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ORIGINAL PAPER

# ASSESSMENT OF WET ACID DIGESTION METHODS FOR ICP-MS DETERMINATION OF TRACE ELEMENTS IN BIOLOGICAL SAMPLES BY USING MULTIVARIATE STATISTICAL ANALYSIS

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Abstract

Evaluation of wet digestion methods based on acids in acid mixtures set at different temperatures and ratios is important to assess accurately the content of trace elements in biological samples. This study presents a comparison of three digestion procedures based on nitric acid--hydrogen peroxide (NH), nitric acid-sulfuric acid (NS), and nitric acid-perchloric acid (NP) at different temperatures and ratios of the acid mixture. This study was conducted on blood serum of healthy volunteers (Hospital of Tanta University, Tanta, Egypt) for comparison of different digestion methods. An inductively coupled plasma-mass spectroscopy ICP-MS was calibrated using the certified standard biological samples IAEA-A-13 and then it was used to determine eight elements, Cr, Cu, Fe, Co, Mn, Ni, Se, and Zn in the digested samples. In order to reduce the experimental variables and group the analyzed trace elements into clusters correlated with the preferred digestion procedures, multivariate statistical analysis based on cluster analysis (CA) and principal component analysis (PCA) was used to select the optimal digestion method for each trace element. The results confirmed that none of the used digestion procedures for the certified biological samples, freeze dried animal blood, have given an accurate assessment for all trace elements, however the most acceptable digestion procedure is the one involving nitric acid/perchloric acid, 4:1 and 4:2 v/v, at a temp. of 120°C. The nitric acid/sulfuric acid procedure, 4:2 v/v, achieved good extraction of trace elements at temp. of 120°C while the nitric acid/hydrogen peroxide procedure, 4:2 v/v, achieved the highest extraction for cobalt and iron.

**Keywords**: cluster analysis, ICP-MS, multivariate statistical analysis, principal component analysis, wet digestion method.

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# INTRODUCTION

Trace elements in biology and medicine have attracted great research interest because of their important roles in the metabolism and growth of living organisms (MALAKAR et al. 2014, MARJANIA et al. 2015, BADRAN et al. 2016). Currently, various methods are used in trace element analysis such as optical spectrometry, atomic mass spectrometry, X-ray Fluorescence Spectrometry, Inductively Coupled Plasma Mass Spectrometry (ICP-MS), chromatography, and neutron and photon activation methods (KYENGER et al. 1998, BECKER et al. 2003, MCCOMB et al. 2014). For many reasons, ICP-MS is the most accurate technique used for determination of the content of trace elements in biological samples (DRESSLER et al. 2011). It is a very powerful and precise method for elemental analysis with detection limits at or below the part per trillion (BALCAEN et al. 2015). In addition, it has a multi-element capability and is able to analyze small or complex samples (SWAMI et al. 2009, MATHEUS et al. 2016). However, using the ICP-MS technique for trace element analysis requires a sample to be in the liquid state, which necessitates digestion procedures to liquidify the analyzed samples (ENDERS et al. 2012). Therefore, sample digestion, in particular acid digestion, is considered as an important and critical process for accurate analysis of trace element content in biological samples. Elemental analysis of biological samples is based on destroying organic matter, the main component of biological samples, then dissolving them using acid digestion. Different acids and acid mixtures based on nitric, sulfuric or perchloric acids have been used for acid digestion of biological samples to analyze trace elements by ICP-MS (VANHHOE 1993, TAKAHASHI et al. 2000, HESU et al. 2004, HANSEM et al. 2009). Also, many studies have used wet oxidation procedures based on mixing oxidant agents such as hydrogen peroxide with nitric, sulfuric and perchloric acids (BADRAN et al. 2014). However, recently used wet acid digestion methods have many limitations such as a significant loss of some trace elements due to possible formation of precipitates containing trace elements during the digestion of biological samples (SOMER et al. 2007). Unfortunately, until now none of the used digestion methods has been approved as a standard official method of digestion of biological samples to measure the content of trace elements using ICP-MS. Therefore, the aim of the present study was to compare the effect of three different digestion procedures based on nitric acid-hydrogen peroxide, nitric acid-sulfuric acid and nitric acid-perchloric acid at different ratios in an acid mixture and at different temperatures on the results of determination of eight chosen trace elements in blood serum samples. To reduce the number of experimental variables and group the analyzed trace elements into clusters correlated with the preferred digestion procedures, multivariate statistical analysis based on cluster analysis (CA) and principal component analysis (PCA) was used so as to analyze and select the optimal digestion method for each trace element.

# MATERIAL AND METHODS

### Serum samples

This study was conducted on blood samples of 9 Egyptian healthy volunteers (Tanta University Hospitals, Gharbia governorate, Egypt). All of them had the same socio-economic status and were diagnosed according to the recommend procedures. The mean age of the volunteers was 41.47 ± 8.75 yr, and their body mass index (BMI) was 22.45 ± 1.0 kg $m^{-2}$. For serum separation, 6 ml of fasting blood samples were withdrawn from the volunteers and preserved in sterilized clean tubes without an anticoagulant agent. The samples were centrifuged at 3000 rpm for 10 min.

### Methods of digestion

Three digestion procedures were applied to digest serum, involving nitric acid-hydrogen peroxide (NH), nitric acid-sulfuric acid (NS), and nitric acid--perchloric (NP) acid mixtures. In a typical procedure, 0.5 ml of serum sample was added to 10 ml of a concentrated acid mixture containing nitric acid and hydrogen peroxide ($HNO_3/H_2O_2$) mixed at a 4:1 v/v ratio and kept in a beaker, whose contents were then heated at 80°C until the volume of the digested sample reached 1.0 ml. After cooling to room temperature, the contents were diluted with deionized water up to 10 ml. After digestion, a trace element analysis was carried out using ICP-MS: Finnigan elements 2). The same steps were carried out for all mixtures, i.e. $HNO_3/H_2O_2$, $HNO_3/H_2SO_4$, and $HNO_3/HClO_4$, at two volume ratios, 4:1 and 4:2 v/v, and at temp. of 80, 100, and 120°C.

### Quality assurance and control

Single standard reference biological sample (IAEA-A-13, freeze dried animal blood) from International Atomic Energy Agency (IAEA) was digested in triplicate and analyzed using the three digestion procedures to support quality assurance and control. The recoveries of elements in the standard reference materials by the three digestion procedures using ICP-MS: Finnigan elements 2) are shown in Table 1. The recoveries of elements by the three digestion procedures ranged from 67% to 96%,. To correct the measurements, a blank was used for each digestion procedure.

### Statistical analysis

Multivariate statistical analysis consisting of cluster analysis (CA) and principal component analysis (PCA) was used to reduce the number of observed variables to a relatively smaller number of components. PCA was applied to achieve optimization of the digestion procedures and to develop an analytical procedure using conventional wet acid digestion for dissolution of chosen trace elements in serum samples using ICP-MS, as well as to find the

Table 1

Recovery (%) of trace elements measured in certified reference material, IAEA-A-13; concentration (μg $g^{-1}$), by using the ICP-MS method

| Elements | Certified value | Nitric acid and $H_2O_2$ | Nitric and sulfuric acid | Nitric and perchloric acid |
|---|---|---|---|---|
| **Br** | 22.00 | 17.86(81%) | 18.12(82%) | 17.75(81%) |
| **Ca** | 286.00 | 223.08(78%) | 223.12(78%) | 231.56(81%) |
| **Cu** | 4.30 | 3.91(91%) | 3.71(86%) | 3.92(91%) |
| **Fe** | 2400 | 1983(83%) | 1920.00(80%) | 1961(82%) |
| **K** | 2500 | 2074(83%) | 2061(82%) | 2070(83%) |
| **Mg** | 99.00 | 78.21(79%) | 78.23(79%) | 78.21(79%) |
| **Na** | 12600 | 11970(95%) | 12000(95%) | 12062(96%) |
| **Ni** | 1.00 | 0.75(75%) | 0.77(77%) | 0.72(72%) |
| **P** | 940.00 | 755(80%) | 761.40(81%) | 770(82%) |
| **Pb** | 0.18 | 0.12(67%) | 0.13(71%) | 0.14(78%) |
| **Rb** | 2.30 | 1.61(70%) | 1.62(70%) | 1.65(72%) |
| **S** | 6500 | 4940(76%) | 5213(80%) | 5112(78%) |
| **Se** | 0.24 | 0.19(79%) | 0.19(79%) | 0.19(81%) |
| **Zn** | 13.00 | 10.54(81%) | 11.00(85%) | 11.71(90%) |

best experimental conditions for all the digestion procedures. Optimization was conducted by changing the main factors influencing the efficiency of digestion such as an acid mixture, and the effect of a temperature on dissolution efficiency for the chosen trace elements in blood serum samples. The multivariate analysis comprised a principal component analysis (PCA) and cluster analysis (CA) performed using statistical packages of SPSS software, version 12.

## RESULTS AND DISCUSSION

Table 1 shows the accuracy of an analysis and recovery of trace element from certified reference material by using three digestion procedures. In all the procedures, $HNO_3/H_2O_2$, $HNO_3/H_2SO_4$, and $HNO_3/HClO_4$, accurate analysis of elements was achieved. However, the recovery of elements was between 70 and 91%, and varied depending on an individual element and digestion procedure. The concentrations of the measured elements were in satisfactory agreement with the certified value. Table 2 shows serum concentrations of eight trace elements: Cr, Mn, Fe, Co, Ni, Cu, Zn and Se, obtained using three digestion methods with different acid mixtures, ratios: 4:1 and 4:2 v/v; and temp. 80, 100, and 120°C.

Table 2

Comparison of trace element concentrations (μg $L^{-1}$) determined by ICP-MS in serum digested by different acid mixtures and at different temperatures

| Type of acid mixture (v/v) | Temperature (°C) | Cr | Cu | Fe | Co | Mn | Ni | Se | Zn |
|---|---|---|---|---|---|---|---|---|---|
| $HNO_3/H_2O_2$ (4:1) | 80 | 0.14 | 122.9 | 60.99 | 0.15 | 0.53 | 0.22 | 244.11 | 942.43 |
| | 100 | 0.14 | 126.56 | 62.13 | 0.16 | 0.59 | 0.25 | 249.01 | 989.38 |
| | 120 | 0.16 | 130.22 | 63.21 | 0.15 | 0.59 | 0.25 | 252.93 | 961.00 |
| $HNO_3/H_2O_2$ (4:2) | 80 | 0.14 | 130.32 | 63.34 | 0.17 | 0.51 | 0.22 | 233.31 | 970.55 |
| | 100 | 0.15 | 123.56 | 61.00 | **0.18** | 0.64 | 0.24 | 252.12 | 952.44 |
| | 120 | 0.15 | 128.59 | **71.62** | **0.18** | 0.57 | 0.24 | 227.07 | 1001.08 |
| $HNO_3/H_2SO_4$ (4:1) | 80 | 0.16 | 128.54 | 58.69 | 0.14 | 0.56 | 0.21 | 259.22 | 979.66 |
| | 100 | 0.15 | 122.00 | 65.67 | **0.18** | 0.56 | 0.23 | 244.00 | 1005.00 |
| | 120 | 0.16 | 131.04 | **67.52** | 0.14 | 0.59 | **0.26** | **260.00** | 1000.00 |
| $HNO_3/H_2SO_4$ (4:2) | 80 | 0.13 | 120.62 | 62.67 | 0.15 | 0.61 | 0.22 | 229.82 | 951.22 |
| | 100 | 0.16 | 133.67 | 60.21 | 0.15 | 0.54 | 0.25 | 252.93 | 961.00 |
| | 120 | **0.17** | **138.56** | 59.54 | 0.14 | **0.64** | **0.26** | **260.39** | **1050.56** |
| $HNO_3/HClO_4$ (4:1) | 80 | 0.15 | 121.56 | 56.44 | 0.17 | 0.49 | 0.22 | 237.67 | 932.88 |
| | 100 | 0.14 | 123.82 | 60.93 | 0.14 | 0.59 | 0.22 | 227.59 | 952.84 |
| | 120 | **0.18** | **139.52** | **67.14** | **0.18** | **0.68** | **0.26** | **274.99** | **1062.44** |
| $HNO_3/HClO_4$ (4:2) | 80 | 0.16 | 123.56 | 62.00 | 0.15 | 0.53 | 0.22 | 254.16 | 952.44 |
| | 100 | 0.15 | 123.82 | 58.93 | **0.18** | 0.59 | 0.25 | 244.11 | 951.22 |
| | 120 | **0.17** | **141.22** | **68.61** | **0.18** | **0.64** | **0.27** | **264.22** | **1055.88** |

The principal component analysis was applied to evaluate the effect of the studied factors, temperature and ratio of an acid mixture, on the element digestion from serum samples. The loadings and scores of the first two principal components can be used to show the effect of these factors on the efficiency of the digestion procedures. Principal component (PC) loadings recognize the extracted elements whose variabilities as a function of the three optimized digestion procedures are highly correlated. Principal component (PC) scores allow us to obtain a significant reduction in the number of optimized procedures. The influence of each factor was evaluated by comparing its effects on the three digestion procedures for element dissolution. For the three digestion procedures, the PCA investigated the effect of three experimental factors, i.e. type of acids, acid ratios in a mixture and temperatures, on the digestion efficiency of eight elements: Cr, Mn, Fe, Co, Ni, Cu, Zn, and Se. To reduce the number of parameters, only principal components with eigenvalues greater than 1 were extracted according to the Kaiser criterion. There were only 2 PCs having eigenvalues greater than one and they accounted for 73.792% of the total variance in the original dataset (Figure 1).

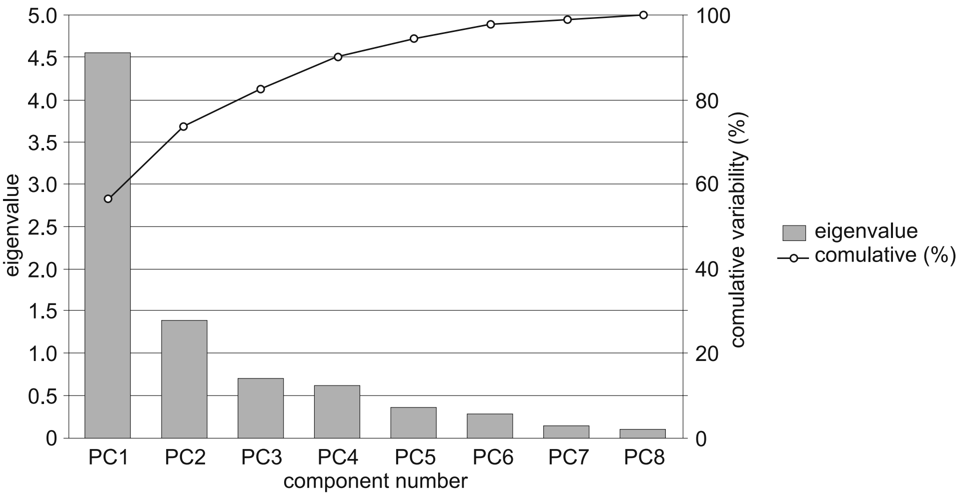


Fig.1. The eigenvalues and the cumulative percentage of variation of the extracted components

The PCA allowed the reduction of 8 variables to two principal components, PC1 and PC2, which explained most of the variance, at 56.617 and 17.174%, respectively.

Table 3 showed the loading of two significant components, eigenvalues, percentage of variation and cumulative percentage of variation explained by a given eigenvalue. PC1 was highly related to Cr, Cu, Mn, Ni, Se, and Zn, indicating that these variables have common patterns. Loadings on PC2 showed that Fe and Co were most important for this component. The concentrations of Cr, Mn, Fe, Co, Ni, Cu, Zn and Se which dissolved by the three

Table 3

Loadings of variables on two Principal Components (PCs) of trace element dissolution in samples using three digestion procedures

| Elements | Component | |
|---|---|---|
| | PC1 | PC2 |
| Cr | **0.846** | -0.316 |
| Mn | **0.734** | 0.096 |
| Fe | 0.520 | **0.657** |
| Co | 0.216 | **0.766** |
| Ni | **0.850** | 0.080 |
| Cu | **0.891** | -0.154 |
| Zn | **0.904** | 0.125 |
| Se | **0.788** | -0.449 |
| Eigenvalue | 4.529 | 1.374 |
| Variance (%) | 56.617 | 17.174 |
| Cumulative variance (%) | 56.617 | 73.792 |

optimized wet acid digestion procedures at 120°C have statistically significant different values of scores.

The representation of data in score plots is an efficient method for differentiation between different optimized wet acid digestion procedures in the data set. The main factors affecting the element dissolution by the three digestion procedures is shown as either positive or negative scores in the principal component score plot. A score plot reveals that digestion procedures having positive scores showed a good dissolution rate, whereas those having negative scores showed a poor dissolution rate. The digestion procedures that were grouped together on the score plot have a similar element extraction rate. In the PCA score plot (Figure 2) all scores for the digestion procedures of 120°C are positive on the first principal component.

The digestion procedures carried out at 120°C using both $HNO_3/HClO_4$, 4:2 v/v, and $HNO_3/HClO_4$, 4:2 v/v, formed a tight cluster. They are characterized by the highest positive scores corresponding to PC1, indicating that these digestion procedures were the most efficient for the determination of the serum trace elements, Cr, Mn, Ni, Cu, Zn and Se, when compared with other procedures and also showed a similar elemental extraction for these elements. But at 120°C, the digestion acid mixture $HNO_3/HClO_4$, 4:1 v/v, was more efficient than the digestion procedure $HNO_3/HClO_{4,}$ 4:2 v/v. This behaviour may be due to the very strong oxidizing power of perchloric acid at

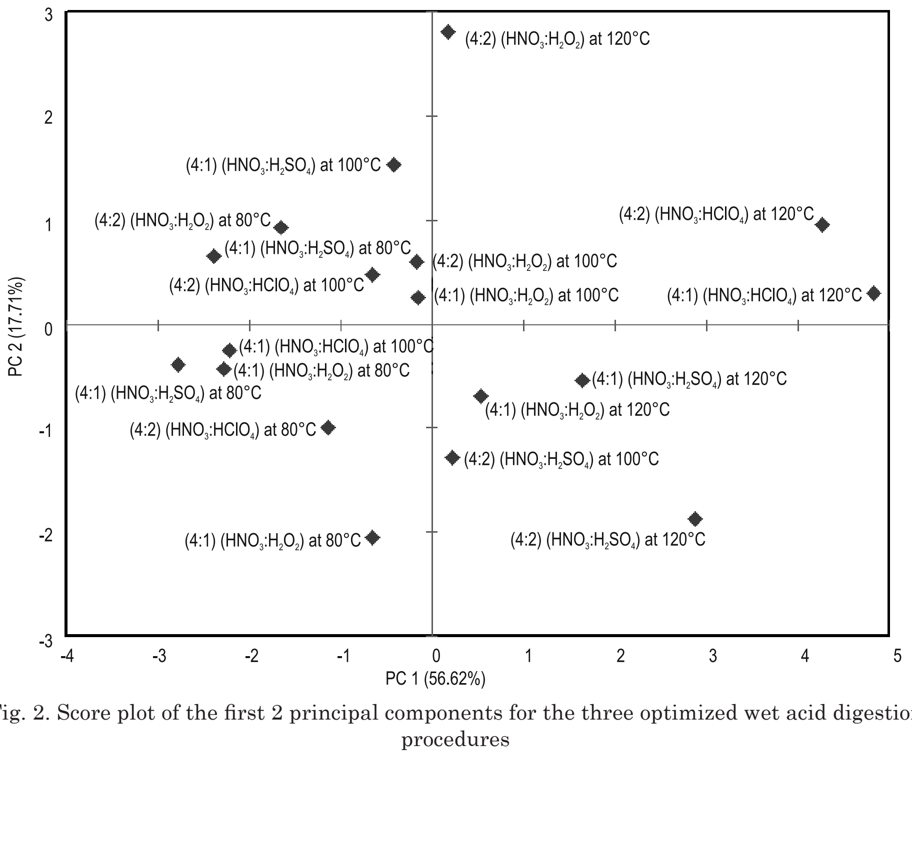


Fig. 2. Score plot of the first 2 principal components for the three optimized wet acid digestion procedures

high temperature, and its addition to nitric acid drastically increases the speed of digestion (Bryce et al. 1995, Conor et al. 2002). In addition, the digestion procedure $HNO_3/H_2SO_4$, 4:2 v/v, showed a good element extraction for Cr, Mn, Ni, Cu, Zn and Se, but this procedure was less efficient than the digestion procedure $HNO_3/HClO_4$ for extraction of these elements. For the digestion procedures carried out at 80°C, $HNO_3/HClO_4$, 4:1 v/v, $HNO_3/H_2SO_4$, 4:2 v/v, $HNO_3/H_2O_2$, 4:1 v/v; and that carried out 100°C, $HNO_3/HClO_4$, 4:1 v/v, are characterized by the highest negative scores corresponding to PC1, indicating that these digestion procedures have a poor extraction rate for these elements. The second component (PC2), which explains 17.17% of the total variance of the origin data set, had a high loading for Co and Fe. The digestion procedure, $HNO_3/H_2O_2$, 4:2 v/v, carried out at 120°C, was characterized by the highest positive score corresponding to PC2. This indicates that this digestion procedure was the most efficient in extracting Fe and Co. On the other hand, the digestion procedure $HNO_3/H_2SO_4$, 4:1 v/v, carried out at 80°C, was characterized by the highest negative scores corresponding to PC2, indicating that this digestion procedure has a poor extraction rate for these elements. It was obvious that the extraction of trace elements from serum samples by using one of the three digestion procedures at a temp. of 80°C or 100°C (Table 2), cannot be recommended where they have either negative first two principal components (PCs) scores or low positive scores. These groups of digestion procedures have the lowest element extraction values; Lodenius et al. (1995) have shown that wet digestion at a lower temperature may result in an incomplete oxidation and incomplete decomposition of the organic matrix. Hierarchical Cluster analysis was performed on the elemental concentrations for the optimized wet acid digestion procedures. Hierarchical clustering analysis (HCA) results are shown in Figure 3. The dendrogram was obtained by calculating the Euclidean distance among the optimized digestion procedures and grouping them by the Ward's linkage method in terms of their nearness or similarity. The dendrogram showed measurements extended up to 14 units that measure the length of the line referring to the degree of dissimilarity between the optimized digestion procedures. The dendrogram revealed that there are three main groups for a dissimilarity index of approximately 7.5. As shown in Figure 3, the hierarchical clusters of the optimized wet acid digestion procedures revealed that there were three main clusters. The first cluster contained the following digestion procedures ${}_{100}NS_{4:2}$, ${}_{120}NH_{4:1}$, ${}_{120}NS_{4:1}$, ${}_{120}NS_{4:2}$, ${}_{120}NP_{4:2}$ and ${}_{120}NP_{4:2}$, the second cluster contained the following digestion procedures ${}_{120}NH_{4:2}$ ,${}_{100}NH_{4:1}$, ${}_{100}NH_{4:2}$, ${}_{100}NS_{4:1}$ and ${}_{100}NP_{4:2}$, while the third cluster contained ${}_{80}NH_{4:1}$ ,${}_{80}NH_{4:2}$, ${}_{80}NS_{4:1}$, ${}_{80}NS_{4:2}$, ${}_{80}NP_{4:1}$, ${}_{80}NP_{4:2}$ and ${}_{100}NP_{4:1}$. Cluster analysis assigns the optimized digestion procedures to clusters so that digestion procedure within each cluster is similar to one another with respect to the concentration of serum trace elements levels (Cr, Mn, Fe, Co, Ni, Cu, Zn and Se).Cluster Analysis (CA) was also used to support and confirm the principal component analysis (PCA) results such that the first cluster revealed that digestion procedures at 120°C had

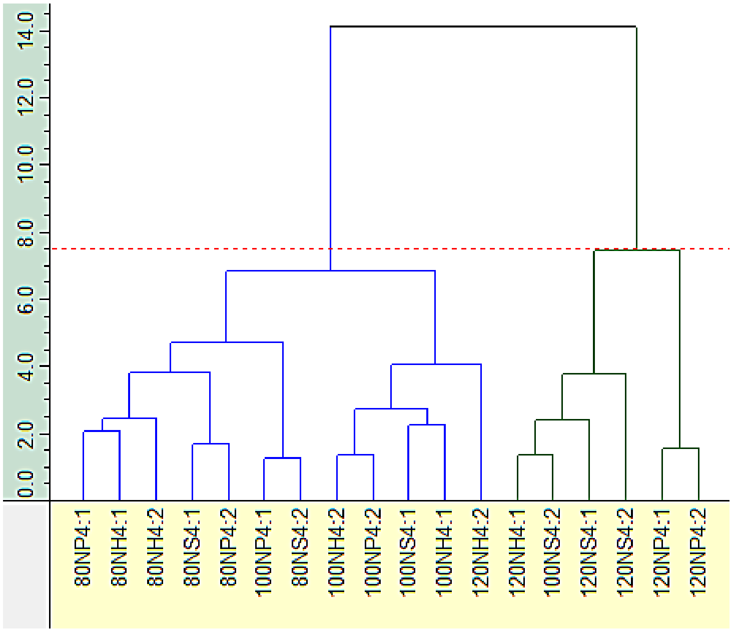


Fig. 3. Dendrogram of cluster analysis of the digestion procedures. The abbreviations NP, NH, and NS correspond to $HNO_3/HClO_4$, $HNO_3/H_2O_2$, and $HNO_3/H_2SO_4$, respectively, under different conditions of volume ratios, 4:1 and 4:2 v/v, and different temperatures, 80, 100, and 120°C

similar potential for extraction of the elements, where the lower the value of the distance cluster, the more significant the association between the digestion methods, and this is supported by the positive part of PC1, which showed high element extraction. The second and third cluster of the digestion procedures, at 80 and 100°C, confirm the analogous behavior of these two procedures. These clusters have negative first two principal components (PCs) scores or low positive scores, which showed low element extraction. Cluster analysis confirms that the variability of the optimized digestion procedures is due to the difference in the element extraction by the digestion procedures between the temperature subgroups.

## CONCLUSIONS

The dissolution of trace elements using wet acid digestion procedures in serum samples using ICP-MS is an essential step in spectroscopic elemental analyses as it can significantly influence the accuracy of results. For the quantification of Cr, Mn, Ni, Cu, Zn and Se in serum samples using inductive coupled plasma mass spectroscopy a digestion solution of nitric-perchloric acid, 4:1 and 4:2 v/v, at temperature of 120°C is recommended, while a digestion solution of nitric acid and hydrogen peroxide 4:2 v/v, at temperature of 120°C is recommened for Fe and Co. The results show that the efficiency for metal dissolution of trace elements from biological samples is dependent on the parameters affecting wet acid digestion procedures.

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