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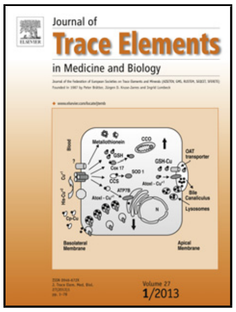

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RESEARCH PAPER

Determination of toxic and essential trace elements in serum of healthy and hypothyroid respondents by ICP-MS: A chemometric approach for discrimination of hypothyroidism

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Highlights

- Determination of essential and toxic trace elements in serum of healthy persons and hypothyroid patients
- An analytical method for the determination of elements by ICP MS was validated.
- Chemometric tools were applied for discrimination of patients with hypothyroidism

Abstract

Inductively coupled plasma-mass spectrometry (ICP-MS) was used to determine three toxic (Ni, As, Cd) and six essential trace elements (Cr, Mn, Co, Cu, Zn, Se) in blood serum of patients with hypothyroidism (Hy group) and healthy people (control group), in order to set the experimental conditions for accurate determination of a unique profile of these elements in hypothyroidism. Method validation was performed with standard reference material of the serum by varying the sample treatment with both standard and collision mode for analysis of elements isotopes. Quadratic curvilinear functions with good performances of models and the lowest detection limits were obtained for ^{52}Cr, ^{66}Zn, ^{75}As, ^{112}Cd in collision mode, and ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{78}Se in standard mode. Treatment of serum samples with aqueous solution containing nitric acid, Triton X-100 and n-butanol gave the best results. Chemometric tools were applied for discrimination of patients with hypothyroidism. All nine elements discriminated Hy group of samples with almost the same discriminating power as indicated by their higher values for this group of patients. Statistically significant correlation ($p < 0.01$) was observed for several elements. Results indicated clear differences in element profile between Hy and control group and it could be used as a unique profile of hypothyroid state.

Keywords: Trace elements, ICP-MS, Serum, Hypothyroidism

Introduction

Trace elements are essential for humans and occupy less than 0.01% of total body weight [1]. The quantification of trace elements in whole blood, serum and urine is used for assessing the health status [2], diagnosis and treatment of various disorders, or occupational and environmental exposure to toxic metals [3].

A very low detection limit (ppt level) and high linear dynamic range of inductively coupled plasma-mass spectrometry (ICP-MS) allow for the determination of many elements and even their isotopic distribution in a wide concentration range within the same analytical run [4]. However, one of the major disadvantages of ICP-MS is the occurrence of mass interferences having the same mass/charge (m/z) ratio as analyte [5]. With the collision/reaction cell technology, many problems with polyatomic interferences can be avoided [6]. The addition of suitable internal standards in clinical samples significantly corrects the matrix-induced ion signal fluctuations [7]. The matrix composition of serum is too complex for direct analysis with ICP-MS and usually calls for sample pretreatment. Very often, the preparation of blood includes procedures with microwave digestion for the complete removal of organic matter with HNO_3 and H_2O_2 [8]. However, digestion procedures are time-consuming and there is a risk of carryover effects in the digestion vessels [9]. In order to avoid time-consuming digestion and the possible contamination risk, another possibility is simple dilution reducing the deposition of solids in the sample introduction system and reducing signal suppression. In order to avoid the deposition of solids in pneumatic nebulizer or central tube in the torch, and to reduce signal suppression, serum samples were usually diluted with appropriate diluents [7]. Some of the suggested diluents were: 0.14 M nitric acid [10], 0.1 mM EDTA, 0.1% Triton X-100 and 10 mM ammonium hydroxide [11], or 0.5% n-butanol with 0.65% nitric acid and 0.01% Triton X-100 [12].

Hashimoto thyroiditis (chronic inflammation of thyroid gland) is the most common autoimmune disease and cause of hipothyroidism (Hy). Trace elements, such as selenium, copper and zinc are essential for normal thyroid hormone synthesis and metabolism [13], and the changes in metal concentrations in body fluids might be associated with different thyroid dysfunction. The most studied trace elements in hypothyroidism were copper, selenium and zinc [14, 15], but there was a lack of information about concentrations of other trace elements, particularly toxic metals and metalloids.

The aims of this study were the optimization of the method for accurate determination of trace elements in serum by application of ICP-MS, and afterward, the classification and discrimination of hypothyroidism according to the toxic and essential trace elements present in serum by application of chemometric technique.

Materials and Methods

Reagents and chemicals

All used chemicals were of analytical grade and were supplied by Merck (Darmstadt, Germany). Analytical grade nitric acid was used after additional purification by double-distillation. Ultrapure water was prepared by passing doubly de-ionized water of Milli-Q system (≥ 18 MΩ). For the calibration, multi-element stock solution (VHG standards; Manchester, UK) containing 10 mg/L of 22 elements was used to prepare intermediate standard solution. Internal Standard Mix (VHG standards, Manchester, UK) Li, Sc (50 µg/L) and Bi, Ga, Y, Tb, In (10 µg/L) was used. The certified reference material “ClinChek® Serum Control lyophilised for trace elements, REC- 8881” was employed for method validation. All glassware was soaked in 10% HNO_3 for minimum 24h and rinsed well with doubly de-ionized water.

Instrumentation

The determinations of elements were carried out in an ICP-Q-MS (iCAP Q, Thermo Scientific X series 2) equipped with flatapole collision/reaction cell. Instrument parameters were optimized using a tuning solution B containing 1 µg/L of Li, Co, In, Bi, Ba, Ce, U in 2% nitric acid. The plasma and nebulizer gas used pure argon (99.999%, Messer, Serbia). The collision cell was filled with pressured helium/hydrogen gas (99.999%). The PFA (Perfluoroalkoxy) concentric nebulizer and standard cyclonic spray chamber were employed. The entire system was controlled with the Qtegra Instrument Control Software. The operating parameters and measurement conditions are given in Table 1.

Sample collection

In this study participated 23 subjects with hypothyroidism (22 women and 1 man, mean age 47±12), treated as out-patients in University Hospital Zemun, Belgrade, Serbia and 70 healthy volunteers (60 women and 10 men, mean age 52±10) as the control group. The control group was chosen by health professionals from the Blood Transfusion Institute of Serbia. Several categories were excluded from the study: smokers, people on dieting, women on oral contraceptives, pregnant women and diabetics. All subjects had not taken vitamin or mineral supplements for at least two weeks before blood sampling. The study was approved by the Ethics Committee of Blood Transfusion Institute of Serbia. All subjects voluntarily participated in the study, and the consent was obtained from each subject.

Sample preparation

Venous blood samples were collected after 8h fasting into trace element free tubes and left at room temperature for 30 minutes. Centrifugation was performed at 3000 rpm and serum samples were placed in trace element free tubes. All samples were stored at -20ºC before the analysis.

All plastic tubes used were checked for contamination by leaching for one week in 10% nitric acid at room temperature. In all cases the concentrations of Cr, Mn, Co, Ni, Cu, Zn, As, Se and Cd were negligible.

The serum samples were prepared in 1:10 (v/v) dilution with three different diluents (treatments). Considered treatments were:

Treatment 1: aqueous solution containing 3% n-butanol, 0.05% nitric acid and 0.1% Triton X-100;

Treatment 2: aqueous solution containing 0.05% ethylenediaminetetraacetic acid (EDTA) and 1% ammonium hydroxide;

Treatment 3: aqueous solution containing 10% acetic acid and 0.1% Triton X-100.

All diluents were prepared immediately before the use to avoid the changes in compositions. The blood serum reference material was prepared according to the manufacturer's instructions and further diluted. The internal standard solution was aspired separated by a second channel of peristaltic pumps, allowing on-line addition to standard and sample solutions.

Statistical methods

The content of elements in serum samples was obtained from calibration curves of each element contained in standard solutions. Limit determination (Limit of detection (LOD) and Limit of quantification (LOQ)) were determined based on standard deviation of blank. Ten determinations were made with blank (at no concentration in the appropriate matrix) to set the limits according to the mean and standard deviation of blank samples.

Descriptive statistics and Mann-Whitney U-test were performed by a demo version of the NCSS statistical software (Hintze, 2001, Number Crucher Statistical System, Kaysville, UT, www.ncss.com).

Principal Component Analysis (PCA) and Partial Least Square-Discriminant Analysis (PLS-DA) were carried out by PLS ToolBox, v.6.2.1, for MATLAB 7.12.0 (R2011a). All data were auto scaled prior to any multivariate analysis. PCA was carried out by using a singular value decomposition algorithm and a 0.95 confidence level for Q and T^2 Hotelling limits for outliers. PLS-DA was performed using the SIMPLS algorithm and validated using a venetian blinds-validation procedure.

Results

Optimization of instrument conditions

The instrument condition of ICP-MS, such as torch position, sample flow rate and plasma power was optimized daily by monitoring the sensitivity (highest signal-to-background ratio) for ^{7}Li, ^{115}In and ^{238}U in tune solution, along with ratios $^{140}Ce^{16}O^{+}/^{140}Ce^{+} < 2.5\%$ and $^{140}Ce^{2+}/^{140}Ce^{+} < 3.0\%$. The gas flow rate of collision gas was tested in the range 3-8 mL/min. A flow rate of 5.0 mL/min proved to be appropriate, with minimal BEC (background equivalent concentration) values for all studied elements.

Selection of the best isotope and mode

CRM of blood serum was prepared in treatment 1 and selection of isotopes was carried out by comparing the obtained results with the certified values. All available isotopes of elements were determined in both modes, standard (STD) and collisional. Six calibrator solutions were used to cover the range of expected analyte concentrations: 0.1-10 µg/L (Cr, Mn, Co, Ni, As, Cd) and 10-100 µg/L (Cu, Zn, Se). Despite the fact that the correlation coefficients, r, were greater than 0.998 and the F-values of the Lack-of-fit test revealed that linear regression model (LRM) adequately fits the calibration data at 95% confidence level (F-values lower than critical ones) (Table S1, Supplementary material), the U-shaped residual plots (Figure S1, Supplementary material) indicated that a curvilinear regression model (QRM) should be preferred over an LRM [16]. Higher values of coefficients of determination, R^2, and lower F-values of the Lack-of-fit test of QRM for each investigated element compared to LRM confirmed the significance of quadratic curvilinear function. Therefore, QRM was chosen as the reference model.

The limit of quantification ranged from 0.003 (Cd) to 2.799 (Cu). LOQ for other elements was: 0.067 (Cr), 0.123 (Mn), 0.007 (Co), 0.057 (Ni), 2.699 (Zn), 0.013 (As), and 1.133 (Se).

The comparison of three treatments

The comparison of three different treatments for selected isotopes and modes were given in Table 2. Treatment 1 (aqueous solution containing 3% n-butanol, 0.05% nitric acid and 0.1% Triton X-100) gave the best results for the following isotopes: ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{78}Se, ^{112}Cd. In order to investigate the influence of different treatments, CRM was prepared in two more treatments: treatment 2 (aqueous solution containing 0.05% EDTA and 1% ammonium hydroxide) and treatment 3 (aqueous solution containing 10% acetic acid and 0.1% Triton X-100). Treatment 3 was good for all selected isotopes, except for ^{60}Ni. On the other hand, the treatment 2 gave the most accurate results only for the most abundant elements in samples (Cu, Zn, Se).

Elements profiles of hypothyroid group vs control group

The summarized parameters of descriptive statistics obtained from the metal content analysis of serum of healthy subjects (Control group – C) and hypothyroid patients (Hy) were presented in Table 3. The ratio of copper with zinc and selenium were also given in Table 3. Copper and zinc, followed by selenium were the most abundant mineral components in all investigated samples.

Although the rest of the studied elements were present in minor quantities and some of them could be detected in trace amounts (µg/L), there were significant variation of their content in serum for healthy and hypothyroid patients. The content of all trace elements were higher in a group of respondents suffering from endocrine disorder. Statistical evaluation of differences in elements content between subjects suffering from hypothyroidism and control subjects were assessed with Mann-Whitney U-test. Statistically significant difference ($p < 0.0001$) between element content in blood samples of comparing groups of respondents was observed for each element (Table 3).

The ranges and selected percentiles (P) of trace elements for the control and Hy group were presented in Tables 4. The comparison with published data was also presented in Table 4.

In addition to univariate data analysis, PCA and PLS-DA were applied to differentiate groups of subjects according to endocrine disorder. A PCA resulted in a four-component model which explained 90.17% of total variance. The first principal component, PC1, accounted for 63.81% of the overall data variance, the second one, PC2, for 14.24% and the third principal component, PC3, for 7.16%. This was very high overall data variance captured by few PCs for the case when the variability among the samples was relatively high (naturally occurring objects, samples), and diverse set of parameters (variables) was considered. Mutual projections of factor scores and their loadings for the first two PCs were presented in Figure 1.

Samples from diverse group of patients were also modeled simultaneously using PLS-DA. In addition to the cross-validation procedure, and in order to determine the degree of accuracy and sensitivity of PLS-DA model, the entire data set was divided into two subsets, calibration set and testing set composed of the randomly selected samples. Calibration set was consisted of 55, out of 70 subjects from control group, and 15 out of 23 subjects from hypothyroid group, while testing set was composed of the rest of the studied patients. Two latent variables (LVs) were selected on the basis of the minimum value of *RMSECV*. Classification and validation results were as follows: R^2_{cal} (0.9741), R^2_{CV} (0.9545), R^2_{PRED} (0.9265), *RMSEC* (0.068), *RMSECV* (0.091) and *RMSEP* (0.103). Scores plot of data for these samples is presented in Figure 2a. The assessment of variables that had the greatest influence on differentiation was done based on Variable Importance in the Projection (VIP) scores. The variables with a VIP score higher than 1 was considered as the most relevant for explaining certain class of subjects. The most important factors that discriminate C and Hy group of objects were Cr, Mn, Co, Zn and Cd (Figure 2b). The standardized regression coefficient that revealed the significance of an individual variable in the regression models was shown in Figure 2c and 2d. Model for hypothyroid patients indicated high positive values of regression coefficients for Cr, Mn, Co, Zn, Cu and Cd and negative values for Ni, As and Se. Contrary, the parameters showed opposite influence on model for the control group.

The correlation study

The correlation between elements was examined by Spearman's rank correlation test for control and hypothyroid group, separately. Statistically significant correlation ($p < 0.01$) was confirmed between Cu and Zn in both groups of objects, the control ($r = 0.65$) and Hy ($r = 0.78$). Our results indicated that Se was correlated with Cu ($r = 0.42$) in the serum of healthy persons, while for Hy patients also with Ni ($r = -0.59$), As ($r = 0.62$) and Cu ($r = 0.65$). Significant correlation was also observed between Co and Mn ($r = 0.58$) in the group of Hy patients.

Discussion and conclusion

Dilution of serum samples with diluent containing nitric acid, Triton X-100 and n-butanol gave the most accurate results for selected isotopes: ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{78}Se, ^{112}Cd. In order to find the best conditions for preparation of serum samples the dilution was varied. For 1:10 (v/v) dilution, after 3h of analysis, good precision (RSD < 5.0%) and low detection limits were observed for all nine elements, without clogging of nebulizer. In addition, the signals of ISs were approximately 100%. Significant signal suppression was observed in 1:5 (v/v) dilutions. It was approximately 60 % of ISs due to high content of organic matter. The similar result was reported earlier [17]. For high dilution factor 1:30 (v/v) the signal suppression was lower and the signal of ISs was from 90-100%. However, the precision and LOD were not improved. Therefore, 1:10 (v/v) dilution was used for further analysis.

Treatment 3 also gave accurate results for all selected isotopes, except for the ^{60}Ni. Kira et al. [18] reported that obtaining of EDTA in a pure form is difficult. However, higher concentrations of elements in treatment 2 could not be attributed to the impurities of EDTA because of blank correction was done.

The comparison of metal content between population groups

The comparison of metal content determined in the present study with those found by other authors in literature was difficult due to the differences in parameters, such as age, sex, time of exposure, geographical factors, tobacco and alcohol intake, food and medication, which could affect the levels of some elements.

Chromium (Cr) is one of the most difficult elements for determination in biological materials by ICP-MS in STD mode. In this study, helium/hydrogen gas was used to reduce ArC^+ background, and the concentrations below 0.42 µg/L for seventy healthy subjects were obtained. The interval from 0.038 to 0.350 µg/L was given as range which was not the result of contamination [19].

Manganese (Mn) is present in most plastics, air dust, and it is a component of medical devices, such as stainless-steel needles. The concentration of Mn in fluids and human tissues is generally low [20] and age independent [21]. The proposed range for Serbian population (0.18-2.82 µg/L) was slightly higher when compared with Italian (0.3-0.9 µg/L) [21] and Belgian population (0.38-1.04 µg/L) [22], but significantly lower when compared with Germany inhabitants (4.8-18.0 µg/L) [23]. The best agreements were obtained with Caroli et al. (0.1-2.9 µg/L) [19] and Goulle et al. (0.63-2.26 µg/L) [12] who had used similar pre-treatment for preparation of plasma samples of healthy persons.

Cobalt (Co) is monitored in serum and urine at vulnerable workplaces as an indicator of external exposure. However, internal exposure by implants significantly increased the level of cobalt in body fluids and, therefore, must be considered. The example was the release Co and Cr from local tissues into blood in patients after HRA (hip resurfacing arthroplasty) [11]. Considering that our volunteers were not exposed to cobalt at workplaces and did not have built-in medical implants, obtained range was from 0.66 to 1.95 µg/L.

Nickel (Ni) is an integral part of sampler and skimmer cones of the most ICP-MS [24]. There is some information that the formation of a salt layer inhibits release elements, such as Ni, Al and Cr from the cones [25]. In spite of that, we used the cones of platinum (Pt) and our range for Ni (1.01-7.78 µg/L) was in agreement with Forrer at el. that also used ICP-MS technique [24].

Copper (Cu) levels in healthy subjects were reported to be 1.1 mg/L in blood serum. Tendency for women to have higher values of Cu is associated with pregnancy and by using oral contraceptive pills [19]. Our results confirmed that female respondents have significantly higher values of copper (763.4 ± 173.5 µg/L) when compared with male respondents (563.5 ± 103.6 µg/L, $p < 0.0001$).

The range obtained for Serbian population (512-1429 µg/L) was in agreement with Caroli et al. (600-1400 µg/L) [19], Minoia et al. (601-1373 µg/L) [21] and with many other authors.

As red blood cells contain a lot of zinc (Zn), its concentration in serum is lower than in whole blood. Many factors, such as medication, stress, fasting or pregnancy influence the level of zinc in serum [19]. The range for zinc (403.6-698.1 µg/L) was lowers in comparison with other publications in different population groups [21, 23], including Czech and Slovak population [26]. However, it was in agreement with studies when authors used double-focusing ICP-MS (420-710 µg/L) [25].

Arsenic (As), as monoisotopic element (m/z = 75), is difficult for determination with ICP-MS without collision gas, as the chloride from the serum forms polyatomic species $^{40}Ar^{35}Cl^{+}$ with argon gas in plasma that overlaps with the same mass [5]. Cornelis et al. [27] and Tuakuila et al. reported that reference range for arsenic heavily depended on dietary intake (particularly meat, seafood and fish) and environmental exposure (water, soil, ambient air etc.). The obtained range for As in Serbian population, who declared to eat fish once or twice a week, was 0.13-3.02 µg/L.

Interpretation of the results for selenium (Se) among the population is difficult, as the geographic abundance and age strongly influence the normal serum level from 30 (new-borns) to 100 µg/L (elderly) [19]. The obtained range for Serbian population (age from 18 to 65 years; 49-101 µg/L) was similar to Belgian population (45-90 µg/L) [27].

Due to the fact that cadmium (Cd) is almost totally bound to erythrocytes, whole blood is better samples for quantification of this metal. The concentration of cadmium in serum was low (~ 0.1 µg/L) [22]. It is known that sex, smoking and drinking habits largely affected the results [19]. Cigarettes contain significant amount of cadmium (1.0-1.7 µg/L) and smokers have > 80 nmol/l of Cd in compassion with non-smokers (< 20 nmol/L) [29]. On the other hand, Cd can enter in the body as a result of industrial exposure or from dietary sources. The obtained results for cadmium in our study were below 0.02 µg/L.

The status of trace elements in hypothyroidism

Several trace elements, Cu, Zn, Se and Mn were investigated in untreated hypothyroidism, but with different conclusions about their serum concentrations [15, 30, 31]. The main limitation was small number of samples. Only the manganese was higher in Hy group then in control group [15, 30, 32] and it was also found in our study. In the paper of Baig et al. [33] the relationship between low cobalt concentrations and hyperthyroidism was reported. Although cobalt was not studied in hypothyroidism, our study demonstrated that the concentration of cobalt was higher in Hy group. For other studied elements, there is no available information about their concentrations. There was also the lack of information about the concentrations of trace element in serum of euthyroid patients. Erdal et al. emphasized that concentrations of trace elements did not change before and after the treatment with levothyroxine (LT4) [14]. It was also concluded in the study of Przybylik-Mazurek et al. [34]. In that sense, our results could indicate unique trace element profile of euthyroid status (Table 4).

Assessment of hypothyroidism based on elements profile and chemometrics

A lot of information (variables) for a great number of samples (objects) obtained in this study required the use of statistical procedures, in order to efficiently extract the maximum of useful information from data. In that sense, PCA was carried out at the exploratory level, not as a classification model, but rather as a hint what could be expected from the current data and to check if there was some logical patterns in the data that might be explained. PLS-DA served as a classification method. The response variable in this supervised technique was categorical, i.e. indicated the groups or categories of the samples, control and hypothyroid group. Obtained mathematical model could be used in further classification of unknown samples.

The results of PCA revealed the existence of two distinctive groups of samples belonging to healthy and hypothyroid patients separated alongside the PC1 (Figure 1a). Subjects from control group were firmly clustered and exhibited small internal variability, while patients from hypothyroid group were dissipated in a broader range. Several subjects exceeded the limits imposed by the Hotteling T^2 95% probability ellipse and could be considered as extreme cases. These samples had higher level of Ni (those in the upper right side of score plot) or Se (subjects in the lower right side of score plot). The loading plot (Figure 1b) revealed that all parameters discriminate Hy group of samples with almost the same discriminating power and indicate their higher values in group of patients suffering from hypothyroidism.

Parameters of PLS-DA models were statistically significant, with high values of R^2_{cal}, R^2_{CV} and R^2_{PRED}, and low difference between *RMSEC, RMSECV* and *RMSEP* values. Hence, data from element content in blood allows accurate prediction of hypothyroidism and provides a useful tool for determination of patients suffering from this disease.

For the fast and reliable determination of ^{52}Cr, ^{66}Zn, ^{75}As, ^{112}Cd in standard mode, and ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{78}Se in collision mode by ICP-MS, the simple 10-fold dilution of serum with nitric acid (0.05%), Triton X-100 (0.1%) and n-butanol (3%) was the best.

For the first time in this study, the range for the trace elements in serum of Serbian population was determined. Significant differences in the concentration of trace elements in serum for control and hypothyroid group were found. It could implicate the disturbance of mineral homeostasis in general. PLS-DA revealed that the most important factors that discriminate both groups of subjects were Cr, Mn, Co, Zn and Cd. Statistically significant correlation for Cu-Zn and Cu-Se indicated the mutual correlation between examined groups. In comparison with the control group, the hypothyroid group had significantly higher values of concentrations for all nine examined elements. These results could provide unique profile of hypothyroidism.

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Declarations of interest

None

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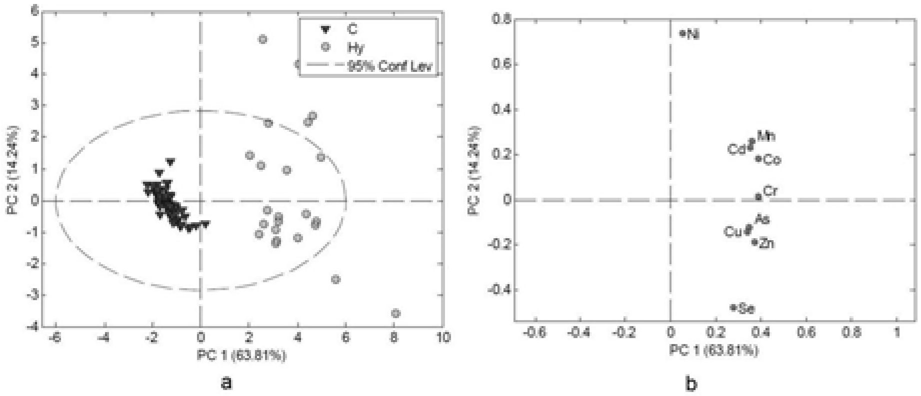

Figure 1. Mutual projections of factor scores and their loadings for the first two PCs

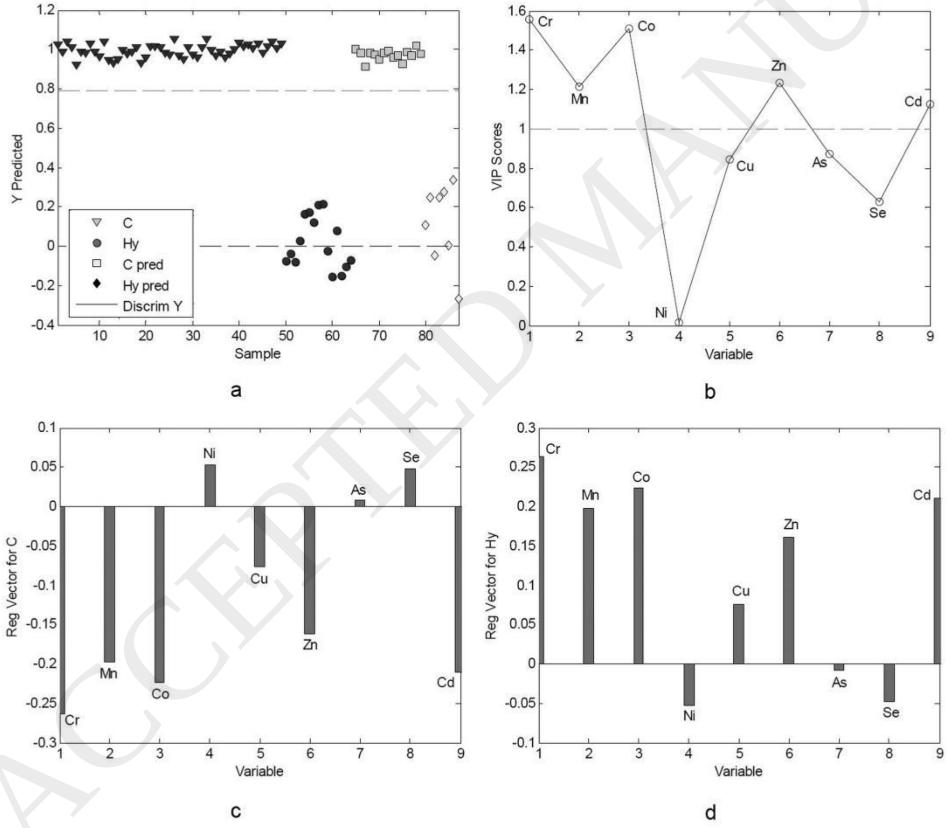

Figure 2. PLS-DA results: a) scores plot of data for examined samples, b) VIP score, c and d) the standardized regression coefficient revealed the significance of an individual variable in the regression model

Tables

Table 1. Operation parameters and measurement conditions

Parameter	**Condition**
RF power	1.35 kW
Gas flow rate	
nebuliser gas flow	0.87 L/min
auxiliary gas flow	0.60 L/min
cool gas flow	13.0 L/min
CCT gas flow (8% H_2/He)	5.0 mL/min
Interface	
sampling cone	1.00 mm, Pt
skimmer cone	0.4 mm, Pt
Ion lens voltages	Optimized for 1 µg/L Li, Co, In, Bi, Ba, Ce, U
Points per peak	3
Dwell time	10 ms
Sample uptake and wash time	30 s
Replicates	6

Table 2. Comparison of different treatments (results are presented as mean ± standard deviation)

Isotope	Mode	Unit	Treatment 1	Treatment 2	Treatment 3	CRM
^{52}Cr	KED	µg/L	6.5 ± 0.2	11.1 ± 0.4	7.0 ± 0.2	5.99-8.99
^{55}Mn	STD	µg/L	8.8 ± 0.2	14.0 ± 0.5	9.6 ± 0.4	7.49-11.20
^{59}Co	STD	µg/L	3.0 ± 0.3	4.6 ± 0.2	3.0 ± 0.3	2.46-3.68
^{60}Ni	STD	µg/L	9.2 ± 0.7	17.3 ± 1.2	12.2 ± 0.8	7.08-10.60
^{65}Cu	STD	mg/L	1.4 ± 0.4	1.5 ± 0.6	1.3 ± 0.6	1.14-1.54
^{66}Zn	KED	mg/L	1.8 ± 0.4	2.1 ± 0.7	1.9 ± 0.5	1.73-2.35
^{75}As	KED	µg/L	72.9 ± 0.5	80.0 ± 0.6	68.4 ± 0.6	50.6-76
^{78}Se	STD	µg/L	108.2 ± 0.3	118.1 ± 0.3	94.3 ± 0.5	84-126
^{112}Cd	KED	µg/L	4.0 ± 0.2	7.0 ± 0.3	4.03 ± 0.3	3.63-5.45

Table 3. Parameters of descriptive statistics obtained for metal content (µg/L) of serum samples

		Cr	**Mn**	**Co**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Cd**	**Cu/Zn**	**Cu/Se**	**Z**
Control group	mean	0.42	0.92	0.98	2.19	718.26	548.42	0.48	75.96	0.02	1.33	9.46	7
	median	0.42	0.91	0.92	1.90	706.20	548.09	0.36	76.44	0.02	1.27	9.18	7
	stdev	0.00	0.25	0.23	1.17	146.60	59.48	0.44	11.07	0.00	0.35	1.94	1
	min	0.42	0.18	0.66	1.01	511.90	403.58	0.13	49.04	0.02	0.87	6.34	4
	max	0.42	2.82	1.95	7.78	1428.99	698.14	3.02	101.35	0.02	3.21	18.26	1
Hypothyroid group	mean	16.41	8.98	3.02	3.40	987.15	1053.56	1.57	98.62	0.50	0.93	10.24	1
	median	15.79	7.71	3.14	0.10	959.97	1060.77	1.27	94.97	0.35	0.95	9.98	1
	stdev	3.65	3.9	0.53	5.94	246.03	113.21	0.63	31.19	0.29	0.18	1.64	2
	min	8.60	4.43	1.92	0.10	548.03	876.44	0.97	57.94	0.12	0.58	6.64	6
	max	22.37	20.42	3.82	21.16	1543.17	1452.70	3.09	202.25	1.15	1.42	12.86	1
Mann-Whitney U-test	P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	0.0003	<0.0001	0.0001	0.0291	<
	H_0	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Accept	R

Table 4. The ranges and percentiles (P) of elements for control group and Hy group

Element	Respondents	Found range (μg/L)	Percentiles (μg/L)			Published data
			P5	P50	P95	
Cr	C group	< 0.42	0.42	0.42	0.42	< 0.30 [22]
	Hy group	8.60-22.37	8.6	15.79	20.70	
Mn	C group	0.18-2.82	0.18	0.91	0.91	0.10-2.90 [23]
	Hy group	4.43-20.42	4.43	7.71	15.13	
Co	C group	0.66-1.95	0.71	0.92	1.40	0.30-1.02 [13]
	Hy group	1.92-3.82	1.92	3.14	3.80	
Ni	C group	1.01-7.78	1.19	1.87	4.36	2.98-7.34 [17]
	Hy group	0.10-21.16	0.10	0.10	17.09	
Cu	C group	512-1429	560.45	706.20	956.44	600-1400 [23]
	Hy group	548-1543	548.03	959.97	1487.79	
Zn	C group	403-698	445.58	548.09	677.49	420-710 [24]
	Hy group	876-1452	876.44	1060.77	1156.08	
As	C group	0.13-3.02	0.15	0.36	1.61	< 0.5-3.6 [24]
	Hy group	0.97-3.09	0.97	1.27	2.55	
Se	C group	49-101	58.80	76.37	96.82	45-90 [25]
	Hy group	58-202	57.94	94.97	156.54	
Cd	C group	< 0.02	0.02	0.02	0.02	< 0.10 [26]
	Hy group	0.12-1.15	0.12	0.35	0.92	