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Novel approaches for correction against the soft matrix effects in the quantitative elemental imaging of human substantia nigra tissue using synchrotron X-ray fluorescence

A.D. Surowka [a,*], P. Wrobel [a], M.M. Marzec [b], D. Adamek [c], M. Szczerbowska-Boruchowska [a,*]

[a] AGH University of Science and Technology, Faculty of Physics and Applied Computer Science, Al. Mickiewicza 30, 30-059 Kraków, Poland
[b] AGH University of Science and Technology, Academic Centre for Materials and Nanotechnology, al. Mickiewicza 30, 30-059 Kraków, Poland
[c] Jagiellonian University, Faculty of Medicine, Department of Neuropathology, Krakow, Poland

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ABSTRACT

The inherent structural heterogeneity of biological specimens poses a number of problems for analytical techniques to assess for the elemental composition of a sample, and this is the case with quantitative X-ray fluorescence (XRF). Differences in density along with any possible variation in thickness upon frequently used freeze drying of thin samples could influence the results of the quantification and therefore underlie one of the most critical matrix effects in XRF, often referred to as the mass thickness effect. In our study, we analyzed substantia nigra tissue samples of various thicknesses mounted onto silicon nitride membranes. The aim was to show up the variation in the mass thickness of the different substantia nigra tissue compartments: the neuromelanine pigmented neurons and neuropil could influence the final quantitative results. In that respect, the main goal was to derive several semi- and fully-quantitative methods to correct for the mass thickness effects using either a membrane *Si* transmission signal or the intensity of incoherently scattered primary X-ray radiation. Also, the pioneer topographic studies on dried substantia nigra tissue specimens demonstrated the drying procedure is accompanied by an around 80% reduction in the samples' thickness. The correction scheme is presented together with the semi-theoretical procedure developed to compute for the mass thickness for substantia nigra tissue structures, and the correction scheme's robustness is also presented.

1. Introduction

The inherent structural heterogeneity of biological specimens poses a number of problems for analytical techniques to assess for the elemental composition of a sample, and this is the case with quantitative X-ray fluorescence (XRF) [1]. The method is based on the emission of various lines of characteristic radiation attributable to different elements upon irradiation of a sample with the primary exciting beam of sufficient energy [2]. For a fully theoretical conversion of the intensities of the characteristic lines of various chemical elements into their mass fractions in a sample, the fundamental parameters approach first developed by *Shermann* in 1955 is now commonly used [3,4]. However, due to the relatively large number of parameters to be known, a quantification based solely on a fully theoretical approach is fairly difficult, though. For this purpose, various simplifications to this formula have been proposed [3,4]. One of these, a thick sample approximation, relies on the assumption that the areal mass of a sample (the mass thickness – M) is infinite [5]:

$$I_{el} = I_0 \frac{\Omega}{4\pi} \frac{\sigma_{XRF}(E_0)}{\sigma_{TOT}(E_0)} \frac{1}{\sin\psi_1} \frac{C_{el}\alpha_{el}(E_0)}{\frac{\alpha(E_0)}{\sin\psi_1} + \frac{\alpha(E_1)}{\sin\psi_2}} \quad (1)$$

Where: E_0, E_1 - energy of the primary exciting beam and characteristic radiation of the *ith* element, respectively; I_{el} - intensity of the characteristic radiation of the *ith* element; C_{el} – weight concentration of the ith element; I_0 - incident photon intensity at the energy E_0; Ω - the detector solid angle; σ_{XRF} and σ_{TOT} - fluorescence and photoionization cross sections, respectively; ψ_1 and ψ_2 – incidence and take-off angles, respectively; α_{el}, α – attenuation coefficients for the *ith* element analyzed and sample matrix, respectively.

However, with another approximation referred to as a thin sample approximation ($M \approx 0$), we can easily quantify the elements in terms of areal masses, which requires an external calibration based on the measurement of the standards of known elemental composition [4]. Nevertheless, one of the main challenges behind the method is to determine the dimensionless mass fractions in biological samples in-situ,

* Corresponding authors.
E-mail addresses: asurowka@agh.edu.pl (A.D. Surowka), magdalena.boruchowska@fis.agh.edu.pl (M. Szczerbowska-Boruchowska).

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which, unlike the areal mass, are thickness- and density-independent [6]. By using the values of the areal masses determined by the external standard approach, the concentration of a specific element (C_i) yields:

$$M = \rho\langle T\rangle \tag{2}$$

$$C_i = \frac{M_i}{M} = \frac{M_i}{\rho\langle T\rangle} \tag{3}$$

Where: M_i - the areal mass of the *i-th* element; M - the areal mass of the sample; ρ – the sample density; $\langle T\rangle$ - the projected thickness.

One can notice that in order to transform the areal mass into the concentration, the product of the sample's density and thickness, called the projected areal mass, must be known [6]. This problem becomes extremely cumbersome when we need to carry out the analysis of biological tissues (human, animal, cell cultures) as they vary in their histological composition, and in their physical properties (density, thickness) thereof [4,7]. Hence, there is a need for developing any readily applicable methods for correction against the mass thickness effect arising from inherent heterogeneity in the structure/topography of biological specimens, and there is also a need to develop any methods for correcting those factors underpinning any variations in instrumental instabilities as well. To get rid of these critical issues, different solutions, fitted in with a number of analytical problems, have been proposed so far. One of the first solutions to tackle the problem was addressed by *Lubecki* many decades ago, and the method to utilize the information on the mass thickness of the samples taken from the intensity of the incoherently scattered X-ray (the Compton peak) radiation was theoretically devised and proposed for practical purposes [8]. Up to now, an increasing number of algorithms to utilize Compton-based correction schemes against the mass thickness effects have been formulated. This is because the Compton intensity, apart from its relation to the mass thickness, is directly proportional to the intensity of the primary exciting beam, and therefore could be used as a versatile way to facilitate the exact error-free determination of elemental concentrations in biological specimens [9]. *Sitko* proposed the use of the coherent to incoherent X-ray intensities to correct for the matrix effects in the samples collected on membrane filters for the fully quantitative assessment of their elemental concentrations using the Energy Dispersive X-ray Fluorescence (EDXRF) scanning microprobe [10]. Recently, *Greaves* et al. carried out a number of investigations into the organic matrix effects using a Total Reflection X-ray Fluorescence spectroscopy (TXRF) of a range of reference biological materials in order to arrive at the quantification of chemical elements where fluctuations in the mass thickness of the organic matrix were a serious concern [11]. The authors developed a semi-mathematical standardization method utilizing the intensity of the incoherently scattered X-ray photons as a kind of internal standard, and this method demonstrated excellent agreement with the reference concentrations [11]. Likewise, using a similar approach, *Marco* et al. have also demonstrated a Compton-based total reflection XRF analysis of homogenized human brain and human serum samples which has been able to get rid of departures from the mass thickness both in the standard and biological samples themselves [12,13]. In turn, the recent work by [14] using energy dispersive XRF and Wavelength Dispersive XRF techniques, presented the theoretical derivations and experimental evidence for the robustness of the Compton correction based on a validation involving the gravimetric measurements of inorganic powders. Turning now into in-situ bio-imaging of elements using scanning Synchrotron Radiation based XRF, *Liu* et al. as well as *Wang* et al. by analyzing the animal models of Alzheimer's disease and hypothyreosis, respectively, have proposed normalization of the results utilizing the intensity of the incoherently scattered radiation [15–18]. Such a solution was to take into account any differences in the mass thickness in various anatomical structures in the mouse brain, and demonstrated that the concentrations determined matched those of previous studies using other analytical approaches [15–18]. Interestingly, a very recent study by *Campos* et al. made use of the Compton signal for a fully quantitative elemental analysis of *Pityrogramma calomelanos,* and allowed for the unbiased estimation of mass fractions of As and P in various parts thereof [19]. In our previous study using the quantitative synchrotron XRF imaging of the substantia nigra pars compacta tissue, the correction of the results using the net Compton intensities was also utilized for showing age-associated variability in the elemental composition of the neurons and surrounding neuropil area [20]. Besides, the X-ray phase contrast imaging has lately emerged as a very popular tool to determine the areal masses of different sample areas. Despite its popularity, however, the method does require additional algorithms to superimpose the X-ray phase contrast images with those acquired by the quantitative synchrotron XRF analysis [6,21].

With the help of the state-of-the art methodology in the quantitative XRF analysis of biological specimens highlighted as above, our contribution has aimed at the development of a simple mass thickness effect correction based solely on synchrotron XRF measurements. The subjects of the studies were substantia nigra tissue sections put on silicon-nitride (Si_3N_4) membranes (Silson Ltd., UK): the substrate that is of a well-defined density, thickness, and perfect homogeneity. Also, since the membrane *Si* is absent in the human brain, we have utilized the $Si-K_\alpha$ line for computation of absorption properties of biological scaffolds. The membranes, as reported, make possible to utilize a wide range of electromagnetic radiation (from mid-IR to hard X-rays) to allow for simultaneous micro-imaging of molecular components (Fourier transform infrared spectroscopy, Raman spectroscopy), chemical elements (synchrotron XRF), and local variation in their oxidation state (X-ray absorption near edge structure) in a biological specimen, which somehow overcomes stringent requirements of different substrates for various spectroscopic methods [22]. Importantly, as almost all already-mentioned techniques were successfully implemented in our research, the utilization of Si_3N_4 seems to be an attractive choice in the case where the same sample area must be scanned by different imaging modalities to allow for quantitative information on biologically active elements to be combined with molecular composition [20,23–25].

In our present study, the substantia nigra tissue was in focus. Substantia nigra is a part of the human dopaminergic system which is responsible for the synthesis and transmission of a neurotransmitter dopamine. Evidence shows the tissue is morphologically heterogeneous, and one can distinguish its two major parts: the neuromelanine pigmented neurons (visible in the optical microscope as dark points without any sort of staining) and surrounding neuropil areas, both suspected to have different mass density which could influence the final quantification using the XRF technique due to their different densities [20,24]. We propose for the first time the normalization involving three different approaches utilizing: the membrane $Si-K_\alpha$ transmission signal or the net Compton signal or the normalized net Compton signal, respectively, to correct for the mass thickness effect in the substantia nigra tissue structures. In what follows, we explain how we presented the physical foundations behind the correction algorithms, and finally determined the corrected elemental mass fractions in the respective histological structures of substantia nigra.

2. Methodology

2.1. Sample preparation

The substantia nigra sample specimens were taken from a human individual during the autopsy, and were then put to the freezer at −80 °C and kept frozen in darkness until the time come for further preparation procedures. Just before the synchrotron XRF experiments, the samples were cryosectioned onto 10 μm, 15 μm, 20 μm and 25 μm thick sections at −20 °C, mounted onto 200 nm thick Si_3N_4 membranes and finally freeze dried in a freezer at the working temperature of around −80 °C. For a topographic analysis of dried specimens, two cryo-sections of 10 μm and 20 μm were mounted onto microscope