Critical evaluation of the use of total reflection X-ray fluorescence spectrometry for the analysis of whole blood samples: Application to patients with thyroid gland diseases

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Abstract

Multielemental analysis of whole blood can provide significant information for the evaluation of nutritional status, diagnosis of certain diseases as well as for the assessment of exposure to potentially toxic metals. However, the quantification of multiple elements in whole blood is not easy partly because of the wide variation in element concentrations (from ng·L^{-1} to g·L^{-1}) and the complex matrix. The aim of this work was to develop a fast, sustainable and reliable analytical method, in combination with low power TXRF, for multielemental analysis of blood samples.

Firstly, a set of experiments were carried out to select the best diluent type and dilution factor using the control material SeronormTM Trace Elements Whole Blood L-1. A critical evaluation of the parameters affecting the sample deposition on the reflector was also carried out including a study of the shape and element distribution of the deposited residue on the reflector by micro X-ray fluorescence spectrometry. Using the best analytical conditions, limits of detection estimated were in the low mg·kg^{-1} range and similar to those obtained using more complex sample treatments such as digestion. Accuracy and precision of the results were in most cases acceptable (Recoveries: 89-102 %, RSD: 6-8%, n=5). Only underestimated values were obtained for light elements such as potassium.

To prove the applicability of the method, several blood samples from control and thyroid diseases patients were analysed. Despite the fact that more samples need to be analysed, it seems that Zn and Br contents in some of the patients are significantly higher compared to control samples.

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1. Introduction

Biomonitoring of trace metals in human body fluids such as whole blood, serum or plasma has become a flourishing field especially after knowing the importance of body metal imbalances in the diagnosis and prevalence of various disorders [1-2]. For instance, development of some chronic diseases such as atherosclerosis, neurological disorders, autoimmune diseases, ageing and cancer are connected to Zn deficiency [3]. However, the quantification of multiple elements in whole blood is not easy partly because of the wide variation in element concentrations (from $ng \cdot L^{-1}$ to $g \cdot L^{-1}$) and the complex matrix [4].

The most commonly used analytical techniques for element determination in blood samples are atomic absorption spectrometry (AAS) [5], inductively coupled plasma with emission spectrometry (ICP-OES) [6] or mass spectrometry (ICP-MS) [7]. Compared to AAS, ICP-MS have some advantages including multielemental capability, very low detection limits and a wide linear dynamic range which allows the determination of major and trace elements at the same sample injection [8]. However, this technique suffers from spectral as well as non-spectral interferences that may degrade the accuracy of the blood analyses [9]. For this reason, usually ICP-MS needs to be carried out using a reaction and/or collision cell to minimize spectral interferences [10] and the use of internal standardization, standard addition or a total sample consumption system are usually employed to overcome blood matrix effects [9, 11]. Moreover, usually an additional treatment such as digestion or dilution of the blood sample has to be used before ICP-MS analysis to obtain quantitative results [4].

Another analytical possibility is the use of total reflection X-ray fluorescence spectrometry (TXRF). TXRF is a well-established analytical technique for multi element determination in various sample types, especially liquids and powdered micro samples [12-14]. To perform analysis under total-reflection conditions, samples must be provided as thin films. For liquid samples, this is done by depositing 5–50 µL of sample on a reflective carrier with a subsequent drying of the drop. However, due to the complexity of blood matrix, to prepare sample as thin layers usually a sample treatment procedure is also needed to carry out the TXRF analysis. In Table 1, a summary of published TXRF methods for the analysis of whole blood samples is displayed. As it is shown in Table 1, blood samples are commonly digested using nitric acid or a mixture of this acid in combination with hydrochloric acid or hydrogen peroxide [15-21]. A further enhancement of detection limits can be achieved using more sophisticated sample treatments involving an iron separation step using methyl isobutyl ketone or metal preconcentration using ammonium pyrrolidinedithiocarbamate [16-17]. Nevertheless such sample treatments are expensive and time-consuming and, in most cases, are not adequate for most clinical laboratories where a large number of blood samples have to be analysed. In this sense, fast and simple procedures for sample preparation with minimal handling are much more desirable [8]. It is also important to highlight that most of the published papers dealing with the analysis of blood samples by TXRF were performed using large scale instruments with high-power X-ray tubes, which demand water-cooling systems and liquid-nitrogen cooled detectors. In recent years, the development and commercialization of benchtop TXRF instrumentation, which offers extremely simple operations with a low-cost compact design, have promoted its application in many different fields [22-25].

In view of this premises, the aim of this work was to develop a fast, sustainable and reliable analytical method, in combination with TXRF, for multielement analysis of whole blood samples. For that, dilution is proposed as a sample treatment alternative to acidic digestion. Firstly, a set of experiments were carried out to select the best diluent type and dilution factor for real blood sample analysis. A critical evaluation of the parameters affecting the sample deposition on the reflector was also carried out including a study of the shape and element distribution of the deposited residue on the reflector by micro X-ray fluorescence spectrometry. Analytical parameters of the method (limits of detection, accuracy and precision of the results) were evaluated using the control material SeronormTM Trace Elements Whole Blood L-1. Finally, to prove the applicability of the method, several blood samples from control and thyroid diseases patients were analysed and zinc concentrations were compared with those obtained by ICP-MS analysis.

2. Materials and Methods

2.1 Reagents and materials

Stock solutions of 1000 mg L^{-1} (ROMIL PrimAg@ Monocomponent reference solutions) were used to prepare internal standard solutions (Mo, V, Co, Ga, Rh) and spiked blood samples (Pb, Se, Cr, Sn, Cd, As, Sb, Ni, Ag). Ultrapure de-ionized water used for dilution of stock solutions and blood samples was obtained from a Milli-Q purifier system (Millipore Corp., Bedford, Massachusetts). A solution of 1% of Triton^{TM} X-100 in water (laboratory grade, Sigma-Aldrich) and a solution of 0.1% $Mg(NO_3)_2$ (laboratory grade, Sigma-Aldrich) were tested as diluting agents to prepare blood samples before TXRF analysis.

Nitric acid (69%, HIPERPUR, Panreac) and hydrogen peroxide (30%, TraceSELECT, Sigma- Aldrich) were used to digest blood samples. Silicone solution in isopropanol (Serva GmbH & Co, Germany) was used to coat all the quartz glass disc reflectors (∅: 30mm, thickness: 0.1 mm) in order to obtain a hydrophobic film so as to facilitate sample deposition. Ethylenediaminetetraacetic acid (EDTA), 1-butanol, ammonia (laboratory grade, Sigma-Aldrich) were used to dilute blood samples before ICP-MS analysis. The reference material Seronorm "Trace Elements Whole Blood L-1" (Sero, Billingstad, Norway; Reference 210105) was used to optimize and validate the developed TXRF method. This lyophilized human whole blood material was reconstituted in 3mL of water, according to the instructions provided in the certificate.

2.2 Studied blood samples

A total of 21 blood samples were collected from controls (n=7) and patients with thyroid gland diseases (n=14) in the age group 26 to 73 years old. The diagnosis of the case population (patients) includes papillary thyroid cancer, follicular thyroid adenoma and other diseases needing surgical intervention. All volunteers signed a written consent to participate in the study and were introduced with the rights to abandon it at any time without further justifications. The blood sampling was done as a part of the study approved by the Institutional and Hospital Ethics committees and observed the ethical principles of the Declaration of Helsinki. Based on the answers given in their questionnaires, volunteers were not exposed to potentially confounding factors (occupational chemical exposure, genetic disorders, anticancer drugs or radiation).). Blood of the target sample were drawn by antecubital venepuncture into K_3EDTA-coated tubes (10 mL).

(Becton Dickinson, USA) and then stored at -20°C until analyses were performed. Samples were then thawed, left to reach room temperature, and treated following the procedures described in section 2.3.

2.3 Sample preparation

2.3.1 Dilution for TXRF analysis

Several tests (diluting agents, dilution factor, internal standard, drying mode on the reflector) were performed to select the best strategy to analyse whole blood samples by TXRF (see section 3.1.2 for specific details). Finally, the experimental procedure followed to prepare whole blood samples was as follows: 0.1 g of blood were spiked with 0.05 g of Ga 100 mg kg^{-1} (internal standard) and diluted up to 0.5 g using a solution of 1% Triton X-100 in water (final sample dilution 1:5). The mixture was homogenized by vortex agitation for 10 s and an aliquot of 10 µL was transferred onto a quartz glass sample carrier and dried using an infrared lamp.

2.3.2 Digestion for TXRF analysis

For comparison purposes, blood samples were also digested using a dry block heater for polypropylene vials (2 mL) with digital electronic control of temperature and time (Tembloc, Selecta). Briefly: 0.1 g of blood sample was mixed with 0.02 g of Ga 100 mg kg^{-1} (internal standard), 0.5 mL nitric acid and 0.25 mL hydrogen peroxide. Then the mixture was heated at 80 °C for 1 h until a clear solution was obtained. The sample deposition volume and drying mode to perform TXRF analysis were the same as for the diluted blood samples.

2.3.3 Dilution for ICP-MS analysis

Cu and Zn concentration levels in blood samples were also determined by ICP-MS after alkali dilution [26]. For that, 0.2 g aliquots of the whole blood were spiked with 0.025 g of a 10 mg kg^{-1} Ge (internal standard) and diluted 1:25 with an alkali solution consisting of 2% (w/v) 1-butanol, 0.05% (w/v) EDTA, 0.05% (w/v) Triton X-100, 1% (w/v) NH_4OH.

2.4 Instrumentation

TXRF analysis were performed using a commercial benchtop TXRF spectrometer S2 PICOFOX (BrukerNano, GmbH, Germany), equipped with a low power tungsten X-ray tube (50 kV, 1 mA) and a Silicon Drift Detector (SDD) with a resolution <150 eV at Mn-K_α. The evaluation of TXRF spectra and calculation of the analyte net peak area were performed using the software (Spectra Plus 5.3, Bruker AXS Microanalysis GmbH, Berlin, Germany) supplied with the equipment. For quantification using TXRF spectra, the software applies a deconvolution routine which uses measured mono-element profiles for the evaluation of peak areas. A measurement time of 2000 s was selected as a compromise between precision and total analysis time. More detailed information about instrumental parameters and measurement conditions can be found in Table 2.

For comparison purposes, Zn concentration in the target blood samples were also determined by ICP-MS (Agilent 7500c, Agilent Technologies, Tokyo, Japan) equipped with an octapole collision cell. The instrumental parameters and measurement conditions are displayed in Table 2. To obtain quantitative

results for Cu and Zn determination, ^{72}Ge was used as internal standard and the collision cell was pressurized with 2 mL min^{-1} He to avoid any polyatomic interference. Moreover, matrix-matched calibration standards (using the same alkali solution employed to dilute blood samples) were used for quantification purposes.

3. Results and Discussion

3.1 TXRF method development

As stated in the introduction section, the main aim of this contribution was the development of a fast, sustainable and reliable analytical TXRF method for multielemental analysis of whole blood samples. For that, several tests were performed to select the best strategy to prepare blood samples by dilution (diluting agent, internal standard, dilution factor) and performing TXRF analysis (sample deposition volume, drying mode, measurement time). All the experiments were carried out using the reference material Seronorm "Trace Elements Whole Blood L-1".

3.1.1 Selection of the internal standard

In TXRF analysis, since samples are prepared as thin films on a reflective carrier, matrix effects are usually neglected and quantification can be performed directly by the addition of an internal standard (IS) to the sample using Expression-1 [27]:

$$C_i = \left(\frac{N_j C_{pi} S_{pi}}{N_{pi} S_i}\right) \quad \text{Expression-1}$$

where C_i: analyte concentration, N_i: analyte net peak area, C_{pi}: IS concentration, S_{pi}: instrumental sensitivity for the IS, N_{pi}: IS net peak area, S_i: instrumental sensitivity for the analyte.

An ideal element to be used as internal standard should not be present in the original sample, not interfere with the target elements and have an adequate XRF response. According to that and after checking the non-presence of V, Co, Ga, Mo and Rh in blood samples, we tested these elements as internal standard candidates. In Figure 1, TXRF spectra for the analysis of the Seronorm reference material with and without the addition of the aforementioned internal standards are displayed. From the results of elemental quantification obtained (data not shown) it was found that there were no statistically significant differences using Ga, Mo and Rh as IS. When using V, results were systematically underestimated and larger uncertainties between replicates were found when using Co as IS surely due to the overlapping of Co-K_α line with Fe-K_β (arising from the blood sample). It is important to remind that the TXRF system used in this study is equipped with a W anode to generate X-rays and allows the determination of Mo and Rh using its K-lines that is not possible when employing the most commonly used Mo anode. For that reason, finally Ga selected as IS for further TXRF analysis since it can be used in both Mo and W TXRF systems.

3.1.2 Sample treatment

Firstly a study was conducted to explore the possibilities of direct analysis of blood samples by TXRF analysis. For that, aliquots (5 and 10 μL) of the whole blood were deposited on reflective quartz carriers and dried under IR lamp. However, after the drying step the residue on the reflector was too thick and it was detached from the surface of the quartz reflector.

For this reason, direct TXRF analysis of blood samples was discarded and dilution was studied as a possible simple sample treatment. Several diluting agents were used for such purpose including ultrapure water, a solution of 1% Triton X-100 and a solution of 0.1% $Mg(NO_3)_2$. These reagents have been previously used in TXRF analysis to obtain homogeneous complex liquid samples and solid suspensions [12, 14, 20] and as chemical modifier for a proper sample deposition on the reflector [28]. In Figure 2, the effect of the sample dilution type on element concentrations and the shape of the dried residue on the quartz reflector are shown. In all cases, a sample dilution factor 1:5 was used. Lower dilution factors were avoided since after the drying step the residue on the reflector was still too thick and it was detached from the surface of the quartz reflector. As it can be seen in Figure 2, in general, better results in terms of accuracy and precision of the obtained results were obtained using a solution of 1% Triton X-100 to dilute blood samples. This fact can be related to the non-ionic nature of this surfactant and the attainment of a more homogeneous suspension, as has been really pointed in other applications dealing with the use of Triton X-100 as dispersing agent [29]. Moreover, using this diluent, a better deposition of the drop on the reflector was assessed, surely due to the reduction of the surface tension of the blood sample on the reflector surface, as it is shown in the images displayed also in Figure 2.

Otherwise, it is interesting to remark that potassium was underestimated regardless the diluent agent used. This fact can be explained probably due to absorption issues arising from the blood matrix. An improvement of potassium results could be surely assessed by using a higher dilution factor as it was already demonstrated in a previous work focussed on the analysis of wine samples by TXRF [22]. However, since the aim of this study was the development of a multielemental method (including trace elements), it was not considered appropriate to increase the sample dilution factor. Finally, TXRF analyses were performed diluting blood samples with a solution 1% Triton X-100 in water using a ratio of 1:5.

Usually in TXRF analysis liquid samples are mixed by vortex agitation for several seconds before sample deposition on the reflector. In order to increase the homogeneity of the diluted blood samples, an additional mixing procedure using a sonication time of 10 minutes, was also tested. However, obtained results (data not shown) demonstrated that the sonication processes did not improve the accuracy and precision of the obtained results and therefore it was discarded for further TXRF analysis.

3.1.3 Sample deposition on the reflector and TXRF analysis

The aim of the sample preparation process in TXRF is to obtain the target sample as a thin layer on a carrier with high reflectivity sample support. Therefore, the choice of an adequate sample deposition volume and a proper drying mode of the drop on the reflective carrier are of significant importance in order to ensure the conditions of total reflection. For that, a study was conducted to evaluate the effect of the sample deposition volume (in the range of 2-10 μL) on the obtained element concentrations. It is interesting to remark that, for all the deposition volumes, the diameter of the sample spot on the reflector was adequate considering the volume spanned by excitation beam and the detector viewing.

As it is shown in Figure 3, similar results were obtained for all the tested volumes in the case of K, Ca and Fe quantification. However, for trace element determination, better results in terms of precision and accuracy were assessed using a sample volume in the range of 5-10 µL. The amount of sample deposited on the reflector affect also the limit of detection (LODs) of the elements to be determined. Better LODs were obtained using a volume of 10 µL (i.e. LOD (Cu0.18 $\mu g \cdot L^{-1}$ (5 µL), 0.06 $\mu g \cdot L^{-1}$ (10 µL), LOD (Zn): 0.14 $\mu g \cdot L^{-1}$ (5 µL), 0.05 $\mu g \cdot L^{-1}$ (10 µL)). Additionally, a study of the shape and homogeneity of the dried residue on the reflector using different sample deposition volumes (2 µL, 5 µL and 10 µL) was performed by µ-XRF. In Figure 4, 2-D mappings for Fe (element present in the blood matrix) distribution in the blood residues are displayed. As it can be seen, it seems that Fe was more accumulated in the centre part of the drop in all cases but a better deposition was obtained using a volume of 10 µL. In view of the obtained results, a volume of 10µL was selected as a suitable deposition volume for TXRF analysis of blood samples. For some applications, the drying mode used to dry the drop on the quartz reflector can have a great effect on element determination [30]. For this reason, a test was conducted drying 10 µL of a diluted aliquot of the control material Seronorm^{TM} (1:5, Blood: Trition 1%) using: (i) an IR lamp, (ii) a hot plate set at 50°C or (iii) under the laminar flow hood at room temperature. It was found that the drying mode did not influence the obtained element concentrations and for that an IR lamp was used in further experiments since it was the faster way to dry the drop on the quartz reflector.

The measurement time is also a parameter that can influence both the repeatability of the obtained results and the limits of detection (LODs). In Figure 5A, as an example, relative standard deviation (RSD) of K, Fe, Zn and Cu concentrations obtained by analysing five times a diluted aliquot of the control material Seronorm^{TM} (1:5, Blood: Trition 1%) are displayed. As it can be seen, the effect of measurement time (500-3000 s) on results repeatability was not relevant for element concentrations in the range of 300-1000 $mg \cdot kg^{-1}$. However, for elements present at lower concentration levels (i.e., Zn and Cu) it was necessary a longer time to assess an acceptable RSD value. In Figure 5B, LODs estimated for the same elements at different measurement times are also displayed. As it is shown, limits of detection improve when increasing the measurement time. So, depending on the concentration level of the elements of interest, measurement time can be increased or reduced. However, since in this application multielemental analysis of blood samples is considered (including trace and ultratrace elements) longer measurement times are required. As it can be seen also in Figure 5B, for measuring times higher than 2000 s the expected improvement in the LODs is be very small, and as a consequence 2000 s was fixed as suitable for TXRF measurements as trade-off between an acceptable limit of detection and total analysis time.

3.2 Analytical figures of merit of the TXRF method

Using the best analytical conditions previously described, LODs for K, Ca, Fe, Cu, Zn, Br and Rb were estimated by analysing the control material Seronorm^{TM}. In order to evaluate LODs for the determination other elements that could be present in whole blood samples using the developed method, an aliquot of the control material Seronorm^{TM} was spiked at the level of 200 $\mu g \cdot L^{-1}$ of Cr, Ni, As, Se, Ag, Cd, Sn, Sb and Pb. Obtained results are summarized in Table 3. For comparison purposes, LODs calculated using a previous digestion of the whole blood sample using a block heater digester (see additional details in section 2.3.2) are also included in Table 3. As it can be seen, for most elements, similar limits of detection were

assessed by using both sample treatments before the TXRF analysis. However, slightly better LODs for light elements (K, Ca) were estimated using a previous digestion of the sample. This fact can be explained probably due to absorption issues arising from the blood matrix using a sample dilution factor 1:5. On the contrary, lower LODs were obtained for volatile elements (As, Br) using a dilution step of the blood sample instead of an open digestion procedure before the TXRF analysis. Despite the fact that LODs estimated using the developed TXRF are higher than those obtained using more sophisticated sample treatments in combination with TXRF including digestion with a subsequent iron separation step using methyl isobutyl ketone (see Table 1) or in comparison with other spectrometric techniques such as ICP-MS (see Table 4), they are suitable to monitor some interesting elements which are present in blood samples which are useful for diagnosis of certain diseases (i.e. Cu and Zn). It is also interesting to remark that the reported limits of detection can be significantly reduced using Mo X-ray tube TXRF systems. In a previous publication we demonstrated that limits of detection for most elements were one order of magnitude lower when using the Mo-TXRF system in comparison with W-TXRF systems using a lower measuring time (600s) [22]. Anyway, despite of the lower sensitivity of TXRF in comparison with ICPMS analysis, TXRF method present some interesting advantages in the field of blood analysis including a simpler sample treatment and quantification approach as well as a reduction of measuring costs (see Table 4).

In order to study the quality of the results obtained using the TXRF method, the blood reference material Seronorm™ Trace elements human whole blood L-1 was analysed and obtained results are summarized in Table 5. As it is shown, good agreement was obtained between experimental results and reference values, demonstrating the usefulness of the developed methodology for the quantification of Fe, Cu, Zn Br and Rb in blood samples.

Global precision of the TXRF method was also tested by analysing five independent replicates of the same reference material mentioned above. Besides, one of the replicates was measured five times and the relative standard deviation (RSD) associated was also calculated. This uncertainty is related to the instrument stability and counting statistics. Therefore, by means of error propagation, the uncertainty due to sample preparation can also be estimated. Results obtained are displayed in Figure 6.

As it is shown, global precision is acceptable for elements present at concentration levels higher than 4 $mg \cdot L^{-1}$ with RSD values between 5.7 to 8.1%. However, for elements present at lower concentrations (<1 $mg \cdot L^{-1}$), RSD values higher than 20% are obtained. From Figure 6 it can also be deduced that uncertainties related to sample preparation step have a significant contribution (>50 %) to the global precision of the obtained results.

3.3 Application to real blood samples

To further prove applicability of the method for real blood samples, a total of 21 blood samples were collected from healthy volunteers (controls) and patients with thyroid gland diseases (additional information in section 2.2) and were analysed by the proposed TXRF method. Results obtained are displayed in Figure 7 and Table 6. As it is shown, in general, similar element concentrations were found for control and patients, except for Zn and Br. To the best of our knowledge, this is the first observation of difference in blood Br levels in patients with thyroid diseases. Higher levels of Zn, but Cu and Se as well, were also found in whole blood of patients with different thyroid diseases [31]. However, the results are

not consistent indicating that on a limited number of volunteers, a clear picture of Zn measured in the blood or serum of patients with thyroid diseases cannot be drawn [32, 33]. Some authors indicate that higher tissue Zn concentration might be associated with other toxic metals such as cadmium in later stages of thyroid cancer [34]. These data highlight the importance of further studies on larger number of volunteers in order to resolve the impact of certain essential and toxic elements in thyroid pathology.

Finally, in order to verify the obtained results, Zn concentrations were compared with those obtained by ICP-MS analysis. As it can be seen in Figure 8, similar results were obtained using both analytical approaches highlighting the suitability of the developed methodology for quantification of Zn in real blood samples in a simple and cost-effective way.

Conclusions

In the present contribution a simple and sustainable TXRF method is proposed for element determination in whole blood samples. The method consists of a dilution 1:5 of the blood sample with Triton 1% in water with a subsequent analysis of 10 µL of the standardized sample by TXRF. The method is also cost-effective since the use of chemical reagents are reduced to a minimum and the TXRF system used is equipped with a low-power X-ray tube and thus, no consumables or cooling media are required for function.

This analytical approach could be useful for quantitative measurement of elements with Z>20 that are found at concentration levels in the low $mg \cdot L^{-1}$ range in blood samples. This includes the determination of halogen elements that are difficult to be measured by other spectrometry techniques (i.e. Br). However, for the determination of toxic elements such as Pb and Cd, present at ultra-trace levels, more sophisticated sample treatments as well as more sensitive techniques are needed.

In contrast to most of the published contribution in this field, quantitative analysis by TXRF is assessed by internal standardization. Therefore, the use of matrix-matched standards for quantitative purposes is avoided.

As study case, the TXRF method has been successfully applied for multielement analysis of blood samples from healthy volunteers (controls) and patients with thyroid gland diseases. Preliminary results showed significant higher Zn and Br contents in some blood samples from patients. However, additional samples should be considered in order to understand the relationship between Zn and Br concentrations and their potential relation to thyroid gland diseases.

To sum up, TXRF method shows considerable potential for the determination of the aforementioned elements in blood samples and it could be of interest to extend its application to other biological fluids (i.e., urine or plasma) in future studies.

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Compilance with Ethical Standards:

Research involving human participants and/or animals. All volunteers signed a written consent to participate in the study and were introduced with the rights to abandon it at any time without further justifications. The blood sampling was done as a part of the study approved by the Institutional and Hospital Ethics committees and observed the ethical principles of the Declaration of Helsinki.

Conflicts of Interest. The authors declare that they have no conflict of interest.

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Table 1. Summary of published TXRF methods for the analysis of whole blood samples.

Elements	Sample treatment			TXRF analysis					LODs	Ref.
	V (mL)	Treatment	IS	V (µL)	System	Anode	Power (W)	Time (s)	(mg/kg)	
P, S, K, Ca, Fe, Cu, Zn, Se, Rb, Sr, Pb	1	Digestion - HNO_3 (MW/PTFE bomb)	Ga	10	EXTRA II	Mo/W	2000	1000-5000	0.02-0.08	15
S, K, Ca, Cu, Zn, Br, Rb, Pb	2	Lyophilisation Digestion - HNO_3/HCl Extraction with MIBK	Ni	10	TX2000	Mo	3000	1000	S, K, Ca: 1.5-4.5 Others: 0.007-1.5	16
Pb	n.a.	Digestion - HNO_3 Extraction with MIBK	Ni	10	TX2000	Mo	3000	n.a.	0.03	17
P, S, K, Ca, Fe, Cu, Zn, Se, Rb, Sr	0.5	Digestion - HNO_3/H_2O_2 (MW)	Y	20	Atomika 8030C	Mo/W	2500	1000	P, S, K, Ca, Fe: 5.4-75 Others: 0.1-1.1	18
S, P, K, Ca, Fe, Br	0.1	Lyophilisation Digestion - HNO_3 (PTFE bomb)	Ga	10	TXRF ISO 2000	Mo	2000	3500	2-32	19
P, S, Cl, K, Ca, Fe, cu, Zn, Se, Br, Rb, Sr, Pb	0.5-3	Dilution (1:1 in water) Digestion - HNO_3/H_2O_2 - MW/PTFE bomb Cold plasma ashing	Ga	10	S2 PICOFOX	Mo	50	600-1000	P, S, K, Ca, Fe: 40-2000[a] Others: 5-60[a]	20
Ni, Pb	n.a.	Digestion : HNO_3	Ga	n.a.	n.a.	Mo	n.a.	n.a.	Ni, Pb: 0.05-1[a]	21
Ca, Fe, Cu, Zn, Br, Rb, Cr, Ni, As, Se, Ag, Cd, Sn, Sb, Pb	0.2	Dilution (1:5 in Triton 1%) Digestion - HNO_3/H_2O_2 (BH)	Ga	10	S2 PICOFOX	W	50	2000	K, Ca: 14-25 Others: 0.2-1.3	This work

[a] LODs in µg/L

Nomenclature: MW (microwave), MIBK (Methyl isobutyl ketone), n.a. (not available), APDC (ammonium pyrrolidinedithiocarbamate), BH (Block heater)

Table 2. Instrumental parameters and measurement conditions.

S2 PICOFOX TXRF benchtop spectrometer	
X-ray tube anode	W
Power	50 W
Optics	Multilayer monochromator (35.0 keV)
Detector	SDD, 10 mm^2 , <150 eV resolution at Mn-K_α
Working environment	Air
Agilent 7500c ICP-MS spectrometer	
RF power	1500 W
Plasma gas flow rate	15 L min^{-1}
Nebuliser gas flow rate	1.12 L min^{-1}
Sampling cone	Ni, 1 mm aperture diameter
Skimmer cone	Ni, 0.4 mm aperture diameter
Integration time for each isotope	0.1 s
Readings per replicate	3
Signal measurement mode	Three points per peak
Isotopes monitored	^{65}Cu, ^{66} Zn, ^{72} Ge (as internal standard)
Collision cell conditions	Pressurized cell: 2 mL min^{-1} He

Table 3. Limits of detection (mg/kg) for the analysis of whole blood samples by TXRF after a dilution or a digestion step (measurement time: 2000 s)

Element	Dilution (1:5)[a]	Block heater digester[b]
K[c]	24.9	22.8
Ca[c]	14.4	5.2
Fe[c]	1.1	0.7
Cu[c]	0.3	0.7
Zn[c]	0.4	0.4
Br[c]	0.4	1.0
Rb[c]	0.2	0.2
Cr[d]	0.6	0.9
Ni[d]	0.6	0.7
As[d]	0.6	15.0
Se[d]	0.2	0.3
Ag[d]	0.4	1.7
Cd[d]	0.3	0.4
Sn[d]	1.0	0.9
Sb[d]	1.3	1.3
Pb[d]	0.2	0.2

[a] Dilution 1:5 (0.2 mL blood in 1 mL of Triton 1% (v/v in water))
[b] Digestion (0.1 mL blood in 0.5 mL HNO_3 and 0.25 mL H_2O_2, T:80 °C, 1 h)
[c] Evaluated analysing the control material Seronorm ™ Trace Elements Whole Blood L-1
[d] Evaluated analysing the control material Seronorm ™ Trace Elements Whole Blood L-1 spiked at the level of 200 μg L^{-1} of each element

Table 4. Comparison of analytical performance of the proposed TXRF method with the ICP-MS reference method for the analysis of whole blood samples.

Characteristic	TXRF method	ICP-MS
LODs	μgL^{-1}- mgL^{-1}[a]	< ngL^{-1}
Multielemental analysis	Yes (including halogens, i.e. Br)	Yes
Sample treatment	Dilution with Triton 1% (1:5)	Digestion or alkali dilution (1:25 or more)
Calibration	External calibration not needed (internal standardization)	External calibration using matrix matched standards
Consumables	Not required	High purity Ar
Cooling media	Not required	Water cooling

[a] TXRF system equipped with a low power (50 W max.) W X-ray tube.

Table 5. Results obtained for the determination of Fe, Cu, Zn, Br and Rb in the commercial blood reference material (Seronorm^{TM} Trace elements whole blood L-1) by means of the developed TXRF method. Results are expressed as mean concentration values of three replicates with the associated standard deviation.

Element	Certified value (mg L^{-1})	Approximate value (mg L^{-1})	TXRF method
Fe		334	320 ± 20
Cu	0.64 ± 0.13		0.6 ± 0.2
Zn	4.3 ± 0.9		4.4 ± 0.4
Br		1.053	0.9 ± 0.1
Rb		1.35	1.2 ± 0.1

Table 6. Descriptive statistics of element content in blood samples from controls and patients with thyroid disorders.

	Controls (n=7)						
	K	Ca	Fe	Cu	Zn	Br	Rb
Mean	1396.4	76.7	457.6	1.0	6.3	3.1	2.6
SD	49.8	8.3	43.2	0.3	0.5	0.4	0.4
COV(%)	4	11	9	27	8	14	15
Min	1317.0	63.4	383.3	0.6	5.4	2.6	2.0
Max	1454.5	88.7	511.6	1.4	6.8	3.9	3.1
	Patients (n=14)						
	K	Ca	Fe	Cu	Zn	Br	Rb
Mean	1487.4	76.5	463.7	1.1	8.3	7.8	2.3
SD	142.3	9.6	49.4	0.2	3.5	8.5	0.4
COV(%)	10	13	11	15	42	110	18
Min	1310.1	68.4	404.8	0.8	5.3	2.5	1.8
Max	1600.9	88.7	496.0	1.3	16.8	27.8	2.9

Fig. 1. TXRF spectra for the control material Sernonorm ™ Trace Elements Whole Blood L-1 using different internal standards.

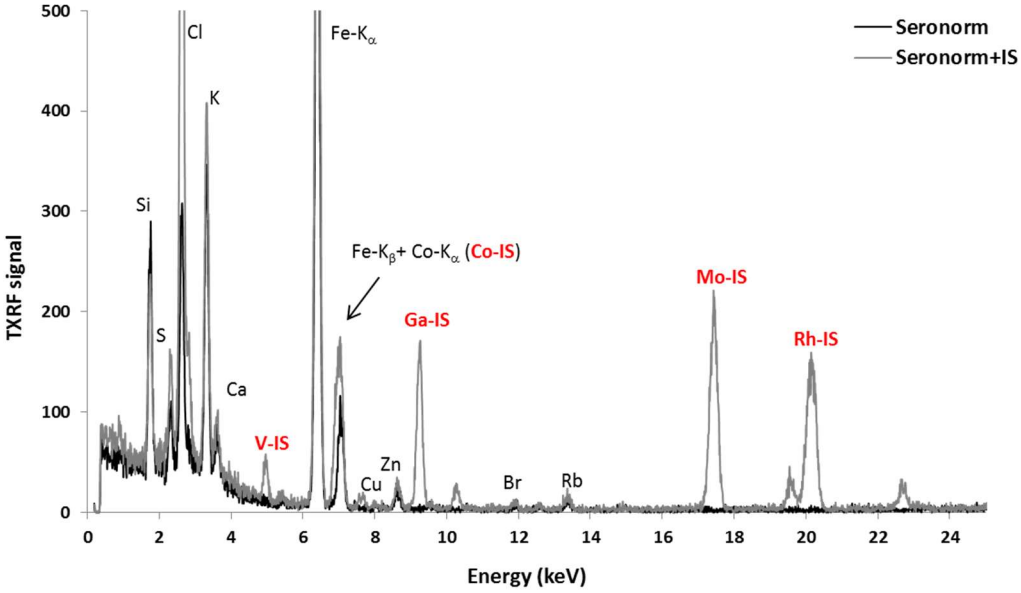

Fig. 2. Effect of sample diluent type (dilution factor 1:5) on element concentrations and shape of the dried residue on the quartz reflector. Results are expressed as mean of triplicate analysis of the control material Sernonorm ™ Trace Elements Whole Blood L-1 with the associated standard deviation.

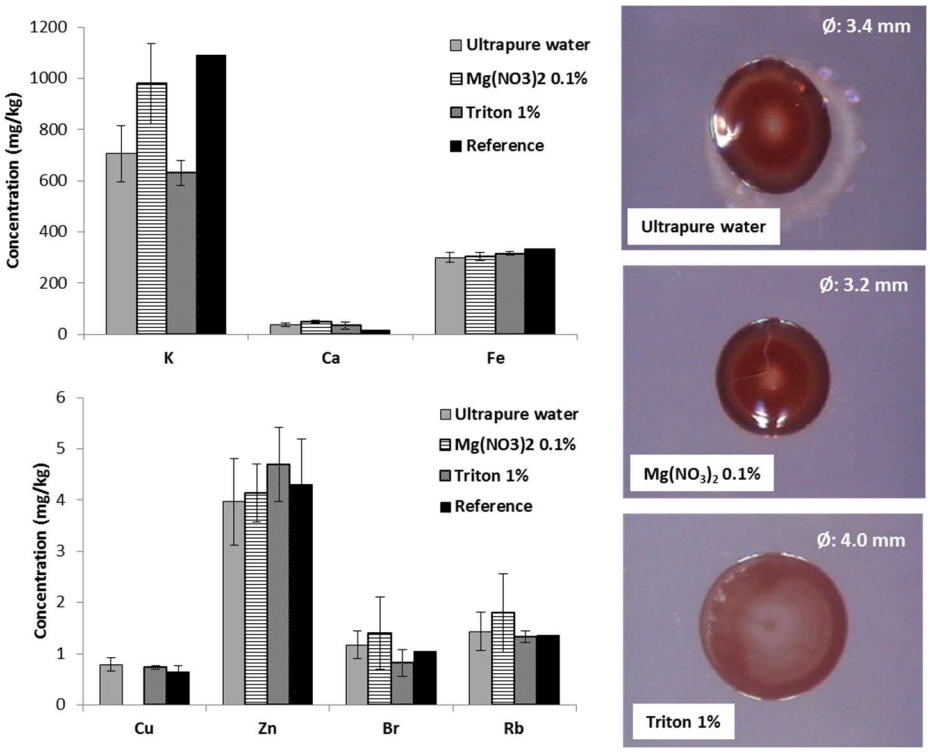

Fig. 3. Effect of sample deposition volume on the reflector on element concentrations. Results are expressed as mean of triplicate analysis of the control material Sernonorm ™ Trace Elements Whole Blood L-1 with the associated standard deviation (Dilution 1:5 with Triton 1%).

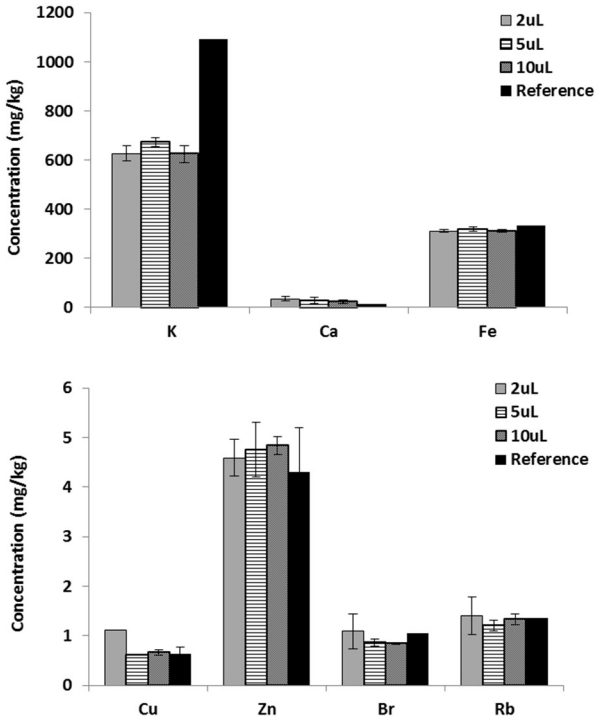

Fig. 4. Effect of sample deposition volume on the shape and iron distribution (µg/cm^2) of the solid residue on the reflector. Analytical conditions: Dilution 1:5 of the control material Sernonorm ™ Trace Elements Whole Blood L-1 with Triton 1%. Mapping conditions (µ-XRF): 50 kV, 1 mA, No primary filter, collimator: 1.0 mm, Grid: 10x10 points, time per point: 100 s.

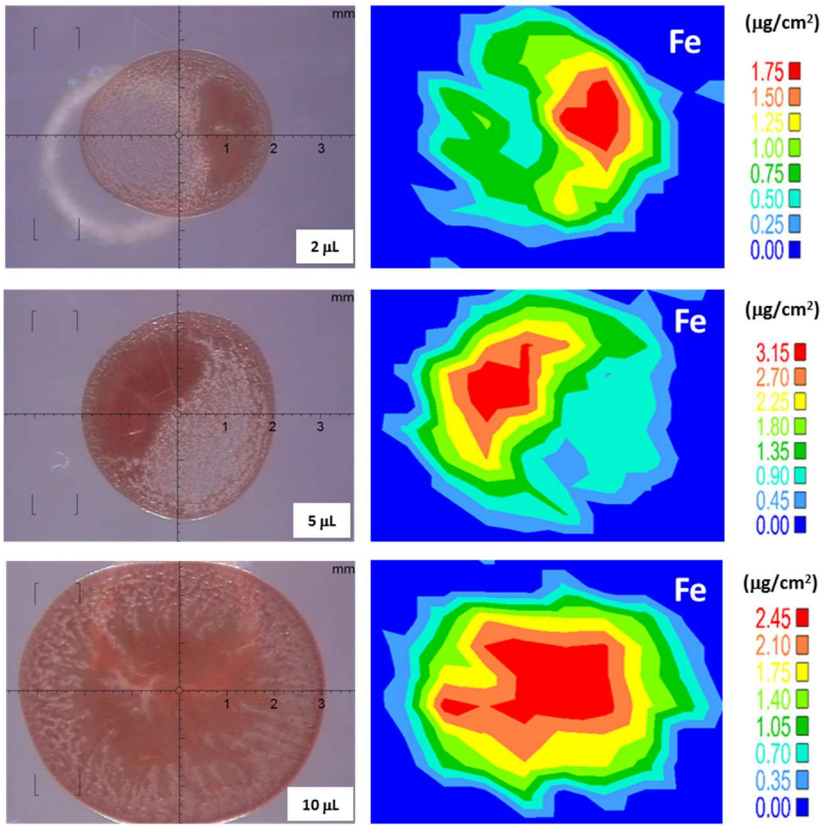

Fig. 5. Effect of measurement time on repeatability of the results (A) and limits of detection (B). Analytical conditions: Dilution 1:5 of the control material Sernonorm ™ Trace Elements Whole Blood L-1 with Triton 1%, 10 µL sample deposition volume. Element concentrations (raw control material Sernonorm ™ Trace Elements Whole Blood L-1): [K]=1091 mgL^{-1}, [Fe]=334 mgL^{-1}, [Zn]=4.2 mgL^{-1}, [Cu]=0.64 mgL^{-1}.

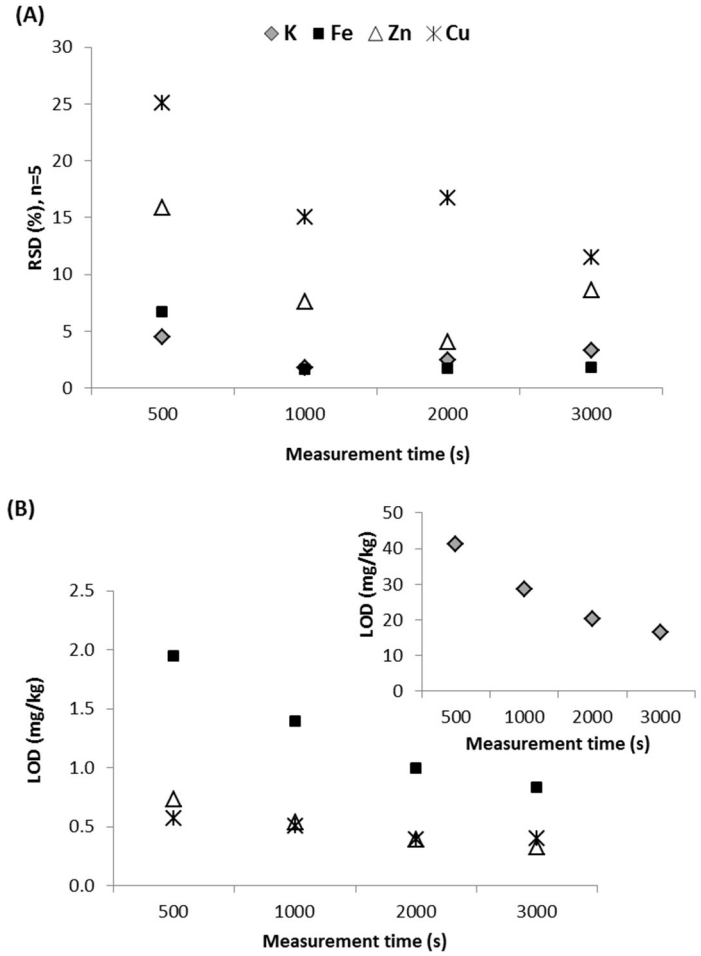

Fig. 6. Contribution of instrument and sample preparation uncertainties to the global precision of the TXRF results.

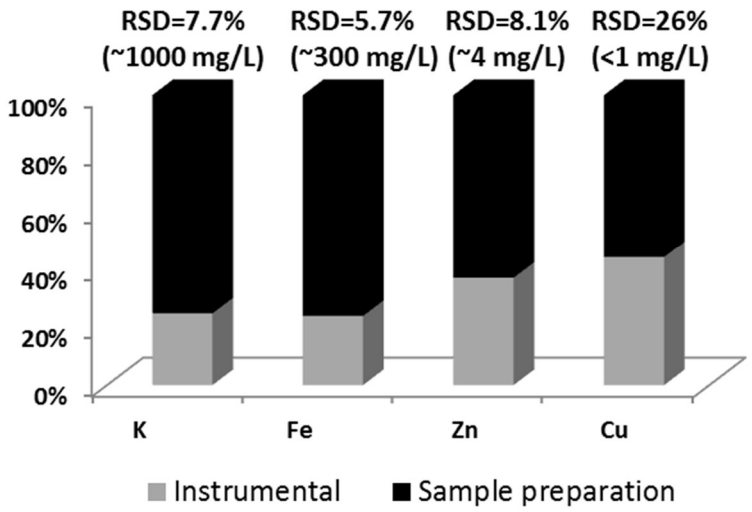

Fig. 7. Multielemental analysis of whole blood samples from controls and patients with thyroid disorders using the developed TXRF method

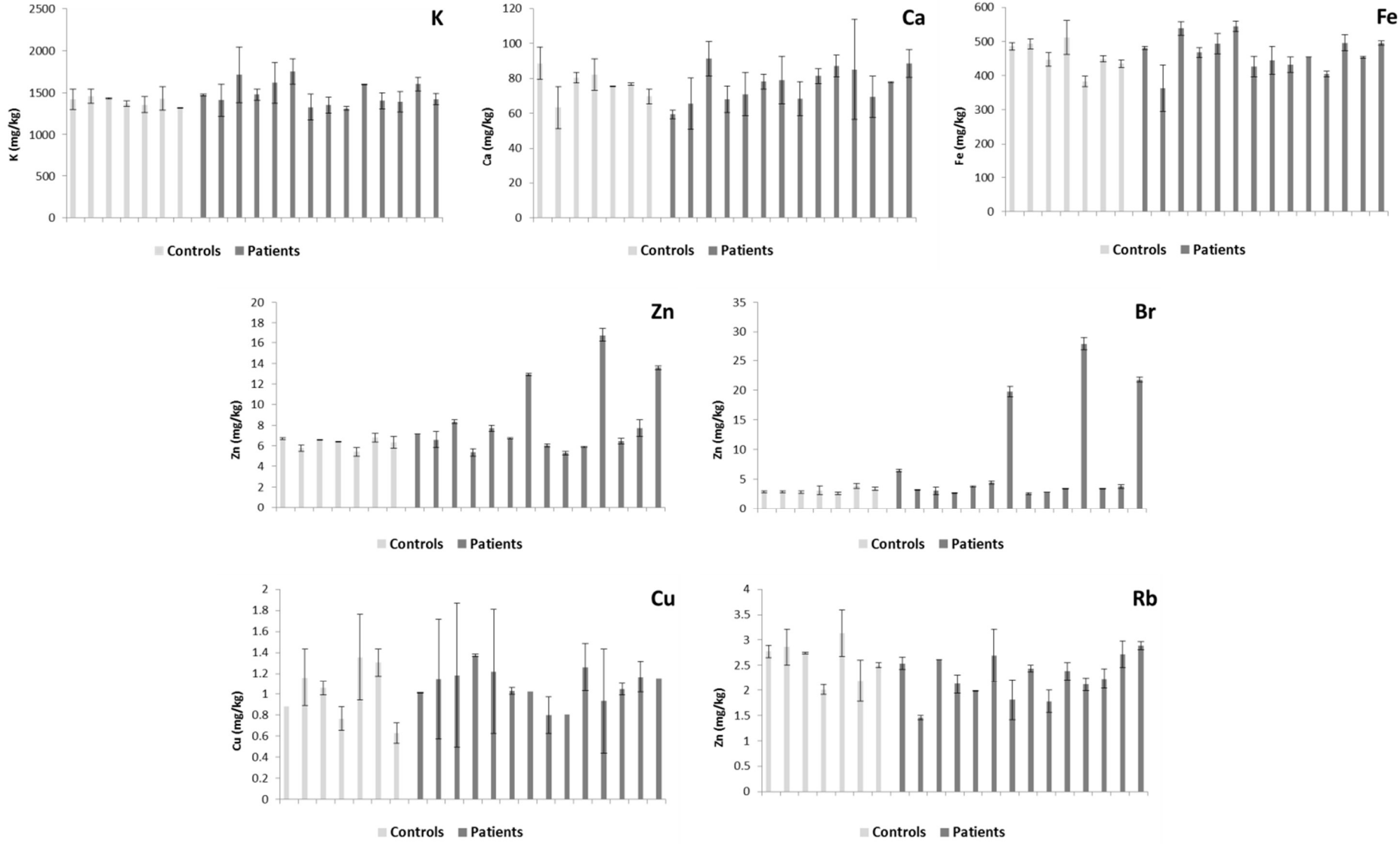

Fig. 8. Comparison of Zn concentration in whole blood samples from controls and patients with thyroid disorders using the developed TXRF method and ICP-MS analysis.