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IN VITRO BIOACCESSIBILITY OF AMINO ACIDS IN DARK CHOCOLATE

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RESUMO – O chocolate é uma fonte importante de aminoácidos livres e ácido gama-aminobutírico (GABA) que desempenham papéis importantes para a saúde humana. Considerando a escassez de informações sobre a bioacessibilidade desses compostos em chocolate amargo, o objetivo deste estudo foi caracterizar seus perfis e bioacessibilidade em chocolate amargo (70% de cacau), por meio de uma simulação *in vitro* das digestões oral, gástrica e intestinal. Todos os aminoácidos livres e o GABA foram detectados e seus níveis aumentaram durante a digestão *in vitro* devido às atividades das enzimas digestivas—pepsina e pancreatina. A proteína do chocolate amargo mostrou ser uma boa fonte de alguns aminoácidos essenciais, como triptofano, soma de fenilalanina e tirosina, isoleucina, histidina, mas limitante para lisina, leucina e treonina.

ABSTRACT – Chocolate is an important source of free amino acids and gama aminobutyric acid (GABA) which play important roles for human health. Considering the scarcity of information on the bioaccessibility of these compounds from dark chocolate, the objective of this study was to characterize their profiles and bioaccessibility in 70% cocoa dark chocolate through an *in vitro* simulation of oral, gastric and intestinal digestions. All free amino acids and GABA were detected, and their levels increased during *in vitro* digestion due to digestive enzymes activities—pepsin and pancreatin. Dark chocolate protein showed to be a good source of some essential amino acids, like tryptophan, sum of phenylalanine and tyrosine, isoleucine, histidine, but limiting for lysine, leucine and threonine.

PALAVRAS-CHAVE: bioaccessibility; aminoácidos livres; triptofano; chocolate; GABA.

KEYWORDS: bioaccessibility; free amino acids; tryptophan; chocolate; GABA.

1. INTRODUÇÃO

The global chocolate consumption is still growing directed by the consumer demands for healthy products, single origin, and unique organoleptic properties. 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, et al., 2016), hydroxycinnamic acids, methylxanthines, alkaloids, (Martini et al., 2018), bioactive amines (Do Carmo Brito et al., 2017), with less scientific reports but not less important, the amino acids (Żyżelewicz et al., 2018).

Free amino acids are responsible for many physiological functions associated to human health. Dietary tryptophan supplementation was reported to induce beneficial effects in Alzheimer's disease in mice (Maitre et al., 2020) and inflammation-mediated tryptophan catabolism has been related to the development of anemia, fatigue and depression in cancer patients (Lanser et al., 2020). There is recent evidence that L-arginine supplementation prevent chronic stress-induced depression (Dong et al2020). On the other hand, phenylalanine intake must be avoided by individuals diagnosticated with phenylketonuria (Almeida 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 was enriched with GABA (280 mg/100 g) from glutamic acid by natural fermentation using Lactobacillus hilgardii K-3 to enhance functional properties (Nakamura et al., 2009).

However, during the digestive process, changes can happen on the profile and levels of free amino acids, thereby affecting taste perception (Kongor et al., 2016; Rotola-Pukkila, Yang & Hopia, 2019) and the availability of these compounds for intestinal absorption. 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 of chocolate as a functional product and the scarcity of information on the bioaccessibility of amino acids from chocolate, the objective of this study was to characterize the profile and levels of free amino acid in dark chocolate and to investigate, for the first time, the *in vitro* bioaccessibility of these compounds, GABA, and ammonia in 70% cocoa dark chocolate, in order to better understand the accessibility of these compounds. Chemical extraction of free amino acids in dark chocolate with 5% trichloroacetic acid was also compared to *in vitro* simulated oral digestion in order to evaluate its application in research of active taste amino acid active in chocolate.

2. MATERIAL AND METHODS

Chocolate (70% cocoa dark chocolate) was produced commercially at a farm from Bahia (Brazil), which is responsible for the whole process including cocoa production and chocolate making. 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), 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 UPLC 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 Gültekin-Özgüven et al. (2016) with a few modifications. The protocol simulated oral, oral+gastric, and oral+gastric+intestinal phases of the digestion process.

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 of α-amylase (30 units/mg)] in a centrifuge tube. The mixture was homogenized and shaken (45 rpm) in an incubator at 37 °C for 5 min. The resulting oral phase extract 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 homogenized and shaken (45 rpm) in an incubator at 37 °C for 2 h. Finally, the oral+gastric phase extract was adjusted to pH 6.5 by adding 900 µL saturated NaHCO3 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), were added. The intestinal digestion mixture was homogenized and shaken (45 rpm) in an incubator at 37 °C for 2 h.

After the conclusion of each phase, the enzymatic reactions were stopped by means of pH (oral phase – pH 2; gastric phase – pH 6.5) and temperature (0 ºC) changes. In addition, the extract was centrifuged at 7,000 g at 4 °C for 10 min (MOD 280R, FANEN Excelsa 4, Sao 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.

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

The amino acids, GABA and ammonia were extracted from the chocolate by three successive extractions of 5 g ground chocolate with 7 mL 5% 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 (Reis et al., 2020). No extraction was needed for the fractions which resulted from the *in vitro* digestions.

The internal standard norvaline (25 pmol in column) was added to the extract, and the volume was brought up in a 25-mL volumetric flask. The extract was centrifuged at 16,000 g at 4 °C/10 min, neutralized with an equal volume of 1 M NaOH and derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl – AQC (Reis et al., 2020).

The levels of bioactive amines were determined using a Waters AcquityTM 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 x 2.1 mm, 1.7 µm, Acquity UPLCTM) 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 bioactive amines were calculated by interpolation in external analytical curves ($R2 \geq 0.996$) and the recovery of the internal standard was also used in the calculations. The results were expressed in mg/100 g of 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).

3. RESULTS AND DISCUSSION

All free amino acids investigated were detected; and serine+aspartic acid and glutamic acid+glutamine were quantified together without affecting negatively the investigation. The total levels of free amino acids were 1,282.31 mg/100 g (Table 1). 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 with less than 4% of the total levels. Free tryptophan levels were similar to 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 et al., 2011).

Table 1 - Levels of free amino acids (mg/100 g) in dark chocolate (70% cocoa mass) before and after *in vitro* digestion assay.

Analyte	Free amino acids (mg/100 g)			
	Chocolate	Oral	Gastric	Intestinal
Alanine[2]	104.46±1.90^{b}	151.05±9.10^{b}	145.94±5.73^{b}	243.07±29.71^{a}
Arginine[3]	86.83±1.13^{d}	127.49±7.66^{cd}	182.56±1.59^{b}	305.75±7.23^{a}
Asparagine	115.60±7.00^{b}	125.75±7.51^{b}	256.84±15.08^{a}	313.08±27.61^{a}
Cysteine	48.83±0.71^{b}	54.07±3.50^{c}	107.40±6.36^{b}	157.20±11.21^{a}
Glutamic acid[1] + Glutamine	8.36±1.13^{c}	11.42±0.69^{c}	58.69±2.84^{b}	231.39±23.70^{a}
Glycine[2]	95.46±0.76^{bc}	100.14±5.97^{bc}	177.74±3.74^{ab}	225.71±5.39^{a}
Histidine[3]	46.97±0.42^{b}	47.27±2.85^{b}	72.74±7.21^{b}	241.41±31.63^{a}
Isoleucine[3]	25.06±5.66^{c}	28.63±6.44^{c}	119.80±3.87^{b}	624.14±38.87^{a}
Leucine[3]	66.26±5.48^{b}	76.62±4.62^{b}	321.76±3.02^{a}	343.34±4.53^{a}
Lysine[4]	34.58±1.49^{c}	33.92±0.87^{c}	63.46±2.35^{b}	174.25±10.47^{a}
Methionine[3]	28.05±3.39^{b}	30.70±1.83^{b}	210.11±46.63^{a}	201.12±2.04^{a}
Ornithine	5.03±0.89^{c}	6.16±0.39^{c}	65.05±0.33^{b}	236.96±6.42^{a}
Phenylalanine[3]	29.60±1.75^{c}	33.44±2.02^{c}	431.77±29.54^{b}	861.73±30.00^{a}
Proline	30.69±1.48^{c}	30.59±1.85^{c}	83.53±4.85^{b}	483.06±15.69^{a}
Serine[2] + Aspartic acid[1]	130.55±1.41^{b}	137.80±8.27^{b}	313.77±7.63^{a}	361.76±17.33^{a}
Threonine[2]	80.08±5.75^{c}	83.72±5.04^{c}	125.49±11.64^{ab}	156.49±2.75^{a}
Tryptophan[3]	12.67±2.19^{c}	17.83±1.07^{c}	163.09±28.65^{b}	238.87±6.90^{a}
Tyrosine[4]	205.90±5.90^{c}	225.38±13.55^{bc}	274.47±14.42^{ab}	288.77±1.91^{a}
Valine[3]	107.33±5.02^{b}	123.72±11.11^{b}	207.26±7.54^{a}	207.78±12.50^{a}
Total of essencial AA	685.33±7.1^{c}	755.30±9.1^{c}	2,097.4±46.2^{b}	3,495.1±79.0^{a}
Total	1,282.3±53.60^{c}	1,445.7±50.5^{c}	3,431.8±64.6^{b}	5,895.9±105.2^{a}
GABA	16.10±0.18^{c}	16.46±0.99^{c}	33.26±3.67^{b}	45.42±5.54^{a}
NH_3	135.54±7.09^{c}	148.90±8.96^{bc}	225.46±22.04^{ab}	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 the equal minuscule letter in the same line have not significantly difference (Tukey test, $p \leq 0.05$). AA: amino acids; C- control test without addition of digestive enzymes.

Free amino acids in chocolate can be inherent to unfermented cocoa, affected by species, soil, climatic, geographical origins (Delgado-Ospina et a., 2020). But they can also be affected by fermentation and roasting. (Marseglia et al., 2014). GABA, detected at 16.10 mg/100 g, was present in fermented and dried cocoa beans from different geographical origins (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). It can be due to the degradation of cocoa free amino acids during fermentation or even from the alkalization of cocoa beans during processing to provide desirable flavors (Alasti et al., 2019).

The total levels of amino acids in chocolate (1,282.3 mg/100 g) increased significantly after oral+gastric (2.7-fold) and also after oral+grastric+intestinal digestions (4.6-fold) reaching levels of 5,915.77 mg/100 g, representing a 1.7-fold increase from the gastric to the intestinal phase. The oral phase did not affect total levels

of amino acids (Table 1). In addition, the control treatments did not affect amino acids. Therefore, the release of amino acids was mainly due to enzymatic activity, both pepsin and pancreatin.

The amino acids found at lower levels at the end of the *in vitro* digestion were threonine, lysine, cysteine, methionine, valine and the non-protein amino acid GABA (Table 1). GABA levels in chocolate before and after the oral phase were similar, however, GABA levels 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 the digestion or even from the hydrolysis or breakdown of its conjugates.

The profile and levels of free amino acids are especially important to chocolate taste perception in the mouth (Rotola-Pukkila et al., 2019). At the end of the oral phase, there was a balance of the levels of sweet (alanine, glycine, serine, threonine) and bitter (arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan and valine) amino acid, around 447 and 468 mg/100 g, respectively. The levels of umami amino acids (aspartic and glutamic acids) found in the analyzed chocolate was around 3-fold lower than sweet and bitter ones, corresponding to approximately 149 mg/100 g (Table 1). Due to the relevance of taste perception on chocolate, this is an area that deserves further studies. In addition, considering the similarity among the free amino acids levels detected and quantified after 5% TCA extraction of dark chocolate and after its *in vitro* oral digestion (Table 1), the chemical extraction could be an adequate method to be performed in future studies.

4. Conclusion

All free amino acids analyzed were detected in dark chocolate (70% cocoa mass). The levels of amino acids increased over the *in vitro* digestion phases, mainly the gastric and intestinal phases. After the *in vitro* intestinal digestion, total amino acids levels increased 4.6-fold and total essential ones, 5.1-fold. Tyrosine was the predominant free amino acid in dark chocolate before digestion but after the intestinal digestion, phenylalanine was the predominant one. Chemical extraction in dark chocolate with 5% TCA showed be adequate to quantify the free amino acids released during oral digestion.

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