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Nutritional Evaluation of the Mineral Composition of Chocolate Bars: Total Contents vs. Bioaccessible Fractions

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Abstract

The total contents and the bioaccessibility of essential (Co, Cu, Fe, Mn, Zn, Cr and Se) and non-essential elements (Al, Ba, Sr, As, Ni and V) were estimated in dark, milk and white chocolates. The analytical determinations were made by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) after microwave-assisted acid mineralization. The bioaccessibility was determined for each type of chocolate by applying an *in vitro* digestion method and the results were in the range of 45-63% for Co, 26-89% for Cu, 4-67% for Fe, 29-89% for Mn, 8-89% for Zn, 28-36% for Cr, 9-89% for Se, 3-5% for Al, 13-55% for Ba, 25-86% for Sr, 42-62% for As and 50-63% for Ni. Although the highest total elemental concentrations were found in dark chocolate, white chocolate exhibited bioaccessible fractions significantly higher (55-89%) when compared to milk (5-63%) and dark (3-50%) chocolate. The results also pointed out that the consumption of milk and dark chocolate contributes to a high daily intake of Cr for children.

Keywords: Bioaccessibility; Chocolate; ICP-MS; Trace elements

Abbreviations: ANVISA: Agência Nacional de Vigilância Sanitária; AOAC: Association of Official Agricultural Chemists; ICCO: International Cocoa Organization

Introduction

Chocolate bars are the most consumed cocoa-derived product worldwide. The regular and moderate consumption of chocolate bars and other cocoa derivatives can benefit health due to their antioxidant activity, high carbohydrate content and presence of some essential metallic elements such as Ca, Cu, Fe, K, Mg, Mn and Zn [1-3]. Despite their positive health effects, the pleasant palatability of chocolate products is probably the main reason for their popularity [4,5]. As a result, between the years 2013 and 2014, the cocoa beans world production was estimated at 4370 thousand tons [6], highlighting its wide consumption and the global importance of the cocoa and chocolate sector. Cocoa, the seeds of the *Theobroma cacao* tree, is the primary basis of chocolate [1,7]. For the production of dark and milk chocolate, cocoa mass or liquor is used, and the tablets are normally classified according to their cocoa content. According to Brazilian legislation, to be considered as a chocolate a food product must contain at least 25% of cocoa liquor or 20% of cocoa butter [8]. Dark chocolates present the highest cocoa contents, varying between 50 and 70%, although some products with 80% or higher cocoa contents can also be found in the market, whereas in milk chocolate bars the cocoa content is normally between 25 and 50%. For the production of white chocolate, cocoa butter is the main ingredient and usually no information about the cocoa content is provided. The chemical composition of chocolate products has already been investigated in some papers [9-11]. Most of studies concerning the presence of elements in cocoa and derivatives are devoted to the determination of their total concentrations, an approach usually adopted to evaluate the presence of essential and non-essential elements in foodstuffs. In a recent paper, Villa et al. [11] determined the total contents of Cd and Pb in samples of chocolate bars, showing that the concentrations of these elements increase linearly with the cocoa content and, as expected, dark chocolates presented the highest concentrations of Cd and Pb, suggesting that the principal source of Cd and Pb in chocolate is the cocoa used in their production, which is also the main source of essential elements in chocolate products [3]. In spite of the important data raised in studies of total elemental concentrations, a more accurate evaluation of the benefits and risks associated with chocolate consumption can be done by evaluating the bioaccessible and bioavailable fractions. The oral bioaccessibility of a food component can be described as the fraction that is released from the food matrix and is soluble in the gastrointestinal tract, being available for intestinal absorption [12,13]. The estimate of the bioaccessible fraction can be used as indicative of maximum bioavailability, the fraction of the ingested compound that could be absorbed by the human organism. Taking this into account, Mounicou et al. [14] studied the bioaccessibility of Cd and Pb from cocoa. According to their results, Cd presented bioaccessibility in the range of 10-50%, and bioaccessible fractions below 10% were obtained for Pb in the main raw material used for the production of chocolate. Bioaccessibility studies have also been conducted for chocolate drink powder [15], in which essential elements presented higher bioaccessibility than potentially toxic elements at trace levels. For chocolate bars, despite their significant consumption, there is still no data available about the bioaccessibility of the elements. Thus, in order to provide a better evaluation of the presence of elements in chocolate bars, the aim of this work was to estimate the bioaccessible fractions of Al, Ba, Cu, Fe, Mn, Sr, Zn, As, Co, Cr, Ni, Se and V in dark, milk and white chocolates.

Materials and Methods

Reagents, samples and certified materials

Deionized water, obtained using a Milli-Q system (Millipore,

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Bedford, MA), with a conductivity of 18.2 mΩ cm, was used throughout. Nitric acid (65%, w/w) and hydrogen peroxide (30%, w/w) were purchased from Merck (Darmstadt, Germany). Standard monoelemental solutions (1000 mg/L) of Al, Ba, Cu, Fe, Mn, Sr, Zn, As, Co, Cr, Ni, Se and V from Sigma Aldrich (TraceCERT®, Fluka Analytica, St. Louis, USA) were used in the preparation of calibration standards. A 100 mg/L solution of Ge (Specsol®, São Paulo, Brazil) was used as internal standard. Pepsin (enzyme activity 944 U/mg of protein), porcine pancreatin (activity equivalent to 4XUS Pharmacopeia specifications/mg pancreatin) and porcine bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts), all acquired from Sigma Aldrich and were used in the digestion simulations. Three chocolate bars from the same brand, labeled by the manufacturer as white, milk and dark (55% of solid cocoa content) were analyzed in this study. They were purchased in a local market of Campinas, State of São Paulo - Brazil in April 2014. A certified reference material of Baking Chocolate (NIST 2384) from the National Institute of Standards and Technology - NIST (Gaithersburg, USA) was used to verify the accuracy of the analytical method. All the glassware and plastic materials were decontaminated with a 10% (v/v) HNO_3 solution for 24 h and rinsed with deionized water prior to use.

Instrumentation

An inductively coupled plasma mass spectrometer (ICP-MS) instrument, model 7700x (Agilent Technologies, Hachioji, Japan), equipped with octapole collision/reaction system third generation (ORS^3) using He as collision gas, was used for the determination of all elements. The operational conditions are shown in Table 1. The analyte solutions used for external calibration were prepared by doing appropriate dilution of standard stock solutions and the internal standard $^{72}Ge^+$ (12.5 µg/L) was added *on line* to compensate the possibility of instrument drift and matrix effects. Liquid argon and helium gas (99.996%), purchased from White Martins (São Paulo, Brazil), were used for all measurements. Sample treatment was performed using a microwave digestion system (ETHOS1, Milestone, Sorisole, Italy) equipped with closed polytetrafluoroethylene vessels and sensors for temperature and pressure control. A pH-meter (model Q400RS), a water bath with linear shaking and temperature control (Dubnoff, model Q226M) and a centrifuge (model Q222TM), all from Quimis® (Diadema-SP, Brazil), were used in the *in vitro* digestion method.

Procedure for *in vitro* digestion method

The *in vitro* digestion method employed was based on the method used by Laparra et al. [16] and adapted for chocolate samples. Briefly, 4.0 g of the chocolate sample were weighed and 50 g of water were added. Next, the pH was adjusted to 2.0 with 6 mol/L HCl and freshly prepared pepsin solution (0.1 g/mL in 0.1 mol/L HCl) was added to provide a proportion of 0.01 g of pepsin/50 g of solution. The sample was incubated in a water bath with shaking, at 37°C, for 2 h. Prior to the intestinal digestion step, the pH of the gastric digests was set to pH 6.5 by adding 1 mol/L NH_4OH. Then, a pancreatin-bile extract mixture was added to provide a proportion of 2.5×10^{-3} g pancreatin and 1.5×10^{-2} g bile/50 g of solution, then incubation at 37°C continued for an additional 2 h. The pH of digests was then adjusted to 7.2 and centrifuged at 4000 rpm for 30 min. In order to achieve a better separation of the soluble and insoluble fraction, the samples were filtered through Whatman filter paper (No. 42) and the soluble fractions were submitted to analysis. Blank digestion assays were run in parallel with the samples. The bioaccessibility of elements is defined as the proportion of the total element content that is available for absorption, and it was calculated as:

$$\text{Bioaccessibility (\%)} = ([\text{Element}]_{\text{soluble fraction}}/[\text{Element}]_{\text{total}}) \times 100 \quad (1)$$

Microwave-assisted acid digestion

Chocolate samples (500 mg) or the soluble content obtained in the *in vitro* digestion (5.0 mL) were put into the microwave vessels and 2.0 mL of deionized water, 2.0 mL of H_2O_2 (30%, w/w) and 3.0 mL of 14 mol/L HNO_3 were added. The vessels were then placed inside the microwave oven, and the heating program was performed over six steps: (1) heat from room temperature to 80°C in 3 min, (2) hold for 3 min at 80°C, (3) heat from 80°C to 140°C in 3 min, (4) hold for 4 min at 140°C, (5) heat from 140°C to 200°C in 7 min, and (6) hold for 15 min at 200°C. The resulting solutions were left to cool, transferred to polyethylene flasks and diluted to 25.0 mL with deionized water for the determination of the total concentration and to 13.0 mL to determine the bioaccessible fraction of the elements.

Method validation and statistical analysis

The method validation was performed by evaluating the accuracy,

Parameters		
Radio frequency power	1550 W	
Sample depth	10.0 mm	
Plasma flow rate	15 L/min	
Nebulizer gas flow rate	1.09 L/min	
Nebulizer pump	0.10 rps	
Spray chamber	Scott (double pass) at 2°C	
Interface	Platinum cones	
Sampling cone	1 mm	
Skimmer	0.4 mm	
Resolution	1.5 u	
Selected isotopes for analytes	$^{27}Al^+$, $^{51}V^+$, $^{52}Cr^+$, $^{55}Mn^+$, $^{56}Fe^+$, $^{59}Co^+$, $^{60}Ni^+$, $^{63}Cu^+$, $^{68}Zn^+$, $^{75}As^+$, $^{80}Se^+$, $^{88}Sr^+$ and $^{137}Ba^+$	
On line internal standard	^{72}Ge (250 µg/L diluted *on line* 20 x)	
ORS^3 *	Collision	
Gas flow	He Mode	HEHe Mode
	4.3 mL/min	10 mL/min
Energy discrimination	3.0 V	7.0 V
* ORS^3: Octapole collision/reaction system third generation		

Table 1: Operating conditions used in the ICP-MS.

precision, LOD, LOQ and the correlation coefficients. A certified reference material (Baking Chocolate, NIST 2384) was used to verify the accuracy for the determination of Fe, Cu, Mn and Zn. For Al, Ba, Sr, As, Co, Cr, Ni, Se and V, analyte addition and recovery experiments were performed. Analytical blanks were prepared following the same procedure used for sample preparation and the LOD and LOQ were calculated as 3 and 10 times the standard deviations obtained in the analysis of the analytical blanks. The analyses for the determination of the total concentrations were performed in triplicate and the *in vitro* digestion assays in quintuplicate. All the results are expressed as the mean of independent experiments ± standard deviation. The differences obtained for the bioaccessible fractions were analysed by using one-way ANOVA with Tukey HSD post-hoc tests (Statistica 7) and differences were considered as significant for $p < 0.05$.

Results and Discussion

Analytical determinations

The determination of elements in the fraction of food soluble in the gastrointestinal tract (bioaccessible fraction) almost always requires the use of a sensitive technique for detection, considering that the concentrations to be determined in these types of assays are often lower than the total concentrations found in the food matrix. Besides the low concentrations, a lot of attention has to be paid to possible interferences caused by the organic compounds of the food matrix, by the enzymes added during the digestion simulation, or by the salts of easily ionized elements that are commonly added in high concentrations during the digestion described for some protocols [12,17]. In the protocol used in this work, there was no addition of salts of easily ionized elements and the main concern remains the elimination of the organic matter of the food matrix and bioactive compounds. So, all the samples were treated by acid mineralization assisted by microwave radiation prior to the analysis made by ICP-MS. The operating conditions to be used in the ICP-MS also has to be carefully evaluated, especially for some elements such as Al, As and Co, which are mono-isobaric and whose analytical determination might be severely hampered by mass interferences, especially from polyatomic and molecular species [18]. Thus, the use of a strategy to minimize possible interferences is frequently mandatory for obtaining reliable results. In this case, the use of an octapole collision/reaction system third generation (ORS^3), using He, as collision gas, and kinetic energy discrimination, was the strategy adopted to reduce spectral interferences. The ORS^3 was used in two different tunes mainly defined by He flow rate: 4.3 (He) and 10 mL/min (High Energy Helium mode, HEHe). The use of He gas in the highest flow rate (HEHe) was necessary for the determination of most elements (As, Cr, Cu, Fe, Mn, Ni, Se and Zn), whereas He at a flow rate of 4.3 mL/min was used for the determination of Al, Ba, Co, Sr and V. The choice of the best He flow rate was made based on the recovery and the repeatability obtained in the *in vitro* digestion assay, considering that the digested portion of chocolate is the most complex matrix to be analysed in this study. In the selected conditions, method validation was performed and the analytical features are presented in Table 2. The results obtained for the CRM were in agreement with the certified levels ($p > 0.05$) for Cu, Fe, Mn and Zn and recoveries in the range 92-122% were obtained for all elements in the addition and recovery experiments, which is in agreement with AOAC recommendations [19]. Analytical curves presented correlation coefficients of $r > 0.999$ or higher and the precision of the measurements were better than 8%. Therefore, considering the low levels to be determined and the complexity of the matrix to be analysed in digestion studies, the analytical method was considered suitable to be applied to the determination of the bioaccessible fractions of metallic elements in chocolate.

Total elemental concentrations in chocolates

The results obtained for total concentrations of Co, Cu, Fe, Mn, Zn, Cr, Se, Al, Ba, Sr, As, Ni and V in the three types of chocolate are shown in Table 3. Concentration ranges of Al, As, Cr, Cu, Fe, Mn, Ni, Co and Zn were consistent with measurements reported in previous papers [20-22] and the contents of Ba, Se, Sr and V were determined by the first time in chocolate bars. As can be seen from the results, there were significant differences in total elemental concentrations between white, milk and dark chocolate, with the latter presenting the highest concentrations of all elements studied, which is probably due to the capability of the cocoa plant to absorb minerals from the soil, accumulating them in the cacao seeds, which in turn are the main ingredient of dark chocolates. The most abundant essential and non-essential elements determined in chocolate bars were Fe and Ni, respectively. Concentrations of As in the three types of chocolate were below the maximum level allowed in Brazil (0.2 mg/kg for cocoa products with less than 40% of cocoa and 0.4 mg/kg for products with a cocoa content higher than 40%) [23]. With regard to other elements studied, there are no maximum admissible levels specifically established for chocolate bars.

Element	Addition experiments		CRM baking chocolate (mg/kg)		LOD	LOQ
	Spiked (µg/L)	Recovery (%)	Certified	Found	(µg/kg)	(µg/kg)
Al	500	92	n. r.*	-	300	1000
Ba	500	106	n. r.*	-	50	170
Cu	500	100	23.2 ± 1.2	22.8 ± 0.2	20	70
Fe	500	114	132 ± 11	131 ± 1	160	530
Mn	500	107	20.3 ± 1.3	19.9 ± 0.2	11	37
Sr	20	96	n. r.*	-	4	15
Zn	500	103	36.6 ± 1.7	36.1 ± 0.3	250	850
As	20	116	n. r.*	-	0.2	0.6
Co	20	99	n. r.*	-	1.5	5
Cr	500	103	n. r.*	-	27	90
Ni	20	102	n. r.*	-	0.9	3.2
Se	20	122	n. r.*	-	0.5	1.9
V	20	106	n. r.*	-	1.3	4.3
* n. r.: not reported						

Table 2: Analytical features obtained in the method validation.

As determined in this study, differences in the chocolate matrix can lead to significantly different elemental concentrations. However, in studies in which only the total concentrations are considered, the effects of the matrix on the nutritional value of food cannot be assessed, nor can risk assessment analysis be made. In this case, the application of a simple *in vitro* digestion method could provide a more reliable approach for the evaluation of the presence of essential and potentially toxic elements in food.

Evaluation of bioaccessible fractions

The bioaccessible fractions (%) determined for essential and non-essential elements in white, milk and dark chocolate [Calculated by Eq. (1)] are presented in Table 4 and Figure 1. The bioaccessibility of some elements (Al, Co, Cr, Ni in white chocolate and V in milk and dark chocolate) are not represented since the concentrations found in the bioaccessible fractions were below the LOD of the analytical method. It was possible to estimate the bioaccessibility of V but only in white chocolate, for which a bioaccessibility of 89% was obtained. The results presented show that the solubility of the elements in the simulated gastric and intestinal juices varies depending on the type of chocolate being analyzed, except for As, for which the bioaccessible fractions obtained were similar in the three types of chocolate ($p < 0.05$). Although the highest total concentrations of all elements were found in dark chocolate, this type of chocolate presented the lowest bioaccessible fractions for the majority of elements, including essential elements (Cu, Fe, Mn, Se and Zn) and some contaminants (As, Ba, Ni and Sr), for which bioaccessible fractions lower than 50% were obtained. An opposite behavior was observed for white chocolate, which presented the lowest total concentrations and the highest bioaccessible fractions. The bioaccessibility of metallic elements in white chocolate was shown to be significantly higher, considering that the bioaccessible fractions of 8 of 12 elements studied exceeded values of 50%. Metallic elements in milk chocolate presented an intermediate bioaccessibility between dark and white chocolates. In this way, in general, bioaccessible fractions for most elements were obtained in the ascending order: dark < milk < white chocolate. The bioaccessibility of metallic elements from foodstuffs depends on several factors such as the type of food, mainly due to the presence of different food components that would interact in different ways with the elements; the gastrointestinal conditions and physical and chemical characteristics of the element under study. The chemical form of the element and its solubility in the aqueous environment of the human gastrointestinal tract, particularly, play important roles in bioaccessibility. In the case of chocolate, the main source of metallic elements is cocoa, and a possible explanation for the differences observed in the bioaccessible fractions, is the form in which cocoa is added to the food matrix. Cocoa butter and cocoa mass or liquor could present elements in different chemical forms, considering that the production process may change the chemical form of some metallic elements. The results suggest that the metallic elements in white chocolate are more easily released from the food matrix and present higher solubility in the human gastrointestinal tract when compared with milk and dark chocolate. Considering the influence of the element bioaccessibility in the nutritional value of food, the contribution of chocolate consumption to the Recommended Daily Intake (RDI) of essential elements (Cr, Cu, Fe, Mn, Se and Zn) were calculated for the three types of chocolate, using the total contents and their bioaccessible fractions, for children of 7-10 years [24], which are important consumers of this type of food. The results obtained are shown in Table 5. Cobalt is not represented since there is no RDI for this element in the Brazilian legislation. In general, a reduction in the contribution of chocolate consumption was observed when the bioaccessible fractions were taken into account. Despite the high total concentrations found in dark chocolate, the consumption of milk chocolate contributes most to the daily intake of Cr, Fe, and Zn, whereas for Se a higher contribution was observed when white chocolate is consumed, considering the bioaccessibility of the elements. In this way, milk and white chocolate contributes more to the ingestion of Cr, Fe, Se and Zn, despite their lower total concentrations. Additionally, the results also

Element	White	Milk	Dark
Al	<LOQ	25.35 ± 1.40	26.7 ± 2.86
Ba	0.46 ± 0.06	1.76 ± 0.13	5.63 ± 0.34
Cu	0.17 ± 0.01	3.30 ± 0.02	12.18 ± 0.20
Fe	1.60 ± 0.23	56.44 ± 0.63	77.06 ± 1.81
Mn	0.23 ± 0.01	4.03 ± 0.04	13.36 ± 0.18
Sr	1.10 ± 0.07	2.54 ± 0.07	6.04 ± 0.34
Zn	3.89 ± 0.19	10.65 ± 0.04	23.06 ± 0.34
As*	6.52 ± 0.29	12.37 ± 0.56	14.06 ± 1.11
Co*	<LOQ	87.50 ± 1.72	358.68 ± 16.37
Cr*	<LOQ	456.73 ± 12.45	515.59 ± 23.65
Ni*	7.6 ± 1.1	195.97 ± 5.36	815.43 ± 15.37
Se*	17.62 ± 2.31	40.46 ± 1.67	70.77 ± 1.48
V*	9.70 ± 0.42	50.46 ± 2.77	76.37 ± 6.44
* Total content in µg/kg			

Table 3: Total content (mg/kg ± s, n=3) of essential and non-essential elements in white, milk and dark chocolate.

Element	White	Milk	Dark
Al	n.d.*	5.3 ± 0.2	2.7 ± 1.1
Ba	54.8 ± 4.2	34.7 ± 2.5	13.3 ± 1.2
Cu	88.8 ± 9.1	35.7 ± 2.4	25.5 ± 0.7
Fe	66.7 ± 8.8	8.3 ± 1.3	3.5 ± 0.4
Mn	88.9 ± 6.8	57.6 ± 4.1	28.6 ± 1.9
Sr	86.0 ± 8.7	56.9 ± 3.8	25.0 ± 1.5
Zn	88.7 ± 7.4	37.0 ± 2.7	7.9 ± 1.1
As	61.6 ± 15.1	61.5 ± 27.4	41.9 ± 8.1
Co	n.d.*	62.9 ± 4.0	45.3 ± 3.4
Cr	n.d.*	35.9 ± 7.5	27.7 ± 5.2
Ni	n.d.*	62.8 ± 7.7	50.1 ± 1.8
Se	89.1 ± 8.1	21.8 ± 2.8	8.7 ± 0.7
V	89.3 ± 5.0	n.d.*	n.d.*
* n.d.: not determined			

Table 4: Bioaccessible fractions (%) of essential and non-essential elements in three types of chocolate bars (n=5).

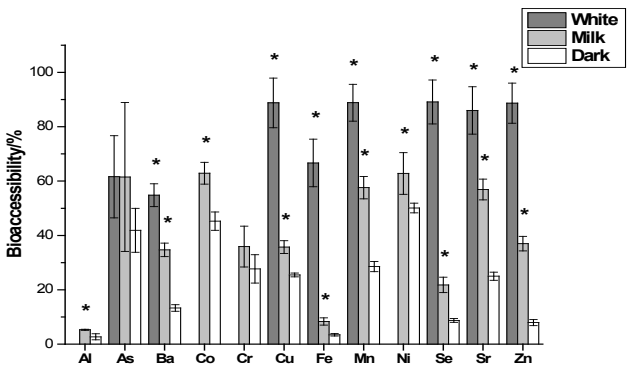

Figure 1: Bioaccessible fractions of essential and non-essential elements in white, milk and dark chocolate. Bars marked with an asterisk indicate significant differences with respect to dark chocolate ($p < 0.05$).

Element	Type of chocolate	Total content	Bioaccessible fraction
Cr	White	n.d.	n.d
	Milk	122	73
	Dark	138	64
Cu	White	1.5	1.4
	Milk	30	11
	Dark	111	29
Fe	White	0.7	0.5
	Milk	25	2.0
	Dark	34	1.4
Mn	White	0.6	0.55
	Milk	11	6.1
	Dark	36	10
Se	White	3.4	3.0
	Milk	7.7	1.7
	Dark	14	1.2
Zn	White	2.8	2.5
	Milk	7.6	2.8
	Dark	17	1.3

n.d.: not determined, RDI for children 7-10 years: Cr: 15 µg; Cu: 440 µg; Fe: 9 mg; Mn: 1.5 mg; Se: 21µg e Zn: 5.6 mg [8].

Table 5: Contribution (%) of chocolate consumption (40 g/day) to the RDI* of essential elements for children (7-10 years).

pointed out that the consumption of milk and dark chocolate would also contribute to a high daily intake of Cr for children, even when the bioaccessible fractions are considered, representing 73 and 64% of the RDI, respectively. Although Cr is considered an essential element, it is important to mention that there are some recent studies that refute the essentiality of Cr (III), mainly due to the lack of experimental evidence of biomolecules that would interact with Cr (III) at a cellular level [25,26]. However, Cr (III), the predominant specie of Cr in foods of animal and plant origin, is not toxic to humans when present at trace levels [27].

Conclusions

According to the results, dark chocolate presented higher total concentrations of essential and non-essential elements than milk and white chocolate, probably due to its high cocoa content. As already demonstrated in another work, the concentrations of some elements in chocolate bars are related to their cocoa content [11]. However, the evaluation of the bioaccessibility showed that the highest bioaccessible fractions for all elements studied were found in white chocolate, indicating that the fractions of the elements released into the gastrointestinal tract have a high dependence on the type of chocolate being considered. The results obtained show that information about bioaccessibility of elements should be considered in conjunction with their total concentrations in studies of risk assessment or nutritional evaluation of foodstuffs.

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