University of São Paulo
"Luiz de Queiroz" College of Agriculture

Biofortification of broccoli seedlings with selenium: influence on bioactive compounds and *in vivo* toxicity

Patricia Bachiega

Thesis presented to obtain the degree of Doctor in Science. Area: Food Science and Technology

Piracicaba
2018

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Bachelor of Pharmacy

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Advisor:
Prof[a]. Dr[a]. **JOCELEM MASTRODI SALGADO**

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Bachiega, Patricia

Biofortification of broccoli seedlings with selenium: influence on bioactive compounds and in vivo toxicity / Patricia Bachiega. - - Piracicaba, 2018.

103 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. *Brassica oleracea* 2. Selenato de sódio 3. Alimentos funcionais 4. Mutagenicidade 5. Micronúcleo 6. Spray dryer I. Título

This work is dedicated to my mother, Artenize, for the education, dedication and love

ACKNOWLEDGMENT

It is difficult to find the best words to describe the immense joy of completing this stage. Getting to the conclusion of this work was not easy; however, it would have been even more difficult if there were not so many people by my side. For this reason, I want to thank with all my heart:

God, for always guiding my ways and showing me in small gestures that all the effort is worth it. For always strengthening me in moments of anguish and giving strength and courage to face each difficulty.

My mother and my grandmother, Artenize and Brunilde. Thanks for always encouraging the search of my dreams and for all the confidence. Thanks for understanding the moments of absence. You are the reason of my living and those responsible for this victory.

My father, Valdir and my brothers, Thiago and Gabriel, for their constant support and encouragement.

My fiancé Rafael Campagnol, my best friend and companion. Thank you for all your understanding, patience and care throughout this phase. Thank you for always being by my side, even so, many miles away and for the unconditional support.

My advisor, Dr. Jocelem Mastrodi Salgado for the opportunities, patience, and friendship throughout these almost 7 years of partnership. Thanks for trusting in my work and allowing this dream to come true.

My co-adviser, Dr. João Ernesto de Carvalho for receiving me in his laboratory, for the teachings and for enabling the accomplishment of the in vivo and in vitro tests.

The “Escola Superior de Agricultura “Luiz de Queiroz” (ESALQ), the “Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA)” and the “Universidade Estadual de Campinas (UNICAMP)” for the opportunity to develop this research.

CAPES for granting the doctoral scholarship.

Professors Solange, Thais, Marília and Severino for providing access to the laboratories to carry out the analyzes.

Sakata Seed South America for the supply of broccoli seeds and Cargill for the supply of maltodextrin.

IBS Mudas for the aid in the cultivation of the broccoli seedlings.

Regina, Fábio, Ana, Jair, Adna, Ivani, Carlota, Mariana and especially to Vana, for the friendship and coffees.

Edson and Professor José Laércio Favarin for lending the freeze dryer.

Professor Valdemar and Rodrigo for the partnership in the analysis of LC-MS/MS.

Prof. Hudson and Eduardo for the immeasurable help in the EDXRF analyzes.

Prof. Marco Zezzi and Eraldo for the help in ICP-MS analysis.

And last but not least, my friends, who were fundamental in this journey:

Maressa, for all the stories that we have lived since the master's degree. For always being by my side and guiding me in all aspects. Thank you for always encouraging me. You are my professional example. I hope we can still share many life and professional experiences.

Little Helena, who taught me how life can be lighter and with every smile and hug made me feel renewed. “Di Paty” loves you very much.

To the friends that Esalq allowed me to meet: Anirene, Fernanda, Nataly, Paulo, Rafael, Samara, Richtier, Mirian, Amanda and Talita for the companionship in the laboratory and in this journey in Piracicaba. For Grazi, Natalia, Fernanda, Linda and Paula help in planting broccoli seedlings. Special thanks to Jessica "morena", Jessica "loira", Gizele and Fúvia for the friendship and for sharing many happy and sad moments during this trajectory. You will always be in my heart.

To Gizele for the companionship and help me so much in this final stretch of the doctorate. Thank you for the positive messages and for always calming me in moments of uncertainty. A lot of success for you.

To Kélin Schwarz for all the help during the experiments and trips to Pirassununga. Thank you for all your patience in clarifying my questions. I wish you every success in this world.

The family CPQBA, Adriana, Gisele, Luciana, Ellen, Lúcia, Karin, Sica, Ilza, Mariana, Tuany, Nubia, Mary Ann, Ana P., Ana Lúcia, Fabi and Livia. Thank you for welcoming me and for the immeasurable help in animal and cell culture experiments and for teaching me so much about research and about life. Thank you for all of the coffees surrounded by conversations and advices. I have a deep admiration for each one of you. I'm sure that, despite the short time, I have gained friends for a lifetime.

Thanks especially to friends Matu, Tu, and Nubs (Mom). I have no words to express my gratitude for you. Thanks for making the routine lighter and for being always by my side in the ups and downs. I will miss the conversations, the laughs (which were not few) and the "gordices" (which "unfortunately" were many). I'm sure your path will be full of achievements. See you in Cuiabá.

Thanks to “Grandma” Sica for helping me and always teaching me. Thank you for the tight hugs that renewed me, for the long conversations and for being an example of dedication to work and family. I'll miss you.

To Karin, for the help of all the design and conduction of the animal experiment, especially on the weekends and holidays. Thank you for your patience in teaching me, for your advices and good laughs.

To the friends Hyanne, Thais and Bruna, who even from afar always gave me strength and cheered for my achievements. To Naiarinha, my sister, for the patience with the long audios sent by WhatsApp and for the countless times to calm my anxiety and anguish. Thanks for always understanding and staying by my side in all the decisions.

The biggest obstacle to discovery is not ignorance; it is the illusion of knowledge.

Daniel J. Boorstin

CONTENTS

RESUMO

Biofortificação de mudas de brócolis com selênio: influência nos compostos bioativos e toxicidade *in vivo*

Os objetivos desse estudo foram avaliar a influência da biofortificação com selênio nos compostos bioativos de brócolis; testar sua toxicidade e mutagenicidade *in vivo* como forma de comprovar sua segurança para consumo; desenvolver micropartículas pela técnica de *spray dryer* e avaliar suas propriedades físico-químicas. As mudas de brócolis foram biofortificadas pela adição de 2 mL de solução de 50 µM de selenato de sódio 15 dias de após a semeadura (DAS) e coletadas aos 30 DAS. As amostras foram analisadas por diferentes métodos para a quantificação de selênio (ICP-MS, XRF portátio e EDXRF de bancada). O método de fluorescência de Raios X por dispersão em energia feito em equipamentos de bancada ou portátil apresentou desempenho adequado para quantificação de selênio aliado à baixo custo e menor tempo de análise. A técnica de biofortificação com selênio aumentou a concentração desse micronutriente nas mudas de brócolis. Além disso, também proporcionou aumento significativo nos compostos bioativos, tais como, clorofila (12%), compostos fenólicos (26%) e flavonoides (19%), vitamina C (32%) e sulforafano (12%), principal composto bioativo com potencial anticarcinogênico do brócolis. Verificada a eficiência da biofortificação com selênio, conduziu-se estudos de toxicidade e mutagenicidade em camundongos fêmeas e machos tratados com diferentes doses de mudas de brócolis biofortificadas com selênio (15, 45 e 70 µg Se/kg peso corporal). Após 40 dias consecutivos de tratamento, observou-se que os animais tratados com mudas de brócolis com selênio nas doses de 45 e 70 µg Se/kg peso corporal provocaram significativas alterações nos pesos relativos dos órgãos vitais (fígado, baço e rins) e reprodutivos (ovário/testículos), com presença de alterações histológicas nos rins e no baço, em camundongos de ambos os sexos. Nos camundongos machos, a dose de 70 µg Se/kg peso corporal provocou aumento da frequência de eritrócitos policromáticos micronucleados. Nas fêmeas este aumento não foi observado, demonstrando maior sensibilidade dos machos ao tratamento. Por fim, através da técnica de microencapsulação por *spray dryer* desenvolveu-se micropartículas a base de extratos etanólicos de mudas de brócolis com e sem selênio. As amostras apresentaram baixa umidade (4-5%) e higroscopicidade (11-12 g de água absorvida 100^{-1} g de micropartícula) e alta solubilidade (99%). No entanto, houve uma redução significativa no teor de compostos fenólicos e atividade antioxidante após o processo. Os resultados desse estudo demonstram a importância da biofortificação do selênio como uma estratégia para aumentar o teor de compostos bioativos no brócolis. Com relação à toxicidade, a dose de 15 µg Se/kg peso corporal não apresentou efeitos tóxicos significativos em machos e fêmeas, porém apresentou pontencial mutagênico em machos. Além disso, comprovou-se que a microencapsulação possibilitou a elaboração de amostras com boas características tecnológicas, porém, estudos de otimização devem ser conduzidos a fim de aumentar o poder de retenção dos compostos bioativos e atividade antioxidante.

Palavras-chave: *Brassica oleracea*; Selenato de sódio; Alimentos funcionais; Mutagenicidade; Micronúcleo; Spray dryer

ABSTRACT

Biofortification of broccoli seedlings with selenium: influence on bioactive compounds and *in vivo* toxicity

The objective of this study was to evaluate the influence of biofortification with selenium in the bioactive compounds of broccoli; to test their toxicity and mutagenicity in vivo to prove their safety for consumption; to develop microparticles by the spray dryer technique and to evaluate its physicochemical properties. The broccoli seedlings were biofortified through the addition of 2 mL of 50 μM sodium selenate solution 15 days after sowing (DAS), and collected 30 DAS. The samples were analyzed by different methods for the quantification of selenium (ICP-MS, benchtop and handheld EDXRF). The energy-dispersive X-ray fluorescence spectrometry made either in bench or in a portable equipment presented a good performance for the quantification of selenium, together with low cost and less analysis time. The selenium biofortification technique increased the concentration of this micronutrient in broccoli seedlings. In addition, it also allowed a significant increase in bioactive compounds, such as chlorophyll (12%), phenolic (26%) and flavonoid (19%) compounds, vitamin C (32%) and sulforaphane (12%), the main bioactive compound with anticarcinogenic potential. The efficiency of biofortification with selenium in the culture was verified, and toxicity and mutagenicity studies were conducted in female and male mice treated with different doses of selenium-biofortified broccoli seedlings. After 40 consecutive days of treatment, broccoli seedlings with selenium at doses of 45 and 70 μg Se/kg body weight resulted in significant changes in the relative weights of vital (liver, spleen and kidneys) and reproductive organs (ovary/testis), with the presence of histological alterations in the kidneys and spleen in mice of both sexes. In male mice, the dose of 70 μg Se/kg body weight increased the frequency of micronucleated polychromatic erythrocytes. In females this increase was not observed, demonstrating greater sensitivity of males to the treatment. Finally, microparticles of ethanolic extracts of broccoli seedlings with and without selenium were developed through the spray drying microencapsulation technique. The samples presented low moisture (4-5%) and hygroscopicity (11-12 g of water absorbed 100/g of microparticle) and high solubility (99%). However, there was a significant reduction in the content of phenolic compounds and antioxidant activity after the microencapsulation process. The results of this study demonstrate the importance of biofortification of selenium as a strategy to increase the content of bioactive compounds in broccoli and that the dose of 15 μg Se/kg body weight had no significant toxic or mutagenic effects. In addition, microencapsulation has been shown to allow the elaboration of samples with good technological characteristics however, optimization studies should be conducted to increase the retention power of bioactive compounds and their antioxidant activity.

Keywords: *Brassica oleracea*; Sodium selenate; Functional foods; Mutagenicity; Micronucleus; Spray dryer

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1. INTRODUCTION

Fruits and vegetables are the main sources of nutrients and bioactive compounds in the human diet. However, these foods do not always have sufficient or bioavailable quantities of these compounds. Statistics show that around the world, about two billion people, or one in three people, have micronutrient deficiencies (RITCHIE; ROSER, 2018). In this context, agriculture has focused not only on food production to reduce hunger, but also on the production of nutrient-rich food to reduce hidden hunger (KENNEDY; NANTEL; SHETTY, 2003). Thus, to meet the need for some micronutrients and to combat their deficiency some strategies are suggested, such as fortification and biofortification. The fortification refers to the addition of nutrients to food products, for example the addition of iodine to salt. However, despite being an effective strategy, it has some disadvantages/limitations, such as limited stability of the additives, interference of the additives in food quality and the high cost for its realization, requiring advanced infrastructure, which makes its use difficult in developing countries (GÓMEZ-GALERA et al., 2010, RAWAT et al., 2013).

Biofortification, in turn, is a more effective, economical and sustainable method. It allows the synthesis or accumulation of micronutrients in a food crop using conventional and transgenic methods, and can be grown and distributed through existing farming practices. When consumed regularly, biofortified food crops can lead to significant improvements in human health, being a great alternative especially for populations that have limited access to varied diets or other interventions (DÍAZ-GÓMEZ et al., 2017; BOUIS; SALTZMAN, 2017). HarvestPlus and its partners have already proven the effectiveness of this method, with more than 20 million rural households in developing countries having access to biofortified crops. In addition, statistics suggest that by 2030 biofortification could reach up to one billion people (BOUIS; SALTZMAN, 2017).

It is important to note that no single intervention strategy can fully address the problems of micronutrient deficiency and that biofortified foods can not deliver as high levels of vitamins and minerals as food supplements or fortified foods. However, the biofortification strategy acts as a complement to existing interventions, aiding in the daily adequacy of micronutrients intake throughout life. Compared to other intervention methods, biofortification has two main advantages: the ability to reach rural populations and long-term cost-effectiveness. Even though, there is a need for an initial investment - unlike other strategies, which require continuous financial disbursements. From the establishment of the cultivation conditions to the costs of the production, monitoring and maintenance are reduced.

In addition, it is possible for developed crops to be applied in other environments and geographies, increasing the benefits of the initial investment (BOUIS et al., 2011; BOUIS, SALTZMAN, 2017).

In Brazil, through the HarvestPlus Latin American and Caribbean (LAC) program, led by the Brazilian Agricultural Research Corporation (EMBRAPA), BIOFORT Brazil was created. This program has developed and released some nutrient-rich varieties of sweet potato (up to 115 ppm provitamin A), maize (up to 7.5 ppm provitamin A), cassava (up to 9 ppm provitamin A), cowpeas (up to 77 ppm iron and 53 ppm zinc) and beans (up to 80 ppm iron and 50 ppm zinc) (SALTZMAN et al. 2013).

Another micronutrient that has stood out in biofortification research is selenium, an essential micronutrient for humans and animals (DENG et al., 2017). When consumed in small doses it plays important biological functions in the organism (MALAGOLI et al., 2015), which has aroused the interest in this compound in the last three decades (RAYMAN, 2012). Through co-translational mechanisms, selenium is incorporated into proteins as part of the amino acid selenocysteine, the twenty-first essential amino acid (ROMAN; JITARU; BARBANTE, 2014). From this amino acid about 25 selenoproteins are formed, of which one third has the main function of minimizing oxidative damage (KUMAR; PRIYADARSINI, 2014). Scientific studies have already demonstrated the essential role of selenium in various functions in the human organism, such as antioxidant defense (AHMAD et al., 2012), immune function (KHOSO et al., 2015), thyroid hormones formation (WICHMAN et al., 2016), fertility and reproduction (MEHDI et al., 2013). In addition, in the last 20 years, a direct relationship between selenium and cancer has been demonstrated (IBÁÑEZ et al., 2011; JAYAPRAKASH; MARSHALL, 2011; LAMBERTO et al., 2013; MORENO et al., 2012; ROMAN; JITARU; BARBANTE, 2014; BACHIEGA et al., 2016).

Selenium compounds may be present in inorganic (selenate, selenite and selenide) and organic forms (methylselenocysteine, selemethionine and selenocysteine) (RAYMAN, 2008). These forms are found in different sources, which may present variations in their content due to the amount of selenium present in the soil. Therefore, the same food can present different amounts of this nutrient when cultivated in different areas (MEHDI et al., 2013).

Although the recommended daily dose of selenium is not high, the sources of this micronutrient carry low amounts of it, which makes selenium deficiency a global concern (VALDIGLESIAS et al. 2010; WAEGENEERS et al., 2013). There are reports stating that selenium deficiency affects 800 million people worldwide (MALAGOLI et al., 2015) and, in

European countries, recommended daily intakes are not achieved (IVORY; NICOLETTI, 2017).

As the main sources of selenium, we can highlight plants and meats. In fruits and vegetables, it can vary from 1 to 60 ng g^{-1} of whole matter, and in cereals this variation is from 20 to 370 ng g^{-1} of whole matter. In meat, meat products and eggs the content of selenium ranges from 100 to 810 ng g^{-1} of whole matter. In dairy products it ranges from 10 to 160 ng g^{-1} of whole matter, and in marine fish from 400 to 1500 ng g^{-1} of whole matter (KUMAR; PRIYADARSINI, 2014). In Brazil, we have one of the main sources of selenium - the Brazil nut (*Bertholetia excelsa* HBK), which stands out with contents ranging from 5.8 to 169.9 μg g of whole $matter^{-1}$ (PACHECO; SCUSSEL, 2007; COMINETTI et al., 2012; ROCHA et al., 2014). However, despite its richness in selenium, this source is still not easily acquired by the Brazilian population (KUMAR; PRIYADARSINI, 2014).

As the vegetables represents the main selenium source for humankind, agricultural crop selenium biofortification becomes even more important as a way to reduce health problems related to this nutrient deficiency (MALAGOLI et al., 2015). Considering this, in 1980 Finland began to perform the genetic improvement of some cultures and to introduce selenium in their fertilization, significantly increasing the selenium levels in the population's blood (ALFTHAN et al., 2015).

The selenate or selenite are the main inorganic forms of selenium used in the soil to biofortification of plants with selenium. This process can be performed by foliar application, soil application, or the combination of both (ZHU et al., 2017). During this process, accumulation capacity and the form of accumulated selenium will also be different among plants, a fact probably related to the expression levels of sulfate transporters. Due to this variation, some species naturally tend to accumulate greater amounts of selenium, being possible to emphasize the species *Allium* spp. and *Brassicas* spp. (HSU et al., 2011; TERRY et al., 2000). Among *Brassicas*, broccoli is classified as the primary accumulator of this nutrient because of its great capacity to accumulate selenium (> 2000 mg kg^{-1}) (RAMOS, 2011). According to Ávila et al. (2013), inflorescences and sprouts of broccoli accumulate significant amounts of Se-methylselenocysteine.

After the production of Se-biofortified plants, an important step is the quantification of this micronutrient in the food matrix. For this purpose, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Energy dispersive X-ray fluorescence (EDXRF) spectroscopy (variant of X-ray fluorescence spectrometry) are the main techniques used. ICP-MS, among the atomic spectrometry techniques, is the most powerful due to its low detection limits. In

addition, it has a rapid detection and ability to measure isotope ratios (POLATAJKO; JAKUBOWSKI, SZPUNAR, 2006; HE et al. 2017). However, this technique requires the complete destruction of the matrix, which increases the time and cost of the analysis. Moreover, the samples digestion with strong acid reagents requires greater caution during sample preparation (TEZOTTO et al. 2013).

On the other hand, EDXRF has shown to be a very promising technique among instrumental analytical methods, being versatile for application in several fields of research, industry and science (VELOSO; SILVA, 2018; ROMANO et al. 2014). This method is based on measuring the energies and fluorescence intensities of each element. X-rays emitted from an ionized atom have characteristic energies for each element, functioning as their fingerprint. Thus, the elements contained in the studied matrix can be identified through the energy of the peaks generated in the spectrum (SILVA et al. 2012; MANSO; CARVALHO, 2007). The main advantages of this technique involve the non-destruction of the sample, the low cost and the short period of analysis (ROMANO et al., 2014), which has encouraged its use in recent years (FLEMING et al., 2015; JOLLY et al., 2017).

As previously mentioned, the biofortification of broccoli with selenium is a great alternative to increase the concentration of this micronutrient in the food. However, in addition to the positive effects mentioned, this process can also promotes some negative events (CHOMCHAN; SIRIPONGVUTIKORN; PUTTARAK, 2017) since, in the plant, it can generate an abiotic stress. This stress can significantly alter the amount of bioactive compounds present in broccoli, since the assimilation of selenium can affect the metabolic pathways of sulfur and nitrogen. Changes in the sulfur pathway may directly affect the nitrogen pathway, resulting in alterations in the synthesis of proteins and amino acids such as methionine, phenylalanine, tyrosine and tryptophan, precursors of glucosinolates, and phenylalanine, a precursor of phenolic compounds (MALAGOLI et al, 2015). Thus, the study of the evaluation of the influence of biofortification on bioactive compounds becomes very important, allowing to determine if the process of broccoli enrichment causes the increase or decrease of bioactive compounds.

Another important issue when referring to biofortification is the safety of the consumption of biofortified foods, since the range between selenium benefits and toxicity is narrow. According to the Food and Nutrition Board (FBN) of the Institute of Medicine of the National Academy (USA), the recommended daily intake and the maximum tolerable selenium intake are 55 and 400 μg day^{-1} respectively (OTTEN, HELLWIG, MEYERS, 2006). However, selenium toxicity is not only dependent on the dose, but also on the form and state

of oxidation (LYONS; PAPAZYAN; SURAI, 2007). The main generalized symptoms of selenium toxicity include skin, mucous and eyes irritated, weight loss and anemia. On the other hand, the most specific symptoms involve garlic odor in respiration and sweat beside irritation in the pharynx, intestine and bronchi (BENKO et al., 2012).

The knowledge that certain agents (physical, biological or chemical) can interact with the genetic material and cause mutations is a long-standing one. The major concern is that these damages result, therefore, in genomic instability and cancer (MALLING, 2004). Therefore, in addition to acute and/or repeated dose toxicity studies, the assessment of the mutagenic potential is a mandatory toxicological evaluation to ensure the safety of the sample. One of the widely used tests for this purpose is the mammalian *in vivo* micronucleus test, which evaluates the potential of the sample to cause cytogenetic damage, resulting in micronuclei (MN) formation containing either whole chromosomes or lagging chromosome fragments (ARALDI et al., 2015). MNs can be originated by the disruption of the mitotic apparatus (aneugenesis) or by chromosomal breaks (clastogenesis) (SAMANTA; DEY, 2012). In adult rodents MNs are formed in the spleen or bone marrow during erythropoiesis. After 6h of final mitosis, erythroblasts originate the polychromatic erythrocytes (PCE), which subsequently undergo a maturation process and give rise to normochromic erythrocytes (NCEs). Thus, the mutagenic agents cause a change in the chromosomes (loss or fragmentation) during the cell division, giving rise to the MNs (KRISHNA; HAYASHI, 2000).

MN is therefore a small fragment of nucleus left behind along the cell division (SYLVIA; BASKARAN; BHAT, 2018). The increase in the frequency of micronucleated PCEs (MNPCEs) is indicative of chromosomal damage and has been used as a marker since 1959 (KRISHNA; HAYASHI, 200; KIRSCH-VOLDERS et al., 2003). The *in vivo* experiment also allows the evaluation of the absorption, tissue distribution, metabolism and excretion influences on the samples toxicity (MORITA et al., 2016).

Besides the aspects evolved in the biofortified food production and the influence these processes on plant metabolism and safe consumption, another important point concerns how to transfer the benefits of bioactive compounds from these biofortified foods to the consumer market. One of the strategies to carry out this transfer is the production of powders with high nutritional value. Due to their stability and ease storage, these powders can be introduced in different matrices, meeting the requirements of the food industries (RAGHAVI; MOSES; ANANDHARAMAKRISHNAN, 2018; SHISHIR; CHEN, 2017).

Among the technologies already available in the food industry we can mention microencapsulation. This technology has as its primary function the protection of process-sensitive compounds (DIAS; FERREIRA; BARREIRO, 2015). Developed approximately 60 years ago, microencapsulation refers to the technique of conditioning liquids, solids or gases in which the release of the active content will occur in a controlled manner under specific conditions (DESAI; PARK, 2005).

Spray drying is one of the most successful microencapsulation techniques in the industry. In this process, liquid foods are transformed into powders in a single-step procedure (TONTUL; TOPUZ, 2017), being more economical than lyophilization (eight times) and vacuum drying (four times). In addition, this process has a low drying time (5-100 s), which contributes to a better preservation of heat-sensitive compounds and attributes such as taste, odor and color (SANTIVARANGKNA; KULOZIK; FOERST, 2007; SOSNIK; SEREMETA, 2015). Moreover, the powders produced in this process are resistant to oxidative and microbiological degradation, since they present low levels of water activity (0.2-0.6) and moisture (2-5%) (SHISHIR et al., 2016; PATIL; CHAUHAN; SINGH, 2014; TAN et al., 2011).

In this context, the present study was conducted aiming the production of broccoli seedlings biofortified with selenium. Employing different analytical methods, the broccoli seedlings products were characterized as their selenium and bioactive compounds contents as well as being evaluated for *in vivo* toxicity and mutagenicity in adition to the development of the microparticles through the spray drying technique.

Thus, this thesis was organized in five chapters. The first is the introduction about the study. In the second chapter, the selenium quantification in the matrix studied was described and using three different methods (benchtop and handheld EDXRF and ICP-MS). In the third chapter, we evaluated the biofortification influence in the bioactive compounds (chlorophyll, phenolic and flavonoid compounds, carotenoids, vitamin C and sulforaphane) profile and quantity from broccoli seedlings. In the fourth chapter, male and female mice were fed broccoli seedlings biofortified with selenium at different doses to assess toxicity through hematological, biochemical and histopathological biomarkers and mutagenicity through the micronucleus test. Finally, the fifth chapter evaluated the possibility of producing microparticles from broccoli seedlings extract by spray dryer and surveyed their physicochemical properties.

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2. BENCHTOP AND HANDHELD ENERGY DISPERSIVE X-RAY FLUORESCENCE (EDXRF) AS ALTERNATIVE FOR SELENIUM DETERMINATION IN BROCCOLI SEEDLING

Abstract

Selenium (Se) has beneficial or toxic effects depending on its concentration, encouraging its determination in food matrices. Inductively coupled plasma mass spectrometry (ICP-MS) is one of the most used techniques due to its high sensitivity. However, due to some disadvantages (high cost, longer analysis time and being destructive) there is a need to search for new alternatives for Se quantification. This study aimed at establishing the instrumental parameters for Se quantification using two energy-dispersive X-ray fluorescence spectrometry (EDXRF) techniques (benchtop and handheld) in Se biofortified broccoli seedlings in comparison to ICP-MS. The results showed that both EDXRF systems and ICP-MS presented similar results for sodium selenate treatments (MC). However, for control (MS) treatments, EDXRF techniques were not able to perform quantification due to the high limit of detection (LOD, 0.6-0.9 mg kg^{-1}) unlike the ICP-MS (LOD, 0.0007 mg kg^{-1}). This study demonstrates that EDXRF system are suitable techniques for the determination of Se in biofortified samples.

Keywords: X-ray fluorescence; Complex matrices; Biofortification; Trace elements; Sodium selenate

2.1. Introduction

Selenium is an essential micronutrient for human beings and the importance of this metalloid in human health has received considerable attention in the last several years (NUNES et al., 2012). It plays important biological functions in the animal organism due to presence in selenoproteins, scavenge free radicals, anti-aging effects and beneficial effects on diabetes and multiple types of cancer (MALAGOLI et al., 2015). Previous studies of our group identified that seedlings biofortified with selenium showed an important cytostatic activity against the glioma cell line U251 (BACHIEGA et al., 2016).

Plants are the most common sources of Se in foods (LI; YU, 2016). In fruits and vegetables, Se content may vary from 1 to 60 ng g^{-1} fresh weight (KUMAR; PRIYADARSINI, 2014). Due to such low concentration in biological samples and the insufficient consumption, Finland started in 1980 the genetic improvement of some crops and

the introduction of Se in their fertilization (ALFTHAN et al., 2015). Since then, there is a high interest in the production of Se-enriched food (NUNES et al., 2012). The biofortification with this micronutrient can also a strategy for increased the bioactive compounds in vegetables, for example, Se-enriched broccoli seedlings presented the higher phenolic compounds (6%) and antioxidant activities (10-16%) compared with control treatments (BACHIEGA et al., 2016).

Due to the low concentration of Se in food, its quantification becomes difficult and there is a need for high sensitivity techniques (LI; YU, 2016; ZUKOWSKA; BIZIUK, 2008). The inductively coupled plasma mass spectrometry (ICP-MS) is one of the most used techniques for quantification of Se (KUBACHKA et al., 2017). However, this technique has some limitations, such as, high cost, highly trained analysts and total sample matrix destruction.

X-ray fluorescence (XRF) technique, especially EDXRF, becomes a promising technique for nutrient plant analysis, offering a simple, fast and non-destructive analysis for nutrient analysis in foods (JOLLY et al., 2017), especially for Se (HANLEY et al., 2017; PALTRIDGE et al., 2012). In addition, the use of the portable X-ray fluorescence (pXRF) system has increased dramatically in the last 10 years in measurement of many elements concentrations in different matrices (BARNETT et al., 2016; FLEMING et al., 2015; FLEMING et al., 2017; MCINTOSH et al., 2017, ROUILLON; TAYLOR, 2016; SHEHAB et al., 2016). The aim of this study was to evaluate the performance of the benchtop and handheld EDXRF techniques in comparison to ICP-MS for Se determination in broccoli seedling.

2.2. Materials and methods

2.2.1. Plant material

IBS MUDAS (Piracicaba, São Paulo, Brazil) provided broccoli seedlings of the cultivar Avenger (Sakata Seed Sudamerica). The broccoli seedlings (Figure 1) were cultivated in black polyethylene trays containing 200 cells filled with commercial substrate (coconut fibre, Amafibra). After sowing, the trays were transferred to an arc-type agricultural greenhouse (8 m wide, 103 m long and 4.5 m height) right foot, covered with 150 μm thick anti-UV polyethylene film and closed sides with anti-fake screens. After 15 days of germination, broccoli seedlings were randomly separated and received the application in each

cell of 2 mL of distilled water (control treatments; MS) or 50 µM sodium selenate solution (MC) and remained for another 15 days in the greenhouse (BACHIEGA et al., 2016). In the 30th day after sowing, broccoli seedlings were collected and only the aerial part of the plant was selected for the analysis. Subsequently, the plant tissue was sanitized (0.5% sodium dichloroisocyanurate for 10 min), freeze-dried (Modelo E-C–Modulyo) and stored at -20 °C until use. Samples of broccoli seedlings were cultivated in August-October 2014 (MS14 and MC14) and August-October 2016 (MS16 and MC16).

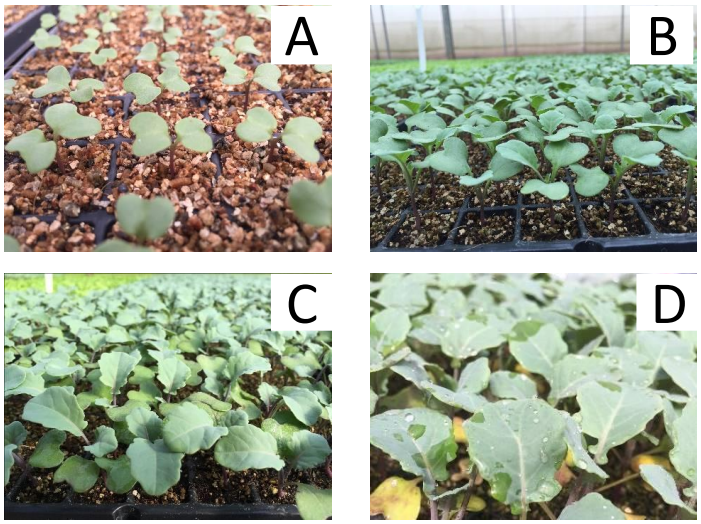

Figure 1. Broccoli seedlings after 7 (A), 15 (B), 23 (C) and 30 (D) days of germination.

2.2.2. Sample preparation for ICP-MS analysis

Initially, aliquots (100 mg) of the broccoli seedlings treatments (MS14, MC14, MS16 and MC16), certified reference material of plankton (BCR 414) and Sea Lettuce (Ulva Lactuca) (BCR 527) were accurately weighed and transferred to poly(tetrafluoroethylene) flasks of microwave oven (DGT 100 Plus). The microwave decomposition of each sample was carried out using a mixture of HNO_3 (4 mL) with H_2O_2 (200 µL) for 7 minutes at 400 W. After decomposition, the samples were transferred to poly(propylene) tubes and volume made up to 50 mL with deionized water except the sea lettuce sample that was completed to 14 mL.

2.2.3. ICP-MS analysis

The operational conditions of ICP-MS quadrupole (model ELAN® DRC-e, PerkinElmer) are shown in Table 1, and the analytical calibration curve ranged from 0.5 to 25 μg L^{-1}. The results were expressed in μg Se g^{-1} dry weight (DW) unit.

Table 1. ICP-MS instrumental parameters used for Se determination.

ICP RF power	1200 W
plasma gas flow rate	15 L min^{-1}
auxiliary gas flow rate	1.1 L min^{-1}
nebulizer flow	0.75 L min^{-1}
isotope monitored	^{78}Se
lens voltage	7.0 V
pulse state voltage	1000 V

2.2.4. Sample and standard preparation for EDXRF analysis

The standard curve of Se was prepared by adding known volumes of a 100 μg Se mL^{-1} working standard solution of Se in a broccoli seedling sample (control treatment). This standard was provided from the dilution (1:10) of 1,000 μg mL^{-1} Se stock standard (Acros Organics, 1 mg mL^{-1}). Subsequently, the standard samples were dried in a laboratory oven at 50 °C and finally were thoroughly homogenized in an agate mortar. The calibration curve concentration ranged from 0 to 250 μg g^{-1}.

Samples and standards (1 g) were weighted into a poly(ethylene) sample cup (23.9 mm opening, cat. no. 1530, Chemplex), gently and manually pressed with an acrylic piston, and sealed with 6-μm-thick Mylar® film (no. 3517, Spex).

2.2.5. Benchtop EDXRF analysis

The benchtop EDXRF measurements were carried out using a Shimadzu EDX-720 spectrometer equipped with a 50 W Rh target X-ray tube using voltage 50 kV voltage and auto-tuneable current for a 30 % maximum detector dead time. A Si (Li) detector was used for sample X-ray spectrum acquisition. To optimize the EDXRF analysis for the Se quantification, 6 filters conditions were tested, being: no filter, silver (Ag), molybdenum (Mo), molibdenium-nickel (MoNi), aluminum (Al) and titanium (Ti). The acquisition time used was 300 s and all analyses were carried out in triplicate under no vacuum.

The X-ray spectrum processing was carried out utilizing the EDX-720 software package provided by Shimadzu.

2.2.6. Handheld XRF analysis

A handheld Bruker AXS, Tracer III-SD model (2 kg, 30 cm long x 10 cm wide x 28 cm height) was also employed. The samples were excited with a 4 W Rh target X-ray tube at 40 kV and 30 µA, and X-ray spectra recorded by a 10 mm^2 X-Flash® Peltier-cooled Silicon Drift Detector (SDD).

At first, the conditions of the Se determination using the handheld XRF were optimized. Five primary filters with distinct thickness and composition were tested: no filter, red filter (30.58 µm Al + 2.54 µm Ti + 2.54 µm Cu), green filter (30.48 µm Al + 2.54 µm Ti + 15.24 µm Cu), yellow filter (30.48 µm Al + 2.54 µm Ti) and blue filter (2.54 µm Ti) to optimize the Se evaluation. The acquisition time used was 300 s and all analyses were carried out in triplicate without vacuum. The sample spectra were processed by the Artax software.

2.2.7. Limits of detection and quantification

ICP-MS limits of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation of 10 readings of the standard solution blank and on the slope of the analytical curve (LOD = 3σ/slope and LOQ = 10σ/slope).

The benchtop and handheld EDXRF LOD and LOQ were calculated according to Equation 1:

$$LOD = \frac{3}{S}\sqrt{I_{BG}} \quad and \quad LOQ = \frac{10}{S}\sqrt{I_{BG}} \qquad (1)$$

where: S is the sensitivity (counts mg^{-1} kg) and I_{BG} is the background intensity under Se Kα characteristic X-ray (counts).

2.3. Results and Discussion

To optimize the determination of Se by both EDXRF systems, the figure of merit of its Kα X-ray characteristic intensity and background under the X-ray peak (noise) square root ratio (GUERRA et al., 2014; ERNST et al., 2014) was evaluated for different primary filters

in both EDXRF systems. Indeed, primary filter absorbs distinctly the polychromatic X-ray excitation beam from the X-ray tube, thus changing its profile, which may improve the analyte X-ray characteristic intensity to noise ratio. The choice of the proper filter depends on the X-ray tube and its operational condition, sample matrix, analyte and filter thickness and composition. Thus, selecting the best filter option is not a straightforward task; the analyst must carry out a preliminary study that will lead to the best choice. Figures 2A and 2B show the effect of several primary filters on the Se Kα X-ray intensity to background square root ratio for the benchtop and handheld EDXRF units.

Regarding the benchtop system (Figure 2A), the Se Kα X-ray intensity (counts) to BG (counts) square root ratio using Ag filter was roughly four times higher than no filter condition. The Ag Kα X-ray absorption edge energy (25.517 keV) is higher than Rh–K lines anode energies (Kα = 20.170 and Kβ = 22.725 keV), therefore absorbing weakly and only partly the high intensity anode K lines. The latter lines from the X-ray tube anode excite Se K shell efficiently, and on the other hand, the Ag filter absorbs more effectively the background of the Se Kα line energy region (16.209 keV) from the X-ray tube, thus increasing significantly the Se Kα X-ray intensity (counts) to BG (counts) square root ratio. Although the Mo filter did not present a ratio value as good as the Ag filter, it shows a meaningful improvement over other conditions. The Mo filter has the K X-ray absorption edge energy just below the Rh anode K lines, thus absorbing efficiently the anode K lines, and as consequence reducing the sensitivity; conversely, this filter dramatically diminishes the background of the Se Kα line energy region as well, resulting the second better option for Se evaluation. Unfortunately, the thicknesses of these filters embedded in the equipment were not disclosed by the manufacturer.

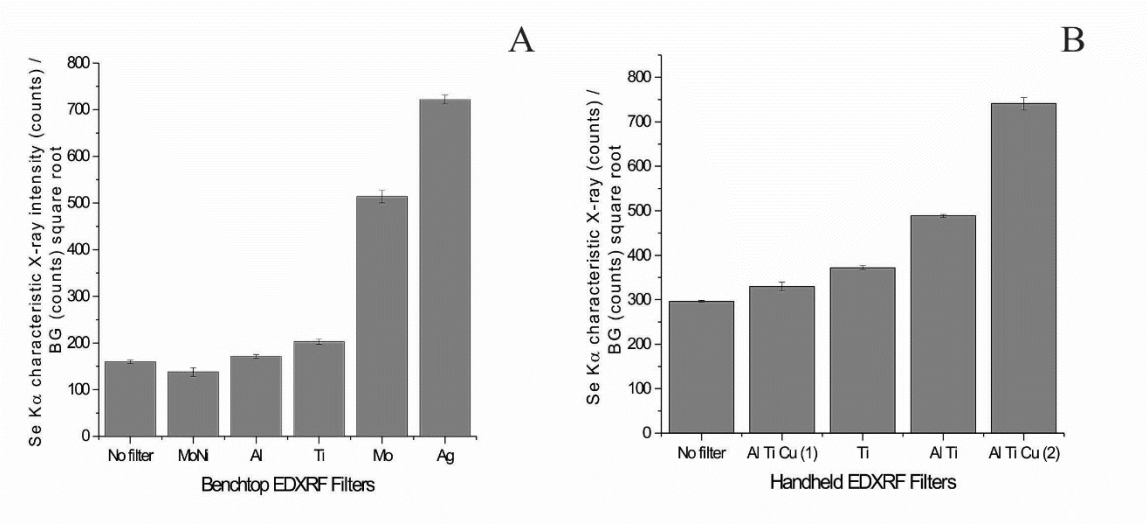

Figure 2. Effect of primary filters on the Se Kα X-ray intensity to background square root ratio for the benchtop (A) and portable (B). Ag primary filter demonstrated better performance in the benchtop facility and Al Ti Cu(2) (green filter) in the handheld device. Results expressed as mean ± standard deviation. Silver (Ag), molybdenum (Mo), molibdenium-nickel (MoNi), aluminum (Al), titanium (Ti) and copper (Cu).

Similarly, for the handheld system (Figure 2B), when using the Al-Ti-Cu(2) (30.58 µm + 2.54 µm + 2.54 µm) filter, the figure of merit gain was approximately twice compared to no filter condition. Although this filter reduces the X-ray excitation intensity, it decreases the background to a larger extent, therefore promoting the best filter option considering all handheld XRF filters available. Figures 2A and 2B highlight the importance of the adequate filter selection for improvement of the EDXRF analysis performance.

There are different strategies for quantification in EDXRF, such as fundamental parameters, external calibration, standard addition, sample dilution and others. In our study, we selected the standard addition approach. For quantitative analysis, the absorption and enhancement matrix effects are corrected since the calibration was performed using the sample itself. Although this procedure may be time consuming and labor intensive to some extent, including the careful pipetting-drying-homogenizing step, once the standards have been prepared they can be used indefinitely because of the non-destructive EDXRF feature. Figures 3A e 3B show the calibration curve for Se Kα characteristic X-ray intensity (counts) versus Se concentration (mg kg^{-1}) for the benchtop and handheld XRF units. The correlation coefficient (R) was higher than 0.999 in both systems, thus being appropriated for Se quantification in broccoli seedling samples. The handheld XRF sensitivity for Se was 22 % higher compared to the benchtop one.

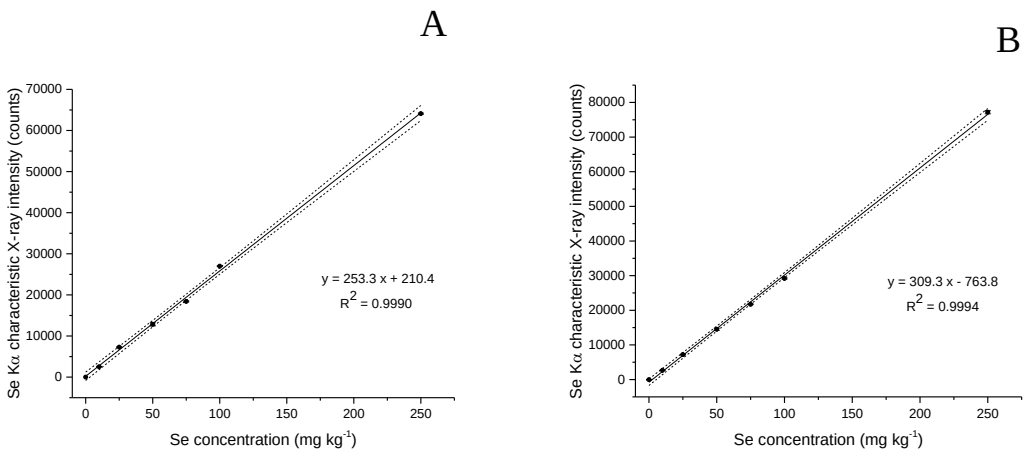

Figure 3. Standard curves for Se Kα intensity (counts) versus concentration (mg kg^{-1}) for the benchtop (A) and handheld XRF (B) units.

Data shown in Figure 4A and 4B demonstrates the broccoli seedling fluorescence X-ray spectra from 10.5 to 13.75 keV for the both X-ray fluorescence units. It can be seen that the Se Kα and Kβ peaks do not have any spectral interference from the broccoli seedling elemental composition. It also shows an impressive Se Kα net intensity and noise ratio for 73 mg kg^{-1} concentration range (tens of mg kg^{-1} level) for both EDXRF systems, in spite of the remarkably handheld XRF low X-ray tube power (4 W).

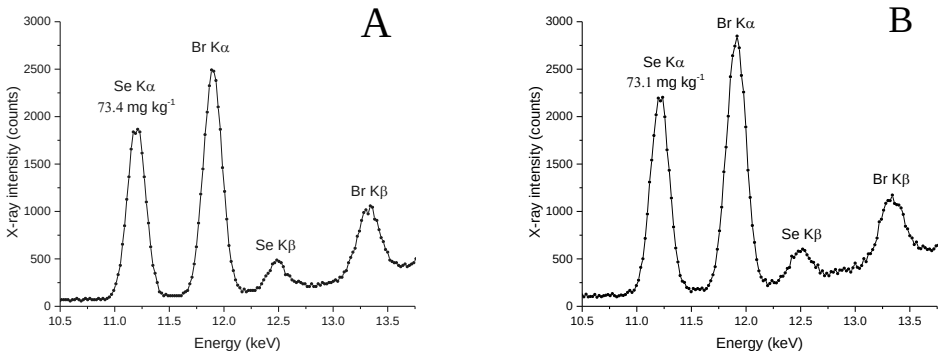

Figure 4. Representative spectra for different elements detected on Se-enriched broccoli seedlings by energy dispersive x-ray fluorescence/ spectrometer from benchtop (A) and handhelXRF (B).

The results of the concentration of Se (mg kg^{-1}) in broccoli by both EDXRF systems and ICP-MS are summarized in Table 2.

Table 2. Concentration of Se (mg kg^{-1}) in selenium-enriched broccoli seedling by benchtop and handheld EDXRF and ICP-MS.

Sample	Benchtop EDXRF	Handheld EDXRF	ICP-MS
MS14	< LOQ	< LOQ	0.60 ± 0.06
MC14	161.4 ± 0.8	163.2 ± 0.6	169.80 ± 20.39
MS16	<LOQ	<LOQ	0.25 ± 0.02
MC16	73.4 ± 1.4	73.1 ± 0.4	61.03 ± 3.15
LOD*	0.9	0.6	0.0007
LOQ*	3.0	2.1	0.0021

(*) for 300 s acquisition time. Results expressed as mean ± standard deviation.

The concentration of Se in the MC and MS samples determined by benchtop and handheld EDXRF units were close to the corresponding values quantified by ICP-MS, taking into account the standard deviation (SD) of measurements. It must be considered that EDXRF SD is due to only instrumental error, then presenting a low SD value, whereas ICP-MS SD encompasses the complete analytical process (including digestion and instrumental reading replicates for each sample). Considering the limits of detection and quantification, it was not possible to determine Se concentration in the samples MS14 and MS16 by EDXRF systems (Table 2). These results clearly pointed to the analytical superiority of the ICP-MS technique compared to both EDXRF systems in terms of LOD, reaching sub-µg kg^{-1} levels.

The benchtop and handheld XRF LOD presented closed values, which range is appropriate for Se determination at mg kg^{-1}. One serious EDXRF limitation for Se quantification in biological sample is its mg kg^{-1} LOD, taking account that Se is seemingly found in biological samples in concentrations < 1 mg kg^{-1} (PALTRIDGE et al., 2012). Nevertheless, both EDXRF instruments were appropriate for Se high content biological samples. Thus, they can be employed in the analysis of Se rich samples such as Brazil nuts (*Bertholletia excels)*, Se fortified food as Se-enriched broccoli seedling.

As a comparison, Li and Yu (2016) used a high energy polarized beam EDXRF unit for Se determination in biological sample, obtaining a Se LOD of 0.1 mg g^{-1} for 1,000 s of acquisition time. Estimating the LOD of our benchtop and handheld approaches for this acquisition time, in which the LOD is inversely proportional to the time square root, the LOD values were of the same order of magnitude compared to the high energy polarized EDXRF system above mentioned. Additionally, Paltridge et al. (2012) reported the Se limit of quantification (LOQ) of 2 mg kg^{-1} for whole grain wheat using a benchtop EDXRF for acquisition time of 60 s. Hanley et al. (2017) found the Se LOD of 4 mg kg^{-1} for Se-enriched dietary supplement for a benchtop one using a acquisition time of 100 s.

Concerning the overall cost of analysis, either benchtop or handheld EDXRF equipament are cheaper than the ICP-MS equipment, demanding less trained operator. Moreover, the running cost of EDXRF is much smaller than ICP-MS due to purge and carrier purified gas required by the last one. According to Paltridge et al. (2012), even in comparison to ICP-OES analysis, that is expected to be lower-cost than ICP-MS, consumables cost per sample was 67 to 100 times lower in EDXRF than in ICP-OES analysis.

Regarding both EDXRF systems, from sample preparation (just weighing) up to running the analysis in triplicate, we can estimate under 40 min as the time analysis per sample. For ICP-MS, including the sample preparation step and the instrumental analysis, we predict a time analysis of 180 min (4.5 times higher). Hanley et al. (2017) evaluate the use of XRF combined with direct analysis in real time high resolution accurate mass-mass spectrometry (DART-HRAM-MS) for Se speciation. The throughput gain using both techniques was of 7 times.

Comparing the benchtop and handheld EDXRF, both systems presented similar analytical performance for Se determination in broccolis seedling (Table 2). Guerra et al. (2014) evaluated P, K, Ca, S, Fe, Mn and Si in pressed pellet sugar cane leaf utilizing identical benchtop and handheld XRF systems. Both systems also showed comparable figures of merit regarding these analytes; additionally, the latter work discussed advantages and drawbacks of each systems. Besides Guerra et al. (2014) viewpoint, we added in our case of study (a) the advantage of handheld Si drift detector which does not require liquid nitrogen for its cooling as the Si(Li) benchtop detector does. Nonetheless, most of benchtop systems are currently equipped with Si drift nowadays. (b) The Shimadzu benchtop allows the use of 1 mm collimator, which ultimately permits a heterogeneous study of the sample. (c) Despites the manual filter change on the handheld unit results in some cautin, it allows making a homemade filter, which permits to select a proper filter composition and thickness dedicated to a particular analyte and matrix. (d) Albeit the quantitative XRF direct analysis without any sample preparation is not an easy work, the handheld one is wide open opportunities for in situ analysis, which may be even coupled to global positioning system technology (WEINDORF et al., 2012), affording real-time results, which includes Se-enriched plants evaluation.

Although ICP-MS showed some disadvantage in terms of cost and analysis time for Se-enriched broccolis compared to both EDXRF systems, the former is a reference technique for Se evaluation in foods in general (OHKI et al., 2016). As mentioned before, normal levels

of Se in food falls in μg L^{-1} or even lower concentration ranges, and only a highly sensitivity technique as ICP-MS are able to cope with.

2.4. Conclusions

The EDXRF systems (benchtop and handheld) presented similar performance compared to the ICP-MS for high Se concentration samples. The choice of the proper filter is an important step in the Se determination by EDXRF. The benchtop and handheld units presented similar Se LOD at 0.6 – 0.9 mg kg^{-1} range for this matrix with no spectral interference for Se evaluation in broccoli matrix. Although EDXRF approaches are not able to determine Se in samples at either μg kg^{-1} or sub μg kg^{-1}, both EDXRF systems offered a fast, accurate and lowcost alternative for Se determination in Se-enriched broccoli seedlings and it can be extended to other foods with Se at mg kg^{-1} range.

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3. CAN BIOFORTIFICATION WITH SELENIUM INCREASE HEALTH-PROMOTING COMPOUNDS IN BROCCOLI (*Brassica oleracea* L. VAR. *ITÁLICA*)?

Abstract

Biofortification of broccoli with Se may alter the profile and amount of bioactive compounds. In this study, the influence of Se treatment (50 µM sodium selenate) on the health-promoting compounds of broccoli was explored. Broccoli seedlings content of 22.43 ± 2.69 µg Se g^{-1} fresh weight (FW). The treatment significantly increased the antioxidant activity, as well as ascorbic acid (12%), total phenolics (26%) and flavonoids (19%), and chlorophyll (12%) contents. Biofortification with Se increased the content of sulforaphane, main bioactive compound of broccoli, by approximately 12%. Furthermore, there was a significant increase in caffeic, *p*-coumaric and *trans*-ferulic acids, and the latter showed moderate activity in the *on-line* HPLC-ABTS assay, contributing 9.71% to the total antioxidant activity. However, biofortification did not influence carotenoids or chlorophyll b. Biofortification of broccoli with Se is a viable alternative to increase the bioactive compounds in this vegetable.

Keywords: Antioxidant; Brassicas; Bioactive compounds; Functional foods; Sodium selenate; Sulforaphane

3.1. Introduction

The perception of human nutrition has changed in recent decades. Awareness that eating not only provides basic nutritional needs but also assists in reducing the risk of chronic diseases has been reviewed (DE VITA et al., 2017). Nowadays, the nutritional recommendations highlight the importance of fruits and vegetables consumption. This fact is related to the presence of bioactive compounds in these foods (SEPTEMBRE-MALATERRE; REMIZE; POUCHERET, 2017).

The Brassicaceae family, to which belongs broccoli (*Brassica oleracea* L. var. *itálica*), is an excellent source of bioactive compounds, especially glucosinolates. Broccoli is appreciated not only for its flavor but also for some of its health-promoting effects, such as antioxidant, antibacterial and especially anticancer properties (ARES et al., 2015; LI et al., 2018). Another element implicated with broccoli anticancer benefits is selenium (Se). This micronutrient increases the expression of selenoproteins, such as glutathione peroxidase, iodothyronine deiodinases, selenophosphate synthetase 2, thioredoxin reductases and

selenoprotein P (CARDOSO et al., 2017; KUMAR; PRIYADARSINI, 2014; SWART et al., 2017).

The major sources of Se are cereals, nuts, meats and seafood. However, their mineral content varies according to the plant species, the stage of development and the biogeochemical factors that influence the selenium availability in the soil (BOLDRIN et al., 2016), affecting the adequate intake of this micronutrient. Therefore, the biofortification technique is appropriate to improve both the Se and the other bioactive compounds contents, generating super plant foods (CHOMCHAN; SIRIPONGVUTIKORN; PUTTARAK, 2017). Among the families studied in Se-biofortification, *Brassica* spp can be highlighted and broccoli is a primary accumulator of this micronutrient (ÁVILA et al., 2013), giving it a great potential as a central part of a Se-enriched diet. However, Se accumulation occurs in the same way as sulphur (S). Thus, the assimilation of Se can affect the metabolic pathways of S and nitrogen, thereby causing changes in the profile and content of bioactive compounds (MALAGOLI et al., 2015). Previous reports have evaluated changes in the glucosinolate profile and/or selenocompounds in mature broccoli plants and sprouts biofortified with Se (ÁVILA et al., 2013; TIAN et al., 2016).

In the current study, the effects of the sodium selenate treatment in broccoli seedlings were evaluated by quantification of different bioactive compounds (Vitamin C, chlorophyll, phenolic compounds, flavonoids, carotenoids and sulforaphane) besides the antioxidant analysis. In support of our aim, we demonstrated that the Se enrcihement which provided a positive modulation in the synthesis of bioactive compounds, increasing by about 12-30% the content of phenolics and total flavonoids, chlorophyll, vitamin C and sulforaphane.

3.2. Materials and methods

3.2.1. Plant material

Avenger broccoli seeds were supplied by Sakata Seed Sudamerica. The broccoli seedlings were produced in black polyethylene trays with 200 cells filled with commercial substrate (coconut fibre, Amafibra) and the experiment was conducted in a completely randomized design. The plants remained for 30 days in an arched agricultural greenhouse. Fifteen days after germination, the seedlings were treated with a single dose of distilled water (2 ml/seedling, control group) or sodium selenite (Na2SeO4, Sigma Aldrich) aqueous solution (50 µM, 2 ml/seedling, Se-enriched group) (BACHIEGA et al., 2016). Fifteen days after

treatment, the aerial part was collected, properly cleaned, lyophilized and stored at -20 °C until use. Samples of broccoli seelings were cultivated in August-Octorber 2014.

3.2.2. Quantification of Se

The total Se contents were determined using an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) quadrupole (model ELAN® DRC-e, PerkinElmer). Freeze-dried samples of 100 mg were decomposed in a microwave oven (DGT 100 Plus), using a mixture of 4 mL HNO3 with 200 µL H_2O_2 for 7 min at 400 W. The conditions were as follows: 1200 W ICP RF power, 15 L min^{-1} plasma gas flow rate, 1.1 L min^{-1} auxiliary gas flow rate, 0.75 L min^{-1} nebulizer flow, 7.0 V lens voltage, 1000 V pulse state voltage, ^{78}Se isotope monitored, and 0.5-25 µg L^{-1} concentration range of the analytical curve (MANEETONG et al., 2013). The results were expressed as µg Se g^{-1} fresh weight (FW).

3.2.3. Sulforaphane content

After heating (45 ± 2 °C, 2.5 h) in a water-bath, aliquots of each broccoli seedlings (1.0 g) in ultrapure water (4 mL, pH 6) were mixed with dichloromethane (20 mL per sample), vortexed for 1 min and stored at room temperature for 1 h (CAMPAS-BAYPOLI et al., 2010). After that, the incubation mixture was filtered through filter paper with anhydrous sodium sulphate. The extract was evaporated to dryness under vacuum at 45 ºC, and the residue was redissolved in acetonitrile (2 mL). Aliquots (20 µL) of each extract were injected into an HPLC system, Shimadzu 20A, equipped with a pump (LC-20 AT), autosampler (SIL-20AHT) and a UV-vis detector (SPD-20A). The analysis was carried out isocratically at a flow rate of 0.6 mL min^{-1}, employing acetonitrile/ultrapure water (30:70, v/v) as the mobile phase. The Waters Spherisorb® column (ODS-C18, 250 mm x 4,6 mm i.d., 5 µm particle size) was thermostated at 36 °C (CAMPAS-BAYPOLI et al., 2010). For quantification, the DL-sulforaphane (>90%, Sigma Aldrich) calibration curve was constructed (2.5 to 80 µg mL^{-1}). The measurement was carried out at 254 nm, and the total time between injections was 20 min., with the results expressed in µg sulforaphane 100 g^{-1} FW. The validation parameters for identification/quantification of sulforaphane were LOD 0.006 µg and LOQ 0.018 µg.

3.2.4. Preparation of extracts

The plant material (1 g) was mixed with 10 mL of 50/50 hydroalcoholic solution, and the extraction was carried out under stirring in a water bath for 30 minutes at 40 °C. After this period, the extracts were centrifuged at 1956.2 *g*, filtered, and rota-evaporated at 40 ºC (Fisatom, Model 801) and stored at -20 °C in amber bottles for light-protection until the analysis.

3.2.5. Total phenolic content

The total phenolic content was estimated using the Folin–Ciocalteu assay (SINGLETON; ORTHOFER; LAMUELA, 1999). Briefly, each extract aliquot (0.5 ml) was mixed with Folin-Ciocalteu reagent (diluted 1:10 in distilled water, 2.5 mL) and sodium carbonate solution (4%, 2 mL). The mixture was vortexed and allowed to stand at room temperature (25 ± 2 ºC) for 60 min. All samples were analysed in a spectrophotometer at 740 nm using a blank group containg just the reagents. Using a standard calibration curve (10-100 µg mL^{-1}) of gallic acid, the total phenolic content was expressed as equivalents of mg gallic acid g^{-1} FW.

3.2.6. Total flavonoid content

The total flavonoid content was determined by a colorimetric method (XU; CHANG, 2007). Briefly, each extract aliquot (0.25 ml) was mixed with distilled water (1.5 mL) and $NaNO_2$ solution (5%, 75 µL) followed after 6 min by the addition of $AlCl_3 \cdot 6H_2O$ solution (10%, 150 µL). After 5 min, NaOH solution (of a 1 M, 0.5 mL) was added in each sample reaction. The absorbance was measured immediately at 510 nm using a spectrophotometer. Using a standard calibration curve (0.02 to 0.10 mg mL^{-1}) of catechin, the flavonoid total contents were expressed as equivalents of mg catechin g^{-1} FW.

3.2.7. LC-ESI-MS/MS analysis of phenolic and flavonoid compounds

The extracts were filtered through a 0.20 µm regenerated cellulose membrane and analysed with Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric

(LC-ESI-MS/MS) using an Agilent (Wilmington, USA) Chromatograph 1200, equipped with a binary pump and auto sampler G1367C.

The chromatographic separations of each sample (10 µL) were carried out using a Waters Spherisorb® column (ODS-C18, 250 mm x 4.6 mm i.d., 5 µm particle size) kept at 30 °C. The mobile phases included ultrapure water/formic acid (99.75/0.25, v/v, phase A) and acetonitrile/ultrapure water/formic acid (80/19.75/0.25, v/v, phase B). The optimized gradient (B in A) started with 10% B, reaching 20% B at 10 min, 30% B at 20 min, 50% B at 30 min and 10% B at 38 min with a constant flow (1 mL min^{-1}).

Using the Quadruple Triple 6430 mass spectrometer as detector, the ESI parameters in the negative ionization mode were stablished as gas flow 12 L min^{-1}, nebulizer pressure 50 psi, gas temperature 350 °C and capillary voltage of 3500 V. Nitrogen at 99.99% was used as a nebulizer and at 99.9999% as a collision gas. For each sample, the optimum conditions of Multiple Reaction Mode (MRM) were determined in the direct infusion mode. Triplicate injections were made for each standard solution and extract, and the software Agilent Mass Hunter was used for data acquisition.

The analytes presents in broccoli extracts were identified by comparing the retention time and m/z values obtained through MS and MS2 with those registered for the standards tested under the same conditions. The identified phenolic compounds and flavonoids were quantified (µg 100 g^{-1} FW) using their peak areas and the corresponding standard calibration curve (0.1 to 2 µg mL^{-1}) obtained in the MRM mode obtained. For all standards, the limits of detection (LOD) and quantification (LOQ) were determined.

For all standards, the curves were performed in the 0.1 to 2 µg mL^{-1} range, and the limits of detection (LOD) and quantification (LOQ) were as follows: Gallic acid monohydrate (>99%, Sigma Aldrich) 0.0340 and 0.1019 µg mL^{-1}; chlorogenic acid (> 95%, Sigma Aldrich) 0.0558 and 0.1675 µg mL^{-1}; caffeic acid (>98%, Sigma Aldrich) 0.0191 and 0.0572 µg mL^{-1}; (-)-epicatechin (>90%, Sigma Aldrich) 0.0197 and 0.0590 µg mL^{-1}; *p*-coumaric acid (>98%, Sigma Aldrich) 0.1220 and 0.3661 µg mL^{-1}; *trans*-ferulic acid (>99%, Sigma Aldrich) 0.0108 and 0.0325 µg mL^{-1}; and rutin hydrate (>94%, Sigma Aldrich) 0.0127 and 0.0381 µg mL^{-1}. The results were expressed in µg 100 g^{-1} FW.

3.2.8. Antioxidant capacity

3.2.8.1. DPPH assay

The radical-scavenging ability of each extract was measured using the stable radical 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) according to the method described by Brand-Williams, Cuvelier and Berset (1995). Briefly, the reaction mixture contained 500 μL of different extracts and 300 μL of DPPH. The absorbance of each reaction mixture was measured at 517 nm using a UV–Vis spectrophotometer (modelo Unico® 2800 UV/VIS – Interprise Brasil) against a blank that did not contain a test sample. Using a calibration curve of Trolox (0 to 200 μM), the results were expressed as μM Trolox g^{-1} FW.

3.2.8.2. ABTS assay

In this assay, the radical-scavenging ability of each extract was measured using the 2,2-azino-bis-3-ethylbenzothiazoline-6 sulfonic acid ($ABTS^{\bullet+}$) radicals prepared in situ by the reaction of ABTS (7 mM) with potassium persulphate (2.45 mM). For this assay, aliquots (20 μL) of each extracts were mixed with of $ABTS^{\bullet+}$ radical solution (2.0 mL). The absorbance of each sample was measured at 734 nm using a UV–Vis spectrophotometer (Unico® 2800 UV/VIS – Interprise Brasil) against the blank, which did not contain a test sample. Using a calibration curve of Trolox (50 to 2000 μM), the results were expressed as μM Trolox g^{-1} FW (RE et al., 1999).

3.2.8.3. FRAP assay

A determination of the total antioxidant activity through the reduction of iron was based on a direct measurement of the ability of the antioxidants (reducing agents) for reducing, under acidic conditions (pH 3.6), the complex Fe^{3+}/tripyridyltriazine (TPTZ) to form Fe^{2+}/tripyridyltriazine. Such complex presents an intense blue colour, with a maximum absorption at 595 nm (OYAIZU, 1999). An aliquot of 90 μL of different extracts was added to 2.7 mL of the FRAP reagent. A calibration curve was built-up from 500 to 2000 mM iron sulphate concentration. The results were expressed in μM ferrous sulphate g^{-1} FW.

3.2.8.4. ORAC assay

This assay was used to monitor the antioxidant action of the samples on the fluorescence decay by peroxyl (ROO·)-induced oxidation of fluorescein. Aliquots (30 μL) of the standard, control, or extracts were transferred to a microplate followed by fluorescein solution (508.25 nM, 60 μL) and AAPH solution (76 mM, 110 μL). All solutions were prepared in potassium phosphate buffer (75 mM, pH 7.4), also used as a blank. The reaction was performed at 37 °C, and the fluorescence was measured every minute over 2 h at 485 (excitation) and 528 (emission) nm using a microplate multireader (Molecular Devices, LLC, Sunnyvale, CA, USA). Using a calibration curve of Trolox (12.5 to 400 μM), the results were expressed as μM Trolox g^{-1} FW (MELO et al., 2015).

3.2.8.5. On-line HPLC-DAD-ABTS radical scavenging activity assay

Using one high performance liquid chromatography (HPLC) coupled with a post-column reaction system, the radical scavenging ability of each sample was analysed by direct detection. Separation of standards (10 μL) and each extract (20 μL) was carried out as described in the section 3.2.6 and for this analysis the chromatographic system was acopled to one diode array detector (DAD, SPD-M10AVp, Shimadzu) and one UV-vis detector (SPD-20AV, Shimadzu). By mixing ABTS solution (7 mM) with potassium persulphate solution (140 mM) at room temperature in the darkness, the $ABTS^{\bullet+}$ stock solution was prepared 16 h before the experiment to stabilize the $ABTS^{\bullet+}$ radicals. The $ABTS^{\bullet+}$ work solution was prepared by diluting the stock solution with pure water to an absorbance of 0.70 ± 0.02 nm. While the sample was separating, the $ABTS^{\bullet+}$ stock solution was delivered via a pulse pump, with a flow rate of 0.8 mL min^{-1}. The mixture was then transported to a DAD detector, and the absorbance was monitored at 734 nm. The presence of the separated molecules was visualized as positive peaks by this detector. Their anti-oxidant capacity was revealed and could be quantified when the corresponding negative peak was detected by the UV-vis detector. The negative chromatograms were aligned with the positive chromatograms, with an offset of – 0.67 min. Using a calibration curve of Trolox (2.34-150 μg mL^{-1}), the results were were expressed in μM Trolox 100^{-1} g FW.

3.2.9. Ascorbic acid content

Aliquots (5 g) of each broccoli seedlings were mixed with oxalic acid solution (1%, w/v, 50 mL), filtered and submitted to titration with 2, 6-diclorobenzenoindofenol (0.02%) for ascorbic acid content determination (BENASSI; ANTUNES, 1988). The results were expressed in mg ascorbic acid 100 g^{-1} FW.

3.2.10. Chlorophyll

The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were determined using a method from Lichtenthaler (1987). Briefly, aliquots (0.5 g) of each broccoli seedlings were homogenized with acetone (80%, v/v, 5 mL) and kept in a dark place until the sample colour changed to white. After centrifugation (15 min), the supernatant absorbance was measured at 645 nm and 663 nm, employing an UV–Vis spectrophotometer (modelo Unico® 2800 UV/VIS – Interprise Brasil). The results were expressed in µg chlorophyll g^{-1} FW.

3.2.11. Carotenoids profile

An analysis of carotenoids was performed according to the procedures described by Dos Reis et al. (2015). The main steps involved the extraction of the pigments with acetone and methanol saponification with 10% KOH overnight at room temperature. The extract was rota-evaporated (Fisatom, Model 801) at < 25 °C and freezer stored (-20 °C) for subsequent quantification. For the HPLC analysis, the extract was diluted with methyl tert-butyl ether (MTBE-JT Baker, CAS. Number 1634-04-4, purity 99.96%), sonicated (Unique, model USC 1400) for 30 s and filtered (Millex LCR 0.45 mm, 13 mm) for injection into an Agilent 1100 Series HPLC (Santa Clara, CA, USA) equipped with a quaternary system and a UV-visible detector. The column was a 250 x 4.6 mm ID, 3 mm, C30 polymeric reverse-phase column (YMC, model CT99SO3-2546WT). The mobile phase gradient (water:methanol:MTBE) (JT Baker, CAS Number 04.04.1634, 99.96% purity) started at 5:90:5, reaching 0:95:5 at 12 min, 0:89:11 at 25 min, 0:75:25 at 40 min, and finally 00:50:50 at 60 min. The flow rate was 1 mL min^{-1} at 33 °C.

Data acquisition and processing were performed using ChemStation® software. The chromatograms were processed at an absorption wavelength of 470 nm, and the compounds

were identified by comparing the sample retention times with the retention times obtained for controls. For quantification, a standard curve was built-up for carotenoids over the following ranges: 1-65 µg mL^{-1} lutein (>95%, Sigma Aldrich); 1-40 µg mL^{-1} zeaxanthin (>95%, Sigma Aldrich); 4-100 µg mL^{-1} cryptoxanthin (>97%, Sigma Aldrich); 2-25 µg mL^{-1} α-carotene (>95%, Sigma Aldrich); and 5-50 µg mL^{-1} β-carotene (>97%, Sigma Aldrich). The results were expressed in µg of carotenoids 100 g^{-1} FW.

The LOD and quantification LOQ were, respectively, as follows: 0.0069 and 0.0115 µg g^{-1} for lutein, 0.0956 and 0.0159 µg g^{-1} for zeaxanthin, 0.0211 and 0.0351 for cryptoxanthin, 0.0197 and 0.0328 µg g^{-1} for α-carotene, and 0.0653 and 0.1089 µg g^{-1} for β-carotene.

3.2.12. Statistical Analysis

All experiments were carried out in triplicate, and the results were expressed as the average ± standard deviation (SD). Statistical analyses were performed using SAS version 9.0 for Windows. After verifying the normality and homogeneity of varience of the data using the Shapiro-Wilk and Box-Cox tests, the difference between the means of the treatments with and without Se was tested using a Student's t-test.

3.3. Results and discussion

3.3.1. Levels of Se in broccoli seedlings

Biofortification with Se was performed using sodium selenate due to its superior efficiency in promoting Se accumulation (ÁVILA et al., 2013; RAMOS et al., 2011a; TIAN et al., 2016). The chosen dose (50 µM) provided a significant absorption of Se affording a Se concentration of 22.43 ± 2.69 µg Se g^{-1} FW after treatment, against 0.10 ± 0.02 µg Se g^{-1} FW in control treatment. These results corroborated our previous evidences (BACHIEGA et al., 2016).

The diet of many populations around the world contains insufficient amounts of Se, and 800 million people are deficient in this micronutrient (MALAGOLI et al., 2015). Considering that the prophylactic dose of Se against cancer is between 50 and 100 µg day^{-1} (SIMONOFF; SIMONOFF, 1991), consumption of only 2 to 5 g of our broccoli seedlings biofortified with Se would be enough to achieve this recommended dose. According to these

results, consumption of Se-enriched broccoli seedlings would contribute to increase human Se status and, furthermore, to prevent certain types of cancer.

3.3.2. Sulforaphane content

The glucosinolates are secondary sulphur-enriched compounds that have different ratings due to the variable chain of their basic chemical structure (CHOMCHAN; SIRIPONGVUTIKORN; PUTTARAK, 2017). Among the sixteen glucosinolates already identified in broccoli, the glucoraphanin represents approximately 81% of the total content. Furthermore, this is the only glucosinolate that, when hydrolysed by the enzyme myrosinase, leads to sulforaphane, a compound with significant anticarcinogenic activity (PÉREZ et al., 2014).

In the present study, the sulforaphane content was higher in Se-biofortified plants (7686.79 ± 305.60 μg 100^{-1} FW) in comparison to the control broccoli seedlings (6890.17 ± 396.72 μg 100^{-1} FW), in agreement with previous studies (ROBBINS et al., 2005; ÁVILA et al., 2013; THIRUVENGADAM; CHUNG, 2015; TIAN et al., 2016).

The increasing in sulforaphane content may be related to, at least, three events described in literature. First, the sodium selenate treatment can increase the myrosinase activity during early days of broccoli growth (TIAN et al., 2016). Second, low levels of selenium can improve the glucosinolates levels by influence on the S uptake and, consequently, enhancing the S-metabolites levels (THIRUVENGADAM; CHUNG, 2015). Finally, Se biofortification can exert an additional effect on the synthesis of amino acids, such as methionine, phenylalanine, tyrosine and tryptophan, which are glucosinolates precursors. Therefore, the variation in the synthesis of these amino acids may influence the production of these compounds (MALAGOLI et al., 2015).

3.3.3. Total phenolic and flavonoid compounds

Phenolic compounds are secondary metabolites of plants produced through the shikimate and phenylpropanoid pathways and their biosynthesis can be induced by biotic and abiotic stresses (GIORGI et al., 2009). For many different species, such as Goji leaves, purple potatoes, wheat and tomato, biofortification with Se resulted in a significant increase in phenolic and flavonoid compounds (CHU; YAO; ZHANG, 2010; DONG et al., 2013; LEI et

al., 2014; SCHIAVON et al., 2013). For broccoli, there are evidences that the total phenolic content will depend on the Se concentration used during the growth. For example, using Majestic broccoli variety, Robbins et al. (2005) observed that Se concentration upto 100 μg per g of broccoli promoted a reduction in phenolic compounds production while lower Se concentration (5 μg per g of broccoli) increased the total phenolic content. More, when Fenglei 60 and Shenglv 120 broccoli cultivars were submitted to the sodium selenate treatment (100 μmol L^{-1}), the total phenolic content reduced by 22.8% and 21.1%, respectively, compared to the control, while no significant variation on the flavonoid total contents was detected (TIAN et al, 2016).

In our experiment, using Avenger broccoli cultivar, we found higher total phenolic compounds concentrations ($p < 0.05$) when broccoli plants were treated with sodium selenate (50 μM) compared to the control (2.98 ± 0.01 e 2.36 ± 0.01 mg gallic acid g^{-1} FW, respectively). Moreover, the same behaviour was noted for total flavonoid contents, been the Se-enriched seedlings (1.92 ± 0.02 mg catechin g^{-1} FW) significantly higher than the control ones (1.61 ± 0.01 mg catechin g^{-1} FW). These results are in agreement with our previous study (BACHIEGA et al., 2016).

Biofortification can promote abiotic stress in broccoli by promoting changes in the metabolic pathways of sulfur and nitrogen. Thus, a possible explanation for phenolic and flavonoid incresead is that biofortification can improve the accumulation of amino acids, such as phenylalanine, the major substrate for phenolic biosynthesis (MALAGOLI et al., 2015; MIMMO et al., 2017).

3.3.4. Phenolic profile by LC-ESI-MS/MS

Using the parameters described in Table 3, we searched for seven phenolic standards (gallic acid monohydrate, chlorogenic acid, caffeic acid, (-)-epicatechin, *p*-coumaric acid, *trans*-ferulic acid and rutin hydrate) in the broccoli seedling extracts using the LC-ESI-MS/MS method.

Table 3. Qualitative (tr, pi, prodi, Frag and CE) otained parameters in LC-ESI-MS/MS analysis of phenolic and flavonoid compounds.

Compound	t_r (min)	pi (m/z)	prodi (m/z)	Frag (V)	CE (V)
Gallic acid monohydrate	4.69	169.01	125	90	13
Chlorogenic acid	12.86	354.10	191.1	45	12
Caffeic acid	14.04	170.03	135.1	45	12
(-)-Epicatechin	15.03	290.08	109.1	45	16
p-Coumaric acid	18.78	164.05	119.1	45	12
trans-Ferulic acid	20.92	193.05	178	87	8
Rutin hydrate	22.69	609.14	301.1	45	36

tr = retention time; pi = precursor ion; prodi = product ion; Frag = fragmentation energy; CE = collision energy.

Among these standards, just *trans*-ferulic, *p*-coumaric and caffeic acids were qualitatively and quantitatively identified in both seedling treatments (Table 4).

Table 4. Phenolic compounds in broccoli seedlings.

	Broccoli seedling* (μg 100^{-1} g FW)		
	Control	Sodium selenate	t-Student test
trans-Ferulic acid	1345.75±13.83	2276.49±13.77	$p < 0.05$
p-Coumaric acid	102.90±5.66	161.88±0.34	$p < 0.05$
Cafeic acid	26.89±2.20	44.20±1.80	$p < 0.05$

Each value is expressed as the mean (triplicate) ± standard deviation (SD).
*Broccoli seedlings: control treatment (distilled water) and sodium selenate (50 μM).
FW: fresh weight

The comparison of individual phenolic compounds demonstrated a positive effect of the Se application on individual phenolic compounds in broccoli seedlings, with a significant increase in the phenolic acids identified ($p < 0.05$). Our results are in aggrement with those described by Oniszczuk and Olech (2016) that resported *p*-coumaric, *trans*-ferulic, *cis*-ferulic, *trans*-sinapic and *cis*-synapic acids as the main phenolic compounds in broccoli seedlings.

3.3.5. Antioxidant evaluation

Research into the antioxidant potential using different analytical methods has grown significantly due to the importance of antioxidant compounds in inhibiting oxidation processes in foods and especially in humans (CÖMERT; GÖKMEN, 2018). Although presenting some limitations and particular characteristics, methods based on synthetic free radicals, such as DPPH and ABTS have been extensively used to determine the antioxidant

activity of different matrices, including food (KARAÇELIK et al., 2015). Moreover, food has complex matrices presenting functional groups with different polarities and chemical behaviours; therefore, no single antioxidant test can determine the real antioxidant power of a sample or determine the active components of plants and/or food products.

In this context, our study selected two experiments based on capture of free radicals (DPPH and ABTS), one based in metal (iron) reduction power (FRAP) and one based on the reactive oxygen species generated in situ (ORAC assay) besides one on-line HPLC-DAD-ABTS experiment to evaluate the influence of Se biofortification on antioxidant activity.

Therefore, the broccoli seedlings biofortified with Se resulted in a better antioxidant performance in DPPH, ABTS, FRAP and ORAC assays (Table 5).

Table 5. Results of antioxidant activity in vitro (DPPH, ABTS, FRAP and ORAC) in broccoli seedlings.

	Treatments*		
Assays	**Control**	**Sodium selenate**	**Student t-test**
DPPH (μM Trolox g^{-1} FW)	5.66 ± 0.02	6.64 ± 0.03	$p < 0.05$
ABTS (μM Trolox g^{-1} FW)	9.42 ± 0.27	13.23 ± 0.24	$p < 0.05$
FRAP (μM ferrous sulphate g^{-1} FW)	118.20 ± 2.43	170.03 ± 1.79	$p < 0.05$
ORAC (μM Trolox g^{-1} FW)	117.63 ± 9.39	387.22 ± 8.27	$p < 0.05$

Each value is expressed as the mean (triplicate) ± standard deviation (SD).
*Broccoli seedlings: control treatment (distilled water) and sodium selenate (50 μM).
FW: fresh weight

Our antioxidant results for Se-enriched broccoli seedlings (Table 5) can be explained by the increase in the total phenolic and flavonoid contents also observed after broccoli biofortification, since these compounds are mainly responsible for the antioxidant activity in this plant. More, for many species such as broccoli (RAMOS et al., 2011b), mustard sprouts (PIEKARSKA et al., 2014), garden cress (FRIAS et al., 2010) and radish (HANLON; BARNES, 2011) the Se biofortification promoted an increased total antioxidant activity.

Bearing in mind the phenolic profile identified in Se-enriched broccoli seedlings (Table 4), we evaluated these samples using the on-line HPLC–DAD–ABTS method. This method has shown higher sensitivity, selectivity, and relative simplicity for antioxidant capacity analysis in various plant extracts (HE et al., 2010; NIEDERLÄNDER et al., 2008). Furthermore, this method allows the demonstration of the individual contribution of each phenolic compound found in the studied matrix into the radical-scavenging ability of each

extract. It is worth mentioning that this work is the first to demonstrate the individual antioxidant contribution of phenolic compounds in Se-biofortification broccoli using the on-line HPLC-DAD-ABTS method.

The chromatograms obtained in our study are shown in Figure 5. The chromatograms (I) and (III), obtained with a C-18 column, correspond to the phenolic compounds, and the chromatograms (II) and (IV), presenting negative peaks, refer to the antioxidant activity belonging to each chemical compound in the sample.

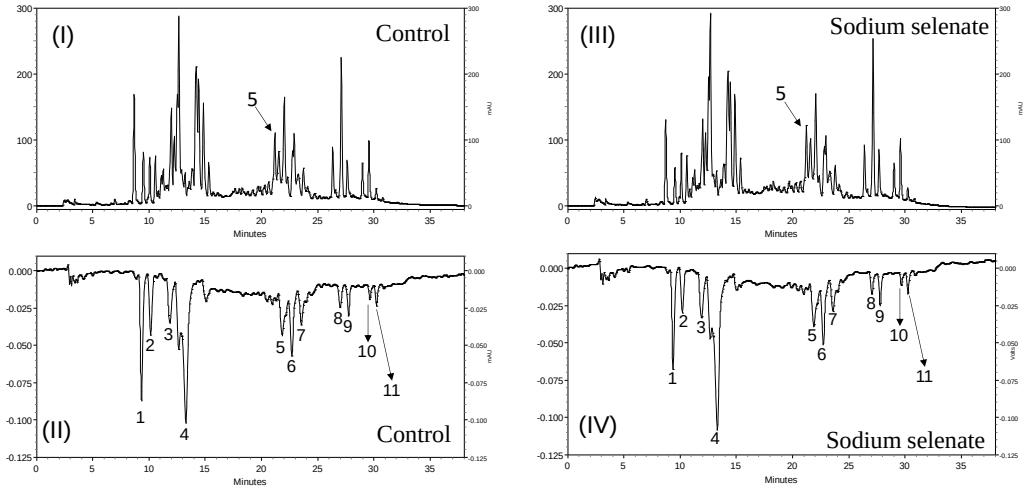

Figure 5. Chromatograms of the phenolic compounds of broccoli seedlings (I; III) and their respective antioxidant activity in an on-line HPLC-ABTS assay at 754 nm (II; IV). Numbered peaks refer to major peaks and/or antioxidant activity.

Eleven compounds had ABTS•+ scavenging capacity and, regardless of the treatment, the samples exhibited similar chromatographic profiles. For both samples, compounds 1 and 4 (see Fig. 5) exhibited relatively high radical scavenging capacity.

From the three identified phenolic acids (*p*-coumaric, *trans*-ferulic and caffeic phenolic) in the sample of broccoli seedlings, only trans-ferulic acid (compound 5) presented antioxidant activity in the on-line HPLC–DAD–ABTS method (Figure 5). In the control treatment, this compound presented 6.96 ± 0.35 µmol Trolox 100 g^{-1} FW representing 9.45% of the total antioxidant activity of this extract. Besides, in the sodium selenate treatment (7.72 ± 0.29 µmol Trolox 100 g^{-1} FW), the contribution was 9.71%. In the experimental conditions employed, the p-coumaric and caffeic acids did not exhibit ABTS•+ scavenging capacities.

3.3.6. Ascorbic acid content

Considering the ascorbic acid content, the Se biofortification of broccoli seedlings promoted a significantly higher amount of ascorbic acid (42.96 ± 1.48 mg ascorbic acid 100 g^{-1} FW, $p < 0.05$) in comparison to broccoli seedling control (32.60 ± 1.71 mg ascorbic acid 100 g^{-1} FW). This increase may be related to the Se role in protecting the plants against various types of biotic and abiotic stresses, acting on the antioxidative defence system and thereby increasing the potential of the plants to combat stressful conditions (HANDA et al., 2016).

3.3.7. Carotenoids and chlorophyll profile

According to Kaulmann et al. (2014), among the plants belonging to the Brassicaceae family, broccoli presents a higher diversity of carotenoids, including β-carotene and lutein. In our study, we evaluated the carotenoid prolife for both broccoli extracts (Table 6).

Table 6. Results of carotenoids and chlorophyll content in broccoli seedlings.

	Treatments*		
Analyte [a]	**Control**	**Sodium selenate**	**Student t-test**
Lutein	5.26 ± 1.05	4.52 ± 0.12	$p = 0.3438^{ns}$
β-carotene	20.78 ± 1.06	22.13 ± 1.30	$p = 0{,}2358^{ns}$
Chloropyll a	189.12 ± 2.16	213.06 ± 3.45	$p < 0.05$
Chloropyll b	111.90 ± 2.31	111.97 ± 1.54	$p = 0{,}9820^{ns}$
Total chloropyll	301.03 ± 4.33	325.03 ± 4.84	$p < 0.05$

a) Each analyte is expressed as the mean (triplicate) ± standard deviation (SD) in μg g^{-1} FW.
*Broccoli seedlings: control treatment (distilled water) and sodium selenate (50 μM).
FW: fresh weight
ns: not significant

Our results suggested that carotenoid content was not influenced by Se biofortification in our experimental conditions (Table 6). Despites the literature report of α-carotene, zeaxanthin and cryptoxanthin presence in organic broccoli (DOS REIS et al., 2015), in our study these carotenoids were in concentrations lower than the experimental LOD.

Moreover, seedling Se-biofortification significantly increased both the total and chlorophyll a amounts whit none effect on chlorophyll b content (Table 6). Similar results

have also been reported in lettuce (HAWRYLAK-NOWAK, 2013) and goji berry leaves (DONG et al., 2013). Some previous studies pointed that Se-biofortification could stimulate the respiration rates and the electrons flow in the respiratory chain, which further accelerates chlorophyll biosynthesis (DONG et al., 2013; GERM; KREFT; OSVALD, 2005).

3.4. Conclusion

Our hypothesis was successfully proven that the sodium selenate as inorganic Se was efficient in producing broccoli seedlings Se-biofortified. While the treatment significantly increased the content of health-promoting compounds, such as sulforaphane, chlorophyll (a and total), phenolic compounds, flavonoids and vitamin C, there was no influence on the carotenoid or chlorophyll b contents. *Trans*-ferulic acid was the only one to present antioxidant activity in the on-line HPLC–DAD–ABTS method. Such results highlight the importance of carrying out studies focusing on biofortification and may help in the investigations of the influence of biofortification with Se in bioactive compounds of broccoli. Se-enriched plants may be an alternative to increase Se levels and to provide significant levels of compounds with health benefits to the human population.

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4. TOXICITY AND MUTAGENICITY OF BROCCOLI SEEDLINGS BIOFORTIFIED WITH SELENIUM

Abstract

Selenium is a contradictory micronutrient. In low doses, this micronutrient presents benefits to human health; however, in larger doses it can be potentially toxic. The broccoli seedlings (Se-biofortified and control) were evaluated for their toxicity and mutagenicity in male and female mice. Animals were orally treated administered by gavage and the doses of broccoli seedlings with selenium were 15, 45 and 70 µg Se/kg-BW. After 40 days of treatment body weight, viscera index, blood biochemical and hematological parameters and histopathological examination were conducted. The mutagenic potential was analyzed by mouse bone marrow micronucleus assay. The results showed that selenium broccoli seedlings at different doses did not cause significant toxicological alterations in body weight gain and hematological parameters of male and female mice. Biochemical parameters indicate significant changes in males in the UR and AST values in the groups treated with 45 µg Se/kg-BW. Significant alterations were observed in the relative weight of the liver, spleen, kidneys and ovary/testis, and histological changes in the kidney and spleen of male and female mice treated with the highest dose of selenium. After micronucleus counting, a significant increase in micronucleus frequency was observed in male mice at dose of 70 µg Se/kg-BW. From this study, we can conclude that selenium-biofortified seedlings at a dose of 15 µg Se/kg-BW have a high potential for selenium supplementation and do not present obvious toxic effects and the toxic effects were observed in doses higher than that proposed as cancer chemopreventive in humans. However, further studies should be conducted to evaluate the mechanisms of toxicity and mutagenicity of broccoli seedlings biofortification with selenium.

Keywords: Biofortification; Brassica oleracea; Sodium selenate; Micronucleus; Safety

4.1. Introduction

Selenium is recognized as an essential micronutrient for humans and animals (XIE et al., 2016). Epidemiological studies have shown the relationship between dietary intake and risk of different types of cancer, such as colon, breast, ovary, rectum, prostate and lung (ROMAN, 2016; LETAVAYOVÁ; VLČKOVÁ; BROZMANOVÁ, 2006; DENNERT et al., 2011). This micronutrient enters the food chain through plants, so the amount of selenium in the soil is a decisive factor in determining the concentration of selenium in these foods (NIEDZIELSKI et al., 2016, LAVU et al., 2016). Thus, biofortification of plants, especially broccoli, has been studied as an efficient strategy to suppress the deficiency of this

micronutrient in populations where its consumption is lower than recommended (ÁVILA et al., 2013; HSU et al., 2011; THOMSON, 2004; MALAGOLI et al., 2015). *In vitro* tests demonstrated that the association of bioactive compounds of broccoli with selenium was effective in increasing the expression of antioxidant enzymes, such as thioredoxin reductase 1 and glutathione peroxidase (WANG et al., 2015; BARRERA et al., 2012; LI et al., 2012). This association also had benefits in reducing colon cancer and increasing pro-apoptotic gene expression *in vivo* (ZENG; DAVIS; FINLEY, 2003; FINLEY et al., 2001).

Despite the proven benefits of selenium to health, this micronutrient is controversial and may exert toxic effects. The first evidence of selenosis (poisoning by high selenium intake) was recorded by Marco Polo when observing that some horses had dystrophic hooves and blindness, being associated with the consumption of wheat grown in soils with high selenium (O'TOOL et al., 1996; LEE; JEONG, 2012). In humans, the main symptoms of selenosis involve skin lesions, neurological abnormalities and brittle nails and hair (LENZ; LENS, 2009). The precise mechanism of selenium toxicity is not yet clearly understood, but some studies suggest that it is attributed to its ability to induce free radicals production, leading to DNA damage and thiol group oxidation (LETAVAYOVA et al., 2006; LEE; JEONG, 2012).

However, the toxicity of selenium is not only related to the dose ingested (400 μg), but also to its chemical form. In general, organic selenium compounds have lower toxicity when compared to inorganic compounds (BENKO et al., 2012, NAGY et al., 2015). In view of these facts, although the biofortification of plants with selenium is an efficient strategy to increase the consumption of this micronutrient by the population, caution should be exercised regarding the safety of these foods. Thus, the present study was designed to investigate the effects of chronic consumption of selenium-biofortified broccoli seedlings under toxicological and mutagenic parameters. We observed that only the dose of 70 μg Se/kg-BW resulted in histopathological changes in the spleen and kidney of male and female mice. Only males showed a significant increase in micronucleus frequency. However, these effects were found in higher doses than that proposed as cancer chemopreventive in humans.

4.2. Materials and methods

4.2.1. Plant material

The broccoli seedlings were produced in an arc-type agricultural greenhouse in the company IBS Mudas (Piracicaba, São Paulo, Brazil). Broccoli seeds (cv. Avenger/ Sakata Seed Sudamerica) were sown in black polyethylene trays with 200 cells filled with coconut fibre substrate (Amafibra). Fifteen days after germination, seedlings were randomily treated with distilled water (2 ml, control group) or sodium selenate (50 µM, 2 mL, Sigma Aldrich, Se-enriched broup). The broccoli seedlings remained for another 15 days in the greenhouse (totaling 30 days of cultivation). Then aerial parts were harvested, sanitized with water, freeze-dried (Modelo E-C–Modulyo) and stored at -20 °C until use. The concentrations of Se was determined by ICP-MS (Chapter 2) in broccoli seedlings affording 0.25±0.04 and 61.03±3.15 µg Se/g dry sample for control and Se-enriched groups, respectively.

4.2.2. Animals

The experimental protocol was reviewed and approved by the University's Committee for Ethics in Animal Use (CEUA-UNICAMP, protocol number 4319-1 and 4319-1(A)/2017). Animals' welfare guidelines were in accordance with both the International Guiding Principles for Biomedical Research Involving Animals (CIOMS-ICLAS, December 2012) as well as with the Brazilian Guideline for the Care and Use of Animals for Scientific and Didactic Purposes (CONCEA, 2013).

Male and female *Swiss* mice weighing 18-25g were obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB), State University of Campinas (UNICAMP, São Paulo, Brazil). After the maintenance period (at least 7 days), animals were group housed in polypropylene cages (5 individuals per cage; length 49 cm, width 34 cm, depth 16 cm) with sterile soft wood bedding (*Pinus* sp.). As a form of environmental enrichment, the animals had access to clean polyvinyl chloride (PVC) tubes and 2-3 sheets of white paper.

The animals were maintained under controlled temperature (22 ± 2°C), with a constant 12 h light–dark cycle, with lights off at 18:00 h. The mice had *ad libitum* access to water and to a commercial diet (Biobase, *Biotécnicas Indústria e Comércio Ltda*., Brazil).

4.2.3. Experimental groups

In the repeat dose oral toxicity study and the bone marrow micronucleus test, the male and female mice were randomly divided into six experimental groups. There were one vehicle control group (PBS, pH 7.0; n = 16/sex), one broccoli seedling control group (n = 10/sex) three Se-enriched broccoli group (15, 45 and 70 µg Se/kg-BW, corresponding to 0.25, 0.74, and 1.15 g broccoli seedlings, n = 10/sex/group). For the micronuclei evaluation, half animals (n = 8/sex) in vehicle control group were challenged with cyclophosphamide (Cyp, 50 mg/kg, i.p.; n = 8), 24 h prior the end of the experiment. All the samples (broccoli seedling with/without Se and cyclophosphamide) were diluted in phosphate buffer in saline solution PBS (pH 7.0) and the administrated volume in oral (v.o.) and intraperitoneal (i.p.) routes was 10 mL/Kg. The Se doses were chosen based on Benko et al. (2012) and Nagy et al. (2015).

4.2.4. Repeat dose oral toxicity study and Mammalian Cell Micronucleus Test

The protocols were based on the OECD Guidelines 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents) and 474 (Mammalian Erythrocyte Micronucleus Test) (OECD, 2008; 2016). All animals were treated by gavage daily for 40 consecutive days and 24h before the end of the experiment, half animals in vehicle group were treated with cyclophosmide monohydrate (50 mg/kg bw; i.p.). During all the experiment, the mice were clinically evaluated every day to evidence toxic signs and to annotate death. Body weights of each mice were measured before treatment and every 3 days during treatment. Food and water consumption per group were also annotated every 3 days. At the end of the period, all animals were anesthetized (ketamina and xylazine, 200 and 20 mg/kg, respectively) for blood collection from the retro-orbital plexus and euthanazied by cervical dislocation for necropsy and bone marrow collect.

4.2.4.1. Hematological and biochemical analysis

At least two blood samples were colletecd from each animal. For hematological analysis, the blood was collected into EDTA anticoagulant tubes and analyzed with Sysmex (model poch-100iV®, EUA). Hematological evaluations included white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT),

mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and blood platelet count (PLT).

For biochemical determinations, the blood was collected into tubes, centrifuged and serum samples were analysed in one automatic clinical analyzer (Reflotron®Plus, Roche, Macromed) by reactive tapes (Testes Reflotron®). Bilirubin (BIL) and creatine (CREA) were determined using the same blood from hematological analysis. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and urea (UR) were determined using serum from non-heparinized blood.

4.2.4.2. Necropsy and histology analysis

After the sacrifice, the mice were examined carefully to observe macroscopic abnormalities. The relative weights of some organs (heart, liver, spleen, lung, kidney, brain and thymus) and reproductive organs (testis or ovary) were measured.

For histopathology, samples of liver, spleen, kidney and testis/ovary were fixed in 10% buffered formalin for 24h and then transferred to 70% ethanol to preserve. After dehydration, fragments were embedded in paraffin prior to the 5 μm microtome sections, stained with haematoxylin and eosin (H&E) and were examined microscopically.

4.2.4.3. Bone marrow collect

From both femurs of each animal, bone marrow was collected by washing with fetal bovine serum. After centrifugation (120 *g*, 5 min), the each cell precipitate was ressuspense and smeared onto at least two slides. After fixation and stain (Leishman's stain), normochromatic erythrocyte (NCE) and polychromatic erythrocytes (PCE) were observed under microscopy (100x; Leica, Model SME). The ratios of PCE/NCE were calculated for each animal by the counting of 500 erythrocytes per animal, and the incidence of micronucleated PCE (MNPCE) was recorded by the examination of 4000 PCE per animal.

4.2.5. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SAS version 9.0 for Windows. Using Shapiro-Wilk and Box-

Cox tests, the normality and homogeneity of variance was tested. In case of homogeneous variance, the data was subjected to one-way analysis of variance (ANOVA); otherwise, it was analyzed by the Kruskal–Wallis nonparametric ANOVA. If either of the tests showed significant differences ($p < 0.05$) among the groups, the data was then analyzed by Dunnett's or Dunn's test.

4.3. Results

4.3.1. Repeat dose oral toxicity study

During the 40 days of experiment, male and female mice did not present any clinical signs of toxicity, including hair loss, irritated skin, mucous membranes and eyes, fatigue and soft or mucoid feces, and all animals appeared healthy at the conclusion. No treatment-related mortality were observed. For female mice there were no statistically significant differences in body weights between vehicle control and treatment groups (Figure 6). However, for male mice the 15 µg Se/kg-BW group presented a significant reduction on body weight gain when compared with vehicle control (Figure 6). Moreover, by monitoring water and food consumption throughout the experiment, there was no change in the intake between the vehicle control and the treated groups for the male and female mice (data not shown).

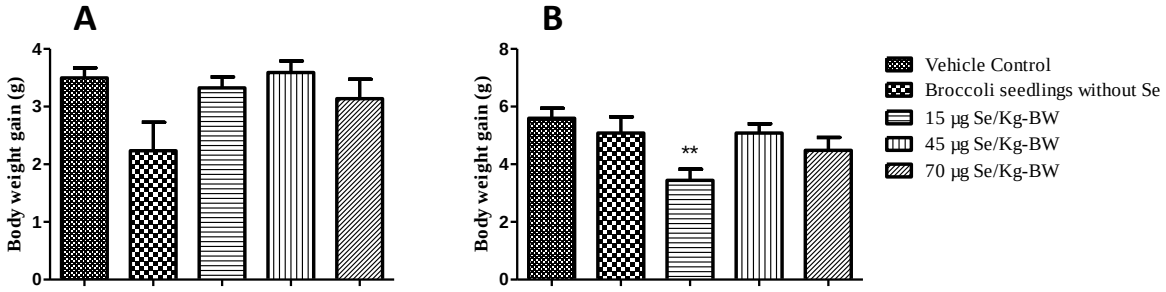

Figure 6. Body weight gain for female (A) and male (B) mice orally administered broccoli seedling without and with Se for 40 days. Values are presented as mean ± SEM; Treatments: PBS (Phosphate buffer in saline solution (NaCl 0.9%), 10 ml/Kg, v.o.); Broccoli seedlings cultivated without Se biofortification (v.o., dose equivalent to 70 µg Se/Kg); Broccoli seedlings cultivated with Se biofortification (0.25, 0.74 and 1.15 g/Kg v.o., corresponding to 15, 45 and 70 µg Se/Kg, respectively).

The hematological profiles of the treated and control groups are presented in Table 7. Feeding of broccoli seedlings control (without Se) and Se-enriched broccoli seedlings, in all three doses, was associated with significant ($p < 0.05$) decreased on MCHC in female groups. According to literature, these varitation althought significant could be considered inside the

normal range (MCHC 25.9 - 35.1 g/dL) for the specie (SUCKOW; DANNEMAN; BRAYTON, 2001). More, we did not find significant changes in hematological profiles in all groups compared with negative control in male mice.

Biochemical evaluation showed that the administration of broccoli seedlings control or Se-enriched did not induce significant effects in parameters indicative of liver (AST, ALT and BIL) or kidney (CREA and UR) toxicity in the female mice (Table 8). However, male mice treated with Se-enriched broccoli seedlings at 45 µg Se/kg-BW showed a 43% increase in UR level ($p<0.05$). Besides, both broccoli seedlings control and Se-enriched broccoli seedlings at 45 µg Se/kg-BW showed a reduction 45 and 46%, respectively, in AST. No differences were found for the other parameters between treated groups and vehicle control group (Table 8).

Table 7. Hematological data for female and male mice orally administered broccoli seedling without and with Se for 40 days[a].

Hematological parameters	Vehicle Control[b]	Broccoli seedlings Control[c]	Se-enriched Broccoli seedlings[d]		
			15 µg Se/kg-BW	45 µg Se/kg-BW	70 µg Se/kg-BW
No. female	8	10	10	10	10
WBC ($x10^3$/µL)	8.51 ± 0.56	7.58 ± 0.94	6.92 ± 0.99	7.73 ± 0.92	7.44 ± 1.0
RBC ($x10^6$/µL)	10.84 ± 0.17	10.73 ± 0.21	10.48 ± 0.20	10.46 ± 0.13	10.71 ± 0.18
HGB (g/dL)	14.98 ± 0.24	14.31 ± 0.23	14.34 ± 0.26	14.17 ± 0.21	14.37 ± 0.25
HCT (%)	53.74 ± 0.59	53.89 ± 0.94	53.26 ± 0.98	52.53 ± 0.66	53.91 ± 0.97
MCV (fL)	49.61 ± 0.50	50.27 ± 0.29	50.86 ± 0.31	50.23 ± 0.27	50.36 ± 0.30
MCH (pg)	13.80 ± 0.16	13.42 ± 0.13	13.71 ± 0.14	13.54 ± 0.09	13.42 ± 0.10
MCHC (g/dL)	27.80 ± 0.38	26.66 ± 0.14*	26.96 ± 0.18*	26.98 ± 0.14*	26.67 ± 0.13*
PLT ($x10^3$/µL)	1659 ± 159.1	1772 ± 67.94	1702 ± 82.54	1611 ± 44.73	1740 ± 65.03
No. male	8	10	10	10	10
WBC ($x10^3$/µL)	9.24 ± 0.79	7.07 ± 0.55	7.10 ± 0.88	7.06 ± 0.58	6.75 ± 0.36
RBC ($x10^6$/µL)	10.38 ± 0.24	9.58 ± 0.51	10.34 ± 0.26	10.62 ± 0.07	10.79 ± 0.18
HGB (g/dL)	13.65 ± 0.29	13.19 ± 0.50	13.77 ± 0.30	13.77 ± 0.19	14.02 ± 0.12
HCT (%)	51.93 ± 1.13	49.12 ± 2.03	52.42 ± 1.14	53.05 ± 0.38	53.64 ± 0.55
MCV (fL)	49.99 ± 0.29	51.62 ± 0.90	50.74 ± 0.50	49.97 ± 0.22	49.76 ± 0.39
MCH (pg)	13.14 ± 0.06	14.09 ± 0.94	13.36 ± 0.27	12.97 ± 0.15	13.01 ± 0.18
MCHC (g/dL)	26.29 ± 0.15	27.12 ± 1.27	26.28 ± 0.30	25.95 ± 0.24	26.15 ± 0.19
PLT ($x10^3$/µL)	1731 ± 121.2	1807 ± 166.3	1740 ± 98.36	1734 ± 74.90	1770 ± 109.1

Abbreviations: WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, blood platelet count and BW, body weight.

[a]Values are presented as mean ± SEM; Treatments: b) PBS (Phosphate buffer in saline solution (NaCl 0.9%), 10 ml/Kg, v.o.); c) Broccoli seedlings cultivated without Se biofortification (v.o., dose equivalent to 70 µg Se/kg-BW); d) Broccoli seedlings cultivated with Se biofortification (0.25, 0.74 and 1.15 g/kg v.o., corresponding to 15, 45 and 70 µg Se/kg, respectively).

$p<0.05$ (*) compared with vehicle control.

Table 8. Effects of broccoli seedlings without and with Se on biochemistry parameters of female and male mice orally treated for 40 days[a].

Biochemistry parameters	Vehicle control[b]	Broccoli seedlings control[c]	Se-enriched Broccoli seedlings[d]		
			15 µg Se/kg-BW	45 µg Se/kg-BW	70 µg Se/kg-BW
No. female	8	10	10	10	10
CREA (mg/dL)	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0
BIL (mg/dL)	4.59 ± 1.38	2.52 ± 0.94	1.69 ± 0.58	1.73 ± 0.26	2.35 ± 0.46
AST (U/L)	237.3 ± 35.87	228.9 ± 21.82	199.9 ± 26.21	244.1 ± 28.55	159.4 ± 23.41
ALP (U/L)	65.55 ± 6.54	55.54 ± 6.54	55.67 ± 4.62	70.05 ± 5.40	53.87 ± 5.82
UR (mg/dL)	39.56 ± 6.2	36.51 ± 4.72	38.92 ± 3.74	38.56 ± 2.52	34.09 ± 1.35
No. male	8	10	10	10	10
CREA (mg/dL)	0.50 ± 0.0	0.52 ± 0.02	0.51 ± 0.01	0.50 ± 0.0	0.50 ± 0.0
BIL (mg/dL)	1.14 ± 0.24	3.88 ± 1.52	4.19 ± 1.35	1.38 ± 0.32	2.22 ± 0.50
AST (U/L)	239.9 ± 43.11	132.4 ± 29.57*	176.4 ± 24.72	128.4 ± 14.45*	204.1 ± 48.34
ALP (U/L)	78.79 ± 22.85	52.99 ± 9.10	71.84 ± 17.01	50.88 ± 5.51	143.0 ± 48.39
UR (mg/dL)	36.35 ± 1.14	34.55 ± 2.40	46.36 ± 3.59	51.90 ± 2.14*	46.80 ± 3.34

Abbreviations: CREA, creatine; BIL, bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; UR, urea and BW, body weight.

[a]Values are presented as mean ± SEM; Treatments: b) PBS (Phosphate buffer in saline solution (NaCl 0.9%), 10 ml/kg, v.o.); c) Broccoli seedlings cultivated without Se biofortification (v.o., dose equivalent to 70 µg Se/kg-BW); d) Broccoli seedlings cultivated with Se biofortification (0.25, 0.74 and 1.15 g/kg v.o., corresponding to 15, 45 and 70 µg Se/kg, respectively).

$p<0.05$ (*) compared with vehicle control.

The relative organ weights of the mice (female and male) orally treated with broccoli seedlings control and Se-enriched ones are shown in Table 9. In general, for both males and females, the treatments increased the relative weight of some organs, except for the liver. For female mice, Se-enriched broccoli seedling treatment significantly ($p<0.05$) increased the relative weight of kidney (at 15 and 45 µg Se/kg-BW), ovary (all doses), spleen and lung (at 70 µg Se/kg-BW). More, the broccoli seedling control (without Se) promoted a significant increase of lung and ovaries relative weight in female mice (Table 9).

Unlike the females, for male mice (Table 9), the liver relative weight had a significant ($p<0.05$) reduction by broccoli seedlings treatments, without or with Se (at 45 and 70 Se/kg-BW). Moreover, the treatment with Se-enriched broccoli seedlings promoted a significant ($p<0.05$) increase in the relative weight of the kidneys (at 15 µg Se/kg-BW), heart (at 15 µg Se/kg-BW) and testicle (at 70 µg Se/kg-BW). The relative weight of the other organs (spleen, lung, thymus and brain) were not altered by the treatments.

Representative microscopic findings in the liver, kidney, spleen, testis or ovary of female and male mice after oral treatments with control and Se-enriched broccoli seedlings for 40 days are shown in Figure 7. The negative control and broccoli seedlings control groups had typical histological structures for the liver, spleen, kidney, testis and ovary (Figure 7). However, the animals treated with Se-enriched broccoli seedling at 70 µg Se/kg-BW presented morphological alterations in the spleen (white pulp and intense edema, Fig 7F) and in the kidney (intense edema and reduction of the renal tubules, Fig. 7I).

Table 9. Relative organ weight of female and male mice treated orally with broccoli seedling without and with Se for 40 days[a].

Relative weights of organs (%)	Vehicle control[b]	Broccoli seedlings control[c]	Se-enriched Broccoli seedlings[d]		
			15 µg Se/kg-BW	45 µg Se/kg-BW	70 µg Se/kg-BW
No. female	8	10	10	10	10
Liver	6.40 ± 0.21	5.90 ± 0.15	6.05 ± 0.23	5.82 ± 0.12	5.99 ± 0.16
Kidney	1.32 ± 0.05	1.40 ± 0.04	1.48 ± 0.03*	1.47 ± 0.04*	1.46 ± 0.02
Spleen	0.46 ± 0.01	0.52 ± 0.04	0.50 ± 0.02	0.49 ± 0.02	0.60 ± 0.04*
Lung	0.85 ± 0.03	1.02 ± 0.04*	0.94 ± 0.03	0.90 ± 0.05	0.93 ± 0.04
Heart	0.54 ± 0.02	0.68 ± 0.05	0.65 ± 0.04	0.66 ± 0.03	0.66 ± 0.03
Thymus	0.50 ± 0.04	0.47 ± 0.03	0.52 ± 0.05	0.44 ± 0.03	0.43 ± 0.05
Ovary	0.14 ± 0.01	0.31 ± 0.04*	0.30 ± 0.04*	0.31 ± 0.04*	0.29 ± 0.03*
Brain	1.15 ± 0.03	1.24 ± 0.07	1.24 ± 0.05	1.21 ± 0.05	1.24 ±0.06
No. male	8	10	10	10	10
Liver	6.35 ± 0.21	5.81 ± 0.11*	5.96 ± 0.4	5.66 ± 0.14*	5.82 ± 0.10*
Kidney	1.49 ± 0.07	1.53 ± 0.04	1.70 ± 0.04*	1.61 ± 0.06	1.65 ± 0.03
Spleen	0.29 ± 0.02	0.35 ± 0.03	0.29 ± 0.02	0.29 ± 0.01	0.33 ± 0.01
Lung	0.72 ± 0.02	0.71 ± 0.03	0.80 ± 0.03	0.75 ± 0.03	0.82 ± 0.03
Heart	0.50 ± 0.01	0.56 ± 0.02	0.68 ± 0.04*	0.61 ± 0.05	0.62 ± 0.03
Thymus	0.29 ± 0.03	0.27 ± 0.03	0.33 ± 0.05	0.37 ± 0.04	0.34 ± 0.05
Testicle	0.62 ± 0.02	0.68 ± 0.02	0.67 ± 0.04	0.70 ± 0.04	0.76 ± 0.04*
Brain	0.94 ± 0.02	0.93 ± 0.03	1.01 ± 0.03	0.94 ± 0.04	0.99 ± 0.04

Abbreviation: BW, body weight.

[a]Values are presented as mean ± SEM; Treatments: b) PBS (Phosphate buffer in saline solution (NaCl 0.9%), 10 ml/kg, v.o.); c) Broccoli seedlings cultivated without Se biofortification (v.o., dose equivalent to 70 µg Se/kg-BW); d) Broccoli seedlings cultivated with Se biofortification (0.25, 0.74 and 1.15 g/kg v.o., corresponding to 15, 45 and 70 µg Se/kg, respectively).

$p<0.05$ (*) compared with vehicle control.

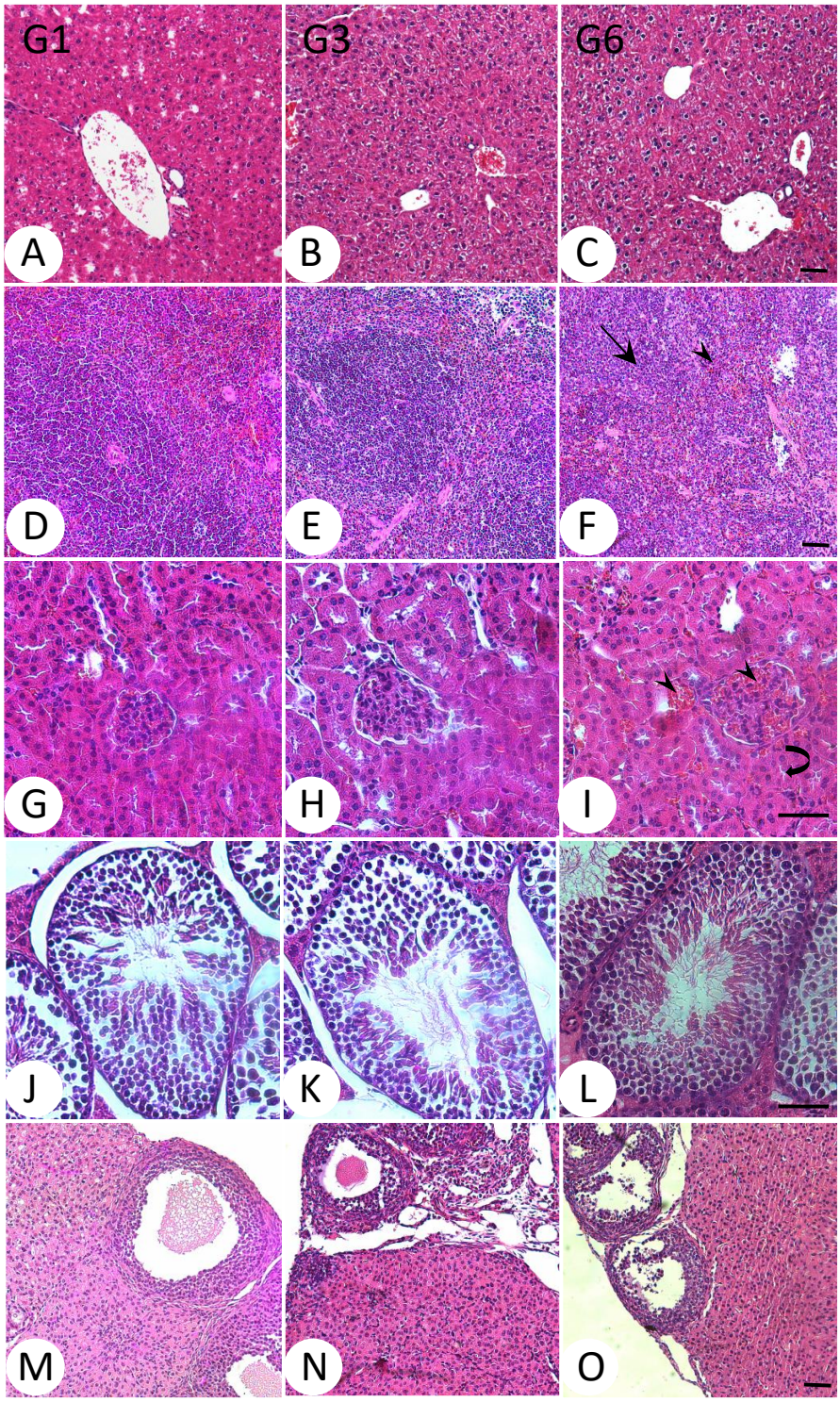

Figure 7. Histopathological analysis of tissues after treatment with broccoli seedlings (control or Se-enriched) for 40 days. (A, D, G, J, M) Vehicle control; (B, E, H, K, N) Broccoli seedlings control; (C, F, I, L, O) Se-enriched broccoli seedlings at dose 70 µg Se/kg-BW. The animals of the vehicle and broccoli seedlings control groups had no histopathological changes in the liver (D and E), kidney (G and H), testis (J and K) or ovary (M and N). Instead, the animals (male and female) treated with Se-enriched broccoli seedlings (70 µg Se/Kg) presented in the spleen (F) disruption of the white pulp (arrow) and intense edema (arrowhead). In the kidneys (I) Se-treated animals also presented renal edema (arrowhead) and reduction of renal tubules (curved arrow) besides no detectable alterations in liver (C), testis (L) or ovary (O). Bar = 50 µm.

4.3.2. Mammalian Cell Micronucleus Test

The micronucleus frequency (MNPCE) and the PCE/NCE ratios are summarized in Table 10. None treatment promoted significant alteration of PCE/NCE ratio in comparison to animals of the vehicle group. Besides, the MN inductor cyclophosphamide was able to increase significantly ($p<0.05$) the micronucleus frequency in both male and female mice indicating the conduction of a valid test.

Considering the broccoli seedlings treatments, female mice did not present significantly changes in the micronucleus frequency independently of treatments (Se-enriched or control broccoli seedlings) compared with the vehicle group. Instead, in male mice, the Se-enriched broccoli seedling treatment at 70 µg Se/kg-BW induced a significant ($p<0.05$) elevation on the micronucleus frequency whem compared to the vehicle control, indicating bone marrow toxicity (Table 10).

Table 10. The effect of broccoli seedlings without or with Se on the micronucleated polychromatic erythroblasts in bone marrow cells of mice.

Sex	Treatment		MNPCE/4000 PCEs (Mean±SEM)	PCE/NCE (Mean±SEM)
Female	Vehicle Control[a]		0.32±0.03	2.37±0.21
	Cyclophosphamide[b]		0.80±0.09*	0.98±0.14
	Broccoli seedlings control[c]		0.48±0.04	1.89±0.11
	Se-enriched Broccoli seedlings[d]	15 µg Se/kg-BW	0.40±0.04	2.05±0.13
		45 µg Se/kg-BW	0.45±0.05	2.23±0.21
		70 µg Se/kg-BW	0.49±0.05	2.30±0.21
Male	Vehicle Control[a]		0.26±0.02	4.19±0.65
	Cyclophosphamide[b]		0.78±0.05*	1.49±0.18
	Broccoli seedlings control[c]		0.32±0.03	3.66±0.52
	Se-enriched Broccoli seedlings[d]	15 µg Se/kg-BW	0.38±0.02	4.01±0.78
		45 µg Se/kg-BW	0.43±0.04	2.85±0.24
		70 µg Se/kg-BW	0.46±0.04*	4.86±0.90

Abbreviations: MNPCE, micronucleated polychromatic erythrocytes; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte.

Treatments: a) PBS (vehicle, 10 ml/kg, v.o., n=8); b) MN inductor (cyclophosphamide, 50 mg/kg, i.p., n=8); c) Broccoli seedlings cultivated without Se biofortification (v.o., dose equivalent to 70 µg Se/kg, n=10); d) Broccoli seedlings cultivated with Se biofortification (0.25, 0.74 and 1.15 g/kg v.o., corresponding to 15, 45 and 70 µg Se/kg, respectively; n=10).

(*) significant ($p<0.05$) compared with vehicle control.

4.4. Discussion

Selenium is an essential nutrient for humans, especially due its ability in reducing cancer risks (LAVU et al., 2016). However, this micronutrient is also considered as a “double-edged sword” because of the narrow range between deficient and toxic levels, highlighting the importance of toxicity studies (POLATAJKO; JAKUBOWSKI; SZPUNAR, 2006).

The growth retardation is the best indicator of the toxic effects of selenium-consumption (ORSKOV; FLYVBJERG, 2000). In the present study, only the male mice treated with broccoli seedlings with selenium at a minor dose (15 µg Se/kg-BW) showed a significant decrease in body weight gain. However, as we observed that changes in body weight gain were not dose-dependent, this effect could not be clearly attributed to direct Se toxic effect.

Hematological parameters are also a good toxicity indicator. After the treatments with broccoli enriched or not with selenium, we could observe that male mice presented no significant changes in these parameters when compared with the vehicle control group, while in female mice the MCHC parameters in all treatments were significantly lower than in the vehicle control. Changes in MCHC values may be related to anemia when associated with changes in MCV, RBC and MCH parameters (EVANS, 2008). In our experiment, the later parameters were inaltered. Moreover, considering expectables values described in literature, the MCHC values found lower in broccoli-treated female mice can be considered inside the normal range for the specie (SUCKOW; DANNEMAN; BRAYTON, 2001).

At high doses, selenium can cause toxicity in all organs, being liver the main target organ (NAGY et al., 2015; BENKO, et al., 2012). After 40 day-treatment with broccoli seedlings, with or without Se biofortification, at different doses, we observed a reduction tendency at the relative liver weight. This alteration was significant only in male mice at doses of 45 and 70 µg Se/kg-BW and the broccoli seedlings control (equivalent dose to 70 µg Se/kg-BW). This reduction was correlated with a significative reduction in AST values observed for male mice treated with broccoli seedlings control (equivalent dose to 70 µg Se/kg-BW) and Se-enriched broccoli seedlings at 45 µg Se/kg-BW. Zhang et al. (2005), Wang et al. (2007) and He et al. (2014) already reported similar toxic liver effects attributed to selenium. However, as broccoli seedlings with low Se level (0.25±0.04 µg Se/g dry sample) also promoted similar liver alterations (reduction in relative liver weight and AST level), other components present in broccoli could contribute to the toxic effect. Moreover,

despites these alterations, the histopathology analysis of the liver revealed a preserved architecture.

According to Nagy et al. (2015), selenium poisoning could also harm the kidneys. In our study, both the female and male mice treated with Se-enriched broccoli seedlings showed significant increasing in the relative kidney weight. More, significant enhanced uremia was also observed in male mice treated with Se-enriched broccoli seedlings at 45 µg Se/Kg-BW besides renal edema and renal tubules reduction in Se-enriched broccoli seedling-treated mice (female and male) at 70 µg Se/kg-BW.

Despite the relate of no toxic effects on spleen by selenium nanocompounds (BENKO et al., 2012), our results demonstrated increase relative spleen weight togheter with spleen pulp disruption and intense edema in female mice treated with Se-enriched broccoli seedlings at 70 µg Se/kg-BW. These results suggested that the matrix in which the Se in included affect the toxicological events.

Recent studies have shown that selenium may affect the reproductive function of male rats (HE et al., 2014). In our study, male mice treated with the highest dose of selenium (70 µg Se/kg-BW) showed a significant increase in the relative testis weight besides none histopatological alteration. To the best of our knowledge, effects of selenium treatment on ovary have not yet been reported. As in males, female mice also showed a significant increase in the relative ovary weight in all doses of selenium toghether with none histopatological alterations.

Finally, the micronucleus test is routinely used *in vitro* and *in vivo* to evaluate the activity of clastogenic and aneugenic chemicals, so the presence of micronuclei represents chromosome mutations (HINTZSCHE et al., 2017). In this assay, cyclophosphamide was used as a positive control due to its ability to produce mutagenicity through alkylating DNA and the free radicals generation after hepatic metabolization (HU; XU; CHEN, 2005). Analysing the parameters MNPCE frequency and PCE/NCE ratio, none mutagenic effect was observed in female mice treated with both broccoli seedling samples while in male mice, at 70 µg Se/kg-BW, Se-enriched broccoli seedling treatement promoted a significant increase (43%) in the micronuclei frequency in comparison to vehicle control.

It is well known that selenium has two faces and its toxicity is not only related to the amount of total selenium ingested, but also is strongly dependent upon the chemical form (JIA; LI; CHEN, 2005; RAYMAN; INFANTE; SARGENT, 2008). In general, elemental selenium and most metallic selenides have low bioavailability and hence relatively less toxicity. In contrast, selenates, selenites and organoselenium (selenocysteine,

methylselenocysteine, Se-methylselenocysteine and selenomethionine) compounds have high bioavailability and consequently can promote toxic effects at large doses (KHANAM; PLATEL, 2016). Studies that carried out the selenium speciation in broccoli indicate that the main species of this micronutrient are selenomethylselenocysteine, methylselenocysteine, selenomethionine and selenocystine (BAÑUELOS et al., 2015, BODNAR; KONIECZKA, 2016).

Moreover, the food matrix also is essential in the bioavailability process (KHANAM; PLATEL, 2016; THIRY et al., 2012). Zeng et al. (2008) demonstrated that the methylselenocysteine bioavailability was lower in broccoli than in the pure compound when tested in the Caco-2 cell model. In addition, the bioavailability processes likewise depends on the organism. Silva, Mataveli and Arruda (2013) evaluated the bioavailability of selenocysteine after Brazil nuts consumption by men and women for 15 days; at the end, only women urine samples have present increased selenocysteine concentration highlighting the influence of sex on the bioavailability processes of selenium in foods. These evidences may explain differences observed in our study between male and female mice.

Furthermore, it is important to remember that the estimated Se dose to promote cancer chemoprevention is between 50 and 100 μg day^{-1} (SIMONOFF; SIMONOFF, 1991). Considering our results, we can estimated the toxic dose of Se-enriched broccoli seedlings using the formula for dose translation from mouse (mouse dose = MS) to human (human estimated dose – HED) through the normalization to body surface area – BSA (REAGAN-SHAW; NIHAL; AHMAD, 2007). By using this formula (HED = MS x K_m^{mouse}/K_m^{human}) and assuming as K_m^{mouse} = 3 (mouse weight: 0.02 Kg and mouse BSA: 0.007 m^2) and K_m^{human} = 37 (human weight: 60 Kg and human BSA: 1.6 m^2), it was possible to estimate a human diary dose of 5.67 μg Se/kg for our Se-enriched broccoli seedlings, which equates to a 340 μg dose of Se for a 60 kg person. Using this calculus, the total estimated toxic dose in human (340 μg/day x 60 Kg) will be at more than 3 times higher than the higher chemopreventive dose (100 μg day^{-1}).

4.5. Conclusion

This is the first report on the safety assessment of selenium-enriched broccoli seedlings. In summary, investigation into oral and mutagenic toxicity of broccoli seedlings with selenium in different doses were performed in male and female mice. Our results showed no important changes in body weight gain and blood count, but toxic effects were observed in

organs such as kidneys, spleen, liver and testis/ovary. In male mice, the frequency of micronuclei increased at dose of 70 µg Se/kg-BW, indicating a possible mutagenic effect. Moreover, these toxic effects were observed in doses higher than that proposed as cancer chemopreventive in humans. More studies are required to get a deep insight into the toxicity and mutagenic mechanisms of broccoli seedlings biofortified with selenium.

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5. THE SPRAY DRYING TECHNOLOGY INFLUENCE ON THE PHYSICOCHEMICAL PROPERTIES OF SELENIUM-ENRICHED BROCCOLI SEEDLINGS EXTRACTS

Abstract

The search for bioactive compounds-enriched foods grows each year due to its potential health benefits. However, many of these compounds have high instability and sensitivity, requiring special processes to preserve them. This study aimed the production of dried extracts from broccoli seedlings biofortified or not with selenium using the spray drying technology. The extracts were spray dried with 30% of maltodextrin (carrier) at the temperature of 150 °C. The powders were characterized by physicochemical properties (moisture, hygroscopicity, solubility, morphology, total phenolic compounds and antioxidant activities). The microparticles had low values of moisture (4-5%) and hygroscopicity (11-12% g of adsorbed water 100 g-1 of microparticle) and high solubility (98-99%), being therefore biochemically and microbiologically stables. In terms of the morphology, the samples presented a wrinkled surface. The total phenolic compounds and antioxidant activities of the extracts were drastically reduced by the spray drying process and requires further studies to optimize microencapsulation processes.

Keywords: Maltodextrin; Sodium selenite; Microencapsulation; Antioxidant and powder technology

5.1. INTRODUCTION

In the Brassicaceae family, broccoli (*Brassica oleracea* L. var. italica), is one of the most consumed vegetables all over the world and has been highly valued due to its richness in bioactive compounds, such as glucosinolates, carotenoids, ascorbic acid, amino acids and phenolic compounds (SÁNCHEZ et al., 2016). Studies demonstrate that the bioactive compounds in broccoli, especially sulforaphane, have significant chemopreventive properties (WANG et al., 2018). Another compound that stands out is selenium due to its potential health benefit. Observational studies have reported an inverse relationship between selenium and risk of different types of cancer (HE et al., 2017; LUBIŃSKI et al., 2018). These potential benefits become promising for the food industry. Nowadays there is a high consumer interest in food containing natural ingredients and the increased demand for these products has been appreciably affecting trends in the global market (BORA et al., 2018; PAULO; SANTOS, 2017). However, due to the sensitivity of these compounds to changes in temperature, pH,

light and oxygen exposure, it becomes difficult to incorporate them into food products (JANISZEWSKA-TURAK, 2017).

Microencapsulation arises as a strategy to solve this problem. This technique is used to protect the active ingredient (called core) through a physical barrier (wall materials), providing consequently durability, stabilization and protection against external factors (light, moisture and heat) (CHRANIOTI; NIKOLOUDAKI; TZIA, 2015). Spray drying is one of the most commonly used microencapsulation techniques and allows the production of particles of good quality. Moreover, it is a economic, flexible and simple process (SHISHIR; CHEN, 2017).

The sulforaphane microencapsulation by spray drying has previously been investigated by Do et al. (2010) and Wu et al. (2014) using, as the wall material, respectively bovine serum albumin cross-linked chemically by glutaraldehyde and maltodextrin, gum arabic, κ-carrageenan and β-cyclodextrin. To the best of our knowledge, there are no other available publications investigating broccoli microencapsulation by spray drying. The objective of the present study was to evaluate the effect of broccoli microencapsulation by spray drying on physicochemical properties of broccoli seedlings biofortificated or not with selenium.

5.2. MATERIALS AND METHODS

5.2.1. Plant material and preparation of extracts

The broccoli seedlings (Avenger; Sakata Seed Sudamerica) were produced in partnership with the company IBS MUDAS (Piracicaba/SP). The samples were produced in black polyethylene trays of 200 cells filled with commercial substrate (coconut fibre) in an arched agricultural greenhouse and separated into two treatments: distilled water (MS) and 50 μM sodium selenate solution (MC). Both treatments were performed with only one application of 2 mL solution (water or sodium selenate) when the seedlings were at 15 days of germination (BACHIEGA et al., 2016). The broccoli seedlings were collected with 30 days of germination. The roots were eliminated, and the aerial part was sanitized, frozen, lyophilized and stored at -20 °C until use.

Aliquots (1 g) of each broccoli seedlings (MS and MC) were extracted with 50% ethanol (10 mL) under continuous shaking in water bath (Modelo Dubnoff SL-157 – Solab) for 30 min at 40 °C. The extracts were centrifuged for 1956,2 *g* during 15 min (Modelo NT

825, Nova Técnica, Brasil), the supernatant was filtered and the solvent was evaporated under vacuum at 50 °C (Modelo 801, Fisatom, Brasil) until a yield of 20%. The extracts were stored in amber bottles at - 20°C and were named extract of MS (ExMS) and extract of MC (ExMC).

5.2.2. Spray drying conditions

Spray drying was performed using a pilot spray dryer apparatus (MSD10, Labmaq do Brasil Ltda). Maltodextin (Maltogill 10) was employed as the carrier agent and was added to ExMS and ExMC at 30% concentration. The solutions were homogenized using a mechanical stirrer (model 752, Fisatom, Sao Paulo, Brazil) with a magnetic bar. Experimental microencapsulation conditions were nozzle size of 1.2 mm, compression air flow of 0.35 L min^{-1}, feed air flow of 2.50 m s^{-1}, feed flow of 10.0 ml min^{-1}, inlet temperature of 150 ± 2 °C and outlet temperature 94 ± 1 °C (WU et al., 2014). The obtained microparticles were named microparticles of MS (MpMS) and microparticles of MC (MpMC).

5.2.3. Physical properties of microparticles

5.2.3.1. Moisture

Moisture of MpMS and MpMC was determined in a moisture analyzer (MB35, Ohaus, Switzerland) using infrared radiation from a halogen source. Results were expressed as a percentage (%).

5.2.3.2. Hygroscopicity

To determine the hygroscopicity, 0.2 g aliquots of the MpMS and MpMC were weighed into Petri dishes and transferred to a desiccator previously conditioned with NaCl saturated solution. The samples remained for one week in the desiccator and the hygroscopicity was measured by the mass of water absorbed by the sample and expressed as g of adsorbed water 100 g^{-1} of microparticle (CAI; CORKE, 2000).

5.2.3.3. Solubility

Using the protocol described by Cano-Chauca et al. (2005), aliquots (0.5 g) of MpMS and MpMC were manually mixed with distilled water (50 mL) at room temperature followed by 30 minutes of shaking in one shaker table (Model ET-1401, Tecnal, Brazil) at 100-120 rpm and centrifugation (3000 *g*, 5 minutes). Aliquot (25 mL) of each supernatant was removed kept in one drying oven (FANEM 315 SE) at 105 °C for 5 hours. Solubility was calculated based on the difference of initial mass of the sample that was solubilized in 25 mL and the final mass present in 25 mL of the supernatant. The results were expressed as a percentage (%).

5.2.3.4. Morphology

The morphology of MpMS and MpMC were evaluated by scanning electron microscopy (SEM) using the TM 3000 Tabletop Microscope (Hitachi, Tokyo, Japan) and the program TM3000 for image acquisition and analysis. Initially, samples were arranged in a double-sided carbon tape (Ted Pella, Inc., Redding, United States) and then fixed in aluminum stubs. The images were captured at 5 kV of acceleration and 1750 mA.

5.2.4. Total phenolic content and antioxidant activities

The quantification of total phenolic content and antioxidant activities were performed in the extracts (ExMS and ExMC) and the corresponding microparticles (MpMS and MpMC). Initially, the microparticles were submitted to a extraction stage to remove maltodextrin. Then, aliquots (1 g) of each microparticles were diluted in methanol (5 mL for the total phenolic content, DPPH and FRAP analyzes; 3 mL for the ABTS assay), shaken for 15 minutes and centrifuged at 1956,2 *g* (Modelo NT 825, Nova Técnica, Brasil) for 5 min. Each supernatant was collected for the analysis.

5.2.4.1. Total phenolic content

Total phenolic content was measured with the Folin–Ciocalteu reagent and results were expressed as mg of gallic acid equivalents g^{-1} dry extract (SINGLETON; ORTHOFER; LAMUELA, 1999). Aliquots (0.5 mL) of each sample (ExMS, ExMC, MpMS, MpMC) were

mixed with Folin–Ciocalteu reagent (diluted 1:10 in distilled water, 2.5 mL) and Na_2CO_3 solution (4%, 2 mL). Thereafter, the absorbance was measured in a spectrophotometer (Unico® 2800 UV/VIS – Interprise Brasil) at 740 nm.

5.2.4.2. DPPH method

The DPPH assay was performed by the reaction of 500 µL of each sample and 300 µL of DPPH. After mixing, the tubes were left to stand and, after 45 min, the absorbance was measured at 517 nm. The same analysis was performed for Trolox solutions in several dilutions, making a standard curve. The results were expressed in µM Trolox g^{-1} dry extract, and the full equivalence values were calculated using the standard curve of Trolox (BRAND-WILLIAMS; CUVELIER; BERSET, 1995).

5.2.4.3. ABTS method

Initially the $ABTS^+$ radical was formed by the reaction of ABTS (7 mM) with 2.45 mM potassium persulphate. After that, 20 µL of samples were reacted with 2 mL of ABTS radical. Trolox was used to construct the standard curve and the absorbance was measured at 734 nm. The results were expressed as µM Trolox g^{-1} dry extract (RE et al., 1999).

5.2.4.4. FRAP method

The FRAP assay was based on the methodology described by Rufino et al. (2006). The analysis was performed by FRAP reagent from the mixture of 0.3 M sodium acetate buffer pH 3.6 with tripiridil-2,4,6-s-triazine (TPTZ) 10 mM in 40 mM and ferric chloride 20 mM in a ratio of 10:1:1, respectively. An aliquot of 90 µL of samples were added to 2.7 mL of the FRAP reagent and placed into water bath at 37 °C for 30 min. Results were measured using a spectrophotometer (Unico® 2800 UV/VIS – Interprise Brasil) at 595 nm and expressed as µM iron sulphate g^{-1} dry extract.

5.2.5. Statistical analysis

Each analysis was performed in triplicate and the results were expressed as mean values ± standard deviation. The data were submitted to the Shapiro-Wilk and Box-Cox tests to verify the normality and homogeneity of varience. The difference between means values was tested by the Student's t test for paired samples using SAS version 9.0 for Windows.

5.3. RESULTS AND DISCUSSION

5.3.1. Physical properties of microparticles

In the present study, ExMS and ExMC were spray-dried using maltodextrin as carrier, resulting in powders that were characterized with regard to the moisture content, hygroscopicity, solubility and morphology, as shown in Table 11. No significant differences ($p>0.05$) were found between the physical properties made with ExMS and ExMC.

Table 11. Physical properties of MpMS and MpMC.

Samples**	Moisture (%)	Hygroscopicity (g of adsorbed water 100 g^{-1} of microparticle)	Solubility (%)
MpMS	4.60±0.31	10.73±0.54	98.99±0.02
MpMC	4.97±0.26	11.75±0.57	99.03±0.05
Student *t*-test	p=0.1681^{ns}	p=0.0626^{ns}	p=0.2388^{ns}

^{ns} not significant
** MpMS: microcapsules prepared with ethanolic extract of broccoli seedlings without selenium; MpMC: ethanolic extract of broccoli seedlings with selenium.

The microcapsules of both broccoli extracts presented low moisture content. This carachteristic can be attributed to the high operation temperature and to the maltodextrin concentration. High temperatures during the process lead to higher heat transfer rate, reducing consequently the moisture content (KURIAKOSE; ANANDHARAMAKRISHNAN, 2010) while higher carrier agent concentrations resulte in an increase in feed solids and in a reduction in total moisture for evaporation (FAZAELI et al., 2012). In general, the moisture content lower than 6% promote inhibition of biochemical and microbiological reactions in a

food system, preventing the product deterioration (TAN et al., 2015). Therefore, our moisture results suggested biochemically and microbiologically stable microcapsules.

Hygroscopicity refers to the ability of a material to attract and hold moisture from the environment and is important for food products, influencing the stickiness and caking during the drying, storage and distribution phases (ZHANG et al., 2018). The MpMS and MpMC produced by spray drying showed very similar ($p > 0.05$) low hygroscopicity. As well as moisture, this parameter can be influenced by the maltodextrin concentration (THIRUGNANASAMBANDHAM; SIVAKUMAR, 2017). This carrier has a low hygroscopicity- therefore, higher ratios of maltodextrin resulted in powder with less hygroscopicity (COMUNIAN et al., 2011). Zotarelli et al. (2017) and Calomeni et al. (2017) were obtained similar results for the spray drying of mango and peanut skins, respectively.

Solubility is another important parameter for the technological evaluation of the microparticles produced by the spray drying technique. The MpMS and MpMC showed a high solubility (Table 11). Thus, the samples could effectively and easily be reconstituted in water. According to Stoll et al. (2016), solubility is influenced by the specific properties of the wall material used and is independent of the core material. Our work used maltodextrin as a wall material, which explains the high solubility of the microparticles. Tupuna et al. (2018) and De Marco et al. (2013) microencapsulated norbixin and bixin and observed that the higher the concentration of maltodextrin, the greater the solubility of the microparticle formed.

The morphology of MpMS and MpMC was investigated by SEM (Figure 7). The microparticles showed a nearly spherical form and most of them presented a wrinkled surface, with the presence of pores and cavities. Only a small part of the microparticles exhibited a smooth surface. These morphology irregularities can be explained due to the fast evaporation of liquid droplets and consequently to the shrinking of the particles (TUPUNA et al., 2018), and have been reported by Ballesteros et al. (2017), Santiago-Adame et al. (2015) and Zhang et al. (2018) using the spray drying process.

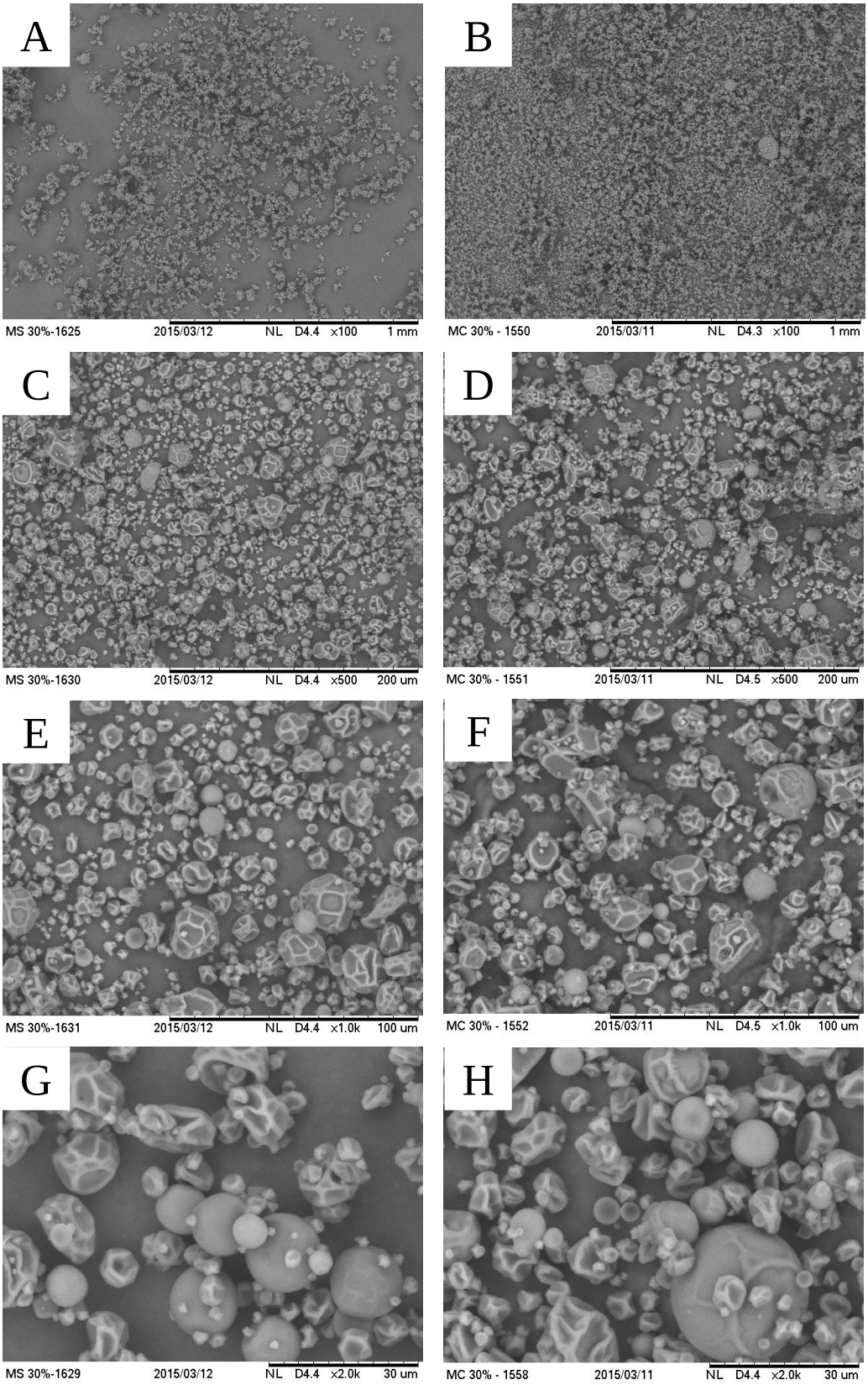

Figure 8. Scanning electron microscopy (SEM) photographs of spray dried ethanolic extracts of broccoli seedling powders: MpMS (A, C, E and G) at x100, x500, x1000 and x2000 magnification; MpMC (B, D, F and H) at x100, x500, x1000 and x2000 magnification.

5.3.2. Total phenolic content and antioxidant activities

The total phenolic contents and antioxidant activities, with different methods, of ExMS and ExMC before and after encapsulation are shown in Table 12. Microencapsulation by spray drying reduced significantly ($p<0.01$) all evaluated parameters. The possible explanation for this reduction may be because of the temperature (150 ºC) employed in the process. According to Mishra, Mishra and Mahanta (2014), the exposure to higher temperatures can adversely affect the structure of phenolics resulting in the breakdown of these compounds. In this way, the antioxidant activities are also reduced, since the phenolic compounds in broccoli are the main responsibles for these activities.

Table 12. Total phenolic compounds and antioxidant activities of MpMS and MpMC.

Samples*	DPPH	ABTS	FRAP	Total phenolic compounds
	(µM Trolox g^{-1} dry extract)	(µM Trolox g^{-1} dry extract)	(µM iron sulphate g^{-1} dry extract)	(mg of gallic acid equivalents g^{-1} dry extract)
ExMS	169.11±1.23	281.40±15.91	3532.00±145.50	70.40±0.22
MpMS	25.34±0.36	41.13±2.12	504.90±16.67	8.57±0.46
Student *t*-test	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
ExMC	161.80±1.45	322.60±11.73	3581.00±280.00	72.54±0.30
MpMC	21.40±0.86	18.35±1.34	431.70±12.83	7.77±0.34
Student *t*-test	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$

The comparison of means was performed between extract and microparticle of each sample separately.
* ExMS: extract of broccoli seedlings without selenium; ExMC: extract of broccoli seedlings with selenium; MpMS: microcapsules prepared with ethanolic extract of broccoli seedlings without selenium; MpMC: microcapsules prepared with ethanolic extract of broccoli seedlings with selenium.

The microstructure of the particles is associated with the capacity of retention of bioactive compounds (TAN et al., 2015). As observed in particle morphology using SEM, the MpMS and MpMC presented some cracks on the surface which leads to a lower retention of bioactive compounds. The cracks present in the surface of the microparticles may increase the contact of the bioactive compounds inside the particles with air and heat, which will cause oxidation and degradation of these compounds (KHA et al., 2014). Thus, the spray drying temperature is directly associated with the bioactive compounds retention.

Maltodextrin is a good carrier for protecting the compounds against oxidation due to its film-forming capacity. However, in our results we observed that the samples

microencapsulated with maltodextrin did not present a significant retention of the bioactive compounds. In this way, besides the influence of temperature, the concentration of the carrier was not able to produce well-formed and completely coated microparticles. Thus, another strategy to increase the retention of bioactive compounds is the combination of other wall materials, such as arabic gum, whey protein concentrate, pectin or fructooligosaccharide, with maltodextrin (CALVA-ESTRADA et al., 2018; CHATTERJEE; BHATTACHARJEE, 2013; IGUAL et al., 2014; KALITA et al., 2018; TAN et al., 2015).

5.4. CONCLUSIONS

The microparticles formed from the ethanolic extracts of broccoli seedlings with or without selenium had good physical properties, such as low moisture and hygroscopicity and high solubility. However, the extracts showed a significant reduction of total phenolic compounds and antioxidant activities after the spray drying process. Thus, it is necessary to study the optimization of the microencapsulation parameters to obtain microparticles with a greater potential of bioactive compounds retention.

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6. GENERAL CONCLUSIONS

Micronutrient deficiency, such as selenium, is a concern worldwide. Studies show that, in several countries, selenium consumption is below the recommended daily dose. As a strategy to reduce the deficiency of this micronutrient, biofortification studies of agricultural crops appear, especially with broccoli. Our work proved that the biofortification of broccoli seedlings with selenium presented a significant potential to increase the content of selenium in this food.

We verified that the benchtop and handheld Energy Dispersive X-Ray Fluorescence techniques were efficient for the quantification of selenium in biofortified samples. Both techniques presented as main advantages the reduced time and cost of analysis. However, they do not have low limits of detection, being a limitation of these techniques.

Although biofortification is efficient in increasing the micronutrient content in plants, this may cause a change in the profile of bioactive compounds. When evaluating the bioactive compounds of broccoli, we observed that ascorbic acid, total phenolics and flavonoids, chlorophyll and sulforaphane showed a significant increase. Thus, this strategy was also effective in increasing the health-promoting compounds in broccoli.

Although selenium has numerous health benefits, in high doses it can be toxic. Thus, to ensure the safe consumption of selenium-biofortified broccoli seedlings, it was evaluated its toxicological and mutagenic potential in different doses. After treatment (40 days), the results demonstrated a toxic effect in organs such as kidneys, spleen, liver, testis/ovary in male and female mice. In addition, dose of 70 µg/kg presented a mutagenic potential in male mice. Only doses of 15 µg/kg showed no toxic or mutagenic effects, which represents a human estimated dose (HED) of 1.22 µg/kg (73 µg/day for a 60 kg person).

Finally, we have prepared broccoli microparticles with and without selenium for future application in the food industry. Although the microparticles produced presented promising technological characteristics, the spray dryer technique had a negative influence on phenolic compounds and on the antioxidant activity. Thus, the importance of future studies to optimize microencapsulation processes is emphasized.