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Chemical and microbiological quality of sugar cane juice influences the concentration of ethyl carbamate and volatile congeners in cachaça

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The aim of this study was to assess the influence of heat treatment of sugar cane juice, supplementation with urea and double distillation on the concentration of volatile congeners (acetic aldehyde, ethyl acetate, *n*-propyl, isobutyl and isoamyl alcohols and acetic acid) and contaminants (methanol, 1-propyl and 2-butyl alcohols, copper, and ethyl carbamate) in cachaça. Samples of fresh sugar cane juice, sugar cane juice submitted to heat treatment and contaminated sugar cane juice were supplemented (or not) with urea and fermented. The washes so obtained underwent single and double distillation. Supplementation with urea stimulated ethyl carbamate formation. The distilled products that originated from contaminated worts presented higher concentration of acetic acid and ethyl carbamate. Double distillation reduced the concentration of contaminants. The best quality pot still cachaça was obtained employing heat treatment of sugar cane juice, nonsupplementation with urea and double distillation.

Keywords: sugar cane spirit; contaminant compounds; ethyl carbamate; urea supplementation

Introduction

Sugar cane spirit is an alcoholic beverage produced by distillation of fermented sugar cane juice that can reach 38–54% alcohol by volume (ABV). Cachaça is the typical and exclusive denomination of Brazilian sugar cane spirit *(1)*, the fourth most consumed distilled beverage worldwide. The production of cachaça reaches approximately 1.5 billion litres a year and involves the direct participation of 450,000 people in Brazil.

The chemical quality of cachaça is related to low concentrations of contaminant compounds and acetic acid, the latter responsible for the acidity of this beverage. The main compounds considered contaminants in cachaça are methanol, *sec*-butanol, *n*-butanol, ethyl carbamate and copper.

Volatile acidity, measured in terms of acetic acid, is a consequence of bacterial contamination during fermentation. Acetic acid bacteria ferment wort and increase its acidity. Good practices of fermentation and distillation are indicated to reduce the concentration of acetic acid in distilled beverages in order to not hinder their quality *(2)*.

Ethyl carbamate (urethane), or carbamic acid ethyl ester, is considered the main contaminant of cachaças since it is a potential carcinogenic compound. The highest exposure to ethyl carbamate is represented by the consumption of distilled beverages *(3)*. Brazilian law established an upper limit of 210 $\mu g\,L^{-1}$ ethyl carbamate in sugar cane spirits *(1)*, which came into force in August 2014 *(4)*. Nevertheless, relatively high concentrations of ethyl carbamate are generally found in Brazilian sugar cane spirits (3,5-9). In a literature review comprising 13 studies on the occurrence of ethyl carbamate in cachaças purchased in the Brazilian market, a total of 536 analytical results provided an average of 380 $\mu g\,L^{-1}$ of this compound in the samples analysed *(10)*. However, taking into account the weighting of data based on the production of cachaças in pot stills (38%) and column stills (62%) *(11)*, the average content of this contaminant was 450 $\mu g\,L^{-1}$.

During alcoholic beverage processing and aging, several precursors may participate in the formation of ethyl carbamate. Among them, the major compounds are hydrocyanic acid (hydrogen cyanide), *N*-carbamoyl phosphates, cyanogenic glycosides, urea and citrulline *(3,12,13)*. Sugar cane is a cyanogenic crop *(14)* and cyanate is another important precursor of ethyl carbamate in cachaça. The ion cyanide is formed by the enzymatic degradation of cyanogenic glycosides present in sugar cane. Cyanide can be oxidized to cyanite, which in turn reacts with ethanol in the presence of the ion copper to form ethyl carbamate *(15,16)*.

Urea, the most common nitrogen source used to fertilize sugar cane crops and occasionally employed to supplement worts, is also a precursor of ethyl carbamate in distilled beverages. The formation of ethyl carbamate in wine has been associated with high concentrations of urea during fermentation *(17)*. The degradation of arginine by yeast results in urea, which reacts with ethanol to form ethyl carbamate. It has been suggested that the thermal decomposition of urea during distillation releases cyanic acid, another precursor of ethyl carbamate in spirits *(15,18)*. Nevertheless, this pathway has not been confirmed experimentally yet.

Another supposition is that ethyl carbamate formation during cachaça storage occurs gradually as a result of the reaction

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between ethanol and urea *(19)*. However, the concentration of urea in cachaça is very low, and although the reaction of ethanol and urea is possible, the amount of ethyl carbamate formed in the spirits during aging would be insignificant *(20)*. That is why no correlation has been observed between urea concentration and ethyl carbamate content in commercial cachaças *(15)*.

Furthermore, lactic bacteria, which comprise approximately 76% of the microbiological contaminants frequently found in the process of production of sugar cane spirits *(21)*, metabolize the amino acid arginine forming citrulline, a compound that is also a precursor of ethyl carbamate in these beverages *(22)*.

Researchers have been searching for technologies intended to eliminate or minimize the formation of this contaminant during the process of fermentation and/or to remove it during the process of distillation. The literature indicates that the method of double distillation can significantly reduce the concentration of ethyl carbamate in distilled spirits *(18,23)*.

Therefore, the present study aimed to assess the influence of heat treatment of sugar cane juice, supplementation with urea and double distillation on the concentration of volatile congeners and contaminants in cachaça.

Material and methods

The juice of sugar cane variety SP 81–3250, diluted with tap water to 18°Brix, underwent three different treatments. The first treatment consisted only of filtering the fresh sugar cane juice using cellulose filter paper (10 μm pore size) (fresh sugar cane juice – FSJ). In the second treatment, the sugar cane juice was boiled (100 °C) for 10 min, cooled and filtered using cellulose filter paper (10 μm pore size) (treated sugar cane juice – TSJ). In the third treatment, the sugar cane juice was filtered using cellulose filter paper (10 μm pore size) and incubated at 30 °C for 48 h for the development of contaminant microorganisms naturally present in sugar cane juice (contaminated sugar cane juice – CSJ) (Fig. 1). The population of contaminant bacteria in the samples of sugar cane juice was determined after serial dilution and culturing onto MRS agar. Bacteria identification was carried out according to the protocol proposed by Ausubel *et al.* *(24)*.

Prior to fermentation, the sugar cane juice samples submitted to the three different treatments were supplemented (or not) with urea at a concentration of 0.1 g L^{-1}, totaling six treatments. All of the samples were inoculated with 3 g L^{-1} (dry matter) *Saccharomyces cerevisiae*, strain CA-11 (LNF Latinamericana, Bento Gonçalves, RS, Brazil), and fermented at 30 °C for 48 h.

After fermentation, all of the washes obtained were distilled in a copper pot still. The monodistilled cachaça (single-distilled sugar cane spirit – SDS) refers to the liquid collected after the 'head' cut (1.5% of the boiler useful volume) and up to 43% (v/v) of ethanol in the distilled beverage. In the double distillation, the first distillation extracted all of the ethanol from the wash, originating the low wines. The low wines underwent the second distillation. The bi-distilled cachaça (double-distilled sugar cane spirit – DDS) refers to the liquid collected after the 'head' cut (1.5% of the boiler useful volume) and until the distilled beverage presented 60% (v/v) of ethanol in the condenser outlet *(25)*.

Triplicates of distilled fractions were analysed to quantify volatile congeners, ethanol, copper, and ethyl carbamate. Ethanol and copper were determined according to official Brazilian methods *(26)*.

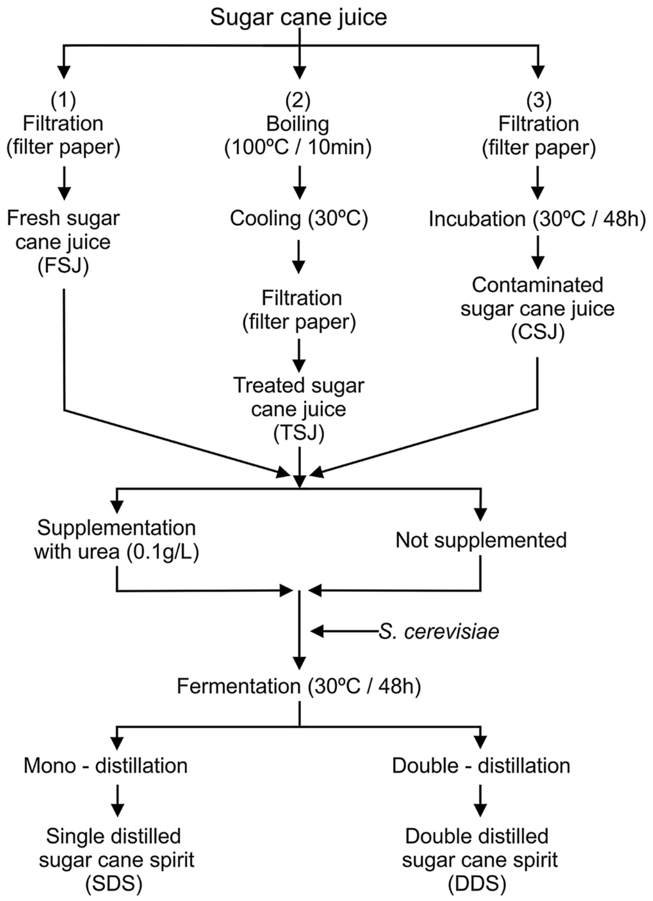

Figure 1. Scheme of the different modalities and steps of the cachaça production process.

The contents of acetic aldehyde, ethyl acetate, methanol, higher alcohols (*n*-propyl, isobutyl and isoamyl), 1-propyl and 2-butyl alcohols and acetic acid were determined using gas chromatography *(27)*. Standards used in this study were all from Merck (Darmstadt, Germany), purity >99%. Samples were spiked with the internal standard (4-methyl-2-pentanol). Aliquots of 1.0 μL were automatically injected into a gas chromatographic system (Shimadzu, QP-2010 Plus, Tokyo, Japan) equipped with a Stabilwax-DA column (crossbond carbowax polyethylene glycol, 30 m × 0.18 mm × 0.18 μm film thickness) and a flame ionization detector. The analyses were performed at a 1:25 split ratio, in triplicate. Nitrogen was used as the carrier gas, at 31.8 cm s^{-1} (flow rate of 1.5 mL min^{-1} and pressure of 252.3 kPa). The temperatures of both the injector and the detector were set at 250 °C. The oven temperature programme was 40 °C for 4 min, followed by an increase to 120 °C at 20 °C min^{-1}, kept for 1 min, then up to 180 °C at 30 °C min^{-1}, and maintained for 4 min.

The samples were analysed for ethyl carbamate in a gas chromatograph coupled to a mass spectrometer, model GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan), using electron impact ionization with an energy of 70 eV and a chromatography capillary column with polar phase (esterified polyethylene glycol – HP-FFAP; 50 m × 0.20 mm × 0.33 μm stationary phase film thickness). The temperatures of the injector and the detector interface were 230 and 220 °C, respectively. The following temperature programme was used in the oven: starting with 90 °C for 1 min; increasing to 150 °C at a rate of 10 °C min^{-1}; followed by heating up to 230 °C at a rate of 30 °C min^{-1}; and maintained at this temperature for 2 min. An aliquot of 1.0 μL was injected using the splitless injection mode, in duplicate. Helium was used as the carrier gas (flow rate of 1.2 mL min^{-1}). Selected ion monitoring acquisition was employed, monitoring the m/z 62 ions for ethyl carbamate and m/z 75 ions for methyl carbamate, used as the internal standard *(27)*. Quantification was performed comparing the chromatographic results of the samples with an analytical curve obtained using an ethyl carbamate stock solution. All reagents were analytical grade. Ethyl carbamate (99%) and methyl carbamate (98%) were purchased from Sigma-Aldrich (St Louis, MO, USA). For the dilutions, ethanol gradient grade for gas chromatography (Merck, Darmstadt, Germany) and ultrapure water (Milli-Q) were used.

The quantification of the compounds was performed based on external calibration curves of six standard concentration points. The concentration range and correlation coefficients (a, b, r^2), as well as the retention indices, detection limit (DL) and quantification limit (QL) of volatile and maturation-related compounds are shown in Table 1. The DL and QL were calculated based on chromatograph baseline noise *(28)*.

The statistical analyses were performed using analysis of variance (one-way ANOVA) and the Tukey test when the ANOVA p-value was <0.05 *(29)*.

Results and discussion

The population of contaminant bacteria was as follows: 10^1 cells mL^{-1} in TSJ; 10^5 cells mL^{-1} in FSJ, 78% lactic bacteria, with a predominance of the genus *Lactobacillus* (60%); and 10^8 cells mL^{-1} in CSJ, 84% lactic bacteria, with a predominance of the genus *Lactobacillus* (67%). CSJ resulted in SDS and DDS presenting the highest concentrations of ethyl carbamate. This result can be attributed to the fact that lactic bacteria can metabolize arginine forming citrulline, a compound that is a precursor of ethyl carbamate in distilled beverages *(22)*.

In all treatments, SDS obtained from samples supplemented with urea presented the highest concentrations of ethyl carbamate (Table 2). TSJ nonsupplemented with urea resulted in the DDS with the lowest content of ethyl carbamate. The correlation between the concentration of urea and the content of ethyl carbamate has already been observed in wine obtained from grape juice *(17)*.

CSJ supplemented with urea originated the SDS with the highest content of ethyl carbamate (285.15 μg L^{-1}), above the limit of 210 μg L^{-1} established by Brazilian law (Table 2). However, double distillation reduced this compound by 71%, and therefore the bi-distilled cachaça of this treatment was below the permitted limit of contaminant (Table 2). All of the remaining treatments resulted in SDS presenting contents of ethyl carbamate below the maximum permitted limit.

Double distillation promoted an average reduction of ethyl carbamate of 74% in DDS, and reached 91% in DDS obtained from TSJ nonsupplemented with urea. These results corroborate those obtained by Riffkin *et al.* *(18)*, who reported that only 1% of the ethyl carbamate formed in the first distillation was distilled in whisky produced in the second distillation. Nagato *et al.* *(30)* analysed commercial cachaças and observed that the samples presenting the lowest concentrations of ethyl carbamate (38–48 μg L^{-1}) were double-distilled spirits. Galinaro and Franco *(14)* found that redistillation reduced the concentration of ethyl carbamate in 15 samples of commercial cachaça by up to 92.5%. Alcarde *et al.* *(23)* also observed that double distillation reduced the concentration of ethyl carbamate in cachaça by 97%.

CSJ supplemented with urea originated the SDS and DDS with the highest concentrations of acetic aldehyde and ethyl acetate (Table 2). In spite of the reduction by 27% with double distillation (Table 3), the content of acetic aldehyde in DDS from CSJ supplemented with urea was approximately three times higher than the maximum limit established by Brazilian law. DDS from this treatment also had higher concentrations of ethyl acetate compared with the respective SDS, probably owing to esterification reactions during the second distillation, stimulated by the high contents of volatile acids in the distilled spirits. Furthermore, supplementation with urea may have stimulated the development of contaminant bacteria during fermentation, microorganisms that generate acetic aldehyde and ethyl acetate as secondary products of their metabolism *(31)*. Even nonsupplemented with urea, spirits obtained from CSJ presented ethyl acetate approximately twice as high as for the remaining treatments.

The different treatments did not affect the content of propanol in the spirits. CSJ produced the SDS with the lowest concentrations

Table 1. Average retention indices (RI), detection limit (DL), quantification limit (QL) of volatile and contaminant congeners, and concentration range and correlation coefficients (a, b, r^2) of the calibration curves in alcoholic solutions (40% alcohol by volume) for quantification of the compounds

Compound	RI (min)	DL[a]	QL[a]	Concentration range[a]	a	b	r^2
Volatile congeners							
Acetic aldehyde	0.29	0.070	0.220	7.5–37.5	5.5900	−1.0200	0.9957
Ethyl acetate	1.41	0.057	0.171	12.5–62.5	2.8792	0.9075	0.9996
n-Propanol	4.43	0.038	0.114	37.5–187.5	2.0471	−0.2444	0.9999
Isobutanol	5.22	0.014	0.042	12.5–62.5	1.7260	−0.1724	0.9998
Isoamyl alcohol	6.72	0.016	0.048	50–250	1.6748	9.1053	0.9999
Acetic acid	9.15	0.530	1.590	37.5–187.5	5.4259	4.1160	0.9997
Contaminant congeners							
Methanol	1.62	0.092	0.276	5–25	4.1394	−0.1620	0.9997
sec-Butanol	4.02	0.049	0.180	2.5–12.5	1.9168	−5.1082	0.9998
n-Butanol	5.99	0.072	0.216	0.75–3.75	1.1168	−1.8596	0.9997
Ethyl carbamate	10.15	0.180	0.550	50–500	64.714	1241.67	0.9984

[a] Milligrams per 100 mL anhydrous ethanol.

Table 2. Contents of ethanol (% v/v), volatile and contaminant congeners (mg per 100 mL AE), copper (mg L^{-1}), and ethyl carbamate (µg L^{-1}) in single-distilled spirit (SDS) and double-distilled spirit (DDS) obtained from fresh sugar cane juice (FSJ), treated sugar cane juice (TSJ) and contaminated sugar cane juice (CSJ) supplemented (urea) and nonsupplemented with urea (non)

Compound	Type of distillation	FSJ		TSJ		CSJ		Limits determined by Brazilian law (1)
		Non	Urea	Non	Urea	Non	Urea	
Ethanol	SDS	43.89	43.50	43.84	43.28	43.28	43.05	
	DDS	67.61	65.06	66.28	66.98	65.98	67.14	
Volatile congeners								
Acetic aldehyde	SDS	33.11	26.53	23.39	25.67	19.49	120.07	max 30
	DDS	23.95	23.36	18.63	16.34	12.72	87.12	
Ethyl acetate	SDS	32.32	25.74	21.65	27.46	41.74	110.63	max 200
	DDS	29.97	22.28	22.76	20.13	44.09	149.49	
Propanol	SDS	57.52	67.26	57.69	64.32	56.84	58.87	
	DDS	54.86	68.37	51.76	62.14	53.35	54.40	
Isobutanol	SDS	64.69	53.36	68.27	56.03	33.63	32.51	
	DDS	67.86	57.49	65.25	59.71	34.18	33,19	
Isoamyl alcohol	SDS	147.32	133.38	158.07	144.28	96.54	123.21	
	DDS	154.46	143.48	151.08	158.87	99.28	121.89	
Superior alcohols[a]	SDS	269.53	254.00	284.03	264.63	187.01	214.59	max 360
	DDS	277.19	269.34	268.10	280.72	186.81	209.48	
Acetic acid	SDS	63.74	51.12	50.22	57.06	220.99	221.81	max 150
	DDS	13.12	12.49	13.20	12.31	42.10	36.28	
Total volatile congeners[b]	SDS	398.70	357.39	379.29	374.83	469.23	667.11	200–650
	DDS	344.23	327.46	322.69	329.49	285.72	482.37	
Contaminant congeners								
Methanol	SDS	3.66	2.52	1.17	2.56	10.74	5.17	max 20
	DDS	0.72	0.58	0.82	2.27	1.32	0.57	
sec-butanol	SDS	0.22	0.20	0.22	0.20	0.30	0.20	max 10
	DDS	< LD[c]	0.18	< LD	< LD	0.22	< LD	
n-butanol	SDS	0.54	0.63	0.67	0.82	0.42	1.23	max 3
	DDS	0.46	0.55	0.52	0.80	0.37	1.14	
Ethyl carbamate	SDS	68.51	82.15	87.63	99.55	150.45	285.17	max 150
	DDS	31.79	55.93	11.37	33.50	47.87	130.44	
Copper	SDS	3.34	2.57	2.67	2.18	8.96	6.55	max 5
	DDS	0.89	0.79	0.87	0.80	1.43	2.83	

[a] Sum of propanol, isobutanol, and isoamyl alcohol.
[b] Sum of aldehydes, esters, high alcohols, and acetic acid.
[c] LD: Limit of detection.

of isobutanol, isoamyl alcohol and consequently superior alcohols. The content of superior alcohols in DDS did not change with double distillation. All of the distilled beverages obtained presented a content of superior alcohols below the maximum permitted limit established by Brazilian law.

Acidity was analysed in the distilled beverages in relation to acetic acid. SDS from CSJ supplemented and nonsupplemented with urea presented acidity above the maximum permitted limit and approximately 4 times higher than the acidity measured in the remaining treatments. Nevertheless, double distillation reduced the acidity of the distilled beverages from these treatments by 81–84%, adjusting them to within the legal limit.

Owing to the high contents of acetic aldehyde, ethyl acetate and acetic acid, the distilled beverages obtained from CSJ supplemented with urea presented the highest content of total volatile congeners. However, double distillation adjusted the DDS of this treatment to the limits permitted by law.

All of the treatments resulted in SDS and DDS with contents of methanol, *sec*-butanol and *n*-butanol below the maximum permitted limits (Table 2). Double distillation reduced these contaminants by 63, 57 and 12%, respectively. Supplementation with urea did not affect the concentration of these contaminants in the distilled spirits.

SDS obtained from CSJ supplemented and nonsupplemented with urea presented contents of copper above the maximum permitted limit. Nonetheless, double distillation significantly reduced this contaminant in the obtained distilled spirits (by 90 and 72% in nonsupplemented and supplemented treatments, respectively), adjusting the resulting DDS to the Brazilian law. Copper exhibits a catalytic effect in the formation of ethyl carbamate *(15,18)*, and the optimum concentration of this element to cause this effect is 0.8 mg L^{-1} *(6)*. The high acidity of distilled spirits from CSJ may have been responsible for the high content of copper in the resulting cachaças, since acetic acid solubilizes the oxidized metallic copper from the pot still, carrying it

Table 3. Approximate reduction (%) of volatile and contaminants congeners of spirits obtained from FSJ, TSJ and CSJ supplemented and nonsupplemented with urea owing to double distillation

Compound	Reduction (%)						
	FSJ		TSJ		CSJ		Average
	non	urea	non	urea	non	urea	
Volatile congeners							
Acetic aldehyde	28	12	20	36	35	27	26
Acetic acid	79	76	74	78	81	84	79
Total volatile congeners[a]	14	8	15	12	39	28	19
Contaminant congeners							
Methanol	80	77	30	11	88	89	63
sec-Butanol	>77	10	>77	>75	27	>75	>57
n-Butanol	15	13	22	2	12	7	12
Ethyl carbamate[b]	70	54	91	78	79	71	74
Copper[b]	83	79	78	76	90	72	80

[a] Sum of aldehydes, esters, high alcohols, and acetic acid.
[b] Considering the distillates standardized at 38% alcohol by volume, minimum value established by the Brazilian law for cachaça.

into the beverage *(32)*. The ethanol content of the SDS ranged from 43.05 to 43.89% (v/v). DDS presented ethanol concentrations ranging from 65.06 to 67.61% (v/v).

Conclusions

Supplementation of sugar cane juice with urea caused the production of higher concentrations of ethyl carbamate in cachaças. Bacteria contamination of sugar cane juice caused distilled spirits with higher contents of acetic aldehyde, ethyl acetate, acetic acid, copper and ethyl carbamate. Double distillation caused a reduction in the content of the chemical contaminants of the beverages, such as ethyl carbamate. The best process to produce good quality cachaça included a heat treatment of sugar cane juice, nonsupplementation with urea for fermentation and double distillation.

We should point out that the effect of double distillation on reducing ethyl carbamate level is specific to pot-distilled cachaça. The impact on column-distilled cachaça is presently under investigation.

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