Comparison of digestion methods and trace elements determination in chocolates with pistachio using atomic absorption spectrometry

METIN GÜLDAŞ

Summary

Digestion methods were validated using standard certified reference materials (BCR-185R, SRM-1577b and BCR-679). The microwave - assisted digestion was found the most reliable and accurate method for chocolate samples. The recovery rates were 96–102 %, 92–98 % and 90–96 % for the microwave digestion, the wet digestion and the dry ashing, respectively. The results obtained using certified reference materials were in a good agreement with certified values. Flame atomic absorption spectrometry (FAAS) was used for the determination of Fe, Cu, Zn, As and Hg. Graphite furnace atomic absorption spectrometry (GFAAS) was also used for the determination of Ni, Cd and Pb in chocolate with pistachio belonging to the same brand sold in different markets in Bursa, Turkey. In total, twelve chocolate samples were analysed for this purpose. Cu, Zn, Fe, Cd, Ni, Pb, As and Hg levels ranged from 9.15 to 10.61 $mg.kg^{-1}$, 14.05 to 16.68 $mg.kg^{-1}$, 2.31 to 3.67 $mg.kg^{-1}$, 0.01 to 0.03 $mg.kg^{-1}$, 0.33 to 1.52 $mg.kg^{-1}$, 0.001 to 0.04 $mg.kg^{-1}$, 0.004 to 0.02 $mg.kg^{-1}$and 0.008 to 0.02 $mg.kg^{-1}$, respectively. The elemental contents of the analysed samples were within the ranges reported by the legal authorities for chocolate and other foods.

Keywords

chocolate; heavy metals; trace elements; digestion methods; atomic absorption spectrometry

Chocolate is a food product with a high energy and nutritive value. It contains large amounts of fat (cocoa butter) and sugar. Cocoa butter melts at the body temperature, thereby contributing to the overall pleasurable, mouthwatering experience of chocolate ingestion [1].

The significance of trace elements and toxicological effects of heavy metals on human health and nutrition have been increasingly studies in recent years [2, 3]. Some elements (such as Cu, Zn and Fe) can act as nutrients and are important for health, while others (such as Ni, Pb, Cd, As and Hg) may be harmful for humans if excessive amounts are consumed [4].

Trace element contents may vary in foods for several reasons. These are the factors such as variety of crop source (cacao, nuts, pistachio etc.), fertilization, feeding, lactation period, genetic origin, seasonal or annual factors, conditions of harvest, storage, processing (e.g. hydrogenation of unsaturated fats by using Ni) and environmental pollution [5]. On the other hand, the maximum permitted levels of metals and other elements in foods are still under consideration in many countries. European Commission has recently specifically requested data for some foods where there is a lack of information about some toxic metals and trace elements [6].

The dietary contribution of Ni has been reported to range from 200 to 900 $\mu g.d^{-1}$ but the tolerable oral intake of Ni may be different for sensitized individuals [7, 8]. Regarding Pb, in addition to the failure of brain functions, it is known to damage liver, organs of the reproductive system and kidney. The toxic symptoms of Cd intoxication are gastrointestinal complaints, respiratory problems, nausea, diarrhea, kidney damage, hypertension and impaired reproductivity [8]. As and Hg are the elements that can cause acute toxicological effects as well as death.

A provisional tolerable weekly intake (PTWI) of the toxic heavy metals has been established at 25 $\mu g.kg^{-1}$, 7 $\mu g.kg^{-1}$ and 1.6 $\mu g.kg^{-1}$ of body weight for Pb, Cd and Hg, respectively [9, 10]. The limit for As in foods is 1 $mg.kg^{-1}$ according to WHO [9] and 0.5 $mg.kg^{-1}$ according to Turkish Food Codex [11]. The maximum permitted levels of Zn and Fe range between 2–50 $mg.kg^{-1}$ and 0.2–25 $mg.kg^{-1}$ in various foods. This level for Cu is 15 $mg.kg^{-1}$ for chocolate [11].

Inductively coupled plasma (ICP) is a new, efficient technique for element analysis, but flame

Metin Güldaş, Uludag University, Karacabey Vocational School, Department of Food Technology, Karacabey 16700 Bursa, Turkey. Tel: +90 224 662 0651, fax: + 90 224 676 5562, e-mail: mguldas@uludag.edu.tr

atomic absorption spectrometry (FAAS) and graphite furnace atomic absorption spectrometry (GFAAS) are still among the most commonly used methods for the determination of Cu, Ni, Pb, Cd, Mo and Zn in biological and food samples [3, 12, 13]. Prior to FAAS or GFAAS analyses, the samples should be first liquefied to a solution. For this reason, a digestion process is needed for solid samples. Dry, wet and microwave digestion are the main methods for trace heavy metals in solid samples. Sample digestion or decomposition is a critical phase in the analysis of products with high organic matter contents such as chocolate.

Problems observed with spectrophotometric analysis are usually associated with the insufficient decomposition of organic matter during digestion [14]. The digestion procedure depends upon the composition of the organic sample investigated, trace elements to be determined and methodology to be used for their determination [15]. With the increase of organic matter, decomposition or digestion of sample becomes more difficult.

In the trace element analysis, standard certified reference materials (CRM or SRM) play an important role in terms of accuracy and reliability. For this reason, analysis of CRM is carried out at first, and then the obtained results are compared with the certified values [15].

As announced in a survey by EU authorities, there is still a need to update data dealing with heavy metal contents in foods [4]. Only a limited number of studies on the determination of elements and heavy metals in chocolate has been published [5, 8, 15]. In this study, some minerals (Cu, Zn and Fe) and toxic heavy metals (Ni, Pb, Cd, As, and Hg) in one brand of chocolate were investigated using FAAS and GFAAS. As well, the most appropriate digestion technique for the extraction process was investigated.

MATERIAL AND METHODS

Sampling and apparatus

Twelve samples of chocolate with pistachio (containing chocolate constituents and at least 15% pistachio) belonging to the same brand were purchased from different supermarkets in the city

Tab. 1. Instrumental parameters for flame atomic absorption spectrometry.

	Elements				
	Fe	Cu	Zn	As	Hg
Lamp current [mA]	12	6	8	14	4
Wavelength [nm]	248.3	324.8	213.9	193.7	253.7
Slit width [nm]	0.2	0.5	0.5	0.2	0.5
Fuel gas flow [$dm^3.min^{-1}$]	2.2	1.8	3	2	–
Type of oxidant	Air/acetylene				–
Type of measurement	Flame	Flame	Flame	Flame + hydride vapour	Hydride vapour
Detection limit [$mg.kg^{-1}$]	0.009	0.004	0.001	0.001	0.001

Tab. 2. Instrumental parameters for graphite furnace atomic absorption spectrometry.

	Elements														
	Cd					Pb					Ni				
Lamp current [mA]	6					10					30				
Wavelength [nm]	228.8					283.3					232				
Slit width [nm]	0.7					0.7					0.2				
Detection limit [$mg.kg^{-1}$]	0.001					0.001					0.001				
Matrix modifier	Palladium 1%					Palladium 1%					–				
Temperature[a] [°C]	100	140	850	1650	2600	100	140	700	1800	2600	100	140	1400	2500	2600
Ramp[a] [$°C.s^{-1}$]	5	15	10	0	1	5	15	10	0	1	5	15	10	0	1
Hold[a] [s]	20	15	20	5	3	20	15	20	5	3	20	15	20	5	3
Air flow[a] [$m.min^{-1}$]	250	250	250	0	250	250	250	250	0	250	250	250	250	0	250

a - Atomization stages.

of Bursa, Turkey. It is normally expected that contents of elements change depending on chocolate brands. Therefore, only one brand was investigated in the present research to prove differences arising from digestion methods and to fix other factors that can cause differences in the contents of elements (brand variety, processing steps, processing conditions etc.).

Determination of Fe, Cu, Zn, As and Hg was made with Shimadzu AA-6701F (Kyoto, Japan) flame atomic absorption spectrometer. Shimadzu Hydride Vapour Generator AAS-HGV-1 (Kyoto, Japan) equipped to FAAS (AA-6701F) was used for As and Hg determination to provide accuracy. Ni, Cd and Pb were measured with Perkin-Elmer Analyst 700 (Überlingen, Germany) graphite furnace atomic absorption spectrometer. Instrumental conditions for FAAS and GFAAS are shown in Tab. 1 and Tab. 2.

Reagents

All reagents were of analytical grade unless otherwise stated. Ultrapure water used throughout the study was supplied by a Milli-Q reagent water system (Millipore, Danvers, Massachusetts, USA: resistivity of 18.2 MΩ cm). Sulfuric, nitric and hydrochloric acids, perchloric acid ($HClO_4$) and H_2O_2 were of suprapure quality (Merck, Darmstadt, Germany). All laboratory glassware was kept overnight in 10% (v/v) HNO_3 acid solution, rinsed with deionized water and dried in a dust-free environment prior to use. For the calibration procedure, standard solutions of metal ions were produced by diluting a stock solution of 1 000 mg.l^{-1} of the elements (CertiPUR; Merck).

Standard certified reference materials BCR-185R (Bovine Liver, Institute for Reference Materials and Measurements, Geel, Belgium), SRM-1577b (Bovine Liver, National Institute of Standards and Technology, Gaithersburg, Maryland, USA) and BCR-679 (White Cabbage, Institute for Reference Materials and Measurements) were used to verify the digestion methods.

Digestion Methods

Dry ashing

The results were subsidiary compared to validate the efficiency of the digestion methods utilized. For the dry ash method, 1–5 g of the sample was weighed and heated in a muffle furnace at (500 ± 25) °C until a white ash was obtained. After cooling, 1 ml of HNO_3 and 10 ml of ultrapure water were added to the material and the mixture was left on the hot plate heater to dissolve completely. The solution was then completed to 50 ml with ultrapure water.

Wet digestion

A mass of 2 g of a homogenized chocolate sample was weighed and then 10 ml of concentrated HNO_3 (65%, v/v) and 15 ml of $HClO_4$ (70–72 %, v/v) were added. The mixture was heated up to (200 ± 10) °C on a hot plate for 3–4 h. The digestion was stopped when a clear solution was obtained. The residue was filtered through Whatman filter paper (No. 589/3). After cooling, the solution was diluted to 100 ml with ultrapure water.

Microwave digestion

A microwave (Berghof Speedway, MVS-3$^+$, Eningen, Germany) was used for the microwave digestion. A mass of 0.25 g of a homogenized chocolate sample was weighed and then 5 ml of concentrated HNO_3 (65%, v/v) and 2 ml of H_2O_2 (35%, v/v) were added. The samples were digested using the pressure microwave system according to the procedure recommended by the manufacturer and the operation conditions as given in Tab. 3.

All samples were analysed in triplicate.

Statistical evaluation

Statistical analyses were done using Statistical Package for the Social Sciences 15.0 for Windows (SPSS, Chicago, Illinois, USA). For the determination of mean differences among the chocolate samples, *t*-test was used.

Tab. 3. Operation programme of the microwave digestion system.

Stages	I	II	III	IV	V
Pressure [Pa]	35×10^5	35×10^5	35×10^5	35×10^5	35×10^5
Temperature [°C]	150	170	195	100	100
Rise time [min]	5	5	5	1	1
Hold time [min]	10	10	20	1	1
Power [Watt][a]	60	75	90	10	10

a – Power consumption during operation as %.

Tab. 4. Certified and measured means using various digestion methods of trace elements in standard certified reference materials.

Element	Certified value	Dry ashing	Recovery [%]	Wet digestion	Recovery [%]	Microwave digestion	Recovery [%]
As [mg.kg^{-1}]	0.033 ± 0.040[a]	0.03 ± 0.090	91	0.031 ± 0.011	94	0.032 ± 0.050	97
Cd [mg.kg^{-1}]	0.544 ± 0.012[a]	0.518 ± 0.110	95	0.530 ± 0.016	97	0.549 ± 0.014	101
Cu [mg.kg^{-1}]	277 ± 2.010[a]	265.9 ± 9.450	96	271.5 ± 5.160	98	274.2 ± 4.760	99
Pb [mg.kg^{-1}]	0.172 ± 0.014[a]	0.165 ± 0.025	96	0.167 ± 0.017	97	0.175 ± 0.017	102
Zn [mg.kg^{-1}]	138.6 ± 1.550[a]	131.7 ± 9.650	95	135.1 ± 7.430	97	141.3 ± 5.870	102
Fe [mg.kg^{-1}]	184 ± 3.190[b]	165.1 ± 8.910	90	169.3 ± 5.630	92	176.6 ± 6.560	96
Ni [mg.kg^{-1}]	27 ± 0.710[c]	25.6 ± 2.180	95	25.9 ± 1.290	96	26.7 ± 1.650	99
Hg [μg.kg^{-1}]	6.3 ± 0.630[c]	5.75 ± 0.960	91	5.98 ± 0.870	95	6.24 ± 0.700	99

Each mean value belongs to 5 replicates with standard deviations. a – BCR-185R Bovine Liver, b – SRM- 1577b Bovine Liver, c – BCR-679 White Cabbage.

RESULTS AND DISCUSSION

Evaluation of Methods

Validation of digestion methods

Although the microwave-assisted digestion is largely used for matrices of different kind, conventional methods such as wet digestion and dry ashing are still being used in many laboratories. Validation of digestion methods is necessary to assure that accurate and reliable results are obtained [16]. In the present research, BCR-185R Bovine Liver, SRM-1577b Bovine Liver and BCR-679 White Cabbage were used as standard certified reference material for the validation process.

As given in Tab. 4, the highest recoveries (96–102%) were obtained using the microwave digestion method. This was followed by wet digestion (92–98%) and dry ashing (90–96 %). The results were in good agreement with the given CRM values. Apparently, the most reliable and consistent results were obtained by the microwave digestion. The variabilities between the certified values and the values obtained from digestion methods were lower than 10% ($SD < 10\%$). It can be suggested that the microwave-assisted digestion provides better mineralization of chocolate samples. This technique is quite rapid and easier compared to the others. Wet digestion and especially dry ashing are the time consuming methods. Another problem arisen from conventional digestion methods, during the longer process, is the formation of oxides or elemental occlusion to siliceous particles in the samples [17, 18]. This might be a reason of the lowest recovery value (90%) of Fe after dry ashing of certified reference materials.

Comparison of digestion methods

Since trace elements are found in foods in small quantities, the significance of the differences between means was taken as 1% ($P < 0.01$). The

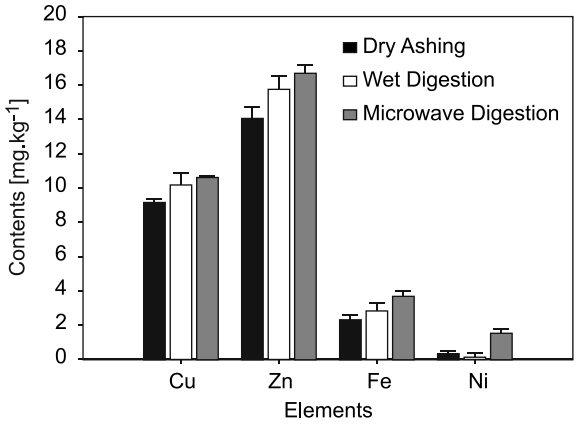

Fig. 1a. Cu, Zn, Fe and Ni contents in chocolates with pistachio (n = 12).

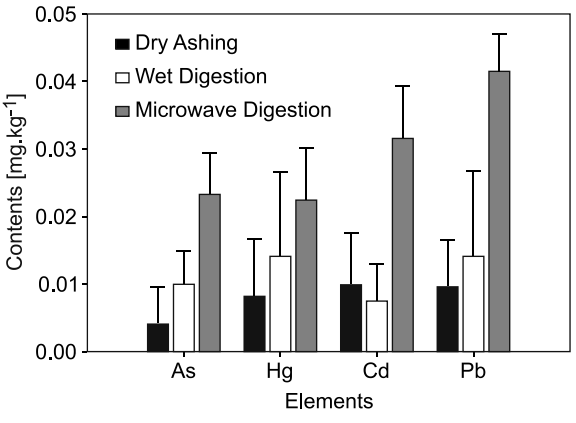

Fig. 1b. As, Hg, Cd and Pb contents in chocolates with pistachio (n = 12).

Tab. 5a. Comparison of wet digestion and microwave digestion.

	Mean difference	Standard deviation	95% confidence interval of the difference		*t*	Significance (2-tailed)
			Lower	Upper		
As	–0.0133[a]	0.0012	–00159	–0.0107	–10.35	0.000
Cu	–0.4358	0.1690	–0.7730	–0.0985	–2.57	0.0012
Hg	–0.0083 [a]	0.0024	–0.0131	–0.0034	–3.42	0.001
Zn	–0.9388 [a]	0.1523	–1.2426	–0.6351	–6.16	0.000
Fe	–0.8477 [a]	0.0961	–1.0395	–0.6560	–8.81	0.000
Cd	–0.0240 [a]	0.0016	–0.0272	–0.0209	–15.14	0.000
Pb	–0.0273	0.0094	–0.0461	–0.0085	–2.90	0.005
Ni	–0.9175 [a]	0.0508	–1.0188	–0.8161	–18.05	0.000

a – Significant at 1% level.

Tab. 5b. Comparison of dry ashing and wet digestion.

	Mean difference	Standard deviation	95% confidence interval of the difference		*t*	Significance (2-tailed)
			Lower	Upper		
As	–0.0058 [a]	0.0012	–0.0082	–0.0034	–4.86	0.000
Cu	–1.0244 [a]	0.1187	–1.2613	–0.7875	–8.62	0.000
Hg	–0.0058	0.0024	–0.0108	–0.0008	–2.33	0.0022
Zn	–1.6952 [a]	0.1674	–2.0292	–1.3612	–10.12	0.000
Fe	–0.5180	0.0944	–0.7064	–0.3296	–5.48	0.000
Cd	0.0024	0.0015	–0.0007	0.0055	1.55	0.125
Pb	–0.0044	0.0023	–0.0092	0.0003	–1.86	0.67
Ni	–0.2725 [a]	0.0254	–0.3231	–02218	–10.72	0.000

a – Significant at 1% level.

Tab. 5c. Comparison of dry ashing and microwave digestion.

	Mean difference	Standard deviation	95% confidence interval of the difference		*t*	Significance (2-tailed)
			Lower	Upper		
As	–0.0191 [a]	0.0013	–0.0218	–0.0165	–14.37	0.000
Cu	–1.4602 [a]	0.1240	–1.7076	–1.2129	–11.77	0.000
Hg	0.0141 [a]	0.0019	–0.0179	–0.0103	–7.41	0.000
Zn	–2.6341 [a]	0.1373	–2.9080	–2.3603	–19.18	0.000
Fe	–1.3658 [a]	0.0668	–1.4991	–1.2325	–20.43	0.000
Cd	–0.0216 [a]	0.0018	–0.0259	–0.0180	–11.91	0.000
Pb	–0.0317 [a]	0.0092	–0.0502	–0.0133	–3.43	0.001
Ni	–1.1900 [a]	0.0482	–1.2862	–1.0938	–24.67	0.000

a – Significant at 1% level.

values that were higher than 1% were statistically not important.

When compared to other digestion methods, highest values of trace elements were obtained using microwave digestion. This method was followed by wet digestion and dry ashing (Fig. 1a, 1b). The values obtained for all the tested techniques are presented in Tab. 5a, Tab. 5b and Tab. 5c.

When wet digestion and microwave-assisted digestion methods were compared (Tab. 5a), the differences between means of Cu and Pb elements were not significant; whereas the differences between means of As, Hg, Zn, Fe, Cd ve Ni elements were significant ($P < 0.01$). In other meaning, the comparison of wet digestion and microwave-assisted digestion techniques for determination of

Cu and Pb revealed no significant difference while statistically important differences were obtained for Hg, Zn, Fe, Cd and Ni ($P < 0.01$).

When dry ashing and wet digestion were compared (Tab. 5b); significant differences were obtained for As, Cu, Zn and Ni. Similar differences ($P < 0.01$) were observed with all the tested elements when dry ashing and microwave digestion methods were compared (Tab. 5c).

Determination of Cu, Zn, Fe, Ni, Pb, Cd, As and Hg in chocolates with pistachio

Using the microwave digestion technique, Cu, Zn, Fe, Ni, Pb, Cd, As and Hg contents in chocolates with pistachio were determined. The element determined in the highest amount was Zn, ranging between 14.05 mg.kg^{-1} and 16.70 mg.kg^{-1}. It was followed by Cu (9.15–10.61 mg.kg^{-1}), Fe (2.31–3.67 mg.kg^{-1}) and Ni (0.33–1.52 mg.kg^{-1}; Fig. 1a, Fig. 1b). From these values, it can be concluded that chocolates with pistachio were especially rich in terms of these nutritive elements such as Cu, Zn and Fe.

SROGI [3] found that Zn, Cu and Ni contents in chocolate sold in Poland were 4.01–11.01 mg.kg^{-1}, 0.85–2.41 mg.kg^{-1} and 1.09–2.23 mg.kg^{-1}, respectively. KARADJOVA et al. [2], reported the highest Cu and Ni values of 6.0 mg.kg^{-1} and 3.7 mg.kg^{-1}, respectively. Furthermore, in various studies; Cu and Fe contents were reported as 0.3–0.7 mg.kg^{-1} and 1.6–2.4 mg.kg^{-1} [1], Cu contents as 0.02–0.03 mg.kg^{-1} [19] and Ni contents as 0.041–8.29 mg.kg^{-1} [8]. According to Turkish Food Codex [11], the maximum limit for Zn is 50 mg.kg^{-1}, for Cu 15 mg.kg^{-1} in chocolate; for Fe 25 mg.kg^{-1} in various foods, and inorganic Sn 200 mg.kg^{-1}. The daily contribution of Ni to the diet was defined as 200–900 μg [6]. It was reported that the recommended daily amount of Zn [20], approximately 75% of Cu and 16% of Fe can be covered by chocolate with pistachio intake [11].

The amount of Ni which may be hazardous at an increased daily intake, or of highly toxic heavy metals such as As, Hg, Cd and Pb, were under the determined limits for chocolate samples (Fig. 1a, Fig. 1b). As did not exceed 25% of the limit value (1 mg.kg^{-1}) [9]. Pb was the highest contained toxic heavy metal in the chocolate samples, but its contents was lower than the limit value (0.04 mg.kg^{-1}) as well.

No information is available to the author's knowledge in the literature on the determination of As and Hg in chocolate. The amount of Pb was reported as 0.10-0.18 mg.kg^{-1} by SROGI [3], 0.07 mg.kg^{-1} by RANKIN et al. [21] and 4.0 mg.kg^{-1} by KARADJOVA et al. [2]. Different Cd values were reported by different authors. SROGI [3] reported that Cd contents varied between 0.01 and 0.05 mg.kg^{-1} in chocolates while KARADJOVA et al. [2] reported 0.123 mg.kg^{-1} in Greek chocolates. Contents with greater variations, between 0.001 and 2.73 mg.kg^{-1}, were determined by DAHIYA et al. [8].

The high nutritive value of chocolate of the studied type is mostly arising from high pistachio contents. The chocolate samples used in this research contained at least 15% pistachio [22]. Pistachio is a rich source of trace elements such as Cu, Zn and Fe. The contents of Cu, Fe and Zn have been determined as 8.5–12 mg.kg^{-1}, 24.0–36.5 mg.kg^{-1} and 17.1–26.9 mg.kg^{-1} in varieties of Siirt and Keten gomlegi of Turkish pistachio, respectively [23].

Although, the contents of As, Hg, Cd and Pb did not exceed the limit values prescribed by international authorities such as WHO or FAO, presence of these elements in the chocolate samples is thought to result from the their contents in main chocolate ingredients (cocoa seeds, pistachio, milk powder) which can be easily contaminated by pesticides and other environmental factors. The source of Ni is probably the hydrogenized vegetable oil (liquid margarine) used in the chocolate production.

The main and selected chocolate ingredients as skimmed milk powder, pistachio, cocoa powder,

Tab. 6. Contents of selected toxic heavy metals in some chocolate ingredients.

	Skimmed dried milk [mg.kg^{-1}]	Pistachio [mg.kg^{-1}]	Cocoa powder [mg.kg^{-1}]	White sugar [mg.kg^{-1}]	Margarine [mg.kg^{-1}]
As	0.089 ± 0.042	0.025 ± 0.016	0.043 ± 0.025	0.035 ± 0.026	0.030 ± 0.018
Hg	0.062 ± 0.005	0.024 ± 0.001	0.061 ± 0.054	0.044 ± 0.005	0.045 ± 0.015
Cd	0.047 ± 0.004	0.076 ± 0.012	0.181 ± 0.078	0.065 ± 0.004	0.029 ± 0.012
Pb	0.041 ± 0.021	0.028 ± 0.004	0.087 ± 0.032	0.041 ± 0.041	0.035 ± 0.018
Ni	–	–	–	–	0.082 ± 0.041

Values of mean of four replicates ± standard deviation are presented

white sugar and hydrogenized vegetable oil that were supplied from local markets were investigated to find out the possible sources of some heavy metals found in chocolate samples (Tab. 6). Arsenic was at the lowest level in pistachio among the other investigated ingredients. Cocoa powder was the ingredient which contained the highest levels of Hg, Cd and Pb. Contents of Pb were high also in other chocolate ingredients. Ni contents were high in the hydrogenized vegetable oil, as expected.

CONCLUSION

The analyses done using CRM and the statistical evaluations showed that microwave digestion is the most reliable method for chocolate samples, although wet digestion can alternatively be used for the analyses of Cu and Pb. Since the mean differences between ash drying and microwave digestion for all investigated elements were significant ($P < 0.01$), dry ashing technique can not be recommended as an alternative method. The microwave digestion technique is recommended for routine analysis of chocolate samples since it is more reliable, lesser time-consuming and cheaper.

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