TIMS and Ion Chromatography: 
$^{44}\text{Ca}/^{40}\text{Ca}$ Analysis in Plants

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Introduction
Characterization of the Co-occurring Geochemical and Biological Processes Affecting Ca Isotopes within Plants

Calcium is an essential mineral nutrient in plants where it is mainly involved in the stabilization of cell walls and membranes.$^{1,2}$ Calcium acts as a counter-ion for inorganic and organic anions in cell vacuoles (intracellular specialised sub-units) and as an intracellular messenger in the cytosol (intracellular liquid phase).$^1$ Calcium is taken up by the plant lateral roots by diffusion and/or by the water mass flux induced by foliar transpiration. Ca is subsequently transported to the leaves by the xylem sap. No translocation (transportation) from foliage to reproductive organs occurs through the phloem and, therefore, Ca supply is mainly supported by the xylem.$^{1-3}$ Along with nutrient elements, Ca is returned to litter and soil with the falling leaves or needles and subsequently mineralized. Ca is thus available again for vegetal uptake from soil solutions of organic-rich forest floor and underlying mineral soils of forested watersheds.$^6$ Mineral reserves prove often insufficient to sustain the nutrient requirements necessary to the development of the forest annual biomass, so that the vegetal Ca cycle constitutes an essential additional nutrient supply to forests.

Over the past decade, Ca isotopes have shown their potential in identifying and characterizing fractionation processes occurring within plants$^{7,8}$ or at the water-soil-plant interface of small forested watersheds (see reference 9 and references mentioned therein). The present study was initially intended at preparing germinated Phaseolus vulgaris L. bean organs to be used in hydroponic experiments designed to characterize the co-occurring geochemical and biological processes affecting the Ca isotopic compositions within plants.$^7,8$ Ca isotopes have been measured in Phaseolus vulgaris L. bean organs prior to and after germination. Due to small natural Ca isotopic fractionation within watersheds ($\sim 1.25\%$/amu), it was mandatory to achieve an external reproducibility in the permil to sub-permil range in order to detect any Ca isotopic variability within the bean organs of Phaseolus vulgaris L. $^{44}\text{Ca}^{40}\text{Ca}$ ratios cannot be measured to high-precision by multiple collector inductively coupled plasma source mass spectrometry (MC-ICP-MS) because of the large $^{40}\text{Ar}$ interference. Instead, Ca isotope analysis was achieved through high-precision mass spectrometry by TIMS and complete chromatographic separation of Ca from elements susceptible to induce isobaric interferences during mass spectrometry analysis (Mg, K, Sr). In particular, special attention has been paid to complete removal of K from the samples of Phaseolus vulgaris L. bean organs, where K/Ca $\leq$ 40.
Analytical Protocol

systematically monitored for at masses 41K+ and 88Sr+. No intensity of 5.0 were subsequently re-dissolved in 1 µL 0.25 N HNO3 and/or cation exchange resin breakdown. The Ca residues eliminate organic impurities originating from the sample.

Phaseolus vulgaris L. bean organs (Figure 1) were crushed in a clean agate mortar and digested in Savillex™ vials in a hot oxidative medium (double distilled HNO3, double distilled HCl, suprapure H2O). Then, following the double spike method introduced for Ca isotope10 and modified for Ca multicollection TIMS analysis11, 1 µg of 40Ca double spike was added to 5 µg of sample prior to Ca purification. This ensured correction for any procedural or instrumental mass discrimination that would occur after addition of the double spike.

Classical Ca-exchange chromatography is not optimized for full recovery and complete separation of Ca from K and Sr (Figure 2A). Instead, the development of an automated high selectivity ion chromatography separation protocol (Thermo Scientific™ Dionex™ ICS-3000 device with a high-capacity carbonate-functionalized column Dionex CS16 and a CSRS-300 cation self-regenerating suppressor) ensures Ca full recovery and eliminates K and Sr tailing (Figure 2B,12).

Non-germinated beans mainly consist of cotyledons, which constitute nutritional reserves for germination. Germinated beans consist of roots (underground part) and shoots (above ground part, other than the cotyledons). Teguments are present in both non-germinated and germinated beans and solely correspond to an envelope around the cotyledons, with no biological influence on germination.

**Phaseolus vulgaris** L. organs resolve sample measurements was 0.12‰ (2 RSD, n = 80). The Ca 40Ca isotope compared to the non-germinated cotyledons (Figure 3). Non-germinated and germinated teguments have an envelope around the cotyledons, with no biological influence on germination.

External reproducibility of δ44Ca based on replicate sample measurements was 0.12‰ (2 RSD, n = 80). The Ca isotopic analysis of Phaseolus vulgaris L. organs resolve natural 40Ca/Ca isotopic variations in the sub-permil range between teguments and non-germinated cotyledons, and in the sub-permil to permil range between non-germinated cotyledons and germinated organs (Figure 3). Non-germinated and germinated teguments have identical Ca isotopic composition. In contrast, germinated cotyledons (87Ca/Ca = 0.45‰) are enriched in the light 40Ca isotope whereas shoot (87Ca/Ca = 0.20‰) and roots (87Ca/Ca = 0.68‰) are depleted in the light 40Ca isotope compared to the non-germinated cotyledons (87Ca/Ca = -0.37‰).

**Ca**^{44} Isotopic Composition Fractionation During the Germination of Phaseolus vulgaris L.

Ca isotopic variability observed in Phaseolus vulgaris L. organs evidences fractionation mechanisms. Ca isotopic variability between teguments and non-germinated cotyledons could result from Ca isotopic fractionation during physiological processes leading to the formation of seeds. As expected, teguments, which do not participate in the germination, do not show Ca isotopic fractionation during this process. On the contrary, Ca isotopic variability between non-germinated cotyledons and germinated cotyledons, shoot and roots, shows that isotopic fractionation occurred during the germination process, i.e during the development of roots and shoots from the cotyledons. The Ca amount of rinsed vermiculite has been checked to be negligible compared to the Ca amount of the bean seeds and, as such, is not expected to contribute the Ca isotopic composition of the bean organs. The dry-mass-weighted calculated average 87Ca of the germinated organs (cotyledon, shoot and roots) equals that of the non-germinated cotyledons (87Ca = +0.37‰), confirming that all the Ca mobilized during germination originated from the cotyledons, not from the germination medium (vermiculite) itself.

**Results**

**Ca**^{44} Isotopic Composition Long-term Reproducibility and Accuracy

**Ca**^{44} Ca isotopic values are expressed as permil deviations relative to the NIST SRM 915a standard solution: δ44Ca = [(44Ca/40Ca)sample/(44Ca/40Ca)SRM915a] -1 × 1000 (ref. 13). The 87Ca/Ca values were corrected for instrument-related mass bias using the exponential fractionation sample and the Newton-Raphson iteration technique.22 Data reduction was performed off-line using the optimization toolbox provided in the commercial Math™ software.

Figures 1 and 2. Elution curves for (A) classical Ca-exchange chromatography of a soil sample (ASW00X, 200–400 mesh Biorad™ resin, 1.5 N HCl as-eluant, recovery yield = 70%); and (B) automated high selectivity ion chromatography of a soil sample (Dionex CS16 cation-exchange column, methanesulfonic acid as eluant, recovery yield = 100%). The overall Ca blank contribution from the HPIC system was ~30 ng i.e., ~0.05% of the sample Ca amount. (Figure 2B modified after ref. 12).

Figure 3. Calcium isotopic variations between germinated (full symbols) and non-germinated (empty symbols) bean organs. Germinated cotyledons present a 44Ca/40Ca composition intermediate to those of non-germinated cotyledons and of roots and shoots.

Figure 4. Thermo Scientific TRITON Plus Thermal Ionization Mass Spectrometer (TIMS).

Figure 5. Thermo Scientific Dionex ICS-5000 Reagent-Free™ HPIC™ system (replacing, since 2010, the Thermo Scientific Dionex ICS-3000 Reagent-Free™ HPIC™ system).
Conclusion

Flexible Analytical Protocols and Measurement Routines for High-precision Ca Isotopic Analysis

Coupling Dionex ICS-3000 and ICS-5000 ion chromatography and TRITON/TRITON Plus TIMS analysis improved the purity of Ca samples and ensured a long-term 2RSD δ44/40Ca external reproducibility of 0.12‰ on plant samples. This unruaveled natural Ca isotopic variability down to the sub-permil range within plant organs and assessed the extent of Ca isotopic fractionation during vegetal germination. Note that the TRITON/TRITON Plus system offers diverse analytical protocols for Ca isotopic analysis, combining different loading techniques and measurement routines. With Ca sample amounts in the µg range, large 40Ca ion beams (>40 V with 40Ca measured on a 1011 Ω resistor amplifier and >400 V with 40Ca measured on a 1010 Ω resistor amplifier) can provide 2RSD external reproducibility on 40Ca/44Ca ratios better than 50 ppm. 17–18

References