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Article

Iron and Zinc Determination in Dietary Supplements by Flame Atomic Absorption Spectrophotometry

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Recent studies have demonstrated that 30% of the world's population suffers of anemia, half of the cases are related to iron deficiency, and the most common treatment is the use of iron supplementation. In this framework, the iron and zinc determination from different dietary supplements was performed by flame atomic absorption spectrophotometry. Concerning the dissolution of supplements, direct acid dissolution, wet digestion, and microwave digestion (MW) techniques were used for sample preparation. The iron and zinc recovery results demonstrated that the MW technique was the most appropriate for all of the supplements with the highest metal recovery yields. Moreover, the method validation parameters referred to a linear range for iron of 0.1–4 mg L^{-1} with a regression coefficient (R^2) of 0.9998 ± 0.002, while for zinc it was 0.01–1 mg L^{-1} (R^2 = 0.9997 ± 0.003). The limit of detection and quantification values were calculated as 0.03 and 0.09 mg L^{-1} for iron and 0.01 and 0.02 mg L^{-1} for zinc, respectively. The accuracy of the method was evaluated from the % recovery yield for iron and zinc, which, respectively, resulted in an oscillate of 99.2% to 102%, and 99.4% to 100.4% for the dietary investigated supplements. The precision of the method was determined by intra-day and inter-day precision with a relative standard deviation that was <2.0%.

Keywords: Iron. Zinc. Dietary Supplement. Validation.

INTRODUCTION

Iron and zinc play essential roles in biological systems and their shortage is responsible for the main micronutrient deficiencies in the world (Burke, Leon, Parminder, 2014). Iron is a fundamental component of various proteins and enzymes, and has a role in vital processes, such as oxygen and electron transport (Burke, Leon, Parminder, 2014; Asperti *et al.*, 2018; Musallam, Taher, 2018). Recent studies have demonstrated that 30% of the world's population suffers of anemia and half of the cases are related to iron deficiency (ID) (Gomez-Ramírez *et al.*, 2018). Particularly, iron deficiency anemia (IDA) occurs during infancy, adolescence, and pregnancy, causing morbidity and maternal mortality (Milman, 2012; Burke, Leon, Parminder, 2014; Kartal, Gursel, 2019) On the other hand, zinc is required for many specific enzymes, metallo-proteins, and the integrity of the immune system, and has importance in DNA and RNA metabolism. Zinc deficiency causes the retardation of growth and development, and morbidity (Hambidge, Krebs, 2007). The common method for the prevention and treatment of micronutrient deficiency consists of adequate dietary supplementation. As a consequence, the common use of dietary supplements requires simple, reliable, sensitive, and fast methods for elemental analyses.

Iron and zinc determination studies from different environmental and biological samples have been performed through potentiometry, flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma-mass spectrometry (ICP-MS), UV-Vis spectrophotometry, fluorometry, and voltammetry (Stephens, Suddeth, 1967; Stookey, 1970; Allen *et al.*, 1978; Mori *et al.*, 1989; Toral *et al.*, 1993; Gao, Siow, 1996; Aleixo, Nobrega, 2003;

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Meddourene *et al*., 2004; Perring, Blanc, 2008; Mao, He, Liu, 2009; Bizzi *et al*., 2010; Bakircioglu, Kurtulus, Ucar, 2011; Elango *et al*., 2021). Concerning iron determination via direct potentiometry, there are few sensors (Mahmoud, 2001). Moreover, many cations do interfere with iron determination using iron selective chemical sensors (Mao, He, Liu, 2009; Mahmoud, 2001). Further complications arise from the fact that most techniques for iron determination require the presence of selective complexing reagents whose employment is limited by the fact that a high salt concentration and ionic strength have negative effects on the stability of the complex between iron and the complexing reagent (Araújo *et al.,* 1997; Marczenko, 1986). Flame atomic absorption spectrophotometry is the most widely used method for metal analyses and does not require previous metal complexation.

It should be noted that the sample preparation step has great importance in the goodness of the analytical results. The above-mentioned elemental analysis techniques demand an effective sample preparation process in order to recover the analyte from dietary supplements with high yields. Literally, the precision and accuracy of method highly related with the sample preparation process (Bizzi, Nóbrega, Barin, 2014).

Despite the increasing supplementation, there are few studies on this topic. Generally, comparisons of the sample preparation techniques have been studied for trace elemental analysis from soil, biological materials, and food samples. For this purpose, direct acid dissolution (DD), wet digestion (WD), dry ashing (DA), and microwave-assisted digestion (MW) techniques are applied to different materials before the elemental analysis, and this step is defined as the bottleneck method due to the possibility of analyte losses, sample contamination, and incomplete sample digestion. Previous studies have demonstrated that, concerning the mentioned samples, the microwave digestion technique is fast and efficient in comparison with the WD and DA methods (Soylak *et al*., 2004). For instance, Somer and Unlu (2006) reported that low recovery yields were caused by volatilization during the digestion process or incomplete digestion. Moreover, the matrice effect of the high residual carbon content and high acidity on the different analyzing methods have been reported (Bizzi *et al*., 2017).

The previously reported iron recovery yields by different digestion process are summarized in (Table I). Soylak *et al*. (2004) compared the dry, wet, and MW of spice samples and found that the MW technique provided the highest recovery yield. On the other hand, Somer and Unlu (2006) demonstrated that, in the case of natural materials, the MW process can lead to low recovery results, independent of the analyzed element; therefore, they proposed the selection of the most appropriate sample preparation technique according to the composition of the analyzed material. In the case of dietary materials, the complexity of pharmaceutical products makes the investigation procedures different from those of other materials that require elaborate sample preparation (Canfranc *et al*., 2001).

Compared to the other sample preparation techniques, the advantages of MW were reported as complete digestion, less reagent consumption, less time request, the avoidance of metal losses by volatilization, less sample contaminations, and minimized residual carbon content and final acid concentration in digests (Doner, Ege, 2004; Korn *et al*., 2008; Reis, Almeida, 2008; Bizzi *et al*., 2014). On the other hand, the decomposition efficiency of MW was related with pressure, temperature, and the use of hydrogen peroxide as an oxygen source. Actually, in MW, the use of H_2O_2 with HNO_3 aided in organic material decomposition and nitric acid regeneration (Bizzi, Nóbrega, Barin, 2014).

Apparently, no previous studies on the comparison of sample treatment techniques on dietary supplements have been published. In the current work, direct acid dissolution (DD), and the wet and MW techniques were applied to 3 different supplements, including different iron oxidation states complexes. Moreover, in the case of Sucrosomial® Iron (SI), the ferrous complex was protected by a phospholipid bilayer and included a non-toxic dose sucrose ester. (Asperti *et al*., 2018; Fabiano *et al*., 2018). The FAAS technique was employed to determine the iron and zinc content and make the validation of the tested procedures. These method validation studies were performed according to the ICH Harmonized Q2 (R1) Validation of Analytical Procedures: Text and Methodology (2005).

TABLE I - Compilation of the results for iron determination from previous studies. Ashing. *Digestion Techniques:* MD = Microwave Digestion, WD = Wet Digestion, DA = Dry Ashing, DD = Direct Dissolution. *Analytical Techniques:* FAAS = Flame Atomic Absoprtion, V = Voltammetry, P = Potentiometry, S = Spectrophotometry, GFAAS = Furnace Atomic Absorption Spectrometry

Digestion Method	%Yield	%RSD	Analytical Method	Materials	References
MD	103	5.7	FAAS	Spice	(Soylak *et al*. 2004)
WD	98	8.8	FAAS	Spice	(Soylak *et al*. 2004)
DA	96	7.7	FAAS	Spice	(Soylak *et al*. 2004)
WD	95-103	4.2	V	Natural Products	(Gao and Siow 1996)
WD	98-100	<0.8	P	Dietary Supplements	(Mahmoud 2001)
MD	99	4.1	S[a]	Multivitamin	(Soriano *et al*. 2007)
DD	98-102	≤0.9	S	Multivitamin	(Tesfaldet, van Staden, and Stefan 2004)
DA	97-103[b]	1.8	FAAS	Dietary Supplements	(Canfranc *et al*. 2001)
MD	99	3.9	FAAS/ GFAAS	Biologic Materials	(Uluozlu *et al*. 2007)

[a]Authors was performed S and FAAS method the results are given with S method. [b]%Yield was calculated as Analytical Recovery by standard addition in placebo material.

MATERIAL AND METHODS

Reagents and chemicals

All of the chemicals used herein were of analytical grade and were employed without further purification. These chemicals included: hydrochloric acid (HCl) and nitric acid (HNO_3) (Sigma-Aldrich, St. Louis, MO, USA), perchloric acid ($HClO_4$) (Merck KGaA, Darmstadt, Germany), and stock solutions of iron (1000 mg L^{-1} in 5% HNO_3) and zinc (1000 mg L^{-1} in 5% HNO_3) (Agilent Technologies, Santa Clara, CA, USA) for preparation of the standard solutions.

Samples

The analyzed dietary supplements were commercially available and purchased from a local pharmacy. Information about the analyzed products are given in Table II.

All of the solutions and samples were prepared using ultrapure water (Tekkim Chemicals, Bursa, Turkey).

TABLE II - The analyzed dietary supplement name, content and producer company

The Supplement	The Producer Company	Content	Mass(mg)	Elemental Content (mg)
Ferrum Haussman Fort	Abdi İbrahim (Turkey)	Iron(III)-hydroxide Polymaltose Complex (IPC); Folic Acid (350µg)	630.2	100(Fe)
FerroZinc	Berko (Turkey)	Iron (II) Fumarate Complex (IFC); Zinc Sulphate monohydrate, Vitamin C(25 mg) and Folic Acid (400µg)	393.4	79.87(Fe)/ 25(Zn)
Sidefer Stick	Generica (Turkey)*	Sucrosomial® Iron, Ferric pyrophosphate covered by a matrix of phospholipids plus sucrose esters of fatty acids (SI); Vitamin C (48 mg), Folic Acid (150 µg), Vitamin B6 (1mg) and Vitamin 12 (2.5 µg)	160	14.46(Fe)

*Junia Pharma (Italy) is a supplier company.

Instruments and Experimental Procedures

The MW procedure was executed using the Speedwave Xpert, DAK-100, MW system (Berghof, Germany). Digestion was done in 3 steps and the conditions are given in Table III.

The amounts of metal ion were evaluated using the flame atomic absorption technique with an Agilent AA 240 instrument in air/acetylene flame. In order to evaluate the method reproducibility, the amounts of iron and the zinc ion were measured using a Perkin Elmer PinAAcle 900T FAAS instrument (Waltham, MA, USA). The instrument parameters are given, for both elements, in Table IV.

TABLE III - The microwave digestion parameters

Step	T(°C)	P(bar)	Time(min)	Power(Watt)
1	170	80	5	800
2	210	80	45	900
3	50	60	10	Ventilation

TABLE IV - FAAS instrumental parameters for the iron and zinc determination

Parameter	Iron	Zinc
Wavelength(nm)	248.3	213.9
Slit width(nm)	0.2	1.0
Light source	Single Fe Hollow Cathode Lamp	Multi Zn, Hollow Cathode Lamp
Power Supply(mA)	5	5
Flame, flow setting (L min^{-1})	Air (10.0), Acetylene (2.0)	Air (10.0), Acetylene (2.0)

Sample Preparation

The sample preparations were performed using 3 different treatment techniques. In the case of acid dissolution and WD, the mentioned supplements were ground and then weighted to equal the supplement mass (Table II) using an analytical balance.

The sample preparation process for the direct dissolution consisted of the addition of 3 mL of concentrated HCl to each sample, followed by the addition of ultrapure water up to a volume of 250 mL. Finally, the mixtures were sonicated for about 1 h.

In the WD technique, the sample treatment was performed by dissolving the samples in a (1:2) $HClO_4$:HNO_3 mixture. An acid mixture of about 15 mL was also added, step-by-step, until the nitrogenous gas exhibition was complete, and then the samples were heated for about 5 h in a water bath (100 °C). After the sample treatment completion, the sample (approximately 2.5 mL) volumes were brought to 250 mL with ultrapure water and the mixtures were sonicated for about 1 h.

The third sample treatment technique was based on MW. Wherein 0.1 g of each dietary supplement was digested in 15 mL of the HNO_3 (65%) and 3 mL of the H_2O_2 (30%) mixtures, as presented in Table III. Then, the volume of the mixtures was brought to 100 mL. Finally, the mixtures were sonicated for about 1 h.

After all of the sample treatment steps, the acquired mixtures were filtered through Whatman No. 42 filter paper (blue band) filter paper.

RESULTS AND DISCUSSION

The linear ranges were determined to be 0.1–4 mg L^{-1} for the iron and 0.05–1 mg L^{-1} for the zinc. The concentration ranges and the analytical curves for both elements are shown in Figure 1.

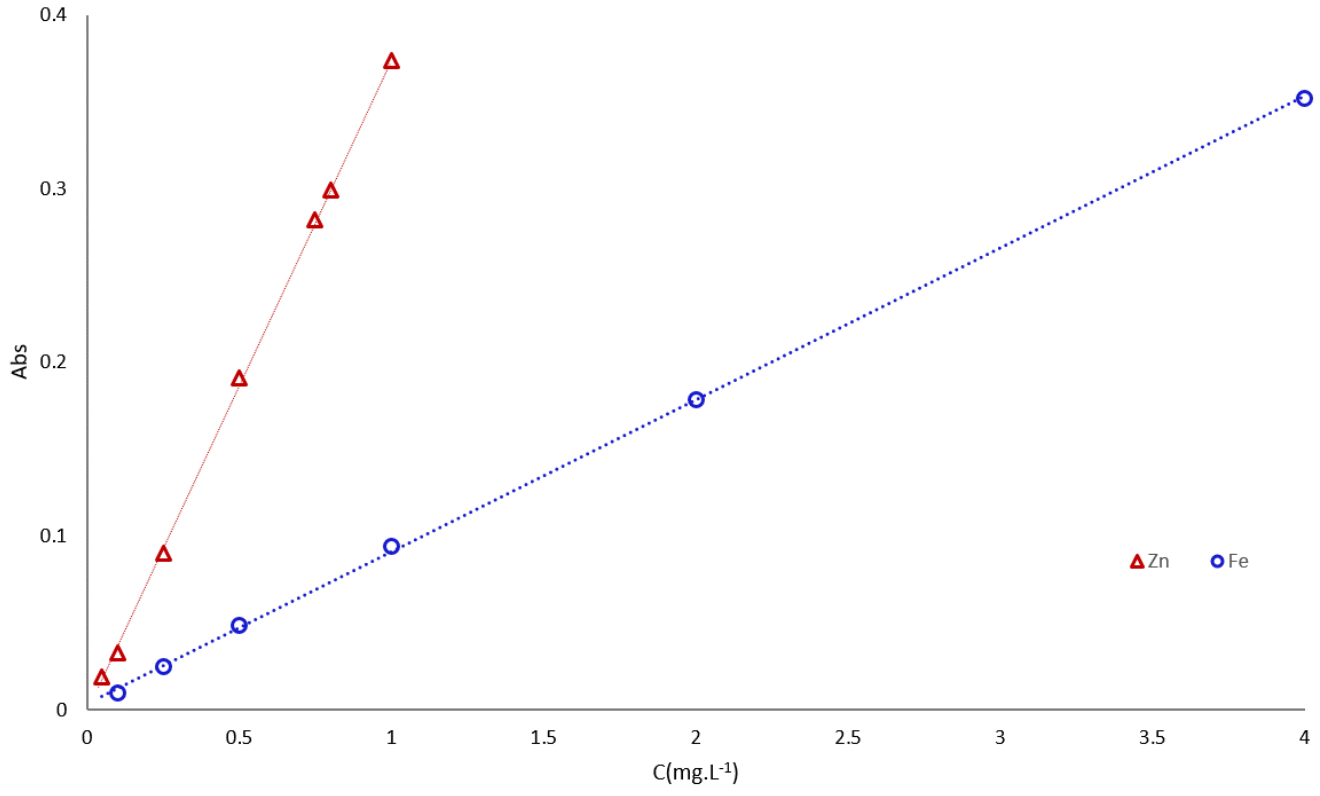

FIGURE 1 - Iron and zinc calibration graph.

The concentration range of both elements was in agreement with the range specified by the Turkish Pharmacopeia (2016), which was adapted from the European Pharmacopeia. Iron determination can be performed in the presence of zinc amounts up to 50 ppm, while the zinc determination can be performed in the presence of a maximum of 100 ppm of iron. The linearity parameters are also summarized in Table V. The limit of detection (LOD) is the lowest analyte (iron, zinc) concentration that can be detected within a certain level of statistical confidence, 3.33, while the limit of quantification (LOQ), the level of confidence is 10.

TABLE V - Linearity parameters of iron and zinc

Regression Parameters	Zinc	Iron
Equation	0.377x-0.002	0.09+0.003
Slope	0.3777±0.003	0.09±0.001
Intercept	-0.002±0.002	0.003±0.002
R^2	0.9997±0.003	0.9998±0.002
LOD	0.01	0.03
LOQ	0.02	0.09

The LOD and LOQ values of the employed methods were calculated using, respectively, Eq. (1) and Eq. (2), where *sd* is a low-concentrated sample standard deviation and *m* is the slope of the calibration curve. Hence, n ≥ 7 individual low-concentrated samples were prepared and measured, one-by-one.

$$LOD = \frac{3.33 \times sd}{m} \quad \text{Eq. (1)}$$

$$LOQ = \frac{10 \times sd}{m} \quad \text{Eq. (2)}$$

The effects of the measurement and delay times on the instrumental LOD, LOD, and regression coefficient were investigated. The relative standard deviation (%RSD) was evaluated for different delay and measurement times from the slope of each calibration curve, and was around 1.2%. On the other hand, the increment of delay and the measurement times induced a reduction of the instrument LOD and LOQ values. The values of LOD, LOQ and R^2 were, respectively, 0.5×10^{-2} mg L^{-1}, 2.1 mg L^{-1}, and 0.99985, with a 5-s delay time and 10-s measurement time. Concerning the zinc, the values were 0.4×10^{-2} mg L^{-1}, 1.1×10^{-2} mg L^{-1}, and 0.9997, respectively.

Furthermore, the effect of the different sample treatment methods on the iron and zinc recovery was investigated. The results, shown in Figure 2, indicated that, on one hand, the recovery yield depended on the nature of the dissolution procedure, while, on the other hand, it depended on the nature of the sample. MW appeared to be the most suitable dissolution method for the presently investigated materials.

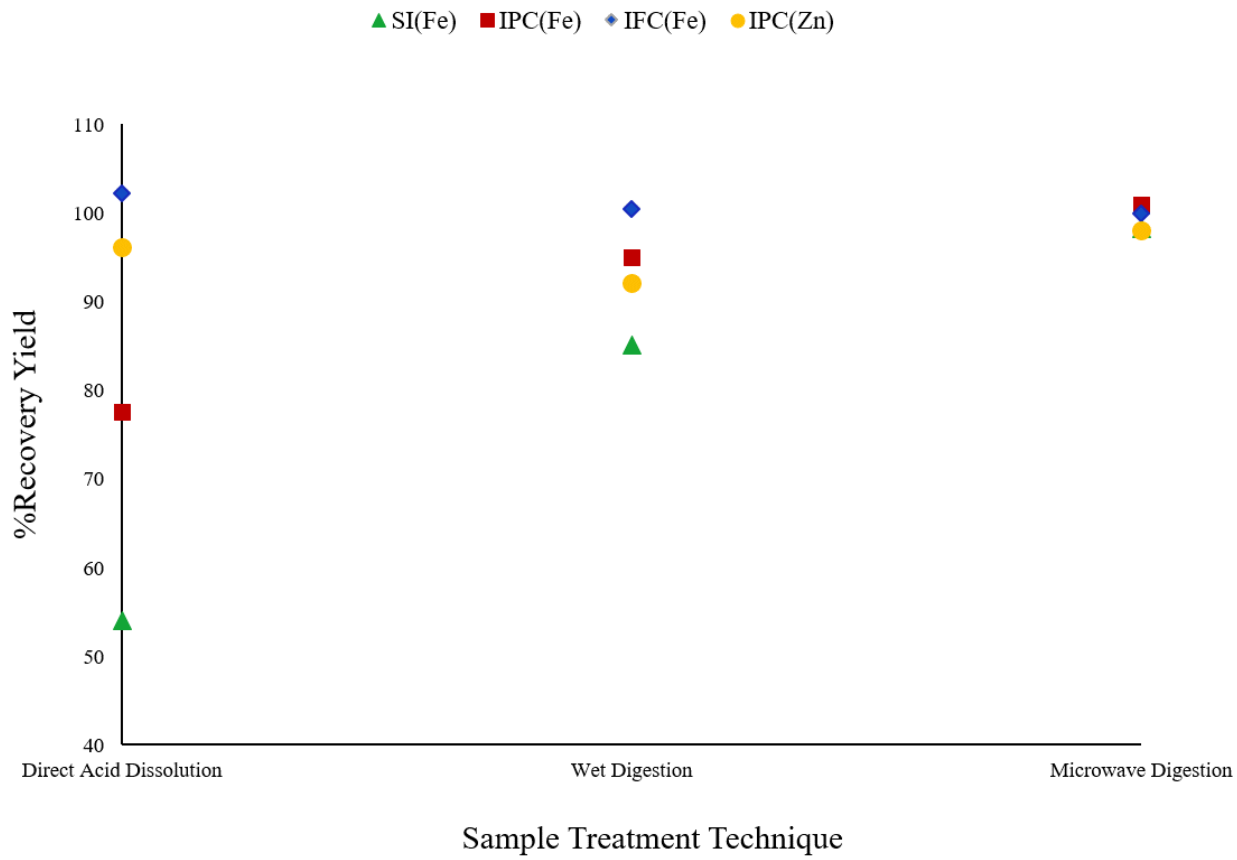

FIGURE 2 - Iron and zinc recovery dependence on the sample preparation technique (n = 9). Delay: 5 s, Measurement: 5 s. For the microwave-digested and wet digested samples %RSD was less than 0.8%, while for the directly dissolved samples, the %RSD was less than 1.8%.

The importance of the sample nature and chemical properties on the digestion efficiency was recognized by Gonzalez *et al.* (2009). Previous studies have pointed out that the DD or WD techniques could be suitable for relatively simple samples, while they seem to be unsuitable for samples that, owing to their complexity, require long dissolution times (Sneddon *et al.*, 2006; Bizzi, Nóbrega, Barin, 2014). Kingston and Jassie (1989) reported the digestion temperatures for carbohydrates, protein, and lipid molecules in concentrated nitric acid as 140, 150, and 160 ºC, respectively. Carrilho *et al.* (2001) observed that the fat present in biological samples had a significant effect on the digestion efficiency. Gonzalez *et al.* (2009) measured the %RCC values of different biological samples after MW in oxidizing media containing 14 mol/L of nitric acid. The obtained %RCC values were 45% for the bovine viscera sample, about 23% for soybeans grains, about 20% for bovine muscle, and 18% for bovine blood. In the case of coffee, Castro *et al.* (2009) determined the %RCC values of samples digested by microwave and conventional heating in a closed vessel. The decomposition yields were higher than 97% using both techniques in the presence of 3.5 mol/L nitric acid, but acid consumption was determined to be higher with the conventional heating system (Castro *et al.*, 2009; Bizzi, Nóbrega, Barin, 2014). Regarding the present work, it appeared that the sample matrices played a key role in digestion efficiency.

Actually, the recovery yield was very high (about 100%) for the 3 dietary supplements when treated using the MW method. By contrast, the DD method displayed the widest dependence on the sample nature, and the recovery yield decreased in the order of: [IPC (100) > IFC

(75) > SI (54)]. The WD method displayed an intermediate behavior [IPC (100) > IFC (92) > SI (85)]. The largest dependence of the recovery yield on the sample treatment technique was displayed by the SI [DD (54) < WD (85) < MW (100)], whereas the IFC showed an intermediate behavior. Somer and Unlu (2006) reported that incomplete digestion or volatilization of the sample during digestion could explain low recovery yields from biological material. In this context, the low recovery yields of the SI preparate herein could be explained in terms of incomplete digestion due to its phospholipid bilayer and sucrose esters content. MW appeared to be the most suitable sample treatment method for the presently investigated materials.

No comparison between the different digestion techniques on dietary supplements has been reported thus far. Previous investigations have shown that MW was the most favorable digestion technique for samples of botanical or biological nature. Sun, Chi, Shiue (2001) compared the MW, hot plate heating, and pressurized digestion techniques to determine heavy metal contents in sediments and concluded that MW was more feasible in order to decompose solid wastes. Furthermore, Soylak *et al.* (2004) investigated the DA, WD, and MW techniques for 12 different species and obtained higher recovery yields using MW. For instance, the recovery yields of zinc were, respectively, 96%, 97%, and 100%, while for iron, they were 96%, 98%, and 103%. Soriano, Netto, and Cassella (2007) determined the MW efficiency of multivitamin/mineral tablets in the presence of diluted HCl and HNO_3 for different digestion times and power levels (W). The best results were obtained with the HNO_3 where the iron and zinc % recovery yields from the commercial sample were determined as between 90% and 104%, and 93% and 105%, respectively, using the optimized FAAS method.

On the other hand, the iron and molybdenum levels in the dietetic materials were determined after the DA process. In order to evaluate the accuracy of the method, the recovery yields of the spiked samples were measured, and their values were determined to be 97.1%–103% and 95.2%–103%, respectively. However, the reported results did not give any information about the digestion efficiency.

Congruently, the zinc content present in the IFC samples was investigated and the %Recovery yields obtained for the different sample treatment techniques are shown in Figure 2. Again, the highest zinc recovery yield was obtained using MW technique, while the lowest was displayed using the WD technique. This finding suggested that volatilization may have occurred during the digestion process (Somer, Unlu, 2006; Reis, Almeida, 2008).

In order to evaluate the robustness of the method, the measurements were also repeated at different premeasurement and measurement times, and the relevant data are presented in Table VI.

For each method, the results were independent of the various combinations of the premeasurement and measurement times, as shown by the consistency of the metal determination value and was confirmed by the t test at a 95% confidence level. The %RSD values were determined as well and are given in Table VI.

TABLE VI - Iron and zinc levels from the microwave-digested samples at different premeasurement times and measurement times

Premeasurement time (S)	Measurement time(s)	SI Fe(mg)	IFC Fe(mg)	IPC Fe(mg)	IFC Zn(mg)
5	3	13.7±0.3	99.9±0.4	77.9±0.5	25.1±0.3
5	5	13.8±0.2	101.8±0.4	79.5±0.4	24.7±0.1.
10	5	14.2±0.2	100.2±0.3	78.8±0.4	24.5±0.1
10	10	14.3±0.2	100.4±0.4	80.2±0.6	25.1±0.2
5	10	14.6±0.2	102.5±0.2	78.1±0.8	24.4±0.1
Avearage		14.1±0.1	101±0.2	78.9±0.2	24.6±0.4
%RSD		0.77	0.16	0.32	1.1

In order to estimate the accuracy of the method, known amounts of iron standards were added to the samples and the iron and zinc contents of the sample were determined by FAAS, both with and without the standard addition. The difference between the 2 results was divided by the added amounts of iron and zinc. Three different spike levels were tested for each supplement and the %Recovery results are given in Table VII.

TABLE VII - The %Recovery of microwave digested dietary supplements for different spike percentages. (a) Iron, (b) Zinc

Sample	%Spike	%Recovery	%RSD
IPC (a)	50	102	0.4
	100	99.2	0.4
	150	99.3	0.4
IFC (a)	50	101.6	0.8
	100	101.8	0.3
	137.5	100.1	0.6
SI (a)	50	101.3	2
	100	100.4	1.9
	150	101.5	1.5
IFC (b)	25	100.2	1.4
	50	100.4	1.6
	75	99.4	0.6

The results demonstrated that the accuracy of method was adequate for the samples to within %RSD values lower than 2.

The instrumental precision was evaluated using the standard iron solution (4 mg L^{-1}). Nine consecutive measurements of the iron content were made, and the calculated relative standard deviation was 0.46%. Moreover, 6 iron standard solutions (4 mg L^{-1}) were prepared individually by the same analyst in the same laboratory on the same day, and the relative standard deviation value was 1.4%, thus indicating a good repeatability of the FAAS method.

In order to evaluate the precision of the method, repeatability and reproducibility studies were performed. In the repeatability study, 5 solutions of each supplement were prepared individually by the same analyst in the same laboratory on the same day. Moreover, intermediate precision studies were carried out as inter-day studies, in the same laboratory by the same analyst on 6 different days. The results of the intra- and inter-day studies are given in Tables VIII and IX.

TABLE VIII - Intra-day repeatability of the results for the investigated dietary supplements (Delay time =5 s, Measurement Times=5 s, n=9 times)

Experiment number	IPC[a] Fe(mg)	IFC[a] Fe(mg)	SI[a] Fe(mg)	IFC[b] Zn(mg)
1	78.1	99.9	11.9	24.6
2	77.6	98.2	12.6	25.1
3	77.7	101.6	12.1	24.8
4	76.4	99.5	12.2	24.8
5	76.6	97.1	12.2	24.5
Average	77.3	99.3	12.2	24.8
Standard Deviation	0.7	1.7	0.2	0.2
%RSD	1.0	1.7	1.9	0.9

[a] *Iron amount(mg)of acid digested dietary supplements,* [b] *zinc amount of microwave digested IFC sample.*

TABLE IX - Intermediate precision results for the investigated dietary supplements (Delay time =5 s, Measurement Time=5 s, n=9 times, A=Same analyst)

Analyst	Day/Dietary Supplement	IPC[a] Fe(mg)	IFC[a] Fe(mg)	SI[a] Fe(mg)	IFC[b] Zn(mg)
A	1	76.8	101.1	12.1	24.8
A	2	78.5	99.4	12.3	24.6
A	3	77.3	99.3	12.2	25.2
A	4	76.3	99.2	11.8	24.7
A	5	76.8	101.8	12.6	24.7
A	6	75.8	100.3	12.3	24.6
Average		77.4	100.2	12.2	24.8
Standard Deviation		1.5	1.1	0.3	0.2
%RSD		1.9	1.1	2.2	0.9

[a] *Iron content(mg) of acid digested dietary supplements,* [b] *zinc amount(mg) of microwave digested IFC sample.*

The method reproducibility was also investigated for the microwave-digested samples in two different laboratories by different analysts and the results are given in Table X.

TABLE X - Reproducibility studies on Microwave digested dietary supplements from two different laboratories

Sample	Lab I	Lab II	%RSD
SI/Fe(mg)	14.2	14.1	0.3
IFC/Fe(mg)	100	101.1	0.8
IPC/Fe(mg)	79.1	78.1	0.9
IFC/Zn(mg)	24.5	24.7	0.6

Tables VIII, IX, and X show that the precision of method for the studied samples was sufficiently good, and the relative standard deviation values were confined to within 2% in all the investigated systems.

In the present study, the determination of iron and zinc from 3 different dietary supplements was undertaken using the FAAS method. In addition, the effects of 3 different sample preparation methods were investigated, and among them, the most efficient was proven to be the MW technique, which displayed the highest iron and zinc recovery yield and the highest rapidity. Finally, concerning the validation studies, the linearity, precision, accuracy, and sensitivity results indicated that the FAAS is a simple, fast, and appropriate method to be used for iron and zinc elemental analysis from dietary supplements.

CONFLICT OF INTEREST

The author has no conflicts of interest to declare.

ETHICAL APPROVAL

The present investigation did not involve animal or human research participants.

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