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GELATIN STANDARDS FOR ELEMENTAL QUANTIFICATION IN BIOLOGICAL X-RAY MICROANALYSIS

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Abstract

Standards for X-ray microanalysis (XRMA) can be prepared simply by dissolving measured amounts of gelatin and an appropriate salt in water, dipping grids in the solution, and allowing them to gel and dry. The present study was intended to assess the uniformity, reproducibility, and stability during irradiation of such standards.

Visually, the gelatin films appear homogeneous, and XRMA measurements of different grid squares are generally in good agreement. The gelatin standards are susceptible to radiation damage, as judged by several criteria. These are: visible damage; variation of peak/continuum ratios with electron dose; anomalous values obtained with very thin standards; and changes in X-ray counts with prolonged irradiation. In general, the gelatin matrix appears to be lost during irradiation, but the elements within the matrix may be lost at the same rate as the gelatin, or faster, or more slowly.

KEY WORDS: **Biological X-ray microanalysis; Elemental standards; Elemental quantification; Radiation damage**

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Introduction

Quantitative elemental analysis of biological specimens using X-ray microanalysis (XRMA) requires calibration with standards containing known concentrations of the elements of interest, in a suitable matrix. Ideal standards should have a chemically defined composition, be homogeneous at the level of resolution used, and should resemble the specimen in its chemical and physical properties (Roomans, 1979b). If the composition of the matrix of the standard differs substantially from that of the specimen, correction factors may be required for quantification (Hall, 1971), and it may also lose mass under irradiation to a different extent from the specimen. Two main types of standards have been developed over the years: those in which the elements are incorporated in an embedding resin (eg, Spurr, 1975; Weakley et al. 1980; Roomans & van Gaal, 1977; Roos & Barnard, 1984), and those in which a protein matrix is used (eg, Ingram & Hogben, 1968; Roomans & Seveus, 1977; Warley et al. 1983; El-Masry & Sigee, 1986). Resin standards are probably more difficult to prepare, and the variety of elements that can be incorporated in them more restricted, but are probably more robust and permanent once prepared. Protein standards are held to resemble the composition of biological tissue more closely, and can incorporate a wide variety of elements, but appear to be more ephemeral than resin standards. Evidently the continued development of new types of quantitative standards for X-ray microanalysis is an indication that the ideal universal type of standard has yet to be invented.

In this paper standards for biological XRMA are described in which the elements of interest are incorporated as a salt in a concentrated gelatin solution. Thin film standards on grids are prepared by dipping grids in this solution, and allowing them to gel and dry. A similar approach has been used by Lupton & Saubermann (1986) for preparing aminoplastic standards. Although these gelatin standards have been used by the author for several years (Sumner 1978b, 1984, 1986), a full description of their properties has not been given hitherto. The experiments to be described in this paper were intended to assess the uniformity, reproducibility, and stability of these standards.

Materials and Methods

Preparation of the standards

The method currently used for preparing the standards differs in detail from that described previously (Sumner, 1978b). The basis for the standards was gelatin powder (BDH Chemicals Ltd, product no 44045), which was weighed out accurately and dissolved in distilled water at 50°C at a concentration of approximately 20%. Accurately weighed quantities of salts containing the elements of interest were added to the gelatin solutions, giving mixtures containing known quantities of both salt and gelatin. These solutions were maintained at 50°C in a water bath for 3 days or more to ensure homogeneity. Sometimes the salts were dissolved in the distilled water first, followed by the gelatin; no obvious differences were found between standards prepared in the different ways.

To make the thin film standards, uncoated nickel grids, held in fine tipped, non-magnetic forceps, were dipped in the salt-plus-gelatin solution, taken out immediately and wiped on both sides with a small (5.5 cm diameter) Whatman No 1 filter paper. They were then left to gel and to dry out completely in a slot of a grid storage box. Before analysis the films were coated with a thin layer of carbon. The mesh size of the grids used was not critical, but "Athene" thin bar 200 mesh grids (Agar Scientific Ltd, catalogue number G2002N) were found to be more convenient than grids with a smaller mesh size, as larger areas could be analyzed, and there was less shading by the grid bars.

Most of the experiments described in this paper were done using standards containing various concentrations of potassium iodide, although similar results have been obtained with standards containing a variety of other salts (see Table 3). When results are described in this paper using standards containing salts other than potassium iodide, these are specifically indicated in the text.

X-ray microanalysis

Analysis of the standards was carried out in a Cambridge Stereoscan 180 scanning electron microscope to which was attached a Link Systems model 290 energy dispersive X-ray microanalysis system. The microscope was operated in the scanning transmission mode, using a specially modified carbon-coated stage (Sumner, 1978a) and a carbon specimen holder, to reduce extraneous X-rays.

Analysis was done using a 25kV accelerating voltage, and with the specimen tilted at 45° to the beam, and set at a constant height. The X-ray detector was on the same level as the specimen, giving a take-off angle of 45°, and was 25mm distant from the specimen. For any one experiment, the specimens were analyzed for a constant live time, usually 150secs. Magnification and probe current (measured using a Faraday cup) were varied as required to vary the radiation dose to the specimen; the standard conditions used were either 2.5nA probe current at 10,000 X magnification, giving a dose of 3.25×10^{-9} coulombs/μm^2, or 1.0nA probe current at 2,000 X magnification, giving a dose of 5.20×10^{-11} coulombs/μm^2, in both cases with the beam scanning over the full raster (12.05 x 9.58μm at 10,000 X, or 60 x 48μm at 2,000 X). The dose/unit area was thus 62.5 X greater in the former case than in the latter.

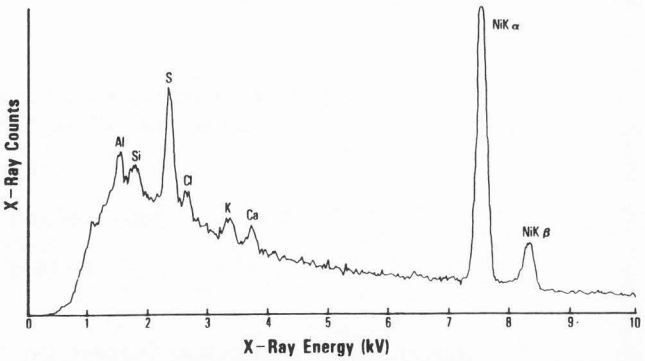

Figure 1 X-ray spectrum of a thin film of gelatin.

Results in this paper are generally expressed as "peak/background" ratios, that is, the ratio between the counts in the characteristic elemental peaks and the counts in a selected region of continuum ("background") in this case between 5 and 6kV. Calculations were made using the Link Systems QUANTEM-FLS programme, which corrects for continuum counts due to the specimen grid, so that the continuum counts should be only those due to the specimen. Since the continuum counts are not strictly proportional to the mass of the specimen, but actually to the mean value of Z^2/A (where Z = atomic number, and A = atomic weight), appropriate corrections have been made to the continuum counts for all standards (Sumner, 1978b). Using this correction, a linear relationship is obtained between peak/background ratios and elemental concentration.

Results

An X-ray spectrum of a gelatin film, containing no added salts, is shown in Fig. 1. The nickel peaks are due to the grid carrying the film, and the aluminium and silicon peaks appear to be instrumental in origin. The gelatin itself therefore contains a substantial amount of sulphur, and small quantities of chlorine, potassium and calcium. Therefore, when using gelatin standards, it is desirable to use several different concentrations of the salts of interest (particularly when these contain elements that are endogenous to the gelatin), as well as gelatin without added salts, and to calculate the regression of peak/background ratios on concentration of added salts. This has been done wherever it is appropriate in this paper, and it is in any case a better practice than relying on values obtained from a single standard.

Uniformity of standards

Standards normally appear structurally homogeneous, without any crystals (Fig. 2), although inhomogeneous gelatin films have been observed rarely. These tend to occur when using an excessively high salt concentration, and also when a standard containing more than one salt is

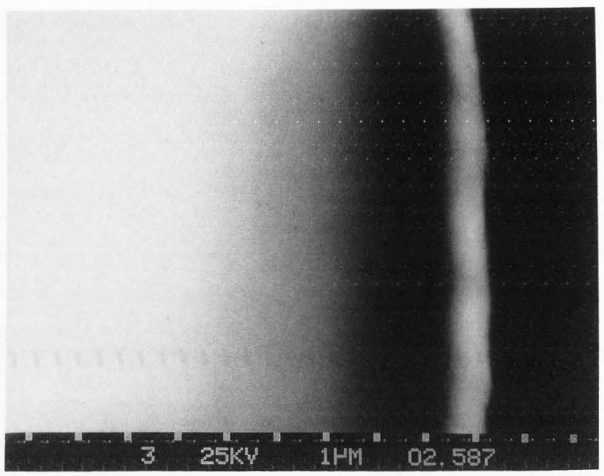

Figure 2 Transmission micrograph of a thin potassium bromide-gelatin standard on a nickel grid, showing structural homogeneity, but thickening at the edge next to the grid bar (right) from which the gelatine film is separated by a narrow gap. Each division of the scale equals 1μm.

Table 1 Variation of peak/background ratios within and between grids for the same standard

GRID (n)	S/BG MEAN± S.D.	K/BG MEAN± S.D.	I/BG MEAN±S.D.
1 (3)	0.64±0.04	1.61±0.06	2.78±0.11
2 (3)	0.66±0.04	1.69±0.17	2.97±0.22
3 (3)	0.61±0.03	1.31±0.08	2.22±0.21
4 (2)	0.61±0.03	1.69±0.02	2.88±0.06
5 (3)	0.64±0.05	1.45±0.08	2.48±0.15
ANALYSIS OF VARIANCE			
$F_{4,9}$*	0.85	8.06	9.39
P	N.S.	<0.05	<0.05

n = number of analyses
S.D. = standard deviation

* degree of freedom for the F statistics

prepared. This was found to be a problem when standards were made containing both potassium chloride and potassium di-hydrogen phosphate. Any such standards must be discarded, but it must be emphasised that there is normally no difficulty in preparing structurally homogeneous standards containing up to at least 10% by weight of a wide variety of salts.

At the same time, the thickness of the standards can be quite variable from one part of the grid to another, as well as being thicker at the edges of each grid square (Fig. 2). So far no way has been found to control the thickness of the gelatin films, which usually consist of thicker and thinner patches arranged randomly, and some parts may be too thick for the beam to penetrate. However, there are usually enough thin areas on the grids to make several independent analyses.

Measurements of peak/background ratios for various elements show reasonably low standard deviations within a grid (Table 1); coefficients of variation are mostly in the range of 5-10% for the standard shown here, consisting of potassium iodide dissolved in gelatin.

Reproducibility

Different grids of the same standard made at the same time generally show similar mean values for peak/background ratios, although an occasional grid may give anomalous values (Table 1). In the example given, grid 3 clearly gives significantly lower values for K/BG and I/BG than the other grids, and the values for grid 5 are also rather low. The fact that these variations are not seen with the S/BG ratios for the different grids indicates that the variations in the potassium and iodine signals are due to inhomogeneities in the solution from which the standards were prepared, in spite of the thorough mixing of these solutions.

Nevertheless, there is good evidence that in general these standards show a high degree of reproducibility. Fig. 3 shows the results from 2 sets of potassium iodide standards made from the same standard solutions several days apart. The regression of K/BG ratio on potassium concentration is virtually identical for the two sets. In Fig. 4, comparison is made between three sets of potassium standards made using three different salts (potassium iodide, potassium bromide, and potassium sulphate). Again there are only very minor differences in the slopes of the K/BG ratio against potassium concentration.

Radiation damage

The gelatin standards appear to be susceptible to radiation damage during analysis, judged by a variety of criteria. Firstly, the films often appear thinner in the area that has been analysed (Fig. 5). This, of course, provides no evidence on the question of differential loss of matrix and specific elements.

The occurrence of radiation damage is also indicated by the finding of lower peak/background ratios for different elements when the same standards are analysed at lower electron doses (Table 2). In all cases except one the ratios are significantly lower when the films are analysed at a probe current of 1.0nA at a magnification of 2000 X (5.20×10^{-11} C/μm^2), compared with 2.5nA at 10,000 X (3.25×10^{-9} C/μm^2), a difference of 62.5X in the electron dose per unit area. These results indicate relatively greater loss of the gelatin matrix with increased electron dose.

Thirdly, anomalous peak/background ratios are obtained in very thin films (Fig. 6), which are those having the lowest background (continuum) X-ray counts. In the case of sulphur (Fig. 6a), the S/BG ratio increases in very thin films, which suggests that in these conditions the gelatin matrix is lost faster than the sulphur. The sulphur is part of the gelatin, but it is not known whether it is predominantly in the form of

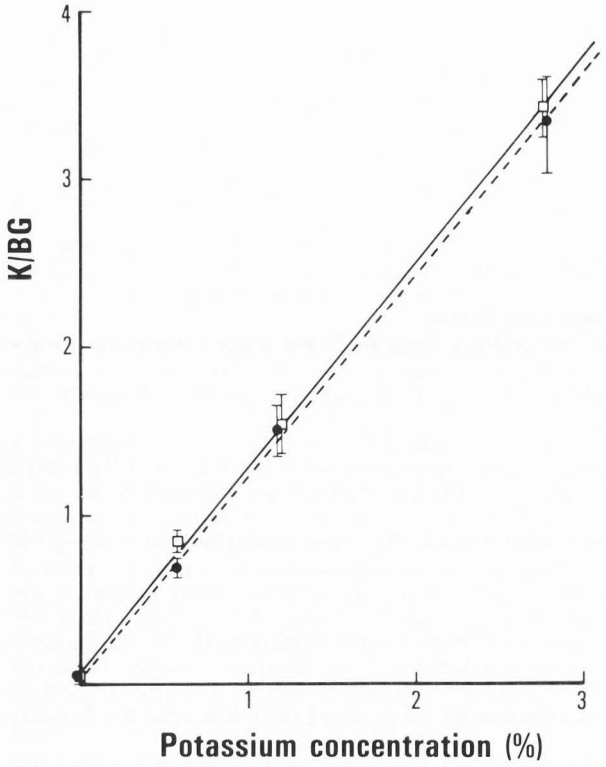

Figure 3 Calculated regression lines for two sets of potassium iodide standards made at different times from the same solutions. K/BG is the ratio of the counts in the potassium peak to the counts in the selected region of continuum.

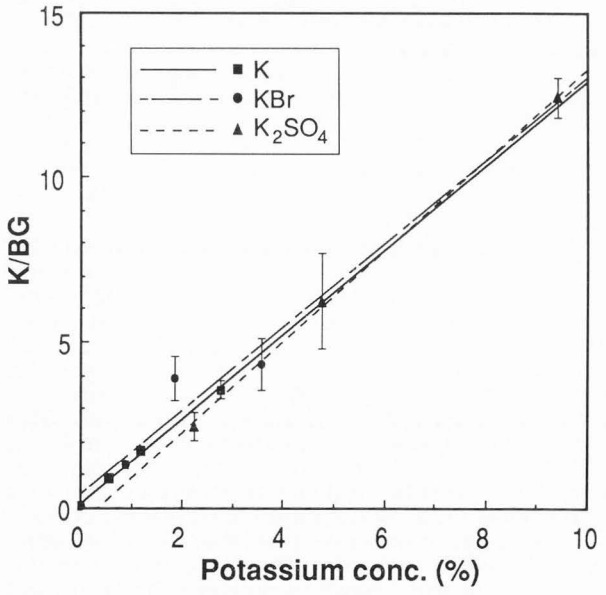

Figure 4 Comparison of standards containing different potassium salts. Calculated regression lines giving slope of K/BG (ratios of counts in potassium peak to counts in selected region of continuum) on potassium concentration for three different salts: K_2SO_4 (triangles), KI (squares), and KBr (circles).

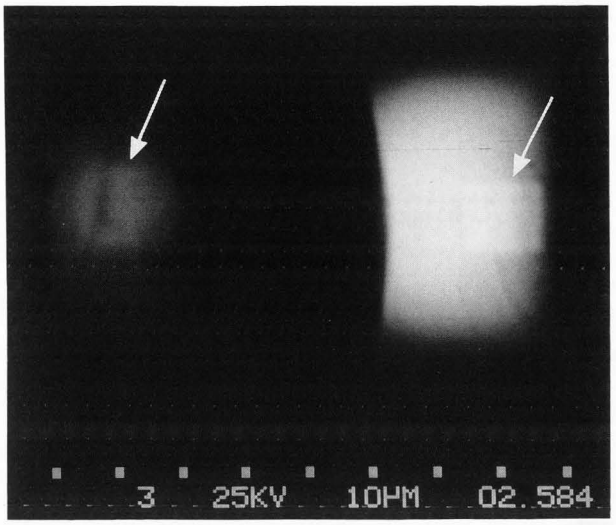

Figure 5 Potassium bromide standard showing radiation damage. The paler rectangles within each grid square (arrows) have been scanned with a raster during the period of analysis and have become thinner through etching. Each division of the scale equals 10μm.

sulphur-containing amino acids, thus forming an integral part of the gelatin matrix, or whether it occurs as inorganic salts. For iodine (Fig. 6b), introduced into the gelatin matrix as potassium iodide, the I/BG ratio is lower in very thin films, indicating that the iodine is lost more readily than the matrix during irradiation.

Fourthly, experiments in which the same area of several gelatin standards was subjected to repeated analyses over a long period of time showed changes both in characteristic elemental counts and in peak/background ratios (Fig. 7). No consistent pattern is discernible. The standard in Fig. 7a, a particularly thin film, shows progressive loss of both matrix and elements, but since all are lost at similar rates, the elemental peak/background ratios remain fairly constant. A relatively much thicker film (Fig. 7b) shows almost constant values, although there is a tendency for the potassium values to rise steadily during irradiation. Another film (Fig. 7c) shows rises for both potassium and iodine, which are particularly marked during the early stages of irradiation. Finally, the film in Fig. 7d shows losses of potassium and iodine, particularly during the early stages of irradiation, but since the gelatin matrix is initially lost at a faster rate the K/BG and I/BG ratios rise during irradiation, with the greatest rise during the early stages.

Discussion

Standards consisting of various salts dissolved in gelatin, and prepared as thin films by dipping and drying, have been used for biological XRMA by the author for more than ten years (Table 3). These standards are much simpler to prepare than standards made from gelatin cryosections (Roomans, 1979b; Hagler et al. 1983; Warley et al. 1983). When correction is made for

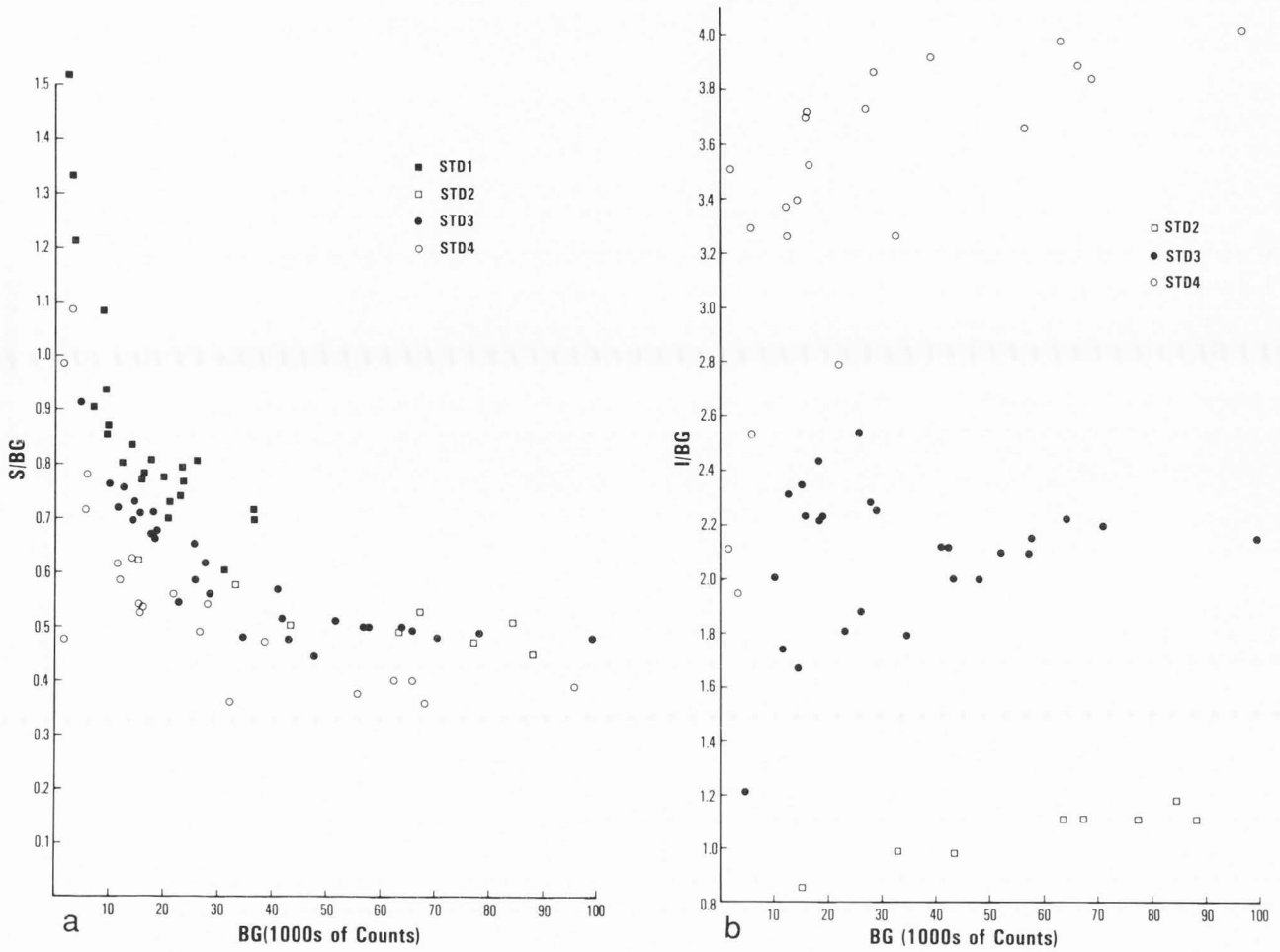

Figure 6 Plots of peak/background ratios against background (continuum) counts (equivalent to specimen thickness) for individual analyses of different points on different potassium iodide standards (Std 1, 0% iodine; Std 2, 1.864% iodine; Std 3, 3.883% iodine; Std 4, 8.959% iodine). **(a)** S/BG ratios; note the higher values at low background counts (thinner films). The sulphur is a component of the gelatin, and not added. **(b)** I/BG ratios; note the lower values at low background counts.

Table 2 Peak/background ratios for standards analysed at two different electron doses

	2.5nA, 10K (3.25×10^{-9} C/μm²)	1.0nA, 2K (5.20×10^{-11} C/μm²)	t	p
STANDARD 2				
S/BG	0.693 ± 0.030 (12)	0.605 ± 0.035 (14)	6.819	<0.001
K/BG	0.848 ± 0.065 (12)	0.712 ± 0.043 (14)	6.378	<0.001
I/BG	1.438 ± 0.100 (12)	1.196 ± 0.093 (14)	6.390	<0.001
STANDARD 3				
S/BG	0.634 ± 0.037 (14)	0.575 ± 0.039 (23)	4.548	<0.001
K/BG	1.541 ± 0.177 (14)	1.330 ± 0.120 (23)	4.328	<0.001
I/BG	2.648 ± 0.323 (14)	2.312 ± 0.215 (23)	3.807	<0.001
STANDARD 4				
S/BG	0.591 ± 0.036 (6)	0.533 ± 0.067 (10)	1.941	>0.05
K/BG	3.423 ± 0.183 (6)	2.861 ± 0.400 (10)	3.212	<0.01
I/BG	5.947 ± 0.326 (6)	5.026 ± 0.652 (10)	3.197	<0.01

Numbers in brackets following the values for mean ± standard deviation are the numbers of analyses.

t = Student's t statistic for the differences between means at the different doses.

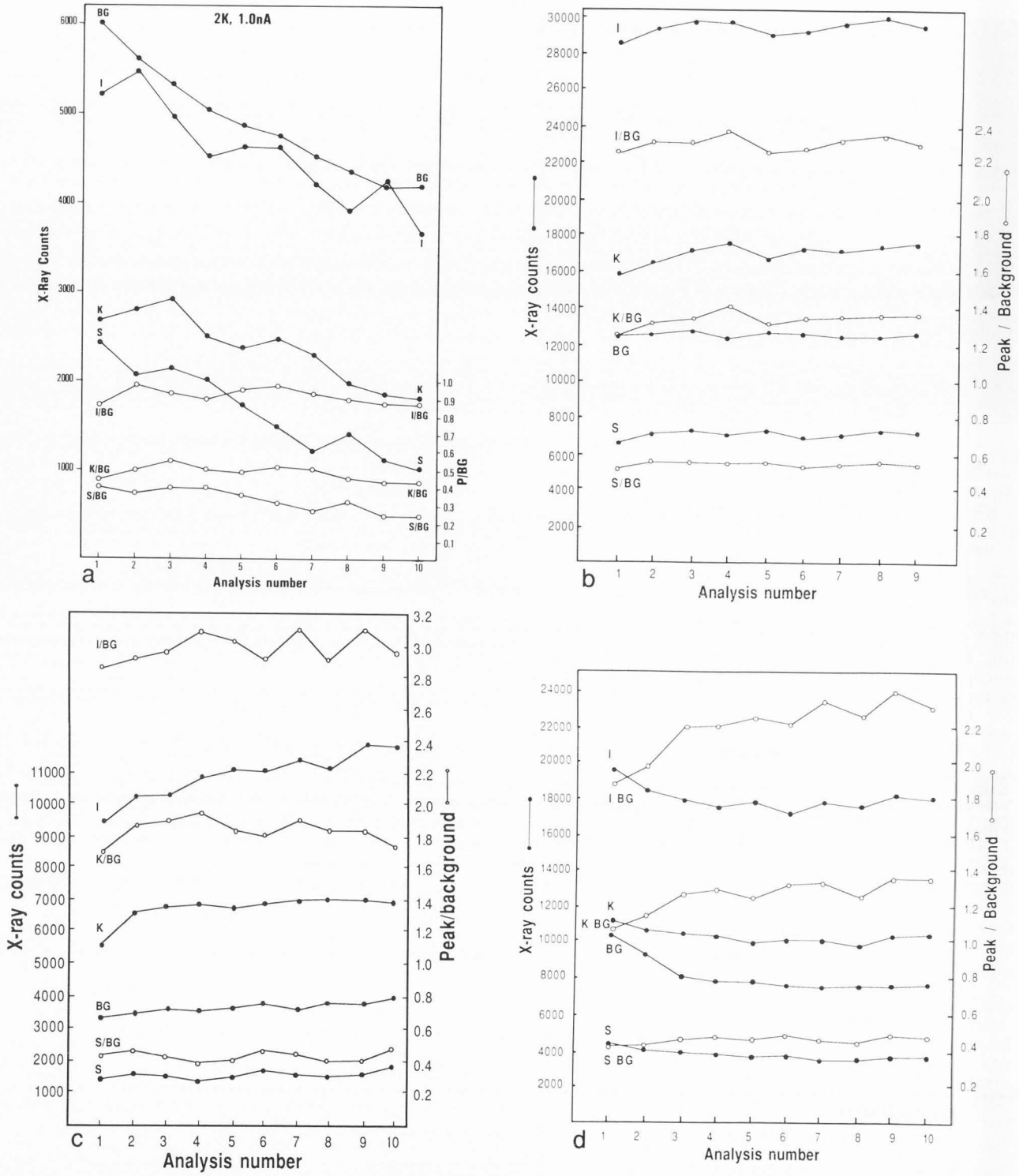

Figure 7 Changes in characteristic elemental counts and peak/background ratios for different potassium iodide standards under different analysis conditions. Ten (in one case, nine) successive analyses were made of the same area for 100sec live time for analysis. (**a**) Standard 2, magnification 2,000 X, 1.0nA probe current (dose/analysis = 3.46×10^{-11} C/μm^2). Mass loss (as measured by continuum counts (BG)), 30%. (**b**) Standard 3, magnification 10,000 X, 2.5nA probe current (dose/analysis = 2.16×10^{-9} C/μm^2). No significant change in continuum counts. (**c**) Standard 4, magnification 10,000 X, 2.5nA probe current (dose/analysis = 2.16×10^{-9} C/μm^2). Increase in continuum counts, possibly due to contamination. (**d**) Standard 3, magnification 2,000 X, 1.0nA probe current (dose/analysis = 3.46×10^{-11} C/μm^2). Mass loss (as measured by continuum counts), 26%.

Table 3 Different elements incorporated in gelatin standard

Salt	Element(s)	Reference
Sodium nitrate	Na	) Sumner, 1978b
Eosin Y	Br	)
Barium acetate	Ba	)
Potassium bromide	Br	) Sumner, 1984
Potassium iodide	I	)
Phosphorylcholine chloride	Cl/P ratio	Sumner, 1986
Chloroquine diphosphate	Cl/P ratio	Sumner, unpublished
Potassium sulphate	K, S	Sumner, unpublished
Cadmium sulphate	Cd/S ratio	Sumner, unpublished

variation of continuum counts with variations in the mean value of Z^2/A, the standards described here show good linearity between peak/background ratios for the elements of interest, and the concentrations of the elements in the standards. Although evidence for such linearity is an essential feature of any reliable standard for biological XRMA, and has been demonstrated by numerous authors, it is only one criterion for assessing the quality of such standards. In this paper, gelatin standards have also been assessed for uniformity, reproducibility, and susceptibility to radiation damage.

Visual inhomogeneity of standards is obviously unacceptable, but a more satisfactory estimate of homogeneity can be obtained from actual analyses. In the present study, coefficients of variation of measurements on different parts of the same standard were in the region of 5%, roughly similar to those reported by other authors both for cryosectioned standards (Roomans & Seveus, 1977; Warley et al. 1983; Saubermann et al. 1981), and for resin standards (Roomans 1979a), although the coefficients of variation quoted by El-Masry & Sigee (1986) for metalloprotein standards appear to be rather higher, while Lupton & Saubermann (1986) reported a somewhat lower coefficient of variation (2.7%) for amino-plastic standards. Evidently the gelatin standards described in this paper show a degree of homogeneity comparable with that of other types of standards described in the literature, although different reports are not necessarily strictly comparable because of differences in experimental design.

Reproducibility of standards does not seem to have been studied by others. The data given in this paper show that standards made from the same gelatin solution on different days give closely similar results (Fig. 3) as do potassium standards made with different salts (Fig. 4). Nevertheless, some variation between individual grids has been found (Table 1), for which at present there is no clear explanation. One possibility, apart from actual differences between standards, could be inaccurate subtraction of continuum counts due to the grid or surrounding parts of the microscope chamber. Even so, the overall coefficient of variation in this case is only a little over 10%, which, while higher than desirable, is probably within acceptable limits.

Radiation damage is a constant problem in biological XRMA, and the analysis of standards is no exception (Hall & Gupta, 1974, 1984; Shuman et al. 1976; Rick et al. 1979; Morgan & Davies, 1982; Cantino et al. 1986). The results described in this paper show clearly that gelatin standards do lose material as a result of irradiation, and that although the results are not entirely consistent, different components of the standard appear to be lost at different rates. It is also clear that the effects of radiation damage are more obvious in thinner standards. It should also be noted that specimens containing halogens, such as the standards described here, are particularly susceptible to loss of the halogens during irradiation (Morgan & Davies, 1982), and other types of compounds might be expected to show greater stability under the beam. Nevertheless, the other data in this paper seem to indicate that the amount of radiation damage sustained within the usual period for an analysis is within acceptable limits, otherwise the consistent results described here would not have been obtained. Hall & Gupta (1974) showed that extensive loss of mass occurred in protein specimens with a dose of $4x10^{-10}$ C/μm^2, after which the specimen stabilized. Similar results have been obtained by others (Rick et al. 1979; Cantino et al. 1986), and on this basis substantial loss would be expected in the standards described here, at least at the higher dose used. Rick et al. (1979) and Cantino et al. (1986) also found extensive loss of sulphur under irradiation, but though this was obviously occurring in the experiment illustrated in Fig. 7a, other experiments were less clear on this point, while the data in Fig. 6a appear to indicate retention of sulphur while the matrix is lost. The reason for these differences is not clear, but may be related to the chemical combination in which the sulphur is found. It should be noted that Shuman et al. (1976) found an increase of sulphur counts under irradiation, which they attributed to migration of the sulphur atoms. Something similar must be happening in the experiments in Figs. 7b & c, where K and I counts rise with time. Rick et al. (1979) reported that X-ray counts for C1,K and Na were steady up to a dose of 10^{-8} C/μm^2, but it is clear that some losses of both K and I occurred in the present experiments at lower doses (see especially Figs. 6b and 7a). Such losses were most obvious in the thinnest standards, which may be attributable simply to the higher surface/volume ratio in such specimens. Roomans (1979a) also reported loss of material, from resin standards, but the rates of loss of the specific element, iodine, and of the organic matrix, were similar, so that the peak/background ratio remained constant for some considerable time. El-Masry & Sigee (1986) reported that metalloprotein standards were stable

with increasing beam current. Kitazawa et al. (1983), using as standards a series of different sulphur-containing proteins, emphasised that analysis should be performed only at low temperatures, to avoid specific loss of sulphur as a result of radiation at room temperature (see also Cantino et al. 1986). Unfortunately equipment for low temperature analysis was not available for the experiments described here, but in principle any precautions to reduce radiation damage are highly desirable. Evidently more detailed study of the susceptibility of different types of standards to radiation damage is urgently needed.

In conclusion, the gelatin standards for biological XRMA described in this paper appear to be easily prepared, and versatile. They can be produced for a wide variety of elements at substantial concentrations, and as regards homogeneity and reproducibility, appear to have properties as good as many of the standards described in the literature. However, in some circumstances they suffer considerable radiation damage, and may be less stable than resin standards. Comparisons with other types of standards would be valuable in making an objective assessment of gelatine standards in relation to other types that have been described in the literature.

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Discussion with Reviewers

A.T. Marshall: Would it not be a good idea to dialyse the gelatine? This will remove exogenous salts.
Author: This was attempted, but was only partly successful, small amounts of sulphur, chlorine, potassium and calcium remaining. Since the sulphur is probably largely protein bound, it would not be removed by dialysis anyway.

T. von Zglinicki: **How is the air-drying process controlled? Air drying is obviously the reason for the inhomogeneities mentioned in samples with higher salt concentrations. Moreover, salts might migrate during air drying, depending on external moisture, temperature, and film thickness.**
Author: **The air-drying process has not been controlled, and therefore results may well depend on the atmosphere conditions at the time the standards are prepared. However, the first event to occur is the gelling of the gelatine, after which it is assumed that the migration of ions is more restricted than in the liquid state. Redistribution of ions during the subsequent drying might therefore be limited. Nevertheless, more detailed studies on the homogeneity of the standards, and the effects of preparation and storage conditions on them, would be desirable.**

D.C.Sigee: The author states that the gelatin films usually consist of thicker and thinner patches arranged randomly. Can he make any suggestion as to why this variation occurs, and does he know what the thickness variation of these patches is? The dense patch of protein seen in Fig 2 appears to be lying adjacent to a grid bar, and not randomly - as generally stated.
Author: Within any one grid square, the gelatin film is normally thicker at the edges and thinner in the centre, as would be expected from the effects of surface tension. The variations in thickness of the films between grid squares do not, however, appear to conform to any clear pattern, and the thickness is probably influenced by such factors as the position where the grid is held by the forceps, the effect of wiping surplus gelatine from the grid, and drainage of the gelatin solution before it gels.

G.M.Roomans: A serious problem in this paper is that no indication is given of the (range of) thickness of the gelatin standard. It is, however, important to know this thickness since at thicknesses >2μm an absorption correction may be necessary, especially for lighter elements (Na). Wouldn't it be possible to compare the continuum of the gelatin standards with that of a plastic section of known (mass) thickness analyzed under the same condition? This would at least give an estimate of the mass thickness of the gelatin standard.
Author: Analyses have been carried out on Araldite sections, the thickness of the analyzed areas being determined by microinterferometry using a Vickers M86 microinterferometer. There was a linear relationship between continuum counts and thickness up to approximately 2μm (the thickest section measured)(correlation coefficient 0.985). On this basis, the continuum counts for a specimen 1μm thick analyzed at 2.5nA and 10000x magnification would be 12363; at 1.0nA and 2000x magnification, 6067. Assuming the density of the gelatin films to be similar to that of Araldite (probably not quite true), the specimens analyzed to give the results in Tables 1 and 2 would average 1.21μm (standard deviation 1.03μm) for those analyzed at 2.5nA, and 1.78± 1.19μm for those analyzed at 1.0nA.

T. von Zglinicki: Local sample thickness in the standards is not known. The background under the peak should be used to correct for thickness effects.
Author: An estimate of local sample thickness can be made (see answer to question by Roomans, above). Although there is no evidence for absorption occurring in the thicker specimens, use of the background under the peak to correct for such effects would be a valuable approach. However, a programme to calculate the background under the peak accurately was not available to us. See also the reply to Sigee, immediately below.

D.C.Sigee: Does any variation in peak/background ratio occur for particular elements in relation to film thickness? If so, could this explain differences between grids - where differences may occur in the overall amount of gelatin deposited.
Author: As shown in Fig 6, variation in peak/background ratio with film thickness does occur, especially with the thinnest films. This has been attributed to radiation damage. With thicker specimens (> about 1μm), peak/background ratios are fairly constant and do not vary systematically with thickness. The data in Table 1 are all obtained from films in this range of thickness. Surprisingly, no indication has been obtained of absorption effects, even in the thickest specimens analyzed.

T. von Zglinicki: Vacuum conditions are not given. Was an anticontamination device used? Was the vacuum constant for all experiments?
Author: No anticontaminator was available, and the vacuum in the specimen chamber, being under automated control, could not be held constant. Analysis of the standards under more controlled conditions, particularly using a cold stage to reduce radiation damage, would indeed be valuable, and is planned for the future when new equipment becomes available to us.

T. von Zglinicki: **As discussed by Cantino et al (1986), background values may depend, among others, on beam current drift, specimen drift and, especially, specimen shrinkage. These effects should be excluded or data given in Table 2 and Fig. 7 should be corrected for these influences.**
A.T. Marshall: **Presumably you have considered the obvious possibilities of instrumental instability**

accounting for the excursions in characteristic and background counts? For example, charging, or some other cause of beam shift, could change the analysed region with time.
Author: Beam current was stable during these experiments, and charging and specimen drift were not observed. In a few cases shrinkage of the irradiated area could be detected as distortion of the surrounding, unirradiated area, but this was rare; most specimens showed an undistorted appearance as in Fig. 5. Errors due to the effects mentioned are therefore likely to be small, but in any case the effects of radiation on these standards seems to be so variable that the precise quantitation of radiation damage does not seem useful.

D.C.Sigee: It might be expected that gelatin films would show close similarity to dried gelatin cryosections - since both are essentially a gelatin matrix containing added salts. Could the author compare these two types of standard in terms of homogeneity and susceptibility to radiation damage?
Author: Such a comparison, and also comparisons with other types of standards, would indeed be valuable, and are planned for the future, when new equipment will be available to the author.

A.T. Marshall: For how long can these standards be stored and reanalysed?
Author: This has not yet been investigated systematically, but the standards appear to be quite stable for at least several weeks and possibly longer when stored at room temperature in the laboratory. If stored in a dessiccator their useable life might be much longer.