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In vitro bioaccessibility of amino acids and bioactive amines in 70% cocoa dark chocolate: What you eat and what you get

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ABSTRACT

Chocolate is an important source of free bioactive amines and amino acids which play important roles in human health. Considering the limited information on the bioaccessibility of these compounds from chocolate, the objective of this study was to characterize their profiles and bioaccessibility in 70% cocoa dark chocolate through *in vitro* simulation of oral, gastric and intestinal digestions. Seven amines were detected; polyamines were predominant before *in vitro* digestion, whereas tyramine, cadaverine and spermidine after digestion. All amines showed high bioaccessibility with slight influence of digestive enzymes. Amines increased after gastrointestinal digestion: tyramine (13-fold), tryptamine (9-fold), others (2.4–4.2-fold) and histamine appeared. All amino acids, GABA and ammonia were detected in chocolate, and their contents increased after *in vitro* digestion due to digestive enzymes (4.6, 2.8 and 2.1, respectively). Dark chocolate protein is a good source of tryptophan, phenylalanine + tyrosine, isoleucine, histidine, but limiting for lysine, leucine, and threonine.

1. Introduction

Chocolate is a food product consumed worldwide by different populations due to its desirable sensory characteristics (Oracz, Nebesny, Żyżelewicz, Budryn, & Luzak, 2019). This cocoa-based product is one of the most promising functional foods, due to its high levels of bioactive compounds, including flavonoids, phenolic acids (Gültekin-Özgüven, Berktas, & Özçelik, 2016; Oracz, Nebesny, & Żyżelewicz, 2019), hydroxycinnamic acids, methylxanthines, alkaloids (Martini, Conte, & Tagliazucchi, 2018), amino acids (Żyżelewicz, Budryn, Oracz, Antolak, Kręgiel, & Kaczmarska, 2018) and, with less scientific reports but not less important, the bioactive amines (Restuccia, Spizzirri, Luca, Parisi, & Picci, 2016; Do Carmo Brito, Chisté, Pena, Gloria, & Lopes, 2017).

Bioactive amines are comprised of polyamines and biogenic amines. The polyamines—spermidine and spermine—show antioxidant activity in food and biological systems through metal chelating and radical scavenging properties, and are associated with reduced blood pressure and low incidence of cardiovascular disease (Muñoz-Esparza et al., 2019). These polycationic molecules accumulate in highly proliferative tissues, being responsible for the maintenance, turnover and integrity of intestinal epithelial cells (Ramos-Molina, Quipo-Ortuño, Lambertos, Tinahones, & Peñafiel, 2019). Biogenic amines play important roles in several biochemical and physiological mechanisms for human nutrition and health. 2-Phenylethylamine and tryptamine are modulators of neurotransmission in the brain and are found in diverse mammalian tissues (Yılmaz & Gökmen, 2020). Phenylethylamine has association with higher cognitive functions, memory, and in the prevention of schizophrenia, depression, attention deficit disorder and Parkinson's disease (Tofalo, Perpetuini, Schirone, & Suzzi, 2016). This catecholamine releasing agent, is a stimulator of the hypothalamus, inducing pleasurable sensations, enhancing mood lifting and sexual drive (Yılmaz & Gökmen, 2020). Tryptamine is a neurotransmitter related to the amino acid tryptophan. It has antioxidant properties because of the noticeable scavenging activity toward radicals, mainly related to the nitrogen atom of the indole ring (Bentz, Lobayan, Martínez, Redondo, & Largo, 2018). Anti-inflammatory activity has been attributed to tyramine and its level in urine of metabolic syndrome patients were inversely correlated with multiple biomarkers of inflammation and cardiometabolic risk (Pastel, Thompson, Abdelmalek, Adams-Huet, & Jialal, 2019). Histamine is also a neurotransmitter and vasodilating amine (EFSA, 2011). However, the intake of foods containing high concentrations of histamine and tyramine can cause adverse effects to human health. Tyramine can cause headache, pupil and palpebral tissue dilatation, and increased blood pressure (Yılmaz & Gökmen, 2020). No

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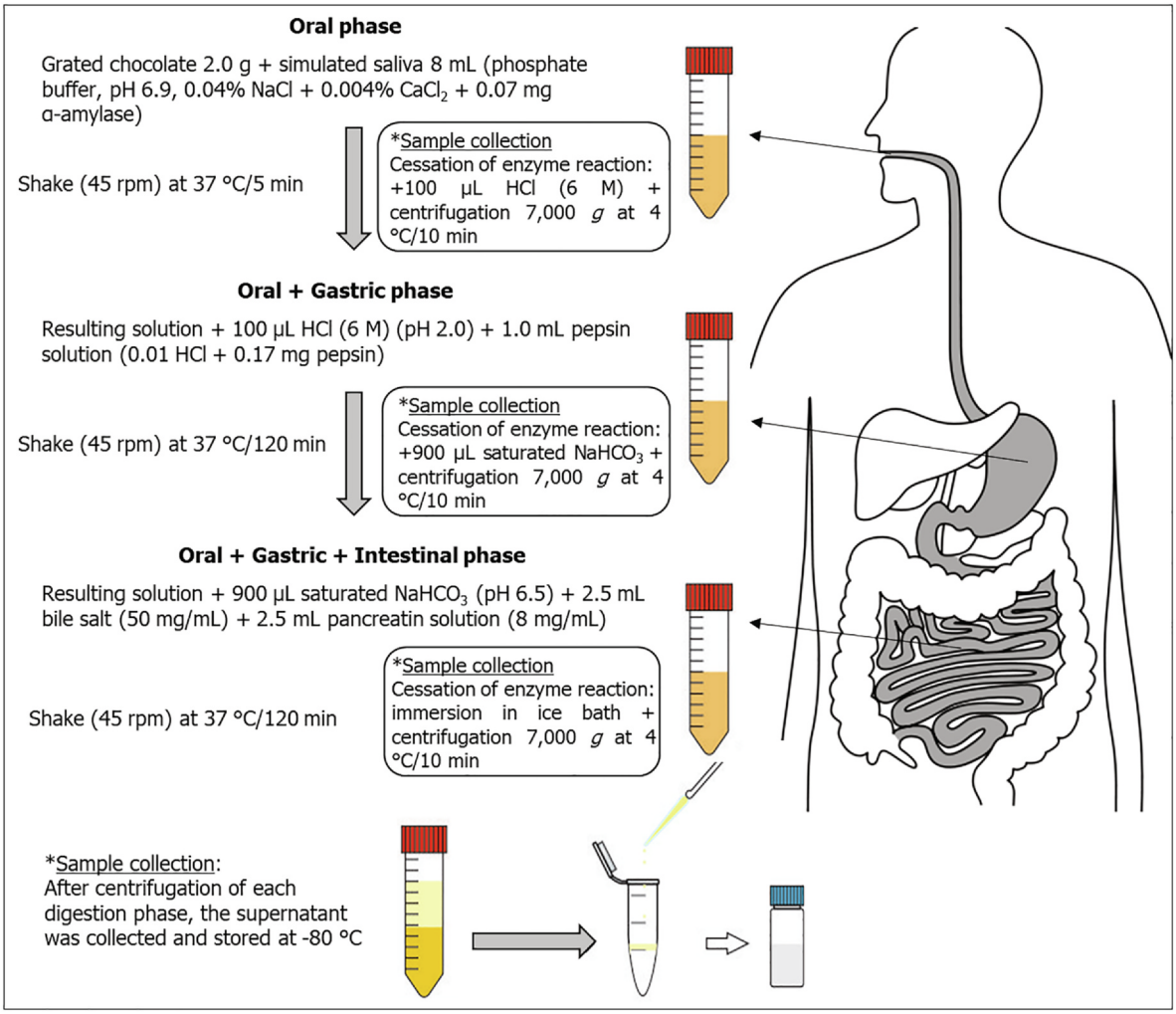

Fig. 1. Sketch map of simulated *in vitro* gastrointestinal digestion of 70% cocoa dark chocolate.

observed adverse effect level (NOAEL) were established as 600 mg tyramine per person per meal for healthy individuals not taking monoamino oxidase inhibitor (MAOI) drugs, but 50 mg for those taking third generation MAOI drugs and 6 mg for those taking classical MAOI drugs (EFSA, 2011). Histamine at high concentrations may lead to hypotension, nausea, migraine, abdominal pain, and heart problems (EFSA, 2011). NOAEL was observed after exposure to 50 mg histamine per person per meal for healthy individuals, but below detectable limits for those with histamine intolerance (EFSA, 2011).

Free amino acids are responsible for many physiological functions associated with human health. Essential amino acids are required for protein synthesis, tissue repair and nutrient absorption. Some individual amino acids are associated with health promotion. Dietary tryptophan supplementation was reported to induce beneficial effects in Alzheimer's disease in mice (Maitre, Klein, Patte-Mensah, & Mensah-Nyagan, 2020) and inflammation-mediated tryptophan catabolism has been related to the development of anemia, fatigue and depression in cancer patients (Lanser et al., 2020).

Gamma-aminobutyric acid (GABA) is a non-protein amino acid which plays physiological and biological roles, such as modulation of synaptic transmission, prevention of sleeplessness and depression, hypertension, diabetes, cancer, oxidation, inflammation, microbial activity and allergy (Ngo & Vo, 2019). GABA can be naturally present in dark chocolate, and chocolate has been enriched with GABA (280 mg/100 g) from glutamic acid by natural fermentation using *Lactobacillus hilgardii* K-3 to enhance its functional properties (Nakamura et al., 2009).

However, during the digestive process, changes can happen to the profile and contents of free amino acids and free bioactive amines, thereby affecting taste perception (Kongor et al., 2016; Rotola-Pukkila, Yang, & Hopia, 2019) and the availability of these compounds for intestinal absorption (Dima, Assadpour, Dima, & Jafari, 2020). There can be polypeptide and protein hydrolysis, and there can also be hydrolysis of conjugates, as reported for *N*-caffeoyl-L-amino acids which break down into caffeic acid and the respective amino acid (Oracz et al., 2019).

Considering the increased interest in chocolate as a functional product and the scarcity of information on the bioaccessibility of free bioactive amines and amino acids from chocolate, the objective of this study was to characterize the profile and levels of free bioactive amines and free amino acid in dark chocolate. In addition, the *in vitro* bioaccessibility of these compounds, GABA, and ammonia in 70% cocoa dark chocolate was investigated for the first-time. The contents of essential amino acids from 70% cocoa dark chocolate protein due to scoring pattern from requirements for adults were also quantified.

2. Material and methods

2.1. Sample and reagents

Chocolate (70% cocoa dark chocolate) was produced commercially at a farm from Bahia (Brazil), which specializes in both cocoa

production and chocolate manufacturing. It consisted of 67% nibs, 3% cocoa butter, 29.6% sucrose and 0.4% soy lecithin; and no additive is used.

Alpha-amylase (Sigma A-3176), bile salts (Sigma B-8756), pancreatin from porcine gastric mucosa (Sigma P-3292), pepsin from porcine gastric mucosa (Sigma P-7012), bioactive amines standards (spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine, serotonin hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride), L-amino acids standards (alanine, arginine hydrochloride, aspartic acid, asparagine, cysteine, glutamic acid, glycine, histidine hydrochloride, isoleucine, leucine, lysine hydrochloride, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and norvaline – internal standard), γ-aminobutyric acid (GABA) and ammonium chloride were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). AccQ.FluorTM pre-column derivatization kit was purchased from Waters (Milford, MA, USA).

The reagents were of analytical grade, except UPLC solvents which were LC grade. Ultrapure-water was from Milli-Q Plus (Millipore Corp., Milford, MA, USA). The organic and aqueous solvents for HPLC analysis were filtered through 0.22 μm pore size HAWP and HVWP membranes, respectively (Millipore Corp., Milford, MA, USA).

2.2. In vitro simulation of oral, gastric and intestinal digestion

The *in vitro* simulation of gastrointestinal digestion was performed as describe by Ortega, Reguant, Romero, Macià, and Motilva (2009) and Gültekin-Özgüven et al. (2016) with a few modifications. The protocol simulated oral, oral + gastric, and oral + gastric + intestinal phases of the digestion process (Fig. 1).

Digestion was initiated by the oral phase, mixing 2.0 g grated chocolate with 8 mL simulated saliva [phosphate buffer solution (0.04% NaCl and 0.004% $CaCl_2$, pH 6.9) containing 0.07 mg α-amylase (30 units/mg)] in a centrifuge tube. The mixture was shaken (45 rpm) in an incubator at 37 °C for 5 min. The resulting oral phase solution was adjusted to pH 2.0 with 100 μL HCl (6 M) and 1.0 mL 0.01 N HCl solution containing 0.17 mg pepsin (2188 units/mg) was added. The mixture was shaken (45 rpm) in an incubator at 37 °C for 2 h. Finally, the oral + gastric phase was adjusted to pH 6.5 by adding 900 μL saturated $NaHCO_3$ solution. Then, 5 mL duodenal juice, including 2.5 mL bile salt solution (50 mg/mL) and 2.5 mL pancreatin solution (8 mg/mL), was added. The intestinal digestion mixture was shaken (45 rpm) in an incubator at 37 °C for 2 h.

After the conclusion of each phase, enzymatic reactions were stopped by means of pH (oral phase – pH 2; gastric phase – pH 6.5) and temperature (0 ^{0}C) changes. In addition, the resulting solution was centrifuged at 7,000 *g* at 4 °C for 10 min (MOD 280R, FANEN Excelsa 4, São Paulo, SP, Brazil) to precipitate and to eliminate insoluble materials. The supernatants were collected and stored at −80 °C, until analysis of amino acids and bioactive amines. The digestion protocol was performed in duplicates. Control treatments were undertaken for each phase, without addition of the respective enzymes.

2.3. Determination of amino acids, bioactive amines, GABA and ammonia by UPLC

The free amino acids, bioactive amines, GABA and ammonia were extracted from 5 g ground chocolate by three successive extractions with 7 mL 5% trichloroacetic acid (TCA) followed by centrifugation at 11,180 *g* at 4 °C/10 min. The supernatants were collected and filtered through Whatman #1 filter into a 25-mL volumetric flask (Do Carmo Brito et al., 2017; Reis, Guidi, Fernandes, Godoy, & Gloria, 2020). The fractions which resulted from the *in vitro* digestions were used directly.

The internal standard L-norvaline (25 pmol in column) was added to the samples (chocolate extract and resulting solutions from digestion), and the volume was brought up in a 25-mL volumetric flask. Derivatization was undertaken under the optimized conditions: an aliquot of the extract (500 μL) was neutralized using 300 μL 0.1 mol/L NaOH. After homogenization, 5 μL neutralized extract was mixed with 30 μL AccQ.Fluor® borate buffer and 15 μL AQC. The mixture was allowed to rest for 1 min and it was heated in a water bath at 55 °C for 10 min. The extract was filtered through PTFE 0.22-μm pore size membrane (Minisart SRP 4®, Sartorius, Gottingen, Germany) and analyzed by UPLC (Marseglia, Palla, & Caligiani, 2014; Reis et al., 2020).

The concentrations of free amino acids, bioactive amines, GABA and ammonia were determined simultaneously using a Waters Acquity™ UPLC system (Waters, Milford, MA, USA) equipped with an AcquityTM tunable ultra-violet (TUV) detector at 249 nm (Reis et al., 2020). A CSH C18 column (50 × 2.1 mm, 1.7 μm, Acquity UPLC™) and a gradient elution of A – 0.01 mol/L sodium acetate (pH 4.80) and B – acetonitrile was used: initial–2.5 min/0–0% B; 2.8–4.5 min/0–3% B; 4.5–10.0 min/3–30% B; 10.0–11.0 min/30–100% B; 11.0–11.75 min/100–100% B; 11.75–12.5 min/100–0% B, and further re-equilibration at initial conditions for another 2.5 min. The injection volume was 2 μL. The concentrations of amino acids, bioactive amines, GABA and ammonia were calculated by interpolation in external analytical curves of each analyte ($R^2 \geq 0.996$). In addition, the recovery of the internal standard (L-norvaline) during the derivatization process was used to correct results regarding the derivatization efficiency. The results were expressed in mg/100 g chocolate.

2.4. Statistical analysis

Each digestion was performed in two replicates. The results were submitted to one-way ANOVA and the means were compared by the Tukey test at 5% significance (Minitab® 16.2.3). Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were applied for the characterization of the simulated *in vitro* digestion phases and the respective control tests. For PCA, two analysis were performed, the first using the contents of individual free bioactive amines as variables in the derivation of the principal components and the second one with the free amino acids, GABA and ammonia. PCA was performed by covariance as the type of matrix. The dendrogram for HCA was obtained by clustering variables (the same used for PCA). McQuitty's linkage was used for the distance matrix and the Euclidian's method to calculate the distance between observations (Minitab® 16.2.3).

3. Results and discussion

3.1. Characterization of the dark chocolate

3.1.1. Bioactive amines

Among the ten amines investigated, seven were present – cadaverine, 2-phenylethylamine, putrescine, spermine, spermidine, tryptamine, and tyramine at total contents of 8.67 mg/100 g (Table 1). Agmatine, histamine and serotonin were not detected. There was predominance of the polyamines—spermine and spermidine (22% of total, each), followed by 2-phenylethylamine, cadaverine and putrescine (~15%), tyramine (6%) and tryptamine (2%).

Information on the occurrence of bioactive amines in dark chocolate is scarce. Restuccia et al. (2016) found lower total contents (1.04 to 6.5 mg/100 g) in five commercial samples of dark 70% cocoa chocolate. The same amines were found, except for cadaverine (not detected), tryptamine (not investigated) and 2-phenylethylamine (only present in one sample). Histamine and serotonin were not detected in our sample, but their presence in dark chocolate has been reported (Restuccia et al., 2016).

Some amines are inherent to unfermented cocoa (spermidine, serotonin, tyramine, putrescine, and tryptamine) and the profile and contents can be affected by cultivar, soil, climatic conditions and

Table 1
Levels of free bioactive amines in 70% cocoa dark chocolate and in solutions resulting from oral, gastric, and intestinal *in vitro* digestion phases and the respective controls*.

Bioactive amines	Free bioactive amines (mg/100 g)						
	Chocolate	C-Oral	Oral	C-Gastric	Gastric	C-Intestinal	Intestinal
Agmatine	nd	nd	nd	nd	nd	nd	nd
Cadaverine	1.32 ± 0.09^{d}	3.39 ± 0.35^{bc}	3.68 ± 0.08^{bc}	3.29 ± 0.28^{c}	4.29 ± 0.30^{b}	3.27 ± 0.02^{c}	5.59 ± 0.28^{a}
Histamine	nd	nd	nd	nd	nd	nd	1.39 ± 0.17
Phenylethylamine	1.39 ± 0.05^{b}	1.74 ± 0.22^{b}	1.46 ± 0.31^{b}	3.35 ± 0.02^{a}	3.77 ± 0.36^{a}	3.88 ± 0.04^{a}	4.11 ± 0.14^{a}
Putrescine	1.32 ± 0.07^{c}	2.48 ± 0.22^{b}	2.64 ± 0.14^{b}	2.53 ± 0.26^{b}	2.88 ± 0.05^{b}	4.32 ± 0.01^{a}	4.75 ± 0.48^{a}
Serotonin	nd	nd	nd	nd	nd	nd	nd
Spermidine	1.93 ± 0.08^{c}	1.40 ± 0.04^{c}	1.52 ± 0.07^{c}	5.39 ± 0.19^{b}	8.98 ± 0.39^{a}	5.74 ± 0.08^{b}	5.05 ± 0.22^{b}
Spermine	1.98 ± 0.01^{c}	1.77 ± 0.02^{c}	1.80 ± 0.14^{c}	4.39 ± 0.19^{b}	5.14 ± 0.14^{a}	4.29 ± 0.05^{b}	4.65 ± 0.15^{b}
Tryptamine	0.15 ± 0.04^{b}	0.23 ± 0.01^{b}	0.17 ± 0.02^{b}	0.28 ± 0.03^{b}	0.30 ± 0.01^{b}	1.27 ± 0.08^{a}	1.35 ± 0.05^{a}
Tyramine	0.58 ± 0.05^{b}	1.58 ± 0.14^{b}	0.83 ± 0.07^{b}	6.87 ± 0.03^{a}	7.26 ± 0.09^{a}	6.48 ± 0.21^{a}	7.63 ± 1.28^{a}
Total	8.67 ± 0.39^{b}	12.59 ± 1.00^{b}	12.11 ± 0.83^{b}	26.10 ± 1.00^{a}	32.62 ± 1.33^{a}	29.26 ± 0.49^{a}	34.52 ± 2.77^{a}

* without addition of digestive enzymes (C-).
nd – not detected.
Mean values ± standard deviations with different letters in the same line are significantly different (Tukey test, $p \leq 0.05$).

geographical origins (Do Carmo Brito et al., 2017; Delgado-Ospina et al., 2020; Gloria, Deus, & Franca, 2019). Amines can change during fermentation, e.g. there can be decreases on total, spermidine, tryptamine, tyramine and serotonin contents, and the production and accumulation of 2-phenylethylamine (Gloria, Deus, & Franca, 2019). During cocoa roasting, there can be lipid oxidation, Maillard reaction and thermal decarboxylation of amino acids with formation of amines, including 2-phenylethylamine (Oracz & Nebesny, 2014), spermine (Delgado-Ospina et al., 2020) and tryptamine (Oracz & Nebesny, 2014). However, there can also be degradation of some bioactive amines, e.g. cadaverine, spermidine and serotonin, especially at high roasting temperatures (Delgado-Ospina et al., 2020).

3.1.2. Free amino acids, GABA and ammonia

All free amino acids investigated were detected. The total contents of free amino acids were 1282.31 mg/100 g (Table 2). Tyrosine was the prevalent amino acid (16%), followed by serine + aspartic acid (10%), asparagine (9%), valine and alanine (~8%, each), glycine and arginine (~7%), threonine (~6%) and leucine (~5%). The other amino acids contributed less than 4% of total contents. Free tryptophan contents were like values reported for fermented, dried, roasted and defatted cocoa beans from different origins, with values ranging from 8.49 to 17.26 mg/100 g (Bertazzo, Comai, Brunato, Zancato, & Costa, 2011).

Free amino acids in chocolate can be inherent to unfermented cocoa, affected by cultivar, soil, climatic, geographical origins (Delgado-Ospina et al., 2020). But they can also be affected by fermentation and roasting. During fermentation, the concentration of hydrophobic amino acids – phenylalanine, leucine, alanine, aspartic acid, and arginine – increase, mainly due to endogenous cocoa proteolytic enzymes, aspartic endoprotease and carboxypeptidase, which

Table 2
Levels of free amino acids in 70% cocoa dark chocolate and in solutions resulting from oral, gastric, and intestinal *in vitro* digestion phases and the respective controls*.

Analyte	Free amino acids (mg/100 g)						
	Chocolate	C-Oral	Oral	C-Gastric	Gastric	C-Intestinal	Intestinal
Alanine[2]	104.46 ± 1.90^{b}	116.45 ± 1.60^{b}	151.05 ± 9.10^{b}	134.75 ± 25.89^{b}	145.94 ± 5.73^{b}	124.93 ± 13.91^{b}	243.07 ± 29.71^{a}
Arginine[3]	86.83 ± 1.13^{d}	161.35 ± 15.52^{bc}	127.49 ± 7.66 ^{cd}	162.15 ± 1.13^{bc}	182.56 ± 1.59^{b}	178.30 ± 22.85^{b}	305.75 ± 7.23^{a}
Asparagine	115.60 ± 7.00^{b}	131.49 ± 7.42^{b}	125.75 ± 7.51^{b}	146.09 ± 20.64^{b}	256.84 ± 15.08^{a}	134.82 ± 15.93^{b}	313.08 ± 27.61^{a}
Cysteine	48.83 ± 0.71^{b}	42.11 ± 2.66^{c}	54.07 ± 3.50^{c}	40.67 ± 2.04^{c}	107.40 ± 6.36^{b}	46.30 ± 7.95^{c}	157.20 ± 11.21^{a}
Glutamic acid[1] + Glutamine	8.36 ± 1.13^{c}	15.71 ± 0.55^{c}	11.42 ± 0.69^{c}	15.16 ± 0.79^{c}	58.69 ± 2.84^{b}	18.11 ± 4.17^{c}	231.39 ± 23.70^{a}
Glycine[2]	95.46 ± 0.76^{bc}	74.95 ± 6.55^{c}	100.14 ± 5.97^{bc}	101.45 ± 37.47^{bc}	177.74 ± 3.74^{ab}	130.18 ± 40.63^{bc}	225.71 ± 5.39^{a}
Histidine[3]	46.97 ± 0.42^{b}	62.88 ± 3.74^{b}	47.27 ± 2.85^{b}	61.65 ± 1.74^{b}	72.74 ± 7.21^{b}	64.30 ± 3.75^{b}	241.41 ± 31.63^{a}
Isoleucine[3]	25.06 ± 5.66^{c}	18.97 ± 2.33^{c}	28.63 ± 6.44^{c}	24.18 ± 7.36^{c}	119.80 ± 3.87^{b}	28.50 ± 6.11^{c}	624.14 ± 38.87^{a}
Leucine[3]	66.26 ± 5.48^{b}	71.19 ± 2.63^{b}	76.62 ± 4.62^{b}	84.56 ± 18.92^{b}	321.76 ± 3.02^{a}	79.85 ± 6.67^{b}	343.34 ± 4.53^{a}
Lysine[4]	34.58 ± 1.49^{c}	20.68 ± 0.50^{c}	33.92 ± 0.87^{c}	21.69 ± 1.43^{c}	63.46 ± 2.35^{b}	21.32 ± 0.53^{c}	174.25 ± 10.47^{a}
Methionine[3]	28.05 ± 3.39^{b}	33.60 ± 1.93^{b}	30.70 ± 1.83^{b}	36.42 ± 3.98^{b}	210.11 ± 46.63^{a}	44.55 ± 11.51^{b}	201.12 ± 2.04^{a}
Ornithine	5.03 ± 0.89^{c}	6.20 ± 1.59^{c}	6.16 ± 0.39^{c}	7.05 ± 1.20^{c}	65.05 ± 0.33^{b}	7.92 ± 1.24^{c}	236.96 ± 6.42^{a}
Phenylalanine[3]	29.60 ± 1.75^{c}	16.02 ± 0.98^{c}	33.44 ± 2.02^{c}	39.39 ± 4.76^{c}	431.77 ± 29.54^{b}	31.91 ± 3.57^{c}	861.73 ± 30.00^{a}
Proline	30.69 ± 1.48^{c}	30.38 ± 1.49^{c}	30.59 ± 1.85^{c}	34.74 ± 6.18^{c}	83.53 ± 4.85^{b}	39.07 ± 6.12^{c}	483.06 ± 15.69^{a}
Serine[2] + Aspartic acid[1]	130.55 ± 1.41^{b}	155.34 ± 6.94^{b}	137.80 ± 8.27^{b}	165.72 ± 14.69^{b}	313.77 ± 7.63^{a}	179.64 ± 19.68^{b}	361.76 ± 17.33^{a}
Threonine[2]	80.08 ± 5.75^{c}	80.80 ± 0.67^{c}	83.72 ± 5.04^{c}	93.90 ± 18.53^{bc}	125.49 ± 11.64^{ab}	88.34 ± 7.87^{c}	156.49 ± 2.75^{a}
Tryptophan[3]	12.67 ± 2.19^{c}	15.17 ± 0.34^{c}	17.83 ± 1.07^{c}	13.98 ± 1.68^{c}	163.09 ± 28.65^{b}	17.67 ± 5.22^{c}	238.87 ± 6.90^{a}
Tyrosine[4]	205.90 ± 5.90^{c}	241.10 ± 2.51^{abc}	225.38 ± 13.55^{bc}	264.58 ± 33.21^{abc}	274.47 ± 14.42^{ab}	267.92 ± 4.72^{ab}	288.77 ± 1.91^{a}
Valine[3]	107.33 ± 5.02^{b}	106.27 ± 8.56^{b}	123.72 ± 11.11^{b}	115.93 ± 13.65^{b}	207.26 ± 7.54^{a}	126.85 ± 15.44^{b}	207.78 ± 12.50^{a}
Total essential AA	685.33 ± 7.1^{c}	708.8 ± 9.1^{c}	755.30 ± 9.1^{c}	796.9 ± 96.4^{c}	2,097.4 ± 46.2^{b}	817.60 ± 43.2^{c}	3,495.1 ± 79.0^{a}
Total	1,282.3 ± 53.60^{c}	1,445.7 ± 33.5^{c}	1,445.7 ± 50.5^{c}	1,564.1 ± 202.8^{c}	3,431.8 ± 64.6^{b}	1,630.5 ± 108.1^{c}	5,895.9 ± 105.2^{a}
GABA	16.10 ± 0.18^{c}	13.76 ± 0.34^{c}	16.46 ± 0.99^{c}	12.43 ± 1.93^{c}	33.26 ± 3.67^{b}	14.14 ± 2.46^{c}	45.42 ± 5.54^{a}
NH_3	135.54 ± 7.09^{c}	158.35 ± 2.66^{bc}	148.90 ± 8.96^{bc}	174.34 ± 22.6^{bc}	225.46 ± 22.04^{ab}	202.02 ± 39.15^{bc}	285.13 ± 19.46^{a}

Taste amino acids: [1] Umami; [2] Sweet; [3] Bitter; [4] Tasteless (Kongor et al., 2016; Rotola-Pukkila et al., 2019).
Mean values ± standard deviations with different letters in the same line are significantly different (Tukey test, $p \leq 0.05$).
AA: amino acids; * without addition of digestive enzymes (C-).

hydrolyze storage proteins (Marseglia et al., 2014). During cocoa roasting there can be losses of free amino acids, in especial lysine, due to loss during Maillard reaction (Żyżelewicz et al., 2018), which is relevant for the formation of the typical chocolate flavor. There is scarce information on the profile and contents of amino acids in dark chocolate; in addition, the number of amino acids analyzed in previous studies was limited (Pätzold & Brückner, 2006; Żyżelewicz et al., 2018). Therefore, this is the most comprehensive study on amino acids in dark chocolate.

GABA was detected at 16.10 mg/100 g, which is lower compared to fermented and dried cocoa beans from different geographical origins –Africa, (South, Central and North) America, Asia and Oceania— (31.7 to 101.2 mg/100 g) (Marseglia et al., 2014), and in 35–99% cocoa chocolate (11.1 to 32.5 mg/100 g) (Pätzold & Brückner, 2006). Ammonia was present in the chocolate (135.54 mg/100 g) and its presence can result from the degradation of free amino acids during fermentation or even from the alkalization of cocoa beans during processing to provide desirable flavors (Alasti, Asefi, Maleki, & SeiiedlouHeris, 2019).

3.2. *In vitro* simulation of gastrointestinal chocolate digestion

3.2.1. *Bioaccessibility of bioactive amines from 70% cocoa dark chocolate after in vitro simulation of gastrointestinal digestion*

To the best of our knowledge, this is the first insight on bioactive amines bioaccessibility from dark chocolate. As indicated in Table 1, total amines increased significantly (4-fold increase) during the *in vitro* gastric digestion but remained stable under oral and intestinal conditions ($p > 0.05$). The increase in total amines during gastric digestion was also observed for control, suggesting that the low pH (2.0) was responsible for it. According to Casal, Mendes, Alves, Alves, Oliveira and Ferreira (2004), low pH values can breakdown conjugated forms of amines with proteins and phenolic compounds.

When considering individual amines, the same ones were present throughout *in vitro* bioaccessibility; except for histamine, which was not detected in chocolate before *in vitro* digestion but was found after the intestinal phase. Agmatine and serotonin were not detected in any *in vitro* digestion simulation. During the oral phase (Table 1), there was only a significant increase in putrescine and cadaverine (2- and 2.8-fold increases, respectively), which was also observed for control. The similar levels of putrescine and cadaverine found in the oral phase and its control suggest that α-amylase did not affect the release of amines from chocolate. However, the solution (phosphate buffer, pH 6.9 containing NaCl and $CaCl_2$) affected the release of the aliphatic diamines – putrescine and cadaverine, through the breakdown of their conjugated forms. These amines can bound to anions by reversible ionic interactions (Nazifi, Sadeghi-alibadi, Fassihi, & Saghaire, 2019).

The gastric phase was responsible for most of the changes on individual amines. Tyramine increased the most (8.7-fold), followed by spermidine (5.9-fold), spermine (2.9-fold) and phenylethylamine (2.6-fold). The increases in tyramine and phenylethylamine were also observed for control, therefore, this change was not affected by pepsin activity, but probably due to the low pH (2.0) typical of this digestion stage. However, the contents of spermine and spermidine were higher, compared to control, therefore, pepsin activity played a role on the increments observed, being responsible for 15% and 40% of the increases in spermine and spermidine, respectively. Polyamines can be found as free bases or conjugated to other molecules, including nucleic acids, phospholipids, phenolic acids, lignin, protein and polysaccharides with negative charges, like pectin (Casal et al., 2004). Pepsin activity and the low pH could have contributed to the hydrolysis of these conjugates and to the breakdown of ionic bonds between amines and conjugates. Taking into consideration that chocolate is a good source of polyphenolic compounds, pectin and proteins, this is a plausive hypothesis (Gültekin-Özgüven et al., 2016; Martini et al., 2018; Żyżelewicz et al., 2018).

During intestinal phase there were significant increases on tryptamine (4.5-fold), putrescine (1.6-fold) and cadaverine (1.3-fold). In addition, histamine was detected for the first time. The changes on tryptamine did not differ from control ($p > 0.05$), suggesting that the pH (6.5) and bile salts were responsible for its release. In fact, as a biological detergent, bile salts can liberate tryptamine from the lipid phase (unpublished data). The changes on cadaverine and histamine were different from control, and, therefore, were affected by pancreatic enzymes, possibly due the hydrolysis and breakdown of their conjugates. Spermidine and spermine contents in the intestinal phase were lower compared to the gastric phase, for both intestinal and control, suggesting restoration of conjugated forms or degradation of polyamines due to polyamine oxidases or other pancreatic enzymes with unspecific action. The pancreatin used, according to Sigma (2020), is a mixture of several digestive enzymes produced by exocrine cells with broad-spectrum protease, composed of amylase, trypsin, lipase, ribonuclease, and protease.

Based on these results, the amounts of free bioactive amines available from dark chocolate after the simulated intestinal *in vitro* digestion were higher compared to the original chocolate. The digestive enzymes increased the levels of spermidine and spermine after the gastric phase and of cadaverine and histamine after intestinal phase. The digestive enzymes also decreased spermidine and spermine after intestinal phase. In addition, the changes in pH, ionic strength or even the period of incubation and shaking increased the levels of cadaverine after the oral phase; of spermine and spermidine after the gastric phase, and of putrescine and tryptamine after the intestinal phase (Table 1). The highest increases were observed for tyramine (13.2-fold), followed by tryptamine (9-fold), cadaverine (4.2-fold), putrescine (3.6-fold), phenylethylamine, spermidine (2.6-fold), and spermine (2.4-fold). In addition, histamine, which was not detected in the chocolate, showed up at 1.39 mg/100 g. Tyramine, cadaverine and spermidine were the predominant amines after the *in vitro* digestion.

This result can be associated with the food matrix, which contributed to the gradual release of amines during *in vitro* digestion, as reported for phenolic compounds in dark chocolate (Martini et al., 2018). The amines can result from conjugated forms with phenolic acid and proteins conjugates (Casal et al., 2004; Oracz et al., 2019). The release of amines was mostly associated with the media (solution), whereas only spermine and spermidine were affected by pepsin and only cadaverine and histamine were affected by pancreatin. It is important to consider that in the presence of the intestinal microbiota, there could be an increase in bioactive amines due to microbial enzymes decarboxylation of free amino acid (Fernández-Reina, Urdiales, & Sánchez-Jiménez, 2018).

The release of cadaverine and putrescine when chocolate was exposed to simulated salivary fluid, can probably result from the presence of lysine- and ornithine-decarboxylase, respectively in the chocolate matrix. These enzymes can lead to the formation of cadaverine and putrescine and can be expressed during fermentation or even during different stages of industrial processing of cocoa beans into cocoa powder. In fact, lysine decarboxylase can be synthesized by *Enterecoccus faecium, Lactobacillus casei, Leuconostoc mesenteroides*; whereas ornithine decarboxylase or agmatine deiminase can be synthesized by *Enterococcus faecium, E. durans, Lactobacillus plantarum*, *Leuconostoc mesenteroides* (Barbieri, Montanari, Gardini, & Tabanelli, 2019). These bacteria were identified during cocoa beans fermentation (Ouattara et al., 2017) or even at different stages of cocoa beans processing (Lima, Velpen, Wolkers-Rooijackers, Kamphuis, Zwietering, & Nout, 2012).

Multivariate analyses were carried out to help understand how the amines were affected by *in vitro* bioaccessibility (Fig. 2a). A two-principal components (PC) model explained 99.1% (Fig. 2b and c) of the variance. According to PC1 loadings, total amines, tyramine, and spermidine were the most affected amines by *in vitro* digestion. PC2 explained 2.0% of the variance, especially due to spermidine and negatively by putrescine, cadaverine and histamine (Fig. 2c).

The chocolate before and after oral digestion and its control

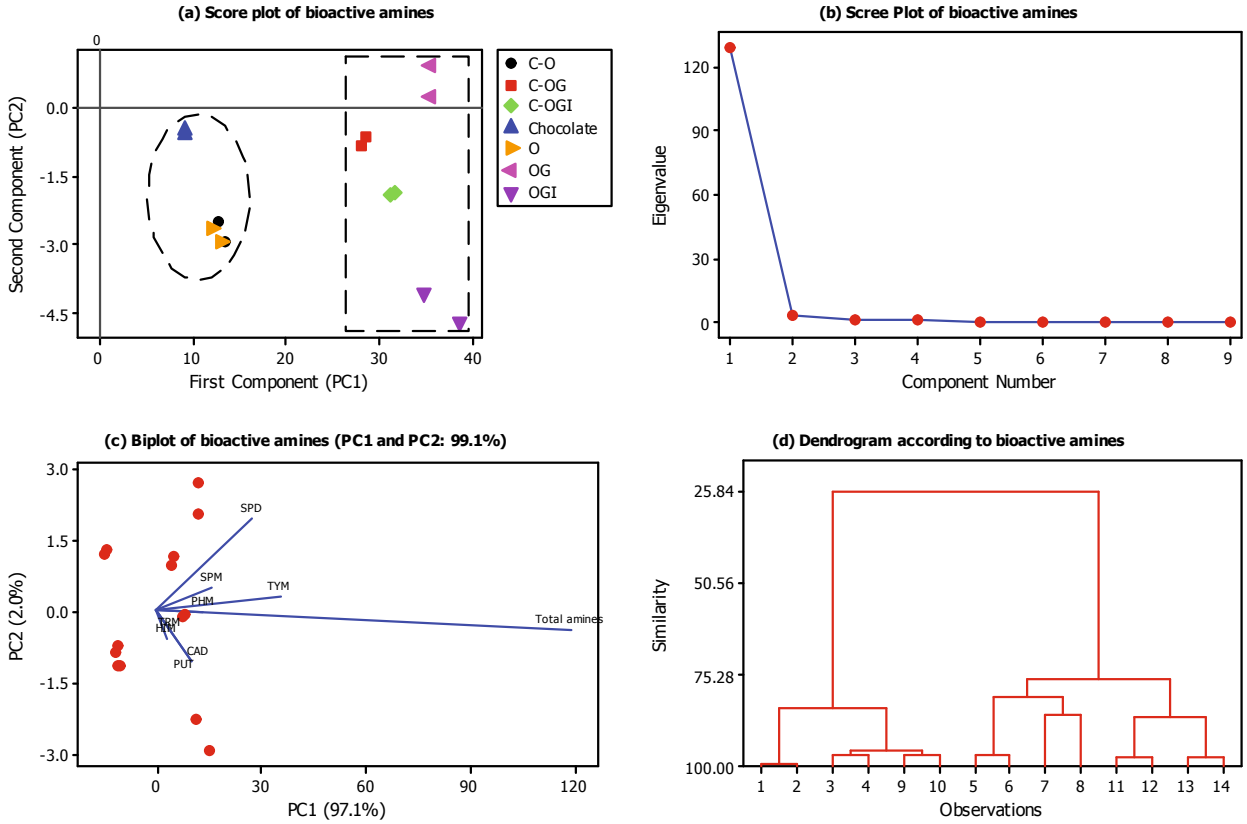

Fig. 2. Principal Component Analyses (PCA) and Hierarchical Cluster Analyses (HCA) of free bioactive amines in 70% cocoa dark chocolate and extracts after oral (O), oral + gastric (OG), and oral + gastric + intestinal (OGI) *in vitro* digestions and the respective controls (without digestive enzymes): **(a)** Score plot (PC1 and PC2); **(b)** Scree plot; **(c)** Biplot (PC1 and PC2: 99.1%); and **(d)** Dendrogram. Legend: CAD – cadaverine, HIM – histamine, PHM – 2-phenylethylamine, PUT – putrescine, SPD – spermidine, SPM – spermine, TRM – tryptamine, TYM – tyramine. Dendrogram observations: 1 & 2 – chocolate; 3 & 4 – after *in vitro* simulation of gastrointestinal digestion. O – 5 & 6 – oral + gastric phases (OG); 7 & 8 – oral + gastric + intestinal phases (OGI); 9 & 10 – control oral phase (C−O); 11 & 12 – control oral + gastric phases (C-OG); and 13 & 14 — control oral + gastric + intestinal phase (C-OGI).

clustered together with up to 84% of similarity (Fig. 2d), whereas the other digestion phases—gastric and intestinal—along with their respective control assays, were grouped with similarity above 76%. The gastric and intestinal controls showed up to 87.0% similarity (Fig. 2d). The dendrogram reinforced the results observed in Table 1, clearly separating chocolate before and after oral phase and the controls.

3.2.2. Bioaccessibility of amino acids, GABA and ammonia from 70% cocoa dark chocolate after in vitro simulation of gastrointestinal digestion

Total amino acids in chocolate (1,282.3 mg/100 g) increased significantly after oral + gastric (2.7-fold) and after oral + gastric + intestinal digestions (4.6-fold) reaching contents of 5,915.77 mg/100 g. The oral phase did not affect total amino acids (Table 2), like amines' results. The control treatments did not affect the contents of amino acids. Therefore, the release of amino acids was mainly due to enzymatic activity, both pepsin and pancreatin. The simulated salivary fluid extracted the same amounts of free amino acids compared to 5% TCA used in the analytical extraction. Furthermore, simulated salivary fluid did not affect total free amino acids, total essential amino acids, GABA and ammonia from chocolate.

In oral + gastric phase, the highest increases in amino acids were found for phenylalanine (14.6-fold), ornithine, tryptophan (12.9-fold), methionine (7.5-fold), and glutamic acid + glutamine (7-fold). The changes in the other amino acids varied from 1.4-fold for alanine up to 4.8-fold for isoleucine and leucine. The highest increases observed for aromatic (tyrosine, phenylalanine, tryptophan) and hydrophobic (leucine and isoleucine) amino acids may have resulted from the greater pepsin specificity for cleavages of peptide bonds between hydrophobic and aromatic residues, in which the carboxyl group is provided by aromatic amino acids (Yu et al., 2018).

The intestinal phase exerted significant impact ($p < 0.05$) on the liberation of several individual amino acids, except for asparagine, glycine, leucine, methionine, serine + aspartic acid, threonine, tyrosine, valine and ammonia. The highest increases in amino acids compared to chocolate were observed for ornithine (47.1-fold), followed by phenylalanine (29.1-fold), glutamine + glutamic acid (27.7-fold), isoleucine (24.9-fold), tryptophan (18.9-fold) and proline (15.7-fold), whereas the increase in the other amino acids was ≤ 5.1-fold. Pancreatin was responsible for the release of these amino acids.

The amino acids found at lower concentrations at the end of the *in vitro* digestion were threonine, lysine, cysteine, methionine, valine, and the non-protein amino acid GABA (Table 2). Thermal or alkaline treatments, common during cocoa beans processing, can negatively affect the digestibility of the chocolate protein by the formation of lysinoalanine, which is formed from the reaction of dehydroalanine with lysine amino groups, resulting in losses of lysine, cysteine, and threonine (Dima et al., 2020). GABA levels in chocolate before and after the oral phase were similar, however, GABA significantly increased after gastric and intestinal phases (2.0- and 2.8-fold, respectively). Considering that GABA is not a chocolate protein component, its increase could be related to the ease extraction from the chocolate matrix after digestion or even from hydrolysis or breakdown of GABA conjugates.

According to multivariate analyses (Fig. 3a), a two-principal components (PC) model explained 99.8% of the variance (Fig. 3b and c). PC1 loadings explained 99.4% of the variance, and total amino acid, total essential amino acids and phenylalanine were the components with the highest impact. PC2 explained 0.4% of the variance, especially due to leucine, total essential amino acids and methionine, and negative

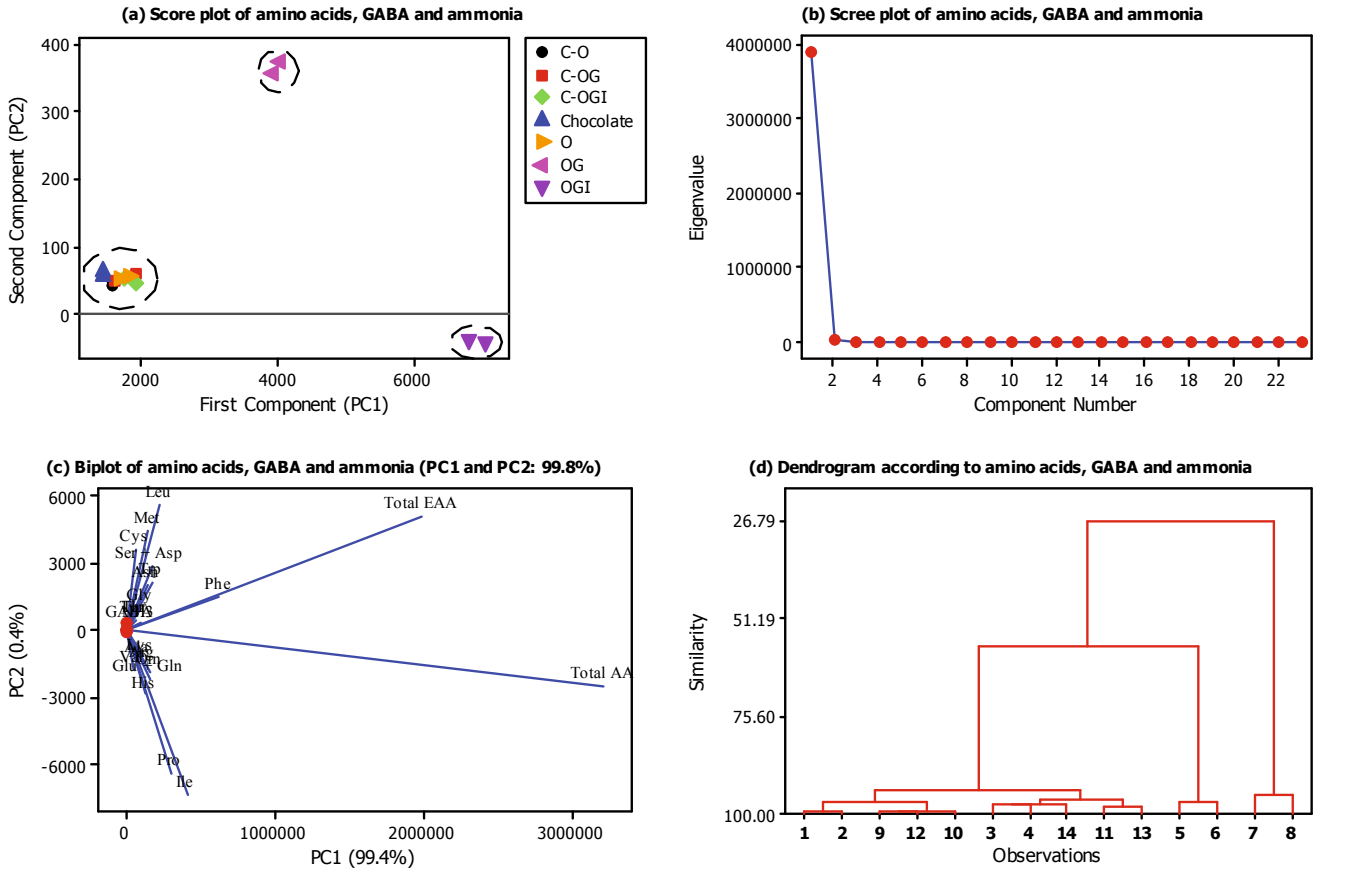

Fig. 3. Principal Component Analyses (PCA) and Hierarchical Cluster Analyses (HCA) of free amino acids, γ-aminobutyric acid (GABA) and ammonia in 70% cocoa dark chocolate and extracts after oral (O), oral + gastric (OG), and oral + gastric + intestinal (OGI) *in vitro* digestions and the respective controls (without digestive enzymes): **(a)** Score plot (PC1 and PC2); **(b)** Scree plot; **(c)** Biplot (PC1 and PC2: 99.1%); and **(d)** Dendrogram. Legend: Ala-Alanine, Arg-Arginine, Asn-Asparagine, Cys-Cysteine, *Epi* – Epinephrine, GABA - γ-aminobutyric acid, Glu + Gln-Glutamic acid + Glutamine, Gly-Glycine, His-Histidine, Ile-Isoleucine, Leu-Leucine, Lys-Lysine, Met-Methionine, NH_3 – ammonia, Orn – Ornithine, Phe-Phenylalanine, Pro-Proline, Ser + Asp-Serine + Aspartic acid, Thr-Threonine, Trp-Tryptophan, Tyr-Tyrosine, Val-Valine, total AA – total amino acids, and total EAA – total essential amino acids. Dendrogram observations: 1 & 2 – chocolate; 3 & 4 – after *in vitro* simulation of gastrointestinal digestion. O – 5 & 6 – oral + gastric phases (OG); 7 & 8 – oral + gastric + intestinal phases (OGI); 9 & 10 – control oral phase (C—O); 11 & 12 – control oral + gastric phases (C-OG); and 13 & 14 — control oral + gastric + intestinal phase (C-OGI).

values of isoleucine and proline (Fig. 3c).

The chocolate before and after oral phase and all the control tests (oral, gastric, and intestinal) were grouped together with up to 96% similarity (Fig. 3d). The simulated *in vitro* oral phase digestion did not affect protein digestion and control, contrary to the behavior observed for amines. However, gastric and intestinal phases were responsible for significant increases in several amino acids, representing two individual groups (gastric and intestinal phases). This clustering differed from that observed for the bioactive amines (Fig. 2d). The dendrogram confirmed the changes on amino acid described in Table 2. After intestinal phase, the contents of some amino acids changed compared to the previous digestion phases (oral and gastric) and to the chocolate before digestion.

3.3. Impact of amino acids on chocolate taste

The profile and levels of free amino acids are especially important to chocolate perception in the mouth (Rotola-Pukkila et al., 2019). At the end of the oral phase, there was a balance on the contents of sweet (alanine, glycine, serine, threonine) and bitter (arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan and valine) amino acids, around 447 and 468 mg/100 g, respectively. The concentration of umami amino acids (aspartic and glutamic acids) found in the chocolate were lower than sweet and bitter ones, corresponding to approximately 149 mg/100 g (Table 2). However, it is important to take into consideration not only the amount of each component, but their perception threshold and other factors, as well. Due to the relevance of taste perception on chocolate, this is an area that deserves further studies.

3.4. Impact of chocolate digestion on nutritional value

The essential amino acids (histidine, isoleucine, leucine, lysine, methionine + cysteine, phenylalanine + tyrosine, threonine, tryptophan, and valine) represented 53.44% of the total content of free amino acids in the chocolate before digestion and 59.42% at the end of the simulated *in vitro* digestion. Therefore, *in vitro* gastrointestinal digestion improved the accessibility of essential amino acids (Table 2). Even though chocolate is not eaten for its protein content, *in vitro* digestion caused a 5.0-fold increase in essential amino acids (Table 2).

The scores of all essential free amino acids for chocolate before *in vitro* digestion were below 1, which is the reference protein pattern (FAO/WHO, 2013). At the end of the *in vitro* digestion, the score was above 1 for histidine, isoleucine, methionine + cysteine, phenylalanine + tyrosine and, especially for tryptophan, which had the highest score, 4.74 (Table 3). However, the score remained below 1 for leucine, lysine, threonine and valine. Lysine was the first limiting amino found in the chocolate.

Table 3
Levels of essential amino acids in 70% cocoa dark chocolate before and after simulation of *in vitro* digestion in relation to scoring pattern from amino acid requirements (mg/g protein) for adults.

Essential amino acids	Scoring pattern (FAO/WHO, 2013)	Amino acid levels (mg/g chocolate protein)			
		Before *in vitro* digestion	Score	After *in vitro* digestion	Score
Histidine	15	5.59	0.37	28.74	1.92
Isoleucine	30	2.98	0.10	74.30	2.48
Leucine	59	7.89	0.13	40.87	0.69
Lysine	45	4.12	0.09	20.74	0.46
Methionine + Cysteine	22	9.15	0.42	42,66	1.94
Phenylalanine + Tyrosine	38	28,03	0.74	136,96	3.60
Threonine	23	9.53	0.41	18.63	0.81
Tryptophan	6	1.51	0.25	28.44	4.74
Valine	39	12.78	0.33	24.67	0.63

Scoring pattern mg/g protein requirement for adults (> 18 years old).
Amount of essential amino acids present in 11.9 g of chocolate. Total protein content (8.4 g/100 g) on the chocolate label was considered for calculation of the chocolate portion. The score was calculated according to FAO/WHO (2013).
Underlined values represent the first limiting amino acid of the sample.

3.5. Functional properties associated with bioactive amines and amino acids in chocolate after in vitro digestion

It is well known that the presence and levels of certain bioactive amines in the diet is relevant due to their health promoting properties. Chocolate contained several health promoting amines and *in vitro* gastrointestinal digestion improved the bioaccessibility of some amines in the intestinal epithelium (Table 1). In fact, there were 2.3- and 2.9-fold increases in the polyamines spermine and spermidine, respectively (Fernández-Reina et al., 2018), which can regulate the immune response and apoptosis, stabilize DNA, RNA, protein synthesis, membrane lipids and nucleic acids through their antioxidant activity (Muñoz-Esparza et al., 2019) and prevent cardiovascular disease (Ramos-Molina et al., 2019). In addition, the accessibility of these amines in the intestine are relevant due to their role in the integrity of intestinal epithelial cells (Fernández-Reina et al., 2018).

Increases in 2-phenylethylamine (~3-fold) were also observed. The presence of phenylethylamine is interesting due to its mood modulation activity, cognitive functions and association with the aphrodisiac properties of chocolate (Yılmaz & Gökmen, 2020). However, at high concentrations, phenylethylamine can lead to hypertension and it can trigger migraine attacks especially in sensitive individuals (EFSA, 2011).

Tryptamine increased 9-fold after the *in vitro* digestion. Tryptamine is also related to mood modulation, and it is considered as a neuromodulator or neurotransmitter, and it has noticeable antioxidant properties, essential to cell protection from free radical damages (Bentz et al., 2018; Yılmaz & Gökmen, 2020).

Tryptophan is important for the adequate development of the human body, and diets rich in tryptophan improve learning and memory in Alzheimer's disease and serotonin formation in the brain (Maitre et al., 2020). This research identified a 18.9-fold tryptophan increase after *in vitro* digestion. Free GABA increased 2.8-fold after *in vitro* digestion. This increase is beneficial as GABA has been associated with several health promoting activities such: anti-hypertension, anti-diabetes, anti-cancer, antioxidant, anti-inflammation, hepato-protection, reno-protection and intestinal protection (Ngo & Vo, 2019).

However, there were significant increases in tyramine, and histamine, which was not detected in the chocolate, was released after intestinal digestion. Histamine and tyramine, at high levels, can cause adverse effects to human health – hypotension, nausea, migraine, abdominal pain and heart problems (EFSA, 2011); and headache, increased blood pressure and hypertensive crisis (Yılmaz & Gökmen, 2020), respectively. NOAEL for histamine is 50 mg per person per meal for healthy individuals, which would be hard to reach, even by eating large amounts of chocolate. However, individuals with histamine intolerance should avoid it (EFSA, 2011). With respect to tyramine, the consumption of 100 g chocolate could result in hypertensive crisis for individuals taking MAOI drugs, both classical and third generation (EFSA, 2011).

These recent findings reinforce the importance of undertaking further studies on amino acid bioaccessibility, considering the additional conversion into bioactive amines by the gut microbiota, and the bioaccessibility, metabolism and absorption of amines (Fernández-Reina et al., 2018). The findings can also bring some insights to the better understanding of some negative and functional effects associated with the consumption of certain kinds of food, such as chocolate. Nowaczewska et al. (2020) reported the availability of several studies, in which chocolate was reported to be a triggering factor in migraineurs. However, all of them had insufficient number of participants." These authors mentioned that serotonin and its precursor tryptophan can possibly be migraine triggers; but no information was provided about tryptamine and histamine as a possible migraine trigger".

4. Conclusion

Dark chocolate (70% cocoa) was a good source of bioactive amines, mainly polyamines followed by phenylethylamine, cadaverine, putrescine, tyramine, and tryptamine. *In vitro* digestion increased accessibility of polyamines (pepsin), histamine and cadaverine (pancreatin). However, during control digestions (without enzymes) there were increases in cadaverine and putrescine (oral phase), phenylethylamine and tyramine (gastric phase), and putrescine and tryptamine (intestinal phase). Histamine, which was not detected in chocolate, was found at the end of digestion. The contents of amino acids increased during *in vitro* digestion, mainly in the gastric and intestinal phases. Digestion improved the scoring pattern of all essential amino acids, reaching values above 1 for tryptophan, phenylalanine + tyrosine, isoleucine, methionine + cysteine and histidine. This research brought better understanding of the bioaccessibility of bioactive amines, amino acids, GABA and ammonia in dark chocolate. An *in vivo*, or even an *in vitro* simulation of the gut microbiota action from different health conditions can help identify the main changes on biogenic amines through the respective amino acid precursors.

CRediT authorship contribution statement

Bruno M. Dala-Paula: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. **Valterney L. Deus:** Methodology, Investigation. **Olga L. Tavano:** Conceptualization, Methodology, Writing - review & editing. **Maria Beatriz A. Gloria:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Supervision, Project administration.

Declaration of Competing 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.

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(Brasília, DF, Brazil).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128397.

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