Low Voltage Energy Dispersive Quantitative X-Ray Microanalysis of Inorganic Light Elements in Bulk Frozen Hydrated Biological Specimens

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Although there are many advantages to working at low voltages and low temperatures [1] [2], the topic discussed here remains one of the most challenging areas of quantitative x-ray microanalysis. The specimens, which have naturally low concentrations of elements, are beset with problems associated with sample preparation, peak overlaps, uncertain analytical algorithms and radiation damage. It is not feasible to use ultra-thin (50-100nm) frozen hydrated sections as they are quickly destroyed by the electron beam and there are problems maintaining the fully hydrated state in thicker (500-1000nm) frozen hydrated sections. This paper will discuss some of the critical issues that arise during low voltage quantitative x-ray microanalysis of frozen hydrated bulk biological material and suggest ways we may hope to improve the analytical procedures.

An accelerating voltage of 5keV is suitable for most studies but there are advantages to working down to 3keV which is now accepted as the lowest practical voltage for x-ray microanalysis [3]. However at 3keV there is not enough energy to produce sufficient x-rays from elements much above $Z=15$ [4]. With frozen hydrated samples, there is a 17 fold decrease in the size of the interactive volume between 10 and 5 keV and a further 15 fold decrease between 5 and 3keV. The sample volume from which x-rays may be detected is smaller than the volume penetrated by the primary electrons It is important to carefully monitor the landing energy of the primary electron beam and avoid any charging. The non-invasive charge balance procedures [5] are only practical for SEM imaging of organic material up to $E2=2keV$ and do not appear to be applicable to x-ray studies carried out at 3keV and above. It is unwise to rely just on a visual examination of the sample as a check that there is no charging. It is far better to check the Duane-Hunt limit, first on the calibration standard and then on the specimen throughout spectral acquisition [6]. The most practical way to avoid charging is to coat the sample with a thin continuous layer of a conducting metal bearing in mind the caveats of peak overlaps, electron and x-ray photon energy attenuation and specimen damage. Because the volume from which the detected x-rays is so small, the coating layer has to be very thin (2-3nm), must not form an oxide layer and must not be contaminated. The metals Be, Al and Cr together with C, despite various advantages and disadvantages, may be used as coating materials [7]. Radiation damage is a real problem. There is nothing that can be done to ameliorate the primary events and secondary physical and chemical reaction associated with radiation damage as these occur anywhere between 10^{-9} to 10^{-18} sec. The tertiary events that centre on the production and movement of free radicals, occur between 10^{-2} to 10^{-4} sec at room temperature, and their diffusion rate is temperature dependent. A study made of radiation damage in the environmental SEM, confirmed that liquid and gaseous water acts as a rich source of small, highly mobile free radicals, of which the hydroxyl (.OH) radical is the most dominant and reactive species [8]. There is an increase in the quantities of reactive species with beam energy. The increased viscosity of ice slows the movement of free radicals, and their rates of reaction decrease with temperature resulting in a 3-8 fold decrease in radiation damage. [1]. The consequences of beam damage in ice, even at 100K, are mass loss, loss of crystallinity, structural damage and the production of volatile materials that could influence the landing energy of the primary electron beam. The two analytical algorithms, ZAF and

P/B ratio method, form the basis of quantitation. At the low voltages we are discussing here, the count rate decreases by x100, the minimum detectable limit is only between 1-10%, and there are severe peak overlap problems with the Ca and K L lines and the C and N K lines [3] [6].

The specimen preparation and analytical procedures currently being used are as follows. Small samples (1-3mm^3) are quench cooled at between 2-10^3 K s^{-1}, cryofractured at 110K at 5 x 10^{-6} torr (667μPa), plasma magnetron sputter coated with 2nm Cr at 120K using high purity argon at 5 x 10^{-3} torr (667mPa) and transferred to the cold stage of the microscope held at 120K and 1 x 10^{-6} torr (133μPa). The sample is kept under high vacuum throughout the whole process and the collected x-rays analysed by the P/B ratio algorithm using a ATW germanium ED detector. At the present time, using a 200s live time and a beam current of between 250-400pA, quantitative analysis to within +/- 5%, has been successfully carried out on plant material, with a total inorganic elemental content of between 2-4% FW, at 5keV for Na, Mg, Al and Si; at 6keV for P and S; at 7keV for Cl; at 8keV for K and at 9keV for Ca, all at a spatial resolution of 50-100nm [9].

The elements C and N make up a substantial part of the 10-12% FW of the sample organic material Their K lines overlap the much smaller L lines of K and Ca and it not possible to use these two lines for analysis and thus reduce the accelerating voltage closer to 5keV for the analysis of all elements Z=11-20. The two L lines could be resolved using a WD detector or the newer bolometric detector, although it remains to be seen whether the two very small peaks could be used to obtain quantitative data. Water, which is 88% oxygen, makes up 80-90% FW of the samples and for quantitation, the O K line is used as a measure of the water (ice) content. The O K line is overlapped by the Cr L line and although considerable progress has been made in measuring the contribution of Cr to the O peak [10] further studies are needed on how to maintain an anoxic vacuum environment in the microscope. We need to calculate the oxidation rates of the other metals that have been used to coat frozen hydrated samples. Although preliminary studies suggest that Be, Mg and Al oxidize at a faster rate than Cr, we need to find out whether Mg might be a better coating material than Cr. We need to look for more accurate ways of continuously measuring the landing energy of the low voltage beam on the specimen. We need more information on the fundamental constants used for quantitative analysis in order to improve the matrix corrections that are used in quantitative analysis [11]. We need to explore the effects on quantitation of reducing the overvoltage from the usual 2 to closer to unity.

References

1. Echlin, P. (1992) Low Temperature Microscopy and Microanalysis, Plenum, New York.
2. Joy, D.C. & Joy, C.S. (1996) Micron 27 247.
3. Joy, D.C. (1999) Microscopy & Microanalysis 5 (Supp 2) 302.
4. Echlin, P. (2001) Microscopy and Microanalysis 7 211.
5. Joy, D.C. & Joy, C.S. (1995) Microscopy and Microanalysis 1 109.
6. Newbury, D.E. (2001) Microscopy and Microanalysis 7 (Supp 2) 702.
7. Goldstein, J. I. et al (1992) SEM and X-ray Microanalysis. Plenum, New York.
8. Royal, C.P. et al (2001) Journal of Microscopy 204 185.
9. Echlin,P. (2000) Microscopy and Microanalysis 6 (Supp 2) 758.
10. Echlin, P. (2000) Proc. Micros. Soc. Southern Africa 30 1.
11. Joy, D.C. (2001) Microscopy and Microanalysis 7 (Supp 2) 664.