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Bachelor of Science in Biomedical Engineering

Study of trace elements concentration in cancerous and healthy Bladder, Colon and Lung tissues

Dissertation for the degree of Master of Science in Biomedical Engineering

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UNIVERSIDADE NOVA DE LISBOA

November 2016

To all my family members, the ones I was born with and the ones I chose.

ACKNOWLEDGMENTS

This project started over 6 months ago and since then many hours have been spent planning all work's steps and general course, investigating previous bibliography, learning laboratory procedures, performing several measurements, analyzing all data and writing the dissertation, in order to finalize it as expected. Obviously this journey was not made on my own, as many other people and institutions helped me fulfill my objectives, either in professional or personal way.

First I would like to thank my supervisor Professor Mauro Guerra for all the patience and hours of sleep that I took from him. Mauro was always present during the entire work, always available to help in any imaginable way, from theoretical approaches to the smallest detail, certifying that my work would follow the right path.

I also want to thank my co-supervisor Professor José Paulo Santos for his extremely rigorous and professional advices that in times of need were a huge help.

I want to thank laboratory supervisor Professor Maria Luísa Carvalho for the great conditions that transformed the laboratory in a healthy work environment. Also for all the available hardware, software and instruments inside the laboratory, which made possible to conduct all the work without leaving the room. And mainly for being a generous person always ready to give a helping hand on anything.

I have to thank Professor João O'Neill in the name of NOVA Medical School for the collaboration and for providing the cancerous and healthy tissues. Although the number of available samples was not the expected, I understand the difficulties in the process.

Regarding institutions, I definitely have to thank Universidade Nova de Lisboa and particularly Faculdade de Ciências e Tecnologia for providing the perfect environment for me to grow not only as a working individual but also as a person with a great set of soft skills that will help me triumph in the professional world. And also a word for the Department of Physics, my own "house", for the complex laboratories, the large auditoriums and the good team of dedicated professors.

Also I would like to acknowledge Professor Sofia Pessanha for her gracious smile that would bright up the workspace and my dear laboratory colleagues with whom I spent many hours, in a nice working atmosphere.

I have to thank a wise friend, Aquiles Gomes, my unofficial supervisor, for his infinite advices on every step of my dissertation, since the beginning till the end. His knowledge in this matter was a great help during the entire work.

At a personal level, firstly and above all, I have to thank my two dear parents that are also two of my best friends, for their never ending support, love, understanding and council, pushing me in the right direction,

since the beginning of times. Without their teachings and perception of the world, I would not be the person that I am, and for that, I am eternally grateful. I consider myself a very lucky person because at my age of 23 years old, I have the privilege of living with my 4 grandparents. They are, at their own way, 4 wells of knowledge and I have the honor of learning from all of them on how to be a better person. My eldest grandfather once told me that one of his great desires was to see his grandson become an engineer so ending this important chapter of my life has a transcendent meaning to me, because there is no greater emotion than the one felt by seeing pride in the eyes of the people we most care about.

And last but not least, an enormous thank you for all my unique, crazy and dearest friends that accompanied me in my everyday life, sharing a bit of their selves and listening to my wanderings. A special thankful word to my Biomedical mates Bernardo, Eduardo, João, Marcos, Miguel and Pedro, who helped me throughout all this college adventure, without whom the experience would not have been the same. Another huge hug and gratitude to my “not normal” friends, which walked side-by-side with me in an unimaginable musical lifetime. Many other friends, which know who they are, deserve an acknowledgment by being there whenever I needed them, asking or not, supporting me through good and bad moments, with whom I have the honor of sharing my personality. That is the essence of working and interacting with others, giving what you have and receiving what you are offered, contributing to others’ happiness and becoming a fulfilled citizen of the world.

ABSTRACT

Cancer is one of the leading causes of death in developed and developing countries, where the incidence continues to increase each year. Annually about 8 million people die due to this disease. Hence, the development of efficient treatments, that fall short nowadays, is highly necessary. Therefore it is imperative to fully understand the biological and physiological processes intrinsic to the carcinogenesis. Trace elements may have an important role in this process, being responsible for healthy cellular growth mechanisms. These elements are responsible for a variety of metabolic processes, knowing, for instance, that they are components of different enzymes and catalysts of chemical interactions in living cells, among many others. At the biological level, they are also responsible for the activation or inhibition of enzymatic reactions and changes in the permeability of cell membranes. In addition, they appear in different concentrations in healthy and cancerous tissues due to biological changes induced by the disease.

In order to measure the elements' concentration and distribution it is necessary to resort to a specific technique, X-ray Fluorescence Spectrometry, a multi elemental analysis that relates X-ray Emission Spectra to specific elements and its concentration. The spectrometer used was M4 Tornado, from Bruker, an instrument that allies non-destructive techniques with high lateral resolution, able to conduct a quantitative and qualitative analysis even when the concentrations are at the µg/g range. The main objective is to correlate the trace element concentrations variation between cancerous and healthy human tissues in order to both evaluate the influence of these variations in cancer development and these elements' expression due to carcinogenesis process.

RESUMO

O cancro é uma das maiores causas de morte em países desenvolvidos e em países em desenvolvimento, onde a incidência aumenta ano após ano. Por ano, morrem cerca de 8 milhões de pessoas devido a esta doença. Deste modo, o aparecimento de tratamentos eficazes, que escasseiam na actualidade, é urgentemente necessário. Para isso, é preciso compreender totalmente os processos biológicos e fisiológicos intrínsecos ao processo da carcinogénese. Elementos traço aparentam ter um papel importante neste processo, visto que são responsáveis pelos mecanismos de crescimento celular controlado. Estes elementos são responsáveis por diversos processos metabólicos, podendo ainda ser componentes de enzimas e catalisadores de interacções químicas em células. Ao nível biológico, são responsáveis pela activação e inibição de reacções enzimáticas e pela alteração da permeabilidade das membranas celulares. Por fim, estes elementos apresentam concentrações diferentes em tecidos cancerígenos e em tecidos saudáveis devido às alterações biológicas induzidas pelo cancro.

De modo a possibilitar a medição da concentração elementar, é necessário recorrer a uma técnica específica, a Espectrometria de Fluorescência de Raios-X, que consiste numa técnica de análise multi elementar que relaciona o espectro de emissão de raios-x com o elemento correspondente e a sua concentração. O espectrómetro utilizado foi o M4 TORNADO da Bruker, que alia a sua altíssima resolução lateral à técnica não destrutiva das amostras. Este é capaz de fazer uma análise quantitativa e qualitativa mesmo à escala das µg/g. O principal objectivo do trabalho é correlacionar a variação das concentrações dos elementos traço entre tecidos cancerígenos e tecidos saudáveis com a finalidade de avaliar tanto a influência destas variações no desenvolvimento cancerígeno como a resposta destes elementos às condições da carcinogénese.

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CHAPTER 1 – INTRODUCTION

Due to the exponential increase of world cancer incidence and mortality, adding the constant failure to prevent it, it's imperative to find new treatments and approach techniques in order to fight this global epidemic. To be able to counter this disease it is necessary to fully understand its biological and physiological processes. One way to do so is to study the concentration of trace elements present in human tissues. These elements appear in minimal quantities in our cells but they take part in important cell mechanisms, such as cellular growth, by being components of enzymes and catalysts that operate in basic cell chemical reactions. Depending on their concentration, they activate or inhibit certain reactions and change the membranes' permeability. In addition, their concentrations vary between healthy and cancerous tissues in response to this disease's biological changes. Therefore, by quantifying these elements and studying their variations it is plausible to observe whether or not the excess or lack of a certain element influences carcinogenesis mechanisms. This will allow the possibility of creating a pattern if these changes are recurrent.

Considering the low concentrations of trace elements in a cell, it is necessary to use a specific technique, such as X-Ray Fluorescence Spectrometry, which allows a multi elemental analysis on trace elements concentration. Some techniques even display their spatial distribution. This technique is based on the interaction between x-rays and matter, in this case, human tissues. The x-rays will excite these tissues and when these return to their normal states they will emit a spectrum of characteristic x-rays that with further analysis will show the specific elements present in that same tissue and their respective concentrations. In this work the chosen XRF technique is EDXRF (Energy Dispersive X-Ray Fluorescence), one of many XRF techniques that highlights the non-destructive method, allowing repeated sample analysis without compromising their composition. The 14 samples of cancerous and healthy tissues, equally divided and paired, will be analyzed through this technique. There are 4 samples from Lung tissue, 4 from Bladder tissue and 6 from Colon tissue. For every cancerous tissue, there is a pair of healthy tissue from the same individual. Unfortunately, the clinical history from each patient was not facilitated, due to bureaucratic problems, which would have helped explaining certain results.

Posterior to this information gathering, a statistical study will take place, measuring the elements' concentrations in all tissues and comparing the results, in order to reach a correlation between them. The results will be compared, mainly between cancerous tissues and healthy ones, although other parameters will be considered, such as the tissues' original organ, the used spectrometer and each tissue's pair. Therefore, if a correlation is found in these variations between cancerous and healthy tissues it will be possible to evaluate the influence or the cause of these variations in cancer development. The objective in future studies will be to discover indicators, measuring trace elements concentrations variations (in blood,

for example) of cancer and other pathologies, which would help improve early diagnosis measures and new, more efficient, treatments.

This work falls into a series of projects that have as a final objective the ability to perform early diagnosis on cancer and predict its development in the human body. There have been several works previous to this one. The first ones tried to test this spectrometry on biological tissues, being able to quantify each element's concentration. Others quantified the amount of lead present in human tissues [1]. Then, some works focused on analyzing cancer and healthy human tissues hoping to find correlations in trace element concentration variations but failed to reach a solid one, mainly due to the short number of available samples. This work will try to find more solid conclusions using the same laboratorial environment and techniques but hopefully with more available samples.

In the future, if these correlations are found, it will be able to create a pattern that will make possible the early detection and state evaluation of carcinogenesis by the quantity of each trace element in each cell. So maybe it will finally be at human reach the definite prevention of cancer.

I've accepted this work with much esteem because I am closely familiar with a cancer diagnosis and know what it can do to a person and his family. The immense emotional cost and mainly the monetary problems, that still present a big barrier in world cancer treatment, are believed to increase with the raise of patient numbers. If we find motivation in our work, we can reach levels that, at first, might seem impossible. So, allying motivation and hard work I intend to add my contribution, even if lowermost, to this noble cause that is hoping to terminate one of the biggest and most severe diseases of the twenty first century.

CHAPTER 2 – STATE OF THE ART

CONTEXTUALIZATION

According to 2012 statistics there were diagnosed 14.1 million new cases of cancer worldwide, which represents a significant raise compared to 2008 results. It's expected that this raise will continue dramatically over the next two decades, resulting in over 20 million new cancer cases. The number of deaths worldwide was about 8 million in 2012. But then again, in the next 20 years this number is expected to rise to 13 million deaths caused by cancer or cancer related problems. Even so, 32.5 million people diagnosed with cancer in the previous 5 years were alive at the end of 2012 [2, 3].

Even though cancer has a worldwide incidence, there are differences in numbers and dominating types of cancer between developed and developing countries. Developed countries show greater incidence in lung, breast, prostate and colon cancer, while in developing countries incidence is higher in stomach, liver, esophagus and cervix cancer. Globally lung and breast cancer have approximately the same number of diagnosed cases, at the top of worldwide incidence. Yet, referring to the number of deaths caused by cancer, lung cancer is responsible for 1.6 million of the 8.2 grand total, much more than breast cancer Although 60% of worldwide cancer incidence is verified in developed countries, more than a half of worldwide deaths were registered in developing countries [4].

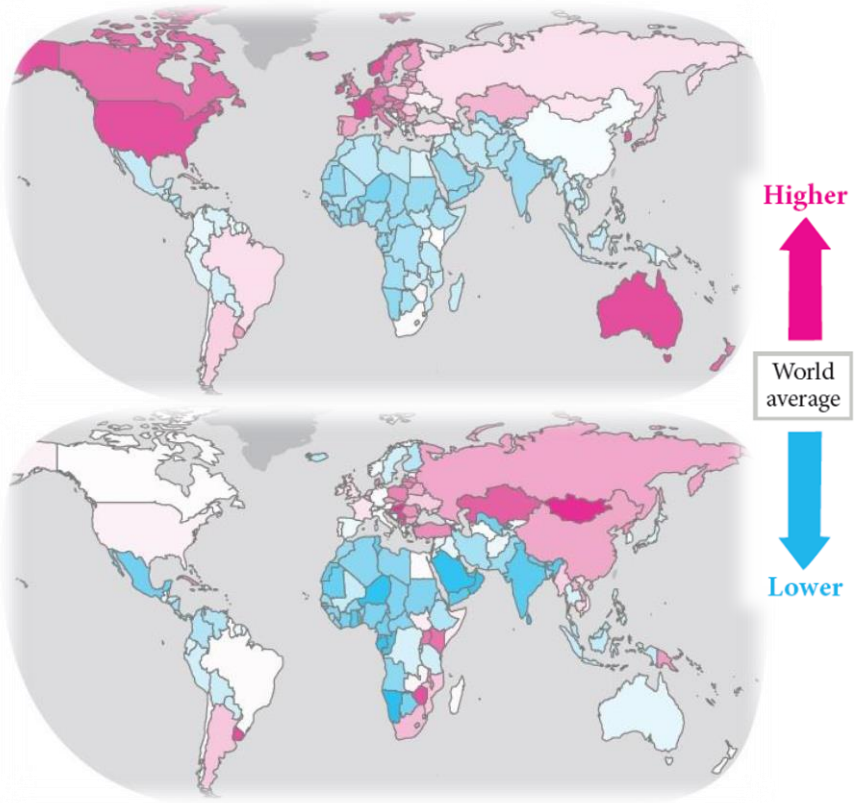


Figure 1 - Worldwide cancer incidence (top) and mortality (bottom) rates per 100,000 population compared to the world average [4].

The increasing worldwide cancer incidence will bring even more severe consequences to developing countries, due to the underworld conditions that these populations live in and to the adoption of new and more industrialized lifestyles, such as smoking, alcohol consumption and improper nourishment. Although it is logical to think that in the future cancer will strike harder on developing countries, the enormous costs associated with this disease and population aging confer alarming factors in developed countries. So, to counter this world tendency, it is necessary to improve treatment and early diagnosis measures [5].

Portugal doesn't escape this tendency and as other developed countries, lung, breast, prostate and colon cancers are abundant and all in expansion. It is predicted that by 2030, 55 thousand new cancer cases will be diagnosed in that year, which is very alarming [6].

CANCER

The ability to improve treatment and diagnosis measures lies on understanding all intervenient factors in the carcinogenesis process, which has several stages and can be originated by genetic alterations or environmental factors. All multicellular organisms' cells replicate from one mother-cell originating two exactly alike cells. There are mechanisms that ensure this replication is well conducted. If these mechanisms do not work as they should, cells will multiply freely transmitting this characteristic to other cells and forming a mass that is called tumor. There are benign and malignant tumors: the first ones are usually removed with relative ease, however, malignant tumors spread throughout surrounding tissues using bloodstream and the lymphatic system as its transportation, relocating in other parts of the human body, compromising its normal functioning. So, cancer is the term used to designate certain pathologies that present uncontrollable cell growth, therefore including a vast range of malignant masses, differentiated by their origin and severity [7, 8, 9].

Lung cancer has the highest worldwide incidence, resulting in 1.8 million new cases in 2012, that is, 1 in 5 patients diagnosed with cancer, and responsible for 1.6 million deaths that same year. 1 in 13 individuals is expected to develop lung cancer in his lifetime, being smoking the ruling factor, causing 22% of cancer related deaths and 71% of lung cancer related deaths. There are other factors, such as environmental and genetic, assuming a secondary role as smoking turned a worldwide practice of the modern society, traduced in more than a thousand million smokers [4, 10, 11].

Colon cancer has the third highest incidence worldwide and is responsible for more than 1.3 million new cases and 700 thousand deaths, according to 2012 statistics. By 2035 it is predicted that these incidence numbers will rise up to 2.4 million new cases. Unlike lung cancer, colon cancer has high prevalence, as the number of people diagnosed within the previous five years that are still alive at the end of a given year is high. Approximately 95 per cent of colon cancers are adenocarcinomas, which originate in glands. There is convincing evidence that consuming alcohol and processed meat increases the risk of developing colon cancer. On the other hand, having a lifestyle with plenty of physical activity and healthy eating and drinking,

helps protect against this type of cancer. Due to the amount of fat in food, incidence is 20 times higher in developed countries than in developing ones. As prevention, it is recommended to consume less fat and much more fiber [12, 13].

In 2012, 430 thousand new cases of bladder cancer appeared, making it the ninth most common cancer worldwide, responsible for 165 thousand deaths that year. Bladder cancer incidence is more than four times higher in men than women and occur mainly in developed countries. The main causes of this type of cancer are smoking, as in lung cancer, exposure to industrial chemicals and drinking contaminated water, especially water that contains arsenic [13].

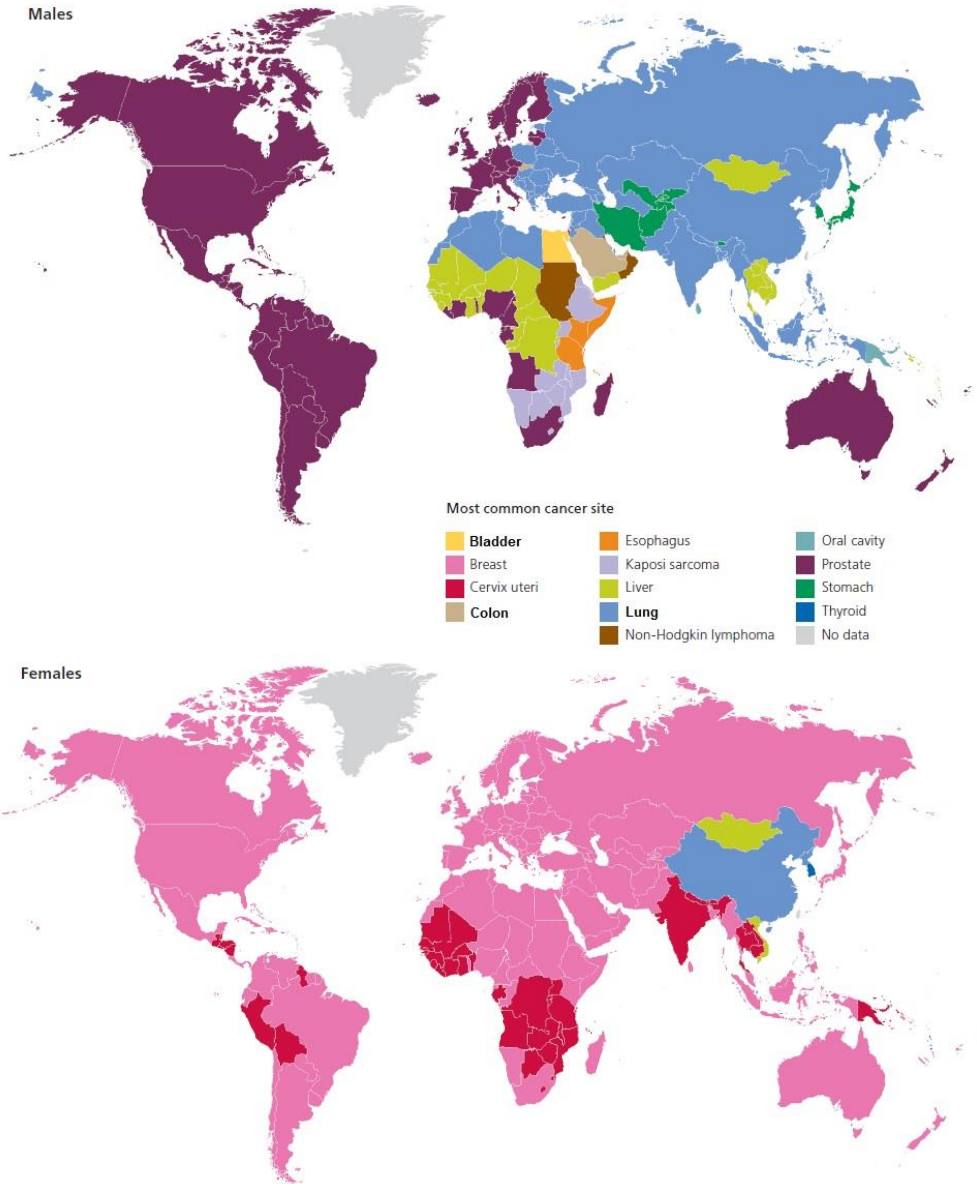


Figure 2 – Most common cancer sites worldwide by sex, according to 2008 statistics [14].

TRACE ELEMENTS

Carcinogenesis processes are not of easy comprehension, therefore effective treatment is lacking. Even so, new studies have been made, measuring trace element concentrations and analyzing their variations, which could provide core information in helping to understand this disease. Trace elements appear in very low concentrations, inferior to 1000 mg/kg (0.1%), which exist in our body, having the particularity of intervening in important processes, varying their concentrations depending on physical and chemical conditions, as well as physiological and pathological states of the organism. It is plausible to connect their variations with several pathologies' development [15, 16].

Some of these elements have active roles in carcinogenesis processes, so it's possible to correlate their concentrations and ratios with different types of tumors. These elements have their own way of interacting with the human organism, and each have a set of characteristics that can influence or be influenced by carcinogenesis. Some of them are associated with the presence of unpaired electrons that allow their participation in redox reactions [17].

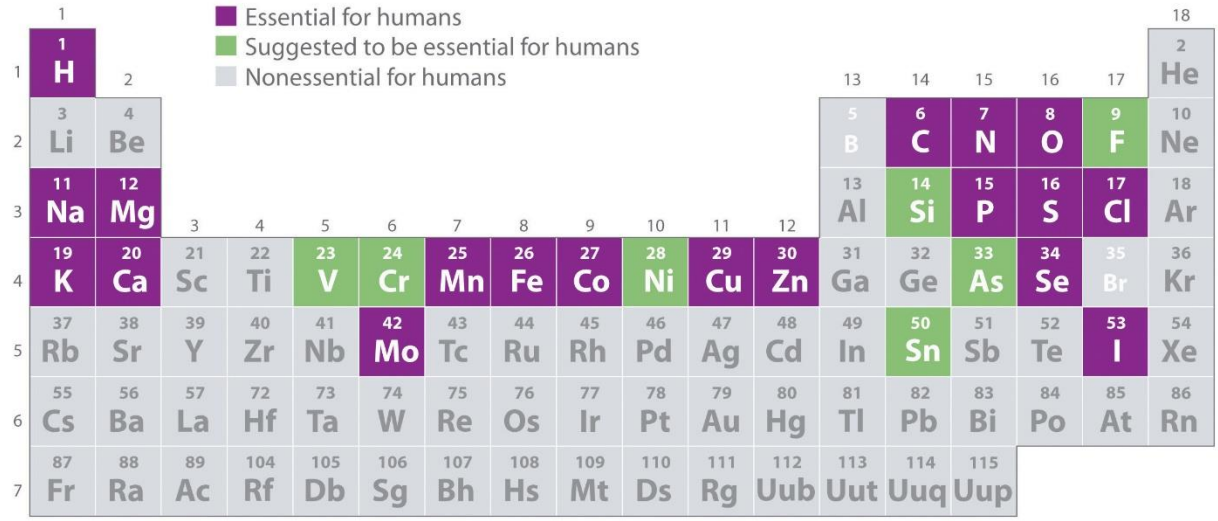


Figure 3 – Periodic table with highlighted elements that are essential or are thought to be essential to human organism [18].

Fe (Iron) is an essential element in the human organism due to its role in many physiological functions, certifying that proteins and enzymes work correctly. It is one of the elements that can influence neoplasia development, by intervening in cellular growth and differentiation processes. Studies reveal that Fe present in each cell could promote cancer development as it catalyzes the production of oxygen radicals, which are thought to be carcinogens.

Zn (Zinc) is responsible for the correct functioning of several enzymes in the human body, for instance, superoxide dismutase, an enzyme that regulates healthy cellular growth and, if not correctly working, related to tumor cellular growth. Zn may have inhibitory effect on phosphodiesterase and activation effect on adenylate cyclase, which underlines its importance in carcinogenesis.

Cu (Copper) plays an important role in several biochemical reactions inside the human organism. Like Zn, Cu is also a cofactor of superoxide dismutase enzyme, preventing the start and development of tumors. In addition, Cu intervenes in angiogenesis that is indispensable for tumor growth.

Se (Selenium) is accountable for glutathione peroxidases, antioxidant enzymes that protects DNA from free-radicals, therefore related with anti-cancerous effects. Se is thought to be a “redox switch” in the activation or inhibition of cellular growth factors. Its raise promotes cytotoxicity in cancerous cells, altering its metabolism, even though many adjacent processes are yet to be understood [19, 20, 21, 22].

PREVIOUS STUDIES

As said before, there have already been made several studies in this matter. Some focused on the quantification of trace elements in biological tissues and others tried to find correlations between trace elements’ concentrations and cancer development.

One of these studies on **lung** cancer presented some important data regarding element concentration variations and linearity between several elements. For example, P (Phosphorus), Ti (Titanium) and Pb (Lead) concentrations appear to be higher in cancerous tissue, roughly doubling the concentration comparing to healthy tissue. Then, there are many elements that indicate lower concentrations in cancerous tissue: some reduced by half, like Ca (Calcium), Fe, Cu and Zn; and others with greater decrease, like S (Sulfur), K (Potassium), Cr (Chromium), Mn (Manganese), Se and Sr (Strontium). These changes, adding the fact that most of these elements represent great importance in biological and enzymatic processes, establish a pattern in lung cancerous tissues. In fact some of these elements have been under rigorous surveillance since they influence carcinogenesis in laboratory animals. It is thought that nutrients and food components, by their antioxidant characteristics may have an effect in cancer development, accelerating it or stopping it. Furthermore, this study showed linear correlation between certain elements concentrations, for instance, between P and S, Ti and Cr, Mn and Fe, Ti and Se. In addition, Mn and Fe, Se and As (Arsenic) appear to have an antagonist relation [23].

Other data confirm that the most important elements in distinguishing between cancerous and healthy **lung** tissues are Fe, Mn and Cu. While for **colon** the most important elements are Ca, Zn e Fe. This work helped confirm previous studies that indicated that Pb, Cu and Zn concentrations are significantly lower in cancerous **lung** tissue than in healthy tissue [24].

Another study proved the increase of Ti, Cr and Mn concentration in **lung** cancerous tissues and the decrease of Sr and Pb. There is also linear correlation between some of the elements in lung tissue, for example P and S. This data is in conformity with previous results, which concluded that Fe, Mn and Cu are the best indicators to differentiate between cancerous and healthy lung tissues. In addition, concentrations of elements in women and men cancer tissues are statistically equal. The same work indicates a correlation between **colon** cancer and the nutrition system. In general, studies treat the correlation between cancer

and specific food, but none treat the elemental content of food and its relation to this disease. Ca concentrations are very similar in both cancerous and healthy tissues and in some studies its use is suggested as prevention of colon cancer. Low Se levels are thought to be a result of the disease rather than the reason for the tumor development, as so, Se levels should be closely monitored. Se is known for its cancer-protecting effect, however, when in subtoxic concentration, Zn prevents Se activity, accelerating tumor development. Only P, S, K, Ti, Se and Rb (Rubidium) have a tendency to accumulate in cancerous colon tissue [12, 25].

Other studies that are related to **colon** tissues, verifying that some elements increased their concentrations in cancerous tissues, for example P. These conclusions were reached due to a deeper statistical analysis. Furthermore, the difference in Se concentrations between healthy (higher) and cancerous (lower) tissue may remit to the antioxidant factor of this element, that helps neutralize free radicals [26].

Further studies used and compared two different techniques, EDXRF and TXRF (Total-reflection X-Ray Fluorescence). This study focused on **colon** cancer showing that Ca and Sr levels are alike in both cancerous and healthy tissues. Moreover, P, K, Cu and Ni (Nickel) were found in higher concentrations in cancerous tissue. On the other hand, Br (Bromine) was decreased in tumor tissue. Zn concentration is either constant or decreased in cancerous tissues. This decrease is also found in other pathologies, for example arteriosclerosis. It can be linked with the alteration of metabolism and modification in the transport and maintenance of essential elements. On the contrary, the increase of some elements can be associated with mechanisms of toxicity whenever these elements are in excess. The results acquired from both techniques are similar, however, TXRF tend to have higher sensitivity for light elements, while EDXRF is more sensitive for heavy elements [27].

More studies related to **colon** cancer tissues revealed a significant increase in K, Rb and Cu concentrations in cancerous tissues comparing to healthy ones, while Zn and Mn appear to have constant concentrations regarding both types of colon tissues. There is also evidence that Ni and Pb have higher concentrations in tumor tissues [28].

A study with a large number of samples, nearly 14.000, discovered, analyzing iron-binding capacity and transferrin saturation, that high body iron storage elevates the risk of developing cancer. These conclusions were only proved for men, while for women there was no significant data [29].

Another work regarding trace-metal analysis was conducted with specific types of cancer in specific human organs. It studied the **lung** bronchogenic carcinoma and the **colon** adenocarcinoma recurring to several samples, some cancerous and others healthy human tissues. After data was analyzed, one could observe that in the bronchogenic carcinoma, Fe appeared with lower concentration in cancerous tissue than in healthy ones. On the contrary, the levels of Zn were higher in cancerous bronchial tissue. As for the colon adenocarcinoma, only Sn (Tin) varied its concentration, appearing lower in cancerous tissues. This study underlined that the observable differences of trace-metal concentrations between cancerous and healthy

tissues belong to specific types of cancer, indicating that analysis cannot group all types of cancer in one class, for interpretation purposes [30].

A work related to patients with **bladder** cancer studied the concentrations of Cu, Zn, Se, Pb and As. After comparing these elements concentrations the data indicated higher levels of Zn in cancerous tissues than in healthy bladder tissues. This element shows a gradual and significant increase in its concentration as bladder cancer evolves from initial to the advanced stages. The other elements failed to have a statistically difference and so conclusions are undefined [31].

A study that conducted an analysis on **breast** tissues reached some conclusions. First it showed that trace elements concentrations were higher in tumor tissues, either benign or malignant, than in healthy breast tissues. This, as other previous studies, reveals that trace elements can be used as tumor biomarkers, once they give useful data that allows the distinction between tumor and healthy tissue. It was also found that Cu has a significant correlation with overall survival, meaning that patients with positive expression of this element had poor chances of survival. Other parameters that influence trace elements expression are age and menstrual status [32].

The biggest problem found in almost every study made so far is defining a reference value of concentrations that would help immensely to standardize variations between trace element concentrations. Other issues remit to inconsistent results, even for similar samples, that create many doubts as these variations are a cause or a consequence of carcinogenesis [20].

CHAPTER 3 – X-RAY SPECTROMETRY

HISTORY

In 1895, Wilhelm Conrad Roentgen accidentally observed in a light-absent room that the radiation from the discharged tubes caused a fluorescent effect on a small black screen painted with barium salts. This was the first time that X-Rays were observed, gaining this denomination because they presented characteristics different from any other kind. These rays are electromagnetic radiation and have wavelengths between 10^{-8} m and 10^{-12} m and energies between 100 eV and 100 keV. They are produced by the deceleration of high-energy particles, through transitions of electrons between atom inner orbitals or by the decay of certain radioactive elements. In the first process X-Rays are emitted in a continuous spectrum, while in the second, as a characteristic spectrum [33, 34].

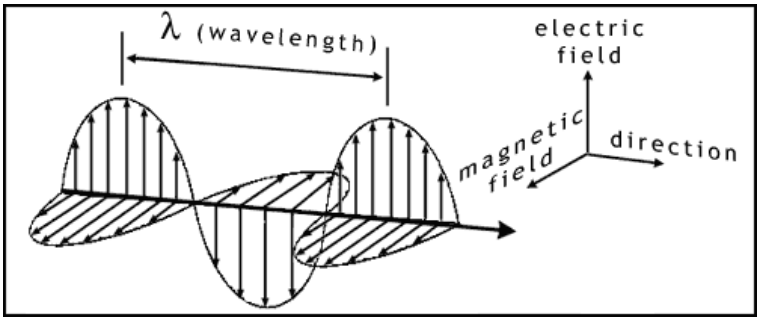


Figure 4 – An electromagnetic wave with a correspondent electric field, magnetic field and direction [35].

Continuous spectrum of radiation occurs when a high-energy charged particle closes in an atomic nucleus, decelerating really fast, emitting a type of radiation called **Bremsstrahlung** (Brake Radiation). In this interaction, the kinetic energy of the electron is transformed in radiation (photon), posteriorly emitted [36].

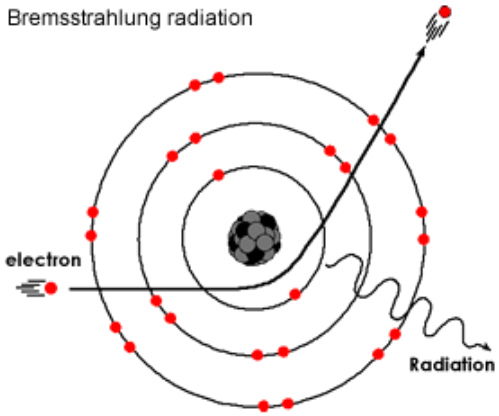


Figure 5 – Scheme of Bremsstrahlung radiation: an electron passes near an atomic nucleus, decelerating instantaneously, emitting continuous X-rays [36].

Characteristic spectrum is a result of electron transitions between atom inner shells. If an incident particle or photon interacts with a bound electron of a certain atom, that electron is ejected, creating a vacancy on the atom inner shells. This is called ionization of an atom. Then, an electron from an outer shell transits to occupy the vacancy, originating the emission of a photon through a characteristic spectrum **(fluorescence)**. This photon has the energy equal to the difference between the binding energies of the two shells in question. If an electron is emitted from an outer shell instead of a photon, it is called **Auger Effect**. The energy of the emitted Auger electron depends on its originating shell [36, 37].

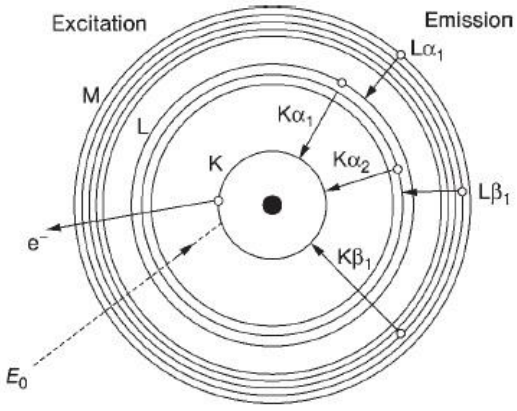


Figure 6 – Scheme of X-ray Fluorescence: an electron from the K shell is ejected from the atom due to an incident photon (E_0). Then, an electron from the L shell occupies its vacancy, emitting characteristic X-rays [38].

This characteristic spectrum emission is called **X-Ray Fluorescence** or **XRF** and is the main principle of several analysis techniques due to the fact that each element emits a characteristic set of x-rays. The energy of these x-rays is related to the element in question, as the intensity is related to its concentration. The interaction between x-rays and matter was intensely studied, as well as its experimental applications. This was a great help in human organism functional analysis [38].

INTERACTION

Depending on the incident photons energy, x-rays interact with matter differently. Four different aspects are seen in this interaction: attenuation, absorption, scattering and transmission. There are also different types of interaction between x-rays and matter, however, only three will be mentioned due to their importance for this work. They are photoelectric absorption, Rayleigh scattering and Compton scattering. Below 100 keV, photoelectric effect prevails, representing more than 80% of all effects for elements with Z (atomic number) > 40. Rayleigh scattering is almost constant over the whole range of energies and atomic numbers, representing roughly 10% of the total effects. While the Compton Effect gains importance at high x-ray energies for low atomic number elements [39].

Photoelectric Effect happens when a photon or other particle interacts with an inner shell electron of an atom. All the photon's energy is transferred to the electron, which is ejected with an energy equivalent to the difference between the photon energy and its binding energy to the atom. To occupy the vacancy left by the ejected electron, another electron from an outer shell transits to the lower level of energy, emitting this energy through characteristic radiation. This is the principle of XRF [40].

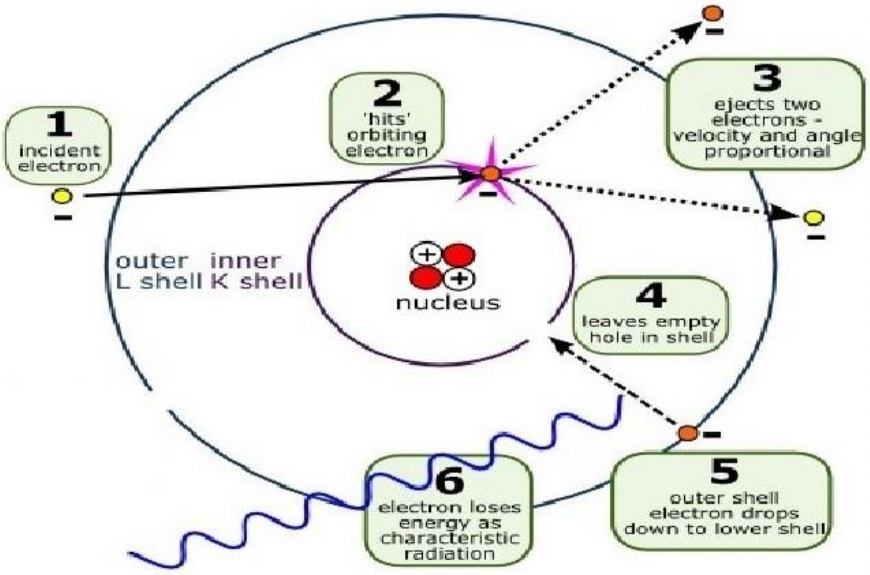


Figure 7 - Scheme of the Photoelectric Effect and its different stages [35].

Rayleigh scattering or Coherent scattering occurs when a photon interacts with electrons or nucleus, interacting with the atom as a whole. This photon is scattered, maintaining its original energy, but altering its direction. The atom is neither excited nor ionized, hence it is a parametric process. This happens mostly for low energies and high atomic number elements [41, 42].

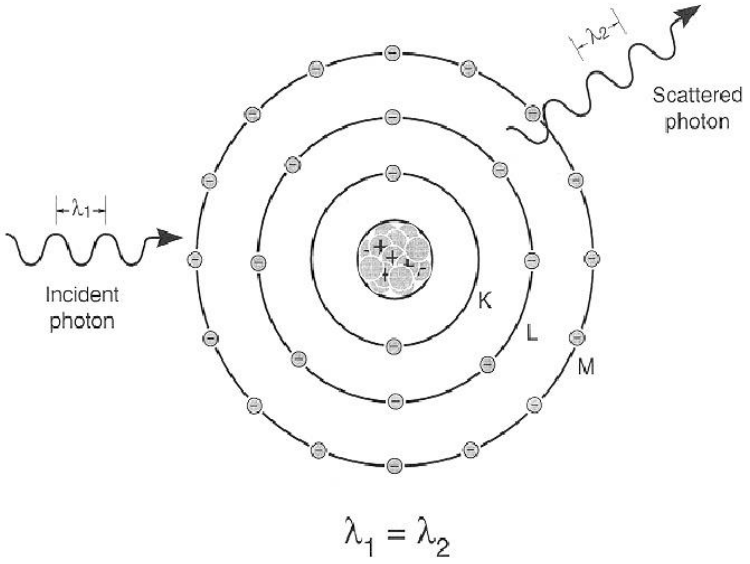


Figure 8 – Scheme of the Rayleigh scattering, evidence of the unaltered wavelength [35].

Compton Effect or Incoherent scattering takes place when a photon collides with an electron and loses some of its energy, being deflected from its original path. Part of the energy is transferred to the electron. The scattered photon is emitted almost isotropically [43].

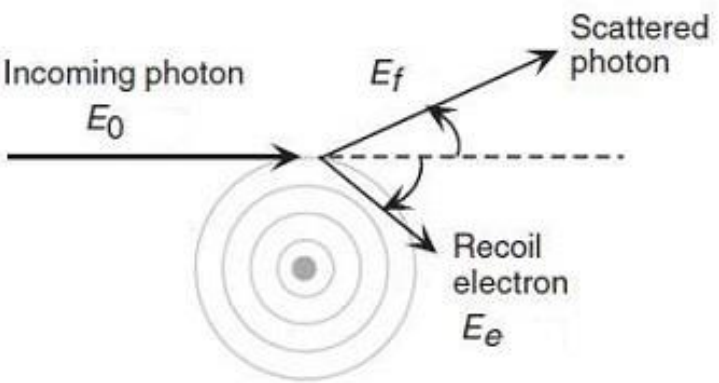


Figure 9 – Scheme of the Compton Effect [35].

There is also the **X-Ray Diffraction** phenomenon when a beam of mono-energetic x-rays irradiates a crystal, resulting in a diffracted beam at definite angles, in agreement with Bragg's law. These last 3 types of interaction increase the detector's background.

The photoelectric effect and the scattering interactions contribute to the attenuation of x-rays in matter. The Lambert-Beer law shows the reduction of the intensity of an x-ray beam that passes through a certain layer. There are multiple variables in the equation, being the most relevant the **mass attenuation coefficient** that depends on the interactions between the beam and the layer, which promotes its attenuation. This coefficient is proportional to the sum of the cross sections for all elementary scattering and absorption process. At the range of XRF energies (< 100 keV), photoelectric effect accounts about 95% of the mass attenuation coefficient, while the scattering interactions comprehend the other 5%. This is due to the fact that photoelectric absorption coefficient is much higher than the sum of the two scattering coefficients [37, 44].

TECHNIQUES

In order to identify trace elements in a sample, the production of characteristic x-ray spectra of those elements is required. For that an excitation source is needed. This fits in the field of **X-Ray Spectrometry**, which uses electromagnetic radiation from radioactive sources, synchrotron facilities or x-ray tubes. All the data in this work will be analyzed through the **XRF Spectrometry** analytical technique, although there are several other X-Ray spectrometry techniques, such as Proton Induced X-Ray Emission (PIXE), for example, which uses a beam of charged particles [37].

XRF analysis falls into a variety of techniques, being **EDXRF (Energy Dispersive X-Ray Fluorescence)** and **WDXRF (Wavelength Dispersive X-Ray Fluorescence)** the two most used ones. In this work it will be EDXRF the chosen technique to analyze the concentrations of trace elements in all samples. This technique allows a multi-elemental analysis with quantitative and qualitative data of each sample. Inside an EDXRF Spectrometer is a Si(Li) (or similar) detector with high resolution connected to a multichannel analyzer, which allows to obtain a spectrum of x-ray energies. While in a WDXRF Spectrometer is a single crystal placed behind the sample that isolates a narrow wavelength band of the excited sample radiation, through its diffracting power. The main problems with this technique, in comparison to EDXRF, are the fact that only one element can be analyzed at once and that it is extremely time consuming due to its point by point acquisition, rather than EDXRF that acquires the entire spectrum simultaneously [45].

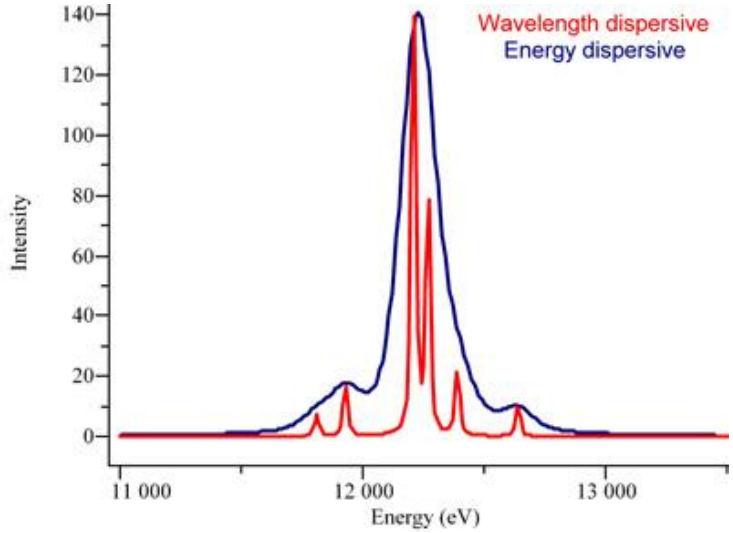


Figure 10 – Difference between EDXRF and WDXRF presented spectrum. Evidence of the EDXRF simultaneous acquisition and the WDXRF point by point one [45].

However, if a more detailed analysis is required, it is recommended the use of an alternative XRF technique, the **µXRF (Micro X-Ray Fluorescence)**. This technique is much similar to the conventional XRF technique, except that it uses X-Ray optics to restrict the excitation beam size or to focus the excitation beam to a small spot on the surface of the sample, in order to analyze its small features [46, 47].

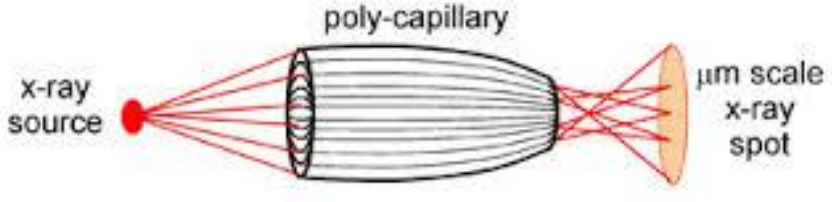


Figure 11 – Scheme of a polycapillary lens restricting the x-rays from the source into a µm scale spot [45].

SPECTROMETERS

In this work two different EDXRF Spectrometers were used, with many different characteristics. **M4 TORNADO** from Bruker and a **Tri-axial Geometry Spectrometer** in-house built. The first one runs a high spatial resolution analysis, recurring to μXRF and returns both an energy spectrum and a map with the spatial distribution of each trace element. The second only returns an energy spectrum with the counts of each energy channel, having the whole sample in consideration.

Commonly EDXRF Spectrometers have a relatively simple composition:

- An excitation source (X-Ray tube)
- Focusing optics (μXRF)
- An X-Ray detector with related electronics
- A multichannel analyzer
- Dedicated software for rapid, automatic analysis of chemical elements

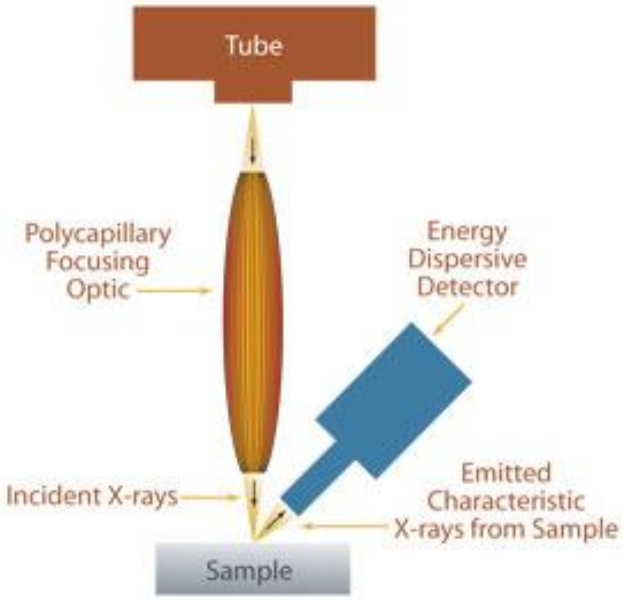


Figure 12 – Simplistic scheme of the M4 TORANDO Spectrometer's composition [48].

This schematic shows, in a simple way, what happens inside the spectrometer. The Tube emits X-rays that are collimated by the optics and impinge on the sample. Then, the sample emits characteristic X-rays, which are captured by the detector. Further analysis takes place in the adjacent software [38].

Commonly, the radiation used in XRF is the one obtained from an anode inside an **X-ray tube**. The tube's cathode emits a beam of electrons that are accelerated by high voltage and focused on the anode. Through ionization, the anode emits a spectrum of characteristic X-rays formed by the characteristic lines of the anode element. The intensity of those lines is proportional to the current applied in the tube. In order to

expect good performance from the X-ray tube, some conditions are required: high voltage to analyze all desired elements; specific currents that vary from one spectrometer to another; good stability versus time and appropriate anode material [37].

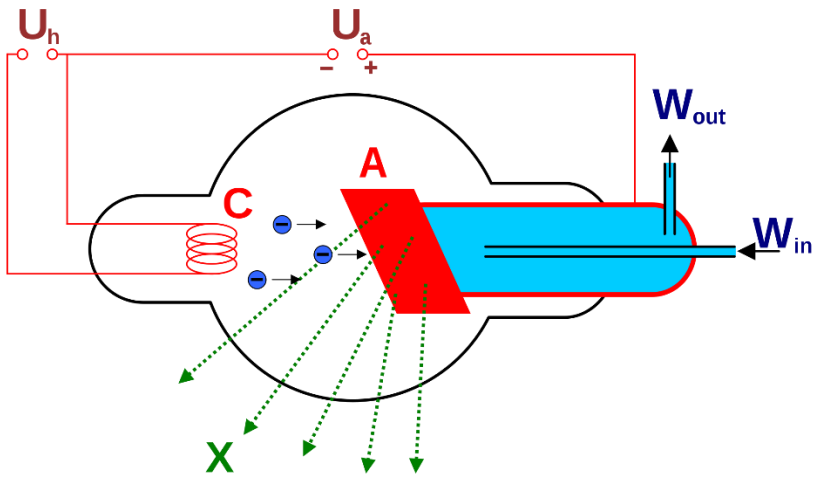


Figure 13 – Simple scheme of an X-ray tube. C – cathode; A – anode; X – Emitted x-rays; U – Applied tension; W – Cooling system (if existent) [48].

The regular EDXRF spectrometer irradiates the sample with X-rays in an area on the order of cm^2, when the sample is homogeneous and sufficiently large. However, if it is small or inhomogeneous the irradiated area must be reduced. If it is desired to reduce it down to an order of 10^{-4} mm^2 or less, μXRF is required, as well as capillary collimators or other kind of X-ray lenses. The most common X-ray lenses can be monocapillary or polycapillary, being the last one the most used in this technique. **Polycapillary X-ray optics** consist of an array of many small hollow glass tubes formed into a desired shape. They collect the X-rays from the X-ray source with a large solid angle and then redirect them, through multiple internal total reflections, to form a focused or a parallel beam [49].

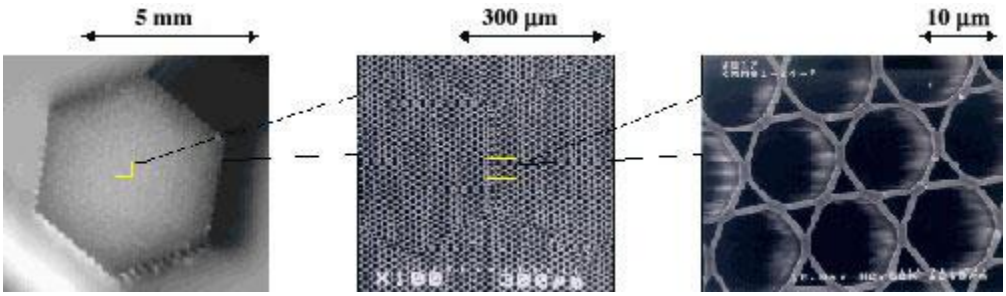


Figure 14 – Polycapillary lens at three different scales [48].

The detector of an EDXRF spectrometer has to be efficient over a very large energy range in order to allow multi-elemental analysis. Basically, the entering X-ray photon interacts with the active detector material,

originating an electronic avalanche and hence a current that is converted into a voltage pulse, by a resistor and a capacitor. Thus, each incoming photon corresponds to an analog voltage pulse, which is then changed to digital by a multichannel analyzer. A detector is characterized by its proportionality, linearity, energy resolution, efficiency and the thickness of its entrance window.

This detector, for laboratory spectrometers, was commonly made of Si(Li), like the one inside the Tri-axial from this work. It consists of a small cylinder of Si with Li to increase its electrical sensitivity. A voltage is applied so Li ions move to a certain layer, which reveals high resistance, forming the depletion area. **The incoming photons interact with this area, creating an electric charge proportional to the photon's energy, which is then processed as mentioned above.** Most recently, there was a new development in low energy X-ray detectors through the use of Si monocrystals with the electrode sets arranged in such a way for maximizing charge collection. These new detectors are labeled Silicon Drift Detectors (SDD), and are now almost ubiquitous in any modern EDXRF setup such as the M4 Tornado [50, 51].

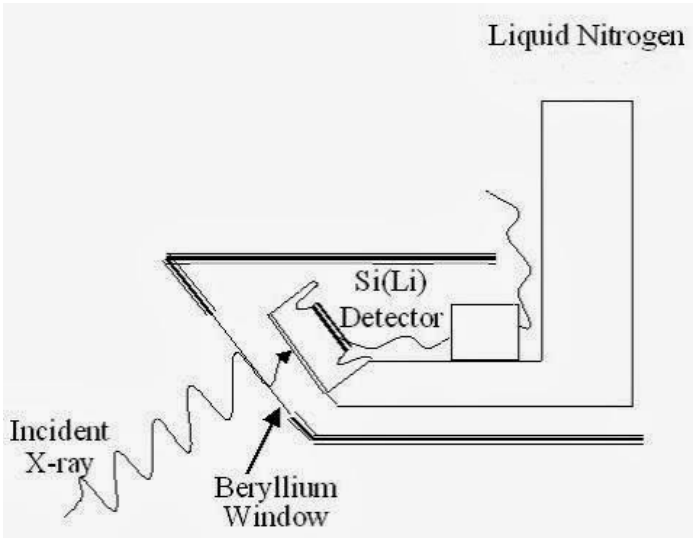


Figure 15 – Typical Si(Li) x-ray detector, commonly used in EDXRF spectrometers [52].

The final **X-ray spectrum** presented by the spectrometer contains: a set of lines for each detectable element in the sample, being their energies and intensities dependent on the composition of the sample; a continuous contribution of the Compton Effect and Rayleigh's Scattering of incident radiation, depending on the excitation source and on the sample; lines due to possible "escape" of incident lines in the detector, depending on the detector and the energies and intensities of the lines; lines due to possible "sum" effects in the detector, depending on the shaping time of the electronics following the detector; possible lines due to elements in some way irradiated in the source shielding or collimators; and determined elements' K-lines, such as Argon, Krypton and Xenon, due to the photoelectric effect in air. This last effect can be avoided by irradiating the sample in a vacuum environment, as some spectrometers allow [53].

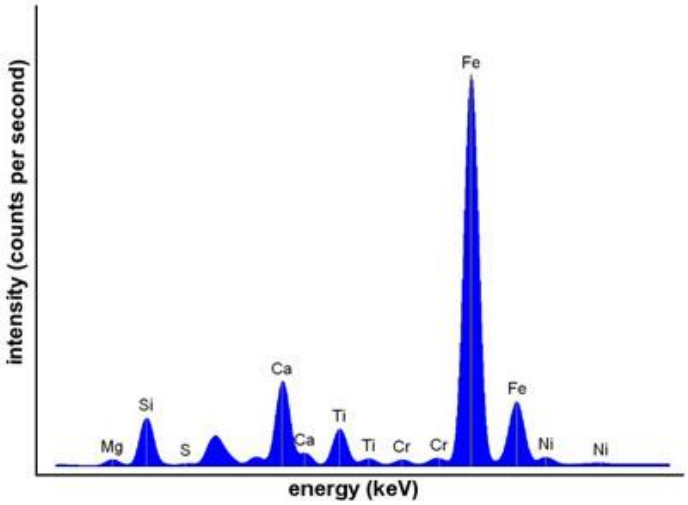


Figure 16 – Typical X-ray spectrum obtained with an EDXRF spectrometer [45].

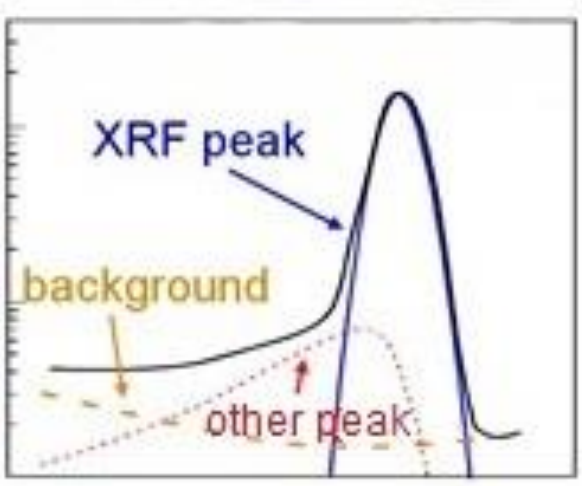


Figure 17 – XRF peaks overlapping may happen in the spectrum. Nevertheless, with software calibration the specific XRF peak can be isolated [45].

Data analysis is based in two different relations. First the qualitative relation that has its principles in the **Moseley's Law**, which relates the wavelength with the atomic number of the studied element. However the screening coefficient value is required and difficult to obtain, therefore, a database with characteristic transition energies of the elements is consulted by the data analysis code.

This allows the identification of a certain element in a sample by analyzing the energy of the characteristic X-rays emitted by it. The Moseley's law also helped the ordering of the periodic table, through atomic number rather than atomic weight, as it was previously [54, 55]

Secondly, the quantitative relation, based on the **Sherman's equation**, which relates the intensity of the X-rays with the concentration of the element. In the spectrometer this is traduced into the number of counts

in an energy channel. The more counts, higher intensity and higher the concentration, although no information can be extracted from the relative intensity between elements in the same spectrum [55].

TORNADO

One of the spectrometers used in this work is the **M4 TORNADO** by Bruker, a μXRF Spectrometer with great speed and accuracy. It allows measurements with information of the composition and element distribution of the sample. It analyzes samples in the solid, liquid or gas state in its vacuum chamber, even if the sample is irregularly shaped. It also presents high element sensitivity and excellent lateral resolution, up to 25 μm, which allows the mapping of the sample, with the spatial distribution of its composing elements [56].

M4 TORNADO has a dedicated software, with a great variety of features that allow a deep analysis of the desired sample. In this work, TORNADO will be used to evaluate the spectrum with the energy counts of each element of interest, to calculate the concentrations of those elements in ppm (parts-per-million) and to observe each element's distribution in the sample. The operating conditions for all analyzed samples were 50 kV and 300 mA. The detector is a solid state SDD with an energy resolution of 145 eV [57].

Figure 18 – M4 TORNADO Spectrometer manufactured by Bruker and adjacent software [58].

TRI-AXIAL GEOMETRY SPECTROMETER

The other spectrometer used in this work is a **Tri-axial Geometry Spectrometer** in-house built, one much older and simpler than the M4 TORNADO. Although it is an antique spectrometer, it presents good speed in analyzing characteristic X-rays emitted from the sample. It consists of a commercial X-ray tube from Philips, equipped with a Mo (Molybdenum) secondary target. The X-ray tube, the secondary target and the sample are arranged in tri-axial geometry, which helps reducing the background radiation due to the polarization of the incident X-ray beam and the almost monochromatic radiation. The X-ray beams emitted are collimated through silver holes, in order to improve detection limits. The characteristic radiation is detected by a Si(Li) detector.

The Tri-axial Spectrometer presents an energy spectrum with the respective counts of each channel. It presents the average concentrations of the whole sample, unlike TORNADO where depending on the chosen area, concentrations can be higher or lower for designated regions of the sample. This allows the identification of the desired trace elements and the measurement of their respective concentrations. The operating conditions were 50 kV and 20 mA and 1000s spectra acquisition [59, 60].

Figure 19 – Tri-axial Geometry Spectrometer in-house built.

EXPERIMENTAL PROCEDURE

The laboratorial work was conducted in *Laboratório de Física Atómica e Molecular da Faculdade de Ciências e Tecnologia,* at *Universidade Nova de Lisboa*, being the laboratory supervisor, Professor Maria Luísa Carvalho.

All samples facilitated by Professor João O'Neill from NOVA Medical School were identified with which organ they were extracted from and either they were cancerous or healthy tissues. Then, all samples were submitted to a sample preparation that will be described below. After being prepared, they were repeatedly analyzed in the two different spectrometers. Since a non-destructive technique is used, samples can be analyzed countless times without affecting their compositions.

In the **Tri-axial Spectrometer** samples were placed in a sample holder next to the X-ray source and before the detector. Then a 1000 seconds measurement was proceeded, resulting in an energy spectrum that was saved and posteriorly quantified into an excel file with each element's concentration.

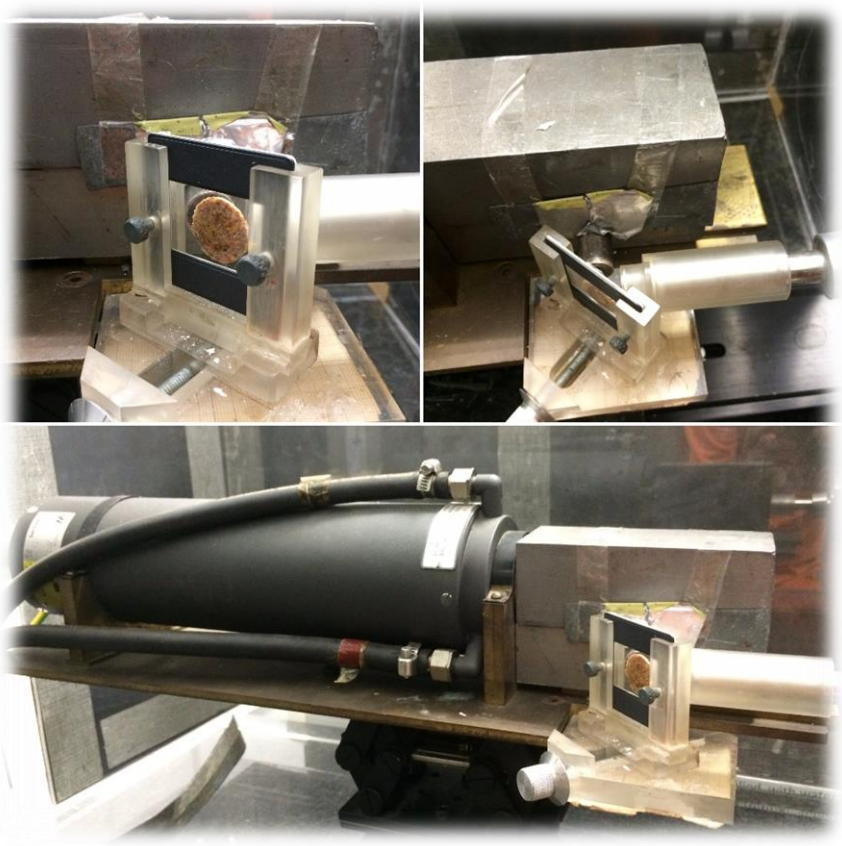


Figure 20 – Position of the sample ready for analysis. Evidence of the tri-axial geometry.

In the **M4 TORNADO** samples were placed in the spectrometer's stage and then placed right below the source and detector. An analysis was then run in an average 5 hour measurement, which resulted in the energy spectrum with peaks from the elements present in the sample and a spatial distribution map, showing where those elements were highly concentrated. The dedicated software allowed the extraction of the desired elements' concentrations in ppm.

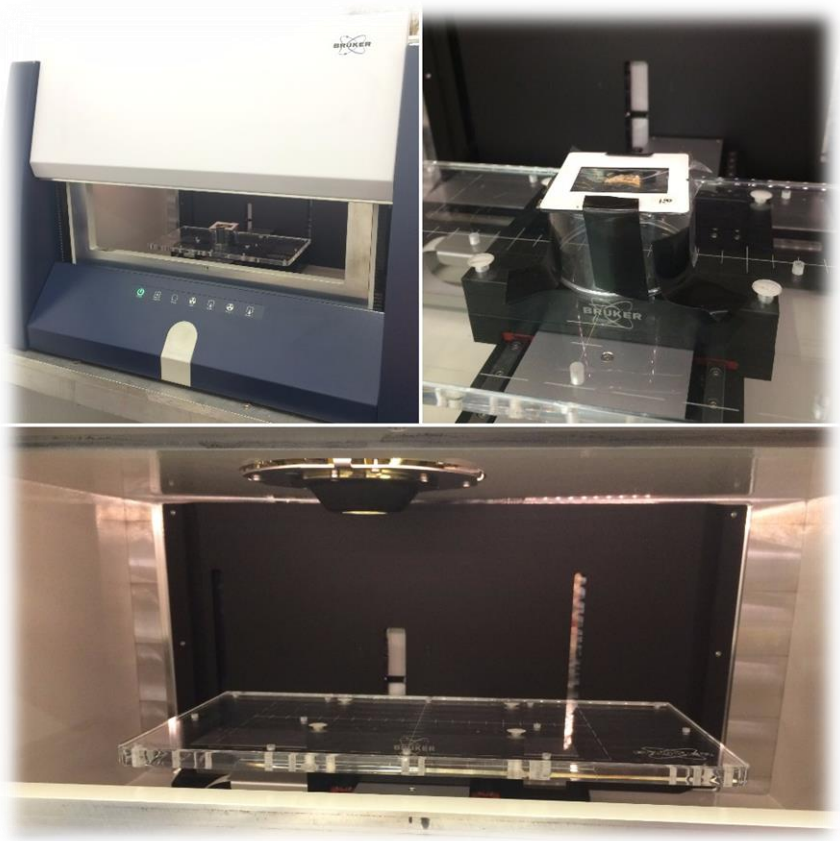


Figure 21 – Sample positioning, moveable stage and M4 TORNADO Spectrometer.

With the results from both spectrometers, a further and deeper analysis on the trace elements concentrations and ratios is now possible.

SAMPLE PREPARATION

Sample preparation was divided into four distinct stages. First the samples were removed from the solutions that they came in and cut in order to be in the desired size to be analyzed in both spectrometers. Then, all samples were lyophilized to remove all water present in them. This is required to lower the self-absorptions of X-rays in the samples, hence decreasing the limits of detection and quantification.

Figure 22 – Lyophilizer manufactured by Edwards.

Posteriorly, samples were glued with a specific glue to a mylar film and put in photograph slides which in turn were placed in petri dishes, helping its transportation and storage. Specific glue and mylar film are chosen due to their composition, made only of hydrocarbons, which aren't detected in XRF, hence not compromising the spectrum for further analysis. Finally, they were numbered and listed by their original organ and whether they were cancerous or healthy tissues.

Figure 23 – Sample glued to mylar film in photograph slides.

Figure 24 – Sample storage and identification.

ANALYSIS DETAILS

After a measurement in the Tri-axial Geometry Spectrometer, the energy spectrum is saved both in the computer that is connected to the spectrometer, in order to view it later, and in a personal computer to analyze the containing information. This information is then inserted in a software that makes use of X-ray fundamental parameters and the Sherman equation [55] for calculation of the respective concentrations and associated errors. This raw information has to be treated, eliminating the elements that were below detection levels or those who have great associated errors, which are due to the fact that their concentration is too close to the detection limits. Finally, it is possible to compare trace element concentrations from different organs and different tissues (cancerous or healthy). Three measurements were made for each sample with this spectrometer, in order to improve statistical study.

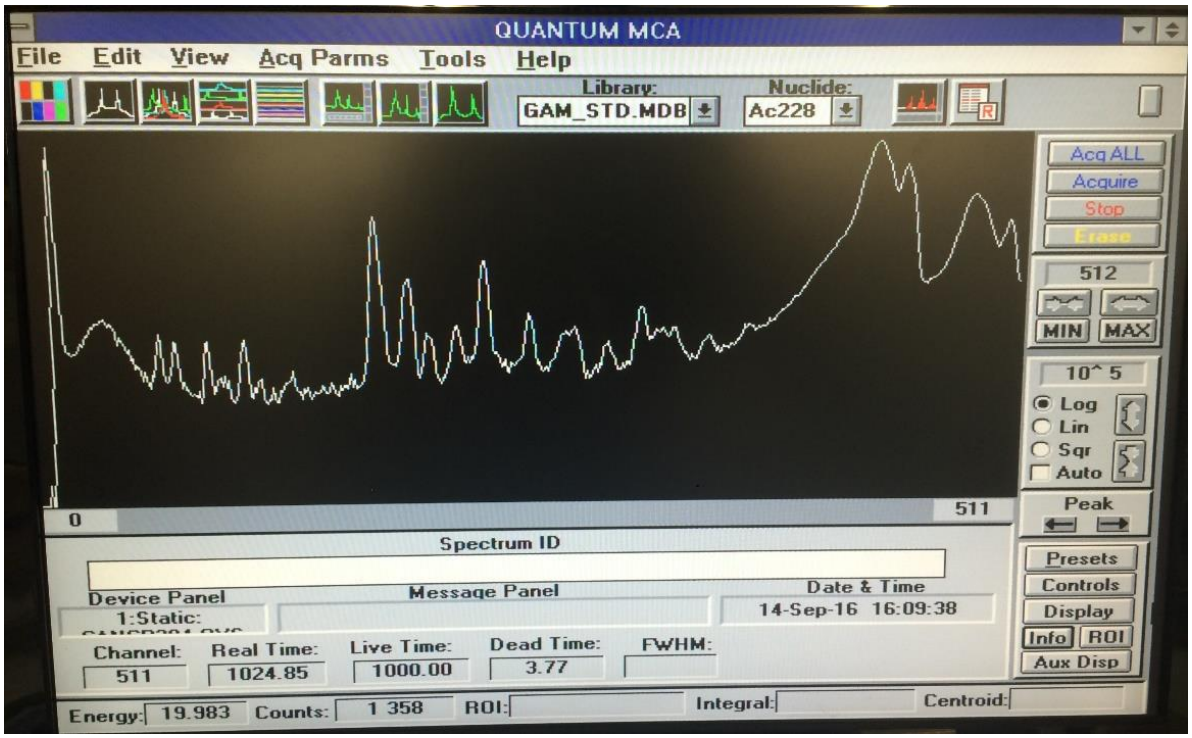


Figure 25 – Tri-axial Spectrometer spectrum showed in the dedicated software Quantum MCA. Energy value and its counts from each channel are observable.

Extracting information from the **M4 TORNADO** is quite different than the Tri-axial. After the measurement is concluded the user is able to see the original sample **(A)**, the distribution of all selected elements in the sample **(B)** and the separated trace elements' map with the spatial distribution of each selected element **(C)**. To observe the correspondent spectrum the user must click in **(D)**:

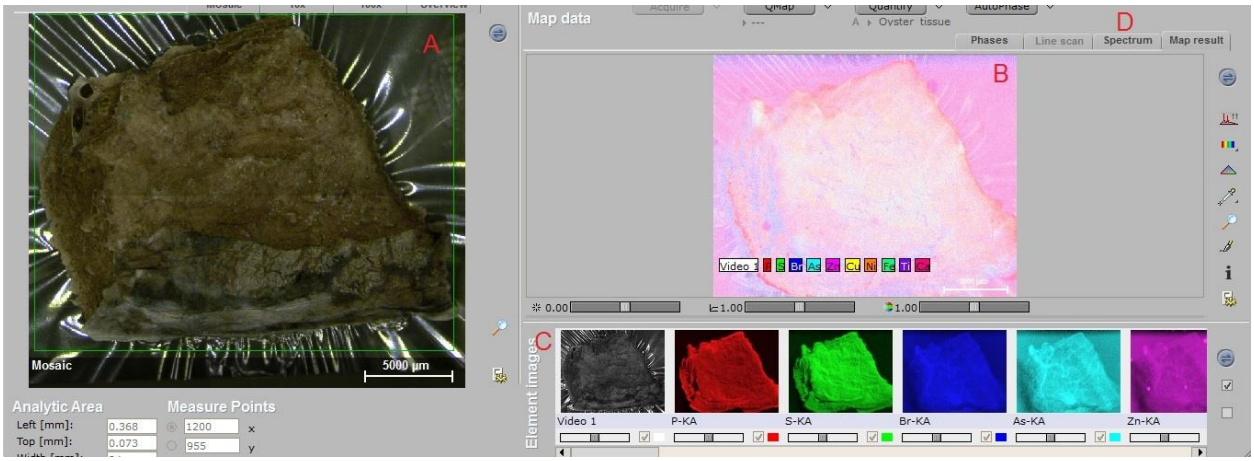


Figure 26 – TORNADO software presenting the trace element distribution map.

The spectrum is then presented. The user must observe the energy peaks in the spectrum in order to identify containing elements and select them in the periodic table **(E)**. The selected elements will appear in colored areas in the spectrum **(F)** where their energy peaks are known to be, allowing the identification of the present elements. That way the user knows which elements are present in the sample.

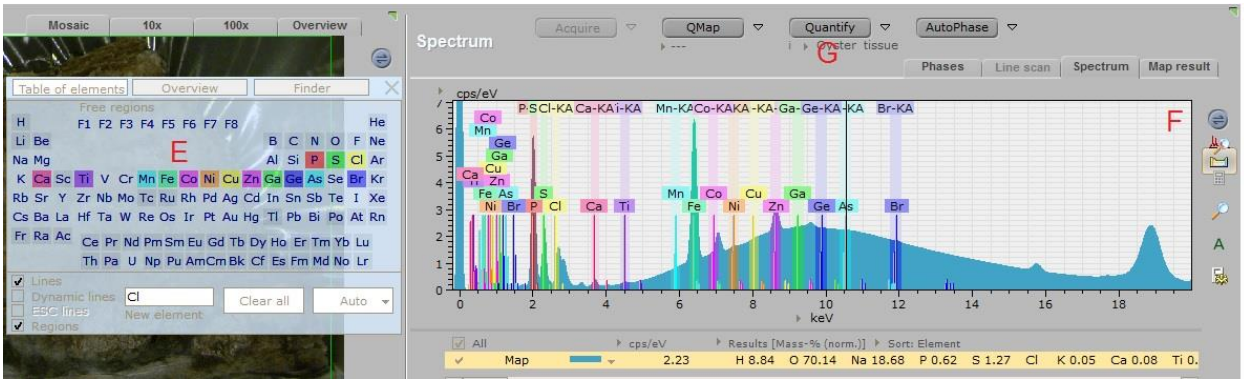
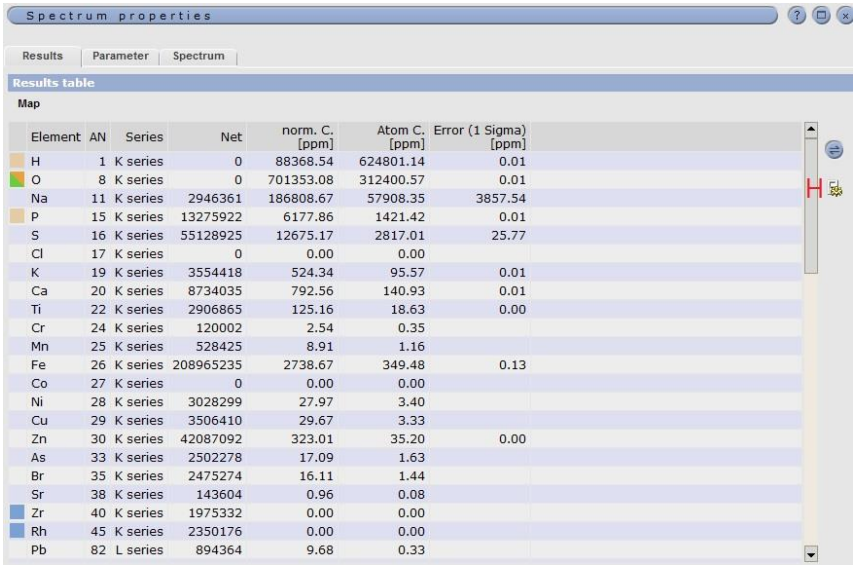


Figure 27 – TORNADO spectrum with trace elements energy peaks and respective identification.

When the user runs the quantify method **(G)**, which is also based on the Sherman equation and the fundamental parameters, only the selected elements are quantified. Both the concentrations and associated errors come in ppm and can be converted into an excel file **(H)**, allowing further statistical analysis. Two measurements were made for each sample with the same objective as stated for the Tri-axial measurements.



Element	AN	Series	Net	norm. C. [ppm]	Atom C. [ppm]	Error (1 Sigma) [ppm]
H	1	K series	0	88368.54	624801.14	0.01
O	8	K series	0	701353.08	312400.57	0.01
Na	11	K series	2946361	186808.67	57908.35	3857.54
P	15	K series	13275922	6177.86	1421.42	0.01
S	16	K series	55128925	12675.17	2817.01	25.77
Cl	17	K series	0	0.00	0.00	
K	19	K series	3554418	524.34	95.57	0.01
Ca	20	K series	8734035	792.56	140.93	0.01
Ti	22	K series	2906865	125.16	18.63	0.00
Cr	24	K series	120002	2.54	0.35	
Mn	25	K series	528425	8.91	1.16	
Fe	26	K series	208965235	2738.67	349.48	0.13
Co	27	K series	0	0.00	0.00	
Ni	28	K series	3028299	27.97	3.40	
Cu	29	K series	3506410	29.67	3.33	
Zn	30	K series	42087092	323.01	35.20	0.00
As	33	K series	2502278	17.09	1.63	
Br	35	K series	2475274	16.11	1.44	
Sr	38	K series	143604	0.96	0.08	
Zr	40	K series	1975332	0.00	0.00	
Rh	45	K series	2350176	0.00	0.00	
Pb	82	L series	894364	9.68	0.33	

Figure 28 – TORNADO quantification with present trace elements and respective concentrations and associated errors.

The concentrations from both spectrometers can now be compared throughout any desired parameters in order to reach the initial objective. This will be this work's core and will be presented in Chapter 4.

CHAPTER 4 – RESULTS AND DISCUSSION

GENERAL CONSIDERATIONS

The number of facilitated samples from NOVA Medical School were not the 20 per organ as expected, for a total of 60; in the end only 14 became available for analysis. These samples were studied by the previously delineated process; this became this work's biggest obstacle, since the size of the available data is at the core of a reliable statistical analysis. To minimize this issue, repeated measurements were conducted for each sample, 3 with the Tri-axial Spectrometer and 2 with the M4 TORNADO, for a grand total of 70 measurements from 14 samples. From the 14 samples: half are from cancerous tissues and the other half are from healthy tissues; 4 are from bladder tissues, 6 from colon tissues and 4 from lung tissues. Each cancerous tissue sample has a healthy tissue sample pair from the same individual. This allows a more reliable comparison between trace elements concentrations from cancerous and healthy tissues, since when other samples from different individuals are considered, the biological diversity would mask correlations in trace elements concentration variations between similar tissues from different patients. Even so, all samples were studied throughout different parameters and ordered in diverse categories. All analysis obey the general-to-specific methodology. The first analysis includes the results from all available samples. The results are, then, differentiated by their corresponding organs and divided by their pairs, allowing a more specific and reliable analysis.

All the results were listed on an excel file with information on their original organ, the spectrometer utilized, the number of the measurement and whether it is cancerous tissue or healthy. The next step compared the results from cancerous tissues with the results from healthy ones. The results from the two spectrometers were separated due to their different quantification methods and associated errors. Therefore for each comparison there will be two graphs, one associated with the results from the Tri-axial spectrometer and the other with the ones from the TORNADO. Some element concentrations might differ from the two spectrometers due to their different quantification procedures and element detection levels. The concentrations tend to be higher in TORNADO because it runs an analysis on the selected and restricted areas that contain the cancerous part of the tissue. This is important due to the biopsy processes in which the identified cancerous tissue can also include parts consisting of healthy cells. On the contrary, Tri-axial runs an analysis on the whole sample, presenting the elements' average concentrations. A detailed comparison of the results obtained by the two referred techniques for the same tissue show discrepancies up to a factor of 5. One plausible explanation is the fact that the Tri-axial spectrometer is programmed to analyze samples with defined size (circular) and thickness, which is not the case the for the samples employed in this work, which have different characteristics among them and compared to the tri-axial's standard sample. Therefore, the results from TORNADO are expected to be more precise.

The concentrations associated errors are represented in each graph's error bars. When there are several measurements for the same sample, a single value is required, therefore a weighted average is made. This average and its associated error were calculated with excel's formulas, based on the general formula of uncertainty propagation, the weighted average being dependent on each measurement's concentration and its associated error. On the other hand, the weighted average's combined error depends on the standard deviation and every error from all considered measurements.

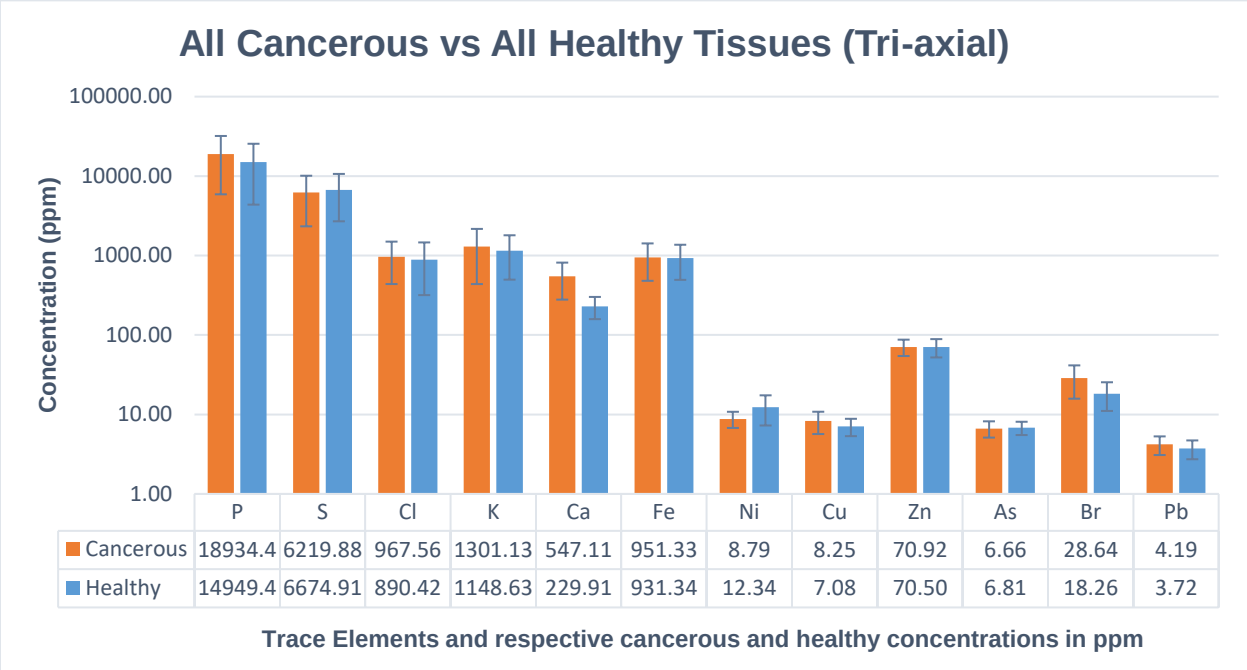
The actual concentrations values have no great importance due to diverse factors, such as the different quantification methods from the two spectrometers, possible laboratorial errors and measurement's uncertainty. The main analysis factor is the concentrations variation from one tissue to another, the concentration in cancerous tissues being compared to healthy ones. The graphs are in logarithmic scale in order to allow the observation of all elements concentrations, as they come in different orders of magnitude. This is the observable core of the entire work, on which the desired correlations will be supported.

In order to verify concentration variations credibly between cancerous and healthy tissues, a relative comparison was conducted on each tissue pair, comparing its cancerous tissue concentration with its corresponding healthy one. Then, the percentage of cases for which the cancerous concentrations increased or decreased when compared to healthy concentrations from the same element was calculated. Later some of the presented results are substantiated with these percentages, to improve their reliability. Only percentages above 68% at a first level and 80% at a second will be shown: 68% due to its similarity with a normal distribution confidence interval at one standard deviation; 80% as a chosen significant statistical value, not too high nor too low in order to overcome the problem of few available samples.

Next the graphs with the trace elements concentrations of each measurement are presented, comparing these concentrations in the cancerous tissues and the healthy ones. The objective is to find correlations between cancerous and healthy concentration ratios, in order to standardize these alterations and find evidence that trace elements have an important role in carcinogenesis. The obtained results will be compared to the ones from previous studies, as described in the state-of-the-art chapter, to verify their validity in case they are similar, or to find new evidence that can be related to carcinogenesis.

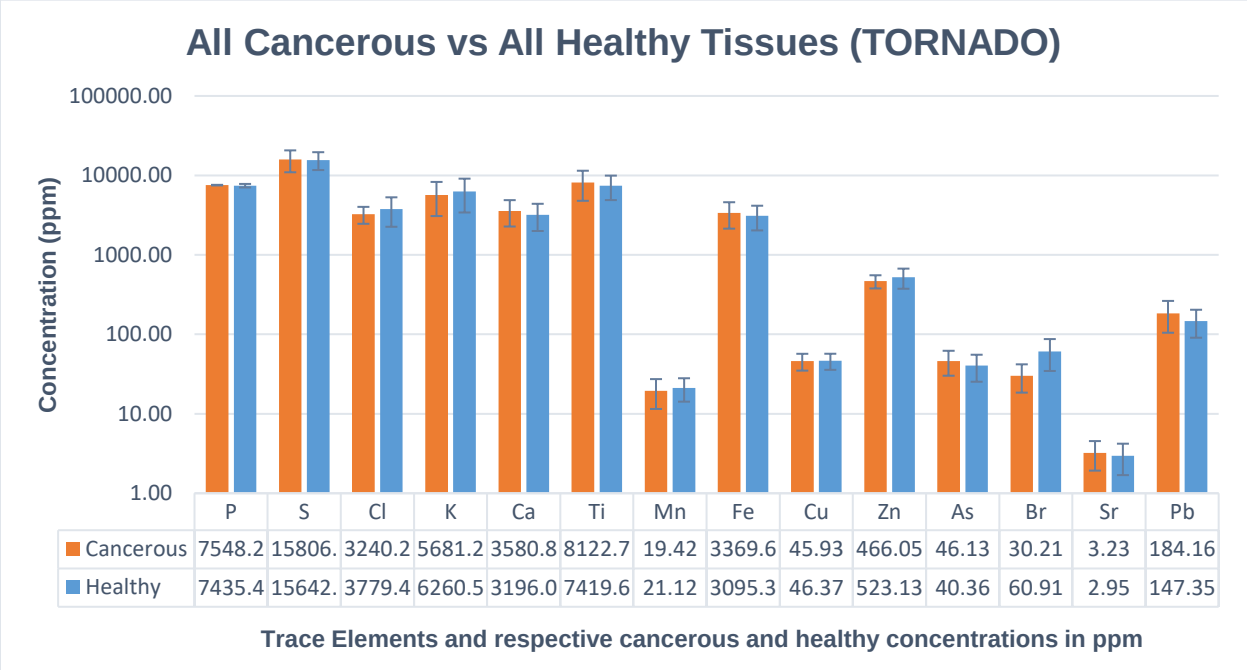
GRAPHIC ANALYSIS

ALL SAMPLES



	P	S	Cl	K	Ca	Fe	Ni	Cu	Zn	As	Br	Pb
Cancerous	18934.4	6219.88	967.56	1301.13	547.11	951.33	8.79	8.25	70.92	6.66	28.64	4.19
Healthy	14949.4	6674.91	890.42	1148.63	229.91	931.34	12.34	7.08	70.50	6.81	18.26	3.72

Figure 29 – Concentrations from all cancerous and healthy samples obtained from Tri-axial spectrometer.

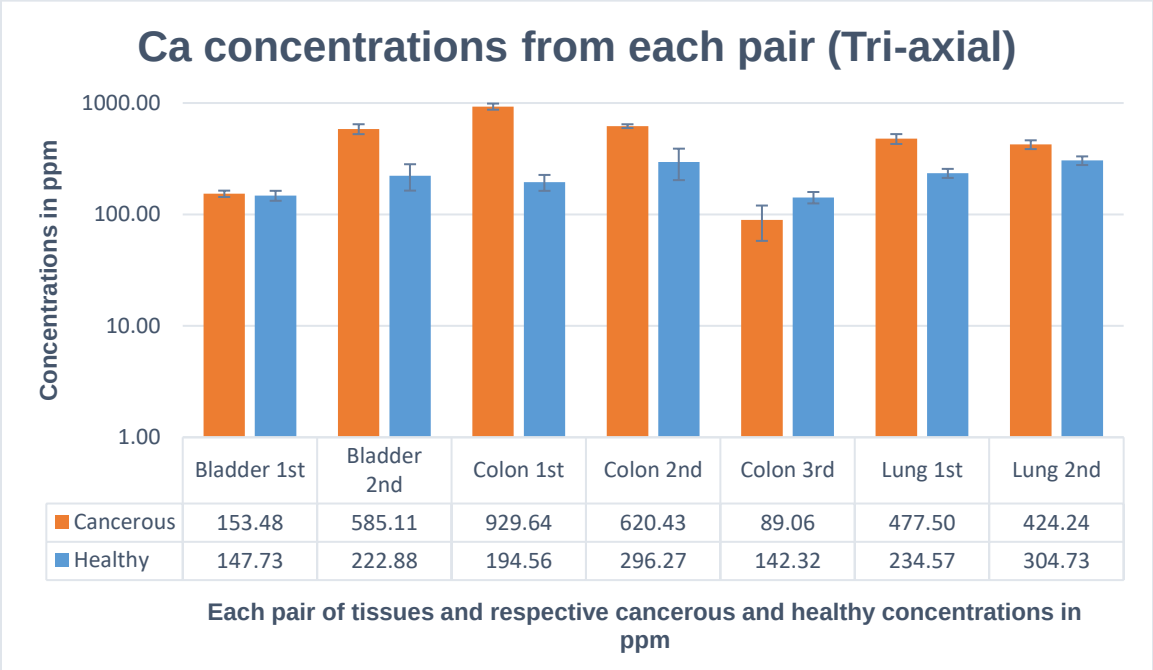


	P	S	Cl	K	Ca	Ti	Mn	Fe	Cu	Zn	As	Br	Sr	Pb
Cancerous	7548.2	15806.	3240.2	5681.2	3580.8	8122.7	19.42	3369.6	45.93	466.05	46.13	30.21	3.23	184.16
Healthy	7435.4	15642.	3779.4	6260.5	3196.0	7419.6	21.12	3095.3	46.37	523.13	40.36	60.91	2.95	147.35

Figure 30 – Concentrations from all cancerous and healthy samples obtained from TORNADO spectrometer.

These first two graphics have all 14 samples and 70 measurements in consideration, from all organs and pairs. This is the most general analysis of this work. Comparing the obtained results from all cancerous and healthy tissues it is observable that one element increases its concentration, other presents a decrease and the rest maintain their concentrations. Generally the results from the Tri-axial spectrometer are in agreement with the ones from TORNADO, however, there are some observable incoherencies between them. This is also explained by their different quantification methods and detection limits.

Several elements may show a small increase or decrease in their cancerous tissue concentrations, however, a difference is significant and valid only when the error bars do not include the same concentration range. Therefore there are only two elements that almost obey to these conditions. **Ca** shows higher concentrations in cancerous tissues and **Br** shows higher concentrations in healthy tissues. The Ca concentration variation is observed in the tri-axial graphic rather than Br variation that is observed in the Tornado graphic. In order to validate these results, the concentrations from each pair of tissues were directly compared, appearing as percentages of the cancerous tissues that show an increase or decrease in their concentrations.



	Bladder 1st	Bladder 2nd	Colon 1st	Colon 2nd	Colon 3rd	Lung 1st	Lung 2nd
Cancerous	153.48	585.11	929.64	620.43	89.06	477.50	424.24
Healthy	147.73	222.88	194.56	296.27	142.32	234.57	304.73

Figure 31 – Ca concentrations from each pair obtained from Tri-axial spectrometer.

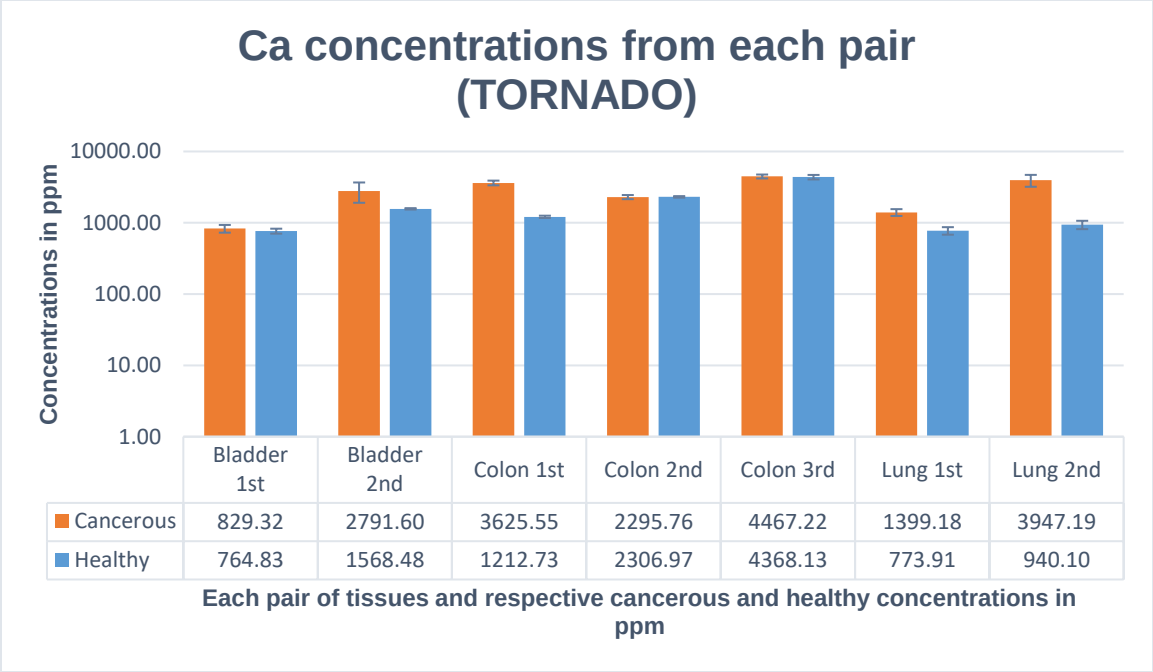


Figure 32 – Ca concentrations from each pair obtained from TORNADO spectrometer.

Thus, it was verified that Ca presented higher concentrations in 86% of all cancerous tissues, observable in the Ca graphics above, in which the concentrations from each pair of tissues are compared.

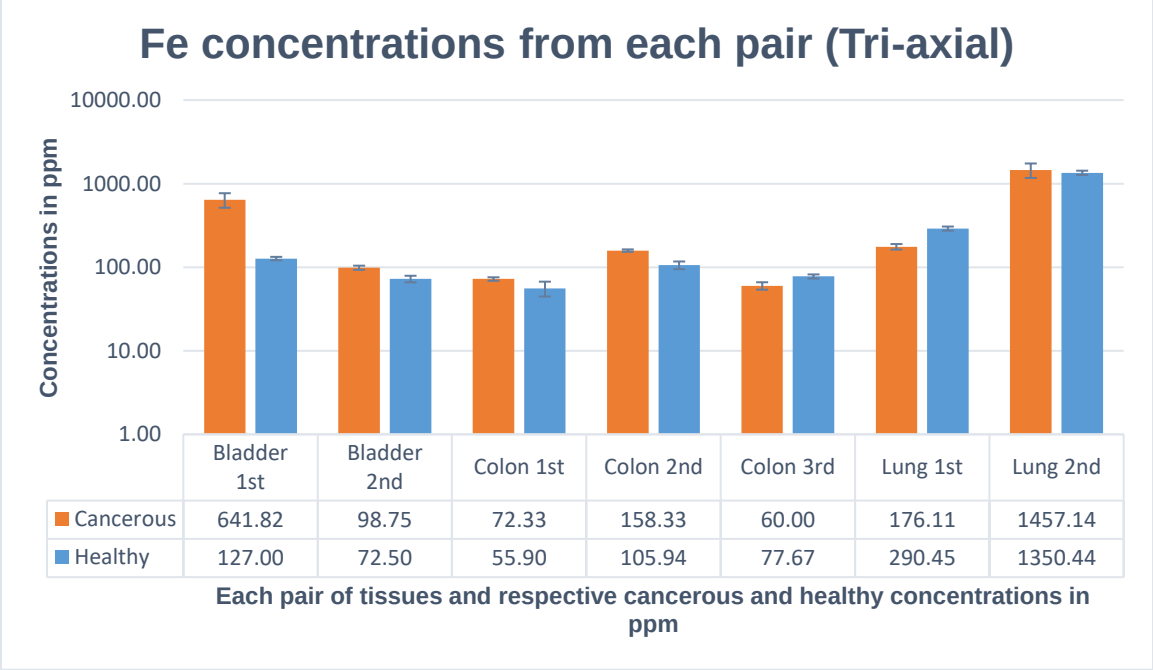


Figure 33 – Fe concentrations from each pair obtained from Tri-axial spectrometer.

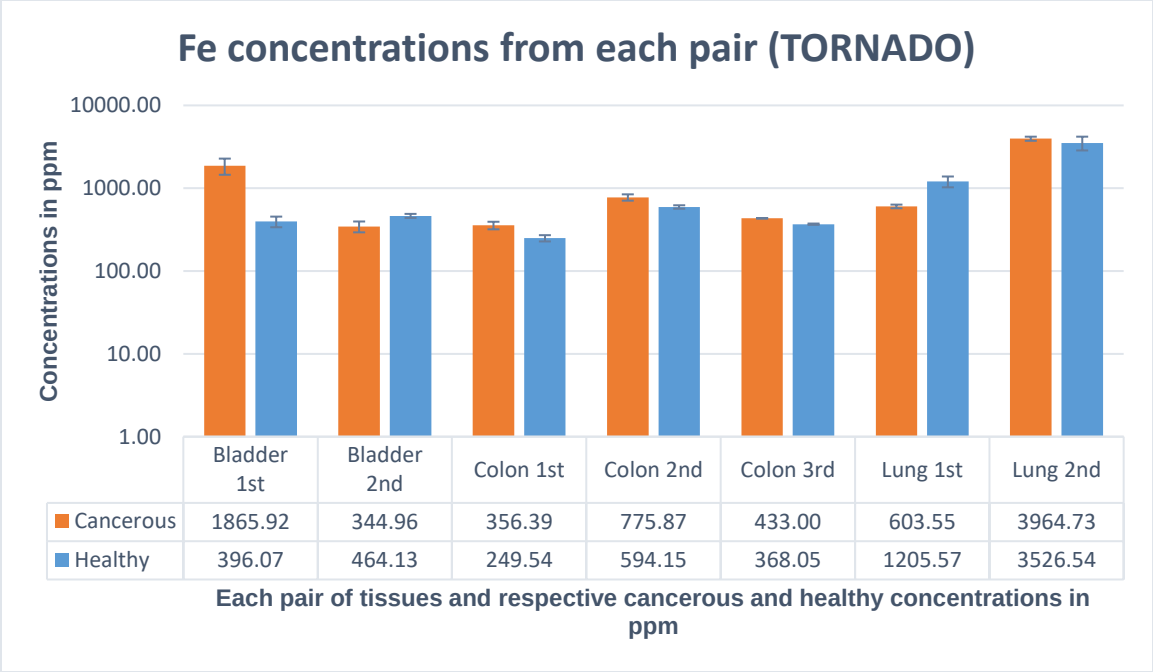


Figure 34 – Fe concentrations from each pair obtained from TORNADO spectrometer.

Although Fe does not show significant differences in its concentrations between cancerous samples, it shows an increase in its concentrations in 71% of the cancerous tissues compared to the correspondent healthy ones. This data is concordant between both spectrometers. This can be explained by the significance of the highest value in the calculation of the weighted average. Even if the majority of the concentrations is higher in cancerous tissues, the weighted average value is similar in both types of tissues, due to the high values of Fe concentration in the 2nd pair of lung tissues, which influences the weighted average. Biological variability is one of the factors that can mask trace elements concentration variations.

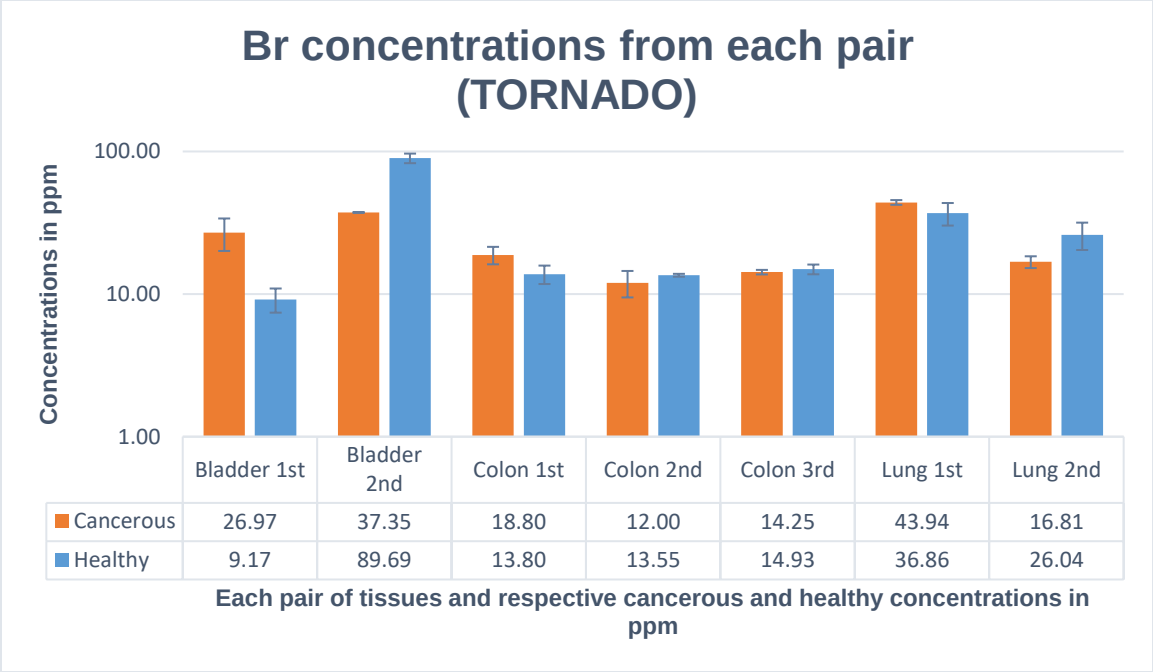


Figure 35 – Br concentrations from each pair obtained from TORNADO spectrometer.

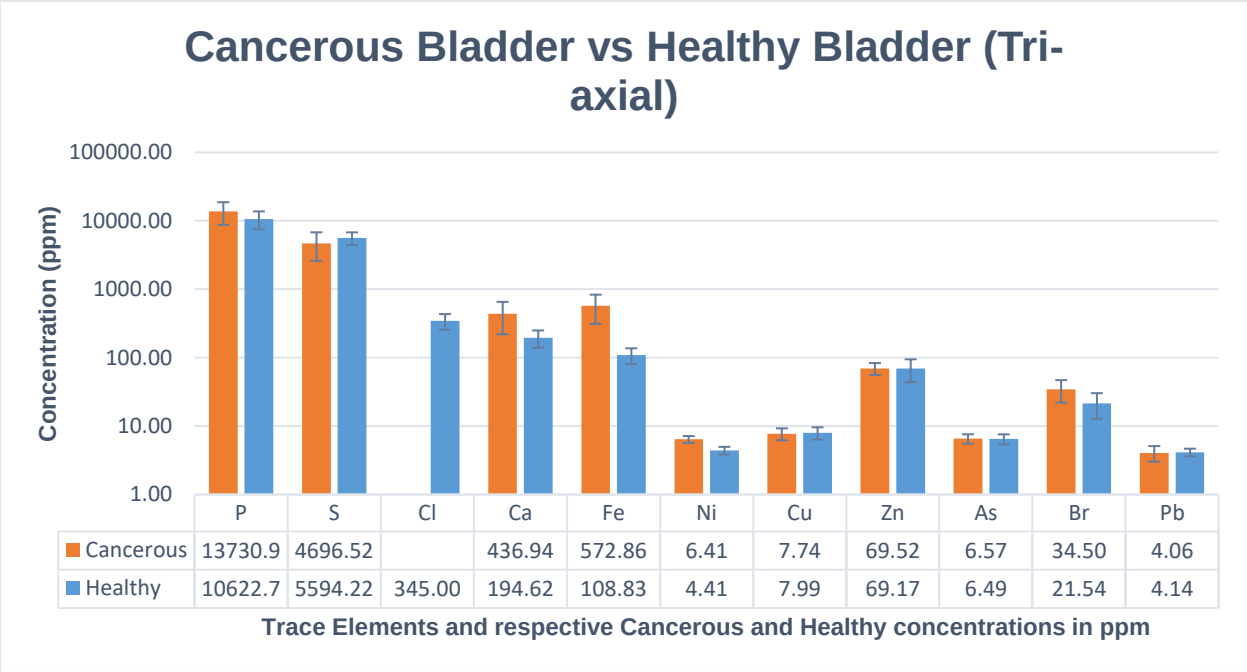
Even though the TORNADO graphic from all samples showed what might be considered an increase in Br healthy tissue concentrations, this graphic that compares each pair of tissue explains that variation. It also discredits this increase due to the fact that almost all concentrations are alike, except the ones from the 2nd pair of bladder tissues, which presents a great increase in healthy tissue concentration that justifies the variation previously observed.

As so, the most significant elements of this first comparison are Ca and Fe, showing an increase in their concentrations in cancerous tissues of the three types studied, when compared to their healthy counterparts. Past studies stated that excess of Fe present in a cell promotes cancer development [19] and that Br concentration decreases in cancerous tissues [27], while comparing colon cancerous and healthy tissues. There are no more relevant relations with results from previous studies due to the fact that those studies focused on specific organs and not gathering all data, like in this work's section.

DIVIDED BY ORGAN

Having compared cancerous and healthy tissues altogether, one concludes there are not many visible variations and the ones that were noted were not substantiated as wanted. Next, all cancerous and all healthy tissue samples will be divided by their original organ, trying to show trace elements concentration variations in specific organs, what is expected to be easier than with all samples in consideration.

BLADDER TISSUES



	P	S	Cl	Ca	Fe	Ni	Cu	Zn	As	Br	Pb
Cancerous	13730.9	4696.52		436.94	572.86	6.41	7.74	69.52	6.57	34.50	4.06
Healthy	10622.7	5594.22	345.00	194.62	108.83	4.41	7.99	69.17	6.49	21.54	4.14

Figure 36 – Concentrations from cancerous and healthy bladder tissue samples obtained from Tri-axial spectrometer.

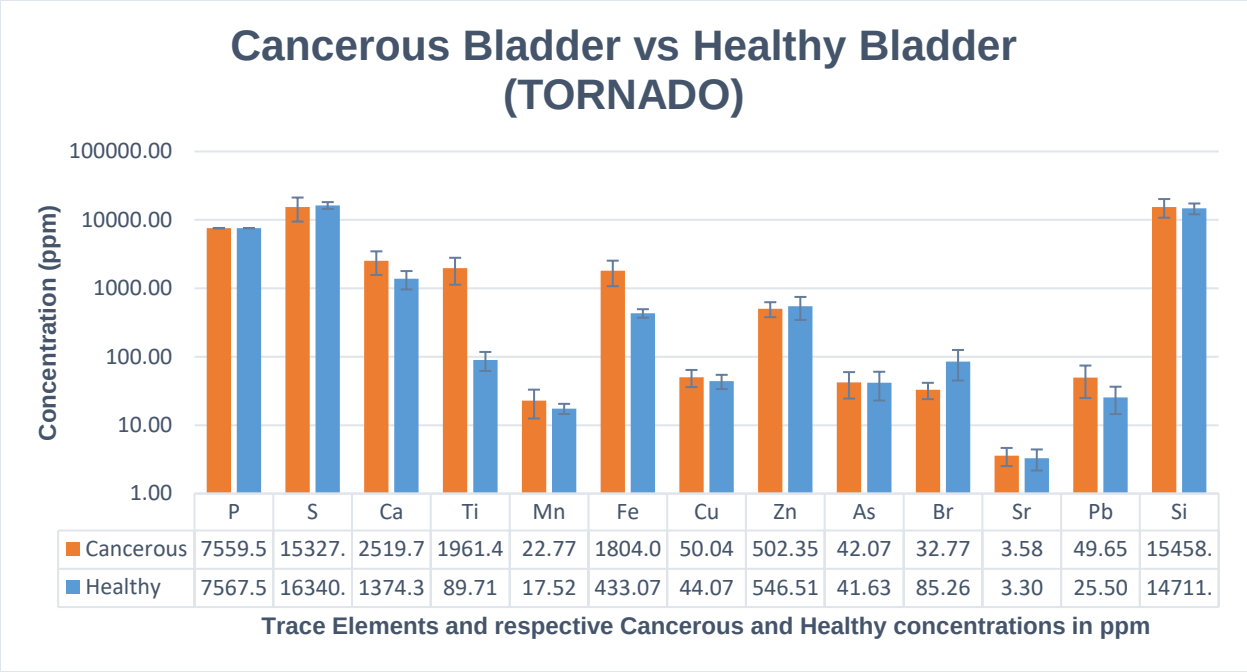


Figure 37 – Concentrations from cancerous and healthy bladder tissue samples obtained from TORNADO spectrometer.

These two graphics have all bladder tissues in consideration. There are 4 samples divided in two pairs of bladder tissues. As analyzed before, one can note that some elements increase their concentrations, others present a decrease and many have their concentrations unaltered, when comparing the average values of cancerous and healthy tissue concentrations.

It is easily verified that **Ca** and **Fe** appear on average in greater concentrations in cancerous tissues, according to both spectrometers. Other elements show variations in their concentrations according to one of the spectrometers, being unaltered or unseen in the other. For instance, Ni and Ti have higher concentrations in cancerous tissues, while Cl and Br show greater concentrations in healthy tissues. Ni and Cl, the two elements analyzed in Tri-axial may show a variation in their concentrations but analyzing each tissue concentration one observes that the validity of these results is questionable, either due to their very low values or to the fact that it is only observed in one measurement of only one type of tissues. Cl for example is a very good example, as it is superimposed on the Rh L-lines of the TORNADO X-ray tube, and thus were not effectively measured with this spectrometer. A similar effect happens with Sr at the Tri-axial, as the Compton peak from the secondary Mo target masks the K-lines of this element.

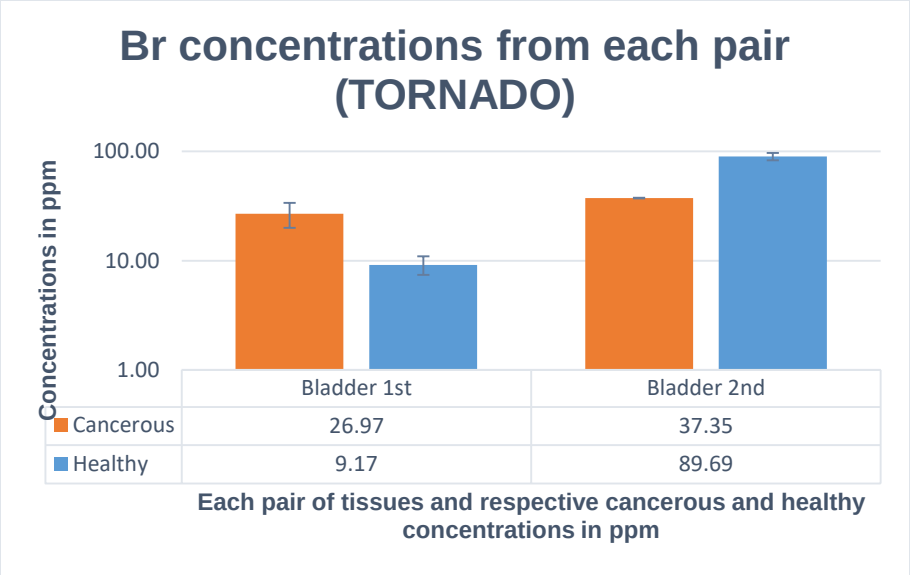


Figure 38 – Br concentrations from each pair of bladder tissues obtained from TORNADO spectrometer.

On the other hand, Ti and Br, analyzed in Tornado, have a different explanation, but also a questionable validity, as both present a variation due to one measurement that appeared with a great offset regarding the rest of the measurements.

Through relative comparison between cancerous and healthy concentrations from each pair of bladder tissue, it is observed that **Ca** and Arsenic present higher concentrations in 100% of all cancerous bladder tissues, according to both spectrometers.

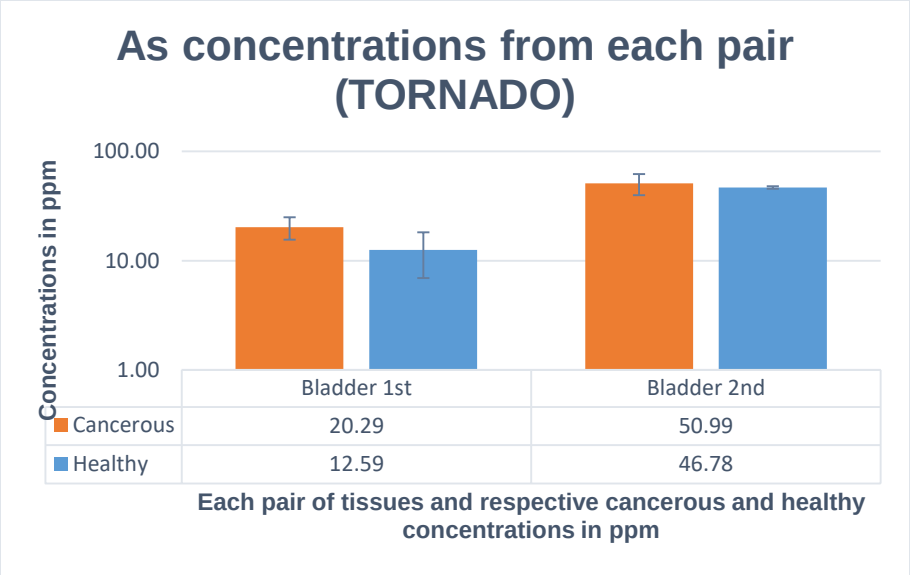


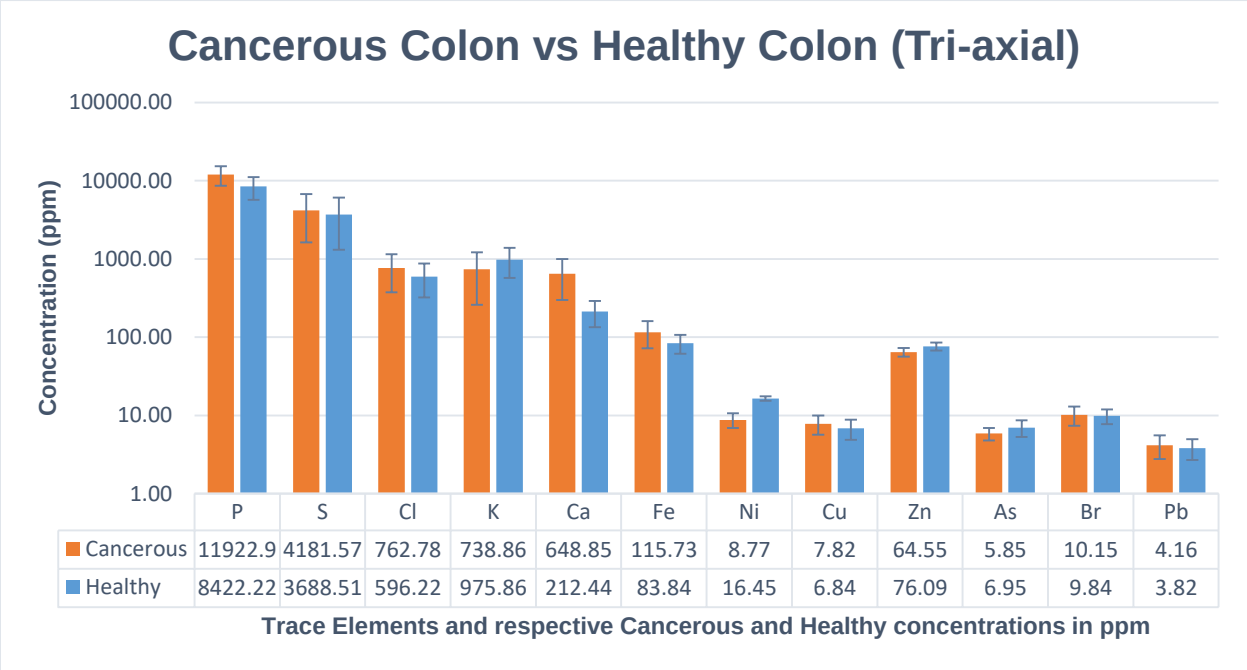
Figure 39 – As concentrations from each pair of bladder tissues obtained from TORNADO spectrometer.

However, looking at the As graphic, one can observe that the concentrations are almost alike, even if higher in cancerous tissues.

Other elements also appear in higher concentrations in all cancerous bladder tissues, despite corresponding to data from only one spectrometer. Fe in Tri-axial's analysis and Cu in Tornado's. These results confirm the increase of Fe concentration in cancerous bladder tissues. As for Cu, its concentrations are very alike, just a little higher in cancerous tissues, which is not significant, although all cancerous tissues have higher concentrations. This is the problem of having few available samples from each organ, which prevents us from drawing more robust conclusions.

The most significant elements in bladder tissues are Ca and Fe, both presenting higher concentrations in cancerous tissues, when compared to healthy ones. There are no results regarding these two elements in previous bladder tissue studies.

COLON TISSUES



	P	S	Cl	K	Ca	Fe	Ni	Cu	Zn	As	Br	Pb
Cancerous	11922.9	4181.57	762.78	738.86	648.85	115.73	8.77	7.82	64.55	5.85	10.15	4.16
Healthy	8422.22	3688.51	596.22	975.86	212.44	83.84	16.45	6.84	76.09	6.95	9.84	3.82

Figure 40 – Concentrations from cancerous and healthy colon tissue samples obtained from Tri-axial spectrometer.

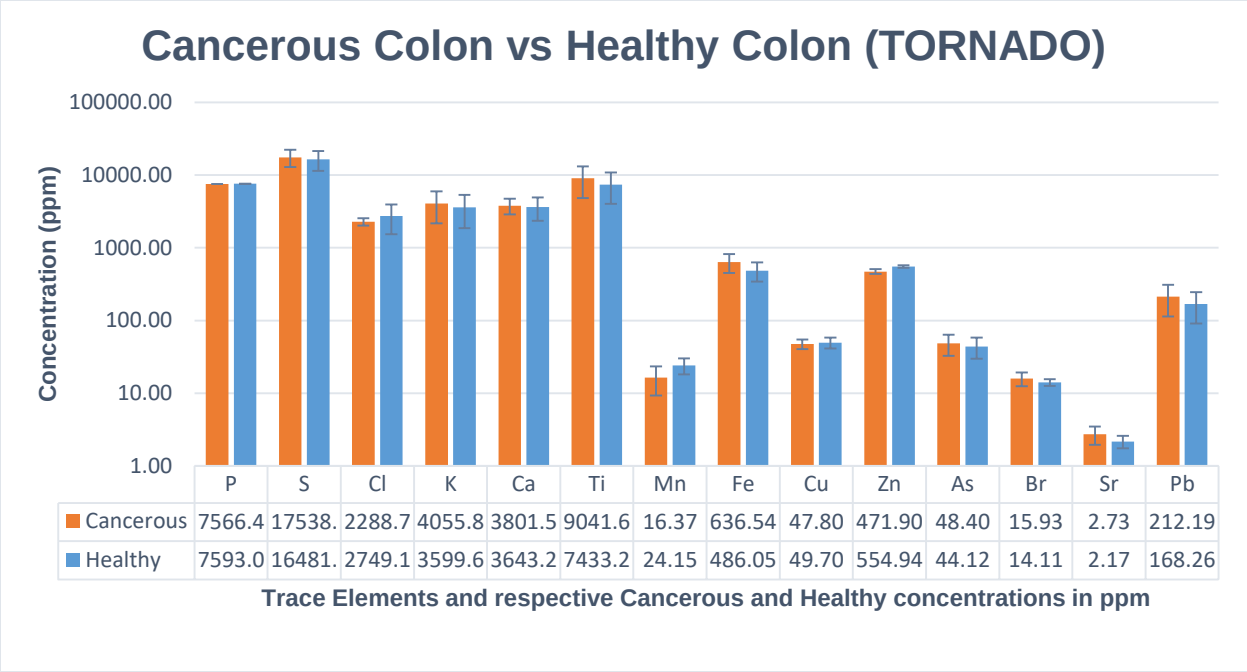


Figure 41 – Concentrations from cancerous and healthy colon tissue samples obtained from TORNADO spectrometer.

These two graphics have all 6 colon tissues, divided in three pairs of cancerous and healthy tissues. Comparing with the previous graphics, colon tissues present fewer variations in trace elements' concentrations.

Analyzing the two graphics it is observable that Ca, once again, increases its concentration in cancerous tissues, comparing to healthy ones. However, this is only seen in the Tri-axial graphic, as well as Ni, which presents higher concentrations in healthy tissues. As for Tornado's graphic analysis, only Zn appears to change its concentration regarding the type of tissue, presenting higher concentration in healthy tissues. This is almost visible in the Tri-axial graphic, although the error bars slightly overlap.

Even if Ni seems to alter its concentration, the results are questionable due to its very low concentrations and the fact that Ni is only found in one of the three colon pairs and very near the technique detection limit.

Relative comparison between cancerous and healthy concentrations from each pair of colon tissues shows that Zn appears in higher concentrations in 100% of all healthy colon tissues, regarding data from both spectrometers. Furthermore, Fe and S also show higher concentrations in all cancerous colon tissues, the first according to Tornado and the second according to Tri-axial. In the case of S, its concentrations are very alike, even if they tend to be higher in cancerous tissues. As for Fe, these variations are more significant, and even in the Tri-axial analysis, it tends to have higher concentrations in cancerous tissues.

Therefore, the most significant elements when comparing colon tissues are Ca, Fe and Zn. The first ones showing significant variations as higher concentrations in cancerous tissues and the last clearly the opposite. These results confirm precisely previous studies that indicated these three elements as the most

important in the distinction of cancerous and healthy colon tissues [24]. In other studies Ca is thought to maintain unaltered its concentration, while Zn either maintains or decreases its concentration in cancerous tissues [27].

LUNG TISSUES

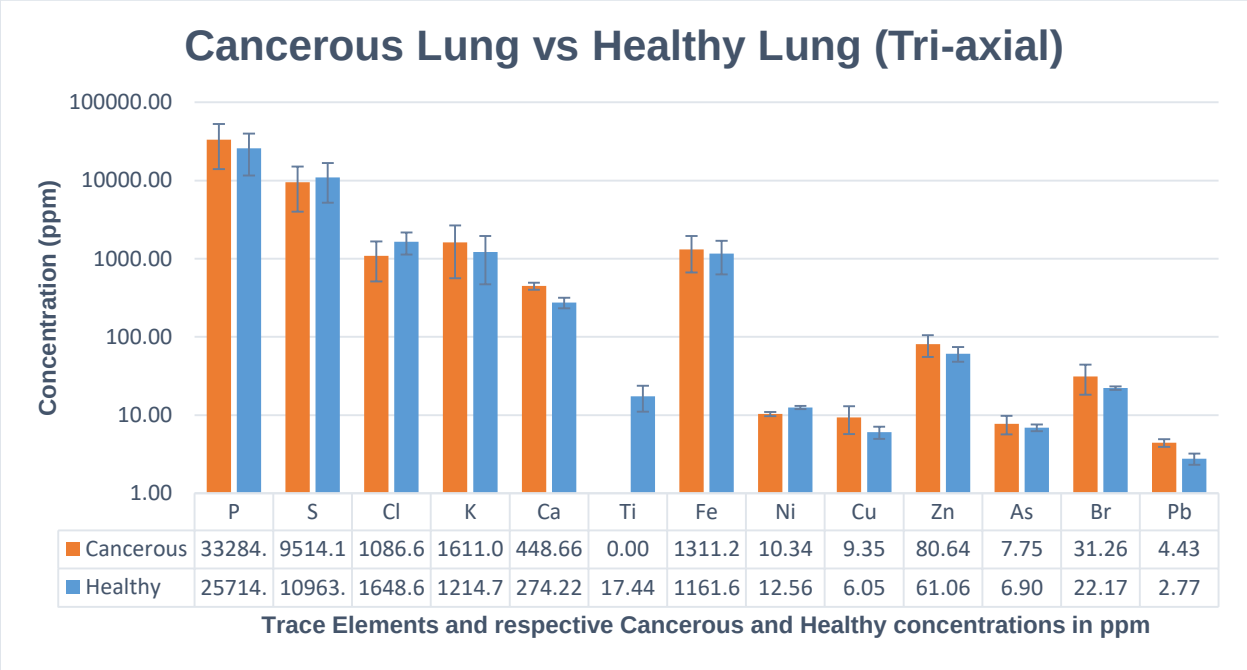


Figure 42 – Concentrations from cancerous and healthy lung tissue samples obtained from Tri-axial spectrometer.

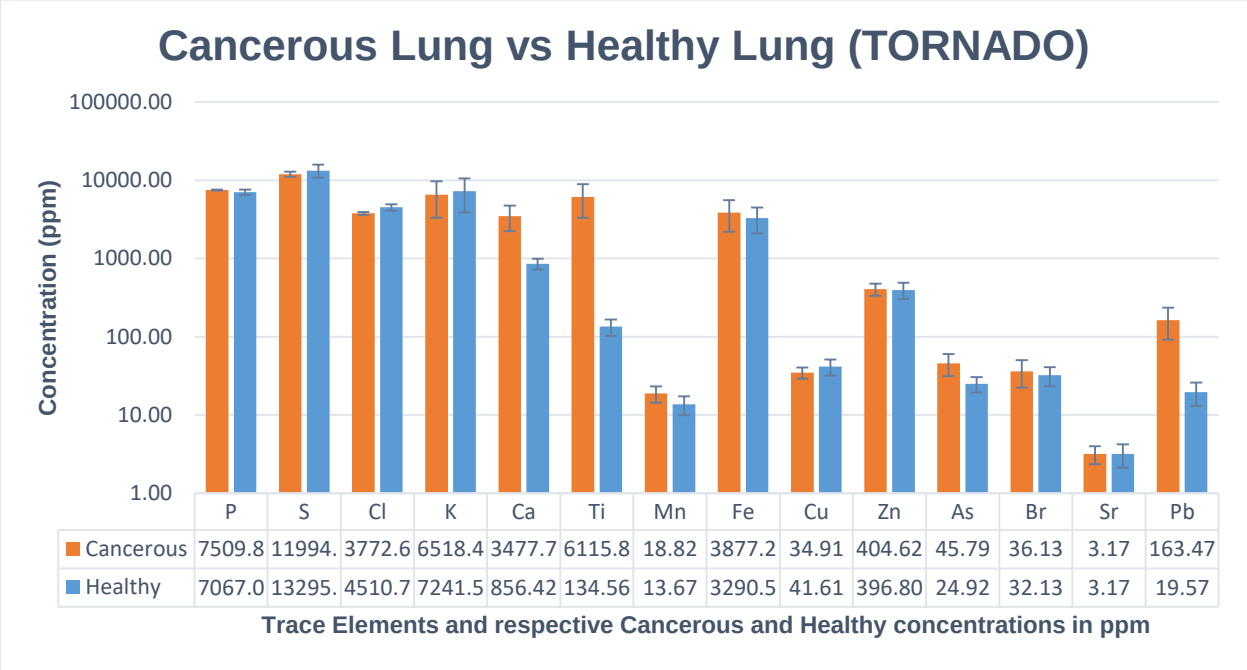


Figure 43 – Concentrations from cancerous and healthy lung tissue samples obtained from TORNADO spectrometer.

These two graphics correspond to the 4 lung cancerous and healthy tissues that are divided in two different pairs. In comparison to the previous graphics, these ones show more different elements that vary their concentrations between cancerous and healthy tissues.

The graphics above show two elements that increase their concentrations in cancerous lung tissues, Ca and Pb, according to data from both spectrometers. Arsenic also has higher concentrations in cancerous tissues, but only according to Tornado. Analyzing this spectrometer's data, it is observable that Cl presents a decrease in its concentration in cancerous tissues.

Ti shows a curious incoherency, showing higher concentration in cancerous tissues according to Tornado but, analyzing Tri-axial graphic, Ti is only found in healthy tissues through all three conducted measurements, which is very odd. A reevaluation of these samples should be performed in the future using other techniques such as PIXE, etc.

Regarding the relative comparison between cancerous and healthy concentrations from each pair of lung tissues, two elements have their concentrations increased in 100% of all cancerous lung tissues, according to both spectrometers. These elements are Ca, as usual, and, surprisingly, Zn. Other elements also show this tendency, however, according to only one of the spectrometers, such as P, Cu and Mn. On the other hand, S and K appear in higher concentrations in 100% of all healthy lung tissues.

Ca, once again, show high concentration variations, with its cancerous tissue concentrations appearing increased. Zn, however, may appear in higher concentrations in cancerous tissues but the differences comparing to healthy tissues are not that significant. In the case of Pb, the variations are high due to the fact that it is only found in one of the pairs of lung tissues (Tri-axial) and that one of the measurements is much higher than all others (Tornado). The rest of the elements described above show a tendency to either increase or decrease their concentrations, however, these variations are not really significant, hence the results do not allow strong conclusions.

The most significant statistical values from concentration variations correspond to Ca, Zn and Pb, all showing variations between cancerous and healthy tissues, appearing higher in cancerous ones. Previous studies show contradictory results regarding Ca and Zn, decreasing their concentrations in cancerous lung tissues. As for Pb, previous results indicated an increase in its cancerous tissue concentration, as well as stated above [23].

DIVIDED BY TISSUE PAIRS (FROM THE SAME PATIENT)

Finally the most specific analysis will compare the cancerous and healthy tissues concentrations from each individual pair of tissues. There are 2 pairs of bladder tissue, 3 pairs of colon tissue and 2 pairs of lung tissue. This analysis promises more reliable data due to the fact that each pair of tissues correspond to the same patient, where biological diversity and other factors cannot interfere. This regarding each element's pair and not between two different pairs. Though, the short number of samples affect once more the significance of the obtained results and correlations found.

In this section the relative comparison method will not be applied for obvious reasons, as the compared results correspond to only one concentration from cancerous tissue and another one from healthy tissue, in contrast with previous analyses that were averages of concentrations from different tissues. However, an alternative method will substantiate the observed variations, where the concentrations from each measurement of the same sample will be displayed, rather than the average of all measurements, in order to enhance each element's tendency to either increase or decrease its concentration in cancerous tissues.

BLADDER TISSUES

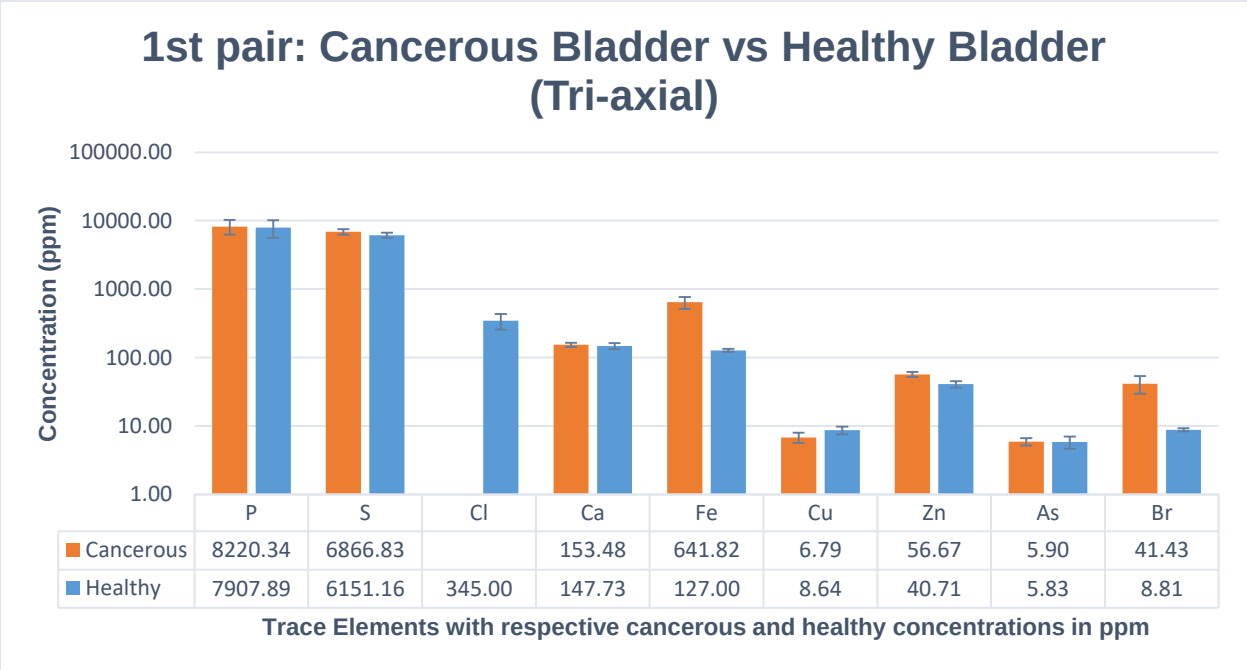


Figure 44 – Concentrations from cancerous and healthy bladder (1st pair) tissue samples obtained from Tri-axial spectrometer.

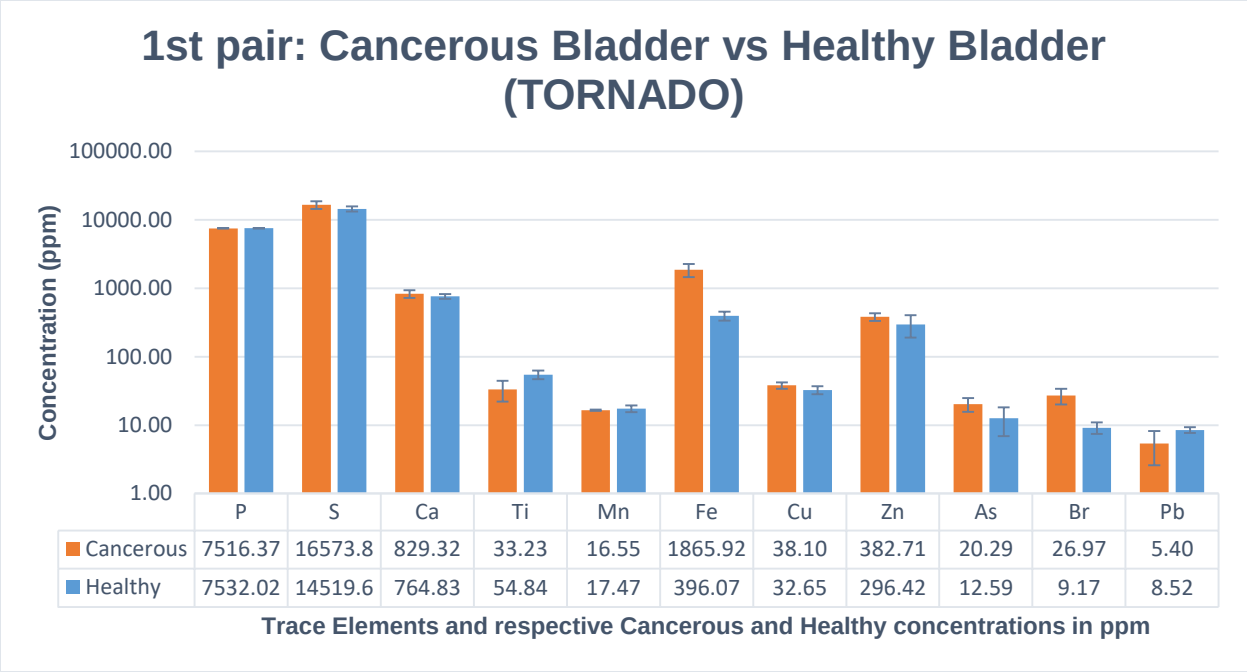


Figure 45 – Concentrations from cancerous and healthy bladder (1st pair) tissue samples obtained from TORNADO spectrometer.

These first two graphics correspond to the 1st pair of bladder tissues, one cancerous and the other healthy, both from the same individual. One can observe that Fe and Br increase their concentrations in cancerous tissues according to both spectrometers data. Zn also have higher concentrations in cancerous tissues when comparing to healthy ones, as shown by Tri-axial's results. Cl and Ti appear to increase their concentrations in healthy tissues according to Tri-axial, the first, and according to Tornado, the second.

Analyzing each measurement's concentration, the variations presented by Fe and Br are significant for both spectrometers.

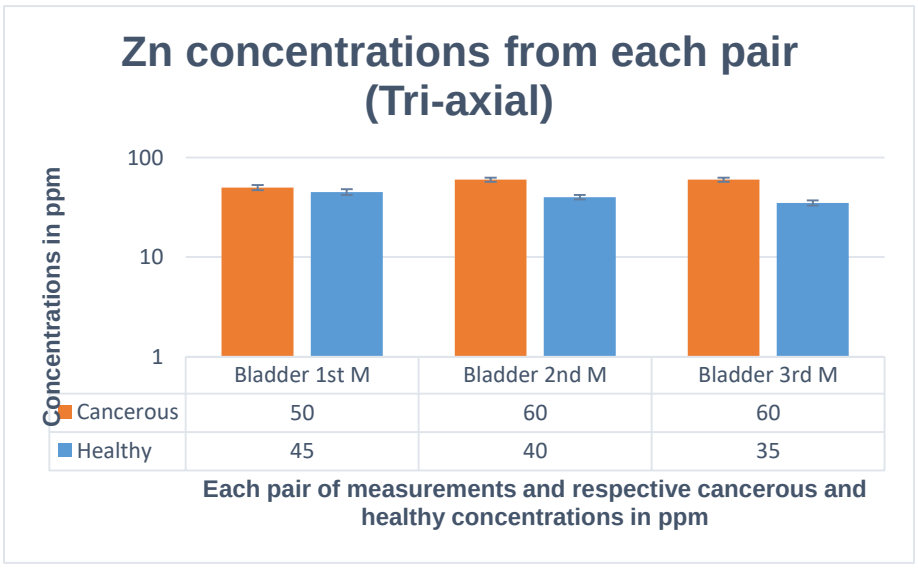


Figure 46 – Zn concentrations from each measurement of bladder (1st pair) tissues obtained from Tri-axial spectrometer.

On the other hand, the variations in Zn concentration are less significant, although the tendency to increase its concentration in cancerous tissues is verified. The variations in Ti concentration are even less significant, being almost alike in cancerous and healthy bladder tissues.

Therefore the most significant elements in this pair of bladder tissues are Fe and Br, both presenting higher concentrations in cancerous tissues. The Fe results are in conformity with the ones from all bladder tissues comparison.

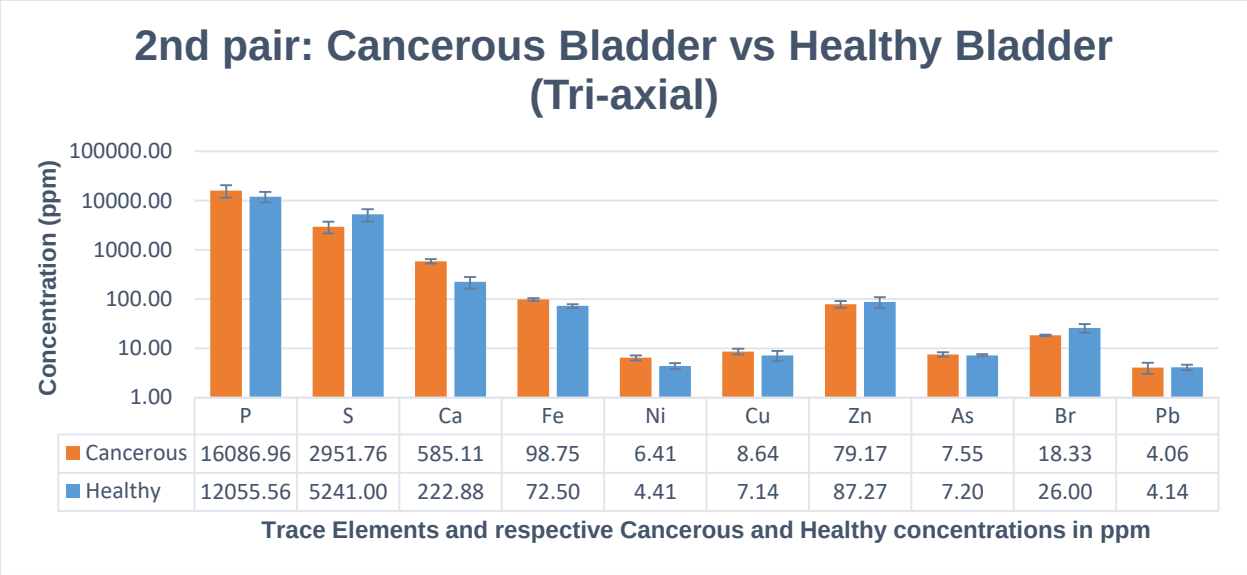


Figure 47 – Concentrations from cancerous and healthy bladder (2nd pair) tissue samples obtained from Tri-axial spectrometer.

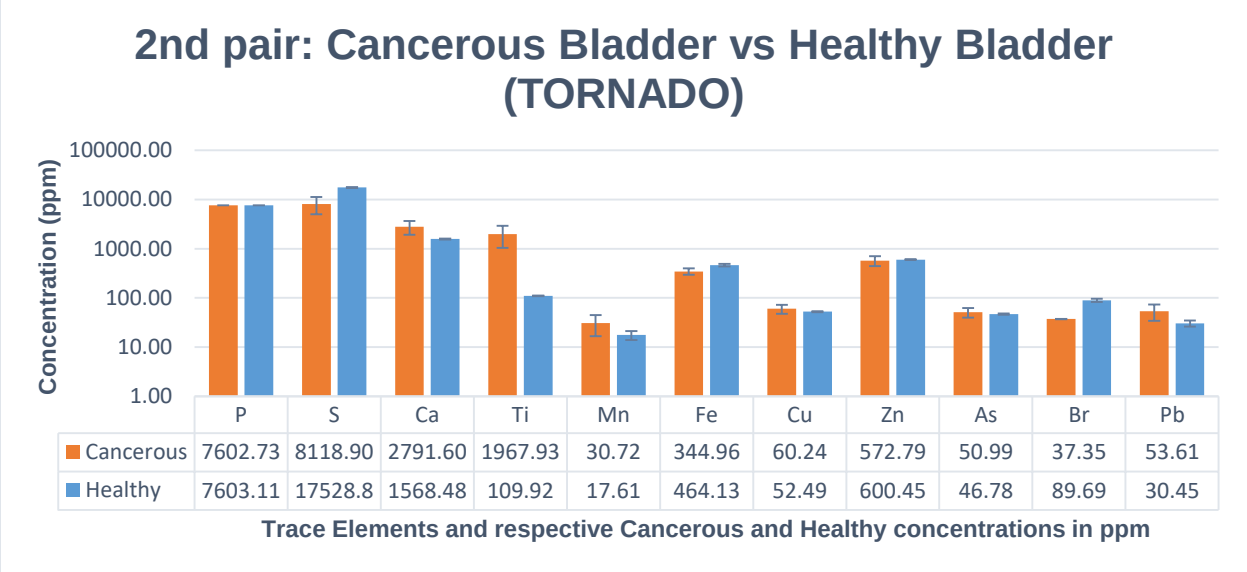


Figure 48 – Concentrations from cancerous and healthy bladder (2nd pair) tissue samples obtained from TORNADO spectrometer.

These next graphics refer to the 2nd pair of bladder tissues. Both spectrometers show that Ca increase its concentration in cancerous tissues, while S and Br present a decrease in the same type of tissue. Other elements also have higher concentrations in cancerous tissues, such as Ni and Ti.

By analyzing each measurement, it is observable that Ca, S and Br concentration variations are consistent and significant.

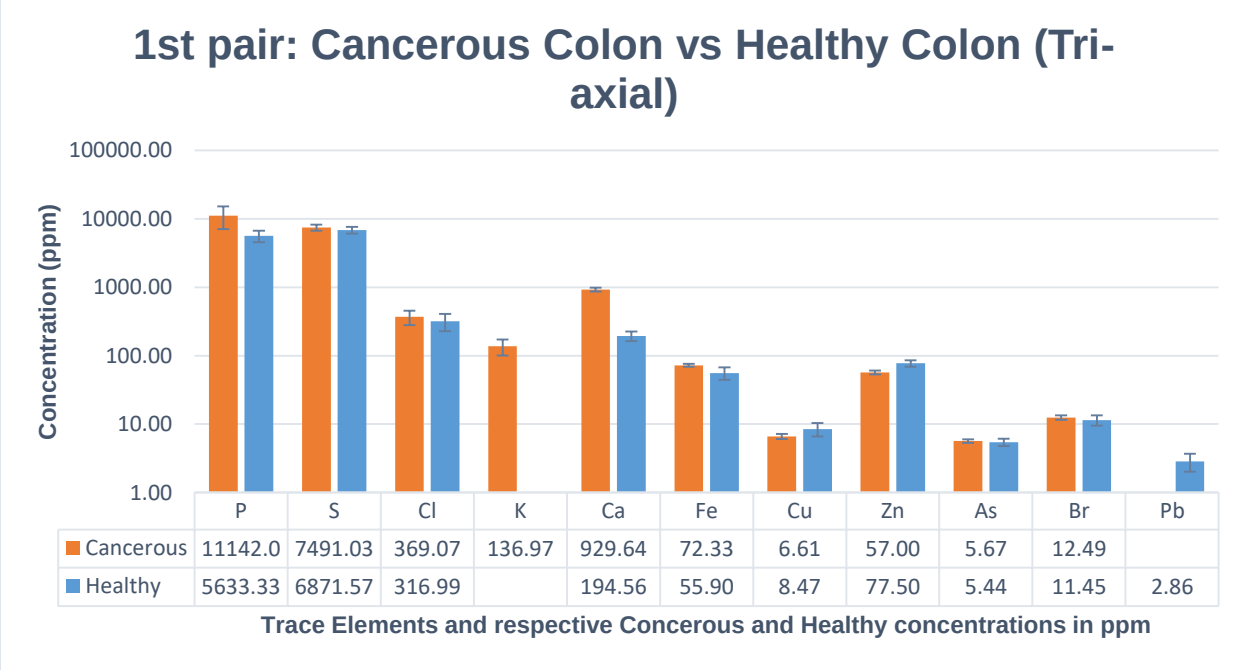
The Fe concentrations are almost all alike, between measurements from the same spectrometer, even if tending to increase (Tri-axial) or to decrease (Tornado).

As for Ni, its concentrations are so close to the technique detection limit that the results are not very reliable. In the case of Ti, one of the measurements showed a great difference comparing to all others, even when comparing with the other from the same tissue, which explains the variation of Ti in the Tornado graphic.

Therefore, Ca, S and Br are the most significant elements according to bladder tissues' 2nd pair. Curiously, Br has the complete opposite result regarding the other bladder tissue pair, which may explain the unaltered concentrations of Br in the comparison of all bladder tissues (Fig. 36).

Combining the results from both bladder pairs, two elements are concordant with the global bladder comparison, **Ca** and **Fe**, both increasing their concentrations in cancerous tissues.

COLON TISSUES



	P	S	Cl	K	Ca	Fe	Cu	Zn	As	Br	Pb
Cancerous	11142.0	7491.03	369.07	136.97	929.64	72.33	6.61	57.00	5.67	12.49	
Healthy	5633.33	6871.57	316.99		194.56	55.90	8.47	77.50	5.44	11.45	2.86

Figure 49 – Concentrations from cancerous and healthy colon (1st pair) tissue samples obtained from Tri-axial spectrometer.

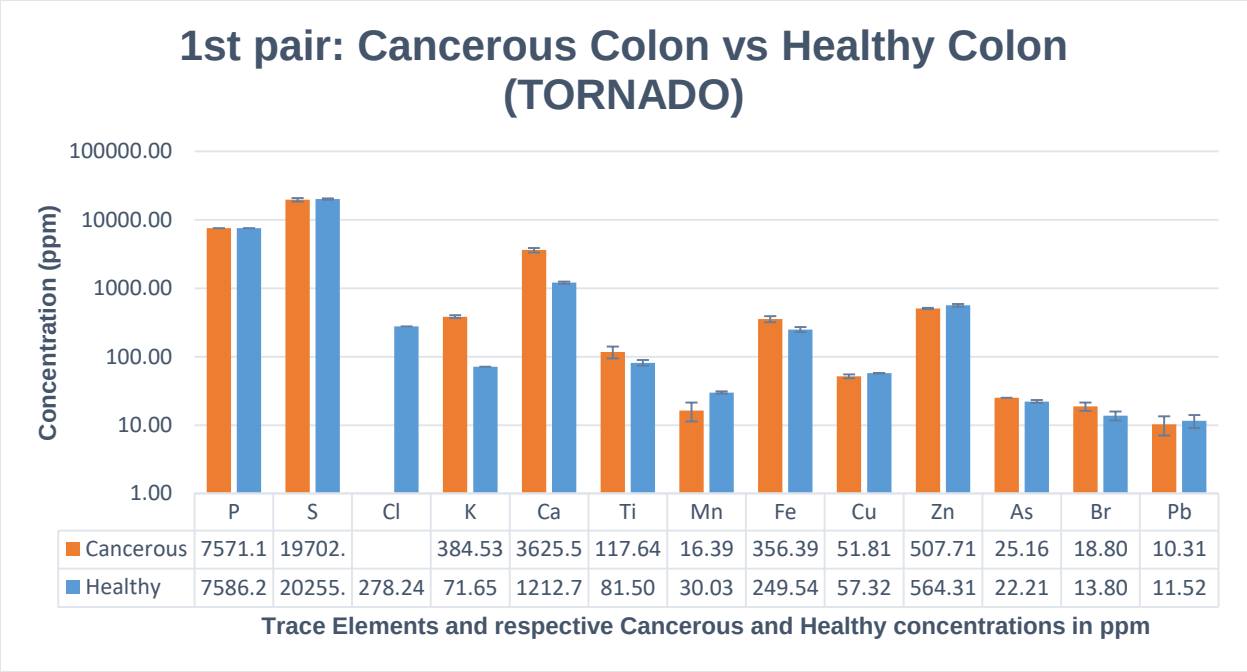


Figure 50 – Concentrations from cancerous and healthy colon (1st pair) tissue samples obtained from TORNADO spectrometer.

The previous graphics have the first pair of colon tissues in consideration. According to both spectrometers' data, it is observable that the concentrations of Ca, K and Fe appear higher in cancerous tissues, while the concentration of Zn is higher in healthy tissues.

Other elements that show an increase in their concentrations in cancerous tissues are P and Ti. On the other hand, Mn and Pb show a decline in their concentrations.

While Ca and K show significant results when comparing each measurement, Fe and Zn, although tending to have higher concentrations in cancerous tissues and healthy ones, respectively, their concentrations are very similar, which questions their variations.

P have consistent results according to Tri-axial, but when comparing cancerous and healthy tissues concentrations in Tornado, its concentrations are alike in both types of tissues.

As for Ti, Mn and Pb, the variations are not significant, either by being very similar or by presenting low concentration values, which questions their validity.

The most significant elements in this colon pair are Ca and K. Comparing to global colon comparison Ca and Zn are the concordant elements with the same variations in their concentrations.

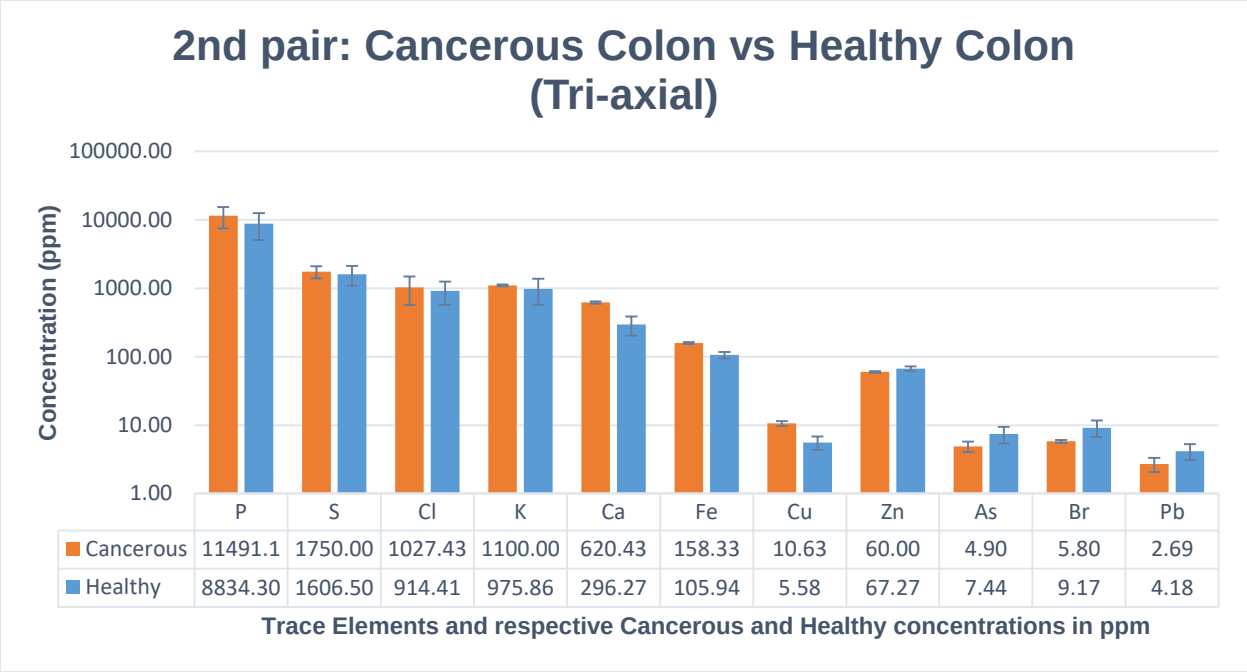


Figure 51 – Concentrations from cancerous and healthy colon (2nd pair) tissue samples obtained from Tri-axial spectrometer.

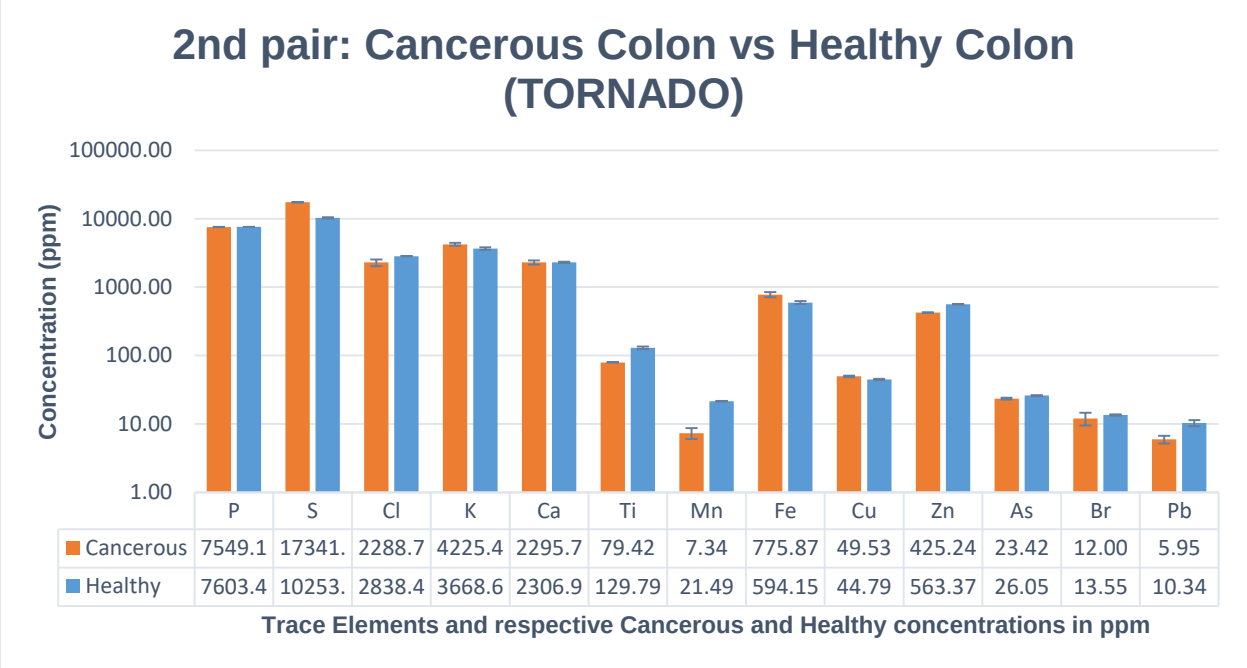


Figure 52 – Concentrations from cancerous and healthy colon (2nd pair) tissue samples obtained from TORNADO spectrometer.

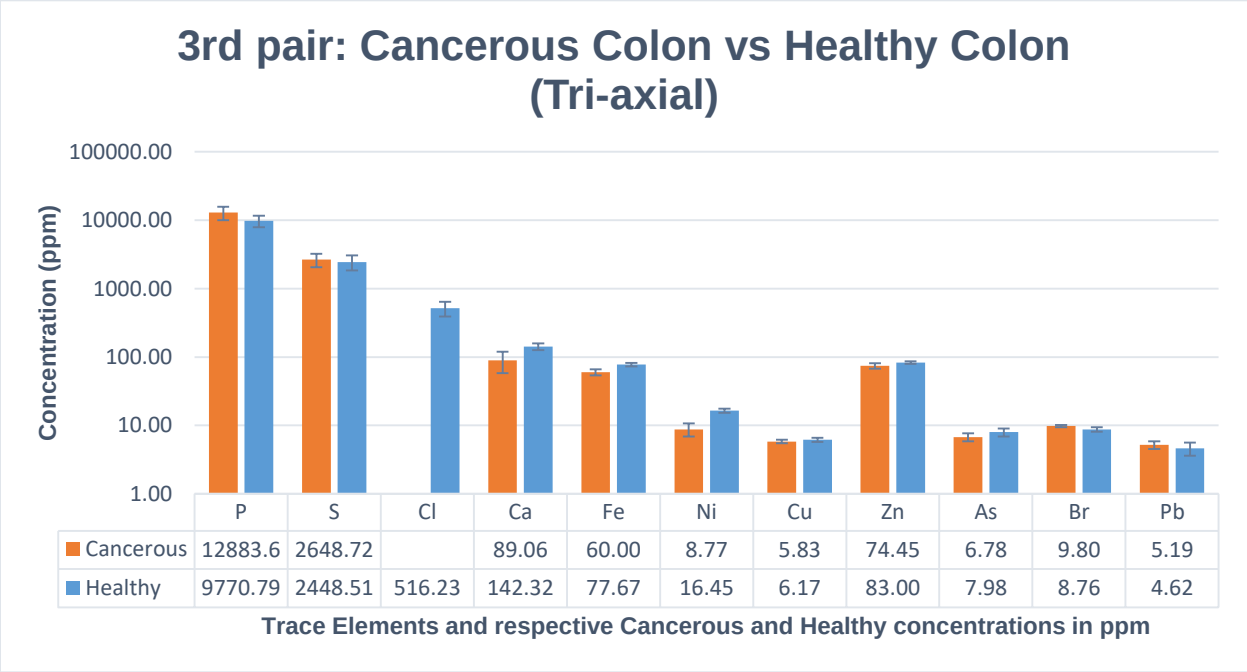
These graphics refer to colon's 2nd pair of tissues and reveal the raise in the cancerous tissue concentrations of **Fe** according to both spectrometers. Regarding only one of the spectrometers other elements tend to have higher concentrations in cancerous tissues, such as Ca, Cu and S. On the other hand, Cl, Ti, Mn, Zn, Br and Pb show higher concentrations in healthy tissues.

Fe variations are consistent and significant, when comparing each measurement's concentration.

Ca, **S**, Cl, Ti and Zn show significant variations in their concentrations according to one of the spectrometers, maintaining their concentration in the other.

Cu, Br, Mn and Pb present questionable results as their concentrations are too low for their variations to be considered significant.

Therefore, the most significant elements found in this pair of colon tissues are Fe, Ca and S. One of them, Ca, has concordant results with the 1st pair of colon tissues' and global colon comparison's variations.



	P	S	Cl	Ca	Fe	Ni	Cu	Zn	As	Br	Pb
Cancerous	12883.6	2648.72		89.06	60.00	8.77	5.83	74.45	6.78	9.80	5.19
Healthy	9770.79	2448.51	516.23	142.32	77.67	16.45	6.17	83.00	7.98	8.76	4.62

Figure 53 – Concentrations from cancerous and healthy colon (3rd pair) tissue samples obtained from Tri-axial spectrometer.

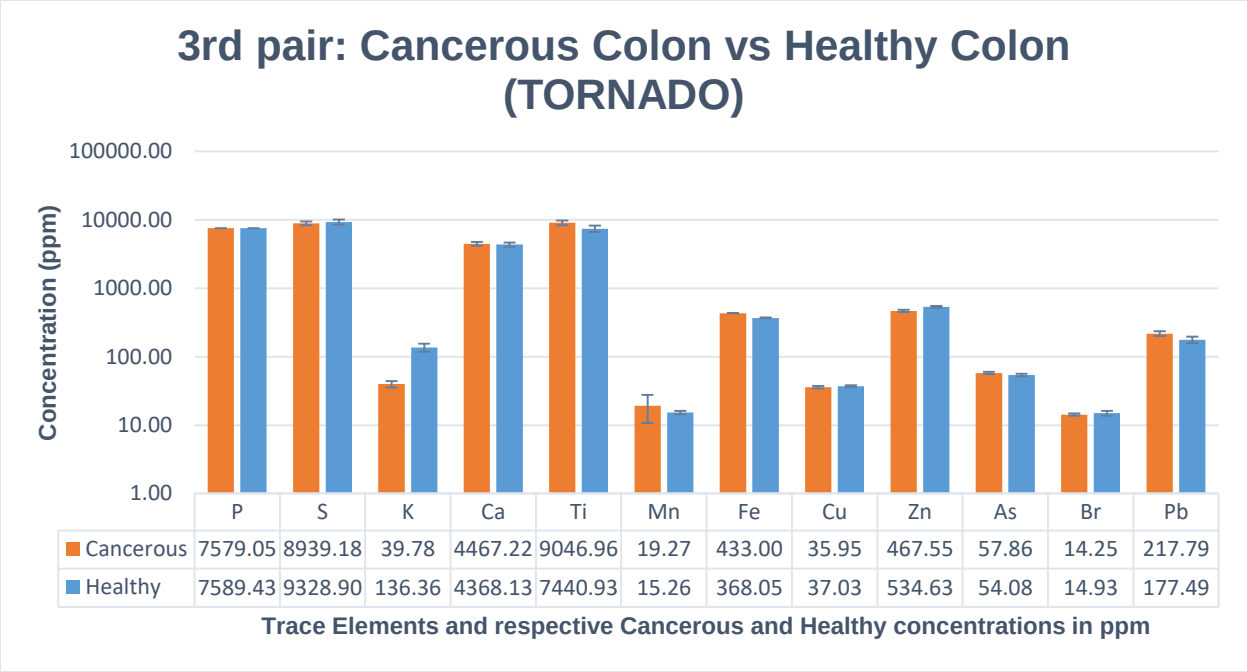


Figure 54 – Concentrations from cancerous and healthy colon (3rd pair) tissue samples obtained from TORNADO spectrometer.

These two graphics have the 3rd pair of colon tissues in consideration. Unlike the two previous pairs this one shows no concordant element's variations between both spectrometers. Furthermore, Fe has incoherent variations between spectrometers, while in the other pairs, it presented a constant raise in the concentrations of cancerous tissues.

This pair's elements that appear in higher concentrations in healthy tissues, according to only one spectrometer are Cl, K, Ca, Ni and Zn, while the only one that has lower concentration in this type of tissue is Pb, which reveals once again incoherency when comparing to other pairs.

The incoherency between Fe variations may be explained through the comparison of each measurement's concentration, by the fact that in Tri-axial its concentrations are much alike, even if it tends to be higher in healthy tissues. As for Tornado's results, Fe variations are more significant, therefore less questionable, showing an apparent increase in its cancerous tissue concentrations.

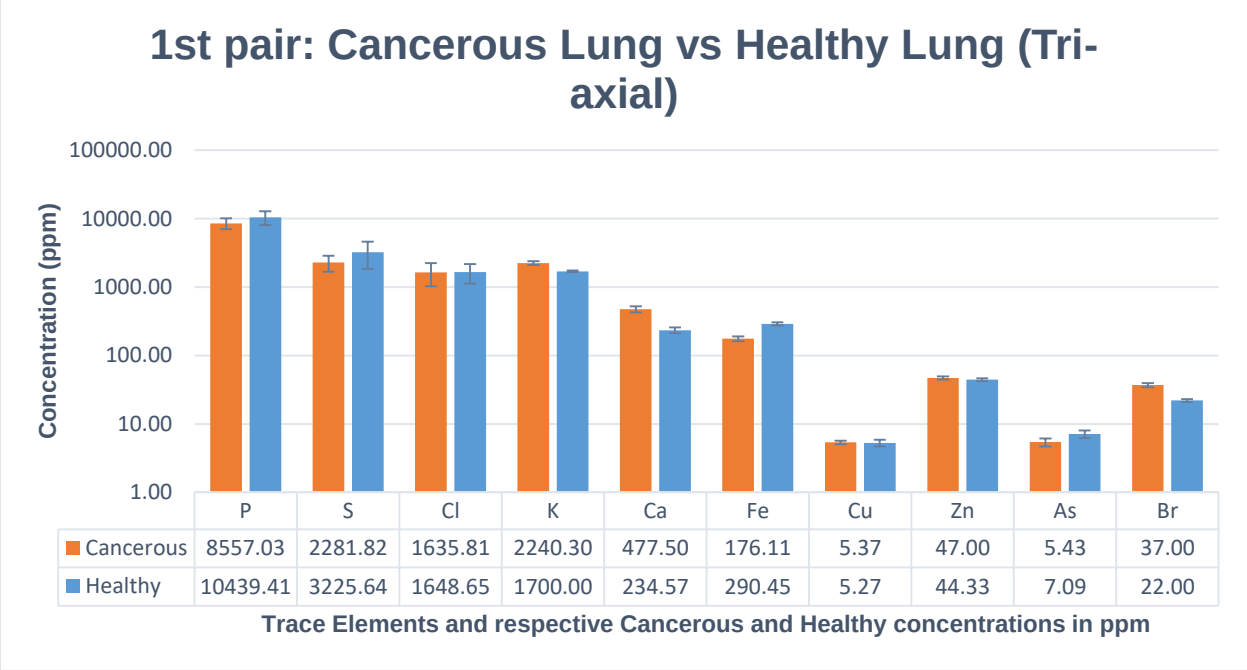
Although Zn shows a tendency to have higher concentrations in healthy tissues, its concentrations are very similar, so its variations are not significant. The same happens for Pb, though its tendency is to show an increase in cancerous tissue concentrations.

Ca, K show relevant variations in their concentrations according to only one of the spectrometers, presenting unaltered concentrations in the other.

Ni presents questionable results as its concentrations reveal very low values, near the detection limit, so its variations are not considered significant.

Therefore, the most significant elements in the 3rd pair of colon tissues are Fe, Ca and K. The first one appearing in higher concentrations in cancerous colon tissues and the other two showing higher concentrations in healthy colon tissues. Although Fe presents similar results with other colon pairs, Ca and K show completely opposite results when comparing with global colon comparison and with other pairs, respectively. This reveals a big contrast between this pair of colon tissues and the other two, confirming the biological variability question between different individuals.

LUNG TISSUES



	P	S	Cl	K	Ca	Fe	Cu	Zn	As	Br
Cancerous	8557.03	2281.82	1635.81	2240.30	477.50	176.11	5.37	47.00	5.43	37.00
Healthy	10439.41	3225.64	1648.65	1700.00	234.57	290.45	5.27	44.33	7.09	22.00

Figure 55 – Concentrations from cancerous and healthy lung (1st pair) tissue samples obtained from Tri-axial spectrometer.

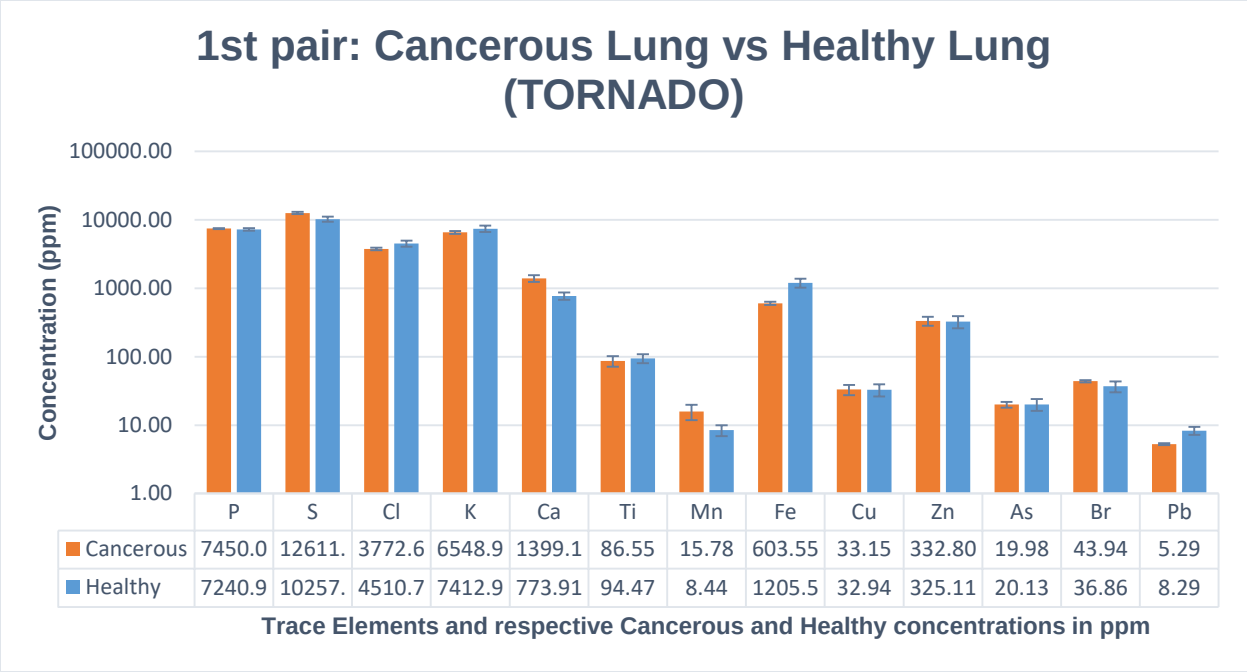


Figure 56 – Concentrations from cancerous and healthy lung (1st pair) tissue samples obtained from TORNADO spectrometer.

The previous two graphics refer to the 1st pair of lung tissues. These tissues show a rise of Ca and Br concentrations in cancerous tissues and a decline in Fe cancerous tissue concentrations, stated by both spectrometers' data. Other elements that have lower cancerous tissue concentrations are Cl and Pb, while K and Mn appear to be higher in cancerous tissues.

Ca and Fe reveal consistent results when comparing each measurement's concentration, validating their variations, while Br, although showing a tendency to have higher concentrations in cancerous tissues, presents very similar concentrations, hence discrediting its variations.

As Br, Cl shows a tendency to have higher concentrations in healthy tissues, however, these concentrations are much alike, so its variations are questionable.

K shows relevant variations in its concentrations according to only one of the spectrometers, presenting unaltered concentrations in the other.

Mn and Pb present questionable results as their concentrations are very low, therefore its variations ae not significant.

As so, Ca, Fe and K are the most significant elements in the distinction between cancerous and healthy lung tissues for this 1st pair. Ca is the concordant element when comparing to global lung comparison, presenting higher cancerous tissue concentrations.

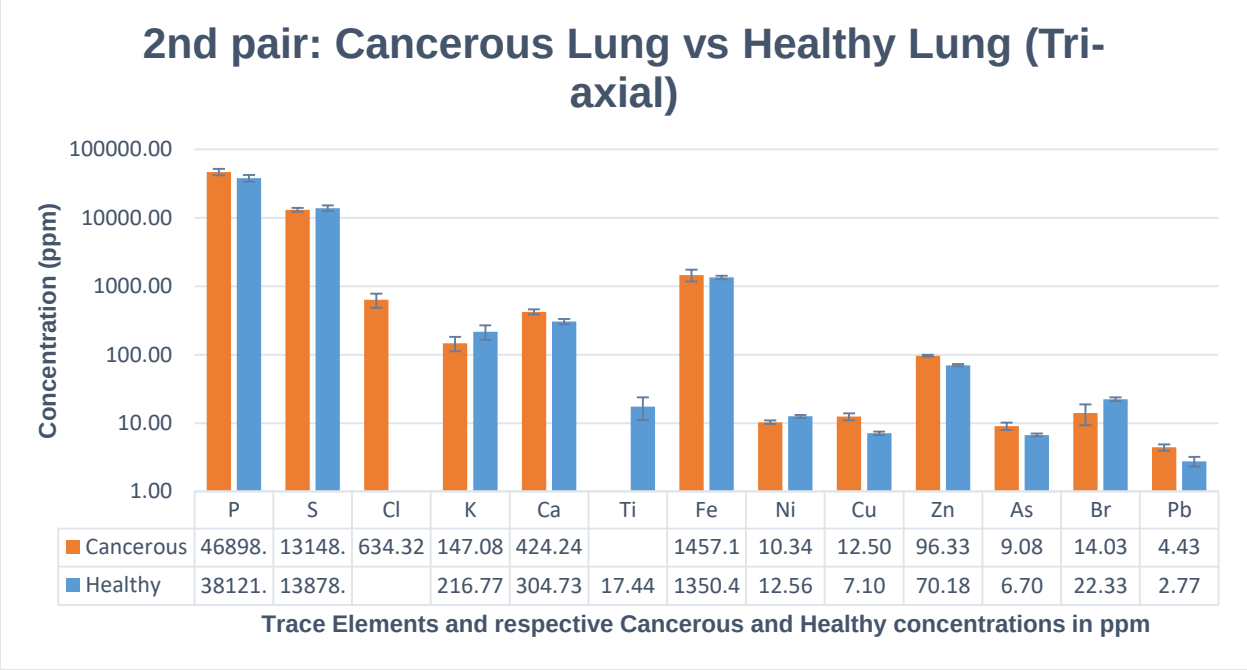


Figure 57 – Concentrations from cancerous and healthy lung (2nd pair) tissue samples obtained from Tri-axial spectrometer.

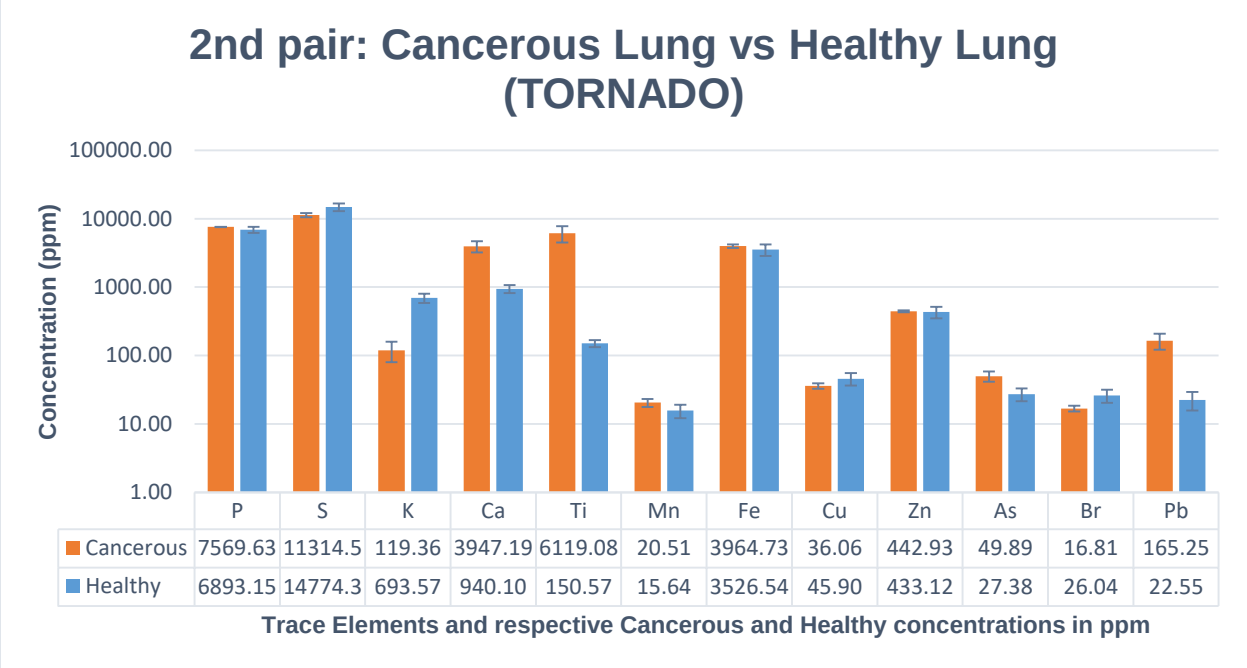


Figure 58 – Concentrations from cancerous and healthy lung (2nd pair) tissue samples obtained from TORNADO spectrometer.

These final two graphics have the 2nd pair of lung tissues in consideration. In these results there are several elements that vary their concentrations similarly according to both spectrometers. As so, Ca, Arsenic and Pb show an increase in cancerous tissue concentrations. On the other hand, Br presents a decline in its concentrations in cancerous tissues. There are other elements that appear to have higher concentrations in cancerous tissues, like P, Cl, Cu and Zn, while S and K appear to have higher concentrations in healthy

tissues. The contradictory element in this pair of tissues is Ti, revealing different variations in its concentrations depending on the used spectrometer.

Comparing each measurement for the same sample, Ca is the most consistent element in this lung pair, having higher concentrations in cancerous tissues.

Even if Tri-axial's data on **Ti** reveals that it is only present in healthy tissues, Tornado states that Ti have a great variation in its concentration, appearing much higher in cancerous tissues, which is odd. There may be a Ti contamination in Tornado spectrometer. Future studies should investigate this subject.

Arsenic, Pb, S and K show significant variations in their concentrations according to one of the spectrometers, revealing no alterations in the other's data.

Br although presenting a tendency to have higher concentrations in healthy tissues, reveals very similar concentration values and so its variations are discredited. The same is verified for P and Zn, only in the opposite type of tissue.

Cl is yet again a curious case, as it is only found in Tri-axial graphic's cancerous tissues and in none other measurement.

Therefore, the most significant elements in this pair of lung tissues are Ca, As, Pb, S and K. While Ca is concordant with 1st lung pair's results, K shows completely opposite variations. Comparing with global lung comparison there are several elements that present the same variations, such as Ca, As and Pb.

It is easily observable that the 2nd pair is much more alike the global comparison than the 1st one, having many more elements in concordance. This remits to the idea that, in this short-numbered samples statistical study, one measurement on a biologically different tissue can compromise general data, and consequently mask probable correlations between trace elements concentration variations.

SELENIUM

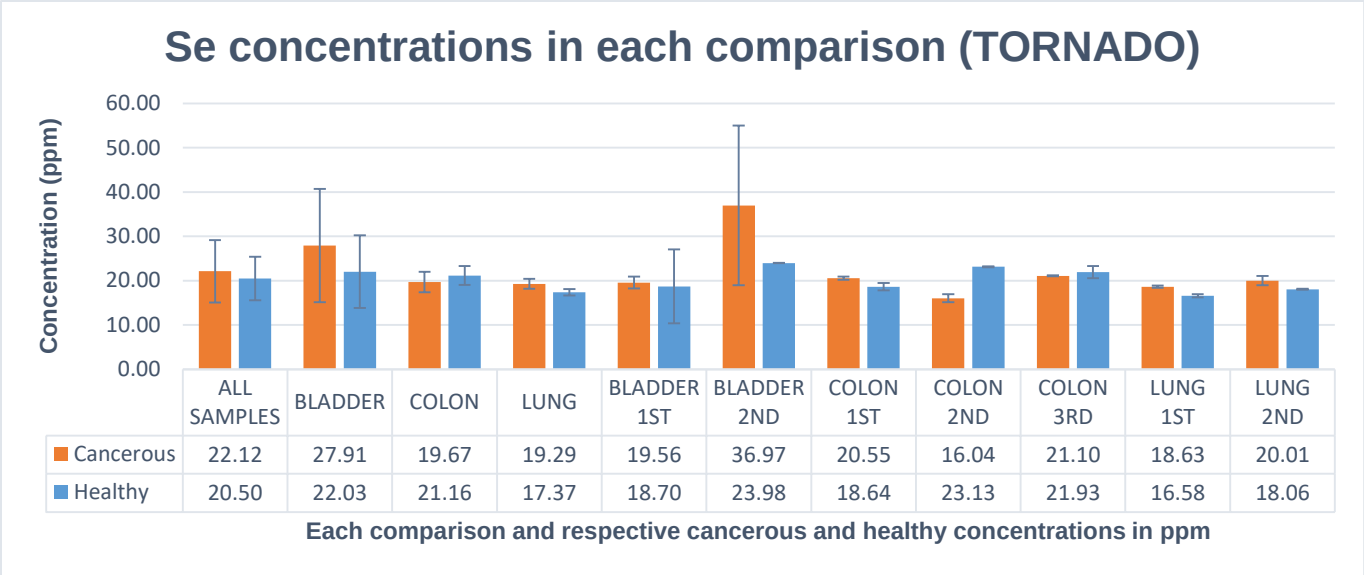


Figure 59 – Se concentrations from each comparison of cancerous and healthy tissues obtained from Tri-axial spectrometer.

Selenium is compared separately due to the fact that it was one of the elements that several previous studies referred as potentially good indicators of carcinogenesis [20] and so concordant results were expected. However, Se was not found in any Tri-axial measurement. In addition, it is present in Tornado's measurements but very near the element's detection limit and almost always similar between cancerous and healthy tissues, which invalidates the possibility of finding variations in the concentration of Se caused by carcinogenesis processes.

The only variation that one may consider is verified in both pairs of lung tissues, which seem to show greater concentrations of Se in cancerous lung tissues. Even so, previous results regarding lung cancer indicate the complete opposite [23].

DISCUSSION

There is a large variety of trace elements in this work when it comes to their expression in cancerous and healthy tissues. Some are almost always higher in the same type of tissues, either cancerous or healthy. Others change their concentrations depending on the organ, being higher in a type of tissue for a certain organ and higher in the other type for a different organ. And finally there are elements that maintain their concentrations independently of the type of tissue, which do not constitute good elements in the distinction between cancerous and healthy tissues, so those will not be considered in these discussion and conclusions.

Pair		Organ		All samples
1st	**Fe**, **Br**	Bladder	**Ca**, **Fe**	**Ca, Fe**
2nd	**Ca**, S, Br			
1st	**Ca**, **K**	Colon	**Ca**, **Fe**, Zn	
2nd	**Ca**, **Fe**, **S**			
3rd	K			
1st	**Ca**, Fe, **K**	Lung	**Ca**, **Zn**, **Pb**	
2nd	**Ca**, **As**, **Pb**, K			

cancerous
healthy

Figure 60 – Schematic with the significant trace elements' tendencies.

Although this statistical study may have questionable validity, it also allows the perception of some tendencies in the variation of several trace elements concentrations. Through this continuous analysis of the acquired data one is able to observe certain trace elements that show a pattern in their concentrations variation, which was one of the prime objectives of this work. It is also perceptible, although less verified, that depending on the organ some elements change their expression whether in higher concentrations in cancerous or healthy tissues, which confirms previous studies that state that one should analyze cancerous and healthy tissues divided by organ and not altogether [30].

CALCIUM (CA) is the most consistent trace element in terms of its concentration variation. It expresses itself in higher concentrations in cancerous tissues almost in all comparisons. This happens when

analyzing: all samples together; each group of samples from bladder, colon and lung tissues; and in 5 of the 7 pairs of cancerous and healthy tissues. Ca only expresses itself differently in the 3rd pair of colon tissues, where it presents higher concentration in healthy tissues and in the 1st pair of bladder tissues, where it maintains its concentration unaltered. Analyzing this data it is easily observed that this element could be a good indicator of carcinogenesis, within the studied organs as its concentration is almost always higher in cancerous tissues. Of course this tendency is a deduction from the analyses conducted in this work and can be contradicted by other studies, due to the poor statistical significance of this one. A demonstration of this is the fact that in previous studies concerning lung cancer, Ca is expected to lower its concentration in cancerous tissues [23], the opposite of what is found in this analysis. However for other organs, Ca has no previous results to compare with. Even so, Ca is one of the most important elements in the distinction of cancerous and healthy colon tissues, therefore these acquired results may be a good indicator to work on in future studies.

IRON (FE) is another element that has consistent data regarding bladder and colon global analysis. Then, on the specific level, Fe has higher concentrations in cancerous tissues in one pair of the bladder tissues and in all pairs of the colon tissues. Furthermore, in one of the lung tissues pair, Fe appears in higher concentrations in healthy tissues. Although a more general analysis indicates a consistent tendency in the variation of Fe concentration, at a specific level it is clear that the variation of Fe concentration is more random and less consistent, except for colon tissues. This element is considered to be one of the most significant ones in the distinction of cancerous and healthy tissues from the colon and lung. In previous lung cancer analysis, Fe showed a decrease in its concentration in cancerous tissues, which happens for one of the lung tissue pairs.

ZINC (ZN) only appears significantly in the global colon and lung comparisons and then in one pair of bladder tissue and in another from colon tissues. Its expression is not constant, being either higher in cancerous tissues or in healthy ones. This is one of the elements that changes its expression regarding its original organ. Generally, Zn has higher concentrations in cancerous lung tissues and higher concentrations in healthy colon tissues. At the specific level, Zn appears in higher concentrations in cancerous tissues from the 1st bladder pair and in higher concentrations in healthy tissues from the 1st colon pair. So, analyzing these variations one may consider that Zn alternates its expression regarding the organ, increasing its concentration in cancerous bladder and lung tissues and decreasing it in cancerous colon tissues. This is just a consideration due to the short number of variations found, not capable of creating a pattern. Previous studies reveal results that are in conformity with ones from this work regarding bladder and colon tissues. While the results from lung tissue comparisons show either conformity or contradiction with previous results [23, 27, 31].

Even though **SELENIUM (SE)** was referenced as a promising indicator of carcinogenesis' presence, results from this work shows that it does not vary its concentration on cancerous tissues, comparing to its healthy

counterparts. Even so, these results are questionable due to very low concentration values and to the fact that Se is only found in Tornado's measurements.

ARSENIC (AS) presents different results regarding the organ that is analyzed. In bladder tissues comparison, Arsenic has higher concentrations in cancerous tissues, while in lung tissues comparison it shows the opposite, being higher in healthy tissues.

PHOSPHORUS (P) has an almost equal expression in all comparisons, maintaining its concentration unaltered. These concentrations present high values almost always, as well as they are very similar between the two types of tissues. Significantly the only variation found in P concentrations are from the 1st pair of colon tissue. Previous studies confirm that P tends to accumulate in cancerous colon tissues [25]. But due to the insufficient number of cases found, these results do not present any relevance for this study.

MANGANESE (MN) is an element that shows very low concentrations, so, even if higher in a determined type of tissue, any correlation found is even more doubtable than the others before, because the lower concentrations are, less accurate they become, due to possible analysis errors. Mn is thought to be one of the beat indicators in the distinction between cancerous and healthy lung tissues. However, this study does not facilitate enough results or even solid ones.

LEAD (PB) is another element that presents very low concentrations in both types of tissue, which may question its results. It also has a characteristic analysis. Pb only expresses itself by being higher in cancerous tissues. However, these variations are only found in lung tissues. In the general comparison as well as in the specific ones, which could be a good indicator for future studies to analyze and confirm. Previous lung studies reveal either concordant or contradictory results [23, 25].

COPPER (CU) does not have any significant expression in this analysis due to its very low concentrations. However, if we consider these concentrations to be valid, Cu appears almost always alike in the two types of tissues. Previous studies also indicate that Cu maintains its concentrations in cancerous and healthy bladder tissues, while in colon tissues it presents an increase in cancerous tissue concentrations. As for lung tissues previous results indicate that Cu is a significantly important element in the distinction between the two types of tissues, appearing in lower cancerous concentrations [24, 27, 31].

SULFUR (S) only shows significant variations at the specific level, appearing in one pair of each organ. However its behavior depends on the organ, as for bladder and lung tissues S has higher concentrations in healthy tissues and for colon tissue it appears in high concentrations in cancerous tissues. Previous studies confirm these results in both colon and lung tissues, indicating that S accumulates in cancerous colon tissues and in lung ones it presents a decrease in cancerous tissue concentrations [23, 25].

BROMINE (BR) appears to have a decrease in its cancerous tissue concentrations, when comparing all samples. However, analyzing each organ separately Br does not show significant variations in its

concentrations, which questions the general variation. Furthermore, at the specific level Br presents different results between organs and even between pairs of the same organ. This contradiction is observed in the bladder tissue pairs, confirming the biological variability stated previously.

POTASSIUM (K) is another element that shows different variations within the same organ tissues, altering its expression from one colon pair to another. The same happens for lung tissues.

Although **CHLORINE (CL)** shows few comparison expression, when it appears, is almost always in higher healthy tissue concentration, rather than cancerous.

The remaining elements have low expression in every comparison or maintain their concentrations disregarding the type of tissue, hence they will not be mentioned. Examples are **NICKEL (NI)** and **TITANIUM (TI)**.

CHAPTER 5 – CONCLUSIONS

Firstly, and a continuous outflow during this work, is that solid statements are improbable to be verified in a short-sample statistical study. Adding it to several factors, such as biological diversity, data is insufficient to find correlations. Even so, throughout this entire work certain trace elements concentration variations between cancerous and healthy tissues are found, described and tentatively explained.

Throughout this analysis one can observe the different types of trace elements in terms of their expression in cancerous and healthy tissues, evaluating those that may have an important role in the distinction between the two types of tissues. Thus, one is capable of choosing the right elements to be used as indicators of cancer development. This was one of the most important objectives of this work. Of course due to statistical difficulties, the validity of these results is questionable, however, some trace elements tendencies are observable and therefore constitute good indicators for further analysis, reserved to future studies.

This work showed certain elements that might be used as indicators of cancer development, due to their concentration variation when carcinogenesis process is present and their similarity to previous studies. Elements such as Ca and Fe show consistent data when varying their concentrations, even if their concentrations are very low, in the case of Pb. Other elements when analyzing organ by organ show evidence that trace elements expression may change due to different organs, even if in the general analysis they maintain their concentrations unaltered. Some of these elements are Zn, As, Br and K. Even others, may show a tendency in their expression but present insufficient results to be considered relevant, such as P, S and Cl. Finally there are elements like Cu, Mn, Ni and Ti that, in this work, show random expression in their concentrations, making impossible to find any correlation. Of course the statistical population is a great obstacle but also biological variability and each patient's clinical history, which can compromise or invalidate any observed tendency and, therefore, correlation.

At laboratorial level, all caution was taken in order to avoid any measurement error, repeating both the measurements and data analysis several times. The obtained results were not as significant as expected, however, as said before, the low statistical population compromised this work results' robustness. All efforts were made to minimize this obstacle but even measuring each sample 5 times, it was not enough to fully standardize trace elements concentration variations.

Even though these results are questionable due to their lack of statistical population, they can contribute to future studies and meta-analysis. They may show interesting trace elements and their tendency to vary depending on the type of tissue. Obviously, it would require further analysis to confirm their validity and present solid results. Further studies in which the clinical history of the patient is available, are required.

Also it could be interesting to simultaneously analyze blood samples from the patients in order to correlate the trace elements found in blood and the pathological stage at which the cancer is at the biopsy time.

Future studies may consider doing a meta-analysis, grouping all available results from all studies ever conducted on this subject. If so, the statistical population would be enough to be capable of finding correlations and patterns in the variation of trace elements concentrations with the grand objective of explaining the influence of these elements on the carcinogenesis process. Thus, one will know when cancer is arising just by quantifying the previously indicated trace elements, what may give human beings, a new tool on cancer diagnosis, which could, hopefully, be a step in the direction of its extinction.

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APPENDIX

This section will present the spatial distribution maps of the trace elements found to be the most significant in the previous chapter.

Due to their low concentrations or homogeneous distribution, the majority of the elements does not present interesting maps. Only a few have associated maps on which one can observe the areas where a determined element has higher concentration. In the case of cancerous tissues, it is possible to predict where the malignant cells are concentrated.

In the case of P and S maps, the tissue is more distinct due to their concentrations' values, much higher than the rest. On the other hand Ca and Fe reveal more detailed maps, on which blood vessels are visible.

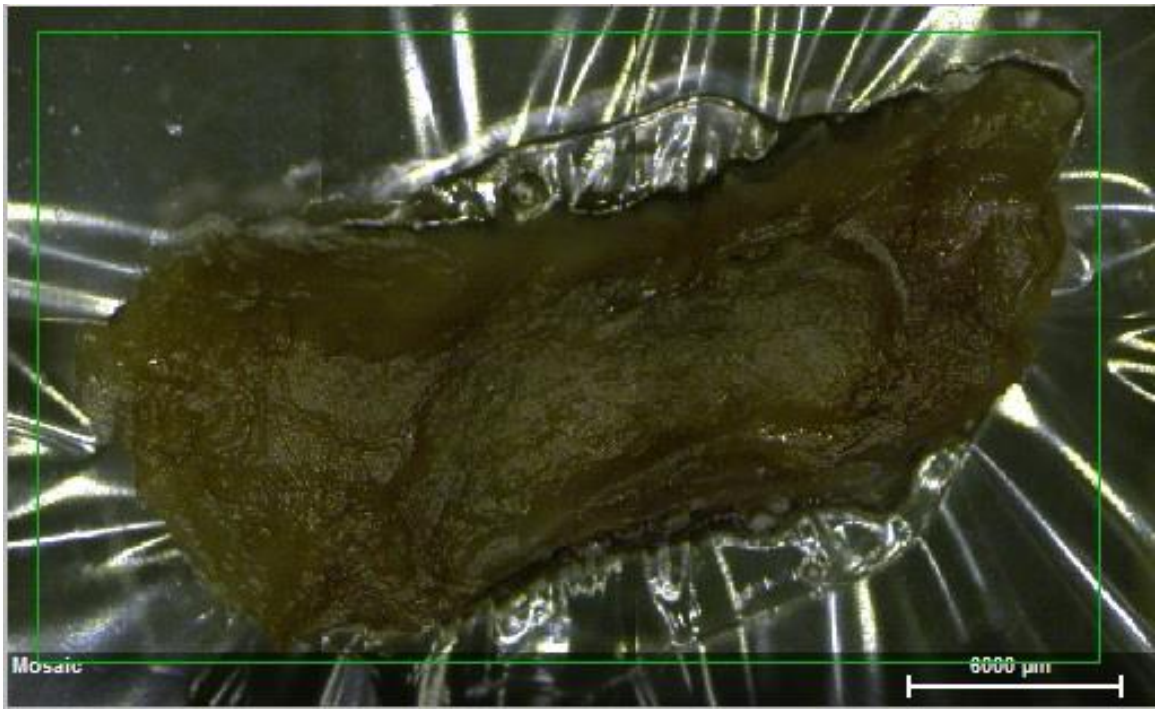

Figure 61 – 2nd pair of cancerous bladder tissue.

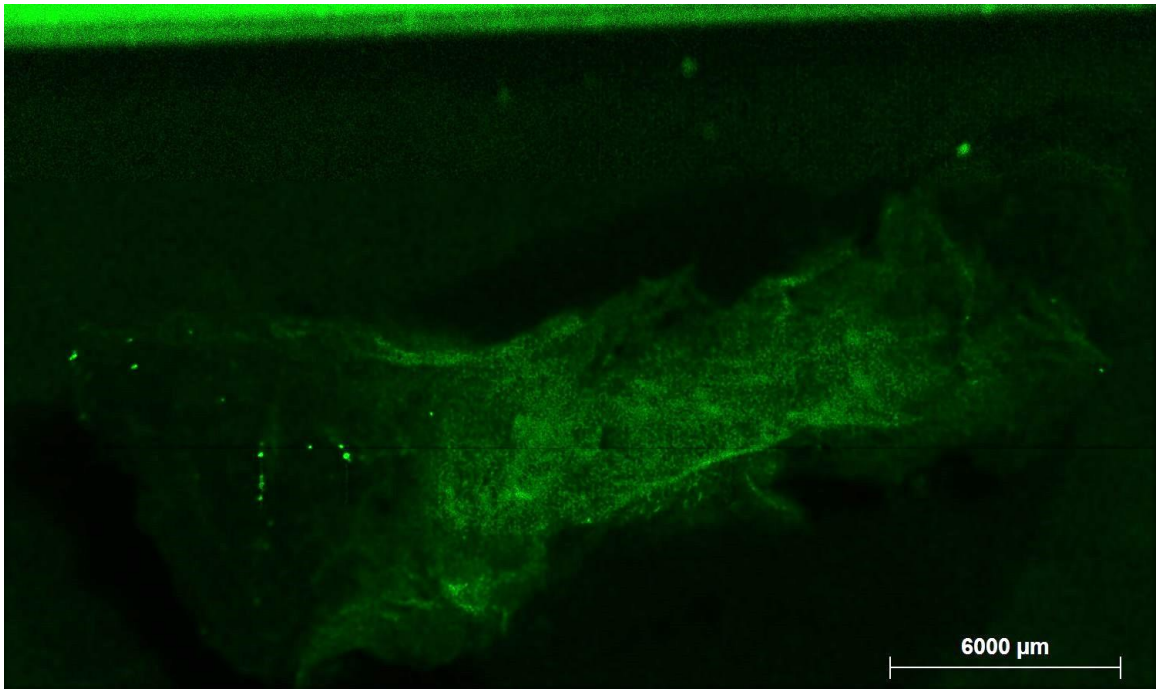

Figure 62 – Spatial distribution of Ca in the 2nd pair of cancerous bladder tissue.

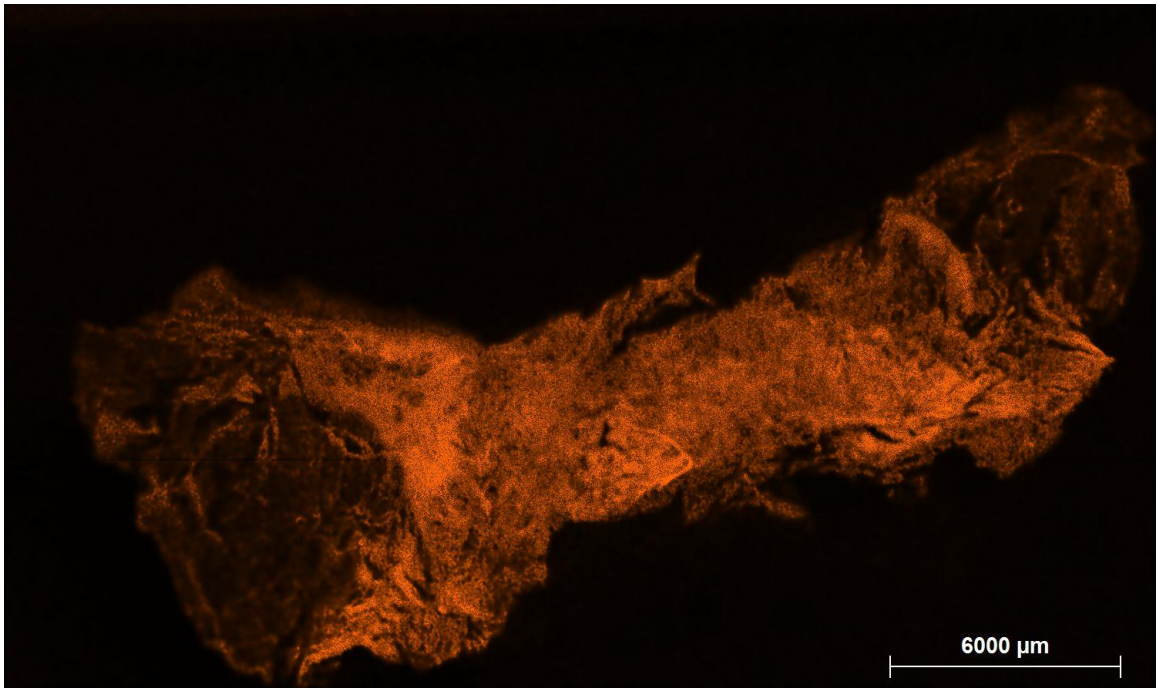

Figure 63 – Spatial distribution of S in the 2nd pair of cancerous bladder tissue.

Figure 64 – 3rd pair of healthy colon tissue.

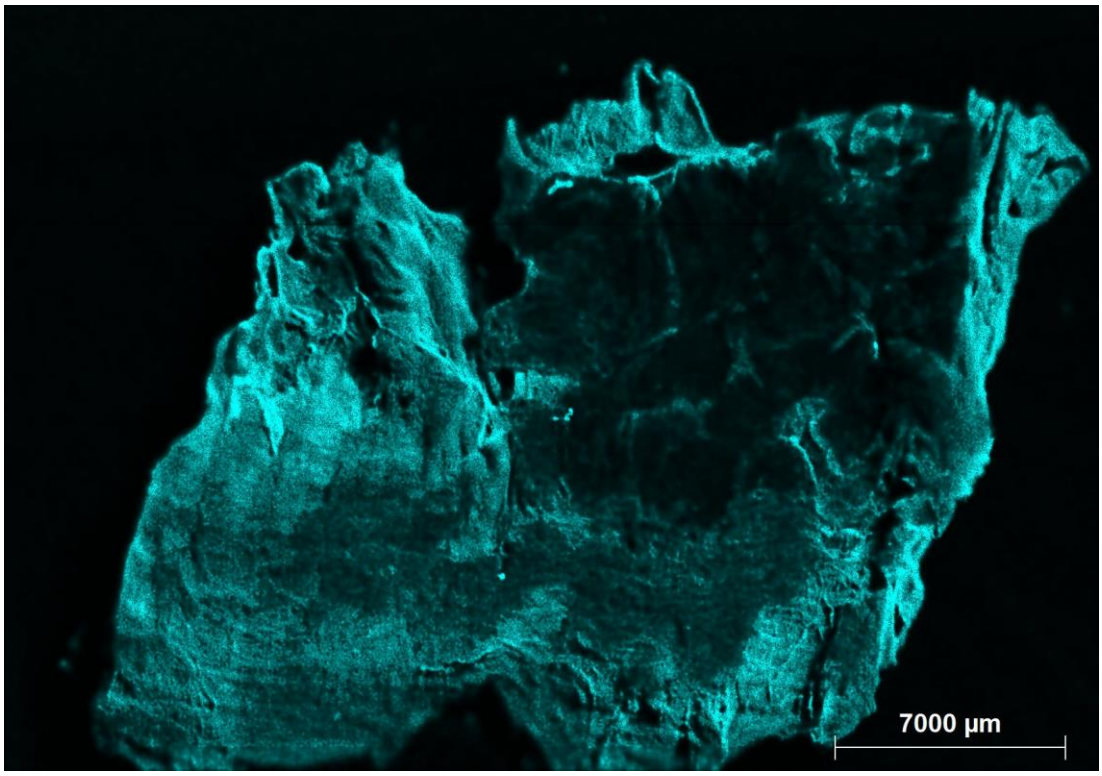

Figure 65 – Spatial distribution of P in the 3rd pair of healthy colon tissue.

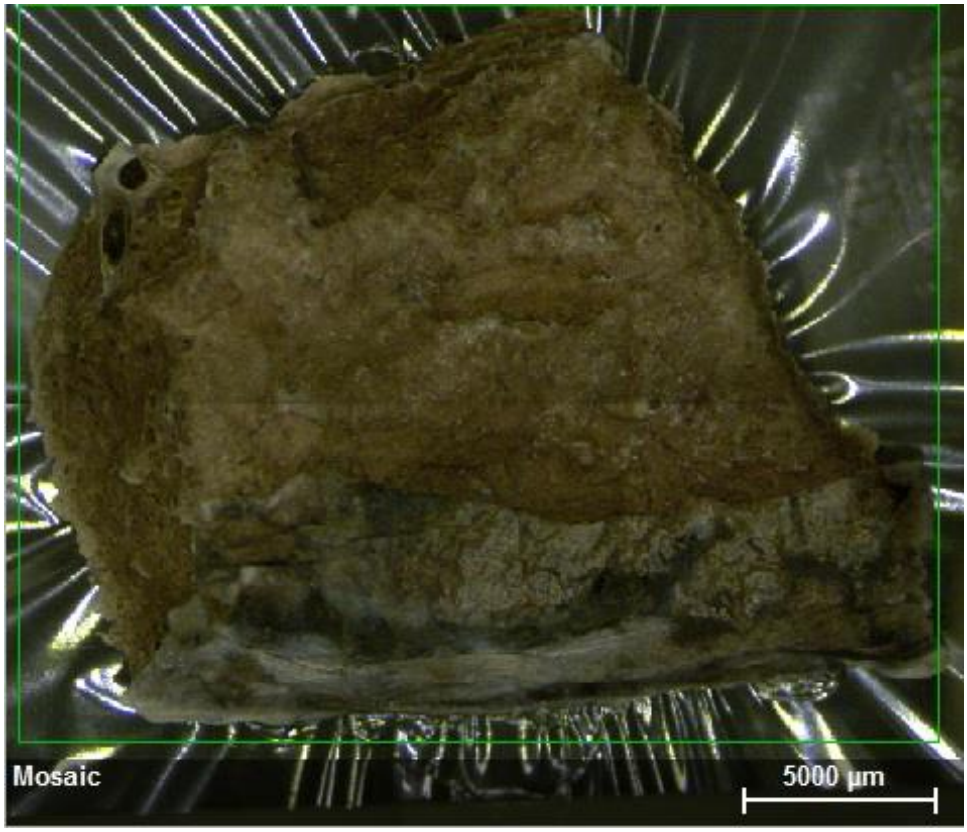

Figure 66 – 2nd pair of healthy lung tissue.

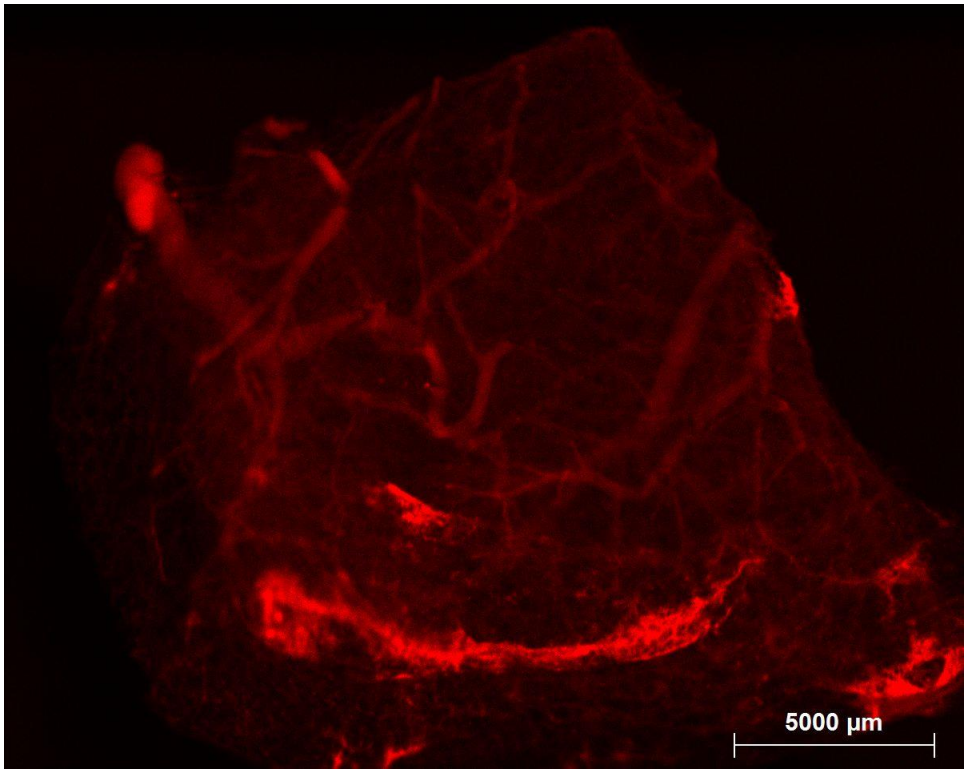

Figure 67 – Spatial distribution of Fe in the 2nd pair of healthy lung tissue.