

27 A 29 DE OUTUBRO DE 2020



ON LINE

7º Simpósio de
Segurança Alimentar

Inovação com sustentabilidade

BIOACTIVE AMINES IN 70% COCOA DARK CHOCOLATE: WHAT YOU EAT AND WHAT YOU GET

B.M. Dala-Paula¹, V.L. Deus², O.L. Tavano¹, M.B.A. Glória^{2,3}

1- Faculdade de Nutrição – Universidade Federal de Alfenas (UNIFAL-MG) – CEP: 37130-001, Alfenas, MG – Brasil. Telefone: (35) 3701-9742 – e-mail: bruno.paula@unifal-mg.edu.br / olga.tavano@unifal-mg.edu.br

2- LBqA and CEDAFAR, Faculdade de Farmácia – Universidade Federal de Minas Gerais (UFMG) – CEP: 31270-901, Belo Horizonte, MG – Brasil. Telefone: (31) 3409-6830 – e-mail: neideus@hotmail.com

3- Departamento de Ciências do Consumo – Universidade Federal Rural de Pernambuco (UFRPE) – CEP 52171-900, Recife, PE – Brasil. Telefone: (81) 99281-6242 – email: mbeatriz.gloria@gmail.com

RESUMO – O chocolate é uma fonte importante de poliaminas livres e aminas biogênicas 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. Dentre as dez aminas investigadas, sete foram detectadas, as poliaminas predominaram no chocolate antes da digestão *in vitro*, enquanto a tiramina e o cadaverina após a digestão. Agmatina, histamina e serotonina estavam ausentes no chocolate antes e após todas as fases da digestão, com exceção da histamina encontrada após a digestão intestinal. A formação de histamina após essa fase pode causar efeitos adversos à saúde em indivíduos sensíveis à histamina ou em uso de medicamentos inibidores da monoamino-oxidase clássicos. Todas as aminas do chocolate amargo apresentaram alta bioacessibilidade, com leve influência das enzimas digestivas.

ABSTRACT – Chocolate is an important source of free polyamines and biogenic amines 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. Out of ten investigated amines, seven were detected and polyamines were the predominant in chocolate before the *in vitro* digestion, while tyramine and cadaverine after digestion. Agmatine, histamine and serotonin were absent in chocolate before digestion and after all digestion phases, with exception for histamine which was found after intestinal phase. The formation of histamine after intestinal digestion can cause adverse health effects in histamine sensitive individuals or who is taking classical monoaminooxidase inhibitor drugs. All amines from dark chocolate showed high bioaccessibility with slight influence of the digestive enzymes.

PALAVRAS-CHAVE: bioacessibilidade; aminas biogênicas; poliaminas; espermidina; tiramina.

KEYWORDS: bioaccessibility; biogenic amines; polyamines; spermidine; tyramine.

1. INTRODUÇÃO

Chocolate is a food product consumed worldwide by different population groups due to its desirable sensory characteristics. The global chocolate consumption is still growing directed by the consumer demands for healthy products, single origin, and unique organoleptic properties (Muñoz et al., 2020). 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), amino acids and, with less scientific reports but not less important, the bioactive amines (Restuccia et al., 2016; Do Carmo Brito et al., 2017).

REALIZAÇÃO



ORGANIZAÇÃO



www.officeeventos.com.br



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. These polycationic molecules accumulate in highly proliferative tissues, being responsible for the maintenance, turnover and integrity of intestinal epithelial cells (Muñoz-Esparza et al., 2019).

Biogenic amines play important roles on 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 (Yilmaz & 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 et al., 2016). This catecholamine releasing agent, is a stimulator of the hypothalamus, inducing pleasurable sensations, enhancing mood lifting and sexual drive (Yilmaz & Gökmen, 2020). Tryptamine is a neurotransmitter related to the amino acid tryptophan. It also has antioxidant properties due its noticeable scavenging activity toward radicals, mainly related to the nitrogen atom of the indole ring (Bentz et al., 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 et al., 2019). Histamine is also a neurotransmitter and vasodilating amine (EFSA, 2011). However, the intake of foods containing high levels of histamine and tyramine can cause adverse effects to human health. Tyramine can cause hypertension, headache, pupil and palpebral tissue dilatation, and increased blood pressure (Barbieri et al., 2019). No 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. Histamine at high concentrations may lead to hypotension, nausea, migraine, abdominal pain and heart problems. 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), reinforcing attention for food consumption, source of histamine.

Considering the increased interest of chocolate as a functional product and the scarcity of information on the bioaccessibility of bioactive amines from chocolate, the objective of this study was to characterize the profile and levels of free bioactive amines in dark chocolate and to investigate, for the first time, the *in vitro* bioaccessibility of these compounds in 70% cocoa dark chocolate, in order to better understand their bioaccessibility.

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), bioactive amines standards (spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine, serotonin hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). AccQ.Fluor™ 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 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% CaCl₂, 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 °C) 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 bioactive amines by UPLC

The bioactive amines were extracted from the chocolate by three successive extractions of 5 g ground chocolate 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 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 Acquity™ UPLC system (Waters, Milford, MA, USA) equipped with an Acquity™ 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 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 bioactive amines were calculated by interpolation in external analytical curves ($R^2 \geq 0.996$) 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).

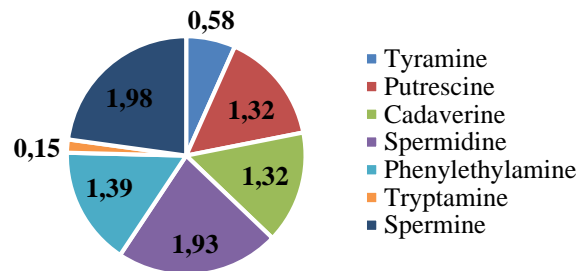
3. RESULTS AND DISCUSSION

Among the ten amines investigated, seven were present – cadaverine, 2-phenylethylamine, putrescine, spermine, spermidine, tryptamine, and tyramine at total levels of 8.67 mg/100 g (Figure 1). Agmatine, histamine and serotonin were not detected. There was predominance of the polyamines—spermine and spermidine (22% of total levels, 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 levels (1.04 to 6.5 mg/100 g) in five commercial samples of dark 70% cocoa chocolate. Histamine and serotonin were not detected in our sample, but their presence in dark chocolate has been reported in the literature (Baranowska & Plonka, 2015; Restuccia et al., 2016).

Some amines are inherent to unfermented cocoa (spermidine, spermine, serotonin, tyramine, putrescine, and tryptamine) (do Carmo Brito et al., 2017; Delgado-Ospina et al., 2020); but amines can change during fermentation, e.g. there can be decreases on total, spermidine, tryptamine, tyramine and serotonin levels, and the production and accumulation of 2-phenylethylamine (Delgado-Ospina et al., 2020).

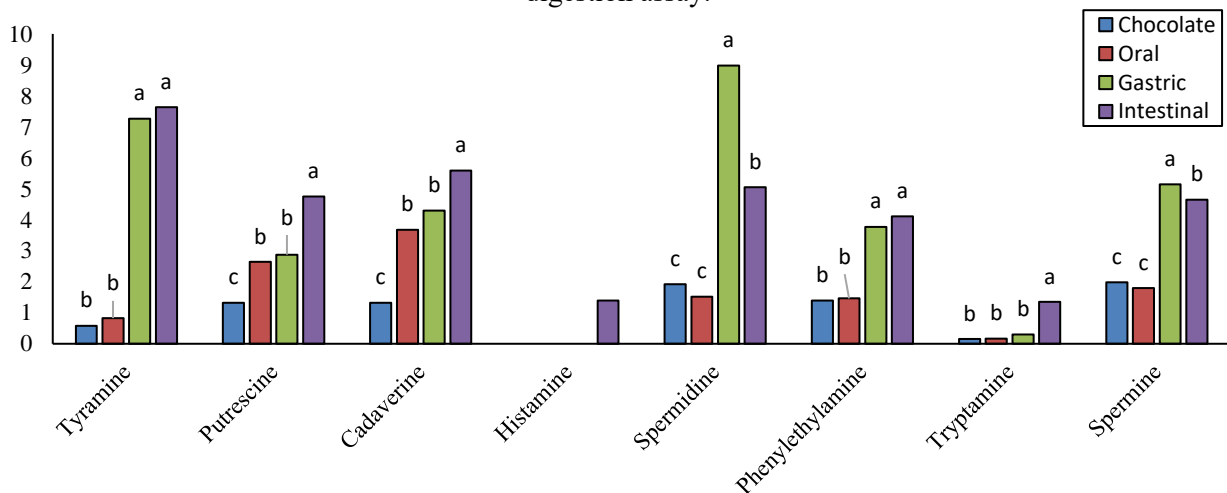
Figure 1. Levels (mg/100 g) of bioactive amines in dark chocolate (70% cocoa mass).



As indicated in Figure 2, total levels of amines increased significantly during *in vitro* gastric digestion (oral+gastric) and remained stable under gastrointestinal conditions (oral+gastric+intestinal), a 4-fold increase. The oral and intestinal phases did not affect total amines ($p>0.05$). The increase on total amines levels during gastric digestion, suggest that the low pH (2.0) and pepsin were responsible for the increased levels of amines. According to Casal et al. (2004), low pH values can breakdown conjugated forms of total amines from proteins and phenolic compounds. When considering the individual amines, the same amines were present throughout *in vitro* bioaccessibility; except for histamine, which was not detected in chocolate before the *in vitro* digestion but was found after the intestinal phase. Agmatine and serotonin were not detected in any *in vitro* simulation digestion in the dark chocolate sample.

Based on these results, the amounts of free bioactive amines available from dark chocolate after *in vitro* digestion were higher than before. The highest increases compared to the original chocolate were observed for tyramine (13.2-fold), followed by tryptamine (9-fold), cadaverine (4.2-fold), putrescine (3.6-fold), phenylethylamine and 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.

Figure 2. Levels (mg/100 g) of bioactive amines in dark chocolate (70% cocoa mass) before and after *in vitro* digestion assay.



This result can be associated with the food matrix, which contributed to the gradual release of amines during *in vitro* simulated 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). Spermine and spermidine were negatively affected by pepsin while cadaverine and histamine were positively affected by pancreatin. In fact, Moreau & Hicks (2004) demonstrated the capacity of pancreatin and cholesterol esterase to hydrolyze putrescine conjugates—diferuloylputrescine and p-coumaroyl-feruloylputrescine—in an *in vitro* model. It is important to consider that in the presence of the intestinal microbiota, there could be an increase in bioactive amines due to microbial free amino acid decarboxylation (Fernández-Reina et al., 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 enzymes in the chocolate matrix, respectively. These enzymes 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 *Enterococcus faecium*, *Lactobacillus casei*, *Leuconostoc mesenteroides*; whereas ornithine decarboxylase or agmatine deiminase can be synthesized by the *Enterococcus faecium*, *E. durans*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* which (Barbieri et al., 2020), which were identified in cocoa beans fermentation (Ouattara et al., 2017).

4. Conclusion

Dark chocolate was a good source of bioactive amines, mainly polyamines followed by phenylethylamine, cadaverine, putrescine, tyramine, and tryptamine. All amines increased significantly ($p < 0.05$) after *in vitro* digestion. Tyramine, cadaverine and spermidine were the predominant ones. Histamine, which was not detected in chocolate, was found at the end of digestion. The bioactive amines showed be relatively stable to *in vitro* simulated gastrointestinal digestion.

The polyamines, phenylethylamine, and tryptamine released from the consumption of a portion of dark chocolate can be related to functional health properties. In other hand, the consumption of approximately 100 g of dark chocolate in a same meal may cause negative health effects to histamine and tyramine sensitive individuals. An *in vivo*, or even an *in vitro* simulation of the gut microbiota action from different health conditions could help to identify the main changes of the biogenic amines through them respective amino acid precursors.

5. REFERÊNCIAS BIBLIOGRÁFICAS

- Baranowska, I., & Plonka, J. (2015). Simultaneous determination of biogenic amines and methylxanthines in foodstuff—sample preparation with HPLC-DAD-FL analysis. *Food Anal. Methods*, 8, 963–972.
- Barbieri, F., Montanari, C., Gardini, F., & Tabanelli, G. (2019). Biogenic amine production by lactic acid bacteria: a review. *Foods*, 8(17). <http://doi:10.3390/foods8010017>.
- Bentz, E.N., Lobayan, R.M., Martínez, H., Redondo, P., & Largo, A. (2018). Intrinsic antioxidant potential of the aminoindole structure: a computational kinetics study of tryptamine. *The Journal of Physical Chemistry*, 122, 6386–6395.
- Do Carmo Brito, B.N., Chisté, R.C., Pena, R.S., Gloria, M.B.A., & Lopes, A.S. (2017). Bioactive amines and phenolic compounds in cocoa beans are affected by fermentation. *Food Chemistry*, 228, 484–490.
- Casal, S., Mendes, E., Alves, M.R., Alves, R.C., Oliveira, M.B.P.P., & Ferreira, M.A. (2004). Free and conjugated biogenic amines in green and roasted coffee beans. *Journal of Agricultural and Food Chemistry*, 52, 6188–6192.
- Delgado-Ospina, J., Di Mattia, C.D., Paparella, A., Mastrocola, D., Martuscelli, M., & Chaves-Lopez, C. (2020). Effect of fermentation, drying and roasting on biogenic amines and other biocompounds in Colombian Criollo cocoa beans and shells. *Foods*, 9, 520.
- European Food Safety Authority - EFSA (2011). Scientific Opinion on risk-based control of biogenic amine formation in fermented foods. EFSA, panel on biological hazards (BIOHAZ). *EFSA J.* 9, 2393. <https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2011.2393>.
- Fernández-Reina, A., Urdiales, J.L., & Sánchez-Jiménez, F. (2018). What we know and what we need to know about aromatic and cationic biogenic amines in the gastrointestinal tract. *Foods*, 7(145). <http://doi:10.3390/foods7090145>.
- Gültekin-Özgülven, M., Berktaş, I., & Özçelik, B (2016). Change in stability of procyanidins, antioxidant capacity and *in vitro* bioaccessibility during processing of cocoa powder from cocoa beans. *LWT – Food Science and Technology*, 72, 559–565.

27 A 29 DE OUTUBRO DE 2020



ON LINE

7º Simpósio de
Segurança Alimentar

Inovação com sustentabilidade

- Martini, S., Conte, A., & Tagliazucchi, D. (2018). Bioaccessibility, bioactivity and cell metabolism of dark chocolate phenolic compounds after in vitro gastro-intestinal digestion. *Journal of Functional Foods*, 49, 424–436.
- Moreau, R.A. & Hicks, K.B. (2004). The in vitro hydrolysis of phytosterol conjugates in food matrices by mammalian digestive enzymes. *Lipids*, 39(8), 769–776
- Muñoz-Esparza, N.C., Latorre-Moratalla, M.K., Comas-Basté, O., Toro-Funes, N., Veciana-Nogués, M.T., & Vidal-Carou, M. (2019). Polyamines in food. *Frontiers in Nutrition*, 6, 108. <https://doi.org/10.3389/fnut.2019.00108>.
- Ouattara, H.D.; Ouattara, H.G., Droux, M., Reverchon, S., Nasser, W., & Niamke, S.L. (2017). Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: species diversity and citrate lyase production. *International Journal of Food Microbiology*, 256, 11–19.
- Pastel, A. Thompson, A., Abdelmalek, L., Adams-Huet, B., & Jialal, I. (2019). The relationship between tyramine levels and inflammation in metabolic syndrome. *Hormone Molecular Biology and Clinical Investigation*, 6(40), 1–7. doi: 10.1515/hmbci-2019-0047.
- Restuccia, D.; Spizzirri, U.; Luca, M.; Parisi, O., & Picci, N. (2016). Biogenic Amines as Quality Marker in Organic and Fair-Trade Cocoa-Based Products. *Sustainability*, 8(9), 8561–12.
- Reis, G.C L., Guidi, L.R., Fernandes, C., Godoy, H.T., & Gloria, M.B.A. (2020). UPLC-UV method for the quantification of free amino acids, bioactive amines, and ammonia in fresh, cooked, and canned mushrooms. *Food Analytical Methods*, 13, 1613–1626.
- Tofalo, R., Perpetuini, G., Schirone, M., & Suzzi, G. (2016). Biogenic amines: toxicology and health effect. *Encyclopedia of Food and Health*, 424–429.
- Yilmaz, C. & Gökmen, V. (2020). Neuroactive compounds in foods: Occurrence, mechanism and potential health effects. *Food Research International*, 128, 1–23.

REALIZAÇÃO



ORGANIZAÇÃO



www.officeeventos.com.br