sieved through screens with openings of 250 and 40 μm and washed with deionized water. The procedure was repeated by adding the materials retained on the screens. In the third extraction two fractions were collected: 40−250 and >250 μm. Both fractions were freeze-dried in a model Beta Crist freeze-dryer (Germany). Cell wall isolates were mounted on aluminum stubs using colloidal graphite cement and sputter coated with gold (300 μm). The samples were observed with a Hitachi S-570 scanning electron microscope at an accelerated voltage of 20 kV. Photos were taken with Polaroid type 55 (P/N) film.

Determination of the Cellulose Crystallinity Index. A lyophilized cell wall preparation (1 ± 0.02 mg) was dispersed in 90 ± 1 mg of KBr, and pellets were prepared for examination. Samples were analyzed using an infrared (IR) spectrophotometer Bomem MB-102. Crystallinity index was calculated from the ratio of absorbances at 1372 cm^{-1} (C−H angular) and 2900 cm^{-1} (C−H axial), according to the method of Gondim Tomaz et al.. (1994).

Light Microscopy. Dehulled beans were fixed in ethanol/formaldehyde/acetic acid/water (30:10:10:10) for 7 days, with three substitutions of the fixative solution. The samples were embedded on paraffin and sectioned (5 μm). Deparaffinized sections were exposed to ammonia vapors and immediately observed under UV light (Yiu et al., 1983). The observations were made using a Nikon microscope model Fluophot, equipped with filters UV 330−380 (excitation) and 410W (absorption). Sections were also stained with phloroglucinol (Parker and Waldron, 1995).

RESULTS AND DISCUSSION

Phenolic Content and Distribution. Through TLC and HPLC it was confirmed that there were no soluble phenolics in the F_1 extract (Figure 1) of the bean flour prepared from either soft or HTC beans. The results from TLC (not shown) gave an indication of the phenolic composition of all the fractions extracted, but a complete separation and the final quantification of the phenolic compounds were performed by HPLC. Table 1 presents the results obtained for the F_2 and F_3 fractions (Figure 1), which consist of hydroxycinnamic acids and derivatives extracted from the cotyledon of both soft and HTC beans. Higher levels of free phenolics (F_2) were found in the HTC beans than in the controls. However, this is not true for the methanol-soluble phenolic esters (F_3): the control samples contained higher levels of phenolics than the HTC beans. Among the phenolics found in Carioca beans were caffeic, *p*-coumaric, ferulic, and sinapic acids, with ferulic acid being the main constituent of the F_2 and F_3 fractions from both control and HTC beans. According to Harborne (1980), phenolic compounds are commonly present in conjugated forms, probably due to potential toxicity of the free forms to the plant tissue. The increase in the free phenolics (F_2) in the HTC beans seems to have been induced by the adverse storage conditions. L-Phenylalanine and L-tyrosine can be deaminated with the formation of *trans*-cinnamic and *trans*-*p*-coumaric acids, both excellent precursors of lignin, according to Goodwin and Mercer (1983). Holhberg and Stanley (1987) described a buildup of aromatic amino acids as a consequence of the hydrolysis of storage proteins during the storage-induced hardening of common beans.

Table 2. HPLC Quantification of Phenolic Acids Bound to Soluble Pectin (SPF)[a]

phenolic acid (μg/g of pectin)	SPF bound phenolics	
	control	HTC
p-coumaric	3.07 ± 0.2	nd
ferulic	6.14 ± 0.1	19.52 ± 0.1
total	9.21	19.52

[a] nd, not detected.

Table 3. Quantification of Phenolics Bound to the Cell Wall[a]

cell wall fraction	ferulic acid	
	control	HTC
0.05 M NaOH	88.23 ± 0.4	15.32 ± 0.2
0.10 M NaOH	79.54 ± 0.3	24.98 ± 0.2
0.50 M NaOH	32.41 ± 01	6.66 ± 0.1
residue	nd	nd
total	200.18	46.96

[a] Results are expressed in μg/g WIRCW. nd, not detected.

Tables 2 and 3 show the results of phenolics bound to the SPF and to the other polymer constituents of the cell wall (WIRCW), respectively. Among SPF-bound phenolics, only *p*-coumaric and ferulic acids were found, wheras only ferulic acid was released from the WIRCW samples from both control and HTC beans. An increase (3 times) of ferulic acid bound to SPF was observed in the HTC samples in relation to the control. Ferulic acid dimerized as diferulic acid has been reported in grasses bound to carbohydrate polymers of the cell wall (Hartley and Jones, 1976). It has been suggested that feruloylated pectins might have roles in physiological and defense processes, such as cell expansion and disease resistance, as well as in the initiation of lignification (Fry, 1983). The possibility of a process involving tannin condensation during the development of HTC beans was also considered, which would lead to a decrease in the extractable phenolic compounds (Stanley, 1992a,b).

When the total amount of phenolics (all fractions analyzed) per gram of cotyledon is taken into account, the sum of the phenolic compounds is 5 times higher for the control than for HTC beans. However, the distribution of the phenolics is not the same in the different fractions analyzed (as shown in Tables 1−3). HTC beans have a higher content of free phenolic acids (4 times) and phenolics bound to pectin (2 times); control

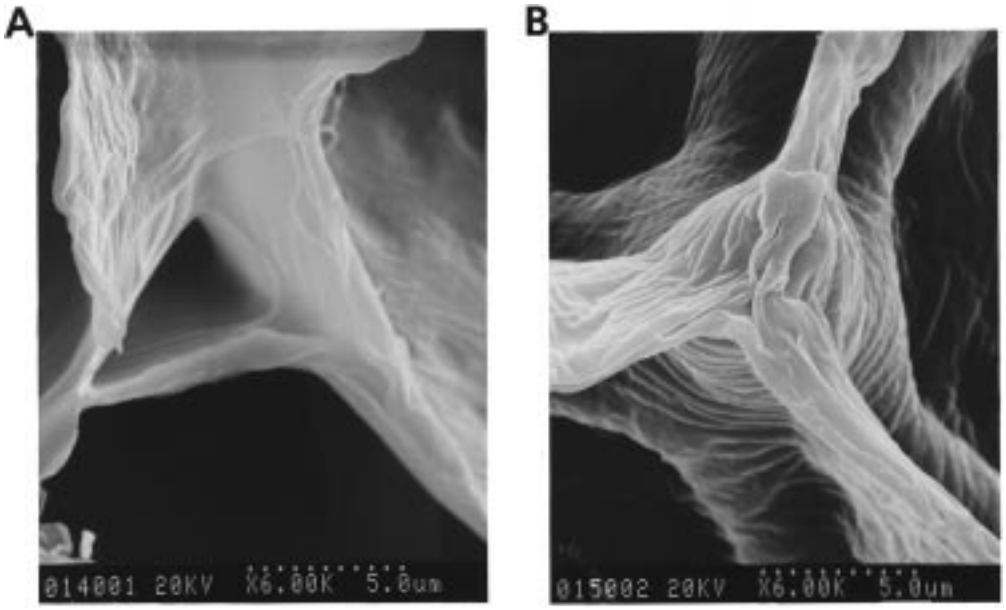

Figure 2. Scanning electron micrographs of isolated cell walls of (A) control and (B) HTC bean cotyledons.

has ≈45 times more phenolics as methanol-soluble esters and 4 times more ferulic acid associated with the WIRCW.

Cell Wall Microscopy. It is known that after excitation by UV, phenolic acids and their esters present in the cell wall emit a blue fluorescence (Harris and Hartley, 1976). Using fluorescence light microscopy, we detected autofluorescence of the bean cotyledons cell walls. The intensity of the fluorescence was limited, and the lack of appropriate laboratory conditions did not allow its measurement (micrographs not shown); nevertheless, no differences were noted between the control and HTC sample. Stanley and Plhak (1989) described a significant increase of the autofluorescence with the hardening process in samples of black beans.

Figure 2 shows the scanning electron micrographs of cell wall isolates. The sample prepared from HTC beans shows a thickening of the walls at the cell junction. Similar results were observed by Hincks and Stanley (1987), while searching for evidence of lignification in beans, and by Bhatty (1990) in poor-cooking lentils. Varriano-Marston and Jackson (1981) had suggested the occurrence of a possible lignification of the middle lamella as an explanation for the HTC of beans. In isolated cell walls of dehulled normal and hard beans, we failed to confirm the presence of lignin (the phloroglucinol−HCl test was negative).

FTIR of Cell Wall Fractions. FTIR spectra of fractions WIRCW, SPF, CDTA, and the final residue R_7 (Figure 1) were examined for some chemical species of interest. Among those are included phenolics (1605 cm^{-1} aromatic C═C skeletal stretching and 1635 cm^{-1} C═C stretching), carbohydrates (1160 to 970 cm^{-1} highly coupled vibrational modes of polysaccharide backbones), carboxylic acid groups on pectins (1610 cm^{-1} COO^- symmetric stretching), carboxylic ester group (1740 cm^{-1} C═O stretching of alkyl ester), and protein (1650 cm^{-1} amide I: C═O stretching + contribution from C−N stretching; and 1550 cm^{-1}, amide II: N−H deformation + contribution from C−N stretching), according to McCann et al. (1992) and Séné et al. (1994). In Figure 3A−D, all sample spectra are presented; for each fraction, spectra of control and HTC are shown in the same figure. For comparison, spectra of standards, pectin, polygalacturonic acid, and microcrystalline cellulose (Avicel) were also taken and are included in Figure 3E−G.

Examination of the spectra of WIRCW, the remaining residue after the extraction of the water-soluble pectins, showed no differences between the control and HTC samples (Figure 3A). It is difficult to compare the spectra with those of the standards (Figure 3E−G) due to overlap of the bands of the various polymers present in the partially extracted cell wall. With fractionation of WIRCW some interesting differences were observed (Figure 3B,C). The SPF of the HTC sample revealed a higher absorption in the region assigned to the pectin salt form (1420 cm^{-1}) than that of the control. The peak centered at 1740 cm^{-1}, which is diagnostic for the ester carbonyl stretching associated with pectins, is defined only in the control sample. The HTC sample showed much higher peaks in the region 1500−1550 cm^{-1} and around 1650 cm^{-1}. To confirm the presence of phenolic compounds, according to Séné et al. (1994), only the doublet 1605 and 1635 cm^{-1} can be used. Our FTIR spectra suggest a higher content of phenolic material associated with the HTC sample, in agreement with the results shown in Table 2, regarding the phenolic content of the SPF: HTC beans contained 3 times more phenolics. Accumulation of phenolics can occur as a result of stress (injury), and these phenolics can later be involved in cross-linking with cell wall components (Selvendran, 1989). It is known that in dicot plants some pectins are esterified with ferulic and *p*-coumaric acids (Fry, 1983).

In Figure 3C the region 1400−1600 cm^{-1} was not analyzed for comparison of samples due to absorbance of CDTA and possible artifacts caused by conformational changes in the presence of the chelator (Séné et al., 1994). The largest difference observed between CDTA fractions was in the carbohydrate IR fingerprint region (1160 to 970 cm^{-1}); a considerably higher amount of carbohydrates was extracted from the HTC sample, indicating a higher content of pectates in the HTC beans. It suggests that with the development of the hardening process there is a decrease in the degree of esterification of the pectin, probably as a result of pectinmethylesterase activity. As a consequence, more

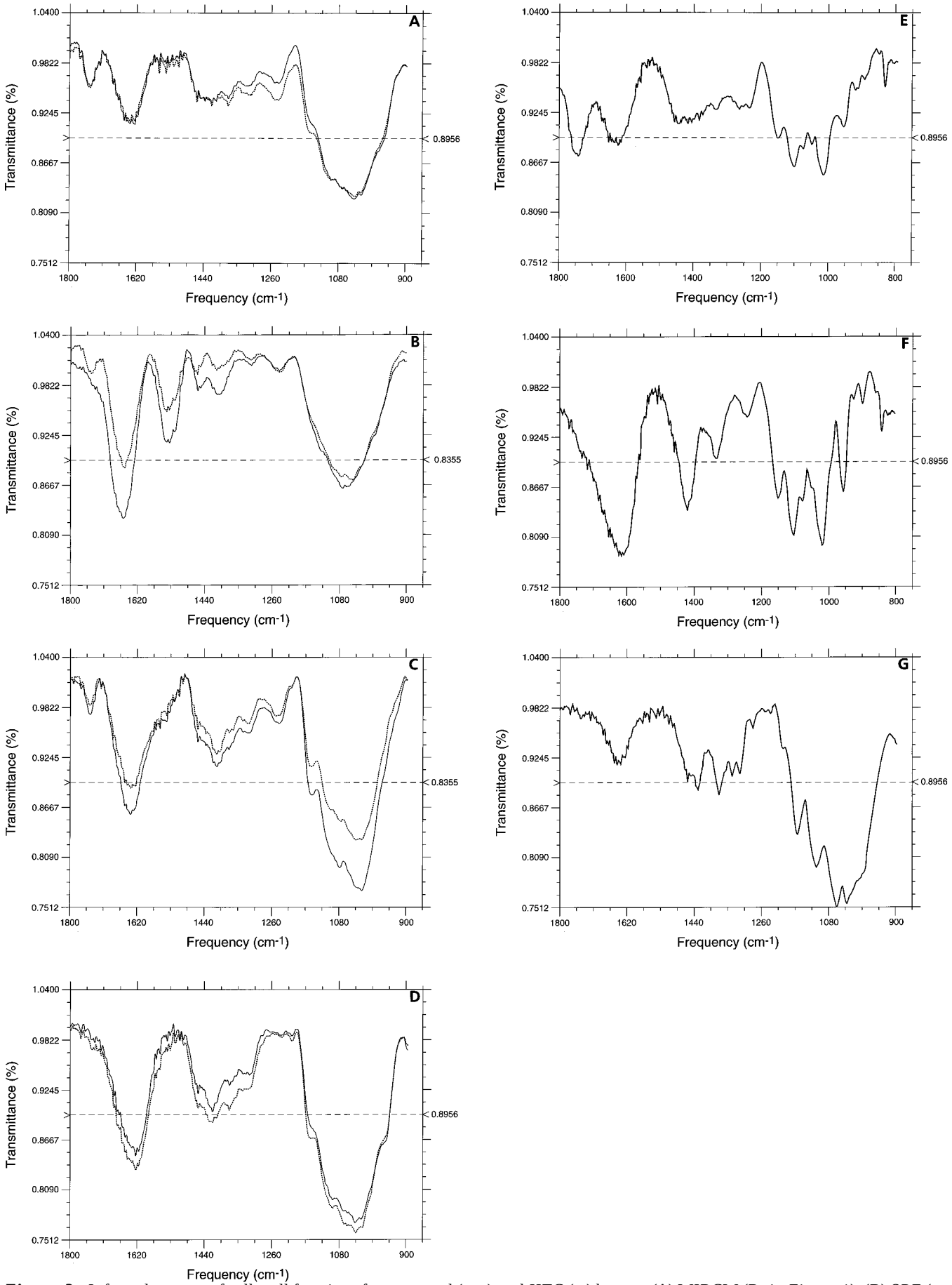

Figure 3. Infrared spectra of cell wall fractions from normal (· · ·) and HTC (—) beans: (A) WIRCW (R_3 in Figure 1); (B) SPF (or S_1 in Figure 1); (C) CDTA fraction (or S_2 in Figure 1); (D) R_7 or final residue; (E−G) standards [(E) pectin, (F) polygalacturonic acid, (G) microcrystalline cellulose (Avicel)].

pectate would be present in HTC beans. Jones and Boulter (1983) and Stanley and Aguilera (1985) suggested the possible involvement of a prior demethoxylation in the hardening process. In previous work (Garcia et al., 1993), the authors demonstrated that in histological sections of HTC beans there was more calcium (detected by fluorescence microscopy with chlorotetracycline) than in sections of soft (control) beans. In addition, the yield of the cell wall fractions is another indication of accumulation of pectates during hardening, and it is consistent with the hypothesis of pectate involvement in HTC. From the fractionation of cotyledons of the HTC beans the yield of SPF was 28% (w/w) lower than that of the control, whereas 35% (w/w) more CDTA-soluble fraction was recovered from HTC beans.

Spectra of the final residue (R_7) are shown in Figure 3D. Overall, both spectra are similar; small differences were observed between the control and HTC samples, namely, higher absorbance in the bands of phenolics (1605 and 1635 cm^{-1}) and protein (1650 cm^{-1}, amide I) due to the control sample. In addition, HTC had a lower absorbance in the carbohydrate fingerprint region.

The cellulose crystallinity indexes obtained were 0.16 (control), 0.25 (HTC), and 0.39 (aged sample). These results suggest an increase in the degree of cellulose crystallinity with the hardening process associated with storage under elevated temperature and humidity, and also with the aging of the bean seeds stored at ambient conditions (variable throughout the storage). The crystallinity index of the HTC beans (0.25) was lower than that of the aged sample (0.39), whereas the cooking times of those samples were 272 and 101 min, respectively, showing that the alterations of cellulose crystallinity alone do not explain the hardening. Increase in cellulose crystallinity is also known to occur upon maturation of lignocellulosics used to feed ruminants (Northcote, 1972; Kerley et al., 1988).

The HTC phenomenon is a complex process that affects different components of the cell, such as the cell wall polymers, phenolics, starch, and protein among others. It is probably a combination of mechanisms that take place in the development of the HTC in common bean. In this paper, we found that cotyledons of HTC Carioca beans overall have less hydroxycinnamic acids than the control. Nevertheless, HTC beans have 3 times more phenolics associated with the soluble-pectic fraction and an increased content of pectates. It is believed that the presence of more ferulic acid bound to soluble pectin, if involved in cross-links, may ultimately contribute to changes in cell adherence, and consequently this would lead to a textural defect by impairing cell separation upon cooking of HTC beans.

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