Phenolics and Antioxidant Properties of Fruit Pulp and Cell Wall Fractions of Postharvest Banana (*Musa acuminata* Juss.) Cultivars

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Banana fruits are important foods, but there have been very few studies evaluating the phenolics associated with their cell walls. In the present study, (+) catechin, gallocatechin, and (−) epicatechin, as well as condensed tannins, were detected in the soluble extract of the fruit pulp; neither soluble anthocyanidins nor anthocyanins were present. In the soluble cell wall fraction, two hydroxycinnamic acid derivatives were predominant, whereas in the insoluble cell wall fraction, the anthocyanidin delphinidin, which is reported in banana cell walls for the first time, was predominant. Cell wall fractions showed remarkable antioxidant capacity, especially after acid and enzymatic hydrolysis, which was correlated with the total phenolic content released after the hydrolysis of the water-insoluble polymer, but not for the posthydrolysis water-soluble polymer. The acid hydrolysis released various monosaccharides, whereas enzymatic hydrolysis released one peak of oligosaccharides. These results indicate that banana cell walls could be a suitable source of natural antioxidants and that they could be bioaccessible in the human gut.

INTRODUCTION

Cell wall-bound phenolics and polymers, such as lignin, are known to strengthen plant cell walls, for example, iso-dityrosine and diferulate linkages in cereal cell walls and lignins in most plants. Preformed defense compounds (phytoanticipins) for resistance against bacterial and fungal pathogens and insect pests include phenolics such as avenacosides and benzoxazinoid glucosides, which are found in cereals, and various nonphenolic compounds in other species (*1−3*).

The *Musa* species (Musaceae) (specifically bananas) are important fruit crops in many tropical countries (*4*). Bananas are not generally considered to be especially rich sources of (poly)phenolics as compared to banana peels or other fruits, but they are a good source of various nutrients (*4*). Recent studies have shown that there are significant levels of total free phenolics in the pulp ranging from 11.8 to 90.4 mg of gallic acid equivalents 100^{-1} g of fresh weight (*5*). An evaluation of banana pulp as a source of cloud components for the juice industry also revealed that bananas have high phenolic content (138 mg of gallic acid equivalents L^{-1}) (*6*). Banana fruit pulp has also been found to have significant levels of total cell wall bound phenolics (ethyl acetate-soluble cell wall phenolics) (4.4 mg of gallic acid equiva-

lents 100^{-1} g of cell wall) and water-soluble cell wall phenolics (29.9 mg of gallic acid equivalents 100^{-1} g of cell wall) (*7*). Various soluble phenolics have also been identified in the pulp of banana fruits: gallic acid, catechin, gallocatechin, and naringenin 7-*O*-neohesperoside (*4*, *8*). Significant levels of tannins have been reported in unripe and ripe fruits, spent pulp, and the fruit juices of different Ugandan bananas (*9*). The total phenolic content has been measured in flour derived from green bananas with and without peels (1401 ± 98 and 747 ± 19 mg of catechol 100^{-1} g, respectively) (*10*). Various diarylheptanoids (**Figure 1**) that induce phase II enzymes and quinone reductase have been identified in French plantain (*Musa × paradisiaca*) fruits (*11*). A triploid banana hybrid (Flhorban 920; AAA group) had higher levels of phenolics at all stages of the fruit development process as compared with a control banana (Grand Naine; AAA group) but showed similar qualities in relation to all other physicochemical parameters (*12*). Banana pulp is also a rich source of various catecholamines, with dopamine being predominant (**Figure 1**) (*13*). Soluble condensed tannins have been identified in banana pulp by HPLC, using a postcolumn derivation procedure, as having the value of 0.14 mg 100^{-1} g of fresh weight (identified as epicatechin, epigallocatechin, and a gallocatechin−catechin dimer) (*14*). In a second HPLC study, using fluorescence detection, the total soluble tannin levels were found to be 4.0 mg 100^{-1} g of fresh weight; they were identified as monomers, dimers, trimers, and
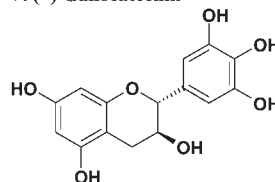
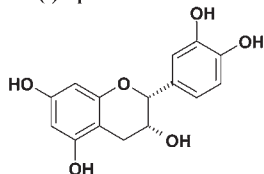*Author to whom correspondence should be addressed (e-mail hojak@usp.br).
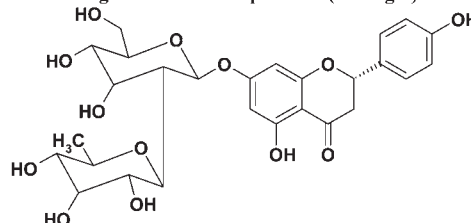
I. Dopamine

II. Gallic Acid
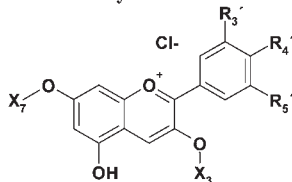
III. *trans*-Ferulic
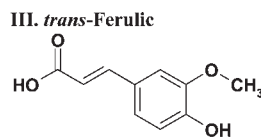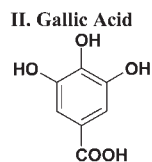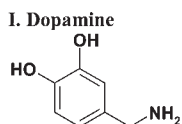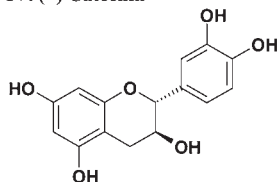
IV. (+) Catechin

V. (+) Gallocatechin

VI. (-) Epicatechin

VII. Naringenin 7-*O*-Neohesperoside (Naringin)

VIII. Anthocyanins

Cy-3-Rha-7-Glc (R3′= R4′= OH, R5′= H, X3 = Rha, X7 = Glc)
Delphinidin-3-Rut (R3′= R4′= R5′= X7 = OH, X3 = Rut)
Cyanidin-3-Rut (R3′= R4′= X7 = OH, R5′= H, X3 = Rut)
Petunidin-3-Rut (R4′= R5′= X7 = OH, R3′= OCH₃, X3 = Rut)
Pelargonidin-3-Rut (R4′= X7 = OH, R3′= R5′= H, X3 = Rut)
Peonidin-3-Rut (R3′= OCH₃, R4′= X7 = OH, R5′= H, X3 = Rut)
Malvidin-3-Rut (R3′= R5′= OCH₃, R4′= X7 = OH, X3 = Rut)
Dephinidin Aglycone (R3′= R4′= R5′= X3 = X7 = OH)

IX. Leucocyanidin
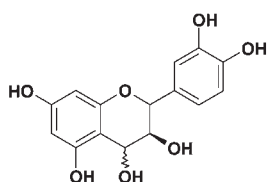
X. *Rel*-(3*S*, 4a*R*)-8-hydroxy-3-(4-hydroxyphenyl)-9-methoxy-4a,5,6,-10b-tetrahydro-3*H*-naphtho[2,1-*b*]pyran
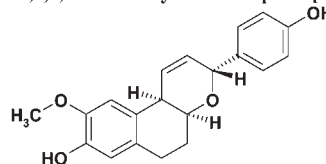
XI. 1,2-Dihydro-1,2,3-trihydroxy-9-(4-ethoxyphenyl)phenalene
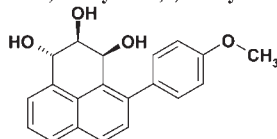
XII. Hydroxyanigorufone
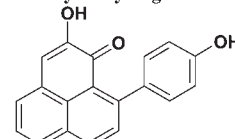
XIII. 2-(4-Hydroxyphenyl)naphthalic anhydride
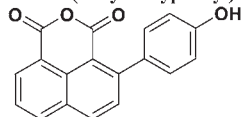
XIV: 1,7-bis(4-hydroxyphenyl)hepta-4(*E*),6(*E*)-dien-3-one
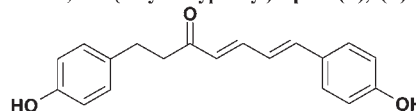
Figure 1. Chemical structures of phytochemicals commonly reported in tissues of various *Musa* species.

oligomers [DP4−6] of the procyanidin type (*15*). Increases in the levels of tannins, as well as the associated increases in flavan-3,4-diols, have been reported to occur in the postinfestation process of banana roots with the nematode *Radopholus similis* (*16*). Soluble anthocyanins (predominantly rutinosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin; **Figure 1**) have been detected in the bracts of bananas and were evaluated as a source of functional food ingredients and as natural colorants (*17−19*). Leucocyanidin has also been identified in unripe plantain bananas (3.3 mg g^{-1}) (*20*). The treatment

of banana roots with elicitors that are derived from the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* leads to the accumulation of both soluble and cell wall bound phenolics with significant increases in vanillic acid, protocatechuic acid, ferulic acid, and sinapic acid (*21*). The increase in hydroxycinnamic acids is associated with an increase in lignin deposition in the cell walls (*22*).

There are very few reports on the specific composition of the cell wall bound phenolics of bananas and, generally, they focus only on the total phenolic values rather than on specific analyses (*7*).
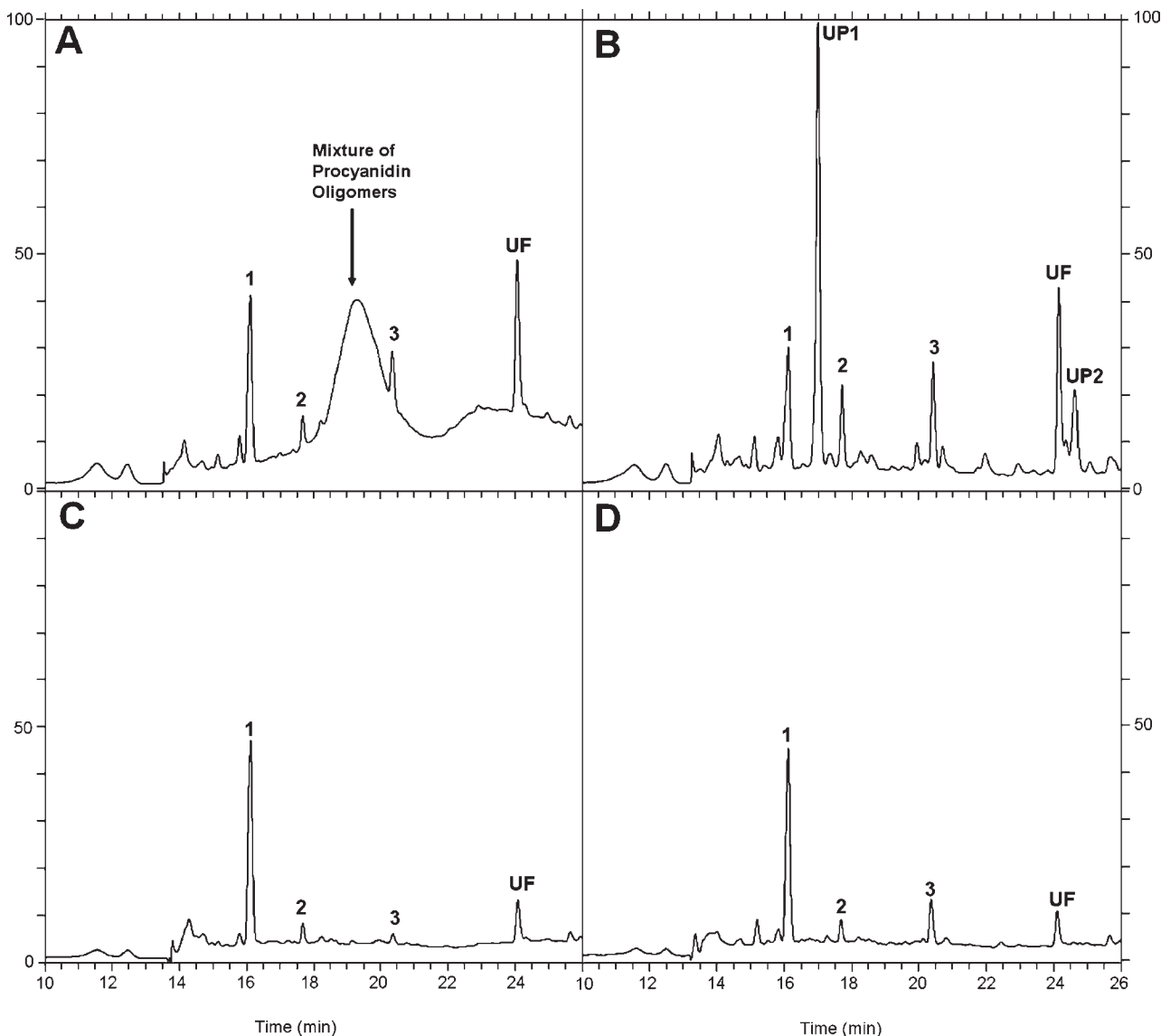
Figure 2. Soluble phenolics in pulp extracts at 270 nm for cv. Mysore at 2 dph (**A**) and 7 dph (**B**) and for cv. Terra at 2 dph (**C**) and 11 dph (**D**). Chromatograms are in the same scale for comparison. Peak ID: 1, gallocatechin; 2, (+)-catechin; 3, (−)-epicatechin; UF, unidentified flavonoid; UP1 and UP2, unidentified phenolics, found only in cv. Mysore samples.

The aims of the current study are (i) to identify and quantify the different classes of phenolics in pulp and cell wall fractions of different banana cultivars on different days after harvest to further understand the changes in phenolic metabolism in relation to fruit ripening and (ii) to measure the antioxidant activities of the different fractions and the pre- and posthydrolysis treatments as part of an evaluation on their potential health effects.

MATERIALS AND METHODS

Reagents. All of the chemicals and reagents were of analytical grade and were obtained from various commercial sources (Sigma/Aldrich, Merck, and Pronalab). The enzymes pancreatin (P-1750), α-amylase (EC 3.2.1.1, A-3176), and amyloglucosidase (EC 3.2.1.3, A-9913) were obtained from Sigma/Aldrich (Madrid, Spain). Pepsin (EC 3.4.23.1, Merck 7190) was obtained from Merck (Darmstadt, Germany). All of the solvents were of high-performance liquid chromatography (HPLC) grade, and all of the water was ultrapure. Gallocatechin, (+)-catechin, (−)-epicatechin, delphinidin (aglycone), and cyanidin 3-*O*-glucoside standards were obtained from Extrasynthese (Genay, France). The Folin−Ciocalteu reagent, gallic acid, and the hydroxycinnamic acids (*p*-coumaric, caffeic, and ferulic) were obtained from Sigma-Aldrich. HPLC calibration curves were constructed

with 10 μL injections of 0.1, 0.05, 0.025, 0.01, 0.005, and 0.0025 mg mL^{-1} using the standards.

Banana Samples. Banana fruit (*Musa acuminata* Juss.) cultivars Terra and Mysore (both in the AAB group), Figo (ABB), Pacovan (AAB), and Nanicão (AAA group) were harvested in the same plantation located in Itapetininga (São Paulo State, Brazil). Bananas approximately 110 days after anthesis (daa) were stored at a temperature of 20 °C and a moisture level of about 90% under control. Bananas were sampled on the basis of their respiration and ethylene production, along the pre- and postclimacteric, including the first stage of senescence. The samples were composed of 10 banana fingers (at least), which were peeled, sliced, immediately frozen in liquid N$_2$, and stored at −80 °C. Representative samples of the stages of ripening (mature-green and ripe) were chosen on the basis of their ethylene, respiration, and starch profiles.

Starch Content. Starch from frozen samples (100 mg) was made soluble in 3 mL of 0.5 M NaOH. After neutralization with 3 mL of 0.5 M acetic acid, an aliquot was precipitated with 4 mL of 80% ethanol. The precipitated starch was hydrolyzed with amyloglucosidase (EC 3.2.1.3) (28 U mL^{-1}), and the resultant glucose was determined by the glucose−oxidase (EC 1.1.3.4)−peroxidase (EC 1.11.1.7)−ABTS (2,2′-azinobis[3-ethylbenzthiazoline] sulfonate) system.

Ethylene and CO$_2$ Emission Measurements. For the ethylene and respiration analysis, the bananas were enclosed in 1.5 L jars (three fingers

Table 1. Quantification of Soluble Specific Phenolics, Total Procyanidins, and Total Phenolics from the Pulp of Banana Cultivars at Different Days Postharvest (dph) and Significance of Changes

cultivar	stage[a] (dph)	specific phenolics[b]				total procyanidins[d]	total phenolics[e]
		gallocatechin	(+)-catechin	(−)-epicatechin	unidentified flavonoid[c]		
Figo	2	61.5 ± 8.9	60.0 ± 5.6	198.4 ± 6.4	57.1 ± 16.2	2.2 ± 0.1	9.0 ± 0.2
	16	37.3 ± 0.6	76.1 ± 11.2	55.9 ± 3.0	41.4 ± 2.6	1.6 ± 0.2	8.1 ± 0.1
		**	**	***	**	*	**
Nanicão	0	487.1 ± 34.7	47.7 ± 0.3	193.5 ± 6.9	42.0 ± 9.1	3.5 ± 0.4	9.3 ± 0.7
	18	542.0 ± 17.7	59.1 ± 2.6	459.8 ± 16.2	43.9 ± 2.8	4.0 ± 0.9	9.9 ± 0.2
		***	**	***	ns	*	ns
Terra	2	450.5 ± 11.2	33.3 ± 3.5	26.7 ± 4.4	36.3 ± 4.9	21.3 ± 0.7	7.2 ± 0.5
	11	418.1 ± 22.6	39.4 ± 2.2	92.4 ± 4.7	29.5 ± 0.4	20.0 ± 0.1	6.5 ± 0.1
		*	*	***	*	ns	*
Mysore	2	355.0 ± 10.0	51.4 ± 1.8	86.8 ± 3.8	117.7 ± 6.8	124.7 ± 2.1	18.9 ± 1.4
	7	255.3 ± 10.9	143.2 ± 7.4	214.3 ± 9.7	119.8 ± 11.4	86.1 ± 0.6	9.8 ± 0.2
		***	***	***	ns	***	***
Pacovan	1	382.7 ± 28.7	82.2 ± 4.9	17.9 ± 1.6	80.4 ± 3.6	3.2 ± 0.1	8.4 ± 1.4
	24	295.2 ± 22.4	69.7 ± 9.4	33.9 ± 6.4	83.1 ± 9.3	1.7 ± 0.2	6.7 ± 0.2
		***	*	**	ns	**	*

[a] The first stage of banana ripening means mature green banana and the second means ripe banana. [b] Individual phenolics are expressed as mean ± SEM μg/100 g of dry weight pulp. No free hydroxycinnamic acids (including ferulic acid), anthocyanidins, anthocyanins, or naringin was detected in the pulp. ***, **, *, significant at 0.001, 0.01, 0.05, respectively; ns, not significant ($P > 0.05$). [c] The unidentified flavonoid (λ_{max} 360 nm) is expressed as mean ± SEM μg of quercetin equivalents/100 g of dry weight pulp. [d] Total procyanidins expressed as mean ± SEM μg of procyanidin oligomers/g of dry weight pulp. [e] Total phenolics expressed as mean ± SEM mg of GAE equivalents/g of dry weight pulp.

per jar; six jars for each cultivar). After 1 h, samples of 10 mL for ethylene analysis and 1 mL for CO_2 analysis were taken from the jars' headspace by using a gastight syringe and injected into a gas chromatograph (HP-6890, Agilent Technologies). A flame ionization detector was employed for ethylene analysis, and a thermal conductivity detector was used for CO_2 analysis. For both gases, the column used was the HP-Plot Q (30 Mts., i.d. 0.53 mm, Agilent Technologies); the injector and detector temperatures were 250 °C, and the runs were isothermic at 30 °C. Fluxes of helium carrier gas were 1 mL min^{-1} for ethylene and 4 mL min^{-1} for CO_2. The injections were made in the pulsed splitless mode for ethylene and in the split mode for CO_2 analysis (50:1). The ethylene and CO_2 standards for synthetic air (Air Liquid Ltd.) were used for the calibration curves.

Extraction and Analysis of Soluble Pulp Phenolics. Samples (three 40 mg replicates) were weighed and extracted using a standard method that has been previously described (*23*). Essentially, two of the replicates were extracted with 1 mL of 70% (v/v) methanol, and a third replicate was extracted with 950 μL of 70% (v/v) methanol and 50 μL of 1 mg mL^{-1} rutin in 100% methanol (internal standard). All of the samples were heated at 70 °C for 30 min with vortex mixing every 5 min to optimize extraction. After extraction, the samples were centrifuged at 4 °C for 20 min at 17000*g*, and the supernatants were transferred to 1.5 mL HPLC vials. These supernatants were used for total phenolic analyses (Folin−Ciocalteu) and reverse-phase HPLC analyses.

Preparation of Banana Pulp Cell Walls. For the cell wall preparations, the frozen banana pulp was powdered in a mortar with liquid N_2, and three 8-g replicates of this powdered pulp were incubated with 200 mL of chloroform/methanol (1:1, v/v) for 1 h at 70 °C for the extraction of pigments, lipids, and various secondary metabolites and also to inactivate cell wall degrading enzymes. The suspensions were centrifuged for 10 min at 10000*g*, and the supernatants were discarded. The remaining residues were washed at least five times with acetone and allowed to dry at 25 °C; they were submitted to the previously described chemical/enzymatic method of extraction used to remove starch and protein and isolate cell wall water-soluble polymers (WSP) and water-insoluble polymers (WIP) (*24*).

Trifluoroacetic Acid (TFA) Hydrolysis of WSP and WIP Fractions. Hydrolysis was carried out using different known weights of samples (between 19 and 40 mg depending on the cell wall material available) in 2-mL screw-cap microtubes with 1 mL of 2 M TFA containing 1 mM butylated hydroxytoluene (BHT) as an antioxidant.

Hydrolysis was done at 80 °C for 2 h; the samples were vortex mixed every 15 min to optimize extraction. After extraction, the samples were left to cool at 20 °C for 10 min and then centrifuged at 4 °C for 30 min at 15500*g* using a Sigma 16K centrifuge. The supernatants were removed with 1-mL disposable syringes and filtered (0.2 μm Whatman universal filter) into 1.5-mL screw-cap HPLC vials. The supernatant that was obtained from the 2-h acid hydrolysis was used for measuring the antioxidant activities and sugar contents. The WIP pellets, still red in color after the first hydrolysis, had an additional 1 mL of hydrolysis reagent added and were rehydrolyzed for a further 16 h at 80 °C.

Enzymatic Hydrolysis of WSP and WIP Fractions. The WSP and WIP fractions of ripe bananas were sequentially incubated with digestive enzymes according to the method of Saura-Calixto et al. (*25*). Briefly, 100 mg of the sample was dissolved in 5 mL of 0.2 M HCl−KCl (pH 1.5) and incubated with 0.1 mL of pepsin (300 mg mL^{-1} solution dissolved in the same buffer) at 40 °C for 1 h, followed by the sequential addition of 4.5 mL of a 0.1 M phosphate buffer (pH 7.5) and 0.5 mL of pancreatin (5 mg mL^{-1} solution in the same buffer). Then, incubation at 37 °C for 6 h occurred. The solution was adjusted to a pH of 6.9 by the addition of 1.0 mL of 0.2 M HCl, and it was incubated with 0.5 mL of α-amylase (120 mg mL^{-1} solution in 0.1 M tris-maleate buffer, pH 6.9) at 37 °C for 16 h. After incubation, the samples were centrifuged at 25 °C for 15 min at 3000*g*, and the supernatant was removed. Exactly 5 mL of a 0.2 M acetate buffer (pH 4.75) was added to the supernatant and incubated with 100 μL of amyloglucosidase (400 U mL^{-1}) for 45 min at 60 °C. The supernatants were stored at −18 °C prior to the antioxidant capacity and total phenolic measurements. For HPLC analysis, the samples were filtered (0.2 μm Whatman universal filter) into 1.5-mL screw-cap HPLC vials.

Quantification of Total Phenolics. The total amount of phenolics was determined using a Folin−-Ciocalteu method that was previously described (*26*). Essentially, 10 μL of the supernatant was diluted with 40 μL of 70% (v/v) methanol in a 10-mL glass screw-cap tube, followed by the sequential addition of 2.5 mL of 1:10 diluted (with ultrapure water) commercial Folin−Ciocalteu reagent (Sigma-Aldrich) and 2.0 mL of 7.5% (w/v) sodium carbonate (in ultrapure water). The tubes were sealed and heated at 45 °C for 15 min in a water bath. The absorbance of each sample was read using 1-cm path length disposable cuvettes in a Hitachi U-2000 spectrophotometer that was set at 765 nm. A calibration curve with different concentrations of gallic acid (2.0, 1.0, 0.5, 0.25, 0.1, 0.05 mg. mL^{-1}) was produced, and the total amount of phenolics was expressed as milligrams of gallic acid equivalents (GAE) per gram.

Article

J. Agric. Food Chem., Vol. 58, No. 13, 2010 **7995**

Table 2. Anthocyanidins in Water-Soluble (WSP) and Insoluble (WIP) Polymer Fractions at Different Days Postharvest (dph) and Significant Differences

cultivar	stage[a] (dph)	cell wall fraction	A1	A2	A3	A4	A5	total
					anthocyanidins[b]			
Figo	1	WSP	0	0	0	0	0	0
	16	WSP	0	0	0	0	0	0
	1	WIP	0	0.58 ± 0.17	0.03 ± 0.01	0.22 ± 0.06	0.06 ± 0.01	0.89 ± 0.25
	16	WIP	0	1.09 ± 0.14	0.07 ± 0.01	0.33 ± 0.06	0.07 ± 0.01	1.57 ± 0.22
				**	*	*	ns	**
Nanicão	0	WSP	0	0	0	0	0	0
	18	WSP	0	0	0	0	0	0
	0	WIP	0	T	0	0	0	0
	18	WIP	0	0	0	0	0	0
Terra	1	WSP	0	0.04 ± 0.01	0	0.04 ± 0.004	0	0.09 ± 0.00
	12	WSP	0	0.04 ± 0.00	0	0.04 ± 0.01	0	0.08 ± 0.01
				ns		ns		ns
	1	WIP	0.25 ± 0.01	7.74 ± 0.70	0.25 ± 0.01	1.87 ± 0.05	0.18 ± 0.01	10.29 ± 0.75
	12	WIP	0.28 ± 0.00	7.51 ± 0.55	0.25 ± 0.01	2.00 ± 0.11	0.24 ± 0.02	10.27 ± 0.67
			ns	ns	ns	ns	ns	ns
Mysore	1	WSP	0	0.48 ± 0.15	0	0.07 ± 0.04	0	0.55 ± 0.19
	7	WSP	0	0.33 ± 0.04	0	0.06 ± 0.00	0	0.38 ± 0.04
				(0.69)		(0.86)		(0.69)
	1	WIP	3.52 ± 0.08	40.09 ± 5.28	0.39 ± 0.06	2.34 ± 0.03	0.22 ± 0.01	46.55 ± 5.93
	7	WIP	4.12 ± 0.54	41.35 ± 8.67	0.22 ± 0.08	2.25 ± 0.32	0.20 ± 0.07	48.14 ± 9.68
			*	ns	**	ns	ns	*
Pacovan	1	WSP	0	0	0	0	0	0
	24	WSP	0	0	0	0	0	0
	1	WIP	0	0.55 ± 0.13	0.02 ± 0.01	0.17 ± 0.02	0.02 ± 0.01	0.75 ± 0.15
	24	WIP	0	0.92 ± 0.34	0.04 ± 0.02	0.29 ± 0.10	0.03 ± 0.01	1.27 ± 0.46
				**	ns	*	ns	**

[a] The first stage of banana ripening means mature green banana, and the second means ripe banana. [b] Anthocyanidins expressed as mean ± SEM μg of delphindin equivalents/100 g of cell wall. A2, delphinidin aglycone; T, trace detected but not accurately quantifiable. ***, **, *, significant at 0.001, 0.01, 0.05, respectively; ns, not significant ($P > 0.05$).

Quantification of Total Condensed Tannins. The total number of condensed tannins was determined using the acid/*n*-butanol method, which was previously described (*27*). Essentially, 75 μL of each extract replicate was added to 1.4 mL of HCl/*n*-butanol (5:95, v/v) in a screw-cap 2-mL microtube, sealed, and heated at 95 °C for 2 h. The samples were centrifuged at 4 °C for 10 min at 15493*g* using a Sigma 2-16K centrifuge. The absorbance of each supernatant was read using 1-cm path length disposable cuvettes in a Hitachi U-2000 spectrophotometer set at 555 nm. A calibration curve was produced using partially purified tannin oligomers that were prepared from the pulp of a locally available banana. The total amount of condensed tannins was expressed as micrograms of procyanidin oligomers per gram.

Preparation of Banana Fruit Condensed Tannin Standard. Banana pulp (500 g) was flash-frozen with liquid nitrogen and then freeze-dried for 5 days (Dura Dry μP from FTS Systems, Stone Ridge, NY). The dried pulp was powdered using a commercial blender. A subsample (75 g dry weight) was weighed into a 500-mL round-bottom flask, and 300 mL of 70% methanol was added. The sample was extracted in a water bath at 70 °C for 1 h with manual shaking for 10 s every 10 min to optimize the extraction. The sample was filtered through muslin cloth and transferred to a 500-mL round-bottom flask; it was rotary evaporated with the water bath set at 40 °C. The postevaporation volume was 80 mL. A silica solid-phase extraction column (60-mL plastic column with a sorbent bed volume of 2-cm diameter, 10-cm height) was sequentially prewashed with 40 mL of 100% methanol followed by 80 mL of ultrapure water. The sample was loaded onto the column using a Vac-elut SPE system. The unbound (loading) and first elution (60 mL of 5% methanol/95% dichloromethane)

were discarded, and the tannins were eluted with 30 mL of 100% methanol. The tannin fraction was transferred to a preweighed 50 mL glass beaker; the solvent was removed using a stream of N_2 gas. The beaker was reweighed and the dry material (yield was 19.2 mg) was resuspended in 100% methanol to produce a 5 mg mL^{-1} stock solution, which was then used for producing the tannin calibration curve.

HPLC Analysis of Soluble Pulp and Cell Wall Bound Phenolics. HPLC analysis was performed using a Thermo Surveyor HPLC (solvent degasser, quaternary pump, thermostatically controlled autosampler [set at 10 °C], a thermostatically controlled column oven [set at 25 °C] and a diode array detector that was set to collect data at 227, 270, 370, and 520 nm and 200−600 nm overall) in combination with a Phenomenex Luna C_{18} (2) (250 × 4.6 mm, 5 μm) with a Phenomenex Securityguard pre-column with a C_{18} cartridge (http://www.phenomenex.com/cms400 min/index.aspx?ID = 31&langtype = 1033); this is a previously optimized multiphytochemical method (*23*): solvent A = 0.1% (v/v) TFA in ultrapure (distilled, deionized) water; solvent B = 0.1% (v/v) TFA in HPLC grade acetonitrile, flow rate = 1 mL min^{-1}. The gradient was previously reported (*23*). Calibration injections (10 μL of 0.1, 0.05, 0.025, 0.01, 0.005, and 0.0025 mg mL^{-1}) were performed using the various commercial phenolic standards. The phenolics in the samples were confirmed by co-injection (spiking) and spectral comparisons with the authentic standards.

Antioxidant Activity Measurements of WIP and WSP. For the DPPH assay, the antioxidant capacity was assessed on the basis of the DPPH free radical scavenging activity assay according to the method of Brand-Williams et al. (*28*). For an evaluation of the antioxidant capacity of the WIP and WSP without hydrolysis, 2−5 mg of the sample was

Table 3. Other Phenolics in Water-Soluble (WSP) and Insoluble (WIP) Polymer Fractions at Different Days Postharvest (dph) and Significant Differences

cultivar	stage[a] (dph)	cell wall fraction	HCA1[b]	HCA2[b]	total procyanidins[c]	total phenolics[d]
Figo	1	WSP	5.1 ± 0.1	4.2 ± 0.0	0	24.48 ± 0.5
	16	WSP	12.3 ± 0.8 **	8.9 ± 0.1 **	0	22.45 ± 0.4 *
	1	WIP	58.8 ± 8.5	55.9 ± 0.5	T	13.41 ± 0.8
	16	WIP	49.7 ± 0.2 **	54.1 ± 1.0 ns	T	16.54 ± 0.2 *
Nanicão	0	WSP	22.7 ± 0.1	13.0 ± 0.1	0	28.64 ± 0.7
	18	WSP	26.6 ± 0.1 *	15.2 ± 0.1 *	0	26.81 ± 0.5 *
	0	WIP	74.5 ± 0.2	42.7 ± 0.4	T	20.65 ± 0.2
	18	WIP	112.9 ± 1.1 ***	60.5 ± 1.0 **	T	15.94 ± 0.2 **
Terra	1	WSP	7.5 ± 0.1	6.49 ± 0.11	T	34.97 ± 0.6
	12	WSP	3.9 ± 0.1 **	3.88 ± 0.05 **	T	39.11 ± 0.4 **
	1	WIP	21.5 ± 0.8	23.48 ± 0.89	37.5 ± 0.1	21.64 ± 0.4
	12	WIP	19.2 ± 1.1 *	22.20 ± 0.17 ns	46.3 ± 0.1 *	19.30 ± 0.0 *
Mysore	1	WSP	3.3 ± 0.2	3.3 ± 0.3	T	32.69 ± 1.4
	7	WSP	40.9 ± 1.9 ***	33.7 ± 1.2 ***	T	38.97 ± 1.3 **
	1	WIP	2.7 ± 0.3	6.9 ± 0.0	204.7 ± 1.0	29.68 ± 1.5
	7	WIP	1.3 ± 0.0 *	4.5 ± 0.3 *	259.4 ± 1.0 **	31.03 ± 1.8 *
Pacovan	1	WSP	4.4 ± 0.1	3.4 ± 0.0	0	24.08 ± 0.4
	24	WSP	9.0 ± 0.1 **	6.5 ± 0.1 **	0	23.36 ± 0.4 ns
	1	WIP	26.2 ± 0.3	26.0 ± 0.1	T	18.14 ± 0.1
	24	WIP	23.3 ± 2.9 *	24.6 ± 2.5 ns	T	18.18 ± 1.1 ns

[a] The first stage of banana ripening means mature green banana and the second means ripe banana. [b] HCA, unidentified hydroxcinnamic acid derivatives; from spectra they are most likely ferulic acid derivatives (see **Figure 4**); expressed as mean ± SEM μg of ferulic acid equivalents/100 g of cell wall. ***, **, *, significant at 0.001, 0.01, 0.05, respectively; ns, not significant ($P > 0.05$). [c] Total procyanidins expressed as mean ± SEM μg of procyanidin oligomer equivalents/g of cell wall); T, trace (detected but not accurately quantifiable). [d] Total phenolics expressed as mean ± SEM mg of GAE equivalents/g of cell wall.

resuspended in 1 mL of methanol. Under agitation, aliquots of 100 μL were added to 1.0 mL of DPPH in methanol (0.5 mM) and reacted during 25 min in the dark. Furthermore, the sample was briefly centrifuged (10000g), and 240 μL of the supernatant was added to the microplate. The absorbance was measured at 517 nm using the Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA). The analyses were done in triplicate. The supernatant of the WIP and WSP that was obtained from the acid hydrolysis was first neutralized to a pH of 7.0 by the addition of 1 M NaOH because acidic conditions cause interference in the DPPH reaction. A 40-μL aliquot of the supernatants that was previously diluted (WIP and WSP from enzymatic and acid hydrolysis) and 200 μL of DPPH (0.5 mM) was added to the microplate; after 25 min, the absorbance was measured at 517 nm. The control consisted of a Trolox solution at different concentrations. The antioxidant capacity was expressed as micromoles of Trolox equivalents per gram of cell wall.

The ORAC (oxygen radical absorbance capacity) assay was performed according to the method of Prior et al. (*29*). The WIP and WSP supernatant that was obtained from enzymatic hydrolysis was initially acidified with 0.5 M perchloric acid (1:1, v/v) and centrifuged at 10000g for 5 min at 25 °C to precipitate the protein. An appropriate dilution was made with 75 mM phosphate buffer (pH 7.0). Twenty-five microliter aliquots of the diluted samples were added to the 96-well microplate, and 150 μL of 40 nM fluorescein (FL) in 75 mM phosphate buffer (pH 7.0) was added to the microplate, followed by 25 μL of 2,2′-azobis(2-amidinopropane) dichloride (53 mM) in 75 mM phosphate buffer (pH 7.0); readings were initiated immediately. The fluorescence measurements were recorded over 80 min at 5-min intervals using a Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT) set to 37 °C having excitation and emission wavelengths set to 485 dnd 525 nm, respectively. The results were

calculated using the differences in the areas under the quenching curves of fluorescein between a control (phosphate buffer instead of the sample) and a sample. Data are expressed as micromoles of Trolox equivalents (TE) per gram of the sample (μmol of TE g^{-1} of cell wall).

Analysis of Sugars Released from Acid- and Enzyme-Hydrolyzed WIP and WSP. The soluble sugars that were released during acid hydrolysis were analyzed using a HPAEC-PAD (DX500, Dionex, Sunnyvale, CA) and a PA10 column (250 × 4 mm, Dionex) with the respective precolumn. An isocratic elution was done using ultrapure water and 300 mM NaOH in the postcolumn for sugar detection. The flow rate was 1 mL min^{-1}, and the column temperature was set at 30 °C. The sugar standards used were fucose, arabinose, rhamnose, galactose, glucose, xylose, and mannose. These initial analyses were qualitative, and no quantification was done. The detection of oligosaccharide that was released from enzymatic hydrolysis was also analyzed by HPAEC-PAD (DX500, Dionex), using a Dionex CarboPac PA-100 column (250 mm × 4 mm, Dionex) with the respective precolumn. The elution was done according to the method of Jacobs et al. (*30*), in which, essentially, a 150 mM NaOH and sodium acetate gradient is used. The flow rate was 1 mL min^{-1}, and the column temperature was set at 30 °C. The oligosaccharide was expressed as the degree of polymerization (DP), and it was estimated according to the retention time and compared with data obtained by the acid hydrolysis of starch.

Statistical Analysis. The data were analyzed using one-way ANOVA. The differences between the means were separated by Duncan's comparison test. The results are presented as means ± standard error of the mean (SEM). The significance level for the separation was set at $P < 0.05$. Statistical analyses were performed using the statistical program called Super-ANOVA v. 1.11 software (Abacus Concepts, Berkeley, CA).

Article

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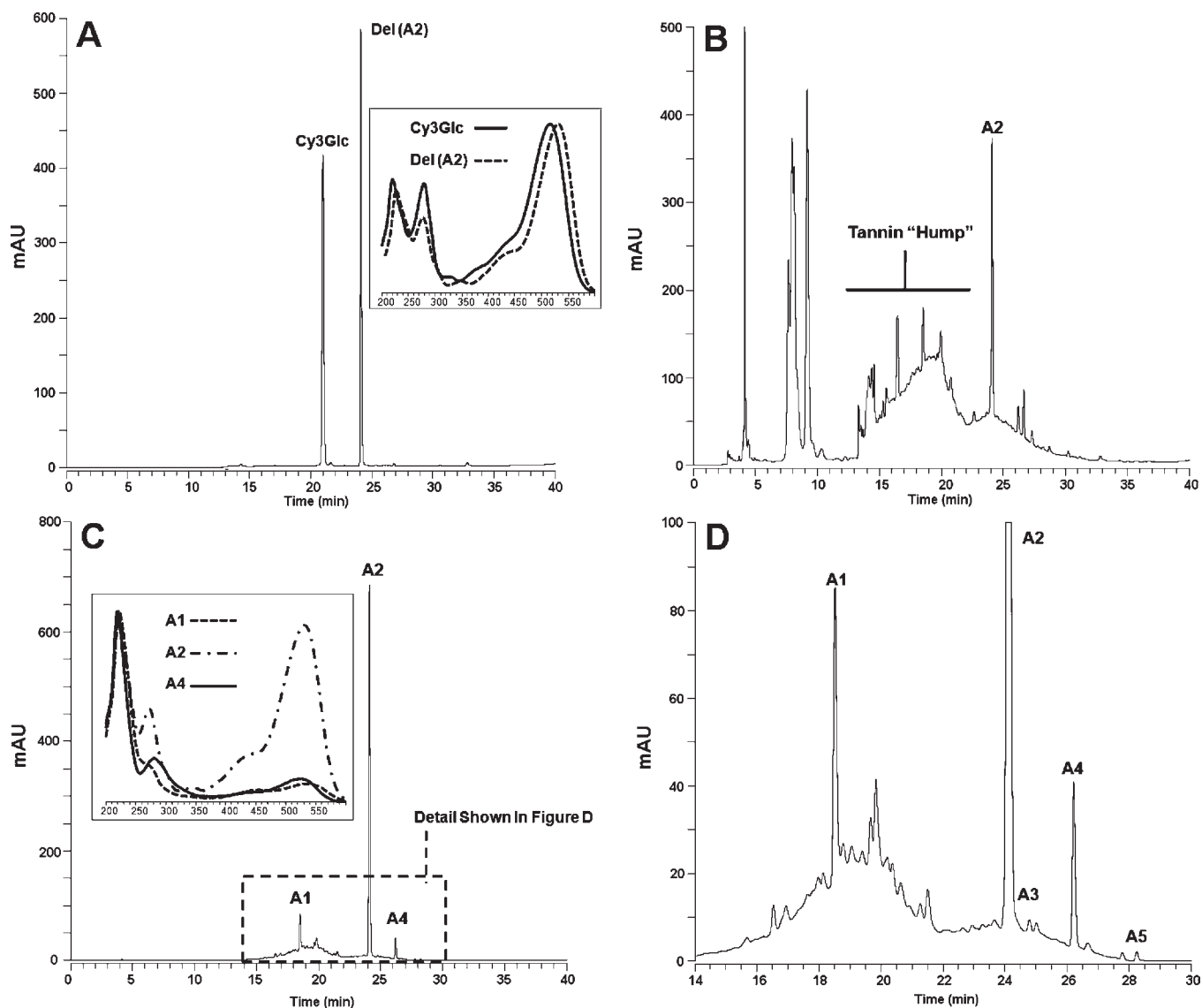
Figure 3. Chromatograms of anthocyanin standards at 270 nm (**A**; inset is UV−visible spectra of each standard) and a sample of 7 days postharvest Mysore WIP posthydrolysis sample (no interfering peaks have come from the antioxidant BHT) at 270 nm (**B**), full range at 520 nm (**C**; inset is UV−visible spectra of anthocyanidins A1, A2, and A4), and detail of anthocyanidin region at 520 nm (**D**). Peak ID: Cy3Glc, cyanidin 3-*O*-glucoside; Del, delphinidin aglycone; A1, A3, A4, and A5. anthocyanidins (from their UV−visible spectra); A2, delphinidin aglycone.

RESULTS AND DISCUSSION

Soluble Phenolics. Various soluble phenolics have previously been identified in the pulp of bananas, such as naringin (naringenin 7-*O*-rhamnoglucoside, various monomeric flavan-3-ols, and commonly gallocatechin and catechin) and oligomeric flavan-3-ols (procyanidins not greater than DP 6) (*4*, *8*). In the current study, four major methanol-soluble phenolics and a mixture of procyanidins were detected in the pulp (**Figure 2**; **Table 1**). Three of the phenolics that were identified were flavan-3-ols; the fourth one was an unidentified flavonoid with a λ_{max} of 360 nm. With the exception of the cultivar Nanicão, the levels of total phenolics decreased after harvesting, especially in cv. Mysore (**Table 1**). The total procyanidin levels also decreased during maturation, and this was of major significance in the Mysore; the exceptions were cv. Terra and cv. Nanicão (**Table 1**). There were significant increases in the levels of (+)-catechin and (−)-epicatechin in cv. Mysore and significant increases in (−)-epicatechin in the cultivars Nanicão, Terra, and Pacovan (**Table 1**). Naringin, previously reported in banana pulp, was not detected in any of the cultivars that were analyzed in the current study. The total phenolics levels

$(6.5−18.9 \, \text{mg of GAE g}^{-1}$ of dry weight) are lower than the lowest values that have been previously reported $(11.8 \, \text{mg g}^{-1}$ of fresh weight) (*5*), but the total phenolic levels previously reported have varied greatly depending on the cultivar and the method of extraction and analysis used (*4−10*).

Cell Wall Anthocyanidins. Acid hydrolysis of the two cell wall fractions (WSP and WIP) led to the release of various cell wall bound simple phenolics and polyphenolics (**Tables 2** and **3**; **Figure 3**). Cell wall bound anthocyanidins were released from the WIP fraction of cv. Mysore and, to a lesser extent, from cv. Terra (**Table 2**). The other cultivars either had very low (cv. Figo and cv. Pacovan) or no detectable anthocyanidins (cv. Nanicão). After the first cell wall hydrolysis treatment, there was a persistently strong red color in all of the WIP pellets (cell wall residues posthydrolysis treatment) even after a further 16 h in the 2 M acid. This pigment suggested that the linkages of some of the anthocyanidins to the cell walls could be through C−C bonds for example, anthocyanidin−C−C−polysaccharide, anthocyanidin−C−C−polyphenolic (lignin)−polysaccharide, and/or anthocyanidin−C−C−suberin. These types of anthocyanidin linkages

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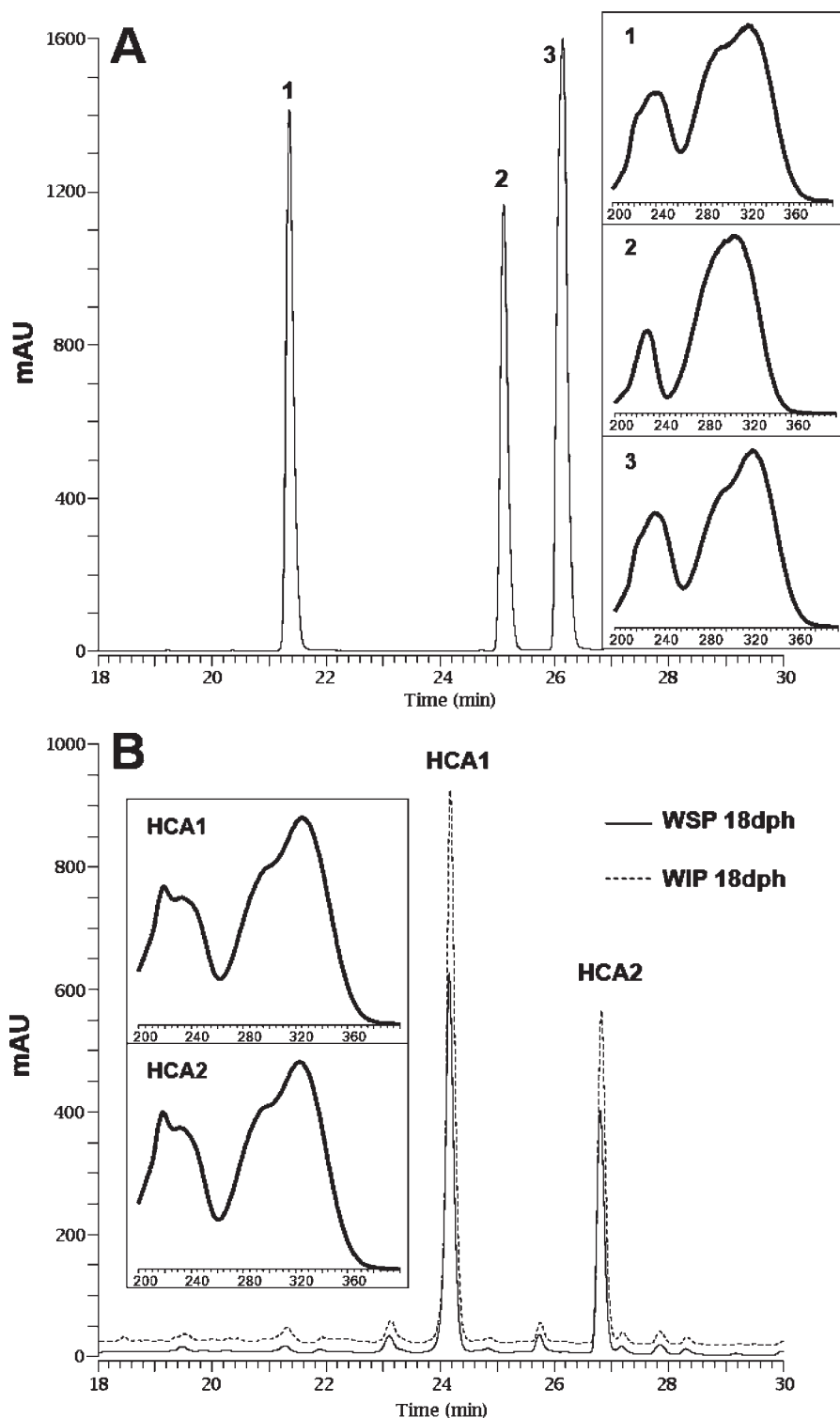
Figure 4. Hydroxycinnamic acid standards at 325 nm (**A**) and cell wall WSP and WIP (**B**) from cv. Nanicão 18 dph. (Inset) UV−visible spectra of standards of caffeic acid (peak 1), *p*-coumaric acid (peak 2) ferulic acid (peak 3), and HCA1 and HCA2 in the WSP sample.

would not be hydrolyzed by acid, as compared with the acid-labile C−O linkages of anthocyanidins to polyphenolics and/or poly-saccharides. Anthocyanidin−cell wall conjugates have been reported in various mosses and liverworts and, more recently, in the periderm cell walls of sweet potato tubers; the postulated linkage is a C−O bond to suberins (*31*). The current paper is only the second one to report the presence of anthocyanidin−cell wall conjugates in angiosperms. It is not clear what the role of these cell wall bound anthocyanidins is, whether it relates to cell wall strengthening functions and/or defense functions against pathogens. Because cv. Mysore and its clones (Mysore Poovan and Malaikali) are classified as resistant or partially resistant to black leaf streak disease and the banana nematode, a role in defense may be possible (*32−34*). This role is supported by the fact that cv. Nanicão (similar to Grand Nain) is considered to be highly susceptible to black leaf streak disease and is lacking these anthocyanidins (*34*).

Cell Wall Hydroxycinnamic Acids. In the current study, two major hydroxycinnamic acid derivatives (HCA1 and HCA2) were released from WSP and WIP by acid hydrolysis (**Table 3**;

Figure 4). Clear differences were seen in the levels of HCA1 and HCA2, depending on the cultivar and the days after harvest. The highest levels of HCA1 were found in the WIP fraction of cv. Nanicão (112.9 μg of ferulic acid equivalents 100^{-1} g of cell wall), and significant levels of HCA1 were also found in the WIP fractions of cv. Figo (58.8 μg) and to a lesser extent in cv. Pacovan (26.2 μg) and cv. Terra (21.5 μg). Interestingly, the levels of HCA1 and HCA2 in the WIP fraction of cv. Mysore were the lowest of all the cultivars, but this cultivar had the highest levels of HCA1 and HCA2 in the WSP fraction (40.9 and 33.7 μg, respectively). The highest levels of HCA2 were found in the WIP fractions of cv. Nanicão (60.5 μg) and cv. Figo (55.9 μg). The lowest levels of HCA2 were found in the WSP fractions of cv. Mysore (3.3 μg) and cv. Pacovan (3.4 μg). Many simple phenolics have been shown to occur in cell walls, including various hydroxycinnamic acids. Banana fruits have previously been shown to contain high levels of *trans*-ferulic acid (5.4 mg 100^{-1} g of fresh weight) and low levels of *p*-coumaric acid (0.46 mg) and caffeic acid (0.20 mg) (*35*). Other parts of the banana plant have also been reported to contain high levels of *trans*-ferulic acid (*36*). Several other simple aromatic compounds have also been detected: vanillic acid, *p*-hydroxybenzoic acid, and syringic acid (*35*). However, none

of these acids was soluble, and these acids were only released after hydrolytic treatment of the tissues; therefore, they are covalently linked to the cell walls. In the current study, the two hydroxycinnamic acid derivatives (HCA1 and HCA2) were predominant;
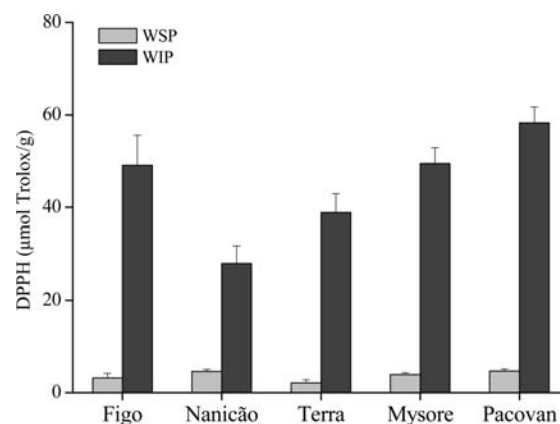
Figure 5. Antioxidant capacity of WSP and WIP fractions without hydrolysis evaluated by the DPPH method.
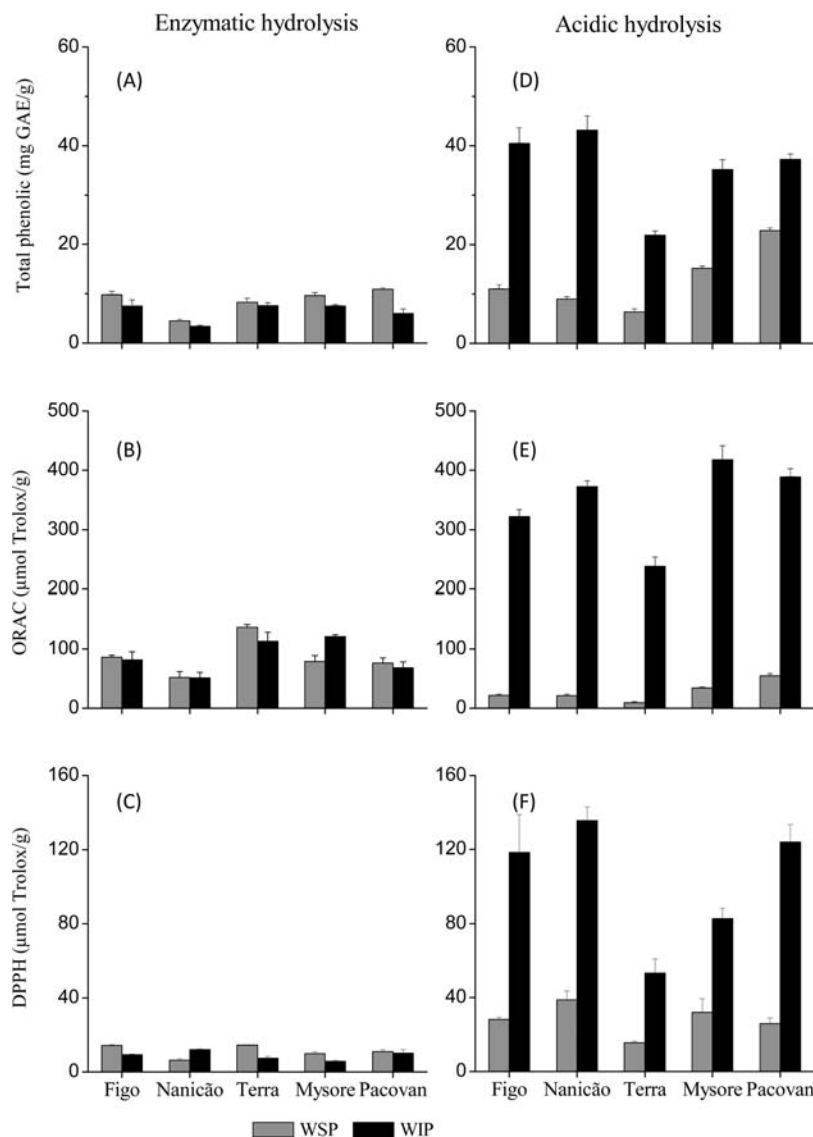
Figure 6. Total phenolic content and antioxidant capacity evaluated with the ORAC and DPPH methods of soluble fraction of WIP and WSP fractions submitted to enzymatic hydrolysis (**A**−**C**) and acid hydrolysis (**D**−**F**).
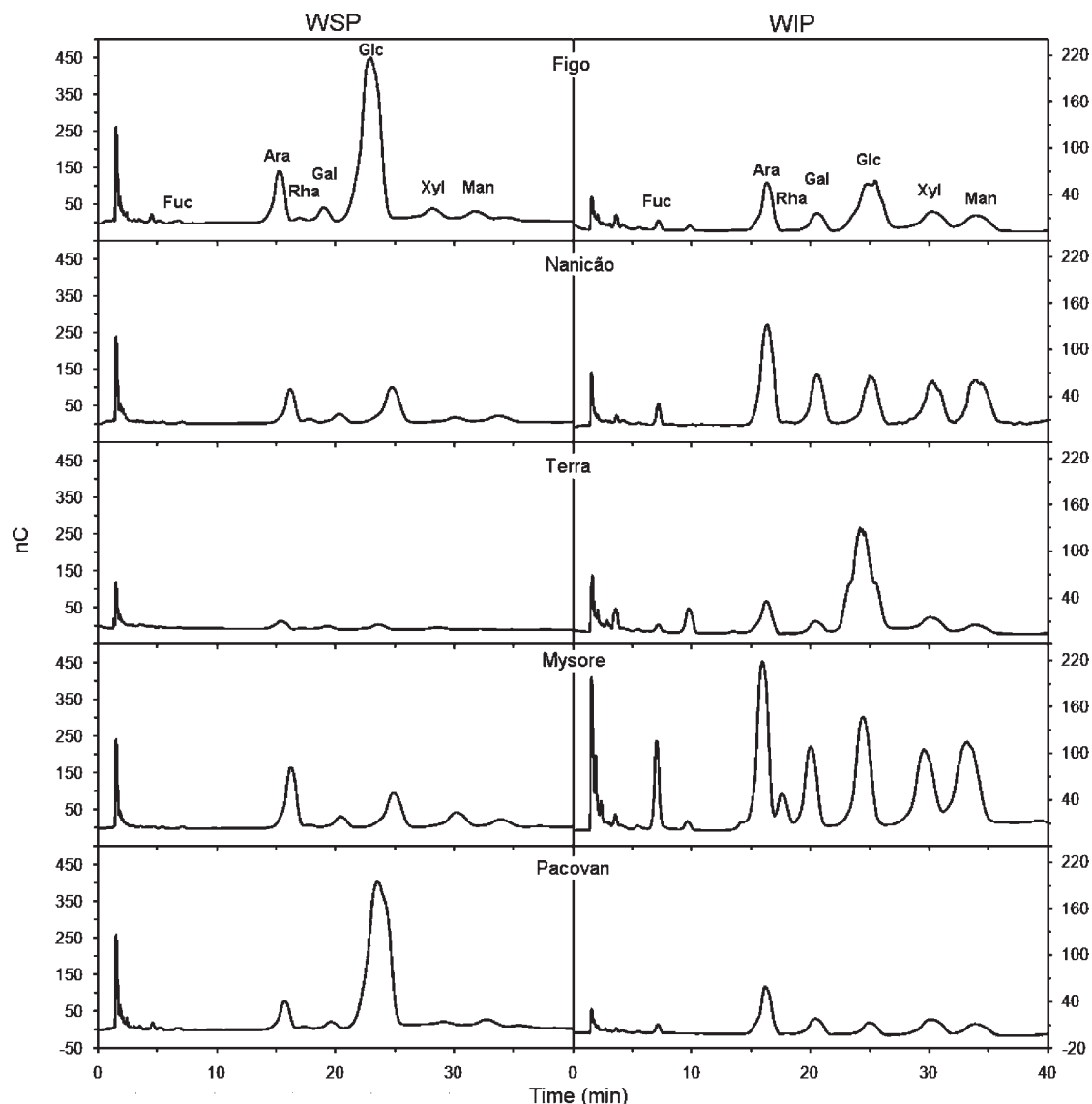
Figure 7. Chromatogram profile of soluble sugar after acid hydrolysis of WIP and WSP. Identified peaks: Fuc, fucose; Ara, arabinose; Rha, rhamnose; Gal, galactose; Glc, glucose; Xyl, xylose; Man, mannose.

on the basis of the earlier studies showing high levels of cell wall bound ferulic acid and the UV−visible spectral comparison of HCA1 and HCA2 with ferulic acid, it seems possible that these are two ferulic acid derivatives, yet this will have to be confirmed by LC-MS.

Cell Wall Total Condensed Tannins. Trace amounts of condensed tannins were detected in the acid- hydrolysates of the WSP fractions of cv. Terra and cv. Mysore; they were not detected in the WSP of any of the other cultivars (**Table 3**). A significant amount of condensed tannins was detected in the acid hydrolysate of the WIP of cv. Mysore and to a much lesser extent in cv. Terra (**Table 3**). Only trace amounts of condensed tannins were detected in the WIP fractions from the other cultivars. There were small but significant increases in the incorporation of condensed tannins into the WIP, and, after harvesting, in cv. Terra and cv. Mysore. Tannins and other aromatic polymers, such as lignins, are associated with increased cell wall strength and defense mechanisms (*1−3*). In banana cell wall, condensed tannins probably fulfill the same function.

Cell Wall Total Phenolics. The highest levels of total phenolics were found in the WSP and WIP fractions of cv. Mysore (38.97 and 31.03 mg of GAE g^{-1} of cell wall, respectively) and cv. Terra

(21.64 and 39.11 mg of GAE g^{-1} of cell wall, respectively) (**Table 3**). These cultivars seem to be particularly rich in all classes of cell wall bound phenolics. There also appeared to be significant increases in the total phenolics in the WSP fractions of cv. Terra and cv. Mysore, indicating further incorporation of phenolics into the cell walls after harvesting. The other cultivars contained lower levels of total phenolics in the WSP and WIP fractions, and changes after harvesting were of low significance with the exception of cv. Nanicão, for which the total phenolics in the WIP decreased (**Table 3**). Many simple phenolics have been identified in plant cells walls, for example, derivatives of simple benzoic and hydroxycinnamic acids. The function of these phenolics is not always clear, but, in several cases, they appear to be involved in cell wall strength and possibly in plant defense mechanisms; this may also be the case for banana fruits either directly as antimicrobial and antifungal agents or as physicochemical barriers to bacterial and fungal pathogens (*1−3*).

Antioxidant Activity of WIP and WSP. The antioxidant activity of WIP and WSP was evaluated using the DPPH method, which was initially carried out without the hydrolysis of these fractions (**Figure 5**). Both intact fractions demonstrated antioxidant activities; the WIP (28−58 μmol of TE g^{-1} of cell wall) was approximately
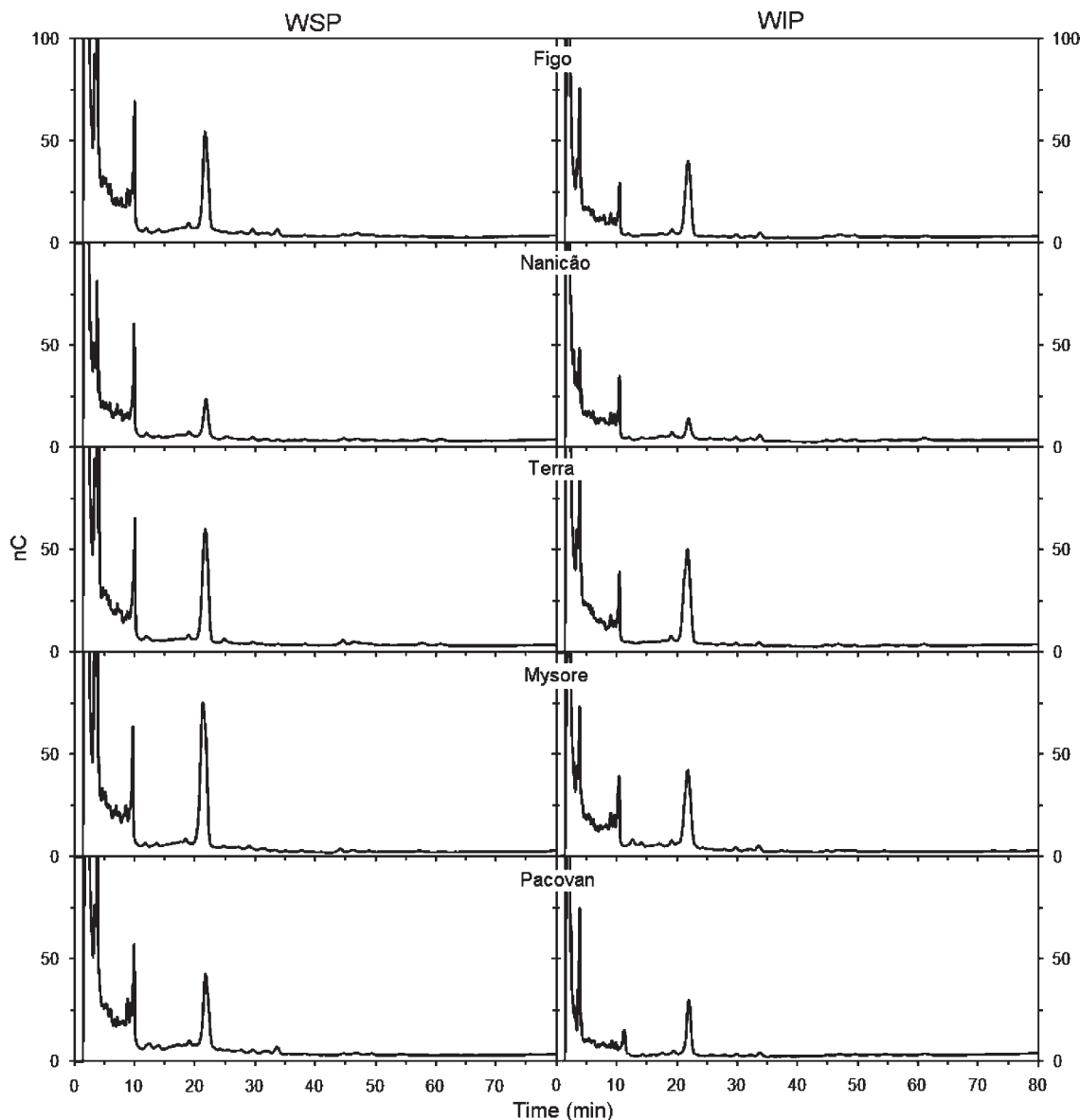
Figure 8. Chromatogram profile of oligosaccharide released after enzymatic hydrolysis of WIP and WSP fractions. The peak corresponds to an oligosaccharide having a degree of polymerization around 10−12 (RT = 21 min).

10 times more active than the WSP (2.1−4.7 μmol of TE g^{-1} of cell wall). The antioxidant activities were then compared using the DPPH assay for the untreated and hydrolyzed (acid or enzyme) WIP and WSP fractions. For WIP, the order of activity was acid hydrolysis > without hydrolysis > enzymatic hydrolysis, which was probably due to the highest release of phenolic compounds after acid treatment (**Figure 6A**−**C**). On the other hand, the antioxidant capacity of WSP was acid hydrolysis > enzymatic hydrolysis > without hydrolysis. After acid hydrolysis, among WIP, the cultivars Nanicão, Figo, Pacovan, and Mysore presented the highest phenolic contents and antioxidant capacities (ORAC and DPPH assays), and a lesser value was observed for cv. Terra. In this case, the antioxidant capacity was more influenced by the higher content of hydroxycinnamic acid derivatives released by the cultivars Nanicão and Pacovan than by the higher content of anthocyanidins that was released by cv. Mysore (**Table 2**). When both fractions were submitted to enzymatic hydrolysis, the WSP released more phenolics and had a higher antioxidant capacity, which was evaluated by ORAC and DPPH assays, than WIP undergoing the same hydrolysis did (**Figure 6D**−**F**). However, enzymatic hydrolysis had a lower phenolic content and lower antioxidant capacity as compared

to acid hydrolysis. From a physiological point of view, the enzymatic hydrolysis data are more representative than the acid hydrolysis data, demonstrating that digestive enzymes could also increase the bioaccessibility of the phenolic compounds from WIP and WSP of bananas. In WIP, in both hydrolysis conditions, a high correlation was observed between the phenolics released and the antioxidant capacity that was measured by DPPH ($R^2 = 0.96$), the phenolics released and the antioxidant capacity that was measured by ORAC ($R^2 = 0.98$), and between antioxidant capacity measured by ORAC and DPPH methods ($R^2 = 0.92$). On the other hand, in both hydrolysis conditions, WSP did not show any correlation between these parameters ($R^2 = -0.05$; $R^2 = 0.46$; $R^2 = -0.52$, respectively), demonstrating that other nonphenolic compounds, such as nonstarch polysaccharides released by enzymatic hydrolysis, could be involved in the antioxidant activity of WSP.

Several groups have shown that there is an underestimation of the phenolic contents and the antioxidant capacities of fruits and vegetables because polyphenols that are associated with dietary fiber (PDF) were not considered. In some fruits, the PDF content was demonstrated to be higher than the free polyphenols content (2−5 times higher) after acid hydrolysis (*37*). This suggests that

these phenolics could be bioaccessible in the human gut (*25*), as demonstrated by in vitro digestion, and make a significant contribution to the overall antioxidant activity of these foods. The bioavailability of the PDF was also demonstrated by increased plasma antioxidant capacity after an acute intake of dietary fiber rich in cell wall bound polyphenols by humans (*38*). In the present study, WIP and WSP are contributory sources of bioactive compounds (upon consumption of bananas), and they could act as antioxidants in an intact form even after digestive degradation.

Sugar Profiling of Acid- and Enzyme-Hydrolyzed WSP and WIP. Some papers emphasize dietary fiber (mainly the in-soluble fraction) as a potential source of bioactive compounds, for example, the formation of short-chain fatty acids, and as having a potential antioxidant capacity role. However, little is known about the soluble fraction. In this study, WSP demon-strated potential antioxidant capacity in its intact form as well as after digestive and acid hydrolysis treatments that released antioxidant compounds. Because the WSP antioxidant capa-city was not correlated with the phenolics released after hydrolysis, it possibly could be correlated with the water-soluble polysaccharides from WSP. The acid hydrolysis of both fractions released six monosaccharides (arabinose, rham-nose, galactose, glucose, xylose, and mannose). Fucose was detected only in WIP. Seven monosacharides were released from the WIP of cv. Mysore and, to a lesser extent, from cv. Pacovan (**Figure 7**). In WSP, the cultivars Figo and Pacovan released all six monosacharides, mainly glucose, whereas cv. Terra had low detectable monosaccharide content (**Figure 7**). On the other hand, enzymatic hydrolysis of both fractions released an oligosaccharide with a mean degree of polymeri-zation (DP) of 10−12 (**Figure 8**), eluting as a single peak at 21 min. The DP was estimated according to the retention time and was compared with data obtained by acid hydrolysis of starch (*30*). The water-soluble pectin polysaccharides derived from apple cell, pectin, and polygalacturonic acid A (PolyGalA) appear to increase the antioxidant activity of ascorbic acid. PolyGalA exerts its strongest effect on ascorbic acid activity, indicating that the homogalacturonan backbone rather than the neutral side chain of pectin polysaccharides is implicated in the observed effect (*39*). In WSP, the oligosaccharide released by enzymatic hydrolysis could be responsible for the antioxidant capacity.

In conclusion, banana pulp contains several different classes of free and cell wall bound phenolics, including anthocyanidins. Anthocyanidins have only been reported in the cell walls of one other angiosperm, the sweet potato. It is clear from these initial studies that a more detailed and careful chemical and enzymatic dissection of banana cell wall fractions, in combination with LC-MS, needs to be done to fully identify the HCA derivatives and identify the linkages of the acid hydrolysis released anthocyani-dins and the remaining cell wall bound anthocyanidins that could not be released by acid treatment. Further studies need to be performed to evaluate the role of the cell wall bound phenolics, for example, comparing the resistance of cv. Nanicão with that of cv. Mysore against economically important banana pathogens and measuring both pre- and postinfection changes in the phenolics. In addition, banana pulp was demonstrated to be a source of bioactive phenolics, mainly flavan-3-ols. The levels of flavan-3-ols changed after ripening, probably due to their in-corporation into condensed tannins and/or catabolic processes. Chemical and in vitro digestion indicated that the cell wall bound phenolics from bananas are bioaccessible and could be a suitable source of natural antioxidants in addition to the free phenolics in pulp.

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