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A REVIEW ON THE EXTENSION OF HALL'S METHOD OF QUANTIFICATION TO BULK SPECIMEN X-RAY MICROANALYSIS

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Abstract

The theoretical background and the experimental data described in this paper justify the application of the Hall's continuum method of quantitation and the use of bulk crystals of known composition as standards, without ZAF correction, for the biological bulk specimen X-ray microanalysis, provided that proper criteria are respected during the realization of such measurements. The most important points are as follows: (i) Only crystals can be selected where the electrostatic charging is negligible or absent. This depends in part on the own characteristics of the crystals, and can also be facilitated by using low accelerating voltage, e.g. 10 kV, well-conducting specimen holders, and fast scanning rates; (ii) Apart from the element of interest (Na, K, Cl, etc.) all other accompanying components must be of low atomic number (11 or lower), in order to assure the similarity to the composition of the biological matrix where C, O, N and H are the most abundant elements. Comparison of the results in brain and liver cell nuclei and cytoplasm revealed that the elemental concentrations of Na and K are identical within the statistical scatter, if the continuum radiation used for the calculation of the peak-to-background ratios is selected under the respective elemental peak, or farther, in a peak-free region of the spectrum.

KEY WORDS: Bulk specimen X-ray microanalysis in biology, freeze-fracture freeze-drying method, selection of standards, Hall's continuum method, crystal standards

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Introduction

The question whether the intracellular monovalent ion contents of the postmitotic cells increase with age was raised in experimental gerontology already during the early seventies. The technique of X-ray microanalysis combined with electron microscopy, developed during the late sixties, seemed to be the most suitable method of choice for such investigations. Therefore, in 1974-75 a special preparative technique (freeze-fracture, freeze-drying, abbreviated as FFFD) and a quantitative bulk specimen X-ray microanalytic method have been developed (Zs.-Nagy and Pieri 1976, Zs.-Nagy et al. 1977). Essential point of this approach is that the Hall's method of quantitation based on the peak-to-background ratios (Hall et al. 1973) can be applied to bulk specimens under certain circumstances, and properly selected crystals of known composition can serve as standards. Later on, this bulk specimen X-ray microanalytic method was applied in a combined way to frozen-hydrated and FFFD specimens, allowing us to determine intracellular water and dry-mass contents (Zs.-Nagy et al. 1982). Advantages and limitations of the bulk specimen method as well as the biological significance of the results obtained have extensively been described and critically reviewed (Zs.-Nagy 1983, 1988, 1989, Lustyik and Zs.-Nagy 1985, 1988, Roomans 1981, Hall 1986, Hall and Gupta 1984, Gupta and Hall 1982, Elbers 1983, Roomans and Wroblewski 1985, Zierold 1986, Von Zglinicki et al. 1986). It has been recognized that "Despite the limitations, imposed by inferior spatial resolution of analysis, X-ray microanalysis of thick biological specimens is of remarkable practical interest" (Roomans 1981), and the bulk specimen technique has been applied for biological purposes also by others (Marshall 1980, Ingram and Ingram 1980, Boekestein et al. 1980, Fuchs and Fuchs 1980, Echlin et al. 1982, Pieri et al. 1981, 1983, 1984, 1986, Echlin and Taylor 1986). Nevertheless, there are still some contested points of the method.

For example, according to Roomans (1981) the "Use of crystal standards can, despite their easy preparation and chemical homogeneity, not be recommended." It should be noted, however,

that this negative view of Roomans (1981) was based on the ZAF-correction method, in which only the net peak intensities were used. Furthermore, the measurements were carried out at a relatively high accelerating voltage (20 kV), which results in a large absorption correction. Under more favorable conditions, such as used in our laboratory (10 kV) and with the use of peak-to-background ratios, much more encouraging results were obtained on several dozens of crystals. Therefore, it seems to be worthwhile to return to this problem and to review the available evidence together with some recently made new observations.

The Concept of Quantitative Analysis

The FFFD preparation technique was found to be suitable to preserve the intra- and extracellular biological gradients of the light elements such as Na, K and Cl in brain and liver cells (Zs.-Nagy et al. 1977, Pieri et al. 1977). Using such preparations, one can collect a large number of X-ray spectra regarding the elemental composition of cell nucleus and cytoplasm in a relatively short time. Once we obtain an X-ray spectrum, a proper method has to be selected for the quantification of the data, otherwise we remain only at the possibility of qualitative statements. The most reliable method according to our experience for the FFFD specimens proved to be the mass-fraction method of Hall et al. (1973). This method had originally been elaborated for thin specimens, nevertheless, its validity for thick specimens has also been demonstrated (Millner and Cobet 1972, 1973) and adopted later on also by others (Marshall 1980, Echlin et al. 1982, Echlin and Taylor 1986). This method is based on the following principle:

$$C_x = A_x \frac{(n_x/n_w)_{sp}}{(n_x/n_w)_{st}} (N_x/\Sigma NZ^2)_{st} \overline{(Z^2/A)}_{sp} \quad (1)$$

where C is the mass fraction of the element (x in the lower index always means the element of interest); A is the atomic weight of the elements; n_x and n_w represent the counts in the peak of element x and the background, respectively; sp and st indicate the specimen and the standard; N stands for the number of atoms of each element in the standard molecule, whereas Z is the respective atomic number.

Equation (1) requires the use of proper standards. However, according to the physical theory, bulk samples would require a correction for the matrix effects, called ZAF (Z = atomic number; A = absorption effects; F = fluorescence effects) correction. The problems of application of the conventional ZAF correction have been treated in detail in previous literature (Russ 1974, Boekestein et al. 1980, 1983a, 1983b, 1984). Although the computer capacities available today could perform this type of correction without any problem, the validity of ZAF correction methods is doubtful for the light elements distributed in an organic matrix because of the unsatisfactory exactness of the available physical constants, etc. (Russ 1974). Therefore, a compromise was needed allowing us to perform the quantitative analysis even without ZAF correction, based on the assumption that the interelement influences in the standards and the biological bulk specimen will not differ considerably. A relatively simple but powerful method has been found for this purpose (Zs.-Nagy and Pieri 1976, Zs.-Nagy et al. 1977), and is summarized below.

Equation (1) implies that the value:

$$Y = (n_x/n_w)_{sp} \overline{(Z^2/A)}_{sp} \quad (2)$$

is directly proportional to the mass fraction of element x (C_x), since all other expressions of the equation (1) are constant for a given case. Comparison of the values of Y belonging to various bulk crystals of known composition allows us to check whether they can be used as standards without ZAF correction or not. The most important criteria in this respect are: (i) the existence of a close linear correlation between Y and C_x, and (ii) the regression line fitted to Y and C_x should pass through zero as close as possible (Zs.-Nagy and Pieri 1976; Zs.-Nagy et al. 1977). In other words, various standard crystals are measured and compared to each other. In this comparison they are considered either as specimens or standards alternatively, and in both relationships the obtainable concentrations for the element of interest must be in the proper range. If the value of Y falls far out from the "good" regression line, the crystal cannot be used as a standard, since correct elemental concentration in it can only be obtained by using a ZAF correction. Using an empirical selection of standard bulk crystals containing potassium, sodium and chloride, it has been established that a good positive linear correlation (r is in the range of 0.9920 - 0.9997) exists between Y and C_x, if the crystals are chosen so that apart from the main element of interest, the atomic number of the other accompanying elements is not larger than Z = 11. In such cases, the accompanying elements were generally C, O, N, and H, i.e., the composition was, in principle, very similar to the biological matrix. For a number of crystals listed in previous papers (Zs.-Nagy and Pieri 1976, Zs.-Nagy et al. 1977) there were no technical difficulties while performing the analysis, however, some of them proved to be unsuitable for various reasons discussed later. Using this possibility, one can calculate a factor (F) from the equation (1) as follows:

$$F = A_x \frac{(N_x/\Sigma NZ^2)_{st}}{(n_x/n_w)_{st}} \overline{(Z^2/A)}_{sp} \quad (3)$$

The value of F was calculated for the standard crystals containing a given element of interest and was averaged. The FFFD specimens display a value of the mean $(Z^2/A)_{sp} = 3.28$ (Hall et al. 1973); this figure was used in each case for the calculations (Zs.-Nagy et al. 1977). The only information we need from the X-ray spec-

trum for this type of analysis is the net peak integral (n_x) as well as the value of n_w, i.e., the "white" counts, or the background integral. In principle, one can take n_w from any part of the spectrum, provided that no significant peak is present there and the same energy range is used also for the standards. Optimally n_w should be determined below the peak of element x, but due to the fact that, especially in the early seventies, the available computer capacities and software did not allow the calculation of n_w below the peak of interest, it was necessary to use the background integral in the range of 4 - 6 keV energy where no peaks occur in the biological specimens. The value of F can be expressed so that $F(n_x/n_w)_{sp}$ gives directly the mass fraction or percental concentration of element x in the specimen. It is important to stress here that values of F are valid for a given instrumental configuration. If parameters such as the specimen-detector distance, the take-off angle of the X-rays, the accelerating voltage, etc. are changed; new measurements must be performed on the standards to obtain the values of F valid for the new situation.

Although this method of quantification proved to be reliable and gave reproducible results in various biological specimens like brain, liver, tumor biopsies, etc. (see for details: Zs.-Nagy 1983, 1988, 1989), there have been critical comments from various authors as to whether the surface geometry such as a very uneven surface, problems of electrostatic charging, etc., can introduce errors in this type of quantitation. Furthermore, the use of background integral in the energy range of 4 - 6 keV instead of the proper background under each elemental peak was also criticized (see the comments of Reviewers in Zs.-Nagy 1983), although on a pure theoretical basis, without experimental checking. Therefore, some new experiments were designed, using an EDAX 9100 system with proper computer capabilities, in order to answer the question whether the use of the n_w under the peak for both the specimens and the standards will give essentially different results of quantitation or not. At the same time further details could be expected regarding the potentially utilizable standard crystals.

Description of the New Experiments

The experiments were carried out on a Philips 515 scanning electron microscope equipped with an EDAX 9100 system. All the analyses were carried out at 10 kV accelerating voltage, at a spot size varying between 50 - 200 nm, using a fast (TV) scanning speed, keeping the count rate at about 400-500 cps for 50 sec. live analysis time. According to various measurements, the real beam current in the specimen is in the order of magnitude of 1-5 pA. Tilt angle was 45^o, and calculating from the data regarding the position of the detector, a take-off angle of 60.7^o was used. Analysis time was equal for both the tissues and the standards.

Biological samples were prepared according to the rules of the FFFD technique described earlier (Zs.-Nagy et al. 1977). Brain and liver tissue of male, spontaneously hypertensive, stroke prone (SHRsp) rats (Okamoto et al. 1974) were selected for these studies, because these tissues contain usually more Na than those of the normal rats (i.e., the relative statistical scatter of the Na-concentrations was lower). Biological samples were mounted on Al-made specimen holders with a silver paint (Dotite, product of Fujikura Kasei Co. Ltd., Japan) as a conducting glue. It is important to stress that the surface of neither the specimens nor the standards was covered by a conductive layer.

Standard crystals were usually mounted on graphite specimen holder with graphite glue, since it has been established empirically that this holder caused less electrostatic charging problems.

The practical realization of the measurement was the following: after having recorded the spectrum, escape peaks were removed, than the spectrum was once smoothed. This was followed by the background subtraction performed usually by the "List" command of the EDAX 9100 program package, utilizing the program-defined points selected automatically on the energy scale. User-defined points were never applied for the background subtraction, i.e., this operation was carried out according to the actual shape of the spectrum. The correctness of this background subtraction was checked in almost all cases also by calculating the net peak integrals of the K_α lines of the spectrum (by means of the "K!" command of the program package). This procedure gave the cps values for the peaks (P), for their own background (B), together with the peak-to-background ratios. This latter ratio will be designated as P/B_n throughout this paper, whereas the other ratio, calculated from the peak integral and the window integral from 4 to 6 keV will be symbolized as P/B_o. The letters "n" and "o" in the lower index here and in the case of all other parameters refer to our "new" and "old" method of calculation, respectively.

On the basis of Equation (2) one can make several predictions. (i) Y_n and Y_o should fall on two different regression lines, the slopes of which display a ratio being equal (within the experimental error) to the ratio ($R_{o/n}$) between the background count under the given peak and the window integral between 4 - 6 keV. (This prediction is valid since there is no absorption edge between the lightest element of interest (Na) and the region of 4-6 keV). (ii) The ratio $R_{o/n}$ of various standards (and of specimens) as plotted against the concentration of the element of interest should fit to a horizontal line (L_h) the slope of which should not differ significantly from zero. (iii) The quantitation of biological samples on the basis of the selected standards may be correct only if the elemental concentrations, obtained for the same element by using F_o and F_n, are identical within the statistical scatter of the results.

This paper demonstrates the results obtained for 2 elements, Na and K. Table 1 and 2 report the numeric values obtained with all relevant data regarding the crystals used as standards. It should be stressed that NaOH and KOH (never tried before) proved to be very good standards, since they display absolutely no charging at 10 kV. Some crystals have a tendency to lose their crystal water in the vacuum; in such cases the operator can notice the increase of the P/B values during the first 10-15 minutes, and a stabilization of them later on. Obviously, in such cases one has to calculate C_x and all other parameters accordingly for the given molecule (Tables 1 and 2). The loss of water changes the physical appearance of the crystals as well: they are of vitreous macroscopic appearance until the crystal water is present, and become white, non-transparent in the vacuum.

Figure 1 and 2 demonstrate how the criteria listed above under (i) and (ii) are met by the experimental data. One of the usual criticism against the validity of this method of selecting standards has always been that the elemental concentrations of our standard crystals are considerably higher than those we are going to measure in biological samples. Therefore, it might be possible that there exists some decline from linearity of the equation describing Y against the concentration of the element of interest. There is, however, a very simple method of excluding this possibility. When performing the regression analysis, the data pair $C_x = 0$ and $Y = 0$ were included in the analysis, extending in this way the covered concentration range to zero. One can see that the values of Y_o and Y_n really fall on separate regression lines passing very near to the origin, and in spite of the relatively low number of crystals used, the data display extremely good linear correlation with the concentration of the element of interest. This finding fully confirms the previous results obtained with different X-ray microanalytic systems (Zs.-Nagy and Pieri 1976, Zs.-Nagy et al. 1977, 1982).

Figures 1 and 2 report also the regression analysis of the ratios (R) between the slopes of the lines belonging to Y_n and Y_o. As theoretically expected, this line for both Na and K, has a slope which is not significantly different from zero and displays an insignificant correlation with the elemental concentration. The equations belonging to R shown in Figures 1 and 2 were calculated together with the respective R values obtained in the cell nuclei and cytoplasm of brain and liver cells. Addition of these latter data do not change the original situation observed for standards, which is a proof for the validity of our method also in the low concentration ranges of the elements, and the applicability of the Hall's continuum method for biological bulk specimens.

The present experimental results are in agreement with our previous ones. Namely, it has been confirmed that crystals may only be used as standards without ZAF correction, if apart from the element of interest, all other elements are of the atomic number 11 or lower (Zs.-Nagy and Pieri 1976, Zs.-Nagy et al. 1977). Examples of this statement are the Na-K-tartarate (good as K-standard) and the NaCl (good as Cl-standard), which cannot be used as Na-standards. In such cases ZAF correction could bring the values near to the regression lines shown in Figure 1 (data not shown). Obviously, the presence of high concentrations of an element heavier than the element of interest can seriously alter the absorbance of the incoming electron beam and this leads to a shift of data far out from the regression lines shown in Figures 1 and 2. Since in the dry biological bulk sample the total concentration of the elements heavier than sodium remain usually below 5 % by weight, one can neglect this interelement effect in the biological X-ray microanalysis.

Tables 3 and 4 give a survey of the results obtained in the biological specimens (brain and liver). It is important to stress that the elemental concentrations calculated on the basis of F_o and F_n are not significantly different from each other in either case of measured cell compartments. It should be stressed that each parameter was calculated individually for each spectrum, therefore, the values of the standard error (S.E.M.) shown in the tables are really significant. In case of liver, the Na-content of the measured cell compartment was sometimes too low and remained below the detectability under the given conditions. Therefore, the number of observations is lower in case of Na (Table 3) than in case of K (Table 4). Obviously, one cannot take these cases as Na-free cells, therefore, we omitted them from consideration. This omission results in a somewhat higher average Na concentration than the real one. It is possible also to apply other methods. One can either include these values in the mean as values just equal or somewhat below the detection limit: in this case one obtains an average value nearer to the reality, or it is possible to consider the omitted values as being equal to 0. In this latter method one obtains an average which is certainly lower than the real average. It is important to stress that in comparison of various experimental data, identical methods should always be applied for the calculation of Na-contents, otherwise one can have misleading differences which may even prove to be statistically significant.

The ratios between the P/B_n and P/B_o in the cell compartments for both elements are plotted on Figures 1 and 2 demonstrating that they are practically equal with the values of F_o/F_n. This fact clearly indicates that the method of choosing standards as described here may deliver useful quantitative information even in the bulk biological specimens, independently from the fact whether we apply the background intensity under the peak or between 4 and 6 keV energy range.

Tables 3 and 4 show also that the monovalents show different concentrations in the nucleus and cytoplasm of the same cells. This is due to the fact that the water content of the nuclei is usually higher than that of the cyto-

Table 1. The basic data of the crystals used as standards for Na. Means ± S.E.M. of n measurements.

Standard	C_{Na}	$N_{Na}/\Sigma NZ^2$	$\overline{Z^2/A}$	Y_o	F_o	Y_n	F_n	F_o/F_n
$Na_3C_6H_5O_7.2H_2O$								
Na-citrate	0.2347	3/1164	3.959	5.97	0.130	50.95	0.0152	8.54
n = 11				± 0.16	± 0.004	± 1.43	± 0.0004	± 0.15
$(CH_2\text{-}COONa)_2$								
Na-succinate+	0.2840	2/646	3.988	6.58	0.143	60.74	0.0154	9.39
n = 23				± 0.11	± 0.002	± 0.86	± 0.0002	± 0.19
HCOONa								
Na-formate	0.3382	1/286	4.206	7.95	0.141	74.47	0.0149	9.43
n = 20				± 0.15	± 0.003	± 0.85	± 0.0002	± 0.22
NaOH	0.5750	1/186	4.650	14.48	0.131	122.28	0.0155	8.47
n = 33				± 0.16	± 0.002	± 1.17	± 0.0002	± 0.11
Mean					0.136		0.0153	8.96
S.E.M.					± 0.003		± 0.0001	± 0.26

Note: +Na-succinate originally contained $6H_2O$, however, this cyrstal water evaporated very quickly (within several minutes) in the vacuum.

Table 2. The basic data of the crystals used as standards for K. Means ± S.E.M. of n measurements.

Standard	C_K	$N_K/\Sigma NZ^2$	$\overline{Z^2/A}$	Y_o	F_o	Y_n	F_n	F_o/F_n
$C_4O_6H_4KNa$								
K-Na-tartarate+	0.1857	1/1014	4.829	10.82	0.056	79.46	0.0077	7.34
n = 10				± 0.23	± 0.001	± 2.42	± 0.0002	± 0.12
$KC_8H_5O_4$								
K-phtalate	0.1912	1/910	4.311	10.49	0.058	78.31	0.0078	7.45
n = 10				± 0.25	± 0.001	± 2.75	± 0.0003	± 0.13
KOH	0.6952	1/426	7.594	38.57	0.059	297.58	0.0077	7.71
n = 25				± 0.23	± 0.000	± 3.28	± 0.0001	± 0.07
Mean					0.058		0.0077	7.50
S.E.M.					± 0.001		± 0.00003	± 0.11

Note: +K-Na-tartarate originally contained $4H_2O$, however, this cyrstal water evaporated rather quickly (within about 15 minutes) in the vacuum.

Table 3. Na concentrations in percent of the dry mass of brain and liver cells of male SHRsp rats of 2 months of age as revealed by FFFD bulk specimen X-ray microanalysis. Elemental concentrations were calculated by using the respective values of F_o and F_n shown in Table 1. Average values of $\underline{n}$ measurements in each cellular compartment $\pm$ S.E.M.

Cell compartment	P/B_{Na} (old)	P/B_{Na} (new)	Ratio n/o	C_{Na} % (old)	C_{Na} % (new)	Signif. (o-n)
BRAIN						
Cell nuclei	0.076	0.67	8.98	1.03	1.02	N.S.
$\underline{n}$ = 25	$\pm$ 0.008	$\pm$ 0.07	$\pm$ 0.25	$\pm$ 0.11	$\pm$ 0.11	
Cell cytoplasm	0.056	0.48	8.92	0.76	0.73	N.S.
$\underline{n}$ = 25	$\pm$ 0.006	$\pm$ 0.05	$\pm$ 0.43	$\pm$ 0.09	$\pm$ 0.08	
LIVER						
Cell nuclei	0.024	0.21	8.95	0.32	0.33	N.S.
$\underline{n}$ = 16	$\pm$ 0.003	$\pm$ 0.03	$\pm$ 0.16	$\pm$ 0.04	$\pm$ 0.04	
Cell cytoplasm	0.020	0.18	8.86	0.27	0.28	N.S.
$\underline{n}$ = 19	$\pm$ 0.002	$\pm$ 0.02	$\pm$ 0.18	$\pm$ 0.03	$\pm$ 0.03	

Note: Parameters were calculated individually for each spectrum. The significance in the last column refers to the differences between C_{Na}(old) and C_{Na}(new) N.S. = not significant.

Table 4. K concentrations in percent of the dry mass of brain and liver cells of male SHRsp rats of 2 months of age as revealed by FFFD bulk specimen X-ray microanalysis. Elemental concentrations were calculated by using the respective values of F_o and F_n shown in Table 2. Average values of $\underline{n}$ measurements in each cellular compartment $\pm$ S.E.M.

Cell compartment	P/B_K (old)	P/B_K (new)	Ratio n/o	C_K % (old)	C_K % (new)	Signif. (o/n)
BRAIN						
Cell nuclei	0.53	3.96	7.48	3.05	3.05	N.S.
$\underline{n}$ = 25	$\pm$ 0.02	$\pm$ 0.18	$\pm$ 0.10	$\pm$ 0.11	$\pm$ 0.14	
Cell cytoplasm	0.47	3.57	7.58	2.73	2.75	N.S.
$\underline{n}$ = 25	$\pm$ 0.01	$\pm$ 0.10	$\pm$ 0.10	$\pm$ 0.05	$\pm$ 0.07	
LIVER						
Cell nuclei	0.36	2.69	7.47	2.08	2.07	N.S.
$\underline{n}$ = 25	$\pm$ 0.01	$\pm$ 0.08	$\pm$ 0.13	$\pm$ 0.05	$\pm$ 0.06	
Cell cytoplasm	0.36	2.64	7.45	2.05	2.03	N.S.
$\underline{n}$ = 25	$\pm$ 0.01	$\pm$ 0.07	$\pm$ 0.15	$\pm$ 0.04	$\pm$ 0.05	

Note: Parameters were calculated individually for each spectrum. The significance in the last column refers to the differences between C_K(old) and C_K(new) N.S. = not significant.

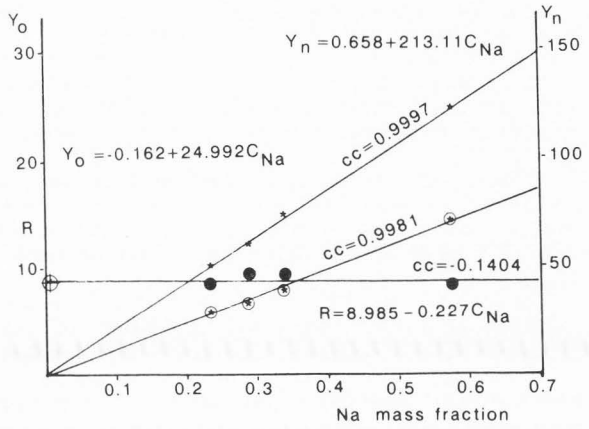

Figure 1. Summary of the linear regression analysis of the parameters Y_o (⊛), Y_n (*) and R (●) regarding Na-standard crystals, as described in the text. Note the difference in vertical scale for Y_o and Y_n. The symbols indicate real measurements. cc = correlation coefficients. The symbol ⊕ shows the zone where values of R of the biological specimens are to be found.

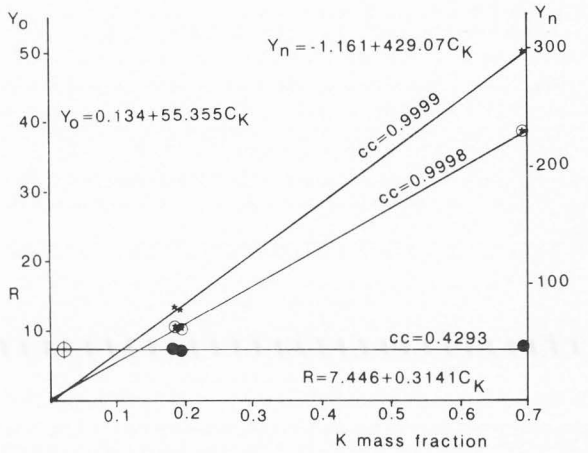

Figure 2. Summary of the linear regression analysis of the parameters Y_o (⊛), Y_n (*) and R (●) regarding K-standard crystals, as described in the text. Note the difference in vertical scale for Y_o and Y_n. The symbols indicate real measurements. cc = correlation coefficient. The symbol ⊕ indicates the zone where values of R of the biological specimens are to be found.

plasm (see for ref.: Horváth et al. 1983), therefore, after freeze-drying one obtains more monovalents for the dry mass of nuclei. This is in agreement with previous findings (Zs.-Nagy et al. 1982, Zs.-Nagy 1983, Lustyik and Zs.-Nagy 1985, 1988).

Conclusion

One can conclude that the application of the Hall's continuum method of quantitation to biological bulk specimen X-ray microanalysis, and the use of bulk crystals of known composition as standards, without ZAF correction are justified, provided that proper criteria are respected.

As one regards the relative error of this type of measurement, one can estimate it from the reproducibility of the values of Y on the standard crystals. The S.E.M. of these parameters is in the range of 0.5 - 3.1 relative % (Tables 1 and 2), and tends to decrease with increasing C_x, which is theoretically expected. Since one can assume a rather homogeneous elemental distribution in the crystals, we can consider the error of the measuring method as within + 3 %, which is acceptable in biological microanalysis.

As it has been explained (Zs.-Nagy 1983, 1988), the relatively simple preparation technique required for such studies represents an advantage when the average intracellular concentrations of monovalent electrolytes should be revealed, in spite of the low spatial resolution of this technique. The FFFD method is sensitive enough to reveal differences in the monovalent electrolyte concentrations in the dry mass of cell nucleus and cytoplasm. The relative standard errors of the K concentrations obtained in this way are impressively low (1-3 relative %, Table 4), and somewhat larger for Na (around 10-12 relative %, Table 3). This latter fact is due in part to the relatively low Na-content of the intracellular space and to the decreasing instrumental sensitivity in this range of the spectrum. However, the biological difference in the intracellular Na-content due to the actual functional state of various cells is certainly contributing to the higher scatter of Na concentrations observed.

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Discussion with Reviewers

K. Zierold: What is the specific advantage of your quantification procedure in comparison to the usually used ZAF method?
Authors: To best of our knowledge, the ZAF method cannot be applied to biological bulk specimens for various reasons mentioned also in this paper. On the other hand, ZAF correction for the bulk crystal standards, although feasible, always gives some doubtful results due to the insufficient precision of the physical constants regarding the low atomic number elements. Therefore, the simplest way was to avoid this correction method, since it was especially a laborious, complicated procedure during the early seventies when only limited computer capacities were available.

K. Zierold: You stress that crystals used as standards should be composed mainly of low atomic number elements (Z smaller than 11). This agrees with the usual condition that standard specimens should be similar to the specimens to be measured. What is the advantage of crystals in comparison to standards such as freeze-dried solutions consisting of organic material (albumin, gelatin, dextran) and elements of known concentration?
Authors: The main advantages of our choice of standards are: the simplicity, the repeatability at any time, the rapidity and the fact that the use of our regression line for the calculation of the true value of F decreases, considerably, the error of the measurements. On the other hand, the standards mentioned by you require an independent method of measuring the concentration of the element of interest, what we do not need.

K. Zierold: I am surprised by your report that you do not observe electrical charging during analysis of uncoated bulk specimens which are rather electric insulators than conductors. Have you checked the absence of charging by observation of the high energy end of the X-ray background? Does it coincide with the accelerating voltage? I can imagine that electrical charging can be prevented by use of a very low current, but then you have to count for a very long time or you get insufficient counting statistics. Please comment!
Reviewer 4: How did you establish that there was no charging? Why did you use different stub materials and glues or pastes for specimen and standard?
Authors: The absence of charging is perfectly controllable: if the background counts of the X-ray spectrum reach the nominal value of the accelerating voltage used, no charging is present. Otherwise the difference between the accelerating voltage and the maximum backround intensity gives the level of charging (it can be even of several kV size). We have checked always very carefully this point. As we described in our various papers including the present one, the effective beam current is several pA in the bulk specimen, and rather high count rate (cps) can be obtained (400 - 500), so that 40 - 50 second analysis time is fully sufficient for obtaining a statistically sound spectrum. This fact may seem to be strange for those authors who use thin sections for microanalysis, since in our case the analyzed bulk volume is much larger than in the sections. The use of various stub materials and glues was justified empirically, when searching the optimum conditions for the SEM imaging and microanalysis.

T. Von Zglinicki: Why was an iterative procedure not used for the evaluation of the mean atomic mass of the specimen?
Authors: Iterative procedure for the evaluation of the mean atomic mass of the specimen would not improve the results, since the elemental concentration differences obtained by F_o or F_n would have been the same order of magnitude, independently from using the uniform value of 3.28 for the biological sample as did Hall et al. (1973), or using somewhat different values. On the other hand, at least some of the main components (C, N, etc.) should have been measured, which was not the scope of the present work.

T. Von Zglinicki: Are not the differences between the F_o values for the Na standards (Table 1) significant? Why? How much could surface roughness contribute to these differences? Surface roughness should influence your old Na measurements in the specimen, too. Why are not there any differences (Table 3)?
Authors: It is true that some of the differences between the values of F_o shown in Table 1 and 2 are statistically significant. This is due to the fact that there exist some differences in the surface morphology and consequently in the absorption characteristics of various standard crystals for the background counts of 4-6 keV. It is important to stress, however, that none of the values of F_o differ significantly from the mean value of F_o. On the other hand, values of F_n show a smaller scatter, i.e., this method is somewhat more accurate than the older one. As regards the biological samples, the surface roughness probably causes much smaller differences in the concentration values than the really existing physiological ones, therefore, they remain invisible.

T. von Zglinicki: Can the authors show that the elemental concentrations they obtained are not influenced by a different response of biological samples and inorganic crystals to radiation?
Authors: Mass loss is certainly taking place under the electron beam in both biological and crystal specimens. However, the effect of this phenomenon is minimized by the fact that the composition of the analyzed volume remains practically identical even if a part of the surface is lost. In thin sections this phenomenon is much more harmful than in bulk ones. An indirect proof for this statement can be that 3- or 4-times repeated measurements on the same place of the bulk specimens do not give different concentrations for the same element.

Reviewer 4: Background subtraction is in the EDAX 9100 program performed for the program parts you mentioned by user-defined points on the energy scale. As this introduces a strong dependence of the resulting P/B data on the specific user, I think it is necessary to specify these points provided that you kept the set of background points constant all the way through.
Authors: User defined points for background subtraction were never used. The EDAX program can perform automatic background subtraction by using the frequency filtering method, if no user defined points are given.

Reviewer 4: How have you cryofixed your specimens and have you observed in your freeze-fractured frozen-dried specimens any damage due to ice-crystal formation?
Authors: The biological samples were cryofixed in isopentane kept at its melting point (-165°C) by liquid nitrogen as described in the cited previous publications. Of course, one can see some ice crystal damage on such preparations. However, this is not compromising the results, since the analyzed microvolume is relatively much larger than the average ice crystal size.

Reviewer 4: Referring to Table 3 and 4 it seems that P/B values were collected more reproducibly in the old (PB_0) situation. Now if you use the background integral from 4 - 6 keV, it is nearby the K K-alfa which means that a possible effect of the difference in X-ray absorption path length due to surface roughness between background and K K-alfa radiation will be relatively small. The effect should be more pronounced in the case of analyzing sodium with the same background integral, especially if you have used a normal beryllium window. However, in this case the spread in the results is almost similar (Table 3), while in Table 4 it appeared that the old situation is even slightly better. Can you comment on these data? How accurate was your (non-linear) background determination beneath the Na and K peaks?
Authors: The theoretical expectations of the Reviewer are certainly justified, but we believe that the relatively low number of determinations may not always reflect them in terms of the spread of the results. It should also be considered that the noise of the microanalytic system may become sometimes more important for a relatively narrow background range (under the peak) than in a wider one (4-6 keV). This may be all the more important, since the analysis time and the total counts are relatively low.