Freeze-substitution methods for Ni localization and quantitative analysis in *Berkheya coddii* leaves by means of PIXE

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Nuclear microprobe is being used in studies of the concentration and distribution of elements in different biological specimens. There are many difficulties related to sample preparation for elemental microanalysis due to possible elemental losses and their redistribution. One of the preparation techniques is freeze-substitution. The crucial issue is the choice of freeze-substitution protocol suitable for elemental and structural analysis.

In the present study leaves of Ni hyperaccumulator *Berkheya coddii* were chosen as a model to investigate the influence of eight freeze-substitution protocols on the Ni content. Freeze-substitution of leaf samples cryofixed by high-pressure freezing was carried out in dry acetone, methanol, diethyl ether and tetrahydrofuran. Dimethylglyoxime was added to all substitution media as a precipitation reagent. The samples were infiltrated and embedded in Spurr's resin. Micro-PIXE analysis of Ni concentration and localization, complemented by proton backscattering for matrix assessment, was performed using the nuclear microprobe at Materials Research Group, iThemba LABS, South Africa. True elemental maps were obtained using GeoPIXE-II software.

The highest Ni content was found in leaf samples substituted in diethyl ether. This concentration was statistically different from results obtained for other media. Dimethylglyoxime did not improve the Ni preservation. In case of diethyl ether medium Ni was mainly localized in the mesophyl tissue, whereas tetrahydrofuran medium preserved Ni in epidermis and vascular tissue. It was impossible to assess Ni distribution in other solvents due to high Ni loss.

These results show that diethyl ether is a suitable substitution medium for Ni in leaves of *Berkheya coddii*. The content and distribution of Ni is in accordance with previous results obtained for freeze-dried leaves of this species.