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Sugar Cane Stillage: A Potential Source of Natural Antioxidants

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ABSTRACT: Biorefinery of sugar cane is the first economic activity of Reunion Island. Some sugar cane manufactured products (juice, syrup, molasses) have antioxidant activities and are sources of both phenolic compounds and Maillard Reaction Products (MRP). The study aimed to highlight the global antioxidant activity of sugar cane stillage and understand its identity. Chromatographic fractionation on Sephadex LH-20 resin allowed the recovery of a MRP-rich fraction, responsible for 58 to 66% of the global antioxidant activity according to the nature of the sugar cane stillage (DPPH test), and a phenolic compounds-rich fraction for 37 to 59% of the activity. A good correlation was recorded between the antioxidant activity of the sugar cane stillage and its content in total reducing compounds amount (Folin-Ciocalteu assay), among them 2.8 to 3.9 g/L of phenolic compounds (in 5-caffeoylquinic acid equivalent). Preliminary experiments by HPLC-DAD-MS allowed to identify several free phenolic acids and gave clues to identify esters of quinic acids.

KEYWORDS: *antioxidant, Maillard Reaction Products, phenolic compounds, sugar cane stillage, biorefinery*

INTRODUCTION

Nowadays, sugar cane is the main crop of production of Reunion Island (French department, Indian Ocean) with about 2 Mt of sugar cane harvested per year. Cane sugar production is the first economic activity with more than 200 000 t of sugar produced per year.[1] The sugar production process from sugar cane starts with a milling step in order to separate the raw juice from the fiber called bagasse (Figure 1). Raw juice is clarified in order to settle the mud. Filtration of the mud makes possible the recuperation of a residual juice called filtrated juice from the scum. The clarified juice is then concentrated by evaporation to a softly viscous solution, called syrup. Sugar is produced by crystallization from the syrup, centrifugation, and drying. Depletion of fossil resources and environmental issues caused by their industrial transformation encourage the use of alternative and sustainable solutions in order to replace petroleum based molecules by biobased molecules. In this context, the industrial transformation of sugar cane has evolved toward biorefinery systems based on the utilization of the whole plant and coproducts: scum is spread into fields as fertilizers, bagasse is burned for producing energy, and molasses are fermented after dilution for producing alcohol leaving a liquid effluent, named stillage. This effluent, considered today as a waste, could become a coproduct of interest in an integrated biorefinery approach. In order to improve the competitiveness of such systems, research efforts have recently focused on the valorization of high valued molecules such as antioxidants.

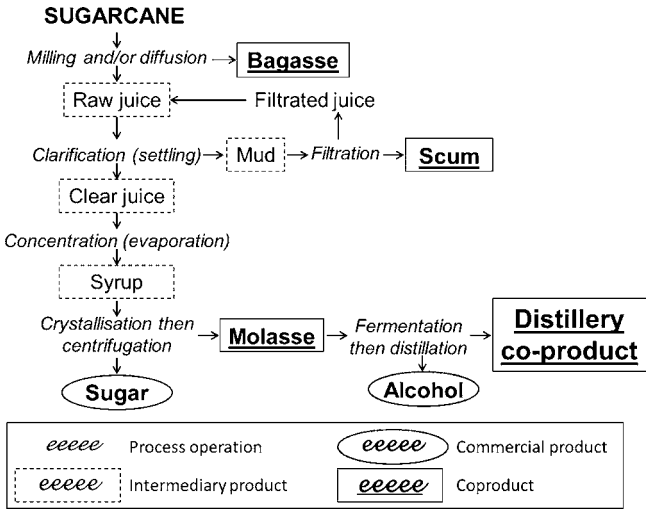

Figure 1. Sugar cane manufacturing process.

Sugar cane manufactured products are well-known sources of phenolic compounds and Maillard Reaction Products (MRP). Indeed, Lionnet [2] showed that about 60% of sugar cane color was due to the clean stalk, which corresponds to the manufactured part, and pointed out a positive correlation between color and response to Folin-Ciocalteu assay, method used to measure the total content of phenolic compounds.[3] However; this method is not a specific method and responds to

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other compounds such as aromatic amines, proteins and reducing sugars,[3] and can also react with MRP. Nevertheless, Lionnet [2] showed that the manufactured part of sugar cane contains phenolic and other reducing compounds up to 5500 mg/kg of dry matter (in caffeic acid equivalent), depending on the sugar cane varieties, sugar cane age, and month of harvest. Later, Kadam et al.[4] completed this knowledge by determining phenolic compounds in barely crushed sugar cane (laboratory scale) from different varieties. Phenolic compounds are thus clearly present in sugar cane.

It has been also shown that many products from sugar cane manufactured process contained MRP, generated during the process due to the presence of both amino compounds and reducing sugars in sugar cane. And the higher the temperature, the higher their production.[5,6] Besides, MRP remain in sugar cane molasses based distillery effluent.[7]

Several studies have highlighted the antioxidant activity of both phenolic compounds and MRP. Indeed, antioxidant activity of phenolic compounds has been widely reviewed in fruits,[8−12] in agro-industrial coproducts and manufactured products.[13−16]

MRP also have antioxidant properties, as shown for coffee,[17−19] vinegar,[20] processed food such as beer,[21] pasta or tomato puree [22] and model systems.[23]

Antioxidant activity of phenolic compounds and MRP has also been measured in sugar cane manufactured products such as juice, syrup, molasses,[24−26] raw cane sugars,[27] and non centrifugated raw brown sugar called kokuto.[28−30] But until now, no study has been run on sugar cane stillage. However, considering the manufacturing process and the chemical composition of previous manufactured products, one could expect those products to contain MRP and phenolic compounds.

The aim of our study was thus to highlight the global antioxidant activity of the distillery effluent, to identify the families of molecules responsible for this activity and to assess their respective contribution. In order to fulfill our goal, it was necessary to first remove insoluble matters from the sugar cane stillage by microfiltration. A MRP-rich fraction and a phenolic compounds-rich fraction were recovered after chromatographic fractionation on Sephadex LH-20 resin and were analyzed for their UV-vis characteristics, their concentration in reducing compounds by Folin-Ciocalteu assay and their antioxidant activity by DPPH assay. Preliminary experiments were also performed to identify some phenolic compounds by high performance liquid chromatography coupled to diode array detection and mass spectrometry (HPLC-DAD-MS).

MATERIALS AND METHODS

Chemicals. Hydrochloric acid (37%, v/v) was purchased from VWR International (Fontenay-sous-bois, France). Formic acid (96%, v/v), D-Glucose (99.5%, w/w), L-Lysine (99.9%, w/w), sodium carbonate (99.9%, w/w), 2,2-diphenyl-1-picrylhydrazyl, zinc acetate (99.99%, w/w), potassium ferrocyanide (98.5%, w/w), *p*-hydroxybenzoic acid (99%, w/w), vanillic acid (>97%, w/w), syringic acid (>95%, w/w), *p*-hydroxybenzaldehyde (98%, w/w), vanillin (99%, w/w), syringaldehyde (>98%, w/w), *p*-coumaric acid (>98%, w/w), sinapic acid (>99%, w/w), 5-caffeoylquinic (chlorogenic acid, >95%, w/w), caffeic acid (>98%, w/w) and protocatechuic acid (>97%, w/w) were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Absolute ethanol (99.9%, v/v), methanol (99.9%, v/v), acetonitrile (99.9%, v/v), sodium hydroxide (98%, w/w) and Folin-Ciocalteu reagent were from Grosseron (Saint-Herblain, France). Ultrapure water was obtained from a Millipore Elix filtration system (Saint-Quentin-en-Yvelines, France).

Sugar Cane Stillage. Sugar cane stillage was supplied by two local cane molasses distilleries of Reunion Island during operating period. One sample was collected in a first distillery and was called "sample A". Two other samples were collected in another factory at two different dates and were called "sample B1" and "sample B2". The dry weight of the sugar cane stillage was around 100 g/L.

Microfiltration of Sugar Cane Stillage. The insoluble matters were eliminated from the sugar cane stillage by microfiltration, as soon as it was supplied. The sugar cane stillage was fed in the microfiltration unit at its initial temperature (75 °C) and the permeate collected. The membrane was a mineral device supplied by Tami (Nyons, France) with mesh of 0.14 μm. All samples were stored at −18 °C before analysis. Their stability was checked by measuring, before and at each stage of defrosting, the total reducing compounds by Folin-Ciocalteu assay.

Maillard Reaction Products Synthesis. Three samples of MRP were synthesized according to ref 23 by heating in sealed vials at 103 °C an aqueous mixture of glucose (0.8 mol/L) and lysine (0.5 mol/L), for 1 and 14 h.

Fractionation of Maillard Reaction Products and Phenolic Compounds by Low Pressure Chromatography. Molecules from microfiltration permeate by were fractionated by Sephadex LH-20 chromatography according to a method adapted from Dupas et al.[18] Briefly, 100 μL of the microfiltration permeate were applied onto a Sephadex LH-20 (VWR International, Fontenay-sous-bois, France) column (length 10 cm; i.d. Five mm). Two column volumes of pH 3 acidified water were then applied (using 37% hydrochloric acid) leading to a Maillard reaction products-rich acidified water fraction, followed by four column volumes of absolute ethanol, leading to a phenolic compounds-rich ethanolic fraction. On both fractions, UV-visible spectrum were drawn and their total reducing compounds content (Folin-Ciocalteu assay) and their antoxidant activity were measured (DPPH assay). Fractionation of the same sample was repeated three times.

UV-Visible Spectrophotometry. UV-visible spectra of the microfiltration permeate and of the two chromatographic fractions obtained by Sephadex LH-20 chromatography were recorded between 200 and 500 nm by a UV-vis Perkin-Elmer apparatus equipped with Lambda 9 software (Shelton, CT), after appropriated dilution in ultrapure water if necessary.

Folin-Ciocalteu Assay. Total reducing compounds were assessed by Folin-Ciocalteu assay according to the protocol of Singleton et al.[3] One milliliter of each sample (microfiltration permeate or chromatographic Sephadex LH-20 fractions) and 5 mL of Folin-Ciocalteu reagent (diluted 10 times in ultrapure water) were stirred for 5 s in a test tube. Four minutes later, 4 mL of a 75 g/L aqueous sodium carbonate solution were added and the whole reacting solution was mixed in a vortex for 5 s at the maximum speed. After 1 h of storage in the dark at room temperature, the absorbance of the solution was read at 760 nm using a UV-vis Genesys spectrophotometer, equipped with VisionLite software. For absorbance superior to 1, an appropriate dilution of the initial sample was performed in ultrapure water and the Folin-Ciocalteu assay was repeated. A calibration curve was established with 5-caffeoylquinic acid (chlorogenic acid) from 0 to 100 mg/L.

DPPH Assay. Antioxidant activity of the microfiltrated permeate and of the chromatographic fractions was assessed by DPPH (2,2-diphenyl-1-pycrylhydrazyl) assay according to Brand-Williams et al.[31] Briefly, several dilutions of the samples were prepared. Seventy seven microlitres of each sample (pure or diluted in water, from a dilution factor of 0.5/100 to 3/100 for the microfiltration permeate and from 20/100 to 80/100 for chromatographic fractions) were incubated with 3 mL of a 6×10^{-5} mol/L DPPH° methanolic solution, and the absorbance of the mixture was regularly measured at 515 nm by a UV-vis Genesys spectrophotometer, until a steady state was reached (20 h). DPPH assay was done once on each repetition of chromatographic fractionation.

Sample Preparation Prior to HPLC-DAD-MS Analysis. Ethanolic chromatographic fractions, obtained as mentioned in 2.5, were concentrated 25 times by ethanol evaporation under reduced pressure (150 mbar, 30 °C). The concentrated fractions were hydrolyzed in mild or severe alkaline conditions, and then purified by precipitation with Carrez reagents, as described by Culhuaoglu et al.[32] Briefly, for mild hydrolysis, 500 μL of the concentrated fraction were added to 5 mL of 2 mol/L aqueous sodium hydroxide and the mixture was magnetically stirred at room temperature for 20 h. For severe hydrolysis, 500 μL of the concentrated fraction were added to 5 mL of 4 mol/L aqueous sodium hydroxide and the mixture was placed at 170 °C for 2 h in a stainless steel reaction vessel. After reaction, the hydrolyzates were acidified to pH between 5 and 6 with 1 mol/L hydrochloric acid. Then, 0.75 mL of the acidified hydrolyzate was mixed with 1 mL of absolute ethanol and 70 μL of Carrez I reagent (aqueous solution of zinc acetate, 1 mol/L) during 2 min and left for 1 min at room temperature. Then, 70 μL of Carrez II reagent (aqueous solution of potassium ferrocyanide, 0.25 mol/L) were added and the solution was stirred during 20 s at room temperature. The mixture was centrifuged during 10 min at 13 500*g*. The supernatant was collected and stored at 4 °C until HPLC-DAD-MS analysis.

HPLC-DAD-MS Analysis. HPLC-DAD-MS analyses were performed on a Thermo Electron Corporation apparatus (Waltham, MA) equipped with a quaternary pump (Spectro system P4000), an autosampler (Spectro system AS300), and a photodiode-array detector (Spectro system UV 6000 LP from Thermo Finnigan). The HPLC system was coupled to a LCQ Deca mass spectrophotometer detector (Thermo Quest) equipped with an electrospray ionization interface (ESI). The UV spectra and mass spectra (recorded in the negative ion detection mode in the 35−2000 uma m/z range) were treated by Xcalibur software. Analyses were performed according to the method developed by Culhuaoglu et al.[32] Compounds were separated at 20 °C by a thermoscientific Hypurity C18 column (150 × 4.6 mm, 3 μm i.d., 190 Å). Solvents for the mobile phase were A, acetonitrile acidified with formic acid at 1‰ (v/v); B, ultrapure water acidified with formic acid at 1‰ (v/v). Elution conditions were the following ones: isocratic 9% of A between 0 and 15 min; linear gradient from 9% to 11% until 22 min; linear gradient from 11% to 18% until 38 min; isocratic 18% of A between 38 and 40 min; linear gradient from 18% to 80% until 41 min; isocratic 80% of A between 41 and 49 min. Standard phenolic compounds were dissolved in methanol at a concentration of 150 mg/L each. Standards and ethanolic chromatographic fractions were filtrated on a 0.45 μm Acrodisc GHP filter before injection of 5 μL.

RESULTS AND DISCUSSION

Global Antioxidant Activity of the Sugar Cane Stillage. Three different samples (A, B1 and B2) were studied for their global antioxidant potential, assessed by radical scavenging DPPH assay, after removing insoluble matters by microfiltration. All of them had an appreciable antioxidant activity whatever was the distillery or the collecting period (Figure 2). The three sugar cane stillages had a similar activity

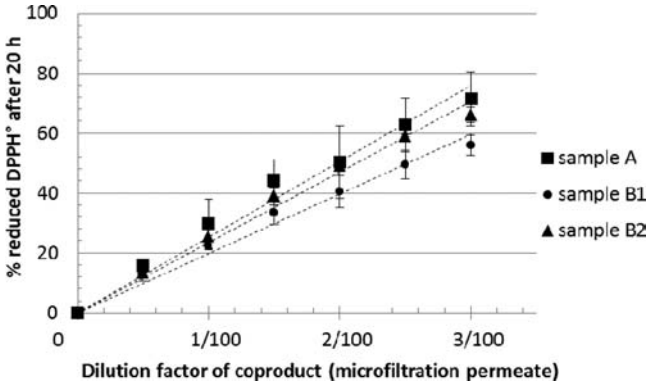

Figure 2. Antioxidant activity of sugar cane stillage microfiltration permeate from two different factories, assesed by DPPH° scavenging assay after 20 min of reaction.

whereas sample A and B1 were not collected in the same factory, and samples B1 and B2 were collected at two different operating times. At a dilution factor of 3/100, which corresponds to a dry matter of the samples of 3 g/L, the three sugar cane stillages reduced between 55 and 70% of DPPH° when the steady-state was reached. Such an antioxidant potential is close to that of other coproducts from industries such as juices extracted from peel of papaya and pulp of orange, containing respectively 0.38 and 0.47 g/L in gallic acid equivalent of phenolic compounds.[33] The evolution of reduced DPPH°, expressed as a percentage, was plotted for each repetition of sugar cane stillage vs the concentration of reducing compounds according to Folin-Ciocalteu assay (Figure 3). A good correlation was obtained using a linear model (R^2 = 0.9601), which confirms the antiradical activity of the sugar cane stillage molecules reacting with Folin-Ciocalteu assay. This result is in agreement with already published studies on other

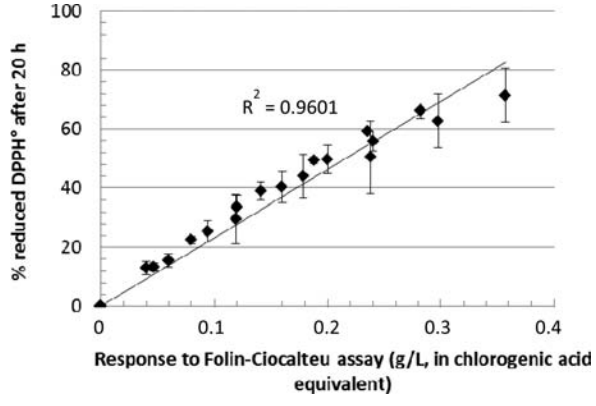

Figure 3. Correlation between the antioxidant activity of the sugar cane stillage microfiltration permeates and their response to Folin-Ciocalteu assay.

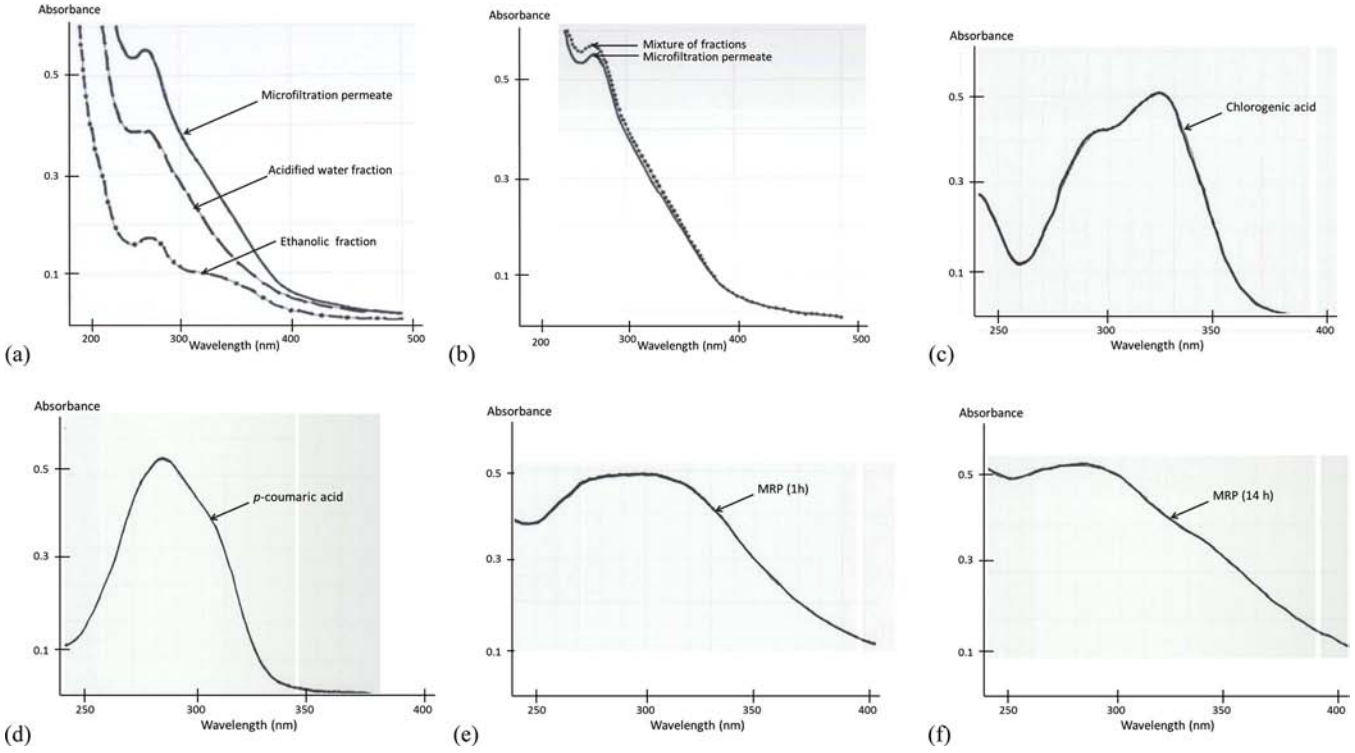

Figure 4. UV-vis spectra of (a) the sugar cane stillage microfiltration permeate (sample B1), the acidified water chromatographic fraction and the ethanolic chromatographic fraction of sugar cane stillage (sample B1), after appropriate dilution allowing to directly compare the spectra; (b) the microfiltration permeate and the mixture of both chromatographic fractions of sugar cane stillage (sample B1), after appropriate dilution; (c) 5-caffeoylquinic acid (chlorogenic acid); (d) *p*-coumaric acid; (e) model glucose-lysine MRP obtained after 1 h of heating at 103 °C; (f) model glucose-lysine MRP obtained after 14 h of heating at 103 °C.

products. Indeed, the analysis of 50 fruits, vegetables and beverages such as apple, bean or coffee, also led to linear correlation, with a R^2 of 0.897,[34] and the analysis of a Kudingcha bitter tea led to a R^2 of 0.829.[35]

Contribution of Two Isolated Antioxidant Fractions. As already mentioned, the antioxidant activity of sugar cane stillages was expected to be due to both phenolic compounds and MRP. Thus, the contribution to global antioxidant activity of each of these chemical classes needed to be assessed. As neither Folin-Ciocalteu assay nor DPPH test is specific to one of these two chemical classes, it was necessary to separate them in order to determine their proportion and measure their activity. Thus, chromatographic fractionation using Sephadex LH-20 was carried out as previously described:[18] the MRP and phenolic compounds were collected as two distinct fractions eluted by acidified water and ethanol respectively. Development and validation of this fractionation method were made through tests with standards: 5-caffeoylquinic acid (chlorogenic acid, 1 g/L), *p*-coumaric acid (1 g/L) accounting for phenolic compounds and three model glucose-lysine MRP for MRP family. The initial standard solutions and their two chromatographic fractions were analyzed according to Folin-Ciocalteu assay. Recovering rates of MRP mixtures were 99.9 ± 3.2% in the acidified water fraction for the glucose-lysine sample heated for 1 h at 103 °C and 90.2 ± 0.6% for the sample heated for 14 h at 103 °C ; their content was inferior to 2% in the ethanolic fraction. Recovering rates of 5-caffeoylquinic and *p*-coumaric acids were, respectively, 101.4 ± 8.0% and 104.0 ± 5.2% in the ethanolic fraction and were inferior to 2% in acidified water fraction. It was then possible to record the UV-visible spectrum of each fraction, to evaluate their respective antioxidant activity and to determine the actual content of phenolic compounds in the sugar cane stillage by Folin-Ciocalteu assay without interfering with MRP.

The spectra of the acidified water and ethanolic fractions showed distinct shapes (Figure 4a): a maximum at 275 nm for the acidified water fraction, and a maximum at 275 nm and a shoulder at 320 nm for the ethanolic fraction. This difference revealed distinct compositions. Comparison with standard solutions of phenolic acids already detected in large amounts in other sugar cane products, e.g. 5-caffeoylquinic and *p*-coumaric acids[25,36] and of MRP mixtures (Figure 4c–f), confirmed that the acidified water fraction contained MRP, whereas the ethanolic fraction were rich in phenolic compounds, probably hydroxycinnamic acids or derivatives. Moreover, the combined spectrum of both fractions (Figure 4 b) showed that the fractionation was quantitatively successful since the mixture of the two fractions was similar to the spectrum of the microfiltration permeate.

DPPH assays, applied to the microfiltration permeate and both chromatographic fractions, showed that the contribution of the MRP rich fraction to the global antioxidant activity of the sugar cane stillage, was stronger than the one of phenolic compounds rich fraction (Figure 5). Nevertheless, this latter was not negligible since it corresponded to more than one-third of the global antioxidant activity of the sugar cane stillage. It was also noted that the addition of the respective contributions of both antioxidant classes was higher than 100% for samples B1 and B2, which could suggest an antagonism between both types of molecules when they are combined in the sugar cane stillage. Thus, there is probably a real interest to separate them before valorisation of the phenolic compounds as antioxidants.

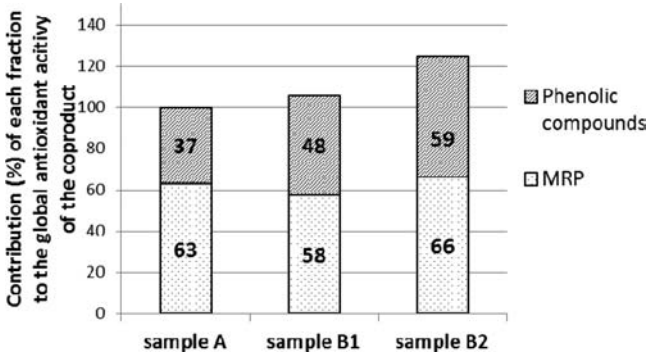

Figure 5. Relative contribution of phenolic compounds and MRP to global antioxidant activity of the sugar cane stillage microfiltration permeates from two different factories.

The amount of total phenolic compounds was assessed by Folin-Ciocalteu method in the ethanolic fraction and allowed to calculate this content in microfiltration permeate. Samples A, B1, and B2 respectively contained 2.9 ± 0.2, 2.8 ± 0.3, and 3.9 ± 0.1 g/L in 5-caffeoylquinic acid equivalent. Considering a dry matter of the sugar cane stillage at about 100 g/L, the content of phenolic compounds in sugar cane stillage was calculated to be around 28−39 g/kg of dry matter, thus demonstrating their competitiveness with well-known polyphenols-rich sugar cane stillages from other industries: 33 g/kg of dry apple peels, 13 g/kg of dried coconut husk, 2.3 g/kg of dry pulp of banana or 0.2 g/L of free polyphenols with low molecular weights for olive mill waste.[14] These analyses also revealed that the concentration of phenolic compounds of the coproduct was not specific to the distillery since samples A and B1 had similar total phenolic compounds amount whereas samples B1 and B2 did not. Numerous parameters could explain the difference between the total phenolic compounds amount of the three samples such as varietal and climatic conditions.[2,4]

Identification of the Main Phenolic Compounds from Sugar Cane Stillage. The ethanolic fraction obtained from Sephadex LH-20 chromatography was analyzed by high performance liquid chromatography coupled to UV-vis diode array detection and mass spectrometry (HPLC-DAD-MS) (Figure 6).

Thanks to a comparison with the UV and mass spectra of standard molecules, several free phenolic acids were identified, among them *p*-hydroxybenzoic acid (retention time rt = 8.87 min; *m*/*z* 137), vanillic acid (rt = 10.75 min; *m*/*z* 167) and syringic acid (rt = 11.87 min; *m*/*z* 197) (Table 1). This finding is consistent with the fact that these phenolic acids are also contained in sugar cane juice, syrup and molasses[36,37] and that they are not expected to be consumed during fermentation or lost during distillation process.[38]

Several quinic acid esters were also identified, among them 5-caffeoylquinic acid by comparison with the standard molecule

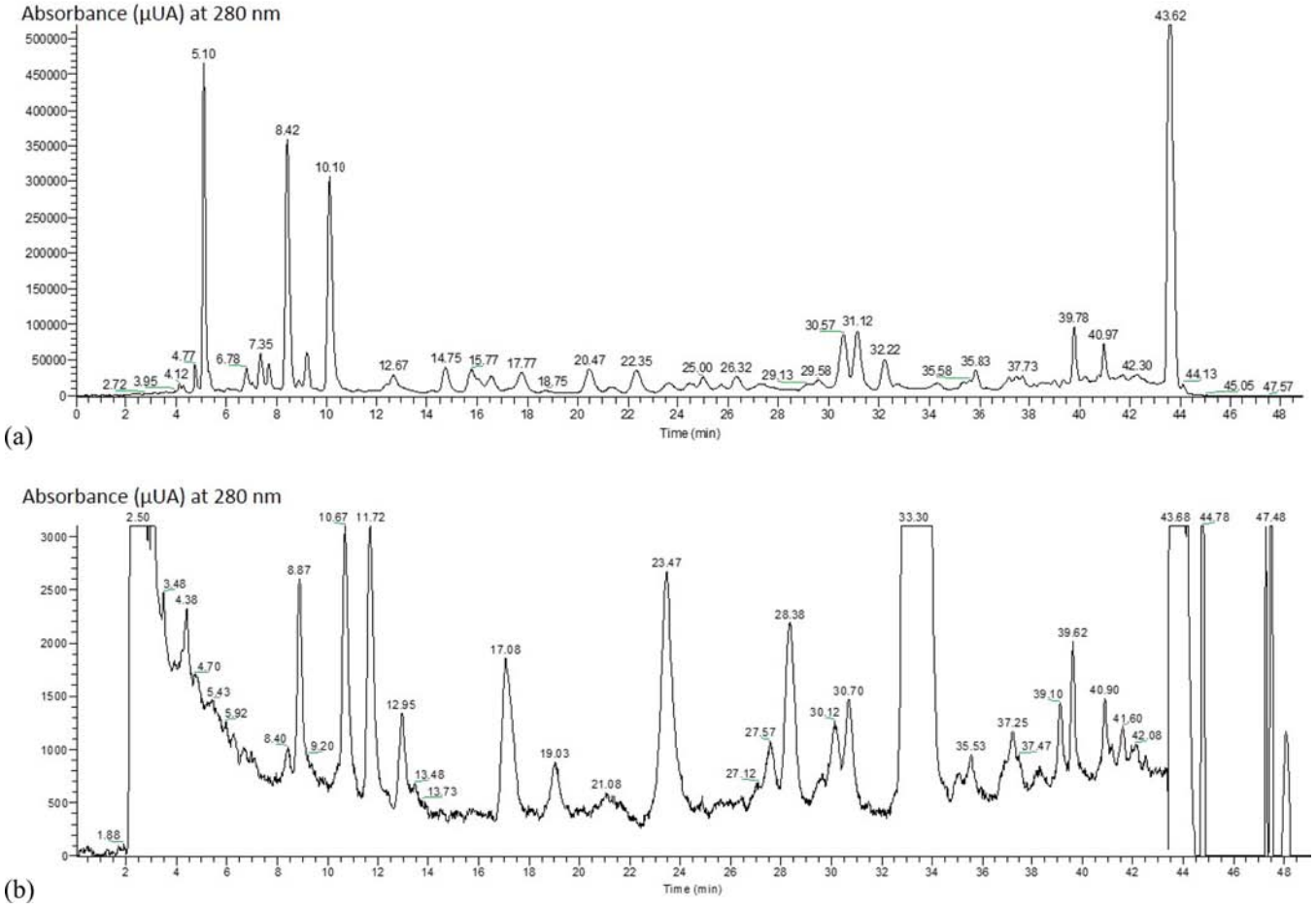

Figure 6. HPLC-DAD-MS chromatogram at 280 nm of the phenolic compounds-rich fraction recovered from chromatographic fractionation of sugar cane stillage microfiltration permeate: (a) before alkaline hydrolysis; (b) after alkaline hydrolysis.

Table 1. Molecules Identified by HPLC-DAD-MS Analysis in the Ethanolic Chromatographic Fraction[a].[45]

rt (min)	mass spectra (m/z)[b]	UV spectra (nm)	proposed molecule
Ethanolic Chromatographic Fraction			
5.10	191 (7), 353 (100)	295 (sh), 325 (max)	3-caffeoylquinic acid
8.42	191 (39), 353 (100)	295 (sh), 325 (max)	*trans* 5-caffeoylquinic acid[c]
8.87	137 (100)	255	*p*-hydroxybenzoic acid[c]
10.10	173 (7), 353 (100)	295 (sh), 325 (max)	4-caffeoylquinic acid
10.75	167 (100)	260 (max), 292	vanillic acid[c]
11.87	197 (100)	274	syringic acid[c]
14.75	191 (20), 353 (100)	325	*cis* 5-caffeoylquinic acid
15.78	191 (24), 337 (100)	280 (sh), 310 (max)	5-coumaroylquinic acid
17.73	173 (2), 337 (100)	280 (sh), 310 (max)	4-coumaroylquinic acid
20.47	191 (15), 367 (100)	293 (sh), 322 (max)	5-feruoylquinic acid
22.37	173 (13), 367 (100)	293 (sh), 322 (max)	4-feruoylquinic acid
Ethanolic Chromatographic Fraction after Mild and Severe Hydrolysis			
8.87	367 (100)	255	*p*-hydroxybenzoic acid[c]
10.67	167 (100)	260 (max), 292	vanillic acid[c]
11.72	197 (100)	274	syringic acid[c]
12.99	nd	282 (max)	*p*-hydroxybenzaldehyde[c]
17.08	nd	280 (max), 311	vanillin[c]
23.47	163 (100)	280 (sh), 310 (max)	*p*-coumaric acid[c]
28.28	193 (100)	293 (sh), 322 (max)	ferulic acid[c]

[a]rt: Retention time. For UV spectra, max: maximum height of peak; sh: shoulder. Esters of quinic acids are numbered as requested by IUPAC nomenclature. [b]Mass spectra corresponding to the $[M-H]^-$ pseudomolecular ion (intensity taken as 100) and to major fragment ions, relative intensities are specified in parentheses. [c]Identification confirmed by comparison with standard molecule

(rt = 8.42 min; m/z 353, 191). Two additional peaks (rt = 5.10 and 10.10 min) showed the same UV-vis spectrum and pseudo molecular ion (m/z 353) as 5-caffeoyl acid and could correspond to 3- and 4-caffeoylquinic acids. According to the respective polarity of these different isomers and to the presence of a fragmental ion at either m/z 191 (due to the loss of caffeoyl group) or m/z 173 (due to the loss of caffeoyl group and then water), the peak eluted at 5.10 min would correspond to 3-caffeoylquinic acid and the one at 10.10 min to 4-caffeoylquinic acid.[39−42] Further experiments by HPLC-ESI-MS^n should be performed in order to confirm these identifications. Another peak (rt = 14.8 min) showed the same pseudo molecular ion (m/z 353) as 5-caffeoylquinic acid but its UV-vis spectrum was slightly different, with the absence of the shoulder at 295 nm. This difference of absorbance could be representative of an isomerization of 5-caffeoylquinic acid in its *cis* isomer,[39] moreover knowing that the *cis* isomer is more hydrophobic than the *trans* one,[43] which explains its higher retention time on the chromatographic column.

Two compounds, eluted at 15.78 and 17.73 min, respectively, showed the same UV-vis spectra as *p*-coumaric acid (shoulder at 280 nm and maximum at 310 nm) and their pseudo molecular ions at m/z 337 lead to identify them as coumaroylquinic acids. The presence of a fragmental ion at m/z 191 may mean that the peak at 15.78 min could correspond to 5-coumaroylquinic acid and the presence of a fragmental ion at m/z 173 may mean the peak at 17.73 min could correspond to 4-coumaroylquinic acid, by analogy with the 5-caffeoylquinic acid. A similar reasoning resulted in identifying 5-feruoylquinic acid at a retention time of 20.47 min and 4-feruoylquinic acid at 22.37 min.[36,41−43] These identifications should also be further confirmed by HPLC-ESI-MS^n analysis.

In order to go further in this analysis, a mild and a severe alkaline hydrolysis were carried out on this ethanolic chromatographic fraction and the produced samples were analyzed by HPLC-DAD-MS. As described by Culhaoglu et al.,[32] mild alkaline conditions make possible the hydrolysis of esters whereas severe alkaline conditions cause the hydrolysis of both ester and ether linked structures. The same phenolic compounds were identified in both hydrolysates (Table 1). Ferulic acid and *p*-coumaric acid, not present in the initial fraction, were detected, which confirmed the presence of corresponding quinic acid ester in the initial fraction. Eventually, *p*-hydroxybenzaldehyde and vanillin were identified by comparison with standard molecules only in hydrolysate which means that the initial sample probably contained esters of these molecules.

Further experiments will be necessary in order to confirm some identification, in particular for hydroxycinnamic derivatives and to quantify each of them. The presence of flavonoids in the samples will also be looked for, this chemical class being already detected in sugar cane juice and molasses.[25,26,36]

In conclusion, sugar cane stillage turned out to have antioxidant properties, with DPPH° scavenging activity comparable to that of other industrial food by products. This activity was found correlated to the content of reducing compounds as determined by the Folin-Ciocalteu method, which confirms the relevancy of this method, as a convenient routine method to predict the antioxidant potential of industrial coproducts. Chromatographic fractionation on Sephadex LH-20 resin of the coproduct microfiltration permeate allowed to separate in a quantitative manner two antioxidant fractions, respectively enriched in phenolic compounds and Maillard Reaction Products. The contribution of MRP fraction to the global antioxidant activity was shown to be stronger than that of the phenolic fraction. This result stresses the interest of such a fractionation process with respect to possible future exploitation of molasses distillery coproducts as a source of high-value biobased molecules. Preliminary analysis by HPLC-DAD-MS allowed to identify several free phenolic acids and gave clues to point out esters of quinic acids. These first original results on a so far hardly investigated coproduct open to the prospect of further in-depth structural investigations and exploration of other functional properties.

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Notes

The authors declare no competing financial interest.

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