METAL DISTRIBUTION AND QUANTIFICATION IN PLANTS LEAVES BY LA-ICPMS

MASTER CIENCIAS ANALÍTICAS Y BIOANALÍTICAS

UNIVERSIDAD DE OVIEDO

MASTER SCIENCES ANALYTIQUES POUR LE VIVANT ET L'ENVIRONNEMENT

UNIVERSITÉ DE PAU ET DES PAYS DE L'ADOUR

Jenifer García Fernández

Supervisors:

Marie-Pierre Isaure

Sandra Mounicou

ACKNOWLEDGMENTS

This research project would not be possible without the support of many people. I would like to express my gratitude to my supervisors Dr. Marie-Pierre Isaure and Dr. Sandra Mounicou, for making me feel involved in this project since the beginning. Really thankful for your comments, your availability at any time, your patience to teach me and the trust in me you always showed. Not only I have learnt a lot in the science field, but also I have had the chance to share my daily work with admirable people.

I also thank to Dr. Stéphanie Huguet, for her assistance and dedication, as well as her technical advices in the research work.

I would like to extend my sincere thanks to all the people who make LCABIE for helping me and making my stage really enjoyable. Some of them are closer to me than others but everyone had always a 'bonjour' smile for me and that is very important when you are far away from home.

I want to make special mention to my office mates at S202, Aníbal, Eirini, Virginia, Silvia, Arnau. If walls could talk… There were many hours spent there, many conversations, many good and bad moments but at the end I have always found the encouragement to follow inside these four walls and their ambiance.

And last but not least, I am really grateful to my family, the one from Pau, my "Spanish community" (Mónica, Silvias, Cristina, Virginia), for making this experience unforgettable, and the one from Asturias, my parents and my boyfriend, for being always my support in spite of the distance.

CONTENTS

1 INTRODUCTION

1.1 Abstract

Hyperaccumulator plants are complex family characterized by taking up high amounts of heavy metals in their roots, translocating them to the shoot to be finally accumulated in above-ground organs, especially leaves. One of their principal interests is due to the possibility of using this type of plants for phytoremediation of contaminated or natural metal-rich soils.

A new approach to quantify locally metals by LA-ICP-MS technique in these plants is presented using external calibration with addition of yttrium as internal standard with SRM NIST of tomato pressed pellets.

On the other hand, a study of the metal distribution in *Arabidopsis halleri* leaves is performed by the same technique and elemental mapping is compared for fresh and freeze-dried leaves, young and mature leaves as well as for thin sections.

1.2 Interest of research

Industrial development has been always accompanied by an increase of the environmental pollution. Toxic elements and compounds are found in higher levels in soil, water and biological tissues, due to the mobility of heavy metals and their ability to get into the animal and human body through the food chain. The most dangerous and also intensively tracked elements are Pb, Cd, Ag, Hg, As, among others.

In the recent years it seems like we are getting more and more conscious about how severe the damage is and what we could do to try to mitigate it as much as possible. That is the reason why the search for eco-friendly techniques and sustainable development has increased a lot.

As it happens very often, if we have a look to the nature itself we discover that some organisms have developed mechanisms for surviving in harsh conditions and what is more remarkable, without causing any damage to their environment. That is the case of plants with high metal uptake ability which are able to accumulate high amounts of toxic species without suffering phytotoxic effects.

There are different kinds of plants with this ability:

- **Metal tolerant plants**, which have the ability to survive and reproduce on soils containing high concentrations of metals in forms that are toxic or inimical to other plants.
- **Metal hyperaccumulating plants**, with the additional property of storing large amounts of metals in their aerial parts (more than 10 ppm typically and 100-1000-fold higher than those found in non-hyperaccumulating species, depending on the element) and another different hallmarks in comparison to previous ones as a faster root-to-shoot translocation and a greater ability to detoxify and sequester heavy metals in leaves.

In this project we have been working with the last type mentioned, the hyperaccumulator ones and *Arabidopsis halleri*, specifically. It is one of the closest relatives to *Arabidopsis thaliana* (Koch et al., 2001), whose genome is entirely sequenced (Meinke et al.,1998; Kaul et al.,2000). This known information added to the huge amount of literature disposed about *Arabidopsis*, makes a study of hyperaccumulator plant's behaviour easier. *A. halleri* is a pseudo-metallophyte, which means that it is found both in polluted and non-polluted areas. It is shown as a Zn hyperaccumulator, but some studies showed that it can also hyperaccumulate cadmium (Dahmani-Muller et al., 2000; Küpper et al., 2000; Bert et al., 2002).

These hyperaccumulator plants are used for a process called phytoremediation which includes all technologies that plants can use to reduce, remove, degrade or immobilize environmental toxins. The most important aims to achieve by this mechanism are to reach maximum accumulation of potentially toxic compounds to hyperaccumulator plants and also, restore area sites to a condition useable for private or public applications.

Phytoremediation (or phytoextraction referring to the step of uptaking contaminants by plant roots and moving them to the aboveground parts of the plant) can be produced in a natural or induced way depending on the conditions of the soil where the plants are growing.

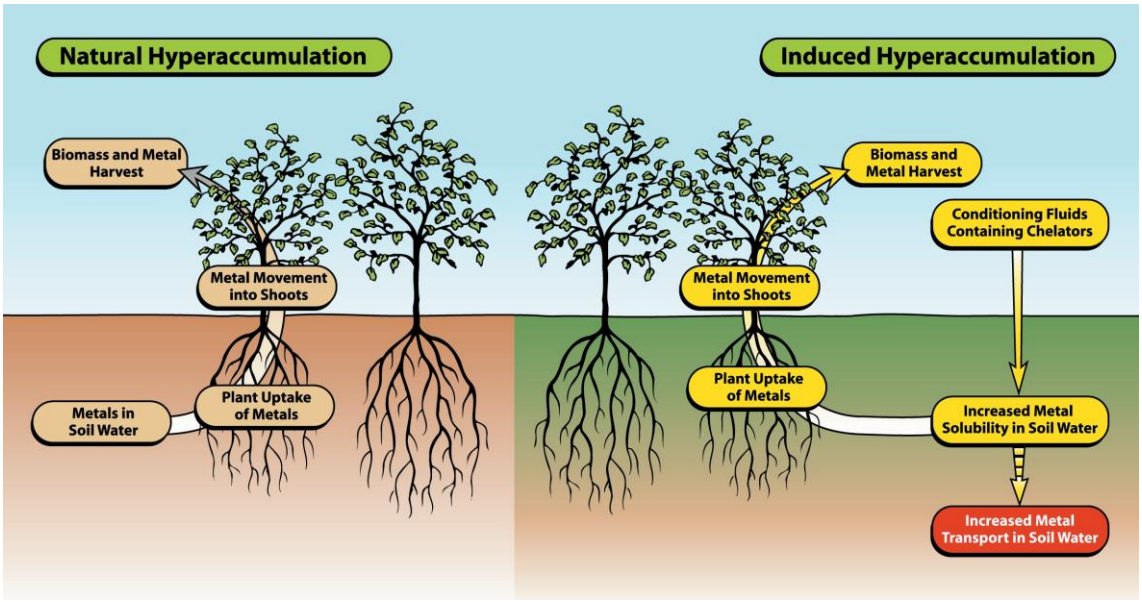


Figure1. Natural and induced phytoextraction.
Source: Pierzynski et al., 2001, Copyright Kansas State University

The advantages offered by this mechanism are:

- The possibility of trapping metal contaminants in mobile chemical forms, which are the most threatening to human and environmental health.
- It is typically less costly than excavation although costs depend on site-specific conditions. An approximate range goes from 16 to 62 € /m^3 of soil treated compared to nearly 600 € /m^3 of excavation processes (J. Wiley & Sons, *Wiley's Remediation Technologies Handbook: Major Contaminant Chemicals and Chemical Groups,2004*).
- It is definitely the most aesthetically pleasant to the public compared to alternate remediation strategies involving excavation/removal or chemical *in situ* stabilization/conversion.

However, there are also some drawbacks and limitations associated with this phenomenon:

- It takes longer than other technologies, and usually many crops are needed to remove all the contaminants to the desired levels.
- There are no so many plants which can be applied for this purpose and the conditions to make them grow are very strict (not under submerged or wetland, for instance). Moreover, in most of cases, plants which are good phytoextraction candidates are not native to the area, so it can become a problem for the ecosystem.
- Accumulation of contaminants in the aboveground part of the plants may suppose a risk for animals eating these plants.
- It is not possible to remove organic contaminants (PCBs, PBDs…) directly from soils and sediments, although there are evidences to ensure that microbial activity associated with plant roots may accelerate the degradation of these contaminants to non-toxic forms.

Over the last decade, comparative physiological, genomic, and proteomic studies of hyperaccumulators and non-hyperaccumulator plants led to a significant progress in understanding of the mechanisms governing metal hyperaccumulation. However, there are still some uncertainties and hypotheses proposed to explain the function of hyperaccumulation.

Our research is focused on going one step further in the understanding of these complex mechanisms developed by such plants to resist metal toxicity and contribute to make this technology more competitive for restoring polluted areas.

1.3 Objectives

The main aim of this research project is to map and quantify metals (Cd, Zn and Pb) accumulated in leaves of Zn and Cd hyperaccumulator plant (*Arabidopsis halleri*) grown in the field. For that purpose, an analytical method using Laser Ablation coupled to Inductively Coupled Plasma Mass Spectrometry (LA-ICP MS) has to be developed and optimized mainly at two levels: (1) setting up a quantification strategy by preparing matrix-matched standards and (2) setting of laser ablation parameters and sampling strategy.

1.4 Context

1.4.1 Metals accumulation

1.4.1.1 Zinc

Some heavy metals like zinc are considered oligo-nutrients and thus essential in small quantities for normal plant development. However, at metalliferous places, such metals can occur at highly elevated concentrations in the soil, either through ancient natural processes or through recent human activities. The total metal content of contaminated sites can be up to 10- to 1000-fold higher than non-contaminated ones. Those extreme conditions explain that even the essential heavy metals become toxic for any organism except for this small number of plant species which have evolved tolerance to such concentrations.

Although there are still some doubts about how these plants can handle such huge concentration of a heavy metal and what they do with it, recent studies using scanning electron microscopy and energy-dispersive x-ray microanalysis documented the cellular distribution of Zn in *A. halleri* tissues (Küpper et al., 2000; Zhao et al., 2000). It is known that in the leaves, Zn was mostly sequestered in the base of trichomes and in mesophyll cells. Trichomes are epidermal hairs present at the surface of plant leaves and their function can be as diverse as the exudation of various molecules, the protection against the wind and sunlight, or the storage of metals (Rodriguez et al., 1983).

Referring to concentration levels, previous studies performed in our lab with *A. halleri* leaves shows that the content of Zn is in the range of 2,5 % Zn, more or less concentrated depending on the age of the leaves (more for mature leaves, less for young ones in most of the cases as we can see in the next figure).

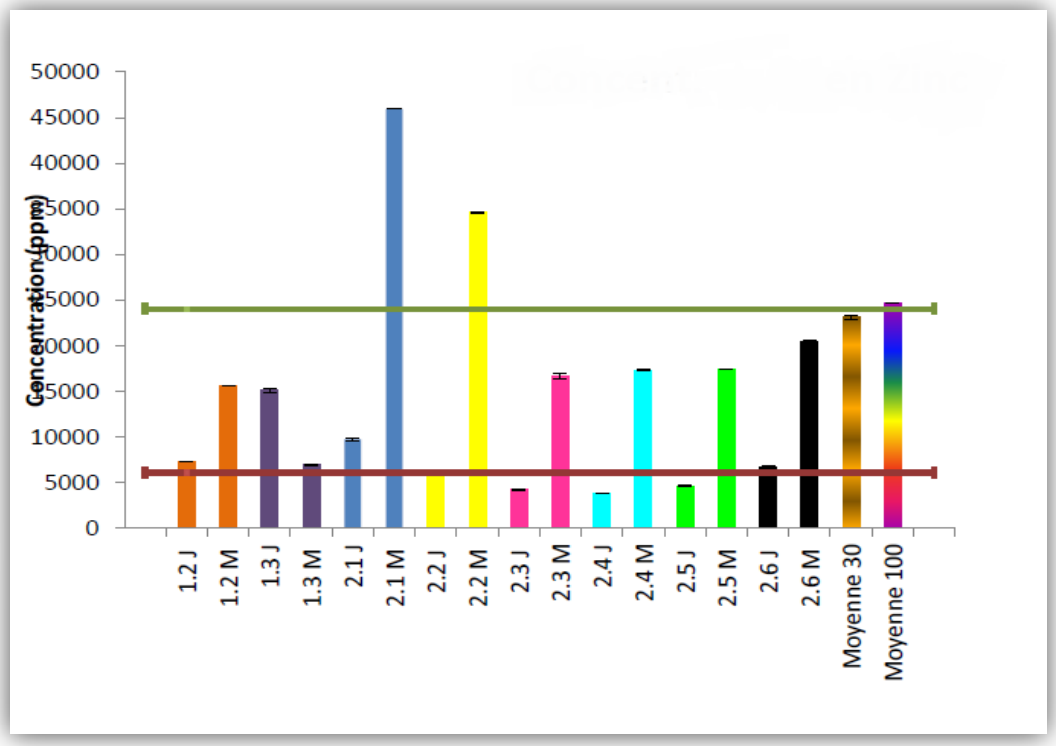


Figure 2. Zn concentration levels for A. halleri leaves (J= young leaves; M=mature leaves)

1.4.1.2 Cadmium

Nowadays, still minimal information is available on cadmium accumulation in plants, as hyperaccumulation of Cd is a very rare phenomenon due to its nonessential nature and high phytotoxicity to plants. Cd hyperaccumulation is defined as the accumulation and tolerance of up to 100 µg Cd g^{-1} in shoots by plants (Baker et al., 2000). It is preferentially partitioned in roots of normal plants but translocated to the aerial parts of the hyperaccumulators.

According to concentration, previous studies performed in our lab confirm that Cd is in the range of 150 ppm, and it fluctuates again depending on the age of the leave.

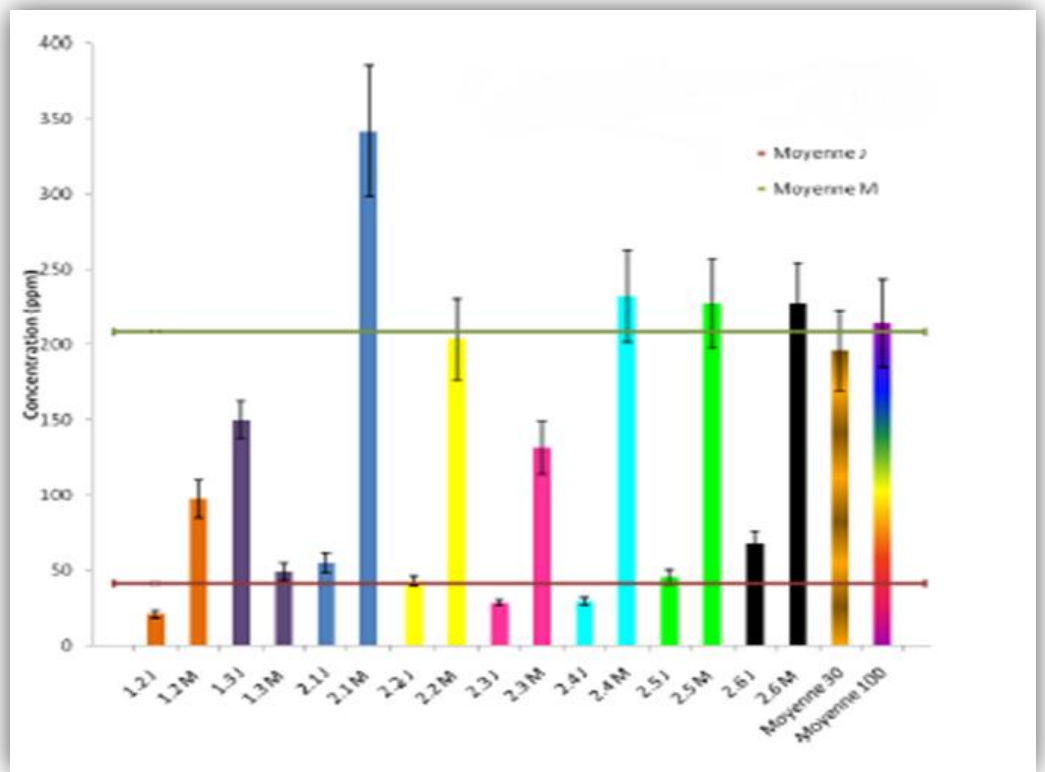


Figure 3. Cd concentration levels for A. halleri leaves (J= young leaves; M=mature leaves)

1.4.1.3 Lead

This element is highly toxic and it is dangerous already at low content (30-300 µg g^{-1}). Lead is accumulated mainly in roots in comparison with its content in the other parts of plant.

1.4.2 LA-ICPMS Technique

1.4.2.1 Introduction

Mass spectrometry is one of the most analytical techniques used today for the determination of element concentrations down to the trace and ultra trace level, for isotope analysis, surface characterization, and structural analysis of organic and bioorganic compounds due to its very high sensitivity and low detection limits (Becker, 2007).

In 1985, a Laser Ablation device was associated for the first time with an ICP-MS for the determination of trace element abundances in solids; since then LA-ICP-MS has been increasing its importance as an analytical tool for all type of sciences. The potential of laser radiation to cause ablation and vaporization when it interacts with solid materials is exploited as a method of sample introduction for mass spectrometry. The different species produced, including particulates, ground-state atoms, excited atoms, and ions, are used for elemental analysis via mass spectrometry. The interaction of laser light with the sample depends on the characteristics of the laser beam and the physical properties of the solid.

Respect to other analytical techniques at trace levels, some of the advantages offered by this technique are:

- Minimal sample preparation.
- High sample throughput (depending on the surface to scan and resolution wanted).
- Relative high spatial resolution at the sub-mm scale (to 20 µm).
- Elemental mapping across the surface of a sample.
- High sensitivity and detection limits below the ppm level.
- Analysis of a wide variety of samples (biominerals, gems, environmental wastes, biological structures, etc).

However, the main disadvantage of techniques that uses laser ablation for sampling is a lack of certified reference materials for calibration and for this reason different calibration strategies have been developed up to now.

1.4.2.2 LA-ICPMS for quantification

If every ablation on every sample transports the same amount of material to the ICP-MS, quantification would be definitely easier. But it is not the case. Actually, composition of ablated mass can be different from the composition of the solid sample and on the other hand, composition of ablated mass and sample can be the same under particular laser conditions. It

is known as elemental fractionation or non-stoichiometric effects and it is one of the main drawbacks of the laser technique.

There are different methods for quantitative analysis:

- External calibration with solid standard (matrix-matched): requires reference materials that are closely matrix-matched to samples to compensate for large differences in ablation yield (from fluctuations in laser output and small differences in target matrix).
- External calibration with solid standard combined to internal standardization: it allows correction for differing ablation yield between sample and reference materials, and it also provides correction for matrix effects and signal drift. Internal standard must have similar ablation characteristics to analytes (generally a major constituent).
- External calibration with solution standards combined to internal standardization: carrier gas flow exiting the ablation cell is mixed with nebulised aerosol prior to entry into ICP. Blank solution is also nebulised during sample ablation for consistent ICP conditions.
- Isotope dilution mass spectrometry combined to LA-ICP-MS: consisting of an addition of the corresponding isotope-enriched spike solutions to the sample. For quantification, only one isotope ratio per analyte must be measured in the isotope diluted sample. This internal standardization corrects all signal variations during analysis, such as instrumental drift or varying mass ablation rates.

Our calibration strategy for quantication has consisted on an internal standardization with Yttrium as well as external calibration with Cd, Zn and Pb. Furthermore data are normalized by the ratio between ^{89}Y and ^{13}C, that represents the content of ablated mass, in order to minimize signal drift.

1.4.2.3 LA-ICPMS Instrumentation

The ICP-MS standard instrument configuration consists of a plasma (luminous volume of partially ionized gas) as ion source, generated from radio frequency magnetic fields induced by a copper coil, wounded around the top of a partly demountable glass torch, with removable injector. Nickel cones, in this case, (sampler and skimmer), represent the interface for extracting ions from the plasma and transferring them in the region of the mass spectrometer.

A quadrupole, which progressively selects ions with specific mass to charge ratio, is used as mass analyser. Ions are detected by a Channel Electron Multiplier (CEM), at the end of which a computer collects and process the data.

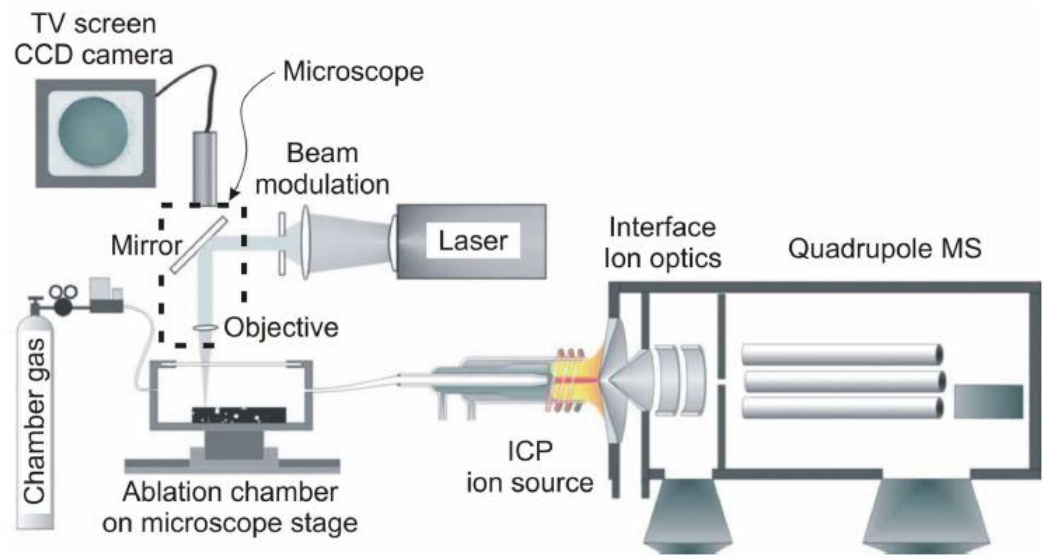


Figure 4. Scheme of a LA-ICP-MS equipment

The laser ablation system consists of an Yttrium Aluminium Garnet ($Y_3Al_5O_{15}$) rod doped with approximately 3 wt% Nd_2O_3, working at wavelength of 213 nm. It is a Q-switched laser characterized by a single large output pulse which releases the energy stored in the cavity as shorter pulse of nanoseconds with both higher average and peak power. The output of a Q-switched Nd:YAG laser is a train (typically 1-20 Hz) of high intensity pulses. The laser beam is highly directional, has a very high wavelength purity and strong spatial and temporal coherence that results in enhanced ablation efficiency and a representative composition of the material ablated. The maximum repetition rate of the laser can generally be used (10-20 Hz normally) with lower pulse frequencies being used to ablate solid samples, to reduce count rates for very concentrated samples or to produce a prolonged stable signal when required.

The laser ablation device is equipped with a video camera for the high resolution sample viewing, ensuring an accurate focusing of the sample and an easier identification of the areas to be analyzed. In our instrumental setup, helium is used as carrier gas to transport the aerosol to ICP-MS.

Data were collected in time-resolved analysis mode.

2 Materials and methods

2.1 Reference materials

As it was said, lack of reference materials is usually a problem for quantification by LA-ICPMS. In this work, we tend to simulate the matrix of *A. halleri* as much as possible by working with two different standards: NIST 1570a (spinach leaves) and NIST 1573a (tomato leaves) reference materials, certified by the National Institute of Standards and Technology.

For both standard reference materials, concentrations of elements of interest are given below.

Table 1. Zn and Cd concentration in NIST reference materials

	Zn (mg/Kg)	Cd (mg/Kg)
NIST 1573a (Spinach leaves)	82 ± 3	2.89 ± 0.07
NIST 1570a (Tomato leaves)	30.9 ± 0.7	1.52 ± 0.04

In the case of lead, it is not given in any case because the consortium didn't find any agreement.

2.2 Quantification strategy

As a quantification strategy we have chosen an external standard calibration with SRM NIST 1573a and Yttrium as internal standard.

2.2.1 Sample preparation

Standard preparation for quantification was performed with SRM NIST 1573a tomato leaves for the standard calibration of the analytical data. 0.5 g of tomato leaves powder was put in solution and mixed with different volumes of an only solution of 80000 mg/Kg Zn, 800 mg/Kg Cd and 200 mg/Kg Pb in order to get a calibration curve with five standards. On the other hand, 1 ppm yttrium solution was prepared and added in the same way for all the standards (volume of 500 µL). After stirring and homogenization they were dried at 60 ºC for

24 hours to be ground into fine powder. Approximately 0.017 g of powder was weighed to make pressed pellets (trying to get similar thicknesses) without any binder under 2-atm pressure.

It was also taken SRM NIST 1570a spinach leaves in order to validate the analytical procedure and the same protocol was applied except for the addition of the multielemental solution.

For *A. halleri* samples, collected on a Zn, Cd and Pb contaminated site, 12 medium size leaves of each plantpot in the lab were taken (to assess the reproducibility of analysis), washed with deionized water and dried in the oven at 55 ºC during 24 h. After the first 2 h, loss of water was already 86 %. Then it still lost a bit more up to the 87 % at the end of the established total period of time. Dried leaves turned to fine powder by grinding and 0.5 g was weighed to add Y solution afterwards. After drying the mixture (oven 60ºC, 24 h) and grinding, pressed pellets were made in the same way.

Figure 5. A. halleri grown in contaminated fields with high concentration levels of Zn, Cd and Pb.

The pelletized materials and plant samples were analyzed for elemental concentrations by LA-ICP-MS and then subjected to further digestion and element determination by ICP-MS to compare concentrations and evaluate quality of measurements.

The protocol followed for digestion has consisted in 1 mL H_2O_2 60 % and 1 mL HNO_3 70% under 3h of digestion program at 80 ºC. After that, samples were filtered with a 25 mm diameter syringe filter of cellulose acetate membrane with 0.45 µm of retention.

2.2.2 Instruments parameters

Quantification and element distribution of selected metals in *A. halleri* were determined using a quadrupole ICP-MS (Agilent 7500) with the laser UP 213 ablation system from New Wave with a lateral resolution in the 12 µm range.

The isotopes ^{13}C, ^{57}Fe, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{89}Y, ^{111}Cd, ^{112}Cd, ^{113}Cd, ^{114}Cd, ^{206}Pb, ^{208}Pb were recorded. Ratio between $^{89}Y/^{13}C$ was used as internal standard to compensate water content effect and the possible inhomogeneity of the pellets.

Figure 6. ICP-MS Agilent 7500

Figure 7. New Wave Research UP Series 213 Laser Ablation

For the calibration by LA-ICP-MS, optimized parameters have been:

Table 2.ICP-MS AGILENT 7500

Carrier gas flow (Ar)		0.73 L min^{-1}
Nebulizer pump		0.15 rps
Ion lenses	Omega Bias-ce	-44 V
	Omega Lens-ce	9.8 V
	Cell entrance	-38 V
	QP Focus	2 V
	Cell Exit	-68 V
Octopole Parameters	OctP RF	198 V
	OctP Bias	-9 V
Reaction cell mode		Off
Integration time		0.1000 sec
RF Power		1600 W

Table 3.New Wave Research UP Series 213 Laser Ablation

Carrier gas flow (He)		0.50 L min^{-1}
Energy output		60 %
	Sample	0.024 mJ
	Fluence	2.15-2.45 J cm^{-2}
Spot size		40 µm
Laser frequency		20 Hz
Scan speed		50 µm sec^{-1}

Ablation was done in raster mode by ablating three successive lines for each sample.

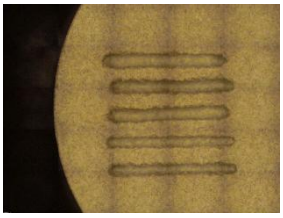


Figure 8. Ablated lines in NIST spinach leaves pressed pellet

Optimized parameters after pellets digestion have been:

Table 4.ICP-MS AGILENT 7500

Carrier gas flow (Ar)		1.1 L min^{-1}
Nebulizer pump		0.15 rps
Ion lenses	Omega Bias-ce	-20 V
	Omega Lens-ce	1.2 V
	Cell entrance	-40 V
	QP Focus	-10 V
	Cell Exit	-80 V
Octopole Parameters	OctP RF	190 V
	OctP Bias	-18 V
Reaction cell mode		On
	H_2 gas	1.7 mL min^{-1}
Integration time		0.1000 sec
RF Power		1600 W

2.3 Chemical mapping strategy

To achieve one of our objectives of localizing where and how the metals were distributed in *A. halleri* leaves, different preparations have been made for young and mature leaves in order to check if there is any difference between elements. At the end, they were all analyzed in imaging mode by LA-ICP-MS.

2.3.1 Sample preparation

Young and mature leaves were cut from *A. halleri* plants grown in the lab. All of them were washed with deionized water and dried with a paper. Some of them were analysed by LA-ICP-MS just after and they correspond to fresh leaves samples.

For freeze-dried treatment, washed leaves were put in the freezer at -20ºC for 6 hours and then put in the lyophilizer at -80ºC and $\sim 32x10^{-6}$ torr over a night. They are the freeze dried samples ready for LA-ICP-MS analysis.

Figure 9. Lyophilizer Cryotec

Finally thin sections were performed using Tissue-Tek OCT Compound, a formulation of water-soluble glycols and resins that provides a convenient specimen matrix for cryostat sectioning at temperatures of -10 ºC and below. It was put in small plastic vials covering the leave and then placed in a container of liquid nitrogen. Once totally cryogenized it was ready to make thin sections (50 µm of thickness) with the cryomicrotome (temperature sample rack: -20 ºC; temperature cold room: -25 ºC) and the chemical mapping by LA-ICP-MS.

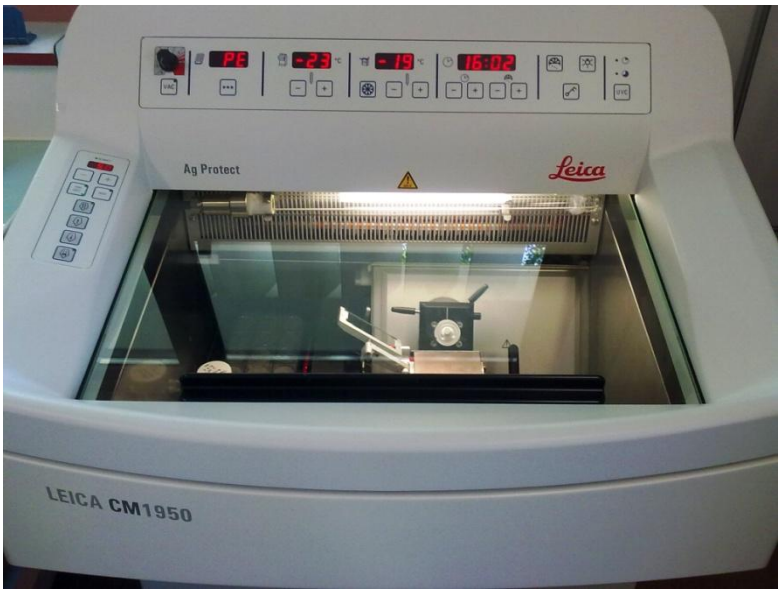


Figure 10. Cryomicrotome LEICA CM1950

2.3.2 Instrument parameters

For imaging studies, optimized parameters in the ICP-MS equipment have been the same as for the quantification strategy while the Laser ablation system parameters have been slightly changed (related to energy output, spot size and scan speed) depending on the size of samples to map.

For fresh and freeze-dried leaves:

Table 5.New Wave Research UP Series 213 Laser Ablation		
Carrier gas flow (He)		0.50 L min^{-1}
Energy output		65 %
	Sample	0.075 mJ
	Fluence	5.80-6.54 J cm^{-2}
Spot size		40 µm
Laser frequency		20 Hz
Scan speed		50-70 µm sec^{-1}
Distance between lines		150 µm

For thin sections:

Table 6.New Wave Research UP Series 213 Laser Ablation		
Carrier gas flow (He)		0.50 L min^{-1}
Energy output		65 %
	Sample	0.005 mJ
	Fluence	4.29-4.43 Jcm^{-2}
Spot size		12 µm
Laser frequency		20 Hz
Scan speed		50-70 µm sec^{-1}
Distance between lines		40 µm

3 Results and discussion

First of all we have set the optimized parameters of the laser with NIST standards pellets and we have established the best isotope intensities according to abundances, polyatomic interferences and significant intensity values.

Table 7. Polyatomic interferences in ICP-MS Atomic Spectroscopy vol.19(5), sept./oct. 1998

Isotope	Abundance, %	Interference
^{110}Cd	12.5	$^{39}K_2{}^{16}O^+$
^{111}Cd	**12.8**	$^{95}Mo^{16}O^+$, $^{94}Zr^{16}O^1H^+$, $^{39}K_2{}^{16}O_2{}^1H^+$
^{112}Cd	24.1	$^{40}Ca_2{}^{16}O^+$, $^{40}Ar_2{}^{16}O_2$, $^{96}Ru^{16}O^+$
^{113}Cd	12.22	$^{96}Zr^{16}O^1H^+$, $^{40}Ca_2{}^{16}O_2{}^1H^+$, $^{40}Ar_2{}^{16}O_2{}^1H^+$, $^{96}Ru^{17}O^+$
^{114}Cd	28.7	$^{98}Mo^{16}O^+$, $^{98}Ru^{16}O^+$
^{206}Pb	24.1	$^{190}Pt^{16}O^+$
^{208}Pb	**52.4**	$^{192}Pt^{16}O^+$
^{64}Zn	**48.89**	$^{32}S^{16}O^+$, $^{48}Ti^{16}O^+$, $^{31}P^{16}O_2{}^1H^+$, $^{48}Ca^{16}O^+$, $^{32}S_2{}^+$, $^{31}P^{16}O^{17}O^+$, $^{34}S^{16}O_2{}^+$, $^{36}Ar^{14}N_2{}^+$
^{66}Zn	27.81	$^{50}Ti^{16}O^+$, $^{34}S^{16}O_2{}^+$, $^{33}S^{16}O_2{}^1H^+$, $^{32}S^{16}O^{18}O^+$, $^{32}S^{17}O_2{}^+$, $^{33}S^{16}O^{17}O^+$, $^{32}S^{34}S^+$, $^{33}S_2{}^+$

These are some of the spectra registered with different isotope intensities:

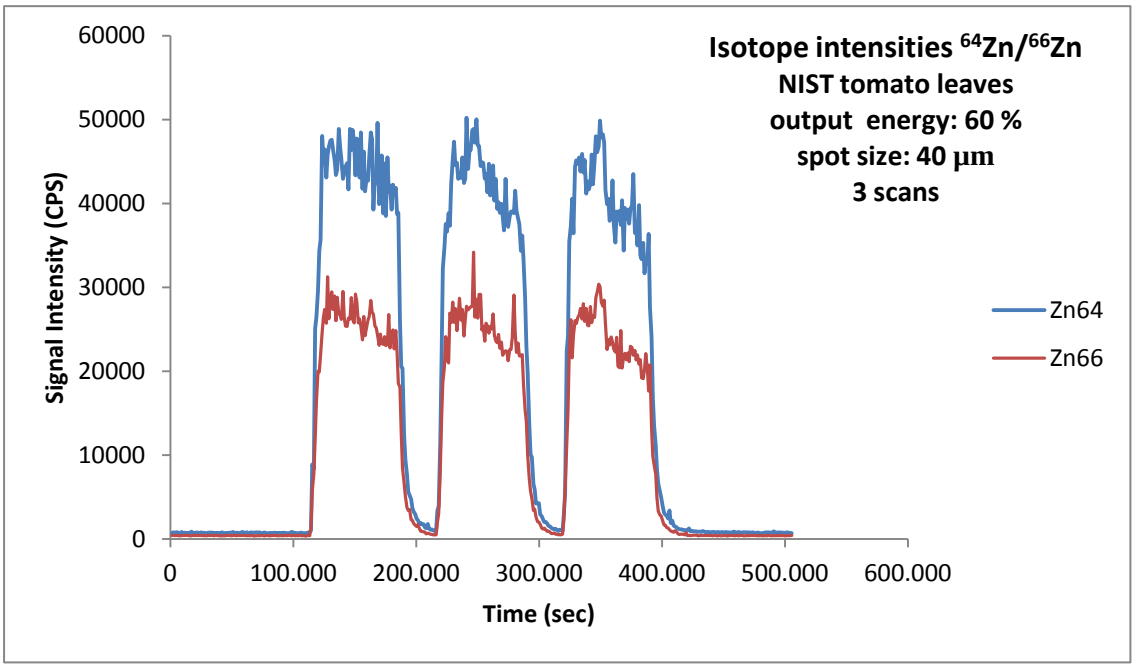


Figure 11. Triplicate analysis of a Tomato leaves NIST ablated pellet. Zn signal Intensity (counts per second) Vs Time (seconds).

As we can see in the graph, ^{64}Zn intensity is higher than ^{66}Zn and it also the most abundant isotope so it was the chosen isotope for data treatment in calibration

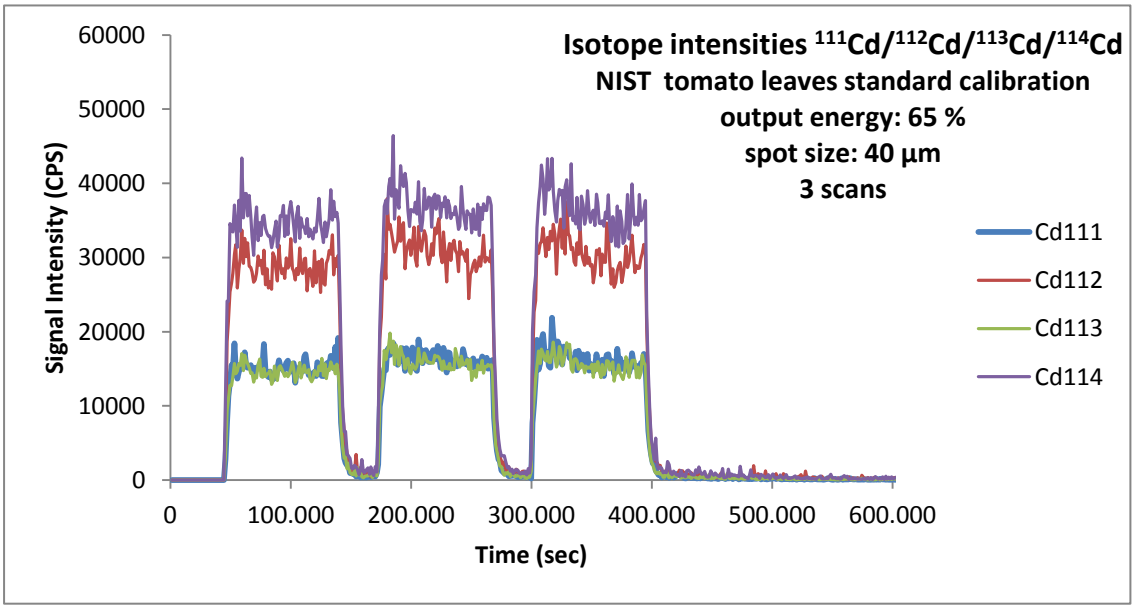


Figure 12. Triplicate analysis of one of the calibration standards ablated pellet. Cd signal Intensity (counts per second) Vs Time (seconds).

In this case, ^{111}Cd is not the most intense but the polyatomic interferences are less probable at this mass (m/z) so it was the chosen isotope for data treatment.

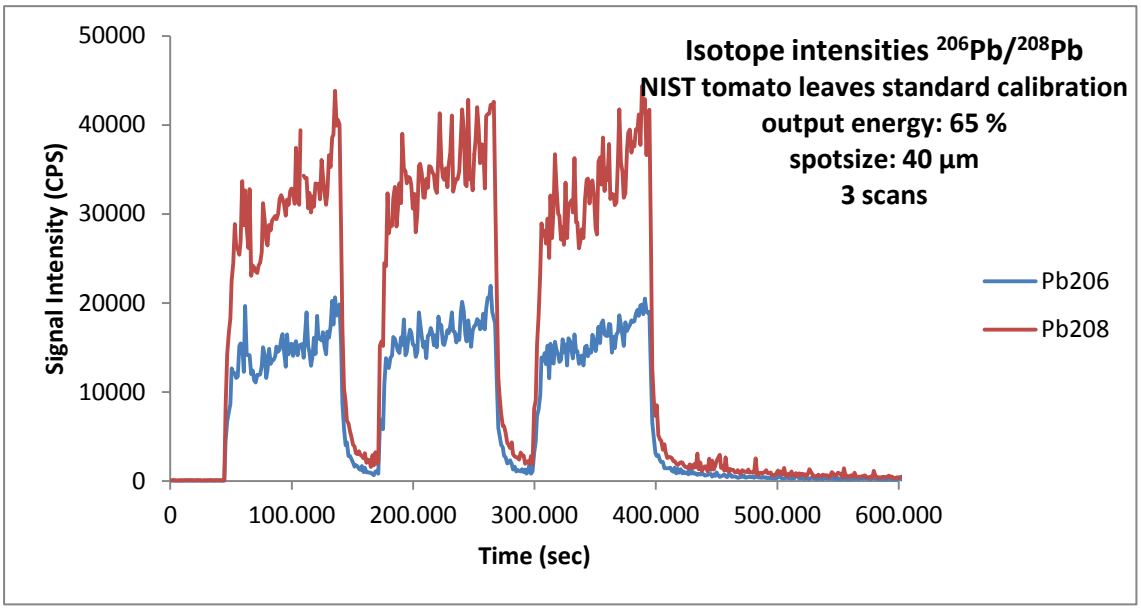


Figure 13. Triplicate analysis of one of the calibration standards ablated pellet. Pb signal Intensity (counts per second) Vs Time (seconds).

In the case of lead, ^{208}Pb is the most intense and also the most abundant and with less polyatomic interferences for our analysis, so all make it the best choice for data treatment.

Here it is also presented ^{89}Y and ^{13}C profiles, since they are the signals that were taken to normalize isotope intensities for the calibration. As Y signal is not always stable, ^{13}C intensity helps to compensate signal drift.

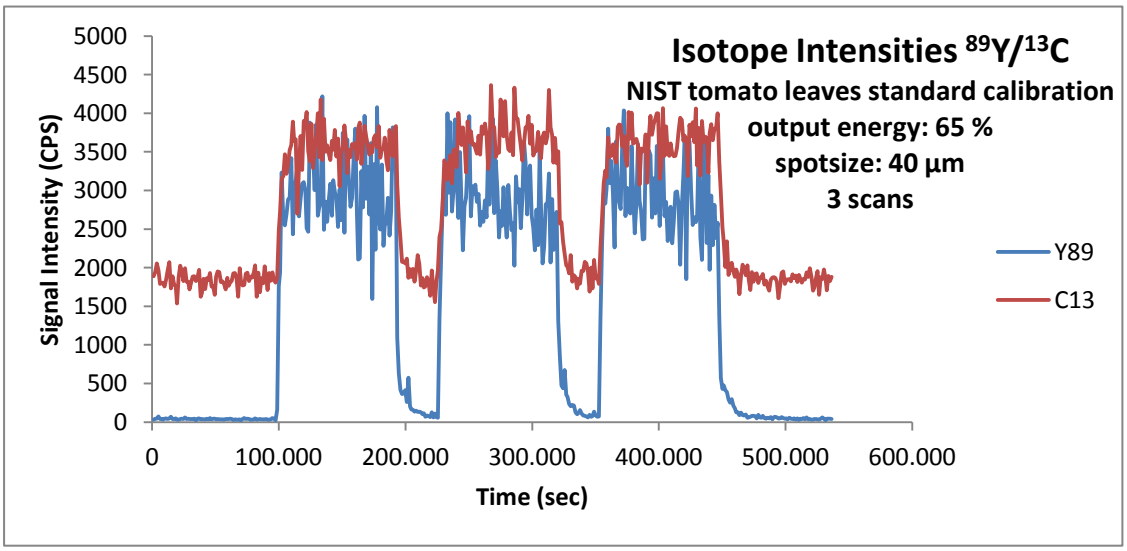


Figure 14. Triplicate analysis of one of the calibration standards ablated pellet. ^{89}Y and ^{13}C signal intensities (counts per second) Vs Time (seconds)

3.1 Calibration

In this study we have tried to introduce Y as internal standard in our calibration and the results obtained with this strategy are recorded in the table below.

Table 8. Calibration data with addition of Y as internal standard corresponding to standards and samples (spinach leaves as reference material and A. halleri leaves).

standard	Zn, mg/Kg	Intensity $^{64}Zn/(^{89}Y/^{13}C)$	Cd, mg/Kg	Intensity $^{111}Cd/(^{89}Y/^{13}C)$	Pb, mg/Kg	Intensity $^{208}Pb/(^{89}Y/^{13}C)$
0	30,9	2125,401	1,52	71,575	0	400,236
1	5030,9	1151434,096	51,52	12124,362	12,5	29855,956
2	10030,9	2526875,025	101,52	23695,248	25	67218,761(*)
3	20030,9	1757919,628(*)	201,52	18652,892(*)	50	65672,664
4	40030,9	7365694,156	401,52	79229,889	100	153093,640
Spinach +yttrium	**1008,937**	190295,567	**5,749**	1150,049	**111,925**	167730,336
***A.halleri* +yttrium**	**2301,740**	434131,118	**14,555**	2911,531	**7,550**	11314,622

(*)Data not included to obtain calibration curve

Having a look to the results obtained for NIST CRM spinach leaves, we detect some alterations in comparison to the real NIST concentration values. So we try another calibration strategy just normalizing intensity signals to the ^{13}C intensity (it represents the amount of ablated mass). Results are presented in table 9.

Table 9. Calibration data with intensity signals normalized to ^{13}C intensity signal.

standard	Zn, mg/Kg	Intensity $^{64}Zn/^{13}C$	Cd, mg/Kg	Intensity $^{111}Cd/^{13}C$	Pb, mg/Kg	Intensity $^{208}Pb/^{13}C$
0	30,9	1,209	1,52	0,041	0	0,228
1	5030,9	85,463	51,52	0,900	12,5	2,216
2	10030,9	183,625	101,52	1,722	25	4,885
3	20030,9	518,154(*)	201,52	5,498(*)	50	19,357(*)
4	40030,9	570,504	401,52	6,137	100	11,858
spinach	**186,440**	1,266	**2,703**	0,042	**1,845**	0,229
A.halleri	**4997,648**	72,466	**24,800**	0,382	**3,612**	0,447

(*)Data not included to obtain calibration curve

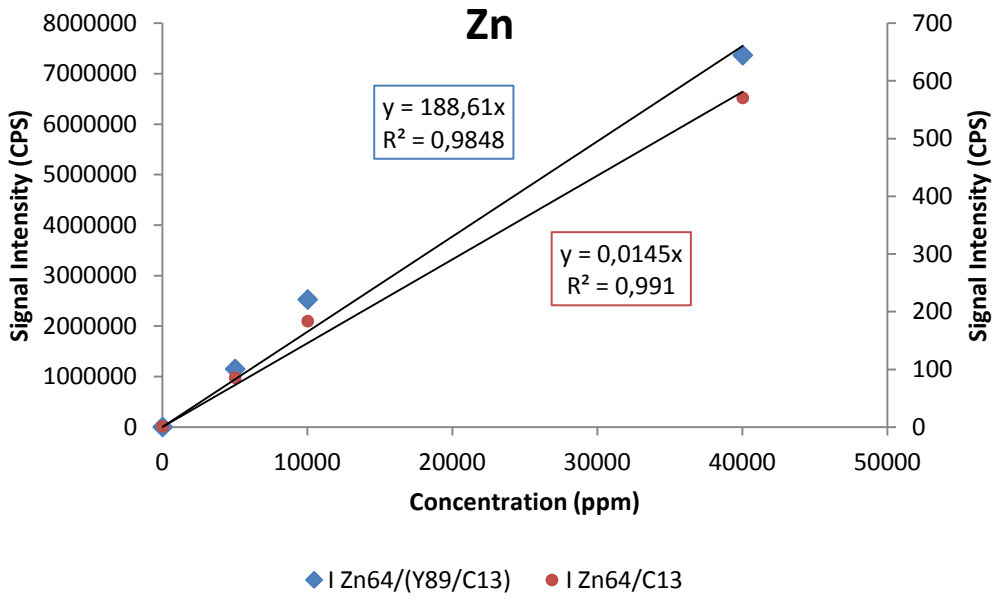


Figure 15. Calibration curves for Zn normalizing isotope intensities by $^{89}Y/^{13}C$ and to ^{13}C respectively.

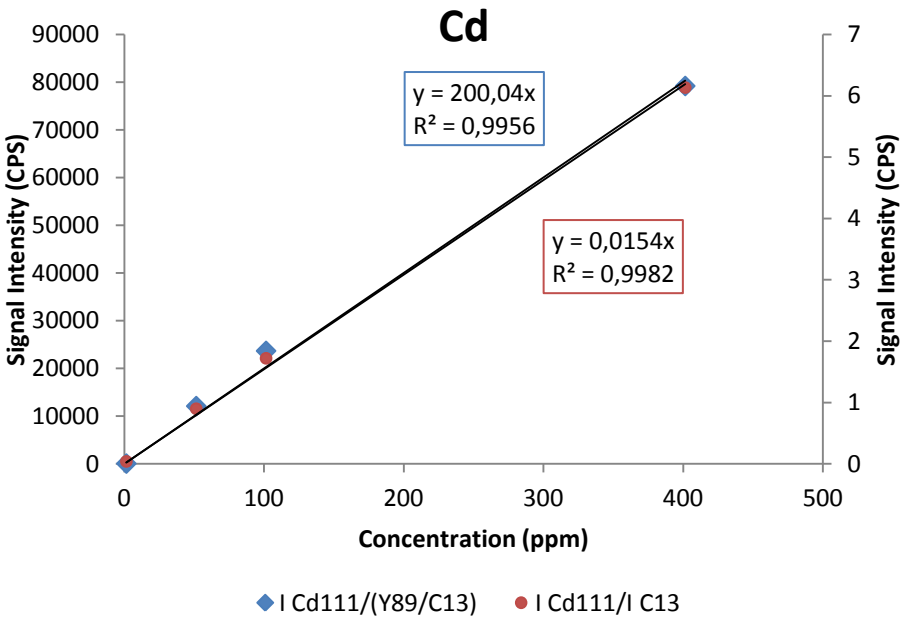


Figure 16. Calibration curves for Cd normalizing isotope intensities by $^{89}Y/^{13}C$ and to ^{13}C respectively.

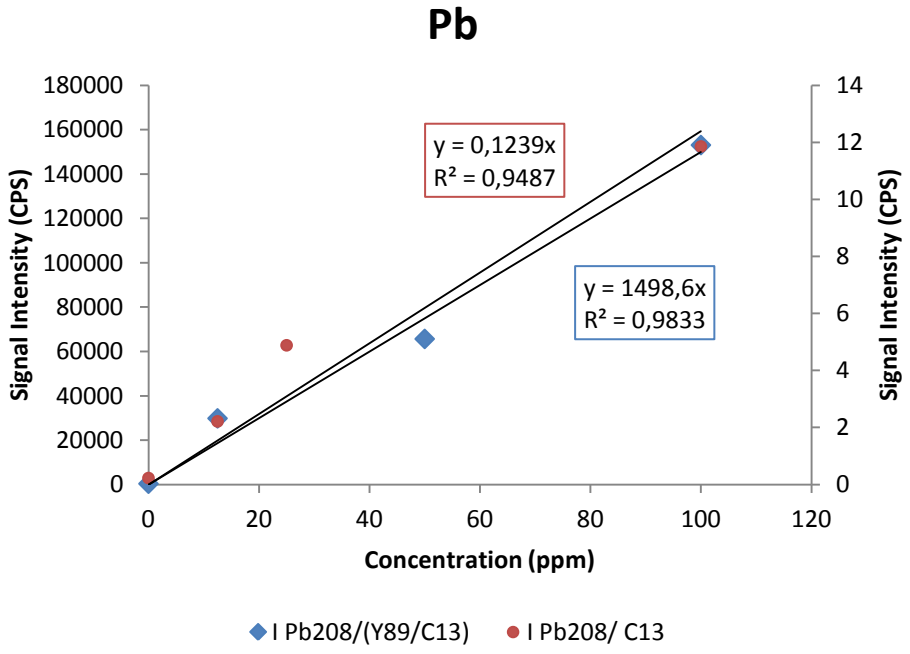


Figure 17. Calibration curves for Cd normalizing isotope intensities by $^{89}Y/^{13}C$ and to ^{13}C respectively.

These results are substantially better for SRM NIST spinach leaves concentration for all the elements (Zn, Cd and Pb), but they differ quite a lot from *A. halleri* concentration values obtained in the first case.

Table 10. Concentration values obtained from different normalization patterns

SPINACH LEAVES	**Zn mg/Kg**	**Cd mg/Kg**	**Pb mg/Kg**
NIST	82	2,89	0
Normalized by $^{89}Y/^{13}C$	1008,937	5,749	111,925
Normalized to ^{13}C	186,44	2,703	1,845
A. HALLERI			
Normalized by$^{89}Y/^{13}C$	2301,74	14,555	7,55
Normalized to ^{13}C	4997,648	24,8	3,612

Digestion of the pellets followed by ICP-MS analysis was performed to compare.

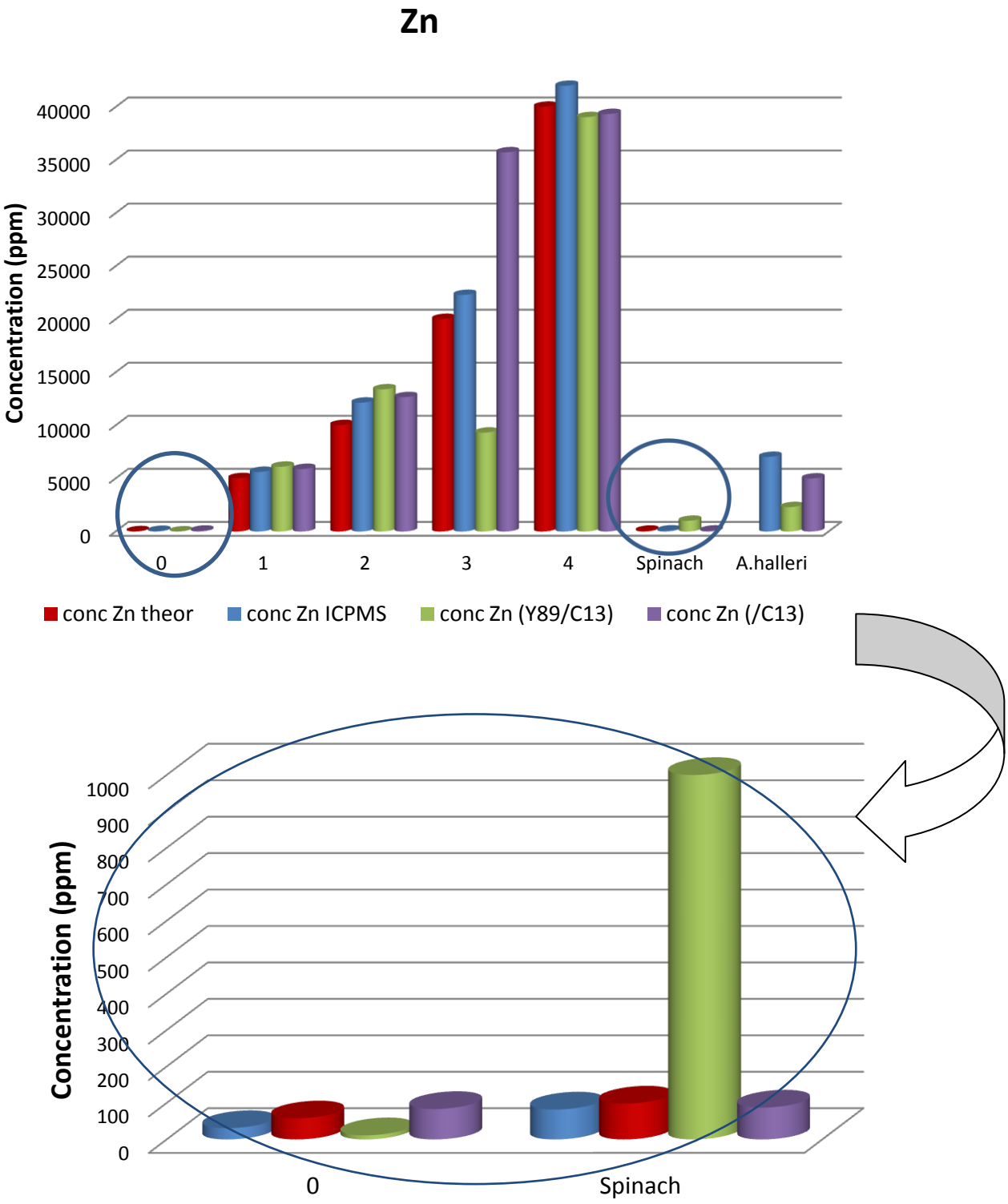


Figure 18. Bar graph comparing different strategies developed for extracting Zn concentration values.

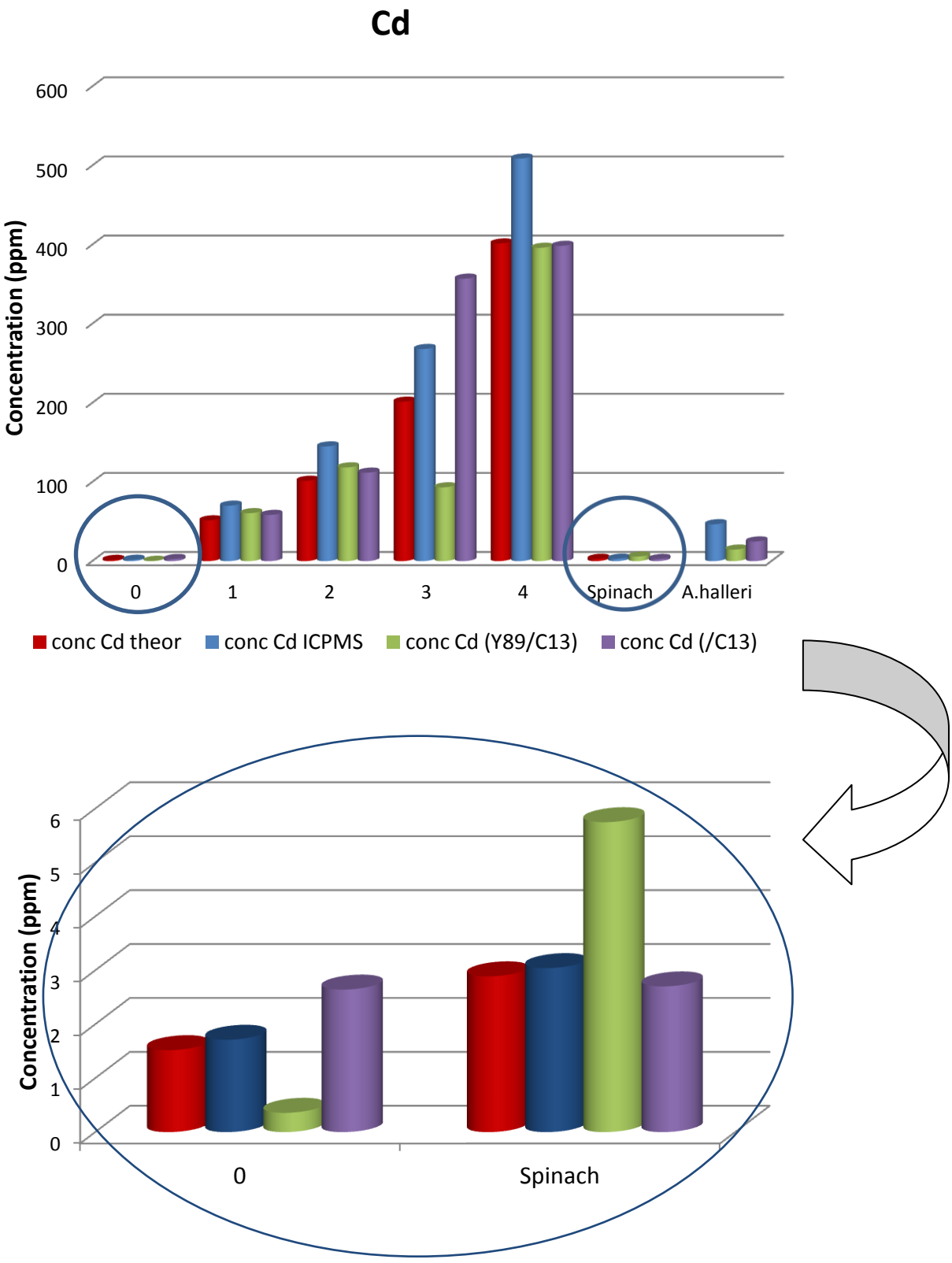


Figure 19. Bar graph comparing different strategies developed for extracting Cd concentration values.

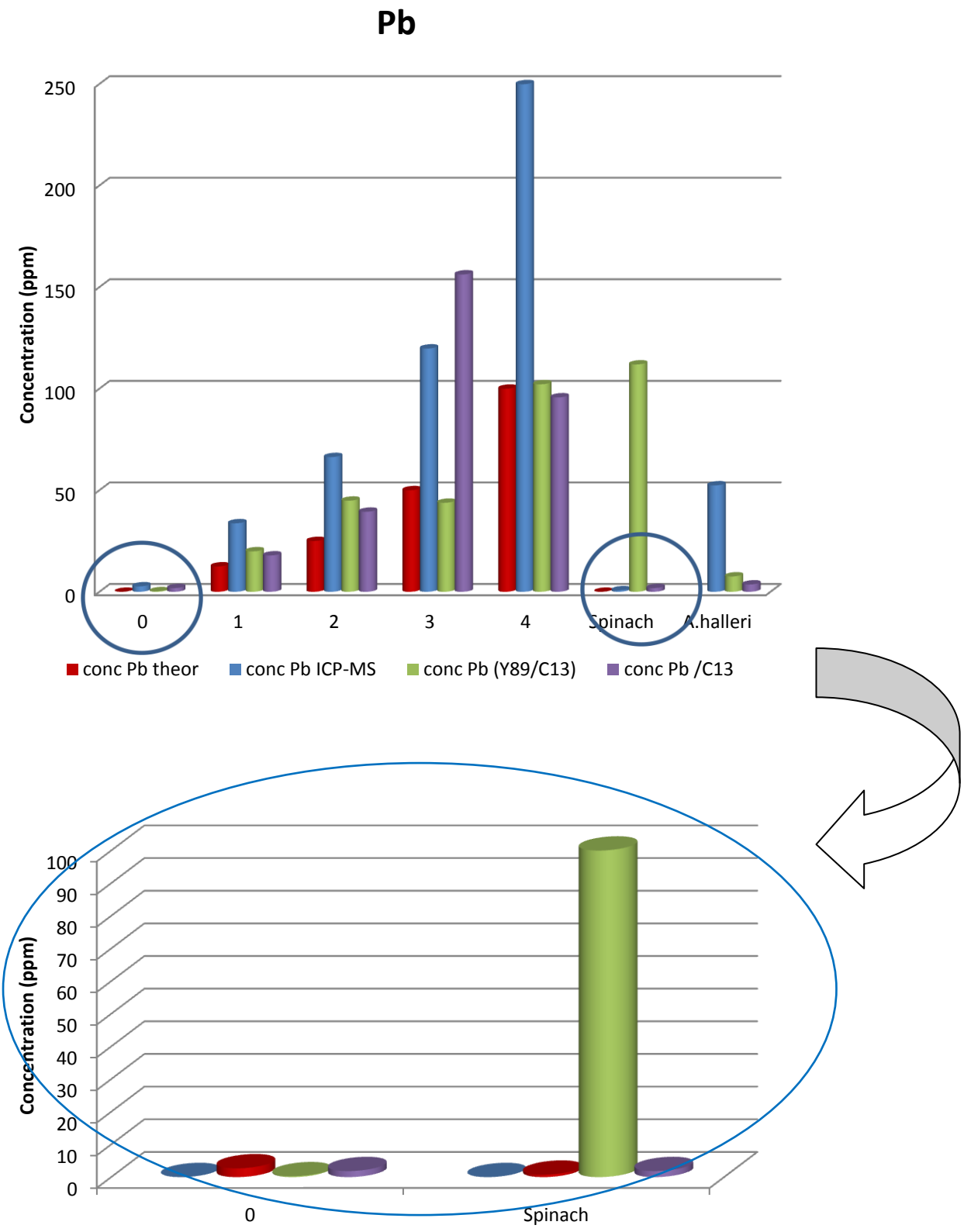


Figure 20. Bar graph comparing different strategies developed for extracting Pb concentration values.

Similar results are obtained for Zn and Cd in the calibration standards regardless of the methodology. Digestion followed by ICP-MS analysis gives higher concentration values than the theoretical ones we expected, due to a possible contamination. In general, calibration with Y as internal standard provides similar concentration values when data are normalized by $^{89}Y/^{13}C$ and when it is only normalized to ^{13}C intensity. Digestion with ICP-MS analysis provides the closest values to theoretical ones in the case of Zn, whereas for Cd, normalization by $^{89}Y/^{13}C$ seems to be the best option. Extremely high value is determined for Pb in spinach by $^{89}Y/^{13}C$ normalization probably because of a contamination of the pellet.

These results confirm that there is no loss of metal in any case by using Y as internal standard, and also that it does not interfere with any other element contained in the leaf.

3.2 Chemical mapping

By means of two-dimensional (2D) imaging of plant tissues, a defined sample area (several cm^2) of whole leaves and thin sections (thickness: 50 µm) was ablated line by line with a focused laser beam. The spot size of the laser beams varied between 12 µm for thin sections and 40 µm for whole leaves samples. Ion images were validated by analyzing at least two isotopes if available ($^{63}Cu^+$ and $^{65}Cu^+$, $^{64}Zn^+$ and $^{66}Zn^+$, $^{111}Cd^+$ and $^{114}Cd^+$, $^{206}Pb^+$ and $^{208}Pb^+$). To quantify chemical elements of interest, external calibration was performed with pressed pellets.

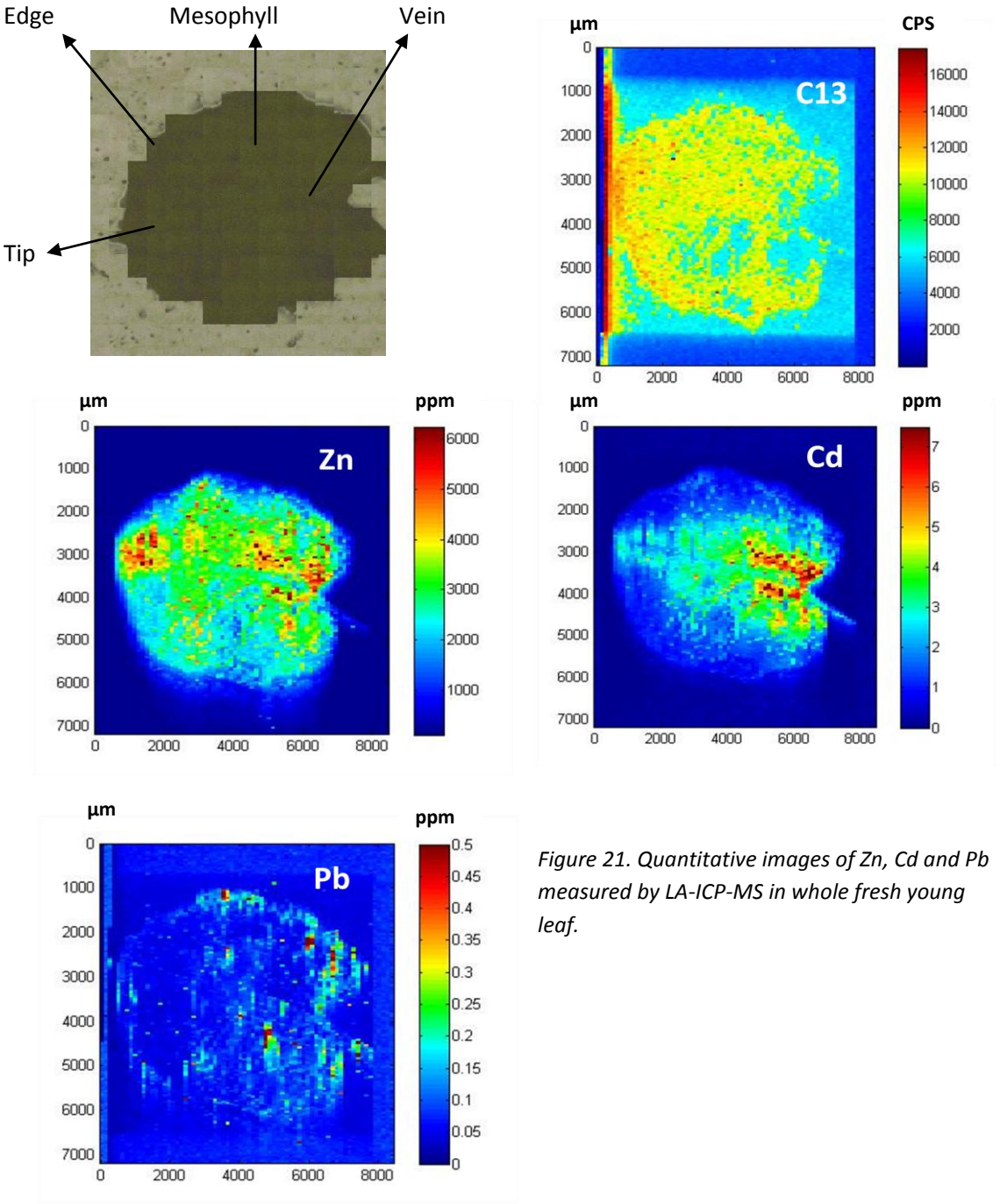


Figure 21. Quantitative images of Zn, Cd and Pb measured by LA-ICP-MS in whole fresh young leaf.

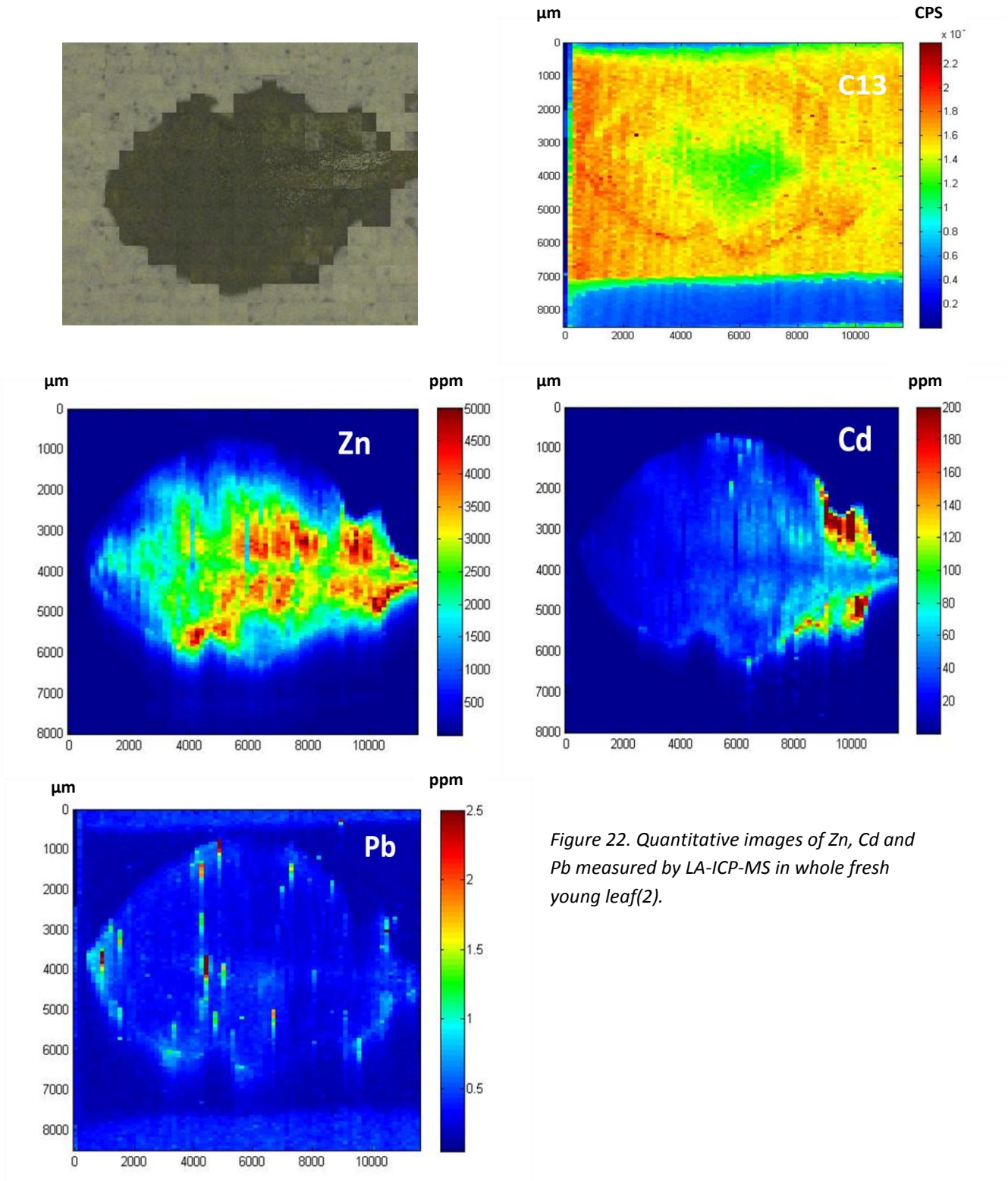


Figure 22. Quantitative images of Zn, Cd and Pb measured by LA-ICP-MS in whole fresh young leaf(2).

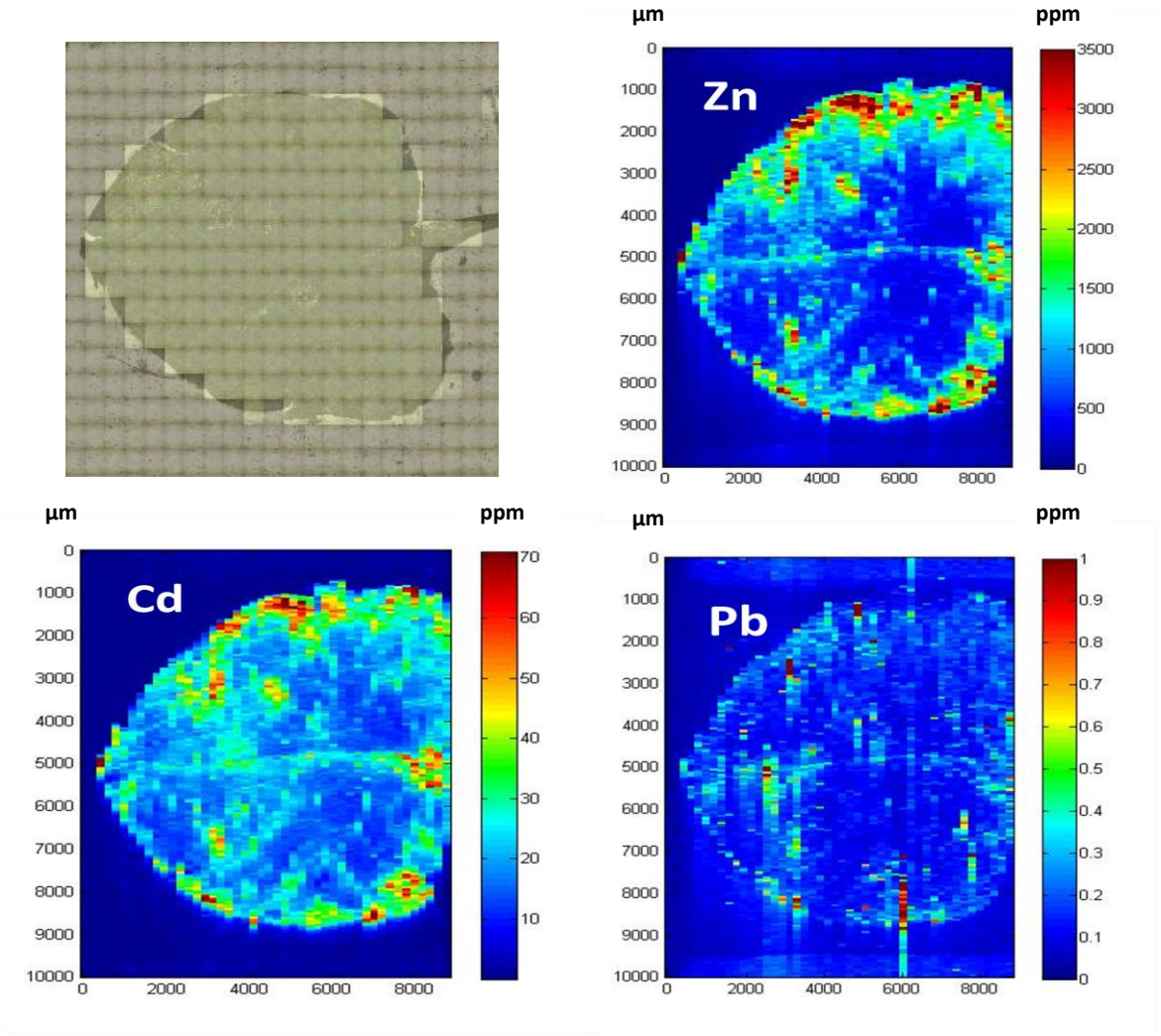
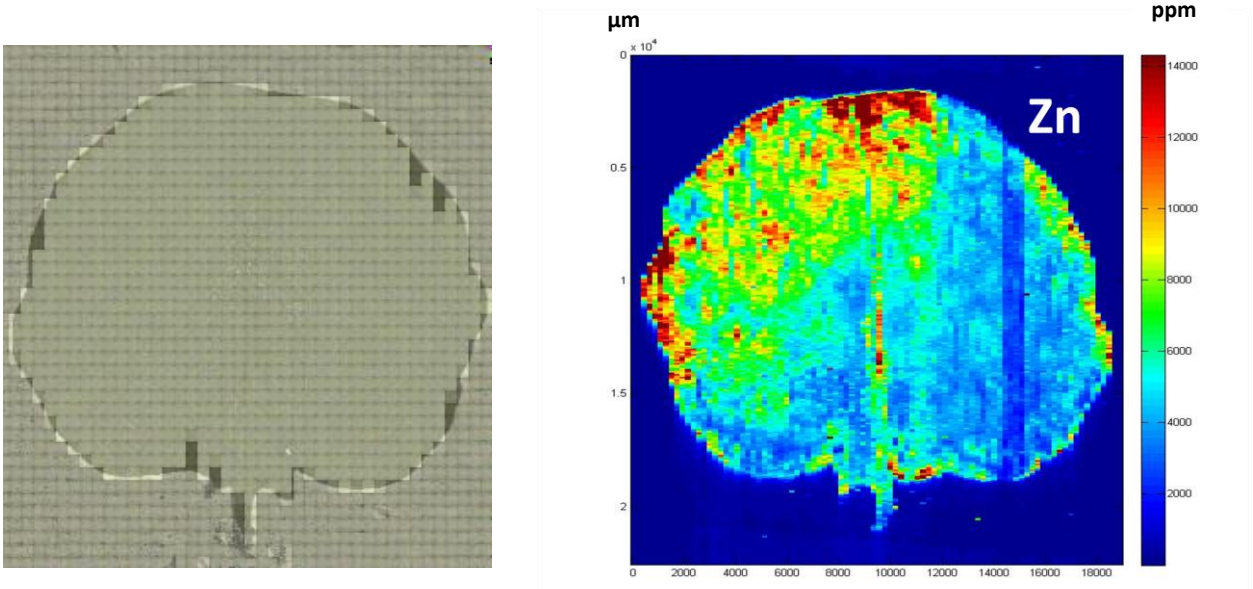


Figure 23. Quantitative images of Zn, Cd and Pb measured by LA-ICP-MS in whole freeze-dried young leaf.



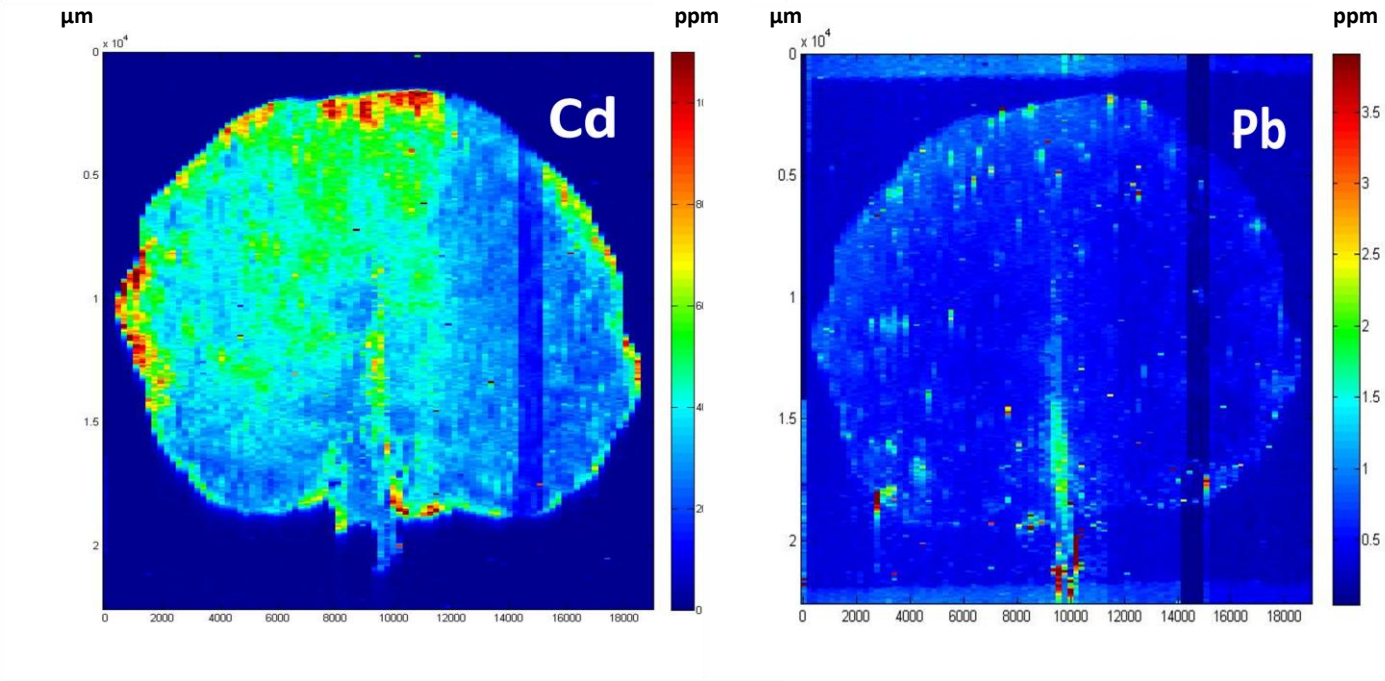
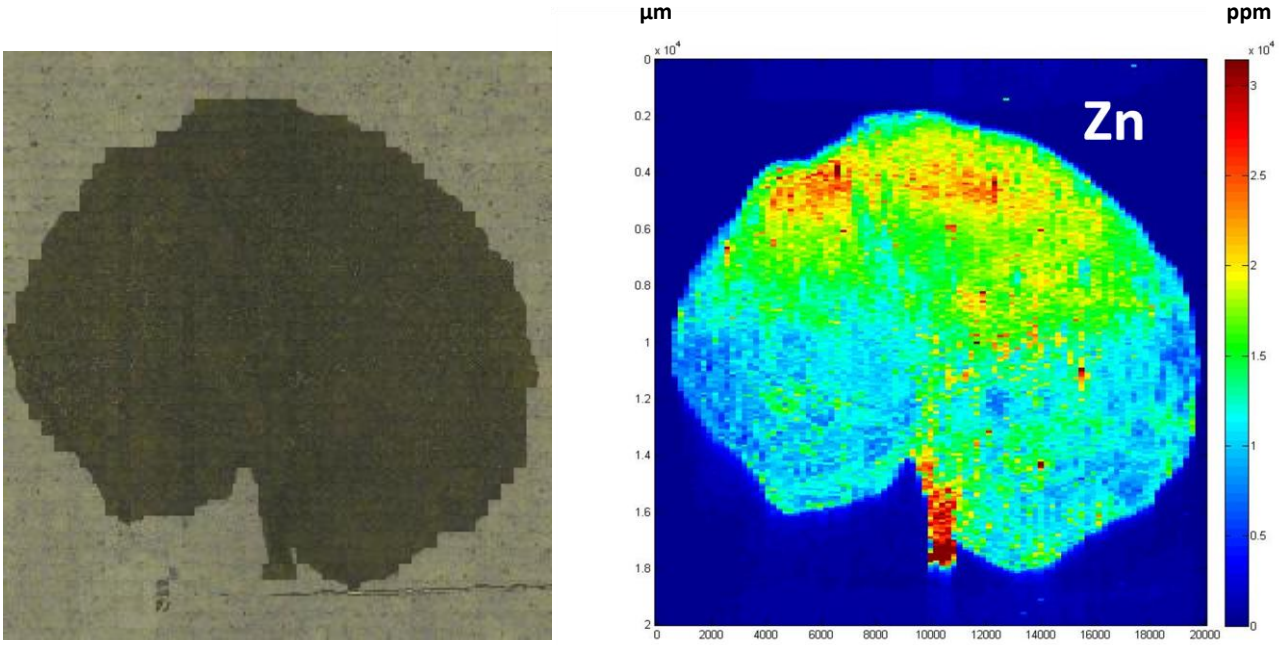


Figure 24. Quantitative images of Zn, Cd and Pb measured by LA-ICP-MS in whole fresh mature leaf.



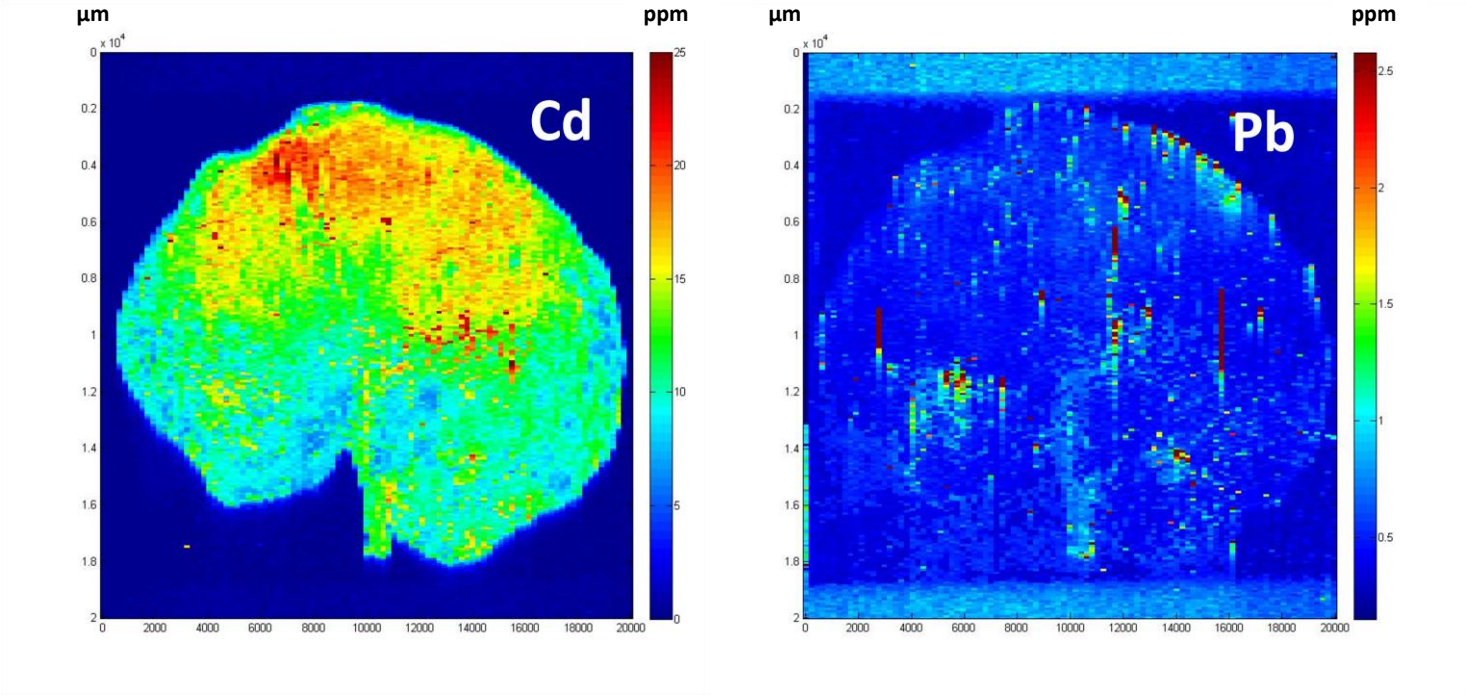


Figure 25. Quantitative images of Zn, Cd and Pb measured by LA-ICP-MS in whole freeze-dried young leaf.

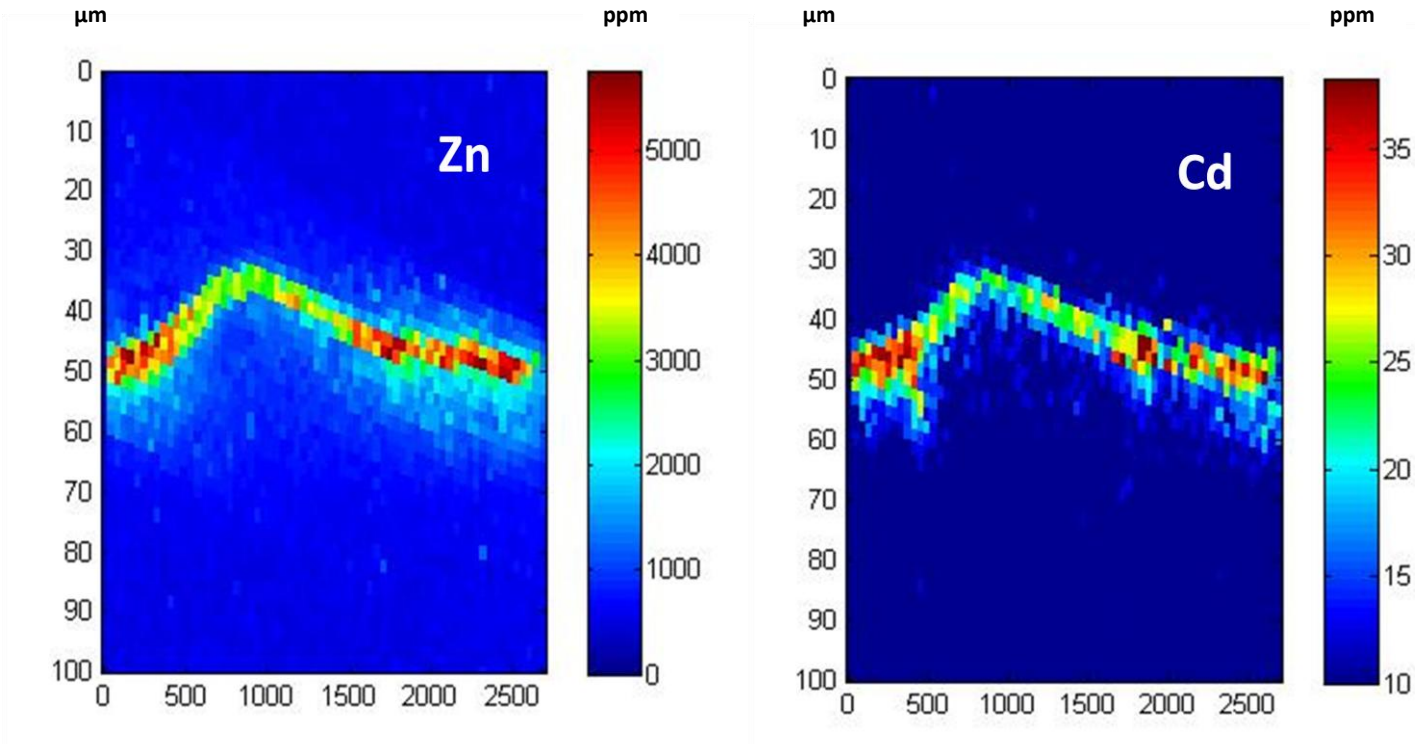


Figure 26. Quantitative images of Zn and Cd measured by LA-ICP-MS in young leaf thin sections.

According to the images recorded, we can assume that Zn and Cd are not concentrated in the main vein but they are in the green part of the leaves, as it was expected for this kind of plants. This idea is confirmed in thin sections. It is also noticed that concentration values differ depending on the leaf preparation, reaching higher values in fresh leaves than in freeze-dried ones. Also it is taken into account the difference in concentration between young and mature leaves, being almost 5 fold higher in mature leaves for Zn, and 10 fold higher for Cd. In the case of Pb, changes are more difficult to evaluate and resolution is not so clear because of its low level in the leaf.

Comparing these results with other techniques like µSXRF (synchrotron micro X-ray fluorescence) distribution of Zn is corroborated, being located in the green part of the leaves and not in the veins. With this technique it is also possible to observe hot spots in trichomes, epidermal hair of leaves, which is unconceivable with LA-ICP-MS because of the larger size of the beam. For imaging studies, µSXRF has a better lateral resolution (5 x 5 µm), is less sensitive and the quantification is very tricky, in comparison to the laser ablation technique.

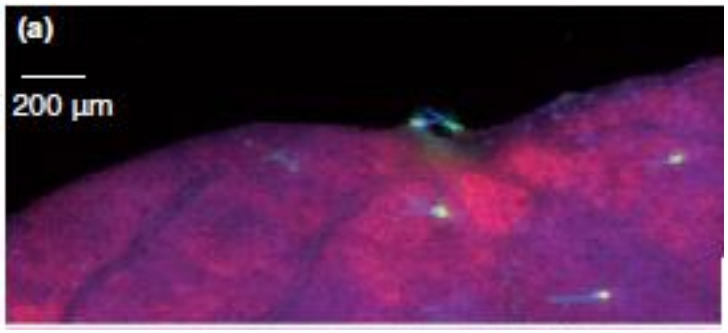


Figure 27. µSXRF map obtained for Zn, Ca and Mn on A. halleri mature leaf.

4 Conclusions

A new approach to quantify metals in plants by LA-ICP-MS was developed and the use of an internal standard (Y) was experimented. Although there are still some limitations in the methodology referring to the pellet homogeneity, possible contamination or changes in the solution pH, it can be postulated as a valid alternative for elemental quantification.

Related to imaging results, LA-ICP-MS is shown as a competitive tool for element distribution studies in environmental samples but until now one of the major drawbacks of this technique was the difficult standardization. Thanks to the quantification strategy developed, the local element concentration could be mapped. That allows us to get a better idea of the differences between leaves from different ages and also notice the variations in detection limits according to the preparation applied to the sample.

5 Future developments

In order to improve quantification strategies development, some of the limitations found in this proposed calibration could try to be overcome checking if the accuracy and the precision of the method are also improved.

It will be also interesting try to optimize thin sections parameters for a better resolution of the chemical map than the one obtained.

To sum up, all efforts are joined in getting reliable quantification strategies and improving lateral resolution of the technique to reach micrometer scale range. It is starting to be a reality in LA-ICP-MS field that will provide a better knowledge of hyperaccumulator mechanism at mesophyll cells scale.

6 Bibliography

J. Sabine Becker, Miroslav Zoriy, Andreas Matusch, Bei Wu, Dagmar Salber, Christoph Palm, and J. Susanne Becker, *BIOIMAGING OF METALS BY LASER ABLATION INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (LA-ICP-MS),* Mass Spectrometry Reviews (2010) 29, 156-175.

M. Galiová, J. Kaiser, K. Novotny, M. Hartl, R. Kizek, P. Babula, *UTILIZATION OF LASER-ASSISTED ANALYTICAL METHODS FOR MONITORING OF LEAD AND NUTRITION ELEMENTS DISTRIBUTION IN FRESH AND DRIED CAPSICUM ANNUUM L. LEAVES,* Microscopy Research and Technique 74, 845−852 (2011).

Johanna Sabine Becker, *State-of-the-art and progress in precise and accurate isotope ratio measurements by ICP-MS and LA-ICP-MS,* J. Anal. At. Spectrom.(2002) 17, 1172–1185.

Y. X. Wang, A. Specht and W. J. Horst, *Stable isotope labelling and zinc distribution in grains studied by laser ablation ICP-MS in an ear culture system reveals zinc transport barriers during grain filling in wheat,* New Phytologist (2011) 189, 428−437.

ClaudiaD'Oriano, Stefania Da Pelo, Francesca Podda and Raffaello Cioni, *Laser-Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS): setting operating conditions and instrumental performance,* Per. Mineral. (2008), 77, 3, 65-74.

Ahmad B. Moradia, Siegfried Swobodab, Brett Robinsona, Thomas Prohaskab, Anders Kaestnerc, Sascha E. Oswaldd, Walter W. Wenzele, Rainer Schulina, *Mapping of nickel in root cross-sections of the hyperaccumulator plant Berkheya coddii using laser ablation ICP-MS,* Environmental and Experimental Botany 69 (2010) 24–31.

Shengke Tian, Lingli Lu, John Labavitch, Xiaoe Yang, Zhenli He, Hening Hu, Ritimukta Sarangi, Matt Newville, Joel Commisso, and Patrick Brown, *Cellular Sequestration of Cadmium in the Hyperaccumulator Plant Species Sedum alfredii,* Plant Physiology, (2011) Vol. 157, pp. 1914–1925.

Benjamin Klug, André Specht and Walter J. Horst, *Aluminium localization in root tips of the aluminiumaccumulating plant species buckwheat (Fagopyrum esculentum Moench),* Journal of Experimental Botany (2011), 1-10.

Wendy Ann Peer, Ivan R. Baxter, Elizabeth L. Richards, John L. Freeman, Angus S. Murphy, *Phytoremediation and hyperaccumulator plants (*2005).

George A. Zachariadis & Parthena C. Sarafidou, *Internal standardization with yttrium spectral lines using axial-viewing inductively coupled plasma atomic emission spectrometry for plant certified reference materials analysis,* Microchim Acta (2009) 166:77–81.

Glenda Willems, Dörthe B. Dräge, Mikael Courbot, Cécile Godé´, Nathalie Verbruggen and Pierre Saumitou-Laprade, *The Genetic Basis of Zinc Tolerance in the Metallophyte Arabidopsis*

halleri ssp. halleri (Brassicaceae): An Analysis of Quantitative Trait Loci, Genetics 176: 659–674 (2007).

Géraldine Sarret, Pierre Saumitou-Laprade, Valérie Bert, Olivier Proux, Jean-Louis Hazemann, Agnès Traverse, Matthew A. Marcus, and Alain Manceau, *Forms of Zinc Accumulated in the Hyperaccumulator Arabidopsis halleri*, *Plant Physiology*, (2002) Vol. 130, pp. 1815–1826.

Markus Tibi and Klaus G. Heumann, *Isotope dilution mass spectrometry as a calibration method for the analysis of trace elements in powder samples by LA-ICP-MS*, J. Anal. At. Spectrom., (2003) 18, 1076-1081.

S. Clemens, *Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants,* Biochimie 88 (2006) 1707–1719.

Nicoletta Rascioa, Flavia Navari-Izzo, *Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting?,* Plant Science 180 (2011) 169−181.