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Analytical Methods

Amino acids profile of sugar cane spirit (cachaça), rum, and whisky

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Abstract

An analytical procedure for the separation and quantification of 20 amino acids in cachaças has been developed involving C18 solid phase cleanup, derivatization with *o*-phthalaldehyde/2-mercaptoethanol, and reverse phase liquid chromatography with fluorescence detection. The detection limit was between 0.0050 (Cys) and 0.25 (Ser) mg L^{-1}, whereas the recovery index varies from 69.5 (Lys) to 100 (Tyr)%. Relative standard deviations vary from 1.39 (Trp) to 13.4 (Glu)% and from 3.08 (Glu) to 13.5 (His) for the repeatability and intermediate precision, respectively. From the quantitative profile of amino acids in 41 cachaças, 5 rums, and 12 whisky samples, the following order of amino acids in significant quantities is observed: Gly = Ser < Cys < Ile < His < Pro = Asp < Asn < Tyr for cachaça; Phe < Glu = Gln = Val = Ala < His = Gly = Thr = Arg = Tyr < Asn = Ser = Lys = Pro < Cys = Asp for rum; and Ala = Asn < Trp < Gln = His = Met = Ile = Cys < Thr < Asp = Leu < Phe = Lys < Ser = Gly = Tyr = Val < Glu = Pro < Arg for whisky samples.

Keywords: Amino acids; Cachaça; Liquid chromatography; Distilled spirits; Derivatization

1. Introduction

The production of cachaça, Brazilian sugar cane spirit, is only overcome by vodka and soju (Cardoso et al., 2004). Nowadays, approximately 20 million liters per year of cachaça are exported. However, this amount represents only about one percent of the cachaça production, which is around 2 billion liters (Cardoso, Bettin, Reche, Lima-Neto, & Franco, 2003; Cardoso et al., 2004).

There are reports in the literature (Hernández-Orte, Cacho, & Ferreira, 2002; Perpète, Santos, Bodart, & Collin, 2005) concerning the presence of amino acids in beer, grape, wine, and other fermented beverages and their relationship with quality parameters such as flavor and appearance.

The quantitative and qualitative analysis of amino acids profile in cachaça has not been reported hitherto. The origin of amino acids in cachaça could be related to their presence in the sugar cane juice as a consequence of the protein hydrolysis in the cellular wall of the yeast during the fermentation process (Pozo-Dengra et al., 2006), from sucrose (sweetener) (Chen, 1985), and by extraction from the wooden cask used for the maturation step (Fengel & Wegener, 1989).

In order to contribute to a better knowledge of the cachaça chemical composition and to identify possible haze precursors, herein, we describe a qualitative and quantitative liquid chromatography method for the determination of 20 amino acids in samples of cachaça, rum, and whisky.

2. Experimental section

The *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol derivatization combined with liquid chromatography using fluorescence detection were applied for the analysis of 20 amino acids in 41 samples of cachaça (22 sweetened and 19 unsweetened) from 10 different states of Brazil, 12 samples of whiskys, and five samples of rum.

2.1. Samples

Cachaça: Espirito de Minas[u] (MG), Pitú[s] (PE), Aguardente 51[s] (SP), Jamel[s] (SP), Colonial[s] (CE), Capitão das Gerais[u] (MG), Catedral[u] (SP), Ypióca Prata[s] (CE), Vila

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Velha[s] (SP), Azuladinha[s] (AL), Pingo de Ouro[s] (SC), Germana[u] (MG), Vale das Águas Quentes[u] (GO), Koloniale[u] (SC), Galembeck[s] (SP), Boazinha[u] (MG), Chapéu de Palha[s] (SP), Tiquara[u] (SP), Colonial Ouro[s] (CE), Serrote[u] (PE), Baronesa[u] (MG), Armazém Viera[u] (SC), Izê[s] (SP), Velha Aroeira[u] (MG), Pinguinha[u] (PR), Box 32[u] (SC), Arara[s] (SP), Jequity[u] (SP), Nega Fulo[u] (RJ), Vat 45[s] (SP), Brasileira[u] (SP), Colonial Prata[s] (CE), Cravo e Canela[s] (SE), Corote[s] (SP), Marquesi[s] (SP), Sapupara[s] (CE), Cana verde & Cia[u] (MG), Lua Cheia[u] (MG), Ypióca Ouro[s] (CE), Delicate[s] (SP), and Old César 88[s] (SP).

Where: s = sweetened, u = unsweetened, and (SP) = São Paulo state, (CE) = Ceará state, (MG) = Minas Gerais state, (PE) = Pernambuco state, (SC) = Santa Catarina state, (GO) = Goiás state, (PR) = Paraná state, (AL) = Alagoas state, (SE) = Sergipe state, and (RJ) = Rio de Janeiro state.

Whisky: Chivas Regal (Scotland), Grant's (Scotland), Four Roses (USA), Ballantines Finest (Scotland), Wild Turkey (USA), Whyte and Mackay Founders Reserve (Scotland), Jack Daniel's (USA), Early Times (USA), Laphroaig (Scotland), Tullamore Dew 12 years old (Ireland), Makers Mark (USA), Natu Nobilis (Brazil).

Rum: Montilla (Brazil), Havana Club añejo 7 years (Cuba), Saint James (Martinique), Havana Club blanco (Cuba), Aniversario (Venezuela).

2.2. Chemicals

Aspartic Acid (Asp), Glutamic acid (Glu), Asparagine (Asn), Serine (Ser), Glutamine (Gln), Histidine (His), Glycine (Gly), Threonine (Thr), Alanine (Ala), Arginine (Arg), Tyrosine (Tyr), Methionine (Met), Tryptophan (Trp), Valine (Val), Phenylalanine (Phe), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Cysteine (Cys) and Proline (Pro) were of analytical grade and obtained from Sigma–Aldrich (USA). The *o*-phthaldialdehyde (OPA), 2-mercaptoethanol (2-ME), and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (USA). Iodoacetic acid (IDA) and sodium borohydride were from Fluka (USA), and Chloramine-T from Carlo Erba (Italy). The solvents of HPLC grade (methanol, ethanol, acetonitrile, and tetrahydrofuran) were from Mallinckrodt (USA) and used without further purification. Bi-distillated and deionized water used in this work was obtained using a Milli-Q system (Millipore, MA – USA).

2.3. Apparatus

A Shimadzu HPLC system consisting of two high pressure pumps (model LC-10AD), a manual Rheodyne injection valve 7725i with a 20 μL loop, a programmable fluorescence detector RF-551, and a SCL-10Avp interface was used for HPLC analysis. The class-VP Shimadzu software version 6.12 was employed for data acquisition and system control.

The chromatographic separation was performed using a Waters Resolve C_{18} column (5 μm particle size, 30 cm × 3.9 mm i.d.). For Pro analysis a short version of a Waters Resolve C_{18} column (5 μm particle size, 15 cm × 3.9 mm i.d.) was used.

2.4. Solutions and sample preparation

Stock solutions of amino acids containing 0.02 mol L^{-1} of each standard was prepared in HCl 0.10 mol L^{-1}. Only the solutions of Glu, Cys, and Pro were daily prepared, in deionized water, at concentrations of 1.00×10^{-2} mol L^{-1}, 2.00×10^{-4} mol L^{-1}, and 5.00×10^{-4} mol L^{-1}, respectively.

The *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol (2-ME) derivatizing solution was prepared as follows: 50.0 mg of OPA dissolved in 4.50 mL of methanol, 50.0 μL of 2-ME and 0.50 mL of 0.40 M potassium borate buffer (pH adjusted to 10 with sodium hydroxide). After mixing, the resulting solution was stored for 24 h in the dark at 4 °C (Gomis, Lobo, Alvarez, & Alonso, 1990; Paramás, Bárez, Marcos, García-Villanova, & Sánchez, 2006; Pripis-Nicolau, Revel, Marchand, Beloqui, & Bertrand, 2001). Every 48 h, 10.0 μL of 2-ME was added to maintain the reagent efficacy (Gomis et al., 1990).

Samples were pre-concentrated as follows: 60.0 mL of sample added 0.50 mL of hydrochloride acid (0.10 mol L^{-1}) was evaporated to dryness on a rotary evaporator at 40 °C, followed by addition of 1.00 mL of the same sample to the residue, this mixture was vortexed and after this, 1.00 mL of trifluoroacetic acid (TFA) 0.10% in water–methanol (70:30 v/v) was added under vigorous stirring.

Afterward, samples were cleanup by solid phase extraction (SPE) on Waters Sep-Pak C_{18} cartridges. The SPE cartridges were previously activated and conditioned by sequential elution with 2.0 mL of methanol, 2.0 mL of aqueous TFA 0.10%, and 1.0 mL of TFA 0.1% in water–methanol (80:20 v/v). The pre-concentrated samples were percolate though the SPE cartridges and eluted with 1.00 mL of TFA solution 0.10% in water–methanol (70:30 v/v). The first 1.00 mL of eluate is rejected and the subsequent 2.00 mL are collected. When necessary, extracted samples were stored at −10 °C prior to analysis.

The derivatization procedure was as follow: 0.10 mL of a standard solution or sample, filtered through a 0.22 μm Millex HV (Millipore) membrane, was mixed with 0.20 mL of the derivatizing solution. The resulting solution was vortexed and allows reacting at room temperature for 60 s. Then an aliquot of 20 μL was withdrawn and injected into the HPLC system. The total conversion of standards and unknown amino acids in samples were guaranteed by the use of a 200 fold-excess of OPA/2-ME in the reaction mixture.

The individual analysis of Cys requires one additional step prior to the derivatization procedure describe above

(Cooper & Turnell, 1982; Pripis-Nicolau et al., 2001). An aliquot of 0.2 mL of iodoacetic acid (IDA) was added to 0.1 mL of pre-treated sample and then submitted to the derivatization procedure.

The IDA solution was prepared by the dissolution of 3.50 g of iodoacetic acid in 50.0 mL of borate buffer at pH 9.5 adjusted with NaOH 4 M and the final volume adjusted to 100 mL (Pripis-Nicolau et al., 2001).

The Pro analysis was carried out after its oxidation to 4-amino-1-butanol. A volume of 0.20 mL of the standard solution or sample was added to 0.20 mL of 13.3 mmol L^{-1} chloramine-T solution preheated at 60°C for 2 min. After 1 min of incubation, 0.20 mL of 0.33 M sodium borohydride was added and the resulting solution kept at 60°C for no longer than 10 min. After cooling at room temperature, 0.10 mL of the resulting solution was immediately used for Pro analysis (Cooper, Lewis, & Turnell, 1984; Wu, 1993).

Table 1
HPLC gradient profile for the amino acids analysis

Flow	Cysteine	Proline		Other amino acids	
		Time (min)	% Solvent B	Time (min)	% Solvent B
1 mL min^{-1}	Isocratic	0	25	0	5
		40	100	65	70
		45	100	70	90
		55	25	75	90
		–	–	85	5

2.5. Analytical conditions

Cysteine analysis: the mobile phase used was a dibasic sodium phosphate buffer 50 mmol L^{-1} pH = 7.4 – acetonitrile (89:11 v/v) solution. Proline analysis: the mobile phase consisted of methanol (solvent B) and aqueous solution of sodium acetate 0.10 mol L^{-1} pH = 7.20 containing 0.50% of tetrahydrofuran and 9.00% of methanol as additive (solvent A).

For the remaining amino acids, the eluting solvent consist of methanol (solvent B) and a sodium phosphate buffer 4.20×10^{-2} mol L^{-1} pH 6.80 (solvent A) prepared daily in an aqueous solution of tetrahydrofuran 1.20%.

The eluting gradients for the HLPC analysis of Pro, Cys, and the remaining amino acids are described in Table 1.

The detection of the isoindole derivatives were carried out with excitation at 340 nm and the emission probed at 440 nm. The exception was for the isoindole derivatives of Cys and Pro where the emission was monitored at 425 nm and 450 nm, respectively.

3. Results and discussion

The optimal experimental conditions were achieved using three independents chromatographic analysis: Cys and Pro were analyzed in individual runs, and the remaining amino acids analyzed in the same chromatographic run, Fig. 1.

Quantifications were carried out by external standard calibration method and the calibration curves were built through linear regression of the data obtained for the mean

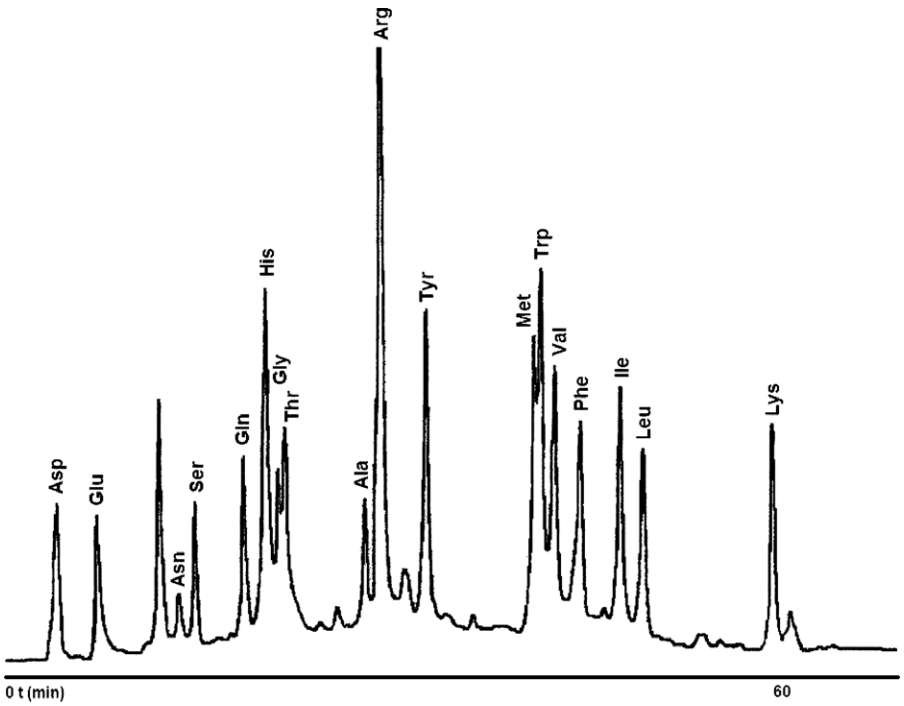

Fig. 1. Liquid chromatographic separation of the amino acids standard as their isoindole derivatives using the analytical conditions described in the experimental section.

Table 2
Linear regression parameters for the amino acids calibration plots

Compound	R.T. (min)	Slope	R^2	Linearity (mg L^{-1})	D.L. (mg L^{-1})
Asp	4.97	62.0	0.997	0.10–6.00	0.15
Glu	8.03	61.1	0.992	0.10–6.00	0.15
Asn	14.3	178.0	0.992	0.10–6.00	0.15
Ser	15.5	95.3	0.993	0.10–6.00	0.25
Gln	19.3	73.0	0.997	0.10–6.00	0.20
His	20.9	579.0	0.991	0.10–6.00	0.10
Gly	21.8	1063.0	0.995	0.10–6.00	0.08
Thr	22.6	134.0	0.990	0.10–6.00	0.15
Ala	28.7	208.0	0.992	0.10–6.00	0.15
Arg	29.9	946.0	0.990	0.10–6.00	0.08
Tyr	33.5	512.0	0.996	0.10–6.00	0.10
Met	41.8	565.0	0.990	0.10–6.00	0.10
Trp	42.4	1187.0	0.994	0.10–6.00	0.08
Val	43.4	1439.0	0.991	0.10–6.00	0.08
Phe	45.4	1025.0	0.991	0.10–6.00	0.08
Ile	48.5	511.0	0.992	0.10–6.00	0.10
Leu	50.3	692.0	0.997	0.10–6.00	0.10
Lys	60.3	248.0	0.991	0.10–6.00	0.10
Cys	4.6	7068.0	0.991	0.0050–0.50	0.0005
Pro	18.4	319.5	0.994	0.10–8.30	0.0016

R.T. = retention time.
D.L. = detection limit.

peak area of each analyte, already converted to isoindole derivatives, after triplicate injection of standard solution in the following range: 0.0050–0.50 mg L^{-1} (Cys), 0.10–8.30 mg L^{-1} (Pro), and mixed amino acid solution from 0.10–6.00 mg L^{-1}. Table 2 presents the linear regression parameters obtained for the calibration plot of each analyte.

The detection limits were estimated visually after successive dilutions (1:1 ratio) of a 5.00 mg L^{-1} mixture of each isoindole derivative. Dilutions were done until the observed signal-to-noise ratio of 3:1 for peak height (International Conference on Harmonization – ICH., 1996).

The precision of the method (Table 3) was evaluated by the relative standard deviations (RSD) of a within-day sequence (repeatability, $n = 7$) and on 10 consecutive days (between-to-day intermediate precision). The RSD values for repeatability varies from 0.61% (Ser) to 13.4% (Glu) with an average value of 5.91%, and the intermediate precision shows relative standard deviations ranging from 3.08% (Glu) to 13.5% (His) with an average value of 8.35%, which is considered satisfactory for trace analysis (Green, 1996).

The analytical method accuracy was evaluated by spiking a previously analyzed sample with the standards (ICH guideline, 1996; Thompson, Ellison, Fajgelj, Willetts, & Wood, 1999). The recovery level was determined according to Eq. (1). and the results are presented in Table 3.

$$\text{Recovery } (\%) = (C_1/C_2) \times 100 \quad (1)$$

where C_1 = measured concentration and C_2 = expected concentration.

Fig. 2 illustrate a typical chromatogram obtained for the amino acid profile in cachaça.

Table 3
Analytical performance parameters obtained for the proposed method

Compound	Precision				Accuracy (recovery index)	
	Repeatability		Intermediate precision		Recovery (±S.D.)	R.S.D. (%)
	Average[a] (±S.D.)	R.S.D. (%)	Average[b] (±S.D.)	R.S.D. (%)		
Asp	5.1 (± 0.56)	11.1	5.42 (± 0.47)	8.64	98 (± 1.00)	1.03
Glu	1.6 (± 0.22)	13.4	1.76 (± 0.05)	3.08	87 (± 1.29)	1.49
Asn	0.39 (± 0.01)	2.32	0.27 (± 0.03)	10.5	97 (± 1.51)	1.56
Ser	5.05 (± 0.03)	0.61	5.34 (± 0.34)	6.49	93 (± 2.98)	3.21
Gln	0.36 (± 0.04)	11.4	0.49 (± 0.05)	10.5	72 (± 1.55)	2.14
His	0.73 (± 0.02)	2.35	0.73 (± 0.09)	13.5	85 (± 3.01)	3.55
Gly	2.2 (± 0.15)	6.83	2.23 (± 0.11)	4.92	89.4 (± 0.62)	1.12
Thr	<L.D.		<L.D.		96 (± 2.51)	2.61
Ala	5.30 (± 0.34)	6.36	4.73 (± 0.47)	9.89	93 (± 6.02)	6.50
Arg	<L.D.		<L.D.		90 (± 1.45)	1.61
Tyr	0.52 (± 0.03)	5.46	0.54 (± 0.03)	6.45	100.1 (± 0.10)	0.90
Met	<L.D.		<L.D.		96 (± 2.62)	2.72
Trp	1.47 (± 0.02)	1.39	1.57 (± 0.07)	4.23	72 (± 1.53)	2.12
Val	<L.D.		<L.D.		90 (± 5.14)	5.71
Phe	0.16 (± 0.01)	6.58	0.18 (± 0.02)	8.99	75 (± 6.07)	8.04
Ile	0.65 (± 0.03)	4.60	0.70 (± 0.04)	6.04	96 (± 0.52)	0.54
Leu	0.69 (± 0.02)	2.92	0.79 (± 0.10)	13.1	94 (± 3.22)	3.41
Lys	<L.D.		<L.D.		69.5 (± 0.70)	1.16
Cys	0.01 (± 0.001)	9.10	0.02 (± 0.002)	11.8	70.5 (± 0.52)	0.74
Pro	1.60 (± 0.07)	4.12	1.85 (± 0.03)	7.10	95.8 (± 0.32)	0.34

S.D. = standard deviation.
R.S.D. = relative standard deviation.
[a] Within-day precision data ($n = 7$).
[b] Day-to-day (10 consecutive days) analysis in duplicate.

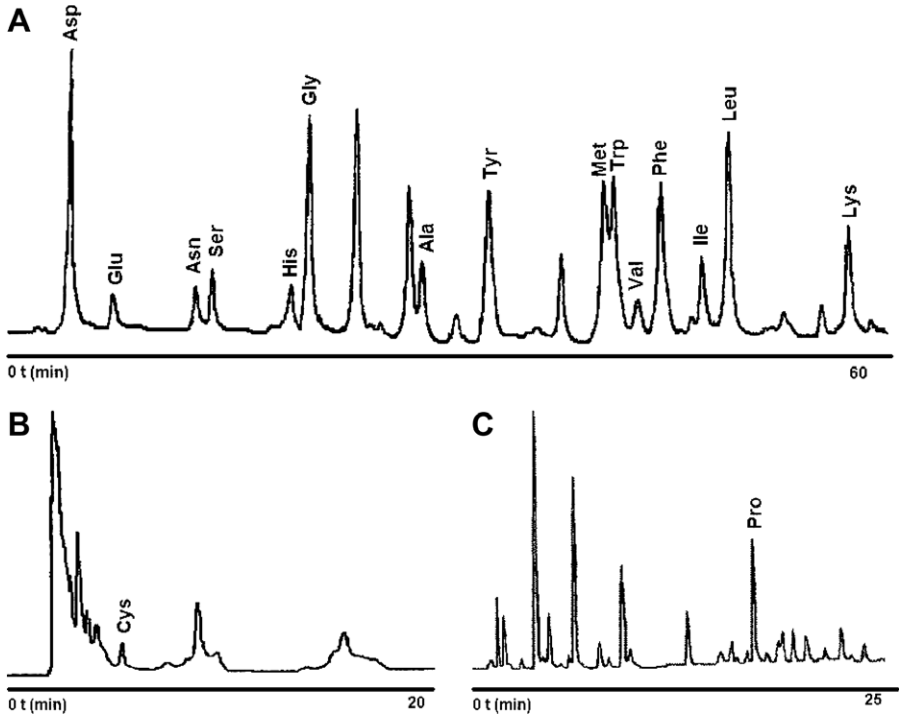

Fig. 2. Chromatogram for the amino acids analysis in a cachaça sample; (A) Analysis of: aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), alanine (Ala), arginine (Arg), tyrosine (Tyr), methionine (Met), tryptophan (Trp), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys), (B) cysteine (Cys), and (C) proline (Pro). For sample preparation and chromatographic conditions, see experimental section.

The quantitative analytical data collected for the analysis of 20 amino acids in 41 samples of cachaça, 05 samples of rum, and 12 samples of whisky is given in Table 4. According to the data, Table 4, there is no uniformity in the qualitative profile of amino acids in the two groups of cachaças (sweetened and unsweetened). Threonine was barely identified in six samples of sweetened cachaça. The order of amino acids, based on occurrence, in increasing rank of frequency are Gln < Glu = Val < Trp = Phe < Thr = Ala = Leu = Lys < Arg < Ile < Ser < Gly < His < Cys < Asp < Asn < Tyr < Pro for sweeted cachaças and Lys = Val < Leu < Trp = Arg < Gln = Phe < Glu = Met = Ile < Cys < Gly = Ala < Pro = Ser < His < Asn < Asp < Tyr for unsweetened cachaças. The increasing order of amino acids in this two groups of cachaças as function of their median content are Cys (0.001 mg L^{-1}) < Gly (0.004 mg L^{-1}) < His (0.013 mg L^{-1}) < Tyr (0.020 mg L^{-1}) < Asn (0.026 mg L^{-1}) < Asp (0.045 mg L^{-1}) < Pro (0.084 mg L^{-1}) for sweetened cachaças and Cys (0.001 mg L^{-1}) < Ile (0.011 mg L^{-1}) < Gly (0.013 mg L^{-1}) < Ala (0.015 mg L^{-1}) < Pro (0.032 mg L^{-1}) < Tyr (0.036 mg L^{-1}) < Ser (0.050 mg L^{-1}) < Asn (0.055 mg L^{-1}) < His (0.064 mg L^{-1}) < Asp (0.065 mg L^{-1}) for unsweetened cachaça.

Despite the observed difference in the order of amino acids, based on occurrence and content, in sweetened and non-sweetened cachaça, the one-way analysis of variance (ANOVA) applied to the full data shown no significant difference ($p = 0.05$) between the amino acid composition of both types of spirit with exception to Cys and Pro, Table 5. Although, the median content of Cys for the two groups of cachaça are numerically equal, the ANOVA test point out that the data by itself is significantly different as observed for Cys incidence in 63.6% of the sweetened cachaça samples and 52.6% for non-sweetened cachaça. Unquestionably, the sucrose added to the distillate in the fining step can contribute to the beverage amino acid content, however, this influence is difficult to establish since the sugar origin, the exact added amount, and possible impurities are not declared by producers.

In rum, comparable quantities of the representative amino acids are present in the following rank of occurrence: Phe < Glu = Gln = Val = Ala < His = Gly = Thr = Arg = Tyr < Asn = Ser = Lys = PRO < Cys = Asp. Notwithstanding, the similarity in the qualitative profile of amino acids in rum and cachaça being expected, since both spirits are originated from sugar cane, the ANOVA test show significant difference ($p = 0.05$) between these two spirits regarding the content and frequency of the amino acids Ser, Ala, Arg, Phe, and Cys. The following occurrence and median contents are observed for rum and cachaça: Ser (frequency: 80.0%; median: 0.149 mg L^{-1}), Ala (frequency: 40.0%; median: < 0.015 mg L^{-1}), Arg (frequency: 60.0%; median: 0.048 mg L^{-1}), Phe (frequency: 20.0%; median: < 0.0080 mg L^{-1}), and Cys (frequency: 100%; median: 0.0060 mg L^{-1}) for samples of rum and Ser (frequency: 53.6%; median: 0.015 mg L^{-1}), Ala (frequency: 41.5 0%; median: < 0.015 mg L^{-1}), Arg (frequency: 46.3%; median: 0.008 mg L^{-1}), Phe (frequency:

Table 4
Amino acid content (mg L^{-1}) of the analyzed spirits

Sample	Asp	Glu	Asn	Ser	Gln	His	Gly	Thr	Ala	Arg	Tyr	Met	Trp	Val	Phe	Ile	Leu	Lys	Cys	Pro
Sweetened Cachaça																				
1	1.690	0.017	0.971	0.372	nd	0.021	0.046	nd	0.133	0.010	0.076	nd	0.027	nd	nd	0.027	0.016	nd	0.002	0.239
2	0.018	nd	0.028	0.033	nd	nd	0.009	nd	0.016	nd	0.020	nd	nd	nd	nd	nd	nd	nd	0.003	1.040
3	nd	nd	nd	nd	nd	nd	nd	0.015	nd	nd	0.018	nd	nd	nd	nd	nd	nd	nd	0.001	1.460
4	0.073	nd	0.018	0.067	nd	0.013	0.024	nd	0.027	nd	0.026	0.023	nd	nd	nd	0.015	0.021	0.014	0.001	0.030
5	1.080	0.085	0.037	0.171	nd	0.022	0.085	nd	0.075	nd	0.064	0.110	0.081	nd	0.044	0.048	0.086	0.032	nd	0.043
6	0.047	nd	0.142	nd	nd	nd	nd	nd	nd	nd	0.013	nd	nd	nd	nd	nd	nd	nd	0.005	1.030
7	0.042	nd	0.046	0.049	nd	nd	0.008	0.122	nd	nd	0.018	nd	nd	nd	nd	nd	nd	0.111	0.001	0.090
8	nd	nd	0.022	nd	nd	nd	nd	nd	nd	nd	0.014	nd	nd	nd	nd	nd	nd	nd	0.001	1.800
9	nd	nd	0.023	nd	nd	0.123	nd	nd	nd	0.015	0.028	0.013	nd	nd	nd	nd	nd	nd	0.001	0.025
10	1.490	nd	0.017	nd	0.031	0.012	0.045	nd	nd	nd	0.033	0.051	nd	nd	nd	nd	nd	nd	0.002	0.051
11	0.070	nd	0.080	0.073	nd	0.050	nd	nd	nd	0.022	nd	0.018	nd	nd	nd	nd	nd	nd	0.003	0.373
12	0.025	nd	0.163	nd	0.172	0.096	nd	nd	0.018	0.017	0.043	0.034	nd	nd	0.01	0.014	0.020	nd	0.001	nd
13	0.663	0.207	nd	0.206	nd	nd	0.103	1.170	nd	0.125	0.210	nd	nd	0.084	0.04	0.200	0.850	0.360	nd	0.170
14	0.033	nd	0.030	nd	nd	0.013	nd	nd	nd	nd	0.017	nd	nd	nd	nd	0.090	nd	nd	nd	0.078
15	nd	nd	0.042	nd	nd	0.025	nd	nd	nd	nd	0.020	nd	nd	0.028	nd	0.010	nd	nd	nd	nd
16	0.202	nd	0.040	nd	nd	0.106	nd	nd	nd	nd	0.017	0.029	nd	nd	nd	0.015	nd	nd	nd	1.570
17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.010	nd	nd	nd	nd	nd	nd	nd	0.001	0.158
18	nd	nd	nd	0.027	nd	nd	0.007	0.055	nd	nd	0.133	nd	nd	nd	nd	nd	nd	nd	nd	0.058
19	0.089	nd	0.039	nd	nd	0.132	nd	nd	0.016	nd	0.033	nd	0.022	nd	0.038	nd	0.053	0.156	0.002	0.050
20	0.131	nd	0.011	nd	nd	0.026	0.027	0.073	nd	nd	0.023	nd	nd	nd	nd	0.013	nd	0.022	0.001	0.008
21	0.106	0.055	nd	0.028	nd	0.061	0.034	0.662	nd	0.047	nd	nd	0.013	0.024	0.023	nd	nd	nd	nd	0.003
22	nd	nd	nd	0.332	nd	nd	0.059	nd	nd	0.010	nd	nd	0.018	0.010	nd	nd	nd	nd	nd	0.104
Median content	0.045	nd	0.026	nd	nd	0.013	0.004	nd	nd	nd	0.020	nd	nd	nd	nd	nd	nd	nd	0.001	0.084
% Frequency	68.2	18.2	72.7	45.5	9.1	59.1	50.0	27.3	27.3	31.8	86.4	31.8	22.7	18.2	22.7	40.9	27.3	27.3	63.6	90.9
Unsweetened Cachaça																				
1	0.793	0.299	0.065	0.846	0.061	0.121	0.256	nd	0.822	nd	0.086	nd	0.247	nd	0.026	0.111	0.115	nd	nd	0.055
2	0.349	0.016	nd	0.465	nd	nd	0.034	nd	0.396	nd	0.035	nd	0.169	nd	nd	nd	0.032	0.031	0.005	0.058
3	0.028	nd	0.016	0.050	nd	0.023	0.096	nd	nd	nd	0.028	nd	nd	nd	nd	nd	nd	nd	nd	0.049
4	2.160	nd	0.140	1.430	nd	0.165	0.241	nd	nd	0.092	0.216	0.817	nd	nd	0.056	0.217	nd	nd	0.001	0.058
5	0.054	0.462	0.035	0.277	nd	0.275	0.454	nd	0.022	nd	0.036	0.089	nd	0.012	0.014	nd	nd	nd	0.002	0.037
6	0.045	nd	0.053	0.057	nd	nd	0.039	nd	0.013	nd	0.028	nd	0.017	nd	0.014	nd	0.019	nd	nd	0.062
7	0.098	0.025	0.059	0.043	nd	0.087	Nd	nd	0.018	nd	0.035	nd	0.022	nd	nd	nd	nd	nd	0.007	0.032
8	0.193	0.065	0.020	0.294	nd	0.027	0.040	nd	0.017	nd	0.069	0.065	nd	nd	nd	nd	nd	nd	0.003	nd
9	0.057	nd	0.677	1.230	nd	1.540	Nd	nd	0.084	0.282	0.622	0.756	nd	nd	0.06	0.231	nd	nd	nd	nd
10	1.330	0.089	0.155	0.163	2.240	0.085	Nd	nd	0.045	0.011	0.035	0.013	nd	nd	nd	nd	nd	nd	0.002	0.049
11	0.034	nd	0.140	nd	0.150	0.064	Nd	nd	nd	0.012	0.043	0.054	0.012	nd	0.011	nd	nd	nd	nd	0.030
12	0.043	nd	0.055	nd	nd	0.012	0.019	nd	nd	nd	0.032	nd	nd	nd	nd	nd	nd	nd	0.008	nd
13	0.065	nd	0.096	nd	0.133	0.035	0.010	nd	nd	nd	0.040	nd	nd	nd	nd	0.015	nd	nd	0.001	nd
14	0.069	nd	nd	0.015	nd	nd	Nd	nd	nd	nd	0.012	nd	nd	nd	nd	nd	0.007	nd	nd	nd
15	0.956	nd	0.390	nd	0.332	0.113	Nd	nd	nd	nd	0.524	0.255	nd	nd	nd	0.150	nd	nd	0.001	nd
16	0.079	nd	0.035	nd	0.074	0.013	Nd	nd	0.009	nd	0.018	nd	nd	nd	nd	nd	nd	nd	nd	0.023
17	0.049	0.049	2.340	nd	nd	0.082	Nd	nd	0.550	0.021	0.071	nd	nd	nd	nd	nd	nd	nd	nd	0.060

18	nd	nd	nd	nd	nd	nd	0.043	nd	nd	nd	0.036	nd	nd	nd	nd	nd	nd	nd	nd	nd
19	nd	nd	0.040	0.181	nd	0.155	0.013	nd	0.115	nd	0.048	nd	nd	nd	nd	0.011	nd	nd	0.006	0.047
Median content	0.065	nd	0.055	0.050	nd	0.064	0.013	nd	0.015	nd	0.036	nd	nd	nd	nd	0.011	nd	nd	0.001	0.032
% Frequency	89.5	36.8	84.2	63.2	31.6	73.7	57.9	0.0	57.9	26.3	100.0	36.8	26.3	5.3	31.6	36.8	21.1	5.3	52.6	63.2
Whiskey																				
1	nd	0.032	nd	0.026	nd	nd	0.050	nd	nd	0.043	0.250	0.005	nd	0.010	0.026	nd	0.062	0.091	0.003	0.012
2	2.030	0.660	nd	0.141	0.061	0.420	0.540	nd	nd	0.373	0.210	nd	nd	0.170	0.011	0.300	0.600	0.066	nd	0.060
3	0.741	0.311	nd	0.150	nd	nd	nd	nd	nd	0.096	0.334	0.021	nd	0.050	0.140	nd	nd	nd	nd	0.007
4	0.509	0.181	nd	nd	nd	nd	0.139	1.720	2.770	0.136	nd	nd	nd	0.050	0.024	nd	nd	0.126	nd	0.011
5	0.663	0.221	nd	0.149	0.145	0.136	nd	1.500	nd	0.060	0.439	nd	nd	0.047	0.027	nd	nd	nd	0.005	0.037
6	0.842	0.211	0.119	2.940	nd	nd	0.743	nd	nd	0.130	1.390	0.653	nd	0.265	0.168	0.422	0.489	1.790	0.004	0.270
7	nd	0.077	nd	0.034	nd	nd	0.018	0.160	nd	0.019	0.032	0.005	nd	0.013	0.011	0.153	0.305	0.026	nd	0.039
8	0.103	0.084	nd	0.029	0.024	nd	0.025	0.290	nd	0.044	0.019	nd	0.015	0.017	nd	nd	0.033	0.037	nd	nd
9	nd	0.124	nd	1.930	nd	0.171	0.117	1.100	nd	0.135	0.266	nd	nd	0.074	nd	nd	nd	0.226	nd	0.006
10	nd	nd	nd	0.493	nd	nd	0.049	nd	nd	0.032	nd	nd	nd	nd	nd	nd	nd	0.058	nd	0.211
11	nd	0.058	nd	nd	0.044	0.30	0.060	0.393	nd	0.130	0.363	nd	0.058	nd	0.028	0.319	0.193	0.133	0.003	0.016
12	0.336	0.029	nd	1.290	nd	nd	0.213	nd	nd	0.086	0.324	nd	nd	0.029	0.017	nd	nd	nd	nd	0.078
Median content	0.220	0.104	nd	0.145	nd	nd	0.055	0.080	nd	0.091	0.258	nd	nd	0.038	0.021	nd	0.017	0.062	nd	0.027
% Frequency	58.3	91.7	8.3	83.3	33.3	33.3	83.3	50.0	8.3	100.0	83.3	33.3	16.7	83.3	75.0	33.3	50.0	75.0	33.3	91.7
Rum																				
1	0.359	nd	nd	1.690	nd	0.055	0.066	0.235	0.390	0.106	0.103	nd	nd	0.033	nd	nd	nd	1.880	0.009	nd
2	1.150	0.183	0.074	1.470	nd	nd	0.267	nd	nd	0.179	0.328	nd	nd	0.050	nd	nd	nd	0.101	0.004	0.035
3	0.081	nd	0.333	0.053	0.241	nd	nd	0.708	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.006	0.717
4	0.040	nd	0.248	nd	0.492	0.076	0.009	0.029	nd	0.084	0.012	nd	nd	nd	nd	nd	nd	0.090	0.010	0.004
5	0.524	0.171	0.605	0.149	nd	0.196	nd	nd	0.834	nd	nd	nd	nd	nd	0.184	nd	nd	0.041	0.004	1.210
Median content	0.359	nd	0.248	0.149	nd	0.055	0.009	0.029	nd	0.084	0.012	nd	nd	nd	nd	nd	nd	0.090	0.006	0.035
% Frequency	100.0	40.0	80.0	80.0	40.0	60.0	60.0	60.0	40.0	60.0	60.0	0.0	0.0	40.0	20.0	0.0	0.0	80.0	100.0	80.0

* The preconcentration factor was 10 for all amino acids with except for Cysteine (7.5).

Table 5
One-way ANOVA test for significant difference in the individual amino acid content between the distillated spirits studied

	Asp	Glu	Asn	Ser	Gln	His	Gly	Thr	Ala	Arg	Tyr	Met	Trp	Val	Phe	Ile	Leu	Lys	Cys	Pro
Sweetened cachaça × unsweetened cachaça	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Y	Y
Cachaça × rum	N	N	N	Y	N	N	N	N	Y	Y	N	–	–	N	Y	–	–	N	Y	N
Cachaça × whisky	N	N	N	N	N	N	N	N	Y	Y	Y	N	N	N	N	Y	N	N	N	N
Whisky × rum	N	N	N	N	Y	N	N	N	N	N	N	–	–	N	N	–	–	N	N	Y

N = Population means are not significantly different ($p = 0.05$).
Y = Population means are significantly different ($p = 0.05$).
– = ANOVA test was not carried out.

26.8%; median: 0.008 mg L^{-1}), and Cys (frequency: 58.5%; median: 0.0010 mg L^{-1}) for samples of cachaça.

The presence of amino acids in raw sugar cane juice and molasses is well documented (Chen, 1985; Waliszewski, Romero, & Pardio, 1997). Furthermore, the percentage of free amino acids in sugar cane as dry solids, changes from 0.01 (Gly) to 0.11% (Asp) for different sugar cane variety with a total protein content of 0.49% (Chen, 1985). Hence, differences in production process, yeasts, sugar cane variety, and maturation process and mostly in the rectification step of the raw distillate may account for the quantitative differences observed for the amino acids content in cachaça (median of total concentration = 0.63 mg L^{-1}) and rum (median of total concentration = 3.18 mg L^{-1}).

Although, the rectification processes is employed in the whisky production, higher quantities of amino acids in whisky (median of total concentration = 3.20 mg L^{-1}) are observed in comparison with cachaça (median of total concentration = 0.63 mg L^{-1}). The sequence of amino acids abundance in whisky samples is Leu < Phe < Pro < Val < Gly < Lys < Thr < Arg < Glu < Ser < Asp < Tyr with the following decreasing order of occurrence: Ala = Asn < Trp < Gln = His = Met = Ile = Cys < Thr < Asp = Leu < Phe = Lys < Ser = Gly = Tyr = Val < Glu = Pro < Arg. A possible reason for the high amino acid content is the raw material used to produce whisky. The malted raw materials such as corn, rye, barley, and wheat used for whisky production exhibit higher dry protein content than sugar cane, with an average content ranging from 8.00 to 13.2% in weight of dry basis for corn and wheat, respectively (Bronsky & Schumann, 1989). However, the difference in raw material cannot be exclusively responsible for the higher content of amino acids in whisky and rum.

The rectification process is more widespread for rum and whisky production than for cachaça, therefore, the distillation step itself would favor a higher amino acid content in cachaça which is not observed. At the first view amino acids by itself are not expected to be transferred during distillation step into the spirit. However, their presence could be explained on basis of the mass transfer through micro drops of liquid phase dragged by alcoholic vapor into the spirit. This is still more evident in pot still apparatus, which has only one theoretical plate and where the foam formed during the distillation significantly increase the contact area between the liquid and the vapor phase. The non-uniform heating on the pot still apparatus, which is usual on small produces, leads to a distillation with unsatisfactory rate control which in turn may increase the dragging phenomena.

Additional uptake of amino acids through extraction from the wood casks by the alcoholic beverage during the aging is feasible (Fengel & Wegener, 1989), however, there are not available reports in the current literature for this specific subject, at least as far we know. Unfortunately, our samples were collected in the market and their traceability is not always the desired one to support sound considerations in this matter.

Furthermore, analysis by ANOVA evidenced significant difference ($p = 0.05$) between whisky and rum only for the presence of His and Pro. The frequency of His for whisky samples was 33.3% with a median content of <0.01 mg L^{-1} and for samples of rum the frequency was 60.0% with a median content of 0.055 mg L^{-1}. In the case of Pro, the frequency for whisky samples was 91.7% with a median content of 0.027 mg L^{-1} and for rum samples the frequency was 80.0% with a median content of 0.035 mg L^{-1}. In other hand, the comparison between whisky and cachaça by ANOVA lead to significant differences ($p = 0.05$) for the presence of Ala, Arg, Tyr, and Phe. This dissimilarity coincides with the difference observed for rum and cachaça in terms of Ala and Arg.

The Arg content measured in cachaças (<0.008 mg L^{-1}) is lower than in rum and whiskies (median levels equal to 0.084 and 0.091 mg L^{-1}, respectively). Indeed, arginine is barely identified occurring in less than 50% of the cachaça samples with a mean content of 0.035 mg L^{-1} for sweetened cachaças and 0.084 mgL^{-1} for unsweetened cachaça. It is well accepted in the literature that Arg is an ethyl carbamate precursor in the fermented most (Tonon & Lonvaud-Funel, 2002; Uthurry, Lepe, Lombardero, & Del Hierro, 2006), mainly when the fermentation medium has a high population of lactic acid bacteria, condition usually found in cachaça production. Thus, it is reasonable to assume that Arg is converted into ammonia, ornithine, ATP, carbamyl phosphate, and urea during the fermentation (Tonon & Lonvaud-Funel, 2002) and therefore will not be transferred to the spirit during the distillation step.

The proline median concentration in cachaça (0.049 mg L^{-1}) and rum (0.035 mg L^{-1}) are similar, and about twice the value found for whiskies (0.027 mg L^{-1}). The content of Pro is quite important for haze and precipitate formation once polyphenols are present (Siebert, 1999; Siebert, 2006). The "beverage defect" (haze and precipitates) could be formed in any type of alcoholic beverage and even in soft drinks (Refsgaard, Schaumburg, & Skibsted, 1996; Wu & Siebert, 2002), and despite being non toxic they are undoubtedly undesirable for sensorial and commercial reasons. According to an analysis performed at our Laboratory in a sample of haze isolated from cachaça, the percent of proline was found to be around 19% in weight indicating Pro as one of the plausible precursors of haze formation in cachaça.

Other relevant aspect observed on the data presented in Table 4 is the low levels and occurrence of the sulfur amino acid Met (median value < LD) in all spirits analyzed. This would be consequence of the decomposition of this amino acid during fermentation and distillation yielding compounds like dimethylsulfide (DMS), which presence is associated with undesirable odor in the beverage (Cardoso, Sobrinho, Lima-Neto, & Franco, 2004).

Encouraged by the slightly difference on the amino acids content observed in Tables 4 and 5 multivariate analysis has been applied to the full quantitative data set of amino acid in the spirits studied. Unfortunately, the exploratory principal components analysis and hierarchical cluster analysis (PCA, HCA) do not provide any clear interpretation.

4. Conclusions

The presence of 20 amino acids in cachaças (with or without sugar addition), rums, and whisky is reported by the first time.

The analyzed rum and whisky samples exhibit higher amounts of amino acid than cachaça.

Proline is the most abundant amino acid in all classes of samples, especially for cachaça. Whisky shows the lower proline concentrations and the highest levels of arginine in comparison with cachaças. Proline, aspartic acid, asparagine, serine, histidine, glycine, arginine, tyrosine, and lysine are the most representatives amino acids founded in the three spirits studied.

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