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Quantification of the cyanogen Dhurrin at high levels in sugar cane varieties and its correlation as a precursor of ethyl carbamate in cachaças

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Abstract: Cachaça, a genuinely Brazilian spirit, contains ethanol, water, and various secondary compounds, along with potential contaminants such as ethyl carbamate (EC), which can depreciate product quality. EC can form from cyanogenic glycosides like Dhurrin, present in sugarcane. This study aimed to quantify Dhurrin in methanolic extracts using High Performance Liquid Chromatography (HPLC), evaluate its variation across different sugarcane varieties, and relate it to the presence or absence of the sugarcane tip (topping level), as well as correlate it with EC concentrations in cachaça. Six sugarcane varieties were evaluated: RB867515, CTC2, CTC11, CTC9003, IACSP95-5000, and SP80-1842. For each, two plant fractions were studied: Po (stalks with tips, including apical meristem and immature leaves) and Co (mature stalks without the tip). Methanolic extracts from these fractions were analyzed to determine Dhurrin concentration, and cachaças were produced from each fraction for EC and ethanol analysis. The results showed that sugarcane tips (Po) contained higher levels of Dhurrin and led to significantly higher EC concentrations in the resulting cachaças. This is likely due to the biochemical composition of the tip, particularly the higher amino acid content in the apical meristem, which favors EC formation. These findings highlight the importance of proper harvesting practices specifically, the removal of sugarcane tips to reduce EC levels in the final beverage.

Keywords: Alcoholic beverage; Control and quality; Cyanogenic agents; Ethyl carbamate; Organic contaminants.
Abbreviations: BP_boiling point; Co_stems without tips; EC_ Ethyl carbamate; HCN_hydrocyanic acid; INCT-Café_National Institute of Coffee Science and Technology; MAPA_Ministry of Agriculture, Livestock and Supply; MP_melting point; Po_stems with tips; UFLA_Federal University of Lavras.

Introduction

Cachaça, a genuinely Brazilian beverage, is one of the most widely consumed spirits throughout the country and the world. Its history was built alongside the history of Brazil, going from being a beverage consumed only by slaves to becoming appreciated by various social classes. According to Ordinance No. 539 of December 26, 2022, of the Ministry of Agriculture, Livestock and Supply (MAPA), cachaça is the typical and exclusive name for sugarcane spirits produced in Brazil, with an alcohol content of 38.0 to 48.0% (v/v) at 20.0 °C, obtained by the distillation of fermented must from sugar cane juice with peculiar sensorial characteristics. Sugars can be added (Brasil, 2022).

Chemically, cachaça is predominantly composed of ethanol and water, in addition to hundreds of secondary compounds such as carboxylic acids, esters, aldehydes and higher alcohols, which add aroma and flavor characteristics to the beverage. In addition to these substances, the formation of organic and inorganic substances, capable of degrading the quality of the final beverage and being unhealthy for the consumer, can occur throughout the production chain (Cardoso, 2020).

Among these contaminants, ethyl carbamate (EC) stands out because it is classified as a possible carcinogen for humans (Group 2A) by the International Agency for Research on Cancer (IARC). It can be found in several foods and fermented beverages consumed daily, such as cheeses, breads, yogurt, wine, soy sauce, whiskey and cachaça, and requires strict control of its limits (IARC, 2010; Gowd et al., 2018; Jung et al., 2021).

The EC formation pathway is not yet well understood, but it is believed that this compound can be formed at various stages of the production of cachaça and distilled spirits. The precursors present in sugarcane varieties have been identified as one of the main routes for the presence of this contaminant in beverages. It is suspected that the EC can result from the presence of cyanogenic glycosides in the raw material, which, depending on the process, can be converted into cyanide and, through the reaction with ethanol, EC forms, as is shown in Figure 1.

Cyanogenic glycosides are formed in the secondary metabolism of various plants. They are derived from amino acids and are present in more than 2500 species of plants, such as barley, rice, apples, mangos, malt, cassava, sorghum and sugarcane. Through enzymatic degradation, they generate cyanohydrin which breaks down into cyanide ions. The oxidation of cyanide ions leads to the formation of isocyanate, which reacts with ethanol to form EC (Vetter, 2000; Santiago et al., 2017; Cressey and Reeve, 2019). The cyanide content in plants varies in concentration in different plant species. Jayasekara and Horagoda (1992) and Melo et al. (2005) observed that the HCN content was 75% higher in in mature *Manihot glaziovii* leaves than in *Manihot esculenta* leaves. They also observed that the concentration of this substance was higher in young than in mature leaves, with a 25% difference in *M. esculenta* and 16% difference in *M. glaziovii*.

Dhurrin is a natural toxin produced mainly by sorghum and most cyanogenic plants in the Poaceae family. Its molecular formula is $C_{14}H_{17}NO_7$, the molar mass is 311.29 g mol^{-1}, density, 1.56 g mL^{-1}; melting point (mp), 200 ºC; boiling point (bp), 586.7 ºC at 760 mm Hg; refractive index, 1.65 (Figure 2). During cyanogenesis, this compound is degraded to form hydrocyanic acid (HCN). Sugarcane (*Saccharum* spp) is the raw material for the production of cachaça. The plant is cyanogenic and can contain Dhurrin. This compound passes into the must, leading to the formation of ethyl carbamate. Currently, there are several varieties of sugarcane available in Brazil, with varying chemical compositions that vary with the species (Halkieret al., 1988; Johansen et al., 2007; Tokpohozin; Fischer and Becker, 2018; Cravo et al., 2019; Guidechem, 2021).

The choice of sugarcane variety for the production of cachaça or distilled spirits is crucial because its characteristics directly influence the final product. Many stills do not perform the ideal trimming of the sugarcane tip during harvest. The tip is composed of immature internodes, apical meristem and leaves that are still green and contain high levels of reducing sugars, amino acids, starch, organic acids, phenolic compounds and total polysaccharides, in addition to low levels of sucrose. These compounds can compromise the quality of the beverage (Dinardo-Miranda et al., 2008). Therefore, a thorough study to identify the effect of removal of the tip (a vegetable impurity that contains these glycosides) during the harvest of sugarcane varieties and verify the influence on the formation of EC is essential because the contamination of beverages by EC can be an obstacle to the sale of distilled spirits (Cardoso, 2020).

The objectives of this study were to propose a method using high performance liquid chromatography to quantify the cyanogenic glycoside Dhurrin in methanolic extracts in relation to the degree of pruning in different varieties of sugar cane and correlate its influence with the formation of EC in cachaça. This study was performed because some producers harvest sugarcane that is not yet fully matured for the production of cachaça.

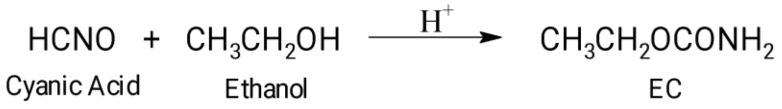
Results and Discussion

Alcohol level analysis of cachaças

The alcohol concentrations obtained from the physical-chemical evaluation of cachaças obtained according to harvesting levels of stems and sugarcane varieties are represented in Figure 3.

The values observed for all the samples were within the maximum and minimum limits allowed by Brazilian legislation for the beverage to be considered cachaça, sugarcane spirits or both (Brasil, 2022). According to Normative Instruction No. 539 of December 26, 2022, sugarcane spirits are a beverage with an alcohol content between 38% and 54% (v/v) at 20 ºC, obtained from the simple alcoholic distillation of sugarcane or by distillation of the fermented must of sugarcane juice, to which up to 6.0 g L^{-1} of sugar, expressed as sucrose, can be added. Cachaça is the typical and exclusive name for sugarcane spirits produced in Brazil, with an alcohol content between 38% and 48% (v/v) at 20 ºC, obtained by the distillation of fermented must from sugarcane juice, with peculiar sensory characteristics, to which up to 6.0 g L^{-1} of sugar, expressed in sucrose, can be added.

Minimum significant differences in the samples as a function of the variety of sugarcane can be seen. Therefore, it is extremely important when cutting the fractions during the distillation process to avoid contamination of the "heart" fraction so as to avoid an excess of some compounds and organic contaminants that are unhealthy. This fact that can be observed in this study regarding alcohol content (Santiago et al., 2016; Alvarenga et al., 2023; Barbosa et al., 2023). The volatile components (water, ethanol, methanol, higher alcohols, acetic acid, esters, aldehydes, carbon dioxide, etc.) are separated from the fixed or non-volatile components (wort solids, yeast cells, minerals, organic and inorganic acids) in the distillation process. The substances are separated according to boiling temperature, affinity with alcohol/water, and alcohol content in the vapor phase, which favor the differentiation of the composition of each fraction collected during the distillation of the beverage (Cardoso, 2020).



$$HCNO + CH_3CH_2OH \xrightarrow{H^+} CH_3CH_2OCONH_2$$

Fig. 1 Reaction of cyanic acid with ethanol to form EC in cachaças.

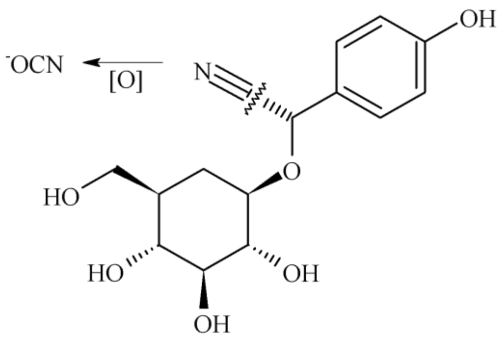


Fig. 2 Chemical structure of the Dhurrim molecule.

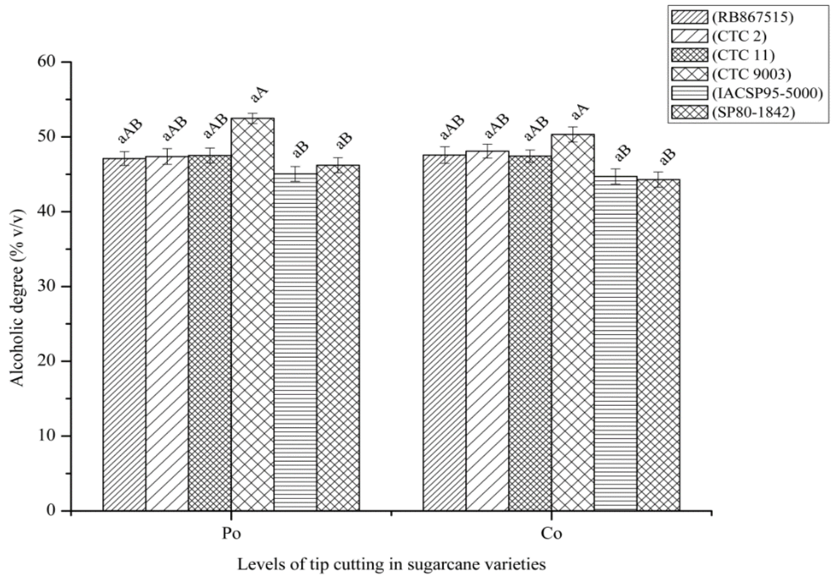


Fig. 3 Assessment of ethanol content according to the variety of cane and cut of the cachaças obtained. The means (+/- standard deviation) followed by the same lowercase letter (between cropping levels) and the same capital letter (between varieties) do not differ from one another using the Tukey Test at the 5% probability level.

Standardization of the Dhurrin detection method

A high degree of separation and response under the chromatographic conditions used can be observed in the chromatogram of the Dhurrin standard solution (Figure 4) after spectrophotometric detection at 232 nm, obtained after injection of 20 µL. The average retention time obtained for the compound was 6.17 ± 0.19 min, a value close to that found by Nicola et al. (2011).

A satisfactory selectivity of the proposed method for detecting Dhurrin in methanolic extracts of tips and stalks in different varieties of sugar cane was obtained using calibration by the addition of the standard. The selectivity can be observed in Figure 5, where similar angular coefficients can be observed when comparing the analytical curves, curve (1) of the standard and curve (2) of the standard/matrix. The curves are parallel, and it can be inferred that there was no interference of the matrix in determining the substance of interest; thus, the method was shown to be selective.

A coefficient of determination (R) of 0.99999 was obtained, which indicates that there is a strong linear correlation between the concentration of the compound and the areas of the peaks analyzed, and proves the linearity of the method (Snyderet al., 1997; Ribani et al., 2004; Harris, 2008; MAPA, 2011; Magnusson and Örnemark, 2014). For Nascimento et al. (2018), the estimate of the analyte concentration is more appropriate when the value of the coefficient of determination is closest to 1. Nicola et al. (2011) obtained a linear regression of R = 0.9999 using a five-point calibration curve in the range of 5 to 100 mg L^{-1} to determine dhurrin.

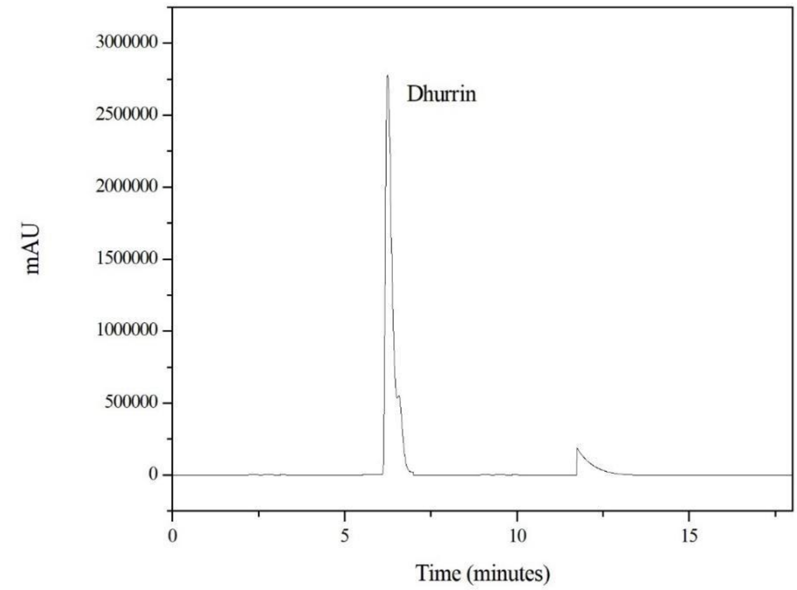


Fig. 4 Chromatogram of the standard Dhurrin solution at a concentration of 0.01 mg mL^{-1}

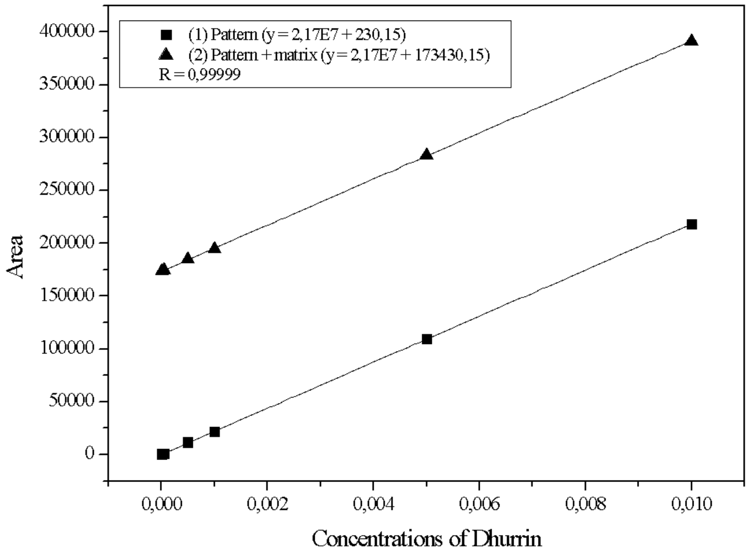


Fig. 5 Equations for the straight lines of the standard curve and standard/Dhurrin matrix.

The detection and quantification limits found for dhurrin were 0.00004 and 0.0001 mg mL^{-1}, respectively. The values obtained were lower than those found by Nicola et al. (2011) and Gustafson et al. (2020). The values found in this study are satisfactory because the concentrations of the analyte were quantifiable in the sugarcane matrix. Differences arose as a result of chromatographic conditions adopted for quantification, such as the equipment, methods or both.

The precision of the methods used was evaluated through the repeatability of three points for hurrin determination, repeated over four different days. The values varied from 0.99 to 3.26%. According to the literature, the CV value should not exceed 5% for the analysis of compounds present in low concentrations, which attests to a positive precision of the analytical method (Ribani et al., 2004; Collins et al., 2006; MAPA, 2011; Magnusson & Örnemark, 2014). The precision obtained corroborates studies in the literature in which cyanogenic glycosides in other matrices were quantified (Burke et al., 2013; Hayes et al., 2016; Emendack et al., 2017; Gustafson et al., 2020). Nicola et al. (2011) studied the presence of Dhurrin in sorghum and obtained values that varied between 0.4 and 1.9%. Madrera and Valles (2021) standardized a method for the detection of two cyanogenic compounds derived from mandelonitrile (amygdalin and R-prunasin) in Rosaceae and Sambucus families and obtained repeatability values ranging from 0.4% to 4.1%.

Regarding the accuracy of the method analyzed in this study, results were found regarding recovery percentages for the determination of Dhurrin in sugarcane extract samples. Values ranged from 96.7% to 116.2%. To be acceptable, recovery values must vary from 70 to 120%, with a ± 20% precision. However, depending on the analytical complexity and the sample, this value can range from 50 to 120%, with precision of up to ± 15% (Ribani et al., 2004; MAPA, 2011; Magnusson and Örnemark, 2014). Thus, a good recovery was obtained for Dhurrin with the method, and it is, therefore, suitable for determining the cyanogenic glycoside. Recovery values close to those found in this study have been reported in the literature. Gustafson et al. (2020) proposed an HPLC method to determine Dhurrin in bicolor sorghum and obtained recovery rates ranging from 92 to 98%. The author reports that the analytical method developed proved to be simple and reliable. Nicola and collaborators (2011) found recovery values ranging from 87% to 98% for Dhurrin determination in sorghum varieties.

Determination of the cyanogenic glycoside Dhurrin in sugarcane extracts and quantification of ethyl carbamate in cachaças

The results obtained for the quantification of Dhurrin and ethyl carbamate in samples of sugar cane extract and cachaça are represented in Figure 6. Dhurrin was found in all the different varieties of sugar cane studied in this work. Dhurrin from Po and Co was detected and quantified in different varieties and in different parts of plants, where significantly different values were observed for some varieties. On the average, the concentration of Dhurrin in the tips (Po) varied from 0.001 to 0.051 mg $mL^{-1.}$ It varied from 0.002 to 0.050 mg mL^{-1} in the sprouted culms (Co).

Dhurrin is a natural toxin produced mainly by sorghum. It constitutes 30% of the dry weight of these plants, but it is absent in the seeds and roots. The distribution of Dhurrin in sorghum means that both production and storage occur in the same location within the same cells. As in sorghum, it is present in most cyanogenic plants of the Poaceae family, including sugarcane (Halkier et al., 1988; Johansen et al., 2007; Tokpohozin et al., 2018; Castada et al., 2020).

Dhurrin biosynthesis in sorghum occurs in the presence of a specific enzymatic system (cytochrome-P450 and UPD-glucosyltransferase enzymes), in which the CYP79A1 enzyme converts tyrosine to (Z)-p-hydroxyphenylacetaldoxime. The CYP71E1 enzyme converts this compound into *p*-hydroxymandelonitrile, and a UDP-glycosyltransferase converts it into Dhurrin (Tokpohozin et al., 2018). Rosa Júnior (2005) reported the presence of Dhurrin in sugarcane leaf extracts at a concentration of 4.3 mg g^{-1} of fresh leaf mass. According to the author, genes that encode the UDP-glucosyltrasferase and dhurrinase enzymes are induced by pest attacks on the plant.

The highest concentrations of the Dhurrin were found in the Po and Co samples from the SP80-1842 variety of sugarcane (Figure 6). They were statistically similar to each other and distinct from the other samples, both with respect to different varieties and to parts of the plant (tip and stalk). The highest concentrations of dhurrin were associated with the presence of the tip, Po, which contained the highest average Dhurrin concentrations (RB867515, Po = 0.02 ± 0.001 mg mL^{-1}, Co = 0.001 ± 0, 0007 mg mL^{-1}; CTC 2, Po = 0.009 ± 0.001 mg mL^{-1}; Co = 0.001 ± 0.0005 mg mL^{-1}; 0008 mg mL^{-1}; CTC9003, Po = 0.021 ± 0.001 mg mL^{-1}, Co = 0.023 ± 0.001 mg mL^{-1}; 0006 mg mL^{-1} and SP80-1842, Po = 0.051 ± 0.001 mg mL^{-1}, Co = 0.050 ± 0.001 mg mL^{-1}). Studies by Cravo et al. (2019) corroborate the present study because the authors report the presence of Dhurrin in different varieties and in different portions of sugarcane. The study was performed using thin layer chromatography, where the presence of the compound under study could be observed.

The results in this study demonstrate that the Dhurrin content is variable among the different varieties of sugarcane studied, and its concentration can vary in different parts of the sugarcane. Tokpohozin et al. (2018) reported that the dhurrin content in sorghum grains also varied with the cultivars studied. Miller and Tuck (2013), when studying cyanogenic glycosides in the foliage of a tropical tree species (*Polyscias australia*), found high levels of Dhurrin in the tips of the youngest leaves, especially in the emerging leaves. The work showed that these high levels can serve as protection from pests and provide a source of nitrogen during plant leaf expansion. The authors reported that the higher concentrations of cyanogenic glycosides in the soft and expanding tissues of leaves and the greater relative investment of nitrogen in the defense of young tissues are consistent with the predictions of the Optimal Allocation Theory of plant defense, which predicts that more valuable and vulnerable parts of the plant are more heavily defended. This fact was proven in this study, where the portion of the plant with the greatest expansion and development (leaves and young stems) contained a higher concentration of Dhurrin.

Yadav et al. (2023) reported that Dhurrin has relevant biological activity against various herbivores as a natural control agent and acts as a substitute for chemically synthesized pesticides. They mentioned that Dhurrin is catabolized into HCN (a respiratory inhibitor) during herbivory, making it useful for sustainable pest management. Thus, it can be inferred that ethyl carbamate can potentially be formed in cachaças because HCN groups can be released by sugarcane throughout the beverage production process.

According to Cheeke (1998) and Melo et al. (2005), cyanide is released from the glycoside by two chemical reactions through the action of endogenous plant hydroxylases. First, ß-glucosidase promotes the breakdown of the glycoside into a sugar and a cyanohydrin, which is an aglycone. The cyanohydrin is then dissociated by the enzyme hydroxynitrilaliase into hydrocyanic acid and benzaldehyde or a ketone. However, in intact plant cells, cyanide is not released because of the spatial separation between the cyanogenic glycoside and the enzymes that degrade it. The glycoside is found in the vacuole and the enzymes in the cytosol. The meeting of the glycoside with the enzymes and the consequent enzymatic degradation occurs when there is physical damage to plant cells.

The localization of cyanogenic glycosides in sorghum was visualized using Raman hyperspectral imaging (Heraud et al., 2018). The authors showed that Dhurrin is mainly found around the cortical tissue of leaves and in the outer layers of cells, which is consistent with its role in defense against herbivores. At the same time, work by Sun et al. (2018) inferred that the cyanogenic potential in sorghum can increase according to conditions of water stress, salt, frost, light, nitrogen fertilization and attack by herbivores and insects, varying according to the genetic history of the crop and its development.

It is clear from Figure 6 that the average EC concentration for the different varieties and pruning levels ranged from 18.17 µg L^{-1} to 65.02 µg L^{-1}. The levels in all the cachaça samples were below the maximum limit allowed by legislation, which is 210 µg L^{-1} (Brasil, 2022). The presence of the Po tip resulted in the highest concentrations of EC, the highest being observed in the SP80-1842 sample. Significantly different results were obtained for all the samples when the cropping levels (Po and Co) were compared.

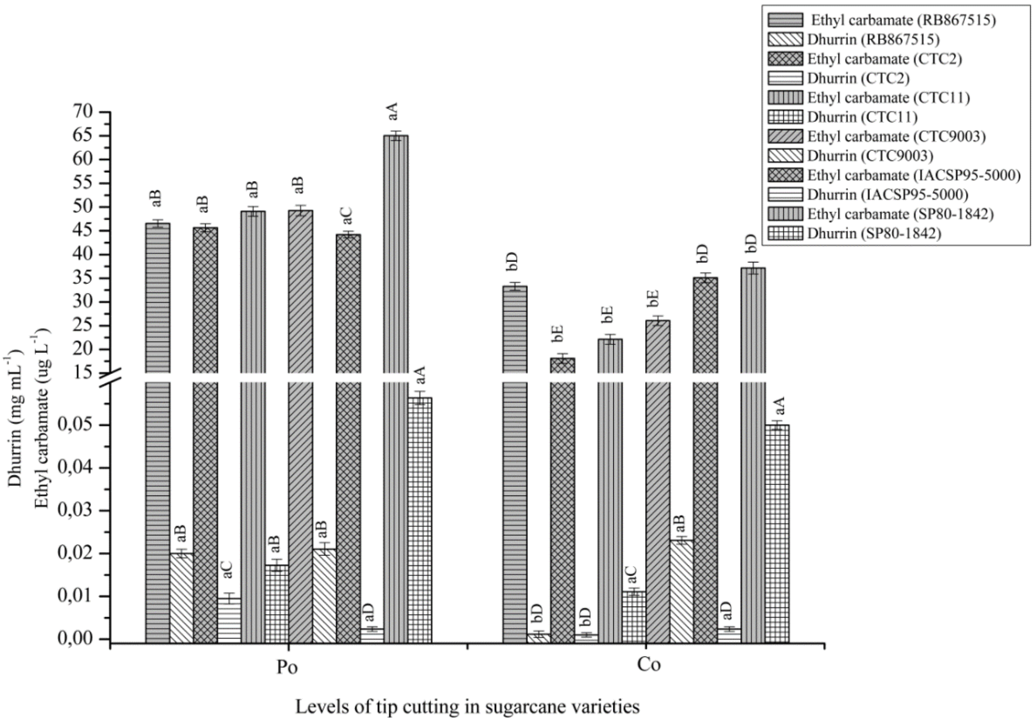


Fig. 6 Average concentrations of Dhurrin and EC in sugarcane extracts and cachaças. The means (+/- standard deviation) followed by the same lowercase letter (between cropping samples) and the same capital letter (between varieties) do not differ from one another by the Tukey test at the 5% probability level.

According to Cardoso (2020), several pathways for the formation of EC have already been established; Due to the complexity of the cachaça production chain, several hypotheses have already been raised and studied regarding its origin from the raw material, fermentation (yeast, temperature and pH), distillation (material, equipment geometry, fraction cutting, heating profile) until storage or aging of the distillate. Bueno et al. (2020) demonstrated that efficient quality control in cachaça producing industries is effective in reducing the concentration of ethyl carbamate. The impact of the use of active yeasts, post-harvest quality, selection of the variety and temperature reduction during distillation were the most critical points in the process of obtaining the distillates described by the authors.

Some authors suggest that the contaminant can be formed from the raw material and also from the fermentation must (Bortoletto et al., 2015; Galinaro et al., 2015; D'avila et al., 2016; Fang et al., 2018; Cravo et al., 2019; Jung et al., 2021) through the metabolism of microorganisms during fermentation, depending on the substrate, type of microorganisms used and process conditions (Borges et al., 2014; Mendonça et al., 2016; Gowd et al., 2018). Another route for formation would be during distillation, depending on the way the process is performed (continuous or discontinuous), the still material, the separation of fractions and the reflux rate during the process (Borges et al., 2014; Bortoletto et al., 2015). Some authors mention that the EC can be formed in the last stage of the process, which involves aging or storage in wooden or stainless steel barrels, depending on the material used, the conditions and the length of storage (Anjos et al., 2011; Santiago et al., 2017; Barbosa et al., 2023). From the results of this study, it can be inferred that the EC concentration was influenced by the variety of sugarcane, cropping levels, and concentration of Dhurrin because statistically different results regarding the proposed variables were observed.

EC can be formed from cyanogenic glycosides at various stages in the cachaça production process through enzymatic degradation and thermal cleavage of these glycosides present in sugar cane. Because these cyanogenic cultures contain HCN bound to sugars, the sugars are released by hydrolysis in a process called cyanogenesis. The concentration and type of cyanogenic glycosides varies between species, cultivars and varieties and especially the different parts of plants. Correlating the Dhurrin glycoside with the concentration of EC in cachaças, one can see the strong relationship found in the study, where the higher concentration of Dhurrin led to a higher concentration of EC in the distillate. This phenomenon can be attributed to the presence of the plant's apical meristem, which, because it is responsible for growth, contains higher concentrations of amino acids than the stem, thereby influencing the formation of EC (Vetter, 2000; Asad et al., 2009; Cuny et al., 2019).

Factors such as climate, nitrogen fertilization and water deficiency can influence the EC concentration, in addition to the age of the plant, because the younger and faster growing the plant, the higher its cyanogenic glycoside content will be (Miller and Tuck 2013; Shlichta et al., 2014; Cuny et al., 2019). Some authors report that fertilization of sugarcane with nitrogenous compounds can contribute to the formation of potential EC precursors, such as urea, citrulline, N-carbamyl phosphate and cyanide (Pelegrin et al., 2020). Nascimento et al. (2022) found that there is a correlation between the incidence of total nitrogen found in sugarcane juice with the formation of EC in cachaças, even if these cachaças were in compliance with current legislation. Machado et al. (2012, 2013) correlated the formation of EC in cachaças with nitrogen fertilization in the sugarcane field, expecting the nitrogen supplied to the sugarcane crop to influence the composition of nitrogenous compounds. In the study, the authors concluded that there was no correlation between the different doses of urea and ammonium nitrate and the concentration of EC, showing that its formation did not occur during sugarcane planting and growth. Borges et al. (2014) evaluated EC levels in different fermented and distilled musts of cachaças produced with different yeasts (spontaneous and selected). The authors concluded that EC levels were lower in the fermentation process and in distillation when performed correctly.

Mendonça et al. (2016) determined the EC concentrations in cachaças produced by different types of fermentation (selected yeasts and spontaneous fermentation) and followed the formation during the production stages. The authors came to the conclusion that the addition of nutrients in the fermentation process led to an increase in the EC concentrations when they used rice bran or a mixture of corn bran and rice bran, a plausible factor given that such nutrients can contain nitrogenous or cyanogenic groups.

Research by Qin and collaborators (2021) on the effect of fermentation of cassava flour on EC levels indicates that, even during fermentation, the cyanogenic glycoside (linamarin) was not completely hydrolyzed. It was present in the fermented liquid and vinasse. The authors' data show that a large part of the glycoside is only broken-down during fermentation to release hydrocyanic acid. However, the authors concluded that the cyanide content in cassava flour decreased during fermentation.

Cravo and collaborators (2019) evaluated the correlation between the presence of cyanogenic groups from different varieties of sugarcane in the formation of EC in cachaças. The authors observed the formation of EC in greater quantities in two samples of cachaça from the SP832847 and SP803280 sugarcane varieties. These results corroborate those found in this study, especially for the variety SP80-1842, which has the same parents as SP80-3280 (SP71-1088 × H57-5028). This fact reinforces the genetic makeup identified in both studies, indicating a higher concentration of EC in these materials.

Research indicates that efficient quality control in beverage producing industries is effective in reducing the concentration of EC in distillates (Bueno et al., 2020). For Pimentel and Andrade (2020), the use of sugarcane tips is harmful. In addition to contaminating the juice with gums, resins, and salts, they can be detrimental to the fermentation process, leading to the formation of contaminants such as butyl alcohol, *sec*-butyl alcohol, acrolein and EC. Furthermore, sugarcane tips are low in sugar, which can hinder the formation of ethanol.

Materials and Methods

Collection and preparation of sugar cane

The sugarcane varieties were collected at the Fazenda Cassiano, located in a rural area of the city of Candeias, Minas Gerais, Brazil, at an altitude of 950 m (latitude 20º 45' 05'' S and longitude 45º 15' 15'' W). The climate of the region was type Cwa, according to the Köppen classification.

The choice of varieties used in this study is justified by their wide application in the production of cachaça. The following varieties were used, RB867515, CTC2, CTC11, CTC9003, IACSP95-5000 and SP80-1842. Plant samples were collected from the first cut (sugarcane plant). Two parts were collected, tips and stems, identified as Po and Co, respectively, for each variety studied. The tips were classified as the first leaves of the cartridge, subsequent leaves and first visible collar and the culms were used as being developed/mature, a classification based on Silva and Silva (2012). After collection, they were sanitized and sent to the Spirits Quality Analysis Laboratory of the Chemistry Department of the Federal University of Lavras (UFLA). The sugarcane samples were dried in a forced-air oven at 38 ºC until completely dry, and the plant materials were crushed in an analytical type IKA model A11Basic mill for approximately one minute. The samples were then sieved on a 200 mesh Granulometric Sieve/Opening 0.075 mm and stored in dark amber glass containers under refrigeration.

Obtaining methanolic extracts from sugarcane samples

The extracts were obtained following the method proposed by Cravo et al. (2019), with some adaptations. The mass of 40 g of crushed/sieved samples was measured and transferred to a round-bottom flask coupled to a condenser, using 500 mL of methanol and left at slow reflux for 6 hours. After extraction, the samples were filtered in a Büchner funnel, and the solvent was evaporated in a Büchi model B-480 rotary evaporator to furnish the dry crude extract.

Chromatographic analysis of the cyanogenic glycoside Dhurrin

The determination of Dhurrin concentration in crude sugarcane extracts was achieved by HPLC, based on the method proposed by Nicola et al. (2011) and Emendack et al. (2017) with modifications. No standardized analysis for its quantification in sugarcane was found in the literature. Thus, a chromatographic method was standardized for the quantification of the analyte under study, in accordance with Ribani et al. (2004), MAPA (2011) and Magnusson and Örnemark (2014).

Samples were prepared using 1 g of each dry crude extract, weighed on an analytical balance (Shimadzu, model AY220). Subsequently, 1 mL of a methanol:water (1:1) solution was added and stirred for ± 2 minutes in a Vortex shaker until complete homogenization. The mixture was filtered through a 0.45 µm polyethylene membrane (Millipore) and injected directly into the chromatographic system.

Chromatographic conditions and standardization of Dhurrin analysis

The external standardization method was employed for quantification. The analytical curve for Dhurrin (Sigma-Aldrich) was obtained from the stock solution in type I ultrapure water and methanol (1:1) at a concentration of 1 mg mL^{-1}. The preparation of concentrations used in the curve varied between 0.00003 and 0.01 mg mL^{-1}. Analyses of extracts were performed at the National Institute of Coffee Science and Technology at UFLA (INCT-Café). A Shimadzu HPLC high-efficiency liquid chromatograph was used, equipped with two high-pressure model SPD-M20A pumps, a model DGU-20A3 degasser, a model CBM-20A interface, a model SIL-10AF automatic injector and a CTO-20A oven. The column used for the separations was the Agilent-Zorbax Eclipse XDB-C18 column (4.6 x 250 mm, 5 µm) connected to an Agilent-Zorbax Eclipse pre-column. Dhurrin detection was performed using an SPD-20A UV-vis detector. A gradient of water (Solvent A) and acetonitrile (Solvent B) was used as the mobile phase, with the following programming: 1 min (10% B); 7 min (30% B) and 9 to 60 minutes (10% B). The wavelength used was 232 nm, the flow rate was 1 mL per minute, and the oven temperature was 35 ºC.

The Dhurrin standard and samples were injected into the chromatographic system in triplicate. The identity of the analytes was confirmed by the retention times, and the peak profiles of the samples were compared to those of the standards.

To guarantee the analytical quality of the results, validation procedures for the applied method were performed using the following parameters: selectivity, linearity, precision, accuracy, limit of detection (LD) and limit of quantification (LQ). Method selectivity was achieved through calibration by the addition of the standard. An analytical curve was constructed with the addition of the substance of interest to the matrix and compared with an analytical curve without the presence of the matrix (Ribani et al., 2004; MAPA, 2011; Magnusson and Örnemark, 2014).

Linearity was obtained by external standardization using analytical curves and determining the coefficients of determination (R) expressed by the straight line equation. To determine LD and LQ, the parameters related to each analytical curve were considered using the following mathematical relationships:

$LD = 3x\ \frac{s}{S}$ and $LQ = 10x\ \frac{s}{S}$

where s is the estimate of the standard deviation of the regression line equation and S is the angular coefficient of the analytical curve (Ribani et al., 2004; MAPA, 2011; Magnusson and Örnemark, 2014;).

Accuracy was expressed through intermediate precision. For this purpose, three points of the Dhurrin analytical curve were analyzed (0.00003; 0.005 and 0.01 mg mL^{-1}) on five different days, and the coefficients of variation (CV) were estimated at the end of successive repetitions. To estimate the precision of the method, the coefficients of variation (CV) of a series of measurements were determined using the following mathematical equation:

$$CV\ (\%) = \frac{s}{CMD}\ x\ 100$$

where, s = estimate of the standard deviation; CMD = average measured concentration (Ribani et al., 2004; MAPA, 2011; Magnusson and Örnemark, 2014).

Accuracy was assessed through recovery tests using three randomly chosen samples. These samples were fortified at three concentration levels (0.00003, 0.005 and 0.01 mg mL^{-1}). Recovery was determined considering the results obtained for each analyte studied using the following mathematical equation (Ribani et al., 2004; MAPA, 2011; Magnusson and Örnemark, 2014):

$$\%\ Recovery = \frac{measured\ concentration}{expected\ concentration}\ x\ 100$$

Production and collection of cachaças

The beverages were produced at the Fazenda Cassiano mentioned above. Twelve cachaças were produced, considering the two degrees of trimming (Po = stalks with tips; Co = stalks without tips and 100% clean at full maturity) from the six sugarcane varieties involved in the study (RB867515, CTC2, CTC11, CTC9003, IACSP95-5000 and SP80-1842).

The juice was extracted using a mill and filtered to remove coarse impurities. The must was obtained by dilution, adjusting the concentration of soluble solids to 16 ºBrix, and the temperature was adjusted to 25-32 ºC, a temperature considered to be optimum for the physiological development of the yeast.

The must was inoculated using CA-11 yeast (dose recommended by the manufacturer: 1 kg per 1000 L of broth). Fermentations were achieved in stainless steel vats (60 liters) at a temperature of approximately 32 °C. The fermentation was terminated when the total soluble solids (TSS content - determined by refractometry) of the broth was lower than one.

Distillation was performed in a simple still with a copper boiler, dome and neck, heated by direct fire to 50 °C. The temperature was increased, the distillation process began at a temperature of 78 °C, while trying not to exceed 90 °C. The head, heart and tail fractions were separated using a Gay Lussac 320 mm Alcohol Density Meter calibrated at 20 °C. The heart fraction was collected with an alcohol content ranging from 38 to 54% at 20 ºC.

The samples were sent to the Spirits Quality Analysis Laboratory, where chromatographic analyses for ethyl carbamate were performed. The ethanol concentration of the beverages was also evaluated to confirm that an adequate cut of the "heart" fraction (cachaça) was achieved in the distillation process.

Analysis of the ethanol content of cachaças

The analysis of the ethanol content in the samples was achieved in accordance with the specifications established by Normative Instruction nº 24 of 09/08/2005 of MAPA (Brasil, 2005). This analysis was accomplished by distilling 250 mL of the samples, and the ethanol concentration was measured using a Gibertini electronic densimeter (DensiMat) at 20 °C.

Analysis of ethyl carbamate (EC) in cachaças

The EC analysis was performed according to the method proposed by Machado et al. (2013) and Santiago et al. (2017) using a Shimadzu HPLC system equipped with two model SPD-M20A high-pressure pumps, a model DGU-20A3 degasser and a model CBM-20A interface. The cachaça samples were derivatized with 0.02 mol L^{-1} xanthydrol solution. The separations were performed using an Agilent-Zorbax Eclipse AAA column (4.6 x 150 mm, 5 µm) connected to an Agilent-Zorbax Eclipse AAA pre-column (4.6 x 12.5 mm, 5 µm), coupled to a model RF-10AXL fluorescence detector (FLD). The excitation and emission wavelengths employed were 233 and 600 nm, respectively. The flow rate used throughout the analysis was 0.75 mL min^{-1}, and the injected volume of samples and standard was 20 μL. Elution was performed in a gradient system, programmed at: 0 to 5 min (40-60% B); 5 to 10 min (60-70% B); 10 to 18 min (70-80% B); 18 to 19.5 min (80-90% B); 19.5 to 25 min (90-40% B); 25 to 30 min (40% B). The mobile phase was composed of 20 mM sodium acetate solution (Solvent A) and acetonitrile (Solvent B). The identification of the EC was achieved by comparing the retention time of the samples with those of the standards, and quantification was accomplished by external standardization.

Statistical analyses

The experimental design used was composed of completely randomized blocks (DIC) with six treatments corresponding to the sugarcane varieties, with the experimental plot consisting of 2 cuts (Po = stems with tips and Co = stems without tips), totaling 12 methanol extracts and cachaças. All the samples were standardized in relation to the period of collection and processing of extracts and distilled beverages for the quantification of Dhurrin, ethyl carbamate and ethanol content. Statistical analyses were performed in two ways: (i) comparison of the six varieties with one another with respect to the compound levels, regardless of the cut; (ii) comparison of the cropping levels with each other for each cultivar. Data normality was verified using the Shapiro-Wilk test, and data were subjected to analysis of variance (ANOVA). When significant differences were detected, the means were grouped using the Tukey test ($p \leq 0.05$). Statistical analyses were performed using the MINITAB® version 18.0 program (Minitab Inc., State College, PA, USA). Data represents the average of technical triplicates.

Conclusion

The method developed for the detection and quantification of the dhurrin cyanogenic glycoside in methanolic extracts from different parts of sugarcane was satisfactory. A variation in Dhurrin content was observed that depended on the sugarcane variety, with the highest concentrations of Dhurrin being associated with the presence of the tip (Po). This portion of the stalk contained the highest average concentration, which resulted in a higher concentration of EC in beverages produced with this raw material. These results suggest that producers should take special care in removing the tips during the sugarcane harvest to minimize the presence of Dhurrin and, consequently, reduce the concentration of EC in the beverages.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All data generated or analyzed during this study are included in this published article.

Author Contribution

Cardoso, MC: Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing. Santiago, WD: Methodology, Conceptualization, Data curation, Formal analysis. Pimentel, GV: Conceptualization, Data curation, Formal analysis. Santos, PA: Conceptualization, Data curation, Formal analysis. Marques, MALSM: Methodology, Visualization. Fernandes, AI: Conceptualization, Data curation, Formal analysis. Rezende, DACS: Conceptualization, Data curation, Formal analysis. Teixeira, A.R: Conceptualization, Data curation, Formal analysis. Santos, WA: Conceptualization, Data curation, Formal analysis. Machado, AMR: Conceptualization, Data curation, Formal analysis. Freire, CS: Conceptualization, Data curation, Formal analysis. Nelson, DL: Visualization, Writing – review & editing.

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