Trace elements and in particular manganese in three Brazilian medicinal plants studied in tea and in hydropony grown plants

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Index

Abstract **6**

1. Introduction to element speciation **8**

2. Motivation **9**

2.1. One element in particular: manganese 9

2.2. Medicinal plant teas 10

2.3. Plants growing under metal stress condition 12

3. Literature survey and the studied plant material **14**

3.1. Jambu = Jam (*Spilanthes oleracea*) 17

3.2. Beldroega = Bel (*Portulaca oleracea*) 18

3.3. Alfavaca = Alf (*Ocimum gratissimum*) 19

4. Methods, materials and calibrations **19**

4.1. High-Performace Liquid Chromatography (HPLC) 20

4.2. Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) 24

4.3. Electrospray Ionization -Mass Spectrometry (ESI-MS) 27

4.4. Matrix assisted laser desorption/ionisation - time of flight - mass spectrometry 29

4.5. Instrumental Neutron Activation Analysis (INAA) 30

4.6. Ultrafiltration 32

4.7. Determination of the total protein concentration by the Bradford test 34

4.8. Determination of the sulfhydryl group concentration by the Ellman test 35

4.9. Materials 36

4.10. Botanical material and extraction method (Tea) 36

4.11. Comparison of methods to grow from seeds 37

4.12. The hydroponic system 38

4.13. Methods to break down cells in plants 40

4.14. Digestion of plant samples 40

4.15. Working scheme 42

5. Detailed results **43**

5.1. Medicinal plant teas 43

5.1.1. Water content in plant leaves 43

5.1.2. Medicinal teas studied by HPLC (SE)-ICP-MS 43

5.1.3. Dry leaves and tea studied by ICP-MS off line and by INAA 46

5.1.4. Extraction efficiencies of manganese, iron, zinc and cobalt in tea 46

5.1.5. Protein and sulfhydryl groups in tea 47
5.1.6. Metals in medicinal teas studied by ICP-MS off-line 49
5.1.7. Separation of lower molecular weight compounds in tea 51
5.1.8. Lower molecular weight compounds studied by MALDI-TOF-MS 57
5.1.9. Lower molecular weight compounds studied by HPLC (RP)-ESI-MS 58
5.2. Metal stress on plants grown in a hydroponic system 62
5.2.1. Comparison of extraction methods 62
5.2.2. Plants grown under manganese stress in the hydroponic system 64
5.2.3. Concentration of zinc, iron and cobalt in stressed plants 66
5.2.4. Manganese analysis by HPLC-ICP-MS 68
5.2.5. Elemental concentration in plant extracts by ICP-MS off line 71
5.2.6. INAA and ICP-MS off line 74
5.2.7. Extraction efficiencies of plants stressed and not stressed 74
5.2.8. Determination of the total protein concentration by the Bradford Reagent 75
5.2.9. Determination of sulfhydryl groups concentration by the Ellman´s Reagent 75
5.2.10. Separation of lower molecular weight compounds in medicinal plant extracts 77
5.2.11. Plant extracts <5kDa studied by HPLC (RP)-ICP-MS 80
5.2.12. Analysis of lower molecular weight compounds by MALDI-TOF-MS 83
5.2.13. Analysis of lower molecular weight compounds by HPLC (RP)-ESI-MS 84
5.2.14. Analysis of higher molecular weight compounds by HPLC (SE)-ESI-MS in the case of crude extracts from leaves of Jam plants grown under stress. 89
6. Final Discussion 91
7. Final conclusion and outlook 97
8. List of chemicals, materials and equipments 99
9. References 101

List of abbreviations

Alf	Alfavaca
Bel	Beldroega
CCA	α-cyano-4-hydroxy-cinnamic acid
Da	Dalton
DHB	Dihydroxybenzoic acid
d_p	Particle diameters
EPA	Environmental Protection Agency
ESI-MS	Electro Spray Ionization Mass Spectrometry
g	acceleration due to gravity
HPLC	High Performance Liquid Chromatograph
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
INAA	Instrumental Neutron Activation Analysis
IUPAC	International Union for Pure and Applied Chemistry
Jam	Jambu
MALDI-TOF-MS	Matrix assisted laser desorption/ionisation – time of flight- mass spectrometry
MMT	methylcyclopentadienyl manganese tricarbonyl
MW	Molecular Weight
NIH	National Institute of Health
PCs	Phytochelatins
Omega-3	Omega-3 fatty acids
RF	Radio Frequency
RP	Reversed Phase
rpm	revolutions per minute
SA	Sinapinic acid
SE	Size Exclusion
SEC	Size Exclusion Chromatography
TPN	Total parenteral nutrition
TFA	Trifluoroacetic acid
UV	Ultra violet
V	voltage

Abstract

Brazil offers many medicinal plants not known in Europe, and only a few significant studies have been performed on these plants. The present study focuses on the correlations between some metals and organic compounds in such plants.

Trace elements, and in particular manganese in three Brazilian medicinal plants (*Ocimum gratissimum*, *Portulaca oleracea* and *Spilhantes oleracea*) were studied in tea and in plants grown in hydroponic system.

Teas were made from dried leaves of these plants and were extracted in the same way as recommended for medicinal use (hot water extraction). The plants grown in a hydroponic system were stressed with different manganese concentrations. Three methods of cell break down were tested. Ultra-Turrax has shown the best results.

In order to gain better information about the molecular size of the compounds that may bind the metals, ultrafiltration of different molecular weight cut off was used. In this way the results from tea and plants grown in the hydroponic system have shown that major concentrations of all studied elements are in molecular weight fractions <5kDa.

The effect of Mn stress on the concentrations of proteins and sulfhydryl groups in the extracts were studied by the Bradford and the Ellmans test, respectively.

The elemental concentrations in fresh plants and tea were studied by ICP-MS off line; INAA was used for validation of the method. The results show good agreement.

Tea and plant extracts were also studied by HPLC-ICP-MS and HPLC-UV-ESI-MS with different columns and eluents. Many elements were detected such as Mn, Mg, Fe, Zn, Sr, S, P, Ni and Co. Interestingly, all elemental chromatograms and mass spectra were similar.

As expected organic acids like citric acid, tartaric acid and maleic acid were detected by HPLC-UV and confirmed by HPLC-ESI-MS. But regrettably no asignment could be worked out for probable Mn binding compounds although the chromatographic peaks (by UV) correlate to the Mn peaks (by ICP), due to lack of sensitivity of the presently developed methods.

Zusammenfassung

In Brasilien gibt es viele Heilpflanzen, die in Europa nicht bekannt sind; nur wenige Studien wurden über solche Pflanzen angefertigt. Diese Arbeit zielt auf Korrelationen zwischen einigen Metallen und organischen Verbindungen in solchen Pflanzen ab.
Spurenelemente und besonders Mangan wurden in drei brasilianischen Heilpflanzen (Ocimum gratissimum, Portulaca oleracea und Spilanthes oleracea) in Tee und in in Hydrokultur gezogenen Pflanzen untersucht.

Die Tees wurden aus getrockneten Blättern in derselben Weise wie in medizinischen Anwendungen bereitet (Heißwasserextraktion). Die in Hydrokultur gezogenen Pflanzen wurden zusätzlich mit Mangan unterschiedlicher Konzentration gestresst. Drei Methoden zum Zellaufschluss wurden getestet; Ultraturraxbehandlung lieferte die besten Ergebnisse.

Um detailliertere Information zur Größe der an Metalle gebundenen Verbindungen zu gewinnen, wurden die Extrakte sequentiell durch Ultrafilter unterschiedlicher Porenweite gefiltert. Sowohl für Tee wie für die Pflanzenextrakte der in Hydrokultur gezogenen Pflanzen wurde gefunden, dass die größte Konzentration aller untersuchten Elemente im Bereich <5kDa zu finden ist.

Mittels Bradford und Ellman-Test wurde der Effekt von Manganstress auf die Konzentration an Proteinen und Sulfhydrylgruppen getestet.
Elementkonzentrationen in Pflanzenextrakten und in Tees wurden mit ICP-MS off-line bestimmt; instrumentelle Neutronenaktivierungsanalyse (INAA) wurde zur Validierung dieser Messungen herangezogen, wobei gute Übereinstimmung gefunden wurde.

Sowohl die Tees als auch die Pflanzenextrakte wurden dann mit HPLC-ICP-MS und HPLC-UV-ESI-MS mit unterschiedlichen Säulen und Eluenten untersucht. Eine Reihe von Elementen wurden quantifiziert, wie Mn, Mg, Fe, Zn, Sr, S, P, Ni und Co. Interessanterweise sind alle erhaltenen Chromatogramme und Massenspektren sehr ähnlich.

Wie zu erwarten war wurden organische Säuren, wie Zitronensäure, Weinsäure und Maleinsäure per HPLC-UV und HPLC-ESI-MS gefunden. Aber leider konnte keine Zuordnung von möglicherweise Mangan bindender Verbindungen getroffen werden obwohl die chromatographischen Peaks - einerseits UV, anderseits ICP – korrelieren. die entwickelten Extraktions- und Anreicherungsmethoden müssen dazu noch verfeinert werden.

1. Introduction to element speciation

Trace elements play an important role in the functioning of life on our planet. While some elements can be highly toxic to various life forms, others are essential. But even essential trace elements can be toxic in higher doses [1][2][3][4]. The influences and effects of trace elements are largely determined by the nature of the ligands around it [5][6], and by their chemical form. For example, Cr (VI) ions are by far more toxic than Cr (III) [7]. Therefore, speciation has become a topic of growing importance, especially with respect to biological effects, like toxicity.

According to the International Union for Pure and Applied Chemistry (IUPAC), speciation analysis is definied as follows [1]: *speciation analysis is the analytical activity of identifying and/or measuring the quantities of one or more individual chemical species in a sample; the chemical species are specific forms of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure; the speciation of an element is the distribution of an element amongst defined chemical species in a system. In case that it is not possible to determine the concentration of the different individual chemical species that sum up the total concentration of an element in a given matrix, that means it is impossible to determine the speciation, it is a useful practice to do fractionation instead. Fractionation is the process of classification of an analyte or a group of analytes from a certain sample according to physical (e.g. size, solubility) or chemical (e.g. bonding, reactivity) properties.*

Speciation analysis provides the information necessary to describe the effects of active species which is not available from the results of total trace element determinations. The proper measurement of elemental speciation requires careful attention to the entire analytical procedure, including sampling, sample storage and preparation. The possible problem of contamination is at least as severe as with conventional elemental analysis, and complications due to loss, degradation or instability of species of interest are much more problematic in speciation measurements.

The interest for trace element speciation has now reached a wider audience and is no longer limited to academic spheres. The industry and governments are getting involved, especially with the advent of new and more stringent regulations [8].

2. Motivation

Brazil offers many medicinal plants of which little is known so far about their heavy metal content and the speciation there-of. Many Brazilian plants will become extinct without having ever been studied due to the indiscriminate extraction of these plants, the use of pesticides in agriculture and the devastation of forests caused by agricultural development, including clearing land for grazing animals. One and a half acres of rainforest are lost every second with tragic consequences for both developing and industrial countries [9][10].

In this thesis, three plants have been chosen to be studied as tea and as grown under manganese stress*: Ocimum gratissimum* (Alfavaca = Alf)*, Portulaca oleracea* (Beldroega = Bel)*, Spilhantes oleracea* (Jambu = Jam). The choice of these plants was based on their medicinal effect and their widespread use by the populations in the Amazon area.

2.1. One element in particular: manganese

Manganese has one stable isotope, ^{55}Mn. The most stable oxidation state of manganese is +2, however oxidation states from +1 to +7 are observed [11].

In general, manganese was chosen because of its importance to living systems and its potential toxicity, as well as because of the fact that few speciation studies of manganese in plants have been performed. The speciation analysis of manganese is of paramount importance because of the versatility of its occurrences and forms and of their respective properties in living systems. Additionally, human beings are coming into more frequent contact with higher doses of this element through the environment or by self-medication, e.g. food supplements and vitamins. The importance of manganese in living systems remains poorly explored [12].

Manganese is an essential trace element to all living systems and can be found everywhere on earth. It is both an activator and constituent of several enzymes [13]; it is a major constituent of several metalloenzymes, hormones and proteins of humans. For plants, manganese is necessary in photosynthesis, nitrogen metabolism and it is an integral part of several enzymes and proteins [14][15].

2.2. Medicinal plant teas

Medicinal plant teas have been used since the old times by humanity for the treatment of ailments ranging from the common cold to cancer [16][17]. During the last century, the use of medicinal plants declined with the development of synthetic drugs, especially in developed countries [18]. Notwithstanding, the use of easily accessible and low-cost medicinal plants continued, co-existing with modern medicine.

Lately, there has been a revival of social interest in the use of medicinal plant products because of their observed and proven efficacy and being relatively free from toxic side effects. Nowadays, plant materials are widely used throughout developed and developing countries as home remedies, nutritional supplements or as raw materials for the pharmaceutical industry, representing a substancial proportion of the global drug market [18].

The proprerties of tea arise from a combination of a large number of constituents. The chemical constituents of tea leaves and manufactured tea is very complex and consists of tanning substances, flavonols, alkaloids, proteins, amino acids, enzymes, aroma-forming substances, vitamins, minerals and trace elements [19]. The composition also depends on the medicinal plant species and the sample preparation.

One important factor for the formation of active constituents in tea of medicinal plants is the trace elemental composition because they are known to play an important role in various metabolic processes [16][19][20]. Therefore, information about their distribution in medicinal teas is of significance [20]. Although there are reports in the literature on the trace element content in medicinal plant teas, we are still far from the point of knowing exactly the mechanisms of action and formation of active constituents for each medicinal plant tea [21]. Even though a direct link between elemental content and its curative capability is yet to be established [21][22][23].

It is interesting to note that the comparison of the elemental content of medicinal with that of non-medicinal plants shows that medicinal plants are richer in element content than non-medicinal plants [23].

The efficacy of medicinal plants for curative purpose is often accounted in terms of its organic constituents. On the other side it is an established fact that there is a strong relationship between the chelation of metals and some chemotherapeutic agents [22]. To be pharmacologically effective, the trace element may need to be combined or chelated with some ligand (for example porphyrin), in order to be physiologically absorbed and thus be able

to prevent or cure impairment caused by deficiency of the element [24].

Trace elements co-exist with numerous organic compounds (many of them are complexing agents) in the tea of medicinal plants. Therefore, the concentrations of the free trace elements are usually very low. Different oxidation states have different functions, toxicity and absorption rates by the body [18].

While some of the biological functions of essential metals are quite well understood, e.g. activation of enzymes or involvement in the synthesis and metabolism of biomolecules, the chemical form of a metal, which naturally occurs in plants, has not been deeply studied [25]. Consequently, there is little knowledge about the potential influence of the metal on the pharmacologically active substances in plants.

The possible influence of metals on pharmacological effects of teas is the most obvious reason why metal species in tea should be known, but there are two other fields in which this knowledge is important. One is the topic of medicinal plants authentication, which is of growing interest, especially in the field of traditional herbal medicine. The other one is the field of chemotaxonomy, e.g. the classification of biological species in relation to their chemical composition, which of course includes metal species [26].

There are already publications dealing with total concentrations of elements in medicinal plant teas. However, total concentration of elements does not provide much relevant information for medical or biological investigations. Elemental speciation of these teas is of major importance to understand their pharmacological action [21][23]. Only a few investigations dealt with the elemental speciation in tea.

One problem in speciation, especially if the species are small metal complexes in a low concentration range, is the question as to whether the species remain intact throughout the whole separation process. As long as the respective species are not fully identified (and no thermodynamic or kinetic data is available) the only way to at least minimize the risk of artefact formation is to use different methods, which should be based on independent separation mechanisms [26].

A few speciation studies [27][28][29][30][31] have been carried out for tea, most of all regarding the speciation of aluminium, which is because of the relatively high concentration of Al in tea, for many people tea being the major source of Al in the diet [32].

Manganese in tea leaves has been reported to amount for 350-900 μg/g [33]. The recommended range of daily dietary intake for an adult is 2-3 mg of manganese. Intake of tea can provide a rich dietary source of manganese to ill people. It is reported that, under

simulated intestinal conditions, a single serving of tea contributes to about 40% of the average daily dietary intake of manganese in a potentially bioavailable form [33].

Manganese deficiency has been observed in a number of animal species [34]. Signs of deficiency of manganese include impaired growth, impaired reproductive function, skeletal abnormalities, impaired glucose tolerance, altered carbohydrate and lipid metabolism [13][34][35]. In humans, demonstration of a manganese deficiency syndrome deserves more studies; just few cases have been reported in literature. Norose et al. [36] described the medical case of a child on long-term total parenteral nutrition (TPN) that lacked manganese. It developed bone demineralization and impaired growth which were corrected by manganese supplementation. Other possible signs of manganese deprivation, e.g. problems with biosynthesis of cholesterol, have been reported [37][38].

Low manganese dietary or low levels of manganese in blood or tissue have been associated with osteoporosis [39][40], diabetes mellitus [41][42] and epilepsy [35][43]. Although manganese insufficiency is not currently thought to cause these diseases, more research may determine whether a suboptimal manganese nutritional status contributes to certain disease processes.

Other metals were also analysed in the present thesis because of their important function to health, e.g. magnesium (production and transfer of energy for protein synthesis, for contractility of muscle and excitability of nerves [44]), zinc (participates in the metabolism of nucleic acids and the synthesis of proteins [45]), and iron (co-factor and activator of enzymes and metallo-enzymes; respiratory pigments and electron transfer for utilization of oxygen [28]).

2.3. Plants growing under metal stress condition

In the last years, some heavy metals, e.g. mercury, were identified as environmental hazards. Unlike most other pollutants, heavy metals can´t be chemically or biologically degraded. The use of microorganisms and plants for the matrix decontamination from these metals is called phytoremediation.

Phytoremediation is an emerging technology that uses various plants to degrade, extract and immobilize contaminants from soil and water. This technology has been receiving attention recently as an innovative, cost-effective alternative to the more established treatment methods used at hazardous waste sites [46].

Manganese complexes have attracted considerable interest recently because of the frequent occurrence of such metal centres in biological systems. Knowledge about the speciation of manganese in phytosystems is essential for understanding environmental and biological pathways of this element, including essential or toxic effects on human nutrition.

The uptake and metabolism of manganese in plants is of special interest, because this represents one of the main links between environmental deposition of pollutants and the food chain. The plant strategies to prevent high concentrations of Mn and the chemical form in which manganese exists in plant tissues are yet not known and phytoremediation might prove to be a possible alternative to detoxify manganese from the environment.

In addition to occurring naturally in the environment, manganese can be introduced by human activity. Industries reported to the U.S. Environmental Protection Agency (EPA) that 4567 tons of manganese had been released into the environment in 1995, approximately 90% of it into the soil [47]. The EPA has identified 1467 toxic compounds in the National Priorities List sites, manganese has been found in at least 603 compounds on this list [48]. Manganese is the fourth most widely used metal in the world, after iron, aluminium and copper. Over 90% is used in steel making, either as ferromanganese or silicomanganese [49]. Manganese is also used in products for construction, machinery, in fireworks, porcelain, glass, dry cell batteries, and in the oil and gas industries [49].

Manganese in higher concentration can be toxic for all forms of life. The disease caused by higher concentration of manganese in human body is called manganism. Manganism is a collection of symptoms that result from excessive exposure to manganese. It occurs because high concentration of manganese injures a part of the brain that helps to control body movements [13]. It has also been called "Parkinson's syndrome" because its symptoms closely resemble those of Parkinson´s disease [50][51][52]. Levy and Nasseta [53] reported that many of the cases diagnosed as Parkison´s disease in the United States and elsewhere are related to manganese exposure.

Manganism is also called welder's-disease [54] because of the high incidence in welders and those exposed to the fumes from welding rods. However, welding rod use is not the only potential source of manganese exposure. In addition to welding, on-the-job exposure to manganese occurs mainly in mining, alloy production, processing, ferro-manganese operations and work with agrochemicals.

The symptoms of manganism are: multiple neurologic problems, reduced white blood cell counts, impotence, asthenia, insomnia, mental confusion, dry throat, coughing, tight chest, dyspnea, rales, flu-like fever, lower-back pain, vomiting, fatigue, headache, speech

disturbances and muscle contractions [53][54][55][56][57]. In worst form, manganism symptoms include tremors, difficulty in walking, facial muscle spasms and psychiatric symptoms, such as irritability, aggressiveness and even hallucinations [34].

The wide range of variability of manganism symptoms and signs suggests that there exist individual (perhaps genetically modulated) susceptibility factors for this disease. Other proposed susceptibility factors include: age, gender and developmental stage. Some of the symptoms of manganism may improve upon certain medical treatments, but the improvements are usually temporary, and the brain injury is permanent [34].

Scotland, USA, Japan, Cuba, Chile, Egypt, India, Australia, Taiwan, Israel, Italy and Norway have reported cases of manganese exposure and neurological disorder [53].

Scientists are still working to understand the diverse effects of manganese toxicity in living organisms.

3. Literature survey and the studied plant material

Plants consist of two main structural components: (1) the root system, which anchor the plant in the soil and let absorb materials from it, and (2) the shoot system consisting of the stem and leaves. The stem supports the plant above the ground, and is the part through which minerals and water absorbed from roots are conveyed to leaves. The mechanisms of metal uptake by the root system, translocation from roots to shoots, and tolerance to toxicity are dependent on the speciation of the absorbed metals, which may be further modified by the plant [28].

Speciation of heavy metals in plants has been attracting considerable interest as a way to understand the internal mechanisms allowing the living organisms to grow in an environment contaminated by heavy metals [28].

It is clear that plants control both the oxidation state and coordination environment of specific elements to maximize their detoxification and/or transport. For detoxification of metals and metalloids, plants directly coordinate the element, using the most chemical appropriate ligand, to form stable non-toxic complexes. Plants require roots to acquire essential minerals such as iron, copper, nickel, zinc, and selenium from the soil. Though these elements are essential, they are also potentially toxic to the plants themselves; so plants possess complex biochemical pathways to control them.

The great efforts made by the living organisms to take up, accumulate, transport, and store essential or toxic metals are realized by surrounding the metal ion by electron-pair

donating biological ligands [28]. The evolution of a metal in a living organism happens by its interaction with the highly complex bioligand coordinating environment.

After the metals have been taken up by the roots, the xylem transports them and they are distributed to the leaves. When the metal concentration increases in a plant, the cell membrane controls the metal efflux and the metals bind to negative charges in various macromolecules that are either soluble or part of cellular structures in the cell. Metals may be accumulated in the cytoplasm, the amount depending on the plant species and metal. Soluble molecules in the cytoplasm, such as organic acids or sulfur-rich polypeptides, like phytochelatins, form complexes with metals and may also function as shuttles to facilitate metal transport into the cell vacuole. The sequestration and translocation of manganese into vacuoles was proposed to enhance manganese tolerance [58].

Phytochelatins (PCs) is probably the most widely studied metal binder in plants. PCs can detoxify metals by forming a metal-PC complex in which the metal is bound to thiol groups of the cysteine units (Figure 1). However, recognition of PCs as the chelators of metals in general, and protectors of plants against their toxic effect requires careful consideration. In fact PC-metal complex formation has been reported mainly for cadmium but experiments with other metals, e.g. Hg, which active the formation of PC-metal complex also have been reported [28]. In literature there are related studies showing that some metals, e.g. selenium, vanadium and cobalt, could not induce the formation of phytochelatins [59][60][61].

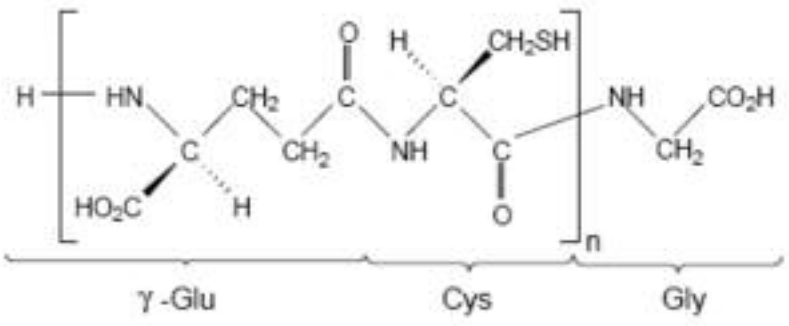
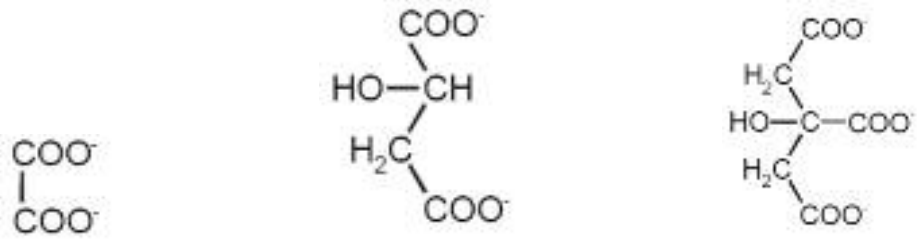


Figure 1. PCs are a class of oligopeptides composed of three amino acids only: cysteine (Cys), glutamic acid (Glu) and glycine (Gly) and in which glutamic acid is linked to cysteine through a peptide linkage. Their general formula is $(\gamma\text{-GluCys})_n$ Gly, where n is between 2 and 11 [28].

Other mechanisms of plant resistance to toxic elements include high turnover of organic acids such as e.g. oxalate, malate, citrate (Figure 2), phytate, succinate and induction

and/or activation of anti-oxidant enzymes, such as e.g. superoxide dismutase or glutathione peroxidase [28].



Oxalate Malate Citrate

Figure 2. Chemical formula of related organic acid anions

Metal complexation leads to a number of sometimes yet poorly characterized metal complexes. The understanding of mechanisms controlling the detoxification can benefit from the identification of the species formed. The thermodynamic stability of the metal complexes strongly depends on their equilibrium constants; thus, not every possible complex can be isolated with a chromatographic column.

Plants – like all other organisms – have to tightly control the intracellular concentration and distribution of toxic (e.g. Cd) and essentially (yet potentially toxic such as manganese) elements. The mechanism of detoxification adopted by plants varies from metal to metal and for a metal from species to species, and it is difficult to reconcile the idea of tolerance by means of any single mechanism [28].

The visible symptoms of manganese toxicity in plants, which vary by species, include brown spots, marginal chlorosis, slow development in growing, necrosis of leaves and crinkled leaves [62][63][64]. The nature of manganese toxicity symptoms varies also by cultivation and leaf age [62], therefore, the results are difficult to compare with the literature data.

In our experiments, after 15 days, manganese excess in Jambu (*Spilanthes oleracea*) causes the formation of brown spots, followed by marginal chlorosis, yellowish, necrosis and crinkled leaves (Figure 3). In Beldroega (*Portulaca oleracea*) the symptom of manganese toxicity was only a slight necrosis on the leaves. In the present study, Alfavaca (*Ocimum gratissimum*) was the most sensitive plant to manganese toxicity; it showed necrosis, brown spots and crinkled leaves.

Excessive manganese accumulation in leaves results in a reduction of photosynthetic activities [65]. Such reduction in photosynthesis is expected to lead to a subsequent reduction in growth. And it was found here too, that all three plants exposed to higher concentration of manganese had a reduction of plant growth.

Other symptoms of manganese toxicity, e.g. leaf shedding and callose formation, was not observed in plants studied here.

Figure 3. Symptoms of manganese toxicity in Jam (*Spilanthes oleracea*) leaves: 1 - Health Jam leaves. 2- Crinkled Jam leaves. 3 - Necrosis. 4 - Marginal chlorosis. 5 - Brown spots. 6 – Yellowish leaves. Alf and bel leaves had similar symptoms.

The brown spots represent local accumulations of oxidized Mn (Mn^{4+}) [62]. The inhibition of manganese uptake or retention of manganese in the roots is not a common plant strategy to maintain normal growth and to suppress the expression of toxicity symptoms in spite of high manganese supply [66]. Stress did not change the concentration of water in plant leaves considerably; water content was between 88% in Jam and 91% in Bel.

3.1. Jambu = Jam (*Spilanthes oleracea*)

Common name (s): Jambu (Brazil), Toothache Plant (U.S.), Paracress (U.S.).
Country of origin: Brazil
Plant family: Asteraceae
Used plant part: Leaves

General information: *Spilanthes oleracea* is native to the Amazon area, Brazil [10][67]. Culinary use of *Spilanthes oleracea* is today almost restricted to tropical Brazil, particularly the provinces of Acre, Amazonas and Pará, where the herb is often used in cooking by indigenous peoples, the leaves are used fresh, added as a whole and eaten as an additional source of vitamins (and flavour) [10]. Analytical investigations have shown that it contains a volatile oil, tannins and alkaloid [8].

Medical use: *Spilanthes oleracea* has been recommended in gastro-intestinal disturbances [6] and digestive functions, to overcome nausea and vomiting [70]. It may also be used in inflammations of the mouth and throat [10]. The natives, indigenous, employed it advantageously in gouty, rheumatism, in uric acid gravel and to remove intestinal worms [68].

It may have received the common name of toothache plant because of its use as a local anesthetic for the teeth [71].

No data about *Spilanthes oleracea* on speciation of metal or even metal contents could be found in the literature.

3.2. Beldroega = Bel (*Portulaca oleracea*)

Common name (s): Beldroega (Brazil), Farfena (Central Oman), Golasiman (Philippines), Purslane (U.S.), Pusley (U.S.), Rigla (Egypt), Vertolaga (Peruvian Amazon).

Plant family: Portulacaceae

Country of origin: The orgin of *Portulaca oleracea* is uncertain

Used plant part: Leaves

General information: *Portulaca oleracea* is identified as an excellent source of Omega-3 fatty acids (Omega-3) [72][73], anti-oxidant vitamins and essential amino acids [74]. Omega-3 fatty acids aid the body in the production of compounds that affect blood pressure, clotting, immune system, prevent inflammation, lower cholesterol, prevent certain cancers and control coronary spasms.

The orgin of *Portulaca oleracea* is uncertain. It can be found growing wild and/or cultivated in many areas of the world. *Portulaca oleracea* can be found growing in cold climate areas (e.g. Canada) as well as in warm areas (e.g. the Caribbean).

Medical use: *Portulaca oleracea* has been used to treat gastro-intestinal disturbances [75], inflammation [70], pneumonia, skin ailments, tumor, hemorrhage [9][76], cancer and heart diseases [77].

There are already some papers on studies on *Portulaca oleracea*, some about the

elemental content [78] but most of these papers are about its pharmacological effects [79][80]. No data about speciation of metals could be found in the literature.

3.3. Alfavaca = Alf (*Ocimum gratissimum*)

Common name (s): alfavaca (Brazil), wild basil (U.S.) and menthe gabonaise (France)
Country of origin: The origin of *Ocimum gratissimum* is uncertain, it is widely naturalized.
Plant family: Lamiaceae/Labiatae
Used plant part: Leaves
General information: *Ocimum gratissimum* is widely distributed in tropical and warm temperature regions [81]. The plant is commonly used in folk medicine in the form of an infusion, decoction, oil or syrup. The genus Ocimum contains around 30 species native to the tropics and subtropics of the world, though a more detailed taxonomic assessment is still needed [82].
Medical use: The plant *Ocimum gratissimum* commonly is used in folk medicine to treat different diseases, e.g. bronchitis [83], cough, diarrhea, fever, headache, nausea, pneumonia, inflammation, rheumatism, scabies, gastro-intestinal disturbances [9][10][84][85], tonic expectorants and against spasms [86].

Most papers found in literature were about the *Ocimum gratissimum* oil [10][83][86]. No paper about speciation of any metal could be found.

This study is the first about the elemental speciation in these plants and their tea.

4. Methods, materials and calibrations

In the last decade a universally accepted approach to speciation analysis has been offered by hyphenated techniques (coupled, hybrid) that are undergoing a rapid and continuous development.

There are many sensitive methods to determine the total concentration of trace elements present, but these do not differentiate between free and bound elements. In order to determine the concentration of the respective species, preliminary separation is required [85]. However, there is one problem in general: separation may change speciation.

The techniques available for species-selective analysis in biological materials are [6]:

Separation:

HPLC (High-performance liquid chromatography)

Size-exclusion

Reversed-phase

Ion-exchange

Electrochromatography

Capillary Zone Electrophoresis

Flatbed gel electrophoresis

Isoelectric Focusing

Immunoelectrophoresis

Detection:

AAS (Atomic Absorption Spectrometry)

ICP-AES (Inductively coupled plasma - Atomic Emission Spectrometry)

ICP-MS (Inductively Coupled Plasma Mass Spectrometry)

INAA (Instrumental Neutron Activation Analysis)

UV (Ultraviolet)

Identification:

ESMS (MS) (Electrospray Tandem Mass Spectrometry)

MALDI-TOF-MS (Matrix Assisted Laser Desorption / Ionization - Time-of-Flight - Mass Spectrometry)

FABMS (Fast Atom Bombardment Mass Spectrometry)

NMR (Nuclear Magnetic Resonance)

For this research the below mentioned techniques were used.

4.1. High-Performace Liquid Chromatography (HPLC)

The principal HPLC separation mechanisms used in bioinorganic speciation analysis include size-exclusion, ion-exchange and reversed-phase chromatography. Because of the

complex nature of metal-protein interactions a combination of these separation mechanisms is needed to identify or classify the trace element species correctly.

Size-exclusion chromatography (SEC) [87] is based on the molecular sieve effect and enables species to be separated according to their size and, to a lesser extent, shape (Figure 4). The average time a substance spends in the pores (determined by its size for a given shape) can usually be related directly to its molecular weight.

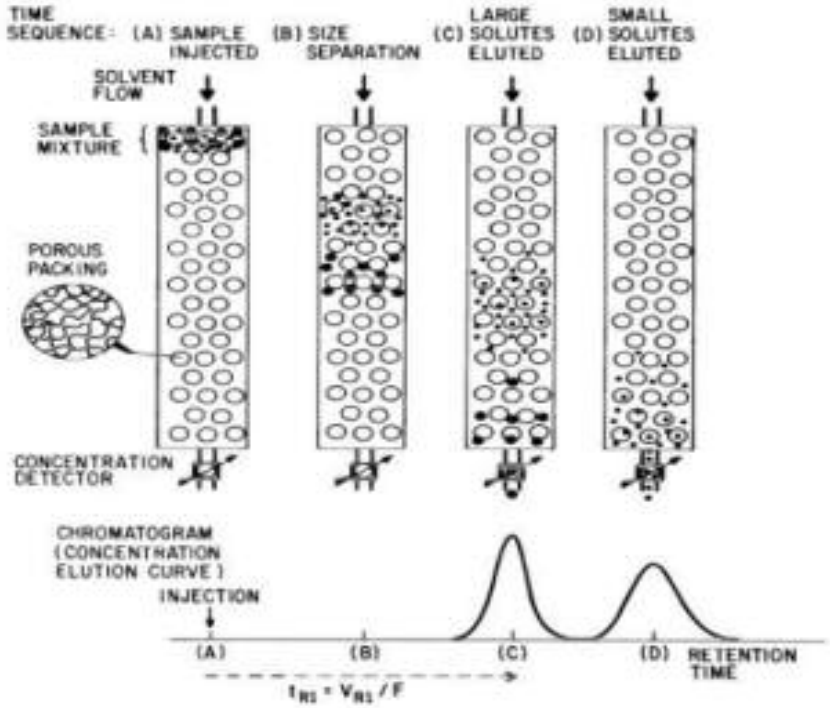


Figure 4. Exclusion chromatography separates molecules on the basis of size [88].

Advantages of size exclusion chromatography are [5][87]: (i) high tolerance to biological matrices; (ii) the possibility of avoiding buffer salts in the mobile phase, and hence the possibility of simplifying the matrix in lyophilized fractions; (iii) the nature of the eluent in the case of gel filtration mostly does not need organic solvents that could denature the analytes. This is of prime interest when working with weak metal-macromolecule complexes, where a slight addition of an organic solvent can change the structure of biomacromolecules and thus the nature of the complex.

The main disadvantage of size-exclusion is its low resolution. It is impossible to resolve two analytes the molecular masses of which differ by not at least 10% [5].

Reversed-phase chromatography (RPC). Analytes in a polar mobile phase, such as water or water-methanol, are chromatographed using a relatively non-polar stationary phase, i.e, porous silica gel surface-modified chemically (covalently) by bonded hydrocarbon chains (C_4-C_{18}). The number of applications for proteins is constantly increasing because of the excellent resolution therefore being able to differentiate proteins, such as metallothioneins varying by one amino acid only [6].

Reversed-phase HPLC seems to be superior to SEC and ion-exchange for the separation of metal-biomolecule complexes because the packing material for RPC is principally free of ligands for metals. Since hydrophobicity of a polypeptide primarily dictates its retention in RP-HPLC, the gradual elution of individual species of a mixture is achieved by decreasing the polarity of the mobile phase by the addition of methanol or acetonitrile [2][87].

The high concentration of organic modifier makes RP-HPLC poorly compatible with ICP-MS. On the other hand, the RP separation conditions are close to ideal for ESI-MS detection, but the use of ion-pairing reagents in the mobile phase dramatically reduces the ESI-MS detection sensitivity [89][90].

High Performance Liquid Chromatography (HPLC) conditions

Size Exclusion Column - SE1: Prontosil 60-10-diol, d_p 10 µm, dim: 300 x 4 mm from Bischoff, mass separation range between 1-100 kDa. The compounds and their molecular weight used for the size exclusion calibration is shown in Table 1, calibration curve is shown in Figure 5. Isocratic separation was performed, flow rate was 0.2 ml/min, 20µl sample were injected, eluent was ammonium acetate buffer, UV-detection was at 240nm.

Compound	Approx. molecular weight (Da)
Albumin, bovine	66.000
Albumin, egg	45.000
Cytocrome C	12.400
Thiourea	76

Table 1. Compounds used for size exclusion calibration of SE1

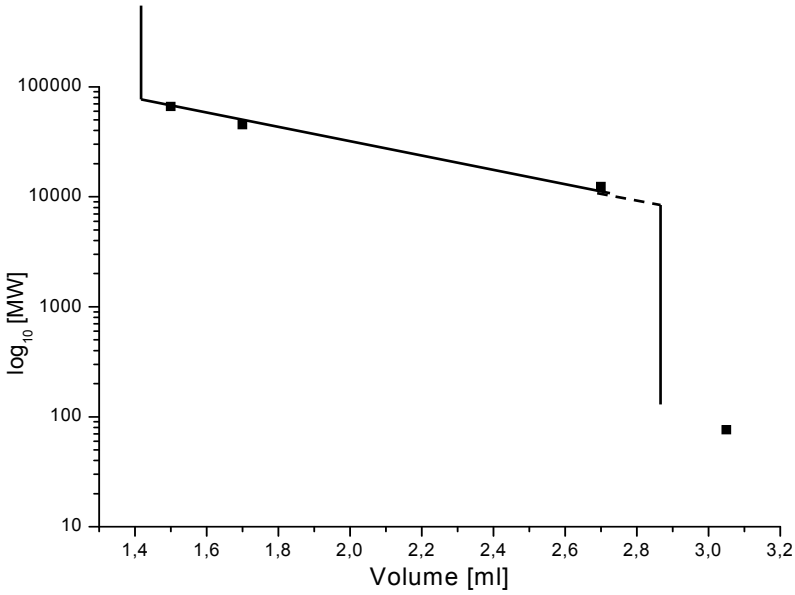


Figure 5. Molecular weight calibration for the size exclusion column SE1. Isocratic separation of the proteins, eluent was ammonium acetate buffer, flow rate was 0.2 ml/min, 20µl sample were injected and UV-detection was at 240nm. The compounds used for size exclusion calibration is shown in Table 1.

Size Exclusion Column - SE2: Polysep-GFC-P 2000, 300 x 7.80mm, from Phenomenex. This column is for lower molecular weight compounds. The column was calibrated using the compounds listed in Table 2 and the calibration curve is shown in Figure 6. The isocratic separation was performed using as eluent water at flow rates of 0.6 ml/min, 20µl sample were injected, and UV-detection was at 215 nm.

Compound	Molecular weight (Da)
Dextran Standard 12000	12.000
Dextran Standard 5000	5.000
Dextran Standard 1000	1.000
Thioureia	76

Table 2. Compounds used for size exclusion calibration of SE2

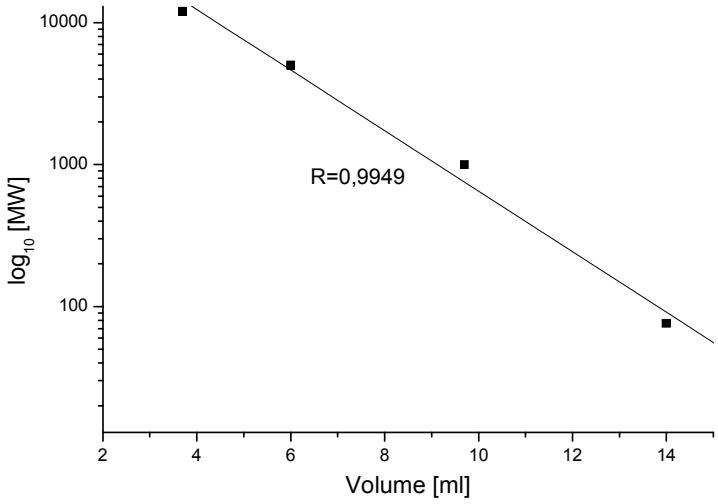


Figure 6. Molecular weight calibration for size exclusion column SE2. Isocratic separation was performed using as eluent water, flow rate was 0.6 ml/min, 20µl sample were injected and UV-detection was at 215 nm. The compounds used for size exclusion calibration are shown in Table 2.

Reversed Phase - RP: Prontosil 120-5-C18-AQ 50µm, 250x4.0 mm from Bischoff. Prontosil C18 AQ is a special reversed phase material for separating a broad spectrum of hydrophilic analytes that show no retention on other reversed phase materials. Strongly polar samples soluble only in water can be separated using Prontosil C18 AQ. The eluent can be water with no added organic solvent.

In this work isocratic separation was performed, the eluent was water (0.1% Trifluoroacetic acid) at flow rate of 0.6 ml/min, 20µl sample were injected. The dead volume (2.9 ml) was measured with water, water/1% acetonitrile, potassium dichromate and sodium nitrate.

4.2. Inductively Coupled Plasma – Mass Spectrometry (ICP-MS)

ICP-MS is one of the most powerful detection techniques for trace elements. The excellent detection limits, typically at the ng kg^{-1} level, make this method very suitable for trace elemental research. The principle of this technique is illustrated in Figure 7. First, the aerosol formed by the introduction system is desolvated in the plasma, the molecules are then atomised and the atoms ionised. This mechanism is not totally understood, but a good model is that ionisation occurs through interactions, namely collisions, between the argon atoms and its electrons, partly free in this plasma, and the analyte [5].

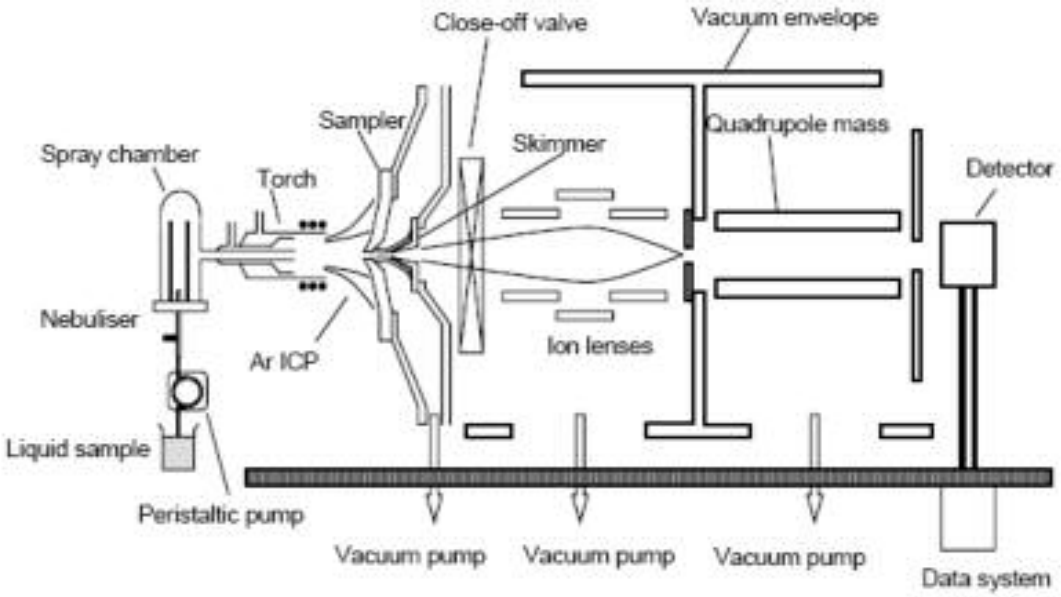


Figure 7. Principal components of a quadrupole ICP-MS. From Chery, 2003 [5], originally from Turner, 1998 [91].

As for all mass spectrometers, the crucial point is the interface between the atmospheric pressure and the low pressure, lower than 1 mPa, which is necessary to ensure a collision-free transmission of the ions, in other words a long enough free path. This interface consists of two coaxial cones with a tiny opening at their vertex, with their axis in the direction of the plasma plume. Only a part of the gas coming out of the plasma is thus allowed to go further, gas consisting of ions, neutrals and electrons. At each step of the ion transmission a vacuum pump is installed.

The pump has to pump away all neutral molecules. After those two cones, when the sampling of the plasma gas is achieved, the ionised atoms are focused by an electrostatic lens, which in the case of negative mode also deflects anions and electrons. Only cations are thus further transported to the filtering device of the instrument, a joined magnetic and electric sector field or a quadrupole. The dynamic electromagnetic field that is present in the quadrupole has the capacity to bring at the exit of the device only ions with a specific mass-to-charge ratio; other ratios are simply destabilised and expulsed from a stable trajectory. The selected ions then leave the quadrupole to migrate towards the detector, in most cases an electron multiplier [5].

One of the great advantages to ICP-MS [6][87][89][92] is its extremely low detection limit for a wide variety of elements. However, this technique suffers from certain

disadvantages [92][93]. First, spectral interference, i.e. the formation of ions with the same nominal mass as the analyte, can cover and hinder the detection of the latter. Furthermore, the matrix, in which an element is present, plays an important role in, among other things, the position of the plasma plume, the ionisation equilibrium and the charge distribution, so that a calibration with the same matrix is preferable. Some biologically important elements, such as sulphur and phosphorus, have high ionization energies and are not as efficiently ionized in the ICP as metals. Moreover, they suffer from a number of polyatomic interferences, and thus they could not be determined by ICP MS in biological matrices. The problem of the poor ionization efficiency also concerns halogens, especially fluorine, which is of interest in drug metabolism studies. Also, some vital transition elements, such as iron or vanadium, are interfered by polyatomic ions. ICP-MS instruments are not stable over a long period of time, which makes mandatory the use of an internal reference or of periodical external standardisation.

Instrumental ICP-MS conditions

A double-focusing sector-field ICP-MS ELEMENT 2 (E2) and VG Elemental PlasmaQuad 3 (PQ3) were used for trace element determination in plant samples under the conditions:

→ ICP-MS ELEMENT 2, nebulizer type: cross-flow, spray chamber: Scott type, double pass, RF power: 1350 W, cooling gas flow rate: 16 L min^{-1}, auxiliary gas flow rate: 1.0 L min^{-1}, nebulizer gas flow rate: 0.93 L min^{-1}, solution uptake rate: 20 µL min^{-1}, mass resolution: medium (4,000 - chromatography online) and low (300 - concentration determination), focus lens potential: 860 V, points per isotope peak: 20, integration time per point: 0.01s.

→ ICP-MS VG Elemental PlasmaQuad 3, nebulizer type: cross-flow, spray chamber: Impact Bead, RF power: 1350 W, cooling gas flow rate: 14 L min^{-1}, auxiliary gas flow rate: 0.85 L min^{-1}, nebulizer gas flow rate: 0.85 L min^{-1}, solution uptake rate: 800-900 µL min^{-1}, mass resolution: 300, focus lens potential: 27 V, points per isotope peak: 1 (peak hopping), integration time per point: 0.1s.

The ICP torch was shielded with a grounded platinum electrode (GuardElectrode™, Finnigan MAT). A low-flow microconcentric PFA (perfluoroalkoxy) nebulizer was used for solution introduction into the ICP-MS. Single element and multielement standard stock solutions for the calibration procedures were obtained from Merck. The solutions were diluted

with deionized Milli-Q water and acidified with subboiled nitric acids of supragrade purity. A cross-flow nebulizer with a Scott double-pass quartz spray chamber cooled to 3°C was applied for sample introduction in to ICP-MS. Mass resolutions of 300 (low resolution) and 4,000 (medium resolution) were applied for the determination of trace elements; the criteria for choosing the appropriate mass resolution for an isotope were possible isobaric interferences and the analyte concentration in the samples.

4.3. Electrospray Ionization -Mass Spectrometry (ESI-MS)

ESI is a method of generating highly charged droplets from which ions are ejected by an ion evaporation process. An electric field is generated at the tip of a sprayer by applying a high voltage, with a close proximity of a counter electrode. Ions of one polarity are preferentially drawn into the drops by the electric field as they are separated from the bulk liquid. The sample solution is passed through an electrically charged needle and the liquid takes the shape of a Taylor cone as it comes under the influence of the flow and the electrostatic field (the force on the ions drags the liquid along while surface tension tries to pull it into a sphere). There is rapid evaporation of the droplet and the capillary into which the droplets fly is heated to aid solvent evaporation. In the case of the IonSpray interface, the sample is dispersed by a nebulizing gas (Figure 8). Negative ions can also be selected [94]. This technique is typically performed either in the infusion mode or in combination with HPLC or capillary electrophoresis [94].

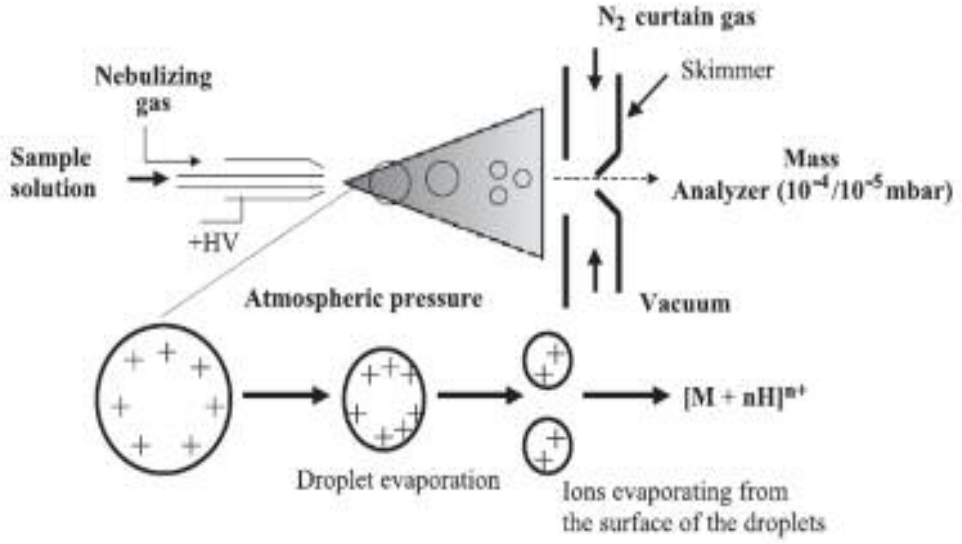


Figure 8. Major components of the ESI source positive mode [94].

ESI is a soft ionisation technique. It is one of the most effective interfaces for liquid chromatography (LC/MS) and capillary zone electrophoresis (CZE/MS). This technique is highly specific and relatively sensitive. ESI-MS has increasingly been applied to the speciation analysis of low-molecular-mass organometallic compounds in recent years. Low-molecular-mass organometallic compounds and redox species are of particular importance in environmental analysis (e.g. organotin and organomercury compounds), and in food and nutrition-related studies (e.g. organoselenium and organoarsenic compounds).

As the analytes very often are polar or ionic compounds, the advantage of coupling liquid-phase separation and ESI-MS detection becomes evident. In contrast to ICP-MS, ESI-MS is able to positively identify unknown or to confirm the presence of known species based on the molecular mass spectra even when authentic reference compounds are not available. Due to the softness of electrospray ionisation and the comparatively low information content of ESI mass spectra obtained at low fragmentor voltage, this challenging task is mostly achieved by MS *n* experiments (performed with either double or triple quadrupole or ion trap instruments) [87][89][95].

A serious inconvenience with the use of electrospray for probing metal-ligand interactions and the determination of metal-ligand stoichiometry is its poor tolerance to nonvolatile salt buffers (e.g. clorines added to buffers reduce the lifetime of the chanel electron multiplier) and other solubilizing agents (e.g. detergents), which are often necessary to maintain the analyte's stability and integrity. An electrospray compatible solvent system containing a volatile salt such as ammonium acetate, ammonium formiate or ammonium carbonate is required [6][87][95].

When a mass spectrometer is used in combination with chromatography a smaller column diameter will favor the detection by electrospray MS, which is concentration sensitive, in comparison with ICP-MS, which is a mass sensitive detector.

Instrumental ESI-MS conditions

The equipment used in this study was an API 150EX (ESI-Q-MS).

The operational parameter values for positive mode: ion spray potential was 5000 V, declustering potential: 35 V, focusing potential: 200 V, entrance potential: 10 V, nebulizer gas: 8 $L \cdot min^{-1}$, temperature: 240 °C.

And for negative mode: ion spray potential was -5000 V, declustering potential: -35 V, focusing potential: -200 V, entrance potential: -10 V, nebulizer gas: 8 $L \cdot min^{-1}$, temperature: 240 °C.

4.4. Matrix assisted laser desorption/ionisation - time of flight - mass spectrometry

MALDI (Matrix Assisted Laser Desorption/Ionisation) is a soft ionisation technique that converts samples from the solid form into the ionized form in gas phase for MS analysis.

In this technique (Figure 9), samples are co-crystallized with a matrix, usually an aromatic organic acid, which absorbs energy from laser pulses and allows a soft desorption ionization of the sample. The sample ions are then analyzed by a time-of-flight mass analyzer. Samples that are analyzed by MALDI are first mixed with a crystalline matrix and spotted on a stainless steel target. Upon drying, it is inserted into the ion source of the mass spectrometer which is under high vaccum. A laser usually (a pulsed nitrogen laser at 337 nm) is fired onto the sample, resulting in a desorption event. The ions are repelled from the target surface and drift through the flight tube and their arrival at the detector is carefully timed, smaller ions fly faster than larger ions. Thus ions are separated according to their mass-depending velocities and therefore time-of-flight.

Increased mass resolution is obtained by bouncing the ions off an electronic mirror (a reflectron) [94]. This increases the focus of the ions. The origin of ions in the MALDI process is still under discussion [96].

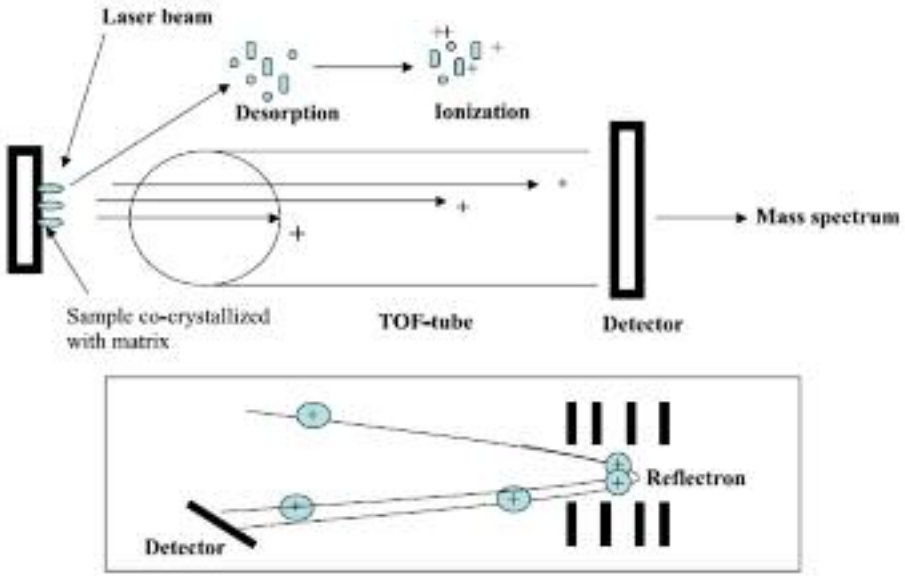


Figure 9. Schematic of MALDI-TOF-MS analysis [94].

MALDI-TOF-MS has advantages over other methods, including high speed of analysis, good sensitivity, and good tolerance towards contaminants [97]. Additionally, it produces mainly molecular ions without fragmentation. These attributes allow for the simultaneous determination of masses in complex samples of low- and high-molecular-weight compounds. MALDI-TOF-MS is well known as a powerful tool for analysis of a wide range of biomolecules, such as peptides and proteins.

Instrumental MALDI-TOF-MS conditions

Three experiments with MALDI-TOF-MS were carried out.

First experiment: Jam, Bel and Alf samples were measured with 3 different matrices, dihydroxybenzoic acid (DHB), α-cyano-4-hydroxy-cinnamic acid (CCA) and sinapinic acid (SA). About 10 mg of the matrices were dissolved in a mixture of pure water and acetonitrile (DHB and CCA, ratio1:1) and pure water with 0.1 % trifluoric acetic acid and acetonitrile in the case of sinapinic acid (ratio 60:40). 20 µl of matrix solution and 20 µl of every sample solution were mixed and 2 µl of the mixture were dropped on the target. After drying, the samples were measured by MALDI-TOF-MS (Micromass TOFSPEC E with pulsed N-Laser (4 ns)) with the measuring modes: linear (limit 0-250000 Dalton) and reflectron mode (0-12000 Dalton), software: Micromass MassLynx V2.3, mode: linear mode, positive polarity, source voltage: 20000 V, extraction voltage: 19950 V, focus voltage: 14500 V, mass range: 0-5000 (stimulate with nitrogen laser, laser energy is depending on the sample, 100 shots per spectrum).

Second experiment: like experiment 1, two matrices (DHB and CCA) were measured with and without samples. Preparation was as the first experiment. Then the mass spectra were compared.

Third experiment: samples were measured without any matrix.

4.5. Instrumental Neutron Activation Analysis (INAA)

Instrumental Neutron Activation Analysis is a sensitive analytical technique useful for performing both qualitative and quantitative multi-elemental analysis of elements in samples from almost every conceivable field of scientific or technical interest. In this process, stable isotopes are converted into radioactive isotopes, which emit electromagnetic radiation at

different and specific energies [98]. When a neutron interacts with the target nucleus, via a non-elastic collision, a compound nucleus forms an excited state. The excitation energy of the compound nucleus is due to the binding energy of the neutron with the nucleus. The compound nucleus will almost instantaneously de-excite into a more stable configuration through emission of one or more characteristic prompt gamma rays. In many cases, this new configuration yields a radioactive nucleus which also de-excites (or decays) by emission of one or more characteristic delayed gamma rays, but at a much slower rate according to the unique half-life of the radioactive nucleus. Depending upon the particular radioactive species, half-lives can range from fractions of a second to several years [98].

The basic essentials required to carry out an analysis of samples by INAA are a source of neutrons, instrumentation suitable for detecting gamma ray spectra, and a detailed knowledge of the reactions that occur when neutrons interact with target nuclei [98].

For many elements and applications, INAA offers sensitivities that are superior to those obtainable by other methods, on the order of parts per billion or better. In addition, because of its accuracy and reliability due to being almost free from matrix interference, INAA is generally recognized as the "reference method" of choice when new procedures are being developed or when other methods yield results that do not agree. Worldwide application of INAA is widespread; it is estimated that approximately 100,000 samples undergo INAA each year. About 70% of the elements have properties suitable for measurement by INAA [98].

Instrumental Neutron Activation Analysis conditions

The TRIGA Mark II research reactor of the University of Mainz was used to irradiate the samples under investigation. Two different measurements were done to account for the medium and short half life elements, as shown in Table 3.

	Irradiation time	Decay time	Detection time
Short	1 hour	15 min	15 min
		1 hour	30 min
Medium	6 hours	1 day	1 hour
		15 days	8 hours

Table 3. Irradiation parameters for the Instrumental Neutron Activation Analysis of medicinal plants at the Triga Mark II Reactor.

Standards of manganese, chromium, zinc, cobalt and iron of appropriate concentrations were used, i.e. according to the level of respective elements in the samples. Samples, each weighing about 150 mg, were taken in pre-cleaned polyethylene vials for short and medium irradiation. The samples along with the standards were irradiated at a thermal neutron flux of $0.7X10^{9}$ $n.cm^{2}.s^{-1}$ for 1 and 6 hours. After appropriate decay, the irradiated samples and standards were transferred to pre-weighed polyethylene vials and re-weighted to determine the exact sample weight. Each element was identified with one or two characteristic gamma emission lines and self absorption and interferences were corrected, when necessary.

4.6. Ultrafiltration

Ultrafiltration is a separation process for different molecular weight compounds. The primary basis for separation is molecular size, although in all filtration applications, the permeability of a filter medium can be affected by the chemical and molecular properties of the sample. Ultrafiltration can only separate reliably molecules which differ by at least an order of magnitude in size. Molecules of similar size cannot be separated by ultrafiltration.

The ultimate aim of ultrafiltration is to maximize recovery of solutes of interest, but there are many membrane characteristics that affect that goal.

Ultrafiltration membranes are rated according to the molecular weight cut off (MWCO). The MWCO indicates that most dissolved macromolecules with molecular weights higher than the MWCO will be retained. An ultrafiltration membrane with a stated MWCO should retain (reject) at least 90% of a globular solute of that molecular weight in daltons. However, for a wider safety margin, the selected cut-off should be well below the molecular weight of the solute to be retained. When solutes are to be exchanged, the cut-off should be substantially above that of the passing solute. A lower MWCO increases rejection but decreases the filtration rate for the same membrane material. Retention and product recovery are a function of a variety of other factors, including the molecular shape and size of the molecule (e.g. linear molecules (e.g., nucleic acids) may find their way through pores that will retain a globular species of the same weight), sample concentration and composition [99].

Another factor affecting the retention characteristics is the potential for membrane fouling, or concentration polarization. This occurs when there is an accumulation of the retained solute on the surface of the membrane. At high concentrations, a gel layer may form that can act as a secondary membrane (Figure 10).

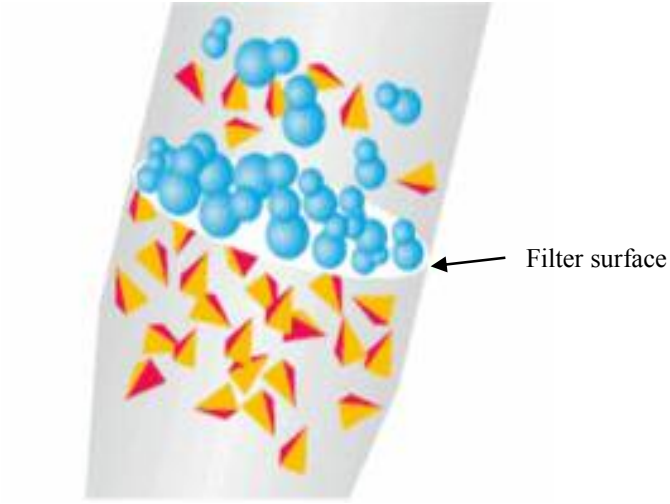


Figure 10. Ultrafiltration separates proteins from soluble salts. “Concentration polarization” slows down filtration. The proteins form a gel layer on the membrane surface [99].

Ultrafiltration with different molecular weight cut off

Filters with regenerated cellulose membrane – ultracel series with different molecular weight cut off values (5, 10 and 30 kDa) from Millipore were centrifuged by Eppendorf Centrifuge 5417R for 40 min, *g*-force was 1800g and temperature of 4ºC. To avoid degeneration, the extracts were kept at a temperature of minus 76°C until further analysis.

The experiment consists in three steps. First, the crude plant extracts have been ultrafiltrated with 30kDa cut-off filters. Fractions that resulted from the first step were then ultrafiltrated with 10 kDa filters, and then with 5kDa, as shown in Figure 11:

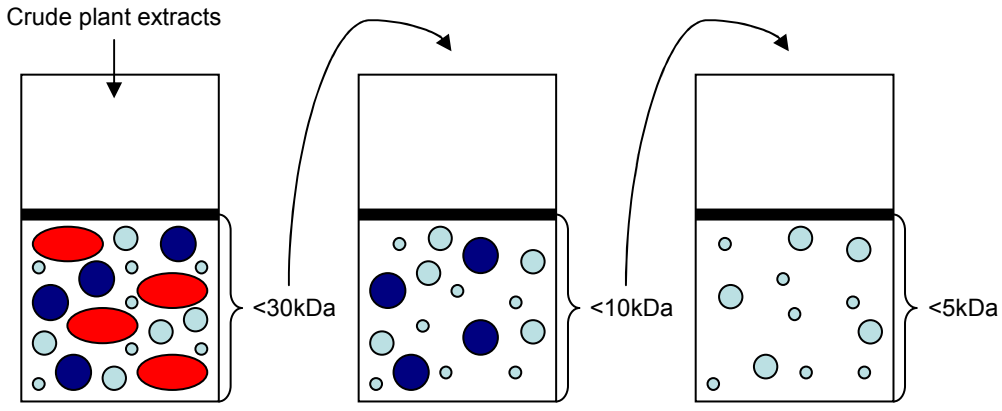


Figure 11. Ultrafiltration scheme: first the crude plant extracts are filtered with filters of a cut-off value of <30kDa, the resulting ultrafiltrate is filtered with filters with a cut-off value of <10kDa, and finally with filters of a cut-off value of <5kDa.

4.7. Determination of the total protein concentration by the Bradford test

Use of Coomassie G-250 Dye in a colorimetric reagent for detection and quantitation of the total protein content was first described by Bradford [100] in 1976. Proteins bind to the Coomassie dye (Figure 12). This results in a spectral shift from the reddish/brown form of the dye to the blue form of the dye. The difference between the absorption of two forms of the dye is greatest at 595nm, so that this is the optimum wavelength to quantify the Coomassie dye protein complex.

The development of color in Coomassie dye based protein assays has been associated with the presence of certain basic amino acids (primarily arginine, lysine and histidine) in a protein. The number of Coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein. Free amino acids, peptides and low molecular weight proteins do not produce a color with Coomassie dye reagents. In general, the mass of a peptide or protein must be at least 3 kDa to be assayed with the reagent [101].

The quantification of unknown proteins in solutions is problematic since calibration then is impossible.

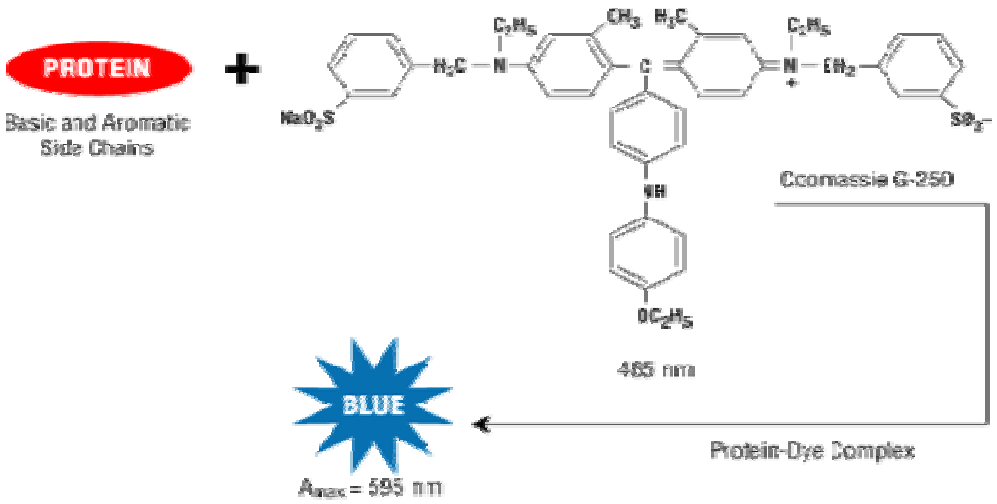


Figure 12. Reactions chematic for the Coomassie dye based protein assay [101].

Bradford Test procedure

To determine the concentration of proteins in solution in this work the Bradford test was applied. Bovine Serum Albumin (BSA) Standards were used for the calibration curve in the linear range of 0.25 and 1.4 mg/ml (Figure 13). Standards and plant extracts with Bradford Reagent have been incubated for 15 min at room temperature. The absorbance was measured at 595 nm by a UV/VIS spectrometer Lambda 2. The protein concentration was determined by comparision of the plant extracts to a standard curve prepared using the BSA standards. Each sample and standard was measured three times.

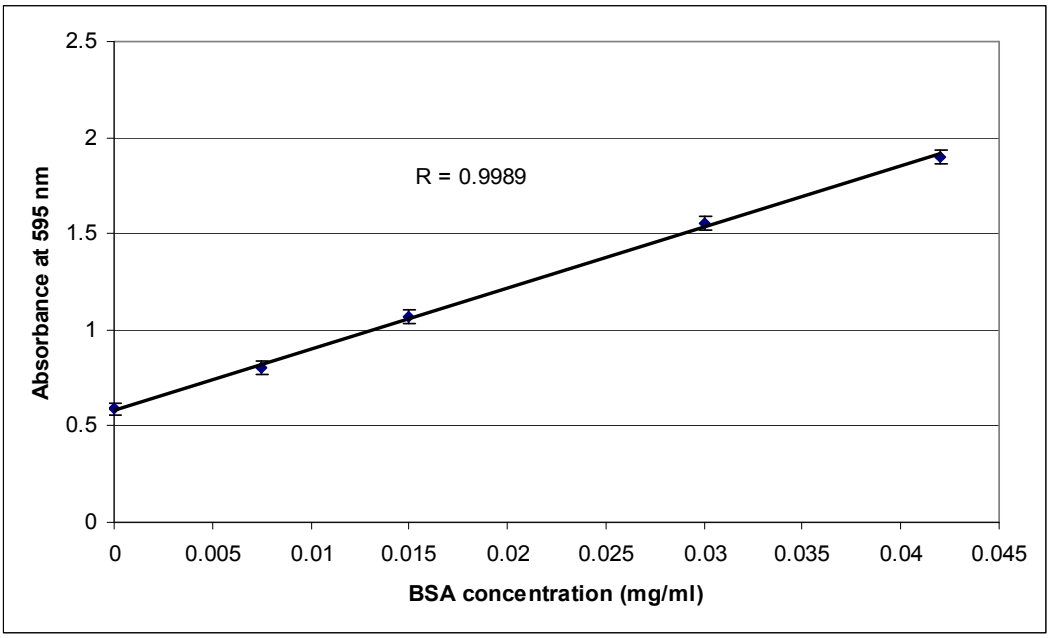


Figure 13. BSA standard curve for the Bradford test. The absorbance was measured at 595 nm.

4.8. Determination of the sulfhydryl group concentration by the Ellman test

In 1959 Ellman introduced 5, 5-dithio-bis-(2-nitrobenzoic acid), also known as DTNB, as a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryl groups. Consequently, Ellman´s Reagent is very useful as a sulfhydryl assay reagent because of its specificity for -SH groups at neutral pH and within short time [102].

Ellman´s test procedure

Sulfhydryl groups were determinated using Ellman Reagent (5, 5-Dithiobis-2-nitrobenzoic acid). The buffer was made with 0.1 M Sodium phosphate, pH 8.0, containing 1mM EDTA. L (+) cysteine was used as standard in a concentration range of 0.25-1.5 mM (Figure 14). A mix of the buffer with standards and samples was incubated for 15 min. The absorbance was measured at 412 nm by a UV/VIS spectrometer. Sulfhydryl groups were estimated in plant extracts by comparison to a standard curve.

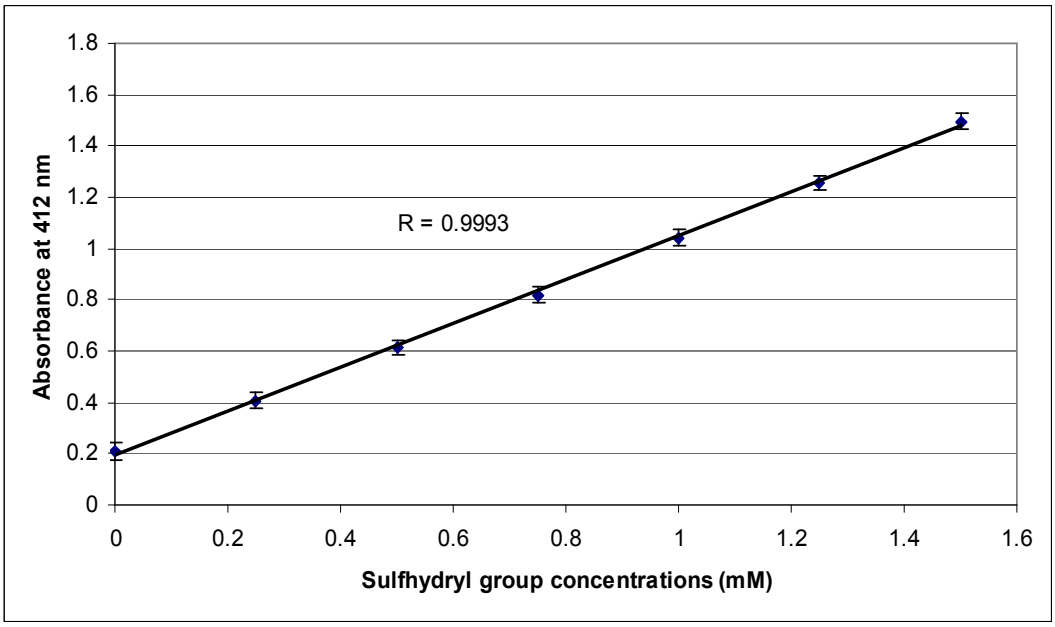


Figure 14. Cysteine standard curve of the Ellman´s test. The absorbance was measured at 412 nm.

4.9. Materials

All the labware (glass, bottles, ceramics, etc.) were soaked in 1 M nitric acid for 2 days to extract residual metals and then rinsed thoroughly with Milli-Q water. All the solutions were prepared with MilliQ purified water from Milli-Q® Ultrapure Water Purification Systems, with conductivity under 0.05 µS. All the reagents and solvents used were of analytical grade or better, in order to avoid contamination.

4.10. Botanical material and extraction method (Tea)

Leaves of *Spilanthes oleracea, Portulaca oleracea* and *Ocimum gratissimum* were collected at EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria), a research institute

in Manaus, in the state of Amazonas, Brazil. Leaves were washed with deionized water, shaked and dried in the shade for 7 days at room temperature, the same procedure used by ill people in the Amazone area to prepare medicinal tea. The dried leaves were brought to Mainz for further analysis.

To 0.500 g of dry plant material 2.5 mL of hot (80 °C) MilliQ purified water was added. The extract was ultrafiltered using filters with different cut off values (see 4.6) and centrifuged for 40 min, *g*-force was 1800g, temperature 4ºC.

4.11. Comparison of methods to grow from seeds

The seeds brought from Brazil were very small, and two methods of growing from seeds have been tested. First: seeds were germinated on wet filter paper (water from the tap) in Petri dishes at room temperature. Second: seeds were put on Lecaton, watering (tap water) 3 times for a week (Figure 15).

The main problem of the first method is that the root "stick" on the filter paper and it is almost impossible to transfer them to the hydroponic system. Also many seeds were not germinating. Lecaton are like "small stones". Although it was not expected that very small seeds could grow on it, because the "stones" are at least 500 times bigger than the seeds, the second method gave the better results.

Seeds of four different plant species have been brought from Brazil but just three germinated with this method.

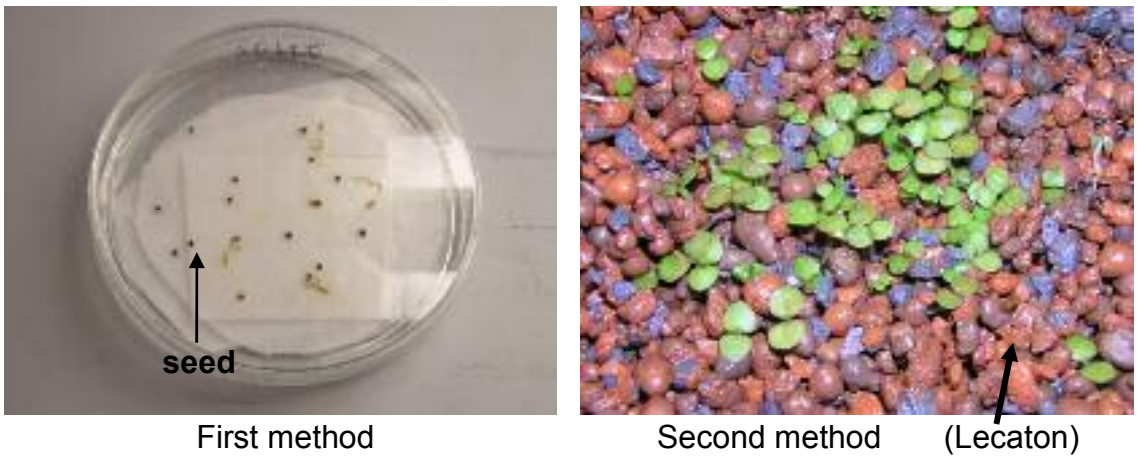


Figure 15. Seeds of same age growing in different methods. First method: seeds were germinated on wet filter paper (water from the tap) in Petri dishes at room temperature. Second method: seeds were put on Lecaton, watering 3 times for a week.

4.12. The hydroponic system

Seeds of the plants studied in this research were brought from Manaus-Amazonas-Brazil. Seeds were germinated and grown for 21 days in 250 ml plastic pots filled with expanded clay (Lecaton, 2 - 5 mm particle size), watering (tap water) three times per week.

Twenty-one days old seedlings of the plants in this study were transplanted into a closed hydroponic system (Figure 16). Plant seedlings were placed in 1L each container with: (i) the basal nutrient solution adapted from the Hoagland solution and (ii) nutrient solution with different manganese concentrations. For the Mn stress, Mn supplied as $MnCl_2\ 4H_2O$ was added to the basal nutrient solution. Plants were exposed to different manganese concentration: 50, 100, 200, 500, 800, 1000 µM.

Basal nutrient solution adapted from the Hoagland solution was 1 mM of $Ca(NO_3)_2\ 4H_2O$, 250 µM of $(NH_4)H_2PO_4$, 1.5 mM of KNO_3, 500 µM of $MgSO_4$, 12.5 µM of H_3BO_3, 0.25 µM of $ZnSO_4\ 7H_2O$, 0.125 µM of $CuSO_4\ 5H_2O$, 0.025 µM of H_2MoO_4, 0.025 µM of $Co(NO_3)_2\ 6H_2O$, 0.025 µM of NaOH, 22.5 µM of $FeSO_4\ 7H_2O$, **2.5 µM of $MnCl_2\ 4H_2O$**, 22.5 µM of EDTA-di-Na-Salt (Titriplex).

The nutrient solutions in the hydroponic systems were aerated continuously and changed once per week for new similar ones.

The nutrient solution had pH = 6.5. The best pH to uptake of manganese is between 5.8 - 6.8 [103].

Plants were grown in a climate-controlled growth chamber in the Institute of General Botany Mainz with the temperature maintained 25°C during a 12 hours light period and 20°C during darkness.

Optimization of the nutrition solution

Solution culture techniques are commonly employed when it is necessary to exert more control over the root environment than is possible with soil-grown plants. The major advantages of hydroponic systems are the good reproducibility of the supply of nutrients and potentially toxic metals, the accessibility of the roots for observation during the experiment, and the ease of harvesting the complete plant.

Hydroponics is the practice of growing plants in either a bath or flow of highly oxygenated, nutrient enriched water. All plants need 16 elements to grow (C, H, O, N, P, K,

Ca, Mg, S, Zn, Cu, Fe, Mn, B, Mo and Cl). However, the most difficult part of the hydroponic culture for three different plant species is the establishment of the basal nutrition solution for all three plants.

There are numerous formulations of nutrient solutions. Hoagland solution formula was choosen as basal nutrient solution. However, just five days after the young plants (21 days grown in lecaton) had been transportated to the hydroponic system, all plants had died.

New experiments adapting the Hoagland solution formula to the plants were made. These experiments were based on a dilution series of the original Hoagland solution: 80, 50, 25 and 10% of the elemental concentration of the original solution. The young plants then were transported to the hydroponic system runing under this lower concentration solution.

The results show that the 25% level of concentration of element of the original Hoagland solution formula was ideal for use as basal nutrition solution for the plants studied here. All plants could survive in this basal nutrient solution without any toxic symptoms for long time.

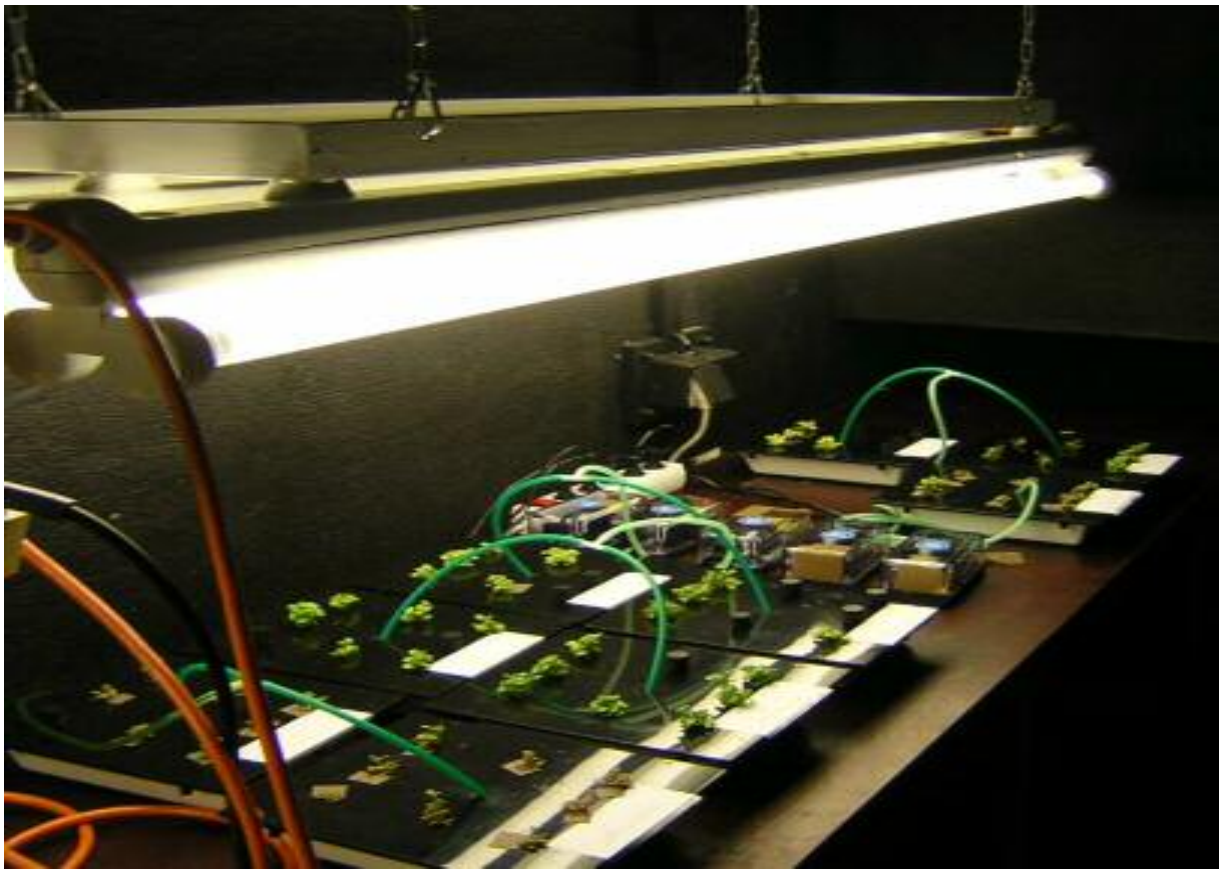


Figure 16. The hydroponic system.

4.13. Methods to break down cells in plants

Three cell break down methods have been compared: Ultra-Turrax, detergent (CelLytic P) and lysozyme.

The diagram of the methods is shown in Figure 17.

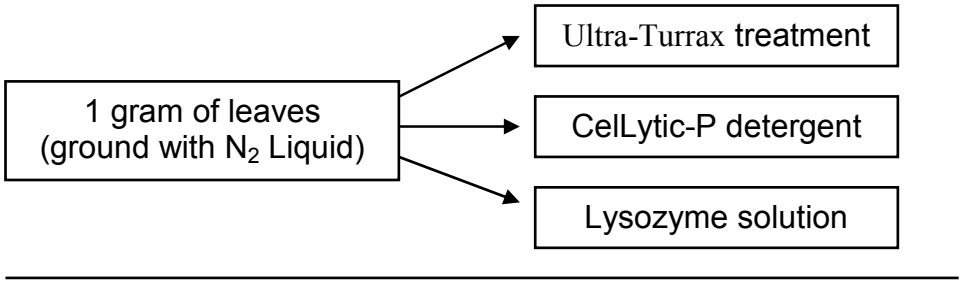


Figure 17. Diagram of comparison of cell break down methods

As a first step: 1 gram of leaves was frozen with liquid nitrogen and grounded using mortal and pestle.

Ultra-Turrax: After the first step, 2 ml of MilliQ purified water was added. The extracts were subjected to Ultra-Turrax treatment for 4 minutes with speed of 6,000 rpm, in an ice bath.

Lysozyme: After the first step, 2 ml of lysozyme solution (200µg/ml) was added.

CelLytic-P (Detergent, Sigma): After the first step, 2 ml of CelLytic P reagent from Sigma was added and ground thoroughly with the pestle. Protease Inhibitor cocktail diluted 1:100 was added to the CelLytic P reagent, as recommended from the manufacturer.

4.14. Digestion of plant samples

To 0.1g of powdered plant samples, 8 ml freshly prepared *aqua regia* was added. After 1 hour, the samples were heated for 10 min in Microwave following the EPA 3051 method, then cooled down to room temperature and diluted up to 25 ml with MilliQ purified water. Blanks were analogously prepared. In order to check the reliability of the analytical methods used here for trace metal analysis, *Olea europaea* (Reference material Nr. 62) and *Platihypnidium riparioides* (Reference material Nr. 61) both from the Community Bureau of Reference (B.C.R.) were also digested and then analysed following the same procedure.

The results obtained on the certified reference materials to validate the digestion results show good agreement for many elements, for most of the elements it was within ±10% of the reported values, as shown in Figure 18 for Mn and Zn.

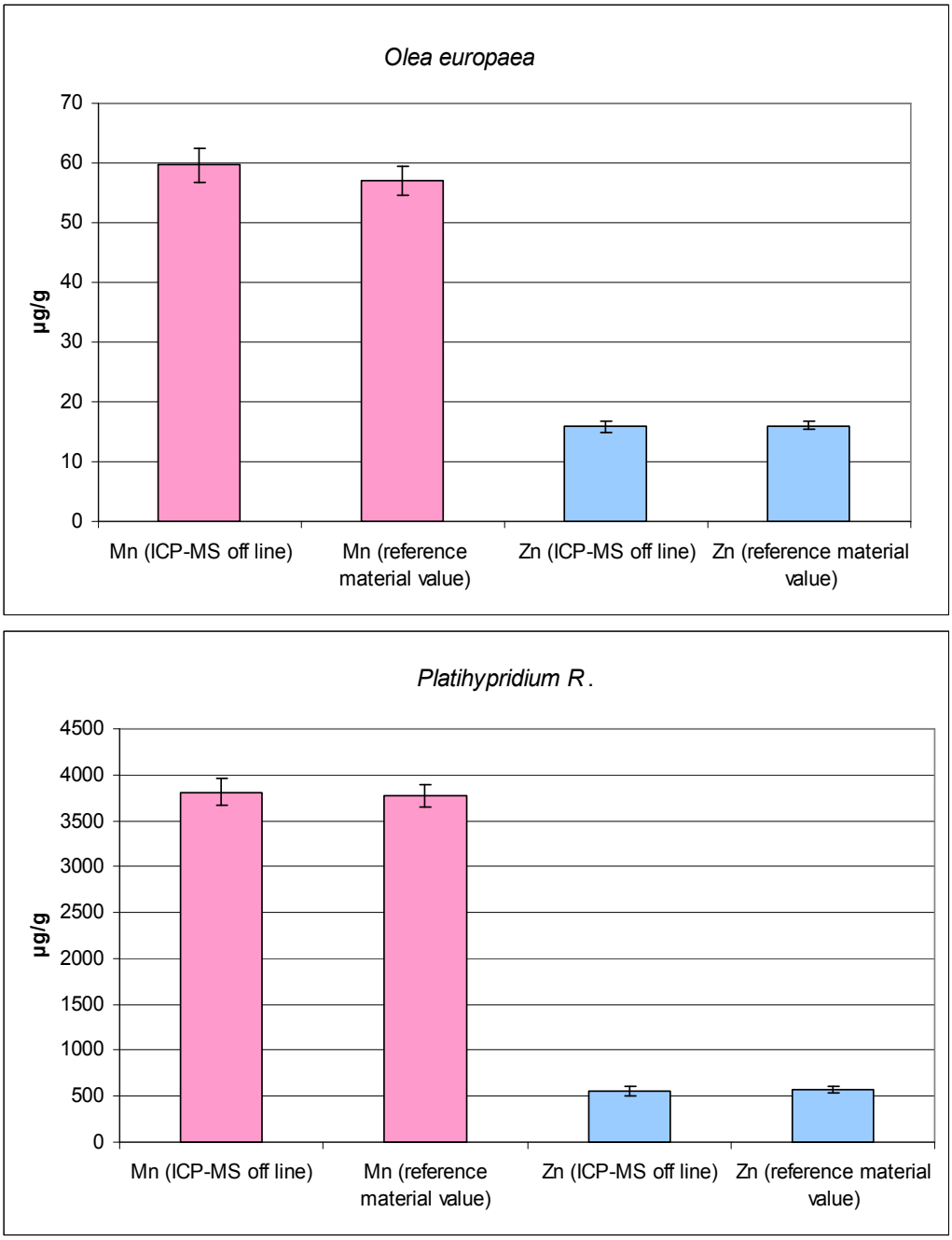
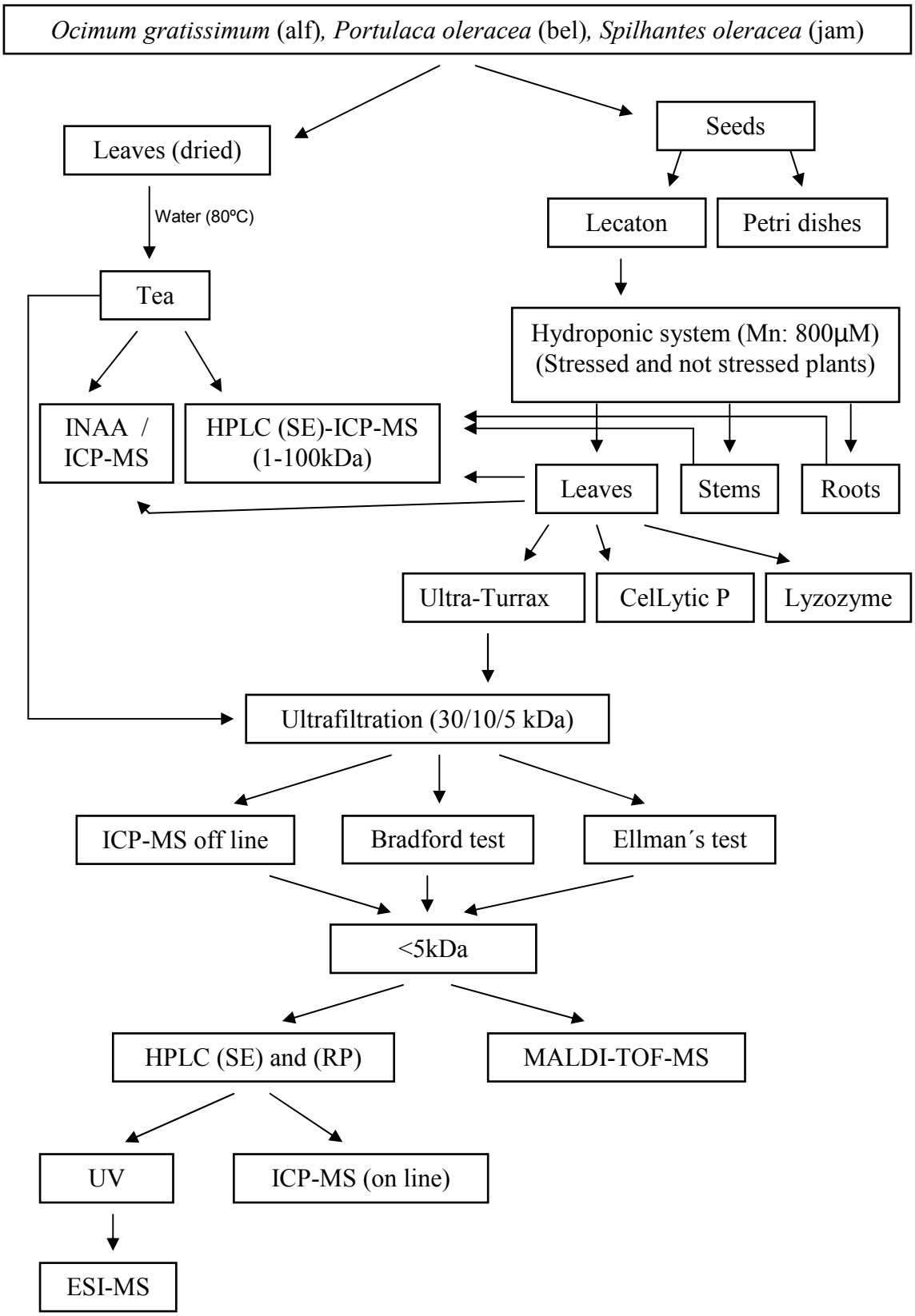


Figure 18. Concentration of Mn and Zn determined by ICP-MS off line, after the digestion, compared to the certified value.

4.15. Working scheme



5. Detailed results

5.1. Medicinal plant teas

This chapter shows the results of some essential elements and their species in medicinal plant teas from *Spilanthes oleracea* - Jambu (Jam), *Portulaca oleracea* - Beldroega (Bel) and *Ocimum gratissimum* - Alfavaca (Alf). The leaves of these plants were extracted in the same way as recommended for medicinal use (hot water extraction). Because the sample preparation was exactly the same for all samples, the results should reflect the chemical differences of the respective plant extracts.

5.1.1. Water content in plant leaves

Before the plant leaves were brought to Mainz, three groups of fresh leaves of approximately three grams were dried until constant weight. The results showed that 85-91 % of the weighted fresh leaves were water as is shown in the Table 4.

Tea	Initial weight/g	Final weight/g	(%) water in fresh leaves
Alf	3.2276	0.4975	85
Bel	3.2589	0.3059	91
Jam	3.2486	0.3802	88

Table 4. Water content in plant leaves. The errors were estimated 5%.

5.1.2. Medicinal teas studied by HPLC (SE)-ICP-MS

Medicinal plant teas contain a large number of compounds of different molecular weights. Many of these compounds can be bioactive and may bind to trace elements, e.g. manganese and zinc. In order to know the molecular size of the compound which bind to

some essential elements, the size exclusion Prontosil-diol column - SE1, with a range of 1 - 100 kDa (according to the manufacturer) was used.

Based on the calibration, the compounds between 1.5 – 3.0 ml elution volume (V) are between 66000 - 76 Da. Those elements with dead volume (V_0) > 3 ml are inorganic species, or bound to low molecular weight, or are retained by interaction with the column particles (not very probable).

This work is focused on manganese speciation in teas; however, the therapeutic effect of medicinal plants for the treatment of various diseases is based on a multitude of essential trace elements, and because of this, other essential elements will be analysed in addition.

The chromatograms of Alf and Jam crude tea (Figure 19) show one intensive manganese peak for each tea. These manganese peaks eluted after the dead volume. This means that not only size exclusion effects are present, but also some affinity retention. At very low intensity two low intensity peaks have also been detected.

In Bel crude tea chromatogram (Figure 19) two intensive manganese peaks could be detected, one around 1.6 ml and the other around 5 ml (later than V_0).

Alf and Jam teas in Figure 19 show similar manganese chromatograms with one intensive peak while the Bel tea chromatogram shows two intensive peaks.

For Sr, all tea chromatogram were similar. For Zn and Fe, Jam and Alf chromatograms were similar.

Chromium and molybdenum chromatograms are shown in Figure 19 for Alf, Bel and Jam. These chromatograms show two peaks for chromium and one peak for molybdenium of each tea, all peaks eluted before the dead volume, and they probably bind to high molecular weight compound (s).

As a conclusion the two different plant species (Alf and Jam) show similar SE chromatogram for Mn, Fe, Zn, Sr, Cr and Mo, however, no precise information about the molecular weight of the compounds to which the elements were bound could be achieved.

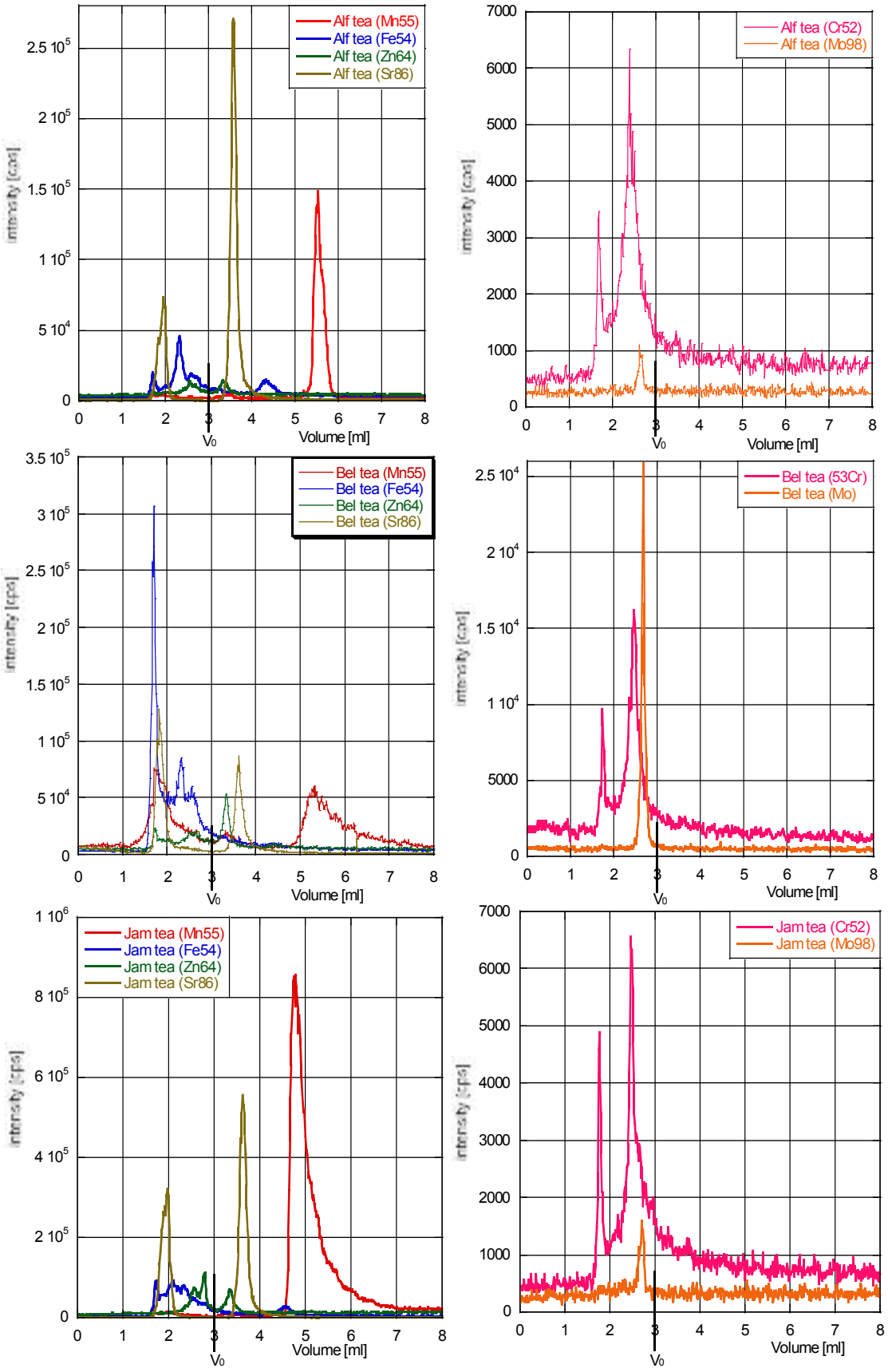


Figure 19. Alf, Bel and Jam crude tea chromatograms determined by HPLC (SE1)-ICP-MS, eluent was amonium acetat buffer, flow rate was 0.2 ml/min, 20µL sample were injected and detected.

5.1.3. Dry leaves and tea studied by ICP-MS off line and by INAA

The dry leaves brought from Amazonas and the teas made from these leaves were studied by ICP-MS off line and by INAA.

The concentrations of Mn, Fe, Zn and Co studied by INAA and ICP-MS off line are shown in Table 5.

	Mn55 (ppm)		Fe54 (ppm)		Zn64 (ppm)		Co59 (ppm)	
Samples	INAA	ICP	INAA	ICP	INAA	ICP	INAA	ICP
Jam (dry leaves)	352.0	348.0	691.0	684.0	729.0	746.0	2.7	3.2
Jam (tea of leaves)	24.5	26.5	64.0	60.0	66.0	50.0	2.6	4.9
Bel (dry leaves)	32.0	30.0	2592.4	2572.0	454.8	449.0	2.4	2.7
Bel (tea of leaves)	5.0	7.0	64.5	70.0	42.5	47.0	2.2	5.0
Alf (dry leaves)	2.0	1.8	1507.5	1499.0	665.3	658.0	0.9	0.7
Alf (tea of leaves)	1.0	0.9	10.0	11.5	21.5	17.5	0.3	0.5

Table 5. Results obtained by INAA and ICP-MS off line for the concentration of manganese, iron, zinc and cobalt in dry leaves brought from Amazonas and tea mass leaves. The errors for ICP-MS were below 10% and the errors of INAA were estimated as 10-15%.

Table 5 shows a very good agreement of the results obtained by INAA and those by ICP-MS off line.

5.1.4. Extraction efficiencies of manganese, iron, zinc and cobalt in tea

With respect to medicinal application it is interesting to compare the extraction efficiency of the tea in three different plants. Taking into account that 1 g dry tea leaves have been brewed by 5 g water (in agreement with the methods people use in the Amazone region), the extraction efficiency as the percentage of the extracted element over element content in the leaves can be calculated and is given in Table 6.

Samples	Mn55 (%)	Fe54 (%)	Zn64 (%)	Co59 (%)
Jam (tea) - INAA	6.9	9.2	9.0	96.3
Bel (tea) - INAA	15.6	2.5	9.3	91.7
Alf (tea) - INAA	50.0	0.7	3.2	33.4
Jam (tea) - ICP-MS	7.6	8.7	6.7	153.0
Bel (tea) - ICP-MS	23.3	2.7	10.5	185.0
Alf (tea) - ICP-MS	50.0	0.8	2.6	71.0

Table 6. Extraction efficiency in % of Mn, Fe, Zn and Co in teas determined by INAA and ICP-MS off line.

As a result, the extraction efficiencies are relatively low, Mn in alf and Co in general being an exception. Co higher than 100% by ICP-MS in bel and jam indicates some impurity or interference.

For the plants studied here, no literature data could be found.

5.1.5. Protein and sulfhydryl groups in tea

The Bradford test was used for the determination of the total protein concentration and the Ellman test for the determination of the concentration of sulfhrydryl groups.

Bel contains a higher concentration of proteins and sulfhydryl groups than Jam and Alf teas. Alf tea has higher protein concentration than jam tea but the opposite is observed with the sulfhydryl groups.

Figure 20 shows that the concentration of protein and sulfhydryl groups decreased with ultrafiltration. Nevertheless, Figure 20 also shows that the detected proteins and sulfhydryl groups are at relatively low molecular weight, mainly below 5 kDa.

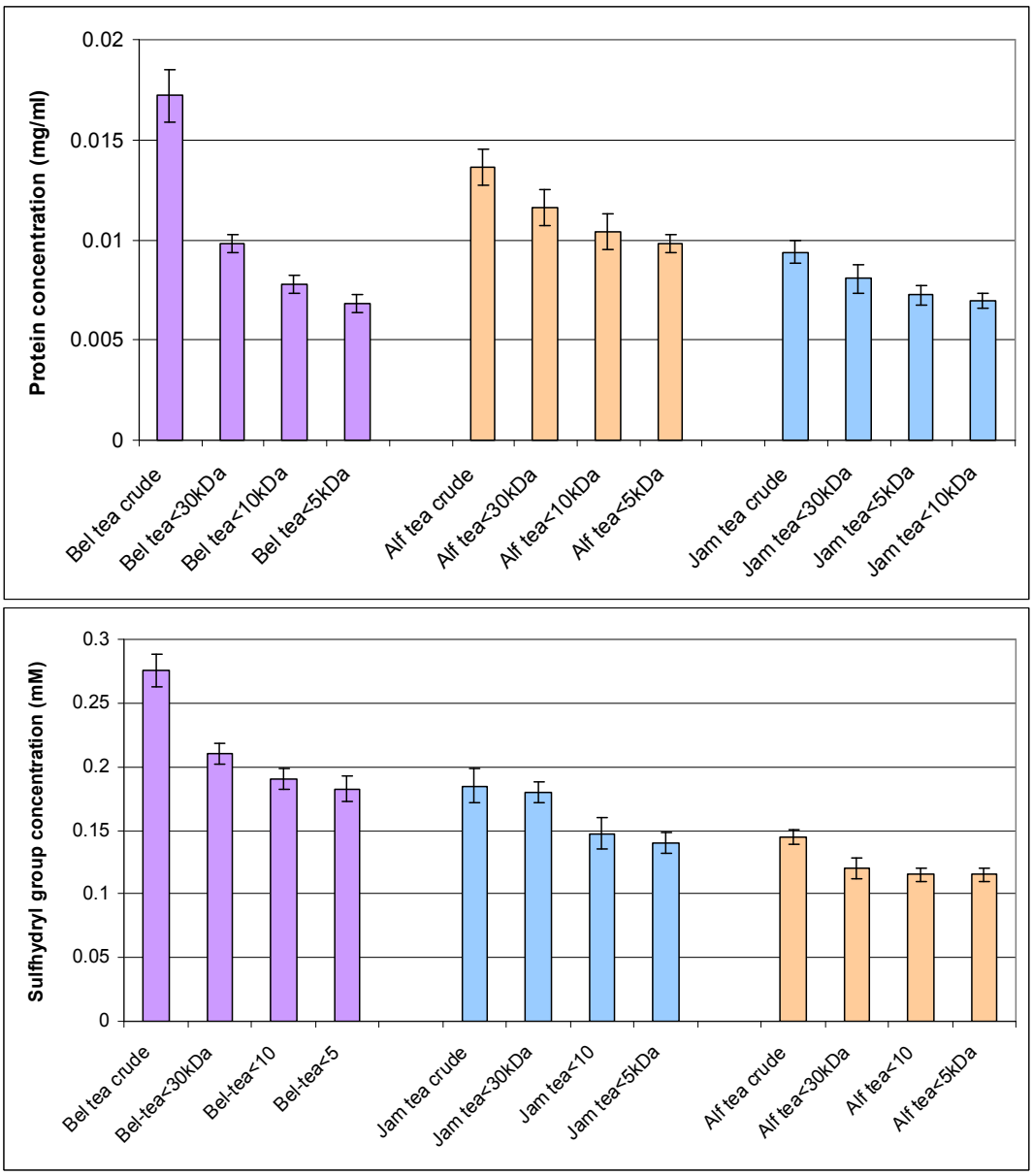


Figure 20. Protein concentrations and sulfhydryl group concentrations of Bel, Jam and Alf teas by the Bradford test and Ellman´s test respectively.

Although literature says that low molecular weight related compounds like aminoacids do not interfere the response of the Bradford test, its response to some low molecular weight compounds was tested. As an example, Figure 21 shows the response to trypsin – the other compounds (amino caprylic acid, D-glutamic acid, aprotinin, L-phynylanine and cyclodextrin) show even lower response.

Comparing this result to Figure 13 (see 4.7) it is obvious that the response of the Bradford test is at least 10 fold higher for poteins. Taking into account that many low molecular weight compounds are present in leaves, the results of the Bradford test should not be taken too serious – the test seems to indicate proteins in tea.

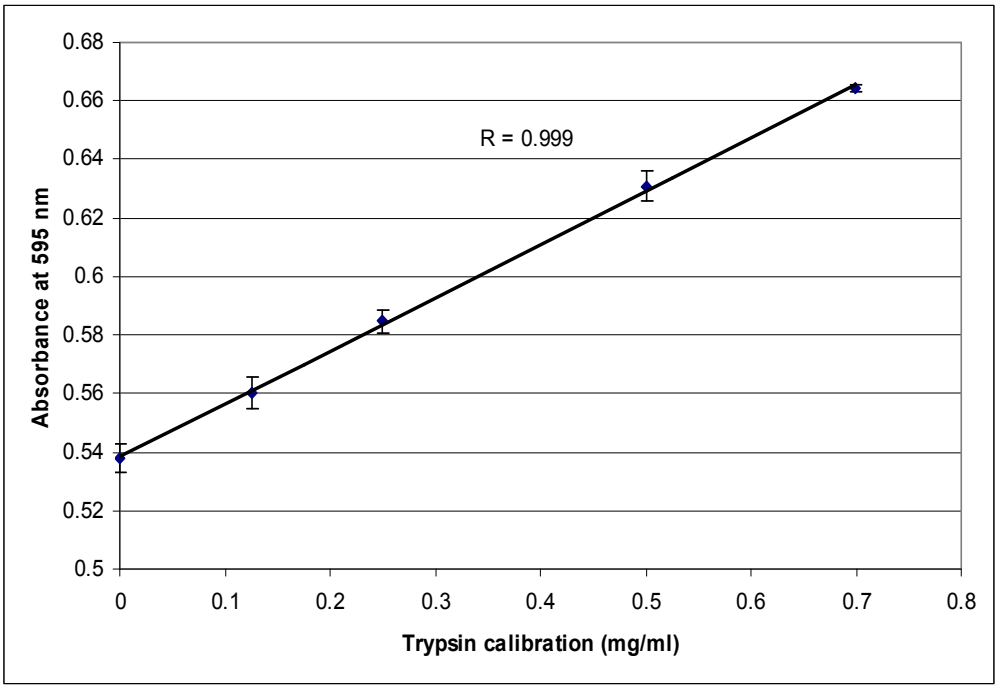


Figure 21. Calibration curve of trypsin measured by the Bradford test.

5.1.6. Metals in medicinal teas studied by ICP-MS off-line

The results presented in Table 5 (see 5.1.3) from ICP-MS off line studied on tea (1g per 5 ml) are further extended to size separated fractions, obtained by ultrafiltration with 30, 10 and 5 kDa exclusion limit. The results are shown in Tables 7 and 8, the tea crude values are identical to those in Table 5.

Table 9 show the relative elemental content after ultrafiltration steps. The results show that higher concentrations of the elements are found in the <5kDa fractions in teas of all plants studied here.

Some rare earth elements have been reported at the ng/g level in tea [19], in the tea of medicinal plants. In those studied here no rare elements could be found in their teas.

The general distribution and the concentration of the elements in alf, bel and jam tea are quite similar. Interestingly, all these tea plants studied here are used for gastro-intestinal disturbances and inflammation. However, it is very difficult to correlate the trace element concentration in tea and their therapeutic effects.

Samples (tea)	(ppm) Mg24	(ppm) Mn55	(ppm) Zn64	(ppm) Fe54
Jam crude	880 ± 70	26.5 ± 1.0	50.0 ± 4.9	60.0 ± 2.0
Jam<30kDa	865 ± 75	26.0 ± 1.5	38.0 ± 1,4	50.0 ± 2.5
Jam<10kDa	860 ± 40	25.5 ± 1.2	36.5 ± 1,2	45.0 ± 1,5
Jam<5kDa	595 ± 40	18.5 ± 0,9	35.5 ± 1,6	45.0 ± 4.0
Bel crude	355 ± 30	7.0 ± 0,5	47.0 ± 3.9	70 ± 4.0
Bel<30kDa	330 ± 30	6.5 ± 0,5	44.5 ± 3.8	50.0 ± 1.5
Bel<10kDa	320 ± 30	6.5 ± 0,5	42.0 ± 3.5	47.5 ± 3.0
Bel<5kDa	300 ± 15	6.0 ± 0,5	41.0 ± 3.2	43.0 ± 3.0
Alf crude	345 ± 25	0.9 ± 0,1	17.5 ± 1.3	11.5 ± 0.9
Alf < 30kDa	325 ± 15	0.9 ± 0,05	15.5 ± 1.3	10 ± 1.0
Alf < 10kDa	300 ± 10	0.9 ± 0,15	14.0 ± 1.2	7.5 ± 0.6
Alf < 5kDa	290 ± 20	0.8 ± 0,05	13.0 ± 1.2	7.5 ± 0.5

Table 7. Concentration of Mg, Mn, Zn and Fe in crude tea and their ultrafiltrates by ICP-MS off-line

Samples (tea)	(ppm) Ni58	(ppm) Cu65	(ppm) Sr86
Jam crude	0.5 ± 0,02	6.0 ± 0.5	6.0 ± 0,4
Jam<30kDa	0.5 ± 0,03	5.0 ± 0.4	5.0 ± 0.4
Jam<10kDa	0.5 ± 0,01	4.5 ± 0.3	4.5 ± 0.3
Jam<5kDa	0.5 ± 0,05	4.0 ± 0.3	4.5 ± 0.4
Bel crude	0.5 ± 0,04	5.0 ± 0.4	6.5 ± 0,5
Bel<30kDa	0.5 ± 0,01	4.5 ± 0.3	5.0 ± 0.2
Bel<10kDa	0.5 ± 0,02	4.5 ± 0.2	5.0 ± 0.3
Bel<5kDa	0.5 ± 0,01	4.5 ± 0.3	5.0 ± 0.4
Alf crude	0.5 ± 0,05	4.0 ± 0.3	5.0 ± 0.3
Alf < 30kDa	0.5 ± 0,05	4.0 ± 0.2	5.0 ± 0.4
Alf < 10kDa	0.5 ± 0,01	4.0 ± 0.3	4.0 ± 0,3
Alf < 5kDa	0.5 ± 0,01	4.0 ± 0.4	4.0 ± 0,2

Table 8. Concentration of Ni, Cu and Sr in crude tea and their ultrafiltrates by ICP-MS off-line.

Tea	Mn55 (%)	Fe54 (%)	Zn64 (%)	Ni58 (%)	Cu65 (%)	Sr86 (%)	Mg24 (%)
Alf crude	100	100	100	100	100	100	100
Alf < 30kDa	100	86	88	100	100	100	94
Alf < 10kDa	100	65	80	100	100	80	86
Alf < 5kDa	**89**	**65**	**74**	**100**	**100**	**80**	**84**
Bel crude	100	100	100	100	100	100	100
Bel < 30kDa	93	71	95	100	90	77	93
Bel < 10kDa	93	68	89	100	90	77	90
Bel < 5kDa	**86**	**61**	**87**	**100**	**90**	**77**	**85**
Jam crude	100	100	100	100	100	100	100
Jam < 30kDa	98	83	76	100	83	83	98
Jam < 10kDa	96	77	73	100	75	75	98
Jam < 5kDa	**70**	**76**	**71**	**100**	**66**	**75**	**68**

Table 9. Relative concentration (%) of the elements in crude tea and their ultrafiltrates.

5.1.7. Separation of lower molecular weight compounds in tea

The results shown in Table 9 show that 61-100% of elements studied here appear in tea sample extracts <5kDa, and therefore these fractions were chosen for further analysis. However, it is expected that some amount of compounds >5kDa may be present in samples <5kDa, as was explained in section 4.6.

Separation of the <5kDa fractions in tea by SEC/UV with the theoretically well suited column SE2 with exclusion limit of 10kDa did not show any separation as shown in Figure 22, since varying the mobile phase and the buffer conditions could not at all improve the separation, the only conclusion from Figure 22 is that UV-absorbing compounds of mass near 5kDa are present.

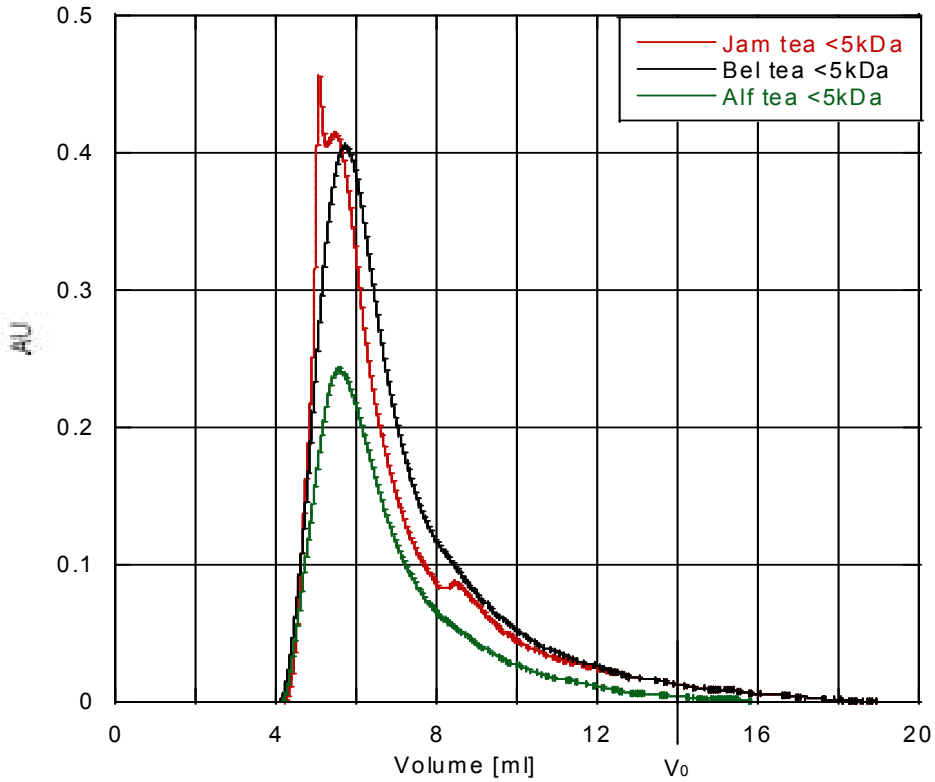


Figure 22. Alf, Bel and Jam tea <5kDa chromatogram. The separation was performed using the size exclusion column SE2 for low molecular weight, UV detection at 215nm, eluent was water, flow rate was 0.6 ml/min, 20µL of the sample were injected.

Reversed phase HPLC gave better results. The column 120-5-C18-AQ 50µm, 250x4.0 mm from Bischoff was used for the separation of the compounds. The <5kDa tea fractions have been studied by HPLC (RP)-UV (215nm), isocratically with mobile phase of 20, 10, 5 and 0% of methanol in water/0.1% TFA. Also some different gradients have been used in order to optimize the separation.

The RP C18-AQ column material is especially designed to be used with pure water as an eluent. After many different experiments to improve the separation, the best results were obtained with water/0.1% TFA as eluent.

Chromatograms of tea (<5kDa) from *Spilanthes oleracea* (Jam), *Portulaca oleracea* (Bel) and *Ocimum gratissimum* (Alf) are shown in Figure 23. The elements were detected by HPLC (RP)-UV and the results show that a great variety of compounds are present in these medicinal plant tea fractions (<5kDa).

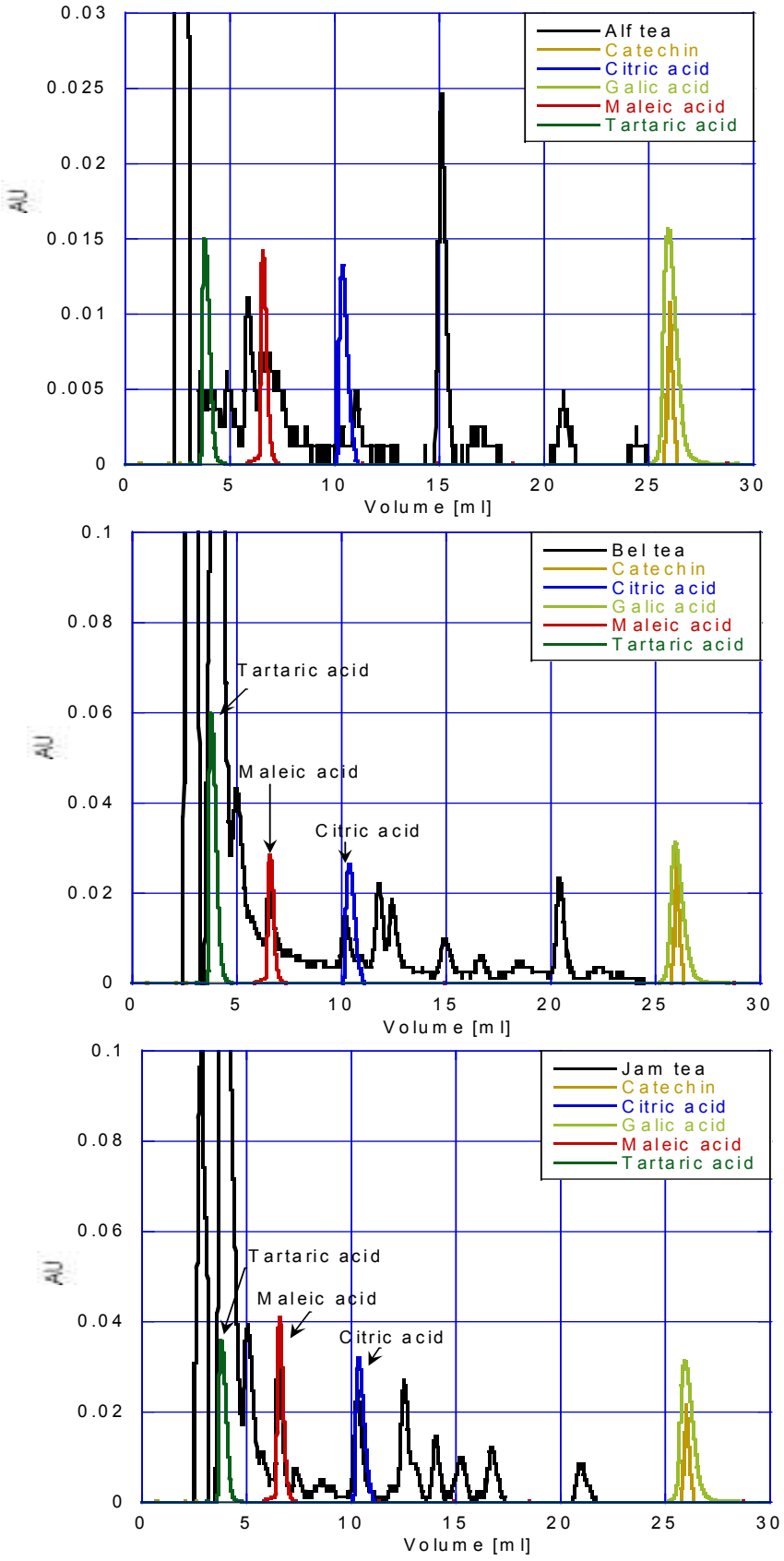


Figure 23. Chromatograms of Alf, Bel and Jam tea <5kDa using the reversed phase column, UV detection at 210nm, eluent was water/0.1% TFA, flow rate was 0.6ml/min, 20µL sample were injected.

To identify some peaks, the chromatograms of organic acid standards of gallic acid, tartatic acid, catechin, maleic acid and citric acid were recorded under the same conditions. For identification in the chromatogram, these acid standards then are overlaid to the chromatograms of the <5kDa fractions.

As a result, in Alf tea no such organic acids could be detected and/or confirmed by ESI-MS. The other observed peaks could not be identified. In Bel and Jam tea <5kDa fractions three organic acids could be detected: tartaric acid, maleic acid and citric acid as show by the chromatograms and have been confirmed with ESI-MS see for example Figure 24. Gallic acid and catechin were not detected in these teas. The remaining peaks could not be identified.

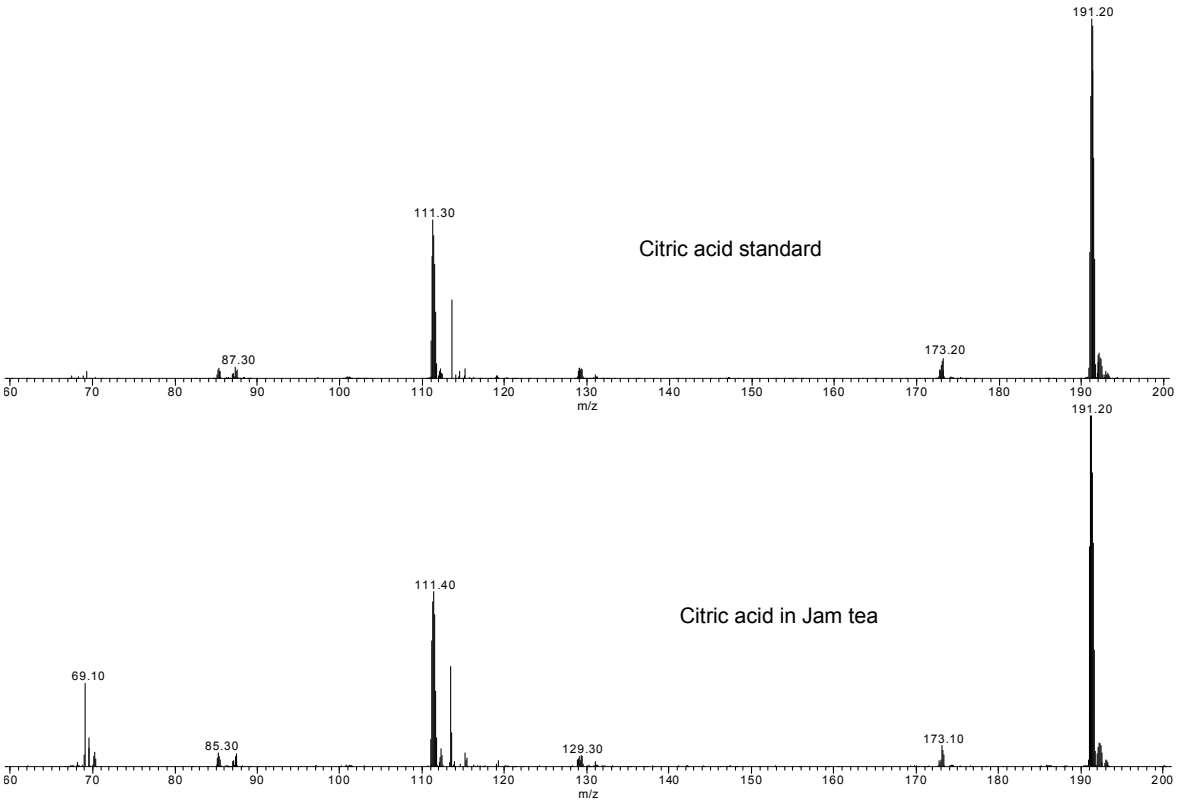


Figure 24. Mass spectrum of the peak around 10.2 ml in Jam tea as shown in Figure 23, and of a citric acid standard by HPLC (RP)-ESI-MS, negative mode, 20µl sample were injected, flow rate was 0.6 ml/min. Both mass spectra show a base peak at *m/z* 191.2 and its fragment ions *m/z* 173.2 and *m/z* 111.3.

Element specific RP chromatograms of <5kDa tea fractions

Figures 25 and 26 show the chromatograms of the elements in Alf, Bel and Jam tea fractions <5kDa detected by HPLC (RP)-ICP-MS.

Magnesium - Alf, Bel and Jam <5kDa tea fraction chromatograms show one intensive magnesium peak at the dead volume (V_0). These peaks may be ascribed to inorganic (ionic) Mg-salts (Figure 25).

Manganese - Besides the typical most probably (inorganic) salt peak at V_0, a slightly retained second peak is observed (Figure 25).

Sulphur and phosphorus - S and P mainly appear at V_0, with some tailing (Figure 25).

Iron - the iron chromatograms for all three plants consist of two peaks. Fe binds to two different compounds. The first peak at V_0 again may be salts, the second peak with different retention volume for the three plants is broad and retained; it might be due to a non or weakly - dissotiated compound, probably organic and / or complex (Figure 25).

Zinc - again strong zinc signals are observed at V_0 but for Bel there is a slightly retained compound at $V_r = 3.14$ ml, again probably a complex (Figure 25).

Cobalt - two Co peaks were detected in Bel tea fraction but only one peak in Alf and Jam tea fractions were detected. The first peak again was at V_0 (Figure 26).

Strontium - Sr could only be detected at V_0, probably due to an inorganic salt (Figure 26).

Copper - the Cu chromatograms in Bel and Jam tea <5kDa fractions show two peaks, whereas in Alf tea just one peak of Cu was detected. Interesting that the V_0 peak is missing in Alf and Jam tea fractions! (Figure 26).

Nickel – the Ni chromatogram of all three plant tea fractions are different. While two peaks could be detected in Bel tea just one peak was detected in Alf and Jam tea fractions, both with quite different peak form. It means two different compounds of nickel can be found in the Bel tea fraction (Figure 26).

In an overall view, the distributions of the anlysed elements are interesting, since in general a similar behaviour of some metals can be observed in tea fractions <5kDa for quite different plant species.

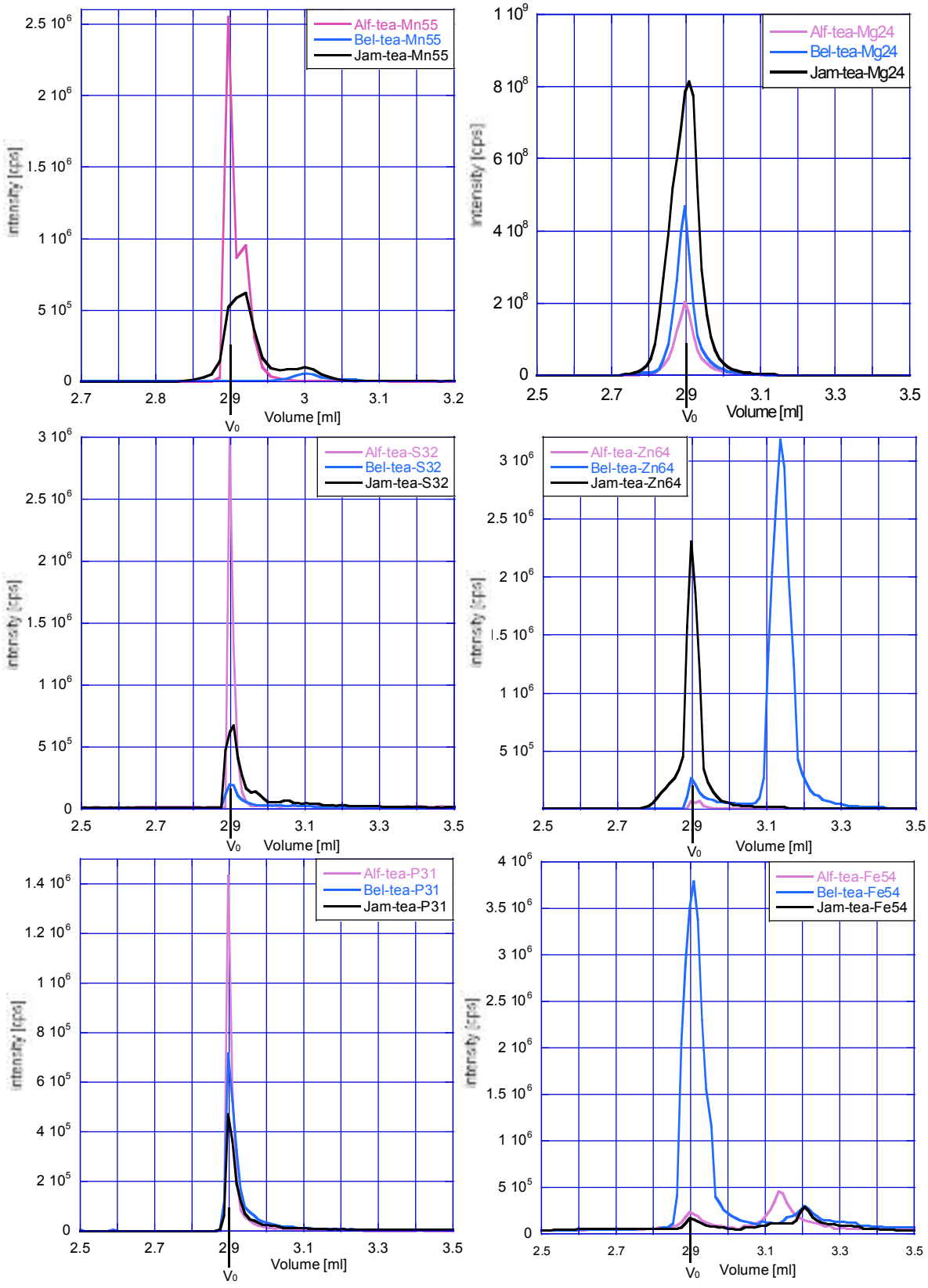


Figure 25. Chromatograms of the Mn, S, P, Mg, Zn and Fe from Alf, Bel and Jam tea <5kDa determined by HPLC (RP)-ICP-MS, isocratic separation, eluent was water/0.1%TFA, flow rate 0.6 ml/min, 20µL sample were injected.

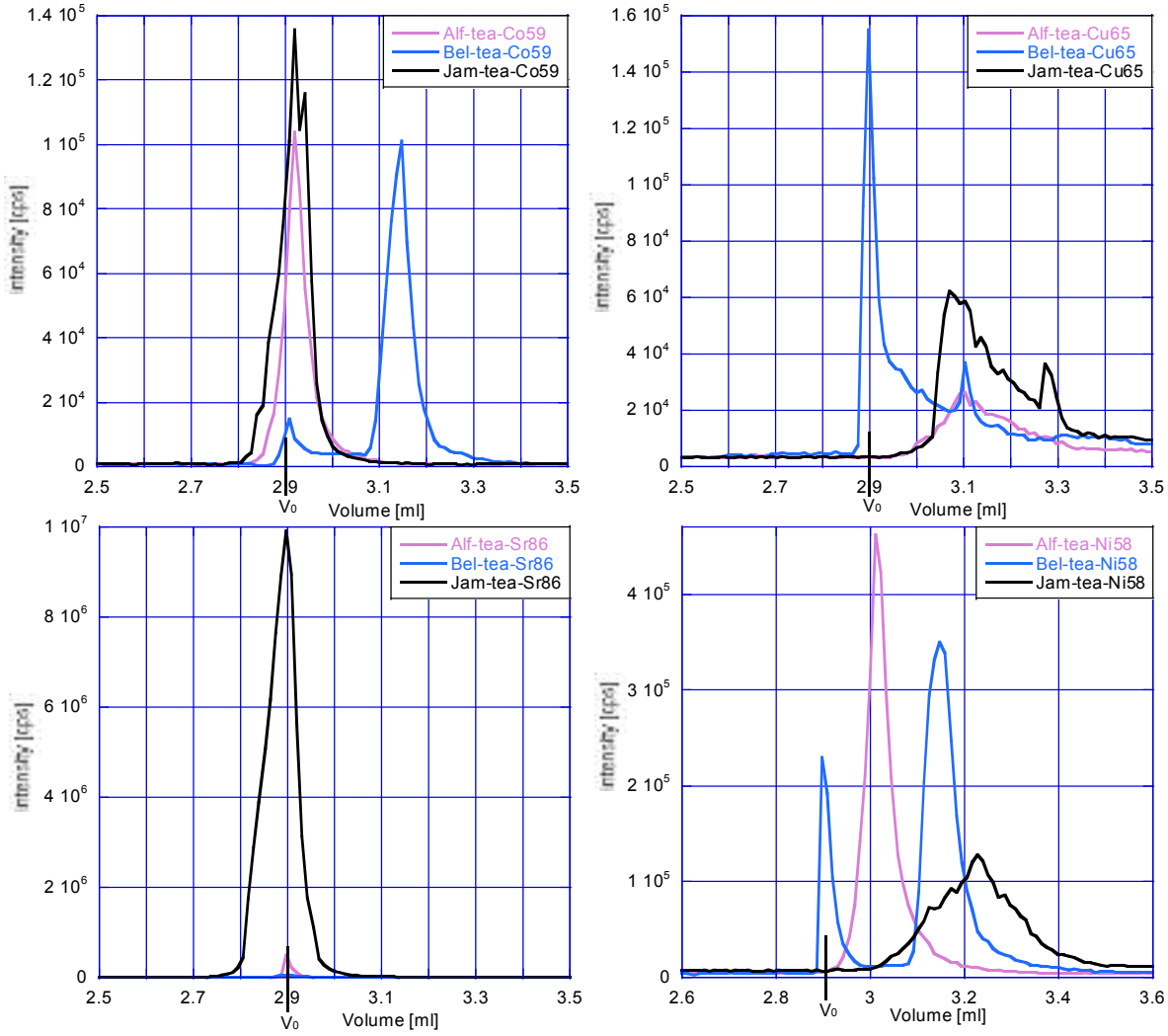


Figure 26. (continuation) Chromatograms of Co, Cu, Sr and Ni from Alf, Bel and Jam tea <5kDa determined by HPLC (RP)-ICP-MS, isocratic separation, eluent was water/0.1%TFA, flow rate was 0.6 ml/min, 20µL sample were injected.

5.1.8. Lower molecular weight compounds studied by MALDI-TOF-MS

MALDI-TOF-MS did not give valuable results. Peaks could only be observed in the mass range of the used matrix or very weakly when no matrix was involved.

5.1.9. Lower molecular weight compounds studied by HPLC (RP)-ESI-MS

The ultrafiltrates <5kDa of Alf, Bel and Jam tea were studied by HPLC (RP)-UV-ESI-MS (detection of the compounds as shown in Figure 23) and by HPLC (RP)-ICP-MS (detection of the elements as shown in Figures 25 and 26) under the same conditions. Comparing the chromatograms of both techniques, the results show that all elements studied by ICP-MS were detected in the same retation volume range of the first peak detected by UV at 210nm. Consequently, the mass spectra of the first peaks from Jam, Bel and Alf tea were recorded and analised (Figure 28, 29 and 30).

The teas were analysed in negative and positive mode by ESI-MS.

Citric acid, maleic acid and tartaric acid were detected in tea fractions <5kDa and of course they were better detected in negative mode of ESI-MS, as the example in Figure 27 shows.

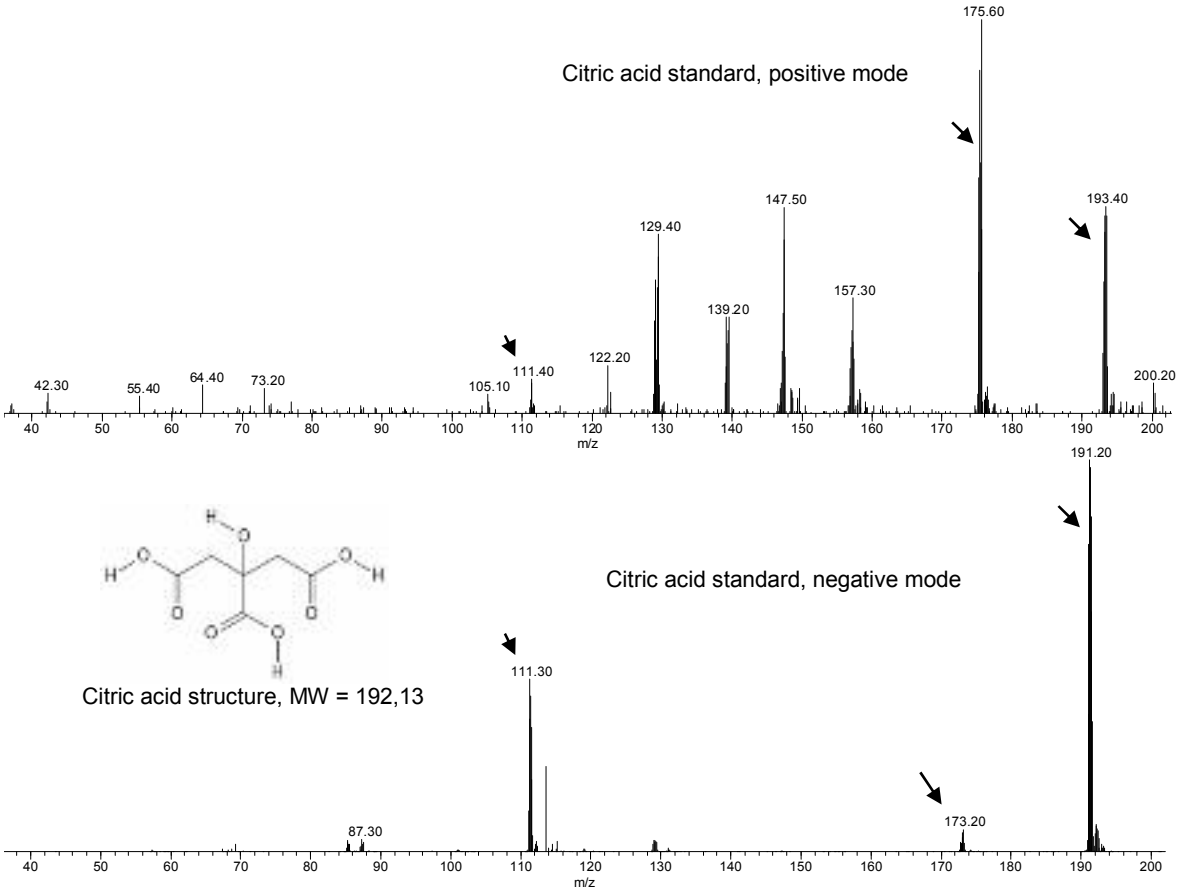


Figure 27. Analysis of citric acid standard (0.2 g/ml) at positive and negative mode by HPLC (RP)-ESI-MS. The mass spectra were studied by HPLC (RP)-ESI-MS under the same conditions, isocratic separation, water/0.1% TFA as eluent, flow rate was 0.6 ml/min, 20µl sample were injected.

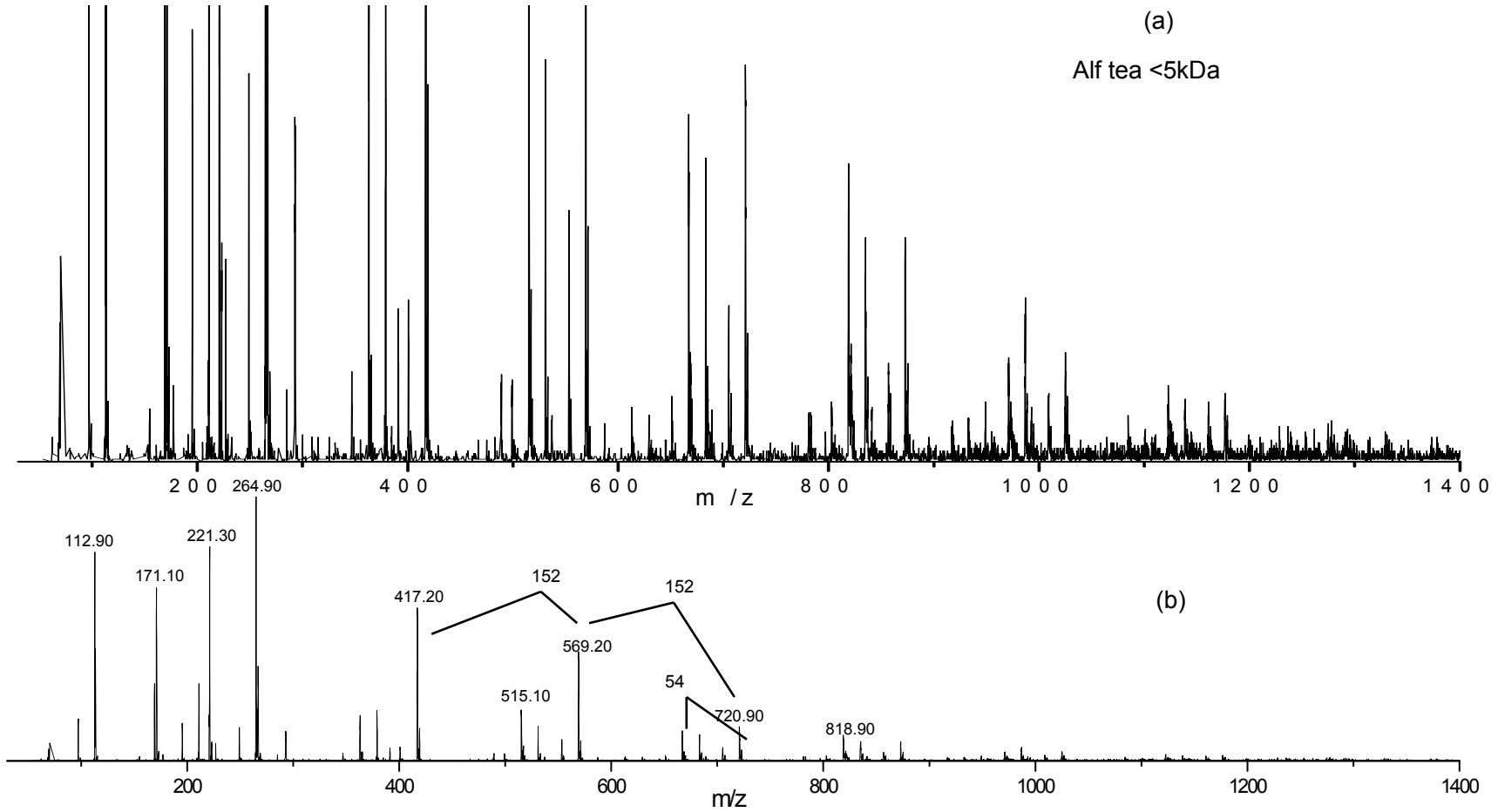


Figure 28. (a) Alf tea <5kDa mass spectra (intensity x 4) of the first peak studied by HPLC (RP)-ESI-MS, negative mode, isocratic separation, eluent water/0.1% TFA, flow rate was 0.6 ml/min, 20µL of the sample was injected. (b) The same mass spectra in normal size.

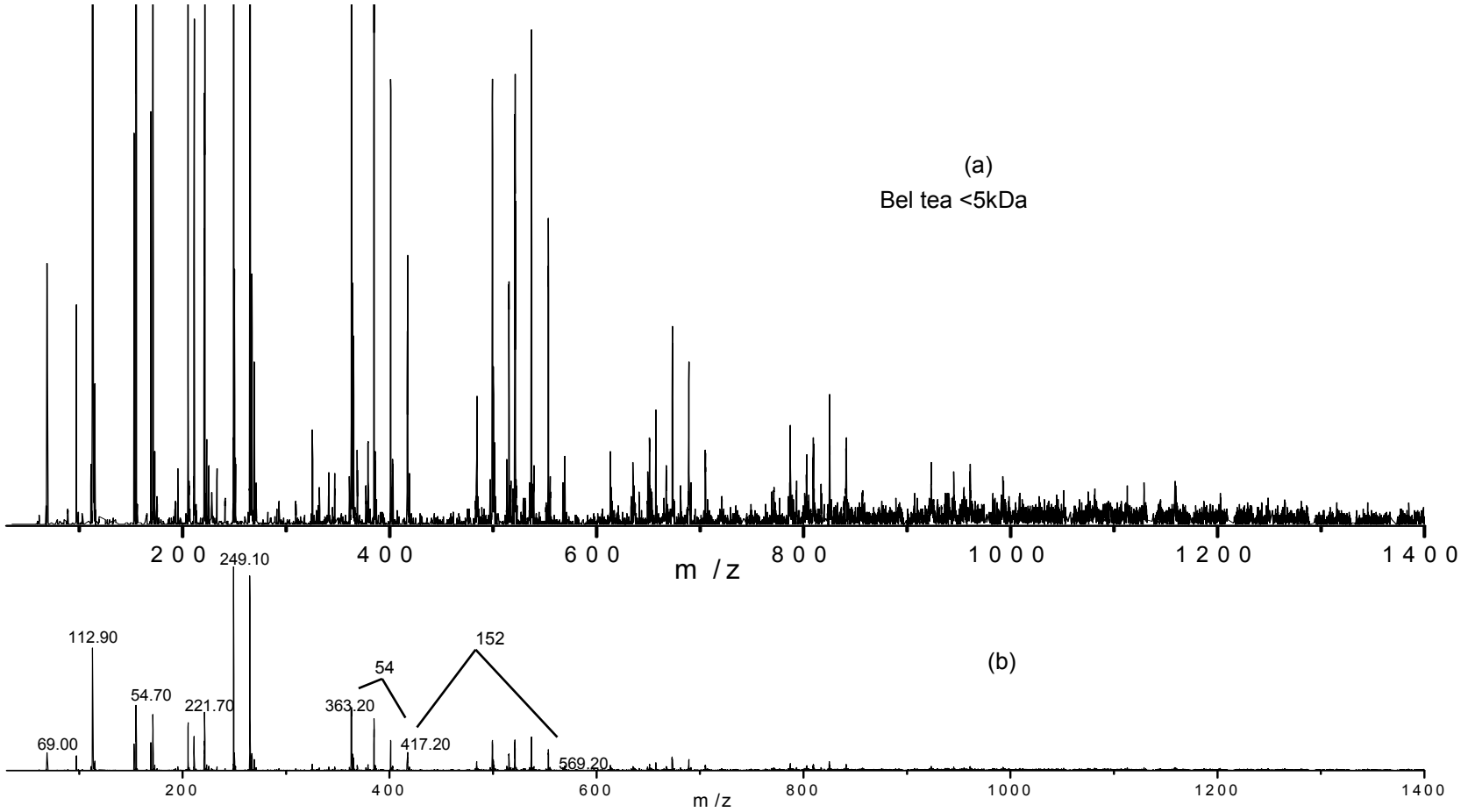


Figure 29. (a) Bel tea <5kDa mass spectra (intensity x 4) of the first peak studied by HPLC (RP)-ESI-MS, negative mode, isocratic separation, eluent water/0.1% TFA, flow rate was 0.6 ml/min, 20μL of the sample was injected. (b) The same mass spectra in normal size.

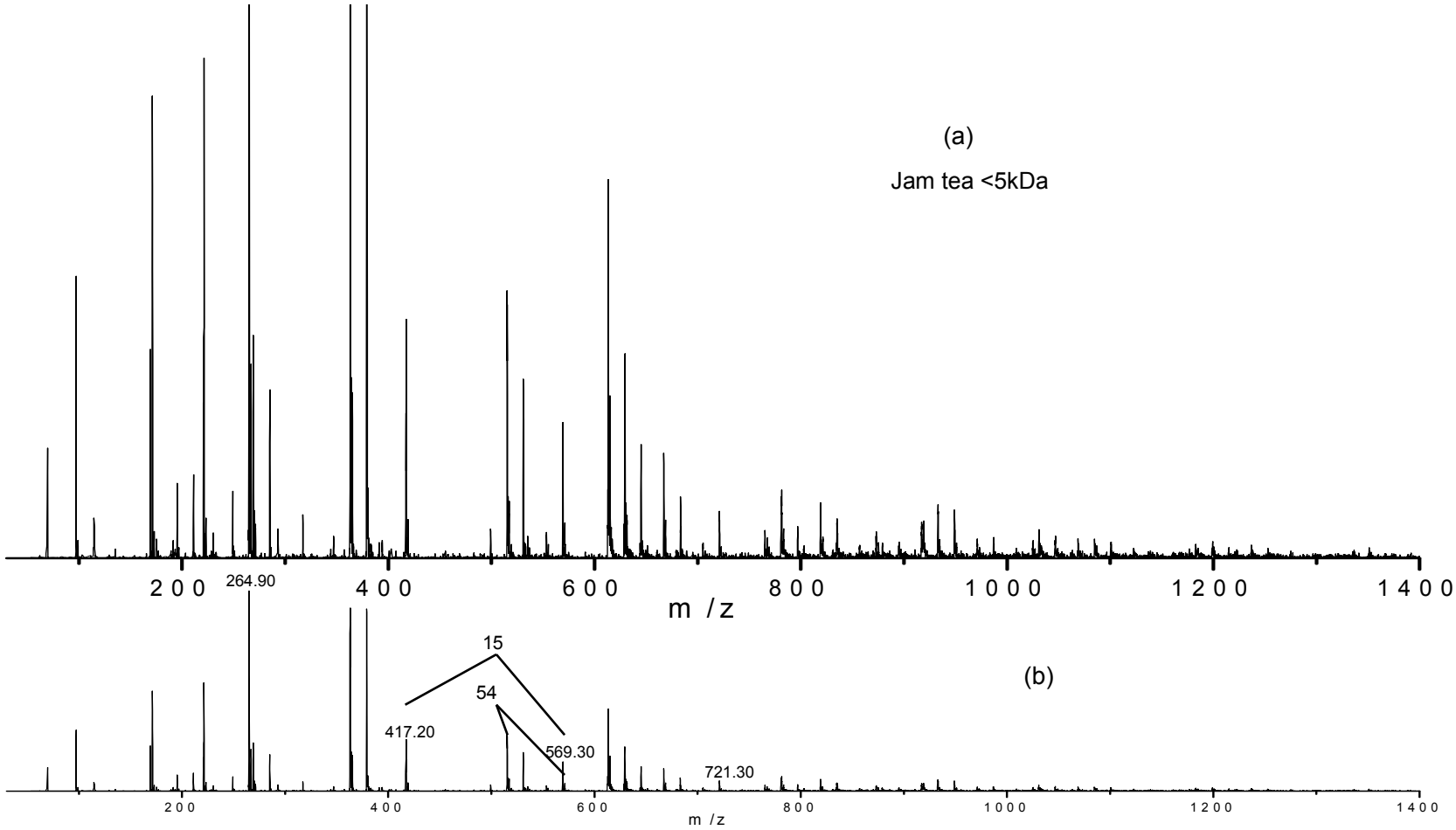


Figure 30. (a) Jam tea <5kDa mass spectra (intensity x 4) of the first peak studied by HPLC (RP)-ESI-MS, negative mode, isocratic separation, eluent water/0.1% TFA, flow rate was 0.6 ml/min, 20µL of the sample was injected. (b) The same mass spectra in normal size.

Interestingly, the results Figures 28, 29 and 30 show that all tea mass spectra were quite similar in spite of the fact that these plants are from different families. In all mass spectra the most intensive peak was at *m/z* 264. Quite distinctly blocks of peaks appear that differ by $\Delta_{m/z}$=152. Within the periods of 152, characteristic differences of $\Delta_{m/z}$=54 and within these periods characteristc differences of $\Delta_{m/z}$=16 and 38 appear.

5.2. Metal stress on plants grown in a hydroponic system

The result of research on some essential elements and their species in medicinal plant extracts from *Spilanthes oleracea* - Jambu (Jam)*, Portulaca oleracea* - Beldroega (Bel) and *Ocimum gratissimum* - Alfavaca (Alf) is shown in this chapter. The sample preparation was exactly the same for all samples. Thus, the results should reflect the different reaction of the considered three plants to stress.

5.2.1. Comparison of extraction methods

As an initial step of manganese speciation, three different methods to break down cells in plant leave samples were studied: CelLytic-P, lysozyme and by Ultra-Turrax (see section 4.13). The results are comparable because Jam leaves had been collected at the same day and the same group of plants exposed to higher manganese concentration. Manganese was measured by ICP-MS off line. Figure 31 shows the results of these methods.

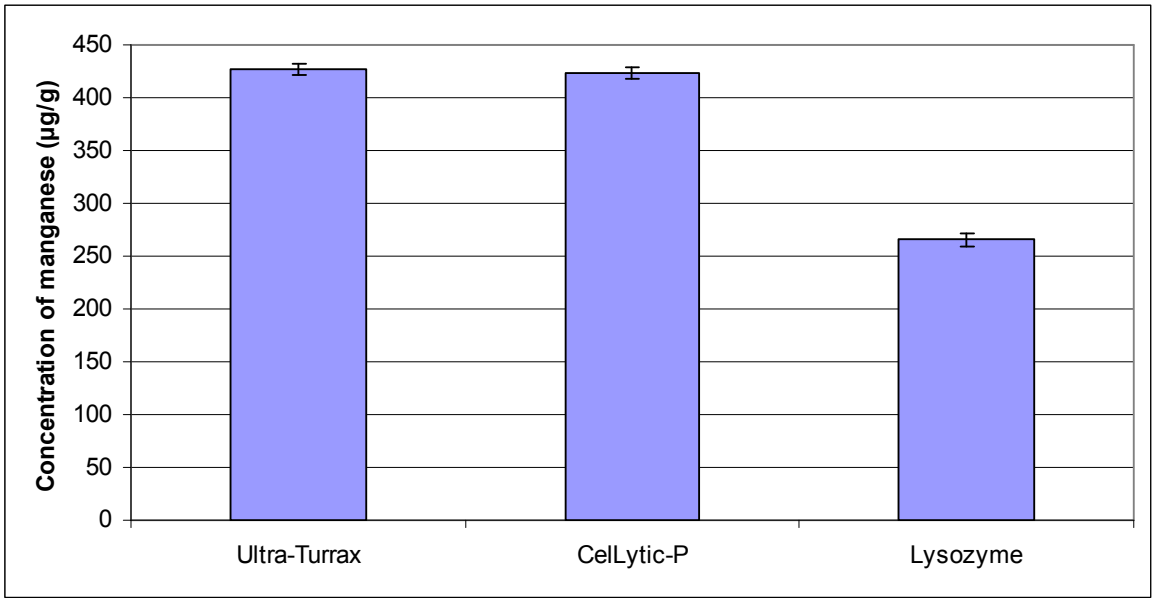


Figure 31. Concentration of manganese in Jam leaves by different methods to break down the cells. Leaf age was 25 days in hydroponic system.

The lysozyme based method was the method that yielded the lowest extracted amount of manganese. Higher concentration of lysozyme did not change the extracted amount of manganese. In addition, lysozyme caused problems in the ESI mass spectra. In literature mainly lyzozyme is used for bacterial cell wall breakdown.

CelLytic-P and Ultra-Turrax treatment extracted practically the same amount of manganese as show in Figure 31.

CelLytic-P is a detergent-based reagent used for the extraction of proteins in leaves. The CelLytic-P reagent offers a convenient method for efficient plant cell lysis and protein solubilisation. A protease inhibitor cocktail is added to avoid protein degradation. The addition of the detergent and the protease cocktail add peaks to the mass spectra. Therefore, Ultra-Turrax treatment looks to be ideal. As the Ultra-Turrax equipment is made of metal, the contamination effect of Ultra-Turrax treatment in relation to cell breakdown of plant material was studied. Manganese was analysed by ICP-MS off-line in blanks prior to and after Ultra-Turrax treatment. The results obtained do not show any significant contaminations induced by the Ultra-Turrax treatment in the case of manganese (Figure 32). The experiment was carried out under exactly the same conditions used for the cell breakdown of all plant samples.

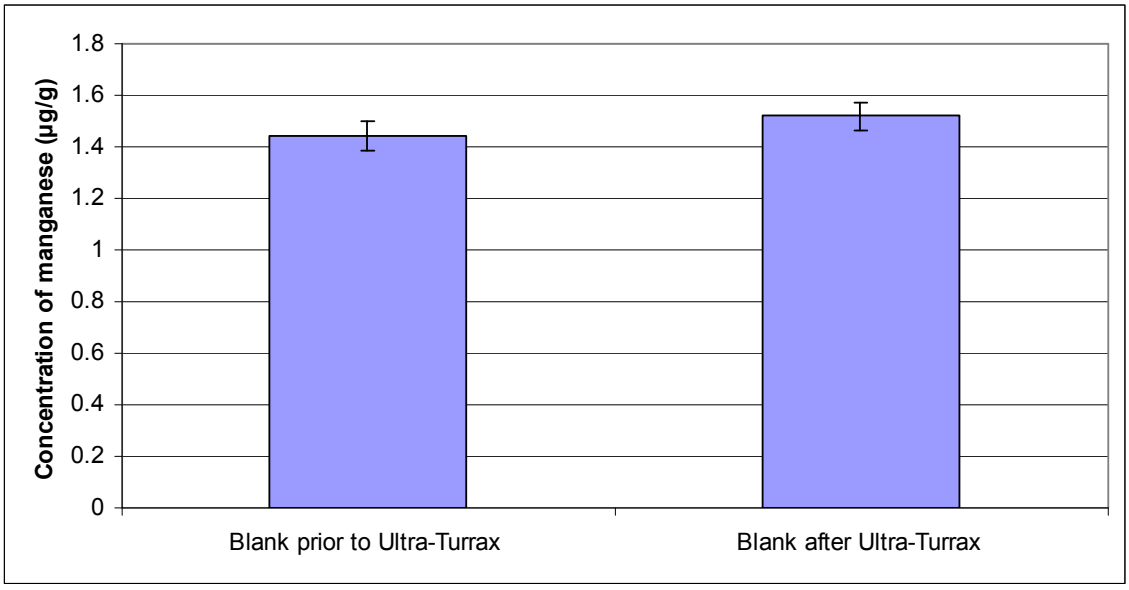


Figure 32. Manganese contents in blank prior and after Ultra-Turrax treatment.

Figure 33 shows the protein concentration determined with the Bradford reagent in Jam leaves extracts prior to and after treatment with Ultra-Turrax.

The result shows that Ultra-Turrax treatment breaks down cells at least sufficiently to release proteins.

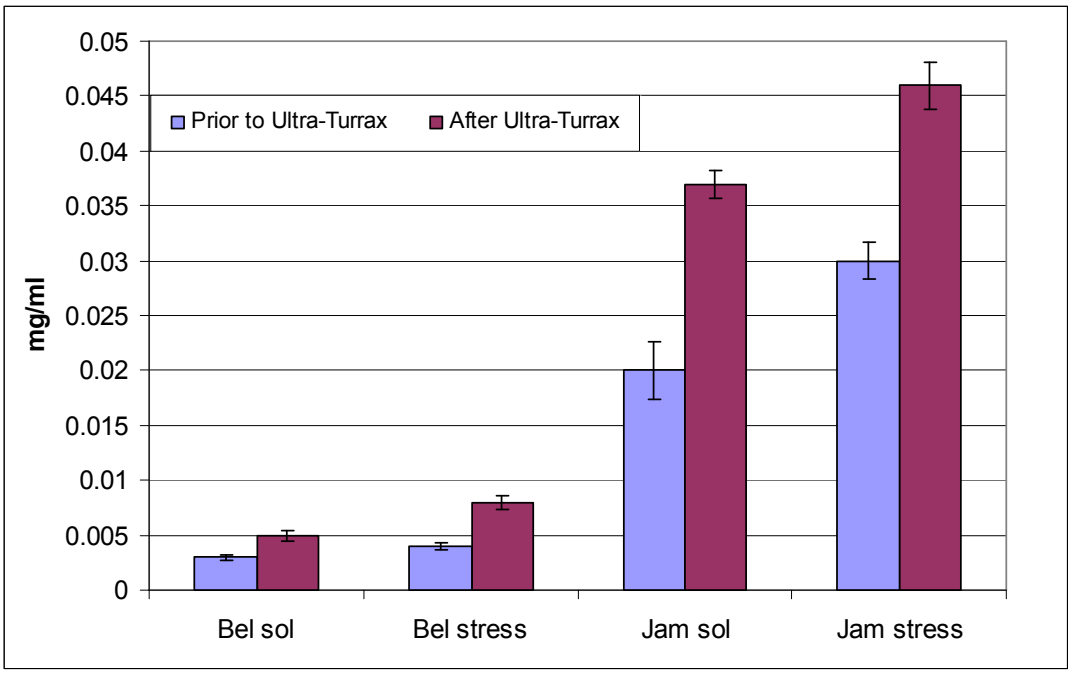


Figure 33. Determination of the protein concentration by the Bradford reagent in Jam leaf extracts prior to and after Ultra-Turrax treatment.

5.2.2. Plants grown under manganese stress in the hydroponic system

For Mn-stress studies the plants were exposed to different manganese concentration: 50, 100, 200, 500, 800, 1000 µM. Visible symptoms of manganese toxicity can be noticed in all plants (*Spilanthes oleracea* (Jam), *Portulaca oleracea* (Bel) and *Ocimum gratissimum* (Alf)) in lower intensity starting from 50 µM of manganese. These symptoms were more visible in alf and less visible in Bel.

The symptoms increase with increasing manganese concentration in the nutrition solution.

Bel and Jam exposed to 800µM of manganese could even survive over one year, of course with symptoms of manganese toxicity. Alf was much more sensitive to manganese concentrations in the solution as compared with Bel and Jam. Alf could not survive in 200µM of manganese concentration in the nutrition solution. Figure 34 shows the results. After once adult (60 days) the plants could survive for months.

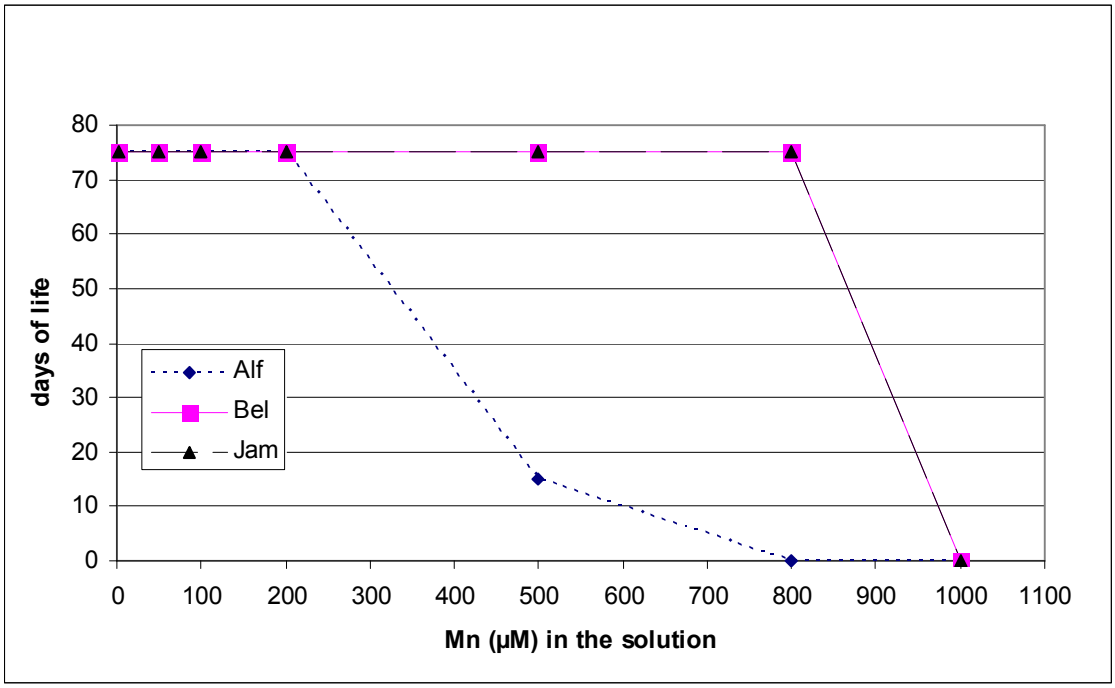


Figure 34. Graphic between days of life of the plants versus concentration of Mn. Errors were estimated below 10%.

Because of the observed high sensitivity of Alf to Mn-stress the stress experiments have only been continued with Bel and Jam.

The concentration of manganese in the optimized basal nutrient solution is 2.5 µM. Thus Jam and Bel could survive concentrations of manganese 320 times higher than in the basal nutrition solution.

Figure 35 shows that the accumulation of manganese in the leaves appear to be cumulative in Jam and Bel where the manganese concentration increased with the age of the leaves. However, even grown under basal nutrient solution (sol) conditions the concentration of manganese in leaves also increased with the age. For both adult plants - after 60 days - grown under stress and no stress, the concentration of manganese did no more increase.

Interesting that Bel accumulated manganese until the adult phase (60 days) while Jam reached the maximum Mn level already after 30 days, stressed or not stressed. Note that the nutrition solution was renewed every week.

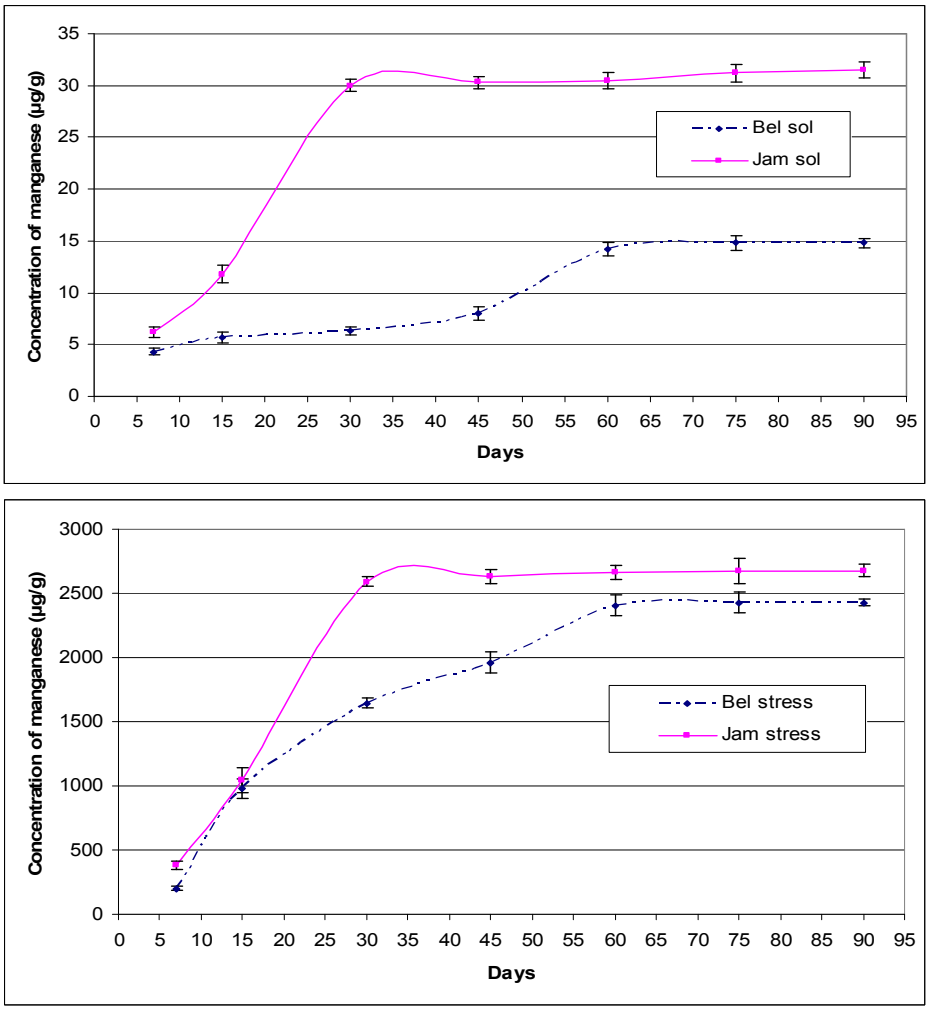
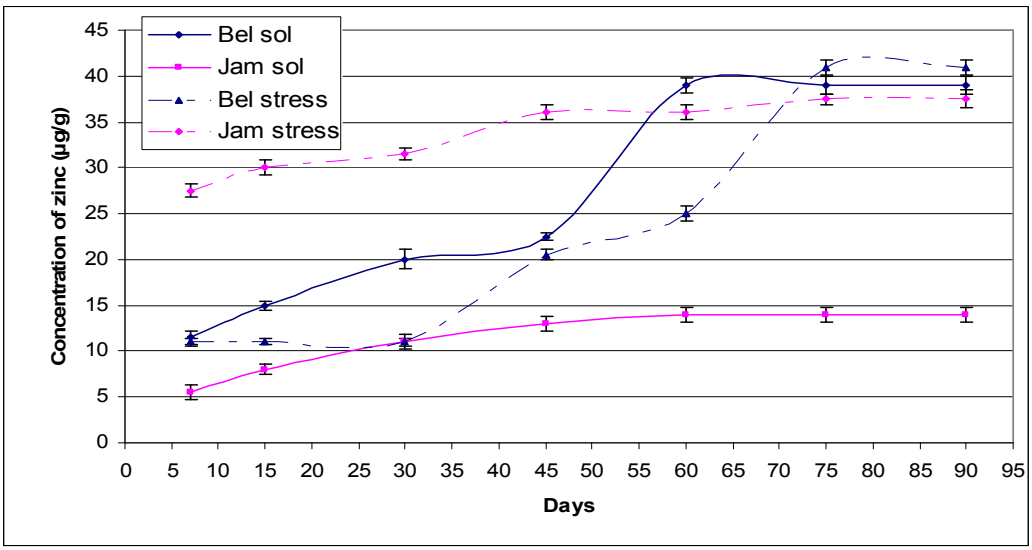


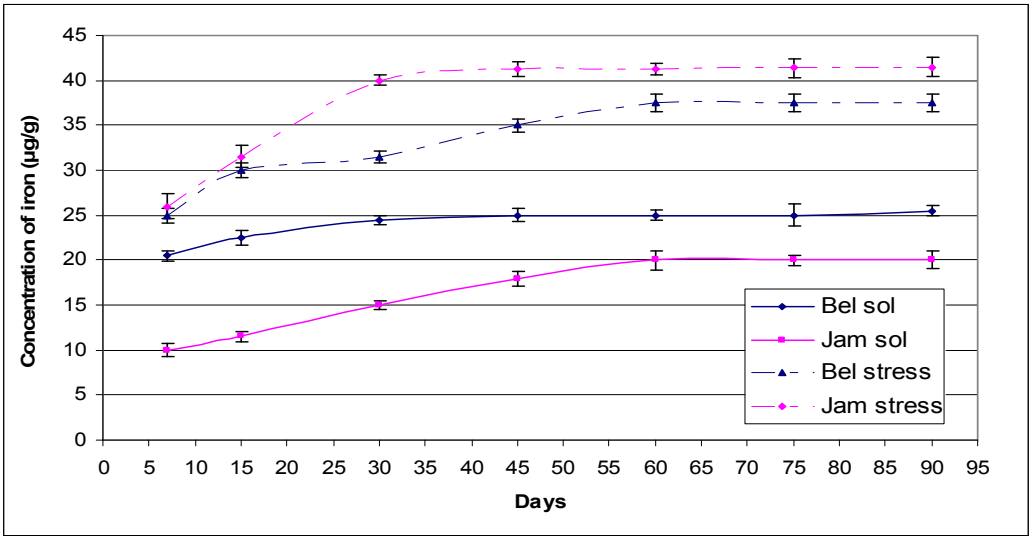
Figure 35. Manganese concentration in Jam and Bel leaves of different age grown under basal nutrient condition (sol) and exposed to 800 µM of manganese stress, determined by ICP-MS off line.

5.2.3. Concentration of zinc, iron and cobalt in stressed plants

Manganese undergoes important interactions with many other nutrition elements. Many papers, for example references [104][105][106], report a direct evidence of a decrease in Mn uptake as a result of increased external concentration of other elements, e.g. Fe, Zn and Cu. Thus manganese concentrations of 800µM may modify the up-take of other elements in the plant. In order to elucidate this, the influence of manganese uptake to zinc, iron and cobalt into the plant were studied comparing plants grown in basal nutrient solution (sol) with plants exposed to 800µM of manganese stress during the growing phase until adult. The concentrations of the elements have been measured by ICP-MS off line.

The results are shown in Figure 36. It appears that Mn stress yields in higher Fe uptake in Bel and Jam and higher Zn uptake in Jam, whereas the effect on Co is small.





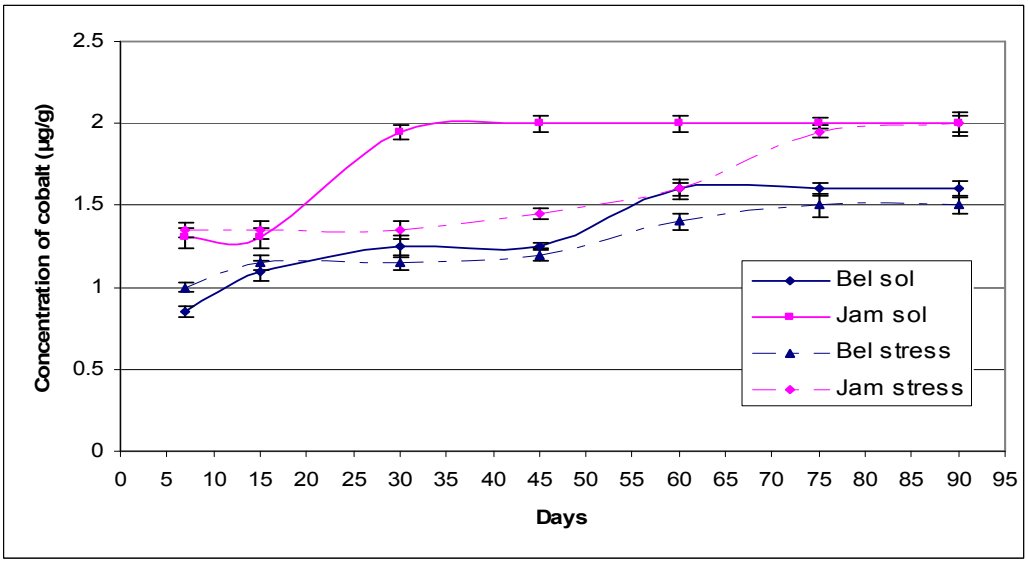


Figure 36. Influence of Mn uptake in leaves of plants grown in nutrient solution (sol) and exposed to 800 µM of manganese (stress) on Zn, Fe and Co in different age of the leaves, by ICP-MS off line.

5.2.4. Manganese analysis by HPLC-ICP-MS

In the previous section it was shown that Bel and Jam exhibit increased Mn-levels when grown under Mn-stress. Therefore, in the following the attempt is made to elucidate in which form or species Mn then is stored in the plants.

As a first step, Mn-HPLC (RP)-ICP-MS chromatogram of crude extracts of leaves, stem and roots have been recorded.

Bel and Jam chromatograms (Fig. 37) look similar, even though these plants are from different families. The chromatograms of Bel and Jam stem show only one Mn peak at V_0. The chromatograms of roots and leaves from Bel and Jam show again this Mn peak at V_0, but in addition a second one at $V \approx 5ml$.

The peak at V_0 indicates Mn in ionic form (transport through the stem!) but the peak at $V \approx 5ml$ must be ascribed to another Mn species, organically or inorganically complexed or bound.

Since leaves show higher Mn concentrations, they have been selected for further studies.

In order to get more information, the crude extracts of leaves from Jam and Bel were injected into the size exclusion column Prontosil-diol - SE1. The chromatograms of Bel and Jam show to be similar, with peaks at different retention volumes and different intensities, indicating large size excluded compounds and small size ones retained by affinity as shown in Figure 38.

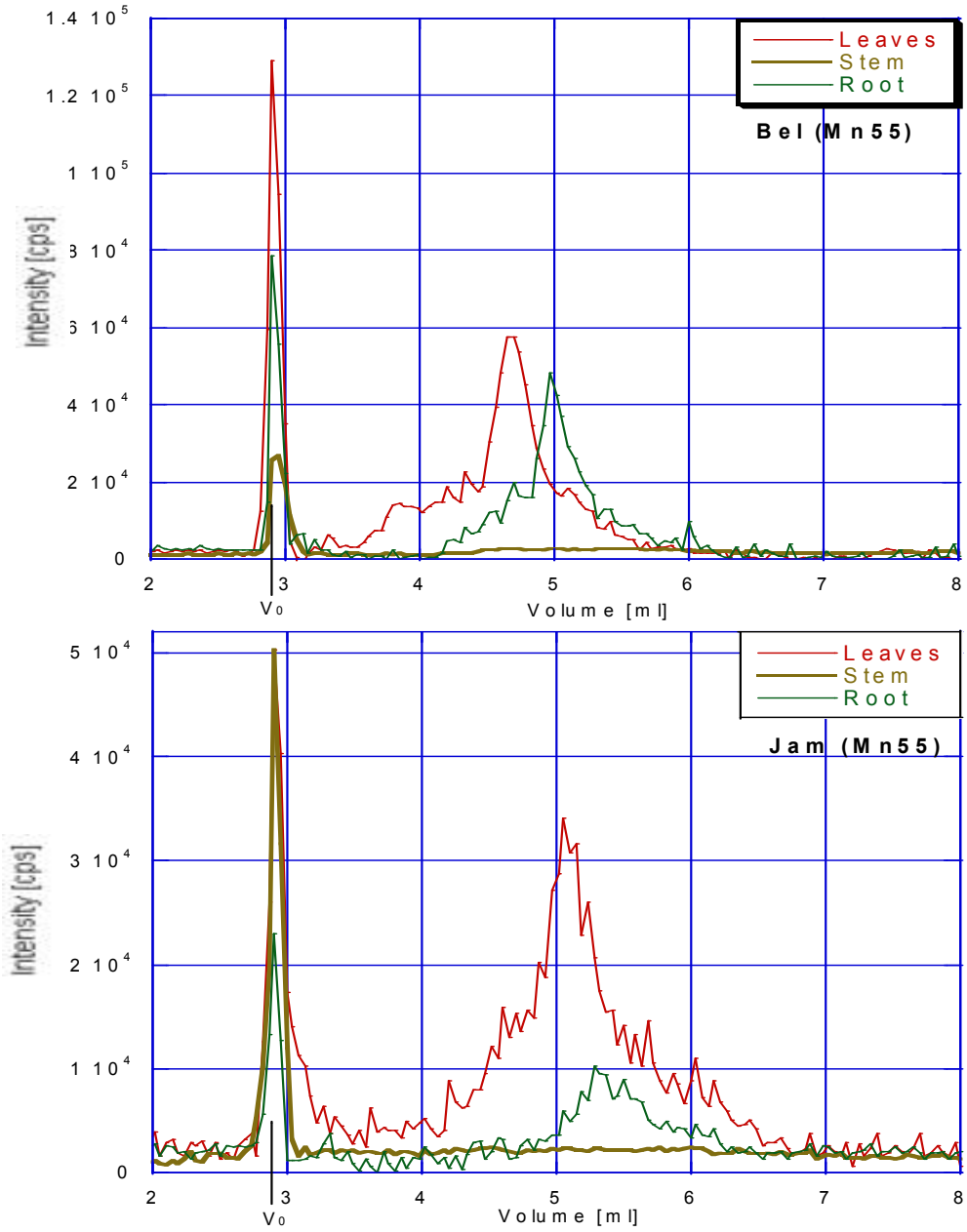


Figure 37. Manganese chromatogram of Bel and Jam crude extracts (leaves, stem and root) from HPLC (RP)-ICP-MS, isocratic separation, eluent was water/0.1%TFA, flow rate was 0.6 ml/min, 20µL sample were injected.

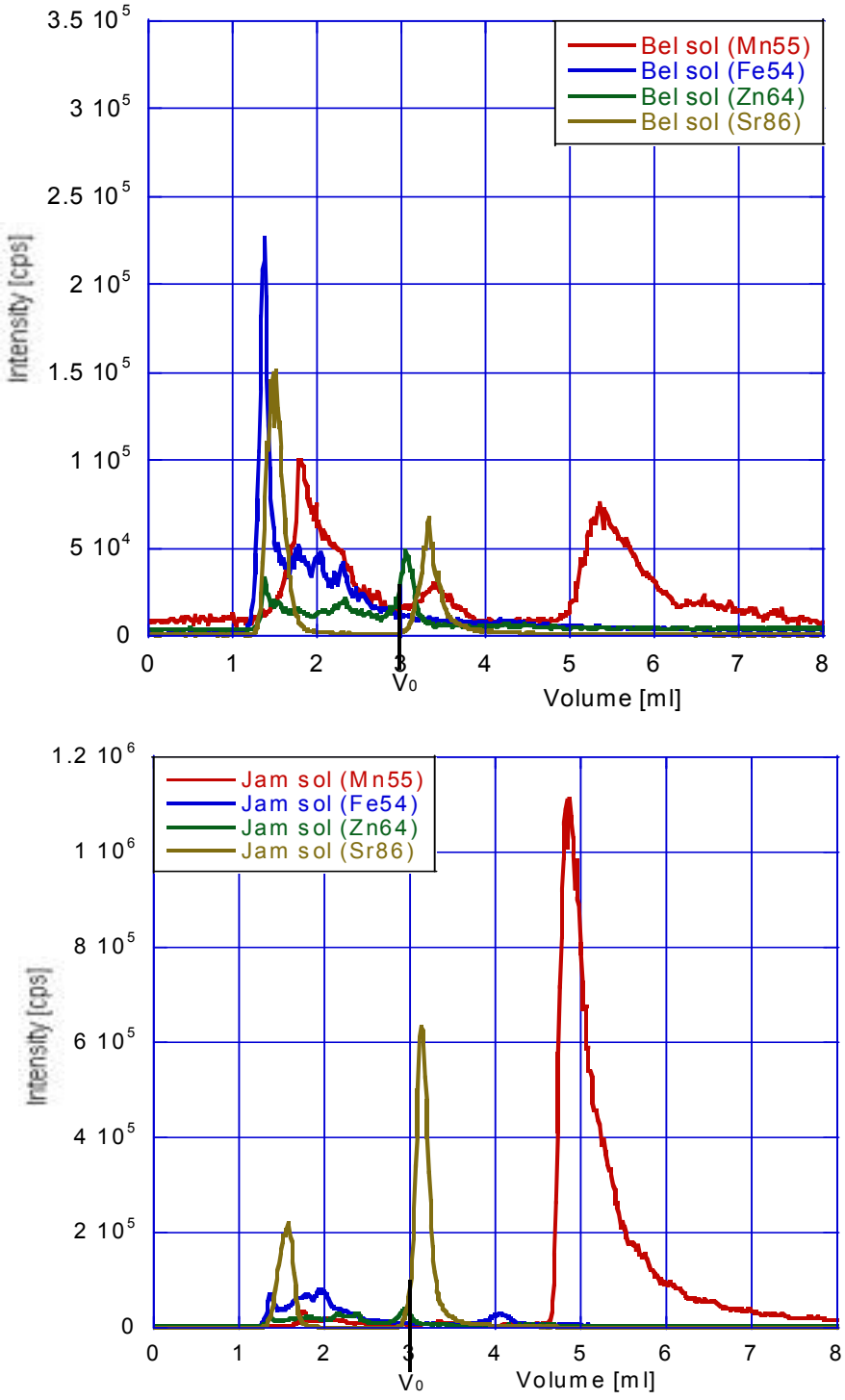


Figure 38. Chromatograms of Bel and Jam crude extracts from leaves grown in basal nutrient solution (sol). The chromatogram was determined by HPLC (SE1)-ICP-MS, isocratic separation, and eluent was ammonium acetate buffer, flow rate was 0.6 ml/min and 20µL sample were injected.

5.2.5. Elemental concentration in plant extracts by ICP-MS off line

Since there are excluded Mn containing fractions in the SE-HPLC (see Fig. 38) the crude leaf extracts were sequentially ultrafiltrated with filters of different molecular weight cut-off (30, 10 and 5 kDa) and then were studied by ICP-MS off line.

The comparison between the stressed (stress) and not stressed adult plants (stress) is shown in Figure 39. Under stress conditions both plants accumulate much more Mn, the relation between stressed and not stressed plant leaves being 85 for Jam and 164 for Bel. But more important is that under stress more than 80% of Mn is found in the fractions below 5kDa.

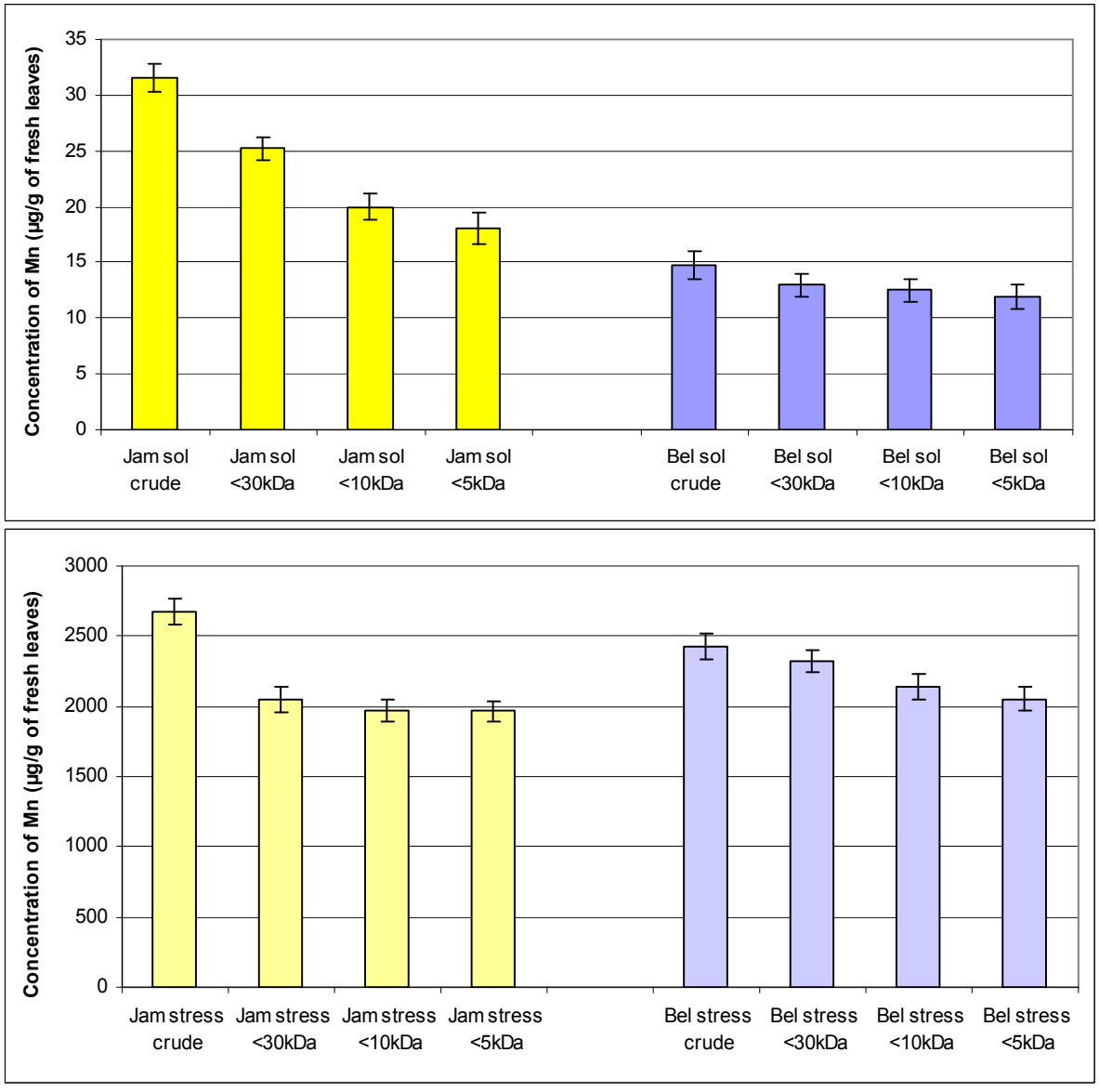


Figure 39. Manganese concentration in crude extracts of Jam and Bel and their ultrafiltrates in fresh plant leaves grown in the nutrient solution (sol) and exposed to 800 µM of manganese (stress).

Figure 40 shows the concentrations of Mg, Fe, Zn and Co in crude extracts and of Jam and Bel stressed and not stressed and in their ultrafiltrates studied by ICP-MS off line.

The observed behaviour does not reveal many interesting features. Just Bel under Mn stress lets Mg go up distinctly and Jam under Mn stress shows increasing Zn concentration which mainly occur in the higher molecular weight region – ultrafiltration in this case shows the Zn concentration decreasing with decreasing filter cut-off, more pronounced than in other cases. Table 10, for a better survey, shows the percentage of Mn in the <5kDa fraction referred to the crude extract.

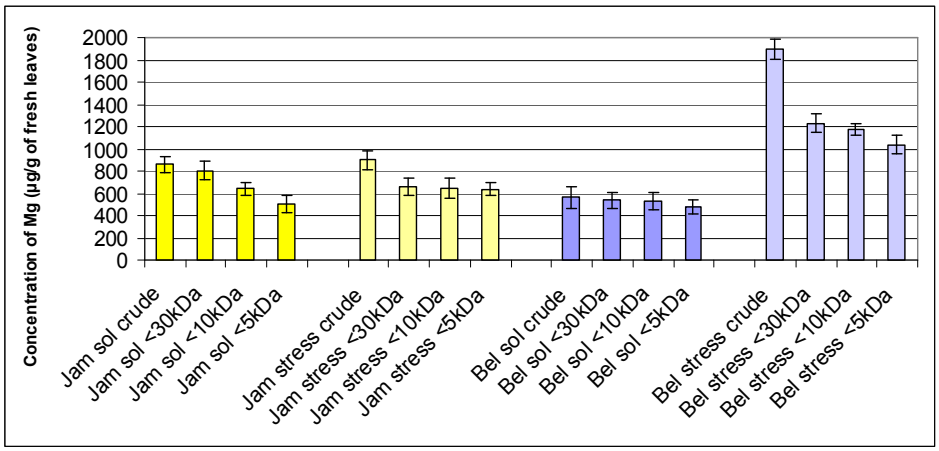
Samples	Mn55 (%)	Mg24 (%)	Fe54 (%)	Zn64 (%)	Co59 (%)
Jam sol <5kDa	58	58	67	80	73
Jam stress <5kDa	74	71	65	33	80
Bel sol <5kDa	81	86	98	91	100
Bel stress <5kDa	85	55	83	64	80

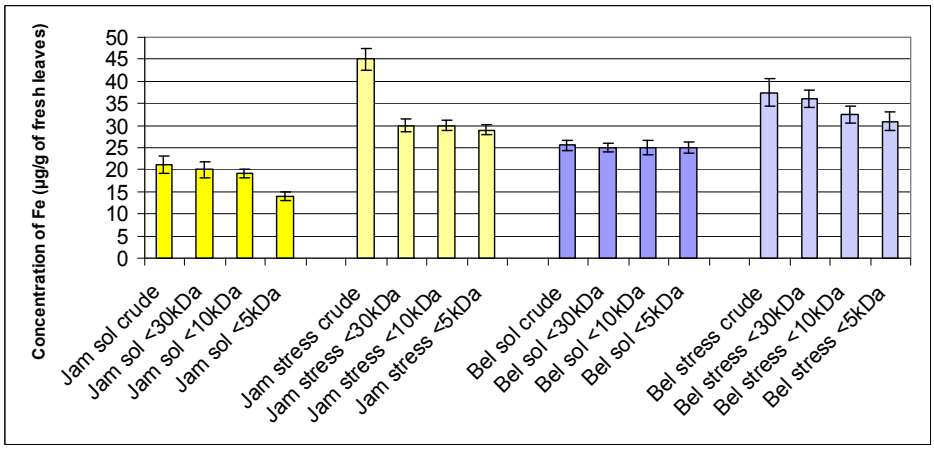
Table 10. Concentration of Mn, Mg, Fe, Zn and Co in % present as low molecular weight compounds

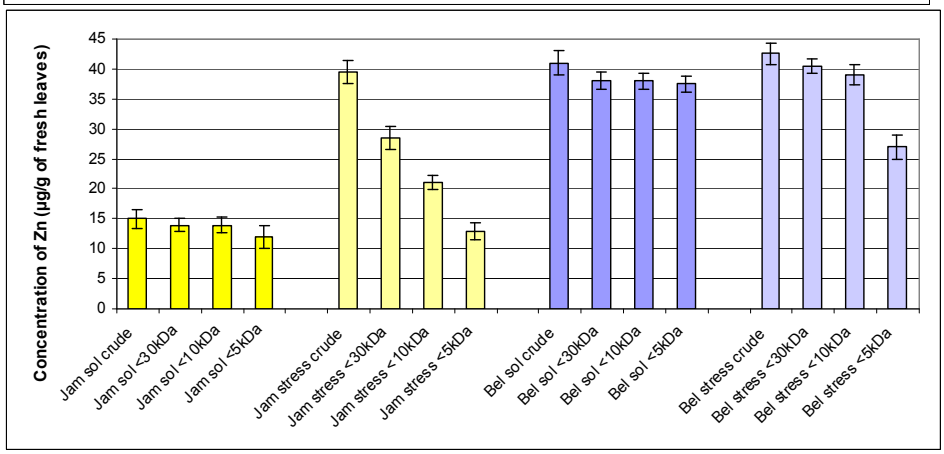
The ratio of the elements in crude extract between the plants stressed and not stressed is shown in Table 11. The results show that Mn stress has little effect on Mg, Fe and Zn, different in Jam and Bel, but no effect on Co.

Samples	Mg24	Fe54	Zn64	Co59
Jam stress / Jam sol	1.00	2.14	2.63	1.00
Bel stress / Bel sol	3.38	1.47	1.04	1.00

Table 11. Ratio of the elements in crude extracts between the plants stressed and not stressed.







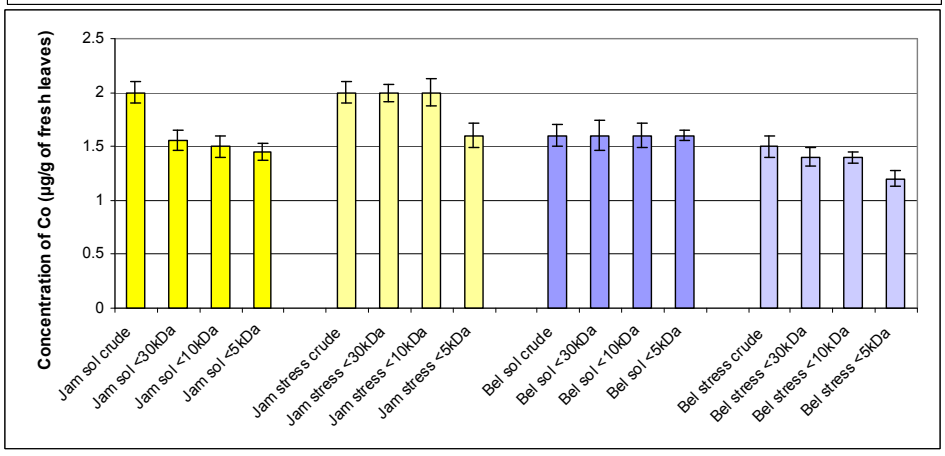


Figure 40. Mg, Fe, Zn and Co concentrations in crude extracts of Jam and Bel and their ultrafiltrates stressed and not stressed by Mn, by ICP-MS off line.

5.2.6. INAA and ICP-MS off line

INAA again was chosen to validate the results from ICP-MS shown before. The comparison of results on leave extracts (stress and no stress conditions) is shown in Table 12. The agreement between both methods is very good.

	Mn55 (ppm)		Fe54 (ppm)		Zn64 (ppm)		Mg24 (ppm)	
Samples	**INAA**	**ICP**	**INAA**	**ICP**	**INAA**	**ICP**	**INAA**	**ICP**
Jam (sol)	32.5	31.5	21.0	20.0	15.0	14.0	782.5	859.0
Jam (stress)	2873.5	2675.0	45.0	41.5	39.5	37.5	831.0	903.5
Bel (sol)	14.5	14.8	27.5	25.5	41.0	39.0	512.5	561.5
Bel (stress)	2567.0	2429.2	40.5	37.5	42.5	41.0	1756.0	1898.0

Table 12. Results obtained by INAA and ICP-MS for Jam and Bel (sol and stress). The errors, for ICP-MS were below 10% and that of INAA were estimated as 10-15%.

5.2.7. Extraction efficiencies of plants stressed and not stressed

The digestion of the fresh plant leaves by a microwave system is described in **4.14.** The result in mg total element content is set to be 100%. And taking the results from Table 12, the extraction efficiencies of the plants can be calculated and are found to be around 50% as shown in Figure 41.

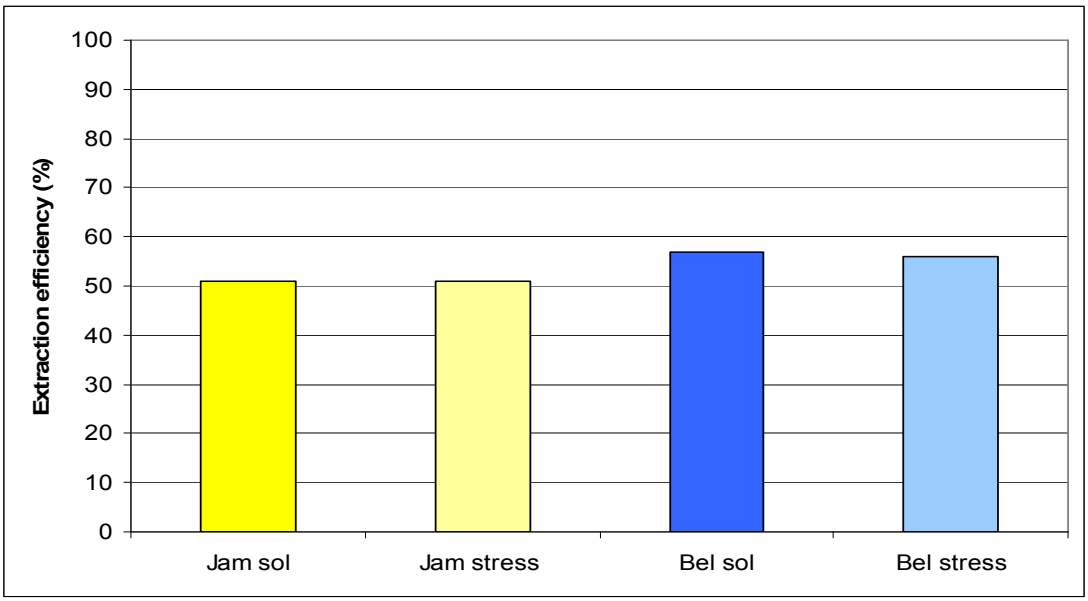


Figure 41. Extraction efficiency for the used brewing process to get the Jam and Bel leaf extracts.

5.2.8. Determination of the total protein concentration by the Bradford Reagent

The Bradford´s test for proteins shows a positive correlation between leaf extracts of plants in the basal nutrition solution (sol) and plant exposed to 800 μM of manganese stress, for both Jam and Bel crude extracts. Figure 42 shows the results. Most interesting is that Mn stress does not induce big changes of the protein content, at least not in the lower molecular weight fraction. In Bel there is some indication for Mn stress induced increase of high molecular weight (>30kDa) proteins. Another important result is that in Jam the lower molecular weight fraction (<5kDa) is much smaller than the >30kDa fraction, this difference not being too pronounced in Bel.

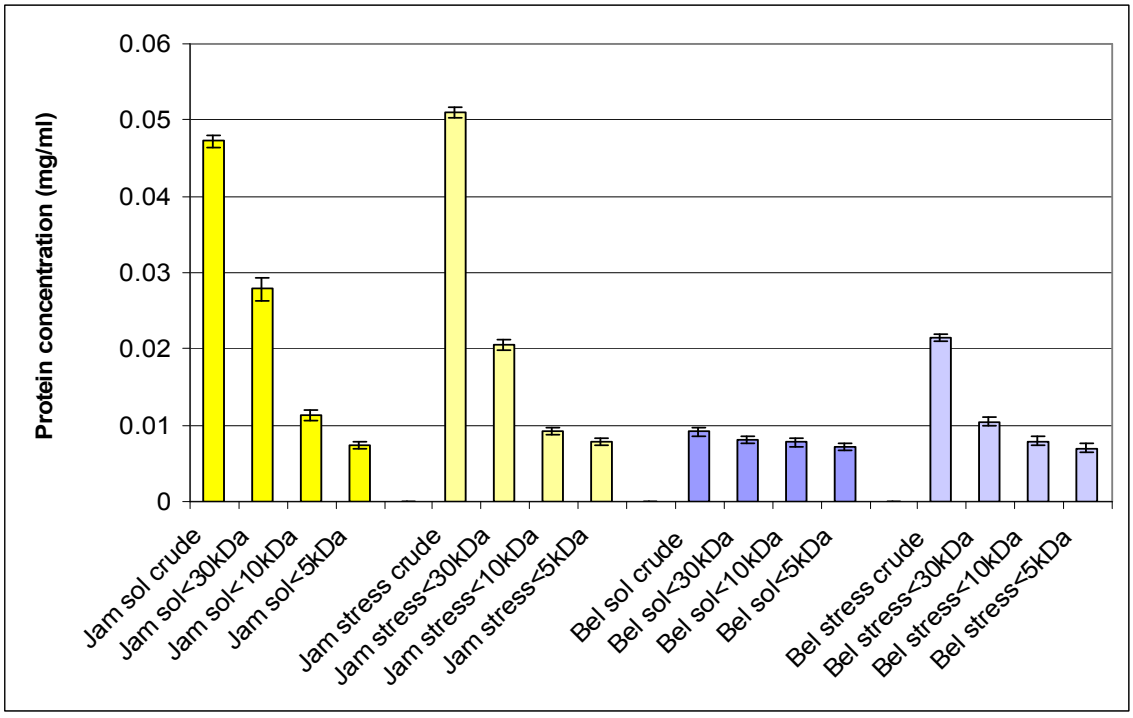


Figure 42. Determination of proteins in Jam and Bel extracts and their ultrafiltrates determined by the Bradford test.

5.2.9. Determination of sulfhydryl groups concentration by the Ellman´s Reagent

Figure 43 shows the sulfhydryl group concentration for Jam and Bel leave extracts and for their ultrafiltrates. The first interesting observation is that for stress and no stress conditions the concentration parttern by ultrafiltration follows closely that of the Bradford

result. A difference can be seen with high molecular weight fractions (>30kDa) for Bel which shows increasing SH- group concentration while decreasing protein concentration if stressed.

The second important observation is that stress does not induce SH- group formation such as were to be expected with the formation of phytochelatins – their production is not induced, which is in agreement with what is known from literature [28].

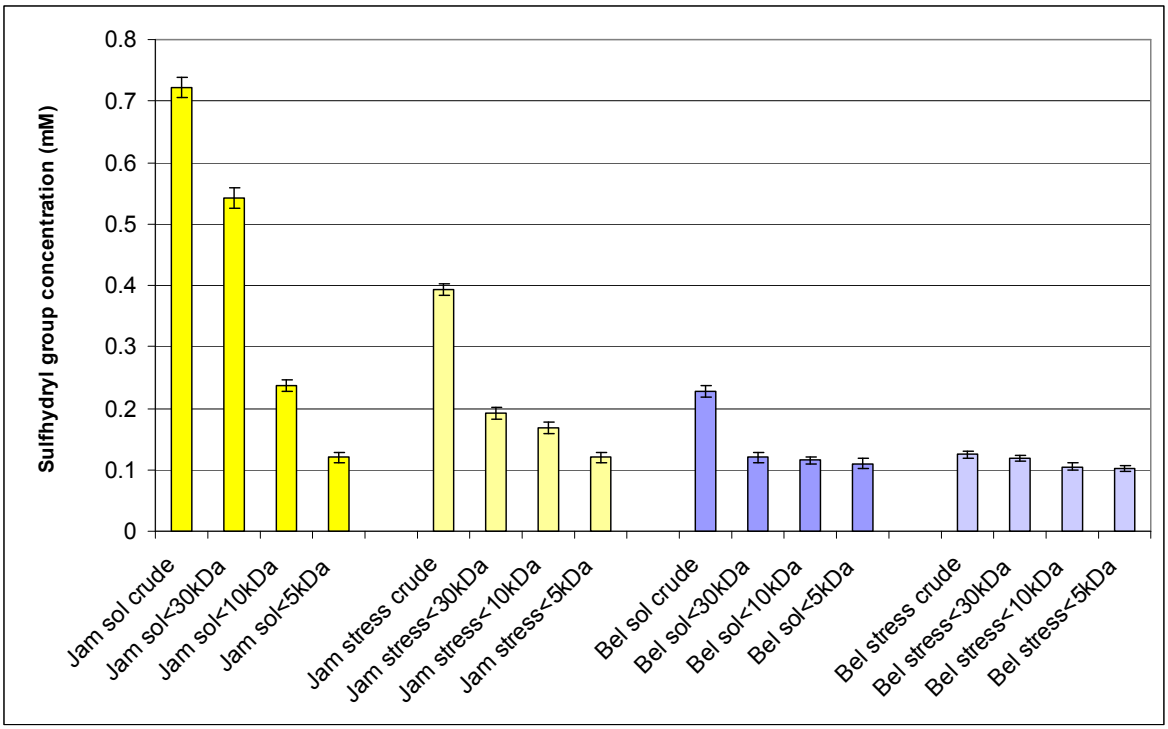
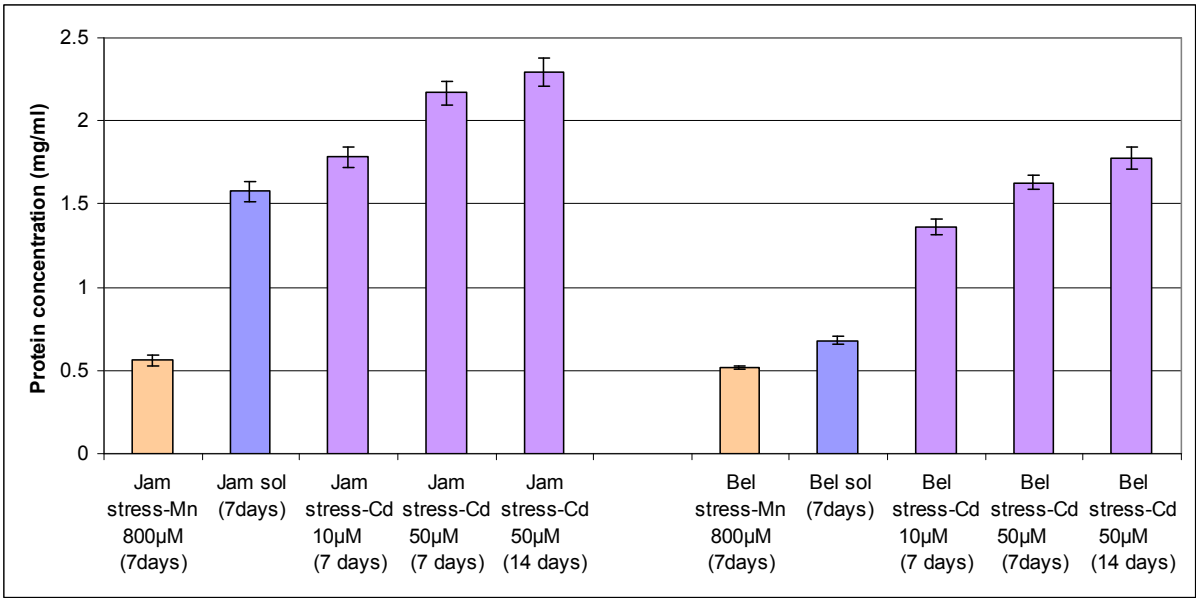


Figure 43. Determination of sulfhydryl group in Bel and Jam extracts and their ultrafiltrates detected by the Ellmans test.

The well-known phytochelatins induction by Cd independently is also observed with Jam and Bel, as Figure 44 reveals for the respective crude extract, where Jam and Bel were exposed to 10 and 50 μM Cd for 7 and 14 days. For comparison, results for Bel and Jam stressed by 800 μM Mn are given: Mn stress lets produce even less protein (and SH group) and thus does not induce phytochelatins production.



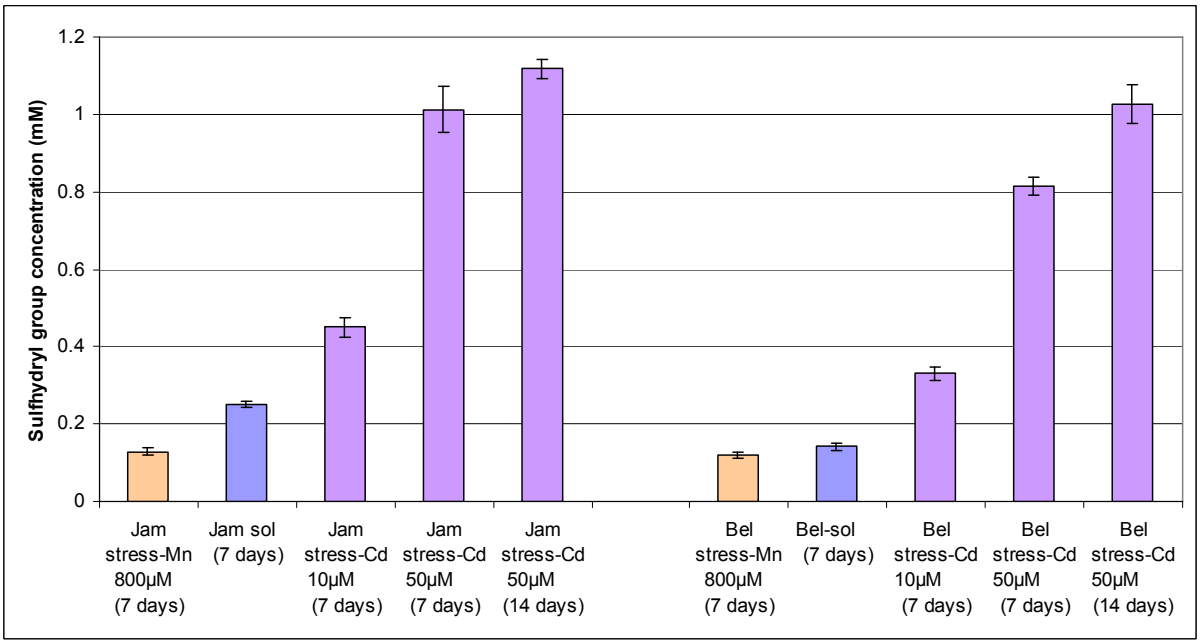


Figure 44. Determination of proteins and sulfhydryl groups by Bradford and Ellman´s test respectively, on crude extracts of Jam and Bel exposed to different concentrations of cadmium.

5.2.10. Separation of lower molecular weight compounds in medicinal plant extracts

As show in Figure 39, manganese species in plant extracts increase drastically after uptake of manganese through the roots. More than 70% of total manganese is found in the fraction <5kDa. For characterization of the respective species two strategies were developed.

The first one is based on size exclusion chromatography (SEC) coupled on-line to ICP-MS. The exclusion limit of the chromatographic column Polysep-GFC-P 2000 - SE2 is 10 kDa, and thus should be ideally suited to separate the lower molecular weight compounds further.

Extracts <5kDa of Bel and Jam (sol and stress) were injected into the HPLC (SEC2)-UV at 215nm and isocratic separation was performed using water as eluent. These conditions were chosen in order to avoid the risk of interactions between the sample material and the separation medium as much as possible. Regrettably, the results obtained with the SE2 column were not satisfactory just like mentioned before in section 5.1.7.

As a second strategy, a reversed phase column has been used for the characterization of the low molecular weight compound(s). Different eluents were tested for the optimization of the system. Methanol with different addition (20, 10, 5 and 0) of water/0.1%TFA was tested isocratically. Also different gradients were tested. However, the best results were obtained with water/0.1%TFA as the eluent under isocratic conditions.

The chromatograms of Bel and Jam extracts (stressed and in basal solution) <5kDa studied by HPLC (RP)-UV at 215 show some differences (Figure 45), and indicate organic acids, which are commonly found in plants. They were indentified by HPLC (RP)-UV and confirmed by HPLC (RP)-ESI-MS.

An interesting observation is that Mn stress lets increase the citric and maleic acid concentration as shown in Figure 45. The other peaks could not be identified, since ESI-MS did not show these peaks. Peaks at 3 ml elution volume are probably due to ionic compounds, together with Figure 37 perhaps also manganese salts.

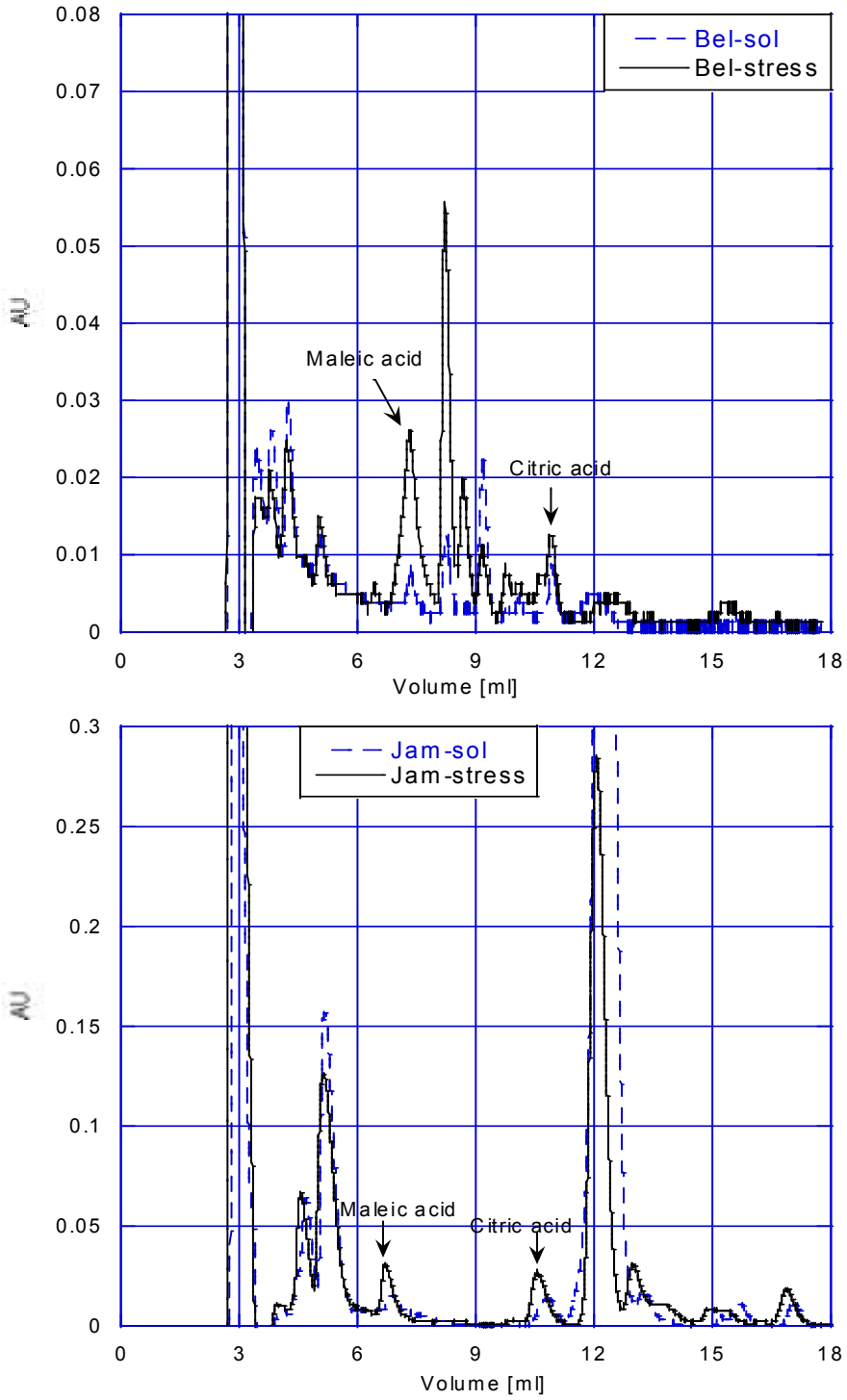


Figure 45. Chromatogram of the <5kDa extracts of Jam and Bel exposed to 800 µM of manganese (continuous line) and Jam grown in basal nutrient solution (dotted line), by HPLC (RP)-UV at 210nm, isocratic separation, water/0.1%TFA as eluent, flow rate was 0.6 ml/min, 20µL sample were injected.

5.2.11. Plant extracts <5kDa studied by HPLC (RP)-ICP-MS

Jam and Bel ultrafiltrates <5kDa (sol and stress) were analysed by HPLC (RP)-ICP-MS and the results are shown in Figures 46 and 47.

Mn

Bel and Jam show one peak at the dead volume under no stress conditions under stress, Bel shows two peaks, Jam three, although the third seems to be broad, with some distortion. Peak intensity goes up by stress.

S

Jam shows one peak at the dead volume, with a slight shoulder, which disappears under stress. Its intensity goes down with stress. Some indication exists for two small peaks at around 3.1 min. Bel shows also one peak at the dead volume, which also disappears under stress where an indication of a broader slightly retained peak appears. Jam shows higher S-content than Bel. This result correlates with the results from the Ellman's test Figure 43.

P

There is only one peak, with a slight indication of a retained shoulder. With Jam, Mn stress lets increase the P content, but with Bel the situation is opposite.

Mg

For both Bel and Jam only one peak at the dead volume is observed, although some slight indication exists for some excluded compound.

Zn

For Bel, the Zn chromatogram does not change much with Mn stress, quite different from Jam, which does not show any peak at V_0, instead a structured retained one which splits to two under Mn stress.

Fe

Iron in Bel shows one peak at V_0 which is more retained under Mn stress and reduced in its intensity. In the position for that latter peak Jam shows its only peak which increases distinctly under Mn stress. This peak seems to be comprised of more than one compound.

Ni

With Bel two peaks, one at V_0, one retained are observed the intensity of which goes down under Mn stress. With Jam no peak is observed at V_0, but one with a running shoulder and slightly retained which does not change its position under Mn stress, but increases slightly its intensity.

Co

Cobalt in Bel shows mainly one retained peak the concentration of which decreases under Mn stress. Little amount of Co is also observed around V_0. In Jam there is only one peak slightly retained and structured, which looses part of its structure under Mn stress.

Cu

Without stress, Jam shows a slightly retained broad peak, but under Mn stress two distinct peaks, one at the dead volume and one retained, with an indication of an even more retained shoulder. Bel under basal conditions shows some structure which under stress changes to exhibit a broad retained band.

Sr

Sr is an interesting candidate. At no stress conditions there is almost no peak at the dead volume, but instead a retained peak. Under Mn stress this latter peak decreases with Jam and increases with Bel, both shifting more towards V_0.

As a general and interesting observation follows that Mn stress exhibits in most cases an effect on the concentration of other elements. Most elements studied here show up at V_0, which may be due to ionic (inorganic?) species, and in a slightly retained peak. More retained peaks could not be found. Under the chromatographic conditions (eluent water, stationary phase RP18) the slightly retained peaks therefore may be ascribed to yet very polar compounds, may be larger polar (charged?) complexes.

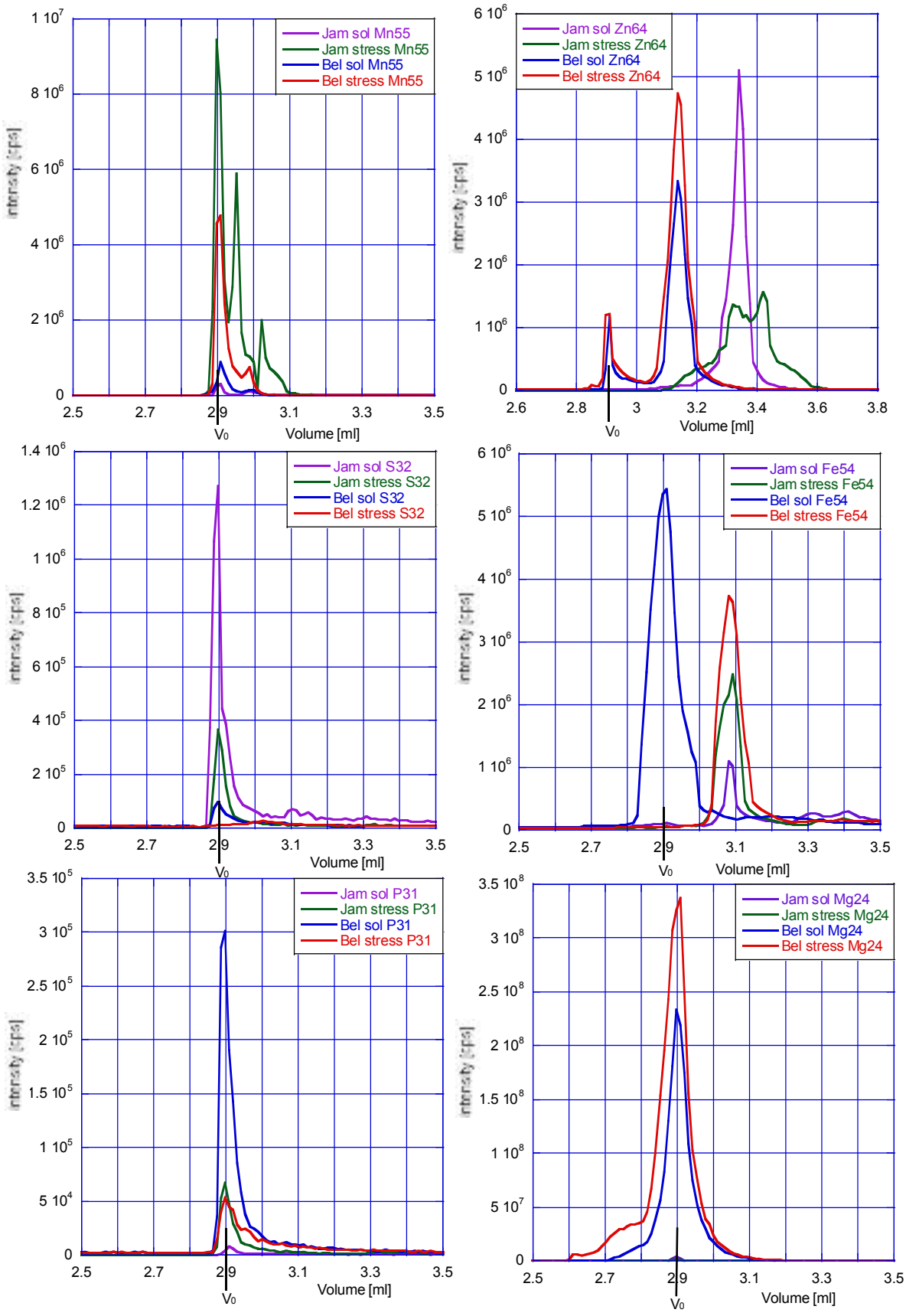


Figure 46. Elemental chromatograms of Jam and Bel (sol and stress) performed by HPLC (RP)-ICP-MS, isocratic separation, water/0.1%TFA as eluent, flow rate 0.6 ml/min, 20µL sample was injected.

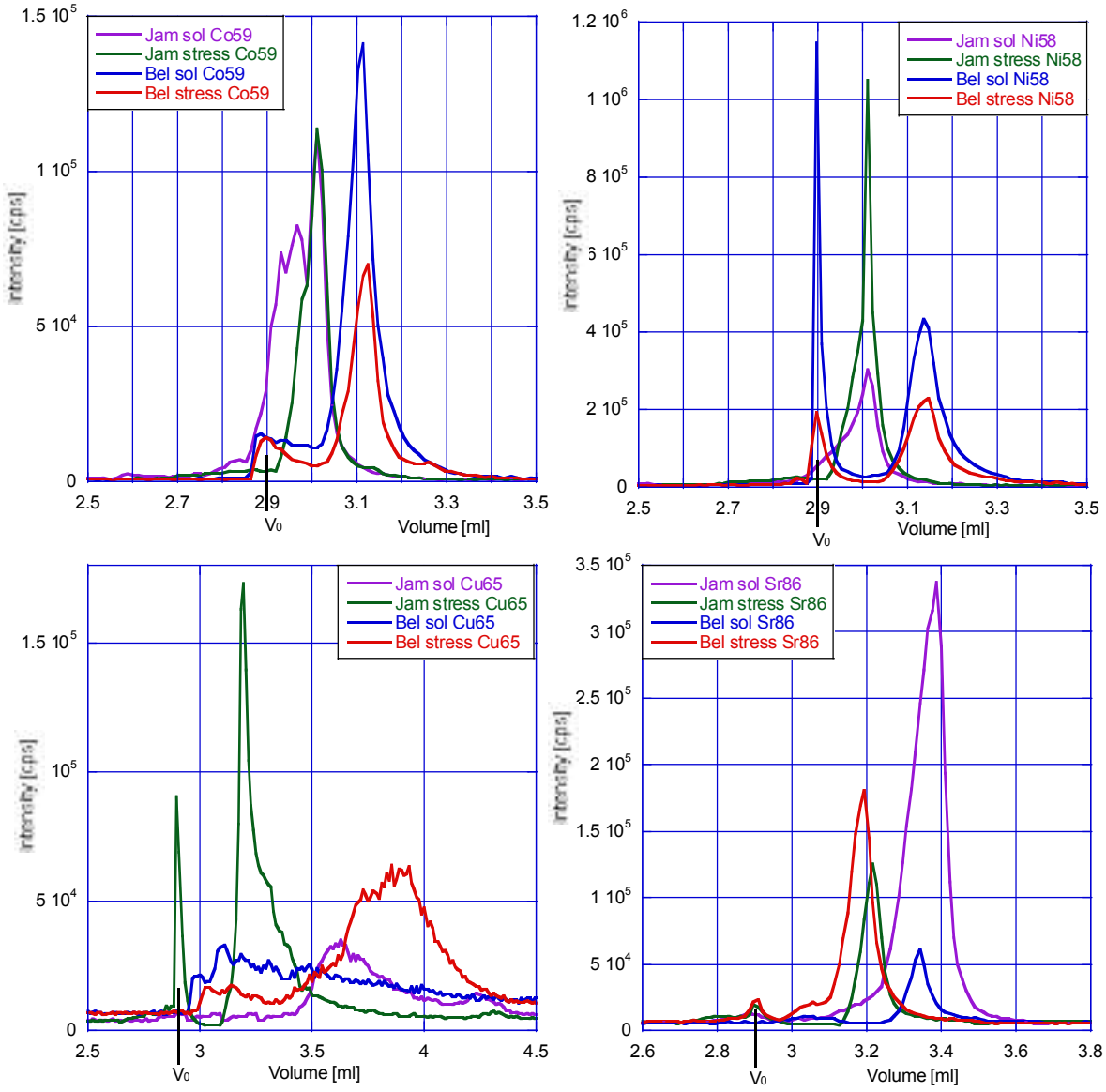


Figure 47. Elemental chromatograms of Jam and Bel (sol and stress) performed by HPLC (RP)-ICP-MS, isocratic separation, water/0.1%TFA as eluent, flow rate 0.6 ml/min, 20µL sample was injected.

5.2.12. Analysis of lower molecular weight compounds by MALDI-TOF-MS

Like the respective experiments with tea, Jam and Bel extract fractions <5kDa, MALDI-TOF-MS did not give valuable results too.

5.2.13. Analysis of lower molecular weight compounds by HPLC (RP)-ESI-MS

The <5kDa extracts also have been studied by HPLC (RP)-ESI-MS. In the ESI-MS mass spectra only the peak at V_0 could be observed, therefore only this first peak of each sample (Jam and Bel - sol and stress) has been analysed in negative mode, positive mode did not give sufficiently intense peaks. Interestingly, the mass spectra of sol and stress for both plants were quite similar as shown in Figures 48, 49, 50 and 51.

The mass spectra of both plants stressed and not stressed are also similar with mass spectra of teas (see Figures 28, 29 and 30), the same m/z peaks and periods were detected. Differences between stress and no stress conditions mainly appear at the high mass side.

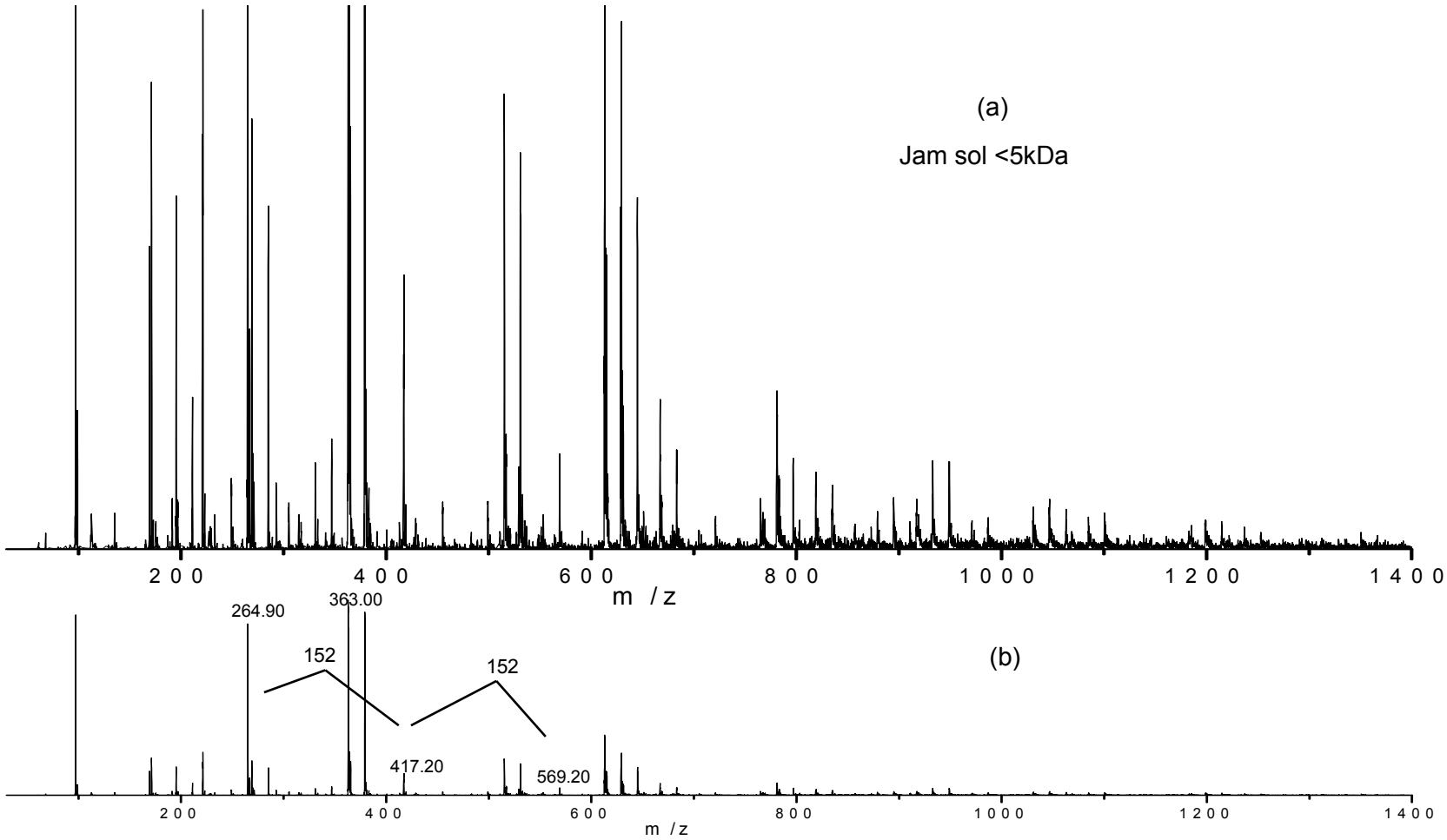


Figure 48. (a) Jam sol <5kDa mass spectra (4 times higher) of the first peak studied by HPLC (RP)-ESI-MS, negative mode, isocratic separation, eluent was water/0.1% TFA, flow rate was 0.6 ml/min, 20µL sample was injected. (b) Jam sol <5kDa mass spectra.

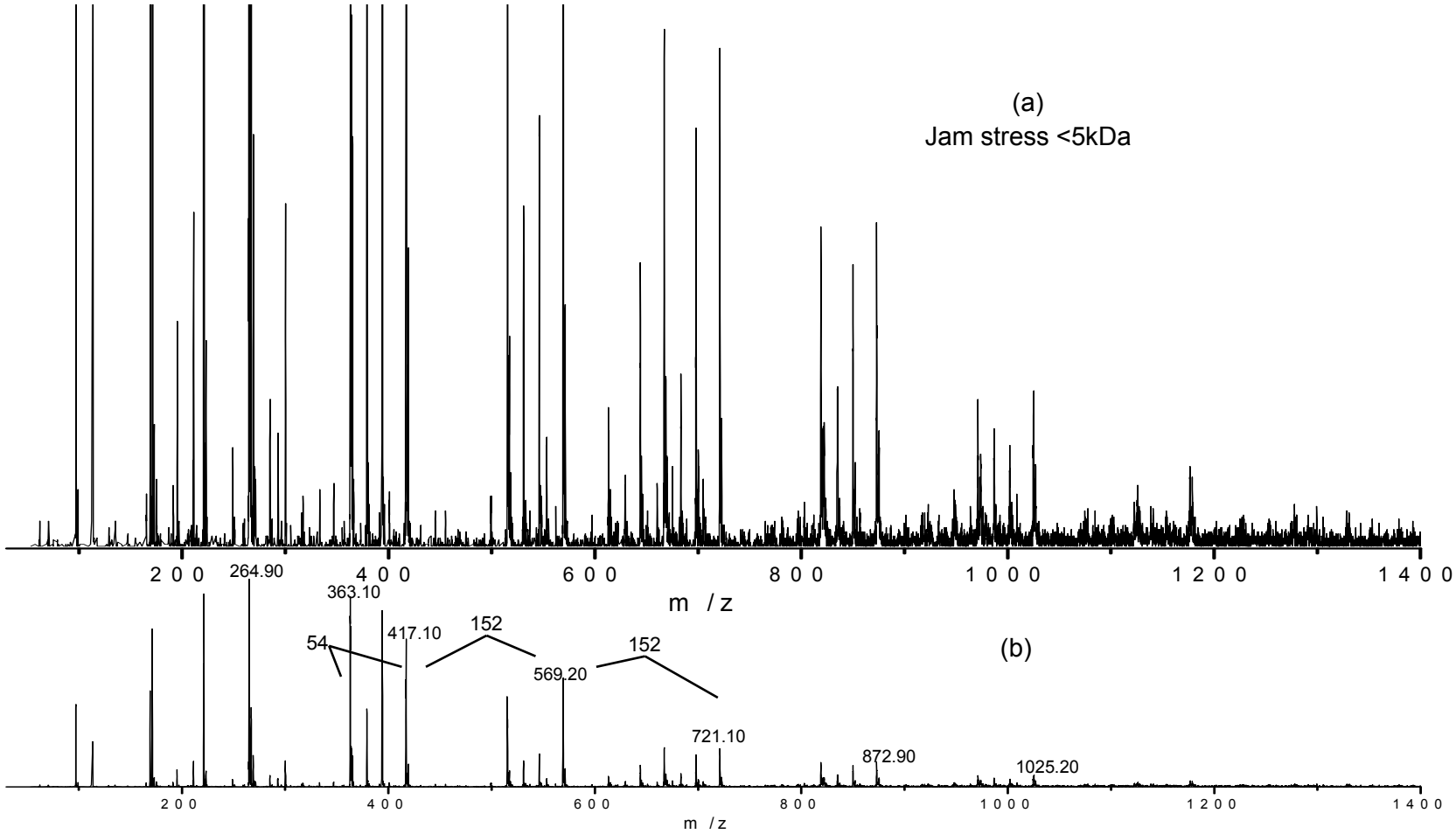


Figure 49. (a) Jam stress<5kDa mass spectra (4 times higher) of the first peak studied by HPLC (RP)-ESI-MS, negative mode, isocratic separation, eluent was water/0.1% TFA, flow rate was 0.6 ml/min, 20μL sample was injected. (b) Jam stress<5kDa mass spectra.

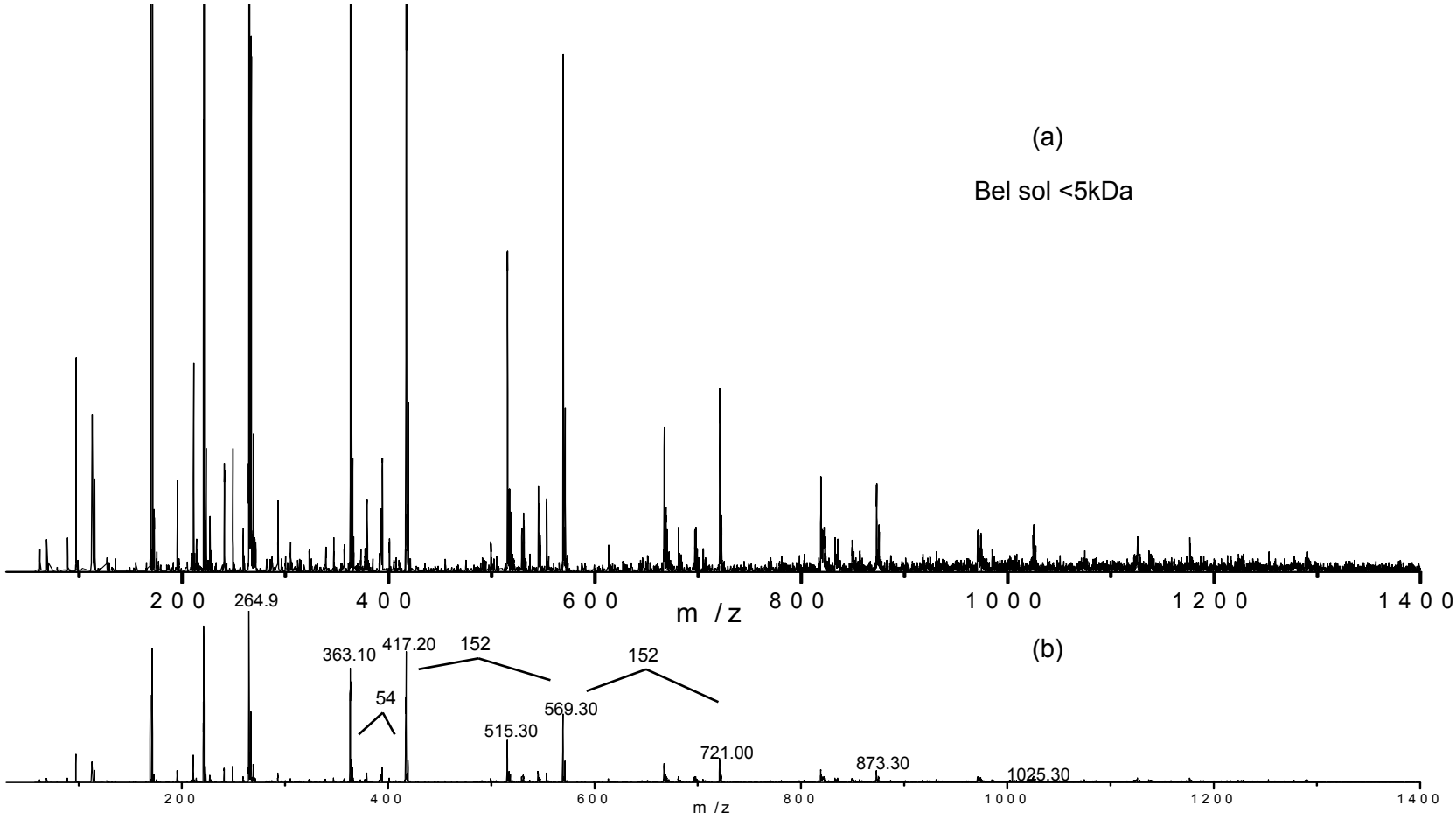


Figure 50. (a) Bel sol<5kDa mass spectra (4 times higher) of the first peak studied by HPLC (RP)-ESI-MS, negative mode, isocratic separation, eluent was water/0.1% TFA, flow rate was 0.6 ml/min, 20µL sample was injected. (b) Bel sol <5kDa mass spectra.

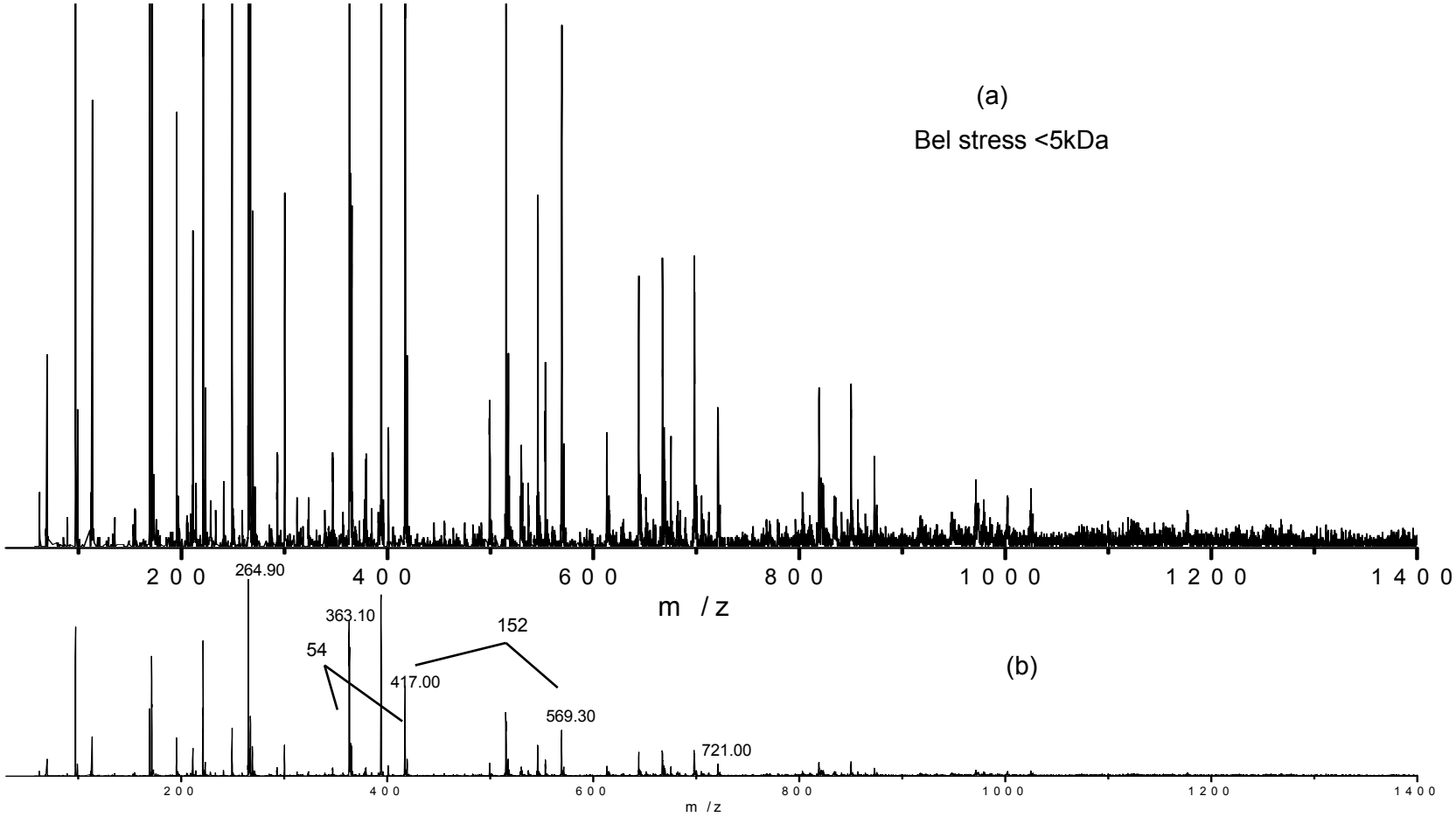


Figure 51. (a) Bel stress <5kDa mass spectra (4 times higher) of the first peak studied by HPLC (RP)-ESI-MS, negative mode, isocratic separation, eluent was water/0.1% TFA, flow rate was 0.6 ml/min, 20µL sample was injected. (b) Bel stress <5kDa mass spectra.

5.2.14. Analysis of higher molecular weight compounds by HPLC (SE)-ESI-MS in the case of crude extracts from leaves of Jam plants grown under stress.

Jam grown under Mn stress conditions shows a marked decrease of the protein as well as of the sulfhydryl group concentration when passing from the crude estract by the <30kDa to the <10kDa fraction, Figures 42 and 43. From this the conclusion must be drawn that there is some amount of larger compounds present, may be proteins which develop under stress. Therefore, SE1-HPLC was performed, with UV-detection at 280 nm. Figure 52 shows the chromatogram.

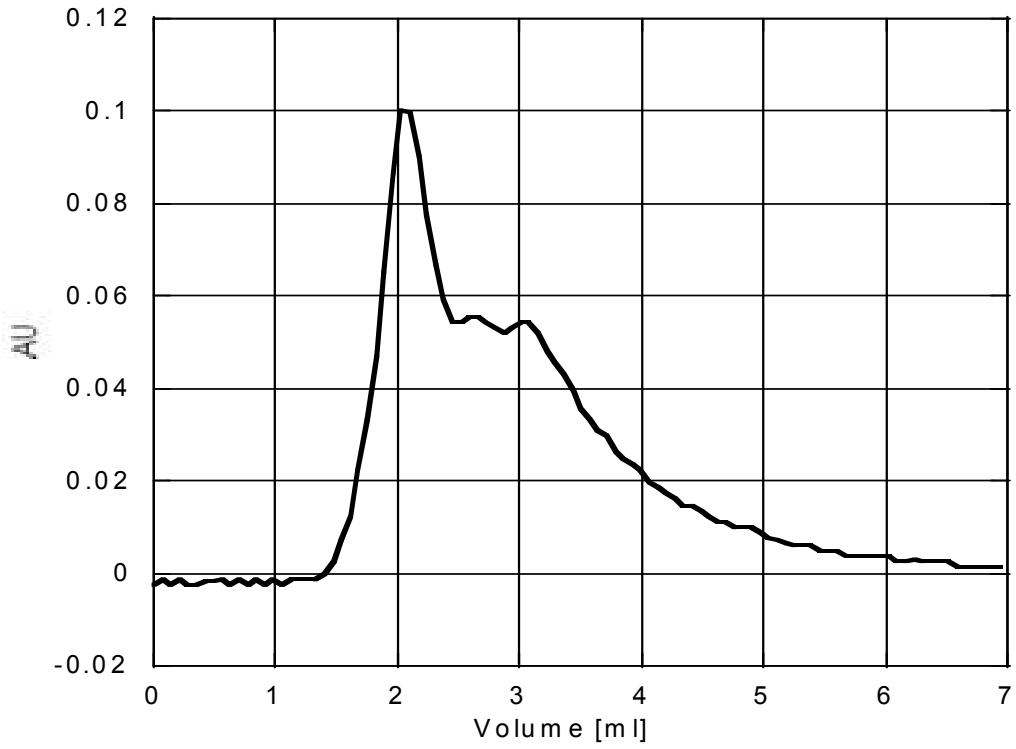


Figure 52. Size exclusion chromatogram detected at 280 nm of Jam crude extract from plants grown under Mn stress.

There is a marked peak at larger masses, presumably beyond 30 kDa, according to the calibration curve Figure 5. Hence, ESI-MS was performed and the mass spectrum in negative mode corresponding to this peak is shown in Figure 53. The mass spectrum mainly shows small fragments below 300 Da, which without further information cannot be interpreted.

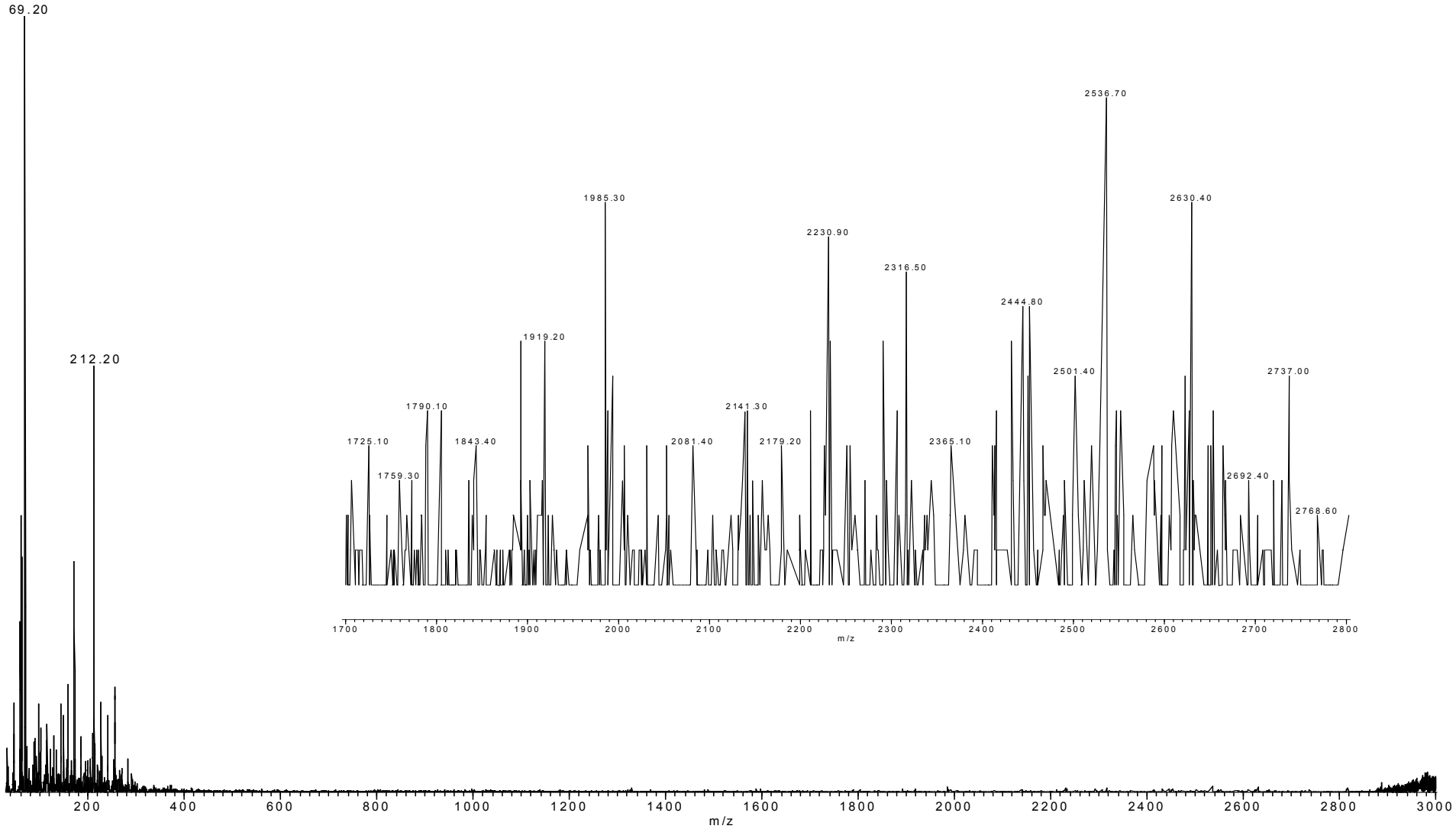


Figure 53. Negative ion mass spectrum of the peak around 2ml in Figure 52 over the whole instrumental range 30 to 3000 Da, and inlarged insert for the range 1700 to 2800 Da.

6. Final Discussion

Comparison between tea and leaf extracts from plants grown under hydroponic no stress conditions

Neutron activation and ICP-MS offline analyses have been performed to determine the elemental concentration in tea and in leaf extracts; the results from both methods presented in sections 5.1.3 and 5.2.6 agree reasonably.

In chapter 5.1.7 fractions <5kDa of standardized tea preparations have been studied by SE and RP-HPLC with UV and with element specific detection. The results may be compared to the results obtained from such fractions of leaf extracts from Bel and Jam grown in hydropony, under no stress conditions. The comparison of the UV detected chromatograms from Figure 23 and Figure 45 show of course citric and maleic acid (in addition identified by their mass spectra), but many other peaks, which have not been identified. Important also is that at V_0 an intensive peak is observed. Under the chromatographic conditions (water as eluent with 0.1% TFA) this intensive peak must be ascribed to highly water soluble and polar compounds or ions.

RP-HPLC with ICP-MS detection then should show for some relevant elements whether they are all found around V_0 or eventually retained. The corresponding figures are Figures 25 and 26 for the tea extracts and Figures 46 and 47 where the no stress (sol) chromatograms are to be considered here. Figure 54 compares the results from these figures for Bel and Jam with respect to the elemtns Mn, Zn, Fe, Cu, Co, and Ni. The two different preparations (tea = hot water extract and ultra turrax assisted extraction of leaves) show some differences with jam, and there in particular with the elements Ni and Zn.

Important to note: no elements could be detected at this level of sensitivity at retention volumes higher than about 3.5 ml. Element peaks at retention volumes higher than 2.9 ml means of course, that these are not pure elemental ion peaks. Such peaks must be ascibed to some complexes, may be inorganic or organic up to smaller proteins, within the limit of the ultrafiltration (< 5kDa).

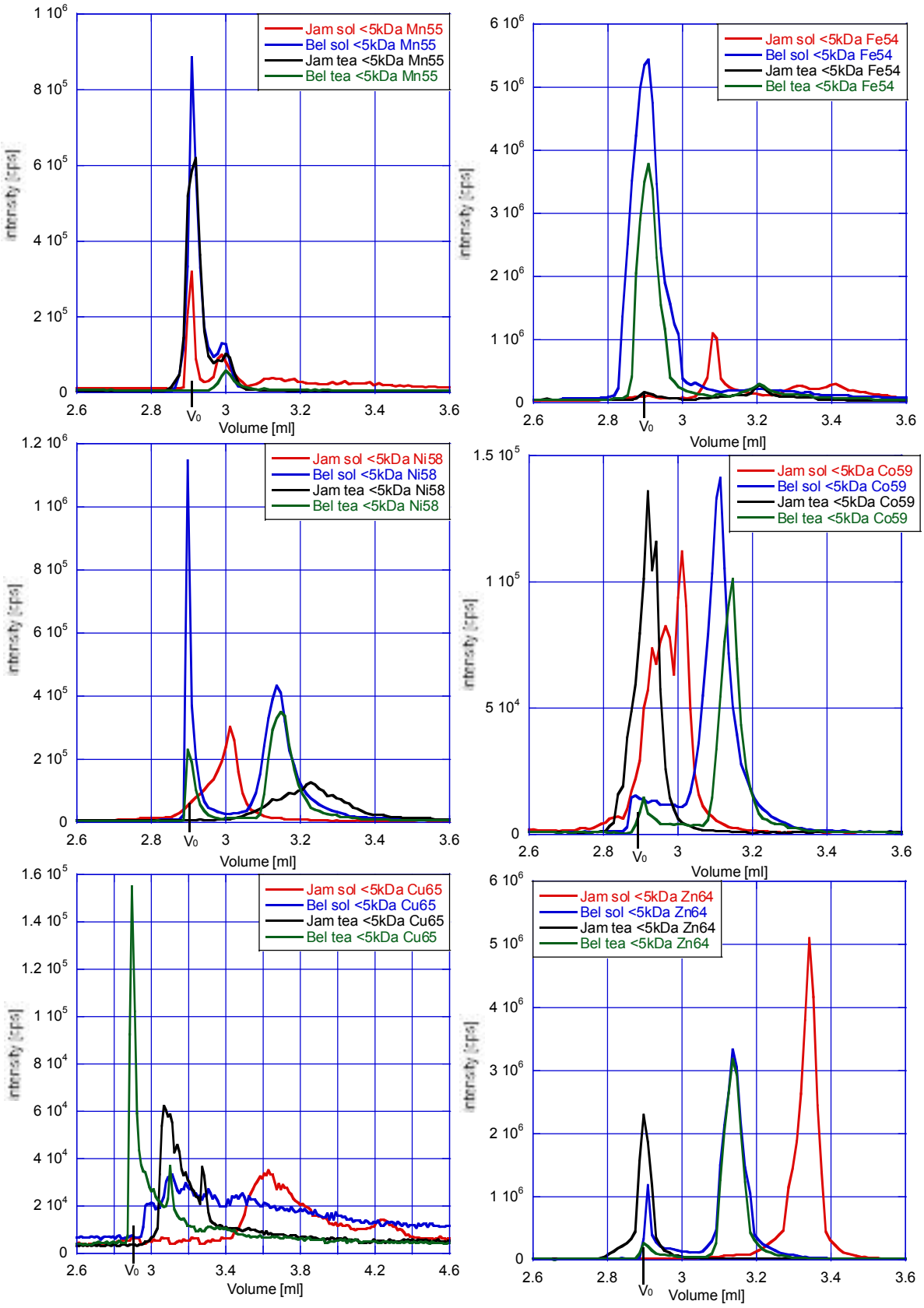


Figure 54. Bel and Jam chromatograms of Mn, Zn, Fe, Cu, Co, and Ni from two different preparations (tea = hot water extract and sol and Ultra-Turrax assisted extraction of leaves).

Figure 55, derived from data already presented in the Figures 19 and 38 shows HPLC SE1 chromatograms detected by ICP MS for Mn, Fe, Zn, and Sr for Bel and Jam teas and crude extracts at no stress conditions. Bel and jam chromatograms are different for all four elements (they are from different plant families!) but tea and extract do not differ essentially. Regrettably, in the case of tea and no stress grown plant leaves the intensities of peaks in the excluded range of the SE1-HPLC are too low as to be detected by ESI-MS.

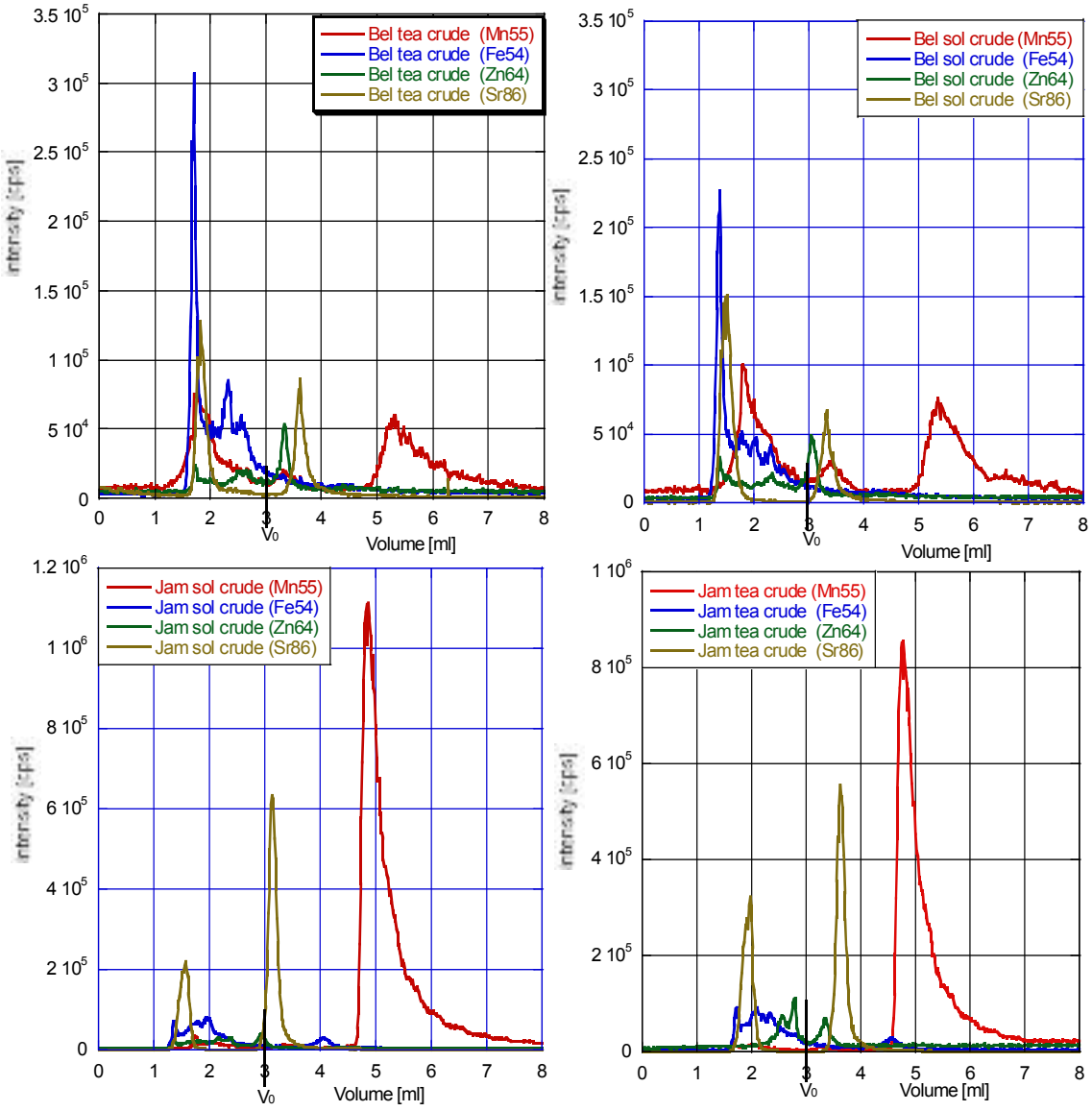


Figure 55. Jam and Bel (tea and sol) crude extract chromatograms obtained by HPLC (SE1)-ICP-MS, eluent was amonium acetat buffer, flow rate was 0.2 ml/min, 20µL of sample were injected.

For further comparison of tea and hydropony grown plant leaf extracts the respective mass spectra of the first peak at V_0 in RP-HPLC are considered, Figures 29, 30 and 48, 50, for Bel and Jam. For the tea extracts it has already been mentioned that mass spectra consist of

blocks which are separated by $\Delta_{m/z}$ = 152. Within the blocks peak series are observed at $\Delta_{m/z}$ = 54, 35 and 18. Besides this structure not much can be detected. In Bel (sol) and Jam (tea), m/z = 264.9 is the most intensive peak. In Bel (tea) m/z = 249.1 is even more intensive, opposite to Jam (tea) where this peak is very weak. Jam (sol) reveals two strong lines at m/z = 363 and 379, much more intensive than in Jam (tea). If the general peak pattern within the periods of $\Delta_{m/z}$ = 152 is studied one realizes that the peaks within the periods always are there but that the pattern may be very different when compared in sol and tea preparations – see for example for bel the group of lines around m/z = 400. For Jam the pattern differences are much less.

In addition the results from the Ellman´s test and from the Bradford test regarding sulfhydryl groups and proteins may be compared. It appears from comparison of the Figures 20 and 42 that the protein content in the crude extract of Jam is considerably higher than that in tea, but that this difference levels off after ultrafiltration to <5kDa. Bel shows a similar behaviour, with differences between the crude extract and the tea being less. The sulfhydryl concentration is comparable in tea and crude extracts for Bel but for Jam is much larger in the crude extract than in the tea. After ultrafiltration to <5 kDa again there is no big difference. As a conclusion, relatively small proteins or polypeptides such like the phythochelatins are yet present after ultrafiltration.

Now the big question arises to which compound(s) the (periodic) mass spectra can be ascribed. The periodic structure together with the intra-period structure allows two interpretations:

1. The whole spectrum is to be ascribed to one larger compound which fragments by stepwise loosing subunits in steps which sum up to a subunit´s m/z = 152, or
2. The spectrum is due to a series of homologuous compounds of different lengths.

Since proteins/polypetides are present in the studied solutions a possible interpretation would be a small protein or a series of homologuous proteins/polypetides. In literature despite of the ample work on phythochelatins I found only one publication [107] where a naturally occurring dipeptide of 2,4-diaminobenzoic acid (M = 152) is described for the first time. Its UV spectrum is reported to show two fused maxima at 363.8 and 336 nm.

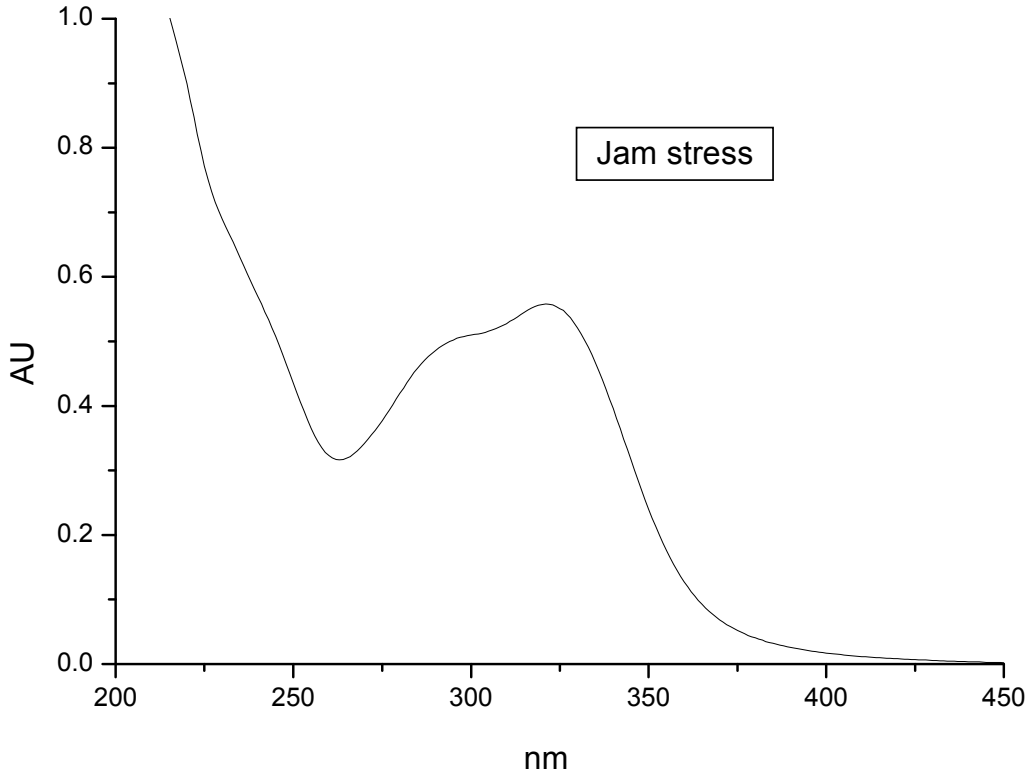


Figure 56. UV absorption of the first peak of Jam stress <5kDa at V_0 in the RP-chromatogram.

The UV spectrum Figure 56 of the first peak of the ultrafiltrated extracts of Jam (stress) at V_0 in the RP-chromatogram also shows two fused peaks at about 280 and 345 nm, thus indicating compounds with very probably aromatic units. Despite of all this similarity we were not able to mount a hypothetic structure of a compound that might give the observed mass spectrum.

In addition, it is astonishing that the mass spectra of the teas of all three compounds show the same basic structuring.

Another principally possible option is lysis products from the cell walls, which consist of sugar derived acids, etc. The relatively high abundance of these structures in plants would explain why the observed mass spectra are showing their structure relatively pure, even when obtained at the dead volume of of a chromatogram where all really polar compounds would appear together and thus would give an overlay of presumably many compounds. Regrettably we again could not reproduce the observed peak blocks and series, with such structures. Various trials to get a MALDI-TOF spectrum did not give positive results so that we cannot decide between the two options pointed out above.

Bel and Jam leaf extracts from plants grown in hydropony under stress conditions

Mn stress during growth in a hydroponic system shows of course a strong direct effect on the Mn concentration in the plant. This was demonstrated in Figure 39 where both Bel and Jam show a strong encrease in the Mn concentration under 800 μM (≈44 mg/l) manganese stress in the nutrition solution. Interesting is that more than 80% of Mn is found in the lower molecular weight fraction <5kDa where it appears at 2mg/g fresh leaf, in both plants. Figure 40 then shows how Mn stress induces concentration changes of other elements: Bel reacts by Mn stress with increased Mg and Jam by encreased Zn concentration. HPLC-(RP)-ICP-MS and HPLC-(SE)-ICP-MS reveal (Figures 37 and 38) that Man is not only present in leaves of Bel and Jam as inorganic material; RP-HPLC shows retained peaks and SE-HPLC shows excluded or larger molecular weight compounds complexed or bound to Mn. This latter observation let us look for multiple charged m/z peaks in the upper mass spectral region of the ESI-mass spectrum in negative mode, see Figure 53. Some weak lines are observed in the high mass side of the spectrum; no such peaks could be detected in positive mode. Although the intensities yet are pretty low, four peaks fulfil the 1/z linearity required in case of a multiply charged series:

z	m/z (observed)
10	1790.1
9	1985.3
8	2230.9
7	2536.7

A linear regression of m/z yields

m/z = 48.2 + 17434 / z, with R=0.99997.

a plot against z-1 yields

m/z = 303.1 + 13435 / (z-1), with R = 0.99977.

A plot against z + 1 yields

m/z = -205.4 + 21930 /(z+1), with R=0.99998.

Considering the intersect, which should be near zero and the R-values, z between 10 and 7 interprets best the peaks under discussion which then means that there is a macromolecule of about 17400 present in the crude extract from leaves of Mn stressed plants. Such a mass is consistent with the observed protein and sulfhydryl group distribution. Since

ICP also shows Mn intensity in the excluded mass region of SE1 it will be worthwhile having a closer look to this mass range by a better worked out size exclusion chromatography on higher concentrated crude stressed and not stressed samples.

Interesting is that Mn stress does not show a considerable effect on the <5kDa protein fraction (see Figure 42). The effect of Mn stress on the sulfhydyl group concentration is even negative in the <5kDa fraction (see Figure 43). Since for some elements, and in particular for Cd, literature reports strong increase of phythochelatin production as a self-protection mechanism [28] the reactions of Jam and Bel on 800 μM Mn stress was compared to the reaction of 10 and 50 μM Cd stress in the nutrition solution over 7 and 14 days. In Figure 44 both the protein and the sulfhydryl group concentration increase highly significantly over the respective Mn stress induced values. This finding agreees with the fact that phytochelatin production under metal stress never has been reported in literature for Mn. This most probably is due to the fact that Mn does not show high affinity to sulfur and therefore to SH groups like it is the case with all the elements that induce phythochelatin production.

7. Final conclusion and outlook

In this thesis three Brazilian plants have been investigated for their elemental content as it shows up in fresh leaf extracts and in the classical medicinal tea preparation. A special focus was on Mn stress in the nutrition solution and its effect on the leaf and tea concentration of other elements, as well as on forms of binding of Mn to organic compounds. While concentrations successfully could be determined by ICP-MS, validated by neutron activation analysis, binding forms could not be worked out, although several correlations between Mn and other peaks under different chromatographic conditions could be shown up. An interesting goal in future would be to further correlate HPLC-(SE) ICP-MS peaks of Mn to respective ESI –MS peaks. This could be achieved by developing a pre-concentration method to achieve the necessary much higher sensitivity in ESI-MS in order to see those higher molecular weight compounds, which iso-elute at least with Mn, as shown in this thesis.

From the standpoint of human toxicology the results achieved in this thesis are highly important. The daily allowed intake of an adult is 2 to 3 mg. On the basis of this value the results of Mn concentrations in leaves cultivated in Manaus, Brazil, must be seen critical. Table 5 shows a content of about 350 ppm Mn in dry leaves of Jam – taking into account the water content then this means about 60 ppm Mn in fresh leaves. Since people not only use Jam in a form of a medicinal tea but also use leaves for the preparation of their meals in case

of certain diseases then about 50 g of Jam leaves already mean a daily intake of 3 mg, the estimated allowed maximum daily rate! The problem is further stressed by the results presented in Figure 39 where under Mn stress conditions Jam shows a total Mn concentration of about 2.5 mg per 1g of fresh leaves! Even when grown in a standard nutrition solution the Mn concentration in Jam is found to be 30 μg per 1 g of fresh leaves, see Figure 39, which amounts to 1.5 mg in 50 g fresh leaves, which people use easily in cooking. So Jam and to a lesser amount Bel as a medicinal plant must be rediscussed under the Brazilian condition where soil Mn concentration is high, in almost any place.

8. List of chemicals, materials and equipments

Chemical	**Purchased from**
Ellman´s Reagent (5-5-Dithiobis-2-nitrobenzoic acid)	Acros Organics
Sodium phosphate	Acros Organics
L(+)cysteine	Acros Organics
Maleic acid 99%	Acros Organics
Lecaton, 2–5 mm particle size	Fibo Exclay
Tartaric acid	Fluka
Citric acid monohydrate	Fluka
Trifluoroacetic acid (TFA)	Fluka
Lysozyme	Fluka
$Ca(NO_3)_2\ 4H_2O$ / $(NH_4)\ H_2PO_4$	Merck
KNO_3 / $MnCl_2\ 4H_2O$	Merck
H_3BO_3 / $FeSO_4\ 7H_2O$	Merck
HCl P.A. / HNO_3 P.A.	Merck
$CuSO_4\ 5H_2O$ / H_2MoO_4	Merck
$Co(NO_3)_2\ 6H_2O$ / NaOH	Merck
$ZnSO_4\ 7H_2O$ / $MgSO_4$	Merck
EDTA-di-Na-Salz	Merck
ICP multi-element standard solution IV	Merck
MilliQ water	Millipore system
CelLytic P	Sigma-Aldrich
Protease Inhibitor cocktail	Sigma-Aldrich
Bradford Reagent	Sigma-Aldrich
(+) Catechin hydrate	Sigma-Aldrich
Bovine Serum Albumin (BSA) Standard	Sigma-Aldrich
Gallic acid monohydrate	Sigma-Aldrich

Material	**Purchased from**
Prontosil 60-10-diol (SE1)	BISCHOFF
Prontosil 120-5-C18- AQ 50µm (RP column)	BISCHOFF
(0,5-10 µl ,100-1000 µl) pipettes	Eppendorf
Filters (regenerated cellulose membrane - ultracel series): 5 kDa (Ultrafree-MC PLCC Centrifugal Filter Unit), 10 kDa (Ultrafree-MC PLGC Centrifugal Filter Unit), 30 kDa (Ultrafree-MC PLTK Centrifugal Filter Unit)	Millipore
Polysep-GFC-P 2000, 300 x 7,80mm (SE column)	Phenomenex

Equipment	**Purchased from**
Centrifuge 5417R	Eppendorf
ELEMENT 2 (ICP-MS)	Finnigan MAT
Ultra-Turrax T18 basic	IKA Works, INC.
Milli-Q® Ultrapure Water Purification Systems - Milli-Q Gradient System (A10)	Millipore
Microwave	Perkin Elmer
UV/VIS spectral photometer Lambda 2	Perkin Elmer
HPLC system	Perkin Elmer
API 150EX (ESI-Q-MS)	Sciex/Perkin Elmer
PlasmaQuad3	VG Experimental

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