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STANDARDLESS ANALYSIS OF BIOLOGICAL TISSUE SECTIONS

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Abstract

The X-ray microanalysis of thin biological samples which are usually supported on a thin organic film or are self-supporting specimens, has required the use of standards which contain the elements of interest. Spectra from the standards are used to calculate the factors for converting X-ray data recorded on the specimen into elemental concentrations. A method is discussed here, in which these factors are evaluated from formulae. The most important physical process to be evaluated is that of characteristic X-ray production in the specimen. The bremsstrahlung production must also be evaluated if the Hall or continuum normalisation (CN) method of quantitation is to be used.

This paper discusses briefly methods of calculating values for the X-ray production cross-sections for both characteristic and bremsstrahlung radiation. The way in which these are incorporated into standardless quantitation methods for biological samples is described. Calculations of some cross-section data are presented for typical analytical conditions.

Key Words: Standardless biological microanalysis, X-ray microanalysis, thin specimens.

Introduction

In any microanalysis, the intrinsic purpose of standard specimens (which may or may not be similar in composition to the specimen), is to "calibrate" the efficiency of generation of characteristic X-rays in the analytical microscope under the same conditions as will be used for analysing the specimens. For the continuum normalisation (CN) method, the standard also provides a calibration for the continuum generation.

In an alternative procedure, the X-ray generation efficiency is deduced from theoretical models so that the factors by which X-ray intensities can be converted into concentrations may be calculated. Such models predict the intensity of X-rays generated in a thin film by a single electron under given experimental conditions. However, it is clear that any theoretical models to be used for quantitation need experimental verification, which implies the use of samples of known composition, *viz* standards. However, using samples, (frequently single element foils), which are easy to prepare, the aim has been to deduce theoretical models of X-ray generation which may then be applied to any sample.

The calculated intensities may need to be modified to account for losses due to absorption of X-rays in the specimen (Goldstein *et al.*, 1977), and due to variations in the efficiency of the detector with X-ray energy (Nicholson and Chapman, 1983). This approach is particularly useful in cases where it is difficult or impossible to obtain suitable standards for the elements of interest.

In this paper the equations of characteristic and bremsstrahlung X-ray production in thin films are presented. The quantitative methods are reviewed on the assumption that the measured intensities require no corrections for absorption in the sample and that the EDS detector efficiency is well known. The method of quantitation using peripheral standards which is based on the K-factor technique (Cliff and Lorimer, 1975) is discussed with the method of adapting this for standardless analysis. The method of continuum normalisation for quantitative analysis of organic samples is briefly reviewed, followed by an outline of

how a standardless version of this method may be developed by calculating bremsstrahlung production cross-sections. The equation for an approximate simple method is presented since this is suitable when the elements of interest are in low concentrations in an organic matrix, which is frequently the case for specimens with soft tissue matrices.

Equations of X-ray Generation in a Thin Sample

The number of background (bremsstrahlung) X-rays B(k)dk, of energy k, detected in the energy interval Δk is

$$B(k)d\Omega\,\Delta k = N^0 \rho\tau It\epsilon_k \sum_r \frac{C_r\sigma_{br}}{A_r}\frac{d\Omega}{4\pi}\Delta k \qquad (1)$$

where σ_{br} is the cross-section for bremsstrahlung production in atoms of type r, into the solid angle $d\Omega$, integrated over the photon energy interval Δk. C_r = the weight fraction of element r, N^0 = Avogadro's No., A_r = atomic weight of atoms type r, $\rho\tau$= mass thickness of the specimen, It = incident dose and ϵ_k = detector efficiency at X-ray energy k, and $d\Omega$ = detector solid angle.

The number of characteristic (peak) X-rays $P_x d\Omega$, of energy k detected from a thin sample is given by

$$P_x d\Omega = C_x \frac{N^0}{A_x}\rho\tau It\sigma_{cx}\frac{d\Omega}{4\pi} \qquad (2)$$

where σ_{cx} is the characteristic ionisation cross-section of the atom, ϵ_x = detector efficiency at X-ray energy of the characteristic line of x. Both σ_{br} and σ_{cx} are functions of the electron energy T_0, and the atomic number of the sample Z. σ_{br} is also a function of the angle between the X-rays detected and the direction of the unscattered electron beam transmitted through the sample.

Determination of X-ray Production Cross-sections

Bremsstrahlung cross-sections

Approximate formulae available for calculating the bremsstrahlung production cross-section σ_{br} have previously been considered, (Chapman *et al.*, 1983) where it was shown that the modified Bethe Heitler (MBH) theory (Koch and Motz, 1959) predicted experimental values well, for elements of $Z < 50$, $k < 20$ keV and $T_0 > 40$ keV. The MBH theory was also shown to be in close agreement with the limited number of exact calculations of Tseng *et al.* (1979). The predictions of the MBH formula were also compared with the exact calculations of Kissel *et al.* (1983), by Adam (1986) who found good agreement down to zero photon energy. Other work (Nicholson *et al.*, 1982), has shown that the MBH formula agrees with theory up to 40 keV photon energy, the useful limit of the energy dispersive silicon (EDS) detector .

The MBH formula is long and not very illuminating to examine, so it is not reproduced in this paper, however, it is given in full in Chapman *et al.* (1983, 1984). The formula is simple to evaluate on a personal computer and has been coded in several high level languages, (FORTRAN, C, Pascal).

Characteristic cross-sections

The principal sources of error in the experimental determination of characteristic ionisation cross-sections using eqn 2 alone are in the values of $\rho\tau$, the mass thickness of the specimen (standard) and, to a lesser extent, the incident number of electrons It, and the solid angle $d\Omega$, of the EDS detector. The problem may be avoided by taking ratios in such a way that the quantities which are difficult to measure accurately, cancel out. For example, this may be done by measuring the peak to background ratio P/B, where the background is measured at the same photon energy as the peak. The result for a single element specimen, will then be given by dividing eqn 2 by eqn 1

$$\frac{P_x}{B_x(k)\,\Delta k} = \frac{\sigma_{cx}}{\sigma_{bx}\,\Delta k} \qquad (3)$$

so that the parameters $\rho\tau$, It, $d\Omega$, and ϵ_x, cancel out. To use eqn 3 to determine σ_{cx}, the P/B is measured experimentally and the bremsstrahlung cross-section σ_{bx} is calculated at the energy of the characteristic line. The cross-section for characteristic *ionisation* σ_{ix} , in atoms of type x may then be evaluated since

$$\sigma_{cx} = \sigma_{ix}\omega_x s_x \qquad (4)$$

where s_x = the partition function which describes the relative intensities of lines from the same shell and ω_x = fluorescence yield. Values of s_x and ω_x are taken from tables so that σ_{ix} may be calculated. To enable cross-sections for other elements for which standards are not available to be calculated, data sets of σ_{ix} are then fitted to a simple functional form, the most frequently used being the Bethe (1930) model which has two fitting parameters b_k and c_k. The ionisation cross-section written in terms of the K shell is then given by

$$\sigma_{ix} = 4\pi e^4 b^K \ln\left[\frac{c_K T_o}{I_K}\right] / T_o I_K \qquad (5a)$$

where I_K is the ionisation energy for the K shell of element x. A parameter $U_K = T_0/I_K$, termed the over voltage, the ratio of the electron energy to the ionisation energy, is introduced to make eqn 5 linear i.e.

$$\sigma_{ix} I_K^2 U_K = 4\pi e^4 b_K \ln(C_K U_K) \qquad (5b)$$

Plotting $\sigma_{ix} I^2_K U_K$ against ln (U_K), is used to determine the best fit values of b_K and c_K. The most recent measurements of cross-section data are from Paterson *et al.* (1989) who extended the range of atomic numbers and electron energies examined by Gray *et al.* (1983), but found very similar values of $b_K = 0.62$ and $c_K = 0.90$. For the peripheral standard method of microanalysis only the ratio of the cross-sections is of importance, so in this case errors in b_K are not significant. Putting in the value for e, the electronic charge and for b_K and c_K above, combining eqn 4 and 5, we get

$$\sigma_{ix} = 6517 \frac{s_x \omega_x}{T_o I_K} \ln\left[\frac{C_K T_o}{I_K}\right] \qquad (5c)$$

where T_0 and I_K are in keV and σ_{ix} has units of barns/steradian (1 barn = 10^{-24} cm^2). Values of s_x may be found in Scofield (1978), Krause, (1979), Schreiber and Wimms, (1981), and the most accurate values of fluorescence yield for the K-shell, ω_K, are those tabulated by Langenberg and Van Eck (1979).

Standardless Quantitation for Organic Specimens

Before detailing how to perform the analysis without the use of standards, it is useful to review briefly how the analysis may be performed using standards. This will clarify where the standard factors are to be replaced by X-ray cross-sections in the analytical formulation.

Ratio Method - Peripheral Standards

The standard may be incorporated with the sample and thus sectioned at the same thickness. This may be done with frozen sections using the peripheral incubation medium (Rick *et al.*, 1979; Dörge *et al.*, 1989). The principal assumptions behind this technique are that the sections are cut uniformly in thickness and that if the sections are freeze-dried before analysis, the shrinkage which occurs on dehydration is uniform. As the composition of the incubation medium before drying is known, it may be used as a standard to quantify the line intensities from the specimen. In the case of the analysis of resin embedded biological samples Hall (1991), has suggested incorporating a "tag" element such as Br, not present in the sample into the resin in a known quantity. The intensity of the tag element line can then be used to determine the proportion of resin in the probed region. If an area of the resin is probed peripheral to the sample, i.e. an area containing no tissue, then this may also be used as an internal standard.

The ratio method requires the knowledge of K-factors (Cliff and Lorimer, 1975) for the tag element and the elements of interest which are determined using standards. For the peripheral standard method the concentration in the sample is given by:

$$\frac{C_x}{C_t} = \frac{P_x}{P_t} * K_{tx} \qquad (6)$$

where C_x is the concentration of the unknown x and C_t is the concentration of the tag element t per unit volume, P_x and P_t are the peak intensities of x and t respectively and K_{tx} is the K-factor. The equation for the peripheral standard method is as eqn 6 (one for each element) with the intensity of the peripheral standard line and the K-factors for the peripheral elements replacing those of the tag element.

To perform this method of analysis without the need of standards to determine the K-factor, we divide eqn 2 evaluated for two elements x and y respectively:

$$\frac{P_x}{P_y} = \frac{C_x}{C_y}\frac{A_y}{A_x} * \frac{\epsilon_x}{\epsilon_y}\frac{\sigma_{cx}}{\sigma_{cy}} \qquad (7a)$$

where σ_{cx}, σ_{cy} are the cross-section for characteristic production in atoms of type x and y, from which it can be seen that the K-factor

$$K_{yx} = \frac{A_x}{A_y}\frac{\epsilon_y}{\epsilon_x}\frac{\sigma_{cy}}{\sigma_{cx}} \qquad (7b)$$

and may be calculated once the production cross-sections are known.

Continuum Normalisation

This method is based on the principle that a region

of the bremsstrahlung spectrum (termed the "white" radiation by Hall, 1971) may be used as a measure of the specimen mass thickness or the total number of atoms in the analysed volume. The basic formula can be expressed:

$$\frac{(C_x)_{spec}}{(C_x)_{stan}} = \frac{(P_x/W)_{spec}}{(P_x/W)_{stan}} * \frac{\left[\sum_r C_r Z_r^2/A_r\right]_{spec}}{\left[\sum_r C_r Z_r^2/A_r\right]_{stan}} \qquad (8a)$$

where Z_x is the atomic number of the element of interest x, $(C_x)_{spec}$, $(C_x)_{stan}$ are the concentrations (or mass fractions) in the specimen and standard respectively and P_x/W is the ratio of characteristic counts from the element to the continuum, again measured on both the specimen and standard where W is the intensity in the bremsstrahlung (white) window. The ratio of

$$(P_x/W)_{spec}/(P_x/W)_{stan} \qquad (8b)$$

removes the need to know the efficiency of characteristic generation in x. The terms in

$$\left(\sum_r C_r Z_r^2/A_r\right) \qquad (8c)$$

often referred to as the G-factor G_x, account for the different bremsstrahlung generation per atom in the different atoms of type r over which the sum is made, and is based on the assumption that bremsstrahlung generation is proportional to Z^2. In most circumstances encountered in biology, (Shuman *et al.*, 1976) this is a good approximation. The limitations imposed by the Z^2 assumption have been evaluated by Nicholson and Chapman (1983). Clearly the G-factors are the mean values of Z^2/A for the sample and standard. Hall (1971) developed this basic equation further to deal with the problem that as the sample is of unknown composition, its mean atomic number can not be calculated accurately. However, if we restrict our attention to specimens which are predominantly organic matrix which contains mostly C, N and O, then $(G_x)_{spec} \cong (G_x)_{matrix}$ which may be calculated. Hall (1973) has shown that its value is not much affected by the matrix composition, so (apart from frozen hydrated specimens which are well approximated by water) the value may be assumed to be a constant evaluated for the composition of dry tissue.

In the standardless development, cross-sections are used to describe the X-ray generation. In this case the bremsstrahlung W, is integrated over the energy range of the white window and summed over all the elements in the sample weighted by their concentrations. Dividing eqn 2 by eqn 1, to give the ratio P/W:

$$(Characteristic/Bremss) = \frac{P_x}{W} = \frac{C_x}{A_x}\epsilon_x\sigma_{cx} / \sum_r \frac{C_r}{W}\epsilon_r\sigma_{cr} \qquad (9a)$$

It should be noted that for many cases, e.g., the bremsstrahlung energy being greater than about 3 keV, ϵ_r will be close to or equal 1. Similarly for the characteristic lines, it is unlikely that apart from those of Na and Mg it will be necessary to calculate ϵ_x. If we assume that the total amount of all the higher atomic number elements (i.e., those with atomic numbers in the range of Na to Ca) is less than 5% weight fraction or 2000 mmol/kg, then to a good approximation all the bremsstrahlung is generated in the matrix elements, so that the denominator of eqn. 9 may be expressed as

$$\sum_r \frac{C_m}{A_m}\epsilon_m\sigma_{bm} \qquad (9b)$$

i.e., the sum is taken over the matrix elements m, alone. This term is simply the average of the bremsstrahlung cross-section weighted over the concentration of the matrix elements. Thus knowing the concentrations of the matrix elements *in the matrix,* we may calculate the mean cross-section for bremsstrahlung production, σ_{bm}, which is analogous to the factor $(G_x)_{matrix}$ and again does not vary much with the matrix composition, so that a good approximation of eqn 9 is

$$C_x = \frac{P_x A_x}{\epsilon_x \sigma_{cx}} \frac{\epsilon_m \overline{\sigma_{bm}}}{W} \qquad (10)$$

The assumptions above will clearly be invalid for mineralised tissues, since their matrices are similar in composition to hydroxy-apatite which is about 40% Ca and about 19% P by weight. However samples in which the matrix is organic matter such as freeze dried tissues, frozen hydrated tissues or resin embedded tissues, other approximations are more likely to limit the analytical accuracy than the assumptions behind equation 10. Table 1 shows some values of σ_{cx} for a range of elements

Table 1. Characteristic Ionisation Cross-sections (barns/sr)

	Na	Mg	Al	P	S	Cl	K	Ca
80	7.34	7.41	7.70	8.07	8.15	8.36	8.45	8.41
100	6.18	6.26	6.52	6.86	6.95	7.15	7.27	7.25
200	3.57	62.1	3.83	4.07	4.15	4.29	4.42	4.43
	0.43	62.1	75.6	89.9	93.4	95.6	97.9	98.5

Detector Efficiency % for 8μm Be window

calculated for an analytical electron microscope operated at a range of electron energies. Values for σ_{bm} at the same electron energies are given in Table 2 for tissue matrices based on the composition used by Hall (1973) and for ice, the former being suitable for use with freeze dried or embedded tissue and the later with frozen hydrated tissue. The cross-sections are integrated over the 9.5 to 14.5 keV range for the EDS situated at 90^0 and 110^0 to the emergent electron beam, i.e. 90^0 and 70^0 to the incident beam which is typical for modern analytical instruments. It is clear that once the cross-section ratios σ_{cx}/σ_{bm}, are calculated (which requires only a few minutes), eqn 9 provides a simple means of quantitation. For the energy range chosen for the white radiation, the efficiency of the EDS will be about 100%, but some correction will be needed for the detector efficiency at low photon energies, particularly at the characteristic line energies of Na and Mg (see Table 1).

Conclusions

Standardless analysis is a viable alternative to using standards for the quantitative analysis of thin specimens. The most accurate analyses are likely to be for the ratio method since the cross-section ratios will be most accurate for elements which are close in atomic number. Here the over all error is likely to be about 5% to 10% relative. Using the CN method, the errors are likely to be higher, up to about 25%, partly because of the uncertainties in the matrix composition and partly due to the difficulty in quantifying mass loss due to radiation damage. However, this is the absolute error in an individual analysis. The relative error between analyses on the same sample will be much smaller and will usually be dominated by statistical errors due to the typically small peak to background ratios encountered. Corrections to the measured counts for detector efficiency may add 3 to 5% to the error.

It is clear that when standards are available for quantitative analysis, it is preferable to measure these as a check of the results obtained by standardless quantitation methods.

Table 2. Matrix Bremsstrahlung Cross-sections (barns/sr), "White" window 9.5 to 14.5 keV

	Angle=90 deg		Angle=110 deg	
kV	Tissue	Ice	Tissue	Ice
80	21.54	20.08	14.76	15.83
100	17.87	16.64	12.16	13.05
200	10.00	9.30	6.47	6.94

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Discussion with Reviewers

T. von Zglinicki: It is of paramount importance to define the underlying assumptions and borderline conditions of the equations used clearly. Also I would like to know the fundamentals of the calculations resulting in the cross-sections given in Tables 1 and 2.

Author: The underlying assumption in fitting the characteristic ionisation cross-sections to a function by optimising the parameters b_k and c_k, is that σ_{ix} really is a smooth function of atomic number Z. Bearing in mind the irregular way in which the atomic shells fill as Z increases this seems a bit unlikely. Further, although experimental data (Paterson *et al.*, 1989) indicate a smooth change of σ_{ix} with electron energy for a single element, there are clear discontinuities between Zs. However, the aim is to be able to interpolate between Zs for elements for which there are no standards, and fitting the data to a polynomial in Z shows no clear trend, nor seems to offer any improvement in accuracy. So we have to live with an overall error of about 10% in calculating absolute characteristic cross-sections.

The assumption made in the Modified Bethe Heitler formula is that the average energy loss of an electron in passing through the specimen is a small fraction of its incident energy. This implies that it is extremely unlikely that any one electron will excite more than one photon. It also implies that the theory is less likely to be accurate as the photon energy generated tends towards the incident electron energy. In practice these conditions are likely to be fulfilled in a modern microscope (of 100

keV or greater electron energy), provided the specimen is thin enough to give a reasonable image. The paper cited restricts $Z<50$. From the point of view of the biologist, this limitation on Z is not likely to be a problem as even for stained specimens, the concentration of such high atomic number elements will be insufficiently high to produce a significant amount of bremsstrahlung.

T. von Zglinicki: Using a "matching" standard for the Hall method, one hopes to cancel the mass loss of the specimen under analysis. Your standardless peak-to-background method (eqn 10) assumes there is no mass loss at all. With the resulting systematic error, is such a method is still really useful for beam sensitive specimens?

Author: I agree that if you completely ignore mass loss due to radiation damage, then the results could be in error of about 25%. However, I am not sure that the technique of using "matching" standards in the hope that the mass loss will be the same as in the specimen is necessarily the best way to proceed although it is certainly better than nothing. There is an argument which favours using mineral standards since these give higher count rates and therefore much better statistical accuracy than organic "specimen like" standards and (mostly) do not suffer from radiation damage.

Perhaps it would be better to measure the damage in some other way (see Hall, 1991 for some suggestions). Alternatively, the use of a cold stage will prevent mass loss, at least until the specimen is warmed up. The change in mass on warming up might be a way getting a value of the typical mass loss for the tissue type.

I did not want to deal with the problems of radiation damage in this paper, regarding the standardless formulation as akin to microanalysis using "perfect" mineral standards.

G.M. Roomans: You state that eqn (9) may be simplified if the sum of the elements in the range of Na to Ca is less than 5%. How important is this limit of 5%, given the fact that in many tissues the sum of these elements is actually between 6 and 6.5%?

T.A. Hall: In your final formula (eqn 10), you use the approximation that all of the specimen bremsstrahlung is generated in the atoms of the organic matrix. Have you estimated the magnitude of the error introduced by ignoring the contributions of the heavier elements? Since the result of an analysis is a set of values for the concentrations of these elements, presumably it would not be difficult to introduce this set into a revised estimation and do an iteration to take account of the effect of the heavier elements.

Author: To determine if there is the critical limit, firstly I calculated mean bremsstrahlung cross-sections for tissue based on the composition of tissue given in Hall 1973, but with no P and S for 80, 100 and 200 keV. I then postulated a composition which was the above to which had been added 50 mmol/kg Na, 300 mmol/kg P 300 mmol/kg S, 200 mmol/kg Cl 500 mmol/kg K and 1 mmol/kg Ca (a total of 1351 mmol/kg). These are somewhat too high to be physiologically realistic, but are equivalent to a total added concentration of 4.7% w/w, if we assume an ionisation coefficient of unity. I also calculated the mean bremsstrahlung cross-sections for these elements added at greater and smaller amounts but in the same relative amounts of Na to Ca.

The cross-section increases linearly with total mmol/kg of high Z elements up to the total of 2000 mmol/kg, (as far as I took the calculation), from which I deduce there is no "cut-off" or critical limit. Of course the way in which the bremsstrahlung increases depends on the relative compositions chosen, but it is interesting to note that for the values I chose about half of the "extra" bremsstrahlung generated in higher atomic number elements is from K.

At the 4.7% w/w of elements Na to Ca, the bremsstrahlung cross-section is about 9% higher than for tissue composition given in Table 2, which would result in an under estimate of the compositions of 9% relative. As suggested by the reviewers, it would be straight forward to set up an iterative calculation to correct for this. However, a quick first approximation could be performed by summing all the initial elemental concentrations and then scaling these up by about 7% for every 1000 mmol/kg in the total. Whether this is worth doing depends on how great this error is compared to others (such as mass loss) in the experiment.

T.A. Hall: You suggest the determination of the characteristic cross-sections by comparing peak count with bremsstrahlung under the peak (your eqn 3). Might it not be better to compare with a broad band of bremsstrahlung from a different region of the spectrum (this could be your 9.5 - 14.5 keV band, but it would not have to be)? It is true that you would have to deal with the small variation in detector efficiency, but you would have the advantage of a much stronger bremsstrahlung signal free of interference from the peak.

Author: An important procedure in comparing experimental and theoretical peak to background ratios is to ensure that the bremsstrahlung originates only from the thin specimen and that instrumental (solid material) bremsstrahlung has been correctly subtracted form the experimental data. To do this we scale and fit a

theoretical bremsstrahlung background for the element concerned over a wide band of photon energies, correcting for variations in detector efficiency if appropriate. In fact the scaling is often done using the 9.5 - 14.5 keV band. This scaled background then serves two purposes; it can be subtracted to remove the background to give the net peak counts and it provides an accurate measure of the bremsstrahlung intensity under the peak. In practice we quote peak to background ratios, where the background is the intensity in a 20 eV band under the peak, but this is quite arbitrary and any range of background could be chosen from the background fitted to the experimental data.