

How to Cite:

Prencipe, S. A., Maddaloni, L., Ruggieri, R., Vieri, S., & Vinci, G. (2022). Quality markers evaluation in chocolates with different cocoa content. *International Journal of Health Sciences*, 6(S6), 7762–7773. <https://doi.org/10.53730/ijhs.v6nS6.11071>

Quality markers evaluation in chocolates with different cocoa content

Sabrina A. Prencipe

Department of Management, Sapienza University of Rome – Via del Castro Laurenziano 9, 00181 (RM)

*Corresponding author email: sabrinaantonia.prencipe@uniroma1.it

Lucia Maddaloni

Department of Management, Sapienza University of Rome – Via del Castro Laurenziano 9, 00181 (RM)

Email: lucia.maddaloni@uniroma1.it

Roberto Ruggieri

Department of Management, Sapienza University of Rome – Via del Castro Laurenziano 9, 00181 (RM)

Email: roberto.ruggieri@uniroma1.it

Simone Vieri

Department of Management, Sapienza University of Rome – Via del Castro Laurenziano 9, 00181 (RM)

Email: simone.vieri@uniroma1.it

Giuliana Vinci

Department of Management, Sapienza University of Rome – Via del Castro Laurenziano 9, 00181 (RM)

Email: giuliana.vinci@uniroma1.it

Abstract--Quality assessment of cocoa-based food products (i.e., chocolate) reached increasing interest by scientific research, as it is rich in bioactive compounds (BCs) (i.e., catechins, methylxanthines, serotonin, etc.), which can have beneficial effects on human health (cardiovascular, immunomodulatory, mood regulator, etc.). Thus, the cocoa content may influence the quality of chocolate products. In this regard, the study aimed at a comprehensive evaluation of different BCs: biogenic amines (BAs), free fatty acids (FFAs), and antioxidant compounds (Total Polyphenols Content, TPC and Antioxidant Activity, AA), in chocolate samples with different cocoa contents (50%, 60%, 70%, 85%, 100%). By means of HPLC-UV/RF analysis, chocolates samples with higher cocoa content showed the highest concentration of serotonin, which represented the 25% of the total BAs

concentration. Considering the total FFAs, unsaturated fatty acids (linoleic, linolenic and oleic) accounted for 30 to 50% in all chocolate samples analyzed. Antioxidant assays showed a great variability within samples, mainly highlighting a gradual increase of TPC and AC as a function of higher cocoa content. The findings of this study provide useful insights into cocoa-based products, showing that cocoa content influences the BCs concentration, thus improving the nutritional characteristics and the quality of the final product.

Keywords---antioxidant compounds, biogenic amines, chocolate, fatty acids, quality markers.

Introduction

Chocolate is a cocoa-based product widely consumed worldwide, which is obtained by processing and transforming seeds of the *Theobroma cacao* L. Chocolate can be a very nutritional component in food and the knowledge of its various medicinal properties represents a stimulus to those involved with its production, processing and consumption. Quality and flavor of cocoa-based products are mainly influenced by the various stages of cocoa processing [1,2]. These processes generally consist of fermentation, drying, roasting, grinding of cocoa beans and manufacturing of cocoa product by mixing of different ingredients (cocoa butter, cocoa mass and powder, sugar and other optional ingredients). According to Directive 2000/36/EC, dark chocolate is defined on the minimum 43% of cocoa content, depending on the percentage of total dry cocoa solids [3].

In recent years, interest in the consumption of dark chocolates with higher cocoa content has increased as a result of a major interest in food quality and safety issues. Eating chocolate, as part of a healthful balanced diet, could potentially provide a beneficial way to improve wellbeing, thus exerting cardio- and neuro-protective effects, modulating inflammatory markers and immune responses, cognitive and mood elevation [4,5]. Furthermore, it also has psychoactive functions based on its composition [1,6]. Therefore, in order to increase the value of the final product, the evaluation of food quality, is highly advisable. In this regard, quality assessment can be carried out through the determination of molecular markers, typical for a sample, which can establish the origin of the sample or the good state of storage and preservation. [7].

Cocoa and its based products (i.e., chocolate) are considered rich in different bioactive compounds (BCs), such as serotonin [8], polyphenols [6], free fatty acids, alkaloids, methylxanthines, etc. [9,10]. Within these compounds, biogenic amines (BAs) are widely considered as food safety markers because of their presence in food and their effect on the human organism. BAs are produced by microbial enzymes that decarboxylate amino acids; however, their occurrence in food can also be related to spoilage and poor preservation. In addition, BAs can induce several physiological reactions, and investigating their content in foods can be a quality and safety index for consumer health and diet formulation [11]. Serotonin is a bioactive monoamine with a broad activity in human brain, playing

a crucial role in modulating mood, appetite as well in muscle contraction and blood pressure regulation [8]. At the same time, the consumption of food containing high concentrations of some biogenic amines (histamine, putrescine, cadaverine, etc.) can cause undesirable toxicological effects, that are similar to those of food poisoning (spasms, nausea, scombroid syndrome, allergic reactions, etc.), and, in extreme cases, cerebral hemorrhage, anaphylactic shock and death [12].

Cocoa beans and cocoa products consist of 12-18% (total dry weight, TDW) of antioxidant compounds (i.e., polyphenols), a broad class of organic compounds produced from the secondary metabolism of plants. They may have antioxidant, anti-inflammatory, antibacterial functions, also providing indications about oxidation and degradation status of food [6, 9]. Furthermore, the nutritional value of cocoa – based products could be also influenced by the composition of cocoa butter [1]. It represented about 45-53% of TDW, in cocoa beans, consisting mainly of saturated (palmitic and stearic acids), monounsaturated (oleic acid), and polyunsaturated (linoleic and linolenic) fatty acids, which were largely investigated in food science, for nutritional labelling, quality control, nutrition and health purposes [4].

However, the chocolate manufacturing processes and the content of cocoa solids may influence the amount of BCs in cocoa-derived products. In this study, a quality marker evaluation in dark chocolates as function of different cocoa content was proposed. The content of eight BAs was evaluated in dark chocolate samples with different cocoa content by means of high-performance liquid chromatography with fluorescence detection (HPLC-FD) and pre-column derivatization with dansyl-chloride. The BAs studied as polyamines were putrescine (PUT), and cadaverine (CAD), spermine (SPM) and spermidine (SPD); whereas, β -phenylethylamine (β -PEA), HIS, SER, and TYR were studied for monoamines. The content of six free fatty acids (FFAs), Myristic, Stearic and Palmitic for saturated FFAs, and Linolenic (ω -3), Linoleic (ω -6) and Oleic were studied for unsaturated FFAs. Thereafter, the evaluation of total polyphenols content (TPC) by means of Folin-Ciocalteu, and antioxidant activity (AA) by means of ABTS and DPPH assays was carried out through UV-Vis spectrophotometric analysis.

Materials and Methods

Chemicals

HIS, SER, SPM, SPD, PUT, β -PEA, CAD, and TYR and dansyl chloride were purchased from Supelco (Bellefonte, PA, USA). The six FFAs – Myristic, Stearic, Palmitic, Oleic, ω -3 and ω -6 acids, and the derivatizing agents, Br-acetophenone and triethylamine were supplied by Sigma-Aldrich (Milan, Italy). Methanol (CH₃OH), n-Hexane (C₆H₁₄), water and acetonitrile (ACN) for HPLC, Folin-Ciocalteu reagent (H₃[P(W₃O₁₀)₄]/H₃[P(Mo₃O₁₀)₄]), ABTS (diammonium salt), DPPH (2,2-diphenyl-1-picrylhydrazyl), potassium persulfate (K₂S₂O₈), sodium bicarbonate (NaHCO₃), gallic acid (C₇H₆O₅), perchloric acid 70% (HClO₄), sodium hydroxide (NaOH), sodium carbonate (Na₂CO₃), ammonium hydroxide (NH₄OH), and acetic acid (CH₃COOH) were supplied by Sigma-Aldrich (St. Louis,

MO, USA).

Instruments

The following instruments were used: Bandelin Sonorex RK100H ultrasonic thermostatic bath, G-Therm AG-System heater, and Whatman (PTFE) 0.45 μm 100 syringe filters (Sigma Aldrich, Milan, Italy), UV-Vis spectrophotometer (Jenway, Stone, UK), NEYA 10R refrigerate centrifuge (Exacta Optech, Modena, Italy). Chromatographic analysis was performed using an ATVP LC-10 HPV binary pump with an RF-10° XL fluorimetric (FD) detector (Shimadzu, Kyoto, Japan) operating to $\lambda_{\text{emission}}=320$ nm, and $\lambda_{\text{excitation}}=523$ nm. A Supelcosil LC-18 column (250 mm \times 4.6 mm, 5 μm) with a Supelguard LC-18 (Supelco, Bellefonte, PA, USA) pre-column were used for the determination of BAs. An SPD-10AVP UV detector (Shimadzu, Kyoto, Japan) operating to $\lambda = 254$ nm, and a Supelcosil LC-18 column (150 mm \times 4.6 mm, 5 μm) with a Supelguard LC-18 (Supelco, Bellefonte, PA, USA) pre-column were used to determine FFAs.

Sampling

Dark chocolate samples were with different cocoa content (50%, 60%, 70%, 85% and 100%) were purchased from local supermarkets. After acquisition, samples were homogenized by grinding and sifting by a sieve with a 0.7 \div 2mm diameters holes. The obtained particle size fraction was collected and stored at refrigerated temperature, $T= -18$ °C until the day of analysis.

BAs Determination in Dark Chocolate samples

The BAs determination was carried out according to a previously reported method with some modifications [13]. About 2.5g of chocolate sample was extracted with 7 mL of 0.6 M HClO_4 , homogenized for 3 min at 200 rpm with a magnetic stirrer, and centrifuged at $2900 \times g$ for 10 min, at $T= 4$ °C. The supernatant was collected in a flask. The residue was added with 7 mL of 0.6 M HClO_4 , mixed, and again centrifuged for 10 min. Then, the second extract was added to the first one, and filtered through a 0.45 μm membrane syringe filter. The final volume was adjusted to 20 mL with 0.6 M HClO_4 . An aliquot of 1 mL of the final extract was then derivatized by adding 200 μL of 2 M NaOH, 300 μL of saturated NaHCO_3 solution, and 2 mL of dansyl chloride solution (10 mg/mL in acetone). After shaking, the samples were left in the dark for 60 min at 45 °C. About 100 μL of NH_4OH was added to stop the derivatizing reaction. The final volume was adjusted to 5 mL by adding ACN. The dansylated extract was filtered using 0.45- μm filter (Whatman® Puradisc filters, Sigma Aldrich, Milan, Italy), injected into the HPLC system, and analyzed with a previous standardized method [13].

For the chromatographic determination of the BAs, a volume aliquot of 20 μL (loop 20 μL) was injected. Analyses were performed by using a Supelcosil LC-18 column (250 mm \times 4.6 mm, 5 μm), Supelco, Bellefonte, PA, USA) coupled with an FD detector ($\lambda_{\text{emission}}=320$ nm, and $\lambda_{\text{excitation}}=523$ nm). The analyses were carried out maintaining a fixed temperature of 30 °C. The solvents used for chromatographic elution were: (A) purified water and (B) ACN. The elution program started with 3 min of isocratic elution (50% A; 50% B) reaching 100% B

after 18 min and ending with another 3 min of isocratic elution. Finally, it also required 5 min to return to the initial isocratic conditions (50% A; 50% B). The flow rate was then kept constant at 1.2 mL/min, for a total analysis time of 30 min. The results were achieved by linear regression through calibration curve for each BAs ranging from 0.1 and 25 mg/L.

FFAs Determination in Dark chocolate samples

FFAs content in chocolate samples was determined according to Fratoddi et al., with some modifications [14]. Briefly, 4 ml of n-hexane was added to 0.1g – homogenized sample, sonicated for 5 min at room temperature and then centrifuged at $2900\times g$ for 10 min, at $T= 25\text{ }^{\circ}\text{C}$. The supernatant was collected in a flask. The residue was added with 2 mL of n-hexane, mixed, and again centrifuged for 10 min. Then, the second extract was added to the first one, and then dry-filled under nitrogen (N_2) flow. The dried state – organic extract was resuspended in 2 ml of n-hexane and filtered through $0.45\mu\text{m}$ PTFE syringe filters. An aliquot of 50 μL of the final extract was then derivatized by adding 50 μl of triethylamine solution (25 mg/ml in acetone), and 50 μl of bromo-acetophenone (20 mg/ml in acetone), in a glass tube. The closed tube was placed in an oven at $T=100^{\circ}\text{C}$ for 15 minutes. The sample was then cooled and 80 μl of a solution of glacial acetic acid (10 mg/mL in acetone) was added, and placed again in the heater at $T=100^{\circ}\text{C}$ for 15 minutes. Thereafter, the tube was cooled and the contents brought to dryness under nitrogen flow. The residue was recovered in 250 μl of an aqueous acetonitrile solution (ACN:H₂O, 70:30 *v/v*) and sonicated in the ultrasonic bath for 15 minutes. The blank was prepared by derivatizing 50 μl of n-hexane, the fatty acid extracting solvent.

For the chromatographic determination of the FFAs, a volume aliquot of 20 μL (loop 20 μL) was injected. Analyses were performed by using a Supelcosil LC-18 column (150 mm \times 4.6 mm, 5 μm), Supelco, Bellefonte, PA, USA) coupled with an UV detector ($\lambda= 254\text{ nm}$). The analyses were carried out maintaining a fixed $T= 43\text{ }^{\circ}\text{C}$. The solvents used for the chromatographic separation were: (A) water purified and (B) acetonitrile. The elution program started with 3 min of isocratic elution (70% A; 30% B) reaching 100% B after 28 min and ending with another 3 min of isocratic elution. Finally, it took 5 min to reinstate the initial isocratic conditions (70% A 30% B). The flow rate was maintained constant at 0.8 mL/min, for a total analysis time of 32 min. The final results were achieved by linear regression with calibration curves different for each fatty acid analyzed: Linolenic acid (0.002 – 0.04 mg/ml), Linoleic acid (0.012 – 0.4 mg/ml), Myristic acid (0.005 – 0.2 mg/ml), Palmitic acid (0.005 – 0.4 mg/ml), Oleic acid (0.08 – 1 mg/ml), Stearic acid (0.1 – 1 mg/ml).

Determination of TPC and AA in Dark chocolate samples

Sample extraction for TPC and antioxidant were prepared according to a previously published method with some modification [13]. After removing the organic fraction (*Section 2.5*), the residue was extracted with 5 ml of methanol in aqueous solution (60:40, *v:v*), homogenized in a ultrasonic bath for 5 min at room temperature, and centrifuged at $2900\times g$ for 10 min, at $T= 25\text{ }^{\circ}\text{C}$. The supernatant was collected in an amber vial. The extraction procedure of total polyphenols was

repeated twice. TPC was determined by the Folin-Ciocalteu method [14], modified for chocolate samples as follows: 1 mL of methanolic extract was added to 0.25 mL of Folin-Ciocalteu reagent and 0.5 mL of aqueous Na₂CO₃ solution (7.5%, w/v) in a 10 mL volumetric flask. The final volume was achieved with purified water. Spectrophotometric analysis was carried out at $\lambda = 750$ nm after 45 min of incubation in the dark at room temperature. The total polyphenols content was expressed as milligrams of gallic acid equivalent (mg GAE) per kg. The final results were obtained through a calibration curve ranging from 5 to 100 mg/l ($R^2 = 0.9998$). Antioxidant activity was determined by means of DPPH and ABTS assays, according to a previously reported method of Preti et al. [13]. The free radical scavenging activity DPPH and ABTS of the chocolate hydroalcoholic extracts was assessed by measuring the absorbance decrease at 515 nm (DPPH), and 734 nm (ABTS). Absorbance was measured in 1 cm path-length cuvettes against methanol in aqueous solution (60:40, v:v), by using a UV-Vis spectrophotometer (Jenway, Stone, UK). Results were expressed as inhibition rate and were calculated based on Equation:

$$I\% = (A_0 - A_f / A_0) \times 100$$

where A_0 is the radical cation's initial absorbance, and A_f is the absorbance after the addition of sample extract.

Results and Discussions

Biogenic Amines Content in Dark chocolate samples

In this study, contents of eight BAs were determined in dark chocolate (DC) samples as function of different cocoa content (50%, 60%, 70%, 85% and 100%). The quantification of BAs in chocolate samples was summarized in Table 1. Chocolates samples showed a great variability in BAs content. BAs detected in all samples at high concentrations were SER (15.8 – 340.98 mg/kg), β -PEA (17.62 – 68.52 mg/kg), SPD (0.36–75.92 mg/kg), and SPM (1.74–49.05 mg/kg), thus agreeing with results from literature [15]. DC with 60% of cocoa content presented the highest amount of PUT (8.45–15.36 \pm 0.02 mg/kg). HIS was present only in three samples (50%, 70% and 100%), within the limit established by European Regulation [16], thus reducing the potential risk to human health. An important highlight should be done about Serotonin, which was detected in all chocolate samples, thus representing the 25% of the total BAs concentration. Dark chocolate samples with higher cocoa content (100%) showed the highest concentration of SER (159.96 – 340.54 \pm 0.93 mg/kg). This could be related to the chemical composition of chocolate samples, thus explaining the plausible physiological mechanism between chocolate and mood.

Results from previous research reporting that many kinds of chocolate, rich in sugar and with low protein, can stimulate the synthesis of serotonin, affecting mood [8,17]. The most diverging data are related to dark chocolate with 60% of cocoa content, presenting the highest amount of CAD (24.42–25.28 \pm 0.1 mg/kg); other studies, for the same cocoa content chocolate samples, never contained levels of CAD higher than 5.3 mg/kg [15]. In this sense, it should be considered that CAD content, considered as a food spoilage maker, could be affected by many

parameters related to hygienic conditions, temperature, pH values of the raw materials or the manufacturing process, as well as the handling and storage conditions.

Fatty acid content in Dark chocolates

The content of six FFAs was investigated in dark chocolates with different cocoa content (50 ÷ 100%). Results are shown in Table 2. Values are expressed as % of FFAs on the % of total free fatty acids (TFFAs) analyzed in this study. Considering the total FFAs, Palmitic (10.9–31.9% ± 0.54 of TFFAs), and Stearic (10.3–40% of TFFAs) were the most abundant saturated FFAs in all tested chocolate samples; while Linolenic (n.d – 8.30% ± 0.52), Linoleic (1.97 – 11.40% ± 0.25), and Oleic (11.97 – 24.48% ± 0.31). The prevalence of C14:0 and C16:0 fatty acids common in almost foods including many oils and fats, as well as in cocoa-based products.

Table 1
Quantitative results of biogenic amines in chocolate samples (mg/kg) ± standard deviation

	Dark Chocolate (50%)	Dark Chocolate (60%)	Dark Chocolate (70%)	Dark Chocolate (85%)	Dark Chocolate (100%)
SER	41.91–89.39 ± 0.25	98.91–143.88 ± 0.34	15.80–127.03 ± 0.1	n.d.–203.18 ± 0.2	16.86–340.98 ± 0.18
TYR	5.37–13.10 ± 0.06	24.42–25.28 ± 0.1	3.15–20.16 ± 0.03	0.76–22.96 ± 0.02	2.60–38.01 ± 0.05
β-PEA	50.07–86.81 ± 0.18	68.52–109.1 ± 0.12	19.23–74.46 ± 0.07	17.62–97.52 ± 0.04	29.64–160.83 ± 0.25
PUT	4.35–7.14 ± 0.01	8.45–15.36 ± 0.02	2.59–7.21 ± 0.01	2.33–13.07 ± 0.02	3.17–23.4 ± 0.01
CAD	7.56–10.32 ± 0.03	16.18–22.16 ± 0.12	6.20–11.1 ± 0.01	5.24–26.31 ± 0.1	6.43–36.20 ± 0.03
HIS	n.d.–40.37 ± 0.11	n.d.	n.d.–91.84 ± 0.21	n.d.	n.d.–154.61 ± 0.07
SPD	11.29–15.33 ± 0.05	18.3–45.44 ± 0.11	0.65–26.16 ± 0.03	0.36–29.46 ± 0.04	0.36–75.92 ± 0.09
SPM	7.04–10.81 ± 0.06	14.71–26.22 ± 0.05	2.45–16.47 ± 0.02	1.74–17.11 ± 0.02	1.74–49.05 ± 0.08

SER: serotonin; TYR: tyramine; β-PEA: β-phenylethylamine; PUT: putrescine; CAD: cadaverine; HIS: histamine; SPD: spermidine; SPM: spermine; n.d.: not detectable

*The ranges obtained from triplicate analysis, referred to the average of the n= 3 samples of DC 50%; n= 3 samples of DC 60%; n= 3 samples of DC 70%; n= 3 samples of DC 85%; n= 3 samples of DC 100.

Considering the fatty acid profiles of chocolates, palmitic acid (C16:0, 3.37–20.13g/100 g), stearic acid (C18:0, 4.10–29.09 g/100 g) and oleic acid (C18:1, 4.10–29.09 g/100 g) were the most abundant [18], thus implying the dependence of the chocolate fatty acid profile on cocoa beans as a raw material. Cocoa beans are the main source of cocoa butter (45–53% of Total Dry Weight, TDW), consisting mainly of saturated (palmitic and stearic acids) and monounsaturated (oleic acid) fatty acids. Furthermore, the determination of the fatty acid profile could be considered as a quality marker for the technological characteristics and the desired nutritional characteristics of cocoa beans and their manufacturing process [19]. Myristic acid was only detected in 50%, 60% and 100% cocoa – dark chocolate (0.04 – 0.97% ± 0.09), thus representing the lowest FFA among the total of FFAs analyzed. This result was in agreement with previously published results of chocolate FFAs content, especially for dark chocolate samples [20]. For polyunsaturated fatty acids, (Linoleic, and Linolenic) accounting for 30% TDW in all chocolate samples analyzed, it is important to highlight, that an increased consumption can have beneficial effects on human health, thus exerting anti-inflammatory, antioxidant, anticancer and cholesterol lowering properties. Therefore, dark chocolate relatively rich in polyunsaturated fatty acids could be of interest for incorporating these bioactive lipids into novel foods designed to produce certain health-related benefits [18].

Total Polyphenols and Antioxidant Activity evaluation

Cocoa beans and cocoa derived-products consist of 12-18% TDW of antioxidant compounds (i.e., polyphenols), which are a broad class of organic compounds produced from the secondary metabolism of plants. In cocoa-derived products such as dark chocolate, polyphenols are widely studied for their beneficial effects (i.e., antioxidant, anti-inflammatory, antibacterial, cardiovascular, etc.) on human health [6]. The TPC assay was carried out to determine the total polyphenols content in the hydroalcoholic fraction [13]. The antioxidant activity was assessed by means of two different in vitro antiradical tests - ABTS and DPPH [13]. In addition, these two radicals have specific free radical scavenging abilities and reducing power that are sensitive to different types of antioxidant compounds occurring in plant food extracts. Consequently, the combining use of these two assays provided an effective assessment of antioxidant activity in chocolate samples. The results are shown in Figure 1.

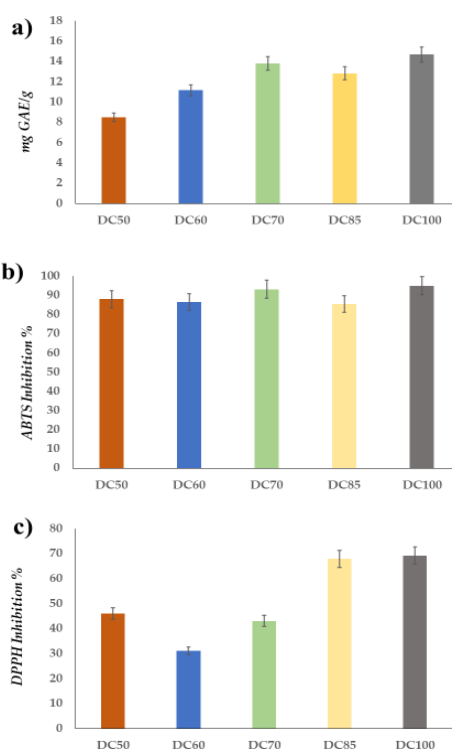


Figure 1. Evaluation of antioxidants in chocolate samples. Histograms of TPC (a) expressed in mg GAE/g \pm standard deviation; Antioxidant Activity by means of ABTS (b) and DPPH (c) expressed as Inhibition % \pm standard deviation. DC50: Dark Chocolate with 50% of cocoa content; DC60: Dark Chocolate with 60% of cocoa content; DC70: Dark Chocolate with 70% of cocoa content; DC85: Dark Chocolate with 85% of cocoa content; DC100: Dark Chocolate with 100% of cocoa content

Table 2
Quantitative results of free fatty acids in dark chocolates with different cocoa content. Values are expressed as % of FFAs on the % of total free fatty acids (TFFAs) analyzed \pm standard deviation

	Dark Chocolate (50%)	Dark Chocolate (60%)	Dark Chocolate (70%)	Dark Chocolate (85%)	Dark Chocolate (100%)
Linolenic (C18:3)	6.84–6.88 \pm 0.2	n.d.–5.27 \pm 0.13	n.d.–3.99 \pm 0.61	n.d.–8.30 \pm 0.56	n.d.–1.02 \pm 0.07
Linoleic (C18:2)	5.11–5.45 \pm 0.4	4.47–11.40 \pm 0.23	1.97–8.12 \pm 0.48	5.73–9.79 \pm 0.08	1.46–2.97 \pm 0.62
Myristic (C14:0)	n.d.–0.97 \pm 0.09	n.d.–0.33 \pm 0.08	n.d.	n.d.	0.04–0.49 \pm 0.17
Palmitic (C16:0)	10.84–21.15 \pm 0.5	12.49–31.90 \pm 0.54	18.43–29.87 \pm 0.22	22.98–27.36 \pm 0.15	14.68–26.88 \pm 0.39
Oleic (C18:1)	11.97–21.74 \pm 0.7	18.43–29.87 \pm 0.32	17.02–37.54 \pm 0.18	24.48–30.72 \pm 0.42	12.51–31.38 \pm 0.05
Stearic (C18:0)	15.65–19.03 \pm 0.4	10.38–18.60 \pm 0.41	10.38–18.60 \pm 0.27	15.53–20.19 \pm 0.34	22.44–39.99 \pm 0.54

*The ranges obtained from triplicate analysis, referred to the average of the $n=3$ samples of DC 50%; $n=3$ samples of DC 60%; $n=3$ samples of DC 70%; $n=3$ samples of DC 85%; $n=3$ samples of DC 100

The results obtained from triplicate analysis referred to the average of the $n=3$ samples of DC 50%; $n=3$ samples of DC 60%; $n=3$ samples of DC 70%; $n=3$ samples of DC 85%; $n=3$ samples of DC 100. For TPC (Fig. 1a), dark chocolate with the highest cocoa content was found to be rich in polyphenols, although each chocolate samples were different in polyphenol content. Dark Chocolate (DC) with 100% showed the highest TPC values (13.33 – 15.91 \pm 0.53 mg GAE/g), followed by DC with 70–85% and 50% of coca content (11.71 – 15.56 \pm 0.34 mg GAE/g, and 8.13 – 8.83 \pm 0.1 mg GAE/g respectively). However, differences between dark chocolates can be ascribed to geographical and plant factors such as soil type, cropping and fermentation conditions, climate and cultivar. As polyphenols are mainly concentrated in non-fat cocoa solids, chocolates with a higher cocoa content are considered to be a better source of these compounds, which have a greater antioxidant activity. [21]. The trend of TPC results agreed with ABTS radical scavenging assay (Fig. 1b) for antioxidant activity, thus providing a high radical inhibition % (I%) by the different cocoa content – chocolates.

The highest result was achieved by DC with 85% of cocoa content (99.20, I%). A similar result was reported by Godočiková et al., [22] who established that the highest antioxidant activity measured by ABTS assay was shown by the sample of chocolate with 90% of cocoa solids, positively correlating with the total polyphenol content. However, the DPPH assay results (Fig. 1c) were significantly lower than those obtained with ABTS assay, for all DC samples analyzed. The results obtained were in consonance with the result of Belščak et al., [23], where DPPH showed lower results than ABTS test, because of its reactivity only with lipophilic antioxidants. In addition, DPPH radical does not react with phenolic acids and, therefore, the antioxidant capacity determined by DPPH and ABTS methods are partially different.

Conclusions

The study investigated quality markers in chocolates as function of different cocoa content. By means of chromatographic analysis, chocolates samples showed a great variability in BAs content. Dark chocolate samples with higher cocoa content (100%) showed the highest concentration of BAs (159.96 – 340.54 \pm 0.93 mg/Kg), thereof SER amount (159.96 – 340.54 \pm 0.93 mg/kg) represented the

25% of the total BAs concentration. HIS was present only in three samples (50%, 70% and 100%), within the limit established by European Regulation. Considering the total FFAs: palmitic (10.9-31.9% Total of analyzed FFAs), and stearic (10.3-40% of TFFAs) were the most abundant in all chocolate samples; while unsaturated fatty acids (linoleic, linolenic and oleic) accounted for 30 to 50% in all chocolate samples analyzed. Therefore, dark chocolate relatively rich in polyunsaturated fatty acids could be of interest for incorporating these bioactive lipids into functional foods intended to exert certain health promoting benefits (anti-inflammatory, antioxidant, anti-cancer, etc.). Even though there were individual differences in the polyphenol content in dark chocolate samples, a good correlation between the antioxidant potency and the declared cacao content was observed, mainly highlighting a gradual increase of TPC and AC as a function of higher cocoa content. Therefore, the chosen bioactive compounds (BCs) resulted to be suitable markers for chocolate quality assessment, showing that cocoa content influences the BCs concentration, thus determining the quality and nutritional characteristics of the final products.

References

1. Afoakwa, E.O., Paterson, A., Fowler M (2007). Factors influencing rheological and textural qualities in chocolate - a review. *Trends in Food Science and Technology*, vol. 18, pp. 290- 298.
2. Badrie, N., Bekele, F., Sikora, E., & Sikora, M. (2015). Cocoa Agronomy, Quality, Nutritional, and Health Aspects. *Critical Reviews in Food Science and Nutrition*, vol. 5, pp. 620-659.
3. Barišić V, Kopjar M, Jozinović A, Flanjak I, Ačkar Đ, Miličević B, Šubarić D, Jokić S, Babić J. The Chemistry behind Chocolate Production. *Molecules*. 2019 Aug 30;24(17):3163. doi: 10.3390/molecules24173163.
4. Belščak, A.; Komes, D.; Horžić, D.; Ganić, K.K.; Karlović, D. Comparative study of commercially available cocoa products in terms of their bioactive composition. *Food Res. Int.* 2009, 42, 707–716. [Google Scholar] [CrossRef].
5. Brito, B.C., Renan, P., Rosinelson, G., & Lopes, A. (2017). Bioactive amines and phenolic compounds in cocoa beans are affected by fermentation. *Food Chemistry*. 228. 10.1016/j.foodchem.2017.02.004.
6. Commission Regulation (EU) No. 1019/2013 amending Annex I to Regulation (EC) No. 2073/2005 as regards histamine in fishery products.
7. Directive 2000/36/EC of the European Parliament and of the Council of 23 June 2000 relating to cocoa and chocolate products intended for human consumption of European Union (EU): Brussels, Belgium, 2003.
8. Fratoddi I., Rapa, M., Testa G., Venditti, I., Scaramuzzo, F.A. and Vinci, G. 2018. Response surface methodology for the optimization of phenolic compounds extraction from extra virgin olive oil with functionalized gold nanoparticles. *Microchem. J.* 138:430.
9. Giacometti, J., Mazor Jolic, S., Josic, D (2015). Cocoa processing and impact on composition. In book: *Processing and Impact on Active Components in Food* (pp.605-612), First Edition, Chapter: 73.
10. Giuggioli, N.R., Sottile, F., Peano, C. (2016). Quality indicators for modified atmosphere packaging (MAP) storage of high-quality European plum (*Prunus domestica* L.) cultivars. *Ital. J. Food Sci.* 28:376. <https://doi.org/10.14674/1120-1770/ijfs.v38>

11. Godočiková L, Ivanišová E, Zagula G, Noguera-Artiaga L, Carbonell-Barrachina AA, Kowalczewski PL, Kačaniová M. Antioxidant Activities and Volatile Flavor Components of Selected Single-Origin and Blend Chocolates. *Molecules*. 2020 Aug 11;25(16):3648. doi: 10.3390/molecules25163648. PMID: 32796548; PMCID: PMC7463549.
12. Guillén-Casla, V., Rosales-Conrado, N., León-González, M.E., Pérez-Arribas, L.V., Polo-Diez, L.M. (2012). Determination of serotonin and its precursors in chocolate samples by capillary liquid chromatography with mass spectrometry detection. *Journal of Chromatography A*, vol. 1232, pp. 158 – 165
13. Hafid, R. N. H., Baso, Y. S., Ramadany, S., Manapa, E. S., & Tamar, M. (2021). The difference of satisfaction level of midwifery students in trying out competency test with computer-based test and web-based test . *International Journal of Health & Medical Sciences*, 4(1), 8-14. <https://doi.org/10.31295/ijhms.v4n1.390>
14. Kalač P. 2014. Health effects and occurrence of dietary polyamines: a review for the period 2005-mid 2013. *Food Chem.* 161:27. 10.1016/j.foodchem.2014.03.102.
15. León-Perez D, Domínguez-Perles R, Collado-González J, Cano-Lamadrid M, Durand T, Guy A, et al. Bioactive plant oxylipins-based lipidomics in eighty worldwide commercial dark chocolates: effect of cocoa and fatty acid composition on their dietary burden. *Microchem J.* (2020) 157:105083. doi: 10.1016/j.microc.2020.105083
16. Martini, S., Conte, A., Tagliazucchi, D (2018). Comprehensive evaluation of phenolic profile in dark chocolate and dark chocolate enriched with Sakura green tea leaves or turmeric powder. *Food Research International*, vol. 112, pp. 1–16.
17. Negro, E.; Williner, M R. (2019). Nutritional quality and origin of fatty acids in chocolates and confectionery baths produced in Argentina. Compliance with the legislation. *Nutrición Clínica y Dietética Hospitalaria*, 39:3, 129-137.
18. Oberrauter, L.-M.; Januszewska, R.; Schlich, P.; Majchrzak, D. Sensory evaluation of dark origin and non-origin chocolates applying Temporal Dominance of Sensations (TDS). *Food Res. Int.* 2018, 111, 39–49. [Google Scholar] [CrossRef] [PubMed]
19. Oracz, J., Nebesny, E (2014). Influence of roasting conditions on the biogenic amine content in cocoa beans of different *Theobroma cacao* cultivars. *Food Research International*, vol. 55, pp. 1–10
20. Preti, R.; Rapa, M.; Vinci, G. Effect of streaming and boiling on the antioxidant properties and biogenic amines content in green bean (*Phaseolus vulgaris*) varieties of different colours. *J. Food Qual.* 2017, 5329070.
21. Restuccia, D., Spizzirri, U., Puoci, F., Picci, N. (2015). Determination of biogenic amine profiles in conventional and organic cocoa-based products. *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment.* 32. 10.1080/19440049.2015.1036322.
22. Samaniego I, Espín S, Quiroz J, Rosales C, Mena P, García-viguera C. Effect of the growing area on the fat content and the fatty acid composition of ecuadorian cocoa beans. *Int J Food Sci Nutr.* (2021) 0:1–11. doi: 10.1080/09637486.2021.1884204
23. Silva NR. Chocolate consumption and effects on serotonin synthesis. *Arch Intern Med.* 2010 Sep 27;170(17):1608.

24. Suryasa, I. W., Rodríguez-Gómez, M., & Koldoris, T. (2021). Get vaccinated when it is your turn and follow the local guidelines. *International Journal of Health Sciences*, 5(3), x-xv. <https://doi.org/10.53730/ijhs.v5n3.2938>
25. Taylor, S.L., Eitenmiller, R.R. (1986). Histamine food poisoning: Toxicology and clinical aspects. *Crit. Rev. Toxicol.*, vol. 17, pp. 91–128.