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Nitrate content, N metabolism and ionome of plant foods: soil-to-plant transfer of chemical species during growth

Thesis presented to obtain the PhD degree in Sustainable Chemistry

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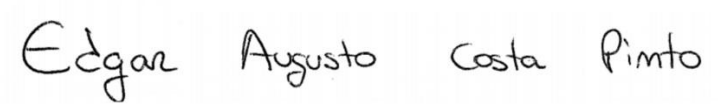

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"Happiness is knowing what you want
and wanting it passionately."

ABSTRACT

Humans need several essential minerals in order to allow the proper functioning of their body. Mineral intake in the human diet comes from plant foods and/or from animal sources. Plant foods are commonly eaten by humans in their diet and are an important source of mineral nutrients. Soils are the main source of mineral nutrients for plant life on earth. Seventeen elements are considered essential for plant growth and development. Non-essential/toxic elements are also present on soils and thus, can also be transferred to plants. The well-balanced contribution of soil mineral components (sand, silt and clay), organic matter, air and water influences the mobility and phytoavailability of chemical species. Intensive cultivation and harvesting of crops for human consumption can deplete the nutrients in soil. The use of chemical fertilizers frequently results in a surplus of primary macronutrients. Nitrate (NO_3^-), used in agriculture as a chemical fertilizer, can accumulate in plant foods, especially in vegetables. Nitrogen (N) metabolism plays a key role influencing the NO_3^- content in plant foods. NO_3^- itself is relatively non-toxic to humans however its reaction products can cause adverse health effects. Moreover, the accumulation of a given element is a complex gene-regulated process comprising its uptake, binding, transportation and sequestration. Many of these genes and physiological processes affect more than one element. Therefore, it is imperative to measure as many elements as possible in a cell, tissue or organism (the ionome).

The main goals of this thesis were to provide new insights about plant foods nutritional value, to understand the soil-to-plant transfer of chemical species during plant growth and how this transfer influences the plant ionome, N metabolism and NO_3^- content. To accomplish these goals, extraction methods for the analysis of vegetables and soil were firstly studied. Afterwards, the soil-to-plant transfer of chemical species and its influence in the nutritional value of lettuces during its growth period was evaluated. Finally, the contribution of the N metabolism for the NO_3^- content of plant foods during their growth period was assessed through the study of key enzymes and the quantification of polyamines content in different plant foods and in lettuces during their growth period in different experimental fields was performed.

Freeze-drying was proposed as a suitable pretreatment for NO_3^- determination in spinach and lettuce, because large number of samples can be lyophilized, the sample size is significantly reduced, a homogenous sample is obtained and the lyophilized sample can be stored for long periods.

An accurate prediction of metal(loid)s content in plants was achieved considering the amount of metal(loid)s extracted by the NH_4NO_3 method and some soil properties (CEC, OM, pH, texture and oxides content).

It was also investigated if the same soil properties play an important role influencing the plant ionome. Soil characterized by high CEC, OM, EC, clay and silt content and by a low pH showed a higher phytoavailable fraction of elements. Significant correlations between different pairs of elements in lettuce shoot revealed similar processes acting on the uptake, translocation, distribution and storage of elements within the plant.

Lettuce nutritional value and the accumulation of toxic elements strongly depend on the lettuce growth stage and soil composition. Overall, higher contents of macrominerals, trace elements and photosynthetic pigments were observed in young lettuces. The general trend observed for all elements and pigments was the decreasing of their contents along the plant growth period. Microgreens can provide a significantly higher intake of essential minerals than mature lettuces. Additionally, the NO_3^- content of microgreens showed to be very low allowing its safe use in children's diet to fulfill their daily mineral requirements, while reducing their exposure to the harmful NO_3^-.

In this thesis it was also described how soil properties/composition and specific enzymes of the N metabolism (nitrate reductase and glutamine synthetase) influenced the NO_3^- content of lettuce during its growth period. Nitrate reductase activity decreased during lettuce growth; consequently, NO_3^- lettuce content increased. Higher soil NO_3^- levels caused NO_3^- accumulation in lettuce, despite the higher nitrate reductase activity during the first stages of plant growth. Higher NO_3^- and NH_4^+ levels were correlated with higher N-Kjeldahl levels in lettuce, suggesting a positive effect in organic N synthesis. Soil composition influenced the mobility of NO_3^- and NH_4^+ within the groundwater-soil-plant system. Loamy sandy soil showed to retain more efficiently the inorganic N, increasing the NO_3^- and NH_4^+ content in soil solution, which results in a higher N content in lettuce, while sandy soils allowed NO_3^- leaching.

The polyamines putrescine, cadaverine, spermidine and spermine have been implicated in several biological processes such as plant growth, and the dietary intake of polyamines influences human health. Ultrasound-assisted benzoylation coupled to dispersive liquid–liquid microextraction was successfully applied to the analysis of polyamines in several plant foods. The low LODs obtained (in the range of 0.019 – 0.045 μg/g) highlight the high sensitivity of the developed method. Good recoveries of polyamines were obtained in the analyses of plant food samples (81.7 – 114.2%). A general decrease in the content of putrescine, spermidine and spermine was observed during plant growth, which may be explained by that the active cells division in the initial stages of lettuce development.

Keywords – Plant foods, nitrate, N metabolism, ionome, soil, chemical species.

RESUMO

O Homem necessita de vários minerais, ditos essenciais, de forma a permitir o normal funcionamento do seu organismo. A ingestão de minerais é realizada através do consumo de alimentos de origem vegetal, e/ou através do consumo de produtos de origem animal. Os alimentos de origem vegetal fazem parte da alimentação humana, constituindo uma importante fonte de nutrientes. O solo é a principal fonte de nutrientes minerais para as plantas. Dezassete elementos são considerados essenciais para o crescimento e desenvolvimento das plantas. Contudo, o solo também pode conter vários elementos não-essenciais/tóxicos que podem ser transferidos para as plantas. A contribuição equilibrada dos vários componentes do solo – componente mineral (areia, limo e argila), matéria orgânica, ar e água – influenciam fortemente a mobilidade e fitodisponibilidade das espécies químicas presentes neste compartimento ambiental. O recurso a práticas de agricultura intensiva de forma a fazer face ao crescente consumo humano tem contribuído para a depleção dos nutrientes presentes no solo. De forma a contrabalaçar este fenómeno, são adicionadas elevadas quantidades de fertilizantes químicos ao solo, o que resulta muitas vezes num excesso de determinados macronutrientes. O nitrato (NO_3^-), frequentemente usado na agricultura como fonte de azoto, pode acumular-se nos alimentos de origem vegetal, especialmente nos vegetais. O metabolismo do azoto nas plantas desempenha um papel crucial no teor de NO_3^- presente nos alimentos de origem vegetal. O NO_3^- por si só não apresenta risco para o Homem, contudo, os seus metabolitos podem causar efeitos adversos na saúde. Além disso, a acumulação de determinada espécie química na planta é um processo regulado a nível genético em todas as suas componenetes: aquisição, ligação, transporte e compartimentação. A maior parte dos genes envolvidos neste processo fisiológico afeta o metabolismo de mais do que uma espécie química. Deste modo, a determinação quantitativa do maior número possível de espécies químicas numa determinada célula, tecido ou organismo (o ionoma) assume um caráter fundamental.

Os principais objectivos desta tese foram aumentar o conhecimento relacionado com o valor nutricional dos alimentos de origem vegetal, perceber o processo de transferência de várias espécies químicas do solo para a planta durante o seu crescimento, e o modo como este processo afecta o ionoma da planta, o metabolismo do azoto e o conteúdo em NO_3^-. Para atingir estes objectivos, foram primeiro estudados métodos de extracção para a análise de vegetais e de solo. Depois, foi avaliado o processo de transferência das espécies químicas do solo para a planta e a sua influência no valor nutricional das plantas ao longo do seu crescimento. Por fim, foi avaliado o papel do metabolismo do azoto no teor de NO_3^- nos vegetais durante o seu crescimento através do estudo de

enzimas específicas envolvidas no metabolismo do azoto, assim como a quantificação de poliaminas em diferentes alimentos de origem vegetal e em várias fases do crescimento de alfaces em diferentes áreas de cultivo.

A liofilização foi proposta como um pré-tratamento adequado de amostras de alface e espinafre para a determinação do NO_3^-. Este procedimento permite o tratamento de um elevado número de amostras de origem vegetal, a redução do tamanho da amostra, a homogeneização da amostra e a armazenagem de amostras por longos periodos de tempo.

Foi realizada uma adequada previsão do conteúdo de vários metais/metalóides no tecido vegetal, considerando a quantidade desses metais/metalóides extraídos do solo pelo método do NH_4NO_3 e algumas propriedades do solo (capacidade de troca catiónica, matéria orgânica, pH, textura e conteúdo em óxidos).

Foi também estudado se as mesmas propriedades do solo exercem uma influência importante no ionoma das plantas. O solo caracterizado por elevada capacidade de troca catiónica, elevado teor de matéria orgânica, elevada conductividade, elevado teor de limo e argila e baixo pH apresentou maior fitodisponibilidade de vários elementos. A obtenção de diversas correlações significativas entre diferentes pares de elementos nas folhas de alface apontou para a existência de processos semelhantes no que se refere à aquisição, transporte, distribuição e compartimentação dos elementos dentro da planta.

O valor nutricional e a acumulação de elementos tóxicos na alface estão fortemente relacionados com a fase de crescimento da planta e a composição do solo. Em geral, foi observado um teor mais elevado de macrominerais, elementos vestigiais e pigmentos fotossintéticos nos estágios iniciais do crescimento das alfaces. A diminuição do conteúdo elementar ao longo do período de crescimento foi a tendência geralmente observada. Deste modo, o consumo de alfaces no seu estágio inicial de desenvolvimento proporciona uma maior ingestão de minerais essenciais, comparado com a ingestão de alfaces maduras. Adicionalmente, o teor em NO_3^- das alfaces no seu estágio inicial de desenvolvimento mostrou-se significantivamente mais baixo, tornando aconselhável o seu uso na dieta das crianças para satisfazer as suas necessidades nutricionais diárias, reduzindo, ao mesmo tempo, a exposição ao NO_3^-.

Nesta tese está ainda descrito como é que as propriedades/composição do solo e a actividade de determinadas enzimas envolvidas no metabolismo do azoto (nitrato redutase e glutamina sintetase) influenciam o teor em NO_3^- das alfaces ao longo do seu crescimento. A actividade da enzima nitrato redutase diminuiu durante o período de crescimento das alfaces, e, consequentemente, o teor de NO_3^- aumentou no mesmo período. Um elevado conteúdo de NO_3^- no solo levou à acumulação de NO_3^- nas alfaces, independentemente da elevada actividade da enzima nitrato redutase nos estágios

iniciais de desenvolvimento das mesmas. Os elevados teores de NO_3^- e NH_4^+ mostraram-se fortemente relacionados, de modo direto, com o elevado conteúdo de N-Kjeldahl nas alfaces, revelando um efeito positivo daquelas espécies químicas na síntese de compostos orgânicos azotados. A composição do solo influenciou a mobilidade do NO_3^- e do NH_4^+ entre os compartimentos ambientais água subterrânea, solo e planta. Solos argilosos mostraram reter eficientemente o NO_3^- e o NH_4^+, permitindo o aumento do conteúdo destas espécies químicas na fase aquosa do solo e o consequente aumento do conteúdo de espécies azotodas nas alfaces. Por sua vez, os solos arenosos permitiram maior lixiviação do NO_3^-.

As poliaminas putrescina, cadaverina, espermidina e espermina estão envolvidas em processos biológicos que ocorrem nas plantas, sendo também relevantes na alimentação humana para promover a saúde. Nesta tese, está descrita uma metodologia analítica baseada na benzoilação assistida por ultrassons acoplada a microextracção líquido-líquido dispersiva e a sua aplicação na análise de vários alimentos de origem vegetal. Os baixos limites de deteção obtidos (entre 0,019 e 0,045 µg/g) realçam a elevada sensibilidade do método desenvolvido. Foram obtidas boas taxas de recuperação (81,7-114,2%) na análise de diferentes alimentos de origem vegetal. Relativamente à análise de alfaces, foi observada uma tendência decrescente para o conteúdo em poliaminas durante o seu período de crescimento que pode ser explicado pela maior actividade ao nível da divisão celular das plantas nos estágios iniciais de desenvolvimento.

Palavras-chave – Alimentos de origem vegetal, nitrato, metabolismo do azoto, ionoma, solo, espécies químicas.

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LIST OF ABBREVIATIONS

Abbreviation	Name
AAS	Atomic absorption spectrometry
ABC	ATP-binding cassette
ABCC	Subfamily C of the ABC family
ACA	Ca^{2+}-ATPase
ACR	Arsenate reductase
ADI	Acceptable daily intake
ADP	Adenosine diphosphate
AES	Atomic emission spectroscopy
ALS	Aluminium sensitive
AMF	Arbuscular mycorrhizal fungi
AMT	Ammonium transporter
ANOVA	Analysis of variance
AQC	6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate
AS	Asparagine synthetase
ATP	Adenosine triphosphate
BCD	Bush-and-chlorotic-dwarf
BCR	Community bureau of reference
CAD	Cadaverine
CAM	Crassulacean acid metabolism
CAX	Ca^{2+}/H^{+} exchanger
CCC	Cation-chloride co-transporter
CCD	Central composite design
CDF	Cation diffusion facilitator
CEC	Cation exchange capacity
CHL	Chlorate resistant
CHX	Cation/H+ exchanger
CLC	Chloride channel
CNBF	4-Chloro-3,5-dinitrobenzotrifluoride
CNGC	Cyclic-nucleotide gated channel
COPT	Copper transporter
CPA	Cation/Proton antiporter
CTP	Cytidine triphosphate
DA-NSCC	Depolarization-activated nonselective cation channel

Abbreviation	Name
DAD	Diode array detector
DEEMM	Diethyl ethoxymethylenemalonate
DLLME	Dispersive liquid-liquid microextraction
DMA	Deoxymugineic acid
DNA	Deoxyribonucleic acid
DNBZ-Cl	3,5-Dinitrobenzoyl chloride
DTPA	Diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EC	European Commission
ECA	Endomembrane-type Ca^{2+}-ATPase
Eh	Redox potential
ENA	Efflux transporter of nicotianamine
EPA	Environmental Protection Agency
EU	European Union
FAD	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization of the United Nations
FRD	Ferric reductase defective
FRDL	Ferric reductase defective-like
FRO	Ferric-chelate reductase oxidase
GC	Gas chromatography
GLR	Ionotropic glutamate receptor
GS	Glutamine synthethase
GSH	Glutathione
GTP	Guanosine triphosphate
HA	H^+-ATPase
HA-NSCC	Hyperpolarization-activated nonselective cation channel
HAK/KT/KUP	K^+/H^+ symporters
HATS	High-affinity transporter system
HF-LPME	Hollow fiber liquid-phase microextraction
HKT/Trk	K^+/Na^+ symporters
HMA	Heavy metal associated
HPLC	High performance liquid chromatography
IC	Ion chromatography

Abbreviation	Name
ICP	Inductively coupled plasma
INAA	Instrumental neutron activation analysis
IREG/FPN	Iron-regulated/Ferroportin
IRT	Iron-regulated transporter
IS	Internal standard
ISO	International Organization for Standardization
KCO	Kir-like K^+ channel
KEA	K^+ efflux antiporter
L	Fresh lettuce
LF	Frozen lettuce
LFd	Freeze-dried lettuce
LO	Oven-dried lettuce
LATS	Low-affinity transporter system
LC	Liquid chromatography
LCT	Low-affinity cation transporter
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
Lsi	Low silicon transporter
MA	Mugineic acid
MAE	Microwave assisted extraction
MATE	Multidrug and toxic compound extrusion
MEKC	Micellar electrokinetic chromatography
MRS2/MGT	Magnesium transporter
MHX	Mg^{2+}/H^+ antiporter
MIP	Major intrinsic protein
MIT	Mitochondrial transporter
MOT	Molybdenum transporter
MS	Mass spectrometry
MT	Metallothionein
MTP	Metal tolerance proteins
NA	Nicotianamine
NADH	Nicotinamide adenine dinucleotide
NAS	Nicotianamine synthase
NAXT	Nitrate excretion transporter

Abbreviation	Name
NHX	Na^+/H^+ antiporter
NIP	Nodulin 26-like Protein
NiR	Nitrite reductase
NR	Nitrate reductase
NRAMP	Natural resistance associated macrophage protein
Nrat	NRAMP aluminium transporter
NRT	Nitrate transporter
NQS	1,2-Naphthoquinone-4-sulfonate
NSCC	Nonselective cation channel
OM	Organic matter
OPA	o-Phthaldialdehyde
OPT	Oligopeptide transporter
PC	Phytochelatin
PCA	Principal component analysis
PCR	Plant cadmium resistance
PCS	Phytochelatin synthase
PDR	Pleiotropic drug resistance transporter
PEZ	Phenolics efflux zero
PGPB	Plant growth-promoting bacteria
PHT	Phosphate transporter
Pi	Inorganic phosphorus
PIC	Permease in chloroplast
PMSF	Phenylmethanesulfonyl fluoride
PPase	Pyrophosphatase
PS	Phytosiderophores
PUT	Putrescine
PVPP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
RSD	Relative standard deviation
RSM	Response surface methodology
SBSE	Stir bar sorptive extraction
SDME	Single drop microextraction
SOS	Salt overly sensitive
S	Fresh spinach
SF	Frozen spinach

Abbreviation	Name
SFd	Freeze-dried spinach
SO	Oven-dried spinach
SPD	Spermidine
SPE	Solid phase extraction
SPM	Spermine
SPME	Solid phase microextraction
SULTR	Sulfate transporter
TLC	Thin-layer chromatography
TPC	Two-pore channel
TPK	Two-pore K^+ channel
TOM	Transporter of mugineic acid family phytosiderophores
UTP	Uridine triphosphate
UV	Ultaviolet
VI-NSCC	Voltage-insensitive nonselective cation channel
VIT	Vacuolar iron transporter
XAS	X-ray absorption spectroscopy
YS	Yellow stripe
YSL	Yellow stripe-Like
WHO	World Health Organization
ZIF	Zinc-induced facilitator
ZIP	(ZRT-IRT)-like protein
ZRT	Zinc-regulated transporter

LIST OF PUBLICATIONS

(Within the scope of this PhD)

Publications in International Peer-Reviewed Journals

1. **E. Pinto**, C. Petisca, L.F. Amaro, O. Pinho, I.M.P.L.V.O. Ferreira (2010) Influence of different extraction conditions and sample pretreatments on the quantification of nitrate and nitrite in spinach and lettuce. Journal of Liquid Chromatography & Related Technologies, 33: 591-602.
2. **E. Pinto**, A.A. Almeida, A.A.R.M. Aguiar, I.M.P.L.V.O. Ferreira (2014) Changes in macrominerals, trace elements and pigments content during lettuce (*Lactuca sativa* L.) growth: Influence of soil composition. Food Chemistry, 152: 603-611.
3. **E. Pinto**, F. Fidalgo, J. Teixeira, A.A.R.M. Aguiar, I.M.P.L.V.O. Ferreira. (2014) Influence of the temporal and spatial variation of nitrate reductase, glutamine synthetase and soil composition in the N species content in lettuce (*Lactuca sativa*). Plant Science, 219-220: 35-41.
4. **E. Pinto**, A.A.R.M. Aguiar, I.M.P.L.V.O. Ferreira. Influence of soil chemistry and plant physiology in the phytoremediation of Cu, Mn and Zn. Critical Reviews in Plant Science, In Press.
5. **E. Pinto**, A. Melo, I.M.P.L.V.O. Ferreira (2014). Sensitive quantification of polyamines in plant foods by ultrasound-assisted benzoylation and dispersive liquid-liquid microextraction with the aid of experimental designs. Journal of Agricultural and Food Chemistry, In Press.
6. **E. Pinto**, A.A. Almeida, A.A. Aguiar, I.M.P.L.V.O. Ferreira (2014). Influence of soil composition in the shoot ionomic profile of lettuce: a holistic approach to understand soil-to-plant element transfer. (submitted).
7. **E. Pinto**, A.A. Almeida, I.M.P.L.V.O. Ferreira (2014). Soil-plant relationship of metal(loid)s: looking for the better strategy to predict Cr, V, Ni, As, Pb, Co, Cd, Sb and U content in lettuce (*Lactuca sativa*). (submitted)
8. **E. Pinto**, A.A. Almeida, A.A. Aguiar, I.M.P.L.V.O. Ferreira (2014). Comparison between the mineral profile and nitrate content of microgreens and mature lettuces (submitted).
9. **E. Pinto**, I.M.P.L.V.O. Ferreira (2014). Changes in the content of polyamines and nitrate during lettuce (*Lactuca sativa*) growth (submitted).

GENERAL SCOPE

What are plant foods? Why are they important for human diet?

Plant foods refer to plants or parts of a plant that are eaten as a food. In this category are included vegetables, fruits, grains and legumes. They constitute one of the most important nutrient sources in human diet since the beginning of civilization. Humans need several essential minerals in order to allow the proper functioning of the body, regarding growth, development and metabolism. Recommended dietary allowances (RDAs), defined as "the levels of intake of essential nutrients that, on the basis of scientific knowledge, are judged by the Food and Nutrition Board to be adequate to meet the known nutrient needs of practically all healthy persons" are established and vary between a few μg/day and 1 g/day. If intakes are low in a certain period of time, deficiency signs may develop. On the other hand, high intakes can result in toxicity.

Deficiencies in mineral macro and micronutrients, including calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), selenium (Se) and iodine (I), affect more than half of the world population. Therefore, it is crucial to develop new approaches that allow us to produce plant foods more efficiently, and with higher nutrient contents and bioavailability in their edible tissues.

What are soils? Why are they important for agriculture?

Soils are functional and diverse natural systems that provide a vital ecosystem to sustain humanity. Soil is composed of five main components: minerals, organic matter, living organisms, gas and water. Soil minerals are divided into three classes according to their size – sand, silt and clay. The percentage of each of these particles in soil is known as soil texture. Soil organic matter is constituted by animal, plant and microbial residues in several stages of decomposition. It is a critical component of soils and is many times used as an indicator of agricultural soil quality.

Soil is usually distinguished in different layers called soil horizons. These horizons are interrelated, and thus cannot be considered as independent, despite their differences. Soil horizons are very complex and diverse However, in general, the surface horizons are richer in life and organic matter. Below, more stable horizons, formed through a range of soil formation processes, are present. Below these, soil layers only partially affected by soil formation processes and/or unaltered layers of parent material are found.

Soils are the main source of nutrients and water for much of the plant life on earth. Seventeen elements (C, H, O, N, P, K, Ca, Mg, S, Fe, Mn, Zn, Cu, B, Mo, Cl and Ni) are considered essential for plant growth and development. They must be available to plants, which take them by root uptake from soils. The natural cycling of nutrients is a ubiquitous

and fundamental process of the water-soil-plant system. Plants and animals take up nutrients from the soil which later returns back, mainly by the decomposition of biomass in soil. This cycling allows the presence of essential nutrients in soil that are necessary for plant growth.

Of the mineral elements, the primary macronutrients (N, P, and K) are needed in high quantities from the soil and usually are in short supply in agricultural soils. Secondary macronutrients (Ca, Mg, S) are required in smaller quantities thus are in sufficient quantities in most soils and are not limiting for crop growth. The micronutrients (Fe, Mn, Zn, Cu, B, Mo, Cl and Ni), also known as essential trace elements, are needed in very small amounts and are usually deficient in most soils. However, when in excess, they can be toxic to plants.

What are the main soil components?

Soil is a complex mixture of mineral components (sand: 0.05-2 mm, silt: 0.002-0.05 mm, clay: <0.002 mm), organic matter (OM), air and water. The well-adjusted combination of these components allows water retention and drainage, oxygen delivery to the root zone, supply of nutrients to facilitate crop growth and ultimately provides the physical support required for plant development. The distribution of these components in a particular soil is influenced by the five factors of soil formation: parent material, time, climate, organisms and topography. Each one of these factors plays a direct and overlapping role in influencing the movement of elements in soil.

The mineral components of soil can exist as single particles, although they are often found associated in larger aggregates, providing structure to soil. These aggregates play a key role in soil processes by influencing the movement of water and air through soil. The cation exchange capacity (CEC) is a measure of the soil's ability to exchange cations between the soil particles and the soil solution. Clay particles have a huge influence on several soil properties such as CEC and structure, due to their high surface area with charged sites. The dominant negative charged clay-sized particles in soils are the aluminosilicates while the most common positively charged clay particles are the iron (Fe), aluminum (Al), and manganese (Mn) (hydr)oxides.

Soil OM includes the partial or well-decomposed residues of organic material present in soil. Surface soils contain between 1 to 6% of organic matter, and this percentage decrease with depth. The presence of OM is crucial as it provides essential nutrients, positively influences soil structure, buffers soil pH and improves water holding capacity and aeration. Moreover, the presence of ionizable functional groups confers to soil OM an ion-exchange capacity that highly influences the mobility of elements on soil.

Soil pH controls various physical, chemical and biological processes and properties that affect elements mobility. Besides, soil pH, which reflects the acidity level in soil, significantly influences the microbial activity and the stability of soil aggregates. At low pH, essential plant macronutrients, including N, P, K, Ca, Mg and S, are less phytoavailable than at pH values near 7. On the other side, certain micronutrients such as Fe, Mn and Zn are more phytoavailable and potentially toxic to plants at low pH values (< 5). Aluminum toxicity is also a common problem for crop growth at low pH (< 5.5).

What happens to chemical species in soil? Why some are retained and others are lost?

The majority of inorganic chemical species can be grouped into two sorptive categories: (i) anionic sorptives that are negatively charged (e.g., NO_3^-); and (ii) cationic sorptives that are positively charged (e.g., the divalent cations Ca^{2+} and Cd^{2+}). The major sorbents in soils are clays, metal-(hydr)oxides and OM. Clays are mainly negatively charged and, in most soils, they represent the largest source of negative charge. Metal-(hydr)oxides are variably charged and adopt anionic, neutral, or cationic forms based on the degree of protonation, which varies as a function of soil solution pH. Hence, these variably charged minerals usually assume a net positive surface charge at low pH and a net negative surface charge at high pH. Soil OM contains a multitude of reactive sites including, among others, hydroxyls (R-OH), carboxylic (R-COOH), sulfhydryl (R-SH) and amino groups (R-NH_2). Variations in the chemical composition, surface area and abundance of these three sorbents significantly influence the sorption characteristics of a given soil.

Soil solution pH can have a marked effect on sorption of elements by influencing both the magnitude and sign of sorptive and sorbent charge. For example, as the soil solution pH increases, sorbent hydroxyl and carboxyl functional groups of metal-(hydr)oxides and OM deprotonate. This increases the negative charge density on the sorbent enabling cation adsorption, while decreasing anion adsorption. Overall, the ability of a soil to retain cations (known as the CEC) generally increases when soil pH also increases.

How agriculture alters the natural cycling of chemical species?

Agriculture has a profound effect in the natural cycling of elements in soil. Intensive cultivation and harvesting of crops for human consumption can greatly deplete nutrients in the soil. In order to maintain soil fertility for high crop yields, soil amendments are typically used by farmers. Nowadays, farmers add several and higher quantities of soil amendments to enhance soil fertility. The use of inorganic chemical fertilizers as well as organic sources of nutrients frequently results in a surplus of primary macronutrients. The

efficiency of fertilizer application and use by crops is not always optimized, and excess nutrients, especially N, can accumulate in plants or be transported via surface runoff or leaching from agricultural fields resulting in surface- and groundwater pollution.

Why is the nitrate problematic?

Nearly 80% of the earth's atmosphere is made of nitrogen (N). Moreover, N is also a crucial constituent of most essential biomolecules. In living tissues, N is the fourth most common element and is an essential piece of the N cycle, which continuously distributes N between organisms and the environment.

Nitrate is a ubiquitous chemical species of the environment and has drawn an important attention in the last years due to its potential to accumulate in vegetables. Nitrate is widely used in agriculture as a chemical fertilizer to substitute the traditional use of livestock manure and in food industry as an approved food additive. Nitrate *per se* is fairly nontoxic, but its metabolites (nitrite, nitric oxide and N-nitroso compounds) confer to nitrate an extra importance because of their potential adverse health effects.

Usually, nitrate enters the human body exogenously from water, vegetables and other food sources. In agriculture, the application of N fertilizers and/or manures can result in increased concentrations of inorganic N-compounds in surface- and groundwater.

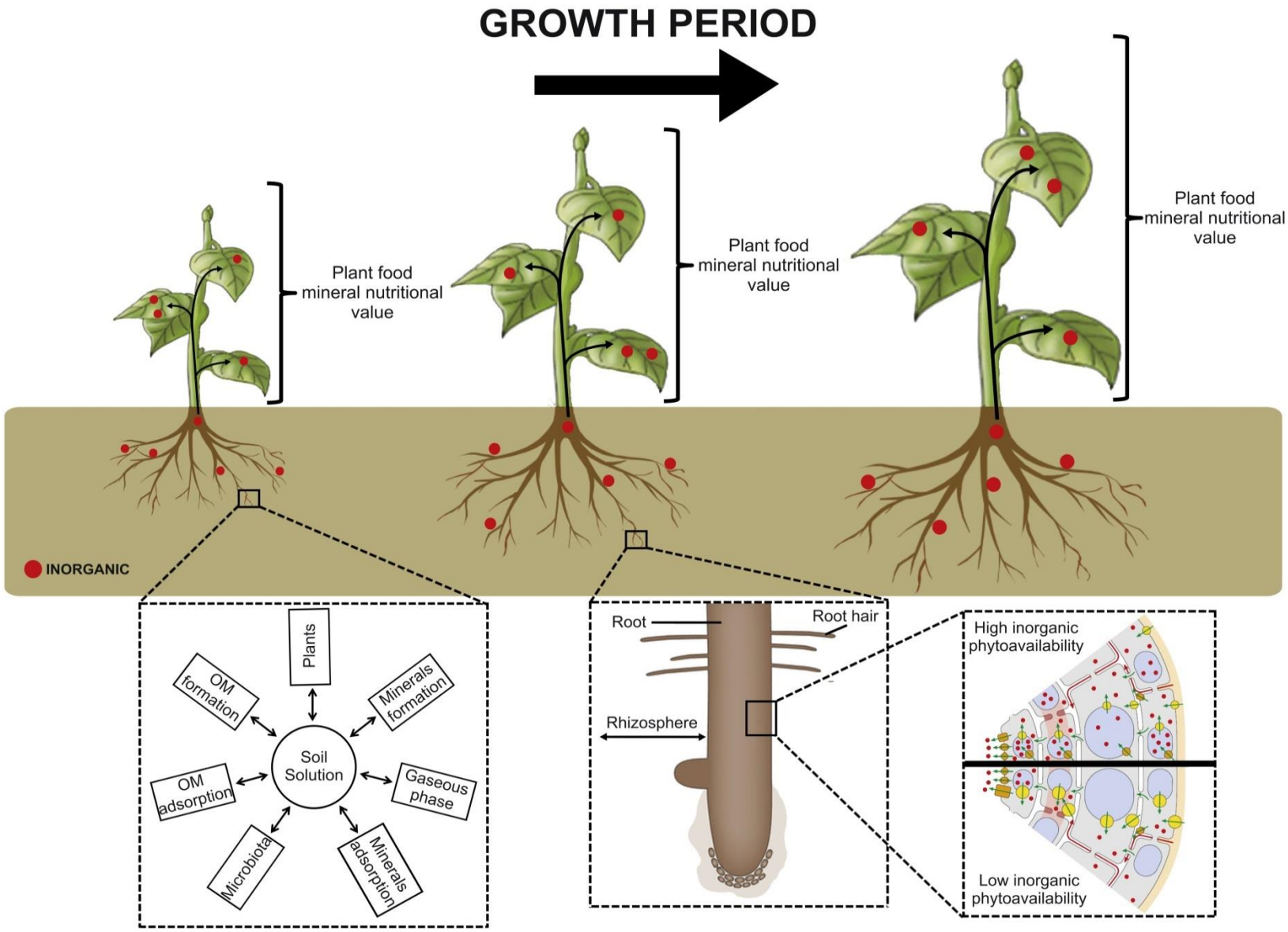
What is the role of N metabolism regarding nitrate accumulation in plant foods?

In aerobic soils, the main form of inorganic N is nitrate. Thus, plants take up nitrate to fulfil their physiological N requirements. Once within the plant tissues, nitrate can be distributed in the root and shoot or can be assimilated. In the process of N metabolism, two important enzymes, nitrate reductase and nitrite reductase, mediate the conversion of nitrate to ammonium. This two-step process is initiated by nitrate reductase, which ensures nitrate conversion into nitrite. Afterwards, the nitrite reductase converts nitrite into ammonium. This process can occur in both roots and shoots. The conversion of ammonium into amino acids and proteins is the main mechanism of ammonium assimilation and detoxification and two enzymes, glutamine synthetase and glutamate synthase, mediates all that process. The high affinity of glutamine synthetase for ammonium enables the assimilation of ammonium at low levels which is important to control the content of ammonium in plant. However, nitrate content is not so easily controlled since, besides providing the amino group to several important plant compounds, nitrate has a major role in osmoregulation. Thus, this anion can be present at higher levels inside some cellular organelles such as vacuoles.

How the plant ionome is affected by soil physicochemical properties?

The broad variation in the soil mineral components provides a huge challenge to farmers to grow their crops. The accumulation of a given element is a complex gene-regulated process comprising its uptake, binding, transportation and sequestration. However, many of these genes and physiological processes affect more than one element. Therefore, it is of huge importance to measure as many elements as possible in a cell, tissue, or organism (the ionome). The elements that share components of their network vary depending on the plant species and environment where they are grown. In fact, the changes observed in the soil chemical environment, caused either by the plant (e.g., root exudates) or by changes in the environment (e.g., rain and drought) are frequently referred as the ones with major impact in the plant ionomic profile. Therefore, several soil properties are thought to greatly influence the phytoavailability of several chemical species which, in turn, will affect the plant ionome.

All of these concerns fall within the topic of food chemistry, an important section of food science, that deals with the composition and properties of food and the chemical changes it undergoes during production, handling, processing and storage. Thus, a food chemist is greatly focused in the study and control of biological substances, such as plant foods, as sources of human food. To know the intrinsic properties of plant foods and understand the means of manipulating them are common interests of both food chemists and biological scientists. The primary interests of biological scientists include reproduction, growth and changes that plant foods undergo under environmental conditions. By contrast, food chemists are concerned primarily with postharvest physiology of plant foods and the changes they experience when exposed to a wide range of environmental conditions. Food chemists share many common subjects with biological scientists, however they also have interests that are clearly different and are of the greatest importance to humanity.


GROWTH PERIOD
Plant food mineral nutritional value
Plant food mineral nutritional value
Plant food mineral nutritional value
INORGANIC
Plants
OM formation
Minerals formation
Soil Solution
OM adsorption
Gaseous phase
Microbiota
Minerals adsorption
Root
Root hair
Rhizosphere
High inorganic phytoavailability
Low inorganic phytoavailability

OBJECTIVES

The main objective of this thesis was to provide new insights about plant food nutritional value, understanding the soil-to-plant transfer of chemical species during plant growth and how this transfer influences the plant ionome, the N metabolism and the nitrate content. To achieve this main goal, several specific goals were established as follows:

- Review the literature to apprehend the influence of soil chemistry and plant physiology in the composition of plant foods;
- Search for the most appropriate sample pretreatment and extraction method for quantification of nitrate and nitrite in vegetables;
- Assess the most suitable soil extraction method to estimate the phytoavailability of essential and non-essential elements as well as to predict their content in plant foods;
- Study the influence of soil physicochemical properties and composition in the plant ionomic profile and the potential interrelations between different elements;
- Monitor changes in the content of chemical species, namely macrominerals, trace elements and pigments in plant foods along its growth period;
- Evaluate the potentiality of using plant foods in their initial stages of development as important sources of mineral nutrients with lower nitrate content in the human diet, especially in infant nutrition;
- Study the influence of soil physicochemical properties in the N metabolism of plant foods, in particular in the nitrate content;
- Evaluate the polyamines content in plant foods during growth; previous developement and validation of an analytical methodology involving ultrasound-assisted benzoylation and dispersive liquid-liquid microextraction.

THESIS ORGANIZATION

According to the proposed objectives and in order to allow a better integration of all the data presented, this thesis was divided in six parts and ten chapters (see page xli).

Part I corresponds to **Chapter 1,** which is entirely dedicated to present a global overview of the soil-plant transfer system. This chapter aimed to describe the current and relevant knowledge regarding the presence of inorganics in soil, plant and water and how they are involved in the processes that take place in these environmental compartments. A detailed description of inorganics behavior in soil and plants is presented, with a special focus in the movement of these chemical species within and between environmental compartments.

Part II, III and IV includes Chapters 2 to 8. These chapters describe the scientific research work developed in the scope of this thesis.

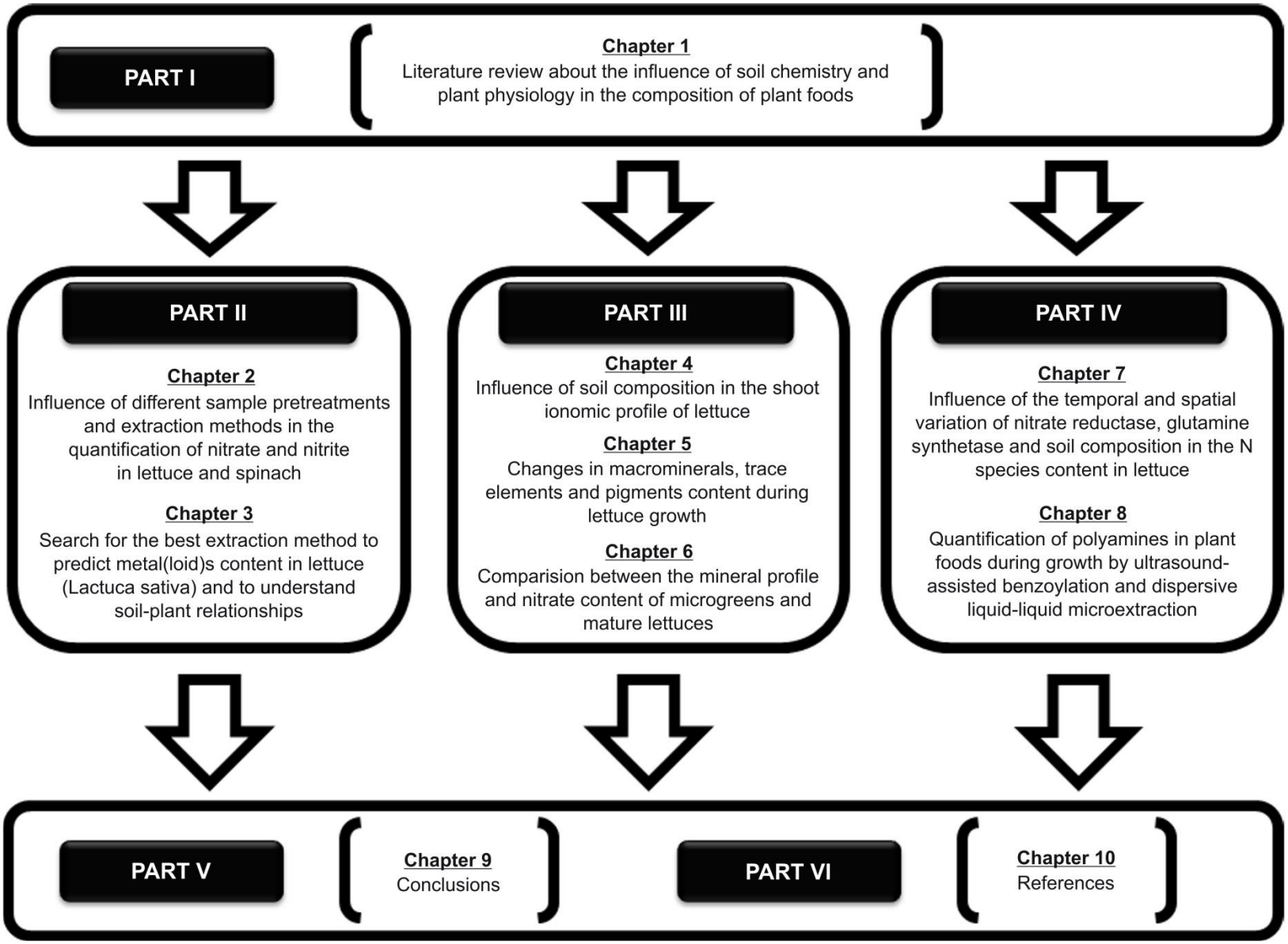
Part II is dedicated to the extraction methods for the analysis of vegetables and soil. This part includes: **Chapter 2,** which describes the influence of different sample pretreatment and extraction methods for the analysis of nitrate; and **Chapter 3**, which describes the study of the most appropriate strategy to predict the soil phytoavailability of inorganics to lettuce

Part III is dedicated to the soil-to-plant transfer of chemical species during lettuce growth and its influence in lettuce nutritional value. It comprises three chapters: **Chapter 4**, describes the study on the influence of soil physicochemical properties in the plant ionomic profile; **Chapter 5** about the mineral content of lettuce along its growth period; and **Chapter 6** describes the mineral profile and nitrate content of microgreens and how these greens can be a richer source of minerals, with a lower nitrate content, compared to mature lettuces.

Part IV is on the N metabolism of plant foods during their growth period. Two chapters are included here: **Chapter 7**, which describes the influence of soil physicochemical properties and specific enzymes involved in N metabolism in the nitrate content of plant foods along their growth period; and **Chapter 8**, which presents the optimization and validation of a ultrasound-assisted benzoylation procedure coupled to dispersive liquid-liquid microextraction for the sensitive quantification of polyamines content in several plant foods, and its application in the analysis of lettuces during their growth period in different experimental fields.

Part V corresponds to **Chapter 9**, which presents the general discussion, the main conclusions and achievements of the work as well as future prospects.

Part VI corresponds to **Chapter 10**, where all the references used throughout the thesis are listed.


PART I
Chapter 1
Literature review about the influence of soil chemistry and plant physiology in the composition of plant foods
PART II
Chapter 2
Influence of different sample pretreatments and extraction methods in the quantification of nitrate and nitrite in lettuce and spinach
Chapter 3
Search for the best extraction method to predict metal(loid)s content in lettuce (Lactuca sativa) and to understand soil-plant relationships
PART III
Chapter 4
Influence of soil composition in the shoot ionomic profile of lettuce
Chapter 5
Changes in macrominerals, trace elements and pigments content during lettuce growth
Chapter 6
Comparision between the mineral profile and nitrate content of microgreens and mature lettuces
PART IV
Chapter 7
Influence of the temporal and spatial variation of nitrate reductase, glutamine synthetase and soil composition in the N species content in lettuce
Chapter 8
Quantification of polyamines in plant foods during growth by ultrasound-assisted benzoylation and dispersive liquid-liquid microextraction
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PART I

Theoretical background

Chapter 1

Literature review about the influence of soil chemistry and plant physiology in the composition of plant foods

1.1. Introduction

Minerals are elements present in the soil that cannot be produced by living organisms, such as plants and animals. However, plants, animals and humans need minerals in order to be perform their physiological functions (1, 2). Plants absorb minerals from the soil, and animals get their minerals from the plants or other animals they eat. Most of the minerals in the human diet comes from plant foods, such as fruits and vegetables, or from animal sources (3-5). Minerals from plant sources may vary widely, depending on the mineral content of the soil in which the plant was grown (6, 7). Minerals are also present in drinking water, particularly in mineral water.

By definition, essential minerals for humans are those minerals whose removal from the human diet or other route of exposure results in "a consistent and reproducible impairment of a physiological function" (4). Human requirements for essential minerals vary from a few μg/day up to about 1 g/day (8). If low intakes in a certain period of time occur, deficiency signs may develop. On the other hand, high intakes can result in toxicity. For most minerals, the range of safe and adequate intake is fairly wide, so deficiency or toxicity is uncommon as long as a varied diet is consumed. This wide-range of safe and adequate intakes exists because humans have homeostatic mechanisms for dealing with both low and high exposures to essential nutrients (1, 5).

The implementation of recommended dietary allowances (RDAs), defined as "the levels of intake of essential nutrients that, on the basis of scientific knowledge, are judged by the Food and Nutrition Board to be adequate to meet the known nutrient needs of practically all healthy persons" was a very important milestone in human nutrition science. RDAs are established by estimating the requirement for the absorbed nutrient, taking into consideration the incomplete utilization of the ingested nutrient (bioavailability) and integrating a safety factor to account for variability among individuals. Assuming this approach, RDA values are greater than the human requirements and, thus, individuals with nutrient intakes below the RDA do not necessarily have an inadequate intake (8).

Plant foods constitute one of the most important nutrient sources in human diet since the beginning of civilization. To grow and develop, plants must take up water and essential mineral nutrients from the soil. Once taken up by plant roots, nutrients can be transported and accumulate in other parts of the plant. The final composition of the edible parts of plants is influenced and controlled by several factors, including soil fertility, plant genetics and the environment in which they grow (9-11). The list of essential minerals for plants is quite similar but not equal to the list for humans. For example, Se, I, Co and Na are essential for humans but not for most plants (12). Therefore, human deficiencies may arise for these elements in populations that depend on plants grown locally where the soil

content of these elements is low. Actually, serious human deficiencies of Se and I exist worldwide (13). For nutrients required by both plants and humans, one can expect that human deficiencies will be less of a problem because the elements will necessarily be present in plant foods. However, the mineral content of plants is sometimes very low to meet human requirements, or the minerals may be present in forms that cannot be efficiently utilized by humans. Examples of both these situations are Ca and Fe, respectively (14-16). Although it is possible in some cases to enhance the nutritional quality of plant foods through agronomic practices and plant breeding, the soil-to-plant and plant-to-human movement of mineral nutrients is a very complex process (3). First of all, soils differ greatly in their mineral composition. Besides, the total mineral content of a soil is not a good indicator of the amount that can be actually taken up by plant roots, since mineral phytoavailability depends on several factors (17-19). Secondly, plants possess physiological mechanisms to regulate the amounts of nutrients taken up from the soil. Hence, one can expect that attempts to alter the overall mineral composition of plant foods will be very complex (9, 20-23). Thirdly, it has been recognized that the concentration of a nutrient in a food is not necessarily a reliable indicator of the value of that food as a source of the nutrient in question. This leads us to the concept of nutrient bioavailability, defined as the amount of a nutrient in ingested food that is available for utilization in metabolic processes. In the case of mineral nutrients, bioavailability is determined mainly by the efficiency of minerals absorption from the human gut (2).

In the last decades, the continuous growth of the world population and the quest for material goods has generated a massive expansion in agricultural production. Demand for food is quickly rising and will continue to rise with increases in global population. World population is expected to grow 47%, reaching 8.9 billion people in 2050 (24, 25). Since the 1960s, intensive irrigation and massive use of chemical fertilizers and pesticides allowed an increase in the food production obtained from the same surface of land. However, the extensive use of mineral fertilizers influences plant food composition and causes serious contamination of soil and water, affecting plant food nutritional value and decreasing the quality of water and land for human purposes (26-28).

One of the major potential hazards to consumer health linked to fertilization is the contribution of N fertilizers to nitrate levels in the food supply (29). Nitrates can be reduced to nitrites which then may react with secondary amines to produce nitrosamines under the influence of gastric acid. Large amounts of nitrate can accumulate in plant foods, particularly in leafy plants, when excessive amounts of N fertilizers are applied as a top dressing during growth (30). Usually, nitrates are absorbed and metabolized by plants; they start to accumulate when the uptake rate is higher than the reduction rate (31). The adequate monitoring of nitrate in plant foods and its lower intake through the human diet is

advisable and has been of major concern for several international organizations (29, 32, 33).

Nowadays, soils contain inorganics of mainly two origins: those that are directly inherited from the lithosphere (known as the mother material) – lithogenic elements; and those that are added to the soil as a direct or indirect consequence of man's activities – anthropogenic elements (18, 34). Both lithogenic and anthropogenic elements are affected by pedogenic processes, which have an essential role in the distribution and speciation of elements in soil. Evidence has suggested that the behavior of inorganics in soils and thus their bioavailability differ according to their origin. Recent findings have clearly stated that regardless of the origin of anthropogenic elements in soils, their phytoavailability is significantly higher compared to those of lithogenic origin. Consequently, it is expected that under similar soil conditions, lithogenic elements will be less mobile and bioavailable than anthropogenic elements (35).

1.2. Soil Chemistry

Soil is a unique component of the biosphere, acting as a geochemical sink for contaminants and a natural buffer controlling the transport of elements and substances to the atmosphere, hydrosphere and biota. The common assumption that soil is simply a combination of unconsolidated material originated from weathering processes is one of the most reductive and incorrect concepts. Soil is a natural resource comprising inorganic and organic components, with particular physical, chemical and biological properties (18).

The composition of soil is very diverse and heterogeneous (Figure 1.1) and is influenced by many factors. However, climatic conditions and parent material have been described as the most important ones. Overall, soil is composed of three phases: (1) solid (inorganic and organic compounds), (2) liquid and (3) gaseous. Besides the chemical composition of the solid components of soil, the mineral structure and the state of dispersion are also important factors that influence soil properties (35).

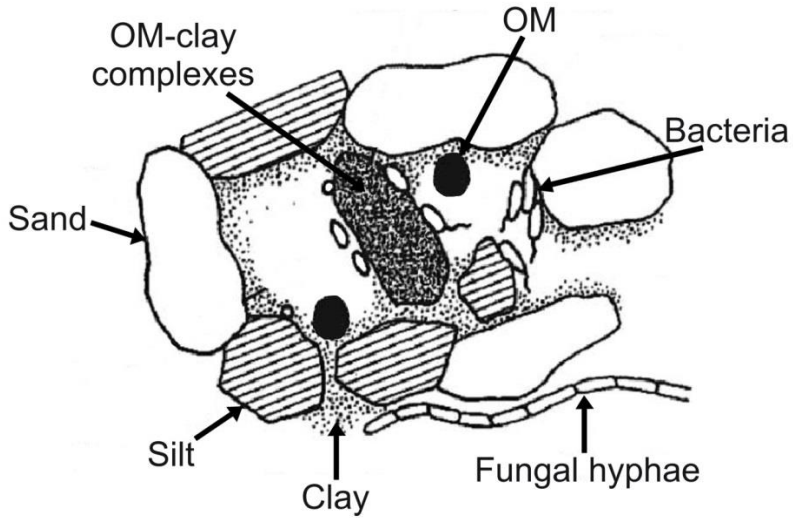


Figure 1.1 – Schematic representation of the complex heterogeneous nature of soil in terrestrial environments. Adapted from Fedotov, Kördel (17).

Inorganics include macro and trace elements and, regardless of their very different contents, both play a key role in soil nutrition and fertility. Studying the link between inorganics and their affinity to other soil constituents is the key to understand the principles governing their behaviour in soils. The natural elemental composition of soil is relatively well-established. The establishment of “normal levels” of inorganics in soils is of utmost interest since background values are needed to make risk assessments regarding soil contamination. Background concentrations of inorganics in soil are almost exclusively influenced by geological and physicochemical properties (36). Recently, it has been emphasized the need to determine local background concentrations, because the parental material highly influence inorganics content. In the last years, several publications reported background levels of selected inorganics in soils of particular countries such as Spain (37), Italy (38), France (39), England (40) and China (41).

The most important role of soil is the production of plant foods which are basic for the survival of humans. Therefore, the maintenance of the ecological and agricultural functions of soil is a top priority. Soil plays a key role in determining the quality of environment Physical, chemical and biological interactions that occur between plants and the surrounding soil are the most complex experienced by land plants and influence plant food composition. Recent years have seen great advances in the understanding of the complexity of some of these interactions, including the processes involved in nutrient and water uptake by roots under ideal conditions as well as when some nutrients are in short supply (18, 42).

1.2.1. Inorganics solubility and soil properties

The solubility and speciation of inorganics in soil defines their mobility and availability. Therefore, understanding the factors that control inorganics solubility and speciation allows a more comprehensive knowledge of the soil – plant transfer processes (43). Inorganics content can vary significantly; however it is well-established that the load at a certain site is the sum of parental material and atmospheric conditions of that place (44, 45).

The aqueous phase of the soil (usually known as “soil solution”) is composed of water with dissolved substances and colloidal suspension, which can be constituted by free ions of various elements, free inorganic salts, organic and complex compounds. Various ion pairs can also occur (46). The knowledge of total composition of soil solution composition is essential for predicting plant uptake of nutrients as well as plant growth. The partitioning of inorganics between the soil (solid phase) and soil solution determines their mobility and bioavailability. The concentration of inorganics in soil solution is a close reflex of their mobility and availability. However, the chemical composition of soil solution is in constant change and it is difficult to predict. The solubility and speciation of inorganics in soil depends on several physical, chemical, microbial, and plant factors (43, 47). As shown in Figure 1.2, the dynamic equilibrium between soil components is controlled by several interactions between the solid, aqueous and gaseous phases of soil and the biota.

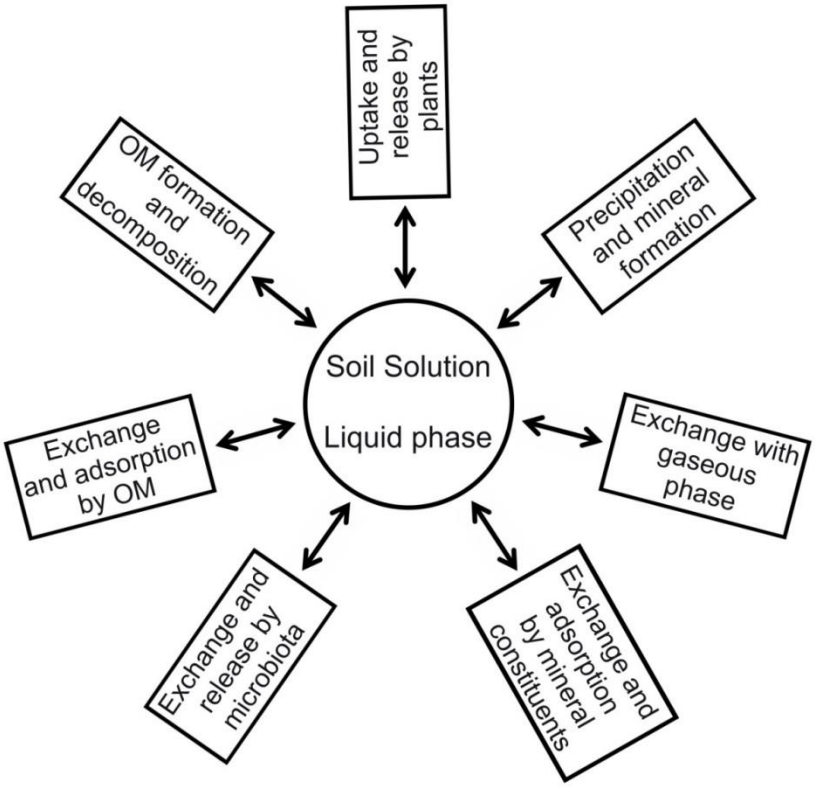


Figure 1.2 – Schematic interactions between soil components and compartments. Adapted from Kabata-Pendias (48).

The stability of minerals and the electrochemical properties of elements govern the mobility of inorganics. As a result, in a waterless environment, physical weathering usually takes place and chemical transformation is poor. On the other hand, in a tropical climate, the high temperature and humidity favours chemical weathering, which results in the formation of more clay minerals. The basic weathering processes can be categorized in: (1) dissolution; (2) hydration; (3) hydrolysis; (4) oxidation; (5) reduction and (6) carbonatization (49). All these reactions are strictly controlled by the chemical equilibrium of a particular soil which is influenced by several intrinsic soil properties.

The main soil parameters that control the processes of sorption and desorption of inorganics and thus the soil solution composition are: (1) pH and redox potential (Eh), (2) cation exchange capacity (CEC), (3) particle size distribution, mainly the fine granulometric fraction (< 0.02 mm), (4) oxides and hydroxides, mainly of Al, Fe and Mn, (5) organic matter (OM) and (6) microorganisms (35, 50). However, other environmental factors such as precipitation, temperature and evaporation can have a pronounced impact in the composition of the soil aqueous phase. High temperatures accelerate physical, chemical and biological processes. Precipitation and high soil moisture increases the migration of water-soluble elements (45, 51, 52).

Since soil pH and Eh control all redox reactions in soil, a strong correlation exists between these soil properties and free ion species content. In normal soil conditions, pH ranges between 5 and 7, and Eh ranges between 300 and 750 mV, except in waterlogged soils where high reduction states are present (53-55). Since the most mobile fractions of ions occur at lower pH, it can be expected that, by increasing soil pH, the solubility of most elements will decrease (Figure 1.3). This can be easily observed worldwide since alkaline and neutral soils have significantly lower concentration of inorganics in soil solution than acid soils (56, 57). Generally, the mobility of inorganics such as Cd and Zn increases with increasing Eh. Therefore, for most elements, oxidizing soil conditions favour inorganics solubility, mobility and availability. However, one should be aware that the diversity of ionic species and their different affinities to form complexes with inorganic and organic ligands make possible the dissolution of each inorganic over a wide range of pH and Eh (58).

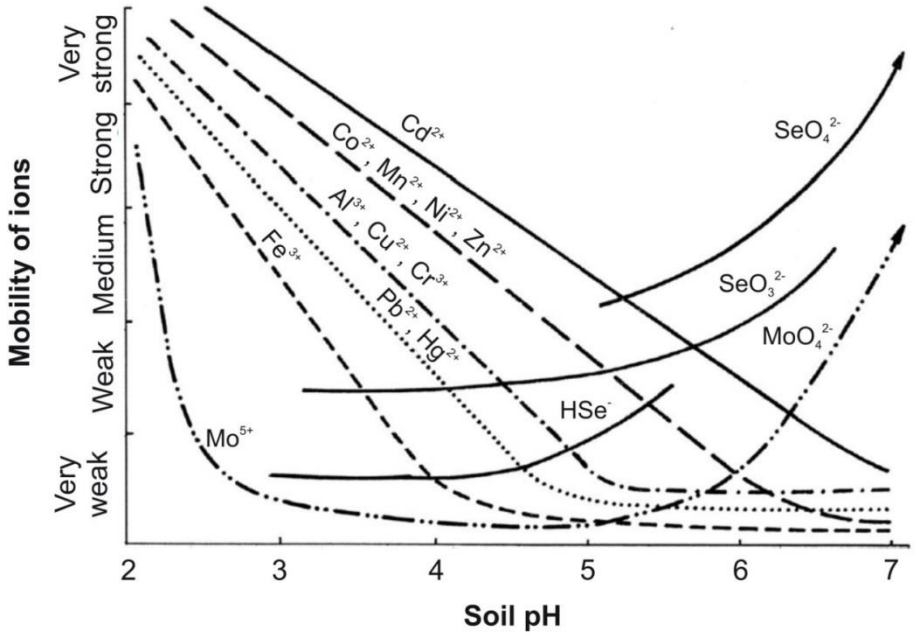


Figure 1.3 – Examples of trends in the mobility of inorganics as a function of soil pH. Adapted from Kabata-Pendias (48).

The affinity of inorganics for soil components is strongly influenced by their electrochemical properties and is closely related to the specific surface area and CEC of minerals. The ability of the solid soil phase to exchange cations (the so-called CEC) is one of the most important soil properties governing the mobility of cations in soil (59). The amount of adsorbed cations compared to the amount in solution is interpreted as the

buffering capacity of soils, whereas adsorption capacity defines the amount of ions needed to occupy all the adsorption sites per unit of soil mass (50, 60).

The CEC of different soils varies widely both quantitatively and qualitatively, and can range from 1 to 100 cmol/kg (37, 56, 61, 62). Surface properties of soil particulates are the most important factors in defining its capacity for cations adsorption. Although total adsorption processes cannot be related simply to CEC phenomena, the amount of adsorbed cations is in accordance with the CEC. Usually, soil particulates with a large surface area show a high CEC value and high adsorption and buffer capacities (63).

The affinity of cations for adsorption (i.e., for binding at anionic sites) is closely related to ionic potential (charge/radius). For example, Co, Fe and Ni ions occupy almost the same percentage of the CEC of various minerals due to the very similar ionic potential. However, some cations may have a higher replacing power than others and can be selectively fixed by the sorbing sites (64).

The size and shape of mineral particles define their ratio of surface to volume and mass, which in turn determine their physical and chemical properties. Therefore, the particle size distribution is considered to be one of the most important soil characteristics and is included in the soil classification systems (65).

Most elements are generally associated with the clay- and silt-size fractions of soil (61). These fractions are mixtures of several aluminosilicate minerals with low amounts of feldspars, quartz, oxides and hydroxides. In certain soils, carbonate, phosphate, sulfides and sulfates may also be present (49).

The surface properties of clay minerals, high active surface areas and presence of electrical charge, seem to be essential for the buffer and sink properties of soil and thus have a major effect on soil physicochemical properties (66-68). The binding processes involved in ions absorption still remain controversial. However, it has been demonstrated that pH and Eh are key factors in the sorption and desorption reactions of cationic elements by clay minerals (69, 70). Clay minerals greatly vary in chemical composition and thus in their properties, especially CEC. The ability of the clays to bind the metal ions is highly correlated with their CEC, and generally higher CEC values result in higher amount of cation adsorbed (59).

Clay minerals usually contain negligible amounts of trace elements as structural components. Their sorption capacities to cations are their most important role. The affinity of trace elements for the clay surface has been studied by many investigators which concluded that cations content increases as particle-size decreases (71, 72).

Several (hydr)oxides occur in soils. The presence of oxides and hydroxides is so common that they sometimes determine the soil colour due to their high pigment content (73). The formation of (hydr)oxides occurs mainly during weathering of minerals, although

pedogenic processes are also involved. Generally, they possess a high surface area which is related with their high affinity for ions adsorption, especially cations (74).

Among (hydr)oxides, Fe and Mn (hydr)oxides are very important since they greatly influence inorganics mobility in soil. However, Al (hydr)oxides can also adsorb a variety of inorganics and in some soils the role of these (hydr)oxides can be even more important than Fe (hydr)oxides (62, 75). The mechanism behind the sorption process involves the isomorphic substitution of divalent or trivalent cations for Fe and Mn ions, cation exchange reactions and oxidation effects at the surface of the (hydr)oxides precipitates (67).

Despite the high sorption capacity of Fe and Mn (hydr)oxides for cations, the presence of variable charges at the surfaces of (hydr)oxides also promotes the adsorption of anions. A high sorption capacity of Fe (hydr)oxides for phosphates, arsenites and selenites is widely observed (69, 76). The sorption by the (hydr)oxides surface is a highly pH-dependent process for both cations and anions (68). For example, the highest adsorption for various ions on Fe (hydr)oxides is observed for pH values between 4 and 5. However, evidence suggests that their sorption capacity also increases significantly with increasing pH and was found to be the highest at a pH 8 (77).

In soil, the OM consists in a mixture of plant and animal products in various stages of decomposition as well as biologically and chemically synthesized compounds. OM is widely distributed in soils and plays a key role in weathering and thus in geochemical cycling of inorganics (78). Most of the OM in soils results from biological decay of the biota residues. Plant roots exudates, composed of a wide diversity of simple organic acids, occur in most soils and also contribute to the OM content. The composition and properties of OM are dependent on several factors such as climatic conditions, soil types and, when applied, agricultural practices (64, 79).

A clear relationship exists between OM and some soil properties such as CEC and pH. OM increases the CEC of soils because OM possesses multiple binding sites for cations which results in a great sorption capacity (66). This is very important regarding metal contamination of the soil since the ability of OM to adsorb toxic metals such as Cd, Cu and Pb significantly reduces their bioavailability, and thus, their harmful consequences (80). OM is of great importance in the mobilization, especially in the leaching of cationic metals. Due to the relatively lower solubility of OM-metals complexes, OM may act as an important regulator of the mobility of cations in soils (81). Despite its typical negative charge, soil OM is also an important sorbent of anions by controlling the partitioning of anionic species into soil OM (82).

It should be noted that the reaction between OM and cations can lead to the formation of water-soluble and/or water-insoluble complexes. OM can act as a reducing agent and assist in the mobilization of some elements. Usually, compounds acting as a reducing

agent form insoluble complexes with a number of cations but also form few soluble species (83, 84). In those cases, pH has a direct role by controlling the solubilization and precipitation of OM-inorganics complexes (85, 86). The solubility of OM-inorganics complexes depends on both the binding strength and the mobility of the complex formed, which is determined mainly by the size of the organic group involved. Therefore, metal binding to a low molecular weight organic compound will increase its mobility in soil (87, 88).

Trace metals in soil tend to accumulate bound to organic compounds and the lower the metal content, the higher the linkage of the OM-metal complex. The commonly used stability constant of a complex can be defined as the equilibrium constant of a reaction that forms a soluble complex or chelate. The stability constants of OM-metal complexes depend mainly on the pH and type of element (89, 90). Overall, the stability of OM-metal complexes increases with the pH increase from 3 to 7. For example, OM-metal complexes of Cu, Fe and Zn are soluble at alkaline pH (between 7 and 12) and precipitate at low pH (below 3) as shown by (91, 92). Therefore, depending one the pH, one could expect that some metals are more tighly bound to OM and thus less bioavailable.

Soils with a high OM content usually display complex interaction between OM and trace elements. Deficiency symptoms may arise in plants grown on soils rich in OM due to the strong retention (low phytoavailability) of essential micronutrients (e.g., Cu, Mn, Mo and Zn) as insoluble OM-metal complexes (81, 93). On the other hand, the application of OM to soil raises the number of microorganisms able to reduce several cations, such as Fe and Mn, leading to an increase in their availability (94, 95).

Living organisms, known as the soil biota, are ubiquitous in soils. Their abundance in topsoils is mainly influenced by both soil and climatic conditions. Among all living organisms, microbiota particularly important because they are engaged in the production, consume and transport processes that take place in the soil ecosystem. Consequently, microbiota is involved in the cycling of chemical elements and is responsible for a vast number of processes ranging from the mobilization to the accumulation of chemical elements in the soil (47).

The most important role of microorganisms is the degradation of plant and animal residues. It has become widely accepted that the quantity of inorganics has an important role in the growth of microorganisms, which in turn influences the biological activity of soils. The microbial activity is highly controlled by soil properties (96). For example, microorganisms are involved in soil OM transformations thus their activities are associated with OM content (97). Since many inorganics, especially metals, are toxic to microorganisms, they have developed efficient mechanisms to detoxify these elements. The most common detoxification mechanisms are processes of oxidation, reduction,

alkylation and efflux of metals. Among these, the processes of alkylation and dealkylation are very effective in controlling the toxicity and behavioural properties of some elements (98).

Even though microorganisms are sensitive to both deficiency and excess of inorganics, they have the capacity to adapt to extreme environments. With the recognition that microbial transformations of inorganics can result in soil fertility and pollution problems, the ecological importance of microorganisms was recognized (99). The physicochemical relationship between microbiota and elements leads to changes in the valence of inorganics and/or conversion into OM-inorganics complexes. And as already highlighted, these processes are key factors controlling the solubility (and thus the bioavailability) of these elements in soils (47, 100).

High levels of toxic elements such as As, Cd, Cr, Hg and Pb are known to reduce the growth and decomposing capacity of soil microorganisms, which reflects in the reduced capacity of soils to decompose organic pollutants (e.g., pesticides) and organic debris. The negative effect of those toxic elements on the decomposition of OM and on the mineralization of N and P compounds is also widely described (97, 101, 102).

It should be emphasized that all the above described soil properties are inter-related and work together to maintain the multiple processes that occur on soil. For example, CEC is influenced by both pH and OM. When soil pH becomes acid, the bioavailability of cations generally increases due to the replacement of cations on soil binding sites by H^+ ions (59). Oxides can interact with clay minerals establishing complexes or aggregates that may play an important role in OM stabilization (103). OM has several binding sites for cations thus a high OM content will result in a higher retention of cations, decreasing their bioavailability (78). Moreover, clayey soils hold more water than sandy soils, and they have more binding sites for ions, especially for cations. However, the relative proportion of clay, silt and sand in soils has also a great influence in the mobility of anionic species. Generally, anions' leaching is higher in sandy soils than in clayey soils mainly due to the slower water movement (49).

It should also be noted that, being a heterogeneous system, soil properties can significantly change within a few centimeters (even millimeters) at both horizontal and vertical directions resulting huge differences among close sites of the soil. Therefore, elements can be readily precipitated and/or adsorbed when small shifts occurs in the soil conditions influencing the solubility and bioavailability of inorganics (18, 104).

1.2.2. Rhizosphere processes

The rhizosphere includes the area surrounding the root (approximately 1 mm) and plays an important role in phytoavailability. The physical, chemical and biological interactions

that take place between roots and the surrounding environment are largely controlled or directly influenced by roots and are often referred as rhizosphere processes. These include water uptake, exudation, nutrient mobilization, rhizosphere respiration and rhizosphere-associated OM decomposition (104, 105).

Root exudation includes the secretion of ions, free oxygen, water, enzymes, mucilage, and a wide variety of primary and secondary metabolites (106). Roots release compounds using at least two potential mechanisms: (1) root exudates are transported across the root cell membrane and are released into the surrounding rhizosphere and (2) root exudates are secreted from root border cells and root border-like cells (107). The compounds released by roots can be divided in two main classes: (1) low molecular weight compounds (e.g., amino acids, phenolics, sugars and organic acids) which are responsible for the great diversity of root exudates, and (2) high-molecular weight exudates (e.g., mucilage and proteins), that are less diverse, but usually represent a larger proportion of the root exudates (79).

Even though that the several functions of most root exudates have not yet been determined, some evidence supports that several compounds present in root exudates have an important role in biological processes occurring in the rhizosphere zone (108). Plant-plant interaction can also occur. These are usually divided in negative or positive interactions (109). The negative interactions that occur between plants (i.e., chemical interference, resource competition and/or parasitism) can be potentially affected by root exudates. Some plant species use the release of phytotoxins that have a direct inhibition effect in the growth of other plant species (i.e., allelopathy) as a mechanism to gain an advantage over their competitors (107). In fact, plants that secrete those potent phytotoxins can reduce the establishment, growth or survival of neighbour plants, thus reducing the overall competition as well as increasing resource availability (110).

Positive plant-plant interactions can also be governed by root exudates. In particular, some root exudates induce defense responses that initiate the production and secretion of a wide variety of low molecular weight terpenes and green leaf volatiles that increase herbivore resistance (111). Some root exudates also induce defense responses in neighbor plants which reduce herbivore populations indirectly by attracting predators and parasites of the undamaged plant (112).

Finally, root exudates may have an important indirect effect on both positive and negative plant-plant interactions by altering the soil chemistry. Specifically, root exudates can increase or decrease soil nutrient availability by altering soil physicochemical properties and soil biological processes (113). Effects of root exudates on soil inorganics availability are usually stronger in the rhizosphere of the plants that produce them, conferring a competitive advantage over neighbour plants that lack the same capacities (114).

The phytoavailability of inorganics is highly dependent on the rhizosphere processes. The solubility and speciation of inorganics in this area is usually distinct from the bulk soil. Plant roots improve soil aeration through the extraction of soil moisture which results in the formation of channels that not only affect water flux, but also provides pathways for the rapid transport of solutes and suspended particles (115, 116). The phytoavailability of inorganics is significantly affected by biological activity that occurs in that area. Plants can release compounds in the rhizosphere zone that play crucial roles in the mobility, uptake, tolerance, sequestration and transport of inorganics (100, 117), as summarized in Figure 1.4.

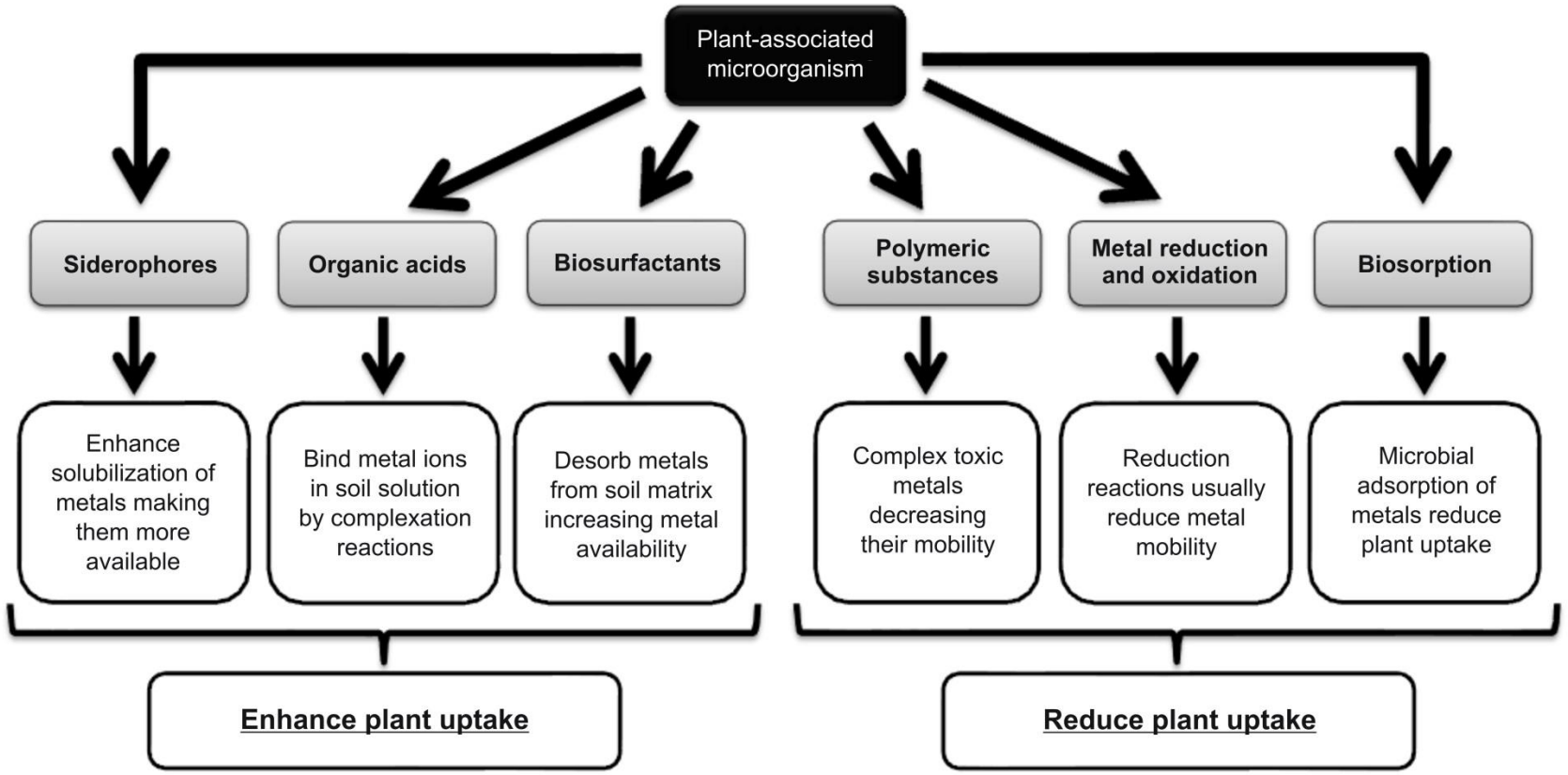


Figure 1.4 – Schematic diagram of the major plant associations and their roles in plant. The production of siderophores, organic acids and biosurfactants generally promotes inorganics uptake. The presence of polymeric substances, a reductive medium and biosorption processes reduce inorganics uptake.

The best evidence that plants use chelators in root exudates to increase micronutrient availability comes from studies on the effects of phytosiderophores (PS) on Fe availability. Despite the fact that Fe is relatively abundant in soil, it is often present as Fe^{3+} insoluble compounds, particularly in soils with alkaline or neutral pH (118). The process of root exudation varies between plant species and is mainly described for Fe. Two main categories can be created according to the plant species: Strategy I in non-graminaceous plants and Strategy II in graminaceous plants (Figure 1.5).

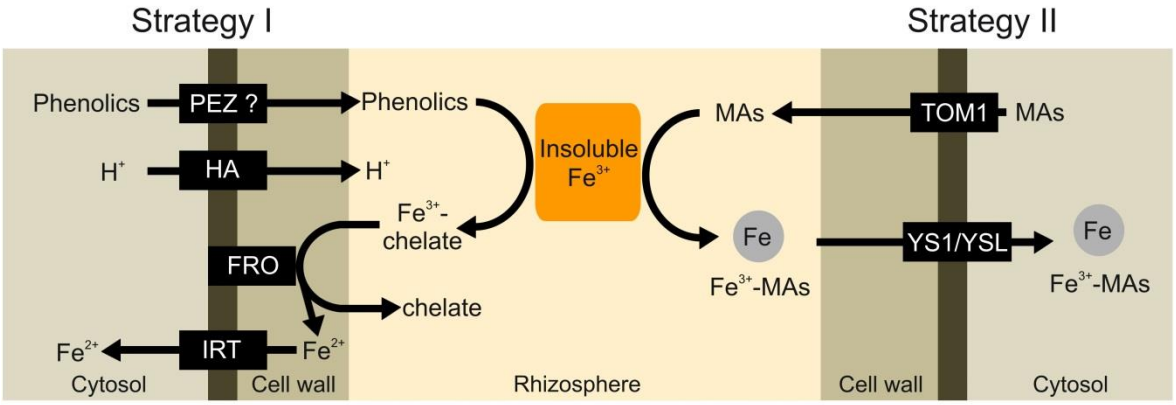


Figure 1.5 – Fe acquisition strategies in plants: (1) Strategy I in non-graminaceous plants (left) and (2) Strategy II in graminaceous plants (right). Black boxes represent the transporters and enzymes that play essential roles in these strategies. Adapted from Kobayashi and Nishizawa (114).

The main processes of Strategy I are the excretion of protons and phenolic compounds from the roots to the rhizosphere, reduction of ferric chelates at the root surface, and the absorption of the resulting ferrous ions across the root by plasma membrane transporters. The H^+-ATPase (HA) transporters are responsible for the efflux of protons to promote the acidification of the rhizosphere (119, 120) and the recently identified OsPEZ1 and OsPEZ2 (phenolics efflux zero) seems to be involved in the secretion of phenolics (121, 122). Non-graminaceous plants depend on ferric reductase oxidase (e.g., AtFRO2) to reduce the ferric chelates at the root surface (123). The exudations of protons may acidify the rhizosphere by up to 2 pH units. Hence, an increase of the solubility of essential and non-essential cations occurs (124, 125). Moreover, both protons and phenolic compounds are thought to help the reducing capacity of ferric chlelates that seem to be involved in the reduction of other inorganics besides Fe (123). The Strategy II used by graminaceous plants, depends on the biosynthesis and secretion of PS such as mugineic acids (MAs) which mobilize Fe and other elements of low phytoavailability (126). Recently, the gene

responsible for MA secretion was identified to be the transporter of mugineic acid family phytosiderophores 1 (OsTOM1 and HvTOM1, for rice and barley, respectively). The MAs secreted into the rhizosphere stimulates the solubilization of Fe^{3+} in the soil (127). Despite being Strategy II plants, graminaceous can also had ferrous transporters (e.g., OsIRT1) allowing the absorption of Fe^{2+} in addition to its Strategy II–based Fe^{3+}-MA uptake (128). However, graminaceous plants are usually very low in ferric-chelate reductase activity at the root surface which suggests that those plants are only adapted to the direct uptake of Fe^{2+} (118). Although the above-described mechanisms were first studied for Fe, the phytoavailability and uptake of other inorganics are thought to be controlled or influenced by similar mechanisms (129).

Organic acids released by plants also play an important role in the uptake of inorganics into the roots as well as in their transport, sequestration and tolerance within plant tissues. In general, organic acids can bind inorganics in soil solution by complexation reactions, making them more available for plant uptake (130, 131). Evidence suggests that although organic acids can act as metal chelators in the rhizosphere, they are more important in P availability than on micronutrients availability (132). Phosphorus, like Fe, is relatively abundant in soils, but in non-available forms. More specifically, P is often present as insoluble Al, Fe and Ca phosphates, especially in soils with high pH (133). Organic acids (e.g., malic, citric and oxalic acids) can form complexes with Al or Fe in aluminium and ferric phosphates, inducing the release of phytoavailable phosphates into the soil solution (134). Furthermore, organic acids may also increase P availability by blocking P binding sites on soil particles or by forming complexes with cations on soil mineral surfaces (135). Despite its role in P availability, it should be emphasize that organic acids exudated by plant roots also have an important role in metal solubility and availability (86).

Plant-microorganisms interactions also are responsible for several intrinsic processes (e.g., carbon sequestration, elements cycling, and ecosystem functioning) (47, 109). Those interactions can positively affect plant growth through a variety of mechanisms such as formation of rhizobia-legumes interactions, establishment of plant growth–promoting rhizobacteria (PGPB) and formation of symbiotic mycorrhizal fungi associations (136). All these plant-microbe associations can potentially release exudates to increase plant tolerance to biotic and abiotic stress as well as to promote soil nutrients availability (100, 137, 138).

The so-called “rhizosphere effect” is no more than the root colonization by microorganisms attracted by nutrients exuded by plant roots. Several authors have stated that the number and activity of microorganisms increase in the vicinity of plant roots and plant roots initiate cross talk with soil microbes that catalyze the root colonization (106). Although the chemical attraction of soil microbes to plant roots (known as chemotaxis) is a

well described mechanism, other mechanisms such as electrotaxis (the use of electric potentials in plant roots to attract microbes) may also affect the root colonization process (109).

One of the most studied positive plant-microbe interactions are the symbiotic associations between rhizobia and leguminous plants that promote the fixation of atmospheric N in root nodules (136). This rhizobia-legume interactions are very specific, allowing only specific rhizobial strains to nodulate with specific host legumes, however nodulation and probably N fixation are not ubiquitous within the legume family (139). Bacteria can locate plant roots through chemical signals exuded from the root, and root exudates such as amino acids and carbohydrates stimulate PGPB chemotaxis (106). Evidence has shown that bacteria grown in the rhizosphere (i.e., rhizobacteria) provide benefits to the plant, resulting in plant growth stimulation (79). Moreover, studies have been demonstrating that some PGPB produce phytohormones, such as cytokinins, auxins and gibberellins, which have a direct role in enhancing plant growth (140).

Arbuscular mycorrhizal fungi (AMF) and plant roots form associations in more than 80 % of terrestrial plants. This symbiotic relationship has several advantages for both plants and fungi since, in one hand, plants increases their nutrients uptake and improve their overall health status and, in other hand, the associated fungi extract useful compounds such as lipids and carbohydrates from the host root necessary to perform their metabolic processes (141). Although the effects of AMF have been almost exclusively attributed to an increased phosphate uptake, evidence indicates that they may also influence other inorganics supply. Studies conducted by Hart and Forsythe (142) and Ortas (143) revealed variable distribution of macro and trace elements in crops inoculated with AMF. The presence of mycorrhizal symbiosis has also been demonstrated in plants, especially in hyperaccumulators, which are being used in the phytoremediation of metal contaminated soils (100).

Siderophores can also be produced by most plant-associated microorganisms in response to low Fe levels in the rhizosphere. These low molecular weight chelators play an important role in enhancing extracellular solubilization of Fe from minerals, making it available to the plant-microbial consortium (144). In addition to Fe, other inorganics, such as Cu, Mn, Ni and Zn, can also stimulate or inhibit siderophore production. Additionally, toxic metals may induce the production of some siderophores that have chelator capacity and can play a role in toxic metal tolerance (145).

Plant-associated microbes can also produce low molecular weight organic acids that can have an important role in the solubilization and mobilization of inorganics in the rhizosphere (100). A study from Li, Ye (146) shows that the inoculation of soils with Cd/Zn rhizobacteria significantly increased the soil phytoavailable content of Zn and Cd when

compared with un-inoculated controls. The authors concluded that the enhanced metal availability was correlated with the increased production of organic acids.

Biosurfactants are another group of important metabolites that have the potential to improve inorganics mobilization and phytoavailability. These amphiphilic molecules have the capacity to form complexes with inorganics at the soil interface and to desorb elements from soil matrix, further increasing their solubility and phytoavailability in the soil solution (147).

Other processes based on plant-microbe associations can promote inorganics mobilization or immobilization. The production of extracellular polymeric substances and glycoproteins (148), redox reactions (149) and biosorption mechanisms (150) all have the capacity to alter the bioavailability and plant uptake of inorganics through metal mobilizing actions.

1.2.3. Soil extraction methods

As soils consist of heterogeneous mixtures of different organic and organo-mineral complexes, clay minerals, (hydr)oxides of Fe, Al, and Mn, as well as a variety of soluble substances, the binding mechanisms for inorganics in a particular soil will depend on the specific soil composition and physical properties. An element may form different species depending on the type and extent of its binding to soil compounds, the reacting surface of a certain mineral and the energy of the internal and external binding sites. In order to evaluate the speciation or binding forms of inorganics in solid materials, several analytical procedures involving single and sequential extractions have been developed. The determination of the different species of inorganics actually present in the samples (i.e., speciation) is broadly used, especially to fulfil two purposes: (1) to assess their bioavailability/potential bioavailability and (2) to predict their mobility within the soil profile and to ground waters (17).

A huge debate has been carried on over the years regarding the suitability of different extraction methods to determine the amounts of inorganics associated with various soil phases. Regardless of all the limitations of these methods, some of them have been widely used. Generally, all the extraction methods are based on the assumption that inorganics, especially cations, exist in the following physicochemical forms:

(1) Water soluble (e.g., soil solution);
(2) Exchangeable;
(3) Organically bound;
(4) Occluded in Fe and Mn oxides;
(5) Definite compounds (e.g., metal carbonates, phosphates, sulfides); and
(6) Structurally bound in silicates and/or other primary minerals (19).

Depending on the physicochemical characteristics of inorganics, their affinity to soil phases controls their fractionation. The soluble and exchangeable fractions constitute the mobile species of inorganics in soils. The other fractions are less mobile. Overall, the mobilization of inorganics from the immobile fractions, or the transformation of mobile to immobile fractions is a slow and controlled process (49, 151).

One widely used approach to study metal mobility in soil relies on the use of single extraction procedures. Several studies have shown the suitability of these extraction procedures for mimicking plant uptake processes in soils. Different extractants are considered according to their role in the release of elements from particular soil phases where they are bounded or associated. Therefore, extractants can be classified according to these soil phases or binding types, which is a very useful strategy in soil chemistry studies to elucidate the mechanisms of metal binding, transformation or release from soils (19). Various concentrations of different reagents and variable soil/solution ratios over broad pH ranges are used in these extraction procedures (17). In consequence, each method gives its own results, and the data comparision is very difficult or even impossible. The main groups of single extractants commonly used are summarized in Table 1.1.

Table 1.1 – Commonly used extractants and the corresponding soil phases with which they interact.

Soil phase extracted	Extraction method
Water-soluble	Water
Exchangeable	Potassium chloride Calcium chloride Ammonium nitrate Magnesium chloride Sodium chloride Calcium nitrate Acetic acid Ammonium chloride
Less readily exchangeable (specifically sorbed)	Acetic acid Citric acid Copper acetate

Organically bound	Sodium pyrophosphate EDTA DTPA
Fe and Mn oxide-sorbed	Hydroxylamine hydrochloride Sodium dithionite Ammonium oxalate – oxalic acid
Residual (non-silicate-bound)	Nitric acid Aqua regia
Mineral lattice	Nitric/hydrofluoric/perchloric acid

The water-soluble fraction is usually negligible for trace cations, however a large amount of macro elements, both cations and anions, are present in this fraction (151). The water-soluble fraction is very important regarding groundwater pollution since water-soluble species (e.g., free ions or ions complexed with inorganic or organic compounds) are highly mobile within soil profiles (17). Obviously, the water extractable fraction contains low amounts of elements compared to other soil extracted fractions.

In general, studies mimicking the process of inorganics uptake by plant roots, soil deficiency/fertility assessment and phytoremediation rely on the use of weak extractants such as dilute solutions of KCl, $CaCl_2$ or NH_4NO_3 because these extractants are able to reasonably simulate the main processes determining inorganics phytoavailability (152, 153). The exchangeable fraction, which includes the weakly adsorbed ions retained on the solid surface by relatively weak electrostatic interaction as well as elements that can be released by ion-exchange processes, is usually the target site of these extractants. By changing the ionic composition, which in turn influences the adsorption-desorption reactions and soil pH, it is possible to cause the mobilisation of metals from this fraction (151). The exchangeable fraction is a measure of the easily released fraction of elements, those that are readily bioavailable. Metals present in the exchangeable fraction typically represent a very small portion (less than 2%) of the total metal content in soil (19).

A less easily exchangeable fraction, known as specifically sorbed species, is not easily displaced by cations such as Ca or K. Those species require the hydrogen ion to displace them from organic or inorganic binding sites and acetic acid (0.5 M) is commonly used to provide the H^+ ion (151). The leaching procedure TCLP Method 1311 used by the US Environmental Protection Agency (EPA) to assess the contamination of sites relies on the use of diluted acetic acid or acetic acid – acetate buffer at pH 5 (154). However, acetic

acid lacks specificity and thus attacks carbonate and silicate phases leading to overestimated results (19).

Oxalate buffers and reductive extractants are usually applied for the differentiation between lithogenic and anthropogenic origin of metals in soils. The results obtained by these methods are useful in studies of soil classification, soil reactivity and metal mobility in soil (155). Extraction of the fraction associated with Fe and Mn (hydr)oxides usually occur by a combination of mechanisms such as co-precipitation, surface complex formation, ion exchange and adsorption (19, 156). The amorphous Fe and Mn (hydr)oxides strongly sorb trace elements, initially in exchangeable forms, that progressively become less mobile. The acid ammonium oxalate method is able to remove organically complexed and amorphous metals forms of Al, Fe and, to a less extent, Mn as well as noncrystalline aluminosilicates from soils. Dithionite and hydroxylamine methods have an extraction capacity very similar to the oxalate method (157).

Chelating agents such as EDTA and DTPA are often used in studies of physicochemical processes in soils, such as soil-plant metals transfer under the action of chelating species released within the rhizosphere (155). These complexing extractants have strong complexing ability, displacing cations from insoluble organic or organo-metallic complexes in addition to those sorbed on inorganic soil components (19).

The application of strong acids such as HNO_3, HCl or a combination of these (e.g., *aqua regia*) gives the “pseudo-total” element content, which is considered the maximum potentially soluble or mobile content of inorganics and, in the case of environmental metal contaminants, usually the inorganics not bound to silicates. To obtain the fraction of elements bound to silicates, hydrofluoric acid (HF) must be used to ensure the solubilization of these compounds (19).

Besides the above-mentioned single extractant methods, the Mehlich 3 method has been widely recognized as a universal soil extractant due to its ability to measure at once elements bound to several solid soil phases. The extraction solution of this method is a combination of acids (acetic acid and nitric acid), salts (NH_4F and NH_4NO_3) and the chelating agent EDTA (158, 159). In this method, several fractions of the soil will be attacked during the extraction procedure, which will result in higher amounts of elements extracted.

Sequential extraction involves the treatment of a soil sample with a series of different extractants in order to sequentially separate different defined fractions of elements. The main advantage of sequential extraction over the single extractant procedures is that the phase specificity is improved. This occurs because each applied extractant reagent has a different chemical nature (e.g., dilute acid, reducing or oxidant agent) and the steps of the procedure are performed in order of increasing “power”. Therefore, a typical sequential

procedure firstly involves the extraction of free species that are already in the soil solution and, eventually, ionic species weakly attached at binding sites of the soil matrix. Secondly, a stepwise attack to the Fe and Mn (hydr)oxides, OM and carbonate phase is performed. Lastly, more refractory soil components are dissolved with the use of additional reagents (151).

Among the several sequential extraction procedures available in the literature the BCR (Community Bureau of Reference) method is the most adopted by investigators (160). This method relies on the use of different extractants in a sequential four steps procedure (Table 1.2).

Table 1.2 – The BCR (Community Bureau of Reference) optimized sequential extraction procedure.

Step number	Extractant	Fraction	Target soil phase(s)
1	0.11 M acetic acid	Exchangeable, acid- and water-soluble	Soil solution, exchangeable cations, carbonates
2	0.5 M hydroxylamine hydrochloride at pH 1.5	Reducible	Fe and Mn (hydr)oxides
3	30 % H_2O_2 + 1 M ammonium acetate at pH 2	Oxidizable	Organic matter and sulfides
4	Aqua regia	Residual	Non-silicate minerals

The above described extraction methods are usually applied to estimate the content of cationic species. However, extraction procedures have also been developed for the determination of anionic species which are also of great importance for soil fertility/pollution assessments (161, 162).

Overall, a great amount of data has been generated regarding the application of chemical extraction and fractionation methods to the assessment of environmental exposure of inorganics in terrestrial environments and on the biological uptake of inorganics. Therefore, studies on the correlation between soil and plant elements content are widely found in the literature (152, 153, 163). It should be taken into account that multiple and complex processes occur at the same time on soil and that the mobility and bioavailability of a given ion depends not only on its activity in the soil solution but also on the activities

of other ions concomitantly present. Soluble major elements significantly influence the amount of soluble trace ions. For example, the aqueous phase of most soils contains high levels of Ca which has an important role in controlling the soluble state of trace elements (61). Therefore, higher than normal concentrations of dissolved metal ions in soil solutions can greatly interfere with the uptake of trace elements by plants. The anionic composition of soil solutions is also very important in governing the trace element status. Anions like chloride, nitrate and phosphate are easily mobile in soils and can react with cationic species resulting in the mobilization of elements along the soil profiles (46).

1.3. Plant physiology

1.3.1. Plant uptake

The absorption of inorganics by plant roots can be achieved by two ways: passive (non-metabolic) and active (metabolic). Passive uptake comprises the diffusion of ions from the external solution into the root endodermis. Active uptake occurs against a chemical gradient and relies in the use of energy. Either way, the uptake of a particular element will most likely be positively correlated with its available pool at the rhizosphere. The central mechanism in the process of root uptake is the path that elements travel through plasma membranes and intercellular spaces of root cells (164, 165).

Recent developments in the field of plant science have been an outstanding contribution to elucidate some of the mechanisms behind inorganics transport. Recently, several gene families were identified that encode putative inorganics transporters opening the way for studies at the gene and protein levels (e.g., regulation of gene expression at the tissue/organ/plant levels, transport activity, subcellular localization) and for functional genomics and reverse genetics approaches to further understand the integrated roles of these transport systems in plants (10, 20, 22, 23, 166).

Inorganics transport across the plant plasma membrane is mediated by an electrochemical gradient of protons generated by plasma membrane H^+-ATPases. This “primary transport system” pumps protons out of the cell creating a pH and electrical potential differences across the plasma membrane (164). At that point, “secondary transport systems” can utilize these gradients for many plant functions such as nutrients influx and efflux (165). Inorganics enter the root either by crossing the plasma membrane of the root endodermal cells (symplastic transport) or by entering the root apoplast through the space between cells (apoplastic transport). They cannot pass through membranes without the aid of membrane transporter proteins. These transporter proteins occur naturally in several plant membranes (tonoplast, endoplasmic reticulum, mitochondria, chloroplasts) because inorganics are either essential nutrients (N, P, Ca,

Mg, Cu, Zn) or are chemically similar to nutrients and are taken up involuntarily (e.g., Cd can be taken up by Zn transporters) (20, 166, 167). Plants possess multiple transporters for most elements. For instance, rice (*Oryza sativa*) has at least seven Cu transporters (168) and *Arabidopsis thaliana* has 14 sulfate transporters (169). Each transporter has unique properties. When a low concentration of nutrients is present in the soil solution, their uptake usually requires the high-affinity transport system (HATS). By contrast, the low-affinity transport system (LATS) is more used when high concentrations of nutrients are present, such as in agricultural soils after fertilization. Furthermore, the abundance of each transporter varies with tissue type and environmental conditions, making the uptake and movement of inorganics in plants a complex process (10, 20, 22, 166).

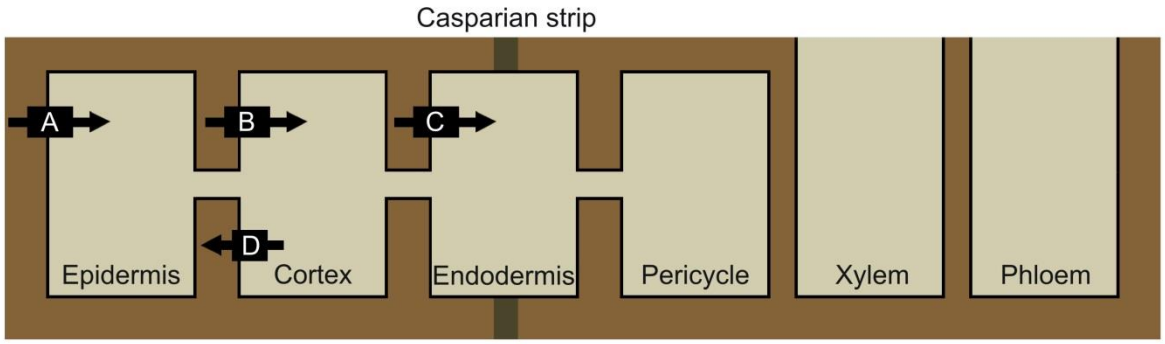
1.3.1.1. Anions

The main inorganic anions present in plants are nitrate (NO_3^-), phosphate (PO_4^{3-}), sulfate (SO_4^{2-}) and chloride (Cl^-). Usually, NO_3^- is the major anion. All together, these anions, in different proportions according to growth conditions, plant species and cell type, balance the positive charges of cations constituting a major part of cell osmoticum. Additionally, NO_3^-, PO_4^{3-} and SO_4^{2-} are essential chemical species that are assimilated to provide nitrogen (N), phosphorus (P) and sulfur (S) for the synthesis of biomolecules by plants (20).

The mechanism of membrane transport of these nutrients depends on the electrical charge and on the direction of transport. Being negatively charged, NO_3^-, PO_4^{3-}, SO_4^{2-} and Cl^- require for their uptake into cells the co-transport of protons to overcome the resting negative membrane potential of the cell. Roots net anions uptake is the balance between anions influx and efflux. However, the term “efflux” comprises both the root-to-soil efflux and root-to-shoot efflux (170, 171). In this section, only the root-to-soil efflux will be addressed. Low amounts of nutrients can also be directly taken up from the leaf surface when deposited from the atmosphere. Foliar fertilizer applications have been widely used in agriculture to overcome nutrient deficiency (172). Some plants such as leguminoses can directly uptake gaseous N_2 from the air by establishing symbiotic interactions with bacteria. These N sources can become very important when N deficient conditions are present. Besides, the acquisition of N and P by plant roots can also be improved by symbiotic associations with mycorrhizal fungi (109).

At neutral/alkaline and aerobic soils, NO_3^- is the predominant N form and it is easily taken up by plants (10). It is widely known that NO_3^- uptake systems are rapidly induced by the presence of NO_3^- in the external environment (173). In fact, the inducible NO_3^- uptake system is very distinct from other nutrients (e.g., P and S) uptake systems which normally are only induced by their substrate deficiency (169, 174). Two NO_3^- uptake systems,

HATS and LATS, provide a regulatory mechanism that ensures increased NO_3^- uptake when substrate becomes available. The HATS and LATS are encoded by genes from the nitrate transporter 1 (NRT1) and NRT2 families, respectively, and require energy provided via a proton-symport mechanism (173). Overall, the members of the NRT1 family comprise the low-affinity NO_3^- transporters with the exception of AtCHL1 (also called AtNRT1.1) and the MtNRT1.3 which are dual-affinity NO_3^- transporters (175, 176). The low-affinity transporter AtNRT1.2 also participates in the uptake of NO_3^- (177). In rice, the orthologue of AtNRT1.1, OsNRT1.1, only shows a low-affinity nitrate transport activity (178). Regarding the NRT2 family, AtNRT2.1, AtNRT2.2, AtNRT2.4, OsNRT2.1, OsNRT2.2, OsNRT2.3 and OsNRT2.4 are included in the HATS (Figure 1.6) (179-181). Among them, both transcriptional and post-transcriptional regulation of AtNRT2.1 has an important role in root high-affinity NO_3^- uptake. It has been showed that the induction of HATS requires the expression of the two-component AtNRT2.1/AtNAR2.1 transport systems enabling the plant to manage with variable N supply (182, 183). A similar mechanism is also observed in rice where OsNRT2.1 and OsNRT2.2 interact with OsNAR2.1 enabling the NO_3^- uptake at both low and high concentrations (184, 185). Regarding NO_3^- efflux, a member of the NRT1/PTR family, nitrate excretion transporter 1 (NAXT1), is mainly expressed in the cortex of mature roots and have an important role in the transport of NO_3^- to the surrounding environment (186).



Transport step	NO_3^-	$H_2PO_4^-$	SO_4^{2-}
A	AtNRT1.1, AtNRT1.2 AtNRT2.1, AtNRT2.2, AtNRT2.4 AtNAR2.1 (NRT3.1)	AtPHT1;1, AtPHT1;2, AtPHT1;3, AtPHT1;4, AtPHT1;8, AtPHT1;9 OsPHT1;6, OsPHT1;10	AtSULTR1;1, AtSULTR1;2
B	AtNRT1;1 AtNRT2.1, AtNRT2.2 AtNAR2.1 (NRT3.1)	OsPH1;1, OsPHT1;6, Os,PHT1;8	AtSULTR1;1, AtSULTR1;2
C	AtNRT1;1		
D	AtNATX1		

Figure 1.6 – Schematic illustration of some of the key transport steps for the uptake and efflux of inorganic N, P and S in the plant (*Arabidopsis thaliana* and *Oryza sativa* were utilized as model plants). The proteins identified for mediating some of these transport functions are given in the table below. The arrows indicate the direction of transport and each letter in the figure corresponds to the list given in the first column of the table.

The availability of inorganic P (Pi) in the soil is usually low due to its precipitation with Al- and Fe-oxides and hydroxides at low pH and with Ca at alkaline conditions (69). In soil solution, Pi can exist in several forms ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) depending on the pH. However, below pH 6, which is the typical soil solution pH, Pi almost exclusively consists of the monovalent $H_2PO_4^-$, while the other phosphate species will be present only in low amounts (10). Studies evaluating the pH effect on inorganic P (Pi) uptake found that uptake rates are higher when pH values are between 5 and 6, in which the $H_2PO_4^-$ species dominates. Therefore, it has been suggested that Pi uptake is achieved in its monovalent form (187). Nine high-affinity Pi transporters have been isolated in *Arabidopsis thaliana*. From those, six different plasma-membrane transporters, AtPHT1;1,

AtPHT1;2, AtPHT1;3, AtPHT1;4, AtPHT1;8 and AtPHT1;9, have been identified in the roots of Arabidopsis as shown in Figure 1.6 (188, 189). Both AtPHT1;2 and AtPHT1;3 transporters have a spatial expression in roots very similar to AtPHT1;1 and AtPHT1;4. However, AtPHT1;1 and AtPHT1;4 are highly expressed compared to AtPHT1;2 and AtPHT1;3 (190). Therefore, the focus is usually given to AtPht1;1 and AtPHT1;4 which play a key role in the acquisition of Pi from the outside environment in both low- and high-Pi conditions (191). Recently, it was showed that AtPHT1;8 and AtPHT1;9 also have a function in Pi acquisition through plant roots on low-Pi environment (189). In rice, 26 potential Pi transporters, named as OsPHT1;1 to OsPHT1;26, have been identified and, from those, eight are functionally characterized (192). OsPHT1;1, OsPHT1;2, OsPHT1;6, OsPHT1;8, OsPHT1;9 and OsPHT1;10 are essential members of the PHT1 family involved in Pi uptake in rice (193-196). Two members of the PHT1 family, OsPHT1;11 and OsPHT1;13, are exclusively induced in roots by inoculation with arbuscular mycorrhiza fungi (197, 198).

In an aerobic environment, SO_4^{2-} is the dominant chemical form of sulfur (S) and thus the main source of S in which plants rely on. However, in anaerobic conditions, other S forms such as FeS, FeS_2 and H_2S can also be present (10). In soil-root interface, the SO_4^{2-} transport system, constituted by members of the SULTR family, is highly expressed in the epidermis, root hairs and cortical cells and are induced or repressed by S availability (169). In *Arabidopsis thaliana*, 14 transporters have been identified but only few of them have been fully characterized (199). Two high-affinity SO_4^{2-} transporters, AtSULTR1;1 and AtSULTR 1;2, play a key role in SO_4^{2-} uptake as showed by the *sultr1;1sultr1;2* double knockout mutant, which is entirely defective in SO_4^{2-} uptake capacity under low-S conditions (Figure 1.6) (200). However, evidence suggests that these two SO_4^{2-} transporters are not functionally equivalent. Independently of the SO_4^{2-} availability in soil, AtSULTR1;2 is the main SO_4^{2-} uptake transporter. In fact, SULTR1;2 seems to be responsible for at least 80% of the SO_4^{2-} uptake (201). Moreover, SULTR1;2 may also have a regulatory function in response to S nutrient status (202). From the 14 rice SO_4^{2-} transporters identified, two of them, OsSULTR1;1 and OsSULTR1;2, are highly expressed in rice root tissues and seem to play a key role in the uptake of SO_4 from soil solution (203).

The majority of worldwide soils contain sufficient levels of Cl^-. Plants take up most of their chloride from the soil solution in the form of the Cl^- anion. Since Cl^- is a high mobile anion within plant tissues, its main function is related to electrical charge balance (204). Although Cl^- is a very important micronutrient for plants, the underlying mechanisms of its transport are not fully understood. Studies performed in root plant cells have demonstrated several different types of anion channels with a potential role in Cl^- fluxes

(205). In terms of active transport, the recent characterized members of the the cation-chloride co-transporter (CCC) family are thought to play an important role in the uptake and homeostasis of several elements, including Cl^- (206, 207). Moreover, some evidence exist that Cl^- influx and efflux can also be mediated by members of the NRT family (208).

The essential element Mo and the beneficial element Se occur in soils mainly in their anionic forms molybdate (MoO_4^{2-}) and selenate (SeO_4^{2-}) and/or selenite (SeO_3^{2-}), respectively. These anionic species can be taken up by plasma membrane transporters present in plant root cells. Furthermore, in the case of Se, organic Se compounds such as selenomethionine can also be taken up actively by plant roots (209, 210). Until now, very few MoO_4^{2-}-specific transporters have been identified. In fact, AtMOT1 is the only Mo-transporter identified to play a key role in the MoO_4^{2-} acquisition from soil and its regulation inside Arabidopsis (211, 212). Since MoO_4^{2-} and SO_4^{2-} are chemical analogues, competition for the same transporters can occur. The close interaction between MoO_4^{2-} and SO_4^{2-} transport in many biological systems suggests that a similar transport system may be involved in the uptake of Mo (213, 214). Plants take up Se because they possess very limited ability to distinguish between Se and S. Selenate, the main form of Se in alkaline and well-oxidized soils, enters root cells symplastically through SO_4^{2-} transporters localized in the plasma membranes of the root cells (214, 215). However, different SO_4^{2-} transporters in a certain plant might have different selectivity for SO_4^{2-} and SeO_4^{2-}. Arabidopsis mutants lacking SULTR1;2 but not SULTR1;1 show significantly enhanced SeO_4^{2-} resistance (216). Furthermore, Arabidopsis *sultr1;1sultr1;2* double mutant shown unequal functional redundancy regarding SeO_4^{2-} uptake and tolerance, suggesting that SULTR1;2 may be the predominant transporter in the acquisition of SeO_4^{2-} into the root cells (217). Regarding SeO_3^{2-}, less is known about the mechanisms responsible for its uptake (210). It seems that SeO_3^{2-} uptake is a metabolic dependent process probably mediated by PO_4^{3-} transporters (218).

Regarding non-essential/toxic anionic species, no specific transporter have been identified as responsible for their transport. Usually, since these species have similar physicochemical characteristics, their transport is mediated by transporters engaged in the mobilization of essential elements (219). Arsenic is present in the soil solution usually in the form of arsenate (As^{5+}), arsenite (As^{3+}), monomethylarsonic acid and dimethylarsinic acid. The bulk of arsenic acid (H_3AsO_4) is dissociated as the oxyanions $H_2AsO_4^-$ or $HAsO_4^{2-}$ under typical soil pH values (pH 4-8). Since these As species are chemical analogues of PO_4^{3-} ions, As^{5+} can be taken up by plant roots through the PO_4^{3-} transporters. In fact, great evidence exists of a competitive behaviour between As^{5+} and PO_4^{3-} influx across root cells. In Arabidopsis, members of the PHT1 family, AtPHT1;1, AtPHT1;4, AtPHT1;8 and AtPHT1;9, were shown to mediate As^{5+} transport (189, 190,

220). In the same way, rice transporter OsPHT1;8 also seems to participate in As^{5+} influx (221). It must be noted that different PO_4^{3-} transporters may have different affinities for arsenate. However, until now, the relative affinities of PO_4^{3-} transporters for As^{5+} and PO_4^{3-} remain poorly characterized (219). In contrast to As^{5+}, arsenous acid (H_3AsO_3) is usually found in its undissociated form at normal pH conditions. Consequently, As^{3+} uptake is performed by plant roots in the form of the neutral molecule $As(OH)_3$. In plants, the nodulin 26-like intrinsic proteins (NIPs), a sub-family of the plant major intrinsic proteins (MIPs) known as aquaporins or water channels, are permeable to As^{3+} (222). For example, the membrane transporters AtNIP1;1, AtNIP1;2, AtNIP5;1, AtNIP6;1 and AtNIP7;1 from Arabidopsis as well as OsNIP2;1 (also known as Lsi1) and OsNIP3;2 from rice were identified as bi-directional As^{3+} channels potentially engaged in As influx and efflux from the root-soil interface (219, 223-226). Even though some NIP aquaporins allow bi-directional transport of As^{3+}, efflux of As^{3+} from the exodermis and endodermis cells in rice roots to the stele is facilitated by the silicon (Si) membrane transporter Lsi2 (226, 227). Recently, the involvement of a member of the NRAMP (natural resistance associated macrophage proteins) family, OsNRAMP1, in As^{3+} influx and mobilization in rice was suggested, rising the complexity of inorganic As uptake and transport in plants (228). Methylated As can also be taken up by plant roots. Since the permeability of the two main methylated As species, monomethylarsonic and dimethylarsinic acids, across the plasma membrane is too low, simple diffusion of these molecules through the lipid layer of the plasma membranes would occur at such a slow rate that their uptake into root cells would be almost irrelevant. Evidence suggests that the rice aquaporin Lsi1 mediates the uptake of undissociated pentavalent monomethylarsonic and dimethylarsinic acids (229).

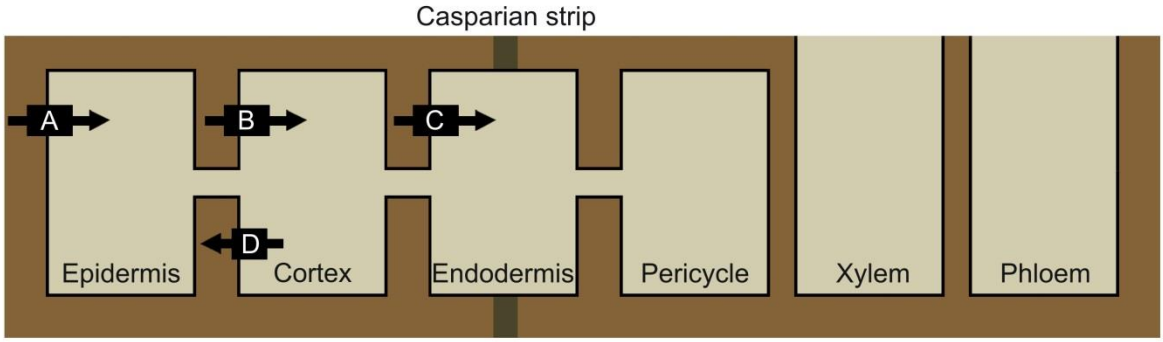
1.3.1.2. Cations

The cationic species, potassium (K), calcium (Ca) and magnesium (Mg) are necessary in large amounts by plants. The trace metals iron (Fe), copper (Cu), manganese (Mn), nickel (Ni) and zinc (Zn) are essential trace elements for plants since they participate in several physiological mechanisms that take place within plant tissues, particularly inside plant cells. Ammonium (NH_4^+) can be an important source of inorganic N to plants in anaerobic conditions. Other cationic elements such as Na, Co and Al are considered to be “beneficial” for plants. Some evidence exists on the beneficial effects of other trace elements (e.g., V and Cr), yet more data is needed to fully clarify their actual importance (12). Usually, the uptake of these major and trace elements is performed by plant roots from the soil solution where their availability can vary by several orders of magnitude (9-

11). Nevertheless, foliar uptake can also be an important source of these elements, especially in crop species (230).

A very distinct pattern in the accumulation of mineral elements is observed between plant tissues, cell types and subcellular compartments which is the result of selective transport systems catalysing the movement of these elements (9, 11). When we think about cation transport, it is important to note that cations can either be essential or toxic depending on the amount present in a certain system/tissue (12). Therefore, plants developed sophisticated regulatory uptake and efflux systems in order to control the intracellular concentrations of cationic species. Similarly to anions transporters, HATS and LATS are present in the several plant tissues providing a complex network regarding cations uptake (9, 11).

Potassium (K) is the most abundant cation in plant cells and plays a vital role in plant growth and development (231). Normally, K content in soil solution ranges between 1 and 10 mM whereas intracellular K levels are maintained at 100 – 200 mM. Therefore, K uptake from the soil is obviously against a concentration gradient (232). Similarly to most mineral nutrients, the main acquisition of K from the outside environment follows a biphasic form, defined as the sum of two distinguishable uptake mechanisms (HATS and LATS) at the root plasma membrane (231). Several genes appear to encode putative K transporters/channels in the plasma membrane of Arabidopsis root cells (Figure 1.7). They are grouped into four families, including HAK/KT/KUP (K^+/H^+ symporters), HKT/Trk (K^+/Na^+ symporters), CHX (cation/H^+ antiporters) and *Shaker* channels (233). The bulk of HATS that mediate K influx are catalyzed by members of the HAK/KT/KUP family (234). In Arabidopsis, 10 of 13 AtKT/KUPs are expressed in root hairs. Five of them were expressed in root tip cells suggesting an essential role of these HATS in K uptake (235). From these 5 AtKT/KUPs expressed in Arabidopsis roots, AtKUP1, AtKUP4 and AtHAK5 are root plasma membranes that seem to be engaged in the K influx (235-238). The physiological importance of HKT/Trk transporters for high-affinity K influx is not yet fully clear. Several plant studies have pointed out that most of HKT transporters function as Na transporters and only a limited number of these transporters are Na^+/K^+ symporters. Therefore, evidence supports a restricted role of the HKT family in K uptake (239, 240). Several members of the CHX family have been identified as K transporters. For example, AtCHX13 and AtCHX17 have been proposed as possible pathways for root K influx (241, 242). Low-affinity K influx seems to be mediated by Shaker K-channels such as AtAKT1 and AtKC1, which are mainly expressed in root epidermal cells (243-245). Plant Shaker channels display extraordinary K selectivity. Consequently, the Shaker K-channels AtAKT1 and AtKC1 as well as the HAK/KT/KUP transporter AtHAK5 are responsible for almost all K^+ influx (234, 246-248).



Transport step	K	Ca	Mg
A	AtKUP1, AtKUP4 AtHAK5 AtCHX13, AtCHX17 AtKT1, AtKC1	DA-NSCCs HA-NSCCs VI-NSCCs	AtMGT1, AtMGT7, AtMGT9
B	AtCHX17 AtKT1, AtKC1	VI-NSCCs	
C			
D			

Figure 1.7 – Schematic illustration of some of the key transport steps for the K, Ca and Mg uptake in plants. The genes identified as putatively being involved in some of these transport functions are given in the table below. The arrows indicate the direction of transport and each letter in the figure corresponds to the list given in the first column of the table.

A peculiar electrochemical gradient exists for Ca in plant roots since cytosolic Ca levels are in sub-μM range, while Ca concentrations in the soil solution are in the mM range (9). Calcium is taken up symplastically through nonselective cation channels (NSCCs) present in the root system. These can be divided according to their voltage dependence into depolarization-activated (DA-NSCCs), hyperpolarization-activated (HA-NSCCs) and voltage-insensitive (VI-NSCCs) types, as shown in Figure 1.7 (249). The DA-NSCCs are thought to be encoded by homologues of the AtTPC1 gene, although this is not yet confirmed (250). Regarding HA-NSCCs, members of the annexin gene family are likely to encode these Ca channels (251). For VI-NSCCs, two gene families, the plasma membrane-localized cyclic-nucleotide gated channels (CNGC) and the ionotropic

glutamate receptor (GLR) homologues are proposed to mediate Ca uptake (252-254). Despite the type of NSCC used to mediate Ca influx, the opening of these channels must be tightly regulated, because changes in cytosolic Ca levels trigger several specific responses to many environmental and developmental stimuli (255).

Magnesium concentration in soil solutions normally ranges from 8.5 to 125 mM. Cytosolic Mg levels are around 0.4 mM, thus Mg influx into root cells can be achieved by Mg-permeable cation channels (9). Despite that, members of the MRS2/MTG gene family expressed in Arabidopsis appear to dominate the Mg uptake across the root plasma membrane (Figure 1.7) (256). A member of the *A. thaliana* Mg transport family, AtMGT1, was expressed in tobacco (*Nicotiana benthamiana*) and showed an increased Mg content (257). Besides AtMGT1, other two MRS2/MTG members, AtMGT7 and AtMGT9, are expressed in Arabidopsis roots and have a key role in Mg uptake (258, 259).

Several Fe species may be taken up by plant roots. The divalent form (Fe^{2+}) is the main absorbed species but Fe^{3+} and Fe-chelates are also taken up. Plants have developed several strategies to enhance Fe uptake. Non-graminaceous plants (e.g., *Arabidopsis thaliana*) depend on ferric reductase oxidase (e.g., AtFRO2) to reduce the ferric chelates at the root surface (123) and then on the iron-regulated transporter (AtIRT1, AtIRT2 and AtIRT3) to absorb the generated Fe^{2+} ions across the root plasma membrane (Figure 1.5) (260-262). Graminaceous plants (e.g., barley, maize and rice) secrete phytosiderophore (PS) compounds to stimulate the Fe solubilization in the soil, and then take up the resulting Fe-PS complexes through Yellow Sripe (YS) and Yellow Stripe-like (YSL) transporters such as HvYS1, ZmYS1 and OsYSL15 (263-266). Besides, in some graminaceous plants (e.g., rice), the divalent Fe can also be taken up by the membrane transporters OsIRT1, OsNRAMP1 and OsNRAMP5 expressed in the plasma membrane of rice (267, 268), although this is not very common (118).

The most bioavailable form of Cu in soils is Cu^{2+}. However, root uptake is often facilitated by reduction of Cu^{2+} to Cu^{+}. The ferric reductase oxidases AtFRO2, AtFRO3, AtFRO4 and AtFRO5, expressed in *A. thaliana*, seem to be involved in this process (269, 270). Copper is likely to enter the cytosol of root cells through a cell surface COPT/Ctr-family transporter. Six members of the Ctr family (COPT1-6) which mediate the influx of Cu have been identified in Arabidopsis. AtCOPT1, AtCOPT2 and AtCOPT3 are expressed in the plasma membrane of roots and have an important role in the acquisition of Cu monovalent ions (271, 272). Furthermore, members of the Zinc-Regulated Transporter and Iron-Regulated Transporter (ZRT-IRT)-like proteins (ZIP) family can also mediate the uptake and transport of Cu in Arabidopsis (272, 273). Members of the YS and YSL family (e.g., ZmYS1 and HvYSL2) are also involved in the transport of Cu-PS complexes (264, 274, 275).

The oxidized forms Mn^{3+} and Mn^{4+} are not bioavailable to plants. It is the reduced form of this element, Mn^{2+}, that is absorbed by root cells (276). The plasma membrane IRT1, expressed in both Arabidopsis and barley, can transport Mn (260, 277). The overexpression of AtIRT2 leads to overaccumulation of Mn in transgenic plants, but the role of this transporter in Mn uptake remains to be clarified (262). The plasma membrane-localized NRAMP1 and NRAMP5 were shown to be high-affinity Mn transporters in Arabidopsis and rice, respectively. Both AtNRAMP1 and OsNRAMP5 have broad selectivity, and their expression is restricted to the root (267, 278). In maize and barley, Mn uptake can also be performed by the ZmYS1 and HvYSL2 transporters, respectively, which were confirmed to be capable of acquiring Mn-PS complexes from the rhizosphere (264, 274).

Zinc is taken up across the plasma membrane of root cells as a free ion and/or complexed with PS (129). The ZIP transporter family includes the best candidates for facilitating Zn influx into the plant cytoplasm. In addition to its role in Fe acquisition, the broad selectivity of AtIRT1 allows this transporter to mediate also the uptake of several divalent metal cations, including Zn (260, 279, 280). This was also demonstrated in rice plants overexpressing OsIRT1, which accumulate elevated levels of Zn in the shoots, roots and mature seeds (281). Like their close homologue AtIRT1, AtIRT2 and AtIRT3 are also able to transport Zn into roots (261, 262). Other ZIP transporters are involved in the uptake of Zn from soil. For example, OsZIP1 and OsZIP3 are likely to play a role in this process (282). In Arabidopsis, AtZIP4 expression is induced upon Zn deficiency, showing its function as a Zn transporter (283). The rice plasma membrane-localized OsZIP8 also seems to be a Zn transporter that functions in Zn uptake (284). A member of the NRAMP family, AtNRAMP1, is also able to mediate Zn influx into the root cells of Arabidopsis (278). Zinc is also taken up from the rhizosphere in the form of Zn–PS complexes by members of the YS and YSL family (264, 274).

At low pH and in waterlogged soils, NH_4^+ is the main form of inorganic N taken up by plants. Therefore, plants like rice that grow in submerged environments are well adapted to the surrounding conditions and thus have a relatively large numbers of NH_4^+ transporters (10). The active specific acquisition of NH_4^+ is mainly performed by members of the ammonium transporter (AMT) family. Six members of this family have been identified in *Arabidopsis thaliana*, which fall into two clades, AMT1 and AMT2 (285). From the AMT1 sub-family, AtAMT1;1, AtAMT1;2, AtAMT1;3 and AtAMT1;5 are the main AMTs responsible for the NH_4^+ uptake from soil (286-288). Furthermore, in maize roots, two rhizodermis-localized transporters, ZmAMT1;1a and ZmAMT1;3, have also showed to be engaged in the NH_4^+ uptake (289). From the sub-family AMT2, the NH_4^+ transporter

AtAMT2;1 was detected in Arabidopsis roots and seems to play a key role in the mobilization of NH_4^+ within plant tissues (290).

Since soil salinity is a global environmental challenge and NaCl is typically the major salt that contributes to that salinity, a huge research activity has been dedicated to the characterization of Na transport and distribution in plants, and particularly to its uptake by plant roots (291). Sodium can enter the root cells by NSCCs, more specifically by the VI-NSCCs. In fact, VI-NSCCs permeability to Na has been demonstrated in several tissues and species resulting in substantial evidence that root Na influx is mediated by these channels (249). Another candidate for mediating Na influx is the low-affinity cation transporter (LCT) 1. However, this function is still not clear and further investigation must be carried out (292). K and Na ions compete with each other to enter plant cells due to their physicochemical similarity. Therefore, it is plausible that K transporters may also be involved in Na acquisition and distribution. However, the involvement of K transporters from both the HAK/KT/KUP and AKT families in Na movement is somewhat contradictory. Some studies support the idea that some members of the HAK/KT/KUP and AKT families are involved in Na accumulation (237, 243, 293, 294) but others fail to confer a specific role to these transporters in Na influx (246, 295). Significant evidence exists for the involvement of K transporters from the HKT families in the Na influx (296, 297). The HKT family can be divided in two different sub-families according to their properties regarding Na and K transport in heterologous expression systems. The sub-family 1, which shows Na-specific transport activity and mediates Na uptake, includes, among others, AtHKT1;1 and its orthologues OsHKT1;1 and HvHKT1;1 expressed in rice and barley, respectively (298-301). The sub-family 2 functions as a K-Na co-transporter and includes, among others, HvHKT2;1, OsHKT2;1, OsHKT2;2 and OsHKT2;4 (302-304). Recently, members of the CCC family were characterized and seem to play an important role is the influx and transport of Na and other elements (206, 207). It should be pointed out that the involvement of a certain transporter may be genotype-specific and not transversal to all plant species (292).

For other essential (Ni) and beneficial elements (Co and Al), the mechanisms of their uptake remain poorly understood, although some transporters have been identified. Nickel is present in the environment usually in the form of Ni^{2+}, which is the most available form to plants. Divalent cations such as Ni, Cu and Fe have very similar physicochemical properties thus they compete with each other`s in biochemical and physiological processes in plants (305). Nickel can enter symplastically into the root cells through AtIRT1 (306). Moreover, Ni can also be taken up in the form of Ni-PS complexes by the ZmYS1 due to its broad selectivity for several cations (264). Similarly, Co can be taken up in its ionic form Co^{2+} through the plasma membrane-localized AtIRT1 (307) and in the

form of Co-PS complex by the ZmYS1 in maize (264). However, other membrane transporter, AtNRAMP1, shows broad selectivity for divalent cations and can also participate in the influx of Co into root cells (278). Aluminium can be taken up by plant root cells although this metal is highly toxic at low concentrations. Few evidence exists of how Al enters root cells in plants. The only plasma-membrane localized transporter identified is the Nrat1 (Nramp aluminium transporter 1), which showed ability to transport Al^{3+} in rice. Nrat1 transports only trivalent Al ion and is localized at the plasma membranes of all cells of root tips except epidermal cells (308).

As already highlighted, toxic elements can enter the plant roots through membrane transporters engaged in the influx of essential elements. Cadmium (Cd) uptake mainly occurs through Ca^{2+}, Fe^{2+} and Zn^{2+} membrane transporters. Calcium shares several physicochemical similarities with Cd and a competitive behaviour for binding sites of NSCCs has been reported for both elements (309-311). Moreover, the non-specific low-affinity cation transporter TaLCT1 was expressed in tobacco and showed a regulatory mechanism on Cd-toxicity by Ca, suggesting that this transporter mediates, at least at some extent, the Cd influx across root cells (312). The ZIP-IRT1 family comprises several non-specific transporters which are well described to mediate Fe influx from the soil. Cadmium can enter Arabidopsis root cells in its divalent form Cd^{2+} through the ZIP transporter AtIRT1 (307). This was also verified for the AtIRT1 orthologues OsIRT1 and HvIRT1, in rice and barley, respectively (277, 281). Cadmium influx across root cells can also be mediated by OsNRAMP1 which is highly expressed in rice roots (268). The uptake of Cd can also occur in the form of Cd-PS complexes by the YSL transporters (275). Regarding Cd efflux, the ABC transporter AtPDR8, highly expressed in the plasma membrane of root hairs and epidermal cells, has been associated to the efflux of Cd^{2+} and/or Cd-conjugates (313).

1.3.2. Root chelation and compartmentation

Once inside plant root cells, inorganics can be stored in vacuoles, transformed and/or translocated to shoot tissues. The accumulation of inorganics in root tissues can cause toxicity by directly damaging the cell structure (which may result in root growth inhibition and root anatomy changes) and/or via replacement of essential nutrients (311, 314). Therefore, plants have developed effective mechanisms to cope with inorganics toxicity (Figure 1.8). Usually, plants prevent the toxic effects of inorganics (e.g., heavy metals) by induction of various cellular mechanisms such as induction of higher levels of metal chelates, compartmentation in vacuoles and adsorption to the cell wall (315, 316). Nicotianamine (NA), glutathione (GSH), phytochelatins (PCs), metallothioneins (MTs), and

organic acids have been increasingly shown in the literature to play a key role in the chelation and sequestration of several metals (317, 318).

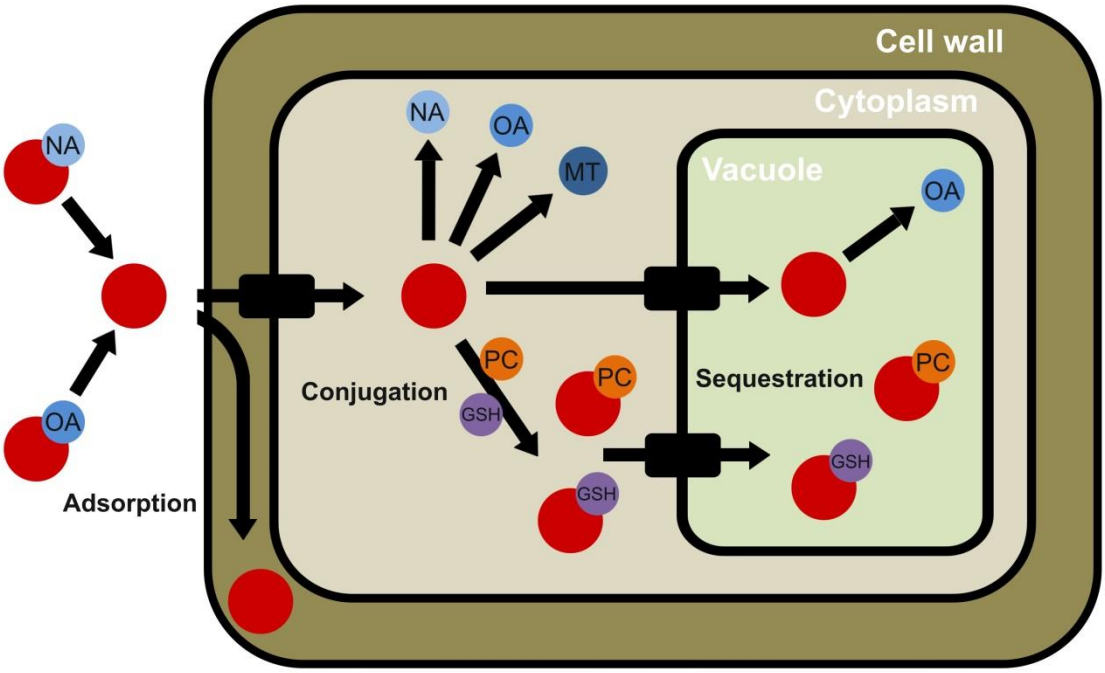


Figure 1.8 – Tolerance mechanisms for inorganics in plant cells. Detoxification usually involves conjugation followed by active sequestration in the vacuole and apoplast, where the toxic inorganics can be less harmful. Chelators shown are GSH: glutathione, MT: metallothioneins, NA: nicotianamine, OA: organic acids, PC: phytochelatins. Active transporters are shown as black boxes with arrows.

Nicotianamine is a low molecular mass compound enzymatically synthesized from three molecules of methionine by nicotianamine synthases (NASs), which have been identified in several plants to play an important role in metal chelation and detoxification (319). Glutathione and PCs have a high affinity for metal cations due to the thiol (–SH) groups on their cysteine residues. After metal(s) exposure, PCs are immediately produced in cells and tissues, though their production is markedly influenced by the type of metal ion present (318). Phytochelatin synthesis was found to be catalyzed by PC synthase in the presence of metal ions such as Cu, Zn, Cd, As and Pb (320). Metallothioneins are also able to bind a variety of trace metals (e.g., Cd, Cu and Zn) and to activate antioxidative enzyme defenses (321-323). Organic acids such as acetate, citrate and histidine also have an important role in the transport, sequestration and tolerance of metals (324, 325).
After root chelation, inorganics may be stored in the vacuole and/or cell wall (Figure 1.8). The vacuole is generally considered to be the main storage site for inorganics in plant cells and vacuolar compartmentation of elements is also a part of the tolerance

mechanism (326, 327). However, cell wall proteins and pectins are also involved in metal chelation and tolerance (328, 329).

1.3.2.1. Anions

Despite the importance of NO_3^- for plant N metabolism and the known role of the vacuole in its storage, the mechanisms involved in its vacuolar transport remained poor understood. Besides that, members of the chloride channel (CLC) family have been characterized to play a role in anions transport. The tonoplast-localized AtCLC-a was proved to be a NO_3^--specific anion/H^+ antiporter (330). Moreover, the same AtCLC-a is also able to mediate the Cl^-/H^+ transport into cell vacuoles (331, 332). AtCLC-b, a second member of the CLC family, was identified in Arabidopsis young roots and was found to act as NO_3^-/H^+ antiporter at the vacuolar membrane (333). A third member of the CLC family, AtCLC-c, was also found to be localized in the Arabidopsis roots and seems to mediate the influx of Cl across the tonoplast membrane (334). Evidence supporting the vacuolar Cl influx and efflux in root cells was observed in several studies (204).

Regarding other inorganic anions, two SO_4^{2-} transporters, AtSULTR4;1 and AtSULTR4;2, mainly expressed in the tonoplast of pericycle and xylem parenchyma cells, mediate the SO_4^{2-} efflux from vacuoles into the cytoplasm of root cells (335). Until now, no SO_4^{2-} transporters and/or channels have been identified to mediate the SO_4^{2-} influx into the vacuoles of root cells (326). In the same way, the transport mechanism responsible for the movement of Pi across the tonoplast of root cells is unclear, however vacuoles are likely to play a key role in the storage of Pi. In fact, roots are generally considered as a source of Pi for other plant parts and become a sink in Pi starvation (336, 337).

It is widely accepted that the anionic forms of molybdenum (MoO_4^{2-}) and selenium (SeO_4^{2-} and SeO_3^{2-}) can be stored in the vacuoles of root cells. Since Se anionic forms are taken up from soils by both non-specific SO_4^{2-} transporters, similar tonoplast-localized transporters can also be responsible for the movement of these anions across vacuoles of root cells (214, 338). In the case of Mo, a specific transporter, AtMOT2, has been shown to be engaged in the efflux of MoO_4^{2-} from vacuoles. Although this transporter was shown to be expressed only in the Arabidopsis leaves (339), one can hypothesize that a similar mechanism occurs in root vacuoles. Until now, no transporter has been identified to mediate the influx of MoO_4^{2-} into vacuoles.

As already mentioned, toxic elements do not possess a specialized transport system. Thus, they are taken up due to their similarity to other essential elements and the low specificity of some membrane transporters. The anionic forms of As, AsO_4^{3-} and AsO_3^{3-}, are mainly acquired by root cells thought phosphate transporters and aquaporins, respectively. Once inside the root cells, As is very toxic to plants and As

metabolization/detoxification must occur. The detoxification process can be divided into four categories: (1) As reduction; (2) As methylation; (3) As complexation with thiol compounds; and (4) vacuolar compartmentation (219).

Arsenic is usually taken up by plants in its pentavalent form (arsenate) yet, it is arsenite that is usually present within plant tissues which indicates that plants have a high capacity to perform As reduction. Arsenate reduction is one of the most important processes occurring within plant cells. Both roots and shoots possess similar reduction capacity, however roots seem to be more important because they are usually the place where higher amounts of As that must be reduced (340-342). Several studies have pointed out that ACR2 proteins play a key role in the reduction of arsenate (343). However, recently it was demonstrated that Arabidopsis *acr2* knockout mutants or overexpression lines do not show significant differences in As speciation when compared with wild-type plants, indicating that other pathways for arsenate reduction exist in plants (344). Arsenic methylation is another important mechanism of the As metabolism. Methylated As species are less toxic than inorganic As and plants rely on this mechanism to cope with the presence of toxic inorganic As species (345). Although little information is known about the intrinsic processes responsible for As methylation in plants, several studies reported the presence of methylated As within plant tissues when only inorganic As was supplied (346, 347) In a recent study, Jia, Huang (348) conducted an elucidative study and showed that microbes at the rhizosphere play a key role in As methylation. Since no methyl transferase gene has been identified in plants to date, evidence suggests that As methylation is a microbe-mediated process that occurs in the rhizosphere (349, 350). Arsenic is a strong inducer of PC synthesis in root cells. In fact, several genes involved in glutathione synthesis, metabolism and transport are up-regulated when rice is exposed to As^{5+} (351, 352). Blocking PC synthesis with l-buthionine-sulfoxime (a specific inhibitor of GSH biosynthesis) leads to low As accumulation and tolerance in plants (353, 354). Arsenic accumulation and tolerance is also improved by increased thiol biosynthesis in transgenic plant roots overexpressing a bacterial γ-glutamylcysteine synthetase gene (355) or the plant PC synthase gene (PCS) (356, 357). Taking into consideration these assumptions and the fact that most of the As^{3+} in plant tissues is complexed with thiol compounds (346), sufficient evidence exists supporting that thiols, mainly PCs, play a key role in As detoxification in plants. Once complexed by thiol compounds, As-complexes need to be sequestered in vacuoles as the final step of As detoxification. Recently, two members of the ABCC subfamily, AtABCC1 and AtABCC2, have been characterized as the major vacuolar transporters of As-PC complexes in Arabidopsis (358, 359).

1.3.2.2. Cations

Inorganic cations can be both essential and toxic chemical species. Evidence suggests that monovalent cations have a crucial role in the osmotic potential of cells. By contrast, divalent cations, like Ca^{2+}, are usually complexed with organic compounds within cells and thus, are responsible for others tasks inside plant tissues (326).

Besides its role in the maintenance of cell osmoticum, K is also required in several biochemical reactions. Since K is less chaotropic than Na, K is preferentially accumulated in plant cells (231). Once in the cytosol, K can cross the tonoplast through transporters or channels. Despite the limited knowledge of the mechanisms that regulate K movement across tonoplast, evidence suggests that transporters are responsible for the influx of K^+ into vacuoles while channels mainly mediate its efflux to the cytosol (326). The Na/H antiporters (NHX) are a class of transporters that catalyzes the electroneutral exchange of Na and/or K for H^+ by using the electrochemical H^+ gradient produced by the H^+–ATPases present in the plasma membrane of cell organelles (232, 234). They belong to the monovalent cation/proton antiporter (CPA1) family of transporters (360). In *Arabidopsis thaliana*, six intracellular NHX members are identified and divided into two groups, vacuolar (NHX1 to NHX4) and endosomal (NHX5 and NHX6) (361). From the four vacuolar NHX transporters, two (NHX1 and NHX2) have been fully characterized and have a crucial role in the regulation of intra-vacuolar K in root cells (362, 363). Moreover, transgenic tomato plants overexpressing AtNHX1 accumulated more K in the vacuole and contain less K in the cytosol than wild-type tomato plants giving a further insight about the important role of NHX1 in K vacuolar compartmentation (364). Regarding K efflux to the cytosol, two families of K-permeable channels, TPC1 (two-pore channel1) and the TPK/KCOs (two-pore K channels), have been identified in the tonoplast of root cells (326). The first class of K channels is the TPC1 which was the first vacuolar channel to be identified and mediates the Ca-dependent K, Na, NH_4^+ and Mg efflux from the vacuole (365). It has been suggested a possible role of TPC1 in Ca efflux from the vacuoles but some contradictory data about this issue exist in the literature (366). The TPK/KCO family constitutes the second class of K channels located in the vacuolar membrane. In *Arabidopsis thaliana*, the AtTPK/KCO family comprises five two-pore K channels (TPKs) and one Kir-type K channels (KCO) (360). From the five TPKs, four of them (AtTPK1, -2, -3, and -5) are localized in the tonoplast as well as the AtKCO3 (367). Characterization of Arabidopsis *tpk1* knockout mutants and TPK1-overexpressing lines demonstrate that TPK1 has an essential role in intracellular K homeostasis by controlling the K vacuolar release (368).

Calcium is usually present at high concentrations in the cytosol of root cells, where it has a pivotal role in the stabilization of cell walls, and also in the vacuole, where its content is

regulated by a larger number of Ca transporters (369). The cytosolic concentration of Ca is around 100 nM while the vacuolar concentration is in the mM range. Hence, vacuolar compartmentation of Ca is an energy-dependent process mediated by two classes of Ca vacuolar transporters (326). The first class includes the P-type Ca-ATPases and includes two Ca transporters, ACA4 and ACA11, localized in the vacuolar membrane. ACA4 occurs mainly on small vacuoles, whereas ACA11 is localized in the large vacuoles of root cells (370, 371). The second class of vacuolar Ca transporters includes the Ca/H exchangers (CAXs). Six CAX transporters have been identified in *Arabidopsis thaliana* which are split into two phylogenetic groups that seems to reflect their functional variation (372, 373). The Type-1B group, which includes CAX2, CAX5 and CAX6, seems to have a broader substrate specificity confirmed by its ability to transport several divalent cations (e.g., Mn and Cd) besides Ca (374). The Type 1A group, including the CAX1, CAX3 and CAX4 transporters, appears to have a higher specificity for Ca transport (375). Arabidopsis *cax1* knockout mutants do not suffer significant changes in their Ca content, however, *cax1cax3* double mutants exhibited reduced Ca concentration in their vacuoles compared to wild-type plants (376-378).

Plants growing on Mg-rich soils use this divalent cation as an osmotic regulator. The MGTs (Mg transporters) are the major transporters of this cation described in plants. In *Arabidopsis thaliana*, ten members with Mg/H antiport activity are identified (326). Two of them, AtMGT2/AtMRS2-1 and AtMGT3/AtMRS2-5, are localized in the vacuolar membrane. Plant lines overexpressing these transporters have shown Mg accumulation (256, 379). Arabidopsis Mg/H exchanger (AtMHX) is another putative vacuolar Mg/H antiporter that seems to mediate the Mg influx across the tonoplast (380). The same mechanism was also observed in the hyperaccumulator *Arabidopsis halleri* expressing an AtMHX homolog (381).

Plants require certain micronutrients for proper growth and development. For example, Fe is involved in numerous redox reactions (114), Cu has a crucial role in electron transfer between photosystem I and II (168), Mn is required as a cofactor for a variety of enzymes, such as the Mn-dependent superoxide dismutase (MnSOD) (276), and Zn acts as a catalytic or structural co-factor in a large number of enzymes and regulatory proteins (129). Micronutrients deficiency is a more common reality that toxicity. Hence, plants usually accumulate extra nutrients to sustain their growth in times of need. To accomplish that, micronutrients are translocated to seeds where they can support the growth of new plantlet. Therefore, most of these micronutrients are not retained in plant roots, so this topic is less relevant to discuss. Only a brief overview will be made here.

The bulk of Fe is found in leaves chloroplasts, where this element is engaged in several biochemical reactions (114). A certain amount of this micronutrient can also be stored in

the leaves vacuoles or mitochondria (326). Iron can also be stored in seeds vacuoles (382). Its storage in root cells is not very common.

Non-tolerant plant species, such as *A. thaliana*, can use chelation as a mechanism to avoid Cu-induced damage in the root cells. In a Cu-rich environment, MTs and PCs are usually up-regulated in response to Cu stress (320, 322, 383). Four Arabidopsis MTs (AtMT1a, AtMT2a, AtMT2b, and AtMT3) have been implicated in the Cu accumulation and tolerance in roots under high Cu levels (321). In addition to the MTs tolerance mechanism, Cu can be sequestered in the vacuole. A member of the HMA (Heavy Metal Associated) family, AtHMA5, mediates the vacuolar Cu influx in Arabidopsis root cells (384, 385). In *A. thaliana*, one of the six COPTs identified is targeted to the tonoplast. An Arabidopsis *copt5* knockout mutant exhibits severe defects in vegetative growth and root elongation, accumulating more Cu in the roots and translocating less Cu to the shoots. These results support the fact that AtCOPT5 would act as a vacuolar Cu exporter (386, 387).

A large pool of Mn (around 80%) is usually observed in leaf vacuoles. Moreover, a significant accumulation of Mn is also present in leaf chloroplasts where this element is required as the catalytic centre for light-induced water oxidation in photosystem II (276). The previously mentioned AtCAX2 and AtCAX4 transporters that are engaged in the transport of Ca have also the ability to transport Mn into the vacuole (374, 376, 388). AtMTP11, which belongs to the cation diffusion facilitator (CDF) family, is implicated in the pre-vacuolar compartmentation of Mn in root cells (389). In Arabidopsis, the export of Mn from vacuoles can be performed by AtNRAMP3 and AtNRAMP4 which were shown to have a crucial role in the Mn homeostasis within plant tissues (390). The P-type ATPases AtECA3, mainly expressed in root tissues, participates in the influx of Mn into the Golgi complex (391).

Once in the root symplast, Zn can be immobilized in the root through transport into vacuoles. Evidence suggests that the speciation of Zn in the root symplast has a direct influence in the extent of Zn immobilization in root vacuoles (129). The vacuolar membrane transporter AtMTP1 has an essential role in detoxification of excessive Zn. When grown in a Zn-rich medium, the mutant line of Arabidopsis that lacks MTP1 was not able to accumulate Zn in vacuoles, unlike wild-type roots (392). The tonoplast-localized AtMTP3 also contributes to basic cellular Zn tolerance and controls Zn partitioning. Overexpression of MTP3 increases Zn accumulation in roots of *A. thaliana* (393). The P_{1B}-type ATPase subfamily HMA plays a key role in the process of metal allocation or detoxification in plants. At least eight (HMA1-HMA8) members are identified in *Arabidopsis thaliana*. AtHMA1 to AtHMA4 belong to the group implicated in divalent cation transport whereas AtHMA5 to AtHMA8 act on monovalent Cu ion transport (394, 395). From the four HMAs involved in divalent cations transport, one of them, AtHMA3, seems

to play a role in the detoxification of Zn by participating in its vacuolar sequestration in root cells (396). It has been speculated that ZIF1 (for Zinc-Induced Facilitator1) is involved in a mechanism of vacuolar Zn sequestration. Specifically, AtZIF1 has been implicated in the transport of Zn complexes (mainly with NA) into the vacuole, since overexpression of ZIF1 leads to strongly enhanced retention of Zn in root vacuoles (319). A member of the ZIP family, AtZIP1, may contribute for the remobilization of Zn from the vacuole to the cytoplasm in Arabidopsis roots (397). Moreover, the tonoplast-localized NRAMP4 also seems to mediate the vacuolar Zn exporter in root cells (398).

Sodium is a ubiquitous cation present in all soils. Nowadays, soils contain high concentrations of Na, which can be readily absorbed by most plants. Since high Na concentrations within the cytosol can be toxic, vacuolar Na storage is essential for plants to survive under high salinity conditions (326). As already described, the NHX family is a Na^+/H^+ antiporter. Although several member of the NHX family are targeted in the vacuole, NHX1-4, are known to be involved in salt tolerance, the endosomal-localized NHX5 and NHX6 also seem to be engaged in this process suggesting a complex interactive coordination between different cell organelles in response to salinity (361, 399, 400).

After uptake into the root symplast, Ni detoxification at the cellular level is achieved by transport of Ni, as a free ion or chelated with appropriate ligands, to the storage sites such as the vacuole and cell wall (401). A recent study showed that the main mechanism that confers Ni tolerance seems to be vacuolar Ni sequestration (402). A member of the IREG/FPN (Iron-Regulated/Ferroportin) family, AtIREG2/FPN2, has been identified in the tonoplast of root cells and mediates vacuolar Ni loading (403). Moreover, this transporter is also involved in the vacuolar sequestration of Co^{2+} in root cells (404). Once inside root cells, toxic Al^{3+} must be detoxified. Illes, Schlicht (405) found an extensive Al^{3+} sequestration into endosomal/vacuolar compartments in root cells. In *A. thaliana*, a half-size ABC transporter, AtALS1, seems to be involved in the vacuolar sequestration of Al^{3+} in root cells (406). In rice, OsALS1 is also localized in the tonoplast and is required for Al^{3+} sequestration into the vacuoles (407).

Among the non-essential cations, Cd has attracted the most interest, because it is a very toxic pollutant and is widely found in worldwide soils (311). Several low-substrate-specificity transporters may contribute to Cd accumulation and tolerance. The CAX transporters that were mentioned above as mediating Ca influx across the vacuolar membranes are also involved in the vacuolar sequestration of Cd (374, 388). In Arabidopsis and rice, HMA3 was shown to be expressed in the tonoplast of root cells and is engaged in the sequestration of Cd, limiting its mobilization to above-ground tissues (396, 408, 409). Besides its sequestration as a free ion, Cd can also be detoxified by metal-binding chelators such as PCs, GSH and MTs (311, 320, 322). Tobacco plants

overexpressing an Arabidopsis phytochelatin synthase gene (AtPCS1) showed higher Cd accumulation in root cells than wild-type plants (410). After chelation with PCs, the Cd-PC complexes are sequestered within the vacuole. Two members of the ABCC-type transporters, AtABCC1 and AtABCC2, that were previously mentioned to mediate As detoxification, are also important vacuolar transporters that confer tolerance to Cd (411). After vacuolar sequestration, Cd can also be exported from the vacuole to the cytosol. Two members of the NRAMP family, NRAMP3 and NRAMP4, are thought to be involved in the vacuolar efflux of Cd (398, 412). Cd-induced MTs production is also a mechanism to cope with Cd toxicity. Arabidopsis *mt1* knockout mutants showed hypersensitive to Cd and accumulated lower levels of this element compared to wild-type plants (413). Moreover, up-regulation of the rice metallothionein OsMT-I-4b was observed in response to Cd, supporting that MTs have a potential role in Cd detoxification (383).

1.3.3. Translocation

Translocation in plants involves a series of steps, as follows: (1) radial transport across the root tissues, which include symplastic transport to pass through the Casparian strip (a waxy coating that prevents solutes from entering the root xylem directly from the soil solution or root apoplast); (2) xylem loading, transport and unloading; (3) xylem-to-phloem transfer; (4) phloem loading, transport and unloading; (5) symplastic movement toward the site of demand; and (6) retranslocation from source or senescing tissue (114, 414).

Although some transporters are highly specialized in the movement of a certain inorganic, others show low substrate specificity and can translocate different elements (311). Some inorganics are chelated by organic acids, which are involved in metal absorption by plant roots, translocation in the xylem and storage in the leaf cells vacuoles (415). Inorganics can also be bound by NA and mobilized by the YSL family transporters (275, 416). It should be emphasized that the inorganics translocation is a complex process involving multiple networks. Specialized proteins can transport elements with similar characteristics (e.g., oxidative state), inducing competition that results in deficiency, toxicity and/or accumulation in the above-ground plant tissues (11, 327).

Transpiration rate is a key variable that determines the rate of inorganics mobilization within plant. Bulk flow in the xylem from root to shoot is driven by transpiration from the shoot, which creates a negative pressure in the xylem that pulls up water and solutes (417). Plant transpiration depends on several aspects of plant species and the surrounding environment. For example, plant metabolic (e.g., C3/C4/CAM photosynthetic pathway) and anatomical differences (e.g., stomatal density) are important factors that affect transpiration rate (418). Plant height and density, as well as environmental

conditions, are also factors to consider (e.g., transpiration is usually higher at high temperature, low relative air humidity and high luminosity) (419, 420).

1.3.3.1. Anions

A significant part of NO_3^- assimilation occurs in plant shoots because the reducing power required for the assimilation processes comes from photosynthesis. For the root-to-shoot NO_3^- translocation occurs, NO_3^- must be loaded into xylem vessels (173). Until now, only one NO_3^- transporter, NRT1.5, has been identified to be involved in this process. AtNRT1.5 is a plasma membrane-localized low-affinity bi-directional NO_3^- transporter expressed in the pericycle cells of Arabidopsis roots. Despite the important role of AtNRT1.5 in the root-to-shoot NO_3^- transport, Arabidopsis *nrt1.5* knockout mutants were also able to translocate NO_3^- to above-ground tissues suggesting that other xylem loading mechanism(s) exist (421). Once in the vasculature, two members of the NRT1 family, AtNRT1.8 and AtNRT1.9, mediate the NO_3^- distribution across the plant shoot. AtNRT1.8, a plasma membrane-localized low-affinity NO_3^- transporter is expressed mainly in the xylem parenchyma cells of Arabidopsis roots. Arabidopsis *nrt1.8* knockout mutants display increased NO_3^- content in xylem sap and increased root-to-shoot NO_3^- translocation, supporting that NRT1.8 has an important role in the movement of NO_3^- from the xylem sap back into the root cells (422). The Arabidopsis NRT1.9 is targeted to the plasma membrane of companion cells of the phloem. AtNRT1.9 is involved in the downward phloem transport of NO_3^- to the plant roots, thereby being involved in the NO_3^- homeostasis in shoot by preventing excessive NO_3^- accumulation in those tissues (423). Recently, a high-affinity transporter from the NRT2 family, NRT2.3a, was identified in rice. OsNRT2.3a was targeted to the plasma membrane of xylem parenchyma cells of the stele and was found to mediate the long-distance NO_3^- transport from root-to-shoot in rice (424). Nitrate can also be remobilized from source (mature) leaves to sink (young) leaves to support the growth of young tissues. In Arabidopsis, a member of the NRT1 family, AtNRT1.7, was proved to be involved in the phloem loading of NO_3^- leaf allowing its transport out of older leaves and into younger leaves (425). A member of the NRT2 family, NRT2.4, was found to be expressed in the phloem parenchyma of leaves and is involved in phloem NO_3^- transport in shoots. Arabidopsis *nrt2.4* knockout mutants displayed decreased NO_3^- content in the phloem exudates of mature leaves, supporting the evidence that NRT2.4 participates in phloem-mediated NO_3^- remobilization (180).

During the plant growth, Cl^- translocation from the root to the shoot via the xylem and its redistribution between tissues via the phloem is known to occur (204). An Arabidopsis member of the CCC family, AtCCC, is expressed in the root and shoot vasculature at the xylem/symplast boundary. Arabidopsis *ccc* knockout mutants showed defective Cl^-

homeostasis, accumulating higher and lower Cl^- amounts in shoots and roots, respectively, than wild-type plants, suggesting that AtCCC is involved in long-distance Cl^- transport (206). A homologue gene in rice, OsCCC1, also seems to be involved in the transport of Cl^- within plant tissues. OsCCC1 was found to be expressed in both the root and shoot indicating that this transporter in involved in Cl^- homeostasis in rice (207).

Most of the Pi taken up by plant roots is rapidly loaded into the xylem for futher distribution in the above-ground tissues. The mobilization of Pi in both xylem and phloem is a fast continuous process with membrane transporters providing a constant loading and unloading of the nutrient into different organs (337). Nowadays, little information is available about the transporters involved in xylem and phloem loading. However, it seems that the PHT1 family is involved in this process. The rice transporter OsPHT1;1, one of the thirteen PHT1 Pi transporters in rice, was found to be abundantly and constitutively expressed in different cells of both roots and shoots. Compared with the wild-type, OsPHT1;1 overexpression lines and knockdown lines exhibit higher and lower Pi content in shoots, respectively, supporting the role of this transporter in the root-to-shoot Pi transport (195). Other two Pht1 transporters expressed in rice, OsPHT1;2 and OsPHT1;6, are also involved in Pi translocation. Rice *pt2* or *pt6* knockdown mutants showed a significantly decreased in the long-distance transport of Pi from roots to shoots (193). Remobilization of Pi is performed by the Arabidopsis transporter PHT1;5. Overexpressing and knockout lines showed altered Pi remobilization which supports the evidence that AtPHT1;5 is implicated in the mobilization of stored Pi out of older leaves (426, 427).

The root-to-shoot transfer of SO_4^{2-} is thought to be mediated by SULTR2;1 and SULTR3;5. In Arabidopsis roots, AtSULTR3;5 was co-localized with AtSULTR2;1 in xylem parenchyma and pericycle cells. Arabidopsis *sultr3;5* knockout mutants showed limited root-to-shoot transport of SO_4^{2-} suggesting that SULTR3;5 is constitutively expressed in the root vasculature and that this transporter, along with SULTR2;1, contributes to the efficient root-to-shoot transport of SO_4^{2-} (428). Once in the shoot, SO_4^{2-} can also be remobilized from the vacuoles of source organs for the growth and development of new plantlet. A member of the high-affinity transporters SULTR1, AtSULTR1;3, seems to mediate the long-distance transport of SO_4^{2-} or sulphur-containing compounds. AtSULTR1;3 is localized in the sieve element-companion cell complexes of the phloem in cotyledons and roots and was demonstrated to play a key role in loading SO_4^{2-} to the sieve tube, initiating the source-to-sink translocation of sulfur nutrient in Arabidopsis (429).

The anionic form of molybdenum (MoO_4^{2-}) seems to be easily mobilized within plant. Until now, little information is available about the root-to-shoot Mo translocation. The molybdate transporter AtMOT1 was also found to be expressed in Arabidopsis shoot, suggesting that this transporter is involved in the whole-plant Mo homeostasis (430). Moreover, it seems

that the root-to-shoot Mo transport is markedly influenced by the presence of SO_4^{2-}, suggesting that Mo translocation may also be mediated by transporters engaged in SO_4^{2-} translocation (214, 431).

The root-to-shoot Se translocation depends on which Se species is available for plant uptake. When SeO_4^{2-} is the main species taken up by plants, Se is easily translocated via the xylem to the shoot. By contrast, when SeO_3^{2-} is taken up, most of the Se accumulates in the roots and low amounts of Se are detected in the xylem sap. The acquired SeO_3^{2-} is readily transformed by plant roots to other Se species (e.g., selenomethionine, selenomethionine Se-oxide, Se-methyl-selenocysteine), which greatly reduces the SeO_3^{2-} content in the shoot (218). Since the SO_4^{2-} transporters are involved in the Se uptake, it has been suggested that the transporters engaged in the root-to-shoot SO_4^{2-} translocation are also able to mediate Se transport to above-ground tissues (214).

Generally, As has low mobility within plant tissues, so its root-to-shoot translocation is very low in non-hyperaccumulator plants (432). For Arabidopsis, 2.6% of the total As taken up by roots was shown to be translocated to above-ground tissues (433). In fact, the mean shoot to root ratio of As content in plants is very low ranging from 0.01 to 0.9 (434). The main explanation for this very low root-to-shoot As translocation results from three processes already described: (1) As^{5+} reduction to As^{3+} in roots, (2) complexation of As with thiols, and (3) sequestration of As in the root vacuoles. The main As species found in the xylem sap is As^{3+} (435, 436). Although no effect on As distribution to the shoots was observed, an *Arabidopsis thaliana pho1* knockout mutant showed reduced Pi xylem loading, suggesting that PO_4^{3-} transporters do not mediate As (as As^{5+}) loading into xylem (433). Even when As^{5+} is the main As species supplied to plant roots, As^{3+} dominates in the vascular tissues (435, 436). This information is consistent with the fact that roots have a high capacity for As^{5+} reduction. There is no evidence that As^{3+}–thiol complexes are present in the xylem sap. In fact, evidence suggests that complexation with thiols decrease the root-to-shoot As^{3+} translocation (219). Rice has an efficient loading of As^{3+} into xylem which is probably the result of the high expression of the Si effluxer Lsi2, which was proved to mediate As^{3+} transport to above-ground tissues (226, 436).

1.3.3.2. Cations

A large fraction of the K taken up by plant roots is translocated to the shoot. Potassium is transported symplastically to the xylem, where the Shaker-like channel AtSKOR, expressed in the root pericycle and stelar parenchyma of Arabidopsis, is thought to be involved in K loading of the xylem (437, 438). The loading of K within the shoot is mainly determined by transpirational water flows and the shoot apoplastic K concentration (9). In addition to the transport of K into above-ground tissues, a large shoot-to-root K flux

through the phloem is maintained. In Arabidopsis, the redistribution of K from leaves to phloem-fed tissues seems to be mediated by the Shaker-like channel AtAKT2/AKT3, which is mainly expressed in the phloem and xylem parenchyma and has been indicated to be involved in phloem loading and unloading (439-441).

Calcium is relatively immobile within plant tissues and thus, it is likely that most of the Ca is sequestered in the root cells vacuoles (10). Until now, no transporters have been identified to be responsible for Ca xylem loading, although it is possible for Ca to be symplastically transported across the root to the xylem (9). Evidence suggests that Ca reaches the xylem apoplastically through regions of the root where the Casparian strips are absent or via the cytosol of unsuberised endodermal cells where Casparian strips are present (442). This assumptions support the observation that Ca mobilization to the shoot occurs mainly from the root apex and/or regions of lateral root initiation, where the Casparian strip is disrupted (443, 444). Once in the vascular system, Ca mobility is also low. Calcium is transported in the xylem as free ions (Ca^{2+}) or complexed with organic acids (9). Additionally, it seems that the rhizosphere Ca concentration has an important effect on the Ca concentration in the xylem sap and transpirational water flows governs Ca delivery within the shoot (444).

Magnesium is loaded into the xylem sap, where it is present as free ions (Mg^{2+}) or complexed with organic acids (9). To date, little information is available regarding membrane transporters engaged in the Mg xylem loading. Recently, it was suggested that members of the MRS2/MGT family could be involved in the Mg transport system (445), but the exact transporter has not been identified yet. As for Ca, evidence suggests that, besides the symplastic pathway, Mg can be loaded into the xylem vessels apoplastically and it seems that the apoplast via is even the preferred pathway of xylem Mg loading (443).

Within plant tissues, Fe has low solubility and high reactivity thus its movement along the plant tissues must be associated with appropriate chelating compounds and proper control of redox states (118). Several lines of evidence have indicated that the main Fe chelators are citrate (416, 446), NA (447) and MAs (448, 449). Citrate has been proved to play a key role in the Fe chelation and mobilization in xylem sap (446). To facilitate the efflux of citrate into the xylem, Arabidopsis relies on the FRD3 (Ferric Reductase Defective 3) transporter which is expressed in the root pericycle cells (450). In rice, an AtFDR3 orthologue, OsFRDL1, is also expressed in root pericycle cells and encodes a citrate effluxer required to load citrate into the xylem sap (451). The already mentioned OsPEZ1 and OsPEZ2, are also responsible for xylem loading of phenolics enhancing the remobilization of Fe along the plant body (121, 122). Although AtFRD3, OsFRDL1, OsPEZ1 and OsPEZ2 can efflux the Fe-chelating molecules, Fe must also be transported

into the xylem. To date, the plasma membrane-localized IREG1/FPN1 present in Arabidopsis stele, is the Fe effluxer identified to mediate the vascular loading of Fe (404). Among the influx transporters, members of the YS and YSL transporters are widely involved in Fe translocation. In barley and maize, the YS1 transporter is able to mobilize Fe-PS complexes (264, 266). Among the 18 YSL transporters present in rice, OsYSL2, OsYSL15, OsYSL16, OsYSL18 were identified as Fe-PS transporters involved in the Fe translocation. More specifically, OsYSL2 is proved to be a Fe-NA transporter, whereas OsYSL15, OsYSL16, OsYSL18 translocate Fe-deoxymugineic acid (DMA) complexes. (263, 447-449). The efflux of NA in rice was found to be facilitated by the ENA1 (efflux transporter of nicotianamine 1) and ENA2, and the efflux of MA is achieved by TOM1 (127). Members of the YSL family are also present in non-graminaceous plants that do not synthesize MAs. It is widely assumed that non-graminaceous plants rely on the YSL transporters to translocate Fe-NA complexes. NA is a precursor and structural analogue of MAs; it has the ability to chelate Fe and other metals and is synthesized by all plants, in contrast to the graminaceous-specific synthesis of MAs (275). The Arabidopsis YSL members AtYSL1, AtYSL2 and AtYSL3 were identified to have a key role in the mobilization of Fe inside the plant (452). Besides their presence in the outer root cells, some FRO genes can also be expressed in root or shoot vascular cells (123), suggesting that Fe reduction can occur within plant tissues with further Fe translocation by members of the IRT and/or NRAMP families (128, 268). The oligopeptide transporters (OPT) seem to play an important role in the mobilization of Fe. OPT3 was markedly expressed in aerial parts of both *Arabidopsis thaliana* and *Thlaspi caerulescens*, including stem and leaf, suggesting that this transporter may be involved in the long-distance Fe transportation (453, 454).

In order to be translocated, Cu must be exported from the root symplast before entering the xylem. Evidence suggests that the export from the root symplast is mediated by an HMA family transporter. In Arabidopsis, AtHMA5 is highly expressed in roots, flowers and pollen and seems to be responsible for root Cu detoxification (384). This phenotype function is the opposite of COPT, corroborating the idea that AtCOPT1 and AtHMA5 transport Cu in opposite directions (273, 385). Cu translocation may involve chelators such as NA and several amino acids (416, 455). Moreover, Cu in the xylem sap of rice seems to be bound to DMA, while in the phloem sap, Cu mainly complexes with NA and histidine (456). Regarding transporters, the recently identified HvYSL2, which is localized in the root endodermis, seems to be involved in the transport of Cu from the cortex to the pericycle cells (274). OsYSL16 is a Cu-NA transporter required for delivering Cu to developing young tissues and seeds through phloem transport (457, 458). In Arabidopsis root, the plasma membrane-localized AtYSL1 and AtYSL3 were proven to be involved in

the root-to-shoot Cu transport (452, 459). In Arabidopsis, AtCOPT6 is expressed in different cell types of different plant compartments, but the bulk of its expression is located in the vasculature suggesting a role in Cu translocation (460). Similarly, OsCOPT6 is not expressed in root but is highly expressed in leaf, stem and sheath (168). Furthermore, the oligopeptide transporter AtOPT3 and its orthologue TcOPT3 seem to participate in the mobilization of Cu (453, 454).

The translocation of Mn involves chelators such as NA, amino acids and carboxylic acids (416, 461). In rice, OsYSL6 is a Mn-NA transporter that is required for the detoxification of excess Mn (462). Likewise, OsYSL2 is involved in Mn translocation in the phloem. In the knockout ysl2 line, rice seeds and shoots contain lower Mn content compared with the wild-type (447). Moreover, the same transporter (YSL2) shows the ability to transport Mn-PS complexes in barley roots (274). In Arabidopsis, AtZIP2 seems to participate in Mn transport into the root vasculature for translocation to the shoot (397). The oligopeptide transporter AtOPT3 seems to play an important role in the long-distance Mn transport (454).

Zinc is exported from root by two members of the P-type ATPase family, HMA2 (expressed in Arabidopsis, rice and barley) and HMA4 (463-465). However, other transporters can also perform the export of Zn to vascular tissues. In Arabidopsis, *pcr2* knockout mutants accumulate Zn in roots, suggesting a role of AtPCR2 in the root-to-shoot Zn translocation (466). Furthermore, it was proved that AtFRD3 is involved in loading Zn into xylem (467). Zn can also be exported from the roots in the form of Zn complexes. Before being exported, Zn must be mobilized to pericycle cells, apparently by HvYSL2 (274). After xylem loading, Zn can be transported to above-ground tissues by the YS and YSL transporters. There is some evidence that AtYSL1, AtYSL2 and AtYSL3 are involved in the transport of Zn (452). Phytosiderophores such as NA and DMA are important in the distribution of Zn within the plant (416, 468, 469). Although no Zn-PS transporters have been identified yet, the presence of these complexes in the xylem and phloem sap suggests that members of the YSL family may be involved in the transport of Zn complexes (317, 470). Moreover, members of the ZIP family are expressed in the vascular bundle, suggesting that they contribute to the distribution of Zn along the plant (282). AtOPT3 and its orthologue TcOPT3 seem to participate in the long-distance transport of Zn (453, 454).

The export of Na to the xylem is assumed to be an active process. In Arabidopsis, SOS1 was proposed to be involved in the loading of Na into the xylem (471). However, at the whole-plant level, the specific role of AtSOS1 still remains uncertain (292). At high Na levels, xylem loading with Na is thought to occur passively through channels, since a higher cytosolic Na content in xylem parenchyma cells and a relatively depolarized

plasma membrane would promote the movement of Na into the xylem sap (472). Several lines of evidence exist supporting the role of AtHKT1;1 and its orthologue OsHKT1;5 in xylem unloading (298, 301, 473). A demonstration of Na efflux from shoot xylem vessels was achieved by overexpressing AtHKT1;1 in the mature root stele of *A. thaliana*, which showed decreases Na^+ accumulation in the shoot (474).

Nickel is translocated from roots to shoots as a free ion (Ni^{2+}) and complexed with several molecules such as histidine and citrate (475, 476). In hyperaccumulator plants, Ni was shown to be translocated also in the form of Ni-NA complexes (477). The transport of NA-metal chelates, required for the xylem loading and unloading, has been assigned to the YSL family. In *Thlaspi caerulescens*, two YSL genes, TcYSL5 and TcYSL7 were found to be expressed around the vasculature of the shoots and in the central cylinder of the roots (478), suggesting that YSL transporters play a key role in the translocation of Ni-NA complexes (275). For Co, little evidence is available regarding its translocation. However, an Arabidopsis *fpn1* knockout mutant showed low Co accumulation in shoot tissues, indicating that ferroportins play an important role in Co homeostasis, more specifically in the root-to-shoot Co translocation (404).

In most plants, Cd accumulates in roots. The capacity to translocate Cd to the shoot is assumed to be a tolerance mechanism developed in hyperaccumulators (311, 479). In *A. thaliana*, members of the HMA family, AtHMA2 and AtHMA4, were found to mediate the transport of Cd to above-ground tissues (465). In *A. halleri*, AhHMA4, a homolog of AtHMA4 also plays a key role in translocation of Cd into shoots (480). In the same way, a homolog of AtHMA2, OsHMA2, is also involved in root-to-shoot Cd translocation in rice (464, 481). In rice, phloem mediates almost 100% of the Cd deposition into grains (482). It has been suggested that xylem-to-phloem Cd transfer can occur at nodes (483) and that phloem Cd transport through a panicle neck shows genotypic variation (461). These evidences suggest the presence and involvement of transporters at nodes for phloem Cd transport into grains. Moreover, remobilization of Cd from leaf blades to grains also seems to be facilitated by phloem transport (484). Recently, a homologue of wheat LCT1 family, OsLCT1, was identified and seems to play a key role in phloem Cd transport (485). It has been shown that several metal–ligand molecules such as NA, GSH and PCs are present in phloem sap of various plant species (275, 486). Moreover, significant levels of Cd and sulfur–Cd complexes were found in the cytoplasm of Arabidopsis companion cells, suggesting that thiols can mediate long-distance transport of Cd through the phloem (487). Cell-specific transcriptome analyses in *A. thaliana* show high expression of phytochelatin synthase (AtPCS1) in companion cells (488). Companion cells and sieve elements (phloem) are connected through highly permeable plasmodesmata (489). Hence, compounds synthesized in companion cells, or transported into companion cells

such as GSH or PCs, may enter the phloem for further transport into sink tissues (490, 491).

1.3.4. Leaves chelation and compartmentation

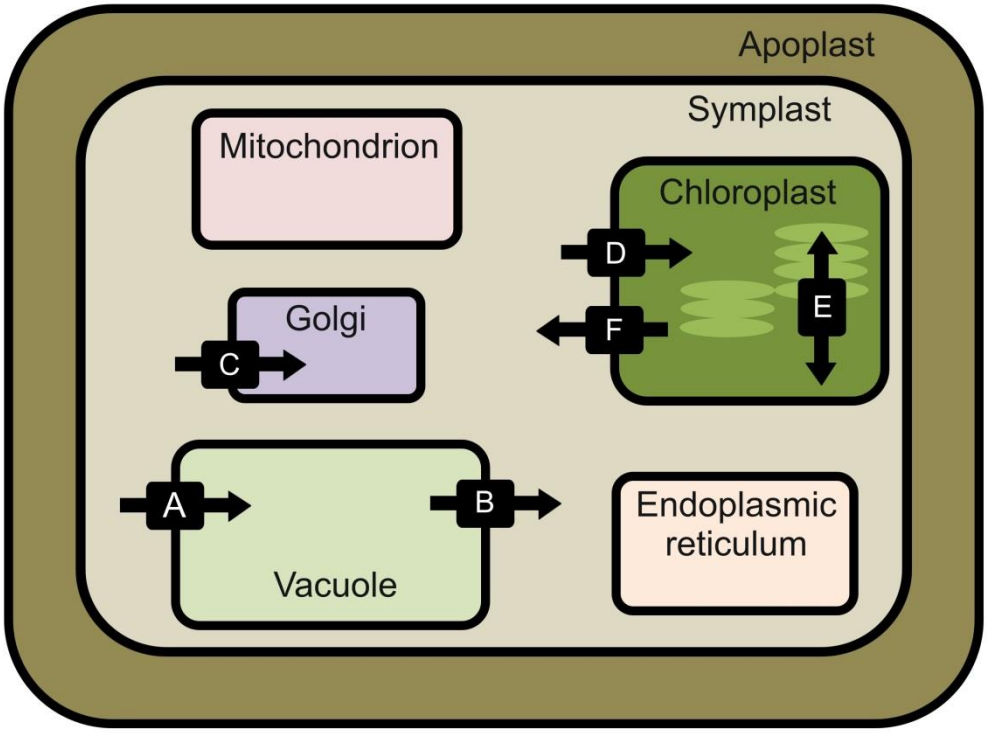
The transport of inorganics into leaf cells from leaf xylem involves another membrane transport step mediated by specific membrane transport proteins. Once inside the leaf symplast, inorganics can be assimilated in the cytosol or stored in cellular organelles. The general rule is that nutrients are assimilated, playing a key role in several physiological mechanisms, and toxic elements are stored in places where they cause the least harm to cellular processes. However, it should be noted that nutrients can also be stored for two reasons: (1) they can become deficient and plant accumulates them for times of necessity; (2) they are at high concentrations and can pose a toxicity problem to plants (10, 23, 166). At the tissue level, toxic elements are generally accumulated in the epidermis and trichomes (492, 493); at the cellular level, they may be accumulated in the vacuoles or cell wall (494).

The distribution of inorganics in leaf tissues is generally asymmetric (495). Toxic inorganics accumulation in leaf vacuoles makes sense because vacuoles do not contain a photosynthetic apparatus, which would be sensitive to toxicity (496). Toxic inorganics storage occurs also in highly tolerant cells such as the leaf epidermis and the vein bundle sheath, as long as the metal import remains under control. Once the inorganics accumulation exceeds the tolerance threshold of the plant, they would be transported to mesophyll cells, which are more sensitive to toxic inorganics than other cell types, allowing photosynthesis to be threatened (497). At present, the physiological mechanisms involved in the sequestration of inorganics between different leaf tissues remain only partially understood. One possible mechanism is the differential expression of inorganics transporters in the plasma membrane and/or tonoplast between mesophyll and other tissues (396, 498).

1.3.4.1. Anions

In Arabidopsis, the low-affinity NO_3^- transporter AtNRT1.4, highly expressed in the leaf petioles, plays a key role in regulating leaf NO_3^- homeostasis and leaf development. Arabidopsis *nrt1.4* knockout mutants showed reduced NO_3^- content in the petiole but the NO_3^- content in the leaf lamina was slightly increased, showing that NRT1.4 regulates NO_3^- distribution in leaves (499). Vacuoles are the main cell organelles responsible for the storage of NO_3^-. Members of the CLC family are the main candidates enrolled in the vacuolar NO_3^- storage (Figure 1.9). The same tonoplast-localized CLC transporters, AtCLC-a, AtCLC-b and AtCLC-c, that were above-described to mediate vacuolar NO_3^-

storage in root vacuoles, are also expressed in leaf cells (330-332, 334, 500). Three other members of the CLC family, CLC-d, CLC-e and CLC-f, are also identified in intracellular compartments of Arabidopsis, although their transport activity has not yet been characterized. Evidence suggests that AtCLC-d could be a Cl^-/H^+ exchanger (500). The physiological role of AtCLC-e has yet to be revealed. Arabidopsis *clc-e* knockout mutants display decreased NO_3^- content and disturbances in photosynthetic electron transfer, but the direct link between these phenotypes and a defect in anion transport remains uncertain (501, 502). As for AtCLC-f, its role in the cell remains indefinite (20).



Transport step	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	MoO_4^{2-}
A	AtCLC-a AtCLC-b ? AtCLC-c			
B	AtCLC-b ?		AtSULTR4;1 AtSULTR4;2	AtMOT2
C	AtCLC-d AtCLC-f ?	AtPHT4;6 ?		
D		AtPHT2;1 ? AtPHT4;1-4;5 ?		
E	AtCLC-e ?			
F		AtPHT2;1 ? AtPHT4;1-4;5 ?		

Figure 1.9 – Schematic illustration of the subcellular localization of transporters involved in anions movement in *Arabidopsis thaliana*. Members of the AtCLC, AtPHT, AtSULTR and AtMOT families are targeted in intracellular membranes: tonoplast (AtCLC-a, AtCLC-b, AtCLC-c, AtSULTR4;1, AtSULTR4;2 and AtMOT2), Golgi complex (AtCLC-d, AtCLC-f and AtPHT4;6), chloroplast envelope (AtPHT2;1 and AtPHT4;1-4;6), and thylakoid membranes (AtCLCe). The arrows indicate the direction of transport and each letter in the figure corresponds to the list given in the first column of the table.

It is clear that a membrane transporter must mediate the Pi influx from the xylem into the leaf cells. However, until now no transporter have been identified (10). Little is known about the transport mechanism responsible for vacuolar Pi storage, however it is widely accepted that vacuoles play an important role (337). Regarding other cell organelles, the Pi transporter PHT2;1 of Arabidopsis was found to be localized in the chloroplast envelope. Analysis of an Arabidopsis *pht2;1* knockout mutant reveals that AtPHT2;1 activity affects Pi allocation within the plant, including the translocation of Pi within leaves (503). Moreover, six members of the PHT4 family were localized in the cell organelles of Arabidopsis. Five of them were targeted to the plastid envelope and one in the Golgi apparatus (Figure 1.9), suggesting a role for PHT4 proteins in the transport of Pi between the cytosol and different cell organelles (504).

So far, no transporter has been identified to be responsible for the leaf uptake of SO_4^{2-} (169). However, it is known that SO_4^{2-} is highly mobile within plant tissues and may be rapidly transported through the xylem to shoot tissues where most is reduced. Despite most members of the SULTR1 family are highly expressed in the root, they are also expressed in the shoot, suggesting that members of this family could be involved in SO_4^{2-} acquisition into leaf cells (202, 429). It is widely accepted that when present in excessive amounts, SO_4^{2-} is deposited in vacuoles. In Arabidopsis, members of the SULTR4 family, AtSULTR4;1 and AtSULTR4;2, were found to be involved in SO_4^{2-} efflux from vacuoles to the cytosol in root and shoot tissues (Figure 1.9) (335). However, the transporter(s) involved in vacuolar SO_4^{2-} influx remain unidentified (169).

Besides its role in Mo uptake from soil, AtMOT1 may also be involved in Mo acquisition into leaf cells (212). An AtMOT1 homologue, AtMOT2, has been targeted to the tonoplast of leaf cells and seems to mediate the vacuolar efflux of Mo (Figure 1.9) (339). Since Se uptake and translocation is mainly mediated by SO_4^{2-} transporters, evidence suggests that the homologues of these transporters may transport Se from the xylem to the leaf symplast (210). However, this assumption has not yet been confirmed.

Since As^{3+} is the main As species present in the xylem sap, evidence suggests that members of the NIP sub-family are involved in the transport of As^{3+} from xylem into leaf symplast (222, 226). Once in the leaf symplast, As^{3+} is detoxified through sequestration of As^{3+} in leaf vacuoles and/or complexation with thiol compounds followed by vacuolar compartmentation (219). Non-hyperaccumulators rely mainly on complexation for As detoxification and tolerance. In fact, two ABCC-type phytochelatin transporters, AtABCC1 and AtABCC2, mediate the sequestration of As-PCs complexes into vacuoles (359). By contrast, hyperaccumulators such as *Pteris vittata* preferentially accumulates inorganic As (As^{3+}) in its leaf vacuoles (432).

1.3.4.2. Cations

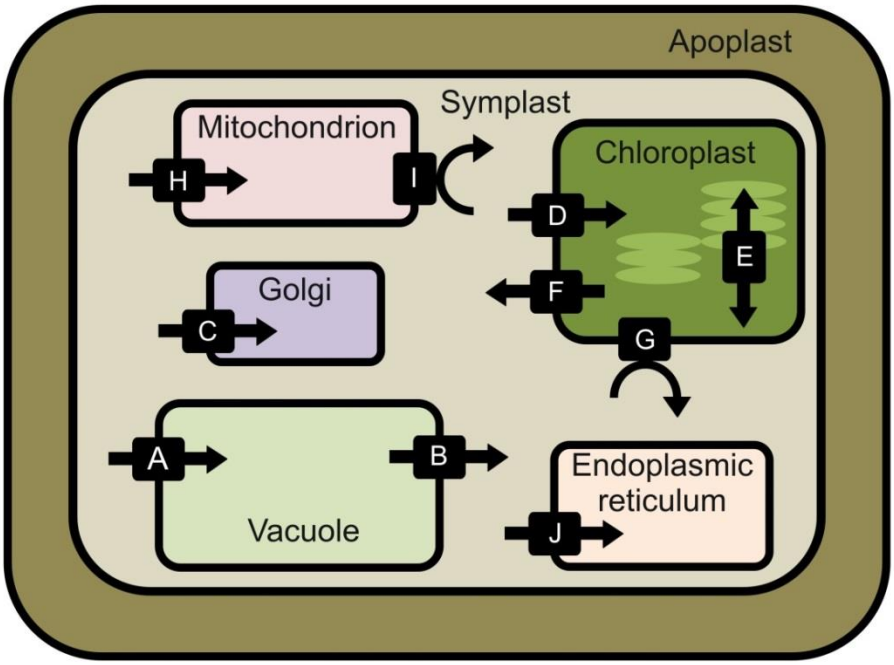
The transport of K from the xylem into the leaf symplast is mediated by membrane transporters and channels. Members of the HAK/KT/KUP family were found to be highly expressed in shoot tissues (231). Specifically, AtKUP1, AtKUP2 and AtKUP4 were expressed in Arabidopsis leaves, suggesting an involvement in K xylem unloading (231, 236). Moreover, Ahn, Shin (235) also showed that other AtKUPs were highly expressed in leaves. In particular, AtKUP6, AtKUP8 and AtKUP9 were highly expressed in young leaves, whereas AtKUP10 and AtKUP12 expression was evident in both older and younger leaves. The Shaker channels AtKT1, AtKT2 and AtKC1 are also highly expressed in the plasma membrane of Arabidopsis leaves, indicating that these cation channels are enrolled in the movement of K between shoot tissues (245, 505). Regarding the intracellular distribution of K in leaves, the vacuolar K transporters AtNHX1 and AtNHX2, expressed in the tonoplast of Arabidopsis leaf vacuoles, mediate the influx of K into this cell organelle (363). To mediate the vacuolar K efflux, several members of the TPK/KCOs family, such as AtTPK1, AtTPK3, AtTPK5 and AtKCO3, are expressed in the vacuolar membrane of leaf vacuoles and mediate this process (367, 368). Regarding other cell organelles, a member of the CHX family, AtCHX23, was found to be expressed in the chloroplast envelope of Arabidopsis leaf cells. Analysis of Arabidopsis *chx23* knockout mutant leaves suggests that CHX23 could mediate the co-transport of K across the chloroplasts envelope of leaf cells (506). Recently, a member of the K efflux antiporters (KEA) has also been identified in Arabidopsis chloroplasts. AtKEA2 was found to be highly expressed in leaves and mediates K transport across the plastid envelope (507).

Within the leaf, Ca follows mainly the apoplastic pathway and tends to accumulate in the mesophyll cells, trichomes and epidermal cells. The cytosolic Ca concentration is very sensitive to the apoplastic Ca concentrations. Thus, Ca is usually stored in leaf vacuoles and/or specific cell organelles to avoid excessive apoplastic Ca accumulation (508). However, before been sequestered in the leaf vacuoles, Ca must be transported across the plasma membrane of leaf cells. Although must members of the NSSC are present in the root cells, several lines of evidence suggest that VI-NSCCs can also be found in leaf cells (249, 509). In the vacuoles of leaf cells, Ca is present as the ionic species Ca^{2+}, in soluble forms (e.g., complexed with proteins and/or organic acids) and/or in insoluble forms (e.g., Ca-oxalate and Ca-phytate) (9, 510). The transport of Ca across the vacuolar membrane of leaf cells is thought to be performed by members of the ACA and CAX families. AtACA11 is targeted to the vacuolar membrane of Arabidopsis leaf cells and mediates Ca transport across the tonoplast of large vacuoles (371). In the CAX family, AtCAX1 is highly expressed in the vacuoles of leaf cells and has an important role in Ca

influx into the vacuole (375, 378). AtCAX2, AtCAX5 and AtCAX6 were also expressed in Arabidopsis leaves, although in a lower extent than AtCAX1 (374).

Members of the MGT/MRS2 family are thought to mediate the leaf uptake of Mg (9, 256). It was shown that AtMGT9 is expressed in leaf cells and thus can be involved in Mg transport (258). Once in the cytosol of leaf cells, Mg can be accumulated in several cell organelles. Evidence suggests that mesophyll cells accumulate the highest vacuolar concentration of Mg in Arabidopsis leaves. To accomplish that, plants rely on members of the MGT/MRS2 family, such as AtMGT2/MRS2-1 and AtMGT3/MRS2-5, which are localized in the tonoplast of Arabidopsis leaf cells and mediate the Mg influx into the vacuole (379). AtMHX is also involved in Mg influx across the tonoplast of leaf cells (380, 381). A member of the MGT/MRS2 family, AtMRS2-11, was expressed in the chloroplast envelope and plays a key role in the Mg transport into this cell organelle (511).

Fe can be transported into the leaf symplast as free ion or complexed with PS. Members of the IRT family are expressed in leaf tissues of Arabidopsis suggesting their involvement in the Fe acquisition (260, 261, 281). Regarding Fe-PS complexes, members of the YSL family seems to mediate the Fe influx into the cytosol of leaf cells (449, 457, 512). Once in the leaf symplast, Fe must be delivered into leaf cells to participate in multiple biochemical processes. The chloroplast is the largest pool of Fe in plant cells accumulating 80 to 90% of cellular Fe (118). The influx of Fe into the chloroplast of *Arabidopsis thaliana* is achieved by the PIC1 (permease in chloroplast 1) localized in the inner envelope membrane of chloroplasts (Figure 1.10) (513). AtFRO7 is localized in the chloroplast envelope and looks to be critical for chloroplast Fe acquisition (514). Mitochondria are another plant sites where Fe is essential. Bashir, Ishimaru (515) identified the rice mitochondrial Fe transporter (MIT), which is essential for rice growth and development. Moreover, AtFRO3 and AtFRO8 are localized in mitochondria membrane, which indicates that these ferric reductase oxidases must be involved in mitochondrial Fe homeostasis (Jeong and Connolly, 2009). The vacuole generally functions as a metal pool to avoid toxicity. The transporters OsVIT1 and OsVIT2 mediate the influx of Fe across the vacuolar membrane into the vacuole (516). Likewise, it seems that the IREG2/FPN2 present in Arabidopsis tonoplast may play a role in the influx of Fe from the cytoplasm into the vacuole (404). On the other hand, both AtNRAMP3 and AtNRAMP4 export Fe from vacuoles (517). In the Golgi complex, the MATE (multidrug and toxic compound extrusion) transporter BCD1 has a role in sustaining Fe homoeostasis by reallocating excess Fe released from stress-induced cellular damage in Arabidopsis (518).



Transport step	Fe	Cu	Mn	Zn
A	OsVIT1/2 AtFPN2		AtCAX2/4 AtCAX5 ? AtMTP11 OSVIT1/2 ?	AtMTP1/3 AtHMA3 AtZIF1 OsVIT1/2 ?
B	AtNRAMP3/4	AtCOPT5	AtNRAMP3/4 AtZIP1	
C	AtBCD1			AtECA3
D	AtPIC1	AtHMA6		
E		AtHMA8		
F		AtHMA1		AtHMA1
G	AtFRO7	AtFRO7 ?		
H	OsMIT	OsMIT ?	OsMIT ?	
I	AtFRO3/8	AtFRO3/8 ?		
J		AtHMA7		

Figure 1.10 – Schematic illustration of the subcellular localization of transporters and reductases involved in Fe, Cu, Mn and Zn movement and reduction, respectively, in *Arabidopsis thaliana* and *Oryza sativa*. Members of the OsVIT, AtFPN, AtNRAMP, AtBCD,

AtPIC, AtFRO, OsMIT, AtCOPT, AtHMA, AtCAX, AtMTP, AtZIP, AtZIF and AtECA families are targeted in intracellular membranes: vacuole (OsVIT1/2, AtFPN2, AtCAX2/4-5, AtMTP1/3/11, AtZIF1, AtNRAMP3/4, AtCOPT5 and AtZIP1), Golgi complex (AtBCD1 and AtECA3), chloroplast (AtPIC1, AtHMA1/6 and AtFRO7), thylakoid membranes (AtHMA8), mitochondrion (OsMIT and AtFRO3/8) and endoplasmic reticulum (AtHMA7). The arrows indicate the direction of transport/reduction and each letter in the figure corresponds to the list given in the first column of the table.

In order to Cu reach the leaf symplast, plasma membrane proteins must exist to mediate Cu xylem unloading and Cu acquisition. It seems that members of the COPT family are responsible for the influx of monovalent Cu (384, 519). However, since Cu is present mainly in the divalent form in the vascular tissues, reduction must occur before it can enter the cell organelles. AtFRO7 (expressed in the chloroplasts) and AtFRO3/8 (expressed in the mitochondria) play a central role in Fe reduction, and it is hypothesized that they also participate in Cu reduction (123). Moreover, Cu can also be transported across the plasma membrane of leaf cells complexed with PS. Members of the YSL family were proved to be engaged in the Cu translocation and delivery to shoot tissues (455-458). Once in the cytosol of leaf cells, Cu is distributed with the aid of plasma membrane-localized transporters among different cell organelles. AtHMA6 (or PAA1), localized in the inner chloroplast envelope, is responsible for the delivery of Cu to chloroplasts (Figure 1.10) (520). AtHMA8 (PAA2), closely related to AtHMA6 (PAA1), is expressed in the thylakoid membrane and supplies Cu to plastocyanin (521). AtHMA1 and HvHMA1, present in the chloroplast envelope, are broad-specificity exporters of metals from chloroplasts and may play a specialized role in Cu mobilization (522). AtHMA7 (RAN1), the first functionally characterized heavy metal ATPase, is responsible for the biogenesis of ethylene receptors by supplying Cu at the endoplasmic reticulum and also for Cu homeostasis in seedling development (523). The rice mitochondrial Fe transporter (MIT) also appears to regulate the influx of Cu, although more studies are needed to confirm this (515). The tonoplast-localized AtCOPT5 is important for Cu export from the vacuole and is involved in the remobilization of Cu ions (386, 387).

Manganese reaches the leaf symplast by crossing the plasma membrane of leaf cells. To do that, members of the IRT family are thought to be expressed in the shoot tissues, mediating the transport of Mn ions into the cytosol of leaf cells (260, 277). After influx into the leaf symplast, Mn must be distributed into various cell compartments. In Arabidopsis, AtECA1 is localized at the endoplasmic reticulum (524). The cation/H^+ exchanger (CAX) transporters AtCAX2 and AtCAX4, which were originally identified as Ca^{2+} transporters, also have the ability to transport Mn into the vacuole (Figure 1.10) (376, 388). Likewise,

vacuolar Mn^{2+}/H^+ antiporter activity in the Arabidopsis *cax2* knockout mutant is significantly reduced, although it is not completely absent, compared with wild-type, suggesting the presence of additional vacuolar transporters (e.g., AtCAX5) that contribute to Mn transport (374). AtMTP11, which belongs to the cation diffusion facilitator (CDF) family, is implicated in the pre-vacuolar compartmentation of Mn as well as in Mn homeostasis mechanisms (389). Other MTPs also contribute to Mn transport (525). Furthermore, it seems that the tonoplast-localized transporters OsVIT1 and OsVIT2 participate in Mn influx to the vacuole (516). Regarding vacuolar export, AtNRAMP3 and AtNRAMP4 are expressed in the tonoplast and can transport Mn and other metals (390). AtZIP1 probably plays a role in Mn vacuolar efflux, based on the increased sensitivity to low Mn and increased accumulation of Mn in roots in the *zip1* knockout line (397). The rice Fe transporter MIT appears to play a role in Mn transport into mitochondria (515). The mechanisms of Mn transport in the chloroplast remain unknown, despite the fact that Mn has a critical role in photosynthesis and the chloroplast is the one of the major sinks for Mn (526, 527).

Zinc enters the leaf symplast with the aid of members of the IRT and ZIP families (129). After reaching the leaf cytosol, the delivery of Zn to plant organelles is also mediated by specific transporters (Figure 1.10). The vacuolar membrane transporters AtMTP1 and AtMTP3 are expressed in the vacuoles of leaf cells and play a key role in detoxification of excessive Zn by accumulating Zn in vacuoles (393, 528). Likewise, AtHMA3 is also involved in vacuolar Zn sequestration (396). In Arabidopsis, AtZIF1 also seems to be expressed in the tonoplast of leaf cells. Therefore, an active role is expected from this transporter regarding the vacuolar Zn sequestration (319). The tonoplast-localized OsVIT1 and OsVIT2 seem to transport Zn into the vacuoles of plant cells (516). In the chloroplasts of both Arabidopsis and barley, HMA1 contributes to Zn detoxification by exporting Zn from the plastids to the cytoplasm (522, 529). It has been proposed that the Golgi-localized transporter AtECA3 may have a role in the mobilization of Zn from the cytoplasm to the Golgi complex (391, 530).

Regarding the beneficial cations Na, Ni and Co, little information is available about the processes regulating their movement into the leaf symplast as well as their distribution in the cell organelles (23). Although their essentiality is not yet proved, evidence suggests that similar membrane transporters should mediate their mobilization within the plant compartments (22, 292).

Since no specific transporter exists for Cd, its movement into the leaf symplast is thought to be mediated by broad-selective transporters (531, 532). Since Cd is very toxic, it tends to accumulate in the vacuole and/or cell walls (494, 498). To enter into the vacuole of leaf cells, members of the CAX and HMA families must mediate the active movement of the

free ion Cd across the tonoplast (374, 388, 408). Moreover, Cd can also enter into the vacuole in the form of Cd-PCs complexes. Two PCs transporters, AtABCC1 and AtABCC2, are involved in the mobilization Cd-PC complexes into the vacuole contributing to the detoxification process (327, 411).

1.3.5. Plant metabolism

By definition, nutrients have specific and essential functions in plant metabolism. The main functions of nutrients such as N, S and P that are constituents of proteins and nucleic acids are evident. Other mineral nutrients (e.g., Ca and Mg) are constituents of organic structures, such as enzyme molecules, where they may be involved in the catalytic function of the enzymes (10). Potassium is one of the few nutrients that are not a component of organic structures. It is rather involved in the osmoregulation, the maintenance of electrochemical balance in cells and their compartments, and the regulation of the enzymatic activity (231). Due to their low concentrations, trace essential elements do not play a direct role in the osmoregulation or similar processes. These elements are redox-active and act as catalytically active cofactors in enzymes, others have enzyme-activating functions, and others fulfil a structural role in stabilizing proteins (22, 23).

1.3.5.1. Macronutrients

In the process of N metabolism, two important enzymes, nitrate reductase (NR) and nitrite reductase (NiR), mediate the conversion of NO_3^- to NH_4^+. This two-step process is initiated by NR, which ensures the NO_3^- conversion into NO_2^- in the cell cytosol. Afterwards, the chloroplast-localized NiR converts NO_2^- into NH_4^+. Nitrate assimilation can occur in both roots and shoots (533, 534). The metabolism of NH_4^+ into amino acids and proteins is the main mechanism of NH_4^+ assimilation and detoxification. In chloroplasts, NH_4^+ from the reduction of NO_2^- by NiR and from the photorespiration is used by two enzymes, glutamine synthetase (GS) and glutamate synthase. The high affinity of glutamine synthetase for NH_4^+ enables the assimilation of NH_4^+ at low concentrations, with the consequent production of glutamine, which will be used by glutamate synthetase to produce glutamate (31). All together, these enzymes assimilate most of the NH_4^+ derived from plant uptake, NO_3^- reduction, N_2 fixation and photorespiration. The glutamate amino group can be transferred to amino acids by a number of different aminotransferases (534). Asparagine synthetase (AS) catalyzes the formation of asparagine and glutamate from aspartate and glutamine. Along with GS, AS is thought to play a key role in primary N metabolism. In addition, the mitochondrial NADH–glutamate dehydrogenase can alternatively incorporate NH_4^+ into glutamate in response to high levels of NH_4^+ under

stress (535). The primary function of N is to provide amino groups to amino acids. N is also key component of nucleotides, where it occurs integrated in the ring structure of purine and pyrimidine bases. Nucleotides are the basic constituents of nucleic acids nevertheless they also have several other important functions such as in energy homeostasis, signaling and protein regulation (10). Furthermore, N is essential in the biochemistry of many non-protein compounds such as co-enzymes, photosynthetic pigments, secondary metabolites and polyamines (7). Among the many N-compounds found in plant, polyamines occupy an important position. There is direct evidence that they have an important role in the regulation of plant growth and development as well as in modulating the defense response of plants to diverse environmental stresses (536). In fact, plant growth and response to stress conditions results many times in significant changes in polyamine content as well as in the activity of their biosynthetic enzymes (537, 538).

It is widely accepted that the bulk of reduced S is produced in shoot chloroplasts. S assimilation is a two-step process where SO_4^{2-} is firstly reduced to SO_3^{2-} and then to S_2^- that is assimilated into the amino acid cysteine (10). In fact, the majority of S is found in the amino acids cysteine and methionine. In cysteine, S appears in the sulfhydryl (–SH or thiol) group which can experience reversible oxidation resulting in the formation of a covalent –S–S– bond if a second –SH group is present. The formation and disruption of these S bridges influence the tertiary and quaternary protein structure influencing the protein activity (539). Under normal conditions, about 2% of the organic S in the plant is present in the water-soluble thiol fraction and 90% of this fraction is in the form of the tripeptide GSH (7). The production of GSH serves as a storage pool and a mobile carrier of reduced S. However, GSH also plays an important role as a general redox buffer (e.g., as a reductant in the detoxification of reactive oxygen species) (540). GSH is also the precursor of PCs, which have high affinity for metal(loid)s, influencing the detoxification process of these compounds, supporting the evidence that SO_4^{2-} and S-organic compounds play a key role in the abiotic stress (318). Another role of inorganic S is represented by sulfolipids. These are usually found in small amounts in chloroplast thylakoids and their function is not fully understood. However, evidence suggests that they are essential for the stabilisation of photosystem components (541).

Unlike N and S, P remains in its oxidized form. After uptake, phosphate remains as inorganic P (Pi) or is esterified through a hydroxyl group to a carbon chain forming, for example, sugars and alcohols. Moreover, Pi may binds to other phosphate groups via pyrophosphate bonds. The formation and disruption of the pyrophosphate bond (i.e., ATP) is one of the central mechanisms in cellular energy homoeostasis (7). ATP is also the base of many pathways such as those for synthesis of nucleic acids. Other energy-rich

phosphonucleotides such as UTP, CTP and GTP also have important roles in nucleic acid metabolism. Moreover, UTP is also a building block of sucrose, starch and cellulose synthesis while CTP acts as energy rich compound during phospholipid biosynthesis (10). Since triphosphate nucleotides form the backbone of DNA and RNA, P is an indispensable component of nucleic acids. In these macromolecules, Pi links two adjacent riboses via esterification (7). P is also a constituent of cellular membranes. Membranes are mainly made up of phospholipids and Pi acts by linking the lipophilic glycerol-fatty acid part to the hydrophilic choline part of the lipid. The negative charge on the phosphate group confers strong hydrophilicity, helping the proper orientation of the membrane. The negative charges are compensated by electrostatic binding of divalent cations (10).

The dominant role of K is the osmoregulation. It role in turgor provision and water homeostasis is obvious in processes such as pressure-driven solute transport in the xylem and phloem, high levels of vacuolar K accumulation and the large fluxes of K that mediate plant movement (231, 232). K is also required in metabolic processes due to its capacity to activate a multitude of enzymes. Specific enzymes such as vacuolar PPase isoforms have been shown to be activated by K. Additionally, many enzymes involved in C-metabolism including pyruvate kinase, phosphofructokinase and ADP-glucose starch synthase have shown K dependence. Ribosome mediated protein synthesis is another key process that requires K (7, 10).

In plants, Ca acts mainly as a structural component and secondary messenger. Ca easilly complexes with negative groups of organic compounds, including phosphates and carboxyls groups of phospholipids, proteins and sugars. This is noticeable in plant cell walls where the cellulose microfibrils are cross-linked by glycans and pectins. Carboxyl groups from opposing pectins are electrostatically coordinated by Ca conferring rigidity to cell walls. An analogous role is observed in cell membranes where Ca coordinates with phosphate groups from phospholipids. Removal or replacement of membrane Ca readily compromises membrane integrity (7, 10).

The best known function of Mg is perhaps the derived from its presence in the chlorophyll molecule, where it coordinates covalently with four N atoms from the porphyrin ring. However, Mg also has further crucial roles in photosynthesis, mainly resulting from its capacity to promote the light reactions in the stroma (10). Most of cellular Mg plays important roles as enzyme cofactors and in the stabilization of nucleotides and nucleic acids. In fact, Mg is indispensable in enzymatic reactions associated with energy transfer and phosphorylation/dephosphorylation. The majority of cell's energy is stored in the high-energy ester and pyrophosphate bonds of phosphosugars as well as in the ADP and ATP molecules. Enzymes like phosphotransferases and ATPases, necessary to release the energy from those molecules, are activated by Mg (7). Moreover, Mg also easily binds to

nucleic acids. Therefore, DNA melting temperatures are significantly higher in the presence of Mg. In RNA, Mg shows a similar role and helps maintaining secondary structure (542).

1.3.5.2. Micronutrients

Micronutrients are elements that are essential for plant growth but at lower amounts compared to primary nutrients such as N, P, S and K. Plants have different needs for certain micronutrients, but the elements that are commonly accepted as essential for plants are: boron (B), chloride (Cl), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn). All plants have to acquire suitable amounts of each micronutrient, a process that involves a metal homeostasis network comprising mobilization, uptake and distribution within the plant, intracellular trafficking, and storage (6). Several micronutrients are redox-active, which is the basis for their occurrence as catalytically active cofactors in many metalloenzymes. Other metals have, in addition to their catalytic role, a structural role in stabilizing proteins. Overall, micronutrients are involved in almost all metabolic and cellular functions in plant, including energy metabolism, cell protection, gene regulation, primary and secondary metabolism, signal transduction, hormone perception, and reproduction. However, it should be noted that when present in higher levels, the same redox properties that make these metal ions essential elements lead to the formation of reactive oxygen species with harmful consequences for the cell (22).

Fe is of great importance for life. Due to its redox-active character, Fe is involved in photosynthesis, N assimilation, mitochondrial respiration, hormone biosynthesis, production and scavenging of reactive oxygen species, osmoprotection and pathogen defense. As much as 80% of the cellular Fe is found in the chloroplasts, which is consistent with its major function in photosynthesis. Depending on the type of Fe ligand, three groups of Fe-containing proteins can be defined: (1) proteins with Fe–S clusters, (2) heme-containing proteins, and (3) other Fe proteins (543). Fe–S clusters have crucial functions in electron transfer, constitute part of substrate binding sites in enzymes, form Fe storage moieties, are involved in transcriptional or translational regulation, can control protein structure in the vicinity of the cluster, and finally they have been shown to be involved in disulfide reduction and sulfur donation. Therefore, Fe–S proteins functions as enzymes, as electron carriers (e.g. ferredoxin), and as regulator proteins (544). The widely known hemo-proteins are the photosynthetic and respiratory cytochromes, involved in electron transfer, and the globins that bind oxygen. Other examples include the oxidative enzymes catalase, peroxidase and NADPH oxidase, involved in the production and/or scavenging of free radicals, and the large group of cytochrome P450 enzymes. In

plants, these latter catalyze mono-oxygenation reactions in biosynthetic pathways (22). The non-heme proteins bind Fe ions directly, i.e., neither as heme nor in the Fe–S form. Among these proteins, ferritins are most prominent. Ferritins are plastidic Fe storage proteins and control the interaction between iron homeostasis and oxidative stress in Arabidopsis (545).

Cu is of greatest importance for life. Among their roles within the cell, it should be emphasized its involvement in the photosynthesis and mitochondrial respiration, in oxidative stress protection, in C and N metabolism and in cell wall synthesis (6). Under physiological conditions, Cu is present in the two oxidation states Cu^{+} and Cu^{2+}, and can interchange between these forms. This allows Cu to function as a reducing or oxidizing agent in biochemical reactions. At the same time, this property also makes Cu potentially toxic since Cu ions can catalyze the production of free radicals, in particular through Fenton reaction, thus leading to the damage of proteins, DNA and other biomolecules (546). Therefore, after uptake most of Cu ions are bound by scavenging proteins like MTs to prevent its accumulation in a toxic form. However, a small amount of Cu avoids this system and becomes captured by Cu chaperones that spare Cu from the detoxification systems and guide it to the target sites in the cell (547). In total, more than 100 Arabidopsis proteins are predicted to be complexed with Cu, forming two groups: Cu binding proteins/chaperones and enzymes (548). Cu has a high affinity to dioxygen molecules which explains why Cu is the catalytic metal in many oxidases. A well-known member of this group is mitochondrial cytochrome c oxidase. Cu is also found in electron carrier proteins like plastocyanin that accounts for about 50% of the plastidic Cu. In fact, more than half of the Cu in plants is found in chloroplasts and participates in photosynthetic reactions (549).

Manganese is essential for plant metabolism and development and is present within plant in three oxidation states (II, III, and IV) in approximately 35 enzymes (550). Manganese has two functions in proteins: (1) serves as catalytically active metal, or (2) exerts an activating role on enzymes. The Mn-containing superoxide dismutase, which protects the cell from the damaging effects of free radicals, the oxalate oxidase and the Mn-containing water splitting system of photosystem II are some examples of the Mn catalytic role (276). Examples of Mn-activated enzymes are isocitrate dehydrogenase, phosphoenolpyruvate carboxykinase and phenylalanine ammonia lyase. Mn activation was observed in enzymes of N metabolism (glutamine synthetase, arginase), gibberellic acid biosynthesis, RNA polymerase activation, and fatty acid biosynthesis (6).

Zn is important as a component of enzymes for protein synthesis and energy production as well as in maintaining the structural integrity of biomembranes (6). More than 1200 proteins are predicted to contain, bind or transport Zn, including a large number of Zn-

fingers containing proteins and transcription factors, oxidoreductases and hydrolytic enzymes such as metalloproteases (548). Most of the Zn enzymes are involved in regulation of DNA-transcription, RNA-processing and translation. Besides transcription factors, several enzymes involved in DNA or RNA synthesis and maintenance are Zn-dependent (129). In chloroplasts, Zn-dependent enzymes display several major functions. The stromal processing peptidase (551) is Zn-dependent in analogy to the mitochondrial system (552). Additional, Zn metalloproteases in these organelles have to destroy the cleaved signal-peptide (553). Moreover, inside the chloroplasts, proteolytic activities are dependent on Zn (554). Zn deficiency also reduces net photosynthesis in plants by disturbing the activity of carbonic anhydrase, Cu–Zn superoxide dismutase and D-ribulose-5-phosphate 3-epimerase (555). In addition to chloroplasts and mitochondria, also the cytoplasm, lysosome and the apoplastic space are compartments with Zn-dependent hydrolytic activities (22).

Ni is essential in numerous prokaryotic enzymes such as dehydrogenases, hydrogenases and methylreductases but is barely used as cofactor in eukaryotes (6). Among plants, it occurs not only in oxidation state II, but also in states I and III. A deficiency symptom in plants is the accumulation of toxic urea that could be explained by the complete loss of urease activity within the cell (22).

Few plant proteins are known to contain Mo. However, these proteins are very important as they are involved in N assimilation, S metabolism, phytohormone biosynthesis and stress reactions (6). Nitrate reductase is the key-enzyme for NO_3^- assimilation whereas nitrogenase is found in N fixing bacteria inside nodules of symbiotically growing species (533).

The uncommon nature of B chemistry suggests the possibility of a wide variety of biological functions for this micronutrient. B is involved in several important processes, such as protein synthesis, respiration, RNA and carbohydrate metabolism, transport of sugars and the metabolism of plant hormones (6). Other B functions are related to cell wall synthesis, lignification, cell wall structure and structural integrity of biomembranes. Up to 90% of the B in plants is found in cell walls (22).

Cl is present in more than 130 organic compounds in plants (22). Since Cl is highly mobile, most of its functions are related to electrical charge balance. In the chloroplast, Cl is a structural constituent of photosystem II (556). Moreover, Cl stimulates the proton-pumping ATPase activity at the tonoplast (6). The open/close process of the guard cells is mediated by the flux of K and anions such as Cl. Therefore, Cl indirectly affects plant growth by stomatal regulation (204).

1.3.5.3. Ionome

The accumulation of a given element involves a complex process controlled by a network of genes critical for its uptake, binding, transportation and sequestration. Many of these genes and physiological processes affect more than one element. Therefore, to understand how elements are regulated, it is necessary to measure as many of the elements contained in a cell, tissue or organism as possible (21). In this context, the study of the plants "ionome", the so-called "ionomics", defined as the quantitative determination of the elemental composition of plant tissues and its changes according to the plant development stage or in response to physiological stimuli or genetic modifications, emerged as a central area in the plant science field (557).

The ionome includes both essential and non-essential elements. Changes in the levels of any given element in a plant can be affected by a range of factors, including changes in the soil chemical environment, caused either by the plant or by changes in the environment; modifications in the plant morphology (e.g., root structure and whether the plant is in the vegetative or reproductive stage of its life cycle); alterations in the plants uptake capacity (e.g., down- or up-regulation of channels or membrane transporters, or, for ions that diffuse into the plant, changes in the biochemical structure of the cell walls); changes in the accumulation of chelators (e.g., organic acids, NA, or GSH); and changes in the amount of the element that is stored in intracellular compartments (e.g., vacuole) (558-560). Very few of these changes exclusively affect a single element, i.e., changes in a transporter that has high specificity for that element. Most of the above-mentioned changes will affect more than one element. Therefore, experiments focused on single elements, which do not take into account the total mineral nutrient content of the plant will not reveal the regulatory networks involved in the homoeostasis of the ionome (21, 557).

Ionomic studies require the use of rapid, sensitive, and accurate multielemental analytical techniques. For several years, the multielemental analysis of plant tissues was performed by atomic absorption spectrometry (AAS), X-ray absorption spectroscopy (XAS) and instrumental neutron activation analysis (INAA) techniques. Later, plasma-based techniques, namely inductively coupled plasma atomic emission spectrometry (ICP–AES) and inductively coupled plasma mass spectrometry (ICP–MS) have become leading instrumental analytical techniques in these studies, because they allow the rapid and simultaneous determination of almost all chemical elements (561).

1.4 Water

Inorganics are present in ground and surface waters, and their sources are associated with both natural processes and man's activities. The two main natural processes that contribute to inorganics loading into waters are weathering of rocks and soil leaching. The anthropogenic sources of inorganics in waters are many times related with agricultural activities. Water pollution by inorganics is a significant aspect regarding both the geochemical cycling of these elements and environmental health. The consequences of inorganics pollution of waters for the human health are a well-known worldwide problem (27).

Clear evidence exists that the transfer of inorganics in the water-soil-plant system plays a key role in elemental cycling. The concentrations of inorganics in waters may vary in several orders of magnitude due to natural and anthropogenic processes that constantly affect the water-soil-plant system (562, 563). Generally, surface waters are more prone to water pollution than groundwaters. However, nowadays, several aquifers are heavy-polluted constituting a serious environmental and health problem (27).

PART II

Extraction methods for the analysis of vegetables and soil

Chapter 2

Influence of different sample pretreatments and extraction methods in the quantification of nitrate and nitrite in lettuce and spinach

Abstract

Plant food samples should be analyzed as soon as possible after collection. However, this is impractical when a high number of samples need to be analyzed at the same time. Freeze, freeze drying and oven drying are the usual procedures applied before the storage of plant foods for NO_3^- and NO_2^- analysis. Additionally, different extraction techniques are often used for their analysis in different matrices. Comparative investigations were carried out with the objective of studying the influence of freeze, freeze drying and oven drying pretreatments before storage and finding the most suitable conditions for quantification of NO_3^- and NO_2^- in lettuce and spinach. A rapid and cost effective RP-HPLC/UV method was validated and used to select the most appropriate extraction procedure to eliminate chromatographic interferences and to evaluate the influence of different sample pretreatments on the accuracy and reproducibility of the results obtained. Activated charcoal was efficient to remove chromatographic interferences from lettuce and spinach matrices. Freeze drying was a suitable alternative for long time storage of plant food samples, because a large number of samples can be lyophilized, the sample size is reduced and low moisture contents helps sample preservation.

2.1. Introduction

Plant foods, in particular vegetables, tend to accumulate nitrate (NO_3^-) ions, thus being a major source of human exposure to this compound, especially if grown under a high application of N fertilizers. NO_3^- contents vary significantly, ranging from 1 to 10.000 mg/kg fresh weight, while nitrite (NO_2^-) levels in fresh vegetables are low (< 2 mg/kg) (30, 96, 564). NO_2^- levels in vegetables may increase during post-harvest storage by the action of indigenous bacteria and/or the presence of nitrate reductase, especially when vegetables are left at room temperature or higher (89, 565).

NO_3^- accumulation in plant foods occurs when crops absorb more than required for their sustainable growth (30). Spinach and lettuce possess a high tendency to accumulate NO_3^-. Consequently, the European Commission (EC) has set maximum levels for NO_3^- in lettuce and spinach (566).

A variety of analytical methods for the determination of NO_3^- and NO_2^- have been developed and applied in the analysis of food, water, plants, and other matrices. NO_2^-, and NO_3^- after reduction to NO_2^-, are routinely determined in food by spectrophotometric methods based upon the ability of NO_2^- to convert aromatic amines into diazonium ions, which, in turn, are coupled to another aromatic compound in order to produce an azo dye (the Griess-Romijn reaction) (179). The most common chromogenic reagent utilizes

sulphanilamide and N-(1-naphthyl)ethylenediamine as the target amine and coupler, respectively, with the reaction product being detected at 540 nm. To promote the NO_3^- to NO_2^- conversion, a variety of reducing agents have been investigated, including, copperized cadmium (567) and zinc (568). More recently, photo-induced reduction was also proposed (569). The automation of the analytical procedures for NO_3^- and NO_2^- determination rely on segmented flow or flow injection analysis, using the traditional colorimetric Griess diazotization method (138, 570). Methods traditionally used to determine NO_3^- and NO_2^- in food typically have low sensitivity, not enough for the determination of trace levels of the analytes and can produce inaccurate results due to sample matrix interferences.

Alternative methods for NO_3^- and NO_2^- determination in foodstuffs have therefore been developed, including spectrophotometric determination after enzymatic reduction (149), polarography (571) and capillary electrophoresis (572). Ion chromatographic methods have also been widely studied for the separation of NO_3^- and NO_2^- in several matrices (91). Compared to ion chromatography, ion-pair HPLC methods offer several advantages, namely a relatively lower cost of instrumentation and columns (141, 573, 574).

NO_3^- and NO_2^- can be unstable and appropriate sampling methods and extraction procedures must be used to obtain reliable results. Extraction into hot water (or borax solution) is the most used process (91, 565). It is recommended that samples are analyzed as soon as possible after collection. However, this may be impractical when the sampling is done at a significant distance from the laboratory or a high number of samples have to be analyzed. It may be necessary to store the samples for a certain period of time before analysis. Freeze, freeze drying and oven drying are the usual procedures described in literature to prepare the samples for storage before NO_3^- and NO_2^- analyses, but information regarding the adequacy of these processes is scarce (96, 101, 117). The main objectives of this study were: (1) to evaluate the influence of freeze, freeze drying and oven drying pretreatments on the NO_3^- and NO_2^- content, (2) to select the most appropriate storage procedure, and (3) to choose and validate an appropriate extraction procedure for NO_3^- and NO_2^- determination in lettuce and spinach matrices.

2.2. Experimental

2.2.1. Reagents and Apparatus

All reagents used were of analytical grade purity. Solvents for HPLC were filtered through 0.22 µm NL 17 filters (Whatman Ltd., Maidstone, UK) and degassed under vacuum for at least 15 min before use. Sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$), and n-octylamine were supplied by Sigma Chemicals Co. (St Louis, MO, USA), methanol (LiChrosolv®) and activated charcoal were from Merck (Darmstadt, Germany). Stock standard solutions of NO_2^- (1000 mg/L) and NO_3^- (1000 mg/L) were prepared from their respective sodium salts previously oven-dried (100 ºC during 1 hour). Calibration standard solutions were obtained by appropriate dilution of the concentrated stock solutions. A few drops of chloroform were added to prevent the development of microorganisms, and the solutions were stored in a refrigerator.

The chromatographic analysis was carried out in an analytical HPLC unit (Gilson, France) equipped with a type 305 pump and a type 7125 Rheodyne injector with a 20 µL loop, a Gilson 118 variable long-wave ultra-violet detector (λ =220 nm) and a Gilson 712 HPLC System Controller Software. The chromatographic separation was achieved using a ACE C_{18} (250 mm × 4.6 mm, 5 µm) chromatographic column and isocratic elution with 0.01 M n-octylamine and 20% methanol at pH 6.6. The flow rate was set at 0.5 mL/min.

A Ultrasonic cleaner (Fungilab, Portugal) and a Metrohm 632 pH-meter were used for eluent preparation. A vortex agitator (Reax Control, Heidolph, Germany) was used for sample preparation.

Prior to analysis, the ion-pair reagent solution was allowed to pass through the HPLC column until a stable baseline signal was obtained. Generally, a stabilization time of 30 minutes was sufficient, and then reproducible retention times were obtained throughout the working day (8-12 hours). At the end of the work, the HPLC column was regenerated overnight by passing a 3:7 water-methanol mixture at a 0.2 mL/min flow rate.

2.2.2. Sampling and sample preparation

Fresh leafy vegetables, spinach (*Tetragonia tetragonioides*) and lettuce (*Lactuca sativa*), were collected from a greenhouse field. Non-edible parts were removed and samples were frozen at – 20 ºC during 6 hours. Then, samples were cut, homogenized and divided in three lots. The first lot was analyzed fresh (less than 24 hours after collection, codified as S or L, for spinach or lettuce, respectively), the second lot was frozen during two weeks (codified as SF or LF, for spinach or lettuce, respectively), and the third lot was freeze dried and sieved through a pore less than 500 µm (these samples were codified as SFd or LFd, for spinach or lettuce, respectively). Additionally, fresh vegetable leafs were taken

and dried in a forced air oven (model WTC Binder 78532, Tuttlingen, Germany) at 70 ºC for 48 h, as described by Castro, Manas (96) The dried leaves were then ground in a mill, sieved through a pore less than 500 µm and codified as SO and LO, for spinach or lettuce, respectively.

Dry matter of fresh, frozen, freeze dried and oven dried samples was evaluated using an oven from Scaltec Instruments (Goettingen, Germany), at 100 ºC.

The use of an effective material to remove interferences from vegetable matrices was tested. Activated charcoal could meet this demand owing to its cheapness and strong adsorption character. The homogenized sample – usually, 0.250 g for fresh and frozen samples and 0.025 g for freeze dried and over dried samples (however, amounts ten times higher were used for evaluation of the quantification limits) – was weighed, put into a 100 mL volumetric flask with an equal amount of activated charcoal, and then 50 mL of deionized water was added. Similar procedure was performed without the addition of activated charcoal. The volumetric flasks were heated for 20 min at 80 ºC, shaken, allowed to cool, and then filled to the mark with deionized water. After filtration through a 0.45 µm syringe filter, the filtrate was analyzed for NO_3^- and NO_2^- by HPLC with UV detection.

2.2.3. Method validation

Each analytical run consisted of replicate analysis of blanks (limit of detection), standard solutions (sensitivity and linear range) and both spiked and un-spiked samples (recovery and precision). Linearity was studied by preparing five standard solutions of NO_3^- and NO_2^- ranging from 0.05 to 20.0 mg/L. Linear regression analysis of analyte concentration *vs.* peak area was performed. The detection limit was calculated as the concentration corresponding to three times the background noise of the blank. Intra-day (3 analytical runs on the same day), and interday tests (6 analytical runs during 7 successive days with at least 24 h intervals) were performed. The precision was characterized by the relative standard deviation (RSD, %).

For recovery studies a series of standard solutions (containing both NO_3^- and NO_2^-) were spiked into spinach and lettuce samples. Spiked and unspiked samples were analyzed in triplicate to evaluate the average recoveries.

2.2.4. Statistical analysis

Data were evaluated through ANOVA and the Duncan test for differences between the means at $p < 0.05$. To check for a normal distribution of the data, standardized skewness and standardized kurtosis values were calculated.

2.3. Results and discussion

2.3.1. Performance of the HPLC method and selection of the extraction conditions

In this study, a simple, efficient and accurate HPLC method, essentially based on the analytical procedures developed by (574), was adapted to the determination of NO_3^- and NO_2^- in solutions obtained from spinach and lettuce extraction. Isocratic elution with a mobile phase containing 0.01 M n-octylamine and 20% methanol, at pH 6.6, enabled NO_3^- and NO_2^- ion-pair chromatographic separation. Under the experimental conditions described, the retention time of the target analytes was very reproducible. The retention times were 11.28 ± 0.03 min and 14.67 ± 0.07 min, for NO_2^- and NO_3^-, respectively. The total running time for one sample analysis was ca. 15 min.

Linearity was obtained over the tested concentration range of 0.05 – 20 mg/L of NO_3^- and NO_2^-. The linear regression equations for the calibration curves were calculated as y = 19718x + 1225.3 and y = 26653x + 3765.2, for NO_3^- and NO_2^- respectively. The correlation coefficients were greater than 0.999 in both cases, proving good linearity. The calibration curves were used to calculate the concentration of NO_3^- and NO_2^- in spinach and lettuce extracted solutions. Results were reported as mg/kg.

The detection limit of NO_3^- and NO_2^-, calculated based on a signal-to-noise ratio of 3, was 0.02 mg/L. Therefore, the method showed good sensitivity and can detect trace levels of NO_3^- and NO_2^- (< 2 mg/kg).

Precision of the measurements was evaluated by intra-day and inter-day analysis. The precision obtained in the repeated determination of a 5 mg/L standard solution of NO_3^- and NO_2^-, indicated by the relative standard deviation (%RSD), is shown in Table 2.1. RSD values were generally lower than 3%.

Table 2.1 – Precision of inter-day and intra-day analysis.

	Precision (%RSD)			
	Retention time (min)		Concentration (5 mg/L)	
	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]
NO_2^-	0.05	2.70	1.42	1.17
NO_3^-	0.25	2.43	2.30	2.64

[a] Intra-day: running three times within 24 hours.

[b] Inter-day: running six times within successive 7 days with at least 24-hour intervals.

The HPLC procedure was applied to the analysis of fresh spinach and lettuce samples and the sample amount used was chosen in order to the concentration of the extraction solution fall within the calibration curve range. Two extraction techniques were assessed: with and without addition of activated charcoal using fresh and spiked vegetable samples. Activated charcoal was efficient to remove interferences from vegetable matrices as can be observed in Figure 2.1a and 2.1b. Several chromatographic peaks were observed in spinach samples extracted without addition of activated charcoal (Figure 2.1a), including one peak near NO_2^-. These interfering peaks were almost removed using activated charcoal as can be observed in Figure 2.1b. Both extraction techniques gave almost similar areas for the NO_3^- peak. Extraction with activated charcoal gave cleaner chromatograms, without loss of NO_3^- and NO_2^- content, thus it was chosen because it could contribute to preserve the chromatographic column.

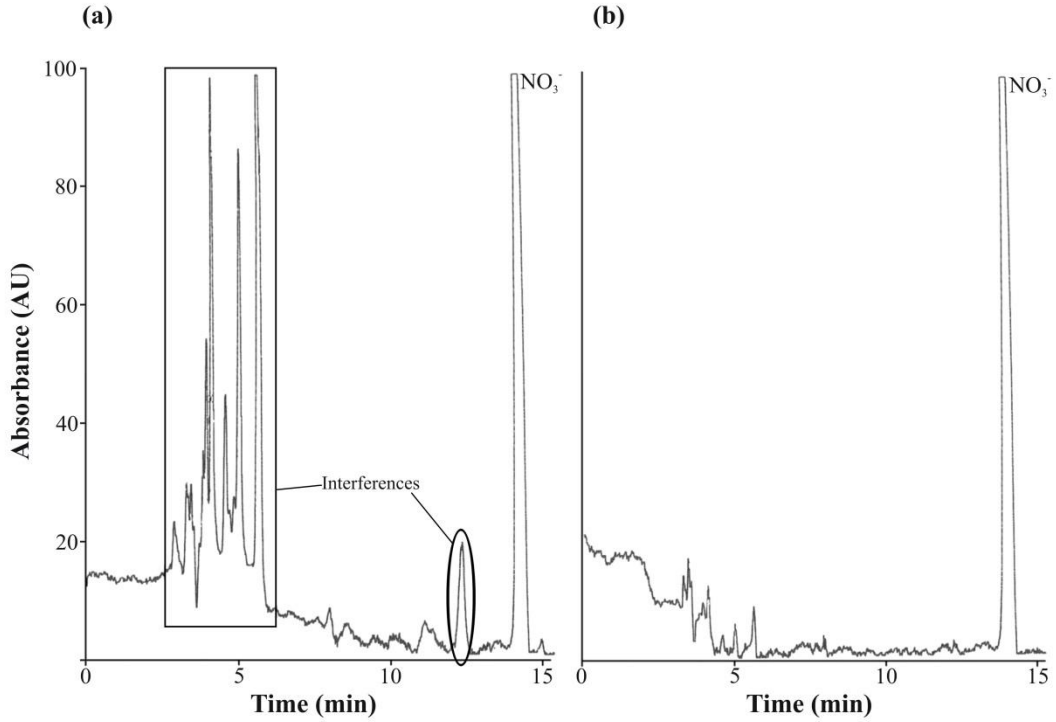


Figure 2.1 – (a) Chromatogram of spinach samples extracted without addition of activated charcoal. (b) Chromatogram of spinach samples extracted with addition of activated charcoal.

Further, recovery studies were performed adding to the sample an equal amount of activated charcoal. The recovery of NO_3^- and NO_2^- in spiked sample extracts was in the range 97.8 – 108.3% and 97.5 – 101.4%, respectively (Table 2.2). This HPLC method was chosen to evaluate the effect of different sample pretreatments because it is fast, sensitive and accurate.

Table 2.2 – Recoveries of NO_3^- and NO_2^- spiked into fresh spinach and lettuce samples extracts.

Spike concentration (mg/L)	Recovery (%) and Standard Deviation[c]	
	NO_2^-	NO_3^-
2.24[a]	108.3 ± 4.9	101.6 ± 1.0
4.25[a]	100.6 ± 3.8	97.8 ± 3.5
4.34[b]	97.5 ± 0.5	101.4 ± 1.0

[a] The content of NO_3^- in unspiked lettuce extracts was 2.91 mg/L; NO_2^- was not detected.

[b] The content of NO_3^- in unspiked spinach extracts was 3.79 mg/L; NO_2^- was not detected.

[c] Mean value of triplicate assays.

2.3.2. Effect of Sample Pretreatment

Results from the determination of the moisture content of spinach and lettuce samples (fresh, freeze dried, oven dried and frozen) are presented in Table 2.3. Fresh lettuce presented a moisture content around 95%, whereas for fresh spinach was around 89%. No significant differences were observed in the moisture content of fresh and frozen samples. Freeze dried and oven dried samples presented around 12% and 15% moisture, for spinach and lettuce, respectively.

Table 2.3 – Moisture content for fresh, freeze-dried, oven dried and frozen spinach and lettuce samples.

Sample pretreatment	Sample ID	Moisture (%)
Fresh (Frozen during 6 h and analyzed within 24 h after collection)	S	88.9
	L	94.7
Freeze-dried (Analyzed 36 h after lyophilisation)	SFd	11.7
	LFd	14.6
Oven-dried (Analyzed after 48 h of forced air oven-drying at 70 ºC)	SO	12.9
	LO	15.0
Frozen (Analyzed after 2 weeks of freezing)	SF	90.4
	LF	94.4

S – fresh spinach; L – fresh lettuce; SFd – freeze-dried spinach; LFd – freeze-dried lettuce; SO – oven-dried spinach; LO – oven-dried lettuce; SF – frozen spinach; LF – frozen lettuce.

The effect of freeze-drying, oven-drying and frozen on NO_3^- content of spinach and lettuce samples is shown in Tables 2.4 and 2.5, respectively. The results indicate that there is a significant variation in the experimentally determined concentration of NO_3^- depending on the sample pretreatment used. Analysis of variance (ANOVA) at the 95% confidence level showed that there is a significant difference between data obtained in both spinach (Table 2.4) and lettuce (Table 2.5). The results for fresh and two weeks frozen samples were closely similar, as indicated by the Duncan test, whereas significant differences were observed for oven-dried and freeze-dried samples. Prasad and Chetty (564) observed minor loss of NO_3^- content in samples frozen for 7 days, which was attributed to some microbial action that took place in the samples during the thawing period (after the samples removal from the freezer).

Freeze-drying and oven-drying of the samples can be a good approach to reduce storage space when a large number of samples must be analyzed. Although, freeze-dried and oven-dried lettuce and spinach samples presented higher changes in NO_3^- content. Oven-dried lettuce containers presented a residue of vegetables exudates that was difficult to remove that probably contained NO_3^-, resulting in lower NO_3^- sample content. Freeze-drying seems to be the most appropriate process for sample preservation during long periods, avoiding changes in sample composition due to the low water content.

Table 2.4 – Statistics for NO_3^- content (mg/kg) differences in spinach samples according to the pretreatments procedures.

Sample	Fresh	Freeze-dried	Oven-dried	Frozen	F test
Mean	1284^a	1118^b	806^c	1272^a	49.3
Std dv.	19.9	37.1	71.7	18.1	
Std err.	9.9	18.6	35.8	10.4	

Letters $^{a-c}$ indicate significant differences at $p < 0.05$ in the Duncan test.

Extraction variables were held constant (extraction temperature 80 ºC and extraction time 20 min) except for sample size, that was 0.25 g for fresh and frozen samples and 0.025 g for freeze-dried and oven dried samples, in order for the concentration of NO_3^- in the extract to fall within the linear range of the calibration curve.

Table 2.5 – Statistics for NO_3^- content (mg/kg) differences in lettuce samples according to the pretreatments procedures.

Sample	Fresh	Freeze-dried	Oven-dried	Frozen	F test
Mean	973[a]	1706[b]	1337[c]	1043[a]	69.1
Std dv.	36.5	49.9	58.4	14.4	
Std err.	21.1	22.3	26.1	22.7	

Letters [a – c] indicate significant differences at $p < 0.05$ in the Duncan test.
Extraction variables were held constant (extraction temperature 80 ºC and extraction time 20 min) except for sample size, that was 0.25 g for fresh and frozen samples and 0.025 g for freeze-dried and oven dried samples, in order for the concentration of NO_3^- in the extract to fall within the linear range of the calibration curve.

NO_2^- was not detected in either fresh spinach or lettuce. NO_2^- was also not detected after samples pretreatment (freeze-dried, oven-dried and frozen samples). This is not surprising because it has been shown that NO_2^- content in fresh vegetable tissues is typically very low and, under frozen storage, the production of NO_2^- is inhibited (565). Our results indicate that freeze-drying and oven-drying also inhibited NO_2^- production.

2.4. Conclusions

Activated charcoal showed to be efficient in the removal of interferences from vegetable matrices in the chromatographic determination of NO_3^- and NO_2^-. The results for each lot of fresh, freeze-dried, oven-dried and frozen samples (lettuce and spinach) indicate the need to standardize appropriate pretreatment procedures. For long storage periods, freeze-drying seems to be the most suitable pretreatment because a large number of samples can be lyophilized, the sample size is reduced and a homogenous sample is obtained. Oven-drying of vegetables material was inappropriate since losses of NO_3^- occur during sample preparation. In the samples analyzed (lettuce and spinach), NO_3^- content was always below the maximum levels set by the European Commission. NO_2^- was not detected in fresh, freeze-dried, oven-dried and frozen samples.

Chapter 3

Searching for the best soil extraction method to predict Cr, V, Ni, As, Pb, Co, Cd, Sb and U content in lettuce (*Lactuca sativa*) and to understand the soil-plant relationship regarding metal(loid)s

Abstract

The influence of soil properties in the phytoavailability of metal(loid)s in a soil-plant system was evaluated. The extractable metal(loid)s content obtained by using different extraction methods was also compared. To perform this study a test plant (*Lactuca sativa*) was grown for 10 weeks in three different soils. Lettuce and rhizosphere soil were sampled at 5 different time points (2, 4, 6, 8 and 10 weeks). Four extraction methods (Mehlich 3, DTPA, NH_4NO_3 and $CaCl_2$) were tested. Significant positive correlations between the soil extractable content and lettuce content were obtained for Cr, V, Ni, As, Pb, Co, Cd, and Sb. Mehlich 3 method resulted in the highest extraction of the studied metal(loid)s. However, the extraction procedure using NH_4NO_3 showed a higher number of strong positive correlations indicating that this method is more appropriate to estimate metal(loid)s phytoavailability and predict plant metal(loid)s content. CEC, OM, pH, texture and oxides content of the soil also showed to influence the distribution of metal(loid)s between the phytoavailable and non-phytoavailable fractions. A reliable prediction model for Cr, V, Ni, As, Pb, Co, Cd, and Sb availability to lettuce was obtained considering the amount of metal(loid) extracted by the NH_4NO_3 method and the main soil properties. This work shows that studies involving the soil-plant system should consider both the soil and plant sampling strategies as key factors to reliably estimate metal(loid)s phytoavailability and to predict lettuce metal(loid)s content.

3.1. Introduction

Metals and metalloids, from now on referred to as metal(loid)s, are ubiquitous components of the lithosphere. Metal(loid)s are distributed in both the solid and aqueous phases of soil and may be involved in a panoply of interactions with other soil components ranging from weak electrostatic sorption to irreversible binding (17). Moreover, terrestrial ecosystems are very complex and heterogeneous systems, where geogenic inputs (e.g. weathering and leaching processes), and anthropogenic activities are part of the complex and interrelated biogeochemical cycles that take place in nature (18). Thus, it is widely recognized that an “universal” approach capable of mimicking the fate of metal(loid)s in soils, and to estimate their bioavailability and uptake by plants is of utmost importance.

According to (575), “environmental bioavailability” is defined as the “fraction of the environmentally available compound which an organism takes up through physiologically driven processes”. This definition includes both the actual available fraction (i.e., the sum of dissolved free ions/molecules plus dissolved complexes) and the potentially available fraction (i.e., ions/molecules that could be released from soil-plant processes such as root exudation). When this definition is specifically applied to plants uptake, it is commonly

referred as “phytoavailability”. Hence, phytoavailability refers to the process of plant uptake of a certain chemical (576).

The use of a single well defined extraction procedure is one of the approaches used to assess the mobile fraction and, thus, the phytoavailability of metal(loid)s in soils. Several extractants are available and can be classified according to the intrinsic mechanism involved in the release of elements from soil. Seven “phases” can be derived as follows: (1) water-soluble; (2) exchangeable; (3) organically-bound; (4) carbonate-bound; (5) Mn and Fe oxide-bound; (6) residual (non-silicate bound); and (7) total (19). The other approach, sequential extraction, relies on the use of a series of extractants, with an increasing extraction “power” to separate metal(loid)s bounded to the different soil phases. The classic procedure starts with the extraction of the metal(loid)s already present in the soil solution and those weakly attached to cation-exchange sites in the soil. Afterwards, a stepwise attack is performed to the organically-bound, to the carbonate-bound and to the Fe and Mn (hydr)oxides-bound metal(loid)s fractions. Lastly, more refractory soil components can be solubilized by using strong acids (151).

In the last years, single and sequential extraction methods have being widely applied to study particular solid-phase associations of metal(loid)s in soils (151-153, 163, 576-578). The data generated in those studies are particularly useful for understanding the physicochemical processes since allows the elucidation of the mechanisms involved in metal(loid)s binding, transformation and/or release from soil. However, none of these extraction procedures proved to be suitable to accurately predict the phytoavailability of metal(loid)s (17). Nowadays, the major challenge in field studies is how to correlate the data on metal(loid)s uptake and accumulation by plant with the results from single or sequential extraction assays. A critical evaluation of reported data regarding this topic may help to estimate the actual or potential environmental exposure of plants to metal(loid)s based on the available extraction methods (152, 153, 577, 579). However, some aspects are still less understood, limiting the interpretation of these studies.

As mentioned before, the actual and potential phytoavailable fractions of metal(loid)s can be considered as the fraction of the total amount of an element present in a specific environmental compartment that, within a defined time period, is either available or can be made available for uptake by plants (19). Therefore, the plant metal(loid)s content should be a direct reflex of the of metal(loid)s phytoavailability in the rhizosphere zone. Several factors that are known to control the phytoavailability of metal(loid)s in the soil are of great importance regarding their soil–plant–human transfer and accumulation (580). Soil properties such as pH, cation exchange capacity (CEC), organic matter (OM) content, particle size distribution and the oxides content are generally regarded as the main parameters that control the distribution of metal(loid)s between the phytoavailable and

non-phytoavailable fractions (71, 81, 581, 582). Moreover, the phytoavailability of soil metal(loid)s is also the result of the root–rhizosphere interactions. The reactions that take place in this interface strongly determine the metal(loid)s speciation and uptake by plants, ultimately affecting the accumulation and overall content of these elements in plants. Plant roots exudates as well as products of the plant-associated bacteria metabolism are involved in many processes operating in the rhizosphere, such as water uptake, nutrient mobilization and rhizosphere-associated OM decomposition (100, 105).

The uptake and accumulation of metal(loid)s by plants is usually assessed by evaluating the total content of these elements in plant tissues (479). In this context, studies on plants that are widely consumed by humans in their diet are seen are of major importance in order to assess the potential transfer of toxic metal(loid)s to the food chain. Lettuce (*Lactuca sativa*) is one of the most consumed vegetables worldwide, representing about 6.5% of the total dietary intake of vegetables by humans (583). Besides its recognized importance in human nutrition, lettuce is recommended by the US Environmental Protection Agency (EPA) as a suitable plant species to determine plant uptake and translocation of toxic substances (584).

Based on the above background, and in order to contribute for a better understanding of the soil-plant relationship regarding metal(loid)s, the focus of this study was to assess the influence of soil properties on the availability of metal(loid)s to lettuce during its normal growth period and the differences in phytoavailability results obtained by using different extraction methods. To accomplish this, a three-step approach was used: (1) the physicochemical properties of three different soils and the soil metal(loid)s extractable content (as assessed by four different extraction methods) was determined; (2) the correlation between the metal(loid)s extractable content in the soil and the plant content at five time points of lettuce growth was studied; and (3) multiple regression analysis was used to identify the most suitable extraction method to estimate metal(loid)s phytoavailability to lettuce and to predict lettuce metal(loid)s content.

3.2. Experimental

3.2.1. Plant and soil sampling

Lettuce (*Lactuca sativa L. var. capitata*) plants (n = 100) with 2 weeks of seed growth were transferred on the same day to three different greenhouse experimental fields : A_1 (41º 26.991 N, 8º 46.335 W), A_2 (41º 25.249 N, 8º 44.936 W) and A_3 (41º 27.435 N, 8º 45.377 W) located in the NW Portugal, in a region between Esposende and Vila do Conde that has an intensive production of fresh plant foods to supply the region North of Portugal. Ten units (or 1 kg) of plants were arbitrarily harvested at five time points (2, 4, 6,

8, and 10 weeks, hereafter referred as T_1, T_2, T_3, T_4 and T_5, respectively) during plant growth according to (585). Simultaneously, soil from the three experimental fields was also sampled from the same locations where plants were harvested. In order to generate a representative sample of rhizosphere soil, single soil samples (n = 25) were combined to generate a composite sample (> 1 kg). Plant and soil samples were stored in plastic containers, previous rinsed with diluted nitric acid (10% v/v) and deionized water, and maintained at 4 ºC on the way to the laboratory.

For physicochemical analysis, plant samples were brushed, thoroughly washed with deionized water, frozen at –80 ºC and then freeze-dried. The dried samples were homogenized by grinding in a blender and sieved through a nylon sieve of 150 µm mesh size.

Pre-treatment of soil samples was performed according to official methods (586). Briefly, soil samples were spread in plastic trays in a layer not thicker than 15 mm and oven-dried during 24h at 40 ºC. Thereafter, samples were crushed and sieved through a 2 mm nylon sieve and stored at 4 ºC until analysis.

3.2.2. Reagents and apparatus

Decontaminated polypropylene laboratory ware, pipette tips (VWR, Radnor, PA), volumetric flasks (Kartell, Milan, Italy) and centrifuge tubes (TRP, Trasadingen, Switzerland) were used to perform the study. Calibration solutions were prepared from AccuTrace™ 10 µg/mL multi-element ICP-MS standard solutions (AccuStandard®, New Haven, CT). High purity HNO_3 (65% w/w, *Trace*SELECT® Ultra, Fluka, L'Isle d'Abeau Chesnes, France) and H_2O_2 (30% v/v, *Trace*SELECT® Fluka, Seelze, Germany) were used as received. All solutions were prepared using ultrapure water (≥18.2 MΩ cm at 25 °C) obtained with a Milli-Q (Milipore, Billerica, MA) water purification system.

A Telstar (Terrassa, Spain) Cryodos-80 freeze-dry system was used to lyophilize the lettuce samples. A Milestone (Sorisole, Italy) MLS 1200 Mega high performance microwave digestion unit equipped with an HPR-1000/10 S rotor was used for total digestion of plant samples and pseudo-total digestion of soil samples. ICP–MS analysis was performed using a VG Elemental (Winsford, UK), PlasmaQuad 3 (quadrupole-based) instrument, equipped with a glass concentric nebulizer (Meinhard® Type A), a water-cooled glass spray chamber with impact-bead, a standard quartz torch and nickel skimmer and sampling cones. For sample introduction, a Minipuls 3 (Gilson, Villiers le Bel, France) peristaltic pump was used. Argon of 99.9% purity (Alphagaz 2™, supplied by Air Liquide, Maia, Portugal) was used as the plasma source. The ICP-MS analysis was carried out under the following instrumental conditions: argon flow rate (13 L/min); auxiliary argon flow rate (0.7 L/min); nebulizer flow rate (0.8 L/min); RF power (1350 W);

scan regions dwell time (200 ms); detection mode (pulse counting). The elemental isotopes (m/z ratios) ^{27}Al, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{75}As, ^{111}Cd, ^{121}Sb, ^{208}Pb, and ^{238}U were monitored for analytical determination and ^{45}Sc, ^{89}Y, ^{115}In, ^{159}Tb and ^{209}Bi were used as internal standards. The instrument was tuned daily for maximum signal sensitivity and stability using ^{115}In as the target isotope.

Certified reference materials (CRM) ISE 918 (sandy soil, supplied by WEPAL, Wageningen, The Netherlands) and BCR 679 (white cabbage, supplied by EC Institute for Reference Materials and Measurements, Geel, Belgium) were used to check the accuracy of the analytical procedures.

3.2.3. Soil samples analysis

The physicochemical soil properties pH, EC, salinity, OM content, CEC, particle size distribution and oxides as well as the total and extractable fractions of meta(loid)s of each composite sample of rhizosphere soil were determined according to official methods of soil analysis. Soil pH was measured in the supernatant of a 1:5 (w/v) suspension made up with a 0.01 M calcium chloride solution. OM content was estimated based on the oxidation of organic matter with potassium dichromate (0.27 M) and sulfuric acid (98% v/v) mixture at 135 °C. CEC was determined according to ISO (587). Particle size distribution was determined according to ISO (588). Total element contents were determined after microwave assisted digestion according to USEPA (589). The extractable fractions of the elements were determined using the following extraction procedures: (1) calcium chloride method [8]; (2) ammonium nitrate method (590); (3) DTPA method (591); and (4) Mehlich 3 method (159). Each composite sample of rhizosphere soil as well as ISE 918 soil reference material was analyzed in triplicates.

3.2.4. Plant samples analysis

A microwave-assisted digestion procedure was performed to obtain the total metal(loid)s content of plants. Samples (500 mg) were directly weighed into the microwave oven PTFE vessels and 5 ml of 65% (v/v) HNO_3 and 2 ml of 30% (v/v) H_2O_2 were added to each vessel. Then, the mixture was submitted to a microwave heating program as follows: 250W for 1 min, 0W for 2 min, 250W for 5 min, 400W for 5 min, and 600 W for 5 min. After digestion, samples were transferred to 25 ml volumetric flasks and the volume was made-up with ultrapure water. Digestion solutions were analyzed in triplicate by ICP-MS. Results were expressed in a fresh weight (fw) basis.

3.2.5. Analytical quality control

Certified reference materials were analyzed by the above-mentioned methods to ensure the quality of the analytical data. For soil, ISE 918 CRM was used and obtained concentrations were in good agreement with certified values: 3.52 ± 0.09 µg/g for As (certified 3.65 ± 0.48 µg/g), 0.25 ± 0.05 µg/g for Cd (certified 0.25 ± 0.03 µg/g), 26.2 ± 1.6 µg/g for Cr (certified 25.3 ± 3.0 µg/g), 7.29 ± 0.24 µg/g for Ni (certified 7.65 ± 0.70 µg/g), 22.0 ± 0.9 µg/g for Pb (certified 21.6 ± 1.2 µg/g), 0.33 ± 0.06 µg/g for Sb (certified 0.30 ± 0.06 µg/g) and 19.2 ± 0.4 µg/g for V (certified 19.6 ± 2.2 µg/g). For plants, a BCR 679 CRM was used and obtained concentrations were: 6.9 ± 0.05 ng/g for As (certified 7.0 ± 0.3 ng/g), 1.69 ± 0.02 µg/g for Cd (certified 1.66 ± 0.07 µg/g), 0.65 ± 0.03 µg/g for Cr (certified 0.6 ± 0.1 µg/g), 26.5 ± 0.1 µg/g for Ni (certified 27.0 ± 0.8 µg/g) and 19.7 ± 1.5 ng/g for Sb (certified 20.6 ± 2.6 ng/g).

3.2.6. Statistical analysis

Data exploration, descriptive statistics calculation, ANOVA, correlation matrix, principal component analysis (PCA) and multiple regression analysis were performed with SPSS for Windows version 22 (SPSS, Chicago, IL). Statistical differences were assumed at $p < 0.05$, unless otherwise noted.

3.3. Results

3.3.1. Soil properties and extraction method efficiency

The main physicochemical properties (pH, CEC, OM, EC, salinity, oxides and particle size distribution) of the three studied soils are shown in Table 3.1. No significant differences were observed for each parameter along the study period (T_1, T_2, T_3, T_4 and T_5). Thus, results in Table 3.1 are the mean and standard deviation of the five individual results obtained. Significant differences between the three soils existed. A_2 soil showed a significantly lower pH and the highest values for CEC, OM, EC, salinity, oxides, clay and silt content. A_3 soil showed the lowest values for OM, EC and Mn oxides. According to their particle size distribution, A_1 and A_3 soils were classified as sandy soils while A_2 soil was a loamy sand soil. The total mean content as well as the Mehlich 3, DTPA, NH_4NO_3 and $CaCl_2$ extractable fractions of metal(loid)s from A_1, A_2 and A_3 soils during the studied period are shown in Table 3.2.

Table 3.1 – Main physicochemical properties of the soils from the three experimental fields (A_1, A_2 and A_3).

	A_1	A_2	A_3
pH	6.8 ± 0.1a	6.7 ± 0.2b	6.8 ± 0.2a
CEC (cmol/kg)	7.9 ± 0.4a	17.2 ± 0.6b	7.9 ± 0.5a
OM (%)	56.3 ± 3.9a	72.8 ± 2.3b	20.7 ± 1.6c
EC (µS/cm)	283 ± 40a	633 ± 69b	196 ± 27c
Salinity (ng/L)	0.13 ± 0.01a	0.30 ± 0.03b	0.09 ± 0.01a
Al oxides (mg/g)	1.11 ± 0.07a	1.58 ± 0.09b	1.12 ± 0.08a
Mn oxides (mg/g)	0.23 ± 0.01a	0.40 ± 0.02b	0.20 ± 0.01c
Fe oxides (mg/g)	2.20 ± 0.10a	4.77 ± 0.17b	2.16 ± 0.11a
Clay (%)	2.0 ± 0.2a	12.2 ± 0.5b	2.2 ± 0.7a
Silt (%)	2.5 ± 0.2a	12.3 ± 0.1b	2.0 ± 0.5a
Sand (%)	95.5 ± 2.9a	75.5 ± 1.8b	95.8 ± 4.7a
Soil classification	Sandy soil	Loamy sand soil	Sandy soil

Data presented as mean ± SD (n = 5 time points during the study period). Differences were tested according to ANOVA followed by Tukey's test. In a line, different letters (a, b and c) indicate significant differences ($p < 0.05$) between the experimental fields.

Table 3.2 – Total and Mehlich 3, DTPA, NH_4NO_3 and $CaCl_2$ extractable content of metal(loid)s in the studied soils (A_1, A_2, A_3).

	Soil ID	Total content	Mehlich 3	DTPA	NH_4NO_3	$CaCl_2$
Cr (µg/g)	A_1	28.2 ± 1.3a	0.13 ± 0.02a	0.028 ± 0.004a	0.013 ± 0.002a	0.011 ± 0.002a
	A_2	18.6 ± 1.5b	0.11 ± 0.03b	0.038 ± 0.009b	0.011 ± 0.004a	0.011 ± 0.004a
	A_3	22.1 ± 1.8c	0.11 ± 0.01b	0.022 ± 0.003c	0.011 ± 0.002a	0.011 ± 0.002a
V (µg/g)	A_1	23.2 ± 1.0a	0.15 ± 0.01a	0.045 ± 0.002a	0.013 ± 0.004a	0.011 ± 0.002a
	A_2	29.3 ± 0.9b	0.89 ± 0.13b	0.078 ± 0.006b	0.024 ± 0.012b	0.018 ± 0.007b
	A_3	24.7 ± 0.8c	0.32 ± 0.01c	0.078 ± 0.008b	0.009 ± 0.001a	0.009 ± 0.001a
Ni (µg/g)	A_1	19.1 ± 0.7a	0.25 ± 0.02a	0.112 ± 0.015a	0.045 ± 0.010a	0.043 ± 0.009a
	A_2	8.47 ± 0.18b	0.25 ± 0.01a	0.112 ± 0.009a	0.047 ± 0.003a	0.044 ± 0.003a
	A_3	15.6 ± 0.8c	0.26 ± 0.02b	0.167 ± 0007b	0.039 ± 0.005b	0.039 ± 0.005b
As (µg/g)	A_1	7.05 ± 0.25a	0.20 ± 0.05a	0.080 ± 0.008a	0.037 ± 0015a	0.036 ± 0.017a
	A_2	6.82 ± 0.16a	0.45 ± 0.06b	0.128 ± 0.042b	0.059 ± 0.018b	0.057 ± 0.018b
	A_3	18.4 ± 1.0b	0.53 ± 0.14c	0.112 ± 0.006c	0.072 ± 0.034b	0.069 ± 0.032b
Pb (µg/g)	A_1	17.0 ± 0.7a	0.89 ± 0.05a	0.29 ± 0.04a	0.0010 ± 0.0005a	0.0009 ± 0.0005a
	A_2	24.2 ± 0.9b	4.57 ± 0.29b	1.73 ± 0.14b	0.0015 ± 0.0007b	0.0013 ± 0.0006b
	A_3	18.9 ±0.7c	1.60 ± 0.11c	0.28 ± 0.01a	0.0009 ± 0.0005a	0.0008 ± 0.0004a
Co (ng/g)	A_1	4541 ± 243a	90.1 ± 2.4a	15.3 ± 1.5a	6.20 ± 1.31a	6.11 ± 1.35a
	A_2	2324 ± 63b	60.1 ± 2.1b	18.8 ± 1.2b	7.36 ± 0.96b	7.15 ± 0.89b
	A_3	6063 ± 252c	155.2 ± 9.2c	17.9 ± 1.6c	6.51 ± 2.38a	6.31 ± 1.50a
U (ng/g)	A_1	3310 ± 223a	55.9 ± 4.5a	11.2 ± 1.2a	1.32 ± 0.13a	1.16 ± 0.17a
	A_2	3983 ± 211b	60.6 ± 3.6b	12.5 ± 0.1b	2.53 ± 0.12b	2.37 ± 0.16b
	A_3	4463 ± 252c	58.9 ± 3.9b	10.4 ± 0.1a	0.79 ± 0.08c	0.85 ± 0.07c
Cd (ng/g)	A_1	1124 ± 91a	19.2 ± 9.1a	11.3 ± 3.9a	1.03 ± 0.55a	0.97 ± 0.48a
	A_2	685 ± 82b	34.7 ± 7.6b	18.9 ± 7.6b	0.85 ± 0.25a	0.92 ± 0.36a
	A_3	2219 ± 119c	75.2 ± 21.1c	32.6 ± 8.3c	1.31 ± 0.72b	1.33 ± 0.62b
Sb (ng/g)	A_1	429 ± 4a	13.1 ± 3.7a	7.87 ± 0.28a	1.93 ± 0.34a	1.61 ± 0.25a
	A_2	256 ± 4b	12.8 ± 3.9a	8.69 ± 0.59b	2.13 ± 0.55a	1.73 ± 0.22a
	A_3	520 ± 6c	21.8 ± 4.9b	7.61 ± 0.20c	1.54 ± 0.40b	1.26 ± 0.17b

Data presented as mean ± SD (n = 5 time points during the study period). Differences were tested according to ANOVA followed by Tukey's test. In a column, different letters (a, b and c) for each element indicate significant differences ($p < 0.05$) between soils.

A PCA was performed using the total content of the 9 studied metal(loid)s and the 12 soil physicochemical properties (pH, CEC, OM, EC, salinity, coarse sand, fine sand, silt, clay, Al-oxides, Mn-oxides and Fe-oxides) as variables. Only factors showing an eigenvalue > 1 were retained. Three principal components (PC) were extracted, which accounted for a total variance of 96.3%. The score plot is presented in Figure 3.1a. PC1 (which explains 68.5% of the total variance) was positively loaded by V, Pb, CEC, OM, EC, salinity, silt, clay and oxides and negatively loaded by Cr, Co, Ni, Cd and Sb. PC2 (which explains 22.8% of the total variance) was positively loaded by As and U and negatively loaded by coarse and fine sand. PC3 (4.9% of the total variance) was positively loaded only by pH (Figure 3.1b). The three soils displayed very distinct characteristics, which resulted in three well separated groups in the PCA score plot. The temporal variation of the selected parameters is shown by different colors of the same symbol (Figure 3.1a).

Figure 3.2 shows the extractable fraction (%) of the studied metal(loid)s obtained by the different extraction methods (Mehlich 3, DTPA, NH_4NO_3 and $CaCl_2$) for the three soils (A_1, A_2 and A_3) along the study period. For all the studied elements, the highest percentage of extractable metal(loid)s was obtained with Mehlich 3 and the lowest with $CaCl_2$. Several extraction ranges were defined for each extraction method in order to clearly understand its performance. For Mehlich 3, three ranges (0.1-1, 1-10, > 10 %) were defined. The first one (0.1-1 %) included Cr and V (A_1 soil); the second (1-10 %) included V (A_2 and A_3 soils), Ni, As, Pb (A_1 and A_3 soils), Co, U, Cd and Sb; and the third (> 10 %) only included Pb (A_2 soil). Regarding DTPA extraction, only two ranges were created (0.1-1 and 1-10 %). The first included Cr, V, Ni (A_1 soil), As (A_1 and A_3 soils), Co and U; and the second included Ni (A_2 and A_3 soils), Pb, Cd and Sb. For NH_4NO_3 and $CaCl_2$ extraction methods, three groups were created (0.001-0.01, 0.01-0.1 and 0.1-1 %). The first one (0.001-0.01) included Pb; the second included Cr, V, U and Cd (A_1 and A_3 soils); and the third included Ni, As, Cd (A_2 soil) and Sb.

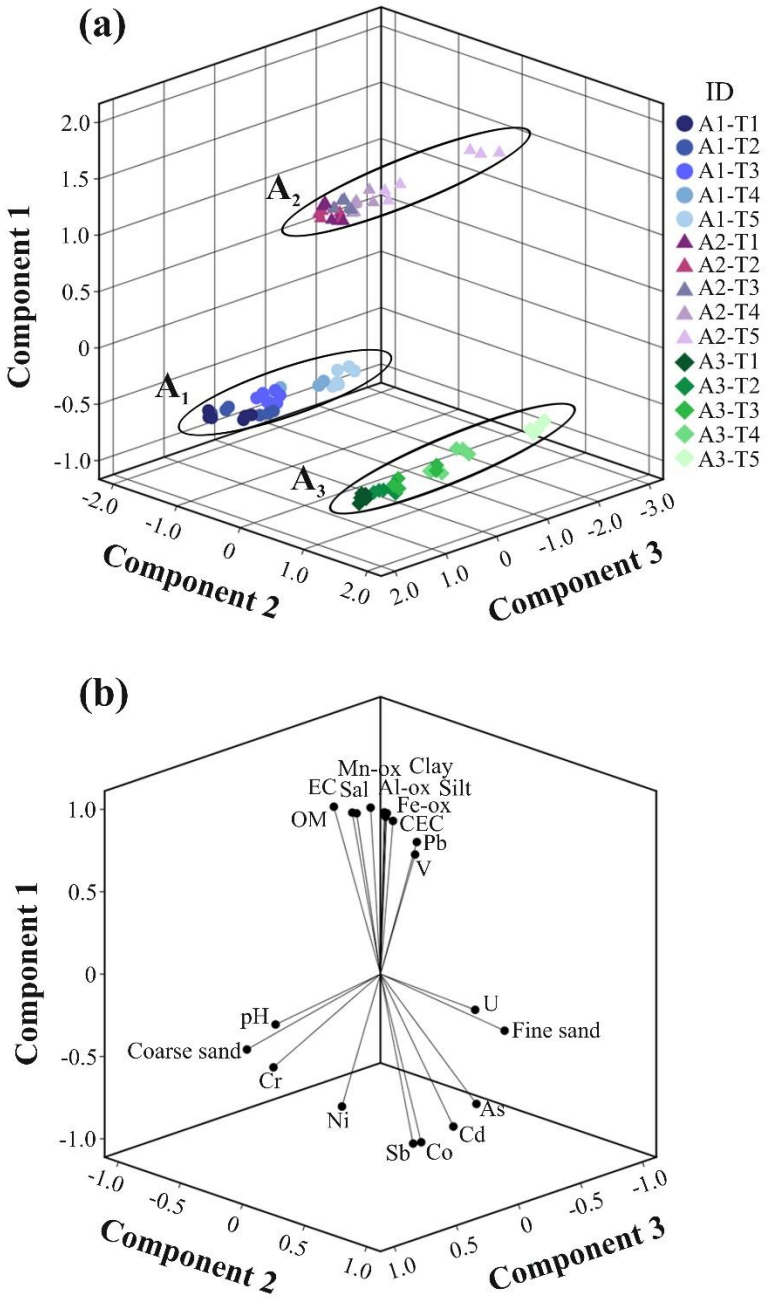


Figure 3.1 – Principal component analysis of the studied soil parameters: total metal(loid)s content and main physicochemical properties (pH, CEC, OM, EC, salinity, coarse sand, fine sand, silt, clay, Al-oxides, Mn-oxides and Fe-oxides). (a) Three dimensional score plot displaying the distribution of A_1, A_2 and A_3 soils. Different colors of the same symbol represent the 5 study time points (T_1, T_2, T_3, T_4 and T_5). (b) Three dimensional loading plot showing the variables with a loading on PC1, PC2 and PC3 higher than 0.5 and the direction of the values increasing.

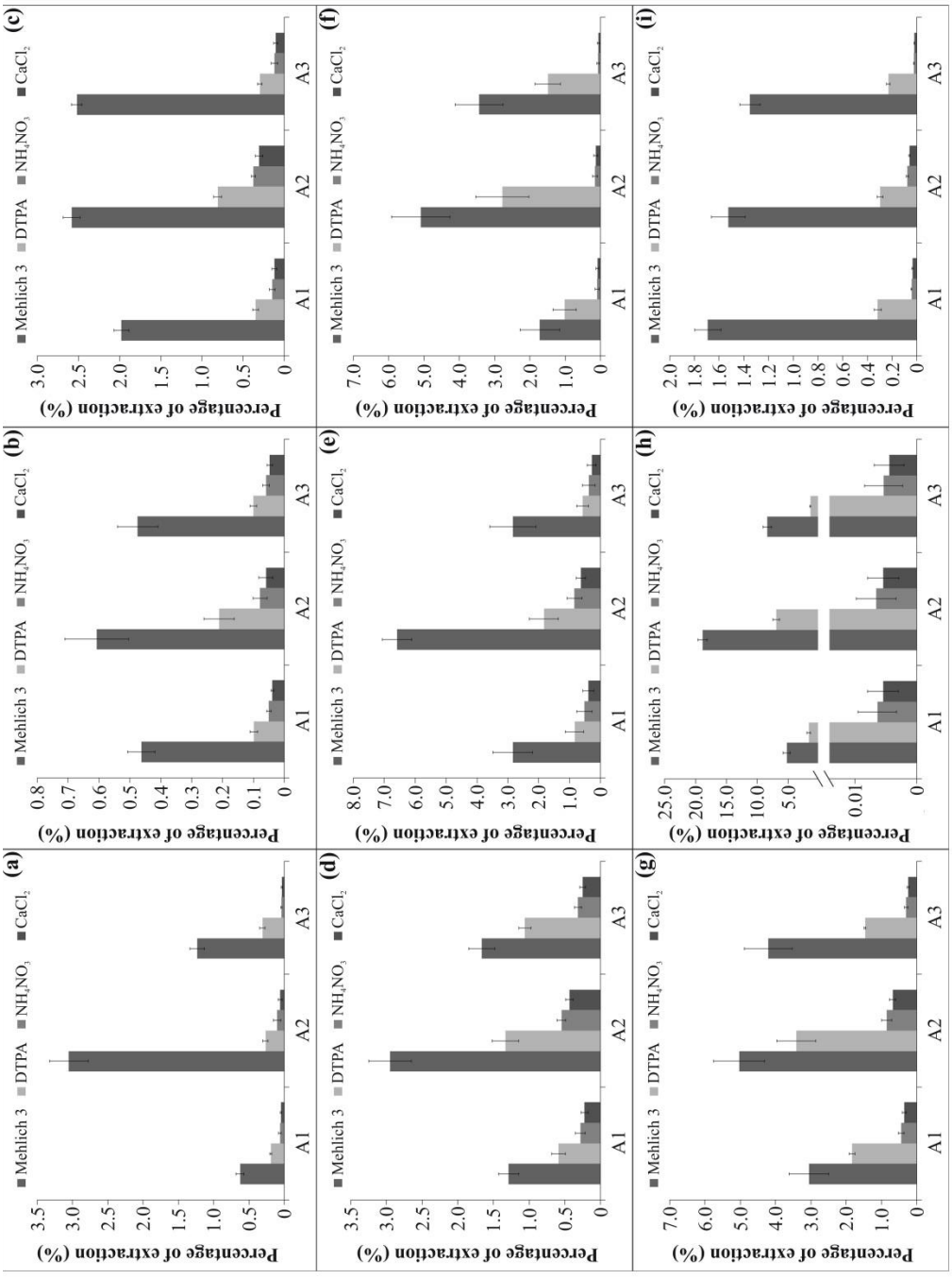


Figure 3.2 – Comparison between the metal(loid)s extractable fraction (%) obtained with the Mehlich 3, DTPA, NH_4NO_3 and $CaCl_2$ extraction methods in the three studied soils (A_1, A_2 and A_3). . (a) V; (b) Cr; (c) Co; (d) Ni; (e) As; (f) Cd; (g) Sb; (h) Pb; and (i) U.

3.3.2. Plant metal(loid)s content

The metal(loid)s content of the lettuces grown in the three soils (A_1, A_2 and A_3) at the different time points (T_1, T_2, T_3, T_4 and T_5) is presented in Table 3.3. Overall, a decrease in the metal(loid)s content was observed along the study period for A_1 and A_3 lettuces. V in A_1 lettuces was the only exception. For A_2 lettuces, different patterns of temporal variation were observed depending on the metal(loid). Cr, V, Pb and U contents increased along the study period whereas Co, Cd and Sb decreased. No significant differences were observed between time points regarding Cr, As and Cd content in A_1, A_2 and A_3 lettuces. A_1 lettuces showed a significantly higher mean content of Ni, Sb and U, A_2 lettuces presented a significantly higher mean content of V and Pb (and a lower mean content of Co) while A_3 lettuces had a higher mean content of Co (and lower mean content of Ni).

Table 3.3 – Metal(loid)s content (ng/g) in lettuces grown in the 3 different soils (A_1, A_2, A_3) at the 5 study time points (T_1, T_2, T_3, T_4 and T_5).

	Soil ID	Sampling time point					
		T_1	T_2	T_3	T_4	T_5	
Cr	A_1	118.3 ± 4.6a*	106.8 ± 0.8b*	93.9 ± 3.3c*	93.6 ± 1.8c*	81.8 ± 2.0d*	95.9 ± 15.6*
	A_2	63.7 ± 1.2a**	67.8 ± 3.6a**	75.1 ± 2.4b**	178.1 ± 7.3c**	227.9 ± 1.6d**	122.5 ± 69.2*
	A_3	150.4 ± 2.1a***	139.4 ± 4.7b***	124.8 ± 1.9c***	102.5 ± 3.7d***	83.4 ± 2.6e*	120.1 ± 25.1*
V	A_1	14.7 ± 1.0a*	19.3 ± 1.4b*	20.8 ± 1.3b*	31.9 ± 2.0c*	33.1 ± 2.1c*	23.9 ± 7.6*
	A_2	17.3 ± 0.9a*	20.8 ± 1.8b*	65.7 ± 4.5c**	77.3 ± 2.2d**	73.1 ± 2.2e**	50.7 ± 26.8**
	A_3	37.6 ± 1.1a**	35.7 ± 2.4a**	39.0 ± 1.9a***	32.0 ± 1.8b*	24.4 ± 2.0c***	34.0 ± 5.9*
Co	A_1	7.33 ± 0.49a*	6.15 ± 0.04b*	4.61 ± 0.13c*	4.53 ± 0.12c*	4.45 ± 0.09c*	5.40 ± 1.21*
	A_2	4.72 ± 0.32a**	4.33 ± 0.08b**	4.21 ± 0.21b**	4.05 ± 0.17b**	3.98 ± 0.03b**	4.27 ± 0.40*
	A_3	13.9 ± 1.1a***	12.2 ± 0.2b***	8.38 ± 0.36c***	8.24 ± 0.22c***	5.84 ± 0.13d***	9.71 ± 3.02**
Ni	A_1	131.1 ± 3.3a*	119.6 ± 2.3b*	86.4 ± 1.9c*	77.9 ± 4.3d*	74.4 ± 2.4d*	97.9 ± 23.7*
	A_2	44.1 ± 1.3a**	39.5 ± 0.6b**	48.1 ± 0.9c**	46.9 ± 1.8c**	47.5 ± 1.7c**	45.2 ±3.5**
	A_3	35.9 ± 2.4a***	35.2 ± 2.0a***	31.7 ± 1.9b***	22.9 ± 1.5c***	18.8 ± 1.3d***	29.1 ± 7.4***
As	A_1	31.9 ± 2.4a*	21.2 ± 1.7b*	18.5 ± 1.4c*	13.0 ± 1.1d*	11.5 ± 0.6e*	19.2 ± 7.5*
	A_2	19.9 ± 1.5a**	14.9 ± 0.9b**	13.4 ± 1.1b**	16.6 ± 1.1c**	19.2 ± 1.7a**	16.8 ± 2.8*
	A_3	27.2 ± 1.3a***	19.7 ± 0.8b***	19.1 ± 1.1b*	14.7 ± 0.9c*	8.59 ± 1.26d***	17.9 ± 6.3*
Cd	A_1	42.5 ± 2.2a*	25.0 ± 0.6b*	11.9 ± 0.6c*	6.84 ± 0.70d*	5.56 ± 0.51d*	18.4 ± 14.2*
	A_2	37.5 ± 1.4a**	20.3 ± 1.3b**	14.3 ± 1.4c**	9.27 ± 0.59d**	8.62 ± 0.37d**	18.0 ± 10.9*
	A_3	48.5 ± 1.9a***	28.3 ± 1.1b***	16.4 ± 1.1c***	14.2 ± 0.5d***	9.65 ± 0.31e**	23.4 ± 14.3*
Sb	A_1	2.27 ± 0.12a*	2.15 ± 0.20b*	1.49 ± 0.15b*	1.32 ± 0.23c*	1.07 ± 0.13d*	1.66 ± 0.51*
	A_2	1.56 ± 0.09a**	1.39 ± 0.09a**	1.29 ± 0.03b**	1.12 ± 0.09c**	0.48 ± 0.05d**	1.17 ± 0.38**
	A_3	1.33 ± 0.17a***	1.17 ± 0.15b***	1.08 ± 0.09b***	0.83 ± 0.11c***	0.69 ± 0.11d***	1.02 ± 0.26**
Pb	A_1	31.6 ± 1.41a*	14.6 ± 0.15b*	13.2 ± 0.76c*	7.46 ± 0.38d*	6.47 ± 0.37d*	14.7 ± 9.3*
	A_2	10.5 ± 0.26a**	11.7 ± 0.91a**	24.4 ± 0.65b**	33.9 ± 1.55c**	35.6 ± 0.95d**	23.2 ± 10.9**
	A_3	10.7 ± 0.77a**	9.43 ± 0.33b***	5.62 ± 0.33c***	5.14 ± 0.32c***	4.82 ± 0.23c***	7.14 ± 2.52***
U	A_1	17.0 ± 1.31a*	16.0 ± 0.57b*	8.81 ± 0.49c*	5.70 ± 0.45d*	4.06 ± 0.25e*	10.3 ± 5.4*
	A_2	1.68 ± 0.23a**	1.89 ± 0.24b**	2.44 ± 0.14b**	5.11 ± 0.51c*	5.27 ± 0.29c**	3.28 ± 1.64**
	A_3	2.36 ± 0.33a***	1.99 ± 0.17a**	0.98 ± 0.07b***	0.71 ± 0.08b**	0.44 ± 0.05b***	1.29 ± 0.78**

Data presented as mean ± SD (n = 3). For each element, differences were tested according to two-way ANOVA followed by Tukey's test. In a column, different number of stars (*, ** and ***) indicate significant differences ($p < 0.05$) in lettuces grown in different soils. In a line, different letters (a, b, c, d and e) indicate significant differences ($p < 0.05$) between the lettuces from different sampling time points.

3.3.3. Correlation between soil extractable content and plant content

Table 3.4 shows, for the different study time points (T_1, T_2, T_3, T_4 and T_5), the significant positive correlations between the total metal(loid)s content in the lettuce shoots and the extractable fraction in the soils obtained with each of the four extraction methods (Mehlich 3, DTPA, NH_4NO_3 and $CaCl_2$). Eighty one significant positive correlations were obtained. Except for U, all the other metal(loid)s showed several positive correlations. The higher number of correlations was obtained for the NH_4NO_3 extraction method (n = 26) and lower with the DTPA method (n = 13). Considering all the study time points, Cr, V, Pb and Cd showed significant correlations in all the methods used; Ni and As in NH_4NO_3 and $CaCl_2$ methods; Co in Mehlich 3, DTPA and NH_4NO_3 methods and Sb in DTPA, NH_4NO_3 and $CaCl_2$ methods.

Table 3.4 – Pearson correlation coefficients (r) for metal(loid)s content in plant and soils (as obtained by different extraction methods) at the 5 study time points (T_1, T_2, T_3, T_4 and T_5).

	Sampling time point	Extraction method			
		Mehlich 3	DTPA	NH_4NO_3	$CaCl_2$
Cr	T_1	-	-	-	-
	T_2	-	-	0.43**	0.48**
	T_3	0.33*	-	0.77**	0.84**
	T_4	0.60**	-	0.91**	0.91**
	T_5	0.83**	0.98**	0.99**	0.93**
	All	0.55**	0.57**	0.87**	0.84**
V	T_1	-	-	-	-
	T_2	0.59**	-	-	-
	T_3	-	0.64**	0.53**	-
	T_4	0.94**	0.73**	0.93**	0.88**
	T_5	0.97**	0.77**	0.98**	0.93**
	All	0.72**	0.59**	0.92**	0.83**
Ni	T_1	-	-	-	-
	T_2	-	-	0.37*	-
	T_3	-	-	-	-
	T_4	-	-	0.94**	0.86**
	T_5	-	-	0.96**	0.96**
	All	-	-	0.55**	0.51**

As	T_1	-	-	-	-
	T_2	-	-	-	-
	T_3	-	-	-	-
	T_4	0.68**	-	0.69**	0.69**
	T_5	0.65**	0.73**	0.75**	0.68**
	All	-	-	0.65**	0.62**
Pb	T_1	-	-	-	0.58**
	T_2	-	-	0.57**	0.57**
	T_3	-	-	0.92**	0.64**
	T_4	0.82**	0.91**	0.97**	0.96**
	T_5	0.97**	0.99**	0.92**	0.92**
	All	0.53**	0.63**	0.80**	0.79**
Co	T_1	0.68**	-	-	-
	T_2	0.79**	-	-	-
	T_3	0.86**	-	-	-
	T_4	0.97**	-	-	-
	T_5	0.98**	0.98**	0.44**	-
	All	0.79**	0.36*	0.33*	-
Cd	T_1	0.51**	0.78**	0.58**	-
	T_2	0.60**	0.68**	0.87**	0.47**
	T_3	0.91**	0.84**	0.88**	0.71**
	T_4	0.88**	0.91**	0.98**	0.73**
	T_5	0.99**	0.99**	0.98**	0.96**
	All	0.59**	0.65**	0.90**	0.87**
Sb	T_1	-	-	-	-
	T_2	-	-	0.41**	0.47**
	T_3	-	-	-	0.51**
	T_4	-	-	0.55**	0.65**
	T_5	-	0.64**	0.91**	0.81**
	All	-	0.42**	0.67**	0.65**

* Significant correlation ($p < 0.05$) was obtained for $0.3 < r < 0.4$

** Significant correlation ($p < 0.01$) was obtained for $r > 0.4$

3.3.4. Selection of an extraction method to predict the metal(loid)s content in plants

Stepwise multiple regression analyses were performed for Cr, V, Ni, As, Pb, Co, Cd and Sb to find how soil properties (CEC, OM, pH, EC, salinity, oxides, coarse sand, fine sand, silt and clay) and extraction methods (Mehlich 3, DTPA, NH_4NO_3 and $CaCl_2$) were correlated with the metal(loid)s content of lettuces along its growth period. U was excluded from this analysis due to the lack of correlation between lettuces and soils content with any of the extraction methods. For each metal(loid) and extraction method, the relevant variables of the multiple regression, the correlation coefficient (r) and the respective estimation error are shown in Table 3.5. To simplify the table, the regression equations were omitted. Overall, the stepwise multiple regression models obtained included three variables. For Mehlich 3 and DTPA methods, pH and OM were the relevant parameters of the multiple regression since they were the first and/or second variables selected by the stepwise method. For the NH_4NO_3 and $CaCl_2$ extraction methods, the first variable selected in the multiple regression was almost always the extractable metal(loid) content, indicating that this was the most relevant variable. Only for As this was not observed. However, pH, OM, CEC, clay and oxides were also relevant variables in the regressions established for these two extraction methods. For all metal(loid)s in the four extraction methods, r was always above 0.50 ($p < 0.01$). Higher r values and lower estimation errors were obtained with the data from NH_4NO_3 extraction method.

Table 3.5 – Stepwise multiple regression parameters of metal(loid)s content in lettuce as a function of soil properties and extractable metal(loid)s content in soil as assessed by the different extraction methods.

Extraction method		Cr	V	Ni	As	Pb	Co	Cd	Sb
Mehlich 3	Relevant variables	Cr Fe-ox CEC	V Clay OM	pH Ni CEC	pH As	OM	Co OM CEC	OM Cd pH	pH Sb
	r	0.64**	0.90**	0.50*	0.67**	0.63**	0.90**	0.80**	0.76**
	Estimation error (µg/g)	34.1	8.69	29.1	4.38		1.31	8.09	0.31
DTPA	Relevant variables	Cr OM CEC	V OM Clay	Ni CEC OM	pH	Pb CEC OM	OM Co pH	pH Cd OM	pH Sb CEC
	r	0.85**	0.76**	0.88**	0.65**	0.86**	0.92**	0.84**	0.86**
	Estimation error (µg/g)	23.5	12.9	15.9	4.47	6.22	1.18	7.29	0.25
NH_4NO_3	Relevant variables	Cr Clay OM	V OM CEC	Ni CEC OM	pH As	Pb OM CEC	Co OM CEC	Cd Clay Mn-ox	Sb pH CEC
	r	0.90**	0.99**	0.97**	0.74**	0.88**	0.96**	0.92**	0.90**
	Estimation error (µg/g)	19.8	3.47	8.31	3.94	5.02	0.87	5.30	0.21
$CaCl_2$	Relevant variables	Cr Clay	V OM Mn-ox	Ni CEC OM	pH As	Pb OM CEC	Co OM CEC	Cd OM Fe-ox	Sb CEC pH
	r	0.86**	0.91**	0.96**	0.73**	0.87**	0.93**	0.89**	0.89**
	Estimation error (µg/g)	22.3	8.28	9.65	4.00	5.28	1.15	6.12	0.21

* Significant correlation ($p < 0.05$) was obtained for $0.4 < r < 0.5$; ** Significant correlation ($p < 0.01$) was obtained for $r > 0.5$

3.4. Discussion

A_2 soil showed the lowest pH and the highest CEC, OM, clay, silt, salinity and oxides content (Table 3.1). These soil properties justify the higher extractable fraction observed in A_2 soil for all the studied metal(loid)s, as shown in Figure 3.2. Soil CEC and pH are important parameters associated with the sorption and retention capacity of several metals. The soil CEC reflects the dynamic equilibrium of cationic species in the soil solution, thus influencing the metal(loid)s phytoavailable fraction. When soil pH becomes acid, metal(loid)s phytoavailability usually increases due to the replacement of cations on soil binding sites by H^+ ions (581). The OM content is another important factor that influences the mobility of metal(loid)s in soils through the formation of soluble/insoluble metal complexes. Both anionic and cationic metal species are prone to be immobilized in soils with high OM content. Although the soil phytoavailable fraction of metal(loid)s includes both the water-soluble and exchangeable fractions, the OM-bound fraction can also become available to plants (81). Soil texture is also an important property, with a major influence in the distribution of metal(loid)s. Soil finer particles fraction, such as clay, have more ion binding sites, and, proportionally, higher metal loadings (71). Soil salinity also has a significant influence in the mobility of metal(loid)s in soil, with metal mobility increasing with soil salinity (592). Metal oxides, particularly Al-, Fe-, and Mn-oxides, are common constituents of soils and also have an important role in metal(loid)s mobility. Due to their high specific surface area, oxide particles may be involved in cation exchange and redox reactions that could influence the distribution of metal(loid)s in soil. Therefore, soil with high content of these oxides present a high scavenging capacity and can accumulate substantial amounts of metal(loid)s (582).

Independently of the soil, the highest and the lowest amount of metal(loid)s were extracted by the Mehlich 3 and $CaCl_2$ methods, respectively (Table 3.2). This is more easily observed in Figure 3.2 where the extractable fraction of each meal(loid) for each extraction method is displayed. Mehlich 3 extraction method uses a combination of acids (acetic acid and nitric acid), salts (NH_4F and NH_4NO_3), and the chelating agent EDTA. This mixture has been considered a “universal” soil extractant due to its ability to extract several elements simultaneously (159). Since this method relies on the use of a combination of acids, salts and a chelating agent, it is expectable that the amount of a metal(loid)s extracted will be significantly higher when compared to methods that just use salts (NH_4NO_3 and $CaCl_2$ methods) or a combination of salts and chelating agents (DTPA method). According to Rao, Sahuquillo (19), diluted salt solutions such as NH_4NO_3 and $CaCl_2$ are only able to extract elements from the water-soluble and exchangeable phases. The DTPA extractant additionally attacks organically-bound elements, and the Mehlich 3

extractant is able to attack even the carbonate-bound and the Mn and Fe oxide-bound elements.

A Pearson correlation was performed in order to verify which extractable content (as obtained by the different extraction methods) was better related with the metal(loid)s content of lettuces (Table 3.4). Except for U, a strong relationship was found between the metal(loid)s content in soils and plants growth in those soils. The extraction with NH_4NO_3 resulted in the higher number of significant correlations (n = 26), followed by the extraction with $CaCl_2$ (n = 23). Moreover, the NH_4NO_3 extraction method produced correlations with higher r values for almost all the studied metal(loid)s. Therefore, it seems to be the most suitable for mimicking metal(loid)s uptake by plants.

The $CaCl_2$ method is widely used in plant uptake studies (576, 593). Since the $CaCl_2$ solution has little effect in the total electrolyte concentration of soil solutions, the amount of extracted metal(loid)s is mainly a reflex of its binding strength (578). However, this method has some drawbacks. Chloride forms stable complexes with several metal(loid)s and may promote the leaching of water-soluble and exchangeable phases, leading to incorrect results (163, 594). Nitrate salts are therefore more advantageous because only ion-exchange processes are involved in the metal(loid)s extraction (576).

The other two extraction methods (Mehlich 3 and DTPA) also resulted in a high number of significant correlations (20 and 14, respectively), however, besides being fewer in number, correlations were also weaker than those obtained by the NH_4NO_3 extraction method. Generally, the DTPA extraction is used in studies on physicochemical processes in soils, such as metal(loid)s mobility under the action of chelating species released within the rhizosphere (153, 155). Significant correlations between results obtained with Mehlich 3 and other extraction methods currently used in laboratories have been obtained (577, 579, 595). As mentioned before, both DTPA and Mehlich 3 methods may cause the release of elements from additional soil phases (less labile elements), compared to NH_4NO_3 and $CaCl_2$ methods. Since the majority of elements associated with those soil phases are not available or even potentially available to plants, an overestimation of the phytoavailability can occur when these methods are used. In our study, the poor relationship between the metal(loid)s content in soil, as assessed by the DTPA and Mehlich 3 methods, and the metal(loid)s content in lettuce is may be seen as the result of an overestimation of the metal(loid)s phytoavailability.

Regarding the correlations between metal(loid)s content in lettuces and soil extracts along the study period (T_1, T_2, T_3, T_4 and T_5), a higher number of correlations were obtained in the later stages (T_4 and T_5) of lettuce growth (Table 3.4). Even for Cd in the NH_4NO_3 method, where correlations were obtained at all the five time points of the study, the highest correlations were at T_4 (r = 0.96) and T_5 (r = 0.98). This trend was also seen for

Pb in the $CaCl_2$ extraction method. These higher correlations in the later stages of plant growth must be regarded as the result of the use of rhizosphere soil to evaluate metal(loid)s phytoavailability. The rhizosphere–plant interface is a very active area because root exudates and microbiota are continuously changing its chemistry (100, 105, 152). Therefore, the mechanisms that take place in the rhizosphere are more pronounced in the last stages of plant growth, influencing the metal(loid)s phytoavailability in that zone. However, despite the lower correlations observed in the initial stages of lettuce growth, a suitable prediction of Cr, V, Ni, As, Pb, Co, Cd and Sb plant content can be made by considering the amount of a particular metal(loid) extracted by the NH_4NO_3 method and soil physicochemical properties such as CEC, OM, texture, pH and oxides content (Table 3.5). By considering 3 factors in the stepwise multiple regression, high r values were obtained (0.90, 0.99, 0.97, 0.74, 0.88, 0.96, 0.92 and 0.90, for Cr, V, Ni, As, Pb, Co, Cd and Sb, respectively), confirming the applicability of this approach.

3.5. Conclusions

The analysis of rhizosphere soil at several time points of lettuce growth proved to be a better approach to predict metal(loid)s plant content. Several significant correlations between lettuces and soil content were obtained for Cr, V, Ni, As, Pb, Co, Cd and Sb. From the four extraction methods used, DTPA and Mehlich 3 methods showed to extract a higher amount of the studied metal(loid)s, but weaker and fewer correlations were obtained. The NH_4NO_3 method showed strong positive correlations for almost all the metal(loid)s studied and thus seems to be the most suitable to estimate metal(loid)s phytoavailability. Soil properties (pH, OM, CEC, clay and oxides content) markedly influence the distribution of metal(loid)s between the phytoavailable and non-phytoavailable fractions. Overall, an accurate prediction of metal(loid)s content in plants can be performed considering the amount of metal(loid)s extracted by the NH_4NO_3 method and some soil properties (CEC, OM, pH, texture and oxides content). This was the first study evaluating metal(loid)s phytoavailability using rhizosphere soil sampled during a plant growth period.

PART III

Soil-to-plant transfer of chemical species during lettuce growth and its influence in lettuce nutritional value and safety

Chapter 4

Influence of soil composition in the shoot ionomic profile of lettuce: a holistic approach to understand soil-to-plant element transfer

Abstract

The effect of soil composition in the shoot ionome of lettuce along its growth was studied using multielemental analytical techniques. Lettuces were grown under similar conditions in three soils that presented marked differences in their physicochemical properties and in the composition of 24 elements (N, P, Ca, Mg, K, Na, Fe, Mn, Cu, Zn, Ni, Mo, Al, Co, Se, Cr, V, As, Cd, Pb, Th, U, Be and Sb). The soil that presented higher CEC, OM, EC, clay and silt and lower pH had a significantly higher phytoavailable fraction of the elements, resulting in a higher elemental content in lettuces grown in that soil. Correlations between different elements in lettuce shoot along its growth period were investigated to identify possible interactions. Positive correlations between Ca and Mg and between N and P, and negative correlations between Ca and K in lettuces grown in the three soils indicate that other factors, such as genetics, may have a more important effect in the shoot ionomic profile of plants than environmental factors. By contrast, the occurrence of positive or negative interactions between Fe, Mn, Ni, Co, Zn and Cd in lettuce shoot was highly dependent on the amount of phytoavailable element in the soil and thus is greatly influenced by environmental factors. This is the first field study that considers the influence of macro and trace element phytoavailability in the shoot ionomic profile of lettuce during its growth period.

4.1. Introduction

All plants are composed by a wide variety of chemical elements that are interrelated in a content-dependent manner. Plant uptake, translocation, distribution and storage of chemical elements involve multiple molecular components such as transporters, channels, chelators and the cellular genes that encode and regulate them (596). Even though a limited number of chemical elements have been established as essential for plants, several studies have shown complex relationships among them, which influence their chemical properties, functions and biochemical behavior (10, 11, 23).

The study of the plants “ionome”, the so-called “ionomics”, defined as the quantitative determination of the elemental composition of plant tissues and its changes according to the plant development stage or in response to physiological stimuli or genetic modifications, has emerged as an important area in the plant science field (557). In this context, environmental pollution assessment is seen as one of the potential applications for ionomics, because the wide range of conditions and pollutants probably results in qualitative and quantitative changes in the plant ionome. Changes in the soil, air and water chemical composition are expected to affect more than one element leading to changes in the ionome (560, 597).

A great increase in the number of studies focused on the ionome of different plant species has been observed in last years. In those studies, the mineral content of plants' roots, stems and leaves was determined and the relationship between them has been investigated. However, the lack of correlation observed between these different plant tissues suggests that researchers interested in ionomics should look for data on element accumulation in a particular tissue as a starting point for further studies (558). There is ample evidence that the seed ionome is influenced by both the direct transport of elements from the root and the element remobilization from the leaves. Therefore, a good correlation between leaf and the seed ionome is unlikely to occur (598). Root ionome is particularly difficult to evaluate because the roots surface in usually contaminated with soil and also because there are several root processes that highly affect its mineral content (100, 599). Therefore, leaf ionome seems to be the best option for investigating the root processes (or even the root ionome) as well as the whole plant ionomic profile. Inductively coupled plasma mass spectrometry (ICP–MS) has become a leading instrumental analytical technique in these studies, because it allows the rapid and simultaneous determination of almost all chemical elements (561).

Reports focusing on soil-to-plant elements transfer usually consider an "endpoint" to measure the content of one or more elements in both soil and plant (17). However, it is known that the elemental content of a certain plant tissue reflects the evolution of those elements in the soil along the plant growth period. Therefore, an holistic approach considering periodic measurements of both soil and plant elemental contents could provide additional data useful to understand soil-to-plant elements transfer as well as interactions between elements within a specific plant tissue.

To our knowledge no report has described the shoot ionomic profile of lettuces grown in soils with different physicochemical properties and composition. Moreover, most of the ionomic studies performed until now have only considered interactions between 16 to 20 elements. Important trace elements such as V, Cr, Sb, Pb and U, which are ubiquitous in the environment, have never been considered in those studies. In this context, the main goals of the present work were: (1) to provide new insights about the influence of the soil physicochemical properties in the phytoavailability of elements; (2) to evaluate the soil-to-lettuce transfer of elements in soils with different properties; and (3) to assess the main elemental interactions in lettuce shoot and which factors (genetic and/or environmental) are responsible for those interactions.

4.2. Experimental

4.2.1. Plant and soil sampling

Lettuce (*L. sativa L. var. capitata*) cultivation as well as plant and soil sampling were performed as described in Chapter 3, section 3.2.1.

4.2.2. Reagents and apparatus

Only polypropylene labware – pipette tips (VWR, Radnor, PA), volumetric flasks (Kartell, Milan, Italy) and centrifuge tubes (TRP, Trasadingen, Switzerland) – was used during the work. A Milestone (Sorisole, Italy) MLS 1200 Mega high performance microwave digestion unit equipped with an HPR-1000/10 S rotor was used for total digestion of plant and soil samples. A K-424 digestion unit and a KjelFlex K-360 distillation unit coupled to a B-414 scrubber, all from Büchi (Postfach, Switzerland), were used to perform the total N determination in soil and plant samples. All solutions were prepared using ultrapure water (>18.2 MΩ.cm at 25 °C) obtained with a Milli-Q RG (Millipore, Billerica, MA) water purification system.

A chromatographic system consisting of a Jasco (Tokyo, Japan) PU-2089_{Plus} gradient pump, a Waters (Milford, MA) IC-PAK^{TM} anion-exchange column (4.6 × 150 mm) and a Waters model 431 conductivity detector was used for anions determination. Data acquisition was done with Borwin PDA Controller Software (JMBS Developments, Grenoble, France). The eluent was a 1.3-mM sodium gluconate / 1.3-mM borax solution adjusted to pH 8.5. Mixed standard solutions of NO_3^- and PO_4^{3-} were prepared from their analytical reagent grade sodium salts.

ICP–MS analyses were performed using a VG Elemental (Winsford, UK) PlasmaQuad 3 (quadrupole-based) instrument, equipped with a Meinhard® Type A concentric glass nebulizer, a water-cooled glass spray chamber with impact-bead, a standard quartz torch and nickel skimmer and sampling cones. For sample introduction, a Minipuls 3 peristaltic pump (Gilson, Villiers le Bel, France) was used. Argon of 99.9% purity (Alphagaz 2^{TM}, supplied by Air Liquide, Maia, Portugal) was used as the plasma source. The ICP-MS analyses were carried out under the following instrumental conditions: argon flow rate - 13 L/min; auxiliary argon flow rate - 0.7 L/min; nebulizer flow rate - 0.8 L/min; RF power - 1350 W; scan regions dwell time - 200 ms; detection mode - pulse counting. The elemental isotopes (m/z ratios) ^{9}Be, ^{27}Al, ^{31}P, ^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{95}Mo, ^{111}Cd, ^{121}Sb, ^{208}Pb, ^{232}Th and ^{238}U were monitored for analytical determinations; ^{45}Sc, ^{89}Y, ^{115}In, ^{159}Tb and ^{209}Bi were used as internal standards. The instrument was tuned daily for maximum signal sensitivity and stability using ^{115}In as the target isotope. Internal standards and tuning solutions were prepared by appropriate

dilution of the corresponding AccuStandard® (New Haven, CT) solutions (ICP-MS-200.8-IS-1: 100 µg/mL of Sc, Y, In, Tb and Bi; and ICP-MS-200.8-TUN-1: 10 µg/mL of Be, Mg, Co, In and Pb). Calibration standards were also prepared from AccuStandard® 10 µg/mL multi-element ICP-MS standard solution (ICP-MS-200.8-CAL1-1). High purity HNO_3 (65% w/w, *Trace*SELECT® Ultra, Fluka, L'Isle d'Abeau Chesnes, France) and H_2O_2 (30% v/v, *Trace*SELECT®, Fluka, Seelze, Germany) were used as received.

AAS analysis were carried out using a Perkin Elmer (Überlingen, Germany) 3100 flame (air-acetylene) instrument for the determination of Ca, Mg, Na, K and Fe. Multi-element calibration standards were prepared from 1000 mg/L single-element standard stock solutions (Sigma-Aldrich, St. Louis, MO) of those elements.

Certified reference materials (CRM) ISE 918 (sandy soil, supplied by WEPAL, Wageningen, The Netherlands) and BCR 679 (white cabbage, supplied by EC Institute for Reference Materials and Measurements, Geel, Belgium) were used to check the accuracy of the analytical procedures.

4.2.3. Plant elemental content

A microwave-assisted digestion procedure was performed to obtain the total content of elements in plant samples. Three replicates of BCR 679 CRM and three replicates of lettuce samples (ca. 500 mg) were directly weighed into the microwave oven PTFE vessels. Then 5 ml of 65% (w/w) HNO_3 and 2 ml of 30% (v/v) H_2O_2 were added to each vessel and the mixture was submitted to the following microwave heating program: 250 W / 1 min, 0 W / 2 min, 250 W / 5 min, 400 W / 5 min, and 600 W / 5 min. After cooling, the vessels content was transferred to 25 ml volumetric flasks and the volume was made-up with ultrapure water. Solutions were then analyzed for Be, Al, P, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sb, Pb, Th and U by ICP-MS and for Ca, Mg, Na, K and Fe by AAS. Nitrogen was determined by the Kjeldahl method (600). Results were expressed on a fresh weight (fw) basis.

4.2.4. Soil analysis

The determination of the physicochemical properties, pH, electrical conductivity (EC), organic matter (OM), cation exchange capacity (CEC) and particle size distribution as well as the procedures for the determination of the total and extractable elemental content of each soil sample were performed according to official methods of soil analysis. Soil pH was measured in the supernatant of a 1:5 (w/v) suspension prepared with ultra-pure water. OM content was determined through an oxidation procedure with a potassium dichromate (0.27 M) and sulfuric acid (98% v/v) solution at 135 °C. CEC was determined

using the ISO (587) method. Particle size distribution was determined according to ISO (588) method. Total elemental content was determined after microwave-assisted digestion according to USEPA (589). Total N content of soil was determined according to ISO (601). The phytoavailable fraction of elements was determined using the ammonium nitrate extraction method described in ISO (590). Nitrate and phosphate were considered to be the phytoavailable fraction of N and P, respectively, and were extracted from soil samples with ultra-pure water at a ratio 1:5 (w/v) at 20 ºC. After 1 h of constant shaking, the solution was centrifuged at 3000 g for 10 min and the supernatant was analyzed for NO_3^- and PO_4^{3-} by ion chromatography. Three replicates of each soil sample and three replicates of ISE 918 soil CRM were analyzed.

4.2.5. Statistical analysis

Data exploration, descriptive statistics calculation, ANOVA, correlation matrixes and principal component analysis (PCA) were performed with IBM SPSS Statistics for Windows, version 22.0 (IBM Corp, Armonk, NY). The statistical significance of the Pearson correlation was set at $p < 0.01$. For the other tests, statistical significance was assumed for $p < 0.05$.

4.3. Results

4.3.1. Physicochemical properties and elemental content of the soils

The physicochemical properties pH, OM, CEC and particle size distribution of the three soils are shown in Table 4.1. A_2 soil had a significantly lower pH value. Significant differences were also found for OM content. A_2 soil presented the highest content (72.8%) of OM and A_3 soil the lowest (20.7%). A_2 soil showed the highest CEC (17.2 cmol/kg), whereas A_1 and A_3 soils presented a quite lower and similar value (7.9 cmol/kg and 7.9 cmol/kg, respectively). A_2 soil also showed a significantly higher EC value (633 µS/cm). Regarding particle size distribution, A_2 soil was classified as loamy sand soil while A_1 and A_3 soils were classified as sandy soils.

Table 4.1 – Main physicochemical properties of the soils samples collected from the three study experimental fields.

Soil ID	pH	OM (%)	CEC (cmol/kg)	EC (µS/cm)	Soil classification
A_1	7.3 ± 0.2a	56.3 ± 3.9a	7.9 ± 0.4a	283 ± 40a	sandy soil
A_2	7.2 ± 0.2b	72.8 ± 2.3b	17.2 ± 0.6b	633 ± 69b	loamy sand soil
A_3	7.3 ± 0.2a	20.7 ± 1.6c	7.9 ± 0.5a	196 ± 27c	sandy soil

Data presented as mean ± SD (n = 5). Differences in soil pH, OM, CEC and EC content were tested according to one-way ANOVA followed by Tukey's test. In each column, values followed by different letters are significantly different at $p < 0.05$.

The total and phytoavailable content as well as the phytoavailable fraction (%) of major and trace elements in samples collected during the study period in A_1, A_2 and A_3 soils are presented in Table 4.2. Regarding the total content, significant differences were observed between the three soils. A_1 soil shown the highest content of N, Na, Cr, Ni, Se and Be and the lowest of V, Pb, Th and U; A_2 soil had the highest content of Al, K, V, Pb and Mo and the lowest of Fe, P, Ca, Mg, Mn, Cu, Zn, Cr, Co, Ni, As, Se, Cd and Sb; A_3 soil shown the highest content of Fe, P, Ca, Mg, Mn, Cu, Zn Co, As, Th, U, Cd and Sb and the lowest of Al, N, K, Na, Be and Mo. Significant differences were also observed regarding the phytoavailable content of the elements. A_1 soil had the highest phytoavailable content of Zn and the lowest of P, Ca, Mg, As and Th; A_2 soil shown the highest phytoavailable content of Al, N, P, Ca, Mg, K, Na, Co, V, Pb, Th, U, Be and Mo and the lowest of Mn, Cu and Zn; and A_3 soil presented the highest phytoavailable content of Mn, Cu, Se and Cd and lowest of Al, N, K, Na, Ni, U, Cd and Sb. Significant differences were also found regarding the phytoavailable fraction of the elements. A_1 soil had the highest phytoavailable fraction of Zn and Pb and the lowest of N, P, Mg, Na, Cu, Cr, Ni and Mo; A_2 soil shown the highest phytoavailable fraction of Fe, Al, N, P, Ca, Mg, K, Na, Mn, Cr, Co, Ni, As, Se, V, Th, U, Be, Cd, Mo and Sb and the lowest of Zn; and A_3 soil presented the highest phytoavailable fraction of Cu and lowest of Fe, Al, Ca, K, Mn, Co, As, Se, V, Pb, Th, U, Be, Cd and Sb.

Table 4.2 – Total and phytoavailable content as well as phytoavailable fraction (%) of elements in A_1, A_2 and A_3 soils.

	Soil ID	Total content	Phytoavailable content	Phytoavailable fraction (%)
Fe (mg/g)	A_1	16.1 ± 0.7a	0.00032 ± 0.00002a	0.0020 ± 0.0001a
	A_2	14.2 ± 0.6b	0.00031 ± 0.00003a	0.0023 ± 0.0003b
	A_3	22.3 ± 0.7c	0.00029 ± 0.00005a	0.0014 ± 0.0002c
Al (mg/g)	A_1	21.5 ± 1.5a	0.00013 ± 0.00001a	0.0006 ± 0.0001a
	A_2	32.7 ± 2.3b	0.00022 ± 0.00003b	0.0007 ± 0.0001a
	A_3	14.6 ± 1.6c	0.00006 ± 0.00001c	0.0004 ± 0.0001b
N (mg/g)	A_1	7.0 ± 0.2a	0.17 ± 0.04a	2.50 ± 0.62a
	A_2	4.1 ± 0.2b	0.41 ± 0.06b	10.1 ± 1.8b
	A_3	3.5 ± 0.3c	0.10 ± 0.03c	2.92 ± 0.86a
P (mg/g)	A_1	3.2 ± 0.1a	0.051 ± 0.003a	1.61 ± 0.11a
	A_2	1.6 ± 0.1b	0.101 ± 0.003b	6.35 ± 0.26b
	A_3	3.7 ± 0.1c	0.063 ± 0.004c	1.70 ± 0.15c
Ca (mg/g)	A_1	17.4 ± 0.8a	0.78 ± 0.11a	4.51 ± 0.53a
	A_2	7.1 ± 0.4b	1.29 ± 0.16b	18.1 ± 2.7b
	A_3	24.6 ± 1.1c	0.89 ± 0.10c	3.59 ± 0.34a
Mg (mg/g)	A_1	3.9 ± 0.1a	0.09 ± 0.01a	2.19 ± 0.29a
	A_2	2.6 ± 0.1b	0.20 ± 0.01b	7.86 ± 0.57b
	A_3	4.0 ± 0.2c	0.12 ± 0.02c	2.84 ± 0.41c
K (mg/g)	A_1	3.8 ± 0.1a	0.17 ± 0.02a	4.40 ± 0.55a
	A_2	4.4 ± 0.2b	0.40 ± 0.04b	9.12 ± 0.79b
	A_3	2.9 ± 0.2c	0.11 ± 0.02c	3.72 ± 0.62c
Na (µg/g)	A_1	751 ± 61a	76.5 ± 4.9a	10.2 ± 0.6a
	A_2	616 ± 79b	194 ± 23b	31.8 ± 4.1b
	A_3	381 ± 38c	40.3 ± 13.7c	10.4 ± 2.8a
Mn (µg/g)	A_1	261 ± 14a	0.22 ± 0.07a	0.08 ± 0.02a
	A_2	143 ± 6b	0.13 ± 0.05b	0.10 ± 0.04a
	A_3	411 ± 29c	0.27 ± 0.07c	0.07 ± 0.01b
Cu (µg/g)	A_1	40.1 ± 4.5a	0.077 ± 0.006a	0.19 ± 0.02a
	A_2	17.2 ± 1.2b	0.055 ± 0.004b	0.32 ± 0.04b
	A_3	130 ± 13c	0.476 ± 0.031c	0.37 ± 0.05c
Zn (µg/g)	A_1	149 ± 5a	0.082 ± 0.011a	0.05 ± 0.01a
	A_2	89.5 ± 2.8b	0.030 ± 0.002b	0.03 ± 0.01b

	A_3	174 ± 9c	0.065 ± 0.022c	0.04 ± 0.01b
Cr (µg/g)	A_1	28.2 ± 2.2a	0.013 ± 0.002a	0.04 ± 0.01a
	A_2	18.6 ± 1.5b	0.011 ± 0.004a	0.06 ± 0.03b
	A_3	22.1 ± 1.8c	0.011 ± 0.002a	0.05 ± 0.01a
Co (µg/g)	A_1	4.54 ± 0.24a	0.006 ± 0.001a	0.13 ± 0.02a
	A_2	2.32 ± 0.06b	0.007 ± 0.001b	0.29 ± 0.04b
	A_3	6.06 ± 0.25c	0.006 ± 0.002a	0.10 ± 0.03c
Ni (µg/g)	A_1	19.1 ± 0.7a	0.045 ± 0.010a	0.24 ± 0.05a
	A_2	8.47 ± 0.18b	0.047 ± 0.003a	0.56 ± 0.09b
	A_3	15.6 ± 0.8c	0.039 ± 0.005b	0.25 ± 0.06a
As (µg/g)	A_1	7.05 ± 0.25a	0.037 ± 0.015a	0.53 ± 0.10a
	A_2	6.82 ± 0.16a	0.059 ± 0.018b	0.85 ± 0.12b
	A_3	18.4 ± 1.0b	0.072 ± 0.034b	0.39 ± 0.18c
Se (µg/g)	A_1	4.49 ± 0.18a	0.023 ± 0.007a	0.51 ± 0.16a
	A_2	3.55 ± 0.20b	0.028 ± 0.008b	0.77 ± 0.22b
	A_3	4.32 ± 0.13c	0.018 ± 0.007a	0.42 ± 0.17a
V (µg/g)	A_1	23.2 ± 1.0a	0.013 ± 0.004a	0.06 ± 0.02a
	A_2	29.3 ± 0.9b	0.024 ± 0.012b	0.08 ± 0.04b
	A_3	24.7 ± 0.8c	0.009 ± 0.001a	0.04 ± 0.01a
Pb (µg/g)	A_1	17.0 ± 0.7a	0.0010 ± 0.0005a	0.006 ± 0.003a
	A_2	24.2 ± 0.9b	0.0015 ± 0.0007b	0.005 ± 0.003a
	A_3	18.9 ± 0.7c	0.0009 ± 0.0005a	0.004 ± 0.003a
Th (µg/g)	A_1	7.61 ± 0.21a	0.0022 ± 0.0001a	0.029 ± 0.002a
	A_2	12.1 ± 0.5b	0.0050 ± 0.0001b	0.041 ± 0.004b
	A_3	17.5 ± 0.3c	0.0046 ± 0.0001c	0.026 ± 0.001c
U (µg/g)	A_1	3.31 ± 0.22a	0.0013 ± 0.0001a	0.039 ± 0.002a
	A_2	3.98 ± 0.21b	0.0025 ± 0.0001b	0.063 ± 0.003b
	A_3	4.46 ± 0.25c	0.0008 ± 0.0001c	0.018 ± 0.002c
Be (µg/g)	A_1	2.11 ± 0.14a	0.0004 ± 0.0008a	0.020 ± 0.003a
	A_2	2.07 ± 0.15a	0.0008 ± 0.0002b	0.038 ± 0.008a
	A_3	1.70 ± 0.07b	0.0003 ± 0.0001a	0.015 ± 0.005b
Cd (µg/g)	A_1	1.12 ± 0.09a	0.0010 ± 0.0006a	0.09 ± 0.05a
	A_2	0.68 ± 0.08b	0.0009 ± 0.0003a	0.12 ± 0.04b
	A_3	2.21 ± 0.12c	0.0013 ± 0.0007b	0.05 ± 0.03c
Mo (µg/g)	A_1	3.17 ± 0.46a	0.021 ± 0.004a	0.66 ± 0.083a
	A_2	3.37 ± 0.31a	0.038 ± 0.006b	1.11 ± 0.153b

	A_3	2.30 ± 0.21b	0.020 ± 0.003a	0.85 ± 0.073c
Sb (ng/g)	A_1	429 ± 4a	1.93 ± 0.34a	0.45 ± 0.07a
	A_2	256 ± 4b	2.13 ± 0.55a	0.83 ± 0.22b
	A_3	519 ± 6c	1.54 ± 0.40b	0.30 ± 0.08c

Data presented as mean ± SD (n = 5). Differences in elemental composition were tested according to ANOVA followed by Tukey's test. In each column, values followed by different letters are significantly different at $p < 0.05$, for each element.

A PCA was performed using the total content of 24 elements and 7 soil physicochemical properties (CEC, OM, EC, coarse sand, fine sand, silt and clay) as variables. Only factors showing an eigenvalue > 1 were retained. Two principal components (PC) were extracted, which accounted for a total variance of 93.1%. The score plot is presented in Figure 4.1a. PC1 explains 64.7% of the total variance. It was positively loaded by Ca, Mg, Fe, Mn, Co, Ni, Cu, Zn, As, Se, Cd, Sb and P and negatively loaded by K, Be, Al, V, Mo, Pb, CEC, OM, EC, silt and clay. PC2 (which accounted for 28.4% of the variance) was positively related to Th, U and fine sand and negatively related to Na, Cr, N and coarse sand (Figure 4.1b).

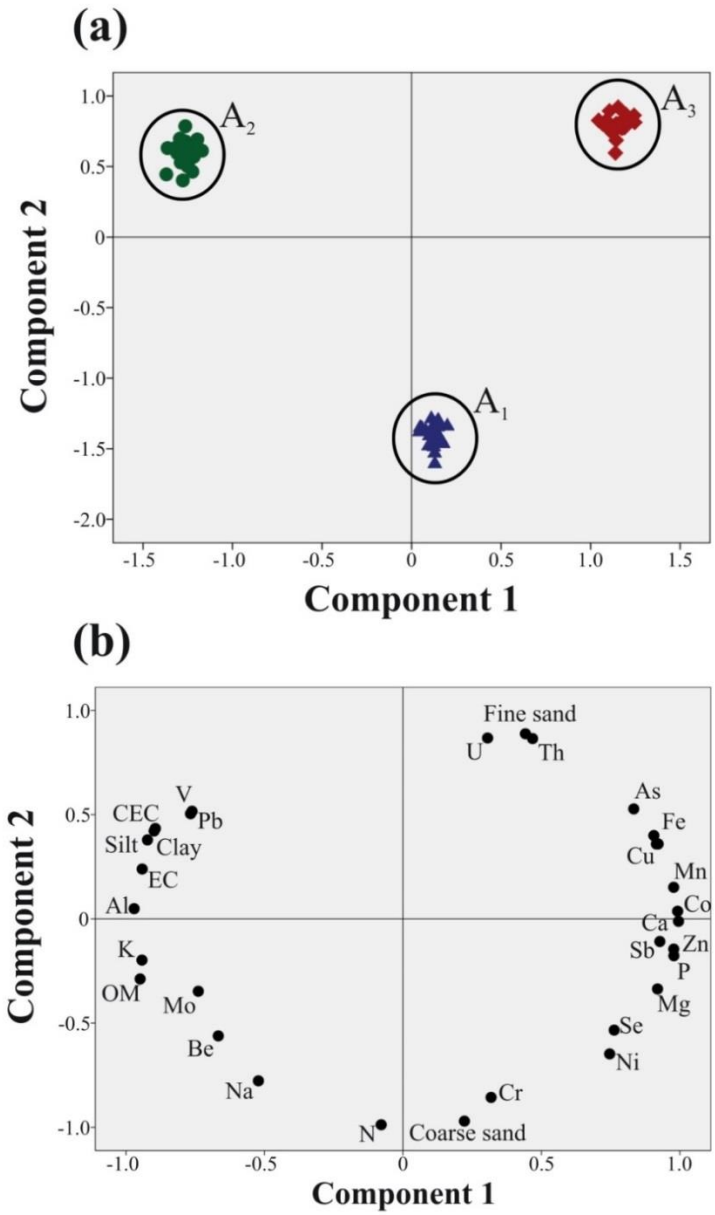


Figure 4.1 – Principal component analysis of soils. (a) Score plot showing the distribution of A_1, A_2 and A_3 soils. (b) Loading plot showing the variables that had a loading on PC1 or PC2 higher than 0.5 and indicating the direction in which the values increased.

4.3.2. Elemental content in lettuce shoot

The macro and trace elements content of lettuces grown in A_1, A_2 and A_3 soils is presented in Table 4.3. Significant differences were observed between lettuces grown in different soils regarding macro elements content. A_1 lettuces had higher Ca content whereas A_2 lettuces shown higher N, P and K content. No significant differences were observed for Mg. Regarding the essential trace elements, no significant differences were observed for Mn and Zn in the A_1, A_2 and A_3 lettuces. A significantly higher content of Fe, Cu and Mo was observed in A_2 lettuces. Ni also exhibited significantly different levels, with

A_1 lettuce showing the highest content and A_3 lettuce the lowest. In the case of the beneficial elements Al, Co, Se and Na, A_2 lettuces showed the highest Al content and the lowest Se content, whereas A_3 lettuces had the highest Co content and the lowest Na and Al content. No significant differences were observed between A_1, A_2 and A_3 lettuces regarding Cr content. However, A_2 lettuces showed higher V content. For toxic elements As, Be, Cd, Pb, Sb, Th and U, significant differences were also observed. A_2 lettuces had a higher Be, Pb and Th content. A_1 lettuces showed the highest U, Be and Sb content while A_3 lettuces had the lowest U and Be content. No significant differences were observed for As and Cd in the studied lettuces.

Table 4.3 – Elemental composition of lettuce shoots (the total content of each element is the mean ± SD of 5 sampling time points during the study period).

	Plant ID	Total content
N (mg/g)	A_1	3.94 ± 0.85a
	A_2	5.03 ± 1.10b
	A_3	4.58 ± 0.95a
P (mg/g)	A_1	1.20 ± 0.22a
	A_2	1.56 ± 0.40b
	A_3	1.39 ± 0.31a
Ca (mg/g)	A_1	1.02 ± 0.17a
	A_2	0.82 ± 0.11b
	A_3	0.77 ± 0.07b
Mg (mg/g)	A_1	0.24 ± 0.03a
	A_2	0.23 ± 0.02a
	A_3	0.23 ± 0.04a
K (mg/g)	A_1	3.62 ± 0.55a
	A_2	4.31 ± 0.44b
	A_3	3.51 ± 0.60a
Na (µg/g)	A_1	654.1 ± 54.0a
	A_2	702.1 ± 52.1a
	A_3	437.2 ± 62.0b
Fe (µg/g)	A_1	8.54 ± 1.36a
	A_2	11.4 ± 5.0b
	A_3	8.82 ± 2.00a
Mn (µg/g)	A_1	5.10 ± 3.78a
	A_2	4.16 ± 2.95a

	A_3	6.41 ± 4.82a
Cu (µg/g)	A_1	245.4 ± 13.7a
	A_2	439.0 ± 22.9b
	A_3	415.5 ± 35.0c
Zn (µg/g)	A_1	4.04 ± 0.45a
	A_2	4.11 ± 0.40a
	A_3	3.78 ± 1.38a
Ni (ng/g)	A_1	97.9 ± 23.7a
	A_2	45.2 ± 3.5b
	A_3	29.1 ± 7.4c
Mo (ng/g)	A_1	47.4 ± 38.7a
	A_2	146.8 ± 70.2b
	A_3	40.5 ± 3.2a
Al (ng/g)	A_1	9.46 ± 4.47a
	A_2	14.2 ± 5.6b
	A_3	2.72 ± 1.05c
Co (ng/g)	A_1	5.40 ± 1.21a
	A_2	4.27 ± 0.40a
	A_3	9.71 ± 3.02b
Se (ng/g)	A_1	35.6 ± 13.4a
	A_2	15.2 ± 5.0b
	A_3	41.2 ± 20.8a
Cr (ng/g)	A_1	95.9 ± 15.6a
	A_2	122.5 ± 69.2a
	A_3	120.1 ± 25.1a
V (ng/g)	A_1	23.9 ± 7.6a
	A_2	50.7 ± 26.8b
	A_3	34.0 ± 5.9a
As (ng/g)	A_1	19.2 ± 7.5a
	A_2	16.8 ± 2.8a
	A_3	17.9 ± 6.34a
Cd (ng/g)	A_1	18.4 ± 14.2a
	A_2	18.0 ± 10.9a
	A_3	23.4 ± 14.3a
Pb (ng/g)	A_1	14.7 ± 9.3a
	A_2	23.2 ± 10.9b

	A_3	7.14 ± 2.52c
Th (ng/g)	A_1	1.74 ± 0.55a
	A_2	2.90 ± 0.97b
	A_3	1.28 ± 0.29c
U (ng/g)	A_1	10.3 ± 5.4a
	A_2	3.28 ± 1.64b
	A_3	1.29 ± 0.78c
Be (ng/g)	A_1	1.30 ± 0.51a
	A_2	1.78 ± 0.37b
	A_3	0.50 ± 0.17c
Sb (ng/g)	A_1	1.66 ± 0.51a
	A_2	1.17 ± 0.38b
	A_3	1.02 ± 0.26b

Data presented as mean ± SD (n = 5 time points during the study period). Differences in elemental composition were tested according to ANOVA followed by Tukey's test. In a column, values followed by different letters are significantly different at $p < 0.05$, for each element

A PCA was performed using the elemental content of lettuce shoots as variables. Three PCs were extracted with eigenvalues > 1, which accounted for a total variance of 89.9%. Figure 4.2 shows the correspondent 3D score and loading plots. PC1 explained 48.1% of the total variance and was strongly loaded (> 0.75) by N, P, Ca, Mg, K, Mn, Zn, As, Se, Cd and Sb; PC2 was high positive loaded (> 0.70) by Fe, Be, Al, Cr, Pb and Th and explained 31.1% of the total variance; PC3 was strongly loaded (> 0.65) by Ni, Co and Cu and explained 10.7% of the total variance. Three main groups are highlighted in the score plot clustering the lettuces grown in the same soil. The temporal variation in the elemental content of A_1, A_2 and A_3 lettuces is also highlighted by the different symbols/colors (Figure 4.2a).

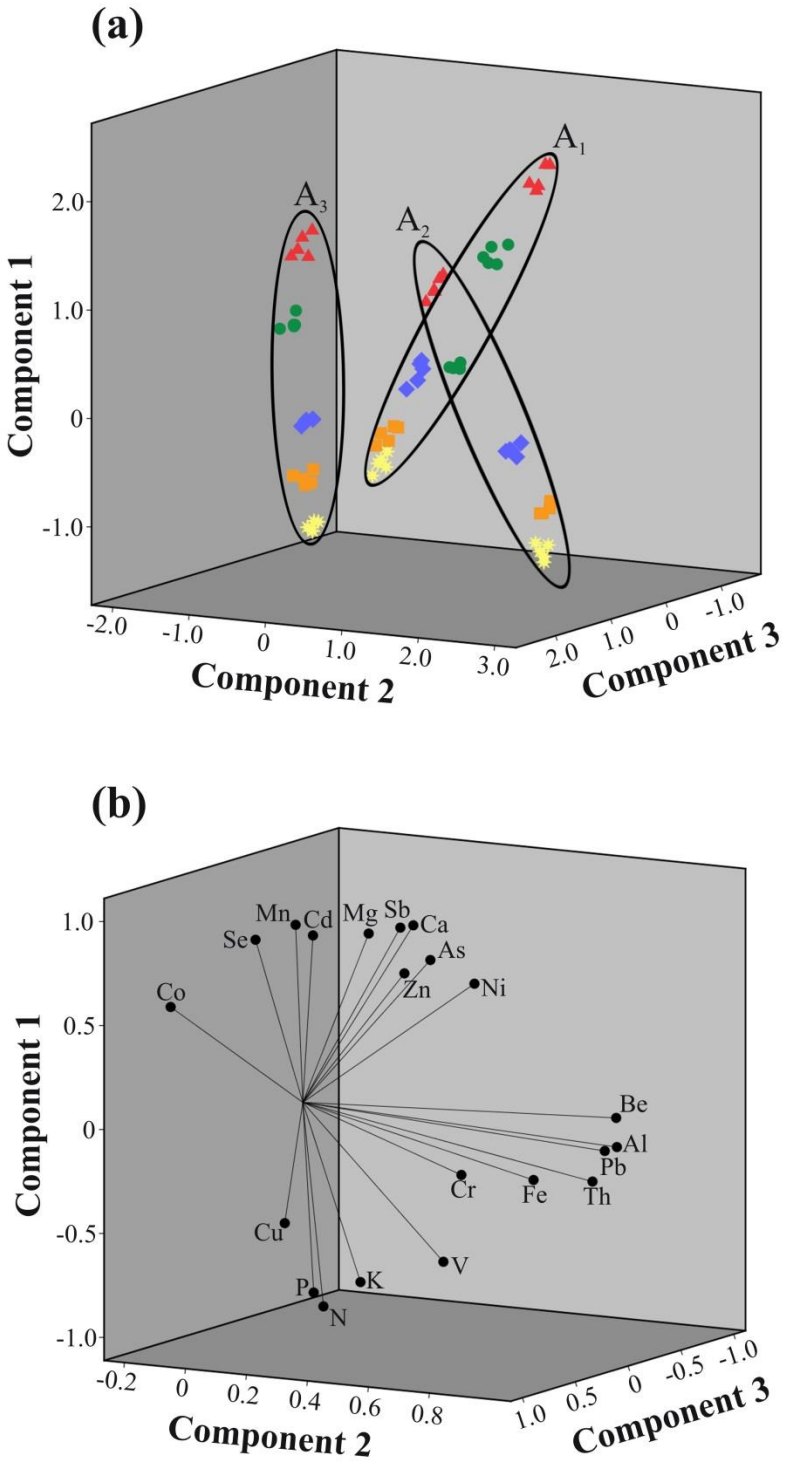


Figure 4.2 – Principal component analysis of the shoot ionome of lettuces: (a) three dimensional score plot displaying the distribution of A_1, A_2 and A_3 lettuces; (b) three dimensional loading plot showing the variables that had a loading on PC1, PC2 and PC3 higher than 0.5 and indicating the direction in which the values increased.

4.3.3. Elemental interactions in lettuce shoot

The correlation between the different elements in lettuce shoot was also investigated in order to identify possible interactions (Figure 4.3). A large number of positively or negatively correlated elements were found. Forty seven positive and 44 negative correlations were obtained between pairs of elements in the lettuces from the three soils. Ca and Mg showed a significant positive correlation in all the plants. By contrast, K showed significant negative relationship with Ca. Generally, N, P and K were positively correlated. Significant interactions between the essential trace elements Fe, Mn, Co, Ni and Zn were also observed in all the studied lettuces. Fe showed significant positive correlations with Mn, Ni, Co, Cu and Zn in A_1 and A_3 lettuces. However, a negative correlation was observed between Fe and Mn, Co and Ni in A_2 lettuces. V showed both positive (A_1 and A_2 lettuces) and negative (A_3 lettuces) correlations with P. As, Cd and Pb showed several negative correlations with essential elements in all the studied lettuces.

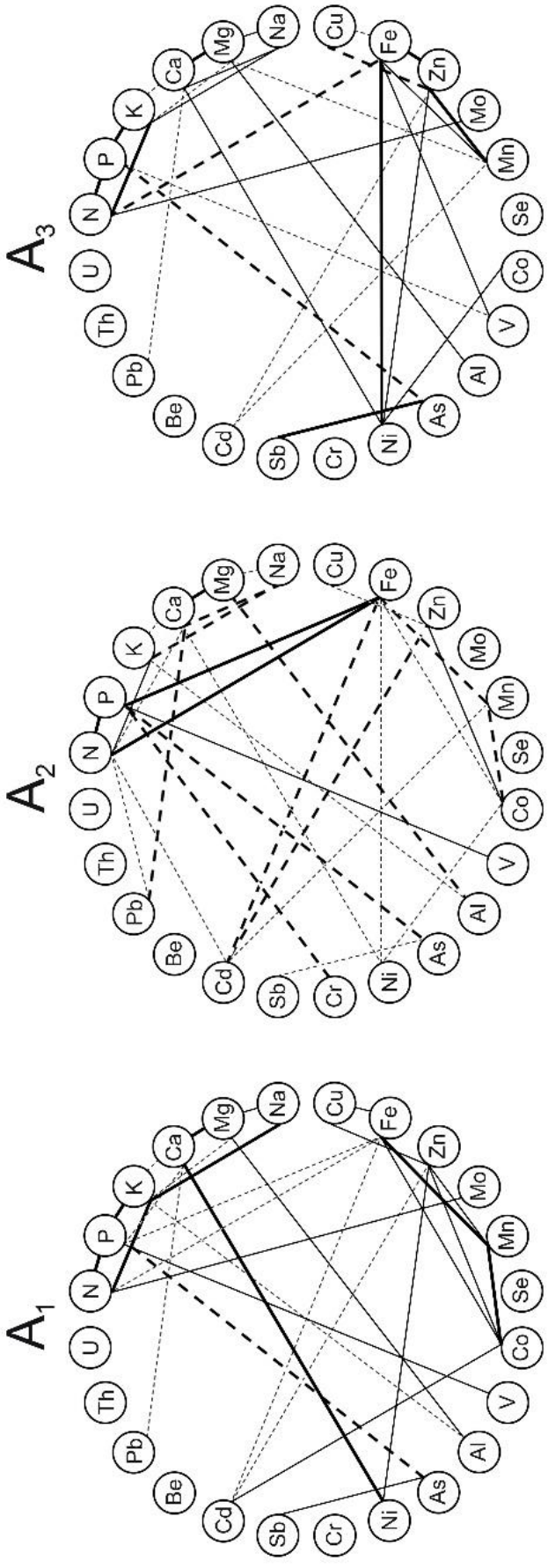


Figure 4.3 – Correlations wheels presenting significant correlations between elements within an experiment. Only significant correlations (r^2 > 0.32, p < 0.01) are displayed on each wheel. Positive correlations are represented by solid lines, negative correlations are represented by dashed lines. Thick lines indicate r^2 > 0.5, thin lines indicate 0.32 < r^2 < 0.5.

4.4. Discussion

The three soils presented very distinct characteristics, which resulted in three well separated groups in the PCA score plot. As referred above, A_2 soil was characterized by the highest CEC, OM, clay and silt values. These soil parameters play a key role in the distribution of most metals between the phytoavailable and the non-phytoavailable fractions (62, 71, 81). As shown in Table 4.2, in general, A_2 soil had the highest phytoavailable fraction of the studied elements. A significantly higher phytoavailable fraction was found for Fe, N, P, Ca, Mg, K, Na, Cr, Co, Ni, As, Se, V, Th, U, Be, Cd, Mo and Sb in this soil. The mobility and phytoavailability of anionic species is also dependent on the same soil properties referred above. NO_3^- was the dominant phytoavailable N species in all the soils due to the neutral pH and aerobic conditions (10). A_2 soil presented a significantly higher NO_3^- fraction, which is related with its particular physicochemical properties (Table 4.2). For example, A_2 soil had a higher OM and clay content, and this helps to reduce NO_3^- loss by leaching due to the slower water movement (602, 603).

Several differences were observed in the elemental content of lettuces from the same soil due to changes in soil physicochemical properties and composition along the study period as shown in Figure 4.2. The mean content of the macro elements Ca, Mg, K, N and P in lettuce shoot was in good agreement with those previously reported in the literature (12). A_2 soil showed a significantly higher phytoavailable content of N, P and K (Table 4.2), positively correlated with the higher content of these elements in A_2 lettuces, suggesting a direct relationship between soil and plant elemental composition. Nitrate, phosphate (the main anionic forms of N and P, respectively) and K are highly mobile in soil and inside the plant tissues, which seems to contribute to the close relationship between the lettuce shoot content and the phytoavailable content of these elements (10). The same pattern was not observed for Ca and Mg. Within plant tissues, Ca has low mobility, thus, the shoot Ca content may not reflect the phytoavailable Ca content in the soil. In fact, until now no transporters have been identified to be responsible for Ca xylem loading. Besides, it seems that part of xylem Ca can arrive to shoot tissues via the apoplast, although Ca mobility is this vascular system is also low (9, 508).

In the present study, no evidence of an antagonistic relationship between Ca and Mg was observed (Figure 4.3). The Ca/Mg ratio in lettuce shoot was always < 5, which is in good agreement with previous reports (604). The Ca/Mg ratio in soils varies greatly, however plants developed a wide range of strategies to handle with these differences (605). Although both Ca and Mg are essential macronutrients for plants, a proper Ca/Mg ratio must be ensured to avoid the inhibitory effect of one element on the absorption of the other (606, 607). We observed significant positive correlations between Ca and Mg in A_1

(r^2 = 0.85), A2 (r^2 = 0.94) and A_3 lettuces (r^2 = 0.90) suggesting that the mechanisms driving Ca and Mg accumulation are related. This correlation between Ca and Mg is widely assumed to occur in several plant species, although it is very clear the existence of different pathways that regulate Ca and Mg homeostasis (9).

Mg may also interact with other cationic species such as K and Na. In our study, an antagonistic effect of Mg on K was evident (Figure 4.3). A significant negative correlation was obtained in A_1 (r^2 = 0.46) and A_3 (r^2 = 0.41) lettuces. According to Ding, Chang (608), a high uptake of K negatively affects the Mg status of plant, inducing Mg deficiency. Regarding Mg interaction with Na, both positive and negative correlations were observed: a positive relationship in A_1 (r^2 = 0.40) and A_3 (r^2 = 0.47) lettuces and a negative relationship (r^2 = 0.37) in A_2 lettuces. Since Na content in lettuces is strongly related with soil salinity, inhibitory effects on plant uptake are usually observed between Na and other cationic species in saline soils (609, 610). A_2 soil showed a significantly higher content of phytoavailable Na which could result in a high Na uptake and the consequent inhibition of Mg uptake and translocation.

Besides the already mentioned antagonistic effect of K on Mg, K content in plants can also be affected by Na. In the present study, a significantly positive correlation between K and Na was observed for A_1 (r^2 = 0.54) and A_3 (r^2 = 0.48) lettuces, whereas A_2 lettuces showed a negative correlation (r^2 = 0.62), as shown in Figure 4.3. Na is able to inhibit K uptake through the suppression of high- and low-affinity K transport systems or even by utilizing these systems for Na influx (232).

In the present study, a significant positive correlation between N and P was found in A_1 (r^2 = 0.80), A_2 (r^2 = 0.82) and A_3 (r^2 = 0.52) lettuces (Figure 4.3). Leaf P is stoichiometrically related with leaf N and restrictions of one element are likely to control N and P partitioning in plants. Hence, leaf N and P are well correlated across organs, species and functional classifications (611, 612). This indicates that although distinct pathways for N and P uptake, transport, storage and transformation exist, the internal content of these elements is strictly correlated because of their essentiality for many physiological processes in plants (10).

Regarding the micronutrients Fe, Cu, Zn, Mn, Co and Ni, significant interactions between Fe, Mn, Cu, Co, Ni and Zn were observed. Fe showed significant positive correlations with Mn, Ni, Co, Cu and Zn in A_1 and A_3 lettuces. However, a negative correlation was also observed between Fe and Mn, Co and Ni in A_2 lettuces (Figure 4.3). Plants have developed several physiological responses to low Fe availability that can influence their ionome. Among them are rhizosphere acidification, exudation of siderophores, reduction of Fe chelates at the root surface and up-regulation of membrane transporters. These

mechanisms have been widely described to influence the uptake and translocation of several elements besides Fe (114).

For toxic elements, several negative correlations were observed. Cd showed negative relationships with N, Fe, Mn and Zn, and As showed negative correlations with P (Figure 4.3). Toxic elements are taken by plants through the same transport systems involved in nutrients uptake. For example, Cd uptake is thought to occur through membrane transporters engaged in the uptake of Ca, Mg, Fe, Cu and Zn (167, 345). Thus, some of these metals can influence the uptake of Cd from soil resulting in interactions between them. In a similar way, As is taken up by plants mainly by the phosphate transporters. When present as As(VI), which is the main As form in aerobic soils, the phosphate transporter system easily takes this As species. Therefore, interactions between As species and phosphate can influence the As content in several plant tissues (345).

4.5. Conclusions

Results from the present study support the idea that environmental conditions, mainly the soil properties, have a key effect in the shoot ionome of lettuce. A_2 soil was characterized by a higher CEC, OM, EC, clay and silt content and a lower pH, which markedly contribute to a higher phytoavailable fraction of elements. A very distinct pattern was also observed in the shoot ionome of A_2 lettuces. The occurrence of significant correlations between different pairs of elements in lettuce shoot shows that similar processes regulate their uptake, translocation, distribution and storage within the plant. For macro elements, a similar pattern of correlations was observed in the lettuces from the three soils, with a positive relationship between Ca and Mg and between N and P, and a negative relationship between Ca and K. This indicates that, for macro elements, other factors (e.g., genetics) may be more important to the ionomic profile of plants than environmental factors. The trace elements Fe, Mn, Ni, Co, Zn and Cd showed both positive and negative correlations in lettuce shoot, depending on soil properties and the amount of phytoavailable elements. In general, toxic elements such as As, Cd and Pb showed negative correlations with essential elements because these elements are taken by plants through transporters involved in nutrients uptake, which results in ion competition. Valuable information was obtained regarding elements uptake, accumulation and interaction by combining soil physicochemical properties, soil total elemental content, soil phytoavailable content and total elemental content of lettuce shoot along plant growth period, which can be used to improve nutrients use efficiency as well as the nutritional quality of foods.

Chapter 5

Changes in macrominerals, trace elements and pigments content during lettuce (*Lactuca sativa*) growth: influence of soil composition

Abstract

Changes in macrominerals, trace elements and photosynthetic pigments were monitored at 5 stages of lettuce growth. Plants were grown in three experimental agriculture greenhouse fields (A_1, A_2 and A_3). Soil composition was also monitored to understand its influence on lettuce composition. In general, the content of macrominerals, trace elements, chlorophylls and carotenoids decreased during lettuce growth, consequently, high nutritional value was observed at younger stages. A_2 lettuces showed an increase of Fe, Al, Cr, V and Pb due to the different soil physicochemical parameters. Multiple linear regression analysis with stepwise variable selection indicated that soil characteristics, namely, pH($CaCl_2$) for Fe and Cr, silt and fine-sand for Al and V, OM for Al and Pb, coarse-sand and CEC for Cr, had a key role determining element bioavailability and plant mineral content. Thus, lettuce nutritional value was strongly dependent of growth stage and soil characteristics.

5.1. Introduction

Lettuce is one of the most consumed vegetables worldwide with a global production of about 24 million tons in 2011 (613). Mean daily consumption of lettuce in Europe is 22.5 g, which represents about 6.5% of the total dietary intake of vegetables (583). Lettuce contains several macrominerals (e.g., K, Na, Ca and Mg) and trace elements (e.g., Fe, Mn, Cu, Zn and Se) which are essential for human nutrition (614). It is also known as a good source of photosynthetic pigments (chlorophylls and carotenoids) and other phytochemicals that benefit nutrition and have a significant role in the prevention of several oxidative stress-related diseases (615).

Although the tender immature greens produced from the seeds of vegetables have gained popularity as a new culinary trend due to its alleged high nutritional value when compared with commercial mature vegetables, no scientific data is available about the changes in nutritional elements during lettuce growth and influence of soil characteristics (616).

From soil, vegetables can take not only nutrients but also potentially harmful substances, such as toxic trace elements (617). Lettuce obtained from agriculture fields usually presents levels of non-essential/toxic elements such as Sb, As, Cd, Pb, Ni and U below the permissible limits. However, even when produced in uncontaminated or poorly contaminated agriculture soils, variable amounts of these toxic elements can accumulate in lettuce since their absorption depends on several soil-to-plant transfer factors (618, 619). The uptake and translocation of elements in plants is mainly affected by factors such as plant species, soil properties (pH, cation exchange capacity, texture and organic matter), environmental conditions and human agricultural practice (62, 71, 81, 580).

Moreover, some elements accumulate preferentially in specific plants. For example, Cd accumulates mostly in lettuce, spinach, carrot and radish, rather than in onion, pumpkin or sweet pea (620). The total content of trace elements in soil is not the best indicator regarding plant foods nutritional value or safety. It is the phytoavailable fraction that is highly correlated with the content of chemical species in plant tissues and thus is of major relevance in the studies of macro and trace elements accumulation (17, 19).

This work provides new insights on macrominerals, trace elements and photosynthetic pigments of lettuce (*Lactuca sativa*) at immature stages and on the changes that occur during lettuce growth, together with the influence of soil composition. It provides valuable information regarding the production of lettuces with increased nutritional value. In particular, (*i*) the contents of macrominerals, trace elements and pigments were monitored at 5 stages of lettuce growth produced in three experimental greenhouse fields, located in an area of intensive horticulture; (*ii*) the physicochemical properties of soil as well as total and bioavailable fractions of major and trace elements were assessed during the study period; and (*iii*) the soil-to-plant transfer factors for trace elements during lettuce growth were estimated.

5.2. Experimental

5.2.1. Plant and soil sampling

Lettuce (*L. sativa L. var. capitata*) cultivation as well as plant and soil sampling were performed as described in Chapter 3, section 3.2.1.

5.2.2. Reagents and apparatus

ICP–MS analysis were performed using a VG Elemental (Winsford, UK) PlasmaQuad 3 (quadrupole-based) instrument with the instrumental conditions and reagents described in Chapter 4, section 4.2.2. The elemental isotopes (m/z ratios) ^{27}Al, ^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{95}Mo, ^{111}Cd, ^{121}Sb, ^{208}Pb, and ^{238}U were monitored. The detection limits were calculated as the concentration corresponding to 3 standard deviations of 10 replicate measurements of the blank (HNO_3 2% v/v).

AAS analysis were carried out using a Perkin Elmer (Überlingen, Germany) 3100 flame (air-acetylene) instrument for the determination of Ca, Mg, Na, K and Fe. Multi-element calibration standards were prepared from 1000 mg/L single-element standard stock solutions (Sigma-Aldrich, St. Louis, MO) of those elements.

Chlorophyll and total carotenoids measurements were performed using a Shimadzu (Kyoto, Japan) UV-1800 spectrometer. The moisture content was determined by using an HR73 Moisture Analyzer from Mettler Toledo (Greifensee, Switzerland).

5.2.3. Lettuce analysis

The microwave-assisted digestion procedure described in Chapter 4, section 4.2.3., was used to obtain total metal content in plant samples. After digestion, samples were transferred into 25 ml volumetric flasks and the volume was made-up with ultrapure water. Solutions were then analyzed by AAS and ICP-MS for total metal content and results were expressed on a fresh weight (fw) basis.

Chlorophylls (a + b) and total carotenoids from 0.2 to 0.3 g samples of frozen leaf material were extracted with 7 ml of acetone (80%, v/v). After centrifugation for 10 min at 3400 g, the supernatant was recovered and diluted 10-fold with acetone 80%. The absorption of the extracts at λ = 470 nm, λ = 645 nm and λ = 663 nm was measured. The concentrations (µg/g fw) of chlorophyll a, chlorophyll b and total carotenoids were then calculated using the equations of Wellburn (621).

5.2.4. Soil analysis

The physicochemical characteristics of each batch of soil samples, i.e., pH, electrical conductivity (EC), salinity, organic matter (OM), cation exchange capacity (CEC), particle size distribution and total elements were determinedas described in Chapter 3, section 3.2.3. Soil pH was measured in the supernatant of a 1:5 (w/v) suspension prepared with ultra-pure water as described in Chapter 4, section 4.2.4.

5.2.5. Transfer factor from soil to lettuce

The soil-to-plant transfer factors (TF) are widely used to assess the ability of plants to transfer elements from soil to edible tissues. TF was calculated for each metal in each experimental field as follows:

$$TF = C_{plant} / C_{soil}$$

where C_{plant} and C_{soil} represent the total metal content, on a dry weight basis, in the edible parts of plants and in the soil, respectively (617).

5.2.6. Statistical Analysis

Data exploration, descriptive statistics calculation, two-way ANOVA, correlation matrix and principal component analysis (PCA) were performed with SPSS for Windows version 21 (SPSS, Chicago, IL). Statistical significance was assumed at $p < 0.05$, unless otherwise noted.

5.3. Results and Discussion

5.3.1. Lettuce composition concerning elements and pigments content

Table 5.1 shows the macrominerals, trace elements and pigments content in lettuces from the three fields (A_1, A_2 and A_3) at the five sampling time points (T_1 – T_5). Mean moisture content was 94.4 ± 0.4%. No significant differences were observed in moisture content of samples from different fields and harvested at different sampling time points. Lettuce composition regarding nutritionally important elements and pigments at commercial maturity state (T_5) fall within the ranges found in literature, except for Na. The mean Na content in mature lettuces (730.6, 640.5 and 313.8 µg/g, for A_1, A_2 and A_3 lettuces, respectively) was 6 to 15 times higher than the value (50 µg/g) described by Kawashima and Soares (614). In five lettuce samples collected at different times of the year these authors reported an average content of 3.18 mg/g for K and 0.47 mg/g for Ca. For Mg, Fe, Mn, and Zn the mean contents were 180, 5, 3 and 3.3 µg/g, respectively. Mean content of Cu was 400 ng/g. Iron content in lettuces from A_1 and A_3 was within the range 5 to 9 µg/g described by Römheld and Nikolic (622), but was significantly higher (18.9 µg/g) in lettuces from A_2. Selenium content was very similar in lettuces from A_2 (11.5 µg/g) and A_3 (11.2 µg/g), but about 2-folds higher in lettuces from A_1 (24.9 µg/g). Nevertheless, Se levels were in agreement with those described by Mazej, Osvald (623) in several plant foods, including lettuce. Chlorophylls and carotenoids content in mature lettuces were also within the range reported by Baslam, Morales (624) in three types of lettuce consumed as salads.

Table 5.1 – Content of macrominerals, trace elements and pigments at the 5 sampling time points (T_1, T_2, T_3, T_4 and T_5) in 3 different fields (A_1, A_2, A_3).

	Field ID	Sampling time				
		T_1	T_2	T_3	T_4	T_5
Ca (mg/g)	A_1	1.31 ± 0.07	1.11 ± 0.01	0.89 ± 0.01	0.90 ± 0.02	0.89 ± 0.02
	A_2	0.98 ± 0.04	0.89 ± 0.01	0.78 ± 0.01	0.72 ± 0.01	0.72 ± 0.01
	A_3	0.89 ± 0.02	0.82 ± 0.02	0.73 ± 0.01	0.73 ± 0.01	0.69 ± 0.01
K (mg/g)	A_1	3.01 ± 0.11	3.15 ± 0.03	3.47 ± 0.06	4.03 ± 0.18	4.42 ± 0.07
	A_2	3.76 ± 0.12	3.89 ± 0.03	4.84 ± 0.03	4.74 ± 0.05	4.34 ± 0.07
	A_3	2.72 ± 0.15	2.99 ± 0.07	3.71 ± 0.08	3.79 ± 0.03	4.33 ± 0.02
Mg (µg/g)	A_1	275.8 ± 7.8	264.9 ± 3.0	215.1 ± 0.1	222.9 ± 2.6	197.6 ± 2.6
	A_2	257.6 ± 6.8	246.0 ± 1.8	223.9 ± 2.6	219.6 ± 3.4	211.8 ± 2.1
	A_3	281.2 ± 8.4	270.8 ± 2.7	214.2 ± 3.8	204.8 ± 1.7	195.1 ± 4.2
Na (µg/g)	A_1	586 ± 36	618 ± 5	651 ± 6	685 ± 11	731 ± 11
	A_2	778 ± 25	718 ± 15	718 ± 12	656 ± 3	641 ± 8
	A_3	712 ± 17	521 ± 3	320 ± 3	319 ± 3	313 ± 4
Fe (µg/g)	A_1	10.6 ± 0.7	9.23 ± 0.43	8.26 ± 0.20	7.55 ± 0.38	7.05 ± 0.25
	A_2	6.34 ± 0.21	7.74 ± 0.50	8.70 ± 0.45	15.5 ± 0.4	18.9 ± 0.6
	A_3	11.7 ± 0.7	9.78 ± 0.34	9.13 ± 0.77	7.10 ± 0.21	6.39 ± 0.30
Al (µg/g)	A_1	15.3 ± 0.4	14.0 ± 0.4	7.41 ± 0.12	4.16 ± 0.05	6.46 ± 0.14
	A_2	8.15 ± 0.47	9.96 ± 0.28	12.4 ± 0.3	17.5 ± 0.2	23.2 ± 1.0
	A_3	4.32 ± 0.11	3.14 ± 0.09	2.89 ± 0.13	1.73 ± 0.03	1.50 ± 0.04
Mn (µg/g)	A_1	10.5 ± 0.3	8.46 ± 0.19	3.17 ± 0.05	2.04 ± 0.02	1.29 ± 0.02
	A_2	8.11 ± 0.38	7.02 ± 0.11	3.11 ± 0.02	1.50 ± 0.02	1.07 ± 0.02
	A_3	13.1 ± 0.1	11.1 ± 0.2	3.92 ± 0.07	2.53 ± 0.03	1.48 ± 0.03
Zn (µg/g)	A_1	4.53 ± 0.17	4.44 ± 0.04	4.11 ± 0.05	3.39 ± 0.04	3.71 ± 0.06
	A_2	4.72 ± 0.25	4.22 ± 0.06	3.80 ± 0.10	3.63 ± 0.04	4.17 ± 0.02
	A_3	5.55 ± 0.06	5.17 ± 0.02	3.44 ± 0.04	2.40 ± 0.04	2.37 ± 0.19
Cu (ng/g)	A_1	271 ± 5	244 ± 3	242 ± 2	235 ± 3	237 ± 5
	A_2	424 ± 14	404 ± 5	455 ± 5	458 ± 3	455 ± 6
	A_3	389 ± 16	367 ± 3	433 ± 5	444 ± 27	444 ± 11
Cr (ng/g)	A_1	118.3 ± 4.6	106.8 ± 0.8	78.3 ± 3.2	94.3 ± 1.8	81.5 ± 2.0
	A_2	63.7 ± 1.2	67.8 ± 3.6	75.1 ± 2.4	178.1 ± 7.3	227.9 ± 1.6
	A_3	150.4 ± 2.1	139.4 ± 4.7	124.8 ± 1.9	102.5 ± 3.7	83.4 ± 2.6
Se (ng/g)	A_1	59.3 ± 3.7	39.9 ± 1.5	27.3 ± 2.1	26.5 ± 0.6	24.9 ± 1.5
	A_2	23.2 ± 1.1	16.2 ± 1.6	9.30 ± 0.8	15.5 ± 1.1	11.5 ± 0.8

	A_3	65.8 ± 3.2	58.2 ± 4.0	45.0 ± 1.7	25.8 ± 1.8	11.2 ± 0.7
Mo (ng/g)	A_1	98.8 ± 2.0	85.2 ± 0.7	35.2 ± 1.5	11.6 ± 0.5	6.45 ± 0.36
	A_2	223.5 ± 4.8	198.1 ± 6.5	183.6 ± 3.2	73.4 ± 3.5	55.7 ± 1.8
	A_3	42.5 ± 1.7	42.7 ± 2.2	42.8 ± 2.4	37.7 ± 2.1	36.7 ± 0.5
V (ng/g)	A_1	14.7 ± 1.1	19.3 ± 1.3	20.8 ± 1.8	31.9 ± 2.5	33.1 ± 2.2
	A_2	17.3 ± 0.9	20.8 ± 1.8	65.7 ± 4.5	72.7 ± 2.2	72.7 ± 2.2
	A_3	37.6 ± 1.1	35.7 ± 2.4	40.2 ± 1.9	32.0 ± 1.8	24.4 ± 2.0
Co (ng/g)	A_1	7.33 ± 0.49	6.15 ± 0.04	4.61 ± 0.13	4.28 ± 0.12	4.63 ± 0.09
	A_2	4.72 ± 0.32	4.33 ± 0.08	3.71 ± 0.21	4.05 ± 0.17	4.53 ± 0.03
	A_3	13.9 ± 1.1	12.2 ± 0.2	8.38 ± 0.36	8.24 ± 0.22	5.84 ± 0.13
Ni (ng/g)	A_1	131.1 ± 3.3	119.6 ± 2.6	86.7 ± 1.9	77.9 ± 4.3	74.4 ± 2.4
	A_2	44.1 ± 1.3	39.5 ± 0.6	48.1 ± 0.9	46.9 ± 1.8	47.5 ± 1.7
	A_3	35.9 ± 2.4	36.2 ± 1.9	31.7 ± 2.0	22.9 ± 1.5	18.8 ± 1.3
As (ng/g)	A_1	31.9 ± 2.4	21.2 ± 1.7	18.5 ± 1.4	13.0 ± 1.1	11.5 ± 0.6
	A_2	19.9 ± 1.5	14.9 ± 0.9	13.4 ± 1.1	16.6 ± 1.1	19.2 ± 1.7
	A_3	27.2 ± 1.3	19.7 ± 0.8	19.1 ± 1.1	14.7 ± 0.9	8.59 ± 1.7
Cd (ng/g)	A_1	42.5 ± 2.2	25.0 ± 0.6	11.9 ± 0.6	6.84 ± 0.70	5.56 ± 0.51
	A_2	37.5 ± 1.4	20.3 ± 1.3	14.3 ± 1.4	9.27 ± 0.59	8.62 ± 0.37
	A_3	48.5 ± 1.9	28.3 ± 1.1	16.4 ± 1.1	14.2 ± 0.5	9.65 ± 0.31
Sb (ng/g)	A_1	2.27 ± 0.12	2.15 ± 0.20	1.49 ± 0.15	1.32 ± 0.23	1.07 ± 0.13
	A_2	1.56 ± 0.09	1.39 ± 0.09	1.29 ± 0.03	1.12 ± 0.09	0.48 ± 0.05
	A_3	1.33 ± 0.17	1.17 ± 0.15	1.08 ± 0.09	0.83 ± 0.11	0.69 ± 0.11
Pb (ng/g)	A_1	31.6 ± 1.4	14.6 ± 0.2	13.2 ± 0.8	7.46 ± 0.38	6.47 ± 0.37
	A_2	10.5 ± 0.3	11.7 ± 0.9	24.4 ± 0.7	33.9 ± 1.6	35.6 ± 1.0
	A_3	10.7 ± 0.8	9.43 ± 0.33	9.43 ± 0.33	5.14 ± 0.32	4.82 ± 0.23
U (ng/g)	A_1	17.0 ± 1.3	16.0 ± 0.6	8.81 ± 0.49	5.70 ± 0.45	4.06 ± 0.25
	A_2	1.68 ± 0.23	1.89 ± 0.24	2.44 ± 0.14	5.11 ± 0.51	5.27 ± 0.29
	A_3	2.36 ± 0.33	1.99 ± 0.17	0.98 ± 0.07	0.71 ± 0.08	0.44 ± 0.05
Chlorophyll (µg/g)	A_1	511± 5	459 ± 10	390 ± 13	344 ± 3	277 ± 7
	A_2	621 ± 11	503 ± 8	454 ± 14	373 ± 5	311 ± 4
	A_3	573 ± 7	487 ± 3	412 ± 86	365 ± 2	301 ± 2
Carotenoids (µg/g)	A_1	75.9 ± 3.6	60.9 ± 2.3	52.9 ± 1.8	47.9 ± 1.5	4.39 ± 1.66
	A_2	73.9 ± 2.7	60.6 ± 1.1	40.9 ± 1.1	36.1 ± 1.5	3.49 ± 1.22
	A_3	81.4 ± 0.9	73.0 ± 1.3	55.3 ± 1.4	50.9 ± 1.2	4.85 ± 1.04

Among the toxic elements, Cd and Pb content in lettuces from the three fields (A_1, A_2 and A_3) were below the maximum levels established by the official entities (566). Although several reports have been published regarding the presence of As, Sb and U in plant foods, no maximum levels have already been established. At the maturity state (T_5), Pb content was 6.47, 35.6 and 4.82 ng/g in lettuces from A_1, A_2 and A_3, respectively. These Pb levels were below the range 48.6 to 63.1 ng/g obtained by the analysis of 2303 leafy vegetables conducted by EFSA (619), and well below the maximum level for Pb (300 ng/g) laid down in EC (566). Uranium content ranged between 0.44 ng/g in lettuces from A_3 and 5.27 ng/g in lettuces from A_2. According to EFSA, more data is needed regarding U content in food at the European level in order to better understand the human exposition (618).

The chlorophyll content decreased in plants in an age-related manner (625). Moreover, both chlorophylls and carotenoids content showed to be dependent on both the total and relative ratio/content of several mineral elements in plants. Magnesium deficiency in plant leaves is usually followed by a decrease in the photosynthesis rate and finally by chlorosis (626). The Mg content decrease in all the lettuces along the study period could have a detrimental role in the decrease of chlorophyll content. The ratio of chlorophylls to carotenoids is an essential factor in maintaining the integrity of the photosynthetic system of plants. Hence, the equilibrium between chlorophylls and carotenoids levels is essential for plant homeostatic processes (627).

Regarding the changes in lettuce composition during growth, Ca, Mg, Mn, Co, Zn, Se, Mo, Cd, Sb, chlorophyll a + b and carotenoids all presented a similar pattern. Independently of the greenhouse field (A_1, A_2 or A_3), their content decreased along the time. Fe, Al, Cr, Ni, As, Pb, V and U also presented a similar pattern: their content increased along the study period in A_2 lettuces but not in A_1 and A_3 lettuces. Potassium levels increased along the time in all the fields. Sodium concentration decreased along the time in A_2 and A_3, but increased in A_1 lettuces. A_2 lettuces had the lower levels of chlorophylls and carotenoids. According to Nayek, Gupta (628), higher levels of toxic elements are correlated with lower amounts of these pigments in plants. In our study, a significant negative correlation ($p < 0.01$) was obtained between chlorophylls/carotenoids and all the toxic elements studied.

A PCA was performed using the content values for the 20 elements and the 2 pigments to reduce dimensionality of data with no significant loss of information and highlight the changes on lettuce composition during growth. The first two components achieved a significant reduction of the dimensionality of the original data set (70.7%). The score plot for this two PCs is presented in Figure 5.1. The variables that presented a loading higher than 0.5 on PC1 or on PC2 are shown on the axis edges, indicating the direction of the values increase. PC1 (accounting for 47.8% of the total variance) received a predominant

loading from Ca, Mg, K, Mn, Co, Zn, As, Se, Cd, Sb, chlorophylls and carotenoids. PC1 was almost entirely related with elements that increased or decreased in all lettuces independently of the experimental field. PC2 was mostly loaded by Fe, Al, V, Cr and Pb and explained 22.9% of the total variance. These elements increased along the growth period of A_2 lettuces, indicating that external factors, namely soil composition or element bioavailability, increased the soil-to-plant transference of these elements.

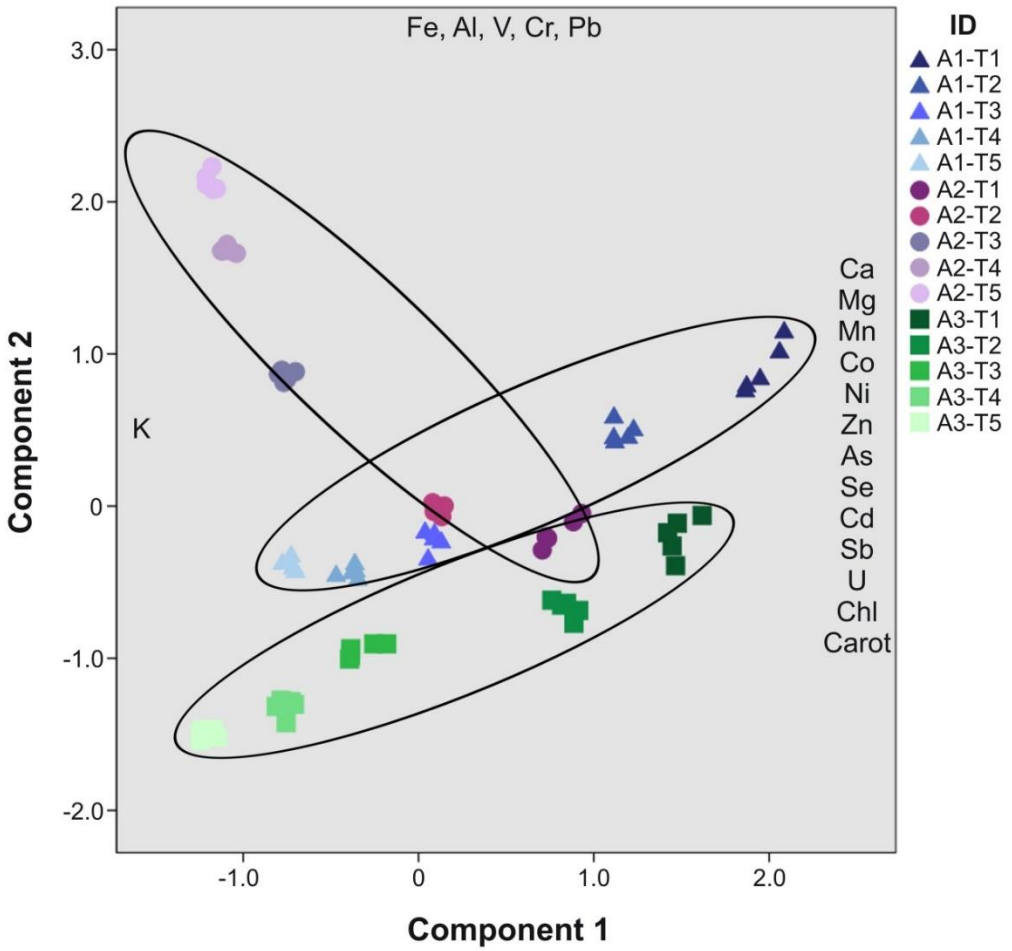


Figure 5.1 – Scores of major and trace elements and pigments of lettuce at 5 sampling time points on 2 principal components. The variables that presented a loading on PC1 or PC2 higher than 0.5 are shown on the axis edges indicating the direction in which the values increased.

5.3.2. Soil characteristics and elements bioavailability

The physicochemical properties (pH, OM, CEC, particle size distribution, EC and salinity) of the soil from the three greenhouse experimental fields are shown in Table 5.2. Soil pH(H_2O) decreased along the study period in all the three fields. At T_1, the soil pH was 7.49, 7.39 and 7.56 for A_1, A_2 and A_3, respectively, and decreased to 7.08, 6.85, and 6.96 at T_5. Soil pH($CaCl_2$) was generally 0.5 units lower compared to the pH(H_2O), but the same trend was observed. The highest content of OM was observed at A_2 (72.6%) and the lowest at A_3 (18.5%), whereas A_1 soil showed an intermediate average OM content (54.5%). A_1 and A_3 soils presented a similar CEC (7.86 cmol/kg and 7.88 cmol/kg, respectively), much lower than the CEC of A_2 soil (17.2 cmol/kg). Highest values of EC were also observed in A_2 (632.9 µS/cm) compared to A_1 (283 µS/cm) and A_3 (195.8 µS/cm) soils. The same behavior was observed for salinity with A_2 soil showing the highest values. Regarding the particle size distribution, A_1 and A_3 soils were classified as sandy soils and A_2 as loamy sand soil.

The total and NH_4NO_3-extractable fraction of major and trace elements in soils collected during the study period in A_1, A_2 and A_3 fields as well as the ratio extractable/total fraction ("bioavailable") are presented in Table 5.3. Regarding the total content, significant differences were observed between the three soils. Ca, Mg, Fe, Mn, Cu and Zn contents were higher at A_3 followed by A_1. For K and Al, A_2 soil showed the highest content and A_3 the lowest. Na content was higher in A_1 and lower in A_3. A_2 soil showed the lowest content of Cr, Co, Ni, As, Se, Cd and Sb, whereas A_3 soil showed the highest content of Co, As, Cd, and Sb. Overall, the total content of the different elements was maintained fairly constant in each field during the study period with a variation below 10% (RSD).

Table 5.2 – Main physicochemical properties of soils collected from the three greenhouse experimental fields (A_1, A_2 and A_3). Data are presented as mean ± SD (n = 5).

Soil ID	Sampling time	pH (H_2O)	pH ($CaCl_2$)	OM (%)	CEC (cmol/kg)	EC (µS/cm)	Salinity (ng/L)	Silt (%)	Clay (%)	Fine Sand (%)	Coarse Sand (%)
A_1	T_1	7.49 ± 0.03	7.10 ± 0.05	58.0 ± 0.7	7.7 ± 0.3	278 ± 18	0.13 ± 0.01	2.5 ± 0.2	2.0 ± 0.2	11.4 ± 1.5	84.0 ± 1.4
	T_2	7.41 ± 0.03	6.98 ± 0.04	55.0 ± 8.1	7.8 ± 0.1	316 ± 32	0.15 ± 0.02				
	T_3	7.38 ± 0.04	6.87 ± 0.03	49.3 ± 1.6	7.9 ± 0.2	319 ± 49	0.15 ± 0.03				
	T_4	7.26 ± 0.01	6.76 ± 0.06	52.2 ± 2.5	7.6 ± 0.4	257 ± 9	0.12 ± 0.01				
	T_5	7.08 ± 0.06	6.64 ± 0.05	57.9 ± 1.6	8.4 ± 0.1	246 ± 13	0.12 ± 0.01				
A_2	T_1	7.35 ± 0.01	6.86 ± 0.01	73.9 ± 2.4	16.3 ± 0.5	620 ± 12	0.30 ± 0.06	12.3 ± 0.1	12.2 ± 0.5	25.5 ± 0.9	50.0 ± 0.9
	T_2	7.29 ± 0.03	6.82 ± 0.05	72.6 ± 1.2	17.8 ± 0.5	670 ± 1	0.32 ± 0.01				
	T_3	7.22 ± 0.01	6.78 ± 0.01	69.9 ± 1.2	17.3 ± 0.3	626 ± 88	0.30 ± 0.04				
	T_4	7.12 ± 0.01	6.65 ± 0.02	71.7 ± 2.3	17.1 ± 0.4	597 ± 41	0.29 ± 0.02				
	T_5	6.75 ± 0.01	6.31 ± 0.04	74.9 ± 1.6	17.4 ± 0.3	651 ± 17	0.32 ± 0.01				
A_3	T_1	7.56 ± 0.08	7.02 ± 0.01	21.9 ± 1.0	8.7 ± 0.1	211 ± 31	0.09 ± 0.02	2.0 ± 0.5	2.2 ± 0.7	40.8 ± 2.4	55.0 ± 2.3
	T_2	7.40 ± 0.06	6.95 ± 0.03	18.5 ± 0.6	7.8 ± 0.1	218 ± 2	0.10 ± 0.02				
	T_3	7.32 ± 0.02	6.86 ± 0.08	16.6 ± 0.6	7.5 ± 0.1	208 ± 4	0.09 ± 0.01				
	T_4	7.28 ± 0.01	6.74 ± 0.04	21.0 ± 2.6	7.9 ± 0.2	182 ± 1	0.09 ± 0.01				
	T_5	6.96 ± 0.07	6.41 ± 0.02	14.5 ± 1.3	7.5 ± 0.4	159 ± 15	0.07 ± 0.01				

Table 5.3 – Total and NH_4NO_3-extractable content of elements in soils from the three experimental fields (A_1, A_2 and A_3) collected during the study period and the bioavailable fraction (extractable to total ratio). Data are presented as maximum and minimum values obtained in the five sampling time points (T_1, T_2, T_3, T_4 and T_5).

	Soil ID	Total content	NH_4NO_3-extractable	Bioavailable (%)
Ca (mg/g)	A_1	18.4 – 16.5	0.96 – 0.65	5.2 – 3.7
	A_2	7.44 – 6.86	1.49 – 1.12	21.8 – 15.0
	A_3	26.1 – 23.9	1.04 – 0.78	4.0 – 3.2
Mg (mg/g)	A_1	4.03 – 3.81	0.10 – 0.07	2.5 – 1.9
	A_2	2.64 – 2.46	0.21 – 0.19	8.5 – 7.1
	A_3	4.30 – 3.86	0.14 – 0.09	3.3 – 2.4
K (mg/g)	A_1	3.91 – 3.72	0.18 – 0.14	5.0 – 3.7
	A_2	4.52 – 4.07	0.44 – 0.36	10.2 – 8.3
	A_3	3.10 – 2.72	0.14 – 0.09	4.5 – 3.2
Fe (mg/g)	A_1	16.8 – 15.3	0.00033 – 0.00029	0.0022 – 0.0018
	A_2	15.1 – 13.8	0.00038 – 0.00029	0.0027 – 0.0019
	A_3	22.9 – 21.6	0.00038 – 0.00025	0.0016 – 0.0011
Al (mg/g)	A_1	23.9 – 20.1	0.00015 – 0.00012	0.0007 – 0.0006
	A_2	35.6 – 31.1	0.00025 – 0.00018	0.0008 – 0.0005
	A_3	16.4 – 13.3	0.00006 – 0.00005	0.0004 – 0.0003
Na (µg/g)	A_1	795.3 – 688.4	82.3 – 70.2	10.5 – 9.9
	A_2	688.2 – 563.9	224.7 – 161.9	32.6 – 28.7
	A_3	415.9 – 343.2	61.9 – 25.5	14.9 – 7.4
Mn (µg/g)	A_1	287.7 – 249.2	0.30 – 0.15	0.11 – 0.06
	A_2	147.9 – 137.4	0.20 – 0.09	0.15 – 0.06
	A_3	441.4 – 386.9	0.34 – 0.18	0.08 – 0.05
Cu (µg/g)	A_1	45.6 – 34.9	0.09 – 0.07	0.22 – 0.17
	A_2	18.7 – 16.1	0.06 – 0.05	0.37 – 0.29
	A_3	146.7 – 117.9	0.51 – 0.43	0.43 – 0.31
Zn (µg/g)	A_1	153.7 – 145.9	0.095 – 0.066	0.06 – 0.04
	A_2	92.1 – 86.0	0.034 – 0.026	0.04 – 0.03
	A_3	184.5 – 162.4	0.094 – 0.041	0.05 – 0.03
Cr (µg/g)	A_1	38.1 – 28.4	0.015 – 0.010	0.04 – 0.03
	A_2	20.5 – 16.9	0.018 – 0.007	0.10 – 0.03
	A_3	25.2 – 20.8	0.015 – 0.008	0.06 – 0.04
Co (µg/g)	A_1	4.92 – 4.33	0.008 – 0.005	0.16 – 0.11

	A_2	2.57 – 2.29	0.008 – 0.006	0.36 – 0.26
	A_3	6.61 – 6.13	0.009 – 0.004	0.14 – 0.06
Ni (µg/g)	A_1	18.7 – 18.1	0.06 – 0.03	0.33 – 0.19
	A_2	9.32 – 8.38	0.08 – 0.06	0.89 – 0.71
	A_3	16.2 – 15.2	0.05 – 0.03	0.30 – 0.17
As (µg/g)	A_1	9.4 – 6.8	0.060 – 0.018	0.63 – 0.27
	A_2	8.1 – 6.8	0.038 – 0.018	0.46 – 0.26
	A_3	19.4 – 17.5	0.123 – 0.026	0.64 – 0.15
Se (µg/g)	A_1	4.58 – 4.35	0.04 – 0.02	0.77 – 0.38
	A_2	3.67 – 3.48	0.04 – 0.02	1.11 – 0.52
	A_3	4.41 – 4.22	0.03 -0.01	0.63 – 0.19
V (µg/g)	A_1	24.5 – 22.3	0.018 – 0.008	0.08 – 0.03
	A_2	28.9 – 28.4	0.034 – 0.008	0.12 – 0.03
	A_3	25.2 – 22.5	0.012 – 0.007	0.05 – 0.03
Cd (ng/g)	A_1	290.0 – 264.0	1.95 – 0.47	0.67 – 0.16
	A_2	193.9 – 160.0	1.43 – 0.57	0.74 – 0.36
	A_3	652.2 – 584.1	2.55 – 0.61	0.39 – 0.10
Sb (ng/g)	A_1	120.5 – 98.0	4.46 – 2.91	3.82 – 2.97
	A_2	60.0 – 49.9	4.15 – 2.13	6.92 – 4.26
	A_3	142.0 – 111.6	3.21 – 1.89	2.26 – 1.70
Mo (ng/g)	A_1	941.5 – 681.9	28.9 – 16.4	3.06 – 2.41
	A_2	951.8 – 779.8	47.0 – 30.9	4.94 – 3.93
	A_3	654.2 – 510.0	22.8 – 15.7	3.65 – 3.08
Pb (µg/g)	A_1	18.7 – 16.1	0.0020 – 0.0006	0.011 – 0.004
	A_2	28.2 – 23.6	0.0024 – 0.0006	0.010 – 0.002
	A_3	19.5 – 17.1	0.0018 – 0.0006	0.009 – 0.003
U (µg/g)	A_1	3.60 – 3.08	0.0014 – 0.0012	0.039 – 0.038
	A_2	4.16 – 3.88	0.0016 – 0.0014	0.039 – 0.037
	A_3	4.73 – 4.29	0.0009 – 0.0007	0.020 – 0.016

Independently of the experimental field, the NH_4NO_3-extractable fraction for most elements (Ca, Mg, K, Na, Mn, Zn, As, Se, Mo Cd, Sb and U) decreased along the study period. In general, the concentration of major and trace elements was higher in the extracts from A_2 soil. The content of Fe, Al, Cr, Ni and Pb in the extracts increased along the study period only for A_2 soil.

Compared to A_1 and A_3, A_2 soil was characterized by a higher CEC, OM, clay and silt content and a lower pH. These soil parameters have a major influence in the extractability of most elements. In general, bioavailable fraction was higher in A_2 soil, although the total element content was lower compared to A_1 and A_3 soils (Table 5.2). CEC and pH are important parameters that control the bioavailability of elements in soil. In a study conducted by Vega, Andrade (62) about the influence of soil properties on the sorption and retention of metals, the authors also founded that CEC and soil pH were the most relevant parameters. When soil becomes acid, the bioavailability of metal cations generally increases due to the replacement of cations on soil binding sites by H^+ ions (580). CEC is also influenced by the OM content. OM content influences the mobility of elements in the soil through the formation of soluble/insoluble metal complexes. Soils with high OM content can immobilize both anionic and cationic species. Although the soil bioavailable fraction of elements includes only the water-soluble and exchangeable fractions, the OM-bound fraction can also become available to plants if the pH and redox potential of soil changes (81). The texture of soil is another important parameter that influences the distribution of elements. Finer particles fraction usually show a proportionally higher metal loadings, thus soils with higher clay content have more biding sites for ionic species, especially for cations (71). Soil salinity is a widespread limitation regarding agriculture use and can cause ion toxicity and nutrient imbalances in crops. Moreover, soil salinity influences the mobility of elements in soil solution (629). A_2 soil showed significantly higher values for both EC and salinity compared to A_1 and A_3 soils.

5.3.3. Elements transfer from soil to lettuces

The TF values of most elements showed a similar behavior through the study period for the three experimental fields. TFs for K increased from T_1 to T_5 (ranging from 15 to 23 for A_1, 16 to 19 for A_2, and 16 to 31 for A_3). Potassium has a major role in protein synthesis, enzyme activation and osmotic function. A high cytosolic concentration of K is required for protein synthesis in growing tissues, thus K in the vacuole not only represents a storage pool but also functions as a crucial osmoticum (232). Therefore, due to its essentiality, K uptake by plants is significantly higher compared to other essential elements. Sodium is chemically similar to K and presented TFs on the same order of magnitude, but with a decreasing trend. This element easily enters plants via non-selective cation transporters.

Together with K, Na can also have an important role in the activation of some enzymes and as an osmoregulator (10, 23).

Ca and Mg showed TFs values ranging from 1 to 3 in A_1 and A_2, and from 0.5 and 2.5 in A_3. A decreasing trend along the study period was observed for TFs of both elements. Ca and Mg are essential macronutrients for plants. Ca has an important function in the structural integrity of membranes and cell walls and Mg is known for its central position in the chlorophyll molecule, playing a key role in photosynthesis (10). Therefore, due to their essentiality, high amounts of these elements are taken up by plants in order to fulfill their nutritional requirements.

TFs were > 1 for Cd and Mo and decreased during the study period. All the other elements studied (Al, Fe, Cr, Co, V, Ni, Cu, Zn, Mn, As, Se, Sb, Pb, U) presented TF values < 1 and the general trend was for a decrease during the study period. The low TFs observed for these elements are understandable considering their (non)-essentiality and low bioavailability in soil. Several elements are required only in very small amounts (micronutrients) and some are non-essential for plants. Therefore, plants only take up low amounts of these elements. Generally, the bioavailable fraction of most of these elements is very low. Even for essential elements (e.g., Fe, Cu and Zn) a very small fraction is actually available for plant uptake. Thus, plants developed specific strategies to improve the uptake of those elements (166). The TFs for Fe, Al, Pb, V and U were very low (< 0.1) because the total fraction of these elements in the soil was very high compared with the content found in lettuce leaves. However, TFs for Fe, Al, Cr, Pb and V showed a distinct behavior in A_2 compared to A_1 and A_3. In A_2, a significant increase of the TF values was observed from T_1 to T_5.

Multiple linear regression analysis with stepwise variable selection was used to estimate the content of specific group of trace elements (Al, Cr, Fe, V and Pb). The total concentration of each element in the soil, the respective extractable fraction and other soil parameters such as pH(H_2O), pH($CaCl_2$), OM, CEC, EC, salinity, silt, clay, fine sand and coarse sand were used as variables. The general formula of the estimation equation is as follows:

$$y = a1\ x1 + a2\ x2 + ... + an\ xn$$

where y is the trace element content and x1, x2,... xn are the soil parameters. The regression equations obtained for Al, Cr, Fe, V and Pb are summarized in Table 5.4. The concentrations of these trace elements in lettuces were significantly correlated with the respective extractable fraction in soil; however, other soil parameters also showed to

influence the elements transfer from soil to lettuces, namely, pH($CaCl_2$) for Fe and Cr, silt and fine-sand for Al and V, OM for Al and Pb, and coarse sand and CEC for Cr.

Table 5.4 – Estimation of the content of a specific group of trace elements by multiple linear regression analysis with stepwise variable selection. Total and extractable content of each trace element in the soil as well as the main physicochemical soil parameters were used as variables.

Element	Relevant parameters	Regression equation	r	Estimation Error
Fe	Fe-extractable fraction pH($CaCl_2$) salinity	y = 26.11 – 0.068 Fe-extractable fraction – 5.79 pH($CaCl_2$) + + 5.4 salinity	0.82	2.01 µg/g
Al	Al-extractable fraction Silt Fine-sand OM	y = – 29.18 + 0.19 Al-extractable fraction – 2.33 silt + 0.52 fine-sand + 0.24 OM	0.93	2.43 µg/g
V	V-extractable fraction Fine-sand Silt	y = – 10.33 + 2.21 V-extractable fraction + 0.60 fine-sand – 0.52 Silt	0.99	3.51 ng/g
Pb	Pb-extractable fraction OM pH (H_2O)	y = 95.35 + 12.481 Pb-extractable fraction + 0.16 OM – 13.94 pH (H_2O)	0.90	4.69 ng/g
Cr	Cr-extractable fraction Coarse-sand pH ($CaCl_2$) CEC	y = 366.91 + 13.20 Cr-extractable fraction – 1.35 coarse-sand – 41.24 pH ($CaCl_2$) – 1.19 CEC	0.94	15.9 ng/g

5.4. Conclusions

This study highlights that lettuce nutritional value and potential toxicity strongly depends on growth stage and soil composition. Higher contents of macrominerals, trace elements and photosynthetic pigments were observed in young lettuces. Only K content increased along the study period (2 to 10 weeks of plants growth) in the lettuces from the three experimental fields. For several trace elements (Al, Cr, Fe, V and Pb) their content increased but only in A_2 lettuces. A_2 soil presented the lowest total content of macrominerals and trace elements but the highest NH_4NO_3-extractable fraction when compared with the other two soils (A_1 and A_3). Additionally, in A_2 soil the bioavailable fraction of Al, Cr, Fe, V and Pb increased along the study period. This soil was classified as loamy sand soil and presented higher CEC, OM and lower pH when compared with A_1 and A_3, which were both sandy soils. The estimation of the concentration of these specific group of trace elements (Al, Cr, Fe, V and Pb) in lettuce by multiple linear regression analysis with stepwise variable selection confirmed that besides element extractable fraction, other soil parameters play a key role in the soil-to-plant transfer, namely, pH($CaCl_2$) for Fe and Cr, silt and fine-sand for Al and V, OM for Al and Pb and coarse-sand and CEC for Cr.

Chapter 6

Comparison between the mineral profile and nitrate content of microgreens and mature lettuces

Abstract

Microgreens are a new class of edible vegetables. They correspond to plants harvested when seed-leaves have fully expanded and before true leaves have emerged, and are gaining increasing popularity as new culinary ingredients. However, studies regarding the differences between microgreens and mature plants are scarce. Thus, the goal of this work was to perform a comparison between the mineral profile and NO_3^- content of microgreens and mature lettuces. Results showed that microgreens present a higher content of most minerals studied (Ca, Mg, Fe, Mn, Zn, Se and Mo) and a lower NO_3^- content than mature lettuces. Therefore, microgreens can be considered as a good source of minerals in human diet and, particularly in children, their consumption can be seen as an important strategy to meet minerals dietary requirements without exposing them to the harmful NO_3^-. This work provides original and important data on the nutritional value of microgreens and shows that their consumption can be a cost-effective answer to the problem of mineral malnutrition.

6.1. Introduction

Mineral malnutrition is a common problem in both developed and developing countries. It is estimated that over 60% of the world's 7 billion people are Fe deficient, over 30% are Zn deficient and 15% are Se deficient. Additionally, Ca, Mg and Cu deficiencies are also common in several developed and developing countries (13). Nowadays, mineral malnutrition is considered to be one of the most important global challenges to mankind that can be prevented (630).

Vegetables are a good source of vitamins and minerals and are known to have protective benefits against oxidative stress-related diseases (631, 632). Several studies have shown that vegetables consumption is associated with a reduction in the development of chronic diseases, such as cancer and cardiovascular disease (633, 634). FAO/WHO stressed the need to increase consumption of fruits and vegetables highlighting that they are important components of a healthy diet (635). Therefore, their production, quality and adequate consumption should be promoted.

However, as mentioned in Chapter 2, vegetables can also be an important source of nitrate (NO_3^-), mainly due to the use of agricultural fertilizers. NO_3^- can accumulate in plants and cause health problems (29, 636). Nitrate itself is relatively non-toxic but about 5% of the ingested NO_3^- is reduced in gastrointestinal tract to the more toxic nitrite anion (NO_2^-). In fact, it is the NO_3^-–metabolites, e.g., NO_2^-, nitric oxide (NO) and N-nitroso compounds, which are considered toxic and can lead to human disorders, namely methaemoglobinaemia. This is of particular concern in infants and children (29, 30) and

several reports of methaemoglobinaemia in children after the consumption of vegetables are available (637-639). Thus, reduction of NO_3^- intake associated to vegetables consumption is advisable.

Microgreens are a new class of edible vegetables harvested at the soil level when cotyledon (seed-leaves) have fully expanded and before true leaves have fully emerged, which usually occurs within 7 to 14 days after germination (640). Their size is usually between 3 and 10 cm in height, depending on the plant species. Over the past few years, microgreens have been gaining increasing popularity as new culinary ingredients due to their wide range of intense flavors, attractive colors and tender textures. Microgreens can be served in salads, soups, sandwiches and as main dishes (616).

Lettuce contains several elements (e.g., Ca, Mg, K, Fe, Mn, Cu and Zn) that are considered essential for humans. Until now, the few studies performed with microgreens have only addressed a limited set of phytonutrients. Sun, Xiao (640) showed that microgreens from Brassica species are good sources of polyphenols. Recently, Xiao, Lester (616) studied 25 commercially available microgreens regarding their content of ascorbic acid, carotenoids, phylloquinone and tocopherols. Comparison between nutritional composition of mature plants and microgreens indicated that the last had higher nutritional densities. Younger leaves of baby spinach (*Spinacia oleracea L.*) also showed to have higher levels of phytonutrients (ascorbic acid, carotenoids, folate, α-tocopherol and phylloquinone) than more mature leaves (641). To our knowledge, no studies are available comparing the nutritional value and safety of microgreens and mature plants. The aim of this study was to compare their mineral profile and NO_3^- content.

6.2. Experimental

6.2.1. Lettuce sampling

Lettuce (*L. sativa L. var. capitata*) seeds were germinated in three greenhouse experimental fields – A_1 (41º 26.991 N, 8º 46.335 W), A_2 (41º 25.249 N, 8º 44.936 W) and A_3 (41º 27.435 N, 8º 45.377 W) – located in the NW Portugal. Ten units (or 1 kg) of lettuce plants were randomly harvested after 2 and 10 weeks of germination. All plants were exposed to the same light intensity and photoperiod from December to February. The samples collection was performed during the same period of the day (9 – 12 am). Plant samples were placed into plastic containers, previous rinsed with diluted nitric acid (10% v/v) and deionized water, and kept at 4 ºC on the way to the laboratory. At the laboratory, plant samples were cleaned with a brush and thoroughly washed with ultra-pure water to remove soil contamination, frozen at –80 ºC and then freeze-dried. The dried samples

were homogenized by grinding in a blender and sieved through a nylon sieve of 150 µm mesh size.

6.2.2. Apparatus and reagents

A Telstar (Terrassa, Spain) Cryodos-80 freeze-drying system was used to lyophilize the lettuce samples. The moisture content was determined by using an HR73 Moisture Analyzer from Mettler Toledo (Greifensee, Switzerland). A Milestone (Sorisole, Italy) MLS 1200 Mega high performance microwave digestion unit equipped with an HPR-1000/10 S rotor was used for acid digestion of plant samples. A K-424 digestion unit and a KjelFlex K-360 distillation unit coupled to a B-414 scrubber, all from Büchi (Postfach, Switzerland), were used to perform the N-Kjeldahl determination. A GFL (Burgwedel, Germany) 1083 shaking water bath was used to carry out NO_3^- extraction. Only plastic labware – pipette tips (VWR, Radnor, PA), volumetric flasks (Kartell, Milan, Italy) and centrifuge tubes (TRP, Trasadingen, Switzerland) – was used during the work. All solutions were prepared using ultrapure water (> 18.2 MΩ.cm at 25 °C) obtained with a Milli-Q RG (Millipore, Billerica, MA) water purification system.

A chromatographic system consisting of a Jasco (Tokyo, Japan) PU-2089_{Plus} gradient pump, a Waters (Milford, MA) IC-PAKTM anion-exchange column (4.6 × 150 mm) and a Waters model 431 conductivity detector was used for NO_3^- determination. Data acquisition was done with Borwin PDA Controller Software (JMBS Developments, Grenoble, France). The eluent was a 1.3-mM sodium gluconate / 1.3-mM borax solution adjusted to pH 8.5. A standard stock solution of NO_3^- was prepared from its analytical reagent grade sodium salt.

ICP–MS analyses were performed using a VG Elemental (Winsford, UK) PlasmaQuad 3 (quadrupole-based) instrument under the instrumental conditions and using the reagents described in Chapter 4, section 4.2.2. The elemental isotopes (m/z ratios) ^{31}P, ^{55}Mn, ^{65}Cu, ^{66}Zn, ^{82}Se and ^{95}Mo and the internal standards ^{45}Sc, ^{89}Y and ^{115}In were monitored. The instrument was tuned daily for maximum signal sensitivity and stability using ^{115}In as the target isotope. Internal standards and tuning solutions were prepared by appropriate dilution of the corresponding AccuStandard® (New Haven, CT) solutions (ICP-MS-200.8-IS-1: 100 µg/mL of Sc, Y, In, Tb and Bi; and ICP-MS-200.8-TUN-1: 10 µg/mL of Be, Mg, Co, In and Pb). Calibration standards were prepared from AccuStandard® 10 µg/mL multi-element ICP-MS standard solution (ICP-MS-200.8-CAL1-1). High purity HNO_3 (65% w/w, *Trace*SELECT® Ultra, Fluka, L'Isle d'Abeau Chesnes, France) and H_2O_2 (30% v/v, *Trace*SELECT®, Fluka, Seelze, Germany) were used as received.

AAS analysis were carried out using a Perkin Elmer (Überlingen, Germany) 3100 flame (air-acetylene) instrument for the determination of Ca, Mg, Na, K and Fe. Multi-element

calibration standards were prepared from 1000 mg/L single-element standard stock solutions (Sigma-Aldrich, St. Louis, MO) of those elements.
Certified reference material (CRM) BCR 679 (white cabbage, supplied by EC Institute for Reference Materials and Measurements, Geel, Belgium) was used to check the accuracy of the analytical procedures.

6.2.3. Lettuce analysis

The extraction of NO_3^- and NH_4^+ from lettuce samples was performed as follows: Freeze-dried plant samples (0.1 to 1 g) were transferred into a 125 ml conical flask, about 100 ml of hot water (70 to 80 ºC) were added and the mixture was heated for 15 min in a boiling water bath with reciprocal shaking. After cooling, the content was transferred to a 200 ml volumetric flask and diluted to the mark with ultra-pure water. Plant extracts were then analyzed by ion chromatography for NO_3^- content. Ammonium was determined by the spectrophotometric method (642). Nitrogen was determined by the Kjeldahl method (600).
The microwave-assisted digestion procedure described in Chapter 3, section 3.2.4., was used to obtain total metal content in plant samples. After digestion, samples were transferred into 25 ml volumetric flasks and the volume was made-up with ultrapure water. Solutions were then analyzed by AAS and ICP-MS for total metal content. All the plant samples were analyzed in quintuplicate. The CRM was analyzed three times. Results were expressed on a fresh weight (fw) basis.

6.2.4. Estimated daily intake of major and trace elements from lettuce

The estimated daily intake (EDI) of minerals resulting from lettuce consumption was calculated. The EDI, which is dependent on both the mineral content of the edible part of the plants ($C_{mineral}$; mg/g fw basis) and the average daily consumption of lettuce ($DC_{lettuce}$), was calculated using the following formula:

$$EDI = DC_{lettuce} \times C_{mineral}$$

where EDI is expressed as mg (or µg)/day and DC was assumed to be 22.5 g/day for adults (583, 617).

6.2.5. Quality control

For the quality control of analytical determination of total metal content, the certified reference material BCR 679 was used. It was analyzed after being subjected to the same pretreatment as for the samples, and the values obtained proved the accuracy of the whole analytical procedure (Table 6.1).

Table 6.1 – Concentrations obtained for minerals determination in BCR 679 (certified reference material), expressed as mg/kg.

	Certified value ± SD	Experimental value (mean ± SD, n = 3)
Ca	7768 ± 655	7998 ± 41
Mg	1362 ± 127	1305 ± 7
P	3307 ± 241	3341 ± 27
Fe	55.0 ± 2.5	54.7 ± 1.4
Mn	13.3 ± 0.5	13.0 ± 0.1
Cu	2.89 ± 0.12	2.80 ± 0.06
Zn	79.7 ± 2.7	80.5 ± 0.5
Mo	14.8 ± 0.5	15.1 ± 0.2

6.2.6. Statistical Analysis

Data exploration, descriptive statistics calculation and *t*-test were performed with IBM SPSS Statistics for Windows, version 22.0 (IBM Corp, Armonk, NY). Statistical differences were assumed at $p < 0.05$.

6.3. Results and Discussion

6.3.1. Mineral content of microgreens and mature lettuces

Microgreens and mature lettuces studied were grown in 3 different greenhouse experimental fields in order to take into account the soil mineral variability. The moisture content of microgreens and mature lettuces was similar (94.8 ± 0.3 and 94.3 ± 0.5 %, respectively). No significant difference was also observed in the moisture content of lettuces from the different fields. Figure 6.1 shows the mean mineral content in both microgreens and mature lettuces from the three fields. Except for Mg, Na and Cu, significant differences were observed between microgreens and mature lettuces for all the other minerals. Mature lettuces presented higher N, P and K content. All the other elements (Ca, Mg, Fe, Mn, Zn, Se and Mo) were present at higher levels in microgreens. The mean content of all the elements in mature lettuces were within the typical ranges described in the literature (614, 643, 644). The mean Na content in mature lettuces (627.9 ± 29.3 μg/g) was almost 13-fold higher than the value reported by Kawashima and Soares (614) for butterhead lettuce (50 μg/g). Regarding microgreens, few data is available on

their mineral content, which limits data comparison. Recently, Santos, Oliva-Teles (645) quantified several minerals (P, K, Ca, Mg, Na, Fe, Mn, Zn and Cu) in ready-to-eat ‘‘baby leaf’’ vegetables. The results obtained in our study are in good agreement with the values reported by those authors.

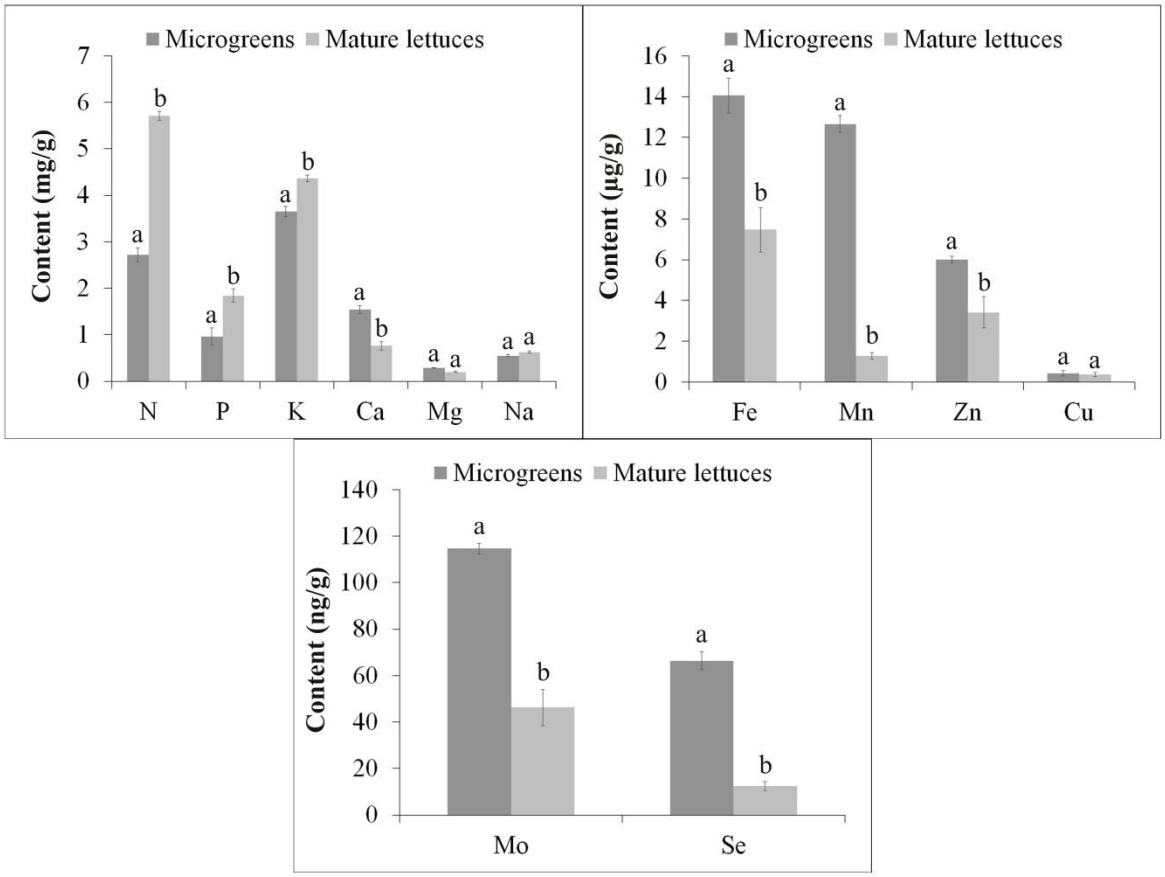


Figure 6.1 – Comparison between the mineral content of microgreens and mature lettuces. Data presented as mean ± SD of the results obtained for the samples of the three experimental fields. Different letters in the bars of each mineral indicate a significant difference at $p < 0.05$, *t*-test.

6.3.2. Estimation of the mineral daily intake from microgreens and mature lettuces

The mean values for the EDI of nutritional minerals resulting from the consumption of microgreens and mature lettuces are shown in Table 6.2. Overall, microgreens showed to provide a higher daily intake of most minerals (Ca, Mg, Fe, Mn, Cu, Zn, Se, Mo) than mature lettuces (EDIs of N, P, K and Na were higher for mature lettuces). Specifically, microgreens consumption can provide twice as much Ca and Fe, almost three-fold as much Mo, five-fold as much Se and nine-fold as much Mn than mature lettuces. This

information is a breakthrough in the field of human nutrition. Our study shows that the consumption of microgreens can be a cost-effective solution for reducing the problem of mineral malnutrition.

Table 6.2 – Estimated daily intake (EDI) of nutritional minerals resulting from the consumption of microgreens and mature lettuces based on an average daily intake of 22.5 g/person.

Element	EDI (mg/day)				
	Microgreen lettuces	Mature lettuces	Microgreen/mature lettuces ratio	RDA/**AI** [a]	
				Men	Women
N	82.1	98.1	0.8	-	-
P	21.7	41.5	0.5	700	700
Ca	34.8	17.2	2.0	**1000**	**1000**
Mg	6.5	4.5	1.4	420	320
K	82.1	98.1	0.8	4700	4700
Na	12.4	14.1	0.9	1500	1500
Fe	0.32	0.17	1.9	8	18
Mn	0.28	0.03	9.3	**2.3**	**1.8**
Zn	0.13	0.08	1.6	11	8
	EDI (µg/day)				
Cu	9.6	8.5	1.1	900	900
Se	1.49	0.28	5.3	45	55
Mo	2.58	1.04	2.5	45	45

[a] RDAs (Recommended Dietary Allowances) and AIs (Adequate Intakes) may both be used as goals for individual intake. RDAs are in regular type and AIs in **bold** type.

6.3.3. Nitrate content of microgreens and mature lettuces

Both NO_3^- and NH_4^+ content was determined in microgreens and mature lettuces of the three fields. As shown in Figure 6.2, higher NO_3^- and NH_4^+ contents were observed in mature lettuces. It is important to know which form of inorganic N (NO_3^- or NH_4^+) is present in plant foods since NO_3^- can accumulate in vegetables, leading to the toxic effects mentioned above. A maximum tolerable level of NO_3^- in vegetables is set at 4500 mg/kg by the EU Regulation (566). NO_3^- content of both microgreens and mature lettuces was below this limit; however, mature lettuces provides four-fold as much NO_3^- than microgreens. The accumulation of NO_3^- in leaf crops (e.g., cabbage, spinach, lettuce) is

reported in the literature (30, 646). Since infants and children are particularly susceptible to methaemoglobinaemia and there are reports describing the development of methaemoglobinaemia in children after the consumption of vegetables (637-639), microgreens may be regarded as a good alternative to reduce NO_3^- intake while increasing the intake of essential minerals.

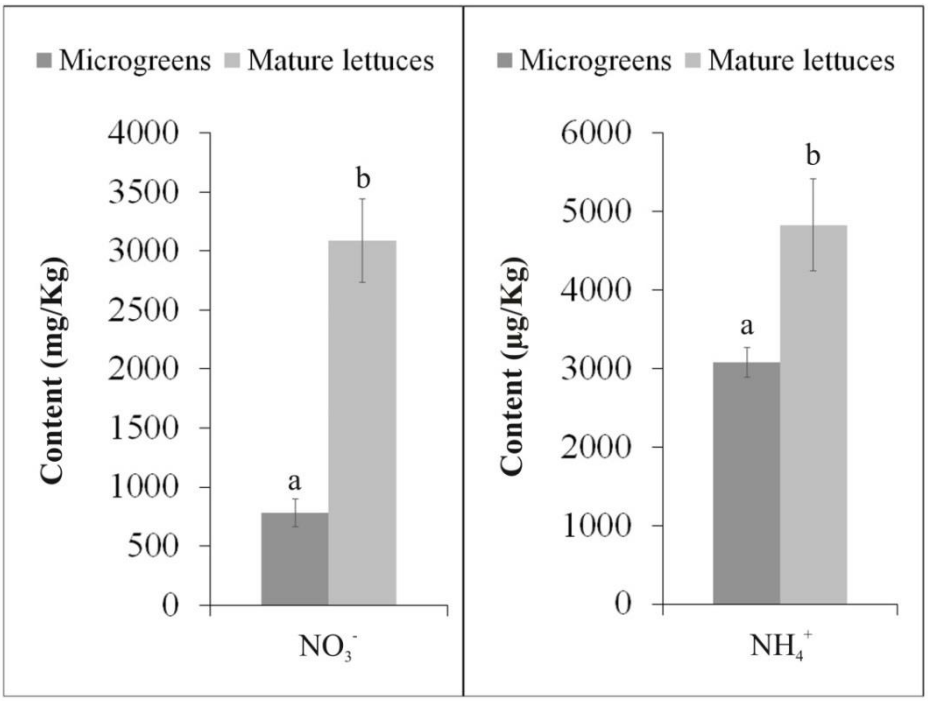


Figure 6.2 – Comparison between NO_3^- and NH_4^+ content of microgreens and mature lettuces. Data presented as mean ± SD of the results obtained for the samples of the three experimental fields. Different letters in the bars of each compound indicate significant difference at $p < 0.05$ (*t*-test).

6.4. Conclusions

This is the first study that compared the mineral profile and NO_3^- content of microgreens and mature lettuces. The results obtained showed that microgreens can provide a significantly higher intake of essential minerals than mature lettuces. Mature lettuces had higher N, P, K and Na content, but Ca, Mg, Fe, Mn, Zn, Se and Mo content was higher in microgreens. Microgreens can be considered a good source of minerals to reduce mineral malnutrition in humans. Additionally, the NO_3^- content of microgreens showed to be very low. Thus, microgreens can be safely used in children's diet to fulfill their daily mineral requirements, while reducing exposure to the harmful NO_3^-. This work provides important data on the nutritional value of microgreens.

PART IV

N metabolism of plant foods during growth

Chapter 7

Influence of the temporal and spatial variation of nitrate reductase, glutamine synthetase and soil composition in the N species content of lettuce (*Lactuca sativa*)

Abstract

Changes in nitrate reductase (NR), glutamine synthetase (GS) and N content in lettuce were evaluated at 5 sampling time points. Soil physicochemical properties and N content were also assessed in order to elucidate the soil-to-plant transfer of inorganic N and potential leaching to groundwater. A decrease of NR activity and an increase of NO_3^- and N-Kjeldahl content in lettuces were observed during plant growth, whereas GS activity and NH_4^+ content increased during the first weeks of lettuce growth and then decreased. Although the temporal changes were similar in lettuces grown in different soils, quantitative differences were observed, indicating that high NO_3^- content in soil causes a higher NO_3^- accumulation in lettuce despite the higher NR activity during the first sampling time points. Higher levels of NO_3^- and NH_4^+ were correlated with higher levels of N-Kjeldahl in lettuce suggesting a positive effect of these N species in the biosynthesis of organic N forms. Soil physicochemical properties influenced the mobility of inorganic N within the groundwater-soil-plant system. Sandy soils with low OM content allowed NO_3^- leaching, which was confirmed by higher NO_3^- levels in groundwater. Therefore, lettuces grown in those soils presented lower N content and the inputs of N to the environment were higher.

7.1. Introduction

Nitrogen (N) supply is a ubiquitous and fundamental piece to sustain the increase of crop yields (647). Since N phytoavailability is low in most terrestrial ecosystems, and that can limit plant growth, most farmers rely on the application of fertilizers to support crops productivity (636). In the year 2000, the total N input in European agriculture was around 24.5 Mton/year. From that amount, N uptake by plants was only about 54%, which leads to total N excess varying from 10 to 13 Mton/year (648). N added to agricultural systems is highly mobile and reactive. Its speciation forms are biologically active in soil, water and atmosphere. Thus, elevated N inputs in soil pose an important pressure on the environment (649).

Plants need larger quantities of N compared to other essential elements. Most plants absorb N soluble forms such as ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), amino acids and urea that are usually available in the soil solution (650). Nevertheless, the preferred form depends generally on the form of N supplied to soil, plant species, environmental conditions, light intensity, soil properties and root zone temperature (31, 651). Overall, plant roots take up NO_3^- and NH_4^+ as the major sources of inorganic N. However, their phytoavailability changes between different environments (10). In aerobic and well-drained soils, NO_3^- is the major form of inorganic N and plants take up NO_3^-

preferentially. By contrast, in environments where nitrification process is inhibited, due to anoxic and/or acid conditions, NH_4^+ predominates and plants favor the uptake of this N form (652).

Nitrate plays a key role in the function and nutrition of plants. On the other hand, NO_3^- accumulation can occur in leaf crops such as lettuce and spinach. Nitrate reductase (NR) and glutamine synthetase (GS) are crucial metabolic enzymes involved in the conversion of simple inorganic N species (653). Nitrate reductase (NR, EC 1.6.6.1) is the first enzyme involved in NO_3^- assimilation by plants. NR reduces NO_3^- into NO_2^- a reaction catalyzed by NAD(P)H that occurs in the cytosol of the cells and acts as a central point in the plant metabolism by governing the flux of N by several regulatory mechanisms (533). In plants, both roots and shoots are able to metabolize NO_3^-. The rate of NO_3^- conversion in shoots and roots depends on environmental factors (e.g., temperature), type and amount of N supply and plant species and age (654). NR is a cytosolic enzyme and has been labeled as the rate-limiting step of the NO_3^- assimilation pathway, which results in the formation of NH_4^+, and has been considered a limiting factor for the plants growth. According to Taghavi and Babalar (655), NR is NO_3^-–inducible and there is a close relationship between NR activity and NO_3^- concentration in plants. Glutamine synthetase (GS, EC 6.3.1.2) is a crucial enzyme in the NH_4^+ assimilation. GS catalyzes the ATP-dependent fixation of the δ-carboxyl group of glutamate to form glutamine, which then provides N groups, directly or through glutamate synthase (GOGAT, EC 1.4.7.1), for the biosynthesis of other N-containing organic molecules. The metabolism of NH_4^+ into organic N-compounds is the main mechanism of assimilation and detoxification of NH_4^+ in plants (656).

To our knowledge, no studies exist regarding the temporal variation of NR and GS activity as a function of the accumulation of N-compounds the during plant growth and the influence of soil N phytoavailability on that activity. Therefore, the aims of this study were: (1) to evaluate the influence of NR and GS activity in the accumulation of NO_3^-, NH_4^+ and organic-N in lettuce grown in three different agricultural soils; (2) to assess the contribution of soil inorganic N to the content of NO_3^- and NH_4^+ in lettuce; and (3) to estimate the influence of the soil composition in the mobility of inorganic N within the soil-plant-groundwater system.

7.2. Experimental

7.2.1. Plant and soil sampling

Lettuce (*L. sativa L. var. capitata*) cultivation as well as plant and soil sampling were performed as described in Chapter 3, section 3.2.1. All plants were exposed to the same light intensity and photoperiod from December to February. The collection of plant samples was performed during the same period of the day (9 – 12 am). Simultaneously, soils were sampled with a stainless steel auger at 0 – 20 cm depth from the same locations where plants were harvested. A zigzag pattern was used for field sampling and all samples were combined to generate a composite sample of each field. Plant and soil samples were placed into plastic containers, previous rinsed with diluted nitric acid (10% v/v) and deionized water, and kept at 4 ºC on the way to the laboratory. Groundwater samples (1.5 L) were also collected from drilled wells located on the three experimental fields.

7.2.2. Sample preparation

For the enzymatic assays, plant samples were cleaned to remove any detritus and leaves from at least five different plants were taken and grinded with liquid N_2 and the resulting powder was stored at –80 ºC until used.

For physicochemical analysis, plant and soil samples were prepared as described in Chapter 3, section 3.2.1.

7.2.3. Reagents and apparatus

All solutions were prepared using ultrapure water (>18.2 MΩ cm at 25 °C) obtained with a Milli-Q (Milipore, Billerica, MA) water purification system. A GFL 1083 shaking water bath (Burgwedel, Germany) was used to perform the extraction of NO_3^- from plant samples. A PU-2089_{Plus} gradient pump (Jasco, Tokyo, Japan) was used in conjunction with an IC-PAK^{TM} anion-exchange column (4.6 × 150 mm). Detection was done with a Waters model 431 conductivity detector. Data acquisition was performed with Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France). The eluent was a 1.3-mM sodium gluconate / 1.3-mM borax buffer solution adjusted to pH 8.5. Standard solutions of NO_3^- were prepared from the analytical reagent grade sodium salt. Ammonium, NR and GS measurements were performed with a Shimadzu (Tokyo, Japan) UV-1800 spectrophotometer. A K-424 digestion unit and a KjelFlex K-360 distillation unit coupled to a B-414 scrubber (all from Buchi, Switzerland) were used to perform the N-Kjeldahl and the total N determination in plant and soil samples, respectively. Moisture content was

determined with the HR73 Moisture Analyzer from Mettler Toledo (Greifensee, Switzerland).

7.2.4. Plant samples analysis

7.2.4.1. Measurement of NR activity

In vitro NR activity was evaluated according to Savidov, Tokarev (657) with some modifications: samples (ca. 0.5 g, fresh weight) were homogenized at 4 ºC with 3 ml of extraction medium containing 25 mM Tris-HCl (pH 8), 1 mM EDTA, 1 mM DTT, 10 mM cysteine, 1 mM PMSF, 5 µM sodium molybdate, 20 µM FAD and 5% (w/v) PVPP. A protease inhibitor cocktail tablets solution (Complete^{TM} Mini, Roche) was prepared according with the manufacture instructions and added to the extraction medium to a final concentration of 1.4 tablets per 10 ml. The resulting homogenate was then centrifuged at 45000 g for 30 min at 4 ºC. Aliquots of supernatant were used for the determination of protein content (658) and the assay of NR activity (0.5 ml). The reaction mixture of the latter consisted of 0.1 M HEPES buffer (pH 7.0), 15.4 mM KNO_3, 0.227 mg/ml NADH and 22 nM FAD. After incubation at 28 ºC for 30 min, the reaction was ended by the addition of 0.1 ml of 1 M zinc acetate and 0.1 ml of 0.3 mM phenazinemethosulfate. After 30 min, the NO_2^- produced was colorimetrically measured at 543 nm after the addition of 1% (w/v) sulphanilamide in 2 M HCl and 0.02% (w/v) N-(1-naphthyl)-ethylene-diammonium dichloride. NR activity was expressed as nkat NO_2/mg protein.

7.2.4.2. Measurement of GS activity

Tissue samples (ca. 0.5 g, fresh weight) were homogenized at 4 ºC with 2 volumes (w/v) extraction buffer (25 mM Trizma [pH 8.0], 10 mM MgCl2, 1 mM DTT and 10% glycerol), and 10% (w/v) PVPP. Afterwards, homogenates were centrifuged 2 times at 12 000 g for 15 min and the supernatant was recovered. GS activity was determined in the supernatant through the transferase assay according to Teixeira, Pereira (659) and expressed as nkat/mg protein.

7.2.4.3. Determination of plant nitrate, ammonium and N-Kjeldahl content

The extraction of NO_3^- and NH_4^+ from plant samples was performed according to ISO [20] with some modifications. Freeze-dried plant samples (0.1 to 1 g) were transferred into a 125 ml conical flask, about 100 ml of hot water (70 to 80 ºC) were added and the flask was heated for 15 min in a boiling water bath with orbital shaking. After cooling, the content was transferred to a 200 ml volumetric flask and the volume was made up to the mark with deionized water. Plant extracts were analyzed for NO_3^- content by ion chromatography. Ammonium content was determined by the spectrophotometric method

(642). Nitrogen was determined by the Kjeldahl method (600). Results were expressed in a fresh weight (fw) basis.

7.2.5. Soil and water samples analysis

The physicochemical characteristics of soil samples, i.e., pH, organic matter (OM), cation exchange capacity (CEC) and particle size distribution were determined as described in Chapter 3, section 3.2.3. Total N content was determined according to ISO (601). NO_3^- and NH_4^+ were extracted from soil samples with 1 M KCl solution at a ratio 1:5 (w/v), for 1 h at 20 ºC. The solution was centrifuged at 3000 g for 10 min and the NH_4^+ was measured in the supernatant by the spectrophotometric method (642). The same extraction procedure was performed using ultra-pure water for NO_3^- determination by ion chromatography.

Nitrate in groundwater was determined directly by ion chromatography.

7.2.6. Statistical analysis

Data exploration, descriptive statistics calculation, ANOVA and correlation matrix were performed with IBM SPSS Statistics for Windows, version 22.0 (IBM Corp, Armonk, NY). Statistical significance was assumed for $p < 0.05$, unless otherwise noted.

7.3. Results

7.3.1. Lettuce leaves biomass and moisture content

Table 7.1 shows the lettuce leaves biomass and moisture content along the studied period in the three soils (A_1, A_2 and A_3). Leaves biomass increased significantly at T_3, T_4 and T_5 in all the studied soils. Only between T_1 and T_2 no significant differences were observed.

Moisture content of lettuces grown in the A_1, A_2 and A_3 soils at the five different study periods was 94.3 ± 0.1%. No significant differences were observed in the lettuce moisture content of samples from the 3 different soils and collected at the 5 different study time points.

Table 7.1 – Leaves biomass and moisture content of lettuces grown in the three soils (A_1, A_2 and A_3) collected along the sampling period (T_1, T_2, T_3, T_4 and T_5).

	Soil ID	Sampling time				
		T_1	T_2	T_3	T_4	T_5
Leaves biomass (g FW/plant)	A_1	1.91 ± 0.04a*	6.69 ± 0.25a*	24.4 ± 1.45b*	56.7 ± 3.53c*	174 ± 7d*
	A_2	1.85 ± 0.03a*	6.84 ± 0.31a*	24.1 ± 1.96b*	58.4 ± 4.36c*	186 ± 12d**
	A_3	2.13 ± 0.09a*	8.23 ± 0.53a*	23.7 ± 1.75b*	59.5 ± 4.31c*	169 ± 8d*
Moisture (%)	A_1	94.4 ± 0.17a*	93.9 ± 0.38a*	94.4 ± 0.35a*	94.0 ± 0.36a*	94.2 ± 0.45a*
	A_2	94.5 ± 0.16a*	94.1 ± 0.43a*	93.9 ± 0.14a*	94.6 ± 0.40a*	94.4 ± 0.31a*
	A_3	94.7 ± 0.13a*	94.3 ± 0.51a*	94.5 ± 0.48a*	94.7 ± 0.41a*	94.1 ± 0.43a*

Data are presented as means ± SD (n = 5). Differences in plant biomass and moisture content were tested according to two-way ANOVA followed by Tukey's test. In a column, means followed by different amount of stars are significantly different at $p < 0.05$. In a line, means followed by different letters are significantly different at $p < 0.05$.

7.3.2. NR and GS activities

The NR activity in lettuces showed a decreasing pattern along the study period as shown in Figure 7.1a. At T_1, A_3 lettuces presented a significantly lower NR activity (2.58 nkat/mg protein) than A_2 lettuces (5.16 nkat/mg protein). However, at T_5 no significant differences were observed between NR activity of A_2 (0.91 nkat/mg protein) and A_3 (0.97 nkat/mg protein) lettuces.

Regarding GS, an increase was observed from T_1 to T_4 in A_1 and A_2 lettuces (Figure 7.1b). In A_3 lettuces, this increase only occurred up to T_3 and then GS activity started to decrease until the end of the study period. At T_1, no significant differences were observed in the GS activity between all the lettuces. Nevertheless, at T_5 the GS activity was significantly higher in A_1 lettuces (6.78 nkat/mg protein) compared to A_2 (4.23 nkat/mg protein) and A_3 (4.72 nkat/mg protein).

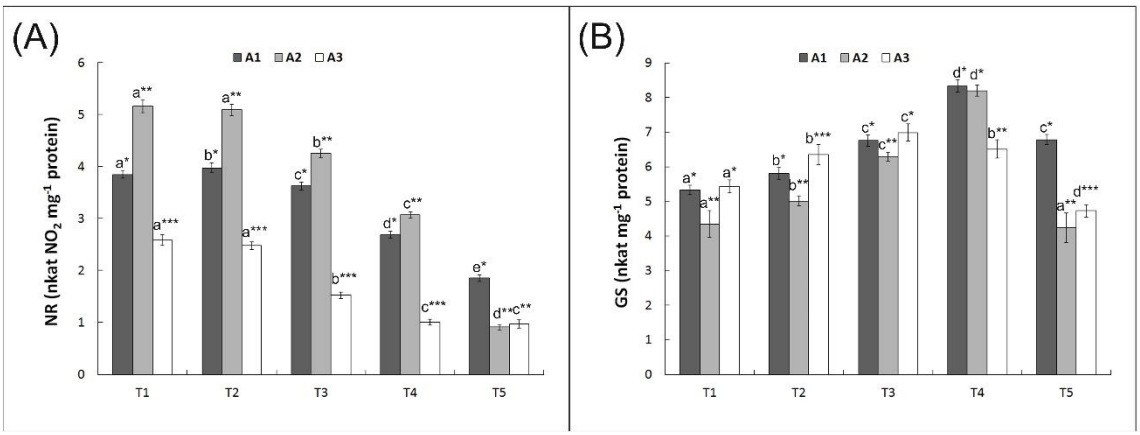


Figure 7.1 – Evolution of NR activity (a) and GS activity (b) along the study period in the three fields. Each value represents the mean of five replicate determinations and error bars indicate SD values. Different letters along the study time points (T_1, T_2, T_3, T_4 and T_5) indicate significant differences at $p < 0.05$. Different number of stars in the study fields (A_1, A_2 and A_3) indicates significant differences at $p < 0.05$ (two-way ANOVA and Tukey's test).

7.3.3. Plant nitrogen content

Figure 7.2 summarizes the evolution of NO_3^- (A), NH_4^+ (B) and N-Kjeldahl (C) content in lettuces along the study period. NO_3^- had a distinct behavior when compared to NH_4^+ and N-Kjeldahl. The NO_3^- content in lettuce leaves increased along the study period ranging from 917 to 2627 µg/g in A_1, from 1322 to 3705 µg/g in A_2 and from 1184 to 2936 µg/g in A_3. Moreover, A_2 lettuces showed a significantly higher NO_3^- content (Figure 7.2a).

The NH_4^+ content of lettuces increased from T_1 to T_3 independently of the soil. Afterwards, the NH_4^+ content decreased until T_5. The NH_4^+ pattern of variation along the study period

was very similar for A_1 and A_3 lettuces. The lowest NH_4^+ content was observed in T_1 and the highest in T_3. A_2 lettuces presented a significantly higher NH_4^+ content in the last three study time points (Figure 7.2b).

An increase of N-Kjeldahl values along the study period was observed in lettuces grown in the three soils. A significant increase was observed from T_1 to T_3 in A_1 and A_3 lettuces. At T_5, A_2 and A_3 lettuces presented a significantly higher N-Kjeldahl content compared to A_1 lettuces (Figure 7.2c).

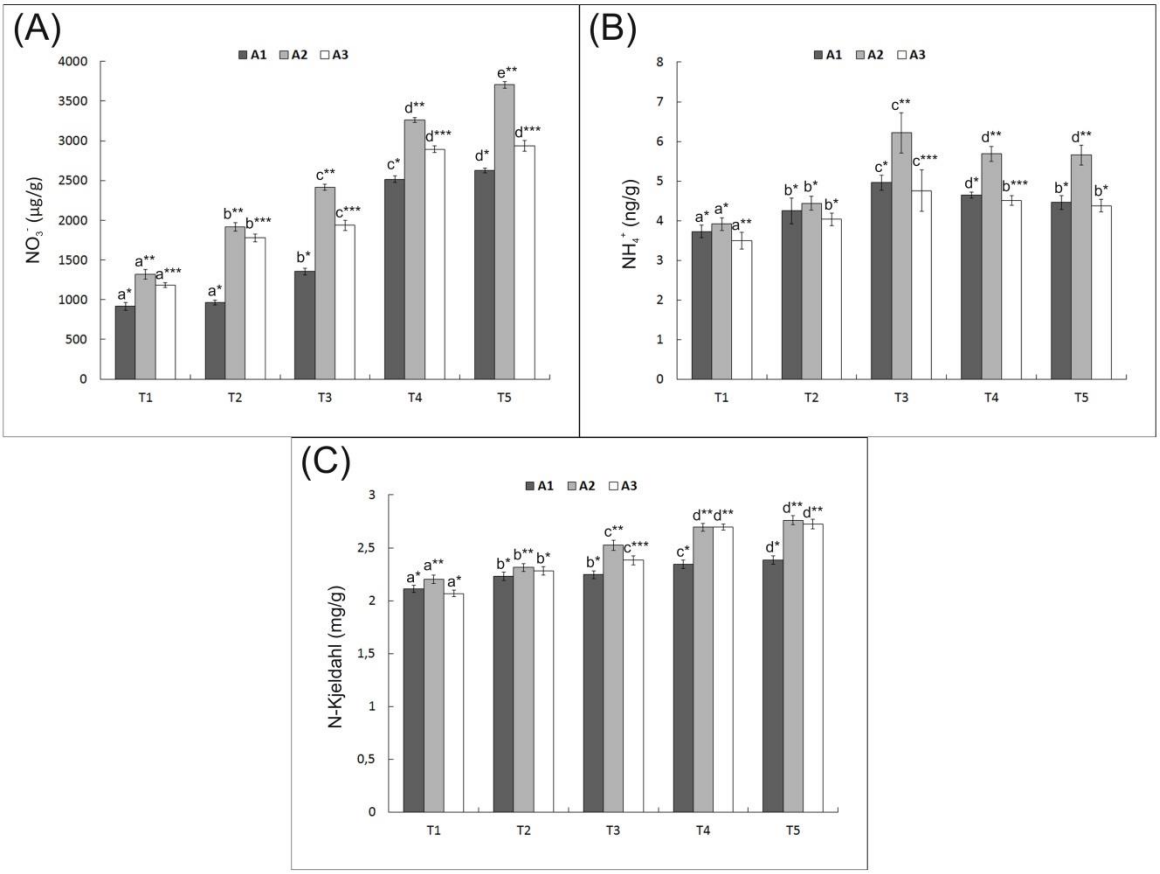


Figure 7.2 – Evolution of NO_3^- (a), NH_4^+ (b) and N-Kjeldahl (c) content in lettuces along the study period in the three fields. Each value represents the mean of five replicate determinations and error bars indicate SD values. Different letters in the study time points (T_1, T_2, T_3, T_4 and T_5) indicate significant differences at $p < 0.05$. Different number of stars in the study fields (A_1, A_2 and A_3) indicates significant differences at $p < 0.05$ (two-way ANOVA and Tukey's test).

7.3.4. Soil nitrogen content and physicochemical properties

Results for NO_3^-, NH_4^+ and total N content in the three soils along the study period are shown in Figure 7.3. NO_3^- content decreased along the study period independently of the soils (Figure 7.3a). A_2 soil had a significantly higher NO_3^- content compared to A_1 and A_3 soils. Regarding NH_4^+ content, a similar pattern was observed for A_1, A_2 and A_3 soils, and increase from T_1 to T_4 followed by a decrease at T_5 (Figure 7.3b). Moreover, the NH_4^+ content was significantly higher in A_2 soil. The NO_3^-/NH_4^+ ratio indicates that NO_3^- content was 300 to 3000-fold higher than NH_4^+ content. The lowest NO_3^-/NH_4^+ ratio was observed in A_3 soil (382 to 989) and the highest in A_2 soil (1672 to 2993). Total N content was significantly different in the three soils with the highest content in A_1 and the lowest in A_3. Despite that, total N content of the study soils did not show significant differences along the study period (Figure 7.3c).

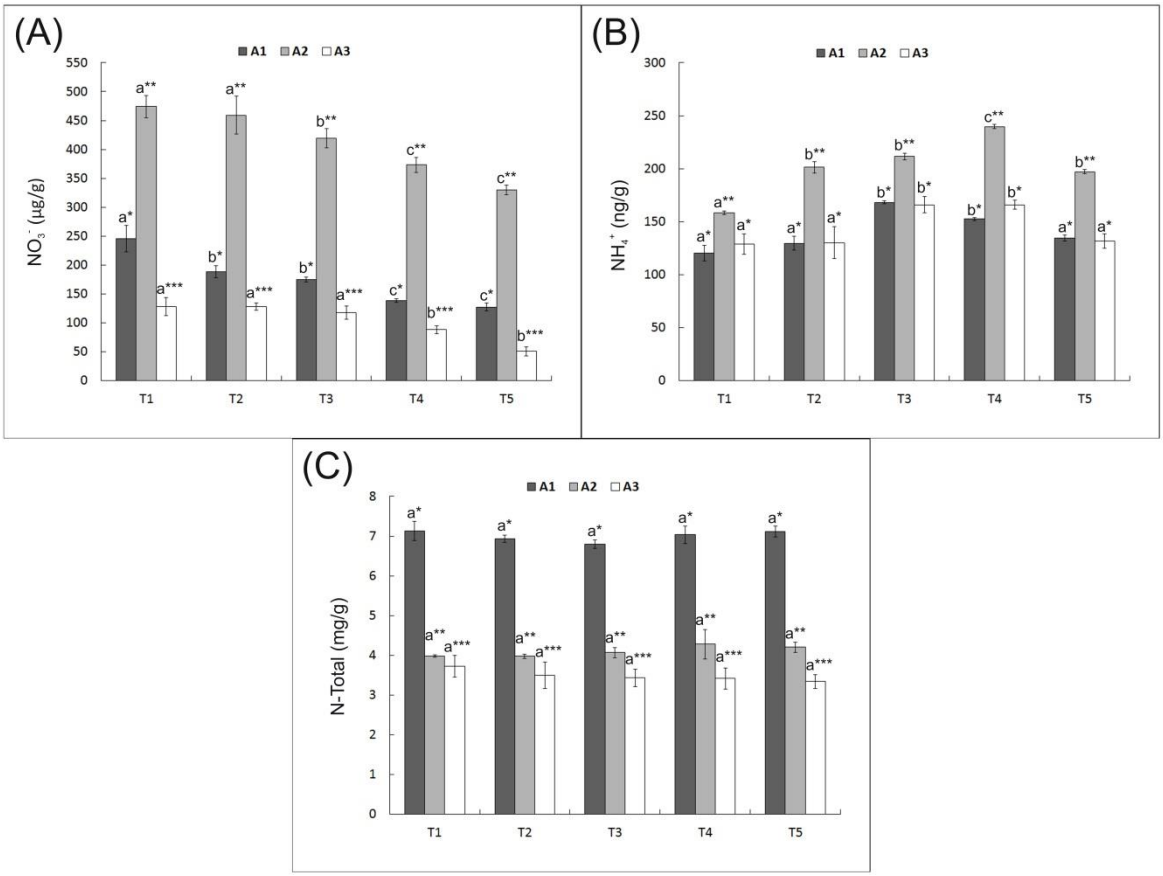


Figure 7.3 – Evolution of NO_3^- (a), NH_4^+ (b) and total N (c) content in the three soils along the study period. Each value represents the mean of five replicate determinations and error bars indicate SD values. Different letters in the study period (T_1, T_2, T_3, T_4 and T_5) indicates significant differences at $p < 0.05$. Different number of stars in the study fields (A_1, A_2 and A_3) indicates significant differences at $p < 0.05$ (two-way ANOVA and Tukey's test).

The soil physicochemical characteristics (pH, OM, CEC, particle size distribution and EC) of the three greenhouse experimental fields are shown in Table 7.2. A_2 soil showed significantly lower pH values. The highest OM content was observed in A_2 soil (72.6%) and the lowest in A_3 soil (18.5%). A_1 and A_3 soils presented a similar CEC (7.86 cmol/kg for A_1 and 7.88 cmol/kg for A_3), which was much lower than for A_2 soil (17.2 cmol/kg). The highest EC was observed in A_2 soil (632.9 µS/cm). Regarding the particle size distribution, A_1 and A_3 soils were classified as sandy soils and A_2 as loamy sand soil.

Table 7.2 – Main physicochemical properties of the studied soils (A_1, A_2 and A_3).

	A_1	A_2	A_3
pH (H_2O)	7.08 – 7.49a	6.75 – 7.35b	6.96 – 7.56a
OM (%)	54.3 – 57.9a	70.9 – 74.8b	19.6 – 21.9c
CEC (cmol/kg)	7.7 – 8.4a	16.3 – 17.8b	8.1 – 8.7a
EC (µS/cm)	266 – 318a	597 – 670b	180 – 218c
Soil classification	sandy soil	loamy sand soil	sandy soil

Data are presented as the minimum and maximum values obtained at the 5 sampling time points (T_1, T_2, T_3, T_4 and T_5). Differences in soil pH, OM, CEC and EC content were tested according to ANOVA followed by Tukey's test. In a column, ranges followed by different letters are significantly different at $p < 0.05$.

7.3.5. Water NO_3^- content

A_1 water showed significantly higher NO_3^- concentrations along the study period (Figure 7.4). Nitrate concentrations in A_2 were very low, ranging from non-detected in T_3 to 1.34 mg/L in T_5. The NO_3^- content of A_3 water decreased along the study period showing its highest value at T_1 (30.4 mg/L) and the lowest at T_5 (20.1 mg/L). Changes in the NO_3^- content along the study period were not significantly in A_2 water.

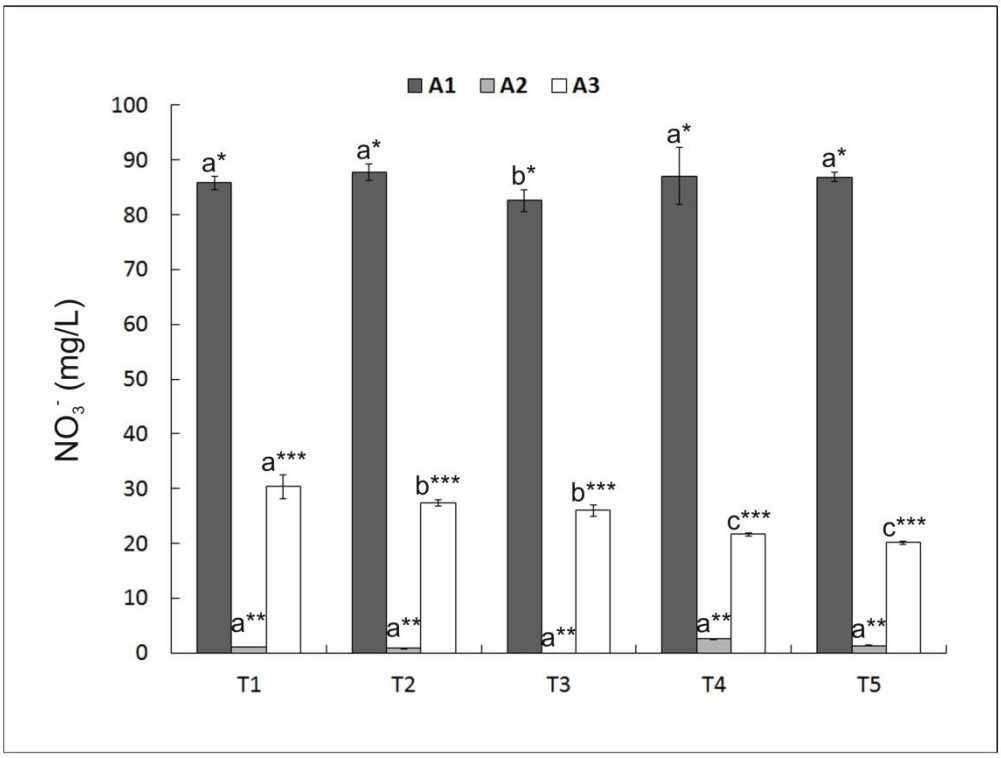


Figure 7.4 – Evolution of NO_3^- concentrations in groundwater collected from drilled wells located on the three experimental fields (A_1, A_2 and A_3). Each value represents the mean of five replicate determinations and error bars indicate SD values. Different letters in the study time points (T_1, T_2, T_3, T_4 and T_5) indicate significant differences at $p < 0.05$. Different number of stars in the study fields (A_1, A_2 and A_3) indicates significant differences at $p < 0.05$ (two-way ANOVA and Tukey's test).

7.4. Discussion

The lettuce leaves biomass increase was similar along the study period in the three soils. Only at T_5, A_2 lettuces showed significantly higher biomass content compared to A_1 and A_3 lettuces (Table 7.1). The NR activity showed its higher value at T_1 and T_2 in all the three experimental fields, which correspond to the study periods were the NO_3^- lettuce content was lower. From T_2 to T_5, the NR activity decreased reaching its lower value at T_5 (Figure 7.1a). A_2 soil presented the highest NO_3^- content and lettuces grown in this soil showed the highest NO_3^- content and NR activity. NO_3^- content increased and NR activity decreased in all lettuces along the study period resulting in a significant negative correlation ($r = -0.609$, $p < 0.01$), as shown in Figure 7.5a. Soil NO_3^- content influenced both NR activity and plant NO_3^- accumulation. The present results are in agreement with other studies that found accumulation of NO_3^- in plants when supplied in amounts above their physiological requirements (660). Nitrate accumulation in lettuce leaves occurred along the study period mainly due to the continuous supply of NO_3^- and the low capacity of

NR activity to reduce the anion in the latter sampling time points. Moreover, since NO_3^- is mainly stored in the plant cells vacuoles and NR is a cytosolic enzyme, the substrate of the NR could be less available, which resulted in a lower activity of the enzyme (661). The NO_3^- inducible character of NR is described in the literature and seems to occur at low NO_3^- levels. This is probably the reason why NR activity kept constant at T_1 and T_2. It indicates that there is a threshold of NO_3^- content necessary to induce NR activity and that it may be mainly related with physicochemical characteristics of soils and the available N. Several reports show that NR activity is sensitive to NO_3^- supply and also to a number of environmental factors such as light intensity, CO_2 concentration, temperature and water availability (662). The NR activity is higher when the rate of leaf expansion is maximal and becomes lower in fully expanded leaves. Therefore, the decrease on the NR activity observed in the present study can be considered as the result of a decrease in the growth rate of lettuce leaves along the study period. Datta and Sharma (663) evaluated the temporal regulation of NR in maize leaves and concluded that the induction of NR during plant growth is strictly regulated. The same decreasing pattern was observed; however, in their study, the temporal scale of the experiment was very short (12 days) compared to the present study (90 days). The relationship between NR activity and NO_3^- content is controversial. Some studies suggest that there is a negative correlation between NR activity and the NO_3^- content in plant tissues, but others indicate that, since NR is induced by NO_3^-, a higher content of this anion in plant results in a positive relationship with the NR activity (661). However, recent studies suggest that NR activity is not induced if NO_3^- concentration surpasses certain levels and can even be affected negatively (664).

In the present study a positive correlation ($r = 0.292$) between the NH_4^+ content and the GS activity in lettuce leaves was found (Figure 7.5b). The NH_4^+ content increased between T_1 and T_3 and the GS activity also increased, which is important to regulate the NH_4^+ content in lettuce leaves. Furthermore, from T_1 to T_3, the GS activity was significantly lower in A_2 lettuces, indicating that a lower depletion of the NH_4^+ content in A_2 lettuce leaves should occur. This behavior was confirmed by the highest NH_4^+ content in A_2 lettuces. Ammonium can be seen as an intermediate product in plant N metabolism. GS has high affinity for NH_4^+ and assimilates it at low concentrations, such as those that result from NO_3^- reduction (656). In this context, GS acts as a regulator of the NH_4^+ content inside the plant. As a result, if the enzyme is inhibited, NH_4^+ may accumulate leading to phytotoxic effects (665). Besides its formation from the NO_3^- assimilation process, NH_4^+ can be originated from soil uptake, N_2 fixation and photorespiration (656). In this study a significant positive correlation ($r = 0.771$, $p < 0.01$) was obtained between soil and plant NH_4^+ content. Overall, the transfer factor (TF – ratio of an individual element in the plant tissue to the bioavailable fraction of the element in soil) of NH_4^+ in lettuce leaves was very

low (0.54) when compared to the TF for NO_3^- (273), indicating that lettuces prefer NO_3^- as N source. Although NH_4^+ assimilation requires less energy NO_3^-, plants usually prefer the uptake of a high proportion of NO_3^- because it can be stored in plant cells without leading to toxic effects, while NH_4^+ is toxic even at low concentrations (666).

As showed in Figure 7.5c and 7.5d, significant positive correlations ($p < 0.01$) were obtained between N-Kjeldahl and NO_3^- ($r = 0.640$) and between N-Kjeldahl and NH_4^+ ($r = 0.917$) in plants, indicating that higher contents of NO_3^- and/or NH_4^+ have a positive effect in the biosynthesis of N organic forms such as amino acids and proteins. Garnica, Houdusse (667) confirmed that the presence of NO_3^- in wheat tissues enhanced protein biosynthesis. However, in another study, Horchani, Hajri (668) showed that NO_3^- nutrition as the only source of N did not have any effect in protein content, whereas NH_4^+ contents above 7.5 mM increase protein content. In the present study, both N sources had a positive effect in the N-Kjeldahl content.

The soil physicochemical characteristics pH, OM, CEC and particle size distribution are usually included in the mobility/phytoavailability assessment. A_2 soil presented very different characteristics when compared to A_1 and A_3 soils. A_2 soil was characterized as a loamy sandy soil with high OM content and CEC, whereas A_1 and A_3 soils were sandy soils with low OM content and CEC. These soil parameters appear to have a major influence in the content of N species in both soil and plants. According to our results, the A_2 soil had a high OM content as well as C/N ratio (> 98) indicating N immobilization. Despite the significantly higher total N content in A_1 soil, the highest NO_3^- and NH_4^+ contents were observed in A_2 soil. This indicates that A_2 soil had the ability to retain more inorganic N, which is the most phytoavailable form of N. The OM content is an important factor that governs the mobility of inorganics in soils through sorption and desorption processes. The C/N ratio is a major indicator of N availability. Generally, a high C/N ratio (> 30) indicates that N is strongly immobilized in the soil-plant system (669). CEC is influenced by the content of OM and soil pH. It is an important parameter that governs the bioavailability of cationic species in soil. Soil cation exchange reactions retain NH_4^+ ions on the surface of clays reducing the NH_4^+ concentration in the soil solution (670). Therefore, the relative proportion of clay, silt and sand in soils has a great influence in the N mobility. Generally, NO_3^- leaching is higher in sandy soils than in clayey soils due to the slower water movement and high rate of denitrification observed in clayey soils (602). A_2 soil had higher clay content and thus was able to reduce NO_3^- loss by leaching processes. By contrast, A_1 and A_3 were classified as sandy soils, thus allowing NO_3^- leaching which was confirmed by the high NO_3^- levels in groundwater. The rate-controlling step in N mineralization is the conversion of organic N into NH_4^+. The conversion of NH_4^+ to NO_3^- is a rapid step and as a result NH_4^+ generally does not accumulate in well-drained soils

(652). This can explain the low NH_4^+ concentrations observed in our study in all soils, when compared to NO_3^- levels. The very high NO_3^-/NH_4^+ ratio observed in all soils gives a good indication of the relative proportion of both elements and clearly indicates that NO_3^- was the dominant N species in the soils used in this study.

7.5. Conclusions

NR activity decreased during lettuce growth; consequently, NO_3^- lettuce content increased. A positive correlation was observed between GS activity and NH_4^+ levels in lettuce leaves, with an increase during the first weeks of lettuce growth and a decrease after that. This pattern was the same in lettuces grown in similar conditions in different soils, however, quantitative differences were observed, indicating that inorganic N from soil influenced both NO_3^- and NH_4^+ content in lettuce. Higher soil NO_3^- levels caused NO_3^- accumulation in lettuce despite the higher NR activity during the first sampling time points. Higher NO_3^- and NH_4^+ levels were correlated with higher N-Kjeldahl levels in lettuce, suggesting a positive effect in organic N synthesis. Soil composition influenced the mobility of inorganic N within the groundwater-soil-plant system. Loamy sandy soil with high OM and CEC retained more efficiently inorganic N, increasing the NO_3^- and NH_4^+ content in soil solution which results in a higher N content in lettuce. Sandy soils with low OM and CEC allowed NO_3^- leaching, which was confirmed by the high NO_3^- levels in groundwater. Consequently, lettuce grown in those soils presented lower N content and higher inputs of N were added to the environment.

Chapter 8

Quantification of polyamines in plant foods during growth by ultrasound-assisted benzoylation and dispersive liquid-liquid microextraction

Abstract

A new method involving ultrasound-assisted benzoylation and dispersive liquid–liquid microextraction (DLLME) was optimized with the aid of chemometrics for the extraction, clean-up and determination of polyamines (PAs) in plant foods. Putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM) were derivatized with 3,5-dinitrobenzoyl chloride (DNBZ-Cl) and extracted by DLLME using acetonitrile and carbon tetrachloride as dispersive and extraction solvents, respectively. Two-level full factorial design and central composite design (CCD) were applied to select the most appropriate derivatization and extraction conditions. The developed method was linear in the 0.5 – 10.0 mg/L range with an $r^2 \geq 0.9989$. Intra and interday precision ranged from 0.8 to 6.9% and 3.0 to 10.3%, respectively, and the limit of detection (LOD) ranged between 0.019 and 0.045 µg/g fresh weight. The novel method was successfully applied to the analysis of six different types of plant foods, with recoveries in the range of 81.7 – 114.2%. The combination of sample freeze-drying with PAs benzoylation and DLLME clean-up and enrichment of sample extracts enabled quantification of low contents of PAs with good precision and accuracy. The present method is inexpensive, simple, sensitive and suitable for the determination of PAs in plant foods. Through the application of the developed method, it was found that: (1) PUT was the most abundant PA present in lettuce; (2) PUT, SPD and SPM contents decrease during lettuce growth; and (3) significant differences exist between the PAs content of lettuces growth in different experimental fields.

8.1. Introduction

The polyamines (PAs) putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM) are low molecular weight organic amines with aliphatic structure, which have been implicated in several biological processes such as plant growth and development (537). These compounds are naturally found in many plant foods and are involved in the regulation of a diverse range of vital cellular processes in eukaryotic cells. In plants, the most common PAs are PUT, SPD and SPM and are usually localized in the cytoplasm and organelles (e.g., vacuoles, mitochondria, and chloroplasts) of plant cells (671, 672).

The dietary intake of PAs influences human health. Their biological role in humans includes participation in cell growth and proliferation, pain sensitization, stabilization of DNA, gene transcription, apoptosis, regulation of the immune response, maintenance of intestinal function and protein synthesis (673, 674). It has been suggested that increased PAs intake has several health benefits such as decreasing age-associated pathologies

and increasing longevity (675). Additionally, a polyamine-deficient diet has also been proposed as an efficient treatment for several human conditions (676).

Plant foods have very low levels of PAs, ranging from a few milligrams to less than 100 mg/kg. Thus, methods to perform PAs quantification in plant foods frequently report results as non-detectable or below the LOD/LOQ due to the insufficient sensitivity (677-679). Plant foods are a very complex and variable matrix (671, 672). Thus, efficient clean-up and pre-concentration techniques are needed to obtain sensitive and accurate quantification of PAs. Since plant foods are solid matrixes, an extraction/deproteinization is also required. The usual deproteinization process is usually performed with diluted strong acids such as perchloric, hydrochloric and trichloroacetic acids (680-682).

Chemical derivatization has become widely accepted in PAs analysis enabling great sensitivity and selectivity to be achieved. Nowadays, there are several derivatization reagents that can be used for UV and/or fluorescence detection, including o-phthaldialdehyde (OPA), dansyl chloride, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), dabsyl chloride, 3,5-dinitrobenzoyl chloride (DNBZ-Cl), 1,2-naphthoquinone-4-sulfonate (NQS), benzoyl chloride, 4-chloro-3,5-dinitrobenzotrifluoride (CNBF) and diethyl ethoxymethylenemalonate (DEEMM) (683, 684). Among them, DNBZ-Cl shows several advantages such as low price, commercial availability and fast reaction times at room temperature (685).

Several methods have been developed for the analysis of PAs, including micellar electrokinetic chromatography (MEKC) (686), gas chromatography (GC) (687), thin layer chromatography (TLC) (688), ion chromatography (IC) (689) and liquid chromatography (LC) (690). Overall, LC is the most used technique in the determination of PAs in the different kinds of food due to its high selectivity, sensitivity and wide applicability.

Many sample preparation techniques have also been developed for clean-up purposes. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are two of the most widely used techniques, however they require large quantities of organic solvents, and are time-consuming (680, 681). Solid-phase microextraction (SPME) and single drop microextraction (SDME) have been increasingly used in the last years because of its eco-friendly nature (687, 691). However, these procedures had some drawbacks such as high price, short durability of the extraction fibers and sample carry-over (692). Dispersive liquid–liquid microextraction (DLLME) is a novel sample preparation technique based on a ternary component mixture consisting in the aqueous sample, a dispersive solvent and an extraction solvent. It presents several advantages such as simplicity, high recovery, quickness and low cost (693).

Growing interest arises from coupling derivatization and DLLME, followed by chromatographic separation (i.e, LC or GC) for the analysis of different kinds of

compounds (694, 695). The potentiality of these techniques to simplify experimental analytical procedures, decrease sample loss, increase method sensitivity and remove interferences is seen as a great improvement in the analytical chemistry field (696).

The development of a derivatization and DLLME procedure requires selection of several variables (concentration of derivatization reagent, reaction time, reaction temperature, dispersive solvent volume, extraction solvent volume and ionic strength) in order to establish the optimum conditions. The optimization of a multivariable system through a one-variable-at-a-time strategy can be time consuming and neglect potential interactions between the different variables. The two-level full factorial design is a useful tool for preliminary screening of experimental factors that need to be optimized, whereas response surface methodology (RSM) aids to understand interactions among the experimental factors that must be optimized in order to determine the optimum operational conditions for the system or to determine a specific parameters range that satisfies the operating specifications (697). Consequently, when applied to derivatization and miniaturized extraction procedures, these chemometric methods can significantly improve the quality of analytical data. However, until now, few studies rely on the use of these methods.

In this work, a novel method was developed for the detection and quantification of PAs (PUT, SPD, SPM and CAD) using ultrasound-assisted benzoylation followed by DLLME and high-performance liquid chromatography – diode array detection (HPLC – DAD). The best conditions for the benzoylation and extraction procedures were found with the aid of experimental designs. The developed method was applied to the analyses of PAs in different types of plant foods and to determine the PAs content of lettuces during its growth period in three different experimental fields.

8.2. Experimental

8.2.1. Plant sampling

Plant food samples (lettuce, carrot, onion, cabbage, tomato and apple) were purchased at local supermarkets. Samples were washed with ultra-pure water, frozen at –80 ºC and freeze-dried. The lyophilized samples were homogenized by grinding in a blender and sieved through a nylon sieve of 150 µm mesh size. Free PAs were determined according to Zhou and Yu (682) with minor modifications. Lyophilized samples (ca. 100 mg) were homogenized with 2.5 mL of 5% (v/v) cold $HClO_4$ and incubated at 4 ºC for 1 h. After centrifugation at 17000 g for 30 min, the supernatant was collected and used as sample extract for the determination of PAs.

Lettuce (*L. sativa L. var. capitata*) plant sampling was performed as described in Chapter 3 section 3.2.1.

8.2.2. Reagents and apparatus

Putrescine dihydrochloride (PUT), cadaverine dihydrochloride (CAD), spermidine trihydrochloride (SPD), 1,7-diaminoheptane (internal standard, IS), spermine tetrahydrochloride (SPM), hydrochloric acid, acetonitrile, methanol, acetone and 3,5-dinitrobenzoyl chloride (DNBZ-Cl) were purchased from Sigma-Aldrich (St. Louis, MO). Boric acid, sodium hydroxide, potassium chloride and sodium chloride were from VWR (Radnor, PA). Perchloric acid ($HClO_4$), carbon tetrachloride (CAS: 56-23-5), chlorobenzene (CAS: 108-90-7), chloroform (CAS: 67-66-3), dichloromethane (CAS: 75-09-2), tetrachloroethylene (CAS: 127-18-4) and trichloroethylene (CAS: 79-01-6) were obtained from Merck (Darmstadt, Germany).

Standard stock solutions were prepared by dissolving each compound in 0.1 M HCl at a final concentration of 1000 mg/L. Working standard solutions were prepared daily from the stock solutions by adequate dilution with 0.1 M HCl. Sodium borate buffer was prepared from dilution of H_3BO_3 and KCl in water (0.5 M each) followed by titration with NaOH (0.5 M) to pH 10. DNBZ-Cl was prepared by dissolving a proper amount of the solid compound in acetonitrile.

Chromatographic analysis was performed with an analytical HPLC unit (Jasco, Tokyo, Japan) equipped with Jasco PU-2080 HPLC pumps, a Column Heater (model 7981; Jones Chromatography, Hengoed, UK), and a MD-2010 Plus multiwavelength detector. The column was a reversed-phase Ultracarb ODS (30) C_{18} (250 mm × 4.6 mm, 5 µm) (Phenomenex, Milford, MA). The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was used for system control and data acquisition. The chromatographic separation was carried out by gradient elution with a two solvents mixture at a 1 mL/min flow rate. Solvent A consisted of 10 mM aqueous sodium formate/formic acid buffer at pH 3 and solvent B consisted of acetonitrile. The linear gradient program was as follow: 0 min, 40% B; 0-5 min, 40% B; 5–11 min, 58% B; 11–15 min, 58% B; 15–20 min, 65% B; 20–23 min, 65% B; 23-28 min, 95% B; 28-30 min, 95% B; 30-40 min, column rinse and re-equilibration. Separations were carried out at 35 ºC. Detection was set at 250 nm.

8.2.3. Derivatization, clean-up and preconcentration

A 2 mL volume of standard solution or sample extract was placed in a 10 mL tube and 0.5 M borate buffer (pH = 10.0) and 2 M NaOH were added in order to adjust pH to 10. After that, 680 µL of DNBZ-Cl in acetonitrile (56 mM) was added to the previous solution which was subjected to vortexing. Afterwards, the mixture was ultra-sonicated for 15 min at room temperature. Benzoylated PAs were extracted by the DLLME method by adding 500 mg of NaCl and 205 µL of carbon tetrachloride (extraction solvent) into the sample solution to form a cloudy mixture. After vortexing for 1 min, the mixture was centrifuged for 5 min at 5000 rpm and the organic phase was aspirated into a microsyringe and collected into a 2 mL glass vial. The organic phase was evaporated in a gentle stream of N_2 and reconstituted in 100 µL of acetonitrile. Finally, 20 µL of this solution was injected into the HPLC system for analysis.

8.2.4. Experimental design

Several trials were conducted to optimize the benzoylation and DLLME conditions for the quantitative analyses of PAs in plant foods using RSM. A two-level (2^k) factorial design was created to screen the main variables affecting the derivatization and extraction procedures. This technique is a powerful tool for screening the key variables in a multivariable system. Two different full factorial designs were created for trials at low (-1) and high (+1) levels of concentration with five central points. The experiments were performed to evaluate the significance of seven variables (DNBZ-Cl concentration, DNBZ-Cl volume, derivatization time, derivatization temperature, dispersive solvent volume, extraction solvent volume and amount of NaCl) under study. In the first full factorial design, the evaluated variables were: DNBZ-Cl concentration (35-65 mM), DNBZ-Cl volume (400-800 µL), derivatization time (15-40 min) and derivatization temperature (20-50 ºC). The screening of the main variables affecting the derivatization procedure was performed without performing the extraction procedure, in order to exclude potential effects arising from it. In the second full factorial design, the variables dispersive solvent volume (400-800 µL), extraction solvent volume (50-250 µL) and amount of NaCl (500-800 mg) were evaluated. The DLLME procedure was carried out after fixing the most suitable derivatization conditions. Afterwards, the significant variables were optimized using a CCD.

Experiments with three independent variables, DNBZ-Cl concentration (X1), dispersive solvent volume (X2) and extraction solvent volume (X3), were conducted by applying a CCD consisting in a complete 20-factorial design with six center points and two axial points on the axis of each design variable at a distance of $\alpha = 1.682$ from the design

center. The response was the ratio between the sum of PAs peak area and the IS peak area. Experiments were carried out as follows: DNBZ-Cl concentration (35-65 mM), dispersive solvent volume (400-800 µL) and extraction solvent volume (100-250 µL). Table 8.1 shows the complete experimental design, which was performed using the Design Expert trial-Version 7 (Stat-Ease Inc., Minneapolis, MN) software.

Table 8.1 – Coded and uncoded values of the independent variables of the central composite design for the method optimization.

Standard order	Independent variables					
	Coded			Uncoded		
	X_1	X_2	X_3	X_1	X_2	X_3
1	-1	-1	-1	35	400	100
2	1	-1	-1	65	400	100
3	-1	1	-1	35	800	100
4	1	1	-1	65	800	100
5	-1	-1	1	35	400	250
6	1	-1	1	65	400	250
7	-1	1	1	35	800	250
8	1	1	1	65	800	250
9	-1.682	0	0	24.8	600	175
10	1.682	0	0	75.2	600	175
11	0	-1.682	0	50	263.6	175
12	0	1.682	0	50	936.4	175
13	0	0	-1.682	50	600	48.9
14	0	0	1.682	50	600	301.1
15	0	0	0	50	600	175
16	0	0	0	50	600	175
17	0	0	0	50	600	175
18	0	0	0	50	600	175
19	0	0	0	50	600	175
20	0	0	0	50	600	175

8.2.5 Method validation

Mixed standard solutions containing PUT, CAD, SPD and SPM were subjected to the derivatization, clean-up and preconcentration procedures described above. Calibration curves were constructed by the least squares linear regression model, using the ratio between peak area of each PA and IS peak area vs the PA concentration. The intraday precision was calculated from the results of five replicate analyses in a single day of standards prepared at three concentration levels (0.5, 2.5 and 10 mg/L); the interday precision was calculated from results obtained in five consecutive days. The LOD and LOQ were calculated based on the calibration curve parameters according to Miller and Miller (698).

Accuracy, based on the relative recovery (RR = [(concentration of the spiked sample – concentration of the unspiked sample)/added concentration] × 100), was estimated from triplicate experiments performed with lettuce, carrot, onion, cabbage, tomato and apple samples spiked with a known volume of a mixed standard solution (2.5 mg/L of each PA). Results of PAs content in plant foods were expressed as µg/g fresh weight.

The stability of the extracts was tested by analyzing the extract of the 5 mg/L standard solution at 0, 1, 2, 6 and 24 h after the derivatization and extraction procedure. During this time period extracts were stored at 4 ºC.

8.3. Results and discussion

8.3.1. Experimental parameters selection

Working standard solutions containing PUT, CAD, SPD and SPM at a fixed concentration (5 mg/L) were used to study the buffer capacity of the derivatization procedure and the different dispersive and extraction solvents on the DLLME procedure. To evaluate the buffer capacity, the standard solutions were diluted in 5% (v/v) $HClO_4$ solution in order to replicate the extraction conditions used in real plant food samples extraction. The obtained extracts presented a pH value around 1. Since the benzoylation reaction requires pH around 10, borate buffers at different concentrations (0.2, 0.5, 0.8 and 1 M) with and without the addition of NaOH (1, 2 and 4 M) were tested. Borate buffer at 1 M was not capable of raising the extract pH to 10 by its own and can easily precipitate, changing its buffer capacity. Therefore, borate buffer at 0.5 M was selected for further experiments, and the pH 10 was achieved by adding NaOH solution (2 and 4 M). Borate buffer (0.5 M) with 2 M NaOH was adequate to obtain the required pH and was used in further experiments.

The adequate choice of the most suitable dispersive and extraction solvents is a crucial step to obtain the maximum extraction efficiency. The dispersive solvent should be

miscible with both aqueous and organic phases, while the extraction solvent must have higher density than water, low water solubility and high extraction efficiency for the target compounds (693). Three dispersive solvents (methanol, acetonitrile and acetone) and six extraction solvents (carbon tetrachloride, chloroform, chlorobenzene, dichloromethane, trichloroethylene and tetrachloroethylene) were evaluated. The 5 mg/L standard solution was diluted in 5% (v/v) $HClO_4$ solution, derivatized and subjected to the DLLME procedure. The use of acetonitrile as dispersive solvent led to higher peak areas for all the tested PAs (Figure 8.1a), indicating that higher sensitivity could be obtained with this solvent. Carbon tetrachloride presented higher extraction efficiency for all the target compounds (Figure 8.1b), thus it was selected as extraction solvent.

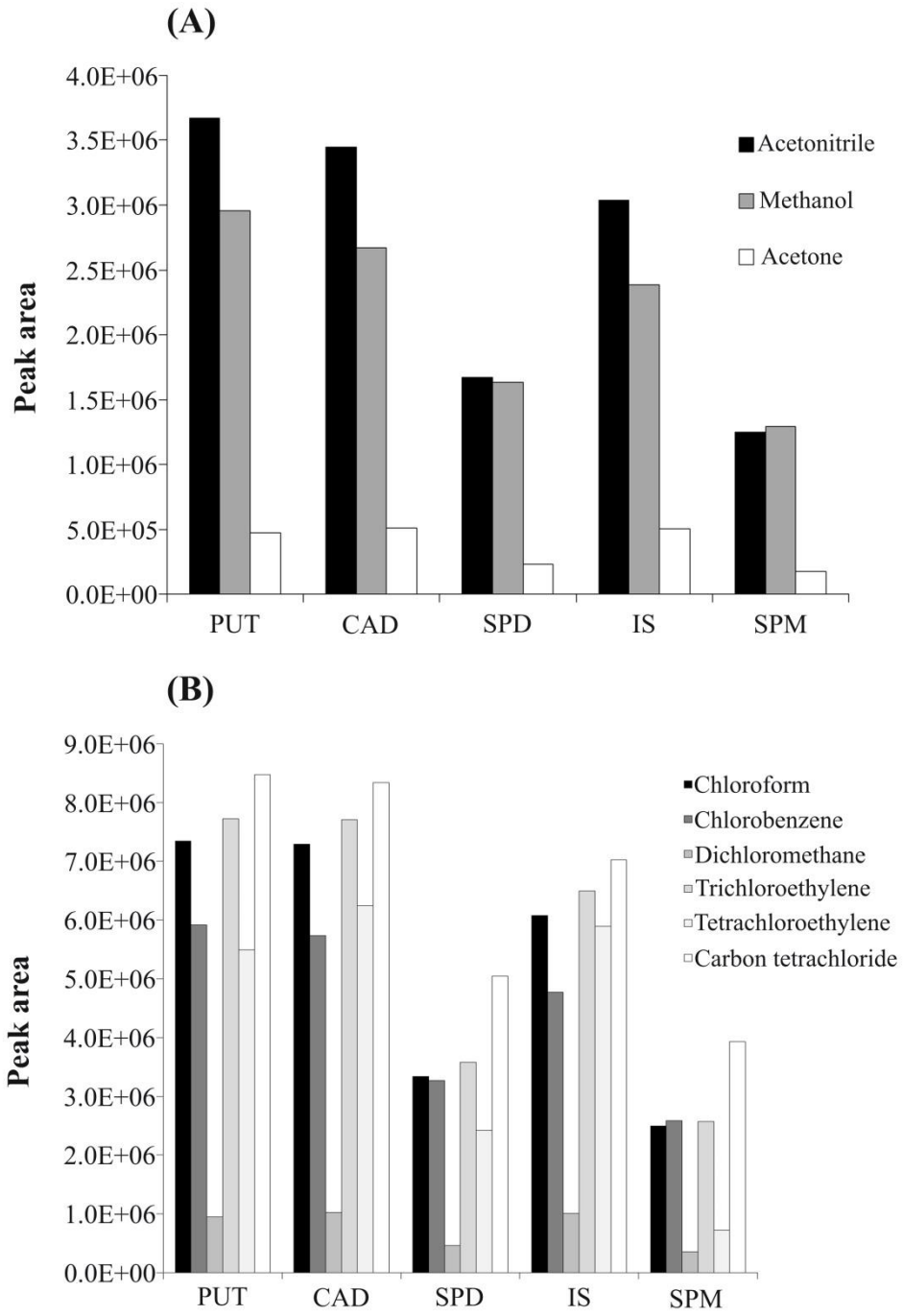


Figure 8.1 – Selection of (a) dispersive and (b) extraction solvents. Derivatization conditions: 2 mL standard solution (5 mg/L); 2 mL of 2 M NaOH plus 0.5 M borate buffer (pH = 10.0); 680 µL of DNBZ-Cl (56 mM); sonication at room temperature for 15 min. Extraction conditions: 500 mg NaCl plus 205 µL of extraction solvent.

8.3.2. Two-level full factorial design

The main effects of the seven studied variables in the screening experiments are presented in Figure 8.2 in the form of a Pareto chart. The same standard solution used to perform the selection of the dispersive and extraction solvents was used in the two steps experimental design (two-level full factorial design for screening and CCD for optimization). The magnitude of the effects is highlighted in an ordered bar-chart where the bar length of the vertical axis is proportional to the significance of the variables. Regarding the derivatization procedure, the significance of the variables DNBZ-Cl concentration, DNBZ-Cl volume, derivatization time and derivatization temperature was evaluated. DNBZ-Cl concentration and DNBZ-Cl volume (indicated in Figure 8.2a by the letters A and B, respectively) as well as the interaction between them (AB) were statistical significant ($p < 0.05$) with a positive effect on the response and thus were selected for further assessment in the CCD. For the extraction procedure, the significance of the variables dispersive solvent volume, extraction solvent volume and amount of NaCl was assessed. The first two variables (indicated in Figure 8.2b by the letters A and B, respectively) as well as the interaction between them (AB) were statistical significant ($p < 0.05$) and therefore were considered for optimization. Since the variables “DNBZ-Cl volume” and “dispersive solvent volume” tested in the first and second full factorial designs, respectively, rely on the use of acetonitrile, the two variables were designated throughout the optimization procedure as just “dispersive solvent volume”. The variables derivatization time, derivatization temperature and the amount of NaCl showed no significant effect on the peak area of PAs and thus were fixed for further experiments at 15 min, room temperature and 500 mg, respectively. Faster reaction times, without the need for heating and with lower amount of reagents involved were achieved without compromising the method performance.

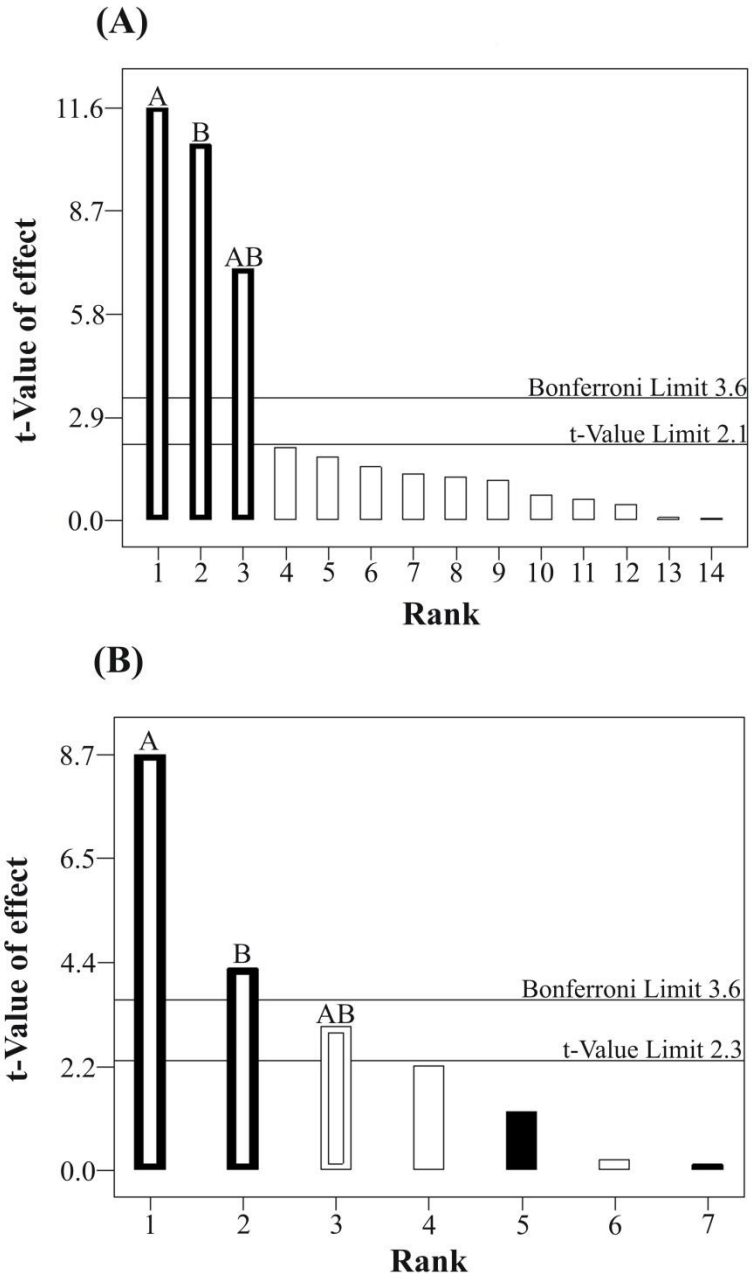


Figure 8.2 – Main effect Pareto chart for the two-level full factorial design of screening experiment for (a) derivatization and (b) extraction procedures.

8.3.3. Central composite design

For CCD, the range of DNBZ-Cl concentration (X1), dispersive solvent volume (X2) and extraction solvent volume (X3) tested are shown in Table 8.1. Twenty experiments were carried out in a random way at different combinations of these parameters using statistically designed experiments. The ratio between the sum of all PAs peak areas and the IS peak area (Y) as a function of X1, X2 and X3 was used as response for CCD design. Regression analysis was conducted to fit the response function and the final model was obtained. Since the CCD approach described the quadratic and interaction effects, the relationship between observed and predicted ratio between the sum of all PAs peak areas and the IS peak area shown that the plotted points gather around a diagonal line, indicating a goodness-of-fit of the model. The values of R^2 pred and R^2 adj were 0.92 and 0.97, respectively, and a ratio of 26.65 was achieved, which indicates an adequate signal.

ANOVA is important in determining the adequacy and significance of the quadratic model. As shown in Table 8.2, the F-value was 66.13, which indicates that the regression model is statistically significant at the 99% confidence level ($p < 0.01$). Only a 0.01% possibility exists that this value is so large that could occur due to noise. Values of Prob > F for X1, X_2, X_3, X_1X_2, X_2X_3, X_1^2, X_2^2 and X_3^2 were lower than 0.05, which indicates that they are significant model terms. The lack-of-fit was 1.29, non-significant as desired, and the quadratic model was valid (699).

Table 8.2 – Analysis of variance (ANOVA) for response surface model for the ratio between the sum of PAs peak area and the IS peak

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Prob > *F*	Remarks
Model	2.54×10^{15}	9	2.83×10^{14}	66.13	< 0.0001	Significant
X_1	2.66×10^{14}	1	2.66×10^{14}	62.24	< 0.0001	Significant
X_2	1.60×10^{15}	1	1.60×10^{15}	375.11	< 0.0001	Significant
X_3	1.28×10^{14}	1	1.28×10^{14}	29.99	0.0003	Significant
X_1X_2	1.02×10^{14}	1	1.02×10^{14}	23.76	0.0006	Significant
X_1X_3	7.64×10^{10}	1	7.64×10^{10}	0.018	0.8963	Not significant
X_2X_3	9.36×10^{13}	1	9.36×10^{13}	21.91	0.0009	Significant
X_1^2	6.28×10^{13}	1	6.28×10^{13}	14.70	0.0033	Significant
X_2^2	2.94×10^{14}	1	2.94×10^{14}	68.80	< 0.0001	Significant
X_3^2	4.18×10^{13}	1	4.18×10^{13}	9.79	0.0107	Significant
Residual	4.27×10^{13}	10	4.27×10^{13}			
Lack of fit	2.40×10^{13}	5	4.81×10^{12}	1.29	0.3949	Not significant
Pure error	1.87×10^{13}	5	3.74×10^{12}			
Total	2.59×10^{15}	19				

Three dimensional surface and contour plots were drawn in order to investigate the interactive effect of two factors on the ratio between the sum of PAs peak area and the IS peak area (Figure 8.3). From the three studied variables (DNBZ-Cl concentration, dispersive solvent volume and extraction solvent volume), two were varying at time while the third remained constant. Figure 8.3a shows the combined effect of DNBZ-Cl concentration and dispersive solvent volume. It shows that, by increasing the dispersive solvent volume, a higher response can be obtained. Figure 8.3b shows the combined effect of DNBZ-Cl concentration and extraction solvent volume, while Figure 8.3c shows the combined effect of dispersive solvent and extraction solvent volumes. The best response was observed using around 50 mM of DNBZ-Cl and 175 µL of extraction solvent and an increase in the dispersive solvent volume results is a better response.

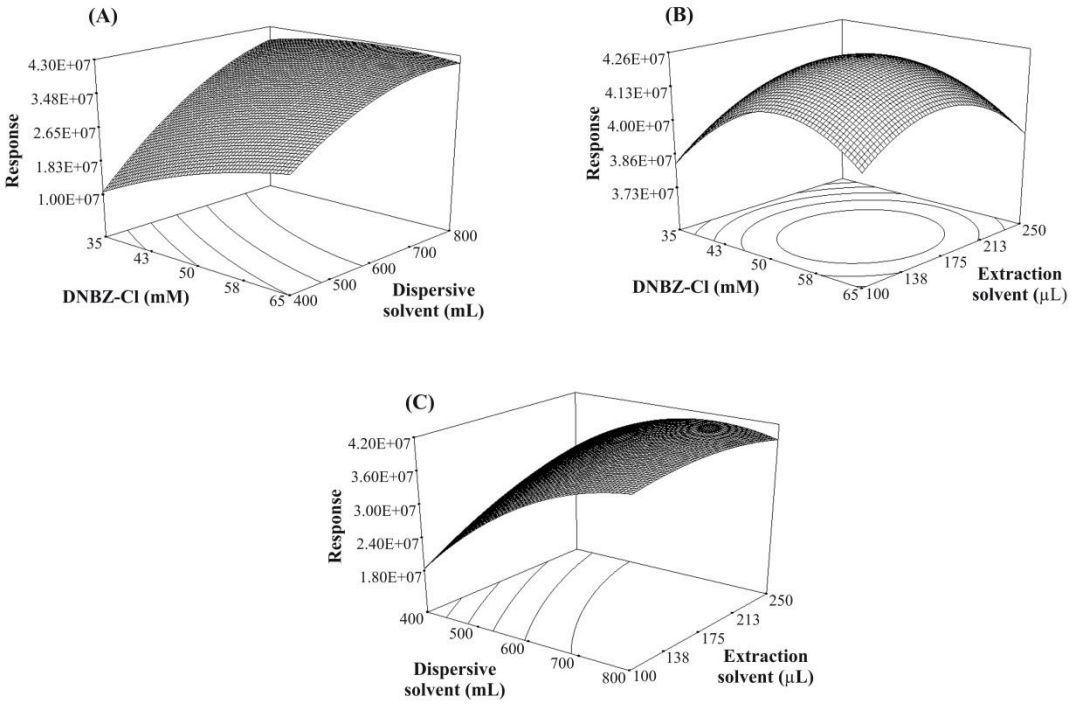


Figure 8.3 – Response surfaces plots for the central composite design: (a) concentration of DNBZ-Cl and dispersive solvent volume; (b) concentration of DNBZ-Cl and extraction solvent volume; (c) dispersive solvent volume and extraction solvent volume.

An evaluation of the optimum combination of the three studied parameters was performed in order to maximize the response while reducing the extraction time. Desirability indices were calculated to maximize the response and the following optimum conditions were selected: 56 mM of DNBZ-Cl, 680 µL of acetonitrile and 205 µL of carbon tetrachloride.

Confirmatory experiments were conducted with the parameters selected by the experimental model. The obtained response of 3.82×10^6 was within the 95% predication response interval (3.45×10^6 – 4.06×10^7) which indicates the adequacy of the obtained RSM. The optimization procedure enabled an enrichment of all PAs in the aqueous extracts resulting in a higher sensitivity, as shown in the chromatograms of Figure 8.4.

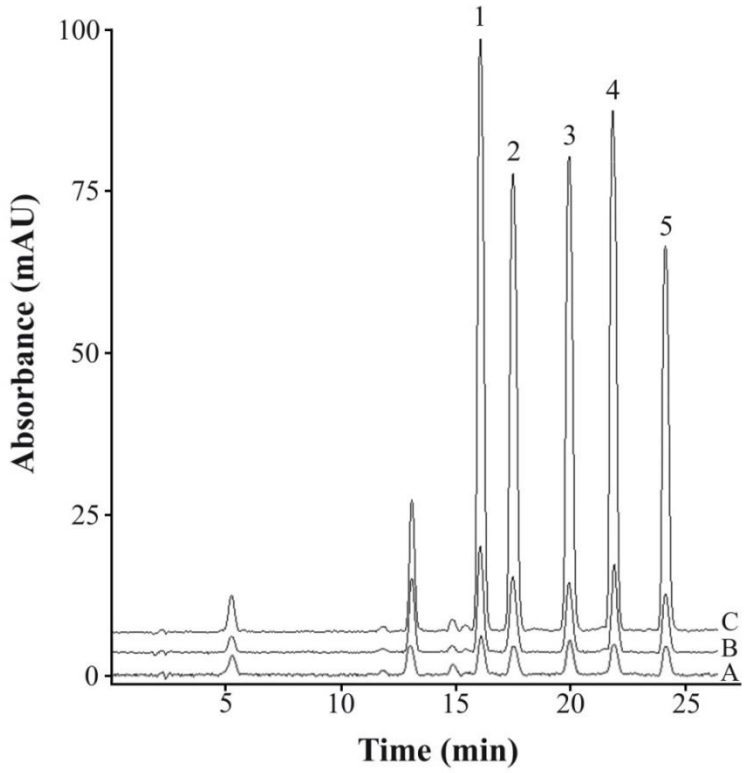


Figure 8.4 – Chromatograms obtained for a 5 mg/L standard solution during the method optimization. (a) Before the optimization procedure. (b) After the derivatization optimization. (c) After the derivatization and DLLME optimization. Peaks: 1, PUT; 2, CAD; 3, SPD; 4, IS; 5, SPM.

The use of the optimized DNBZ-Cl concentration (56 mM) ensured a fast and efficient reaction. However, it also yielded some unwanted by-products, which undesirably affected the analysis. To overcome this problem the use of ultra-sonication during the derivatization reaction was tested and it proved to be effective in lowering the amount of by-products as also observed by Jia, Ryu (694). Thus, this procedure was adopted for further PAs analysis.

8.3.4. Method validation

Linearity, precision, limit of quantification (LOQ) and limit of detection (LOD) were performed to validate the optimized analytical method as shown in Tables 8.3 and 8.4. Method validation was performed with standard solutions following the steps described in section 8.2.5. Linearity of the calibration curves was obtained in the 0.5 – 10.0 mg/L interval for all the PAs, with correlation coefficients (r^2) of 0.9989 or higher (Table 8.3). Mean moisture content of the analyzed plant foods was Intra and interday precision for peak areas, expressed as % RSD, ranged from 0.8 to 6.9% and from 3.0 to 10.3%, respectively (Table 8.4).

Table 8.3 – Linear range, regression equation, correlation coefficient (r^2), LOD and LOQ of the optimized method.

Analyte	Linear range (mg/L)	Regression equation	r^2	LOD (µg/g)[a]	LOQ (µg/g)[a]
PUT	0.5 – 10	Y = 0.2701x + 0.0216	0.9997	0.019	0.065
CAD	0.5 – 10	Y = 0.2524x + 0.0196	0.9993	0.034	0.115
SPD	0.5 – 10	Y = 0.2601x + 0.0062	0.9992	0.036	0.121
SPM	0.5 – 10	Y = 0.203x + 0.0297	0.9989	0.045	0.148

[a] LOD and LOQ were expressed as µg/g of plant foods fresh weight. A mean moisture content of 90% was assumed.

Table 8.4 – Intra and interday precision for peak areas at three concentration levels. Data presented as % RSD.

Compound	Intraday (n = 3)			Interday (n = 3 × 5 days)		
	0.5 mg/L	2.5 mg/L	10 mg/L	0.5 mg/L	2.5 mg/L	10 mg/L
PUT	4.1	3.1	1.6	5.1	4.0	3.5
CAD	3.9	2.2	0.8	4.6	3.9	3.0
SPD	5.7	5.5	4.1	7.8	6.7	4.1
SPM	6.9	6.8	5.3	10.3	8.2	6.6

LOQ and LOD were expressed as µg/g of plant foods fresh weight considering a mean moisture content of 90%. The LOQ of PUT, CAD, SPD and SPM were 0.065, 0.115, 0.121, 0148 µg/g, respectively. The LOD were 0.019, 0.034, 0.036 and 0.045 µg/g, respectively for PUT, CAD, SPD and SPM. A comparison between our LOD and LODs of different methods described in the literature for several matrices is summarized in Table 8.5. The LOD achieved with our method was significantly lower when compared with those methodologies due to the efficiency of clean-up and preconcentration procedures. It was 28- to 111-fold lower than the MECK–UV method employing derivatization with benzoyl chloride applied to plant foods (677, 700). In fact, our method was 6-fold more sensitive for the quantification of PUT and SPM than one of the most sensitive methods described, which relies on the use of SPE and tandem mass spectrometry for the quantification of PAs in fish (701), and lower than the LOD reported in plant analysis (678, 702). The stability of the derivatized and extracted PAs was very good over 24 h, with a mean RSD of only 2.2%. This is a clear advantage over OPA and dabsyl derivatization procedures.

Table 8.5 – Comparison of methods used for polyamines analysis in foods.

Derivatization reagent	Sample pretreatment	Sample extraction	Separation method	Detection mode	LOD (µg/g)	Type of sample	References
-	0.1 M MSA	-	IC	CD	0.15 - 0.50	Fish	(20)
-	5% TCA	SPE	LC	MS/MS	0.07 - 0.75	Fish	(32)
OPA	5% TCA	-	LC	FLD	0.2 - 0.4	Plant food	(34)
OPA	0.6 M $HClO_4$	-	IP-LC	FLD	0.05 - 0.70	Plant food	(9)
OPA	5% TCA	LLE	LC	FLD	1.5	Fish	(21)
OPA	0.6 M $HClO_4$	-	IP-LC	FLD	1.0 - 1.5	Meat	(35)
AQC	0.6 M $HClO_4$	-	UPLC	UV	0.5 - 4.4	Cheese	(36)
AQC	0.6 M $HClO_4$	-	UPLC	UV	1.7 - 16.2	Cheese	(37)
Dansyl-Cl	0.6 M $HClO_4$	MSPD	LC	UV	0.5 - 0.8	Plant food	(33)
Dansyl-Cl	0.6 M $HClO_4$	LLE	UPLC	UV	0.36 - 1.12	Cheese	(38)
Dabsyl-Cl	0.4 M $HClO_4$	LLE/SPE	LC	UV	0.10 - 0.88	Meat	(39)
Benzoyl-Cl	0.1 M HCl	SPE	LC	ELSD	1.1 - 2.8	Cheese	(12)
Benzoyl-Cl	0.6 M $HClO_4$	LLE	MECK	UV	1.0 - 2.1	Food	(31)
DNBZ-Cl	5%$HClO_4$	DLLME	LC	DAD	0.019 - 0.045	Plant food	Current method

8.3.5. Plant food samples analysis

To assess the applicability of the proposed method, PUT, CAD, SPD and SPM were determined in six plant food samples (lettuce, carrot, onion, cabbage, tomato and apple). Typical chromatograms are shown in Figure 8.5. All the four PAs, including CAD, which is not usually detected in plant foods, could be quantified (Table 8.6). Generally, PUT was the most abundant PA with values ranging between 3.52 and 34.5 µg/g.SPM was the second most abundant PA, with mean contents of 1.31, 5.85, 6.29, 5.52, 1.87 and 1.23 µg/g in lettuce, carrot, onion, cabbage, apple and tomato, respectively. Mean content of SPD in the analyzed plant food samples ranged between 0.24 and 1.17 µg/g whereas mean contents for CAD were 0.16, 0.23, 1.86, 0.20, 0.42 and 0.18 µg/g in lettuce, carrot, onion, cabbage, apple and tomato, respectively. The recovery (%) of PAs was also evaluated in the six different types of common plant foods (lettuce, carrot, onion, cabbage, tomato and apple) to assess the accuracy of the present method for different types of samples. Obtained recoveries were between 81.7 and 114.2 % for the studied PAs (Table 8.6). For each concentration and type of plant food, a Student's *t*-test was performed. The comparison between the expected and the experimental concentration showed no statistical significant difference ($p > 0.05$), thus confirming the accuracy of the method when used in the analysis of these different types of matrixes. The RSD from three independent extractions with and without PAs fortification ranged from 1.14 to 9.97%, indicating that reproducible results can be obtained.

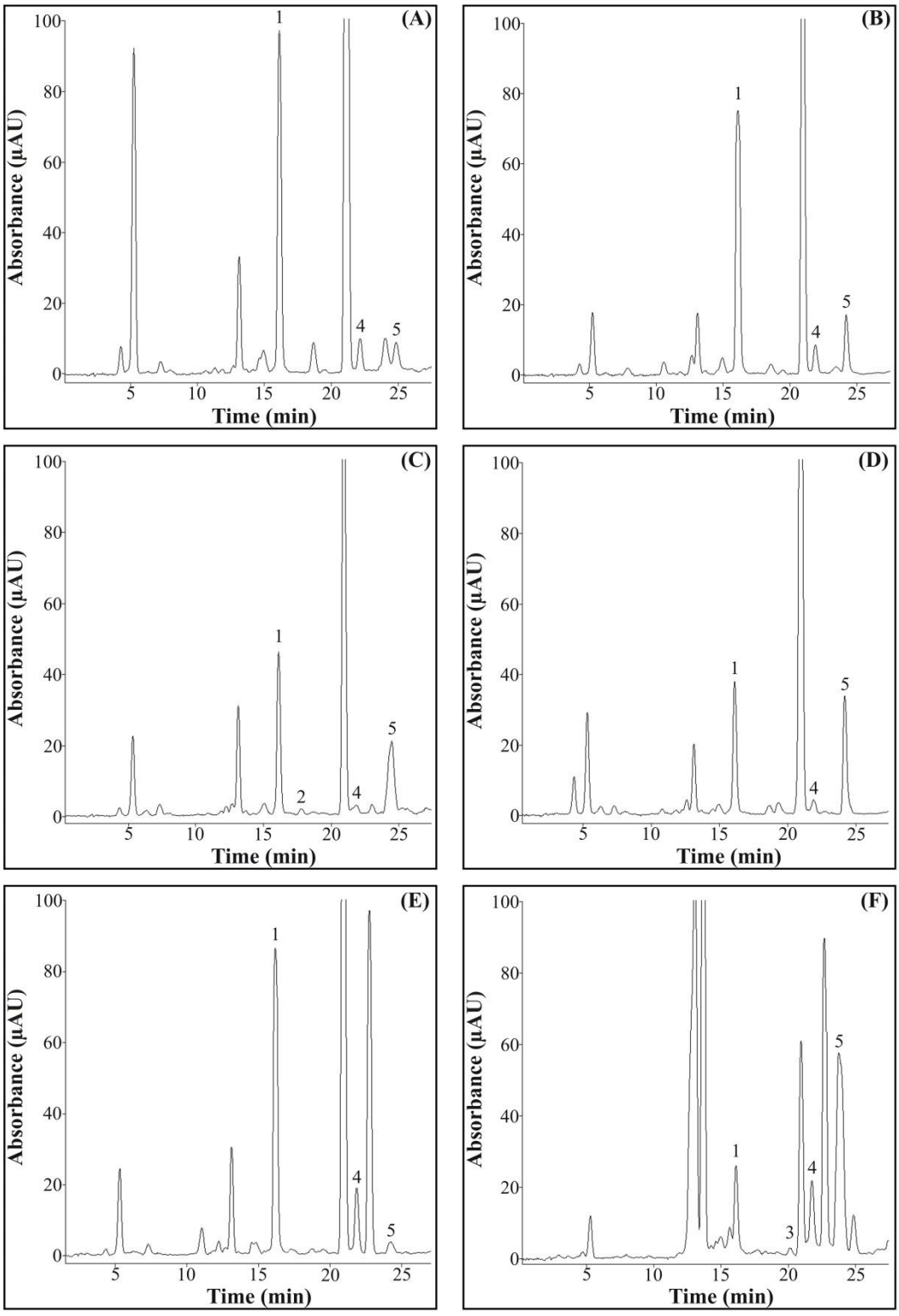


Figure 8.5 – Representative chromatograms of plant food samples: (a) lettuce, (b) carrot, (c) onion, (d) cabbage, (e) tomato and (f) apple. Peaks: 1, PUT; 2, CAD; 3, SPD; 4, IS; 5, SPM.

Table 8.6 – Un-spiked concentration, spiked concentration, relative standard deviation (RSD) and relative recovery (RR) of plant food samples. Data are presented as mean (n = 3).

Plant food sample	Spiking (mg/L)	Compound											
		PUT			CAD			SPD			SPM		
		Found (µg/g)	RSD (%)	RR (%)	Found (µg/g)	RSD (%)	RR (%)	Found (µg/g)	RSD (%)	RR (%)	Found (µg/g)	RSD (%)	RR (%)
Lettuce	0	3.66	3.83		0.16	6.25		0.76	3.95		1.31	5.34	
	2.5	6.35	3.46	107.4	2.72	4.41	102.6	3.14	5.10	95.2	3.61	4.99	91.8
Carrot	0	29.0	1.38		0.23	4.35		1.17	4.27		5.85	5.47	
	2.5	31.2	2.88	88.6	2.61	4.21	95.4	3.68	4.89	100.2	8.58	6.64	109.2
Onion	0	6.39	3.91		1.86	5.38		0.24	4.17		6.29	5.56	
	2.5	9.00	5.67	104.4	4.21	2.14	93.8	3.00	6.67	110.5	8.51	5.76	88.8
Cabbage	0	4.29	3.03		0.20	5.00		0.65	6.15		5.52	5.62	
	2.5	6.70	3.43	96.2	2.75	2.55	101.8	3.04	3.95	95.4	7.76	5.67	89.6
Apple	0	3.52	1.14		0.42	7.14		0.58	8.62		1.87	6.95	
	2.5	5.68	3.52	86.4	2.84	3.52	97.0	2.83	7.42	90.2	3.91	9.97	81.7
Tomato	0	34.5	3.19		0.18	5.56		0.90	6.67		1.23	8.13	
	2.5	37.4	3.48	114.2	2.76	2.90	103.3	3.25	4.62	94.2	3.51	6.55	91.2

8.3.6. Temporal variation of PAs content of lettuces grown in different experimental fields

Table 8.7 shows the content of PUT, SPD and SPM in lettuces grown in A_1, A_2 and A_3 experimental fields. The results show that there were significant differences in the PAs content during the plant growth period. PUT was the most abundant PA in all the studied lettuces, whereas SPD was the PA always present at lower levels. Lettuces from A_1 field showed significantly higher levels of all the three studied PAs while lettuces from A_2 field presented the lower levels. During the initial stage of lettuce development (T_1), PUT, SPD and SPM contents were significantly higher, and decreased until the last sampling time point (T_5). The higher content of PUT over SPD and SPM observed in this study is somewhat contradictory with previous data, as reported by Kalac and Krausova (671). In this review, the PUT reported content ranges between 3.3 and 4.8 mg/kg and the SPD content between 4.2 and 8.3 mg/kg. Only lettuces grown in the A_2 field showed a PUT content within this reported range. Regarding the temporal variation, the higher PAs content observed in the younger lettuces is consistent with the idea that cells are actively dividing in the initial stages of plant development, while the decrease in the PAs content during the lettuce growth indicate that tissue growth is gradually reducing (703).

Table 8.7 – Putrescine (PUT), spermidine (SPD) and spermine (SPM) content in lettuces grown in three different experimental fields (A_1, A_2 and A_3) at different sampling time points (T_1, T_2, T_3, T_4 and T_5).

Polyamine	Sampling time point	Experimental field		
		A_1	A_2	A_3
PUT	T_1	14.39 ± 0.34a*	7.48 ± 0.45a**	9.55 ± 0.30a***
	T_2	10.12 ± 0.42b*	5.52 ± 0.28b**	8.36 ± 0.45b***
	T_3	9.42 ± 0.39b*	5.41 ± 0.16b**	7.85 ± 0.12b***
	T_4	8.16 ± 0.25c*	4.63 ± 0.27c**	6.65 ± 0.24c***
	T_5	6.35 ± 0.33d*	3.84 ± 0.15c**	5.92 ± 0.34d*
SPD	T_1	1.68 ± 0.13a*	0.86 ± 0.03a**	1.26 ± 0.02a***
	T_2	1.42 ± 0.10b*	0.70 ± 0.04b**	1.05 ± 0.05b***
	T_3	1.27 ± 0.06b*	0.57 ± 0.02c**	0.95 ± 0.08c***
	T_4	0.94 ± 0.09c*	0.46 ± 0.02d**	0.81 ± 0.05d*
	T_5	0.81 ± 0.08c*	0.38 ± 0.02d**	0.66 ± 0.03e***
SPM	T_1	3.78 ± 0.11a*	1.62 ± 0.08a**	3.06 ± 0.19a***
	T_2	3.33 ± 0.17b*	1.50 ± 0.05a**	2.52 ± 0.15b***
	T_3	3.12 ± 0.14b*	1.36 ± 0.03a**	1.90 ± 0.10b***

	T_4	2.59 ± 0.12c*	1.05 ± 0.09b**	1.15 ± 0.04c**
	T_5	1.45 ± 0.05d*	0.71 ± 0.03c**	0.93 ± 0.05d***

Data presented as mean ± SD (n = 3). Differences were tested according to two-way ANOVA followed by Tukey's test. In a column, different letters (a, b, c, d and e) indicate significant differences ($p < 0.05$) between the different sampling time points. In a line, different number of stars (*, ** and ***) indicate significant differences ($p < 0.05$) between lettuces grown in different experimental fields.

8.4. Conclusions

Ultrasound-assisted benzoylation coupled to DLLME was applied to the analysis of PAs in plant foods. Experimental designs were successfully used to screen relevant derivatization and DLLME variables and to select the optimum conditions, performing a minimal number of assays, to obtain the maximum PAs chromatographic response and improve method sensitivity. The LODs of PAs were in the range of 0.019 – 0.045 µg/g, highlighting the high sensitivity of the developed method comparing when compared to other proposals from the literature. Moreover, the developed method is inexpensive and easy to perform, and the derivatized PAs acetonitrile extracts could be stored for 24 h at 4 ºC without significant decomposition. The developed method was successfully applied in the determination of PAs in six different types of plant foods with a wide range of PAs contents (from 0.16 to 34.5 µg/g). The good recoveries obtained in plant food samples (81.7 – 114.2%) showed that the developed method has wide applicability. Moreover, it involves equipment easily available in quality control laboratories. Good precision was also obtained in the six different plant foods analysis. The monitoring of PAs content during lettuce growth in three different experimental fields indicate that a general trend for decreasing levels is observed for the PUT, SPD and SPM. PUT was the most abundant PA, while SPD was the less one. Significant differences were observed between PAs content in lettuces grown in different experimental fields.

PART V

Chapter 9

Final Remarks

9.1 General discussion of results

A general discussion of the experimental results was organized according to the pre-defined goals:

- The pretreatment of plant material for the analysis of inorganics is a critical step to obtain accurate analytical data and reliable interpretation of plant analysis results. The usual stepwise procedure involves plant cleaning, drying, particle-size reduction, storage and organic matter destruction. The process of water removal from plant tissues in order to stop enzymatic reactions and to stabilize the sample is very important also when the analysis of inorganics is intended. In the present work, the best sample pretreatment procedure regarding NO_3^- determination was investigated. Freeze-drying was proposed as a suitable pretreatment procedure for NO_3^- determination in the analyzed plant foods (spinach and lettuce). A large number of samples can be lyophilized, the sample size is significantly reduced, a homogenous sample is obtained and the lyophilized sample can be stored for long periods before analysis.
- The selection of the most adequate method to collect representative soil samples that will be used to estimate the elemental content available to plants is another critical issue. The reliability with which a particular soil sample reflects the conditions of plants growing in that soil is highly dependent on the way the sample is collected and handled. Most of the studies looking for the estimation of the phytoavailability of both essential and non-essential elements in soil rely on the analysis of bulk soil in a given time period. Moreover, to estimate a specific range of phytoavailable elements, several soil extraction methods have been used. In this study, the analysis of rhizosphere soil (instead of bulk soil) at five time points (instead of a single time point) during lettuce growth proved to be a reliable approach to estimate plant metal(loid)s content. Four soil extraction methods (Mehlich 3, DTPA, NH_4NO_3 and $CaCl_2$) were compared regarding their capacity to estimate the soil metal(loid)s phytoavailability and, lastly, to estimate the plant metal(loid)s content. Although, DTPA and Mehlich 3 methods showed to extract a higher amount of the studied metal(loid)s, the NH_4NO_3 method resulted in strong soil-plant positive correlations for almost all the metal(loid)s studied. It seems to be the most suitable method to estimate metal(loid)s phytoavailability. Additionally, several soil properties (pH, OM, CEC, clay and oxides content) also showed to markedly influence the distribution of metal(loid)s between the phytoavailable and non-phytoavailable fractions. An accurate prediction of metal(loid)s content in plants was obtained considering the amount of metal(loid)s extracted by the NH_4NO_3 method and the soil properties CEC, OM, pH, texture and oxides content.

NH_4NO_3 extraction method was the most suitable method to study the elemental phytoavailability in soil thus it was used in all the further studies presented in this thesis.

- The broad variation in the physicochemical properties of soils constitutes a major challenge in agriculture. The accumulation of a given element is the result of a complex gene-regulated process that governs the uptake, binding, transportation and sequestration of that element by the plant. On the other hand, evidence suggests that important inter-elemental relationships exist. In this context, it is important to measure as many elements as possible in plant tissues (the so-called plant "ionome"). Since it was previously highlighted the importance of several soil properties in the phytoavailability of metal(loid)s, the effect of the same soil properties in the plant ionome was also investigated. The obtained results support the idea that environmental conditions, particularly the soil properties, have a key effect in the shoot ionome of lettuce. Soil characterized by higher CEC, OM, EC, clay and silt content and by a lower pH presented significantly higher phytoavailable fractions of the studied elements. A very distinct pattern was also observed in the shoot ionome of lettuces grown in this soil. Significant correlations between different pairs of elements in lettuce shoot indicate that similar processes may be engaged in the uptake, translocation, distribution and storage of those elements within the plant. For macro elements, a similar pattern of correlations was observed in lettuces from the three soils, with a positive relationship between Ca and Mg and between N and P, and a negative relationship between Ca and K. This indicates that, for macro elements, other factors (e.g., genetics) may be more important than environmental factors in defining the ionomic profile of plants. The trace elements Fe, Mn, Ni, Co, Zn and Cd showed both positive and negative correlations in lettuce shoot, depending on soil properties and the amount of phytoavailable elements. The toxic elements As, Cd and Pb showed negative correlations with essential elements, showing that these elements are taken by plants through transporters involved in essential elements uptake, which results in inter-element competition.
- As already noted, the availability of a certain element for plant uptake can greatly change with soil properties. These changes can be more significant in particular stages of the plant growth, affecting its actual elemental content. Besides being recognized by the US Environmental Protection Agency (EPA) as a suitable plant species to determine the plant uptake and translocation of toxic substances, lettuce is also an important food in the human diet. Therefore, the study of the content of both essential and non-essential elements in lettuce is of great importance in the food

science field. The work developed highlighted that lettuce nutritional value (and potential toxicity) strongly depends on growth stage and soil composition. Overall, higher contents of macrominerals, trace elements and photosynthetic pigments were observed in young lettuces. The normal trend was for a decreasing in all elements and pigments content along the plant growth period. The only exceptions were the trace elements Al, Cr, Fe, V and Pb, which showed increased contents along lettuce growth period. It was also shown that particular soil properties (CEC, OM and pH) have an important effect regarding the elemental phytoavailability along the plant growth period.

- Microgreens have emerged as a new class of edible vegetables. Young lettuces have a higher content of minerals than mature ones. Moreover, leafy vegetables are able to accumulate NO_3^- during the growth period, potentially posing a problem for human health. Thus, it was considered of major relevance to study if microgreens can be an important source of minerals in human diet, while reducing the intake of harmful NO_3^-. The results obtained showed that microgreens can provide a significantly higher intake of essential minerals than mature lettuces. Mature lettuces presented higher N, P, K and Na content, but Ca, Mg, Fe, Mn, Zn, Se and Mo content was higher in microgreens. Microgreens can therefore be considered a good source of important minerals. Additionally, the NO_3^- content of microgreens showed to be very low allowing its safe use, particularly in children's diet, to fulfil the daily mineral requirements while reducing their exposure to the harmful NO_3^-.
- High levels of NO_3^- in vegetables result mainly from excessive nitrogen (N) supply. N is a ubiquitous and fundamental nutrient to sustain the increase of crop yields. Most farmers rely on the application of N-containing fertilizers to support crops productivity. NO_3^- plays a key role in the function and nutrition of plants. However, accumulation can occur in leaf crops, such as lettuce. NO_3^- itself is relatively non-toxic to humans, but its reaction products and metabolites (e.g., NO_2^-, NO and nitrosamines) have raised alarm due to their adverse effects on human health. In this context, it was studied how soil properties/composition and specific enzymes (nitrate reductase and glutamine synthetase) involved in the N metabolism influence the NO_3^- content during plant growth. Nitrate reductase activity decreased during lettuce growth (consequently, NO_3^- lettuce content increased). A positive correlation was observed between glutamine synthetase activity and NH_4^+ levels in lettuce leaves, with higher levels during the first weeks of lettuce growth and a posterior steady decrease. This pattern was similar in lettuces grown in similar conditions (although in different soils). However, quantitative differences were observed, indicating that inorganic N soil

content influences both NO_3^- and NH_4^+ content in lettuce. Higher NO_3^- levels in the soil caused NO_3^- accumulation in lettuce, despite the higher nitrate reductase activity during the first stages of plant growth. Higher NO_3^- and NH_4^+ levels were correlated with higher N-Kjeldahl levels in lettuce, suggesting a positive effect in organic N synthesis at the plant tissues. Soil composition also showed to influence the mobility of inorganic N within the groundwater-soil-plant system. Loamy sandy soil with high OM and CEC showed to retain inorganic N more efficiently, which results in increased NO_3^- and NH_4^+ content in soil solution and, lastly, in a higher N content in lettuce. Sandy soils with low OM and CEC allowed NO_3^- leaching, which was confirmed by the high NO_3^- levels in groundwater. Consequently, lettuce grown in these soils presented lower N content and higher inputs of N ato the environment were observed.

- The polyamines (PAs) putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM) have been implicated in several biological processes, including plant growth and development. On the other hand, the dietary intake of PAs presents human health effects. Studies describing the evolution of PAs content during plant growth in different soils are scarce. Thus, it was found important to obtain additional data regarding this topic. In the first part of this study the optimization and validation of a sensitive method to quantify PAs was performed. Ultrasound-assisted benzoylation coupled to dispersive liquid–liquid microextraction (DLLME) was successfully applied to the analysis of PAs in several plant foods. Experimental designs were used to screen relevant derivatization and DLLME variables and to select the optimum conditions (performing a minimal number of assays) in order to obtain maximum PAs response and to improve method sensitivity. The low LODs obtained (in the range of 0.019 – 0.045 µg/g) reflect the high sensitivity of the developed method. The present method was also inexpensive and easy to perform and the derivatized PAs extracts (acetonitrile based) proved to be stable for 24 h at 4 ºC. PAs were determined in six different types of plant foods with a wide range of PUT, CAD, SPD and SPM contents (from 0.16 to 34.5 µg/g). The good recoveries of PAs obtained in the analyses of plant food samples (81.7 – 114.2%) shown that the developed method has wide applicability. Good precision was also obtained. In the second part of this work, the temporal and spatial monitoring of PUT, SPD and SPM content was performed. A general decrease in the content of PUT, SPD and SPM was observed during plant growth, which can be explained by cells active dividing in the initial stages of lettuce development. Significant differences were also observed between lettuces grown in the three different experimental fields.

9.2 Conclusions

The work described in this thesis provides an additional understanding regarding the influence of the soil-to-plant transfer of chemical species in the nitrate content, N metabolism and ionome of plant foods and consequently gives information regarding its nutritional value in the human diet.

A careful literature review was conducted in order to understand the underlying mechanisms responsible for the solubility, speciation, availability, uptake, translocation, transformation and accumulation of chemical species in plants. In this context, a broad and highly specialized background was obtained, enabling the performance of field studies.

Two main topics regarding extraction methods for the analysis of vegetables and soil were studied. Firstly, the influence of different sample pretreatment procedures and extraction methods in the content of nitrate and nitrite in plant foods was studied. The obtained results allowed to choose an appropriate storage procedure (freeze drying) to be applied to the high number of plant food samples collected in the field studies. Secondly, the most appropriate soil extraction method and sampling strategy to better predict the phytoavailability of several elements was investigated. By sampling rhizosphere soil together with the use of NH_4NO_3 as soil extraction solution, reliable and accurate data was obtained.

Afterwards, the soil-to-plant transfer of chemical species during lettuce growth and its influence in lettuce nutritional value was studied. Several soil properties (CEC, OM, pH, particle size distribution and oxides content) showed to play a key role in the distribution of chemical species between the phytoavailable and non-phytoavailable fractions. This, in turn, showed to markedly influence the temporal and spatial ionomic profile of lettuces. Overall, lettuces in the initial stages of growth presented higher content of essential minerals and pigments, and lower content of nitrate. Consequently, the use of younger lettuces (microgreens) as a safe and healthy source of important minerals for the human diet is of interest.

The N metabolism of plant foods during growth was also assessed. Since NO_3^- is a well-recognized contaminant that can accumulate in plant foods, in particular vegetables, the influence of soil composition and the activity of the enzymes involved in plant N metabolism were studied along the lettuce growth. Both soil composition and enzymes showed to play a key role in NO_3^- accumulation in lettuces along their growth. Lettuces grown in soils with higher NO_3^- levels accumulate more NO_3^-. Nitrate reductase activity decreases along the lettuce growth, which was also important in the process of NO_3^- accumulation. Lastly, since polyamines are important compounds involved in the plant

growth and development, a high sensitive method was optimized and validated to study the temporal variation of polyamines content in lettuces (and other plant foods) grown in three different experimental fields. Polyamines content decreased during the lettuce growth period independently of the field where they grow. However, quantitatively differences were observed between the polyamines content of lettuces grown in the different fields.

9.3 Future perspectives

Since global population continues to rise, demand for more and highest quality food will also continue to rise. Nowadays, the major global challenge is to produce more food in the same area of land, while reducing environmental impacts. The biofortification of crops through the application of fertilizers, combined with breeding varieties with an increased ability to acquire mineral elements, has been claimed as an immediate strategy not only to increase mineral content in edible crops but also to improve yields on infertile soils. During the last few years, several attempts have been made to increase nutrients phytoavailability, while reducing plant exposition to harmful chemical species. Because of its higher mineral content when compared to mature plants, microgreens can also regarded as an important source of mineral nutrients.

In recent years, the study of the plant ionome and N metabolism has opened new horizons in the plant and food science fields. Key processes involved in the homeostasis of chemical species within plant tissues have been identified. Several genes (e.g., NRT, PHT, COPT, ZIP, NAS, YSL, HMA) have been functionally cloned, and their importance in the accumulation of chemical species has been established. More broadly, genetic engineering has opened a new range of opportunities for understanding soil-plant relationships and the homeostasis of chemical species in plant. Much remains to be learned about some chemical species. In addition, an integrative approach using diverse plant species will lead to a better understanding of the phytoavailability, uptake, transport and distribution of chemical species. Although there is limited evidence for coordination of the regulatory networks that control the homeostasis of chemical species in plants, evidence suggests that there is some crosstalk. Because different chemical species use the same transporters, there is clear evidence for important interactions.

There is significant evidence that the regulation of ion homeostasis is mediated by membrane transporters. More work is necessary to transfer genes into edible crop to improve food quality. There is a need to be cautiously optimistic about the application of biofortification, taking into account the limits of this strategy. Bringing this technology into common practice is also a challenge.

PART VI

Chapter 10

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10. References

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