Spectrochimica Acta Part B: Atomic Spectroscopy

Energy Dispersive X-Ray Fluorescence quantitative analysis of biological samples with the external standard method

--Manuscript Draft--

Manuscript Number:	SAB_2020_119R2
Article Type:	15th Rio Symposium:Research Paper
Keywords:	EDXRF; human soft tissue; quantification; CRM
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Abstract:	Trace elements are present in minute amounts in the human body but contribute to its proper functioning, by participating in several biological processes. Imbalance of the concentrations of these elements can lead to the development of pathologies, including cancer. As such, the determination of trace element content in tumour tissues and its comparison with normal ones may be helpful for a better understanding of carcinogenesis. In this work, we address the collection and preparation of biological samples for Energy Dispersive X-Ray Fluorescence (EDXRF) analysis, and present a model for the quantification of trace elements, based on the external standard method of quantification. The model was used for the quantification of iron, copper, and zinc in a set of paired samples (normal and tumour tissues). The obtained results show the validity of the method and variations of the elemental concentrations in the diferent tissues.
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Opposed Reviewers:	
Response to Reviewers:	

Dear editor,

I am sending for your consideration the manuscript entitled "Energy Dispersive X-Ray Fluorescence quantitative analysis of biological samples with the external standard method" authored by Patrícia M. S. Carvalho, Sofia Pessanha, Jorge Machado, Ana Luísa Silva, João Veloso, Diogo Casal, Diogo Pais and José Paulo Santos. This paper is submitted as a contribution for the virtual special issue of the 15th Rio Symposium on Atomic Spectrometry.

In this paper we analyzed samples of human soft tissue belonging to four sets of paired samples (tumor tissue and surrounding normal tissue) from four cadavers identified with tumor pathology. Quantification of unknown, real, samples is not a straightforward procedure in X ray Fluorescence (XRF), mainly due to dark matrix identification and effects in intermediate and infinitely thick samples. This way, we systematically compared the concentrations obtained using compensating methods with the most popular quantification method in XRF, the Fundamental Parameters Method. The obtained results showed strengths and drawbacks in most methods and, for these samples with a dark matrix composed of H, C, N and O, the method that proved most accurate was method VI - comparison with CRM correcting the fluorescent intensities with correction with Rayleigh-Compton. We also demonstrated variations of trace element concentrations: increase of copper in lung tumor tissue and of zinc in prostate tumor tissue; decrease of iron in all the tumor tissues, of copper in tumor tissue of the prostate, ovary, and colon, and of zinc in tumor tissue of the lung, ovary and colon. We also quantified bromine, using method I - Fundamental Parameter approach despite its lower accuracy, and determined that in all tumor tissues there is a decrease in Br concentration.

We hope that you find the work interesting and valuable enough to uphold the standards of SAB.

Looking forward to your answer,

Sofia Pessanha

Dear professor Margaretha de Loos-Vollebregt,

We are very grateful to the reviewers and editorial office for their valuable corrections to our manuscript and we have rewritten the manuscript in accordance with them, with changes highlighted in yellow shade.

The point to point answers to the reviewers' queries are detailed below.

We hope that this improved version of the manuscript can be considered for publication in Spectrochimica Acta Part B.

Best regards,

On behalf of the remaining authors,

Sofia Pessanha

Editor and Reviewer comments:

Reviewer #1:

The manuscript has been modified but without improving the clarity of the scientific message and is not technical sound. Still, this reviewer has difficulty in figuring out the samples that have been used. The provides answers to comments are sometimes really strange:

"Indeed, as the reviewer highlights, fixation, alcohol, or other sample treatments can cause matrix-alterations and interfere with quantitative calculations. However, the cadavers used in this study were embalmed according to a technique developed at our institution using exclusively intra-arterial perfusion of a solution composed of aliphatic alcohols: Diethylene glycol and Monoethylene glycol (90:10), reference [10] of the manuscript. Subsequently, cadavers were kept in refrigeration cameras at 4ºC with no further exposition to other fixative or preservative chemicals."

Aliphatic alcohols are fixatives... and the technique (intra-arterial perfusion on a cadaver!) developed in your institution is really unusual: you should explain more on the characteristics of your samples if you don't want to show pictures.. and histological data.

The technique for cadaver embalming, developed by Goyri-O'Neill et al [J. Goyri-O'Neill, D. Pais, F. Freire de Andrade, P. Ribeiro, A. Belo, A. O'Neill, S. Ramos, 314 C. Neves Marques, Improvement of the embalming perfusion method: the innovation and the results by light and scanning electron microscopy., Acta Medica Portuguesa 26 (2013) 188-94], mimics "normal vessels in cardiac output

with recoil and variation of systolic and diastolic pressure, reducing the flow resistance and expanding the extent and scope of perfused tissues" Also, the used embalming solution (diethylene glycol and monoethylene glycol) ensures that the decomposition processed is halted, requiring only that the cadavers be kept at 4ºC. Results from pathological analysis of different tissues collected from the various embalmed cadavers (immediately after embalming, and more than one month after) show that the technique ensures tissue integrity.
Regarding the samples collected for this study, no histological data is available as the tissues were identified and excised by a senior surgeon and professional anatomist, based on macroscopic inspection of gross features. Nevertheless, we include pictures of the collected tissues for clarification.

Figure 1. Lungs from cadaver A. Tumour tissue was removed from the left lung, and normal tissue from the right lung.

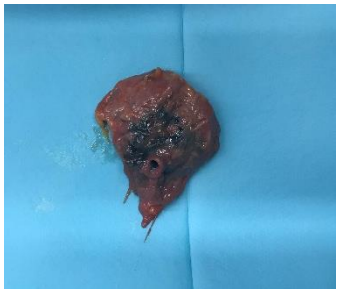


Figure 2. Prostate from cadaver B. Normal tissue was removed from the edges of the sample; the remaining tissue was altered because of the pathology.

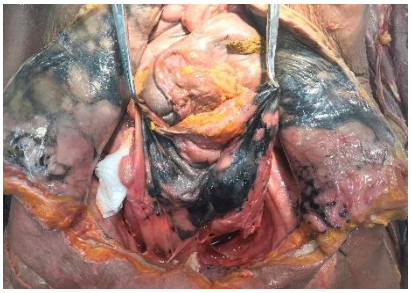


Figure 3. Abdominal cavity of cadaver C. The normal ovary (identifiable with the help of a gauze) was collected for analysis, and tumour tissue was collected from the tumour growth (blackened tissue on the image).

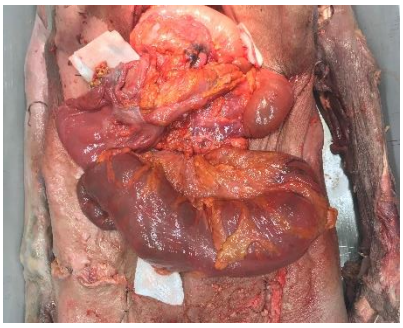


Figure 4. Abdominal cavity of cadaver D. Normal tissue was collected from the top and tumour tissue from the bottom.

For further clarification of the readers, we have included, in section 3.1, the following paragraph regarding the embalming method:

“The authors also acknowledge that any method of cadaveric preservation that involves the addition of substances to prevent cadaveric decay may alter the elemental composition of tissues.[22, 23] However, it is unlikely that these processes affect the comparison of healthy and tumoral tissues, since both types of tissues are permeated homogenously by the fixative solution used at our institution [10], Moreover, recent and independent reviews have highlighted the usefulness of the embalming technique used in the present work in preserving the normal microscopic and macroscopic features of tissues and organs over protracted periods of time [22, 23, 10].”

We have also deleted the following sentence, on section 2.1:

This way, we were able to bypass the need of _fixatives that ensure tissue preservation but may alter its characteristics.

Confusing information:" kept in refrigerated cameras at 4ºC",..... than "After collection, the samples were stored in individual plastic containers and kept at 0ºC for a few hours to avoid tissue degradation, before being prepared for lyophilization"

The cadavers used in the study are kept in refrigerated cameras at 4ºC; the tissue samples that were collected from those cadavers were kept at 0ºC from the moment of collection until preparation for XRF analysis.

"The characteristics of the Energy Dispersive X-ray Fluorescence (EDXRF) technique, such as its non-destructive character (i.e., samples are unaltered for further analysis/treatments)," but you use lyophilizated samples!!!

The sentence that is making the reviewer uncomfortable is in the introduction section, were we are discussing XRF technique in its general characteristics, not in this specific application. In general, it is widely established that XRF is considered a non-destructive technique, and it is described as such in the major handbooks of the technique:

“X-Ray fluorescence analysis (XRF) has developed into a well-established multi-elemental analysis technique with a very wide field of practical applications, especially those requiring nondestructive analytical methods.” Preface of Handbook of Practical X-Ray Fluorescence Analysis, B. Beckhoff, B. Kanngießer N. Langhoff, R.Wedell, H.Wolff (Eds.)

“Among the advantages of XRF analysis are the facts that the method is nondestructive and allows direct analysis involving little or no specimen preparation.” page 402 Handbook of X-Ray Spectrometry: Methods and Techniques René Van Grieken and Andrzej Markowicz (Eds.)

“An important advantage of the use of u-XRF in this respect is that the method is nondestructive, so that surface enrichment or other changes in the microheterogeneity of the material that may be induced by the use of other microbeam methods can be avoided.” page 692 Handbook of X-Ray Spectrometry: Methods and Techniques René Van Grieken and Andrzej Markowicz (Eds.)

“In view of the nondestructive nature of m-XRF and the minimal specimen preparation required, it is an ideal tool for the analysis of material found at crime scenes and for the investigation forgeries.” page 710 Handbook of X-Ray Spectrometry: Methods and Techniques René Van Grieken and Andrzej Markowicz (Eds.)

“The measured sample itself is not influenced by the measurement procedure i.e. the analysis within the measuring system is non-destructive and the samples can be archived for further investigations. However, the analyzed material often has to be ‘destructively’ prepared by cutting, grinding, deformation or polishing in case of the analysis of large sample areas to get homogeneous samples which then represents the material that has to be characterized or to get a sample that fits into the sample holder of the instrument.” Page 1 [M. Haschke, Laboratory Micro-X-Ray Fluorescence Spectroscopy: Instrumentation and Applications, 1ª ed. Springer International Publishing, 2014.]

The reviewer is correct by stating that the samples were lyophilized, but this is the actual process that alters the sample by dehydration, not the XRF analysis itself. The lyophilized sample can still be re-analysed by EDXRF and other techniques, such as TXRF or ICP for elemental analysis.

"are unaltered for further analysis/treatments " this is wrong! you cannot use FTIR or Raman for instance, ... and you cannot believe on molecular analyses performed after irradiation.

We understand the points made by the reviewer, but if this was the case, the whole study would have been designed differently and such analysis would have been performed before the pelletizing process.

From these and other points also noted by reviewer 3, the present reviewer continuous to recommend rejection.

Reviewer 3 was satisfied with the revised version of the paper so, the authors cannot rebuttal the reviewer’s opinion on such points.
We think that there is perhaps a misunderstanding between our definitions of “non-destructive” in analytical techniques and our is based on the handbooks of X-Ray Fluorescence.

Reviewer #4:

The work should be considered valuable. To improve its quality, authors should include two pioneering publications in the bibliography

1. Advances in X-Ray Analysis (AXA) Vol 43, pp 540-546 , APPLICATION OF MICRO X-RAY FLUORESCENCE SPECTROMETRY FOR LOCALIZED AREA ANALYSIS OF BIOLOGICAL AND ENVIRONMENTAL MATERIALS

2.A procedure using polychromatic excitation and scattered radiation for matrix correction in x-ray microfluorescence analysis , Vol 24, Issue 6, November/December 1995, pages 320-326

https://doi.org/10.1002/xrs.1300240607

In first publication, the methods used for XRF elemental analysis of biological samples (pellets) with the use of X-ray micro-beam generated by X-ray tube were described.

The authors are thankful for the suggestion of reviewer #4 and have included the suggested publications on the manuscript in the following sentence

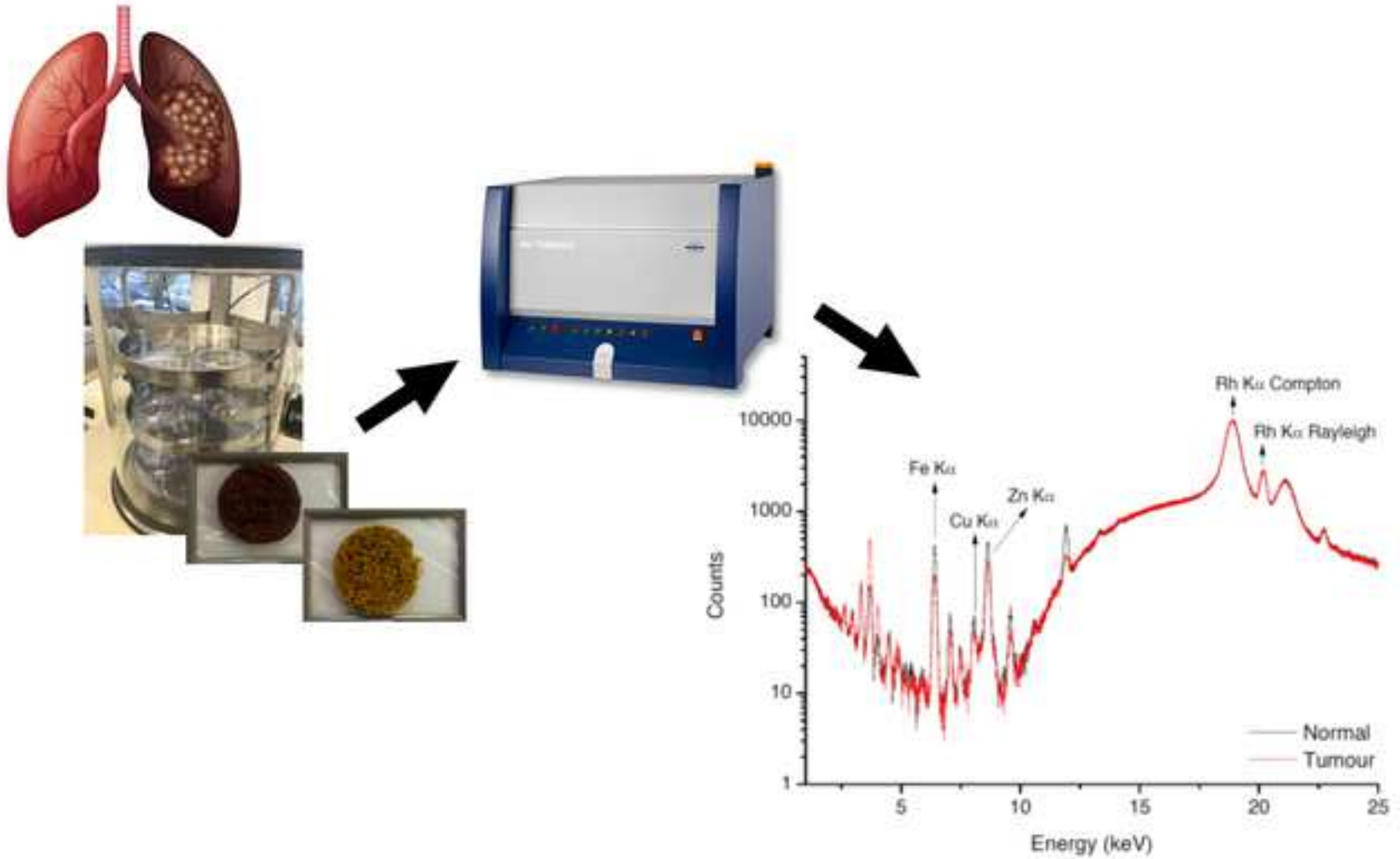
Two pioneering publications, by Lankosz et al. [19] and by Sieber et al. [20], show the suitability and advantages of using matrix correction methods relying on the scattered radiation, when analysing environmental and geological material with micro-XRF spectrometers.

HIGHLIGHTS

- Variations of elemental concentration in soft tissues related to carcinogenesis;
- Elemental concentration in soft tissues assessed by EDXRF analysis;
- Accuracy of quantification methods in EDXRF was compared and improved;
- Fe, Cu, and Zn accurately quantified in paired samples of normal and tumour tissue;

Figure

Click here to access/download;Graphical Abstract;graphical_abstract_final.png



Energy Dispersive X-Ray Fluorescence quantitative analysis of biological samples with the external standard method

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Abstract

Trace elements are present in minute amounts in the human body but contribute to its proper functioning, by participating in several biological processes. Imbalance of the concentrations of these elements can lead to the development of pathologies, including cancer. As such, the determination of trace element content in tumour tissues and its comparison with normal ones may be helpful for a better understanding of carcinogenesis.

In this work, we address the collection and preparation of biological samples for Energy Dispersive X-Ray Fluorescence (EDXRF) analysis, and present a model for the quantification of trace elements, based on the external standard method of quantification.

The model was used for the quantification of iron, copper, and zinc in a set of paired samples (normal and tumour tissues). The obtained results show the validity of the method and variations of the elemental concentrations in the different tissues.

Keywords:
Energy Dispersive X-Ray Fluorescence, quantitative analysis, external standard method, trace elements, cancer

1. Introduction

The total percentage of trace elements (Mn, Fe, Cu, Zn, Se, Co, Mo, I) in the human body does not exceed 1%. Their concentration ranges from tenths to hundreds of μg/g, but they play crucial roles in the normal functioning of the organism, by participating in many essential processes like the activation, inhibition, and promotion of enzymatic reactions. Excess or deficiency of these elements may lead to the development of pathologies, including cancer. For example, copper and zinc are cofactors of the superoxide dismutase enzyme that if not regulated causes cell damage;

iron is responsible for the formation of reactive oxygen species that trigger oxidative stress and consequently, cell damage [1]. Therefore, it is relevant to study trace element content in different tissues, both normal and tumour, in order to establish possible correlations between trace elements and factors like age, sex or cancer stage, leading to a better understanding of carcinogenesis.

The characteristics of the Energy Dispersive X-ray Fluorescence (EDXRF) technique, such as its non-destructive character (i.e., samples are unaltered for further analysis/treatments), sensitivity at ppm levels and high detection limits, make it a suitable option for these analyses. T. Magalhães et al. analysed carcinoma tissues with EDXRF and reported increased or constant levels of Fe and Cu and decreased levels of Zn [2]. Silva et al showed increased concentrations of Fe, Cu, and Zn in breast cancer tissues analysed with EDXRF [3].

EDXRF quantitative analysis of samples requires the conversion of the intensity of the measured characteristic radiation to the concentration of the analytes present in the samples. Many methods, both empirical and theoretical, have been develop for quantitative analysis, because of the complexity of the issue. Several factors must be considered, namely the sample characteristics (e.g., composition, shape, thickness) and the characteristics of the spectrometer system (e.g., geometrical setup, spectral distribution of the excitation radiation) [4, 5, 6].

The fundamental parameter (FP) method is a theoretical method, based on the equations derived by Sherman [7] and later improved by Shiraiwa and Fujino [8]. It calculates the theoretical fluorescence intensities and compares them with the measured ones, iteratively, until a match is obtained. Even though the FP method can be used to analyse a multitude of samples, using any reference material for calibration or none at all (standardless analysis), it does not consider all physical processes in the sample (e.g., tertiary fluorescence, radiation scattering) and its accuracy is reduced by the uncertainties of the atomic parameters needed for the calculation (e.g., mass attenuation coefficients, cross-sections). Moreover, when the sample is composed of undetectable low-Z elements (H, C, O, N) quantitative analysis is hampered.

When studying biological tissues, the major difficulty in the quantification process is dealing with the aforementioned undetectable low-Z elements, i.e., the dark matrix of the tissues. As such the FP method is not the best option for the quantification. To compensate for the dark matrix effects, it is best to employ methods relying on the scattering of the primary radiation, such as the external standard method [4]. It is a compensation method that consists on the determination of the elemental concentration of an unknown specimen by comparing its fluorescence intensity with one reference specimen whose elemental concentration is accurately known [9]. A set of certified

reference materials (CRM) with matrices similar to the unknown samples is analysed and for each element of interest, a calibration curve of concentration versus fluorescence intensity is built. It is essential that the chosen CRMs have a matrix similar to the unknown samples, and that the elements of interest are present in its constitution. Furthermore, the CRMs and the unknown samples must be prepared in similar ways and analysed under the same exact experimental conditions.

2. Materials and methods

2.1. Sample collection and preparation

Samples were collected from deceased patients donated through the Corpses Donation Office at the Department of Anatomy of NOVA Medical School for research and educational purposes. The subjects were embalmed using exclusively intra-arterial perfusion of a solution composed of aliphatic alcohols (diethylene glycol and monoethylene glycol), and then were kept in refrigerated cameras at 4°C, with no further exposition to other fixatives or preservative alcohols, to ensure tissue preservation over time [10].

For this observational study, four sets of paired samples (tumour and normal tissue) were collected from four deceased patients identified with tumour pathology (see table 1). The identification and excision of the tumours and corresponding normal tissues was based on macroscopic inspection of gross features; in cases of doubt, the tissues were not included in the study.

Table 1: Summary of the relevant characteristics of the selected subjects, and collected tissues for the study.

Tag	**Sex**	**Diagnosis**	**Collected sample**
A	Female	Lung neoplasia	Tumour material from the left lung, and normal tissue from the right lung (unaffected)
B	Male	Prostate neoplasia with bone metastasis	Tumour and normal material
C	Female	Ovarian carcinoma and peritoneal carcinomatosis	Tumour material and one normal ovary
D	Male	Colon neoplasia with metastasis	Tumour and normal material

After collection, the tissue samples were stored in individual plastic containers and kept at 0°C for a few hours to avoid tissue degradation, before being prepared for lyophilization.

The samples were lyophilized using a Modulyo Freeze Dryer system (Edwards, UK) operated at −60°C and 20 Pa; total lyophilization time was of two days for prostate and ovarian tissues and three days for lung and colon tissues.

The lyophilized samples were powdered in a mechanical mill and in a pestle and mortar. The obtained powder was pressed into pellets that were glued onto a Mylar film and placed on a sample holder. Due to the low amount of available tissue, only one pellet per tissue sample was made. Also, because of some characteristics of the tissues, e.g., high fat content, it was difficult to obtain a fine and homogeneous powder, compromising the homogeneity and surface of the pellets.

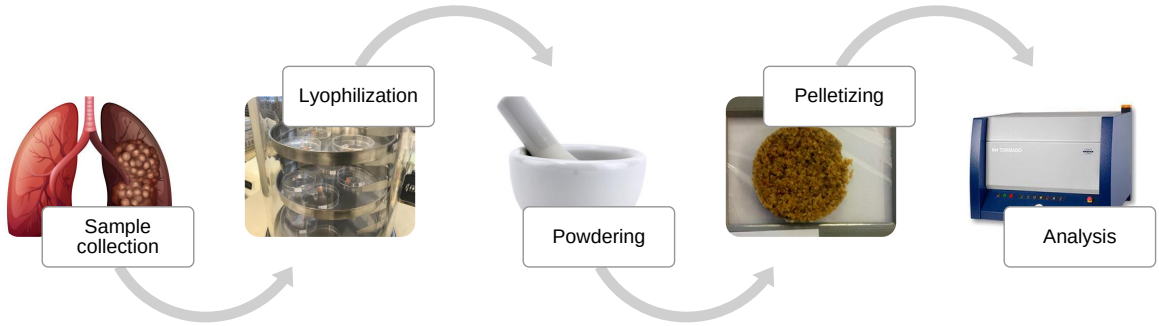


Figure 1: Steps for the collection, preparation, and analysis of samples.

2.2. Certified reference materials

The calibration curves for the quantification methods were built using six certified reference materials, available in powder form, and analysed as pellets. These CRMs were chosen accordingly to the elements present in its constitution and to the similarity of the matrix in respect to the unknown samples: NIST SRM 1577a Bovine Liver, IAEA MA-A-2 Fish Flesh Homogenate, ERM-BB186 Pig Kidney, ERM-BB184 Bovine Muscle, BCR-185R Bovine Liver, and NRC-CNRC TORT-2 Lobster Hepatopancreas Reference Material for Trace Metals. Additionally, 2 CRMs were used for the validation of the methods: NIST SRM 1566 Oyster Tissue, and NRC-CNRC DORM-4 Fish Protein certified reference material for trace metals.

Works by Hodoroaba and Rackwitz [11], and by Pessanha et al. [12] have shown that the ratio between the scattering peaks is proportional to the average atomic number, making it an attractive analytical feature. This fact is particularly interesting when dealing with samples of unknown composition and light matrices, such as human tissue. If we assume that the matrices of the CRMs and of the tissues are similar, the main elements in their compositions are the same and

the respective concentration values are close. As such, the mean-Z values are approximate and consequently, so are the Compton-to-Rayleigh ratios, as is the case.

2.3. Experimental setup

EDXRF analysis of the samples and of the CRMs was performed with a benchtop micro energy dispersive X-ray fluorescence (μ-EDXRF) system, the M4 TORNADO (Bruker, Germany). This spectrometer system allows the analysis of small and large samples of various kinds, and uses poly-capillary X-ray optics with spot sizes under 25 μm for Mo $-$ K_α radiation. The excitation of samples is achieved with a peltier-cooled X-ray tube with a rhodium target, and detection is achieved with a silicon drift detector (SDD) with a sensitive area of 30 mm^2 and energy resolution $<$ 145 eV for the K_α line of Mn (5.90 keV).

All of the pellets were analysed under the same conditions. The X-ray tube was operated at 50 kV and 400 μA and a combination of three filters (100 μm Al/50 μm Ti/25 μm Cu) was used to reduce background and improve the detection limits [13]. To ensure that the analysis was as representative as possible of the average composition of the samples and to surpass the issue of the heterogeneity of the pellets, area acquisition was performed: in each pellet three different 6×6 mm^2 areas were analysed with a 35 μm step and a time per step of 12 ms/pixel, yielding an acquisition time of 300 s.

2.4. Quantification methods

To determine the best method for the quantification of elements in biological tissues, we tested six different methods. A brief description of all the methods is presented in the following sections and summarized in table 2.

Table 2: Summary of the used quantification methods.

Method	**Description**
I	Fundamental parameter approach with the MQuant software.
II	Direct plot of fluorescence peak integrals versus certified concentrations.
III	Fluorescence peak integrals corrected with the Compton peak integral.
IV	Fluorescence peak integrals corrected with the Rayleigh peak integral.
V	Fluorescence peak integrals corrected with the Compton/Rayleigh ratio.
VI	Fluorescence peak integrals corrected with the Rayleigh/Compton ratio.

2.4.1. Fundamental parameters method

For the standardless approach, MQuant, an in-built software of the M4 TORNADO system, was used. This software allows spectra deconvolution, peak fitting and quantification based on the fundamental parameters method. For the quantification, it is possible to input the known matrix of the analysed samples.

Quantification method I was accomplished considering a dark matrix of approximately 9.7% H, 10.7% C, 73.1% O, and 2.5% N. These values were obtained by normalizing to 96% the fraction weights of the composition of soft tissue (ICRU Four-Component) available from NIST [14].

2.4.2. External standard method

Spectra analysis for the external standard method was accomplished with Root-CERN, a framework for data processing and statistical analysis [15]. Characteristic fluorescence and scattering peaks, and background were estimated by methods based on the Sensitive Non-Linear Iterative Peak (SNIP) clipping algorithm [16]. Each peak was fitted to a Gaussian function and numerically integrated.

In order to obtain accurate quantification results when applying the external standard method, we must obtain a smooth calibration curve of concentration versus intensity. This is not always the case, as the fluorescence intensity is affected by matrix effects, such as attenuation of the emitted X-rays or enhancement due to secondary excitation [6]. To overcome this drawback, and to compensate for particle size, surface texture effects and packing density, common problems with heterogeneous samples such as human tissues, the intensity of the characteristic peaks can be corrected with the intensity of the scattered radiation of the characteristic lines of the X-ray tube, Compton and Rayleigh peaks, as they are similarly affected by the elements of the matrix and by experimental variation [9, 17, 18]. Two pioneering publications, by Lankosz et al. [19] and by Sieber et al. [20], show the suitability and advantages of using matrix correction methods relying on the scattered radiation, when analysing environmental and geological materials with micro-XRF spectrometers.

Five approaches (methods II to VI) based on the external standard method were tested. Method II consisted in obtaining calibration curves by plotting the fluorescence peak integral versus the certified concentrations. For methods III to VI, the calibration curves were obtained by plotting corrected fluorescence peak integrals versus the certified concentrations, in order to understand the impact of correction with the scattering peaks from the excitation radiation coming from the X-ray tube's rhodium anode.

Methods III and IV consisted on correcting the fluorescence peak integrals with the Compton peak integral and the Rayleigh peak integral, respectively. The corrected peak integrals, $I_{\mathrm{c,Compton}}$ and $I_{\mathrm{c,Rayleigh}}$, are given by equations 1 and 2, where I_{K_α} is the fluorescence peak integral, and I_{Compton} and I_{Rayleigh} are the integrals of the Compton and Rayleigh scattering peaks.

$$I_{\mathrm{c,Compton}} = \frac{I_{\mathrm{K}_\alpha}}{I_{\mathrm{Compton}}} \tag{1}$$

$$I_{\mathrm{c,Rayleigh}} = \frac{I_{\mathrm{K}_\alpha}}{I_{\mathrm{Rayleigh}}} \tag{2}$$

For methods V and VI, the Compton-to-Rayleigh ratio ($R_{\mathrm{Compton/Rayleigh}}$) and the Rayleigh-to-Compton ratio ($R_{\mathrm{Rayleigh/Compton}}$) were calculated and used to correct the fluorescence peak integrals, following equations 3 and 4, where $I_{\mathrm{c,C/R}}$ and $I_{\mathrm{c,R/C}}$ are the corrected integrals.

$$I_{\mathrm{c,C/R}} = \frac{I_{\mathrm{K}_\alpha}}{R_{\mathrm{Compton-to-Rayleigh}}} \tag{3}$$

$$I_{\mathrm{c,R/C}} = \frac{I_{\mathrm{K}_\alpha}}{R_{\mathrm{Rayleigh-to-Compton}}} \tag{4}$$

3. Results and discussion

3.1. Validation of the method

The certified concentration values of Fe, Cu, and Zn of the validation samples, 1566 Oyster tissue and DORM-4 Fish protein, are displayed on table 3, as well as the detection limits that were calculated through equation 5, where C_{i} is the concentration of the element, N_{b} is the background count rate, and N_{p} is the count rate of the corresponding fluorescence peak [13].

$$\mathbf{DL} = 3\frac{C_{\mathrm{i}}\sqrt{N_{\mathrm{b}}}}{N_{\mathrm{p}}} \tag{5}$$

The results of the quantification of the samples with the different methods are shown on table 4.

Table 3: Certified concentration values ($\mu g.g^{-1}$) of the validation samples, SRM 1566 - oyster tissue and DORM-4 - fish protein, and calculated detection limits ($\mu g.g^{-1}$) for an acquisition time of 300 s.

	Oyster tissue	**Fish protein**	**DL**
Fe	195 ± 34	343 ± 20	2
Cu	63 ± 3.5	15.7 ± 0.46	1
Zn	852 ± 14	51.6 ± 2.8	1

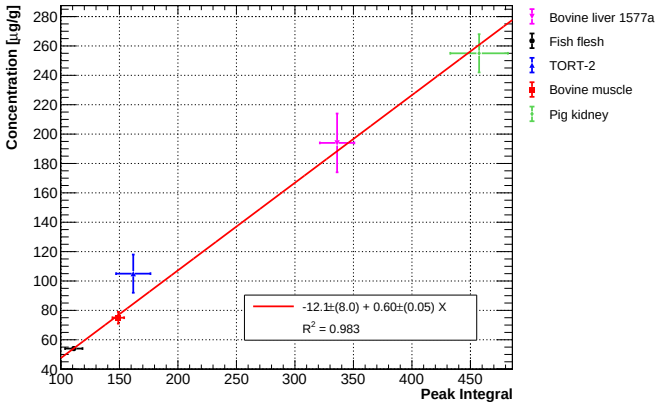
Table 4: Validation of the quantification method: calculated concentrations (μg.g^{-1}) of Fe, Cu, and Zn using the different approaches presented in section 2.4; and relative difference to the certified value (Δ %).

	Fe				**Cu**				**Zn**			
	Oyster	Δ	**Fish protein**	Δ	**Oyster**	Δ	**Fish protein**	Δ	**Oyster**	Δ	**Fish protein**	Δ
I - FP	210 ± 5	8	320 ± 25	7	50 ± 5	21	16 ± 1	2	480 ± 20	44	50 ± 5	3
II - Direct plot	200 ± 20	3	350 ± 45	2	65 ± 5	3	15 ± 5	5	930 ± 80	9	60 ± 10	16
III - Compton peak	200 ± 20	3	700 ± 100	104	60 ± 10	5	40 ± 5	155	990 ± 90	16	145 ± 15	181
IV - Rayleigh peak	190 ± 20	3	600 ± 100	75	60 ± 10	5	35 ± 5	123	960 ± 120	13	135 ± 25	162
V - C/R ratio	220 ± 30	13	400 ± 60	17	70 ± 10	11	15 ± 5	5	990 ± 100	16	65 ± 15	26
VI - R/C peak	190 ± 20	3	310 ± 50	10	60 ± 10	5	15 ± 5	5	900 ± 95	6	50 ± 10	3

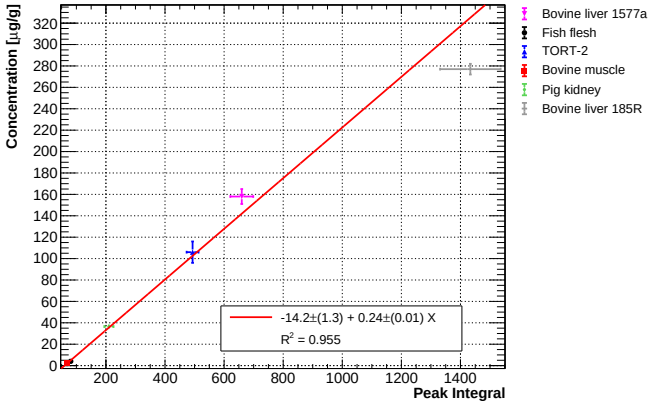
From the obtained results, it is concluded that methods III and IV, correction with the Compton peak and with the Rayleigh peak, respectively, yield the less accurate results: concentrations of Fe, Cu, and Zn in the fish protein CRM deviate from the certified value by values close to or greater than 100%. These are followed by method V, correction with the Compton-Rayleigh ratio, with some of the calculated concentrations values deviating from the certified concentrations by more than 10%. The most suitable quantification method is method VI, correction with the Rayleigh-Compton ratio, as it yields concentration values with deviations of 3%, 5%, and 6% for iron, copper, and zinc, respectively, in the oyster CRM; for the fish protein CRM, the calculated concentrations of Fe, Cu, and Zn deviate from the certified by 10%, 5%, and 3%. However, the results from method II, direct plot of the fluorescence peak integral versus concentration, are also satisfactory, since its results, for both validation CRMs, are deviated from the certified values by 5% or less for iron and copper; for zinc, the calculated values deviate from the certified ones by 9% (oyster CRM) and by 16% (fish protein CRM).

Considering the obtained results, method VI, correction with Rayleigh-Compton ratio, was selected for the quantification of Fe, Cu, and Zn in biological samples. The calibration curves are shown in figure 2.

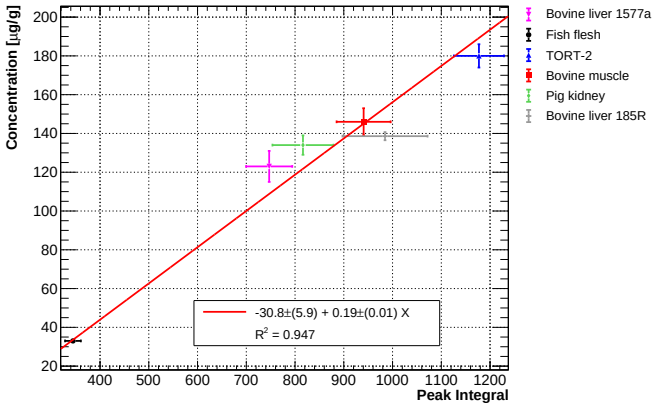
There are many difficulties when quantifying unknown samples, such as the choice of the appropriate quantitative method, and the choice of the certified reference materials to be used as external standards. Here, we concluded that the methods based on correcting the fluorescence peak integrals with the scattered radiation improve the accuracy of the quantification, when compared to the fundamental parameter approach. However, the external standard method limits the elements that we can quantify, because of the need of CRMs that must have a matrix similar to the unknown samples and the elements that we want to quantify present in their composition. Sometimes not many materials meet our requirements, resulting in calibration curves with a limited concentration range that increase uncertainties in the quantification. In fact, this is main reason to only determine Fe, Cu, and Zn concentrations in this study: the available CRMs were not enough to build calibration curves for other elements, such as bromine, an element whose concentration is known to vary from normal to tumour tissues [21]. The fundamental parameter method may be used to overcome this limitation, as it is a standardless approach that does not require the use of CRMs. Even though the results may present considerable deviations from the real values because of the dark matrix effects, it is possible to determine the order of magnitude of the concentration of all the elements present in the sample and detected by the used spectrometer, and to gauge comparisons



(a) Fe calibration curve.



(b) Cu calibration curve.



(c) Zn calibration curve.

Figure 2: Calibration curves for the quantification of iron, copper, and zinc, obtained by plotting the certified concentrations versus the integrals of the fluorescence peaks, corrected with the Rayleigh/Compton ratio. The y error bars correspond to the uncertainties of the certified concentration values of each CRM, and the x error bars, to the quadratic combination of the uncertainty from the calculation of the corrected peak integral and of the standard deviation of the measurements. The biggest contribution to the x error bars is the standard deviation of the measurements, which may be a direct result of the heterogeneity of the pellets.

between tumour and normal tissues.

The authors also acknowledge that any method of cadaveric preservation that involves the addition of substances to prevent decay may alter the elemental composition of tissues [22, 23]. However, it is unlikely that these processes affect the comparison between normal and tumour tissues, since both types of tissues are permeated homogeneously by the fixative solution used at our institution [10]. Moreover, recent and independent reviews have highlighted the usefulness of the embalming technique used in the present work in preserving the normal microscopic and macroscopic features of tissues and organs over protracted periods of time [22, 23, 10].

3.2. Quantification of the biological tissues

Figure 3 shows two fluorescence spectra resulting from the analysis of normal ovarian tissue, and from the analysis of tumour tissue. A careful observation of the spectra allows identifying several characteristic fluorescence peaks, such as the Fe K_α, the Cu K_α, and the Zn K_α peaks, and peaks resulting from the scattering (Compton and Rayleigh) of the primary radiation from the X-ray tube anode. Furthermore, peaks from other elements present in the samples are also identifiable. These elements were not quantified because of limitations of the external standard method, as mentioned on section 3.1.

Quantitative analysis, performed by representing the calculated concentrations in histograms depicting the concentrations of Fe, Cu, and Zn in the analysed tissues (figure 4), highlights possible variations of trace element concentration between normal and tumour tissues.

Regarding the variation of the mean concentration values, in tumour lung tissue there is a 4.5% decrease in iron and a 39% decrease in zinc compared to normal tissue, and there is an increase of 137% in copper concentration. In prostate tumour tissue, iron and copper concentrations decrease 26% and 40%, respectively, compared to normal tissue; zinc concentration increases by 46%. In ovarian tumour tissue, there is a decrease of 46%, 49%, and 40% in iron, copper, and zinc concentration, respectively, compared to normal tissue. Finally, in colon tumour tissues, copper and zinc concentrations decrease 8% and 26%, respectively, compared to normal tissue, whereas iron concentration increases 48%.

Theodorakou and Farquharson [24] reviewed X-ray techniques used for the analysis of human tissues and reported XRF studies where higher concentrations of Zn in colon cancer tissues were determined, while no significant variations of Fe and Cu concentrations between tumour and normal tissues were determined. Our study is in agreement with the results for the variation of Cu between

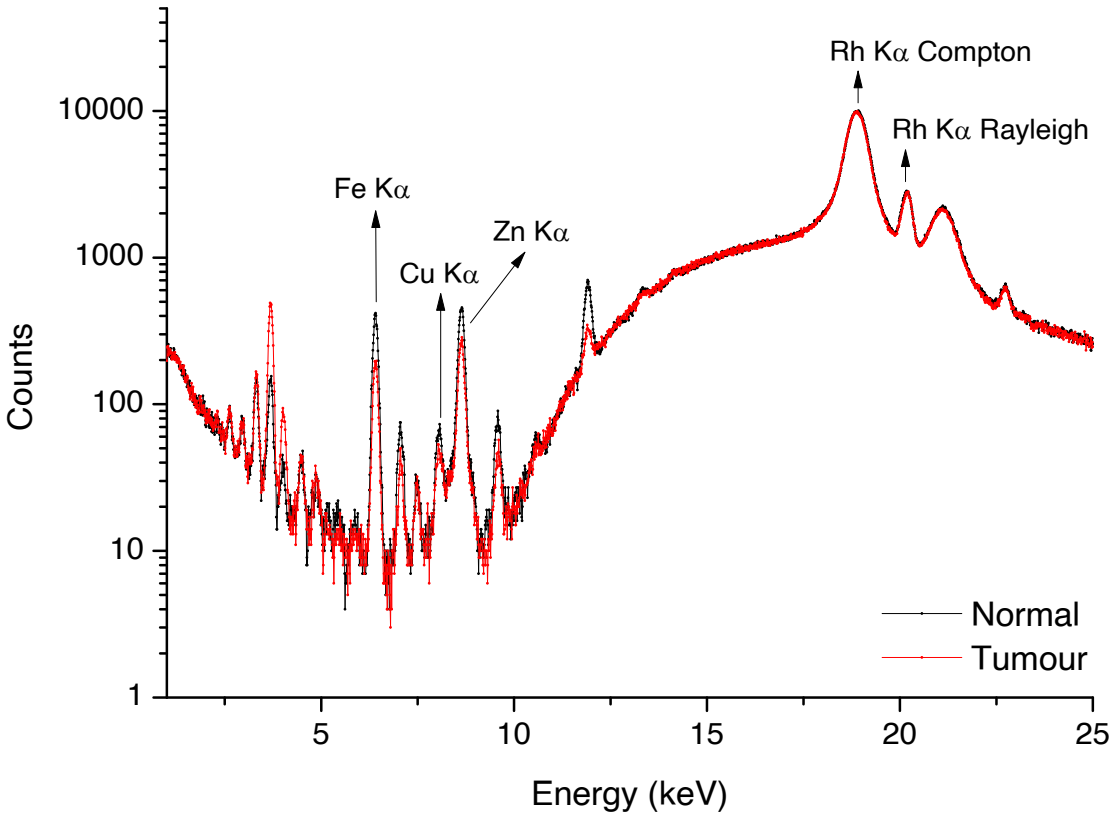
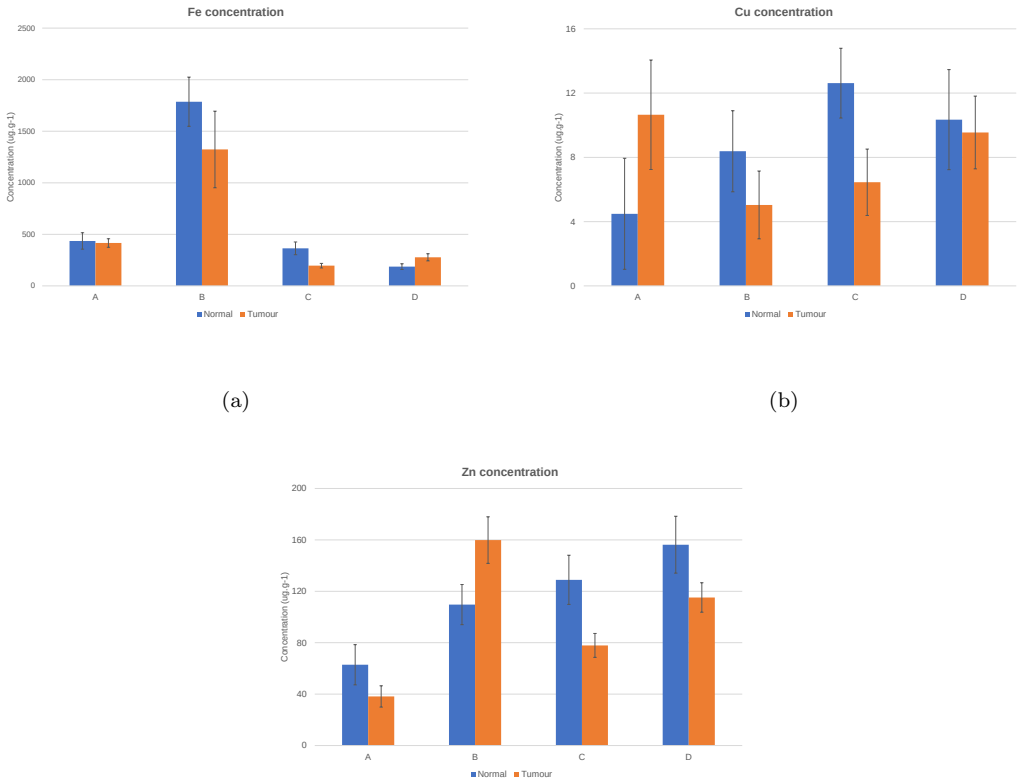


Figure 3: Original fluorescence spectra of the ovarian tissue samples (C): normal tissue (black), and tumour tissue (red). Several characteristic peaks can be identified in both spectra (e.g., Fe K_α, Cu K_α, and Zn K_α), as well as Compton and Rayleigh scattering peaks from the X-ray tube radiation.

normal and tumour tissues. However, in tumour tissues we have determined increased concentration of Fe and decreased concentration of Zn, compared to normal tissues. The calculated concentration of Fe in normal colon tissues is within the concentration range noted by Carvalho et al, $105 - 195$ μg/g [21].

In the case of lung tissues, a study by Kubala-Kukús et al [25] reported decreased concentrations of Zn and Cu in tumour tissues compared to normal tissues, and increased Fe content in tumour tissues. The same was reported by Majewska et al [26]. We have found no variation of Fe content between normal and tumour tissues of the lung, but the calculated Fe concentration for normal tissue is within the reported value range, $64 - 890$ μg/g, as well as the concentration of Cu in the same tissue, $0.99 - 3.1$ μg/g. The same applies to the concentration of Fe and Cu in tumour lung tissues, whose ranges are, respectively, $199 - 416$ μg/g and $2 - 6.10$ μg/g, as reported by Carvalho



(a) (b)

(c)

Figure 4: Histograms depicting the variation of the concentration of iron (4a), copper (4b), and zinc (4c) in the paired samples of normal (blue bars) and tumour (orange bars) tissues. The error bars are due to the standard deviation of the measurements, and to the uncertainties associated with the fit parameters of each calibration curve.

et al [21].

Regarding prostate tumour tissues, our results are in compliance with the increased Zn concentration in tumour tissues highlighted by Yaman [27]. Kwiatek et al [28] analysed prostate tissues by SRIXE and found a decrease of iron and an increase of copper and zinc in adenocarcinomas with Gleason score 3; in adenocarcinomas with Gleason score 5, an increase of Fe and Zn was determined, and the variation of Cu was not significant. In our study, we determined a decreased of the concentration of Fe and Cu in tumour tissues. Also, the value for the concentration of Zn in tumour prostate tissue is within the range reported by Carvalho et al, $149.2 - 176$ μg/g [21].

Al-Ebraheem et al [29] studied ovarian tissues and determined increased levels of Cu and Zn in ovarian tumour tissues compared to normal adjacent tissue, for Fe, no significant variations were found. The findings are contrary to ours - decreased concentration of Fe, Cu, and Zn in tumour tissues compared to normal tissue. On the other hand, Yaman et al [30] used atomic absorption

spectrometry to analyse cancerous and noncancerous human endometrial and ovary tissues. They have found higher levels of Cu in tumour ovarian tissues, but no significant differences between the levels of Fe and Zn in cancerous and noncancerous ovarian tissues.

Nevertheless, we must consider that the existing studies show large discrepancies between them. These might be due to many factors, such as the various used techniques, different experimental conditions, and even biological aspects related to the human organism (e.g., diet, exposure to potentially toxic substances). Besides, all studies have a common setback: the reduced number of available samples, preventing a thorough analysis and inference of conclusions regarding the role of trace elements in cancer.

As mentioned in section 3.1, the used method limits the elements that can be quantified, as is the case of bromine. Existing studies have already reported the variation of its concentration between normal and tumour tissues of various organs. So, we decided to quantify bromine in the analysed paired samples using the fundamental parameter approach (method I). This way we can assess the order of magnitude of bromine concentration in the various tissues and the concentration variation. The results are shown in figure 5.

In all the analysed tissues, bromine concentration is of a few tenths μg/g. Also, the mean concentration values decrease in all tumour tissues: in lung by 33%, in prostate by 30%, in ovary by 74%, and in colon by 42%. These results are merely indicative and may have significant deviations from the actual concentration values but they point us to the need of improving the external standard method, so that more elements can be accurately quantified.

In order to gain statistical relevance and draw conclusions regarding the role of trace elements in carcinogenesis, a larger set of samples is needed. It would then be possible to understand if the found concentration variations can be generalized to a population, and to correlate trace element concentrations with factors like age, sex, and stage of disease.

4. Conclusions

With this study we compared six different approaches for the quantification of trace elements in paired samples of tumour and normal human tissues, based on the fundamental parameter method and on the external standard method. The choice of a quantification method depends on many factors, including the type of samples one wishes to quantify. For our purpose, we found that the external standard method with correction of the characteristic peak integrals yielded the best results. This comes from the correction of the characteristic peak integrals with the scattered

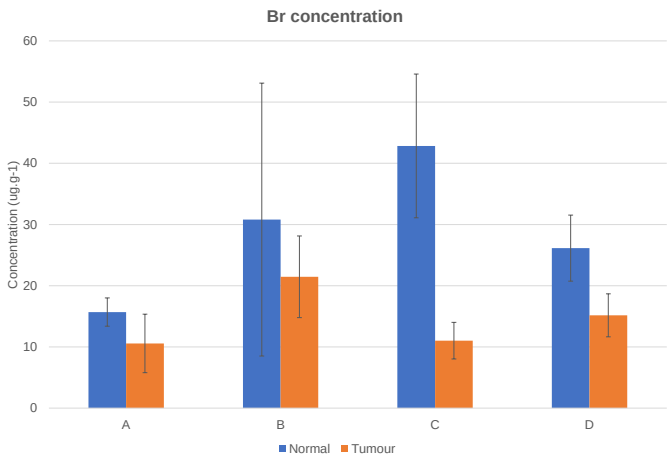


Figure 5: Histogram depicting the variation of the concentration of bromine (Br) in the paired samples. The concentration values were calculated with method I and the error bars are due to the standard deviation of the measurements that is greatly affected by the heterogeneity of some pellets.

radiation, that compensates for matrix effects, sample heterogeneity, and experimental variations. However, the method prevents the quantification of all the detected elements because of the CRMs' requirements. In order to quantify the majority of the elements, the fundamental parameter method can be used, but the accuracy of the results is compromised.

Even though the external standard method with compensation with the scattered radiation yielded the most accurate results, it must be improved. As of now, limitations concerning the available CRMs stop us from quantifying all the elements present in an unknown sample. Furthermore, the CRMs must cover a wide concentration range so that uncertainties related to the extrapolation of the calibration curve can be reduced.

We quantified the concentrations of iron, copper, and zinc in normal and tumour tissues of the lung, prostate, ovary, and colon. We also demonstrated variations of trace element concentrations: increase of copper in lung tumour tissue and of zinc in prostate tumour tissue; decrease of iron in all the tumour tissues, of copper in tumour tissue of the prostate, ovary, and colon, and of zinc in tumour tissue of the lung, ovary and colon. We also quantified bromine, using the fundamental parameter approach in spite of its low accuracy, and determined that in all tumour tissues there is a decrease in its concentration.

Our results may indicate a role of trace elements in carcinogenesis but a more statistically relevant analysis is necessary. This drawback could be solved with a larger set of samples, that

would allow us to generalize findings to a population and to correlate trace element content with factors like age, sex, and even with cancer prevalence trends in certain geographical areas.

Acknowledgements

This work was partially supported by project PTDC/FIS-AQM/32536/2017 through FEDER and FCT (Portugal) programs, and by the research centre grant UID/FIS/04559/2019 to LIBPhys-UNL from the FCT/MCTES/PIDDAC, Portugal. Patrícia M. S. Carvalho acknowledges the support of FCT (Portugal) under contract No. PD/BD/128324/2017.

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Fig.1

Fig.2 a

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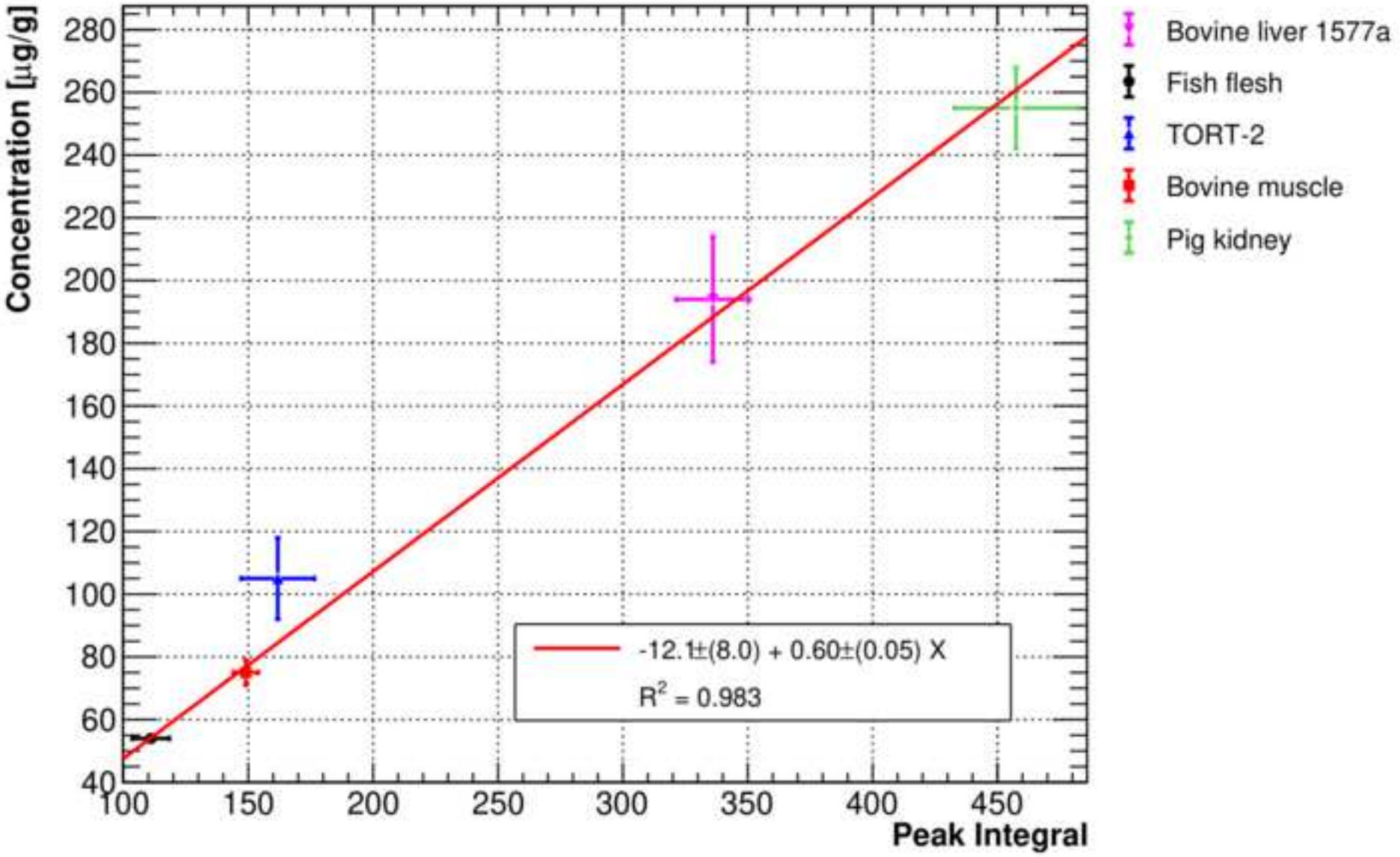

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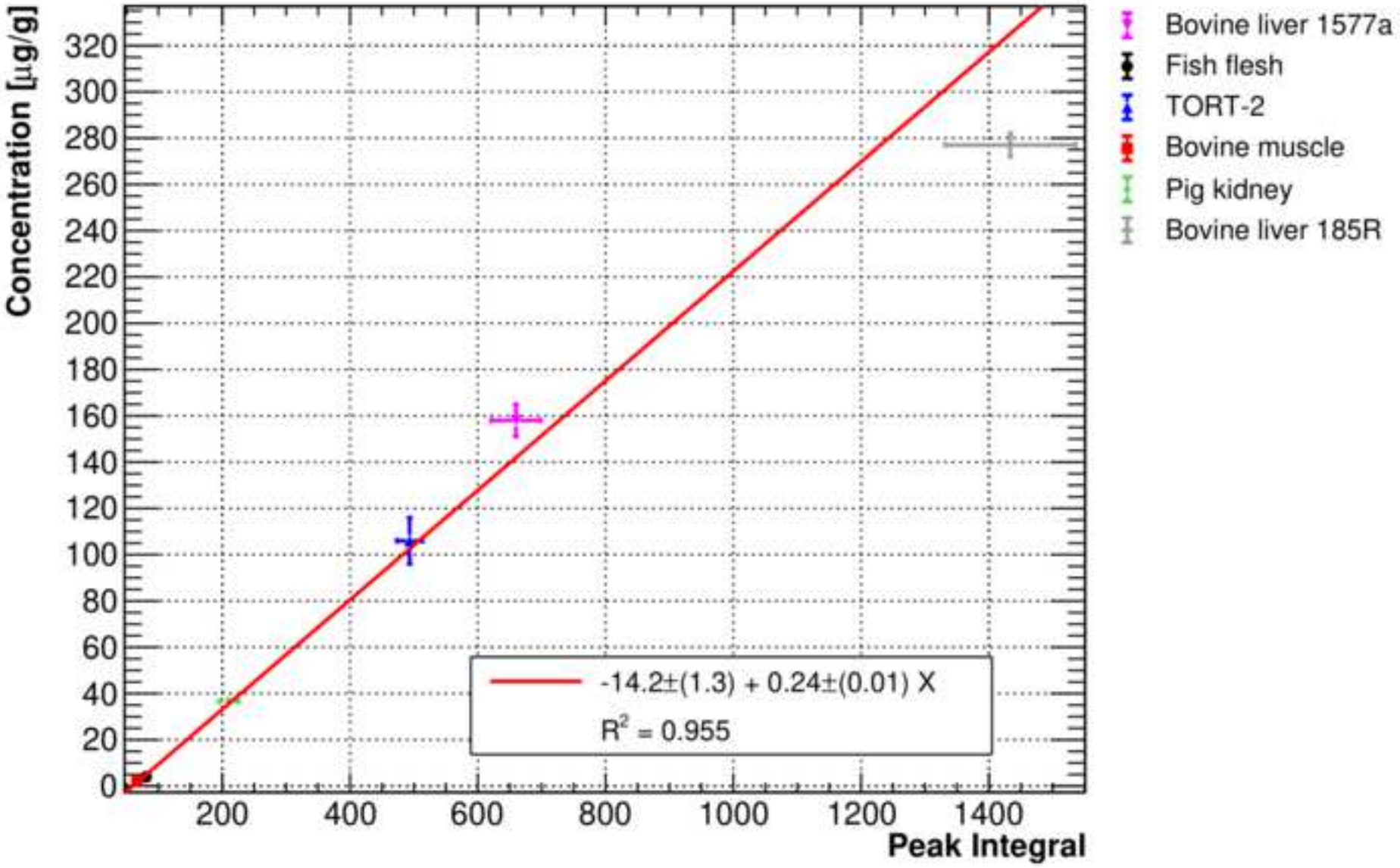

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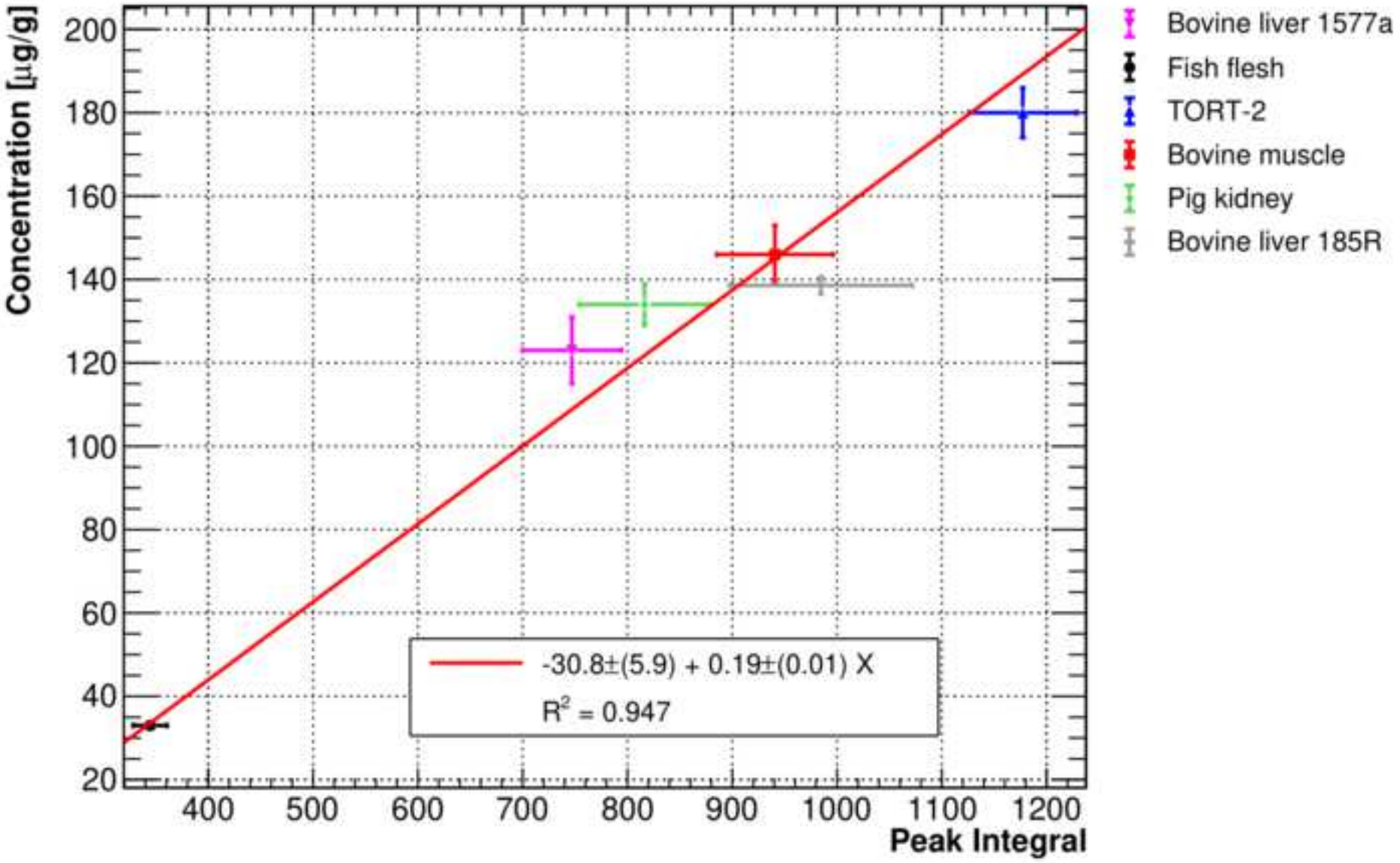

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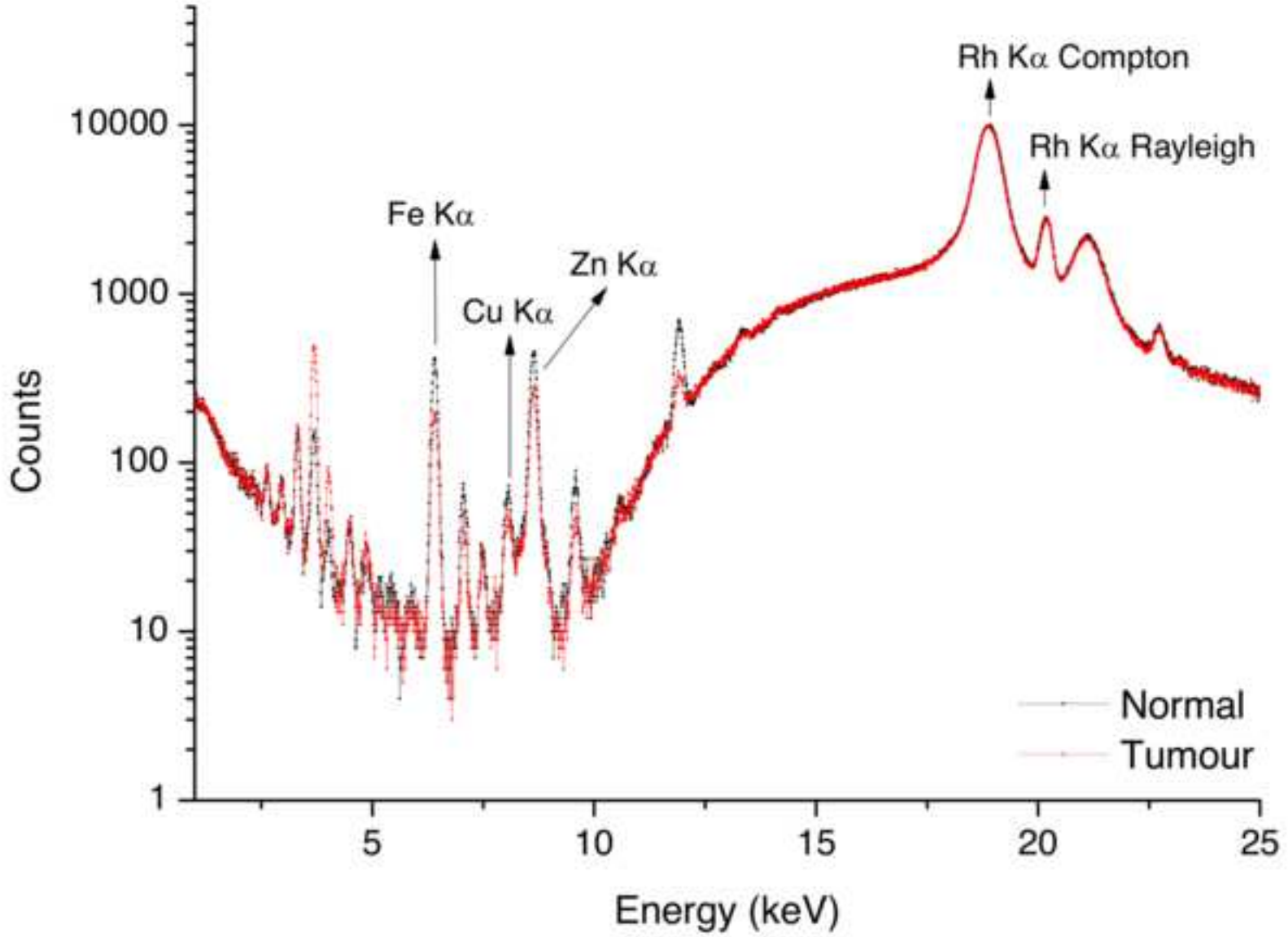

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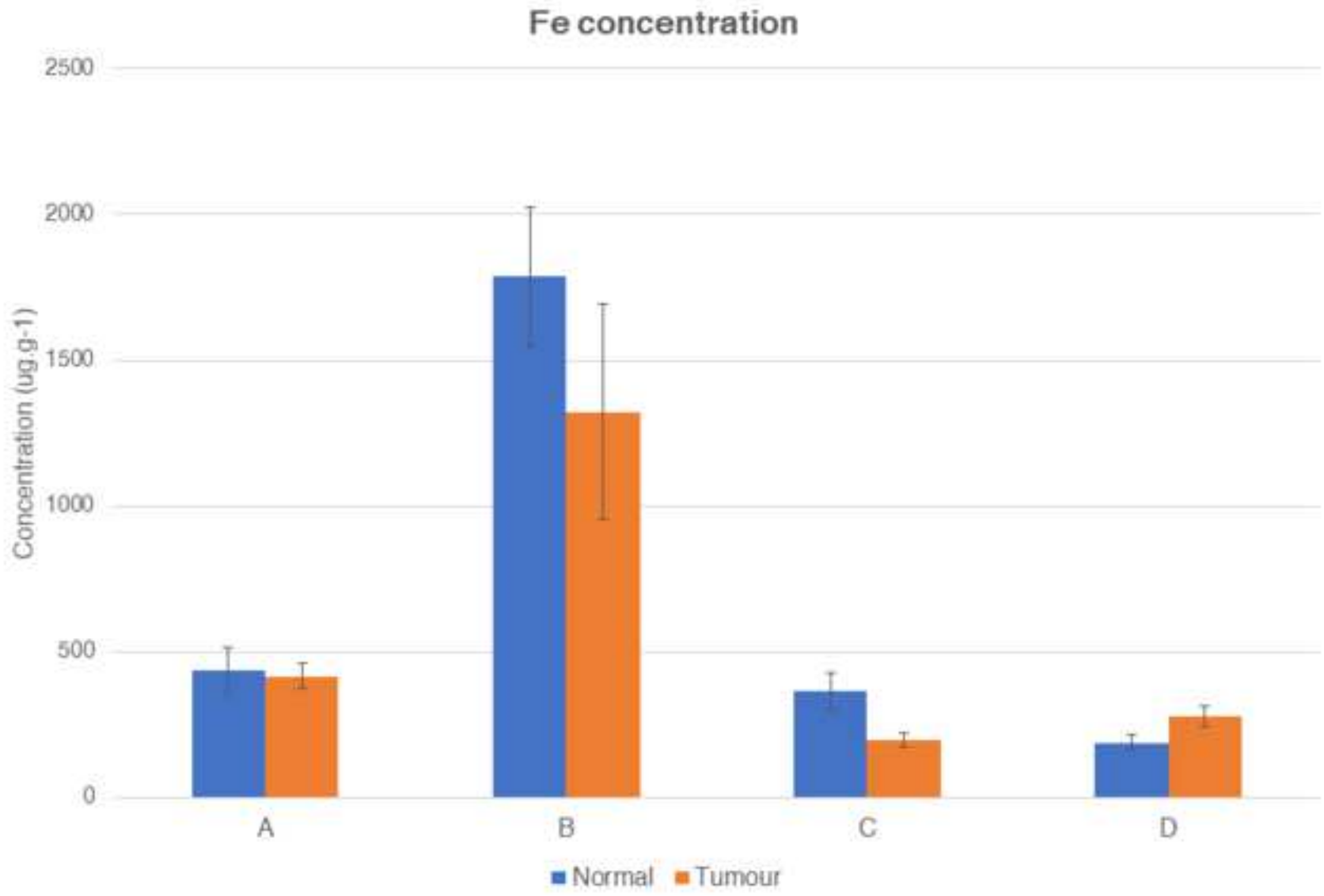

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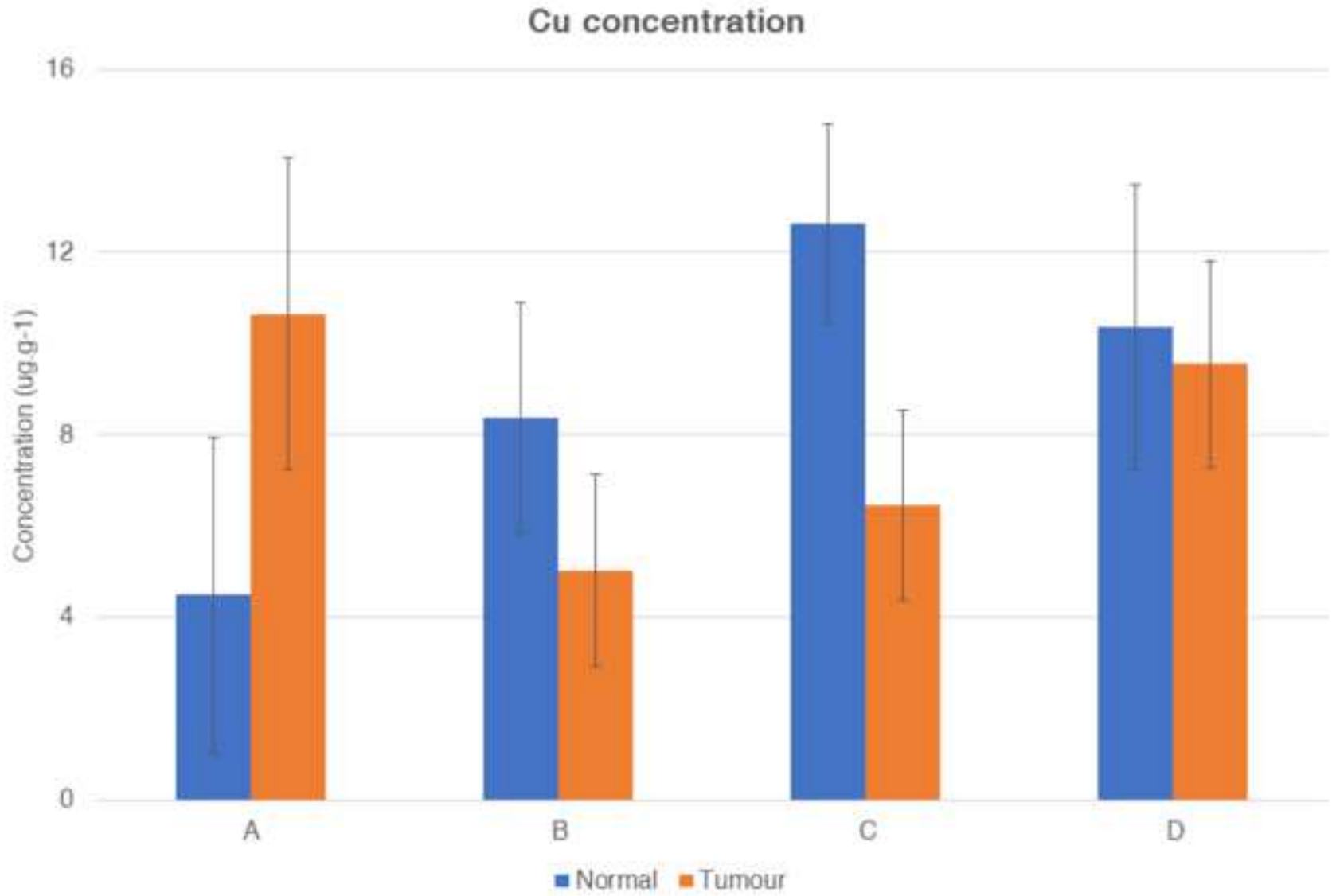

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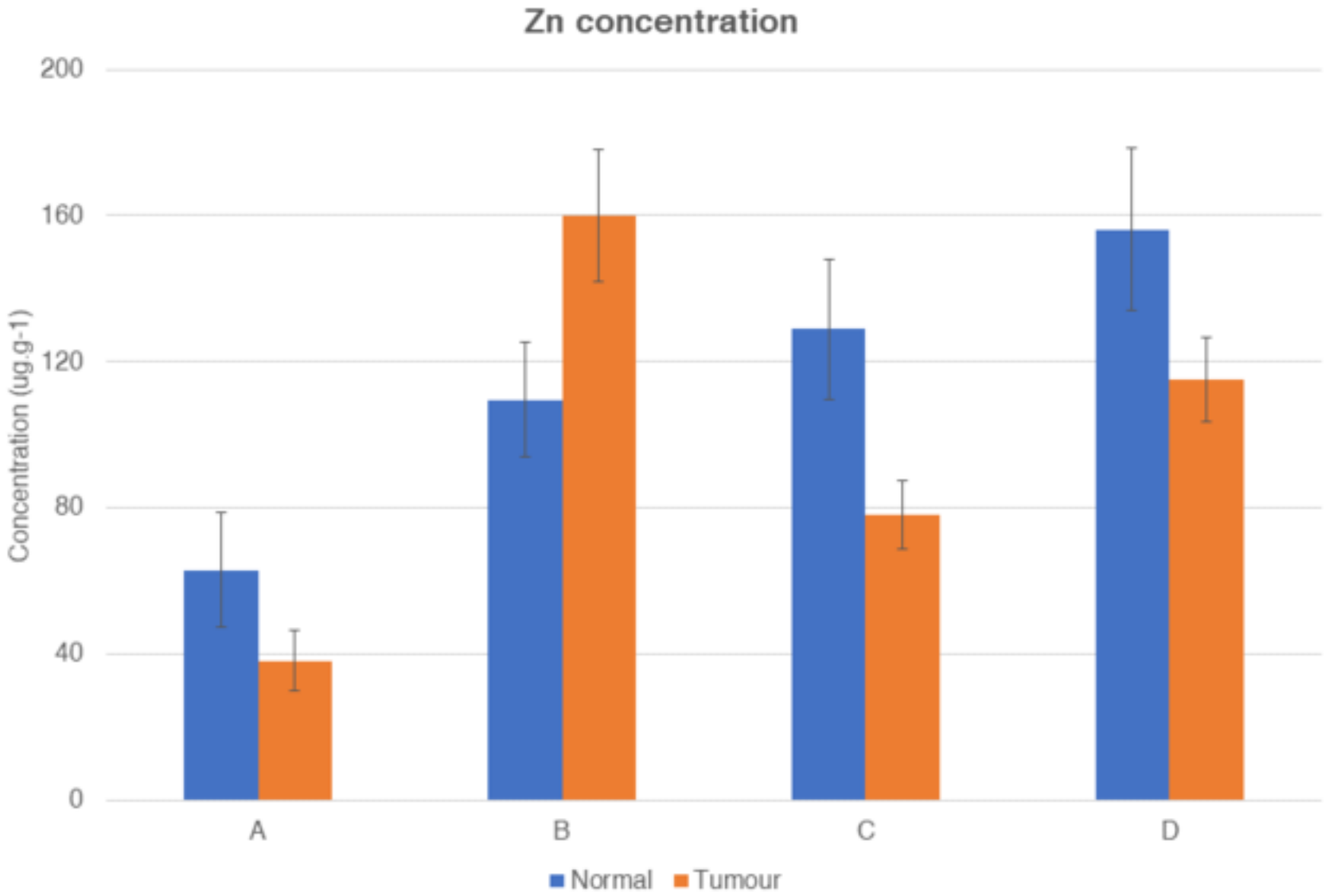
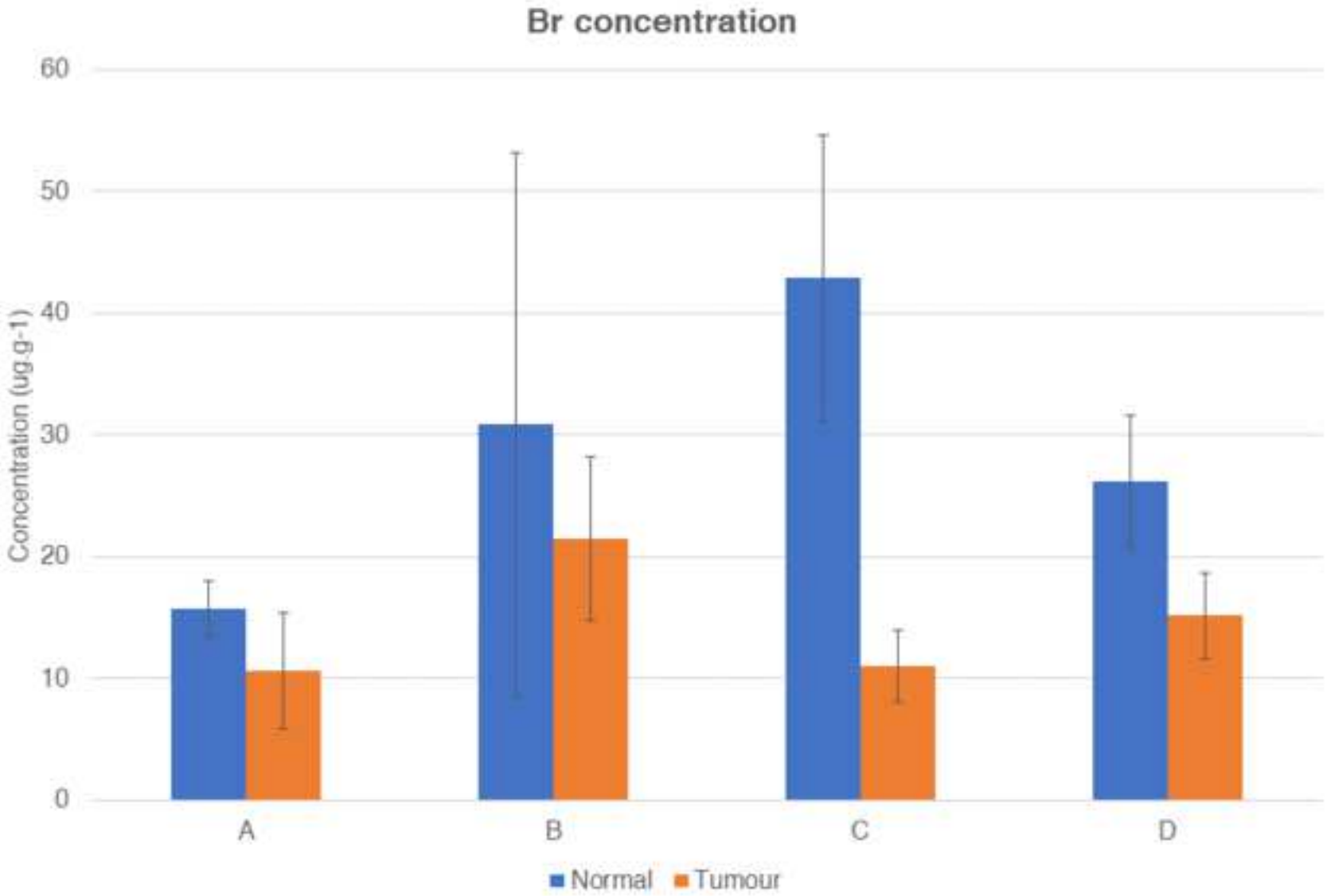


Fig.5



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author Statement

Patrícia M. S. Carvalho – Formal analysis, Writing - original draft; Writing - review & editing
Sofia Pessanha - Conceptualization, Writing - review & editing
Jorge Machado - Formal analysis, Writing - review & editing
Ana Luísa Silva - Formal analysis, Writing - original draft
João Veloso - Writing - original draft
Diogo Casal - Methodology, Writing - review & editing
Diogo Pais - Supervision
José Paulo Santos – Conceptualization, Supervision