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BEAM SENSITIVITY OF GLOBOID CRYSTALS WITHIN SEED PROTEIN BODIES AND COMMERCIAL PREPARED PHYTATES DURING X-RAY MICROANALYSIS

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Abstract

Magnesium, potassium, calcium and phosphorus are stored in seed tissues in spherical particles called globoid crystals. The main component of globoid crystals is phytin, a salt of myo-inositol hexaphosphoric acid. Based on chemical similarity, commercially available phytates may be suitable standards for quantitative energy dispersive X-ray (EDX) microanalysis of globoid crystals. The stability of globoid crystals and commercial phytates was different when analyzed under identical conditions. Phytates in globoid crystals from Cucurbita maxima cotyledons, were relatively stable in the electron beam during EDX microanalysis at room temperature, but there was a loss of potassium. No loss of potassium occurred during analysis at low temperature, even with repeated analyses on the same spot. Commercial phytates showed considerable beam damage in the form of raised mounds at the sites of analyses. The extent of the damage was much reduced with analysis at low temperature. Although there was some variation in the peak-to-background ratios of potassium and sodium with various analytical conditions, there was no differential loss of potassium or sodium as occurred with EDX analysis of globoid crystals. Reasons for differences between in situ and isolated phytates are unclear. Provided analyses are carried out at low temperature, commercial phytates have potential as standards but further research is required to determine how to control moisture content during sample preparation and analysis.

Key Words: Seeds, globoid crystals, phytates, energy-dispersive X-ray microanalysis, cryogenic preparation, low temperature analysis, beam damage, peak-to-background ratios.

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Introduction

Seed tissues generally have water contents of less than 10% while most vegetative plant tissues have water contents of 80% or higher. While all plant tissues contain some P, K, Mg and Ca, seed tissues have these elements concentrated into discrete, spherical particles called globoid crystals (Lott, 1980). Thus energy-dispersive X-ray (EDX) microanalysis of dry seed tissues presents a different set of problems than analysis of other fully hydrated plant or animal components.

The EDX analysis of frozen, hydrated plant tissues commonly produces low count rates and the specimens are susceptible to beam damage. Such tissues are often analyzed by EDX analysis at low beam currents and large raster sizes of 1 µm² or more (Echlin et al., 1982; Marshall, 1987). Dry seed tissues vary in their sensitivity to the electron beam and generally those with high oil content, such as the seeds of Cucurbita with 35 to 50% oil, cannot be analyzed without fixation, defatting or freezing. The globoid crystals, which are rich in mineral nutrients, are comparatively stable in the electron beam and produce high count rates compared to the other constituents of seed tissues. Most Cucurbita globoid crystals are one micrometer or less in diameter so that fairly small raster sizes must be used to analyze individual globoid crystals.

In rapidly frozen tissues, the elements are retained in situ but when frozen bulk samples are fractured the surfaces tend to be irregular. Accurate quantitative EDX analysis of samples with uneven topography is difficult. An additional problem with globoid crystal analysis arises from the fact that the globoid crystals vary in the extent to which they protrude from the surface of the fracture and therefore in their accessibility for analysis. Because of these problems we have, to date, made only semi-quantitative comparisons of element concentrations. As an alternate procedure, Cucurbita seeds can be defatted leaving wall material and protein bodies which are more tolerant of exposure to the electron beam. Globoid crystals within protein bodies of ground, defatted seed tissues can be spread evenly on tape attached to a stub for analysis at both room temperature and at low temperature. This type of preparation is more suitable for
quantitative analysis with appropriate standards.

Globoid crystals consist mainly of phytin which is a K, Mg and Ca salt of myo-inositol hexaphosphoric acid (Lott, 1984). How the phytic acid salts are packaged into the spherical particles remains unknown but, based on chemical composition, all commercially available phytates should be appropriate standards for quantitative analysis of globoid crystals. In this study, globoid crystals and commercial phytates of known composition have been analyzed at various conditions to determine their stability during EDX analysis. This investigation was required before commercial phytates could be routinely used for quantitative microanalysis.

Materials and Methods

Source of Seeds and Phytates

Seeds of Cucurbita maxima cv. Warted Hubbard were obtained from fruits grown in the field at the Royal Botanical Gardens in Hamilton, Ontario. The plants were grown from seeds acquired from Stokes Seeds Ltd., St. Catharines, Ontario. The following commercial phytates were purchased from Sigma Chemical Co.: sodium phytate (12 Na), dimagnesium tetrapotassium phytate, potassium phytate (mono-K), and calcium phytate (2 Ca).

Preparation of Seeds and Phytates for X-ray Microanalysis

Seed coats were removed to obtain embryos. One millimetre square blocks of central cotyledon tissue were mounted in Tissue Tek in wells of an EMSCOPE SP2000 specimen holder and were frozen in melting nitrogen. The specimens were then fractured and chromium coated by evaporation in the EMSCOPE cryo-preparation chamber (stage and knife) cooled to about -182°C. The specimens were then fractured and chromium coated by evaporation in the EMSCOPE cryo-preparation chamber (stage and knife) cooled to about -182°C. The samples were transferred under vacuum into the microscope chamber.

Hexane, a solvent used by Bair and Snyder (1980) to extract crude lipids from soybeans, was used to defat the cotyledons of 25-30 embryos per batch of seeds. The pooled cotyledons were extracted three times for about 24 hours for each extraction. The defatted cotyledons were air-dried and then ground with a pestle and mortar. Cotyledon fragments were brushed onto double-sided tape on an EMSCOPE SP2000 specimen holder. To reduce charging, the pieces of tape were cut only two to three millimetres in diameter, and the perimeter of the tape was secured to the metal of the holder by a border of glue mixed with colloidal graphite. The samples were carbon coated at room temperature in an Edwards Evaporator or in the cold in the EMSCOPE preparation chamber. Some samples were coated with chromium in the EMSCOPE chamber either at room temperature or at about -182°C.

The phytates were analyzed either as provided by Sigma or after drying at 105°C for 2 hours. The phytates were brushed onto double-sided tape on the EMSCOPE specimen holders and coated as described above for defatted cotyledon tissue.

X-ray Microanalysis

Samples were analyzed in an ISI DS-130 scanning electron microscope (SEM) using a PGT System 4 X-ray Analyzer (Princeton Gamma Tech, Princeton, NJ). The fresh cotyledon samples were analyzed at 15 kV while the defatted and ground cotyledons and commercial phytates were analyzed at 12 kV. The probe current was set to 0.65 nA using a Faraday cup and checked periodically during the analysis. Detector distance, working distance and tilt angle were set to give a take-off angle of 34°. The collection time was 60 seconds for all analyses. The dead time was between 5 and 15%. The defatted tissue and commercial phytate samples were analyzed at about 25°C (298K), left within the microscope while the cryo-stage was cooled with liquid nitrogen, and then different regions of the same samples were analyzed at -160°C (113K). In the analysis of seed tissue the beam was placed on individual globoid crystals and the analysis was performed in the raster mode. For phytate analysis relatively smooth and level areas of the particles were analyzed.

X-ray counts for the elements measured were obtained by integrating peaks at the following window widths: Na, 0.936 to 1.112 keV; Mg, 1.146 to 1.358 keV; P, 1.899 to 2.125 keV; K, 3.187 to 3.437 keV; and Ca, 3.562 to 3.818 keV. Since none of the samples contained both K and Ca no peak deconvolution was required. The background was modeled using the PGT System 4 background subtract program which joined points on either side of each peak. The points were selected to produce an adequate fit of the background.

Both the globoid crystals and commercial phytates were analyzed at several raster sizes. Rasters of 0.05 μm² and 0.07μm² were considered small rasters and were in the range used in the analysis of globoid crystals within frozen, fractured cotyledons. Rasters of 0.4 μm² were used to reduce the electron dose and were the largest suitable size for analysis of individual globoid crystals. Sodium phytate was also analyzed with a very large raster of 65 μm² and with a raster of 0.07 μm² which was set to slow scan instead of the usual fast scan rate. To test the effectiveness of cooling on the stability of the specimens in the electron beam, two consecutive analyses were made on the same spot on both globoid crystals and commercial phytates. Peak-to-background ratios, defined as counts above the background divided by background counts, were calculated for all elements measured. Ratios of the elements to phosphorus were calculated from the peak-to-background ratios. The ending of the continuum was monitored to ensure artifacts did not arise from excessive charging. Statistical comparisons were made using Minitab Statistical Software.

Results

Figure 1 shows a fracture through a mesophyll cell from the centre of a frozen, fractured and chromium-coated squash cotyledon. This type of cell generally contained less than 6% moisture (Ockenden and Lott,
X-ray Microanalysis of Globoid Crystals and Phytates

Table 1. EDX analysis of globoid crystals from defatted cotyledons.

<table>
<thead>
<tr>
<th>Temp. of Analysis</th>
<th>Peak-to-background (P/B) ratios</th>
<th>Mg P/B</th>
<th>P P/B</th>
<th>K P/B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>3.86 ± 0.96*</td>
<td>0.33 ± 0.05</td>
<td>0.19 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>-160°C</td>
<td>3.59 ± 0.88</td>
<td>0.33 ± 0.04</td>
<td>0.37 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD (SD = Standard Deviation); N = 66.

Figures 3 and 4. Isolated protein body from defatted cotyledons carbon coated and viewed at 12 kV at room temperature. Globoid crystal is found at the narrow end. Figure 4 shows the site of EDX analysis (arrow) at room temperature with a 0.05 µm² raster. Bar = 1 µm.

1988a) and consisted of protein bodies surrounded by lipid vesicles. Within the protein bodies are the spherical globoid crystals which can be easily identified for EDX analysis. A typical EDX spectrum from a globoid crystal in frozen and fractured cotyledon tissue had peaks of Mg, P and K but little to no Ca (Fig. 2). Globoid crystals were stable during analyses at low temperature. Analyses of globoid crystals within frozen bulk samples routinely produced higher peak-to-background ratios for K than for Mg for most cotyledons analyzed. No loss of K was detected during analyses even when two consecutive analyses were carried out on the same spot.

A protein body isolated from a defatted, ground cotyledon is shown before and after EDX analysis of the globoid crystal at room temperature (Figs. 3 and 4). The beam damage was localized and there was no raised mound or obvious damage to the globoid crystal. Even less damage was visible if the samples were cooled to -160°C prior to analysis. The globoid crystals analyzed at room temperature had markedly lower K peak-to-background ratios than those analyzed at low temperature (Table 1). The peak-to-background ratios for Mg and P were slightly lower with analysis in the cold than with analysis at room temperature and this was subsequently found to be the case for most analyses in the cold. However, the ratios of Mg P/B to P P/B were the same for both conditions. The peak-to-background ratio for K was 43% lower with room temperature analyses.
than with analyses in the cold. This was also reflected in the lowered ratio of K to P. The above analyses were carried out on samples coated with carbon at room temperature, but the results were essentially the same for samples carbon coated at low temperature and for chromium coated samples. Thus it appeared that the loss of K from globoid crystals occurred mainly as a result of X-ray analysis and was not due to the coating procedure. All subsequent analyses of the globoid crystals were carried out on carbon coated specimens.

Table 2 shows the results of analyzing another batch of globoid crystals from defatted cotyledons at two raster sizes and with two consecutive analyses made on the same spot on the globoid crystal. The peak-to-background ratios for Mg and P were again lower with analyses at low temperature than with analyses at room temperature. However, there was no significant difference between the ratio of Mg P/B to P P/B at the two temperatures. The smaller raster size of 0.05 \( \mu m^2 \) produced higher P/B ratios for both Mg and P than the larger raster but in a number of cases the differences were not significant. Repeat analyses on the same spot gave slightly higher P/B ratios for the second analyses for Mg and P but the differences were generally not significant. In all cases the differences were not present when the P/B ratios for Mg and P were expressed as a ratio to each other, indicating that the changes were similar for the two elements.

In contrast to Mg and P, the K P/B were markedly lower with all analyses at room temperature than for all analyses at low temperature. The decrease in K was similar at the two raster sizes used; the K P/B ratio was more than 30\% lower than the values for low temperature analysis. Repeat analysis on the same spot at room temperature further reduced the K P/B ratio to less than 50\% of the low temperature values. Potassium P/B ratios were not significantly different at all conditions of analysis in the cold. Low ratios of K P/B to P P/B for room temperature analyses confirmed that K was lost differentially from the area of analysis.

Analysis of commercial phytate particles is shown in Figs. 5 to 16. The specimens illustrated in these micrographs were all carbon coated at room temperature but the damage was similar for chromium coated samples. All micrographs of Na phytate are grouped together (Figs. 5 to 10) for ease of comparison of the site of analysis at different conditions. Commercial Na phytate particles, shown prior to analysis in Fig. 5, were very variable in shape and size. Figure 6 shows the same Na phytate particles as shown in Fig. 5 after EDX analysis at room temperature using a 0.07 \( \mu m^2 \) raster. Beam-damage mounds had formed at the sites of analyses. The influence of temperature upon beam damage can be seen by comparing the small size of the beam-damage mounds from analyses at low temperature (Fig. 7) with the much larger mounds found after room temperature analyses (Fig. 6). The moisture contents of the commercial phytates ranged from about 3\% for K phytate to 13\% for Na phytate. When dried phytates were analyzed at room temperature they were less sensitive to the electron beam than the phytates analyzed without drying. The reduction of the beam damage to the site of analysis is illustrated for Na phytate (compare Fig. 6 with Fig. 8). The damage mound on the left in Fig. 9 was formed during a single EDX analysis at room temperature on Na phytate (not dried) while the slightly larger mound on the right was formed after two consecutive analyses were made on the same spot. Increasing the raster size from 0.07 \( \mu m^2 \) to 0.5 \( \mu m^2 \) to reduce the electron dose did not reduce the apparent damage. For Na phytate (Fig. 10), the beam-damage mound from the use of a larger raster was bigger than the beam-damage mound after analysis at the same spot on the Na phytate (Fig. 10) at the smaller raster size. When a very much larger raster size (65 \( \mu m^2 \)) was used on Na phytate (Fig. 11), a shallow rectangular mound outlined the area of analysis.

Some examples of beam damage to particles of the other three phytates analyzed are shown in Figs. 11 to 16. Ca phytate was particularly sensitive to the beam at room temperature (Fig. 11) and less sensitive to analysis at low temperature (Fig. 12). Similar reduction in beam-damage mounds with lower temperature can be seen for dimagnesium tetrapotassium phytate (compare Fig. 13 with Fig. 14). Figure 15, and the enlarged view in Fig. 16, show K phytate analyzed at room temperature under various conditions. Repeat analysis on the same spot on K phytate produced a larger damaged area than the single analysis. Repeat analyses at low temperature (not shown) did not noticeably increase the damaged area. With K phytate, there was little difference in the damage at the different raster sizes and even the largest raster produced damage as severe as that produced during analysis with a smaller raster. Figure 16 also shows the very small mound formed when the particle was cooled and the analysis was carried out at low temperature.

As was found with globoid crystals, P/B ratios tended to be slightly lower for low temperature analyses of commercial phytates (not dried) than for room temperature analyses (Table 3). However, when the ratios of K, Mg and Ca to P were compared there was no significant difference between the analyses at the two temperatures. This was not the case for Na phytate. The Na P/B from room temperature analyses was 25\% higher than the Na P/B from analyses at low temperature while the P P/B was only about 3\% higher. The net effect was a higher Na/P ratio for the room temperature analyses than for analyses at low temperature. Dried Na phytate did not produce anomalously high Na P/B ratios with room temperature EDX analysis. The P/B ratios for Na and P for the dried Na phytate were similar for both room and low temperature analysis. Although drying should have produced slightly higher P/B ratios for Na phytate, these ratios were similar to the P/B ratios obtained for Na phytate (not dried) analyzed in the cold.

A different batch of Na phytate particles analyzed at room temperature (Table 4) did not produce the anomalously high Na P/B ratios, but analyses of K phytate
X-ray Microanalysis of Globoid Crystals and Phytates

Table 2. Analysis of globoid crystals in defatted cotyledon tissue at room temperature and at low temperature using small and large raster sizes and duplicate analyses on the same spot. In each row, values (mean ± SD; SD = Standard Deviation) followed by the same letter name are not significantly different at the 95% significance level. N = 20.

<table>
<thead>
<tr>
<th></th>
<th>Analysis at 28°C</th>
<th>Analysis at -160°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raster 0.05 µm²</td>
<td>Raster 0.4 µm²</td>
</tr>
<tr>
<td></td>
<td>Anal. 1</td>
<td>Anal. 2</td>
</tr>
<tr>
<td>Mg/P</td>
<td>3.62ac ± 0.59</td>
<td>3.82a ± 0.61</td>
</tr>
<tr>
<td>K/P</td>
<td>2.02a ± 0.74</td>
<td>1.27b ± 0.40</td>
</tr>
<tr>
<td>P/P</td>
<td>11.31a ± 1.43</td>
<td>12.41 ± 1.58</td>
</tr>
<tr>
<td>Mg/P</td>
<td>0.32a ± 0.04</td>
<td>0.31a ± 0.05</td>
</tr>
<tr>
<td>K/P</td>
<td>0.19a ± 0.05</td>
<td>0.10 ± 0.03</td>
</tr>
</tbody>
</table>

Table 3. Peak-to-background (P/B) ratios for commercial phytates analyzed at room temperature and in the cold using a small raster (0.07 µm²).

<table>
<thead>
<tr>
<th></th>
<th>25°C</th>
<th>-160°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na Phytate</td>
<td>N = 52</td>
<td>N = 52</td>
</tr>
<tr>
<td>Na</td>
<td>13.61 ± 1.08*</td>
<td>10.94 ± 0.81</td>
</tr>
<tr>
<td>Mg</td>
<td>2.64 ± 0.17</td>
<td>2.63 ± 0.20</td>
</tr>
<tr>
<td>K</td>
<td>7.34 ± 0.62</td>
<td>6.78 ± 0.54</td>
</tr>
<tr>
<td>Ca</td>
<td>16.24 ± 0.90</td>
<td>15.81 ± 0.80</td>
</tr>
<tr>
<td>P/B</td>
<td>0.84 ± 0.07</td>
<td>0.70 ± 0.07</td>
</tr>
<tr>
<td>Ca Phytate</td>
<td>N = 26</td>
<td>N = 60</td>
</tr>
<tr>
<td>Na</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mg</td>
<td>2.02 ± 0.01</td>
<td>2.01 ± 0.01</td>
</tr>
<tr>
<td>K</td>
<td>0.56 ± 0.07</td>
<td>0.54 ± 0.06</td>
</tr>
<tr>
<td>Ca</td>
<td>0.41 ± 0.03</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>K/P</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.02</td>
</tr>
</tbody>
</table>

Table 4. Peak-to-background (P/B) ratios for single analyses (Anal. 1) and two consecutive analyses on the same spot (Anal. 2) of Na and K phytates analyzed with a raster of 0.07 µm².

<table>
<thead>
<tr>
<th></th>
<th>25°C</th>
<th>-160°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na Phytate</td>
<td>N = 26</td>
<td>N = 26</td>
</tr>
<tr>
<td>Na</td>
<td>9.67 ± 0.83*</td>
<td>11.53 ± 2.10</td>
</tr>
<tr>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>16.20 ± 0.93</td>
<td>15.84 ± 1.22</td>
</tr>
<tr>
<td>P/B</td>
<td>0.60 ± 0.07</td>
<td>0.74 ± 0.19</td>
</tr>
<tr>
<td>K Phytate</td>
<td>N = 26</td>
<td>N = 26</td>
</tr>
<tr>
<td>Na</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td>4.45 ± 0.41</td>
<td>5.01 ± 0.49</td>
</tr>
<tr>
<td>P</td>
<td>21.64 ± 1.46</td>
<td>23.71 ± 2.14</td>
</tr>
<tr>
<td>P/B</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.02</td>
</tr>
</tbody>
</table>

*Mean ± SD (SD = Standard Deviation)
Figure 5. Particles of carbon coated commercial sodium phytate before EDX analysis.

Figure 6. Na phytate particles after three EDX analyses showing mounds formed at the sites of analyses (arrows). Each analysis was carried out at room temperature with a 0.07 $\mu m^2$ raster at 12 kV for 60 seconds.

Figure 7. Na phytate particle with mounds (arrows) formed during analysis with a 0.07 $\mu m^2$ raster at low temperature.

Figure 8. Dried Na phytate with mounds formed during analysis with a 0.07 $\mu m^2$ raster at room temperature.

Figure 9. Na phytate particle with a mound on left (small arrow) produced by a single room temperature analysis with a 0.07 $\mu m^2$ raster and mound on right (large arrow) produced after a second analysis on same spot.

Figure 10. Na phytate analyzed at room temperature using different raster sizes of 0.07 $\mu m^2$ (A), 0.5 $\mu m^2$ (B) and 65 $\mu m^2$ (C). Damaged area for C has been indicated by white lines.

Bars = 10 $\mu m$ (Figs. 5-8); 5 $\mu m$ (Figs. 9-10).
Figure 11. Ca phytate particles, carbon coated and analyzed with a 0.07 $\mu m^2$ raster at room temperature, showing mounds at sites of analyses (arrows).

Figure 12. Reduced damage mounds (arrows) on Ca phytate after analyses at low temperature with same raster size.

Figures 13-14. Dimagnesium tetrapotassium phytate particle. Figure 13: Analyzed at room temperature with a 0.07 $\mu m^2$ raster with damage mounds (arrows) at sites of analyses. Figure 14: Reduced damage (arrows) after analyses at low temperature with the same raster size as used for Fig. 13.

Figures 15-16. K phytate particle, with an enlarged view in Fig. 16. Figure 15: Top left arrow showing damage after a single EDX analysis at room temperature with a 0.07 $\mu m^2$ raster. The other three arrows show room temperature analyses with larger raster sizes as described for Fig. 16. Figure 16. After both room and low temperature analyses. Single analysis at room temperature with 0.07 $\mu m^2$ raster is shown at A. Damaged area B was produced after two consecutive analyses with 0.07 $\mu m^2$ on same spot. Raster size at C was 0.5 $\mu m^2$ and at D was 65 $\mu m^2$. Specimen was then cooled to -160°C and analyzed with a 0.07 $\mu m^2$ raster producing a very small mound (arrow).

Bars = 10 $\mu m$ (Figs 11-14), 50 $\mu m$ (Figs. 15-16).
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Table 5. Peak-to-background ratios for Na phytate analyzed at room temperature using various raster sizes and scan rates.

<table>
<thead>
<tr>
<th></th>
<th>Fast Scan</th>
<th>Slow Scan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65 µm²</td>
<td>0.5 µm²</td>
</tr>
<tr>
<td></td>
<td>N = 19</td>
<td>N = 17</td>
</tr>
<tr>
<td>Na</td>
<td>10.87 ± 0.52 *</td>
<td>8.87 ± 0.60</td>
</tr>
<tr>
<td>P</td>
<td>14.19 ± 0.88</td>
<td>15.74 ± 0.85</td>
</tr>
<tr>
<td>Na P/B</td>
<td>0.77 ± 0.05</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td>P P/B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD (SD = Standard Deviation)

produced higher K P/B ratios for room temperature analyses than for analyses at low temperature. Two consecutive analyses at room temperature on the same spots on both Na phytate and K phytate particles (Table 4) resulted in increases in the Na P/B and K P/B ratios respectively. The effects on P P/B were different in these two phytates. Duplicate analyses on the same spot on cooled K phytate produced similar P/B ratios for the two analyses. Despite the variability of Na and K with room temperature analyses of these commercial phytates, there was no evidence that either element was being lost from the site of analysis.

The ratios of elements to each other (Tables 3 and 4) can be compared to ratios calculated for the commercial phytates from available quantitative data. Based on molecular weights, Na phytate with 12 sodium atoms had a Na/P ratio of 1.48. Using a P value of 0.44 (Russ, 1972) as a rough correction of the collected counts for Na the ratio of Na/P became 1.22 instead of 0.70 (analysis at low temperature). Based on the concentrations of Mg of 4.8% and of K of 13.8% for K Mg phytate the Mg/K ratio was calculated as 0.35; this compared reasonably well to the 0.39 ratio based on peak-to-background ratios. When a P value of 0.6 was used for Mg (Russ, 1972) or correction factors for Mg and P (Van Steveninck et al., 1990) were used on the counts for these two elements, Mg was too high relative to K. Ca phytate had two ions of Ca per phytate molecule hence a ratio of Ca/P of 0.43; the ratio from EDX analysis was 0.39. Potassium phytate contained 5.5% K and 24.7% P giving a ratio of K/P of 0.22; the value from peak-to-background ratios was 0.18.

Table 5 shows the peak-to-background ratios obtained from analysis of Na phytate at room temperature at different raster sizes and scan rates. The largest raster of 65 µm² (electron dose of about 4 ke·nm⁻²) produced a higher Na P/B and Na/P ratio than the 0.5 µm² raster (approximate electron dose of 500 ke·nm⁻²) and the 0.07 µm² raster (approximate electron dose of 3500 ke·nm⁻²). The P/B ratios from analyses at the two smaller raster sizes were not significantly different from each other. There appeared to be no advantage in using a slow scan rather than a fast scan rate since the P/B ratios were the same and the visual damage was also similar.

From neutron activation analyses (NAA) of fresh cotyledon tissue of the same batch of seeds as the defatted seeds, carried out as part of a separate study (Ockenden and Lott, 1988b), it is known that the ratios of Mg/P, K/P and Mg/K are 0.42, 0.51 and 0.84 respectively. Using the peak-to-background ratios for K phytate and globoid crystals, and the concentrations of 5.5% K and 24.7% P for the commercial phytate, the concentrations of K and P in the globoid crystals were calculated as 5.4% and 12.9% respectively. These concentrations gave a K/P ratio of 0.42 which was lower than the 0.51 ratio from NAA. Given that there is 4.8% Mg and 13.8% K in the commercial K Mg phytate, the P concentration can be as low as 17% or as high as 23% depending on differences in assumptions about the stoichiometry of the phytate. Using this phytate as standard, the elemental concentrations in globoid crystals are 5.7% for Mg, 6.6% for K and from 12.6 to 17.2% for P. Ratios of Mg/P, K/P and Mg/K based on these concentrations are 0.45 or 0.33, 0.52 or 0.38 and 0.86 respectively. The ratios to P are either very close or lower than the ratios from NAA. The ratio of Mg/K is very similar to that from NAA analysis.

**Discussion**

There is considerable variation in size, structure and complexity of seed tissues and of individual globoid crystals within these tissues so that there is no single ideal approach to mineral nutrient analysis in seeds. Despite the problems with the use of quantitative EDX analysis with frozen, fractured cotyledon blocks, this method is good for analyzing relatively large globoid crystals within individual protein bodies. Fixed tissue needs to be used when the globoid crystals are very small. The advantage of using defatted tissue is the ability to analyze the tissue without fixation. This method also allows for the sampling of globoid crystals from a batch of many pooled seeds to derive a representative elemental distribution. From our earlier studies with NAA analyses of defatted and undefatted embryo tissues (Ockenden and Lott, 1988b), it was found that hexane defatting of intact embryos did not remove the elements being measured.

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When frozen and fractured cotyledon samples were used for EDX analysis, chromium coating was far superior to carbon in reducing charging. The isolated protein bodies from defatted tissue were less prone to charging than frozen bulk specimens of seed tissue, so that carbon coating gave sufficient protection. Since there is known to be some absorption of X-rays by the chromium coats (Echlin and Taylor, 1986; Marshall, 1987), carbon coating was used for most of the analyses of isolated protein bodies and commercial phytates.

Peak-to-background ratios are accepted as a means of minimizing the effects of uneven surface topography on X-ray collection (Marshall, 1980; Boekstein et al., 1984). The use of peak-to-background ratios instead of peak integrals was initially proposed for particle analysis by EDX spectroscopy (Statham and Pawley, 1978). Globoid crystals are discrete particles different from the surrounding seed tissues, hence peak-to-background ratios have been used throughout this study to present EDX analysis data.

While globoid crystals yielded much higher count rates than the surrounding matrix, it was found in earlier studies (Ockenden and Lott, 1990) that unless a certain level of counts was generated there was an excessive variation in peak-to-background ratios. Count rates of 1000 to 2000 cps generated at least 10,000 counts for P and gave reproducible results for the same samples. The minimum beam current that gave these count rates with globoid crystals was about 0.6 nA. With the necessity of using small raster sizes to target individual globoid crystals, the electron dose used was in the kiloelectron range, namely, 3500 ke\textsuperscript{-}\textsuperscript{2} for the small raster of 0.07 \textmu m\textsuperscript{2}. Echlin (1991) has stated that for EDX analysis of bulk samples electron doses of 3000 ke\textsuperscript{-}\textsuperscript{2} may be needed.

Why the peak-to-background ratios were consistently lower at low temperature than at room temperature is not known. Charging of samples may increase on cooling and this can lead to a decrease in X-ray intensity leading to a corresponding lowering in the peak-to-background ratios (Marshall, 1987). In our study there was no apparent difference in charging and the end of the continuum was the same for room temperature and low temperature analyses. Roinel (1982) found that the initial count rates for microdroplets of inorganic salts were lower at low temperature than at room temperature but then rose as the 1200 second analysis progressed.

Although there are many studies and reviews of electron beam damage on a variety of materials, none have specifically dealt with phytates. Phytic acid consists of C, H, O and P (Johnson and Tate, 1969). Beam damage to organic compounds could result in the breaking of bonds and removal of the C, O and H as volatile degradation products (Hall and Gupta, 1983). Oxygen has been reported as being very sensitive to the electron beam (Ingram and Ingram, 1980) and the phytic acid molecule has 24 oxygen atoms which make up more than 50% of molecular weight of the molecule. In addition, phytates can be hydrated with varying amounts of water; for example, sodium phytate may have from three to forty-four molecules of water associated with one molecule (Cosgrove, 1966). Reduced beam damage with dried phytates is compatible with reports on the relationship between water content and electron beam damage (Egerton, 1982; Talmon et al., 1986). The type of mound produced at the site of analysis with phytate particles is similar to that illustrated by Marshall (1982) with protein gels. The anomalously high room temperature peak-to-background ratios for Na and K in the phytates may be due to a loss of the lighter elements and a collapse of the particles in such a manner that additional Na and K were brought to the site of analysis. The cooling of the specimens slowly within the microscope seemed to give adequate protection from electron beam damage as attested to by the similarity in the repeat analyses on the same spot for both globoid crystals and commercial phytates.

The loss of K and Na during EDX analysis has been reported for a variety of inorganic materials such as glass (Varshneya et al., 1966), zeolite (Treacy and Newsam, 1987) and microdroplets of Na and K chlorides (Roinel, 1982). It is believed that K and Na may be lost as a result of volatilization; Morgan and Davies (1982) found that elements with lower boiling points were more readily lost from microdroplets during EDX analysis than those with higher boiling points. It has also been suggested that K and Na are not removed from the sample but rather migrate out of the site of analysis. Hodson and Marshall (1971) found that K migrated out of the irradiated area in collodion membranes. What mechanism is involved in the loss of K from globoid crystals is not known. Why no loss is detected with commercial phytate particles is also not known but it may be related to the collapse of the particle structure as discussed in the previous paragraph. The spherical shape of the globoid crystal could result in lateral migration of K out of the small area of measurement on top of the globoid crystal but volatilization cannot be ruled out.

This study has shown that phytate that exists in vivo within globoid crystals is more stable in the electron beam than particles of commercial phytate salts. Thus these chemically similar materials must be structurally different. In addition, commercial phytates with different cation complements showed differences in sensitivity to the beam. How phytates are packaged into the spherical particles remains unknown. The few studies made on isolated globoid crystals, mainly from cereal grains (Lasztity and Lasztity, 1990) and from legumes (Sharma and Dieckert, 1975) confirm that the main components are phytic acid salts. There is a small amount of protein and carbohydrate generally making up less than 2% of the globoid crystal but it is possible that in some cases these may be contaminants rather than genuine constituents. Lui and Altschul (1967) working with cottonseed globoid crystals suggested that part of the globoid crystals may also be made up of crystalline water. Powder X-ray diffraction on globoid crystals
from rice by Ogawa et al. (1975) showed no evidence of crystalline structure for globoid crystals. It is not known how labile the cation binding is within the structure of globoid crystals and how much it may be modified during the various procedures used in globoid crystal extraction. Cucurbita maxima globoid crystals have not been isolated and characterized chemically.

Isolated phytates also appear to be very complex and variable in their chemical structure. Although phytic acid had been studied for many years, the exact structure of the molecule remained controversial (Brown et al., 1961; Cosgrove, 1966; Johnson and Tate, 1969). The reason for the controversy appears to be related to the fact that the stereochemistry of phytate can be quite variable depending on the conditions of the investigation (Costello et al., 1976). Conformation of the phytate molecules can be modified by pH, concentration and the type of solvent and the type of cations in the complex. With these changes, phytate could also exist in an amorphous or crystalline state. Studies with various cations have shown that ions are not always bound to the phosphate groups in stoichiometric proportions (Marini et al., 1985; Martin and Evans, 1986; Thompson and Erdman, 1982). This has led to conclusions that phytate molecules may be linked to each other by bridges as suggested for ferric phytate (Thompson and Erdman, 1982) or in even larger polynuclear complexes, (Ca$_{16}$:phytate)$_n$, as postulated for calcium phytate (Marini et al., 1985). If such inter-linking of phytate molecules existed within globoid crystals, it would help to explain the greater stability of these particles in the electron beam as compared to the particles of commercial phytate salts.

The results of EDX analyses are presented in two ways, as peak-to-background ratios and ratios of the peak-to-background for a given element such as Na, K, Mg or Ca to the peak-to-background ratio of P. The ratios to phosphorus are important in the study of mineral nutrient storage in seeds because phytic acid always contains six P atoms per molecule while the associated cations vary. Comparing element ratios to each other instead of comparing absolute concentrations avoids the need to know exactly the moisture content of both the globoid crystals and the commercial phytates. The four commercial phytates analyzed here varied in the ease with which they picked up moisture and it is not known how easily the moisture content can be changed by the vacuum conditions both during coating and analysis. Zs.-Nagy and Casoli (1990) mention the loss of water, during analysis, from crystals of various salts used as standards for bulk specimens.

Van Steveninck et al. (1987, 1990) have used phytates as standards for analysis of phytate deposits in roots and have applied a number of correction factors based on their analytical system. In general, phytates are not yet in common use as standards especially for seed tissues. For Cucurbita cotyledons Mg K phytate is the most suitable standard once the phosphorus concentration is accurately determined. Sodium phytate was analyzed because it has received wide study for its chemical properties but it is not suitable as a standard for globoid crystals which contain little or no Na.

Commercial phytates are highly sensitive to the electron beam under conditions necessary for analysis of the globoid crystals in seeds. The damage is aggravated at higher moisture contents but is markedly reduced by analysis at low temperature. The globoid crystals are less sensitive to the electron beam than the commercial phytate particles but have a tendency to lose K when analyzed at room temperature. It appears from this study that with low temperature analysis, these differences between the phytates are sufficiently reduced that quantitative analysis becomes feasible. In order for EDX analysis to be used quantitatively the moisture content of globoid crystals relative to that of commercial phytates needs to be known. Until this aspect has been studied further, one can express the quantitative data as ratios of elements to each other. The quantitative results of this study have been compared to NAA data. Although NAA analysis was carried out on whole cotyledon tissue, it is believed that most of the elements measured are concentrated within the globoid crystals. Thus actual concentrations are different but ratios of elements to each other should be similar for whole cotyledon tissue and globoid crystals.

The commercial phytates we analyzed are only a few members of a large family of compounds which have the same core structure but may be associated with a variety of biologically important ions. Thus phytates have potential as EDX analysis standards and also may provide an interesting test system for the study of the interactions of the electron beam with the specimen.

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References


X-ray Microanalysis of Globoid Crystals and Phytates


Discussion with Reviewers

S. Craig: The defatted tissue was analyzed at 25°C then frozen, by cooling the cryo-stage, for analysis at low temperature. This is a very slow cooling rate.
While their data show improved retention of elements at low temperature, would the authors comment on the likely influence of rapid cooling, as is normally used for low temperature SEM, on elemental analyses?

Authors: We began this work using the traditional rapid freezing in nitrogen slush so that we have compared the fast and slow freezing procedures. The relatively low water contents of the globoid crystals and phytates obviate the need for rapid freezing.

R. Storey: Did the authors determine the amount of Na, K, P and Mg extracted by the hexane which was used to defat the cotyledons?

Authors: Comparison of NAA analysis of defatted and undefatted cotyledon tissue showed that these elements were not removed by hexane.

R. Storey: Did the authors investigate the effect of reducing beam current and extending the count time on the stability of globoid crystals and commercial phytates to electron beam damage?

Authors: We have analyzed Na phytate using 30 kV and pA currents. The obvious beam damage was much reduced with no mound formation but the count rates were very low and total counts were low even with longer collection times. The results we present are for conditions which are suitable for EDX analysis of both globoid crystals and commercial phytate particles.

R. Storey: The globoid crystal is less than 1 µm in diameter. What effect will the relatively large electron interaction volume at 12 and 15 kV have on EDX analysis of the isolated globoid crystals embedded in the protein body and the globoid crystals in situ?

Authors: The globoid crystals are much more electron dense than the surrounding protein or oil. When we analyze the protein or oil we obtain a very low count rate whereas we obtain a very high count rate from the analysis of globoid crystals. As a result of this we believe that a very high percent of the counts from analysis of a globoid crystal comes from the globoid crystal itself and not from surrounding materials. This is particularly true since only the protruding, easily identifiable globoid crystals are analyzed in situ.

J.S. Greenwood: You have briefly alluded to the use of P values, originally designed for use in "standardless" quantitative analyses (Russ, 1972), as correction factors. Use of the P value for Na brought the Na/P ratio of Na phytate more in line with the lot chemical analysis supplied by the manufacturer. Yet for the other elements investigated, straight ratios of the peak-to-background values seemed to approximate the actual chemical composition. Would using the P values for these other elements affect this approximation? Should P values be used for all estimations?

Authors: The Russ (1972) correction factors were calculated for thin sections viewed in TEM rather than a bulk sample viewed with SEM as used in our study.

When the P value for Mg is applied to our values we get a higher Mg/K ratio than expected from the concentrations given by the manufacturer. When the quantitative results are compared for values with and without the P corrections, the percentages and the ratios of minerals to each other are very similar.

H.T. Horner: Do you think your results would be similar for other inorganic crystals such as calcium carbonate, calcium oxalate and the various calcium phosphates?

Authors: Although we have not done a systematic survey of beam sensitivity of various salts other than the phytates, we have analyzed some phosphates and some minerals such as calcite. None of these salts were as sensitive as the phytates which contain the organic component in form of a sugar alcohol. Previous analyses of calcium oxalate crystals in various seed tissues also showed that these compounds were less sensitive to the electron beam than commercial phytates (see e.g., Spitzer E, Lott JNA, Protein bodies in umbelliferous seeds. II. Elemental composition, Can. J. Bot. 60, 1392-1398, 1982).

H.T. Horner: Movement of elements in the crystalline matrix due to electron beam exposure is an interesting possibility. If this occurs, do you think that the elements are permanently displaced?

Authors: In case of the phytate particles the mound formation suggests destruction of the matrix, hence little chance of movement of the displaced element back to its original site. In case of the globoid crystal, the apparent damage is much less so it is conceivable that given an appropriate stimulus, the displaced element could be returned to the same area if not to the same location on the phytate molecule.

H.T. Horner: You used two extreme temperatures to test beam damage. If one could analyze for damage at every 5°C, do you believe the results would be described by a straight inclined line or do you think there is a narrow temperature range where there is a change from little beam damage to much greater beam damage?

Authors: This is a very interesting question and one that can be answered through experimentation. At this time we have no evidence that would help us select one of the two possibilities that you propose.