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Removal of Pigments from Sugarcane Cells by Adsorbent Chromatographic Column

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Abstract

Pigments are widely distributed in sugarcane plant cells and have been associated with color in sugarcane juice for white sugar production. Chlorophylls, phenolics and flavonoids compounds are some of those pigments. Mechanized harvesting increases the adding of cane tops to processing and the study of these compounds allows us to understand their impact on the final product. The aim was to separate and quantify these compounds obtained from sugarcane top ethanolic extracts. The chromatographic occurred in adsorption resin. The quantification was made in spectrophotometric systems. These methods allow separation and quantification of compounds and even observation of polarities of molecules compared to the eluents.

Introduction

The types and amounts of pigments in sugarcane juice are dependent on a number of factors such as the variety and maturity of the raw material, climatic conditions, agricultural practices, time between cutting and processing, quantity of impurities, soil conditions and grinding types [1].

Sugarcane contains many colorant compounds that could be extracted from its juice [2]. In 1971, a study on sugarcane juice identified chlorogenic acid, cinnamic and flavones as colored compounds [3].

Those colored substances (pigments) are derived from plants and can be found in raw sugar after processing [4]. The presence of a several colorant compounds, such as chlorophylls *a*, *b*, *c*1, *c*2, *d* and *f* [5-7], have been described for agricultural and food chemistry aspects, however, the literature has widely described chlorophylls *a* and *b* [8]. Other colored substances described are anthocyanins [9], flavonoids [9-12] and phenolic acids [13-17].

Pigments are widely distributed in the sugarcane plant cell [17]. Authors have associated these pigments with color in sugarcane juice for white sugar production [1,18-21]. During the clarification process, plant pigments decompose to form polyphenolic compounds with subsequent enzymatic browning [1].

Sugar mills in Brazil remove these pigments, as well as other impurities, for the bleaching action of sulfur dioxide during the sulphitation process [22,23]. The clarified sugarcane juice as matter for sugar production is concentrated up to white crystals [23,24].

Chromatography is a versatile technique widely used for the separation of chemical compounds in a suspension or solution [25] and this technique can also be used for separation of pigments from plant extracts [26].

Adsorption is a low-cost technique depending on the used adsorbent and operation [27]. Chemical compounds can be separated through identification and quantification [28,29]. Adsorption resins have a specific area and pore diameter for rapid diffusion of ions and improved extraction kinetics with an increase in the complexing capacity [30]. Dowex™ Optipore™ SD-2 has been used in the food industry to remove color, flavor and odor in sweeteners as it has pore structures to maximize the load, as well as the high mechanical, chemical and thermal capacity [31].

Previously, we studied the effect of color to evaluate the sugar quality without or with different treatments, such as gamma radiation and electron beam [32,33], Fenton-like reaction [34], ozone [35,36] and hydrogen peroxide [20,36,37]. These studies evaluated the isolation of pigments in sugarcane juice as potential purification process and isolation system of pigments, which have been associated by antioxidant activities [35,38], by absorption chromatography column, as described by Lima and Aguiar [39].

Table 1: Product specifications of adsorbent resin used for syrup treatment.

Parameters	Descriptions*
Matrix	Macroporous styrene-divinylbenzene copolymer
Functional group	Tertiaryamine
Typical surface area	800 m^2/gL

*Dow (2015)

Material and Methods

Material

All the analyses were performed at the Hugot Sugar Technology Laboratory, Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba, SP - Brazil. The reagents were purchased at Sigma-Aldrich Co. (São Paulo, Brazil). The water used to prepare the solutions was Milli-Q grade (Millipore Co.; São Paulo, Brazil). The resin Optipore SD-2 was donated by Dow Chemical do Brazil (São Paulo, Brazil) and its specification used in this study are shown in Table 1.

Experimental procedure

The chromatographic assays were carried out in a vertical glass jacket column (dimension: 1.18" φ × 11.81" L; FGG Ltd., Brazil) and working volume of 180.0 ml. The 55.55% of the internal volume was filled with SD-2 Dow™ Optipore™ adsorbent resin, about 100.0 ml (density equal to 1.04 g/ml). The assay was carried out at room temperature. The extract used was concentrated sugarcane top leaves from RB855453 variety, about 69.0 ml, extracted by 96% ethanolic solution (v/v). After the extract passed through the column and fractions were collected, 69.0 ml of ethanol (≥ 99.5%, v/v), 138.0 ml of methanol (≥ 99.8%, v/v), and 138.0 ml of hexane (≥ 98.5%, v/v) and 138.0 ml of chloroform (≥ 99.8%, v/v) were added, respectively. Each fraction volume was 14.0 ml, totaling 33 fractions. All fractions were analyzed between 200 and 600 nm in UV-Mini 1240 spectrophotometer (Shimadzu Co., Kyoto, Japan) and some fractions were selected to analyze phenolic acid, flavonoids and chlorophylls.

The resin was washed with Milli-Q water and NaCl at 10% (w/v) and dried in an oven at 65 °C overnight.

Chlorophyll analysis: From the maximum absorption profiles, fractions 3 to 11, 18 to 20 and 26 to 31 were selected and subjected for the UV Mini-1240 spectrophotometric analysis (Shimadzu Inc.; Kyoto, Japan) at 645, 652 and 663 nm to determine the concentration of chlorophylls *a*, *b* and total. The chlorophyll concentrations were calculated by equations and the results were expressed by mg of chlorophyll per gram of fresh material [40].

$$\text{Chlorophyll } a = \frac{((12{,}7 \times A_{663}) - (2{,}69 \times A_{645})) \times V}{1000 \times W}$$

$$\text{Chlorophyll } b = \frac{((22{,}9 \times A_{645}) - (4{,}68 \times A_{663})) \times V}{1000 \times W}$$

$$\text{Total chlorophyll} = \frac{A_{652} \times 1000 \times (V/1000 \times W)}{34{,}5}$$

Where: A_{663}: absorbance at 663 nm; A_{645}: absorbance at 645 nm; A_{652}: absorbance at 652 nm; V: final volume of ethanolic extract of chlorophyll (mL); W: mass of plant matter (g).

Total phenolic contents: The spectrophotometric Folin-Ciocalteu phenol reagent was used to measure total phenolic in the samples. For calibration, curves 0, 0.04, 0.08, 0.12, 0.16 and 0.2 ml of solution (0.1 mg/ml tannic acid) were separately mixed with 1.0, 0.96, 0.92, 0.88, 0.84 and 0.8 ml of distilled water, respectively. Next, 0.5 ml of 10 fold dilute Folin-Ciocalteu reagent (note: recently prepared) was mixed and allowed 30 min at room temperature. Before 2.5 ml of 20% (w/v), sodium carbonate was mixed and the absorbance at 725 nm was read in UV Mini-1240 spectrophotometer (Shimadzu Inc.; Kyoto, Japan). Similarly, 0.5 ml of samples was used to determine total phenolic contents, without the distilled water [41] (JULKUNEN-TIITTO with modifications).

Aluminum chloride colorimetric method for flavonoid analysis: For calibration, curves 0, 10, 15, 20, 30, 40, 45 and 50 μL of the solution (0.05 grams of rutin dissolved in 70% (w/v) ethanol) were separately mixed with 4.8, 4.79, 4.785, 4.78, 4.77, 4.76, 4.755 and 4.75 ml of 70% (v/v) ethanol, respectively. Next, 0.1 ml of 2% (w/v) aluminum chloride (≥99.0%) and 0.1 ml of 1 M sodium acetate (≥99.0%) was added. After 40 min at room temperature (26±1 °C), absorbance was measured at 415 nm with in UV Mini-1240 spectrophotometer (Shimadzu Inc.; Kyoto, Japan). Similarly, 0.5 ml of samples was used to determine flavonoid contents and the quantity of 70% ethanol was 4.3 ml [42] (MABRY, et al. with modifications).

Results and Discussion

The spectroscopic analysis of extract sugarcane tops showed that eluates along the chromatographic run were characterized as polar to nonpolar due to the characteristics of the added eluents, ethanol followed by methanol, hexane and chloroform, respectively.

Figure 1 shows that the chlorophylls are presented in the final fractions of eluates as phenolics and flavonoids in peaks in the early and final fractions.

Chlorophylls *a*, *b* and total

Chlorophylls *a* and *b* in a raw ethanolic extract of sugarcane tops were separated from the phenolic compounds and flavonoids with eluted nonpolar organic solvents such as hexane and chloroform.

The behavior of both chlorophylls along the chromatographic run was the same, however at different concentrations (Figure 2). Chlorophyll *a* showed higher concentrations compared to chlorophyll

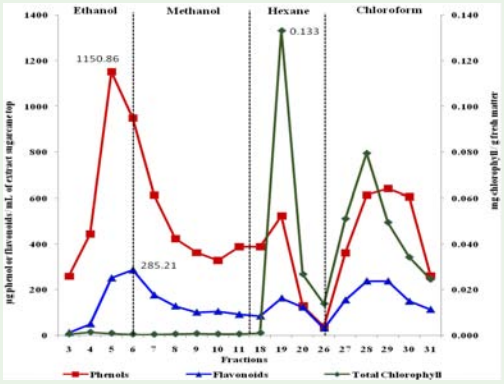

Figure 1: Phenols, flavonoids and total chlorophyll concentrations in sugarcane tops.

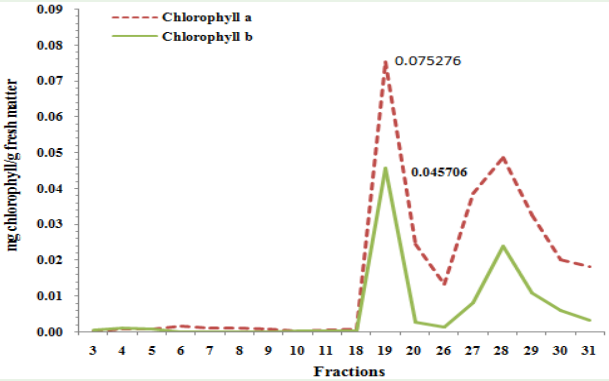

Figure 2: Chlorophyll *a* and *b* concentrations in fresh sugarcane tops.

b in the same fraction. [43] TOMO, et al. reported that chlorophyll *a* is the majority found in nature and that it performs fundamental role in the donation of electrons in photochemical reactions of photosynthesis.

The maximum concentration of chlorophyll *a* was 0.0752 mg/g, while chlorophyll *b* was 0.0457 mg/g fresh material (Figure 2). The addition of eluents with decreasing gradient polarity to the column showed an increase in the concentration of chlorophylls, characterizing them as nonpolar. This feature is justified by the presence of the isoprene tail in the molecules (Figure 3).

Plant pigments, such as chlorophyll, xantophyll and carotene are responsible for two thirds of color in raw sugar [44] and a small amount can be found in polymeric colorant in raw sugar if they are not removed or destroyed in the clarification process [45].

Total phenolic acids and flavonoids

The highest concentration of phenols in the early fractions compared to the final fractions of the chromatography eluate shows a characteristic of polar phenolics in the first fractions, as the eluents used were ethanol and methanol, that is, the polar pigments are loaded in and out on the first fractions by the characteristics of the eluents. Furthermore, there are nonpolar phenolic compounds and this can be observed in the final fractions of the chromatographic run in which hexane and chloroform were used as eluents.

Different phenolic compounds are widely identified in a sugarcane juice allowing studying their molecules (Figure 4; [17]).

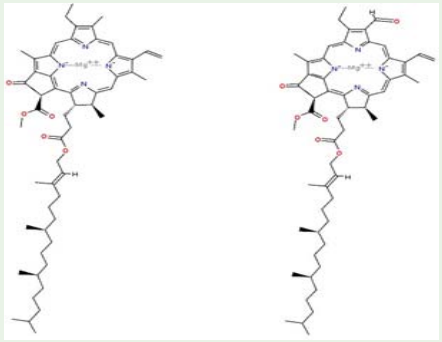

Figure 3: Chemical structures of chlorophylls *a* and *b* [8].

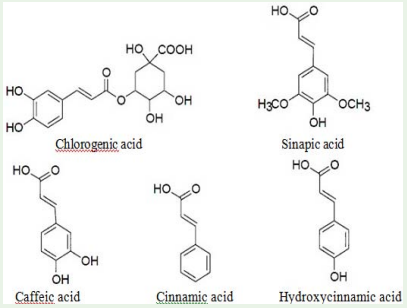

Figure 4: Phenolics compounds of sugarcane juice [17].

The phenolic compounds are responsible for the increase in color in different types of sugars, when present in the sugarcane juice, due to their molecular characteristics. Phenolic compounds in the presence of amino acids initiate the reaction known as Maillard, thus, leading to the production of dark compounds that crystallize with crystal sugar, increasing the product color [46]. Melanoidins are a small part removed from the clarification process and may constitute up to 60% of the color of the clarified juice [47]. Dark colored polyphenols originate when they are exposed to Fe^{+3} ions in acidic solutions due the continuous contact of sugarcane juice with metal surfaces [1].

With the onset of mechanized harvesting, the adding of sugarcane tops to processing increased. The increase of 1% of tops causes a 1.3% increase of color in a sugarcane juice [48].

The profile of flavonoids attached to phenolics, since the types of flavonoids are phenolics, is at lower concentration in sugarcane juice.

Flavonoids such as apigenin, tricine, glycosylated luteolin, orientin, vitexin, schaftoside and swertisin are found in sugarcane juice, however, some steroids and polycosanols can also be found in other parts of sugarcane, such as stalk wax [17]. This pigment group is rather critical because it can cause up to 30% of color when raw sugar production occurs at pH 7.0 [4] and not a large quantity is removed when the clarification process occurs [1].

Therefore, glycosylated flavonoids have been identified in a sugarcane juice and leaves such as diosmetin-8-C-glucoside, tricin-7-O-neohesperoside, 4 ‘, 5’-dimethoxy-8-luteoline C glucoside, tricin-7-O-rhamnosylgalacturonide, tricin-7-O-glucoside, schaftoside, isoschaftoside, vitexin, orientin [44,49-51], swertisin, tricin-7-O-neohesperoside-4’-O-rhamnoside, tricin-7-O-methylglucuronate-4-O-rhamnoside, tricin-7-O-methylglucuronide, tricin-7-O- beta-(6’-methoxycinnamic) -glucoside, luteolin-8-C-glucoside rhamnosyl,

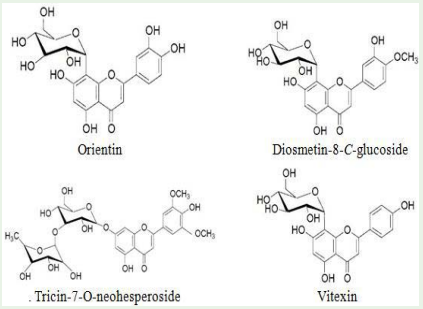

Figure 5: Flavone glycosides from sugarcane juice and leave [52].

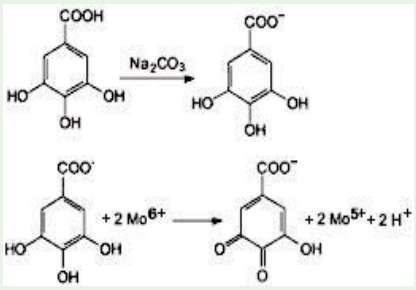

Figure 6: Folin-Ciocaulteau reaction [53].

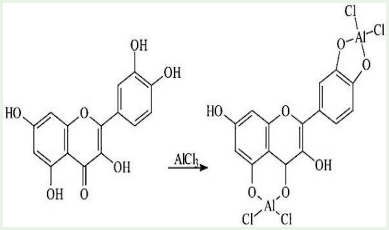

Figure 7: Flavonoids complexation with $AlCl_3$ [54].

tricin-4'-O- (erthroguaicylglyceryl) -ether [11]. Some of these molecules can be observed in Figure 5.

Phenolic and nonpolar flavonoid potentially have aglycone characteristics such as the chlorogenic acid, cinnamic acid, hydroxycinnamic acid, sinapic acid, chlorogenic acid, apigenin, luteolin and tricin [13].

Methodological interferences

The analysis of total phenolics, using the Folin-Ciocalteu reaction spectrophotometry, occurs with deprotonation hydroxyls and consequent formation of carbonyl through reduction of Mo^{+6} to Mo^{+5} (Figure 6) [53].

For the analysis of flavonoids, the complexation of aluminum occur originating aluminum chloride at 2% methanol, between carbonyl-hydroxyl and hydroxyl-hydroxyl (Figure 7) [54].

At the end of the chromatographic run, there are three peaks corresponding to phenolics, flavonoids and total chlorophyll. However, observing chlorophyll *a* and *b* molecules, we can state that deprotonation of hydroxyls and aluminum complexing are not possible since these molecules have no hydroxyls and carbonyls in places and amounts that promote these reactions. Therefore, the analytical method of phenolics and flavonoids did not affect determination of chlorophylls.

Conclusion

The extraction of pigments was effective in 96% ethanol, once the analysis resulted in phenolic, flavonoid and chlorophyll concentrations along the eluted fractions and SD-2 Dow™ Optipore™ adsorption resin, together with eluents. These allowed separating all compounds by polarity, where a decrease in the polarity of the pigments molecules occurred during the chromatographic run.

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