Understanding the partitioning and concentration of trace elements in the plant organs of some food crops: influence of the plant allometry and of the growth stage

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Preface

Global food security has become a primary concern during the last decades. Agriculture is central for human beings in order to produce enough foodstuffs to cover food needs with an adequate amount of nutrients and, thus, to fulfil healthy growth and development of humans.

The Industrial Revolution (which began in the second half of the 18th century) represented a transition to new manufacturing processes. Specifically, the impact on agriculture and food supply was of outstanding importance. Advances in the cultivation systems and in the use of technology to enhance crop growth and food production permitted a great social and economic development in advanced countries, decreasing the human mortality in part thanks to a rapid and direct access to essential nutrients. However, some other problems arouse as a consequence of the fast development of intensive agriculture. Nowadays, more than 10 % of the global land is used for agricultural purposes, resulting frequently in a more or less degradation of ecosystems with negative impacts on living organisms and other natural resources (such as soil and water). This creates new uncertainties about the sustainability of current agricultural production practices and about their impacts for the human being and the environment. So, agricultural production worldwide and the way in which crops are produced become extremely important when food security is in question.

This led to the development of more environmentally-friendly agricultural practices, a production systems which among others, avoid or reduce the use of synthetic chemical inputs for plant growth and favour the production of crops containing little hazardous compounds, for the benefits of both the environment and the human beings.

For an appropriate crop production, apart from the major nutrients such as carbon, oxygen, hydrogen, nitrogen, phosphorus, potassium, calcium, magnesium, and so on, plants also need several elements as essential micronutrients in proper quantities for normal physiological growth and development. The distribution and concentration of these elements in the plant tissues is physiologically regulated depending on the effects of these elements on the vital functions. If the regulation is not possible due to low or excessive availability of these elements, plants would suffer of several internal disorders, deficiencies, toxicities and could even die. Some other elements which have not known functions in plants, which are generally highly toxic to life, can also be taken up by plants and distributed in the plant parts, including the edible ones, therefore entering the food chain and threatening the food quality. In this way, the use of contaminated inputs in the intensive agriculture is known to possibly increase the concentrations of these non-essential elements in the growth substrate and, thus, be a potential risk for plants health and food consumption.

The edible parts change from crop to crop, depending on the nature and life cycle of the plant. Hence, the edible part of a plant can be the underground organs (as in the case of potatoes, carrots, onions and so on), the shoots (as it occurs for example with chards, spinaches, lettuces) or the fruit (e.g. tomatoes, peppers and eggplants). The way by which a chemical element, essential or not, reaches the edible part of a plant must be known in the perspective of guaranteeing the food quality and security.

Plants take up elements mainly by the root system (from the soil solution or from a nutrient solution) or more marginally, by leaves, through stomata from atmospheric depositions. The natural soil is the most popular way to grow plants, either in open-air fields or in greenhouses under controlled conditions of light, humidity and temperature. However, the water-culture is an alternative for some crops because it allows controlling and optimizing the inputs and amount of nutrients taken up by plants, and it enables to grow plants regardless of the season.

The elements that are taken up can be sequestrated or they can be more or less translocated to other plant organs, as part of the mechanisms of regulation of the concentration of essential and non-essential elements in the vital compartments of the plant. Moreover, both the partitioning of an element between plant tissues and the remobilization/retranslocation during the life cycle of the plant are highly influenced by the plant species, its growth stage and the environmental conditions in which they grow up (element availability, temperature, wind, light intensity and humidity above all).

This PhD Thesis has focused on the accumulation of essential (possibly toxic at high concentrations) and non-essential (generally highly toxic at low concentrations) trace elements, in the context of the food quality and safety. Consequently, the objectives of the work were defined as follows:

i) to investigate the uptake, accumulation and translocation of essential and non-essential elements between organs of plants with different edible parts.

ii) to study the influence of the type of agricultural practice (conventional *vs*. organic) on the accumulation of trace elements in the different organs of two vegetables (Swiss chards and tomatoes) along their whole production cycle.

iii) to model the partitioning of a particularly worrying toxic element, cadmium, between the plant organs of an important crop, the sunflower, at its different reproductive stages.

The document contains ten Chapters grouped in three different Sections. First, a general **introduction (Section I)** reviews the key ideas already outlined in this preface: the current state of the agriculture in the world and the main types of crops produced in Europe are discussed, together with a brief explanation of the different media used for plant growth and development (soil and water-culture methods) and a comparison of the main agro-ecosystems used nowadays worldwide. The role of chemical elements in plant nutrition is also explained, focusing on the essentiality of macro and micronutrients and on the toxicity of non-essential trace elements. Finally, a general description of the physiology of trace elements homeostasis in higher plants is presented.

The experimental methods and the results obtained during this Thesis work have been divided into two Sections. The **Section II** relates to an open-air field experiment where two different crops, Swiss chards (as leafy vegetable) and tomato plants (as fruit vegetable), were grown in parallel by using two different cultivation practices (organic *vs*. conventional). The objective was to follow the time course of accumulation of nutrients and of toxic trace elements in the plant organs, taking into account the fertilization practices. A common brief **introduction** to this study is included, followed by the detailed description of the experiment performed and the materials and methods used (**Chapter 1**). The results are presented and discussed in **Chapter 2** for Swiss chards and in **Chapter 3** for tomato plants.

As a natural consequence of the results obtained for tomatoes in the field experiment, the need to develop a tool to estimate the average ripening stage of a set of tomatoes, e. g., those tomatoes collected from a single plant in a specific sampling campaign, arose. In **Chapter 4**, the development of a methodology to discern between tomatoes with different ripening stages is explained, using chromaticity values previously obtained by image analysis of calibrated pictures of the sample collected with a standard camera. The general conclusions of the Section II are summarized in **Chapter 5**.

The last Section (**Section III**) is focused on the widely spread very toxic element, cadmium (Cd). Sunflowers were cultivated in a greenhouse, using a standard nutrient solution enriched with Cd. The objective was to understand and model the partitioning of Cd in sunflower, paying a particular attention to the role of plant transpiration and of the allometry of plants organs, and of the remobilization of previously stored Cd from vegetative parts to the seeds.

This section starts with a brief **introduction** followed by a detailed description of the experiment performed and the materials and methods used (**Chapter 6**). The understanding and modelling of the partitioning of the Cd recently taken up between sunflower organs at reproductive stages is presented in **Chapter 7**, while **Chapter 8** concentrates on the origin of the Cd allocated to grain: the uptake by roots and the remobilization from reserves is discussed. The conclusions obtained in the experiments of Section III are presented in **Chapter 9**.

This document finishes with some **Final remarks and perspectives** of the PhD Thesis, followed by a list of already published submitted and/or pending **Articles and Communications to Scientific Conferences** which derive from the results of this Thesis and several **Annexes** with the supporting information for the Chapters presented above.

This Thesis has been carried out with the consent of the University of the Basque Country (UPV/EHU) and the University of Bordeaux, within the framework of the Cross-Border Euroregional Campus of International Excellence IdEx Bordeaux - Euskampus. The thesis has been performed in a co-tutelle basis between the following two partners that have complementary skills and expertises:

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Abbreviations

A

AAS, Atomic Absorption Spectrometry.

ABC, ATP-binding cassette.

AG , aboveground organs.

AMF, arbuscular mycorrhizal fungi.

ANOVA, Analysis of Variance.

ATP, Adenosine triphosphate.

ATSDR, Agency for Toxic Substances and Disease Registry.

B

BAF, bioaccumulation factor.

BBCH, Biologische Bundesanstalt, Bundessortenamt and Chemical industry.

BHT, butylated hydroxytoluene.

C

CA, Correlation Analysis.

CDF, cation diffuser facilitator.

CEC, cation exchange capacity.

CIE, Commission Internationale de l´Eclairage.

D

DMA, dimethylarsinic acid.

E

ERM, European Reference Materials.

EPA, see US EPA.

F

FAO, Food and Agriculture Organization of the United Nations

FIBL, Research Institute of Organic Agriculture.

FTIR, Fourier transform infrared spectroscopy.

G

GDD, growing degree days.

GET, Géosciences Environnement Toulouse.

GF, graphite furnace.

H

HEDTA, Hydroxyethylethylenediaminetriacetic Acid.

I

IARC, International Agency for Research on Cancer.

ICP, inductively coupled plasma.

IFOAM, International Federation of Organic Agriculture Movements.

INRA, Institut national de la recherche agronomique.

IR, infrared.

IRT, iron-regulated transporter.

ISO, International Organization for Standardization.

L

LC, liquid chromatography.

LCTI, low-affinity cation transporter.

LOD, limit of detection.

M

MES, 2-(N-morpholino)ethanesulfonic acid.

MMA, monomethylarsonic acid.

MT, metallothionein.

N

NA, nicotianamine.

NIST, National Institute of Standards and Technology

NPK, nitrogen-phosphorus-potassium.

Nramp, natural resistance-associated macrophage protein.

NRCS, Natural Resources Conservation Services.

O

OM, organic matter.

OC, organic carbon.

P

PC, principal component.

PCA, Principal Component Analysis.

PCs, phytochelatins.

PTFE, polytetrafluoroethylene.

PVDF, polyvinylidene fluoride.

R

RMSEC, root mean-squared error in the calibration.

RMSEV, root mean-squared error in the validation.

S

SRM, Standard Reference Material.

T

TF, translocation factor.

U

UPV/EHU, University of the Basque Country.

USDA, United States Department of Agriculture.

US EPA, United States Environmental Protection Agency.

Y

YSL, yellow-stripe-like.

Z

ZIP, zinc-regulated transporter, iron-regulated transporter protein.

ZRT, zinc-regulated transporter.

SECTION I

Introduction

Introduction

1. CURRENT STATE OF THE AGRICULTURE IN THE WORLD, CULTURE METHODS AND AGRICULTURAL SYSTEMS

1.1. THE AGRICULTURE AND MAIN CROPS IN EUROPE

Human being has transformed the world through anthropogenic activities. Nowadays, about 52 million km^2 of the land surface area are used for agricultural purposes and other 3.5 million km^2 corresponds to urban and industrial areas.[1-3] These three anthropogenic systems are responsible for the occupation of a third of the global Earth surface. About 12 % of the emerged surface area is used for cultivation,[4] being widely present in Europe, where about 47 % of the land cover corresponds to this activity.[5]

Human being has to produce several crops to sustain the food needs of all the people worldwide. As can be observed in Figure 1, Europe was one of the lands par excellence for growing barley, maize, wheat and sunflower during the nineties. The acquisition of these data from various census organizations (Food and Agriculture Organization of the United Nations (FAO), the United States Department of Agriculture (USDA) and National Institutes) and subsequent processing is explained in detail in several papers of the same authors.[3,4] According to current dataset (until September 2014), the most important crops found in agricultural productions of Europe are vegetables, grain crops and wine crops.[5]

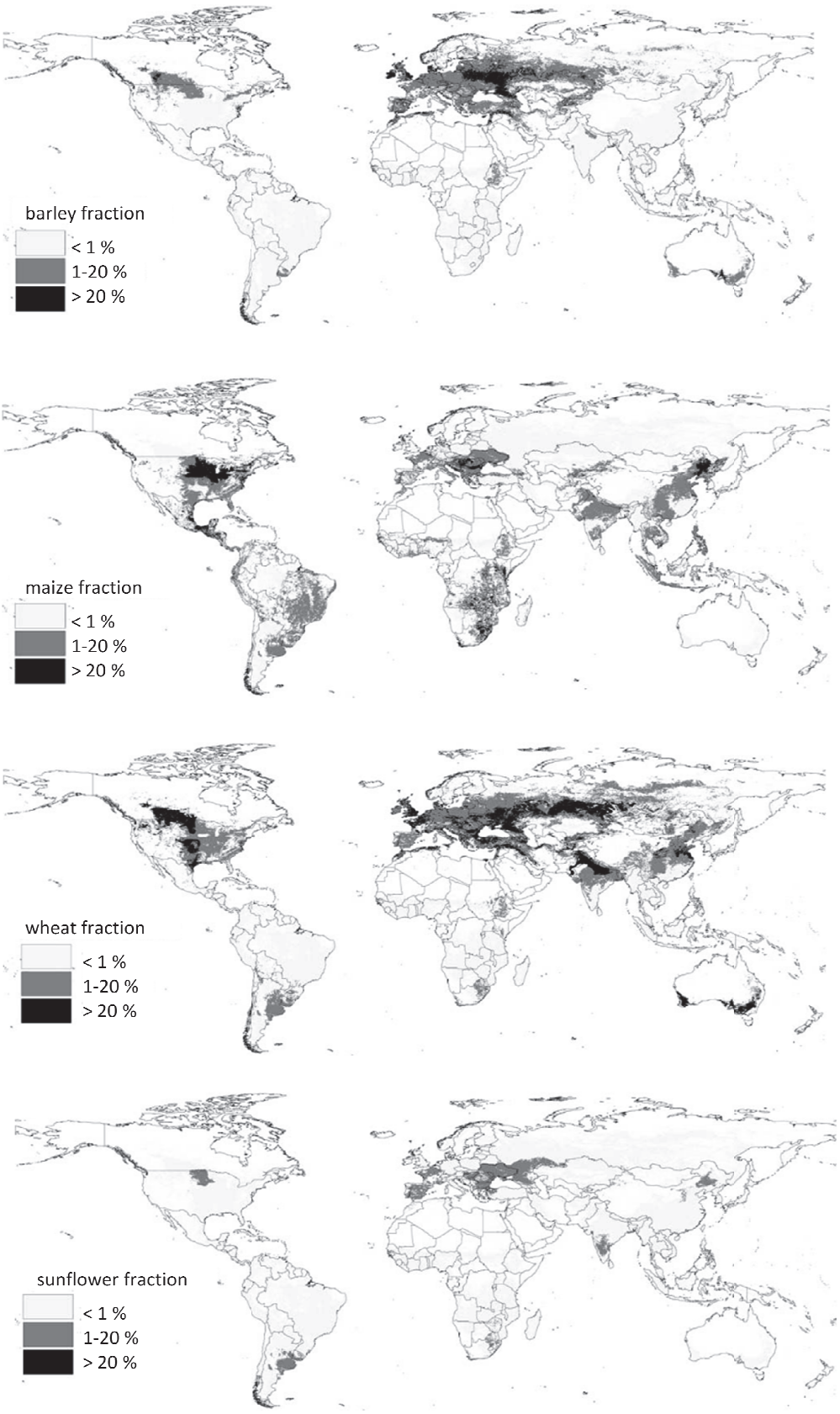

Figure 1. Global distribution of some major crops. Maps from 2004, and adapted from Leff et al.[4]

1.1.1. VEGETABLE CROPS

The favourable climatic and topographic conditions of Europe permit the cultivation of several vegetables. Vegetables can be roughly divided into three main groups, according to plant organ which is eaten: leafy vegetables, fruit vegetables and root + tuber vegetables (Figure 2).[6,7]

Leafy vegetables have gained a significant place in the food pyramid, being a source of essential elements and of other bioactive compounds. They play an important role in human nutrition and in diet and diseases prevention.[8] Some of the most popular leafy vegetables grown up in Europe are lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), and chard (*Beta vulgaris*).[9]

Fruit vegetables refer to those vegetables for which the edible part is the reproductive part. Some examples of fruit vegetables are tomato plant (*Solanum lycopersicum*), green bean plant (*Phaseolus vulgaris*) and green pea plant (*Pisum sativum*). Europe is one of the main global producers of tomatoes in the world. As an example, the European Union produced about 15 million tonnes of tomatoes in 2013, with Italy and Spain being the main producers.[5]

Figure 2. Examples of leafy, fruit and root + tuber vegetables produced in Europe. From left to right: chards (*Beta vulgaris*), tomato fruits (*Solanum lycopersicum*) and carrots (*Daucus carota*).

Finally, vegetables which edible part corresponds to the underground organ are known as root vegetables. The most important root vegetables produced in Europe are carrots (*Daucus carota*) and onions (*Allium cepa*), with a production of about 5 and 6 million tonnes, respectively.[5]

1.1.2. GRAIN AND CEREAL CROPS

Grain crops, including cereal crops, are widely produced around the world, being the most widely grown arable crops in the European Union.[4,10] The edible part of the grain crop is, as its name indicates, the grain or the seed (Figure 3).

In Europe several grain crops are produced, such as wheat (common wheat and durum wheat), rye, barley, oats, mixed grain other than maslin, grain maize, sorghum, triticale, and other cereal crops such as buckwheat, millet, canary seed and rice.[5]

Within this list, common wheat (*Triticum aestivum*) is the most important cereal produced in Europe (44.5 % of the total cereal production in 2013), followed by grain maize (*Zea mays*) and barley (*Hordeum vulgare*), with 21.5 % and 19.6 % of the total cereal production in 2013, respectively.

On the other hand, sunflowers seeds (*Helianthus annuus*) are one of the main oilseeds cultivated in Europe, with a production of about 9 million tonnes in 2013. The outstanding countries in sunflowers seeds production in 2014 were Romania (2128 tonnes), Bulgaria (2009 tonnes), Turkey (1638 tonnes), France (1559 tonnes) and Hungary (1555 tonnes).

Figure 3. The most important grains and oilseeds produced in Europe. From left to right: common wheat (*Triticum aestivum*), grain maize (*Zea mays*), barley (*Hordeum vulgare*) and sunflower (*Helianthus annuus*).

1.1.3. WINE CROPS

The European Union is the largest wine producer in the world (Figure 4). According to the European Commission´s Directorate-General of Agricultural and Rural Development, Europe was responsible for the 65 % of the global worldwide wine production, for the period 2009-2014,[11] the principal grapes producers in Europe being Italy (32 %), Spain (30 %) and France (17 %), according to European statistics (percentage of EU-28 total harvested production-tonnes).[5]

Some of the most important wines produced in these three European countries (Figure 4) are the Lambrusco (a fruity and frizzy red Italian wine), the red wine from Bordeaux, Rioja wine from Spain and the wine called “txakoli” (from the Basque Country), normally produced with green grapes which gives some acidity to the wine.

Figure 4. Some famous wines produced in Europe. From left to right: Lambrusco (Italy), Cabernet Sauvignon (France), Piérola Rioja (Spain) and Gorka Izagirre txakoli (Basque Country).

1.2. SOIL AND WATER-CULTURE METHODS FOR PLANT GROWTH

Soil is a dynamic medium in continuous change due to human activities and natural processes. One of the most accepted definition of soil comes from the Natural Resources Conservation Services (NRCS) of the Unites States Department of Agriculture (USDA),[12] who states that the soil is "(i) the unconsolidated mineral or organic material on the immediate surface of the Earth that serves as a natural medium for the growth of land plant and (ii) the unconsolidated mineral or organic matter on the surface of the Earth that has been subjected to and shows effects of environmental factors of: climate (including water and temperature effects), and macro- and microorganisms, conditioned by relief, acting on parent material over a period of time".

The optimum soil for agricultural purposes is formed by a perfect combination of sand (0.05 - 2 mm), silt (0.002 - 0.05 mm) and clay particles (< 0.002 mm), organic matter, air and water.[13] This combination allows the growth of plant by providing a physical support, by making possible the water retention and drainage, and by allowing the oxygenation of the root system and the bioavailability of nutrients to be taken up by plants.

Besides, during the past two centuries the water-culture has been widely developed, in greenhouses above all, as a modern way of plant growth and nutrition.[14,15] The water-culture method is a kind of nutriculture, that is, a method for plant growth in a medium other than natural soil. The origin of the water-culture technique relied on studies from plant physiology, botany and chemistry, with the outstanding discovery that plants, formed by water and several chemical elements, need air, water and soil for their growth and development.[16,17]

According to Hoagland and Arnon,[15] soil and water-culture methods are not strictly different, based on certain aspects:

i. The obtained yields are not strikingly different under comparable conditions.
ii. Plant growth and water requirement are the same in both cultures.
iii. Nutritional quality of the product is the same.
iv. Nutrient deficiencies, insect attacks, and diseases present similar problems.
v. Climatic requirements for plant growth and development are the same.

These similarities have been far and wide criticized by many other authors, claiming that the hydroponic culture can produce much higher crop yields,[18] avoid nutrient deficiencies and toxic metals pollution and reduced labour input when the hydroponic culture is automated.[19] Moreover, with the water-culture technique, plants can be spaced closer than in a rich soil.[15]

The water-culture systems are usually developed in greenhouses under controlled conditions of temperature and humidity.

As an example, plants can be grown either in a floating platform, all of them being in contact with the same nutrient solution, or in individual devices, with an automatic pump supplying the nutrient solution and oxygen (Figure 5). In any case, a continuous supply of nutrient solution and flow of oxygen (known as *bubble*) is needed. Moreover, the nutrient solution has to be protected from light to avoid the growth of algae not wanted during plant growth.

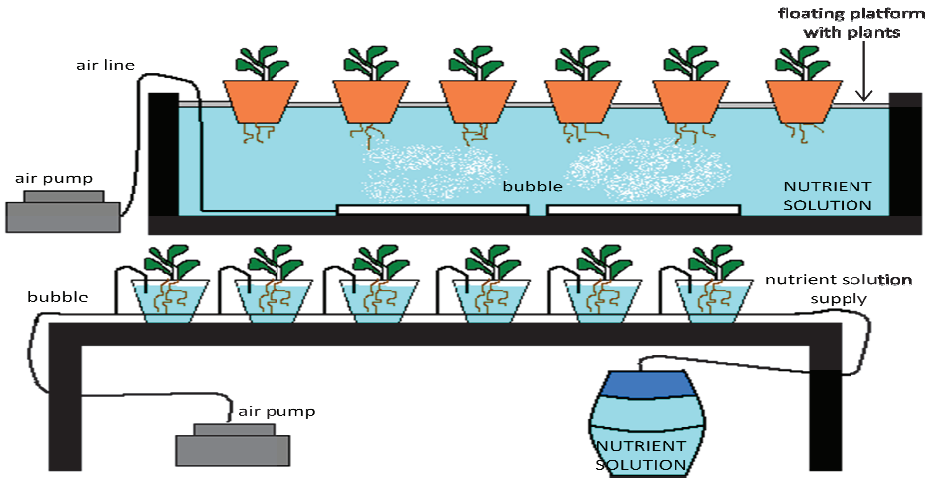

Figure 5. Scheme of two typical water-culture systems. In the upper system plants are in a floating platform inside a big container full of nutrient solution. In the lower system plants are grown up with an individual dose of nutrient solution, supplied by an automatic pump.

In general, the concentration of nutrients (which determines the kinetics of uptake) in the nutrient solution is adapted to provide the necessary amount of them for plants growth and development. However, during its preparation many factors have to be taken into account. According to Hoagland et al.,[15] the concentration of chemical elements in the solution tends to change as the plant grows up, due to the uptake by the root system. This mechanism can be faster or slower depending on the roots and on the nutrients availability in the nutrient solution.

Moreover, during plant growth the pH of the nutrient solution can change, due to the difference in uptake between the cations and the anions. A change in the pH can in turn affect the bioavailability of some nutrients. On the other hand, the nutrient solution must be adequately aerated to provide sufficient oxygen for the root respiration while discarding the CO_2. Hence, during a water-culture of plants, the physic-chemical characteristics of the nutrient solution have to be monitored to make sure that there are enough nutrients available and that the pH and the aeration are also correct. This can be done by continuously renewing partly the nutrient solution or by making periodic total changes of the solution.

Although many growers prefer to develop their own nutrient solutions, there exists a well-known and commonly used one for plant growth (first published by Hoagland and Arnon in 1938 and modified in 1950) and successfully used either in crop productions or in scientific experiments.[15]

The absolute concentrations of the Hoagland nutrient solution can be modified to optimize the quantities of salts that are used, depending on the plant requirements.[20,21]

1.3. AGRICULTURAL SYSTEMS IN SOIL CULTIVATIONS

People around the world depend on crops and livestocks for food. No other activity has transformed humanity, and also the Earth, as much as agriculture did. In the Basque Country, agriculture is present in all the villages, with different kinds of crops, such as tomatoes, lettuces, peppers, potatoes and so on.

Between the 1940s and the late 1960s the agriculture production increased around the world, a phenomenon known as the *Green Revolution.*[22] This was the start of the use of synthetic fertilizers and pesticides in order to increase the yields. These practices are nowadays known as conventional agriculture.

Because of the *Green Revolution*, agriculture has met the food needs of the populations from the industrial countries, in spite of the latter has doubled during the last four decades and although less and less people are farmers. However, the environmental costs became more and more worrying, [23] including soil degradation,[24] loss of crop genetic diversity,[25] release of greenhouse gases, eutrophication of rivers, streams, lakes and coastal marine ecosystems, contamination of groundwaters[26] and increased use of pesticides and their concentrations in foodstuffs.[27]

As global food security and environmental protection have become a primary concern, another sustainable agricultural practice is needed to avoid or reduce the negative environmental impacts. This is the beginning of the organic farming, considered as an environmentally-friendly agricultural practice.

The expected differences between these two agricultural systems are mainly regarding the use of fertilizers (organic *vs*. synthetic) and pesticides (use no pesticide or biopesticides *vs*. synthetic ones).[28]

1.3.1. CONVENTIONAL AGRICULTURE

The conventional agriculture has been widely used during the last decades, this production system being the main practice used in almost all the world thanks to the industrial revolution and to the *Green Revolution*. However, nowadays there is not a general accepted definition of what is conventional agriculture. The Department of Agriculture of the United States of America (USDA) claimed that this agricultural practice, variously called “conventional farming”, “modern agriculture” or “industrial farming” can vary from farm to farm and from country to country, always reaching remarkable gains in productivity and efficiency.[29]

In general, the conventional agriculture worldwide meets a set of characteristics: rapid technological innovation and large capital investments in order to apply production and management technology; large-scale farms; single crops grown continuously over many seasons and using a large tract of land; production of high-yield hybrid crops; use of fungicides, insecticides, pesticides and synthetic fertilizers, especially those containing nitrogen; high labour efficiency; and dependency on agribusiness.[23,26,29,30]

1.3.2. ORGANIC AGRICULTURE

The demand for safe food, in parallel to an increased environmental awareness, has resulted in an increasing demand for organic products.[31] Moreover, the rapid development of the organic sector worldwide has been a key to consider organic agriculture as an alternative choice for the conventional agriculture.

The most widely accepted definition of organic agriculture comes from the International Federation of Organic Agriculture Movements (IFOAM):[32]

> *"Organic agriculture is a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects.*
>
> *Organic agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved."*

Other authors have developed their own definition, but always based on the same basic standards, affirming that organic agriculture is characterized by the absence of synthetic fertilizers to enhance plant growth and development and by the improvement of soil fertility and the crop nutritional quality, with fewer detrimental effects on the environment.[23,33-36]

Most of these practices used in organic agriculture are well described by Hole et al.,[37] but they are not specific to organic farming, and some of them can be used in conventional systems. Briefly, all those authors affirm that organic agriculture entails the following practices: prohibition or reduced use of chemical pesticides according to the Legislation(s) in force; prohibition of synthetic fertilizers; mechanical weeding; farmyard and green manuring; minimum tillage; intercroping and undersowing; small field size and, finally, crop rotation.

On the other hand, some disadvantages of organic agriculture are also well documented.[38,39]

Organic agriculture is labour intensive, needs constant attention and skills, needs abundance of natural inputs, presents higher abundances of pests and weeds, and in transition periods (2 or 3 years) often yield reductions occur, which makes it necessary to have more arable lands than in conventional agriculture to produce the same amount of products. This may results in more deforestation and loss of biodiversity. These statements continue to be discussed by many authors.[37,40]

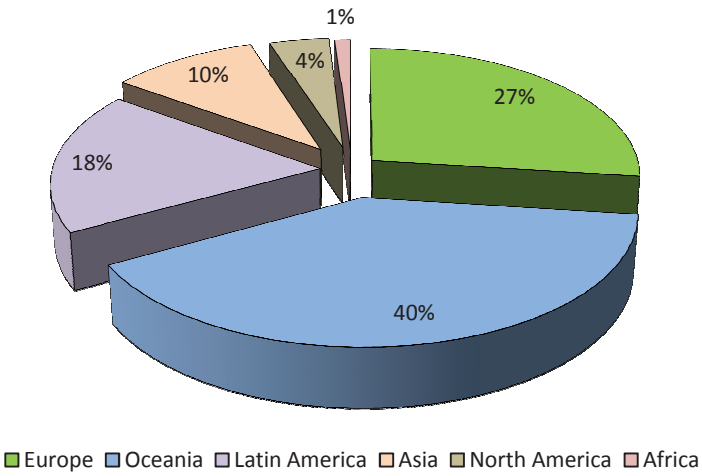

Figure 6. Percentage of the global organic agricultural land used in each continent. Source: FIBL statistics, 2015.[41]

A recent clinical report claimed that current evidence does not support any meaningful nutritional benefits or deficits from eating organic compared with conventionally grown foods, and there are no well-powered human studies that directly demonstrate health benefits or disease protection as a result of consuming an organic diet.[42] However, an updated meta-analysis of the literature reported that organic crops have lower Cd content and lower presence of pesticide residues compared to conventional practices but for As and Pb, no differences were evidenced.[43]

According to IFOAM, there are 4 principles of organic agriculture,[44] which are the roots from which organic agriculture grows and develops. They express the contribution that organic agriculture can make to the world. Briefly, these principles are:

The principle of Health

"Organic agriculture should sustain and enhance the health of soil, plant, animal, human and planet as one and indivisible."

The principle of Ecology

"Organic agriculture should be based on living ecological systems and cycles, work with them, emulate them and help sustain them."

The principle of Fairness

"Organic agriculture should build on relationships that ensure fairness with regard to the common environment and life opportunities."

The principle of Care

"Organic agriculture should be managed in a precautionary and responsible manner to protect the health and well-being of current and future generations and the environment."

Together with IFOAM, the Research Institute of Organic Agriculture (FIBL) is responsible for compiling global organic agriculture data.[41] Their last data confirmed that about 43 million of hectares in the world were used for organic purposes during the period 2004-2013, with Oceania and Europe being the major contributors, with 40 % and 27 % of the global organic agricultural land, respectively (Figure 6).

2. CHEMICAL ELEMENTS AND THEIR RELATIONSHIP WITH PLANT NUTRITION

Regarding the plant mineral nutrition and biochemical functions, chemical elements can be roughly classified by essential and non-essential elements.[45]

2.1. ESSENTIAL ELEMENTS AND THEIR ESSENTIALITY IN HIGHER PLANTS

Essential elements are elements necessary for crop growth and development and, therefore, having a biological function. Depending on the function carried out for the plant, a given amount of each element is required. More precise criteria to state if an element is essential or not were firstly established in 1939 by Arnon and Stout.[46] Thereby, an element is essential for plants if:

a) the element is required for the plant to complete its life cycle,
b) no other element can correct the deficiency caused by that element, and
c) the element is directly involved in the nutrition and metabolism processes of the plant, being required for specific functions.

In addition to these three criteria to be fulfilled by any essential element, there is a fourth requirement to be added to the list:

d) the essential element has to be absolutely necessary for a great number of plant species.

According to the literature, in 1954 it was established that plant species need at least sixteen highlight elements for their growth, reproduction and maturation.[47] However, this number can vary from crop to crop and from author to author.

The essential elements can be, in turn, be classified based on the amount or concentration needed by plant tissues (Table 1). Thereby, three main groups are identified: structural nutrients, macronutrients and micronutrients. Structural nutrients are nutrients necessary in all living organisms, being part of their structure and organization. These elements are oxygen (O), carbon (C) and hydrogen (H). Macronutrients (such as calcium (Ca), nitrogen (N), potassium (K), phosphorous (P), magnesium (Mg) and sulphur (S)) are essential elements required by plants in higher quantities or concentrations, while micronutrients are needed in smaller quantities or concentrations (see their range of concentrations in Table 1). The main micronutrients are iron (Fe), manganese (Mn), boron (B), zinc (Zn), copper (Cu), molybdenum (Mo) and chlorine (Cl).

During the last years more elements have been added to the list, due to the amount of plant species studied and the application of the four criteria of essentiality.[48,49] Nickel is an example of an element recently added to the essential micronutrients list for higher plants, especially for cereals and legumes.[50]

Table 1. Essential elements needed for higher plants growth, ranges of concentration[51] (in percentage (%) or in mg kg^{-1}), principal forms for uptake[52] and year[45,53] and person(s) of the discovery[45,52].

Element	Ranges of concentration	Principal forms for uptake	Year discovered	Discovered by
Structural nutrients (%)				
O	45	H_2O, O_2	1804	T. de Saussure
C	45	CO_2	1882	J. Sachs
H	6	H_2O	1882	J. Sachs
Macronutrients (%)				
Ca	0.1 -6	Ca^{2+}	1856	F. Salm-Horstmar
N	0.5 - 6	NH_4^+, NO_3^-	1872	G. K. Rutherford
K	0.8 - 8	K^+	1890	A.F.Z. Schimper
P	0.15 - 0.5	$H_2PO_4^-$, HPO_4^{2-}	1903	Posternak
Mg	0.05 - 1	Mg^{2+}	1906	Willsatter
S	0.1 - 1.5	SO_4^{2-}, SO_2	1911	Peterson
Micronutrients (mg kg^{-1})				
Fe	20 - 600	Fe^{2+}, Fe^{3+}	1860	J. Sachs
Mn	10 - 600	Mn^{2+}	1922	J.S. McHargue
B	0.2 - 800	H_3BO_3	1923	K. Warington
Zn	12 - 250	Zn^{2+}	1926	A.L. Sommer and C.B. Lipman
Cu	2 - 50	Cu^{2+}	1931	C.B. Lipman and G. Mackinney
Mo	0.1 - 10	MoO_4^{2-}	1938	D.I. Arnon & P.R. Stout
Cl	10 - 80000	Cl^-	1954	T.C. Broyer et al.
Ni	0.05 - 5	Ni^{2+}	1987	P.H. Brown et al.

Essential elements play important roles in functioning as summarized in Table 2. Most of the nutrients are involved in photosynthesis, respiration and transpiration, the three major basic functions needed for plants growth and development. Moreover, many nutrients work together in plants or are used for the same process, so that a deficiency in any of them will stop the plant process and result in a serious nutritional problem.

Additionally, if an essential nutrient is present in too high concentrations, it will provoke a toxic effect on plants, being harmful for their growth. The general relationship between the concentration of essential elements in plant and the plant growth or yield is explained in Figure 7, assuming that all other elements are not limiting. The optimum tissue concentration for growth is indicated as a plateau.

Table 2. Major functions of essential elements in higher plants, adapted from Epstein and Bloom.[51]

Element	Major functions
Structural nutrients	
O	respiration; water and nutrient uptake; synthesis of glucose and fructose; photosynthesis process
C	photosynthesis process; composition of all types of organic compounds like carbohydrates, organic acids, fats, proteins, enzymes, hormones etc.
H	composition of all types of organic compounds like carbohydrates, organic acids, fats, proteins, enzymes, hormones etc.
Macronutrients	
Ca	contribution to cell wall stability and integrity of the plasma membrane
N	major constituent of amino acids (proteins), amides, nucleic acids (e.g. DNA), nucleotides, polyamines and chlorophylls
K	enzymes activation; stomatal activation; water, nutrients and sugars transport; protein synthesis
P	integral in nucleotides, nucleic acids and in all metabolites necessary for energy acquisition
Mg	enzymes activation; synthesis of amino acids and cell proteins; constituent of chlorophyll
S	constituent of several amino acids (proteins), and coenzymes; necessary for chlorophyll formation
Micronutrients	
Fe	chlorophyll development; enzymes activation; constituent of enzymes and proteins (heme proteins, ferredoxin, Fe-S proteins)
Mn	enzymes activation; constituent of superoxide dismutase enzymes; nitrogen metabolism
B	contribution to cell wall stability; sugar transport; germination
Zn	enzymes activation; constituent of metalloenzymes
Cu	lignin synthesis; carbohydrate and nitrogen metabolism
Mo	nitrogen metabolism; enzymes activation; proteins synthesis
Cl	gas exchange; water-balance control; protection against diseases
Ni	constituent of urease enzyme

A deficiency or a toxicity of any element in plant will cause a reduction in plant growth without visible symptoms, being present only for strong deficiencies and toxicities, as reported by McCauley et al.[54]

2.2. NON-ESSENTIAL ELEMENTS AND THEIR POTENTIAL TOXICITY IN HIGHER PLANTS

Non-essential elements are elements which plant does not really need to develop and complete the growth cycle. Some non-essential elements are nowadays considered as very toxic (Table 3), with no known biological function and, thus, are toxic even if they are present in low concentrations.[55]

Due to their properties and lack of functions in plants, the toxic elements can cause several problems in plants tissues and, in addition, enter in the food chain by food consumption.[56]

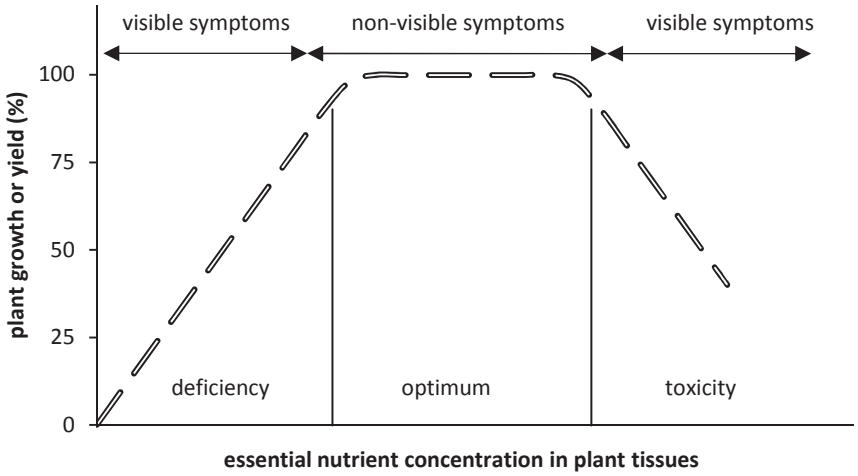

Figure 7. Relationship between essential nutrient concentration in plant tissues and plant growth or yield (%).

There are a large variety of natural and anthropogenic sources of toxic elements in the environment. The crustal material is the main natural source in the environment, by weathering, dissolution, erosion or emission into the Earth´s atmosphere by volcanic activity.[57] These sources correspond to the 80 % of emission. Forest fires and biogenic sources (e.g. released from vegetation) act more as a recycling (accounting for 10 % emission each) and do not increase the stock as the previous mechanisms do.[58]

The anthropogenic sources are many and varied, the major source being mining and smelting. Mining releases toxic elements to the fluvial environment as tailings and to the atmosphere as metal-enriched dust while smelting released them to the atmosphere as a result of high-temperature refining processes.[59]

Other important man-made sources released into the atmosphere include fossil-fuel combustion (primarily coal), municipal waste incineration, cement production and phosphate mining.[60] Important sources of toxic elements for the terrestrial and aquatic environment include discharge of sewage sludges, landfills, use of commercial fertilizers and pesticides, irrigation practices, animal waste and wastewater discharge.[60] Emissions to water are only about twice those relative to air. There are also household sources of toxic elements, such as chemical wastes, fireplaces and building materials.

One of the most impressive environmental disasters caused by the human being occurs in Minamata, where in 1932 several drains containing mercury were discharged into Minamata Bay, in Japan.[61]

The mercury released to the bay was transformed into metylmercury and bioaccumulated in fishes, causing several environmental and health problems during the following decades, known as the Minamata Syndrome (detected in 1952).

The Bhopal disaster, in 1984, is another outstanding disaster.[62] A leakage of methyl isocyanate was produced in a pesticide factory in this region of India, causing such environmental damage that the place of accident remained seriously polluted by toxic heavy metals during lots of years.

The more known and highlighted toxic trace elements are As, Pb, Hg, Cd and Cr(VI) (Table 3), according to the Agency for Toxic Substances and Disease Registry (ATSDR).[63] Each one is accumulated and transported differently inside the plants, also causing several physiological and nutritional problems for the plants but also for the health of humans exposed to them.

Table 3. Classification of the most important elements according to the Priority List of Hazardous Substances from the Agency for Toxic Substances and Disease Registry (ATSDR)[63] published in 2013 and the monographs of the International Agency for Research on Cancer (IARC).[64]

Element	CAS number	IARC group [(a)] (year)	ATSDR ranking [(b)] (in 2013)
As	007440-38-2	1 (2012)	1
Pb	007439-92-1	2B (1987)	2
Hg	007439-97-6	3 (1993)	3
Cd	007440-43-9	1 (2012)	7
Cr(VI)	018540-29-9	1 (2012)	17
Co	007440-48-4	—	51
Ni	007440-02-0	2B (1990)	57
Zn	007440-66-6	—	75
Cr	007440-47-3	—	78
CH_3Hg^+	022967-92-6	2B (1993)	116
Cu	007440-50-8	—	118
Ba	007440-39-3	—	131
Mn	007439-96-5	—	139
Se	007782-49-2	3 (1987)	145
Al	007429-90-5	—	179
V	007440-62-2	—	197
Ag	007440-22-4	—	219
Tl	007440-28-0	—	274

[(a)] Group 1: Carcinogenic to humans (117 agents); Group 2B: possible carcinogenic to humans (287 agents); Group 3: not classified as carcinogenic to humans (503 agents).

[(b)] The ATSDR ranking is not a list of "most toxic" substances, but rather a prioritization of them based on a combination of their frequency, toxicity and potential for human exposure at "National Priority List" sites of the Environmental Protection Agency (EPA).

2.2.1. ARSENIC

Arsenic (As) is a natural abundant metalloid found in the Earth (Figure 8). It has been defined as a group 1 (carcinogenic to humans) and is placed in the highest health hazard category by the IARC.[65] This heavy metal can be naturally dispersed into soil and water through the weathering of rocks and minerals by lixiviation, and volcano activity.[66] Anthropogenically, it appears in soils due to mining processes, agricultural inputs (insecticides, herbicides, defoliants and contaminants of fertilizers) and food additives, among others.[56]

The mobility and bioavailability of As depends on its chemical form and speciation. The redox forms of inorganic arsenic are As(III) and As(V), the first one being generally less mobile but most toxic to living organisms.[67] As (III) dominate in reducing soils while As(V) is dominating under aerobic conditions.

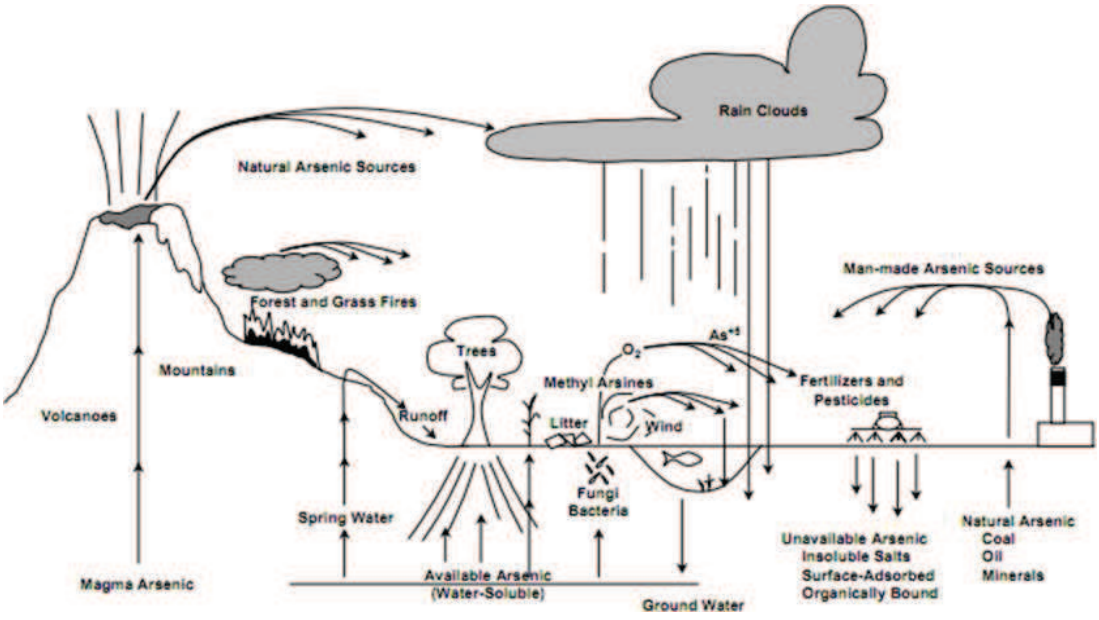

Figure 8. Natural and anthropogenic cycling of As. Source: Asxban Technologies.[68]

In general, plants take up As(III) and As(V) through the phosphate transport channels. Because of their chemical similarity, As(V) competes with phosphate for root uptake and interferes with metabolic processes like ATP synthesis and oxidative phosphorylation.[69,70]

In plant metabolism, As(V) is generally reduced to As(III) and/or biotransformed to less toxic organic compounds such as dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), or as inorganic As(III) complexed with thiol groups of enzymes.[70] As is generally little translocated to aboveground plant organs except for the hyperacumulators.[71] The toxicity from As is visible at cellular level, causing the damage of the plasma membrane, followed by a electrolyte leakage.[72] The plant photosynthesis and transpiration processes can be also reduced.[73]

2.2.2. LEAD

Lead (Pb) is another wide spread toxic pollutant which has no known functions in biological systems. It is listed in the International Agency for Research on Cancer (IARC) inside the group 2B (possible carcinogenic to humans).[74]

Apart from the natural weathering processes (Figure 9), Pb can be released into the environment from mining and smelting activities, Pb containing paints, gasoline and explosives, industrial smokes, fertilizers and pesticides used in agriculture lands and municipal sewage sludges rich in Pb.[75]

Pb from the soil solution can enter the root cell through passive diffusion by using ion channels, including that of Ca which is a strong competitor.[76] Once inside the roots, most of the Pb is bound to ion exchangeable sites in the cell walls and, consequently, extracellularly precipitated as phosphates and/or carbonates.[77]

Some authors claimed that most of the absorbed Pb remains sequestrated in roots, which makes the root the first barrier for the Pb translocation to the above ground plant parts.[78,79] However, other studies have demonstrated that great amounts of Pb can also enter the plant via leaves, depending on the aerial sources and the leaf morphology.[80,81]

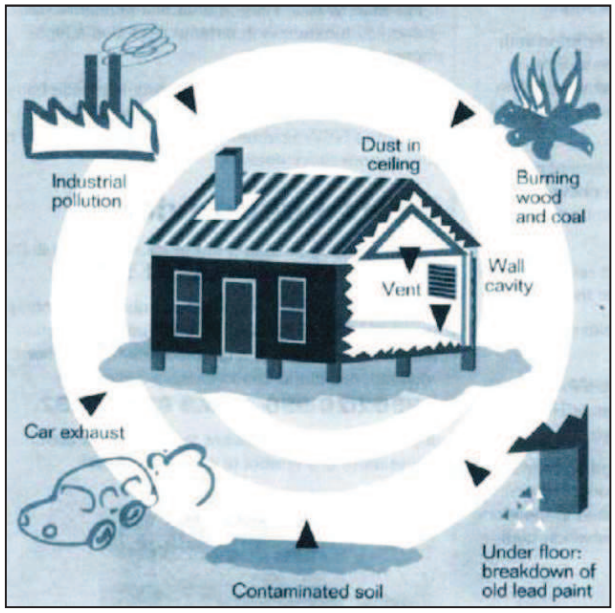

Figure 9. Anthropogenic releases of Pb. Source: The Lead Group.[82]

Pb can cause several physiological, biochemical and structural negative effects in plant tissues.[83] Some visual symptoms are rapid inhibition of root growth, stunted growth of the plant and chlorosis. Moreover, Pb can decrease the germination percent, the root-to-shoot ratio and, consequently, the dry mass of the whole plant.[84]

At the cellular level, due to the high affinity of Pb with organic compounds including proteins, Pb inhibits some enzymes activity (e.g. enzymes responsible for the photosynthesis process, N_2 assimilation, sugar metabolism and energy generation), disturbs mineral nutrition and alters the cell membrane permeability, even causes cell death.[76,77] However, its effects depend on the concentrations found inside the plants.

2.2.3. MERCURY

Mercury (Hg) is defined as a group 3 (not classified as carcinogenic to humans) by the International Agency for Research on Cancer (IARC).[85]

Hg is naturally and anthropogenically emitted to the environment. It can be transported to surface waters by soil erosion and circulated into the atmosphere by a natural degassing of the Earth´s crust and oceans (Figure 10). The man-made emissions are due to coal burning, gold mining activities and other ones related with the agricultural land, with the use of sewage sludge, fertilizers, pesticides and manures.[86,87]

Hg is can be found in the environment in several chemical forms and oxidation states,[88-90] such as elemental Hg (Hg^0), inorganic Hg (Hg^{2+}), associated with some ions (such as Hg_2Cl) and associated with organic ligands(e.g. CH_3Hg^+). In general, the bioavailability of Hg in soils is low, and plants tend to immobilize it in the root system, being little translocated to aboveground organs. The most dangerous form of Hg with regard to human and environmental exposure due to its toxic properties is methylmercury (CH_3Hg^+),[91,92] in which Hg forms a covalent bond with carbon.

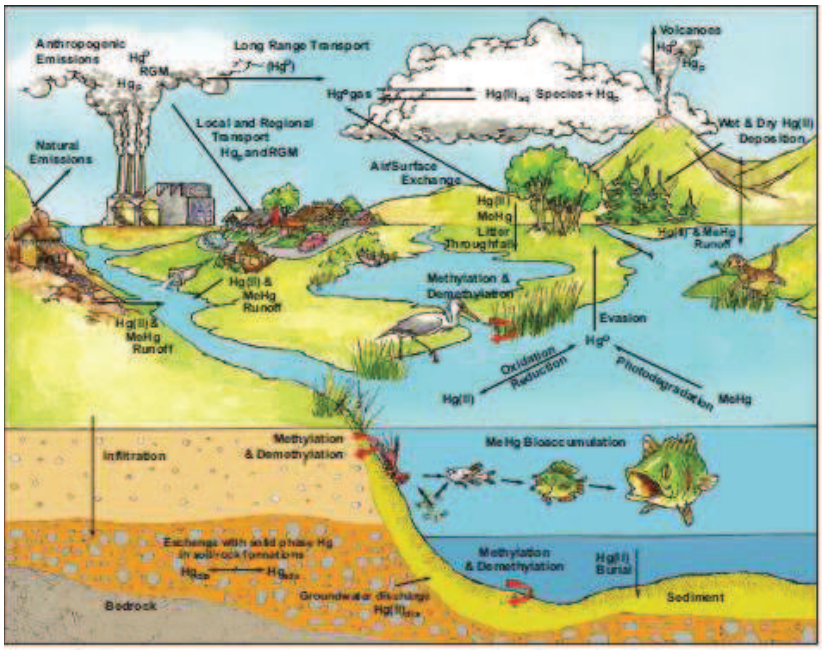

Figure 10. Cycle and natural and anthropogenic releases of Hg. Source: Government of Canada.[93]

If Hg is taken up in this organic form the uptake process is enhanced and then Hg can be greatly accumulated in stems and leaves, due to its facility to be absorbed by cell membranes. Most of the current exposure to methylmercury for the population is generally through the consumption of contaminated fish.[94] Shekar et al.[95] affirm that high concentration of Hg in plant tissues entails a decrease in growth parameters, such as percentage of germination, plant height, number of flowers per plant, number of petals and sepals, pollen viability (%), average number of fruits, fruit girth and fruit weight.

In general, the accumulation of Hg in plant tissues has strong phytotoxic effects. It can reduce seed viability and cause several injuries in fruits.[96] At the cellular level, Hg can damage important molecules (such as enzymes and polynucleotides), can substitute to essential metal ions associated to proteins (e.g. the central atom of the chlorophyll, Mg), can denature or inactivate proteins and also can disrupt cell membranes.[96]

Some physiological mechanisms of plant are then affected by Hg accumulation in tissues. Photosynthesis, transpiration rate, water uptake and chlorophyll synthesis are reduced by the change in the permeability of cell membranes, permitting the entrance of Hg into the plasma membranes.[97]

2.2.4. CADMIUM

Cadmium (Cd) is a non-essential element which have been found to be carcinogenic, mutagenic and teratogenic for a large number of animal species, being included in the group I (carcinogenic to humans) by the the International Agency for Research on Cancer (IARC).[98] Cd is a major environmental contaminant of air, water and soil. Naturally occurring Cd levels are generally low. For cultivated soils, one major source of Cd is through the P fertilizers which can contain significant amount of Cd as contaminant.[99] Other anthropogenic sources of Cd for soils are mining and refining, industrial and municipal wastes and coal combustion (Figure 11).

Excess Cd causes a number of toxic symptoms in plants, such as growth retardation, inhibition of photosynthesis, induction and inhibition of enzymes, altered stomatal functioning and water status, efflux of cations and generation of free radicals.[100] Many authors[100,101] reported that the plant growth can be affected by Cd. Its toxicity starts by a reduced growth of the plant. Particularly, it can affect the percentage of germination, the plant height, the root length, the number of flowers per plant, the number of petals and sepals, the pollen viability, the average number of fruits, and the fruit girth and fruit weight, as for Hg.

Plants mainly take up Cd through root absorption, mainly through Zn and Fe transporters following the electrochemical gradient at the plasma membrane level. Cd uptake by root cells has been reported to also involve the low affinity cation transporter of Ca (LCT1).[55]

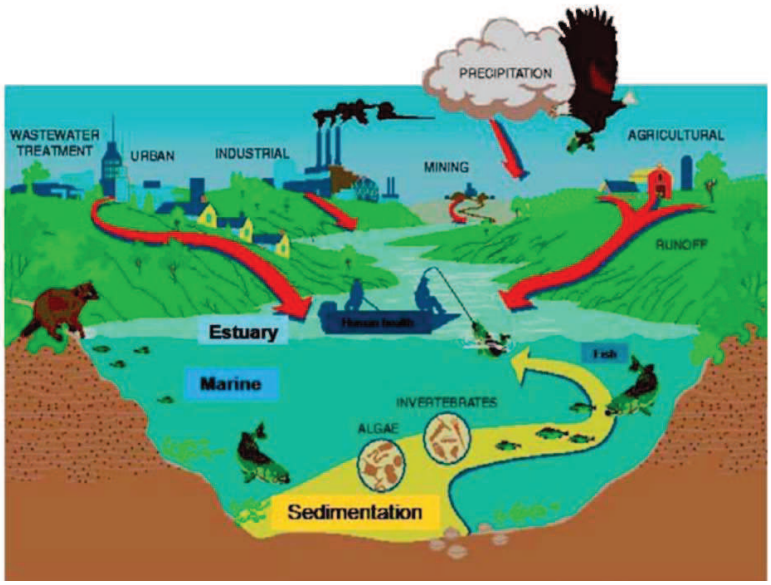

Figure 11. Natural and anthropogenic sources of Cd. Figure adapted from the Crankshaft Publishing.[102]

Cd is generally sequestrated in root cell but can also move into the xylem stream toward aboveground organs. Cd has a high affinity with thiol groups and inhibits enzymes. Detoxification of Cd by plants includes sequestration in vacuoles as complexes.[100]

2.2.5 CHROMIUM

Chromium (Cr) is one of the most harmful elements in the environment, but it is classified as a group 3 (not classified as carcinogenic to humans) by the International Agency for Research on Cancer (IARC).[103] The biological effects of Cr depend on its oxidation state: Trivalent Cr (Cr(III)) is the most innocuous and stable oxidation state of Cr,[104] whereas hexavalent (Cr(VI)) compounds, known as hexavalent Chromium, are considered as the most toxic to humans and animals, and is a major pollutant in the environment,[105,106] due to its high oxidizing potential, solubility, and mobility across the membranes in living organisms and in the environment (Figure 12). It has been reported that Cr(III) play a vital role in some metabolic processes in living organisms, although its essentiality is not totally accepted nowadays.[107,108]

In the environment, natural Cr is generally occurring as Cr(III), while the whole of the Cr(VI) comes from anthropogenic sources, such as cement-producing plants, textile industries, and coal and oil combustion, among others.[109]

Cr(III) occurs as oxides, hydroxides or sulphates, and is much less mobile than the Cr(VI) because it is strongly bound to organic matter in soil and aquatic environments.[106] Cr (VI) is a strong oxidizing agent and in the presence of organic matter in soil it is reduced to Cr(III).

This transformation is faster in acid environments such as acidic soils.[110] Plants may also be able to reduce Cr(VI) to Cr(III), a detoxification reaction that is very likely to occur in roots, and that may be catalyzed by Cr reductases similar to those found in bacteria.[106] In addition, Cr(III) can be also oxidized to Cr(VI) in the presence of an excess of oxygen, being transformed again to the more toxic form.[111]

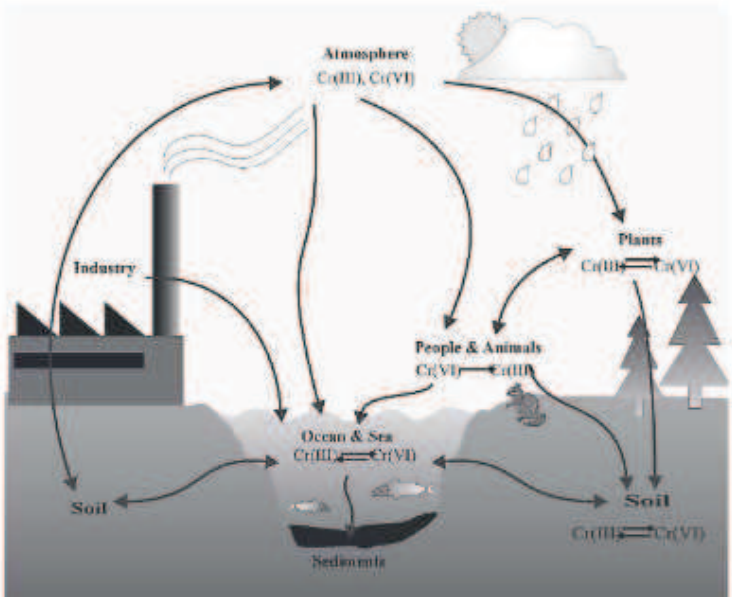

Figure 12. Cr circulation in the polluted environment. Source: Bielicka et al.[108]

Cr enters plants by reduction and/or complexation with root exudates, such as organic acids, which also increase the solubility and mobility of Cr through the root xylem.[112] Once the metal has been absorbed, both Cr(III) and Cr(VI) can enter into the root cells by the symplast pathway where Cr(VI) is reduced and accumulated in the cortex.[113] Even though Cr is poorly translocated to aerial parts, its mobility is mainly governed by its oxidation state.[114] Moreover, it has been reported that Cr(VI) reduces the uptake of some essential elements such as Fe, K, Mg, Mn, P and Ca.[115]

Difference in accumulation of Cr among plant organs has been observed. Roots accumulate 10 to 100 times more Cr than leaves and other tissues.[116-118] Toxic effects of Cr in the plant growth include the alteration of germination and roots and aerial organs growth, the modification of photosynthesis and the water relations[113] and also chlorosis and necrosis in plants.[106]

3. THE PHYSIOLOGY OF ELEMENTS HOMEOSTASIS IN HIGHER PLANTS

3.1. SOIL PROPERTIES AND ELEMENTS BIOAVAILABILITY IN THE UPTAKE MECHANISM

Soil is the main source of trace elements for plants both as nutrients and pollutants, the soil-plant transfer being an important part of the cycling of these elements.[119]

Roots take up elements from the soil, being the magnitude of the uptake inversely proportional to the strength of the association between the element and the soil. Roughly, there are five different levels of association with decreasing mobility,[120,121] as follows:

i) dissolved in the soil solution,
ii) sorbed by non-specific bounds (electrostatic),
iii) sorbed by specific bounds (covalent bound),
iv) precipitated (oxides, hydroxides, carbonates, phosphates), and
v) included in minerals (not available for plants or microorganisms).

Plants are able to mobilize elements in most of these situations. They can even dissolve some minerals. However, they take up metals mostly from the most exchangeable fractions, the dissolved and the non-specifically sorbed ones.[122] Therefore, the magnitude of the uptake depends on the bioavailability of the elements, which in turn depends on the physic-chemical characteristics of the soil, in particular pH, texture, type and content of organic matter, mineral composition, cation exchange capacity (CEC) and complex formation.[119,123-125]

The bioavailability of elements to plants refers to the amount of metal accessible to be taken up by plants in the environment, in the free ionic form.[119] It is governed by the existing pseudo-equilibrium between aqueous and solid soil phases, rather than by the total metal content.[126] Predicting the mobility in soil and bioavailability of elements has been a “hot topic” for years in both agricultural and environmental studies.

Among soil properties, pH is the variable which most influences the metal mobility and bioavailability, due to its strong effects on the solubility of metals in soil solution.[127] The existence of a negative correlation between soil pH and metal mobility and availability to plants is well documented.[128,129] According to these studies, when soil pH decreases, a dramatic increase in metal desorption from soil constituents and dissolution in soil solution is observed for Cd, Pb and Zn.

At lower values of pH trace elements are most available, but the toxicity effects for plant growth also increase.[130] It is now well established that the plant can modify the pH (and consequently, metal bioavailability) in the rhizosphere, by releasing protons (H^+), mainly as a consequence of the cation-anion uptake balance (increase or decrease) and also in response to nutrient deficiency (acidification to solubilise a nutrient).[131,132]

Moreover, plants are also able to respond to toxic concentrations of elements either by immobilizing them with their root exudates or with some mycorrihzal associations.[133] In many plant species the primary barrier against the entrance of toxic elements (or against the incorporation of an excessive concentration of essential elements) into plant tissues operates at the root level.

The *rhizosphere* term was firstly coined by the agronomist and plant physiologist Lorenz Hiltner in 1904.[134] This term is used to describe the plant-root interface, defined as the volume of soil surrounding living plant roots subjected to the direct influence of root activity. The rhizosphere differs within plant species, existing differences in the nature of their exudates, in the nutrients acquisition strategies, in the rhizospheric microbes and in the root system architecture. Apart from the plant functioning, the rhizosphere also depends on the soil characteristics.[135]

In addition to soil pH, organic matter content is another important factor that noticeably influences the ability of soils to retain trace elements in an easily exchangeable form.[122] Organic matter also supply organic ligands that stabilize metals in the soil solution by formation of complexes.

3.2. ELEMENTS UPTAKE FROM THE RHIZOSPHERE

Uptake of essential and non-essential elements by roots from the rhizosphere is the first step for their accumulation in aerial parts.[136] Plant roots take up trace elements from the soil solution mainly by advection with water or by diffusion.[55]

In general, elements are first bound by the root cell wall, an ion exchanger of relatively low affinity and low selectivity. Afterwards, secondary transport systems (such as channel proteins and/or H^+-coupled carrier proteins) mediate and drive the uptake of elements across the plasma membrane of the root cell.[137] The electrochemical gradient, which is negative on the inside of the plasma membrane, provides a strong driving force for the uptake of cations through secondary transporters.

Different types of cation transporters (Table 4) are implicated in the uptake by roots and often more than one transport system exist for one element.[138-142]

a) The Nramp (natural resistance-associated macrophage proteins) family, H^+ coupled transporters which pump some divalent cations such as Cu^{2+}, Fe^{2+}, Mn^{2+} and Cd^{2+}.
b) The ZIP (ZRT, IRT-like proteins) family, plant transporters of Fe^{2+}, Mn^{2+}, Zn^{2+} and Cd^{2+}.
c) P-type or CPx-type ATPases, plant transporters implicated in the transport of Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} across the plasma membrane.
d) CDF (cation diffuser facilitator) family, involved in the transport of Co^{2+}, Mn^{2+}, Zn^{2+} and Cd^{2+}.
e) Cation/H^+ antiporters, physiologically exchange Na^+ or H^+ with Ca^{2+} or Cd^{2+}.
f) ABC (ATP-binding cassette) family, the largest protein family found in all living organisms, acts as ATP-driven pumps and is able to detoxify metals and metalloids such as As^{3+}, Cd^{2+} and Hg^{2+}.

Furthermore, there exists a lack of specificity regarding the transporters and, thus, biologically non-essential and/or toxic elements can enter in the roots cells by the same transporters that are used for essential nutrients uptake.[143] Once inside the cell, non-essential elements can displace and interfere with the function of those essential elements, as occurs in the case of Cd with Zn and Fe transporters, and due to their similar chemical properties, they are taken up and translocated to aerial parts by similar pathways.[144-147]

At intracellular level, plants can also catalyze redox reactions and alter the chemistry of these metal ions allowing their accumulation in non-toxic forms, their mobility and bioavailability.[148]

3.3. SEQUESTRATION IN ROOTS, RADIAL TRANSPORT AND XYLEM LOADING

After the metal is taken up by root cells, several mechanisms govern the fate of elements in plant tissues:[136,149] i) sequestration of metals inside root cells, ii) chemical transformation of elements in cells and iii) xylem loading for the translocation to aboveground organs.

3.3.1. COMPARTMENTATION IN THE VACUOLE

Plants have a wide range of detoxification and tolerance mechanisms that appear to be involved primarily in avoiding toxic concentrations at sensitive sites within the cells, thus preventing damaging effects.[140] As a result, nutrients in excess and non-essential elements are often stored in roots vacuoles so that physiological processes are less affected.[150]

Thereby, to protect themselves from metal poisoning, plant cells must have developed a mechanism by which the metal ion, entering the cytosol of the cell, is immediately inactivated by complexation and stored in vacuoles, minimizing the harmful effects of metal ions to vital cellular processes.[133,151] Vacuoles are assumed to be one of the main storage sites for metal sequestration.[77,152,153] Transport of metal ions into roots vacuoles decreases their availability for transfer to the shoot via the xylem. Several members of families of proteins are implicated in this process, either in roots or in leaves (Table 4), contributing to basal metal tolerance.[140,142,154]

There are two main families of metal-binding ligands dedicated to complex metals: phytochelatins (PCs) and metallothioneins (MTs). The first convincing experimental evidence for the function of PCs in higher plants came from the work of Steffens et al.[155] PCs are an important component of the metal detoxifying mechanisms of higher plants, catalyzed by a 3,-glutamylcysteine dipeptidyl transpeptidase (PC synthase), a constitutive, metal-activated enzyme. They are metal-binding peptides, forming stable metal-phytochelatin complexes and storing metal ions in the vacuole.[156]

The metal-PC complex is transported from the cytosol to the vacuole by either metal/H^+ transporters or ATP-dependent ABC transporters.[142,157]

Table 4. Specificity, cellular location and main tissue expression of some metal transporters involved in plants homeostasis (adapted from Hall et al.[140]).

Name	Specificity	Cellular location	Main tissue expression
Nramp family			
AtNramp1	Fe^{2+}, Mn^{2+}		roots
AtNramp2	-		roots
AtNramp3	Fe^{2+}, Mn^{2+}, Cd^{2+}		roots, shoots
AtNramp4	Fe^{2+}, Mn^{2+}	vacuole	roots, shoots
OsNramp1	Fe^{2+}, Mn^{2+}		roots
OsNramp2	Mn^{2+}		leaves
OsNramp3	Mn^{2+}		roots, leaves
LeNramp1	Fe^{2+}		root vascular parenchyma
ZIP family			
IRT1	Fe^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}		roots
IRT2	Fe^{2+}, Zn^{2+}		roots
OsIRT1	Fe^{2+}	vacuole	roots
LeIRT1	Fe^{2+}		roots
TcZnT1	Zn^{2+}, Cd^{2+}		roots, shoots
ZIPs1-3	Zn^{2+}		roots
ZIP4	Zn^{2+}	plastids	roots, shoots
GmZIP1	Zn^{2+}	peribacteroid membrane	root nodules
ATPases			
RANI	Cu^{2+}	post-Golgi chloroplast	whole plant
PAA1	Cu^{2+}		
CDF family			
ZAT(AtMTP1)	Zn^{2+}	vesicular/vacuolar	whole plant
TcZTP1	Zn^{2+}	intracellular membranes	roots, leaves
TgMIP1	Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}	vacuole	leaves
ShMTP1	Mn^{2+}	intracellular membranes	roots, leaves
cation/H^+ transporters			
CAX2	Ca^{2+}, Mn^{2+}, Cd^{2+}	vacuole	roots
ABC family			
AtABCC1	Cd^{2+}, As^{3+}	vacuole	roots
AtABCC2	Hg^{2+}, As^{3+}		roots

However, PC-metal complexes do not only serve as an important component of intracellular metal detoxification mechanisms in plants by translocating metals across the tonoplast and sequestering in vacuoles. These complexes can also undergo long-distance transport from roots to shoots.[133]

MTs are ubiquitous low-molecular-weight, cysteine-rich proteins that bind to metals and are also important in metal detoxification. MTs were originally identified in animals by their ability to protect against cadmium toxicity.[158] Their mode of action is often connected to their ability to scavenge reactive oxygen species (ROS),[139] and they also play an important role in other cellular processes, including the regulation of cell growth and proliferation and DNA damage repair.[159]

3.3.2. RADIAL TRANSPORT ACROSS THE ROOT SYSTEM AND XYLEM LOADING

Trace elements not sequestrated in vacuoles can be loaded into the xylem in the stele of the root, which is reached by the apoplastic route (through cell walls and intercellular spaces) and/or by the symplastic route (inside the cell cytoplasm through plasmodesmata).[124]

The apoplast is the extracellular free space made of the cell walls outside the plasma membrane (Figure 13). Water and solutes are transported across the apoplast pathway from cell wall to cell wall. The symplast consist on a continuum cytoplasm connected via plasmodesmata, so that water molecules and dissolved low molecular mass substances can diffuse radially.

Regarding the apoplastic route, the deposition of lignin and suberin in the endodermis cell wall (Casparian strip) prevents ions to reach the stele and trace elements have to be internalized into the cytoplasm to be then effluxed into the stele, increasing the ion concentration.[160] However, a bypass to this apoplastic barrier can exist when the endodermis is disrupted by the growth of lateral roots. Passage cells with no or little barrier can also facilitate the apoplastic route till the stele.[161,162]

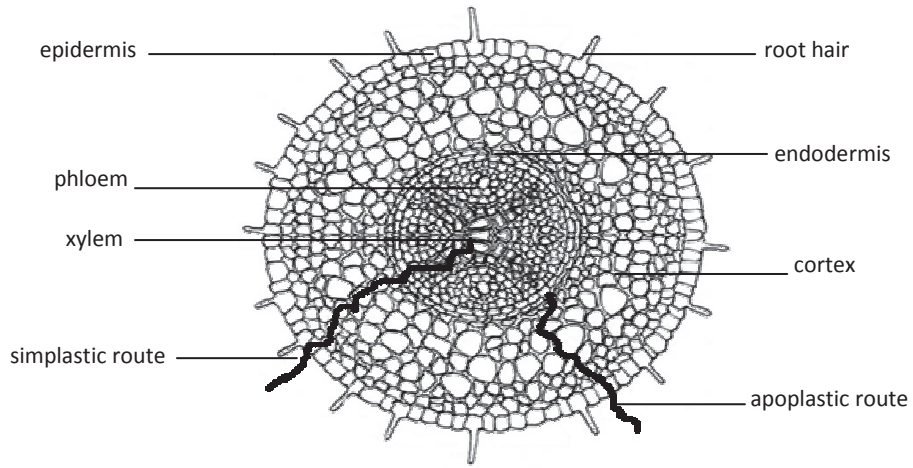

Figure 13. Cross-section of a root with its general parts. The symplastic and apoplastic routes are also indicated.

The radial transport can be facilitated by nicotianamine (NA), a well-known vital metal-chelating ligand of micronutrients for homeostasis during plant growth and for translocation within aerial organs, forming stable metal-NA complexes able to cross the root system.[163,164]

Once within the stele, water and ions are loaded into xylem vessel by diffusion and they move to the shoots by the transpiration stream,[165,166] a process known as long-distance transport.[167]

3.4. LONG-DISTANCE TRANSPORT AND SEQUESTRATION IN SHOOTS

The vascular system of plants comprises both the xylem and phloem to transport elements through the plant (Figure 14).

The xylem vessel are dead cells and consists on a tubular shape without transversal walls, allowing the continuous flow of water and solutes in the interconnected tubes to reach the plant aerial organs.[167] The ascending movement is generated by two mechanisms: the root pressure originates in the loading of solutes and the gradient in water potential between roots and shoots.[165,167,168] This generates a flux of water towards the xylem pushing the flow upwards.

The root pressure contributes mainly when plant transpiration is low (young growth stage, low photosynthesis, and dark periods). Otherwise, when stomata are opened, the difference in the water vapour pressure between the atmosphere and the leaves makes water to be lost and due to the cohesion of the liquid in the xylem, the sap moves upwards.

The transport of ions into the xylem vessel is generally a tightly controlled process mediated by transporters which act as efflux pumps excreting trace elements in the apoplast.[136,169] The general metal transporters involved in xylem loading have yet to be identified. However, some of them, such as P-type ATPases have been recently identified in rice for xylem loading of Cu, Zn and Cd.[170,171]

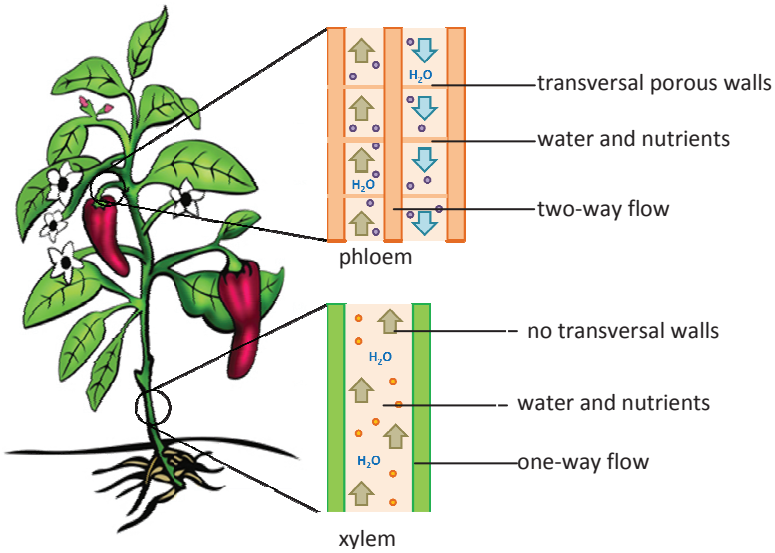

Figure 14. Scheme of the vascular system in higher plants. The differences between the xylem and phloem vessels are indicated.

The phloem, by contrast, is made of living cells, composed basically of sieve elements, several parenchyma cells, fibres and sclereids, being an elongated and tubular shape with thin transversal walls. Phloem transports sugars and nutrients from source organs to sink organs both upwards and downwards depending on the requirements.[172]

During the long-distance transport, trace elements can be complexed with certain low-molecular-weight ligands, such as histidine, nicotianamine and citrate.[154,173] The metal-chelate complexes can reduce concentration of metals inside the xylem and facilitate metal transfer into the transpiration stream.[174] However, this process depends strongly on the pH of the vascular bundles. In the xylem with acid pH the complexation is not always possible, increasing the mobility of the trace element and preventing its sorption onto the cell walls. In the phloem, by contrast, trace elements are more likely to be complexed because of higher pH of the sap.

3.5. XYLEM-TO-PHLOEM EXCHANGE AND PHLOEM LOADING

During their long-distance transport, metals can be unloaded from the xylem and transferred to organ cells of the different plant organs, where they can be sequestrated in vacuoles or involved in the cell metabolism for micronutrients. For plant organs not connected to the xylem like the filial tissues of seeds or when the xylem is interrupted in the reproductive organs, trace elements have to be loaded into the phloem by direct transfer from the xylem.[144,175,176]

The movement of elements in the phloem vessel occurs by bulk flow, resulting from a pressure gradient from the source tissues (leaf, stem, etc.) to the sink ones (in general plant organs with no or reduced photosynthesis),[177] which is generated by loading molecules and elements such as carbohydrates and potassium into the phloem at source tissues. The final unloading of the elements at the sinks decreases the pressure and maintains a gradient. Elements will not be present in concentrations high enough to create a pressure gradient on their own, and thus will move with water and sugars in the phloem.[144]

Several transporters (efflux pumps and influx transporters) are involved in the phloem loading with elements binding capacity.[178] All of them are not identified but the yellow stripe-like (YSL) family of metal transporters play a central role for some trace elements, as occurs with mugenic acids, glutathione, phytochelatins (PCs), metallothioneins (MTs) and nicotianamine (NA).[144,179] As an example, proteins that transport micronutrient-NA complexes have been identified in recent years.

3.6. PHLOEM UNLOADING AND REMOBILIZATION MECHANISMS

Although xylem loading and long-distance transport of elements from roots to shoots is likely to be an important mechanism governing the partitioning of elements between plant organs, their remobilization from one organ towards other via phloem may be also of importance.[180]

Hence, the supply of elements to the developing fruits or grains can be originated from i) direct uptake from the soil and further translocation through xylem and phloem vessels, and ii) from the remobilization/retranslocation of stored elements in shoots during the reproductive period.[136,181-183]

In some reproductive organs, there exist a discontinuity between the maternal and the filial tissue (e.g. in sunflower, the husk is connected to the xylem, but not the almond). Thus, to enter the filial tissue, an element will first has to be present in the phloem (or connected surrounding cells) of the maternal tissue, remaining in the apoplast and, secondly, the element has to be pumped by the filial tissue by direct transport.[154] For the reasons described further above, the phloem unloading step is likely to be a major bottleneck in this process. Unfortunately, very little is known concerning transporters involved in phloem unloading mechanisms.[184,185]

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SECTION II

Accumulation and translocation of essential and non-essential elements by Swiss chards and tomato plants

Introduction

Global food security has become a vital issue to ensure human health and proper nutrition. Consequently, during the last decades more and more concern has been focused on the optimization of agricultural techniques all over the world, with the aim of improving the nutritional value of crops and the crops yield, and reducing their toxic content.

During the *Green Revolution*, developed from 1940s to the late 1960s, chemical fertilizers, insecticides and pesticides started to be used for intensive agriculture, enhancing crop yield and farming production.[1] Food needs in advanced countries were fulfilled, but several damages to the environment were caused: soil degradation,[2] loss of crop genetics diversity,[3] release of greenhouse gases, eutrophication of aquatic ecosystems, ground water depletion[4] and increased pesticide concentration in foods.[5]

The accumulation of essential and non-essential elements in plants is known to depend on several factors, such as the agricultural technique used, the soil properties, the type of plant, the harvesting time, and the environmental conditions, among others.[6,7] Conventional practices that use chemical fertilizers, insecticides, and fungicides have been partially substituted by other more environmentally friendly techniques, as organic farming. Moreover, it has been reported that the use of organic techniques in crops production reduces the input of toxic elements in the food chain.[8-10]

The percentage of agricultural land used for organic farming around the world has greatly increased during the last decade, being Oceania (with 17.3 million hectares, 40 % of the world´s organic agricultural land) and Europe (11.5 million hectares 27 %) the outstanding continents.[11]

Internationally, it is regulated by *Codex Alimentarius* Guidelines,[12] while in Europe exists a Council Regulation that sets out the principles, aims and overarching rules of organic production and defines how organic products have to be labelled.[13]

The International Federation of Organic Agriculture Movements (IFOAM), a recognized farming association, states that organic agriculture is a production system that excludes inputs with adverse effects, sustaining the health of soils, ecosystems and people and enhancing ecological processes, biodiversity and cycles.[14] The key of organic farming is known to be the absence of synthetic mineral fertilizers, pesticides and fungicides, and the use of crop rotation and plant biodiversity.[10] Moreover, the use of synthetic fertilizers as a convenient way to increase fruit production and crop yields, can in counterpart be a possible source of some toxic elements such as Cd in P fertilizers.[8,9]

There exists a controversy about the fact that the amount of toxic substances in plants increases or not with the use of certified organic fertilizers versus conventional fertilizers.[15,16] A recent meta-analysis of the literature reported that organic crops had lower Cd content compared to those obtained after conventional practices, but for As and Pb no significant difference was identified.[17]

Generally, plant roots take up both essential and non-essential elements from the soil. These elements can be immobilized in the root system or translocated to the aerial parts of the plants in variable proportions and following different mechanisms, depending on the element and on the plant species.[18-20] The amount of elements taken up by plant roots mostly depends on their bioavailability in soil, which itself depends on the chemical nature of the element and on the physic-chemical conditions of the soil.[21] High bioavailability of elements results in an important uptake and possible high translocation to aboveground tissues, including the edible parts. This results in a potential risk for consumers if non-essential elements are significantly accumulated in edible parts.[6]

Some essential nutrients, such as micronutrients, are incorporated into plants through absorption by root membrane transport proteins, such as NRAMP (natural resistance associated macrophage protein), ZIP (zinc-regulated transporter, iron-regulated transporter protein), YSL (yellow-stripe-like) and ATPases.[20,22,23] However, some undesirable ions can also be accumulated in plant tissues due to the lack of specificity of these membrane transporters, which results in the contamination of the food chain with subsequent risk for human health. This problem has already been described, for example, for Cd in tomato plants.[24]

The associated benefits and risk of elements taken up by plants from the soil is an important issue as illustrated by Johnson et al.[7] and Hazrat et al.,[25] who reviewed metal levels required in plant tissues for beneficial and toxic effects and their main harmful effects on human health, respectively.

Swiss chard (*Beta vulgaris* subspecies *cicla*) is one of the most popular vegetable around the Mediterranean area.

Its unique nutritional composition includes polyphenol compounds, flavonoids such as syringic acid and many betalain pigments, which have been shown to provide antioxidant, anti-inflammatory, and detoxification support, and blood sugar control.[26,27]

Some research has been carried out to investigate the uptake and translocation of elements in Swiss chards, outstanding its high potential of elements transfer from soil to the edible foliage. As an example, Peris et al.[28] reported that leafy vegetables (such as Swiss chards) present significant heavy metal contents and pointed out the importance of the farming technique for this kind of vegetables regarding the accumulation of beneficial and toxic elements. Moreover, Madejón et al.[29] highlighted the slightly high content of Zn, As and Pb in the leaves of Swiss chards.

Tomatoes (*Solanum lycopersicum*) are one of the most-consumed vegetables worldwide, representing a significant part of the human diet. Their nutritional properties (major source of antioxidant compounds, such as carotenoids and phenols, with low levels of fat, calories, and cholesterol) make them beneficial for human health, by reducing the risks of cardiovascular disease and certain types of cancer, specially the cancers of the prostate, lung, and stomach.[30,31]

Several studies have been recently carried out to compare the accumulation of minerals in tomato tissues when using organic or conventional farming techniques. Recently, Kelly and Bateman[32] found significant differences in the concentrations of Mn, Ca, Cu, and Zn in tomato fruits produced in the United Kingdom using different farming techniques. Other authors, however, reached the opposite conclusion working with tomato fruits obtained in a local market.[33] On the other hand, the elemental composition of plant tissues is known to vary during the growth stages of the crop, depending on the age of the plant, which importantly governs the translocation or remobilization of elements between organs.[34]

The accumulation and translocation of elements in plants has been largely investigated during the last years,[35,36] but most often considering a single harvest. Systematic field experiments carried out in open-air plots are scarce, and the concentration of inorganic elements in plant tissues is known to fluctuate during plant growth and development, in relation with the age of the plant and with the storage and remobilization of elements among organs.[34] Additionally, organic and conventional fertilizers are expected to result in a different bioavailability and accumulation for both nutrients and toxic elements and therefore in different nutritional properties and risk for consumers.

Due to the inconsistencies existing in the literature and the lack of controlled field experiments to investigate the effect that the farming technique has on the time course of accumulation and translocation of chemical elements in Swiss chards and tomato plants, we performed a 5-month experiment in a rural area of the Basque Country, in which the elemental composition of Swiss chards and tomato plants were monitored till the time these vegetables are commonly harvested.

The plants were cultivated in two open-air plots, where conventional and organic practices were carried out. The objectives of the work were i) to study the accumulation of nutrients and non-essential elements in different plant organs, paying special attention to the edible parts, and ii) to investigate the effect of the farming technique and the harvesting time on the uptake and translocation of essential and non-essential elements in the plants.

The following three chapters report the details of the experiments carried out, as well as the results obtained for Swiss chards and tomato plants. The next one concentrates on the proposal of a methodology to discern among tomatoes with different ripening stages by image analysis of standard pictures. The last chapter summarizes the most important conclusions obtained in this Section.

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Chapter 1

MATERIALS AND METHODS

1.1. IMPLEMENTATION OF THE PLOTS AND AGRICULTURAL TREATMENTS

The 5-month experiment was carried out in an open-air garden located in Beotegi, a rural area of the Basque Country (43° 5.370' N, 3° 4.590' W), Spain, at 370 meters over the sea level with no greenhouse protection. Two plots of 25 m^2, separated each other by 35 meters, were selected in the garden (Figure 1.1) for conventional and organic practices, respectively.

Four soil samples were collected in each plot in February 2013, 4 months before plantation, for soil characterization. Soils were randomly selected and collected from 15 to 30 cm depth, using a garden spade and removing stones and other remains.

On the 5th June 2013, each plot was planted with twenty-five Swiss chard seedlings (*Beta vulgaris* subsp. c*icla* var. sima) and with twenty-five tomato seedlings (*Solanum lycopersicum* var. jack). Seedlings had 2 to 4 leaves and were obtained from a local producer (Camino Sociedad Civil, Llodio, Spain).

Different certified agricultural products were applied in the garden. In the plot intended for conventional practice, 0.25 kg m^{-2} of a synthetic chemical fertilizer [NPK 15.15.15 (15); Fertiberia, S.A.] was applied once 25 days before plantation, which corresponds to a total dose of 938 g of total N, P_2O_5 and K_2O for Swiss chards and tomato plants growth.

Figure 1.1. Location of the garden through Google Earth program (picture taken in February 2013). The plot intended for organic treatment is marked in green, while the plot planned for conventional treatment is marked in blue. The garden lines are marked in orange.

An insecticide (Epik 20 SG; Sipcam Jardín S.L.) and a fungicide (Galben M.; Sipcam Jardín S.L.) were also added (200 mL m^{-2} in total) 7 and 36 days after plantation as phytosanitary treatment to minimize pest attacks.

The plot intended for organic practice was supplied with two applications of an organic fertilizer (natural horse manure, Abonos Naturales Hermanos Aguado, S.L.; product approved and certified by CAEE as ecological product; C qualification), 10 and 2 days before plantation, corresponding to a total of 6.48 kg m^{-2}, which corresponds to a dose of 7452 g of total N, 648 g of P_2O_5 and 1458 g of K_2O. A total of 0.67 g m^{-2} of powdered copper sulphate (Desarrollo Químico Industrial, S.A.; product approved and certified by SHC for organic farming) was sprinkled onto the aerial parts of the tomato plants 7 and 36 days after plantation as a fungicide, particularly against the mildew. In the periphery of the organic plot, protective plants (*Tagetes patula*) were planted as natural repellent to avoid pests and insect attacks. Both plots were maintained until October 2013.

During the cultivation, a vegetative pruning in an axis of tomato plants was performed, removing the emerging auxiliary shoots so as to favour the vigour of the plants and to improve fruit growth and quality.

1.2. SAMPLING CAMPAIGNS

Six sampling campaigns were carried out every three weeks (26th June, 15th July, 6th August, 26th August, 16th September and 21th October), covering the whole production cycle of the plants, as is shown in Figures 1.2 and 1.3, for Swiss chards and tomato plants, respectively.

Four Swiss chards and four tomato plants, randomly selected in advance, were harvested at each sampling campaign. In situ, each plant was separated in different parts immediately after harvesting.

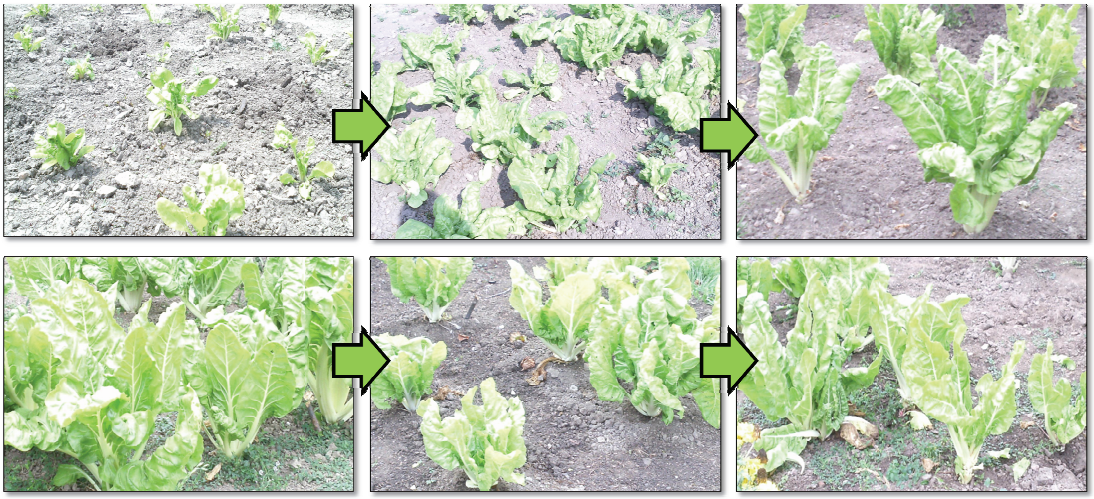

Figure 1.2. Growth stages of Swiss chards and sampling campaigns carried out during the field experiment.

In the case of Swiss chards, the different organs were roots, stalks and leaf blades, whereas for tomato plants, they were roots, stems, leaves, flowers and fruits. In tomato plants, due to phenology, flowers and fruits could only be collected after starting the flowering stage. Leaves and flowers were not available in October due to the senescence of the plants. For each sampling date, three plants were subjected to chemical analysis, while the fourth one was used to estimate the water content of the different plant parts.

During the first, third and fifth samplings, and two weeks after the sixth sampling campaign, soils were also collected at the root zone of the first two sampled Swiss chards and tomato plants.

All samples were preserved in pre-labelled zip bags and kept in portable coolers at low temperature during their transportation to the laboratory. Once in the laboratory, samples were stored in dark at 4 °C until pretreatment, which was carried out on in the same day of sampling.

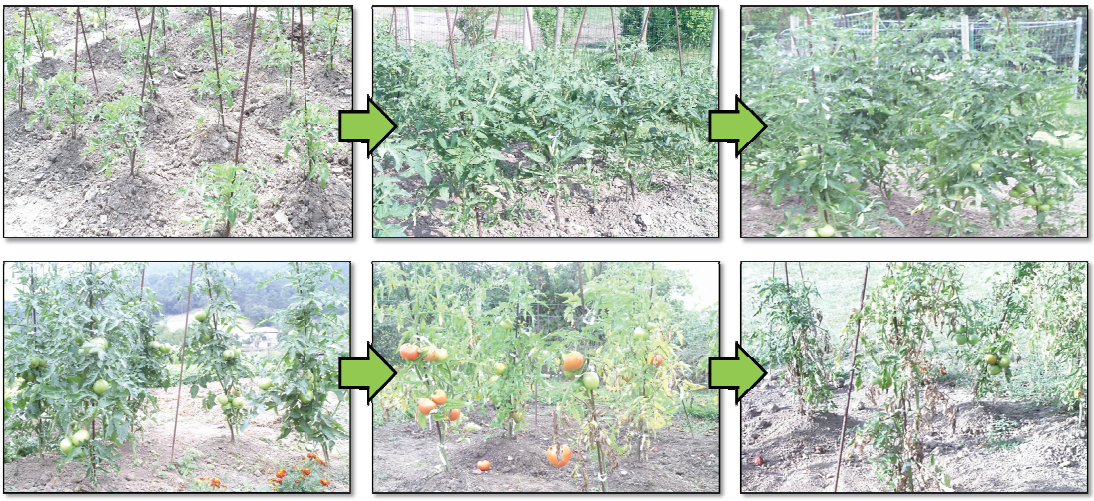

Figure 1.3. Growth stages of tomato plants and sampling campaigns carried out during the field experiment.

Meteorological data (maximum, minimum and mean temperature (°C), mean and maximum daily wind velocity (km h^{-1}), precipitation for 24 hours (mm), relative humidity (%) and total solar irradiation (W m^{-2})) for the whole duration of the experiment were obtained from the C051 meteorological station (43º 1.9271´ N; 3º 0.237´ W) of the Basque Meteorological Agency.[1]

Growing degree days (GDD, °C) were used as thermal time to establish the growth stage of the crop during the 139 days of the experiment. The GDDs were calculated according to Equation 1, where T_{base} (6 °C for Swiss chards and 10 °C for tomato plants) is the base temperature of the considered plant and T_{max} and T_{min} are the maximum and minimum daily temperature, respectively, and *n* is the number of days after transplanting.

$$\mathrm{GDD} = \sum^{\mathrm{n}} \frac{\mathrm{Tmax+Tmin}}{2} - \mathrm{Tbase} \qquad (1)$$

1.3. SAMPLE PRETREATMENT

Soil samples used for chemical analyses were air-dried using a laminar-flow hood, sieved to particle size under 2 mm, and finally ground and homogenized in a planetary ball mill (Pulverisette 6, Fritsch, Germany).

Plant samples were thoroughly washed with Ellix-quality water (Millipore) and Milli-Q water (18.2 MΩ cm, Milli-Q Element A10 purification system, Millipore) to remove dirt and damaged and dead tissues, dried with cellulose paper and weighed using a Sartorius TE412 balance (± 0.01 g).

Plant samples used to estimate the water content (one per plot and sampling) were dried in an oven at 80 °C until constant weight. The water content of each plant part was calculated as the difference in weight between the non-dried and dried samples. The water content values were used to estimate the dry mass (DM, kg) of each plant sample.

Parts of the plants used for chemical analysis (three plants per plot and sampling) were lyophilized (0.01 mbar, -80 °C, 48 h) in a FreeZone Plus (Labconco) freeze-dryer and ground in a planetary ball mill. All dried samples (soils and plants) were stored in glass vials at 4 °C in the dark until analysis.

1.4. CHARACTERIZATION OF SOILS

Soils collected in February 2013 were submitted to several physicochemical analyses to determine the content in organic matter (OM), organic carbon (OC), total calcium carbonate ($CaCO_3$) and total nitrogen (N), as well as their pH, cation exchange capacity (CEC) and texture. Samples were air-dried in a laminar-flow chamber. All the analyses were performed on the fraction below 2mm, following the ISO reference analytical methods described by Cools et al.[2] The soil OC, $CaCO_3$ and N contents were measured after dry combustion of the sample.

The pH was measured in a 1:5 (v:v) suspension of dried soil sample in water. CEC was determined in the fraction below 2 mm according to the method of Metson,[3] using a 1M ammonium acetate solution at pH 7. For the soil texture determination, samples were sieved at different standard fractions (coarse sand, 0.2 - 2 mm; fine sand, 0.2 mm-50 µm; coarse silt, 20 - 50 µm; fine silt, 2 - 20 µm; and clay, < 2 µm), according to the procedure defined in the French norm NF X31-107,[4] and the results obtained were plotted on the USDA textural classification triangle.

1.5. ANALYSIS

All glass material used during the analytical procedure was washed with tap water and soap and left in a 10 % nitric acid (Panreac) bath for at least 24 hours. Afterwards, it was rinsed twice with Ellix-quality water and once with Milli-Q water and stored in clean plastic zip bags until use. HNO_3 (69 %) and HCl (36 %) used for acid extraction were Tracepur-quality and supplied by Merck (Darmstadt, Germany).

About 0.5 g (weighed in a Mettler AJ150 balance, ± 0.0001 g) of dry soil or plant sample were acid digested using a microwave digestion system Multiwave 3000 (Anton Paar, Graz, Austria), equipped with 100 mL fluorocarbon polymer (PTFE) microwave vessels in an 8XF-100 microwave digestion rotor. The EPA 3051A method[5] was followed for soils, using a mixture of 9 mL of HNO_3 (69 %; Merck, Tracepur) and 3 mL HCl (36 %; Merck, Tracepur) instead of 10 mL of HNO_3. In the case of plant samples, a mixture of 6 mL of HNO_3 and 2 mL of H_2O_2 (30 %; Sigma-Aldrich, TraceSelect Ultra) was used for digestion. The heating programs used for plant and soil samples are indicated in Table 1.1.

Table 1.1. Heating program used for the acid digestion of plant and soil samples.

Plant samples				Soil samples			
Steps	**Power (W)**	**Ramp (min)**	**Hold (min)**	**Steps**	**Power (W)**	**Ramp (min)**	**Hold (min)**
1	250	2:00	6:00	1	900	5:00	7:00
2	400	1:00	4:00	2	600	1:00	4:30
3	600	1:00	4:00	3	0		15:00
4	0		15:00	4			

After digestion, samples were filtered using 0.45 µm syringe-filters with PVDF membrane (Olimpeak, Teknokroma, Barcelona, Spain) and quantitatively transferred to polyethylene pre-cleaned vials. The extracts were adjusted to about 50 g with Milli-Q water using a Mettler AJ150 balance (± 0.0001 g). All vials were stored at 4 °C in the dark until analysis. Procedural blanks were processed together with soil and plant samples, for blank correction and estimation of limits of detection (in mg kg^{-1}, LOD), as the average plus three times the standard deviation of the replicate analysis of procedural blanks (Table 1.2).

Table 1.2. Limits of detection (LOD) for different elements in plant ([a]) and soil ([b]) samples.

Element	LOD [a] (mg kg^{-1})	LOD [b] (mg kg^{-1})
Na	18	-
Mg	2.8	2.7
Al	3.6	2.6
K	6.6	-
Ca	69	-
Ti	0.29	0.67
V	0.078	0.41
Cr	0.088	0.14
Mn	0.49	0.13
Fe	2.1	1.7
Co	0.040	0.012
Ni	1.3	0.58
Cu	0.24	0.046
Zn	16	14
As	0.044	0.079
Se	0.52	2.5
Sr	0.46	0.78
Mo	0.19	0.094
Ag	0.076	0.15
Cd	0.022	0.024
Sn	0.14	0.23
Sb	0.20	0.0051
Ba	0.092	0.13
W	0.52	0.17
Hg	0.77	0.49
Tl	0.014	0.0093
Pb	0.069	0.12

Elemental analysis of the extracts was carried out by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, NexION^{TM} 300X, PerkinElmer Inc., Ontario, Canada) in a class 100 clean room. The concentrations of Na, Mg, Al, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Ag, Cd, Sn, Sb, Ba, W, Hg, Tl and Pb were measured in all samples except in soil samples, where Na, K, and Ca were not measured. The experimental conditions used in the ICP-MS measurements are summarized in Table 1.3. A 1000 µg L^{-1} of commercial solutions (Alfa Aesar, Specpure®, Plasma standard solution, Germany), acidified with sub-boiling 69 % HNO_3 or 37 % HCl, were used to prepare gravimetrically the calibration standards, using a Mettler-Toledo XS205 balance (Columbus, OH, with ± 0.00001 g of precision).

Be, Sc, Ge, In, Re and Bi were used as internal standards (10 ng g^{-1}) to quantify chemical elements in samples and to correct for any instrument drift during analysis. The argon used for the plasma was supplied by Praxair (99.99 %, Spain).

Appropriate dilutions of the extracts were carried out gravimetrically. The HNO_3 concentration of all the samples was adjusted to 1 % before injecting them into the ICP-MS. Due to the differences in the concentration range of some elements measured in this study, a second dilution was required so as to determine elements that were out of the calibration range.

Table 1.3. Experimental conditions used in the measurements by ICP-MS.

Parameter	Value
Nebulisation flow	0.90-1.00 L min^{-1}
Plasma flow	18 L min^{-1}
Auxiliary flow	1.2 L min^{-1}
Radio frequency power	1600 W
Helium flow	4.0 mL min^{-1}
Dwell time	50 s
Integration time	1000 ms
Sweeps per replicate	20
Replicates	3
Analysis characteristics	
Plasma	Argon
Internal standards (10 ng g^{-1})	Be, Sc, Ge, In, Re and Bi
Analysis mode	Kinetic Energy Discrimination (KED)
Collision gas	Helium

The accuracy and reproducibility of the method were checked by the analysis of 31 replicates of the ERM-CC141 (loam soils)[6] and 48 replicates of the SRM 1570a (trace elements in spinach leaves)[7] certified reference materials (Table 1.4). The measured concentrations (in mg kg^{-1}) were corrected according to the real humidity content of each reference material. Afterwards, the mean and standard deviation values for each element were calculated so as to check the accuracy and reproducibility of the method, obtaining satisfactory results (relative standard deviations below 10 % and recoveries between 70 and 105 %), either for plant or soil samples.

1.6. DATA PROCESSING

To rationalize further discussions, the elements considered in this field experiment were divided into three main groups: macronutrients (Mg, K, and Ca), micronutrients (Na, Mn, Fe, Co, Cu, Zn, Ni, Mo, and Se), and non-essential elements (Sr, Ag, Ba, W, Al, Ti, V, Cr, As, Sn, Sb, Cd, Tl, Hg, and Pb).

Table 1.4. Relative standard deviations (RSD) and recoveries obtained for each reference material used for plant ([a]) and soil ([b]) samples.

Element	SRM 1570a [a] (n = 48)		ERM-CC141 [b] (n = 31)	
	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
Na	8	-	-	-
Mg	7	-	8	-
Al	9	70	9	-
K	9	-	-	-
Ca	10	-	-	-
Ti	9	-	10	-
V	10	71	9	-
Cr	10	-	8	76
Mn	8	97	9	87
Fe	8	-	10	-
Co	9	83	10	73
Ni	10	87	10	85
Cu	9	91	9	80
Zn	8	98	9	105
As	10	105	10	105
Se	10	91	10	-
Sr	7	-	10	-
Mo	10	-	9	-
Ag	10	-	10	-
Cd	6	94	7	93
Sn	10	-	10	-
Sb	10	-	10	-
Ba	7	-	7	-
W	10	-	10	-
Hg	10	105	10	105
Tl	7	-	8	-
Pb	9	-	8	102

The concentrations of Ni, Sn, Sb, Ag, Se, W, and Hg for plant samples and of W and Hg for soil samples were below the detection limit in more than 80 % of the samples (see Table 1.2 and Annex A) and, thus, were consequently not considered in the discussion.

The data processing for Swiss chards and tomato plants was carried out separately. For Swiss chards, the plant data set consisted of 108 lines (2 farming techniques × 6 sampling campaigns × 3 plants × 3 plant parts), 3 categorical variables (sampling campaign, farming technique and plant part) and 20 numerical variables (corresponding to the concentrations of the chemical elements).

The soil data matrix used included 12 lines (2 farming techniques × 3 sampling campaigns × 2 replicates), 2 categorical variables (sampling campaign and farming technique) and 22 numerical variables (the concentrations of 22 chemical elements).

In the case of tomato plants, the plant data set included 156 samples (2 farming techniques × 6 sampling campaigns × 3 plants × 5 plant parts; leaves and flowers from the last sampling and fruits from the first two samplings were not available), 3 categorical variables (sampling campaign, farming technique and plant part) and the concentrations of 20 elements. The soil data set consisted of 12 samples (2 farming techniques × 3 sampling campaigns × 2 soils replicates), 2 categorical variables (sampling campaign and farming technique) and the concentrations of 22 elements.

The uptake of each element from the soil and its partitioning between roots and shoots are highly dependent on the type of element, as it is already reported,[8] and can be efficiently characterized by means of the Bioaccumulation Factor (BAF) and Translocation Factor (TF). Both factors were independently calculated for each sampling campaign and farming technique. Moreover, the first factor was calculated for the 17 elements for which concentrations were above the detection limits of the method in both soil and plant samples.

The BAF refers to the ratio between the mean element concentration (mg kg^{-1} in dry mass) in plant tissues and that in the soil (Equation 2). A value of BAF higher than 1 indicates that the plant is able to concentrate the element compared to the concentration found in the soil, illustrating a strong extraction of the element from the soil.

$$BAF = \frac{\text{mean element concentration in plant tissues}}{\text{mean element concentration in soil}} \qquad (2)$$

The TF was used to follow the root-to-shoot translocation, defined as the concentration of the element accumulated in aboveground organs in comparison with the concentration in roots (Equation 3). A TF value higher than 1 suggests that translocation of the element from the roots to the aerial parts is greater than the allocation of element to shoots compared to roots. It is an indicator of the mobility of the element within the plant.

$$\text{TF} = \frac{\text{element concentration aboveground}}{\text{element concentration in roots}} \qquad (3)$$

Principal Component Analysis (PCA), Analysis of Variance (ANOVA) and Correlation Analysis (CA) were also carried out, using RStudio v. 0.98.1102 (RStudio, Inc., USA) and The Unscrambler® v. 9.2 (CAMO Software AS). Data were centred and scaled before treatment when necessary.

1.7. REFERENCES

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Chapter 2

RESULTS AND DISCUSSION:

ACCUMULATION AND TRANSLOCATION OF ESSENTIAL AND NON-ESSENTIAL ELEMENTS BY ORGANICALLY AND CONVENTIONALLY GROWN SWISS CHARDS (*Beta vulgaris* subsp. *cicla*) CULTIVATED IN OPEN-AIR PLOTS

2.1. SOIL CHARACTERIZATION BEFORE THE EXPERIMENT

Before starting the experiment, soil samples were collected and analyzed according to the procedures described in Sections 1.2, 1.3 and 1.4 in order to identify possible initial significant differences between the two plots.

The results indicated that there were no significant differences between the two plots (ANOVA, $p > 0.05$) for the physic-chemical parameters and elements concentrations (Annex A). Thus, the two plots were considered as being initially similar. Swiss chards were grown in a basic loam soils, characterized by a high content in nutrients and organic matter, which are considered among the best soils for agricultural purposes, with an optimum combination of sand, silt and clay particles.[1]

The pH in both plots varied between 8.12 and 8.18 and the CEC between 11.8 and 13.2 cmol+ kg^{-1}. Moreover, the content of OM (35 – 44 g kg^{-1}), OC (20 – 25 g kg^{-1}), $CaCO_3$ (370 – 390 g kg^{-1}) and N (1.90 - 2.45 g kg^{-1}) was also very similar in both plots.

2.2. BIOMASS PRODUCTION AND WATER CONTENT OF PLANTS DURING THEIR GROWTH

The water content of roots, stalks and leaf blades of 12 Swiss chards (one per plot and sampling campaign) was calculated as described in Section 1.3.

In general, the aerial parts of chards presented higher water content than roots during the experiment (82.6 ± 6.3 % for roots, 93.5 ± 1.8 % for stalks and 89.6 ± 2.8 % for leaf blades). A two-way ANOVA, crossing samplings and treatment by plant part, indicated that organically grown leaf blades had more water content (91.2 ± 2.0 %) than conventionally grown ones (88.1 ± 2.7 %), regardless of time ($p < 0.05$). Additionally, the water content of roots decreased progressively with time (from June to October; 90.66 ± 0.73 %, 87.3 ± 2.3 %, 84.8 ± 2.0 %, 78.9 ± 5.5 %, 80.8 ± 3.0 % and 73.21 ± 0.68 %), regardless of the treatment ($p < 0.05$), probably due to (i) the difficulty to maintain an appropriate supply of water when the temperature rose and (ii) the increasing contribution of structural tissues with plant age.

The dry biomass (in kg) of each plant part was calculated from water content and the total biomass (in fresh weight) collected at each harvesting time. Whatever the sampling time, no significant differences were observed between the conventional and the organic plots for the roots and the aboveground dry biomass due to a large variability (two-way ANOVA, $p = 0.72$ for roots, $p = 0.50$ for stalks; $p = 0.67$ for leaf blades). The results obtained have been plotted in Figure 2.1 against GDD (°C). In both plots root biomass strongly increased between 868 and 1140 GDD ($p < 0.001$) and then remained stable. For stalks and leaf blades, the dry mass increased more steadily and reached a maximum at 1388 GDD, and seemed to decrease thereafter for the organic practice ($p < 0.01$).

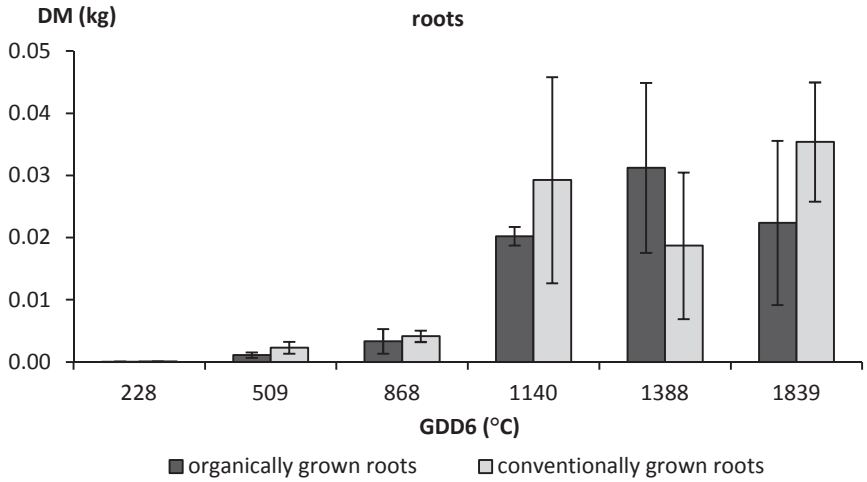

Figure 2.1. Total biomass production (in dry mass, DM, kg) of roots, stalks and leaf blades of Swiss chards grown up using conventional (grey) and organic (black) fertilizer. In each sampling campaign, three Swiss chards were collected from each plot (n = 3), as replicates.

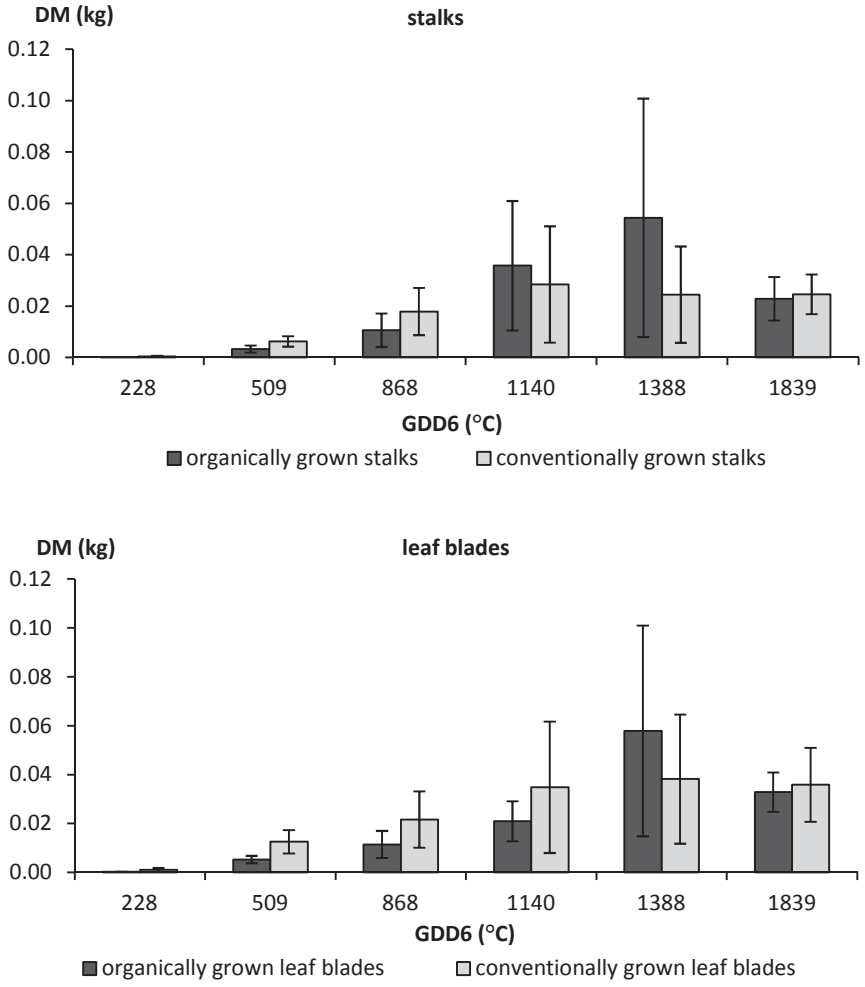

Figure 2.1 (*cont.*). Total biomass production (in dry mass, DM, kg) of roots, stalks and leaf blades of Swiss chards grown up using conventional (grey) and organic (black) fertilizer. In each sampling campaign, three Swiss chards were collected from each plot (n = 3), as replicates.

2.3. CONCENTRATION OF ELEMENTS IN PLANT AND SOIL SAMPLES

The concentrations (in mg kg^{-1} in dry weight) of 27 (for plant samples) and 24 (for soil samples) elements measured in the digested samples are provided in Annex A.

The Principal Component Analysis (PCA) models finally selected for plant and soil samples explained, respectively, 75.9 % (PC1: 41.9 %; PC2: 18.0 %; PC3: 10.5 %; PC4: 5.5 %) and 83 % (PC1: 42.3 %; PC2: 24.6 %; PC3: 9.1 %; PC4: 7.5 %) of the total variance. The results obtained will be further discussed in the next sections.

Statistical analysis showed that plant part was the most significant factor influencing the accumulation of almost all chemical elements in Swiss chards, followed by the sampling campaign and, finally, the farming technique (see Annex B). No element exhibited, however, a simple dependence on a unique factor.

2.3.1. PARTITIONING OF CHEMICAL ELEMENTS WITHIN SWISS CHARDS

According to the scores and loadings plots of the model obtained after PCA of the whole data matrix, the components 2 and 3 of the PCA discriminated correctly the clusters for each plant part (Figure 2.2), accounting for 18.0 and 10.5 % of the total variance, respectively. ANOVA showed that the differences in the mean concentrations between the plant organs were significant for all elements. This was especially marked for Mg, K, Al, Na, Mn, Cu, Fe, Co, Mo, Sr, Ba, V, As, Tl, Cd and Pb, with *p* values below 0.001 (Annex B).

Roots were relatively less concentrated in Na, K, Ca, Sr and Ba and relatively more concentrated in Al, V, Pb, Tl, Co and Fe (Figure 2.2), while the aerial part of Swiss chards presented significantly higher concentrations of macro and micronutrients. Compared to stalks, leaf blades were more concentrated in Mg, Mn, Fe, Cu, Zn and Mo, consistent with the photosynthesis activity, but they were also richer in Cd. By contrast, stalks were richer in K, which ensure the turgescence of tissues and thereby, likely contribute to the rigidity of shoots.[2]

Na and K are involved in the osmorregulation while Ca is required for cell division elongation and integrity.[2] Sr and Ba, although non-essential, tend to behave like Ca due to their chemical similarity and this is probably why they were highly translocated to aboveground, which may be a problem for food safety if the soil bioavailability is important, or in case of contamination of the soil by radioactive isotopes of these elements.

The concentration of Cd (0.019 ± 0.013 mg kg^{-1} of Cd, in fresh weight) was rather high in leaf blades, but always below the threshold defined by the Commission Regulation (EC) No 629/2008[3] to be consider as toxic for human consumption (0.20 mg kg^{-1} of Cd, in fresh weight, for leafy vegetables).

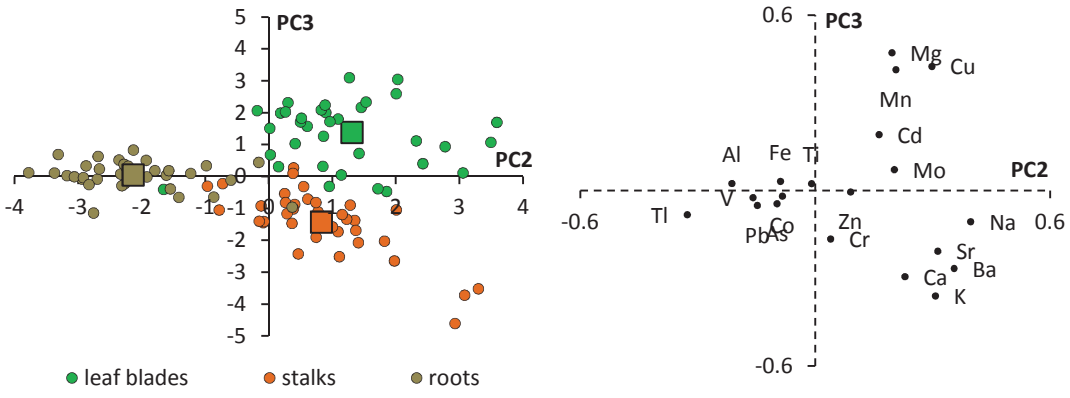

Figure 2.2. Scores (left, n = 108) and loading plots (right, n = 20) obtained after PCA of the whole data matrix for Swiss chards (76 % of explained variance with 4 PCs). Samples are grouped by plant part. The barycentre of each formed cluster is marked by square dots.

It is well established that Cd is absorbed by plant roots due to the lack of specificity of Zn, Fe, Cu and Mn transporters,[4,5] explaining why Cd can be found in green parts of the plant, as it has been already reported.[6-9]

2.3.2. BIOACCUMULATION FACTORS AND TRANSLOCATION FACTORS

The Bioaccumulation Factor (BAF) and Translocation Factor (TF) for each element and for each plant organ were calculated according to the Equations 2 and 3 of Chapter 1. For some elements it was not possible to calculate the BAF values because the concentration in soil was not measured (Na, K and Ca) or because the concentration in the plant organs was below the detection limit (Ni, Sn, Sb, Ag, Se, W and Hg).

The BAFs obtained for each aboveground organ of Swiss chards have been summarized in Figure 2.3. Each bar in the figure represents the mean of three plant samples, for each sampling campaign and farming technique. The concentration in soil used in the calculation was the mean of the concentrations found in the six soil samples collected in each plot (distinguishing between conventional and organic soils) along the experiment with Swiss chards.

BAF values over 1 in both edible parts were obtained for Mg and Mo (stalks and blades), Zn (stalks), and Cu and Cd (leaf blades). Significant differences were also observed in BAF values when comparing organically and conventionally grown edible parts. Mn ($p < 0.01$) and Cu ($p < 0.001$) were significantly more accumulated in organically grown stalks, while in the conventional plot Cd ($p < 0.05$) was more presented. Compared to the conventionally grown leaf blades, organically grown ones accumulated more Mn ($p < 0.05$) and Cu ($p < 0.05$) and less Cd ($p < 0.05$).

Except for Cd in young conventionally grown leaf blades, the BAF values for all non-essential elements were below 1, indicating that these elements were not noticeably concentrated in edible parts of chards, and therefore were relatively little mobile in the soil-plant system, as was observed in Section 2.3.1.[5,10]

As it was previously outlined, the BAF for Cd was relatively high being sometimes above 1, in particular for young leaf blades grown on the conventional plot. This illustrates the quite high mobility and accumulation of Cd in some of the edible plant organs. Once taken up by roots, part of the Cd is sequestrated in roots while the rest is loaded into the xylem stream and moved aboveground, where it is allocated to aerial parts of plants. On its way upwards, like many other trace elements, Cd can be unloaded from the xylem and loaded into the phloem and may therefore return to roots.[11] Because in Swiss chards at the vegetative growth stages there is no developed stem, there is less opportunity for Cd to be sequestrated in the stem or to exchange from the xylem to the phloem and this might explain the high BAF observed in our study.

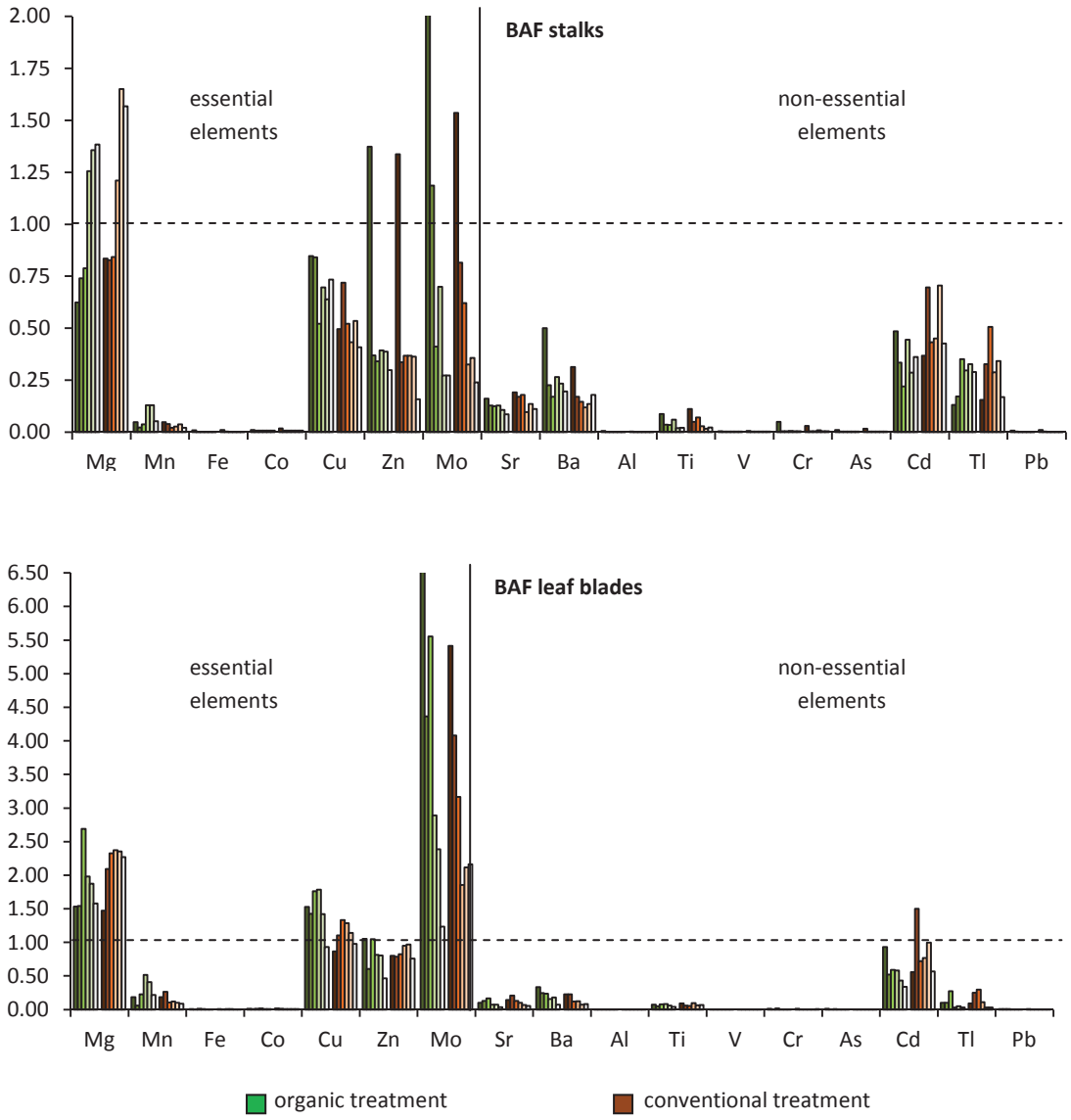

Figure 2.3. Bioaccumulation Factors (BAF) of elements measured in Swiss chards and in soils during the field experiment. Each bar represents the mean value (n = 3) of each element for each sampling campaign. Organic and conventional treatments are marked in green and brown, respectively.

Furthermore, the BAF was greater in the conventional plot possibly because the P fertilizers can be contaminated by Cd and also because synthetic fertilizers can increase the solubility of Cd due to changes in the soil pH or in the ionic strength of the soil solution.[12]

TFs for each element were also calculated separately for stalks and leaf blades, as is shown in Figure 2.4, as the mean of the three replicates for each plot and each sampling campaign. TFs help to understand the BAF, so that they indicate if the root-to-shoot translocation of the elements was important. Some elements, such as Na, K, Ca, Sr and Ba, showed high TF values in both stalks and leaf blades, consistent with the general partitioning shown in Section 2.3.1.

TFs for toxic trace elements were close or below 1, showing that these elements were more importantly sequestrated in roots than in aboveground organs, particularly in mature plants, except Cd.[10,13] This confirms that Cd is quite mobile in plants as it behaves similar to Zn.[14]

The TF for leaf blades were higher than for stalks, showing that leaf blades are richer in inorganic elements than stalks (Figure 2.4). For nutrients, this is consistent with the high metabolic activity of leaf blades compared to stalks. Moreover, as stalks are a structural organ, their main function is to drive nutrients and water to the leaf blades.

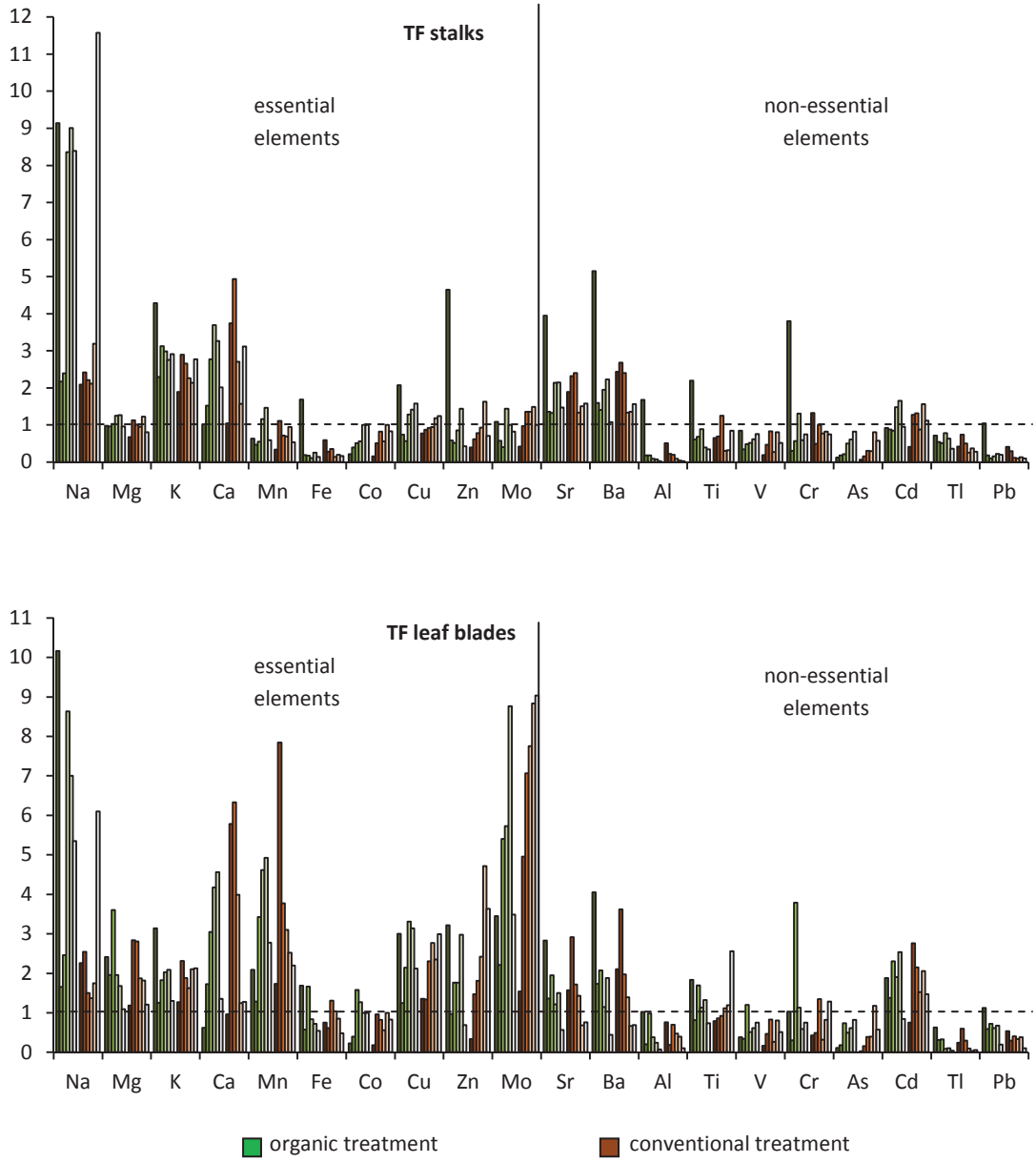

Figure 2.4. Translocation Factors (TF) of elements measured in Swiss chards during the field experiment. Each bar represents the mean value (n = 3) of each element for each sampling campaign. Organic and conventional treatments are marked in green and brown, respectively.

In particular, Mg, Mn, Fe, Cu, Zn and Mo, together with the toxic Cd, presented higher TF values in leaf blades, compared to stalks, during the whole experiment ($p < 0.001$). These elements are mainly allocated by the transpiration stream in the xylem and, at the growth stages considered, storage in the stalks is much less compared to allocation to the green leaf blades.

The higher TF for Cd in leaf blades indicates that this toxic element, following a similar trend that nutrients, was less stored in the stalks than in leaf blades. Regarding the risk for consumers, the possible intake of Cd by the leaf blades could be avoided discarding the leaf blades when preparing this vegetable.

The fertilizer used had no significant effect on the TF of elements, except for Na, which was generally more translocated to organically grown leaf blades ($p < 0.05$).

2.3.3. ELEMENTS WITH SIMILAR ACCUMULATION AND TRANSLOCATION TRENDS

Pearson correlation coefficients (r) between the concentrations of elements in Swiss chards were calculated so as to identify similar behaviours among elements (n = 108; 6 sampling campaigns x 3 replicates x 3 plant parts x 2 farming techniques). The whole correlation matrix for Swiss chards during the experiment is shown in Table 2.1. Because we have n = 108 values for each element (6 sampling campaigns x 3 replicates x 3 plant organs x 2 farming techniques), the Pearson correlation coefficient between two element concentrations differs significantly from zero at 95 % confidence level if it is higher than 0.19, in absolute value.

All the correlations between Al, Ti, V, Cr, Fe, Co, Zn, As and Pb, on the one hand, and between Na, Ca, K, Sr and Ba, on the other hand, were positive and statistically significant at 95 % confidence level. This means that for each of these two groups, the elements behaved in the same way, their concentration in tissues increasing or decreasing together depending on the plant organ, on the age and on the treatment.

The strongest correlations were found between Fe, Co and some toxic elements (Fe and Co, r = 0.84; Fe and As, r = 0.81; Co and As, r = 0.96; Fe and Pb, r = 0.92; Co and Pb, r = 0.87; Fe and V, r = 0.89; and Co and V, r = 0.93) and also between other toxic elements (V and Al, r = 0.90; V and As, r = 0.90; and V and Pb, r = 0.90). The correlations between Co, Fe and As may have a geochemical origin as Fe and As are associated in arsenopyrite while As and Co are associated in cobaltite. This group of elements was mainly sequestrated in roots and poorly transferred to aboveground organs, which is a good point concerning a safe consumption of Swiss chards.

A lower but still strong correlation was found between Sr and Ba (r = 0.65), Ca and Ba (r = 0.57) and Ca and Sr (r = 0.54), likely because Sr and Ba are two alkaline earth metals closely related for their physic-chemical properties, also close to those of Ca, as indicated before. These last elements were significantly accumulated in the edible aerial parts of Swiss chards.

Table 2.1. Correlation matrix obtained for 20 elements detected in Swiss chards (n = 108; r_{crit} = 0.19).

	Na	Mg	Al	K	Ca	Ti	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Mo	Cd	Ba	Tl	Pb
Na	1.00																			
Mg	0.14	1.00																		
Al	-0.39	0.02	1.00																	
K	0.52	-0.11	-0.29	1.00																
Ca	0.20	0.02	0.21	0.36	1.00															
Ti	-0.14	0.13	0.71	-0.02	0.34	1.00														
V	-0.25	0.02	0.90	-0.12	0.32	0.70	1.00													
Cr	-0.01	-0.01	0.71	0.11	0.51	0.67	0.76	1.00												
Mn	0.10	0.48	0.16	0.00	0.11	0.32	0.20	0.18	1.00											
Fe	-0.28	0.13	0.88	-0.18	0.46	0.66	0.89	0.75	0.30	1.00										
Co	-0.19	0.06	0.80	-0.03	0.39	0.82	0.93	0.75	0.29	0.84	1.00									
Cu	0.31	0.53	0.02	-0.04	0.11	0.15	0.02	0.11	0.65	0.20	0.07	1.00								
Zn	-0.08	0.23	0.56	0.05	0.67	0.65	0.66	0.66	0.38	0.82	0.77	0.30	1.00							
As	-0.18	0.05	0.75	-0.02	0.40	0.74	0.90	0.67	0.23	0.81	0.96	0.00	0.78	1.00						
Sr	0.36	0.14	0.01	0.35	0.54	0.17	0.07	0.27	0.12	0.16	0.13	0.27	0.29	0.10	1.00					
Mo	0.24	0.17	0.35	0.08	0.28	0.48	0.33	0.53	0.31	0.38	0.40	0.39	0.40	0.33	0.18	1.00				
Cd	0.06	0.47	0.36	0.00	0.36	0.50	0.46	0.43	0.34	0.45	0.52	0.39	0.53	0.48	0.39	0.46	1.00			
Ba	0.43	0.06	0.05	0.43	0.57	0.26	0.06	0.48	0.18	0.20	0.14	0.22	0.41	0.10	0.65	0.45	0.24	1.00		
Tl	-0.41	-0.18	0.40	-0.38	-0.17	0.06	0.29	0.09	-0.26	0.22	0.15	-0.32	-0.04	0.14	-0.01	-0.19	-0.04	-0.21	1.00	
Pb	-0.32	-0.01	0.86	-0.17	0.42	0.67	0.90	0.71	0.17	0.92	0.87	0.06	0.76	0.84	0.12	0.35	0.40	0.15	0.32	1.00

2.3.4. THE TIME EFFECT ON THE ELEMENTAL COMPOSITION OF SWISS CHARDS

The results of PCA (Figure 2.5) indicate that the effect of the time on the elemental composition of Swiss chards with important. It was illustrated by components 1 and 3 of the model, which accounted for 41.9 and 10.5 % of the total variance.

The first sampling in June was strongly discriminated from the others, which were similar. This was confirmed by one-way ANOVA, which established that the first sampling was significantly different for all the elements.

The element concentrations of the samples collected during the first campaign in June were higher than those from the rest of the samplings, with the only exception of Mg, for which the average concentration increased with time. The fact that the addition of fertilizers (conventional or organic) was done in May and June (close to the first sampling date) is a possible explanation of this sharp decrease in the element concentrations in Swiss chards. Moreover, the plant capacity to take up elements may have also decreased with time, due to the aging of the root system,[15] resulting in a progressive dilution of the elements in plant tissues.

However, although younger plants were richer in essential nutrients than older ones, non-essential and toxic elements seemed to follow the same pattern (Figure 2.6).

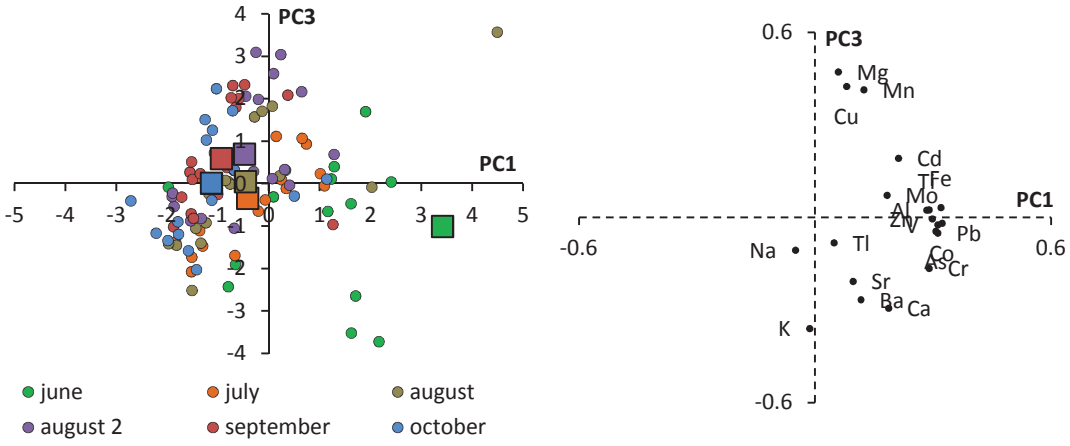

Figure 2.5. Scores (left, n = 108) and loading plots (right, n = 20) obtained after PCA of the whole data matrix for Swiss chards (76 % of explained variance with 4 PCs). Samples are grouped by sampling campaign. The barycentre of each formed cluster is marked by square dots.

As examples, the maximum concentrations of Mo in all plant organs, and in both treatments, were at the beginning of the experiment, together with Cd and Pb but only in conventional leaf blades. After carrying out Pearson correlation analysis ($\alpha = 0.05$, $r_{crit} = 0.47$, in absolute value), a significant negative correlation between Mo, Cd and Pb and thermal time (expressed as GDD) was detected.

This outlines that consuming very young Swiss chards is more risky than consuming older plants regarding the toxic Cd and Pb. The opposite behaviour was noticed for Mg in roots and stalks of both plots, increasing its concentration with GDD. Some of these significant correlations between the element concentrations and thermal time (expressed as GDD) are shown in Figure 2.6.

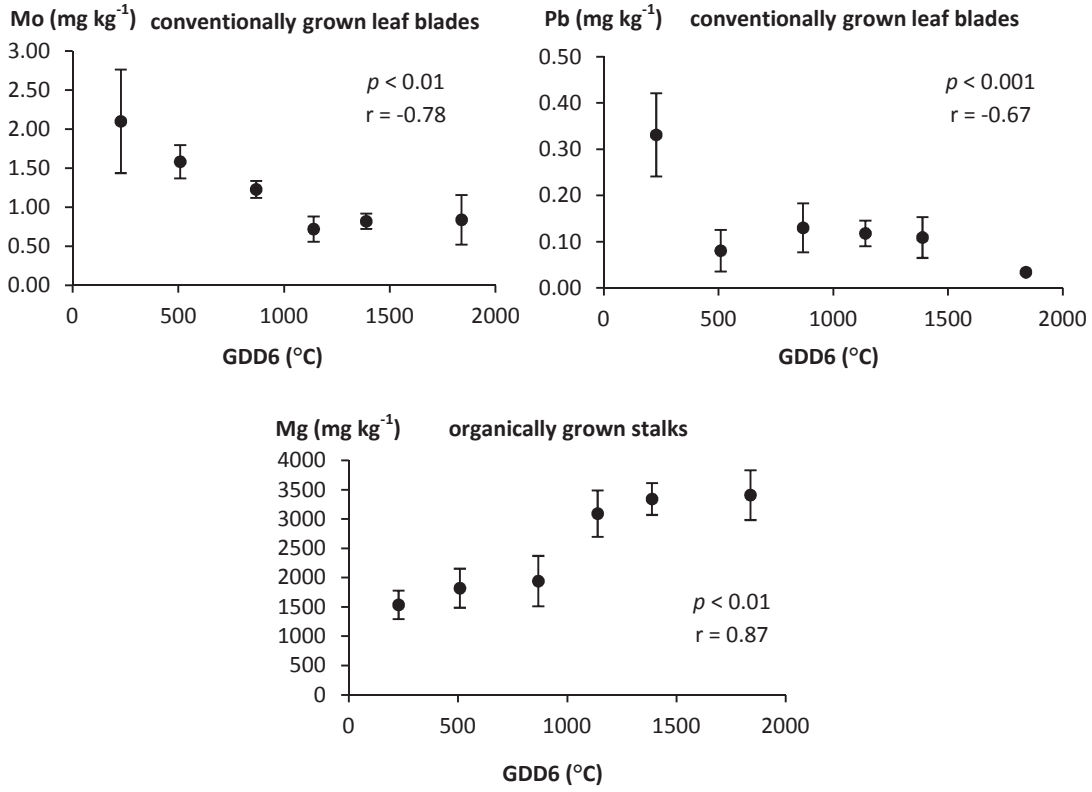

Figure 2.6. Variations in the concentrations (in mg kg^{-1}) of some elements in aboveground organs of Swiss chards against thermal time (expressed as GDD).

2.3.5. THE EFFECT OF THE CULTIVATION PRACTICE ON THE ELEMENTAL COMPOSITION OF SWISS CHARDS

The two types of practices (organic and conventional) were discriminated by the component 4, which accounted for only 5.5 % of the total variance (Figure 2.7). This illustrates the low effect of this variable in the elemental composition of the plants. In general the differences for the element concentrations between the two treatments were weak and not significant, except for Cd ($p < 0.001$), Ba ($p < 0.01$), Mn ($p < 0.01$), Mo ($p < 0.05$), Na ($p < 0.05$) and K ($p < 0.05$).

Compared to the organic plot, the concentration of Cd in the conventional one was significantly higher, which is likely to result from the contamination of the chemical P fertilizer by Cd.[16] Ba was also more concentrated in Swiss chards from the conventional plot.

By contrast, K and some micronutrients (Na, Mn and Mo) were less concentrated in conventionally grown plants than in organically grown ones, possibly because these elements were added in the manure. These results support previous studies carried out in other plants showing that organically grown crops contain less amount of Cd and higher amount of some other nutrients.[9,17]

During the whole experiment, no element exceeded the current European legislation about food consumption and human toxicity in conventionally grown chards.[18]

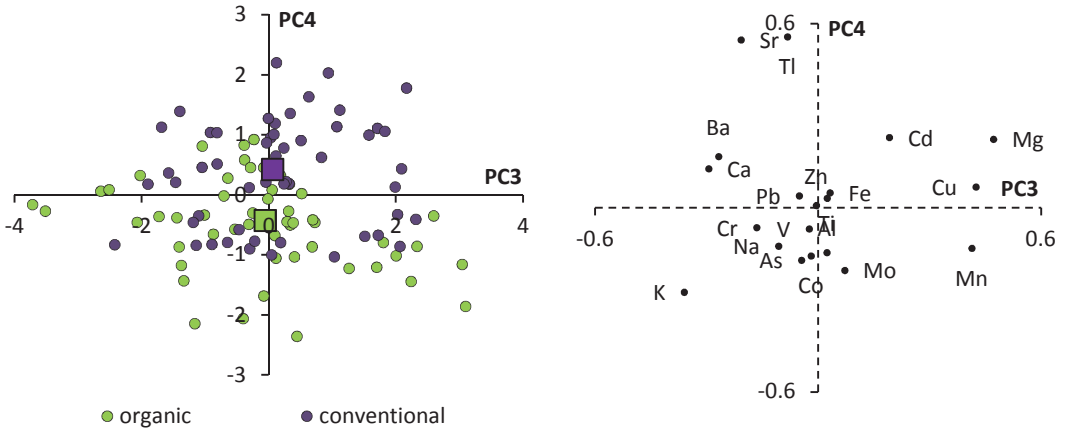

Figure 2.7. Scores (left, n = 108) and loading plots (right, n = 20) obtained after PCA of the whole data matrix for Swiss chards (76 % of explained variance with 4 PCs). Samples are grouped by farming technique. The barycentre of each formed cluster is marked by square dots.

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Chapter 3

RESULTS AND DISCUSSION:

ACCUMULATION AND TRANSLOCATION OF ESSENTIAL AND NON-ESSENTIAL ELEMENTS BY TOMATO PLANTS (*Solanum lycopersicum*) CULTIVATED IN OPEN-AIR PLOTS UNDER ORGANIC OR CONVENTIONAL FARMING TECHNIQUES

3.1. CHARACTERIZATION OF SOILS BEFORE PLANTATION

The characterization of soils before plantation was carried out following the procedure described in Section 1.4. The results obtained have already been summarized in Section 2.1. Briefly, for all the variables determined to characterize the soils, no significant differences were observed between the two plots (ANOVA, $p > 0.05$), growing plants in loam soils adequate for agricultural purposes.[1]

3.2. WATER CONTENT AND BIOMASS PRODUCTION DURING THE LIFE CYCLE OF PLANTS

The water content in tomato plants at each growth stage was calculated according to Section 1.3. The total dry mass produced at each sampling campaign was, therefore, obtained from the water content and from the total fresh weight measured for each plant organ.

Farming technique did not result in significant differences in the water content of plants ($p > 0.05$).

In general, considering all the plants from both the conventional and organic treatments, aerial parts of tomato plants presented higher water content than roots (81.9 ± 5.6 % for roots, 83.5 ± 2.4 % for leaves, 86.2 ± 3.6 % for flowers, 89.1 ± 2.9 % for stems, 92.8 ± 1.4 % for fruits).

A decrease in water content with time was observed for the roots ($p < 0.05$) and leaves ($p < 0.01$) (for roots, from June to October, 89.8 ± 1.0 %, 86.7 ± 3.1 %, 82.3 ± 3.3 %, 77.1 ± 2.9 %, 80.8 ± 1.1 % , and 75.0 ± 1.0 %; for leaves, from June to September, 84.82 ± 0.26 %, 86.2 ± 1.4 %, 84.0 ± 1.4 %, 80.8 ± 0.6 %, and 81.5 ± 2.2 %), which was attributed to (i) the difficulty to maintain an appropriate supply of water when the temperature rose and (ii) the increasing contribution of structural tissues with plant age.

The dry biomass measured for each plant part plotted against thermal time is presented in Figure 3.1. In general, an increase of biomass with time was observed for all the plant parts, with a p value below 0.05 for roots, $p < 0.01$ for fruits, and $p < 0.001$ for stems, leaves and flowers. For roots, the biomass reached a plateau, while for the aboveground parts, the biomass decreased after the 972 GDD sampling campaign due to the senescence of the plants.

In addition to the general increase in plant biomass, in general, significant differences were detected between the two treatments, organic and conventional. As it can be observed in Figure 3.1, conventionally treated plants produced significantly more aboveground biomass than organically grown ones ($p < 0.05$ for roots, $p < 0.001$ for stems, $p < 0.001$ for leaves, $p < 0.001$ for flowers, and $p < 0.01$ for fruits). The difference was more pronounced in the case of stems, leaves and flowers: in the conventional plot, dry biomass increased with time, while no significant change was observed in the organic one ($p < 0.001$).

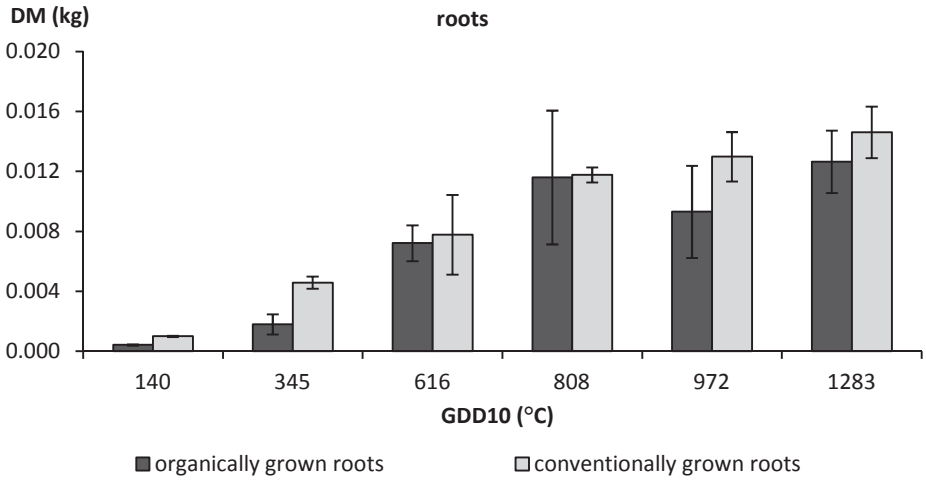

Figure 3.1. Biomass production (in dry mass, DM, kg) of roots, stems, leaves, flowers, and fruits in conventionally (gray) and organically (black) grown tomato plants. Each bar represents the mean dry matter (n = 3) obtained at each sampling campaign.

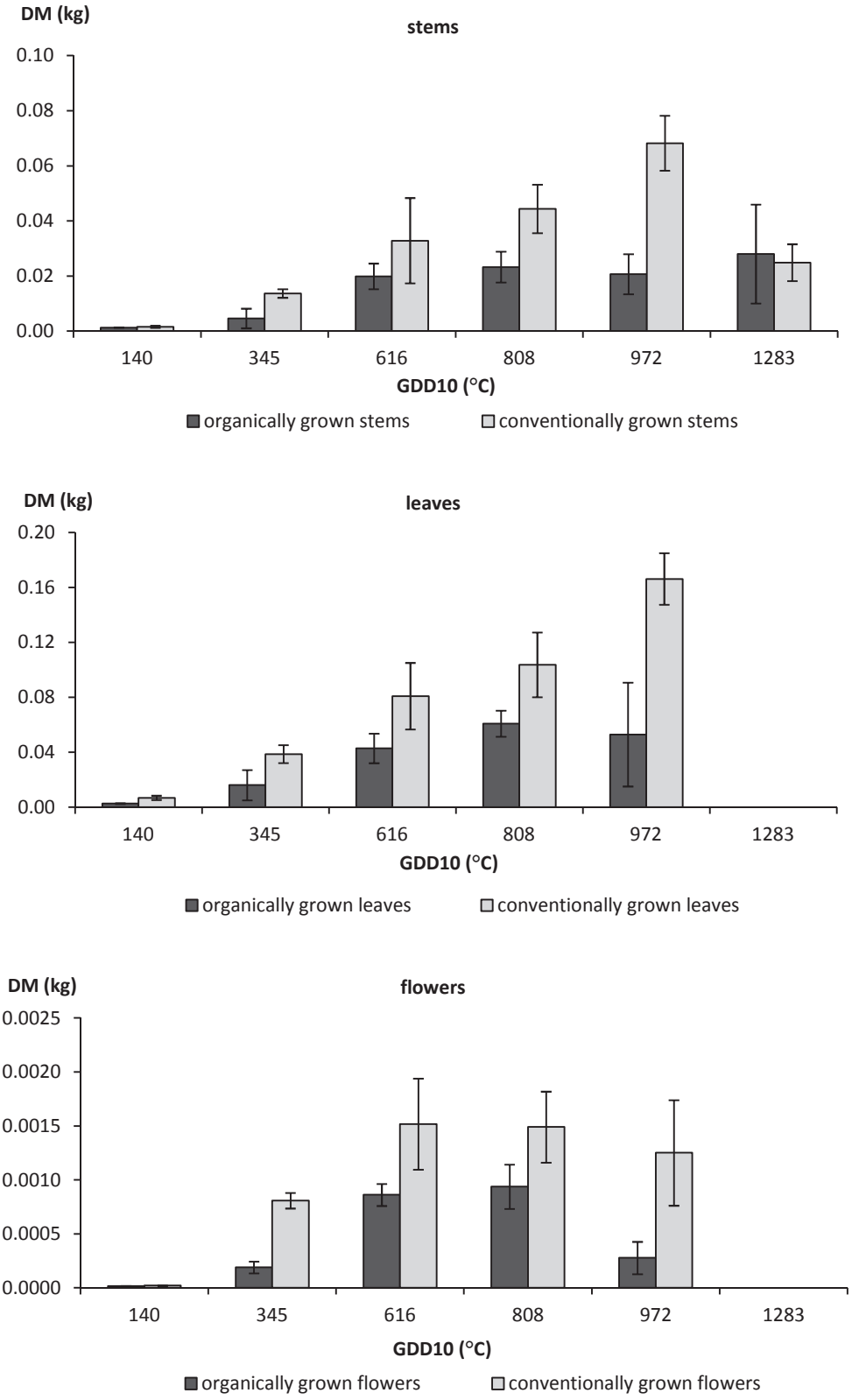

Figure 3.1. (*cont.*) Biomass production (in dry mass, DM, kg) of roots, stems, leaves, flowers, and fruits in conventionally (grey) and organically (black) grown tomato plants. Each bar represents the mean dry matter (n = 3) obtained at each sampling campaign.

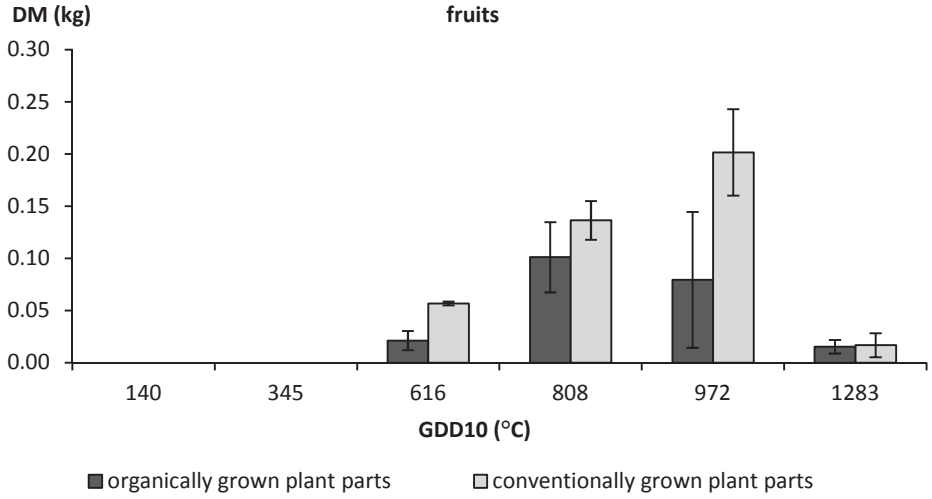

Figure 3.1. (*cont*.) Biomass production (in dry matter, DM, kg) of roots, stems, leaves, flowers, and fruits in conventionally (grey) and organically (black) grown tomato plants. Each bar represents the mean dry matter (n = 3) obtained at each sampling campaign.

3.3. ELEMENT CONCENTRATIONS IN PLANT AND SOIL SAMPLES

The concentrations (in mg kg^{-1} in dry weight) of the elements measured in the samples (27 elements in plant samples and 24 in soil samples) are given in Annex A.

The data sets for plants and soils were individually analyzed by Principal Component Analysis (PCA). The models finally selected for plants (PC1, 32.6 %; PC2, 16.2 %; PC3, 11.0 %; and PC4, 9.5 %) and soils (PC1, 35.1 %; PC2, 27.2 %; PC3, 11.5 %; and PC4, 10.0 %) explained, respectively, 69.3 % and 83.8 % of the total variance of the data matrix.

The factor which affected most significantly the concentration of elements in tomato plants was the plant part, followed by the sampling campaign and, finally, the farming technique (see Annex B). Except Sr (which concentration depended exclusively on the plant part), no element was completely dependent on a single factor.

The influence of each factor, plant organ, sampling campaign and fertilizer treatment, on the accumulation of the elements in tomato plants is individually and deeply discussed in Sections 3.3.2, 3.3.3 and 3.3.4 of this Chapter, respectively.

3.3.1. ELEMENT UPTAKE FROM SOIL BY TOMATO PLANTS

As a first approach, the capacity of tomato plants to absorb elements from soil was investigated by the mean of the BAFs, which are plotted in Figure 3.2. Each bar represents the mean of the BAFs for the three replicates, by sampling and by fertilization treatment.

For some elements it was not possible to calculate the BAF values because the concentration in soil was not measured (Na, K and Ca) or because the concentration in the plant parts was below the detection limit (Ni, Sn, Sb, Ag, Se, W and Hg).

As it can be observed in Figure 3.2, most of the elements were not noticeably absorbed from the soil and accumulated in tomato plants (BAF < 1). However, Mg, Cu, Zn, Mo and Cd presented BAFs higher than 1 in some cases. It must be noted that no toxic element was strongly accumulated in plants except Cd.

Cu was directly spread in leaves of organically grown tomato plants at the beginning of the experiment, which very probably explains the extremely high BAF values obtained for this element during the first sampling campaigns. For this reason, Cu will not be further considered for the discussion of the results as its origin is not the uptake from the soil.

Many studies have focused on the mobility of Cd from soil to crops because it has been shown that this metal can be noticeably absorbed from the soil and further translocated to the aerial parts of plants. Our results confirm this behaviour in the case of tomato plants.[2-4]

The highest concentrations of Cd were found in leaves (0.46 ± 0.21 mg kg^{-1} in dry weight), significantly higher than for the rest of the tomato plant organs (flowers 0.226 ± 0.097 mg kg^{-1}, stems 0.168 ± 0.056 mg kg^{-1}, roots 0.137 ± 0.036 mg kg^{-1} in dry weight) also including the fruits (0.062 ± 0.046 mg kg^{-1} in dry weight), which had the lowest Cd concentration, an important result from the viewpoint of safe consumption of tomatoes.

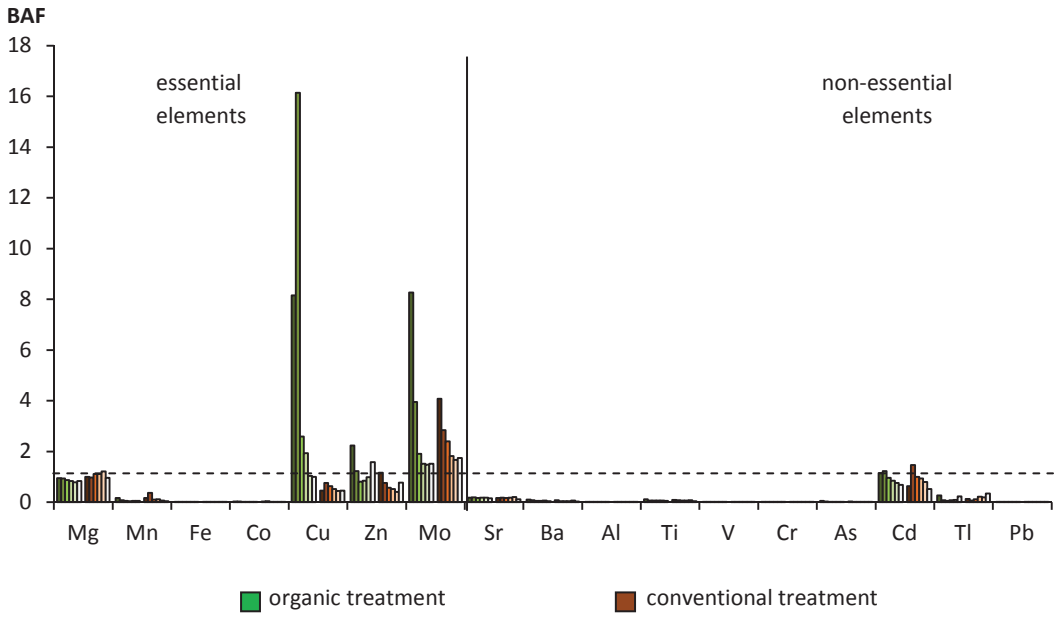

Figure 3.2. Bioaccumulation factors (BAF) obtained for each element along the whole life cycle of tomato plants. Each bar of the figure represents the mean BAF value (n = 3) obtained for each element at each plot and sampling campaign. The use of organic and synthetic fertilizer is illustrated in green and brown, respectively.

3.3.2. ELEMENTS PARTITIONING WITHIN TOMATO PLANTS

PCA of the plant dataset resulted in five clusters (one per plant part) well differentiated by components 1 and 2 of the model (accounting for 32.6 and 16.2 % of the total variance, respectively) (Figure 3.3). This model clearly discriminated between roots and aboveground organs. Fruit samples resulted in a well defined cluster, a fact already observed by other authors.[5]

ANOVA indicated the strong influence of the plant organ on the partitioning of elements in tomato plants, with *p* values below 0.001 for all the elements (see Annex B). It must be highlighted that the plant part was the only factor that strongly affected the concentration of Sr measured in plant tissues.

In general, most of the non-essential elements (Al, V, Cr, As, Tl and Pb), together with Na, Fe and Co, were more strongly accumulated in roots, limiting the translocation to the aboveground parts of the plant and, thus, also limiting the potential risk for consumers.

The barycenters of the clusters of the aboveground plant parts followed a line from negative to positive scores on both PC1 and PC2. Following the plant part cluster distribution on this line, leaves were the most enriched in Mg, Ca, Mn, Cu, Zn, Mo and Cd while fruits were characterized by low concentrations of the majority of the elements, except K for which they were the richest (Figure 3.3).

The ranking is consistent with the fact that leaves require both macronutrients (Ca, Mg) and micronutrients (Cu, Zn, Mn, Mo) for their high metabolic activity, while tomato fruits require high levels of K for turgor pressure and sink strength for photoassimilate.[6,7] Tomato fruits also presented high concentrations of Mg and Ca, which is in agreement with other studies carried out on tomato fruits.[5,8,9]

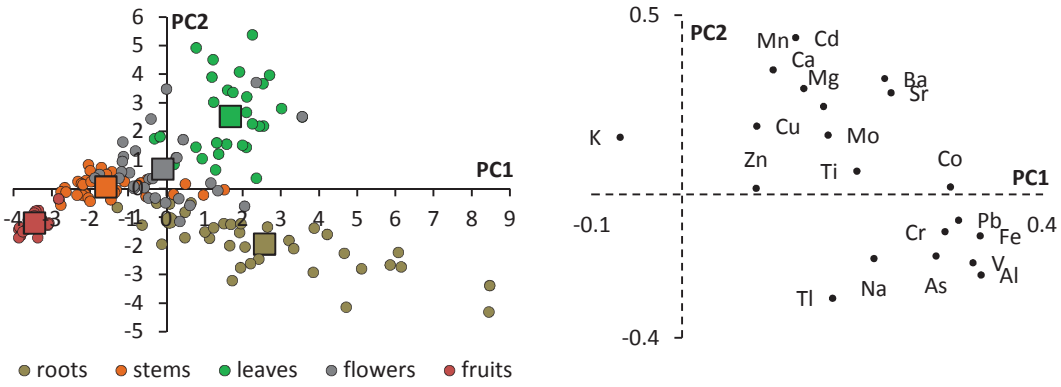

Figure 3.3. Scores (left, n = 156) and loading plots (right, n = 20) obtained after PCA of the whole plant dataset for tomato plants (69.3 % of explained variance; PC1, 32.6 %; PC2, 16.2 %; PC3, 11.0 %; and PC4, 9.5 %). Samples are grouped by plant part. The barycenter of each cluster is marked by a square.

The main function of stems, apart from its structural role, is to transport elements via xylem and phloem, behaving as a passageway for element partitioning, explaining why they did not show any specificity regarding their concentration in inorganic elements (Figure 3.3).

Among non-essential elements, only Sr, Ba, and Cd were noticeably translocated to leaves. The mobility of Sr and Ba can be related to their chemical similarities with Ca, while that of Cd was likely due to the lack of specificity of plant membrane channels and transporters involved in Zn, Cu, Ca, Fe, and Mn homeostasis.[10,11] This indicates an important Cd sequestration in leaves and stems as it was previously reported elsewhere.[12]

The possible existence of significant correlations between element concentrations in the whole plant data set (Table 3.1) was checked by the calculation of the corresponding correlation matrix (n = 156; 20 elements; r_{crit} = 0.15, an absolute value).

Consistent with the loadings plot (Figure 3.3), two groups of highly and positively correlated elements were identified. Na, Fe, Co, Al, V, Cr, As, Tl, and Pb on the one side, and, Ca, Mg, Mn, Sr, Ba and Cd, on the other side. Interestingly, the elements in the first cluster are those more strongly sequestrated in roots and, consequently, translocated to aboveground parts to a lesser extent. Very strong and positive correlations were found between Al and V (0.86), V and Fe (0.86), Al and Fe (0.81), and V and Cr (0.81). The toxicity of most of the elements of this cluster is high,[13] and their sequestration in roots may be one mechanism of detoxification to prevent diseases in plants.[11,14]

The elements in the second cluster are primarily accumulated in leaves. Earth alkaline elements with similar chemical characteristics are included in this group, which may explain a similar accumulation and translocation mechanisms of these metals inside the tomato plants. Strong correlations were also found among Sr, Ba and Ca (Sr and Ba, r = 0.74; Sr and Ca, r = 0.48; Ca and Ba, r = 0.48).

When treating each plant organ separately as individual data matrices, some of the correlations between pairs of elements improved considerably. In roots (n = 36; 20 elements; r_{crit} = 0.33, an absolute value) the strongest correlations were found between Al and V (0.92), Al and Cr (0.81), Al and Fe (0.89), V and Cr (0.86), and V and Fe (0.85). A similar situation was found in leaves (n = 30; 20 elements; r_{crit} = 0.36, an absolute value) for Ca and Sr, for example, with a Pearson correlation coefficient of 0.89.

The concentrations of some elements in fruits were also highly correlated (Table 3.2), as it has already been reported by other authors.[9,15] The highest Pearson correlation coefficient was obtained between Mg and K (0.93), two elements strongly accumulated in tomato fruits. Cd in fruits is also presented (although at very low concentrations) and positively correlated with that of essential elements, which is something to be taken into account in terms of food quality and risk assessment.

Table 3.1. Correlation matrix obtained for 20 elements detected in tomato plants (n = 156; r_{crit} = 0.15).

	Na	Mg	Al	K	Ca	Ti	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Mo	Cd	Ba	Tl	Pb
Na	1.00																			
Mg	0.40	1.00																		
Al	0.48	0.06	1.00																	
K	-0.29	-0.19	-0.17	1.00																
Ca	0.00	0.36	0.12	0.05	1.00															
Ti	0.04	0.16	0.40	0.08	0.33	1.00														
V	0.39	0.09	0.86	-0.27	0.01	0.32	1.00													
Cr	0.30	0.10	0.71	-0.16	0.03	0.30	0.81	1.00												
Mn	0.03	0.29	-0.05	0.14	0.20	0.05	-0.03	0.12	1.00											
Fe	0.36	0.17	0.81	-0.22	0.05	0.38	0.86	0.78	0.12	1.00										
Co	0.31	0.26	0.64	-0.05	0.23	0.31	0.59	0.51	0.47	0.67	1.00									
Cu	-0.16	-0.02	0.04	0.18	0.16	0.16	0.13	0.11	0.05	0.16	0.16	1.00								
Zn	0.08	0.14	0.24	0.07	0.59	0.37	-0.05	-0.14	-0.07	-0.05	0.17	-0.08	1.00							
As	0.54	0.11	0.71	-0.04	0.16	0.29	0.60	0.41	0.01	0.59	0.60	0.07	0.26	1.00						
Sr	0.28	0.63	0.29	-0.20	0.32	0.16	0.37	0.37	0.42	0.38	0.31	0.21	-0.01	0.11	1.00					
Mo	0.13	0.07	0.22	0.29	0.39	0.40	0.17	0.13	0.21	0.27	0.24	0.50	0.24	0.42	0.11	1.00				
Cd	0.08	0.63	-0.08	-0.01	0.41	0.07	0.03	0.11	0.71	0.13	0.33	0.17	-0.10	-0.08	0.70	0.13	1.00			
Ba	0.08	0.35	0.27	0.08	0.48	0.34	0.30	0.36	0.34	0.34	0.29	0.35	0.05	0.26	0.74	0.42	0.53	1.00		
Tl	0.80	0.19	0.50	-0.32	-0.06	0.01	0.36	0.19	-0.16	0.26	0.25	-0.17	0.28	0.47	0.13	-0.02	-0.16	-0.11	1.00	
Pb	0.34	0.19	0.70	-0.09	0.25	0.44	0.65	0.61	0.03	0.64	0.54	0.15	0.22	0.56	0.36	0.27	0.08	0.41	0.33	1

However, the concentrations of this toxic element in fruits were generally low (0.0041 ± 0.0029 mg kg^{-1}, in fresh weight), being below the maximum level set by the European Commission for fruit vegetables (0.05 mg kg^{-1}, in fresh weight).[16]

Table 3.2. Pearson correlation coefficients obtained for some elements in tomato fruits (n = 24), significant at 95 % confidence level (r_{crit} = 0.40). The best correlation, exiting between Mg and K, is marked in bold type.

	Na	Mg	K	Fe	Cu	Zn	Mo	Cd
Na	1.00							
Mg	0.73	1.00						
K	0.47	**0.93**	1.00					
Fe	0.68	0.66	0.52	1.00				
Cu	0.40	0.43	0.39	0.84	1.00			
Zn	0.64	0.81	0.71	0.81	0.61	1.00		
Mo	0.73	0.84	0.74	0.85	0.66	0.78	1.00	
Cd	0.81	0.83	0.70	0.85	0.71	0.84	0.85	1.00

3.3.3. THE TIME EFFECT ON THE ELEMENTAL COMPOSITION OF TOMATO PLANTS

The model obtained after PCA of the whole plant data set (Figure 3.4) showed a clear difference between the elemental composition of the plants collected in June sampling campaign and the rest when the scores on components 1 and 3 were plotted (accounting for 32.6 and 11.0 % of the total variance, respectively). This significant difference ($p < 0.001$) could be due to (i) the fertilization carried out before plantation, which likely increased the bioavailability of some elements, and/or (ii) higher root absorption at the early growth stages. The fourth and fifth samplings, which correspond to the fructification period, were indistinguishable, with very similar barycenters coordinates.

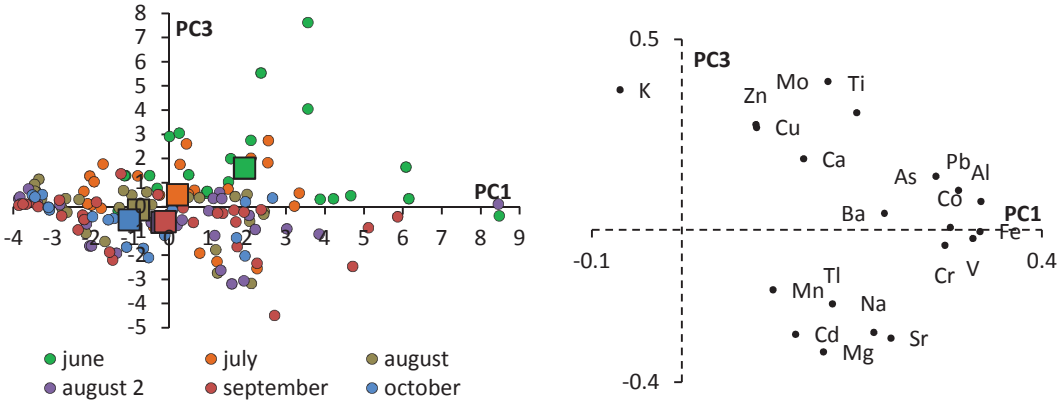

Figure 3.4. Scores (left, n = 156) and loading plots (right, n = 20) obtained after PCA of the whole plant dataset for tomato plants (69.3 % of explained variance; PC1, 32.6 %; PC2, 16.2 %; PC3, 11.0 %; and PC4, 9.5 %). Samples are grouped by sampling campaign. The barycenter of each cluster is marked by a square.

ANOVA of the whole plant data set showed that, for some elements, the concentration in the whole plant (mean of the concentrations found in different plant organs) significantly change between sampling campaigns, with *p* values below 0.001 for Na, K, Ca, Mn, Fe, Co, Cu, Zn, Mo, Ba, Al, As, V, Cr, Cd, Tl and Pb; below 0.01 for Ti; and below 0.05 for Sr (see Annex B).

Looking in detail at the results obtained for each plant organ, it was observed that while the concentration of Na and Sr increased significantly with time in the non-edible parts of conventionally grown plants (Na: $p < 0.05$ in roots, $p < 0.05$ in stems, $p > 0.05$ in leaves, $p < 0.01$ in flowers; Sr: $p < 0.01$ in roots, $p < 0.05$ in stems, $p < 0.01$ in leaves, $p < 0.001$ in flowers), the concentration of Mo decreased significantly in all the non-edible plant tissues. A lower element bioavailability from soil, a decrease in root absorption capacity due to aging, or a re-translocation or turnover processes from vegetative tissues might be responsible of this trend found for Mo.

Table 3.3. Pearson correlation coefficients obtained between element concentrations in plant parts and GDD. The strongest correlations are marked in bold type (significant at 95 % confidence level: for roots and stems, n = 18, $r_{crit} = 0.47$; for leaves and flowers, n = 15, $r_{crit} = 0.51$; for fruits, n = 12, $r_{crit} = 0.58$).

	Organically grown tomato parts					**Conventionally grown tomato parts**				
	Roots	**Stems**	**Leaves**	**Flowers**	**Fruits**	**Roots**	**Stems**	**Leaves**	**Flowers**	**Fruits**
Na	-0.59 [a]	-0.58 [a]	-0.34	0.72 [a]	0.14	0.52 [a]	**0.81**	**0.61**	**0.81**	0.46
Mg	-0.26	**0.61**	**0.52**	-0.11	-0.03	0.05	**0.81**	**0.84**	0.05	0.30
Al	0.11	NA	0.04	-0.26	NA	-0.13	NA	0.23	0.38	NA
K	-0.37	-0.65 [a]	**-0.90**	-0.47	-0.07	**-0.78**	0.58 [a]	**-0.87**	**-0.60**	0.03
Ca	0.09	-0.47 [a]	**0.81**	**-0.76**	0.25	0.22	0.02	**0.90**	-0.57 [a]	-0.52
Ti	-0.09	**-0.78**	0.09	-0.20	NA	-0.16	-0.14	**0.56**	-0.15	NA
V	0.15	NA	-0.24	NA	NA	-0.15	NA	0.26	NA	NA
Cr	0.22	NA	0.13	NA	NA	-0.19	NA	0.15	NA	NA
Mn	**-0.71**	-0.65 [a]	**-0.82**	**-0.81**	0.06	**-0.76**	-0.73 [a]	-0.50	-0.43	0.51
Fe	-0.24	-0.70 [a]	-0.35	**0.69**	-0.12	-0.42	-0.57 [a]	0.12	**0.76**	0.23
Co	**-0.77**	-0.38	-0.48	**-0.55**	NA	**-0.65**	-0.22	-0.34	**-0.60**	NA
Cu	**0.80**	**-0.73**	**-0.78**	-0.41	-0.17	**0.56**	-0.26	**-0.59**	-0.42	-0.11
Zn	**0.74**	**0.70**	0.05	**-0.80**	0.10	**0.49**	-0.19	**-0.72**	**-0.88**	0.39
As	-0.69 [a]	NA	**-0.58**	-0.63 [a]	NA	-0.59 [a]	NA	-0.06	-0.60 [a]	NA
Sr	**0.71**	0.40	0.49	**0.58**	0.33	**0.55**	**0.63**	**0.72**	**0.84**	-0.26
Mo	**-0.76**	**-0.81**	**-0.78**	**-0.73**	-0.11	**-0.54**	**-0.62**	**-0.82**	**-0.79**	0.37
Cd	-0.36	0.01	0.14	-0.49	0.11	-0.23	0.15	0.44	-0.17	0.18
Ba	**-0.69**	**-0.56**	0.27	**-0.60**	0.05	**-0.75**	-0.33	0.46	-0.22	-0.03
Tl	-0.24	NA	NA	NA	NA	0.71 [a]	NA	NA	NA	NA
Pb	-0.16	-0.41	-0.08	-0.41	NA	-0.45	-0.39	**0.72**	**0.59**	NA

NA: concentration below the detection limit (see Table 1.2).

[a] false positives detected after analysis of the results.

For the rest of elements in the non-edible tissues there were no clear trends in their concentrations between sampling campaigns.

Furthermore, the possible existence of correlations between elements concentrations in each plant part taking into account the thermal time (expressed as GDD) was also investigated (Table 3.3). The most important correlations were obtained for non-edible parts.

Interestingly, some elements behaved in the same way within plant tissues in both plots. K, Mn, Co, Mo and Ba were negatively correlated with thermal time in the root system, while Zn and Sr presented the opposite behaviour. The strong correlations obtained for Sr and Mo in both plots for most of the plant organs should be highlighted: Sr was positively and significantly correlated with time, while Mo concentrations decreased in all non-edible parts, with the strongest Pearson correlation coefficient obtained in conventionally grown leaves (r = -0.82, $p < 0.01$).

As an example, the variation in the concentrations of some elements with GDD is plotted in Figure 3.5. The first two graphs show, respectively, the increase in Na concentration in leaves and the decrease in Mo concentration in stems with thermal time, both in conventionally grown plants. The other two graphs correspond to the variations in Cd and K concentrations in tomato fruits with thermal time.

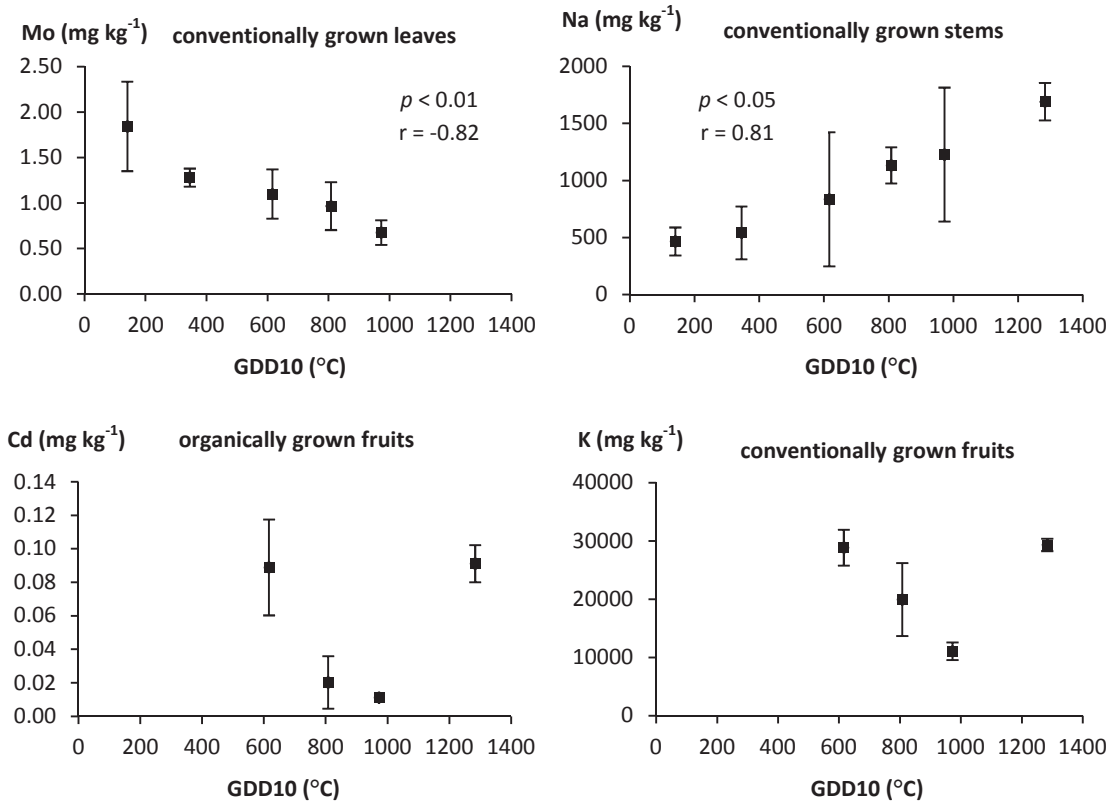

Figure 3.5. Concentrations (in mg kg^{-1}) of Mo, Na, Cd and K in different plant parts against thermal time (expressed as GDD).

As can be observed, in both cases the concentration found in fruits collected during the last campaign was unexpectedly high, breaking the decreasing trend observed within the previous samplings. This was due to the fact that at the last sampling campaign, most of the plants to be harvested had already lost most of their mature fruits due to their very advanced ripening stage. Therefore, most of the remaining fruits (those which were finally collected and analyzed) were at the early stage of their ripening, little and medium-sized green and green-red tomatoes.

The results observed suggest that the concentrations of Cd, K, and the rest of elements that presented a similar trend (Na, Mg, Fe, Cu, Zn and Mo) decrease with the ripening stage of the fruit, which is in good agreement with the results reported by other authors.[15] This may indicate that the accumulation of those elements takes place in the early stages of the fruit formation, while other organic nutrients and basically water are incorporated all along of the ripening. Other studies, however, concluded that most of the elements increase their concentrations in ripe tomatoes, although it depends on the tomato variety studied.[17]

These apparent discrepancies show that the concentration of elements in mature tomato fruits can fluctuate a lot, likely depending on the bioavailability of the elements in soil, the variety used and the timing of harvest during the ripening.

3.3.4. THE EFFECT OF THE CULTIVATION PRACTICE ON THE ELEMENTAL COMPOSITION OF TOMATO PLANTS

The farming techniques were weakly differentiated by the components 2 and 3 of the model, accounting for 16.2 and 11.0 % of the total variance, respectively.

In general, samples from the conventional treatment showed negative scores on PC2 and PC3 and those from the organic one presented positive scores (Figure 3.6). The concentrations of Mn, Co, Na, Mg, Cd and Tl ($p < 0.001$) were significantly higher in conventionally grown plants, whereas higher concentrations of Mo, Zn, K and Ba ($p < 0.001$) were measured in organically grown ones. As Cu was spread in tomato leaves, it was not taken into account to study the effect of the practice in our experiment, as indicated before.

In good agreement with our results, it has been reported that higher concentrations of Zn and lower concentrations of Mn in plant organs are characteristics of organic agricultural practices, probably due to the presence of arbuscular mycorrhizal fungi (AMF) in organically fertilized soils.[18-20] Other authors have reported that the concentration of Mn in soil notoriously increases after the application of organic manure, thus increasing the bioavailability for the plant.[21,22]

On the other hand, high concentrations of Cd in conventionally grown tomato plants have already been observed and can be related by the application of chemical P fertilizers, which can be contaminated with Cd.[23]

If we focus on the edible part of the tomato plant, there were no significant differences ($p > 0.05$) in the elemental composition of organically and conventionally grown fruits. At this point, contradictory results have been reported in the bibliography. De Souza Araújo et al. obtained similar conclusions in a recent experiment,[5] while Kelly and Bateman observed significant differences between organically and conventionally treated tomato fruits.[19] The discrepancies might be explained by the use of different tomato varieties, types of fertilizer, or other cultivation and climatic factors, the most important being likely the soil characteristics, that governs the bioavailability of the elements.

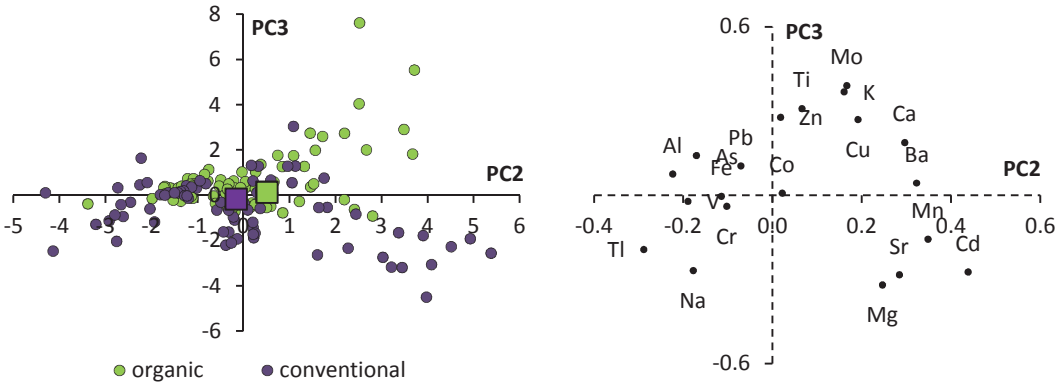

Figure 3.6. Scores (left, n = 156) and loading plots (right, n = 20) obtained after PCA of the whole plant dataset for tomato plants (69.3 % of explained variance; PC1, 32.6 %; PC2, 16.2 %; PC3, 11.0 %; and PC4, 9.5 %). Samples are grouped by farming technique. The barycenter of each cluster is marked by a square.

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Chapter 4

USE OF CHROMATICITY VALUES OBTAINED FROM CALIBRATED PICTURES TO DISCERN AMONG TOMATOES WITH DIFFERENT RIPENING STAGE

4.1. INTRODUCTION

Studies on the nutritional composition of fruits and vegetables have considerably increased during the last years in the fields of food science and safety, as the consumption of these products has been associated with the preservation of a good health and with the prevention of diseases.[1]

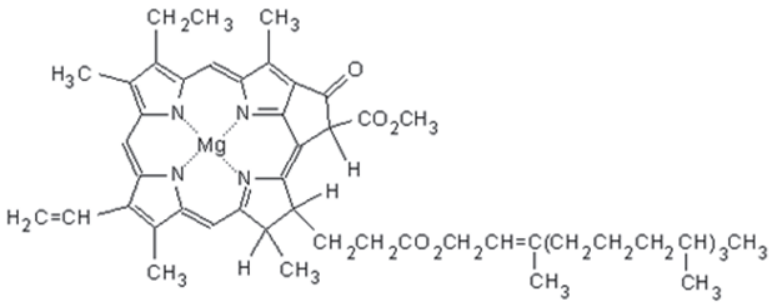
Particularly, studies carried out on tomato (*Solanum lycopersicum*) are relatively abundant as it is one of the most extended worldwide agricultural crops.[2] Tomato is the second most consumed culinary vegetable after potato (*Solanum tuberosum*), and it is commonly an integral part of the human diet.[3,4] According to the last statistics from the Food and Agriculture Organization (FAO) and the United Nations, more than 161 million tons of tomatoes are produced per year over the world.[5] Most of this volume is used in food industry to manufacture products such as tomato sauce, tomato juice and ketchup, although a significant volume is still purchased as whole tomato fruits by consumers.[6]

The nutrient content and chemical composition of tomato fruits mainly depend on the cultivar, on the postharvest conditions and on the ripening stage of the fruit.[2,7] In general, the tomato fruit is composed of water, soluble solids, insoluble solids, organic acids and macronutrients, above all.

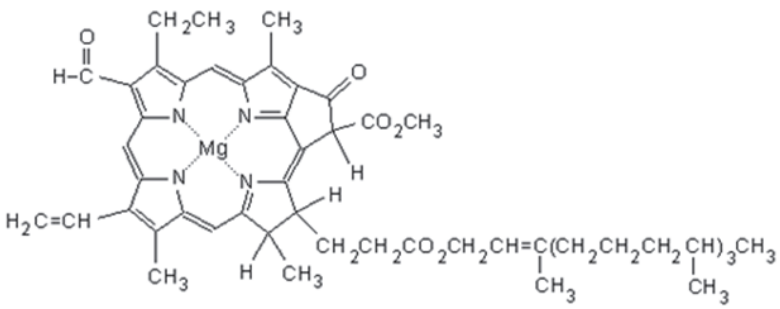
Soluble solids are mainly sugars, including equal amounts of glucose and fructose with a small amount of sucrose. Insoluble solids are mostly constituted of fibers, like hemicelluloses, celluloses and pectins. Organic acids are mainly citric and malic acids. Finally, the most important macronutrients found in tomato fruits are K, Mg, Ca and P, together with other compounds such as vitamins and carotenoids. Thus, tomatoes are recognized as health simulating fruits due to their low content in fat, calories and cholesterol, whereas they have high antioxidant properties thanks to the presence of some compounds such as carotenoids and phenolic compounds.[2,8,9]

The ripeness of tomato fruits involves various physical and chemical changes including changes in colour, texture, flavour and aroma.[10] During ripeness, the degradation of chlorophyll and the concomitant synthesis and storage of carotenoids, particularly, lycopene, results in a colour change from green to red.[11] The colour of the tomato fruit affects not only the buying decision of the consumer, but also the quality of the final product regarding the nutritional value. If the colour is unappealing, consumers are often unlikely to purchase the product.

Chlorophylls are a family of pigmented compounds widely found in vegetables and plants. They are green photosynthetic pigments which help plants convert energy from light into organic carbon. In tomato fruits, two main chlorophylls are found (chlorophyll a and chlorophyll b), mainly in the early stages of ripeness (Figure 4.1).[12]



chlorophyll a



chlorophyll b

Figure 4.1. Molecular structure of chlorophylls found in tomato fruits: chlorophyll a and b.

Carotenoids constitute other family of pigmented compounds widely distributed in biological systems, being recognized as potentially beneficial for human health due to the negative correlation existing between the consumption of products with a high content in carotenoids and risk of some types of cancer.[6,7,13,14] Three carotenoids are mainly found in tomato fruits (Figure 4.2): lycopene, which dominates, is a strong antioxidant and responsible for the red color of the fruit; β-carotene, which shows provitamin A activity; and lutein, which has a protective effect on the eye from photooxidation.[15]

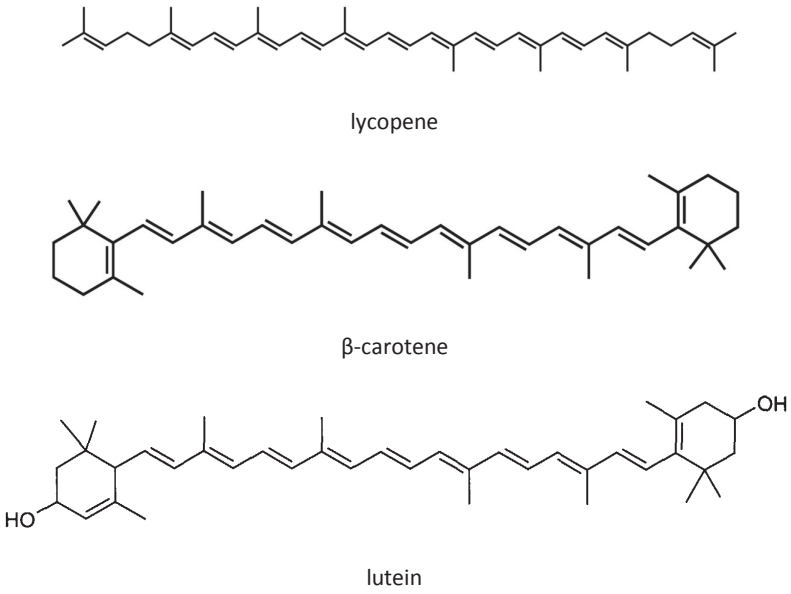


Figure 4.2. Molecular structure of carotenoids found in tomato fruits: lycopene, β-carotene and lutein.

The harvesting and the storage of the fruit is also crucial due to the fact that the metabolism of tomato fruits continue when the fruit has reached a optimum ripening-red stage and finally deteriorates to a point where it becomes valueless, affecting the final nutrient composition of the fruit.[16] Harvest at an appropriate ripening stage is crucial to ensure an optimal tomato quality.[17] In fact, in the case that tomato harvest is successful, in order to extent its nutritional value, tomatoes have to be maintained in darkness at low temperature.[1]

As previously mentioned, along the ripening process, the concentration of pigments in tomato fruits varies and a concomitant and gradual change in colour from green to red occurs. Consequently, the ripening stage of a tomato fruit can be estimated by colour analysis, as an alternative to the chemical analysis. Indeed, the analysis of pigments often entails destructive analytical techniques. The quantification of carotenoids, specifically lycopene, is generally performed after their extraction in organic solvents followed by liquid chromatography (LC).[18,19] However, this analytical method is time-consuming, relatively expensive and requires complicate procedures for the sample preparation and the further quantification of the pigments.

Spectrophotometric techniques have also been used as cheaper and faster alternatives to estimate the concentration of pigments in tomato fruits.[7,20-24] However, an extraction step is also required prior to analysis and, as the absorbance of the whole solution is measured, spectral interferences must be resolved by an appropriate multivariate calibration technique.

Other spectroscopic techniques (e.g. IR and Raman) are also good alternatives for the determination of carotenoids and chlorophylls in vegetables. The measurement can be made directly on the surface of the sample, so that sample preparation is minimum. They are easy-to-use, non-destructive, cheaper than chromatography, and allow measurements in situ. When combined with multivariate calibration methods, infrared spectroscopy (IR),[25] Fourier transform infrared spectroscopy (FTIR),[26] fiber optic visible reflectance spectroscopy[27] or Raman spectroscopy[28] provide very reliable results. Their use, however, is still limited and not generalized.

The estimation of the ripening stage of fruits through the measurement of colour is an innovative method that has been widely used during the last decades. The colour of a tomato fruit can be measured either by colorimetry[29,30] or digital image analysis after appropriate calibration.[31,32] This last option is fast, cheap, safe and does not require any specific sample preparation.[33-35]

However, human identification of colours is quite complex and sensations like brightness, intensity, lightness and others could modify the perception of the primary colours (red, blue, green) and their combinations (cyan, yellow, purple, etc). In fact, colour definition results from an interaction between light (the region of the electromagnetic spectrum visible for the human eye, from 400 to 700 nm), object (which can absorb, reflect, transmit or emit the light) and the viewer (the human).[36]

In 1931 the International Commission on Illumination (CIE, Commission Internationale de l´Eclairage) introduced the CIE colour system, a mathematical system to be able to express the colour in exact quantitative and numerical terms.

In 1976, an improvement of this system was developed (CIELab colour space, Figure 4.3) where the colour is defined close to human perception and all possible colours were included in the colour sphere, defined by three orthogonal components (XYZ), which correspond to the chromaticity values *L*, *a*, and *b*.[37]

L value is the measurement of the lightness or the amount of light reflected, *a* value indicates the intensity of the red (positive values) or green (negative values) coloration, and *b* value shows the intensity of the yellow (positive values) or blue (negative values) coloration. Two more chromaticity values that result from the combination of the previously mentioned *a* and *b* parameters can be used to measure colour: the chroma (*C*, Equation 1), related to the intensity or saturation of the colour, and hue angle (*H*, Equation 2), related to the hue, the name of the colour (red, yellow, green, etc.).[30,38]

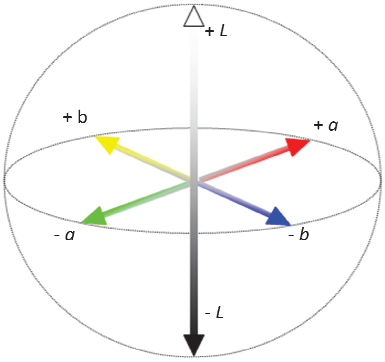


Figure 4.3. The three-dimensional CIELab colour space system.

When using image analysis, the picture of an object previously taken by a standard camera should be properly calibrated and corrected to be able to obtain the *L*, *a* and *b* (or *L*, *C* and *H*) values of the object for each pixel of the image. This is done by using a ColorChecker card with known values of the colour components (process explained in Section 4.2.4).

$$C = \sqrt{(a^2 + b^2)} \qquad (1)$$

$$H = arctan(b/a) \qquad (2)$$

The aim of this work was to propose a methodology to discern among tomatoes with different ripening stage using chromaticity parameters obtained after image analysis of the pictures taken with a standard digital camera. Furthermore, based on that methodology, we also aim to produce a simple and intuitive relative scale from zero to ten to sort tomato fruits according to their ripening stage, avoiding cumbersome and expensive chromatographic or spectroscopic analysis.

4.2. MATERIALS AND METHODS

4.2.1. SAMPLING

The tomatoes (*Solanum lycopersicum,* variety jack) used in this study were obtained from a greenhouse culture in Urduliz (43.370618 N; 2.950849 W), a small village in Biscay, Basque Country (Figure 4.4).

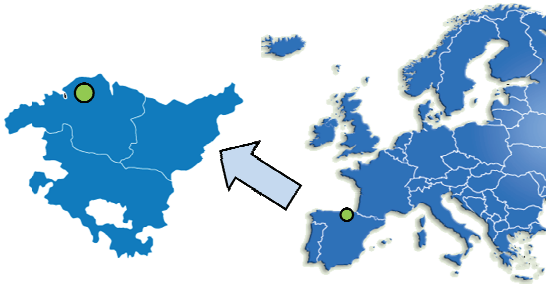


Figure 4.4. Location of Urduliz in Biscay, Basque Country.

Tomato plants were grown hydroponically and under controlled conditions of light and temperature. A single sampling campaign was carried out in May 2014, by selecting 23 tomato fruits from different plants and taking into account a broad range of ripening stages (from the greenest to the reddest).

4.2.2. SAMPLE PRETREATMENT

All glassware material used was firstly thoroughly cleaned with tap water and soap, rinsed twice with Milli-Q water (18.2 MΩ cm, Milli-Q Element A10 purification system, Millipore, USA) and dried at room temperature. Afterwards, it was soaked in an acetone bath for at least 24 h, rinsed with clean acetone and dried again at room temperature.

Immediately after harvesting, the tomatoes were transported to the laboratory at 4 °C and protected against light. Once in the lab, the tomatoes were cleaned with tap water, in order to remove traces of dirt, successively rinsed with Ellix-quality water (Millipore, USA) and Milli-Q water and carefully dried with cellulose paper.

Pictures of each whole tomato fruit were immediately taken according to the procedure described in Section 4.2.4, before going on with the next steps. Afterwards, each whole fruit was individually cut into small pieces and ground to a homogenized fresh puree with a regular electric grinder (BV 6500, Orbegozo, Spain). Pictures were also taken for a portion of the puree following the procedure described in Section 4.2.4. The rest of the puree was kept in zip bags and stored in the freezer at -80 °C until lyophilisation of the sample. From sampling to this point, the process was performed within the same working day. The lyophilisation process (Telstar Cryodos, Spain) was carried out at -52 °C during 72 h. Freeze-dried samples were then ground to a fine powder using a Planetary ball mill Pulverisette 6 (Fritsch, Germany), until obtaining a homogenized powder of each tomato fruit. Pictures of a portion of each powder were also taken as explained in Section 4.2.4. The rest of the powder was stored at 4 °C in darkness until chemical analysis, which was carried out in the next 24 h.

4.2.3. CHEMICAL ANALYSIS

The chemical analysis of the dry powder samples of tomatoes was done according to the procedure described by Fish et al.,[39] slightly modified.

Triplicate analysis of each dry powder sample was carried out as follows: between 0.4 and 0.6 g (± 0.0001 g, Mettler AJ150 balance) of samples were weighed in 40 mL amber screw-top vials. Then, 5 mL of 0.05 % (w/v) butylated hydroxytoluene (BHT, ≥ 99 % GC, Aldrich, USA) in acetone (99.8 % HPLC, Poch, Poland), 5 mL of ethanol (96 % PA, Panreac, Spain), and 10 mL of n-hexane (95 % HPLC, Poch, Poland) were individually added to each vial. Samples were laid in a rectangular container filled with ice and, then, they were stirred on an orbital shaker (Ovan, Spain) at 700 rpm for 15 min.

After shaking, 3 mL of Milli-Q water were added to each vial and the samples were again shaken for 5 additional min in the same experimental conditions as before. Afterwards, vials were left at room temperature from 5 to 10 min to allow phase separation. Once the liquid-liquid extraction was finished, the upper hexane layer was carefully separated and stored in a clean amber vial in the freezer at 4 °C until analysis. Procedural blanks were also carried out in the same way.

The absorbance in the visible spectrum (from 400 to 700 nm, every 1 nm) of each extract was immediately measured at room temperature in a UV-VIS spectrophotometer (MultiSpec-1501, Shimadzu, Japan), using a 1 cm path length quartz cell and n-hexane as a blank sample. Samples were diluted when the recorded absorbance was over 2 in any of the wavelengths considered. Each spectrum was baseline corrected for the possible drift, first, using The Unscrambler v. 9.7 software (Camo software AS, Norway) to achieve a horizontal spectra using the option of linear baseline correction and then, using The Excel software (Microsoft Corporation, USA) to correct the offset by subtracting the mean absorbance value registered from 700 to 800 nm to the absorbance measured at each wavelength.

The resulting spectra were blank corrected and finally was normalized (the absorbance measured at each wavelength was divided by the weight of dry powder used in the extraction process). The dilution factor was also taken into account when necessary. Each sample was analyzed in triplicate and the mean absorbance values of the three spectra were further considered as characteristic of each tomato sample.

4.2.4. ANALYSIS OF TOMATO PICTURES

Pictures were taken for the whole tomato fruit and the corresponding fresh puree and powder (summary shown in Figure 4.5), following the process explained below.

All the pictures were taken using a standard digital camera (Canon EOS 1000D). Each picture includes the tomato and a X-Rite ColorChecker Passport card (X-Rite Inc., USA) placed on the background for the colour calibration, as shown in Figure 6.6. It was checked that the camera image plane was levelled and parallel to the plane of the sample and of the ColorChecker card.

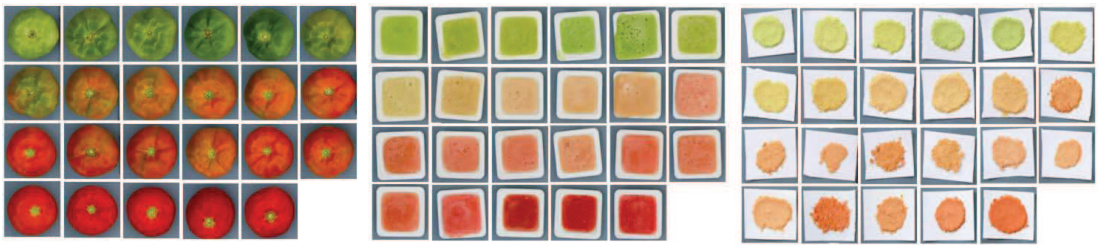


Figure 4.5. Summary of the pictures obtained for the whole tomato fruits (pictures from the top), and for the corresponding fresh purees and dry powders, respectively, sorted from the greenest (tomato 1) to the reddest (tomato 23).

A neutral grey paper that completely filled the viewfinder was selected as background for all the pictures. Once the samples were placed, a photo was taken by taking care that the exposure was correct. The camera was set in the raw mode to have uncompressed picture files. Six pictures were taken for each individual whole tomato fruit (one for the top, another one for the bottom and four additional for the sides). The fresh puree samples were places into white plastic holders to a thickness of about 1 cm and the powder was spread on a piece of white paper as shown in Figure 4.6.

All pictures were calibrated before further use. Calibration is necessary for any standard camera to guarantee the correct and accurate rendering of the colours. Cumbersome experimental procedures and expensive dedicated hardware for picture collection described in others works[35] can be avoided if pictures taken with a standard digital camera are properly calibrated.

Figure 4.6. Procedure followed for obtaining each picture of whole fruit, fresh puree and dry powder. Pictures not calibrated.

Therefore, a camera profile that characterizes the camera sensor[40] was obtained using the program Colour-Checker Passport v. 1.0.2 (X-Rite Inc., USA) and the photo information was modified with this profile in Photoshop v. 12.1 (Adobe Photoshop CS5 Extended, Adobe Systems Inc., USA). Photographs were calibrated with a JavaScript plug-in of Photoshop ACR Calibrator CC24 and checked with a JavaScript plug-in "Read_Colors_CC24".[41] In the calibration process, the chromaticity values of the 24 patches of the X-Rite ColorChecker Passport card previously known were compared with those of the patches shown in the picture taken of the sample and of the ColorChecker card. In this way, the script opens the picture multiple times modifying the Photoshop colour sliders and selecting the settings that best fit all of them.

A visual layer of the ColorChecker card colours was created to check that the calibration had been successful as it showed the theoretical and measured colours together. Once the calibration finished, the raw calibrated picture was saved in JPG format to be used in the following steps.

Each picture in JPG format was opened in Photoshop program to estimate the representative chromaticity values (L, a and b, which could be taken for each point of the picture) of each sample.

After discarding the background and those parts of the picture affected by shadows and reflections, 15 points (3 x 3 pixels each point) were randomly selected on each sample picture and their *L*, *a* and *b* values were measured. The average of each chromaticity parameter was considered to be representative of the colour of each sample in the picture. The corresponding *C* and *H* parameters were also calculated according to Equations 1 and 2.

4.2.5. CONSTRUCTION OF A REGRESSION MODEL TO PREDICT THE ABSORBANCE OF A TOMATO EXTRACT FROM THE CHROMATICITY VALUES

Mathematical models to predict the absorbance of a tomato sample at a given wavelength (A_λ) from the chromaticity values obtained from the pictures were constructed by regression analysis.

The wavelength for which the absorbance was to be predicted were 445, 472, 503 and 665 nm, which correspond to the maximum of absorption for the chlorophylls and for the carotenoids (Section 4.3.2). Models were built for the chromaticity values (*L*, *a, b)* and (*L*, *C, H)* of the whole tomato, of the puree and of the powder.

The dataset corresponding to the 23 tomato samples has been split in two subsets: i) the calibration set (tomatoes 1, 2, 4 to 11, 13 to 20, 22 and 23; Figure 4.5), used to construct the model and ii) the validation set (tomatoes 3, 12 and 21; Figure 4.5), to test the model.

In the calibration step, one set of 20 (A_λ, *L*, *a, b)* or (A_λ, *L*, *C, H)* data were used to construct the mathematical model shown in Equation 3, where Y is the predicted absorbance at a given wavelength (A_λ), X_i are the chromaticity parameters considered in each model (*L*, *a* and *b*, or *L*, *C* and *H*), and k_i are the parameters to be adjusted by regression analysis.

$$Y = k_0 + k_1 \cdot X_1 + k_2 \cdot X_2 + k_3 \cdot X_3 + k_{12} \cdot X_1 \cdot X_2 + k_{13} \cdot X_1 \cdot X_3 + k_{23} \cdot X_2 \cdot X_3 + k_{123} \cdot X_1 \cdot X_2 \cdot X_3 + k_{11} \cdot X_1^2 + k_{22} \cdot X_2^2 + k_{33} \cdot X_3^2 \quad (3)$$

The model was adjusted to the data by a backward stepwise regression. Hence, a first set of k_i parameters were obtained. Then, the term of the model for which the parameter k_i was not significantly different from zero ($p > 0.05$), was removed from the model, and this was again adjusted. This process was repeated until all the adjustable parameters were significantly different from zero ($p < 0.05$).

Regressions were carried out by the NLREG software (v. 6.3, by Phillip H. Sherrod). The final model was test by the external validation method using the validation set (tomatoes 3, 12 and 21). In this step, the value of absorbance predicted by the model for each sample was compared with the experimental value of absorbance.

The criteria used to assess the quality of the models were as follows: i) the percentage of variance explained in the calibration step (the highest, the best), ii) the root mean-squared error in the calibration (RMSEC) and in the validation (RMSEV) steps (Equation 4, where y_i is the observed absorbance, $\hat{y}_i$ is the predicted absorbance, and N is the number of samples; the lowest, the best), iii) the slope and the determination coefficient of the y_i *vs.* $\hat{y}_i$ plot (the closest to one, the best), and iv) the values and distribution of the residuals (the lowest and the most homogeneously distributed, the best).

$$RMSEC\ (or\ RMSEV) = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{N}} \qquad (4)$$

4.3. RESULTS AND DISCUSSION

4.3.1. PRELIMINARY EXPERIMENTS

4.3.1.1. REPEATABILITY OF THE CHEMICAL ANALYSIS

Figure 4.7 shows from the literature, the typical absorption spectra of the most important photosynthetic pigments present in tomato fruits. As it can be observed, the maximum values of absorbance in the visible spectra of the pigments responsible for the colour change during the tomato ripening process are between 400 - 550 nm (where chlorophylls and carotenoids largely overlap) and between 650 - 700 nm (chlorophylls) spectral ranges.

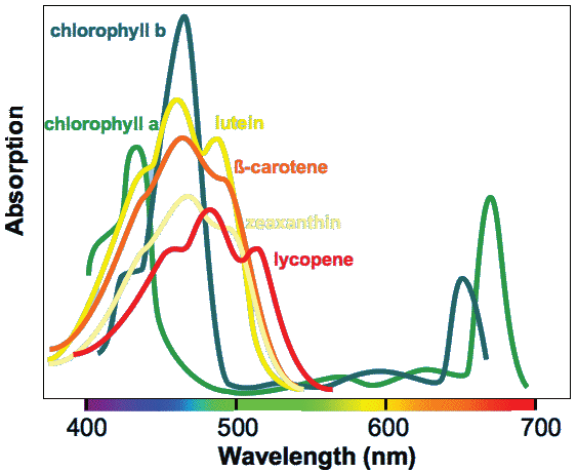


Figure 4.7. VIS absorption spectra of photosynthetic pigments present in tomato fruits. Adapted from Lichtenhaler et al.[42]

The spectra obtained after the extraction (Section 4.2.3) of three categories of tomatoes differing for the colour (green orange and red) are shown as examples in Figure 4.8. Triplicate analysis of each sample was carried out in order to estimate the repeatability of the whole procedure. The obtained repeatability (expressed as relative standard deviation, RSD) was below 10 % in all the cases.

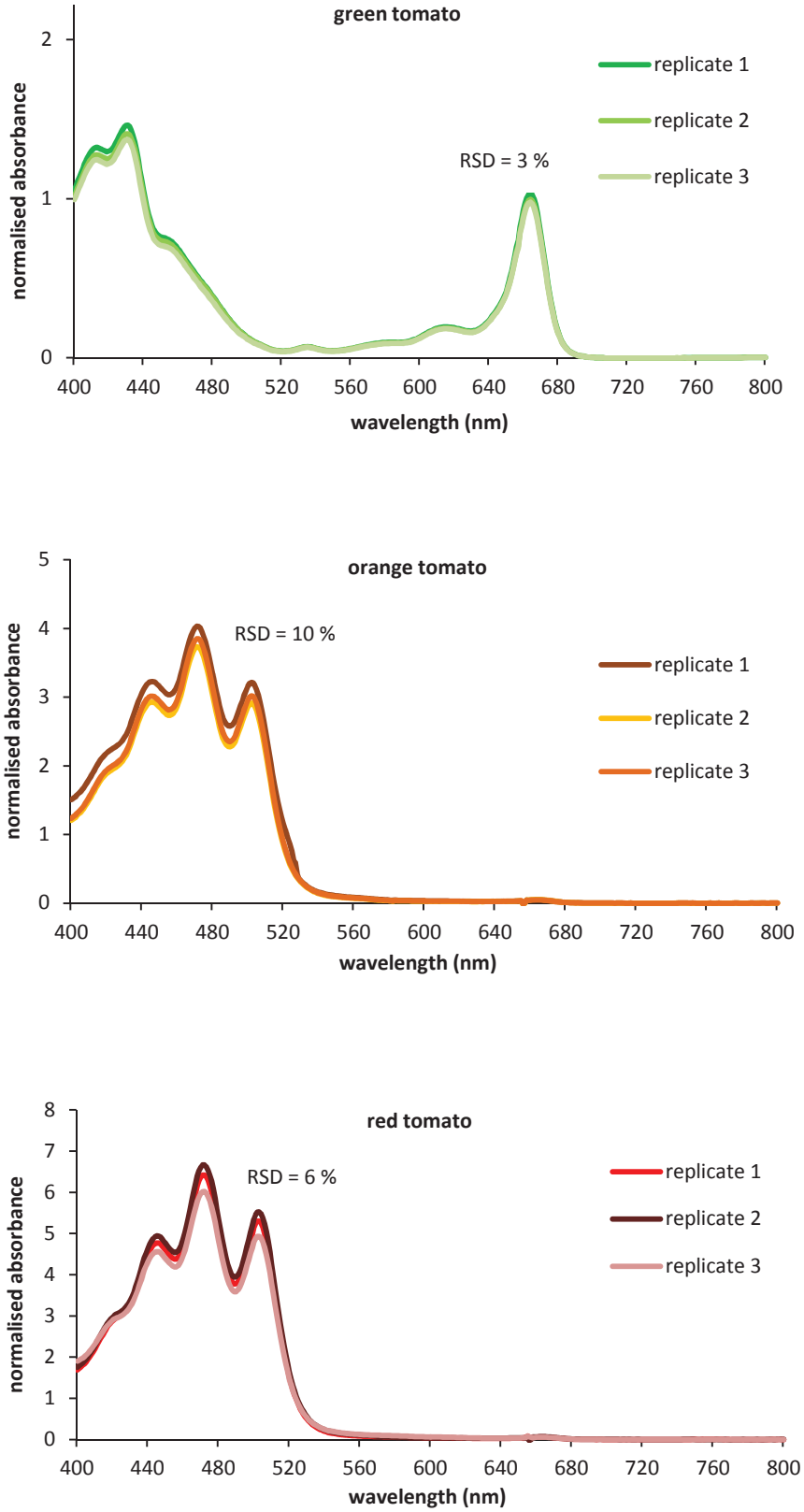


Figure 4.8. Examples of spectra obtained after chemical extraction and analysis (Section 4.2.3, three replicates per sample) of three categories of tomatoes at different ripening stage (2, green tomato; 10, orange tomato; 19, red tomato; see Figure 4.5)

4.3.1.2. DEGRADATION OF THE PIGMENTS WITH TIME

The pigments studied in this work (chlorophylls and carotenoids) are light-sensitive and have been reported to be degraded relatively quickly after the extraction process.[1,17,31] In order to check the stability of these compounds after their extraction from the freeze-dried sample to an organic phase, two subsamples of one green tomato and another two subsamples of one red tomato (selected from the 23 fruits used in this study) were subjected, in triplicate, to the extraction procedure described in Section 4.2.3.

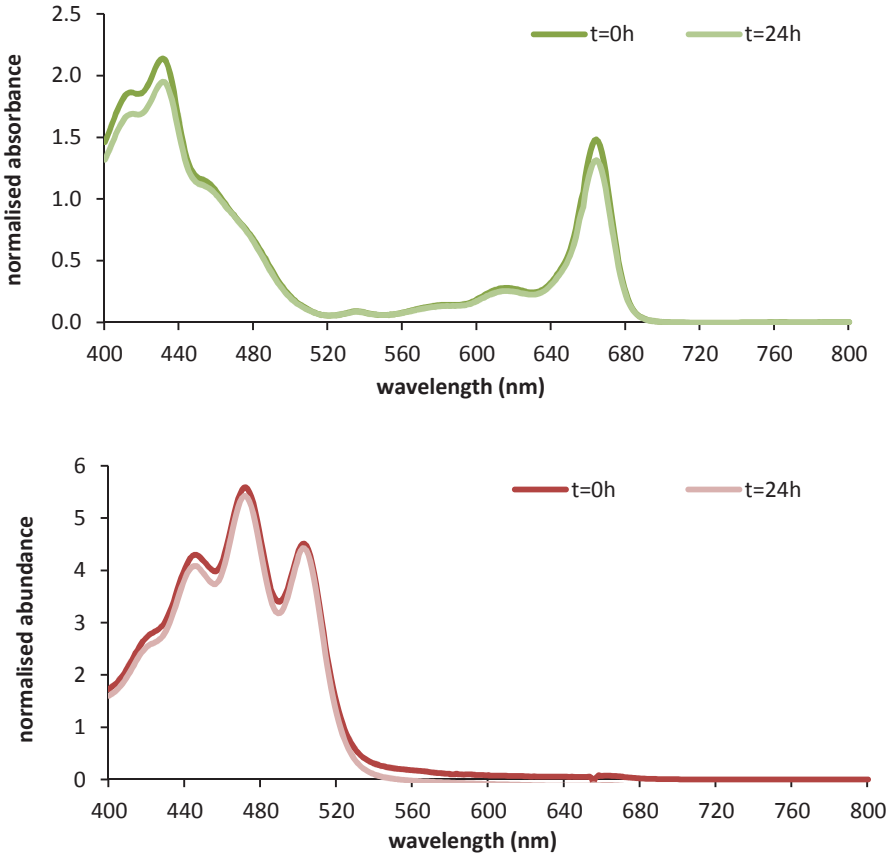


Figure 4.9. Average spectra (n = 3) of extracts of one green tomato (5) and one red tomato (17). The extracts have been stored in dark at 4 °C for different times, namely, 0 and 24 h.

For one of the subsamples, the spectrum was measured immediately after the organic extract was obtained (t = 0), while for the other one, the obtained extract was stored in dark at 4 °C for 24 h, and then the spectrum was measured (t = 24). The results (average of three measurements in each case) are shown in Figure 4.9. As it can be observed, similar spectra were obtained when comparing the absorbance values obtained at t = 0 and t = 24, being the difference between spectra within the limits previously estimated (Section 4.3.1.1) for the repeatability of the chemical analysis. It can consequently be concluded that pigments, if appropriately stored, remain stable at least 24 hours after their extraction to the organic phase.

4.3.1.3. RELIABILITY OF THE IMAGE CALIBRATION PROCESS

An independent technique (Imatest program) was used to check the reliability of the image calibration process by JavaScript plug-in of Photoshop ACR Calibrator CC24, described in Section 4.2.4. This technique is based on the comparison of the distances between the standard chromaticity values (*a* and *b*) of the ColorChecker card with those values obtained from a picture (calibrated and non-calibrated) of the ColorChecker card.

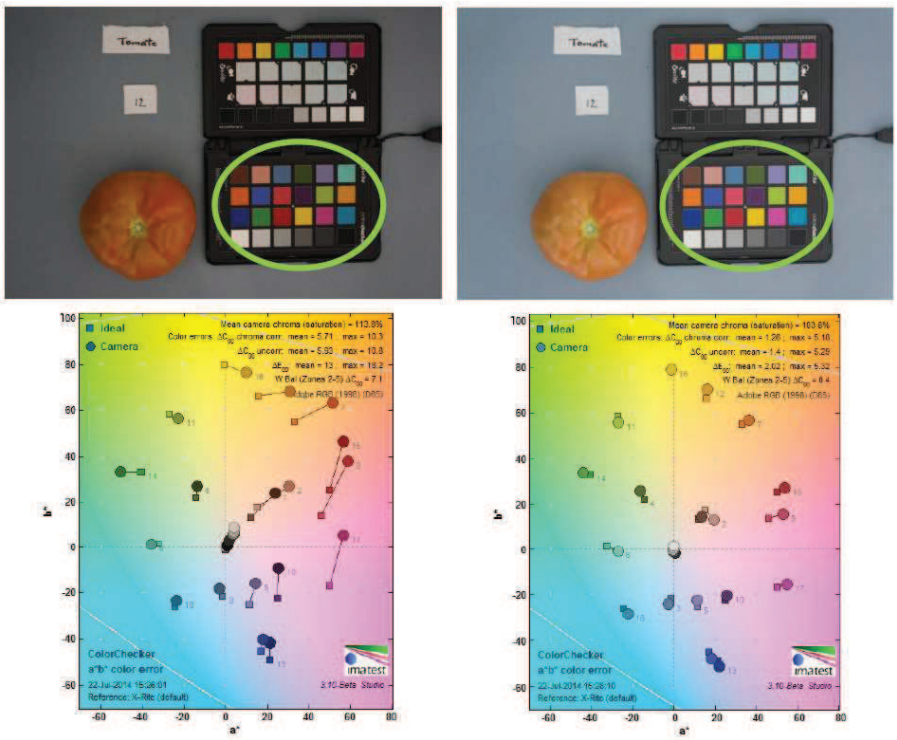


Figure 4.10. Picture of the ColorChecker card before (left) and after (right) calibration and the corresponding results obtained with the Imatest program (standard *a* and *b* values are indicated with squares, while those obtained from the pictures are shown with circles).

As can be observed in Figure 4.10, significant differences were found between the *a* and *b* values obtained before and after pictures calibration, outstanding the importance of the calibration process when determining the colour of an object by this technique. As expected, the *a* and *b* values obtained after calibration process were closely related to the standard values of the ColorChecker card, which indicates the success and need of the calibration process.

4.3.1.4. REPEATABILITY OF THE PICTURE COLLECTION PROCEDURE

Three independent pictures of each sample type of whole tomato, fresh puree and dry powder of the same tomato were taken according to the procedure described in Section 4.2.4 to study the repeatability of the picture collection and calibration processes themselves.

The results are shown in Table 4.1. As can be observed, a good repeatability was obtained for all the chromaticity values (the relative standard deviation was always below 4 %) in all the samples, which confirms the reliability of the experimental procedure to collect chromaticity data.

Table 4.1. Chromaticity values (n = 15) obtained from three independent pictures (replicates) for each sample type of tomato number 20. SD: standard deviation; RSD: relative standard deviation.

	Replicate 1	Replicate 2	Replicate 3	Mean	SD	RSD (%)
Whole tomato						
L	44.8	45.3	45.1	45.07	0.25	1
a	46.1	48.3	48.1	47.5	1.2	3
b	42.9	42.2	42.7	43.60	0.36	1
Fresh puree						
L	56.7	57.3	55.8	56.60	0.75	1
a	38.3	38.8	38.1	38.40	0.36	1
b	25.1	25.7	25.8	25.53	0.39	1
Dry powder						
L	70.0	71.2	69.8	70.33	0.76	1
a	23.7	25.7	24.8	24.7	1.0	4
b	28.5	30.1	29.7	29.43	0.83	3

4.3.1.5. SELECTION OF THE PLANES OF OBSERVATION

Three whole tomato fruits at different ripening stages (green, orange and red) were selected and 6 photos of each tomato were taken corresponding to the top, to the bottom and to the four sides (Figure 4.11). The chromaticity values of each picture were obtained according to the procedure described in Section 4.2.4 (Figure 4.12). Since considering only the top and the bottom simplifies the picture collection process, they were studied to check if comparable chromaticity information was obtained when comparing these two planes with the mix of the 6 planes of observation of the same sample.

Figure 4.11. Pictures of three selected tomatoes (1, green tomato; 16, orange tomato; 22, red tomato; see Figure 4.5) from six different planes of observation (the top, the bottom and the four sides).

No significant differences (t-student, $\alpha = 0.05$; $t_{crit} = 1.98$) were observed between the average of the chromaticity values obtained from the top and bottom pictures of the sample (two planes of observation) and the top, bottom and side pictures of the sample (six planes of observation).

However, the chromaticity information obtained from the top and the bottom pictures was significantly different ($p < 0.05$). Consequently, on the whole both pictures from the top and the bottom are enough and necessary to characterise the colour of each tomato fruit.

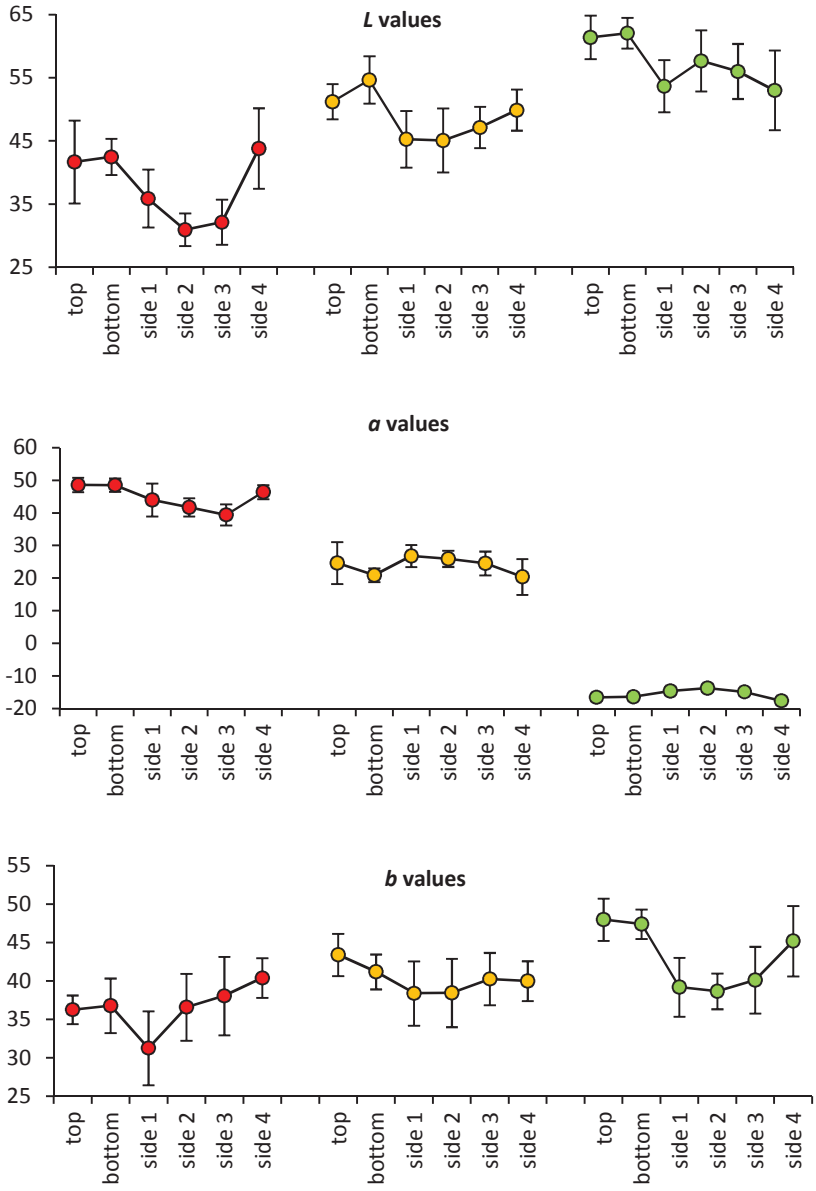


Figure 4.12. Chromaticity values (*L*, *a* and *b*) obtained from the pictures of three different tomatoes (1, green tomato; 16, orange tomato; 22, red tomato; see Figure 4.11). Average (circle) values and their standard deviation (bars) of 15 points randomly selected in each picture are indicated.

4.3.2. MEASUREMENT OF THE ABSORPTION SPECTRA CHARACTERISTIC OF EACH TOMATO AND WAVELENGTH SELECTION

The 23 tomatoes were processed according to the procedure described in Section 4.2.3 in order to obtain their characteristic absorption spectra. The results obtained are shown in Figure 4.13.

The spectra of the 23 tomatoes (Figure 4.13) clearly changed according to the ripening stage. Four relative maximums of absorbance were identified in tomato fruits. The reddest tomatoes exhibited higher absorbance values at 445, 472 and 503 nm, which is related to a high carotenoid content, whereas the absorbance values in the 560 - 700 nm range, corresponding to chlorophylls, were close to zero. The greenest tomatoes showed an opposite kind of spectra with high absorbance values in the chlorophylls range and a low absorbance values in the carotenoids range.

In other words, a decrease in chlorophylls (665 nm) and a concomitant increase in carotenoids (445, 472 and 503 nm) can be observed as the colour of the tomatoes changes from green to red. Thus, these four maximums of absorbance will be used for constructing the mathematical model predicting the ripening stage of tomatoes.

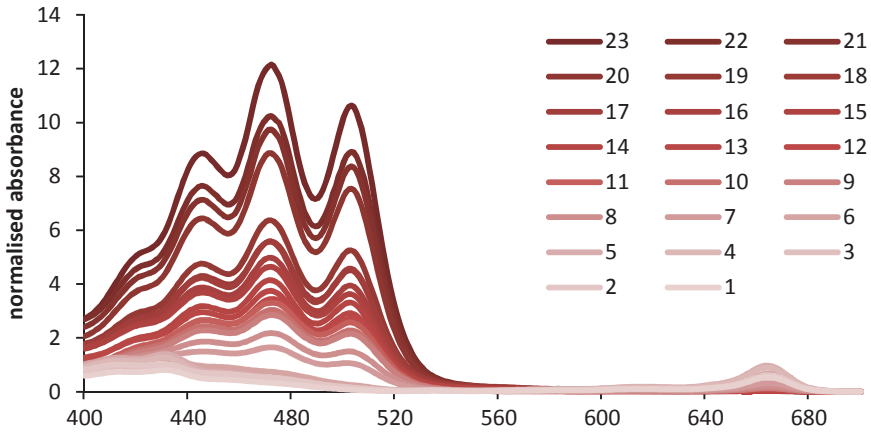


Figure 4.13. Visible spectra (n = 3) of the extract of the powder of the 23 tomatoes considered in this study, sorted (from the greenest to the reddest) by the value of absorbance obtained at 503 nm. Samples from 13 to 23 were previously diluted prior to absorbance measurement.

4.3.3. MEASUREMENT OF THE CHROMATICITY PARAMETERS CHARACTERISTIC OF EACH TOMATO SAMPLE

The chromaticity parameters *L*, *a* and *b* were measured for each picture taken from the whole fruit, from the fresh puree and from the dry powder (see Figure 4.5) according to the procedure described in Section 4.2.4 and the results obtained from the preliminary image experiments (Section 4.3.1.3, 4.3.1.4 and 4.3.1.5). The chromaticity parameters *C* and *H* were also calculated according to Equations 1 and 2.

Table 4.2. Chromaticity values (*L, a, b, C* and *H*) obtained from the pictures (top and bottom) of the whole tomato fruit (n = 30). Tomatoes sorted from the greenest (tomato 1) to the reddest (tomato 23).

	Whole tomato				
	L	***a***	***b***	***C***	***H***
1	62 ± 3	-16 ± 1	48 ± 2	50 ± 2	109 ± 1
2	58 ± 6	-16 ± 2	50 ± 2	53 ± 2	108 ± 2
3	60 ± 7	-16 ± 2	46 ± 5	48 ± 5	109 ± 3
4	55 ± 8	-18 ± 2	46 ± 4	49 ± 4	111 ± 3
5	50 ± 10	-17 ± 2	45 ± 7	49 ± 7	111 ± 3
6	62 ± 8	-14 ± 2	44 ± 6	46 ± 6	107 ± 3
7	59 ± 7	-4 ± 7	44 ± 6	44 ± 6	95 ± 9
8	58 ± 8	8 ± 9	42 ± 4	43 ± 5	80 ± 10
9	56 ± 4	10 ± 8	42 ± 5	44 ± 6	80 ± 10
10	58 ± 6	10 ± 10	44 ± 3	46 ± 5	70 ± 10
11	56 ± 7	13 ± 7	45 ± 5	47 ± 5	74 ± 8
12	53 ± 3	31 ± 7	43 ± 5	53 ± 6	54 ± 6
13	51 ± 4	39 ± 4	43 ± 3	58 ± 4	48 ± 4
14	50 ± 4	31 ± 6	42 ± 5	52 ± 6	53 ± 6
15	49 ± 5	30 ± 5	43 ± 5	52 ± 5	55 ± 5
16	53 ± 4	23 ± 5	42 ± 3	48 ± 4	62 ± 5
17	51 ± 4	37 ± 3	45 ± 4	58 ± 3	50 ± 3
18	53 ± 6	31 ± 5	41 ± 4	51 ± 5	53 ± 5
19	49 ± 3	43 ± 2	43 ± 3	60 ± 2	45 ± 2
20	45 ± 6	47 ± 4	42 ± 3	63 ± 4	42 ± 3
21	44 ± 2	51 ± 3	43 ± 3	67 ± 3	40 ± 2
22	42 ± 5	49 ± 2	37 ± 3	61 ± 2	37 ± 2
23	42 ± 3	50 ± 2	42 ± 3	65 ± 3	40 ± 2

As a result of the image analysis, one set of chromaticity values (*L*, *a*, *b*, *C* and *H*) that characterizes the colour of each of the 23 tomatoes was obtained, from the whole fruit, from the puree and from the dry powder (Tables 4.2, 4.3 and 4.4, respectively). Samples have been sorted from the greenest to the reddest according to the maximum absorbance measured at 503 nm (Figure 4.13).

As expected, the *L* parameter slightly decreased with colour change from green to red, the *a* parameter displayed negative values in green tomatoes and positive values in red ones, and the *b* parameter remained rather constant for all the tomatoes. As the tomato colour turn from green to red, the *C* parameter slightly increased while the *H* parameter exhibited a clear decrease. All these tendencies were clearly observed either for processed (fresh puree and dry powder samples) or non-processed (whole tomato) ones.

Table 4.3. Chromaticity values (*L, a, b, C* and *H*) obtained from the pictures of the fresh puree (n = 15). Tomatoes sorted from the greenest (tomato 1) to the reddest (tomato 23).

	Fresh puree				
	L	***a***	***b***	***C***	***H***
1	71 ± 1	-16 ± 1	44 ± 2	47 ± 2	109 ± 1
2	69 ± 2	-13 ± 1	44 ± 2	46 ± 2	106 ± 1
3	71 ± 1	-11.7 ± 0.5	41 ± 1	43 ± 1	106 ± 1
4	71 ± 1	-15 ± 1	37 ± 3	40 ± 3	113 ± 2
5	69 ± 2	-16 ± 1	41 ± 3	43 ± 3	111 ± 2
6	72 ± 2	-11 ± 1	41 ± 2	43 ± 1	105 ± 1
7	75 ± 1	-0.5 ± 0.5	27 ± 2	27 ± 2	91 ± 1
8	71 ± 1	5 ± 1	30 ± 2	30 ± 2	81 ± 1
9	75 ± 1	7 ± 1	22 ± 2	23 ± 2	73 ± 3
10	74 ± 1	8 ± 1	22 ± 2	24 ± 2	71 ± 3
11	71 ± 1	10 ± 1	27 ± 2	29 ± 2	69 ± 1
12	69 ± 2	18 ± 2	21 ± 2	28 ± 2	49 ± 4
13	60 ± 1	26 ± 1	29 ± 2	39 ± 2	48 ± 3
14	64 ± 1	24 ± 2	25 ± 1	34 ± 2	46 ± 3
15	64 ± 1	23 ± 1	25 ± 1	34 ± 1	47 ± 2
16	67 ± 3	17 ± 2	26 ± 2	31 ± 2	56 ± 3
17	61 ± 1	30 ± 2	28 ± 2	41 ± 2	43 ± 3
18	64 ± 1	24 ± 2	28 ± 2	37 ± 2	49 ± 2
19	57 ± 2	33 ± 1	29 ± 1	44 ± 1	41 ± 2
20	57 ± 2	38 ± 2	25 ± 2	46 ± 2	33 ± 2
21	47 ± 2	38 ± 1	27 ± 1	47 ± 1	35 ± 2
22	47 ± 1	43 ± 1	28 ± 2	51 ± 1	33 ± 2
23	51 ± 3	43 ± 1	25 ± 1	49 ± 1	30 ± 1

4.3.4. CONSTRUCTION OF A MATHEMATICAL MODEL TO ESTIMATE ABSORBANCE FROM CHROMATICITY VALUES

As a result of the experimental measurements one set of (*L*, *a*, *b*, *C*, *H*, A_{400}, A_{401}, …A_{700}) data was obtained for each tomato sample (whole tomato fruit, fresh puree and dry powder). Data sets of (*L*, *a*, *b*) or (*L*, *C, H*) at each of the four wavelengths considered (Section 4.3.2) were subjected to regression analysis according to the procedure described in Section 4.2.5.

4.3.4.1. RESULTS FROM THE CALIBRATION STEP

24 mathematical models were finally obtained after considering each sample type (whole tomato fruit, fresh puree and dry powder), two types of chromaticity parameters (*L*, *a* and *b*, or *L*, *C* and *H*) and four wavelengths previously selected (445, 472, 503 and 665 nm).

Table 4.4. Chromaticity values (*L, a, b, C* and *H*) obtained from the pictures of the dry powder (n = 15). Tomatoes sorted from the greenest (tomato 1) to the reddest (tomato 23).

	Dry powder				
	L	***a***	***b***	***C***	***H***
1	81 ± 1	-8. 1± 0.5	28 ± 1	30 ± 1	106 ± 1
2	82 ± 1	-6 ± 1	28 ± 1	29 ± 1	103 ± 1
3	81 ± 1	-7 ± 1	31 ± 1	32 ± 1	102 ± 1
4	79 ± 1	-9.3 ± 0.5	27 ± 1	29 ± 1	109 ± 1
5	78 ± 2	-9.7 ± 0.5	28 ± 1	30 ± 1	109 ± 1
6	81 ± 1	-6 ± 1	32 ± 1	33 ± 1	100 ± 1
7	81 ± 1	-0.5 ± 0.6	29 ± 1	29 ± 1	91 ± 1
8	77 ± 1	4 ± 1	32 ± 2	32 ± 2	83 ± 1
9	79 ± 2	7.1 ± 0.5	26 ± 2	27 ± 2	75 ± 1
10	80 ± 1	6.9 ± 0.5	26 ± 1	27 ± 1	75 ± 1
11	77 ± 1	9 ± 1	28 ± 3	30 ± 2	72 ± 2
12	74 ± 1	14 ± 1	29 ± 1	32 ± 1	63 ± 2
13	76 ± 1	15 ± 1	28 ± 2	32 ± 2	61 ± 2
14	75 ± 1	15 ± 1	26 ± 1	30 ± 1	59 ± 2
15	71 ± 2	18 ± 2	31 ± 2	35 ± 2	60 ± 3
16	75 ± 3	13 ± 1	29 ± 3	32 ± 3	66 ± 2
17	77 ± 1	14.1 ± 0.5	22 ± 1	26 ± 1	57 ± 2
18	78 ± 1	14 ± 1	24 ± 2	27 ± 2	60 ± 3
19	78 ± 2	15 ± 1	25 ± 1	29 ± 1	59 ± 2
20	70 ± 3	24 ± 3	29 ± 2	37 ± 3	51 ± 4
21	74 ± 1	16 ± 1	27 ± 1	31 ± 1	59 ± 2
22	73 ± 1	23 ± 1	29 ± 1	36 ± 1	52 ± 1
23	67 ± 2	28 ± 2	33 ± 2	43 ± 2	49 ± 2

Based on the criteria used to evaluate the models and after a stepwise regression procedure, the final best models by kind of sample and by wavelength are summarized in Table 4.5, together with their corresponding adjustable parameters (and their standard deviations) and with the total variance explained in each case.

As it can be observed, the use of *L*, *C* and *H* chromaticity values allowed us to obtain better results in the case of the whole tomato fruit and the dry powder, whereas *L*, *a* and *b* were better only in the case of fresh puree.

In order to assess the quality of the models, the RMSEC values (Equation 4), as well as the slopes and determination coefficients of the "observed *vs.* predicted absorbance" plots were calculated using the 20 tomatoes considered in the calibration step (Table 4.6). Note that the RMSEC values obtained at different wavelengths are not comparable since they have been calculated in absolute terms and the absorbance values measured at each wavelength are considerably different.

Table 4.5. Adjustable parameters (and their standard deviations) of the mathematical models obtained in the calibration step of the regression analysis for each case studied (WF, whole fruit; FP, fresh puree; DP, dry powder; var = explained variance).

Sample	**WF**	**FP**	**DP**	**WF**	**FP**	**DP**
X_i	***LCH***	***Lab***	***LCH***	***LCH***	***Lab***	***LCH***
λ (nm)		**445**			**472**	
k_0	-50 ± 10	100 ± 20	-	-100 ± 20	140 ± 20	-40 ± 20
k_1	1.0 ± 0.2	-4 ± 1	-	2.4 ± 0.5	-5 ± 1	0.8 ± 0.3
k_2	1.1 ± 0.2	-	-	2.2 ± 0.3	-	-
k_3	$(-40 \pm 8)\times10^{-3}$	2 ± 1	-	-0.5 ± 0.2	2 ± 1	-
k_{12}	$(-21 \pm 4)\times10^{-3}$	$(13 \pm 3)\times10^{-3}$	$(49 \pm 8)\times10^{-4}$	-0.05 ± 0.01	$(18 \pm 5)\times10^{-3}$	-
k_{13}	-	-	-	-	-	$(-5 \pm 2)\times10^{-3}$
k_{23}	-	$(-23 \pm 6)\times10^{-3}$	$(-10 \pm 2)\times10^{-3}$	$(8 \pm 3)\times10^{-3}$	$(-32 \pm 9)\times10^{-3}$	$(-8 \pm 3)\times10^{-3}$
k_{123}	-	-	-	-	-	-
k_{11}	-	$(29 \pm 7)\times10^{-3}$	-	-	0.04 ± 0.01	-
k_{22}	-	-	$(6 \pm 1)\times10^{-3}$	-	-	$(13 \pm 2)\times10^{-3}$
k_{33}	-	$(-24 \pm 9)\times10^{-3}$	$(12 \pm 3)\times10^{-4}$	-	-0.03 ± 0.01	$(31 \pm 8)\times10^{-4}$
var (%)	95.3	96.7	94.8	96.8	96.7	96.5
λ (nm)		**503**			**665**	
k_0	-90 ± 20	130 ± 20	-40 ± 10	-12 ± 4	-70 ± 10	-
k_1	2.3 ± 0.5	-5 ± 1	0.7 ± 0.2	0.2 ± 0.1	1.5 ± 0.2	-
k_2	2.0 ± 0.3	-	-	0.3 ± 0.1	1.4 ± 0.2	-
k_3	-0.4 ± 0.2	2.1 ± 0.8	-	-	-0.27 ± 0.05	-
k_{12}	-0.05 ± 0.01	$(16 \pm 4)\times10^{-3}$	-	$(-26 \pm 8)\times10^{-4}$	$(-16 \pm 2)\times10^{-3}$	-
k_{13}	-	-	$(-5 \pm 2)\times10^{-3}$	$(-10 \pm 2)\times10^{-4}$	$(26 \pm 5)\times10^{-4}$	$(-6 \pm 1)\times10^{-4}$
k_{23}	$(8 \pm 3)\times10^{-3}$	$(-30 \pm 8)\times10^{-3}$	$(-8 \pm 2)\times10^{-3}$	-	$(27 \pm 4)\times10^{-4}$	-
k_{123}	-	-	-	-	-	-
k_{11}	-	$(38 \pm 9)\times10^{-3}$	-	-	$(-8 \pm 1)\times10^{-3}$	$(23 \pm 6)\times10^{-5}$
k_{22}	-	-	$(13 \pm 2)\times10^{-3}$	$(-12 \pm 5)\times10^{-4}$	$(-7 \pm 1)\times10^{-3}$	
k_{33}	-	-0.03 ± 0.01	$(29 \pm 7)\times10^{-4}$	$(4 \pm 1)\times10^{-4}$	-	$(36 \pm 6)\times10^{-5}$
var (%)	96.8	96.6	96.4	98.9	98.8	96.6

All the models summarized in Table 4.5 are of similar (and relatively high) quality. In all the cases, the explained variance is greater than 94.8 % (Table 4.5), the R^2 of regression between the predicted and the observed values in the calibration step is always higher than 0.95 and the corresponding slope is not significantly different from 1 (Table 4.6). The residuals are also homogeneously distributed around zero in all the cases (data not shown). The use of non-processed samples (whole fruit) resulted in models of similar quality to that of the models obtained from processed (fresh puree and dry powder) ones.

Table 4.6. Slope and intercept (mean and standard deviation values), Root Mean Square Error of Calibration (RMSEC), and determination coefficients (R^2) of the "observed *vs*. predicted absorbance" regression obtained for the models summarized in Table 4.5 for the calibration step (n = 20).

	Slope	Intercept	R^2	RMSEC
445 nm				
whole tomato fruit	0.984 ± 0.050	0.15 ± 0.20	0.95	0.243
fresh puree	0.989 ± 0.042	0.11 ± 0.17	0.97	0.174
dry powder	0.982 ± 0.052	0.17 ± 0.21	0.95	0.274
472 nm				
whole tomato fruit	0.988 ± 0.043	0.13 ± 0.22	0.97	0.344
fresh puree	0.987 ± 0.044	0.14 ± 0.23	0.97	0.357
dry powder	0.986 ± 0.045	0.12 ± 0.19	0.96	0.379
503 nm				
whole tomato fruit	0.986 ± 0.043	0.10 ± 0.18	0.97	0.275
fresh puree	0.985 ± 0.044	0.11 ± 0.19	0.97	0.289
dry powder	0.984 ± 0.044	0.12 ± 0.20	0.96	0.307
665 nm				
whole tomato fruit	0.994 ± 0.024	0.0027 ± 0.0095	0.99	0.001
fresh puree	0.993 ± 0.026	0.003 ± 0.010	0.99	0.001
dry powder	0.981 ± 0.043	0.0086 ± 0.016	0.97	0.001

This is an important point since it will allow us working in the future directly with non-processed tomatoes, which will considerably shorten the process to obtain the chromaticity data.

4.3.4.2. RESULTS FROM THE VALIDATION STEP

In the validation step, first, the absorbance values characteristic of the three samples previously selected to be part of the validation set (tomatoes 3,12 and 21) were predicted at a given wavelength using the models of Table 4.5. Then, the predicted absorbance values were compared with the experimentally observed ones, and finally, the corresponding errors of prediction (RMSEV value) were calculated.

The results are shown in Table 4.7. Again, as they have been calculated in absolute terms, the RMSEV values are comparable within the same wavelength but not among them.

As can be observed, the lowest values of RMSEV were always obtained for the models corresponding to the whole tomato fruits, followed by fresh puree and, finally, dry powder. The only exception was found at 665 nm, in which the dry powder offered the lowest values of RMSEV, followed by the whole fruit and the fresh puree.

Table 4.7. Observed and predicted absorbance values (with the corresponding Root Mean Square Error of Validation (RMSEV), n = 3) obtained for the models summarized in Table 4.5. The 3 samples differing in ripening stage were used (3, green tomato; 12, orange tomato; 21, red tomato; see Figure 4.5).

		Tomato number	Observed absorbance	Predicted absorbance	RMSEV
445 nm	whole tomato	3	0.69	0.67	0.674
		12	2.7	3.3	
		21	7.1	8.1	
	fresh puree	3	0.69	0.83	0.927
		12	2.7	3.7	
		21	7.1	8.4	
	dry powder	3	0.69	0.51	1.814
		12	2.7	3.6	
		21	7.1	4.1	
472 nm	whole tomato	3	0.52	0.28	0.894
		12	3.4	4.3	
		21	9.8	11.0	
	fresh puree	3	0.52	0.65	1.226
		12	3.4	4.9	
		21	9.8	11.3	
	dry powder	3	0.52	0.66	2.712
		12	3.4	4.2	
		21	9.8	5.1	

4.3.4.3. SELECTION OF THE BEST MATHEMATICAL MODEL

As already stated in the introduction, the final objective of this work is to propose a methodology that allows us to discern among tomatoes with different ripening stage using chromaticity values obtained by image analysis of standard pictures. A minimum manipulation of the sample is, of course, desirable in order to minimise experimental efforts, to save time and money, and to minimise the possibility of pigments degradation. Thus, the process to select the best model must necessarily include the selection of the wavelength, the selection of the sample type, and the selection of the chromaticity values to be used in future works (shown in Table 4.5).

The spectra of tomato extracts present relative maximums of absorbance at 445, 472, 503 and 665 nm (Section 4.3.2). The absorbance values at 445 and 472 nm result from the concentration of both chlorophylls and carotenoids in the extract, whereas those at 503 and 665 nm, specifically depend on the presence of one family of pigments, respectively, carotenoids or chlorophylls. Consequently, in order to facilitate a possible estimation of the pigment content in the future, the models obtained at 445 and 472 nm were not further considered.

Table 4.7 (*cont.*). Observed and predicted absorbance values (with the corresponding Root Mean Square Error of Validation (RMSEV), n = 3) obtained for the models summarized in Table 4.5. The 3 samples differing in ripening stage were used (3, green tomato; 12, orange tomato; 21, red tomato; see Figure 4.5).

		Tomato number	Observed absorbance	Predicted absorbance	RMSEV
503 nm	whole tomato	3	0.16	-0.08	0.820
		12	2.7	3.4	
		21	8.4	9.6	
	fresh puree	3	0.16	0.24	1.137
		12	2.7	3.9	
		21	8.4	9.9	
	dry powder	3	0.16	0.22	2.482
		12	2.7	3.3	
		21	8.4	4.1	
665 nm	whole tomato	3	0.54	0.66	0.095
		12	0.047	0.084	
		21	0.085	-0.022	
	fresh puree	3	0.54	0.710	0.186
		12	0.047	0.161	
		21	0.085	-0.166	
	dry powder	3	0.54	0.62	0.052
		12	0.047	0.066	
		21	0.085	0.054	

On the other hand, the power to discriminate among different ripening stages at 503 nm is considerably higher than at 665 nm, since the difference in absorbance between a green and a red tomato is much higher at 503 than at 665 nm. Consequently, only the models obtained for 503 nm were finally retained. Moreover, a model that estimates the absorbance at 503 nm would potentially allow the estimation of the lycopene concentration, as it has been already reported, because 503 nm is in the range of the maximum absorption of lycopene.[39]

At 503 nm, the best model was that for the whole tomato fruit. Compared to the model at 503 nm for the fresh puree and the dry powder, it explained a higher amount of variance, had the highest coefficient of determination and the lowest values of RMSEC and RMSEV (Tables 4.6 and 4.7). The model is not significantly biased as shown by the slope and the intercept of the observed *vs*. predicted regression (Table 4.7). Finally, working with the whole fruit extremely simplifies the manipulation of the sample for the acquisition of the chromaticity values.

Thus, the final model selected was that obtained working with the whole tomato fruit at 503 nm and using *L*, *C* and *H* chromaticity values (Equation 5).

$$A_{503} = (-90 \pm 20) + (2.3 \pm 0.5) \cdot L + (2.0 \pm 0.3) \cdot C + (-0.4 \pm 0.2) \cdot H + (-0.05 \pm 0.01) \cdot L \cdot C + ((8 \pm 3) \cdot 10^{-3} \cdot C \cdot H \quad (5)$$

4.3.5. DEFINITON OF A RELATIVE SCALE TO DISCERN AMONG TOMATOES WITH DIFFERENT RIPENING STAGE

Working with regression model, the absorbance values can be predicted with a confidence interval so as to decide if several tomato fruits are equivalent or not in terms of ripening. However, with the intention of obtaining intuitive results, so they can be quickly and simply understood, the option to define a relative scale to sort the tomatoes according to their ripening stage is proposed.

The absorbance values at 503 nm (A) of the 23 tomato fruits used in this study were predicted using the final selected model (Equation 5) and the corresponding *L*, *C*, and *H* chromaticity values. Then, the absorbance values were range-normalized (A´) according to Equation 6, where A_{max} and A_{min} are the maximum and minimum absorbance values predicted by the model, respectively (Table 4.8).

$$A' = \frac{A - Amin}{Amax - Amin} \times 10 \quad (6)$$

In this way, a relative scale from zero to ten was obtained (Figure 4.14), in which the greenest tomato has a score of zero (tomato number 1) and the reddest one (tomato number 23) a score of ten. As an example, four more tomatoes (2, 10, 19 and 22) have been located in the scale according to their ripening stage.

The scale can be used in the future to sort new tomatoes according to their ripening stage. Pictures of the top and bottom of the tomatoes should be taken, their characteristic chromaticity values should be calculated according to the procedure described in Section 4.2.4, and the absorbance at 503 nm should be estimated by means of Equation 5. The value of A´ calculated according to Equation 6 should give us the score of the tomato and, consequently, its relative position in the scale of the Figure 4.14.

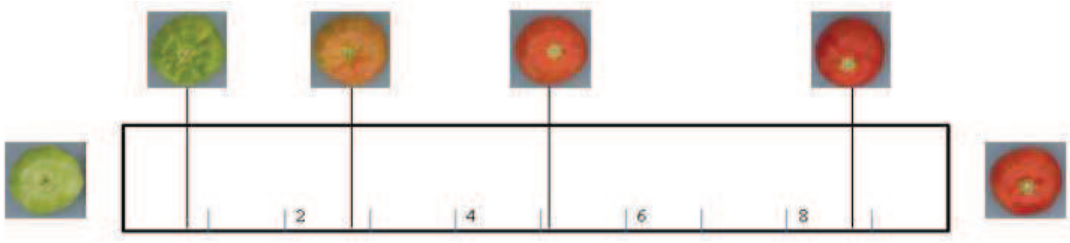


Figure 4.14. Relative scale to estimate the ripening stage of tomatoes (tomatoes 1, 2, 10, 19, 22 and 23 were selected as examples; see Figure 4.5).

Table 4.8. Absorbance at 503 nm (A) predicted by the model (Equation 5) for each tomato used in this study, and range-normalized absorbance (A´) calculated according to Equation 6. Whole tomato fruits sorted from the greenest (tomato 1) to the reddest (tomato 23).

Tomato number	A	A´
1	-0.20	0.00
2	0.56	0.72
3	-0.09	0.10
4	0.10	0.29
5	-0.06	0.13
6	0.12	0.30
7	0.92	1.07
8	2.24	2.32
9	2.16	2.25
10	2.66	2.72
11	2.30	2.38
12	3.37	3.40
13	4.07	4.07
14	3.92	3.92
15	4.06	4.06
16	3.07	3.11
17	3.85	3.86
18	3.50	3.52
19	5.12	5.07
20	7.87	7.69
21	9.61	9.34
22	8.89	8.66
23	10.3	10.00

4.4. REFERENCES

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Chapter 5

Accumulation and translocation of essential and non-essential elements by Swiss chards and tomato plants

CONCLUSIONS

Swiss chards and tomato plants were grown up in two open-air plots covering the whole production cycle of the plants, with the aim of studying the accumulation and translocation of essential and non-essential elements in different organs, and the effect that the cultivation method and the harvesting time had on the accumulation and translocation rates.

First of all, it is important to highlight that the concentrations of the regulated elements (Cd, Pb) measured in the edible parts of the plants collected during the whole field experiment were always below the threshold values defined in the European Legislation currently in force (Commission Regulation No 1881/2006), which regulates the composition of foods and beverages regarding contaminants in order to avoid risks for human health.

Most of the toxic elements, together with Fe, and Co (and also Na in tomato plants) were mainly immobilized in roots, which limited the presence of these elements in the edible parts of the plants. Aboveground organs were richer in essential elements, although some non-essentials, such as Sr, Ba, and Cd, were highly accumulated in leaves of both Swiss chards and tomato plants.

In the case of chards, as leaves are the edible part of the plant, the concentration of Cd in blades and stalks is an important variable to keep under control in order to guarantee a safe consumption of this vegetable. On the other hand, tomato fruits presented low concentrations of non-essential and toxic elements. Thus, it can be concluded that eating raw tomatoes should not represent a real threat to human health in terms of the bioaccumulation of toxic metals through diet.

Although the elemental composition of the conventionally and organically grown plants (both Swiss chards and tomato plants) did not drastically differ, some differences were however observed when comparing plants grown up using these different cultivation methods. In general, the organic practice favoured the accumulation of essential elements, while the conventional one produced plants with higher concentrations of some toxic elements, such as Tl and Cd. The elemental composition of tomato fruits, however, did not depend on the farming technique used.

Regarding the effect of the harvesting time, in general, the elemental composition of plants changed with the time, although no clear tendencies were observed for tomato plants. The only exceptions for these plants were observed in the cases of Na and Sr (with progressively increasing concentrations with time) and Mo (whose concentration showed a decreasing trend with time). The elemental composition of the plants (both Swiss chards and tomato plants) collected at the first sampling campaign was significantly different than that of the plants collected in the rest of the sampling campaigns, probably due to i) the addition of the fertilizer a few days before plantation, which likely increased the availability of some elements in soil, and/or ii) a higher root absorption at the early growth stages. Younger Swiss chards, for example, were richer in most of the elements considered, whether essential, non-essential or toxic elements.

When investigating the dependence with time of the elemental concentrations measured in fruits of tomato plants, we observed that concentrations did not correlate with the age of the plant, but rather with the age of the fruits. It became obvious that it was important to have a tool to estimate the age of a single tomato or a set of tomatoes (in this case, the set of tomatoes collected from a single plant). This can be done by estimation of the ripening stage.

Following this reasoning, a cheap, fast, simple and intuitive method was proposed to discern among tomatoes with different ripening stage, by using the chromaticity parameters obtained after image analysis of pictures collected with a standard digital camera. Image analysis of pictures of the whole non-processed tomato fruit allowed colour characterization with the required accuracy and precision. Manipulation of the sample was kept to a minimum, thus extremely simplifying the procedure before image analysis. Two pictures taken from the top and the bottom of the sample were enough to characterize chromatically a tomato. Chromaticity values were used to predict the corresponding value of absorbance at 503 nm (maximum absorbance of lycopene), which allowed the estimation of the ripening stage.

The proposed methodology is susceptible to be applied directly in the field to decide about the appropriate harvesting time according to the needs of the producer.

Moreover, as the mathematical model has been developed using the absorbance at the wavelength characteristic of lycopene, the method may also be used to estimate the lycopene concentration.

The proposed methodology might be also used to estimate the ripening stage of other varieties of tomato previous re-calibration and validation of the mathematical model. However, as the amount of samples used in our study in the calibration step was relatively low in comparison with the number of parameters to be estimated in the regression process, the uncertainty associated to the predicted absorbance was relatively high. This point requires further investigation in the future.

In summary, the methodology based on the measurement of chromaticity values presents important advantages in terms of simplicity, time, cost and reagents when compared with chromatographic- or spectrometric-based approaches, nowadays considered as reference procedures for the estimation of the ripening stage of tomato fruits through the measurement of the lycopene concentration and/or other chemical compounds.

Before finishing this Section, it should be highlighted that the results obtained for both Swiss chards and tomato plants indicate that the plant organ is a key factor governing the content in nutrients but also in non-essential and toxic trace elements, pointing out the need for understanding the rules of partitioning that are tackled in the next Section of this Thesis, in which the distribution of Cd between different organs of sunflowers is specifically investigated.

SECTION III

Cd uptake and allocation to the different organs of sunflowers

Introduction

Plants need to take up inorganic elements from the environment for their own growth and development.[1] However, some of them happen to be nonessential and even toxic.[2,3] Plants have response mechanisms to cope with toxic elements, including the decrease in root absorption or the sequestration in organs to prevent these elements to disturb vital functions.[4,5] Moreover, plants are able to avoid soil contamination by phytoextraction of these toxic elements.[6] A general ranking of metals toxicity in plant tissues has been already described by Johnson et al.,[7] showing that Cd, Cr, Hg and Pb are the elements with the highest toxicity at low concentrations.

Cd is one of the most studied toxic elements in environmental biology and soil science, due to its possible high mobility in soil and due to its high toxicity for animals and plants.[8,9] In fact, Cd is considered as being one of the most potential carcinogenic elements for humans, according to the International Agency for Research on Cancer (IARC),[10] and being a metal, it cannot be degraded in the environment.

Cd is accumulated in plant organs mainly as the result of its uptake from the soil solution by the root system and through its translocation to aerial parts, as the result of the lack of specificity of metal transporters involved in the uptake and distribution of essential nutrients.[11-13] Cd has been reported to be absorbed into the symplasm of root cells by ZIP, Nramp and LCTI family transporters mediating the uptake of Zn, Fe and Ca.[11,14,15] Once taken up by roots, Cd is partially sequestrated in vacuoles as a detoxification mechanism, mainly in roots but also along its route to the different plant organs.

This sequestration, at least in roots, has been reported to result from the energy-dependent pumping of Cd from the symplasm into the vacuoles by heavy metal ATPases including *HMA3*.[16]

The radial transport of Cd towards the xylem can occur by the symplastic route through the plasmodesmata or by the apoplastic route in the free space of the root cell walls. In the latter case, the radial transport of Cd is generally restricted by the apoplastic barriers consisting in the impregnation of the cell walls by lignin and suberin.[5] The apoplastic barriers can be developed at the root periphery (the exodermis) and they also separate the root cortex from the stele where the xylem and phloem are located. Therefore, Cd has to be internalized into the symplasm of the cortex or endodermis cells to reach the stele, where the heavy metal ATPases efflux pumps (*HMA2* and *HMA4*) of the parenchyma cells excrete the Cd into the stele apoplast. Then, Cd diffuse into the xylem sap, and is transported upwards[11,16] by the transpiration stream or by the root pressure when transpiration is null or very low, for example at night and at night/day/night transitions.[17]

Once in the xylem sap, Cd can reach the phloem sap due to exchange of solutes between these two saps mediated by the parenchyma cells of the vascular bundles, which pump Cd from the xylem sap and which mediate the symplastic transport of the metal towards the companion cells of the phloem, more particularly at the level of the node in cereals[18,19] or at the level of the ray of parenchyma cells in roots of dicots or in leaves.[20-22] Therefore, Cd in the phloem can reach the non-transpiring organs, and can therefore return back to the roots or be transported to developing organs having no or little transpiration like young leaves or fruits and seeds.[11,13] For the filial tissues of the grains, which are not connected to the maternal tissue by vascular bundles, Cd is assumed to be unloaded from the phloem by the maternal parenchyma cells, which then excrete the metal in the apoplast from which it is pumped by the filial cells,[13] presumably like the other nutrients.[23]

Hence, from the knowledge of the current literature, the major key points controlling the allocation of Cd to an organ can be inferred as follows. For roots, the balance between the loading of the xylem and the sequestration in the vacuole is a major point of control as it has been demonstrated for different plant species.[24-27] For roots, transpiration may also be of importance. Indeed, if there is a significant bypass of the apoplastic barriers due to their immaturity at the root tip or due to the endodermis disruption at the level of the root branch emergence, Cd can directly move to the xylem by the apoplastic route.[28] This flow is expected to be positively correlated to the plant transpiration. Furthermore, transpiration can influence the active loading of the xylem by increasing the gradient of Cd at the parenchyma cells/xylem sap interface.

For a transpiring organ, it is reasonable to assume that the major force driving the Cd to it is the transpiration stream, but at the level of this organ the sequestration will also depend on the unloading of the xylem as well as on the loading of the phloem with Cd, which will contribute to export the metal to other sinks.

For a non-transpiring organ, the allocation of Cd to it is likely controlled by the flow of the phloem sap towards that sink (the so-called sink strength for assimilate) and by the regulation of transporters involved in the xylem-to-phloem exchanges and in the unloading of the phloem.

The relationship between transpiration and Cd accumulation has been investigated in several plant species, either by manipulation of the transpiration through modifying the relative humidity or by adding exogenous abscisic acid or by just observing changes in the transpiration rates.[28-33] The role of transpiration on the uptake and partitioning of Cd between roots and shoots is not clear. When it is significant, which is not always observed,[33] the effect of transpiration may have been confounded with some other effects, such as i) changes in the availability of Cd in soils, ii) changes in the plant growth and allometry induced by the applied treatments,[31,32] and iii) genetic differences in plant varieties for the sequestration of Cd in roots.[28]

The uptake, accumulation and translocation of Cd have been widely studied in durum wheat, sunflower and rice[16,24,34-36] because of the importance of these plants for the safety of the food chain. Sunflower is also potential valuable specie for the phytoextraction of Cd from polluted soils because it produces an important aboveground biomass, which in the meanwhile, accumulates significant amount of Cd compared to other crops.[36,37] It is important to understand the rules of Cd partitioning, both for the phytoextraction perspective and for the food safety. In particular, the rules of Cd allocation at the plant organ levels are required in the perspective of modelling the accumulation of this toxic metal in crops.

Therefore, the objective of this study was to i) investigate and model the uptake and partitioning between sunflower organs of the Cd recently taken up (followed by the isotope labelling of Cd), at two reproductive stages (the flower bud and the grain filling stages) and under two treatments aimed at changing the plant transpiration, and ii) study the origin of the Cd allocated into seeds of sunflowers, namely uptake by roots and the remobilization from plant reserves. We performed a greenhouse experiment with sunflowers grown in nutrient solution with Cd at natural abundance, till maturity. Five harvestings were performed during the late vegetative and reproductive stages of sunflowers. Among them, at the flower bud and grain filling stages, the nutrient solution was enriched with ^{111}Cd for 3 days and the plants were exposed or not to fans to increase the transpiration. It was hypothesized that i) transpiration would increase the allocation to aboveground organs and therefore decrease the sequestration in roots, ii) allocation of Cd to a given plant organ will depend on its relative biomass with respect to other compartments that would act as competitive sinks, following the source-sink relationships for carbonaceous assimilates.

The following chapters report the modelling of Cd recently taken up with a particular focus on the effect of transpiration and plant allometry. The last experimental chapter concentrates on the possible role of remobilization of Cd for the allocation of the metal to the seeds.

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Chapter 6

MATERIALS AND METHODS

6.1. PLANT CULTIVATION

The study was conducted on the early flowering sunflower variety *Helianthus annuus* var. ES Biba (Euralis Semences, France), which has a medium height and size of seed and a rapid growth of grain.

Seeds were washed in a glass container with deionised water for 10 min. Afterwards, seeds were individually brushed so as to remove the coating containing the pesticides. Cleaned seeds were then treated with a 3 % H_2O_2 solution (Sigma-Aldrich, TraceSelect Ultra, USA) for another 10 min, washed again with deionised water and put in cleaned glass containers onto previously moistened cellulose paper. The containers were placed for four days at 26 °C. The moisture and the germination of seeds were regularly checked.

The germinated seeds were individually placed into 1.5 mL microtubes, the bottom of which was cut to allow the root growth. The microtubes were inserted into a perforated polystyrene tray floating onto nutrient solution (composition described below) aerated by bubbling and installed in the greenhouse (Figure 6.1). After four days, the seedlings were enough developed to be individually transferred into 17 L capacity polypropylene cubic pots, previously filled with 14 L of nutrient solution.

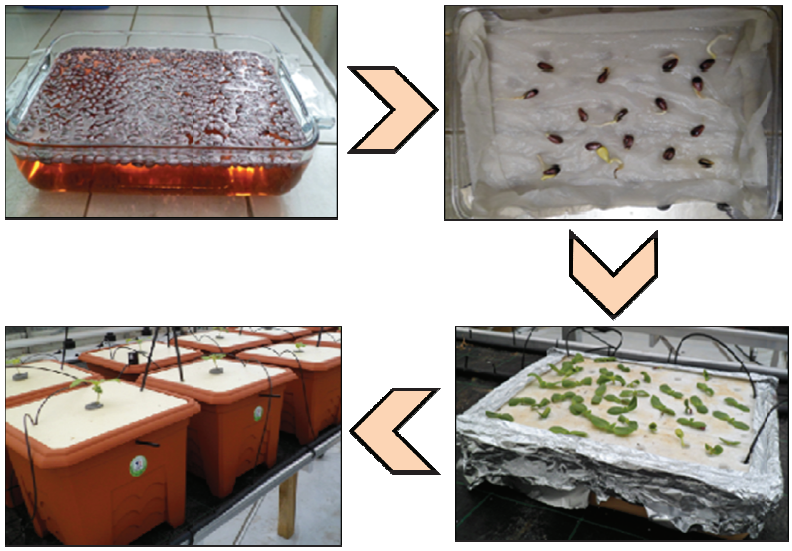

Figure 6.1. Cleaning, germination, pre-culture and transplantation steps (stages BBCH 00, BBCH 05, BBCH 07 and BBCH 09, respectively, a universal scale proposed by Lancashire et al.[1]).

Seedlings were surrounded by a piece of polyurethane foam (a slice of a foam tube used to insulate heater tubes) and inserted into a 4 cm diameter hole, itself drilled into a square polystyrene cover placed on the top of the container to avoid the formation of algae formation in the nutrient solution. The cover also included two additional 3 mm diameter holes for the capillary tubes that supplied the nutrient solution and the air used for aeration/homogenization by bubbling.

Thirty-five plants were grown in a modified half-strength Hoagland nutrient solution[2] with the following composition: 0.5 mM KH_2PO_4 (ISO grade, Merck Millipore), 2.5 mM KNO_3 (ISO, Reag. Ph Eur grade, Merck Millipore), 2.5 mM $Ca(NO_3)_2{\cdot}4H_2O$ (≥ 98 %, AnalaR NORMAPUR grade, VWR Chemicals), 1 mM $MgSO_4{\cdot}7H_2O$ (≥ 99.5 %, AnalaR NORMAPUR grade, VWR Chemicals), 46.25 µM H_3BO_3 (≥ 99.8 %, AnalaR NORMAPUR grade, VWR Chemicals), 1 µM $MnCl_2{\cdot}2H_2O$ (EMSURE grade, Merck Millipore), 10 µM $ZnSO_4{\cdot}7H_2O$ (ACS, ISO Reag. Ph Eur grade, Merck Millipore), 2 µM $CuSO_4{\cdot}5H_2O$ (ACS, ISO Reag. Ph Eur grade, Merck Millipore), 0.20 µM $Na_2MoO_4{\cdot}2H_2O$ (EMSURE grade, Merck Millipore), 71.63 µM $FeNaEDTA{\cdot}3H_2O$ (EDTA 70.2 %, Alfa Aesar), and 200 nM $Cd(NO_3)_2{\cdot}4H_2O$ (EMSURE grade, Merck Millipore).

In order to buffer the pH at 6 (adjusted with KOH), 2 mM of 2-(N-morpholino)ethanesulfonic acid (MES) hydrate (≥ 99 %, Panreac AppliChem) was added to the nutrient solution, and 139 µM of HEDTA (≥ 98 %, Sigma-Aldrich), was also added to buffer the availability of trace elements during their uptake by roots.[3]

The free ion activities of the trace elements as determined by modelling with the software CHEAQS Pro version (P2014.1) were: pCd^{2+} = 11.03, pCu^{2+} = 14.44, pMn^{2+} = 6.81, pZn^{2+} = 10.14, pFe^{3+} = 17.15.

During the cultivation a 3 mL aliquot of the nutrient solution of each pot was sampled twice a week to control the total Cd concentration. Furthermore, the pH, conductivity and dissolved oxygen were also monitored in each pot with a mobile digital multimeter (Multi 3430 SET F, WTW, Germany).

Based on these measurements, the nutrient solution was delivered to each pot by a computer-controlled pump every hour from 7:00 am to 10:00 pm with a flow rate between 1 and 3 L plant^{-1} day^{-1} in order to both compensate the loss due to the plant transpiration and to limit the variations of the concentration of Cd (260 ± 93 nM), of the pH (6.18 ± 0.17), of the conductivity (1380 ± 280 µS cm^{-1}) and of the dissolved oxygen (8.33 ± 0.54 mg L^{-1}) (Figure 6.2). This was achieved by supplying nutrient solution in excess by the capillary tube dipping at the bottom of the pot and by overflowing the excess thanks to a 3 mm hole located at the upper part of the side of the pot.

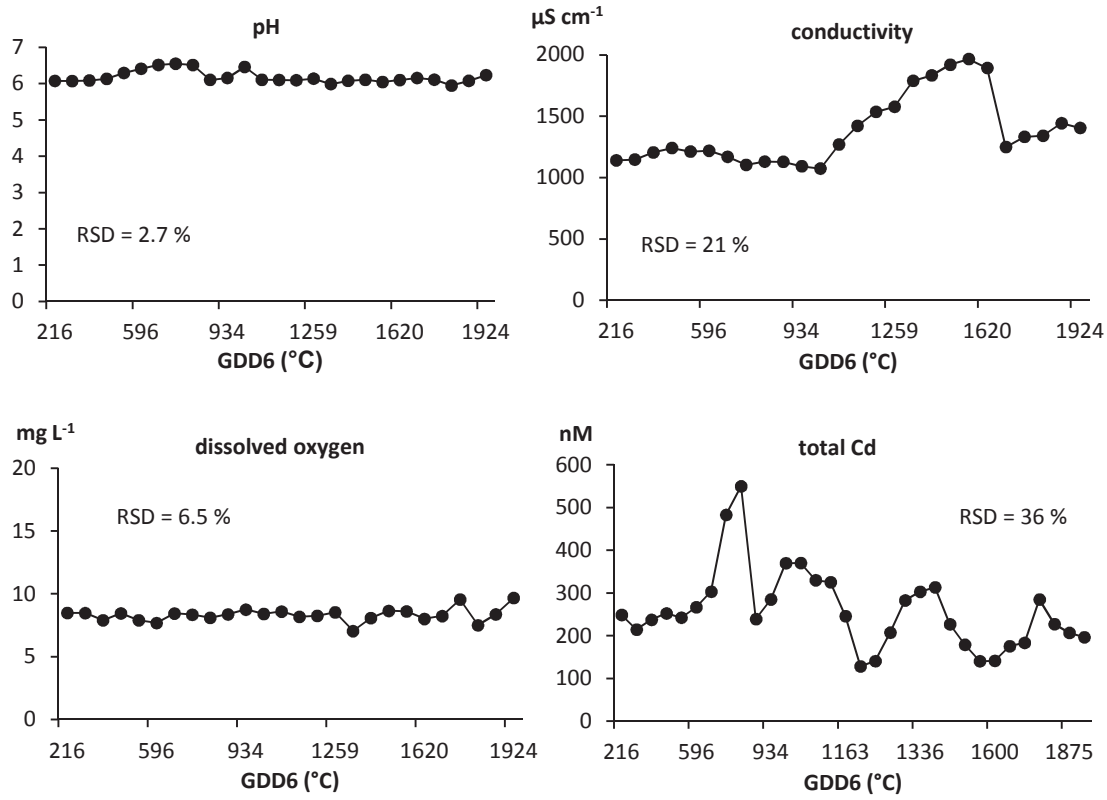

Figure 6.2. Time course of the pH, dissolved O_2 concentration (mg L^{-1}), conductivity (µS cm^{-1}) and total Cd concentration (nM) of the nutrient solution during the growth of sunflowers from transplantation of the seedlings to seed maturity.

Plants were grown under natural light + twelve additional lamps (400 W each) functioning for 8 h per day until the flower bud stage (daily maximum photosynthetically active radiation typically between 1200 - 1400 µmoles cm^{-2} s^{-1}). Except some little frequent peaks around 40 °C, the air temperature was maintained in the range of 15 - 35 °C by a fog and by opening the roof of the greenhouse. The air relative humidity was between 30 - 80 %.

The climatic data were recorded every 30 min by a data logger + computer system (CR23X Campbell Scientific, UK). The growing degree days (GDD, °C) were used as a thermal time scale according to Equation 1:

$$\mathrm{GDD} = \sum^{n}(\mathrm{Temp} - \mathrm{Tbase}) \qquad (1)$$

where T_{emp} is the average air temperature in the greenhouse and T_{base} = 6 °C is the base temperature for sunflowers growth.

Starting at flowering and until no more pollen was produced, the flowers were brushed daily with a soft brush to facilitate the pollination. A unique brush was used to favour cross-pollination.

6.2. TRANSPIRATION TREATMENT AND EXPOSURE OF PLANTS TO ^{111}Cd

At the flower bud and grain filling stages (800 GDD, stage BBCH 57 and 1600 GDD, stage BBCH 75, respectively), ten plants per growth stage were exposed to ^{111}Cd (Figure 6.3). The five control plants were left as is whereas four fans were installed in front of the five "high transpiration" plants. This gave a factorial design crossing the growth stage (flower bud and grain filling) with the transpiration treatment (control, high) with five replicates by combination. For the exposure to ^{111}Cd, the root system of the plant was first drained, and the whole plant was weighted (± 0.001 g). Then the root system was bathed for 10 min into 5 L of 5 mM $CaCl_2$ at 2 °C to remove the extracellular Cd with the natural abundance signal (12.8 %) sorbed onto the apoplast.[4]

Afterwards, the root system was drained and installed into a pot with new nutrient solution having the same composition as during the previous cultivation period, but where the ^{111}Cd abundance was 92.69 ± 0.34 % (certified values shown in Table 6.1, Trace Sciences International Corp., Ontario, Canada).

Figure 6.3. Plants exposed and not exposed to fans to increase transpiration, at flower bud (left, BBCH 57) and grain filling (right, BBCH 75) stages.

Table 6.1. Theoretical abundances of stable isotopes of Cd (%).

	^{106}Cd	^{108}Cd	^{110}Cd	^{111}Cd	^{112}Cd	^{113}Cd	^{114}Cd	^{116}Cd
Natural	1.25	0.89	12.49	12.8	24.13	12.22	28.73	7.49
Enriched	0.02	0.02	0.5	95.5	2.12	0.59	1.1	0.15

The exposure to ^{111}Cd and to the fans lasted 72 h during which the nutrient solution level of each pot was daily checked and maintained by adding manually new labelled solution, the total amount added being determined by weighing.

6.3. PLANT SAMPLING

Plant sampling was carried out at five different growth stages (Figures 6.4 and 6.5), two of which corresponded to the transpiration treatment and exposure to ^{111}Cd (see Section 6.2). At each sampling, the root system of each plant was bathed for 10 min into 5 L of 5 mM $CaCl_2$ at 2 °C to remove the extracellular Cd.[4] After having drained the root system, the whole plants and the labelled nutrient solution remaining in the pot were weighted (± 0.001 g).

In the case of plants labelled with ^{111}Cd (stages BBCH 57 and BBCH 75), the total uptake of solution during the 72 h of exposure could be calculated from the small difference in the weight of the nutrient solution in the pots between the start and the end of the exposure to ^{111}Cd and from the amount of solution added. The total amount of water transpired was calculated as the difference between the nutrient solution taken up during the three days of exposure to ^{111}Cd and the water incorporated into the growth of the plant during the 72 h of exposure to the ^{111}Cd.

Figure 6.4. Sunflowers at each harvesting time. In order of growth, (from left to right and from top to bottom) flower bud (BBCH 57), early grain filling (BBCH 71), middle grain filling (BBCH 75), late grain filling (BBCH 85) and maturity (BBCH 89) stages.

This quantity of water incorporated in the plant growth was obtained from the change in the plant fresh weight during the exposure to ^{111}Cd and from the whole plant water content, determined as explained below. The transpiration was normalized by the leaf area (see below) or by the root dry mass.

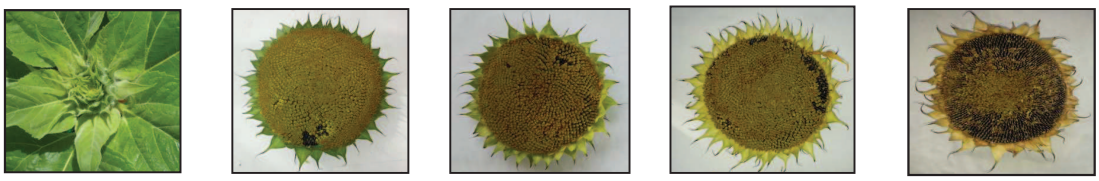

Figure 6.5. Heads of sunflowers at each harvesting time. From left to right: flower bud (BBCH 57), early grain filling (BBCH 71), middle grain filling (BBCH 75), late grain filling (BBCH 85) and maturity (BBCH 89) stages.

At each sampling, sunflowers were divided into the following plant parts: the roots, the taproot (main root + the hypocotyle), the stem, the leaves (common parts at all growth stages), the flower bud (sampling 1 only), and the receptacle, the flowers (sepals + petals of tubular flowers), and the seeds from samplings 2 to 5. Seeds were divided into non-developed seeds (seeds under development or aborted), empty seeds (no almond inside), husks of the full seeds (husks of the seeds with an almond) and the almonds.

A test carried out before the experiment revealed that Cd was not significantly accumulated in the petals of the ligulated flowers (≈ 0.006 mg kg^{-1} in dry mass) and therefore, because some of them were lost during the cultivation, the remaining ones were discarded. The plant leaf area (in cm^2) was measured at harvest using a planimeter (LI-3100C Area Meter, LI-COR, USA).

Each plant part was individually weighed (± 0.01 g) to obtain the fresh weight, and dried in an oven at 50 °C until constant weight, which allowed us to determine the water content and dry weight of each plant organ.

6.4. CHEMICAL ANALYSES

Dried plant samples were ground and homogenized by using a planetary ball mill (PM 400, Retsch, Germany). Around 0.25 g of powdered samples was accurately weighed (± 0.0001 g) in Teflon digestion tubes (50 mL) and a mixture of 4 mL H_2O_2 and 1 mL HNO_3 (69 %, Merck, Tracepur, Germany) was added to the powder.

The mixture was left at ambient temperature in a laminar flow hood for 12 h for preliminary digestion. Then, the digestion was carried on in a mid temperature graphite digestion block system (DigiPREP MS, 115V/230V, SCP Science, Canada) with the following heating program:

In a first step, the temperature was increased to 60 °C in 27 min and held constant for 10 min. In a second step, the temperature was increased to 90 °C in 30 min and kept constant during 15 min. Finally, the temperature was increased to 120 °C in 30 min and maintained for 60 min.

After cooling, the samples were filtered on paper disks and their volume was adjusted to 50 mL with ultrapure (type 1) water (18.2 MΩ cm, Synergy® Water Purification System, Merck Millipore, USA). The extracts were stored in darkness at ambient temperature. The total Cd and Zn concentrations in the digested solutions were determined by atomic absorption spectrometry (AAS, PinAAcle 900T, Perkin Elmer) equipped with a graphite furnace (GF). The operating conditions for phase separation and the heating program of graphite furnace are shown in Table 6.2. The aliquots of nutrient solution were also analyzed in the same way after being diluted and acidified at 2 % with HNO_3.

Procedural blanks and replicates of a certified reference material (NIST SRM 1573a – tomato leaves) were also processed as samples for blank correction, estimation of limits of detection (in mg kg^{-1}) and quality control, with satisfactory results (for Cd: repeatability of 2 % and recovery of 93 %; for Zn: repeatability of 5 % and recovery of 89 %, mean values of the whole experiment).

Table 6.2. GF-AAS operating conditions and limits of detection (LOD, in mg kg^{-1}) obtained for Cd and Zn.

Parameter	Value
Injection volume	20 µL
Flushing gas	Nitrogen
Nitrogen flow	250 mL min^{-1}
Wavelength (nm)	228.80 (Cd); 213.86 (Zn)
Light source (type)	C-EDL (Cd); C-HCL (Zn)
Lamp current (mA)	230 mA (Cd); 30 mA (Zn)
Sample vaporization	Graphite-coated tube
Background correction	D_2
Furnace temperature program	2 min
Drying stage	110 – 600 °C
Atomization stage	600 – 1600 °C
Clean-out stage	2450 °C
Cd LOD	0.015 mg kg^{-1}
Zn LOD	0.78 mg kg^{-1}

All the stable isotopes of Cd (^{106}Cd, ^{108}Cd, ^{110}Cd, ^{111}Cd, ^{112}Cd, ^{113}Cd, ^{114}Cd and ^{116}Cd) were measured in each digestion solution at the flower bud and grain filling samplings by high resolution ICP-MS at the GET laboratory (Géosciences Environnement Toulouse) in Toulouse, France (http://www.get.obs-mip.fr/). The uncertainties of each isotope measurements were between 1 and 4 %, and the detection limits are indicated in Table 6.3, estimated as the average plus three times the standard deviation of the replicate analysis of procedural blanks.

A standard solution of 250 ng kg^{-1} Cd with natural abundances of all the stable isotopes of Cd was measured every 8 samples to control and correct the drift and sensitivity of the analyses. The isotopic ratios between ^{111}Cd and the rest of stable isotopes were calculated, as shown in Figure 6.6. The isotopic ratios of ^{111}Cd were correct for ^{110}Cd, ^{113}Cd and ^{114}Cd.

Table 6.3. Limits of detection (LOD, in ng kg^{-1}) for each stable isotope of Cd in the high resolution ICP-MS.

Isotope	LOD (ng kg^{-1})
^{106}Cd	25.1
^{108}Cd	16.8
^{110}Cd	3.0
^{111}Cd	1.5
^{112}Cd	13.2
^{113}Cd	6.4
^{114}Cd	5.2
^{116}Cd	7.5

The ratio between the amount of Cd derived from the labelling (q_L) and the amount of Cd initially in the sample before the labelling (q_N) was calculated from the ^{114}Cd/^{111}Cd ratio of the sample following the Equation 2:[5,6]

$$\frac{q_N}{q_L} = \frac{M_N}{M_L}\frac{{A_L}^{111}}{{A_N}^{111}}\frac{R_L - R_{PL}}{R_{PL} - R_N} \qquad (2)$$

where M_N and M_L are the molar mass (g mol^{-1}) of the Cd initially in the sample (natural abundance) and derived from labelling, respectively, ${A_N}^{111}$ is the natural abundance of ^{111}Cd and ${A_L}^{111}$ is the abundance of ^{111}Cd in the enriched source, R_N is the natural ^{114}Cd/^{111}Cd ratio and R_L is the ratio in the enriched solution, and R_{PL} is the ^{114}Cd/^{111}Cd ratio in the plant sample.

From the equation 2, the amount of Cd derived from labelling in the plant sample can be calculated, as follows (Equation 3):

$$q_L = \frac{1}{(q_N/q_L)+1} q_{tot} \qquad (3)$$

where q_{tot} is the total amount of Cd in the plant sample ($q_L + q_N$), determined by atomic absorption spectrometry.

To check if the ^{114}Cd/^{111}Cd ratios were correctly determined in the digestion solutions for the different plant organs, known amounts of ^{111}Cd were added to non-enriched digestion solutions to obtain ^{114}Cd/^{111}Cd ratios ranging from 2.2445 to 0.2878.

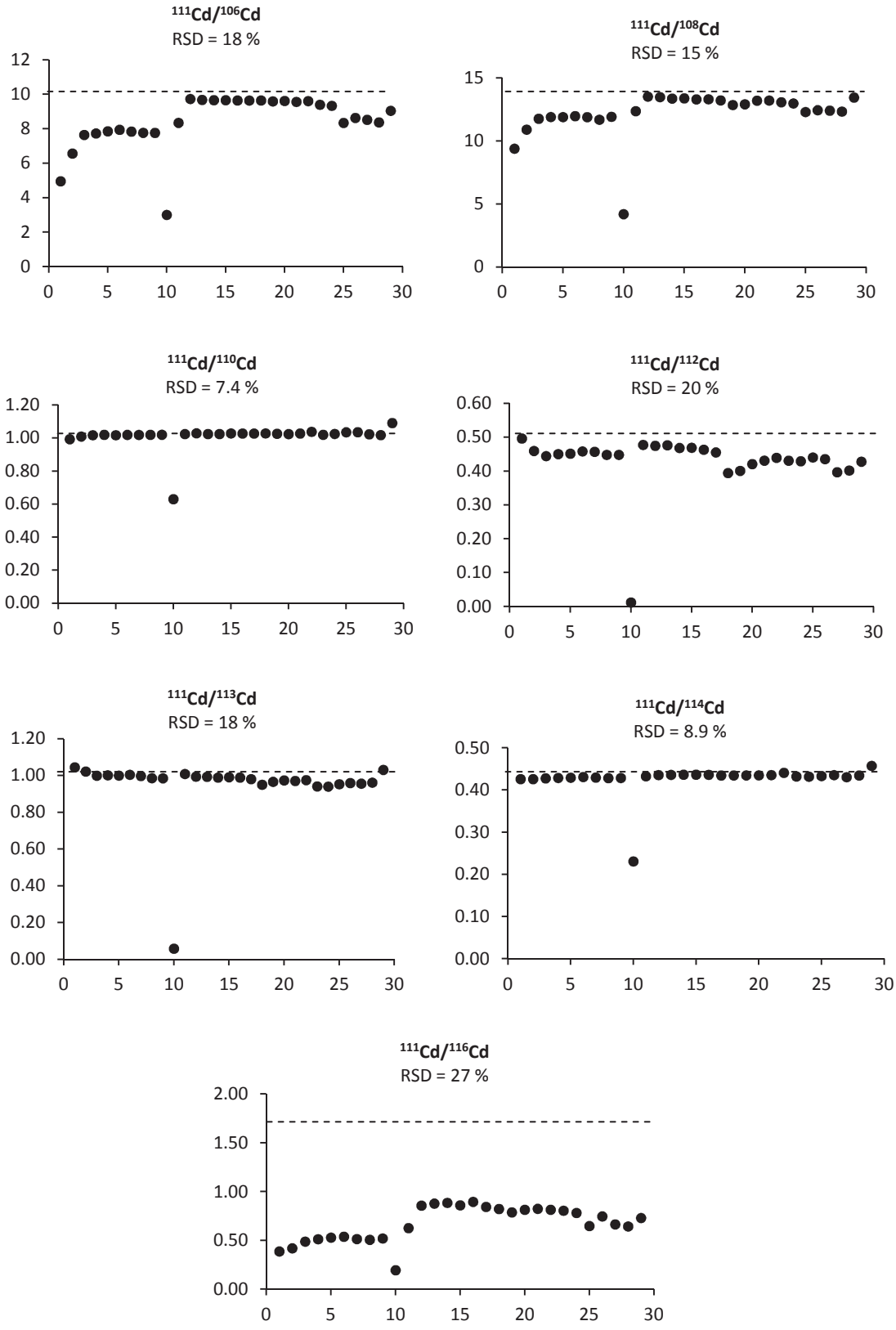

Figure 6.6. Observed isotope ratios with ^{111}Cd. The dashed line corresponds to the ratio expected from the natural abundances (see Table 6.1). The standard 10 was considered an outlier probably due to an error in the sample preparation.

The observed $^{114}Cd/^{111}Cd$ ratios against theoretical ones are plotted in Figure 6.7, obtaining a single regression line valid for all plant organs, with a determination coefficient (R^2) of 0.98.

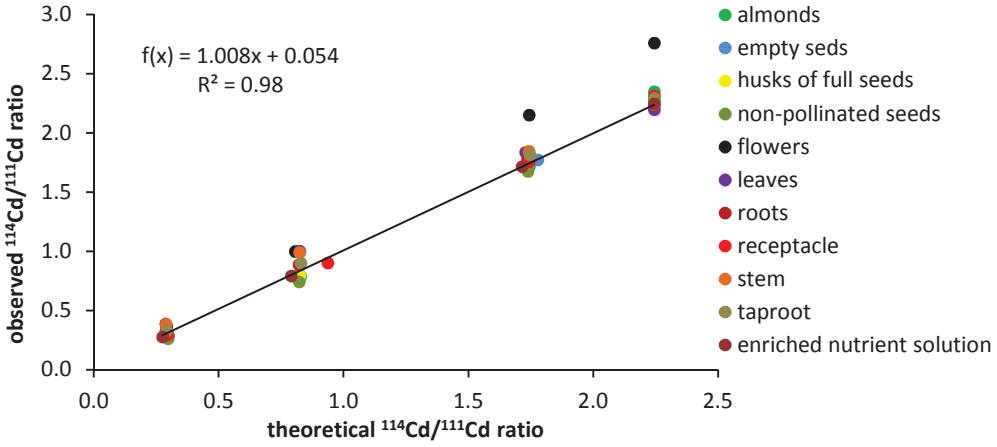

Figure 6.7. Observed $^{114}Cd/^{111}Cd$ ratios against theoretical ones. The slope ($p < 0.001$) and the intercept ($p < 0.05$) values of the regression line do not significantly differ from 1 and 0, respectively.

6.5. MODELLING OF Cd ALLOCATION BETWEEN PLANT ORGANS

We developed a compartment model for the allocation of the Cd derived from the labelling (thereafter the "Cd recently taken up") between the different plant parts (Figure 6.8). The Cd recently taken up by the root system, which corresponded to the labelled Cd recovered in each of the whole plant, was first partitioned between the sequestration in roots and the exportation to the taproot, which in turn sequestrated a fraction of it and exported the remaining.

After the taproot we tried three scenarios, i) sequestration in the stem and then in the leaves, ii) sequestration in the leaves and then in the stem or iii) sequestration in the stem and in the leaves in parallel. For this latter case, the stem and the leaves competed with each other for the sequestration of the Cd recently taken up not sequestrated in the roots and in the taproot.

Afterwards, the Cd recently taken up not sequestrated in the roots, in the taproot, in the stem and in the leaves was allocated to the receptacle, and the Cd recently taken up not sequestrated by the receptacle was partitioned between the flowers, the non-developed seeds, the empty seeds and the full seeds (almonds + their husks) in parallel. Finally, the Cd recently taken up of the full seeds was partitioned between the husks and the almonds. The general flow chart for the allocation of the Cd recently taken up is shown in Figure 6.8.

The amount q_i (µg) of labelled Cd sequestrated in a given compartment *i* was assumed to be a linear function of the amount of Cd recently taken up exported by the previous compartment (E_i) and of the absolute or relative biomass of the compartment (DM_i). This gives two possible equations:

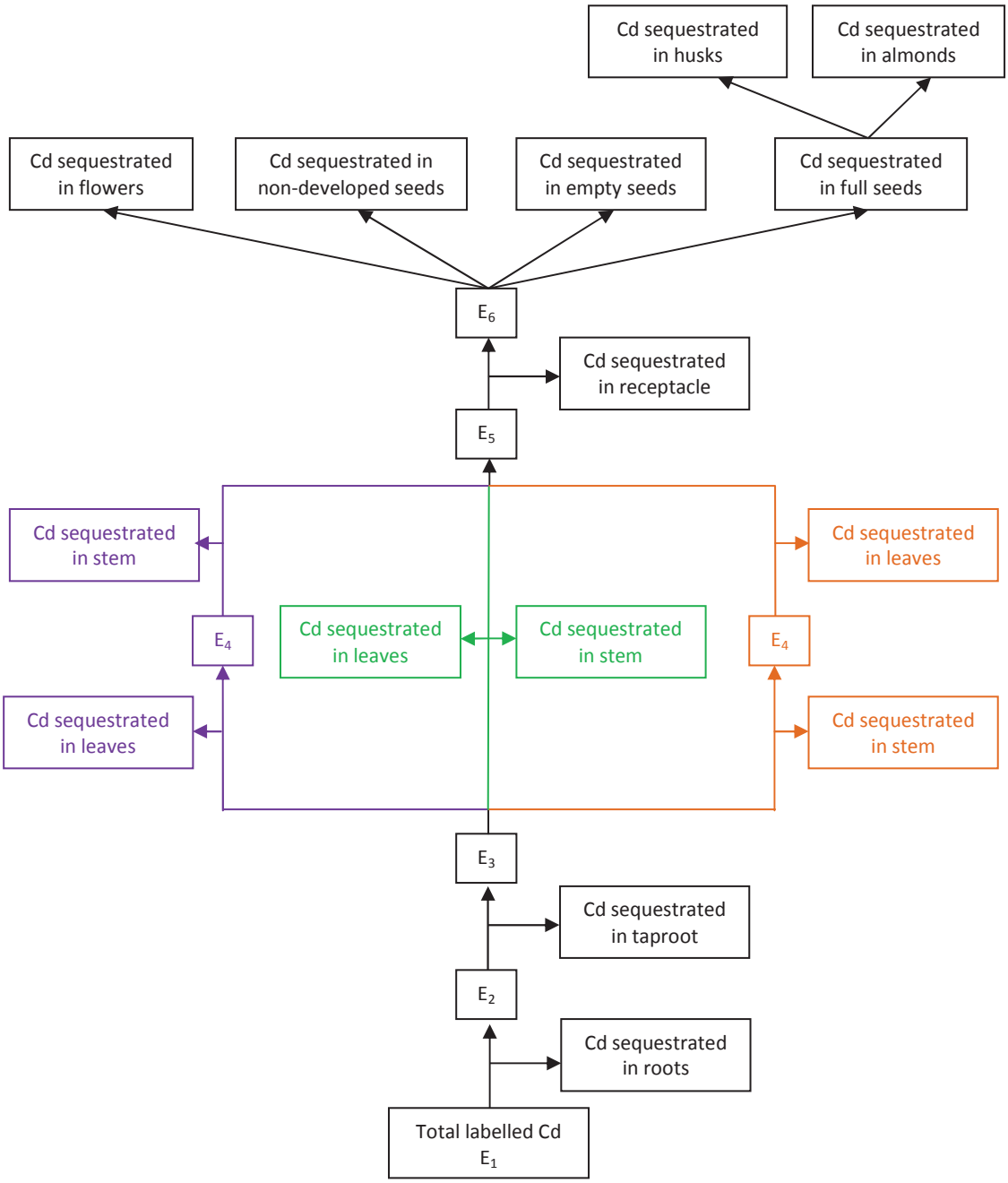

Figure 6.8. Flow chart for the allocation of the Cd recently taken up between different plant organs. Roots, taproot and receptacle are compartments in series while flowers and seeds are organs in parallel. Stem and leaves could be considered organs in series or in parallel depending on the model tested (the colours indicate the three scenarios tested). E_i corresponds to the amount of labelled Cd exported by the previous compartment.

If there are no competitive sinks, the amount of Cd sequestrated in a compartment is (Equation 4):

$$q_i = k_i \, DM_i \, E_i \qquad (4)$$

where k_i is an adjustable parameter.

If there are competitive sinks, the amount of Cd sequestrated in a compartment is (Equation 5):

$$q_i = k_i \frac{DM_i}{DM_i + DM_c} E_i \qquad (5)$$

where DM_c is the biomass of the competitive compartment(s).

Considering the relative biomass allowed us to take into account the competition between the compartment *i* and some other compartments. The two models were fitted to the observed values of labelled Cd in a given compartment, considering the different possible competitive plant organs.

6.6. DATA PROCESSING

As a whole, the data set obtained consisted of 325 plant samples (5 plants per sampling + 5 extra plants for each transpiration treatment). The categorical variables taken into account were the transpiration treatments, the growth stages (expressed as GDD) and the plant organs.

Analysis of Variance (ANOVA) was used to test the significance of the effect of the transpiration treatment, of the growth stage and of the plant organ on different variables. Data were log-transformed when necessary to obtain normal distributions for the errors.

Linear Regression Analysis was used to fit the different compartment models to the observed values of Cd recently taken up (derived from the labelling with ^{111}Cd). Data processing was carried out by using the software R 3.2.2 (R Core Team, 2015) with RStudio v. 0.99.441 (RStudio, Inc., USA).

6.7. REFERENCES

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Chapter 7

RESULTS AND DISCUSSION:

SHORT-TERM PARTITIONING OF Cd RECENTLY TAKEN UP BETWEEN SUNFLOWERS ORGANS (*Helianthus annuus*) AT FLOWERING AND GRAIN FILLING STAGES: EFFECT OF PLANT TRANSPIRATION AND ALLOMETRY

7.1. RESULTS

7.1.1. PLANT BIOMASS AND TRANSPIRATION

Whatever the growth stage, the fans had no significant effect ($p > 0.05$) on the total plant and organ biomass, the surface area of green leaves, the change in the plant fresh weight during the three days of labelling, and the plant water content (Table 7.1). By contrast, the use of fans resulted in a significant increase ($p < 0.05$) in the plant water uptake and transpiration, which were both enhanced by on average 20 %.

All the plant variables mentioned above changed significantly with the growth stage. Between the flower bud and grain filling stages, the biomass of all organs increased as well as the surface area of the green leaves but the water uptake by root mass unit, the transpiration, the plant water content and the change in biomass during the duration of the labelling decreased significantly ($p < 0.01$) (Table 7.1).

Table 7.1. Effect of the growth stage (flower bud and grain filling) and of the transpiration treatment (control and high) on plant growth variables (n = 5, mean and standard deviation values are given). Different lowercase letters indicate significant differences between the control and the high transpiration treatments for each growth stage whereas different uppercase letters indicate significant differences between the two growth stages for each transpiration treatment (ANOVA, no letter $p > 0.05$, ^{a,A} $p < 0.05$, ^{b,B} $p < 0.01$, ^{c,C} $p < 0.001$).

	Flower bud		**Grain filling**	
	Control	**High**	**Control**	**High**
Water uptake (L H_2O g $root^{-1}$)	0.37^{aB} ± 0.050	0.436^{aB} ± 0.039	0.235^{aB} ± 0.039	0.312^{aB} ± 0.048
Transpiration (L H_2O m^{-2} day^{-1})	2.87^{cC} ± 0.15	3.60^{cC} ± 0.25	1.75^{aC} ± 0.30	2.18^{aC} ± 0.13
Leaves area (cm^2)	4730^{B} ± 770	4390^{B} ± 510	7400^{B} ± 1300	7300^{B} ± 1300
Water content (%)	92.48^{C} ± 0.42	92.25^{C} ± 0.28	85.83^{C} ± 0.72	85.61^{C} ± 0.54
Total plant dry weight (g)	53.9^{C} ± 8.0	54.2^{C} ± 7.2	179^{C} ± 45	204^{C} ± 24
Change in plant fresh weight (g)	221^{C} ± 40	194^{C} ± 38	-158^{C} ± 46	-145^{C} ± 35
Roots dry weight (g)	11.6^{A} ± 1.4	11.4 ± 2.4	16.6^{A} ± 3.6	15.5 ± 3.8
Taproot dry weight (g)	4.98^{C} ± 0.59	5.71^{C} ± 0.56	14.4^{C} ± 3.2	14.85^{C} ± 0.85
Stem dry weight (g)	20.7 ± 3.8	20.9^{C} ± 2.6	43 ± 27	65.0^{C} ± 6.6
Leaves dry weight (g)	16.6^{C} ± 2.7	16.1^{C} ± 2.2	30.5^{C} ± 4.3	30.3^{C} ± 4.2
Flower bud dry weight (g)	0.094 ± 0.043	0.130 ± 0.046	-	-
Receptacle dry weight (g)	-	-	23.2 ± 5.8	24.9 ± 4.2
Flowers dry weight (g)	-	-	5.34 ± 0.81	5.30 ± 0.54
Non-developed seeds dry weight (g)	-	-	2.4 ± 1.4	2.0 ± 1.8
Empty seeds dry weight (g)	-	-	3.3 ± 1.4	1.88 ± 0.69
Husks of full seeds dry weight (g)	-	-	16.3 ± 5.9	18.9 ± 2.5
Almonds dry weight (g)	-	-	23.8 ± 5.7	25.1 ± 6.4
Belowground:aboveground ratio	0.447 ± 0.044	0.461 ± 0.029	0.216 ± 0.046	0.174 ± 0.012

The change in the biomass during the three days of exposure to ^{111}Cd became negative at the grain filling stage, indicating a shift from a net gain to a net loss when the plants became older. The belowground to aboveground biomass ratio decreased by half between the two growth stages.

7.1.2. TOTAL AND RECENTLY TAKEN UP Cd IN THE WHOLE PLANTS AND IN THE DIFFERENT PLANT ORGANS

7.1.2.1. TOTAL Cd

The transpiration treatment had no significant effect on the concentration of total Cd (mg kg^{-1}) and on the amount of total Cd (µg) in the whole plants and in the plant organs (Tables 7.2 and 7.3), except in the case of the stem for which the fans decreased significantly the amount of Cd at the flower bud stage ($p < 0.05$), without affecting the concentration.

Table 7.2. Effect of the growth stage and of the transpiration treatment on the concentration of total Cd in the plant organs (n = 5, mean and standard deviation values are given). Different lowercase letters indicate significant differences between the control and the high transpiration treatments for each growth stage whereas different uppercase letters indicate significant differences between the two growth stages for each transpiration treatment (ANOVA, no letter $p > 0.05$, ^{a,A} $p < 0.05$, ^{b,B} $p < 0.01$, ^{c,C} $p < 0.001$).

	Total Cd concentration (mg kg^{-1})			
	Flower bud		**Grain filling**	
	Control	**High**	**Control**	**High**
Total plant Cd	0.316 ± 0.021	0.267 ± 0.024	0.124 ± 0.010	0.119 ± 0.016
Roots	0.62^{C} ± 0.17	0.518^{C} ± 0.085	0.280^{C} ± 0.046	0.260^{C} ± 0.021
Taproot	0.32^{C} ± 0.10	0.243^{C} ± 0.031	0.1051^{C} ± 0.0062	0.1100^{C} ± 0.0093
Stem	0.108^{A} ± 0.016	0.080^{C} ± 0.015	0.063^{A} ± 0.039	0.0353^{C} ± 0.0028
Leaves	0.385 ± 0.063	0.367 ± 0.027	0.350 ± 0.058	0.351 ± 0.010
Flower bud	0.146 ± 0.035	0.127 ± 0.017	-	-
Receptacle	-	-	0.0462 ± 0.025	0.0477 ± 0.0051
Flowers	-	-	0.0834 ± 0.052	0.079 ± 0.015
Non-developed seeds	-	-	0.071 ± 0.018	0.080 ± 0.015
Empty seeds	-	-	0.0646 ± 0.0063	0.077 ± 0.012
Husks of full seeds	-	-	0.050 ± 0.032	0.0427 ± 0.0053
Almonds	-	-	0.094 ± 0.010	0.103 ± 0.011

By contrast, between the two growth stages, for most of the plant organs, the total Cd concentration decreased significantly by around 50 % ($p < 0.05$), except for leaves ($p > 0.05$), for which the Cd concentration remained stable (Table 7.2). The general ranking of the plant organs for the concentration of Cd was roots > leaves > taproot > stem for the vegetative organs and for the head organs, which were in general less concentrated in Cd, the ranking was flower bud > almonds > flowers ≈ non-developed seeds > empty seeds > husks of full seeds ≈ receptacle.

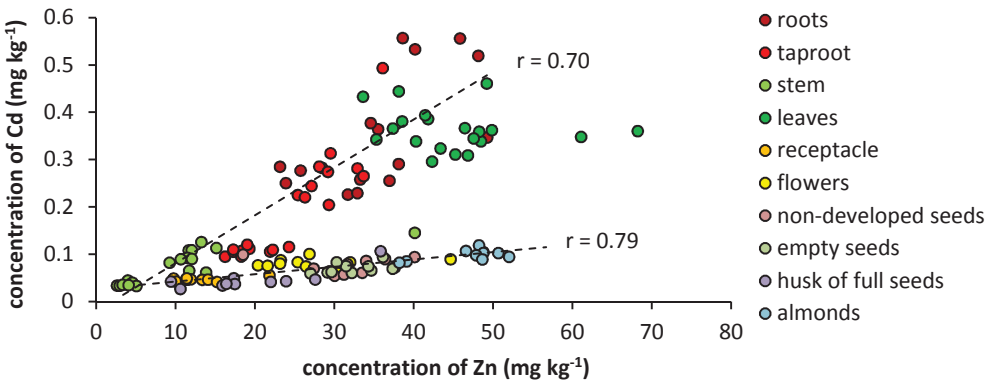

Figure 7.1. Correlation between the concentrations of Cd and Zn in different organs. A linear relationship was observed i) for the receptacle, flowers and parts of seeds (n = 60; at 95 % confidence level, $r_{crit} = 0.25$, r = 0.79) and ii) for the roots, taproot, stem and leaves (n = 80; at 95 % confidence level, $r_{crit} = 0.22$, r = 0.70).

Table 7.3. Effect of the growth stage and of the transpiration treatment on the total amount of Cd in plant organs (n = 5, mean and standard deviation values are given). Different lowercase letters indicate significant differences between the control whereas the high transpiration treatments for each growth stage and different uppercase letters indicate significant differences between the two growth stages for each transpiration treatment (ANOVA, no letter $p > 0.05$, ^{a,A} $p < 0.05$, ^{b,B} $p < 0.01$, ^{c,C} $p < 0.001$).

	Total amount of Cd (µg)			
	Flower bud		**Grain filling**	
	Control	**High**	**Control**	**High**
Total plant Cd	17.5^{A} ± 4.2	15.0^{B} ± 3.1	23.9^{A} ± 3.9	23.8^{B} ± 3.2
Roots	7.1^{B} ± 1.6	6.0 ± 1.8	4.54^{B} ± 0.48	4.01 ± 0.87
Taproot	1.63 ± 0.76	1.39 ± 0.22	1.52 ± 0.40	1.64 ± 0.20
Stem	2.26^{a} ± 0.71	1.66^{a} ± 0.37	2.07 ± 0.69	2.30 ± 0.28
Leaves	6.4^{A} ± 1.8	6.0^{C} ± 1.2	10.7^{A} ± 2.6	10.6^{C} ± 1.4
Flower bud	0.0128 ± 0.030	0.0163 ± 0.0058	-	-
Receptacle	-	-	1.08 ± 0.29	1.18 ± 0.16
Flowers	-	-	0.447 ± 0.084	0.415 ± 0.064
Non-developed seeds	-	-	0.159 ± 0.090	0.14 ± 0.10
Empty seeds	-	-	0.208 ± 0.086	0.149 ± 0.073
Husks of full seeds	-	-	0.93 ± 0.88	0.80 ± 0.12
Almonds	-	-	2.22 ± 0.58	2.56 ± 0.61

It is noteworthy that for the organs of the head of the plant (receptacle, flowers and seeds) there was a single linear relationship between the concentrations of Cd and of Zn in the tissues (r = 0.79, Figure 7.1), which can be interpreted by the fact that for all these organs Cd and Zn moves together and in one direction flow, upwards likely by phloem with a little contribution of xylem. By contrast, for the roots, taproot, stem and leaves, the more scattered and different relationship between Cd and Zn (r = 0.70, Figure 7.1) could indicate that the flows are likely to be bidirectional, i.e. upwards in the xylem corresponding to the uptake and downwards due to the reallocation through the phloem.

7.1.2.2. Cd RECENTLY TAKEN UP

The fans had little effect on the distribution of the Cd derived from labelling thereafter called the Cd recently taken up (Table 7.4). The only significant changes were i) a decrease in the uptake of Cd by root mass unit at the flower bud stage by almost a factor of 2 ($p < 0.05$) and ii) at the grain filling stage a slight increase in the sequestration of the Cd recently taken up in roots ($p < 0.05$) and a decrease in the allocation to leaves and to the empty seeds ($p < 0.05$).

Again, the growth stage had a much stronger effect on the partitioning of the Cd recently taken up (Table 7.4).

Between the flower bud stage and the grain filling stage, the uptake of labelled Cd decreased more markedly (by a factor of 2) when it was normalized by the root dry mass ($p < 0.01$). Also, between the flower bud and the grain filling stages, the allocation of the Cd recently taken up to roots ($p < 0.05$) and to stem ($p < 0.001$) decreased and the allocation to leaves increased ($p < 0.001$). The changes were particularly strong for the leaves and for the stem.

Roots were the major sink for the Cd recently taken up, accumulating around 50 to 70 % of it (Table 7.4). Moreover, a strong relationship was observed between the total amount of Cd recently taken up and the part of it recovered in roots, by growth stage (Figure 7.2, n= 10, at 95 % confidence level, $r_{crit} = 0.63$), showing the decrease in the uptake and in the sequestration of labelled Cd in roots when plants became older. At the flower bud stage, the taproot and the leaves were the second most important sinks but at the grain filling stage, leaves became the only second sink before the taproot. The stem was then the smallest sink among the vegetative parts.

The head organs (receptacle, flowers and seeds) were very small sinks for the Cd recently taken up, with the almonds being the strongest sink, containing about 2 % of the total labelled Cd.

Table 7.4. Effect of the growth stage and of the transpiration treatment on the total amount of labelled Cd taken up and on its partitioning between plant organs (expressed as % of labelled Cd recovered, n = 5, mean and standard deviation values are given). Different lowercase letters indicate significant differences between the control and the high transpiration treatments by growth stage whereas different uppercase letters indicate significant differences between the two growth stages by transpiration treatment (ANOVA, no letter $p > 0.05$, ^{a,A} $p < 0.05$, ^{b,B} $p < 0.01$, ^{c,C} $p < 0.001$).

	Flower bud		**Grain filling**	
	Control	**High**	**Control**	**High**
Total labelled Cd uptake (µg)	$4.9^{B} \pm 1.1$	3.2 ± 1.5	$2.54^{B} \pm 0.51$	2.35 ± 0.35
Total labelled Cd/root dry mass (µg Cd g root^{-1})	$0.43^{aB} \pm 0.13$	$0.275^{aB} \pm 0.062$	$0.156^{B} \pm 0.034$	$0.157^{B} \pm 0.040$
Labelled Cd in % of the total recovered				
Roots	$71^{A} \pm 12$	65.9 ± 7.3	$51.0^{aA} \pm 6.5$	$60.6^{a} \pm 5.4$
taproot	9.8 ± 6.4	13.2 ± 3.0	9.3 ± 1.8	11.5 ± 2.1
Stem	$9.4^{C} \pm 2.6$	$8.3^{C} \pm 3.4$	$3.6^{C} \pm 1.6$	$3.1^{C} \pm 1.3$
Leaves	$9.4^{C} \pm 3.8$	$12.6^{A} \pm 3.8$	$30.0^{aC} \pm 7.6$	$20.8^{aA} \pm 4.7$
Flower bud	0.0283 ± 0.0086	0.048 ± 0.026	-	-
Receptacle	-	-	1.37 ± 0.61	0.77 ± 0.36
Flowers	-	-	0.28 ± 0.18	0.24 ± 0.10
Non-developed seeds	-	-	0.30 ± 0.19	0.17 ± 0.16
Empty seeds	-	-	$0.48^{a} \pm 0.24$	$0.202^{a} \pm 0.081$
Husks of full seeds	-	-	0.91 ± 0.31	0.768 ± 0.048
Almonds	-	-	2.09 ± 0.75	1.96 ± 0.80

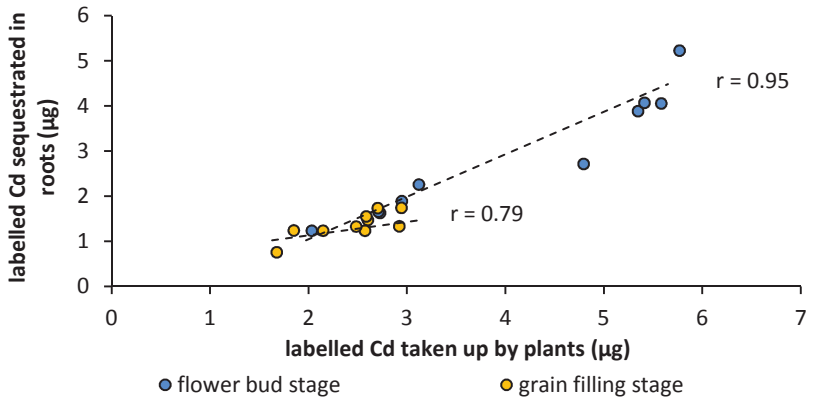

Figure 7.2. Correlation between the Cd recently taken up and the part of it allocated to roots, in µg (n = 10; at 95 % confidence level, r_{crit} = 0.63; r = 0.95 at flower bud stage; r = 0.79 at grain filling stage).

The other important sinks included the receptacle and the husks of the full seeds. The full seeds (the almonds and their husks) contained 67 % of the labelled Cd allocated to the head organs at the grain filling stage.

7.1.2.3. EFFECT OF THE ALLOMETRY BETWEEN PLANT ORGANS ON THE PARTITIONING OF THE Cd RECENTLY TAKEN UP

The amount of Cd taken up during the labelling that was accumulated by a given plant organ was regressed as a function of the biomass of the plant organ, taking into account when necessary the biomass of one or several other plant parts acting as a competitive sinks (see Equations 4 and 5 and Figure 6.8 of Chapter 6).

The objective was to find for each plant organ, a single model that fits all the data for the two transpiration treatments and for all growth stages. All the models tested are listed in Annex C, while the best models are presented in Figures 7.3 and 7.4. They were selected according to several criteria: the goodness of fit by the R^2 of the regression, the value of the intercept that should not be significantly different from zero ($p > 0.05$) according to Equations 4 and 5 of Chapter 6, and the sense it had with respect to the current knowledge about the plant physiology.

For roots, the potential competitive sinks tested for the sequestration of the labelled Cd (Equation 5 of Chapter 6) were one of the followings: the taproot, the stem, the leaves, the head organs (receptacle, flower bud, flowers, empty seeds, full seeds, non-developed seeds), and all plant organs excluding roots. The best model explaining the amount of labelled Cd sequestrated in roots was that considering the relative biomass of roots compared to leaves (R^2 = 0.94, Figure 7.2) and was much better than the model with only the root biomass (R^2 = 0.53, Annex C). The other good models were those considering the taproot or the stem as a competitive sink (R^2 = 0.91), with an intercept also not significantly different from zero. The models with the other plant organs included as a competitive sinks gave poor fits with R^2 values of 0.87 (Annex C).

For the taproot, the competitive sinks tested were the stem, the leaves, the head organs (receptacle, flowers and seeds), and all the aboveground organs excluding or not the leaves. The sequestration of the labelled Cd in the taproot was not correlated to the biomass of this organ alone ($R^2 = 0.01$). The best model was based on the relative biomass of the taproot with all aboveground organs except the leaves as competitive sinks ($R^2 = 0.67$, Figure 7.3).

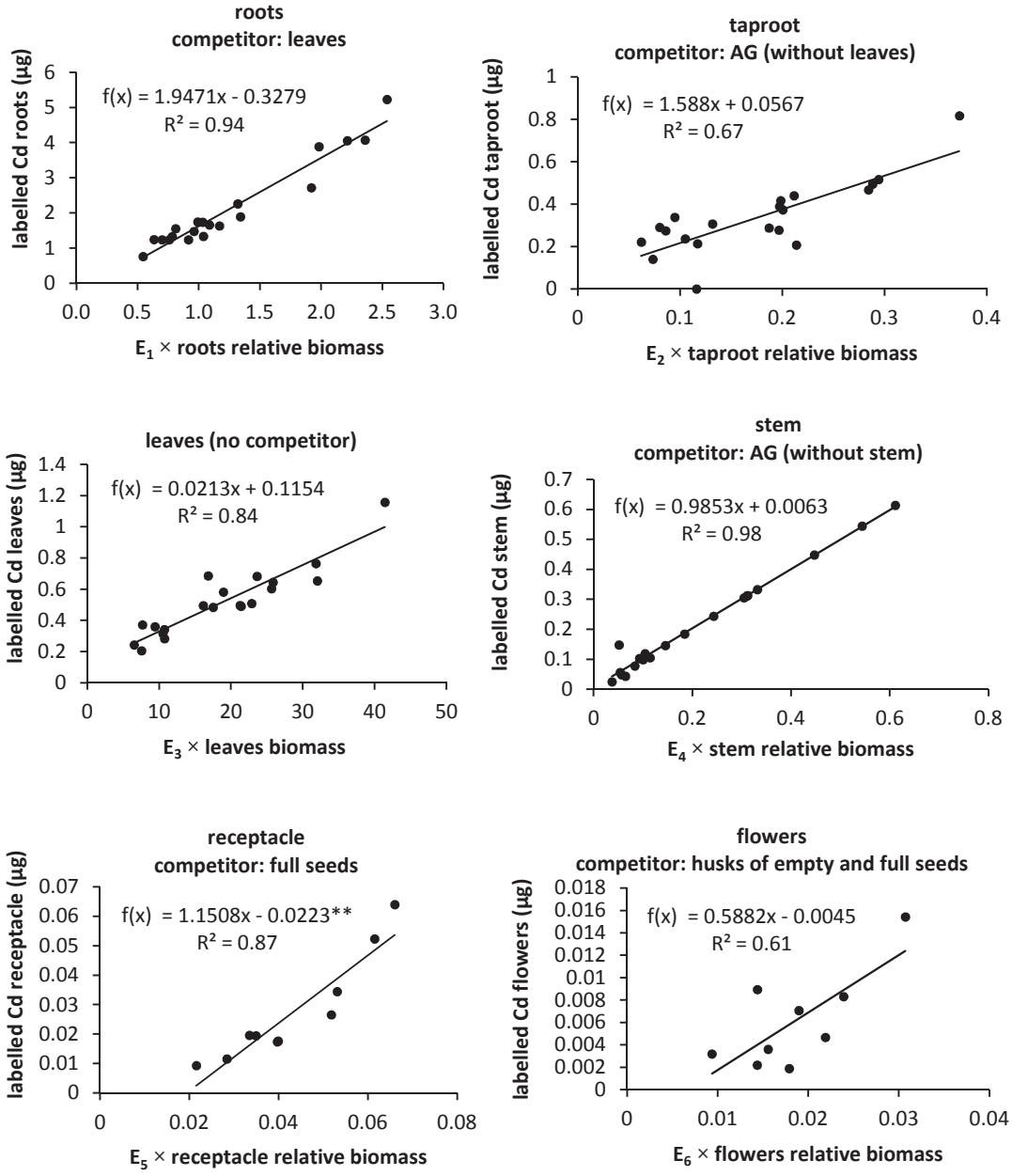

Figure 7.3. The best regression models selected for explaining the partitioning of labelled Cd in each plant organ, following Equations 4 and 5 of Chapter 6 (AG = aboveground organs). E_i indicates the amount of labelled Cd exported by the previous plant organ, according to the general flow chart of labelled Cd described in Figure 6.8. The significance of the intercept is coded as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

It is remarkable that considering the leaves biomass in the aboveground organs decreased slightly the goodness of fit ($R^2 = 0.63$, Annex C), which indicates that the leaves were not influential. The other models had R^2 from 0.09 to 0.55 when considering the leaves and the stem alone as a competitive sink, respectively.

After the taproot, the stem and leaves were considered in parallel or in series. When the stem was considered first, the possible competitive sinks were the leaves, the head organs or both of them together. Conversely, when the leaves were set first, the possible competitive sinks were the stem, the head organs or both of them together (Figure 6.8 of Chapter 6, Annex C).

When the stem was set just after the taproot, the best model was the biomass of the stem with the biomass of the head organs as competitive sink ($R^2 = 0.79$) and then, the best model for leaves after the stem gave a $R^2 = 0.80$ with a positive intercept significantly different from zero (0.23, $p < 0.001$). When leaves were set after the taproot, the best model was obtained when only their biomass was in the model with no competitive sinks ($R^2 = 0.84$). Then, the next best model when the stem was set after the leaves gave $R^2 = 0.98$. Therefore, for the stem and leaves, the best models obtained were when leaves were set before the stem (Figure 7.3). In this case, other models gave lower R^2 (from -0.05 for the stem without competitive sink to 0.29 when considering the stem as competitive sink for the leaves).

For the receptacle, the competitive sinks tested were the flowers, the empty seeds, the full seeds (the almonds and the husks) and the non-developed seeds. The sequestration of the labelled Cd in the receptacle was explained by its relative biomass with the husks of the full seeds as a competitive sink ($R^2 = 0.88$), the intercept being only slightly significantly different from zero (-0.018, $p = 0.02$). Considering the full seeds (husks + almonds) instead of only the husks of the full seeds gave similar results ($R^2 = 0.87$), with an intercept value of -0.022 ($p = 0.01$). The other models gave R^2 from 0.29 to 0.81 (Annex C).

For the allocation of the remaining labelled Cd, namely the amount that was not sequestrated in the roots, taproot, leaves, stem and in the receptacle, we assumed that the rest of the plant organs (the flowers, the non-developed seeds, the empty seeds, and the full seeds) were in parallel after the receptacle (Figure 6.8). For the flowers, the best model was that obtained when the husks of the full seeds and the empty seeds were considered together as competitive sinks ($R^2 = 0.61$). If the almonds were also included as a competitive sink (corresponding as a whole to the full and empty seeds), the goodness of fit decreased ($R^2 = 0.52$) and also if the non-developed seeds were additionally included ($R^2 = 0.46$).

The labelled Cd sequestrated in the non-developed seeds was very well explained by the biomass of this compartment ($R^2 = 0.96$). However, the fit was better when the biomass of the full seeds ($R^2 = 0.99$) or of the almonds ($R^2 = 0.97$) was set as a competitive sink (Figure 7.4).

Regarding the Cd recently taken up that was sequestrated in the empty seeds, two models gave similar results (Figure 7.4): the biomass of the empty seeds alone ($R^2 = 0.91$) or with the biomass of the almonds as a competitive sink ($R^2 = 0.92$).

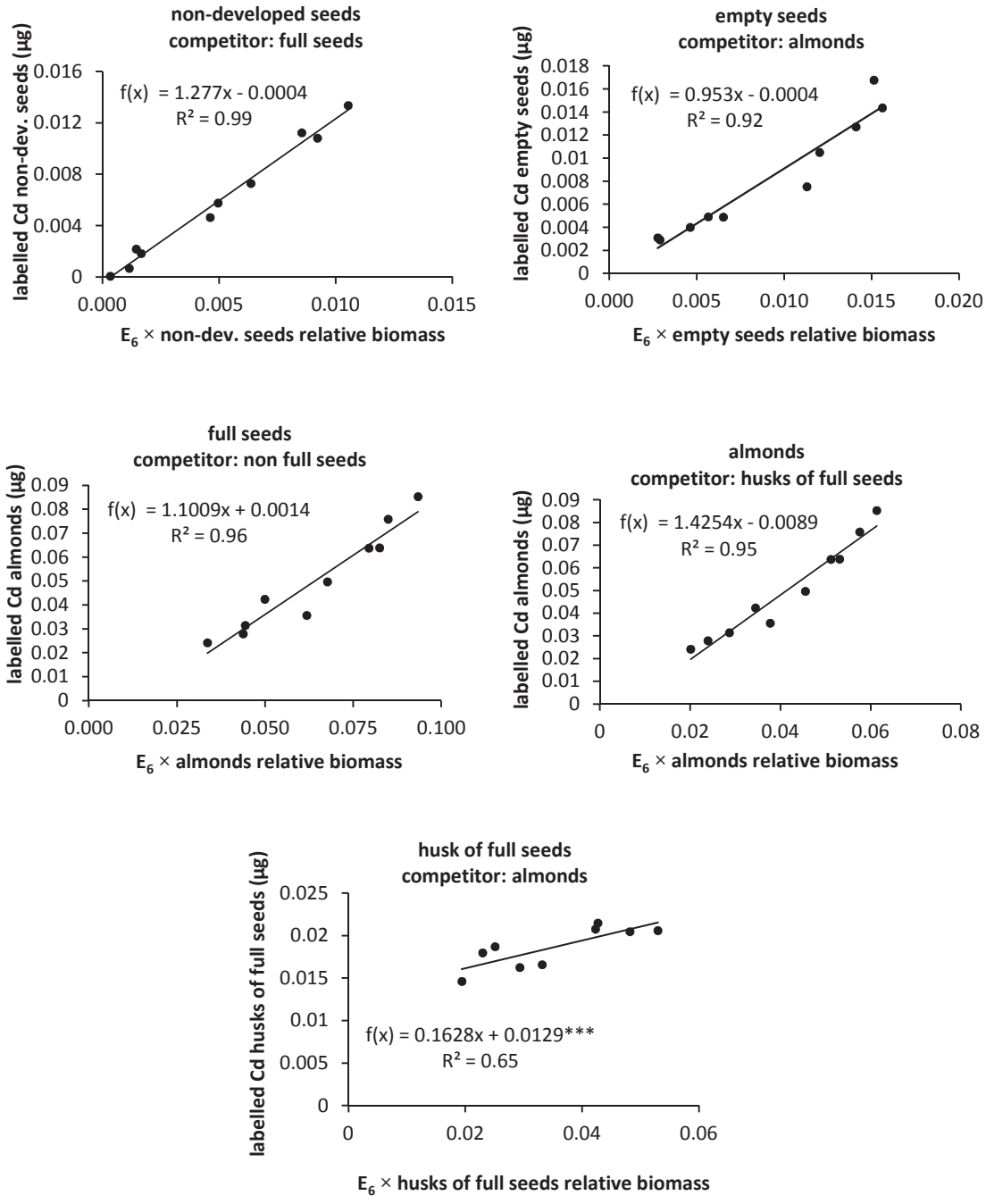

Figure 7.4. The best regression models selected for explaining the partitioning of the labelled Cd in the different seed compartments considered in the study, according to Equations 4 and 5 of Chapter 6. E_i indicates the amount of labelled Cd exported by the previous plant organ, according to the flow chart of the labelled Cd described in Figure 6.8. The significance of the intercept is coded as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

For the labelled Cd allocated to the full seeds (almonds + husks), the best model corresponded to the relative biomass of the almonds with the biomass of the empty + non-developed seeds +flowers as a competitive sinks (R^2 = 0.96, Figure 7.4). The model considering only the biomass of the full seeds was worse (R^2 = 0.87) indicating that the driving force of Cd import to the full seeds may likely be the almond.

Finally, the partitioning of the labelled Cd of the full seeds between the husks and the almonds was better explained if the biomass of the complementary part of the full seeds was considered as a competitive sink (Figure 7.4, Annex C), namely the almond and the husk, respectively.

7.2. DISCUSSION

7.2.1. NO STRONG EFFECT OF TRANSPIRATION ON THE PARTITIONING OF THE Cd RECENTLY TAKEN UP

The lower transpiration and water uptake at the grain filling stage compared to the flower bud stage (Table 7.1) was attributed to a decrease in the conductance for water in leaves as they become old[1] and to the decrease in plant growth[2] as illustrated by the change in the plant biomass during the exposure to the fans, which shifted from a net increase to a net decrease (Table 7.1).

It has been reported that the wind velocity could increase the plant transpiration and, thus, the sap flow in the xylem.[3,4] Exposing the plants to fans was expected to enhance the plant water loss by reducing the water vapour pressure at the leaf surface and indeed, the transpiration was observed to increase by 20 % on average. The total or partial closure of stomata can prevent an excessive water loss with in counterpart a reduction of the photosynthesis and a reduction in growth. This may have happened at the flower bud stage (Table 7.4). Indeed, we also observed that the uptake of labelled Cd per root mass unit, which requires energy,[5] was reduced by 35 % (p = 0.04) by the high transpiration treatment. However, this possible negative effect of the transpiration on the photosynthesis was weak because, for both growth stages, the transpiration treatments had no significant effect on the plant water content, biomass partitioning, and change in biomass during the three days of labelling.

The Cd not sequestrated in roots is loaded into xylem vessels and then it is allocated to aboveground organs.[6,7] If the transpiration affects the loading of the xylem, it will therefore also affect the partitioning of Cd to aboveground as indicated by some studies in other plants.[8,9] Cd can reach the xylem by two routes, namely the symplastic and apoplastic routes. For the symplastic (intracellular) route, Cd is transported into the symplasm from cell to cell through plasmodesmata. For the apoplastic (extracellular) route, Cd moves in the free space of the cell walls. The apoplastic barriers can be developed at the root periphery (the exodermis) and in the endoderm they also separate the root cortex from the stele where the xylem and phloem are located.

Thus, due to the impossibility of crossing the apoplastic barriers of the endoderm (the Casparian strips), Cd has to be internalized into the symplast to reach the stele and then the xylem.[10-13]

In the stele, efflux pumps belonging to the family of the Heavy Metal ATPases (*HMA2* and *HMA4*) excrete the Cd from the symplast of the parenchyma cells into the xylem apoplasm,[14,15] allowing the loading of the xylem by diffusion and mass flow. This active loading of the xylem is not directly dependent on the transpiration stream, as it is controlled by the activity of the pumps.[16]

Therefore, for the symplastic route and also for the apoplastic route in presence of the corresponding barriers, transpiration is expected to weakly affect the loading of the xylem and therefore the exportation of Cd to aboveground organs, which is likely controlled by the pumps involved in the loading of the xylem. One exception may be in case of a significant apoplastic bypass in roots i.e. when Cd directly reaches the xylem by the apoplastic route i) at the root tip where the apoplastic barriers are not mature, ii) at the lateral root emergence were they are disrupted, or finally iii) due to passage cells that have a lower resistance in their cell wall due to the lack of suberin deposition.[10,17] In that case, the xylem loading by this apoplastic bypass strongly depends on the transpiration stream. It is relatively non-selective for divalent cations[18] and can therefore be effective for Cd.

In our study, the transpiration did not have any effect on the sequestration in roots of the Cd recently taken up and consequently on its root-to-aboveground translocation, except at the grain filling stage, when the sequestration was increased by the high transpiration treatment (Table 7.4). These observations suggest that in our case the loading of Cd into the xylem was predominantly by the symplastic pathway with no or a minor importance of the apoplastic bypass. Similar conclusions were obtained for rice, durum wheat and eggplant.[16,17,19,20] Moreover, in our work, a minor contribution of the apoplastic bypass is also consistent with the low level of Cd exposure (200 nM ^{111}Cd in the nutrient solution, pCd^{2+} = 11.03). Indeed, because it is related to advection, the apoplastic route may become significant at high Cd exposure, leading to a correlation between transpiration and Cd allocated to aboveground organs, as observed by Liu et al.[8] in *Phytolacca americana* exposed to 10 and 100 µM $CdCl_2$.

As shown in the results, the transpiration treatment did not affect the partitioning of the labelled Cd between the aboveground plant organs (Table 7.4). The exception is a decrease in the allocation to leaves and empty seeds at the grain filling stage. Furthermore, we observed that almonds, which are non-transpiring, contained around 6 % of the aboveground labelled Cd, showing that the tracer also moved into the plant by the phloem sap. Altogether, these observations indicate that the allocation of Cd to the different plant organs is not directly and solely governed by the xylem stream.

The exchanges of elements between the xylem and the phloem sap are well documented.[21]

In rice and more generally in cereals, these xylem-phloem exchanges are important at the level of the nodes,[15,22,23] playing an important role in signalling to roots the nutritional status of the plant and in allocating nutrients to plant organs that are only connected to the phloem, like the seeds, or that have a low transpiration stream, like developing tissues.[24] In dicots, parenchyma cells organized in rays also operate exchanges between the xylem and the phloem[25-27] and some intervascular transfers have also been shown for boron in *Arabidopsis thaliana*.[28] In rice, it has been shown that the exchanges of Cd and Zn between the xylem and the phloem involve the same transporters *OsHMA2*, and therefore, at least at low Cd exposures, Cd follows the allocation of Zn to the grain and to developing leaves by the phloem flows after the transfer from the xylem.[24]

It is unknown if the same mechanisms of exchanges between the xylem and the phloem during the transport also operate in sunflowers but the fact that in our study, the partitioning of the Cd recently taken up between head organs (receptacle, flowers, non-developed seeds, empty seeds, husks of full seeds and almonds) did not depend on the transpiration but was strongly correlated with that of Zn ($r = 0.79$, Figure 7.1), suggests that the allocation of Cd may also be in part governed by the exchanges between the xylem and the phloem during the transport. Another possibility could be that Cd was unloaded from the xylem in leaves where it would have been loaded into the phloem by similarity with Zn, following the source-sink relationships for Zn.

7.2.2. PARTITIONING OF THE Cd RECENTLY TAKEN UP DEPENDS ON THE ALLOMETRY BETWEEN PLANT ORGANS

Contrary to the transpiration treatment, the growth stage strongly affected the accumulation and partitioning of the Cd recently taken up (Tables 7.2, 7.3 and 7.4). When shifting from the flower bud to the grain filling stage, the concentration of the total Cd decreased in roots and stems, which was consistent with the fact that in parallel the allocation of the labelled Cd to these organs also decreased. By contrast, the Cd concentration in the taproot declined between both growth stages although there was allocated a constant percentage of the labelled Cd. For leaves, the concentration of the total Cd remained stable but the allocation of the labelled Cd increased strongly.

These discrepancies between the time course of the Cd concentration and that of the Cd allocation for a given plant organ suggest that the allocation of the Cd recently taken up does not always follow the changes in biomass and/or that Cd is remobilized from or toward the plant organs as it was observed for durum wheat.[29] In our work, we concentrated on the relationships between the allocation of the Cd recently taken up to a plant organ and its biomass, taken into account the possible influence of the biomass of other plant organs.

7.2.2.1. SEQUESTRATION OF THE Cd RECENTLY TAKEN UP IN ROOTS

The allocation of Cd to roots has been demonstrated, for different plant species, to be dependent on the balance between the loading of the xylem and the sequestration in the vacuole.[19,30-32]

Roots retained 68 % of the labelled Cd at the flower bud stage and 56 % at the grain filling stage (Table 7.4). They were the major sink of Cd likely acting as a detoxification barrier[14-16] against the contamination of shoots and the negative effects on photosynthesis, as it was frequently observed in non-hyperaccumulators.[31,33,34] We observed a clear relationship between the total uptake and the sequestration of labelled Cd by roots (Figure 7.2), which suggests that both the uptake and the sequestration processes were controlled by the same mechanisms, likely the assimilate import from shoots as it will be explained thereafter.

The relative biomass of roots compared to leaves was the best model explaining the sequestration of the Cd recently taken up in roots (Figure 7.3, Annex C), suggesting that leaves were an important variable. As discussed previously, transpiration is unlikely to be the mechanism mediating the influence of leaves. By contrast, the rate of assimilates allocated to roots by the phloem might be the mechanism controlling both the uptake and the sequestration of Cd, as indicated before. Indeed, according to the literature for other species like rice and *Arabidopsis*, the uptake requires energy to allow Cd to enter the root cells through the ZIP and Nramps family transporters.[14]

Sequestration of Cd in root vacuoles is reported to be mediated by ATPases pumps that load the vacuoles and that unload the xylem and load the phloem to redirect Cd towards the roots.[15] Hence, an important phloem flow towards the roots means two things: i) a high energy availability for the transporters involved in the uptake of Cd and in the exchange of Cd from xylem to phloem and ii) a high flow of Cd returning back to the roots in the phloem. This hypothesis could explain why, in our work, at the grain filling stage, when the phloem flow was likely redirected to grains, the uptake and sequestration of Cd by roots was the lowest whereas at the flower bud stage, when much more assimilates were allocated to roots since the grains were not yet growing, the uptake and sequestration of Cd by roots were the highest.

Further investigations are required to examine if the most relevant rule for modelling the sequestration of Cd in roots may be the rate of assimilates allocated to roots instead of the relative biomass of roots and leaves.

7.2.2.2. SEQUESTRATION OF THE Cd RECENTLY TAKEN UP IN THE TAPROOT

Some Cd may have been directly taken up by the taproot from the nutrient solution and this may be a reason why the goodness of the fits were a little bit lower than for the other plant organs (Figure 7.3, Annex C).

Based on the literature,[15,35] the sequestration of the Cd recently taken up in a particular tissue requires that it is unloaded from the xylem by the parenchyma cells of the vascular bundles and further internalized into the vacuoles of the cells. However, once unloaded from the xylem, besides being sequestrated into the cells, Cd can also be loaded into the phloem and allocated to other sinks.

The balance between the sequestration of Cd and its loading into the phloem is likely to be controlled by the flow of the phloem towards the competitive sinks, which is for the taproot the aboveground organs, except the leaves that are on average a source of assimilates. Hence, when the aboveground sink organs develop, the flow of the phloem towards them is enhanced. This may stimulate the loading of Cd in the phloem along with Zn and therefore reduce the sequestration of the toxic metal in the taproot. This would be one possible explanation for the negative effect of the aboveground biomass except leaves on the sequestration of Cd in the taproot.

7.2.2.3. SEQUESTRATION OF THE Cd RECENTLY TAKEN UP IN LEAVES, STEM AND RECEPTACLE

Regarding the rule of priority, the Cd exported from the taproot can be allocated i) first to the stem, or ii) first to leaves or iii) to the stem and leaves in parallel (Figure 6.8). The best fit was obtained when the leaves were set before the stem.

For the sequestration of the Cd recently taken up in leaves, the best model indicated that they only depended on their biomass without any influence of other competitive sinks. This could suggest that once leaves get the Cd from roots and taproot through the xylem stream, they 'pull' the Cd to the stem and to the head organs, likely by mass flow with the flux of assimilates in the phloem. This hypothesis allows explaining why the other compartments have no influence on the sequestration of Cd in leaves. Furthermore, contrary to carbonaceous assimilates, Cd is not enough concentrated in the phloem to influence its loading at the sources (leaves) when it is unloaded at the sinks, which would correspond in this case to a 'pull' hypothesis.[36]

In the stem, parenchyma cells are strongly involved in the unloading of solutes from the xylem, in the radial transport to the phloem and also in the storage in the stem cells.[25] As for the taproot, the labelled Cd allocated to the stem can be assumed to be depended on the competitive sinks also branched onto the phloem. Hence, the sequestration of Cd in the stem is well explained when the compartments located after the stem were considered as competitive (Figure 7.3, Annex C).

The same rationale holds for the sequestration of the labelled Cd in the receptacle. As for the stem, the sequestration in the receptacle is limited by the competitive sinks located after it, which 'pull' the assimilates and with them the Cd by attracting the phloem flow. Because the full seeds (husks + almonds) are the major competitive sinks after the receptacle, the sequestration of Cd in this organ depends on its relative biomass compared to the biomass of the full seeds (Figure 7.3).

7.2.2.4. SEQUESTRATION OF THE Cd RECENTLY TAKEN UP IN FLOWERS, NON-DEVELOPED SEEDS, EMPTY SEEDS AND FULL SEEDS

During the grain filling period, the phloem sap flowing from the receptacle have to split to fuel each flower unit (sepal, petal, ovary and embryo),[37] and consequently it makes sense to put the flowers, the non-developed seeds, the empty seeds and the full seeds in parallel (Figure 6.8).

For flowers (sepals and petals of tubular flowers), the competitive sinks were found to be the husks of empty and full seeds. As the strands of vascular bundles have to be divided between different parts of the flower units, namely the sepal, petals and ovary,[37] the allocation of Cd to the sepals and petals (harvested as 'flowers') can be assumed to depend on the sink strength of the ovary, which becomes the husk at maturity (Figure 7.3).

The non-developed seeds are seeds that have undergone a limited development likely due to a restriction in assimilate availability, and empty seeds are ovary that failed to be fertilized by the pollen.[37] They are very similar since most of the tissues for these parts are the husk. The main common competitive sink was found to be the almonds (Figure 7.4, Annex C), which was the driver of the Cd accumulation in the full seeds as discussed below. This makes sense under the hypothesis that the allocation of Cd followed the flow of the phloem sap as almonds are the main sink of assimilates. Following the same reasoning, as expected, the flowers, non-developed seeds and empty seeds on the receptacle compete for the allocation of the Cd recently taken up to the full seeds, which represent the main sink because they contain 8.6 % of the aboveground labelled Cd (Figure 7.4, Annex C).

7.2.2.5. PARTITIONING OF THE Cd RECENTLY TAKEN UP BETWEEN THE ALMOND AND THE HUSK

From what is known in the literature, the filial tissues of the seeds (in this work the almonds) are not connected to the maternal tissues (the husks) by vascular bundles. Therefore, Cd has to be unloaded from the phloem by the maternal parenchyma cells, which then excrete the metal into the apoplast from which the almond pumps it, presumably together with the other nutrients.[35,37,38]

These two steps for the partitioning support the best model found in this study, namely that the Cd allocated to the almonds depends on the biomass of the husks and conversely. However, the goodness of fit was better for the allocation of Cd to the almonds than for the husk (Figure 7.4), which suggests that the almonds were the 'pulling' driver of Cd between both parts of the full seeds.

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Chapter 8

RESULTS AND DISCUSSION:

PLANT GROWTH AND Cd PARTITIONING DURING THE REPRODUCTIVE GROWTH STAGE OF SUNFLOWERS (*Helianthus annuus*)

8.1. RESULTS

8.1.1. PLANT GROWTH

8.1.1.1. PLANT WATER CONTENT

The water content of plant organs at each growth stage was calculated from the difference in weight between the non-dried and dried samples, as a percentage of the fresh weight (Table 8.1). The total water content per plant was merely the mean water content of all the organs weighed by the biomass of individual plant parts.

In general, the total water content of plants significantly decreased ($p < 0.001$) with time. However, not all plant organs lost water content during the experiment. The roots, the taproot, the stem and the receptacle maintained their water content from sampling to sampling ($p > 0.05$), whereas the water content of the leaves, the flowers and the seeds (mean water content of seeds compartments) decreased significantly with time, more markedly at maturity ($p < 0.001$).

Table 8.1. Water content (in percentage, %) obtained at five growth stages (from 802 to 1972 GDD) for the total plant and different plant organs (n = 5, mean and standard deviation values are given). Significance values of the differences between two consecutive dates are indicated (ANOVA, no letter $p > 0.05$, ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$).

	GDD				
	802	**1418**	**1601**	**1715**	**1972**
Total plant	92.45 ± 0.42	85.6^{c} ± 1.0	85.87 ± 0.70	79.7^{c} ± 1.1	65.8^{c} ± 2.5
Roots	94.13 ± 0.68	94.13 ± 0.65	94.79 ± 0.29	94.85 ± 0.41	94.85 ± 0.17
Taproot	84.62 ± 0.48	83.7 ± 1.8	85.0 ± 1.0	86.28 ± 0.52	84.3 ± 1.6
Stem	93.11 ± 0.30	86.8^{c} ± 1.1	86.68 ± 0.79	86.04 ± 0.78	85.0 ± 1.3
Leaves	87.04 ± 0.44	84.08^{c} ± 0.71	82.8 ± 1.7	82.1 ± 1.6	75.2^{c} ± 1.5
Receptacle	-	88.6 ± 1.1	88.93 ± 0.70	89.50 ± 0.83	88.9 ± 1.3
Flowers	-	79.37 ± 0.84	74.9^{a} ± 4.1	70.8 ± 5.0	21.7^{c} ± 1.5
Seeds	-	82.3 ± 1.8	59.2^{c} ± 1.5	48.3^{c} ± 3.0	26.3^{c} ± 4.6

8.1.1.2. TIME COURSE OF PLANT BIOMASS

The whole plant biomass was harvested at five growth stages. At the first sampling of the experiment, no seeds were available for harvesting (Figure 8.1). The stem was always the plant organ having the highest biomass, followed by the leaves, the receptacle and the roots. After 1418 GDD, the full seeds (husk + almonds) became an important compartment as they had the highest biomass of the reproductive organs (flowers, non-developed seeds, empty seeds, husks of full seeds and almonds). At maturity (1972 GDD), the dry mass of the reproductive organs onto the receptacle was 44.0 ± 8.1 g per plant and partitioned between 5 % of flowers, 7 % of non-developed seeds, 9 % of empty seeds and 80 % of full seeds (Table 8.2).

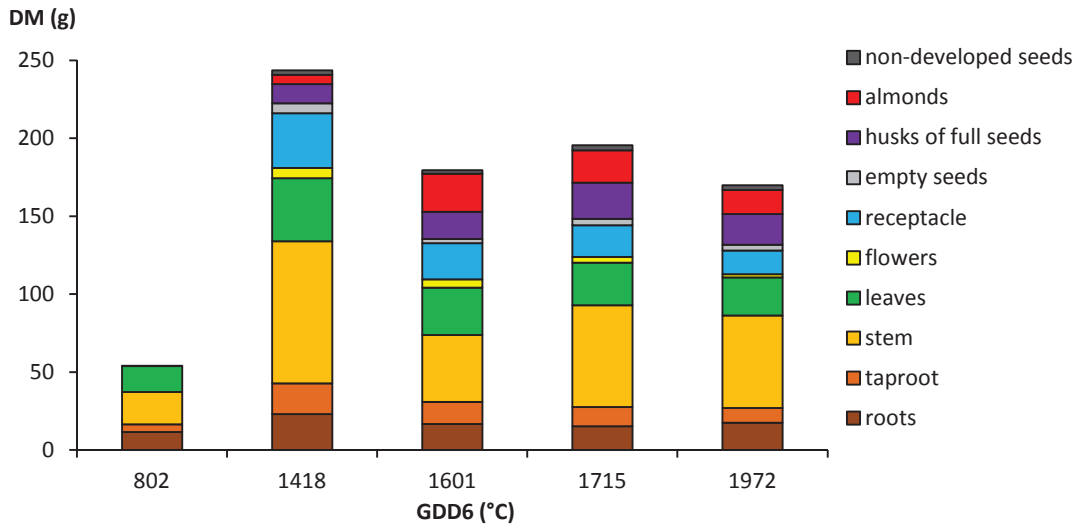

Figure 8.1. The time course of total plant biomass and of plant organs (in dry mass, DM).

The dry biomass of the full seeds was 35 g per plant and partitioned between 56 % for husks and 44 % for almonds. Non-developed seeds were always a minor compartment, with a dry mass ranging between 0.13 and 1.9 % of the whole.

Sunflower plants were the smallest at the flower bud stage (802 GDD), with a total dry mass of 53.9 ± 8.0 g per plant (Table 8.2). The dry biomass of the whole plant increased strongly until 1418 GDD ($p < 0.001$), reaching the maximum of 243 ± 25 g per plant, just when grain began to develop and then, decreased significantly till maturity. On average, 30 % of the biomass of the plant at 1418 GDD was lost at maturity (Figure 8.1, Table 8.2). Moreover, between 1418 and 1601 GDD the loss of biomass from the vegetative parts (roots, taproot, stem, leaves and receptacle, - 82 g, $p < 0.05$), was much higher than the corresponding increase (+ 17.7 g) of the biomass of the reproductive compartments (flowers, non-developed seeds, empty seeds and full seeds) (Table 8.2).

Roots biomass was 22 % of the whole biomass at 802 GDD and this percentage dropped to 8-10 % at the late reproductive stages (> 802 GDD, $p < 0.001$). This gave underground-to-aboveground biomass ratios of 0.22 at 802 GDD to 0.08-0.13 at later stages, outstanding the important growth of stem, leaves, receptacle and seeds. Among the aerial vegetative parts (stem, leaves and receptacle), the most important losses of biomass happened between 1418 GDD and 1601 GDD (for the stem, 53 %; for the leaves, 25 %; and for the receptacle, 34 %) (Figure 8.1, Table 8.3).

Table 8.2. Biomass production (DM, g), Cd concentration (CCd, mg kg^{-1}) and amount of Cd (QCd, µg) obtained at five growth stages (from 802 to 1972 GDD) for the total plant, the vegetative organs (roots, taproot, stem, leaves and receptacle) and the reproductive organs (flowers, non-developed seeds, empty seeds and full seeds) (n = 5, mean and standard deviation values are given). Significance values of the differences between two consecutive dates are indicated (ANOVA, no letter $p > 0.05$, ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$).

	GDD				
	802	**1418**	**1601**	**1715**	**1972**
Total plant					
DM (g)	53.9 ± 8.0	243^{c} ± 25	179^{c} ± 10	195 ± 12	170 ± 17
CCd (mg kg^{-1})	0.316 ± 0.021	0.186^{a} ± 0.015	0.124 ± 0.010	0.196 ± 0.013	0.228 ± 0.016
QCd (µg)	17.5 ± 4.2	47.3^{c} ± 4.2	23.9^{c} ± 3.9	35.2^{c} ± 5.1	35.8 ± 2.9
Vegetative organs					
DM (g)	53.9 ± 8.0	209^{c} ± 29	127^{a} ± 12	140 ± 22	126 ± 20
CCd (mg kg^{-1})	0.316 ± 0.021	0.217^{a} ± 0.020	0.154^{a} ± 0.013	0.235^{a} ± 0.017	0.275 ± 0.021
QCd (µg)	17.5 ± 4.2	42.9^{c} ± 6.3	19.9^{c} ± 4.0	27.3^{c} ± 4.6	29.5 ± 4.7
Reproductive organs					
DM (g)	-	34.3 ± 3.4	52^{b} ± 10	55 ± 10	44.0^{a} ± 8.1
CCd (mg kg^{-1})	-	0.132 ± 0.031	0.074^{a} ± 0.018	0.160^{a} ± 0.020	0.150 ± 0.025
QCd (µg)	-	4.36 ± 0.35	4.04 ± 0.93	7.9^{c} ± 1.4	6.3^{a} ± 1.1

Among the reproductive compartments (flowers and seeds), the husks of full seeds and the almonds first accumulated biomass and then they lose a little bit of it at the senescence stage (1972 GDD) (Figure 8.1, Table 8.3). The strongest biomass accumulation for almonds was between 1418 GDD and 1601 GDD (+ 17.8 g, $p < 0.001$), whereas for the husk the biomass accumulation increased more steadily over a larger duration until 1715 GDD ($p < 0.001$).

Table 8.3. Biomass production (DM, g), Cd concentration (CCd, mg kg^{-1}) and amount of Cd (QCd, µg) obtained at five growth stages (from 802 to 1972 GDD) for different plant organs (n = 5, mean and standard deviation values are given). Significance values of the differences between two consecutive dates are indicated (ANOVA, no letter $p > 0.05$, ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$).

	GDD				
	802	**1418**	**1601**	**1715**	**1972**
Roots					
DM (g)	11.6 ± 1.4	23.1^{c} ± 1.6	16.6^{c} ± 3.6	15.4 ± 2.8	17.6 ± 2.3
CCd (mg kg^{-1})	0.62 ± 0.17	0.52^{a} ± 0.11	0.280^{c} ± 0.046	0.384^{c} ± 0.021	0.560^{c} ± 0.012
QCd (µg)	7.1 ± 1.6	11.9^{c} ± 2.1	4.54^{c} ± 0.48	5.86^{c} ± 0.38	9.95^{c} ± 0.43
Taproot					
DM (g)	4.98 ± 0.59	19.7^{c} ± 3.8	14.4^{c} ± 3.2	12.2^{c} ± 3.7	9.43^{c} ± 0.37
CCd (mg kg^{-1})	0.32 ± 0.10	0.121^{b} ± 0.067	0.1051 ± 0.0062	0.206^{a} ± 0.087	0.22 ± 0.11
QCd (µg)	1.63 ± 0.76	2.32^{c} ± 0.58	1.52^{c} ± 0.40	2.52^{c} ± 0.23	2.05^{c} ± 0.17
Stem					
DM (g)	20.7 ± 3.8	91^{c} ± 11	43^{c} ± 27	65^{c} ± 10	59.4 ± 8.6
CCd (mg kg^{-1})	0.108 ± 0.016	0.084 ± 0.019	0.063 ± 0.022	0.050 ± 0.038	0.059 ± 0.027
QCd (µg)	2.26 ± 0.71	9.21^{c} ± 0.67	2.07^{c} ± 0.69	3.20^{c} ± 0.37	3.46 ± 0.29
Leaves					
DM (g)	16.6 ± 2.7	40.4^{c} ± 5.7	30.5^{c} ± 2.3	27.4 ± 1.9	24.4 ± 3.7
CCd (mg kg^{-1})	0.385 ± 0.063	0.405 ± 0.048	0.350^{a} ± 0.058	0.48^{a} ± 0.12	0.513 ± 0.067
QCd (µg)	6.4 ± 1.8	17.0^{c} ± 2.7	10.7^{c} ± 2.6	13.2 ± 3.7	11.9 ± 2.7
Receptacle					
DM (g)	-	35.0 ± 6.7	23.2^{c} ± 5.8	20.2 ± 4.9	15.1 ± 3.7
CCd (mg kg^{-1})	-	0.0704 ± 0.0049	0.046^{b} ± 0.025	0.123^{b} ± 0.067	0.142 ± 0.073
QCd (µg)	-	2.43 ± 0.49	1.08^{c} ± 0.29	2.49^{c} ± 0.27	2.15 ± 0.13

8.1.2. CONCENTRATION OF Cd IN THE DIFFERENT PLANT ORGANS

In general, the highest concentrations of Cd were found in roots and leaves, the lowest were for the stem, whereas the taproot, the receptacle and the reproductive organs were intermediate (Figure 8.2, Table 8.3).

Between 802 and 1601 GDD, the Cd concentration decreased significantly for all vegetative organs except for leaves and stem (Table 8.3). The decrease in the Cd concentration in roots was more marked between 1418 and 1601 GDD, whereas for the taproot, the decrease was more pronounce between 802 and 1418 GDD. The concentration of Cd for the seed compartments also decreased more markedly between 1418 and 1601 GDD, being significant ($p < 0.001$) for all of them (Table 8.3).

Table 8.3 (*cont.*). Biomass production (DM, g), Cd concentration (CCd, mg kg^{-1}) and amount of Cd (QCd, µg) obtained at five growth stages (from 802 to 1972 GDD) for different plant organs (n = 5, mean and standard deviation values are given). Significance values of the differences between two consecutive dates are indicated (ANOVA, no letter $p > 0.05$, ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$).

	GDD				
	802	**1418**	**1601**	**1715**	**1972**
Flowers					
DM (g)	-	6.71 ± 0.42	5.34^{c} ± 0.81	3.75^{c} ± 0.67	2.12^{c} ± 0.11
CCd (mg kg^{-1})	-	0.099 ± 0.021	0.083 ± 0.052	0.165^{b} ± 0.067	0.161 ± 0.086
QCd (µg)	-	0.67 ± 0.10	0.447 ± 0.084	0.623 ± 0.048	0.340^{a} ± 0.083
Non-developed seeds					
DM (g)	-	3.0 ± 1.9	2.4 ± 1.4	3.30 ± 0.49	3.15 ± 0.37
CCd (mg kg^{-1})	-	0.144 ± 0.067	0.071^{c} ± 0.018	0.180^{c} ± 0.025	0.163 ± 0.023
QCd (µg)	-	0.44 ± 0.11	0.159^{c} ± 0.090	0.60^{c} ± 0.18	0.45 ± 0.12
Empty seeds					
DM (g)	-	6.3 ± 1.8	3.3^{c} ± 1.4	4.3 ± 1.5	3.8 ± 1.2
CCd (mg kg^{-1})	-	0.1300 ± 0.048	0.0646^{c} ± 0.0063	0.124^{c} ± 0.037	0.132 ± 0.043
QCd (µg)	-	0.845 ± 0.049	0.208^{c} ± 0.086	0.527^{b} ± 0.046	0.490 ± 0.068
Husks of full seeds					
DM (g)	-	12.3 ± 2.7	16.3 ± 2.9	23.2^{c} ± 1.8	19.6^{b} ± 1.7
CCd (mg kg^{-1})	-	0.109 ± 0.067	0.050^{c} ± 0.032	0.133^{c} ± 0.086	0.116 ± 0.095
QCd (µg)	-	1.34 ± 0.13	0.93^{b} ± 0.18	2.94^{c} ± 0.24	2.23^{c} ± 0.37
Almonds					
DM (g)	-	6.0 ± 1.2	23.81^{c} ± 0.72	20.7^{b} ± 1.8	15.4^{c} ± 1.6
CCd (mg kg^{-1})	-	0.178 ± 0.067	0.094^{c} ± 0.010	0.161^{c} ± 0.037	0.176 ± 0.027
QCd (µg)	-	1.06 ± 0.11	2.22^{c} ± 0.58	3.22^{c} ± 0.18	2.75^{c} ± 0.21

From 1601 to 1972 GDD, the Cd concentration increased for all plant organs except for the stem but more importantly for roots and leaves. The Cd concentration in the full seeds (husks + almonds) ranged between 0.07 mg Cd kg^{-1} plant at 1601 GDD and 0.15 mg Cd kg^{-1} plant at 1972 GDD (not shown). Moreover, at maturity stage, the almonds were 1.5 times more concentrated in Cd than the husk (0.18 *vs*. 0.12 mg Cd kg^{-1} plant).

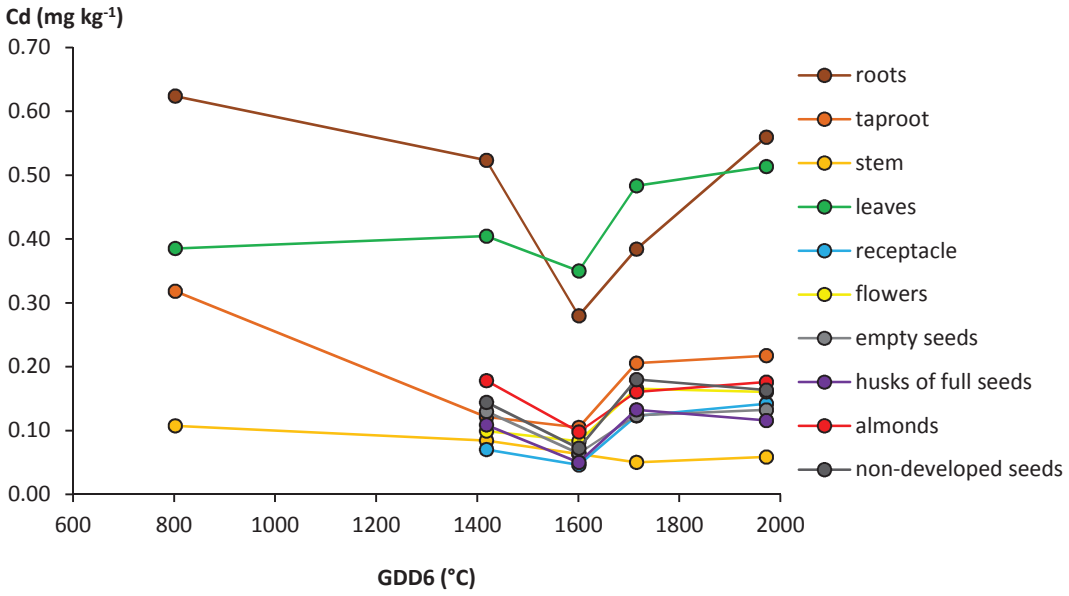

Figure 8.2. The time course of the concentration of Cd (in mg kg^{-1}) in the plant organs.

8.1.3. AMOUNT OF Cd IN THE PLANT ORGANS

8.1.3.1. WHERE IS Cd?

The amount of Cd in each plant organ (in µg) was calculated from the concentrations of Cd measured by AAS (in mg kg^{-1}) and the dry mass obtained for each organ (in kg). The total uptake of Cd per plant was obtained from the sum of Cd recovered in each organ.

In general, about 30 % of the total Cd taken up by plants was sequestrated in underground organs (roots and the taproot), whereas the rest of the Cd was translocated to aboveground organs (Figure 8.3). Most of the Cd was recovered in leaves and roots whereas, the stem, the receptacle, the full seeds and the taproot were intermediate (from the most to the least: leaves (37 %) > roots (25 %) > stem (13 %) > receptacle (6 %) ≈ full seeds (6 %) ≈ taproot (6 %)). Flowers, empty seeds and non-developed seeds contained a minor part of the plant Cd: at the final sampling (1972 GDD) they were only 4 % of the plant Cd but 20 % of the Cd in the reproductive organs (flowers, non-developed seeds, empty seeds and full seeds, Table 8.3).

At the late grain filling stages (≥ 1601 GDD), the amount of Cd in the almonds or in the husks of full seeds was similar to that found in the receptacle or in the stem. At 1715 GDD, the amount of Cd contained in the full seeds was similar to that recovered in roots (Figure 8.3, Table 8.3). At the final sampling, corresponding to maturity (1972 GDD), the full seeds contained 5.0 of the 35.8 µg Cd recovered in the whole plant (14 %, average values, Table 8.3).

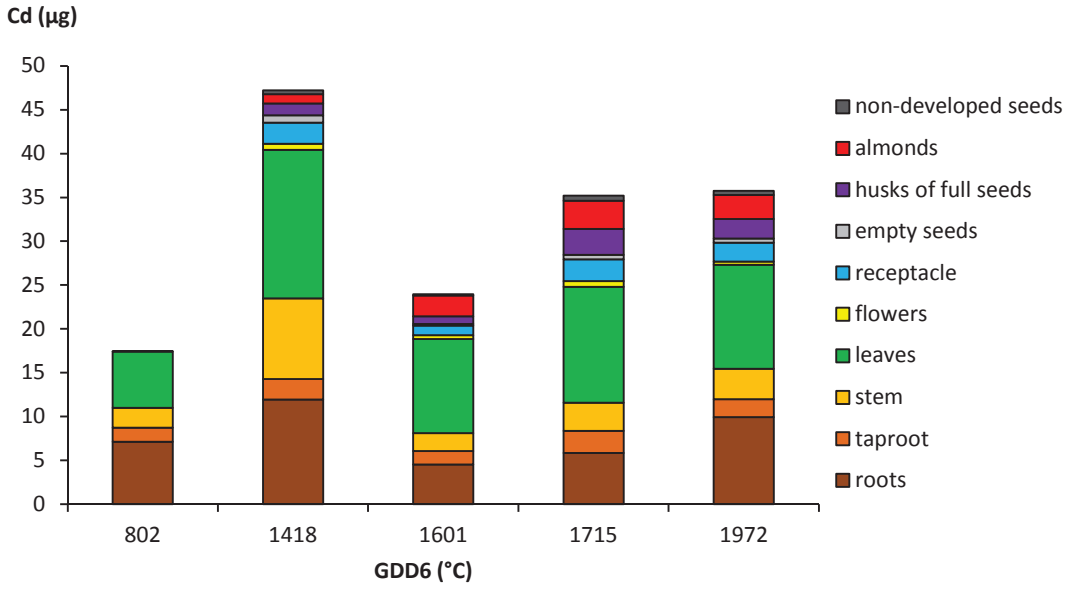

Figure 8.3. The time course of the amount of Cd (in µg) in the whole plant and in plant organs.

8.1.3.2. TIME COURSE OF ACCUMULATION OF Cd

Our results indicated a significant variation in the Cd recovered with time, the maximum amount of Cd being at early grain filling stage (1418 GDD, Figure 8.3, Table 8.2), with an average value of 47.3 µg Cd per plant. Then, 50 % of this Cd was not recovered at the following sampling (1601 GDD). This loss of Cd between 1418 and 1601 GDD also corresponded to 63 % of Cd accumulated between 802 and 1418 GDD. At the end of the experiment (> 1601 GDD), compared to 1418 GDD, the net loss of Cd for the whole plant was reduced to around 24 %, as the result of a new accumulation between 1601 GDD and 1715 GDD (Figure 8.3, Table 8.2).

Both the vegetative and the reproductive compartments accumulated a significant amount of Cd between 802 and 1418 GDD, being 6 times more the amount of Cd accumulated in the previous group of organs (Table 8.2). Among the vegetative organs, the greatest accumulation of Cd was found in leaves followed by the roots, by the stem, by the receptacle and by the taproot. For the reproductive parts, the strongest accumulations of Cd were observed for the husk of full seeds, followed by the almonds, the empty seeds, the flowers and the non-developed seeds (Table 8.3).

Between 1418 and 1601 GDD, the loss of the plant Cd was due to a net loss of Cd (50 % less, mean value) from all vegetative organs ($p < 0.001$), whereas the changes in the amount of Cd in the reproductive organs were not significant (Figure 8.3, Table 8.2). Regarding the reproductive organs, the almonds tended to accumulate Cd ($p < 0.001$), like during the previous period, contrary to the husk of full seeds, which shifted from accumulation to loss of Cd ($p < 0.01$, Table 8.3). All other reproductive organs also lost Cd significantly ($p < 0.001$) except flowers.

Between 1601 GDD and 1715 GDD, on the whole both the vegetative and the reproductive compartments accumulated Cd significantly ($p < 0.001$, Table 8.2). In detail, the increase in Cd was marked but not significant for leaves and significant for roots, for the taproot, for the stem and for the receptacle ($p < 0.001$). Among the reproductive organs, the amount of Cd increased significantly for the full seeds and non-developed seeds ($p < 0.001$), followed by empty seeds ($p < 0.01$). This accumulation was stronger for the husks compared to the almonds.

Between 1715 and 1972 GDD, roots still accumulated Cd significantly ($p < 0.001$) whereas the amount of Cd in the rest plant organs was stable or even tended to decrease (Table 8.3).

8.2. DISCUSSION

8.2.1. GENERAL PLANT GROWTH AND Cd CONCENTRATION IN SEEDS

Growing sunflower plants in nutrient solution till maturity and having a correct development of full seeds with Cd concentrations similar to the ones observed in the field was challenging. More particularly, hand pollination and fertilization of the ovules was a critical step during the greenhouse experiment. Seeds represented on average 42 g per plant, in dry mass (Table 8.3), which is a little less compared to the typical range of 46 to 52 g per plant calculated from the yield of that cultivar in the field assuming a water content of 10 % and a final plant density of 6.6 plant m^{-2} (http://www.myvar.fr/variete/497/es-biba.html). This is probably due to a slightly greater proportion of seeds without almonds in our study (18 % of empty seeds + non-developed seeds) compared to what can be obtained in the field.[1] Cultivated sunflowers are generally 80 - 90 % auto-fertile but higher flower fertilization is generally obtained by cross pollination between different plants or cultivars.[2] Although we repeated the hand pollination three times per day and although we cross-pollinated the plants, the fertilization of ovules may have not been as successful as in the field, being however satisfactory.

The Cd concentration in the seeds was on average of 0.13 mg Cd kg^{-1} (Table 8.3), which is typically in the range of the concentrations observed in the field in France[3] and elsewhere.[4] This was achieved by growing the plants in a nutrient solution with a low concentration of Cd^{2+} buffered by a ligand,[5,6] which on the whole, was able to reproduce the typical low availability of Cd in non-polluted agricultural soils.[7,8]

8.2.2. LOSS OF BIOMASS DURING GRAIN FILLING PERIOD

Three growth phases were roughly distinguished in this experiment (Figure 8.1). From transplantation until 1418 GDD, the vegetative growth dominated and the reproductive phase started as illustrated by the development of the receptacle and of the seeds, mainly the husks.

Between 1418 and 1601 GDD, the seeds strongly developed at the expense of the biomass of the stem, of the receptacle and of the leaves. These plant organs were therefore assumed to be sources of remobilized carbonaceous assimilates for the developing seeds, based on similar observations reported by Hall et al.[9] in sunflowers, who found in parallel that water soluble carbohydrate in the stem, in the receptacle and in the taproot decreased sharply in parallel with the growth of the seeds.

Starting from 1418 GDD, the whole plant biomass decreased with time and the loss of senescent leaves, which occurred at late stage (> 1715 GDD) and was of minor importance (typically the first two basal leaves may have been lost), was little responsible of it. This decline in plant biomass was mainly attributed to i) a small loss in the root biomass and ii) respiration for the maintenance of existing tissues and for the growth of the seeds. This is an indication that photosynthesis was not sufficient to cover the costs for the intense growth of seeds and for the respiration maintenance of the plant and, consequently, that biomass was remobilized. The decreased with time observed for the underground-to-aboveground biomass ratios indicated a significant increase of aboveground biomass while the root biomass remained more or less stable. This decrease of the underground-to-aboveground ratios during plant development is classical and has been also reported by other authors.[10]

The third phase extended between 1601 and 1972 GDD. Plant and organ biomass were first stable and then decreased, more markedly for the taproot, for the receptacle and for the almonds (Figure 8.1, Table 8.3). It cannot be excluded that the slight decreases in biomass could be due to fortuitous smaller individuals at 1601 and 1972 GDD but it is unlikely because plants were chosen randomly. At these later growth stages when almonds were likely to mainly synthesize oil, the costs for almonds were probably less than between 1418 and 1601 GDD. These costs could have been covered by the remaining photosynthesis between 1601 and 1715 GDD but probably no longer between 1715 and 1972 GDD, when senescence was important, as demonstrated by the yellowing leaves. Interestingly, Hall et al.[9] also reported that the C-reserves decreased first rapidly and then more slowly similarly to the decrease in the biomass of the whole plant observed in our work.

8.2.3. LOSS OF PLANT Cd

The uptake of Cd from the nutrient solution occurs thanks to some transporters located in the plasma membrane of the root cells. This system is designed to take up essential elements (e.g. Zn), but the uptake competition between both essential and toxic ions has been demonstrated.[11-13] The strong correlation observed between Zn and Cd in our study (n= 25, r = 0.65 in roots and taproots, r = 0.90 in stems and leaves, r = 0.80 in flowers, r = 0.35 in receptacle and seeds compartments) support the hypothesis that Cd entered in the plant using the same transporters than those used for some nutrients uptake, especially Zn.[14]

After the uptake, most of the toxic elements are known to be in part sequestrated in root-cell vacuoles to avoid phytotoxicity, but Cd mobility in plants permits its translocation through the transpiration stream via xylem vessels.[15-18] Moreover, it is known that elevated Cd can also reach young growing organs without a high transpiration and this explains the accumulation of Cd in the new plant organs at the beginning of the reproductive stage.[19]

Our results indicated a significant variation in the amount of Cd in plants with time ($p < 0.01$), reaching the maximum amount of Cd at 1418 GDD (Figure 8.3, Table 8.2). Particularly, it is remarkable that almost half of the Cd accumulated by the plants between 802 and 1418 GDD was not recovered at 1601 GDD (Figure 8.3 and Table 8.2). Most of the net loss of Cd was assumed to come from leakage from roots, probably due to i) senescence and roots decay and ii) differences in sequestration capacity with time, as has been already reported.[10]

This roots turnover could have released Cd in the nutrient solution, which is consistent with the fact that the root system began to turn brown at the 1601 GDD sampling. Unfortunately, the amount of Cd released was too small to make the concentration of Cd in the nutrient increase significantly, and further taking into account that part of the solution was daily renewed by overflowing. The fact that vacuoles are an important compartment for the storage and detoxification of Cd[20,21] could explain why the concentration of Cd in roots decreased along with the loss of the metal. Indeed, during senescence, the permeabilization of membranes[22] would have released Cd with a negligible loss in the root dry weight, mainly constituted by the apoplast, leading to a decrease in the root Cd concentration.

It cannot be excluded that the other vegetative organs (e.g. leaves and stems) had also exported Cd towards roots with a further release in the nutrient solution. Literature gives evidences that Cd can be transported by the phloem towards the roots[23,24] and, in fact, in our work, between 1418 and 1601 GDD, the leaves, the stem and the receptacle tended to lose Cd significantly (Table 8.3). One supporting point to this hypothesis is that the reproductive organs were apparently not the corresponding sink of the Cd lost by the aerial vegetative organs, as they did not shown any net accumulation of Cd (Table 8.2).

8.2.4. Cd IN SEEDS: UPTAKE *vs*. REMOBILIZATION

Cd can be accumulated in seeds through i) direct uptake from the nutrient solution by roots and further translocation to aboveground organs via xylem and xylem-to-phloem exchanges to reach the seeds, and ii) remobilization from other organs including the leaves and the stem during plant maturation.[12,25,26]

A remobilization of Cd from shoots to seeds via phloem has been reported by some authors,[25,27-29] in particular when Cd was applied as a spike.

On the other hand, when Cd was applied for a longer duration, net remobilization of the metal was not evidenced.[5] For Zn, it was shown that the importance of remobilization versus the new uptake for the allocation to the grain of barley was depending on the Zn status of the plant.[30]

In our work, around half of the amount of the Cd recovered in the reproductive organs was accumulated between 1601 and 1715 GDD, i.e. after the biomass of this compartment was built up between 802 and 1601 GDD, after the loss of Cd by vegetative parts, which occurred between 1418 and 1601 GDD, and also in parallel with the new increase in the amount of Cd in the vegetative parts between 1601 and 1715 GDD (Table 8.2). Altogether, these observations support that a significant part of the Cd recovered in the reproductive organs, namely that accumulated between 1601 and 1715 GDD, might have been originated from the new Cd recently taken up. Furthermore, between 1418 and 1601 GDD, while a loss of the biomass from the vegetative organs could explain the growth of the reproductive organs, a simultaneous remobilization of Cd from the vegetative compartments towards the reproductive organs was not suspected because the amount of Cd in the latter did not increase (Table 8.2).

In detail, it cannot be excluded that for the husks and almonds of the full seeds, which accumulated both biomass and Cd between 1418 and 1601 GDD, part of the Cd was originated in the remobilization of Cd from the vegetative organs, along with the remobilization of biomass (Table 8.3). However, the concentration of Cd in the full seeds (husks and almonds) during their intense growth at 1601 GDD decreased compared to the previous period (≤ 1418 GDD), which does not support that Cd mainly accumulates from remobilization. By contrast, this decrease in the Cd concentration of full seeds is consistent with the hypothesis that Cd was originated from the uptake, which was not enough important to follow the dry mass accumulation (Table 8.2) due to a slightly lower Cd concentration in the nutrient solution (Figure 6.2) and/or a decrease in the root activity.

The fact that apparently, in our work, Cd in seeds mainly came from uptake is not surprising in the light of what is known about Zn. Remobilization of Zn is caused by low N plant status,[30,31] which induces senescence and therefore the breakdown of Zn-containing compounds and the cell membrane permeabilization, including the tonoplast of vacuole where excess of Zn can be stored. Because of the alkaline pH of the phloem sap, Zn has to be bound to ligands such as nicotianamine to be mobile and reach the developing seeds.[11,32] This remobilization can compensate the decrease in the uptake of Zn, which results from the lower activity of roots because assimilates are mostly allocated to the developing seeds.[33]

Additionally, a low availability of Zn for root uptake also stimulates the remobilization of this element.[31] However, other authors reported that, under low bioavailability of Zn for uptake, the total Zn uptake was greatly reduced whereas the partitioning within different plant organs of *Brassica napus* did not change, with no apparent remobilization of Zn.[34]

In our study, N supply was maintained during the whole experiment, including during the intense growth of seeds between 1418 and 1601 GDD. The plants remained green till the late grain filling period and senescence was likely delayed by the high N availability.

A delayed senescence in our work could have had two main consequences: i) little release of Cd by senescence from storage compartments and ii) little availability of amino acids derived from the breakdown of proteins, and consequently less availability of N-rich ligands for binding to Cd and for making the metal mobile in the phloem. Indeed, nicotianamine has been reported to poorly bind to Cd which should therefore require other ligands to be mobile.[32] Furthermore, in our study, the availability of Cd in the nutrient solution was also maintained for the root uptake. Hence, as both N and Cd were continuously supplied during the grain filling period, remobilization and redistribution of Cd was probably not favoured and of little importance. Similarly, Harris and Taylor[5] did not found evidences of a net remobilization of stored Cd towards the grain of durum wheat grown in nutrient solution when N and Cd availability were also maintained.

In any case, the importance of root uptake, root-to-shoot translocation and further remobilization of Cd in seeds remains under dispute for field grown plants where senescence may occur more rapidly than in rich nutrient solution.

8.3. REFERENCES

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Chapter 9

Cd uptake and allocation to the different organs of sunflowers

CONCLUSIONS

A water-culture of sunflowers was successfully conducted with a correct development of seeds with a Cd concentration similar to the ones observed in the field.

Our study showed that increasing the transpiration of sunflower plants did not generally affect the partitioning of Cd between the plant organs. Furthermore, except for leaves, the partitioning of the Cd recently taken up to a given plant organ could be explained by the relative biomass of this organ compared to one or several competitive sinks.

Based on these main conclusions, we developed a conceptual model for the partitioning of the Cd recently taken up that was able to fit our experimental observations and that was also consistent with the current knowledge about the plant physiology regarding Cd.

For roots, which are the first sequestration compartment for Cd on the way to aboveground tissues, the sequestration was related to the biomass of leaves. We hypothesized that the activity of the transporters (transport by active efflux pumping of Cd towards the xylem) controlled the allocation of Cd to aboveground organs, and were themselves regulated by the availability of assimilates from leaves, explaining why transpiration had no effect on the Cd sequestration in roots and its allocation to aboveground organs.

The Cd not sequestrated in roots is transported to aboveground organs by the xylem. For organs with low or null transpiration (e.g. taproot, stem, receptacle and seeds), the main process for the allocation would be the unloading of the Cd from the xylem and the distribution by the phloem sap, which could explain why different plant organs that are sink for assimilates could also compete for Cd. Thus, the sequestration of the Cd in these organs with low or null transpiration depended on the sink strength of the other plant compartments.

Through the delivery of assimilates, leaves could control the sequestration/exportation of Cd from roots and the allocation of Cd to other plant organs. As a result, the amount of Cd sequestrated in leaves was dependent only on their biomass with no competitive compartments.

The conceptual model obtained put the leaves and the source-sink relationships for carbonaceous assimilates as the main factors controlling the partitioning of the Cd recently taken up, which requires further experimental confirmation.

During the whole life cycle of sunflower plants, roots and leaves were the main sinks for the total Cd, even if their sequestration capacity could decrease during seeds production. This resulted in a significant net loss of Cd at the plant scale during the grain filling period, mainly from roots. This was not reported in durum wheat for example (*Harris, N.S. and Taylor, G.J. BMC Plant Biology 2013, 13:103*). Therefore, it has to be clarified if some plants can lose significantly amount of Cd during grain filling, through leaks from the root system and if this Cd only comes from Cd previously stored in roots or if it also comes from reallocation from shoots. This may have important consequences on the accumulation of Cd in the edible plant organs, including seeds.

A small net remobilization of Cd was observed in some vegetative aerial parts but it was not possible to show that some remobilized Cd was redirected to seeds, which does not mean that it did not happened. The constant supply of N by the nutrient solution delayed the senescence of the plants and, thus, likely reduced the importance of remobilization of nutrients and also of Cd previously stored. If the nutritional status of the plant is actually a key point governing the magnitude of the remobilization of Cd versus its new uptake, this may be a mean for acting on the Cd concentration in seeds. De-favouring the remobilization of Cd and favouring its new uptake during grain filling may help to reduce the concentration of the metal in seeds because, in general, the bioavailability and mobility of Cd in soil is expected to be less during grain filling compared to the vegetative growth, in particular because of lower soil moisture, among other reasons.

Final remarks and perspectives

This Thesis has been carried out in co-tutelle within the framework of the Cross-Border Euroregional Campus of International Excellence IdEx Bordeaux - Euskampus. The co-tutelle has proven to be a beneficial and positive experience for me and my supervisors, reaching a multidisciplinary level thanks to the cooperation and internationalization of both universities. I had the opportunity to work with two different scientific groups (IBeA and ISPA) and topics (analytical chemistry and plant ecophysiology), both with different philosophies and working methodologies. In this way, the Thesis has been the opportunity to gain the knowledge of the two scientific groups. However, it has not been an easy task due to the evident differences between France and the Basque Country, especially concerning administrative issues, that have hindered to some extent a fluent coordination. Finally, the requirement to spend at least nine months in France has resulted in a unique opportunity to develop my research career, and it provided a stimulating and enriching personal experience that has allowed me to know and live with other culture and social realities.

This PhD Thesis has focused on the accumulation of essential (possibly toxic at high concentrations) and non-essential (generally highly toxic at low concentrations) trace elements, according to the following objectives:

i) to investigate the uptake, accumulation and translocation of essential and non-essential elements between organs of plants with different edible parts.

ii) to study the influence of the type of agricultural practice (conventional *vs*. organic) on the accumulation of trace elements in the different organs of two vegetables (Swiss chards and tomatoes) along their whole production cycle.

iii) to model the partitioning of a particularly worrying toxic element, cadmium, between the plant organs of an important crop, the sunflower, at its different reproductive stages.

Reaching these objectives is of a vital importance in the field of food safety and plant ecophysiology, because they can help to identify the factors involved in the allocation of the elements between the plant organs as well as to gain knowledge about the elements partitioning. This information can be used to perform an optimum food crop by minimizing the possible entrance of toxic elements in the plants, ensuring the production of safe food and preserving thus the health of potential consumers.

The experiments of this Thesis have been carried out under very different conditions (open-field with natural soil *vs*. greenhouse with nutrient solution). Working either in the field or in the greenhouse allowed us to familiarize with both cultivation methods. Obviously, the experiments had to be adapted to the specific growth and maintenance conditions in each case.

In the open-field experiment, the soil of the garden was used as source of nutrients and non-essential elements for plant growth, being closer to most usual agricultural practices. However, in natural conditions it is more difficult to control all the variables involved in the experiment and thus, reproducibility of the experiment becomes difficult. During the experiment plants are directly exposed to meteorological conditions, which can be daily monitored but hardly kept under control. Harvesting the whole plant organs becomes a hard task (specially the roots, but also other plant organs which are lost during the production cycle of the plants). Cleaning the plants in the laboratory is also not easy, due to the high amount of biomass usually collected. Moreover, working in the field increases the risk that plants are affected by pests.

In the greenhouse experiment, a nutrient solution was used for growing plants. In this way, the experimental variables (e.g. the transpiration rate, the temperature and humidity, the availability of nutrients, the composition of the nutrient solution, etc.) can be more easily controlled and/or, if necessary, manipulated. Consequently, future reproduction of the experiment could be easier and more reliable. In the greenhouse experiment, the biomass could be totally harvested and the presence of pests and contaminants can be easily minimized and controlled. By contrast, the production of really big plants makes harvesting more difficult and leads to problems concerning the storage, manipulation and pretreatment of samples. Likewise, a huge amount of surplus of nutrient solution is required to maintain the nutrient availability relatively constant, which corresponds to high reagents expenses, and to difficulties to manage the surplus. The latter requires being cleaned to remove any toxic element detected and, before this treatment, it occupies a lot of room for storage.

In the field experiment more than 25 elements were considered at the same time. Some of them are major or minor nutrients, while other ones are non-essential and even toxic metals. The behaviour of each element concerning its uptake and allocation in different plant organs is so various that catching all the information for all elements becomes very difficult. This has been one of the reasons to focus more specifically on Cd in the greenhouse experiment, and also an important lesson for the future.

The accumulation of elements resulted to be plant-dependent, and in sunflower, the allometry of the plant organs was the most important factor determining the partitioning of elements between plant organs, as it was shown by the modelling of the Cd recently taken up between sunflower organs. Except for sunflower leaves, the partitioning of the Cd recently taken up to a given plant organ could be explained by the relative biomass of this organ compared to one or more competitive sinks.

Although it was plant-dependent, some similarities were observed when comparing the elements partitioning between different vegetables. Most of the toxic elements were immobilized in roots, whereas aboveground organs were richer in essential nutrients, which is a good point for food safety.[1] Other works support our results, since it has been proven that plant roots are organs that can sequestrate toxic metals as a detoxification mechanism against metal toxicity.[2,3] However, Sr, Ba and Cd could easily reach the edible parts of the plants. According to the results of the greenhouse experiment, in sunflowers, the root-to-shoot translocation of Cd was well explained by the biomass of leaves, and we hypothesized that leaves controlled the activity of some non-specific transporters (active efflux pumping towards the xylem) involved in the homeostasis of some essential micronutrients, such as Zn and Fe, as it has been already reported.[3,4] The further distribution of Cd between aboveground organs (except leaves) could depend on its unloading from xylem and on its further loading in the phloem, probably following the competition between organs for the allocation of assimilates. As in leafy vegetables leaves are the edible part of the plant, they are more risky to consumers, due to the high mobility and high toxicity of Cd. This high mobility of Cd, including its uptake and further translocation to leaves, has been also observed in other leafy plants, such as spinach and lettuce.[5,6]

The concentration of Cd in tomato leaves was higher than that found in leaf blades of Swiss chards, showing that the accumulation depends on the plant species. In terms of food safety this is not of practical use for tomatoes, since the fruit is the only edible part of that plant. We have found that the fruits of tomatoes are richer in macronutrients such as K, Mg and Ca, which is in good agreement with other studies,[7-9] indicating that these macronutrients, except Ca, are highly mobile in the phloem.[10,11]

It has to be also highlighted that during the whole field experiment the allocation of Cd and Pb in edible organs of our plants resulted in concentrations below the threshold values defined in the European legislation (Commission Regulation (EC) No 1881/2006),[12] concerning the consumption of safe foods: for Cd, in fresh weight, 0.20 mg kg^{-1} for stalks and leaf blades, and 0.05 mg kg^{-1} for tomato fruits, while for Pb, in fresh weight, 0.30 mg kg^{-1} for stalks and leaf blades, and 0.10 mg kg^{-1} for tomato fruits.

The root uptake and remobilization mechanisms are also of major interest for the allocation of elements to a plant organ, being directly affected by the plant species.[10,13] Uptake of essential and non-essential elements by roots from the rhizosphere is the first step for the accumulation in aerial parts.[3] The root uptake is known to be highly dependent on the mobility and bioavailability of elements in the growth substrate,[14-16] which is one or even the first factor affecting the content of elements in plants. The bioavailability of elements needs to be considered prior to the plantation of a crop, following, for example, the sequential extraction procedures described by many authors[17-19] as an estimation of the bioavailability, keeping in mind that this is unlikely to reflect always what the plant can take up. In our field experiment, many elements were considered, with different chemical behaviours and nature in soil. Since the bioavailability of elements in the field experiment was not previously characterized (we only determined the total content), the need to perform speciation analyses in soil arises as a must in future investigations.

In our study, remobilization or redistribution of Cd between plant organs was suspected during the reproductive stages, but the remobilization was apparently not the source of the Cd for the seeds, which does not indicate that it did not happen. This suggests that, under high N and Cd availability, the root uptake of Cd prevails over remobilization for the allocation to the seeds. A possible remobilization of mineral nutrients in different plants is well-reported, including for Cu, Fe, K, Mo, Zn, N, P and S in senescent leaves of *Arabidopsis Thaliana*.[20] However, regarding trace elements, remobilization is not clearly understood and is not always evident. For example, Mailliard et al.[10] did not observed Zn remobilization when the availability of Zn for uptake was limited in hydroponically grown *Brassica napus*. Similar results were obtained by Harris and Taylor,[21] who did not find evidences of a net remobilization of stored Cd towards the grain of *Triticum durum* grown in nutrient solution when N and Cd availability was maintained. However, under N deficiency, leaf Fe remobilization has been observed in *Hordeum vulgare* senescent leaves.[22] In the field, remobilization may occur more importantly, due to the faster senescence of the plants exposed to the environmental conditions and also probably because of a lower bioavailability of elements (e.g. Zn and Fe).[23]

In our study, we found that increasing the transpiration did not affect the partitioning of Cd at the reproductive stages of sunflowers.

However, the hypothesis of a partial closure of stomata needs to be further investigated, as it may have reduced the change in transpiration due to the exposure to the fans and it is possible that the increase in transpiration was not strong enough to affect the partitioning of Cd. Moreover, the apoplastic route, which links transpiration with the translocation of Cd to aboveground organs, may have been too weak to be evidenced. For testing this hypothesis, plants can be exposed to higher concentrations of Cd, in order to favour the potential apoplastic bypass which makes the translocation of Cd to aboveground depend on the transpiration stream.

The influence of the harvesting time on the allocation of elements within the plant is also important, but seems to be more connected with the plant allometry: the concentration of the elements tended to decrease with time probably due to i) remobilization from vegetative tissues, ii) uncoupling between the growth and the allocation of elements, leading to a possible dilution of elements with time, and iii) a senescence and roots decay as well as differences in sequestration capacity with time, as it has been already reported.[24,25] Hence, the highest concentrations of essential and non-essential elements were observed at the beginning of the growth cycle. As an example, eating young stalks and leaf blades not only provides the consumer with a high nutrient content, but also with a relatively high amount of Cd. In a similar way, the elemental concentrations that we have measured in fruits of tomatoes were related to the age of the fruits rather than to the age of the whole plant. This is true for Na, Mg, K, Fe, Cu, Zn, Mo and Cd. Our results are in good agreement with those reported by other authors,[26] although other publications obtained the opposite result.[27] These discrepancies can be due to the variety used, the bioavailability of the elements in the growth substrate and/or to the environmental and growth conditions.

As the need of estimating the age of tomatoes (in our work, the set of fruits collected from a single tomato plant) became evident, we developed a method to assess the ripening stage using chromaticity values obtained after image analysis of pictures of the samples. If the correlation between the element concentration and the ripening stage is mathematically characterized, the measurement of colour may also be helpful to estimate the change in element concentration with time.

The use of a standard camera combined with image analysis to estimate tomato colour has a number of advantages in comparison with other methods of measurement. The use of spectrophotometric techniques[28,29] requires a previous extraction of the pigments to an organic solvent and subsequent measurement of the absorbance. Another option consists in measuring colour by a colorimeter.[30,31] Like standard cameras, they are easy to transport, simple to handle, require low maintenance and, if properly manipulated, provide us with *Lab* chromaticity values, which is the closest mode to the way the human eye perceives the colours. However, the colorimeters require a smooth surface for the measurement,[32] and the surface of tomato fruits is not usually smooth.

Moreover, the calibration of a colorimeter turns out to be more complex and less systematic than that of a camera. The use of a standard camera also provides us with the possibility to take raw pictures directly in the field, permitting the calibration of the pictures to be carried out some days after, if the same colour profile is used.

This methodology opens a door for the future, for example, concerning the possibility to control the ripening stage of tomato fruits directly in the field, thus allowing us to decide the optimum harvesting time for each individual tomato.

However, the methodology proposed for the estimation of the ripening stage also presents some important drawbacks that should be addressed in the future. In fact, a low amount of samples (20 tomatoes in the calibration set and 3 tomatoes in the validation set) was used in its development. This was a result of i) the limited time available to analyse the samples, due to the duration of the extraction procedure and the relatively fast photodegradation of the pigments contained in the extract, and ii) the limited availability of human and technical resources. As the number of adjustable parameters in the model to be estimated by regression analysis is relatively high, the uncertainty associated to the predicted absorbance is also high. Consequently, we foresee that a future refinement of this methodology will be required, by increasing the amount of samples used in the regression analysis. Moreover, only three basic ripening stages were roughly considered in this study (green, orange and red). The selection of more ripening stages according to the external colour of fresh tomatoes (namely, green, breaker, turning, pink, light red and red, established in 1991 by the United States Department of Agriculture (USDA),[33] may help in discerning more accurately between tomatoes and, consequently, between the plants used in the field experiment.

The cultivation method turns out to be the less influential factor in the absorption and allocation of elements by plants, although several significant trends have been found for different variables. The organic practice favoured the accumulation of essential elements (e.g. K and Mo), while the conventional one produced plants with higher concentrations of some toxic elements, such as Tl and Cd. A significant accumulation of Cd in crops explained by the application of chemical P fertilizers contaminated by this metal has been previously reported.[34] In our study, the elemental composition of tomato fruits did not depend on the farming technique used, as observed by other authors.[7] However, this topic remains currently under dispute, as other authors have found significant differences between fruits grown up under organic and conventional conditions, either in field experiments or in greenhouse.[26,35,36] The probable reasons for the differences between farming techniques are likely governed by the bioavailability of the elements in soil, which needs to be characterized in future works, as previously indicated.

This Thesis in co-tutelle has been a unique opportunity to investigate the mechanisms involved in the accumulation of elements by plants from a multidisciplinary approach combining the areas of chemistry, agronomy and plant physiology. Although considerable progresses have been done in the way to answer the questions initially presented in the objectives of this work, some important new issues have raised. They pose exciting challenges that should be addressed in the future in order to fully understand the partitioning and concentration of trace elements in the plant organs of some food crops.

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Articles and Communications to Scientific Conferences

All the results showed in this PhD Thesis have been already published or will be published in scientific journals indexed in international bibliographic databases.

Scientific articles already published in international scientific journals:

- ❒ Liñero, O.; Cidad, M.; Carrero, J.A.; Nguyen, C.; de Diego, A. Accumulation and translocation of essential and nonessential elements by tomato plants (*Solanum lycopersicum*) cultivated in open-air plots under organic or conventional farming techniques. *Journal of Agricultural and Food Chemistry* 2015, *63 (43)*, 9461-9470. DOI: 10.1021/acs.jafc.5b03878.

Scientific articles in preparation:

- ❒ Liñero, O.; Cidad, M.; Carrero, J.A.; Nguyen, C.; de Diego, A. Accumulation and translocation of macro, micronutrients and non-essential elements by organically and conventionally grown Swiss chards (*Beta vulgaris* subsp. *cicla*) cultivated in open-air plots.
  To be submitted to: Chemosphere.
- ❒ Cidad, M.; Liñero, O.; Arana, G.; Nguyen, C.; de Diego, A. Use of chromaticity values obtained from calibrated pictures to discern among tomatoes with different ripening stage.
  To be submitted to: Food Control.

- Liñero, O.; Cornu, J-Y-; Candaudap, F.; Pokrovsky, O.; Bussiére, S.; Coriou, C.; Thunot, S.; Robert, T.; de Diego, A.; Nguyen, C. Short-therm partitioning of Cd recently taken up between sunflowers organs at flowering and grain filling stages: effect of plant transpiration and allometry.
  To be submitted to: Plant and Soil.
- Liñero, O.; Cornu, J-Y-; Bussiére, S.; Coriou, C.; Thunot, S.; Robert, T.; de Diego, A.; Nguyen, C. Time course of Cd accumulation in seeds of sunflower (*Helianthus annuus*) in nutrient solution until maturity.
  To be submitted to: Environmental and Experimental Botany.

Some results of this PhD Thesis have been also presented in International Scientific Conferences,as follows:

Oral presentations:

- Vegetables and eating parts of plants. Chemistry, eco-physiology and agronomy.
  I Bordeaux - Euskampus Symposium, November 19th & 20th 2014. Bordeaux, France.
  Scientific Session: nutrition; 20 minutes of oral presentation.
- Accumulation and translocation of macro, micronutrients, non-essential and toxic elements by Swiss chards (*Beta vulgaris* subsp. *cicla*) grown up in open-air plots under organic or conventional farming techniques.
  2nd Annual Food Analysis Congress, September 15th & 16th 2015. Cambridge, United Kingdom.
  Scientific Session: contaminants; 45 minutes of oral presentation.

Poster presentations:

- Uptake of trace elements by food plants: influence of metal availability in soil, accumulation in edible parts and implications for food safety.
  I Bordeaux - Euskampus Symposium, November 19th & 20th 2014. Bordeaux, France.
- Accumulation and translocation of essential and non-essential elements by vegetables grown up in open-air plots under organic or conventional farming techniques.
  II Bordeaux - Euskampus Symposium, November 26th & 27th 2015. Donostia – San Sebastián, Spain. First prize winning poster.

Annexes

Annex A

Results for the field experiment

Elements concentrations in Swiss chards and tomato plants

Elements concentrations in soil samples

Table A1. Concentrations (mg kg^{-1}) of 27 elements measured in roots of Swiss chards (n = 3; S1 - S6 = samplings; LOD values in Table 1.2).

	Organically grown roots of Swiss chards						Conventionally grown roots of Swiss chards					
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Na	8200 ± 6000	15900 ± 2200	10900 ± 1900	2730 ± 260	2700 ± 1300	5500 ± 3700	6500 ± 1400	8000 ± 1800	6400 ± 620	9800 ± 1300	10000 ± 3700	3180 ± 860
Mg	3400 ± 2300	1950 ± 300	1880 ± 130	2480 ± 170	2750 ± 560	3560 ± 200	3280 ± 930	1856 ± 94	2072 ± 91	3184 ± 88	3500 ± 1100	4800 ± 1000
Al	220 ± 180	77 ± 17	93 ± 61	122 ± 75	78 ± 23	97 ± 54	180 ± 210	76 ± 32	47.2 ± 4.6	125 ± 18	80 ± 32	120 ± 83
K	26000 ± 18000	27000 ± 10000	17500 ± 4000	17200 ± 5600	17800 ± 6800	31600 ± 9900	34200 ± 3200	21000 ± 2900	11790 ± 570	14500 ± 5600	13000 ± 6000	16000 ± 4000
Ca	17000 ± 15000	9300 ± 3900	5800 ± 1500	2140 ± 120	2330 ± 270	5800 ± 1600	33000 ± 24000	6900 ± 4300	3200 ± 1100	2590 ± 120	18000 ± 21000	8000 ± 2000
Ti	3.1 ± 3.3	0.65 ± 0.16	0.55 ± 0.16	0.80 ± 0.25	0.50 ± 0.14	0.60 ± 0.16	2.0 ± 2.1	0.61 ± 0.15	0.48 ± 0.14	0.74 ± 0.16	0.47 ± 0.12	0.52 ± 0.43
V	0.41 ± 0.38	0.118 ± 0.025	0.132 ± 0.092	0.115 ± 0.079	0.075 ± 0.031	0.074 ± 0.060	0.40 ± 0.39	0.144 ± 0.092	0.052 ± 0.023	0.1453 ± 0.0095	0.056 ± 0.030	0.116 ± 0.082
Cr	0.67 ± 0.61	0.1467 ± 0.0069	0.105 ± 0.064	0.077 ± 0.057	0.092 ± 0.045	0.088 ± 0.076	0.36 ± 0.40	0.17 ± 0.14	< LOD	0.138 ± 0.014	0.061 ± 0.029	0.090 ± 0.080
Mn	53 ± 39	13.8 ± 5.3	18.6 ± 2.4	32 ± 14	25.5 ± 8.9	24.2 ± 4.9	47 ± 41	10.7 ± 2.2	8.6 ± 1.5	11.8 ± 1.7	13.1 ± 3.9	12.1 ± 1.9
Fe	230 ± 240	100 ± 26	140 ± 100	120 ± 87	67 ± 14	84 ± 50	280 ± 350	92 ± 35	64.1 ± 5.2	97 ± 14	77 ± 26	110 ± 54
Co	0.27 ± 0.19	0.0511 ± 0.0041	0.060 ± 0.041	0.050 ± 0.031	< LOD	< LOD	0.29 ± 0.24	0.057 ± 0.032	0.028 ± 0.013	0.046 ± 0.023	< LOD	0.027 ± 0.012
Ni	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cu	8.8 ± 6.2	12.8 ± 2.2	11.4 ± 3.6	6.02 ± 0.85	5.01 ± 0.15	5.19 ± 0.66	8.0 ± 1.3	10.6 ± 2.6	7.3 ± 1.1	5.8 ± 1.1	8.3 ± 6.4	4.3 ± 1.1
Zn	91 ± 74	43.70 ± 0.39	47.0 ± 9.8	32.3 ± 5.8	19.3 ± 4.3	49.2 ± 4.9	200 ± 180	40.5 ± 9.5	34.4 ± 6.6	29.5 ± 4.7	29 ± 27	21 ± 14
As	1.8 ± 1.3	0.134 ± 0.050	0.142 ± 0.090	0.071 ± 0.049	0.043 ± 0.018	0.030 ± 0.014	2.7 ± 2.7	0.162 ± 0.071	0.075 ± 0.015	0.085 ± 0.035	0.033 ± 0.018	0.053 ± 0.035
Se	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Sr	57 ± 42	60 ± 17	58.6 ± 8.0	37.04 ± 0.65	30.6 ± 1.5	36.2 ± 3.5	64 ± 29	42.7 ± 6.3	47 ± 13	42.0 ± 5.6	71 ± 55	41.3 ± 2.7
Mo	6.0 ± 4.1	0.68 ± 0.15	0.35 ± 0.12	0.26 ± 0.15	< LOD	0.130 ± 0.064	1.6 ± 1.1	0.320 ± 0.037	0.23 ± 0.12	< LOD	< LOD	< LOD
Ag	0.20 ± 0.15	< LOD	< LOD	< LOD	< LOD	< LOD	0.49 ± 0.53	< LOD	< LOD	< LOD	< LOD	< LOD
Cd	0.32 ± 0.24	0.084 ± 0.016	0.0578 ± 0.0065	0.064 ± 0.023	0.040 ± 0.014	0.079 ± 0.024	0.24 ± 0.15	0.150 ± 0.033	0.094 ± 0.025	0.141 ± 0.014	0.137 ± 0.057	0.102 ± 0.019
Sn	0.09 ± 0.04	0.1405 ± 0.0027	0.118 ± 0.043	< LOD	< LOD	< LOD	< LOD	0.1432 ± 0.0051	0.091 ± 0.039	< LOD	< LOD	< LOD
Sb	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ba	12.5 ± 8.8	9.22 ± 0.72	7.3 ± 2.7	8.6 ± 3.8	7.1 ± 3.0	11.1 ± 2.1	9.0 ± 7.4	4.230 ± 0.062	4.2 ± 1.3	5.76 ± 0.65	8.6 ± 6.6	8.0 ± 1.5
W	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hg	0.92 ± 0.49	< LOD	< LOD	< LOD	< LOD	< LOD	1.9 ± 2.3	< LOD	< LOD	< LOD	< LOD	< LOD
Tl	0.075 ± 0.062	0.069 ± 0.021	0.163 ± 0.050	0.090 ± 0.032	0.105 ± 0.035	0.172 ± 0.048	0.096 ± 0.053	0.098 ± 0.061	0.219 ± 0.039	0.269 ± 0.077	0.200 ± 0.036	0.128 ± 0.019
Pb	0.71 ± 0.60	0.346 ± 0.058	0.44 ± 0.28	0.25 ± 0.12	0.160 ± 0.037	0.22 ± 0.12	0.99 ± 0.94	0.41 ± 0.20	0.313 ± 0.046	0.348 ± 0.015	0.279 ± 0.068	0.352 ± 0.086

Table A2. Concentrations (mg kg^{-1}) of 27 elements measured in stalks of Swiss chards (n = 3; S1 - S6 = samplings; LOD values in Table 1.2).

	Organically grown stalks						Conventionally grown stalks					
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Na	28700 ± 6900	34400 ± 4500	25200 ± 4500	22600 ± 2500	22400 ± 7200	24800 ± 3800	13100 ± 5700	18600 ± 1100	14200 ± 1400	20440 ± 850	29700 ± 2200	35400 ± 6900
Mg	1540 ± 240	1820 ± 330	1940 ± 430	3100 ± 400	3340 ± 270	3410 ± 430	2090 ± 180	2070 ± 580	2110 ± 540	3000 ± 900	4130 ± 730	3920 ± 920
Al	83 ± 14	12.5 ± 6.2	12.1 ± 5.9	7.0 ± 4.6	4.8 ± 2.6	< LOD	34 ± 13	15.4 ± 5.0	9.02 ± 0.47	11.1 ± 1.6	4.2 ± 4.1	< LOD
K	53900 ± 5100	55500 ± 3000	53800 ± 8000	48800 ± 3700	47000 ± 12000	85300 ± 1500	65000 ± 13000	60000 ± 4100	31300 ± 4300	29900 ± 2900	26000 ± 11000	44000 ± 14000
Ca	41000 ± 29000	13400 ± 3500	15100 ± 3600	8000 ± 2800	7600 ± 1300	10800 ± 2700	38000 ± 37000	16300 ± 3900	13400 ± 4700	7000 ± 4000	12500 ± 3300	24300 ± 6800
Ti	0.905 ± 0.068	0.378 ± 0.012	0.349 ± 0.034	0.63 ± 0.48	0.199 ± 0.093	0.21 ± 0.12	0.96 ± 0.67	0.42 ± 0.13	0.61 ± 0.25	0.24 ± 0.17	< LOD	0.195 ± 0.087
V	0.057 ± 0.031	< LOD	< LOD	< LOD	< LOD	< LOD	0.06 ± 0.04	< LOD	< LOD	< LOD	< LOD	< LOD
Cr	0.57 ± 0.23	< LOD	< LOD	0.068 ± 0.041	< LOD	< LOD	0.34 ± 0.22	< LOD	< LOD	0.103 ± 0.053	< LOD	< LOD
Mn	12.9 ± 2.7	5.91 ± 0.71	10.0 ± 2.2	35.4 ± 8.9	35.4 ± 6.1	14.2 ± 2.1	14.1 ± 9.1	11.5 ± 2.3	5.94 ± 0.92	7.92 ± 0.70	11.1 ± 5.9	6.32 ± 0.72
Fe	97 ± 21	18.2 ± 5.2	16.2 ± 5.5	9.44 ± 0.12	15.6 ± 6.5	9.2 ± 1.2	130 ± 130	23.3 ± 4.7	22.9 ± 7.5	13.8 ± 1.3	13.4 ± 6.0	13.4 ± 8.5
Co	0.028 ± 0.014	< LOD	< LOD	< LOD	< LOD	< LOD	0.052 ± 0.056	< LOD	< LOD	< LOD	< LOD	< LOD
Ni	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cu	9.37 ± 0.76	9.31 ± 0.84	5.8 ± 1.2	7.7 ± 1.5	7.1 ± 1.1	8.12 ± 0.31	6.2 ± 1.4	8.92 ± 0.82	6.5 ± 1.4	5.4 ± 1.4	6.63 ± 0.46	5.06 ± 0.60
Zn	96 ± 32	25.7 ± 4.0	23.70 ± 0.51	27.5 ± 4.7	27.0 ± 1.3	20.8 ± 3.6	99 ± 122	24.8 ± 5.6	27.2 ± 7.7	27.2 ± 4.7	26.9 ± 2.9	11.7 ± 6.4
As	0.085 ± 0.026	< LOD	< LOD	< LOD	< LOD	< LOD	0.15 ± 0.16	< LOD	< LOD	< LOD	< LOD	< LOD
Se	0.36 ± 0.17	< LOD	< LOD	< LOD	< LOD	< LOD	0.49 ± 0.40	< LOD	< LOD	< LOD	< LOD	< LOD
Sr	100 ± 10	79 ± 17	77 ± 22	79 ± 28	65.9 ± 8.5	52 ± 34	112 ± 18	99 ± 28	105 ± 13	57 ± 38	80 ± 17	65 ± 16
Mo	3.38 ± 0.42	0.40 ± 0.18	0.140 ± 0.082	0.24 ± 0.13	< LOD	< LOD	0.60 ± 0.26	0.32 ± 0.11	0.241 ± 0.066	0.126 ± 0.058	0.138 ± 0.078	< LOD
Ag	0.24 ± 0.10	< LOD	< LOD	< LOD	< LOD	< LOD	0.25 ± 0.36	< LOD	< LOD	< LOD	< LOD	< LOD
Cd	0.107 ± 0.019	0.074 ± 0.015	0.0484 ± 0.0021	0.098 ± 0.063	0.063 ± 0.014	0.079 ± 0.052	0.100 ± 0.069	0.189 ± 0.028	0.118 ± 0.013	0.1225 ± 0.0073	0.192 ± 0.046	0.116 ± 0.043
Sn	< LOD	0.093 ± 0.043	< LOD	< LOD	< LOD	< LOD	0.126 ± 0.050	0.091 ± 0.040	< LOD	< LOD	< LOD	< LOD
Sb	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ba	32.8 ± 8.0	14.8 ± 3.0	11.2 ± 7.2	17 ± 11	15.3 ± 5.7	13 ± 10	21 ± 16	11.3 ± 1.9	9.8 ± 2.1	7.9 ± 6.3	9.0 ± 2.3	12.0 ± 2.0
W	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hg	0.96 ± 0.54	< LOD	< LOD	< LOD	< LOD	< LOD	1.2 ± 1.4	< LOD	< LOD	< LOD	< LOD	< LOD
Tl	0.0286 ± 0.0045	0.037 ± 0.014	0.0765 ± 0.0065	0.065 ± 0.016	0.071 ± 0.050	0.063 ± 0.034	0.0339 ± 0.0067	0.071 ± 0.043	0.110 ± 0.028	0.063 ± 0.016	0.075 ± 0.024	0.037 ± 0.016
Pb	0.216 ± 0.059	0.063 ± 0.025	< LOD	< LOD	< LOD	< LOD	0.365 ± 0.288	0.104 ± 0.029	< LOD	< LOD	< LOD	< LOD

Table A3. Concentrations (mg kg^{-1}) of 27 elements measured in leaf blades of Swiss chards (n = 3; S1 - S6 = samplings; LOD values in Table 1.2).

	Organically grown leaf blades						Conventionally grown leaf blades					
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Na	31000 ± 5000	26300 ± 6400	27000 ± 15000	23600 ± 3600	15700 ± 1100	18200 ± 1900	14300 ± 1500	18900 ± 3900	9600 ± 1100	13100 ± 1700	16070 ± 850	19400 ± 5200
Mg	3773.5 ± 9.0	3800 ± 530	6600 ± 3900	4900 ± 1700	4620 ± 900	3890 ± 520	3700 ± 160	5240 ± 570	5820 ± 300	5900 ± 2600	5890 ± 160	5680 ± 410
Al	55.5 ± 8.6	15.6 ± 5.0	53 ± 23	35.8 ± 10.0	18.6 ± 5.2	5.6 ± 3.9	56 ± 16	11.7 ± 1.3	33.2 ± 6.6	58.8 ± 5.8	35 ± 28	8.4 ± 1.7
K	38100 ± 5700	31600 ± 6800	30830 ± 940	32730 ± 510	33400 ± 4900	38300 ± 1800	44000 ± 11000	48300 ± 3100	22200 ± 2500	22200 ± 2800	23600 ± 3300	33400 ± 3900
Ca	16000 ± 6500	14500 ± 1700	17400 ± 2600	8900 ± 1100	10800 ± 3700	8000 ± 2900	25000 ± 6700	22600 ± 2600	17700 ± 4500	10300 ± 4800	10500 ± 2600	9680 ± 660
Ti	0.749 ± 0.019	0.496 ± 0.063	0.83 ± 0.48	0.87 ± 0.14	0.620 ± 0.065	0.42 ± 0.24	0.799 ± 0.044	0.510 ± 0.030	0.46 ± 0.35	0.820 ± 0.092	0.57 ± 0.18	0.58 ± 0.28
V	< LOD	< LOD	0.067 ± 0.048	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cr	0.155 ± 0.065	< LOD	0.21 ± 0.19	0.060 ± 0.028	< LOD	< LOD	0.117 ± 0.087	< LOD	0.060 ± 0.027	< LOD	< LOD	0.068 ± 0.041
Mn	50.6 ± 6.3	16.15 ± 0.61	63 ± 68	141 ± 35	112 ± 15	59 ± 51	56 ± 12	79 ± 18	31.7 ± 4.2	35.8 ± 6.6	30.0 ± 8.3	26.0 ± 3.2
Fe	101.8 ± 5.7	56.6 ± 1.2	123 ± 63	73.5 ± 4.9	48.3 ± 8.9	31 ± 26	100 ± 31	49.7 ± 3.7	84.1 ± 8.8	100 ± 14	67 ± 29	44.7 ± 3.6
Co	0.034 ± 0.012	< LOD	0.042 ± 0.037	0.0477 ± 0.0062	< LOD	< LOD	0.046 ± 0.025	0.035 ± 0.013	< LOD	< LOD	< LOD	< LOD
Ni	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cu	16.9 ± 6.8	15.8 ± 3.7	19 ± 13	19.8 ± 1.7	15.74 ± 0.92	10.3 ± 9.2	10.7 ± 1.6	13.7 ± 1.1	16.6 ± 1.0	16.0 ± 2.4	14.2 ± 1.5	12.14 ± 0.69
Zn	73.6 ± 6.8	42.3 ± 3.3	73 ± 53	57 ± 12	56.3 ± 8.9	33 ± 22	59 ± 39	58 ± 12	61.1 ± 4.8	70.5 ± 6.6	71.6 ± 6.9	56.1 ± 5.1
As	0.096 ± 0.018	< LOD	0.068 ± 0.019	< LOD	< LOD	< LOD	0.046 ± 0.022	< LOD	0.030 ± 0.014	0.031 ± 0.015	0.030 ± 0.014	< LOD
Se	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Sr	62.5 ± 5.8	79 ± 22	104 ± 95	45 ± 11	46 ± 12	22 ± 22	85 ± 24	123 ± 19	77.0 ± 5.3	60 ± 33	39 ± 14	31.4 ± 2.0
Mo	10.3 ± 1.2	1.49 ± 0.32	1.9 ± 1.3	0.99 ± 0.12	0.814 ± 0.024	0.42 ± 0.29	2.10 ± 0.66	1.58 ± 0.21	1.23 ± 0.11	0.72 ± 0.16	0.820 ± 0.098	0.84 ± 0.32
Ag	0.134 ± 0.014	< LOD	< LOD	< LOD	< LOD	< LOD	0.086 ± 0.084	< LOD	< LOD	< LOD	< LOD	< LOD
Cd	0.205 ± 0.047	0.114 ± 0.015	0.13 ± 0.11	0.128 ± 0.068	0.095 ± 0.017	0.074 ± 0.059	0.152 ± 0.028	0.408 ± 0.048	0.196 ± 0.034	0.208 ± 0.079	0.271 ± 0.090	0.154 ± 0.066
Sn	0.164 ± 0.015	0.14495 ± 0.00073	0.159 ± 0.014	< LOD	< LOD	< LOD	0.129 ± 0.053	0.1439 ± 0.0051	0.095 ± 0.047	< LOD	< LOD	< LOD
Sb	< LOD	< LOD	< LOD	< LOD	0.0132 ± 0.0058	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ba	21.9 ± 2.8	16.2 ± 4.8	16 ± 15	10.2 ± 5.6	11.8 ± 2.0	4.9 ± 4.1	15. 1 ± 5.5	15.3 ± 5.6	8.01 ± 0.47	8.2 ± 5.1	4.9 ± 2.5	5.5 ± 1.1
W	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Tl	0.0224 ± 0.0045	0.0218 ± 0.0092	0.060 ± 0.060	< LOD	0.0105 ± 0.0057	< LOD	0.096 ± 0.0024	0.055 ± 0.030	0.0645 ± 0.0083	0.024 ± 0.020	< LOD	< LOD
Pb	0.281 ± 0.086	0.23 ± 0.30	0.21 ± 0.10	0.124 ± 0.046	0.104 ± 0.029	< LOD	0.331 ± 0.090	0.081 ± 0.045	0.130 ± 0.053	0.118 ± 0.028	0.109 ± 0.044	< LOD

Table A4. Concentrations (mg kg^{-1}) of 24 elements measured in soils of Swiss chards ([a] n = 4; [b] n = 2; S1 – S5 = samplings; LOD values in Table 1.2).

	Organically treated soils of Swiss chards					Conventionally treated soils of Swiss chards				
	February [a]	S1 [b]	S3 [b]	S5 [b]	October [a]	February [a]	S1 [b]	S3 [b]	S5 [b]	October [a]
Mg	2719 ± 34	2500 ± 150	2425.0 ± 7.1	2460 ± 110	2502 ± 37	2788 ± 37	2490 ± 210	2366 ± 89	2655 ± 12	2430 ± 110
Al	18490 ± 200	15170 ± 600	13290 ± 260	15260 ± 990	17500 ± 780	19440 ± 740	14340 ± 2300	13047 ± 89	16160 ± 500	16400 ± 2200
Ti	12.6 ± 1.1	11.6 ± 3.1	10.0 ± 1.4	9.6 ± 3.9	15.5 ± 2.3	14.6 ± 2.6	10.0 ± 2.5	6.16 ± 0.18	9.7 ± 1.1	14.2 ± 4.4
V	14.44 ± 0.31	12.87 ± 0.52	11.241 ± 0.024	12.7 ± ± 1.2	20.49 ± 0.92	15.03 ± 0.55	12.0 ± 1.4	11.33 ± 0.69	13.24 ± 0.12	20.0 ± 2.0
Cr	12.70 ± 0.38	12.08 ± 0.82	10.399 ± 0.040	11.52 ± 0.75	16.54 ± 0.59	13.47 ± 0.28	11.36 ± 0.96	10.29 ± 0.84	12.280 ± 0.054	16.2 ± 1.5
Mn	281.0 ± 6.4	277.9 ± 9.2	269 ± 16	273 ± 19	271 ± 11	301 ± 14	296 ± 14	288 ± 11	305.6 ± 7.4	285.5 ± 8.9
Fe	12462 ± 68	11710 ± 700	11530 ± 240	11430 ± 200	15500 ± 2900	13260 ± 200	12360 ± 600	12510 ± 170	12868.7 ± 2.9	14030 ± 290
Co	3.03 ± 0.12	2.971 ± 0.057	2.91 ± 0.12	2.98 ± 0.15	4.193 ± 0.078	3.108 ± 0.080	2.97 ± 0.10	3.11 ± 0.33	3.17 ± 0.14	4.421 ± 0.050
Ni	6.94 ± 0.39	7.53 ± 0.27	7.234 ± 0.402	7.086 ± 0.032	9.06 ± 0.51	7.10 ± 0.27	7.44 ± 0.25	7.45 ± 0.64	7.78 ± 0.35	9.319 ± 0.080
Cu	9.98 ± 0.69	10.92 ± 0.26	11.861 ± 0.055	10.40 ± 0.51	16.7 ± 3.6	12.34 ± 0.53	12.24 ± 0.22	12.56 ± 0.98	12.45 ± 0.42	15.17 ± 0.23
Zn	61.3 ± 6.5	69.8 ± 1.4	72.40 ± 0.80	67.1 ± 7.1	57.0 ± 1.8	70.9 ± 5.8	77.3 ± 2.7	73.7 ± 9.6	71.2 ± 4.1	69.3 ± 8.1
As	9.90 ± 0.26	9.30 ± 0.22	8.59 ± 0.41	9.21 ± 0.22	9.00 ± 0.81	10.18 ± 0.51	9.42 ± 0.49	9.30 ± 0.22	9.98 ± 0.55	8.97 ± 0.40
Se	10.2 ± 3.4	14.35 ± 0.88	13.1 ± 1.1	12.3 ± 2.4	< LOD	11.2 ± 2.0	12.2 ± 2.9	14.26 ± 0.48	13.2 ± 2.0	< LOD
Sr	680 ± 23	630 ± 10	595 ± 18	625 ± 15	579 ± 12	637.1 ± 9.0	591.7 ± 6.3	567 ± 58	602.0 ± 6.6	574 ± 35
Mo	0.457 ± 0.028	0.334 ± 0.032	0.3550 ± 0.0077	0.334 ± 0.015	0.320 ± 0.029	0.409 ± 0.085	0.48 ± 0.19	0.432 ± 0.063	0.247 ± 0.030	0.2941 ± 0.0088
Ag	0.117 ± 0.052	< LOD	< LOD	< LOD	< LOD	0.220 ± 0.038	0.14930 ± 0.00061	0.111 ± 0.055	0.14708 ± 0.00070	0.133 ± 0.040
Cd	0.157 ± 0.023	0.246 ± 0.015	0.207 ± 0.046	0.207 ± 0.032	0.178 ± 0.010	0.234 ± 0.052	0.308 ± 0.025	0.236 ± 0.048	0.2720 ± 0.0055	0.2204 ± 0.0077
Sn	3.246 ± 0.060	2.898 ± 0.062	2.584 ± 0.010	2.86 ± 0.15	2.754 ± 0.094	3.84 ± 0.25	3.16 ± 0.28	2.98 ± 0.33	3.38 ± 0.17	2.95 ± 0.19
Sb	0.363 ± 0.015	0.3355 ± 0.0032	0.378 ± 0.034	0.296 ± 0.059	0.323 ± 0.016	0.431 ± 0.023	0.357 ± 0.053	0.20 ± 0.20	0.344 ± 0.032	0.304 ± 0.090
Ba	80.71 ± 0.97	66.7 ± 2.3	61.3 ± 1.2	68.8 ± 6.0	61.8 ± 2.1	85.5 ± 1.5	66.2 ± 6.1	61.6 ± 5.7	72.9 ± 1.9	61.1 ± 4.5
W	0.1824 ± 0.0089	< LOD	< LOD	< LOD	< LOD	0.189 ± 0.016	< LOD	< LOD	< LOD	< LOD
Hg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Tl	0.2906 ± 0.0079	0.224 ± 0.010	0.195516 ± 0.000072	0.235 ± 0.023	0.2274 ± 0.0097	0.3077 ± 0.0047	0.217 ± 0.037	0.191 ± 0.024	0.245 ± 0.022	0.218 ± 0.024
Pb	29.4 ± 1.7	29.66 ± 0.87	27.7 ± 1.7	28.53 ± 0.73	24.54 ± 0.82	43.8 ± 4.0	34.54 ± 0.62	33.9 ± 3.0	34.845 ± 0.091	31.19 ± 0.85

Table A5. Concentrations (mg kg^{-1}) of 27 elements measured in roots of tomato plants (n = 3; S1 - S6 = samplings; LOD values in Table 1.2).

	Organically grown roots						Conventionally grown roots					
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Na	3430 ± 610	770 ± 210	440 ± 110	600 ± 200	1100 ± 300	660 ± 210	1330 ± 480	680 ± 230	840 ± 460	1470 ± 230	2700 ± 1100	1870 ± 510
Mg	3540 ± 290	1930 ± 170	1620 ± 180	1860 ± 210	2390 ± 280	2546 ± 46	2830 ± 160	1677 ± 93	2070 ± 300	2240 ± 160	2840 ± 370	2240 ± 340
Al	170 ± 110	116 ± 24	107 ± 63	135 ± 40	147 ± 21	173 ± 65	212 ± 22	143 ± 45	158 ± 20	220 ± 110	224 ± 45	125 ± 39
K	18410 ± 510	21400 ± 3400	14600 ± 1200	12900 ± 1400	15600 ± 1600	17580 ± 990	27000 ± 4200	16400 ± 3500	13000 ± 3900	9650 ± 500	11800 ± 5100	9100 ± 1600
Ca	21500 ± 6300	19500 ± 5600	15100 ± 5000	13550 ± 530	18300 ± 3800	24200 ± 2300	17900 ± 3000	16200 ± 2500	1500 ± 2200	17500 ± 1000	20600 ± 1900	17700 ± 4000
Ti	1.01 ± 0.44	0.48 ± 0.13	0.80 ± 0.10	0.58 ± 0.17	0.73 ± 0.10	0.80 ± 0.21	0.99 ± 0.15	1.10 ± 0.44	0.75 ± 0.09	1.28 ± 0.81	1.14 ± 0.29	0.65 ± 0.12
V	0.17 ± 0.11	0.087 ± 0.0044	0.089 ± 0.056	0.151 ± 0.035	0.175 ± 0.022	0.144 ± 0.057	0.248 ± 0.051	0.179 ± 0.071	0.200 ± 0.010	0.28 ± 0.13	0.292 ± 0.054	0.128 ± 0.032
Cr	0.18 ± 0.11	0.059 ± 0.026	0.10 ± 0.10	0.146 ± 0.054	0.243 ± 0.041	0.143 ± 0.038	0.35 ± 0.10	0.18 ± 0.14	0.191 ± 0.043	0.41 ± 0.31	0.347 ± 0.059	0.093 ± 0.046
Mn	47 ± 11	16.4 ± 3.7	10.7 ± 3.7	8.8 ± 2.3	9.23 ± 0.85	10.1 ± 2.9	29.1 ± 3.9	16.15 ± 0.82	11.3 ± 1.0	13.6 ± 3.7	15.9 ± 2.0	7.58 ± 0.94
Fe	210 ± 130	135 ± 31	110 ± 51	129 ± 34	146.8 ± 6.2	139 ± 55	212 ± 31	198 ± 49	147 ± 24	240 ± 100	197 ± 29	93 ± 23
Co	0.128 ± 0.025	0.0849 ± 0.0049	0.070 ± 0.020	0.079 ± 0.013	0.0772 ± 0.0065	0.043 ± 0.020	0.1566 ± 0.0092	0.169 ± 0.025	0.131 ± 0.014	0.162 ± 0.039	0.149 ± 0.019	0.059 ± 0.011
Ni	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cu	6.30 ± 0.66	8.2 ± 1.1	8.8 ± 1.5	10.0 ± 1.1	12.2 ± 1.1	11.0 ± 1.6	4.75 ± 0.35	5.48 ± 0.69	7.9 ± 1.5	9.72 ± 0.58	11.6 ± 1.3	6.9 ± 1.2
Zn	105 ± 11	80 ± 10	80 ± 16	113 ± 14	123 ± 31	228 ± 20	91.6 ± 4.0	70.1 ± 4.3	82 ± 12	88 ± 15	81 ± 29	127 ± 26
As	1.10 ± 0.11	0.32 ± 0.11	0.166 ± 0.046	0.186 ± 0.064	0.231 ± 0.032	0.186 ± 0.041	0.95 ± 0.34	0.325 ± 0.096	0.301 ± 0.056	0.320 ± 0.090	0.46 ± 0.12	0.202 ± 0.080
Se	0.51 ± 0.43	0.73 ± 0.50	< LOD	< LOD	< LOD	< LOD	< LOD	0.80 ± 0.62	< LOD	< LOD	< LOD	< LOD
Sr	108 ± 12	106.5 ± 9.5	107 ± 24	108 ± 11	143 ± 27	178 ± 28	101.0 ± 1.2	81.2 ± 7.7	109 ± 16	120 ± 12	152 ± 19	118 ± 33
Mo	5.3 ± 1.3	2.67 ± 0.94	0.86 ± 0.26	0.81 ± 0.19	0.81 ± 0.24	1.00 ± 0.11	1.53 ± 0.80	0.657 ± 0.022	0.783 ± 0.094	0.543 ± 0.095	0.82 ± 0.22	0.50 ± 0.18
Ag	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cd	0.160 ± 0.024	0.141 ± 0.021	0.121 ± 0.026	0.100 ± 0.017	0.0821 ± 0.0066	0.1483 ± 0.0084	0.145 ± 0.022	0.207 ± 0.040	0.134 ± 0.020	0.128 ± 0.016	0.115 ± 0.016	0.1656 ± 0.0082
Sn	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Sb	0.0137 ± 0.0068	0.016 ± 0.011	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ba	5.57 ± 0.58	4.11 ± 0.30	2.9 ± 1.0	2.66 ± 0.64	3.00 ± 0.15	3.09 ± 0.74	5.72 ± 0.26	3.25 ± 0.36	2.98 ± 0.39	2.82 ± 0.67	3.54 ± 0.86	1.81 ± 0.44
W	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hg	< LOD	< LOD	< LOD	< LOD	< LOD	8.2 ± 7.4	< LOD	< LOD	< LOD	< LOD	< LOD	0.98 ± 0.23
Tl	0.1431 ± 0.0055	0.047 ± 0.016	0.019 ± 0.020	0.029 ± 0.013	0.0458 ± 0.0015	0.096 ± 0.016	0.081 ± 0.018	0.0306 ± 0.0043	0.054 ± 0.033	0.127 ± 0.012	0.130 ± 0.092	0.197 ± 0.045
Pb	0.55 ± 0.17	0.394 ± 0.0077	0.32 ± 0.12	0.322 ± 0.090	0.381 ± 0.034	0.477 ± 0.090	1.19 ± 0.85	0.66 ± 0.16	0.568 ± 0.084	0.77 ± 0.26	0.72 ± 0.10	0.417 ± 0.042

Table A6. Concentrations (mg kg^{-1}) of 27 elements measured in stems of tomato plants (n = 3; S1 - S6 = samplings; LOD values in Table 1.2)

	Organically grown stems						Conventionally grown stems					
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Na	1620 ± 440	340 ± 130	420 ± 60	370 ± 100	331 ± 43	450 ± 130	470 ± 120	540 ± 230	840 ± 590	1130 ± 160	1230 ± 590	1690 ± 160
Mg	2000 ± 280	2220 ± 300	2140 ± 370	2150 ± 740	2450 ± 490	3240 ± 520	2330 ± 130	2110 ± 390	2610 ± 800	3050 ± 240	3360 ± 910	4280 ± 360
Al	118 ± 23	48 ± 27	3.3 ± 2.5	< LOD	< LOD	< LOD	65 ± 21	20.0 ± 5.9	< LOD	< LOD	< LOD	5.6 ± 6.6
K	34700 ± 3400	45000 ± 11000	27100 ± 1000	10900 ± 2400	14900 ± 3500	22700 ± 3600	39000 ± 13000	31200 ± 1300	18500 ± 3800	7000 ± 1200	7500 ± 4200	24200 ± 4600
Ca	25600 ± 4300	16300 ± 1700	13900 ± 970	17800 ± 4400	17700 ± 2100	15500 ± 3600	18700 ± 2800	19200 ± 3000	16000 ± 3000	23400 ± 2400	20300 ± 4600	17200 ± 3500
Ti	0.89 ± 0.16	0.85 ± 0.18	0.33 ± 0.17	0.467 ± 0.041	< LOD	0.31 ± 0.15	0.43 ± 0.25	0.54 ± 0.18	0.24 ± 0.17	< LOD	0.380 ± 0.084	0.44 ± 0.26
V	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cr	0.27 ± 0.11	< LOD	< LOD	< LOD	< LOD	< LOD	0.14 ± 0.11	< LOD	< LOD	< LOD	< LOD	< LOD
Mn	32.7 ± 4.6	11.2 ± 1.7	5.8 ± 1.2	3.48 ± 0.37	4.8 ± 1.4	9.6 ± 1.6	29.3 ± 2.8	19.78 ± 0.93	8.08 ± 0.70	8.74 ± 0.71	6.9 ± 1.0	12.39 ± 0.44
Fe	86 ± 20	30.8 ± 3.3	19.7 ± 7.2	11.7 ± 2.4	13.8 ± 1.1	21.1 ± 1.2	71.7 ± 9.3	24.8 ± 2.5	15.0 ± 3.8	11.5 ± 2.8	15.4 ± 1.6	27.9 ± 7.3
Co	0.064 ± 0.018	0.045 ± 0.025	< LOD	< LOD	< LOD	0.0466 ± 0.0073	0.0597 ± 0.0066	0.0501 ± 0.0063	0.027 ± 0.012	< LOD	< LOD	0.0601 ± 0.0022
Ni	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cu	39 ± 20	33 ± 17	11.7 ± 1.4	11.34 ± 0.58		8.19 ± 0.82	4.28 ± 0.82	6.99 ± 0.77	6.66 ± 0.87	5.08 ± 0.31	3.81 ± 0.52	4.8 ± 1.7
Zn	97 ± 15	90 ± 15	99 ± 39	112 ± 22	125 ± 26	167 ± 29	94 ± 14	84.2 ± 3.8	72.9 ± 5.2	77 ± 20	57 ± 14	92 ± 30
As	0.196 ± 0.038	0.035 ± 0.022	< LOD	< LOD	< LOD	< LOD	0.101 ± 0.030	0.030 ± 0.013	< LOD	< LOD	< LOD	< LOD
Se	0.92 ± 0.78	1.5 ± 1.1	0.57 ± 0.29	0.37 ± 0.20	0.49 ± 0.40	< LOD	0.66 ± 0.35	0.45 ± 0.33	0.79 ± 0.50	< LOD	0.64 ± 0.38	< LOD
Sr	121 ± 14	86.9 ± 4.4	82.0 ± 8.3	102 ± 22	107 ± 24	142.8 ± 3.6	105 ± 12	71 ± 12	90 ± 23	114 ± 37	123 ± 21	144.1 ± 3.0
Mo	1.97 ± 0.48	1.29 ± 0.17	0.63 ± 0.27	0.46 ± 0.12	0.57 ± 0.13	0.47 ± 0.16	0.80 ± 0.24	0.738 ± 0.088	0.48 ± 0.12	0.47 ± 0.14	0.425 ± 0.059	0.52 ± 0.13
Ag	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cd	0.133 ± 0.012	0.1572 ± 0.0059	0.140 ± 0.033	0.120 ± 0.017	0.118 ± 0.032	0.159 ± 0.020	0.115 ± 0.027	0.258 ± 0.075	0.246 ± 0.049	0.201 ± 0.023	0.152 ± 0.024	0.219 ± 0.027
Sn	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Sb	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ba	6.220 ± 0.070	3.54 ± 0.83	2.94 ± 0.66	2.48 ± 0.31	2.84 ± 0.67	3.66 ± 0.27	4.7 ± 1.0	1.97 ± 0.11	2.16 ± 0.27	2.26 ± 0.49	2.81 ± 0.11	2.82 ± 0.14
W	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Tl	< LOD	< LOD	< LOD	0.028 ± 0.018	< LOD	0.045 ± 0.013	< LOD	< LOD	0.038 ± 0.018	0.067 ± 0.015	0.041 ± 0.019	0.106 ± 0.020
Pb	0.241 ± 0.070	0.128 ± 0.025	0.077 ± 0.043	0.097 ± 0.059	0.087 ± 0.049	0.144 ± 0.037	0.72 ± 0.83	0.173 ± 0.042	0.155 ± 0.074	0.145 ± 0.056	0.1211 ± 0.0094	0.185 ± 0.020

Table A7. Concentrations (mg kg^{-1}) of 27 elements measured in leaves of tomato plants (n = 3; S1 - S6 = samplings; LOD values in Table 1.2)

	Organically grown leaves					Conventionally grown leaves				
	S1	**S2**	**S3**	**S4**	**S5**	**S1**	**S2**	**S3**	**S4**	**S5**
Na	705 ± 91	210 ± 130	390 ± 140	460 ± 220	333 ± 42	430 ± 170	620 ± 200	1220 ± 600	1250 ± 400	1070 ± 470
Mg	1580 ± 240	2270 ± 130	2550 ± 430	2560 ± 570	2270 ± 430	2050 ± 270	2830 ± 140	4310 ± 910	4830 ± 560	5500 ± 2000
Al	92 ± 25	66 ± 13	73 ± 24	76 ± 16	91 ± 18	46 ± 24	27 ± 14	39 ± 17	40.0 ± 7.9	52 ± 12
K	33600 ± 3600	33200 ± 2900	27000 ± 4800	15300 ± 2800	15200 ± 1400	35700 ± 5500	30600 ± 5300	16700 ± 5600	9800 ± 1100	13200 ± 7000
Ca	20200 ± 2700	27800 ± 1700	33400 ± 6400	39000 ± 12000	40600 ± 3800	21800 ± 1800	32700 ± 1300	42000 ± 2600	40900 ± 4900	47900 ± 6400
Ti	0.83 ± 0.29	1.27 ± 0.46	1.13 ± 0.26	1.070 ± 0.087	1.02 ± 0.19	0.65 ± 0.27	0.40 ± 0.11	0.693 ± 0.030	0.76 ± 0.14	0.86 ± 0.11
V	0.179 ± 0.018	0.140 ± 0.025	0.171 ± 0.032	0.149 ± 0.030	0.149 ± 0.041	0.101 ± 0.031	0.053 ± 0.025	0.072 ± 0.029	0.089 ± 0.019	0.113 ± 0.029
Cr	0.240 ± 0.013	0.196 ± 0.029	0.216 ± 0.038	0.192 ± 0.035	0.27 ± 0.10	0.136 ± 0.027	0.17 ± 0.10	0.117 ± 0.063	0.144 ± 0.038	0.189 ± 0.057
Mn	65.5 ± 3.9	35.4 ± 6.3	24.0 ± 3.0	20.4 ± 7.3	25.2 ± 2.8	96 ± 12	276 ± 22	108 ± 29	91 ± 48	46 ± 12
Fe	147 ± 20	136.2 ± 8.2	133 ± 23	124 ± 17	132.3 ± 26.8	114 ± 36	80.6 ± 2.3	90 ± 13	96.3 ± 8.4	117 ± 10
Co	0.062 ± 0.015	0.1029 ± 0.0049	0.065 ± 0.018	0.046 ± 0.023	0.0522 ± 0.0057	0.0673 ± 0.0083	0.164 ± 0.025	0.090 ± 0.016	0.077 ± 0.027	0.0643 ± 0.0026
Ni	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cu	348 ± 92	584 ± 14	128 ± 83	80 ± 83	38 ± 34	8.77 ± 0.84	13.4 ± 2.8	8.4 ± 3.8	7.5 ± 1.3	4.81 ± 0.55
Zn	24.5 ± 3.1	24.2 ± 3.0	28.3 ± 3.5	24.2 ± 1.6	24.9 ± 7.2	40.5 ± 2.1	52.2 ± 2.7	40.6 ± 8.8	33.6 ± 7.2	21.0 ± 1.8
As	0.192 ± 0.020	0.245 ± 0.093	0.135 ± 0.078	0.128 ± 0.066	0.101 ± 0.041	0.087 ± 0.064	0.058 ± 0.015	0.07191 ± 0.00061	0.070 ± 0.024	0.0754 ± 0.0076
Se	0.43 ± 0.30	< LOD	0.81 ± 0.95	< LOD	< LOD	< LOD	0.57 ± 0.54	< LOD	< LOD	< LOD
Sr	136 ± 24	213 ± 30	220 ± 46	214 ± 80	219 ± 32	152 ± 13	235 ± 18	250 ± 17	231 ± 30	263 ± 34
Mo	7.7 ± 2.6	2.98 ± 0.60	1.7 ± 1.0	1.5 ± 1.2	1.36 ± 0.39	1.84 ± 0.49	1.28 ± 0.10	1.10 ± 0.27	0.97 ± 0.26	0.68 ± 0.14
Ag	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cd	0.283 ± 0.014	0.372 ± 0.063	0.352 ± 0.099	0.36 ± 0.15	0.325 ± 0.060	0.245 ± 0.058	0.77 ± 0.24	0.64 ± 0.12	0.67 ± 0.10	0.63 ± 0.16
Sn	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Sb	< LOD	< LOD	0.022 ± 0.021	< LOD	< LOD	< LOD	0.0147 ± 0.0085	< LOD	< LOD	< LOD
Ba	7.34 ± 0.68	8.7 ± 2.6	8.64 ± 0.62	8.3 ± 3.2	9.4 ± 2.4	6.7 ± 1.2	5.85 ± 0.28	7.1 ± 1.7	6.6 ± 1.1	9.2 ± 2.5
W	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hg	< LOD	< LOD	< LOD	5.0 ± 4.5	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Tl	< LOD	< LOD	< LOD	0.0102 ± 0.0052	< LOD	< LOD	< LOD	0.021 ± 0.012	0.0232 ± 0.0070	0.018 ± 0.019
Pb	0.518 ± 0.065	0.476 ± 0.044	0.40 ± 0.10	0.470 ± 0.099	0.51 ± 0.14	0.302 ± 0.052	0.183 ± 0.016	0.354 ± 0.052	0.363 ± 0.047	0.451 ± 0.039

Table A8. Concentrations (mg kg^{-1}) of 27 elements measured in flowers of tomato plants (n = 3; S1 - S6 = samplings; LOD values in Table 1.2)

	Organically grown flowers					Conventionally grown flowers				
	S1	**S2**	**S3**	**S4**	**S5**	**S1**	**S2**	**S3**	**S4**	**S5**
Na	253 ± 17	159 ± 14	374 ± 40	474 ± 62	390 ± 110	147 ± 14	231 ± 51	480 ± 140	650 ± 140	510 ± 140
Mg	2490 ± 110	3047 ± 77	3250 ± 250	2800 ± 160	2370 ± 660	2490 ± 220	2890 ± 320	3264 ± 87	2590 ± 280	2650 ± 240
Al	83 ± 26	28 ± 13	44 ± 15	60 ± 11	46 ± 18	39 ± 22	2.4 ± 1.1	37 ± 10	47 ± 21	44 ± 23
K	31600 ± 1800	21000 ± 14000	28900 ± 5300	11500 ± 1400	20500 ± 8500	28600 ± 1200	22900 ± 2200	23200 ± 9800	11600 ± 3400	18500 ± 8300
Ca	152000 ± 55000	50000 ± 37000	16400 ± 2200	23310 ± 420	15200 ± 1900	41700 ± 5500	13600 ± 1800	15200 ± 2400	18900 ± 1800	18200 ± 2000
Ti	2.7 ± 3.2	0.61 ± 0.40	0.90 ± 0.12	1.21 ± 0.33	1.45 ± 0.72	1.42 ± 1.84	0.69 ± 0.20	1.08 ± 0.32	0.858 ± 0.033	0.94 ± 0.21
V	< LOD	< LOD	0.082 ± 0.038	0.186 ± 0.065	0.086 ± 0.041	< LOD	< LOD	0.074 ± 0.033	0.081 ± 0.039	0.097 ± 0.053
Cr	< LOD	< LOD	0.176 ± 0.049	0.300 ± 0.049	0.16 ± 0.12	< LOD	< LOD	0.103 ± 0.051	0.261 ± 0.046	0.137 ± 0.085
Mn	35.1 ± 6.9	23.0 ± 1.9	17.3 ± 1.9	18.4 ± 5.7	14.7 ± 2.3	42 ± 12	120 ± 41	25.4 ± 4.0	50 ± 41	14.1 ± 3.5
Fe	35.8 ± 8.0	91 ± 33	94.6 ± 7.5	165 ± 37	160 ± 110	54.4 ± 0.8	83.0 ± 6.7	113.9 ± 5.0	137 ± 48	160 ± 72
Co	0.077 ± 0.025	0.131 ± 0.027	0.0623 ± 0.0054	0.064 ± 0.015	0.0513 ± 0.082	0.0806 ± 0.0145	0.143 ± 0.044	0.0630 ± 0.0078	0.063 ± 0.027	0.032 ± 0.021
Ni	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cu	81 ± 37	310 ± 270	30.6 ± 5.0	37 ± 21	11.0 ± 2.8	7.44 ± 0.17	16.3 ± 1.5	12.7 ± 2.2	7.2 ± 1.7	5.58 ± 0.73
Zn	380 ± 62	141 ± 98	44.4 ± 2.4	31.6 ± 5.2	57 ± 35	204 ± 20	73.5 ± 7.1	49.2 ± 9.4	30.6 ± 4.6	19 ± 10
As	0.28 ± 0.15	0.22 ± 0.22	0.039 ± 0.015	0.089 ± 0.035	0.045 ± 0.021	0.22 ± 0.09	< LOD	0.031 ± 0.015	0.030 ± 0.014	0.044 ± 0.019
Se	2.1 ± 3.2	0.78 ± 0.91	0.38 ± 0.21	< LOD	< LOD	0.45 ± 0.34	0.54 ± 0.24	< LOD	< LOD	< LOD
Sr	54.4 ± 4.3	52 ± 10	73.9 ± 7.9	120.6 ± 5.5	70 ± 20	55.1 ± 6.7	49.7 ± 9.5	73.3 ± 7.2	98 ± 19	93.0 ± 1.8
Mo	5.0 ± 1.8	2.60 ± 0.24	1.94 ± 0.85	1.60 ± 0.79	1.64 ± 0.69	1.80 ± 0.27	1.48 ± 0.20	1.48 ± 0.15	1.06 ± 0.24	1.03 ± 0.27
Ag	0.71 ± 0.12	0.20 ± 0.24	< LOD	< LOD	< LOD	0.343 ± 0.047	< LOD	< LOD	< LOD	< LOD
Cd	0.27 ± 0.16	0.221 ± 0.029	0.177 ± 0.067	0.176 ± 0.035	0.157 ± 0.040	0.188 ± 0.059	0.38 ± 0.17	0.255 ± 0.065	0.244 ± 0.055	0.193 ± 0.040
Sn	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Sb	< LOD	< LOD	< LOD	0.0134 ± 0.062	< LOD	< LOD	< LOD	0.017 ± 0.012	< LOD	< LOD
Ba	6.9 ± 1.6	2.72 ± 0.89	3.03 ± 0.61	3.95 ± 0.81	2.33 ± 0.92	4.55 ± 1.15	1.25 ± 0.22	1.83 ± 0.28	2.45 ± 0.59	3.08 ± 0.58
W	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hg	2.59 ± 0.44	0.84 ± 0.79	< LOD	3.4 ± 2.7	< LOD	1.27 ± 0.18	< LOD	< LOD	< LOD	< LOD
Tl	0.0162 ± 0.0094	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Pb	0.64 ± 0.46	0.30 ± 0.16	0.169 ± 0.019	0.402 ± 0.080	0.258 ± 0.045	0.1424 ± 0.0088	< LOD	0.176 ± 0.055	0.232 ± 0.092	0.35 ± 0.26

Table A9. Concentrations (mg kg^{-1}) of 27 elements measured in fruits of tomato plants (n = 3; S1 - S6 = samplings; LOD values in Table 1.2)

	Organically grown fruits				Conventionally grown fruits			
	S3	**S4**	**S5**	**S6**	**S3**	**S4**	**S5**	**S6**
Na	244 ± 81	266 ± 68	33 ± 21	329 ± 62	480 ± 230	350 ± 280	33 ± 11	950 ± 99
Mg	1383 ± 66	1130 ± 71	450 ± 120	1411 ± 65	1220 ± 160	810 ± 310	373 ± 55	1604 ± 50
Al	3.4 ± 1.4	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
K	36300 ± 1700	29000 ± 3000	12800 ± 2700	35700 ± 1100	28900 ± 3100	19900 ± 6300	11100 ± 1500	29300 ± 1100
Ca	980 ± 150	797 ± 95	650 ± 240	1140 ± 260	870 ± 170	950 ± 170	560 ± 300	580 ± 250
Ti	0.63 ± 0.16	0.26 ± 0.19	< LOD	0.194 ± 0.085	< LOD	0.199 ± 0.094	< LOD	< LOD
V	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cr	< LOD	< LOD	0.065 ± 0.035	< LOD	< LOD	0.071 ± 0.047	0.092 ± 0.082	< LOD
Mn	7.36 ± 0.39	2.0 ± 3.1	11.1 ± 7.8	5.94 ± 0.40	6.98 ± 0.61	5.43 ± 0.45	8.7 ± 2.9	8.79 ± 0.29
Fe	28.6 ± 4.0	5.9 ± 8.3	13.7 ± 6.2	20.8 ± 2.1	31.1 ± 3.1	19.7 ± 4.8	10.9 ± 1.5	36.8 ± 3.2
Co	< LOD	< LOD	0.028 ± 0.014	< LOD	< LOD	< LOD	< LOD	< LOD
Ni	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cu	8.51 ± 0.54	2.3 ± 3.8	4.94 ± 0.88	5.85 ± 0.72	8.22 ± 0.90	6.2 ± 1.5	5.1 ± 1.2	7.6 ± 1.3
Zn	22.2 ± 1.7	< LOD	< LOD	21.8 ± 2.4	18.5 ± 9.4	11.1 ± 5.4	< LOD	26.6 ± 1.5
As	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Se	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Sr	4.19 ± 0.65	1.0 ± 1.3	2.70 ± 0.83	5.0 ± 1.5	3.18 ± 0.68	3.53 ± 0.77	1.71 ± 0.65	2.8 ± 1.2
Mo	0.63 ± 0.22	0.25 ± 0.27	< LOD	0.514 ± 0.076	0.549 ± 0.089	0.29 ± 0.17	< LOD	0.83 ± 0.10
Ag	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cd	0.089 ± 0.029	0.020 ± 0.016	< LOD	0.091 ± 0.011	0.102 ± 0.027	0.050 ± 0.034	< LOD	0.122 ± 0.013
Sn	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Sb	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ba	0.458 ± 0.068	0.263 ± 0.056	4.5 ± 4.7	0.159 ± 0.047	0.303 ± 0.082	0.366 ± 0.085	1.81 ± 0.41	< LOD
W	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Tl	< LOD	< LOD	< LOD	0.0385 ± 0.0021	< LOD	< LOD	< LOD	0.0378 ± 0.0025
Pb	< LOD	< LOD	< LOD	0.092 ± 0.099	< LOD	< LOD	< LOD	< LOD

Table A10. Concentrations (mg kg^{-1}) of 24 elements measured in soils of tomato plants ([a] n = 4; [b] n = 2; S1 – S5 = samplings; LOD values in Table 1.2).

	Organically treated soils of tomato plants					Conventionally treated soils of tomato plants				
	February [a]	S1 [b]	S3 [b]	S5 [b]	October [a]	February [a]	S1 [b]	S3 [b]	S5 [b]	October [a]
Mg	2719 ± 34	2570 ± 220	2456 ± 35	2517 ± 11	2502 ± 37	2788 ± 37	2393 ± 55	2400 ± 48	2490 ± 150	2430 ± 110
Al	18490 ± 200	14600 ± 1400	13100 ± 2000	15280 ± 500	17500 ± 780	19440 ± 740	13380 ± 410	13580 ± 240	15450 ± 610	16400 ± 2200
Ti	12.6 ± 1.1	12.9 ± 4.9	9.750 ± 0.096	11.4 ± 1.8	15.5 ± 2.3	14.6 ± 2.6	8.1 ± 1.4	8.70 ± 0.81	10.46 ± 0.80	14.2 ± 4.4
V	14.44 ± 0.31	12.19 ± 0.11	11.5 ± 1.2	12.56 ± 0.36	20.49 ± 0.92	15.03 ± 0.55	12.131 ± 0.013	12.054 ± 0.033	13.22 ± 0.27	20.0 ± 2.0
Cr	12.70 ± 0.38	11.427 ± 0.029	10.94 ± 0.88	11.72 ± 0.26	16.54 ± 0.59	13.47 ± 0.28	11.07 ± 0.37	11.435 ± 0.049	12.09 ± 0.42	16.2 ± 1.5
Mn	281.0 ± 6.4	267.9 ± 9.9	253.9 ± 7.8	255.0 ± 7.0	271 ± 11	301 ± 14	305 ± 25	277 ± 13	289 ± 13	285.5 ± 8.9
Fe	12462 ± 68	12100 ± 1600	11120 ± 450	11590 ± 33	15500 ± 2900	13260 ± 200	12965 ± 99	12310 ± 240	12810 ± 880	14030 ± 290
Co	3.03 ± 0.12	3.01 ± 0.44	2.972 ± 0.089	3.0119 ± 0.0031	4.193 ± 0.078	3.108 ± 0.080	3.40 ± 0.11	3.156 ± 0.014	3.278 ± 0.081	4.421 ± 0.050
Ni	6.94 ± 0.39	7.5 ± 1.3	7.280 ± 0.042	7.663 ± 0.0021	9.06 ± 0.51	7.10 ± 0.27	8.24 ± 0.14	7.844 ± 0.042	7.938 ± 0.066	9.319 ± 0.080
Cu	9.98 ± 0.69	11.8 ± 1.5	18.54 ± 0.35	13.2 ± 2.7	16.7 ± 3.6	12.34 ± 0.53	13.55 ± 0.51	13.62 ± 0.37	14.13 ± 0.57	15.17 ± 0.23
Zn	61.3 ± 6.5	64.9 ± 6.2	70.3 ± 3.2	68.50 ± 0.26	57.0 ± 1.8	70.9 ± 5.8	98 ± 27	95.9 ± 8.3	84.3 ± 1.2	69.3 ± 8.1
As	9.90 ± 0.26	8.6 ± 1.1	9.2 ± 1.3	9.30 ± 0.15	9.00 ± 0.81	10.18 ± 0.51	9.83 ± 0.63	10.0814 ± 0.0013	10.27 ± 0.15	8.97 ± 0.40
Se	10.2 ± 3.4	11.9 ± 1.8	12.47 ± 0.69	11.24 ± 0.99	< LOD	11.2 ± 2.0	12.9 ± 2.3	10.51 ± 0.55	13.0 ± 5.2	< LOD
Sr	680 ± 23	621 ± 22	561 ± 51	609.4 ± 2.5	579 ± 12	637.1 ± 9.0	634 ± 30	597 ± 19	604 ± 28	574 ± 35
Mo	0.457 ± 0.028	1.102 ± 0.015	0.397 ± 0.013	0.313 ± 0.011	0.320 ± 0.029	0.409 ± 0.085	0.462 ± 0.042	0.381 ± 0.017	0.255 ± 0.025	0.2941 ± 0.0088
Ag	0.117 ± 0.052	< LOD	< LOD	< LOD	< LOD	0.220 ± 0.038	0.109 ± 0.051	0.110 ± 0.053	0.1588 ± 0.0042	0.133 ± 0.040
Cd	0.157 ± 0.023	0.191 ± 0.033	0.207 ± 0.034	0.148 ± 0.038	0.178 ± 0.010	0.234 ± 0.052	0.274 ± 0.026	0.267 ± 0.044	0.2853 ± 0.0018	0.2204 ± 0.0077
Sn	3.246 ± 0.060	4.2 ± 2.1	2.50 ± 0.29	2.826 ± 0.070	2.754 ± 0.094	3.84 ± 0.25	3.34 ± 0.36	3.104 ± 0.060	5.1 ± 2.5	2.95 ± 0.19
Sb	0.363 ± 0.015	0.24 ± 0.12	0.312 ± 0.020	0.32041 ± 0.00034	0.323 ± 0.016	0.431 ± 0.023	0.322 ± 0.029	0.369 ± 0.025	0.434 ± 0.023	0.304 ± 0.090
Ba	80.71 ± 0.97	63.4 ± 2.8	59.4 ± 5.0	64.7 ± 2.0	61.8 ± 2.1	85.5 ± 1.5	66.2 ± 1.8	66.28 ± 0.63	70.09 ± 0.39	61.1 ± 4.5
W	0.1824 ± 0.0089	< LOD	< LOD	< LOD	< LOD	0.189 ± 0.016	< LOD	< LOD	< LOD	< LOD
Hg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Tl	0.2906 ± 0.0079	0.219 ± 0.012	0.186 ± 0.032	0.222 ± 0.013	0.2274 ± 0.0097	0.3077 ± 0.0047	0.2033 ± 0.0092	0.207 ± 0.013	0.232 ± 0.011	0.218 ± 0.024
Pb	29.4 ± 1.7	26.3 ± 2.8	24.9 ± 2.4	26.66 ± 0.46	24.54 ± 0.82	43.8 ± 4.0	37.46 ± 0.73	37.1 ± 3.5	71 ± 45	31.19 ± 0.85

Annex B

Statistics for the effect of the plant part, of the sampling campaign and of the farming technique on the concentration of elements in Swiss chards and tomato plants in the field experiment

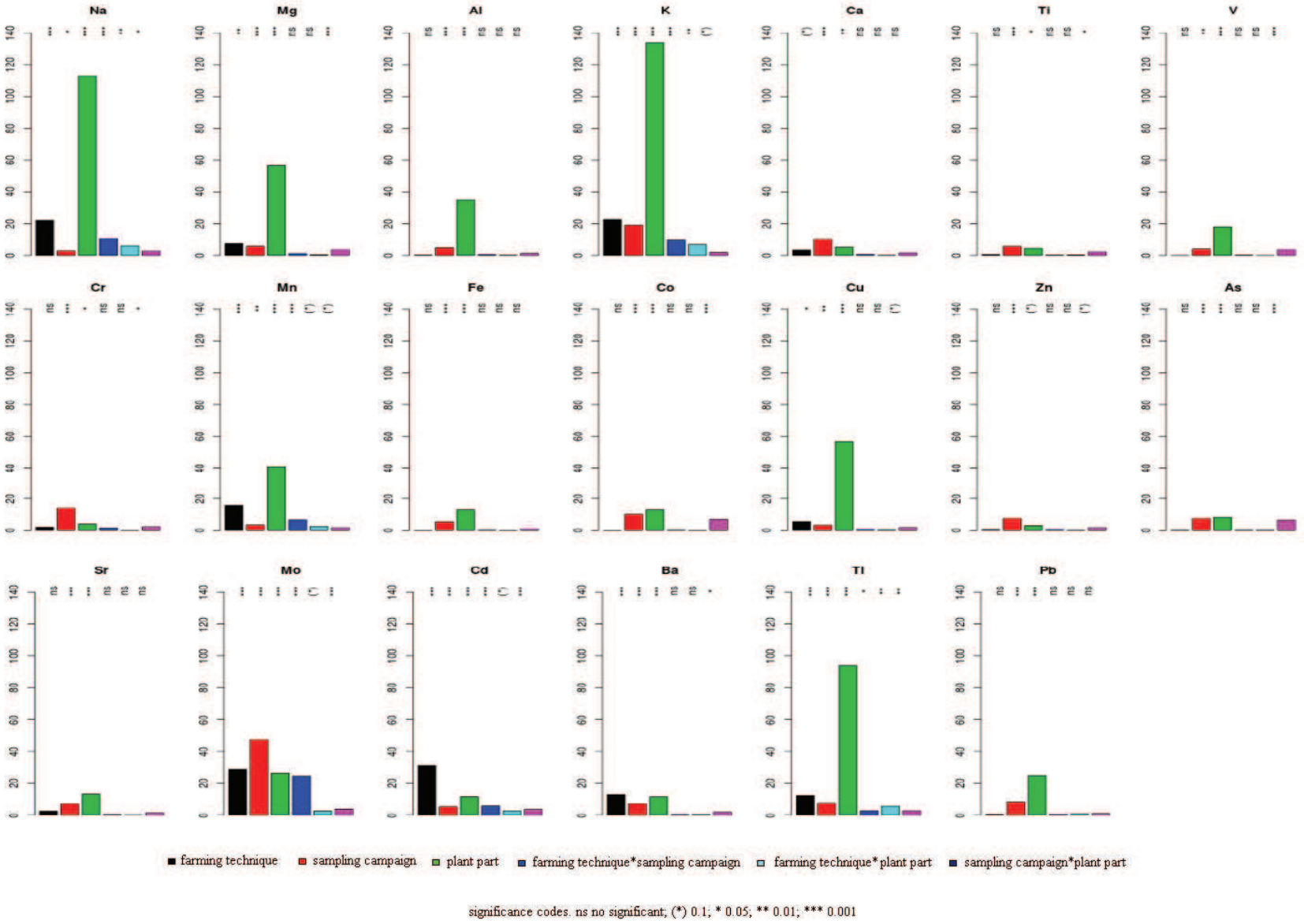

Figure B1. Fisher statistics (Y axis) obtained for each considered factor (plant part, sampling campaign and farming technique) and their interactions after ANOVA of element concentrations in Swiss chards. The significance of the effects of each factor is presented by star codes.

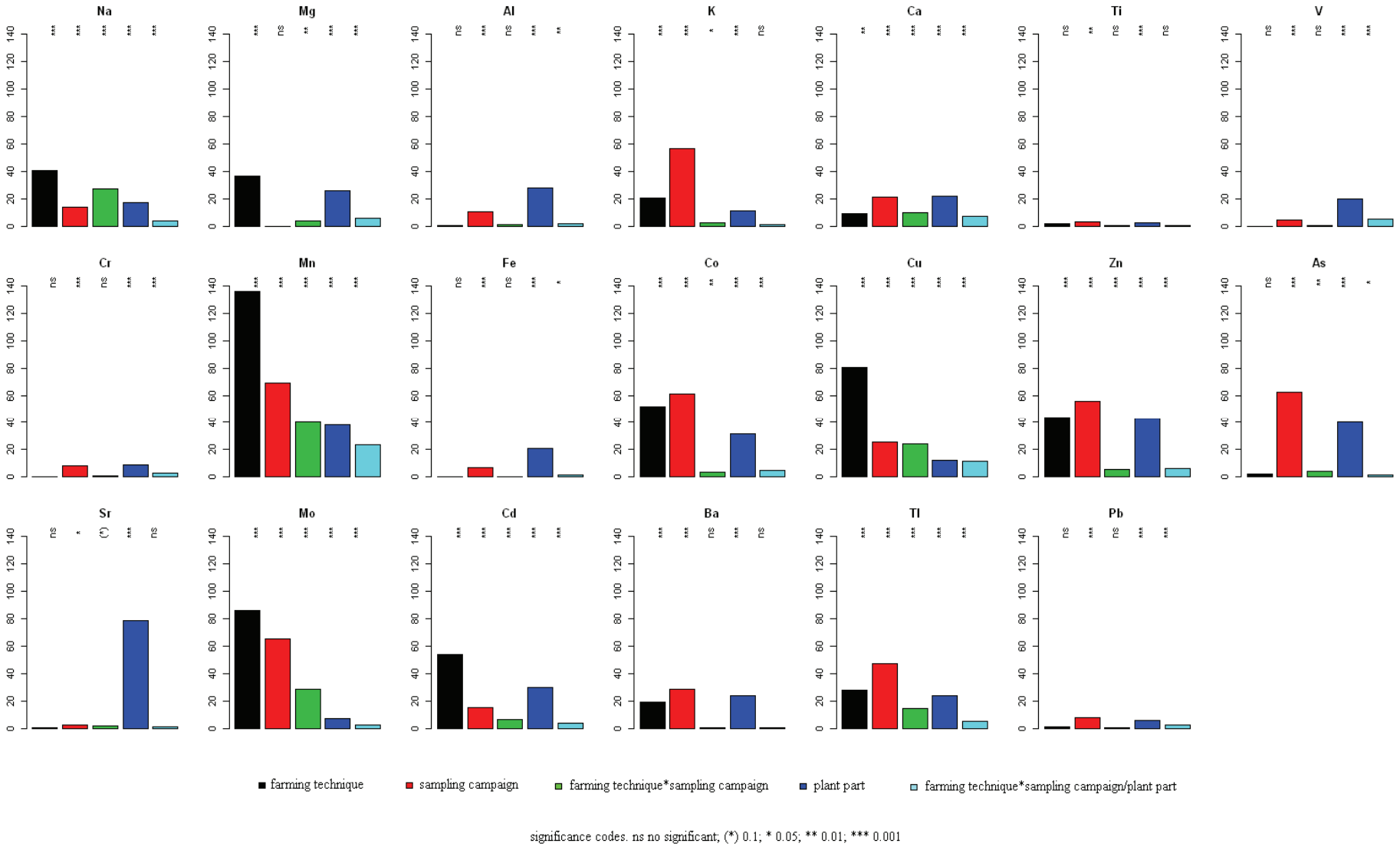

Figure B2. Fisher statistics (Y axis) obtained for each considered factor (plant part, sampling campaign and farming technique) and their interactions after ANOVA of element concentrations in tomato plants. The significance of the effects of each factor is presented by star codes.

Annex C

Sunflowers experiment

Models tested for the allocation of the Cd recently taken up to each plant organ depending on competitive sinks

Table C1. Models tested for the allocation of the Cd recently taken up to each plant organ depending on competitive sinks. The values of R^2, the slope, the intercept and their significances (*p*) are shown (AG = aboveground organs).

Plant organ	Competitive sink	R^2	Slope	*p*	Intercept	*p*
Roots	-	0.53	0.052	< 0.001	-0.16	0.8
	Taproot	0.91	1.10	< 0.001	-0.10	0.6
	Stem	0.91	1.9	< 0.001	0.3	0.07
	Leaves	0.94	1.95	< 0.001	-0.33	0.06
	AG	0.87	2.5	< 0.001	0.65	< 0.001
	Non-roots	0.87	2.8	< 0.001	0.63	0.001
Taproot	-	0.01	-0.003	0.6	0.37	< 0.001
	Stem	0.55	1.9	< 0.001	-0.087	0.3
	Leaves	0.09	0.66	0.1	0.13	0.3
	AG	0.63	2.85	< 0.001	-0.0042	0.6
	AG (without leaves)	0.67	1.59	< 0.001	0.057	0.2
Stem (just after the taproot)	-	0.18	-0.004	0.04	0.33	< 0.001
	Leaves	-0.054	0.042	0.9	0.19	0.1
	AG (without stem)	0.53	0.93	< 0.01	-0.12	0.1
	AG (without stem and leaves)	0.79	0.55	< 0.001	-0.09	0.03
Leaves (just after the taproot)	-	0.84	0.0213	< 0.001	0.12	0.007
	Stem	0.29	1.1	0.008	0.17	0.2
	AG (without leaves)	-0.045	0.16	0.7	0.47	< 0.001
	AG (without leaves and stem)	-0.054	-0.029	0.9	0.52	< 0.001
Stem (after the leaves)	-	-0.05	-0.002	0.8	0.23	0.02
	AG (without stem)	0.98	0.99	< 0.001	0.0063	0.2
Leaves (after the stem)	-	0.80	0.019	< 0.001	0.23	< 0.001
	AG (without leaves)	0.02	0.37	0.2	0.39	0.002
Receptacle	-	0.29	0.011	0.06	-0.0026	0.9
	Flowers	0.76	0.52	< 0.001	-0.021	0.06
	Almonds	0.81	0.95	< 0.001	-0.026	0.02
	Husk of full seeds	0.88	0.65	< 0.001	-0.018	0.02
	Empty seeds	0.72	0.45	0.001	-0.019	0.1
	Non-developed seeds	0.66	0.43	0.003	-0.018	0.2
	Full seeds	0.87	1.15	< 0.001	-0.022	0.01

Table C1 (*cont*.). Models tested for the allocation of the Cd recently taken up to each plant organ depending on competitive sinks. The values of R^2, the slope, the intercept and their significances (*p*) are shown (AG = aboveground organs).

Plant organ	Competitive sink	R^2	Slope	*p*	Intercept	*p*
Flowers	-	0.13	0.015	0.2	-0.0005	0.9
	Non-developed seeds	-0.08	0.038	0.6	0.0041	0.4
	Husk of full seeds	0.59	0.47	0.006	-0.0035	0.3
	Almonds	0.43	0.81	0.02	-0.0062	0.2
	Full seeds	0.55	1.2	0.008	-0.0054	0.2
	Empty seeds	0.11	0.11	0.2	0.00019	1
	Non-flowers	0.46	1.10	0.01	-0.0038	0.2
	Husks of empty and full seeds	0.61	0.59	0.005	-0.0045	0.2
	Full and empty seeds	0.52	1.10	0.007	-0.0043	0.1
Non-developed seeds	-	0.96	0.033	< 0.001	-0.0007	0.4
	Empty seeds	0.48	0.2	0.02	-0.0019	0.5
	Husk of full seeds	0.91	0.5	< 0.001	0.00025	0.7
	Almonds	0.97	0.83	< 0.001	-0.0007	0.2
	Full seeds	0.99	1.28	< 0.001	-0.0004	0.4
	Non-non-developed seeds	0.98	1.5	< 0.001	-0.0004	0.3
Empty seeds	-	0.91	0.036	< 0.001	-0.0002	0.7
	Non-developed seeds	-0.11	0.025	0.8	0.0067	0.2
	Husk of full seeds	0.64	0.53	0.003	0.0016	0.4
	Almonds	0.92	0.95	< 0.001	-0.0004	0.7
	Full seeds	0.79	1.4	< 0.001	0.00042	0.8
	Non-empty seeds	0.80	1.7	< 0.001	0.00014	0.9
Full seeds	-	0.69	0.032	0.002	0.019	0.1
	Almonds	0.86	0.023	< 0.001	0.017	0.04
	Almonds/empty seeds	0.85	0.87	< 0.001	-0.0043	0.7
	Almonds/non-developed seeds	0.87	0.87	< 0.001	-0.0045	0.6
	Almonds/non-full seeds	0.96	1.10	< 0.001	0.0014	0.8
	Non-full seeds	0.87	0.014	< 0.001	0.016	0.6
Almonds	-	0.78	0.022	< 0.001	0.011	0.2
	Husk of full seeds	0.95	1.43	< 0.001	-0.0089	0.1
Husk of full seeds	-	0.43	0.003	0.03	0.015	< 0.001
	Almonds	0.65	0.16	0.01	0.013	< 0.001