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Monte Carlo simulation for an assessment of standard validity and quantitative X-ray microanalysis in plants

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Abstract. Protein matrices with known concentration of elements are used as standards in X-ray microanalysis of biological materials. The aim of the study was to assess if protein standards (gelatine) could be applied for quantitative X-ray microanalysis of carbohydrate material (cellulose) of plant cell walls by means of Monte Carlo simulations. Plant roots grown in 133Cs contaminated soil and gelatine with known elemental concentration were prepared according to freeze-drying protocols. The atomic fractions of gelatine and cellulose were used to simulate spectra and then compared to experimental ones. There was a good agreement in bremsstrahlung generation between gelatine and cellulose matrices. The experimental and simulated spectra of gelatine revealed a difference in background shape at low energies but corrected if specimen was tilted to -10° or the matrix density increased to 0.95 g/cm³. Minimum detection limit (MDL) simulated for 133Cs La in cellulose was also in agreement with that experimentally calculated for gelatine standard. Protein standard resembles carbohydrate materials and can be used for quantitative analysis of plant cell walls. However, using Monte Carlo simulations to predict analytical conditions for biological materials and elemental MDL, differences in topography and/or gelatine densities should be taken into consideration.

1. Introduction

Biological specimens, especially bulk soft tissues do not meet the requirements of quantitative X-ray microanalysis as provided for bulk geological and/or metallurgical specimens. ZAF and PhiRoZet correction methods to calculate elemental concentrations in unknown specimens in relation to a standard require highly polished, homogeneous and compact structure of materials [1]. Biological specimens could also be polished using glass or diamond knives of ultra-microtomes but tissues have to be embedded in resins. Unfortunately, quantitative analysis of chemically processed materials is limited to elements that tightly bind to the tissue organic matrix. Better results are obtained for frozen tissues [2]. Polishing of material surface in frozen-hydrated state is also possible but the measurements and calculations of their elemental composition are difficult and require cryo-equipment [3, 4]. Thus, many of soft biological materials are prepared using freeze-drying.

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techniques [5] and the fully quantitative analysis of dried tissues is based on appropriate standard and quantification methods.

Biological standards must resemble the investigated material in matrix composition and density, and should be prepared and measured in the same manner as unknown material [5, 6]. Soft organic materials like animal tissues (liver, spleen, kidney) are often compared to protein standards. The investigator dissolves element salts of interest in a gelatine or albumin matrix. Both proteins adequately simulate tissues and correction procedures for matrix differences in quantitative analysis are not crucial [7, 8]. Moreover, quantification of elements present in tissues or cells rely generally on the peak-to-background method [1] that minimizes effects of specimen heterogeneity and topography. However, such estimation cannot be valid since differences between ionisation and continuous radiation cross sections exist. There is also discrepancy in absorption of characteristic and continuous X-rays coming from a specimen to the detector [9].

The Monte Carlo simulation relays on the numerical generation of random histories of electron interactions with matter that can be averaged if the number of histories is very large. The Monte Carlo approach might be helpful to calculate ionisation-depth distribution function (ϕ(ρz)) [10], layer thickness on a substrate [11], particle inclusions [12], secondary fluorescence [13], backscattered electrons for contrast [14], and resolution enhancement [15], as well as to establish the influence of topography [9] and conductivity of specimen [16] on results of quantitative analysis of elements. Some simulation packages enable to calculate full spectra of specimens under investigation [17, 18]. Unfortunately, there is little data about the application of the Monte Carlo approach to determine experimental conditions for tissues or cells or their properties.

The aim of the present study is to apply Monte Carlo simulations to test the hypothesis that protein standards based on gelatine are useful for quantitative analysis of cellulose material (carbohydrate), the main component of plant cell walls. It also tests the applicability of simulations in biological X-ray microanalysis. Additionally, the work focuses on the influence of plant material topography on generation and emission of X-ray intensities, since plant tissue is significantly different in geometry from a gelatine specimen. The experimental model is root vascular tissue of *Medicago truncatula* treated with $^{133}$Cs. An estimation of minimum detection limit for caesium is provided by means of Monte Carlo simulation and compared to that experimentally determined.

2. Material and methods

2.1. Gelatine standard preparation

Three groups of standards based on gelatine matrix were prepared following the procedure described previously [6]. The first group of standards contained 20 % of pure gelatine dissolved in deionized water to establish impurities of commercially available protein (Sigma-Aldrich Chemie GmbH). The second group of standards contained the same amount of gelatine but dissolved in water solution of elements usually found in biological soft tissues – Na, Mg, S, Cl and K. The third group contained biological elements as mentioned above and $^{133}$Cs to establish the minimum detection limit for Cs Lα. All groups of gelatine, thoroughly dissolved and mixed at 60 °C were cooled in a refrigerator to solidify. Then, small pieces of gelatine were cut out with a razor blade and quickly plunge frozen in isopentane (Fluka ChemiCAD) precooled to -145 °C with liquid nitrogen. Frozen standards were cut in cryomicrotome into 30 µm sections at -30 °C (Shandon OT, Astmoor) and placed into precooled adhesive graphite laminas (Agar Scientific). Finally, they were dried in Edwards lyophilizer (ETD4, Edwards High Vacuum) overnight at -30 °C and 10⁻² Torr. The temperature inside the lyophilizer chamber was equilibrated to room temperature and the samples were kept in low pressure conditions for 3 h. Dried specimens were coated with 15 nm layer of carbon to prevent charging (JEE 4B, Jeol Ltd.). The main weight fraction of pure gelatine matrix taken for Monte Carlo simulations was estimated previously using RBS measurements [19] – H (8.67 %), C (53.43 %), and O (27.43 %) with remaining parts for N and S. The final fraction of gelatine matrix and elements dissolved was the following: H (6.90 %), C (42.80 %), N (7.50 %), O (28.80 %), Na (2.10 %), Mg (1.10 %), S (2.86 %),
Cl (3.80 %), and K (4.10 %). The standard with $^{133}$Cs however, contained H (7.40 %), C (45.80 %), N (6.80 %), O (28.00 %), Na (1.20 %), Mg (0.60 %), S (3.70 %), Cl (1.90 %), K (2.20 %) and Cs (2.60 %). For a comparison to gelatine, the weight fraction of pure cellulose matrix was established on the basis of its formula $(C_6H_{10}O_5)_n$ - H (6.08 %), C (44.68 %), and O (49.25 %).

2.2. Plant material preparation

Medicago truncatula seedlings were cultivated in soil contaminated with 400 µg g$^{-1}$ of $^{133}$Cs for 3 months. Roots were harvested, washed in distilled water to remove soil grains and gently dried with filter paper. Young parts of roots were dissected and cut into pieces of about 5 mm long. Then, root samples were pooled (10 - 15 pieces of roots), immersed in a OCT compound (Sakura Finetek Europe) and quickly plunge frozen in isopentane precooled to -145 °C with liquid nitrogen. Further steps of plant roots processing were the same as described above for gelatine standards.

2.3. Analytical conditions

The specimens were analysed using a Jeol JSM 5410 scanning electron microscope equipped with Noran energy-dispersive X-ray spectrometer (Noran Instruments Inc.) with 30 mm$^2$ Si(Li) crystal covered with Norvar ultra-thin window. Elemental analyses were performed at plant cell walls of vascular tissues in point mode, whereas gelatine standard in raster mode to minimize the influence of surface topography. Analytical conditions chosen for both specimens were the following: 10 kV of accelerating voltage, take-off angle 25°, 30 mm of detector working distance, 0.033 sr solid angle, tilt angle 0°, and 350 pA of a probe current. The final count rate of the detection system did not exceeded 2000 cts/s and measurements were performed during 100 s of live time.

2.4. Minimum detection limit for Cs L$\alpha$

The minimum detection limit for Cs L$\alpha$ line was calculated on the basis of gelatine standard with known concentration of caesium (see above). The equation proposed by Goldstein and Yakowitz [20] was used.

2.5. Monte Carlo simulations

The Win X-ray software for Monte Carlo simulations of electron scattering applied in this study predicts X-ray spectra and was extensively described by Gavin et al. [17]. Mott tabulated cross-sections [21] were chosen for the calculation of elastic scattering of electrons. The electron energy loss was based on continuous slowing down [22] whereas effective ionisation cross-section for characteristic photons was calculated with Casnati et al. [23]. Mass absorption coefficients proposed by Thinh and Leroux [24] were introduced into the simulation software to assess the absorption of X-rays passing through the specimens. Additionally, the absorption effect of a contamination layer on the surface of the detector as well as the adjustment factors for background, K and L X-ray intensities were determined after comparison of simulated spectra to experimental ones after the analysis of kaersutite standard (SPI Supplies).

All simulation conditions for the Win X-ray software were compatible with parameters characteristic for the microscope and the detector used. The composition of examined materials (see above) incorporated into the software revealed their putative densities: pure gelatine – 0.70 g/cm$^3$, pure cellulose – 0.84 g/cm$^3$ and gelatine with the biological elements – 0.77 g/cm$^3$, and gelatine with caesium – 0.80 g/cm$^3$. Finally, the spectra of specimens related to all matrices mentioned above were simulated for 10,000 electron trajectories and compared to spectra generated during analyses of plant cell walls and gelatine standards.

3. Results and discussion

Cell walls of higher plants are composed of a mixture of polysaccharides, mainly consisting of cellulose. As a carbohydrate polymer, cellulose can be summarized as $(C_6H_{10}O_5)_n$ what means that the weight fraction of each element of the compound is the following: H (6.08 %), C (44.68 %), and
O (49.25%). A density of cellulose material calculated on the basis of that composition is 0.84 g cm$^{-3}$. Gelatine is a mixture of many unknown proteins with H, C and O as a main fraction as well as N and S that are constituents of amino acids. To perform simulations of full spectra for the gelatine standard, the composition previously established by Tylko et al. [19] was taken into consideration: H (8.67%), C (53.43%), and O (27.43%) with the remaining fraction of nitrogen and sulphur. The calculated density of pure gelatine was 0.70 g cm$^{-3}$. However, commercially available gelatine also contains contaminants – Na, Mg and Cl.

The comparison of spectra obtained after simulation of cellulose and gelatine are presented in figure 1. In spite of the strong discrepancy between densities of both matrices, the spectra generated by Win X-ray software fit to each other surprisingly well. Thus, it shows that gelatine might be an appropriate standard for quantitative analysis of elements in cellulose material. However, Monte Carlo simulations of both matrices assumed that surfaces of the materials are flat and compact in structure. It is not true for both experimental specimens. Processing of gelatine solution followed by freeze-drying procedures revealed porosity and roughness of its surface (figure 2a). It is in agreement with the previous results [5]. Since the structure of freeze-dried gelatine is comparable with a structure of majority of soft tissues, cell walls of *M. truncatula* prepared in the same manner do not resemble standard at all (figure 2b).

![Figure 1. A comparison of spectra simulated for gelatine (red) and cellulose (black) matrices. The compositions of both matrices are presented above together with their densities calculated on the basis of atomic fractions. Pure gelatine contains S K$\alpha$ line due to sulphur presence in amino acids. Good agreement between bremsstrahlung intensity confirms that protein standard might be adequate for quantitative analysis of carbohydrate material.](image)

Topography of gelatine might be a source of fluctuation in X-ray intensities measured in a point mode, especially for light elements [4]. Gavin and Lifshin [9] have shown such effects using Monte Carlo simulation when the rough surface of NiAl alloy was probed over the specimen. Strong absorption of Al K$\alpha$ and Ni L$\alpha$ was evident in case of the analysis performed on the back face of the material. Regions facing the EDS detector however, revealed an increase of intensities from Al K$\alpha$ and Ni L$\alpha$ in relation to intensities simulated for flat surface. In our opinion, an average intensity in the range of low energies from all geometries will be measured when spectra are obtained in the raster...
lls are interiors of xylem cells responsible for water transport to higher parts of the plant.

Figure 2. SEM micrographs represent structures of gelatine (left) and plant material (right) prepared with freeze-drying procedure. Gelatine surface is rough and the material seems to be porous. It is a consequence of ice crystal formation during freezing and shrinkage effect due to water evaporation. Plant roots exhibit spongy-like structure of vascular tissue with cross-sectioned cell walls (CW) of different thickness. Empty spaces (X) surrounded by the walls are interiors of xylem cells responsible for water transport to higher parts of the plant.

mode of analysis instead of the point mode. Thus, the scanning area of 620 µm² over the gelatine specimen was chosen.

The spectra collected from analyses of pure gelatine as well as gelatine with known elemental composition fit well with that generated with Win X-ray software but not in the region of energies from C to Si (figure 3a). The intensities for Na and Mg are lower in experimental spectra in comparison to simulated ones. These results suggest absorption of low energy X-rays probably due to topography of the specimen even if the large scanning area was applied. To confirm that surface topography might increase X-ray absorption at low energies, we simulated spectra with a tilt angle of -10°. The experiment confirmed this hypothesis (figure 3b). In spite of specimen topography, its density could also influence X-ray generation and emission towards the detector [3]. Simulations of gelatine spectra with increasing density of the specimen revealed that gelatine of 0.95 g cm⁻³ fit experimental spectra in the same manner as was observed for spectra simulated at tilt angle of -10° (figure 3c). Further experiments should be performed to establish if density and/or topography of gelatine influence on absorption of low energy X-rays.

The raster mode of analysis was not possible for dried plant material due to the specimen topography (figure 2) and the point mode of analysis had to be performed. The thickest cell walls of plant tissues that exhibited perpendicular arrangement in relation to the electron beam were chosen for measurements. Monte Carlo simulations of electron trajectories in cellulose matrix has showed that thickness of plant cell walls examined is close to the range of lateral resolution of the electron interaction volume. In consequence, cell walls might be described as small particles rather than bulk specimen due to lower mass density for incoming electrons and preferential take-off angle for emitted X-rays. In consequence, decrease in continuum and characteristic radiation yield is observed together with the increase of low energy X-rays detection [25, 26]. Point mode measurements of the cell walls revealed, however, that the shape and the structure of the walls could be treated as bulk specimen. Simulated and experimental spectra fit well to each other (figure 4) but again, a tilt angle of -10° or density of 0.95 g cm⁻³ had to be applied to correct the intensities in Na to Si emission energy region.
Figure 3. Representative comparisons of spectra obtained after Monte Carlo simulations and EDS analysis of gelatine in SEM. Spectrum A was obtained from gelatine analysed and simulated at tilt angle of 0°. It showed discrepancies at Na to Si emission energy region (arrow) that was corrected efficiently when simulation was performed at tilt angle of -10° (B) or gelatine density increased to 0.95 g cm⁻³ (C).
Medicago truncatula was grown in soil contaminated with $^{133}$Cs to establish the plant's potential to accumulate caesium. Cell walls of the plant root were investigated since these parts of tissue component were found to sequestrate heavy metals [27]. X-ray microanalysis of plant cell walls have showed the presence of Cs Lα peak at 4.29 keV what confirmed plant ability to accumulate caesium (figure 4). However, the intensity of Cs Lα line seemed to be close to the minimum detection limit (MDL) of the method. It required calculating MDL for Cs Lα line to confirm or reject the peak appearance in the spectra. Thus, gelatine standard with known concentration of caesium was measured and MDL calculated on the basis of Goldstein and Yakowitz [20] equation at 0.20 wt%. Monte Carlo simulation of gelatine has showed comparable result if MDL calculated from that equation was 0.13 wt% of Cs.

Concluding, the preliminary experiments presented above have showed that Monte Carlo simulations could be applied to predict X-ray generation and emission in biological materials. Additionally, a choice of standard for fully quantitative analysis might be validated on the basis of simulations as revealed when the gelatine matrix was compared to a cellulose specimen. It also helped to establish minimum detection limits for trace elements in biological matrices. Differences between simulated and experimental spectra of gelatine were found in the region of low X-ray energies. It suggests that the topography of standard and/or discrepancies between calculated and real gelatine densities are sources of differences.

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