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ORIGINAL PAPER

Design of experiment approach to optimize the high resolution ICP-OES method for biomonitoring Zn level in human blood samples

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Abstract

The study developed an analytical procedure for the determination of Zn in human blood by taking into account spectral and chemical interferences. The ICP's operational settings including radio frequency power, the plasma, and nebulizer gas flow rates, as well as the sample introduction rate and the digestion conditions were established using the response surface methodology (RSM). The paper also analyzes the usefulness of two wet decomposition methods – a closed system with microwave heating in Teflon bombs and a semi-closed system with heating in a heating block. The best ICP operating conditions were as follows: plasma and nebulizer gas flow rates: 14 and 0.5 L min⁻¹ respectively, radio frequency power 1400 W and sample introduction rate 1.5 mL min⁻¹. Three analytical lines most commonly used in the quantitative analysis of Zn by the ICP-OES technique were also tested for their applicability to biological material (whole human blood). Our results showed that the Zn signal overlapped with the Fe signal at 213.856 nm, and this line should be avoided in Zn assays of samples with high Fe content. There was also a significant effect of chemical interference from the digested sample matrix. The elaborated methodology was validated based on certified material and verified using real blood samples. Calibration with the matrix of most abundant elements provided high-accuracy determination.

Keywords: Zn, ICP-OES, optimization, validation, blood, interferences, matrix matching, matrix

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INTRODUCTION

Zn is an important microelement necessary for the proper functioning of living organisms. It is responsible for the function of over 300 enzymes in the human body (Praharaj et al. 2021, Pykhtieieva et al. 2021). An appropriate Zn concentration reduces the risk of heart failure and respiratory infections, ensures proper functioning of the reproductive system and maintains normal neuronal metabolism (Praharaj et al. 2021). Zn is also responsible for skin health and wound healing (Szczepanik et al. 2021). Low Zn levels may lead to depression, exacerbate symptoms of Alzheimer's disease and Parkinson's disease, and cause anemia (Kožlicka, Przysławski 2007). On the other hand, excess Zn can accumulate in various organs and negatively affect the respiratory, nervous, and digestive systems (Pykhtieieva et al. 2021). Therefore, monitoring the Zn level is important for human health and for maintaining homeostasis in the organism.

Inductively coupled plasma optical emission spectrometry (ICP-OES) is a powerful technique of the greatest practical importance because of high throughput, low detection limit, and a relatively low cost of analysis compared to the other methods for elemental determination. It has been applied in numerous studies to monitor levels of metal ions in various human tissues (Alrobaian, Arida 2019, Pokorska-Niewiada et al. 2021, Yadav et al. 2021).

However, methodology used varies significantly. It should be mentioned that the conditions of analysis have a great impact on the results, which may additionally be affected by misidentification or overestimation of the results due to overlapping spectral lines from different elements (spectral interferences). In some cases, insufficient precision and trueness can also be a problem e.g. as a result of inhomogeneity of aerosol introduced into plasma as well as decreased sensitivity due to high concentrations of the other elements in the sample (matrix effects).

Therefore, the aim of our study was to investigate the impact of various ICP-OES working parameters on Zn signal intensity and stability using the response surface methodology RSM. Moreover, the influence of matrix effects was assessed, taking into account potential spectral and chemical interferences on the Zn recovery. The RSM with the design of experiment (DoE) approach was also applied to optimize digestion conditions as an important part of sample preparation for elemental analysis. The elaborated procedure was validated and verified using matrix certified reference material, and applied to the determination of Zn in human blood samples.

MATERIALS AND METHODS

Chemicals

65% HNO₃ Suprapur, 30% H₂O₂ Suprapur were purchased from Merck (Darmstadt, Germany). Zn 1000 µg mL⁻¹, Mg 1000 µg mL⁻¹, K 50000 µg mL⁻¹, Na 50000 µg mL⁻¹, Ca 50000 µg mL⁻¹, and Fe50000 µg mL⁻¹ (SCP Science, Canada) were used as standard solutions. Deionized water (DI) was obtained from the Direct-Q® 3 UV system (Merck, Germany). Seronorm™ Trace Elements Whole Blood L-1 was from SERO (Norway). Matrix-matched solutions were prepared in 0.5% nitric acid. The final elemental concentrations were as follows: 30 mg L⁻¹ of K, 640 mg L⁻¹ of Na, 20 mg L⁻¹ of Ca, 4 mg L⁻¹ of Mg and 100 mg L⁻¹ of Fe.

Equipment

The measurements were performed using a high-resolution inductively coupled plasma optical emission spectrometer PlasmaQuant – PQ 9000 Elite (Analytik Jena, Germany). The final operating conditions were as follows: radio frequency power was 1400W, sample introduction rate was 1.5 mL min⁻¹, and plasma gas, auxiliary gas and nebulizer gas flow rates were 14 L min⁻¹, 0.5 L min⁻¹, 0.5 L min⁻¹, respectively. Zn was determined using analytical line length of 206,200 nm. Digestion was carried out using a DigiPREP MS heating block (SCP SCIENCE, Canada) in closed 50 mL DigiTUBEs (SCP Science).

Experimental design for response surface methodology and statistical analysis

The following ICP-OES working parameters were taken into consideration: RF power, plasma and nebulizer gases flow rates and sample flow rate. The complete experimental plan includes 400 experiments for all possible combinations of the factors levels: five settings of RF power (P) (1100, 1200, 1300, 1400, 1500 W), four levels of plasma gas flow (G) (12, 13, 14, 15 mL min⁻¹), 4 nebulizer gas flow (N) (0.4, 0.5, 0.6, and 0.7 mL min⁻¹), and five sample flow rate (F) (0.5, 1.0, 1.5, 2.0, 2.5 mL min⁻¹). The input variables for regression model building were given in code from 1 to 5 depending on the levels of the factors. Based on the Fedorov exchanged algorithm, the 42 combinations of factors settings were generated by D-optimal designs.

To optimize the digestion, a Box-Behnken design with 4 variables were applied: temperature (temp., 60-120°C range), acid concentration (acid conc., 20-80% range), amount sample (sample conc., 20-50% range), time (time, 30-120 min). The response of variables was the level of recovery (%). The project consisted of 29 combinations with 5 central points.

The statistical models obtained were verified using ANOVA; additionally the Shapiro-Wilk test was applied to verify the normality of the distribution

of the residuals. The analyses were performed using Statistica ver. 13.3 software (TIBCO Software Inc. 2017, Palo Alto, CA, USA).

Validation and real sample analysis

Calibration curves were prepared using solutions with and without the matrix over a range of concentrations 0-1000 $\mu\text{g L}^{-1}$. LOD and LOQ were calculated based on 3 and 10 times of the standard deviation for the blank solution (0.5% HNO_3), respectively.

The trueness was determined by Seronorm™ Trace Elements Whole Blood L-1 analysis. 3.0 mL of deionized water was added to a blood sample, and the sample was dissolved by rolling for 30 minutes. 2 mL of the material was taken for digestion. Blood samples were provided by the Department of Vascular Surgery and Angiology of the Medical University of Lublin. The study was approved by the Independent Ethics Committee of the Medical University of Lublin (no KE-0254/174/2021).

Blood samples (2 mL) were heated at 120°C for 90 min with the mixture composed of 1.8 mL of 65% HNO_3 and 3.4 mL of DI water. Digested samples were then cooled down to room temperature and filled up to 10 mL with DI water. Each blood sample was digested in three independent cycles.

RESULTS AND DISCUSSION

Spectral interferences

Three wavelength values: 206.200, 213.856, and 202.548 nm can be applied to Zn analysis. 206.200 nm followed by 202.548 nm are the most commonly used (Lech, Dudek-Adamska 2009, Lech, Lachowicz 2009, Kilinc et al. 2020, Jablan et al. 2021). The highest Zn signal is obtained at 202.548 nm; however, when choosing the analytical line, it is important to consider not only the signal intensity, but also potential interferences from other matrix components.

At this stage of the study, a matrix matching solution was used to record the Zn signal with different analytical lines to verify potential interferences. The composition of the matrix corresponded to the approximate concentrations of Na, K, Fe, Mg and Ca in digested blood samples.

At a wavelength of 213.856 nm, the signal from Fe overlapped with the Zn line (Figure 1a), which may yield false positive results. Analysis of Zn at 213.856 nm in the matrix matched solution had a systematic error (bias) of 6-9 $\mu\text{g L}^{-1}$.

On the other hand, the high concentration of Mg in blood samples is the main reason why the wavelength of 202.548 nm should be avoided. According to the literature, Mg gives a strong signal at 202.58 nm and can therefore interfere with the measurement of Zn (Ghazi et al. 2005, Yong 2018, Müller et al. 2020). However, Mg interference with the Zn line was not

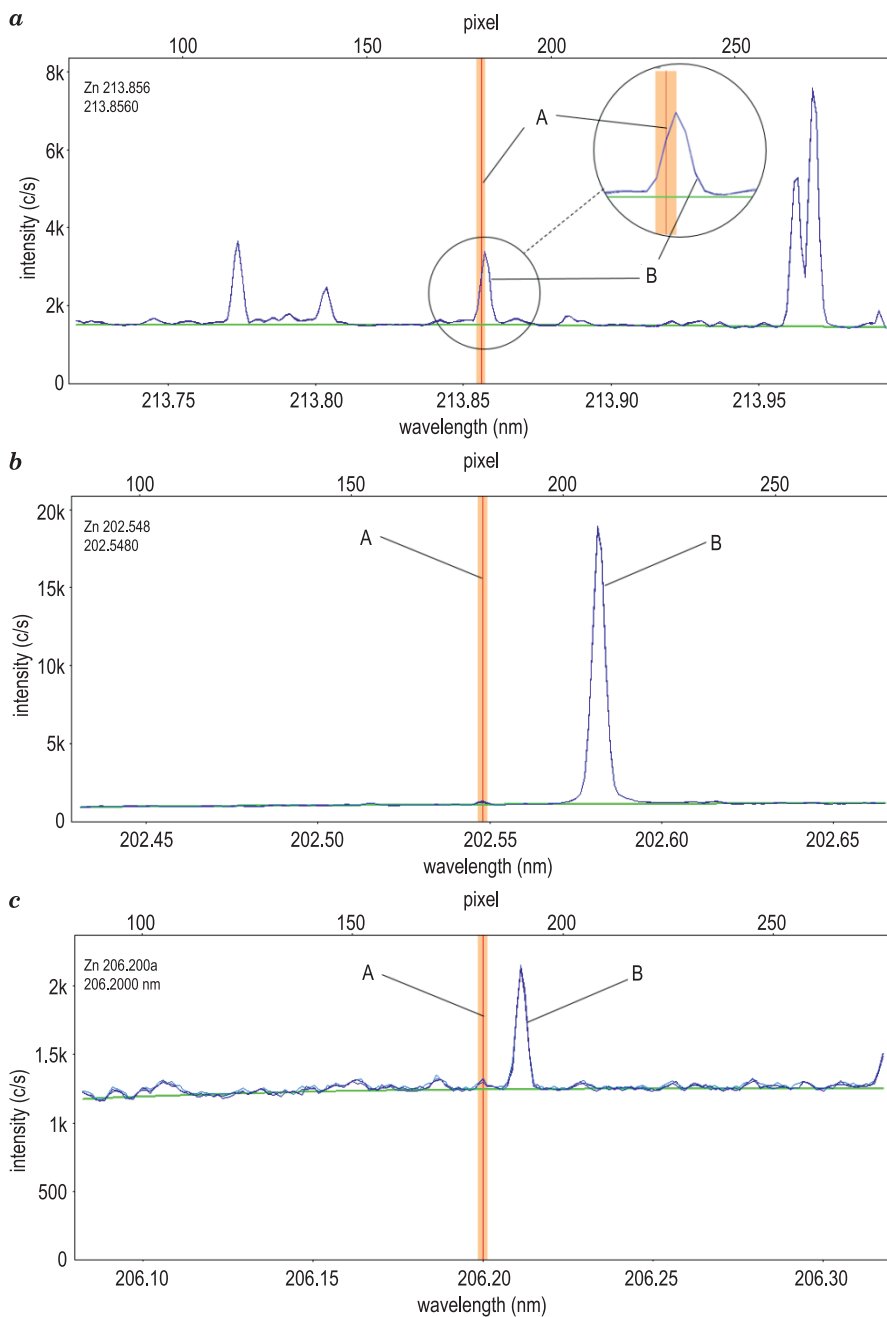


Fig. 1. Zn signal recorded in a matrix matched solution: (a) A – 213.856 nm; B – overlapped signal from Fe, (b) A – 202.548 nm, B – Mg signal, (c) A – 206.200; B – Fe signal

observed in the high resolution ICP-OES (Figure 1b). At 206.200 nm (Figure 1c), the Zn spectral line was located close to the Fe line, but both signals were sufficiently separated. Taking into account these findings, a wavelength of 206.200 nm or 202.548 nm can be applied for the determination of Zn and the line at 213.856 nm should be avoided in Fe-rich samples. The potential spectral interferences mentioned above can have a greater impact on results when measured with lower-resolution instruments.

Optimization of operating conditions using response surface methodology

Important operating parameters in ICP include the radio frequency (RF) power, plasma and nebulizer gas flow, and sample introduction rate. In our study, the effect of the aforementioned factors on the signal intensity was checked for a 100 $\mu\text{g L}^{-1}$ Zn standard solution. The regression model used to evaluate the relationship between the parameters and signal was verified using ANOVA (Table S1). After eliminating nonsignificant components, four linear and two quadratic factors were introduced to the model (Table 1).

Reduction of the model by removing insignificant model terms [RF Power (Q)], [Nebulizer gas flow (Q)] slightly improved the model in relation to the signal to noise ratio or predicted R square (Adeq Precision). The model thus obtained explained 93% of dependent variable variations. Upon the Pareto chart and the length of horizontal bars, the importance of parameters decreased in the order: [Nebulizer gas flow (L)] > [RF Power (L)] > [Sample flow rate (L)] > [Sample flow rate (Q)] > [Plasma gas flow (Q)] > [Plasma gas flow (L)]. However, comparing the relative significance of the factors,

Table 1

Fit statistics of the models and regression coefficients of the ICP-OES factors for signal level obtained

Summary of fit	Before model reduction		After model reduction		<i>p</i>
R square	0.9344		0.9344		
R square adjusted	0.9232		0.9232		
R square predicted	0.8872		0.9017		
Adeq Precision	31.7647		36.9926		
Factor	Effect	Std. Error	T ratio		
Nebulizer gas flow (L)	-20366.4	1346.825	-15.1218		0.000
RF Power (L)	6794.9	525.864	12.9214		0.000
Sample flow rate (L)	11108.5	1261.306	-15.1218		0.000
Sample flow rate (Q)	-4406.2	1106.513	-3.9821		0.000
Plasma gas flow (Q)	5336.1	1802.415	2.9605		0.005
Plasma gas flow (L)	-2509.0	1095.980	-2.2893		0.028

the Nebulizer gas flow and RF Power showed the greatest impact, followed by the Sample flow rate. In Figure S1 (Supplementary material) the predicted response is shown compared to the actual signal values. Based on the utility profile module available in Statistica software, the combination of the tested factors' settings that maximize the level of signal was performed. Response surface plots of the signal level, obtained as a function of the combined impact of the various tested factors, are shown in Figure 2.

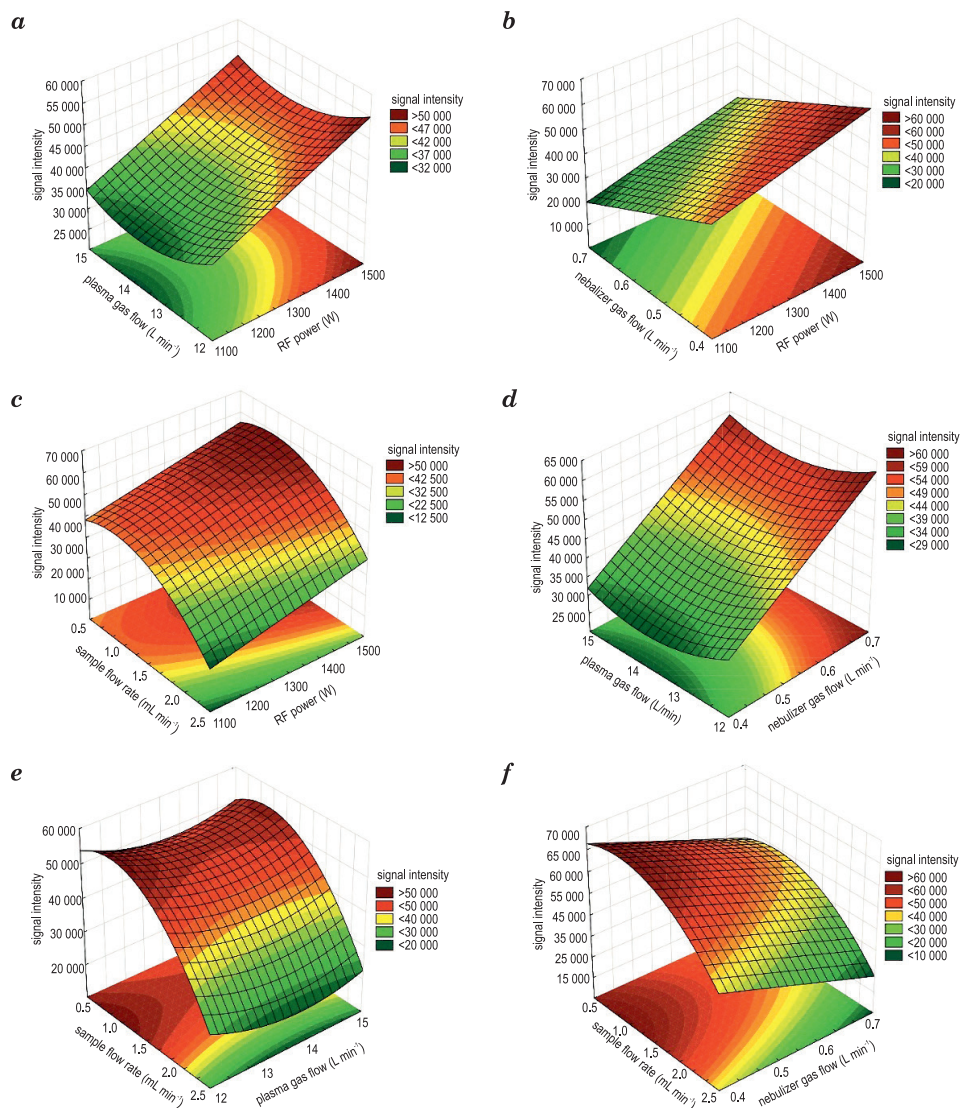


Fig. 2. 3D response surface plots of the signal level, obtained as a function of the combined impact of plasma gas flow ver. radio frequency power (*a*), nebulizer gas flow ver. radio frequency power (*b*), sample flow rate ver. radio frequency power (*c*), plasma gas flow ver. nebulizer gas flow (*d*), sample flow rate ver. plasma gas flow (*e*), sample introduction rate ver. nebulizer gas flow (*f*).

The optimum combination values of the estimated parameters were 1361 W of RF power, 14.57 L min⁻¹ of plasma gas flow rate, 0.46 L min⁻¹ of nebulizer gas flow rate, and 1.56 mL min⁻¹ of sample introduction rate (Figure 3). Finally, the values were rounded to the nearest value possible to set in the ICP apparatus, and were as follows: radio frequency power 1400 W, plasma gas flow 14 L min⁻¹, nebulizer gas flow 0.5 L min⁻¹ and sample introduction rate 1.5 mL min⁻¹.

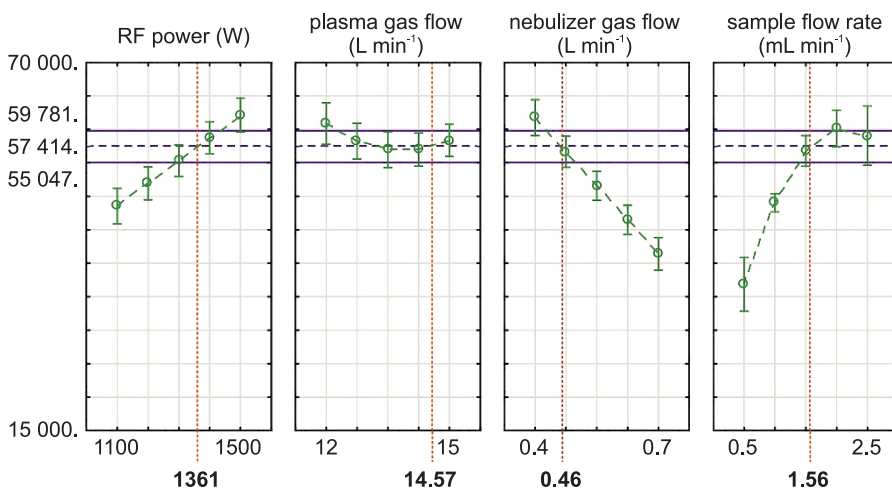


Fig. 3. Profiles of approximated values and optimal factors settings for ICP-OES analysis

Another parameter that can be regulated in the ICP is the auxiliary gas flow rate. However, as our investigation showed (Figure S2), the appropriate working range of flow rate was narrow (0.4-0.5 L min⁻¹) because the Zn signal intensity decreased significantly above 0.5 L min⁻¹ while at lower flow rate values the residues were deposited on the torch, especially when samples with high content of salts such as blood were analyzed.

Chemical interferences

Chemical interferences mainly depend on the type of matrix, since the analyte may undergo different processes (ionization, molecule formation) in the plasma. This type of interference is particularly important when the sample contains high concentrations of easily ionized elements. Therefore, the impact of different concentrations of the most prevalent elements present in blood (Na, Fe, K, Ca) on Zn recovery was determined. The results are shown in Figure 4.

As can be seen, the presence of high concentrations of the elements caused attenuation of the analyte signal, resulting in reduced Zn recovery. Considering the elemental concentrations in the digested blood samples, only sodium can cause chemical interference, resulting in a signal decrease of about 10%. However, blood samples after digestion contain all the ele-

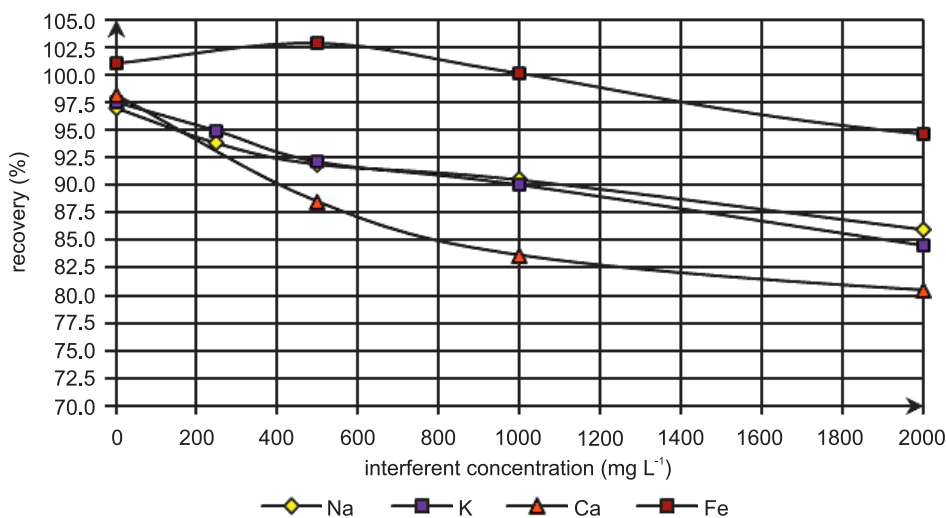


Fig.4. Recovery of Zn in a solution containing different concentrations of Ca, Fe, Na and K

ments for which potential interference on Zn signal was analyzed. Chemical interference caused by this matrix may be amplified synergistically compared to the interference from single elements. Therefore, Zn quantification should be performed based on a calibration curve prepared using matrix-matched calibration solutions. The manuscript presents the composition of matrix-matched solution that significantly increases the trueness of the results obtained.

Validation of the analytical procedure

Validation of the analytical procedure was carried out according to ICH (European Medicines Agency 1994). The calculated LOD ($3 \times \text{SD}/\text{slope}$) and LOQ ($10 \times \text{SD}/\text{slope}$) values were $0.11 \mu\text{g L}^{-1}$ and $0.33 \mu\text{g L}^{-1}$ for the blank solution without the matrix, and $0.28 \mu\text{g L}^{-1}$ and $0.84 \mu\text{g L}^{-1}$ for the matrix-matched solution. LOQ values were experimentally verified according to ECHA requirements using standards with sufficiently low concentrations, selected on the basis of the estimated limit of quantification from the blank measurements (European Chemicals Agency 2016), and the combined standard uncertainty (U) was calculated (Table S2). Values with the expanded uncertainty below 10% are considered as acceptable LOQ values (Thompson et al. 2002, White 2008). As shown in Table S2, the experimentally determined LOQ value was $2.0 \mu\text{g L}^{-1}$ for both the matrix-free solution and the matrix-matched solution.

Linearity and calibration equations are presented in Table S3. The trueness and precision of the method were verified by analyzing the CRM – Seronorm Trace Elements Whole Blood L-1 (Table 2).

The precision did not exceed 1% in both cases (the acceptable value for

Table 2

Trueness and precision established based on certified reference material ($n=5$)

Declared content – interval ($\mu\text{g L}^{-1}$)	Average declared content ($\mu\text{g L}^{-1}$)	Matrix	Determined content ($\mu\text{g L}^{-1}$)	Average recovery (%)	CV (%)
380 – 460 (98.5% – 101.5%)	420	absent	372	88.6	0.53
		present	417	99.2	0.49

trace analysis is below 5%). However, the determined value was below the declared content in CRM when the quantification was carried out using a calibration curve without the matrix-matched solution. This confirmed that the presence of the other elements attenuated the Zn signal.

As shown above, preparing a calibration curve using a matrix-matched solution improves trueness of measurements. However, such an approach is not common practice. Only three papers have described the use of matrix-matched solutions (Kollander et al. 2010, Heitland and Köster 2021, Jablan et al. 2021) and none of them specified their composition.

Optimization of digestion procedure

Sample preparation is another challenge in Zn analysis, due to the common occurrence of the element resulting in a high risk of contamination. Therefore, the digestion technique in an open mode used in some reports (Massadeh et al. 2009, Csikós et al. 2021, Heitland and Köster 2021) should be avoided.

A closed-vessel PTFE coated graphite block was used in this study as it works with disposable vessels and disposable filtration systems, thus reducing the risk of contamination.

The digestion procedure was optimized using the response surface method. The reduction of the model by removing quadric and interaction non-significant components improved the model and increased fit statistics (Table 3).

The model explained 84% of dependent variable variations. The temperature was the most important parameter affecting the digestion. After temperature, the importance of parameters decreased in the order: [Sample conc.] > [interaction between Temp. and Acid conc.] > [Temp. (Q)] > [Acid conc. (L)] > [interaction between Acid conc. (L)] and [Sample conc. (L)]. Surprisingly, duration of digestion in 30-120 min range (Time) did not show any significant impact on the per cent recovery. The interaction between temperature and acid concentration proved to be a significant element of the model. As can be seen, the effectiveness of digestion increased with the increasing acid concentration within the temp. range of 60-100°C; however, digestion turned out to be more effective at a lower concentration of acid above 100°C (Figure 5). The optimum combination values of estimated parameters were 90 min, 120°C, 35% of acid concentration (35% v/v of 65% HNO₃ in oxidation

Table 3

Fit statistics of the models and regression coefficients of the digestion factors for the recovery level obtained

Summary of fit	Before model reduction		After model reduction	<i>p</i>
<i>R</i> square	0.8671		0.8412	
<i>R</i> square adjusted	0.7239		0.7856	
<i>R</i> square predicted	0.1158		0.5997	
Adeq precision	9.9473		15.8970	
Factor	Effect	Std. Error	T ratio	
Temp. (L)	17.077	1.308	13,053	0.000
Sample conc. (L)	-7.677	1.211	-6.338	0.003
Temp. (L) ver. acid conc. (L)	-14.746	2.569	-5.739	0.004
Temp. (Q)	-7.745	1.657	-4.673	0.009
Acid conc. (L)	6.019	1.308	4.6008	0.010
Acid conc. (L) ver. sample conc. (L)	7.425	2.099	-5.739	0.024
Time (L)	2.305	1.211	1.9028	0.129

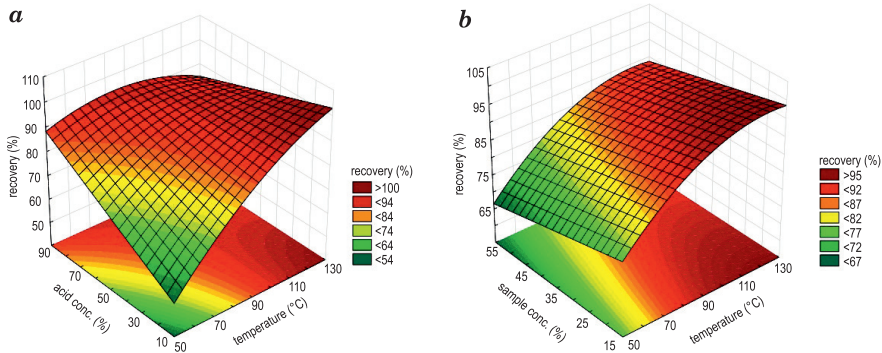


Fig. 5. 3D response surface plots of the recovery, obtained as a function of the combined impact of acid concentration ver. temperature of digestion (a), sample concentration ver. temperature of digestion (b)

mixture), and 27.5% of sample concentration in digestion mixture (27.5% v/v sample and 72.5% v/v of an oxidizing mixture of the above composition) (Figure 6).

In our study, the nitric acid and water mixture was found to be effective in digesting blood samples. This is the most common oxidizing mixture applied for digestion. Sometimes, nitric acid can be partially replaced with hydrogen peroxide or hydrochloric acid (Stanisławska-Głubiak et al. 2009, Parzych 2014) although then the digestion should be supported with micro-

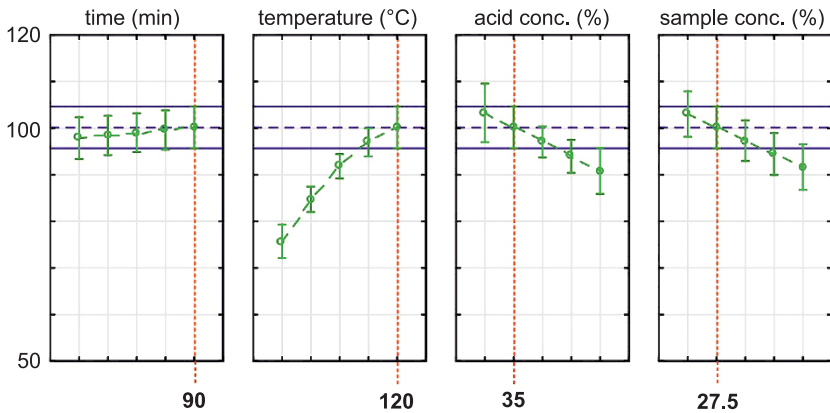


Fig. 6. Profiles of approximated values and optimal factor settings for the digestion procedure

wave radiation (MAE) – Akay et al. (2016). The MAE technique is very effective but requires cleaning reusable vessels, which prolongs the procedure. Furthermore, such cleaning is not always effective and Zn residues in a vessel could have impact on the results (Figure S3).

Analysis of Zn in real blood samples

To verify the elaborated ICP procedure, blood samples were digested in three independent cycles and Zn was analyzed using established ICP working parameters. No spectral interference was observed for the wavelength selected for Zn analysis in blood (Figure S4). The CV value for the measurements was below 1.1%, which indicated high precision of the method. Only slight differences between the Zn concentrations in blood samples

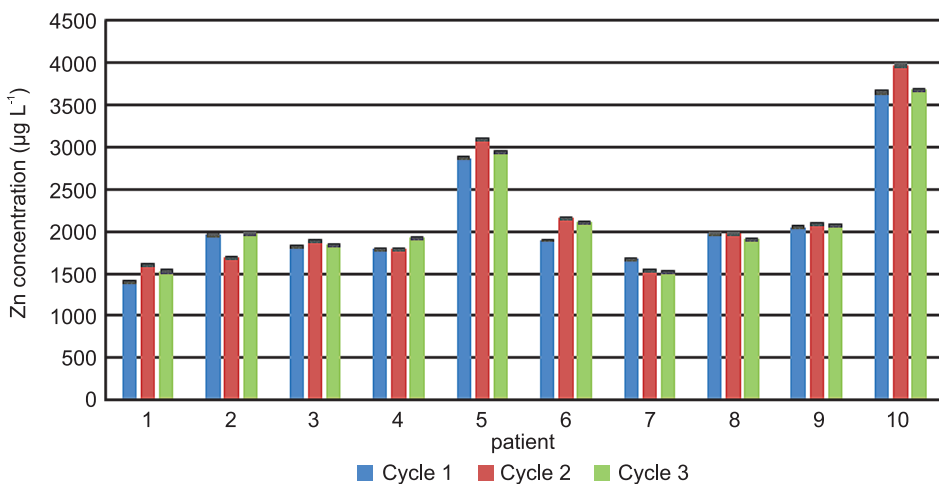


Fig. 7. Zn concentration in the blood samples determined in three independent mineralization cycles

obtained in three independent cycles demonstrate adequate reproducibility of the procedure (Figure 7).

CONCLUSIONS

Our report shows the impact of particular parameters on the Zn quantification results, and highlights the key factors that have to be considered to ensure high precision, trueness and sensitivity of measurements.

ICP-OES is a useful technique for the determination of Zn in biological samples; however, ICP-OES working parameters have a significant impact on the intensity and stability of the Zn signal, and the response surface method is useful for establishment of the optimal analysis conditions. The highest sensitivity was achieved using the following settings: plasma and nebulizer gas flow rates: 14 and 0.5 L min⁻¹ respectively, radio frequency power 1400 W, and sample introduction rate 1.5 mL min⁻¹. It was also found that at 213.856 nm, the signal from Fe overlapped with the Zn line, and therefore this line should be avoided in analyses of Zn in samples with high Fe content. The interference of Mg with the Zn line was not observed in the high resolution ICP-OES. Moreover, to obtain the highest trueness of Zn determination, the calibration curve should be prepared using a solution with the matrix matched to the real sample.

Author contributions

J.S. – conceptualization, methodology, validation, investigation, resources, writing – original draft, visualization, funding acquisition, M.W. – writing – review and editing, supervision, project administration, S.D. – methodology, software, formal analysis, data curation, visualization, A.T. – investigation, A.S.K- investigation, I.S. – project administration.

All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors ensure that they have neither professional nor financial connections related to the manuscript sent to the Editorial Board.

Supplementary material

The manuscript contains supplementary material available only online: <https://jsite.uwm.edu.pl/issue/view/4-2023/>

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