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Short Report

Estimation of the Bioaccessibility of Metallic Elements in Chocolate Drink Powder using an *in vitro* Digestion Method and Spectrometric Techniques

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As frações bioacessíveis de Al, Ba, Cd, Cr, Cu, Mg, Mn e P em amostras de achocolatado foram estimadas usando um método de digestão *in vitro* e técnicas espectrométricas de análise. Como método de digestão *in vitro* foi utilizado um procedimento de três etapas, nas quais são simulados os processos digestivos que ocorrem na boca, no estômago e no intestino com a preparação de fluidos digestivos sintéticos. Para o tratamento da amostra e do quimo (porção solúvel) obtido no método de digestão *in vitro*, utilizou-se mineralização assistida por radiação micro-ondas, considerando os altos teores de matéria orgânica. Os teores de Al, Ba, Cu, Mg, Mn e P foram quantificados por espectrometria de emissão óptica com plasma indutivamente acoplado, enquanto os teores de Cd e Cr foram quantificados por espectrometria de absorção atômica com forno de grafite. Considerando os teores totais dos elementos estudados, as frações bioacessíveis obtidas foram: 5 ± 3% para Al, 9 ± 2% para Ba, 13 ± 3% para Cd, 5 ± 2% para Cr, 29 ± 4% para Cu, 54 ± 3% para Mg, 11 ± 3% para Mn e 31 ± 6% para P. De uma maneira geral, elementos que desempenham funções essenciais no organismo humano apresentaram maior bioacessibilidade quando comparados aos elementos potencialmente tóxicos em níveis traços.

The bioaccessible fractions of Al, Ba, Cd, Cr, Cu, Mg, Mn and P in chocolate drink powder were estimated using an *in vitro* digestion method and spectrometric techniques. A three-step procedure was used as an *in vitro* digestion method, in which the digestive process occurring in the mouth, stomach and intestine are simulated with the preparation of synthetic digestive fluids. Mineralization assisted by microwave radiation was used for the treatment of the sample and the chyme (soluble portion) obtained in the *in vitro* digestion method, considering their high organic content. Aluminum, Ba, Cu, Mg, Mn and P were quantified by inductively coupled plasma optical emission spectrometry, whereas Cd and Cr were quantified by graphite furnace atomic absorption spectrometry. Considering the total content of these elements in chocolate drink powder, the bioaccessible fractions found were 5 ± 3% for Al, 9 ± 2% for Ba, 13 ± 3% for Cd, 5 ± 2% for Cr, 29 ± 4% for Cu, 54 ± 3% for Mg, 11 ± 3% for Mn and 31 ± 6% for P. In general, elements that perform essential functions in the human organism presented higher bioaccessibility when compared with potentially toxic elements at trace levels.

Keywords: bioaccessibility, metallic elements, chocolate, *in vitro* digestion method, spectrochemical analysis

Introduction

The intake of food provides several essential compounds and elements to humans such as carbohydrates, proteins, lipids, vitamins and some metallic species. On the other hand, the ingestion of food can also be the major route of exposure to potentially toxic compounds and elements. However, regarding food safety, it is important to mention that not all of the ingested compounds are effectively used by the human organism, considering that during the digestive process the compounds can be subjected to biotransformation processes. In order to perform their action, the ingested compounds need to be released from their matrices, be soluble in the human gastrointestinal tract and be absorbed to then exert the majority of their effects, whether essential or toxic.

In the context of risk assessment, the bioaccessible fraction of a compound/element can be understood as the

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quantity of a compound/element that is released from its matrix into the gastrointestinal tract, becoming available for intestinal absorption. The concept of bioavailability is more comprehensive, referring to the fraction of a compound or element that is absorbed by the human organism from the gastrointestinal tract, reaches the bloodstream and then is used in biological functions.[1-4]

Thus, the determination of the bioaccessible and bioavailable fractions of compounds or elements in foodstuff plays an important role in nutritional and toxicological studies. In spite of their importance, the majority of metallic element determinations in foodstuffs are aimed at the determination of total contents, which is not sufficient to evaluate either availability or risk. In these cases, the species of an element and its bioaccessible and bioavailable fractions need also to be considered.[1,5,6]

Studies of bioaccessibility and bioavailability can be performed *in vivo*, with humans or animals, or by performing *in vitro* tests. *In vivo* studies are costly and often limited by ethical questions.[2] As an alternative, several *in vitro* methods[7-9] have been proposed in order to assess the bioaccessible and bioavailable fractions of compounds from foodstuffs. In these studies, the main challenge is to achieve *in vitro* conditions as similar as possible to *in vivo* conditions. For this, some requirements have to be considered in the simulation of the human digestion process such as pH, residence time, temperature, agitation, chemical and enzymatic composition of the digestive fluids, frequency of peristaltic movements, microbiota, among others.[1,10]

In the majority of works,[2,7,8,11] the *in vitro* digestion methods are initiated by adding a pepsin suspension in acid medium to the food sample, followed by incubation for 1-2 h. The next step accomplishes the simulation of intestinal digestion, which is frequently made by adding pancreatine and bile salts to the food suspension and elevation of pH to 6-8 with the addition of a bicarbonate solution. Although this simple approach provides important data, it is important to mention the complexity of the human digestion process and the large number of factors involved, since some of those not considered in this model might have significant influences on the digestion of food, such as the presence of other enzymes and the overall composition of the fluids present in the human gastrointestinal tract.

Among the reports in the literature,[7-9,11] one of the most complete static *in vitro* digestion method has been proposed by Versantvoort *et al.*.[9] This method is a three-step procedure that simulates the digestive process occurring in the mouth, stomach and intestine, considering the chemical and enzymatic compositions of the digestive fluids, including organic and inorganic compounds. This method and its modifications have already been applied to the determination of the bioaccessible fraction of several food compounds.[12,13] Hur *et al.*,[12] for instance, applied this digestion model for the study of the type of emulsifier and micro-structural changes that occur in lipids in the human gastrointestinal tract. Whereas Maulvault *et al.*[13] used this approach to study the bioaccessibility of As, Cd and Hg in samples of scabbard fish and edible crab, showing the importance of the food matrix and cooking to the bioaccessibility of these elements.

Studies of the bioaccessibility of metallic elements in chocolate products are rare in the literature. Some studies have already been performed for cocoa samples, regarding mainly the bioaccessibility of Cd and Pb,[14,15] for which bioaccessible fractions of 15 and 5%[15] were obtained under *in vitro* gastrointestinal conditions, but studies applied to chocolate derivatives were not described before.

The choice of the analytical technique in this type of study depends on the level of the element to be determined, often requiring the use of analytical techniques with high detectability, especially for elements or compounds that present low bioaccessibility or bioavailability, considering that in these cases, the analytical determinations are generally performed in a solution containing a small fraction of the total content of the element. As examples, Laparra *et al.*[8] used hydride generation atomic absorption spectrometry (HG AAS) for the determination of the bioaccessibility of arsenic in samples of edible seaweed, while Domínguez *et al.*[11] studied the bioavailability of iron salts in infant formulas using inductively coupled plasma optical emission spectrometry (ICP OES). In general, the majority of studies use spectrometric techniques in the analytical determination of elements in foodstuffs due to their characteristics, such as good detectability, robustness and relatively low cost.[16]

In Brazil, particularly, *in vitro* studies of bioaccessibility and bioavailability of metallic elements are scarce, with only a few studies[17,18] on this topic. Thus, the data are still insufficient and there are many foods consumed on a daily basis by the Brazilian population for which there is no information about the bioaccessibility or bioavailability of their components. Considering that, the main goal of this study was to estimate the bioaccessibility of Al, Ba, Cd, Cr, Cu, Mg, Mn and P in chocolate drink powder, a type of food very popular in Brazil that can represent an important source of metallic elements in the diet, especially for children.

Experimental

Instrumentation

An inductively coupled plasma optical emission spectrometer (Perkin-Elmer, model Optima 3000 DV,

Norwalk, CT, USA) was used in the analytical measurements of Al, Ba, Cu, Mg, Mn and P. This spectrometer is equipped with a solid-state segmented array charge coupled device (SCD) as a detector and an Echelle grating. A crossflow nebulizer, a Scott spray chamber and a quartz demountable plasma torch with an internal diameter of 2.0 mm were used. Shear gas (N_2) was used in the interface to strip off the cool plasma area. The operating conditions used for the determination of the total contents of the analytes in the sample were made based on a previous method optimized for this purpose.[19] For the measurements performed in the chyme, the operating conditions used are shown in Table 1, using the axial configuration for all analytes.

Table 1. Operating conditions used for the determination of Al, Ba, Cu, Mg, Mn and P in the chyme by ICP OES

Parameter	Value
Applied radio frequency power / kW	1.3
Nebulization gas flow rate / (L min^{-1})	0.8
Auxiliary argon flow rate / (L min^{-1})	0.5
Argon flow rate / (L min^{-1})	15
Sample flow rate / (L min^{-1})	1.0
Read delay / s	30
Wavelength / nm[a]	Al(I): 308.215; Ba(II): 233.527; Cu(I): 324.752; Mg(II): 279.077; Mn(II): 260.568; P(I): 213.617

[a](I) atomic line, (II) ionic line.

The analytical measurements for Cd and Cr were made in a graphite furnace atomic absorption spectrometer (GF AAS, Perkin-Elmer, model AAnalyst 600, Norwalk, CT, USA) equipped with an auto sampler (model AS-800), a longitudinal Zeeman system for background correction and pyrolytic graphite tubes with transversal heating and integrated L'vov platform. For Cd, the analytical measurements were made at 228.8 nm with an electrodeless discharge lamp operated at 230 mA as a radiation source. For Cr, the wavelength used was 357.9 nm and a hollow-cathode lamp, operated at 25 mA, was used. The measurements were made using 20 µL of the sample and 5 µL of the modifier. As chemical modifiers, 50 µg $NH_4H_2PO_4$ + 3 µg $Mg(NO_3)_2$ were used for Cd and 15 µg $Mg(NO_3)_2$ for Cr. The temperature programs used for the determination of these analytes are shown in Table 2.

Reagents and materials

Analytical grade reagents were used for the preparation of all solutions, except for the extracts from animal and fungal origin, α-amylase from *Aspergillus oryzae* (86250), mucin from porcine stomach type II (M2378), albumin from bovine serum (BSA, A7906), pepsin from gastric mucosa (P7000), pancreatin from porcine pancreas (P1625), lipase from porcine pancreas Type II (L3126) and bile extract from porcine (B8631) were all purchased from Sigma Aldrich. All solutions were prepared with deionized water (18.2 MΩ cm) obtained from a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). The laboratory glassware was washed and kept overnight in a 10% v v^{-1} HNO_3 solution and rinsed with deionized water prior to use.

Argon of 99.996 % purity (White Martins, São Paulo, SP, Brazil) was used in the analytical measurements.

A sample of a conventional chocolate drink powder, representing the most consumed brand in Brazil, was acquired from a supermarket in Campinas city (São Paulo State, Brazil). According to the information reported by the producer on the product label, the sample is composed of cocoa powder, sugar, maltodextrin, soy lecithin as an emulsifier, antioxidant, flavoring, ascorbic acid, gluten, traces of milk, vitamins and minerals.

In vitro digestion method

An *in vitro* digestion method based on the work of Versantvoort *et al.*[9] was used for estimating the bioaccessible fractions of metallic elements with some adaptations. The method is a three-step procedure where the human digestive process occurring in the mouth, stomach and intestine are simulated, using the synthetic

Table 2. Temperature programs used for the determination of Cd and Cr in chocolate drink powder by GF AAS

Step	Temperature / °C		Ramp time / s	Hold time / s	Argon flow rate / (mL min^{-1})
	Cd	Cr			
Drying	110	110	5	30	250
Drying	130	130	15	30	250
Pyrolysis	400	1400	10	20	250
Atomization	1400	2100	0	5	0
Cleaning	2450	2500	1	3	250

Table 3. Composition of the synthetic digestive fluids used in the *in vitro* digestion method[9]

	Composition / (g L^{-1})			
	Salivary fluid	Gastric fluid	Duodenal fluid	Bile
Inorganic components	KCl: 1.8	NaCl: 5.5	NaCl: 14.0	NaCl: 10.5
	KSCN: 0.4	NaH_2PO_4: 0.5	$NaHCO_3$: 6.7	$NaHCO_3$: 11.6
	NaH_2PO_4: 1.8	KCl: 1.6	KH_2PO_4: 0.16	KCl: 0.8
	$NaSO_4$: 1.1	$CaCl_2$: 0.8	KCl: 1.13	HCl: 0.03%
	NaCl: 0.6	NH_4Cl: 0.6	$MgCl_2$: 0.1	$CaCl_2$: 0.4
	$NaHCO_3$: 3.4	HCl: 1.3%[a]	HCl: 0.04%[a]	
			$CaCl_2$: 0.4	
Organic components	Urea: 0.4	Glucose: 1.3	Urea: 0.2	Urea: 0.5
	Uric acid: 0.03	Glucuronic acid: 0.04		
		Urea: 0.2		
		Glucoseamine Hydrochloride: 0.7		
Natural components	α-Amilase: 0.6	BSA: 2.0	BSA: 2.0	BSA: 3.6
	Mucin: 0.05	Pepsin: 5.0	Pancreatin: 18	Bile: 60
		Mucin: 6.0	Lipase: 3.0	
pH	7	1	8	8

[a]Concentrations in percentage (v v^{-1})

digestive fluids whose compositions are shown in Table 3. All fluids were prepared daily by appropriate dilutions of organic and inorganic stock solutions and adding the compounds from animal and fungal origin to the solutions.

In the simulation, 2.25 g of chocolate drink powder were first incubated with 1.5 mL of the salivary fluid for 5 min. Subsequently, 3.0 mL of gastric fluid were added and incubation was performed for 2 h. In the final step, 3.0 mL of duodenal fluid, 1.5 mL of bile and 500 μL of a 1 mol L^{-1} bicarbonate solution were added and the mixture was left under incubation for an additional 2 h. All the incubations were performed in a water bath (Dubnoff, Quimis, Diadema, SP, Brazil) at 37 ± 2 °C and under agitation (80 rpm). The pH was monitored in every step, registering values of 8, 3 and 8 in the digestion steps occurring in the mouth, stomach and intestine, respectively.

Subsequently, the mixtures obtained by the *in vitro* digestion method were put in an ice bath for 30 min and then centrifuged for 30 min at 2000 rpm for the separation of the soluble (chyme) and insoluble (pellet) part. The chyme was taken for analysis. This procedure was performed in triplicate, and a blank experiment using the digestive fluids was run in parallel.

Sample treatment

For the determination of the total content of the elements, 0.5 g of the sample, 3.0 mL of 65% m m^{-1} nitric acid (Merck, Darmstadt, Germany), 1.5 mL of 30% m m^{-1} hydrogen peroxide (Merck) and 3.5 mL of deionized water were placed in closed polytetrafluoroethylene vessels and submitted to the heating program described in Table 4. After cooling, the samples were transferred to 25.0 mL volumetric flasks and the volume was made up with deionized water.

Table 4. Temperature program used for the mineralization of chocolate drink powders

Ramp time / min	Hold time / min	Temperature / ºC
6	4	80
3	5	140
5	10	200

For the treatment of the chyme, 5.0 mL of the chyme obtained in the *in vitro* digestion method, 3.0 mL of 65% m m^{-1} nitric acid and 1.5 mL of 30% m m^{-1} hydrogen peroxide were added. The mixture was then submitted to the heating program described in Table 4. In this case, the volume was made up to 10.0 mL due to the lower contents of the elements found in the chyme.

Results and Discussion

The analytical determinations of Al, Ba, Cu, Mg, Mn and P were performed by ICP OES, considering that

these elements are normally found in this type of sample in mg L^{-1} levels.[19,20] Cd and Cr are generally found in cocoa derivates in lower levels,[14,15] so the determination of these elements were performed by GF AAS.

Due to the high number of organic compounds added to the sample of chocolate drink powder during the *in vitro* digestion, especially in the model that considers the overall composition of the digestive fluids, it was necessary to use a sample treatment for the chyme, considering the influence of the organic content on analytical determinations made by spectrometric techniques. The analysis of the chyme without sample treatment was not possible using either ICP OES or GF AAS.

The bioaccessible fractions were calculated based on the mass of the elements that were extracted from the sample of chocolate drink powder during the *in vitro* digestion method and were soluble in the medium representing the human gastrointestinal tract. For this, the following equation was used:

$$\text{Bioaccessible fraction (\%, m m}^{-1}) = \frac{\text{Soluble content}}{\text{Total content}} 100 \quad (1)$$

where the soluble content denotes the mass of the element determined in the chyme and the total content refers to the total mass of the element in the digested sample, obtained from the determination of the total content of the element in the sample. All bioaccessible fractions were expressed as mass percentages.

Table 5 shows the total contents of the elements in the studied sample of chocolate drink powder, the soluble contents obtained in the chyme and their respective bioaccessible fractions.

Table 5. Total and soluble contents of metallic elements in chocolate drink powders and their bioaccessible fractions (n = 3, mean ± standard deviation)

Element	Total content	Soluble content (chyme)	Bioaccessible fraction / %
Al	33 ± 2 mg kg^{-1}	0.37 ± 0.19 mg L^{-1}	5 ± 3
Ba	3.2 ± 0.3 mg kg^{-1}	0.06 ± 0.02 mg L^{-1}	9 ± 2
Cd	45.4 ± 0.7 ng g^{-1}	1.4 ± 0.3 µg L^{-1}	13 ± 3
Cr	327 ± 20 ng g^{-1}	4.3 ± 1.7 µg L^{-1}	5 ± 2
Cu	4.6 ± 0.1 mg kg^{-1}	0.30 ± 0.04 mg L^{-1}	29 ± 4
Mg	1376 ± 27 mg kg^{-1}	168 ± 10 mg L^{-1}	54 ± 3
Mn	7.5 ± 0.7 mg kg^{-1}	0.19 ± 0.04 mg L^{-1}	11 ± 3
P	1139 ± 19 mg kg^{-1}	80 ± 16 mg L^{-1}	31 ± 6

According to the results presented in Figure 1, the bioaccessible fractions for all studied elements were considerably less than 100%, considering the total concentration of the elements in the sample of chocolate drink powder. This means that only a fraction of these elements is released from this matrix in the human gastrointestinal tract and, hence, is available to be absorbed by human organism.

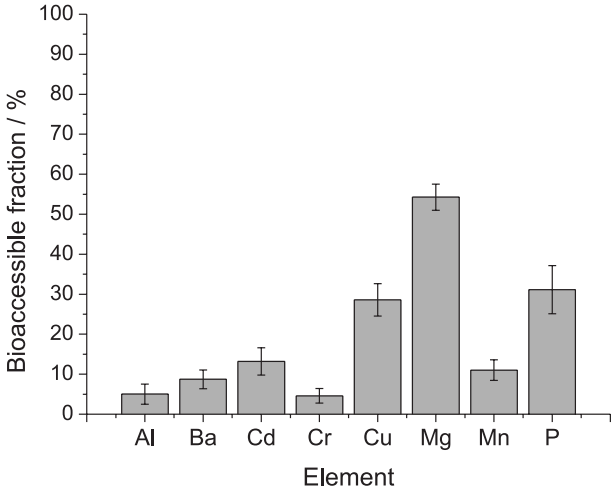

Figure 1. Bioaccessible fractions (%) of Al, Ba, Cd, Cr, Cu, Mg, Mn and P in chocolate drink powder.

Among the studied elements, Mg presented the highest bioaccessible fraction, considering that more than 50% of the mass of this element present in the sample was extracted during the *in vitro* digestion method, indicating that the major portion of the Mg present in this matrix is soluble in the human gastrointestinal tract. Other elements that perform essential functions in human body, such as P and Cu, presented bioaccessible fractions from the chocolate drink powder of approximately 30%, whereas Mn exhibited a bioaccessibility of 11%. Potentially toxic elements for human health at trace levels, such as Al, Ba, Cd and Cr, were less bioaccessible from this matrix; in general, less than 15% of the total content of these elements were extracted during the *in vitro* digestion method.

An important aspect related to the bioaccessibility of elements in foodstuffs is the presence of ligands, which is especially critical for some elements such as Fe, Mn, Co, Cu, Cr, Ni and Zn, which are generally present in organisms as complexed cations.[21] In these cases, the formation of insoluble complexes in the human gastrointestinal tract can prevent the accessibility of elements. Among the compounds acting as chelators in the human gastrointestinal tract, one of the most important is phytate, which is able to bind to elements such as Ca, Zn, Mg and Fe at physiological pH, with seeds, like cocoa, being good sources of these compounds.[22]

Although it is possible that the presence of the phytates found in cocoa derivatives may lower the bioaccessibility

of Mg, the quantities of these compounds normally found in the human diet were shown to have no influence on magnesium absorption,[23] in agreement with the results obtained in this study, considering the high bioaccessibility of this element in the investigated sample.

The presence of phytates can also have a significant influence on the bioaccessibility of other elements. It is known, for example, that the presence of phosphates can contribute to the low bioaccessibility of Al due to the formation of a precipitate of [$AlPO_4$],[24] also in agreement with the results obtained in this study.

In addition to the presence of phytates, other compounds found in cocoa derivatives, such as lignin and cellulose, can be related to the low bioaccessibility obtained for the other elements. These compounds are also able to form stable complexes that are not soluble in the human gastrointestinal tract.[14,15] In this way, the bioaccessibility of metallic elements is highly dependent on the type of food and its composition, showing the importance of bioaccessibility studies in different food types.

The application of *in vitro* digestion methods still presents some challenges and limitations. For other elements found in chocolate drink powders, such as Fe and Zn, for instance, no reproducible data were obtained for the determinations performed in the chyme. This is probably due to the high contents of these elements found in the compounds of animal origin used in the experimental part, mainly in mucin, considering that high blank values were obtained for these elements. This limitation was already reported by Jovaní *et al.*,[25] highlighting the poor reproducibility of the results as one of the principal limitations of the *in vitro* digestion methods that use enzymes from animal origins.

Another critical point of this type of experiment is the precision of the determinations due to the high number of steps and complexity of the *in vitro* simulation of the human digestion process. A coefficient of variation of 25% is generally accepted,[26] although satisfactory standard deviations were obtained for the majority of the elements in this study.

Conclusions

The application of an *in vitro* digestion method and the use of spectrometric techniques such as ICP OES and GF AAS allowed estimating the bioaccessibility of Al, Ba, Cd, Cr, Cu, Mg, Mn and P in chocolate drink powder.

Although this is the first step of a more extensive work, the preliminary data showed that all the studied elements presented bioaccessible fractions smaller than 100%, showing that only a fraction of the total content of the elements has the potential to be absorbed by the human organism. In general, elements that perform essential functions in the human organism presented higher bioaccessibility than potential toxic elements present at trace levels.

The digestive process of humans is complex and difficult to simulate by applying *in vitro* experiments. However, good estimates of the bioaccessible fractions of the metallic elements were obtained for a chocolate drink powder, which can be useful in the evaluation of risk assessment and toxicological studies.

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