NUKLEONIKA 2012;57(3):389–399

ORIGINAL PAPER

Major, minor, and trace elements in whole blood of patients with different leukemia patterns

**Ali Khuder,
Mohammed A. Bakir,
Amin Solaiman,
Haisam Issa,
Khozama Habil,
Ali Mohammad**

Abstract. The elemental sensitivity method for X-ray fluorescence analysis was applied to determine S, Cl, K, Ca, Fe, Cu, Zn, Br, and Rb in the whole blood of leukemia patients and healthy volunteers. Leukemia samples were classified according to type, growth, and age of participants. Student's t-test results showed that, the mean concentration of the studied elements was significantly lower in leukemia patients than that in controls. Strong mutual correlations ($r > 0.50$) in the whole blood of leukemia patients were observed between S-Ca, K-Fe, K-Ca, Fe-Zn, K-Zn, K-Rb, Fe-Rb, Zn-Rb, S-Cl, S-K, Ca-Fe, Cl-Ca, and Ca-Rb; whereas, S-K, S-Ca, S-Cl, Cl-K, Cl-Ca, Fe-Zn, Zn-Rb, Fe-Rb, K-Fe, and Zn-Br exhibited strong relationships ($r > 0.50$) in the whole blood of controls, all were significant at $p < 0.05$. Significant differences between grouping of studied elements in the control group and all classified leukemia groups, except younger age-group, were obtained using principal component analysis. The study indicated appreciably different patterns of element distribution and mutual relationships in the whole blood of leukemia patients in comparison with controls.

Key words: leukemia • principal component analysis • Student's t test • whole blood • X-ray fluorescence

Introduction

Until recently, clinical recognition was limited to very few of the elements, but the recent studies showed that all chemical elements could be involved in physiological processes in varying degrees. For example, the analysis of the light elements in body fluids displayed an important tool for biomedical studies due to the fundamental importance of low-Z elements in a number of biomedical and physiological processes in humans (a number of enzymes, hormones, and cell-signalling molecules depend on low-Z elements for their activation) [5, 14]. On the other hand, trace elements have been attracting a great deal of attention in the field of human health. Several trace elements were determined in body fluids and tissues for the diagnosis and monitoring of various disorders, nutritional deficiencies, and occupational or environmental exposure [11, 21, 24]. Moreover, any change in the environment as well as in the human body itself could change the trace metal composition of any organ or tissue and as a consequence, some diseases could be produced [23]. Therefore, it seems reasonable to assume that the trace and the other essential elements could influence mechanisms, having responsibility for the development of various cancerous diseases, e.g. cervical, uterine myoma, thyroid and leukemia [1, 5, 6, 19]. In addition, many studies reported that various elements inter-dependently work through the mutual interactions in human body [3–6, 8, 19, 20, 22, 25]. Most of these studies were carried out to find the correlation

A. Khuder✉, H. Issa
Department of Chemistry,
Atomic Energy Commission,
P. O. Box 6091, Damascus, Syria,
Tel.: +963 11 213 2580, Fax: +963 11 611 2289,
E-mail: cscientific@aec.org.sy

M. A. Bakir, K. Habil, A. Mohammad
Department of Radiation Medicine,
Atomic Energy Commission,
P. O. Box 6091, Damascus, Syria

A. Solaiman
Al-Mouasat Hospital,
Damascus, Syria

Received: 3 January 2012
Accepted: 6 June 2012

between the trace elements in the examined biomedical samples, while, only a limited number of the studies was devoted to elucidate the correlations of major-to--trace elements in human body [5, 8]. Therefore, further information about the distributions and correlation of major-to-trace elements in human body is highly required for elucidation of their biological roles and functions on the multielement basis.

Human blood is one of the biological fluids, which is frequently used for medical diagnosis because it is easily collected from man [19]. For the purpose of multielement determination in blood samples, different analytical methods were developed, e.g. ICP-AES (inductively coupled plasma atomic emission spectrometry) and ICP-MS (inductively coupled plasma mass spectrometry) [8], PIXE (proton induced X-ray emission) [13], SRTXRF (total reflection X-ray fluorescence induced by synchrotron radiation) [5], and EDXRF (energy dispersive X-ray fluorescence) [9, 12].

Among the previously mentioned analytical techniques, the EDXRF method was the most advantageous because it could make simple, fast, and simultaneous determination of many elements in a wide concentration range [2, 7]. In addition, EDXRF with a combination of different radionuclide sources showed a reliable determination of some elements such as Fe, Rb, Mo, In, Sn, I, Cl, K, and Ca in leukemia patients' bloods [7]. Better detection limits were achieved by EDXRF with X-ray tubes as the excitation source, where detection limits of less than 1 μg/g were attainable for many elements using EDXRF with modern software to produce clean spectra, net peak intensities, and accurate corrections for inter-element matrix effects and spectral tube impurities [16].

The present study was carried out to determine several elements with a wide range of concentrations, i.e. S, Cl, K, Ca, Fe, Cu, Zn, Br, and Rb, in blood of leukemia patients and controls using EDXRF technique. The EDXRF data were statistically estimated using Student's t-test and a multivariate method of principal component analysis. The mutual relationships, the possible linking between the studied elements, as well as the essential factors affecting the distribution patterns of the elements were studied.

Experimental

Study population

A total of 80 leukemia patients, ages between 14 and 93 years, were included in this study on voluntary basis. Average age of the patients was 44.2 years. Subjects were selected from the patients admitted in Al-Mowasat hospital in Damascus city, Syria. Prior to the sample collection, the protocol of the study was approved by the Human Ethical Committee of the respective hospital. Diagnosis of leukemia was confirmed histologically. Thirty six healthy volunteers were participated in this study in order to determine the normal concentrations of various elements in blood. All volunteers were healthy and not receiving any medication. Average age of the volunteers was 36.2 years. Four ml of blood samples were collected from the patients and the healthy volunteers each in suitable glass tubes. All samples were labelled with relevant codes related to the donor's name, age, social and health status. All data were recorded and compiled on regular forms at the time of sampling.

Sample preparation

Each of blood sample was subjected to a freeze-drying during 36 h by a freeze-dry system (LABCONCO, Freezone 4.5) and weighed before and after the freeze-drying process. Masses of 0.200 g of the freeze-dried samples were pressed into the form of pellets with a diameter of 2 cm using a hydraulic press machine (PW 40, WEBER Co, Germany) at 15 tons pressure. The pellet samples were kept in tightly closed Petri dishes for further XRF analysis.

Materials

The metal foils of Ti, V, Cr, Fe, Co, Ni, and Zn, with dimensions of 25 × 25 mm and 1 mm thickness each from Alfa Aesar Co in addition to the pressed pellets of the single element of S (purity: 99%, Merck) and the stoichiometric compounds of KH_2PO_4 (purity: 99.5%, Merck), KCl (purity: 99.5%, Riedel-de Haen), $CaCO_3$ (purity: 99%, Riedel-de Haen), As_2O_3 (purity: 99.5%, Merck), RbCl (purity: 99%, AppliChem) and $SrCO_3$ (purity: 98.0%, Fluka) were used for the XRF calibration of K_α lines. The pellets were pressed with a 2 cm diameter and around 1.0 g/cm^2 surface density each.

Measurement conditions

The XRF measurements were performed using an EDXRF instrument, which was equipped with a 2 kW Mo tube and a Si(Li) semiconductor detector with an energy resolution of 160 eV at 5.9 keV. The operating conditions differed, depending on the mode of X-ray excitations. However, these were: (35 mA and 40 kV), (10 mA and 35 kV), and (20 mA and 35 kV) by using Ti-, Cu- and Mo--secondary targets, respectively; taking into the account, that the previously mentioned secondary targets were used for the determination of (S, Cl, K and Ca), (Fe), and (Cu, Zn, Br, and Rb), respectively. The live time was 2000 s for each of the X-ray excitation modes.

XRF analysis

XRF spectra was evaluated using the AXIL-QXAS software package which was developed in the International Atomic Energy Agency (IAEA) [10]. This program was designed for the qualitative and quantitative analysis of XRF spectra. AXIL program has the ability to determine the net peak area for each selected group of elements during the spectrum fitting. In the present work, the net peak areas of K_α lines of the elements in the standards were used in the calibration sensitivity of XRF technique. The sensitivities of the characteristic lines from the standards were then used to determine

the concentrations of elements in the unknown samples using QXAS (quantitative X-ray analysis system) program.

Sample mass for XRF analysis

In order to make a definition of the thickness of the pressed pellets, the attenuation of the X-ray intensities by the blood samples was determined. For this purpose, a set of blood samples with masses of 0.1000 g, 0.1515 g, 0.1876 g, 0.1950 g, and 0.2530 g were pressed into the form of pellets with diameters of 2 cm each. Then, each pellet in the set was covered by a pure Ti foil (purity 99.9%). The Ti foils were cut with a surface area suitable for the pressed pellets. The pellets with Ti foils were then exposed to the X-ray excitation of Mo-XRF mode for 100 s live time. The Ti foils without pellets were also exposed to XRF for the same time. Finally, the peak area of Ti (K_α) in the obtained spectra was evaluated by means of the AXIL program.

Validity of XRF method

The accuracy of XRF method was checked by the analysis of IAEA A-13 standard sample, while Brown Bread BCR no. 191 certified reference material was used for estimation of only Cl accuracy. To establish the reproducibility of the instrumental technique, five different standard samples with a mass of 0.200 g each were prepared in a similar way as for the unknown samples and analyzed with the previously mentioned operating conditions.

Statistical analysis

For statistical purposes, leukemia patients were divided into subgroups relevant to the type of the affected cells: (i) lymphocytic leukemia (LL), which affected lymphoid cells, $n = 33$; and (ii) myeloid leukemia (ML), which affected myeloid cells, $n = 47$; or into subgroups relevant to the growth of leukemia: (iii) chronic leukemia (CL), which grows slowly, $n = 45$; and (iv) acute leukemia (AL), which grows quickly, $n = 35$. According to the previously mentioned definitions, LL subgroup was either: (v) chronic (CLL), $n = 21$; or: (vi) acute (ALL), $n = 12$. Similarly, ML was either: chronic (CML), $n = 24$; or: acute (AML), $n = 23$.

For further identification of possible sources and grouping of the studied elements in the whole blood, leukemia patients were divided into subgroups relevant to the age of volunteers. Number of the age-subgroups, which approved the suitability for such studies, were only 2 with ranges of 14–28 years ($n = 23$) and 29–93 years ($n = 57$).

Student's t-test, Pearson correlations, and multivariate technique of principal component analysis were used to identify the significant differences between the studied subgroups, investigate the mutual relations between each pair of determined elements, and find sources and grouping of studied elements in the whole blood of the leukemia patients, respectively. Overall, the results obtained for leukemia groups were compared with those obtained for the controls.

Results and discussion

XRF analysis

A typical XRF spectra from leukemia blood samples excited by means of Ti-, Cu-, and Mo-secondary targets are shown in Figs. 1a–1c. The EDXRF technique revealed that elements such as S, Cl, K, Ca, Fe, Cu, Zn, Br, and Rb were presented in all analyzed blood samples.

The elements found in spectra of the blood samples were determined by the elemental sensitivity (ES) method for XRF. For this purpose, standards of single elements and stoichiometric compounds in the form of foils and pellets were used for the determination of the sensitivities, which were calculated using the following equation [10]:

$$S = I_p \cdot f / t \cdot I \cdot C$$

where S is the sensitivity; I_p is the net peak area of a characteristic line; f is the absorption correction factor; t is the live time of the spectrum; I is the current of the

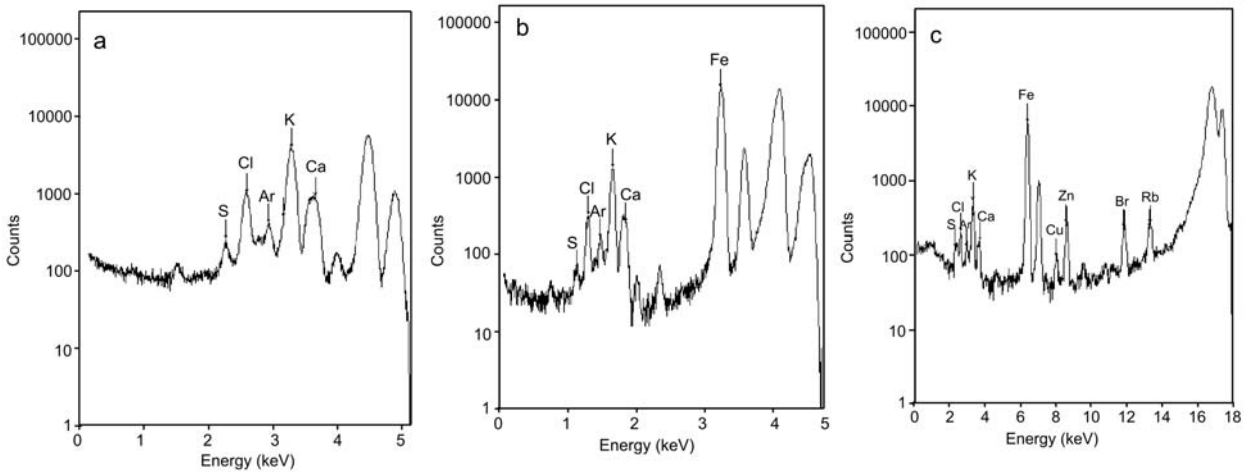

Fig. 1. Typical spectra of a leukemia blood sample excited by X-ray Mo tube with (a): Ti-, (b): Cu-, and (c): Mo-secondary targets. The operating conditions are shown in Table 1. X-ray fluorescence K_α lines of the elements are shown.

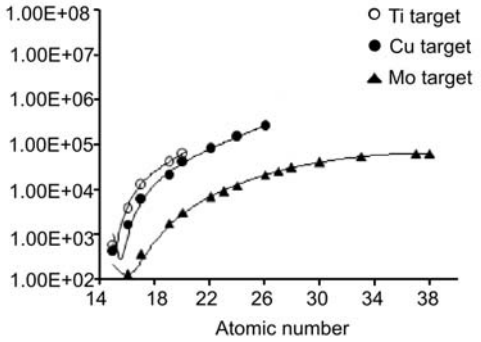

Fig. 2. Calibration sensitivity curves obtained by X-ray excitation of elements using different secondary targets. The operating conditions are shown in Table 1.

X-ray tube; *C* is the concentration of the element producing the characteristic line in the standard.

The calibration curves of the obtained sensitivities against the atomic number of the studied elements were constructed (Fig. 2). The results showed that the excitation of K_α lines of the light elements, e.g. S, Cl, K, and Ca, by Ti-secondary target led to sensitivity values higher than those obtained using Cu- and Mo-secondary targets. While the higher sensitivity values of Fe were obtained by X-ray excitation using Cu-secondary target. The results showed also the priority of Mo-secondary target mode to excite Cu, Zn, Br, and Rb.

Sample mass for XRF

The concentrations of the elements in the unknown blood samples were estimated using the elemental sensitivity (ES) approach for XRF analysis [10]. For this purpose, information about the blood-sample masses or the density of the pressed pellets (g/cm^2) was required. In principle, a sample is considered thin if the inter-element effects are negligible, causing no significant errors in the quantization; while, it is considered infinitely thick if it attenuates more than 99% of the intensities of the excitation. A sample with a thickness between the previously mentioned two definitions is considered as an intermediately thick sample [10]. In order to establish a definition of the thickness of the pressed pellets, the attenuation of the X-ray intensities of Mo-secondary target mode caused by a set of blood samples was determined. The experimental results showed that the pellets with the surface area < 0.081 g/cm^2 were able to attenuate < 99% of the X-ray intensities. Accordingly, the masses chosen (around 0.0637 g/cm^2) for the analysis of all blood samples satisfied the criteria of samples having intermediate thickness.

'Dark matrix' estimation

In order to accurately determine the elements in the blood pellets using the ES-XRF method, a suitable composition of non-detectable light elements ('dark matrix') was to be given. This information was required for the absorption correction calculations. In the instance of Ti- and Cu-secondary targets, the chosen 'dark matrix' was consisted of oxygen (O), while carbon (C) was entered as an element by difference. In the instance of Mo-secondary target, the chosen 'dark matrix' was consisted of silicon (Si) and oxygen (O), while, hydrogen (H) remained as the element by difference. It is important to clarify that the element by difference was selected so that the absorption of the 'dark matrix' was represented adequately. The reality of the chosen 'dark matrix' for each applied XRF mode was studied by the analysis of IAEA A-13 animal blood (Brown Bread BCR no. 191 standard reference for Cl determination) standard sample using QXAS program for XRF. To achieve the optimal data of XRF analysis, the concentrations of elements were verified by changing the percentage of oxygen entered and the hydrogen as the element by difference; a constant percentage of Si was entered only to the 'dark matrix' of spectra obtained by Mo-XRF mode. Comparison between the concentrations obtained by changing the percentage of entered 'dark matrix' was carried out by averaging the squares of the accuracy values for the elements determined and calculating the square root (root mean squares = rms) to obtain pooled of accuracy ($A\%$), taking into the account that the accuracy was estimated as the relative difference between the obtained concentrations and the certified values. Data of the verified concentrations, the accuracies, the 'dark matrices' and their mean Z, and the rms values for each secondary target mode are presented in Table 1. The results showed that by the analysis using Ti-secondary target, the rms values of S, K, and Ca were minimized to 1.59% (2.57% for Cl) vs. a 'dark matrix' with a mean Z of 5.93 (6.94 for Cl). By the analysis of Fe using Cu-secondary target, the rms values were minimized to 4.40% vs. a mean Z of 5.96. While, by applying Mo-secondary target for the analysis of Cu, Zn, Br, and Rb, the rms values were minimized to 5.51% vs. a mean Z of 6.63.

Validity of ES-XRF method

The suitability of ES-XRF method for the determination of the elements in the blood samples was checked as follows:

1. By estimation of the accuracy and precision. The accuracy of the optimized ES method for XRF analysis was estimated by the analysis of five different subsamples (n = 5) of the IAEA A-13 animal blood and Brown Bread BCR no. 191 standard. The results in Table 2 showed that the elemental concentrations were in good agreement with the certified values with errors less than 8.6%. The precision of the ES-XRF method was evaluated in terms of the relative standard deviation (RSD = SD/Ci) × 100, where SD is the standard deviation and Ci is the mean of i element concentrations. The results showed that the precision was better than ± 9.9% for all determined elements.
2. By determination of the lower limits of detection (LLD) of the elements in the A-13 standard reference sample, using the following equation [12]:

$$m_{\min} = \frac{3}{S} \cdot \sqrt{\frac{I_b}{t}}$$

where $m_{\min}$ is the minimum detectable mass or concentration, S is the sensitivity (net peak counts per second

Table 1. Verification of concentrations (μg/g) obtained by the analysis of IAEA standard reference material animal blood A-13 using elemental sensitivity method for XRF technique with different secondary targets[a]

Secondary target	Component	(C1)[b]	(A1%)[c]	(C2)[b]	(A2%)[c]	(C3)[b]	(A3%)[c]	(C4)[b]	(A4%)[c]	(C5)[b]	(A5%)[c]	Certified values
Ti	S	6 163	–5.19	6 327	–2.66	6 493	–0.11	6 615	1.76	6 825	5	6 500
	K	2 442	–2.31	2 511	0.42	2 580	3.2	2 649	5.97	2 719	8.76	2 500
	Ca	280	–2.10	288	0.62	296	3.46	293	2.37	312	9.09	286
Matrix[d]	O%	1		3		5		7		9		
	Mean Z	5.89		5.93		5.97		6.00		6.04		
Pooled	rms[e]		3.5		**1.59**		2.72		3.84		7.84	
Ti	Cl[f]	15 163	–8.1	15 813	–4.16	16 076	–2.57	17 120	3.76	17 773	7.72	16 500
Matrix[d]	O%	45		50		55		60		65		
	Mean Z	6.76		6.85		6.94		7.03		7.13		
Pooled	rms[e]		8.1		4.16		**2.57**		3.76		7.72	
Cu	Fe	2 166	–9.75	2 258	–5.93	2 279	–5.04	2 510	4.60	2 594	8.10	2 400
Matrix[d]	O%	1		5		6		7		10		
	Mean Z	5.86		5.93		5.95		5.96		6.02		
Pooled	rms[e]		9.75		5.93		5.04		**4.60**		8.10	
Mo	Cu	4.18	–2.88	4.31	0.33	4.15	–3.53	4.9	13.9	5.15	19.8	4.3
	Zn	11.3	–13.2	11.6	–10.5	11.9	–8.46	13.1	0.62	13.8	6.12	13
	Br	21.1	–3.98	20.7	–5.89	20.7	–5.89	22.4	1.79	23.3	5.94	22
	Rb	2.22	–3.34	2.26	–1.69	2.26	–1.58	2.42	5.02	2.5	8.66	2.3
Matrix[d]	O%	10		15		25		35		45		
	Si%	2		2		2		2		2		
	Mean Z	6.33		6.43		6.63		6.82		7.02		
Pooled	rms[e]		7.23		6.06		**5.51**		7.43		11.6	

[a] XRF measurements of S, Cl, K, and Ca; Fe; Cu, Zn, Br, and Rb were carried out by the following operating conditions: Ti-secondary target with 35 mA and 40 kV; Cu-secondary target with 10 mA and 35 kV; Mo-secondary target with 20 mA and 35 kV, respectively. Mass of standard sample was 0.200 g. Collection time was 2000 s.

[b] C1, C2, C3, C4, and C5 are the concentrations obtained according to the given dark matrix.

[c] A1, A2, A3, A4, and A5 are the accuracies estimated by the difference between the obtained and the certified values.

[d] The 'dark matrix' entered for running QXAS-AXIL program, while, carbon (C) was chosen as an element by difference.

[e] rms is the root mean square of elemental accuracies.

[f] Data obtained were for Brown Bread BCR No. 191 certified reference material.

Data in bolds are the optimal pooled accuracies obtained by changing the percentages of entered matrices.

Table 2. Analysis of IAEA standard reference material animal blood A-13 by XRF technique[a]

Element	Certified values		Obtained concentration, $n = 5$ (μg/g)	LLD[c] (μg/g)	A[d] (%)	RSD[e] (%)
	Confidence interval (μg/g)	Concentration (μg/g)				
S	6 000–7 000	6 500	6 127 ± 601	105	–5.7	±9.8
Cl[b]	NA	16 500	16 076 ± 536	67.8	–2.6	±3.3
K	2 100–2 700	2 500	2 289 ± 215	8.11	–8.4	±9.4
Ca	226–332	286	296 ± 24	1.02	3.5	±8.1
Fe	2 200–2 500	2 400	2 510 ± 157	1.50	4.6	±6.3
Cu	3.7–4.8	4.3	4.15 ± 0.34	0.851	–3.5	±8.2
Zn	12–14	13	11.9 ± 1.1	0.660	–8.5	±9.2
Br	19–24	22	20.7 ± 2.0	0.333	–5.9	±9.7
Rb	1.7–3.1	2.3	2.26 ± 0.19	0.320	–1.7	±8.4

[a] XRF measurements of S, Cl, K, and Ca; Fe; Cu, Zn, Br, and Rb were carried out by the following operating conditions: Ti-secondary target with 35 mA and 40 kV; Cu-secondary target with 10 mA and 35 kV; Mo-secondary target with 20 mA and 35 kV, respectively. Mass of standard sample was 0.200 g. Collection time was 2000 s.
[b] Data obtained were for Brown Bread BCR no. 191 certified reference material.
NA: not available.
[c] is the lower limits of detection.
[d] is the accuracy, which is estimated by the difference between the obtained and the certified values.
[e] is the precision, which is estimated by a relative standard deviation.

per mass or concentration); I_b is the intensity of the background of the respective peak (counts per second); and t is the counting time (s).

The results in Table 2 showed that S, Cl, K, Ca, Fe, Cu, Zn, Br, and Rb were with concentrations much higher than the obtained LLD values, reflecting again the suitability of XRF technique to analyze the blood samples.

Distribution of elements

The XRF analysis showed the possibility to determine nine elements in the whole blood of healthy volunteers and leukemia patients. Of these, S, Cl, and K were classified as major elements, Fe as a minor element, while Ca, Cu, Zn, Br, and Rb as trace elements. Table 3 displays concentrations of the determined elements in the whole blood of the leukemia patients and the control groups, in addition to concentrations of the reference values. The highest concentration was observed for Cl (2380 μg/ml), followed by S (1387 μg/ml), K (1372 μg/ml), Fe (356 μg/ml), Ca (68.7 μg/ml), Zn (4.32 μg/ml), Br (1.61 μg/ml), Cu (0.909 μg/ml), and Rb (0.737 μg/ml) in leukemia patients. In comparison, the dominant element in the whole blood of the control group was also Cl (3454 μg/ml), followed by K (2227 μg/ml), S (1894 μg/ml), Fe (515 μg/ml), Ca (75.6 μg/ml), Zn (5.49 μg/ml), Br (2.17 μg/ml), Rb (1.13 μg/ml) and Cu (0.955 μg/ml). Overall, the results showed that, the levels of S, Ca, Fe, Cu, Zn, and Rb in the control group were comparable to those obtained for the reference values, while the concentrations of K, Cl, and Br were slightly higher than the reference values. The most probable reason for this behaviour was related to the regional factors, where the elemental levels could be influenced by feeding habits, chemical characteristics of the soils, etc., and therefore, could vary widely from place to place [15]. The results showed the following decreasing concentration order of determined elements in leukemia patients: Cl > S > K > Fe > Ca > Zn > Br > Cu > Rb, while the following was for the control group: Cl > K > S > Fe > Ca > Zn > Br > Rb > Cu. Thus, the results confirmed a similar decreasing order obtained for the determined elements in both groups (patients and control), except for K and Rb. On the other hand, the results in Table 3 showed a significant decrease in concentration for all studied elements in leukemia patients in comparison to those concentrations obtained for the controls; taking into account that the data were estimated at 99% level for all elements, except Ca and

Table 3. Comparative results between whole blood of control group and leukemia patient group

Element	Reference values (μg/ml)	Control group mean value (μg/ml), $n = 36$	Leukemia patient group (μg/ml), $n = 80$	Student's t-test	Difference[a]
S	(1 680–1 930)[b]	1 894 ± 156	1 387 ± 64	–20.86	Significant
Cl	3 020[c]	3 454 ± 274	2 380 ± 116	–23.85	Significant
K	(1 450–1 920)[b]	2 227 ± 209	1 372 ± 97	–22.75	Significant
Ca	(57.5–78.0)[b]	75.6 ± 4.6	68.7 ± 2.0	–6.73	Significant
Fe	(482.1 ± 12.0)[d]	515 ± 35	356 ± 30	–13.66	Significant
Cu	(1.00 ± 0.05)[d]	0.955 ± 0.057	0.909 ± 0.044	–2.06	Significant
Zn	(6.20 ± 0.17)[d]	5.49 ± 0.51	4.32 ± 0.38	–7.87	Significant
Br	(1.70 ± 0.20)[b]	2.17 ± 0.21	1.61 ± 0.14	–10.22	Significant
Rb	(1.17–5.98)[b]	1.13 ± 0.10	0.737 ± 0.066	–15.45	Significant

[a] Analysis of variance at the 99% confidence level for all elements, except Ca and Cu which were estimated at 95%.
[b, c, d] corresponding to Refs. [17], [26], and [18], respectively.

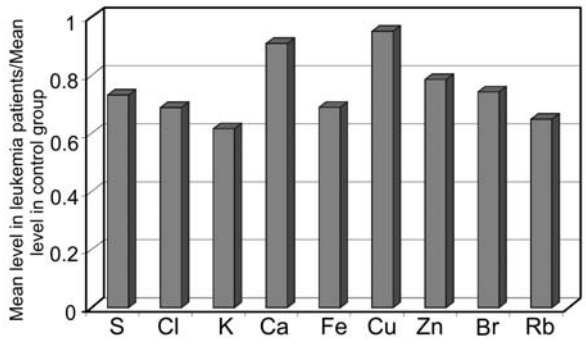

Fig. 3. Comparative mean of ratios of the element levels in whole blood of leukemia patients and controls.

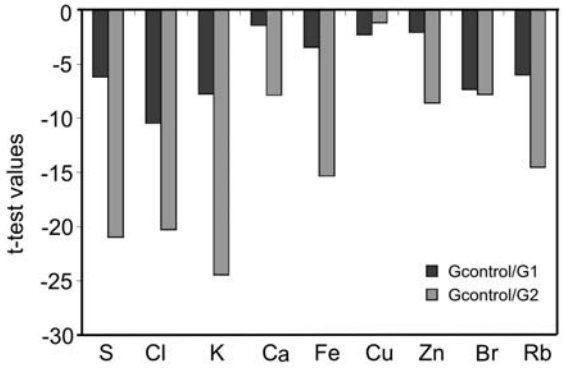

Fig. 4. Significant differences between concentrations of the elements in whole blood of control group ($G_{control}$) and leukemia age-groups (G1: 14–28 years, n = 17; G2: 29–93 years, n = 57). Student's t-test was estimated at 95% confidence level.

Cu at 95% level. Evaluation of the decreasing ratios was also estimated by dividing the concentrations of the elements in leukemia patients by those obtained for control group. However, the results of the comparative means of ratios of the determined elements in the whole blood of leukemia patients and control groups (Fig. 3) showed the following decreasing order: Cu (0.952) > Ca (0.910) > Zn (0.786) > Br (0.743) > S (0.732) > Fe (0.690) > Cl (0.689) > Rb (0.649) > K (0.616). This reveals the lowest and the highest affected elements in the whole blood of leukemia patients were Cu and K, respectively.

Presence of significant differences in the concentrations of the studied elements in leukemia subgroups was also estimated using the Student t-test. The results showed that the concentrations of Fe, Br, and Rb in the lymphocytic leukemia, (i)-subgroup, were significantly higher than the concentrations of the same elements in the myeloid leukemia (ii)-subgroup. Student's t-test values estimated at a 95% level for the previously mentioned elements were: 2.079, 2.266 and 2.677, respectively. On the other hand, the concentrations of Cl, K, Cu, and Zn in the acute leukemia patients (iv)-subgroup, were significantly lower at a 95% level than the concentrations of the same elements in the chronic leukemia patients (iii)-subgroup, with the Student's t-test values: –4.26, –2.36, –2.56, and –4.07, respectively. It is also important to mention hereby that no significant differences were found between the concentrations of the studied elements in the following subgroups: ALL/AML, ALL/CLL, ALL/CML, AML/CLL, AML/CML, and CLL/CML.

To study the effect of age factor on the distribution of the elements in blood samples, two ranges of age were selected from 28 to 35 years (n = 19) and from 36 to 50 years (n = 17) for the controls, while the other two ranges: from 14 to 28 years (G1, n = 23) and from 29 years to 93 years (G2, n = 57) were selected for the leukemia patients. The results showed no significant differences between the concentrations of the elements in the two age-groups of the controls, indicating the homogeneous distribution of the elements regardless of the age of the participants. Figure 4 demonstrated two cases based on the comparative results of concentrations of the elements in the leukemia age-subgroups (G1 and G2) and the control group. In the first case, when G1 was compared with the control group, a significant decrease for all element concentrations, except for Ca, was observed, while, in the second case, when G2 was compared with the control group, a significant decrease was observed for all element concentrations, except for Cu. It should be added that all significant differences were estimated at a 95% level.

Correlation study

The data on metal-to-metal correlations in the controls and the leukemia patients groups are given in Tables 4 and 5, respectively. The results showed very strong positive correlations in the control group: S-K (r = 0.93), S-Ca (r = 0.91), S-Cl (r = 0.90), Cl-K (r = 0.89), Cl-Ca (r = 0.89), Fe-Zn (r = 0.74), Zn-Rb (r = 0.70). Another notably strong correlations were observed for Fe-Rb, K-Fe, and Zn-Br. In addition, some significant correlations were also noted between Cu-Zn, S-Fe, Br-Rb, Fe-Br, and K-Rb. The previously mentioned results revealed that the studied elements had mutual correlations with each others, indicating the importance of presence of these elements in the human whole blood.

Table 4. Pearson correlation coefficient matrix of several elements in whole blood of control group, n = 36

Element	S	Cl	K	Ca	Fe	Cu	Zn	Br	Rb
S	1.00								
Cl	**0.90**	1.00							
K	**0.93**	**0.89**	1.00						
Ca	**0.91**	**0.89**	**0.89**	1.00					
Fe	**0.46**	0.29	**0.53**	**0.50**	1.00				
Cu	0.04	0.12	0.05	0.13	0.27	1.00			
Zn	0.22	0.18	0.29	0.30	**0.74**	**0.49**	1.00		
Br	0.09	0.11	0.12	0.20	**0.42**	0.17	**0.50**	1.00	
Rb	0.25	0.22	**0.37**	0.29	**0.69**	0.27	**0.70**	**0.45**	1.00

Bold values are significant at $p < 0.05$.

Table 5. Pearson correlation coefficient matrix of several elements in whole blood of leukemia patients, $n = 80$

Element	S	Cl	K	Ca	Fe	Cu	Zn	Br	Rb
S	1.00								
Cl	**0.65**	1.00							
K	**0.65**	**0.26**	1.00						
Ca	**0.90**	**0.51**	**0.76**	1.00					
Fe	**0.42**	–0.01	**0.83**	**0.57**	1.00				
Cu	0.14	0.19	0.02	0.10	–0.06	1.00			
Zn	**0.33**	0.04	**0.74**	**0.48**	**0.75**	0.07	1.00		
Br	–0.13	–0.05	0.01	–0.05	0.11	0.14	0.08	1.00	
Rb	**0.39**	–0.06	**0.69**	**0.50**	**0.69**	0.02	**0.66**	0.10	1.00

Bold values are significant at $p < 0.05$.

Very strong positive correlations were also found in the leukemia group: S-Ca ($r = 0.90$), K-Fe ($r = 0.83$), K-Ca ($r = 0.76$), Fe-Zn ($r = 0.75$), K-Zn ($r = 0.74$). Another notably strong correlations were observed for K-Rb ($r = 0.69$), Fe-Rb ($r = 0.69$), Zn-Rb ($r = 0.66$), S-Cl ($r = 0.65$), S-K ($r = 0.65$), Ca-Fe ($r = 0.57$), Cl-Ca ($r = 0.51$), and Ca-Rb ($r = 0.50$). In addition, some significant correlations were also noted between Ca-Zn, S-Fe, S-Rb, S-Zn, and Cl-K. The obtained correlation study revealed some common origins of the studied elements in the whole blood of leukemia patients. It could also be concluded that important relations were found for S, Cl, K, Ca, Fe, Zn, and Rb while, such relations were not found for Cu and Br in the whole blood of leukemia patients.

Furthermore, the inter-relationship between pairs of the studied elements in the whole blood of control and leukemia patients groups revealed a marked difference in metal-to-metal correlations (Tables 4 and 5). However, the values of the correlation coefficients obtained for each pair of the major elements (S-Cl, S-K, and Cl-K) in the control group were extremely decreased in comparison with the values obtained for the same pairs of the elements in the leukemia patient group. The range and the mean concentrations of the previously mentioned elements were lower in the leukemia patients than in the healthy volunteers. Most likely due to some metabolic process whereby these elements are depleted and replaced by other elements in the whole blood of leukemia patient group as compared with the control group. It could be concluded hereby that S, Cl, and K are less bioavailable in leukemia patients, which may lead to a number of physiological disorders. Other significant differences were observed in the cases of K, Ca, and S, which showed significant correlations with the trace levels of Zn and Rb in the leukemia patients; but similar correlations were not investigated in the control group. Likewise, S, Ca, Zn, and Rb exhibited almost comparable correlations with Fe in both groups. Similar comparable relations were found for Ca with S and K each, and for Zn with Rb.

Principal component analysis

In the present study, multivariate technique of principal component analysis (PCA) [19] was employed to identify possible sources and grouping of studied elements in the whole blood of the leukemia patients and compare these results with those obtained for the controls. Three important factors: type, growth, and age of leukemia patients, which were expected to affect the source identification and grouping of elements, were studied.

Regarding to the type factor, the whole blood samples of patients were divided into two subgroups: lymphatic (LL) and myeloid (ML) leukemia. On the other hand, the same samples were divided into other two subgroups regarding to the growth of the leukemia disease: chronic (CL) and acute (AL) leukemia. Accordingly, PCA was employed for the previously mentioned leukemia subgroups and the results were compared to those obtained for the control group. The principal

Table 6. Principal component loadings[a] of several elements in whole blood of $G_{control}$[b], LL[c] and ML[d] groups

	PC1			PC2			PC3		
	$G_{control}$	LL	ML	$G_{control}$	LL	ML	$G_{control}$	LL	ML
S	**0.97**	0.26	0.48	0.17	**0.89**	**0.83**	–	0.03	–0.03
Cl	**0.93**	–0.14	–0.04	–0.03	**0.88**	**0.90**	–	–0.00	0.02
K	**0.94**	**0.72**	**0.89**	0.64	0.57	0.35	–	–0.08	–0.06
Ca	**0.92**	0.37	0.66	0.28	**0.83**	0.68	–	–0.01	0.00
Fe	0.35	**0.85**	**0.95**	**0.79**	0.14	0.09	–	–0.16	–0.03
Cu	0.02	0.14	–0.13	–0.01	0.16	0.34	–	**0.80**	**0.80**
Zn	0.11	**0.90**	**0.87**	**0.93**	0.00	0.08	–	0.06	0.09
Br	0.05	0.15	0.08	0.59	0.14	–0.32	–	–0.69	**0.82**
Rb	0.18	**0.80**	**0.89**	0.60	0.15	0.03	–	0.08	–0.09
Eigen values	4.38	3.93	4.57	1.89	1.70	1.66	–	1.15	1.33
Total ariance (%)	48.7	43.7	50.8	21.0	18.8	18.4	–	12.8	14.7
Cumulative variance (%)	48.7	43.7	50.8	71.6	62.5	69.2	–	75.3	83.9

[a] Higher loadings are shown in bold.
[b, c, d] are the control ($n = 36$), the lymphocytic leukemia ($n = 33$), and the myeloid leukemia ($n = 47$), respectively.

Table 7. Principal component loadings[a] of several elements in whole blood of $G_{control}$ [b], CL[c] and AL[d] groups

	PC1			PC2			PC3		
	$G_{control}$	CL	AL	$G_{control}$	CL	AL	$G_{control}$	CL	AL
S	**0.97**	0.37	0.39	0.17	**0.89**	**0.88**	–	–0.01	0.06
Cl	**0.93**	–0.07	–0.27	–0.03	**0.88**	**0.84**	–	0.00	–0.23
K	**0.94**	**0.86**	**0.82**	0.64	0.41	0.43	–	–0.04	0.09
Ca	**0.92**	0.55	0.55	0.28	**0.78**	**0.73**	–	0.06	0.24
Fe	0.35	**0.92**	**0.90**	**0.79**	0.06	0.25	–	0.03	0.10
Cu	0.02	–0.05	–0.14	–0.01	0.27	0.16	–	**0.80**	**–0.90**
Zn	0.11	**0.88**	**0.90**	**0.93**	0.09	–0.12	–	0.00	–0.12
Br	0.05	0.10	0.29	0.59	–0.24	–0.25	–	**0.79**	–0.38
Rb	0.18	**0.87**	**0.85**	0.60	0.12	0.00	–	0.07	–0.00
Eigen values	4.38	4.39	4.13	1.89	1.67	1.90	–	1.26	1.06
Total variance (%)	48.7	48.8	45.9	21.0	18.6	21.1	–	14.0	11.8
Cumulative variance (%)	48.7	48.8	45.9	71.6	67.4	67.0	–	81.4	78.9

[a] Higher loadings are shown in bold.
[b, c, d] are the control (n = 36), the chronic leukemia (n = 45), and the acute leukemia (n = 35), respectively.

component (PC) loadings, extracted by using varimax normalized rotation on the metal data-set, are shown in Tables 6 and 7 for (LL and ML) and (CL and AL) leukemia subgroups, respectively. In addition, PC loadings for the control group were added in the previously mentioned two tables. The results showed that two PCs were extracted with eigen values > 1 for the control group, commutatively explaining more than 69%; while, three PCs were extracted with eigen values > 1 for LL, ML, CL, and AL leukemia subgroups; commutatively explaining more than 75%, 83%, 81%, and 78% of total variance for the previously mentioned subgroups, respectively. In the case of control group, PC1 showed higher loadings for the light elements: S, Cl, K, and Ca; while, for all studied leukemia subgroups, PC1 showed higher loadings for K, Fe, Zn, and Rb. An important difference emerging here was that K as a major element in all leukemia subgroups shared PC1 with the minor and trace elements (Fe, Zn and Rb). Such association was absent in the control group. PC2 showed higher loadings for S, Cl, and Ca in most leukemia subgroups, while for control group, Fe and Zn contributed maximum loadings to PC2. The maximum loadings of Cu and Br (or only Cu) were shown in PC3 for all leukemia subgroups, while these findings were absent in the control group. It could be concluded that the PCA demonstrated significantly different grouping of elements in leukemia patients and controls. However, in the leukemia patients groups, the role of the light elements (S, Cl, and K, and Ca) was changed by existing a new mutual dependence between K and other minor and trace elements, such Fe, Zn, and Rb. In addition, the trace elements of Cu and Br in leukemia patients have different sources as compared with the healthy volunteers.

Regarding to the age factor, PCA was also employed to identify possible sources and grouping of studied elements in the whole blood of the leukemia age-groups. The PC loadings are shown in Table 8 for control and G1 and G2 leukemia age-groups. Three PCs were extracted with eigen values > 1 for G1 and G2 leukemia age-subgroups; commutatively explaining more than 82% and 75% of total variance, respectively. In the case of the control group and the G1 leukemia age-group, PC1 showed maximum loadings for S, Cl, K, and Ca; while, for the G2 leukemia age-subgroup, PC1 showed maximum loadings for K, Fe, Zn, and Rb. An important difference emerging here was that K as an essential metal in leukemia G2 age-subgroup shared a common PC with the minor and the trace elements (Fe, Zn and Rb). Such association was absent in the control

Table 8. Principal component loadings[a] of several elements in whole blood of the control and leukemia age-groups

	PC1			PC2			PC3		
	$G_{control}$ [b]	G1[c]	G2[d]	$G_{control}$	G1	G2	$G_{control}$	G1	G2
S	**0.97**	**0.96**	0.22	0.12	0.17	**0.92**	–	0.06	0.02
Cl	**0.93**	**0.86**	–0.20	0.07	–0.03	**0.82**	–	–0.25	0.07
K	**0.94**	**0.73**	**0.83**	0.21	0.64	0.37	–	0.01	0.00
Ca	**0.92**	**0.93**	0.45	0.21	0.28	**0.80**	–	0.03	0.02
Fe	0.35	0.43	**0.89**	**0.77**	**0.79**	0.02	–	0.28	0.05
Cu	0.02	0.02	–0.11	0.41	–0.01	0.27	–	**–0.96**	**0.77**
Zn	0.11	0.11	**0.82**	**0.93**	**0.93**	0.02	–	–0.10	0.03
Br	0.05	–0.28	0.25	0.53	0.59	–0.18	–	0.39	**0.76**
Rb	0.18	0.41	**0.83**	**0.77**	0.60	0.04	–	–0.09	0.10
Eigen values	4.38	4.56	3.63	1.90	1.89	2.00	–	1.01	1.15
Total variance (%)	48.7	50.6	40.3	21.1	21.0	22.2	–	11.3	12.8
Cumulative variance (%)	48.7	50.6	40.3	69.8	71.6	62.5	–	82.9	75.3

[a] Higher loadings are shown in bold.
[b, c, d] are the groups of control participants with ages: 28–50 years (n = 36), leukemia patients with ages: 14–28 years (n = 17), and leukemia patients with ages: 29–93 years (n = 57), respectively.

group and also in the G1 leukemia age-subgroup. PC2 shows higher loadings for S, Cl, and Ca in G2 leukemia age-subgroup, while for the control and the G1 leukemia age-groups, Fe and Zn contributed maximum loadings to PC2. The maximum loadings of Cu and Br were shown in PC3 for the G2 leukemia age-subgroup. The negatively maximum loading was shown for Cu in PC3 for the G1 leukemia age-subgroup, while these findings were absent in the control group. It could be concluded that the PCA demonstrated significantly different grouping/origin of the elements in the older--age of leukemia patients and controls. However, in older leukemia patients group, the major element, K, shared common PC with minor and trace elements and therefore, showed mutual dependence, while, the trace elements of Cu and Br revealed independent sources compared to the other major, minor and trace elements. The results showed that S, Cl, K, and Ca shared the common PC in both of the control and the leukemia younger age-groups, indicating that the light elements had the ability to continue their important role in constructing of enzymes, hormones, and cell-signalling molecules in leukemia younger-age patients.

Conclusions

In conclusion, the ES-XRF method was successfully applied for the determination of nine major, minor and trace elements (S, Cl, K, Ca, Fe, Cu, Zn, Br, and Rb) in leukemia patients and control groups. The results revealed marked differences in the distribution and correlations of the studied elements in the whole blood of leukemia patients compared with the healthy volunteers. On the average basis, all the studied elements were significantly lower in the whole blood of leukemia patients than controls.

PCA showed different apportionment mechanisms of major, minor, and trace elements in whole blood of leukemia patients and controls which supported that the body metabolism in the patients is being significantly affected by the studied elements. In addition, the PCA results showed serious effects of the type, the growth and the older-age factors on redistribution of the element levels in whole blood of leukemia patients. On the other hand, PCA showed similar apportionment mechanisms of all studied elements, except Cu and Br, in younger--age and control groups, confirming the high stability of leukemia younger-age patients vs. any change in grouping of certain elements in the whole blood.

Acknowledgment. The authors thank Prof. I. Othman, Director General of AECS, for his encouragement and keen interest in this work. Thanks are also due to the head of the Chemistry Department, Dr. T. Yaseen, for his valuable advice and discussion.

References

1. Al-Sayer H, Mathew TC, Asfer S *et al.* (2004) Serum changes in trace elements during thyroid cancers. Mol Cell Biochem 260:1–5
2. Bamford SA, Węgrzynek D, Chinea-Cano E, Markowicz A (2004) Application of X-ray fluorescence techniques for the determination of hazardous and essential trace elements in environmental and biological materials. Nukleonika 49:87–95
3. Bárány E, Bergdahl IA, Bratteby LE *et al.* (2002) Trace elements in blood and serum of Swedish adolescents: relation to gender, age, residential area, and socioeconomic status. Environ Res 89:72–84
4. Bocca B, Alimonti A, Petrucci F *et al.* (2004) Quantification of trace elements by sector field inductively coupled plasma mass spectrometry in urine, serum, blood and cerebrospinal fluid of patients with Parkinson's disease. Spectrochim Acta B 59:559–566
5. Canellas CGL, Carvalho SMF, De Jesus EFO, Anjos MJ, Lopes RT (2006) Trace and major elements in serum of patients with chronic myelogenous leukemia. J Radioanal Nucl Chem 269:631–634
6. Cunzhi H, Jiexian J, Xianwen Z, Jingang G, Shumin Z, Lili D (2003) Serum and tissue levels of six trace elements and copper/zinc ratio in patients with cervical cancer and uterine Myoma. Biol Trace Elem Res 94:113–122
7. Ekincia R, Ekinci N (2004) An alternative method for the determination of element concentrations in schizophrenic, lung cancer and leukemia patient bloods. J Quant Spectrosc Radiat Transf 86:379–385
8. Hasegawa T, Inagaki K, Haraguchi H (2001) Multielement correlation analysis of major-to-trace elements in human blood serum for medical diagnosis as studied by ICP-AES and ICP-MS. Anal Sci 17;Suppl:i979–i982
9. Hong NT, Ha HV (1996) Application of EDXRF to the determination of lead and other trace elements in the body fluids of industrial workers in Vietnam. X-Ray Spectrom 25:3–14
10. IAEA (2005) Quantitative X-ray Analysis System. QXAS, Doc. Version 2.0. International Atomic Energy Agency, Vienna
11. Khalique A, Shah MH, Jaffar M, Shaheen N, Tariq SR, Manzoor S (2006) Scalp hair metal analysis in the assessment of the occupational exposure of arc welders. Toxicol Environ Chem 88:697–704
12. Khuder A, Bakir MA, Karjou J, Sawan MK (2007) XRF and TXRF techniques for multielement determination of trace elements in whole blood and human hair samples. J Radioanal Nucl Chem 273:435–442
13. Kumar A, Kennedy J, Sasikala K, Jude ALC, Ashok M, Moretto Ph (2002) Trace element analysis of blood samples from mentally challenged children by PIXE. Nucl Instrum Methods Phys Res B 190:449–452
14. Majewska U, Braziewicz J, Banaś D *et al.* (1999) Some aspects of statistical distribution of trace element concentrations in biomedical samples. Nucl Instrum Methods Phys Res B 150:254–259
15. Marco LM, Jiménez E, Hernández EA, Rojas A, Greaves ED (2001) Determination of Zn/Cu ratio and oligoelements in serum samples by total reflection X-ray fluorescence spectrometry for cancer diagnosis. Spectrochim Acta B 56:2195–2201
16. Marques MI, Carvalho ML, Oblad M, Amorim P, Ramos MT (1993) Energy-dispersive XRF analysis of trace elements in Nerium oleander for pollution monitoring. X-Ray Spectrom 22:244–247
17. Martines T, Lartigue J, Avila-Perez P *et al.* (2004) Determination of trace elements in blood samples by TXRF analysis. J Radioanal Nucl Chem 259:511–514
18. Mumtaz M, Siddique A, Mukhtar N, Mehboob T (1999) Status of trace elements level in blood samples of different age population of Karachi (Pakistan). Turk J Med Sci 29:697–699

19. Pasha Q, Malik SA, Shah MH (2008) Statistical analysis of trace metals in the plasma of cancer patients versus controls. J Hazard Mater 153:1215–1221
20. Rahil-Khazen R, Bolann BJ, Ulvik RJ (2002) Correlations of trace element levels within and between different normal autopsy tissues analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES). Bio--Metals 15:87–98
21. Ren Y, Zhang Z, Ren Y, Li W, Wang M, Xu G (1997) Diagnosis of lung cancer based on metal contents in serum and hair using multivariate statistical methods. Talanta 44:1823–1831
22. Rostkowska-Nadolska B, Pospiech L, Bochnia M (1999) Content of trace elements in serum of patients with carcinoma of the Larynx. Arch Immunol Ther Exp 46:321–325
23. Shtangeeva I, Kulikov V (1995) Study of chemical element behavior in health and disease by means of neutron activation analysis and multivariate statistics. Nutrition 11:592–594
24. Wang T, Fu J, Wang Y, Liao C, Tao Y, Jiang G (2009) Use of scalp hair as indicator of human exposure to heavy metals in an electronic waste recycling area. Environ Pollut 157:2445–2451
25. Zaitseva NV, Zemlianova MA, Kir'ianov DA (2001) Identification of specific features of the development of iodine deficiency in children exposed to low-intensity ecological factors (in case of the Perm Region). Vestn Ross Akad Med Nauk 6:39–45
26. Zamboni CB, Oliveira LC, Medeiros JAG *et al*. (2007) Determination of reference values of elements in whole blood using nuclear methodology. Int Nuclear Atlantic Conf – INAC 2007 Santos, SP, Brazil