Free and Conjugated Polyamines and Phenols in Raw and Alkaline-Clarified Sugarcane Juices

Roberto de Armas,[†] Maritza Martinez,[†] Carlos Vicente,[‡] and María-Estrella Legaz*[,‡]

Department of Plant Physiology, Faculty of Biology, Havana University, Havana, Cuba, and Department of Plant Physiology, Faculty of Biology, Complutense University, 28040 Madrid, Spain

Sugarcane juice contains a lot of sucrose associated with several monosaccharides, defined as low molecular mass carbohydrates (LMMC), as well as some polysaccharides and glycoproteins, which are defined as mid and high molecular mass carbohydrates (MMMC and HMMC, respectively). These three categories of carbohydrates can be separated by size-exclusion chromatography through Sephadex G-10 and Sephadex G-50 columns, but elution profiles change drastically after juice clarification performed by adjusting the pH value of the juice to 8.0. In addition, polyamines and some phenolics are currently associated with carbohydrate preparations, and the distribution pattern of these conjugates also changes after clarification. Polyamine levels generally decrease after juice clarification. Cadaverine is completely removed from the different carbohydrate preparations, whereas spermidine is the main polyamine occurring in association with sugarcane carbohydrates, as free or acid-soluble form in LMMC preparation or as acid-soluble and -insoluble forms in both MMMC and HMMC preparations. Polyamines, presumably spermidine, conjugate to *p*-hydroxybenzoic acid in LMMC, mostly to caffeic acid in MMMC, and to syringic acid in HMMC preparations. HMMC-associated polyamines appear in both acid-soluble and -insoluble fractions. Syringic acid also occurs in the LMMC preparation, but juice clarification changes it from acid-soluble to free form, and it coelutes with sucrose.

Keywords: *Carbohydrates; juice; phenols; polyamines; sugarcane*

INTRODUCTION

Sugarcane juice contains soluble substances and solids in suspension that can modify the color of the final product (Pauletti et al., 1996). Juice clarification using alkaline chemicals (Laluce et al., 1993) or flocculating agents (Tong et al., 1984) is essential to obtain a white, good-looking, high-quality product. Flavonoids (Laucas, 1986) and phenolics (Patton, 1992) have been extensively considered as the main metabolites having influence on the sugar color. The major concentration of colorants has been found on the longitudinal, *b*-axis of the sugar crystal; ∼25% of the colorants are adsorbed on its surface and, in addition, polyphenol concentration increases with the crystal size (Sanyal et al., 1992). The major flavonoids in sugarcane are flavones, all of them derivatives of either naringenin, tricin, apigenin, or luteolin (Laucas, 1986; De Armas et al., 1998). They are biogenetically related, because the flavones apigenin and luteolin are produced by oxidation of the flavonone, naringenin. One of the enzymes responsible for this conversion has been purified and characterized from several higher plants (Britsch et al., 1981; Spribille and Forkmann, 1984) and requires molecular oxygen, Fe^{2+}, and 2-oxoglutarate as cofactors. Flavonoids are also present in raw sugar obtained from sugarcane, in which they constitute a significant portion of the contaminating colorants (McGhie, 1993). In addition, some feruloyl conjugates to sucrose have been reported (Meurer et al., 1984). However, they practically disappear from the refined, final product. Phenolics have a very low solubility in water and, as a side effect, they produce conjugates with several compounds, including polyamines, proteins, and polysaccharides. Thus, separation of the aqueous phase after sucrose crystallization and the previous precipitation of macromolecules during clarification could easily remove the major amount of phenolics from the syrup. Phenolics conjugated to polyamines have often been described in higher plants and related to reproductive stages (Martin-Tanguy, 1985). Bokern et al. (1995) reported the isolation and identification of four hydroxycinnamic acid amides from pollen of *Quercus dentata* as spermidine derivatives. Conjugation mainly affects tyramine conjugated to ferulic and hydroxycinnamic acids (Fleurence and Negrel, 1989; Louis and Negrel, 1991) or spermidine and spermine conjugated to hydroxycinnamic acids (Hedberg et al., 1996).

The color of sugarcane juice due to the presence of phenolics can be increased by the action of polyphenol oxidase, a catechol oxidase-type enzyme (Bucheli and Robinson, 1994) and peroxidase. Sugarcane polyphenol oxidase is more active with chlorogenic acid than with other phenols, and its activity depends on oxygen availability.

Because anionic phenols and polycationic polyamines have been revealed as abundant in several varieties of sugarcane plants, the aim of the present study was, first, to compare the concentration of phenolic compounds and polyamines in both raw and clarified sugarcane juices; second, to find the occurrence of polyamine−phenolic conjugation and, consequently, to

* Author to whom correspondence should be addressed (fax 34 91 394 5034; e-mail melegaz@eucmax.sim.ucm.es).
[†] Havana University.
[‡] Complutense University.

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determine if the clarification procedure changes the pattern of this conjugation.

MATERIALS AND METHODS

Plant Material and Juice Processing. Stalks from 11-month-old plants of *Saccharum officinarum* var. Jaronu 60-5, field-grown, were mechanically crushed immediately after being cut, and the crude juice was filtered through filter paper and adjusted to pH 8.0 by the addition of a saturated solution of sodium carbonate. The juice was then centrifuged at 20000*g* for 15 min at 4 °C. The pellet was discarded, and sodium azide was added to the supernatant to a final concentration of 0.02% (w/v). This preparation was defined as clarified juice. Alternatively, the crude juice was centrifuged at 2000*g* for 15 min at 4 °C. The pellet was discarded, and sodium azide was added to the supernatant as above. This preparation was defined as centrifuged juice.

Both clarified and centrifuged juices were then chromatographed through a column of Sephadex G-10 (15 × 2.5 cm), pre-equilibrated with saturated sodium carbonate for clarified juice or with distilled water for centrifuged juice (Legaz et al., 1990). Elution was carried out with distilled water containing 0.02% (w/v) sodium azide. The pH was adjusted to 8.0 for clarified juice elution. Fractions (1.0 mL) 1−27 were discarded. Fractions 28−35 were collected and considered as a mixture of both high molecular mass carbohydrates (HMMC) and mid molecular mass carbohydrates (MMMC). Fractions 36−57, mainly composed of sucrose, were also collected and considered as low molecular mass carbohydrates (LMMC). This last preparation is composed of sucrose and some monosaccharides (molecular mass varying from 180 to 343). After separation, 3.0 mL of a mixture of the fractions 27−35 was chromatographed through a Sephadex G-50 column (30 × 2.5 cm), pre-equilibrated as described above. Fractions 49−85 contained HMMC, whereas MMMC eluted in fractions 86−157. MMMC contained polymers with a molecular mass of ~9.0 kDa, whereas HMMC was a heterogeneous fraction containing macromolecules with a maximum molecular mass of 870 kDa (Martinez et al., 1990). Eluted fractions were monitored for carbohydrates according to the method of Dubois et al. (1956) and for protein according to the method of Lowry et al. (1951).

Extraction and Analysis of Polyamines. Polyamines were analyzed as their dansyl derivatives by high-performance liquid chromatography (HPLC) using the method described by Escribano and Legaz (1988). Samples of 2.5 mL of juice were mixed with 6.0 mL of 5% (w/v) cold perchloric acid (PCA) containing 150 μL of 5 mM *n*-butylamine as internal standard (Figure 1). The mixtures were stored overnight at 4 °C in plastic tubes and then centrifuged at 48000*g* for 20 min at 2 °C. The supernatant (first supernatant) contained free (S) and nonliberated acid-soluble (SH) polyamines, whereas the precipitate (first precipitate) contained nonliberated acid-insoluble (PH-polyamines). This first precipitate was washed four times with 5% (w/v) cold PCA, resuspendend in 6.0 mL of 1.0 M NaOH containing 150 μL of 5 mM *n*-butylamine, and, finally, stored for 12 h at 4 °C. Aliquots of 2.0 mL of both the first supernatant and the first resuspended precipitate were hydrolyzed with 2.0 mL of 12 M HCl for 18 h at room temperature. After this, hydrolysates were centrifuged at 30000*g* for 15 min at 2 °C. Pellets were discarded, and a second supernatant from the first one and a third supernatant from the first precipitated were obtained. The second supernatant contained both free (S) and liberated, acid-soluble (SH) polyamines, whereas the third supernatant contained acid-insoluble polyamines. Both the second and third supernatants were dried at 40 °C under a stream of air and then resuspended in 1.5 mL of 5% (w/v) cold PCA to be later centrifuged at 6000*g* for 15 min at 2 °C. Precipitates were discarded and supernatants (fourth and fifth supernatants, respectively) were used for derivatization.

Aliquots of 0.2 mL of the first, fourth, and fifth supernatants were dansylated with 0.4 mL of 75 mM dansyl chloride in

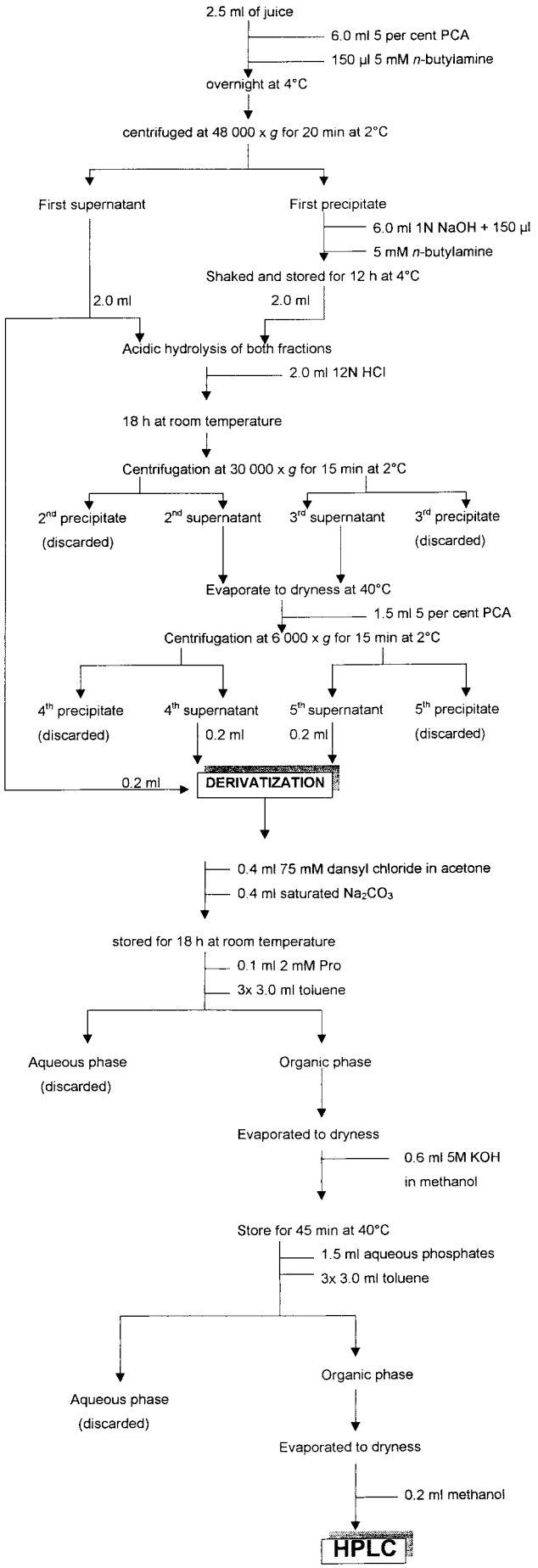

Figure 1. Scheme for extraction and derivatization of polyamines from sugarcane juice.

acetone in the presence of 0.4 mL of saturated sodium carbonate. Dansylation was carried out in a vial hermetically sealed for 18 h at room temperature. Proline (0.1 mL of 2.0 mM) was then added to destroy the excess dansyl chloride. Dansylated polyamines were extracted from the mixtures with 3 × 3.0 mL of toluene (HPLC grade). The toluene phase was dried at 40 °C under a stream of air. Once the derivatization procedure was concluded, samples were cleaned by adding 0.6 mL of 5.0 M KOH in methanol (HPLC grade), according to the method of Seiler and Knödgen (1979). Mixtures were left to stand for 45 min at 40 °C, and then 1.5 mL of an aqueous mixture containing 200 mg of KH_2PO_4 and 200 mg of Na_2HPO_4 was added. Polyamines were extracted again with 3 × 3.0 mL of toluene, as described above. The organic phase was dried, and dry residues were redissolved in 200 μL of methanol (HPLC grade) to be chromatographed.

Polyamines were eluted from a MicroPak MCH-5N cap Varian (15 cm × 4 mm) reverse phase column at 40 °C using a methanol/water gradient (Escribano and Legaz, 1988). Detection was performed by fluorescence intensity measurements.

Extraction and Analysis of Phenolic Acids. An aliquot of 2.0 mL of each polyamine fraction, S, SH, and PH, was first extracted twice with 4.0 mL of chloroform/acetonitrile (60:40 v/v) and second with diethyl ether/ethyl acetate (65:35 v/v). Organic phases were mixed and carried out to dryness under air flow. Residues were redissolved in 0.1 mL of acetonitrile and used for HPLC analysis. HPLC separation was carried out by using a Varian 5000 liquid chromatograph equipped with a Vista CDS 401 computer. Several methods including methanol, orthophosphoric acid (Babic et al., 1993), or tripotassium citrate (Trugo and Macrae, 1984) in the mobile phase were previously assayed. Even the method developed by Torres et al. (1987) consisting of a gradient of acetonitrile and aqueous acetic acid did not provide good separations. The best analysis conditions were as follows: column, reverse phase Nucleosil 5 C-8 (12 cm × 2.6 mm i.d.); injection, 10 μL; mobile phase, solvent A, acetonitrile/water (2:98 v/v), and solvent B, acetonitrile/acetic acid/water (20:2:78 v/v); gradient, from 95% A + 5% B, flow rate 1.0 mL min^{-1} (time = 0), to 30% A + 70% B, flow rate, 1.0 mL min^{-1} (time = 30 min), 20% A + 80% B, flow rate, 1.2 mL min^{-1} (time = 45 min); temperature, 22 °C; aufs, 0.005; detector, UV at 270 nm; internal standard, salicylic acid, 0.5 mg mL^{-1}. Protocatechuic, *p*-hydroxybenzoic, syringic, cinnamic, *p*-coumaric, ferulic, and caffeic acids from Sigma Chemical Co. and chlorogenic acid from Fluka were used as standards. A chromatographic trace of a mixture of standards, including the retention time values of each one, is shown in Figure 2.

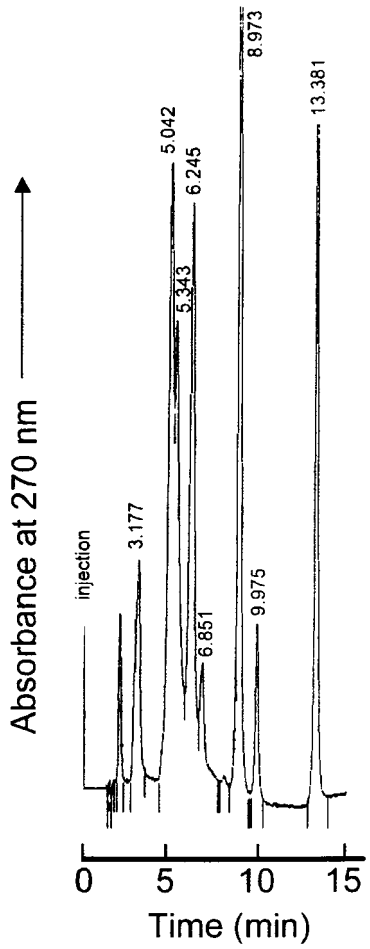

Figure 2. Chromatographic trace of a mixture of standard phenols dissolved in acetonitrile at a concentration of 0.5 mg mL^{-1}. Numbers near peaks give the retention time of the compounds (3.177 min = protocatechuic acid; 5.042 min = *p*-hydroxybenzoic acid; 5.343 min = chlorogenic acid; 6.245 min = caffeic acid; 6.851 = syringic acid; 8.973 min = *p*-coumaric acid; 9.975 min = ferulic acid; and 13.381 min = cinnamic acid).

RESULTS

Separation of LMMC, MMMC, and HMMC from unclarified juice is shown in Figure 3. After the centrifuged juice was chromatographed through a Sephadex G-10 column, a mixture of MMMC and HMMC of a mass of 31.5 mg of total carbohydrates was recovered from fractions 28−35, being 8.0 mL in total volume (Figure 3A). Mixed MMMC and HMMC recovered from the chromatographic separation of the clarified juiced represented 62.5 mg of total carbohydrates (Figure 3B). The pattern of protein elution was always bimaximal. The main protein peak eluted from fractions 15−23 of the centrifuged juice, fractions in which the absence of carbohydrates was assessed. The second protein maximum, eluted from fractions 29−43, was coincident with the major part of carbohydrate forms in the juice, HMMC, MMMC, and LMMC (Figure 3A). Size exclusion chromatography of the clarified juice caused the main peak of protein to show a shift toward fractions 25−35, coeluting with the mixture of HMMC and MMMC, whereas the second peak of protein coeluted with LMMC (Figure 3B).

An aliquot (3.0 mL) of both samples, fractions 28−35, was then chromatographed through a Sephadex G-50 column; 0.135 mg of HMMC and 9.66 mg of MMMC were recovered from the mixture of polysaccharides obtained from centrifuged juice (93.2% of the total carbohydrates loaded onto the column, as is shown in Figure 4A). However, 0.475 mg of HMMC and 5.5 mg of MMMC were recovered from the mixture of polysaccharides obtained from the clarified juice (∼30% of the total carbohydrates loaded onto the column, as shown in Figure 4B). Very low amounts of protein coeluted with both HMMC and MMMC obtained from centrifuged juice. However, a broad peak of protein eluted from fractions 75−109, a zone in which the complete absence of polysaccharides was found (Figure 4A). The pattern of protein elution after a similar sample obtained from clarified juice was chromatographed through Sephadex G-50 was very different. Protein eluted in two clear-cut, broad peaks, absolutely coincident with HMMC and MMMC, respectively (Figure 4B). The amount of protein coeluting with HMMC was ∼6 times higher than that recovered from fractions containing MMMC.

Soluble polyamines (S-PAs) were detected preferentially in samples containing LMMC. They were not detected in samples containing exclusively HMMC

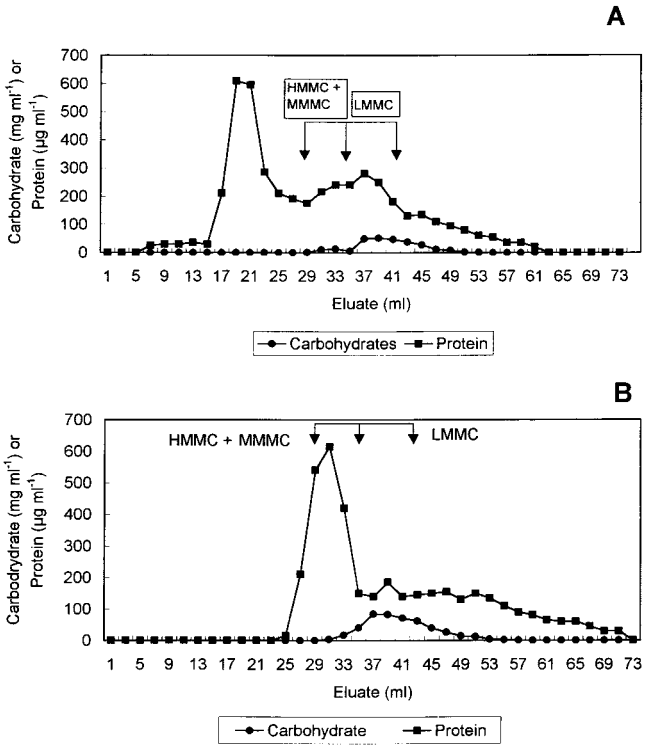

Figure 3. Elution profiles of both carbohydrates and protein from centrifuged juice (A) or clarified juice (B) of sugarcane chromatographed on a Sephadex G-10 column (A) to separate LMMC and a mixture of MMMC and HMMC.

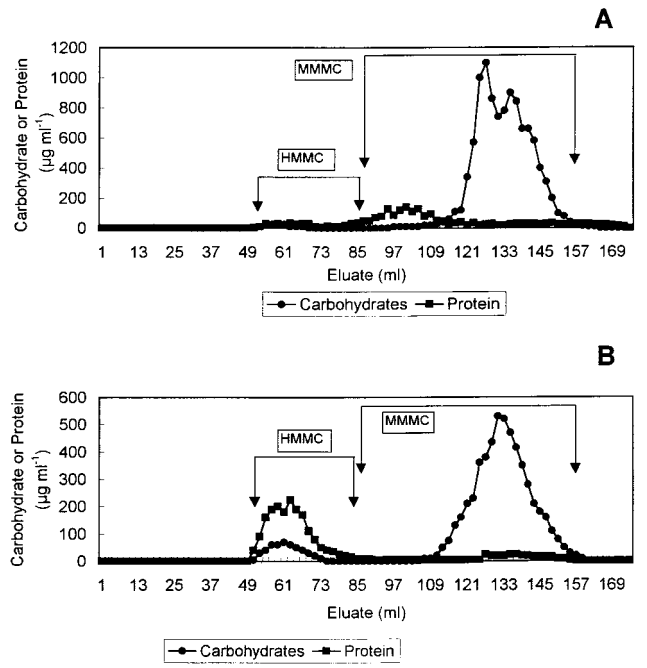

Figure 4. Elution profiles of carbohydrates and protein from the polysaccharide-containing fraction, eluted from the Sephadex G-10 column loaded with centrifuged (A) or clarified (B) juices, chromatographed on a Sephadex G-50 column to separate MMMC and HMMC.

obtained either from centrifuged or from clarified juices. Moreover, S-PAs were detected only in very small amounts (or even undetected, such as S-SPM) as components of samples consisting of MMMC, with the exception of S-SPD (Figure 5). SH-PAs were revealed as minor constituents of HMMC and MMMC or undetected in some samples, such as SH-CAD and SH-SPM

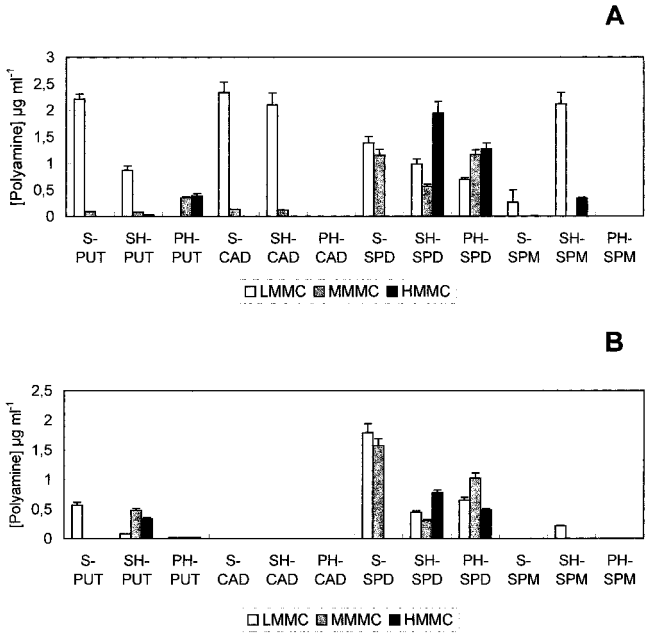

Figure 5. Quantitation of S-, SH-, and PH-polyamines associated with LMMC, MMMC, and HMMC preparations obtained from centrifuged (A) and clarified (B) sugarcane juices. Values are the mean of three replicates, and each experiment was repeated twice. Vertical bars give standard error when it can be drawn.

in both HMMC and MMMC obtained from clarified juice. PH-CAD and PH-SPM were never detected. However, PH-PUT and, especially, PH-SPD were preferentially associated with MMMC and HMMC samples, although both juices and LMMC preparation also contained small amounts of these acid-insoluble polyamines (Figure 5). It is noticeable that juice clarification absolutely removed CAD (S-, SH-, and PH-CAD) from the juice. SPD was always the major polyamine occurring in sugarcane juice.

Centrifuged juice contained three phenolic compounds from the benzoic acid series and three cinnamic acid derivatives. The major part of these phenols appeared to be associated with preparations containing SH-PAs and some of them associated with the preparation containing PH-PAs. Chlorogenic acid, a caffeic acid ester, occurred only as soluble, unconjugated component of the centrifuged juice but disappeared from carbohydrate-enriched preparations (Figure 6). LMMC preparation contained only *p*-hydroxybenzoic and syringic acids as components of the first series of phenols, cinnamic acid traces, and small amounts of both *p*-coumaric and caffeic acids associated with the preparation containing SH-PAs polyamines (Figure 6A). The MMMC preparation from centrifuged juice was revealed as the lowest phenol-containing sample with only syringic, *p*-coumaric, caffeic, and ferulic acids being present in the preparation. All of them appeared to be associated with SH-PAs (Figure 6B). The HMMC preparation had a similar phenolic content but also contained cinnamic acid, so the practically total amount of this compound contained in the centrifuged juice was recovered in this preparation (Figure 6C).

After clarification, a lot of syringic acid occurred in the S-fraction from LMMC in which *p*-hydroxybenzoic acid was the main phenol associated with the SH-fraction (Figure 7A). MMMC contained only cinnamic, *p*-coumaric, and caffeic acids as components of the SH-fraction in a concentration <0.2 μg mL^{-1} (Figure 7B).

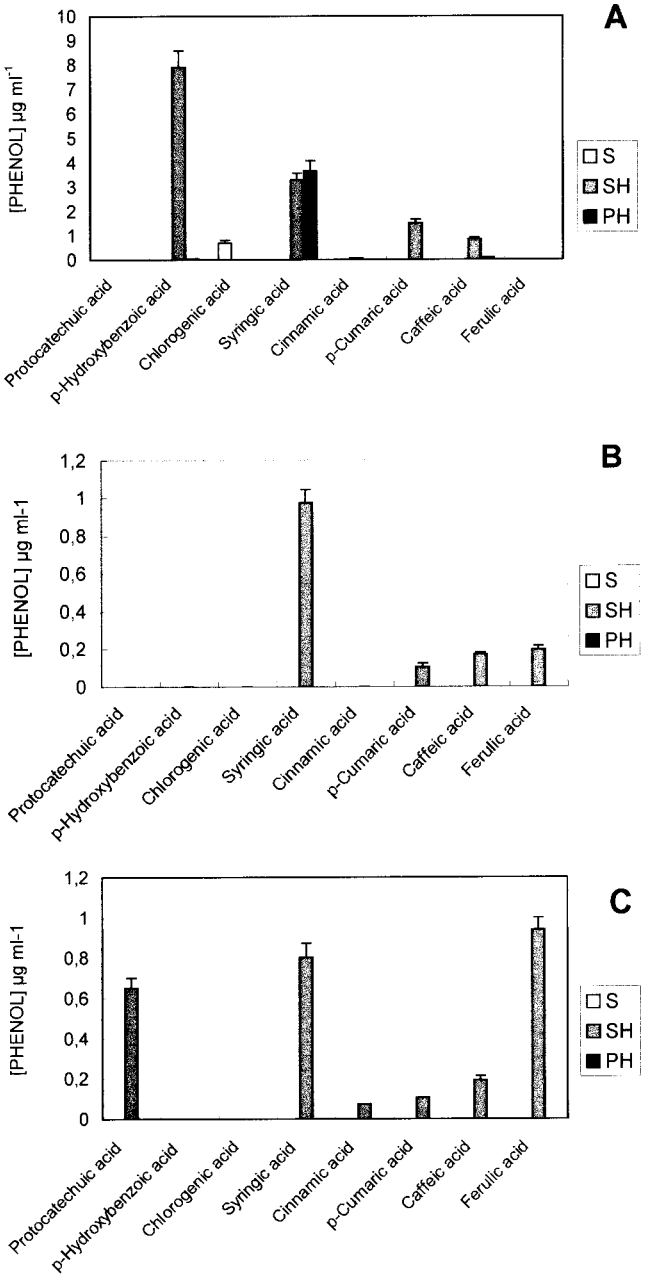

Figure 6. Quantitation of phenolics associated with S-, SH-, and PH-polyamines occurring in LMMC (A), MMMC (B), and HMMC (C) preparations obtained from centrifuged sugarcane juice. Values are the mean of three replicates, and each experiment was repeated twice. Vertical bars give standard error when it can be drawn.

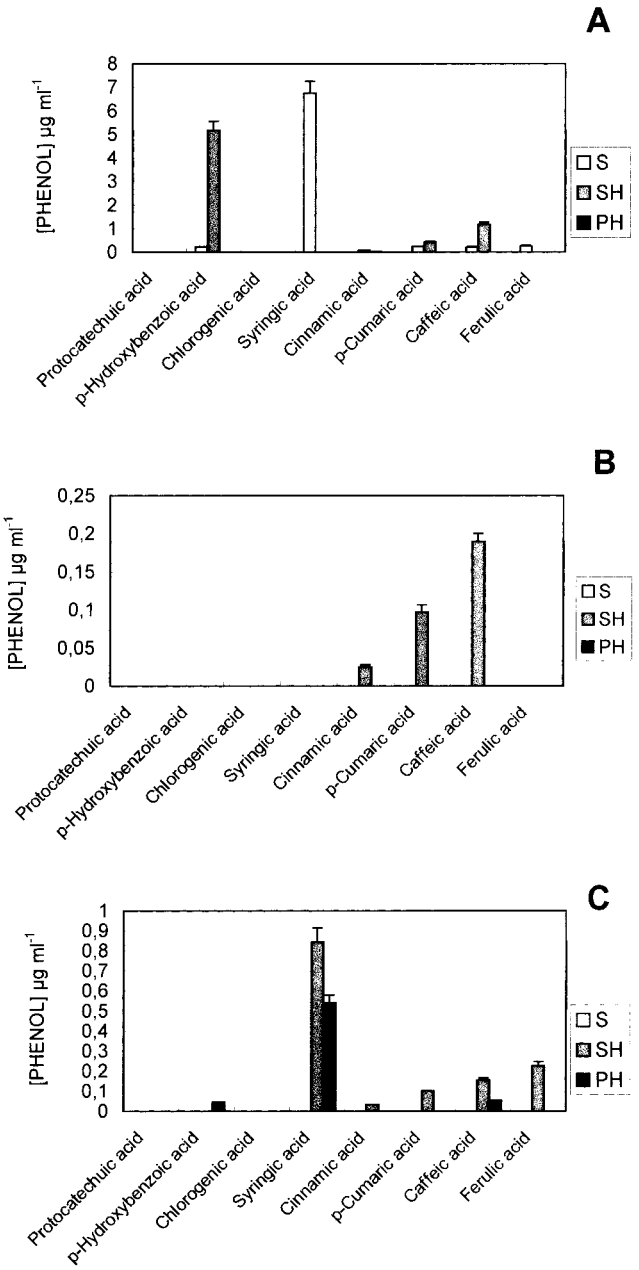

Figure 7. Quantitation of phenolics associated with S-, SH-, and PH-polyamines occurring in LMMC (A), MMMC (B), and HMMC (C) preparations obtained from clarified sugarcane juice. Values are the mean of three replicates, and each experiment was repeated twice. Vertical bars give standard error when it can be drawn.

Strikingly, *p*-hydroxybenzoic, syringic, and caffeic acids appeared as components of the PH-fraction obtained from the HMMC preparation, in addition to some other phenols occurring in the SH-fraction (Figure 7C).

DISCUSSION

Polyamines appear as soluble polycations (Morris and Harada, 1980) in the physiological, acidic pH range of sugarcane juice, and they are mainly recovered as free (S) compounds in the LMMC preparation, where most abundant free polyamines are PUT and CAD (Figure 5A).

The classification of conjugated polyamines in SH- or PH-groups is based on their solubility in acid. Acid-soluble conjugated polyamines (SH-) appear as associated with small molecules, such as sugars, phenols, and amino acids, and PH-conjugated polyamines are associated usually with macromolecules (Tiburcio et al., 1990). As shown in Figure 5, this classification is not completely valid for sugarcane juice, in which there are some PH-SPD in the LMMC preparation, maybe joined to sugars, and SH-SPM in the HMMC preparation, joined to macromolecules.

Adjusting the juice pH to 8.0 and centrifuging it later during the clarification process significantly change the elution profiles of protein and carbohydrates. Part of the macromolecules are removed, and the structure of HMMC also changes. In the HMMC preparation, proteins and carbohydrates coelute in an amount higher than that obtained at pH 5.2 (Figure 4B). Because an appreciable amount of polyamines is re-

moved in these conditions, it is feasible that the ionic interaction between polyamines and proteins increases at pH 8.0 (Slocum et al., 1984) and both coprecipitate together.

The polyamine more strongly retained in HMMC and MMMC fractions, even after the clarification process, is SPD (Figure 5B), probably due to the occurrence of three amino groups in the molecule to do the corresponding linkages. On the other hand, both HMMC and MMMC are glycoproteins (Legaz et al., 1995), and SPD may be strongly retained by a covalent linkage to the protein moiety (Legaz et al., 1998), as has been reported by Icekson and Apelbaum (1987) for other plant proteins. Another hypothesis deals with the conjugation of polyamines to phenolics. The conjugation of polyamines to hydroxycinnamic acids has been widely reported (Martin-Tanguy et al., 1978). In the results described above, some interactions between polyamines with *p*-hydroxybenzoic acid, syringic acid, and the hydroxycinnamic acids *p*-coumaric and caffeic acids occur in MMMC and HMMC preparations, but a very strong interaction with syringic acid is also possible (Figures 5, 6B, and 6C).

The clarification process seems to increase the interaction between syringic acid and HMMC. In this case, the reported interaction appears in the pH-fraction (Figure 7C), where SDP conjugates are present, too (Figure 5B). Thus, it is possible to hypothesize that syringic acid, the only methylated phenolic acid studied, reinforces a solvophobic effect (Tan and Carr, 1997; Vailaya and Horvath, 1997) which facilitates the protein agglutination (shown in Figure 4B) and may produce a greater retention of SDP and syringic acid in SH- and PH-conjugates. This agglutination moves syringic acid from SH-MMMC to SH- and PH-HMMC fractions (Figure 6B). The MMMC preparation, at pH 8.0, retains only SH-hydroxycinnamic acids, as is usually reported (Bendech de Cantuú and Kandeler, 1989; Cabanne et al., 1977; Kaur-Sawhney et al., 1988).

Other changes in syringic acid content following the clarification process are observed in the LMMC preparation, in which all SH- and PH-conjugates are liberated to the S-fraction. It is feasible that alkaline conditions facilitate unconjugated syringic acid to be formed. The fact that syringic acid was one of the most retained phenols after clarification could be very important. This phenol has been reported as the most abundant in raw sugars (De Armas et al., 1998), and its occurrence could be related to sugar color problems derived from biological oxidations (Bucheli and Robinson, 1994) that affect the quality of the industrial sugar.

It is shown that clarification does not eliminate important amounts of SDP and syringic acid fractions of carbohydrates studied here. Also, hydroxycinnamic acids (*p*-coumaric, caffeic, and ferulic acids) are retained, mainly as SH-conjugates. Conjugation of polyamines to phenolics has often been described as a defense mechanism against infection of several higher plants by viruses and fungi (Fleurence and Negrel, 1989; Louis and Negrel, 1991). In relation to sugarcane plants, Lloyd and Naidoo (1983) were able to isolate glycosidic substances from fresh bud scales and to establish a linear correlationship between resistance rating to smut and concentration. These substances were previously identified as flavonoids, which behave as inhibitors of teliospore germination (Lloyd and Pillay, 1980). These conjugates might act also as a polyamine reserve, which would be liberated for physiological purposes during growth and development of the plant (Martin-Tanguy, 1985), but a metabolic function of these conjugates cannot be discarded, as derived from the work of Martin-Tanguy et al. (1988), Mizusaki et al. (1973), and Tiburcio et al. (1985).

ABBREVIATIONS USED

CAD, cadaverine; HMMC, high molecular mass carbohydrates; HPLC, high-performance liquid chromatography; LMMC, low molecular mass carbohydrates; MMMC, mid molecular mass carbohydrates; PAs, polyamines; PCA, perchloric acid; PH, acid-insoluble polyamines; PUT, putrescine; S, free, soluble polyamines; SH, acid-soluble polyamines; SPD, spermidine; SPM, spermine.

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