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Determination Essential and Toxic Metals in Bitter, Milk and White Chocolate Samples from Eight Different Manufacturers

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Abstract: Chocolate is consumed by people of all ages in every part of society. Chocolate is a very rich source of many essential metals for the body but it also may involve toxic metals. Therefore, the accurate and precise method should be selected and applied to determine the essential and toxic metals in the chocolate samples. In present study, Na, K, Mg (II), Ca(II), Cu(II), Fe(III), Zn(II), Cd(II), Cr(III), Pb(II) and Ni(II) metals were determined by using flame atomic absorption spectrometry (FAAS) in 24 different chocolate samples of 3 different types including 8 different brands of bitter, milk and white. The microwave-assisted digestion system was also used for the chocolate samples digestions. All results were given at 95% of confidence level. In addition, the accuracy of the method was also investigated by the analysis of a standard reference material (spinach leaves, NIST-1570a).

Sekiz Farklı Üreticiden Temin Edilen Siyah, Sütlü ve Beyaz Çikolata Örneklerindeki Gerekli ve Zehirli Metallerin Tayini

Anahtar Kelimeler
Siyah çikolata,
Sütlü çikolata,
Beyaz çikolata,
Esansiyel metal,
Zehirli metal

Özet: Çikolata, toplumun her kesiminden her yaştan insan tarafından tüketilen bir besindir. Çikolata, vücut için gerekli metaller için zengin bir kaynaktır, ancak içeriğinde toksik metaller de bulunabilmektedir. Bu yüzden çikolata örneklerindeki gerekli ve toksik metalleri belirlemek için doğru ve kesin yöntem seçilmelidir. Bu çalışmada 8 farklı markanın siyah, sütlü ve beyaz olmak üzere 3 farklı tipteki toplam 24 farklı çikolata örneğinde Na, K, Mg (II), Ca (II), Cu (II), Fe (III), Zn (II), Cd (II), Cr (III), Pb (II) ve Ni(II) tayinleri alevli atomik absorpsiyon spektrometre (FAAS) kullanılarak gerçekleştirilmiştir. Çikolata numunelerinin çözünürleştirilmesi için ise mikrodalga çözme sistemi kullanılmıştır. Çalışmada tüm sonuçlar % 95 güven seviyesinde verilmiştir. Ek olarak, yöntemin doğruluğu ıspanak yaprağı standart referans maddesinin (NIST-1570a) analizi ile test edilmiştir.

1. Introduction

Chocolate is produced by cocoa beans obtained from cocao tree (*Theobroma cacao*). It is one of the foods widely consumed by people of various ages around the world as it is found in various foods such as ice cream, cake and biscuit [1].

There are three main types of chocolate such as, bitter, milk and white depending on the milk, sugar, cocoa bean, cocoa butter and condiment materials [2]. Among these types, bitter chocolate has the least amount of added fat and the largest cocoa mass. Milk and white chocolates contain high amounts of cocoa butter and milk. While these two types generally have similar components, white chocolate does not contain cocoa mass [3].

The properties and definitions of cocoa and chocolate products are specified in the EC Directive 2000/36 [4]. Despite its high sugar and fat content, chocolate also contains vitamins, carbohydrates, polyphenols and minerals, such as calcium, copper and iron etc. [5]. For example, it is known that dark chocolate

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contains at least 56 elements. While potassium, magnesium, calcium and phosphorus are the most abundant macro elements, iron is also the most abundant micro element. Furthermore, these elements (macro and micro) play an important role in the proper functioning of living organisms [6]. In addition, it is known that heavy metal contents in chocolate samples are also increased towards high concentrations due to the heavy metal content of cocoa [4]. Therefore, it is necessary to determine the metal content in chocolate samples.

Nowadays, many different analytical techniques are used to determine the metal content of food samples, such as flame atomic absorption spectrometry [7], graphite furnace atomic absorption spectrometry [8], cold vapour atomic absorption spectrometry [9], inductively coupled plasma optical emission spectrometry [10], inductively coupled plasma mass spectrometry [11] and X-Ray fluorescence spectroscopy [12]. Among these techniques, FAAS is the preferred techniques in metal determinations due to its advantages, such as simplicity, speed, accurate and precision measurement [13]. Before the analysis, it is an important issue in the digestion of food products with high organic matter content, such as chocolate. Microwave digestion is a fast and reliable method that can be used in the digestion of such foods [14].

In this study, the metal contents of bitter, milk and white chocolate samples were determined. For this, the chocolate samples were digested by microwave digestion system and Na, K, Mg (II), Ca (II), Cu (II), Fe (III), Zn (II), Cd (II), Cr (III), Pb (II) ve Ni(II) were determined by FAAS. Three different measurements were made to determine the concentration of the elements in the chocolate samples and the results were given at 95% of confidence level. The accuracy of the method was verified by the experiment performed on the standard reference material (spinach leaves, NIST-1570a).

2. Material and Method

2.1. Apparatus

The determination of all studied metals in this study were carried out using a flame atomic absorption spectrometer (ATI Unicam 939 model) and the operating conditions in the determination step were given in Table 1.

The microwave digestion system (CEM, Mars 6 240/50 model) was used to digest the chocolate samples and standard reference material. The digestion process for all samples was as follows: 15 min/heating, 15 min/thawing (200°C), 15 min/cooling.

Table 1. Operation conditions for metal determination

Metals	Wavelenght (nm)	Slit width (nm)	Lamb current (mA)
Ca	422.7	0.5	6.0
Mg	285.2	0.5	4.0
Cu	324.8	0.5	5.0
Fe	248.3	0.2	15.0
Zn	213.9	0.5	10.0
Cd	228.8	0.5	8.0
Cr	357.9	0.5	12.0
Pb	217.0	0.5	10.0
Ni	232	0.2	15.0

*Na and K measurement were performed in emission mode of FAAS.

2.2. Reagents

All solutions were prepared with analytical reagent grade. The resistivity of 18.2 MΩ·cm of ultra-pure water was obtained by PURIS, Expe-up water purification system. The concentrated HNO_3 and H_2O_2 solutions were purchased from Merck. The stock aqueous solution (1000 mg/L) of Na, K, Mg, Ca, Cu, Fe, Zn, Cd, Cr, Pb and Ni were prepared by dissolving their salts, such as NaCI (Sigma-Aldrich), KNO_3 (Merck), $Mg(NO_3)_2{\cdot}6H_2O$ (Riedel-de Haën), $CaCI_2{\cdot}2H_2O$ (Sigma-Aldrich), $CuSO_4{\cdot}5H_2O$ (Sigma-Aldrich), $FeCI_3{\cdot}6H_2O$ (Merck), $ZnCI_2$ (Merck), $Cd(NO_3)_2{\cdot}4H2O$ (Aldrich), $Cr(NO_3)_3{\cdot}9H_2O$ (Merck), $Pb(NO_3)_2$ (Merck) and $Ni(NO_3)_2{\cdot}6H_2O$ (Merck), respectively.

2.3. Collection and preparation of chocolate samples

A total of 24 chocolate samples from 8 different brands of bitter, milk and white chocolate varieties were purchased from Burdur city markets at Turkey and websites.

During the digestion of the chocolate samples, about 1.0 g of chocolate samples was firstly placed in teflon vessels and 5 mL of concentrated HNO_3 and 3 mL of concentrated H_2O_2 were added in the vessels. In the experimental studies, the determination of Cu(II), Cd(II), Cr(III), Pb(II), and Ni(II), which were found in trace levels in chocolate samples, were directly performed in FAAS without any dilution. However, for the determination of Na, K, Mg(II), Ca(II), Zn(II), and Fe(II) metals, the necessary dilution processes (5-250 fold) based on metals were carried out to enter their concentrations in the linear range of calibration graphs. Afterwards, their concentrations found in the calibration graphs were multiplied by dilution factors based on metals to find their exact concentrations.

In addition to this, 0.0732 g of standard reference material (spinach leaves, NIST-1570a) was digested with 10 mL of concentrated HNO_3 using the digestion procedure described in Section 2.1.

3. Results and Discussion

In this study, the concentrations of Na, K, Mg (II), Ca(II), Cu(II), Fe(III), Zn(II), Cd(II), Cr(III), Pb(II) and Ni(II) in the chocolate samples after digestion procedure were determined by FAAS. The standard solutions were prepared between 0.5 and 10 mg/L depending on metals and the linear equations of calibration graphs were obtained by using the absorbance and intensity values of standard solutions as shown in Table 2. The concentrations of metals in the chocolate samples were calculated by these linear equations depending on metals.

Table 2. Linear equations obtained from calibration graphs

Metals	Lineer equation	R^2
Na	y = 5.6718x + 4.6974	0.993
K	y = 3,498x + 1.7449	0.997
Ca	y = 0.0138x + 0.0015	0.999
Mg	y = 0.0317x + 0.0129	0.999
Cu	y = 0.0357x + 0.0027	0.996
Fe	y = 0.0182x +0.0067	0.998
Zn	y = 0.109x + 0.0015	0.994
Cd	y = 0.061x + 0.008	0.999
Cr	y = 0.012x + 0.006	0.990
Pb	y = 0.036x -0.004	0.999
Ni	y = 0.0193x- 0.0004	1.000

3.1. Na and K amounts in the chocolate samples

Sodium (Na) and potassium (K) are both considered essential mineral nutrients [15]. The amounts of Na in the chocolate samples were shown in Table 3. In this study, the concentration of Na was found to be the lowest in a bitter chocolate sample marked with 5 whose concentration was 429±7 mg/kg, whereas concentration in white chocolate sample marked with 8 was found to be the highest with the value of 3936±281 mg/kg.

Table 3. Amount of Na in the chocolate samples, $\bar{x} \pm \frac{ts}{\sqrt{N}}$, N:3

Brand	Bitter	Chocolate type milk	White
1	2166±47	2749±50	3477±50
2	638±14	3131±129	3271±76
3	496±10	2158±75	3811±82
4	647±25	2927±47	3070±57
5	429±7.0	3440±25	3228±80
6	440±40	2290±278	2393±62
7	1087±12	3237±97	2833±72
8	697±14	2624±50	3936±281

The amounts of K in the chocolate samples were given in Table 4. While the concentration of K was found to be the lowest in a milk chocolate sample marked with 2 whose concentration was 5331±87 mg/kg, that of K was found to be the highest with the value of 17972±57 mg/kg in a bitter chocolate sample marked with 1.

Table 4. Amount of K in the chocolate samples, $\bar{x} \pm \frac{ts}{\sqrt{N}}$, N:3

Brand	Bitter	Chocolate type milk	White
1	17972±57	6892±336	6101±80
2	6502±85	5331±87	6003±430
3	12315±55	6142±72	6245±80
4	17381±258	7779±80	8849±85
5	11411±67	9094±70	6832±119
6	10571±147	7430±72	7337±70
7	6084±169	8530±42	8477±70
8	8903±94	7322±122	9025±70

3.2. Mg(II) and Ca(II) amounts in the chocolate samples

Magnesium (Mg) is an essential metal for the body that acts as a cofactor of more than 300 enzymatic reactions. It was reported that some diseases, such as hypertension, diabetes, osteoporosis, glucose intolerance and myocardial infarction may be associated with magnesium deficiency [16]. The amounts of Mg(II) in the chocolate samples were shown in Table 5. The content of Mg(II) was in a range of between 291±20 and 4805±249 mg/kg, where the lowest concentration was found in a white chocolate sample marked with 2, and the highest concentration was found in bitter chocolate marked with 6.

Table 5. Amount of Mg(II) in the chocolate samples, $\bar{x} \pm \frac{ts}{\sqrt{N}}$, N:3

Brand	Bitter	Chocolate type milk	White
1	3276±42	665±86	408±42
2	1831±75	436±42	291±20
3	2178±87	704±112	1256±117
4	1618±221	2090±159	546±50
5	4363±250	1319±70	840±82
6	4805±249	800±124	544±59
7	1836±124	1198±122	458±97
8	4542±234	947±85	636±70

Calcium (Ca) is another essential mineral which is the most abundant in the body. Ca is essential for growth and bone density, also keeps the heart pumping, muscles moving and nerves in communication [17]. The amounts of Ca(II) in the chocolate samples were given in Table 6. The amounts of Ca(II) were in a range of between 995±77 and 35436±880 mg/kg, where the lowest concentration was found in a bitter chocolate sample marked with 6, and the highest concentration was found in white chocolate marked with 5.

3.3. Cu(II) amount in the chocolate samples

Determination of the copper (Cu) in the chocolate samples is an important issue for its compounds because they are widely used as fungicides in cocoa cultivation. In addition, Cu plays a role in a variety of biological activities in the body and it is necessary for a strong immune system. For this reason, it is important to determine the amount of it in the chocolate samples for nutritional adequacy [18]. The amounts of Cu(II) in the chocolate samples were given in Table 7. The content of Cu was determined in only bitter chocolates. The concentration of Cu was in a range of between 6.9±1.0 mg/kg (brand 2) and 15.8±0.7 mg/kg (brand 4).

Table 6. Amount of Ca(II) in chocolate samples, $\bar{x} \pm \frac{ts}{\sqrt{N}}$, N:3

Brand	Bitter	Chocolate type milk	White
1	4090±191	4351±186	4165±291
2	1236±34	1951±37	4127±395
3	1293±142	5745±283	6856±270
4	1398±57	5831±263	4175±286
5	1675±40	7933±184	35436±880
6	995±77	5034±375	7135±288
7	1870±57	7457±181	5660±181
8	1051±70	5816±286	6855±201

Table 7. Amount of Cu in chocolate samples, $\bar{x} \pm \frac{ts}{\sqrt{N}}$, N:3

Brand	Bitter	Chocolate type milk	White
1	14.4±0.6	N.D.[a]	N.D.
2	6.9±1.0	N.D.	N.D.
3	9.1±1.0	N.D.	N.D.
4	15.8±0.7	N.D.	N.D.
5	11.8±0.7	N.D.	N.D.
6	15.6±0.5	N.D.	N.D.
7	7.30±0.7	N.D.	N.D.
8	9.4±1.5	N.D.	N.D.

[a]N.D.= Not detected

3.4. Fe(III) amount in the chocolate samples

Iron (Fe) is one of the major trace metals and its deficiency causes anaemia. On the other hand, excess amount Fe intake in the body may be associated with important ailments, such as heart disease, liver disease and cancer [19]. The amounts of Fe(III) in the chocolate samples were shown in Table 8. The concentration of Fe was in a range of between N.D. (not detected) and 363±50 mg/kg, where the highest concentration was found in bitter chocolate marked with 5.

Table 8. Amount of Fe in chocolate sample, $\bar{x} \pm \frac{ts}{\sqrt{N}}$, N:3

Brand	Bitter	Chocolate type milk	White
1	8.9±1.0	24.7±0.5	N.D.[a]
2	67.9±7.0	20.5±1.5	7.6±1.2
3	222±10	85.4±3.0	21.3±1.0
4	83.7±7.5	47.1±5.0	18.5±1.5
5	363±50	81±7.0	14.1±1.0
6	89±6.0	14.8±2.0	7.9±1.5
7	114±22	32.6±1.0	10.6±2.0
8	128±4.0	32.3±1.0	N.D.

[a]N.D.= Not detected

3.5. Zn(II) amount in the chocolate samples

Zinc (Zn) is an important trace element which is involved in many biochemical events in the biological systems. Its deficiency can cause some diseases, such as eye and skin lesions, diarrhea, impaired wound healing and growth retardation [20]. In this study, the concentration of Zn was in a range of between 4.6±0.2 and 65±1.0 mg/kg, where the lowest concentration was found in a white chocolate sample marked with 2, and the highest concentration was found in bitter chocolate marked with 6. The amounts of Zn(II) in the chocolate samples were given in Table 9.

Table 9. Amount of Zn in the chocolate samples, $\bar{x} \pm \frac{ts}{\sqrt{N}}$, N:3

Brand	Bitter	Chocolate type milk	White
1	39±1.5	10±0.2	8.5±0.2
2	46±1.5	11±1.5	4.6±0.2
3	29.7±0.5	9.5±0.7	12.4±0.5
4	57±1.5	37±2.5	8.9±1.2
5	44±1.2	25±1.5	11±0.2
6	65±1.0	61±2.0	10±0.2
7	45±3.5	33±2.0	11±0.7
8	35±1.5	30±2.5	12±0.5

3.6. Cd(II), Cr(III), Pb(II) and Ni(II) amounts in the chocolate samples

Cadmium (Cd) is another dangerous metal even at trace levels and it was reported that exposure of it can cause liver damage or kidney failure [21].

Chromium III (Cr(III)) is an essential micronutrient for the body. It has a beneficial effect on the metabolism of carbohydrates, lipids and certain proteins, and increases to insulin activity in diabetic patients [22].

The presence of lead (Pb) in cocoa and its derivatives has been investigated for many years. Pb is harmful to the human body even at trace level. Calcium metabolism may be affected after Pb enters the human body. It can cause abdominal and headache, hypertension, central nervous system disorder, and kidney dysfunction [21].

Nickel (Ni) amount in the chocolate samples has been determined by researchers in the different studies. The chocolate samples can be contaminated during the chocolate production process with Ni. Moreover, cocoa butter is another essential ingredient that may contain high concentrations of Ni. While trace amounts of Ni act as an activator of some enzymes, it may cause bronchial bleeding and accumulate in the lungs at high levels of Ni [23].

In this study, the amounts of Cd(II), Cr(III), Pb(II) and Ni(II) in all chocolate samples were not detected because the amounts of these metals were lower than the value of LOD for FAAS detection.

3.7. Validation of method

The accuracy of the method was evaluated to determine Manganese (Mn) in a certified reference material (spinach leaves, NIST-1570a). The accuracy of the method was calculated from three replicate measurements at 95% confidence level. The Mn value was found to be 68.9±4.0 mg/kg (recovery: 91%), which was in good agreement with certified value (75.9±1.9 mg/kg), in which relative error was found to be -9%.

4. Discussion and Conclusion

In this study, the amounts of Na, K, Mg(II), Ca(II), Cu(II), Fe(III), Zn(II), Cd(II), Cr(III), Pb(II) and Ni(II) in three different types of chocolates (bitter, milk and white) purchased from 8 different producers were investigated. Based on experimental results, the investigated chocolate samples can be considered as a good source of essential metals. Because, essential metals, which are Na, K, Mg(II), Ca(II), Cu(II), Fe(III), Zn(II) in the chocolate samples might significantly contribute to healthy nutrition. In addition, it was found that bitter chocolate was especially richer for essential metals. The differences between the results may be due to the contents of the different brands of chocolate samples which were not used in the same proportions during the production. Furthermore, some comparisons were performed for the maximum allowable limits of metals in chocolates and/or their raw materials with obtained results in this study. For example, according to the European Legislation, the maximum tolerable levels for Pb(II) and Cd(II) in cocoa powder should be 1 mg/kg and 0.3 mg/kg, respectively [4]. In our study, Pb(II) and Cd(II) amounts in the investigated chocolate samples do not have a hazardous effect because the amount of them was lower than the value of LOD for FAAS detection. Secondly, ıt has been stated that the average content of Ni in chocolate types can be 3.8 mg/kg in the literature [24]. In our study, Ni(II) amounts were also lower than the value of LOD for FAAS detection.

Table 10. Comparison of investigated metals with literature data

Metals, mg/kg	Other studies			This study
	Bitter	Milk	White	
Na	45.6-270 [5] 43-5590 [25] 45-435 [26]	484-1095 [5] 634-1281 [26]	728-5001 [5]	429-2166 (B)[a] 2158-3440 (M)[b] 2393-3936 (W)[c]
K	4885-8574 [5] 4950-12700 [25]	4379-6546 [5]	3555-7442 [5]	6084-17972 (B) 5331-9094 (M) 6003-9025 (W)
Mg	1083-2775 [5] 855-1610 [25] 1104-1457 [26]	580-821 [5] 460-693 [26]	147-265 [5]	1618-4805 (B) 436-2090 (M) 291-1256 (W)
Ca	653-903 [5] 118-919 [25] 449-1421 [26]	1584-2449 [5] 1422 – 2447 [26]	2056-2467 [5]	995-4090 (B) 1951-7933 (M) 4127-35436 (W)
Cu	7.6-19.5 [5] 9.5-17.7 [25] 8.51 - 15.05 [26] 1.4-4.4 [18]	3.0-5.4 [5] 3.09 - 6.10 [26] 1.7-4.2 [18]	<0.16 [5]	6.9-15.8 (B) [d]N.D. (M) N.D. (W)
Fe	57-227 [5] 47-211 [25] 86.6 – 150 [26] 15 -51 [18]	20-62.7 [5] 20.1 - 88.1 [26] 3 – 60 [18]	<1.6-7.9 [5]	8.9-363 (B) 14.8-85.4 (M) N.D.-21.3 (W)
Zn	14-33 [5] 16.3-28.9 [25] 18.5 - 25.9 [26] 3.2-19 [18]	8.40-13.4 [5] 9.11 - 17.0 [26] 4 – 27 [18]	4.8-6.8 [5]	29.7-65 (B) 9.5-61 (M) 4.6-12.4 (W)
Cd	0.007-0.040 [4] 0.022-0.242 [25] 0.02-0.19 [18]	0.01-0.14 [18]	-	N.D. (B) N.D. (M) N.D. (W)
Cr	< 0.6-2.8 [5] 0.23-1.69 [25]	<0.6 [5]	<0.6 [5]	N.D. (B) N.D. (M) N.D. (W)
Pb	0.019-0.895 [4] 0.25-0.86 [25] 0.06-1.4 [18]	0.029-0.25 [18]	-	N.D. (B) N.D. (M) N.D. (W)
Ni	5.0-10.2 [5] 1.90-5.90 [25] 0.2-5 [18]	3.2-5.1 [5] 0.2-2 [18]	3.6-9.6 [5]	N.D. (B) N.D. (M) N.D. (W)

[a]B: Bitter chocolate, [b]M: Milk chocolate, [c]W: White chocolate, [d]N.D.: Not detected.

Furthermore, according to Turkish Food Codex (TFC), the maximum allowable Fe, Zn and Cu values in food samples were indicated as 0.2-25 mg/kg, 2-50 mg/kg and 15 mg/kg, respectively [14]. While the obtained amount of Fe in our study was higher than the literature values, that of Zn and Cu were in good agreement with the literature values. Finally, the obtained results were compared with other studies in the literature and results were given in Table 10.

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Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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