

DETERMINATION OF IODINE AND ITS COMPOUNDS IN IODINE ENRICHED PLANTS

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Doctoral Dissertation
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DOLOČANJE JODA IN NJEGOVIH SPOJIN V Z JODOM
OBOGATENIH RASTLINAH

Doktorska disertacija

Supervisor: Prof. Dr. Vekoslava Stibilj

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On ne doit rien craindre dans la vie – il suffit de comprendre.
Marie Skłodowska-Curie

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Abstract

Iodine is an essential element for humans and other mammals, important for normal growth and development. It is a component of thyroid hormones, which regulate metabolism and thus many important biochemical reactions. The main natural iodine source is seafood but the majority of iodine comes into the body by iodized salt, which has been introduced to reduce iodine deficiency disorders. However, the consumption of salt must be reduced and new/additional sources of iodine for human nutrition must therefore be found, one of which could be iodine enriched plants.

However, before such plants can be introduced into nutrition, it is important to know the iodine content, in order to control iodine intake. In the first part of the thesis, therefore, a method for iodine determination by ICP-MS after alkaline microwave extraction was optimized. Firstly, Schöniger combustion was compared to microwave extraction, which was chosen for further work. The temperature program and ratio between water and tetramethylammonium hydroxide (TMAH) were optimized in the sample preparation step. For iodine determination, the concentration of TMAH in the measured solution was optimized, external calibration was compared to the standard addition technique for quantitative iodine determination and the need to use an internal standard was evaluated. In addition to evaluation of LODs, linearity and repeatability, the independent method k_0 -INAA was used to confirm the trueness of the obtained results, because of the lack of biological materials with certified iodine value.

Due to possible differences in the bioavailability, mobility and toxicity of different species of the elements, speciation studies are also important. Thus, a method for iodine speciation by HPLC-ICP-MS was also optimized, with special interest in the extraction procedure and stability of the species. In the first step, the suitability of an ultrasonic probe for extraction of iodine species was evaluated and, because of some difficulties using the ultrasonic probe, a second step – optimization of the enzymatic extraction for iodine speciation analysis – was performed with a water bath with longitudinal shaking. Iodate interconversion into iodide was observed in the presence of the enzyme or plant material and conditions to prevent this transformation could not be found, so the method is appropriate for determination of MIT, DIT and inorganic iodine. The best enzyme for extraction of iodine species was selected, dissolving enzyme in water or in buffer was tested and the optimal temperature and time were selected. Column recovery, limits of detection, linearity, repeatability and procedure of recovery were also evaluated.

Iodine content was determined in pea, pumpkin and buckwheat sprouts, grown from seeds soaked in solutions of different iodine forms and combinations of iodine and selenium forms. Furthermore, iodine content was determined in different parts of pea plants, pumpkin seeds, buckwheat achenes and chicory leaves, foliar sprayed with the same solutions as used for seed soaking. In all of the investigated plants or parts of plants, elevated iodine concentrations were observed, compared to controls, except in pumpkin seeds. Additionally, in some of the investigated parts of plants, only treatment with one of

the forms resulted in higher iodine content. Comparing all selected plants, each of them responded differently to the addition of iodide and iodate and there was no general trend of better uptake of one or another of the forms.

Selenium also had various effects on iodine uptake; in some cases it did not affect iodine uptake, in some cases it had a synergistic effect, while in others an antagonistic effect, which could not be explained.

Iodine compounds were determined in iodine enriched pea, pumpkin and buckwheat sprouts. The results showed that iodine was mostly present in inorganic form and small amounts of MIT were also observed. DIT was not present in the analyzed plant samples or it was in concentrations lower than the detection limit. Some unknown iodine species were also observed in traces. Good results in terms of extractible iodine (around 75% on average) and identified species (around 90% on average) were observed. Buckwheat sprouts grown from seeds soaked in a solution of iodine and selenium combinations were also analyzed and the results showed no influence of selenium presence on iodine compounds.

Povzetek

Jod je za ljudi in ostale sesalce esencialen element, pomemben za normalno rast in razvoj. Je del ščitničnih hormonov, ki uravnavajo metabolizem in posledično veliko pomembnih biokemijskih reakcij. Primarni naravni vir joda je morska hrana, vendar pa večino joda zaužijemo z jodirano soljo, ki so jo posamezne države uvedle, da bi zmanjšale število obolenj za boleznimi, povezanimi s pomanjkanjem joda. Ker pa bi morali ljudje zmanjšati vnos soli v telo, je pomembno, da najdemo nove oz. dodatne vire joda v prehrani in eden od njih bi lahko bile z jodom obogatene rastline.

Preden pa bi lahko takšne rastline uvedli v prehrano, moramo poznati vsebnost joda, da lahko spremljamo vnos joda v telo. V prvem delu disertacije smo zato optimizirali metodo za določanje joda z ICP-MS po bazični mikrovalovni ekstrakciji. Najprej smo primerjali Schönigerjev razkroj z mikrovalovno ekstrakcijo, ki smo jo tudi izbrali za nadaljnje delo. V prvem koraku, pripravi vzorca, smo optimizirali temperaturni program in razmerje med vodo in tetrametilamonijevim hidroksidom (TMAH) za ekstrakcijo. Pri naslednjem koraku, določitvi joda, smo optimizirali koncentracijo TMAH v merjeni raztopini, primerjali metodo standardnega dodatka z eksterno umeritveno krivuljo za kvantitativno določitev joda in ovrednotili potrebo po uporabi internega standarda. Poleg določitve meje detekcije, linearnosti in ponovljivosti, smo zaradi pomanjkanja bioloških referenčnih materialov s certificirano vrednostjo za jod uporabili tudi neodvisno metodo k_0 -INAA, s katero smo potrdili pravilnost dobljenih rezultatov.

Določitev različnih oblik elementov, ki so prisotne v vzorcih, je pomembna zaradi potencialnih razlik v biološki dostopnosti, mobilnosti in toksičnosti. Zato smo optimizirali tudi metodo za določanje jodovih spojin s HPLC-ICP-MS, s posebnim poudarkom na postopku ekstrakcije in stabilnosti spojin. V prvem delu smo ugotavljali primernost ultrazvočne sonde za ekstrakcijo jodovih spojin, ker pa smo pri tem postopku naleteli na več težav, smo v drugem delu – optimizaciji encimske ekstrakcije za določanje jodovih spojin – uporabili vodno kopel z longitudinalnim stresanjem. Ugotovili smo, da se jodat v prisotnosti encima ali rastlinskega materiala pretvarja v jodid. Ker nismo uspeli najti pogojev, ki bi to pretvorbo preprečili, je metoda primerna za določitev MIT, DIT in anorganskega joda. Izbrali smo najprimernejši encim za ekstrakcijo jodovih spojin, preverili, ali je boljše raztopiti encim v vodi ali v pufru in izbrali optimalno temperaturo in čas ekstrakcije. Poleg tega smo določili izkoristek kolone, meje detekcije, linearnost, ponovljivost in izkoristek celotnega postopka.

Vsebnost joda smo določili v kalicah graha, buč in ajde, ki smo jih vzgojili iz semen, predhodno namakanih v raztopinah različnih oblik joda ali kombinacijah joda in selen (1000 mg I/L in 10 mg Se/L). Jod smo določili tudi v različnih delih odraslih rastlin graha, bučnih semenih, zrnju ajde in listih radiča, ki smo jih škropili z enakimi raztopinami, kot smo jih uporabili za namakanje semen. V vseh rastlinah ali delih rastlin, ki smo jih preučevali, so bile koncentracije joda povišane v primerjavi s kontrolo, razen v bučnih semenih. V nekaterih analiziranih delih rastlin je samo tretiranje z eno od oblik joda vodilo

v povečanje vsebnosti joda. Pri rastlinah nismo opazili splošnega trenda boljšega privzema ene od oblik joda, saj se je vsaka vrsta na dodatek joda odzvala drugače.

Tudi vpliv selena na privzem joda je bil pri vsaki rastlini drugačen. Pri nekaterih selen ni imel vpliva na privzem joda, pri nekaterih je ta imel sinergistični učinek medtem ko smo pri nekaterih opazili antagonistični učinek, česar nismo mogli razložiti.

Jodove spojine smo določili v z jodom obogatenih kalicah graha, buč in ajde. Rezultati so pokazali, da je večina joda prisotna v anorganski obliki, majhen delež je bil tudi v obliki MIT. DIT v analiziranih rastlinah ni bil prisoten ali pa so bile njegove koncentracije nižje od mej zaznave. Opazili smo tudi neznane jodove spojine, ki pa so bile prisotne v sledovih. Z optimizirano metodo smo dosegli visok delež ekstraktibilnega joda (povprečno približno 75%) in identificiranih spojin (povprečno približno 90%). Jodove spojine smo določili tudi v kalicah ajde, katerih seme je bilo namakano v kombinacijah joda in selena, vendar pa so rezultati pokazali, da prisotnost selena ne vpliva na jodove spojine.

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Abbreviations

AcH	... Acetic acid
AEC	... Anion Exchange Chromatography
CH ₃ I	... Methyl iodide
CE	... Capillary Electrophoresis
CPS	... Counts Per Second
DIT	... DiIodoTyrosine
DW	... Dry Weight
GC	... Gas Chromatography
HPGe	... High Performance Germanium Detector
HPLC	... High Performance Liquid Chromatography
ICP-MS	... Inductively Coupled Plasma Mass Spectrometry
ICP-OES	... Inductively Coupled Plasma Optical Emission Spectroscopy
ICP-QQQ	... Inductively Coupled Plasma tandem mass spectrometry
IDD	... Iodine Deficiency Disorders
INAA	... Instrumental Neutron Activation Analysis
ISE	... Ion Selective Electrode
I(–I)	... Iodide
I(V)	... Iodate
IS	... Internal Standard
I ₂	... Elemental iodine
LC	... Liquid Chromatography
LOD	... Limit of Detection
MeOH	... Methanol
MIT	... MonoIodoTyrosine
m/z	... Mass to charge ratio
RDI	... Recommended Daily Intake

RNAA	... Radiochemical Neutron Activation Analysis
RPC	... Reversed Phase Chromatography
SEC	... Size Exclusion Chromatography
Se(IV)	... Selenite
Se(VI)	... Selenate
TMAH	... TetraMethylAmmonium Hydroxide
TSH	... Thyroid Stimulating Hormone
T3	... 3,5,3'-triiodothyronine
T4	... 3,5,3',5'-tetraiodothyronine, thyroxin
UV	... UltraViolet
XRF	... X-Ray Fluorescence

Chapter 1

Introduction

Iodine (I) was first discovered in 1811 by the French chemist, Bernard Courtois, during the production of KNO_3 for Napoleon's army. Two years later, Gay-Lussac named the element "iode" after the Greek word for "violet-colored", due to the color of its vapor (Holleman & Wiberg, 2001). It is a non-metallic element from the halogen family that is a purple-black crystalline solid with a slight metallic luster under normal conditions (Cotton, Wilkinson, Murillo, & Bochmann, 1999; Greenwood & Earnshaw, 1997). It is positioned between bromine and astatine in group 17 of the periodic table, with the atomic number 53 (Elvers & Bellussi, 2011). Some of the iodine atomic, chemical and physical properties are summarized in Table 1.1.

Table 1.1: Some of the atomic, chemical and physical properties of iodine (Elvers & Bellussi, 2011; Greenwood & Earnshaw, 1997; Holleman & Wiberg, 2001).

Atomic number	53
Relative atomic mass	126.9
Electronic configuration	$[\text{Kr}]4\text{d}^{10}5\text{s}^25\text{p}^5$
Melting point [$^{\circ}\text{C}$]	113.6
Boiling point [$^{\circ}\text{C}$]	185.2
Oxidation states	-1, 0, +1, +3, +4*, +5, +7
Stable isotopes	^{127}I
Radioactive isotopes	35 isotopes, masses between 117 and 139

* ... *unstable oxidation state*

Iodine can form organic and inorganic compounds and can be found in oxidation states -1 in combination with more electropositive partners (iodide, I^- or $\text{I}(-\text{I})$), 0 (elemental iodine, I_2) and +1, +3, +5 and +7 in combinations with more electronegative partners (the most common in oxidation state +5 – iodate, IO_3^- or $\text{I}(\text{V})$). Oxidation state +4 also exists in the form of dioxide, IO_2 but it is thermodynamically unstable (Elvers & Bellussi, 2011; Holleman & Wiberg, 2001).

There are 36 known isotopes of iodine. The only stable isotope naturally present in all compartments of the environment is ^{127}I . Of the radioactive isotopes, ^{129}I is the only natural radioisotope, which is a product of spontaneous fission of ^{238}U in the lithosphere. It can also be formed in nature by the interaction of cosmic rays with ^{129}Xe in the upper atmosphere (Osterc, Stibilj, & Raspor, 2011). The half-life of ^{129}I is the longest of all iodine radioisotopes, 1.6×10^7 years (Elvers & Bellussi, 2011). The most important iodine radioisotopes are ^{131}I ($t_{1/2} = 8$ days) and ^{125}I ($t_{1/2} = 60$ days), due to their use in radioactive tracer work and radiotherapy (Elvers & Bellussi, 2011).

1.1 Iodine in the Environment

Biogeochemical cycling of iodine in the environment is influenced by interdependent biological, geological and chemical processes. It includes exchanges of the element between different compartments of the ecosystem that are conditional on the mobility of iodine, which is further dependent on iodine species (Osterc et al., 2011). Due to the number of valence states in which iodine can exist, its chemical reactivity and various inorganic and organic compounds, the chemistry of iodine is relatively complex (Whitehead, 1984).

Figure 1.1 shows a scheme of iodine movement in the environment. The major pathways of iodine cycling are volatilization of iodine compounds into the atmosphere, accumulation in living organisms and sorption to soil and sediment. Conversions between different iodine species are controlled by the biological activity of microorganisms, algae and phytoplankton and photochemical and chemical interactions (Osterc et al., 2011).

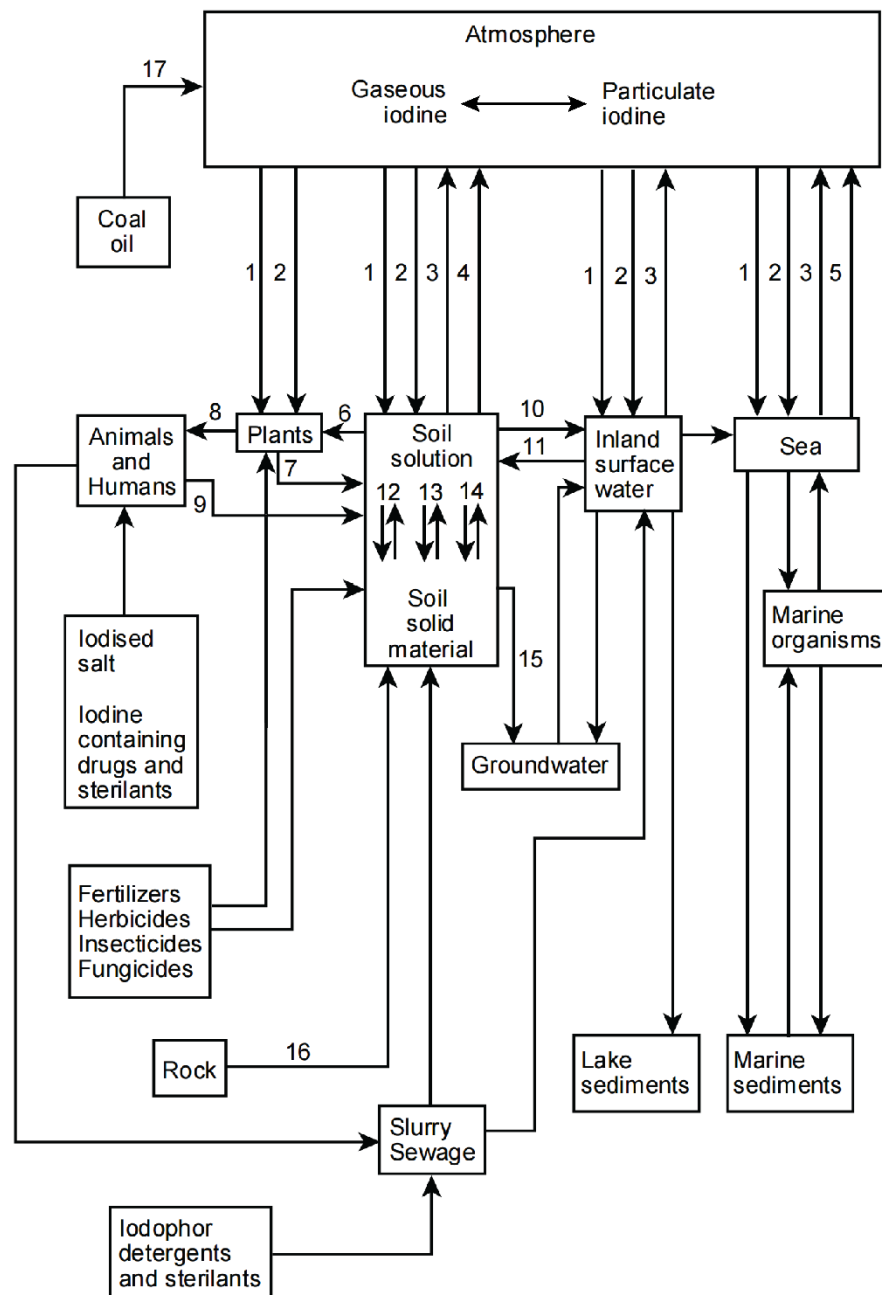
Iodine was present during the primordial development of the Earth. Large amounts of it were later leached from the surface soil by rain and snow or carried by wind, rivers and floods into the sea (Hetzel & Maberly, 1986). Iodine was also removed from the surface soil, by glaciations. The oceans have thus become the primary source of iodine (Hetzel & Maberly, 1986). Iodine in seawater is in equilibrium with iodine in marine sediments. The exchange of iodine between water and sediment is conditional on the formation and decomposition of organic material (Osterc et al., 2011). In the ocean, marine algae, bacteria and phytoplankton produce volatile iodomethane (CH_3I), which is the main way by which iodine enters the atmosphere. Other ways are weathering of iodine-rich rocks, volcanic activity, volatilization of CH_3I from plants and volatilization of elemental iodine, produced by oxidation of iodide with sunlight in the marine boundary layer (Osterc et al., 2011).

Iodine undergoes photochemical reactions in the air. It is present in gaseous organic or inorganic form. Combination with water and particles can also occur. The inorganic and particulate forms of iodine precipitate from the atmosphere by wet (rain, snow, sleet) and dry (gravitational settling, wind turbulences) deposition onto soil, plants and into surface waters (ATSDR, 2004; Whitehead, 1984).

Retention of iodine in soils is dependent on the pH, moisture, porosity and composition of soil. Bound to organic material and iron or aluminum oxides, it can remain in the soil for 4000 years. Depending on the bioavailability, it can be taken up by plants (see section 1.2.2) and then eaten by animals or humans, which is how iodine enters the food chain (Osterc et al., 2011; Whitehead, 1984).

In surface waters, iodine is present mainly as dissolved iodate, iodide and a small amount of organic iodine compounds. Although iodate is a thermodynamically favorable form in waters, the kinetic barrier prevents direct oxidation of iodide to iodate. Iodide is thus mainly converted into organic forms (primarily CH_3I) by microbial action. Volatilization of CH_3I from surface waters into the atmosphere is promoted by low vapor pressure and limited solubility in water (Osterc et al., 2011).

Iodine concentrations, typical of certain compartments of the environment, are summarized in Table 1.2.



*1 = deposition in rainfall; 2 = dry deposition (including absorption by plant leaves); 3 = volatilization; 4 = suspension of dust; 5 = suspension of marine aerosols; 6 = uptake by plant roots; 7 = decomposition of plant residues; 8 = consumption of food; 9 = decomposition of animal excreta and residues; 10 = run-off; 11 = irrigation; 12 = non-specific adsorption/desorption; 14 = immobilization/mineralisation involving soil organic matter; 15 = leaching; 16 = weathering; 17 = combustion of fossil fuels

Figure 1.1: Scheme of iodine movement in the environment (modified from Whitehead, 1984).

Table 1.2: Iodine concentrations in the environment (Hetzel & Maberly, 1986; Osterc et al., 2011).

Marine sediments	1–10 µg/g
Seawater	45–60 µg/L
Air	10–20 ng/m ³
Surface waters	0.1–18 µg/L
Rainwater	0.1–15 µg/L
Groundwater	~1 µg/L
Drinking water	≤ 8 µg/L

1.2 Iodine in Biological Systems

1.2.1 Iodine and its role for humans

“An element is essential when a deficit intake consistently results in an impairment of a function from optimal to suboptimal and when supplementation with physiological levels of this element, but not of others, prevents or cures this impairment” (Mertz, 1981). “Trace elements are essential components of biological structures but, at the same time, they can be toxic in concentrations beyond those necessary for their biological functions” (Fraga, 2005). Iodine corresponds to both definitions and is therefore classified as an essential trace element for humans and other mammals.

Iodine is a constituent of the thyroid hormones, thyroxine (3,5,3',5'-tetraiodothyronine, T4) and 3,5,3'-triiodothyronine (T3) (Hetzel & Maberly, 1986). Thyroid hormones regulate the metabolism and thus many important biochemical reactions: protein synthesis and enzymatic activities. They influence cellular oxidation and thermoregulation, they are involved in the absorption of glucose and galactose, decrease the level of cholesterol in serum etc. (Institute of Medicine of the National Academies, 2002; Koeppen & Stanton, 2010; Osterc et al., 2011). The major target organs are the developing brain, muscle, heart, pituitary and kidney (Institute of Medicine of the National Academies, 2002; Osterc et al., 2011). By production of thyroid hormones, the thyroid gland controls the rate of metabolism, i.e., how quickly the body burns energy (carbohydrate, fat, protein, vitamin and mineral metabolism) and the sensitivity of the body to other hormones (Osterc et al., 2011). The synthesis of T3 and T4 is schematically shown in Figure 1.2.

In addition to T3 and T4, a number of other iodinated molecules are present in the human body – moniodotyrosine (MIT), diiodotyrosine (DIT) and reverse triiodothyronine (rT3), which are mainly bound to proteins in the thyroid and blood (Osterc et al., 2011).

After ingestion, iodine is rapidly and almost wholly absorbed in the stomach and duodenum (> 90%). In the gut, iodate is reduced and absorbed through the gastrointestinal tract as iodide. Organic compounds in which iodine is bound are usually digested and the released iodide is absorbed. However, about 75% of the intake of thyroxine is absorbed intact. Absorbed iodide is then removed from the blood circulation by the thyroid gland and kidney (Institute of Medicine of the National Academies, 2002; Osterc et al., 2011; Zimmermann, Jooste, & Pandav, 2008). There are 15–20 mg of iodine in the body of a healthy adult, 70–80% of which is found in the thyroid (Zimmermann et al., 2008).

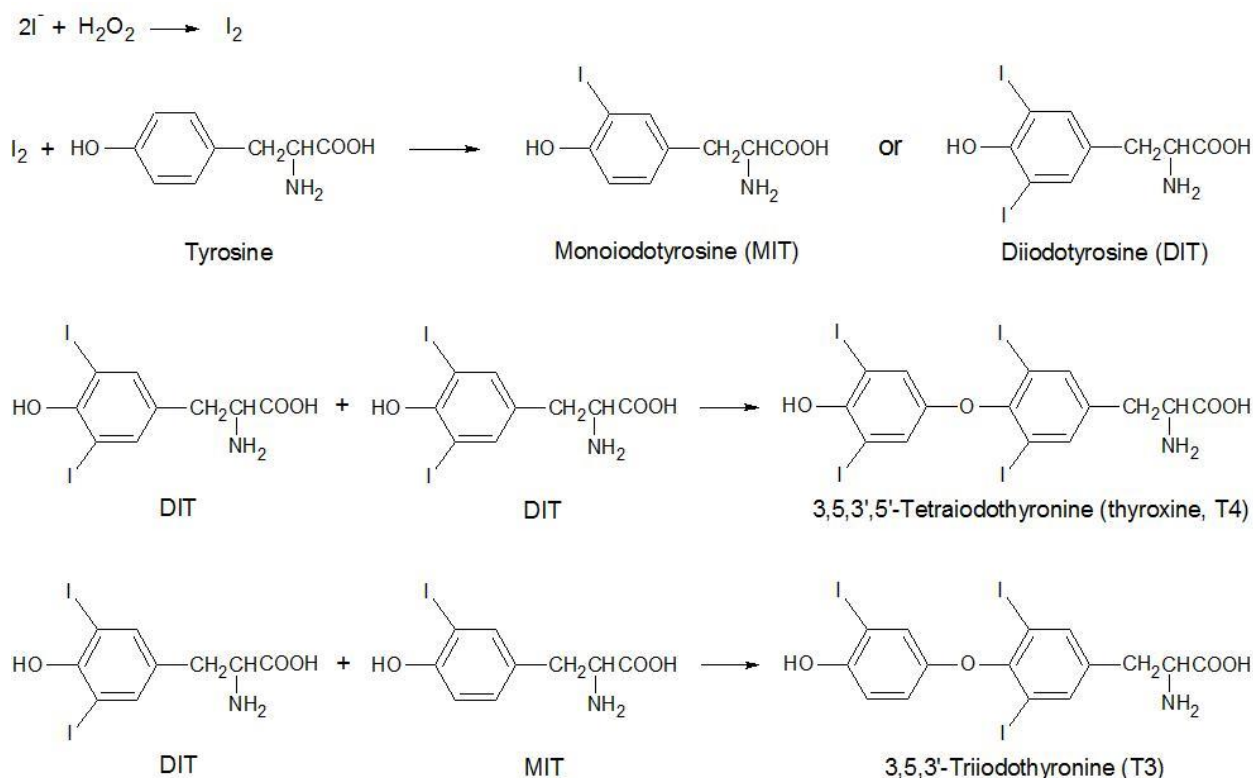


Figure 1.2: Scheme of synthesis of thyroid hormones T3 and T4 (Koeppen & Stanton, 2010).

The recommended daily intake (RDI) of iodine is 150 µg/day (German Nutrition Society, Austrian Nutrition Society, Swiss Society of Nutrition Research, & Swiss Nutrition Association, 2002). Humans mostly get iodine from food, with a smaller amount also entering the body with water (ATSDR, 2004). However, the natural iodine content in most foodstuffs and beverages is low (Zimmermann et al., 2008). Since high amounts of iodine are present in the oceans, the primary natural source of iodine in a normal, balanced diet is sea food – fish, shellfish and edible seaweeds (Osterc et al., 2011). However, the majority of iodine enters the body through a manmade source, iodized salt. Iodine is also added to animal fodder to increase the iodine content in milk and eggs (Osterc et al., 2011; Zimmermann, 2011). Another important source of iodine is bakery products, since iodates are used as conditions for dough (Osterc et al., 2011). If there are any limitations in consuming table salt, e.g., due to blood hypertension, food supplements are alternative sources (Osterc et al., 2011).

Despite the different iodine sources available, according to a study from 2007 (De Benoist, McLean, Andersson, & Rogers, 2008), two billion people worldwide still suffer from insufficient iodine intake. Iodine deficiency has many adverse effects on growth and development in humans, which are collectively called iodine deficiency disorders (IDDs) (Zimmermann, 2008). IDDs are one of the most common preventable human health problems, including mental retardation, hypothyroidism, goiter, cretinism and varying degrees of other growth and development abnormalities (Institute of Medicine of the National Academies, 2002). IDDs are a consequence of inadequate iodine intake, resulting in insufficient thyroid hormone production (Institute of Medicine of the National Academies, 2002; Zimmermann, 2008).

On the other hand, iodine excess occurs when the intake of iodine is too high, and this is also risky. It generally happens due to high iodine levels in salt or poor salt monitoring and can cause iodine induced hypo- or hyperthyroidism (Andersson, de Benoist, & Rogers, 2010; Delange, de Benoist, Pretell, & Dunn, 2001). Some effects may occur at an iodine intake far above the recommended values, while others have been observed at intakes that are still within the range of tolerable doses (Bürigi, 2010). However, the response to excess iodine can vary greatly among individuals (Bürigi, 2010). Although people with a normal thyroid gland may respond to excess iodine, they can usually tolerate a chronic excess of 0.03–2 g per day without clinical symptoms of thyroid dysfunction (Bürigi, 2010). It is more problematic in the population with a history of iodine deficiency, when an increase of iodine intake occurs suddenly (Andersson et al., 2010; Bürigi, 2010).

To prevent the adverse effects of iodine deficiency or excess in the population, the control of iodine status is very important. It is a continuous process, which requires sustainable monitoring (Hetzel & Maberly, 1986; Osterc et al., 2011). Iodine status must be evaluated before any program of iodine fortification, so the effects can be followed and the epidemiology of thyroid dysfunctions in a population can be linked to different levels of iodine intake (Osterc et al., 2011). The following methods are generally used for assessment of iodine status: palpation, urinary iodine concentrations, the goiter rate and TSH or thyroglobulin blood levels (Zimmermann, 2008).

1.2.2 Iodine in plants

Plants play an important role in providing sufficient iodine intake for humans, since they are the first level of the food chain (Smoleń, Kowalska, & Sady, 2014). Iodine is not known to be an essential element for plants but, according to some literature data, can be considered to be beneficial (Borst Pauwels, 1961; Kabata-Pendias & Pendias, 2001). Accumulation of iodine in crops is dependent on the form, concentration and plant tissue involved (Mackowiak & Grossl, 1999). There are generally two ways of iodine entering a plant – the first is sorption of deposited iodine from the atmosphere through the leaves, while the second is uptake of iodine from soil through the roots (Whitehead, 1984).

Gaseous forms of iodine may enter the stomata of plant leaves, where they are incorporated into leaf mesophyll tissue. Due to differences in the extent of stomata opening, the uptake of atmospheric iodine is greater in higher atmospheric humidity (Fuge, 2005; Whitehead, 1984). However, elemental iodine seems to have a short lifetime in the atmosphere, but the absorption of other gaseous forms (e.g., methyl iodide or hypoiodous acid), which may persist longer, is low. Dry deposition of gaseous and particulate iodine depends on the nature of the vegetation, whereby the major role is played by the properties of the leaf surface. Absorption also strongly varies between different plant species (Whitehead, 1984). Translocation of iodine absorbed through the leaves into other parts of plants is quite slow (Fuge, 2005).

Iodine in soil can be strongly bound to other components of the soil and is thus not bioavailable. Soil composition plays an important role in the bioavailability of iodine. A high iodine content in soil cannot therefore be directly linked to the iodine content in plants growing on it (Fuge, 2005; Whitehead, 1984). Generally, the major pathway of different elements in plants is from the soil through the root system, followed by translocation into the upper parts (Fuge, 2005). However, mechanisms of iodine transport in plants are still unclear. It has been suggested that iodine is mostly transported through the xylem, while phloem mobility and, consequently, transport into the grains is low (Herrett, Hatfield, Crosby, & Vlitos, 1962; Mackowiak & Grossl, 1999). The opposite trend was observed by

Landini, Gonzali & Perata (2011) in tomato fruits, which are relatively isolated from the xylem transport. In the experiment with radioactive ^{125}I added into soil or hydroponic system they found elevated iodine content in fruits from treated plants and thus concluded the transport into fruits depends on phloem transport. To clarify iodine movements in plants, further research is therefore needed.

The relative importance of each of the two sources of iodine in plants – sorption through the leaves and uptake through the roots – as well as the influence of environmental conditions on them, are uncertain. However, it is likely that both sources significantly contribute to the iodine content in plants.

Due to the important role of various plants in nutrition, improvement (in terms of iodine content) has been proposed for vegetables through biofortification. The term biofortification has been defined as “the process of increasing the bioavailable concentrations of essential elements in edible portions of crop plants through agronomic intervention or genetic selection” (White & Broadley, 2005). The efficiency of biofortification depends on the capacity of the plant for iodine accumulation in the edible parts. Plant species that can be used in such programs must therefore be defined (Blasco et al., 2008).

For iodine, biofortification studies are usually performed with two different forms of iodine – iodide and iodate – searching for the form that is better uptaken and accumulated in the edible parts of different vegetables. However, the results are not uniform. The uptake is dependent on the plant species, concentrations, how it is grown, the manner of addition of iodine etc.

It is generally suggested that iodide is the form that is more readily absorbed. Iodate has a higher valence and molecular weight and therefore has to be reduced to iodide prior to absorption. Accumulation is thus limited by the process of reduction, which requires energy (Blasco et al., 2008; Voogt, Holwerda, & Khodabaks, 2010; Whitehead, 1973, 1975). However, in some studies, treatments with iodate resulted in a higher iodine content than treatments with iodide. Most such studies were performed by fortification of the soil and they concluded that iodide is more mobile in soil and therefore rinsed to a greater extent than iodate, resulting in a higher concentration of iodate remaining in the soil (Borst Pauwels, 1961). On the other hand, experiments performed in solution culture have generally resulted in a better uptake of iodide, which can be explained by the reduction of iodate prior to absorption (Borst Pauwels, 1961; Whitehead, 1975). Another explanation of the differences in uptake of iodide and iodate was given by Weng et al. (2008). They found better uptake of iodate when applied to the soil in low concentrations (0.1 mg I/L or lower), while iodide was better uptaken when higher concentrations were added to the soil (0.5 mg I/L or more). The different behaviors were explained by the response of iodine species to active (occurs when nutrition concentration around roots is low and requires energy) and passive (occurs when nutrition concentration is high) uptake mechanisms. The active mechanism requires energy and is important when the nutrient concentration around the roots is low, while passive transport is the predominant mechanism in the case of high nutrient concentrations. Their results showed iodate to be uptaken more effectively by the active mechanism, while iodide is more favorable for the passive mechanism (Weng et al., 2008).

Although it has not been explained, low doses of iodine can have a positive effect on plant growth, but at higher doses it can become toxic (Borst Pauwels, 1961). However, despite the general stimulating effect on plant development, it is difficult to say whether or not iodine has any specific function in plants (Borst Pauwels, 1961). The nature of the

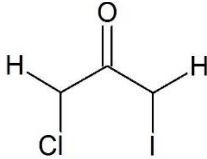
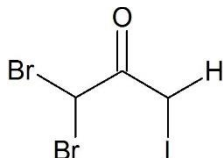
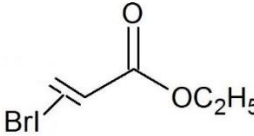
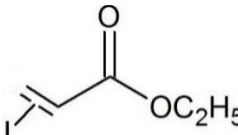
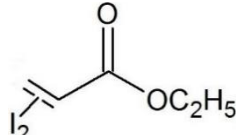
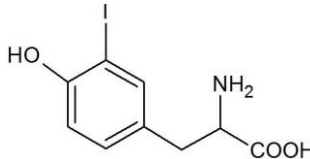
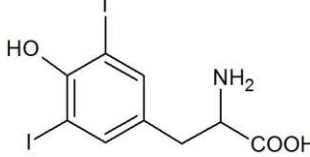
crop is very important, since some are more favorably influenced than others. Additionally, plants can bear iodate in a higher concentration than iodide (Borst Pauwels, 1961). The sensitivity of plants to iodine is not uniform for different plant species. Some of them show no adverse effect at iodine doses up to 1 $\mu\text{g/g}$ of soil, while even doses of 0.01–0.10 $\mu\text{g/g}$ of soil are toxic for others (Borst Pauwels, 1961).

The mechanisms of phytotoxicity of iodine are poorly researched. It is possible that it results from excessive iodine accumulation in plant tissues. Another explanation in the case of iodide is intracellular oxidation to elemental iodine (I_2), which can iodinate cell components, including chlorophyll, and thus inhibit the photosynthetic process (Mynett & Wain, 1973). Toxicity can appear as margin chlorosis in older leaves or a dark green color in younger leaves (Kabata-Pendias & Pendias, 2001). Kiferle, Gonzali, Holwerda, Ibaceta & Perata (2013) observed leaf chlorosis, epinasty and visible wilting of tomato plants when they applied iodine (as KI or KIO_3) in a concentration higher than 10 mM to soil. At a concentration of 5 mM, some discoloration and necrotic areas, limited to the basal leaves, were also observed. It is still not clear which iodine form, iodide or iodate, is more toxic to plants, since contradictory data are found in the literature (Kiferle et al., 2013; Mackowiak & Grossl, 1999; Weng et al., 2008). Additionally, the literature data show that a higher uptake is not necessarily linked to the lower toxicity of a particular iodine form. Kiferle et al. (2013) reported that iodate showed greater phytotoxicity in tomato plants and treatment with this form also resulted in lower uptake. On the other hand, Mackowiak & Grossl (1999) and Weng et al. (2008) found iodide to be more toxic in terms of biomass reduction for rice and Chinese cabbage, respectively, although this form was generally better uptaken. However, in some studies (Dai, Zhu, Huang, Zhang, & Song, 2006; Lawson, Daum, Czauderna, Meuser, & Härtling, 2015; Voogt et al., 2010), neither iodide nor iodate influenced plant biomass, regardless of the iodine accumulation in plant tissues.

1.2.2.1 Iodine compounds in plants

Data about iodine species in the tissues of terrestrial plants are rare or non-existent. However, iodine speciation has been performed in various algae and seaweed samples, which biologically belong to the same kingdom. In addition to inorganic iodine forms, MIT and DIT, which are present in samples of plant, animal and human origin, various other species were found in algae and seaweed samples (Table 1.3). However, due to the different environment in which they grow, the same species are not necessarily also present in terrestrial plants.

Table 1.3: Iodine species found in algae and seaweed samples (adapted from Moreda-Piñeiro, Romarís-Hortas, & Bermejo-Barrera (2011)).

Name	Structure
Iodide	I^-
Iodate	IO_3^-
Iodomethane	CH_3I
Chloriodomethane	CH_2ICl
Dibromiodomethane	$CHIBr_2$
1-chloro-3-iodoacetone	
1,1-dibromo-3-iodoacetone	
Ethyl bromoiodoacrylate	
Ethyl iodoacrylate	
Ethyl diiodoacrilate	
Monoiodotyronine	
Diiodotyronine	

1.3 Correlation Between Iodine and Selenium in Plants

Selenium (Se) is another essential element for humans, since it is a component of several selenoproteins. It is a cofactor of the glutathione peroxidases family of enzymes, which protect against oxidative stress (Navarro-Alarcon & Cabrera-Vique, 2008). There is a well-known correlation between iodine and selenium in the human body. Se is a cofactor of the enzyme iodothyronine deiodinase, which is responsible for conversion of the thyroid hormone T4 into the metabolically active form T3 (Navarro-Alarcon & Cabrera-Vique, 2008; Osterc et al., 2011). In the case of selenium deficiency, the conversion of T4 into T3 might be impaired, and even people with sufficient iodine intake can have symptoms similar to iodine deficiency (Arthur, Nicol, & Beckett, 1993; Schomburg & Köhrle, 2008).

Due to the close relationship between iodine and selenium in humans, and the common deficiency of both elements, it has been suggested that biofortification should be approached simultaneously (White & Broadley, 2005). However, there is a great lack of information about the iodine and selenium influence on plants (Smoleń et al., 2014). Possible interactions between I and Se in plant uptake processes and metabolic processes within plants have still not been sufficiently investigated (Smoleń et al., 2016; Zhu, Huang, Hu, Liu, & Christie, 2004). Additionally, the effect of simultaneous application of I and Se on plant growth, development and yield is still unknown (Smoleń et al., 2016). Due to the lack of information about different approaches to achieving enrichment with I and Se, selecting the most efficient method is also an important issue (Smoleń et al., 2014).

In the first study conducted by Zhu et al. (2004), the uptake of iodine and selenium in their oxidized forms, iodate and selenate, by spinach plants grown in a hydroponic system was investigated. Iodine was added in concentrations of 0, 10 and 50 $\mu\text{mol/L}$ and selenium in concentrations of 0, 10 and 20 $\mu\text{mol/L}$. The total time of growth was 33–34 days. Their results showed no influence on root biomass when I in concentrations of 10 $\mu\text{mol/L}$ was applied in combination with selenium, regardless of its concentration. However, at a higher iodine concentration, root biomass was reduced. In addition, when the two elements were applied simultaneously, a reduction of shoot biomass was observed.

Both iodine and selenium were mainly accumulated in spinach leaves. When iodine was added in a concentration of 50 $\mu\text{mol/L}$, the addition of selenium slightly inhibited the accumulation of iodine in shoots, while no effect was observed in roots. After the addition of 10 $\mu\text{mol I/L}$, Se did not affect iodine accumulation. The authors of the study therefore concluded that there are no strong interactions between iodine and selenium in plant uptake processes and metabolism (Zhu et al., 2004).

Smoleń et al. (2014) published the results of a study about possible simultaneous biofortification of lettuce with iodate (1 mg I/L) and selenate (0.5 or 1.5 mg Se/L) using the hydroponic system of nutrient film technique (NFT) and/or foliar application (5 times with 0.05% I solution (3.94 mmol I/L) and 0.005% Se solution (0.633 mmol Se/L)). The total time of growth was between 11 and 13 weeks. Their results showed no influence of iodine and selenium treatment on the biomass of lettuce.

When the application of iodine and selenium was done separately (NFT or foliar application), the results showed no effect of selenium on iodine content. However, when the two applications were performed one after another (NFT and foliar application), selenium decreased the iodine content in lettuce leaves, compared to iodine treatment alone (Smoleń et al., 2014).

Another study by Smoleń et al. (2016) is the only one so far to have investigated two different forms of each element – iodide, iodate, selenite and selenate. Carrots were grown on a field in fertilized soil using the following combinations: control, KI, KIO₃, Na₂SeO₃, Na₂SeO₄, KI + Na₂SeO₃, KI + Na₂SeO₄, KIO₃ + Na₂SeO₃, KIO₃ + Na₂SeO₄. Iodine and selenium were applied twice: incorporated into soil before planting and as a top dressing, in total doses of 5 kg I/ha and 1 kg Se/ha. They observed no influence of iodine and selenium treatment on the carrot biomass.

Regardless of the forms, their results showed an antagonistic effect of selenium on iodine uptake but to a greater extent in the case of Se(IV).

In view of the results of the mentioned studies (Smoleń et al., 2016, 2014; Zhu et al., 2004), there might be some interaction between iodine and selenium uptake and/or metabolism. However, the data obtained in those studies are not sufficient to understand what happens in a plant when those elements are added and further studies are needed. The possibility of each plant responding differently can also not be excluded.

1.4 Determination of Iodine in Biological Samples

1.4.1 Determination of iodine concentrations

Validated analytical methods for iodine determination in biological samples are required in order to determine iodine content accurately (Shelor & Dasgupta, 2011). Monitoring iodine in humans is important to prevent iodine deficiency or excess in the population. Determination of iodine content in other biological samples, such as plants and other potential food samples, is of high significance in providing the appropriate nutritional iodine intake. However, iodine concentrations in most foods, as well as in biological samples, are low and determination has been a challenging analytical problem for a long time (Haldimann, Alt, Blanc, & Blondeau, 2005). It is associated with difficulties in sample decomposition (Mahesh, Deosthale, & Narasinga Rao, 1992; Stibilj, 1994). Additionally, problems during analytical procedures can be caused by adsorption, volatilization, contamination, interference by other halogen elements, high blank values etc. (Stibilj, 1994). Another problem in iodine determination is quality control due to the very limited number of reference materials with certified values for iodine content.

1.4.1.1 Sample preparation

Sample preparation is a crucial step in an analytical procedure and represents the main problem (Shelor & Dasgupta, 2011). Analysis of iodine in different matrixes has been a challenge due to low concentrations (usually below 1 µg/g). In addition, volatilization of some forms of iodine can occur and can result in losses of the analyte. A suitable sample digestion procedure must therefore be employed to overcome this problem (Moreda-Piñeiro et al., 2011; Shelor & Dasgupta, 2011; Tinggi, Schoendorfer, Davies, Scheelings, & Olszowy, 2012).

Wet digestion using concentrated acids in open vessels is not widely applicable; alkaline solutions (e.g. ammonia, sodium hydroxide, tetramethylammonium hydroxide (TMAH) and water soluble tertiary amines solutions) are rather used in sample preparation approaches (Shelor & Dasgupta, 2011; Tinggi et al., 2012). The use of TMAH for sample digestion in closed vessels has been applied by many researchers. Alternatively, procedures with oxidizing acid mixtures can also be used but the oxidizing potential must be high enough for oxidation of volatile I⁻ to non-volatile IO₃⁻ (e.g., 65% HNO₃ mixed with 30%

H₂O₂ 5:1 (v/v) was used by Shah, Wuilloud, Kannamkumarath, & Caruso (2005)). However, acidic procedures are time consuming, the values of blanks are usually high and interferences in the determination step can occur (Fecher, Goldmann, & Nagengast, 1998; Larsen, Knuthsen, & Hansen, 1999; Mesko et al., 2010; Vanhoe, Van Allemeersch, Versieck, & Dams, 1993; Vtorushina, Saprykin, & Knapp, 2009). Another option for organic samples is burning in a closed vessel filled with oxygen (Schöniger combustion), after which the iodine is absorbed in an alkaline medium as iodide (Shelor & Dasgupta, 2011). Alkaline fusion can also be employed, usually using KOH (Hou et al., 1999). However, combustion in microwave systems is the most widely used method nowadays (Mesko et al., 2010; Moreda-Piñeiro et al., 2011; Shelor & Dasgupta, 2011; Tinggi et al., 2012). If iodine is to be determined in liquid samples, such as water or biological fluids (e.g., milk, blood, etc.), no extensive sample preparation is needed, since dilution or dispersion in an alkaline solution is sufficient (Gélinas, Krushevskaya, & Barnes, 1998; Shelor & Dasgupta, 2011).

1.4.1.2 Detection

Currently, two methods are almost exclusively used for determination of iodine in biological samples – the catalytic spectrophotometric method and inductively coupled plasma mass spectrometry (ICP-MS) (Shelor & Dasgupta, 2011). Other methods that have been used for iodine determination are inductively coupled plasma optical emission spectrometry (ICP-OES), neutron activation analysis (NAA), X-ray fluorescence (XRF), atomic absorption spectrometry, AAS, electrochemical and potentiometric probes, gas and liquid chromatography (GC and LC, respectively) etc. (Moreda-Piñeiro et al., 2011; Shelor & Dasgupta, 2011). The nature of the sample (matrix), the concentration range that can be determined, effectiveness, cost, reproducibility, interferences, time of analytical procedure, number of samples, availability of instrumentation, simplicity of the method and sample preparation etc., are factors that influence the decision on which method should be used for a particular application (Moreda-Piñeiro et al., 2011; Shelor & Dasgupta, 2011; Stibilj, 1994).

Some of the studies in which iodine was determined in plant materials using different methods are listed in Table 1.4. Where stated in the papers, the LOD and linear range are also listed in Table 1.4.

1.4.1.2.1 Catalytic spectrophotometric method

Two different reactions catalyzed by iodine are in use. The first, the Sandell-Kolthoff (S-K) reaction, uses the reduction of yellow Ce(IV) to colorless Ce(III) by As(III), which is oxidized to As(V) (Moreda-Piñeiro et al., 2011; Sandell & Kolthoff, 1937; Shelor & Dasgupta, 2011). The reaction is normally very slow but can be catalyzed by trace amounts of iodide. Iodate also catalyzes the reaction but to a much smaller extent. Organoiodine cannot catalyze this reaction without previous decomposition, which usually involves perchloric acid and thus special care is required due to the risk of explosion (Shelor & Dasgupta, 2011). The reaction is monitored by measuring the decrease of absorbance in two possible ways; the first is recording the complete absorbance profile with time, while the second, used more often, is measuring the absorbance after a preset time interval after mixing all the compounds (Shelor & Dasgupta, 2011). Determination of iodine can be obstructed by high concentrations of thiocyanate (SCN⁻), traces of metal ions that bind iodide (e.g., silver and mercury) and compounds that easily undergo oxidation (nitrite, ascorbic acid, ferrous iron) (Shelor & Dasgupta, 2011).

Another reaction catalyzed by iodide is reduction of thiocyanate by nitrite in the presence of ammonium iron(III) sulfate. The decrease in the orange color of the solution can be monitored spectrophotometrically. If iodine is present in the form of iodate, a previous reduction step with ascorbic acid is required (Moxon & Dixon, 1980; Shelor & Dasgupta, 2011).

1.4.1.2.2 Inductively coupled plasma mass spectrometry

ICP-MS is a relatively new technique that can be used for trace element determination in a variety of samples, including biological samples. The main advantages of ICP-MS are the wide range of sample types that can be analyzed, high sensitivity, large dynamic range, small amount of sample needed and multi-element analyses (Moreda-Piñeiro et al., 2011; Shelor & Dasgupta, 2011). Iodine has a relatively high first ionization potential (10 eV to form I^+) and is consequently only partially ionized ($\sim 25\%$). Due to the low ionization, iodine is not one of the most sensitive of elements that can be determined by ICP-MS. However, the sensitivity of ICP-MS in iodine determination is still superior to that of other techniques (Shelor & Dasgupta, 2011). Most samples require sample digestion prior to analysis, for which microwave-assisted digestion, combustion in oxygen or wet ashing in closed vessels are the most commonly used (Shelor & Dasgupta, 2011).

1.4.1.2.3 Inductively coupled plasma optical emission spectrometry

For ICP-OES, plasma is used for atomization and excitation/ionization. Light emitted from the excited atom is detected at a specific wavelength for the element of interest. Lower sensitivity may be achieved by ICP-OES compared to ICP-MS and so ICP-OES is not so widely used for iodine determination. Additionally, the most intense emission lines of iodine (178.276 nm and 183.038 nm) are very close to phosphorus emission lines (178.284 nm and 183.480 nm), causing difficulties in determination (Minnhagen, Svendenius, & NIST ASD Team; Shelor & Dasgupta, 2011). Since phosphorus is present in most biological samples in much higher concentrations than iodine, even when using high resolution monochromators, large errors cannot be avoided. Alternatively, the iodine emission line at about 183 nm can be measured so that interferences are avoided, although some sensitivity is lost. Another possibility is to isolate iodine from the matrix prior to determination (Shelor & Dasgupta, 2011).

1.4.1.2.4 Neutron activation analysis

NAA is an analytical method for quantitative and qualitative analysis of several trace elements in various sample matrixes (Moreda-Piñeiro et al., 2011). In terms of technique, there are two main types of NAA: instrumental (INAA) and radiochemical (RNAA). In INAA, the sample is irradiated and the induced gamma activity that originates from the reaction $^{127}I(n, \gamma)^{128}I$ is measured on a gamma spectrometer after a suitable decay time, without any chemistry performed after irradiation. This type is therefore nondestructive. In RNAA, a suitable chemical reaction step is carried out to isolate the elements of interest, individually or in groups, from the matrix. After irradiation, this type is thus destructive (Guinn & Hoste, 1980). However, due to the chemical separation from the matrix, higher sensitivity, low matrix influences and high efficiency can be achieved by RNAA. When iodine concentrations in the sample are higher and there is a low concentration of interfering elements, such as Br, Cl, Na and Mn, which induce high activities after irradiation, separation is not needed and INAA can be used instead (Rao & Chatt, 1993; Stibilj, 1994). However, NAA is not very commonly used due to the equipment, since a

nuclear reactor and special conditions for laboratories are required (Moreda-Piñeiro et al., 2011).

1.4.1.2.5 Atomic absorption spectrometry

Although some applications have been described for iodine determination by AAS, direct determination is difficult since no commercial lamp is available and the main resonance lines lie in the vacuum UV region, where air is an important interference and thus inert gas purging of the optical path is required (Moreda-Piñeiro et al., 2011; Shelor & Dasgupta, 2011). However, good limits of detection can be achieved by indirect analysis. A reaction of iodine (mainly in the form of iodide) with metallic species is performed and the metal fraction that has reacted with iodine is then measured (Moreda-Piñeiro et al., 2011; Shelor & Dasgupta, 2011).

1.4.1.2.6 Electrochemical methods

Iodine can be determined by ion selective electrode (ISE) the linear responses of which are between 0.02 and 1 mM. Commercial iodine ISE is based on insoluble silver salt membranes, which respond to high levels of other anions that form insoluble silver salts (halides, pseudohalides). Electrochemical detection (e.g., amperometric) is popular due to the fast response, and selectivity and sensitivity for the target analyte (Shelor & Dasgupta, 2011).

1.4.1.2.7 Chromatography

After conversion of all iodine forms into iodide, it can also be determined by chromatographic methods, e.g., gas chromatography (GC), after appropriate derivatization (Moreda-Piñeiro et al., 2011; Shelor & Dasgupta, 2011).

Table 1.4: Methods used for iodine determination in plant samples.

Reference	Method	Samples ^b	LOD	Linear range
Weng et al. (2008)	CSM ^a	Chinese cabbage	-	-
Bellanger, Tressol, & Piel (1979)	CSM (S-K) ^a	Hay, hay silage, maize silage, beet pulps, wheat grains, barley grains, maize grains, peanut cake, soya cake, sunflower cake, apple tree leaves, palm tree leaves, olive tree leaves, vine leaves, orange tree leaves, cotton leaves	-	-
Borst Pauwels (1961)	CSM (S-K) ^a	Buckwheat, tomato, white clover, flax, perennial ryegrass, mustard, barley, wheat, oats, spinach, turnip and fodder beet	-	-
Johnson & Butler (1957)	CSM (S-K) ^a	White clover, perennial ryegrass, short rotation ryegrass	-	-
Mackowiak & Grossl (1999)	CSM (S-K) ^a	Rice	-	-
Knapp, Maichin, Fecher, Hasse, & Schramel (1998)	ICP-MS ^c	Diet soya [*]	10 ng/L	
Hasegawa, Tsukada, Kawabata, Takaku, & Hisamatsu (2015)	ICP-MS	Radish	-	-
Kiferle et al. (2013)	ICP-MS	Tomato fruits	-	-
Tagami, Uchida, Hirai, Tsukada, & Takeda (2006)	ICP-MS	Potato, radish, spinach, komatsuna, grass	0.013 µg I/L or 0.007 mg I/kg	0.01–100 µg I/L
Voogt et al. (2010)	ICP-MS	Lettuce	-	-

Reference	Method	Samples ^b	LOD	Linear range
Smoleń et al. (2014), Smoleń, Strzetelski, Rożek, & Ledwożyw-Smoleń (2011) and Smoleń & Sady (2012)	ICP-OES	Spinach and lettuce	-	-
Smoleń et al. (2016)	ICP-OES	Carrot	0.5 µg I/dm ³	-
Dai et al. (2006)	INAA	Spinach	-	-
Zhu et al. (2004) and Zhu, Huang, Hu, & Liu (2003)	INAA	Spinach	-	-
Bowen (1959)	RNAA	Tomato seeds	0.1 ng	-
Johansen & Steinnes (1976)	RNAA	Kale, wheat flour	-	-
Stibilj (1994)	RNAA	Asparagus flour, asparagus tips, red pepper, carrot, tomato, celery, peas, kale, parsley, onion, leek, potato [*]	1 ng/g	-
Blasco et al. (2008)	AAS	Lettuce	-	-

^a ... CSM – Catalytic Spectrophotometric Method, S-K – Sandell-Kolthoff reaction

^b ... Plant samples are stated in table, ^{*} means other samples (e.g., of animal origin) were also analyzed

^c ... Comparison between CSM (S-K) and ICP-MS was described but CSM(S-K) was only used on samples of animal origin

- ... data not given in paper

1.4.2 Determination of iodine compounds

Determination of the total concentration of elements provides no information about bioavailability, bioaccumulation or mobility. Determination of the species of an element is thus very important in order to assess the behavior of the element in the environment or biological system (Gonzalvez, Cervera, Armenta, & De la Guardia, 2009).

There are three important definitions referring to speciation in chemistry, according to the International Union for Pure and Applied Chemistry (IUPAC, 2014):

- *Chemical species of an element* is a “specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure”.
- *Speciation analysis* is “analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample”.
- *Speciation* is the “distribution of an element amongst defined chemical species in a system”.

If elemental speciation is not possible, fractionation can be used instead (Cornelis, Caruso, Crews, & Heumann, 2003), defined by IUPAC as a “process of classification of an analyte or group of analytes from a sample according to physical (e.g., size, solubility) or chemical (e.g., bonding, reactivity) properties” (IUPAC, 2014).

1.4.2.1 Sampling and sample preparation

Sampling is considered to be one of the most critical steps, which can cause irreversible errors. The sampling procedure must therefore preserve the information about the species and species equilibria from the original sample (Michalke, 2003).

Sample storage prior to analysis should be as short as possible, although long-term storage often cannot be avoided. If required, freeze drying or shock-freezing at -80°C are recommended (Caruso, Klaue, Michalke, & Rocke, 2003; Michalke, 2003).

In iodine speciation, sample pre-treatment is needed to isolate iodine species from the samples (Moreda-Piñeiro et al., 2011). This step should be as simple as possible in order to reduce the steps in which contamination or species conversions can occur (Michalke, 2003). Sample preparation depends on sample type, species of interest and the separation and/or detection system. For liquid samples, such as aqueous samples, simple treatments such as dilution can be sufficient, but for solid samples, more complex methods are required (Moreda-Piñeiro et al., 2011). Enzymatic hydrolysis procedures are very applicable as sample pre-treatment in iodine speciation studies in biological samples. Specific bonds of bio-molecules (proteins) are broken by enzymatic activity, allowing selective release of the analyte without changing the chemical species (Moreda-Piñeiro et al., 2011). Since iodine can be bound to compounds of low or high molecular weight (iodinated proteins) or integrated in proteins, usually in the form of the iodinated amino acids, MIT and DIT, enzymatic hydrolysis is required (Romarís-Hortas, Bermejo-Barrera, & Moreda-Piñeiro, 2013; Shah et al., 2005). Enzymes operate at mild temperature and pH conditions and should not therefore cause interconversions and/or degradations of target analytes (Romarís-Hortas et al., 2013).

Various enzymes were evaluated for iodine speciation during method development by Romarís-Hortas et al. (2013). Using an ultrasonic bath for extraction of seaweed samples

(Wakame) and HPLC-ICP-MS in reversed phase mode, they tested the following enzymes: cellulase, β -glucosidase, α -amylase, lipase, pepsin, pancreatin and a mixture of pancreatin, lipase and β -glucosidase. Since similar amounts of inorganic iodine were determined regardless of the enzyme used, their results suggested that the major part of inorganic iodine is leached from the samples by the action of buffer solution and ultrasonication. On the other hand, MIT and DIT were not determined in the extracts after extraction using α -amylase, β -glucosidase, cellulase or lipase, while both species were determined when pepsin, pancreatin or a mixture of three enzymes (pancreatin, lipase and β -glucosidase) were used. These results proved organic iodine species to be associated with proteins in seaweed. Using the mixture of pancreatin, lipase and β -glucosidase, they obtained the lowest amounts of DIT and inorganic iodine, compared to pancreatin and pepsin. They chose pancreatin for further experiments, since they obtained the highest concentration of MIT in the extracts, while amounts of DIT and inorganic iodine were comparable to those extracted with pepsin.

1.4.2.2 Separation and detection of iodine compounds by hyphenated techniques

Hyphenated speciation techniques are combined systems including separation and detection of the species (molecules) or specific elements present in these species (Michalke, 2003). During separation, species equilibrium can be drastically altered and species transformations are likely to occur, so the total separation time must be shorter than the velocity of species transformation (Michalke, 2003). There are some great advantages in using element-selective detection, such as extended variability and wider applicability (Michalke, 2003).

The choice of separation technique is based in the physicochemical properties of the analytes, such as volatility, charge and polarity (Caruso & Montes-Bayon, 2003). Liquid chromatography (usually high performance liquid chromatography, HPLC) is the most commonly used, although gas chromatography and capillary electrophoresis (CE) can also be used for the separation step (Moreda-Piñeiro et al., 2011). The latter is a high-resolution and fast separation technique, which enables the separation of neutral molecules, and positive and negative ions in a single run (Caruso et al., 2003; Caruso & Montes-Bayon, 2003). Used mainly on human samples, thyroid hormones, their precursors as well as inorganic iodine species can therefore be separated by CE in a very short time (a few minutes) (Moreda-Piñeiro et al., 2011).

Although less selective detectors such as UV spectrophotometry and electrochemical detectors have been used for iodine speciation studies, ICP-MS has been proposed as the most convenient and selective detection system and is therefore used the most (Moreda-Piñeiro et al., 2011).

1.4.2.2.1 Liquid chromatography

HPLC is the most commonly used technique for separation of iodine species, operating in three different modes – anion exchange chromatography (AEC), reversed phase chromatography (RPC) and size exclusion chromatography (SEC) (Moreda-Piñeiro et al., 2011).

Reversed phase chromatography is the preferred method over normal phase chromatography to use with ICP-MS detectors. Due to the polar mobile phase, it is better for plasma stability, safety and waste disposal (Caruso et al., 2003; Caruso & Montes-Bayon, 2003). Although inorganic forms of iodine can be detected, RPC is more suitable

for separation of organic iodine species (Moreda-Piñeiro et al., 2011). However, iodide and iodate cannot be separated by RPC and additional procedures must be applied, usually using AEC (Romarís-Hortas et al., 2013).

In ion exchange chromatography, two different modes, cation- and anion-exchange, are used. The latter is more commonly applied in iodine speciation. It is useful when iodide and iodate have to be separated in addition to organic iodine compounds (e.g., MIT and DIT), which can also be detected (Moreda-Piñeiro et al., 2011).

For separation of compounds with high molecular weight (e.g., proteins), size exclusion chromatography is used, and iodine bound to high and low weighted molecules can therefore be determined (Caruso & Montes-Bayon, 2003; Moreda-Piñeiro et al., 2011). Coupling of SEC to an element-specific detector is often used for the first screening of an unknown sample (Caruso & Montes-Bayon, 2003; Cornelis et al., 2003).

1.4.2.3 Separation of iodine compounds in algae and seaweed samples

Iodine compounds have not yet been determined in samples of terrestrial plants. However, for iodine speciation in algae and seaweed samples, different RPC and AEC columns were used. Some of them are listed in the Table 1.5.

Table 1.5: Reversed phase and anion exchange columns that have been used for iodine speciation in algae and seaweed samples.

Reference	Column	Species*	LOD
Han, Cao, Cheng, Liu & Xu (2012)	RPC – Diamonsil C18 (4.6 mm × 150 mm, 5 µm)	I ⁻	6.1 ng/g
		MIT	7.9 ng/g
		DIT	24 ng/g
Romarís-Hortas et al. (2013)	RPC – Zorbax Eclipse XDB-C8 (4.6 mm × 150 mm, 5 µm) with Phenomenex C8 guard column (4 mm × 3.0 mm)	Inorganic iodine	3.2 ng/g
		MIT	2.4 ng/g
		DIT	3.4 ng/g
	AEC – Dionex IonPac AS7 (250 mm × 4 mm, 10 µm) with Dionex IonPac AG7 guard column (50 mm × 4 mm, 10 µm)	I ⁻	-
		IO ₃ ⁻	
		MIT	
Shah et al. (2005)	AEC – Ion Pac AS-11 (250 mm × 2.0 mm, 13 µm)	I ⁻	0.12 µg/L
		IO ₃ ⁻	0.2 µg/L
	RPC – Alltima C18 (150 mm × 4.6 mm, 5 µm)	I ⁻	-
		MIT	
		DIT	
		DIT	

* ... Species that can be determined by the applied method using the stated column

- ... Not stated in the paper

Chapter 2

Research Aims and Hypotheses

2.1 Goals of the Thesis

The goals of the thesis were:

- to develop/optimize and validate a method for iodine determination in plant samples by ICP-MS after microwave-assisted extraction,
- to develop, optimize and validate a method for iodine compounds determination by HPLC-ICP-MS after enzymatic extraction,
- to determine total iodine concentrations in different plant species (peas, buckwheat, pumpkin, chicory) after two different biofortification approaches with iodine and selenium solutions (breeding sprouts after seed soaking and foliar spraying during growth on the field),
- to investigate which approach can be used for enrichment of plants with iodine,
- to investigate which form of iodine is better uptaken by plants and whether there is any similar trend for different plant species,
- to investigate whether the form of iodine in plants is the same as the form of iodine in the soaking/spraying solution or whether any transformation processes occur during uptake, translocation and growth,
- to study the distribution of iodine in selected enriched plants,
- to determine iodine compounds in selected fortified plants,
- to investigate whether the presence of selenium in the solution for soaking/spraying has any effect on iodine uptake, what this effect is and whether the trend is similar in different plant species.

2.2 The Hypotheses

- Both investigated biofortification approaches increase iodine concentrations in selected parts of a plant.
- Sprouts contain higher amounts of iodine after soaking seeds in iodine solutions than the selected parts of mature plants after foliar spraying in the blooming period or in the rosette phase (3–4 leaves).

- Uptake of iodine in the form of iodide is higher than in the form of iodate.
- Selenium in inorganic forms does not affect iodine uptake by plants.
- The majority of uptaken iodine is in the same form as in the soaking/spraying solution, part of it is transformed to iodinated amino acids (monoiodotyrosine and diiodotyrosine). After enzymatic extraction, iodine is not completely extracted and part of it remains in the sample residue. Only some species are identified of the extracted iodine.

2.3 Description of Plants Selected for Iodine and Selenium Enrichment

To achieve the goals of the study and to confirm or disprove the hypotheses, several plant species were selected for biofortification study with iodine (and selenium). Plants were divided into two sections – sprouts and adult plants. The following plants were investigated: green peas (*Pisum sativum* L., cultivar ‘Petit Provençal’), pumpkin (*Cucurbita pepo* L., cultivar ‘Slovenska Golica’) and buckwheat (*Fagopyrum esculentum* Moench, cultivar ‘Darja’) as sprouts and adult plants and chicory (*Cichorium intybus* L. var. *foliosum*, cultivar ‘Leonardo’) as adult plants only.

Although adult plants are well known in human nutrition, sprouts, young plants formed from seeds during sprouting, gained attention as recently as the last decades of the past century. Sprouts are a great source of protein, vitamins, minerals and other nutrients important for human health. They are consumed at the beginning of their growth and so, compared to adult plants, the nutrient content is much higher. Sprouts of adzuki bean, alfalfa, broccoli, buckwheat, clover, mungo bean, mustard, radish, red cabbage and soybean are the most popular in human nutrition. Due to the fat content and fatty acid composition, protein content and amino acid composition and vitamin content, sprouts are interesting for investigation (Márton, Mándoki, Csapó-Kiss, & Csapó, 2010).

Legumes, of which peas are one, are a primary plant protein source. Plants from the leguminous family are used in both human and animal nutrition (Dahl, Foster, & Tyler, 2012). In addition to being a source of protein, peas contain high amounts of starch, fiber, vitamins, minerals and phytochemicals (phenolic compounds, of which the major ones are tannins, phenolic acids and flavonoids, phytates, saponins and oxalates). Due to these constituents, peas have many health benefits, such as maintaining metabolic, cardiovascular and gastrointestinal health, weight management etc. (Dahl et al., 2012).

Pumpkins can be cultivated at a wide range of altitudes in various parts of the world (Yadav, Jain, Tomar, Prasad, & Yadav, 2010). Although fruits and green parts are also edible, nutritionally the most important part are seeds, which have been used in traditional medicine for centuries, for the treatment of problems of the kidney, urinary tract and against tapeworms (Stibilj, Kreft, Smrkolj, & Osvald, 2004; Yadav et al., 2010). They are high in protein content, essential fatty acids and linoleic acid, vitamins of B group and E, nutritional plant sterols and also contain relatively large amounts of K, Ca, Cr, Na, Mg, P, Mn, Fe, Zn, Cu, Mo and Se (El-Adawy & Taha, 2001; Yadav et al., 2010). Some of the compounds present in pumpkin seeds have biological activity and medicinal properties. Some of the health benefits of pumpkin seeds are anti-diabetic activity, antioxidant activity, anti-carcinogenic effect, antimicrobial activity etc. (Yadav et al., 2010).

Buckwheat is an important functional food with a high content of polyphenols, proteins, balanced amino acid composition and relatively high fiber content. It is also rich in vitamin B1 and B2 and contains antioxidants (tocopherols, phenolic substances such as rutin, quercetin, quercitrin, phenolic acids etc.) and large amounts of available Zn, Cu and Mn (Bonafaccia, Marocchini & Kreft, 2003; Eggum, Kreft & Javornik, 1980; Holasova et al., 2002; Ikeda & Yamashita, 1994; Luthar, 1992; Steadman, Burgoon, Lewis, Edwardson & Oberndofr, 2001). Buckwheat is a pseudocereal that does not contain gluten and is therefore suitable for nutrition of patients with coeliac disease (Wieslander & Norbäck, 2001). Rutin, present in buckwheat, benefits human health by antioxidative activity, antihypertensive activity, anti-inflammatory activity etc. (Suzuki et al., 2015).

Chicory is a bushy perennial herb, used as a salad vegetable. It is important in human nutrition since it is resistant to low temperatures and can therefore be cultivated in winter time, when most other vegetables are not available (Germ, Stibilj, Osvald & Kreft, 2007; Rossetto et al., 2005). The beneficial health effects of chicory, which mainly arise from flavonoids, are protection against cancer, cardiovascular diseases and aging (Rossetto et al., 2005).

All of the plants selected for biofortification in this study are cultivated in Slovenia. According to the Statistical Yearbook of the Republic of Slovenia 2013 (Statistical Office of the Republic of Slovenia, 2013), chicory production is the highest among the plants selected for enrichment in this study (Table 2.1). In fact, the production of chicory was in 9th place among all vegetables produced in Slovenia in 2012 and peas were in 23rd place (Statistical Office of the Republic of Slovenia, 2013).

Table 2.1: Statistical data of production of selected plants in Slovenia in 2012 (Statistical Office of the Republic of Slovenia, 2013).

	Production area (ha)	Production (t)
Peas	57	186
Pumpkin (for oil)	4512	2542
Buckwheat	1676	1340
Chicory	202	2562

Chapter 3

Materials and Methods

3.1 Chemicals and Standards

In all the procedures, ultra-pure water (18 M Ω cm) from the Milli-Q purification system (Millipore Corporation Bedford, MA, USA) was used, unless otherwise stated. For the extraction of total iodine, 25% tetramethylammonium hydroxide in water (TMAH, Acros Organics) was used. Methanol (MeOH, HPLC-PLUS-gradient, Carlo Erba Reagents) was added to the mobile phase for the anion exchange column and was also used to prepare some of the stock solutions. NH₄NO₃ (p.a., Kemika, Zagreb or Alkaloid-Skopje) was used for the mobile phase for the anion exchange column. Acetic acid (AcH, Panreac) was used in mobile phases for reversed-phase chromatography. KH₂PO₄ (p.a., Merck) and NaOH (p.a., Carlo Erba Reagents) were used to prepare buffer for enzymatic extraction.

The following enzymes were used in speciation analysis: pancreatin from porcine pancreas (4 $\times$ USP specifications, P1750, Sigma), protease from *Streptomyces griseus* (Type XIV, $\geq$ 3.5 units/mg solid, P5147, Sigma), α -amylase from porcine pancreas (Type VI-B, $\geq$ 10 units/mg solid, A3176, Sigma), lipase from porcine pancreas (Type II, L3126, Sigma).

Iodine standards were prepared from KI (> 99%, Sigma-Aldrich), KIO₃ (p.a., Kemika, Zagreb or Sigma-Aldrich), moniodotyrosine (MIT, 97%, Acros Organics) and diiodotyrosine (DIT, 99%, Acros Organics). Stock solutions of iodide and iodate were prepared in water, while MIT and DIT were prepared in a solution containing 0.01 M NaOH/MeOH in a ratio of 1:1. All standard solutions were stored at 4 °C. Stock solutions (1000 μ g I/g) were diluted with water to 1 μ g I/g. Further dilutions were performed daily as needed. Tellurium ICP standard (traceable to SRM from NIST, Merck) was used as internal standard. The original standard (1000 μ g Te/mL in 2–3% HNO₃) was diluted to 1 μ g Te/g. Before adding to samples prior to measurement, it was further diluted to 100 ng Te/g.

For method optimization as well as further accuracy and precision checking, two different plant reference materials were used – BCR 129 (Hay powder) and NIST SRM 1573a (Tomato leaves). For method optimization, two reference materials of animal origin were also used – BCR 150 (Skim milk powder) and NIST SRM 1549 (Non-fat milk powder).

3.2 Samples

Plants were cultivated at the Biotechnical Faculty, University of Ljubljana. Two different approaches were used to enrich plants with iodine and/or selenium. Sprouts were grown in a greenhouse after soaking seeds in solutions of different iodine and/or selenium forms. Adult plants were grown in an experimental field and were treated in the blooming period (pea, pumpkin and buckwheat) or in the rosette phase (chicory, 3–4 leaves) with the same iodine and/or selenium solutions. In all experiments, two different forms of each element were used – iodide I(–I) as potassium iodide (KI), iodate I(V) as potassium iodate (KIO_3), selenite Se(IV) as sodium selenite (NaSeO_3), and selenate Se(VI) as sodium selenate (NaSeO_4). Iodine was applied in concentration 1000 mg I/L and selenium in concentration 10 mg I/L, unless otherwise stated. Each treatment was performed in 2–4 replicates, depending on the experiment and each replicate was analysed in two parallels. Samples were lyophilized prior to iodine determination. The lyophilized sample was taken as dry sample and the results are thus calculated on dry weight (DW) basis.

Throughout the thesis, samples will be designated in short as “plant treatment”, e.g., pea sprouts grown from seeds soaked in a solution of I(–I)+Se(VI) will be written as “pea sprouts I(–I)+Se(VI)” or buckwheat achenes from buckwheat plants treated with I(V) will be written as “buckwheat I(V)”. In the case of pumpkin or buckwheat sprouts, when different concentrations were used, it will be added, e.g., “pumpkin sprouts I(V)-1000+Se(VI)-10”. If the form of element is not specified, it refers to both forms, e.g., I(–I)+Se means both the combination I(–I)+Se(IV) and I(–I)+Se(VI).

3.2.1 Sprouts

3.2.1.1 Pea sprouts

In the experiments conducted in June and July 2013, 161.6 ± 0.2 g and 161.4 ± 0.1 g of pea seeds (*Pisum sativum* L., cultivar ‘Petit Provençal’), respectively, were soaked in 200 mL of water (control) or solutions of iodine and/or selenium (Figure 3.1A) for 8 h. Iodine was present in the soaking solutions in the form of I(–I) or I(V) and selenium in the form of Se(IV) or Se(VI), in concentrations of 1000 mg I/L (7.9 mmol I/L) and 10 mg Se/L (0.13 mmol Se/L). Seeds were soaked in the following solutions: I(–I), I(V), Se(VI), I(–I)+Se(VI), and I(V)+Se(VI) in the first experiment and I(–I), I(V), Se(IV), I(–I)+Se(IV) and I(V)+Se(IV) in the second. After soaking, seeds were evenly distributed on plastic plates ($\varnothing$ 32 cm), covered with filter paper (Figure 3.1B) and put in a bright air-conditioned room. Each treatment was performed in duplicate in each experiment. During germination and growth, plates were moisturized with tap water as required. In the first experiment, sprouts were grown for 14 days (average day/night temperatures were 21 °C/15 °C) and in the second experiment for 12 days (average day/night temperatures were 22 °C/18 °C). The results from the two experiments were combined and average concentrations were calculated from all replicates of the same treatment, regardless of the experiment in which they were grown. Sprouts were sampled when two extended true leaves had developed (Figure 3.1C). Seed germination was $96 \pm 2\%$ in the first experiment and $95 \pm 2\%$ in the second. After counting and weighing, plants were divided into sprouts (stem with leaves), roots and seed residue (Figure 3.1D) and separately lyophilized at -50 °C and 0.05 mbar (Gamma 1-16 LSC, Martin Christ, Osterode am Harz, Germany), milled and homogenized in a planetary micro mill (MM 200, Retsch, Hann, Germany). Samples were stored until analysis at -20 °C. Detailed growing conditions were described by Jerše et al. (2017).



Figure 3.1: Procedure of breeding pea sprouts – soaking of seeds (A), distribution on plates with moist filter paper (B), sprouts before sampling (C) and sprout with marked division for further analysis (D). Photo: Nina Kacjan Maršić.

3.2.1.2 Pumpkin sprouts

Pumpkin (*Cucurbita pepo* L., cultivar ‘Slovenska Golica’) sprouts were grown in a growth chamber in February and March 2014. Seeds were soaked in the following solutions: water (control), 250 mg I(-I)/L, 1000 mg I(-I)/L, 250 mg I(V)/L, 1000 mg I(V)/L, 2.5 mg Se(IV)/L, 10 mg Se(IV)/L, 2.5 mg Se(VI)/L, 10 mg Se(VI)/L, 1000 mg I(-I)/L + 10 mg Se(IV)/L, 1000 mg I(-I)/L + 10 mg Se(VI)/L, 1000 mg I(V)/L + 10 mg Se(IV)/L and 1000 mg I(V)/L + 10 mg Se(VI)/L. Two experiments were performed; in the first, each element was applied alone, while in the second, combinations of the two elements were added. Controls were present in both experiments. Each treatment was made in 1–3 replicates. Seeds (0.2 g) were soaked for 4 h and placed into rolled filter paper (Figure 3.2A). The average temperature in the growth chamber was 22 °C and humidity 70–80%. Sprouts were sampled as whole plants when two cotyledons had fully developed (10–11 days after soaking, Figure 3.2B), lyophilized at –50 °C, milled and homogenized in a planetary micro mill and stored at –20 °C until analyzed. Detailed growing conditions were described by Germ et al. (2015).



Figure 3.2: Pumpkin sprouts growing in rolled filter paper before sampling (A) and after sampling (B). Photo: Nina Kacjan Maršić.

3.2.1.3 Buckwheat sprouts

In two experiments, one conducted in June and the second in July 2015, seeds (0.024 ± 0.002 g) of common buckwheat (*Fagopyrum esculentum* Moench, cultivar ‘Darja’) were soaked for 4 h in solutions of different iodine and/or selenium forms. The concentration of selenium in the soaking solution was 10 mg/L in both experiments, while the concentration of iodine in the first experiment was 1000 mg/L and in the second 1500 mg/L. Control sprouts were grown in both experiments. Each treatment was performed in triplicate. After soaking, seeds were distributed on plates and grown for 13 days in the first experiment and 10 days in the second (Figure 3.3). The temperature during growth was 22–24 °C. Sprouts were sampled as whole plants when two true leaves had developed, lyophilized, milled and homogenized in a planetary micro mill and stored at –20 °C until analyzed. Between 71 and 89% of seeds germinated. The growing conditions were also described by Germ et al. (2015).



Figure 3.3: Buckwheat sprouts before sampling. Photo: Nina Kacjan Maršić.

3.2.2 Adult plants

3.2.2.1 Pea

Pea seeds (*Pisum sativum* L., cultivar ‘Petit Provençal’) were sown in March 2015 and iodine and selenium were applied by foliar spraying at the beginning of the blooming period (56 days after sowing). Both iodine (1000 mg/L) and selenium (10 mg/L) were present in the spraying solutions, in two forms in the following combinations: I(–I), I(V), Se(IV), Se(VI), I(–I)+Se(IV), I(–I)+Se(VI), I(V)+Se(IV) and I(V)+Se(VI), each in triplicate. Control plants were sprayed with water. The arrangement of the blocks on the field is shown in Figure 3.4A. Detailed site description and growing conditions have been described by Jerše et al. (n.d.) Plants were sampled in two parts – in the first part, 19 days after I and Se application, pods were sampled and in the second part, 29 days after treatment, whole plants were sampled, which were then divided into pods, leaves, stems and roots (Figure 3.4B). Pods from both samplings were further divided into pod shells and seeds, but iodine content was only determined in those from the first sampling. Samples were lyophilized (Gamma 1-16 LSC or Gamma 1-16 LSCplus, Martin Christ, Osterode am Harz, Germany), milled and homogenized in a planetary micro mill – leaves, seeds and pod shells or in a rotor speed mill (Pulverisette 14, Fritsch, Idar-Oberstein, Germany) – stems. Roots were cut into small pieces (approx. 2–3 mm long) before lyophilization. Samples were stored at –20 °C until analysis.



Figure 3.4: Pea plants – arrangement of the blocks on the field (A) and sampled plants after the last picking of the pods (B). Photo: Lara Škof.

3.2.2.2 Pumpkin

Pumpkin seeds (*Cucurbita pepo* L., cultivar ‘Slovenska Golica’) were sown in April 2014 and transplanted one month later. Plants were foliar sprayed with I and/or Se solutions (the same solutions as for pea plants) when plants had developed three to four leaves (3 weeks after transplanting, at the beginning of blooming). Each treatment was performed in three replicates. Plants growing on the field are shown in Figure 3.5. Pumpkin seeds were sampled approx. 3.5 months after foliar spraying, lyophilized, milled in a knife mill (Grindomix GM 200, Retsch, Hann, Germany) after freezing in liquid nitrogen, and stored at –20 °C until analysis.



Figure 3.5: Pumpkin fruits approx. two weeks before sampling. Photo: Marjetka Pirc.

3.2.2.3 Buckwheat

Seeds of common buckwheat (*Fagopyrum esculentum* Moench, cultivar ‘Darja’) were sown in July 2014. Foliar spraying with iodine and selenium solutions (the same as for pea plants) was performed in the blooming period (Figure 3.6), 4 weeks after sowing, each treatment in four replicates. Achenes (in other studies sometimes also called seeds or grains) were sampled in the mature phase, 7 weeks after foliar spraying, lyophilized, milled in a knife mill (M20, IKA® Werke, Staufen, Germany) and stored at -20°C until analysis.



Figure 3.6: Buckwheat plants when foliar sprayed (just before blooming). Photo: Janja Turk.

3.2.2.4 Chicory

Chicory (*Cichorium intybus* L. var. *foliosum*, cultivar 'Leonardo') seedlings (approx. 1 month old) were transplanted to the field in July 2015 and foliar sprayed with iodine and selenium solutions (the same as for pea plants) 2 weeks later, in the rosette phase (3–4 leaves), each in three replicates. Outer leaves were sampled during the growth period (5 weeks after foliar application, at the beginning of head formation), and inner leaves (heads) were sampled in the phase of technological maturity (11 weeks after spraying, Figure 3.7). Samples were lyophilized, milled and homogenized in a planetary micro mill (Pulverisette 7, Fritsch, Idar-Oberstein, Germany) and stored at -20°C until analyzed.



Figure 3.7: Chicory - before the second sampling (A) and weighing of the heads (B). Photo: Nina Kacjan Maršić.

3.3 Procedures

3.3.1 Iodine content in plant samples

3.3.1.1 Determination of iodine content in plant samples by ICP-MS

For determination of the iodine content Schöniger combustion and extraction in microwave system were performed.

The combustion of 0.12 g of sample was done in an oxygen atmosphere in a Schöniger flask (2 L) containing 15 mL of 0.1 or 0.5% TMAH as an absorption solution. After combustion and absorption (mixing for 25 min), the solution was quantitatively transferred to polyethylene tubes and diluted to 40 mL with TMAH (the same concentration as in the sorption solution).

For microwave alkaline extraction of plants, 15 g of milled sample was weighed into a vessel and 10 mL of water and 2 mL of TMAH were added. Vessels were closed and placed in a microwave system (Ethos I, Milestone, Italy or UltraWave, Milestone, Italy). The following temperature program was used in the Ethos I microwave system: 20 min ramp to 200°C , 5 min hold on 200°C and cooling to a temperature below 35°C . In the UltraWave system, the initial closing pressure was 40 bar and the temperature program was set to 10 min ramp to 200°C and 10 min hold on 200°C . After cooling, the pressure

was released (8 bar/min) when the temperature in the reactor dropped below 40 °C at. Quartz and Teflon vessels were used in Ethos 1, and disposable glass vessels in UltraWave. After microwave-assisted extraction, extracts were quantitatively transferred into 30 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) and diluted with water. Extracts were stored at 4 °C until analysis. Before measurement, extracts were filtered through 0.45 µm PVDF syringe filters (Millex-HV, Millipore) and diluted with water so that 0.1% TMAH was present in the final solution. If samples needed to be further diluted, TMAH was added so that the concentration of TMAH in the measured solution was always the same. Before measurement, a tellurium internal standard was added in the final concentration in a measured solution of 1 ng Te/g of solution.

Iodine (^{127}I) determination was performed by an ICP-MS or ICP-QQQ instrument. The latter operated in single quadrupole mode. Operating conditions are summarized in Table 3.1. To check accuracy and precision, reference materials BCR 129 (Hay powder) and NIST SRM 1573a (Tomato leaves) were analyzed each time that the analysis was performed. The procedure is schematically presented in Figure 3.8.

Table 3.1: ICP-MS/ICP-QQQ operating conditions.

	ICP-MS	ICP-QQQ
<i>Nebulizer</i>	MicroMist	MicroMist
<i>Plasma conditions</i>		
RF power [W]	1500	1550
Plasma gas flow rate [L/min]	15	15
Carrier gas flow rate [L/min]	0.90 ^a	1.05 ^a
Makeup gas flow rate [L/min]	0.10 ^a	0.00 ^a
<i>Octopole cell</i>		
He gas flow rate [mL/min]	3	3
H ₂ gas flow rate [mL/min] ^b	4	4
<i>Measurement</i>		
Monitored m/z	^{127}I (analyte), ^{125}Te (internal standard)	^{127}I (analyte), ^{125}Te (internal standard)
Integration time/point [s]	0.2	1
Number of points/mass	3	1

^a ... parameters were changed daily as required to optimize sensitivity; values close to those in the table were generally used

^b ... used only when testing the best mode of the octopole cell

3.3.1.2 Determination of iodine content in plant samples by k_0 -INAA

k_0 -INAA was used as a reference method for iodine determination due to the lack of reference materials. Samples (approx. 0.2 g) were compressed in a pellet with diameter 10 mm. Each pellet was irradiated with the Al-0.1% Au standard (IRMM-530R alloy) for 5 minutes in a TRIGA reactor with a thermal neutron flux of $1.1 \times 10^{12} \text{ cm}^{-2}\text{s}^{-1}$. The gamma activity of induced radionuclide ^{128}I ($t_{1/2} = 24.99 \text{ min}$, $E = 442.9 \text{ keV}$), which originates from the reaction $^{127}\text{I}(n, \gamma)^{128}\text{I}$ with thermal neutrons, was measured on an absolutely calibrated HPGe detector (Canberra, USA) with 45% relative efficiency after 10 and 30 min of cooling (Jaćimović, Smodiš, Bučar & Stegnar, 2003). The procedure is

shown in brief in Figure 3.8. On the basis of gamma activity of induced radionuclide in plant sample the limit of detection (LOD) of k_0 -INAA was 0.19 $\mu\text{g I/g}$.

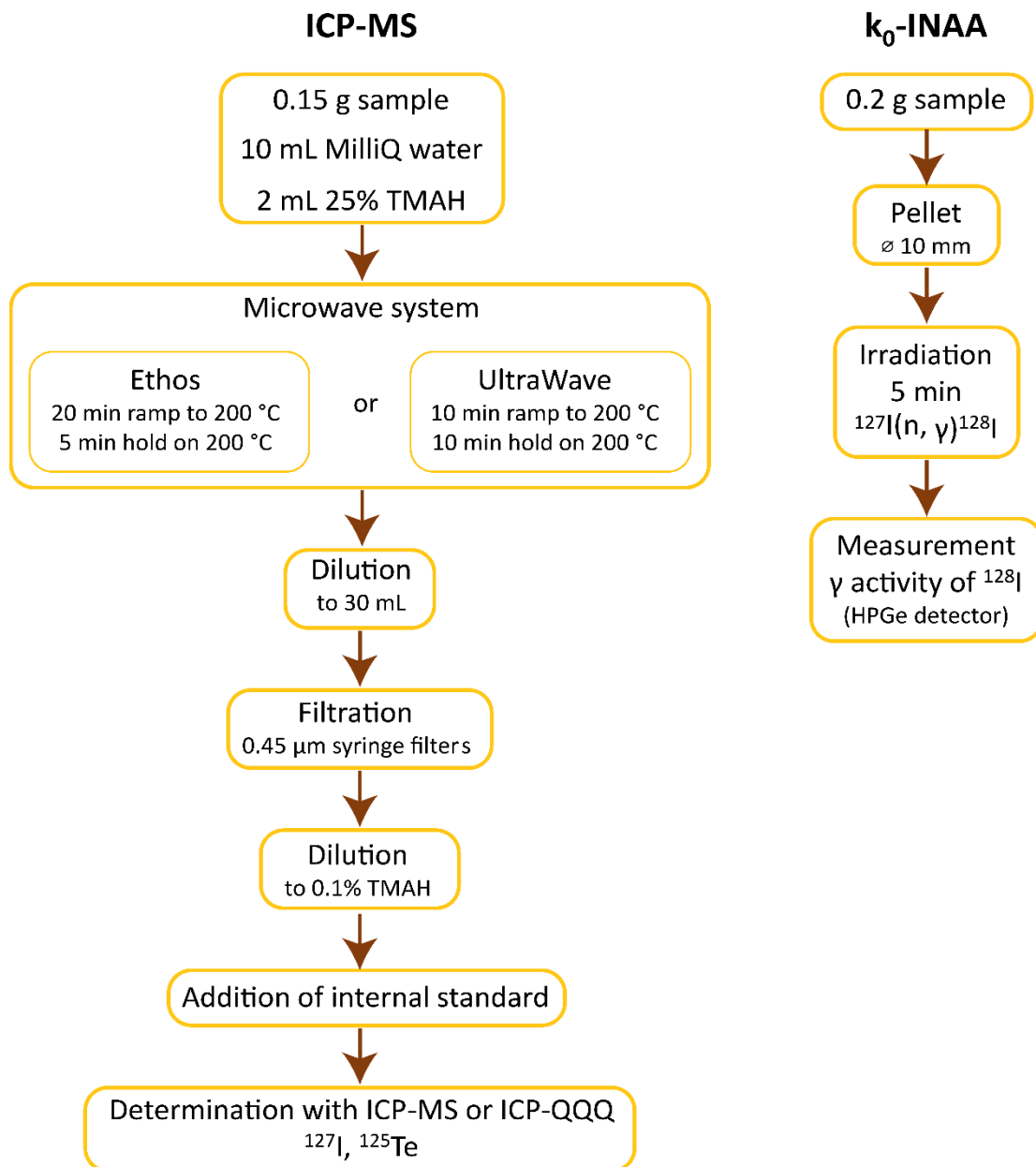


Figure 3.8: Procedures for determination of total iodine concentrations in plant samples.

3.3.2 Iodine compounds and their content in plant samples

3.3.2.1 Enzymatic extraction with an ultrasonic bath

Enzymatic extraction using an ultrasonic bath was performed according to Romarís-Hortas et al. (2013). A milled algae sample (0.2 g) was mixed with 7 mL of pancreatin (pancreatin from porcine pancreas ($4 \times$ USP specifications, P1750, Sigma)) solution containing 40 mg of enzyme in a 0.2 M KH_2PO_4 /0.2 M NaOH buffer at pH 8. After ultrasound irradiation for 12 h at the temperature set to 50 °C (temperature was not controlled during the extraction), the mixture was centrifuged at 3000 rpm for 15 min. The extracts were filtered through 0.2 μm CA syringe filters before injection into the HPLC-ICP-MS system (HPLC system Flexar, Perkin Elmer and ICP-MS system NexION 300X, Perkin Elmer) with a reversed phase column (RPC) Zorbax Eclipse XDB-C18 (4.6×150 mm, 5 μm ; Agilent) and Phenomenex AQ C18 (4×3.0 mm) guard column. The operating conditions are summarized in Table 3.2 (Part 1). Blank samples were prepared in each experiment and concentrations were calculated based on a standard addition technique. Iodine in extracts was determined by ICP-MS after dilution with Milli-Q water (without further microwave extraction with TMAH).

3.3.2.2 Enzymatic extraction with an ultrasonic probe

The extraction procedure involved mixing 0.2 g of milled sample and 7 mL of buffer (0.2 M/0.2 M KH_2PO_4 /NaOH, pH = 8) solution containing 100 mg of enzyme in centrifuge tubes, unless otherwise stated. The mixture was irradiated by ultrasound using an ultrasonic probe (Sonics Vibra cell, model CV18, 130W, 20 kHz, titanium tip) at 70% amplitude in 5 cycles of 1 min with 2 min breaks, unless otherwise stated. During the extraction, the centrifuge tubes were placed in ice to prevent heating of the mixture. After centrifugation at 3000 rpm for 15 min (room temperature), the extract solution was separated from the sample residue and filtered through 0.2 μm CA syringe filters. Prior to analysis by the HPLC-ICP-MS system, extracts were stored at -20 °C. Samples were prepared in duplicate. Blank samples were prepared in each experiment.

Analysis was performed by HPLC-ICP-MS (Perkin Elmer). Zorbax Eclipse XDB-C18 (4.6×150 mm, 5 μm ; Agilent) RPC with a Phenomenex AQ C18 (4×3.0 mm) guard column was used for separation of iodine species (inorganic iodine, MIT and DIT) in algae samples. An anion exchange column IonPac® AS7 (4×250 mm) with an IonPac® AG7 (4×50 mm) guard column was previously used to check the presence of iodate in extracts. Operating conditions are listed in Table 3.2 (Part 1).

Iodine in extracts was determined by ICP-MS after dilution with Milli-Q water (without further microwave extraction with TMAH).

3.3.2.3 Enzymatic extraction in a water bath with longitudinal shaking

A milled sample (0.2 g) was weighed in centrifuge tubes and mixed with 7 mL of buffer solution (0.2 M/0.2 M KH_2PO_4 /NaOH, pH = 8) containing 40 mg of pancreatin. Tubes were placed in a water bath with longitudinal shaking (SW22, Julabo, Germany) set to 37 °C and 200 rpm for 12 h. In the first 4–6 h, the tubes were rotated by 180° every half an hour to prevent the sample from sticking to the tube walls. After extraction, the tubes were immediately placed into ice to stop enzymatic activity and centrifuged at 4 °C and 11000 rpm for 15 min. The extract solution was separated from the sample residue (sediment), filtered through a 0.45 μm syringe filter and stored at -20 °C until analyzed.

Prior to injection into the HPLC column, extracts were filtered through a 0.22 μm PVDF syringe filter and diluted with enzyme/buffer (blank) solution if required. Blank samples were prepared in each experiment. The procedure is schematically shown in Figure 3.9.

Analyses were performed with the HPLC system (Agilent 1260 Infinity, Agilent Technologies) hyphenated with inductively coupled plasma tandem mass spectrometry (ICP-QQQ, Agilent 8800, Agilent Technologies) operating in single quadrupole mode. An anion exchange column IonPac[®] AS7 (4 $\times$ 250 mm) with an IonPac[®] AG7 (4 $\times$ 50 mm) guard column was used for separation of iodine species (iodide, iodate, MIT and DIT) in sprouts samples. The operating conditions are summarized in Table 3.2 (Part 2). For determination of iodine concentrations in the extracts, an aliquot of extract was mixed with 25% TMAH and water to achieve the same ratio between sample, water and TMAH as used for determination of iodine concentrations in samples. After extraction in a microwave system, samples were diluted and filtered. Prior to analysis by ICP-MS, samples were again diluted as required and a tellurium internal standard was added, as stated in section 3.3.1.1.

Table 3.2: Instrumental parameters of HPLC and ICP-MS/ICP-QQQ.

	Part 1 ^a	Part 2 ^b
ICP-MS		
Radiofrequency power [W]	1600	1600
Plasma gas flow rate [L/min]	16	15
Carrier gas flow rate [L/min]	1.2	0.8
Option gas ^c	0.01 L/min ^d	10% ^e or 0.01 L/min ^d
Monitored m/z	¹²⁷ I ¹²⁵ Te (internal standard)	¹²⁷ I
AE-HPLC		
Column	IonPac [®] AS7 (4 mm i.d. $\times$ 250 mm $\times$ 10 μm)	IonPac [®] AS7 (4 mm i.d. $\times$ 250 mm $\times$ 10 μm)
Precolumn	IonPac [®] AG7 (4 mm i.d. $\times$ 50 mm $\times$ 10 μm)	IonPac [®] AG7 (4 mm i.d. $\times$ 50 mm $\times$ 10 μm)
Injection volume	20 μL	20 μL
Mobile phase	175 mM NH ₄ NO ₃ in 15% (v/v) MeOH	175 mM NH ₄ NO ₃ in 5% (v/v) MeOH
Mobile phase flow gradient	0.0–8.0 min: 0.5 mL/min	0.0–8.0 min: 0.5 mL/min
	8.0–9.0 min: 0.5 $\rightarrow$ 1.5 mL/min	8.0–9.0 min: 0.5 $\rightarrow$ 1.2 mL/min
	9.0–17 min: 1.5 mL/min	9.0–17 min: 1.2 mL/min
	17–18 min: 1.5 $\rightarrow$ 0.5 mL/min	17–18 min: 1.2 $\rightarrow$ 0.5 mL/min
	18–20 min: 0.5 mL/min	18–20 min: 0.5 mL/min

	Part 1 ^a	Part 2 ^b
RP-HPLC		
Column	Agilent Zorbax XDB-C18 (4.6 × 150 mm, 5 µm)	
Precolumn	Phenomenex AQ C18 (4 × 3.0 mm)	
Injection volume	20 µL	
Mobile phases	A: 0.2% (v/v) acetic acid in water B: 1% (v/v) acetic acid in 85/15 water/methanol C: 0.2% (v/v) acetic acid in methanol	
Mobile phase composition gradient	0.0–3.0 min: 98% A, 2% B, 0% C 3.0–3.5 min: 98 → 90% A, 2 → 0% B, 0 → 10% C 3.5–6.0 min: 90% A, 0% B, 10% C 6.0–6.5 min: 90 → 80% A, 0% B, 10 → 20% C 6.5–8.0 min: 80% A, 0% B, 20% C 8.0–8.5 min: 80 → 60% A, 0% B, 20 → 40% C 8.5–14 min: 60% A, 0% B, 40% C 14–15 min: 60 → 98% A, 0 → 2% B, 40 → 0% C 15–16 min: 98% A, 2% B, 0% C	
Flow rate [mL/min]	0.75	
Postcolumn internal standard	¹²⁵ Te (10 µg/L)	

^a ... Parameters used in the first part of the study – extraction with ultrasonic probe and comparison with extraction using ultrasonic bath

^b ... Parameters used in the second part of the study – extraction with water bath with longitudinal shaking, comparison with extraction using ultrasonic bath and determination of iodine compounds in pea, pumpkin and buckwheat sprouts

^c ... option gas is 20% O₂ in Ar

^d ... Set in Perkin Elmer NexION 300X instrument

^e ... Set in Agilent 8800 instrument

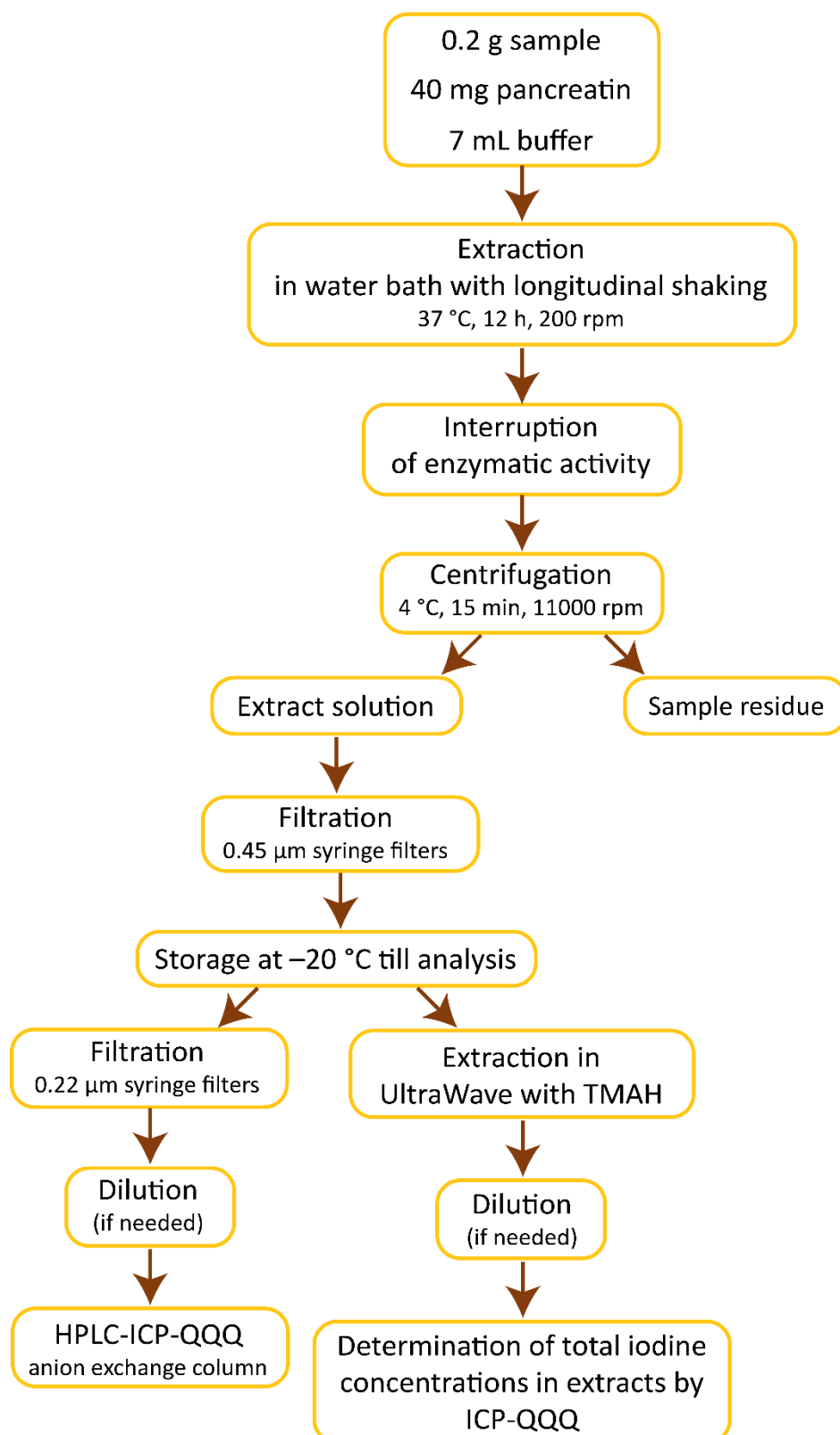


Figure 3.9: Procedure for determination of iodine compounds in plant samples.

3.4 Statistical Data

Statistical analysis was performed using Statgraphics Centurion XVI (Statgraphics, Herndon, VA, USA). Analysis of variance (ANOVA) was used to assess the effect of treatments on iodine and selenium content, possible interactions between these elements during uptake and the effect on other measured parameters. Differences between treatments were estimated using Tukey's (HSD) *post-hoc* test at a significance level $p = 0.05$.

Chapter 4

Results and Discussion

4.1 Optimization and Validation of Analytical Procedure for Determination of Iodine Concentrations in Plant Samples

4.1.1 Sample combustion and alkaline extraction

4.1.1.1 Schöniger combustion

Schöniger combustion was often used as a sample preparation procedure in the past (Gelinas, Iyengar & Barnes, 1998; Knapp et al., 1998; Schramel & Hasse, 1994). We thus wanted to test the method to see whether it can be used for preparation of plant samples for further iodine determination by ICP-MS.

The results obtained by ICP-MS showed that in 0.1% TMAH, counts per second (CPS) in blank samples were around 6000 after Schöniger combustion. The calculated LOD was 0.3 µg I/g, making this type of sample preparation useless for iodine determination in plants. However, LODs of around 0.05 µg I/g were determined after Schöniger combustion (0.2 g of sample, 10 mL of 0.05 M NaOH solution) by Schramel & Hasse (1994).

In addition to being a time consuming procedure, another disadvantage of Schöniger combustion is analytical error due to the adsorption and desorption effect caused by the high ratio of the flask surface to the sample amount (Knapp et al., 1998).

4.1.1.2 Microwave extraction procedure

Due to the high CPS of blanks and LODs as well as time consuming procedure, we decided rather to optimize microwave extraction for further work.

4.1.1.2.1 Temperature program

The temperature programs listed in Table 4.1 were tested using 5 mL of water + 5 mL of 25% TMAH added to the sample for extraction. The first two temperature programs were chosen according to the microwave system manufacturer's recommendations regarding the temperature for use in work with quartz vessels – 180 °C is recommended as the highest temperature for digestion, while the limit is 200 °C for a short time. The third program was found in the literature, applied to algae samples (Romarís-Hortas et al., 2011). However, the power of the microwave oven was insufficient for the set of 20 vessels and

the actual temperature did not increase as set in the program. Consequently, the real temperature in the vessels reached 200 °C later than set in the program and the step of holding on maximum temperature was shorter than 5 min. Although the results were acceptable, the ramp time was extended to 20 min to prevent the risk of incomplete extraction and to avoid working the microwave oven at maximum power for an extended time.

Table 4.1: Temperature programs tested for extraction of iodine from samples.

Program 1	15 min ramp to 180 °C
	15 min hold on 180 °C
	5 min ramp to 200 °C
	5 min hold on 200 °C
Program 2	20 min ramp to 180 °C
	15 min hold on 180 °C
	5 min ramp to 200 °C
	10 min hold on 200 °C
Program 3	10 min ramp to 200 °C
	5 min hold on 200 °C
Program 4	20 min ramp to 200 °C
	5 min hold on 200 °C
Program 5	10 min ramp to 200 °C
	10 min hold on 200 °C

The results for iodine content showed no differences in extraction of plant samples when using different temperature programs, nor when using different microwave systems (data not shown). The fourth program (Table 4.1) was therefore chosen for further work in Ethos 1, since it was the shortest in which the actual temperature increased properly with the set temperature program. The fifth program (Table 4.1), was used in the UltraWave system since the power was not problematic. It was chosen over the third program due to the longer time at the highest temperature and therefore lower possibility of incomplete extraction.

Quartz vessels were originally used for the extraction but Teflon vessels were also tested for this application. Glass vessels were additionally tested in the UltraWave system. No differences in the results were obtained, so quartz vessels were used in Ethos 1 and disposable glass vessels with UltraWave. Teflon vessels can also be used but particular attention has to be paid in order to avoid a memory effect, since iodine can be captured in the pores of the Teflon net at higher temperatures.

4.1.1.2.2 Water : TMAH proportion

The initial extraction medium was 25% TMAH (3 mL) but the obtained results were not usable due to the high CPS for blank samples and unrepeatable results. For further work, several combinations found in the literature were tested: 5 mL of water + 5 mL of 25% TMAH (Romarís-Hortas et al., 2011), 10 mL of water + 2 mL of 25% TMAH (Tinggi et al., 2012) and 10 mL of water + 1 mL of 25% TMAH (Smoleń et al., 2014). By Smoleń et

al. (2014), the combination of 10 mL of water + 1 mL of 25% TMAH was not used in a microwave system but 0.5 g of the samples were incubated in this solution for 3 hours at 90 °C in closed tubes. A mixture of 10 mL of water + 0.5 mL of TMAH was additionally tested in our study. The agreements of obtained results with certified values in BCR 129 ($0.167 \pm 0.024 \mu\text{g I/g}$), BCR 150 ($1.29 \pm 0.09 \mu\text{g I/g}$) and NIST SRM 1549 ($3.38 \pm 0.02 \mu\text{g I/g}$) and with informative values in NIST SRM 1573a ($0.85 \mu\text{g I/g}$) are summarized in Figure 4.1. The best agreement was observed when using 5 mL of water + 5 mL of TMAH or 10 mL of water + 2 mL of TMAH. A slightly lower percentage was found after extraction performed with mixtures of 10 mL of water + 1 mL of 25% TMAH or 10 mL of water + 0.5 mL of 25% TMAH, indicating incomplete extraction of iodine or losses after opening the vessels due to an insufficient amount of base (TMAH). Since the consumption of the organic base is lower and higher accuracy in diluting the extracts can be assured, the procedure using 10 mL of water + 2 mL of TMAH was chosen for further work. In spite of the variation of the final volume in the vessels for the extraction, with different proportions between water and TMAH, no differences were observed in the results, since the lowest volume (10 mL when the proportion 1:1 was used) and highest volume (12 mL when the 1:5 ratio was used) gave the same results.

Although the main focus of this method development was optimization of the method for plant materials, two reference materials of animal origin were additionally used for the optimization for two reasons – firstly, the lack of plant reference materials with certified value for iodine and, secondly, to check whether the method can also be used for iodine determination in samples of animal origin.

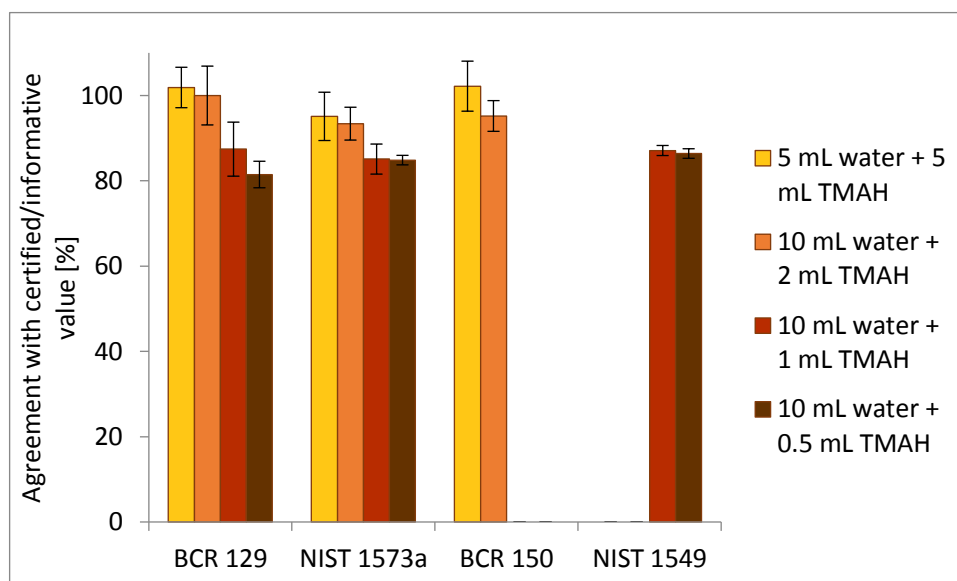


Figure 4.1: Agreement between obtained and certified/informative values of iodine in reference materials after extraction with different H₂O + TMAH proportions, with standard deviation (n = 5).

4.1.2 ICP-MS determination procedure

Before measurement, tellurium as an internal standard was added offline to each tube in a final concentration of 1 ng Te/g of solution. Iodine (¹²⁷I) determination was performed in no gas, helium and hydrogen modes (collision/reaction gas). For qualitative analysis, external calibration was compared to the standard addition technique. The latter was also

used for calculation of the yield of extraction. Different TMAH concentrations were used for iodine determination – 0.1, 0.5 and 1%. The results were calculated with and without an internal standard.

4.1.2.1 External calibration and standard addition technique

For quantitative analysis, an external calibration technique was used. A stock solution of potassium iodate with a concentration of 1 mg I/g was prepared in NH_3 and H_2O in a 1:4 proportion after drying the KIO_3 at 105 °C for 2 h. The working standards were prepared daily by dilution of the stock solution with water. At least five standard solutions were prepared each time, from the approx. limit of detection (0.01 ng I g/g of solution) up to 100 ng I/g of solution. Final calibration standards were prepared in the TMAH solution, the same percentage as in the sample solutions (0.1, 0.5 or 1%).

For the standard addition technique, 0.1 mL of KI or KIO_3 solution was added before extraction, in the following concentrations: 0.35 and 0.7 μg I/mL for BCR 129, 1.5 and 3 μg I/mL for NIST SRM 1573a and 2 and 4 μg I/mL for BCR 150. Reference materials were spiked when a 5 mL of water + 5 mL of TMAH combination was used for the extraction. In addition to comparison of the results calculated from the external calibration and standard addition, the extraction yield was calculated.

The results proved that negligible analyte loss occurred during the analytical procedure, regardless of the iodine form (I^- or IO_3^-), since good agreement between concentrations calculated from standard addition or external calibration was observed (Table 4.2). In the case of BCR 150, the results calculated from the calibration curve are in even better agreement with certified values than those determined from standard addition. External calibration can therefore be used for calculation of the results.

Table 4.2: Comparison between values calculated from the calibration curve and standard addition (average $\pm$ standard deviation).

	c_1 [μg I/g]	c_2 [μg I/g]	c_3 [μg I/g]	c_r [μg I/g]
BCR 129	0.170 ± 0.008	0.193 ± 0.017	0.143 ± 0.007	$0.167 \pm 0.024^*$
NIST SRM 1573a	0.826 ± 0.041	0.826 ± 0.014	0.871 ± 0.042	0.85
BCR 150	1.318 ± 0.076	1.512 ± 0.086	1.524 ± 0.086	$1.29 \pm 0.09^*$

c_1 ... calculated from calibration curve ($n = 4$)

c_2 ... calculated from standard addition of KI ($n = 5$)

c_3 ... calculated from standard addition of KIO_3 ($n = 5$)

c_r ... certified or informative value of iodine in reference material

* ... expanded uncertainty with a coverage factor $k=2$ (level of confidence of about 95%)

The extraction yield represents the ratio between real and theoretical concentrations. The real concentration in samples (BCR 129, NIST SRM 1573a and BCR 150) with standard addition was calculated using external calibration. The theoretical concentration was calculated as the sum of the certified/informative value in reference materials and the amount of iodine added (as I^- or IO_3^-). Yields with standard deviation of 10 determinations are shown in Table 4.3. The highest deviation from 100% was observed for BCR 129, which contained the lowest iodine content (0.167 ± 0.024 μg I/g). For all reference materials used, high extraction efficiency was found when calculated both with and without an internal standard (IS).

Table 4.3: Extraction yields with standard deviation for different reference materials and added iodine forms, calculated with and without internal standard ($n = 10$).

	Extraction yield [%]	
	no IS	with IS
Hay powder (BCR 129) + KI	109 ± 7	96 ± 12
Hay powder (BCR 129) + KIO ₃	105 ± 8	90 ± 9
Tomato leaves (NIST 1573a) + KI	100 ± 5	94 ± 4
Tomato leaves (NIST 1573a) + KIO ₃	101 ± 6	91 ± 7
Skim milk powder (BCR 150) + KI	97 ± 12	93 ± 3
Skim milk powder (BCR 150) + KIO ₃	99 ± 8	91 ± 6

4.1.2.2 Internal standard and concentration of TMAH in the solution for measurement with ICP-MS

Before measurement, samples were diluted to 0.1, 0.5 or 1% TMAH solution. Results were calculated twice – once with raw counts for iodine and once with ratios of analyte CPS to internal standard CPS. The results (Figure 4.2) showed very good agreement between obtained and certified/informative values, regardless of the TMAH concentration or use of internal standard. There was also good agreement between the results determined in different TMAH solutions, as well as between results calculated with and without internal standard.

The use of an internal standard is recommended due to the matrix correction and variations in instrument response. The sensitivity can vary not only between different days but also within the same, especially larger, set of measurements. The results within one set of measurements (50–230 measurements) in the present study were comparable when concentrations were calculated with or without the internal standard. Although these results (Figure 4.2) suggested that the matrix correction is not necessary, we concluded that it is better to use the internal standard in further work since it allows better control over the stability of the signal of the instrument. In addition, comparison of results obtained over a longer time period is more accurate, since the repeatability is more than 15% better when using an internal standard (see section 4.1.2.5). Tellurium was selected since it is close to iodine in m/z ratio (125 for Te, 127 for I) as well as first ionization potential (9 eV for Te, 10 eV for I).

The limits of detection were comparable between various percentages of TMAH in the solution and were less than 20 ng I/g of sample, based on 0.15 g in a 30 mL final solution. However, the lowest values of blank samples were obtained when 0.1% TMAH solution was measured (≈ 200 CPS), in comparison with 0.5% (≈ 600 CPS) and 1% TMAH (≈ 1100 CPS). A 0.1% TMAH solution was therefore chosen as optimal for further work.

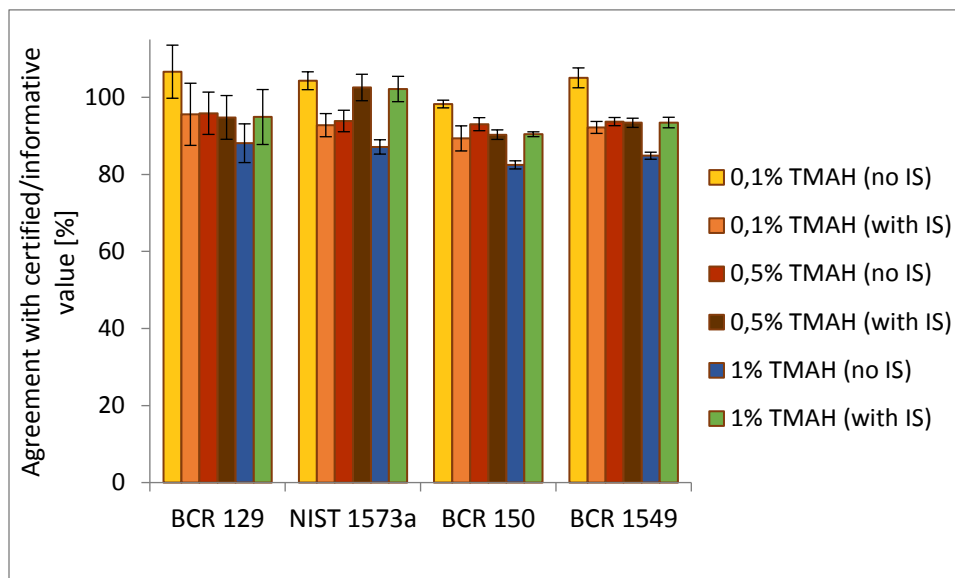


Figure 4.2: Agreement between obtained and certified/informative values in certain reference materials after measurement in a solution containing different amounts of TMAH, with and without internal standard, with standard deviation ($n = 5$).

4.1.2.3 Collision or reaction mode

Measurement by ICP-MS can be performed in our laboratory in three different modes regarding the octopole cell gas – collision mode with He, reaction mode with H_2 or no gas mode (without gas in the cell). All three modes were tested for iodine determination. The lowest LODs and blank values were achieved in collision mode (30% lower than in reaction mode and 50% lower than in no gas mode). Collision mode was consequently selected as the most suitable for iodine determination by ICP-MS.

4.1.2.4 Estimation of measurement uncertainty

The measurement uncertainty of the result was evaluated according to the “Guide to the expression of uncertainty in measurement” (JCGM, 2008). In the guide, two types of evaluation of uncertainties are defined: type A – evaluation by statistical analysis of a series of observations and type B – by means other than statistical analysis of a series of observations (JCGM, 2008). The mathematical model shown in Table 4.4 was used for calculation of the result, as well as evaluation of the measurement uncertainty. The contributions to the uncertainty of the results in the method used for iodine determination are shown in Figure 4.3.

Table 4.4: Equations for calculation of iodine content in plant samples and iodine concentration in stock solution.

$$[I_{sample}] = \frac{(CPS_{sample} - CPS_{blank})}{a} \times \frac{m_{extract\ sol.} \times m_{measured\ sol.}}{m_{sample} \times m_{aliquot}} \times \eta$$

$$[I_{stock}] = \frac{m_{KIO_3} \times w_{KIO_3} \times M_I}{M_{KIO_3} \times m_{stock\ sol.}}$$

[I] = I content in sample or stock solution

a = slope of calibration line

CPS = average counts per second of sample or blank solution

m = mass of sample, mass extract solution, mass aliquot, mass measured solution, KIO₃ or stock solution

η = recovery

w = purity

M = molar mass

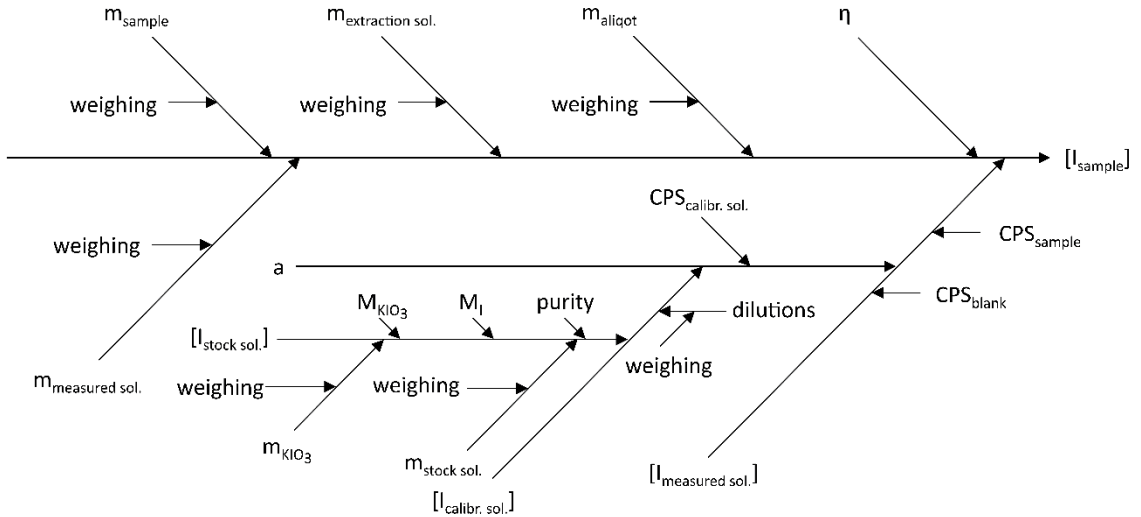


Figure 4.3: Cause and effect diagram of different uncertainty contributions to the result.

The measurand was defined as the concentration of iodine in $\mu\text{g I/g}$ of dry weight (DW) in the plant material (BCR 129, NIST SRM 1573a and iodine enriched kohlrabi leaves sample). The following contributions to uncertainty were estimated:

The uncertainty of balance was determined by weighing three different control check weights – 0.1 g, 1 g and 20 g (type A). The uncertainty of the weight that was the closest to the mass determined was used as the uncertainty of a particular result. These uncertainties refer to sample mass, mass of the extract solution, mass of aliquot of extract solution, mass of measured solution and masses of standards, which were weighed for each dilution for preparation of the calibration curve. Due to the low contribution of mass uncertainty, the uncertainties that refer to dilution were neglected.

The uncertainty of the number of counts per second (CPS) of sample and blank includes all uncertainties of the ICP-MS system and was evaluated as type A. One sample was measured 5 times to determine the uncertainty of the number of CPS of the sample and 8 blanks were measured to determine the uncertainty of number of CPS of the blank. Calibration solutions were measured twice and the uncertainty in the calibration slope was calculated using the LINEST function in Excel. An intercept was not used in the calculation but the CPS of blanks was used instead.

The uncertainty of recovery was calculated from 5 independent measurements by the standard addition technique (type A).

The combined uncertainty was calculated using an internally prepared Excel template (Figure 4.4). Figure 4.5 shows contributions to the uncertainty budget for iodine determination in reference materials BCR 129, NIST SRM 1573a and iodine enriched plant samples.

BCR-129			sample mass, g	mass of extract, g	aliquot mass, g	mass of solution, g	recovery	calibration slope	Count-Blank
I-127 (by ICP-MS)	Value		0.19740	30.2643	0.24900	10.0200	0.94	10.8583	0.2794
	Z propag.		1.000	1.000	1.000	1.000	1.000	1.000	1.000
	St.unc.		0.000012	0.000006	0.000012	0.000006	0.03	0.0166	0.0536
	Z*std unc.(%)		0.006%	0.000%	0.005%	0.000%	3.191%	0.153%	19.184%
sample mass, g	0.19740		0.19741						
mass of extract, g	30.2643			30.2643					
aliquot mass, g	0.24900				0.24901				
mass of solution, g	10.0200					10.02001			
recovery	0.94						0.970		
calibration slope	10.8583							10.8749	
Count-Blank	0.2794								0.3330

Result (I, mg/kg)		1.69E-01		0.169	0.169	0.169	0.169	0.164	0.169	0.142
differences				-1.03E-05	-3.35E-08	-8.14E-06	-1.01E-07	-5.23E-03	-2.58E-04	-2.72E-02
u(C)^2	7.67E-04	u(I)^2		1.06E-10	1.12E-15	6.63E-11	1.02E-14	2.73E-05	6.65E-08	7.40E-04
u(C)		2.77E-02								
u(C)/X		1.64E-01								
(u(I)/u(C))^2	(k=1)	1.000E+00		1.38E-07	1.46E-12	8.64E-08	1.33E-11	3.56E-02	8.67E-05	9.64E-01
index(%)	100%			0.00%	0.00%	0.00%	0.00%	3.56%	0.01%	96.43%

Estimated unc. for I-127 obtained by ICP-MS at the IJS, Ljubljana, Slovenia

BCR-129	Unit	Value	St. unc.	RSD, %	Unc. Contr.	Index (%)
sample mass, g	g	0.19740	0.00001	0.006%	-1.03E-05	0.00%
mass of extract, g	g	30.2643	0.0000	0.000%	-3.35E-08	0.00%
aliquot mass, g	g	0.249000	0.000012	0.005%	-8.14E-06	0.00%
mass of solution, g	g	10.02000	0.00001	0.000%	-1.01E-07	0.00%
recovery		0.94	0.03	3.191%	-5.23E-03	3.56%
calibration slope		10.8583	0.0166	0.153%	-2.58E-04	0.01%
Count-Blank		0.2794	0.0536	19.184%	-2.72E-02	96.43%
Result	mg/kg	0.169	0.028	16.4%	(k=1)	100%

Note:
Fill only yellow cells

Figure 4.4: Template used for calculation of combined standard uncertainty (with an example for BCR 129).

For BCR 129, which has the lowest iodine content, the major uncertainty contribution (> 96%) was due to the counts of the sample and blank, while a smaller share was from the uncertainty of the procedure recovery. On the other hand, the greatest contribution to the combined uncertainty for NIST SRM 1573a and iodine enriched plant samples was from the uncertainty of the procedure recovery and a smaller share from the uncertainty of counts (Figure 4.5, Table 4.5). For all three samples, other uncertainties were negligible.

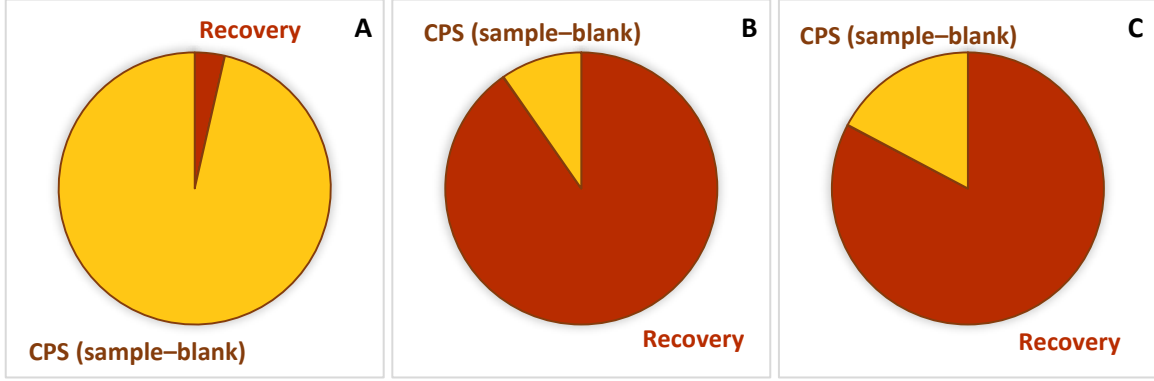


Figure 4.5: Contributions to the combined measurement uncertainty obtained for BCR 129 (A), NIST SRM 1573a (B) and iodine enriched plant samples (C).

Expanded uncertainty U ($k=2$) of the mean value was calculated as follows:

$$U = 2 \times u_{\text{mean}} \quad (1)$$

where u_{mean} is combined standard uncertainty of the mean calculated as:

$$u_{\text{mean}} = \sqrt{\frac{(St.dev.)^2}{n} + u_c} \quad (2)$$

where $St.dev.$ is standard deviation of the results obtained from n replicates and u_c is overall method combined standard uncertainty (see Table 4.5).

For one result of particular sample type, we obtained the following results, given with the uncertainty ($2 \times u_c$, $k = 2$, approx. level of confidence 95%) as well as with relatively expressed uncertainties stated in the brackets: 0.169 ± 0.055 $\mu\text{g I/g DW}$ (32.8%) for BCR 129, 0.73 ± 0.05 $\mu\text{g I/g DW}$ (6.5%) for NIST SRM 1573a and 1.88 ± 0.13 $\mu\text{g I/g DW}$ (6.8%) for iodine enriched plant sample. Results for 7–8 measurements of iodine content in reference materials with the expanded uncertainty U are summarized in Table 4.6. The results showed large measurement uncertainty for reference material with low iodine content. However, above the certain iodine content in sample or reference material, the uncertainty seemed to be constant (6.5–6.8%). In addition, satisfactory of our data was evaluated by calculation of statistical parameter $E_n\text{-score}$ (ISO 13528:2015, 2015), which is calculated as follows:

$$E_n = \frac{X_{\text{lab}} - X_{\text{ref}}}{\sqrt{U_{\text{lab}}^2 + U_{\text{ref}}^2}} \quad (3)$$

where X_{lab} is the laboratory value, U_{lab} is the expanded uncertainty of laboratory value, X_{ref} is the certified value and U_{ref} is the expanded uncertainty of the certified value.

As shown in Table 4.6, $E_n\text{-score}$ is < 1.0 , which confirm that our results are satisfactory.

Table 4.5: Contributions to the uncertainty budget (standard uncertainties, $k = 1$) for iodine determination in BCR 129, NIST SRM 1573a and iodine enriched plant samples.

Uncertainty contribution	BCR 129			NIST SRM 1573a			Sample		
	Value	St. unc.	Index (%)	Value	St. unc.	Index (%)	Value	St. unc.	Index (%)
Sample mass [g]	0.1974	0.000012	0.00	0.1476	0.000012	0.00	0.1485	0.000012	0.00
Mass of extract [g]	30.2643	0.000006	0.00	30.7755	0.000006	0.00	31.0067	0.000006	0.00
Mass of aliquot [g]	0.2490	0.000012	0.00	0.6120	0.000012	0.00	0.5970	0.000012	0.00
Mass of measured solution [g]	10.0200	0.000006	0.00	10.0340	0.000006	0.00	10.0260	0.000006	0.00
Recovery	0.94	0.03	3.56	0.94	0.03	90.25	0.94	0.03	82.69
Calibration slope [g/ μ g]	10.858	0.017	0.01	10.594	0.009	0.07	10.594	0.009	0.06
CPS (sample-blank)	0.2794	0.0536	96.43	2.2201	0.0227	9.68	5.7398	0.0822	17.25
Result (iodine content and u_c [μg/g])	0.169	0.028		0.73	0.02		1.88	0.06	

Table 4.6: Obtained values (average $\pm$ expanded uncertainty U , $k = 2$) for iodine in reference materials determined under optimized conditions.

	Certified ^a /informative ^b	Obtained value	n	Agreement [%]	<i>En-score</i>
	value [μ g I/g]	[μ g I/g]			
BCR 129	0.167 ± 0.024^a	0.167 ± 0.058	8	100	0.00
NIST SRM 1573a	0.85^b	0.79 ± 0.06	8	93	-
BCR 150	1.29 ± 0.09^a	1.23 ± 0.09	7	95	-0.47

4.1.2.5 Quality assurance and method validation

The correlation coefficients for the calibration curves (linear function) in 0.1% TMAH were 0.9995 or higher, indicating a good linear response through the range of studied concentrations (from LOD up to 100 ng I/g of solution). A similar linear range was also found by other researchers, since Tagami et al. (2006) found the linear response of an instrument to be in a range between 0.01 and 100 ng/mL, Schramel & Hasse (1994) reported a linearity of the calibration curve up to 100 ng/mL and G  linas et al. (1998) even beyond 100 ng/mL.

The repeatability of measurements, calculated as the relative standard deviation of calibration line slopes within five months, was 7.5%. The repeatability was calculated from slopes obtained with an internal standard.

In every series of measurement, iodine was measured in at least five individually prepared blank samples. Limits of detection (LODs) were calculated as the 3-fold standard deviation of blanks for each set of measurements individually. Under optimized conditions of extraction and detection, limits of detection were 3–20 ng I/g of sample for 0.15 g of sample in 30 mL of final solution. A comparable LOD of 20 ng I/g of samples (0.3 g of food sample in 50 mL final solution) was reported by Tinggi et al. (2012), while Romar  s–Hortas et al. (2011) obtained a slightly higher LOD of 25 ng I/g based on 0.1 g of algae samples in 50 mL final solution.

For all analysed reference materials, E_m -score was below 1 indicating good agreement between obtained and certified/informative values for iodine (Table 4.6).

4.1.2.6 Comparison with an independent method

Due to the lack of plant reference materials with certified value for iodine, an independent method was used to prove that the optimized method gives accurate results for different plant materials. Very good agreement between the results for iodine content obtained with the optimized method using ICP-MS after alkaline microwave-assisted extraction and k_0 -INAA was observed (Figure 4.6). This proved that the method optimized in the present study can be used for a variety of different plant samples in a wide concentration range. Since good agreement was found between the two methods, it is evident that losses occurring during the sample preparation procedure (microwave extraction) were negligible, and that a negligible amount of iodine was lost during filtration through the PVDF syringe filters prior to measurement with ICP-MS. However, most of the results obtained by ICP-MS were slightly lower than those obtained by k_0 -INAA. This is probably due to small losses occurring during the sample preparation procedure that is necessary before ICP-MS analysis, but not when using k_0 -INAA. Another possible explanation for slightly higher results obtained by k_0 -INAA may be the presence of other induced radionuclides (typically ^{24}Na ($t_{1/2} = 897.6$ min), ^{38}Cl ($t_{1/2} = 37.24$ min) and ^{56}Mn ($t_{1/2} = 154.74$ min)) with higher gamma energies, which increase background near gamma energy of 443 keV (^{128}I).

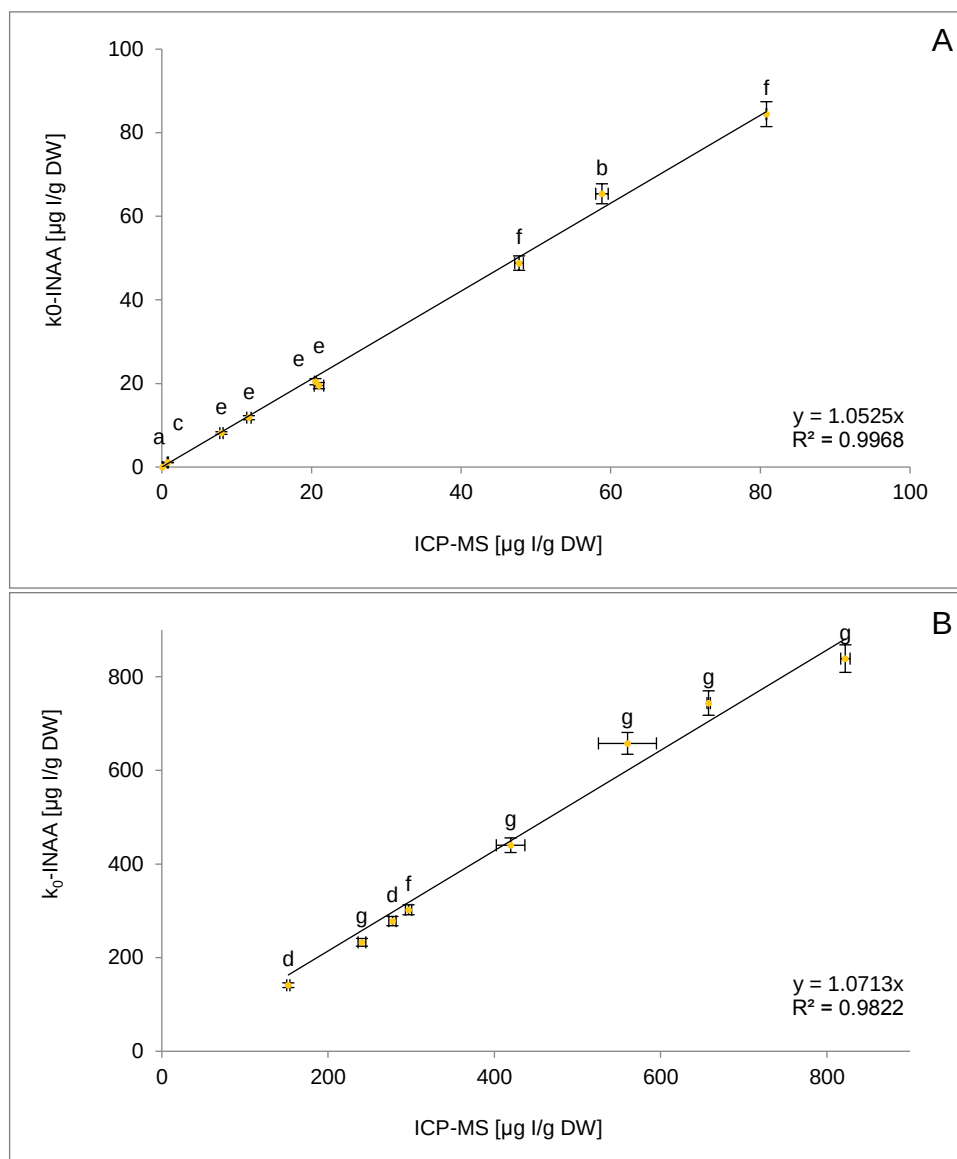


Figure 4.6: Comparison of the results obtained with ICP-MS (with standard deviation) and the independent method k_0 -INAA (with combined standard uncertainty, $k = 2$). Lower concentrations (< 100 $\mu\text{g I/g}$) are shown on Figure 4.6A and higher (> 100 $\mu\text{g I/g}$) on Figure 4.6B. Letters next to the points indicate sample type:

- a – pea seeds,
- b – leaves of pea plants,
- c – buckwheat achenes,
- d – pea sprouts,
- e – seed residues remaining after pea sprouts growth,
- f – pumpkin sprouts,
- g – buckwheat sprouts.

4.2 Optimization of the Analytical Procedure for Determination of Iodine Compounds in Plant Samples

Optimization of the analytical procedure for determination of iodine compounds in plant samples was performed in two parts. In the first part, we investigated whether or not an ultrasonic probe is suitable for enzymatic extraction of iodine species from plant samples. In the second part, the procedure for enzymatic extraction in a water bath with longitudinal shaking was optimized.

4.2.1 Optimization of enzymatic extraction with an ultrasonic probe

The following parameters were optimized in the extraction procedure using an ultrasonic probe: buffer pH, mass of enzyme, amplitude of ultrasonic probe and time of sonication. In addition to iodine standards, samples of brown algae *Fucus Virsoides* were used, since the matrix is more similar to samples for which the method was already developed using an ultrasonic bath (Romarís-Hortas et al., 2013).

Algae were sampled in 2009 at three different locations on the Slovenian coast – Žusterna, Portorož and Debeli Rtič and the determined iodine content was between 274 and 464 $\mu\text{g I/g}$ (Osterc & Stibilj, 2012). After harvesting, samples were rinsed with seawater to remove sand, sediment and other particles and organisms and then with tap water. Freeze drying was performed at $-54\text{ }^{\circ}\text{C}$ for 172 h (Alpha 1-4, Christ) and samples were milled in a Pulverisette 14 (Fritsch, Germany) at a rotational speed of 18000 rpm (Osterc & Stibilj, 2012).

Iodine content in the algae samples were determined by radiochemical neutron activation analysis (RNAA) in 2009 and published by Osterc & Stibilj (2012). For comparison, iodine content in some of the samples was determined again in 2015 by ICP-MS after an alkaline microwave-assisted extraction, using the method by Romarís-Hortas et al. (2013). The results obtained by the two methods are listed in Table 4.7 and good agreement indicated no losses of iodine during storage.

Table 4.7: Iodine concentration in *Fucus Virsoides* determined by ICP-MS (average $\pm$ mean absolute error of 2 parallels) and RNAA (average $\pm$ measurement uncertainty at 95% confidence, $k = 2$).

Location/date of sampling	ICP-MS	RNAA ^a
	[$\mu\text{g I/g DW}$]	[$\mu\text{g I/g DW}$]
Žusterna/1.4.2009	283 ± 4	274 ± 9
Debeli Rtič/15.7.2009	405 ± 21	417 ± 9
Portorož/26.11.2009	417 ± 14	438 ± 12

^a ... Results by Osterc & Stibilj (2012)

4.2.1.1 Optimization of buffer pH

Extraction was performed as described in section 3.3.2.2, using 40 mg of enzyme and testing pH of 6, 7, 8 and 9. The mixture was sonicated for 3 min with 40% amplitude.

In the algae samples used for optimization, iodide and MIT were the only species that were identified by RP-HPLC-ICP-MS. The results showed lower concentrations of iodide extracted at pH 6 and 7 than at 8 or 9. Compared to neutral pH, approx. 21% more iodide

was extracted at pH 8 and 9, but no differences were observed between these two. The highest amount of MIT was extracted at pH 6. However, due to the higher extractability of iodide, pH 8 was chosen for further experiments. Romarís-Hortas et al. (2013) also chose pH 8 as optimal for extraction of iodine species in their study of iodine speciation in seaweed.

4.2.1.2 Optimization of mass of enzyme

Enzyme masses of 0, 20, 40, 60 and 100 mg were used for the extraction of samples in 7 mL of buffer at pH 8. Samples were sonicated for 3 min with 40 % amplitude.

From the tested masses of enzyme (0–100 mg), the highest amount of MIT was extracted when using 100 mg of enzyme, approx. 9-fold more than without enzyme. With 100 mg of enzyme, the average iodide concentration was similar to those obtained with 40 and 60 mg of enzyme (on average 26% higher than without enzyme). Due to better results for MIT, 100 mg was therefore chosen as optimal of those tested.

4.2.1.3 Amplitude optimization

The sample was mixed with 7 mL of buffer at pH 8, containing 100 mg of pancreatin. The mixture was sonicated for 3 min using 20, 30, 40, 50, 60, 70, 80 and 90 % amplitude.

The results showed that the highest amount of MIT was extracted using an amplitude of 70%, more than 2-fold higher than when using the lowest amplitude, 20%. One of the best results for iodide was also obtained using 70%, which extracted 42% more iodide than when using 20%. The lowest amounts of both iodine compounds, iodide and MIT, were extracted using 20% amplitude. Extraction using amplitudes of 80 and 90% were also performed and the obtained concentrations of iodide were slightly higher, but the same results were achieved with a longer time of sonication. In order to reduce the risk of species transformations due to higher amplitude, 70% was selected.

4.2.1.4 Time of sonication

Optimization of the time of sonication was performed in two steps. In the first step, the algae sample, in 7 mL of pancreatin solution containing buffer at pH 8 and 100 mg of pancreatin, was sonicated with an amplitude of 70% for 1, 2, 3 or 5 minutes. In the second experiment, two different modes were used: 1 cycle of 5 min of sonication or 5 cycles of 1 min of sonication with 30 s, 1 or 2 min breaks between each cycle. The second part of the experiment was performed with a mixture of iodine species standards (I^- , MIT and DIT) or with I^- standard alone in buffer at pH 8 containing 100 mg of pancreatin, to evaluate possible losses and/or species transformations.

In the first experiment, when the algae sample was sonicated for 1, 2, 3 or 5 minutes, the amounts of iodide and MIT were highest using 5 minutes, so this time was chosen as optimal. Longer times were not tested. However, iodine species might be volatile and since the solution became very hot during the extraction in spite of keeping it in ice, the next step was extraction using only iodine standards to prove that there were no losses or conversions between species during the extraction procedure. In addition to 5 minutes of sonication, which had been chosen in the previous experiment, 5 cycles of 1 min of sonication with 30 s breaks between each cycle were used. Extraction was performed using standards of iodide, MIT and DIT (separately) in buffer with enzyme. The results are shown in Figure 4.7A. The first column is the theoretical value that should be obtained (calculated amount of added standard). The results for iodide were much lower than

expected and we presumed that this was because of losses. In the next experiment, only iodide standard was extracted and the breaks between each cycle of sonication (1 min) were longer – 1 or 2 min. For comparison, the solution without sonication was also measured. The first column in Figure 4.7B shows the expected value (known concentration of standard), the second column shows the amount of iodide in the solution that was not sonicated. Since there was no significant difference between sonicated and non-sonicated solutions, this proved that there were no losses during extraction. However, the obtained concentrations were lower than the theoretical concentration. The experiment was repeated using a post-column internal standard (tellurium, 50 $\mu\text{g/L}$) and calibration curve prepared in buffer instead of in mobile phase A, as in previous experiments, and the results (Figure 4.7C) were in better agreement with the theoretical value. However, concentrations were higher than expected when using the internal standard, and it is therefore better to use a standard addition to correct for matrix effects (see section 4.2.1.5). Although the results were comparable using 1 or 2 min breaks, 5 cycles of min sonication with 2 min breaks were chosen as a better option, in order to prevent overheating of the solution.

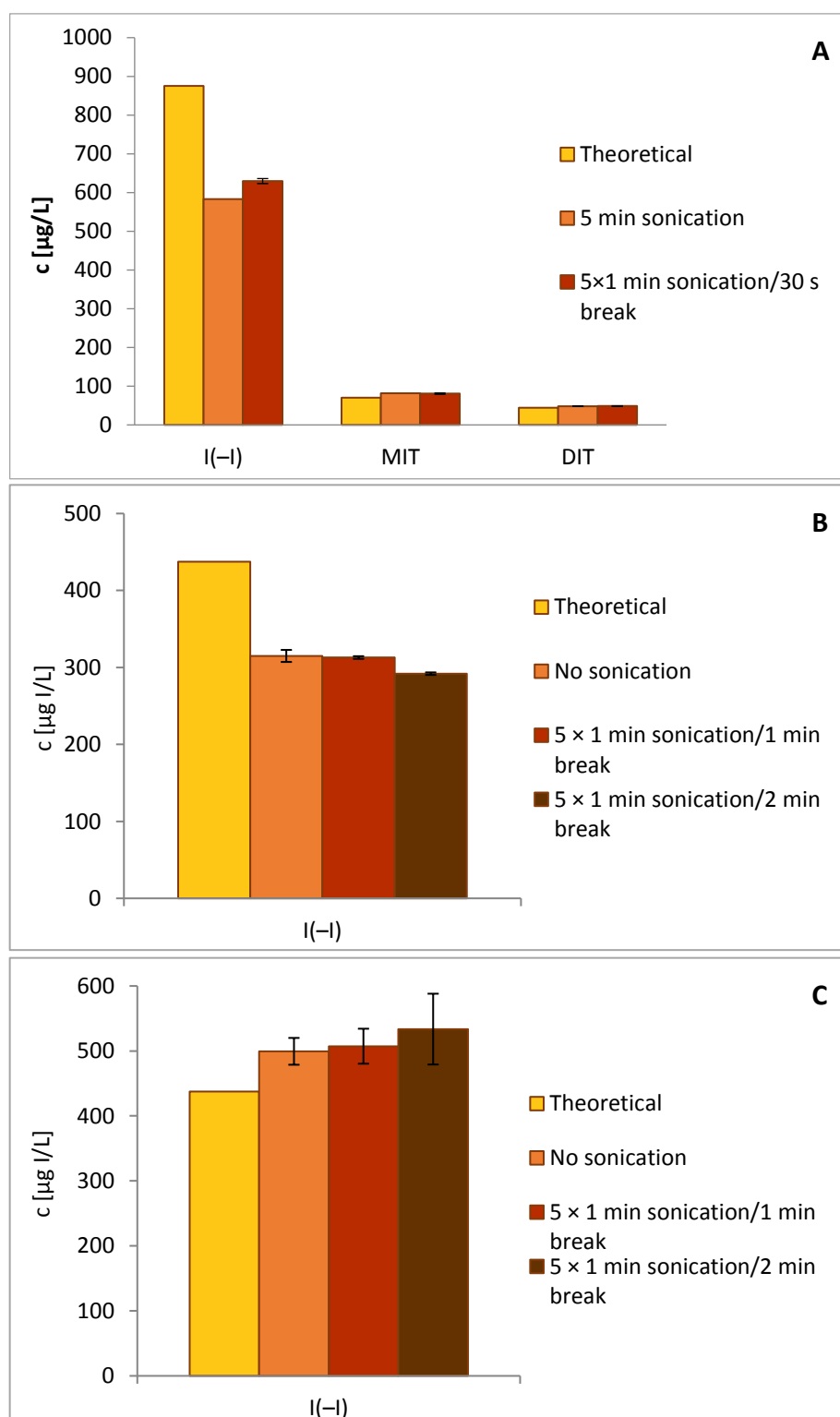


Figure 4.7: Results for time optimization with iodine standards (given as average with standard deviation of 3 parallels);

A: different modes of sonication with or without breaks, concentrations determined based on calibration curve (standards prepared in mobile phase A (Romarís-Hortas et al., 2013)),

B: sonication of iodide standard, concentrations determined based on calibration curve (standards prepared in mobile phase A (Romarís-Hortas et al., 2013))

C: sonication of iodide standard, post-column internal standard used, concentrations determined based on calibration curve (standards prepared in buffer)

4.2.1.5 Yield of extraction

For determination of the extraction yield, samples were prepared in triplicate and standard additions of I⁻ (0.875 and 1.313 µg I/L), MIT (0.070 and 0.105 µg I/L) and DIT (0.018 and 0.026 µg I/L) were added before the extraction. Concentrations stated in brackets are final concentrations in the mixture for extraction.

It was shown in this experiment that a matrix effect is present in extracts of algae samples and a standard addition must therefore be used prior to measurement for quantification of iodine species instead of external calibration. It was also proven that there were no losses during the extraction procedure. The analytical recovery for all three species was between 97 and 114%. The relative standard deviations of the three replicates for iodide, MIT and DIT were 3.8%, 11.3% and 16.1%, respectively.

4.2.2 Iodine speciation in algae samples and comparison between extraction using an ultrasonic probe and extraction in an ultrasonic bath

Iodine species were determined in extracts of algae samples after enzymatic extraction using an ultrasonic probe, as well as an ultrasonic bath. To calculate concentrations in algae samples, a standard addition technique was used (standards were added after extraction in one of the samples). The results are summarized in Table 4.8.

The obtained values for extracted iodine were relatively low after both methods, which suggests that a large amount of iodine is strongly bound to the components in the sample residue or incorporated in proteins that cannot be hydrolysed with the proposed extraction methods. However, higher amounts of iodine were extracted when using an ultrasonic bath (37–71% of the iodine in samples) than when using an ultrasonic probe (12–48% of the iodine in samples). The same trend can be observed for MIT concentrations but, in the majority of the samples, iodide concentrations were higher when using an ultrasonic probe. However, during method optimization, a lack of batch-to-batch repeatability was observed when using an ultrasonic probe. We therefore concluded that the method developed by Romarís-Hortas et al. (2013) is better for extraction of iodine species from algae samples, in spite of the longer time, since the repeatability of the results was proved within a day (RSDs of 8 samples were 9% for MIT and 4% for DIT). In contrast to our results, Romarís-Hortas et al. (2013) reported high amounts of extracted iodine in seaweed (76–96%). As shown in Table 4.8, neither iodine content nor extractability of iodine from *Fucus Virsoides* were dependent on the sampling season.

A low percentage of identified species was also observed in brown algae *Fucus Virsoides* (26–35%), which was not the case in the study by Romarís-Hortas et al. (2013), since they identified 83% and 106% of iodine species in the brown seaweeds, Wakame and Kombu, respectively, while in other types (red and green seaweeds) 50–92% of species were identified. The range between 50 and 106% of identified iodine species suggests that this is strongly dependant on the seaweed species.

Table 4.8: Concentrations of iodine species in algae samples (in $\mu\text{g I/g}$ of dry weight (DW) sample) after two different extraction procedures.

Location of sampling	Iodine [μg I/g DW] ^a	Ultrasonic probe				Ultrasonic bath			
		Extracted iodine ^{b,c}	MIT ^b	Iodide ^b	Σ ^{e,f}	Extracted iodine ^{b,c}	MIT ^b	Iodide ^b	Σ ^{e,f}
		[μg I/g DW]	[μg I/g DW]	[μg I/g DW]	[μg I/g DW]	[μg I/g DW]	[μg I/g DW]	[μg I/g DW]	[μg I/g DW]
<i>Sampling date: 1.4.2009</i>									
Portorož	464 ± 10	119 ± 4 (26)	4.5 ± 0.1	93.4 ± 2.0	97.9 (82)	197 ± 12 (42)	7.4 ± 0.1	62.3 ± 3.5	69.73 (35)
Žusterna	274 ± 9	104 ± 9 (38)	1.6 ± 0.1	86.7 ± 2.1	88.3 (85)	145 ± 1 (53)	2.4 ± 0.3	35.9 ± 1.3	38.3 (26)
Debeli Rtič	328 ± 6	107 ± 11 (33)	2.1 ^d	83.7 ^d	85.8 (80)	141 ± 12 (43)	3.4 ± 1.0	38.4 ± 0.6	41.8 (30)
<i>Sampling date: 15.7.2009</i>									
Portorož	451 ± 10	-	1.2 ± 0.1	305.8 ± 4.7	307.0 (-)	320 ± 4 (71)	0.8 ± 0.2	88.0 ± 0.3	88.8 (28)
Žusterna	324 ± 7	-	0.9 ± 0.1	135.4 ± 2.7	136.3 (-)	171 ± 4 (53)	1.8 ± 0.1	49.2 ± 0.5	51.0 (30)
Debeli Rtič	417 ± 9	97 ± 4 (23)	2.4 ± 0.1	94.5 ± 1.1	96.9 (100)	186 ± 10 (45)	4.9 ± 0.1	51.4 ± 0.4	56.3 (30)
<i>Sampling date: 26.11.2009</i>									
Portorož	438 ± 12	92 ± 10 (21)	3.5 ^d	37.2 ± 0.2	40.7 (44)	178 ± 8 (41)	4.4 ± 0.5	43.4 ± 1.3	47.7 (27)
Žusterna	387 ± 8	48 ± 3 (12)	1.7 ± 0.1	24.2 ± 0.3	25.9 (54)	142 ± 10 (37)	3.8 ± 0.1	36.7 ± 0.8	40.5 (29)

a ... determined by Osterc & Stibilj (2012)

b ... the results are given as an average concentration with mean absolute error of 2 parallels in $\mu\text{g I/g}$ of sample

c ... values in brackets represent the percentage of extracted iodine

d ... it was possible to determine the area of the peak in one of the replicates only

e ... sum of identified species

f ... values in brackets represent the percentage of identified extracted iodine

- ... not determined

4.2.3 Optimization of enzymatic extraction in a water bath with longitudinal shaking and study of stability of iodine species

When using an ultrasonic probe, we struggled with difficulties in controlling the temperature of the extract solution, contamination and the repeatability of the extraction and we therefore decided rather to use a water bath with longitudinal shaking in the second part of the study. Many ultrasonic baths have an option to set the temperature, but the temperature of the water inside the bath usually increases more during sonication. Since an ultrasonic probe is much more powerful, this is an even bigger problem, because the temperature increases quite fast directly in the sonicated solution and the risk of transformation of compounds and/or analyte losses is higher. Although extraction in a water bath with longitudinal shaking is as time consuming as in an ultrasonic bath, or even more so, the temperature control is much more accurate and, consequently, the conditions are more repeatable between different sets of samples.

Optimization of extraction for determination of iodine species in terrestrial plants was performed with special attention paid to the stability of the iodine species. Since no plant reference material with certified or at least informative values for iodine compounds is commercially available, optimization was performed with buckwheat sprouts.

An anion exchange column was used, since we were also interested in separation of inorganic iodine species, iodide and iodate, which cannot be separated with a reversed phase column, which is usually used for iodine speciation studies (Han et al., 2012; Romarís-Hortas et al., 2013; Shah et al., 2005).

Initially, the method developed by Romarís-Hortas et al. (2013) described in section 3.3.2.1 was used with minor changes. The mass of the sample, volume and composition of the buffer and mass of enzyme were retained but different enzymes were tested during the optimization and dissolving the enzyme in buffer was compared to dissolving in water. Unless otherwise stated, the extractions described in the following sections were performed in a water bath with longitudinal shaking. The results obtained with the optimal parameters in a water bath with longitudinal shaking were then compared with results obtained after extraction using an ultrasonic bath.

The composition of the mobile phase was also changed (175 mM NH_4NO_3 in 5% methanol instead of 15%) and the program was slightly modified so that the highest flow was 1.2 mL/min instead of 1.5 mL/min. The mix of standards was prepared in pancreatin/buffer solution and injected into the HPLC-ICP-MS system. As shown in Figure 4.8, compounds were very well separated.

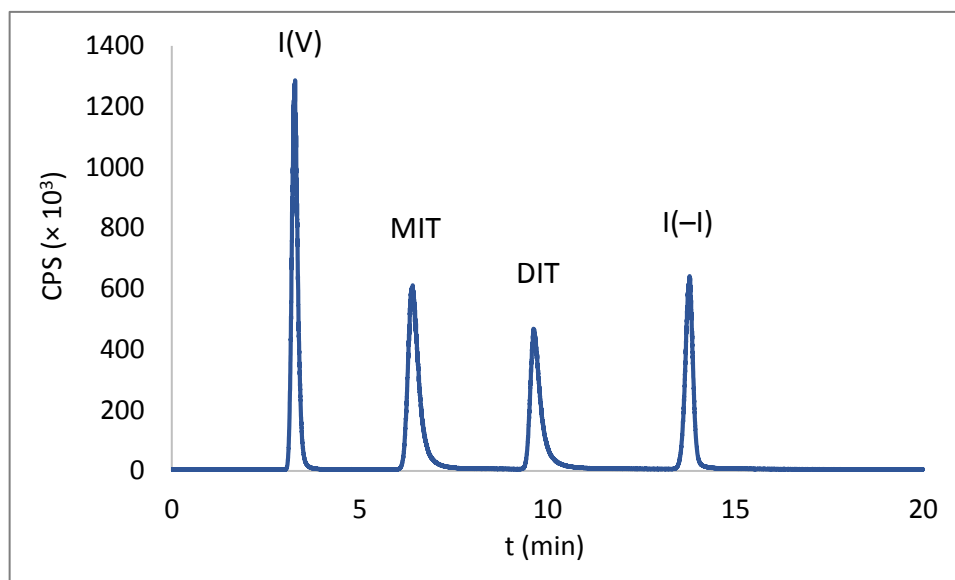


Figure 4.8: Separation of four iodine standards (200 ng I/g solution) prepared in the enzyme/buffer solution.

4.2.3.1 Effect of enzyme, temperature and bath on iodate stability

Standards were separately added to buckwheat sprouts prior to extraction (results shown in Figure 4.9). In the extract, complete transformation of iodate into iodide was observed (top chromatogram in Figure 4.9). Some experiments were therefore performed in order to find conditions to prevent this transformation.

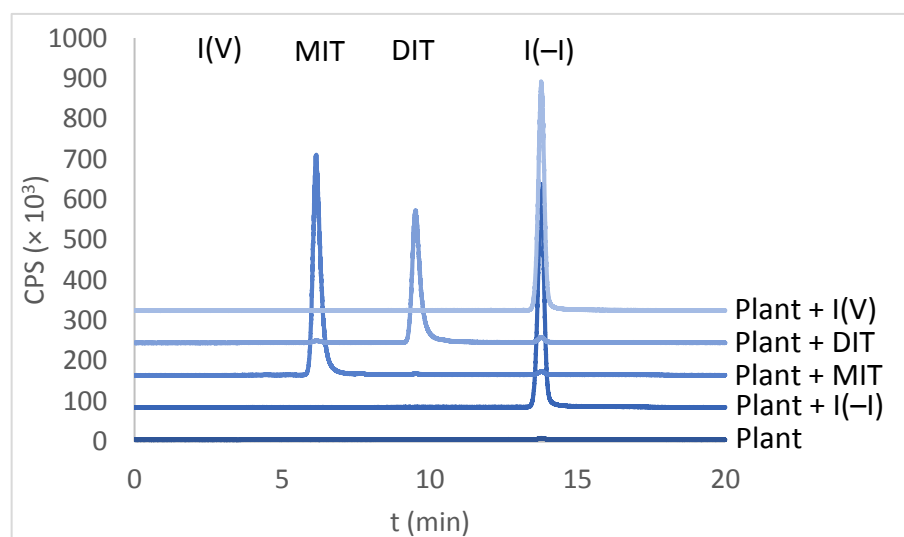


Figure 4.9: Chromatograms of plant extracts with separately added standards (200 ng I/g solution) prior to extraction.

Iodate standard (200 ng I/g of buffer solution) was added to a mixture of buckwheat sprouts, buffer and various enzymes – pancreatin, protease, α -amylase, lipase or without enzyme. With each enzyme, extraction was performed in an ultrasonic bath at 50 °C for 12 h and in a water bath with longitudinal shaking at 25 °C, 37 °C and 50 °C and 200 rpm for 12 h.

However, iodate was completely transformed into iodide after all the experiments. The only exception was when iodate standard was extracted in buffer without enzyme or plant material. Similarly, Shah et al. (2005) reported that in 0.1 M NaOH (without enzyme or sample), they observed no losses or interconversions of iodide and iodate. Based on these results, we assumed that plants and enzymes contain some compounds that reduce iodate. The transformation of iodate was very fast, since it was observed to be partly transformed when mixed with the enzyme solution approx. one minute prior to injection. The same transformation was also previously observed with standard addition after extraction in an ultrasonic bath. Since none of the tested conditions prevented the transformation, we further developed a method appropriate for determination of MIT, DIT and inorganic iodine. However, in the study by Shah et al. (2005), low concentrations of iodate ($4.16 \mu\text{g/g}$) were determined in samples of Wakame seaweed, but the extraction was carried out in 0.1 M NaOH without enzyme for 30 min at room temperature. In this sample, the iodine concentration was $226 \pm 11 \mu\text{g/g}$ and the extractability in NaOH was $98 \pm 3\%$.

4.2.3.2 Enzymes

Iodine content was determined in pancreatin, protease, α -amylase and lipase after extraction in buffer solution for 12 h at 50°C and 200 rpm.

The results (Figure 4.10) showed small amounts of iodide present in the buffer, as well as in all the enzymes (one LOT of each only). Especially when analysing samples with a low content of species present in the enzymes, it is important to subtract the amount of iodine from the enzyme, otherwise the calculated results will be incorrect.

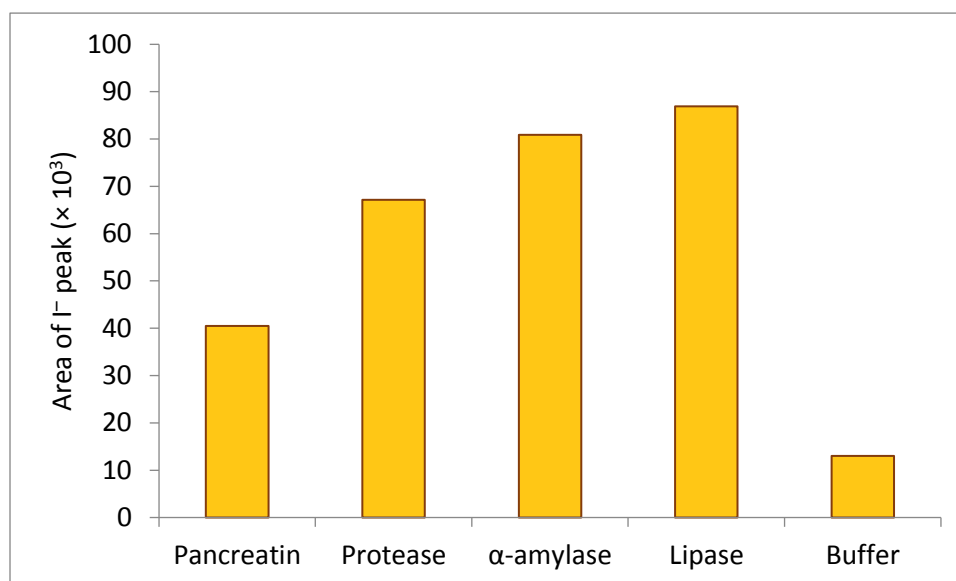


Figure 4.10: Comparison of iodide peak areas obtained in different enzymes dissolved in buffer.

The effect of enzyme on species transformation was studied after extraction of buckwheat sprouts with added iodine standards (MIT, DIT and I⁻) with pancreatin, protease, α -amylase and lipase in buffer solution for 12 h at 50°C and 200 rpm. The same extraction was also performed for sample in buffer solution without enzyme added.

The results for iodine standards were very similar, regardless of the enzyme used for the extraction (not shown). No transformations were observed except transformation of

iodate to iodide. Due to the lowest iodide content, pancreatin was therefore chosen as optimal for further experiments. In the study by Romarís-Hortas et al. (2013), pancreatin was also chosen from a number of other enzymes (α -amylase, β -glucosidase, cellulase, lipase, pepsin and a mixture of pancreatin, lipase and β -glucosidase), based on extraction efficiency for inorganic iodine, MIT and DIT from seaweed samples.

4.2.3.3 Enzyme dissolved in buffer or in water

In this experiment, extraction of buckwheat sprouts with added iodine standards (200 ng I/g of buffer solution, four different standards added separately) was performed with pancreatin dissolved in buffer and in water. Extraction was performed for 12 h at 50 °C and 200 rpm.

Incomplete separation of peaks for DIT and iodide was observed when the enzyme was dissolved in water (Figure 4.11A), while good separation and much narrower peaks were observed when the enzyme was dissolved in buffer (Figure 4.11B). Buffer was thus used in all further experiments.

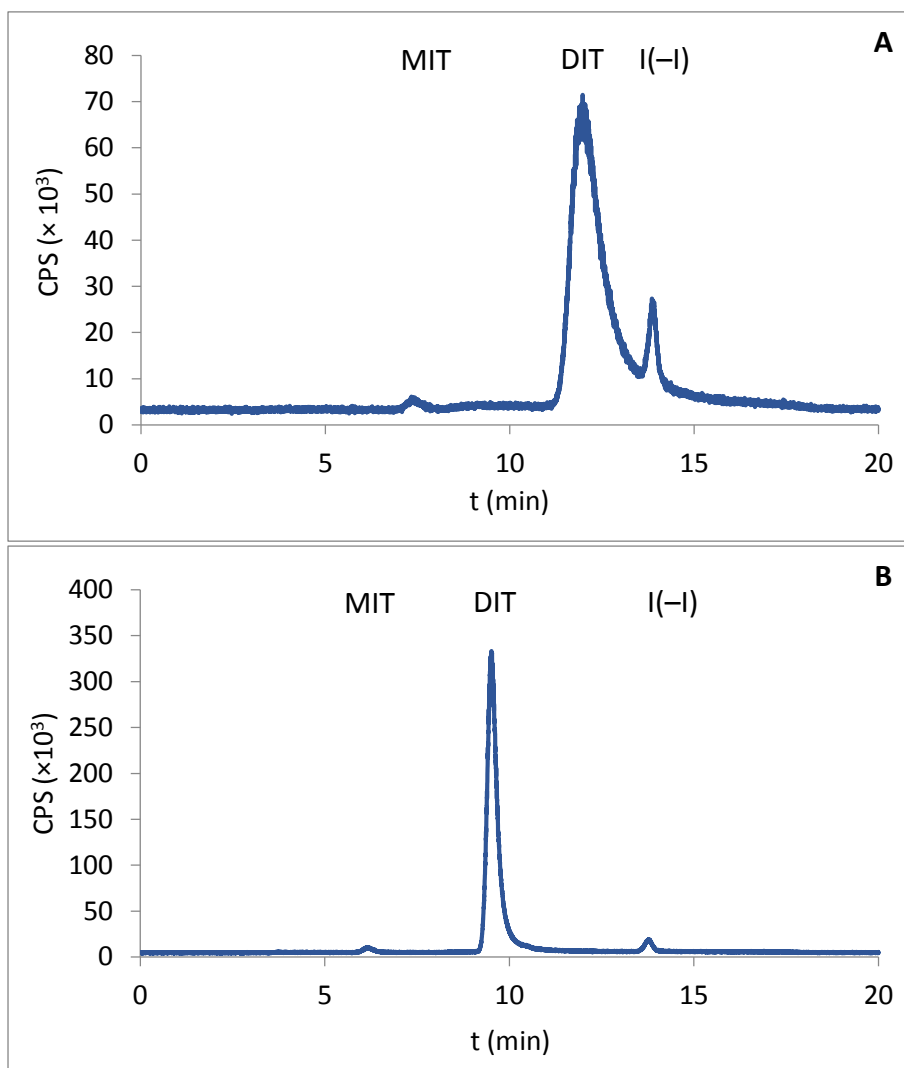


Figure 4.11: Chromatograms of injected DIT standard, extracted with enzyme dissolved in water (A) and in buffer (B).

4.2.3.4 Temperature

Three different temperatures of the bath were tested for the extraction – 37 °C, 50 °C and 60 °C. Extraction was performed with buckwheat sprouts with added standards (200 ng I/g of buffer solution, four different standards added separately). The time of extraction was 12 h and shaking was set to 200 rpm.

Different temperatures of the bath did not cause any transformations of iodide. The same amounts were also observed when iodate standard was added, which was detected as iodide and which proved the complete transformation of iodate into iodide. However, with increasing temperature, transformations of MIT into iodide and DIT into MIT and iodide were observed (Figure 4.12). From the tested temperatures, 37 °C was therefore chosen as optimal.

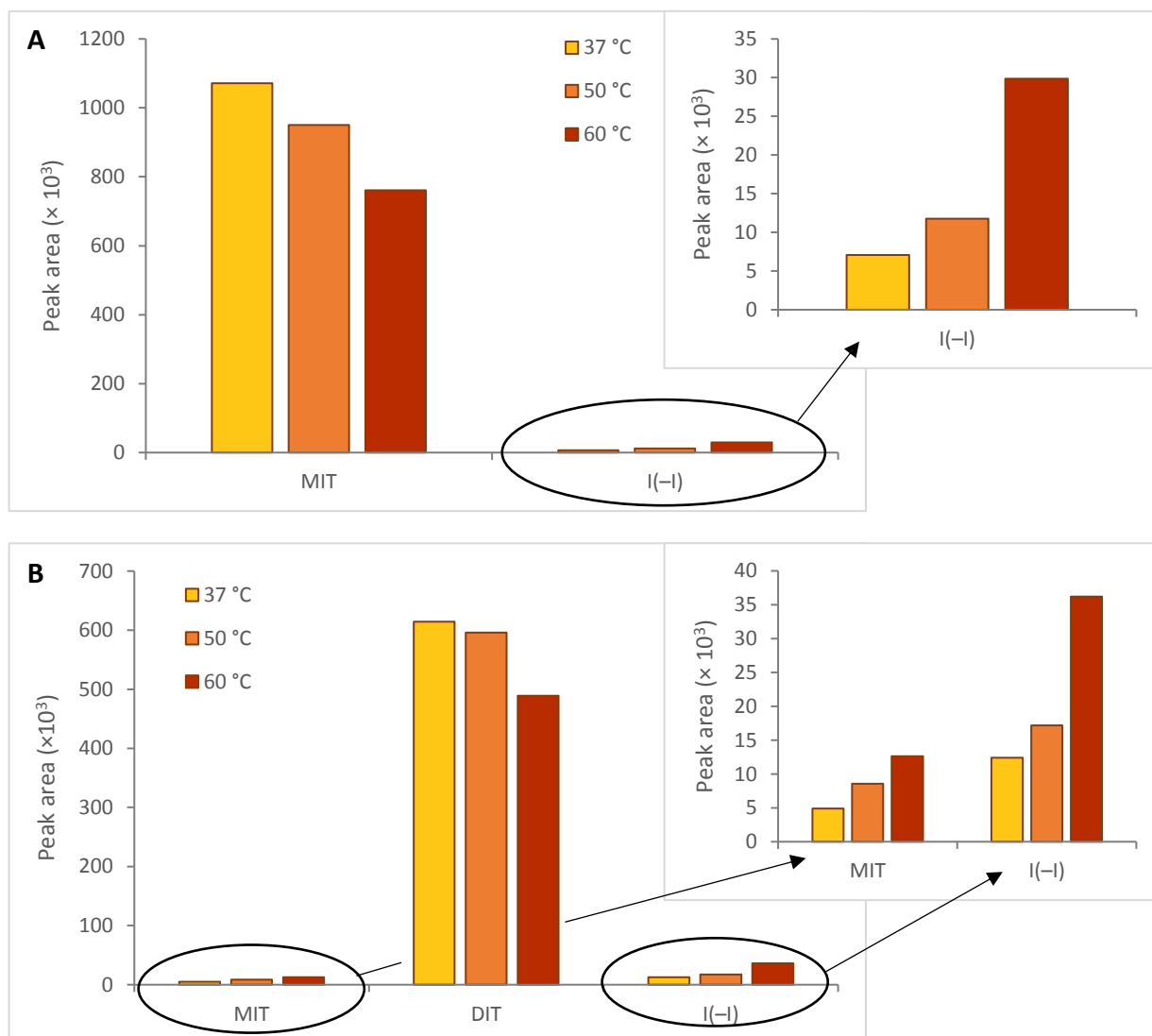


Figure 4.12: Influence of bath temperature on MIT (A) and DIT (B) (both 200 ng I/g) transformations.

4.2.3.5 Time of extraction

Buckwheat sprouts with added standards (200 ng I/g of buffer solution, four different standards added separately) were extracted at 37 °C and 200 rpm for 12 or 24 h. For iodide and iodate (detected as iodide), recoveries were between 78 and 81% and did not vary greatly between 12 and 24 hours. On the other hand, lower recoveries were observed for MIT and DIT when extraction was performed for 24 hours, compared to 12 h of extraction. After 12 h, recoveries for MIT and DIT were approx. 65 and 51%, respectively, while after 24 h recoveries dropped to 35 and 42%, respectively. It is possible that some adsorption/desorption processes occurred during the extraction and MIT and DIT were adsorbed onto the particles/compounds present in the sample residue in the second 12 hours.

4.2.3.6 Method validation

Method validation was performed with the optimized parameters. Column recovery was determined as the peak area of standard obtained after separation on a column compared to peak areas obtained without a column. The ratios were expressed in percentage as column recovery, in which the peak area obtained without a column was taken as 100%. Standards for determination of column recovery were prepared in the mobile phase in a concentration of 200 ng I/L. The obtained column recoveries for four species, iodate, MIT, DIT and iodide, were 87%, 84%, 73% and 69%, respectively.

Limits of detection (LODs) and quantification (LOQs), determined as 3-/10-fold standard deviation of the blank (enzyme in buffer solution) and calculated as the concentration per g of solution and per g of sample, are summarized in Table 4.9. Although calculated LODs and LOQs were very low, they were determined in blanks (enzyme in buffer) without plant matrix. The LODs/LOQs for samples might therefore be higher. However, peaks representing approx. 0.25 ng I/g of measured solution were integrated without difficulty for all three compounds. LODs/LOQs found in the literature vary from 0.05 ng/g to 1 µg/g. LODs of 6.1, 7.9 and 24 ng/g for iodide, MIT and DIT, respectively, were reported by Han et al. (2012) for seawater samples. Also for seaweed samples, Romarís-Hortas et al. (2013) obtained LODs of 1.1 and 4.3 ng/g of seaweed sample for MIT and DIT, respectively, while in another paper by Romarís-Hortas, Bermejo-Barrera & Moreda-Piñeiro (2012), LODs for iodide, MIT and DIT were 3.2, 2.4 and 3.4 ng/g of seaweed sample, respectively. Shah et al. (2005) determined LOD for iodide in seaweed samples of 0.12 µg/L. In extracts of dog food samples, Wilson, Yanes & Kemppainen (2016) found LOQs of 15 ng iodide/g, 5.1 ng MIT/g and 3.8 ng DIT/g, while Fernandez Sanchez & Szpunar (1999) reported an LOQ of 1 µg/L for iodide in milk samples.

Linearity was checked in the range between 20 and 200 ng I/g for three iodine standards – MIT, DIT and iodide – which were prepared in the enzyme solution. It was daily determined at 3 points in a range into which concentrations in samples fitted. Since iodate was transformed into iodide very quickly, it was not added to the mixture of standards in the enzyme solution. The obtained coefficients of determination (R^2) were in a range from 0.9801–0.9995, 0.9841–0.9996 and 0.9857–0.9999 for MIT, DIT and iodide, respectively, but usually higher than 0.99 for all three species. The repeatability was calculated from the slopes of the calibration lines (6301 ± 666 mL/ng for MIT, 3404 ± 382 mL/ng for DIT and 3388 ± 598 mL/ng for iodide) obtained over the course of 2 months. Expressed as relative standard deviations, repeatabilities were 10.6%, 11.2% and 17.6%, respectively.

For determination of the stability of iodine compounds, the enzymatic extracts of buckwheat sprouts from the experiment described in section 4.2.3.5 were measured after extraction, one day and one week later and the recovery was calculated and compared.

The recovery of the whole procedure for each standard was determined as the ratio between the concentration calculated from the peak area using the calibration curve and the concentration of the standard that was added before the extraction, calculated from the mass of added standard, known concentration of standard and dilution factor. The average recovery for MIT, DIT and iodide obtained from measurements after extraction were 65%, 51% and 79%, respectively. Stability was examined for 1 day and 1 week and was calculated as the relative standard deviation between the recovery obtained from results after extraction and after 1 day or 1 week. The differences after 1 day were 11% for MIT, 8% for DIT and 7% for iodide and after 1 week 12% for MIT, 10% for DIT and 8% for iodide. Extracts were stored in the meantime at -20 °C. The results showed good stability of all iodine species, even 1 week after extraction.

Table 4.9: LODs and LOQs for three iodine species.

	MIT	DIT	Iodide
LOD (ng/g extract)	0.04	0.06	0.04
LOD (ng/g sample)	1.44	2.07	1.36
LOQ (ng/g extract)	0.14	0.20	0.13
LOQ (ng/g sample)	4.81	6.92	4.54

4.2.4 Iodine speciation in pea sprouts and comparison between extraction using a water bath with longitudinal shaking and an ultrasonic bath

The results for iodine species in pea sprouts, obtained by the same method and under the same conditions were compared. The only difference was the bath used for extraction – a water bath with longitudinal shaking (described in section 3.3.2.3) and an ultrasonic bath with water recirculator to assure constant temperature. The sample (0.2 g) was mixed with 40 mg of pancreatin and 7 ml of $\text{KH}_2\text{PO}_4/\text{NaOH}$ buffer at pH 8. Extraction was performed for 12 h at 37 °C, but an ultrasonic bath was used instead of a water bath with longitudinal shaking. Samples were analysed by the HPLC-ICP-MS system (Perkin Elmer) using an IonPac® AS7 (4 × 250 mm) with an IonPac® AG7 (4 × 50 mm) guard column. The concentration of extracted iodine was determined by ICP-MS after dilution of extracts (without previous microwave extraction with TMAH). Comparison of the results obtained after the two extraction procedures is shown in Table 4.10. A high amount of iodine was extracted with both baths used for extraction (> 73%). Reasonable agreement for concentrations of inorganic iodine was found after the two approaches applied. However, when extracting in an ultrasonic bath, the obtained concentrations of MIT were much higher (> 600%) than when a water bath with longitudinal shaking was used. These results suggest that an ultrasonic bath is more powerful in the hydrolysis of proteins, in which MIT is presumably incorporated (Romarís-Hortas et al., 2013; Shah et al., 2005). On the other hand, the conditions in a water bath with longitudinal shaking are milder and the enzyme itself cannot hydrolyse as much protein as it can in combination with ultrasonication and so less MIT is released in the solution. However, after extraction in water bath with longitudinal shaking almost all of the extracted iodine was identified, while after extraction in ultrasonic bath only 51–65% of the extracted iodine species were identified.

Table 4.10: Comparison of the results obtained in pea sprouts after enzymatic extraction in a water bath with longitudinal shaking and when using an ultrasonic bath with water recirculation (concentrations given in $\mu\text{g I/g DW} \pm$ standard deviation of 4–8 determinations or mean absolute error of 2 determinations).

Sample	Water bath with longitudinal shaking					Ultrasonic bath with water recirculation			
	Iodine [$\mu\text{g I/g DW}$]	Extracted iodine ^a [$\mu\text{g I/g DW}$]	MIT [$\mu\text{g I/g DW}$]	Inorganic iodine [$\mu\text{g I/g DW}$]	Σ^b [$\mu\text{g I/g DW}$]	Extracted iodine ^a [$\mu\text{g I/g DW}$]	MIT [$\mu\text{g I/g DW}$]	Inorganic iodine [$\mu\text{g I/g DW}$]	$\Sigma^{b,c}$ [$\mu\text{g I/g DW}$]
Control	3.8 ± 0.2^d	2.9 ± 0.2^f (77)	0.07 ± 0.03^f	2.5 ± 0.5^f	2.5 (86)	2.8 ± 1.3^g (74)	0.4 ± 0.1^g	1.0 ± 0.2^g	1.4 (51)
I ⁻	214.5 ± 16.0^e	156.7 ± 6.3^f (73)	2.1 ± 0.2^f	163.6 ± 2.0^f	165.7 (106)	237 ± 30^d (110)	19.9 ± 1.6^d	133.6 ± 21.7^d	153.5 (65)
IO ₃ ⁻	188.2 ± 1.4^e	146.1 ± 0.1^f (78)	3.0 ± 0.6^f	142.0 ± 14.7^f	145.1 (99)	200 ± 14^g (106)	18.6 ± 1.6^g	107.6 ± 22.9^g	126.1 (63)

^a ... values in brackets represent the percentage of extracted iodine

^b ... sum of identified species

^c ... values in brackets represent the percentage of identified extracted iodine

^d ... standard deviation of 6 determinations

^e ... standard deviation of 8 determinations

^f ... mean absolute error of 2 determinations

^g ... standard deviation of 4 determinations

4.3 Iodine and its Compounds in Enriched Sprouts

4.3.1 Pea sprouts

No visible toxic signs such as leaf chlorosis, necrosis or plant drying were noticed during the growth of seedlings. However, treatments with iodine and selenium significantly influenced the height and mass of pea sprouts. When iodine was present in the soaking solution, regardless of the form and presence of selenium, sprouts were significantly shorter and the mass was reduced in comparison to control sprouts (Jerše et al., 2017).

4.3.1.1 Iodine content and distribution in pea plant

Iodine content was determined in sprouts, roots and seed residues. The results, given as average (2–4 replicates, each measured in two parallels) with standard error, are summarized in Table 4.11. Uptake is an approximation, calculated as the amount (mass) of iodine in a particular part of the plant divided by the theoretical mass of iodine in the seed after soaking. An assumption of the same passage of water and iodine into seeds was used in the calculation. The obtained dry masses of sprouts, roots and seed residues were 10.4%, 8.4% and 16.8%, respectively, regardless of the treatment. The mass proportions of plant parts used for the calculations were on average 38.3% for sprouts, 28.3% for roots and 33.4% for seed residues. Statistical evaluation of the iodine content in sprouts, roots and seed residues is presented in Figure 4.14.

Table 4.11: Iodine contents in pea sprouts, roots and seed residues (average with standard error in $\mu\text{g/g}$ DW) and calculated uptakes (in %).

Treatment	Sprouts		Roots		Seed residues	
	c	uptake	c	uptake	c	remaining
Control	3.2 ± 0.4^b	-	26.3 ± 0.5^b	-	12.3 ± 1.6^c	-
I(-I)	211 ± 13^c	5	355 ± 22^c	5	460 ± 14^b	17
I(-I)+Se(IV)	186 ± 4^a	5	341 ± 31^a	5	468 ± 13^a	21
I(-I)+Se(VI)	247 ± 9^a	6	306 ± 21^a	5	566 ± 10^a	23
I(V)	188 ± 8^c	6	334 ± 15^c	6	419 ± 6^c	19
I(V)+Se(IV)	152 ± 6^a	4	275 ± 2^a	4	498 ± 9^a	21
I(V)+Se(VI)	213 ± 3^a	5	326 ± 32^a	5	522 ± 21^a	20

^a ... standard error of 4 determinations

^b ... standard error of 6 determinations

^c ... standard error of 8 determinations

The results showed a large increase in iodine content in all parts of plants grown from treated seeds in comparison with control plants. Regardless of the form of iodine in the soaking solution, sprouts contained the lowest amounts of iodine ($200 \mu\text{g/g}$ DW on average) while the highest iodine content was found in seed residues ($489 \mu\text{g/g}$ DW on average). As can be seen from Figure 4.14, the iodine contents in seed residues were very similar after soaking in solutions of I(-I) and I(V). We therefore assumed that components in seed residues, such as starch, proteins, polyphenolic substances etc., are responsible for retaining iodine in both forms. It is also possible that oxidation/reduction processes occurred and iodine was transformed into elemental iodine that can be bound to starch. However, further investigations are needed to define which substance(s) is/are responsible for binding or

transforming iodine. The observation of a high iodine content in seed residues may also be explained biologically. Since iodine is not an essential element for plants, it is not required in such high amounts and, consequently, it remains in the seed residues. In sprouts and roots, slightly lower uptake was observed after soaking in I(V) than in I(-I) but the differences were not significant.

Although the concentrations of iodine in plants were high, the uptake was quite low (on average 5% for sprouts and roots and 20% remained in seeds). The sum of the calculated percentage of uptake in sprouts and roots and percentage of iodine remaining in seed residues was around 30%. Three possible reasons were found for the low uptake: (1) volatilization of iodine during plant growth, (2) rinsing of iodine from seeds with water for moistening filter paper during growth and/or (3) much slower passage of iodine into seeds than the passage of water.

4.3.1.2 Iodine compounds

Iodine compounds were determined in 2 of the replicates of pea sprouts I(-I), I(V) and control sprouts. Peaks for MIT and inorganic iodine were found in all chromatograms, regardless of the treatment (Figure 4.13). Some unidentified peaks were also found in the chromatograms but the areas were small and they were not considered in the calculation. The samples of pea sprouts I(-I) and I(V) were 50-fold diluted before injection.

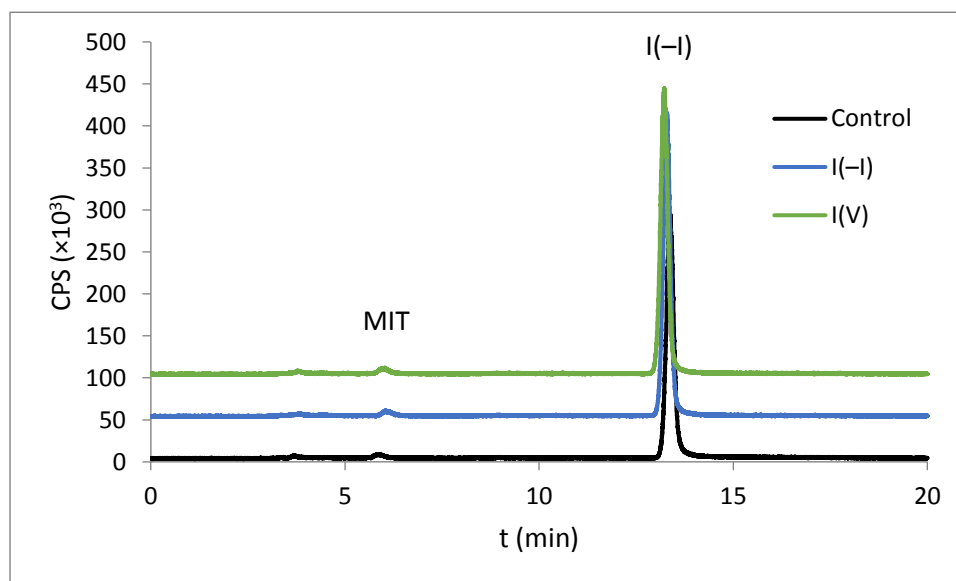


Figure 4.13: Chromatograms of control pea sprouts, pea sprouts I(-I) and I(V).

In pea sprouts, between 73 and 78% of total iodine was extracted from the samples (Table 4.12), and 22–27% remained in the sample residue. From the extracted iodine, 86–106% of iodine species were identified. The highest amount of identified iodine was present as inorganic iodine (97.3–98.7%) and the remainder was in the form of MIT (1.2–2.7%).

Table 4.12: Concentrations (given in $\mu\text{g I/g DW}$ sample) of iodine compounds (average $\pm$ mean absolute error, $n = 2$) in analyzed pea sprouts.

Treatment	Iodine [$\mu\text{g I/g DW}$]	Extracted iodine ^a [$\mu\text{g I/g DW}$]	c (MIT) [$\mu\text{g I/g DW}$]	c (inorg. I) [$\mu\text{g I/g DW}$]	$\Sigma^{\text{b,c}}$ [$\mu\text{g I/g DW}$]
Control	3.8 ± 0.2	2.9 ± 0.2 (77)	0.07 ± 0.03	2.5 ± 0.5	2.5 (86)
I(–I)	214.5 ± 16.0	156.7 ± 6.3 (73)	2.07 ± 0.23	163.6 ± 2.0	165.7 (106)
I(V)	188.2 ± 1.4	146.1 ± 0.1 (78)	3.03 ± 0.55	142.0 ± 14.7	145.1 (99)

^a ... values in brackets represent the percentage of extracted iodine

^b ... sum of identified species

^c ... values in brackets represent the percentage of identified extracted iodine

4.3.1.3 The effect of selenium on iodine content

Selenium had different effects on iodine uptake, as shown in Figure 4.14. In sprouts, Se(IV) had no influence on the uptake of I(–I) but decreased the uptake of I(V), while Se(VI) did not affect the uptake of iodine in any form.

In roots, selenium, regardless of its form, had no significant effect on the uptake of I(–I). The uptake of I(V) in roots was decreased in the presence of Se(IV) but remained at the same level when Se(VI) was added.

Selenium seemed to increase the retention of iodine in seed residues in three out of four combinations. The only exception was I(–I)+Se(IV), in which selenium had no impact on the amount of iodine remaining in seed residues.

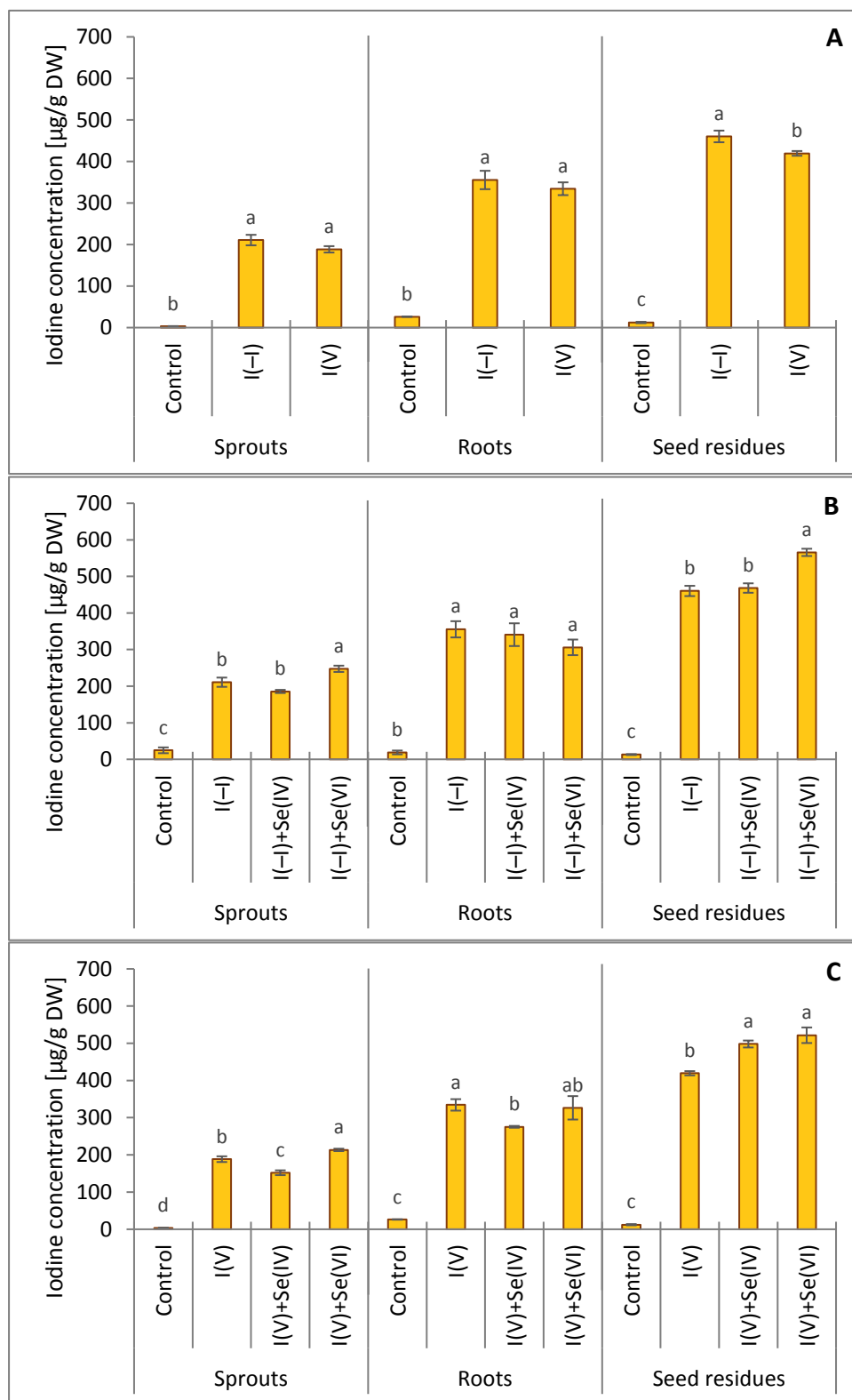


Figure 4.14: Iodine content (average with standard errors, $n = 2-4$) in pea sprouts, roots and seed residues. Letters above the columns indicate statistically significant differences ($p = 0.05$) inside the group (marked with line).

A: Comparison of iodine concentrations in sprouts, roots or seed residues I(-I) and I(V).

B: The influence of selenium on the uptake of I(-I) from the soaking solution.

C: The influence of selenium on the uptake of I(V) from the soaking solution.

4.3.2 Pumpkin sprouts

A different approach was used for germination of pumpkin sprouts than for other sprouts. Seeds were placed on moist paper, which was rolled and placed in a growth chamber in a beaker to which water was added as required. Germination between 33 and 60% was achieved (data not shown). The rest of the seeds remained ungerminated and the same was also observed for controls. Even on germinated and developed sprouts, certain deformations were observed. However, none of the treatments influenced the mass of sprouts.

4.3.2.1 Iodine content

Iodine content was determined in whole sprouts. The results are given as the average with standard error (Table 4.13).

Table 4.13: Iodine content (average $\pm$ st. error) in pumpkin sprouts (1–3 replicates, each determined in 1–2 parallels; n is stated in brackets).

Treatment	c [$\mu\text{g I/g DW}$]
Control	0.83 ± 0.10 (6)
I(–I)-250	57 ± 6 (6)
I(–I)-1000	279 ± 19 (6)
I(–I)-1000+Se(IV)-10	314 ± 1 (3)
I(–I)-1000+Se(VI)-10	323 ± 12 (4)
I(V)-250	49 ± 4 (6)
I(V)-1000	217 ± 13 (6)
I(V)-1000+Se(IV)-10	288 ± 25 (6)
I(V)-1000+Se(VI)-10	266 ± 28 (4)

The iodine content in pumpkin sprouts increased after soaking the seeds in iodine solutions, compared to controls. As expected, a lower iodine concentration in the soaking solution resulted in a lower iodine content in the sprouts. The ratio between concentrations in the soaking solution (1:4) is similar to the ratio between the iodine content in sprouts (1:4.9 for iodide and 1:4.4 for iodate). When 250 mg I/L solution was used, no statistically significant differences in iodine content in sprouts were observed. On the other hand, when soaking in a solution of 1000 mg I/L, a higher iodine content was found when iodine was present in the form of iodide. These results suggest a higher toxicity of iodate for plants.

4.3.2.2 Iodine compounds

Iodine speciation was performed in pumpkin sprouts I(–I)-1000, I(V)-1000 and control sprouts, in 2 replicates of each treatment. Inorganic iodine and MIT were identified in the chromatograms (Figure 4.15) and some small peaks were not identified and were not considered in the calculation. Samples of pumpkin sprouts I(–I) and I(V) were 50-fold diluted before injection.

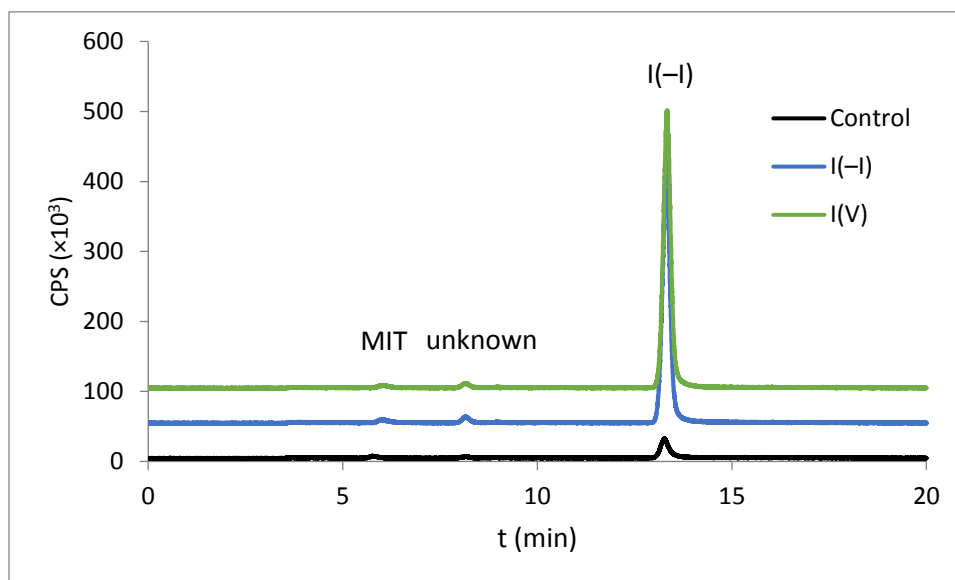


Figure 4.15: Chromatograms of control pumpkin sprouts, pumpkin sprouts I(-I) and I(V).

Between 68 and 77% of total iodine was extracted and, in the extracts, 17 to 103% of iodine species were identified (Table 4.14). The lowest percentage of identified species was found in control pumpkin sprouts (17%), while in sprouts I(-I) and I(V), 93–103% of the species were identified. The highest amount of iodine was in inorganic form (83.3–99.1%), while the remaining iodine was present as MIT (0.8–16.6%). In control sprouts, a larger share of iodine was present in the form of MIT than in pumpkin sprouts I(-I) or I(V) (Table 4.14).

Table 4.14: Concentrations (given in $\mu\text{g I/g DW}$ sample) of iodine compounds (average $\pm$ mean absolute error, $n = 2$) in analyzed pumpkin sprouts.

Treatment	Iodine [$\mu\text{g I/g DW}$]	Extracted iodine ^a [$\mu\text{g I/g DW}$]	c (MIT) [$\mu\text{g I/g DW}$]	c (inorg. I) [$\mu\text{g I/g DW}$]	$\Sigma^{\text{b,c}}$ [$\mu\text{g I/g DW}$]
Control	0.8 ± 0.3	0.8^{d} (68 ^e)	0.02^{d}	0.1^{d}	0.1 (17)
I(-I)	250.7 ± 11.4	177.2 ± 9.4 (71)	1.80 ± 0.08	162.9 ± 14.6	164.7 (93)
I(V)	237.4 ± 2.3	182.3 ± 4.2 (77)	1.58 ± 0.29	185.9 ± 3.8	187.5 (103)

^a ... values in brackets represent the percentage of extracted iodine

^b ... sum of identified species

^c ... values in brackets represent the percentage of identified extracted iodine

^d ... due to the low concentration, determination was possible only in one replication

^e ... calculated based on the iodine concentration in the sample in which iodine compounds were determined, not average iodine concentration

4.3.2.3 The effect of selenium on iodine content

Combinations of iodine and selenium in the soaking solution were applied only with higher concentrations of both elements, 1000 mg I/L and 10 mg Se/L. Except for the combination I(-I)+Se(IV), for which statistical analysis did not show significant differences, selenium positively affected the uptake of iodine (Figure 4.16). The highest iodine content was observed after soaking in the solution I(-I)+Se(VI), which seems to be a less toxic form for pumpkin sprouts.

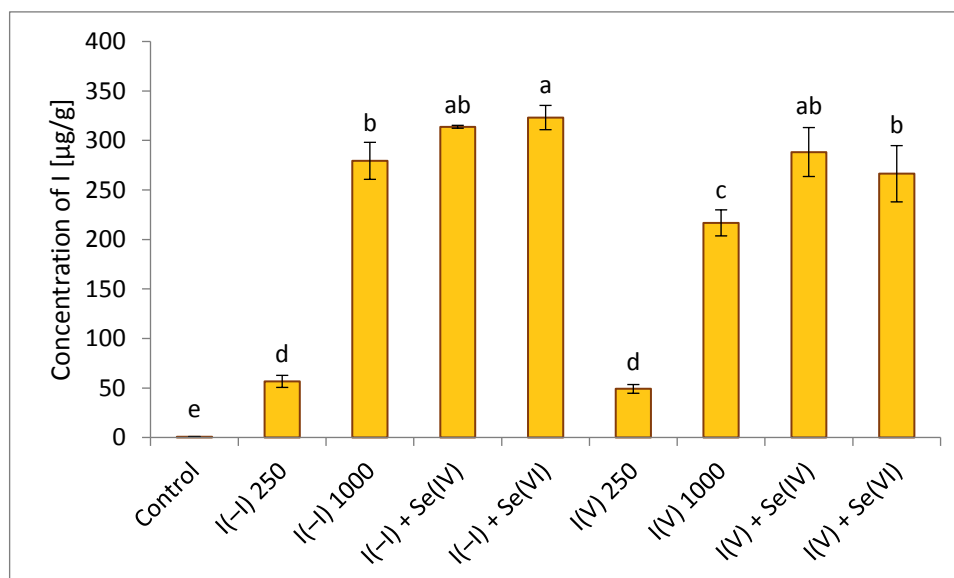


Figure 4.16: Iodine content (average with standard error, $n = 3-6$) in pumpkin sprouts with indicated statistically significant differences ($p = 0.05$, letters above the columns).

4.3.3 Buckwheat sprouts

In some cases, treatments significantly affected the germination, mass and height of sprouts. Except for seeds soaked in a solution of 1000 mg I(V)/L, for which much lower germination was observed (54%), between 70 and 90% of seeds germinated in both experiments, conducted in June and July. Iodate significantly lowered the germination in both experiments, while other treatments with iodine did not affect germination. However, treatments with 1000 mg I(-I)/L, 1000 mg I(V)/L or I(V)+Se(VI) in both concentrations of iodine significantly lowered the mass of sprouts. Sprout height was negatively affected by 1000 mg I(-I)/L, 1000 mg I(V)/L and 1000 mg I(-I)/L+Se combinations in the soaking solution, while I(V) in combination with selenium, regardless of the iodine concentration, had no influence on sprout height. I(-I) in a concentration of 1500 mg I/L even increased sprout height, while other treatments with 1500 mg I/L had no statistically significant effect on sprout height (data not shown).

4.3.3.1 Iodine content

Whole sprouts were taken as samples for analysis. Iodine contents in buckwheat sprouts, given as the average (2-3 replicates, each determined in 2 parallels) with standard error, are summarized in Table 4.15.

Table 4.15: Iodine content (average $\pm$ st. error) in buckwheat sprouts (n is stated in brackets).

Treatment	c (I) [$\mu\text{g/g DW}$]
Control	4.3 ± 0.3 (4)
I(-I)-1000	5 ± 1 (4)
I(-I)-1000+Se(IV)	20 ± 1 (6)
I(-I)-1000+Se(VI)	20 ± 1 (6)
I(-I)-1500	167 ± 18 (6)
I(-I)-1500+Se(IV)	255 ± 31 (6)
I(-I)-1500+Se(VI)	125 ± 11 (6)
I(V)-1000	217 ± 13 (6)
I(V)-1000+Se(IV)	114 ± 5 (6)
I(V)-1000+Se(VI)	3.7 ± 0.4 (6)
I(V)-1500	1042 ± 55 (6)
I(V)-1500+Se(IV)	332 ± 20 (6)
I(V)-1500+Se(VI)	339 ± 11 (6)

Enrichment of buckwheat sprouts did not conform to all expectations. When seeds were soaked in 1000 mg I(-I)/L solution, the iodine content in the sprouts remained at the same level as in control sprouts. Compared to control sprouts, a more than 50-fold higher iodine content was found in buckwheat sprouts I(V)-1000. A similar amount of iodine was also found in buckwheat sprouts I(-I)-1500, in spite of a higher iodine concentration in the soaking solution. The highest enrichment was achieved using a 1500 mg I(V)/L soaking solution, the iodine content being elevated more than 260-fold compared to the control.

4.3.3.2 Iodine compounds

Iodine speciation was performed in 2 replicates of buckwheat sprouts of the following treatments: control, I(-I)-1000, I(V)-1000 and combinations I-1000+Se. Inorganic iodine and MIT were found in the chromatograms (Figure 4.17). Some small, unidentified peaks were also observed but the unknown compounds were present in traces. Some of the samples were diluted as required before injection: buckwheat sprouts I(V) 25-fold, I(-I)+Se 5-fold and I(V)+Se(IV) 20-fold. The remaining samples did not require dilution.

Between 74 and 89% of the total iodine was extracted, 11–26% remained in the sample residue. Identified species represented between 32 and 116% of the extracted iodine. The lowest percentage of iodine species was identified in control sprouts (32% on average), while more than 77% was found in all sprouts from treated seeds. Regardless of the treatment, more than 99% of the iodine was in inorganic form, the remaining iodine being in the form of MIT (Table 4.16).

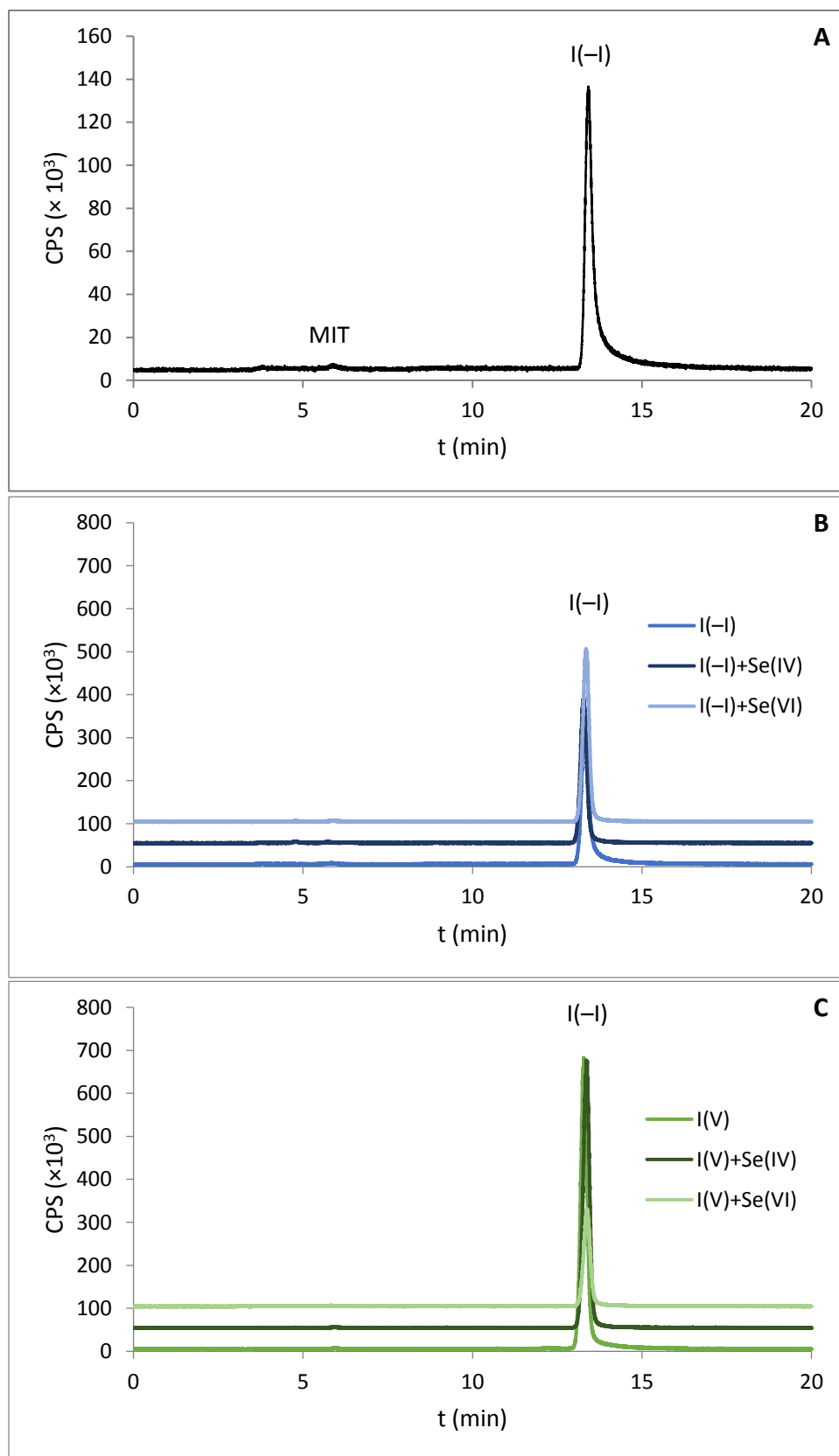


Figure 4.17: Chromatograms of control buckwheat sprouts (A), buckwheat sprouts $I(-I)$ and $I(-I)+Se$ (B) and buckwheat sprouts $I(V)$ and $I(V)+Se$ (C).

Table 4.16: Concentrations (given in $\mu\text{g I/g DW}$ sample) of iodine compounds (average $\pm$ mean absolute error, $n = 2$) in analyzed buckwheat sprouts.

Treatment	Iodine [$\mu\text{g I/g DW}$]	Extracted iodine ^a [$\mu\text{g I/g DW}$]	c (MIT) [$\mu\text{g I/g DW}$]	c (inorg. I) [$\mu\text{g I/g DW}$]	$\Sigma^{\text{b,c}}$ [$\mu\text{g I/g DW}$]
Control	4.1 ± 0.4	3.0 ± 0.4 (74)	0.009 ± 0.001	0.9 ± 0.1	0.9 (32)
I(-I)	4.6 ± 1.6	3.4 ± 1.4 (74)	0.02 ± 0.01	2.5 ± 0.9	2.5 (77)
I(-I)+Se(IV)	19.1 ± 0.3	15.3 ± 0.1 (80)	0.039 ± 0.001	15.1 ± 0.1	15.1 (99)
I(-I)+Se(VI)	21.3 ± 0.1	17.5 ± 0.4 (82)	0.06 ± 0.02	18.7 ± 0.1	18.8 (108)
I(V)	196.1 ± 5.2	155.8^{d} (77 ^e)	0.30^{d}	171.5^{d}	171.8 (110)
I(V)+Se(IV)	121.4 ± 0.8	107.5 ± 0.7 (89)	0.15 ± 0.01	124.5 ± 0.2	124.7 (116)
I(V)+Se(VI)	3.38 ± 0.02	2.5 ± 0.1 (74)	0.012 ± 0.001	2.3 ± 0.1	2.3 (93)

^a ... values in brackets represent the percentage of extracted iodine

^b ... sum of identified species

^c ... values in brackets represent the percentage of identified extracted iodine

^d ... due to contamination, determination was possible only in one replication

^e ... calculated based on the iodine concentration in samples in which iodine compounds were determined, not average iodine concentration

4.3.3.3 The effect of selenium on iodine content and its compounds

Regardless of the presence of selenium in the soaking solution, no enrichment was achieved with 1000 mg I(-I)/L (Figure 4.18). The same results were also observed when seeds were soaked in solution I(V)-1000+Se(VI). It is possible that an error occurred and iodine was not present in the soaking solution. However, in the case of iodide, it is more likely that it was transformed into a volatile form by components present in buckwheat sprouts and was volatilized during growth. Se(IV) in the soaking solution negatively affected the uptake of I(V)-1000, but to a lesser extent than Se(VI) and both forms of selenium had the same

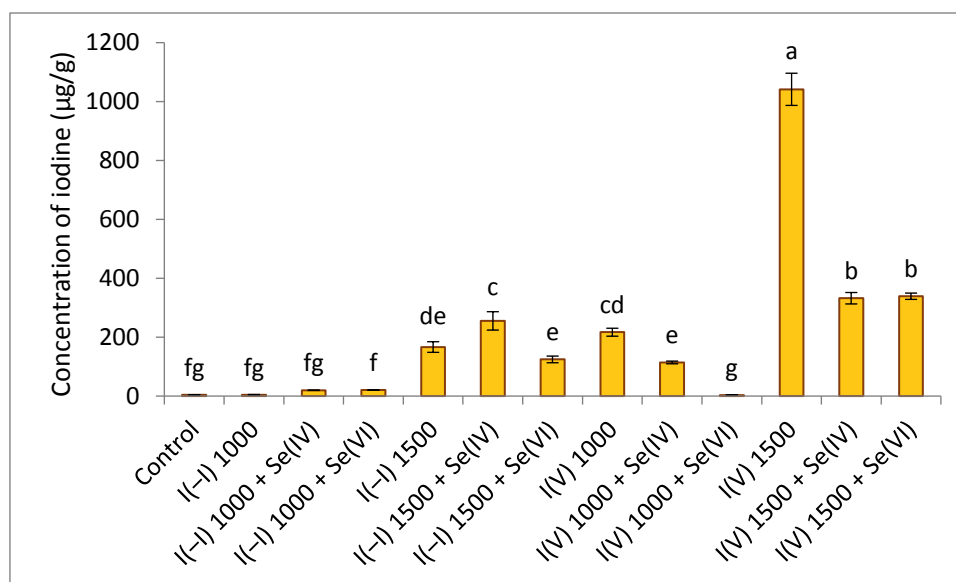


Figure 4.18: Iodine content (average with standard error, $n = 4-6$) in buckwheat sprouts with results of statistical analysis (letters above the columns indicate statistically significant differences, $p = 0.05$).

negative effect on the uptake of I(V)-1500. The opposite trend was found for the impact of selenium on the uptake of I(-I)-1500, since Se(VI) had no effect while Se(IV) even increased its uptake.

No effect of selenium on iodine species was observed in the analysed buckwheat sprouts. In all sprouts, regardless of the treatment, more than 99% of identified iodine was in inorganic form, while the remainder was present in the form of MIT (Table 4.16).

4.4 Iodine Content in Enriched Adult Plants

4.4.1 Pea plants

In comparison with control plants, treatments with I or I+Se did not significantly influence plant characteristics such as the number of pods per plant, pod mass per plant, fresh seed mass per plant, pod yield, fresh seed yield or yield of dry matter of pea seeds. However, some significant differences were found regarding pod characteristics. Pod mass was lower after treatment with I(-I)+Se(IV) and I(V)+Se(VI) solutions, while pod length was negatively influenced by all I+Se combinations except I(V)+Se(IV), which resulted in longer pods. After spraying with I(V) solution, a significant decrease in the mass of seed per pod and single seed weight was observed. No differences were found between control and treated plants for the other pod characteristics – number of seeds per pod, share of seeds per pod mass and dry matter of seeds (Jerše et al., n.d.)

4.4.1.1 Iodine content and distribution in different parts of pea plants

Iodine content was determined in different parts of plants – seeds, pod shells, leaves, stems and roots – in order to study the distribution of the element through the plant. The results for iodine content are summarized in Table 4.17.

Table 4.17: Iodine content in different parts of pea plants (average of 2–3 replicates, each determined in 2 parallels $\pm$ standard error in $\mu\text{g I/g DW}$); the number next to the plant part represents when it was sampled (1 – first sampling, 2 – in second sampling).

Treatment	Pod shells 1	Seeds 1	Leaves 2	Stems 2	Roots 2
Control	0.042 ± 0.003^b	0.014 ± 0.001^b	0.6 ± 0.1^b	0.16 ± 0.01^b	0.58 ± 0.03^b
I(-I)	1.9 ± 0.1^b	0.094 ± 0.004^b	45.6 ± 6.4^b	14.1 ± 2.0^b	4.3 ± 0.5^b
I(-I)+Se(IV)	3.2 ± 0.3^b	0.104 ± 0.007^b	46.8 ± 4.1^b	14.6 ± 0.4^b	7.3 ± 0.7^b
I(-I)+Se(VI)	1.9 ± 0.1^b	0.069 ± 0.003^b	28.7 ± 1.2^b	8.9 ± 0.7^b	3.0 ± 0.2^b
I(V)	0.6 ± 0.1^b	0.018 ± 0.001^b	4.7 ± 0.5^b	2.1 ± 0.2^b	3.5 ± 0.5^b
I(V)+Se(IV)	0.59 ± 0.04^b	0.019 ± 0.003^b	5.5 ± 0.6^b	2.2 ± 0.2^b	4.9 ± 0.9^b
I(V)+Se(VI)	0.58 ± 0.02^b	0.014 ± 0.001^b	5.4 ± 0.7^b	2.1 ± 0.2^b	3.1 ± 0.5^b

^a ... standard error of 4 determinations

^b ... standard error of 6 determinations

In all parts of pea plants, treatment with I(-I) resulted in increased iodine content in comparison to control plants. Treatment with I(V) had a significant impact on final amounts of iodine only in pod shells and roots, while in other parts the iodine content remained at the same level as in control plants (Figure 4.19). When treated with I(-I), plants stored the highest amounts of iodine in leaves and/or stems, while I(V) was mostly transported to and stored in roots.

The lowest amount of iodine among all plant parts was found in seeds, the edible part of pea plants. The explanation of these results might be found in a paper by Herrett et al. (1962), suggesting iodine is mainly transported via xylem, while only a little or even no transport occurs through the phloem. Consequently, transport and uptake in seeds is not likely to happen. Similarly, slow transport of iodine from leaves to the rest of the plant was mentioned also by Fuge (2005).

Spraying with a solution of I(V) did not influence the iodine content in pea seeds, while spraying with iodide resulted in almost 7-fold higher concentrations than in control seeds.

4.4.1.2 The effect of selenium on iodine content

Selenium affects iodine uptake in various ways (Figure 4.19). In the combination I(-I)+Se(IV), selenium showed a synergistic effect on iodine uptake in pod shells, leaves and roots, but no effect on final iodine concentrations in seeds and stems. On the other hand, when Se(VI) was added to I(-I) spraying solution, a lower iodine content was observed in seeds and stems, comparable content in pod shells and roots, and even higher content in leaves. However, the addition of selenium to I(V) spraying solution had no impact on the final iodine concentration in any part of the plants. These results indicate that there are some interactions between iodine (in the form of I(-I)) and selenium during their uptake and allocation in plants. Both forms of selenium seem to retain iodine in the leaves and Se(IV) also promotes transport of iodine into pod shells and roots. Se(VI) had less impact on iodine transport in plants.

In seeds, the highest iodine concentration was determined when the combination (-I)+Se(IV) was applied, although there were no significant differences between this treatment and I(-I) alone. On the other hand, Se(VI) had an antagonistic effect on I(-I) uptake/transport to seeds and, compared to individual I(-I) treatment, approx. 25% lower iodine concentrations were determined.

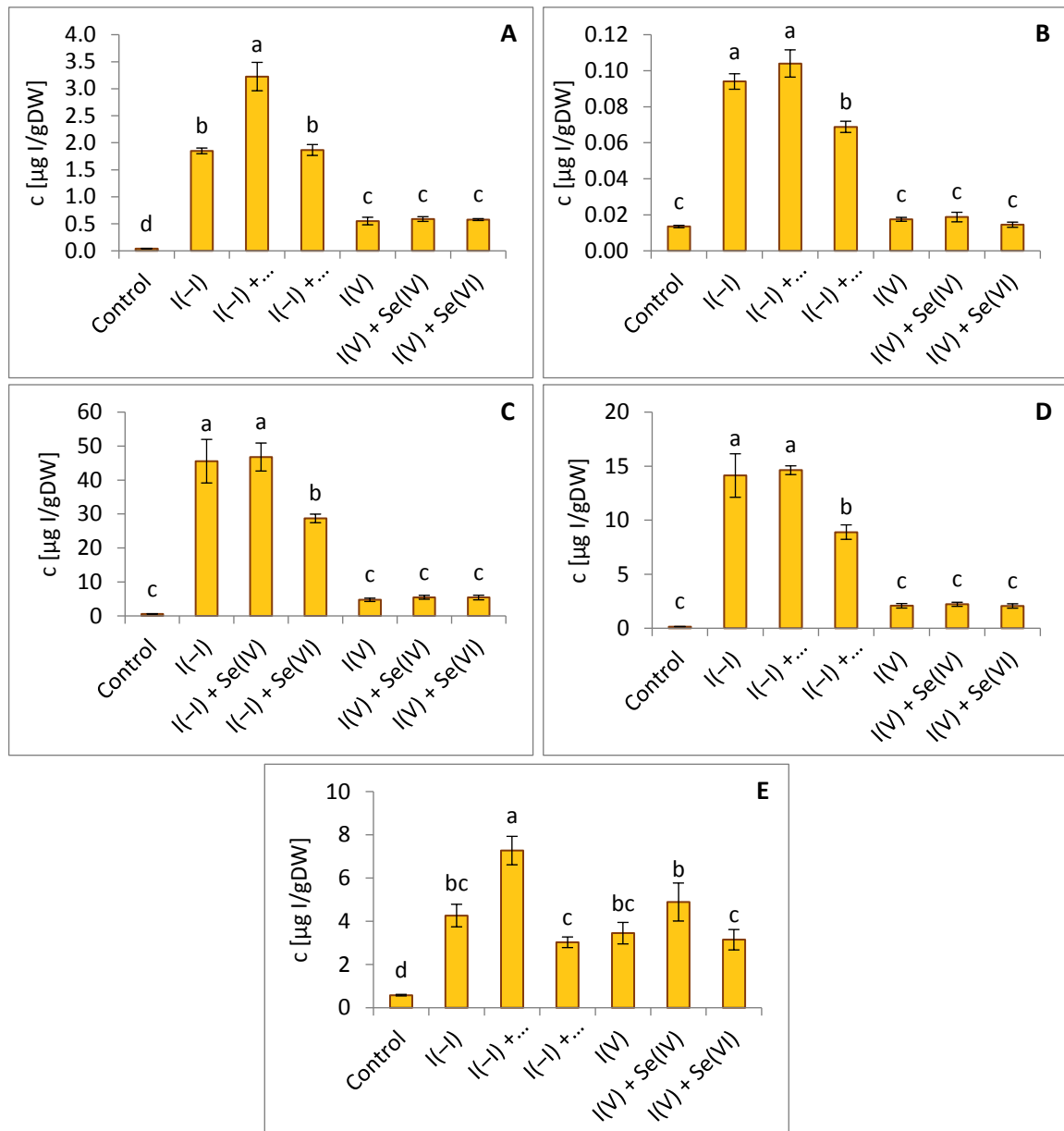


Figure 4.19: Comparison of iodine contents in different parts of pea plants (average with standard error, $n = 4-6$). Letters above the columns indicate statistically significant differences ($p = 0.05$).

A: Pod shells

B: Seeds

C: Leaves

D: Stems

E: Roots

4.4.2 Pumpkin seeds

Application of iodine or a combination of iodine and selenium significantly affected the yield of pumpkin fruits. All treatments resulted in a higher yield of pumpkin fruits compared to the control (data not shown). On the other hand, only the yield of pumpkin seeds I(V) was significantly higher than the yield of control seeds, while after all other treatments, the yield of pumpkin seeds remained at a comparable level to that of the control (data not shown).

4.4.2.1 Iodine content

The results of iodine content in pumpkin seeds are listed in Table 4.18 as an average of 2–3 replicates, each determined in 1–2 parallels with standard error.

Table 4.18: Iodine content (average $\pm$ st. error) in pumpkin seeds (n is stated in brackets).

Treatment	c [$\mu\text{g I/g DW}$]
Control	0.030 ± 0.010 (4)
I(–I)	0.009 ± 0.001 (6)
I(–I) + Se(IV)	0.031 ± 0.003 (6)
I(–I) + Se(VI)	0.016 ± 0.001 (6)
I(V)	0.008 ± 0.002 (5)
I(V) + Se(IV)	0.015 ± 0.003 (6)
I(V) + Se(VI)	0.020 ± 0.004 (6)

Neither I(–I) nor I(V) treatment resulted in an elevated iodine content in pumpkin seeds. In both cases, the average iodine content was even significantly lower (Figure 4.20) than in control seeds, but the differences were attributed to natural variability. The reason for the low iodine content may also be the distribution over the high mass of fruits (on average 0.7 t seeds/ha for control plants and 2.5 t of seeds/ha for treated plants), developed between spraying and sampling (approx. 3.5 months). Additionally, the low amount of iodine in seeds can be explained by low iodine mobility through the phloem, as suggested by Herrett et al. (1962). The reasons for high errors in the results may be found in the very low concentrations (close to the LOD, $0.008 \mu\text{g I/g DW}$) or in the high amount of fat present in pumpkin seeds, which may cause matrix effects that were not specifically studied.

4.4.2.2 The effect of selenium on iodine content

Statistical analysis (Figure 4.20) showed some significant differences in iodine content between individual treatments with iodine and simultaneous treatments with iodine and selenium. In the case of I(–I), Se(IV), there was an increased iodine content in pumpkin seeds, while Se(VI) had no influence. The opposite trend was observed for I(V), in which only Se(VI) affected the iodine content in pumpkin seeds. According to statistical analysis, after I(–I)+Se(IV) and I(V)+Se(VI) treatments, the iodine content was comparable to the content in control seeds, while the amount of iodine was lower after all other treatments. However, the statistical results are not very reliable since the iodine concentrations in seeds were very low, close to the limit of detection and therefore the chances of error are higher. The differences were therefore attributed to natural variability.

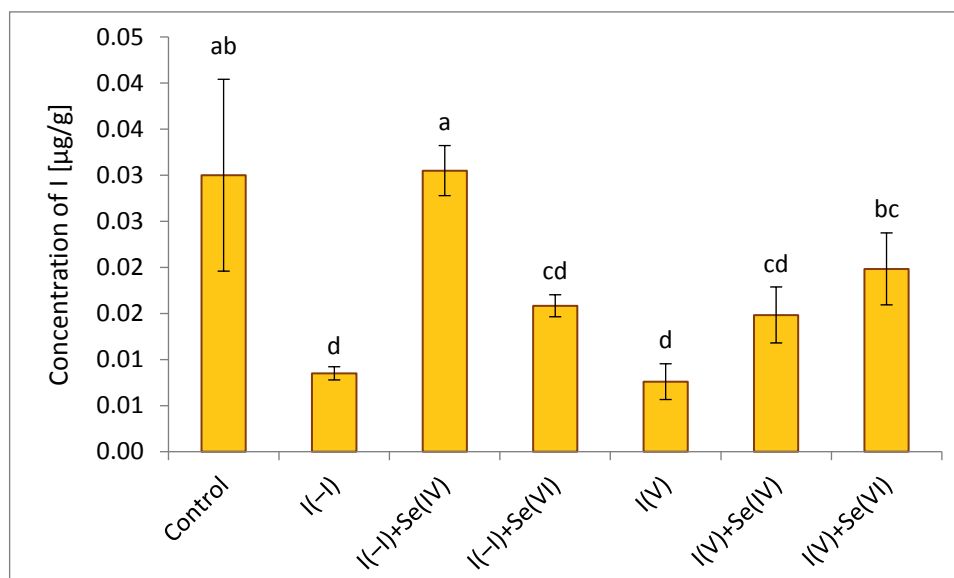


Figure 4.20: Iodine content (average with standard error, $n = 4-6$) in pumpkin seeds with results of statistical analysis (letters above the columns indicate statistically significant differences, $p = 0.05$).

4.4.3 Buckwheat achenes

None of the measured morphological parameters – height of plants, dry mass of above ground part of plants, dry mass of roots and dry mass of achenes – were affected by the addition of iodine or iodine and selenium. In none of those parameters were statistically significant differences found compared to control plants (data not shown).

4.4.3.1 Iodine content

The iodine contents in buckwheat achenes (average of 4 replicates, each determined in 1–2 parallels with standard error) after different treatments are summarized in Table 4.19.

Table 4.19: Iodine content (average $\pm$ st. error) in buckwheat achenes (n is stated in brackets).

Treatment	c [$\mu\text{g I/gDW}$]
Control	0.03 ± 0.01 (6)
I(-I)	0.33 ± 0.02 (8)
I(-I) + Se(IV)	0.37 ± 0.02 (7)
I(-I) + Se(VI)	0.53 ± 0.03 (8)
I(V)	0.29 ± 0.05 (8)
I(V) + Se(IV)	0.61 ± 0.03 (8)
I(V) + Se(VI)	0.35 ± 0.04 (8)

The results showed a much higher iodine content in treated achenes than in control achenes. Regardless of the iodine form in the spraying solution, more than 8-fold higher concentrations were determined in achenes from treated plants. These results clearly show that iodine was transported from leaves to achenes and thus disagree with the findings of Herrett et al. (1962). However, the results from our study are similar to those obtained by

Landini, Gonzali & Perata (2011) in tomato fruits, which are relatively isolated from the xylem. Since they found elevated iodine content in the fruits from treated plants, they concluded the iodine transport to be dependent on phloem transport.

4.4.3.2 The effect of selenium on iodine content

Regardless of the form of the two elements, selenium did not negatively affect iodine uptake (Figure 4.21). Se(IV) did not affect the uptake of I(-I), while Se(VI) had a synergistic effect. The trend was just the opposite for I(V), since no influence was observed when Se(VI) was added but an increased iodine content in achenes was found after I(V)+Se(IV) treatment.

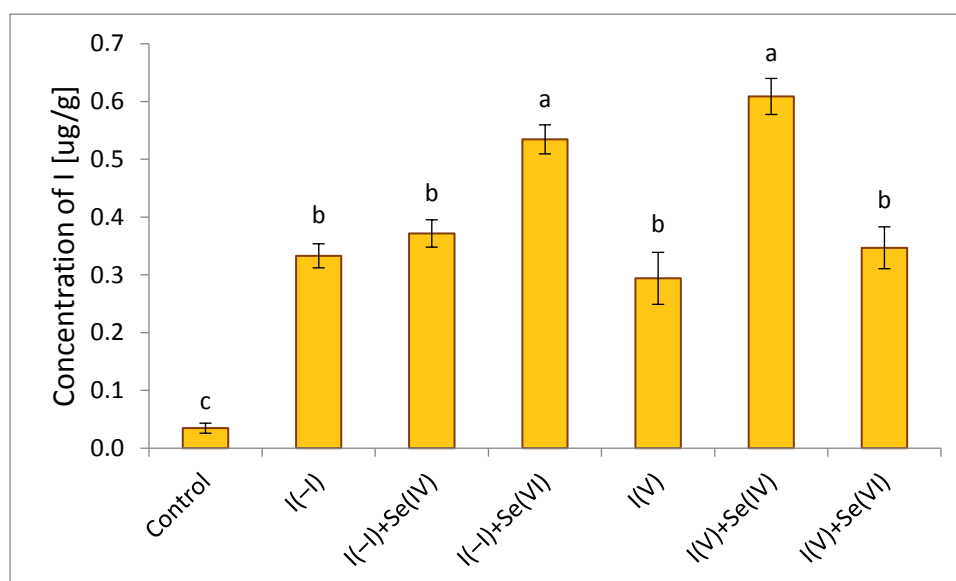


Figure 4.21: Iodine content (average with standard error, $n = 6-8$) in buckwheat achenes with results of statistical analysis (letters above the columns indicate statistically significant differences, $p = 0.05$).

4.4.4 Chicory

The average mass of the aerial part of plants and mass of the marketable part of the chicory did not significantly differ between treatments. The highest amount of rotten or damaged leaves was removed from plants treated with I(-I), while the lowest after treatment with I(V)+Se(VI) (Kroflič et al., 2017).

4.4.4.1 Iodine content

Iodine content was determined in outer chicory leaves, sampled 22 days after foliar application of iodine and selenium and in inner chicory leaves (heads) sampled 66 days after I and Se application. The results, given as an average of 2–3 replicates, each determined in 2 parallels, with standard error, are listed in Table 4.20.

Table 4.20: Iodine content (average $\pm$ st. error) in chicory leaves (n is stated in brackets).

Treatment	Outer leaves ^a	Inner leaves ^b
	c [$\mu\text{g I/g}$]	c [$\mu\text{g I/g}$]
Control	0.11 ± 0.02 (4)	0.021 ± 0.002 (4)
I(-I)	0.32 ± 0.01 (4)	0.055 ± 0.004 (4)
I(-I) + Se(IV)	0.29 ± 0.01 (4)	0.074 ± 0.004 (4)
I(-I) + Se(VI)	0.73 ± 0.04 (6)	0.015 ± 0.003 (4)
I(V)	0.68 ± 0.28 (4)	0.063 ± 0.010 (6)
I(V) + Se(IV)	0.22 ± 0.01 (4)	0.074 ± 0.014 (6)
I(V) + Se(VI)	0.19 ± 0.01 (4)	0.037 ± 0.002 (4)

^a ... sampled in the phase of formatting the heads, 22 days after foliar spraying

^b ... heads, sampled 66 days after foliar spraying

Generally, leaves from the second sampling (inner leaves) contained from 3- up to 49-fold lower amounts of iodine than leaves from the first sampling (outer leaves), regardless of the treatment. The same was also observed for control plants. This can be partly explained by the age of the leaves (outer leaves were older than inner leaves). The differences may also partly arise from the fact that spraying was performed in the rosette phase and iodine was translocated into the outer leaves during growth. However, the majority of inner leaves were developed after sampling of the outer leaves and hence the majority of added iodine was no longer present in the plants. Voogt et al. (2010) also observed higher iodine concentrations in outer leaves of hydroponically grown lettuce. However, in their experiment, performed in winter and in summer, iodine was added to the hydroponic system and was available for plants for a longer time period. The differences in iodine content between outer and inner leaves were probably due to the age of the leaves, since they did not report sampling of outer and inner leaves separately.

In the outer leaves, treatment with I(-I) resulted in slightly higher iodine content than in control plants but the enrichment was not significant. A higher iodine content was obtained after treatment with I(V), but high standard error was observed between different replicates.

In inner leaves, the iodine content was significantly higher than in control plants, regardless of the iodine form used for foliar spraying. Although a slightly higher iodine content was observed after treatment with iodate, no significant differences were found between the two iodine treatments.

4.4.4.2 The effect of selenium on iodine content

In outer leaves, Se(VI) showed a synergistic effect on the uptake of I(-I), while Se(IV) had no impact. On the other hand, both selenium forms acted antagonistically on the uptake of I(V). Statistical analysis (Figure 4.22) showed that only treatment with I(V) or the combination I(-I)+Se(VI) increased the iodine content in comparison with control plants, while concentrations remained at the same level as in control leaves after all other treatments.

In contrast to the results for outer leaves, Se(VI) had a significantly antagonistic effect on I(-I) uptake in inner leaves. Se(IV), similar as in outer leaves, had no impact on the

uptake of I(-I). The same effect of selenium was also observed in the case of I(V), the uptake of which was not influenced by Se(IV) but was decreased in the presence of Se(VI).

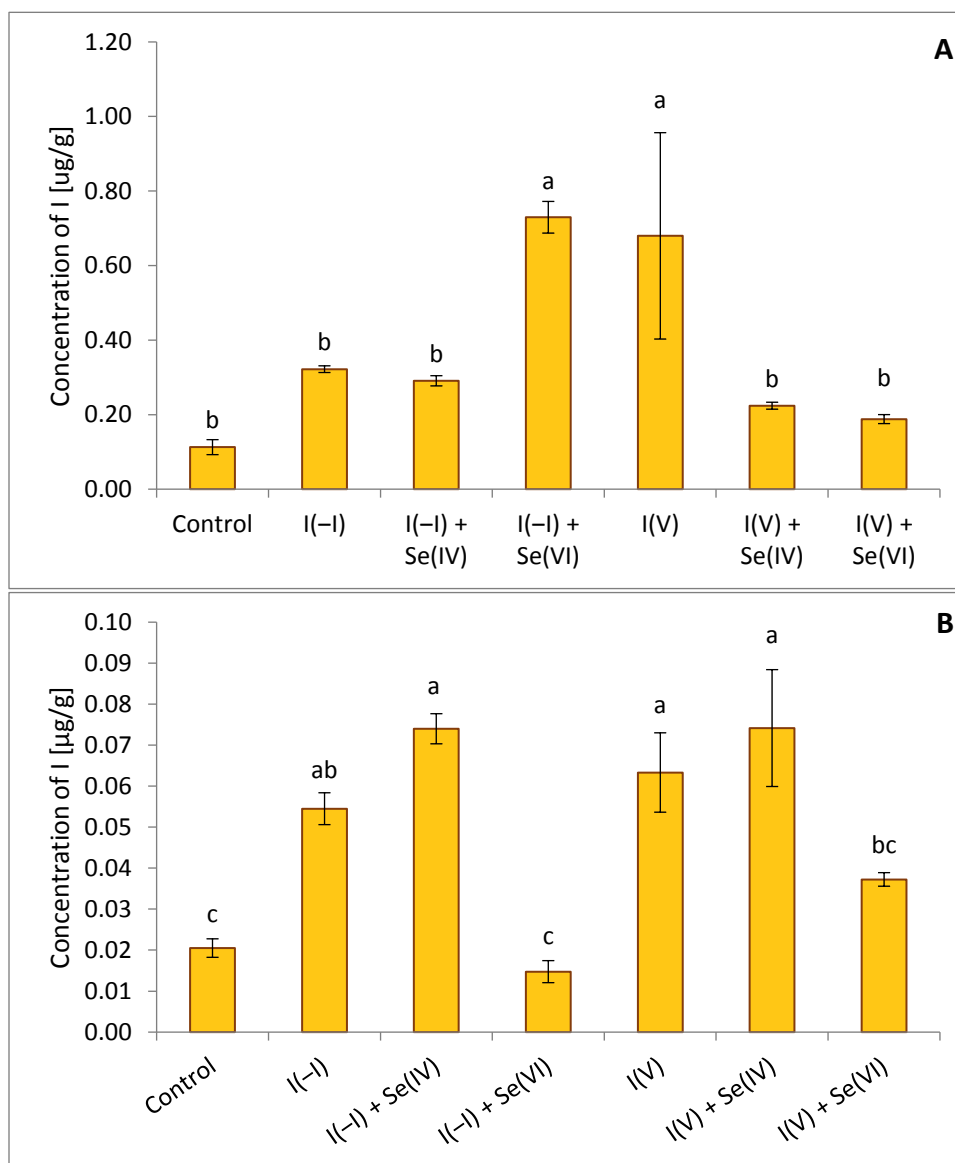


Figure 4.22: Iodine content (average with standard error, $n = 4-6$) in outer (A) and inner (B) chicory leaves with results of statistical analysis (letters above the columns indicate statistically significant differences, $p = 0.05$).

4.5 Comparison of the Effect of Seed Soaking and Foliar Spraying on the Investigated Plant Species

4.5.1 Sprouts

In this section, only sprouts grown from seeds treated with 1000 mg I/L and 10 mg Se/L are compared, so that the growing conditions are as similar as possible. Additionally, only pea sprouts are included in the comparison, without roots and seed residues.

Seed germination is a critical step in the plant life cycle, which starts with the uptake of water by imbibition of dry seeds and results in the expansion and elongation of the seed embryo (Hermann et al., 2007). Processes involved in seed germination and further sprout development can be negatively affected by abiotic stress, e.g., salinity (Anaya, Fghire, Wahbi, & Loutfi, 2015) or high concentrations of minerals in the soaking solution (Pongrac et al., 2016). However, in comparison to soil fertilization or foliar spraying, the approach of soaking seeds is environmentally safer since there are negligible chances of contamination of the environment.

Soaking seeds in solutions of different iodine forms had some negative effects on sprouts. Regardless of iodine form, the height and mass of pea sprouts were reduced and the same was observed for buckwheat sprouts. For pumpkin sprouts, no differences were observed in the mass of the sprouts, although certain deformations of leaves were observed.

4.5.1.1 Iodine content

The lowest natural iodine content (control sprouts) was found in pumpkin sprouts (less than 1 µg I/g DW), while control pea and buckwheat sprouts contained higher amounts of iodine, 3 and 4 µg I/g DW, respectively. Soaking seeds in solutions of I(-I) or I(V) resulted in various iodine contents in sprouts and each plant species responded differently (Table 4.21). Pea sprouts I(-I) contained 211 µg I/g DW and pea sprouts I(V) 188 µg I/g DW and statistical analysis showed no significant differences. Pumpkin sprouts I(-I) contained higher amounts of iodine (279 µg I/g DW) than pumpkin sprouts I(V) (217 µg I/g DW). On the other hand, only buckwheat sprouts I(V) were successfully enriched with iodine, while concentrations in sprouts I(-I) remained at the same level as in control sprouts. Although it is possible that an error occurred when preparing the soaking solutions, it is more likely that some compounds present in buckwheat sprouts transformed iodide into a volatile form, which was lost during growth. Overall, the highest enrichment, regardless of iodine form in the soaking solution, was achieved in pumpkin sprouts, which naturally contained the lowest amounts, but after soaking the seeds in iodine solutions the highest amounts of iodine.

Table 4.21: Comparison of the uptake of different iodine forms added in the same concentration in investigated sprouts. The iodine content (average $\pm$ st. error in $\mu\text{g I/g DW}$) is stated below the form in the soaking solution.

Sprouts	Uptake of iodine form	
	<i>Content in $\mu\text{g I/g DW}$</i>	
Pea sprouts	$\text{I(-I)} = \text{I(V)}$	
	211 ± 135	188 ± 8
Pumpkin sprouts	$\text{I(-I)} > \text{I(V)}$	
	279 ± 19	217 ± 13
Buckwheat sprouts	$\text{I(-I)} < \text{I(V)}$	
	5 ± 1	217 ± 13

To the best of our knowledge, pea, pumpkin and buckwheat sprouts have not yet been included in any other study of iodine enrichment of plants. Additionally, iodine enrichment has not been investigated on any other sprouts. However, Zhu, Huang, Hu, & Liu (2003) studied the uptake of iodine by spinach, grown in solution cultures. Spinach was grown for 33–34 days in total before sampling. Iodine was added as I(–I) or I(V) in the following concentrations: 0, 1, 10, 50 and 100 μM . They found a higher iodine content in shoots after all treatments, compared to the control. When iodine was applied in a concentration of 1 μM , better uptake of I(V) was observed, while after the addition of all other concentrations (10–100 μM), I(–I) treatment was found to have resulted in a higher iodine content in spinach leaves. In a study by Dai, Zhu, Huang, Zhang & Song (2006), iodine enrichment of spinach was also investigated. After germination on filter paper, germinated seeds were transferred into pots. I(–I) and I(V) were supplied to the soil in concentrations of 0, 0.5, 1.0 and 2.0 mg I/kg soil. Plants were grown for 7 weeks. Iodine uptake by spinach was well related to the concentration of iodine added to the soil and, regardless of these concentrations, spinach shoots contained a higher iodine content after treatment with I(V). These results were in agreement with those obtained for buckwheat sprouts from our study, which also contained a higher iodine content when seeds were treated with I(V), regardless of the concentration in the soaking solution. On the other hand, pea and pumpkin sprouts showed different trends, since no differences were found between I(–I) and I(V) for pea sprouts, while I(–I) was better uptaken by pumpkin sprouts, which is in agreement with the findings of Zhu et al. (2003). However, the results are difficult to compare directly, since completely different approaches were used to grow and enrich the plants.

4.5.1.2 The effect of selenium on iodine uptake

There is no general trend regarding selenium impact on iodine uptake in sprouts after soaking seeds in different I+Se solutions. Again, each plant species responded differently (Table 4.22). The only combination to which all investigated sprouts responded in the same way was I(–I)+Se(IV) in which no effect of Se(IV) on iodine uptake was observed. In the combination I(–I)+Se(VI), selenium did not affect the uptake of iodine in buckwheat sprouts, while a synergistic effect was observed for pea and pumpkin sprouts. A similar effect of Se(VI) was also observed in combination with I(V), the uptake of which was increased in pea and pumpkin sprouts. However, decreased uptake of I(V) in the presence of Se(VI) was observed in buckwheat sprouts. When Se(IV) was combined with I(V), an antagonistic effect on iodine uptake was found in pea and buckwheat sprouts, while there was the opposite effect in pumpkin sprouts.

Table 4.22: Effect of added selenium forms to iodine spraying solution on the uptake of different iodine forms in sprouts.

Sprouts	Iodine form	Effect of Se	
		Se(IV)	Se(VI)
Pea sprouts	I(-I)	-	↑
	I(V)	↓	↑
Pumpkin sprouts	I(-I)	-	↑
	I(V)	↑	↑
Buckwheat sprouts	I(-I)	-	-
	I(V)	↓	↓

↓ addition of Se to soaking solution decreased iodine uptake in sprouts, compared to I treatment alone

- addition of Se did not affect iodine uptake in sprouts, compared to I treatment alone

↑ addition of Se to soaking solution decreased iodine uptake in sprouts, compared to I treatment alone

No literature is available on simultaneous I and Se enrichment of sprouts. However, Zhu et al. (2004) studied the interaction between (iodate) and Se (selenate) uptake by young spinach plants (total time of growth was 33–34 days) in a hydroponic system. Three different concentrations, 0, 10 or 50 $\mu\text{mol/L}$ of both elements were used. They reported that at a high concentration of iodine in the growth solution, the addition of selenium slightly decreased the iodine content in spinach shoots. No interaction at lower doses was observed in their study. Compared to plants from our study, their results are in agreement with ours for buckwheat sprouts, in which Se(VI) negatively affected the uptake of I(V), regardless of the concentrations. Just the opposite trend was observed for pea and pumpkin sprouts, in which a synergistic effect of Se(VI) on I(V) uptake was found. However, in addition to differences in plant species, the approach used was completely different. The concentrations applied in our study were much higher (7.88 mmol/L, compared to 0.05 mmol/L in the study by Zhu et al. (2004)) but seeds were soaked for a short time (a few hours), while in their experiment, seedlings were exposed to a nutrient solution containing iodine and selenium for 21 days.

4.5.1.3 Iodine compounds

Inorganic iodine and MIT were determined in all analysed samples. A peak of DIT was not observed in any chromatogram. Some unknown peaks occurred in each sample type but they could not be identified.

Similar amounts of iodine were extracted from all sample types, 68–78% of the total amounts of iodine in samples of control sprouts and sprouts I(-I) and I(V) (Table 4.23). In all sample types, the lowest amount of iodine species was identified in control sprouts. The highest amount of iodine was present in inorganic form, regardless of the treatment, a minority was in the form of MIT. The lowest percentage was found in the form of MIT in buckwheat sprouts (1% or less, regardless of the treatment), while up to 2.9% and 15.1% was found in pea and pumpkin sprouts, respectively.

Table 4.23: Comparison of the results of iodine speciation in sprouts (in %).

Plant/treatment	Extracted iodine ^a	Identified species ^b	MIT ^c	Inorganic iodine ^c
<i>Pea sprouts</i>				
Control	77	86	2.9	97.1
I(-I)	73	106	1.3	98.7
I(V)	78	99	2.1	97.9
<i>Pumpkin sprouts</i>				
Control	68	17	15.1	84.9
I(-I)	71	93	1.1	98.9
I(V)	77	103	0.8	99.2
<i>Buckwheat sprouts</i>				
Control	74	32	1.0	99.0
I(-I)	74	77	0.7	99.3
I(V)	77	110	0.2	99.8

^a ... % of extracted iodine in relation to iodine content in sample

^b ... % of identified species in extracts in relation to extracted iodine

^c ... % of MIT/inorganic iodine in relation to identified species

To the best of our knowledge, iodine species have not yet been studied in any terrestrial plant samples. However, iodine speciation has been performed in different seaweed samples by HPLC-ICP-MS (Table 4.24). Romarís-Hortas et al. (2013) investigated iodine compounds in five seaweed species, Nori, Dulse, Sea Lettuce, Wakame and Kombu, using ultrasound-assisted enzymatic extraction followed by HPLC-ICP-MS (reversed phase column) separation and determination. They found inorganic iodine, MIT and DIT in all samples. The lowest amount of iodine was present in the form of DIT, 7% in Nori and 0.05% or less in other samples. The prevailing form of iodine was iodide in all the samples except Nori, in which the majority of iodine was in the form of MIT (71%). Han et al. (2012) performed iodine speciation in two different Nori samples after ultrasound assisted-extraction and ion-pair reversed phase HPLC-ICP-MS. They used TMAH and water for the extraction of iodine species using an ultrasound wave at room temperature immediately after purging with a nitrogen stream for 5 min. The mobile phase used in the HPLC system was composed of 2.0 mM tetraethylammonium hydroxide and 10 mM L-phenylalanine in 1% methanol at pH 8.9. Of four iodine species that they were able to determine – iodate, iodide, MIT and DIT – iodide and MIT were present in both seaweed samples, while DIT was determined in one of the samples. Iodate was not detected in any of the seaweed samples. In both samples, iodine was mostly present in the form of iodide. Shah et al. (2005) studied iodine species in Wakame samples by HPLC-ICP-MS with a reversed phase column and in Wakame and Kombu with an anion exchange column. The extract for the RPC was prepared using Tris-HCl buffer solution at pH 8, CaCl₂, protein extract and enzyme Proteinase K. The solution was kept at 50 °C and continuously stirred for 12 h. In samples analyzed by RPC, they found iodide, MIT and DIT, and some unidentified peaks were also present in the chromatograms. For analysis with AEC, they mixed the samples with 0.1 M NaOH and carried out extraction at room temperature for 30 min with constant stirring. While in Kombu samples they only found iodide, a small amount of iodate was found in Wakame samples, although iodide was predominantly in inorganic form. Similar to the results in the mentioned studies, the majority of iodine was in inorganic form in algae samples from this study, but MIT was also determined. However, DIT was below the LOD in enzymatic extracts of *Fucus Virsoides*. In spite of the different environments in which they grow, iodine is present in both terrestrial plants and seaweed samples in

predominantly inorganic form. In all samples, MIT was also determined, while DIT was present in the majority of the algae samples but was below the LOD in terrestrial plants.

Table 4.24: Comparison of the literature data of iodine speciation in seaweed samples with the results from the present study.

Reference	Samples	Enzyme	Method	Species found ^a
Our study	Buckwheat sprouts	Pancreatin	HPLC-ICP-QQQ (AEC) ^b	Inorganic iodine, MIT
	Pea sprouts			
	Pumpkin sprouts			
Romarís-Hortas et al. (2013)	<i>Fucus Virsoides</i>	Pancreatin	HPLC-ICP-MS (RPC) ^b	Inorganic iodine, MIT
	Nori	Pancreatin	HPLC-ICP-MS (RPC)	Inorganic iodine, MIT , DIT
	Dulse	Pancreatin	HPLC-ICP-MS (RPC)	Inorganic iodine, MIT, DIT
	Sea lettuce			
Han et al. (2012)	Wakame	-	HPLC-ICP-MS (ion-pair RPC) ^c	Iodide, MIT, DIT^d
	Kombu			
Shah et al. (2005)	Nori	Proteinase K	HPLC-ICP-MS (RPC)	Iodide, MIT, DIT
	Wakame	-	HPLC-ICP-QQQ (AEC)	Iodide, iodate^e
	Kombu			

^a ... prevailing form written in bold

^b ... DIT could be determined but was below the LOD in sample extracts

^c ... Iodate could be determined but was below the LOD in sample extracts

^d ... determined in one of two samples

^e ... Iodate was present only in Wakame

4.5.2 Adult plants

In this section, the edible parts of the selected plants – pea seeds, pumpkin seeds buckwheat achene and chicory heads – are compared.

Foliar spraying with a solution of different iodine forms had no influence on buckwheat and chicory plants. No influence was observed for pea plants either, but there were some negative effects on pod characteristics, which were greater in the case of treatment with iodate than in the case of iodide. For pumpkins, a higher mass of fruits was observed but the yield of seeds after iodine treatments was comparable to controls.

4.5.2.1 Iodine content

All of the investigated plant parts that are normally consumed contain very low amounts of iodine, which is probably due to the distribution over the whole mass of the plant. Due to the time period between spraying and sampling, it is also possible that some iodine was transformed into volatile forms and had been volatilized. Additionally, the low iodine content in pea and pumpkin seeds can be explained by the presumed low iodine mobility through the phloem (Herrett et al., 1962). However, buckwheat achenes were enriched in iodine up to 9.5-fold, showing transportation of iodine from leaves into achenes, which agree with the findings by Landini et al. (2011).

In the edible parts of adult plants, different responses to iodine treatments (iodide or iodate) were again observed for each plant species (Table 4.25). In pumpkin seeds, buckwheat achenes and inner chicory leaves, no significant differences in iodine content were observed when plants were treated with I(–I) or I(V). On the other hand, pea seeds uptook higher amounts of iodine in the case of treatment with I(–I). However, these comparisons can only be figurative and the fact that the investigated plants belong to various families of plants, namely Fabaceae, Cucurbitaceae, Polygonaceae and Cichoriaceae, has to be considered.

Table 4.25: Comparison of the uptake of different iodine forms added in the same concentrations, into edible parts of the investigated plant species. Iodine content (average $\pm$ st. error in $\mu\text{g I/g DW}$) is stated below the form in the soaking solution.

Plant part	Uptake of iodine form	
	<i>Content in $\mu\text{g I/g DW}$</i>	
Pea seeds	I(–I) > I(V)	
	0.094 ± 0.004	0.018 ± 0.001
Pumpkin seeds	I(–I) = I(V)	
	0.009 ± 0.001	0.008 ± 0.002
Buckwheat achenes	I(–I) = I(V)	
	0.33 ± 0.02	0.29 ± 0.05
Chicory heads	I(–I) = I(V)	
	0.055 ± 0.004	0.063 ± 0.010

The results from studies investigating iodine fortification of a few other species have been published (Table 4.26). In a study by Blasco et al. (2008), lettuce was grown in nutrient solution and iodine was added as KI or KIO₃ in concentrations of 10–240 $\mu\text{mol/L}$. A higher iodine content was found in lettuce leaves after the addition of I(–I), compared to the addition of I(V). Similar results were also published by Voogt, Holwerda & Khodabaks (2010) for hydroponically grown lettuce. Iodine was added to the containers as KI or KIO₃, in concentrations between 13 and 129 $\mu\text{g I/L}$. Better uptake of iodide, compared to iodate, was also found in studies by Mackowiak & Grossl (1999), Kiferle, Gonzali, Holwerda, Ibaceta & Perata (2013) and Hasegawa, Tsukada, Kawabata, Takaku & Hisamatsu (2015). Mackowiak & Grossl (1999) investigated iodine uptake in rice grown in nutrient solution (1–100 $\mu\text{mol I/L}$), while tomato fruits grown in soil in plastic pots and root-treated with I(–I) in concentrations 1, 2 and 5 mmol I/L or I(V) in concentrations of 0.5, 1 and 2 mmol I/L were studied by Kiferle et al. (2013). Hasegawa et al. (2015) studied iodine uptake and translocation in radish plants grown in an artificial climate experimental chamber, to which iodine was foliar applied at concentrations of 0.2, 0.5 and 1 g I/L. Another study, performed by Smoleń et al. (2016), also resulted in higher iodine content in field grown carrots after treatment with I(–I) rather than I(V). The total dose of iodine added to the soil was 5 kg I/ha. In our study, only pea seeds showed the same trend of better uptake of I(–I), while other plants' response was not the same as those from the described papers. Two studies (Weng et al., 2008; Zhu et al., 2003) reported better uptake of iodate when applied in low concentrations, while the opposite trend was found at higher concentrations. These results were observed for Chinese cabbage in a hydroponic experiment (addition of 0.05–5.0 mg I/L) (Weng et al., 2008) and for hydroponically grown spinach (iodine added in concentrations of 10–100 $\mu\text{mol I/L}$) (Zhu et al., 2003). In our study, plants were sprayed with one concentration only of iodine and, consequently, we

cannot compare which form is better uptaken at low and which at high iodine concentrations in the spraying solution. On the other hand, Dai et al. (2006), Smoleń & Sady (2012) and Lawson, Daum, Czauderna, Meuser & Härtling (2015) found better uptake of I(V) compared to I(-I) in spinach in a pot experiment (0.5–2 mg I/kg soil), spinach in a pot experiment using pre-sowing fertilization (1 mg I/dm³ soil) or fertigation (approx. 1.1 mg I/dm³ soil) and in kohlrabi, butterhead lettuce and radish grown in fertilized soil (1–15 kg I/ha). In three out of four different plants from this study, the uptakes of I(-I) and I(V) into the edible part of these plants were comparable. This trend was only observed by Lawson et al. (2015) for kohlrabi and butterhead lettuce, in which they did not find significant differences between I(-I) and I(V) uptake after foliar application of iodine (0, 1 × 0.5, 2 × 0.5, 1 × 1.0 or 2 × 1.0 kg I/ha). These results evidently indicate that each species responds in its own way to the addition of iodine and there is no general trend of better uptake of any one of the iodine forms.

Table 4.26: Comparison of our data relating to uptake of I(-I) and I(V) in edible parts of plants with the literature.

Reference	Plant	Growth	Concentrations	Results
Our study	Pea (seeds)	Field experiment	1000 mg I/L (foliar application)	$I(-I) > I(V)$
	Pumpkin (seeds), buckwheat (achenes), chicory (inner leaves)	Field experiment	1000 mg I/L (foliar application)	$I(-I) = I(V)$
Blasco et al., 2008	Lettuce (leaves)	Pot filled with vermiculite, nutrient solution renewed every 3 days	10–240 μmol I/L (added to nutrient solution)	$I(-I) > I(V)$
Voogt et al., 2010	Lettuce (outer and inner leaves)	Hydroponic system	13–129 μg I/L (added to nutrient solution)	$I(-I) > I(V)$
Weng et al., 2008	Chinese cabbage (leaves)	Pot experiment/soil fertilization	0.5–5 mg I/L (applied to soil)	$I(-I) > I(V)^b$
Dai et al., 2006	Spinach (leaves)	Pot experiment/soil fertilization	0.5–2 mg I/kg of soil (applied to soil)	$I(-I) < I(V)$
Smoleń & Sady, 2012	Spinach (leaves)	Pot experiment	1 mg I/dm ³ of soil (applied to soil by pre- sowing or fertigation)	$I(-I) < I(V)$
Zhu et al., 2003	Spinach (leaves)	Hydroponic system (nutrient solution changed twice a week)	1–100 μmol I/L (added to nutrient solution)	$I(-I) > I(V)^b$
Hasegawa et al., 2015	Radish (root tubers)	Experimental chamber (vermiculite + nutrient solution)	0.2–1 g I/L (foliar application)	$I(-I) > I(V)$

Reference	Plant	Growth	Concentrations	Results
Kiferle et al., 2013	Tomato (fruit)	Pots with soil in growth chamber/soil fertilization	0–10 $\mu\text{mol I/L}$ (applied to soil)	$I(-I) > I(V)$
Lawson et al., 2015	Kohlrabi ^a , radish ^a , butterhead lettuce ^a	Field experiment	1–15 kg I/ha (applied to soil)	$I(-I) < I(V)$
Lawson et al., 2015	Kohlrabi ^a , butterhead lettuce ^a	Field experiment	0.5, 1 kg I/ha (foliar application)	$I(-I) = I(V)$
Smoleń et al., 2016	Carrot (roots)	Field experiment/soil fertilization	5 kg I/ha (total dose applied to soil before sowing and as top- dressing)	$I(-I) > I(V)$
Mackowiak & Grossl, 1999	Rice (seeds)	Tubes with nutrient solution	1–10 $\mu\text{mol I/L}$ (added to nutrient solution)	$I(-I) > I(V)$

^a ... part of plant not specified

^b ... when applied in lower concentrations, $I(-I) < I(V)$

4.5.2.2 The effect of selenium on iodine uptake

The different effects on iodine uptake are presented in Table 4.27. Similar to sprouts, in all plants except in pumpkin seeds, Se(IV) did not affect I(–I) uptake. However, pumpkin seeds contained very low concentrations and no enrichment was observed, regardless of the treatments, so we attributed the differences to natural variability. Se(VI) increased the uptake of I(–I) in buckwheat achenes, while decreased it in inner chicory leaves and pea seeds. The uptake of I(V) into pea seeds and inner chicory leaves was not affected by Se(IV). In buckwheat achenes, this combination showed a synergistic effect. The addition of Se(VI) to the I(V) spraying solution negatively influenced the uptake of iodine in inner chicory leaves but no impact was found for pea seeds and buckwheat achenes.

Table 4.27: Effect of selenium forms on the uptake of different iodine forms in edible parts of the investigated plant species.

Plant part	Iodine form	Effect of Se	
		Se(IV)	Se(VI)
Pea seeds	I(–I)	-	↓
	I(V)	-	-
Pumpkin seeds	I(–I)	*	*
	I(V)	*	*
Buckwheat achene	I(–I)	-	↑
	I(V)	↑	-
Chicory leaves – inner	I(–I)	-	↓
	I(V)	-	↓

↓ *addition of Se to the spraying solution decreased iodine uptake in edible plant part, compared to I treatment alone*

- *addition of Se did not affect iodine uptake in edible plant part, compared to I treatment alone*

↑ *addition of Se to spraying solution decreased iodine uptake in sprouts, compared to I treatment alone*

* ... *Differences attributed to natural variability.*

As already mentioned in section 4.5.1.2, the results of only three studies have so far been published about simultaneous fortification of sprouts with iodine and selenium. Zhu et al. (2004) and Smoleń et al. (2014) investigating the interaction between I and Se in spinach and lettuce. In the first study (Zhu et al., 2004), spinach was grown in a solution culture in which I(V) and Se(VI) were added in concentrations of 0, 10 or 50 µmol/L. Selenium slightly decreased the iodine content in spinach shoots when iodine was added in a higher concentration (50 µmol/L). Smoleń et al. (2014) used the same I and Se forms in an experiment with lettuce grown in a hydroponic system of nutrient film technique (1 mg I/L and 0.5 or 1.5 mg Se/L) and foliarly (5 times with 0.05% solution of I and 0.005% solution of Se). The lettuce was grown for 11–13 weeks. Their results showed no effect of selenium on iodine content when each application was used separately, but when the two applications were used one after another, selenium decreased the iodine content in lettuce leaves compared to iodine treatment alone. A reduced iodine content was also observed in chicory leaves when the combination I(V)+Se(VI) was applied. However, no effect of Se(VI) on I(V) uptake was observed in pea seeds or buckwheat achenes.

In the most recent study by Smoleń et al. (2016), carrots were field-grown in soil, fertilized with two forms of each element – I(–I), I(V), Se(IV) and Se(VI) and their combinations. I and Se were applied twice: before planting the seedlings and as a top dressing, in a total dose of 5 kg I/ha and 1 kg Se/ha. They found an antagonistic effect of selenium on iodine uptake in storage roots, regardless of the form. However, the effect was greater when selenium was added as Se(IV) rather than Se(VI). Although in our study the addition of Se to the iodine spraying solution decreased the iodine content in the edible parts of plants in some cases (pea seeds I(–I)+Se(VI) and inner chicory leaves I(–I)+Se(VI) and I(V)+Se(VI)), this is not a general trend, since after other treatments, selenium had no influence on iodine uptake, or even increased it.

4.5.3 Comparison between sprouts and adult plants

In all cases listed below, the iodine content was much higher in sprouts than in any part of adult plants. This can be ascribed to the much lower mass of plant in which the iodine was accumulated. However, we cannot say to what extent, if any, the approach of biofortification – soaking of seeds or foliar spraying – is responsible for the differences. This could be further investigated by transplanting the sprouts into soil and sampling the same part at the same stage of growth.

4.5.3.1 Peas

In pea sprouts and roots of pea sprouts, iodide and iodate were uptaken to the same extent. A similar trend was also observed in the roots of adult plants, but in leaves, a higher iodine content was found when treated with iodide.

In sprouts, Se(VI) had a synergistic effect on the uptake of both iodine forms, while Se(IV) did not affect iodide uptake, but decreased the uptake of I(V). Similarly, Se(IV) had no effect on iodine content in leaves of adult pea plants, but Se(VI) even decreased it. Se(VI) had no influence on iodine content in leaves after spraying with iodate. In the roots of pea sprouts, only Se(IV) decreased the iodine content after soaking in iodide solution, while other combinations had no effect compared to iodine treatment alone. In roots of adult plants, a higher iodine content was observed after spraying with I(–I)+Se(IV), while after other combinations, the iodine content was comparable to iodine treatments alone.

4.5.3.2 Pumpkins

Regardless of iodine form in the soaking solution, the iodine content in pumpkin sprouts was increased compared to control sprouts. However, higher amounts of iodine were found in sprouts after soaking in a solution of iodide. On the other hand, foliar spraying with iodine solutions did not result in elevated iodine concentrations in seeds, regardless of the iodine form.

The addition of selenium to the spraying solution also had no influence on the iodine content in pumpkin seeds. Although some differences compared to iodine treatment alone were observed, they were attributed to natural variability, since the iodine content was still lower than in control seeds. In contrast to pumpkin seeds, the iodine content in pumpkin sprouts was influenced by selenium presence in the soaking solution. Both forms of selenium increased the uptake of iodate, and Se(VI) synergistically affected the uptake of I(–I). No difference compared to treatment with iodine alone was observed only in a combination of I(–I)+Se(IV).

4.5.3.3 Buckwheat

The iodine content was elevated, compared to the control in both buckwheat sprouts and buckwheat achenes. In sprouts, the iodine content was higher when seeds were soaked in a solution of iodate, while no significant differences were observed in buckwheat achenes after treatment with different iodine forms.

Selenium, regardless of its form, had no influence on the uptake of I(-I) in buckwheat sprouts but it had an antagonistic effect on the uptake of I(V). On the other hand, the uptake of I(-I) was not influenced by the presence of Se(IV) and was increased by Se(VI), while the opposite trend was observed for the uptake of I(V) – Se(IV) increased it while Se(VI) had no effect.

The results for the iodine content in sprouts cannot be directly compared to those in adult plants due to the completely different stages of growth in which plants were sampled. However, all the results from these comparisons suggest that not only plant species but also the way of growing and the approach used for biofortification have an impact on the final iodine content in selected part of plants.

4.6 Iodine Enriched Plants for Human Nutrition

To provide sufficient amounts of iodine for normal thyroid function, WHO recommends that the daily iodine intake should be 150 µg for an adult person.

4.6.1 Sprouts as potential food supplements

The iodine content in most of the enriched sprouts was very high (up to 323 µg I/g DW in sprouts from seeds soaked in a solution containing 1000 mg I/L). They could thus be used in iodine food supplements. The percentage of RDI was therefore calculated for the case of consuming 0.5 g of dry sprouts, on the assumption that all iodine present in the sprouts would be absorbed in the body. In Table 4.28, the results are listed for sprouts the seeds of which were soaked in 1000 mg I/L (and 10 mg Se/L). For comparison, the percentage of RDI consumed by the same amount of control sprouts is added in the table.

With 0.5 g of enriched sprouts, the amount of iodine generally does not exceeds RDI. This amount of dry sprouts would thus be appropriate for iodine food supplements.

Several different multivitamin-mineral food supplements with declared values for iodine were analysed by (Osterc & Stibilj, 2006). The declared values ranged from 30 to 155 µg I/g, while the measured values indicated an iodine content between 15 and 231 µg I/g. The iodine content in approx. 2/3 of the sprouts from this study, enriched by soaking the seeds in a solution containing 1000 mg I/L, is thus in the concentration range determined in commercially available food supplements.

Table 4.28: Calculated parts of RDI (150 µg I/day) consumed with 1 g of dry sprouts.

	Pea sprouts	Pumpkin sprouts	Buckwheat sprouts
Control	1%	0.3%	1%
Treated I(-I) ^a	62–82%	93–108%	2–7%
Treated I(V) ^b	51–71%	72–96%	1–72%

^a ... Sprouts from seeds soaked in I(-I) and I(-I)+Se solutions

^b ... Sprouts from seeds soaked in I(V) and I(V)+Se solutions

4.6.2 Edible parts of adult plants as functional food

We wanted to know what share of the recommended daily intake for iodine is consumed with a portion of 100 g of pea seeds, pumpkin seeds, buckwheat achenes or chicory leaves. The results are listed in Table 4.29. For pumpkin seeds, the calculation was made on the basis of dry seeds. For other plants, the percentage of RDI was calculated on a fresh mass basis, since they are usually consumed fresh. Pea seeds contained on average 31% of dry mass, which did not significantly differ among the treatments. For buckwheat achenes and chicory, the dry mass was not determined in our study and literature data (Souci, Fachmann & Kraut, 2000) on the amount of water content was used in the calculation – 14% of water (86% of dry mass) for buckwheat achenes (the data was stated for buckwheat flour) and 94.1% of water (5.9% dry mass) for chicory. For all plants, the percentage of RDI was calculated based on the consumption of a portion of 100 g. The highest amount of iodine, covering between 17 and 35% of iodine RDI, would be consumed by enriched buckwheat achenes, while very low percentages would be consumed with all other plants (< 3% RDI). However, in most cases, a higher share of RDI would still be consumed with a portion of the treated parts of the investigated plants than with the control.

Table 4.29: Calculated shares of RDI (150 µg I/day) consumed with a 100 g portion of fresh pea seeds, pumpkin seeds, buckwheat achenes or chicory leaves.

	Pea seeds	Pumpkin seeds	Buckwheat achenes	Chicory leaves (inner)
Control	0.3%	2.0%	2%	0.1%
Treated I(-I) ^a	1.4–2.1%	0.5–2.1%	19–30%	0.1–0.3%
Treated I(V) ^b	0.3–0.4%	0.5–1.3%	17–35%	0.2–0.3%

^a ... Edible parts from plants foliar sprayed with I(-I) and I(-I)+Se solutions

^b ... Edible parts from plants foliar sprayed with I(V) and I(V)+Se solutions

Chapter 5

Conclusions

Iodine has been known to be an essential element since the early 1900s. Although its role in human health, and especially growth and development, is well known, there are still about 2 billion people worldwide that have an insufficient iodine intake and suffer from IDD. The highest amounts of iodine are naturally present in seafood, while terrestrial crops are usually low in iodine content. Iodized salt has been introduced into human nutrition in order to improve iodine status in the population, but it is now recommended that the intake of salt itself is reduced. New and/or additional iodine sources must therefore be found. Iodine biofortification of terrestrial plants has recently been researched as a possible source of iodine in human nutrition. Various plants have been investigated in order to find the most appropriate species for enrichment.

However, before the introduction of enriched plants into nutrition, the iodine content must be known, to avoid excessive iodine intake. Iodine analysis is associated with several difficulties, e.g., volatilization and, consequently, loss of the analyte, memory effects etc. In addition, one of the major problems in iodine analysis in biological samples is a great lack of reference materials. In the first part of the thesis, therefore, a method for iodine determination was optimized. Microwave extraction in alkaline media was shown to have much lower blank values and LODs than with Schöniger combustion. A combination of 2 mL of 25% TMAH and 10 mL of Milli-Q water resulted in the best ratio between agreement with certified/informative values of iodine in reference materials and consumption of chemicals. Negligible matrix effects were observed and so external calibration was used for quantitative iodine determination instead of the standard addition technique. However, an internal standard was used, since better repeatability was achieved over a longer time period. Measurements were performed in 0.1% TMAH solution, due to the lowest blank values compared to 0.5 and 1% TMAH. Comparison with an independent method (k_0 -INAA) showed very good agreement of the results, proving their trueness.

In addition to iodine content in plants, iodine speciation is also important, due to possible differences in the bioavailability, mobility and toxicity of different iodine species. Iodine speciation has so far only been performed in algae and seaweed samples, so a method was optimized for iodine speciation in terrestrial plants. For determination of iodine compounds, enzymatic extraction in a water bath with longitudinal shaking was chosen as a better option over techniques using ultrasound, especially due to the more repeatable conditions for extraction and control of the temperature. Alternatively, an ultrasonic bath can be used with a water recirculator to ensure a constant temperature during the extraction. Pancreatin was the enzyme with the lowest iodine content and was used for further experiments. Both enzyme and plant material caused interconversion of iodate into iodide so, using the optimized method, we were able to separate and determine MIT, DIT

and inorganic iodine. Optimal results of extraction were achieved with extraction for 12 h at 37 °C. Separation and detection of iodine compounds were performed by HPLC-ICP-QQQ in single quadrupole mode with an anion exchange chromatographic column.

Both optimized methods were then applied to determine iodine and its species in iodine enriched plants. Iodine was determined in pea, pumpkin and buckwheat sprouts, as well as in different parts of pea plants, pumpkin seeds, buckwheat achenes and chicory leaves. Iodine compounds were determined in iodine enriched sprouts.

Two different approaches were used for iodine enrichment of plants – sprouts were grown from seeds previously soaked in iodine and/or selenium solutions, while adult plants were foliar sprayed during growth on the field. The first approach was very successful in terms of iodine enrichment, since all sprouts contained high amounts of iodine. Due to the high iodine content, these sprouts could be used in food supplements. On the other hand, no or little enrichment was achieved in the edible parts of adult plants after foliar spraying; enrichment strongly depended on the plant species. No enrichment was observed in pumpkin sprouts, while enrichment was achieved in other plants (edible parts) at least after treatment with one of iodine forms, though to a much lesser extent than in sprouts. The hypothesis of both investigated approaches increasing iodine content in selected parts of plants was therefore only partly confirmed. The hypothesis that sprouts contain higher amounts of iodine after soaking seeds in iodine solutions than selected parts of mature plants after foliar spraying was confirmed.

No unambiguous conclusion can be reached in relation to which iodine form is better uptaken at the same concentration added. Each plant responded differently and even the same plant after different approaches of iodine addition and/or in a different stage of growth did not necessarily show the same trend of iodide and iodate uptake. However, these results are in agreement with previous observations of other authors in relation to different responses of iodide and iodate uptake of various plant species. The hypothesis of better uptake of iodine in the form of iodide than in the form of iodate could not be generally confirmed, although it was true for some plants, namely pumpkin sprouts and a few parts of pea plants – seeds, pod shells, stems and leaves. The hypothesis was not confirmed for all other investigated plants (or plant parts).

Although we were unable to investigate whether iodine remains in a plant in the same form in which it was added or it is transformed into other forms, with a particular focus on transformations of iodide to iodate or *vice versa*, we proved that the majority of iodine remained in inorganic form. Part of the iodine was transformed and detected as MIT and, in some cases, traces of unknown species were also present. DIT was not found in any of the analyzed samples. Of the hypothesis that the majority of uptaken iodine is in the same form as in the soaking solution and part of it is transformed into iodinated amino acids, only the second part was confirmed. Part of the iodine was indeed transformed into iodinated amino acids, mainly MIT. Only traces of DIT, lower than the LOD, might be present. However, the first part of the hypothesis can be neither confirmed nor rejected, since we were unable to determine iodate with the optimized method due to interconversion into iodide. The hypothesis that iodine is not completely extracted and part of it remains in the sample residue, and from the extracted iodine only some of the species are identified, was confirmed. On average, 76% of iodine was extracted, the remainder of the iodine probably remaining in the sample residue. In iodine enriched sprouts, almost all of the extracted iodine was identified. Some unknown peaks were present in the chromatograms, but these represent traces of unidentified compounds. However, lower amounts of identified species were observed in control plants, suggesting that naturally present iodine can be

transformed into unknown species that cannot be detected by the described method, while iodine uptaken during the soaking of seeds is mainly in inorganic form and iodinated amino acids.

Iodine distribution among the plants was studied in both young (sprouts, roots and seed residues) and adult (seeds, pod shells, stems, leaves and roots) pea plants. Of the analyzed parts of young plants, seed residues contained the highest amounts of iodine and sprouts (green part) the lowest amounts, but very small differences were observed in iodine content after soaking in iodide or iodate solution. In adult plants, after spraying with iodate the content was elevated only in roots and slightly in pod shells, while in all other parts it remained similar to the iodine content in controls. However, enrichment was achieved after spraying with iodide and, of the analyzed parts iodine, the content was the highest in leaves, followed by stems, roots and pod shells, while the lowest iodine amounts were found in seeds. Generally, the highest iodine content in both young and adult plants was found in the part to which it was added (seed residue in the experiment with sprouts and leaves in the field experiment with adult plants).

The addition of selenium to the soaking/spraying solution can result in a synergistic, an antagonistic or no effect on iodine uptake. There was no general trend of the same form having the same effect in specific plant species. Additionally, no such trend was observed for plants enriched using the same approach (i.e., all sprouts or all adult plants). The hypothesis of inorganic selenium forms not affecting iodine uptake can only be partly confirmed. Selenium did not significantly increase or decrease the iodine content in selected part of plants in approx. half of the samples, while the effect was significant in the other half. In samples in which selenium influenced iodine uptake, it had a synergistic effect in approx. 60% and the effect was antagonistic in the remaining 40%.

Selenium had no effect on iodine compounds present in buckwheat sprouts. Regardless of whether there was selenium present in the soaking solution, more than 99% of identified iodine species were in inorganic form, with the remainder (less than 1%) being in the form of MIT.

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- Jerše, A., Kacjan-Maršič, N., Šircelj, H., Germ, M., Kroflič, A., Stibilj, V. (2017). Seed soaking in I and Se solutions increases concentrations of both elements and changes morphological and some physiological parameters of pea sprouts. *Plant physiology and biochemistry*, 118, 285–294.
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Unpublished Conference Contribution

- Germ, M., Kacjan-Maršič, N., Šircelj, H., Golob, A., Jerše, A., Stibilj, V. (2015). Concentration of iodine in buckwheat sprouts increased after iodide and iodate treated seeds. In *Proceeding of 12th International Congress on Engineering and Food*. Québec City, Canada.
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Treatise, Preliminary Study, Study

Vreča, P., Stibilj, V., Jaćimović, R., Jerše, A., Zuliani, T., Horvat, M. (2014). Priprava referenčnega materiala tal slovenskega izvora: poročilo o razvojnem projektu MIRS za leto 2013. *IJS delovno poročilo*.

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Submitted Articles Related to the Thesis

Jerše, A., Kacjan Maršić, N., Kroflič, A., Germ, M., Šircelj, H., Stibilj, V. Is foliar enrichment of pea plants with iodine and selenium appropriate for production of functional food? Food Chemistry, revised version resubmitted on July 19th 2017.

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Jerše, A., Jaćimović, R., Kacjan Maršić, N., Germ, M., Šircelj, H., Stibilj, V. Determination of iodine in plants. Microchemical Journal, submitted on August 30th 2017.

Biography

Ana Jerše graduated from the Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia with a bachelor of science degree in chemistry. She finished her BSc study with a diploma thesis entitled “Passive sampling for determination of pharmaceutical residues in an aqueous environment”, for which she won the Krka Award.

During her undergraduate study, she was a Jožef Stefan Institute scholarship holder for four years. She worked via the student employment service at the Department of Environmental Sciences, where she also performed the experimental part of her BSc.

After graduation, she was a young researcher at the Department of Environmental Sciences, Jožef Stefan Institute for three and a half years, as well as a postgraduate student of Ecotechnology at the Jožef Stefan International Postgraduate School. As a young researcher, she published 1 original scientific article as first author, 1 as coauthor and submitted 3 original scientific articles as first author. She participated at eight conferences in Slovenia and other European countries.

During the period of her young researcher position, she gained expertise in the determination of iodine and its compounds by ICP-MS and HPLC-ICP-MS, as well as different sample preparation procedures. She will finish her PhD study with the defense of her doctoral dissertation entitled “Determination of iodine and its compounds in iodine enriched plants”.