Article

Multivariate Optimization of Microwave-Assisted Digestion of Copper and Zinc from Powder Milk

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Neste trabalho, um método fácil e rápido de digestão assistida por micro-ondas combinado com espectrometria de absorção atômica por chama (FAAS) foi desenvolvido para determinações de zinco e cobre em amostras de leite. A potência da irradiação, o volume de ácido nítrico, a temperatura e o tempo de digestão foram selecionados para a otimização deste trabalho. Os resultados do planejamento fatorial completo de 2 níveis (2^4) baseado em análise de variância (ANOVA) e diagrama de Pareto mostraram que todos os fatores são estatisticamente significantes. As condições ideais das extrações dos analitos em amostras de leite foram obtidas por metodologia de superfície de resposta (RSM) baseada no planejamento experimental Box-Behnken. Uma condição de compromisso entre as melhores condições para cada analito foi selecionada para a aplicação do método proposto, na determinação de zinco e cobre em amostras de leite. Estes valores foram 110 °C, 450 W, 4,0 mL e 21,0 min, para temperatura, potência da irradiação, volume de ácido nítrico e tempo de digestão, respectivamente.

In the present study, an easy and fast microwave-assisted digestion method combined with flame atomic absorption spectrometry (FAAS) was developed for zinc and copper determinations in milk samples. The irradiation power, volume of nitric acid, temperature and digestion time were selected for optimization in this work study. The results of 2-level full factorial (2^4) design based on analysis of variance (ANOVA) and Pareto chart showed that all of factors were statistically significant. The optimal conditions for the analytes extraction in milk samples were obtained by response surface methodology (RSM) based on Box-Behnken experimental design. The conditions of work were accepted as an agreement between optimal values that were found for the analytes. These values were 110 °C, 450 W, 4.0 mL and 21.0 min for temperature, irradiation power, volume of nitric acid and digestion time, respectively. This procedure was applied to the zinc and copper determinations in milk samples.

Keywords: zinc, copper, Box-Behnken design, microwave-assisted digestion, milk samples

Introduction

Milk provides all macronutrients (such as carbohydrates, lipids and proteins) and all micronutrients. Therefore, it is known as an almost complete nutritional product in the human diet.[1] This is especially true in the case of early childhood since milk is a unique food source in the first months of life of the baby and the growing children diet containing a high proportion of milk and dairy products. A sufficient intake of milk is also recommended for adults whereas calcium source for maintaining mass of the bone to prevent fractures and osteoporosis.[1,2] It is need to develop the exact analytical methods for analysis of zinc and copper in the milk samples for the quality control.

Copper is categorized as an essential element due to the fact that its deficiency affects iron metabolism in human infants, resulting in various clinical syndromes.[3] The infant milk intake normally fulfils this requirement, but the presence of a high copper concentration is toxic and causes acrodynia. Zinc is an integral part of enzymes such as carbonic anhydrase and alkaline phosphatase. Dairy products are reported to contribute up to 5% of the daily intake and hypogonadism and dwarfism resulting from its deficiency have been reported in the literature.[3] Multi-element control of micro- and macro-contaminants and/or constituents in complex matrices, such as milk, usually requires sample clean-up. This represents an important stage in analysis because it must be able to disaggregate the fat and protein matrix with minimum reagent addition. One of the most common procedures involves

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precipitation of the milk proteins with trichloroacetic acid,[4] dry ashing[5] and wet digestion[6] are also currently used. The preference for microwave radiation over other digestion methods for biological samples is evident in the literature.[7] The sequence analysis time can be reduced by using a microwave oven, thus leading to greater in analytical sampling rate.[8]

The response surface methodology (RSM) is a good tool for determining experimental factor settings[9] and assesses the relationship between experimental and observed results.[10] It is a mathematical tool that can aid to find the optimum conditions for a reaction with the minimum number of experiments to obtain statistically acceptable results.[11] The Box-Behnken is an experimental design that have also been used by other researchers for optimization studies.[12-14]

In this work, the microwave-assisted digestion system was used for the extraction of zinc and copper from milk samples and their determination by flame atomic absorption spectrometry (FAAS). The optimization of the irradiation power, digestion time, volume of nitric acid and temperature is described using a Box-Behnken matrix.

Experimental

Apparatus

The measurements were performed with a Konik Won M300 (Barcelona, Spain) flame atomic absorption spectrometer equipped with a conventional pneumatic nebulizer, and nebulization chamber was used for the analysis. Hollow cathode lamp for determination of copper and zinc was used. The most sensitive wavelengths for copper at 324.8 nm and for zinc at 213.9 nm were used with bandwidths of 1.2 and 0.5 nm for copper and zinc, respectively. The flame composition was acetylene (flow rate of 1.5 L min^{-1}) and air (flow rate of 10.0 L min^{-1}), and the burner height was set to 8 mm. The nebulizer flow rate aspiration was kept between 5.0 and 5.5 mL min^{-1}. The microwave digestion was carried out by an Ethos SEL apparatus (Milestone, Sorisole, Italy).

Materials

Nitric acid used was of the highest purity available from Merck (Darmstadt, Germany). Reagent grades of salts (all from Merck) were of the highest purity available. A stock standard solution containing 1000 mg L^{-1} of each element was used in preparing calibration standards. The calibration solutions were prepared from stock solution using deionized water immediately before analysis. The powder milk samples were purchased from local market in Iran.

Microwave-assisted procedure

The microwave-assisted digestion technique was adopted for the mineralization of the milk samples after some preliminary experiments. This method uses less reagent volumes and shorter digestion time.[1] Milk powder sample aliquot (0.5 g) was weighed and then transferred to a closed-vessel. Then, appropriate amount of nitric acid was added. According to a preliminary Box-Behnken experimental matrix, the digestions of samples were carried out at different conditions: digestion time from 16 to 30 min, volume of nitric acid from 2 to 6 mL, temperature ranging from 80 to 120 °C and irradiation power from 300 to 500 W. The resulted solution was transferred to 10 mL volumetric flask and brought up to the mark with doubly distilled water. The concentrations of zinc and copper in the solutions were determined by FAAS. The procedure was performed in triplicate.

Statistical analysis

The data were analyzed by the Pareto chart and the analysis of variance (ANOVA) was applied to examine the statistical significance of factors. A level of the probability of 0.05 was considered statistically significant. The Box-Behnken experimental design was used for final optimization. The MiniTab 14 software was applied for statistical analysis.

Factorial design

The factors chosen considering the microwave-assisted digestion system were: power (100-200 W), volume of nitric acid (4-8 mL), time (5-15 min) and temperature (60-80 °C). A two-level full factorial design (2^4) was performed in triplicate to determine the effect of each variable and its interactions in the procedure of extraction. Table 1 shows the experimental design and results derived from each run. ANOVA was used to investigate the significant of the effects. The Pareto chart (Figure 1) also shows the statistically significant factors. In the interpretation of this chart, it should be noted that the lengths of the bar are proportional to the absolute value of the estimated effects. A bar crossing this vertical line corresponds to a factor or a combination of factors that have a significant influence on response. This chart demonstrates that all of the factors were significant.

Table 1. Experimental design of the results obtained in function of the µg g⁻¹ of zinc

Trial No.	Power / W	Temperature / °C	time / min	Volume / mL	Amount / (µg *per* 0.5 g)
1	100	60	5	4	66.86
2	300	60	5	4	68.38
3	100	80	5	4	62.98
4	300	80	5	4	66.52
5	100	60	15	4	59.30
6	300	60	15	4	65.37
7	100	80	15	4	63.54
8	300	80	15	4	75.04
9	100	60	5	8	53.15
10	300	60	5	8	58.32
11	100	80	5	8	54.19
12	300	80	5	8	67.03
13	100	60	15	8	47.20
14	300	60	15	8	58.64
15	100	80	15	8	54.49
16	300	80	15	8	79.25
17	200	70	10	6	62.38
18	200	70	10	6	62.51
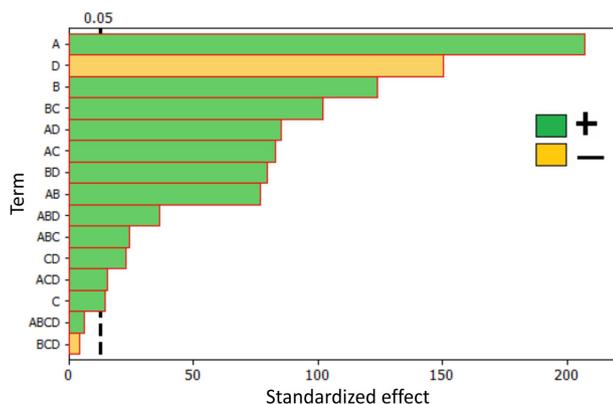
Figure 1. Pareto chart of main effects obtained from 2^4 full factorial designs. The vertical line defines the 95% confidence interval (A: power, B: temperature, C: time and D: volume of nitric acid).

Results and Discussion

Box-Behnken design

Factorial design demonstrated that the factors: power, temperature, volume of nitric acid and digestion time in the studied levels need a final optimization.

Therefore, a Box-Behnken experimental design was performed. In this study, the 3-levels, 4-factorial Box-Behnken experimental matrix was used to investigate the parameters that affected the digestion of these element: volume of nitric acid (2-6 mL), power (300-500 W), extraction time (16-30 min) and temperature (80-120 °C) were input variables, the factor levels were coded as −1 (low), 0 (central point) and 1 (high). The design of real experiments is given in Table 2.

The system behavior can be explained by the following quadratic equation:[15]

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i \chi_i + \sum_{i=1}^{k} \beta_{ii} \chi_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} \chi_i \chi_j + \varepsilon \qquad (1)$$

Here Y is the process response or output (dependent variable), k is the number of the patterns, i and j are the index numbers for pattern, β_0 is the free or offset term called intercept term, $\chi_1, \chi_2, \ldots$ and χ_k are the coded independent variables, β_i is the first-order (linear) main effect, β_{ii} is the quadratic (squared) effect, β_{ij} is the interaction effect, and ε is the random error or allows for description or uncertainties between predicted and measured value.

RSM modeling

In this study, multiple regression analysis was performed with analysis of the RSM for a quadratic response surface model. The response surface analysis allowed the development of an empirical relationship in which the response variable (Y) was assessed as a function of temperature (T), power (P), volume of nitric acid (V) and digestion time (t), four first-order effects (linear term in T, P, V and t), four second-order effects (quadratic terms in T^2, P^2, V^2 and t^2) and six interaction effects (interactive terms in TP, TV, Tt, PV, Pt and Vt). Table 2 shows the matrix and

Table 2. Design matrix in the Box-Behnken model, observed and predicted values

Trial No.	Power / W	Temperature / °C	time / min	Volume / mL	Observed, Y_a^a / (µg per 0.5 g)		Predicted, Y_p / (µg per 0.5 g)	
					Zn	Cu	Zn	Cu
1	300	80	23	4	69.66	2.98	68.26	3.28
2	500	80	23	4	75.28	3.36	75.47	3.48
3	300	120	23	4	72.47	1.94	70.61	2.34
4	500	120	23	4	91.66	5.62	91.38	5.84
5	400	100	16	2	71.71	4.14	68.47	4.37
6	400	100	30	2	64.85	3.98	63.08	4.15
7	400	100	16	6	68.36	3.61	68.45	3.95
8	400	100	30	6	70.23	4.29	71.80	4.57
9	300	100	16	4	68.65	4.31	66.56	4.12
10	500	100	16	4	74.57	3.92	75.87	4.09
11	300	100	30	4	62.32	2.79	60.86	2.44
12	500	100	30	4	77.58	6.15	79.52	6.17
13	400	80	23	2	69.47	3.95	70.63	4.22
14	400	120	23	2	71.47	3.24	72.37	3.36
15	400	80	23	6	68.64	2.94	67.59	2.65
16	400	120	23	6	85.43	5.38	84.11	4.93
17	300	100	23	2	65.76	1.12	69.73	0.81
18	500	100	23	2	77.62	5.92	76.60	5.43
19	300	100	23	6	64.12	3.43	66.97	3.58
20	500	100	23	6	90.20	2.70	88.06	2.66
21	400	80	16	4	64.48	4.86	66.09	4.56
22	400	120	16	4	70.68	5.24	73.01	4.99
23	400	80	30	4	63.36	4.57	62.86	4.48
24	400	120	30	4	73.97	5.51	74.19	5.47
25	400	100	23	4	63.30	4.53	64.30	4.80
26	400	100	23	4	65.05	4.82	64.30	4.80
27	400	100	23	4	64.56	5.06	64.30	4.80

[a]Average of triplicate extraction.

the amount (µg per g) of zinc and copper. The equations below explain the relationship of the four variables that are, P, t, V, T and Y (amount of zinc and copper in the samples).

For zinc:
$$Y = 459.538 - 1.3\,(P) - 4.116\,(T) - 2.353\,(t) - 27.135\,(V) + 0.001\,(P)^2 + 0.013\,(T)^2 - 0.01\,(t)^2 + 1.0\,(V)^2 + 0.002\,(T)(P) + 0.003\,(P)(t) + 0.018\,(P)(V) + 0.008\,(T)(t) + 0.092\,(T)(V) + 0.156\,(t)(V) \quad (2)$$

For copper:
$$Y = 9.05968 + 0.03\,(P) - 0.16\,(T) - 0.93634\,(t) + 2.07542\,(V) - 0.00009\,(P)^2 - 0.0005\,(T)^2 + 0.00554\,(t)^2 - 0.20302\,(V)^2 + 0.00041\,(T)(P) + 0.00134\,(P)(t) - 0.00691\,(P)(V) + 0.001\,(T)(t) + 0.01969\,(T)(V) + 0.015\,(t)(V) \quad (3)$$

The critical points in the surface response model are founded by solving these equations systems for the condition of $\frac{\delta(Y)}{\delta(T)} = 0$, $\frac{\delta(Y)}{\delta(P)} = 0$, $\frac{\delta(Y)}{\delta(V)} = 0$ and $\frac{\delta(Y)}{\delta(t)} = 0$. The way of calculating these critical points was published in previous study.[16] The calculated values for the critical point are as

follows: temperature of 105.0 °C, irradiation power of 490.0 W, volume of nitric acid of 3 mL and irradiation time of 20.0 min for zinc, and temperature of 113.0 °C, irradiation power of 399.0 W, volume of nitric acid of 4.5 mL and irradiation time of 20.1 min for copper. The working conditions were established as a compromise between optimum values which were found for each analyte. These values were 110 °C, 450 W, 4.0 mL and 21.0 min, for temperature, irradiation power, volume of nitric acid and time, respectively.

The summary of ANOVA is shown in Table 3. According to ANOVA, the F-value suggests that most of the differences in the response can be explained using the regression equation. The associated p-value is used to estimate whether F-value is large enough to show statistical significance. If the p-value is lower than 0.05, it then demonstrates that the model is statistically significant.[17] The results in Table 3 shows that the regression, linear, interaction and quadratic terms are significant. ANOVA also shows a term for residual error that measures the amount of variation in the response data left unexplained by the model. Analysis shows that the form of the model chosen to explain the relationship between

Table 3. ANOVA analysis for zinc and copper extractions

Source	Sum of squares	Degree of freedom	Mean of square	*F*-value	*p*-value
Zinc					
Regression	1500.856	14	107.204	16.5	< 0.0001
Linear	451.86	4	112.965	17.38	< 0.0001
Square	406.766	4	101.691	15.65	< 0.0001
Interaction	196.998	6	32.833	5.05	0.008
Residual	77.988	12	6.499		
Lack-of-fit	76.358	10	7.636	9.37	0.1
Pure Error	1.63	2	0.815		
Adjust-R^2	0.90				
Copper					
Regression	37.3063	14	2.66474	17.63	< 0.0001
Linear	5.5879	4	1.39699	9.24	0.0001
Square	8.7786	4	2.19465	14.52	< 0.0001
Interaction	16.6188	6	2.76980	18.33	< 0.0001
Residual	1.8136	12	0.15113		
Lack-of-fit	1.6727	10	0.16727	2.37	0.333
Pure Error	0.1409	2	0.07043		
Adjust-R^2	0.90				

the factors and the response is correct.[17] The non-significant value for lack-of-fit (> 0.05) shows that quadratic model is statistically significant for the response.

The three dimensional (Figure 2) response surface plots were used to determine the individual and cumulative effect of the variable and the reciprocal interaction between the variable and the dependent variable. The high R^2 values showed that the quadratic equation can represent the system under the given experimental domain.

This is also evident from the fact that the parity plot depicted in Figure 3 indicates a satisfactory correlation between the observed and predicted values of zinc and copper extraction efficiency.

As seen in Figure 3, the fact that the points cluster around the diagonal line shows a good fit of the model since the deviation between the observed and predicted values was less, similarly reported by Yetilmezsoy *et al.*[18] The significance of each coefficient was determined by student's *t*-test and *p*-values that are listed in Table 4.

The *p*-value is used as a tool to check the significance of each coefficient. The greater the magnitude of the *t*-value and the lower of the *p*-value, the most significant the factors are in the regression model.[18] Based on the sum of squares obtained from ANOVA, the percentages of contributions (PC) for each term were calculated and tabulated in Table 4.

$$PC = \frac{SS}{\sum SS} \times 100 \qquad (4)$$

where *SS* is sum of squares.

The proposed procedure is compared to microwave digestion and FAAS, as recommended for foods in the AOAC Official Methods of Analysis (AOAC 999.11).[19] The achieved results for proposed method were 180.1 ± 1.4 and 5.3 ± 0.9 μg g^{-1} for zinc and copper, respectively. The achieved results for AOAC method were 181.5 ± 1.1 and 5.7 ± 1.0 μg g^{-1} for zinc and copper, respectively.

Analytical application

The optimized microwave-assisted digestion procedure was applied to the determination of zinc and copper in milk samples. The results obtained are showed in Table 5. Total concentrations of zinc and copper in milk samples were measured by FAAS. Three different milk samples were analyzed. Table 5 presents the total concentration of zinc and copper for 3 milk samples analyzed. Zn and Cu were present in all the samples and their concentrations ranged from 100.5 to 182.4 and 3.6 to 5.2 μg g^{-1}, respectively.

The accuracy of the method was confirmed by the determination of zinc and copper in the reference sample, namely SRM 8433-Corn Bran from NIST (National Institute of Standards and Technology). The achieved results were 18.47 ± 1.6 and 2.43 ± 0.9 μg g^{-1} for zinc and copper, respectively. The certified values are 18.6 and 2.47 μg g^{-1} for zinc and copper, respectively. The statistical comparison by *t*-test showed no significant difference between the certificated values and the experimental results.
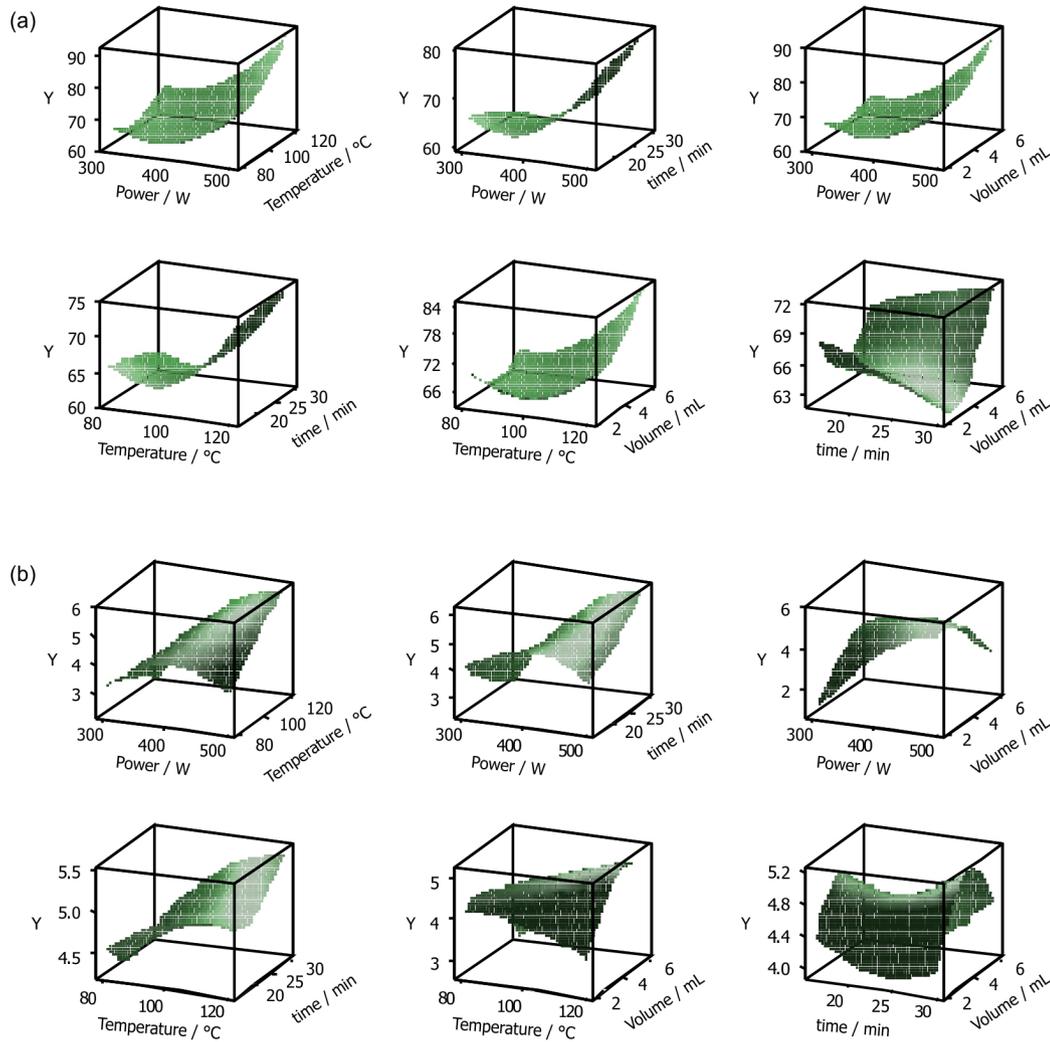
Figure 2. Response surface obtained from Box-Behnken design for extraction efficiency of zinc (a) and copper (b).

Table 4. Multiple regression results and significance of the components for the quadratic model

Factor	Parameter	SE		t-value		p-value		SS		PC	
		Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu
Intercept	β_0										
P	β_1	0.120	0.018	−6.678	1.886	< 0.0001	0.084	289.86	0.54	21.10	1.86
T	B_2	0.657	0.100	−5.508	−1.484	0.0001	0.164	197.16	0.33	14.35	1.15
t	B_3	1.605	0.245	−1.466	−3.825	0.1684	0.002	13.96	2.21	1.02	7.64
V	B_4	5.104	0.778	−5.317	2.667	0.0002	0.021	183.71	1.07	13.37	3.71
P^2	B_{11}	0.001	0.00002	6.247	−5.166	< 0.0001	< 0.001	253.58	4.03	18.46	13.94
T^2	B_{22}	0.003	0.0004	4.738	−1.193	0.0005	0.256	145.91	0.22	10.62	0.74
t^2	B_{33}	0.023	0.003	−0.449	1.614	0.6613	0.133	1.31	0.39	0.10	1.36
V^2	B_{44}	0.276	0.042	3.752	−4.824	0.0028	< 0.001	91.48	3.52	6.66	12.15
PT	B_{12}	0.001	0.0001	2.662	4.244	0.0207	0.001	46.04	2.72	3.35	9.41
Pt	B_{13}	0.002	0.0003	1.832	4.823	0.0919	< 0.001	21.81	3.52	1.59	12.15
PV	B_{14}	0.006	0.0009	2.789	−7.112	0.0164	< 0.001	50.55	7.65	3.68	26.43
Tt	B_{23}	0.009	0.001	0.865	0.72	0.4040	0.485	4.86	0.08	0.35	0.27
TV	B_{24}	0.032	0.005	2.901	4.051	0.0133	0.002	54.69	2.48	3.98	8.57
tV	B_{34}	0.091	0.014	1.712	1.08	0.1126	0.301	19.05	0.18	1.39	0.61

SE: Standard error; SS: sum of squares; PC: percentage of contributions; P: power; T: temperature; t: time; V: volume.
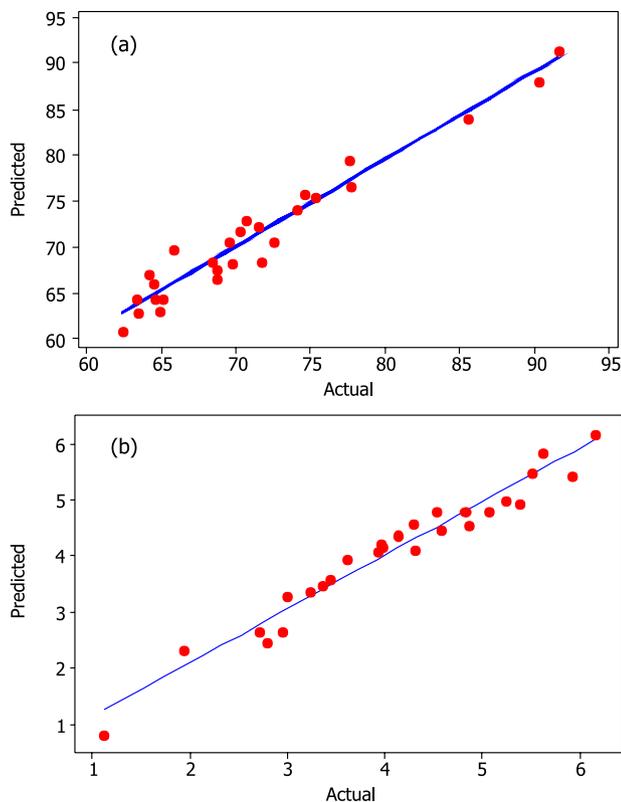
Figure 3. Parity plots show the correlation between the observed and predicted values for zinc (a) and copper (b). Actual and Predicted in μg *per* 0.5 g.

Table 5. Determination of zinc and copper in the powder milk

Sample[a]	Amount / (μg g^{-1}) ± RSD / %	
	Zinc	Copper
Humana powder milk 1	182.4 ± 1.3	5.2 ± 0.9
Humana powder milk 2	171.5 ± 1.1	6.9 ± 1.1
Nan powder milk 1	154.3 ± 1.3	3.8 ± 1.2
Nan powder milk 2	160.7 ± 1.5	4.1 ± 1.0
Nursoy powder milk 1	100.5 ± 1.0	3.9 ± 1.4
Nursoy powder milk 2	104.8 ± 1.2	3.6 ± 0.8

[a]These samples were selectively purchased from a local market (Zabol, Iran); RSD: relative standard deviation.

Conclusion

Microwave-assisted digestion method was developed for the fast extraction of zinc and copper from milk. The working conditions were established as a compromise between the optimum values found for copper and zinc. These values were 110 °C, 450 W, 4.0 mL and 21.0 min, for temperature, irradiation power, volume of nitric acid and time, respectively. Response surface methodology (RSM) analysis showed good correspondence between observed and predicted values. The application of a Box-Behnken matrix became possible, fast, economical and efficient way of an optimization strategy of the proposed

procedure.[20,21] With respect to other methods proposed for the determination of copper and zinc in milk samples, our method offers the advantages of simplicity and the short time necessary: 21 min *versus* 5 h for dry digestion,[22] and from > 4 h for wet digestion.[23]

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