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**Chemical forms of calcium in Ca,Zn- and Ca,Cd-containing grains  
excreted by tobacco trichomes**

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1 **Abstract**

2

3 Tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants exposed to toxic levels of zinc and cadmium  
4 excrete metals through their leaf trichomes (epidermal hairs) as Zn,Ca- and Cd,Ca-containing  
5 grains. Little is known about the nature and formation mechanism of these precipitates. The  
6 chemical, crystalline, and non-crystalline compositions of individual grains produced by tobacco  
7 were studied by scanning electron microscopy coupled with energy dispersive X-ray analysis  
8 (SEM-EDX), micro-X-ray diffraction ( $\mu$ XRD) and calcium K-edge micro X-ray absorption near  
9 edge structure ( $\mu$ XANES) spectroscopy. Zinc is predominantly incorporated in calcite, and  
10 cadmium in calcite and vaterite. Aragonite, which occurs occasionally, does not seem to contain  
11 trace metals. In addition to being precipitated in its three possible polymorphic forms, calcite,  
12 aragonite and vaterite, calcium also is speciated as amorphous  $\text{CaCO}_3$  and possibly organic Ca in  
13 some grains. Most often, a particular grain consists of two or more crystalline and non-crystalline  
14 phases. The observed variability of intra- and inter-grain elemental and phase composition  
15 suggests that this biomineralization process is not constrained by biological factors, but instead  
16 results from thermodynamically and kinetically controlled reactions. This study illustrates the  
17 potential of laterally resolved X-ray synchrotron radiation techniques ( $\mu$ XRD and  $\mu$ XANES) to  
18 study biomineralization and metal immobilization processes in plants.

19

20 **Keywords:** biomineralization, detoxification, micro-XANES, micro-XRD.

## 21 **Introduction**

22 Recently, a novel original mechanism of Zn and Cd detoxification was described in tobacco  
23 (*Nicotiana tabacum* L. cv. Xanthi). Tobacco exposed to Cd excreted Cd,Ca-containing grains  
24 through leaf trichomes (1, 2), and a similar excretion of Zn,Ca-containing grains was observed  
25 under Zn exposure (3). Trichomes are specialized epidermal structures. In tobacco, they are  
26 glandular and excrete various organic substances including nicotine and resins. Such  
27 detoxification process may have implication in human health since smoking is one of the  
28 principal routes of exposure to heavy metals, and also in phytoremediation as tobacco is a  
29 candidate species for phytoextraction.

30 The morphology, elemental composition, mineralogy as well as Zn and Cd speciation in the  
31 grains were investigated by scanning electron microscopy coupled with energy dispersive X-ray  
32 analysis (SEM-EDX), micro-X-ray fluorescence ( $\mu$ XRF), micro-X-ray diffraction ( $\mu$ XRD), Zn  
33 K-edge micro extended X-ray absorption fine structure ( $\mu$ EXAFS) (3) and Cd L<sub>III</sub> edge micro X-  
34 ray absorption near edge structure ( $\mu$ XANES) spectroscopy (Isaure et al., in prep.). Calcium was  
35 always the major component.  $\mu$ XRD analyses revealed the presence of calcite, and less  
36 frequently vaterite and aragonite, two other CaCO<sub>3</sub> polymorphs. Calcium oxalate mono and  
37 dihydrate were found occasionally. Calcite and vaterite were substituted by cations, probably Zn,  
38 Cd and possibly Mn and Mg. Zn  $\mu$ EXAFS confirmed the occurrence of Zn-substituted calcite,  
39 and evidenced Zn associations with other phases including organic compounds, silica and  
40 phosphate (3). Cd  $\mu$ XANES showed that cadmium was a Ca substituent in calcite and vaterite  
41 and/or sorbed on the surface of these minerals (Isaure et al., in prep.).

42 The mechanism of formation of these grains remains unclear. Biogenic minerals may result from  
43 a controlled biomineralization process leading to well defined minerals and shapes. They may  
44 also be “biologically induced”, which means that an organism promotes the precipitation, but  
45 does not control the crystallization process (4). In the present case, grains could result from the  
46 exudation of a liquid containing the various metals and substances, and precipitation of the  
47 various solid phases due to water evaporation. Alternatively, they might be formed intracellularly  
48 and excreted thereafter as suggested by Choi et al. (5). The purpose of this study is to better  
49 characterize the structure and composition of the grains. Grains excreted by tobacco under  
50 various Zn, Cd and Ca exposure conditions were investigated by SEM-EDX,  $\mu$ XRD and Ca K-

51 edge  $\mu$ XANES spectroscopy. Results of these complementary approaches are compared, and a  
52 hypothesis for the formation of the grains is proposed in light of these data.

53

## 54 **Experimental**

### 55 *Materials*

56 Plant culture and grain collection procedure have been described previously (3). Briefly, tobacco  
57 plants were grown in hydroponic conditions and exposed for 5 weeks to Zn or Cd, with or  
58 without a supplement of Ca (Table 1). A control condition with Ca only was also tested. Then,  
59 grains were collected by washing the leaves in ultrapure water and centrifugating the suspension.  
60 Several Ca-containing reference compounds were used for the Ca XANES data analysis.

61 Calcite, vaterite and Cd-containing vaterite were synthesized at room temperature according to a  
62 modified protocol of Paquette and Reeder (6, 7). Briefly, an aqueous solution (500 mL)  
63 containing 10 mM  $\text{CaCl}_2$  and 1.8 M  $\text{NH}_4\text{Cl}$  was placed in a glass reactor, an EPPENDORF tube  
64 containing solid ammonium carbonate was allowed to float at the surface of the solution, and the  
65 reactor was closed and kept unstirred.  $\text{NH}_4\text{Cl}$  was used as a background electrolyte to provide a  
66 high ionic strength. Initial pH was 4.9. The decomposition of ammonium carbonate produces  
67  $\text{NH}_3(\text{g})$  and  $\text{CO}_2(\text{g})$ , which dissolve into the solution, simultaneously increasing pH and  
68 alkalinity. The supersaturation of the solution induces the formation and growth of  $\text{CaCO}_3$   
69 crystals. Continuous sublimation of  $\text{NH}_3(\text{g})$  buffers the solution around  $\text{pH} = 7.9$ . After 13 days,  
70 the reactor contained rhombohedral crystals and spherical particles attached on the surface of the  
71 glass. The two types of particles were separated and characterized by XRD. Rhombohedral  
72 crystals corresponded to calcite, and spherical particles to vaterite. For the synthesis of Cd-  
73 containing vaterite, the same procedure was used except that after 13 days, when crystal size  
74 amounted to 150-200  $\mu\text{m}$  in diameter, the  $\text{CaCl}_2$ - $\text{NH}_4\text{Cl}$  solution was progressively doped with  
75 0.1 M  $\text{CdCl}_2$  to a total concentration of 100  $\mu\text{M}$  Cd in order to incorporate Cd as a Ca substituent  
76 in vaterite (6, 7). The progressive addition of  $\text{CdCl}_2$  kept the solution undersaturated with respect  
77 to otavite ( $\text{CdCO}_3$ ). After 7 days, the particles were collected and separated. Spherical particles  
78 were identified as Cd-containing vaterite by XRD and  $\mu\text{XRF}$ . The Cd content was a few tens  
79 ppm based on  $\mu\text{XRF}$  analysis.

80 Aragonite was a natural specimen. The XANES spectrum for synthetic amorphous  $\text{CaCO}_3$  was  
81 provided by Y. Politi (8). Ca oxalate monohydrate (whewellite) was purchased from Aldrich. A

82 solution containing 0.5 M  $\text{Ca}(\text{NO}_3)_2$  at pH 2.6 was used as a reference for aqueous  $\text{Ca}^{2+}$ . The  
83 phase purity of all crystalline samples was checked by XRD prior to XANES analysis.

84

#### 85 *Electron microscopy*

86 The morphology and the chemical composition of the grains were studied by SEM-EDX using a  
87 Jeol-JSM 840A equipped with a Kevex Si(Li) detector. The chamber pressure was  $10^{-6}$ - $10^{-5}$  Torr,  
88 and the accelerating voltage 20 kV. Grains were mounted on kapton tape or on carbon tape, then  
89 fixed on carbon stubs and coated with carbon. Images were taken at a magnification of 500 to  
90 10000. EDX spectra were recorded on prominent spots of the grains to optimize the detection.

91

#### 92 *Micro-XRF, $\mu$ XRD and $\mu$ XANES*

93 The  $\mu$ XRF,  $\mu$ XRD and Ca K-edge  $\mu$ XANES measurements were performed on beamline 10.3.2  
94 of the Advanced Light Source (ALS, Berkeley, CA) (9). The grains were spread on kapton tape  
95 and analyzed at room temperature and pressure. First, each grain was mapped by  $\mu$ XRF. Then,  
96  $\mu$ XANES and  $\mu$ XRD and data were recorded on the Zn-richest region for the Zn and Zn + Ca  
97 treatments, on the Cd-richest region for the Cd and Cd + Ca treatment, and on the Ca-richest  
98 region for the Ca treatment.  $\mu$ XRF data were collected at 10 keV with a beam size of  $5 \times 5 \mu\text{m}$ .  
99 The same X-ray spot size was used for Ca  $\mu$ XANES. Fluorescence X-ray yield was measured  
100 with a 7-element Ge solid-state detector. The spectra were recorded between 3900 to 4400 eV.  
101  $\mu$ XRD patterns were recorded at an incident energy of 17 keV with a beam size of  $16 \times 7 \mu\text{m}$ .  
102 More experimental details are given in (3). Ca XANES reference spectra were recorded at room  
103 temperature on beamline ID21 at the European Synchrotron Radiation Facility (ESRF, Grenoble,  
104 France). Each spectrum is the average of two to three spectra, each about 15 minutes long.

105

#### 106 *XRD and XANES data treatment*

107 The XRD data treatment was performed as described previously (3). Briefly, after calibration with  
108 alumina, two dimensional XRD patterns were integrated to one-dimensional patterns for peak  
109 assignment. For substituted calcite crystals, the unit cell parameters  $a$  and  $c$  were refined, and the  
110 stoichiometry of the substituent was estimated from these two parameters using the Vegard law  
111 (10) with calcite, smithsonite ( $\text{ZnCO}_3$ ) and otavite ( $\text{CdCO}_3$ ) as end-members for the Ca-Zn and Ca-

112 Cd solid solutions. This approach cannot be used for substituted vaterite because of the absence of  
113 a CdCO<sub>3</sub> structural analogue of vaterite.

114 XANES spectra were processed using WinXAS (11). All spectra were energy calibrated  
115 with respect to calcite (inflection point for this reference set to 4042.6 eV). The collected scans  
116 were averaged; background subtracted and normalized using linear (pre-edge) or cubic (post-  
117 edge) polynomials. XANES spectra were then fitted by linear combinations using calcite,  
118 aragonite, vaterite, Cd-containing vaterite, amorphous CaCO<sub>3</sub>, aqueous Ca<sup>2+</sup> and Ca oxalate  
119 monohydrate reference spectra. The quality of the fits was quantified by the normalized sum-  
120 squares residuals  $NSS = \sum(\mu_{\text{experimental}} - \mu_{\text{fit}})^2 / \sum(\mu_{\text{experimental}})^2 \times 100$ , in the 4000-4150 eV  
121 range, where  $\mu$  is the normalized absorbance. An energy shift of  $\pm 0.5$  eV maximum and a  
122 correction of slope were allowed to account for the energy resolution of the monochromator and  
123 for possible inconsistencies in data processing. For some spectra, linear combination (LC) fitting  
124 did not provide satisfactory results, and over-absorption effects were suspected based on the  
125 comparison of these spectra with the standards. Therefore, each spectrum was fitted with and  
126 without a correction of over-absorption using a simple model (12). This model assumes that the  
127 smooth part of the resonant absorption is a constant fraction of the non-resonant background  
128 (which is a reasonable assumption in the XANES region) and that the sample is infinitely thick,  
129 *i.e.*, totally absorbs the incident beam (which is probably true considering the energy range and  
130 the size and global composition of the grains). The equation used is:

$$131 \quad y_{\text{experimental}} = (1 + a) / (1 + a \cdot y_{\text{corrected}}) \quad (1)$$

132 where  $y$  is the normalized resonant absorbance, which equals 0 below the edge and

133 oscillates around 1 above the edge, and  $a = \mu_{\text{resonant}} / (\mu_{\text{non resonant}} + \mu_{\text{fluorescence}})$

134 where  $\mu_{\text{resonant}}$ ,  $\mu_{\text{non resonant}}$  and  $\mu_{\text{fluorescence}}$  are the three components of the absorbance  $\mu$ .

135 Equation (1) can be solved as:

$$136 \quad y_{\text{corrected}} = y_{\text{experimental}} / (1 + a(1 - y_{\text{experimental}})) \quad (2)$$

137 The over-absorption parameter  $a$  equals 0 in the absence of over-absorption effect, and  
138 increases with this effect. Because of the sample heterogeneity, the precise composition of the  
139 sample is not known, so  $a$  is an adjusted parameter.

140

## 141 **Results**

142 The morphology of the grains as observed by SEM varied considerably. The grains varied from  
143 10  $\mu\text{m}$  to 150  $\mu\text{m}$  in diameter. Each grain is an aggregate of different types of particles. The first  
144 type is micro faceted crystallites (Insets in Fig. 1a and 1b) and the second type is globular  
145 structures of various sizes (from 2 to 50  $\mu\text{m}$ , Fig. 1c, 1d and 1e). Some grains such as Zn<sub>2</sub> (Fig.  
146 1c) seem visually less crystalline. The elemental composition of the grains varies from one grain  
147 to another, and within the same grain. Ca was always the major component regardless of the plant  
148 metal treatment conditions. Minor elements include Mg, Si, P, Cl, K, and Zn or Cd depending on  
149 the plant treatment (Fig. 1). The high Si peaks observed in Fig. 1a to 1d are attributed to kapton  
150 tape on which the grains were mounted. Silicon is not detected in the grain presented in Fig. 1e  
151 (mounted on carbon tape). However, Si was found in small amount in some grains (not presented  
152 in this study), as well as Mn.

153 Our previous study using  $\mu\text{XRD}$  (3) showed that grain ZnCa<sub>3</sub> contained substituted calcite and  
154 aragonite, that grain ZnCa<sub>4</sub> contained substituted calcite, and that grain Zn<sub>2</sub> did not contain  
155 crystalline phases. The two-dimensional  $\mu\text{XRD}$  patterns for grains ZnCa<sub>5</sub> and Zn<sub>3</sub> show that the  
156 Bragg reflections for these two grains consist of small arcs of Debye-Scherrer rings, indicating  
157 that the grains are composed of micrometer-size mosaic crystals (Fig. 2b). All reflections  
158 correspond to substituted calcite, and the major Ca substituent is Zn based on  $\mu\text{XRF}$  (Fig. 2a).  
159 Note that although Ca is the major element, the Ca  $K\alpha$  peak has a low intensity relative to the Zn  
160  $K\alpha$  peak due to air absorption and the lower fluorescence yield of Ca *vs.* Zn. The refined unit cell  
161 parameters *a* and *c* for grain ZnCa<sub>5</sub> and Zn<sub>3</sub> were 4.97 and 16.97  $\text{\AA}$ , and 4.95 and 16.83  $\text{\AA}$ ,  
162 respectively, compared to 4.9896 and 17.0610  $\text{\AA}$  for pure calcite. The calculated formulae using  
163 the Vegard law were  $\text{Ca}_{0.95}\text{Zn}_{0.05}\text{CO}_3$  and  $\text{Ca}_{0.89}\text{Zn}_{0.11}\text{CO}_3$ , respectively. The difference in the Zn  
164 stoichiometry coefficient obtained using parameters *a* and *c* was  $\pm 0.01$ . The  $\mu\text{XRD}$  results for  
165 the Cd-containing grains will be presented separately together with Cd  $L_{\text{III}}$ -XANES data (Isaure  
166 et al., in preparation). Briefly, the grains CdCa<sub>2</sub> and Cd<sub>10</sub> contain micrometer crystals of  
167 substituted calcite and finer (nanometer sized) crystals of substituted vaterite. Grain CdCa<sub>3</sub>  
168 contains nano-crystals of substituted vaterite only. Finally, no crystalline phases were detected in  
169 grain CdCa<sub>6</sub>. For Cd- substituted calcite, the Vegard law provides an imprecise estimation of Cd  
170 stoichiometry because the contrast in ionic radius between Cd and Ca is small (0.95  $\text{\AA}$  for  $\text{Cd}^{2+}$   
171 and 1.00  $\text{\AA}$  for  $\text{Ca}^{2+}$  compared to 0.74  $\text{\AA}$  for  $\text{Zn}^{2+}$ ) (13). For instance, the substitution rate for  
172 grain CdCa<sub>2</sub> could be anywhere between 30 and 80%. It was not possible to calculate the



173 stoichiometry of Cd in vaterite because of the absence of a Cd end member (see the Experimental  
174 section).

175 Calcium being the major element in the grains, Ca K-edge  $\mu$ XANES spectroscopy was then  
176 performed to get some insights on the composition of the grains, and test for the presence of  
177 amorphous Ca phases. Figure 3a shows the XANES spectra for several Ca reference compounds.  
178 The four  $\text{CaCO}_3$  species (calcite, vaterite, aragonite and amorphous  $\text{CaCO}_3$  (ACC)) have clearly  
179 distinct spectra, which enables their identification (14, 15). However, Ca XANES has no  
180 sensitivity to substitutional impurities as seen for instance for low Cd-containing vaterite (Cd  
181 content lower than 1%, as estimated by  $\mu$ XRF) and pure vaterite exhibiting nearly identical  
182 spectra. The spectra for aqueous  $\text{Ca}^{2+}$ , Ca oxalate monohydrate and ACC share some similarities.  
183 However, the lower part of the edge is shifted to lower energy for ACC spectrum, and the  
184 maximum of the white line (zero value of the first derivative) increases from 4049.5 eV (ACC) to  
185 4049.9 eV (Ca oxalate monohydrate) and to 4050.5 eV (aqueous  $\text{Ca}^{2+}$ , Fig. 3b). The aragonite  
186 spectrum is distinct from the three previous spectra in displaying a shoulder at 4046 eV (arrow in  
187 Fig. 3), and a minimum between 4060 and 4070 eV.

188 Figure 4 shows the Ca  $\mu$ XANES spectra for the grains. They were fitted by LC using the standard  
189 spectra presented above, and results are presented in Table 2. The ZnCa3 spectrum presents  
190 strong similarities with the aragonite spectrum, but markedly lower amplitude. The drop in  
191 amplitude suggests some over-absorption effect (12) due to a high Ca content of the analyzed  
192 spot. The fit without over-absorption correction (see materials and Methods) did not provide  
193 satisfactory result ( $NSS = 0.065$  %, with 68% vaterite + 25% aragonite). Introducing an over-  
194 absorption correction greatly improved the fit ( $NSS = 0.014$  % with aragonite as only component,  
195 Fig. 4). The  $a$  value found (1.38) corresponds to a very strong over-absorption effect. The fit was  
196 not improved significantly by introducing a second component ( $NSS$  decreased by less than  
197 10%). Thus, the spot analyzed by  $\mu$ XANES likely contains aragonite as the sole Ca species.  
198 The grains ZnCa4 and ZnCa5 were fitted with 98% calcite. The fit was slightly improved with a  
199 correction of over-absorption ( $NSS = 0.015$  and  $0.021$  %, compared to  $0.020$  and  $0.035$  %, respectively),  
200 and the proportions were unchanged. Adding a second component did not improve  
201 significantly the fits ( $NSS$  decreased by less than 10%).

202 For grain Zn2 and CdCa6, fits without over-absorption correction were not satisfactory ( $NSS =$   
203  $0.048$  and  $0.091$  %, respectively, for two-component fits). Fair one-component fits with over-

204 absorption correction were obtained with ACC ( $NSS = 0.021$  and  $0.024$  %, respectively), but the  
205 match was not optimal for the first oscillation (around  $4080$  eV). Ca oxalate monohydrate and  
206 aqueous  $Ca^{2+}$  provided weaker fits ( $NSS = 0.086$  and  $0.053$  % for Zn2, and  $0.076$  and  $0.043$  % for  
207 CdCa6, respectively). Adding a second component did improve the reproduction of the first  
208 oscillation. Several fits of equivalent qualities were obtained with ACC as major component  
209 (about  $80$  and  $70$  % of total Ca) and various species as secondary component.  $NSS$  values were  
210 more than doubled if ACC was excluded (Table 2 and Fig. 1 in Supplemental Information).  
211 Therefore, grains Zn2 and CdCa6 likely contain ACC as major species, and an additional species  
212 whose nature remains unknown.

213 For the other grains (Zn3, CdCa2, CdCa3 and Cd10), over-absorption correction did not improve  
214 the fits. Calcite was the major form in grains Zn3 and CdCa2, and fits of equivalent quality were  
215 obtained with ACC, aqueous  $Ca^{2+}$  and Ca oxalate monohydrate as minor species. Aqueous  $Ca^{2+}$   
216 is unlikely in solid-state material. ACC and Ca oxalate monohydrate are more likely. In the  
217 absence of reference spectra for Ca bound to organic ligands other than oxalate, this latter  
218 compound may be considered as a proxy for Ca bound to organic compounds in general.  
219 Therefore, this pool is referred to as “organic Ca and/or ACC” in Table 3. Vaterite was found in  
220 grains CdCa3 and Cd10. For this latter grain only, no satisfactory fit was obtained with two  
221 components ( $NSS = 0.046$  %), so a third component was introduced. Again, a contribution of  
222 organic Ca and/or ACC was found in grains CdCa3 and Cd10.

223  
224 The speciation of Ca was then compared with the mineralogy and morphology of the grains  
225 (Table 3). The percentages were rounded to the nearest ten for clarity. Except for two grains,  
226 ZnCa3 and CdCa2, the same crystalline species were identified by  $\mu$ XRD and Ca  $\mu$ XANES. Zn-  
227 substituted calcite and Cd-substituted vaterite were identified by  $\mu$ XRD in grains ZnCa3 and  
228 CdCa2, respectively, but not by Ca  $\mu$ XANES spectroscopy. This difference likely results from  
229 the larger beam size and higher penetration depth of X-rays in XRD measurements relative to  
230  $\mu$ XANES ( $16 \times 7 \mu\text{m}$  vs  $5 \times 5 \mu\text{m}$ , and a few tens of  $\mu\text{m}$  at  $17$  keV vs. a few  $\mu\text{m}$  at  $4$  keV). Thus,  
231 XRD probes a different material volume than Ca  $\mu$ XANES even when conducted on the same  
232 spot.

233

## 234 **Discussion and Conclusion**

235 Results showed that beside crystalline calcium carbonates (calcite, vaterite and aragonite), the  
236 tobacco grains contained ACC as well. Whewellite (Ca oxalate monohydrate) did show up in  
237 some XANES fits, but its presence could not be firmly attested. Ca oxalate mono- and dihydrate  
238 have several XRD peaks in common with calcite, but peaks at distinct positions as well. These  
239 two minerals were positively identified by  $\mu$ XRD in other tobacco grains based on the presence  
240 of these specific peaks (3). In the present study, no such peaks were found on the XRD patterns.  
241 However, it is still possible that coarse crystals of Ca oxalate mono or dihydrate are present but  
242 produce non-specific peaks only, or that Bragg conditions were not met for these coarse crystals.  
243 Therefore, the presence of Ca oxalate mono- and dihydrate cannot be ruled out. Other  
244 undetermined organic ligands may also complex Ca. In addition, mixed mineral and organic Ca  
245 compounds such as organic matter-containing calcite (16) may occur.

246 The Ca  $\mu$ XANES analysis showed that strong over-absorption effects may take place and  
247 decrease dramatically the amplitude of the spectra. We show that this effect is far from negligible  
248 when identifying and quantifying Ca species. For instance, for grain ZnCa3, the uncorrected  
249 spectrum was fitted by 68% vaterite + 25% aragonite, and the corrected spectrum was fitted with  
250 100% aragonite. Note that the LCF without over-absorption correction was relatively bad, which  
251 alerted us on the possibility of an over-absorption effect.

252 In the environment, ACC is thermodynamically unstable and rapidly transforms into vaterite and  
253 then calcite (17). However, it is found as a stable compound in plants (e.g., cystoliths) and  
254 animals (e.g., cuticle of crustaceans, spicules of ascidiae, granules in molluscs) (18). ACC is  
255 probably stabilized by proteins, magnesium and phosphorus in these organisms (18). In our case,  
256 Mg and P were found in the grains.

257 Biologically controlled biomineralization leads to well defined mineral species and shapes.  
258 Here, the variety of morphologies and of CaCO<sub>3</sub> crystal structures, and their coexistence with  
259 amorphous and possibly organic forms, supports the hypothesis of a biologically induced (as  
260 opposed to biologically controlled) biomineralization. This study illustrates the potential of  
261 microfocused X-ray techniques to study biomineralization processes, and strategies of metals  
262 immobilization developed by plants to cope with metal toxicity.

263

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**Table 1.** Tobacco culture conditions and name of the grains investigated

Treatment conditions	Cation Concentrations in the Nutrient Solution (mM)			Grain Names	Techniques and References				
	Ca	Zn	Cd		SEM-EDX	$\mu$ XRD	Zn $\mu$ EXAFS	Cd $\mu$ XANES	Ca $\mu$ XANES
Zn	0.28	0.25	0	Zn2	(3) and this paper	(3)	(3)	-	this paper
				Zn3	-	this paper	unpubl.	-	
Zn + Ca	3.28	0.25	0	ZnCa3	(3) and this paper	(3)	(3)	-	this paper
				ZnCa4	(3) and this paper	(3)	(3)	-	this paper
				ZnCa5	-	this paper	unpubl.	-	this paper
Cd	0.28	$0.08 \cdot 10^{-3}$	0.025	Cd10	(a)	(a)	-	(a)	this paper
Cd + Ca	3.28	$0.08 \cdot 10^{-3}$	0.025	CdCa2	(a)	(a)	-	(a)	this paper
				CdCa3	(a)	(a)	-	(a)	this paper
				CdCa6	(a)	(a)	-	(a)	this paper
Ca	3.28	$0.08 \cdot 10^{-3}$	0	Ca3	this paper	-	-	-	-

(a) Isaure et al., in prep.

**Table 2. Ca  $\mu$ XANES Results**

Treatment	Grain Name	$a^1$	Proportion of Ca species (%) Determined by Linear Combination Fitting the $\mu$ XANES Spectra					Sum	NSS (%) <sup>2</sup>		
			Calcite	Vaterite	Aragonite	Amorphous CaCO <sub>3</sub>	Aqueous Ca <sup>2+</sup>			Ca oxalate 1 H <sub>2</sub> O	
<b>Zn+Ca</b>	ZnCa3	1.38			97 ± 2			97	0.014 <sup>3</sup>		
	ZnCa4	0.12	98 ± 2					98	0.015 <sup>3</sup>		
	ZnCa5	0.17	98 ± 2					98	0.021 <sup>3</sup>		
<b>Zn</b>	Zn2	0.27			22 ± 10	76 ± 15			97	0.011 <sup>3</sup>	
		0.27				77 ± 15		20 ± 15	97	0.016	
		0.27				79 ± 15	17 ± 15		96	0.019	
		0.42				(excluded)	55 ± 15	45 ± 15	100	0.050	
	Zn3			62 ± 5			29 ± 5			91	0.009 <sup>3</sup>
				70 ± 5					22 ± 5	92	0.016
				71 ± 5				21 ± 5		92	0.018

<b>Cd+Ca</b>	CdCa2		85 ± 5		13 ± 5		98	0.012 <sup>3</sup>
			87 ± 5			10 ± 5	97	0.014
			89 ± 5		9 ± 5		98	0.015
	CdCa3		77 ± 5		21 ± 7		98	0.031 <sup>3</sup>
			79 ± 5			20 ± 7	99	0.036
			82 ± 5		16 ± 7		98	0.037
	CdCa6	0.38		74 ± 15		25 ± 15	99	0.015 <sup>3</sup>
		0.42		68 ± 15	32 ± 15		100	0.019
		0.28		(excluded)	73 ± 15	25 ± 15	98	0.040
<b>Cd</b>	Cd10		29 ± 7	42 ± 8		25 ± 5	96	0.022 <sup>3</sup>
			25 ± 7	37 ± 8		34 ± 5	96	0.025
			28 ± 7	42 ± 8			26 ± 5	96

<sup>1</sup> Coefficient of over-absorption. <sup>2</sup> Residual between fit and experimental data  $NSS = \frac{\sum(\mu_{\text{experimental}} - \mu_{\text{fit}})^2}{\sum(\mu_{\text{experimental}})^2} \times 100$  in the 4000-4150 eV range, where  $\mu$  is the normalized absorbance. <sup>3</sup> Fit shown in Fig. 4. The error bars on the percentages correspond to the variation needed to increase  $NSS$  by 20%.



**Table 3. Summary of the information obtained on each grain<sup>1</sup>**

<b>Treatment</b>	<b>Grain Name</b>	<b>Ca Speciation (<math>\mu</math>XANES Results)<sup>1</sup></b>	<b>Mineralogy (<math>\mu</math>XRD Results)</b>	<b>Aspect of the Grain (SEM Results)</b>
<b>Zn+Ca</b>	ZnCa3	100% aragonite	Aragonite, Zn-subst. calcite (Ca <sub>0.93</sub> Zn <sub>0.07</sub> CO <sub>3</sub> )	faceted
	ZnCa4	100% calcite	Zn-subst. calcite (Ca <sub>0.87</sub> Zn <sub>0.13</sub> CO <sub>3</sub> )	faceted
	ZnCa5	100% calcite	Zn-subst. calcite (Ca <sub>0.95</sub> Zn <sub>0.05</sub> CO <sub>3</sub> )	not observed
<b>Zn</b>	Zn2	80% ACC + 20% undetermined	No diffraction peaks	rounded
	Zn3	70% calcite + 30% organic Ca and/or ACC	Zn-subst. calcite (Ca <sub>0.89</sub> Zn <sub>0.11</sub> CO <sub>3</sub> )	not observed
<b>Cd+Ca</b>	CdCa2	90% calcite + 10% organic Ca and/or ACC	Cd-subst. calcite, Cd-subst. vaterite	not observed
	CdCa3	80% vaterite + 20% organic Ca and/or ACC	Cd-subst. vaterite	rounded
	CdCa6	70% ACC + 30% undetermined	No diffraction peaks	not observed
<b>Cd</b>	Cd10	30% calcite + 40% vaterite + 30% organic Ca and/or ACC	Cd-subst. calcite, Cd-subst. vaterite	rounded + faceted

<sup>1</sup>The error bar on the percentages varies from 5 to 15% depending on the species (see Table 2)

## Figure Captions

Figure 1: SEM imaging for the grains ZnCa3 (a), ZnCa4 (b) Zn2 (c), CdCa3 (d) and grain Ca3 (e), and EDX analyses were performed on the spots marked by a black cross.

Figure 2:  $\mu$ XRF spectrum (a) one- and two-dimensional  $\mu$ XRD patterns (b) for grain ZnCa5 and Zn3. All XRD peaks were attributed to substituted calcite. The positions of three peaks for pure calcite are indicated by arrows.

Figure 3: Ca K-edge XANES spectra for the reference compounds used in the linear combination fits (a) and zoom on the main peak for aqueous Ca<sup>2+</sup>, Ca oxalate monohydrate and ACC (b).

Figure 4: Ca K-edge  $\mu$ XANES spectra for the tobacco grains and corresponding linear combination fits. Five spectra (Zn2, CdCa6, ZnCa3, ZnCa5 and ZnCa4) were corrected for over-absorption.

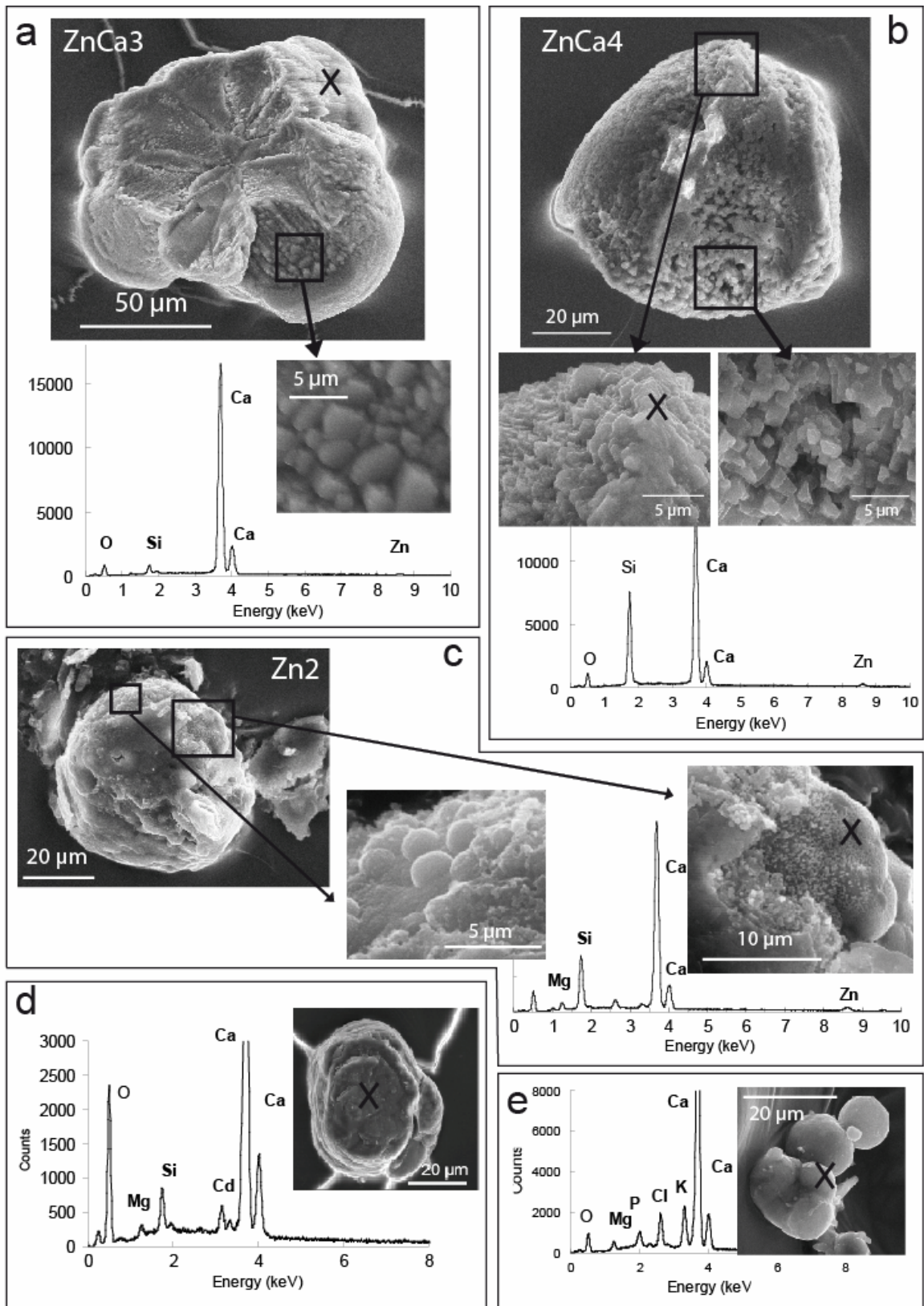


Figure 1

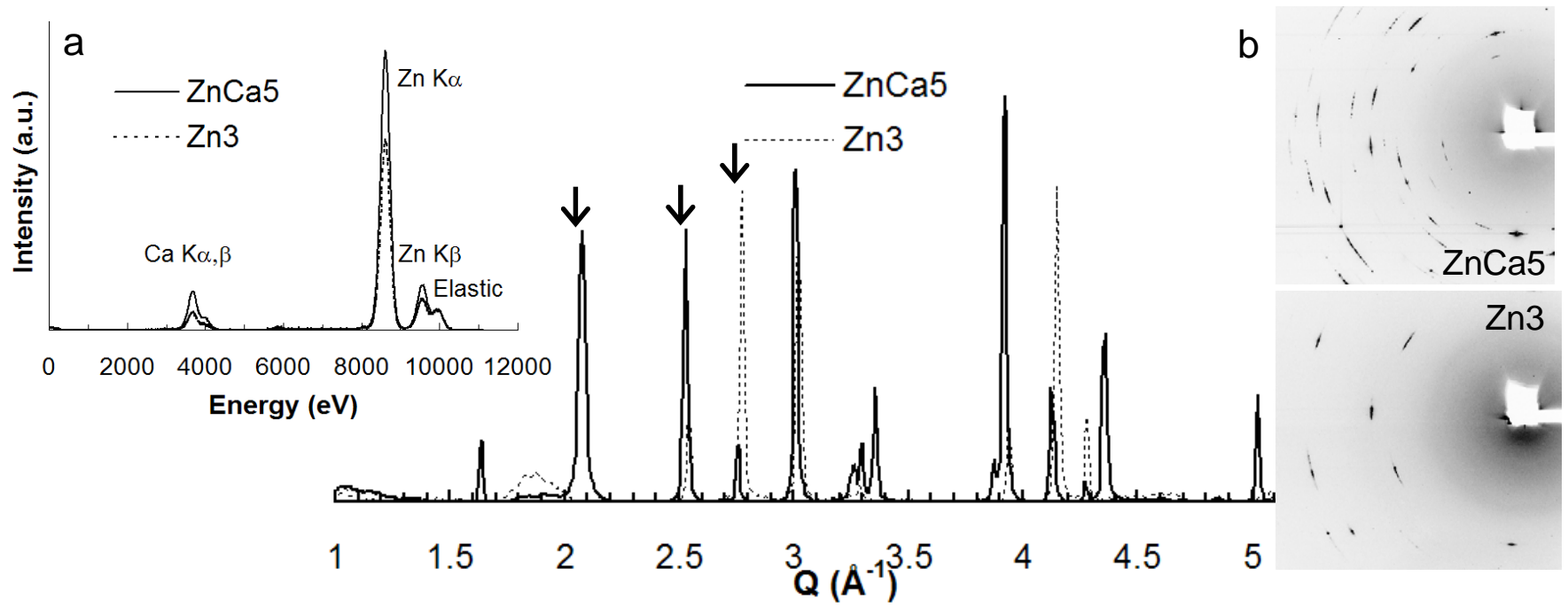


Figure 2

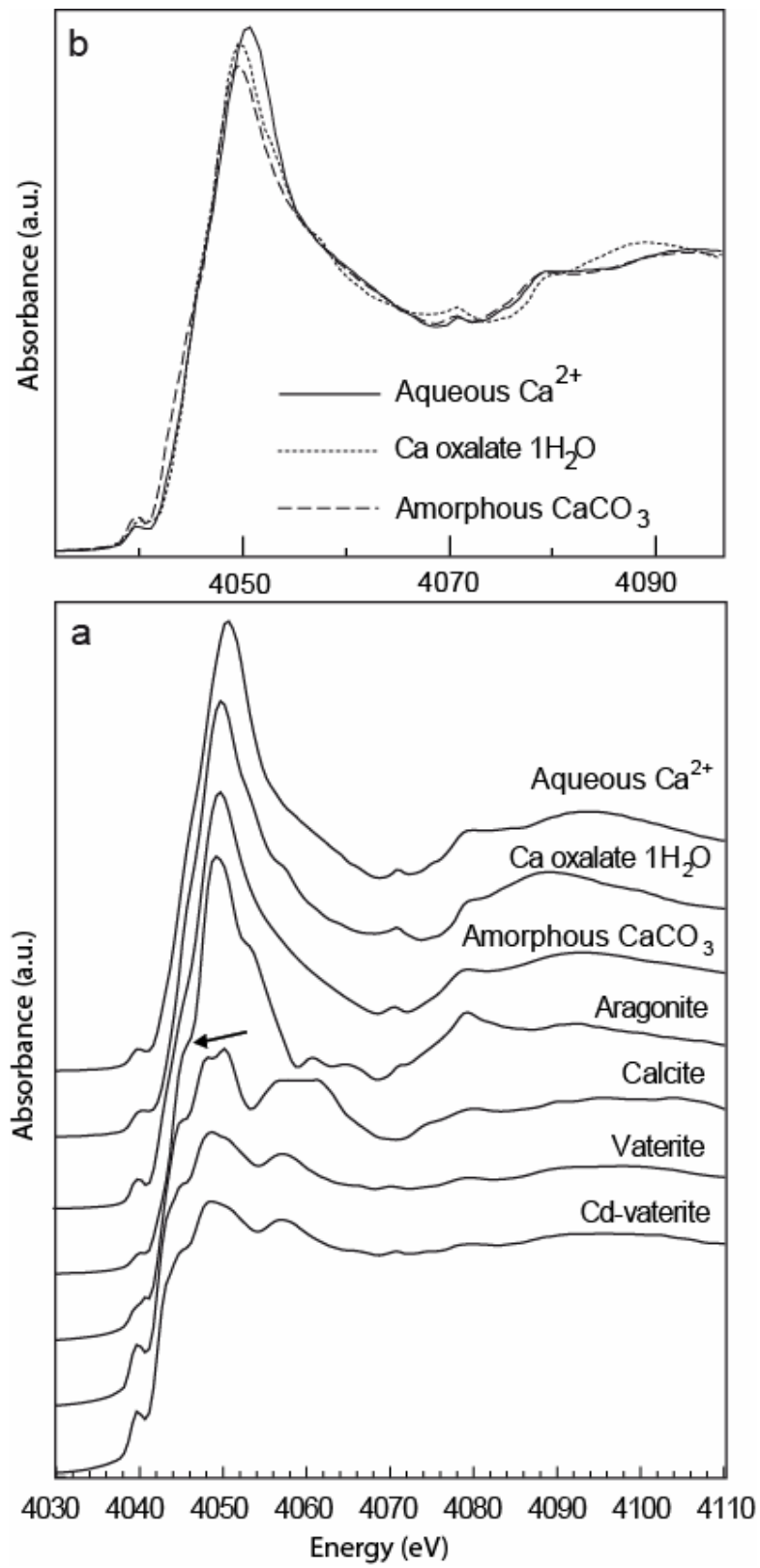


Figure 3

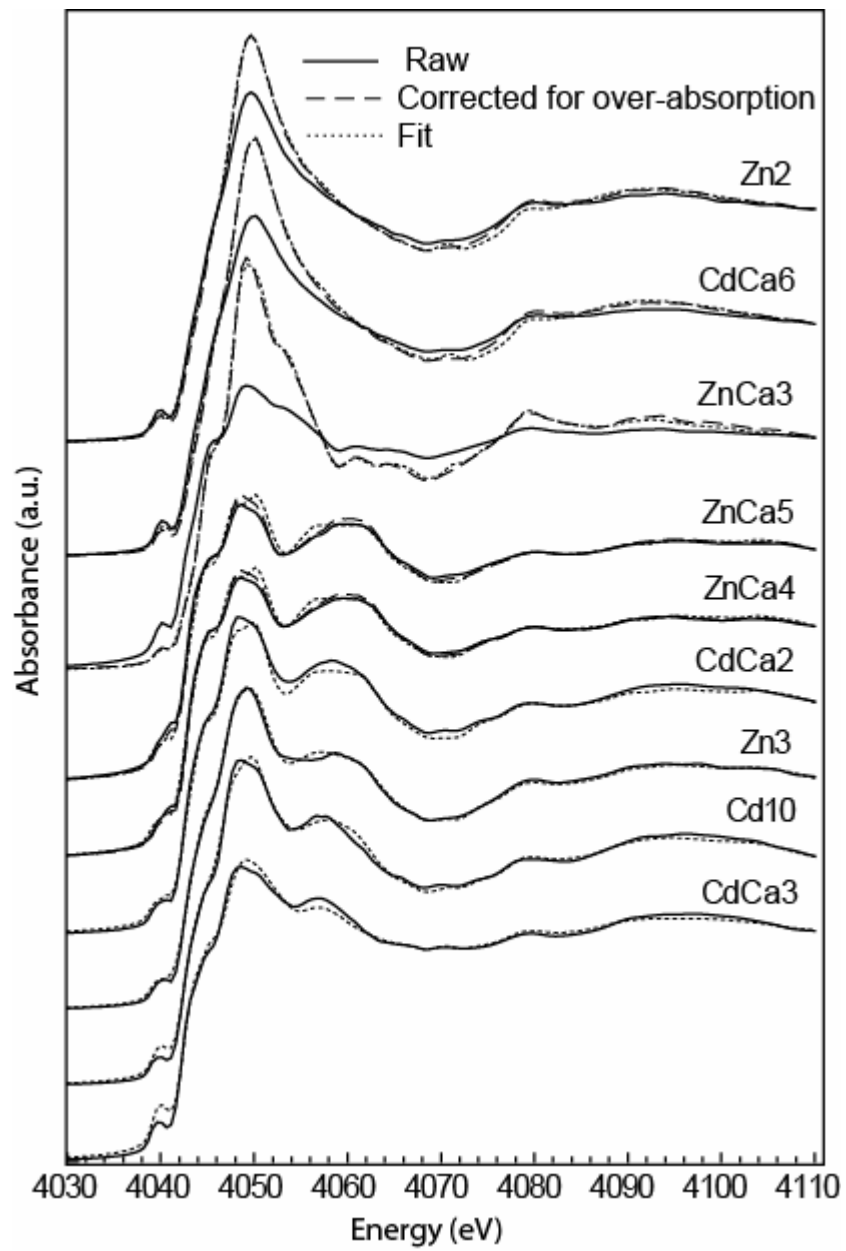


Figure 4

## Supplemental information

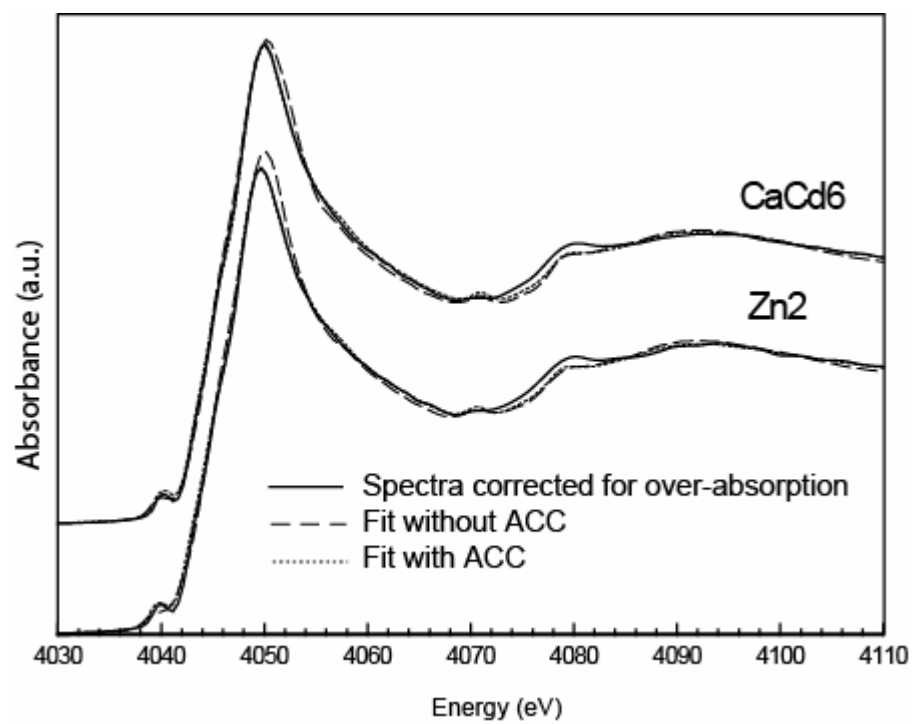


Figure 1: Comparison of the best two-component fits obtained for CaCd6 and Zn2 with and without ACC.