Microchemical Journal 137 (2018) 258–265

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

# Metalloprotein and multielemental content profiling in serum samples from diabetic and hypothyroid persons based on PCA analysis

Ernesto R. Verni [a], Keaton Nahan [b], Alicia V. Lapiere [c], Luis D. Martinez [a], Raúl A. Gil [a,*], Julio A. Landero-Figueroa [b]

[a] Laboratorio de Espectrometría de Masas, Instituto de Química de San Luis (CCT-San Luis). Ejército de los Andes 950, Bloque III, San Luis D5700HHW, Argentina
[b] Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221, USA.
[c] Área de Análisis Clínicos, Departamento de Bioquímica y Cs. Biológicas, Facultad de Química Bioquímica y Farmacia, UNSL, Ejército de los Andes 950 Bloque I, San Luis D5700HHW, Argentina

ARTICLE INFO

*Article history:*
Received 21 August 2017
Received in revised form 30 October 2017
Accepted 31 October 2017
Available online xxxx

*Keywords:*
Diabetes
Hypothyroidism
Metalloproteins
PCA
Trace elements

ABSTRACT

Diabetes and hypothyroidism are both metabolic diseases with great incidence worldwide. Metalloproteins and metals play key roles in normal glucose metabolism and thyroid hormone synthesis, which are altered in their respective pathologies. The aim of this work was to establish the corresponding multielemental and metalloprotean profiles in a control group (n = 20) compared with a diabetic (n = 20) or hypothyroidism group (n = 20), by exploring a multivariate principal components model. Classification to discriminate these groups was possible based in the quantification of 23 elements (Mg, Al, K, Ca, V, Cr, Zn, Fe, Se, Rb, Pb, Cu, Mn, Co, Ni, U, Sr, Mo, Sb, Ba, Tl, Cd, Ag), and alternatively on the metalloprotein profiles obtained by SEC-ICPMS. Determinations were assessed by means of QQQ-ICP and SEC-ICPMS for total and metalloprotean content, respectively. Samples were classified using Principal Component Analysis chemometric tool. Results showed that there were statistical differences in transitional elements concentrations, such as Zn, Cu, Co, Mn, V, and Cr. For the metal associated protein study, the expression of the fractions of the same transitional elements also were statistically different when compared between control vs diabetic patients, and control vs hypothyroid patients. Se levels showed no differences in both studies among groups. This screening study demonstrates that mass spectrometry methods and data analysis with chemometrics tools may be valuable in order to find possible biomarkers in serum samples of diabetic and hypothyroid patients. Future proteomics analysis are necessary to complete these findings.

© 2017 Elsevier B.V. All rights reserved.

## 1. Introduction

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces [1]. Hyperglycemia, or raised blood glucose, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels [1]. In 2012 diabetes was the direct cause of 1.5 million deaths and high blood glucose was the cause of another 2.2 million deaths, and by 2014, 8.5% of adults aged 18 years and older had diabetes [2]. Hypothyroidism is the result of inadequate production of thyroid hormone or inadequate action of thyroid hormone in target tissues. It develops with a great variety of symptoms, altogether effecting general metabolism and dysfunction in multiple organ systems. Primary hypothyroidism is the main manifestation of hypothyroidism, but other causes include central deficiency of thyrotropin-releasing hormone (TRH) or thyroid-stimulating hormone (TSH). Subclinical hypothyroidism (SCH) manifests when there is evidence of primary hypothyroidism with an elevated TSH but a normal free thyroxine (FT4) level [3].

The increasing evidence indicating the potential for antioxidant metals as adjunct therapy for diabetes supports the concept that most of these metals play an important role in the physiological mechanisms related to glycemic control and redox homeostasis [4]. For example, Zn, Mg and Mn are cofactors of hundreds of enzymes, and Zn is involved in the synthesis and secretion of insulin from the pancreatic beta cells [5–15]. Similarly, Cr enhances the insulin receptor activity on target tissues, especially in muscle cells [16–20]. Understanding of the essential role of Se in the synthesis of thyroid hormones, metabolism and action, as well as for normal thyroid function, increased substantially during the last decades [21–23]. High content of Se found within the thyroid tissue is consequence of the expression of several Se-dependent enzymes [22–25].

*Abbreviations:* SEC-ICPMS, size exclusion chromatography inductively couple plasma mass spectrometry; QQQ-ICP, triple quadrupole inductively couple plasma mass spectrometry; OS, oxidative stress; ROS, reactive oxygen species; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; SCH, subclinical hypothyroidism; HbA1C, glycosylated hemoglobin; MARS, multi affinity removal system; PCA, principal component analysis.

* Corresponding author.
*E-mail addresses:* erverni@gmail.com (E.R. Verni), ragil@unsl.edu.ar (R.A. Gil).

https://doi.org/10.1016/j.microc.2017.10.021
0026-265X/© 2017 Elsevier B.V. All rights reserved.

The study of the metallome and metal homeostasis in biological environments brings challenges regarding the development of improved detection methods with high sensitivity, and robust enough to be performed in physiological conditions. There is not much research on accurate quantification of metal and metalloprotein species in serum samples of diabetic nor hypothyroid individuals. Nevertheless, most speciation studies were focused on revealing functionality and profiling of metalloprotein content in neurodegenerative processes [26–33], while others paid their attention on other diseases such as acute and chronic arthritis [34], Wilson's disease [35], cutaneous melanoma [36], degenerative joint diseases [37], among others.

A number of studies were carried out in order to classify diabetic, hypothyroid and control serum samples based on their total elemental and metallopreotean content. To this aim, total elemental and metalloprotean profiles were evaluated with QQQ-ICP and high-performance liquid chromatography (HPLC) coupled to QQQ-ICP, respectively. The functional metalloproteins are usually less abundant than the non-active albumin; effective depletion of this abundant fraction allows observing small differences in metalloprotean contents. For this reason, affinity chromatography was used to remove albumin along with gamma immunoglobulin, removing approximate 70% of proteins from the plasma samples.

Quantitative data from total content and integrations of peak areas were processed using chemometrics methods to elaborate the corresponding profiles, which allowed a statistical classification of samples.

## 2. Experimental

### 2.1. Instrumentation

The human Multiple Affinity Removal System column 4.6 × 50 mm (MARS) was purchased from Agilent Technologies (Santa Clara, CA, USA) to deplete HSA and IgG. For the size exclusion chromatography separation, a TSK Gel 3000SW 7.5 × 300 mm (Tosoh Bioscience, Germany) was used. The pre-concentration of samples was done by centrifugation (Sorvall instruments RC5C, Buckinghamshire, England). An Agilent 8800 QQQ-ICP equipped with a plasma frequency RF generator and an octopole collision/reaction system (ORS3) with collision/reaction cell (CRC) was coupled with an Agilent 1100 LC (Agilent Technologies, Santa Clara, CA, USA). An Agilent 1100 series HPLC system equipped with a binary pump, vacuum membrane degasser, thermostated auto sampler, column oven, and diode array detector with a semi-micro flow UV–Vis cell was used for all chromatographic analysis (Agilent Technologies, Santa Clara, CA, USA). The QQQ-ICP was controlled by the Mass hunter version 4.1 from Agilent Technologies, while the HPLC system was controlled using Chemstation software (Agilent Technologies, Santa Clara, CA, USA).

### 2.2. Reagents and solutions

Methanol, ammonium acetate, bovine serum albumin, and formic acid were analytical grade purchased from Sigma (St. Louis, MO, USA). Ammonium bicarbonate and acetic acid were purchased from Fluka (St. Louis, MO, USA). Nitric acid (trace metal grade) was purchased from Fisher scientific (Pittsburgh, PA, USA). Double deionized (DDI) water was prepared by passing distilled water through a NanoPure (18 MΩ) treatment system (Barnstead, Boston, MA, USA) and was used to prepare all solutions used in the experiments. For calibrating the SEC column, the SEC standard (Bio-Rad Laboratories, Hercules, CA) mixture of molecular weight markers ranging from 1300 to 670,000 Da was used. For the sample concentration after the MARS system, spin concentrators for proteins (3 kDa MWCO, Amicon Ultra Centrifugal Filters) from Millipore (Billerica, MA, USA) were used. Buffer A and buffer B mobile phases for MARS column and 5 kDa MWCO were purchased from Agilent (Agilent Technologies, Santa Clara, CA, USA).

### 2.3. Procedures

#### 2.3.1. Serum samples

Diabetic and hypothyroid patients who volunteered for this project were enrolled under an informed consent approved by an Ethics Committee Board from the Universidad Nacional de Rosario (Argentina). Blood draws were performed in a private Biochemical Clinic from San Luis, Argentina, were every medical history was recorded (data not shown). The samples were collected into metal free collection tubes, and placed in a thermostatic bath (37 °C) for an hour and then centrifuged for 10 min at 2000*g*. in order to obtain the serum. After this, the serum was pipette out into polypropylene cryovials and stored in −80 °C until further analysis.

For the present work, sixty serum samples were collected during a 3-month period: Twenty (20) samples from diabetic patients, twenty (20) from hypothyroid patients, and twenty (20) from individuals with no ostensible symptoms or disease, which were set as controls. In Table 1 are presented some clinical and biochemical characteristics for the selected cases. Limitations of our study lie on the fact that study subjects are ambulatory patients and, for the diabetic ones, glycosylated hemoglobin (HbA1C) determination was not available for monitoring and control. In addition, most of these patients are under treatment (metformin for diabetic and levothyroxine for hypothyroid ones), therefore, it is not expected significant variations on some of the clinical features, specially blood glucose and TSH, in comparison with a non-treated individual. However, there was found subtle differences between groups that are consistent with the diagnosis. For example, average concentration of blood glucose found in diabetic is higher than hypothyroid and control ones, as expected.

#### 2.3.2. Multi affinity removal system

In order to determine the content of functional metalloproteins in serum samples, and subsequently develop the corresponding profiles, the first step was the removal of albumin and immunoglobulin gamma. Albumin has no metals as prosthetic groups or cofactors and, by definition [38], is not considered metalloprotein with specific biological function, yet it will bund a wide range of metals un-specifically. The MARS column is specially designed to remove the unwanted fractions from the serum. First, the HPLC system was flushed with isopropyl alcohol for 10 min, followed by flushing with water for 2 h. After that, 2 blanks with and without column were injected into the system. The mobile phase used in the affinity chromatography to remove the HSA and IgG is a solution of

**Table 1**
Clinical characteristics from volunteer subjects. Complementary biochemical determinations are presented, such as blood glucose and TSH, which are specific biomarkers of diabetic and hypothyroidism diagnosis, respectively.

| | Healthy control (n = 20) | Diabetic (n = 20) | Hypothyroid (n = 20) |
|---|---|---|---|
| Age (years) | 46 | 44 | 44 |
| Median (range) | (29–57) | (29–57) | (27–70) |
| Gender (% female) | 55 | 40 | 60 |
| Body Mass Index (kg $m^{-2}$) | 22 | 26 | 24 |
| Median (range) | (13–28) | (14–34) | (17–28) |
| Creatinine (mg $dL^{-1}$) | 0.81 | 0.99 | 0.78 |
| Median (range) | (0.5–1.1) | (0.45–1.67) | (0.45–1.14) |
| Cholesterol (mg $dL^{-1}$) | 189 | 197 | 196 |
| Median (range) | (139–256) | (113–235) | (113–250) |
| Triglycerides (mg $dL^{-1}$) | 118 | 147 | 129 |
| Median (range) | (56–280) | (55–289) | (59–280) |
| TSH (IU $L^{-1}$) | 2.14 | 2.75 | 3.00 |
| Median (range) | (1.5–2.89) | (1.62–4.6) | (0.7–7.7) |
| Blood glucose (mg $dL^{-1}$) | 80 | 130 | 79 |
| Median (range) | (56–136) | (100 − 200) | (56–100) |

proprietary composition with a high concentration of nonvolatile salts. Plasma samples of 200 μL each were diluted four times with buffer A and 400 μL were injected into the system. The first fraction between 1.5 and 7.5 min was collected for further separation and the HSA and IgG retained on the column were eluted using buffer B. This process was carried out twice per sample in order to have enough protein concentration in the depleted fraction. After the fraction collection from the MARS system, the volume was reduced using a 3 kDa MWCO spin filter, the 3 mL collected were reduced to 300 μL by spinning for 35 min at 5500*g* at 4 °C. The 3 kDa spin filters were passivated before being used, by adding a passivation solution, of 0.2 mg $mL^{-1}$ bovine serum albumin for 2 h. After 2 h, the passivation solution was removed and rinsed with water thoroughly. Passivation of the spin filters was meant to increase the recovery by saturating the active sites in the membrane.

### 2.3.3. Determination of metalloproteins fractions (size exclusion chromatography-ICPMS)

Following the previous separation, size exclusion chromatography was utilized. SEC allows removal of low molecular weight compounds present in the plasma, while preserving size-based fractions of the proteins in the samples. As mentioned earlier, the mobile phase used in affinity chromatography contains considerable amounts of nonvolatile salts, so using a volatile buffer in the SEC process is necessary to prepare the fractions collected. The concentrated samples were then introduced into the HPLC system using a TSK SW3000 column, with a pre-column of 0.45 μm. The mobile phase used was 50 mM ammonium acetate at pH 7.4, with 0.5% methanol, at a flow rate of 0.5 mL $min^{-1}$. Addition of methanol decreases the non-specific interaction of the sample with the stationary phase. The mass spectrometer was coupled to a LC Agilent 1100, and the fast response of the RF generator allows the use of organic solvents without plasma disturbances. The instrumental conditions were: RF power 1500 W, gas flow carrier 1.02 L $min^{-1}$, gas flow distribution 0.12 L $min^{-1}$, sampler and skimmer nickel cones, and reaction gas flow (He) of 3.2 mL $min^{-1}$.

For the HPLC coupling to QQQ-ICP a short PEEK line of 0.17 mm of ID was used. The effluent coming from the UV–Vis detector of the LC instrument was used, which was immediately introduced into the ICP nebulizer. LC detection software monitored the appearance of peaks corresponding to the protein fractions at 280 nm. Similarly, ICP software, thanks to an integrative tool, reproduced the same chromatogram simultaneously, but detecting the signal of appearance of protein bound to a metal in its respective mass to charge (*m*/*z*) ratio spectrum. In Fig. 1 it is shown the typical SEC-ICPMS chromatogram for the method proposed. It was based on an isocratic run (mobile phase of ammonium acetate buffer 0.05 mol $L^{-1}$. 0.5% methanol. pH 7.4) at a flow rate of 0.5 mL $min^{-1}$. Limitations in the number of elements to determine were defined by the integration time spectra. Because of this restriction, only 14 elements were assessed in real samples, as in the molecular weight standard and blanks. Each run (42 min long) involved the injection of 50 μL of the concentrated samples. $^{51}V$, $^{52}Cr$, $^{55}Mn$, $^{56}Fe$, $^{59}Co$, $^{60}Ni$, $^{63}Cu$, $^{64}Zn$, $^{75}As$, $^{78}Se$, $^{85}Rb$, $^{98}Mo$, $^{114}Cd$, and $^{208}Pb$ were measured in the protean fractions, and all vials were placed in the HPLC autosampler cooled at 4 °C. To establish the relationship between the retention times of the different metalloprotean fractions and their corresponding molecular weight, a gel filtration standard (GFStd) was used (Bio-Rad Laboratories, California, USA).

### 2.3.4. Total elemental analysis

Serum samples were digested as follows: to 100 μL aliquot of serum sample, 200 μL of 30% nitric acid and 50 μL of internal standard (composed of $^{45}Sc$, $^{72}Ge$, $^{115}In$ and $^{209}Bi$), were added, and heated for 12 h (overnight) in a digital dry bath (SH1004, Southwest Science, New Jersey, USA) at 140 °C; then, without removing the samples from the bath, 100 μL of 31% hydrogen peroxide were added for another 4 h. After cooling, the volume was brought to 3 mL with deionized water and later introduced to the QQQ-ICP system. The instrumental operating conditions were as follows:

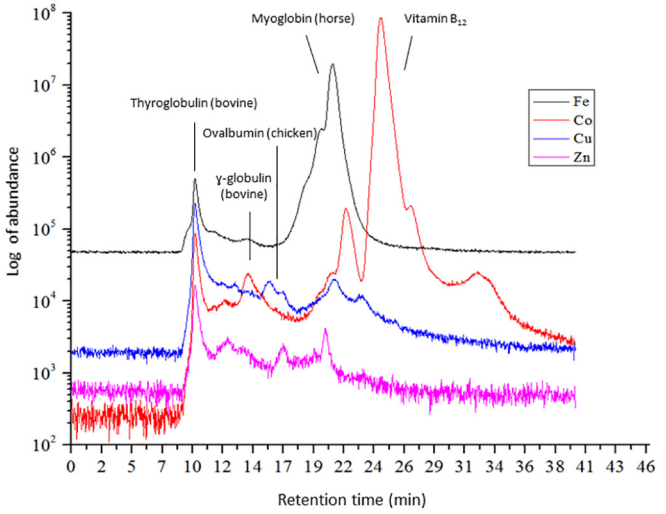


**Fig. 1.** SEC-ICPMS chromatogram of the gel filtration standard. For demonstration purposes, only 4 from the original 14 elements' signals are shown. The standard, containing 5 proteins of known molecular weight, was ran in the beginning and end of each run, in order to evaluate loss of sensitivity due to long run times.

forward power 1500 W, carrier gas flow rate 0.95 L $min^{-1}$, make-up gas flow rate 0.10 L $min^{-1}$, nickel sampling and skimmer cones, collision/reaction cell gas He, 4.5 mL $min^{-1}$. The following isotopes were monitored for total element study: $^{24}Mg$, $^{27}Al$, $^{39}K$, $^{40}Ca$, $^{51}V$, $^{52}Cr$, $^{55}Mn$, $^{56}Fe$, $^{59}Co$, $^{60}Ni$, $^{63}Cu$, $^{64}Zn$, $^{78}Se$, $^{85}Rb$, $^{88}Sr$, $^{98}Mo$, $^{107}Ag$, $^{114}Cd$, $^{121}Sb$, $^{137}Ba$, $^{205}Tl$ $^{208}Pb$ and $^{238}U$. Calibration was performed using the Claritas (SPEX Certiprep) multi-element standard solution (5% nitric acid (v/v)) at element concentration levels: 0; 0.1; 0.3; 0.5; 1.0; 2.0; 5.0; 10; 25 and 40 mg $L^{-1}$ and with addition of the internal standards (5.0 mg $L^{-1}$ Sc, 5.0 mg $L^{-1}$ Ge, 5.0 mg $L^{-1}$ In and 5.0 mg $L^{-1}$ Bi).

For validation purposes of the analytical procedure, a reference material sample of drinkable water was determined simultaneously with the samples (26 elements ICP Trace Metals in Drinking Water Standard in 2% HNO3 + Tr HF, High Purity Standards, South Carolina, USA), where recoveries were above 77% (see Table 2).

### 2.3.5. Statistical analysis

All descriptive statistical analysis (hypothesis testing, errors, correlation, etc.) were performed with STATISTICA software (version 10, Stat Soft Inc., Tulsa, OK, USA). Calculation of the integration and development

**Table 2**
Recovery study from several trace elements in Drinking Water Standard (DWS) by means of QQQ-ICP. Values went from 77% for Ag to 93% for Ni. The DWS was determined in order to validate the analytical procedure.

| Element | DWS conc. [ppb] | Proposed method [ppb] | Recovery (%) |
|---|---|---|---|
| Cu | 20 | 18 | 89 |
| Mn | 40 | 35 | 88 |
| Ag | 2 | 2 | 77 |
| Zn | 75 | 61 | 81 |
| Cd | 10 | 9 | 90 |
| Fe | 90 | 83 | 92 |
| Mo | 110 | 98 | 89 |
| Na | 22,000 | 20,012 | 91 |
| Pb | 20 | 17 | 84 |
| Ni | 60 | 56 | 93 |
| Cr | 20 | 18 | 88 |
| K | 2500 | 2158 | 86 |
| Tl | 10 | 8 | 81 |
| Co | 25 | 22 | 86 |
| Mg | 8000 | 7316 | 91 |
| Se | 11 | 9 | 83 |
| V | 35 | 30 | 87 |

**Table 3**
Determination of total element content by means of QQQ-ICP. Concentrations are expressed in μg $L^{-1}$ except in * (μg $mL^{-1}$). SD: standard deviation. Comparative values of concentrations between groups are significant with levels of $p < 0.05$, being C-D: Control vs Diabetics, and C-H: Control vs Hypothyroid. N.S.: non-significant.

| Elements | Control | | Diabetic | | Hypothyroid | | Detection limits | Statistics | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | | C-D | C-H |
| Mg* | 13.930* | 5.296* | 17.143* | 3.026* | 16.858* | 4.889* | 0.080 | $p < 0.05$ | N.S. |
| Al* | 9.22* | 14.50* | 3.04* | 0.53* | 5.57* | 9.24* | 0.27 | N.S. | N.S. |
| K* | 144.61* | 42.71* | 156.11* | 101.12* | 147.43* | 111.45* | 0.89 | N.S. | N.S. |
| Ca* | 24.29* | 7.772* | 24.69* | 4.74* | 25.37* | 5.98* | 2.247 | N.S. | N.S. |
| V | 6.6 | 5.07 | 1.2 | 0.6 | 1.9 | 2.0 | 0.002 | $p < 0.05$ | $p < 0.05$ |
| Cr | 170.1 | 173.02 | 17.0 | 8.3 | 14.0 | 10.0 | 0.003 | $p < 0.05$ | $p < 0.05$ |
| Mn | 109.0 | 87.43 | 18.4 | 7.7 | 50.3 | 116.5 | 0.02 | $p < 0.05$ | N.S. |
| Fe* | 8.782* | 6.792* | 2.228* | 0.984* | 2.659* | 1.145* | 0.303 | $p < 0.05$ | $p < 0.05$ |
| Co | 31.3 | 73.60 | 0.8 | 0.7 | 0.6 | 0.7 | 0.015 | N.S. | N.S. |
| Ni | 316.3 | 231.65 | 33.1 | 29.0 | 40.2 | 76.3 | 0.018 | $p < 0.05$ | $p < 0.05$ |
| Cu | 1454.5 | 871.67 | 884.6 | 276.8 | 919.6 | 365.4 | 0.06 | $p < 0.05$ | $p < 0.05$ |
| Zn | 1865.1 | 1040.89 | 623.9 | 177.8 | 696.7 | 299.9 | 0.225 | $p < 0.05$ | $p < 0.05$ |
| Se | 54.1 | 11.13 | 51.3 | 13.6 | 49.3 | 14.6 | 0.076 | N.S. | N.S. |
| Rb* | 29.88* | 8.68* | 28.53* | 15.75* | 31.32* | 19.09* | 1.069 | N.S. | N.S. |
| Sr | 89.0 | 39.52 | 53.0 | 20.3 | 59.0 | 29.3 | 0.004 | $p < 0.05$ | $p < 0.05$ |
| Mo | 37.7 | 79.95 | 5.7 | 7.3 | 3.1 | 1.3 | 0.009 | N.S. | N.S. |
| Ag | 296.9 | 348.09 | 72.0 | 110.1 | 28.7 | 53.1 | 0.009 | $p < 0.05$ | $p < 0.05$ |
| Cd | 45.3 | 44.07 | 3.1 | 1.7 | 2.4 | 3.1 | 0.01 | $p < 0.05$ | $p < 0.05$ |
| Sb | 78.6 | 213.79 | 8.7 | 15.2 | 3.6 | 7.1 | 0.005 | N.S. | N.S. |
| Ba | 736.2 | 1460.85 | 50.2 | 40.0 | 36.2 | 29.7 | 0.015 | $p < 0.05$ | $p < 0.05$ |
| Tl | 0.4 | 1.20 | 0.1 | 0.3 | 0.1 | 0.1 | 0.001 | N.S. | N.S. |
| Pb | 713.9 | 574.42 | 45.4 | 33.1 | 45.9 | 43.7 | 0.013 | $p < 0.05$ | $p < 0.05$ |
| U | 1.5 | 1.06 | 0.5 | 0.4 | 0.9 | 1.0 | 0.0007 | $p < 0.05$ | N.S. |

of the chromatograms were performed using Origin Pro software (version 9.0, OriginLab Corporation, Northampton, MA, USA). Classification analysis by principal component analysis (PCA) was achieved using quantification data (concentrations) from total elemental determination and integrations of the corresponding chromatographic peaks of metalloprotean fractions, in order to display the statistical discrimination between groups (plots scores), and confirm those variables who managed to sort out hypothyroid, diabetic and control groups (loadings plots). To accomplish this, data was processed with THE UNSCRAMBLER X software (version 10.2, CAMO AS, Oslo, Norway).

## 3. Results and discussion

### 3.1. Elemental content assessment

A total of 23 elements were analyzed by QQQ-ICP from 60 serum samples from diabetic, hypothyroid and control patients. Accuracy of the analytical method was evaluated by analyzing the drinking water reference material. The mean concentrations and standard deviations for each group are shown in Table 3. As observed, there is a significant difference in the concentration of trace elements between groups. It must be stressed out that, despite these differences, the concept of metallome is dynamic rather than static, and it fluctuates over time and it is influenced by a number of genetic and environmental conditions. Therefore, it can be inferred that drastic variations in the concentrations of certain elements, such as V, which decreases almost 4 times its concentration between groups, has no direct impact on the overall homeostasis.

In the case of higher concentration elements, such as Mg, Fe, Cu and Zn, there was significant differences between control vs diabetic, which is consistent with previous studies [12,39–45]. There is no evidence, for the best of our knowledge, involving studies of multielemental content in thyroid pathologies, though the importance of Se in the synthesis of thyroid hormones, which was mentioned above. For Ni and Cr,

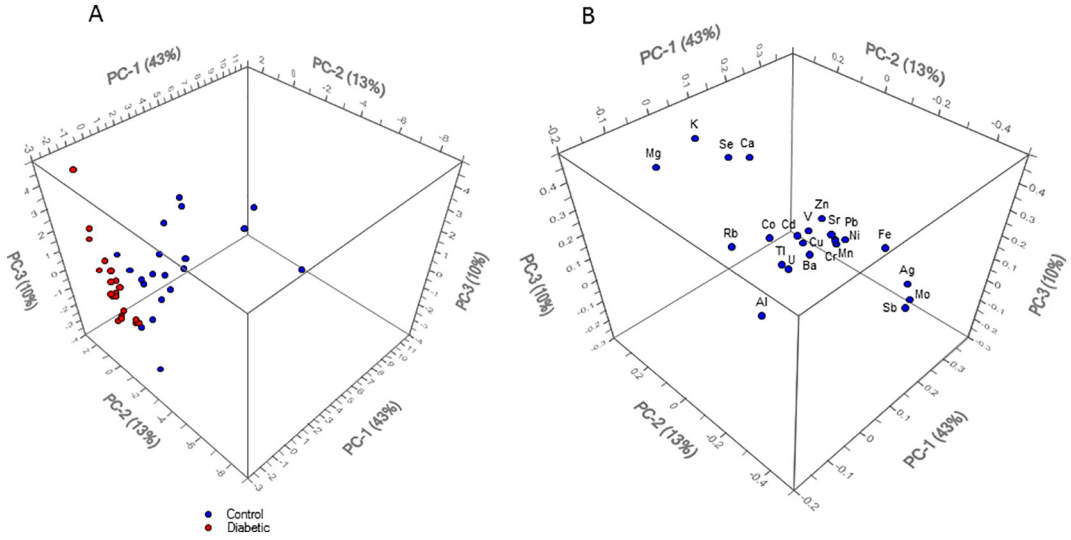


**Fig. 2.** (A) Scores plot of total element content of control samples (blue marked) against diabetic samples (red marked). (B) The loadings plot shows the most relevant variables which have influence on the proposed model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

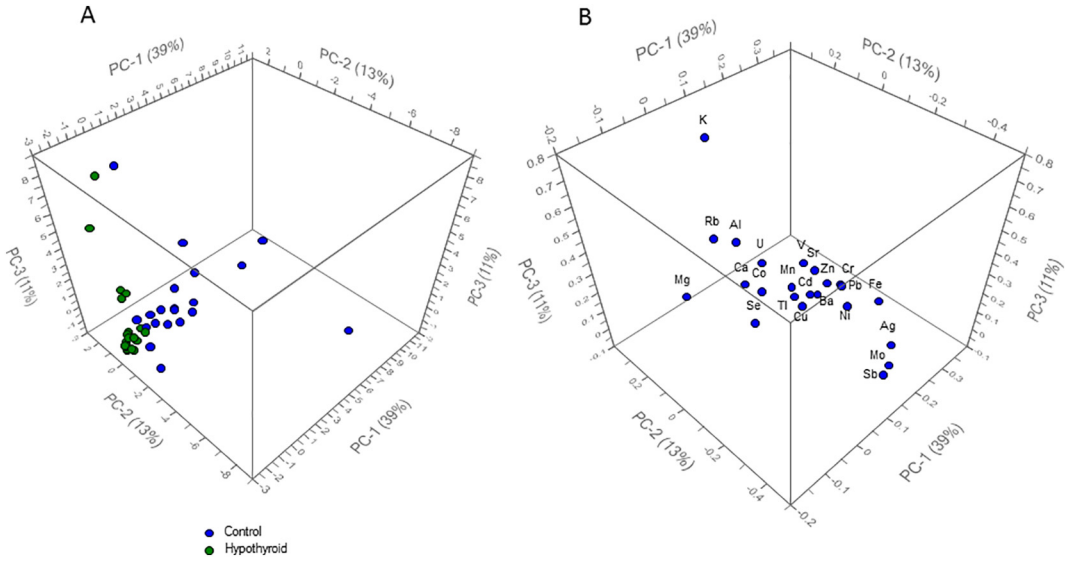


**Fig. 3.** (A) Scores plot of total element content of control samples (blue marked) against hypothyroid samples (green marked). (B) Loadings plot of the variables that classified the samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentrations in control individuals are 2–3 orders of magnitudes higher than diabetic and hypothyroid, which are not consisted with literature.

A Spearman correlation study between elements in each group was performed (see Supplementary material), in order to find associations between variables, in this case metal concentrations. In the control group, a trend of transition elements (V, Cr, Zn, Fe, Ni, Mn) to undergo variations in their concentrations directly proportional with each other was evidenced, often reaching values of $r > 0.8$. This could indicate a synergic effect in absorptive capacity of the mentioned elements. For diabetic and hypothyroid samples, such behavior is less evidenced, indicating only some specific interactions, for example, V–Fe ($r = 0.48$) and Cr–Fe ($r = 0.58$) in diabetic samples. Interestingly, an essential element that showed no significant variations was Se, with $r \approx 0$, which means that its concentration varied independently from the rest.

Principal component analysis (PCA) is a chemometric tool that allows classifying and finding from a large number of variables new "hidden" ones, which have influence over a certain system. In the case of multielemental determination, the analysis of the variables (metals) allows us to classify serum samples according to the elemental profile. First, it was necessary to "weight" (normalize) the variables due to high differences in orders of magnitudes of concentrations between them (for example, Zn is 200-fold more in serum than V). The criterion $weight = 1/\sqrt{SD}$ was applied for this model. Then, validation mode was established using *cross-validation*, which meant repeating and calculating the arithmetic mean obtained from the evaluation measures on different partitions. Finally, all variables and concentration data were introduced in the software, sorted by groups: control samples were analyzed and compared against diabetic ones; then, the same procedure was applied to hypothyroid samples.

Fig. 2A shows the scores plot which represent the 20 control and 20 diabetic serum samples, distributed according to their multielemental content. Both groups are remarkedly separated, probing acceptable classification in a first approach. To properly understand the behavior of both groups, analysis of the loadings plot (Fig. 2B) is required. Pb, Fe, Ni, Mn, Cd, V, Zn and Cr concentrations had the highest influence on the principal component 1 (PC1), followed by Cu and Co. Interesting, Se was located near coordinate origin of the components, thus, its influence over the proposed model is limited as a variable, consistent with the fact that it did not experienced significant differences in concentrations between groups. In order to determine data scattering, explained variance rate was used. In this case, using at least 5 PC, an estimated variation of approximately 80% was reached, an acceptable value for a biological model.

Following the same data analysis, classification between control and hypothyroid samples was achieved (see Fig. 3A). With an estimated explained variance of 39% in the first component, and a cumulative variance of 82% using 6 PC, the model allowed to discriminate between the two groups. The loadings plot in Fig. 3B showed a similar distribution pattern to that observed in the control vs diabetic model. Transition elements had more influence on component 1, which again, confirmed the significant variations in concentrations between these groups, except in the case of Se.

### 3.2. Metalloprotean profile assessment

Once the chromatograms were obtained after separation of the protean fractions by SEC-ICPMS, data was processed by integrating the corresponding area for each peak. For this purpose, a calibration curve was prepared (Fig. 4) from the gel filtration standard. Extrapolating the retention times of the peaks, and calculating the inversed common logarithm of molecular weights, the latter could be estimated and the fractions expressed in terms of molecular weight (in Daltons).

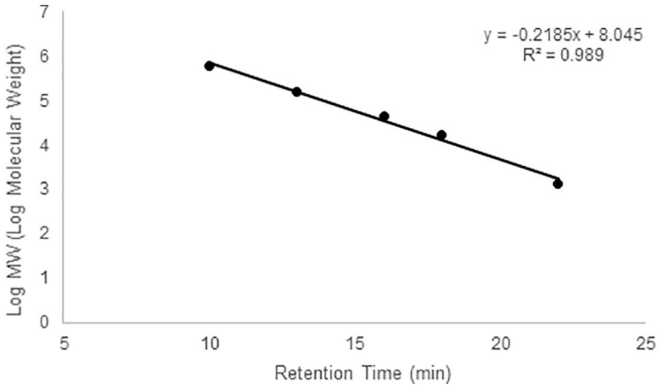


**Fig. 4.** Calibration curve of the gel filtration standard (GFStd). The standard contains bovine thyroglobulin (670,000 Da), bovine γ-globulin (158,000 Da), chicken ovalbumin (44,000 Da), horse myoglobin (17,000 Da), and Vitamin $B_{12}$ (1350 Da). The GFStd was ran in the beginning and end of each chromatographic run in order to check signal fluctuations and normalize data.

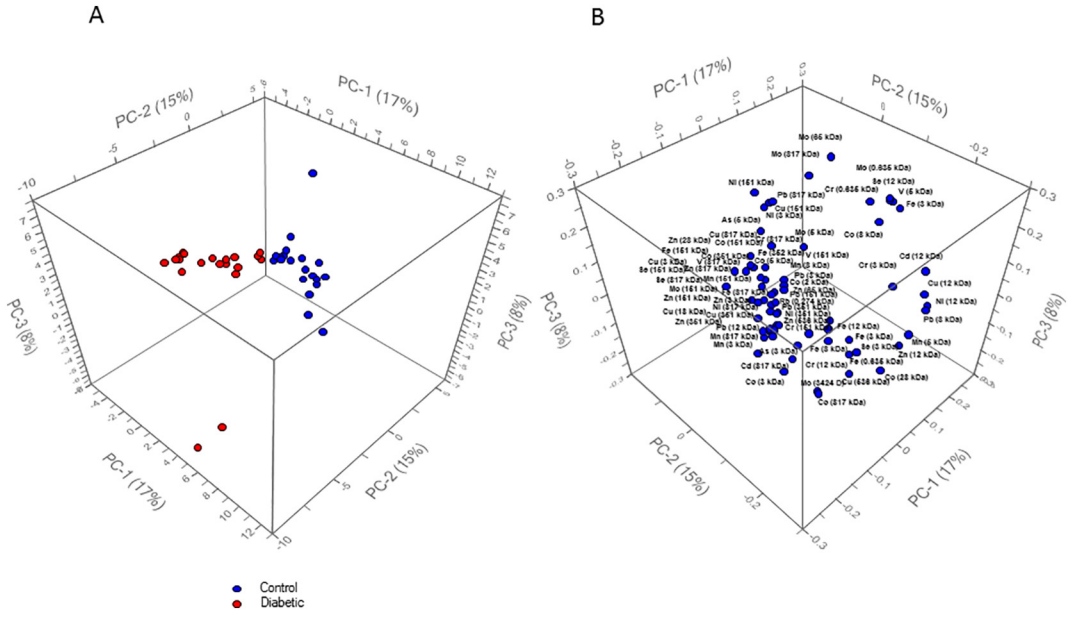


**Fig. 5.** (A) Scores plot of metalloprotean fractions (integrations) of control samples (blue marked) against diabetic ones (red marked). (B) The loadings plot which shows the metalloprotean fractions which their corresponding molecular weight (variables). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Since differences in peak areas among fractions were found, normalization was necessary, along with the evaluation of loss of sensitivity in each sets of runs. For both purposes, the average integrations of the peaks of GFStd (in the beginning and end of each run) were calculated. Finally, data was introduced into the software to perform the classification analysis.

In Fig. 5A it is shown the score plot from control and diabetic metalloprotean integrations. There was a clear separation between the two groups, despite the presence of some outliers, where PC1 evidenced a 17% of explained variance. The use of 6 components was sufficient to explain the model, with an 85% explained variance. Remarkably, not only acceptable separation between the two groups was achieved, but also it has been probed that separation of fractions was reproducible, even for long-term chromatographic runs. From an analytical point of view, this was an advantage, since achieving plasma stability in a coupling system is usually difficult to achieve. The loading plot in Fig. 5B showed that fractions of Cu and Zn (817 kDa), Fe and Co (352 kDa), As and Mo (5 kDa), V (152 kDa), and Pb (3 kDa) had the greatest influence on PC1 component. If interpolating both graphs, the expression of these fractions is decreased in diabetic patients compared to controls.

Identical analysis procedure was performed when comparing metalloprotean fractions from control and hypothyroid samples, as shown in Fig. 6A, where classification was also accomplished, with accumulative explained variance of 80% in the first 6 components. As seen in Fig. 6B, the metalloprotean fractions which had more influence in PC1 were Co and Fe (352 Da), Cu (817 kDa), V (152 kDa), Mo (521 kDa), and Pb (342 kDa), all expressed in a larger extent in control samples.

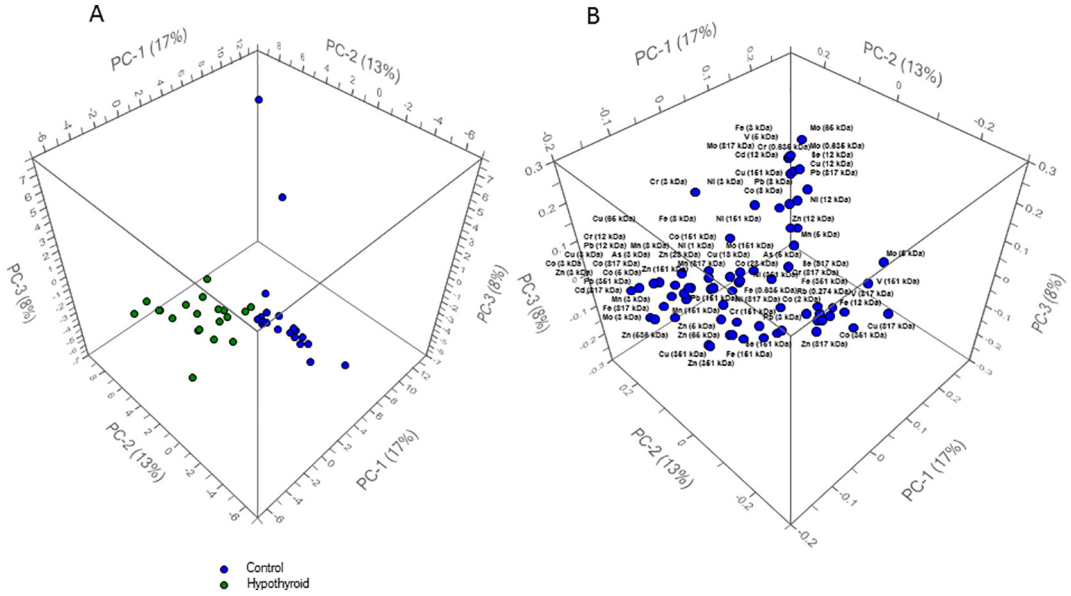


**Fig. 6.** (A) Scores plot of metalloprotean fractions of control samples, blue marked, against hypothyroid ones, which are green marked. (B) As the previous plot, this loadings plot shows which metalloprotean fractions, associated with their respective metals, have more influence on the classification model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.3. Elemental homeostasis

Classification models revealed that selenium, a nonmetal which plays a crucial role in the synthesis of thyroid hormone, showed no relevance in any of them. Both total serum concentrations and metalloprotean fractions expression suffered no variations between groups. On the other hand, there is no evidence, in the best of our knowledge, of pathological processes of the thyroid gland caused by differences in intake and assimilation of Se, but rather of other elements, especially iodine.

Transition elements (V, Fe, Co, Cu, Zn and Mo), essential from the biochemical point of view, suffered a significant variation in their concentrations and expression of its metalloprotean fractions. In diabetic patients, hyperglycemia might be affecting the proper assimilation of many of these essential trace elements. In turn, in hypothyroid patients, the high concentration of TSH could influence by some mechanism, yet unknown, in the elemental homeostatic balance. Nevertheless, these conjectures lack biochemical basis for support. It is important to establish that individuals selected for this study did not show acute symptoms at the time of analysis, so it follows that any irregularity in the homeostatic balance might be hindered by a compensatory mechanism.

## 4. Conclusions

Classification of diabetic and hypothyroid serum samples based on their multielemental and metalloprotean profiles was achieved. This study showed that transitional elements were, both their concentration as metal and metalloprotean species, remarkably altered. Further analysis with larger sample size, might be used in a suitable supervised model (PLS-DA, for instance) for prediction of unknown samples. It should be stressed that clinical prognostic is still far, and our findings are the basis for new research that, in turn, might be focused on the role of selected metals in the diseases. Further studies regarding protein expression should be carried out to establish definitive statements concerning the metabolism of metal species (absorption, transportation, excretion) with detailed information, for example, about nutritional habits of each individual, environmental factors, genetics, and pathophysiological backgrounds.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2017.10.021.

## Acknowledgements

We would like to acknowledge the National Scientific and Technical Research Council (CONICET, Argentina), Ministry of Science, Technology and Productive Innovation (MinCyT, Argentina). We are also grateful to Fulbright and Bunge & Born Foundation. We specially thank to Agilent Technologies for instrumental and technical QQQ-ICP support.

## References

[1] W.H. Organization, Definition, Diagnosis and Classification of Diabetes Mellitus and Its Complications. Part 1: Diagnosis and Classification of Diabetes Mellitus, 1999.
[2] W.H. Organization, Global Report on Diabetes, 2016.
[3] J.P. Almandoz, H. Gharib, Hypothyroidism: etiology, diagnosis, and management, Med. Clin. N. Am. 96 (2012) 203–221.
[4] M. Valko, H. Morris, M.T.D. Cronin, Metals, toxicity and oxidative stress, Curr. Med. Chem. 12 (2005) 1161–1208.
[5] E.N. Baker, T.L. Blundell, J.F. Cutfield, S.M. Cutfield, E.J. Dodson, G.G. Dodson, D.M. Hodgkin, R.E. Hubbard, N.W. Isaacs, C.D. Reynolds, et al., The structure of 2Zn pig insulin crystals at 1.5 A resolution, Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 319 (1988) 369–456.
[6] M. Barbagallo, R.K. Gupta, L.M. Resnick, Cellular ionic effects of insulin in normal human erythrocytes: a nuclear magnetic resonance study, Diabetologia 36 (1993) 146–149.
[7] F. Chimienti, A. Favier, M. Seve, ZnT-8, a pancreatic beta-cell-specific zinc transporter, Biometals 18 (2005) 313–317.
[8] M. de Lordes Lima, T. Cruz, J.C. Pousada, L.E. Rodrigues, K. Barbosa, V. Cangucu, The effect of magnesium supplementation in increasing doses on the control of type 2 diabetes, Diabetes Care 21 (1998) 682–686.
[9] H.W. de Valk, Magnesium in diabetes mellitus, Neth. J. Med. 54 (1999) 139–146.
[10] M.F. Dunn, Zinc–ligand interactions modulate assembly and stability of the insulin hexamer—a review, Biometals 18 (2005) 295–303.
[11] N.L. Eibl, H.P. Kopp, H.R. Nowak, C.J. Schnack, P.G. Hopmeier, G. Schernthaner, Hypomagnesemia in type II diabetes: effect of a 3-month replacement therapy, Diabetes Care 18 (1995) 188–192.
[12] C. Ekmekcioglu, C. Prohaska, K. Pomazal, I. Steffan, G. Schernthaner, W. Marktl, Concentrations of seven trace elements in different hematological matrices in patients with type 2 diabetes as compared to healthy controls, Biol. Trace Elem. Res. 79 (2001) 205–219.
[13] T.G. Kazi, H.I. Afridi, N. Kazi, M.K. Jamali, M.B. Arain, N. Jalbani, G.A. Kandhro, Copper, chromium, manganese, iron, nickel, and zinc levels in biological samples of diabetes mellitus patients, Biol. Trace Elem. Res. 122 (2008) 1–18.
[14] G.J. Naga Raju, P. Sarita, G.A. Ramana Murty, M. Ravi Kumar, B.S. Reddy, M.J. Charles, S. Lakshminarayana, T.S. Reddy, S.B. Reddy, V. Vijayan, Estimation of trace elements in some anti-diabetic medicinal plants using PIXE technique, Appl. Radiat. Isot. 64 (2006) 893–900.
[15] P.D. Zalewski, S.H. Millard, I.J. Forbes, O. Kapaniris, A. Slavotinek, W.H. Betts, A.D. Ward, S.F. Lincoln, I. Mahadevan, Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc, J. Histochem. Cytochem. 42 (1994) 877–884.
[16] K. Schwarz, W. Mertz, A glucose tolerance factor and its differentiation from factor 3, Arch. Biochem. Biophys. 72 (1957) 515–518.
[17] K. Schwarz, W. Mertz, Chromium(III) and the glucose tolerance factor, Arch. Biochem. Biophys. 85 (1959) 292–295.
[18] C.M. Davis, J.B. Vincent, Chromium oligopeptide activates insulin receptor tyrosine kinase activity, Biochemistry 36 (1997) 4382–4385.
[19] M.C. Davis, B.J. Vincent, Chromium in carbohydrate and lipid metabolism, J. Biol. Inorg. Chem. 2 (1997) 675–679.
[20] J.B. Vincent, Mechanisms of chromium action: low-molecular-weight chromium-binding substance, J. Am. Coll. Nutr. 18 (1999) 6–12.
[21] L. Schomburg, J. Kohrle, On the importance of selenium and iodine metabolism for thyroid hormone biosynthesis and human health, Mol. Nutr. Food Res. 52 (2008) 1235–1246.
[22] C. Schmutzler, B. Mentrup, L. Schomburg, C. Hoang-Vu, V. Herzog, J. Kohrle, Selenoproteins of the thyroid gland: expression, localization and possible function of glutathione peroxidase 3, Biol. Chem. 388 (2007) 1053–1059.
[23] J. Kohrle, F. Jakob, B. Contempre, J.E. Dumont, Selenium, the thyroid, and the endocrine system, Endocr. Rev. 26 (2005) 944–984.
[24] R.C. Dickson, R.H. Tomlinson, Selenium in blood and human tissues, Clin. Chim. Acta 16 (1967) 311–321.
[25] J. Aaseth, H. Frey, E. Glattre, G. Norheim, J. Ringstad, Y. Thomassen, Selenium concentrations in the human thyroid gland, Biol. Trace Elem. Res. 24 (1990) 147–152.
[26] P. Kodali, K.R. Chitta, J.A. Landero Figueroa, J.A. Caruso, O. Adeoye, Detection of metals and metalloproteins in the plasma of stroke patients by mass spectrometry methods, Metallomics 4 (2012) 1077–1087.
[27] D.J. Hare, A. Grubman, T.M. Ryan, A. Lothian, J.R. Liddell, R. Grimm, T. Matsuda, P.A. Doble, R.A. Cherny, A.I. Bush, A.R. White, C.L. Masters, B.R. Roberts, Profiling the iron, copper and zinc content in primary neuron and astrocyte cultures by rapid online quantitative size exclusion chromatography-inductively coupled plasma-mass spectrometry, Metallomics 5 (2013) 1656–1662.
[28] P. Adam, S. Křížková, Z. Heger, P. Babula, V. Pekařík, M. Vaculovičová, C.M. Gomes, R. Kizek, V. Adam, Metallothioneins in prion-and amyloid-related diseases, J. Alzheimers Dis. 51 (2016) 637–656.
[29] D.J. Hare, A. Rembach, B.R. Roberts, The emerging role of metalloproteomics in Alzheimer's disease research, Methods Mol. Biol. (2016) 379–389.
[30] J.S. Valentine, P.A. Doucette, S.Z. Potter, Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis, Annu. Rev. Biochem. (2005) 563–593.
[31] X. Huang, R.D. Moir, R.E. Tanzi, A.I. Bush, J.T. Rogers, Redox-active metals, oxidative stress, and Alzheimer's disease pathology, Ann. N. Y. Acad. Sci. (2004) 153–163.
[32] J.D. Doecke, S.M. Laws, N.G. Faux, W. Wilson, S.C. Burnham, C.P. Lam, A. Mondal, J. Bedo, A.I. Bush, B. Brown, K. De Ruyck, K.A. Ellis, C. Fowler, V.B. Gupta, R. Head, S.L. Macaulay, K. Pertile, C.C. Rowe, A. Rembach, M. Rodrigues, R. Rumble, C. Szoeke, K. Taddei, T. Taddei, B. Trounson, D. Ames, C.L. Masters, R.N. Martins, Blood-based protein biomarkers for diagnosis of Alzheimer disease, Arch. Neurol. 69 (2012) 1318–1325.
[33] B.R. Roberts, T.M. Ryan, A.I. Bush, C.L. Masters, J.A. Duce, The role of metallobiology and amyloid-β peptides in Alzheimer's disease, J. Neurochem. 120 (2012) 149–166.
[34] M.F. Moyano, L. Mariño-Repizo, H. Tamashiro, L. Villegas, M. Acosta, R.A. Gil, ICPMS analysis of proteins separated by Native-PAGE: evaluation of metaloprotein profiles in human synovial fluid with acute and chronic arthritis, J. Trace Elem. Med. Biol. 36 (2016) 44–51.
[35] J.M. Walker, R. Tsivkovskii, S. Lutsenko, Metallochaperone Atox1 transfers copper to the NH2-terminal domain of the Wilson's disease protein and regulates its catalytic activity, J. Biol. Chem. 277 (2002) 27953–27959.
[36] B.E.G. Rothberg, M.B. Bracken, D.L. Rimm, Tissue biomarkers for prognosis in cutaneous melanoma: a systematic review and meta-analysis, J. Natl. Cancer Inst. 101 (2009) 452–474.
[37] K. Lund Olesen, K.B. Menander, Orgotein: a new anti inflammatory metalloprotein drug: preliminary evaluation of clinical efficacy and safety in degenerative joint disease, Curr. Ther. Res. Clin. Exp. 16 (1974) 706–717.
[38] H. Haraguchi, Metallomics as integrated biometal science, J. Anal. At. Spectrom. 19 (2004) 5–14.

[39] H. Zhang, C. Yan, Z. Yang, W. Zhang, Y. Niu, X. Li, L. Qin, Q. Su, Alterations of serum trace elements in patients with type 2 diabetes, J. Trace Elem. Med. Biol. 40 (2017) 91–96.
[40] E. Nasli-Esfahani, F. Faridbod, B. Larijani, M.R. Ganjali, P. Norouzi, Trace element analysis of hair, nail, serum and urine of diabetes mellitus patients by inductively coupled plasma atomic emission spectroscopy, Iran. J. Diabetes Lipid Disord. 10 (2011) 1–9.
[41] M. Humayun, A. Khalid, A. Ali, S. Ahamad, A. Javed, To study the levels of serum chromium, copper, magnesium and zinc in patients with diabetes mellitus type 2, Pak. J. Med. Health Sci. 5 (2011) 368–372.
[42] C.C. Lin, G.J. Tsweng, C.F. Lee, B.H. Chen, Y.L. Huang, Magnesium, zinc, and chromium levels in children, adolescents, and young adults with type 1 diabetes, Clin. Nutr. 35 (2016) 880–884.
[43] F. Bozkurt, R. Tekin, S. Gulsun, O. Satici, O. Deveci, S. Hosoglu, The levels of copper, zinc and magnesium in type II diabetic patients complicated with foot infections, Int. J. Diabetes Dev. Countries 33 (2013) 165–169.
[44] S. Sinha, S. Sen, Status of zinc and magnesium levels in type 2 diabetes mellitus and its relationship with glycemic status, Int. J. Diabetes Dev. Countries 34 (2014) 220–223.
[45] C.C. Lin, H.H. Huang, C.W. Hu, B.H. Chen, I.W. Chong, Y.Y. Chao, Y.L. Huang, Trace elements, oxidative stress and glycemic control in young people with type 1 diabetes mellitus, J. Trace Elem. Med. Biol. 28 (2014) 18–22.