Scanning Microscopy

Volume 7 | Number 3 Article 12

9-27-1993

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Schrage, N. F.; Benz, K.; Beaujean, P.; Burchard, W. -G.; and Reim, M. (1993) "A Simple Empirical Calibration of Energy Dispersive X-Ray Analysis (EDXA) on the Cornea," *Scanning Microscopy*: Vol. 7 : No. 3 , Article 12.
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Scanning Microscopy, Vol. 7, No. 3, 1993 (Pages 881-888)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/93$5.00+.00

A SIMPLE EMPIRICAL CALIBRATION OF ENERGY DISPERSIVE X-RAY ANALYSIS (EDXA) ON THE CORNEA

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(Received for publication July 25, 1993, and in revised form September 27, 1993)

Abstract

Monitoring of the corneal electrolyte content is important for the study of chemical eye burns. This paper describes quantitative measurements on gelatin standards, corneas and a cornea homogenate with an energy dispersive X-ray analyzer (EDX) in the scanning electron microscope (SEM). Ten micrometers thick cryosections were freeze-dried and mounted on solid carbon supports. The applied quantification procedure was a local peak background analysis with a specifically designed computer program. Similar chemical and physical properties of gelatin, cornea homogenate, and cornea were proven by EDX-analysis and wet chemical analysis. Gelatin standards with known concentrations of different added salts showed linear correlations with a correlation coefficient higher than 0.95 for all considered elements. The local background generation on carbon supports was the same for gelatin standards and corneal tissue. The results demonstrate that quantitative EDX analysis of semi-thin samples, mounted on neutral carbon supports, can be reliably used for the assessment of the corneal mineral composition.

Key Words: Cornea, mineral composition, electrolytes, quantitation, local peak-background-method, scanning electron microscopy, microprobe, energy dispersive X-ray analysis, experimental ophthalmology, eye burns.

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Introduction

Chemical burns of the eye are a very common ophthalmological accident, and severe eye burns generally have a poor prognosis. The treatment of the chronic inflammatory reaction includes topical application of steroids and reepithelializing drugs. More recently, surgical procedures such as keratoplasty, perdiotomy and peridektomy, and tenonplasty (Reim, 1989, 1990) have been successfully used to improve the prognosis. In previous studies, we noticed changes in the mineral composition of excised corneas and conjunctiva after alkali burns, as well as various types of calcifications (Schrage and Reim, 1990; Schirner *et al.*, 1990). To examine the effects of chemical eye burns and the subsequent topical treatment in further detail, we need a reliable standardised method for the analysis of the corneal mineral content. This method should be simple and applicable to different types of samples, such as, rabbit corneas and excised material from patients treated in our department.

Quantitative energy dispersive X-ray microanalysis (EDXA) on thin sections of biological material is a well established procedure (Hall, 1979; Hall and Gupta, 1982; Roomans, 1980, 1988; Rick *et al.*, 1982). Bulk specimens can be analysed with local peak to background ratios (P/B) of EDXA measurements (Boekestein, 1984). Qualitative analysis of semi-thin corneal sections with a histological standard section size of 5 to 10 μm has been performed by Robinson and Streeten (1984). Warner and Coleman (1975) described the quantitative analysis of semi-thin corneal samples mounted on Al containing supports. A disadvantage of that method is the marked difference in the background spectrum of the Al support, compared to the tissue sample matrix. Small differences in sample thickness can distort the results, and elaborate analytical adjustments are required. Calibrated EDXA measurements of samples mounted on neutral highly polished carbon holders (also described by Sumner, 1978; Robinson and Streeten, 1984) have been performed by Roomans and colleagues (Wroblewski *et al.*, 1978, 1983; Versura *et al.*, 1989). They found that the penetration of the electron beam in freeze-dried muscle was less than 14 μm at 20 kV (Wroblewski *et al.*, 1978), and 16 μm thick samples were used to prevent excitation of

Table 1. Mineral composition (mean values in mmol/kg dry weight and variance coefficient of 3 measurements) of a homogenate of 1000 pig corneas. Analysis by atomic absorption spectroscopy, ion-electrophoresis, and inductive coupled plasma analyzer.

Element	concentration	Variance (%)
Na	485.43	3.92
Mg	4.81	5.16
P	23.47	2.65
S	223.6	1.15
K	55.91	1.4
Ca	2.94	7.79

Table 2. Mineral contents of selected elements (in mmol/kg) in the cornea. Data from de Azavedo and de Jorge (1965); Davson (1948); and Midelfart (1987, 1990). d.w. = dry weight; w.w. = wet weight.

Author →	de Azavedo		Davson	Midelfart
Cornea →	human		ox	ox[1]/rabbit[2]
Element	d.w.	w.w.	w.w.	d.w.
Na	943.2	148.5	115±0.7	537.2±16[1]
Mg	181.31	34.54		
P	113.56	21.72		
Cl			79.6±1.2	355.7±23.5[2]
K	58.75	11.42	23.4±0.8	47.7±3.7[1]
Ca	1309.02	251.27		

the specimen holder. Analysed tissues were human muscle biopsies (Wroblewski *et al.*, 1978), human oral mucosa and rat salivary gland (Wroblewski *et al.*, 1983).

A section thickness of more than 12 μm is problematic for corneal tissue. Our aim therefore was to quantify EDXA-measurements on semi-thin corneal sections of sizes similar to those of Warner and Coleman (1975), but to use neutral carbon specimen holders as stabilizing factor for the continuum shape and as correction for variations in specimen density. Gelatin standards with added amounts of different salts and a cornea homogenate of chemically analysed elemental composition were used for calibration; the cornea homogenate also served as internal reference in the scanning electron microscope (SEM) during EDXA.

Materials and Methods

Cornea homogenate

For the calibration of EDXA on corneas, 1000 pig corneas were homogenized and their mineral composition established by wet chemical analysis. The corneal epithelium of these eyes was removed with a hockey knife and the corneas were excised with a 10 mm trephine. The corneal buttons were snap-frozen in liquid nitrogen and 5 corneae at one time were freeze-milled under liquid nitrogen at 90 K for 15 minutes in a cryo-bullet-mill (Retsch, M2000), resulting in a fine white homogeneous powder. After 200 milling processes, the

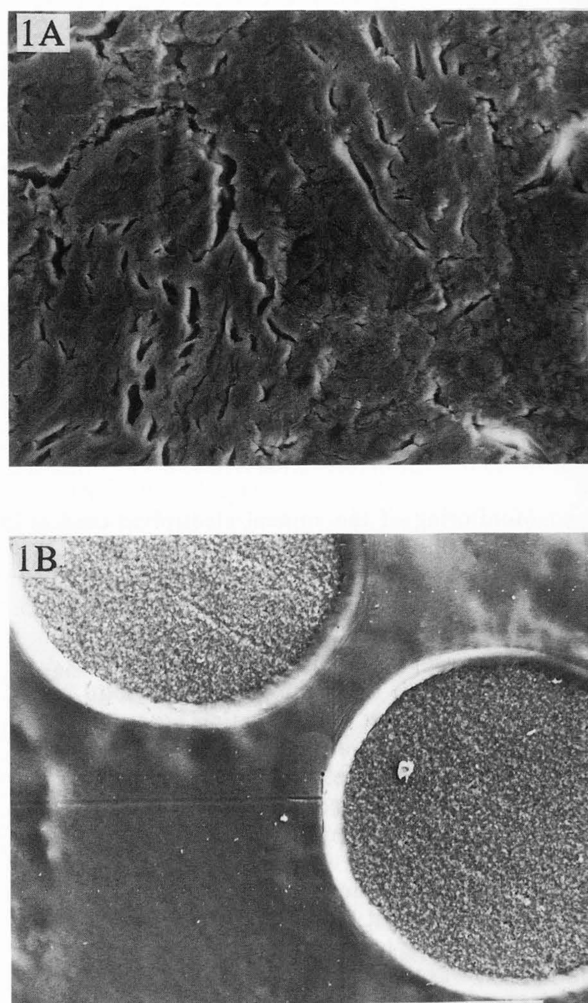

Figure 1. Scanning electron micrographs showing the surface of cornea homogenate (**Fig. 1a**) and a gelatin standard (**Fig. 1b**). In **Fig. 1b** an air bubble and the underlying carbon support are seen. Bar= 100 μm.

samples, which remained frozen during the whole procedure, were combined and lyophilized (WKF 2, Lyophilizer). The dried powder was agitated for 48 hours to achieve homogenization. Wet chemical analysis was carried out against external standards of Na, Mg, K, Ca, Cl, P, S and a reference substance: milk powder (Promochem milk powder A11), containing a certified amount of the mentioned elements. Two and a half grams of corneal powder, subsequently named cornea homogenate, were hydrolysed in 25 ml (65%) HNO_3 and 2.5 ml (100%) H_2O_2 in a microwave decompositor at 300 W for 5 minutes and 600 W for 2 minutes. Nitrous gases were blown off, and the samples were then boiled at 373 K with 15 ml 30% H_2O_2 to denitrify them. The element analysis was carried out by ion-exchange-chromatography, inductive coupled plasma analyser, and flame photometry. Chlorine could not be measured because of interferences from nitrates. The results for

cornea homogenate, given in Table 1, served as internal reference for calibration of the EDXA, in addition to the gelatin standards as described below (the analyses were carried out by: Chemisches Lebensmitteluntersuchungsamt der Stadt Aachen).

For EDX-calibration, samples of corneal homogenate were resuspended to 20 mass-per cent with bidistilled water. Rehydration time was 48 hours at 277 K. These samples were frozen in liquid nitrogen and prepared as described below under "**Preparation**".

Gelatin standards

As proposed by Roomans (1979, 1988), we used cryosectioned gelatin samples as standards. A weighed amount of gelatin was mixed with a weighed amount of the compound (Na, Mg, P, S, Cl, K, Ca) in the appropriate quantity of water, heated to 323 K and poured into clean disposable plastic Petri dishes. The solutions quickly solidified at room temperature and small buttons of 4 mm diameter were frozen in liquid nitrogen. The final electrolyte concentrations were: Na: 1207; Mg: 695.6; P: 344.8; S: 689.9; Cl: 689.9; K: 172.4; and Ca: 695 mmol/kg. The chosen concentrations were higher than the expected values for dried corneal stroma tissue (Table 2; de Azavedo and de Jorge, 1965; Davson, 1948; Midelfart, 1987, 1991).

Rabbit corneas

Ten chinchilla bastard rabbits of 1.5 to 2 kg weight received a lethal injection of pentobarbital, and the healthy right eyes were enucleated. The cornea with adherent sclera was excised and frozen between two cornea-shaped steel blocks at 55 K. All experiments with rabbits were carried out in accordance with the international instructions for animal research.

Preparation

All samples were cut with a cryomicrotome at 240 K into slices of 10 μm and mounted on special polished vitreous carbon supports of highly sintered graphite (CTI-Technik). For thickness control experiments, additional slices of between 4 and 25 μm were mounted on vitreous carbon or brass platelets at 277 K (Fig. 1). Short thawing times and immediate refreezing were used to guarantee fixation on the specimen holders. Ice crystals of a mean diameter of 5 to 15 μm formed at the edges of corneal specimens. The frozen hydrated samples were subsequently lyophilized over 24 hours. Finally, all samples were coated with a thin layer of carbon to provide electrical conductivity.

Energy dispersive X-ray microanalysis (EDXA)

The quantitative analysis was performed on a JEOL 35 CF SEM, equipped with an ORTEC energy-dispersive detector system (Si-Li-detector crystal of 163 eV energy resolution) with an 8.2 μm beryllium window. Fixed parameters were the working distance of 24.8 mm and the take-off angle of 15.5°. The probe current was calibrated every 500 seconds (in a 0.2 mm hole as a "Faraday's" cage in the specimen holder) to 2.0 nA. All measurements were done on areas of 350 μm^2, with a live time (LT) of 100 seconds. To eliminate possible errors due to changes in the take-off angle, the focus-control of the microscope was set in a fixed position throughout all measurements, and the required part of the specimen was brought into focus using the height control on the stage.

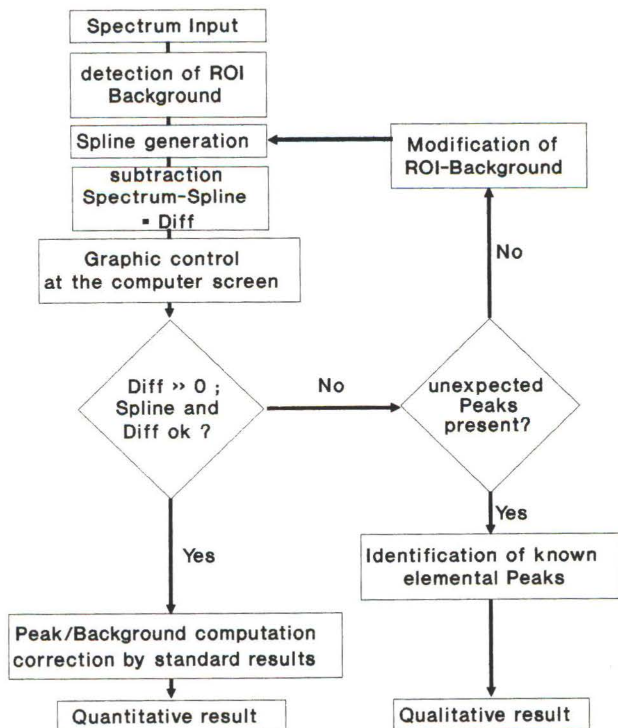

Figure 2. Flow Chart of the program EDXBIO. ROI = region of interest, Diff = difference spectrum.

Treatment of data

All analytical data were transferred to a MS-DOS® compatible microcomputer and calculated with a specifically designed program (EDXBIO) written in Turbo-Pascal (Borland)®. This program performs a P/B analysis, in contrast to the net-peak analysis of the commercially available program on the ORTEC system. The EDXBIO program performs a single step bipartite spline interpolation for continuum estimation over regions of interest without peaks (Figures 2 and 3). The used spline is continuous to the second derivative. Peak detection is performed in regions of specific elemental energies. The first part of the spline ranges from 0 to 1.74 keV (Si Kα-spectral-line) and is fixed at a point below the Si-peak which is a stable blank value of the Si-Li detector, only dependent on the total continuum. The local P/B ratio is constant at 0.35 at this point provided the sample does not contain Si; it is defined by the internal fault of the detector. The mean of 5 channels (=50 eV) around the detected Si-peak in the analysed spectrum is computed and its local P/B value (Boekestein, 1984; Roomans, 1988) is taken as point of the estimated continuum and as intersection between both parts of the spline.

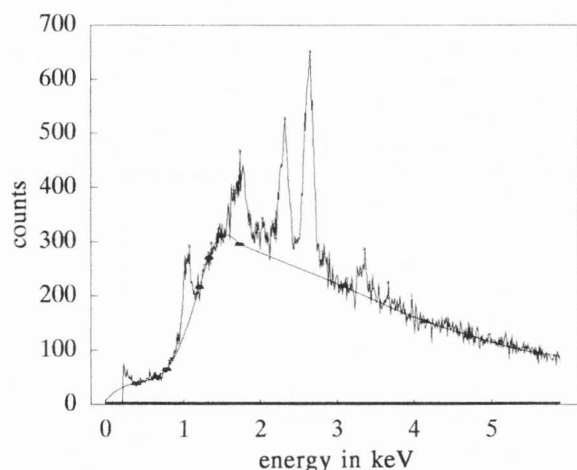

Figure 3. X-ray analysis (X-ray energy in keV versus the number of counts) of cornea homogenate (serrated line). Crosses indicate the points of detected peaks. Continuum estimated by single step bipartite spline interpolation = smooth line. Bars indicate the points of detected minima in regions of interest for spline input. Note the point at 1.74 keV (Si-Kα-line) where a fixed P/B-ratio of 0.35 is used as point of the continuum.

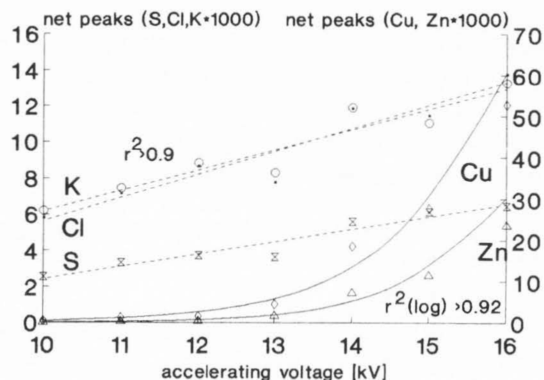

Figure 4. Influence of accelerating voltage from 10 to 16 kV on the net peak development of lyophilized 10 μm gelatin sections containing 0.15 mol/l KCl wet weight. Measuring conditions: 350 μm2, probe current 0.2 nA. Each point represents mean values of 5 measurements. Linear increase for Kα peaks of S, Cl, K and an exponential increase for Kα-peaks of Cu and Zn of the underlying brass specimen holder.

The second part of the continuum is estimated in the same way, using the "Si-continuum point" as the starting point. All P/B values are computed from net peaks and their local continuum. To obtain accurate results, the sample has to be free of Si, otherwise the estimated continuum values are too high. The quantitation routine is not performed if the difference between continuum and original spectrum exceeds defined negative values (Figure 2). The Si content of the corneal samples was assessed prior to the EDXA with a Si-free

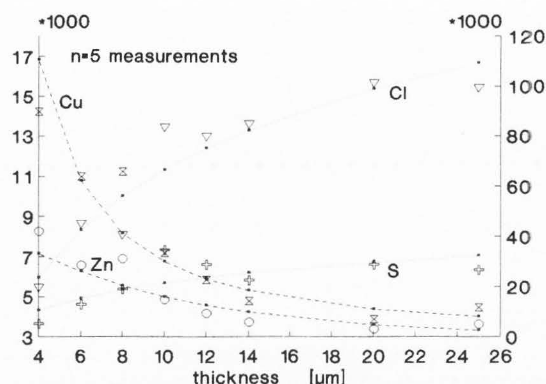

Figure 5. Influence of specimen thickness on the peak generation of lyophilized gelatin sections containing 0.15 mol/l KCl wet weight on brass supports. Linear increase for Kα peaks of S, Cl, and an exponential-like decrease for Cu Lα-P/B from the underlying brass specimen holder. The slightly different slope of S and Cl is due to different absorptions for their specific energies in the detection system. These differences are constant.

Germanium detector (Tracor, Z-Max). We found less than 0.01 g Si/100 g dry weight, which is in good agreement with Oksala (1954) who reported Si contents of about 1.36 mg/100 g wet weight in ox corneas.

The complete program listing of EDXBIO is available from the authors.

Results

In order to assess the influence of different accelerating voltages on the results, we analysed 10 μm slices of gelatin containing 0.15 mol/kg wet weight KCl with accelerating voltages increasing from 10 to 16 kV. The mean values of 5 measurements of the net peaks (Kα) of K, Cl and S increased approximately linearly, whereas the Cu and Zn Kα-signals increased non-linearly (Fig. 4). The optimal slice thickness was established by measuring gelatin samples of between 4 and 25 μm thickness at 15 kV accelerating voltage. Repeated measurements were performed at different sites of the specimens. The computed P/B-ratios showed a linear increase of S and Cl (Kα-peaks) with high variances for thicknesses larger than 12 μm and a logarithmic-like decrease of Cu Lα-P/B-ratio to 0, indicating a complete loss of Cu-Lα-peak for a thickness over 20 μm (Fig. 5). To evaluate whether gelatin and cornea have similar physical properties in the EDX-analysis, we performed ten repeated 50 seconds LT measurements at a defined location on 10 μm sections of cornea homogenate and gelatin containing 0.15 mol/l KCl wet weight. The results for cornea homogenate showed a slight increase of Cl, S and K Kα net peaks in the beginning and subsequently nearly stable results. The Cu-Kα net peak increased linearly over time from 8000 to 12000 counts (Fig. 6).

On the gelatin samples we found very stable values for Kα-peaks of Cl, K, and S, and a slight increase of Cu during the first 3 measurements (Fig. 7).

Table 3. Comparison of local background values at the specific energies of the different elements (± standard deviation of n = 10 measurements).

local background	keV/element 1.04/Na	1.25/Mg	2.01/P	2.31/S	2.62/Cl	3.31/K	3.69/Ca
glass carbon	142 ± 9	246 ± 11	251 ± 5	241 ± 2	229 ± 2	194 ± 5	174 ± 6
cornea rabbit	144 ± 18	251 ± 28	264 ± 17	255 ± 14	242 ± 11	207 ± 8	185 ± 9
cornea homogenate	149 ± 16	263 ± 25	274 ± 20	259 ± 18	242 ± 16	202 ± 13	181 ± 11
gelatin	153 ± 22	269 ± 27	280 ± 18	265 ± 13	248 ± 10	207 ± 5	184 ± 6

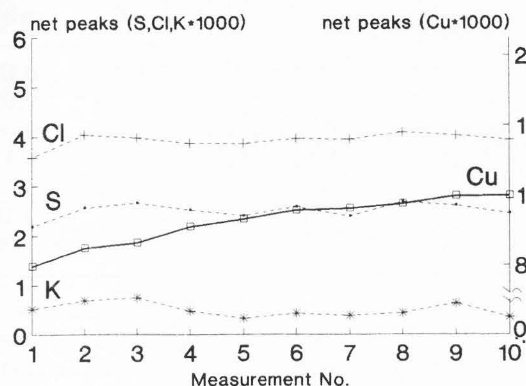

Figure 6. Repeated EDX-measurements on cornea homogenate for 50 seconds LT. Constant increase of Cu and initial increase of Cl, S, K, and stable Cl, S and K, values after 3 measurements. Net peaks S, Cl, K-Kα (left Y-axis) and Cu-Kα (right Y-axis).

The absolute Cu-values differed for 10 μm slices of gelatin and cornea homogenate mounted on copper supports. The results indicate differences in the continuum generation of both substances, which are most likely due to the higher total electrolyte content of the more compact corneal sample.

The cornea homogenate sample was included in our experiment to avoid possible errors due to differences in the physical properties of the specimens.

Continuum similarity

The main EDXA measurements were carried out on 10 μm samples with an accelerating voltage of 15 kV, 100 seconds LT and 2 nA probe current. To assess whether the carbon support in fact produces a neutral background, we compared local background values of gelatin, cornea homogenate, and rabbit cornea samples with those of an empty glass carbon specimen holder (Table 3). The differences between the local background values in the four conditions were less then 10%, with the largest difference between glass carbon and gelatin standards. High amounts of salts increase the local background values because of the higher number of atomic nuclei. For normal rabbit corneas, the difference between cornea and glass carbon was always less than 6.3%.

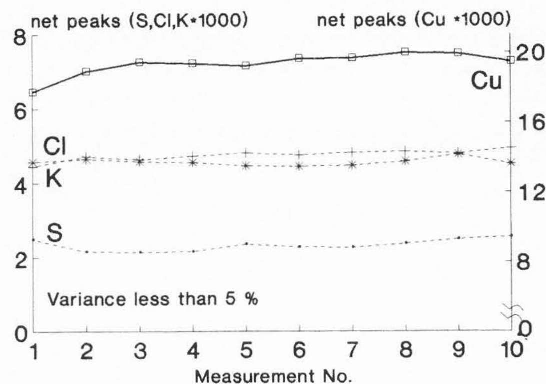

Figure 7. Repeated EDX-measurements on gelatin containing 0.15 mol/l KCl wet weight, for 50 seconds LT. Note: constant increase of Cu, initial increase of Cl, S, and K, and stable Cl, S, and K values after 3 measurements. X-axis indicates the number of measurement, the left Y-axis shows S, Cl, and K-Kα, and the right Y-axis shows Cu-Kα. Variances gelatine < 6%.

To improve the correlation between weighed salt concentrations in gelatin standards and net peaks, we used the local continuum method proposed by Boekestein (1984). With the commercially available procedure of the ORTEC analyser we found acceptable correlations for elements with peaks of energies higher than 2.0 keV. Poor correlations for Na, P, and S is the main problem because of insufficient continuum estimation with the ORTEC program at energy levels below 2 keV. The EDXBIO computer program improves the approximation of the continuum in this region and ensures that the difference between the acquired spectrum and the computed continuum shows only peaks of nearly Gaussian shape and no background. With this program, we found linear correlations for all measured elements (Table 4). The results of the EDXA of the cornea homogenate are given in Table 5. For a comparison between Tables 1 and 5, it is important to recognise that the lowest theoretical P/B value (element not present) = 0, i.e. peak and background are of the same value (no peak). In reality, these values are in a range of 1.09, due to electronic noise.

Twenty rabbit corneas (Fig. 8) were each analysed in the middle of the corneal stroma on 4 fields of 350 μm^2, the results are presented in Table 6.

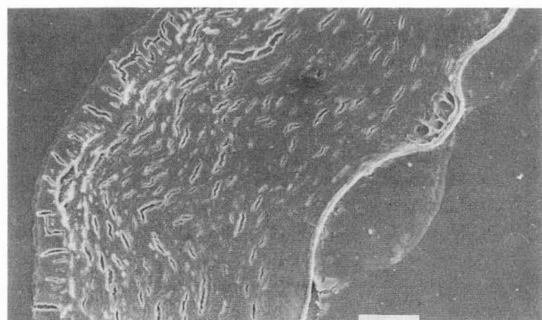

Figure 8. Scanning electron micrograph of a section of a healthy rabbit cornea: epithelium at the left side, followed by stroma, and descemet's membrane. Some endothelial cells can be seen. Bar = 100 μm.

Discussion

The reported results demonstrate that quantitative analysis with EDXa systems can be performed on 10 μm corneal tissue sections, mounted on neutral supports of highly polished vitreous carbon. A thickness of less than 12 μm avoids problems, such as changing yield of X-ray and non-homogenous shrinking of corneal tissue, in the handling of corneal specimens. The neutral support is the main difference to the method of Warner and Coleman (1975) who used Al-containing supports and controlled thickness by measuring the specific signal from the support.

Following Hall and Gupta (1982) and Boekestein (1984), the time of measuring was fixed as 100 seconds "live time (LT)". Shorter measuring times decreased the yield of counts and accuracy, whilst longer times did not increase the precision (Boekestein, 1984), but caused problems due to alteration of the material. At 100 seconds LT, local energies did not exceed 5 to 10 nA /μm^2 and obvious tissue damage was avoided (Hall, 1979). Nevertheless, repeated measurements on cornea homogenate showed a constant loss of matrix, indicated by an increasing signal from the specimen holder (Cu signal in Fig. 6). We therefore, restricted the measurements to fields without previous irradiation. Changes of the probe current strongly affect the yield of counts and the net peak count (Dörge *et al.*, 1978). The probe current was recalibrated every 500 seconds, even though the local P/B method is not sensitive to this error (Boekestein, 1984). Sample thickness was set at 10 μm; corneal tissue becomes rigid after lyophilisation and flat preparations thicker than 12 μm are difficult to obtain. Furthermore, whilst the yield of counts increases with specimen thickness above 12 μm, the precision of measurement decreases (Fig. 4), because of problems with the adherence of the specimens. Increasing the accelerating voltage resulted in higher yields of counts for all analysed elements. Voltages over 13 kV excited the brass specimen holder in the calibration experiments, as indicated by the increased Cu and Zn X-ray signal (Fig. 3). At an accelerating voltage of 15 kV, the supports contributed to the continuum signal, a finding also reported by Warner and Coleman (1975).

Corneal tissue and gelatin are comparable in EDXA, unless some small differences in their physical stability and behaviour in the SEM, as shown by similar effects of measurement, repeat and cause similar local background values at the specific elemental energies (Table 2). Errors due to differences between gelatin

Table 4. Calibration lines obtained in gelatin standards. Measuring conditions: 15 kV accelerating voltage, 100 seconds live time and 350 μm^2 area. r^2 = correlation coefficient squared, A = intercept of linear function, B = slope of linear function, σ = standard deviation. n = 4 measurements under standard conditions. The correlations between concentrations of analysed elements and local peak/background values in gelatin standards are linear. The constant should be = 0 for a concentration = 0. The gelatin contains blank values of Na, S and traces of Mg, P and Cl.

Element	r^2	A	σ(A)	B	σ(B)
Na	0.96	0.36	0.116	0.535	0.05
Mg	0.95	0.11	0.12	0.69	0.09
P	0.98	0.18	0.05	1.10	0.06
S	0.96	0.93	0.21	1.18	0.11
Cl	0.99	0.16	0.10	1.13	0.05
K	0.97	0.03	0.06	1.32	0.10
Ca	0.98	0.23	0.19	1.28	0.08

Table 5. Measurements on cornea homogenate (n = 10; area = 350 μm^2; accelerating voltage = 15 kV; probe current = 2 nA). Background integral stability can be proven with a coefficient of variance of 2%.

Element	P/B	Coefficient of Variance
Na	0.87	± 11.0%
Mg	0.09	± 2.8%
P	0.29	± 3.1%
S	0.92	± 5.6%
Cl	1.69	± 4.1%
K	0.37	± 6.5%
Ca	0.19	± 3.2%

Table 6. Measurements on n = 20 healthy rabbit corneas, composition (± coefficient of variance) of the middle stroma in EDXA-measurements. n.m.c. = no measurable content.

Element	mmol/kg dry weight (cornea)
Na	436.22 ± 116.26
P	39.20 ± 19.55
S	341.80 ± 69.03
Cl	401.82 ± 79.0
K	80.29 ± 26.45
Ca	n.m.c.

standards and cornea homogenate in the excitation of the support (Figs. 6 and 7) are avoided by the neutral background of the carbon support, as wells as by controlled sample thickness; comparison between cornea homogenate and gelatine standards showed similar results for similar mineral concentrations.

For quantification of the data, blank values from cornea homogenate and concentration dependent curves of gelatin standards were obtained through repeated measurements on detectable peaks of defined concentration. Linear correlations between concentrations and P/B-ratios were found for all considered elements (Table 3). The appropriate equation (Roomans, 1980, 1988) is:

$$\text{Concentration}_{(\text{sample})} = \text{blank value} + \text{constant} * \text{P/B-ratio}_{(\text{sample})}$$

The analysed volume in our study included both sample and support (Fig. 3). Differences between the background generation at the specific elemental energies in corneal tissue and the blank graphite support were less than 6.3% for all considered elements (Table 2). Because of a stable shape of the continuum, a fast estimation with a single step bipartite spline interpolation was possible. The quantitative analysis with local P/B-ratios gave reliable and precise results.

Data on the elemental composition of the cornea have been compiled by several authors (Davson, 1948; de Azavedo and de Jorge, 1965; Otori, 1967; Midelfart, 1987, 1991). Differences between our data and chlorine and sodium values obtained by Midelfart (1991) may be partly due to species differences; Maurice and Riley (1970) and Otori (1967) found higher Na contents in hydrated rabbit cornea than in ox cornea. The chlorine values found by us and those reported by Midelfart (1991) differ by less than a single standard deviation, which may not be significant. The higher standard deviations in corneal samples can be explained by the preparation procedure. Freezing and short thawing times lead to ice crystal formation, and thereby, to electrolyte shifts, which can be recognised at the edges of epithelial layers by a nearly complete loss of the sodium and potassium gradients. These shifts were proven to be present in comparison to qualitative analysis of directly frozen un-sectioned corneae, where the potassium gradient from cellular to intercellular compartments remained high. It may be possible to avoid this effect by changes in the preparation methods. Nevertheless, the presence of a phosphorus peak sufficiently confirms cellular elements such as epithelial cells. Magnesium, potassium, and calcium showed P/B values of less than 1.5 in the corneal stroma and therefore could not be analysed quantitatively. Our finding of a low calcium concentration in the rabbit cornea is in contrast with the high calcium content of the human cornea, as reported by de Azavedo and de Jorge (1965). However, it is in line with the calcium levels found in corneas of pig, calf, ox and rabbit (Maurice and Riley, 1970). There are, as yet, no published data on the total sulphur content of the cornea, but many proteins and glycosaminoglycans such as chondroitin-sulfate are known as carriers of sulphates in the cornea.

Conclusion

This study shows that quantitative EDX-analysis of lyophilized corneas with a histological standard section size of 10 μm mounted on neutral carbon holders can be performed in the SEM. The preparation and handling of the specimen is easy. A new program which computes local P/B ratios for elements in a fast single step estimation of the continuum is used. The whole specimen can be analysed and viewed as a histological section, and the analysis is not limited to the region of a drilled hole (Dörge *et al.*, 1978; Wroblewski *et al.*, 1983). Direct comparison with histological samples of similar location and presentation is possible.

Acknowledgements

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (AZ: Re 152/27-1). The authors wish to thank the laboratory teams of the Department of Ophthalmology and the "Gemeinschaftslabor für Elektronenmikroskopie" at the RWTH-Aachen for discussion and technical support.

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Discussion with Reviewers

G.M. Roomans: You state that the absolute Cu values differed for 10 μm slices of gelatin and cornea homogenate because the higher salt content of the homogenate causes a higher background. However, according to Table 3, the background intensity for gelatin is, if anything, slightly higher than that of the cornea homogenate, although the measurements seem to have been carried out for the same (live) time and with the same beam current.

Authors: To explain this difference two things have to be mentioned. In Figures 6 and 7, the copper Lα line and not the local background at a specific line was analysed. This Cu line is of low energy and more susceptible for secondary effects as fluorescence and backscattered electrons. The concentrations of chlorine and sulfur were similar in both specimens, but potassium was added in a higher amount to the gelatine sample. Concentration differences and the described effects might cause the absolute difference in the copper line.

There is no contradiction to the statement in Table 3 because on the neutral carbon support these background generation differences did not occur for low and high concentration of added salts in the gelatin standard. To exclude a background generation difference on cornea for different salt concentrations, we carried out an experiment with cornea homogenate prepared with distilled water as described above and with added amounts of salt like described for gelatin standards. In this experiment, the background generation was the same for both samples and lies within the range of Table 3.

A.T. Marshall: In preparing the corneal homogenate, the lyophilized cornea is ground up, and suspended in water and frozen. Might this procedure result in some loss of Na, K and Cl?

Authors: We examined resuspended corneal homogenate by means of chemical analysis and EDXA, so that the error must be the same for both methods.

A.T. Marshall: Why not dialyse the gelatine and avoid the necessity of having a "blank"?

Authors: From tests with agarose purum, we knew that no sulfur contamination occurs, therefore, we were sure even with a "blank" to have a simply concentration dependent correlation.

A.T. Marshall: Please explain why is it necessary to inject methocel, etc., when removing the cornea?

Authors: Trephination of the cornea needs a well configured eye even when a first brake with outflow of aqueous humour occurs. With a gel substance as stabilizer the trephination is easier and less traumatic for the cornea.

A.T. Marshall: Do you think that the continued increase in copper signal in sections of corneal homogenate could be due to collapse of the section (thinning) without continued mass loss? The same phenomenon could happen with gelatine sections. This interpretation could be consistent with the observed low precision with net peaks.

Authors: We think that this is the explanation for the Cu line increase. Therefore, we use only measurements without previous irradiation of the sample.