**THE FEASIBILITY OF USING X-RAY FLUORESCENCE ANALYSIS OF IRON, COPPER, AND ZINC IN THE CENTRAL NERVOUS SYSTEM IN A RODENT MODEL OF DYSMYELINATION**

**THE FEASIBILITY OF USING X-RAY FLUORESCENCE ANALYSIS OF IRON, COPPER, AND ZINC IN THE CENTRAL NERVOUS SYSTEM IN A RODENT MODEL OF DYSMYELINATION**

By

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 **Analysis of Iron, Copper, and Zinc in the**

 **Central Nervous System in a Rodent Model**

 **of Dysmyelination**

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**ABSTRACT**

Trace elements are involved in many biological processes and serve important functions to maintain the normal development of the central nervous system (CNS). In the CNS, iron (Fe), copper (Cu), and zinc (Zn) are some of the most important elements that play critical roles as catalysts, cofactors, and structural components for many cellular enzymes and proteins. The deficiency or excess of these metals may lead to various neurological disorders. Demyelination is a condition of loss of myelin and leads to neurological diseases like Multiple Sclerosis. Myelin consists of transition metals and hence it would be interesting to study concentrations of these elements in normal and demyelinated models. X-Ray Fluorescence (XRF) is a popular non-destructive technique applied in trace element studies. The principle involves exciting a sample and detecting characteristic X-rays, which provide information on elemental concentrations in the sample. In the present studies the feasibility of XRF for trace element studies was explored. A total of 120 samples of brain and spinal cord tissues were collected from Long Evans (control) and Long Evans Shaker (dysmyelinated)–an incomplete formation of myelin sheaths–rats at ages of 3 weeks and 16 weeks. The samples were excited using x-rays from an Energy Dispersive X-Ray Diffraction (EDXRF) set-up. The spectral data was collected using an Silicon Drift Detector (SDD) and the resultant data were analysed to see if statistically significant changes in concentrations were present in the samples. The results were discussed and suggestions for future work were made.

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Chapter 1

**INTRODUCTION**

Several trace elements are present in the human body, the concentrations of which are believed to be some of the leading causes of various disorders. The common sites of trace element deposits include but are not limited to brain, liver, kidney, bone, and skin. Measurements of trace elements in these sites are important for the diagnosis of diseases that may result due to either excess or deficiency of these elements. The human brain is one site that demands high levels of metal concentration and becomes a promising area of neurological research. These metal levels vary from region to region in the brain, and any abnormal presence of these metals would lead to neurological diseases. The subject of this thesis relates to the role of trace elements in diseases of the central nervous system (CNS), with a focus on those that are linked to dysmyelination of cells in the brain and spinal cord. In the present study, the feasibility of measuring the trace elements iron (Fe), copper (Cu), and zinc (Zn) in CNS tissues of dysmyelinated rodents using X-ray Fluorescence (XRF) analysis is investigated. The goal is to gain better understanding of the role of these elements in the aetiology of CNS diseases that associate with a dysmyelination process since both metal dyshomeostasis and myelin breakdown are observed in many neurodegenerative disorders.

**1.1 Importance of iron, copper, and zinc in CNS function**

 Iron (Z=26), copper (Z=29), and zinc (Z=30) belong to the class of transition metals and are found to play a prominent and diverse role in the normal functioning and development of the CNS. The distribution of these elements is dependent on brain region, subcellular location, age, species, genetic and environmental factors (Prohaska, 1987). These elements are considered as cofactors, catalysts and structural components for many cellular enzymes and proteins in the CNS due to their unique biochemical properties. They also play roles in the homeostatic regulatory mechanisms maintaining their sufficient concentrations in the CNS (Ponka and Richardson, 1997). They take part in neuronal activities such as neurotransmission, neurogenesis, synaptogenesis, neurotransmitter biosynthesis, neurite outgrowth, oxidative phosphorylation and oxygen transport (Hung *et al*., 2010). The metals support different aspects of the metabolism of neurons and glia (Nikonov12 *et al*., 2013), maintain redox balance in the brain and prevent specific diseases and deaths that are a result of deficiencies or toxicities in humans or animals (Aguirre *et al*. 2005). Each of these elements has its own unique form, distribution, and function in the CNS and the imbalance of one element ion will disturb the homeostasis of other elements downstream. Balanced metal levels are strictly maintained in cells and entry of the trace metals to the brain is limited by the blood-brain barrier (BBB), a highly regulated and selective transport system. Cell functioning gets disrupted due to a fall in metal concentration whereas high levels that known as cytotoxicity can cause oxidative damage to cells. Hence, a proper balance needs to be maintained for normal functioning of the CNS (Que *et al*., 2008). Keen *et al*. (1985) studied the effects of dietary Cu and Zn in male rats. They concluded that dietary Zn affects tissue Cu levels primarily when dietary Cu is deficient, whereas dietary Cu has no effect on tissue Zn. However, both Zn deficiency and Cu deficiency affect tissue Fe levels. Carl *et al*. (1990) in their study characterized the elemental concentrations in the brain tissues of rats and found an association between epilepsy-prone rats and altered tissue trace element concentrations. Carpenter (2001) studied the effects of transition elements on the human nervous system and suggested a relation between elevated levels of these elements in brains of patients with Alzheimer and Parkinson’s diseases. Oladiji (2003) investigated the effects of iron deficiency on the levels of iron, copper, and zinc in the brain, liver, kidney, heart, and lungs of 40 albino rats (*Rattus norvegicus*). He concluded that these elements interact with each other and with other nutrients to such an extent that the margin between the levels at which the effects on the organisms are beneficial and toxic may be quite small or even overlap. Serpa *et al*. (2006) analyzed the elemental concentrations in brain structures such as temporal cortex, entorhinal cortex, visual cortex, and hippocampus of young, adult and old Wistar rats. They found that in the entorhinal cortex, Zn decreases with age in the temporal cortex, the Fe level increases with aging in the visual cortex, and in the hippocampus, Fe, Cu and Zn increase with age. They also suggested that the increase of Fe with aging in the hippocampus is a major factor involved in oxidative stress which is one of the main reasons for neuronal death in Parkinson's disease. Fayed (2010) determined the level of Fe, Cu, and Zn in the brain of female albino rats treated with vincamine (an indole alkaloid found in the leaves of Vinca minor plant), and found 50% reduction in Fe concentration, which could contribute to diminished Fe-mediated damage particularly for advanced age animals. More recently Moldovan *et al.* (2015) studied the altered transition metal homeostasis in the cuprizone model of demyelination and concluded that the metal levels may affect the myelin level in rats.

**1.1.1 Iron (Fe)**

Iron is one of the most abundant trace metals in biology and also the most abundant in myelin (Connor *et al*., 1996). It plays a significant role in the functioning of the CNS. It helps to maintain oxygen levels in the brain and maintain the integrity of oligodendrocytes and myelin. Its great ability to gain (ferric to ferrous, or Fe3+ to Fe2+) or lose (Fe2+ to Fe3+) electrons easily makes iron an essential cofactor for enzymes involved in ATP synthesis (Hung *et al.,* 2010). Fe has a critical role in myelin production (John *et al*., 1993). Though iron is found abundantly in various types of cells in the CNS, astrocytes are the primary storage units (Madsen *et al.,* 2007). Accumulating evidence denotes that impaired iron metabolism is an initial cause of neurodegeneration (Hung et al., 2010) and several studies of iron regulatory proteins using transgenic rodents (LaVauta *et al*., 2001; Rouault, 2006) suggest that the dysregulation of iron may be a pathogenic factor for neurodegeneration. Decrease in iron level in the brain affects normal cell division of brain cells such as neuronal precursor cells, astrocytes, and oligodendrocytes (Ke Y and Qian, 2007). Iron deficiency in early child development leads to neurological abnormalities (Lozoff and Georgieff, 2006). Anaemia, a condition of iron deficiency, is quite common in pediatric patients and manifests in several neurological disorders such as developmental delay, stroke, breath-holding episodes, pseudotumor cerebri, and cranial nerve palsies (Yager and Hartfield, 2002). However, iron accumulation in the brain is toxic and is correlated with neurodegeneration (Underwood, 1977). Iron toxicity results in an extensive amount of reactive oxygen species (ROS), which reacts with hydrogen peroxide (H2O2) to cause oxidative damage and affect the brain (Koppenol *et al*.,1978). The CNS disorders that have been proposed to have an iron-dependent component include Friederich’s Ataxia (Barnham and Bush, 2008), Restless Leg Syndrome (Clardy *et al*., 2006), Ischemic/Hemorrhagic Stroke (Hua *et al*., 2007), and Multiple Sclerosis (Abo-Krysha and Rashed, 2008). Furthermore, detailed studies of neurodegeneration disorders suggest that an iron gradient formed in brain tissues is fundamental for triggering neuronal toxicity (Zhou *et al*., 2001; Morgan *et al*., 2006; Harris *et al*., 1999; Curtis *et al*., 2001).

**1.1.2 Copper (Cu)**

Cu as the third most abundant trace metal in the body (Willis MS *et al*., 2005) following Fe and Zn, and is known to be a redox-active metal which is required for survival, growth, and normal development of the CNS. It is an important catalytic cofactor in redox chemistry for proteins that carry out fundamental biological functions including functions in the brain (Hordyjewska *et al*., 2014). The level of copper is relatively high in the brain, mostly concentrated in the gray matter (Prohaska, 1987), and is essential for brain metabolism as well as in the absorption and use of iron. Cu is distributed in two oxidation states: cupric Cu2+ andcuprous Cu+. It also promotes phospholipid formation, purine metabolism, mitochondrial respiration, and development of the nervous system (Boullata, 2013). Abnormal levels of copper in the body may cause health risks. Acquired copper deficiency has been recognized as a cause of myelopathy, which appears with symptoms and signs of cerebellar, spinal cord, and peripheral nerve disease (Rounis *et al*. 2010). Similarly, copper accumulation also causes neuronal toxicity and oxidative stress (Kozlowski *et al.,* 2009) since it is considered an excellent catalyst for the production of reactive-oxygen species (ROS) such as hydroxyl radicals (Halliwell, 2006), Copper dysregulation has also been implicated in neurodegenerative diseases such as AD and Wilson diseases. It is well known among copper researchers that it is impossible to synthesize myelin if dietary copper intake is inadequate (Klevay, 2013). However, the precise molecular mechanisms for most of the noticeable features of human copper deﬁciency are unknown (Prohaska, 2014).

**1.1.3 Zinc (Zn)**

Zn is one of the most productive transition metals with high concentrations in mammalian brain cells (Frederickson, 1989). It is an essential metal for cell growth and cell division, neuronal survival and for the function and structure of a myriad proteins in the CNS. It is also known to be a bio-factor metal that contributes to early neonatal brain development and maintenance of normal functions of the brain in adults (Gower-Winter *et al*., 2012). Studies have shown that zinc also plays a key role in the regulation of gene expression, enzymatic activity, cell signalling, modulation of neurotransmitter activity, and myelinating oligodendrocytes in the CNS (Law *et al*., 2003). At normal levels, Zn acts as an active neuroprotectant against neurotoxic cell death and plays key neuromodulator roles in the regulation of gene expression, enzymatic activity, cell signaling, the modulation of neurotransmitter activity in the CNS (Gower-Winter *et al*., 2012), and in myelinating oligodendrocytes. It is reported that Zn2+-myelin basic protein and Zn2+-proteolipid protein complexes are essential for maintaining the integrity of the myelin sheath (Law *et al*., 2003). However, Zn deficiency can cause neuronal death (Brewer, 2012) and can also increase cell oxidant production, affect the antioxidant defense system, and trigger oxidant-sensitive signals (Omata *et al*., 2013) in the neuronal cells of the brain. Accumulation of zinc causes pathologic dysfunction and cytotoxicity. Some studies (Frederickson *et al*., 1988; Stork and Li, 2006) have suggested a striking correlation between zinc accumulation in neurons and cell death. Variations in zinc homeostasis have been reported in many neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease, as well as in transient forebrain ischemia, seizures, and traumatic brain injury.

**1.2** **Myelin and associated disorders**

 Myelin is a sheath made up of proteins and fatty substances that cover the axons of the neurons to protect and insulate them, facilitating the axonal conduction of nerve signals. The production of myelin is termed ”myelination,” and it is a complex process driven by oligodendroglia cells that supply the myelin to ensheath the axons and compact the myelin sheath. Oligodendrocytes (OL), myelin-forming glial cells, are the major target in all known white matter diseases and a single oligodendrocyte cell may ensheath up to 60 separate axons (Miller, 2002). Oligodendrocyte cells have the highest iron content and hence excess of these levels may contribute to the increased intracellular oxidation whereas inadequate levels result in deficient myelination. However, demyelination is the phenomenon of the destruction of myelin formation resulting in the opening and exposure of the nerve and axons to harm and damage, impeding the conduction of nerve signals, whereas dysmyelination refers to an incomplete formation of myelin sheaths. Dysmyelination can cause permanent neurologic deficits due to the lack of myelin, making the white matter appear grossly gray rather than the shiny white associated with normal myelin tissues. Dysmyelination is marked by the immediate defect in structure and function of myelin sheaths that often arises from genetic mutations affecting the biosynthesis and formation of myelin. The contingency between transition metals and myelination in the pathogenesis of neurodegenerative diseases is explained earlier. In several neurodegenerative disorders of which multiple sclerosis (MS) is the most common form, both impairment of trace element homeostasis and myelin breakdown are observed. The chronic demyelinating disease MS initially displays reactive microglia clusters in the form of white matter lesions (Goldmann & Prinz, 2013) and are marked by acute inflammatory attacks, blood-brain-barrier (BBB) disruption, immune T-cell, astrocyte glial infiltration, axonal damage, and acute demyelination. After the initial stages, most patients undergo continuous neurological decline including degeneration of neuronal structures (Korn 2008). Since the LES rodent model shows similar CNS autoimmune traits, these animals were selected for experiments in the present study.

**1.3 Long Evans Shaker (LES) rat model**

The LES rat is an autosomal recessive mutant model of dysmyelination. These rats tend to have little myelin in the CNS and are a well-described CNS myelin mutant. This mutation leads to a total disruption in the development of myelin sheaths in the LES rat in advanced age. Though most of its axons are not myelinated, they still have the ability to survive up to 4 or 5 months, which makes this a very useful model for long-term studies. LES is a severe model that exhibits a phenotype characterized by tremor, seizure, and early death. The first experimental description of LES rats was reported in 1995 by Delaney *et al*. Initially, full body tremors are observed by 10 to 14 days of age which later decrease in intensity and eventually become a rocking motion as the rat attempts to ambulate. At the 5 to 14 weeks age, seizures are observed and are found roughly 4-5 times a day, each lasting for 30-45 seconds followed by a 45-60-second period of disorientation and lethargy. Initial seizures are characterized by a stimulus which vanishes with age (Delaney *et al*., 1995). However, neurological disorders in these rats start to show up from 5-14 weeks; the myelin is gradually lost and just a little myelin is present from 8 to 12 weeks throughout the CNS (Kwiecien JM *et al*., 1998). More studies have been carried out recently at the Medical Physics Department at McMaster University where the LES rat model was used as an experimental animal for further ascertaining the effects of varying concentrations of transition elements in the nervous system (Moldovan, 2012; Lobo, 2013). In the present studies, levels of transition metals Fe, Cu, and Zn are determined in the LES and its control counterpart, the LE.

**1.4 Present study**

 The objective of the current study is to examine the feasibility of using the X-ray Fluorescence (XRF) principle to determine trace elemental concentrations in different brain regions and spinal cord tissues of rats. As mentioned earlier, the three transition elements of interest are iron, copper, and zinc and experiments were carried out in a rodent model of dysmyelination (which is biologically represented in the brain of Long Evans Shaker [LES] rats). Similar studies were carried out earlier and the principle used was Neutron Activation Analysis (NAA) (Lobo, 2013). Since XRF is also a non-destructive, and highly sensitive technique, its efficacy in measuring the characteristic emissions and determining the elemental content of the brain and spinal cord tissues is explored. XRF data was collected for two strains of three-week- and 16-week-old rat brain and spinal cord tissues. The data was analysed to obtain elemental information. Statistical analysis was carried out to observe trends and significance of the data.

**1.5 Thesis layout**

 The present thesis focuses on the feasibility of XRF technique to measure trace elements and compare the levels between two strains of rat tissues. In the first chapter, the importance of the elements of interest in terms of their concentration levels in the CNS tissue responsible for neurological disorder due to deficiency or cytotoxicity is described. The second chapter deals with the technique of XRF and different features of a typical XRF spectrum. The physics involved in the present experiments is discussed in detail. The third chapter outlines the methods followed in performing the experiments and analyzing the resultant data. Sample preparation, description of the experimental set up, data collection, peak-fitting procedures, and statistical data analysis methods are discussed in this chapter. The fourth chapter details all the results obtained and discusses the same. Conclusions and future work are presented as the fifth chapter.

 Chapter 2

**X-Ray Fluorescence Spectroscopy**

 The process of identifying and analyzing emitted X-rays is called “X-ray Fluorescence Analysis” (XRF). XRF is a method widely used for the measurement of the elemental composition of materials. It has a key role in compositional studies with a beneficial impact on environmental, industrial, pharmaceutical, forensic, medical, and scientific research applications (Walter and Huapeng 2008). Many in vivo studies have been carried out using XRF due to its accuracy and precision. It is described as a powerful tool for obtaining results from the elemental analyses of biological samples in human and animal studies (Farquharson and Bradley, 1999; Carew, 2001; Abu, Atiya, 2012). The XRF technique is useful and valuable due to its ability to identify small amounts of elements in small tissue samples. Several studies (Takahashi *et al*., 2000; Carew, 2001; Abu, Atiya, 2012; Desouza, 2014) have reported successful identification and quantification of various minor and trace elements in biological specimens using XRF analysis.

Unlike other elemental analysis techniques such as Atomic Absorption Spectrometry (AAS), Inductively Coupled Plasma Atomic Emission Spectroscopy (ICPAES), and Inductively Coupled Plasmas Optical Emission Spectrometry (ICPOES), XRF is a non-destructive method.

Depending on the application, XRF can be produced by not only using X-rays but also other primary excitation sources like gamma rays, alpha particles, protons, or high-energy electron beams. The incident X-ray photon interacts with the sample via different methods. The mode of interaction mainly depends on the energy of the incident photon, sample matrix, and source-sample geometry.

**2.1 Interaction of radiation with matter**

 There are three major interactions of photons with matter of importance in this study, namely photoelectric absorption, Rayleigh and Compton scattering. The interaction probabilities depend on the energies of the incident photon and the atomic number (Z) of the element. At low energies of the incident photon beam, photoelectric absorption is dominant whereas scattering probability increases for medium energies. The incident beam as well as the fluorescent X-ray beam gets attenuated as they pass through matter. These are the major types of interactions of the incident photons in XRF experiments. The attenuation of X-rays and the interaction mechanisms relevant to XRF spectroscopy in the present studies are discussed below.

**2.2 Attenuation of X-rays**

When X-rays interact with matter through any of the interaction processes, they undergo attenuation depending upon the energy of the incident radiation, atomic number of the target (absorbing) material, and density of the target. The transmitted intensity 'I' is given by Beer-Lambert's law as:

I = Io e-µx

where Iois the intensity of the incident beam, µ is the linear attenuation coefficient of the target material, and x represents the material thickness. To avoid dependence on the density of the sample, the mass attenuation coefficient (µm) is used instead of the linear absorption coefficient (µ). The equation now becomes:

I = Io e-(µm t)

where µm = µ/*ρ* and t= *ρx*. *ρ* is the density of the target material in g/cm3, and t is the mass thickness expressed in g/cm2.

 Interaction with target atoms attenuates the incident photon beam. However, the probability of attenuation varies for different processes and mainly depends on the energy of the incident beam and the atomic number of the target element. Figure 2.1 shows the mass attenuation coefficient as a function of photon energy in Fe. Different interaction mechanisms by which the incident beam is attenuated are plotted. The photoelectric cross-section for any element is highest for photon energies just above the absorption edges. Hence for efficient XRF spectroscopy, photon energies just above the absorption edge of the elements of interest have to be selected. At low energies, the cross sections for coherent and Compton scattering are much smaller than the cross section for photoelectric absorption for an element, provided the energy is above the K-edge.



**Figure 2.1** µm as a function of energy (MeV) in Fe (NIST XCOM accessed 10th November, 2015)

 The loss of the signals plays a significant role in such experiments like ours, which could severely affect, the minimum detection limit (MDL) of the system. Hence, avoiding the loss of fluorescence signal due to attenuation is required. Therefore in this research, since the X-rays generated in the sample undergo attenuation including both scattering and absorption, the tissue thickness is maintained small. For X-ray experiments, the mean free path in the air ranges from 35-150 cm, hence keeping the sample as close as to the detector reduces attenuation.

**2.3 Photoelectric absorption**

 In the photoelectric absorption process, the incoming X-ray photon interacts with the inner shell electrons of the target atom resulting in a complete absorption of the photon. It is probable that an inner-shell electron of the atom is ejected from the atom leaving a vacancy in that shell (or sub-shell). This process is referred to as ionization of the atom. The electron utilizes a part of the energy received to overcome the binding energy (the amount of energy that is required to remove one of an atom’s electrons from the atom), and the rest of the energy as its kinetic energy. The electron thus emitted is called a photoelectron. The energy of the ejected photoelectron, Ee, is given by the formula:

Ee = Eγ – Eb

Where Eγ is the incident photon energy and Eb is the binding energy of the electron. Hence, photoelectric absorption only occurs when the energy of the incident photon (hv) is greater than the binding energy of the electron, also called the absorption edge (Knoll, 2000). The electric charge of the nucleus, the volume of the atom, and its electronic structure impact the magnitude of the binding energy. The excited atom returns to its steady state by electron rearrangement within an extremely short time (∼10-15 s). The atom in the excited state can return to the ground state by various processes of which the dominant ones are Fluorescence and Auger electron emission.

**2.3.1 Fluorescence**

 When an electron from a higher atomic shell transitions into a lower one to fill the vacancy, the difference in their level energies is emitted in the form of X-radiation that is characteristic of the atom. This is a radiative process, and the phenomenon is called fluorescence. Electrons from the L, M, and other shells can fill a vacancy in the K shell provided the transition rules are satisfied. The process is represented in Figure 2.1. Only those transitions from outer (sub) shells that conform to the electric dipole radiation selection rules are allowed.



**Figure 2.2** Mechanism of X-ray fluorescence of an atom (Flood, 2012)

 Each X-ray emitted during the fluorescence process is designated depending on its origin and destination by Manne Siegbahn. The characteristic X-ray lines are labeled as K, L, M, or N according to atomic shells. Another notation alpha (α), beta (β), or gamma (γ), is made to mark the X-rays that originated from the transitions of electrons from various higher shells. Hence, a Kα X-ray is produced from a transition of an electron from the L to the K shell, and a Kβ X-ray is generated from a transition of an electron from the M shell. Each shell has several orbits of electrons with higher and lower binding energy, necessitating a supplementary level of designation– α1, α2 or β1, β2, etc.–for the destination lower shell. The Kα peak is actually a combination of Kα1 and Kα2 and in systems with poor resolution, Kα1, α2 doublet is observed as the Kα peak. Table 2.1 presents the characteristic X-ray lines and their corresponding IUPAC Siegbahn names (Vo-Dinh 2003; Janssens 2004). The intensities of characteristic X-rays from an atom follow the inequality K > L > M>. Hence, K X-rays are best suited for XRF experiments as they are less attenuated compared to L and M X-rays. Probability of transition is higher for adjacent shells (K and L shells) and hence to fill a vacancy in a K shell, an electron from the L shell has a greater probability than one from the M shell. Therefore, Kα radiation will be more intense than Kβ radiation. Also the energy difference between the K and M shell is greater than that between the K and L shell, which dictates Kβ X-ray, is higher in energy than the Kα X-ray. The photoelectric cross-section (σ) is directly proportional to Zn

$σ ∝ Z^{n}$/ $E^{3}$

where Z is the atomic number and exponent n varies between 4 and 5 over the X-ray energy region of interest. Hence, the cross-section increases with increase in atomic number. Also, τ is inversely proportional to the energy of the incident beam. Hence, as mentioned earlier, the process is more probable at low energies, with the condition that the incident beam energy is greater than the binding energy of the inner shell electron. More specifically, the photoelectric interaction is most likely to occur if the energy of the incident photon is equal to or exceeds the binding energy of the electron with which it interacts. X-ray fluorescence is characterized as a selective absorption process followed by spontaneous emission. Hence, for better XRF results, an efficient absorption process is required. Therefore, the sensitivity of XRF experiment for each element increases at energy just above the K-edge of that element when the exciting radiation has its maximum intensity (Hodak, 2008).

|  |  |  |  |
| --- | --- | --- | --- |
| Series | IUPAC name | Siegbahn name | Relative Intensity |
| K-linesL3-LinesL2-linesL1-linesM-lines | K- L3K- L2K-M3K-M2L3-M5L3-M4L3-N5, 4L3-M1M3-N1L2-M4L2-N4L2-M1L2-O1L1-M3L1-M2L1-N3L1-N2M5-N7M5-N6M5-N6 | K α1K α2Kβ1Kβ3L α1L α2Lβ2, 15L$l$Lβ6Lβ1L γ1L$η$L γ6Lβ3Lβ4L γ3L γ2M α1M α2Mβ | 100$∼$50$∼$17$∼$8100$∼$10$∼$25$∼$5$∼$1100$∼$20$∼$3$∼$3100$∼$70$∼$30$∼$30 |

**Table 2.1**: the characteristic X-ray lines and their corresponding IUPAC Siegbahn names (Vo-Dinh 2003; Janssens 2004)

**2.3.2 Auger electron emission**

 The atom in an excited state can also de-excite by an alternative process called the *Auger process*. It is a radiationless (a non-radiative) transition in which, instead of the emission of a characteristic X-ray in the electronic transition from one of the upper levels to a lower vacant shell, the difference in energy () is taken up to eject a second electron from one of the higher shells so that the atom gets doubly ionized. This process is a competing process to the XRF. It is more likely to happen in elements with a low Z than in high-Z. The kinetic energy of the Auger electron is the difference between the atomic ionization energy and the binding energy of the shell from which it was ejected. In the low-Z materials, the probability of the Auger electron emissions is higher than the characteristic X-rays emission.

 **2.3.3 Fluorescence yield**

XRF and Auger’s process are competing mechanisms in atomic de-excitation following the photoelectric absorption process. The ratio of fluorescence X-rays emitted from the target to the incident X-rays absorbed in the sample is called the fluorescence yield and is denoted by 'ω.' increases with atomic number Z and also varies from one electron shell to another as Figure 2.2 shows. The fluorescence yield from the K shell $(ωκ) $is considered as an important parameter in KXRF studies. It is defined as the number of K X-rays emitted from the target in all directions divided by the number of vacancies in the K shell. The X-ray energies and K shell fluorescence yield for the elements of interest in the present studies are given in Table 2.2. 

 **Figure 2.3** Fluorescence yield 'ω.' increases with atomic number Z and also varies from one electron shell to another (Laing, 1981).

|  |  |  |  |
| --- | --- | --- | --- |
| Element (Z) | Kα energy (keV)a | Kβ energy (keV)a | Fluorescence yield (ωk)b |
| Fe (26) | 6.40 | 7.06 | 0.340 |
| Cu (29) | 8.04 | 8.91 | 0.440 |
| Zn (30) | 8.64 | 9.57 | 0.474 |

 **Table 2.2** The X-ray energies and K shell fluorescence yield for the elements of interest (Thompson and Vaughan, 2001), http://www.nist.gov/data/PDFfiles/jpcrd136.pdf reference for (b) and http://xdb.lbl.gov/Section1/Table\_1-2.pdf for (a)

**2.4 Scattering**

Scattering is another interaction mechanism that leads to either partial or no transfer of incident photon energy to an electron in the absorbing material. The interaction in which loss of incident photon energy is observed due to scattering is known as incoherent or Compton scattering. The energy of the scattered photon is less than that of the incident photon and difference in energy is transferred to the interacting electron. This mode of scattering is most important for energy absorption in the range of 100keV to 10 MeV for soft tissues. The other mechanism in which the incident photon energy is conserved, i.e., is the same before and after scattering, is known as coherent or Rayleigh scattering.

**2.4.1 Compton scattering**

The incident photon transfers part of its energy to the electron, which is assumed to be initially free and at rest in the target and gets deflected through an angle. The electron called the Compton electron gets ejected and the atom recoils. The kinetic energy of the Compton electron is given as:

$$KE\_{e}=E\_{γ}-E\_{γ^{'}}$$

where $E\_{γ}$is the incident photon energy and $E\_{γ^{'}}$is the scattered photon energy. The energy of the scattered photon is calculated as:

$$E\_{γ^{'}}=\frac{E\_{γ}}{1+\frac{E\_{γ}}{m\_{e}c^{2}}\left(1-cosθ\right)}$$

where $m\_{e}c^{2}$ is the rest mass energy of the electron (511 keV) and θ is the scattering angle.

The energy of scattered photon depends on the scattering angle $\left(θ\right),$ where maximum energy transfers to the electron at 1800 and minimum energy transfers at 00. Then, the energy of the recoil electron Ek is:

Ek= $E\_{γ}$- $E\_{γ^{'}}$= $E\_{γ} \frac{\frac{E\_{γ}}{m\_{e}c^{2}}\left(1-cosθ\right)}{1+\frac{E\_{γ}}{m\_{e}c^{2}}\left(1-cosθ\right)}$

The Compton scattering probability is independent of atomic number Z because the interaction is with an electron, which is assumed to be at rest, and the probability is proportional to Z/A, which is approximately 0.5 for most materials. However at high incident energies, the probability of Compton scatter varies inversely with the incident photon energy. The Compton scatter cross section is also dependent upon the scattering angle and the incident photon energy.

**2.4.2 Coherent scattering**

 The coherent scattering process is also called Rayleigh scattering. In this event, the incident photon is scattered by the whole atom, and consequently it only changes direction. The scattered photon possesses the same energy as the incident one and hence this scattering is also called elastic scattering. The target atom is neither excited nor ionized in this mechanism. . The probability of coherent scattering is high for low-energy photons and in high-Z elements. When coherently scattered photons interfere constructively, X-ray diffraction occurs.

 In the present studies, the main type of interaction for the X-ray photons is via photoelectric process as the energy of the beam is greater than the binding energy of the K-shell electron in Fe, Cu and Zn. There is also a probability of secondary fluorescence of Fe X-rays on excitation by Cu and Zn X-rays as the energies of Cu and Zn X-rays are sufficient to cause atomic excitation in Fe. Apart from the photoelectric process, Compton scattering is also likely to occur and to depend on the geometry of the source-sample-detector, the spacing of the Compton peak is determined. Coherent scattering of photons is also observed which occurs due to the interaction of X-ray photons with the sample. Coherent scattered peaks are found at energies of the photons from the X-ray tube, which in the present studies correspond to Molybdenum X-rays.

**2.5 Types of XRF techniques**

 The XRF technique mainly can be divided into energy dispersive XRF (EDXRF) and wavelength dispersive XRF (WDXRF). In EDXRF analysis, the fluorescent X-rays reach the detector without any obstacles in between and hence a complete energy spectrum can be obtained. The resolution of EDXRF spectrometers typically depends on the detector employed and ranges from 150-600eV. The main advantage of an EDXRF system is its high efficiency. In WDXRF, the fluorescent X-rays from the sample is diffracted using a crystal and only selective wavelengths of radiation enter the detector. The spectrometer is based on the principle of Bragg's diffraction, and the geometry is arranged according to the Bragg's law. WDXRF spectrometers offer high resolution. In the present experiments, an EDXRF spectrometer is employed due to its ease of use, compact size and analytical flexibility.

 Chapter 3

 **Methodology**

In the present study, samples of brain and spinal cord tissue of LE (control) and LES rats were analyzed for Fe, Cu, and Zn using the principle of X-ray fluorescence. This chapter provides details on the procedure followed in conducting experiments on the samples and methods of data analysis employed to obtain information from raw data provided by the XRF system. The first step in the procedure was to collect samples and prepare them for experiments. Once the samples were ready, the XRF system was properly adjusted to obtain spectral data. The data were then converted into a readable format and peak fitting was carried out to obtain elemental concentrations. The results were then statistically analyzed to observe trends in concentrations in various specimen tissues. Each step is explained in detail hereunder.

**3.1 Tissue sample preparation**

**3.1.1: Extraction of tissue samples**

 Brain and spinal cord were harvested intact from age-matched LE and LES animals and stored at -85C without tissue fixation, to avoid any alteration in the metal levels in the samples, until their further dissection. In order to study the levels of transition elements regionally, all brain samples were appropriately dissected for five regions, namely cortex, cerebellum, thalamus, striatum, and hippocampus. In normal tissue, myelin assists in identifying the regional borders. However, due to missing myelin in the mutant LES brains, it was more difficult to distinguish among the required brain regions visually. Nevertheless, the dissection specialist experienced no problem in the separation of the desired regions. At the time of dissection, the brains and spinal cords were first thawed; the distinct regions were then dissected using sterilized surgical blades and tweezers.

**3.1.2 Sample holders**

 Several issues such as absorption of solution by the walls of the container, leaching from the walls, loss through volatilization, and degradation through photochemical or biological activity (Valkovic, 1989) have to be taken care of in trace elemental studies, as any of these problems will lead to an underestimation of the concentrations of elements. The sample holders must also be free from contamination. For this reason, custom made high-density polyethylene (HDPE) sample holders was used to hold the tissue samples. The sample holders were cylindrical in shape with a recess cut into the end with 4.2mm diameter and 2mm depth. The thickness of the recess wall was 100 μm to enable lesser attenuation of the incident and emitted X-rays. The sample holder dimensions are shown in Figure 3.1.



 **Figure 3.1** Dimensions of a sample holder (Darvish, 2012)

The dissected samples were then transferred to the sample holders using a micro spatula and then sealed in place with a small piece of XRF film before securing using an O-ring. To avoid any sample contamination, the holders as well as the O-ring were pre-cleaned by storing in nitric acid overnight followed by washing with de-ionized water in an ultrasound bath for approximately three hours. Then the empty sample holders were stored in 70% ethanol until the dissection time when they were filled with tissue. In order to avoid degeneration of the tissue samples in the holders, the samples were stored in a freezer unit until the XRF measurements were made. The samples were kept frozen at -80ºC and allowed to thaw for a few minutes prior to the measurement.

**3.1.3 Sample collection and number of samples**

 Tissues from LE control and age-matched LES mutant rats were used to study the elemental concentrations. Two groups were investigated: three-week LE and LES rats and 16-week LE and LES rats with n = 5 (number of samples) for each strain. The rats of the desired ages were obtained from the Central Animal Facility at McMaster University where they were bred and raised. A total of 120 samples were used in this study out of which 100 samples were extracted from five brain regions; cortex, cerebellum, hippocampus, thalamus, and striatum; the remaining 20 samples were taken from the lumbar region of the spinal cord. Number of samples included 25 brain region samples and five spinal cord tissue samples from each of the 3-week and 16-week LE and LES groups. Region-wise detail of the collected samples from brain and spinal cord is tabulated in Table 3.1.

|  |
| --- |
| **Table 3.1** Collection of samples from brain and spinal cord regions of  control (LE) and shaker rats (LES) |
| # of samples from brain region | # of samples from each spinal cord |
|  25 Control rats (LE) 3 weeks old | 5 Control rats (LE) 3 weeks old |
|  25 Shaker rats (LES) 3 weeks old | 5 Shaker rats (LES) 3 weeks old |
|  25 Control rats (LE) 16 weeks old | 5 Control rats (LE) 16 weeks old |
|  25 Shaker rats (LES) 16 weeks old | 5 Shaker rats (LES) 16 weeks old |
| *Total: 100 samples* | *Total: 20 samples* |

**3.2 XRF set up**

 A careful selection of X-ray source and instrumentations is necessary for the success of any XRF experiment. The system used in this study to investigate the metal levels is shown in Figure 3.2. It was a goniometer-based system, which allows the sample to be rotated to a particular angle. This system has a molybdenum (42Mo) -target X-ray micro-focus tube (also known as the analyzer) that produced a mono-chromatic beam of approximately 17.5 keV. This energy of the incident beam would be sufficient to excite all elements with a K absorption edge less than 17 keV and hence suitable for elements of interest Fe, Cu, and Zn, which have their absorption edges in the range of 5 keV to 10 keV. The X-ray source was focused to deliver a 1.8 mm × 1.8 mm beam of approximately 3.5 × 106 photons/s onto the sample. This beam was achieved by using a multilayer X-ray optics device. To collect the signals from the elements of interest from the irradiated sample, a silicon drift detector (SDD) was used. The SDD employed in the present studies has an active area of 10mm2 and was placed 5mm away from the end of the sample. Being this close allows for a high solid angle and hence maximise the collection of the XRF. A 90° angular geometry between the incident beam and the SDD was used so that the Compton peak appears at almost the same energy as the incident peak and hence for present work does not interfere for the elements of interest. The sample was rotated during measurement in order to allow the incident X-ray beam to probe all of the tissue in the sample. This procedure aids in improving the homogeneity in sample measurements.



**Figure 3.2** A setup of XRF system coupled with a silicon drift detector (SDD) (Mersov. Et al, 2014)

**3.3 Data collection**

 In order to achieve Fe, Cu, and Zn XRF response adequate for statistical purposes, long measurement times were required because elements in biological cells are present in very low concentrations < 1ppm to $\~$ 40 ppm. The X-ray tube was operated at a maximum voltage of 50 kV with maximum current of 500 mA and a counting time of 26100 s (during daytime), and with maximum current of 320 mA and a counting time of 52200 s (overnight). It was decided to reduce the current during overnight measurements in order to reduce instantaneous power and hence an optimum current setting was selected. Therefore, in order to maintain the same flux for day and night measurements the samples were counted overnight for double the counting time during the day. It was observed that a sample counted with a maximum current setting of 500 mA for 7 hours during the day yielded the same number of counts on the X-ray peak as that counted with a maximum current setting of 320 mA for 14 hours during the night. Hence, these voltage and current settings were chosen to ensure approximately the same total X-ray beam intensity during day and overnight assay measurements. To record the energy dispersive XRF spectrum, a software package (QM100) supplied by Bruker-AXS was used. These spectra can be exported in Excel format for processing and analysis purposes. In the present studies, the elements of interest Fe, Cu, and Zn, were identified by the Kα photo-peaks at energies 6.04, 8.04, and 8.64 keV respectively.

**3.4 Peak fitting**

 Each collected spectrum was analysed using Peak Fit software (version: 4.12). Prior to fitting, background subtraction was performed for each measurement to isolate the fluorescence signal and the spectra were smoothed using a Savitsky–Golay procedure that is aimed at suppressing any statistical fluctuations, which may result from the uncertainty of the contents of each channel. Thereafter each peak for the element of interest in the spectrum was adjusted separately with the relevant Gaussian function peak and the amplitude, FWHM; then the integrated areas under each peak were determined.

 In order to avoid inhomogeneity in sample data and to account for any incident photon intensity variations enabling direct comparisons to be made between all spectra, normalisation of Kα photo peak area with total scatter peak area was carried out. Normalisation process primarily avoids effects due to changes in incident flux. The normalisation factor is determined as the ratio of photo peak area to the total scatter peak area. It is a dimensionless quantity and is a useful parameter in calibration procedures. Kα photo peak area represents the number of detected characteristic Kα X-rays from a particular element and hence is proportional to the number of atoms / concentration in the sample. The scatter peak counts mainly arise due to the scatter of incident X-rays by the sample, as the beam is a focussed one. Both events resulting in a photopeak as well as scatter peak arise from the incident beam and hence a ratio of the areas under these peaks will also be proportional to the elemental concentration in the sample. All spectra from LE and LES samples were fitted and normalisation factors were determined.

**3.5 Statistical analysis**

 The sample size in the present experiments is small. The maximum number of samples for one strain of rat tissue samples was 5. The data were assumed to be normally distributed and homogeneous. For the statistical analyses of the XRF data generated in the present study, SPSS software (version 22) was employed. All variables–the mean, the standard error (SE) and the standard deviation (SD) values–were determined by performing descriptive statistical analyses.

 To test the significance levels (P-Value) of any difference between Fe, Cu, and Zn concentrations in LE and LES brain samples, a one-way ANOVA test was carried out with 5% significance level using Primer of Biostatistics program. The one-way analysis of variance (ANOVA) is used to determine whether there are any significant differences between the means of two or more independent (unrelated) groups. The ANOVA test was applied in this study with two factors namely the "rat type", which are LE and LES rats, and "brain region", which are (cortex, cerebellum, etc.,) for the data with similar age group (three weeks and 16 weeks). The obtained p-values were compared and discussed in the next chapter. A p-value > 0.05 indicates no statistical differences between groups. However a p-value < 0.05 indicates a statistical difference between groups. The interest in this study is on the observation of the significant elemental differences between the matched groups of different ages for each respective region or part of the CNS. A one-way ANOVA is an omnibus test statistic. It just tells us that at least two groups are different from each other. One major limitation of ANOVA is it does not yield any information about which two groups are exactly different in means. For this purpose, a post hoc pairwise analysis (Tukey HSD) was carried out. Post hoc tests facilitate additional exploration of the differences among means in order to understsnd which groups are significantly different from each other. Tukey’s test is one of the post hoc procedures that was found accurately maintains alpha levels at their intended values as long as statistical model assumptions are met. It was designed for a situation with equal sample sizes per group, but can be adapted to unequal sample sizes as well.

For Spinal Cord data a T-independent test was applied because we just have two matched groups for each age. A t-test is the best choice to test differences between two independent means specially when the variances are normally distributed and sample size is small as spinal cord data in this study. The obtained p-values for spinal cord were compared and discussed in the next chapter as well.

 Tables 1-3 of Appendix 1 show the statistics of raw data for Fe, Cu, and Zn measurements in brain regions and spinal cords at 3 and 16 weeks of age for the animals under investigation.

**RESULTS AND DISCUSSION**

**4.1 Measurement of elemental contents**

 Figure 4.1 shows an example of the XRF spectrum generated in the present study. The figure is plotted with count per second against energy. It was reasonable to use count per second as samples were counted for different times following day and overnight measurements. Clear Kα X-ray peaks of Fe, Cu, and Zn are observed at energies 6.04, 8.04, and 8.64 keV respectively. The small spikes beside each Kα peak represent the corresponding Kβ X-ray peak. The peaks highlighted on the higher energy side correspond to the total scatter peak as a result of scattering of Mo X-rays by the sample. By observing the height of each X-ray peak, a rough estimate of the peak areas can be done. As can be seen from the figure, the resolution of the SDD detector is excellent and typically ranges from 150-600 eV. The Y-axis gives an estimate of the elemental concentration. Even though the exact level cannot be determined without calibrating the system, the Y-axis data is proportional to the actual concentration.

 **Figure 4.1** An example of XRF spectrum from one tissue

 Calibration of the system needs to be carried out in order to obtain the actual concentrations of the elements. Preliminary investigations revealed that the standard samples were contaminated with zinc; one of the elements of interest. It was not possible during the course of study to secure standard samples free of zinc. However, the analysis was carried out using normalisation procedures. Normalisation factor throws light on the concentration of the element. Though it does not provide the actual concentration of the element, it helps us to identify differences in concentration if any. The primary goal of the present studies was to find if myelinated rats show differences in elemental concentration. Hence, normalisation procedure can be applied. Normalized counts were calculated as the ratio of the Kα X-ray peak area of the element to the total scattered peak area for that element.

 As a further example, Figures 4.2 and 4.3 illustrate two spectra involved in the normalization process after fitting procedure, one for the region from 5-10 keV including elements of interest and the other for the scattering region from 14-18 keV including the Si escape peak (the effect related to the characteristic X-ray generated by the interaction of incident radiation within the detector), tail due to incomplete charge collection at the detector surface, Compton, and coherent scatter peaks.



 **Figure 4.2** Peak fitting in XRF spectrum for ROIs from 5-10 keV along with

 Kα and Kβ X-rays from Fe, Cu and Zn



 **Figure 4.3** Scattering region showing Si escape peak, tail, Compton and coherent scattering peaks

**4.2 Statistical analysis**

**4.2.1 Results from brain tissue samples**

 The first step in the statistical analysis was to check whether there are significant differences between groups in our experiments. 100 samples were tested using one-way ANOVA, and the p-value was determined. Then a pair-wise (TukeyHDS) was applied to find the significant differences between matched groups. The results of ANOVA are represented in tables and bar graphs as a mean value ± standard deviation, for n = 5.

**4.2.1.1 Iron (Fe)**

 Figures 4.4 and 4.5 with tables 4.1 and 4.2 represent the results for iron concentration in LE and LES rats at three weeks and 16 weeks of age respectively.

**Figure 4.4** Brain tissue composition for Fe from three-week-old LE and LES rats. The error bars shown are standard deviation

 **Figure 4.5** Brain tissue composition for Fe from 16-week LE and LES rats. The error bars shown are standard deviation

 As discussed in Chapter 3, a high normalisation factor indicates the higher concentration of the element in the sample. From the histograms for three-week- and 16-week-old LES rats, it can be observed that the concentrations were showing an increasing trend for LES rats. For each tissue type, the concentration of Fe for 16-week rats LE/LES is higher than their counterparts at three weeks of age. Within groups, i.e., at three weeks of age, the concentrations of Fe in LE and LES rats were not statistically different (p>0.05). The results are shown in Table 4.1

|  |  |
| --- | --- |
|  | Fe in all brain regions (ANOVA test) |
| 3w LE and LES | .062 |
| 16w LE and LES | **.001** |

**Table 4.1** p-values for Fe concentration in brain tissues

 One-way ANOVA for LE and LES for all regions of brain determine a p-value of 0.062 at three weeks of age indicating no statistically significant difference at 3weeks age. The same was 0.001 at 16 weeks of age suggesting statistical significance. However, the Tukey test indicates no statistically significant difference between like regions at the same age for control and LES rats. As ANOVA results showed significant differences for 16-week-old rats, Tukey's test was carried out for this age factor with comparisons for same brain region in LE and LES rats. The data is presented in Table 4.2 indicate that iron concentrations do not change significantly in dysmyelinated rats.

|  |  |
| --- | --- |
| Brain region | Sig. value |
| Cortex | 0.835 |
| Cerebellum | 1.000 |
| Hippocampus | 0.701 |
| Thalamus | 0.999 |
| Striatum | 0.935 |

**Table 4.2** p-values for Fe concentration in brain tissues at 16 weeks of age for LE and LES rats from Tukey's test.

 Iron is an important component for myelination. It is available in plenty in different cells of the CNS with astrocytes and oligodendrocyte glial cells being the prominent ones. Astrocytes act as sites of iron storage whereas oligodendrocytes produce high levels of ferritin (Rouault & Cooperman, 2006). Accumulations of ferritin in oligodendrocytes could correspond to the general task of ferritin to store iron in a non-toxic form (Quintana & Gutierrez, 2010). However in LESrats, the actively myelinating oligodendrocytes exhibit structural changes which indicate an abnormal accumulation of metabolites that are involved in myelin formation. There is also a possibility of progressive accumulation of membranous material due to failed attempts at myelination. The metabolite accumulation does not stop at the end of the neonatal stage for LES rats. Oligodendrocyte requires high levels of iron during myelination and after completion requires less iron. They maintain stable iron level later on through iron efflux mechanism. (Magaki *et al.,* 2007). The Presence of ferrous iron in the ferritin cores from oligodendrocytes is a source of oxidative stress that will eventually lead to myelin damage. It has been observed that patients suffering from neurological diseases accumulate iron in the brain. Studies reveal increased activity of antioxidant proteins in AD patients (Quintana & Gutiérrez, 2010). Hence, a higher level of iron in LES rats is not surprising. However, in the present studies, the difference is not that significant and can be due to a smaller sample size and number. The slight variations in the normalisation factors for different regions may be due to variations in mass of the samples used, which would indirectly effect the concentrations of the element.

**4.2.1.2 Copper (Cu)**

 Table 4.3 and Figures 4.6 and 4.7 represent the results for copper concentration in LE and LES rats at three week and 16 weeks of age respectively.

|  |  |  |
| --- | --- | --- |
|  | Cu in all brain regions (ANOVA test) | Tukey HSD test |
| 3w LE and LES | .589 | The only significance is (.009) between LE-Cortex 16w and LES –Cortex 16w |
| 16w LE and LES | **.002** |

**Table 4.3** p-values for Cu concentration in brain tissues

 **Figure 4.6** Brain tissue composition for Cu from three-week-old LE and LES rats. The error bars shown are standard deviation

 **Figure 4.7** Brain tissue composition for Cu from 16-week LE and LES rats. The error bars shown are standard deviation

 No statistically significant difference was observed in levels of copper in different brain regions of LE and LES rats at three weeks. However in 16-week rats, p < 0.05 was observed in the cortex (p value=0.009) region of the brain. This indicates a change in copper concentrations in this region of the brain. In the cortex tissue sample, the concentration of copper in LE rats at three weeks and 16 weeks was not noticeably different. But a significant increase in these levels was observed for LES rats at 16 weeks as compared with those at three weeks. The copper concentrations varied significantly in the LE rats, increasing considerably at 16 weeks of age. LES rats at 16 weeks of age tend to exhibit no such increase when compared with those at three weeks of age.

 Copper plays important non-enzymatic functions in nerve myelination. It also plays and an essential role in brain development, shown by the presence of demyelination and by neurodegeneration in patients affected by Menkes disease (Crisponi *et al.*). Duflou *et al*. (1989) measured the concentration of trace elements in 50 different regions of 12 normal human brains by particle-induced X-ray emission (PIXE) and established that copper is accumulated in the substantia nigra, locus coeruleus, dentate nucleus, and cerebellum. Our results are in agreement with these findings. Some studies have demonstrated that patients with neurodegenerative diseases had elevated Cu concentrations in their plasma suggesting a direct or indirect involvement of Cu overload in the progression and/or the aetiology of neurologic diseases (Squitti *et al*., 2007).

**4.2.1.3 Zinc (Zn)**

 Table 4.4 and Figures 4.8 and 4.9 represent the results for zinc concentration in LE and LES rats at three week and 16 weeks of age respectively.

|  |  |
| --- | --- |
|  | Zn in all brain regions (ANOVA test)  |
| 3w LE and LES | **.033** |
| 16w LE and LES | .**000** |

**Table 4.4** p-values for Zn concentration in brain tissues

**Figure 4.8** Brain tissue composition for Zn from three-week-old LE and LES rats. The error bars shown are standard deviation

 **Figure 4.9** Brain tissue composition for Zn from 16-week LE and LES rats. The error bars shown are standard deviation

 For rats at three weeks and 16 weeks of ages, no significant difference in concentration was observed in the different regions of the brain although significant P values (0.033) for three-week-old and (.000) for 16-week-old were observed among regions but no significances were found between matched regions. However, Zinc concentration was found to be lower in the LES rats as compared with the controls.

 One explanation of the lower Zn content observed in the present study could be due to the samples. The samples in the present study are dysmyelinated rats, in which a lack of myelin is the main feature. Myelin is rich in Zn and hence lack of myelin indicates lower Zn levels. Studies (Popescu *et al*., 2009; Duflou *et al*., 1989) indicate that most of the brain regions that are rich in iron and copper content are low in zinc content. Hence our observation of lower zinc content is not surprising. *In vivo* studies of zinc distributions in the brain have shown relatively high levels in the dentate gyrus within the hippocampus where zinc is thought to act as neuromodulator agent (Corniola *et al.,* 2008; Franklin *et al*., 1992; Law *et al.,* 2003; Yanik *et al.,* 2004).

 Tukey's test however suggests no significant changes in zinc concentration for 3 weeks and 16 weeks old rats as in Table 4.5.

|  |  |  |
| --- | --- | --- |
| Brain region in LE and LES | 3 weeks | 16 weeks |
| Cortex | 1.000 | 1.000 |
| Cerebellum | 1.000 | 0.804 |
| Hippocampus | 0.882 | 0.296 |
| Thalamus | 1.000 | 1.000 |
| Striatum | 0.494 | 0.999 |

**Table 4.5** p-values for Zn concentration in brain tissues at 3 and 16 weeks of age for LE and LES rats from Tukey's test.

 A key point to mention is that the sample holders are made of HDPE and some have been found to have some zinc contamination. As one reason for an overestimation of zinc, this plays an important role in determination of significant differences especially at trace levels. Care should be taken to avoid/reduce this contamination for better statistical results.

**4.3 Results from spinal cord tissue samples**

 The elemental analyses of spinal cord tissues in LE and LES rats at the ages of three and 16 weeks for Fe, Cu, and Zn are shown in Table 4.6 and in Figures 4.10-4.12 respectively.

**Table 4.6** p-values for Fe, Cu and Zn concentration in spinal cord tissues

|  |  |  |  |
| --- | --- | --- | --- |
|  | Fe | Cu | Zn |
| 3w LE and LES | .940 | .144 | .746 |
| 16w LE and LES | .188 | **.018** | **.045** |

**Figure 4.10** Spinal cord tissue composition for Fe from three- and 16-week-old LE and LES rats. The error bars shown are standard deviation

**Figure 4.11** Spinal cord tissue composition for Cu from three- and 16-week-old LE and LES rats

 **Figure 4.12** Spinal cord tissue composition for Zn from three- and 16-week-old LE and LES rats. The error bars shown are standard deviation

 No statistical significance (p<0.05) in iron concentration was found between the matched groups at either age. Though not statistically significant, the concentration for LES rats at 16 weeks was higher than for LE rats at the same age. This observation is in agreement with the results from similar experiments (Lobo, 2013) on spinal cord tissues carried out using neutron activation analysis (NAA). It is evident from the histograms that the overall Fe level in the spinal cord tissues of the 16-week-old LES rats was higher as compared to LE rats. As discussed earlier, iron concentrations tend to increase in dysmyelinated rats due to damage to the myelin sheath, which results in metabolite accumulation. The present technique, XRF, was able to provide results relating to the levels of iron that are in agreement with the actual biological processes that lead to neurological disorders.

 Figure 4.11 shows levels of copper in the spinal cord tissues of the LE and LES rats at three and 16 weeks. In the three-week LES rats, the Cu content appeared to be slightly elevated as compared to LE rats at the same age. However, in the 16-week LES rats, a significant increase (p<0.05) in Cu level (i.e., p = 0.018) was found compared to the age-matched controls (LE). Neurological disorders are often associated with copper concentration in the brain. Cu is essential for brain metabolism as well as in the absorption and use of iron. High levels of Cu lead to oxidative stress, which in turn causes demyelination. Additionally, elevated Cu concentrations in plasma were found to play a key role in the progression of certain neurological diseases (Squitti et al., 2007). Hence, our observation of higher levels of copper in dysmyelinated rats is not surprising.

 The analyses of spinal cord tissues for Zn levels in the three-week- and 16-week-old animals are shown in Figure 4.12. It can be seen that there is a significant difference (p<0.05) in Zn levels (p = 0.045) between the 16-week matched groups. With large error bars, the concentration of zinc in the control LE rats seems to remain almost the same at three and 16 weeks of age while they tend to decrease in LES rats. This observation is however not in agreement with similar studies carried out using NAA (Lobo, 2013). Though a statistically significant difference was seen in the concentrations of zinc, the levels of zinc were higher in the LES rats as opposed to our observation. Again, contamination plays an important role and hence our studies, though they show a statistically significant difference in levels, fail to agree with the trend from NAA studies.

 On the whole, the XRF technique was found to be a suitable technique for trace elemental studies. At three weeks of age, only zinc concentrations were statistically significant between LE and LES rats in the striatum region of the brain. At 16 weeks of age, significant differences in trace element concentrations were observed. The cortex was found to exhibit changes in iron and copper concentrations. Iron and zinc concentrations were found to change in the hippocampus whereas the thalamus showed changes in the levels of copper. For spinal cord tissues, copper and zinc concentrations were found to change significantly at 16 weeks of age.

Chapter 5

**Conclusions and Future work**

 XRF is a popular non-destructive technique for trace element studies. In the present studies, the energy dispersive XRF technique was applied to study the concentrations of iron, copper, and zinc in Long Evans (control) and Long Evans Shaker (dysmyelinated) rats. The results were analyzed and presented in the previous chapter. The following conclusions can be drawn from our study.

 The primary goal of the present study was to check for the feasibility of using the XRF technique for determining trace elemental levels in control and dysmyelinated brain and spinal cord tissues of rats. The results obtained indicate that the technique is suitable for such studies. Clear high-intensity peaks were observed for each element. The resolution was higher as an SDD was employed versus a Si (Li) detector. The present set-up is well suited for Fe, Cu, and Zn as their binding energy is just below the peak energy of photons from the X-ray tube. The results obtained in the present study are in agreement with those presented in the literature. Our results are also in agreement with the results of similar experiments carried out on spinal cord tissues using neutron activation analysis except for zinc.

No statistical difference was found in the content of iron and zinc in different regions of the brain. This could be due to small sample size though the difference in normalisation factors were observed for LE and LES rats at different ages. Increasing the number of samples will facilitate better statistics. Larger samples increase the chance of significance being more reliable. A smaller sample size results in large standard deviation and resultant error thereby making the data less reliable. Under this perspective, the results obtained in this study cannot be considered perfect or error free. Therefore for the generation of more reliable data, a large sample size should be employed, i.e., a greater number of animals should be used for analyses in future studies. Normality was assumed for the present data. With larger sample size, normality tests can also be carried out, and analysis protocol can be designed accordingly.

 In the present study, the actual concentrations could not be obtained, as the system was not calibrated. The problem with calibration is the contamination of standard samples with elements of interest. It is always valuable to know the actual levels of trace elements in units of either µg/g or mg/g. Hence, steps need to be taken to calibrate the system by utilising contamination-free standards. It is also recommended to carry out the spiking technique to determine the level of zinc contamination. The accuracy of the analytical technique can also be tested using process. Spiked and un-spiked samples are tested and the difference in values is essentially the spike concentration.

Before using the data generated in the present study for making a generalized assessment of elemental concentrations in LE and LES rats investigated, the method employed here definitely needs improvements for which further work is required to be undertaken. This will help in developing an effective system to measure the elemental levels in rats and human studies using X-ray fluorescence technique. The available data on transition metals levels in the brain regions of rodents are limited, and the results of this study should add to the present data base for future research addressing the study of neurological diseases in human due to demyelination.

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**APPENDIX 1**

 **RAW DATA:**

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| **Table 1**: The mean, SE and SD values of normalized area under K-peak (ratio of k-value peak area of element/total scatter area) for all the variables for the brain regions at 3-weeks |
| Brain region | Element | Type of rat |  Mean |  SE |  SD |
| Cortex | Fe | LE | .00048598 | .000015939 | .000035640 |
| LES | .00048713 | .000026461 | .000059169 |
| Cu | LE | .00026042 | .000013503 | .000030195 |
| LES | .00023148 | .000025388 | .000056770 |
| Zn | LE | .00176113 | .000061174 | .000136788 |
| LES | .00177595 | .000055456 | .000124003 |
| Cerebellum | Fe | LE | .00058864 | .000040036 | .000089523 |
| LES | .00066307 | .000031991 | .000071535 |
| Cu | LE | .00026612 | .000030710 | .000068670 |
| LES | .00025010 | .000024822 | .000055504 |
| Zn | LE | .00186834 | .000128302 | .000286892 |
| LES | .00180204 | .000010282 | .000022992 |
| Hippocampus | Fe | LE | .00063804 | .000052016 | .000116311 |
| LES | .00061025 | .000053620 | .000119899 |
| Cu | LE | .00029634 | .000029527 | .000066023 |
| LES | .00023848 | .000021445 | .000047953 |
| Zn | LE | .00204837 | .000168912 | .000377699 |
| LES | .00186262 | .000063720 | .000142482 |
| Thalamus | Fe | LE | .00054562 | .000036148 | .000080830 |
| LES | .00060921 | .000080207 | .000179349 |
| Cu | LE | .00021197 | .000011728 | .000026226 |
| LES | .00025688 | .000045728 | .000102250 |
| Zn | LE | .00166113 | .000093932 | .000210038 |
| LES | .00162233 | .000036139 | .000080809 |
| Striatum | Fe | LE | .00051888 | .000033283 | .000074423 |
| LES | .00057742 | .000025147 | .000056231 |
| Cu | LE | .00025418 | .000021218 | .000047446 |
| LES | .00028031 | .000023100 | .000051652 |
| Zn | LE | .00166725 | .000012963 | .000028985 |
| LES | .00193480 | .000099656 | .000222837 |

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| --- |
| **Table 2**: The mean, SE and SD values of normalized area under K-peak (ratio of k-value peak area of  Element/total scatter area) for all the variables for brain regions at 16-weeks |
| Brain region | Element | Type of rat |  Mean |  SE |  SD |
| Cortex  | Fe | LE | .00057684 | .000023180 | .000051832 |
| LES | .00070041 | .000037412 | .000083655 |
| Cu | LE | .00024982 | .000008005 | .000017899 |
| LES | .00037738 | .000037590 | .000084053 |
| Zn | LE | .00206275 | .000070026 | .000156582 |
| LES | .00212679 | .000081422 | .000182064 |
| Cerebellum | Fe | LE | .00088427 | .000040909 | .000091475 |
| LES | .00087195 | .000030560 | .000068335 |
| Cu | LE | .00030995 | .000031490 | .000070413 |
| LES | .00037627 | .000029983 | .000067043 |
| Zn | LE | .00162109 | .000070841 | .000158405 |
| LES | .00178393 | .000045767 | .000102339 |
| Hippocampus | Fe | LE | .00083087 | .000051918 | .000116093 |
| LES | .00068903 | .000025196 | .000056340 |
| Cu | LE | .00033780 | .000017930 | .000040092 |
| LES | .00030564 | .000018458 | .000041274 |
| Zn | LE | .00234063 | .000073808 | .000165040 |
| LES | .00209641 | .000060122 | .000134436 |
| Thalamus | Fe | LE | .00088240 | .000092838 | .000207591 |
| LES | .00082884 | .000046570 | .000104134 |
| Cu | LE | .00035105 | .000012700 | .000028398 |
| LES | .00027981 | .000018872 | .000042200 |
| Zn | LE | .00159252 | .000080055 | .000179009 |
| LES | .00159368 | .000062570 | .000139911 |
| Striatum | Fe | LE | .00077750 | .000094650 | .000211644 |
| LES | .00067405 | .000042354 | .000094706 |
| Cu | LE | .00028402 | .000013778 | .000030809 |
| LES | .00028927 | .000019318 | .000043197 |
| Zn | LE | .00185037 | .000090409 | .000202160 |
| LES | .00192244 | .000035427 | .000079217 |

|  |
| --- |
| **Table 3**: The mean, SE and SD values of normalized area under K-peak (ratio of k-value peak area of element/total scatter area) for all the variables for spinal cord at 3- and16-weeks |
| Group’s Name | Element | Type of rat |  Mean |  SE |  SD | P Value |
| SC (3W) | Fe | LE | .00074217 | .000071681 | .000160285 | .940 |
| LES | .00073417 | .000073768 | .000164951 |
| Cu | LE | .00024723 | .000017513 | .000039160 | .144 |
| LES | .00028733 | .000017542 | .000039226 |
| Zn | LE | .00719157 | .003031570 | .006778797 | .746 |
| LES | .00602030 | .001728906 | .003865951 |
| SC (16W) | Fe | LE | .00081279 | .000098202 | .000219587 | .188 |
| LES | .00102536 | .000110547 | .000247191 |
| Cu | LE | .00027511 | .000029120 | .000065114 | .018 |
| LES | .00036644 | .000009510 | .000021266 |
| Zn | LE | .01041934 | .002511020 | .005614811 | .045 |
| LES | .00399539 | .001007591 | .002253041 |